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Structural and metabolic relationship between the molybdenum cofactor and urothione

(pterin/sulfite oxidase/xanthine dehydrogenase)

JEAN L. JOHNSON AND K. V. RAJAGOPALAN

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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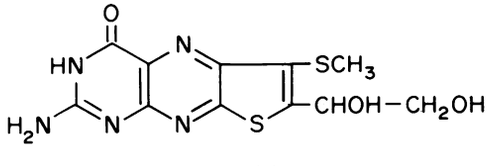
ABSTRACT **The molybdenum cofactor isolated from sulfite oxidase (sulfite:ferricytochrome *c* oxidoreductase, EC 1.8.2.1) and xanthine dehydrogenase (xanthine:NAD$^+$ oxidoreductase, EC 1.2.1.37) in the presence of iodine and KI (form A) has been shown to contain a pterin nucleus with an unidentified substituent in the 6 position [Johnson, J. L., Hainline, B. E. & Rajagopalan, K. V. (1980) *J. Biol. Chem.* 255, 1783–1786]. A second inactive form of the cofactor was isolated aerobically but in the absence of iodine and KI. The latter cofactor derivative (form B) is highly fluorescent, has a visible absorption band at 395 nm and, like form A, contains a phosphate group. Cleavage of the phosphate ester bond with alkaline phosphatase exposes a glycol function that is sensitive to periodate. Oxidation of form B with alkaline permanganate yields a highly polar compound with properties of a sulfonic acid, suggesting that the active molybdenum cofactor might contain sulfur. The sulfur-containing pterin urothione characterized by Goto *et al.* [Goto, M., Sakurai, A., Ohta, K. & Yamakami, H. (1969) *J. Biochem.* 65, 611–620] had been isolated from human urine. The permanganate oxidation product of urothione, characterized by Goto *et al.* as pterin-6-carboxylic-7-sulfonic acid, is identical to that obtained from form B. Because urothione also contains a periodate-sensitive glycol substituent, a structural relationship is suggested. The finding that urine samples from patients deficient in the molybdenum cofactor are devoid of urothione demonstrates a metabolic link between the two molecules.**

Previous reports from this laboratory (1–3) have documented progress towards characterization of the molybdenum cofactor present in sulfite oxidase (sulfite:ferricytochrome *c* oxidoreductase, EC 1.8.2.1), xanthine dehydrogenase (xanthine:NAD$^+$ oxidoreductase, EC 1.2.1.37), and nitrate reductase (NADH) (NADH:nitrate oxidoreductase, EC 1.6.6.1). Structural studies on an oxidized, fluorescent derivative (form A) of the cofactor obtained by denaturation of the molybdoenzymes in the presence of KI and I_2 have established that the cofactor contains a novel pterin (2). Form A as isolated is a phosphate ester which can be dephosphorylated by treatment with alkaline phosphatase (3). In this paper, we describe the oxidative modification of the cofactor to a second fluorescent species (form B). As documented below, the chemical properties of form B are strikingly similar to those of an unusual sulfur-containing pterin isolated from human urine more than 40 years ago and termed urothione



urothione

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(4). The observed structural relationship between the two molecules prompted an investigation of urothione excretion in normal individuals and in molybdenum cofactor-deficient patients (5). The finding that urothione is present in urine from normal individuals but is undetectable in the urine of cofactor-deficient patients provides evidence that urothione is in fact the metabolic excretory product of the molybdenum cofactor. A possible structural basis for this metabolic relationship is presented.

MATERIALS AND METHODS

Chicken liver sulfite oxidase was purified as described (6, 7). Sephadex G-25 and QAE-Sephadex were from Sigma, Florisil resin was from Fisher, alkaline phosphatase from chicken intestine was from Worthington, and pterin-6-carboxylic acid and isoxanthopterin were from Aldrich.

Absorption spectra were recorded on a Perkin-Elmer 575 spectrophotometer. Uncorrected fluorescence spectra were obtained with an Aminco–Bowman SPF spectrofluorometer. Fluorescent bands on columns and plates were visualized with a UVL-56 long-wavelength Blak-Ray lamp.

Thin-layer chromatography was done on analytical microcrystalline cellulose (MN 400) from Brinkmann. HPLC was performed on a Laboratory Data Control chromatograph with fluorescent (LDC) and UV (Altex) detection. A C-18 reverse-phase column (0.46 × 25 cm, Alltech) was run isocratically in 20% methanol at a flow rate of 2 ml/min.

