Biochemical Pharmacology, Vol. 24, pp. 1799–1805. Pergamon Press, 1975. Printed in Great Britain.

INTERACTION OF AFLATOXIN B_{2a} WITH AMINO ACIDS AND PROTEINS*

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(*Received* 19 *July* 1974; *accepted* 24 *January* 1975)

Abstract—Interaction of aflatoxin B_{2a} (B_{2a}), the hemiacetal of aflatoxin B_1, with amino acids and proteins has been demonstrated by spectrophotometric analysis, spectrophotometric titration, equilibrium dialysis, separation of the interaction complex from free reactants by thin layer chromatography (TLC) at pH 7·0, and by the isolation of stable conjugates upon reduction with sodium borohydride ($NaBH_4$). At pH 7·8, the spectrum of B_{2a} shifted to a longer wavelength (from 370 to 400 nm) when either glycine or ovalbumin was present, but did not shift in the presence of *N*-acetylglycine. Likewise, both glycine and ovalbumin altered the dissociation constant (p*K*) of the phenolic group of B_{2a} but *N*-acetylglycine did not. The empirical formula for the complex was B_{2a} (phe)$_2$ when ^{14}C-phenylalanine (phe) was used in the interaction. An apparent equilibrium constant for the B_{2a}(phe)$_2$ system was found to be $1{\cdot}82 \times 10^7$ l. M^{-1} at pH 7·0. The complex was stable at neutral and slightly alkaline pH but dissociated in acid, in contrast to the reduced adduct which was relatively stable in acidic conditions. The interaction was also pH-dependent with a higher degree of interaction as basicity increased. The results indicated that the α- and ε-amino groups of amino acids and proteins and the aldehyde groups of the phenolate ion form of B_{2a} were essential for the reaction. The data support the proposed Schiff base formation mechanism for the interaction.

Aflatoxin B_1(B_1), a potent carcinogen, is the major toxic metabolite produced by *Aspergillus flavus* and *A. parasiticus*. The biochemical and biological as well as the toxicological properties of B_1 have been extensively reviewed [1, 2]. The toxin is readily converted to its hemiacetal, designated as aflatoxin B_{2a}(B_{2a}), by addition of a water molecule to the vinyl ether double bond of the B_1 terminal ring in acid [3] or under ultraviolet light [4]. Recent investigations indicated that B_{2a} is also one of the major B_1 metabolites in several animals [5, 6].

Although it has been reported that B_{2a} is less toxic than B_1 [3], it reacts readily with amino acid and protein [7], and inhibits deoxyribonuclase I (DNase I) activity [8]. It was suggested that such an interaction might be related to the acute toxicity of B_1 because of the conversion of B_1 to B_{2a} in animal liver *in vivo* [9]. Due to the existence of a phenolate ion form of B_{2a} in basic solutions, in which two free aldehyde groups are generated, it was suggested that a Schiff base was formed between the aldehyde groups in B_{2a} and amino groups in amino acid and proteins [7]. Nevertheless, such complexation has not been characterized. In this paper, a more detailed study of the interaction of B_{2a} with amino acids and proteins is presented.

MATERIALS AND METHODS

Materials. DL-Glycine, DL-phenylalanine, DL-proline, *N*-α-acetyllysine, *N*-acetylcysteine, *N*-acetylglycine, calf-thymus DNA (sodium salt) and beef pancreatic DNase I were purchased from Sigma Chemical Co. (St. Louis, Mo.). Crystalline bovine serum albumin (BSA) and ovalbumin were purchased from Schwarz-Mann (Orangeberg, N.Y.). Sodium borohydride ($NaBH_4$) was purchased from Fisher Scientific Co. (Fair Lawn, N. J.). Labeled ^{14}C-phe (1·3 mCi/m-mole) was purchased from Tracerlab (Waltham, Mass.) and ^{3}H-$NaBH_4$ (700 mCi/m-mole) was purchased from New England Nuclear Co. (Boston, Mass.). Plastic sheets (20 × 20 cm) precoated with carboxymethylcellulose (CMC) MN300CM were purchased from Brinkmann Instruments, Inc. (Westbury, N. Y.). Adsorbosil-5 was purchased from Applied Science Laboratories, Inc. (State College, Pa.) and Sephadex G-10 was purchased from Pharmacia Fine Chemicals, Inc. (Piscatawnay, N. J.). All organic solvents used in this study were of analytical grade.

Preparation of B_{2a} and B_{2a} derivatives. Aflatoxin B_{2a} was prepared by the method of Pohland *et al.* [3]. Acetylated B_{2a} was produced by acetylation of B_{2a} with acetic anhydride in pyridine; reduced B_{2a} was prepared by reduction of B_{2a} with $NaBH_4$ as described by Ashoor and Chu.†

Thin-layer chromatography (TLC). Glass plates coated with Adsorbosil-5 (Silica gel) to a thickness of 250 μM and plastic sheets precoated with carboxymethylcellulose (CMC) were used for TLC. The developing systems were chloroform–methanol (90:10, v/v), butanol–acetic acid–water (40:15:10, by vol.) for Adsorbosil-5, and 0·1 M sodium phosphate buffer, pH 7·0, for CMC. The developed plates were air-dried and examined under ultraviolet light again. The major spots in both cases were located, and their R_f values were calculated.

