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Production of Uric Acid and Existence of Xanthine Oxidase in Rat Plasma[1)]

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The experiments, using rat blood, were carried out on increase of uric acid in plasma by preincubation of blood and degradation of adenine nucleotides in erythrocyte and in plasma, by chromatographic separation on a Sephadex G-10 column, and also on existence and characteristics of xanthine oxidase in plasma.

A system for the production of uric acid from adenine nucleotides and the existence of xanthine oxidase were demonstrated in rat plasma from these experimental results. Xanthine oxidase found in plasma has different characteristics from the enzyme present in liver, and rather resembles the enzyme isolated from milk (EC 1.2.3.2).

Keywords——catabolism; adenine nucleotides; uric acid; xanthine oxidase; *l*-epinephrine; allopurinol; erythrocyte; plasma; rat

Xanthine oxidase is known to occur widely in mammalian tissue, cows milk and mammalian liver being particularly good sources of the enzyme.[3)] However, there has as yet been no evidence for the presence of xanthine oxidase in blood or plasma, and according to the generally accepted idea of purine catabolism in animals, most uric acid present in plasma is produced from hypoxanthine in the liver.[4)]

However, we have recently demonstrated two phenomena in rats which conflict with the hitherto held view on purine catabolism. One of these, which we reported in our previous paper,[5)] is the induction of hyperuricemia through excessive utilization of adenosine triphosphate (ATP) by exogenous enhancement of the α-action of catecholamines. This mechanism of induction of hyperuricemia is far from the generally accepted consideration that hyperuricemia is induced by the over synthesis of purine nucleotides. The second phenomenone, which we report here, is the production of uric acid and the existence of xanthine oxidase in plasma. This suggests the possibility that hyperuricemia can be induced through the catabolic pathway of purine nucleotides in plasma, and gives a new direction to the investigation on hyperuricemia.

Materials and Methods

Preparation of Plasma and Erythrocyte Membrane——Blood was collected by decapitation in heparinized centrifuge tubes from 7 week old male Wistar strain rats, and plasma was separated by centrifugation at 3000 rpm for 10 minutes. The residue was suspended in ice-cold saline and centrifuged again. This washing was repeated three times, then the residue was suspended in water, mixed mechanically for 5 minutes, and centrifuged at 10000 rpm for 10 minutes. This procedure for destruction and washing of erythrocytes were repeated five times. The final residue was homogenized with water and used as the erythrocyte membrane preparation.

The material of intact erythrocyte was prepared from the residue washed with saline. The residue was suspended in Krebs-Ringer bicarbonate buffer, centrifuged at 3000 rpm for 10 minutes, and resuspended in buffer.

1) This work was presented at the 46th Annual Meeting of Biochemical Society of Japan, Nagoya, September, 1973.
2) Location: *Fukushima-ku, Osaka, 553, Japan.*
3) R.C. Bray, "The Enzymes," Vol. 7, Ed. by P.D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York and London, 1963, p. 533.
4) M. Tachibana, *Tampakushitsu Kakusan Koso*, **14**, 1310 (1969).
5) Y. Yonetani, T. Douzaki, and Y. Ogawa, *Chem. Pharm. Bull.* (Tokyo), **25**, 441 (1977).

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Separation of Purine Components in Plasma——Plasma was mixed with an equal volume of 0.6 N perchloric acid and centrifuged at 10000 rpm for 5 minutes. The supernatant fluid was adjusted to pH 7.0 with 10 N potassium hydroxide solution, filtered through a glass filter, and the precipitate was washed well with water. The filtrate was concentrated under reduced pressure to less than 10 ml, then the volume was adjusted to 15 ml after refiltration and washing of the precipitate. This sample was subjected to chromatographic separation on a Sephadex G-10 column.

The samples prepared were stored in a deep freezer until being separated by column chromatography. Sephadex G-10 was suspended in water and packed into a 2.2×100 cm column. After the column was washed with water, the sample was put on the column and eluted with water until about 800 ml of eluent was collected in 10 ml fractions. Each fraction was examined by ultraviolet (UV) spectrum.

