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Binding of Proteins to Polysaccharides by Means of Cyanogen Halides. Studies on Cyanogen Bromide Treated Sephadex

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Methods for irreversible fixation of biologically important molecules to water-insoluble carriers have attracted considerable interest in connection with a great range of problems. Polysaccharides have proved to be particularly effective as carriers for the fixation of proteins. Treatment of polysaccharides with cyanogen halides results in activated products having the ability to take up proteins under mild conditions. This paper deals with cyanogen bromide activation of Sephadex. The cyanogen bromide treatment leads to carbamates and imido-carbonates of the sugar moiety. The formation of cyclic 5-membered imido-carbonates from cyanogen bromide and vicinal trans hydroxyl groups in D-glucopyranose residues of Sephadex is discussed.

Treatment of polysaccharides with cyanogen halides in alkaline aqueous solution leads to derivatives that are directly useful for the covalent fixation of proteins.[1,2] By means of such chemically reactive polysaccharide derivatives, various water-insoluble adsorbents for specific adsorption or affinity chromatography [3,4] and water-insoluble enzymes [5] have been prepared. The preparation of protein-conjugates by means of the cyanogen halide method proceeds in two steps:

1. Activation. Cyanogen halide treatment of the polysaccharide to convert the latter to a highly reactive intermediate. 2. Coupling. Reaction of the intermediate with a protein. The organic chemical structures previously suggested to be involved [1] have been rather hypothetical. This paper contains stronger evidence for the presence of the proposed structures. Nitrogen-containing structures obtained by cyanogen bromide treatment of epichlorohydrin cross-linked dextran (Sephadex®) have been investigated in more detail.

Reactions between cyanogen bromide and alkohols or phenols in alkaline aqueous solution have not been studied so far, presumably because of the rapid hydrolysis of cyanogen bromide under these conditions. However, a

great deal is known about reactions between alcoholates and phenolates and cyanogen bromide. The formation of cyanates should be considered, although in older work imido-carbonates were actually obtained along with carbamates and triazines. The first synthesis of a cyanate by means of a cyanogen halide was accomplished as late as in 1960.[6] Since then, a great number of cyanates have been synthesized.[7] The cyanates are very reactive, especially toward nucleophilic reagents. Water yields carbamates and alcohols yield, in the presence of alkali, imido carbonates.[8]

RESULTS

Activation step. Sephadex G200 (200 mg, purchased from Pharmacia Fine Chemicals, Uppsala, Sweden) was suspended in 12 ml of water and was allowed to swell for a couple of hours. 8 ml of a freshly prepared cyanogen bromide solution ($c = 25$ mg CNBr/ml) was added to the suspension. The suspension was quickly adjusted to the desired pH and maintained there by addition of 2 M sodium hydroxide. The suspension was stirred during the reaction and the temperature kept at 23 – 25°. The reaction time was 6 min. The activated gel was rapidly washed on a G3 glassfilter with 300 ml cold 0.1 M sodium bicarbonate and briefly with water. The gel was shrunk by water/acetone mixtures of increasing acetone concentration, and finally with acetone. The product was kept over phosphorus pentoxide in vacuo for 24 h at room temperature.

Table 1. Changes in IR absorption pattern upon cyanogen bromide treatment of Sephadex® G200 in the region 1650 – 1850 cm^{-1} as compared with untreated Sephadex® G200. Changes in IR absorption pattern upon acid and alkaline hydrolysis of the activated product. Relative absorption intensity indicated within parenthesis.

	Frequency cm^{-1}	Proposed structures
Sephadex	No absorption	–
Sephadex * (Cyanogen bromide activated Sephadex)	1720 (v.s.)	Carbamates and cyclic 5-membered imido carbonates
	1670 (v.s.)	Other kinds of imido carbonates
Acid hydrolyzed Sephadex *	1820 with shoulder at 1840 (m)	Cyclic 5-membered carbonates
	1760 (v.s.)	Other kinds of carbonates
(pH 3; 1 h)	1720 (s.)	Carbamates
Alkaline hydrolyzed Sephadex * (pH 10; 36 h)	1720 (v.s.)	Carbamates
Alkaline hydrolyzed Sephadex * (pH 11; 24 h)	No absorption	–

Elementary analysis of the activated products revealed considerable amounts of nitrogen, but only trace amounts of bromine. The amount of nitrogen introduced depends strongly on the pH, at which the reaction is carried out; the higher the pH, the more nitrogen is introduced. We have especially investigated products activated at pH 11. Sephadex G200 activated at pH 11 is 5 – 6 % in nitrogen and < 0.2 % in bromine. The IR-spectrum of cyanogen-bromide-treated Sephadex was compared with the spectrum of unreacted Sephadex (KBr-pellets; Perkin Elmer spectrophotometer 257) (Table 1). In the frequency region 1650 – 1850 cm^{-1} Sephadex has no absorption, but activated Sephadex is characterized by series of overlapping absorption bands. In addition, characteristic changes in the IR-absorption pattern of Sephadex occur in the region 750 – 925 cm^{-1} as a consequence of the activation reaction. These latter may be due to changes in ring vibrations of the gluco-pyranose systems of dextran.[9]

Coupling process. The ability of the activated Sephadex and also of the various hydrolyzed products to react with glycyl-leucine was investigated. The coupling was performed in the following way.

