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Use of various types of column reactors for flow-injection analysis

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ABSTRACT

Two or three different kinds of immobilized enzymes can be aligned in a minireactor so that sequential enzymatic reactions are carried out from upstream to downstream during flow-injection analysis. A lactate oxidase–catalase reactor, used as a precolumn for removing pre-existing lactate in serum before the lactose dehydrogenase (LDH) reaction, was useful for the determination of serum LDH activity, which did not require any blank correction. A sequential glutamate dehydrogenase–glutamate oxidase reactor was also useful for a novel chemiluminometric determination of ammonia. On the other hand, a co-immobilized creatininase–creatinase–sarcosine oxidase reactor, in spite of containing creatininase which catalyses the reversible reaction, was the most efficient for the determination of serum creatinine.

INTRODUCTION

The usefulness of enzymes with biological recognition as reagents for clinical analysis has been well documented. As an enzyme is a biocatalyst, an enzyme reagent should be capable of being used repeatedly if it could be separated from other reactants and recovered from the reaction mixture after each reaction cycle. This requirement can easily be fulfilled by employing immobilized enzymes as reagents [1].

A bioreactor may be defined as a reactor whose operation depends on the biological function of its reaction elements; immobilized enzymes are now being used as such elements of a reactor. A column reactor is packed with porous glass beads on to which the enzyme is immobilized. The combined use of an immobilized enzyme column reactor and a chemiluminometric detection in a flow-injection analysis (FIA) [2] system has made it possible to minimize the sample volume and analysis time [3]. One enzyme alone or two or more different kinds of enzymes are used as reagents in the determination of the constituents of biological fluids, especially blood and urine [4]. For example, one enzyme alone is used for glucose or uric acid and three kinds of

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enzymes are used for urea, creatinine and total cholesterol.

When a reactor with two or more kinds of immobilized enzymes is used for clinical analysis, the order of the respective immobilized enzymes in a minicolumn has a great influence on the efficiency of the column reactor in a FIA system, because the solution is always forced to flow from upstream to downstream unidirectionally through the reactor, without a noticeable backward flow of solutes. In this work, we compared the efficiencies of three different types of column reactors, sequential, mixed-bed and co-immobilized enzyme reactors, with two or three kinds of immobilized enzymes in clinical analysis.

EXPERIMENTAL

Apparatus

A FIA chemiluminometric system with a bioreactor was prepared using a luminophotometer, a 1-μl sample injector, an AutoAnalyzer II proportionating pump (Technicon Instruments, Tarrytown, NY, USA) and a Chromatopack C-2RAX data processor (Shimadzu, Kyoto, Japan) (Fig. 1).

Materials

The enzymes used were glucose oxidase (GOD) (*Aspergillus niger*), urease (jack beans), glutamate dehydrogenase (GLDH) (*Proteus species*), creatininase (CNH, creatinine amidohydrolase) (*Pseudomonas species*) and creatinase (CRH, creatine amidinohydrolase) (*Pseudomonas species*) from Toyobo (Osaka, Japan), pyranose oxidase (*Polyporus obtusus*) from Takara Shuzo (Kyoto, Japan), lactate oxidase (LOD) (*Pediococcus* sp.) from Toyojozo (Tokyo, Japan), catalase (beef liver) from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan), L-glutamate oxidase (GLXD) (*Streptomyces* sp.) from Yamasa Shoyu (Choshi, Chiba, Japan) and sarcosine oxidase (SAO) (*Corynebacterium* sp.) from Kyowa Medex (Tokyo, Japan). NADPH was obtained from Kohjin (Tokyo, Japan). All other chemicals were of analytical-reagent grade. Porous alkylamine glass (particle size 125–175 μm, pore size 50 nm, 0.17 mequiv. functional amino groups per gram) was obtained from Pierce (Rockford, IL, USA).