Spectrophotometric analyses. Spectrophotometric measurements were made using a Beckman model DU spectrophotometer. Absorption spectra were obtained by scanning the appropriate solutions at a

* This work was supported by the College of Agricultural and Life Sciences, The University of Wisconsin, and by Public Health Service Research Grant No. CA 15064 from the National Cancer Institute, by Grant No. ES 00656 from the National Institute of Environmental Health Sciences, and by a grant from the Brown–Hazen Fund of the Research Corp.

† S. H. Ashoor and F. S. Chu, manuscript in preparation.

range of 200–600 nm in a Beckmann Acta III automatic recording spectrophotometer with a light path of 1 cm. Spectrophotometric titrations were carried out in a Radiometer Automatic Titrator with a Radiometer Titrigraph type SBR2 and type SBU 1 syringe burette as described by Chu [10]. Fluorescence measurements were made in an Aminco Bowman spectrophotofluorometer at appropriate activation and emission wavelengths.

Radioactivity measurements. Except where otherwise specified, an appropriate amount of extracted solution (generally less than 0·5 ml) or a radioactive spot scraped from a thin-layer plate was mixed with 10 ml of Bray's solution [11]. The solution was subjected to counting in a Packard Tri-Carb model 5017 liquid scintillation spectrometer for 10 min.

Reaction of B_{2a} and reduced B_{2a} with amino acids and their N-acetyl derivatives. An appropriate amount of either B_{2a} or reduced B_{2a} dissolved in methanol was mixed with an equal amount of the amino acid or the *N*-acetyl derivative dissolved in 0·05 M sodium phosphate buffer, pH 7·2. The reaction mixture was incubated at 37° for 20 min and divided into halves. One-tenth ml of freshly prepared $NaBH_4$ solution ($2{\cdot}6 \times 10^{-2}$ M) in phosphate buffer was added dropwise to one half and both halves were kept at 4° for an additional 30 min. Excess $NaBH_4$ was then destroyed by the addition of two drops of 0·1 N HCl, and the two solutions were brought to equal volume by the addition of phosphate buffer. Equal volumes of both reaction mixtures were subjected to TLC using the butanol acetic acid water system as described before. In certain cases, spots of some reduced products were scraped off and eluted with a small volume of phosphate buffer for use as standards.

For confirmation purposes, B_{2a} was reacted with ^{14}C-phe (1·3 μCi/μmole) and the mixture was reduced with $NaBH_4$. Aflatoxin B_{2a} was also reacted with unlabeled phe, with the subsequent reduction with ^{3}H-$NaBH_4$ ($9{\cdot}8 \times 10^{6}$ cpm/0·1 ml). The reaction conditions in both cases were similar to those described above. The reaction mixtures were analyzed by TLC in the butanol–acetic acid–water solvent system. All major spots were scraped off and subsequently eluted with phosphate buffer. The radioactivity of each solution was counted.

Testing the stability of the B_{2a} amino acid product. The stability of B_{2a} phe adduct was tested in two ways. First, phe was reacted with B_{2a} followed by either reduction or non-reduction as indicated before. Methanol in the mixtures was evaporated under vacuum. Both mixtures were then acidified with 0·1 N HCl (0·1 ml acid per 1·0 ml mixture) to a pH value of approximately 2·0 and extracted 5 times with equal volumes of chloroform. The combined chloroform extracts were concentrated under vacuum and then spotted along with the aqueous layers for TLC analysis, using both solvent systems mentioned before. Second, appropriate amounts of purified ^{14}C-phe B_{2a} (reduced) adduct were hydrolyzed in 6 N HCl at 110° in evacuated and sealed tubes for 2 and 24 hr. The hydrolysates were evaporated to dryness at room temperature, redissolved in equal volumes of phosphate buffer and examined by TLC analysis. The R_f values of all spots were calculated and the radioactivity of each spot was measured.

Reaction of B_{2a} with proteins. One mg of B_{2a} suspended in a few drops of methanol was added to 10 mg of each protein (ovalbumin, BSA and DNase) in 3 ml phosphate buffer. The reaction mixtures were incubated at 37° for 30 min, followed by the addition of two drops of antifoam B and 0·1 ml $NaBH_4$ solution ($1{\cdot}3 \times 10^{-2}$ M) and then incubated for another 30 min at 4°. Two drops of 0·1 N HCl were added to the mixture with gentle stirring to destroy excess $NaBH_4$. Control protein solutions were reduced with $NaBH_4$ in a similar manner. All protein mixtures were passed through a 2 × 50 cm Sephadex G-10 column using 0·05 M phosphate buffer, pH 7·2, as eluent. Absorbance of fractions was measured at 280 and 400 nm. The protein fractions were pooled together, dialyzed for 48 hr against a large volume of water at 4°, and freeze-dried. The activity of DNase samples was measured by the method of Kunitz [12].

