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A Method for Coupling Biologically Important Molecules to Polysaccharides

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This report describes a new, gentle method for coupling insoluble polysaccharides, by covalent bonds, to proteins, peptides, or amino acids. The method involves the use of organic cyanates as activating agents, under alkaline conditions of pH 8–11.

The increasing number of papers dealing with the coupling of biologically important molecules to soluble and particularly insoluble polymers gives a growing realisation of the usefulness of such systems for different purposes.

Several methods for covalently bonding amino acids, peptides, and proteins to various polysaccharides have been described. Reactive derivatives such as diazotized *m*-aminobenzyloxymethylether of cellulose [1–7] and *p*-aminobenzylcellulose,[8–10] carboxymethylcellulose azide,[10–12] bromoacetylcellulose,[13] carboxymethylcellulose activated with dicyclohexylcarbodiimide,[14–16] 2-hydroxy-3-(4-isothiocyanatophenoxy)-propyl Sephadex,[17] and cyanogen halides activated polysaccharides [18] have been used for the coupling of different molecules to hydrophilic polymers.

THE CYANATE METHOD

This paper reports a new gentle method for coupling particularly insoluble polysaccharides with proteins, peptides or amino acids. The method involves (1) the formation of a reactive derivative of the polysaccharide by treating the carbohydrate with an organic cyanate for a few minutes under alkaline conditions and (2) reacting the formed intermediate with the protein, peptide, or amino acid preferably in slightly alkaline solution (pH 8–12).

Cross-linked dextran (Sephadex), cellulose Avicel, and Sepharose have been used as models for the insoluble polysaccharides and insulin antibodies, glycylleucine, glycyltyrosine, and glycine as examples of biological molecules.

Most of the organic cyanates tested as activating agents were prepared according to the procedure given by Grigat and Pütter.[19] This method of

preparation is very simple and usually no difficulties arise if the product is not rendered unstable by negative substituents; *e.g.*, when the aromatic nucleus contains two or more nitro groups or three or more chlorine atoms, the method, however, fails to give cyanates owing to the increased tendency to trimerization. This trimerization may also be caused by the presence of traces of acids or bases. Thus, it was preferable to distil the product immediately after the reaction was completed. In some cases where distillation was not possible and attempts to recrystallize the product also failed the crude material was used as activating agent.

The procedure used for the activation is similar to the procedure for cyanogen bromide activation.[18] Sephadex (Cellulose, Sepharose) and the cyanate were stirred for a few minutes in distilled water. The pH was then adjusted to a value between 8 and 11 and maintained constant during the activation. In the working up stage excess cyanate was eliminated by carefully washing the product with acetone. For the screening of the different cyanates we have used the following conditions for activation: pH 10.7, an activation time of 6 min, and a temperature of 20°C. The reactive derivatives were then reacted with insulin antibodies and the maximum uptake of iodine-labelled insulin (^{125}I-insulin) of the Sephadex-antibody complex was measured. Only small stocks of antiserum have been available and therefore it has been necessary to carry out the testing in small batches.

Figs. 1 and 2 show the results for the different batches but no direct comparison between the figures can be made. In Figs. 1b and 2a, b the value for a BrCN activation has been included as a reference. The heights of the columns show the coupling ability of cyanate-activated Sephadex. Many cyanates are comparable with BrCN.

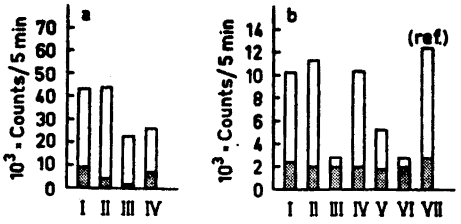


Fig. 1a, b. Uptake of ^{125}I-insulin on insulin-antibodies coupled to cyanate-activated Sephadex.

