0273-2289/93/3801-02—0015$03.20

Colorimetric Determination of Reactive Primary Amino Groups of Macro- and Microsolid Supports

PHILEMON E. TYLLIANAKIS,[1]
SOTIRIS E. KAKABAKOS,[1] GREGORY P. EVANGELATOS,[1]
AND DIONYSSIS S. ITHAKISSIOS*,[1,2]

[1]Institute of Radioisotopes and Radiodiagnostic Products, Radioimmunochemistry Laboratory, N.C.S.R. "Demokritos," 153 10, Athens, Athens, Greece; and [2]Department of Pharmacy, Pharmaceutical Technology Laboratory, University of Patras, Patras, 261 10, Greece

Received August 31, 1992; Accepted October 8, 1992

ABSTRACT

The several factors that could affect the sensitivity and the accuracy of the determination of solid-supported amino groups using 2-iminothiolane (Traut's reagent) and 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) are described. The authors found that by using 0.1*M* phosphate buffer, pH 8.0, instead of ethanol as solvent for the reaction of the solid supports with the 2-iminothiolane, using 0.1*M* phosphate buffer adjusted at pH 7.27 instead of 8.0 as diluent of 5,5'-dithiobis-(2-nitrobenzoic acid), and selecting carefully the concentration of the latter reagent, it was possible to produce a very sensitive assay capable of quantitatively determining the surface amino groups of very different types of samples. The assay is well adapted for quantitative determination of amino-carrying plastic beads, permitting the determination of nanomolar quantities. In addition, the assay is well suited for microparticulated solid supports (e.g., AH-Sepharose).

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Colorimetric determination, of reactive amino groups; Traut's reagent; Ellman's reagent; solid supported amino groups; solid-phase amino groups; amine-carrying solid supports; amino groups, of macrosolid supports; amines carrying plastic beads; amino groups, of microsolid supports.

INTRODUCTION

The covalent immobilization of biomolecules onto solid supports carrying functional groups offers several advantages, the most important of which is higher binding stability over the adsorptive techniques (*1,2*). The most common supports used in immunoassays are those carrying amino groups (*3–6*), although other kind of groups (e.g., carboxy, hydrazido, and so forth) have been used successfully (*7,8*). The quantitation of the reactive amino groups of a solid support could be very useful for optimizing the coupling conditions of ligands, for selecting the appropriate solid support, and for monitoring the number of the amino groups inserted on a solid support during manufacturing.

This article focuses on the quantitative determination of amino groups on plastic supports. In a previous paper (*5*), we observed that these materials offered significant technical advantages that improved the analytical characteristics of the immunoassays developed as compared to plastic supports without functional groups.

The methods described in the literature (*9–13*) for the quantitation of the solid-supported amino groups have been developed especially for microparticulated solid phases (e.g., gels). Among them, we selected for our purposes the method of Ngo (*11*), because this seems to be more specific, simple, and convenient. According to the method, the solid supports were reacted with an excess of 2-iminothiolane (ITL, Traut's reagent) (*14*) and, after being thoroughly washed, were then reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (*15*) to quantify the sulfhydryl groups generated from the reaction of solid-phase amino groups with Traut's reagent. The quantitation was carried out through the extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB), produced in the solution by the cleavage of DTNB, at 412 nm. We failed to obtain accurate, precise, and reliable results using plastic beads carrying amino groups as samples by following the protocol proposed in the paper. Preliminary experiments showed that this failure was associated with the relatively poor sensitivity of the assay, and with several physicochemical parameters associated with both the plastic supports and the detection reagent (DTNB). We reinvestigated several parameters of the assay, and propose a protocol that increases the sensitivity and expands its versatility. The proposed protocol is appropriate for measurement of amino groups on both macro- and microparticulated solid supports.

MATERIALS AND METHODS

Chemicals

2-Iminothiolane (ITL), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiotreitol (DTT), and 4-dimethylaminopyridine were from Sigma Chemical Co., St. Louis, MO. AH-Sepharose 4B was a product of Pharmacia, Uppsala, Sweden. Dylark and AminoDylark beads (6.4 mm in diameter) were from Sekisui Chemical Co., Kita-ku, Osaka, Japan. The polystyrene beads (6.4 mm in diameter) were obtained from Precision Plastic Ball Co., Chicago, IL. All other reagents and solvents used were of analytical grade and were purchased from Merck, Darmstadt, Germany, except as otherwise indicated.