In a separate experiment, the B_{2a} ovalbumin reaction mixture along with a control mixture was reduced with ^{3}H-$NaBH_4$ ($2{\cdot}9 \times 10^{5}$ cpm/0·1 ml) and treated as indicated above. The total radioactivity of both samples was measured.

Effect of pH on B_{2a} interaction with proteins. This was carried out by reacting B_{2a} ($1{\cdot}3 \times 10^{-5}$ M) with either ovalbumin ($1{\cdot}19 \times 10^{-5}$ M) or lysozyme ($3{\cdot}67 \times 10^{-5}$ M) in appropriate buffers of different pH values (acetate for pH 4·8 and 6·0, phosphate for 7·0 and 8·0, and bicarbonate for 9·1; final concentration, 0·06 M) with subsequent $NaBH_4$ reduction. The solutions were then dialyzed against distilled water in a dialysis tank for 48 hr with a change of fresh distilled water twice a day. The pH of the solutions after dialysis was about pH 7·0. The fluorescence intensity of each solution was measured at the activation and emission wavelengths of 390 and 440 nm respectively. Interaction of reduced B_{2a} at pH 8·0 was also tested under similar conditions.

Equilibrium dialysis. Interaction of B_{2a} with proteins without reduction with $NaBH_4$ was carried out at pH 7·5 using an equilibrium dialysis method similar to that described by Chu [13]. Two ml of the protein solutions at a concentration of $1{\cdot}8 \times 10^{-3}$ M was dialyzed against $1{\cdot}72 \times 10^{-6}$ M B_{2a} dissolved in 20 ml of 0·05 M phosphate buffer at pH 7·5. The concentration of free and bound B_{2a} was determined fluorometrically by comparing with a standard curve in the range of $1{\cdot}0 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-8}$ M at activation and emission wavelengths of 390 and 440 nm respectively. The competition of interaction between B_{2a} with glycine and proteins was carried out in a similar manner by incorporation of 1×10^{-3} M glycine in the equilibrium dialysis.

RESULTS

Spectrophotometric analyses. The spectra of B_{2a} and of B_{2a} in the presence of glycine or ovalbumin at pH 7·8 are shown in Fig. 1a. The difference between these spectra is shown in Fig. 1b. In the presence of glycine or ovalbumin, the B_{2a} spectrum shifted to a longer wavelength with maximum changes occurring at 370 and 400–410 nm. No changes in the B_{2a} spectrum were observed when *N*-acetylglycine was present. These results indicate that complexation had

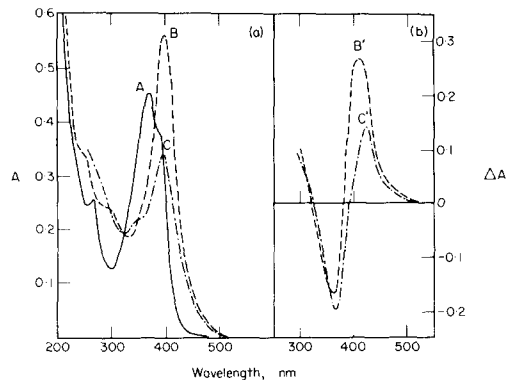

Fig. 1. Effect of glycine and ovalbumin on the absorption spectrum of B_{2a}. The absorption spectra of B_{2a} (curve A, ——), B_{2a} + glycine (curve B, – – –), and B_{2a} + ovalbumin (curve C, · · · ·) are shown in panel a, and the difference in spectra is shown in panel b. Curves B' and C' represent B minus A and C minus B respectively. The concentrations of B_{2a}, glycine and ovalbumin were $2{\cdot}1 \times 10^{-5}$ M, $1{\cdot}3 \times 10^{-4}$ M and $2{\cdot}8 \times 10^{-5}$ M respectively. The final solution was in 0·05 M phosphate buffer, pH 7·8.

occurred between B_{2a} and glycine or ovalbumin but did not occur with *N*-acetylglycine.

Spectrophotometric titrations of B_{2a} in the presence or absence of excess amounts of glycine, *N*-acetylglycine, or ovalbumin, and titrations of reduced B_{2a} and acetyl B_{2a} in the presence or absence of excess amounts of glycine were carried out at 400 nm to study the changes in the dissociation of the phenolic group of B_{2a} and its derivatives due to complexation. The various titration curves are shown in Fig. 2, and the different apparent p*K* values of B_{2a} and its derivatives are shown in Table 1. The apparent p*K* of B_{2a} was shifted to a higher value (from 7·10 to 8·10) when either glycine or ovalbumin was present, whereas no

