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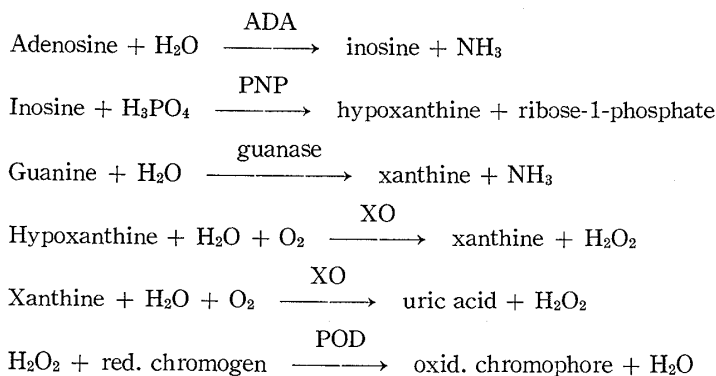
## A New Method for the Assay of Purine Metabolic Enzymes<sup>1)</sup>

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We have established new colorimetric methods for the assay of adenosine deaminase, purine nucleoside phosphorylase and guanase activities in serum, based on the formation of hydrogen peroxide with xanthine oxidase as a coupling enzyme.



The proposed methods were found to be precise and convenient. Under the assay conditions, the mean levels of adenosine deaminase, purine nucleoside phosphorylase and guanase activities in the sera of normal subjects were  $5.8 \pm 2.2$  I.U./l,  $3.7 \pm 2.1$  I.U./l and  $0.5 \pm 0.3$  I.U./l, respectively.

**Keywords**—xanthine oxidase; adenosine deaminase; purine nucleoside phosphorylase; guanase; hydrogen peroxide; colorimetric method

It has been reported that the activities of adenosine deaminase (ADA) and guanase, which play an important role in the purine metabolic system, are increased in sera of patients with hepatic diseases and cancer.<sup>2)</sup>

Available methods for the assay of above two enzymes can be classified as follows: (1) spectrophotometric assay based on the decrease of substrate,<sup>3)</sup> (2) colorimetric assay based on the liberation of ammonia<sup>4)</sup> and (3) spectrophotometric assay based on the formation of uric acid with xanthine oxidase (XO) as a coupling enzyme.<sup>5)</sup> Of these methods, the ammonia methods has been used for the assay of enzyme activities in serum.

On the other hand, the methods available for the assay of purine nucleoside phosphorylase (PNP) activity are spectrophotometric assays based on the decrease of substrate<sup>6)</sup> or the formation of uric acid.<sup>7)</sup> However, these methods have not been used for the assay of enzyme activity in serum.

In this paper, we describe new colorimetric methods for the assay of ADA, PNP and guanase activities based on the formation of hydrogen peroxide with XO as a coupling enzyme.

### Materials and Methods

**Materials**—PNP and adenosine were purchased from Boehringer Mannheim Co. Ltd., peroxidase (POD) and xanthine from Sigma Chemical Co. Ltd. and guanine, 3-methyl-2-benzothiazolinone hydrazone

hydrochloride (MBTH), N,N-dimethylaniline (DMA) and sodium azide from Wako Pure Chemical Industries, Ltd. (Japan). XO was purified from bovine milk by the method of Nathans *et al.*<sup>8)</sup>

**Preparation of Reagents**—The substrate solution for the assay of ADA was prepared by dissolving 20 mg of adenosine and 4 mg of sodium azide in 60 ml of 0.1 M phosphate buffer (pH 7.5). The substrate solution for the assay of PNP was prepared by dissolving 20 mg of inosine and 4 mg of sodium azide in 60 ml of 0.1 M phosphate buffer (pH 7.5). The substrate solution for the assay of guanase activity was prepared by dissolving 6 mg of guanine and 6 mg of sodium azide in 60 ml of 90 mM phosphate buffer (pH 6.2). The color reagent solution was prepared by dissolving 9.6 mg of MBTH, 0.3 ml of DMA and 400 units (one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 and at 20°) of POD in 100 ml of 0.2 M McIlvaine buffer (pH 3.5).

**Standard Assay of ADA Activity**—One hundred  $\mu$ l of the enzyme solution (PNP 87.5 mU/ml and XO 300 mU/ml) was added to 1.2 ml of the substrate solution and the mixture was preincubated at 37° for 5 min. Fifty  $\mu$ l of serum was added to the above incubation mixture and the whole was incubated at 37° for 30 min. One-half ml of 0.2 M citric acid solution and 2 ml of the color reagent solution were added to the enzyme reaction mixture, and the mixture was incubated at 37° for 10 min. The absorbance of the resulting solution was measured at 600 nm. With every batch, a blank was prepared by omitting the substrate from the incubation mixture. The counts from the blank were subtracted from the experimental counts. One unit of ADA activity was defined as the amount which produces 1  $\mu$ mol of hypoxanthine per min under the assay conditions.