Fig. 1a. Activating agent: I Phenyl cyanate. II 4-Nitrophenyl cyanate. III 2-Nitrophenyl cyanate. IV 3-Chlorophenyl cyanate. Fig. 1b. Activating agent: I Phenyl cyanate. II 4-Methoxyphenyl cyanate. III 4,4-Dichlorophenyl cyanate. IV 2-*t*-Butylphenyl cyanate. V 2,6-Dimethylphenyl cyanate. VI 1-Adamantyl cyanate. VII Cyanogen bromide (ref.).

The shaded part of the column represents unspecific absorption of ^{125}I-insulin.

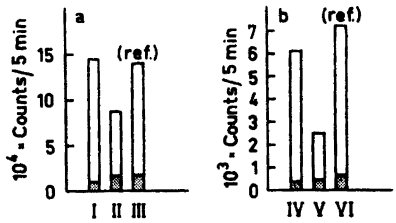


Fig. 2a, b. Uptake of ^{125}I-insulin on insulin-antibodies coupled to cyanate-activated Sephadex.

Fig. 2a. Activating agent: I 2,2,2-Trichloroethyl cyanate. II 2,4,6-Tri-*t*-butyl cyanate. III Cyanogen bromide (ref.). Fig. 2b. Activating agent: IV Naphthyl cyanate. V 1,4-Phenylene cyanate. VI Cyanogen bromide (ref.).

The shaded part of the column represents unspecific absorption of ^{125}I-insulin.

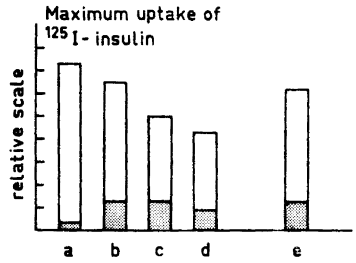


Fig. 3. Maximum coupling capacity after activation at different activation time. Unspecific absorption of ^{125}I-insulin is represented by the shaded part of the column. Column. Activation with PhOCN, time in min. a: 1.5. b: 3. c: 6. d: 12. e: Standard activation with BrCN. *Note:* The results above are taken from different coupling experiments and put on the same scale by use of standard activated Sephadex.

It seems reasonable to believe that the activation ability could be improved if other more suitable conditions were used. We have therefore varied the conditions of the activation with phenyl cyanate (PhOCN) and found that a short activation time should be used (Fig. 3). To find out the most suitable pH range Sephadex was activated with PhOCN at different pH values (Fig. 4). The optimum pH is between 10 and 11. A few attempts to activate Agarose and Cellulose have been made. Standard condition were used and for comparison the results of the activation with BrCN are included (Fig. 5).

STRUCTURE OF THE CYANATE ACTIVATED SEPHADEX

The reaction between cyanates and carbohydrates seems to be rather complex but the kind of bond between the carrier and the protein and its surroundings may be of considerable interest.

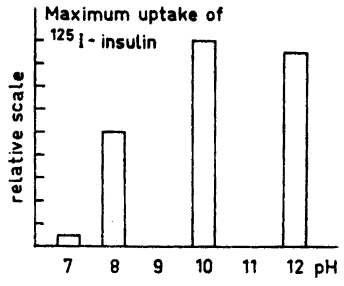


Fig. 4. Activation of Sephadex with PhOCN at different pH. Results from different coupling experiments are put on the same scale by comparison with coupling with standard activated Sephadex.

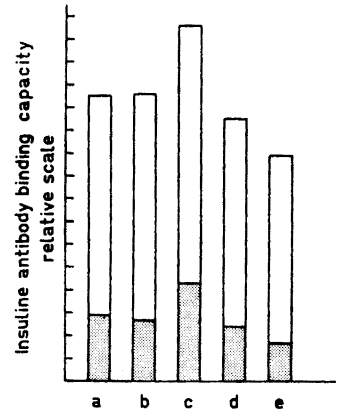


Fig. 5. Activation of Sepharose and Cellulose.
Polymer and activating agent: a, Sephadex (ref.), BrCN. b, Cellulose Avicel, BrCN. c, Cellulose Avicel; PhOCN. d, Sepharose 6B T 3623, BrCN. e, Sepharose 6B T 3623, PhOCN.
The shaded part of the column represents unspecific absorption of ^{125}I-insulin.