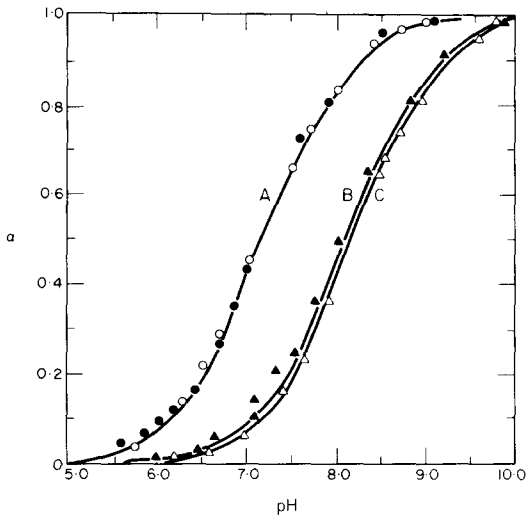

Fig. 2. Spectrophotometric titration curves of B_{2a} (A), B_{2a} + *N*-acetylglycine (A), B_{2a} + ovalbumin (B) and B_{2a} + glycine (C). Theoretical curves were calculated from p*K* values of 7·10, 8·05 and 8·1 for A, B and C respectively. The symbols along the curves (○, ●, △, ▲) represent the experimental data for B_{2a}, B_{2a} + *N*-acetylglycine, B_{2a} + ovalbumin and B_{2a} + glycine respectively. α Was calculated according to Chu [13].

Table 1. Apparent dissociation constant (p*K*) of phenolic group of B_{2a} and B_{2a} derivatives in the presence or absence of amino acid or protein

Amino acid or protein added	B_{2a}	B_{2a} derivative: Reduced B_{2a}	B_{2a} derivative: Acetyl B_{2a}
No amino acid added	7·10	6·10	7·60
+ Glycine	8·10	6·20	7·40
+ *N*-acetylglycine	7·10	ND*	ND
+ Ovalbumin	8·05	ND	ND

* ND = not determined.

shifting was observed in the presence of *N*-acetylglycine. The dissociation of the phenolic group of reduced B_{2a} or acetyl B_{2a} was not altered in the presence of glycine. These results indicate the formation of a complex between B_{2a} and glycine or ovalbumin which resulted in changing the p*K* of the phenolic group of B_{2a}. They also indicate that the amino group of glycine and the aldehyde groups of B_{2a} are essential for the formation of such a complex, since *N*-acetylglycine did not form a complex with B_{2a}, and reduced B_{2a} and acetyl B_{2a} failed to complex with glycine.

TLC analyses of the complexation. Chromatography of B_{2a}–^{14}C-phe mixture on CMC at pH 7·0 resulted in three well separated spots (Fig. 3), two of which were identified as phe (R_f, 0·87) and B_{2a} (R_f, 0·35). The third spot (R_f, 0·20) was radioactive (44·6 per cent of total radioactivity), fluorescent and ninhydrin negative and was considered as that of the reaction complex between ^{14}C-phe and B_{2a}. In order to determine the empirical formula of this complex, ^{14}C-phe was mixed with B_{2a} at different molar ratios (0·44

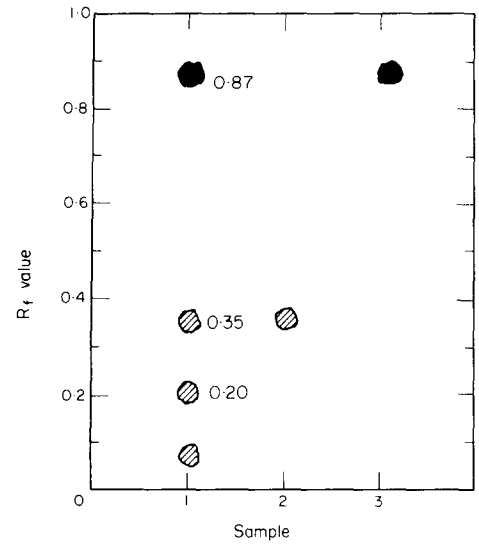

Fig. 3. Thin-layer chromatograms of the ^{14}C-phe–B_{2a} reaction mixture (without $NaBH_4$ reduction) on CMC sheets developed in 0·1 M phosphate buffer, pH 7·0. Samples 1, 2 and 3 represent reaction mixture, B_{2a} standard and phe standard respectively. Solid spots indicate ninhydrin-positive spots: the shaded spots indicate fluorescence spots under ultraviolet light. Values in parentheses represent the R_f values of the various spots. Radioactivity was distributed between phe (R_f, 0·87) and the reaction complex (R_f, 0·20).