Reagents

ITL solution (40 m*M*) was prepared in 0.1*M* sodium phosphate buffer, pH 8.0, containing 40 m*M* 4-dimethylaminopyridine. DTNB solution (0.5 m*M*) was prepared in 0.1*M* sodium phosphate buffer, pH 7.27, containing 0.5*M* NaCl and 0.01*M* EDTA. The pH was readjusted to 7.27 under vigorous stirring using 0.05*M* NaOH solution, and then the solution was filtered successively through 0.45- and 0.22-μm HV filters (Millipore Corporation, Bedford, MA). Washing solutions:

A Sodium phosphate buffer (0.1*M*), pH 7.27;
B DTT (1.0 m*M*) containing 10 m*M* EDTA;
C NaCl (1*M*) containing 10 m*M* EDTA; and
D Sodium phosphate buffer (0.1*M*), pH 7.27, containing 1 m*M* EDTA.

Methods

Pretreatment of the Samples

The plastic beads were washed three times with distilled water and degassed, whereas the microparticulated solid supports (dry powder or gel) were first washed four times with 10× their packed volume of 0.5*M* NaCl solution and twice with 10× their packed volume of degassed 0.1*M* sodium phosphate buffer, pH 7.27.

Determination of the Amino Groups of Solid Supports

Four milliliters of freshly prepared ITL solution were added to a glass vial containing either 10 plastic beads or 250 μL of the packed washed gel. The vial was stoppered, vortexed, and incubated by end-over-end shaking for 30 min (this step was repeated once in case of gel). Then, the ITL solution was removed, and the beads were washed successively with 2×10 mL of each one of the washing solutions A, B, C, and D. Four milliliters of the DTNB solution were added to the washed plastic beads,

whereas the washed gel was diluted with washing solution D to a final vol of 5 mL, vortexed, and 1 mL of the suspension was transferred to a glass tube. The tube was centrifuged, the supernatant was aspirated, and 4 mL of the DTNB solution were added. The solids were incubated with the DTNB solution by end-over-end incubation for 30 min at room temperature. Nonspecific value (background) for each material was estimated following the same procedure, but ITL was eliminated.

Measurements and Calculations

The measurements were carried out by reading the adsorbance of the solutions at 412 nm after the reaction without further dilution, using as blank the DTNB solution. When we used concentrations of DTNB higher than those reported under the Reagents section (0.5 m*M*), appropriate dilutions were carried out to obtain OD values lying inside the working range of the spectrophotometer (usually 20-fold). For the calculations, we used a TNB^{-2} molar absorption coefficient value of $14{,}150 M^{-1} \cdot cm^{-1}$ (*16*).

The quantity of amino groups of each support was calculated by the following formula:

$$(OD_{+ITL}) - OD_{-ITL} / 250 \times 14{,}150 \text{ mol of } -NH_2/\text{tube} \quad (1)$$

where OD_{+ITL}, specific value; OD_{-ITL}, nonspecific value; 250, factor dependent on the reaction volume; 14,150, molar absorption coefficient of TNB^{-2}.

Comparison Method

For comparative studies throughout this work, we followed the protocols described by Ngo (*11*) for the determination of amino groups on microparticulated solid supports.

Amination of Polystyrene Beads

The addition of amino groups on the surface of polystyrene beads was carried out by the method of Chin and Lanks for the amination of polystyrene tissue flasks (*17*). Briefly, the beads were first nitrated using "mixed acid" solution (47% v/v nitric acid in concentrated sulfuric acid), and then the nitro groups generated on the plastic surfaces were reduced to amino groups by treatment with 6% (w/v) sodium hydrosulfite in 2*M* KOH. We prepared several batches of aminated polystyrene beads varying the duration of the nitration step. For the purposes of this work, we used three different batches called hereafter API, APII, and APIII. These were prepared by nitration for 1, 2, and 4 h, respectively. All the other steps of the amination procedure were the same for all three kind of beads.

