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Binding of Covalent Proteins to Polysaccharides by Cyanogen Bromide and Organic Cyanates. I. Preparation of Soluble Glycine-, Insulin- and Ampicillin-dextran

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A method for the preparation of soluble dextran derivatives by using the cyanogen bromide method and the cyanate method is described. Glycine, insulin and ampicillin have been coupled to dextran.

Several authors have described methods for the attachment of proteins and other compounds of biological importance to insoluble polymeric carriers. The conjugates obtained by these methods have found several applications, for example in radioimmunological assays, as immunosorbents and in enzymatic reactions.

Axén *et al.*[1] prepared soluble dextran-enzyme conjugates by coupling the enzyme to cross-linked dextran (Sephadex) by the cyanogen bromide method, followed by solubilization of the conjugates by digestion with dextranase. We have found that soluble conjugates between dextran as carrier and proteins, peptides or amino acids can be prepared directly if the activation of dextran is carefully controlled. The methods used for the coupling are the cyanogen bromide method, first reported by Axén *et al.*,[2] and the cyanate method, reported by the present authors.[3]

EXPERIMENTAL

Commercial dextran fractions from Pharmacia AB, Uppsala, have been used. The cyanogen bromide (BrCN) was purchased from Fluka AG, and the cyanates were prepared according to Grigat and Pütter.[4] The molecular weights ($\bar{M}_w$) have been determined by the light scattering method. The amino acid analyses were made according to Spackman *et al.*[5] The equivalent weights have been determined by titration with sodium hydroxide after ion exchange with Amberlite IR-100. The products were dialyzed and then freeze-dried (Method A) or concentrated *in vacuo* to about 10 %, precipitated with ethanol or acetone, and dried *in vacuo* at about 50°C (Method B).

1a. *Activation of dextran by BrCN. General procedure.* To a stirred solution of dextran (10 g) in water (1 l), BrCN was added in a maximum of three portions (2, 2, and 1.5 g, respectively) at intervals of 15 min. The mixture was stirred vigorously, and pH 10.7 maintained by the addition of 5 M or 33 % NaOH from an automatic syringe or peristaltic pump, connected to the relay of a pH-meter. About 30 min after the addition of the last portion of BrCN, the solution could be used for coupling or for the isolation of the activated dextran (Method A or B).

1b. *Activation of dextran by BrCN. Special procedure.* To a stirred solution of dextran (20 g, $\bar{M}_w$ 4400) in water (500 ml), BrCN (10 g) was added in one portion. The mixture was stirred vigorously, and pH 10.7 maintained by the addition of 33 % NaOH as above. After 30 min, the solution was divided in portions of 50 ml, which were treated as shown in Table 2, and glycine (1 g) was coupled to each portion as described below.

2. *Coupling of glycine to BrCN-activated dextran. General procedure.* Glycine (2 g) was added to a solution of activated dextran (1 g in 100 ml), and the pH adjusted to 9.2

Table 1. Activated dextran isolated after dialysis and lyophilization.

No.	Starting materials			Products	
	Dextran		BrCN	N %	$\bar{M}_w \times 10^{-3}$
	$\bar{M}_w \times 10^{-3}$	g	g		
1	10	20	4	2.15	17
2	10	20	4+4	2.18	27
3	10	20	4+4+4	3.91	–[a]
4	20	20	4	1.20	38 ± 10 %
5	20	20	4+4	2.53	240 ± 15 %
6	20	20	4+4+4	–[b]	–
7	40	10	2+2	2.91	300
8	77	15	3+3+1.5	2.80	750 ± 20 %

[a] Precipitation occurred during dialysis.
[b] Precipitation occurred during activation.

Table 2. BrCN-activated dextran treated in different ways before coupling with glycine.

No.	Treatment of BrCN-activated dextran	Time of coupling h	Glycine content μmol/g
1	Direct coupling	2	1980
2	Direct coupling	7.5	2195
3	Direct coupling	28	2190
4	a. Neutralization b. Dialysis, 24 h c. Coupling	24	337
5	a. Neutralization b. Dialysis, 24 h c. Precipitation (EtOH) d. Coupling	75	243
6	a. Neutralization b. Precipitation (EtOH) c. Coupling	24	1207

Table 3. Coupling of glycine to cyanogen bromide activated dextran.

