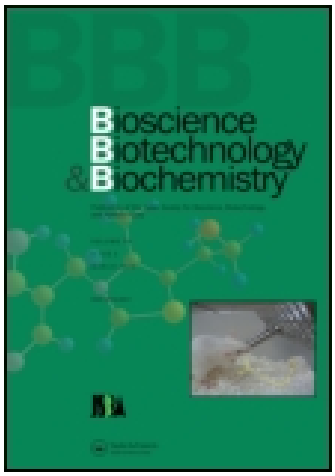

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Multifunctional Flow-injection Biosensor for the Simultaneous Measurement of Creatinine, Glucose, and Urea

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An amperometric flow-injection biosensor system was developed for the simultaneous measurement of creatinine, glucose, and urea, with a single sample injection and one detector. The principle for the amperometric detection of urea and creatinine was based on coupled reactions of three sequentially aligned enzyme reactors, urease or creatinine deiminase/glutamate dehydrogenase/L-glutamate oxidase. Inclusion of a split point and two confluence points between the injector and detector (oxygen electrode) resulted in a flow-injection system composed of three channels. Triple peak recording was obtained by putting two delay coils of different lengths at the channels involving urease and glucose oxidase. The system gave linear calibrations for creatinine, glucose, and urea in the ranges of 0.2—5, 0.2—10, and 0.5—20 mM, respectively. The assay procedure was simple and one run was completed within 4 min. The system was reproducible within 5—8% of relative standard deviation.

In the course of an investigation on developing multifunctional biosensor systems based on flow-injection analysis (FIA),*[1] we tried to apply the principle of splitting and confluence of the flow stream to obtain a multiple peak recording with a single sample injection and one detector. Thus two-channel FIA biosensors were developed for the assay of creatinine in urine using different enzyme systems, with the simultaneous compensation for endogenous ammonia.[1,2] We have also developed a bifunctional FIA system for the simultaneous measurement of urea and creatinine.[3] In both systems double peak recording was obtained by putting a Teflon tube (delay coil) at one of the two channels, which differentiates the arrival of sample zones at the detector.

In this paper we extend the same principle to the development of a three-channel FIA biosensor system for the simultaneous measurement of three components, creatinine, glucose, and urea. Each of them is a very important disease-related substance that is routinely assayed in clinical chemistry. The principle for amperometric detection of urea and creatinine has been described.[1,3] Ammonia produced from the hydrolysis of urea by urease or of creatinine by creatinine deiminase (CRDI) is further converted by glutamate dehydrogenase (GLDH) to L-glutamate, which is finally oxidized by L-glutamate oxidase (GLOD). The corresponding consumption of dissolved oxygen is then detected with an oxygen electrode. Glucose oxidase (GOD) is used for the measurement of glucose.

Materials and Methods

Materials. Urease (EC 3.5.1.5) from a Thermophilic *Bacillus* sp. was purchased from Wako Pure Chemical Industries (Osaka, Japan); CRDI (EC 3.5.4.21) was from Sigma (St. Louis, MO, U.S.A.); GLDH (EC 1.4.1.3) from beef liver and NADH were from Oriental Yeast (Osaka); GLOD (EC 1.4.3.11) from *Streptomyces* sp. was from Yamasa Shoyu (Choshi, Japan). Controlled Pore Glass (CPG, 120—200 mesh, 500 Å pore size), propylamine-CPG and succinate-CPG were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other chemicals were commercially available and of the highest grade.

Reagents. The standard solutions and buffers were prepared with double-distilled water. The working solution to run the FIA system was 67 mM sodium-potassium phosphate buffer (pH 8.0) containing 0.1 mM EDTA, 5 mM 2-oxoglutarate, and 0.3 mM NADH. Standard solutions of urea, creatinine, and ammonium sulfate were refreshed every week.

Immobilization of enzymes. CPG was used as the support for enzyme immobilization. All enzymes were separately immobilized on propylamine-CPG pretreated with glutaraldehyde[4] except GLDH, which was coupled on succinate-CPG activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide by the method of Cho and Swaisgood.[5] The enzyme preparations were packed into glass tubes (i.d. 3.4 or 2.4 mm) for use in FIA system. One cm of the tube of i.d. 3.4 mm corresponded to 36 mg of dry CPG and contained 3.8 units of CRDI, 10.2 units of GLDH, 3.5 units of GLOD, and 12.2 units of GOD. The amount of CPG contained in a tube of i.d. 2.4 mm was half of a tube of i.d. 3.4 mm. When not used the immobilized enzymes were stored in phosphate buffer (pH 7.5) at 4°C.

