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Covalent Binding of Proteins to Polysaccharides by Cyanogen Bromide and Organic Cyanates. III. Structural Studies on the Linkage Region

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The reaction between cyanogen bromide activated methyl 4,6-*O*-benzylidene-α-D-glucopyranoside, Sephadex, or dextran and amino acids or peptides has been investigated. The coupling between the activated carbohydrate and the amino compound involves formation of isourea groups. The simultaneous release of ammonia is independent of the coupling reaction. Part of the ammonia is derived from hydrolysis of carbamate groups but most of it is derived from other groups in the activated carbohydrate.

Proteins can be covalently linked to polysaccharides if the latter are first treated with cyanogen bromide [1] or organic cyanates.[2] Previous proposals [3,4] concerning the nature of the linkage region in such complexes are not unambiguous. We have previously discussed the nature of the activated complex.[5–8] In the present communication, the bonds between the carbohydrates and the fixed amino compounds are re-examined.

EXPERIMENTAL

Apparatus and methods. IR-spectra in KBr were recorded with a Unicam 200 instrument. The amino acid analyses were made according to Spackman *et al.*[9] For the amino acid directly linked to the modified carbohydrate, this analysis gave low values. Corrections were therefore made assuming that the analysis of the second amino acid in a dipeptide linked to the carbohydrate was correct. The ammonia was determined by the Kjeldahl method. The molecular weights were determined by gel chromatography.[10] The melting points were determined in a pre-heated melting point apparatus. As the compounds investigated were amorphous, and further melted under decomposition, these values are not very accurate. Concentrations and dryings were performed under reduced pressure.

Materials. Sephadex® G-25 Superfine and Dextran T10 ($\overline{M}_w$9300, $\overline{M}_n$5700) from Pharmacia Fine Chemicals, Uppsala, were used. The cyanogen bromide (BrCN) was purchased from Fluka AG. Pre-coated TLC-plates Silicia Gel F_{254} and solvent system $CHCl_3/CH_3OH$ 1:1 were used. Cyanogen bromide activated Sephadex (SxOH*) was prepared as previously described.[2] Cyanogen bromide activated methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (MBGP*) was prepared as described previously except that $CHCl_3$ was exchanged for CH_2Cl_2 as extraction solvent.[6] In the experiments performed, the nitrogen content of MBGP* varied from 2.0 to 4.5 %. Sephadex-carbonate was prepared as described for cellulose-carbonate.[11] IR 1750 cm^{-1}(>C=O, acyclic), 1800 cm^{-1} (>C=O, cyclic).

1. *Coupling of glycine to MBGP** (*Ia*). To MBGP* (2.0 g, N 2,4 %) in acetone (40 ml) was added an aqueous solution (40 ml) of glycine (4.0 g) and triethylamine (2 ml). The mixture was stirred for 0.5 h at room temperature, evaporated to dryness and dried for 1 h at 80°. The residue was pulverized, dried again to ensure that all triethylamine had been removed, and thereafter stirred in water (50 ml) at room temperature for 20 min. The solid product was filtered off, washed with boiling acetone (10 ml), dried and precipitated from water (50 ml). After 20 h in a refrigerator (4°) the precipitate was filtered, dried at 110° for 0.5 h giving an amorphous product (0.2 g) that was hygroscopic. M.p. 176–177°d. (Found: C 52.2; H 6.0, N 6.9; glycine 2.25 mmol/g. Calc. for $C_{17}H_{22}O_8N_2$:

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C 53.5; H 5.7; N 7.25; glycine 2.40 mmol/g). The product was homogeneous on TLC. The glycine value is low, due to incomplete hydrolysis, and is not corrected. An IR spectrum showed a double peak at 1680, 1700 cm^{-1} (C=N, stretching).

2. *Coupling of glycylglycine to MBGP* (Ib).* To MBGP* (2.0 g, N 2.7 %) in acetone (30 ml) was added an aqueous solution (30 ml) of glycylglycine (2.0 g) and triethylamine (2.0 ml). The mixture was stirred for 20 min at 20° and evaporated at 50° for 3 h, whereupon the product was washed on a filter with acetone (3 × 20 ml) and finally dried for 1 h at 80°. Two precipitations from hot water (50 ml) and drying at 80° for 2 h afforded an amorphous product (0.4 g) which was homogeneous on TLC. M.p. 187°d. (Found: C 51.4; H 5.7; N 9.0; O 32.5; glycine 4.25 mmol/g. Calc. for $C_{19}H_{25}O_9N_3$: C 51.9; H 5.7; N 9.6; O 32.8; glycine 4.55 mmol/g). The glycine value is low for the same reason as above, and is not corrected. An IR spectrum showed a double peak at 1665, 1690 cm^{-1} (C=N, stretching).

