Journal of Chromatography, 420 (1987) 13–24
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3738

PULSE INJECTION ANALYSIS OF THE BINDING OF SERUM PROTEINS TO POROUS POLYMER GELS MODIFIED WITH FORMYL GROUPS

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(First received December 1st, 1986; revised manuscript received April 6th, 1987)

SUMMARY

Highly porous spherical polymer gels were modified with formyl groups by a modified Friedel-Crafts reaction and the interaction of serum proteins with the modified gels was examined by pulse injection analysis. The introduction of formyl groups into the polymer greatly increases its protein-binding capacity, and the protein bound to the gel is not eluted by washing with acid, alkali or urea solution. The effects of temperature and the percentage of formyl group substitution on the binding capacity indicate that the binding process can be interpreted as initial approach of the protein to the polymer surface, caused by the hydrophobic interaction, followed by formation of a stable Schiff base between the polymer gel and the protein. Theoretical treatment of the elution behaviour of the protein from the polymer-packed column is also examined, with the assumption that there are three kinds of binding site in the polymer gel: surface, macropore and micropore regions. These polymers are shown to be useful for the removal of proteins from biological samples in clinical assays using immobilized enzymes.

INTRODUCTION

The analysis of interactions between proteins and polymers is very important in the field of both biomedical materials and biotechnology [1, 2]. In the former case, anti-coagulant (biocompatible) materials for artificial organs, such as blood vessels, hearts, kidneys, skins and bones, have been extensively studied, and in the latter case polymers that bind proteins have been investigated as carriers of immobilized enzymes. We have reported previously the usefulness of polyoxyphenol gels, which are the products of the addition condensation of polyoxyphenols such as hydroquinone with formaldehyde, for the removal of proteins from biological samples such as blood and urine for clinical assays [3].

Polyoxyphenol gels rapidly and strongly bind proteins. Because of the smaller

0378-4347/87/$03.50

surface area of the gels (80 m^2/g), however, the total amount of protein bound to the gels is not so large (80 mg of bovine serum albumin to 1 g of the formaldehyde–hydroquinone gel at pH 5.5). To enhance fully the ability of the polymers to bind proteins, a highly porous structure of the polymer gels with large surface areas is considered to be essential [4]. In this report we examine pulse injection analysis of the removal of serum proteins from their solution using columns packed with highly porous spherical polymers (MCI Gel CHP-20P) modified with formyl groups. Using this technique, the protein solution is injected onto the column at appropriate intervals, and the protein reversibly bound to the gel in the column is continuously rinsed by the mobile phase. Therefore only the protein irreversibly bound to the gel is retained on the column, which means that the irreversible binding can be analysed.

Formyl groups are known to react with amino groups of proteins at moderate pH and have been widely used as reactive groups in the carriers of immobilized enzymes [5], columns for biospecific affinity chromatography [6] and latex particles for immunological diagnoses [7].

EXPERIMENTAL

Materials

Bovine serum albumin (BSA, fatty acid free, Sigma, St, Louis, MO, U.S.A.) and bovine serum γ-globulin (Sigma) were used without further purification. The standard human serum (Hyland control serum type I) was purchased from Travenol Labs. (Bannockburn, IL, U.S.A.). Glucose oxidase (GOD, *Aspergillus niger*, 116 U/mg, Grade II) and peroxidase (POD, horse radish, 109 U/mg, Grade III) were obtained from Toyobo (Osaka, Japan). Highly porous spherical poly(styrene–co-divinylbenzene) beads for chromatography (MCI Gel CHP-20P, 35–75 μm, mean radius of pores 500 Å, surface area 500 m^2/g) was purchased from Mitsubishi Chemical Industries (Tokyo, Japan), washed several times with hot methanol and, after filtration, dried in vacuo. The range of diameters of the pores of the CHP-20P is probably wide. For comparison, Biobead S-X4 (Bio-Rad, 200–400 mesh, exclusion limit 1400) was also used. Deionized water was distilled just prior to use.