For isolation of form A of the molybdenum cofactor, chicken liver sulfite oxidase (2 mg/ml) in 0.01 M Tris·HCl (pH 7.0) was adjusted to pH 2.5 with HCl. A 1% I_2/2% KI solution was prepared by dissolving the solids in a minimal volume of water and then diluting to the required final volume. The iodine solution was added to the acidified sulfite oxidase at a ratio of 1:20 (vol/vol), and the sample was heated for 20 min in a boiling water bath, cooled, and centrifuged at 35,000 × *g* for 10 min to remove hemin, which dissociates from the protein during the boiling procedure. The clarified solution was applied to a column (2.5 × 34 cm) of Sephadex G-25 (fine) equilibrated with 0.01 M acetic acid and was eluted with the same solvent. The fluorescent fractions were pooled, adjusted to pH 8 with concentrated NH_4OH, and applied to a column of QAE-Sephadex (acetate form) equilibrated with H_2O. The column was washed with H_2O and 0.01 M acetic acid, and the fluorescent material was eluted with 0.01 M HCl. Isolation of form B of the molybdenum cofactor was carried out under identical conditions but without the iodine solution.

Phosphate was quantitated by the method of Ames (8). Alkaline phosphatase cleavage was carried out on samples at pH 8–9 in the presence of 7.5 mM $MgCl_2$.

Oxidation with alkaline permanganate was done by adding excess potassium permanganate to samples in 0.1 M NaOH and heating in a boiling water bath for 50 min. Ethanol was added to destroy residual permanganate, and the samples were cooled

and centrifuged to remove insoluble MnO_2.

Oxidation with periodate was carried out with 10 mM sodium metaperiodate on samples in 0.01 M acetic acid. At this concentration of periodate, the reactions for both form B (dephospho) and urothione were complete in <1 min at room temperature. The reaction mixtures were chromatographed on HPLC, and the isolated pterin derivatives were taken to dryness by rotoevaporation. The residues were dissolved in 1 ml of 0.1 M NaOH for spectral studies and then neutralized with HCl.

The periodate products were allowed to react with 0.5 ml of 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl) at 100°C for 10 min (9). The samples were chromatographed on HPLC with 40% methanol, and the isolated hydrazones were taken to dryness and then dissolved in 800 μl of H_2O. NaOH (200 μl of a 1 M solution) was added to demonstrate the orange hydrazone color.

RESULTS

Denaturation of the molybdoenzymes sulfite oxidase, xanthine dehydrogenase, or nitrate reductase with 6 M guanidine·HCl in the presence of KI and I_2 releases the same low molecular weight fluorescent compound (molybdenum cofactor form A), which is converted to pterin-6-carboxylic acid by alkaline permanganate oxidation (2). Form A also was released by boiling any of these enzymes at pH 2.5 for 20 min in the presence of KI and I_2. The fluorescence and absorption spectra of form A released from sulfite oxidase in this manner and purified by chromatography on Sephadex G-25 and QAE-Sephadex are shown in Fig. 1.

When the denaturation in boiling acid (pH 2.5) was carried out in the absence of KI and I_2, quite a different fluorescent species (form B) was produced. The fluorescence and absorption spectra of form B after purification on Sephadex G-25 and QAE-Sephadex are shown in Fig. 1. The low-energy absorption band at 380 nm characteristic of form A shifted to 395 nm in the case of form B, with corresponding changes in the fluorescence excitation spectra. Significant differences between the two species were also evident in the UV regions of the absorption spectra.

Like form A (3), form B contains a terminal phosphate residue, which could be removed with alkaline phosphatase. Quantitation of organic phosphate indicated an extinction coefficient ϵ of 12,000 $M^{-1}cm^{-1}$ at 395 nm for form B of the cofactor. Exposure to 1 M HCl at 100°C for 1 hr converted 33% of form B to the dephosphorylated form, which we call form B (dephospho). Form B (dephospho) was sensitive to periodate oxidation, yielding an aldehyde derivative that produced an unstable intense orange dinitrophenylhydrazone when treated with 2,4-dinitrophenylhydrazine and subjected to alkaline conditions. The HPLC elution profiles for form B, form B (dephospho), and the periodate product of form B (dephospho) are shown in Fig. 2.