3. *Sephadex-carbamate* was prepared by treating Sephadex-carbonate (0.5 g) with liq. NH_3 (2 ml) for 15 min. After evaporating excess NH_3, the product was washed with water (15 ml), dioxane, (20 ml), ether (20 ml) and finally dried at 50°. 0.53 g. IR 1715 cm^{-1} ($-CONH_2$). N 4.5 %.

4. *Hydrolysis of Sephadex-carbamate.* Sephadex-carbamate (0.250 g, from exp. 3) was stirred in 0.5 M aqueous $NaHCO_3$ (10 ml) at 23°. After various intervals, the reaction mixture was centrifuged, and the supernatant removed, the residue washed with water (10 ml), centrifuged, and the supernatant removed. The washing procedure was repeated twice. The NH_3 present in the combined supernatants was determined. After 1, 2, 4, 22, 28, and 51 h; 2.8, 5.6, 9.2, 26.8, 29.6, and 42.8 μmol NH_3 per g Sephadex-carbamate, respectively, were releazed.

5. *Hydrolysis of Sephadex-carbamate in the presence of different amino compounds.* The Sephadex-carbamate (3.0 g, from exp. 3) was reacted with methanol (5 ml) in 0.5 M aqueous $NaHCO_3$ (10 ml) for 18 h at room temperature to remove remaining carbonate groups. The reaction was followed by IR. The product was filtered and washed with water, water-methanol 1:1, methanol, ether and finally dried at 40° for 20 min. This product (0.250 g) in 0.5 M aqueous $NaHCO_3$ (10 ml) was stirred with glycine (0.075 g), glycylglycine (0.132 g) or glycyl-L-leucine (0.188 g), respectively, for 25 h at 20°. The pH of the solutions was adjusted to 8.4 by adding solid Na_2CO_3. The working up and the determination of NH_3 was carried out as in Exp. 4. After corrections for small amounts of impurities in the amino compounds used the NH_3 liberated was found to be 16 – 28 μmol per g Sephadex-carbamate.

6. *Hydrolysis of SxOH*.* SxOH* (N 1.9 %, 0.250 g) was treated as in Exp. 4. After 0.5, 1, 2, 6, and 21 h, 54, 72, 84, 120, and 132 μmol NH_3 per g SxOH*, respectively, were released.

7. *The reaction of amino compounds with SxOH*.* A. To glycine, glycine ethylester, and glycyl-L-leucine (1.00 g), respectively, in 0.5 M aqueous $NaHCO_3$ (2 ml) was added SxOH* (0.4 g) and the mixtures were stirred for 20 h at room temperature. After filtration, the products were washed with 0.5 M $NaHCO_3$, 0.01 M HCl, 1 M NaCl solution, water, water-acetone (3:1, 1:1, 1:3), acetone, and dried for 2 h at 40°. A blank, containing SxOH* but no amino compound was run parallel with the other samples. The results are given in Table 1.

B. To glycine, glycine ethylester and glycine-L-leucine (0.40 g), respectively, in 0.5 M aqueous $NaHCO_3$ (2.5 ml) was added SxOH* (0.40 g). The mixtures were stirred for 15 h at room temperature and worked up as above. A blank was run as above. The results are given in Table 2.

Table 1. Binding of amino compounds to SxOH*.

Amino compound	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product[a] % A	*B*	C
Glycine	7.3	1390(1348)[b]	7.3	5.4	5.4
Glycine ethyl ester.HCl	6.9	1276(1238)[b]	7.0	5.2	5.2
Glycyl-L-leucine	7.0	658, 680 gly leu	7.1	6.2	6.2
—	6.0				

[a] A, Calc. as isourea derivatives. B, Calc. as imidocarbonates. C, Calc. as *N*-substitued carbamates. [b] The values are corrected for incomplete hydrolysis. Non-corrected values in parentheses.

Table 2. Binding of amino compounds to SxOH*.

Amino compound	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product[a] % A	B	C
Glycine	7.6	1646(1463)[b]	7.6	5.3	5.3
Glycine ethyl ester.HCl	7.4	1575(1401)[b]	7.2	5.0	5.0
Glycyl-L-leucine	6.6	673, 768 gly leu	7.1	6.1	6.1
–	6.0				

[a, b] Compare Table 1.

Table 3. Binding of amino compounds to SxOH*.