Extraction of Xanthine Oxidase from Plasma——Plasma protein was precipitated by 60% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 10000 rpm for 10 minutes, then suspended in 0.005 M phosphate buffer (pH 7.0), and dialyzed overnight against cold water. The inner solution was fractionated with solid ammonium sulfate. The precipitate at from 25% to 50% saturation was collected and dialyzed again as above. The inner solution was adjusted to pH 5.0 with 5 N acetic acid and the supernatant fluid separated by centrifugation was then neutralized with 1 N sodium hydroxide solution. The precipitate at 50% saturation with ammonium sulfate was collected by centrifugation and redissolved in the minimum volume of water. The solution was left at room temperature for a few minutes, the precipitate produced was removed by centrifugation at 15000 rpm for 10 minutes, and the supernatant fluid was dialyzed overnight against cold water. The inner solution was poured onto a column of diethylaminoethyl (DEAE)-Sephadex A-50 buffered with 0.005 M phosphate buffer (pH 7.0), and the column was washed and eluted with 0.005 M phosphate buffer (pH 7.0) until the proteins was not adsorped onto the DEAE–Sephadex A-50. To the pooled eluent was added solid ammonium sulfate to 50% saturation, and the precipitate was collected by centrifugation and redissolved in a little water.

Procedure for Determination of Xanthine Oxidase Activity——One ml of 0.1 M Tris(hydroxymethyl)-aminomethane–hydrochloric acid buffer (pH 8.5) was pipetted into a spectrophotometer cell, and 1.0 ml of 3×10^{-4} M xanthine solution dissolved in sodium hydroxide solution and neutralized with hydrochloric acid was added. As soon as possible after the addition of the diluted enzyme solution, the optical density of the mixture was measured at 290 mμ continuously. One unit of enzyme activity was defined as the amount giving a rise in optical density of 0.001 per minute.

Results

Fractionation of Plasma Components by Sephadex G-10 Column

Though Dowex resins have generally been employed for the chromatographic separation of purine compounds, there are small amounts of plasma components that cannot be separated quantitatively by them. For this reason, we have used Sephadex G-10 for the chromatographic separation. Although this procedure was suitable for the quantitative separation of small amounts of components, the same component in different samples was often eluted in different elution volumes, if the component was conjugated with other component.

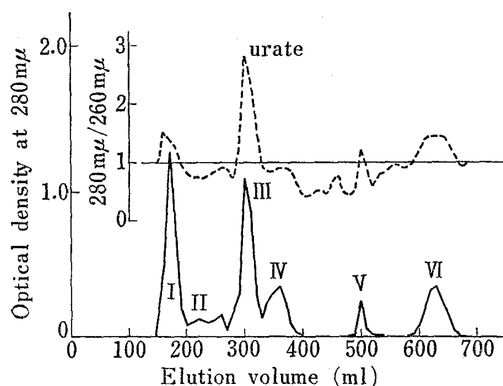

Fig. 1. Components of Rat Plasma through Sephadex G-10 Column

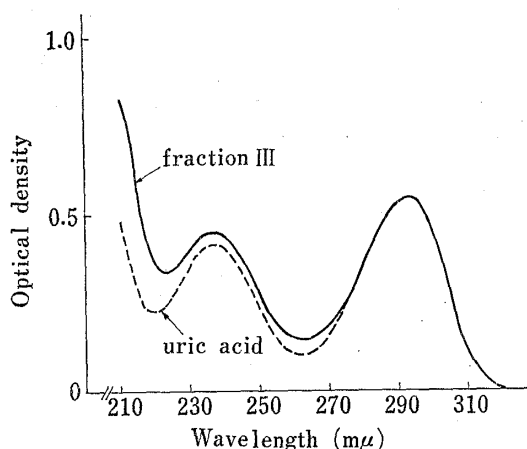

Fig. 2. UV Spectrum of Fraction III of Rat Plasma

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The elution pattern shown as optical density at 280 mμ is illustrated in Fig. 1. The separation was done on 55 ml of deproteinized solution prepared from pooled plasma which was separated immediately after decapitation.

