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Adduct of Tyrosine and the Oncogen 3-Acetoxyxanthine†

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ABSTRACT: A major product of the reaction of L-tyrosine (I) with the activated oncogen 3-acetoxyxanthine (II) at neutral pH has been characterized as 8-xanthinyl-4′-*O*-tyrosine (III). A similar product is formed with tyrosylglycine. Another major product was the hydantoin of tyrosine. 8-Xanthinyl-4′-*O*-tyrosine is hydrolyzed in acid to yield tyrosine and uric acid but is stable in base. The synthesis of 8-phenoxyxanthine is also described. A metabolite identical with III in several chromatographic systems was found in the urine of two rats after administration of radioactive 3-hydroxyxanthine. This represented about 0.05% of the urinary radioactivity.

The potent oncogen 3-hydroxyxanthine (Brown et al., 1973) is activated in vivo to a sulfate ester (Stöhrer et al., 1972) which reacts with cellular nucleophiles and is believed to be the proximate oncogen. This active ester reacts with methionine (Stöhrer and Brown, 1970) and tryptophan (Stöhrer et al., 1973) forming xanthine-substituted diastereoisomers with the latter. In this communication we describe two products from the reaction of 3-acetoxyxanthine with tyrosine. The reaction of the oncogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with aromatic amino acids has been studied by Poirier et al. (1967). Those authors isolated and characterized two adducts of tyrosine and the carcinogen. Both structures, based on color tests, contain linkages to the 3-carbon on tyrosine and are in that respect different from the tyrosine–xanthine adduct described here.

Experimental Section

8-Xanthinyl-4′-O-tyrosine (III). L-Tyrosine (500 mg, 2.75 mmol) was dissolved in 10 ml of H_2O (80 °C) with the addition of 10 N NaOH to bring the pH to 8.0. Acetoxyxanthine (1.0 g, 3.7 mmol) (Birdsall et al., 1971) was then added in portions over a period of 10 min. The solution was stirred and the pH was kept at 8.0 by the continued addition of 10 N NaOH. After completion of the reaction, 10 ml of a slurry of Sulfopropyl-Sephadex C-25 (Pharmacia Fine Chemicals, Piscataway, New Jersey), equilibrated with 0.1 N HCl, was added and stirred for another 10 min, after which the entire slurry was placed on top of a column containing 200 ml of Sulfopropyl-Sephadex. Elution with 0.1 N HCl yielded 0.72 mmol of uric acid in the eluate fraction 175–350 ml, 0.93 mmol of 3-hydroxyxanthine in the fraction 350–600 ml, an undetermined amount of xanthine together with about 1.6 mmol of unreacted tyrosine in the fraction 690–1070 ml, and 0.8 mmol of III in the fraction 1070–1700 ml. This fraction was concentrated in vacuo and re-chromatographed as above. It was then concentrated again and chromatographed over 100 ml of Dowex 1 formate. Elution with 0.1 N formic acid yielded an unknown in the fraction 91–125 ml and pure III in the fraction 185–600 ml. This fraction after evaporation and recrystallization from 0.1 N formic acid yielded 67 mg of needles of III.

Anal. Calcd for $C_{14}H_{13}N_5O_5 \cdot H_2O$: C, 48.14; H, 4.33; N, 20.05. Found: C, 48.04; H, 4.19; N, 19.91.

8-Xanthinyl-4′-O-tyrosylglycine (V). Tyrosylglycine (500 mg, 1.95 mmol) was dissolved in 12 ml of H_2O and reacted with 2 g of acetoxyxanthine as above, but at 37 °C. Workup was as above but the product (0.25 mmol) was eluted in the fraction 1170–1800 ml on the C-25 column. Re-chromatography on C-25 and finally Dowex 1 formate gave 65 mg of a yellow powder.