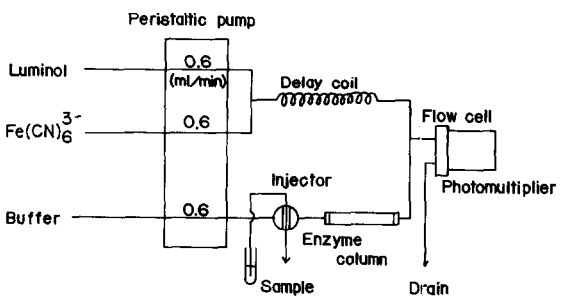


Fig. 1. Flow diagram of a FIA chemiluminescence system for glucose, ammonia, urea and creatinine, using immobilized enzyme column reactors. Buffers: 10 m*M* phosphate buffer, pH 7.5 for glucose and pH 8.5 for creatinine; 10 m*M* phosphate buffer (pH 7.5) containing 0.54 m*M* EDTA disodium salt, 0.14 m*M* α-oxoglutarate and 330 μM NADPH for ammonia and urea.

Reagents

For the determination of chemiluminescence, a 0.7 m*M* luminol working solution and a 20 m*M* potassium hexacyanoferrate(III) working solution were prepared as described previously [3]. A 10 m*M* phosphate buffer (pH 7.5) was used for the determination of glucose and a 10 m*M* phosphate buffer (pH 8.5) for creatinine. For the determination of lactate dehydrogenase (LDH) activity, a 10 m*M* phosphate buffer (pH 7.5) containing 1.2 m*M* sodium pyruvate and a 0.12 m*M* NADH solution prepared by dissolving NADH in 5.0 m*M* phosphate buffer (pH 8.5) were used [5]. For ammonia [6] and urea [7] determinations, a 10 m*M* phosphate buffer (pH 7.5) containing 0.54 m*M* EDTA disodium salt, 0.14 m*M* α-oxoglutarate and 330 μM NADPH was used.

Preparation of immobilized enzymes

The enzymes were immobilized by coupling them to porous alkylamine glass beads with glutaraldehyde through Schiff base formation according to Weetall's method [8].

Analytical procedure

The enzyme-bearing glass beads were packed in a small plastic tube according to a procedure described previously [9]. Fig. 1 illustrates the flow diagram for the FIA system using an immobilized enzyme column reactor and chemiluminescence detection. As a considerable amount of light was emitted by admixing luminol and hexacyanoferrate(III) solutions only, a delay coil of suitable length was in-

troduced to ensure the decay of such transient emission before the mixture came into contact with hydrogen peroxide. Analysis was performed at room temperature and within 10 s. The sample volume used was 1 μl, which was injected with a rotary valve sample injector. The total chemiluminescence emission was determined by measuring the area under the light–time curve.

RESULTS AND DISCUSSION

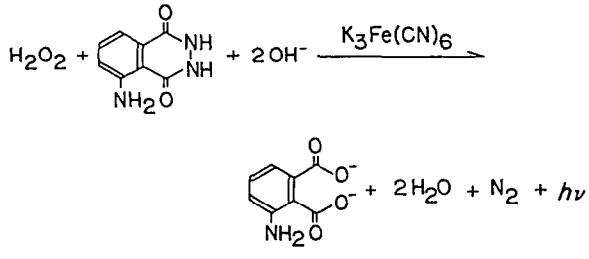
Size of column reactor required for determination of serum glucose

Glucose is a typical constituent of serum and can be determined by using one enzyme alone, *e.g.*, pyranose oxidase or GOD. Each enzyme catalyses the following reactions:

$$\text{D-glucose} + O_2 \xrightarrow{\text{pyranose oxidase}} \text{D-glucosone} + H_2O_2$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2$$

The chemiluminescence emitted as a result of the reaction of the hydrogen peroxide produced by each oxidase with a mixture of luminol and hexacyanoferrate(III) was determined with a luminophotometer:



The smallest amounts of pyranose oxidase and GOD required to determine glucose in serum were examined. Fig. 2 shows the linearity of the glucose calibration graphs depending on the length of the pyranose oxidase column and the GOD column of 0.5 mm I.D. The length of the columns was varied from 2.0 to 4.0 mm for the pyranose oxidase column and from 4.0 to 8.0 mm for the GOD column. The widest linear range was obtained with a 4.0 mm column for pyranose oxidase and an 8.0 mm column for GOD. For example, the linear range obtained with a pyranose oxidase column measuring 2.0 × 0.5 mm I.D. became too narrow, because the amount of glucose in a 1-μl sample exceeded that of immobilized pyranose oxidase packed in the column. Therefore, when glucose standard solutions diluted twofold beforehand were determined with a pyranose oxidase column measuring 2.0 × 0.5 mm I.D., the calibration graph obtained was linear over a wide range (Fig. 3).

