A Chemical Adduct of Tryptophan and the Oncogen 3-Acetoxyxanthine†

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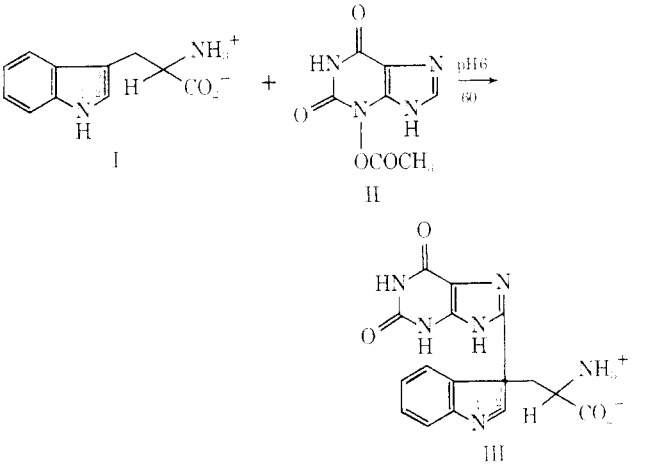
ABSTRACT: Two products of the reaction of L-tryptophan with the activated oncogen 3-acetoxyxanthine at neutral pH have been characterized as a pair of diastereoisomers with the structures of [3-(2-amino-2-carboxyethyl)-3-(8-xanthinyl)]-indolenines (structure III, Figure 1). The two isomers can be isolated together in 25% yield. Simultaneously, a portion of the tryptophan is oxidized to unidentified colored products and about 30% of the tryptophan is recovered unchanged. A metabolic experiment with [8-^{14}C]-3-hydroxyxanthine in a rat indicates that 0.25% of the total urinary radioactivity accompanies one of the isomers of III (Figure 1) in three sequential chromatographic systems.

The potent oncogen 3-hydroxyxanthine (Brown *et al.*, 1973) is activated *in vivo* to a sulfate ester (Stöhrer *et al.*, 1972) which reacts with methionine to yield 8-methylmercaptoxanthine as a urinary metabolite (Stöhrer and Brown, 1970) and 8-methylmercaptoxanthine can be released from tissue proteins (Stöhrer *et al.*, 1972). In this communication we describe two of the products of the reaction of L-tryptophan and 3-acetoxyxanthine, a chemically prepared model of the activated oncogen (Wölcke *et al.*, 1969; Birdsall *et al.*, 1971).

The reaction of the oncogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with aromatic amino acids has been studied by Poirier *et al.* (1967) and Lin *et al.* (1969). A tryptophan adduct of unknown structure was isolated *in vitro* and shown to be different from all of the polar dyes derived from liver proteins of rats after feeding of the precarcinogen (Lin *et al.*, 1969). In a reaction of quinoline *N*-oxide, which resembles the precarcinogen 4-nitroquinoline *N*-oxide, with indole in the presence of an acid chloride, Hamana and Kumadaki (1970) obtained 3-(2-quinolyl)indole. Reaction of oncogens with the aromatic amino acids of proteins could have specific affects on cellular control mechanisms as aromatic amino acids may play a role in the specific binding of proteins to DNA (Helene, 1971).

Experimental Section and Results

To a solution of 1.0 g of L-tryptophan and 3×10^6 cpm of [3-^{14}C]-DL-tryptophan (6.8×10^6 cpm/μmol) in 50 ml of H_2O (pH 7.5) was added, at 60° for 30 min in small portions with stirring, 1.6 g of acetoxyxanthine (Birdsall *et al.*, 1971), while the pH was maintained at 6.0 by the addition of 10 N NaOH. After an additional 30 min at 60°, 5 ml of Dowex 50 [H^+] was added and stirring was continued for another 30 min. The entire slurry was loaded on top of an ion exchange column (100 ml, Bio-Rad AG 50W-X8, 200–400 mesh; 1.7 mequiv/ml) that was previously equilibrated with 0.2 M NH_4OAc (pH 5.3) and eluted with that buffer. Three major fractions were eluted. The first 180 ml of eluent contained uric acid, 3-hydroxyxanthine, and brown decomposition products of tryptophan with 15–20% of the total radioactivity. The next fraction, 180–370 ml, contained both isomers of III in about 30% yield based on tryptophan, while



the unreacted tryptophan was eluted in the fraction 370–550 ml. Separation of the isomers of III was achieved on a subsequent chromatography on 105 ml of AG 50 [H^+]. Elution with 280 ml of H_2O followed by 720 ml of 1 N HCl eluted additional 3-hydroxyxanthine and xanthine. Elution with 2 N HCl yielded isomer IIIa in fraction 600–1200 ml, 2 N HCl, and isomer IIIb in fraction 1500–2200 ml, 2 N HCl. Evaporation *in vacuo* to dryness, dissolution in 5 ml of H_2O, and addition of NaOH to pH 6 yielded 0.21 g of IIIa and 0.26 g of IIIb, respectively, in crystalline form.

