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Note

Quantitative determination of alkylamino side-chains coupled to agarose beads

Comparison of methods

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In affinity chromatography, the concentration of ligand covalently bound to the support is of critical importance for adsorption and elution of the biochemical material to be purified. The exact determination of the amount of bound alkylamino groups is of great interest for two reasons: firstly, it affords the possibility of fitting the conditions of cyanogen bromide activation for the preparation of affinity matrixes bearing a chosen concentration of ligand; and secondly, it allows an estimation of bound ligand by determining alkylamino groups before and after ligand coupling.

Among the spectrophotometric methods for assaying alkylamino side chains, the most widely used is that involving trinitrobenzenesulphonic acid (TNBS). In addition to the qualitative test first introduced by Inman and Dintzis[1], Failla and Santi[2] described a quantitative assay by direct spectral analysis of solubilized agarose whose functional groups were converted to chromophores with TNBS. Recently, Schmitt and Walker[3] proposed a method for the measurement of coupling capacity of solid-phase sequencing supports, based on the formation of a Schiff's base between the free amino groups and 2-hydroxy-1-naphthaldehyde with subsequent displacement of this base by benzylamine and spectrophotometric determination of the new soluble Schiff's base in the supernatant.

Faced with the problem of determining covalently bound amino groups on Sepharose, we decided to compare these two methods, and the results are reported in this paper.

MATERIALS AND METHODS

Sepharose 4B and CL 4B were obtained from Pharmacia (Uppsala, Sweden). 2,4,6-Trinitrobenzenesulphonic acid dihydrate, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), diamines and benzylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). 2-Hydroxy-1-naphthaldehyde was a product of Fluka (Buchs, Switzerland), and dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,-20-dione) a product of Roussel-Uclaf (Paris, France). [1,4-^{14}C]Spermidinetrihydrochloride (120 mCi/mmol) and [1,2(n)-^{3}H]dexamethasone (28 Ci/mmol) were obtained

from the Radiochemical Centre (Amersham, Great Britain). All other compounds were reagent-grade chemicals.

Ultraviolet absorbance spectra were recorded with a Beckman Model 25 spectrophotometer.

Radioactivity was measured in a Intertechnique liquid scintillation counter.

Cyanogen bromide activation and coupling of the alkylamino group

We employed the procedure of March *et al.*[4] with a minor modification: activation was carried out in phosphate buffer of 5 *M*, pH 11.9. By varying the concentration of cyanogen bromide, activation could be controlled in order to prepare gels of a higher or lower degree of substitution.

Reduction of cystamine Sepharose

Reduction of cystamine Sepharose by sodium borohydride was carried out according to Harding[5] and subsequent assay of liberated thiols with Ellman's reagent[6].

TNBS assay[2]

To 0.5 ml of a 3-fold diluted suspension of the derivatized Sepharose were added 10 ml of borate buffer (0.1 *M*, pH 9.0) containing 15 mg of TNBS. After stirring for 2 h, the agarose was washed with water until the washings showed no absorbance at 340 nm. The gel was diluted to 5 ml with 50% formic acid and heated at 100 °C. When all of the Sepharose was solubilized, the concentration of the amine picrate complex was calculated from the absorbance at 340 nm using $\varepsilon = 1.4 \cdot 10^4$ l/mol·cm.

Aldehyde assay[3]

A 0.5-ml amount of packed derivatized agarose was washed with DMF and transferred into a screw-capped 10-ml test-tube. A 5-ml volume of 1.2 *M* 2-hydroxy-1-naphthaldehyde in DMF was added and the suspension was shaken by inverting it on a rotator. After agitation for 14 h, the mixture was centrifuged and the agarose washed with DMF (four 10-ml volumes), then with ethanol until the absorbance at 270 nm was zero (usually four or five 10-ml volumes). The packed agarose was finally diluted to 10 ml with 0.4 *M* benzylamine in ethanol and the suspension was stirred for 15 h. The absorbance of the supernatant was measured at 420 nm and the concentration of amine calculated using $\varepsilon = 1.09 \cdot 10^4$ l/mol·cm.