Component III in this figure was identified to be uric acid from the ultraviolet spectrum (Fig. 2). Further identification was performed on this component, as the elution volume of uric acid dissolved in water was shown to be at 250 ml, *i.e.*, less than that of component III. The eluent was concentrated to a small volume and the substances showing ultraviolet absorbance were identified by DEAE-cellulose paper chromatoraphy. The paper was developed to 20 cm in 0.6 M ammonium formate solution by the ascending method and the substances were detected under UV light.

It was found that component III did not contain any UV absorbing substance except for uric acid, but a large part of component III was recognized at the front of paper as a visible yellow spot. The presence of contaminants is also suggested by the UV spectrum in Fig. 2, which shows a slightly higher absorbance in the short wave range than uric acid does. In order to identify the contaminants, the fractions containing component III were collected in several repeat experiments, and concentrated under reduced pressure. Component III was isolated as a crystalline precipitate by the addition of ethanol to this solution, and was recrystallized from water–ethanol. The UV spectrum of this material was like as that in Fig. 2, but the content of uric acid in it was only 0.3%. Element analysis and the infrared spectrum of the substance also indicated that it was mainly composed of inorganic material. This inorganic substance did not give any ash on analysis, but produced a brown precipitate on addition of silver nitrate solution, and gave the information of presence of sodium and potassium on atomic absorption spectrometry. No purine compounds were identified among the other components separated in the column chromatography.

On the other hand, elution of authentic purine compounds indicated components of the respective elution volumes from the column; ATP was detected at 170 ml, mononucleotides at 180—190 ml, and xanthosine, uric acid, inosine, hypoxanthine, xanthine and adenosine respectively at 200, 250, 340, 480, 500 and 720 ml. However, like uric acid, most of these compounds were eluted at the different positions, if they were present in plasma.

Increase of Uric Acid in Plasma by Preincubation of Blood

A part of pooled blood used in the previous experiment was incubated at 37° for an hour before separation of plasma. Determination of this preincubated plasma by Sephadex G-10 column showed a remarkable increase in component III, as indicated in Fig. 3. The quantities of the other components were near to those in Fig. 1. Component VII in Fig. 3 cannot be distinguished from component VI in Fig. 1, though the elution volume is obviously different from each other. Component VI altered to component VII during storage of the material frozen, and the UV spectra of these components are indentical. The spectra also resembled those of components IV and V.

DEAE-cellulose paper chromatography showed that each of these components contained a small amounts of substances having UV absorbances, corresponding to hypoxanthine, xanthine, and uric acid, the proportions of these compounds being different in each component. Though these components are also considered to be the substances containing purine compounds, their purine contents were so small as we cannot isolate them, and the total amounts of optical density at 280 mμ in the components IV and VII were kept constantly during the preincubation of blood.

Component I is not a known purine compound, though it was readily isolated from the concentrated residue by the addition of ethanol as a substance giving a spot by DEAE-cellulose paper chromatography, and it was slightly diminished by preincubation of blood. The UV spectrum of this component showed several shoulders in the range 260 mμ to 300 mμ, resemble to component VII. Component II was trace, the character could not be identified.

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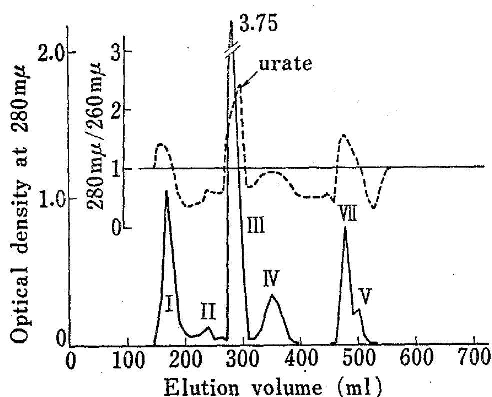

Fig. 3. Components of Rat Plasma prepared from Preincubated Blood through Sephadex G-10 Column

preincubation: 37° for an hour

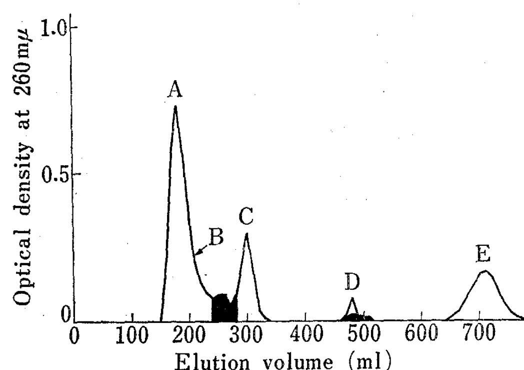

Fig. 4. Elution Pattern of Metabolites of ATP by Rat Erythrocyte Membrane from Sephadex G-10 Column