As reported (2), treatment of form A of the molybdenum cofactor with permanganate in alkaline solution led to the rapid appearance of pterin-6-carboxylic acid, identified by its fluorescence and absorption spectra and by its mobility on cellulose thin-layer plates in several solvents. The conversion to pterin-6-carboxylic acid provided evidence for the presence of an alkyl substituent in the 6 position of the pterin in form A of the oxidized molybdenum cofactor. As described below, attempts to convert form B derived from the cofactor to pterin-6-carboxylic acid were without success.

Chromatography of a solution of permanganate-treated form B on a cellulose thin-layer plate with 4% sodium citrate revealed two major fluorescent products—a green-fluorescing species (product 1) with an R_F of 0.84 and a purple-fluorescing species (product 2) with an R_F of 0.59. Under these conditions, pterin-6-carboxylic acid appeared as a blue fluorescent band with an R_F of 0.41, and isoxanthopterin migrated as a purple-fluorescing spot with an R_F of 0.28. The two fluorescent permanganate oxidation products of form B were eluted from the cellulose with 1 M NH_4OH, and their fluorescent spectra were recorded. The green-fluorescing species had excitation maxima at 270 and 380 nm and an emission maximum at 470 nm. Its extremely polar character was confirmed by HPLC, and its anionic nature was established by its strong affinity for QAE-Sephadex. The purple-fluorescing species showed 345/415-nm (excitation/emission) fluorescence in ammonia, and its spectrum was indistinguishable from that of isoxanthopterin in this solvent. However, the fluorescent spectrum of product 2 was drastically altered in acidic solvents, whereas that of isoxanthopterin remained unchanged. The spectral features and thin-layer chromatographic mobility data suggested that product 2 was an isoxanthopterin derivative with a polar, acidic substituent. The production of a 7-substituted pterin derivative from form B of the molybdenum cofactor pterin was surprising, especially because form A yielded exclusively the 6-substituted molecule.

It has been proposed from EPR (10) and x-ray absorption extended fine-structure (11) data that the molybdenum in various molybdoenzymes is coordinated to sulfur and oxygen ligands. Whereas certain of these ligands may be provided by the

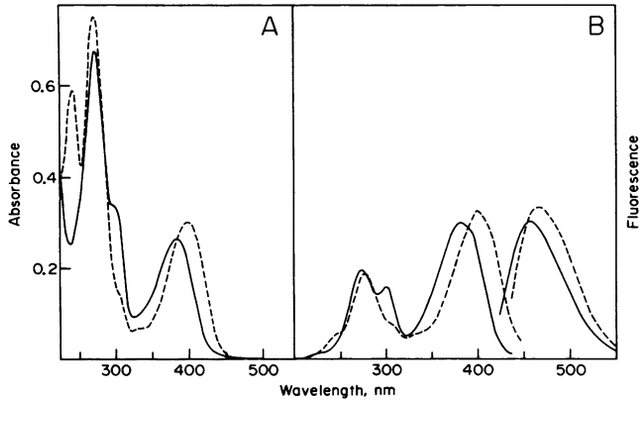


FIG. 1. Absorption (*A*) and fluorescence (*B*) spectra of form A (——) and form B (– – –) derivatives of the molybdenum cofactor. Absorption spectra were recorded in 0.1 M NaOH, and fluorescence spectra, in 1 M NH_4OH.

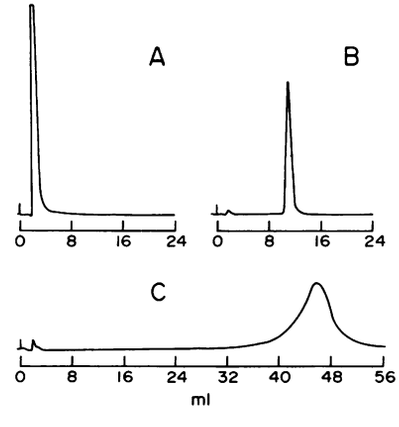
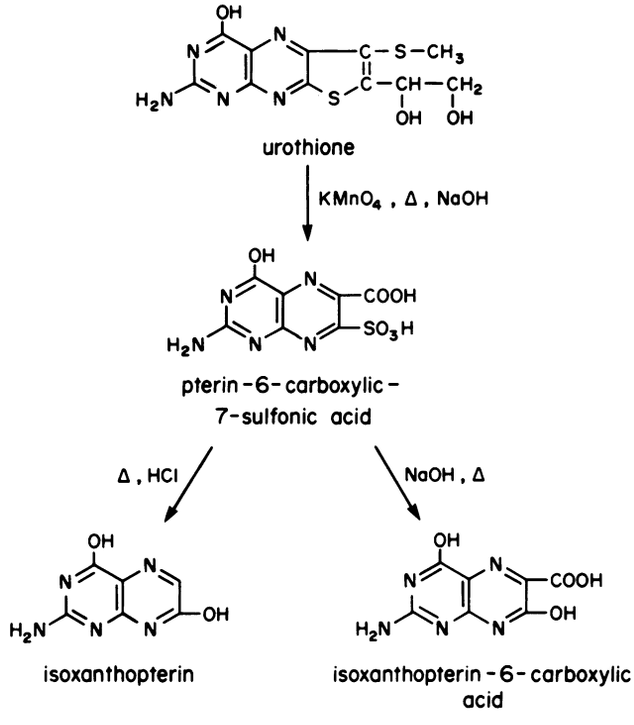