The IR-spectra of Sephadex activated by different cyanates show some puzzling features. In most cases strong to medium absorption is observed at 1670 cm^{-1} and 1700 – 1710 cm^{-1}. Nitrogen is always incorporated in the reactive derivative from about 7.5×10^{-4} to 32×10^{-4} mole/g or 1 – 4.5 % N. When phenyl cyanate was used as the activating agent we found in addition some other peaks of low-medium intensity. These peaks could not be eliminated by repeated washing with water or acetone, and even after treatment with 0.2 M HCl for 2 h these peaks persisted. The IR-absorption of pure phenyl cyanate in the range 1700 – 650 cm^{-1} was compared with the IR-absorption of phenyl cyanate activated Sephadex. As well as the previously mentioned strong bands at 1670 cm^{-1} and 1710 cm^{-1}, peaks at 1585, 1490, 1460, 1210, 840, 770, and 690 cm^{-1} were found. These bands are not observed in the spectrum of Sephadex (SxOH) but phenyl cyanate absorbs strongly at 1585, 1485, 1460, 1190, 1165, 780, 750, and 680 cm^{-1}.

To have a better understanding about how much of the organic part of the cyanate was incorporated during the activation, Sephadex was activated with phenyl cyanate for various periods of time and the product analysed for nitrogen and phenol content. The product contains 17 % of phenol and 4.6 % of nitrogen after about 30 min activation (Fig. 6).

Because of the observed similarity between the spectra of PhOCN activated Sephadex and of PhOCN itself and other evidence mentioned above, we conclude that phenyl or phenoxy groups are incorporated in the Sephadex matrix by covalent bonding.

We also believe that *p*-methoxyphenyl groups are incorporated during activation with *p*-methoxyphenyl cyanate, although only two additional peaks are observed in the IR-spectrum (1510 cm^{-1} and 1210 cm^{-1}). *p*-Methoxyphenyl

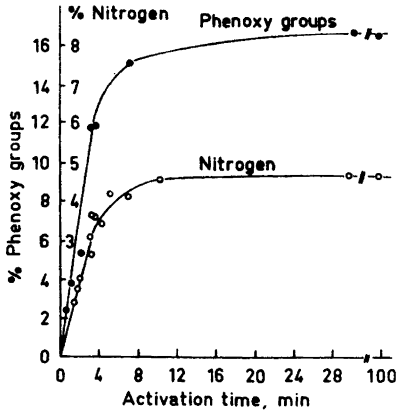


Fig. 6. Activation of 1 g Sephadex G-25 Superfine with 2 g of phenyl cyanate.

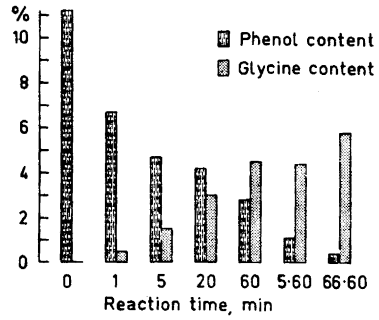


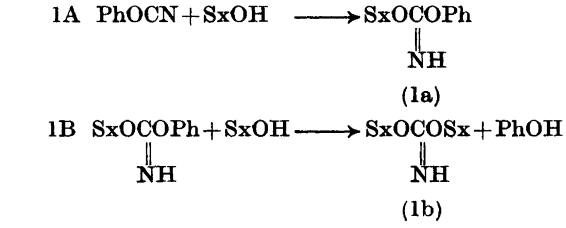
Fig. 7. The glycine and phenol content of glycine-Sephadex, obtained after different reaction times between glycine and activated Sephadex G-25 Superfine.
Note: Phenyl cyanate activated Sephadex was shaken with 0.5 M $NaHCO_3$ for less than 3 min before glycine was added.

cyanate absorbs strongly at these frequencies. Other absorption bands are probably masked by the strong and broad bands of Sephadex itself.

