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10) This unusual splitting pattern is under investigation.

[Chem. Pharm. Bull. 29(5)1451—1455(1981)]

An Enzymic Method for the Determination of Inorganic Phosphate in Serum[1)]

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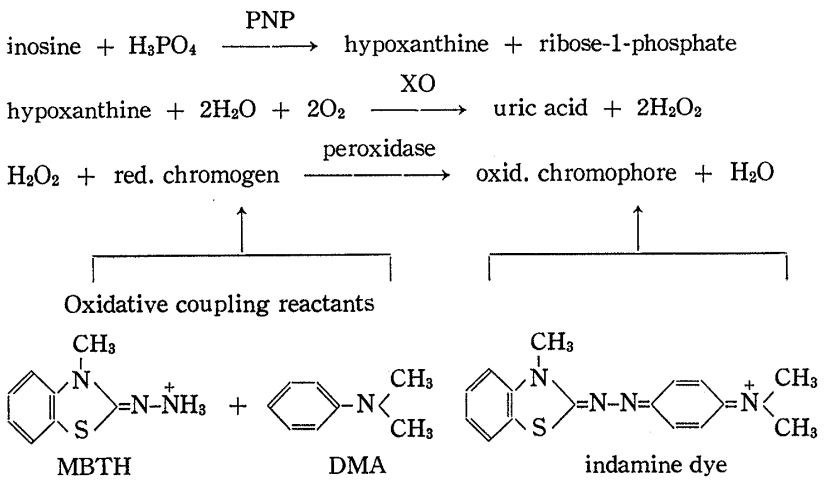
(Received November 19, 1980)

We have established an enzymic method for the determination of inorganic phosphate in serum, based on the formation of hydrogen peroxide with purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO).



The method was evaluated for precision, accuracy, sensitivity and specificity, and was concluded to be useful as a clinical test for the determination of inorganic phosphate in serum.

Keywords——inorganic phosphate; purine nucleoside phosphorylase; hydrogen peroxide; enzymic determination; xanthine oxidase

The determination of inorganic phosphate in serum can be useful in the diagnosis of renal function, parathyroid function, bone diseases, and so on.[2] Inorganic phosphate is increased in the sera of patients with hypoparathyroidism, giantism, and hypervitaminosis D, and decreased in the sera of patients with osteomalacia and hyperparathyroidism.[2]

The methods employed for the determination of inorganic phosphate can be classified into chemical methods and enzymic methods. The chemical methods are based on the principle of converting inorganic phosphate to phosphomolybdate, and in the acidic region, adding reducing agents to form a complex with this phosphomolybdate.[3-5] The methods currently used for the estimation of inorganic phosphate are the Fiske-Subbarow method[3] and some modified methods.[4] However, these methods require deproteinization by centrifugation after the addition of trichloracetic acid solution or dialysis.

In recent years, some sensitive methods using detergents have been reported.[5] However, these chemical methods have the limitation of requiring acidic conditions, leading to hydrolysis of unstable organic phosphates in biological materials.

On the other hand, some enzymic methods using glyceraldehyde-3-phosphate dehydrogenase,[6] phosphorylase a[7] and purine nucleoside phosphorylase[8] have been reported. These methods are spectrophotometric methods and so biological materials, for example uric acid and albumin in serum, cause high blank values. In fact, enzymic methods have not been used in clinical tests.

In this paper, we describe a colorimetric method for the determination of inorganic phosphate in serum based on the principle of forming hydrogen peroxide in the presence of purine nucleoside phosphorylase and xanthine oxidase.

Materials and Methods

Materials——Purine nucleoside phosphorylase (PNP) was purchased from Boehringer Mannheim Co. Ltd., peroxidase (POD) from Sigma Chemical Co., Ltd. and inosine, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), N,N-dimethylaniline (DMA) and sodium azide from Wako Pure Chemical Industries, Ltd. (Japan). Xanthine oxidase (XO) was purified from bovine milk by the method of Nathans and Kirby-Hade.[9]

Standard Assay of Inorganic Phosphate——A color reagent solution was prepared as follows before use; 9.6 mg of MBTH, 0.3 ml of DMA and 400 units of POD (one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 and at 20°) were dissolved in 100 ml of 0.2 M McIlvaine buffer (pH 3.5).

One ml of 0.2 M Tris-HCl buffer (pH 6.8) containing 0.3 mM sodium azide, 0.2 ml of 7.4 mM inosine solution and 10 μl of serum were mixed and preincubated at 37° for 5 min. One hundred μl of the enzyme mixture (PNP 625 mU/ml and XO 700 mU/ml) was added to the above incubation mixture and the whole was incubated at 37° for 30 min. One-half ml of 0.4 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.