**Standard Assay of PNP Activity**—One hundred  $\mu$ l of the enzyme solution (XO 300 mU/ml) was added to 1.2 ml of the substrate solution and the mixture was preincubated at 37° for 5 min. Fifty  $\mu$ l of serum was added to the above incubation mixture and the whole was incubated at 37° for 30 min. One-half ml of 0.2 M citric acid solution and 2 ml of the color reagent solution were added to the enzyme reaction mixture, and the mixture was incubated at 37° for 10 min. The absorbance of the resulting solution was measured at 600 nm. With every batch, a blank was prepared by omitting the substrate from the incubation mixture. The counts from the blank were subtracted from the experimental counts. One unit of PNP activity was defined as the amount which produces 1  $\mu$ mol of hypoxanthine per min under the assay conditions.

**Standard Assay of Guanase Activity**—One hundred  $\mu$ l of the enzyme solution (XO 500 mU/ml) was added to 1.0 ml of the substrate solution and the mixture was preincubated at 37° for 5 min. One hundred  $\mu$ l of serum was added to the above incubation mixture and the whole was incubated at 37° for 60 min. One-half ml of 0.1 M citric acid solution and 2 ml of the color reagent solution were then added, and the mixture was further incubated at 37° for 10 min. The absorbance of the resulting solution was measured at 600 nm. With every batch, a blank was prepared by omitting the substrate from the incubation mixture. The counts from the blank were subtracted from the experimental counts. One unit of guanase activity was defined as the amount which produces 1  $\mu$ mol of xanthine per min under the assay conditions.

**Conventional Assay of ADA and Guanase Activities**—ADA and guanase activities were determined by the method of Okuda *et al.*<sup>4)</sup> One unit of these enzyme activities was defined as the amount which produces 1  $\mu$ g of ammonia with 1 ml of serum after incubation at 37° for 60 min.

## Results

### Effects of Concentrations of Various Reagents and Coupling Enzymes on the Final Absorbance

The concentrations of reagents in the color reagent solution and coupling enzymes for the assay of ADA activity were investigated. The results are shown in Fig. 1.

The absorbance at 600 nm was maximum with concentrations of above 125  $\mu$ g of MBTH, 2  $\mu$ l of DMA and 2 unit of POD per tube. On the other hand, the concentrations of PNP and XO required to obtain a maximum absorbance in the standard assay system for ADA activity were 1.75 mU and 10 mU per tube, respectively.

### Calibration Curve

It was observed that the calibration plots for ADA, PNP and guanase activities were all linear, passing through the zero point. The absorbance at 600 nm was about 0.7 with 30 I.U./l ADA and PNP and 15 I.U./l guanase.

Kinetic studies were also carried out with human serum having the following activities: ADA 8.8 I.U./l, PNP 5.4 I.U./l and guanase 1.8 I.U./l. The correlation was linear, passing through the zero point, up to a serum volume 150  $\mu$ l, and the time course of these enzyme activities were all linear up to 90 min. It was concluded that these conditions were suitable for the assay of ADA, PNP and guanase in this system.

