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An Estimation Method for Aminoalkyl Groups Coupled to Agarose Beads

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A spectrophotometric method for the estimation of aminoalkyl ligands immobilized on agarose gel was developed. The quantitative trinitrophenylation of amino groups on the gel was performed with 2,4,6-trinitrobenzenesulfonate (TNBS) in 75 mM sodium borate. The amount of TNBS consumed during the reaction was calculated from the concentration of remaining TNBS, which was obtained from the absorbance at 252 nm. The quantity of aminoalkyl groups on the gel was determined on the basis of equimolecular TNBS consumption.

Keywords——amino group; affinity chromatography; agarose gel; 2,4,6-trinitrobenzenesulfonic acid; spectrophotometry

The exact estimation of the amount of aminoalkyl groups bound to garose gel is of critical importace in relation to the adsorption and elution of biochemical materials to be purified by affinity chromatography.[1] Spectrophotometric determination[2] was carried out by solubilizing trinitrophenylaminoalkyl gel (TNP-AA-gel: TNP, trinitrophenyl), which was obtained by the reaction of aminoalkyl gel (AA-gel) with 2,4,6-trinitrobenzenesulfonate (TNBS). However, if the full solubilization cannot be performed, the method cannot be used because the insoluble particles interfere with the determination. Lustenberger *et al.*[3] and Schmitt *et al.*[4] reported a spectrophotometric method based on the formation of the Schiff's base between the amino group and 2-hydroxy-1-naphthaldehyde. However, this method also suffers from the disadvantage that it is time-consuming, because a long reaction time is necessary. A simple method, which is based on the reaction of the gel with TNBS and, after removal of the reacted gel, spectrophotometric analysis after coloration by the reaction of glycine with unreacted TNBS, was very recently reported by Antoni *et al.*[5] This paper describes an alternative method, which is based on direct spectrophotometric measurements of unreacted TNBS without the coloration with glycine.

Materials and Methods

Materials——Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). Cyanogen bromide was prepared from sodium cyanide and bromine.[6] Aminoalkyl gels were prepared by the method of Sharma *et al.*[7] Activation was carried out in phosphate buffer (5 M, pH 11.9) of very high buffer capacity. The concentration of cyanogen bromide was 100 mg per ml of packed gel. Coupling was carried out by using diamine at a concentration of 1 mmol per ml of packed gel.

Other compounds and solvents were of the best grade commercially available. Spectral measurements were carried out with a Shimadzu 180 spectrophotometer.

Standard Procedure of Determination——Two ml of 75 mM sodium borate containing a specified amount of TNBS was added to wet AA-gel (200 mg), and the suspension was occasionally shaken for a specified time at room temperature. The resultant TNP-AA-gel was filtered by using a Pasteur capillary pippete packed with sanitary cotton at the tip, and washed with the above borate solution. The filtrate and washing were combined. The combined solution was adjusted to a suitable volume with the borate solution, and the absorbance was measured at 252 nm. The concentration of TNBS was calculated using $e = 1.12 \times 10^{4)}$ 1/mol·cm. The amount of TNBS consumed during the reaction was obtained by subtracting the obtained value from the initial concentration of TNBS.

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Spectral Analysis of Solubilized TNP-AA-gel——Washed TNP-AA-gel obtained as above in the pipette was transferred to a test tube, and diluted to 3 ml with a solution of 6 N HCl and acetic acid (1 : 1 v/v). The suspension was incubated at 75 °C for a specified time. The insoluble gels were filtered with a sintered glass filter, and washed with the acidic solution. The absorbance of the combined solution of the filtrate and washings was measured at 343 nm against the acidic solution. The concentration of the TNP–amine complex was calculated from the absorbance using $e = 1.4 \times 10^4$ l/mol·cm.

Time Course of Solubilization of TNP-AA-gel——The insoluble gel beads in suspension was determined by turbidimetry.[8] Agarose solution (final concentration 0.05%) was added to the suspension incubated with acidic solution as described above. After agitation, turbidity was recorded as absorbance against water at 650 nm.

Determination of Picrate——Picrate produced by the solvolysis of TNBS in 75 mM borate solution (medium for trinitrophenylation of gels) was determined by absorbance measurements at 410 nm. Picrate concentration was calculated using $e = 0.90 \times 10^4$ l/mol·cm.

Results and Discussion

Time Course of TNBS Consumption in the Trinitrophenylation

Aminooctyl Sepharose 4B (AO-Sepharose) was trinitrophenylated with TNBS. The time course of TNBS consumption was studied, and is shown in Fig. 1. No consumption of TNBS was observed after the reaction time of 80 min. Therefore, the reaction time was fixed at 2 h in subsequent trinitrophenylation.