E_{max}: A (5′-IMP) 252 mμ, B (5′-AMP) 260 mμ, C (inosine) 252 mμ, D (hypoxanthine) 252 mμ, E (adenosine) 262 mμ

■: from enzyme material

Degradation of Adenine Nucleotides in Erythrocyte

As mentioned above, plasma uric acid increased when the blood was preincubated, despite the lack of materials leading to uric acid in the plasma. This result suggests the possibility that uric acid is produced from components not supplied by the plasma.

Nakao, *et al.*[6] have reported that adenine nucleotides contained in human erythrocytes are gradually degraded to hypoxanthine during the cold storage of erythrocyte preparation. Though the erythrocyte obtained from rats also contained adenine nucleotides, we could detect only a small amount of xanthosine after incubation at 37° for one hour. However, when the erythrocyte preparation was incubated with ATP as substrate, large amounts of 5′-adenosine monophosphate (5′-AMP) and 5′-inosine monophosphate (5′-IMP) were recognized in the reaction mixture. On the other hand, the erythrocyte membrane preparation was almost free from endogenous purine compounds, and it gave the various catabolites of ATP, when it was used as the enzyme material in the catabolic reaction of ATP. The medium components of this reaction were applied for the determination of sodium-potassium-activated ATPase.[7]

As shown in Fig. 4, incubation of ATP at 37° for one hour decomposed it to 5′-AMP, 5′-IMP, adenosine, inosine and trace of hypoxanthine. After chromatographic separation, the catabolites were identified by their UV spectra and by DEAE-cellulose paper chromatography. The results gave support to those described in the previous section that purine compounds are supplied by erythrocytes into plasma during incubation of blood, but uric acid is not produced in the erythrocytes.

Degradation of Purine Compounds in Plasma

The results described above suggest that rat plasma contains a system for the production of uric acid, although such an idea is very different from the generally held view of purine catabolism. This consideration was studied with pooled plasma collected from 40 rats. Fifteen ml of plasma was incubated with 3 ml of 0.006 M substrate solution at 37° for one hour, and the metabolites were determined in the same way as described on the plasma components.

Fig. 5-a illustrates the elution pattern of plasma components without substrate. A little discrepancy on the relative amounts of respective peak to the result indicated in Fig. 1 is due to the difference of rats used in both experiments, because the relative amounts of plasma components being not always kept constantly in rats. Fig. 5-b shows that with xanthine as substrate, the elution pattern differed markedly from the control (Fig. 5-a). UV spectrum

6) M. Nakao, M. Tachibana, and H. Yoshikawa, *J. Biochem.* (Tokyo), **48**, 672 (1960).
7) M. Saito, *Tampakushitsu Kakusan Koso*, **16**, 723 (1971).

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and DEAE-cellulose paper chromatography show that component III here is composed mainly of uric acid with little of xanthine. The content of uric acid in this component was calculated from the optical densities at 260 mμ and 280 mμ. It is apparent from this result that xanthine was oxidized to uric acid in the plasma. In a similar experiment, hypoxanthine was also oxidized in plasma, but the metabolite was mostly xanthine, which eluted at the same position as component III, as shown in Fig. 5-c. When the substrate was inosine, adenosine, 5′-IMP and 5′-AMP, the final metabolite was uric acid, and the fraction of component III contained inosine in addition to uric acid. Fig. 5-d shows that the metabolites from 5′-AMP were adenosine, inosine and uric acid. A slight amount of hypoxanthine was also detected from the fraction containing inosine by DEAE-cellulose paper chromatography.

