the carbon chain is biosynthesized using the normal fatty acid pathway; (2) the secondary hydroxyl group is put on the chain after the chain is fully synthesized *via* the hydration of a cis double bond; and (3) the fatty acid chains are chlorinated after the chains are fully synthesized.

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Enzymatic O-Methylation of *N*-Hydroxyxanthines by a Rat Kidney Enzyme†

Gerhard Stöhrer

ABSTRACT: An enzymatic activity in rat liver or kidney cytosol catalyzes methyl transfer from *S*-adenosylmethionine to the *N*-hydroxy oxygen of five out of six *N*-hydroxyxanthines tested. The methylated products were compared to authentic compounds in the case of 3-methoxyxanthine and 1-methoxyxanthine; the latter was first synthesized for the purpose of this comparison. 3-Methoxyxanthine was identified as a urinary metabolite of the oncogen 3-hydroxyxanthine in the rat.

Methylated purines, first reported as nucleic acid components and urinary metabolites 74 years ago, are now known to be the products of highly specific purine methylation of polynucleotides (Borek and Srinivasan, 1965). Since then, three instances of direct purine methylation have been reported. These reactions are N-methylation of derivatives of 2-aminopurine (Remy, 1959) by bacterial transmethylases and S-methylation of thiopurines and thiopyrimidines by an enzyme occurring in mammalian tissues (Remy, 1963). Adenine is specifically methylated to 1-methyladenine, a growth hormone in the starfish ovary (Shirai *et al.*, 1972). A *N*-hydroxyoncogen, *N*-hydroxy-2-acetylaminofluorene, is O-methylated by an enzyme from rat liver (Lotlikar, 1968). There are a few more unspecific methylations, among them N-methylation of tryptophan derivatives (Axelrod, 1971) but methylation does not seem to be a general mode of metabolism of extraneous chemicals. This paper reports an enzymatic methyltransfer reaction which is catalyzed by enzymes in the liver and the kidney of rats and for which a number of *N*-hydroxypurines, oncogenic as well as nononcogenic, are the substrates. The methylation product from the oncogen 3-hydroxyxanthine is 3-methoxyxanthine which is identical with a major urinary metabolite of 3-hydroxyxanthine in the rat.

Materials and Methods

S-Adenosyl[*methyl*-^{14}C]methionine was obtained from Amersham/Searle (specific activity 34 Ci/mol), of high radioactive purity as judged by paper chromatography in several systems.

Xanthine derivatives were prepared according to the published procedures (see Table I).

Experimental Section

Enzyme Preparations. Homogenates of rat liver or rat kidney with 4 volumes of 1.15% KCl were centrifuged for 30 min at 100,000*g* and the supernatant was passed over Sephadex G-25, previously equilibrated with 0.1 M potassium phosphate (pH 7.5), in such a way that 10 volumes of resin bed was used for each volume of supernatant. After color appeared one volume of eluent was used, either directly or after conversion into an acetone powder.

Enzyme Acetone Powder. The eluate from the Sephadex column was dropped directly into 20 volumes of acetone at −15° with stirring. The supernatant acetone was decanted after 10 min and the precipitate was stirred for another 10 min with the same amount of fresh acetone at −15°. The precipitate was quickly collected with suction, washed with ether

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previously passed over activated alumina, and transferred while it was still wet with ether to a desiccator connected to a vacuum pump.

Assay for Methylation. See legend of Table I. Micropreparative incubations contained 6 μmol of xanthine derivative, 30 mg of powder preparation of the enzyme, 4 μmol of *S*-adenosylmethionine (1 Ci/mol), and 250 μmol of KH_2PO_4 (pH 7.5) in a total of 5 ml. Incubation was carried out at 37° for 1 hr, after which the protein was precipitated by rapid boiling, followed by chilling and decantation of the supernatant solution.

Urinary Metabolites. Intraperitoneal injection of 3-hydroxyxanthine and collection and work-up of metabolites have been described (Stöhrer and Brown, 1970). For the current experiment 0.4 μmol of 3-hydroxyxanthine (25 Ci/mol) was injected in a single dose and the urine was collected after 8 hr.

