DEXTRAN DERIVATIVES

II. Synthesis of N-Aminoacyl Derivatives of Carboxymethyldextran

A. E. Vasil'ev, A. B. Livshits, G. Ya. Rozenberg, and N. K. Kochetkov

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The synthesis of O-aminoacyl derivatives of dextran by the condensation of N-acylamino acids with dextran under the action of dicyclohexylcarbodiimide (DCHC) has been reported previously [1]. In these derivatives the amino acids are attached to the dextran by a relatively labile ester bond, which is inconvenient under the conditions of chemical and biochemical hydrolysis [2]. Consequently, it was necessary to synthesize aminoacyl derivatives of dextran with the more hydrolytically resistant amide bond.

This paper describes the preparation by the acid chloride and the carbodiimide methods (the latter gave good results) of N-aminoacyl derivatives of carboxymethyldextran (CMD) in which the amino acid is attached to the polysaccharide molecule by an amide bond through the carboxyl group.

We obtained the initial CMD in the form of the Na salt by the alkylation with chloroacetic acid in an alkaline medium of the Russian-made "clinical" dextran "Polyglucin", see [3]. Depending on the reaction conditions (time, temperature, ratio of the reagents), the degree of substitution of the CMD varies from γ 3 to γ 140*:

$$[C_6H_7O_2(OH)_3]_m \rightarrow [C_6H_7O_2(OH)_{3-n}\cdot(OCH_2COONa)_n]_m.$$

Na salt of CMD

The acid chloride of CMD (II) was obtained from the Na salt of CMD [1], $\gamma = 56$, and thionyl chloride in a heterogeneous medium, like the acid chloride of carboxymethylcellulose [4]. The acid chloride of CMD (II) was condensed with the ethyl ester of glycine in ether, again in a heterogeneous medium, with the formation of the ethyl ester of glycyl-CMD (III). From the results of elementary analysis, the degree of substitution of the carboxyl groups of CMD by aminoacyl residues was only 5%.

$$[C_6H_7O_2(OH)_{3-n}(\underset{I}{OCH_2COONa})_n]_m \rightarrow [C_6H_7O_2(OH)_{3-n}(\underset{II}{OCH_2COCl})_n]_m \rightarrow$$

$$\rightarrow [C_6H_7O_2(OH)_{3-n}(\underset{III}{OCH_2CONHCH_2COOEt})_n]_m.$$

Since the acid chloride method leads to a derivative with a low degree of substitution and, apparently, to the formation of a nonhomogeneous polymer, for the synthesis of N-aminoacyl derivatives of CMD we subsequently used the carbodiimide method of condensation developed previously for the synthesis of N-aminoacyl derivatives of uronic acids [5, 6].

In aqueous pyridine, the condensation of esters of amino acids and uronic acids under the action of dicyclohexylcarbodiimide (DCHC) takes place specifically with the formation of amides and not esters, thanks to which there is no necessity for the protection of the hydroxyl groups of the sugars. The reaction of CMD with hydrochlorides of esters of amino acids IV may take place in the following way:

$$[C_6H_7O_2(OH)_{3-n}(OCH_2COOH)_n]_m \xrightarrow[DCHC,\ \mathcal{E}t_3N]{H_2NCHRCOOR'\ (IV)} [C_6H_7O_2(OH)_{3-n}(OCH_2COOH)_{n-p}(OCH_2CONHCHRCOOR')_p]_m$$

IV, V a R = H, R' = Me

b R = CH_2–(imidazole), R' = Me

V c R = H, R' = CH_2Ph

*γ represents the number of substituting groups in 100 anhydroglucose units.

The condensation was carried out in aqueous pyridine at 20° C for 48 hr. The Na salt of CMD [1] was previously converted into the acid by its passage through KU-2 ion-exchange resin, and an equivalent amount of triethylamine and a threefold excess of DCHC were used. At a ratio of pyridine to water of 3:1 the reaction mixture was heterogeneous and the degree of substitution of the carboxymethyl groups of the CMD by amino acid residues was 15.1% for the methyl ester of glycine (from elementary analysis for nitrogen). At a pyridine to water ratio of 1.5:1 (homogeneous medium, pH 7.0), the degree of substitution of the carboxymethyl groups of the CMD was 100%. The modified polymer obtained was desalted by gel filtration through Sephadex G-50 in water.

In addition to the methyl ester of glycyl-CMD (Va) with a degree of substitution of 100% that has been mentioned, the methyl ester of (N^{α}-L-histidyl)-CMD (Vb) with a degree of substitution of 11.4% and benzyl esters of glycyl-CMD (Vc) with various degrees of substitution were obtained by this method. The solubility of the methyl esters of the (N-aminoacyl)-CMDs (Va, b) is practically independent of the degree of substitution, while that of the benzyl esters (Vc) does depend on the degree of substitution. When all the carboxyl groups were aminoacylated, a benzyl ester (Vc) completely insoluble in water, partially soluble only in dimethyl sulfoxide, and insoluble in other organic solvents was obtained.