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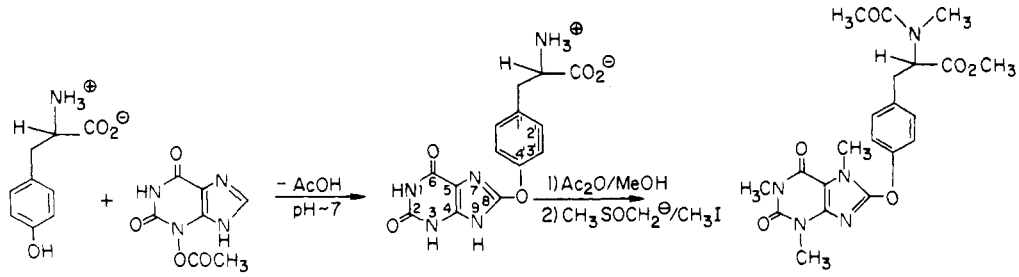

I II III IV

Anal. Calcd for $C_{16}H_{16}N_6O_6 \cdot 4.5H_2O$: C, 40.94; H, 5.36; N, 17.90. Found: C, 40.92; H, 5.09; N, 17.85.

8-Phenoxyxanthine (VI). 3-Acetoxyxanthine (100 mg) was added in portions to 0.5 ml of liquefied phenol in 2 ml of H_2O, the pH was adjusted to 8.0 with NaOH, and the solution was stirred at 80 °C. After cooling, the solution was mixed with 1 ml of a slurry of Dowex 50, H^+, 200–400 mesh, X8 ion-exchange resin (Bio-Rad Laboratories, Rockville Centre, New York), and the mixture was placed on top of a column containing 50 ml of this resin. Elution with 1.1 N HCl yielded 0.017 mmol of uric acid in the eluate fraction 38–70 ml, phenol containing an undetermined amount of 3-hydroxyxanthine in fraction 115–215 ml, 0.190 mmol of xanthine in fraction 250–300 ml, and 0.193 mmol of 8-phenoxyxanthine in fraction 440–650 ml. The last fraction was evaporated in vacuo and the flask was dried in a desiccator over KOH. The residue was dissolved in 2 ml of hot water, filtered, and neutralized with NaOH to pH 6 to yield 23 mg of white crystals.

Anal. Calcd for $C_{11}H_8N_4O_3 \cdot H_2O$: C, 50.38; H, 3.84; N, 21.37. Found: C, 50.74; H, 3.57; N, 21.69.

4-Hydroxybenzylhydantoin (VII). The uric acid fraction from the chromatography of III as described above was evaporated and was re-chromatographed on 80 ml of Sephadex A-25 equilibrated with 0.05 M tetraethylammonium acetate, pH 7.0. Elution with a gradient of 250 ml of this buffer to 250 ml of the same buffer at pH 4.5 yielded 4-hydroxybenzylhydantoin, 0.11 mmol in fraction 48–80 ml followed by uric acid in fraction 320–350 ml. The fraction containing VII was evaporated in vacuo and recrystallized from methanol-H_2O to yield needles, mp 257 °C. Its identity was confirmed by mass, ^1H NMR, and uv spectra and chromatographic comparison with the authentic material (Burrows et al., 1949).

Acetylation and Permethylation of III to IV and of Xanthine to Caffeine. Derivatization was achieved by the method of Das and Schmid (1972) which involves acetylation with acetic anhydride and methanol followed by methylation with methyl iodide and dimethyl sulfoxide anion. The reaction product was extracted with CH_2Cl_2 and the extract passed over a 1-ml column of silica gel equilibrated with ether. The oils resulting after evaporation were used for the mass spectra. Xanthine yielded caffeine under the same conditions.

Results and Discussion

The Structure of the Tyrosine–Xanthine Adduct. Elemental analysis shows that the adduct, III, is composed of one moiety each of tyrosine and xanthine. The uv spectrum is dominated by the purine portion of the molecule and is typical of an 8-alkoxyxanthine. Table I compares the uv spectra of III with those of 8-ethoxyxanthine (Birdsall et al., 1971), for which no spectra have been published, and with those of the newly synthesized 8-phenoxyxanthine.