Analytical Ion-Exchange Chromatography. A column (7.4 cm high, containing 5.5 ml of Dowex AG 50W-X8 minus 400 mesh; 5.1 mequiv/dry g) was eluted with 1.1 N HCl. Elution volumes were reproducible within 5%. Spectral comparisons at pH 0 and 13 were made between unknown and authentic samples after chromatography and showed the identity of all pairs of compounds thus characterized.

Preparative Ion-Exchange Chromatography. A chromatography column, 2.5 × 19 cm of the above resin, with a heating tape above the resin bed, permitted heating of about 30 ml of supernatant eluent. During elution this supernatant eluent was kept at a constant volume and at ~80°. This permitted a very high flow rate (~6 ml/min) and the handling of less soluble chemicals. The elution peaks were sharp even with high loads. The reaction mixture was adsorbed with stirring and heating on 5 ml of the resin. The loaded resin was then layered on top of the column. The effluent was monitored with a modified ultraviolet flow cell of 0.5-mm light path.

Paper Chromatography. Paper chromatography was done with Schleicher & Schuell No. 947 and 3% aqueous ammonium bicarbonate ascending. All *N*-hydroxyxanthines and their *O*-methyl derivatives have an R_F of 0.6–0.7 in this system. For adenosylmethionine, butanol–acetic acid–water (5:1:4) was used as solvent, descending.

Photochemical Deoxygenation.[1] Aqueous neutral solutions of products **1–4** containing about 1 μmol/ml were irradiated in quartz cuvets placed next to a "Spectroline" low-pressure mercury lamp. Dependent on the concentration, irradiation from 3 to 7 hr was required to deoxygenate 90% of the starting material.

Radioactivity Determinations. Liquid scintillation counting with external standard for quench correction was used throughout. For column chromatography, a Packard scintillation flow cell filled with 2 ml of anthracene was modified to eliminate all non-Teflon surfaces in contact with the eluent. The cell was installed in the flow system after the ultraviolet flow cell (Instrumentation Specialties Co., Lincoln, Neb.) and an event marker, activated by the fraction collector correlated the ultraviolet and the radioactivity charts. Paper chromatograms were scanned for radioactivity on a paper chromatogram scanner (Baird-Atomic, Inc., Valley Stream, N. Y.).

Melting points are not corrected. C, H, and N microanalyses were within, at most, 0.3% for I–III as the anhydrous free bases and IV as the monohydrochloride.

[1] Experimental conditions were based upon a personal communication from F. L. Lam and J. C. Parham.

TABLE I: Enzymatic Methylation of *N*-Hydroxypurines.[a]

Xanthine	0
3-Hydroxyxanthine[b]	5.5
1-Hydroxyxanthine[c]	25
7-Hydroxyxanthine[d]	23[e]
7-Methyl-3-hydroxyxanthine[f]	11
8-Methyl-3-hydroxyxanthine[f]	0
8-Aza-3-hydroxyxanthine[g]	4

[a] Substrate activity is expressed as per cent conversion of [^{14}C]adenosylmethionine to [^{14}C]methoxyxanthines. The incubation mixture contained in a total of 1 ml: 50 μmol of KH_2PO_4 (pH 7.5), 0.25 μCi of *S*-adenosylmethionine, 6 mg of protein, and 0.6 μmol of the xanthine derivative. Incubation at 37° was for 1 hr. [b] Wölcke and Brown (1969). [c] Parham *et al.* (1967). [d] Zvilichovsky and Brown (1972). [e] Sum of two products, one may be a derivative of uric acid. [f] Birdsall *et al.* (1971). [g] Cresswell *et al.* (1965).

Chemical Methylation of 1-Hydroxyxanthine to 1-Methoxy-3,7-dimethylxanthine (I). 1-Hydroxyxanthine (160 mg) in 2 ml of dimethylacetamide was stirred with 500 mg of powdered K_2CO_3 and 420 mg of CH_3I at room temperature. After 1 day a new product was detected by analytical chromatography and after 3 days the starting material was consumed. Upon filtration and evaporation, 90 mg of needles was obtained from methanol–ether, mp 220–222° (without decomposition).

