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Biochemistry

Tryptophan analogues form adducts by cooperative reaction with aldehydes and alcohols or with aldehydes alone: Possible role in ethanol toxicity

[mixed acetals/hydroxyethylation/indole/bis(indolyl)ethane]

JAMES E. AUSTIN AND HEINZ FRAENKEL-CONRAT*

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

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ABSTRACT **Previous work showed that the exocyclic amino groups of nucleic acid components react quickly at ambient temperature with acetaldehyde and ethanol to yield mixed acetals [R-NH-CH(CH_3)-O-C_2H_5]. We now find that the same type of reaction occurs readily with the nitrogen of 3-substituted indoles (e.g., indole-3-acetic acid and *N*-acetyltryptophan), analogues of the amino acid tryptophan. In contrast, unsubstituted indole reacts very rapidly at the carbon in ring position 2 or 3 with acetaldehyde to form bis(indolyl)ethane without ethanol entering into the reaction. Product structures have been confirmed by fast atom bombardment MS and ^1H NMR. The former reaction occurs optimally in 30–50% aqueous solution below pH 4. It also proceeds more slowly and with reduced yields in aqueous media at more neutral pH. This reaction may be of biological concern, as it supplies a mechanism for protein modifications with possible toxic effects in human tissues where ethanol is metabolized.**

Acetaldehyde reacts readily under physiological conditions with the exocyclic amino groups of nucleosides, and this labile adduct becomes stabilized by condensation with ethanol (1). We have initiated an investigation to establish whether the same ternary reaction occurs with protein amino groups. While the nucleoside reaction was suspected of being mutagenic, there are grounds to believe that such protein modifications might contribute to the toxicity of ethanol.

Primary amino groups (-NH_2), such as the N-terminal and lysine ε-amino groups of proteins, react rapidly with acetaldehyde to form Schiff bases [-N=CH(CH_3)], which are unable to react further with alcohols. This well-known reaction has received considerable attention from researchers studying ethanol-induced hepatic injury and toxicity (2–5) but is distinct from the cooperative reaction discussed here. Our research focused instead on the secondary amino groups (>NH) that are present in proteins in the imidazole group of histidine, the indole group of tryptophan, and N-terminal proline. To avoid the formation of multiple products through Schiff base formation at the primary amino group of free amino acids, we used *N*-acetyltryptophan and indole-3-acetic acid (IAA) as models for tryptophan in proteins.

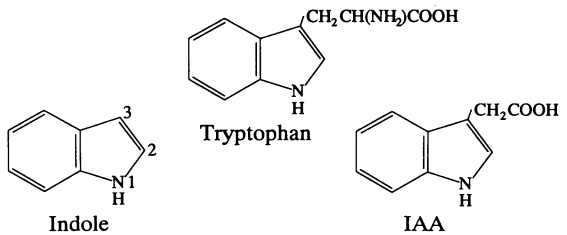

Table 1. Formation of IAA N-acetal

Time, hr	Acetal formed, %			
	80% water	50% water	33% water	10% water
0.5	5	3	3	—
1	12	14	11	—
2	17	27	—	—
24	26	59	62	15

Reaction mixtures contained about 3 mM IAA and equal amounts of acetaldehyde and ethanol. Product was detected after 3 days in reaction mixtures containing as much as 99.9% water buffered to neutral pH.

Both of these compounds yielded reaction products when treated simultaneously with acetaldehyde and ethanol. No products were detected when either of these reagents was used alone. Structural analysis by fast atom bombardment (FAB) MS and ^1H NMR determined that it was on the >NH group, rather than the neighboring ≥CH group, that condensation with the primary aldehyde took place, followed by that with the alcohol to produce stable derivatives:

$$\text{IAA} + CH_3CHO \rightarrow \text{IAA}{>}\text{N-CH}(CH_3)\text{-OH} + C_2H_5OH$$

$$\rightarrow \text{IAA}{>}\text{N-CH}(CH_3)\text{-O-}C_2H_5$$

When attempting to use unsubstituted indole for the same purpose, we found rapid formation of a product that precipitated in aqueous reaction mixtures. MS data support a proposed bis(indole) structure of this product formed by primary addition of aldehydes to the indole ring, producing an -OH group that readily reacts with a second indole to yield a bis(indolyl)alkane (see Fig. 5 *Inset*).

METHODS AND MATERIALS

All reagents were commercial preparations.

TLC was done on silica plates (Eastman Kodak 13181) with 1-butanol/ethanol/water, 80:10:25 (vol/vol), as solvent.