Flow-injection manifold. Figure 1 is a schematic diagram of the three-channel FIA system for the simultaneous measurement of creatinine, glucose, and urea. The system involved a peristaltic pump (Atto Co., Tokyo, Japan), an injection valve (Sanuki Industries Co., Tokyo), enzyme reactors, a galvanic oxygen electrode equipped with a flow cell (Able Co., Tokyo), a potentiostat (Hokuto Electronics Ltd., Tokyo), and a 3-pen recorder (Toa Electronics Ltd., Tokyo). The flow stream was split into three channels with a four-direction connector after sample injection. The channels involving urease and CRDI were rejoined before the GLDH reactor. The joined channels were further joined with that involving GOD before the oxygen electrode. To separate the peaks corresponding to each channel, two delay coils of different lengths were used. The working solution was propelled into the system at a total flow rate of 2.0 ml/min. The distribution of working solution was controlled by the size of enzyme columns in the three channels at a ratio of 2:2:1 in the order of CRDI, GOD, and urease channels.

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*1 *Abbreviations*: FIA, flow-injection analysis; CRDI, creatinine deiminase, GLDH, glutamate dehydrogenase; GLOD, L-glutamate oxidase; GOD, glucose oxidase; CPG, controlled pore glass.

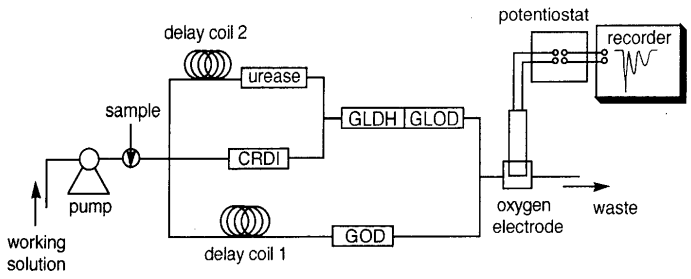

Fig. 1. Schematic Diagram of the FIA System for the Simultaneous Measurement of Creatinine, Glucose, and Urea.

Size of enzyme reactors and delay coils (i.d. × length, mm): GOD, 2.4 × 46; CRDI, 3.4 × 20; urease, 3.4 × 23; GLDH, 3.4 × 30; GLOD, 3.4 × 20; delay coil 1, 0.8 × 1200, delay coil 2, 0.8 × 1500). Enzyme reactors and oxygen electrode were kept at 25°C with a thermostat. When not used, the enzyme reactors were removed from the system and stored at 4°C. Injection volume of samples was 50 μl. Total flow rate was 2.0 ml/min, which was distributed over the three channels at the rates of 0.8, 0.8, and 0.4 ml/min for glucose, creatinine, and urea channels, respectively. Working solution: 67 mM sodium–potasium phosphate buffer (pH 8.0) containing 0.1 mM EDTA, 0.3 mM NADH, and 5.0 mM 2-oxoglutarate.

Calculation of flow rates through the CRDI and urease channels. Since it is difficult to measure the real flow rates directly through the urease and CRDI channels, flow rates through these two channels were calculated by the following procedures. First, the GLDH/GLOD column is removed and the ratio of flow rates through CRDI channel to urease on ($r_{c/u}$, which is a function of the sizes of delay coil 2 and the CRDI and urease columns, and is independent of other factors); then the GLDH/GLOD column is set in place and the total flow rate through the two channels (Q_{cu}) is measured; the respective flow rates through the CRDI and urease channels are thus calculated as

$$Q_c=(1+r_{c/u})^{-1}r_{c/u}Q_{cu}\,;\quad Q_u=(1+r_{c/u})^{-1}Q_{cu}\,.$$

Results and Discussion

Choice of working solution

Urease from a thermophilic *Bacillus* sp. used in this study showed far lower activity (4.5 units/mg powder) than that from jack bean (100 units/mg powder), which is exclusively used for almost all analytical purposes. This enzyme, however, has the advantages that its K_m, 0.44 mM, is much lower than that from jack bean, 10.5 mM,[6] and that its pH optimum of activity is on the alkaline side, showing a common value near pH 8.0 with GLDH and GLOD (Fig. 2). The optimum pH of GOD is on the acid side (Fig. 2B). The immobilized form of this enzyme, however, was found to be stable over a wide area of pH from 5.5 to 8.5 (data not shown). This makes it possible to use a single working solution to run the FIA system. Therefore, 67 mM phosphate buffer of pH 8.0 containing 0.1 mM EDTA (added as enzyme stabilizer) was used as the base buffer of working solution. The optimum concentrations of NADH and 2-oxoglutarate were 0.3 and 5.0 mM, respectively.[1] It seemed uneconomical to use a single working solution to run the system because the oxidation of glucose by GOD does not need NADH and 2-oxoglutarate. However, inclusion of units to supply these two substances only to urease and CRDI channels will greatly complicate the FIA system. Moreover, this study emphasized investigating the probability of a three-channel FIA system based on the principle of splitting and confluence of flow.