Conventional Methods for the Determination of Inorganic Phosphate——Inorganic phosphate in serum was measured by the Fiske-Subbarow method[3] and by an enzymic method using glyceraldehyde-3-phosphate dehydrogenase.[6a]

Results

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

The concentrations of reagents in the color reagent solution and of the enzymes for the assay of inorganic phosphate were investigated in the standard procedure. The results are shown in Fig. 1.

The absorbance at 600 nm was maximum with concentrations of above 125 μg of MBTH, 2 μl of DMA and 2 units of POD per tube. On the other hand, the lowest concentrations of PNP and XO required to obtain maximum absorbance were 25 mU and 50 mU per tube, respectively.

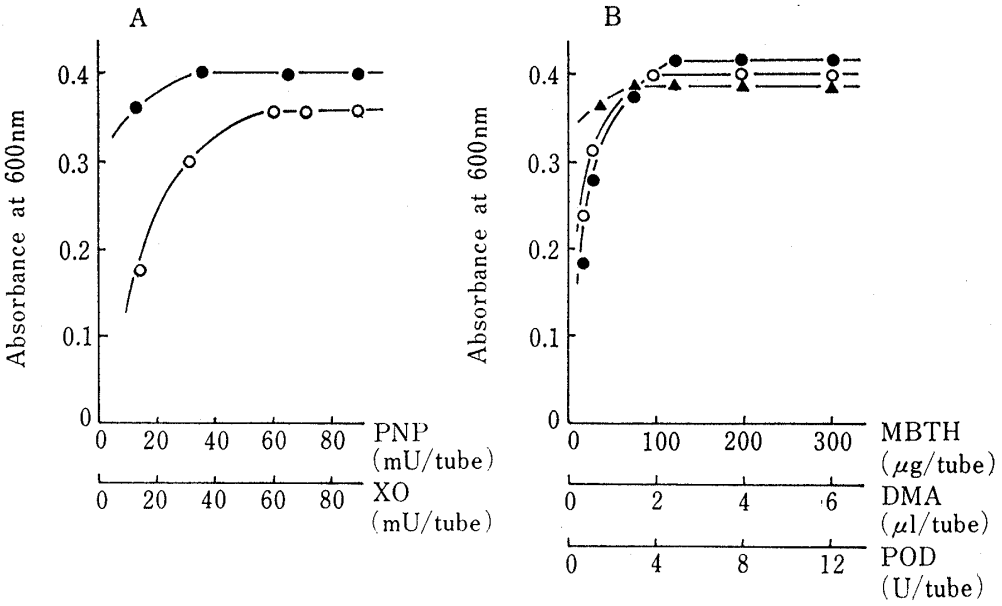


Fig. 1. Effects of the Concentrations of Enzymes and Color Reagents on the Final Absorbance

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

Calibration Curve

The calibration plots for inorganic phosphate were linear, passing through the zero point, and the absorbance at 600 nm was 1.0 with 7.8 μmol/ml of inorganic phosphate.

Kinetic studies were also carried out with human sera having 1.82 μmol/ml or 0.99 μmol/ml of inorganic phosphate. There was a linear relationship between the absorbance and the serum volume, and the enzyme reaction reached a plateau at 15 min in the standard method.

Recovery of Inorganic Phosphate in Serum

The recovery of 40 nmol per tube of inorganic phosphate in serum was assayed by the standard method. The average recovery of inorganic phosphate was 98.1±2.5%.

Effects of Various Compounds in Serum on the Assay

Table I shows that glucose, uric acid, ascorbic acid, bilirubin and albumin had no effect on the standard method.

Table I. Effects of Various Compounds in Serum on the Proposed Method

Compound	Amount (mg/dl)	Relative amount of phosphate (%)
None		100
Glucose	100	97
	200	97
Uric acid	5	100
	10	99
Ascorbic acid	1	100
	5	99
Bilirubin	1	99
Albumin	1000	97
	2000	98
	5000	99

Reproducibility and Day-to-Day Precision

Within-day precision for the determination of inorganic phosphate in serum by the standard method was tested, and the coefficient of variation was 1.27% (1.05 μmol/ml inorganic phosphate in serum). Further daily studies of precision were also carried out and the coefficient of variation was 2.23% (1.08 μmol/ml inorganic phosphate in serum).

Comparison of the Proposed Method and the Conventional Methods

Inorganic phosphate in various sera was determined by the standard method and the conventional methods, and the results are compared in Fig. 2. The correlation coefficients between the proposed method and the Fiske-Subbarow method or an enzymic method using glyceraldehyde-3-phosphate dehydrogenase were 0.994 and 0.998, and the linear regression equations were $Y=1.08X-0.095$ and $Y=0.977X+0.048$, respectively, indicating that there was good agreement between the proposed method and the conventional methods.