UV spectra were obtained from a Varian Cary 219 spectrophotometer.

HPLC separations of eluates or total reaction mixtures were performed on a Beckman Ultrasphere 5-μm ODS semipreparative column (250 × 10 mm) with a mobile phase of 75% methanol/25% water and a flow rate of 2 ml/min at ambient temperature. A Hewlett Packard diode-array detector and Chemstation were used for data collection and analysis.

Positive-ion FAB (FAB+) MS was conducted on a Kratos MS-50 mass spectrometer.

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Abbreviations: IAA, indole-3-acetic acid; FAB+, positive-ion fast atom bombardment.

*To whom reprint requests should be addressed.

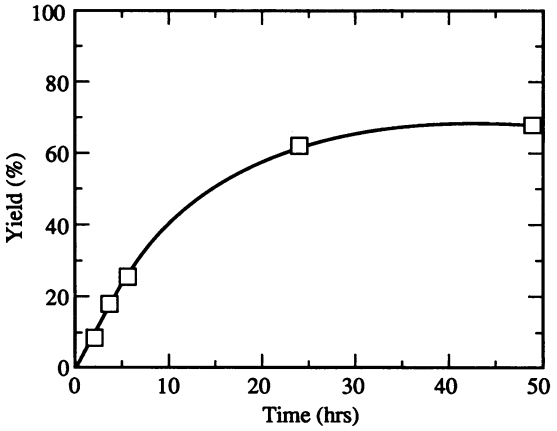

FIG. 1. Reaction rate and product yield for 3 mM IAA treated with acetaldehyde/ethanol/water (1:1:1) at pH 3.

^{1}H NMR spectra were recorded for a 1-mg/ml solution of the IAA adduct in acetone-*d6* (99.96%; Cambridge Isotope Laboratories, Cambridge, MA) on a Bruker AM 500 spectrometer. Spectra were referenced to the residual acetone-*d5* peak set to 2.04 ppm. For the TOCSY (total correlated spectroscopy) experiment, a sweep width of 4545 Hz was used and a 2048 × 128 data matrix was collected using two scans per increment. F1 was zero-filled to 256 points prior to transformation. The mixing time was 13 ms (6).

RESULTS

Reaction of IAA and *N*-Acetyltryptophan with Acetaldehyde and Ethanol. Reaction mixtures consisting of 0.1% IAA in equal amounts of acetaldehyde, ethanol, and water were allowed to react for 18 hr at ambient temperature (22–25°C). TLC clearly separated spots of similar UV absorbance at 5 and 6 cm from the origin. When dilute [^{14}C]ethanol was used, the radiolabel was detected only in the spot at 6 cm. When acetaldehyde was omitted, no spot was seen at 6 cm. An HPLC system was also able to separate the two components

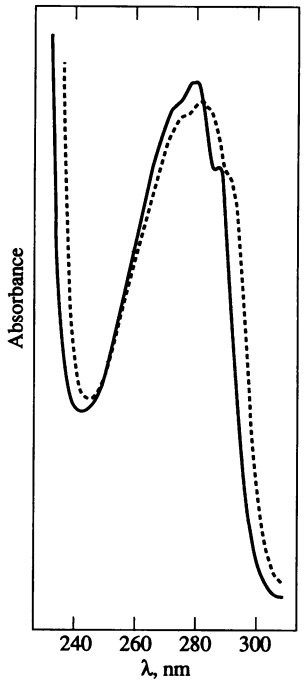

FIG. 2. UV spectra at neutral pH of IAA (——) and its N-acetal adduct (- - -). Their molar extinction coefficients are about 6000.

of this reaction mixture. IAA had a retention time of 7 min compared with 10 min for its acetaldehyde/ethanol product.

Reaction rates and product yields were determined by HPLC analysis of reaction mixtures of varying composition. Data shown are for reactions performed at approximately pH 4, a pH resulting from the acetaldehyde (Table 1; Fig. 1). Aqueous reaction mixtures with pH from 5 to 7.4 and containing as much as 99.9% water also yielded small amounts of product after a few days.