RESULTS AND DISCUSSION

To our knowledge, all of the methods described in the literature for the quantitation of solid-supported amino groups have been developed for microparticulated solid supports with large available surfaces (e.g.,

Table 1
Determination of the Reactive Amino Group Content of Solid Supports Using Different Solvents of ITL[a]

Solid support	Reaction of solids with ITL in			Units
	Ethanol	Ethanol 60%[b]	Buffer[c]	
Dylark	0.0	0.0	0.0	nmol/bead
AminoDylark	1.5±0.2[d]	1.6±0.1	2.1±0.2	nmol/bead
Polystyrene	0.0	0.0	0.0	nmol/bead
API[e]	1.0±0.1	1.2±0.1	1.6±0.1	nmol/bead
APII	2.3±0.2	2.4±0.1	3.4±0.2	nmol/bead
APIII	3.9±0.3	4.2±0.2	5.5±0.2	nmol/bead
Sepharose 4B	0.0	0.0	0.0	μmol/mL gel
AH-Sepharose 4B	1.4±0.1	6.3±0.2	6.5±0.3	μmol/mL gel

[a] All the other conditions were as described under Materials and Methods.
[b] Ethanol 60% v/v in 0.1 M $NaHCO_3$, pH 8.5.
[c] 0.1 M sodium phosphate buffer, pH 8.0.
[d] Mean value of six independent determinations (± SD).
[e] The API, APII, and APIII represent polystyrene beads nitrated for 1, 2, and 4 h, respectively, and then reduced to generate surface amino groups.

gels). We selected the most promising method (*11*) to determine the surface amino groups of plastic beads. However, the small quantity of amino groups existing on a limited surface area and physicochemical characteristics of the plastic supports, such as the adsorption of DTT on ethanol-treated beads, did not permit their accurate determination. Therefore, we reinvestigated several parameters of this assay and here propose a modified protocol, using Traut's and Ellman's reagents. This protocol expands its versatility, and can be used for the determination of amino groups existing on such different materials as gels or plastisc beads.

We found that the most critical parameters that affect the amino group determination were the ITL's solvent, the concentration of DTT used in one of the washing solutions, and the concentration as well as the pH of the DTNB solution.

ITL's Diluent

In the protocol described by Ngo, for the determination of solid-phase amino groups, the solvent used was dry ethanol. However, in order to avoid several effects associated with the use of ethanol and to ensure slight alkaline environment for the reaction of ITL with the amino groups, we used as its solvent a 0.1*M* phosphate buffer, pH 8.0. As is shown in Table 1, when the reaction of ITL with the plastic beads or with AH-Sepharose (dry powder) took place in dry ethanol (according to Ngo's protocol for dry solid supports), the values obtained, under the optimum conditions, were significantly lower than those obtained using the phosphate buffer as diluent. The higher effect was observed using AH-Sepharose (almost

80% less amino groups determined), whereas the lower one was obtained using AminoDylark beads (25%). The decrease observed using API, APII, and APIII was approx 30%.

Using 60% v/v final ethanol concentration (according to the protocol of Ngo for wet gels), similar amino group values were determined for AH-Sepharose using either ethanol or buffer as diluent for ITL. Using ethanol even at 60% v/v concentration did not improve the values obtained for the plastic beads.

We considered the possibility that low swelling of AH-Sepharose in ethanol may significantly reduce the accessibility of the amino groups to ITL, resulting in low estimation when Ngo's protocol for dry solid supports was used. On the other hand, we do not have any solid explanation for the lower estimation observed when this protocol was used to determine the amino groups on the plastic supports. We postulate that a slight decrease in the available surface area may also exist in this case, causing steric hindrance effects.