Monomethyl Derivatives (II, III, and IV). 1-Hydroxyxanthine (450 mg) (Parham *et al.*, 1967) in 5 ml of 2 N NaOH was stirred with 1 equiv of CH_3I for 2 hr, after which time 1 more equiv of CH_3I was added. After 2 more hr the mixture was evaporated, neutralized with 1 N HCl, and again evaporated. The dry residue was extracted with methanol for 1 day in a Soxhlet extractor. The extract was evaporated and chromatographed over 80 ml of Dowex 50 and successive fractions contained: 68 mg of 1-hydroxy-3-methylxanthine (III) needles from H_2O, mp 340° (dec, gas); 190 mg of 1-methoxyxanthine (II) prisms from methanol ether, mp 305° (dec, gas); 19 mg of 1-hydroxy-9-methylxanthine (IV) needles from ethanol, mp 295° (dec, gas).

Other physical constants are given in Table II.

Results and Discussion

3-Methoxyxanthine as Urinary Metabolite in the Rat. In addition to the metabolites of the oncogen 3-hydroxyxanthine so far identified (Stöhrer and Brown, 1970), several minor and a major metabolite remained unknown. This major metabolite of 3-hydroxyxanthine, which represented about 10% of the total radioactivity excreted in the urine in the current experiment, is 3-methoxyxanthine. Figure 1 is the chromatogram of urinary metabolites of [8-^{14}C]-3-hydroxyxanthine. The radioactivity of the peak centered at 245 ml coincides with authentic 3-methoxyxanthine (Birdsall *et al.*, 1970). On the same column but with more dilute acid as eluent (0.05 N HCl), 3-hydroxyxanthine and 3-methoxyxanthine reverse their sequence of elution (Stöhrer *et al.*, 1972b). Again, 3-methoxyxanthine coincides with the radioactivity as it does on further paper chromatography.

Heat Decomposition of 3-Methoxyxanthine to Xanthine. 3-Methoxyxanthine decomposes to xanthine at 127° in the

TABLE II: Physical Data on Some Methylated Xanthines.

	Nmr δMe	δ_{H_8}	Dowex 50[a] V_e (ml)	pK	pH	λ (ε × 10^{-3})
1-Methoxy-3,7-dimethylxanthine (I)	3.43 3.86 3.90	8.02	87	−0.3	7.0	272 (9.85)
1-Methoxyxanthine (II)	3.84	8.04	35	+0.4	4.0 8.0	268 (9.9) 278 (8.3)
1-Hydroxy-3-methylxanthine (III)	3.48	8.08	28	+0.2	5.0 12.0	270 (10.1) 284 (9.9)
1-Hydroxy-9-methylxanthine (IV)	3.68	7.90	24.5	+1.4	7.0 11.0	275 (8.1) 282 (6.9)
3,7-Dimethylxanthine	3.35 3.87	7.98	52	0.3[c]		
3-Methylxanthine[b]	3.39	7.97	33	0.8[c]		
9-Methylxanthine	3.64	7.64	29.5	2.0[c]		
7-Methylxanthine	3.85	7.88	43	0.8[c]		
Xanthine		7.90	27	1.2[c]		

[a] From analytical Dowex 50 column. [b] Prepared according to L. B. Townsend and R. K. Robins (1962). [c] pK data according to Lichtenberg *et al.* (1971).

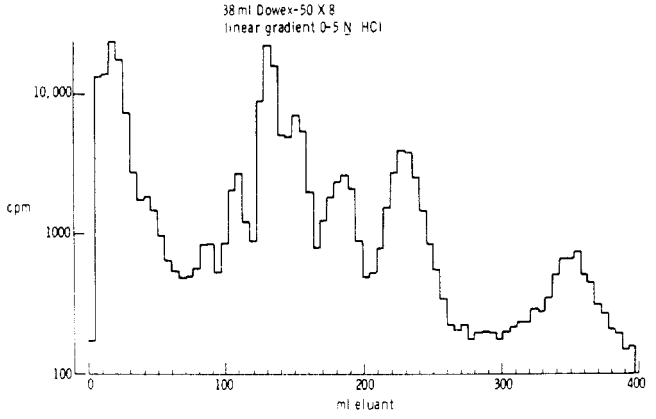

FIGURE 1: Dowex 50 chromatogram of urinary metabolites of 3-hydroxyxanthine. A column (0.9 × 50 cm, Bio-Rad AG 50W-X8, 200–400 mesh) was eluted with a linear gradient made up of 200 ml of H_2O and 200 ml of 5 N HCl.

