# The Determination of Alkaline Phosphatase Activity in Serum by Means of Flow-through Analysis with an Ion-selective Electrode

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In order to determine continuously the alkaline phosphatase activity in a serum sample, a potentiometric method using a newly prepared phosphate ion-selective electrode has been established by the authors for the first time. The alkaline phosphatase activity was determined by measuring the concentration of the phosphate ion which was liberated by the enzymatic hydrolysis of disodium phenyl phosphate in the presence of alkaline phosphatase. The measurement of the phosphate ion with an ion-selective electrode was seriously interfered with by protein and the halide ion, but the interference could be removed by covering the electrode surface with a cellulose acetate membrane and by separating the chloride ion from a serum sample by gel chromatography. The present method was applied to the determination of alkaline phosphatase activity in serum; the results obtained showed a satisfactory correlation with those obtained by the conventional method. The method was found to be useful for practical diagnosis based on the activity of alkaline phosphatase.

The present study was carried out with the object of establishing a simple, rapid, and economical method by which alkaline phosphatase activity(APA) in a transparent as well as an opaque sample solution could be determined continuously with a phosphate ion-selective electrode(PISE) by the use of an arbitrary substrate.

As can be seen from the following examples, the determination of APA is very important in various fields, such as biochemistry, analytical chemistry, clinical examination, and diagnosis: 1) APA in serum is significant for the diagnoses of cancer of the liver and malignant bone tumor, since there seems to be a close relationship between APA and the beforementioned diseases, and 2) the exact determination of APA is indispensable for the enzyme immunoassay, in which alkaline phosphatase is used as a labeling enzyme.

In the conventional determination of APA, the methods proposed by Bodansky,[1] Kind-King,[2] and Bessey-Lowry[3] have been used. All these methods are based on the absorptiometric determination of phosphoric acid,[1] phenol,[2] and *p*-nitrophenol,[3] which are formed by the enzymatic hydrolysis of the corresponding substrates in the presence of alkaline phosphatase. Nowadays, complicated autoanalyzers designed on the basis of these measurement principles are commercially available. These methods have, however, the following defects: 1) the measurement becomes complicated, since coloring and deproteinizing agents are added; 2) the fluctuation of the blank value is large, since the substrate is unstable; 3) the apparatus is complicated and expensive; 4) the method is not applicable to opaque samples, and 5) the concentration range suitable for the measurement is narrow, less than twenty times.

Though ion-selective electrodes have been used in several papers[4–11] for a simple and rapid estimation of enzyme activity, no reports have been published pertaining to the potentiometric determination of APA with a PISE. From this standpoint, the authors have newly made a PISE and applied it to the continuous determination of APA in serum with a simple and inexpensive apparatus; favorable results were obtained.

## Experimental

*Reagents.* The buffers and the base solution were prepared from analytical-grade reagents without further purification. A substrate solution was prepared as a $1.0\times10^{-2}$ mol/dm$^3$ disodium phenyl phosphate in a buffer solution (pH 10.15) consisting of disodium carbonate (3.18 g/dm$^3$) and sodium hydrogencarbonate (1.68 g/dm$^3$). The alkaline phosphatase was obtained from Sigma, and its standard solution was prepared by diluting 1.1 U/mg of alkaline phosphatase (Type 1) with distilled water. Sephadex G-25 was used as a packing agent in gel chromatography. Serum samples were offered by the Kyoto Prefectural University of Medicine; their APA values were certified to be around the normal values.

*Apparatus.* A PISE[12] in which trisilver phosphate was used as the active substance of a soild membrane was built up newly by the authors in order to estimate the amount of phosphate ions. A solid membrane composed of trisilver phosphate and disilver sulfide in the weight ratio of 4:1 was found to be the best with regard to the mechanical strength, the electromotive force of the electrode, the plasticity of the membrane, and the life of the electrode. The electrode responded to the phosphate-ion concentration in the range of $1.0\times10^{-5}$—$1.0\times10^{-1}$ mol/dm$^3$. The electrode has a serviceable life of at least six months. The determination of the phosphate ions was found to be seriously affected by chloride, thiocyanate, and sulfite. The other instrumental units used were as follows: 1) an Atto Perista mini-pump, type SJ 1211; 2) a thermostat manufactured by Netsudenshi Kogyo; 3) a Horiba 2535—05 T reference electrode, and 4) a model VP6521A recorder (Matsushita Communication Industrial Co., Ltd.).