FIG. 2. HPLC elution profiles of form B (*A*), form B (dephospho) (*B*), and the periodate product of form B (dephospho) (*C*). Fluorescence detection was used.

protein, it seemed possible that some of the sulfur ligands are components of the molybdenum cofactor itself. If the cofactor were in fact a sulfur-containing pterin, the acidic species obtained by permanganate oxidation of form B could be a sulfonic acid derivative. A search of the literature for sulfur-containing pterins focused our attention on an unusual compound of unknown function isolated from human urine in 1940 (4) and termed urothione. The complete structure of urothione was established by Goto *et al.* in 1969 (12) and is shown in Scheme I. According to Goto *et al.* (12), permanganate oxidation of urothione, which is itself nonfluorescent, produces a highly acidic, green-fluorescing species identified as pterin-6-carboxylic-7-sulfonic acid. Heating this latter molecule with 4 M HCl for 2 hr resulted in hydrolysis of the 7-sulfonic acid function with concomitant elimination of the carboxyl group at C-6, yielding isoxanthopterin. Heating the carboxysulfopterin in NaOH led to the formation of 6-carboxyl-isoxanthopterin. Because, in the present studies, permanganate oxidation was carried out in alkali at elevated temperatures, it appeared possible that form B of the cofactor, like urothione, was oxidized to the sulfonic acid, which in turn was partially degraded to yield isoxanthopterin-6-carboxylic acid.



Scheme I

To test this hypothesis and verify the possible relationship between form B of the cofactor and urothione, we set about to isolate urothione from human urine. Although published purification procedures for urothione started with 1,000 liters of urine and yielded ≈20 mg of product, we relied on improved chromatographic and analytical procedures to scale down the isolation by several orders of magnitude. Starting with 1 liter of urine, the initial steps in the procedure of Goto *et al.* (12) were used, including acidification, filtration, and the first chromatography on Florisil resin. In lieu of repeated chromatography on cellulose and Florisil columns, the sample was adjusted to pH 8 with NH_4OH and applied to a column of QAE-Sephadex. The column was washed with H_2O and then with 0.01 M acetic acid. Aliquots of each fraction were assayed for the presence of urothione by monitoring the appearance of green fluorescence (pterin-6-carboxylic-7-sulfonic acid) after boiling with alkaline permanganate. Fractions positive in the sulfonic acid assay were pooled and subjected to rotoevaporation. When the volume was reduced to about 2 ml, an orange-yellow precipitate was formed, which was collected by centrifugation and dissolved in 0.1 M NaOH. The absorption spectra in base and in acid (Fig. 3) were identical to those reported by Goto *et al.* (12) for urothione. The HPLC elution profile of the sample indicated that urothione was eluted with 30 ml of 20% methanol as an UV-absorbing nonfluorescent species. The minor, fluorescent contaminants eluted ahead of urothione were removed by subjecting the entire sample to HPLC. The yield of pure urothione from 1 liter of urine was 200 μg based on an $\epsilon_{398\ nm}$ of 11,400 $M^{-1}cm^{-1}$ (13).