Delay coil and flow distribution

Among various factors affecting the separation of three peaks, delay coil (length, inner diameter, and material) and the distribution of flow over the three channels are the most important ones. To minimize sample dispersion the delay

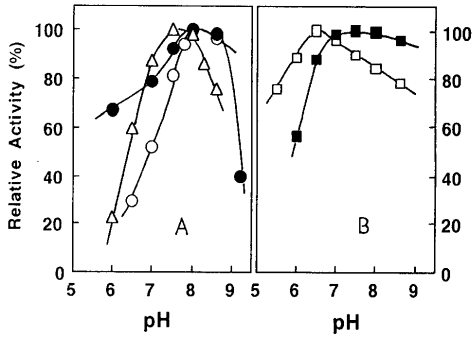

Fig. 2. pH Dependences of the Immobilized Enzymes.

A: (○) urease; (●) CRDI; (△) GLDH. B: (■) GLOD; (□) GOD.

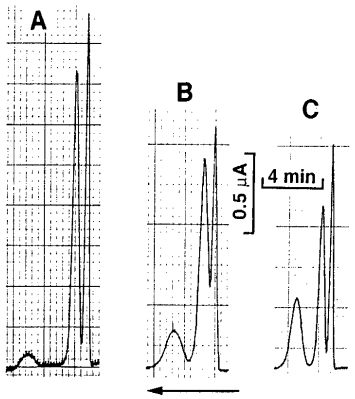

Fig. 3. Effects of Delay Coil and Flow Distribution on the Separation of Peaks.

Each response was obtained from an injection of a mixture of creatinine, glucose, and urea (5 mM). Length of delay coil 2, 3.0 m in A and B, and 1.5 m in C; flow rates (ml/min) in the order of the first (creatinine), second (glucose), and third peak (urea), 0.76, 0.79, 0.41 in A; 0.67, 0.69, 0.62 in B; and 0.79, 0.80, 0.40 in C. Among the enzyme columns only the length of urease reactor was varied for the control of flow distribution. Length of urease reactor, 21, 18, and 23 mm in A, B, and C, respectively. See Fig. 1 for other conditions.

coils should be as thin and short as possible. The minimum length of delay coil needed for the complete separation of two peaks depends on the relative flow rates of working solution through those two channels. Distribution of flow over the three channels were controlled by the size (length and inner diameter) of enzyme reactors. When flow rates through the first and second channels were adjusted to be approximately the same (0.8 ml/min), 1.2 m of Teflon tubing with inner diameter of 0.8 mm was found to be enough for the complete separation of the first and second peaks.[1] Teflon tubing was superior to silicon for the separation, perhaps due to the lower friction resistance, which is one of the factors causing sample dispersion, between the inner wall of the Teflon tube and the flow stream.

If working solution is distributed evenly over the three channels, the assay sensitivity becomes lower in the order of the first, second, and third peak, due to the increasing residual time of the sample zone in the FIA system. The longer delay coil (delay coil 2) should be set in the urease channel because the concentration of urea is usually much larger than the other two compounds in biological samples and two mol of ammonia are formed from one mole of urea.

To find the optimum length of delay coil 2, the responses of the system to a sample containing all three analytes (5 mM) were investigated under different lengths of delay coil 2 and flow distributions. When the delay coil 2 was 3.0 m, and the flow rate through the urease channel was

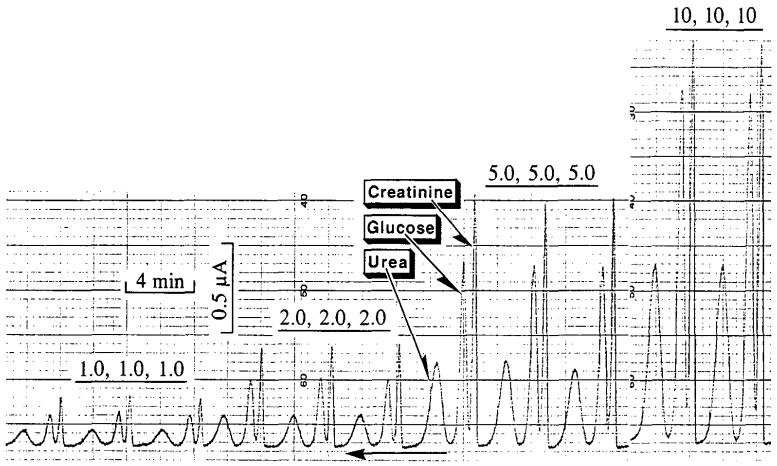

Fig. 4. Flow-injection Response of the Three Channel FIA Biosensor System to Standard Mixtures of Creatinine, Glucose, and Urea.
The concentrations were given in mM at the top of peaks. Chart direction was from right to left as indicated by the arrow below the chart. See Fig. 1 for other conditions.