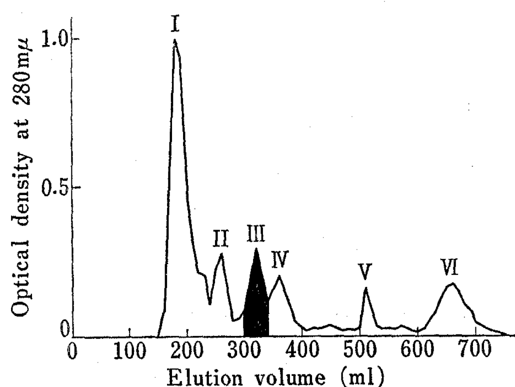

Fig. 5-a. Components of Preincubated Rat Plasma through Sephadex G-10 Column

preincubation: 37° for an hour
Component III (black part) is composed of uric acid, and components IV, V, and VI are purine mixture.

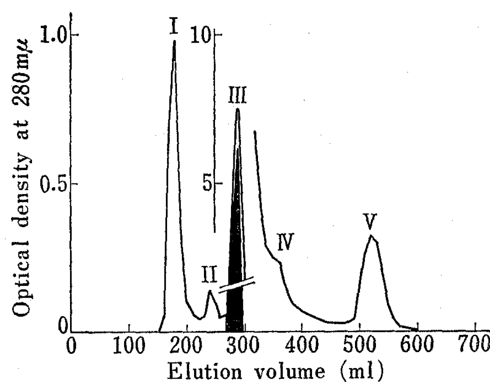

Fig. 5-b. Components of Preincubated Rat Plasma with Xanthine through Sephadex G-10 Column

preincubation: 37° for an hour
Black part shows the portion of uric acid in component III, which is composed of uric acid and xanthine.

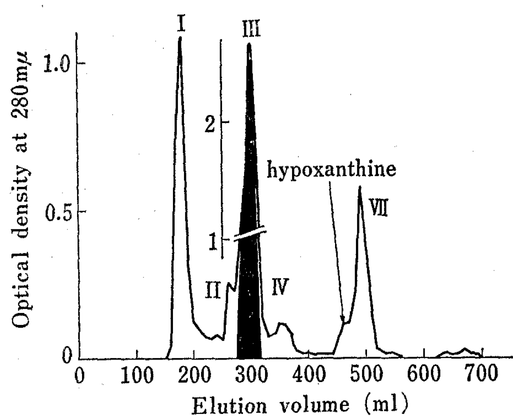

Fig. 5-c. Components of Preincubated Rat Plasma with Hypoxanthine through Sephadex G-10 Column

preincubation: 37° for an hour
Black part shows the portion of xanthine in component III, which is composed of xanthine and hypoxanthine.

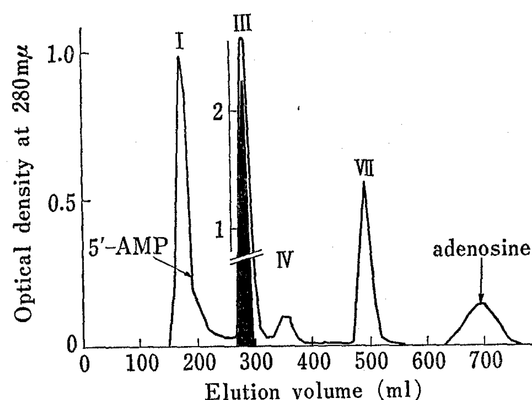

Fig. 5-d. Components of Preincubated Rat Plasma with 5′-AMP through Sephadex G-10 Column

preincubation: 37° for an hour
Black part shows the portion of uric acid in component III, which is composed of uric acid and inosine.

The metabolite composition on progressive catabolism of 5′-AMP was also investigated. Though initially inosine was the main purine component of peak III, this was gradually replaced by uric acid as catabolism progressed. The results indicate that several purine compounds are degraded to uric acid in plasma, and that component III contains various

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purine compounds, though it consists of uric acid in most cases. Xanthosine was also eluted in the fraction of component III. This nucleoside was not metabolized in plasma, although a trace of this compound was detected from erythrocytes incubated.

With respect to the other plasma components, component I was reduced when substrates were added. Though the total optical density at 280 mμ of the component IV, V, VI and VII remained constant in the respective experiments, component VII could not be detected when xanthine was added as substrate, while there was an increase in component V.