Amino compound	Released ammonia μmol/g	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product, %[a] A	B	C
Glycine	252	6.5	680(632)[b]	6.6	5.7	5.7
Glycine ethyl ester.HCl	212	6.5	509(473)[b]	6.4	5.7	5.7
Glycyl-L-leucine	210	6.2	133, 144 gly leu	6.2	5.8	5.8
–		6.0				

[a, b] Compare Table 1.

C. Solutions of glycine, glycine ethylester and glycine-L-leucine (0.25 g), respectively, in 1 M aqueous $NaHCO_3$ (10 ml) were adjusted to pH 8.4 by addition of solid Na_2CO_3. SxOH* (0.250 g) was added and stirring was continued for 18 h at room temperature. The reaction mixtures were centrifuged, the supernatants removed, and the residues washed with water, centrifuged, and the supernatants removed. The washing procedure was repeated twice. The NH_3 content of the combined supernatants was determined. The products were washed and dried as above. The results are given in Table 3.

D. The reactions between SxOH* (N 5.65 %, 0.250 g) and glycine, glycine ethylester, alanine, glycylglycine, glycyl-L-leucine, glycyltyrosine (1 mmol) and aqueous ammonia (68 μmol), respectively, in 0.5 M aqueous $NaHCO_3$ (10 ml) at 25° were allowed to proceed for 24 h. Excees CO_2 was removed at 50° during 5 min. The pH of the buffer solutions was 8.2 – 8.3. The working up procedure was as described in Exp. C. The results are given in Table 4.

8. *Dextran cross-linked by BrCN (dextran*).* A. Dextran 10 (45 g) was dissolved in water (300 ml) in a 500 ml three-necked flask provided with a stirrer and a pH-electrode. BrCN (7.9 g) was added in portions over 90 min. The pH was maintained at 10.5 by adding 5 M NaOH. After neutralization with 5 M HCl, the solution was dialyzed against tap water for 3 days and thereafter in distilled water for 2 days. The solution was then concentrated to 200 ml at 65°, the cross-linked dextran precipitated, using 2.5 l ethanol, and dried for 2 h at 40°. $\overline{M}_w$ 229 000, $\overline{M}_n$ 17 000. N 1.40 %.

B. Conditions and working up procedure as above. BrCN (8.9 g) was added in portions over 90 min. $\overline{M}_w$ 1 080 000, $\overline{M}_n$ 23 000.

9. *Reaction of glycine and glycylglycine with dextran*.* A. Glycine (1 mmol) and dextran* (from exp. 8B, 0.5 g) were dissolved in 1 M aqueous $NaHCO_3$ (10 ml). After 15 h at 20°, the reaction mixture was dialysed for 5 days and freeze-dried. $\overline{M}_w$ 111 000, $\overline{M}_n$ 13 000, glycine 37.5 μmol/g. A parallel experiment using the same reaction conditions, was performed with dextran * (from exp. 8B, 0.5 g). $\overline{M}_w$ 148 000, $\overline{M}_n$ 17 000.

B. Glycylglycine (1 mmol) and dextran* (from exp. 8A, 0.5 g) were dissolved in 0.5 M aqueous $NaHCO_3$ (10 ml). After 23 h at 25°, the reaction mixture was dialysed for 5 days

Table 4. Binding of amino compounds to SxOH*.

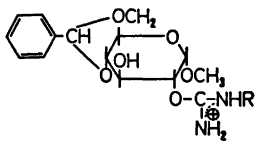
Amino compounds	Released ammonia μmol/g	Nitrogen in the product %	Amino acid μmol/g	Calc. nitrogen in the product, %[a] A	B	C
Glycine	304	5.9	701(637)[b]	5.8	4.8	4.8
Glycine ethyl ester.HCl	292	5.7	542(493)[b]	5.6	4.8	4.8
Alanine	288	5.4	387(352)[b]	5.5	4.9	4.9
Glycyl-L-leucine	280	5.6	275, 309 gly leu	5.7	5.2	5.2
Glycyl-L-tyrosine	320	5.5	399, 438 gly tyr	5.8	5.2	5.2
Glycylglycine	288	5.5	529(503)[b]	5.7	5.3	5.3
Ammonia[d]	484	5.2				
—	268	5.1				
—	—	5.65[c]				

[a,b] Compare Table 1. [c] Nitrogen content before treatment. [d] 272 μmol/g added to the system.

and freeze-dried. $\overline{M}_w$ 57 000, $\overline{M}_n$ 11 000, glycine 51 μmol/g. A parallel experiment, using the same reaction conditions, was performed with dextran* (from exp. 8A, 0.5 g). $\overline{M}_w$ 71 000, $\overline{M}_n$ 15 000.