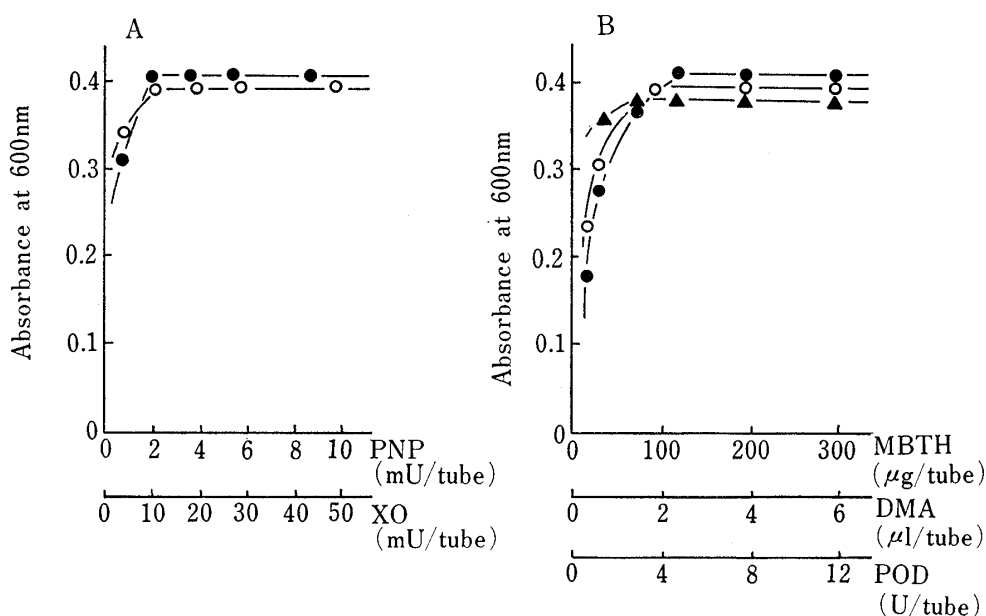


Fig. 1. Effects of the Concentrations of Enzyme and Color Reagent on the Final Absorbance

A) PNP (—●—); XO (—○—).  
B) MBTH (—●—); DMA (—○—); POD (—▲—).

TABLE I. Effects of Various Serum Compounds on Assay Values for Adenosine Deaminase, Purine Nucleoside Phosphorylase and Guanase Activities

Compound	Amount (mg/dl)	Remaining activity (%)		
		ADA	PNP	guanase
None		100	100	100
Glucose	100	100	100	99
	200	100	100	100
Uric acid	5	100	100	99
	10	100	99	97
Ascorbic acid	1	99	101	101
	5	98	98	99
Bilirubin	1	99	100	100
Albumin	1000	94	94	91
	2000	92	92	91
	5000	92	92	90

### Effects of Various Compounds in Serum on the Assay

As shown in Table I, albumin caused slight inhibition in these enzyme assays, while glucose, uric acid, ascorbic acid and bilirubin had no effect.

### Reproducibility and Day-to-Day Precision

Within-batch precision for the determination of these enzyme activities in the standard assay system was determined by analysis of same serum twenty times, and the coefficients of variation for ADA, PNP and guanase assays were  $1.1 \pm 0.38\%$ ,  $1.4 \pm 0.45\%$  and  $2.6 \pm 0.82\%$ , respectively ( $n=5$ ). Further daily studies of precision were carried out for twenty days and the coefficients of variation were  $0.7 \pm 0.32\%$ ,  $2.5 \pm 0.57\%$  and  $2.7 \pm 0.60\%$ , respectively ( $n=5$ ).

### Comparison of ADA and Guanase Activities Determined by the Proposed Method and the Conventional Method

The ADA activity determined by the proposed method and that determined by the con-

ventional method for the same sample were compared; the correlation coefficient between the two methods was 0.879. In the case of guanase activity, the correlation coefficient was 0.840.

### ADA, PNP and Guanase Activities in Healthy Human Serum

ADA, PNP and guanase activities in sera of normal subjects were measured. The normal ranges of serum ADA, PNP and guanase activities were  $5.8 \pm 2.2$  I.U./l,  $3.7 \pm 2.1$  I.U./l and  $0.5 \pm 0.3$  I.U./l (mean  $\pm$  S.D.), respectively.

### Discussion

There are some reports on the assay of ADA, PNP and guanase activities by spectrophotometric methods or ammonia methods. Although the ammonia methods by Okuda *et al.*<sup>4)</sup> are convenient for the assay of ADA and guanase activities in serum, they have the defect of requiring centrifugation. The spectrophotometric methods are also not really suitable for the assay of enzyme activities in serum, because in end-point assay, we observed relatively high blank values due to the uric acid or albumin in serum, and for rate assay, these methods do not offer satisfactory sensitivity.

There is no report on the assay of ADA, PNP and guanase assay based on the detection of hydrogen peroxide with XO as a coupling enzyme, but a spectrophotometric method has been reported, based on the detection of uric acid using XO.<sup>5,7)</sup> This may be workable because the electron transport of XO is of mixed type, forming hydrogen peroxide as well as superoxide anion radical, and this radical is a strong inhibitor of POD.<sup>9)</sup>

The proposed method involves stopping the enzyme reaction in the acidic region, then developing the color. Superoxide anion radical formed by XO was stoichiometrically converted to hydrogen peroxide by the addition of an acidic solution, because the superoxide anion radical is unstable in an acidic medium.<sup>10)</sup>

In conclusion, it was shown that the time courses of these enzyme activities and the calibration plots based on them were linear, passing through the zero point. The reproducibility of the assays was excellent and they were hardly affected by various compounds in the serum. The proposed methods were concluded to be precise, simple, rapid and convenient, and were satisfactory from a kinetic point of view.

### References and Notes

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