After activation of Sephadex with 2,2,2-trichloroethyl cyanate, peaks at 1700 and 1670 cm^{-1} and additional small peaks at 1215 and 810 cm^{-1} are observed. The chlorine content (3.67 %) shows that the 2,2,2-trichloroethyl groups are present to an extent of 3.5×10^{-4} mole/g in the activated product. The nitrogen content (1.06 %) represents 7.6×10^{-4} mole/g.

Most cyanates tested as activating agents gave reactive products, but no correlation between activity and the height of the IR-peaks could be observed. For example the activation with 1-naphthyl cyanate gave a product with rather low absorption at 1700 cm^{-1} but high antibody binding capacity. The reverse was true for the activation by means of 2,6-dimethylphenyl cyanate.

The reaction between Sephadex (SxOH) and PhOCN leading to the activated or reactive derivatives of SxOH may be written as follows [20]



It is conceivable that both the products 1a and 1b are reactive derivatives. It is evident that product 1a is not the only one since the molar ratio N/PhOH is about 2 at any stage of activation (Fig. 6). Several structures are possible for the imido carbonate groups in the product 1b. We have observed that the molecular weight rises during the activation of dextran. In one experiment the molecular weight ($\bar{M}_w$) increased from 40 000 to 270 000 and in other experiments the product precipitated from the water solution. This would suggest that at least some of the imido carbonate groups form cross-links between different dextran chains (Fig. 8). Large ring structures (Fig. 9)

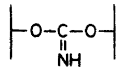


Fig. 8. Imido carbonate groups forming cross-links between different dextran chains.

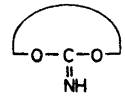


Fig. 9. Large ring imido carbonate structures in dextran or Sephadex.

and six-membered rings at the ends of the chains are also likely to be formed (Fig. 10). Doane *et al.* have recently prepared a reactive dextran carbonate, containing chiefly *trans* 5-membered cyclic carbonate groups.[21] By analogy we would suggest that cyclic imidocarbonate groups constitute the essential part of the reaction product 1b (Fig. 11).

The reaction between phenyl cyanate activated Sephadex and glycine has been studied (II).

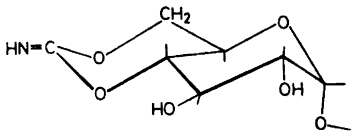


Fig. 10. Six-membered imido carbonate structures of the ends of some dextran chains.

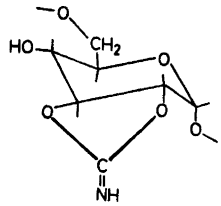


Fig. 11. Five-membered imido carbonate rings between C_2 and C_3 position in the glucose unit of dextran or Sephadex.

II. $SxOCOPh + NH_2CH_2COOH \longrightarrow SxOCNHCH_2COOH + PhOH$ (with NH)

Also the reaction time has been varied and the phenol and glycine content analyzed. Fig. 7. shows a pile diagram where the content of phenol and glycine is plotted against the reaction time. From this experiment it can be concluded that most of the phenoxy groups of the reactive derivative of reaction (IA) is exchanged for the glycine, and that about 80 % of the incorporated glycine is incorporated after appr. 1 h. This is in fairly good accordance with the results of the experiments with insulin antibodies (Experimental, 3b). It was found that this coupling reaction was complete after 4 h and that about 90 % of the totally bound antibodies were incorporated after 1 h. The temperature during the coupling was 23° in this experiment and 4° during the coupling of glycine which might explain the small difference in the rate of the reaction.

To the reactive derivative of Sephadex two peptides have also been coupled, namely glycylleucine and glycyltyrosine. The products contained 13 % and 12 % of the peptides, respectively.

EXPERIMENTAL

The organic cyanates except 2,4,6-tri-*t*-butylphenyl cyanate were prepared according to the procedure given by Grigat and Pütter.[19] All except 2-nitrophenyl cyanate and 1-adamantyl cyanate are known compounds (Table 1).