No.	Starting materials			Reaction time h	Products				
	Dextran		Glycine g		N %	Glycine μmol/g	Titration μmol/g	$\bar{M}_w \times 10^{-3}$	pK_a
	$\bar{M}_w \times 10^{-3}$	g							
1[a]	40	10	20	22	4.80	1320	610	59	4.09
2	40	20	40	16	5.37	1200	688	175	4.25
3	40	30	60	40	5.21	1150	710	74	4.10

[a] In the general activation procedure 2+2 g of BrCN were used.

by the addition of solid Na_2CO_3. The solution was kept at room temperature for different times, and the product isolated using Method B (Table 3).

3. *Metal salts of glycine-dextran. (a) Fe(II) salt.* Glycine-dextran (5 g) (Table 3, No. 2) was dissolved in water (75 ml), and a solution of $FeCl_2.4H_2O$ (1.3 g) in water (25 ml) was added with stirring over 10 min. The brownish-red solution was dialyzed overnight and freeze-dried. Found: Fe 1.80 %. $\bar{M}_w$ 150 000 ± 30 %. *(b) Ca(II) salt.* The procedure was as above (3a), except that the iron(II) salt was replaced by $CaCl_2$ (1.6 g). Found: Ca 1.32 %.

4. *Activation of dextran by* p*-methoxyphenylcyanate and coupling with glycine.* Dextran (5 g, $\bar{M}_w$ 40 000) was dissolved in water (500 ml), and two 1 g portions of *p*-methoxyphenylcyanate were added at an interval of 1 h. 2 M NaOH was added from an automatic burette to maintain pH 10.7. After the addition of the second portion, the stirring was continued for 190 min. The mixture was filtered and the filtrate extracted 3 times by methylene chloride. Glycine (10 g) was added, and pH adjusted to 9.2 with solid Na_2CO_3. After stirring for 16 h, the product was isolated following the Method A. Found: N 1.96 %, glycine 370 μmol/g, titration value 206 μmol/g, $\bar{M}_w$ 40 000.

5. *Activation of dextran with 2,2,2-trichloroethylcyanate and coupling with glycine.* Dextran (5 g, $\bar{M}_w$ 40 000) was dissolved in water (500 ml). 2,2,2-Trichloroethylcyanate (1 g) was added, and the reaction maintained at pH 10.7 by automatic addition of 2 M NaOH. After stirring for 2 h, another portion of 1 g was added. The last 1 g portion was added after another hour. After a total activation time of 5 h, the mixture was filtered, glycine (10 g) added to the filtrate, and the pH adjusted to 9.2 with solid Na_2CO_3. After standing for 24 h, the product was isolated using Method A. Found: N 1.95 %, glycine 440 μmol/g, titration value 288 μmol/g.

6. *Insulin-dextran.* Dextran (1 g, $\bar{M}_w$ 40 000) was activated, using two 0.2 g portions of BrCN. One hour after the start of the activation, insulin (Fluka AG, 23 – 25 I.U./mg 0.5 g) was added. Solid Na_2CO_3 was used to adjust pH to 9.2. The mixture was stirred overnight and then ultrafiltered in a "Diaflo" apparatus (Filter UM 1, $\bar{M}_w$ cut off 10 000). The ultra filtration was repeated twice, diluting the residue with about 300 ml of water. The yield of lyophilized products was 1.2 g. The insulin dextran was separated from free insulin by gel chromatography on a Sephadex G-75 column. Eluent: 1 M HOAc. Bed dimensions: 80 × 2.5 cm. Sample size: 280 mg dissolved in 7 ml of eluent. The samples from the high molecular weight peak were pooled and freeze-dried. Yield: 120 mg. Insulin content: 29 μmol/g (based on the determination of aspartic acid, glutamic acid and leucine).

7. *Ampicillin-dextran.* Dextran (2 g, $\bar{M}_w$ 40 000) was activated and reacted with ampicillin [(α-aminobenzyl)-penicillin] according to the general procedures, using only two BrCN portions in the activation step, and a dextran: ampicillin ratio 1:1. Working up procedure A. Yield 2.4 g. Found: S 310 μmol/g.

RESULTS AND DISCUSSION

When a dextran solution is treated with cyanogen bromide under the same conditions as used in the cyanogen bromide activation of Sephadex,[2] a heavy precipitate is formed within 1 or 2 min. However, soluble activated dextrans can be prepared by using dilute solutions and adding the cyanogen bromide in portions.