Activated polymer (50 mg) was suspended in 5 ml sodium bicarbonate solution (0.5 M), and 20 mg glycyl-leucine was added. The reaction was run for 16 h at 23°. The conjugates were carefully washed, shrunk with acetone and dried. The coupling occurs *via* the amino-group of the peptide.[1] The content of glycyl-leucine was determined by amino acid analysis after acid hydrolysis (6 M hydrochloric acid at 110° for 24 h). Since, in this case, glycine participates directly in the coupling, the recovery of glycine is low. The calculated amount of fixed glycyl-leucine is therefore based on the leucine value (Fig. 1).

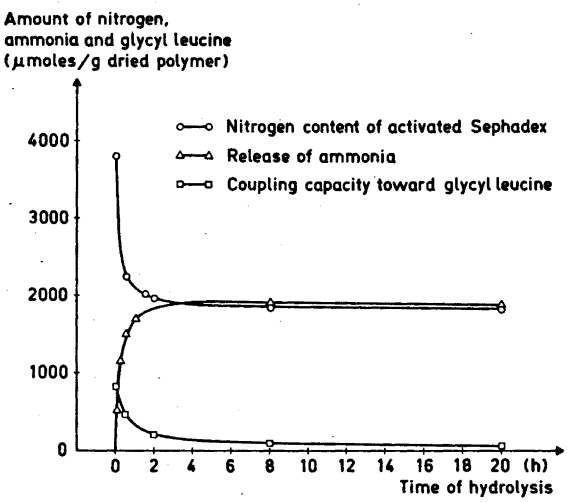


Fig. 1. Nitrogen content of cyanogen bromide activated (at pH 11) and acid hydrolyzed (at pH 3) Sephadex C200. The decrease of bound nitrogen coincides with the release of ammonium ions into the supernatant. Coupling activity toward glycyl-leucine of the cyanogen bromide-activated product and the various hydrolysis products.

Alkaline hydrolysis of activated Sephadex. Hydrolysis of activated Sephadex for 36 h at pH 10 at room temperature gives a product the IR-spectrum of which differs from that of unreacted Sephadex by an absorption peak at 1720 cm^{-1}. The product has no coupling capability toward glycyl-leucine. The spectrum is identical with that of carbamylated Sephadex, prepared by treatment of Sephadex with urea in boiling pyridine. Prolonged hydrolysis at pH 11 restores the original Sephadex spectrum.

Hydrolysis of activated Sephadex for 24 h at pH 9 reduces the coupling capacity to 60 % of the initial value, but the nitrogen content is almost unchanged. Consequently, the reactive groups are relatively stable under weakly alkaline conditions. The observed decrease in coupling ability seems to depend on transformation of the "coupling structures", with conservation of nitrogen to inert carbamate structures. Imido carbonates do add water under alkaline conditions to yield carbamates.[10]

Acid hydrolysis of activated Sephadex. About 50 % of the total nitrogen content of activated Sephadex (activation pH 11) is readily hydrolyzable under mildly acid conditions. The decrease in nitrogen content of the polymer is accompanied by a corresponding increase in the ammonium ion concentration of the supernatant. (The ammonia distilled from the supernatant solution, which had been made alkaline with sodium hydroxide, was taken up in hydrochloric acid and determined by titration.) The decrease in fixed nitrogen is accompanied by a concomitant decrease in coupling ability (Fig. 1). Upon acid hydrolysis for 24 h at pH 3, the residual coupling ability was only 5 – 10 % of the initial value.

Doane *et al.*[11] have described the preparation of dextran carbonate (by means of ethyl chloroformate and triethylamine in dimethyl sulfoxide and dioxane). The product contained, among other carbonates, 5-membered carbonate rings fused *trans* to the glucopyranose ring systems. Doane assigned an IR-band at 1825 cm^{-1} to such strained 5-membered carbonates. We have prepared Sephadex carbonate in an analogous way. We obtained a product with IR-absorption at 1820 cm^{-1}, having a shoulder at 1840 cm^{-1}. The product also absorbed at 1760 cm, where linear or 6-membered cyclic carbonates or larger cyclic carbonates absorb.