The DTT contained in one of the washing solutions was used to keep the sulfhydryl groups in a reduced state. We found that this step was necessary for AH-Sepharose, since a 10–15% decrease in values was obtained when DTT was not included in the washings. On the other hand, very low concentrations of DTT (0.1 m*M*) were adequate to obtain maximum values using plastic beads. During this study, we found another significant effect of the interaction of ethanol with the plastics. As is shown in Fig. 1, the treatment of the plastic beads with absolute or 60% ethanol significantly increased the nonspecific value of these materials in a manner related to the concentration of DTT used in the washing step that followed the reaction with ITL. This effect indicates that the ethanol-treated beads adsorbed significantly higher quantities of DTT, compared with the untreated beads, producing high nonspecific values. Using Sepharose or AH-Sepharose as samples, we found that nonspecific values were negligible and independent of the ITL's diluent as well as of DTT concentrations up to 10 m*M*.

We found also that ethanol could not be used as diluent for ITL when plastic beads carrying surface-grafted amines, diamines, or synthetic polymers (e.g., polyphenyl-lysine) were used as samples, because it eluted the amine-carrying layer (data not shown).

We were able to adopt a common protocol for both the plastic beads and the AH-Sepharose by dissolving the ITL in phosphate buffer, pH 8.0, and using 1.0 m*M* DTT solution in the washing steps. This protocol was free of the problems associated with the use of ethanol and could also be used with all types of samples. We found that for the plastics, a 30-min incubation with ITL was adequate for accurate determination of amino groups, whereas for AH-Sepharose, two repetitive steps were needed to obtain maximum measurements.

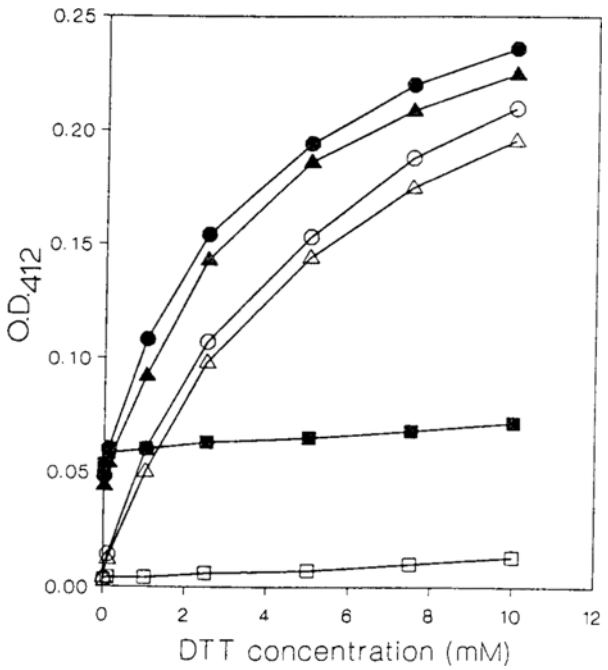

Fig. 1. Effect of the concentration of DTT (in washing solution B) on the specific (closed symbols) and on the nonspecific value (open symbols) of Amino-Dylark beads reacted with ITL in dry ethanol (○, ●), in 60% v/v ethanol (△, ▲), and in 0.1*M* phosphate buffer, pH 8.0 (□, ■). The OD values of the DTNB solution used as blank have been eliminated in all cases.

DTNB Solution

We found that mainly two factors related to the DTNB solution could significantly affect the determination of amino groups. The first was the pH of the DTNB solution. A 30% lower solution blank was obtained when a final pH of 7.27 was used instead of pH 8.0. This finding is in accordance with the results of Riddles et al. (*16*), and is owing to the lower alkaline hydrolysis rate of the DTNB at the lower pH.

The second factor, which—according to our findings—intervenes to the accurate determination of the amino groups, was the concentration of the DTNB. As is shown in Fig. 2, when the ratio DTNB/$NH_{2\ max}$ (amount of DTNB added to maximum amino groups determined) ranged between 3 and 120, a plateau of maximum amino groups measurements was obtained. This was true for all quantities of solid-supported amino groups tested. Using ratios lower than 1, the values obtained were lower than those of the plateau. However, these values were higher than those expected concerning the amount of DTNB added. This could be explained considering