Structural Evidence from Mass Spectra. The high molecular weight of the adduct III together with its high content of dissociable protons prevented the measurement of a conventional mass spectrum (a plasma desorption mass spectrum is described below). A derivative of III, the 1,3,7,*N'*-tetramethyl *N'*-acetyl methyl ester IV was therefore prepared. Three main features of the mass spectrum of IV are: (1) The mass of 443 (25.6%, intensity of base peak *m/e* 370) agrees with structure IV. (2) A part of the fragmentation pattern is consistent with the formation of the ion of 1,3,7-trimethyluric acid ($M^+ = 210$, 6.9%), including such fragments as $M_{IV}^+ - CH_3N = 414$ (6.3%) and $M_{IV}^+ - CH_3NCO = 386$ (6.8%). This pattern of fragmentation has been studied with caffeine (Spiteller and Spiteller-Friedmann, 1962), and we have confirmed it with the methylation product of xanthine. (3) Fragmentation in the amino acid moiety is seen by fragments $M_{IV}^+ - [CH_3CO - N - CH_3] = 371$ (40.9%), $M_{IV}^+ - [CH_3CONH - CH_3] = 370$ (100%), and $M_{IV}^+ - [CH_3OCO - CH - NCH_3 - COCH_3] = 299$ (30.5%).

A direct mass spectrum of III has been possible with the new technique of Californium 252 plasma desorption mass spectroscopy (Macfarlane et al., 1974; Macfarlane and Torgerson, 1976; a PDMS of III is published in the latter article) which can measure highly involatile samples. Positive and negative ion spectra can be measured with this system. The best results for III were obtained with the negative ion spectrum and the following strong peaks could be assigned:

$M^- - H + Na$	= 353
$M^- - H$	= 330
$M^- - H - H_2CO_2$	= 284
$M^- - H - H_2CO_2 - NH$	= 269
$M^- - H_2N - CH - CO_2H$	= 257
M^- – uric acid	= 163

The *m/e* 163 fragment and several related fragments in the positive ion spectrum as well as in the mass spectrum of IV indicate that the bond between the xanthine and the tyrosine

TABLE I.

	pH	λ_{max}	$\epsilon \times 10^{-3}$	λ_{min}	$\epsilon \times 10^{-3}$	p*K*
8-Ethoxy-xanthine	1.0	274	(11.0)	244	(3.65)	6.6 ± 0.4
	7.4	278	(10.6)	250	(5.2)	10.9 ± 0.4
	13.0	287.5	(10.0)	259	(4.35)	
	Isosbestic point:			253	(5.2)	
8-Phenoxy-xanthine	1.0	275	(14.95)	246	(4.45)	6.4 ± 0.3
	7.4	279	(14.30)	250	(6.10)	10.8 ± 0.3
	13.0	287	(11.10)	260	(6.20)	
	Isosbestic point:			255	(6.55)	
8-Xanthinyl-4'-*O*-tyrosine	1.0	276	(17.25)	246	(4.10)	6.0 ± 0.3
	7.4	281	(14.5)	254	(6.85)	9.8 ± 0.3
	13.0	287	(11.2)	266	(5.95)	
	Isosbestic point:			255	(6.85)	

moieties is C-8 to the phenolic oxygen. On fragmentation this oxygen remains with xanthine to form uric acid.

The same type of fragmentation is also observed with 8-phenoxyxanthine where the peak $M_{VI}^+ -$ uric acid which is phenyl = 77 (5.0%) is the second strongest fragment after $M^+ - CONH = 201$ (18.2%), and no phenol fragment is observed. The mass spectrum unequivocally assigns the bond between xanthine and tyrosine as an ether bond as in 8-phenoxyxanthine.

Proton Magnetic Resonance Spectrum. The ^1H NMR spectrum of III is very similar to that of tyrosine with only subtle changes of the chemical shifts and the coupling constants of the AA'BB' system of the four aromatic protons. This also rules out any substitution of a carbon-bound proton as is involved in the structures for the tyrosine–MAB adducts proposed by Lin et al. (1969). Those structures were based chiefly on the positive folin tests given by both tyrosine–MAB adducts. However, tyrosine–xanthine also gives a positive Folin test even though no free phenolic group is present.

pH Dependence of the Reaction. There is an optimal pH for the formation of III, around 5.2, as determined by paper chromatography. At that pH value, 21% of III was formed in a micro-reaction containing 0.65 mg of II, 10^4 cpm of D,L-[1-^{14}C]tyrosine (7.3 mCi/mmole) reacted for 10 min at 37 °C in 0.3 ml of a buffer 0.1 molar with respect to H_3PO_4, H_3BO_3, and CH_3CO_2H, and adjusted with 10 N NaOH to pH values between 2.5 and 11.0. The preparative reaction was done at a higher pH solely for reasons of solubility. At pH's over 8, formation of one or more unknown products exceed the formation of III.