Existence and Characteristics of Xanthine Oxidase in Plasma

When unpurified plasma was employed as the enzyme material, it was hardly possible to detect the existence of xanthine oxidase by any of several procedures used for the detection of this enzyme. In the partially purified preparation, however, an increase of optical density at 290 mμ was detected by the method described, and the activity gradually increased with further purification of the material. The final preparation through DEAE-Sephadex A-50 did not show any UV absorbance after the removal of protein with perchloric acid.

The character of plasma xanthine oxidase was studied with this final preparation, being compared with the character of liver xanthine oxidase and milk xanthine oxidase. Liver enzyme was prepared in the same way as described for that from plasma, except that this enzyme was eluted from the DEAE-Sephadex A-50 column with 0.005 M phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. Milk enzyme used was highly purified commercial preparation. Their enzyme activities were determined in room temperature according to the increase of optical density at 290 mμ due to the formation of uric acid from xanthine.

The reaction mixtures were incubated at 37°, and samples of the mixture were taken at intervals and the reaction in them stopped with 1.0 ml of 10% perchloric acid. After centrifugation at 3000 rpm for 10 minutes, the supernatant fluid was taken and its UV spectrum was measured to determine the metabolites formed. As shown in Fig. 6-a, xanthine was gradually oxidized to uric acid both by xanthine oxidsae from liver and that from plasma, but the reaction with liver enzyme was more rapid than that with plasma enzyme.

The difference in characteristics of the enzyme depending on its source was apparently evidenced in the metabolism of hypoxanthine, which gave uric acid by the liver enzyme, and xanthine by plasma enzyme (Fig. 6-b). This result by plasma xanthine oxidase corresponded well with the data mentioned above for incubation with plasma. This enzyme material did not exhibit aldehyde oxidase activity against N-methylnicotinamide as substrate,[8] but a similar oxidative pattern of hypoxanthine was shown by milk xanthine oxidase, as shown in Fig. 6-c. These results suggested that milk-type xanthine oxidase is present in rat plasma, and that purine compounds can be oxidized to uric acid in plasma.

Effects of *l*-Epinephrine and Allopurinol on Plasma Components

In our previous report,[5] we described the induction of hyperuricemia in rats by the intraperitoneal injection of *l*-epinephrine. A group of rats like that employed in the experiment shown in Fig. 1 was injected with 1.0 mg/kg of *l*-epinephrine and sacrificed 30 minutes later. A volume of pooled plasma, the same as in the control experiment described previously, was analyzed by Sephadex G-10 column and the result was compared with that in Fig. 1.

The results in Fig. 7 show that the amount of component III, containing uric acid, was greatly increased, as it was in preincubated blood. Component I was reduced to below the control level soon after *l*-epinephrine was administered, but recovered gradually with overshoot. As in the previous experiments, the components IV to VII remained at a constant level even in this experiment.

8) K.V. Rajagopalan and P. Handler, "Methods in Enzymology," Vol. 9, Ed. by W.A. Wood, Academic Press, New York and London, 1966, p. 364.

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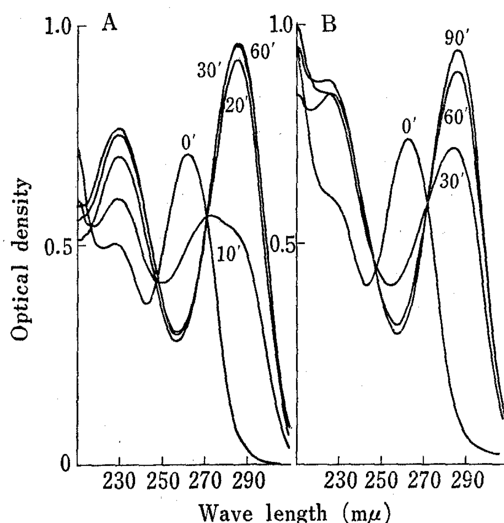

Fig. 6-a. Progressive Oxidation of Xanthine by Rat Xanthine Oxidase

A: liver xanthine oxidase, 26.8 units/tube
B: plasma xanthine oxidase 26.1 units/tube