Preparation of formylated gels

The CHP-20P beads (5 g) were dispersed in 65 ml of dry dichloromethane and degassed using an aspirator under continuous stirring. Then 3 g of aluminium chloride (Nakarai Chemicals, Kyoto, Japan) were dissolved in the solution. Using a droppping-funnel, 3 ml of dichloromethyl methyl ether (Aldrich) were slowly added to the solution [8, 9]. The mixture was stirred for 30 min at room temperature. After the evolution of hydrogen chloride stopped, 30 ml of 5% hydrochloric acid solution was added dropwise at 0 °C, and the mixture was continuously stirred for 3 h. The modified polymer beads were filtered and washed with methanol and a weakly alkaline solution (pH 9). The slightly yellow polymer beads obtained were passed through the standard sieves and the particles with diameters in the range 44–74 μm were collected and used in the protein binding experiments. The

TABLE I

CHARACTERISTICS OF FORMYLATED POLYMER BEADS

Polymer	Percentage of formyl group
AL-CHP-20P I	5.2
AL-CHP-20P II	6.1
AL-CHP-20P III	7.8
AL-CHP-20P IV	8.9
AL-S-X4	12.4

presence of formyl groups in the gel was confirmed by the new absorption band at 1690–1700 cm^{-1} (due to the C=O stretching of the carbonyl group that is introduced into aromatic rings) using an infrared spectrophotometer (IR-440, Shimadzu, Kyoto, Japan).

The percentages of the substituted formyl groups in the styrene unit of the polymer beads were estimated by elemental analysis using the oxime-forming method [10] (Table I).

Analysis of protein binding to polymer gels

The experiments to study the binding of proteins to polymer gel were carried out by the batch and column methods described elsewhere [11].

In the column method, 50 μl of the protein solution (15 mg/ml in the case of BSA and 10 mg/ml in the case of γ-Gl) were injected onto the polymer-packed glass column (55.5 × 4.7 mm I.D.), which was fixed in an automatic column changer (AC-3, Kyoto Chromato, Kyoto, Japan) and connected to a conventional liquid chromatography apparatus. An automatic sample injector (EA-25, Kyoto Chromato) was used.

Enzymatic assays of low-molecular-mass compounds after pretreatment of the samples

The enzymic assay of glucose in the control serum was examined. GOD and POD, which had been treated with sodium periodate and dialysed for two days, were separately immobilized by the glutaraldehyde activation method [12] on to the porous glass beads (FPG-700L, 80–120 mesh, mean pore diameter 774 Å, specific surface area 29 m^2/g; Wako Pure Chemicals, Osaka, Japan) modified with aminopropyl groups [13]. The serum sample (10 μl) was injected automatically every 1 or 2 min. The serum passed through the polymer-packed column fixed in the automatic column changer and then through the GOD column (15 mm × 4 mm I.D., 2.1 mg of GOD per column) (Fig. 1). The eluted solution was mixed with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution and then passed through the POD column (10 mm × 4 mm I.D., 1.4 mg of POD per column). The increase in the absorbance at 450 nm of the eluted solution was followed using a UV detector (S-310A Model II, Soma Optics, Tokyo, Japan), and the peak area was estimated using an electronic integrator (Chromatopak C-R1B, Shimadzu, Kyoto, Japan).

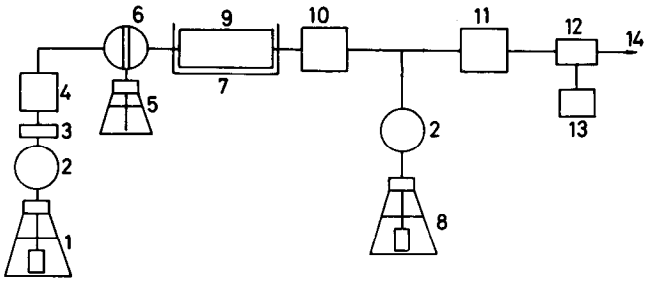

Fig. 1. Schematic representation of the instrument used to estimate the concentration of glucose in serum using the immobilized enzymes and the pretreatment column. 1 = Buffer solution; 2 = pump; 3 = filter; 4 = damper; 5 = sample solution; 6 = automatic sample injector; 7 = automatic column changer; 8 = ABTS solution; 9 = column packed with formylated CHP-20P; 10 = GOD (glucose oxidase) column; 11 = POD (peroxidase) column; 12 = UV detector; 13 = integrator; 14 = waste.