Hydrolysis of Xanthinyltyrosine (III) and Xanthinyltyrosylglycine (V). Xanthinyltyrosine, III, was dissolved in 6 N HCl, the solution was heated to 100 °C in a quartz cuvette, and spectra were taken every 5 min. The uv absorption maximum at 270 nm rose slightly (~5%) during 40 min while shifting to 272 nm. After about 45 min, the absorption at 272 increased by another 20% and then slowly decayed. These unexpected spectral changes are attributed to the uric acid formed as an intermediate in the hydrolysis. Uric acid, under the same conditions of hydrolysis, also displayed a similar biphasic spectral change. Its absorption maximum at 283 nm also increased slightly for about 10–20 min and then grew by 50% while shifting to 273 nm in the next 10 min. The moment of change from the slow to the fast reaction was not reproducible. Attempts to isolate a product were without success and the identity of that product remains unknown.

The hydrolysis of the phenol ether bond relative to the hydrolysis of the peptide bond was studied with the tyrosylglycine derivative, V. The hydrolysis procedure chosen was that of Liu and Chang (1971). Hydrolysis for 7.5 h resulted in 95% cleavage of the peptide bond and was complete after 24 h. Even after 7.5 h, the phenol ether bond was completely hydrolyzed and the uric acid further hydrolyzed as has been noted for purines (Salser and Balis, 1967). The hydrolysis of V thus yielded 2 equiv of glycine and 1 equiv of tyrosine.

The phenol ether bond of III has so far proved resistant to basic hydrolysis and basic hydrolysis or enzymatic hydrolysis may be the route to analyze proteins containing tyrosine-bound xanthine moieties.

The Origin of 4-Hydroxybenzylhydantoin. CD spectra indicate that the material still has the L configuration. It was also formed in both preparative and analytical runs using radioactive D,L-tyrosine. A 50-mg sample of tyrosine did not contain any hydantoin when chromatographed over SP-25. We do not know how this product arises except that the cyclic urea moiety is not derived from the 8-^{14}C of 3-acetoxyxanthine. In a recent paper we have shown that uric acid is oxidatively degraded by 3-acetoxyxanthine (Stöhrer and Salemnick, 1975). This and the acetylating activity of 3-acetoxyxanthine (Stöhrer and Salemnick, 1975) could conceivably lead to the formation of a hydantoin.

Metabolic Experiments. Two male Sprague-Dawley rats (200 g) were injected intraperitoneally with 8.5×10^6 cpm of [8-^{14}C]-3-hydroxyxanthine (42×10^6 cpm/μmol). After 17 h the urine contained 80–90% of the injected radioactivity. Portions of the urines, mixed with uv-marker compounds, were then chromatographed on a column containing 41 ml of SP-25 equilibrated and eluted with 0.1 N HCl. A peak of radioactivity, coinciding symmetrically with marker tyrosine–xanthine (III), was eluted between 205 and 265 ml of eluate. This amounted to 0.11 and 0.24% of the urinary radioactivity. On paper chromatography in system A (1-butanol–acetic acid–water, 4:1:5, ascending), this material was resolved into two peaks, corresponding to 35 and 34% of total radioactivity each. The first peak (R_f 0.38) co-chromatographed with added tyrosine–xanthine (III). After elution from the paper chromatogram, this material was acetylated in MeOH–acetic anhydride and both the uv-absorbent material and the radioactivity migrated at R_f 0.70 in system A and R_f 0.72 in system B (cellulose phosphate paper, Whatman P81, was washed with 0.1 N HCl, partially dried and developed, ascending, with 0.1 N HCl), both characteristic of *N*-acetyl-III. Another portion of the radioactive material R_f 0.38 was treated with 6 N HCl at 100 °C for 1 h. Here the radioactivity shifted to R_f 0.53 in A and R_f 0.88 in B, consistent with the acid hydrolysis of III. The second radioactive peak R_f 0.16 in system A corresponds to an unidentified product in the reaction producing III. It also formed an acetyl derivative with acetic anhydride but was stable in 6 N HCl.