RESULTS AND DISCUSSION

TNBS method

When performed under the conditions described by Failla and Santi[2] (gel hydrolysis with 50% acetic acid by heating at 75 °C for 2 h) difficulties may be encountered in solubilization, as has been pointed out by other workers[7]. Only underivatized Sepharose 4B gives a clear solution. With Sepharose CL 4B or any derivatized Sepharose, cross-linking prevents total hydrolysis. Increasing the temperature to 100 °C proved ineffective even after several hours, as indicated in Fig.1a.

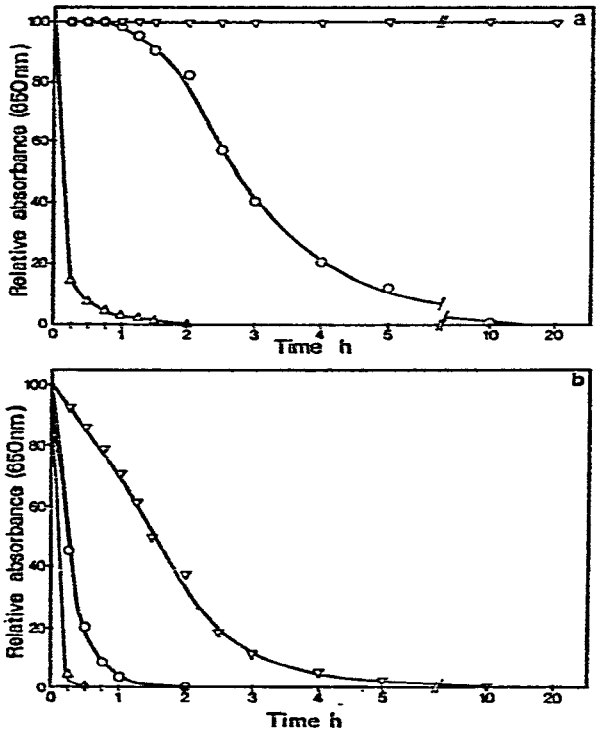

Fig. 1. Kinetics of hydrolysis. A 10-ml volume of packed gel was diluted to 50 ml with either 50% acetic acid (a) or 50% formic acid (b). The screw-capped test-tubes were placed in boiling water and aliquots of homogeneous solutions were taken after various lengths of time, as indicated. The amount of agarose beads in suspension was determined by turbidimetry. To 1 ml of suspension was added 1 ml of 5% PEG 6000. After agitation, the turbidity was recorded as absorbance against water at 650 nm. Results are expressed as a percentage of zero-time absorbance, which is directly proportional to the amount of agarose beads in suspension. Experiments carried out with Sepharose 4B (△), Sepharose CL 4B (○) and Sepharose CL 4B coupled to diaminodipropylamine (▽) (about 2 μmol alkylamino residue/ml of packed gel).

On the other hand, the use of formic acid (50%) instead of acetic acid allows full solubilization. In Fig. 1b, the kinetics of hydrolysis for different kinds of gels are illustrated, indicating that the reaction time for hydrolysis increase with the degree of cross-linking of Sepharose.

Another fact must be considered: whichever acid is used, light-absorbing decomposition products appear and lead to an increase in the absorption measured at 340 nm. This increase remains small as long as solubilization is not complete, but becomes substantial as soon as all of the Sepharose is dissolved. Fig. 2 shows the percentage of gel hydrolysis and the increase in A_{340} as functions of time of exposure to acid.

It therefore became obvious that good results using the TNBS assay could be obtained only if several precautions are taken. Firstly, each assay must be carried out in parallel with a control measurement on the same batch of derivatized Sepharose omitting the TNBS, but under similar conditions of hydrolysis. Secondly,