Theoretical treatment of binding of protein to polymer gels [11, 14]

In general, proteins adsorbed on hydrophobic polymeric materials cannot be readily eluted by washing with the same solvent as that of the protein solution; washing with alkaline or acidic solution is necessary to elute the adsorbed protein [15]. As will be described later, proteins bound to the formylated polymer gel cannot be eluted even by washing with acid, alkali, urea or surfactant solutions. This is because of the irreversible binding between the gel and the protein. Therefore, we assumed the binding scheme of proteins to the polymer gel to be as follows:

$$C + S \xrightarrow{k} CS \tag{1}$$

where C, S, CS and k are the protein examined, the binding site in the polymer gel, the protein bound to the polymer and the apparent reaction rate constant of the protein binding, respectively. For simplification, we assumed that binding sites in the same region are equivalent and that the bound protein did not influence the reactivity of neighbouring binding sites. We also assumed that the distribution of the protein concentration in the injected pulse of protein solution had a rectanglar shape [concentration C_0 (mg/ml), volume ΔV (ml)], and that the pulse passed through the column [length L (cm)] at a flow-rate of u (ml/s). The actual distribution of the protein in the pulse at the inlet of the column was slightly different from that at the outlet. However, the simplification that the distribution of the protein concentration was constant throughout the column did not significantly affect the theoretical elution profile. In a small region of the column [$\Delta x = L/m$ (cm)] containing an amount of the gel w (cm^3), q mg of protein are bound to the polymer:

$$dq/dt = kC(q_e - q) \tag{2}$$

where q_e, q, and k are the amount of protein bound at equilibrium, the amount of bound protein, and the apparent reaction rate constant, respectively. It should be noted that k involves a factor of diffusion in and around the polymer matrix [22]; q is equal to q_0 at $t = 0$. Then eqns. 3 and 4 can be easily derived.

$$q = (q_e - q_0)\{1 - \exp(-kC\Delta t)\} + q_0 \tag{3}$$

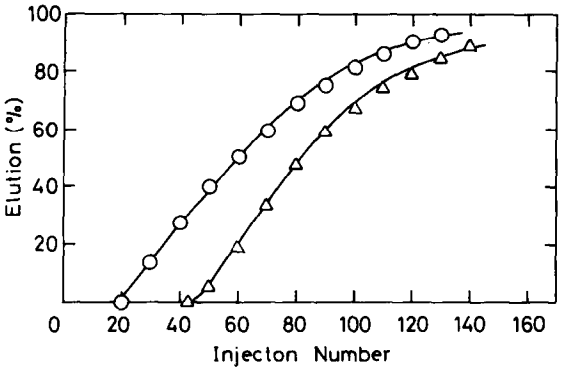

Fig. 2. Elution profiles of BSA from polymer-packed column. Curves: ○ = CHP-20P; △ = AL-CHP-20P-II, 25 °C. BSA concentration 15 mg/ml at pH 5.5

$$\Delta q = (q_e - q_0)\{1 - \exp(-kC\Delta t)\} \quad (4)$$

As will be described later, we assumed here that there were at least three kinds of protein-binding site in the porous polymer gel: in the surface (region 1), macropore region (region 2) and micropore region (region 3). Thus the total amount of bound protein, q_{total}, is the sum of the protein bound to these three regions ($q_{total} = q_1 + q_2 + q_3$). Considering the material balance in the jth region of the column, we have

$$C(i,j) = \{C_0 \Delta V - w \sum_{h=1}^{3} \sum_{k=1}^{j} q_h(i,k)\}/\Delta V \quad (5)$$

$$Q_h(i,j) = \sum_{k=1}^{i} q_h(k,j) \ (h=1,2,3), \ Q(i,j) = \sum_{h=1}^{3} Q_h(i,j) \quad (6)$$

$$q_h(i,j) = \{q_{eh} - Q_h(i-1,j)\}\{1 - \exp(-k_h C(i,j-1)\Delta t)\} \quad (7)$$

and the boundary conditions are

$C_0 = C(i,0)$ (concentration at the inlet)

$Q_h(0,j) = 0$

Using eqns. 5–7 we estimated the concentration at the exit of the column as $C(i, m)$. The division number of a column, m, is fixed at 30 because theoretical elution curves obtained are almost the same whether the value of m is 30 or more.

RESULTS AND DISCUSSION

Elution profile from glass column packed with formylated gels

The proteins injected into the polymer-packed column were initially retained on the column. After several tens of injections of the protein solution, the protein was eluted gradually from the column (Fig. 2). Compared with the usual break-

TABLE II

INFLUENCE OF FORMYL GROUPS ON THE ELUTION OF BSA FROM POLYMER-PACKED COLUMN

Conditions: 25 °C, pH 5.5, 0.03 *M* phosphate buffer.