RESULTS

1. *Studies with model compounds.* When MBGP* was reacted with glycine or glycylglycine in water-acetone, using triethylamine as catalyst, only one type of addition products could be isolated. These are isourea derivatives (I) according to evidence given below.



R: $-CH_2COO^{\ominus}$ $-CH_2CONHCH_2COO^{\ominus}$

Ia Ib

The products obtained contained impurities from the activation of MBGP and were purified by various washing procedures and repeated precipitations from hot aqueous solution. The purified products are amorphous and are only soluble in hot water and methanol, indicating a dipolar structure. The elemental analyses ruled out a carbamate or imidocarbonate structure but were in accordance with the proposed isourea formula (I). This was also confirmed by the characteristic double peak at 1660 – 1700 cm^{-1} in the IR-spectrum.[12] No attempts to separate the 2- and 3-isomers were performed.

II. *The reactions of amino compounds with SxOH* and dextran**. The products obtained by reacting SxOH* with amino compounds in dilute alkaline solution have been given structures represented schematically in II – IV.[3,13,14]

SxOCNHR (O) II SxOCNHR (NH) III $(SxO)_2C=NR$ IV

If products of type III or IV are formed at least one molecule of ammonia per molecule amino compound incorporated into the Sephadex matrix would be liberated. As shown in Table 4, the amount of ammonia liberated during the coupling procedure is 280 – 320 μmol/g SxOH* and the amount of amino compound incorporated 309 – 701 μmol/g SxOH*. Under the same conditions but with no amino compound, SxOH* releases 268 μmol/g SxOH* due to partial hydrolysis of carbamate and other groups in the SxOH* matrix. Thus, only a few percent of the released ammonia may come from reactions leading to *N*-substituted carbamates (III) and *N*-substituted imidocarbonates (IV), which shows that the main component is the isourea derivative (II).

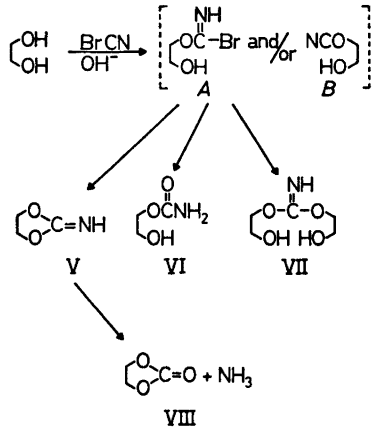
The carbamate groups in SxOH* are only slowly hydrolyzed under the weak alkaline conditions used, as was shown from studies with Sephadex-carbamate, prepared by reacting Sephadex-carbonate with liquid ammonia. The Sephadex-carbamate was hydrolyzed and the ammonia released determined. Only 15 – 30 μmol of ammonia per g Sephadex-carbamate was obtained (Exp. 4 – 5), thus demonstrating that only a small part (5 – 10 %) of the ammonia liberated could then arise from hydrolysis of carbamate groups in SxOH*. The main part of the ammonia liberated during the coupling procedure must originate from the hydrolysis of other groups in the SxOH* matrix. Thus, SxOH* was hydrolysed in 0.5 M aqueous $NaHCO_3$, and the ammonia determined as a function of time. Most of the ammonia was liberated within the first 6 h, giving after 21 h totally 132 μmol of ammonia per g SxOH* (Exp. 6). It may be noted that this SxOH* only contained 1.9 % N, while, in most experiments performed, the nitrogen content was 5.6 – 6.0 %.

Dextran * was obtained by reacting concentrated low molecular weight dextran solutions with BrCN in alkaline media. The product was kept in water for several days to make sure that the unstable five-membered *trans*-cyclic imidocarbonate groups,[5] which also are formed, were completely hydrolysed. The dextran* was then reacted with some amino compounds and the molecular weights and the amino acid contents of the products determined (Exp. 9A and B).

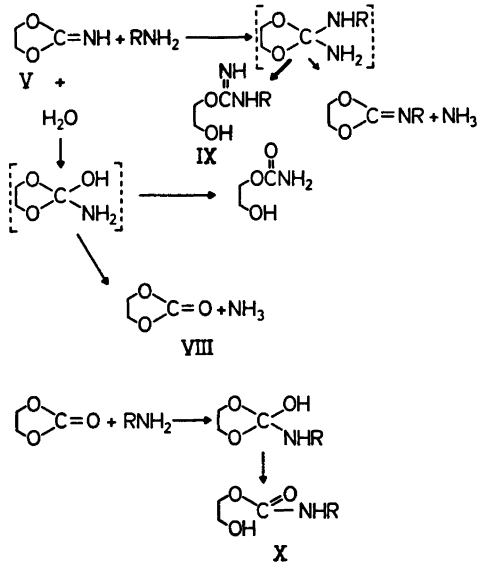
DISCUSSION

The reaction of BrCN in dilute alkaline solution with vicinal *trans*-hydroxyl groups as in Sephadex or dextran is represented schematically in Scheme 1. The structures A[15] and B are proposed as intermediated leading to end-products with the structures V – VIII.