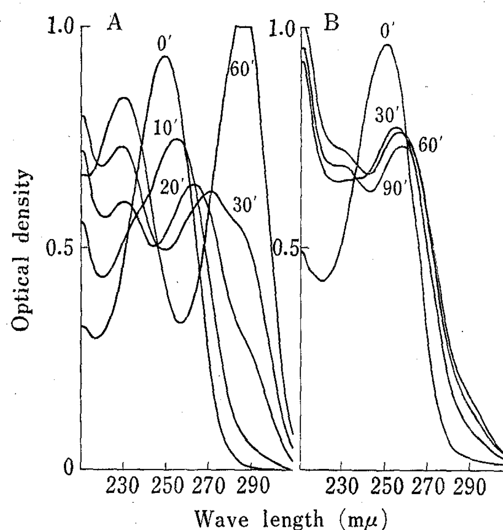

Fig. 6-b. Progressive Oxidation of Hypoxanthine by Rat Xanthine Oxidase

A: liver xanthine oxidase, 26.8 units/tube
B: plasma xanthine oxidase, 26.1 units/tube

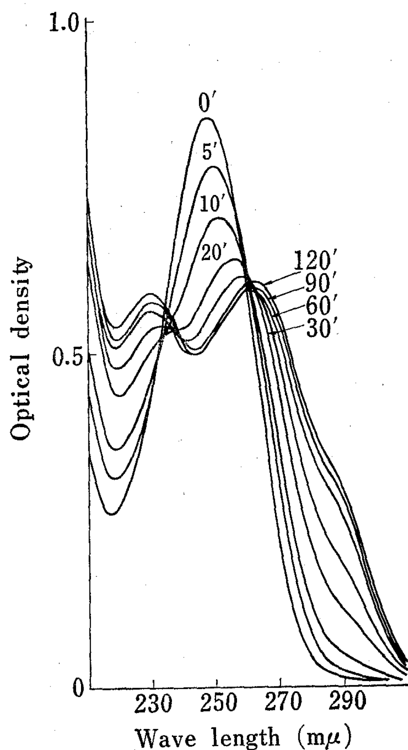

Fig. 6-c. Progressive Oxidation of Hypoxanthine by Milk Xanthine Oxidase

milk xanthine oxidase: 22.0 units/tube

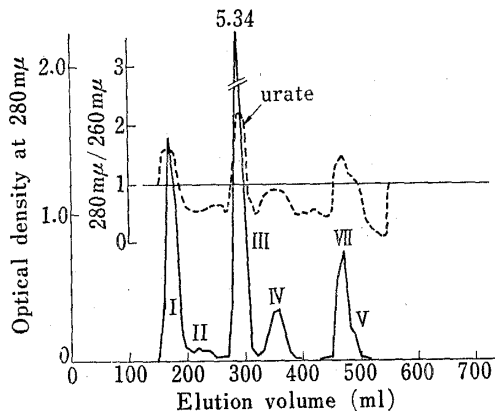

Fig. 7. Plasma Components of *l*-Epinephrine Administered Rats through Sephadex G-10 Column

Rat blood was collected a half an hour after intraperitoneal administration of 1.0 mg/kg *l*-epinephrine.

The effect *in vivo* of the xanthine oxidase inhibitor, allopurinol, was investigated. This agent was orally administered 250 mg/kg 24 hours before sacrifice and *l*-epinephrine was injected intraperitoneally 30 minutes before. As shown in Table I, an unidentified compound appeared at the similar position as component III in the allopurinol treated rats, this designated III': The DEAE-cellulose paper chromatogram showed that component III' contains two compounds with UV absorbance. The minor compound was identified with uric

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Table I. Effects of *l*-Epinephrine and Allopurinol on Plasma Components in Rats

Treatment	Fraction I	III	III′
	optical density at 280 mμ		
Control	1.49	1.16	—
Epinephrine	1.95	5.09	—
Allopurinol	2.52	—	1.21
Allopurinol+Epinephrine	3.63	—	3.98

allopurinol: 250 mg/kg, *p.o.*, 24 hrs before sacrifice
l-epinephrine: 1.0 mg/kg, *i.p.*, 0.5 hr before sacrifice
40 ml of deproteinized plasma was used in each group.

acid, but the major one could not be identified from its *Rf* value, 0.36, those of uric acid, xanthine, and hypoxanthine being 0.29, 0.44, and 0.66, respectively.