The method of isolation of the activated dextran is critical. Precipitation with acetone afforded only partially water-soluble products. Isolation by dialysis, followed by lyophilization, however, yielded soluble products. The molecular weight of the activated dextran is higher than that of the original dextran (Table 1), demonstrating that cross-linking has occurred. This increase in molecular weight is considerable when the incorporation of nitrogen exceeds 2.5 %, and explains the decreased solubility of the product.

From the experiments summarized in Table 2, it is evident that the reaction between activated dextran and glycine is complete after 7 h. The experiments also demonstrate that a considerable part of the activity is lost when the activated dextran is isolated or purified by dialysis. In order to obtain maximum coupling efficiency, the activated dextran should therefore be used as soon as possible.

The UV-spectrum of the activated dextran shows a peak at 216 nm, which is shifted to 235 nm after reaction with glycine. The absorption, typical of carboxylate ions, is also observed in the IR-spectrum of the glycine-dextran. The nitrogen content of an activated dextran (Table 1, No. 7) increased from 2080 μmol/g to 3430 μmol/g after reaction with glycine (Table 3, No. 1). The difference, 1350 μmol/g, compares well with the amount of glycine determined by amino acid analysis, 1320 μmol/g. Thus about 65 % of the nitrogen containing groups in the activated dextran have reacted with glycine. The ratios of the $\overline{M}_w$ values of the original dextran, the activated dextran and the glycine-dextran were 1 : 7.5 : 1.5, indicating that the cross-linkages had been ruptured during reaction with glycine.

A glycine-dextran containing about 2200 μmol/g of glycine was prepared under somewhat different conditions (Table 2, No. 2). This high glycine content may be due to the higher concentration and the lower M_w (4400) of the dextran used.

Only about 50 % of the coupled glycine residues can be titrated. For one preparation (Table 3, No. 2), a value of 688 μmol/g was obtained, which agrees well with the amount of bivalent cations (330 and 324 μmol/g) found in its calcium and iron(II) salts, respectively. The explanation of these observations must await further studies.

Axén [2] found that ammonia was released when alanine ethylester was coupled to cyanogen bromide activated dextran. As most of the nitrogen is retained in the reaction with glycine, these two compounds appear to react by different mechanisms.

We have recently reported the use of cyanic acid esters (cyanates) as activating agents for Sephadex.[3] Cyanates can also be used as activating agents for dextran, but with less satisfactory results than those obtained using cyanogen bromide. The titration values for the glycine-dextrans prepared

by this method again correspond to about half the amount of glycine found by amino acid analysis (Experimental, 4 and 5).

Dextran was activated with cyanogen bromide and coupled with insulin. Ultrafiltration of the reaction mixture, followed by lyophilization and gel chromatography on Sephadex G-75 yielded the *insulin-dextran*, containing 18 % (29 μmol/g) of insulin. Preliminary experiments with rats showed that the blood-sugar lowering effect of this preparation was 200 times less than that of free insulin on a weight basis. Hence the bound insulin was 36 times less active than free insulin.

An *ampicillin-dextran*, containing 320 μmol/g, was prepared by analogous methods and was found to have a low but significant bacteriostatic activity.

Proteins and peptides containing free amino groups and also other amino compounds may be coupled to dextran by the present method. Thus a *histamine-dextran*, containing 5 % histamine residues, has been prepared. The composition of the product can be varied within wide limits. *Bovine serum albumin* was attached to a dextran of low molecular weight, affording a product which contained about 90 % protein. Thus the method is simple and mild enough to permit the fixation of labile compounds.

It seems reasonable to assume that a covalent linkage is formed between the activated dextran and the attached molecule. The results of gel chromatography and dialysis experiments support this assumption. Axén *et al.*[2] have demonstrated that the linkages formed, using the cyanogen bromide method, withstand, from a biological point of view, extreme pH-values and high ionic strength.

It has been proposed that the activation of polysaccharides by cyanogen bromide or cyanates introduces imidocarbonate groups which react readily with a wide range of substances bearing amino groups. In order to shed more light upon these problems, a study on the reaction between cyanogen bromide (and cyanates) and simple model substances is in progress in our Laboratories. Preliminary results strongly support the assertion that most of the reactive groups in activated dextran and Sephadex consist of 5-membered *trans*-cyclic imidocarbonate groups.[6]

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