dry state (Birdsall *et al.*, 1970) and in boiling dimethylformamide.[2] This decomposition by heat can be a specific test for the 3-methoxy substituent in 3-methoxyxanthine, since *N*-methylxanthines are stable under the conditions, and the reaction was used to further characterize the urinary metabolite. The radioactive unknown and authentic 3-methoxyxanthine were mixed and purified by column chromatography and subsequent paper chromatography. The specific activity of this diluted radioactive sample was 82,000 cpm/μmol. An aliquot of this preparation was evaporated, dissolved in 1 ml of dimethylacetamide, and heated to 110° for 1 hr, after which it was evaporated and chromatographed on 10 ml of Dowex 50 using 0.1 N HCl as eluent. Sixty per cent of the 3-methoxyxanthine had been converted into xanthine which had a specific activity of 75,000 cpm/μmol. In such experiments it was noted that low concentrations of 3-hydroxyxanthine are not completely stable in boiling dimethylformamide, although high concentrations appear to be. However, at the lower temperature used here 3-hydroxyxanthine was sufficiently stable, and in a control experiment with 3-methoxyxanthine and [8-^{14}C]-3-hydroxyxanthine the xanthine formed had, after purification, only 15% of the specific activity.

Exchange of the 8-carbon (Cavalieri and Brown, 1949; Balis, 1957) does not appear to be a significant reaction under these conditions.

Enzymatic Methylation. The transmethylating enzyme is in the cytosol of both rat liver and rat kidney, with apparently two- to fivefold higher activities in kidney. The methyl donor is *S*-adenosylmethionine and cofactors, such as divalent cations or glutathione, do not stimulate the reaction.[3] An acetone powder of the enzyme preparation, stored at 4°, retained its activity for at least a month. After incubation of 3-hydroxyxanthine with enzyme and *S*-adenosyl[*methyl*-^{14}C]methionine, a chromatogram of the reaction mixture shows two major radioactive components (Figure 2a). The first, about 20% of the total radioactivity, is an unknown nonbasic material which is not retained on Dowex 50 (H^+). On subsequent paper chromatography with 3% ammonium bicarbonate, it is resolved into two peaks with R_F 0.85 and 0.94, respectively. These apparently are products of the spontaneous breakdown of adenosylmethionine since controls with boiled enzyme showed the same result. The second radioactive peak, centered at 15 ml, is 3-methoxyxanthine and represents about 5% of the total radioactivity. Methionine, adenosylmethionine, and more basic breakdown products were not eluted under the conditions used.

Characterization of the Reaction Product. 3-Methoxyxanthine was characterized as described for the urinary metabolite. In this case the heat decomposition yielded radioactivity that could be volatilized, presumably formaldehyde. A sample with 15,200 cpm/2.3 μmol of 3-methoxyxanthine, including

[2] T.-C. Lee, private communication.

[3] We have previously noted that small molecules can be carried along with the protein fraction on a Sephadex G-25 column (Stöhrer *et al.*, 1972a) and the requirement for cofactors is thus not positively excluded.

the unlabeled 3-methoxyxanthine added as a marker in the original isolation, was dissolved in 0.5 ml of dimethylacetamide and heated in a sealed tube at 120° for 45 min. The solvent was then lyophilized into a micro cooling trap, the condensate containing 4100 cpm. Chromatography of the residue showed that both 3-methoxyxanthine and its accompanying radioactivity had disappeared.

Specificity of the Transmethylating Enzyme. Five derivatives of 3-hydroxyxanthine were used to compare their susceptibility as substrates with that of 3-hydroxyxanthine. Even though the *N*-methoxy derivatives were not known, the enzymatic formation of an O-methylated product could be recognized by the latter's chromatographic behavior. The results (Table I) show a wide spread of substrate activity. The 1- and 7-hydroxyxanthines are considerably more susceptible to methylation than 3-hydroxyxanthine. Curiously, 8-methyl-3-hydroxyxanthine is not a substrate, and xanthine is negative as expected.