Proof of the existence of the reactive groups V, the carbamate groups VI, and the carbonate groups VIII was presented in studies on a model compound, MBGP.[6] The acyclic imidocarbonate structures VII are formed mainly by cross-linking two glucose units in different dextran chains. This is indicated by the reduced swelling capacity of the SxOH* and by the increase in molecular weight when low molecular weight dextrans are treated with excess of BrCN [2] (Exp. 8A and B). The amount of VIII in SxOH* is low since its characteristic IR-absorption at 1800 – 1830 cm^{-1} is very weak. Under favourable activation conditions, it has been found that about 50 % of the nitrogen incorporated in the activated products is due



Scheme 1.



Scheme 2.

to imidocarbonate groups (V).[5] Thus, it can be concluded that the reactive groups V and the carbamate groups (VI) are the main structures present in BrCN activated carbohydrates, containing vicinal *trans*-hydroxyl groups and that V and VI both have IR-absorption at 1715 cm^{-1}.

The reaction of V with an amino compound in $NaHCO_3$ solution and the side reaction the alkaline hydrolysis of V, is shown in Scheme 2. The first step is presumably a nucleophilic addition of the amino compound to V, giving a five-membered unstable intermediate which subsequently rearranges to the stable isourea derivative IX. Investigations with a model compound (MBGP) have resulted in the isolation and characterization of only one type of addition product, the isourea derivative. Only a few percent of the total amount of ammonia anticipated, if *N*-substituted imidocarbonates are the only reaction products, has been found. It may also be pointed out that the amount of NH_3 released is rather constant and thus independent of the amount of amino compound incorporated in the Sephadex matrix (Tables 3 and 4). Furthermore, as shown in Tables 1 – 2, particularly where the amount of amino compound in the products is high, the calculated nitrogen content for the isourea structure agrees fairly well with the value found.

The hydrolysis of V under the coupling conditions used may compete with the addition of the amino compound to SxOH*. Thus, V may by addition of water be transformed into a reactive intermediate (Scheme 2), giving either the carbamate or the carbonate, the latter formed with concomitant release of ammonia.

Several products obtained by reacting SxOH* with amino compounds have been examined by IR-spectroscopy. No absorption at 1800 – 1830 cm^{-1}, characteristic of the carbonate group, has been found. If, however, some carbonate groups are formed, they may react with the amino compound, giving as end-products *N*-substituted carbamates (X), which as discussed above can only be present in a few percent.

When dextran*, in which the reactive *trans*-cyclic imidocarbonate groups have been removed, is treated with amino compounds under mild basic conditions, the molecular weight decreases more than when the dextran* is treated similarly but in the absence of the amino compounds. This indicates that the acyclic structures VII, which are likely to be the remaining reactive groups, react at least to some extent with the amino compounds giving structures IX and X. Stable, low molecular weight acyclic imidocarbonates, analogous to XI, are known,[16] and the formation of such structures cannot be excluded. The main part of the decrease in molecular weight arises from the hydrolytic cleavage of the acyclic imidocarbonates (VII) with the formation of carbamates (VI). Small amounts of the acyclic carbonates (XII) may also be formed. These may then react with amino compounds to give the *N*-substituted carbamates (X).

It has been shown that some of the ammonia released during the coupling procedure may arise from hydrolysis of carbamates and from the formation of imidocarbonates or carbonates. However, the main part was demonstrated to originate from hydrolysis of other groups in the SxOH*. At present we can only speculate as to which groups are responsible and suggest that during the activation procedure part of the imidocarbonate groups (V) may have reacted with BrCN to give cyanamide groups. During the mild alkaline conditions of the coupling reaction, nitrogen compounds may be released from these groups and give ammonia during the strong alkaline conditions of the Kjeldahl analysis.

The ammonia released from SxOH* may react with imidocarbonate groups in the SxOH*, yielding isourea groups. However, from the experiments with added ammonia (Table 4), it is evident that this reaction is of little importance.

XI

XII

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