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A COLORIMETRIC METHOD FOR MONITORING ACTIVATION OF SEPHAROSE BY CYANOGEN BROMIDE

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SUMMARY

A colorimetric method has been developed for monitoring the activation of polysaccharides with cyanogen bromide. The method is based on the "König" reaction of cyanate esters with pyridine and barbituric acid to yield a red-purple colored complex with λ_{max}, 575 nm and an ϵ_M value of 15,000 $M^{-1}cm^{-1}$. Besides providing a means for a quantitative measurement of activation of the gel with CNBr, the method also enables us to determine (a) the extent of washing the activated gel required for the removal of the unreacted reagent; and (b) the amount and the decay of the reactive groups remaining after the coupling of the ligand to the activated gel.

INTRODUCTION

Before polysaccharides can be used as solid supports for affinity chromatography (1) or enzyme immobilization, they must first be activated. The most widely used method for polysaccharide activation is the reaction with cyanogen bromide (CNBr) which was first described in 1967 (2), and although over 10 years have passed, no easy and direct method has been available to monitor the activation reaction itself. Consequently the success of an activation was judged by coupling a small model peptide, like Gly-Leu, to the matrix (3-5). However, since the "coupling capacity" varies not only with the ligand used, but also with the specific coupling procedure employed, it seems preferable to establish an unambiguous measure of activation by determination of the amount of active groups present on the polysaccharide. Especially with the commercial availability of CNBr activated Sepharose the need for a quick test for reactivity of these preparations has become evident.

In 1904, König (6) reported that the reaction between pyridine and cyanogen halides can lead to colored products. This reaction was carefully investi-

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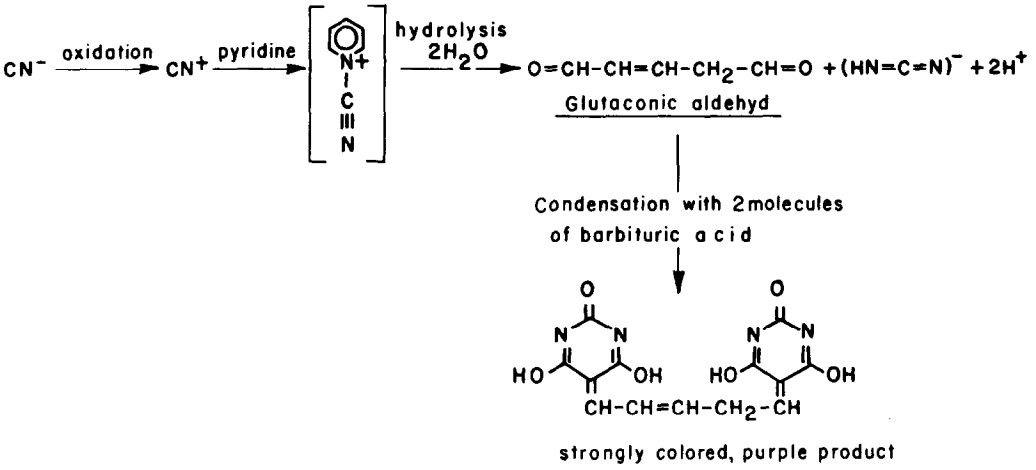
gated (7) and adapted for the quantitative determination of CN^- (8), by using barbituric acid as color producing reagent, whose analytical value was only recently restressed (9) (Scheme 1). The vital step in the entire sequence is



Scheme 1

the attack of CN^+ and the subsequent cleavage of the pyridine ring to yield glutaconic aldehyde. In contrast to earlier views (7,8) we now show that the cleavage of the pyridine ring can be brought about not only by CN^+ but also by cyanate ester (R-O-CN) derivatives. Therefore the above reaction does not only allow the determination of CN^- and (CN^+), but offers a possibility to estimate the amount of cyanate ester groups present in a sample of CNBr activated polysaccharide.

MATERIALS AND METHODS

Sepharose 4B and CNBr-activated Sepharose 4B were obtained from Pharmacia. Cyanogen bromide was from Merck. For all quantitative experiments, the CNBr was purified by sublimation in a cold-finger apparatus. All other chemicals, especially pyridine and barbituric acid (Merck) were carefully purified prior to use. Pyridine was redistilled and barbituric acid recrystallized several times from water.

Activation of Sepharose was performed by using CNBr either as solid (1,2) or dissolved in organic solvents (10).

Preparation of the Qualitative Test Reagent. 12 ml pyridine is mixed slowly with 2.5 ml concentrated analytical hydrochloric acid, 0.5 g of barbi-

turic acid is added and the volume made up to 20 ml with distilled water. After stirring for about 10 min a clear colorless solution is obtained. When stored in a cold and dark place it will be stable for several weeks, depending on the purity of the materials used. For qualitative tests a slight coloration of the test reagent is of no importance.