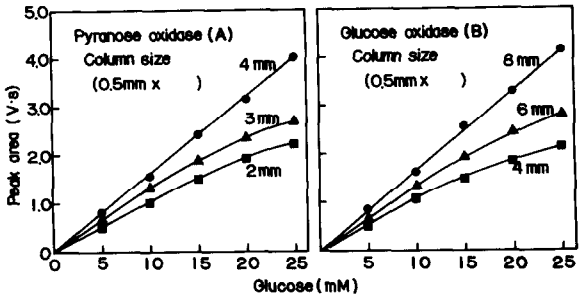


Fig. 2. Linearity of glucose calibration graphs depending on the length of (A) a pyranose oxidase column and (B) a GOD column, both of 0.5 mm I.D., obtained using the system shown in Fig. 1.

Typical recordings obtained with the glucose standard solutions and serum samples are shown in Fig. 3. The recordings were sufficiently discrete to ensure almost no carryover between consecutive assays. Serum glucose results obtained with the pyranose

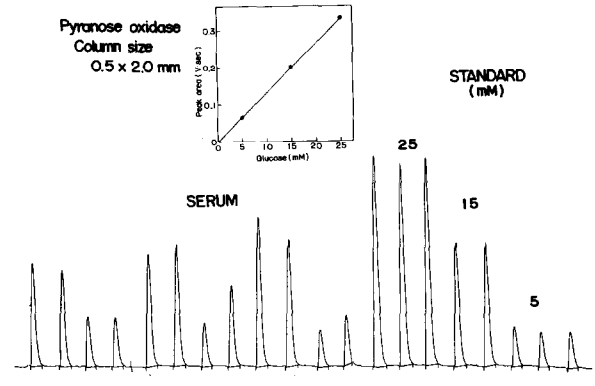


Fig. 3. Determination of glucose, using a FIA chemiluminescence system with an immobilized pyranose oxidase reactor measuring 2.0 × 0.5 mm I.D. Chart recorders traces for the glucose standard solutions and serum samples and plots for calibration (inset) are shown.

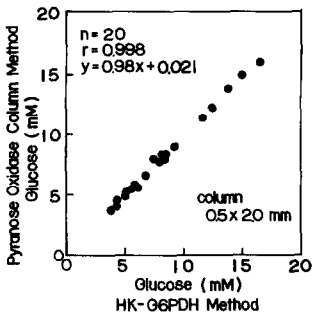


Fig. 4. Correlation between the results of a serum glucose assay obtained by the chemiluminometric method, using an immobilized pyranose oxidase reactor (2.0 × 0.5 mm I.D.) in the FIA system, and results obtained by the soluble hexokinase (HK)–glucose-6-phosphate dehydrogenase (G6PDH) method, using a CentrifiChem Model 400 centrifuge automatic analyser.

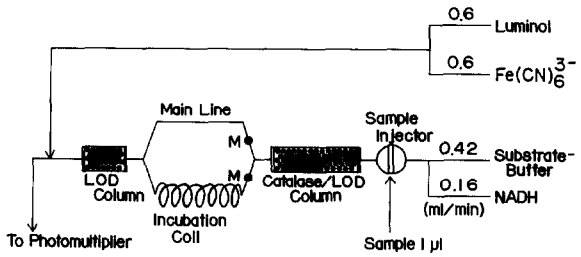


Fig. 5. Flow diagram of a FIA chemiluminescent system for the determination of LDH activity, using an immobilized LOD column reactor and an immobilized LOD–catalase reactor. M = Solenoid-controlled valve. LOD–catalase column: co-immobilized or sequential enzyme column measuring 40 × 2.0 mm I.D., with a mass ratio of LOD to catalase of 3:2. LOD column: 20 × 1.0 mm I.D.

oxidase column (2.0 × 0.5 mm I.D.) method compared well over a wide range of glucose concentrations with the results obtained by the soluble hexokinase (HK)–glucose-6-phosphate dehydrogenase (G6PDH) method [10] using a CentrifiChem Model 400 centrifugal automatic analyser (Fig. 4).