*Procedure.* The determination of APA was done with the apparatus shown in Fig. 1. A 0.5-cm$^3$ serum sample is added with a pipette to the top($l$) of a $290\times8$ mm i.d. column($a$), which is then charged with Sephadex G-25 and cooled at 0 °C in a thermostat. By passing the substrate solution through the column at the rate of 0.5 cm$^3$/min, chloride and alkaline phosphatase are separated and eluted out of the column in the order shown in Fig. 3. The portion containing alkaline phosphatase is sent to a Teflon-made reaction tube by way of a four-way cock($c$) at the rate of 0.5 cm$^3$/min by means of the pump($b_1$). Since

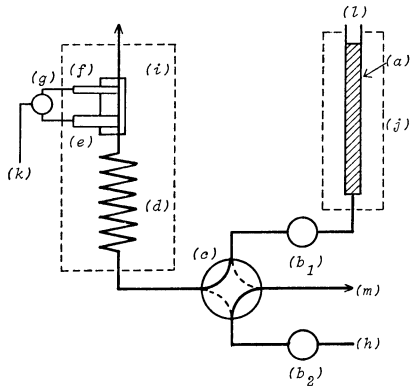


Fig. 1. Schematic flow diagram.
*a*: Gel column, $b_1$: pump, $b_2$: pump, *c*: four-way cock, *d*: reaction tube(Teflon), *e*: indicator electrode, *f*: reference electrode, *g*: voltmeter, *h*: inlet, *i*: thermostat (37.0 °C), *j*: thermostat (0 °C), *k*: recorder, *l*: sample, and *m*: exit.

the reaction tube, 10 m long and 1 mm i.d., is being held at 37.0±0.2 °C in a thermostat(*i*), the enzymatic hydrolysis of phenyl phosphate in the presence of alkaline phosphatase proceeds to form phosphate ions. The concentration of phosphate ions is measured with a voltmeter(*g*) as an electromotive force of a cell consisting of a PISE(*e*) and a reference electrode(*f*), and its value is recorded with a recorder (*k*). By turning a four-way cock(*c*) at a definite time after the addition of a serum sample, the portion containing chloride is removed by way of the exit(*m*), while the substrate solution is sent to the reaction tube by means of the pump($b_2$) by way of the inlet(*h*) at the rate of 0.5 cm³/min. The APA in serum is estimated from the calibration curve on the basis of the peak height on a recorder chart.

## Results and Discussion

*Interference of Serum Proteins.* Ion-selective electrodes for chloride and silver ions in which disilver sulfide is used as a constituent in an active solid membrane have been reported[13,14] to be interfered with by about 6% proteins consisting of albumin, globulin, and so on in serum. Since the present PISE is made of trisilver phosphate and disilver sulfide, it was expected to be interfered with by serum proteins. As can be seen from Fig. 2, the present PISE was interfered with by albumin in bovine serum (Fig. 2(d)). Therefore, the PISE is not applicable to the determination of APA in serum without removing the interference of protein.

It has been already reported[15] that the interference of protein can be removed by covering a electrode with a semi-permeable membrane. Several membranes were tested, but the best result was obtained when a cellulose acetate membrane (0.0203 mm thick, 2.4 nm pore size) was used; the interference of albumin was thus almost completely removed (Fig. 2(b)). In all the following experiments, the PISE covered with a cellulose acetate membrane was used.

*Removal of Chloride.* As can be foreseen from the solubility product of silver chloride and silver thiocyanate, the PISE is seriously interfered[12] with by chloride and thiocyanate ions. Since the concentration of chloride in serum is about 0.1 mol/dm³,

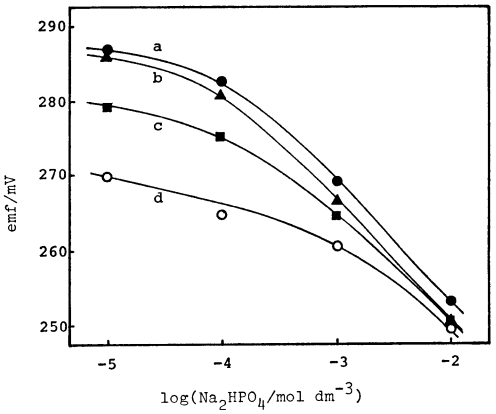


Fig. 2. Response of the PISE in sample solutions containing albumin.
a: Albumin free, b: covered with cellulose acetate, c: covered with nitrocellulose, and d: not covered with a membrane.