IIIa: Anal. Calcd for $C_{16}H_{14}N_6O_4 \cdot H_2O$: C, 51.61; H, 4.33; N, 22.7. Found: C, 51.68; H, 4.24; N, 22.89. R_F (BuOH–HOAc–H_2O, 4:1:5, asc) 0.30; R_F (tryptophan) 0.49; R_F (acetonitrile–H_2O, 3:1, asc) 0.17; R_F (tryptophan) 0.45; uv λ_{max} nm ($\epsilon \times 10^{-3}$) pH 0, 235 sh (10.0), 274 (12.3); pH 5.0, 235 sh (9.9), 275 (12.6); pH 8, 240 (12.2), 279 (12.3); pH 11, 243 (15.0), 283 (11.9); CD θ (295 nm, pH 7.4) +0.28 deg cm^2 μmol^{-1} (Figure 1).

IIIb: Anal. Found: C, 51.41; H, 4.29; N, 22.89. R_F (BuOH–HOAc–H_2O) 0.38; R_F (acetonitrile–H_2O) 0.28; uv λ_{max} nm ($\epsilon \times 10^{-3}$) pH 0, 235 sh (10.1), 274 (12.6); pH 5, 235 sh (9.8), 275 (12.8); pH 8, 241 (12.2), 279 (12.4); pH 11, 244 (15.1), 283 (11.8); CD θ (295 nm, pH 7.4) −0.30 deg cm^2 μmol^{-1}. Analytical data were obtained from nonradioactive samples derived from L-tryptophan.

† Contribution 54 in a series on Purine *N*-Oxides from the Memorial Sloan-Kettering Cancer Center, New York, New York 10021. *Received August 29, 1973.* This investigation was supported in part by funds from the American Cancer Society (Grant No. BC-32) and the National Cancer Institute (Grant No. CA 08748).

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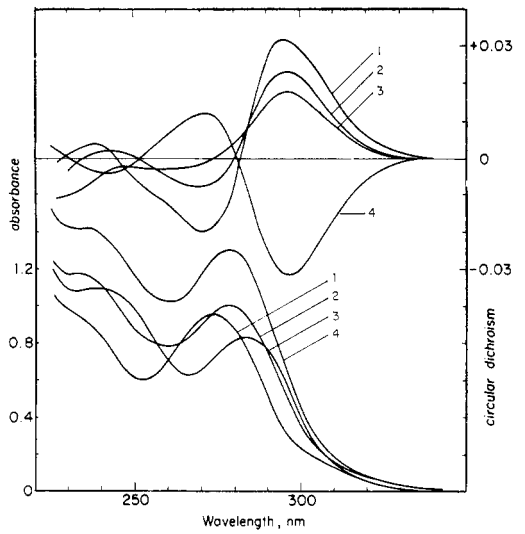

FIGURE 1: Circular dichroism (measured on a Cary 60 recording spectropolarimeter equipped with CD attachment 6002 at 23°, corrected against buffer–blank and expressed in deg cm^2 μmol^{-1}; upper set of spectra) and absorbance (lower set of spectra) for [3-(2-amino-2-carboxyethyl)-3-(8-xanthinyl)indolenines]: (1) isomer IIIa, pH 1.0; (2) isomer IIIa (pH 7.4); (3) isomer IIIa, pH 11.0 (solutions 1–3 are of the same concentration); (4) isomer IIIb, pH 7.4.