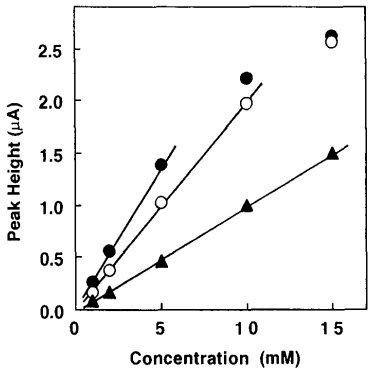

Fig. 5. Calibration Curves of the Three Channel FIA Biosensor System.
(●) Creatinine; (○) glucose; (▲) urea. See Fig. 1 for experimental conditions.

about half of those other channels, the third peak was extremely low and was far apart from the second peak (Fig. 3A). When the flow rate through the urease channel increased, the third peak became higher and appeared immediately following the second peak (Fig. 3B), but the response time was still long. In C, the flow distribution was virtually the same as in A but delay coil 2 was shortened to 1.5 m; as a result the width of the third peak fairly decreased. Therefore, the minimum length of a delay coil depends on the distribution of flow over the corresponding two channels. It seems that a short delay coil with low flow rate is preferable to a long delay coil with high flow rate through the channel in question. The following experiments were done under the same conditions as in Fig. 3C.

FIA response and calibration

Three peaks were recorded from an injection of sample containing all three compounds. The first peak represented the response to creatinine, the second that to glucose, and the third to urea. Figure 4 gives the FIA responses to standard mixtures of urea, creatinine, and glucose under the best conditions shown in Fig. 1. The response time for the first, second, and third peaks were 0.5, 1.0, and 2.0 min, respectively. One run was completed within 4 min. The corresponding calibration curves are shown in Fig. 5. The linear calibration ranges for creatinine, glucose, and urea were 0.2—5, 0.2—10, and 0.5—20 mM, respectively. The sensitivities were lowered and the upper limit of linear response rose from the first to the third peak. That is, the linear response range shifted to the high concentration side in the order of the first, second, and third peak. When conversion of substrate is near complete, the maximum analyte concentration that can be detected in an FIA system based on the oxygen-mediated oxidation is limited by the dissolved oxygen level (max. 0.25 mM at 25°C) in the working solution. Therefore this shift implies an increased substrate dilution from the first to the third channel, due to the differentiated dilution effects of the splitting and confluence of the flow stream among the three channels, and the diffusion effects of analytes in the system.

The response of the FIA system was reproducible within 5—8% of the relative standard deviation for 12 repeated injections (analyte concentration, 2.0 mM). The reproducibility was somehow lower than that of the two channel systems.[1,3] This may be caused by a fluctuation of flow distribution over the three channels. If we consider the splitting of the sample zone as a kind of dilution, then the dilution factors will be squared at the confluence point. That is, the total dilution factor is inversely proportional to the square of the flow rate through the corresponding channel. For instance, in this system the dilution factors caused by the splitting and confluence of flow were $(2.0/0.8)^2=6.25$ for creatinine and glucose, and $(2/0.4)^2=25$ for urea. Thus a little fluctuation in flow distribution may result in a large change in the relative dilution factors for each analyte. Stabilization of flow distribution, however, is possible through the improvement of the FIA system. From the opposite point of view, we can make use of the dilution effects of splitting and confluence of the flow stream to regulate the calibration range for each channel.

Few studies on the multi-functionalization of biosensors have been reported. An enzyme sensor for the measurement of three compounds for fish freshness estimation was developed,[7] based on separation by column chromatography and sequential elution of the analytes. However, the principle for separation was specific to those compounds and the procedure of assay was complicated and time consuming (more than 15 min for a run). Matumoto *et al.* have developed multichannel FIA systems for the simultaneous measurement of three[8,9] or four components,[10] using a 16-way switching valve with three injection loops, and a multichannel flow-through cell composed of

three independent compartments within which three sets of electrodes are separately included. To some extent, they are the accumulation of several single-channel FIA systems and are not considered as multiple peak recordings or multifunctional because each peak is virtually provided by a different injection and detector, so that the information level is not enhanced compared to conventional flow-injection systems.[11] The advantages of our strategy for multi-functionalization are obvious. First, the operation procedure is almost as simple as that of an FIA system for single component, and the assay is very rapid. Secondly, this strategy is universal for all compounds that can be detected with common detectors. Finally, the relative sensitivities of each channel could be regulated. This is especially significant in the assay of samples containing greatly different concentrations of components of interest. For example, the amounts of urea and creatinine are differentiated by a factor of more than 10 in normal body fluids. It is not practical to measure the two substances simultaneously in such samples with an FIA system equally sensitive to them. Improvement of this biosensor system involving the regulation of the calibration range for each channel and the removal of interfering endogenous ammonia will enable us to use it for the simultaneous measurement of three components in biological samples such as urine.

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