108. Research on Xanthurenic Acid. XX

The Isolation of Crystallized 8-Methylether of Xanthurenic Acid from the Urine of Rhesus Monkey and the Purification of Synthesized Xanthurenic Acid

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Introduction. It has been reported by Kotake et al.^{1,2)} that xanthurenic acid (XA) shows diabetogenic activity in albino rats, and that XA-excretion increases in the urine of diabetic patients.

Kotake and Katô³⁾ have also reported that 8-methylether of XA (MXA), which is intermediate synthesis of XA, inhibits the diabetogenic activity of XA; while in 1956 Price⁴⁾ found MXA in human urine, which was also ascertained in our laboratory.

It is not only an interesting suggest to analyse the cause and development of experimental diabetes but also an approach to a new road of the study on human diabetes that XA shows diabetogenic activity in albino rats and MXA inhibits the activity.

Since no reports on MXA excretion of rabbits, rats, or dogs were seen, the authors detected MXA in the urine of rhesus monkey and succeeded in crystallization of MXA from the monkey urine, and thus confirmed MXA to be a metabolite of tryptophan.

By the way, it is important that purity of XA, used for examining the diabetogenic activity, is extremely high. And so at this time we succeeded in purifying the synthesized XA, using cellulose column liquid partition chromatography, which we also reported here.

Material and method. One male *M. facicularis*, 2 years old, weighing 2 kg, and one female, 2 years old, weighing 1.8 kg were kept in the special metabolism cages of monkey and fed the following synthetic diet with vegetables and fruits.

Casein	22%	Yeast	2%
McCillum salt	6%	Agar agar	3%
Butter	10%	Sugar	5%
Starch	52%		

No vegetables and fruits were given from two days before the collection of urine.

The urine of monkeys administered 1 g/day of DL-tryptophan was collected in a urine collection bottle contained 50 ml of 1N HCl through a funnel, and frozen until used.

Isolation of quinoline compounds from urine was made by cation

exchange column chromatography.

Twenty percent of a 24 hour urine was sampled and added 20 ml of HCl and diluted with distilled water to 100 ml. This was then passed through a column of Dowex 50 $\times$ 12 (H⁺) (1.0 cm in diameter $\times$ 4.0 cm long). This column was then washed successively with 50 ml of 0.2 N HCl, 100 ml of 0.5 N HCl, and quinoline compounds were then eluted with 400 ml of H₂O.

The effluents which were added equivolume of Palitzsch's buffer⁵⁾ (pH 8.4) (borax-boric acid NaCl buffer) were determined by the spectrophoto-fluorometer (Hitachi EPU 2A).

The same apparatus was used for absorption spectro-analysis.

The authentic XA was synthesized by Frust-Olsen's method⁶⁾ and purified by our method (m.p. 286.5°C), and MXA was purified by the same method (m.p. 240.5°C). KA was of commercial one (Merk) (m.p. 283.0°C).

We reported here our purification method of synthesized XA, using cellulose column liquid partition chromatography.

Synthesized XA dissolved in solution which consisted of 1% ammoniacal methanol, butanol, benzene, water (2:1:1:1).

The above solution was chromatographed cellulose column, developing by the same ammoniacal M. B. B. W. (2:1:1:1) (as a solvent) under the ultraviolet light. MXA shows very strong white-blue fluorescence and XA shows no fluorescence but detectable yellow zone.

The fraction of XA was concentrated and dried under reduced pressure, and the residue was dissolved in hot 20% hydrochloric acid solution, and crystallized under low temperature.

After crystallization repeated twice, prismatic crystals were obtained.

Fig. 1. Crystals of XA

Recovery was 75% to 80%.

Diabetogenic activity of XA, which has been purified with the

above treatment, was examined on rat and mouse D/D strain.

In the groups injected 100 mg/kg or more of this purified XA, continued hyperglycemia was distinctly shown after injection.

Experiments and results. Paper chromatography: Twenty litres of the effluent was concentrated to 20 ml under reduced pressure under 60°C, and using Toyo Rosi filter paper No. 51A it was developed with Mason-Berg solvent system with 1% NH_4OH ; butanol:acetic acid:water (4:1:5) at room temperature for chromatography. The results were as shown in the following table. (Color number was illustrated according to JIS standard color map.)