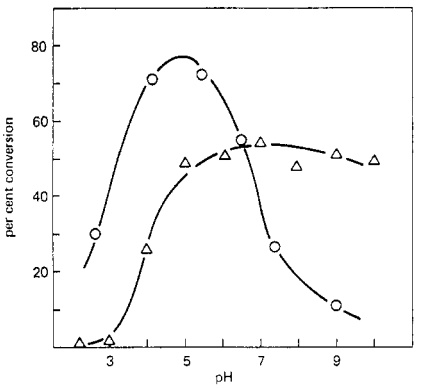

FIGURE 2: Dependence of the formation of xanthine adducts IIIa plus IIIb (○) and "tryptophan oxidation products," (△) on pH; 3 mg of 3-acetoxyxanthine dissolved in 0.05 ml of Me_2SO and 30,000 cpm of [β-^{14}C]-L-tryptophan (122 × 10^6 cpm/μmol) were reacted for 30 min at 37° in 0.3 ml of buffer (0.1 M each of H_3PO_4, HOAc, and glycine, pH adjusted with 10 N NaOH to pH values from 2.3 to 10.0). The yield of IIIa and IIIb was determined from their radioactivity after paper chromatography (BuOH–HOAc–H_2O, 5:1:4, asc.). The yield of "oxidation products" was determined in the first 7.0 ml of H_2O eluate of small 1.0-ml columns of Dowex 50 [H$^+$] over which the reaction mixture was chromatographed. A control experiment involved [^{14}C]tryptophan added to a reaction mixture of 3-acetoxyxanthine in buffer pH 7.0 after preincubation for 30 min, and was negative for both types of products.

Structures of IIIa and IIIb. Elemental analyses show that each isomer contains one moiety each of tryptophan and xanthine; the same conclusion is reached by the use of radioactive samples of each precursor. Both IIIa and IIIb give a positive ninhydrin test.

A weak, but definite Ehrlich test (Kupfer, 1964) suggests an unsubstituted C-2 in the pyrrolidine ring. High resolution nuclear magnetic resonance (nmr) spectra (Varian 220, spectra taken in Me_2SO-d_6, with Me_4Si as internal standard) are almost identical and show a side chain methylene at δ 2.73 and a methine at 3.52. Four aromatic protons in two multiplets are centered at δ 6.60 and 7.06, and a vinylic proton is at 5.83 for the H-2 of the indolenine. No absorption for an H-8 of xanthine is seen. Compounds IIIa and IIIb exhibit mirror image circular dichroism (CD) curves (Figure 1) while their uv absorbance spectra are identical. This indicates that IIIa and IIIb are a pair of diastereoisomers and that a new center of optical activity has been formed. This center at C-3 provides the only structure compatible with all the data. The multiple Cotton effect (Figure 1) is about 20 times larger in amplitude than that of tryptophan (Myar and MacDonald, 1967) where the optically active amino acid group is separated from the indole chromophore while the newly created optically active center in III is part of an asymmetric indolenine.

Reaction with Tryptophylglycine (IV). Reaction of tryptophylglycine with acetoxyxanthine under the conditions described yielded two compounds on AG 50 [H$^+$] chromatography, each with uv spectra like III. The first isomer was isolated and had the following properties. *Anal.* Calcd for $C_{18}H_{17}N_5O_5 \cdot 3H_2O$: C, 46.45; H, 4.98; N, 21.06. Found: C, 46.27; H, 4.35; N, 20.66. Uv λ_{max} nm (ϵ × 10^{-3}) pH 7.4, 242 (12.1), 279 (12.5); CD (pH 7.4) θ_{293} +0.135 deg cm^2 μmol^{-1}; θ_{268} 0; θ_{245} +0.105 deg cm^2 μmol^{-1}.

Although not perfect, the analysis shows the C/N ratio for a tryptophylglycine derivative. Retention factors on the two paper chromatographic systems previously used are almost identical with those found for IIIa but on a 10-ml Dowex 50, 2 N HCl test column IV is eluted at 95 ml while IIIa is eluted at 105 ml and IIIb at 210 ml (elution peaks).

Reaction with Indoleacetic Acid. Similarly, a product has been isolated by chromatography from the reaction of indoleacetic acid and 3-acetoxyxanthine. The product, probably a pair of enantiomers, was eluted from AG 50 [H$^+$] with H_2O (rather than with 2 N HCl as for IIIa and IV) and its uv characteristics again are like those of III.

pH Optima of Reactions. Figure 2 shows a pH optimum near 5.0 for the formation of adducts IIIa and IIIb. In the same reaction, acidic oxidation products are formed at a broad pH optimum that extends from ~4.5 to 10.0. These oxidation products, although not yet characterized, are similar to some of the colored, ninhydrin positive, products obtained by Benassi *et al.* (1965) from the oxidation of tryptophan with performic acid. A major acidic material has an R_F value on paper close to tryptophan in several systems, but there are other products which may explain the apparently greater than 100% combined conversion in the pH range 4.5–6 and less than the 100% above pH 7. The acidic products may be oxidized in the benzene ring, since benzene-aromatic character itself leads to strong binding on aromatic ion exchange resins such as Dowex 50.