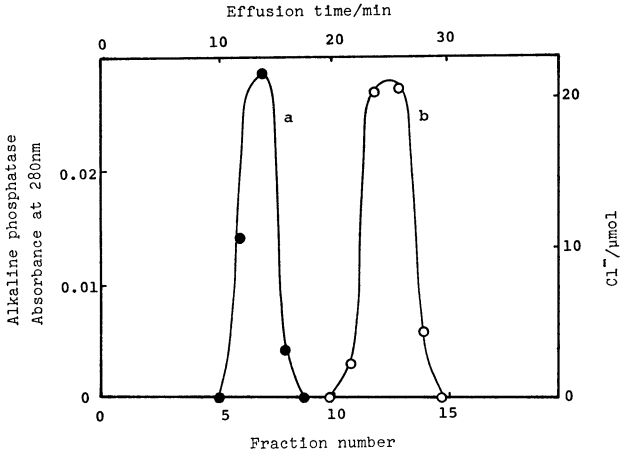


Fig. 3. Gel separation of alkaline phosphatase from a chloride ion on a Sephadex G-25 column.
a: Alkaline phosphatase, b: chloride ion.

the removal of chloride is indispensable for the determination of APA in serum.

The separation of alkaline phosphatase from chloride was examined by means of gel chromatography. A 0.5-cm³ sample solution containing sodium chloride (0.1 mol/dm³) and alkaline phosphatase (1 mg/cm³) was treated in order to separate its components by passing a buffer solution through the column at the rate of 0.5 cm³/min; the results are shown in Fig. 3. Here the flow rate of the buffer solution was chosen to be maximal so long as the gel particle was not crushed. The amounts of alkaline phosphatase and chloride in each 1-cm³ fraction were determined by the absorption at 280 nm and by the precipitation titration based on Mohr's procedure respectively. On the basis of Fig. 3, the following procedure is recommended for the separation of alkaline phosphatase from chloride: the eluent obtained from the column (Fig. 1(*a*)) in 0—16 min is introduced into the reaction tube(*d*), and the eluent obtained after 16 min is drawn out of the exit(*m*) by turning a four-way cock(*c*). By this procedure, the portion of chloride-free alkaline

phosphatase is sent to a measuring chamber equipped with a PISE.

*Calibration Curve.* The hydrolysis of disodium phenyl phosphate in the presence of alkaline phosphatase proceeds in accordance with Eq. 1:

$$C_6H_5OPO_3Na_2 + H_2O \xrightarrow{\text{phosphatase}} C_6H_5OH + Na_2HPO_4. \quad (1)$$

It is possible to estimate the APA by measuring the formation rate of phosphate ions, since the APA is generally represented by the reaction rate of Eq. 1. If the reaction of Eq. 1 proceeds as zero-order with respect to the concentration of disodium phenyl phosphate, the reaction rate of Eq. 1 is given by the concentration of phosphate ions at a definite time. To examine whether or not the reaction of Eq. 1 proceeds as a zero-order one, a PISE was dipped in a $1.0\times10^{-2}$ mol/dm$^3$ disodium phenyl phosphate solution which contained 5.5—110 U/dm$^3$ alkaline phosphatase and was held at 37.0 °C in a thermostat; the relationship between the reaction time and the concentration of phosphate ions was thus obtained (Fig. 4). It may be concluded from Fig. 4 that the reaction of Eq. 1 proceeds as zero-order with respect to the concentration of disodium phenyl phosphate, since there is a linear relationship between the reaction time and the concentration of phosphate ions for the APA values of more than 20 U/dm$^3$. The present results, obtained at $10^{-2}$ mol/dm$^3$ disodium phenyl phosphate, seem to be quite reasonable considering that the Km value in the reaction of Eq. 1 is around $10^{-3}$ mol/dm$^3$.[16]

A calibration curve (Fig. 5) was obtained as a peak height against each standard solution of alkaline phosphatase. The curvature corresponding to 2.2—22 U/dm$^3$ alkaline phosphatase in the calibration curve is attributed to: 1) the deviation of an electrode potential from the Nernst equation at $10^{-4}$—$10^{-5}$ mol/dm$^3$ phosphate ion (Fig. 2(a)); 2) the deviation of the enzyme reaction rate from a zero-order one for the APA values less than 20 U/dm$^3$, 3) the broadening of a phosphate ion peak by the diffusion and/or mixing in a flowing solution, etc. For the practical determination of the APA, the part of the straight line in the calibration curve can be used satisfactorily, since the APA in serum is usually more than 20 U/dm$^3$.

*Effect of Serum Matrix.* To examine whether or not the present method can be applied to the determination of the APA in serum, different APAs were added to 0.5 cm$^3$ control serum and their APAs were estimated by the present method; the result thus obtained are shown in Fig. 6. Figure 6 shows that, there was a good relationship between the activities of the added alkaline phosphatase and the potentiometrically measured ones. The value at the intersection of the straight line on the abscissa in Fig. 6 corresponded exactly to the APA in the control serum. It became clear from the results that the present method could be satisfactorily applied to the determination of the APA in serum.

