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Enzymatic Formation of Xanthurenic Acid 8-Methyl Ether, an Endogenous Carcinogen, in Animal Tissues

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An enzyme activity forming xanthurenic acid 8-methyl ether, a carcinogenic tryptophan metabolite, was found in the organs of various animals by using a new method of analysis. After incubation of a 105000*g* supernatant of the tissues with xanthurenic acid and *S*-adenosylmethionine at pH 10.0, the xanthurenic acid 8-methyl ether was separated by high-performance liquid chromatography on a LiChrosorb RP-18 column and determined fluorometrically. Potent enzyme activity was found in the liver and kidney of pig and wild boar, and in bovine liver. Low activity was found in the liver and kidney of sheep, deer, and monkey. Our data suggest that the ether may be formed from xanthurenic acid by this enzyme, because the enzyme activity was high in porcine liver, and pigs excrete xanthurenic acid 8-methyl ether in the urine.

Keywords——xanthurenic acid; *S*-adenosylmethionine; xanthurenic acid 8-methyl ether; high-performance liquid chromatography; *O*-methylating enzyme; tryptophan metabolite; carcinogen endogenous

Xanthurenic acid 8-methyl ether (MXA) is an intermediate metabolite of tryptophan and is suspected to be carcinogenic. It was reported that MXA induced bladder cancer when tested by the bladder implantation technique[1,2] and induced malignant tumors of the lymphoreticular system in mice upon *s.c.* administration.[2] MXA was first identified in human urine,[3] and has been detected in urine of pig[4] and monkey,[5] but not rodent.[4] However, the pathway of its formation has not been clarified yet. Roy *et al.* suggested that 3-methoxykynurenine might be a precursor of MXA, but 3-methoxykynurenine had not been identified in urine.[6] Leklem *et al.* assumed that MXA was formed from xanthurenic acid (XA) in humans on the basis of an oral administration experiment with XA-4-^{14}C.[7] On the other hand, Ohira *et al.* reported a weak MXA-forming activity from XA in rat liver,[8] but MXA was not identified in the urine of rats. Roy *et al.* could not detect such enzyme activity in liver homogenate from pigs, which excrete MXA in the urine.[6]

In this paper, we detected enzyme activity catalyzing the formation of MXA from XA and *S*-adenosylmethionine (AdoMet) (Fig. 1) in porcine liver by using a new, simple and sensitive method for the determination of MXA by reverse-phase high-performance liquid chromatography (HPLC), and we partially characterized the enzyme. The tissue distribution and species difference of the enzyme were also studied in various animals.

Materials and Methods

Chemicals and Animals——XA was obtained from Sigma Chemical Company and purified by the method of Kotake *et al.*[5] MXA was synthesized following the reports of Furst *et al.*[9] and Price *et al.*[3] AdoMet, LiChrosorb RP-18 (5 μm) and trypsin were obtained from Sigma Chemical Company, Merck and Boehringer Mannheim GmbH, respectively. The organs of pig, cow and sheep were obtained from a slaughterhouse. We also used livers and kidneys obtained from wild boar and deer caught by hunters. The organs of monkeys were provided by Dr. K. Takahashi

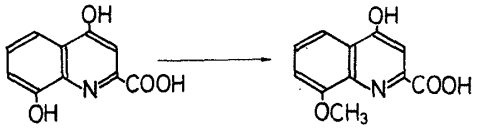

Fig. 1. Formation of MXA from XA and AdoMet

and K. Asaoka (Primate Research Institute, Kyoto University).

Preparation of the Supernatant——A 2 g (wet weight) portion of each organ was homogenized in 10 ml of ice-cold 0.154 M KCl with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 11000 *g* for 30 min at 4 °C, and the supernatant was centrifuged at 105000 *g* for 60 min. The 105000 *g* supernatant fraction was used for the determination.