Hence the combined use of a column reactor and chemiluminometric detection in a FIA system made the determination of serum glucose possible with a very small reactor (2.0 × 0.5 mm I.D.).

Usefulness of a precolumn in the determination of serum LDH activity [5]

The LDH activity in serum was determined by measuring, with a LOD column reactor, the lactate produced according to the LDH reaction using pyruvate as a substrate:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

$$\text{lactate} + \text{O}_2 \xrightarrow{\text{LOD}} \text{pyruvate} + \text{H}_2\text{O}_2$$

As lactate pre-exists in serum, this pre-existing lactate should be removed before a serum sample is introduced into an incubation coil for the LDH reaction. This could be achieved by placing a precolumn of immobilized LOD–catalase in the position shown in Fig. 5. This catalyses the reactions

$$\text{lactate} + \text{O}_2 \xrightarrow{\text{LOD}} \text{pyruvate} + \text{H}_2\text{O}_2$$

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2$$

Columns of three different types, which were used as precolumns for removing pre-existing lactate in serum, were prepared with immobilized LOD and immobilized catalase. The size of the column, 40 × 2.0 I.D. mm, was identical for each type. Column type I, a co-immobilized enzyme column, contained glass beads to which LOD and catalase were co-immobilized. Column type II, a mixed-bed enzyme column, was prepared by packing a minicolumn with a mixture of two kinds of glass beads, one bearing LOD and the other catalase, each immobilized individually. Column type III, a sequential enzyme column, was prepared by packing a minicolumn with LOD–glass beads and catalase–glass beads to make two layers. The weight ratio of LOD to catalase in columns of the three different types was 2:3, 1:1, 3:2 and 2:1, respectively, in terms of total mass of enzyme protein.

The capacity of the three types of columns for removing pre-existing lactate was examined by sequential use of these columns and a LOD column (20 × 1.0 mm I.D.) by injecting standard solutions of lactate into the system. The results obtained are summarized in Table I. For the mass ratio, the co-immobilized and sequential enzyme columns, which showed almost the same capacity, had a higher capacity than the mixed-bed enzyme column. As the former columns, which measured 40 × 2.0 mm I.D. and had a mass ratio of LOD to catalase of 2:1, had a higher LOD to catalase ratio than other columns, hydrogen peroxide, which was not removed by the LOD–catalase columns, eluted from these columns in place of pre-existing lactate at a concentration

TABLE I

REMOVABILITY OF LACTATE BY A LOD–CATALASE COLUMN (40 × 2.0 mm I.D.)

Method	LOD to catalase mass ratio	Removability (mmol/l)
Co-immobilized	2:3	4.40
	1:1	5.36
	3:2	6.04
	2:1	5.89
Mixed-bed	2:3	3.52
	1:1	4.30
	3:2	5.20
	2:1	4.84
Sequential	2:3	4.48
	1:1	5.53
	3:2	6.00
	2:1	5.75

higher than the *ca.* 5.9 m*M* of serum lactate. Therefore, the co-immobilized and the sequential enzyme columns that measured 40 × 2.0 mm I.D. and had a mass ratio of LOD to catalase of 3:2 were selected as the best columns, having the capacity to remove lactate up to 6.0 m*M*, whereas those columns measuring 20 × 2.0 mm I.D. had the capacity to remove lactate up to 3.3 m*M*.

We thus succeeded in developing a new system of highly sensitive FIA–chemiluminometry for the determination of serum LDH activity which did not require any blank correction when an LOD–catalase reactor was used as a precolumn.