The melting points and boiling points are not corrected. The Sephadex used is Sephadex G-25 Superfine, (Pharmacia AB, Uppsala, Sweden). Dextran 40 has a molecular weight of about 40 000.

1. *Preparation of the reactive derivative by cyanates. General procedure.* One gram of the polymer (Sephadex, Cellulose, Sepharose) was stirred in 40 ml of distilled water for 3 min, whereupon 2 g of the compound containing at least one cyanate group was added. The pH-value was adjusted to and maintained at pH 10.7 by automatic addition of an aqueous 2 M solution of sodium hydroxide. The reaction was continued for 6 min, whereupon the reaction product was filtered off, washed carefully with water, acetone, and water, and then shrunk with acetone and dried.

The reactive derivative may also be prepared under somewhat different conditions, given in Figs. 3, 4, and 6.

2. *Preparation of the reactive derivative by means of cyanogen bromide.* The freshly prepared cyanogen bromide solution (60 ml, conc. 10 g BrCN/100 ml H_2O) was put into the reaction vessel and Sephadex, Sepharose or Cellulose (3 g) was added with stirring. The polysaccharide was allowed to swell for 3 min during which period the

Table 1. Physical data for the organic cyanate compounds.

Compound	M.p. °C	B.p. °C	Literature data		Ref.
			M.p. °C	B.p. °C	
Phenyl cyanate		50—2/1.0		82—3/10	19
4-Nitrophenyl cyanate	49— 59[a]		66		19
2-Nitrophenyl cyanate	86—100[a]				
3-Chlorophenyl cyanate		88—93/3.2		81—5/2	19
4-Methoxyphenyl cyanate		94—7/1.1	33—34		19
2,4-Dichlorophenyl cyanate	56—59		66—67		19
2,6-Dimethylphenyl cyanate		74—7/1.3		70/0.5	19
2-*t*-Butylphenyl cyanate		86—92/1.2		75/0.9	19
1-Naphthyl cyanate		156/5		161/11	19
2,2,2-Trichloroethyl cyanate		62—66/1		70—1/5	19
1,4-Phenylene dicyanate			110—111		19
1-Adamantyl cyanate[a]					
2,4,6-Tri-*t*-butyl cyanate[a]					22

[a] Crude product.

pH was 3—4 (which was the pH of the BrCN solution). The pH-value was adjusted to and maintained at pH 10.7 by automatic addition of 5 M sodium hydroxide solution. At the end of the reaction (8 min) the reaction mixture was transferred to a glass filter and the product carefully washed with water until the filtrate was neutral. The product was then shrunk by addition of acetone, dried and stored at −20°C.

3 a. *Binding of insulin antibodies to particles of Sephadex, Cellulose or Agarose. General procedure.* The reactive derivative (100 mg) was swollen in sodium carbonate-sodium bicarbonate buffer (pH 9.2), 400 µl, for 10 min. 100 µl of the solution of antibodies in the same buffer as above were added to the reactive derivative in a test tube and shaken at +4°C for 2—3 days. A few ml of 0.1 M $NaHCO_3$-solution were added and the mixture was then incubated with iodine-labelled insulin (^{125}I-insulin) in excess. After washing the product the maximum uptake of ^{125}I-insulin was measured in a scintillation counter. The results are usually given as counts per minute (cpm).

3 b. *Binding of insulin antibodies to particles of Sephadex. Variation of time.* The procedure given in 3 a, using phenyl cyanate, was followed, except that the reaction was performed at 23° and at different lengths of time (1, 2, 4, 6, and 24 h). The uptake of ^{125}I-insulin by the product was measured as described above. After 1 h the activity was 4500 cpm and it increased only slightly, to a constant value, 4800 cpm, after 4 h.

4. *Binding of glycylleucine and glycyltyrosine to the reactive derivative of Sephadex.* The reactive derivative of Sephadex (0.20 g) and 0.08 g of glycyltyrosine or glycylleucine was agitated in 1 ml of Na_2CO_3-$NaHCO_3$ buffer (pH 9.2) for two days at 4°C. The product was filtered off and washed with 0.5 M $NaHCO_3$, 0.01 M HCl, 1 M NaCl, and finally with water. The washed product was shrunk with acetone and dried. The content of glycylleucine bound to Sephadex was 13 % and that of glycyltyrosine 12 %.