In all cases the presumed *O*-methyl derivative was eluted from the Dowex 50 column close to the unreacted starting *N*-hydroxypurine. On subsequent paper chromatography of the isolated radioactive peaks, the radioactivity preceded the starting material slightly. The exception is 7-hydroxyxanthine for which the radioactive product formed also elutes close to the starting material. However, on subsequent paper chromatography the radioactivity travels with a retention factor of 0.43 and 7-hydroxyxanthine with an R_F of 0.65. This product may not be 7-methoxyxanthine but may be a methylated uric acid, formed by a rearrangement such as that observed (Goldner *et al.*, 1964) with 7-methoxytheophylline.

Isolation and Identification of 1-Methoxyxanthine. 1-Hydroxyxanthine is the best substrate for the methyl-transferring system and it was selected for isolation and positive identification of the product. A sample of radioactive adenosylmethionine was diluted with nonradioactive adenosylmethionine, and after paper chromatographic purification the specific activity was 1.1×10^6 cpm/μmol. A larger scale enzymatic incubation mixture was chromatographed, first on 10 ml of Dowex 50 with 0.05 N HCl, then on 5 ml of Dowex 50 with 1 N HCl and finally on 10 ml of Dowex 50 with 0.1 N HCl. Each time the radioactivity was concentrated and rechromatographed. After the three purifications about 0.25 μmol of 1-methoxyxanthine was isolated, which matched the authentic compound in every chromatographic as well as spectral comparison. Its specific activity was 0.99×10^6 cpm/μmol in good agreement with that of the adenosylmethionine.

Chemical Methylation of 1-Hydroxyxanthine. Direct methylation of 1-hydroxyxanthine under acidic conditions is known to lead to the 7,9-dimethyl derivative (Wölcke and Brown, 1969). Direct O-alkylation was achieved with an alkyl halide and potassium carbonate in dimethylformamide in the case of 1-hydroxyhypoxanthine (F. Lam, unpublished data). This reaction when used with 1-hydroxyxanthine leads to the fully methylated derivative, 1-methoxy-3,7-dimethylxanthine as the only product formed. Methylation in 2 N NaOH with methyl iodide led preferentially to 1-methoxyxanthine with lesser amounts of 1-hydroxy-3-methylxanthine and 1-hydroxy-9-methylxanthine.

Radiolysis for Structure Determination. A major difference between 3-methoxyxanthine and the two 1-methoxyxanthine derivatives described here is the considerably greater heat stability of the latter. For example, 1-methoxy-3,7-dimethylxanthine melts at 225° without decomposition. We therefore used radiolysis, a general method developed by F. Lam and J. C. Parham (unpublished data), to remove *N*-hydroxy and

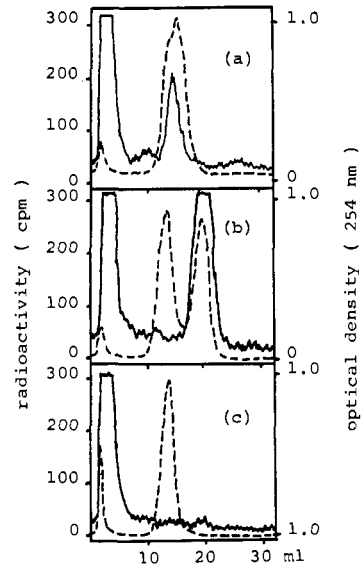

FIGURE 2: Dowex 50 chromatograms of reaction mixtures from enzymatic methylation. Broken lines represent ultraviolet absorption, solid lines represent radioactivity (see Experimental Section). Each run contains nonradioactive 3-methoxyxanthine (centered at ~13 ml) as a reference marker. In chromatogram a 3-hydroxyxanthine is the substrate and unchanged substrate plus 3-methoxyxanthine are eluted at 12–20 ml. Radioactive 3-methoxyxanthine accompanies the marker. In b 7-methyl-3-hydroxyxanthine (at 20 ml) is the substrate and radioactive 7-methyl-3-methoxyxanthine is coincident with it. In a control (c) xanthine, which elutes together with the marker, does not yield a radioactive product.