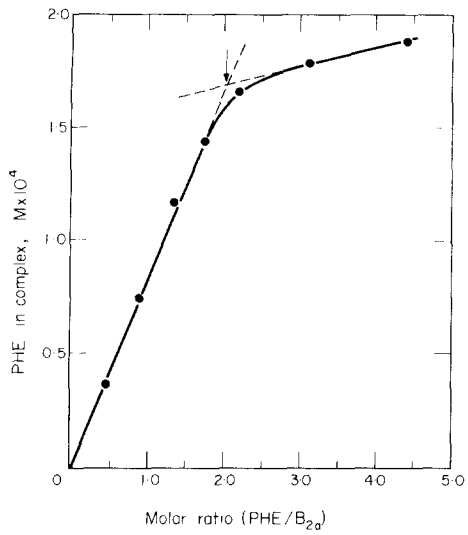

Fig. 4. Effect of the phe:B_{2a} molar ratio on the formation of phe B_{2a} complex. B_{2a} in methanol (3.0×10^{-3} M) was mixed with various amounts of ^{14}C-phe solution (1.31×10^{-2} M) in 0.05 M phosphate buffer, pH 7.2, to a total volume of 0.15 ml. Mixtures were incubated at 37° for 20 min, and 5 μl of each was spotted and chromatographed as indicated in Fig. 3. The amount of bound phe was determined by counting the radioactivity of scraped reaction complex spots.

to 4.0) and the mixtures were subjected to TLC on CMC. The amount of phe in the complex was determined by measuring radioactivity in the complex spot and the various amounts of phe were plotted against the corresponding molar ratios as shown in Fig. 4. The amount of phe in the complex increased almost proportionally with the increase of the phe:B_{2a} molar ratio up to 2.0 and was almost constant thereafter. Extrapolating these results indicated that the complex contained 2 moles of phe/mole of B_{2a} with an empirical formula of $B_{2a}(phe)_2$. With this information, it was possible to determine the overall apparent equilibrium constant, K, which is defined as follows:

$$B_{2a} + 2\,phe \rightleftarrows B_{2a}(phe)_2$$

$$K = \frac{[B_{2a}(phe)_2]}{[B_{2a}][phe]^2}$$

The various values of K and pK for the $B_{2a}(phe)_2$ complex obtained are shown in Table 2. The average apparent K was 1.82×10^7 l. M^{-1} and the average pK was 7.24 as determined at pH 7.0.

Chromatography of B_{2a} and the ^{14}C-phe mixture on Adsorbosil-5 developed in the butanol acetic acid water system resulted in only two spots, identified as B_{2a} and phe, which contained all of the added radioactivity. Apparently, the complex was dissociated under these acidic conditions.

Reduction of B_{2a} amino acid complexes with $NaBH_4$. In order to obtain a more stable covalent adduct and to confirm the possible formation of a Schiff base between B_{2a} and an amino acid, B_{2a} was reacted with different amino acids and *N*-acetyl derivatives of amino acids with subsequent reduction with $NaBH_4$. A typical chromatogram on silica gel (Adsorbosil-5) is shown in Fig. 5. The unreduced reaction

Table 2. Determination of apparent equilibrium constant, K, of $B_{2a}(phe)_2$ complex at pH 7.0

Free phe ($M \times 10^5$)	Free B_{2a} ($M \times 10^5$)	$B_{2a}(phe)_2$ ($M \times 10^5$)	$K \times 10^{-7}$ (l. M^{-1})	pK
8.77	5.77	0.84	1.90	7.28
12.96	5.27	1.33	1.50	7.18
9.86	16.20	3.80	2.40	7.38
14.51	14.16	5.80	1.96	7.29
13.80	13.80	6.20	2.40	7.38
20.82	12.92	7.09	1.26	7.09
20.65	12.80	7.20	1.30	7.11
			1.82*	7.24*

* Mean value.

mixtures showed only two spots, identified as B_{2a} and the corresponding amino acid, whereas the reduced mixture showed an extra spot (fluorescent, ninhydrin negative, with a different R_f value in each of the reduced mixtures) in addition to the spots of reduced B_{2a} and the amino acid. When *N*-acetylglycine, *N*-acetylcysteine and proline were reacted with B_{2a} with subsequent reduction with $NaBH_4$, the new spot was absent. When reduced B_{2a} was reacted with amino acids with subsequent reduction with $NaBH_4$, no additional spots were observed. In order to confirm that the extra spot resulting from reduction of the amino acid B_{2a} mixture was of the reduced amino acid B_{2a} complex, ^{14}C-phe and ^{3}H-$NaBH_4$ were used as labeling reagents. The distribution of radioactivity among the different spots in reduced and unreduced mixtures is shown in Table 3. In the ^{14}C-phe and B_{2a} mixtures, radioactivity was distributed between phe and the extra unknown spot upon reduction with $NaBH_4$, whereas it was located only in the phe spot in the unreduced mixture. When ^{3}H-$NaBH_4$ was used for reduction, radioactivity was distributed between the extra unknown spot and that of reduced B_{2a}; no radioactivity was detected in the phe spot. These results indicate that the extra spot was that of the stable adduct formed between an amino acid and B_{2a} upon reduction with $NaBH_4$.