Usefulness of a sequential enzyme column rector in FIA chemiluminometry for ammonia [6] and its application [7]

The chemiluminometric method for the determination of ammonia using GLDH and GLXD is based on the following sequence of reactions:

$$NH_3 + \alpha\text{-oxoglutarate} + NADPH + H^+ \xrightarrow{GLDH} \text{L-glutamate} + NADP^+$$

$$\text{L-glutamate} + O_2 \xrightarrow{GLXD} \alpha\text{-oxoglutarate} + NH_3 + H_2O_2$$

Ammonia is converted into L-glutamate by the GLDH reaction, and the L-glutamate formed is further oxidized by GLXD to produce hydrogen peroxide, which can be determined by a chemiluminometric method using luminol and hexacyanoferrate (III). It is essential that the GLDH and GLXD reactions proceed in sequence in this order, otherwise one of the final products, ammonia, could be utilized as the substrate for GLDH so that no stoichiometry could be obtained between ammonia and hydrogen peroxide. The overall apparent conversion of ammonia into hydrogen peroxide was attainable only by employing a flow-type system such as FIA with an enzyme reactor containing both immobilized GLDH and GLXD aligned in a serial manner such that the reactants were always forced to flow from upstream (GLDH) to downstream (GLXD). Such flow minimized the possibility of back-diffusion of ammonia produced by GLXD.

An immobilized GLDH–GLXD reactor for the determination of ammonia was prepared by packing two kinds of enzyme-bearing glass beads, one bearing GLDH and the other GLXD, in a plastic tube, (20 mm × 2 mm I.D.) to make two layers, 12 mm long for GLDH and 8 mm for GLXD. The GLDH–GLXD reactor method showed linearity up to 12 m*M* ammonia in the system shown in Fig. 1. Fig. 6 shows the pH optima of soluble and immo-

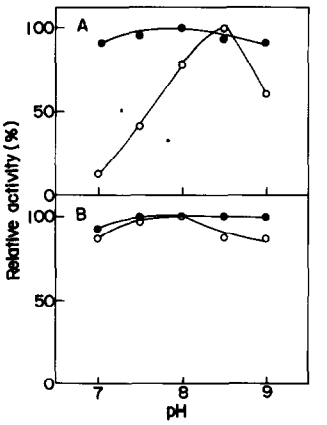


Fig. 6. pH profiles of GLDH and GLXD. (A) Soluble enzymes; (B) immobilized enzymes. ○ = GLDH; ● = GLXD. The activity of GLDH was determined by measuring the change in absorbance of NADPH at 340 nm, using 0.3 m*M* NH_4Cl as the substrate. The activity of GLXD was determined at a substrate (L-glutamate) concentration of 0.3 m*M*. The activity of immobilized GLDH or GLXD was determined with the same FIA system as that shown in Fig. 1, except that each column contained a single enzyme instead of two enzymes.

bilized GLDH–GLXD. The pH profile for immobilized GLXD was almost the same as that for soluble GLXD, whereas the pH profile for immobilized GLDH showed a very significant expansion toward the acidic side as compared with that for soluble GLDH [6]. The apparent K_m value for L-glutamate of immobilized GLXD, 0.28 m*M* at pH 7.5, was found to be fairly close to that of the soluble enzyme, which was 0.22 m*M* at pH 7.5, while the apparent K_m value for ammonia of immobilized GLDH, 0.08 m*M* at pH 7.5, was much lower than that of the soluble enzyme, which was 3.3 m*M* at pH 7.5 [6]. Therefore, pH 7.5 was selected for the optimum reaction of the two enzymes with maximum chemiluminescence intensity.

Serum urea nitrogen is one of the most common items requiring clinical analysis. We attempted to determine urea in serum by placing an additional immobilized urease column just in front of the GLDH–GLXD reactor. The urease–GLDH–GLXD column used measured 30 × 1.0 mm I.D., and was prepared by packing three kinds of enzyme-bearing glass beads in three layers, 2 mm long for urease, 20 mm for GLDH and 8 mm for GLXD. Typical recordings obtained with the urea standard solutions using the urease–GLDH–GLXD reactor are shown in Fig. 7. Linearity was obtained up to 30 m*M* urea. The recordings were reproducible and were discrete enough to ensure almost no carryover between two assays, in spite of the progress of three kinds of enzymatic reactions in a long column.