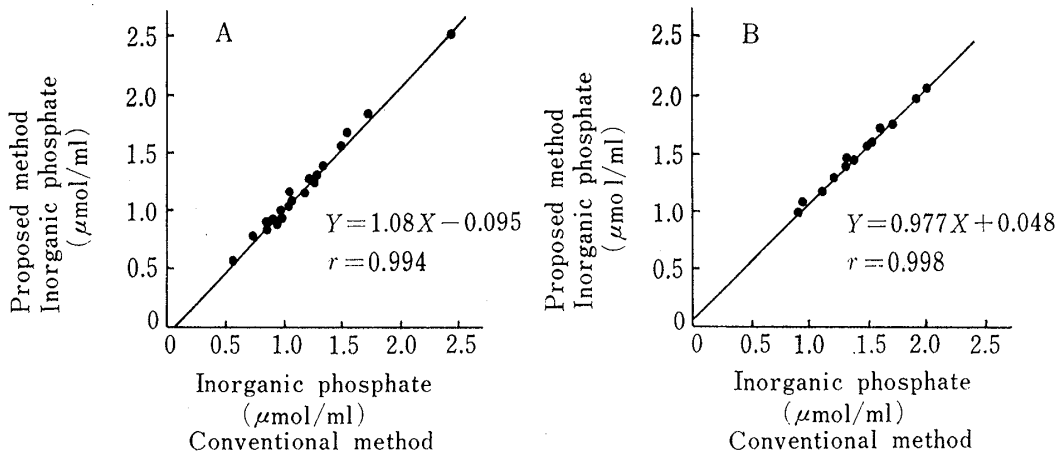


Fig. 2. Method Comparison

Inorganic phosphate in serum was measured by the proposed method and by conventional methods as follows: A, Fiske–Subbarow method; B, enzymic method with glyceraldehyde-3-phosphate dehydrogenase.

Discussion

There are various reports on the determination of inorganic phosphate by chemical methods or enzymic methods. Although the Fiske-Subbarow method and its modifications have been used for the determination of inorganic phosphate in serum and have been applied as routine tests by using an autoanalyzer, they have the defect of requiring centrifugation or dialysis for deproteinization.

Recently some enzymic methods have been reported, but these methods are not suitable for clinical tests, because they have relatively high blank values due to the biological materials in the serum, and are not satisfactory as regards sensitivity. The methods with glyceraldehyde-3-phosphate dehydrogenase, phosphorylase a and purine nucleoside phosphorylase are based on the production of NADH (molar extinction coefficient $\varepsilon=6.22\times10^3$), NADPH ($\varepsilon=6.22\times10^3$) and uric acid ($\varepsilon=9.0\times10^3$).

We have examined a new method for the determination of inorganic phosphate in this study. The calibration plot was linear and passed through the origin, and there was a linear relationship between the absorbance and the serum volume. The reproducibility was excellent and various compounds in the serum had no effect on the results. The amounts of inorganic phosphate determined by the proposed method and the conventional methods were in good agreement.

Moreover, the proposed method is more sensitive than the conventional enzymic methods, because the molar extinction coefficient of oxidized chromophore in the proposed method

($\varepsilon=3.5\times10^4$) is higher than that of the reaction products in the other enzymic methods.

In the proposed method, we added sodium azide to the substrate mixture to inhibit catalase present in biological materials. The concentrations of hypoxanthine and xanthine are very low in serum (1—2 μg/ml serum) and these compounds seem to have no effect on the proposed method in view of the good recovery.

Therefore it was concluded that the proposed method is precise and convenient.

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[Chem. Pharm. Bull. 29(5)1455—1458(1981)]

Calcitonin increases Serum Glucose Concentration independently of Insulin Secretion in Rats

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(Received November 12, 1980)

The effect of calcitonin (CT) on serum glucose and insulin secretion was investigated in rats. The subcutaneous administration of CT (80 MRC mU/100 g body weight) produced a significant increase in serum glucose, while it did not significantly alter serum insulin in fed rats. In addition, a marked elevation of insulin secretion after a single intraperitoneal administration of glucose (0.1 g/100 g) in fasted rats was not significantly altered by the treatment with CT. When both CT and somatostatin (250 μg/100 g) were subcutaneously administered simultaneously, the serum glucose level increased significantly compared with that of rats given somatostatin alone. The progressive increase in serum glucose caused by CT was significantly inhibited by the subcutaneous administration of insulin (0.1 U/100 g). These results indicate that CT increases serum glucose concentration independently of insulin secretion in rats.

Keywords——calcitonin; insulin; somatostatin; serum glucose; hyperglycemic effect of calcitonin

It is reported that calcitonin (CT) inhibits glucose uptake stimulated by insulin in the diaphragm muscle of rats, and that this effect of CT is not mediated by hypocalcemia.[1,2] Ziegler *et al.*[3] found that CT provoked a significant impairment of glucose assimilation and insulin output in man. Also, Passeri *et al.*[4] reported that the stimulation of insulin secretion by an intravenous glucose load in man was inhibited by intravenous infusion of CT. On the other hand, it is reported that CT impair glucose tolerance, failing to produce any significant