The increase in the amount of component III′ seen when *l*-epinephrine was administered in addition to allopurinol may be considered to be due to an increase in the minor compound, uric acid, because a shift of the maximum UV absorbance to a longer wave was observed. Allopurinol also caused a marked rise in the amount of component I, an increase further augmented by additional treatment with *l*-epinephrine. The components IV to VII were kept a constant level by these treatments.

On the other hand, allopurinol had an inhibitory effect on the catalytic action of plasma xanthine oxidase *in vitro*. In the incubation of hypoxanthine or 5′-AMP with plasma, the production of xanthine from hypoxanthine and of uric acid from 5′-AMP were completely inhibited in the presence of allopurinol at a similar concentration as the substrate. Although the formation of component III′ was not observed with the addition of substrate, this component was produced even *in vitro* if plasma was incubated without substrate but with the addition of allopurinol. However, allopurinol was not metabolized by the plasma xanthine oxidase preparation under the conditions used for the oxidation of substrates mentioned in the previous section, so component III′ may not be a metabolite of allopurinol,[9] but be an unknown compound.

Discussion

The induction of hyperuricemia by epinephrine, reported in our preceding paper,[5] gave us important suggestion on the source of plasma uric acid. Earlier workers had demonstrated two possible considerations for the induction of hyperuricemia by catecholamines, one the activation of liver xanthine oxidase,[10] the other a disturbance of uric acid clearance in the kidney.[11]

In our view, it seemed reasonable that this hyperuricemia was induced by the enhanced utilization of ATP through the α-action of catecholamine. On the other hand, no enhancement of the activity of liver xanthine oxidase was observed during epinephrine treatment in rat. So, it is difficult in this hyperuricemia to accept the generally held view which shows that plasma uric acid is mainly produced in the liver. Therefore, we tried to prove the presence of uric acid production system in blood.

Our experimental results on the change of plasma components in administration of epinephrine, suggested that place of uric acid production elicited by epinephrine might exist in plasma, and the existence of xanthine oxidase in plasma was also confirmed.

9) T. Yoshimura, *Taisha* (in Japanese), **5**, 195 (1968).
10) R. Imaizumi, H. Yagi, Z. Shibuya, H. Matsumoto, Y. Watari, K. Sano, and K. Nakajima, *Shinryo* (in Japanese), **10**, 510 (1957).
11) F.E. Demartini, *Arth. Rheum.*, **8**, 823 (1965).

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Detailed chemical information on the substances contained in the plasma components by Sephadex G-10 column chromatography should be obtained in further studies. Particularly, components I and III′ are clearly important in the purine catabolism in plasma. Component I always diminished and recovered with overshoot when an increase of components at position III was recognized, either *in vivo* or *in vitro*. Moreover, this was markedly increased by treatment with allopurinol. Though this component was isolated from aqueous solution by addition of ethanol, its infra-red spectrum gave no evidence for the presence of purine compounds. So it is considered that this component is not a purine compound, but is closely associated to the change of purine compounds in plasma. Component III′ was also unidentified, because of being a trace amount in a mixture containing massive amount of inorganic compound.

Another problem of interest remaining is why several purine compounds were detected in the same fraction as uric acid after addition of different substrates *in vitro*. This problem may subject to the further investigation on a form of purine compounds when they are present in the plasma, because Sephadex column has a character being able to separate easily the conjugated form of compound. Then, if plasma uric acid exists as a conjugated form to an unknown component, the conjugation may be related to the solubility of uric acid in plasma.

The characteristic of plasma xanthine oxidase appeared in the present paper shows that the significance of this enzyme must be considered not only quantitatively, but also qualitatively in comparison with the enzyme in the liver. We are studying further on the distribution of plasma xanthine oxidase in animals, and it is observed that this enzyme is contained in plasma of mice, guinea-pigs, dogs and human.

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