Trinitrophenylation at Various Concentrations

The TNBS consumption in the trinitrophenylation of AO-Sepharose was studied as a

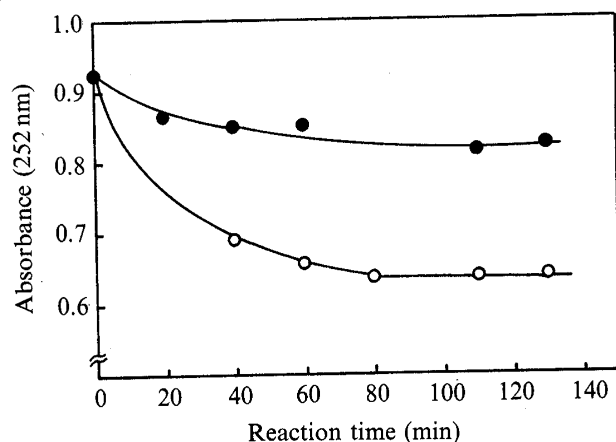

Fig. 1. The Time Course of Trinitrophenylation of Aminoalkyl Gel

Two types of aminooctyl Sepharose 4B (150 mg wet gel), which were obtained at different concentrations of cyanogen bromide, were trinitrophenylated with TNBS (10 μmol) in 75 mM sodium borate (2.0 ml). The aminooctyl contents were 1.1 (—●—) and 2.5 μmol (—○—) per 150 mg of wet gel.

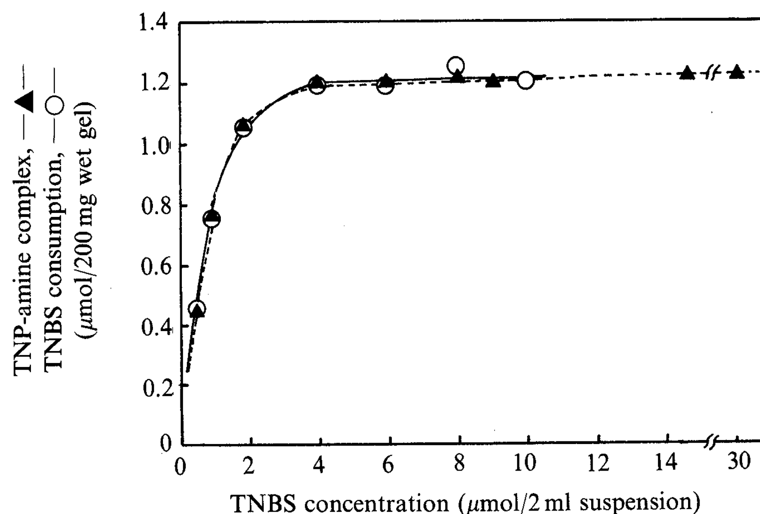

Fig. 2. The Trinitrophenylation of Aminooctyl Sepharose with Various Concentrations of TNBS

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function of the concentration of TNBS. As shown by the solid curve of Fig. 2, the consumption is roughly proportional to the initial concentration of TNBS up to 4 μmol/2 ml gel suspension, after which the consumption remains constant with further increasing concentration of the reagent. The consumption at the constant value (1.2 μmol/200 mg wet gel) is equivalent to the content of aminoalkyl groups of the gel sample, if the reaction occurs quantitatively at the plateau of the curve and the reagent is not consumed except for the reaction. These assumption were shown to be valid by studies of TNP-AA-gel solubilization as described below.

Determination by TNP-AA-gel Solubilization

A different spectral determination was carried out in order to verify the validity of the present method. Failla *et al.*[2] showed that determination was possible by spectral analysis (at 340 nm) of TNP-AA-gel solution provided that a solubilizing solvent was chosen which resulted in minimal interference by light-absorbing decomposition products of the gel. The reported solubilization method, which included incubation with 50% acetic acid for 2 h at 75 °C, failed to solubilize the TNP-AA-gels prepared here. Similar observations have been reported by other workers.[3,7] We found that a mixture of an equal volume of 6 N HCl and

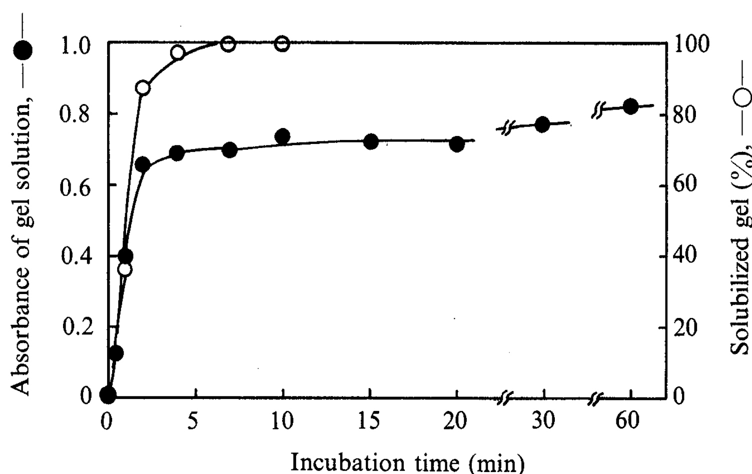

Fig. 3. The Solubilization Course of TNP-Aminohexyl Gel

TNP-aminohexyl gel derived from 200 mg of wet aminohexyl gel was solubilized by incubation with a solution of 6 N HCl and acetic acid (1 : 1, v/v). The solution was monitored for turbidity of the gel suspension. The formation of interfering compounds during incubation was monitored by measuring the absorbance at 343 nm of the gel filtrate.