Polymer	Percentage of formyl group	BSA bound★ (mg per column)	Start of elution★★	50% Elution★★
CHP-20P	0	15.0	21	60
AL-CHP-20P I	5.2	17.3	24	80
AL-CHP-20P II	6.1	33.0	45	82
AL-CHP-20P III	7.8	33.8	46	78
AL-CHP-20P IV	8.9	24.8	34	85

★Total amount of BSA irreversibly bound to the column prior to the start of the elution from the column.

★★Number of injections.

through curves in the frontal analysis, the elution curves in Fig. 2 are initially a little sharper. This is probably because only proteins irreversibly bound to the polymer can be retained on the column, and the binding equilibria of proteins to the gel can be neglected.

We defined the binding capacity of the column by noting the number of the injections at which the protein started to be eluted and also the number of the injections of which 50% of the injected protein had been eluted. The former value in particular shows the usefulness of the polymer beads for the pretreatment of the biological samples. Fig. 2 shows the elution behaviour of BSA from various kinds of polymer-packed columns. In the case of CHP-20P with a large surface area and a pore size large enough for the penetration of BSA, the injected BSA was retained in the gel for up to 21 injections, probably because of the hydrophobic interaction between BSA and the polymer. By the introduction of formyl groups into the polymer gel the binding capacity of the beads was observed to increase greatly; this can be attributed to the formation of a Schiff base between the injected protein and the formyl groups in the gel.

In the case of S-X4 with an exclusion limit of 1400, the binding capacity of the protein was also increased by the introduction of the formyl groups. The total amount of bound BSA went up from 0.10 mg per column to 0.21 mg per column, the start of elution was after seven rather than two injections, and 50% elution was reached after nine rather than four injections. The absolute capacity of the gel, however, was still very small even after the introduction of formyl groups, mainly because the pores were too small for BSA to penetrate. This result also provides evidence that the pores of AL-CHP-20P are large enough for BSA molecules to penetrate.

Table II shows the elution pattern of BSA from the column packed with polymers containing various percentages of formyl group. By the introduction of formyl groups into the beads the binding capacity at first increased, but too many formyl groups caused it to decrease. Since the formyl group increases the hydrophilicity of the polymer, this result might suggest that the process involved in the

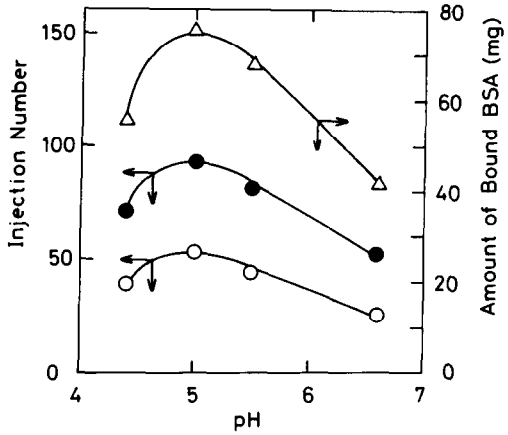

Fig. 3. Effect of pH on the elution at 25 °C of BSA from column packed with AL-CHP-20P-II. Curves: ○ = start of elution; ● = 50% elution; △ = total amount of BSA bound.

binding of proteins to the polymer gel was the approach of proteins to the polymer surface via the hydrophobic interaction between the polymer gel and the proteins, followed by chemical reaction between the formyl groups and the amino groups (mainly ε-amino groups of the lysine residues) of the protein.

To confirm this hypothetical binding process we examined the effect of pH on the binding capacity of the polymer beads. Fig. 3 shows that in the weakly acidic region (pH 5) the binding capacity of the formylated polymer beads shows a maximum. The isoelectric point of BSA has been reported as 4.7–4.9 [16] and, in this region, BSA is highly hydrophobic. In the pH region slightly higher than the p*I* of the protein, the hydrophobic property of the protein is still satisfactorily high and the small amount of reactive free amino groups remained in the protein molecule. This result also supports the hypothesis described above. Such behaviour has also been reported in the interaction of proteins with polyoxyphenol gels [3], and with tannin-containing cellulose [17]. The protein bound to the polymer could not be eluted by washing with 0.1 *M* hydrochloric acid, sodium hydroxide 6 *M* urea or 20% (v/v) ethanol. In general, the Schiff base between small molecules is not stable in the acidic or basic conditions examined here, whereas the Schiff base formed between the hydrophobic polymer gel and the protein might be highly stabilized by the densely surrounding polymer matrix and the protein molecule.