Reaction of Tyrosine with Other Oncogens. As has been mentioned, tyrosine is substituted by the active ester of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Among the purine *N*-oxides, all oncogenic esters react with tyrosine while the nononcogenic esters do not. Only the products formed with 3-acetoxyxanthine are identified, but the chromatographic properties of products with other purine esters suggest similar structures. The sulfate ester of 3-hydroxyxanthine (Stöhrer and Salemnick, 1975) yields the same product as does the acetate ester.

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Purification and Translation of an Immunoglobulin λ Chain Messenger RNA from Mouse Myeloma†

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ABSTRACT: Here we describe the 500-fold purification of an mRNA encoding an immunoglobulin λ light chain derived from the mouse myeloma tumor, RPC-20. Purification involves the isolation of membrane-bound polysomes, oligo(dT)-cellulose chromatography, and sucrose gradient centrifugation under conditions favoring denaturation of polynucleotide complexes. The mRNA purified in this way directs the cell-free synthesis of a polypeptide which is five or six amino acids longer than the mature form of RPC-20 light chain. In addition to directing the synthesis of a precursor-like polypeptide, the mRNA migrates on electrophoresis as a band containing approximately 1150 nucleotides, about 500 more than required to encode the mature form of the light chain.

Considerable insight has been gained into the genetic representation of immunoglobulin light chains from studies using purified κ light chain mRNAs and their complementary reverse transcripts (Faust et al., 1974; Honjo et al., 1974; Rabbitts, 1974; Stavnezer et al., 1974). These studies indicate that the κ constant region is represented relatively few times in the mouse genome. However, because of extensive sequence diversity in the variable regions of mouse κ chains and uncertainties in predicting the extent of cross-hybridization between κ gene sequences, it has not been possible to calculate unambiguously the number of variable region sequences in the mouse genome. This is so despite several recent studies which provide important hints of a relatively small or unique number of κ variable region genes (Leder et al., 1974a,b; Tonegawa et al., 1974a,b; Rabbitts and Milstein, 1975; Rabbitts et al., 1975).

Since quantitation of the variable region gene sequences has strong predictive value in terms of distinguishing between the germ line and somatic mutation hypotheses (Gally and Edelman, 1972; Wigzell, 1973; Cohn et al., 1974; Hood et al., 1974), it remains an important research goal. In this respect, the λ class of mouse light chain offers rather special advantages (to be discussed in the following article, Honjo et al., 1976) for determining the genetic representation of variable gene sequences. In the present report we describe the purification, characterization, and translation of an mRNA encoding an immunoglobulin λ chain derived from the RPC-20 myeloma tumor. This mRNA is employed in an accompanying study for the quantitation of λ chain constant and variable region genes (Honjo et al., 1976).

Materials and Methods

(*a*) *Preparations of Myeloma Polysomes and Myeloma mRNA.* Dr. M. Potter kindly supplied the myeloma tumors, which were grown, harvested, and stored as described (Swan et al., 1972). The polysomes were prepared according to a slightly modified procedure of Swan et al. (1972). One hundred grams of dissected tumor were homogenized as described (Swan et al., 1972), except that buffer A contained 50 mM Tris-HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 10^{-4} M cycloheximide, 0.1% diethyl pyrocarbonate, and 0.88 M sucrose. The homogenate was centrifuged through a discontinuous sucrose gradient composed of 7 ml of 1.5 M sucrose and 15 ml of 1.75 M sucrose, each containing buffer A lacking diethyl pyrocarbonate. After centrifugation at 25 000 rpm (81 500*g*) for 60 min in a Beckman SW27 rotor, the polysomes, which had been concentrated in the 1.5 M sucrose layer, were harvested. The RNA was extracted, followed by two successive purifications on oligo(dT)-cellulose and two sucrose gradient centrifugations, as described by Honjo et al. (1974). The second oligo(dT)-cellulose chromatographic step was modified slightly. The column was washed with 0.25 M KCl–0.01 M Tris-HCl (pH 7.5) instead of 0.18 M KCl–0.01 M Tris-HCl (pH 7.5).

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