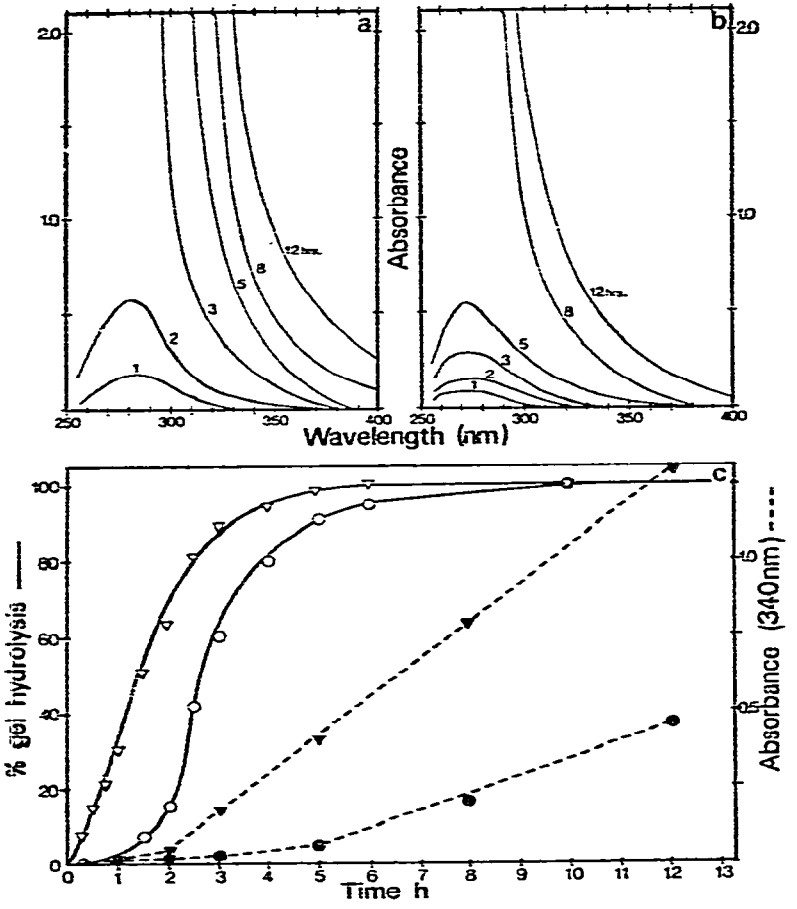

Fig. 2. UV spectra and effect of time of exposure to acid. In the course of measurement of the kinetics of hydrolysis (see Fig. 1) aliquots were taken after various lengths of time. After centrifugation, UV spectra of the supernatants were recorded (a, b). The percentage of gel hydrolysis measured from zero-time absorbance (see legend to Fig. 1) and the absorbance at 340 nm of the supernatant are shown as functions of time of exposure to acid at 100°C (c). Results for Sepharose CL 4B coupled to diaminodipropylamine in 50% formic acid (a) (▽, ▼) and Sepharose CL 4B in 50% acetic acid (b) (○, ●).

solubilization must be monitored carefully in order to stop the reaction as soon as the solution is clear, before the release of interfering by-products. This is possible with formic acid, 5–6 h generally being sufficient to solubilize derivatized agarose. In spite of this, for gels of a low degree of derivatization (less than 0.5 μmol of alkylamino group per millilitre of packed gel) the blank values are considerable, precluding good results.

Aldehyde method

Extension of the method of Schmitt and Walker[3] to alkylamino derivatized agarose required only a re-examination of the kinetics of displacement of the Schiff's

base with benzylamine. The results of this study are presented in Fig. 3. A displacement of 90% occurs in 1 h and the reaction is complete after about 15 h. A longer reaction time (24 h) gave no difference in the absorbance values obtained. Treatment of underivatized agarose gave no appreciable absorption at 420 nm. Moreover, this method avoids hydrolysis of the matrix, and it is possible to perform a subsequent assay on the same sample after extensive washing with ethanol and DMF.

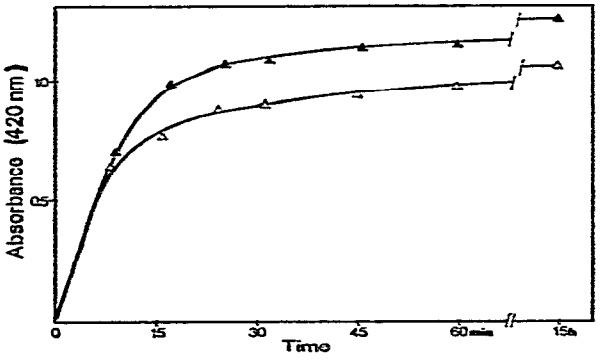

Fig. 3. Displacement by benzylamine at room temperature for two different batches of Sepharose coupled to diaminodipropylamine after reaction with 2-hydroxynapthaldehyde.

Comparison of the two methods

Comparison of the results obtained by these methods is difficult. The lack of a standard method for the determination of the alkylamino side-chain led us to approach the problem from different directions.