The effect of temperature on the binding of BSA to the polymer gel was also examined. When the temperature was increased from 25 to 35 °C, the injection numbers at the start of elution and at 50% elution increased slightly (start of elution, 46→69; 50% elution, 78→87). In general, in the physical adsorption of protein on the polymer an increase in temperature decreases the binding capacity of the polymer [18]. The increase of the binding capacity of the polymer gel with temperature observed by us confirmed that the binding of the protein to the formylated polymer gel was a chemical process.

To confirm this binding mechanism, we further examined the elution pattern

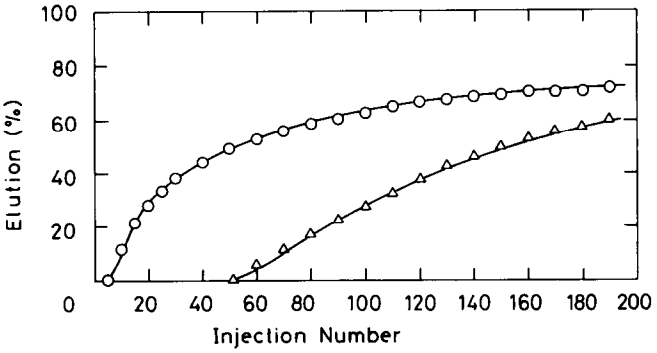

Fig. 4. Elution profiles of γ-globulin from polymer-packed column at 25°C and pH 7.0. γ-Globulin concentration, 10 mg/ml. Curves: ○ = CHP-20P; △ = AL-CHP-20P.

of two kinds of polypeptide, poly-L-lysine (PLL) and poly-L-glutamic acid (PLG) (40 mg/ml, 50 μl in each injection). Both PLL and PLG were eluted quantitatively from the column in the acidic pH region (pH 5.5). In the weakly alkaline region (pH 8.0) PLG was eluted quantitatively, whereas PLL was partially retained on the column (30%), which suggests a contribution of free amino group to the binding of proteins to the formylated polymer gels.

Fig. 4 shows the elution of γ-globulin from the column packed with unmodified or formylated CHP-20P. Formylation increased by a factor of about ten the injection number at which γ-globulin started to be eluted. The curve in Fig. 4 shows a steep increase followed by a slow increase, which indicates that the penetration of γ-globulin into the pores of the unmodified CHP-20P gel is strongly restricted by the γ-globulin molecules already bound on the pore surface of the CHP-20P. Since the F_c chain of immunoglobulin G (IgG), the most representative protein in the γ-globulin fraction, is more hydrophobic than that of F_{ab} chains [19], IgG probably binds to the unmodified CHP-20P by the F_c chain, and twoF_{ab} chains stretch to the centre of the pores, which greatly restricts the diffusion of other globulin molecules into the pores. In the case of the formylated CHP-20P gel, however, F_{ab} chains (amino ends) of γ-globulin might be directed at the polymer surface and the F_c chain might stretch to the centre of the pores, which would reduce the restriction or the diffusion of proteins [20, 21].

Theoretical analysis of elution curves

A theoretical analysis of the elution curves of BSA exemplified in Fig. 2 was carried out. The ratio of eluted protein to injected protein, $A(i,m)$, is $C(i,m)/C_0 \cdot 100$, and Fig. 2 shows plots of i versus $A(i,m)$. From the time dependences of the amount of bound BSA by the batch experiment (Fig. 5), we found that the initial rapid binding was followed by a slow binding step and then by an extremely slow binding. We attributed these three steps to the presence of three kinds of binding site on the gel: surface, macropore and micropore regions. If we assumed a one-step or a two-step binding mechanism, we could not fit the elution curve obtained in the pulse injection experiments with the theoretical one satisfactorily well for the umodified and formylated CHP-20P gels (Fig. 6a and b).