The IR-spectrum of acid-hydrolyzed cyanogen bromide-activated Sephadex was characterized by a strong absorption peak at 1720 cm^{-1} previously assigned to carbamates, but also showed the above-mentioned carbonate absorptions. It is particularly interesting to note the presence of absorption at 1820 and 1840 cm^{-1}, characterizing the strained 5-membered carbonates.

Model experiment. Doane *et al.*[12] have described the preparation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate. In a model experiment, we obtained from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside by means of cyanogen bromide a multi-component product, having spectrometric properties very similar to those of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate: *trans*-cyclic O(C=O)O at $\nu = 1810$, 1830, and 1840 cm^{-1}, but also absorption from linear carbonates and carbamates. The product was synthesized in the following way: cyanogen bromide treatment at pH 11 for 3

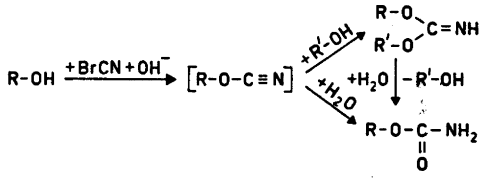
min of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside; extraction by ethyl acetate; evaporation to an oily product ($\nu = 1720$ cm^{-1}); hydrolysis at pH 4 for 15 min; extraction with ethyl acetate; a microcrystalline product was obtained upon evaporation.

Functional groups responsible for the protein coupling ability of cyanogen bromide activated Sephadex. We propose that the Sephadex carbonates mentioned in the previous section are formed during the mild acid hydrolysis from the corresponding C=N structures, that is, from the corresponding imido-carbonates. The IR-spectrum (film) of ethyl imido carbonate is characterized by a strong absorption at 1680 cm^{-1}. The absorption band of activated Sephadex at this wave number, and which disappears during the course of acid hydrolysis, is therefore assigned to an imido-carbonate structure, either linear or cyclic of larger ring size, perhaps even 6-membered. The >C=N vibration of the 5-membered ring carbonates is probably hidden in the strong and broad carbamate peak at 1720 cm^{-1}. Acid hydrolysis decreases the intensity of the 1720-peak slightly. The >C=N-vibration of ethylene imido carbonate hydrochloride is given by Addor to be 1720 cm^{-1}.[13]

Together, these findings afford strong evidence for the presence of imido carbonate groups, forming 5-membered ring systems fused in *trans* fashion to the glucopyranose residues.

After prolonged acid hydrolysis, there is a small residual coupling capacity that cannot be attributed to the imido-carbonate structures. The formed strained 5-membered carbonates are also reactive toward model peptides under the given coupling conditions, but not very efficiently (tested toward Sephadex-carbonate prepared according to Doane). Cyanogen bromide will trimerize under alkaline conditions to triazines, and tri-halo-triazines do react with polysaccharides under the given experimental conditions. The low but significant halogen content of the activated product might indicate the presence of a low level of bromo-triazine groups, which should also take part in the coupling reaction.[14,15]

CONCLUSION



The experimental results provide strong evidence for the applicability of the above reaction scheme to the activation of Sephadex by cyanogen bromide under alkaline aqueous conditions. A cyanate structure is proposed as an intermediate. Aliphatic cyanates are very reactive, but those of bulky residues may be more stable. Possibly an IR-peak, which irregularily appears in activated and rapidly shrunken and dried Sephadex, and which slowly disap-

pears again, reflects the presence of cyanate substituents. The cyanate intermediate presumably reacts with a neighbouring hydroxyl group to give an imido carbonate or adds water to form carbamates. Under the alkaline conditions of the activation process, carbamates may also be produced from imido carbonates. The carbamates, being inert and neutral, will not take part in coupling reactions, while imido carbonate substituents are responsible for the coupling of peptides and proteins. Other kinds of imido carbonates indeed do react readily with amines.[10,16] We have found that ethyl imido carbonate reacts with amino acids and their derivatives, including smaller peptides, under the same conditions as are used for the coupling to activated Sephadex. *N*-Substituted carbamates, isoureas, and imido carbonates have been isolated. 5-Membered cyclic imido carbonates of vicinal *trans* hydroxyl groups should be particularly reactive and probably play an important role in the fixation of proteins to cyanogen bromide activated Sephadex.

Recently, the use of cyanates for the activation of polysaccharides has been demonstrated.[17] The organic chemical basis of the cyanate activation is obviously very similar to the above discussed cyanogen bromide reaction.

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