Firstly, we employed a radioactively labelled side-chain, such as [1,4-^{14}C]-spermidine. Unfortunately, the amount of diamine incorporated, as measured by counting ^{14}C, was 11.8 μmol per millilitre of packed gel, whereas the result of free amino group determination was 4.1 μmol of primary amino groups per millilitre of gel for the aldehyde assay and 4.8 μmol/ml for the TNBS assay. This indicates about 60% cross-linking and casts doubt on the results of Sharma and Slaunwhite[8], who reported good agreement between the results obtained by the TNBS method and radiometric analysis.

Thereby, it became necessary to use indirect methods in order to verify the validity of the determination of alkylamino side-chains.

One possibility was to determine the concentration of alkylamino residues before and after coupling of the ligand and to measure the incorporation of ligand by another method. Such experiments were carried out and the results are summarized in Table I. It appears that the amount of radioactively labelled ligand agrees closely with the differential analysis of the alkylamino side-chain performed using the 2-hydroxy-1-naphthaldehyde method and contrasts with the deceptive results obtained by TNBS assay.

TABLE I

RESULTS OF QUANTITATION OF ALKYLAMINO SIDE-CHAIN BEFORE AND AFTER COUPLING OF THE LIGAND

All results are expressed in μmol/ml of Sepharose. Three different batches of Sepharose CL 4B activated with CNBr were coupled with diaminodipropylamine, and steroid (dexamethasone) was coupled to the matrix 4 weeks later.

2-Hydroxynaphthaldehyde method		*Amount of ^{3}H-labelled steroid coupled to the matrix*	*TNBS method of Failla and Santi*[2]	
Before coupling of the ligand	*After coupling of the ligand*		*Before coupling of the ligand*	*After coupling of the ligand*
0.13	0	0.16	0.90	0.10
0.50	0.10	0.41	1.50	0.40
0.85	0.25	0.65	2.20	0.70

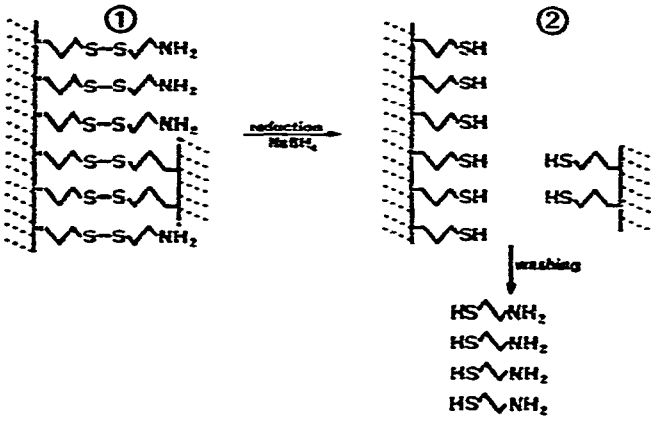

Fig. 4. Principle of checking validity of assay of alkylamino side-chains. 1, Cystamine agarose; 2, after borohydride reduction, amount of thiol liberated is equivalent to the amount of alkylamino group lost by the matrix.

TABLE II

RESULTS OF QUANTITATION OF ALKYLAMINO SIDE-CHAIN ON CYSTAMINE SEPHAROSE CL 4B BEFORE AND AFTER BOROHYDRIDE REDUCTION

All results are expressed in μmol/ml of packed gel. 1 week (I), 2 weeks (II) and 4 weeks (III) after activation. Determinations of amines were carried out in quadruplicate, and thiol assay is the mean of five determinations.

Expt. No.	*2-Hydroxynaphthaldehyde method*		*Amount of cystamine liberated*	*TNBS method*	
	Before borohydride reduction	*After borohydride reduction*		*Before borohydride reduction*	*After borohydride reduction*
I	6.60	1.04	5.51	9.19	2.36
II	6.43	0.74	5.46	9.85	1.89
III	5.63	0.58	5.29	8.31	0.95

Another approach is represented in Fig. 4. In this instance, the difference between amino groups before and after borohydride reduction must agree with the amount of thiol residues released in the supernatant. The results of different experiments are reported in Table II. Again, the aldehyde method proved far better than the TNBS assay.

In conclusion, our results confirm the criticisms of the TNBS assay made by other workers[7,9]. On the other hand, the results obtained by the aldehyde method are good, even with minimally derivatized Sepharose, and allow the determination of bound ligand by differential assay before and after coupling.

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