The molar ratio of phe:B_{2a} in the reduced adduct was determined by TLC analysis of the reduced ^{14}C-phe B_{2a} mixture. The amount of phe in the adduct was determined by measuring the radioactivity of the adduct spot. The amount of B_{2a} in the adduct was

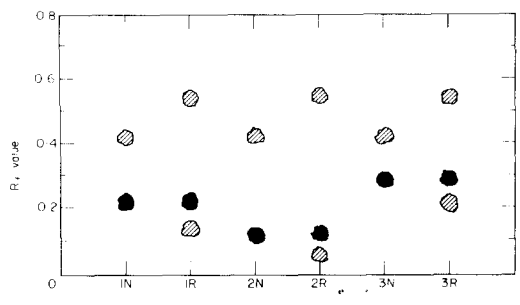

Fig. 5. Thin-layer chromatograms of glycine (1), *N*-ε-acetyllysine (2), and phe (3) reaction mixture with B_{2a} before reduction (N) and after reduction with $NaBH_4$ (R) on Adsorbsil-5. Solid spots were ninhydrin positive and the shaded spots were fluorescent under ultraviolet light. The solvent system was butanol acetic acid water, 40:15:10, by vol.

Table 3. Distribution of radioactivity among spots of thin-layer chromatograms of phe-B_{2a} reaction mixtures*

TLC system†	Reaction mixture	R_f	Identified compound	Radio-activity (%)
A	phe + B_{2a}	0·29	phe	98·6
		0·41	B_{2a}	1·4
A	phe + B_{2a}	0·20	unknown	49·1
	(reduced with	0·29	phe	48·3
	$NaBH_4$)	0·55	reduced B_{2a}	2·6
A	phe + B_{2a}	0·20	unknown	66·0
	(reduced with	0·29	phe	1·0
	^3H-$NaBH_4$)	0·55	reduced B_{2a}	33·0
B	phe + B_{2a}	0·24	unknown	41·9
	(reduced with	0·54	reduced B_{2a}	4·2
	$NaBH_4$)	0·87	phe	53·9

* Either ^{14}C-phe or ^3H-$NaBH_4$ was used for labeling.
† System A: Adsorbosil-5 coated plates developed in butanol–acetic acid–water (40:15:10, v/v); system B: CMC sheets developed in 0·1 M phosphate buffer, pH 7·0.

obtained by measuring the amount of reduced B_{2a} (equivalent to the amount of unreacted B_{2a}) spectrophotometrically, using ϵ_{362}^{MeOH} of 27,000,* and subtracting this amount from the total amount of B_{2a} added. In this manner, the molar ratio of phe to B_{2a} in the reduced adduct was 2·03, which is in agreement with the molar ratio of the unreduced adduct previously determined.

Stability of the B_{2a}–amino acid reduction adduct. When the acidified, unreduced phe–B_{2a} reaction mixture was extracted with chloroform, all the B_{2a} was transferred to the chloroform layer, whereas the aqueous layer did not show any fluorescence and gave only a phe spot on TLC. However, after chloroform extraction, the aqueous layer of the acidified reduced mixture of phe–B_{2a} gave three spots: reduced B_{2a} (less soluble in chloroform than B_{2a}), phe, and the reduction adduct. The chloroform extract of the acidified solution of the reduced phe–B_{2a} adduct did not show any fluorescence, whereas the aqueous layer showed a fluorescent, ninhydrin-negative spot with the same R_f value as the original adduct. These results indicate that the reduced B_{2a}–phe adduct was more stable in acidic conditions than the unreduced one.

Table 4. Stability of $B_{2a}(^{14}C\text{-phe})_2$ adduct to 6 N HCl hydrolysis at 110°

Time of hydrolysis (hr)	Distribution of radioactivity in the hydrolysates* (%) Unknown	Original adduct	phe	Reduced B_{2a}
2	1·7	87·0	9·8	1·5
24	0·3	53·1	46·3	0·3

* Hydrolysates were chromatographed on CMC sheets developed in 0·1 M phosphate buffer, pH 7·0. The R_f values were 0·07 for the unknown spot, 0·24 for the $B_{2a}(phe)_2$ adduct, 0·54 for reduced B_{2a} and 0·87 for phe.

* S. H. Ashoor and F. S. Chu, manuscript in preparation.

The stability of the reduced adduct was also tested by acid hydrolysis of two radioactive preparations of the reduced B_{2a} phe, using either ^{14}C-phe or ^3H-$NaBH_4$ for labeling. The results of the acid hydrolysis of the B_{2a} ^{14}C-phe adduct are given in Table 4. Approximately 87 and 53 per cent of the adduct were unaffected after 2 and 24 hr of hydrolysis in 6 N HCl at 110°. Similar results were obtained when the ^3H-labeled adduct was tested.

Interaction of B_{2a} with proteins. The binding of B_{2a} with various proteins at 6° and pH 7·5 was demonstrated by the equilibrium dialysis technique. Under these conditions, approximately 2, 3, 4 and 13 moles of B_{2a} were bound to 1 mole of lysozyme, DNase I, ovalbumin and BSA respectively. All of the bound B_{2a} was readily extracted with chloroform from all proteins tested except for BSA where 3–4 moles of B_{2a} was still bound to the protein. Interaction of B_{2a} with these proteins was inhibited by the presence of excess amounts of glycine in the dialysis solution.