Reaction mixtures were also prepared using 1-, 3-, and 4-carbon-chain aldehydes and alcohols. After 24 hr of reaction at ambient temperature, these alternative mixtures were

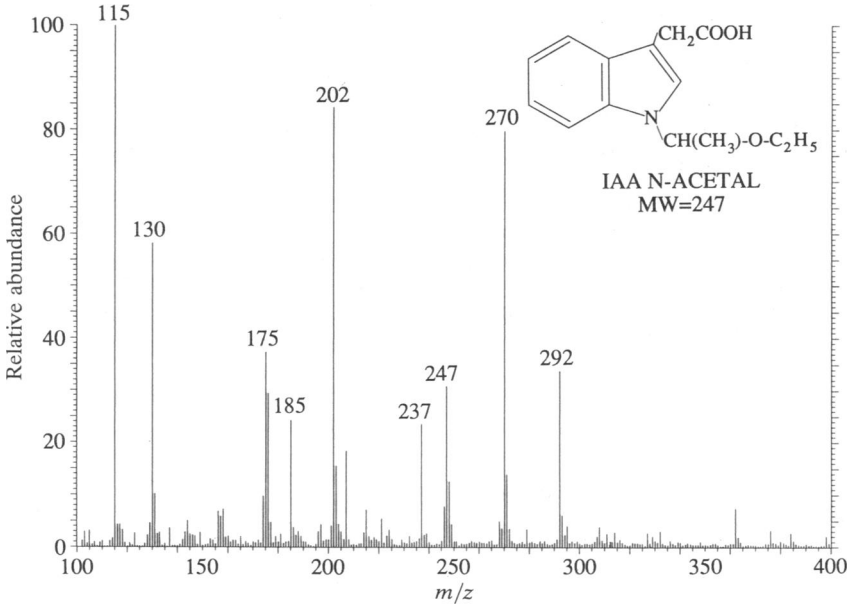

FIG. 3. FAB+ MS of the IAA N-acetal in a thioglycerol/glycerol matrix. The parent ion (M^+) has m/z 247. The m/z 270 and 292 peaks represent the addition of sodium ions present in the matrix to the parent ion, $(M + Na)^+$ and $(M + 2Na - H)^+$, respectively. Three other peaks represent fragments of the parent: 202, $(M - COOH)^+$; 175, IAA^+; 130, $(IAA - COOH)^+$. The remaining major peaks are characteristic of the matrix. MW, molecular weight.

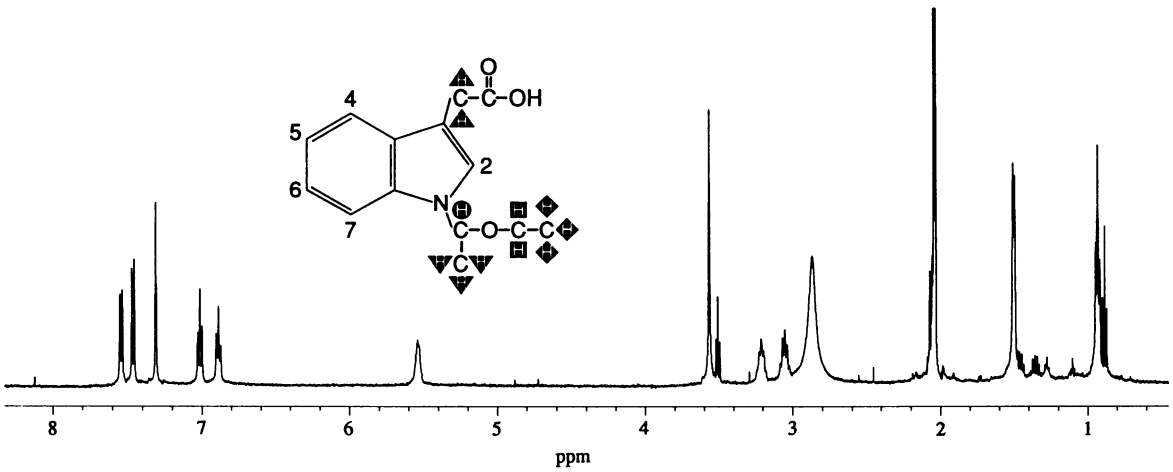

FIG. 4. ^{1}H NMR spectral data confirming the proposed structure of the IAA N-acetal adduct. Assignments for the one-dimensional spectrum shown here are as follows: from left to right, ring protons at positions C-7 and C-4 (doublets), C-2 (singlet), C-6 and C-5 (triplets) and exocyclic protons ● (quartet), ▲ (singlet), ■ (pair of quintets), water (2.85 ppm), residual acetone (2.04 ppm), ▼ (doublet) and ◆ (triplet). A long-range coupling between the C-2 proton and the ▲ methylene protons (average $^4J_{H-H}$ = 0.45 Hz) was detected, since decoupling at ▲ resulted in a sharpening of the C-2 proton singlet resonance of about 0.9 Hz. Addition of excess ^2H_2O to the sample resulted in no loss of intensity of the resonance at 7.31 ppm, confirming it to be a C-bound proton rather than an exchangeable N-bound proton. Other assignments were confirmed by a two-dimensional TOCSY (total correlated spectroscopy) experiment with a short mixing time.