Serum urea results obtained using the urease–

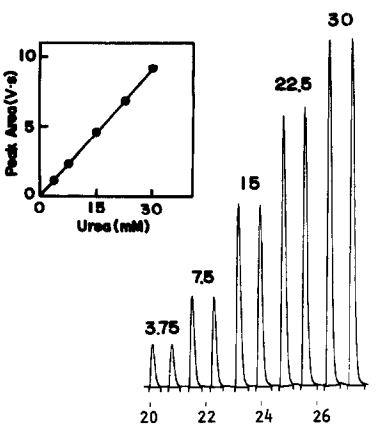


Fig. 7. Analytical trace of a chemiluminometric FIA standard assay for urea and a calibration graph (inset). Time in min.

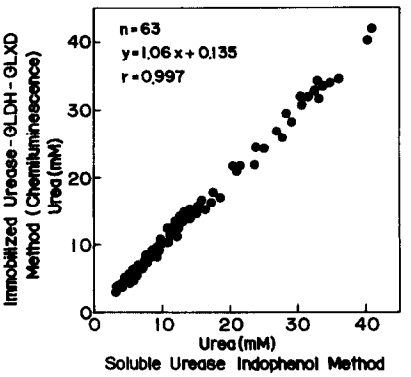


Fig. 8. Correlation between the results of serum urea assay obtained by the chemiluminometric method using an immobilized urease–GLDH–GLXD column reactor and the results obtained by the urease–indophenol method, using a Hitachi Model 726 discrete automatic analyser.

GLDH–GLXD reactor method compared well over a wide range of urea concentrations with results obtained by the urease–indophenol method [11] (Fig. 8). There was an analytical bias due to ammonia and L-glutamate present in serum in the GLDH–GLXD reactor method [5]. The concentrations of ammonia and L-glutamate in normal human serum are known to be *ca.* 0.05 and 0.17 m*M*, respectively [12]. For the analysis of human serum samples, therefore, an immobilized urease–GLDH–GLXD reactor had to be designed to produce ammonia at levels at least 20 times higher than serum levels of ammonia and/or L-glutamate. The urease portion of the reactor ensures the production of a sufficient amount of ammonia from urea, so that the method does not require any blank correction.

The urease–GLDH–GLXD reactor was found to retain 80% of its original activity for 4 weeks, even after at least 1000 runs.

Usefulness of a co-immobilized enzyme column reactor in the FIA chemiluminometric method for determination of serum creatinine [13]

The chemiluminometric method for determination of serum creatinine using CNH, CRH and SAO is based on the following sequence of reactions:

$$\text{creatinine} + H_2O \overset{\text{CNH}}{\rightleftharpoons} \text{creatine}$$

$$\text{creatine} + H_2O \xrightarrow{\text{CRH}} \text{sarcosine} + \text{urea}$$

$$\text{sarcosine} + O_2 \xrightarrow{\text{SAO}} \text{glycine} + \text{HCHO} + H_2O_2$$

As CNH, unlike CRH and SAO, catalyses the reversible reaction [14], it might be considered that the reaction rate of creatinine is influenced by the concentration of pre-existing creatine in serum. When using soluble CNH, CRH and SAO in combination, the concentration of creatinine in serum can be determined by subtracting the concentration of pre-existing creatine in serum, which is obtained after the sequential reactions of CRH and SAO are completely finished, from the concentration of creatinine plus creatine in serum, which is obtained after the sequential reactions of CNH, CRH and SAO are completely finished. However, in a FIA system with an immobilized CNH–CRH–SAO reactor through which a serum sample passes in *ca.* 1 s, it is impossible to convert all the creatinine in serum to hydrogen peroxide by the sequential reactions of the three enzymes, especially when the reactor contains CNH which catalyses the reversible reaction. Therefore, we examined a column reactor from which we could determine serum creatinine by measuring the reaction rate of CNH independently of the concentration of pre-existing creatine in serum in a FIA system, even when the reversible reaction was included in the sequential reactions of the three enzymes.

Four different types of equal-sized columns, measuring 30 × 2.0 mm I.D., were prepared with immobilized CNH, CRH and SAO. Column type 1 was a co-immobilized enzyme column, column type 2 was a sequential enzyme column, column type 3 was a mixed-bed enzyme column and column type 4 was a mixed-bed–sequential enzyme column, prepared by packing the upstream 70% of a minicolumn with a mixture of CNH–glass beads and CRH–glass beads at a mass ratio of 2:5, and the downstream 30% with immobilized SAO. The mass ratio of CNH, CRH and SAO in the four types of columns was 2:5:3, in terms of total mass of enzyme protein. Standard solutions of sarcosine, creatine, creatinine and creatinine containing 0.1 or 0.5 m*M* creatine were injected into the columns, and the intensity of the chemiluminescence emitted was recorded.