TABLE I. Results of Quantitation of Aminoalkyl Sepharose

Carbon number of substituent	Present method (μmol)[a,b]	TNP-AA-gel solubilization method	
		(μmol)[a]	Incubation time[c] (min)
2	0.33	0.31	2
4	ND[d]	ND	3
6	1.57	1.59	8
8	1.24	1.21	12
10	1.20	1.20	12

a) The value for 200 mg of wet gel.
b) The mean of four determinations at the plateau of the TNBS consumption curve (see Fig. 2).
c) Wet gels (200 mg) were incubated with a solution (3.0 ml) of 6 N HCl and acetic acid (1 : 1, v/v) at 75 °C.
d) Not determined.

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acetic acid, among several solvents tested, solubilized the gels with incubation at 75 °C. A typical time course of solubilization of TNP-AA-gel, as well as of the formation of interfering products from the gels, is shown in Fig. 3. Full solubilization was performed with incubation times from 2—12 min (Table I). A prolonged incubation yields the products gradually, producing a gradual rise in the absorption curve. Therefore the incubation was stopped as soon as the solution was clear, before the release of the products, and the clear solution thus obtained was used for the spectral analysis. The analytical results for each TNP-aminooctyl gel (TNP-AO-gel), obtained from TNBS consumption experiments, are shown by the dotted curve of Fig. 2. The trinitrophenylation increases with increasing concentration of TNBS up to a plateau; there is no further increase in the range of 4—30 μmol of TNBS. The concentration of TNP–amine complex obtained at the plateau, at which the reaction is quantitative, is equivalent to the content of aminoalkyl groups on the sample gel. The content obtained here is quite consistent with that obtained by the TNBS consumption method. Similar results were obtained with AA-gels having a different length of aminoalkyl arm (Table I).

The curve obtained by the TNP-gel solubilization method coincides well with that obtained by the present TNBS consumption method (see Fig. 2). These findings indicate that TNBS is consumed only for trinitrophenylation. In fact, picrate produced by the hydrolysis of TNBS under the reaction conditions (incubation for 15 min at 20 °C) was found to be negligible (1.4%) by direct spectral analysis.

Assay with the Present Method

A sample of wet gel (200—300 mg) is trinitrophenylated with various amounts of TNBS for 2 h. The amount of TNBS consumed in the reaction is calculated from the remaining TNBS concentration in the reaction mixture (see Materials and Methods for the detailed procedure). The consumption curve is prepared against the initial concentration of TNBS as in Fig. 2, and the content of aminoalkyl groups on the gel is read from the plateau in the curve. The amount of TNBS added to the gel should range up to an 8-fold excess of the reagent relative to the amount of aminoalkyl groups. Use of a large excess of the reagent results in deviations of the plots. The reagent solution can be kept in a refrigerator for a week.

Determination of Gel Samples of Various Weights

AA-gel samples of various weights were determined by use of the present method. A linear response was observed from 50—300 mg of the gels, as shown in Fig. 4. This indicates

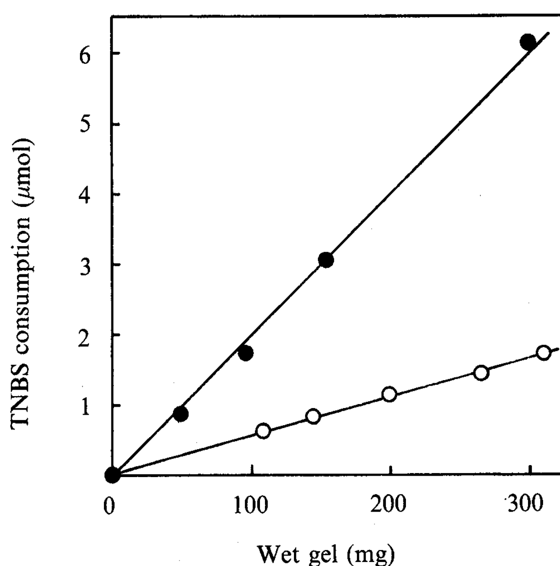

Fig. 4. The Trinitrophenylation of Gel Samples of Various Weights

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that the analytical values obtained can be normalized when the gel weight in the sampling is scattered in the above range.

The present method, which is based on spectrophotometric measurements of TNBS after reaction of the gel with TNBS, is simple, fairly rapid, and useful especially when the TNP-AA-gel solubilization method cannot be applied because of lack of an adequate solubilizing solvent.

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