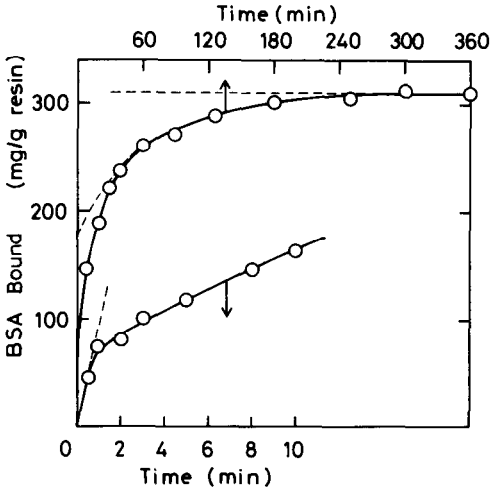

Fig. 5. Time dependence of amount of BSA bound to formylated CHP-20P examined in the batch experiment at 25 °C and pH 5.5. Initial BSA concentration, 1.44 mg/ml.

However, when we assumed a three-step binding mechanism, the curve-fitting method yielded the binding parameters via eqns. 5–7 (Fig. 6c). The parameters evaluated are shown in Table III. The introduction of formyl groups increased both q values and k values. We were also able to evaluate three kinds of apparent binding rate constant, k_1', k_2' and k_3', from the batch experiment (Table III), and these parameters are in good agreement with those for formylated CHP-20P estimated by the pulse injection method.

It should be mentioned that, though the binding of proteins to polymer resin is an intrinsically distributed process, the lumped parameter model assumed here neatly interprets the elution profile of proteins.

As stated before, diffusion of a protein molecule as it approaches the gel might be restricted by protein molecules already bound on the gel. This might partly

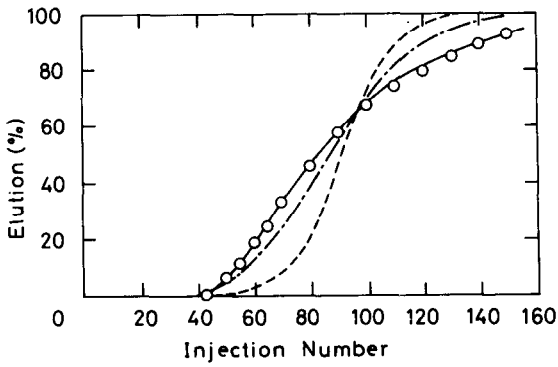

Fig. 6. Theoretical curves for elution of BSA from AL-CHP-20P-packed column, assuming several binding steps. (a) (- - -) One-step binding; $q_e = 303$ mg/g, $k = 0.0025$ ml/mg s. (b) (-·-) Two-step binding; $q_{e1} = 22.7$ mg/g, $q_{e2} = 280$ mg/g, $k_{e1} = 0.028$ ml/mg s, $k_{e2} = 0.0015$ ml/mg s. (c) (—) Three-step binding. Parameters are shown in Table III.

TABLE III

BINDING PARAMETERS OF BSA TO POLYMER-PACKED COLUMNS AT 25°C

$k_1' = 0.039$, $k_2' = 0.0016$ and $k_3' = 0.00047$ ml/mg s for AL-CHP-20P in the batch experiment.

Polymer	Δt (s)	ΔV (ml)	C_0 (mg/ml)	k_1 (ml/mg s)	k_2 (ml/mg s)	k_3 (ml/mg s)	q_{e1} (mg/g resin)	q_{e2} (mg/g resin)	q_{e3} (mg/g resin)
CHP-20P	14.5	0.26	2.87	0.050	0.0016	0.00060	18	100	100
AL-CHP-20P	16.5	0.30	2.53	0.057	0.0028	0.00065	27	123	162

account for the small slope at large injection number in Figs. 2 and 4 [20, 21]. We are currently examining the effect of restricted diffusion on the elution profile of proteins from the column [23].

Assay of serum glucose after pretreatment

In order to examine the usefulness of the porous and formylated polymer gels, we carried out an enzymic assay of the glucose in control human serum using immobilized enzyme columns. Fig. 7 shows the observed peak areas that correspond to the concentration of glucose in the serum: it is evident that the pretreatment of the injected serum using the polymer-packed column increased the peak area by the removal of proteins from the sample. In the case of an aqueous glucose solution, the peak areas detected by this method were in good agreement with those of the serum sample after pretreatment with the formylated polymer beads. In the presence of proteins in the sample solution the peroxidase immobilized on the porous glass receives an electron not only from ABTS but also from the proteins [24], which reduces the peak area. The decrease in peak area could be partly attributed to the inactivation of immobilized enzymes by the serum proteins sticking to the surface of the glass beads. By removal of the protein such a decrease