5. *Binding of glycine to phenyl cyanate activated Sephadex.* The procedure given in 4 was followed but the reaction time was varied. The phenol content was analyzed after hydrolysis of the product and the glycine content determined by the procedure of Spackman, Moore and Stein. See Fig. 7.

6. *p-Methoxyphenyl cyanate activated Dextran 40.* Dextran 40 (15 g) was dissolved in water (1 l) and 3 cyanate portions (3 g) were added to the solution at 30 min intervals. The pH was maintained at 10.7 with 2 M NaOH. About 30 min after the addition of the last portion of the cyanate, the precipitate was filtered off and washed with water. The solution was extracted with methylene chloride 3 times and once with ether, whereupon the solution was dialyzed against tap water for one week. The solution was

concentrated to 125 ml, precipitated with acetone and dried *in vacuo* at 50°C. Yield 16.1 g. The product contained 1.56 % nitrogen and the molecular weight was 270 000.

7. *Phenyl cyanate activated Dextran 40.* Dextran 40 (2 g) was stirred with phenyl cyanate (2 g) in water (25 ml) at pH 10.7. After about 2 min gel formation was observed which indicated that crosslinking had occurred. More than 8 h of stirring with 2 M NaOH was necessary to dissolve this gel.

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REFERENCES

1. Gurvich, A. E. *Biokhimiya* **22** (1957) 977.
2. Gurvich, A. E., Kapner, R. B. and Nezlin, R. S. *Biokhimiya* (English Transl.) **24** (1959) **129.**
3. Gurvich, A. E., Kuzovleva, O. B. and Tumanova, A. E. *Biokhimiya* (English Transl). **26** (1961) 803.
4. Gurvich, A. E., Kuzovleva, O. B. and Tumanova, A. E. *Biokhimiya* (English Transl.) **27** (1962) 210.
5. Nezlin, R. S. *Biokhimiya* **24** (1959) 289.
6. Nezlin, R. S. *Biokhimiya* **24** (1959) 486.
7. Gurvich, A. E. and Drizlikh, G. I. *Nature* **203** (1964) 648.
8. Campbell, D. H., Luescher, E. and Lerman, L. S. *Proc. Natl. Acad. Sci. US* **37** (1951) 575.
9. Malley, A. and Campbell, D. H. *J. Am. Chem. Soc.* **85** (1963) 487.
10. Mitz, M. A. and Summaria, L. J. *Nature* **189** (1961) 576.
11. Epstein, C. J. and Anfinsen, C. B. *J. Biol. Chem.* **237** (1962) 2175.
12. Lilly, M., Money, C., Hornby, W. and Crook, E. M. *Biochem. J.* **95** (1965) 45P.
13. Patchornik, A. *Israel patent* No. 18207.
14. Weetall, H. H. and Weliky, N. *Nature* **204** (1964) 896.
15. Weliky, N., Weetall, H. H., Gilden, R. V. and Campbell, D. H. *Immunochemistry* **1** (1964) 219.
16. Weetall, H. H. and Weliky, N. *Biochim. Biophys. Acta* **107** (1965) 50.
17. Axén, R. and Porath, J. *Nature* **210** (1966) 367.
18. Axén, R., Porath, J. and Ernback, S. *Nature* **214** (1967) 1302.
19. Grigat, E. and Pütter, R. *Chem. Ber.* **97** (1964) 3012.
20. Grigat, E. and Pütter, R. *Angew. Chem.* **79** (1967) 219.
21. Doane, W. M., Sascha, B. S., Stout, E. I., Russel, C. R. and Rist, C. E. *Carbohyd. Res.* **8** (1968) 266.
22. Stroh, R. and Gerber, H. *Angew. Chem.* **72** (1960) 1000.

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