In order to obtain a more stable B_{2a}–protein conjugate, the solutions were also reduced with $NaBH_4$. Under the conditions used, the degree of interaction was higher than that obtained from equilibrium dialysis. Approximately 8, 9 and 12 moles of B_{2a} were covalently bound to 1 mole of ovalbumin, DNase and BSA respectively. Reduction of the ovalbumin–B_{2a} mixture with ^3H-$NaBH_4$ resulted in a radioactive ovalbumin–B_{2a} adduct ($6{\cdot}62 \times 10^5$ cpm/μmole), whereas the control mixture (no B_{2a} present) did not show any radioactivity. The reduced B_{2a}–DNase adduct was completely inactive compared to the control mixture which retained its activity.

Spectrophotometric analysis of the reduced B_{2a}–protein adducts revealed that the phenolic group of the B_{2a} was not involved in the interaction. The adducts showed a bathochromic shift in basic solutions comparable to that of B_{2a} (from 345–350 nm to 395–400 nm at pH 8·0).

The effect of pH on the interaction of B_{2a} with protein was clearly demonstrated by the capability of interaction of B_{2a} with lysozyme and ovalbumin at different pH values. As the pH value of the reaction mixture increased, the degree of interaction was also increased, as shown in Fig. 6. The reaction appeared to be completed at pH 10·0. The degree of interaction followed a pattern similar to the degree of dissociation of the phenolic group of B_{2a}. Reduced B_{2a} did not react with either protein at pH 8·0, as judged fluorometrically.

DISCUSSION

In the present study, interaction of B_{2a} with amino acids and proteins has been demonstrated not only by the finding that amino acids or proteins induced a significant change in the B_{2a} spectrum and the dissociation constant of the phenolic group of B_{2a}, but also by the separation of a B_{2a} amino acid complex, i.e. $B_{2a}(phe)_2$ from the reactants in CMC-TLC as well as by the binding of B_{2a} with proteins in equilibrium dialysis. The $B_{2a}(phe)_2$ complex appeared to be relatively stable in neutral or slightly alkaline conditions,

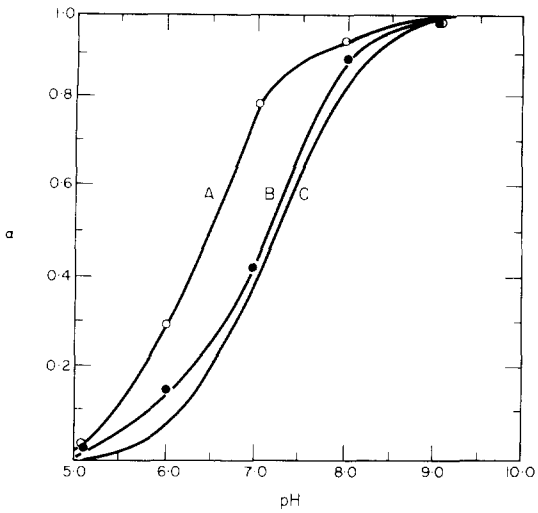

Fig. 6. Effect of pH on the interaction of B_{2a} with lysozyme (curve A) and ovalbumin (curve B). α Represents either the degree of dissociation of the phenolic group B_{2a} (curve C) calculated according to the method described by Chu [13] or the degree of binding of B_{2a} to lysozyme or ovalbumin (α = amount of B_{2a} bound to protein/total B_{2a}).

but was unstable in acid. Although the apparent equilibrium constant of $B_{2a}(phe)_2$ was estimated as $1{\cdot}82 \times 10^7$ liter M^{-1}, the actual equilibrium constant in solution would be higher, since binding of the reactants and the complex with the absorbents may have occurred. Interaction of other amino acids with B_{2a} may give similar equilibrium constants under identical conditions.

The involvement of the amino groups in the interaction between amino acids and B_{2a} was evident from the following observations: (1) neither the complex nor the reduced adduct gave a ninhydrin reaction; (2) neither the B_{2a} spectrum nor the dissociation constant of the phenolic group in B_{2a} was altered in the presence of *N*-acetylglycine; and (3) binding of B_{2a} with protein in the equilibrium dialysis was inhibited in the presence of glycine because of competition for the ligand. The amino acid proline failed to interact with B_{2a}, indicating that the imino group was not involved in the reaction. Our results agree with the observations reported by Patterson and Roberts [7].