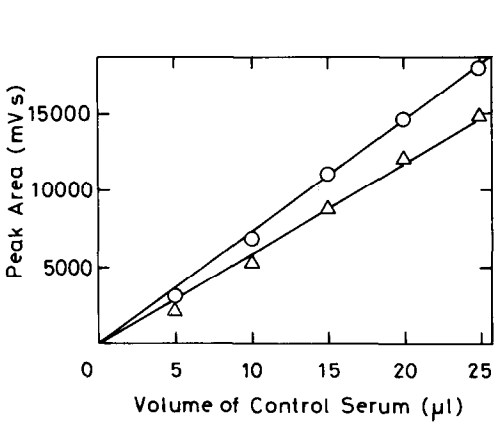

Fig. 7. Plots of peak area vs. concentration of glucose in serum. Curves: △ = without pretreatment; ○ = with pretreatment.

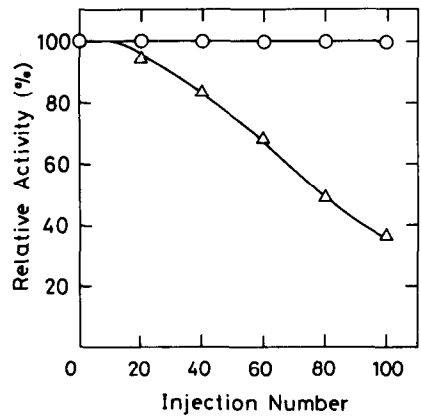

Fig. 8. Operational stability of the assay system. Curves: △ = without pretreatment; ○ = with pretreatment.

in the peak area could be avoided, and the peak area is equal to that of glucose solution whose concentration is the same as that of the serum sample injected. Using the guard column packed with formylated CHP-20P we carried out the assay of glucose in 100 samples (Fig. 8). Using the automatic column changer with many disposable columns packed with formylated resin, we can carry out continuous pretreatment of the serum samples by setting the number of samples that can be pretreated by one polymer-packed column.

In conclusion, the introduction of formyl group into the highly porous spherical polymers increased the capacity of the polymer to bind proteins. The application of these polymer beads to the pretreatment of serum samples in clinical assays yields reliable and reproducible analytical data. Theoretical results indicate that there are at least three kinds of binding site: namely surface, macropore and micropore regions in the polymer gel.

SYMBOLS

C	protein, or concentration of protein solution (mg ml^{-1})
CS	protein bound to polymer gel
C_0	initial concentration of protein solution with a rectanglar shape in the distribution (mg ml^{-1})
$C(i,j)$	concentration of protein in the ith injected solution after passage through the jth region of a column (mg ml^{-1})
k	apparent binding rate constant (ml mg^{-1} s^{-1})
k_h	apparent binding rate constant in the site h ($h=1,2$ or 3) of resin estimated by the pulse injection method (ml mg^{-1} s^{-1})
k'_h	apparent binding rate constant in the site h ($h=1,2$ or 3) of resin estimated by the batch experiment (ml mg^{-1} s^{-1})
L	length of a column (cm)
m	division number of a column
q	amount of protein bound to the minute region of the column (mg cm^{-3})
q_e	maximum amount of protein bound to the minute region of the column (mg cm^{-3})
q_{eh}	maximum amount of protein bound to site h ($h=1,2$ or 3) of resin (mg cm^{-3})
q_h	amount of protein bound to site h ($h=1,2$ or 3) of resin (mg cm^{-3})
$q_h(i,j)$	amount of protein bound to site h ($h=1,2$ or 3) in the jth region of the column during passage of the ith injected protein solution (mg cm^{-3})
q_0	amount of protein already bound (mg cm^{-3})
$Q(i,j)$	total amount of protein bound in the jth region of polymer column after passage of the ith injected protein solution (mg cm^{-3})
$Q_h(i,j)$	total amount of protein bound to site h ($h=1,2$ or 3) of resin in the jth region of column after passage of the ith injected protein solution (mg cm^{-3})
S	binding site in polymer gel
Δt	contact time of protein with polymer gel (s)

u	flow-rate of protein solution through the polymer-packed column (ml min^{-1})
V	volume of the column (cm^3)
ΔV	volume of protein solution passing through the column (cm^3)
w	volume of polymer gel in the jth region of the column (one mth of the total volume of polymer gel in the column) (cm^3)
Δx	length of the jth region of the column (cm)

ACKNOWLEDGEMENT

This work was supported by the Institute of Physics and Chemistry, Tokyo, Japan.

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