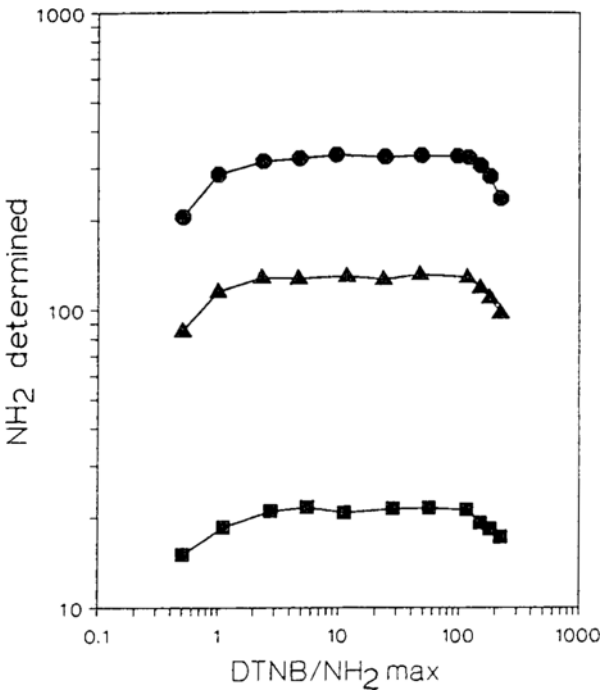

Fig. 2. Amino group values estimated using as samples 20 AminoDylark beads (■), 20 μL of AH-Sepharose (▲), and 50 μL of AH-Sepharose (●) plotted vs. the ratio of the amount of DTNB added to the maximum amino group value determined in each case. The conditions of the experiment were as described under Materials and Methods.

that 1 mol of DTNB produces more than 1 mol of TNB when the sulfhyhryl groups, coupled to the solid supports, are in excess of DTNB. A similar finding has already been reported for cysteine (*16*). Using ratios of DTNB/$NH_{2\ max}$ higher than 120 has generally resulted in significant underestimation of the amino groups. As shown in Fig. 3, the TNB in the sample produced by the reaction of DTNB with the sulfhydryl groups inhibits the alkaline hydrolysis of DTNB, and therefore, causes a different hydrolysis rate between the sample and the blank tube. Using a DTNB solution of 1.5 m*M* at pH 7.27 and 10 AminoDylark beads (DTNB/$NH_{2\ max} = 300$), a 30% lower estimation of the amino groups was obtained compared with that achieved using 0.15 m*M* DTNB solution (DTNB/$NH_{2\ max} = 30$) when the measurement was carried out at 30 min after the addition of DTNB. Although this effect was evident even when diluted DTNB solutions were used (DTNB/$NH_{2\ max} < 120$), it was practically negligible. This was because of the relatively low amounts of the TNB produced, in this case by the alkaline hydrolysis, compared with that produced from the specific reaction. When high excess of DTNB was used, the value of amino groups

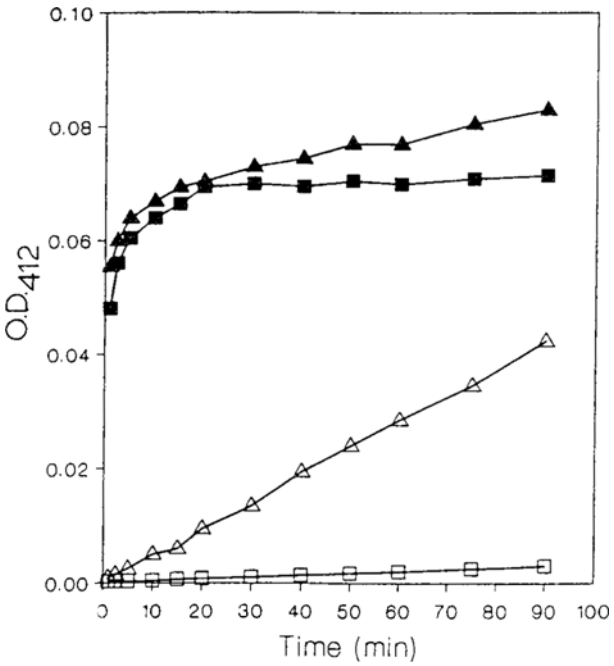

Fig. 3. Kinetic study of DTNB hydrolysis in the blank tube (open symbols) and in the presence of ITL-treated AminoDylark beads (closed symbols). Ten AminoDylark beads in 4 mL of DTNB solution were used. The concentrations of DTNB solutions used were 1.5 m*M* (△, ▲) and 0.15 m*M* (□, ■). All the values have been corrected against the absorbance of DTNB solution at zero time.