Samples of urothione (70 nmol) and form B of the molybdenum cofactor (60 nmol) were boiled separately for 50 min in 0.1 M NaOH with excess $KMnO_4$. The residual $KMnO_4$ was destroyed with ethanol, and the samples were cooled and centrifuged to remove the MnO_2. The clear supernatant solutions were adjusted to pH 6 with HCl and chromatographed on the reverse-phase HPLC column in 20% methanol adjusted to pH 3.6 with acetic acid. Under these conditions, the permanganate product 2 was partially protonated and retarded on the column, so that the breakthrough fractions contained only the permanganate product 1. The absorption spectra of product 1 from form B and from urothione in 0.1 M NaOH and 0.1 M HCl were recorded (Fig. 4) and found to be identical to those described by Goto *et al.* (12) for pterin-6-carboxylic-7-sulfonic acid.

The identity of the pterin-6-carboxylic-7-sulfonic acid isolated from urothione and that recovered from form B of the cofactor was further established by demonstrating conversion to isoxanthopterin by heating for 2 hr in 4 M HCl and to isoxanthopterin-6-carboxylic acid (permanganate product 2) by heating for 2 hr in 2 M NaOH. The spectral properties of isoxanthopterin-6-carboxylic acid reported by Matsuura *et al.* (14) indicated 80–90% conversion of urothione to this compound by the procedures described.

The glycol functions present in urothione and in form B (dephospho) of the cofactor make these molecules susceptible to cleavage by periodate. The products of periodate oxidation of urothione and form B (dephospho) were prepared and purified

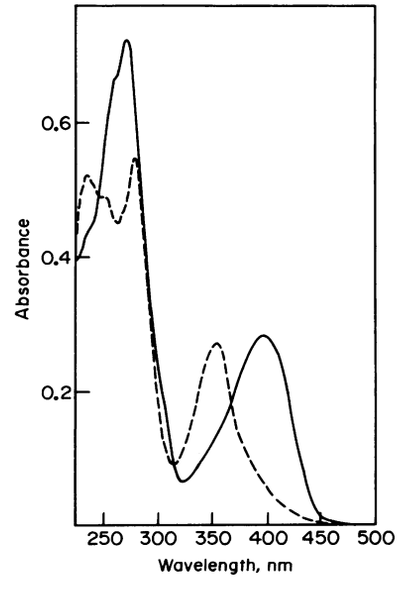


FIG. 3. Absorption spectra of urothione in 0.1 M NaOH (——) and in 0.1 M HCl (– – –).

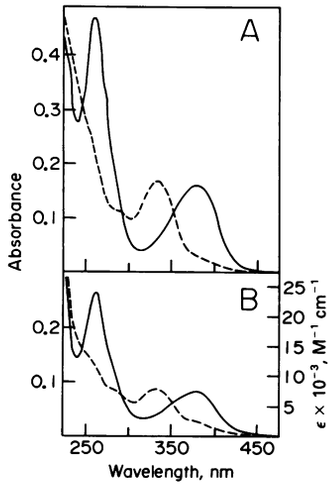


FIG. 4. Absorption spectra of pterin-6-carboxylic-7-sulfonic acid obtained by permanganate oxidation of form B (*A*) and urothione (*B*). Spectra were recorded in 0.1 M NaOH (——) and in 0.1 M HCl (– – –). The scale on the right axis is to allow comparison to extinction values reported by Goto *et al.* (12) for pterin-6-carboxylic-7-sulfonic acid: in 0.1 M NaOH, $\epsilon_{263\ nm}$ = 22,300 and $\epsilon_{379\ nm}$ = 7,510 $M^{-1}cm^{-1}$; in 0.1 M HCl, $\epsilon_{283\ nm}$ = 6,580 and $\epsilon_{333\ nm}$ = 7,960 $M^{-1}cm^{-1}$.

by HPLC. The products differed in their elution behavior in that the urothione product required 22 ml of 40% methanol, whereas the product from form B was eluted at 10 ml. In addition, the urothione periodate product, like its parent molecule, was nonfluorescent, whereas form B and its periodate product were intensely fluorescent. Nevertheless, additional evidence for the relatedness of urothione and form B can be adduced from the similarity of the absorption spectra of their periodate products (Fig. 5).

The striking structural similarities between urothione and form B of the cofactor pterin suggested that urothione might in fact be the excretory product of the molybdenum cofactor. The ideal system for testing this hypothesis was fortunately available, in the form of urine samples from patients previously identified as deficient in active molybdenum cofactor (5). These individuals show a combined deficiency of sulfite oxidase and xanthine dehydrogenase because of a lack of functional molybdenum cofactor and, as a result, show increased levels of sulfite and xanthine in plasma and urine and very low levels of uric acid. Analysis of liver samples obtained by biopsy or *postmortem* has shown that the liver tissue samples from patients lacked the specific pterin of the molybdenum cofactor, which was detectable in control liver samples by oxidative conversion to form A (manuscript in preparation).