Interaction of B_{2a} with amino acids or proteins is based on the hypothesis that B_{2a} undergoes a structural change in basic conditions to form a phenolate ion with two free aldehyde groups [3]. The importance of the phenolate ion structure of B_{2a} was demonstrated by the finding that the increase in binding of B_{2a} to lysozyme or ovalbumin followed a pattern similar to the degree of dissociation of B_{2a} (Fig. 6). However, the phenolic group of B_{2a} was not involved in the interaction because the isolated adducts still exhibited a bathochromic shift (from 345–350 nm in acidic to 395–400 nm in basic solutions), a characteristic of phenolic group dissociation. The relevance of the aldehyde groups in the phenolate form of B_{2a} in the interaction was shown from the fact that reduced B_{2a}, in which aldehyde groups were reduced by $NaBH_4$ to hydroxyl groups,* failed to interact with amino acids or proteins as shown from the spectrophotometric titrations (in which glycine did not alter the p*K* of reduced B_{2a}; Table 1) and from TLC and fluorometric analyses. Thus the present data indicate that amino groups of amino acids or proteins and aldehyde groups of B_{2a}, in the phenolate ion form, are essential for the interaction between these compounds. Since it has been shown that 2 moles of phe had reacted with 1 mole of B_{2a}, both aldehyde groups must be involved in the interaction.

Interaction of amino groups with aldehydes has often been interpreted as formation of a Schiff base. Classically, the existence of such a base is proved by the formation of a more stable covalent adduct upon reduction with a mild reducing reagent such as $NaBH_4$ [14]. Thus, for example, reductive alkylation has been frequently used in the modification of amino groups in proteins by reaction of the proteins with formaldehyde followed by reduction with $NaBH_4$ [15]. Reduction of solutions containing amino acids or proteins and B_{2a} in the present study has indeed resulted in the formation of a stable adduct. Although we have unsuccessfully tried to determine the molecular weight by mass spectroscopy, labeled experiments

* S. H. Ashoor and F. S. Chu, manuscript in preparation.

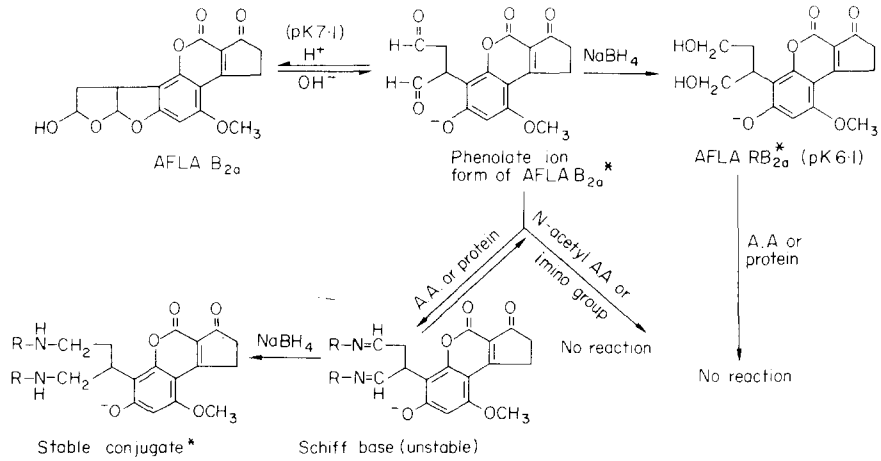

Fig. 7. Proposed mechanism for the interaction of B_{2a} with amino acids or proteins. The asterisk indicates compounds that exhibit a bathochromic shift.

with ^{14}C-phe showed that the adducts contained 2 moles of phe per mole of B_{2a}. With the available evidence reported in the present investigation, the interaction of amino acids or proteins with B_{2a} would be consistent with the mechanism shown in Fig. 7.

Modification of amino groups in proteins has been shown to alter the biological function of certain enzymes [15]. Although it has been found that B_{2a} is less toxic than B_1 [3], the biological significance of the interaction of B_{2a} with proteins or amino acids cannot be overlooked because it has been found that B_{2a} is one of the major B_1 metabolites [5, 6]. It is possible that B_{2a} reacts with certain protein(s) or enzymes at the cellular level. Although B_{2a} is shown to be less toxic than B_1 by either injection or feeding, thus causing nonspecific interaction with proteins or amino acids, manifestation of the interaction of B_{2a} with cellular enzyme(s) right after the conversion of B_1 to B_{2a} may play a role in aflatoxicosis. For example, it has been reported that B_1 was metabolized to B_{2a} which was subsequently bound to the microsomes [16]. Interaction of B_{2a} with DNase *in vitro* has led to the inactivation of the enzyme [8]; however, it is not known whether inactivation was due to the interaction of B_{2a} with amino group in the active center of the enzyme, or the alteration of the enzyme gross conformation. Considerable evidence regarding the possible formation of an active B_1 derivative, i.e. B_1-2,3-oxide, has been presented by Miller, Garner *et al.* [17 19] recently. It has been suggested that this hypothetical epoxide could then either be reacted with macromolecules or subsequently be reduced to B_{2a}. The significance of the interaction of the hypothetical aflatoxin epoxide with macromolecules and the actual role of the interaction of B_{2a} in systems *in vivo* warrant further investigation.

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