Preparation of the Quantitative Test Reagent. 8 ml pyridine is mixed slowly with 1.6 ml concentrated analytical HCl under cooling, 50 mg barbituric acid is added and the volume made up to 10 ml with water. The quantitative test reagent should be freshly prepared prior to use.

Qualitative Test for Presence of Free CNBr or Cyanate Esters on Activated Polysaccharides. To 10-20 mg of dry polysaccharide, or to 0.1-1.0 ml of swollen polysaccharide, or to several drops of the aqueous washings of freshly activated material, 1-2 ml of qualitative reagent is added. After slight shaking even the presence of less than 5 nmoles of CNBr or cyanate ester can be detected by the formation of a red-purple color, which develops within 30 seconds and becomes maximum after 10 min.

Preparation of Calibration Curve. Large, single crystals of freshly sublimed CNBr were used to prepare standard solutions (approximately 1-10 μmoles/ml) of the reagent containing CN^+ ions. After reaction with the freshly prepared quantitative reagent (10 ml) at 40°C for 15 min, the spectral properties of the colored complex were found to be: λ_{max}, 575 nm; ε_M, 15,000 $M^{-1}cm^{-1}$. Calibration curves ($A_{575\ nm}$ *vs.* concentration of CN^+) were found to be linear up to 60 nmoles/ml of the CN^+ ion.

Quantitative Test for Cyanate Esters on Polysaccharides. To a sample of polysaccharide, containing between 1 to 20 μmoles of cyanate ester groups, 10 ml of quantitative reagent are added. The mixture is kept at 40°C in a water bath under vigorous stirring for 15 min, then filtered on a glass-sinter. The solid material is washed with plenty of H_2O. The purple color tends to stick stubbornly to the polysaccharide, however, washing with a few ml of DMF will remove the absorbed color, leaving the polysaccharide with a faint pink tinge. The filtrate, combined with all washings is made up to any convenient volume* with distilled water and the absorbance of the final solution is read at 575 nm.

RESULTS

Freshly activated preparations of Sepharose, Sephadex and cellulose were found to yield strong red-purple color upon reaction with pyridine and barbituric acid. However, Sephadex and cellulose lost this feature after storage for 15 days at 4°C in contrast to the maintenance of such property in Sepharose preparations under similar conditions. No color formation could be observed with the following model compounds: urea, benzonitrile, succinimide, ethyl carbamate, diethyl carbonate and diethylimidocarbonate. Characteristic red-purple color was noted with CNBr, and phenylcyanate. These observations

* For samples of about 10 mg of freshly activated Sepharose, which contained about 10 μmoles of cyanate groups, a dilution to 500 ml proved to be convenient.

suggest that cyanate ester functions in the activated polysaccharides are responsible for the color formation and provide further evidence for the earlier proposal for the participation of reactive cyanate ester intermediate in the reaction with the ligand (11).

In order to assess the amount and the stability of reactive groups generated upon treatment with CNBr, Sepharose 4B was activated by the above described procedures. Part of the activated Sepharose was used for immediate analysis, while the remainder was stored at 4°C following its shrinkage with acetone and drying. The reactivity of the stored material was monitored periodically by the quantitative test. Commercial activated agarose preparations were likewise investigated. These studies showed that the amount of reactive groups in activated Sepharose preparations depended on the state of CNBr used in the activation step. Thus, preparations obtained immediately after activation with solid CNBr were found to contain 1,700 μmoles of reactive groups per g dry gel compared to a much lower value (930 μmoles/g dry gel) in samples derived from activation with solutions of CNBr in organic solvents. Activation of Sepharose with solid CNBr (1,2) appears to be the desirable procedure for the preparation of affinity gels containing large amounts of ligand. The number of reactive groups generated was governed by the concentration of CNBr used in the activation step. The number of reactive groups in the gel was found to decrease on storage, with nearly 50% loss in potency after 15 days of standing at 4°C. Commercially available preparations exhibited the same potency (440 μmoles/g dry gel) as the laboratory preparation that had been stored for 15 days.

The stability of cyanate ester on Sepharose 4B at room temperature was examined using suspensions of activated Sepharose in different media. Aliquots of samples, taken at desired intervals, were assayed for their cyanate ester content. These studies revealed that cyanate ester is relatively stable, especially in acidic media, a finding contrary to the earlier views. The experimentally determined decay half-lives are summarized in Table 1.

Table 1: Experimental half life for cyanate groups in Sepharose

Suspension medium	*Half life (min)*
Distilled water	30
0.15 M $KHCO_3$, pH 8.2	22
0.1 M K_2CO_3, pH 10.0	7
10^{-3} M HCl, pH 3.0	60

The kinetics of the activation reaction was investigated by activating a large quantity of Sepharose 4B according to the procedure of March *et al.* (10). At specific intervals, samples were withdrawn from the reaction mixture and analysed. The results indicate a peak in cyanate ester groups about 90 seconds after initiation of the reaction. The data are presented in Table 2.