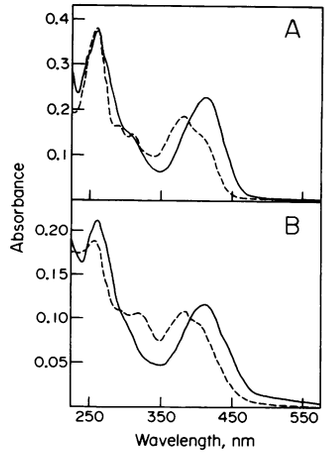


FIG. 5. Absorption spectra of the pterin products obtained by periodate oxidation of form B (*A*) and urothione (*B*). Spectra were recorded in 0.1 M NaOH (——) and in 0.1 M HCl (– – –).

The amount of urothione obtained from 1 liter of control urine (200 μg) coupled with the high sensitivity of HPLC for detection of UV-absorbing materials suggested that quantitation of this compound could be accomplished in a 10-ml sample of urine simply by scaling down the purification procedure. Pooling of appropriate fractions eluted from QAE-Sephadex provided a level of urothione sufficient for UV detection on the HPLC, and its strong retention on the reverse-phase column aided in achieving resolution from other fluorescent and UV-absorbing species. Fig. 6 illustrates the HPLC elution profiles obtained from two samples of control urine and urine from five of the molybdenum cofactor-deficient patients. The urothione peak at 30 ml, easily detected in the control urine, was totally absent in the samples from the cofactor-deficient patients. The peaks at 28.5 ml in two of the patient samples (G and H) were not urothione, as verified by injection of a urothione standard with each sample (data not shown). Quantitation of urothione by this procedure indicated an average value in adult controls of 143 μg/liter (n = 10; range = 38–273) and in children ranging in age from 3–5 yrs of 104 μg/liter (n = 4; range = 42–221).

To further verify the absence of urothione in the urine of cofactor-deficient patients, samples pooled from QAE-Sephadex were boiled for 15 min with 5 mM $KMnO_4$ in 0.1 M NaOH. Excess permanganate was destroyed, and the samples were cooled, centrifuged, and adjusted to pH 3.5 with HCl for injection into the HPLC. The breakthrough fraction from several control samples was collected, and in each case its fluorescence spectrum was found to be identical to that of pterin-6-carboxylic-7-sulfonic acid. Even though conversion of urothione to the sulfonic acid derivative is not quantitative, the yield of sulfonic acid in the control samples was proportional to the amount of urothione originally present in that sample. Significantly, no pterin-6-carboxylic-7-sulfonic acid was identified in samples from patients with molybdenum cofactor deficiency.

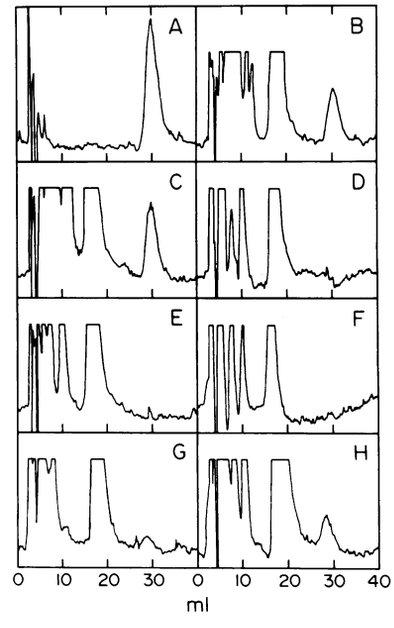
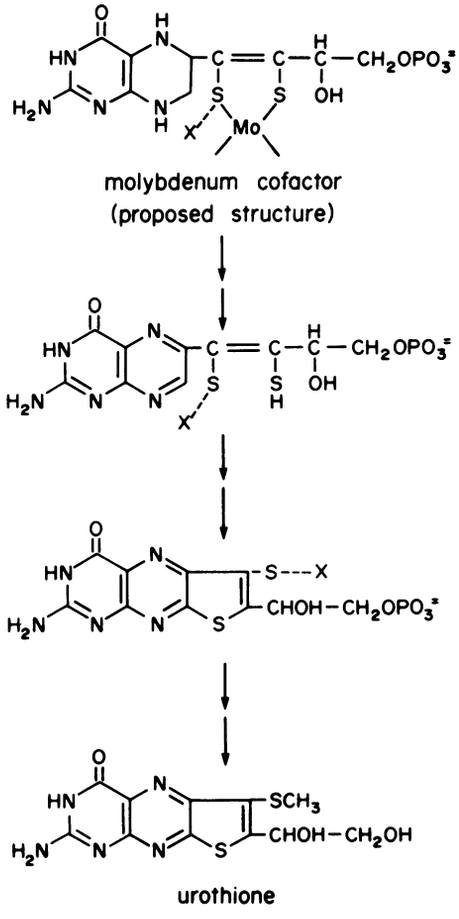