Concentration of *O*-Methylating Enzyme in the Supernatant——A dialysis tube filled with the 105000 *g* supernatant was placed in sucrose for 2 h. The concentrated supernatant was dialyzed in 0.154 M KCl to remove the sucrose. The concentrated supernatant was stored at −20 °C for further analysis.

Enzyme Assay——Each mixture for assay contained 0.3 mM AdoMet and 5 mM XA in 40 mM glycine–NaOH/40 mM NaCl buffer (pH 10.0). The reaction was initiated by the addition of 15 μl of the concentrated supernatant (1—50 mg protein/ml) to the reaction mixture (50 μl) at 37 °C. After incubation of the mixture for 30 min, 100 μl of cold methanol was added and the whole was cooled with ice. The reaction mixture was centrifuged at 3000 rpm for 15 min at 4 °C and 10 μl of the supernatant was applied to the column. Protein was assayed by the method of Lowry.[10]

High-Performance Liquid Chromatography (HPLC)——A Waters model 6000 A solvent delivery system, model U6K sample injector, and Hitachi fluorescence moniter (ex-filter 360 nm, em-filter 440 nm) were employed in these studies. The stainless steel column (150 × 4 mm i.d.) was packed using a pressurized slurry technique with LiChrosorb RP-18 (stored in methanol when not in use). The solvent system was a 1 : 3 (v : v) mixture of methanol and buffer solution (pH 3.2; 3.2 ml of 1 M acetic acid, 1 ml of 0.1 M sodium acetate, 0.5 ml of 0.2 M perchloric acid and 0.71 g of sodium sulfate were diluted with distilled water to 75 ml). Then, 10 μl of sample solution was injected onto the column using a Hamilton 701 microliter syringe. The sample was eluted at a flow rate of 0.5 ml/min (1000—2000 psi). Retention times of XA and MXA were 6 and 14 min, respectively.

Effect of Trypsin and Temperature——The concentrated supernatant (23.3 mg/ml) was incubated with trypsin (2.5 mg/ml) in 50 mM Tris-HCl (pH 7.5) at 37 °C for 30 min, and the enzyme activity of the mixture was assayed. The concentrated supernatant (23.3 mg/ml) and the reaction mixtures were kept at 0 or 37 °C for 30 min, or heated on a boiling water bath for 5 min.

Results

Determination of MXA by HPLC

The standard curve was linear in the range of 2.3—22.8 pmol/10 μl (Fig. 2). At concentrations of 6.8, 11.4 and 22.8 pmol/10 μl, the coefficients of variation were 6.2, 1.9 and 1.4% ($n=4$), respectively. The recovery was 100.7% when 342 pmol of MXA was added to the reaction mixture (50 μl) and taken through the procedure given under "Enzyme Assay" without incubation.

Enzymatic Formation of MXA by Porcine Liver

MXA formation by homogenates and cell fractions of porcine liver is shown in Table I. The 105000 *g* supernatant was the most active for MXA production. The chromatogram of assay mixture with the concentrated supernatant is shown in Fig. 3. The retention times of the fluorescence peaks of MXA and XA produced in the enzyme assay system agreed with those of authentic MXA and XA, respectively. When XA, AdoMet or enzyme was omitted from the incubation mixture, the fluorescence peak of MXA did not appear.

Determination of the Enzyme Activity

The enzyme activity was studied using the concentrated supernatant of porcine liver. Figure 4 shows the effect of pH on the enzyme activity in 40 mM glycine–NaOH/40 mM NaCl buffer (pH 9.5—10.5). The optimal pH was 10.0.