With all five kinds of standard solutions, the chemiluminescence was most intense in the co-immobilized CNH–CRH–SAO colomn and least intense in the mixed-bed CNH–CRH–SAO column. The chemiluminescence intensity of the mixed-bed CNH–CRH–sequential SAO column was much less with the standard creatinine solutions than with the standard sarcosine or creatine solutions, compared with other types of columns.

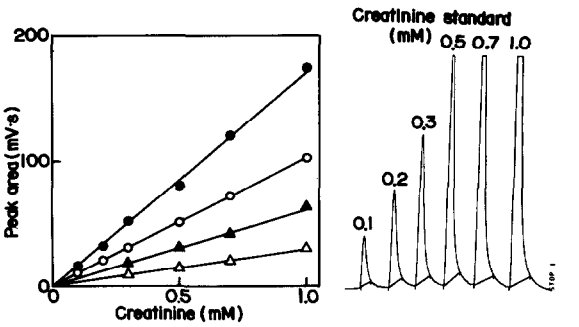


Fig. 9. Calibration graph (left) and recorder trace (co-immobilized CNH–CRH–SAO column) (right) for creatinine obtained with a FIA chemiluminescence system using immobilized enzyme columns. ● = Co-immobilized CNH–CRH–SAO column; ○ = sequential CNH–CRH–SAO column; ▲ = mixed-bed CNH–CRH–sequential SAO column; △ = mixed-bed CNH–CRH–SAO column.

The slope of the calibration graph for the co-immobilized CNH–CRH–SAO column when exposed to the standard creatinine solutions with 0.5 m*M* creatine was almost the same as the slope when exposed to the standard solutions with creatinine alone (Figs. 9 and 10). In contrast, the slope of the

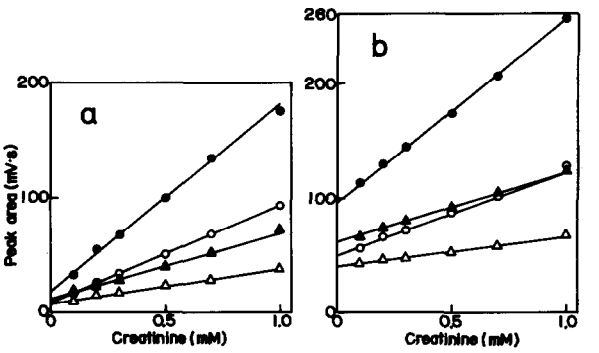


Fig. 10. Calibration graphs for creatinine, containing (a) 0.1 and (b) 0.5 m*M* creatine, obtained with a FIA chemiluminescence system using immobilized enzyme columns. ● = Co-immobilized CNH–CRH–SAO column; ○ = sequential CNH–CRH–SAO column; ▲ = mixed-bed CNH–CRH–sequential SAO column; △ = mixed-bed CNH–CRH–SAO column.

calibration graph for the sequential CNH–CRH–SAO column when exposed to the standard creatinine solutions with 0.5 m*M* creatine was less steep than the slope when exposed to the standard solutions with creatinine alone. Creatine influenced the sequential column but not the co-immobilized column (Figs. 9 and 10). Hence the co-immobilized CNH–CRH–SAO column was the most efficient. This is probably because in a co-immobilized enzyme column, CNH, CRH and SAO are in close proximity, so that creatine is hydrolysed by CRH as soon as it appears on the surface of CNH, so that the whole process, particularly the CNH reaction, proceeds quickly. We also postulate that the three enzymes are distributed uniformly in both the mixed-bed and co-immobilized columns, but the greater distance between each enzyme in the mixed-bed column than in the co-immobilized column adversely affects the successive enzyme reactions in the mixed-bed column, judging from the fact that a serum sample passes through the column in *ca.* 1 s.

ACKNOWLEDGEMENTS

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