P. P. C.		P. P. C. spot					
Effluent	Authentic sample	R _f	Diazo	$\text{Fe}(\text{NH}_4)_2\text{SO}_4$	Resorsinol	BaSO_4	4N NaOH (Fluores)
MXA	B	F ₁	0.56	—	—	—	17-15-5
	A	F ₁	—	—	—	—	Light Blue
	W	F ₂	0.52	+	+	—	8-19-4
1% NH_4OH	B	F ₁	0.77	—	—	—	17-15-5
	A	F ₁	—	—	—	—	Light Blue
	W	F ₂	0.40	+	+	—	8-19-4
M B W	B	F ₁	—	—	—	—	—
	A	F ₁	—	—	—	—	—
	W	F ₂	—	—	—	—	Yellow

pH and fluorescence intensity: Fluorescence intensity of MXA at 460 $\text{m}\mu$ was decreased at pH value under 4 and above 11. When Britton-Robinson⁷⁾ buffer was sprayed to the spot corresponding to the authentic MXA on the filter paper, changes of fluorescence intensity were just agreed with those of the authentic MXA. Therefore, the relation between fluorescence intensity and pH of the effluent was the same as the authentic MXA.

Fluorescence spectra of filter paper: The spot corresponded to MXA on the filter paper was cut out and extracted with water. Thus its extracted solution was developed again with B.A.W.

Fig. 2. Fluorescence spectrum of extract on the filter paper

(4:1:5) using Toyo Rosi filter paper No. 54 (10 cm×40 cm) and the spot corresponded to MXA was cut out again and extracted with Palitzsche's buffer (pH 8.4), and then a piece of Toyo Rosi filter paper No. 51A was immersed in the extract, and was dried in incubator at 37°C. Fluorescence spectrum was measured by spectrofluorometer. Its fluorescence spectrum was almost identical with synthetic MXA.

The extraction from filter paper and the absorption spectra analysis: Twenty litre effluent from cation exchange resin was concentrated to about 20 ml, which was developed by paper chromatography at room temperature, using Toyo Rosi filter paper No. 54 (10 cm×40 cm). The solvent was B. A. W. (4:1:5).

The spot corresponding to MXA was cut off, and was concentrated after extraction with Palitzsche's buffer.

The absorption spectra of the spot corresponded to MXA obtained with P. P. C. were identical with those of the authentic MXA and the change of its spectra by alkali treatment was the same as the authentic sample (Fig. 3).

Fig. 3. U-V spectrum of the extract from P. P. C.

Fig. 4. Fluorescence spectrum of the extract from P. P. C.

Fluorescence spectra: Its fluorescence spectra in Palitzsche's buffer pH 8.4 were identical with those of synthetic MXA and further by 4N alkali treatment maximal fluorescence was shifted from 460 mμ to 410 mμ as well as those of the synthetic sample (Fig. 4).

Isolation of crystallized MXA in monkey's urine: The effluent of cation exchange chromatography was concentrated to dryness and dissolved in ammoniacal Mason-Berg solvent, and separation and puri-

Fig. 5. Crystals of MXA from urine

Fig. 6. Infrared spectra of MXA

fication of MXA was carried out on cellulose column liquid chromatography by ammoniacal M.B.B.W. as a solvent. Then the band corresponding to MXA was eluted and its solvent was evaporated by heating. After the residue was dissolved completely in hot HCl and adequate water was added. Upon cooling it overnight in the icebox, crystals of MXA appeared. The microscopic photograph and infrared spectra of the isolated crystallized MXA were completely identical with those of synthetic MXA.

Administration of DL-tryptophan: After oral supplementation with 0.5–1.0 gr of DL-tryptophan, 48 hour urine was collected. In the effluent eluted from strong cation exchange resin its fluorescence intensity indicated the increment more than 100% at 460 m μ . Its

fluorescence spectra were identical with those of synthetic MXA as shown in Fig. 7, and increasing fluorescence was thought as increasing of MXA excretion.

Discussion. For the first time, Price et al. found MXA qualitatively to be excreted in human urine and recently in pig urine, too.

Especially it is interesting that this substance possesses the inhibitory action against diabetogenic activity of XA, and the phenomenon of O-methylation in animal body, which, to the author's knowledge, has previously been reported a little, arouses biochemically much more interest. The crystallization of MXA of monkey urine will give a reliable evidence that it is a trustworthy metabolite of tryptophan.

In concluding this report, the authors wish to express their heartfelt gratitude to Dr. Yashiro Kotake for his helpful suggestion and encouragement.

Fig. 7. Excretion of MXA, determined by fluorometric method

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