As shown in Fig. 5, Mg^{2+} inhibited this enzyme. The time course of MXA formation by a reaction mixture containing 15 μl of the concentrated supernatant (0.11 mg protein) was linear

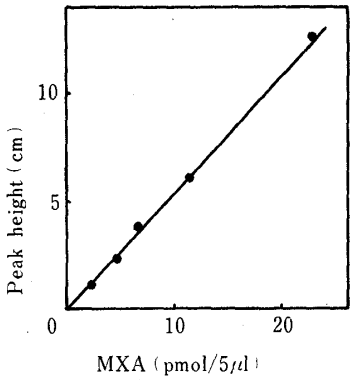

Fig. 2. Standard Curve for Determining MXA by HPLC and Fluorometry

Five μl of synthetic MXA was injected onto the column.

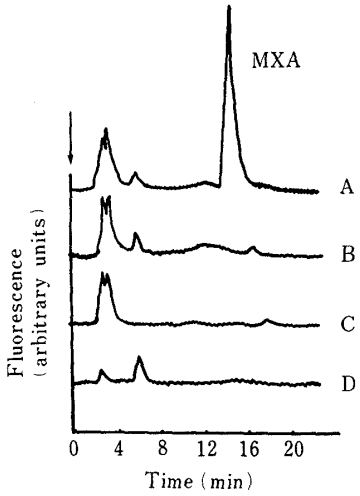

Fig. 3. Chromatogram of an Enzyme Assay Mixture

The enzyme assay mixture contained 15 μl of the concentrated supernatant (3.7 mg protein/ml) from porcine liver, 0.1 mM AdoMet, and 1.0 mM XA (chromatogram A). Chromatograms B, C and D show mixtures omitting 0.1 mM AdoMet, 1.0 mM XA, and concentrated supernatant, respectively.

TABLE I. MXA Formation by the Homogenate and Cell Fractions of Porcine Liver

Fraction	MXA formation[a] (pmol/mg/min)
Whole homogenate	2.3
11000 *g* supernatant	3.7
precipitate	0.6
105000 *g* supernatant	4.9
precipitate	0.7
Concentration of 105000 *g* supernatant[b]	13.2

a) The mixture for assay contained 0.1 mM AdoMet and 1.0 mM XA in 40 mM glycine–NaOH/40 mM NaCl buffer (pH 10.0).
b) 105000 *g* supernatant concentrated in sucrose for 2 h.

up to 45 min (Fig. 6). MXA formation paralleled the enzyme concentration in the range of 2.5—20 μl of the supernatant (7.3 mg protein/ml) (Fig. 7). The relationship between MXA formation and concentration of XA or AdoMet is shown in Fig. 8. Apparent K_m values for XA and AdoMet were 2.0×10^{-2} and 2.6×10^{-5} M, respectively.

Therefore, the determination of the enzyme activity was done at pH 10.0, without addition of Mg^{2+}, and after incubation for 30 min.

Effect of Trypsin and Temperature

The enzyme activities after incubation with and without trypsin were 0 and 10.4 pmol/mg/min, respectively. The enzyme activities after incubation at 0 and 37 °C for 30 min, and heating on a boiling water bath for 5 min were 0, 12.7 and 0 pmol/mg/min, respectively.

Thus, the enzyme was inactivated by heating, and trypsin treatment.

Enzyme Activity in Various Organs of the Pig

Table II shows the enzyme activity for MXA formation from XA in various organs of the

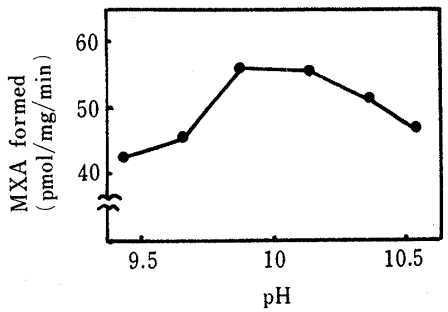

Fig. 4. Effect of pH on MXA Formation

The enzyme assay mixture (total volume 50 μl), containing 15 μl of the concentrated supernatant (7.3 mg protein/ml) from porcine liver, 0.3 mM AdoMet, and 5.0 mM XA in 40 mM glycine-NaOH/40 mM NaCl buffer (pH 10.0), was incubated at 37 °C for 30 min.