Metabolic Experiments. A male Sprague-Dawley rat, 100 g, was injected i.p. with 45 × 10^6 cpm of [8-^{14}C]-3-hydroxyxanthine (43 × 10^6 cpm/μmol). After 14 hr the urine, collected cold under toluene, contained 22 × 10^6 cpm of radioactivity. The total urine plus nonradioactive markers of both IIIa and IIIb were chromatographed over 5 ml of Dowex 50 [H$^+$] with 2 N HCl, while both uv absorbance and radioactivity were monitored in the effluent (technique and apparatus, Stöhrer (1972)). One per cent of the radioactivity was eluted coincident with the marker compound IIIa. Subsequent paper chromatography with BuOH–HOAc–H_2O showed that this fraction consisted of several radioactive component. A quarter of the total activity on the paper chromatogram, corresponding to 0.25% of the total injected, coincided with the marker, and on subsequent paper chromatography with 3% ammonium bicarbonate as solvent (R_F IIIa 0.80) that amount again coincided with the marker.

Discussion

The reaction described gives a comparatively high yield of a xanthine adduct with tryptophan. The structure is drastically altered from the flat indole system to a highly three-dimensional indolenine, which has an analogy to the indolenine alkaloids condifoline and tubifoline (Klyne *et al.*, 1966). In parallel to this substitution reaction several other products, probably oxidation products resulting from the oxidative reactivity of 3-acetoxyxanthine (G. Stöhrer, in preparation), are formed from tryptophan. The formation of these so far uncharacterized products is favored at physiological pH (Figure 2).

Preliminary metabolic experiments showed that compounds IIIa and IIIb cannot be found in alkaline protein digests, but they are not stable under these conditions. The results show that IIIa may be present in the urine to an extent of not over 0.25% of the total radioactivity, but this cannot yet be confirmed by a suitable derivatization.

Acknowledgments

The authors wish to thank Dr. M. Sonnenberg for advice with the CD measurements, Miss Carla Schiller for capable assistance, and Mr. Dieter Ziegler, Rockefeller University, for the 220-Hz nmr spectra.

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Adsorption of Polyadenylate and Other Polynucleotides to Unmodified Cellulose†

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ABSTRACT: Under conditions of high ionic strength, single-stranded polyadenylate (poly(A)) binds to microcrystalline cellulose or a contaminant thereof and can be eluted by lowering the ionic strength of the solution. Single-stranded DNA behaves like poly(A) in these respects but pyrimidine polyribonucleotide homopolymers do not. Likewise, poly(A)·poly(U) hybrids have no affinity for cellulose preparations. In high ionic strength solution excess poly(U) removes most of the bound poly(A) from the poly(A)–cellulose complex but does not affect the bound single-stranded DNA. Poly(U) can be used to obtain substantial separation of poly(A)-containing RNA from nucleic acid mixtures which contain single-stranded DNA by selectively eluting the poly(A)-containing material from the cellulose-bound mixture of the two. An explanation of the binding phenomenon is proposed.

In high ionic strength solution polyadenylate (poly(A)) and other single-stranded purine polyribonucleotide homopolymers adsorb to unmodified and certain partially esterified celluloses. At pH values greater than 6.2 they can be eluted from the adsorbing surface by decreasing the ionic strength of the environment (Sullivan and Roberts, 1971, 1973; Kitos *et al.*, 1972; DeLarco and Guroff, 1973; Lee *et al.*, 1971; Edmonds *et al.*, 1971). Under similar conditions DNA also binds to cellulose and certain of its derivatives (Nygaard and Hall, 1964; Gillespie, 1968; Kitos *et al.*, 1972). Pyrimidine ribonucleotide homopolymers and heteropolymeric RNA adsorb either poorly or not at all (Kitos *et al.*, 1972).

Poly(dT)–cellulose (Edmonds *et al.*, 1971; Aviv and Leder, 1972; Swan *et al.*, 1972), Millipore filters (Lee *et al.*, 1971; Rosenfeld *et al.*, 1972; Yogo and Winner, 1972), and unesterified cellulose (Sullivan and Roberts, 1971, 1973; Kitos *et al.*, 1972; Schutz *et al.*, 1972, 1973) have been used for isolating poly(A)-containing RNAs from complex mixtures. Nitrocellulose membranes have been used extensively in hybridization studies (Gillespie and Spiegelman, 1965; Gillespie,

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