DISCUSSION

The reaction between (CN^+) or (R-O-CN) and pyridine in the presence of barbituric acid leading to the formation of a purple color (λ_{max}, 575 nm) is both very sensitive and very selective (8,9). With the exception of amines, which react with glutaconic aldehyde and prevent the formation of the colored complex, no interfering substances have yet been found. In contrast to the original procedures (8,9), the reaction with polysaccharides requires a high concentration of pyridine. Salts, which may be present in the suspension medium tend to precipitate and reduce the sensitivity of the test.

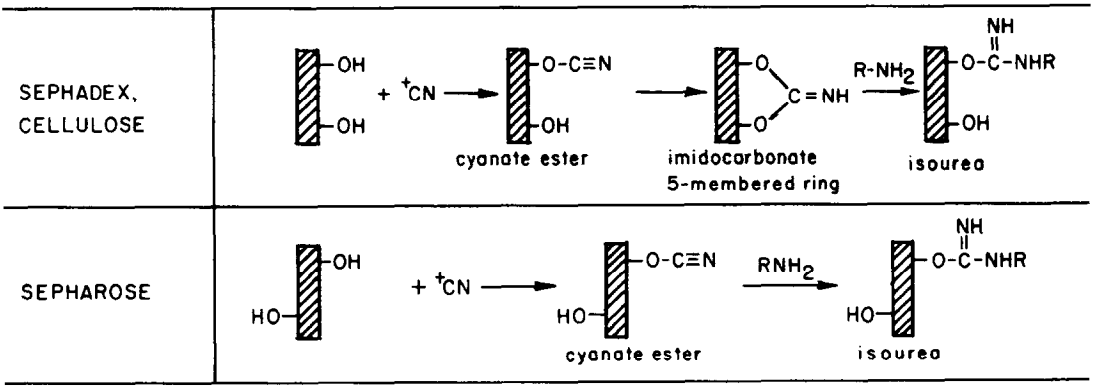
In the past, studies on the mechanism of activation involved the use of either Sephadex or cellulose as the polysaccharide model (12,13). The important difference between dextran or cellulose and agarose is that the molecular structure of the former substances allows for the formation of 5-membered imidocarbonate rings while that of agarose permits the formation of 6-membered rings only. Since the 5-membered rings are more energetically favored than the 6-membered rings, it is conceivable that imidocarbonates are the most predomi-

Table 2: Kinetics of CNBr activation

Sample No.	*Time of withdrawl (min)*	*(μmoles cyanate/g dry wt.) Sepharose*	*Nitrogen incorporated (μmoles/N/g dry wt.) Sepharose*
1	0.5	16	-
2	1.0	150	-
3	1.5	380	450
4	2.0	400	-
5	3.5	270	530
6	4.5	240	-
7	5.7	160	-
8	7.0	80	500
9	12.0	10	-
10	15.0	∿1	-
11	21.0	0	490

nant "active species" in activated Sephadex and cellulose. On the other hand, cyanate ester functions are the preponderant "active species" in Sepharose (Scheme 2). This view derives support from the following:

MECHANISM OF ACTIVATION AND COUPLING



Scheme 2

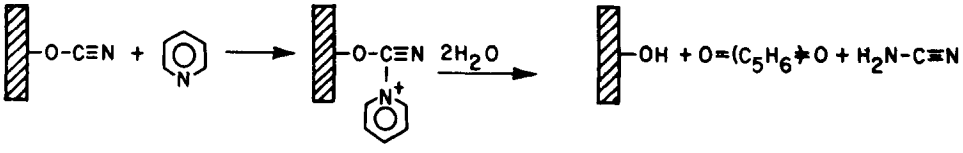
(i) Approximately 70% of the nitrogen incorporated in the freshly activated preparation of Sepharose exists as cyanate esters. These esters are more reactive than imidocarbonates and hence coupling of the ligand can be expected to proceed via cyanate ester function. Furthermore, even after 2 weeks of storage, activated Sepharose possesses nearly 50% of its active groups as

cyanate esters, while under similar conditions, no such group could be detected in activated Sephadex. Yet, both types of polysaccharides are capable of coupling amino containing compounds.

(ii) March *et al.* (10) have reported that coupling of the ligand occurs optimally after 90 seconds of activation. A study of the formation of cyanate esters has revealed that its concentration is maximal after 1-2 min of activation. The close correspondence between the two observations strongly suggests that cyanate esters are responsible for most of the coupling capacity of the activated Sepharose.

Although the binding capacity of Sepharose increases with its cyanate ester content, it has not been possible as yet to establish a simple direct correlatio between the concentration of cyanate ester groups and the coupling capacity towards a specific ligand. Hence, the current procedure can be used only for the quantitative and qualitative determination of "active groups" present but not for the prediction of the coupling capacity.

Other practical applications of the procedure are the following: (a) the ability to remove unwanted reactive groups by treatment of activated Sepharose with pyridine (Scheme 3); (b) the means to ascertain the precise amount of



Scheme 3

washing needed following activation of Sepharose by simply testing several drops of washings for free CNBr with our reagent.

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