analyzed on the same HPLC system. Reaction products (data not shown) were detected in all cases, except when the aldehyde was formaldehyde,† and product retention time increased along with the length of the reagents' carbon chains. The yields decreased with increasing alcohol and aldehyde chain length (methanol/acetaldehyde → butanol/butyraldehyde).

For preparative purposes, a mixture containing 20 mg of IAA in 0.5 ml of 40% water/40% ethanol/20% acetaldehyde was allowed to react at ambient temperature for 18 hr. Aliquots of this mixture were separated by TLC into the 5- and 6-cm components, which were eluted for further study. HPLC analysis determined that the product was pure with a yield of >60%. It was found to be stable in sodium citrate buffer, pH 7.4. Comparison of UV spectra showed a red shift of about 2 nm with a diminished 290-nm hump relative to IAA (Fig. 2). The molecular weight of the adduct was determined by FAB+ MS to be 247, equal to that calculated for the proposed indole-3-ethylcarboxy 1-ethyl acetal (Fig. 3).

One issue still remained to be resolved, however. Previous work had indicated that the carbon at position 2 of the five-membered ring may also be a reactive center, in some cases more reactive than the nitrogen at position 1 (8). To determine whether the observed reaction was taking place on N-1 or C-2, a series of NMR experiments was conducted. The results provided sufficient structural information to conclude that the reaction takes place on N-1 and not on C-2 (Fig. 4).

Very similar results (data not shown) were obtained with *N*-acetyltryptophan (HPLC retention time of product, 8.5 min, compared with 6 min for the starting material; yield was 50% in 18 hr with the same reagent concentration; no product was obtained with acetaldehyde alone).

Reaction of Indole with Acetaldehyde. A 0.2% solution of indole in equal amounts of water, ethanol, and acetaldehyde formed a product with 60% yield after 1 hr of reaction at ambient temperature. Omitting the ethanol (50% acetaldehyde in water mixture) had no effect. Decreasing the concentration of acetaldehyde to 0.1% increased the reaction rate and percent yield of product.

HPLC of organic reaction mixtures determined that the product had a retention time of 8 min compared with 6 min for indole. Aqueous reaction mixtures, ranging in pH from 3 to 5 with diminishing acetaldehyde concentration, quickly took on a yellow tint and in several minutes became turbid. The resulting precipitate, when redissolved in ethanol, represented almost the entire amount of UV-absorbing material, about 80% of which was the indole product. The product's UV spectrum showed a red shift of a few nanometers and some character changes (Fig. 5). Its molecular weight was

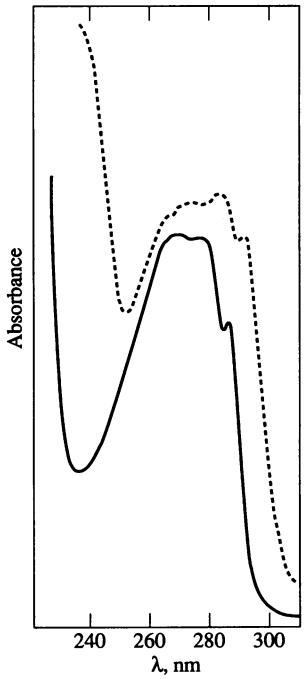

FIG. 5. UV spectra at neutral pH of indole (—) and bis(indolyl)ethane (- - -). Their molar extinction coefficients are about 6400. The overall absorbance of the product is higher due to the yellow tint of the reaction mixtures.

†It might be noted that in the course of the much earlier systematic study of the reactions of formaldehyde with proteins, 3-substituted indoles were found to yield stable *N*-methylol derivatives; this discrepancy requires further study. The dimerization of unsubstituted indoles at position C-2 or C-3 by an aldehyde bridge was first reported in the same paper (7).