determined greatly depended on the interval between the addition of DTNB and the measurement of the absorbance. Even lower estimation of the amino groups could be observed when a high volume of samples was tested. It is remarkable that the difference between the hydrolysis rate in the sample and in the blank tube was much more obvious when the pH of the DTNB solution was 8.0.

Taking into consideration all these findings, we adopted a 0.5 m*M* DTNB solution at pH 7.27 in our protocol. This gave us the capability to measure, without any final dilution, amino groups ranging between 5 and 300 nmol/sample, whereas the range was extended up to 600 nmol when final dilutions were used. It was possible to determine such low amounts of amino groups as those existing on the surface of 3 AminoDylark beads up to such concentration as this contained in 50 μL of AH-Sepharose. When an unknown sample is going to be used (e.g., when no information is given by the manufacturer about the amino groups content), we propose that the DTNB concentration that will be used has to be estimated performing an experiment using full range of DTNB concentrations.

Cleavage of the TNB Bound to the Solid Supports

We tested another approach for determining the solid-phase amino groups that consisted of the removal of TNB and excess of DTNB by washing the solid supports after the reaction, followed by their treatment with excess DTT to release the bound TNB. A similar procedure has already been described for the determination of sulfhydryl content of proteins with DTNB (*16*).

Using this approach, we obtained a value that was 10% of that obtained by the usual protocol. We were able to increase these values when an oxidative agent (chloramine-T) was included in some of the washing solutions used after the reaction with DTNB. However, in any case, the values observed did not exceed 50% of the values obtained by the usual protocol. It seems that the TNB coupled to the solid phase was removed during the washings, in the absence of excess DTNB, resulting in significant underestimation of the amino groups, and thus, we do not propose this procedure.

General Comments

As was expected, using our method, we did not detect surface amino groups on materials without functional groups, such as polystyrene beads, Dylark beads, and Sepharose. We quantitated the amino groups on the surface of aminated polystyrene beads. We found that the longer the duration of the nitration step, the higher the values of the amines. This is in accordance with the findings of Chin and Lanks (*17*), who, using differential infrared spectroscopy, observed the same phenomenon for polystyrene tissue flasks. AminoDylark is prepared from ethylenediamine and a partial hydrolysate of Dylark, and retains amino functions (*18*). The number of amino groups existing on the surface of the beads is not given by the manufacturer. Our determination gave a value of 2.1 ± 0.2 nmol of amino groups/bead. This value seems to be reasonable with respect to the available surface of the beads, since it is similar to the above-mentioned values of the aminated polystyrene beads of same diameter (6.4 mm).

Following the protocol proposed in this article, we estimated an amino group value of 6.5 ± 0.3 μmol/mL for AH-Sepharose, which compared very well with the value of 6.4 ± 0.2 μmol/mL obtained using Ngo's protocol for wet solid supports. Although this value is lower than the mean value given by the manufacturer (8.0 ± 2.0 μmol/mL gel), determined using 2-hydroxy-1-naphthaldehyde (*10*), it lies inside the expected range of 6.0–10.0 μmol/mL of gel. In a recent publication, Ukeda et al. (*13*) reported a value of 4.4 μmol/mL for AH-Sepharose using Ngo's protocol. They ascribed the lower value obtained to the specificity of Traut's reagent as well as to steric hidrance effects. They did not mention which one of Ngo's protocols was used. We postulate that this low value could be partly

attributed to the factors described in the present article that affect the accuracy of the amino group determinations.

CONCLUSION

In this article, we describe the effect of several factors that could affect the determination of solid-supported amino groups using the colorimetric method of Ngo. We also propose a modified protocol, using Traut's and Ellman's reagents, that increases the sensitivity and expands the versatility of the assay allowing the measurement of amino groups on such different materials as gels or plastic beads.

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