N-methoxy groups from purines. By this method, 1-methoxy-3,7-dimethylxanthine was deoxygenated to yield theobromine. In the same way 1-methoxyxanthine yielded xanthine, 1-hydroxy-3-methylxanthine yielded 3-methylxanthine, and 1-hydroxy-9-methylxanthine yielded 9-methylxanthine, proving the position of the methyl group in each case.

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Isolation and Fractionation of Yeast Nucleic Acids. I. Characterization of Poly(L-lysine) Kieselguhr Chromatography Using Yeast Nucleic Acids†

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ABSTRACT: Yeast nucleic acids have been investigated for their fractionating properties on poly(L-lysine)-coated kieselguhr (PLK) columns. Nucleic acids have been shown to bind to these columns, and in turn may be eluted with an increasing gradient of buffered saline. As the salt molarity passing over the PLK–nucleic acid complex is increased, nucleic acid species are eluted in the following order; degraded RNA, rRNA, and DNA. This has been shown by assaying the alkali lability of the various fractions obtained when total nucleic acid mixtures were fractionated, and also by fractionating previously purified yeast DNAs and RNAs. Poly(lysine) kieselguhr has also been shown to be capable of removing nucleic acids selectively from the supernatant of mechanically disrupted yeast cells which have undergone no prior purification. This property can be used to extract and fractionate yeast nuclear and mitochondrial DNA from RNase-treated cell supernatants. It has been shown that some separation can be achieved between n- and mtDNA. DNA prepared in in this way is pure of protein and degraded RNA contaminants and may be used in DNA–RNA hybridization experiments directly.

Yeast contains protein RNA, nDNA,[1] and mtDNA[1] in the ratio of approximately 200:100:1:0.1 (Bhargava and Halvorson, 1971), which accounts for the historical designation of RNA as yeast nucleic acid. Such large excesses of RNA (and protein) makes the purification of DNA much more difficult from yeast than from bacteria. The standard methods of isolating DNA are multistep procedures which start with the deproteinization of cell lysates using either denaturing agents such as chloroform (Marmur, 1961), or detergents and salt precipitation (Kay *et al.*, 1952). The nucleic acids are precipitated from solution using ethanol and the RNA can then be removed from the redissolved nucleic acid mixture by digestion with RNase. Because of the large excess of RNA in yeast, several RNase treatments are usually necessary to degrade all the RNA, and if high purity of the DNA is required, preparative CsCl density gradient centrifugation must be used (Schweizer *et al.*, 1969). Recently hydroxylapatite chromatography has been used as the final stage of purification and fractionation of yeast DNA rather than CsCl centrifugation (Bernardi *et al.*, 1970).

These standard methods rely on the successive removal of contaminating components from crude cell lysates until one is left with DNA of the desired level of purity. This approach has been necessary up to now because of the lack of a truly selective means of extracting DNA from a crude cell lysate.

The method of PLK chromatography (Ayad and Blamire, 1968), although only used here on a small scale, has been shown to selectively remove nucleic acids from the supernatants of mechanically disrupted yeast cells. The nucleic acids may be fractionated by elution of the PLK complex with a salt gradient. This paper concerns the methodology of fractionation of yeast nucleic acids by PLK chromatography.

Materials and Methods

Strain. The haploid wild-type grande yeast strain *Saccharomyces cerevisiae* A664a/18A *a*, ur_3 (kindly donated by Dr. B. Dorfman) was used throughout this work.

Growth and Radioactive Labeling. The strain was maintained and routinely subcultured on agar slants containing 1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, and 2% agar. Liquid cultures were grown in the same media (but lacking

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[1] Abbreviations used are: nDNA and mtDNA, nuclear and mitochondrial DNA, respectively; TG buffer, 0.5 M sodium thioglycolate–0.1 M Tris (pH 8.8); 0.4 M buffered saline, 0.4 M NaCl–0.02 M KH_2PO_4 (pH 6.8); 4.0 M buffered saline, 4.0 M NaCl–0.02 M KH_2PO_4 (pH 6.8); YPB, 0.1 M Hepes (*N*-2-hydroxyethylpeperazine-*N*-2-ethanesulfonic acid)–0.1 M NaCl–0.03 M $MgCl_2$ (pH 7.4). PLK, poly(L-lysine)-coated kieselguhr.