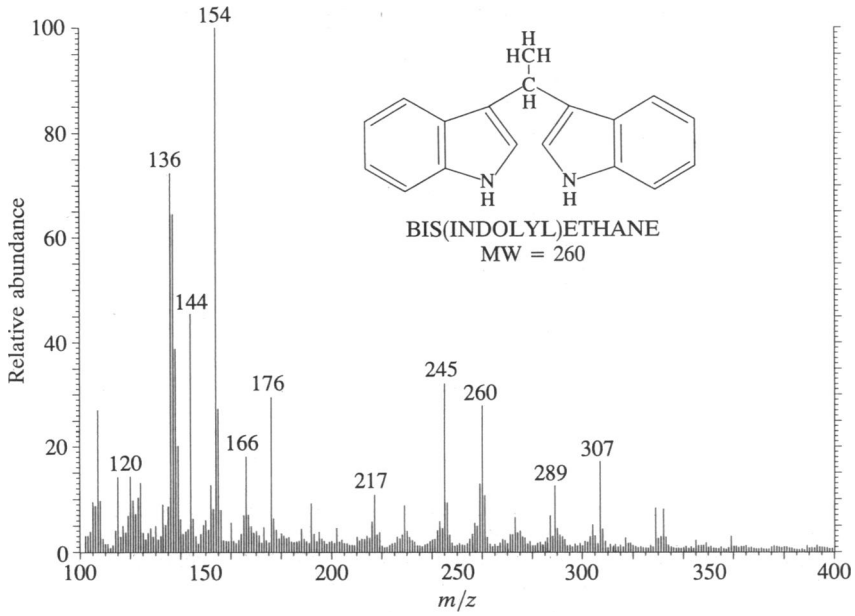

FIG. 6. FAB+ MS of bis(indolyl)ethane in a nitrobenzyl alcohol matrix. The parent ion (M^+) has m/z 260. The m/z 245 peak represents the $(M - CH_3)^+$ fragment. The m/z 144 peak represents the $(M - \text{indole})^+$ fragment. The remaining major peaks in the spectrum are characteristic of the matrix. MW, molecular weight.

determined by FAB+ MS to be 260, consistent with the proposed bis(indolyl)ethane structure (Fig. 6).

DISCUSSION

Acetaldehyde is the first metabolic product of ethanol, as well as an intermediate in other metabolic processes. It is well known that acetaldehyde readily reacts with amines, and in the case of the exocyclic nitrogens of nucleosides, the primary product [R_2>N-CH(CH_3)-OH] has a very reactive hydroxyl group, which rapidly condenses with alcohols to give stable mixed acetals [R_2>N-CH(CH_3)-O-C_2H_5] at ambient temperature (1).

We are now studying the reactivity of protein amino groups with acetaldehyde and ethanol. The strongly basic primary amino groups of lysine residues, N termini, and amino acids are known to react rapidly with acetaldehyde to produce Schiff bases [R-N=CH(CH_3)], which have been intensely studied and would not be able to interact with alcohols. However, much less is known about the reactions of the secondary ring nitrogens of tryptophan and histidine with these reagents. The present study deals with the reactions of models for tryptophan lacking its primary amino groups: IAA and indole.

It was found that IAA (as well as *N*-acetyltryptophan) reacted in the same manner as the nucleosides. Acetaldehyde alone does not give a stable derivative, but the presence of ethanol results in a condensation with that intermediate adduct stabilizing the N-C linkage, and stable mixed acetals are formed rapidly at ambient temperature in reaction mixtures below pH 4. The question of whether such substitutions occur at the >NH or the neighboring ≥CH, a recurring one in the indole literature, is settled here in favor of the >NH group by NMR analysis of the product.

Since these N-acetal adducts are stable under physiological conditions, the reaction that produces them could play a role in ethanol toxicity through the modification of tryptophan-containing proteins in human tissues where ethanol is metabolized. It would seem of interest, therefore, to investigate the reaction of acetaldehyde and ethanol with such proteins.

Indole, which lacks a substituent at the 3 position of the ring, behaves quite differently under the same conditions. It has long been known that in this heterocyclic compound the C-3 ≥CH group, and to a lesser extent the ≥CH at position 2, is more reactive than the >NH group. Thus, aldehydes are known to add preferentially to these carbons (Indole ≥C-CR_2-OH). Yet the high reactivity of both the resulting hydroxyl and the indole C-2 or C-3 position leads readily to condensation of these groups, forming bis(indolyl)alkanes even in the presence of alcohols. That the alcohol does not compete with indole for condensation with the primary addition product may be accounted for by the insolubility of the bis(indolyl)alkanes, which within minutes precipitate from the solution at ambient temperature.

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