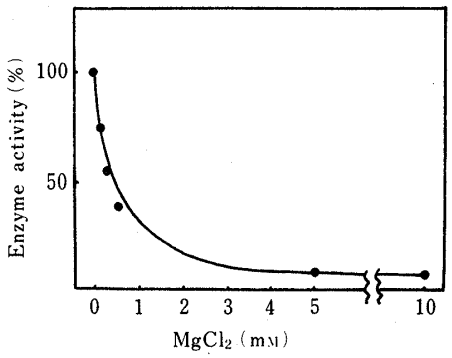

Fig. 5. Effect of $MgCl_2$ on MXA Formation

The enzyme assay mixture (total volume 50 μl), containing 10 μl of the concentrated supernatant (22.0 mg protein/ml) from porcine liver, 0.3 mM AdoMet, various concentration of $MgCl_2$, and 5.0 mM XA in 40 mM glycine–NaOH/40 mM NaCl buffer (pH 10.0), was incubated at 37 °C for 30 min.

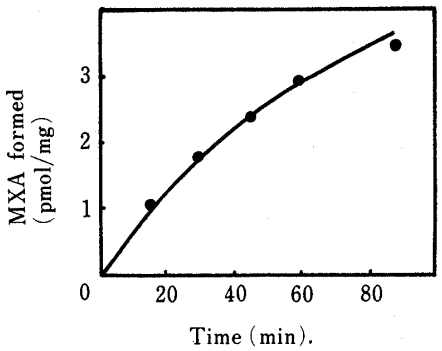

Fig. 6. Time Course of MXA Formation

The enzyme assay mixture was the same as in Fig. 4.

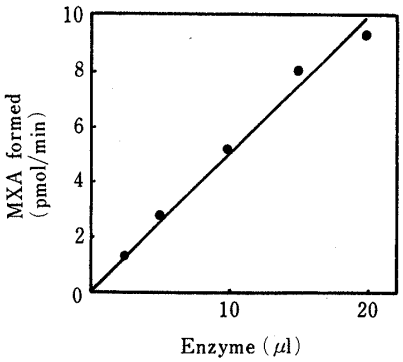

Fig. 7. Effect of the Amount of Enzyme on MXA Formation

The enzyme assay mixture was the same as in Fig. 4 except for the amount of enzyme.

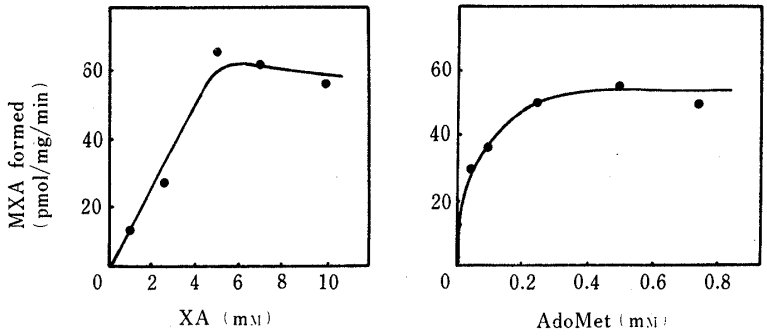

Fig. 8. Effect of Concentration of XA or AdoMet on MXA Formation

The enzyme assay mixture was the same as in Fig. 4 except for the concentration of XA or AdoMet.

pig. The enzyme activity was highest in the renal cortex and in the liver. The activity was detectable in a number of other organs.