FIG. 6. HPLC elution profiles of isolated urothione (*A*) and urine samples from two control individuals (*B* and *C*) and five patients deficient in the molybdenum cofactor (*D–H*). Detection was at 254 nm.

DISCUSSION

The molybdenum cofactor in sulfite oxidase, xanthine dehydrogenase, and nitrate reductase can be oxidized *in vitro* to two fluorescent derivatives, form A and form B. Partial characterization of form A led to the conclusion that the native cofactor contains a reduced pterin ring system with a position-6 alkyl substituent and a terminal phosphate ester (2, 3). Studies on the structure of form B and its chemical and metabolic relationship to urothione provided corroborative evidence for the pterin nucleus in the active molybdenum cofactor. The presence of sulfur in the side chain is clearly established through the characterization of the form B permanganate product as pterin-6-carboxylic-7-sulfonic acid. The presence of a glycol function is evident from the periodate sensitivity of alkaline phosphatase-treated form B. The metabolic link between the molybdenum cofactor and urothione, indicated by the absence of urothione in urine from molybdenum cofactor-deficient patients, implies the presence of two sulfurs and at least four carbons in the side chain of the active cofactor.

The information summarized above may be assembled in a proposed model for active molybdenum cofactor as shown in Scheme II (top structure). In this scheme, the pterin nucleus



Scheme II

is shown in the tetrahydro state, although chemical evidence for this level of reduction of the pyrazine ring is not yet available. Similarly, the presence of the double bond in the side chain and the coordination of molybdenum to the vicinal sulfur functions are, at this point, conjecture. The sulfur on C-1 of the side chain may or may not be methylated in the active cofactor molecule. Examination of form B by NMR and other techniques to be published elsewhere suggests that form B (dephospho) differs from urothione by the absence of the SCH_3 function. Clearly if form B retained the SCH_3, such a function could be assigned to the native molecule. Its absence in form B leaves it unclear as to whether the sulfur, which is very likely to be present in the native cofactor, is methylated in the active molecule or is methylated *in vivo* only after release from a degraded molybdoenzyme as a signal for excretion.

Given the proposed structure for active molybdenum cofactor shown in Scheme II, a hypothetical pathway for conversion of active cofactor to urothione could be elaborated. According to this scheme, the molybdenum cofactor is freed of its enzymic environment, loses molybdenum, and undergoes at least partial oxidation of the pyrazine ring. These steps allow a free SH group to attack the C-7 of the pterin with formation of the thiophene ring. Final modification for excretion would include dephosphorylation and perhaps (as discussed) methylation.

Although structural characterization of the active molybdenum cofactor remains incomplete, the basic chemical nature of an essential component (the pterin moiety) and its major functional groups have been identified through studies of inactive, oxidized degradation products. Final resolution of the structure will depend on more detailed analyses of these compounds as well as on isolation and characterization of the labile active cofactor itself.

The authors thank Dr. Sybe K. Wadman of University Children's Hospital, Utrecht, The Netherlands, and Professor J. M. Saudubray, Hopitaux de Paris, France, for making available samples of urine from five patients deficient in the molybdenum cofactor. Dr. E. C. Taylor of Princeton University provided us with a sample of deoxyurothione, a synthetic analog of urothione, which was extremely valuable for preliminary studies prior to the isolation of urothione from urine. The authors also acknowledge the detailed studies of M. Goto *et al.* (12) in isolating and establishing the structure of urothione at a time when it was merely a minor urinary component of unknown function. Their persistence provided information which has contributed immensely to the resolution of the structure of the molybdenum cofactor. Mr. Ralph D. Wiley skillfully prepared the large quantities of chicken liver sulfite oxidase necessary for these studies. This work was supported by Grant GM 00091 from the National Institutes of Health.

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