*Serum Samples.* The relationship between the APA values in serum samples measured by the present method and those measured by means of a Hitachi autoanalyzer, type 400, based on absorptiometry was examined (Fig. 7). As can be seen from Fig. 7, there was a fairly good correlation between them; the correlation factor was calculated to be 0.88.

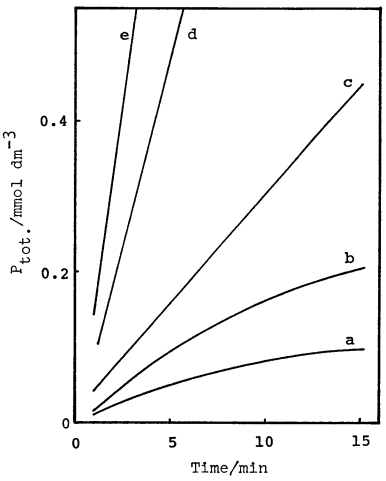


Fig. 4. Relationship between reaction time and $P_{tot.}$. Measured at 37.0 °C in a $1.0\times10^{-2}$ mol/dm$^3$ disodium phenyl phosphate solution containing alkaline phosphatase.
a: 5.5 U/dm$^3$, b: 11 U/dm$^3$, c: 22 U/dm$^3$, d: 55 U/dm$^3$, e: 110 U/dm$^3$. $P_{tot.}=H_3PO_4+H_2PO_4^-+HPO_4^{2-}+PO_4^{3-}$.

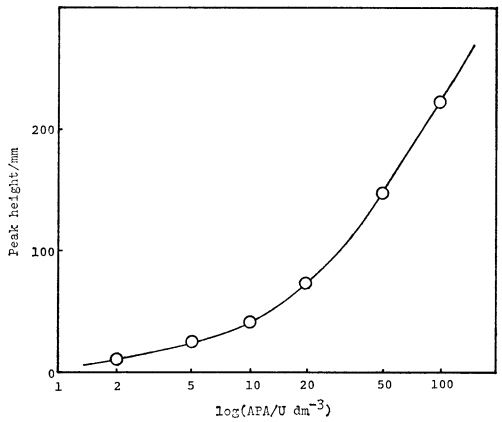


Fig. 5. Calibration curve.

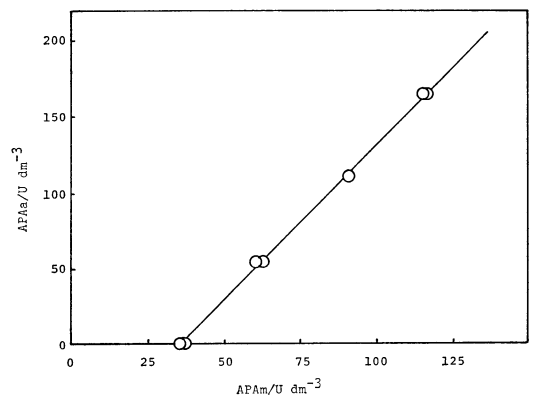


Fig. 6. Relationship between potentiometrically measured APA(APAm) and added APA(APAa).

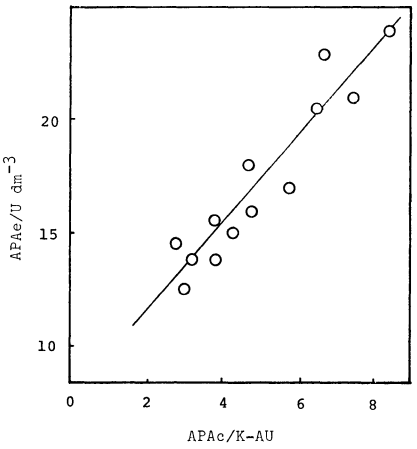


Fig. 7. Correlation between the APAs in serum samples measured by the PISE method(APAe) and by the conventional method(APAc).

Judging from the facts that: 1) the APA in serum sample used in this experiment corresponds to that of alkaline phosphatase in a healthy human, and 2) the APA in a liver patient is much larger than that value, the present method can be said to be sensitive enough for a practical diagnosis based on the APA.

According to the present method, it takes about 30 min for the analysis of one sample. However, many samples can be treated effectively and continuously by arranging a number of columns in parallel with column(*a*), because the time-consuming step in the present method is the separation of alkaline phosphatase from chloride ions. A system by which information concerning APA is obtained from a much smaller serum sample is now being developed.

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