Species Difference of Enzyme Activity in the Liver and Kidney

The kidney and liver of several mammalian species could methylate XA (Table III). In

TABLE II. MXA Formation in Various Organs of the Pig

Organ	MXA formation (pmol/mg/min)
Kidney renal cortex	53.7±2.2
Kidney renal medulla	4.5±1.1
Kidney renal pelvis	4.2±0.8
Liver	33.8±8.3
Lymphonodi paroticidi	9.4
Brain cerebrum	8.7
Brain cerebellum	8.1
Lymphonodi mesenterici	6.9
Renal gland	5.8
Spleen	5.6
Lung	5.4
Bladder	4.6
Pancreas	0.8

Results shown are the means±S.D. ($n=4$ in the kidney and $n=11$ in the liver).

TABLE III. Species Difference of MXA Formation in the Liver and Kidney

Species	MXA formation (pmol/mg/min)	
	Liver	Kidney
Cow[a]	33.0	1.4
Wild boar	10.2	21.5
Sheep	3.6	3.2
Deer	2.8	2.2
Japanese monkey	0.8	1.2
Rhesus monkey	1.2	1.3

a) Data are the means of three experiments.

the wild boar, the enzyme activity in the renal cortex was higher than that in the liver, as was the case in the pig. In the cow, the enzyme activity in the liver was higher than that in the kidney. In the sheep, deer and monkey, low activity was found in the liver and kidney. In the rabbit, mouse, rat and guinea pig, no enzyme activity was observed in the liver, and only low enzyme activity (0.2—0.9 pmol/mg/min) was found in the kidney. No activity was observed in cat liver or kidney.

Discussion

It was reported that some metabolites of tryptophan, *i.e.*, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, XA, *etc.*, induced bladder cancer in mice[1,2,11,12] or rats[13] in studies by the bladder implantation technique. Boyland *et al.* called these substances endogenous carcinogens and suggested that bladder cancer might be caused by such compounds in urine.[12] Recent reports suggest that tryptophan and its metabolites have tumor-promoting activity.[14] MXA was the strongest carcinogen in the bladder implantation experiments with tryptophan metabolites[1] and was also found to induce tumors of the lymphoreticular system upon *s.c.* administration.[2] However, the pathways of formation of this interesting metabolite have not been clarified. This paper shows that the organs of pigs (which excrete MXA in the

urine) contain an enzyme able to form MXA from XA.

Price *et al.* isolated and identified MXA in human urine by chromatography on Dowex 50 W (H^+), Dowex-1-formate and paper.[3] Benassi *et al.* also separated MXA in human urine on an Amberlite IR-120 column and by two-dimensional paper chromatography; the spots were eluted and determined fluorometrically.[15] Leklem *et al.* separated MXA by Price's method and determined it by measuring the fluorescence of the eluate.[7] In a study of enzymic *O*-methylation, Ohira *et al.* separated MXA on a Dowex AGI column and identified MXA by paper or thin layer chromatography.[8] In this paper, we separated and measured MXA by HPLC with a fluorimetric detector. This method is very sensitive and rapid. In assays of *O*-methyltransferases, an isotopic assay with AdoMet-methyl-^{14}C has generally been used. However, the method described here does not require the use of any radiochemical.

Our data suggest that MXA was formed enzymatically, because MXA was not formed when the supernatant was heated or treated with trypsin, and the activities of the supernatants of organs were nondialyzable. Further, MXA in urine may be formed from XA because the enzyme activity is high in the organs of pigs, which excrete MXA, while it is low in those of rodents, in which urinary MXA cannot be detected. Other *O*-methylating enzymes include catechol methyltransferase (EC 2.1.1.6),[16] acetylserotonin methyltransferase (EC 2.1.1.4)[17] and phenol-*O*-methyltransferase (EC 2.1.1.25).[18] However, the enzyme reported in this paper is different from catechol methyltransferase in optimal pH and the effect of Mg^{2+}. Acetylserotonin methyltransferase has been detected only in the pineal and Harderian glands and in the retina of several species of mammals and birds. Further, phenol-*O*-methyltransferase is distributed in microsomes, while the enzyme forming MXA is distributed in the supernatant. Therefore, it is likely that MXA is formed by a new enzyme.

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