To obtain a water-soluble product, a benzyl ester of glycyl-CMD (Vc) with a lower degree of substitution was synthesized. This compound dissolved in water but, in contrast to the methyl esters Va and Vb was not precipitated by ethanol from aqueous solutions, and we therefore used dioxane.

Hydrolysates of the esters V gave a positive ninhydrin reaction.

The structure of the esters of the (N-aminoacyl)-CMDs (V) was confirmed by their IR spectra. These compounds showed characteristic absorption in the 1740–1748 cm^{-1} region (ester and carboxyl groups) and at 1670 cm^{-1} (amide group and carboxylate ion). All the bands were fairly broad, which made it impossible to distinguish the absorption caused by each of the groups mentioned.

A change in the CMD under the action of DCHC is not excluded, since CMD contains —OH and —COOH groups. Consequently, we checked the absence of changes in the basic characteristics of CMD under the action of DCHC under the conditions of a blank experiment. However, in spite of the isolation of the theoretical amount of dicyclohexylurea, no ester bonds were formed in the CMD. The IR spectra of the initial CMD and of the reaction product were practically identical, and the unchanged nature of the Sephadex G-200 fractionation curves (Fig. 1), the similar values of the sedimentation coefficients (for CMD, $S_{19} = 2.71 \times 10^{-13}$; for CMD + DCHC, $S_{19} = 2.79 \times 10^{-13}$) and their behavior on electrophoresis (Fig. 2a, b) show that the macromolecular structure of the CMD was essentially unchanged.

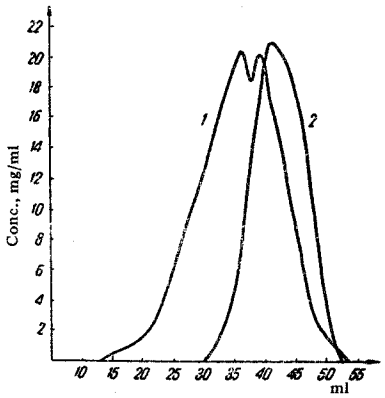

Fig. 1. Fractionation curves on Sephadex G-200 of carboxymethyldextran (CMD) before and after treatment with dicyclohexylcarbodiimide: 1) CMD; 2) CMD + DCHC. Fractionation in water.

A similar pattern is found in a comparison of the fractionation curves of CMD and the methyl ester of glycyl-CMD (Va) on Sephadex G-50 (Fig. 3), and also in a comparison of electrophoresis for the same compounds (see Fig. 2b). Thus, in this case, also, the macromolecular structure of the compounds is basically retained.

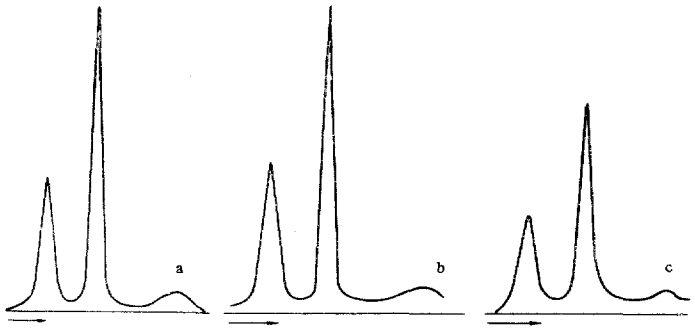

Fig. 2. Electrophoregrams: a) carboxymethyldextran (CMD); b) CMD + DCHC; c) methyl ester of glycyl-CMD (veronal buffer, pH 8.6, $\mu \sim 0.1$).

Derivatives with free carboxyl groups were obtained from the methyl esters Va and Vb by alkaline hydrolysis and from the benzyl ester Vc by hydrogenolysis. As has been shown on the basis of the hydrolysis of the ethyl ester of glycylgalacturonic acid, the amide linkage is stable under the hydrolysis conditions [6]. The alkaline hydrolysis of the methyl ester of glycyl-CMD (Va) was carried out at 20° C in 1 N NaOH:

$$[\,C_6H_7O_2(OH)_{3-n}(OCH_2COOH)_{n-p}(\underset{Va}{OCH_2CONHCH_2COOMe})_p\,]_m \rightarrow$$
$$\rightarrow [\,C_6H_7O_2(OH)_{3-n}(\underset{VI}{OCH_2COOH})_{n-p}(OCH_2CONHCH_2COOH)_p\,]_m.$$

The benzyl group was eliminated from the benzyl ester of glycyl-CMD (Vc) by hydrogenation over Pd/C in water:

$$[\,C_6H_7O_2(OH)_{3-n}(OCH_2COOH)_{n-p}(\underset{Vc}{OCH_2CONHCH_2COOCH_2Ph})_p\,]_m \rightarrow$$
$$\rightarrow [\,C_6H_7O_2(OH)_{3-n}(OCH_2COOH)_{\underset{VI}{n-p}}(OCH_2CONHCH_2COOH)_p\,]_m.$$

In both cases, titration of the (N-glycyl)-CMD (VI) showed that the ester groups had been converted completely into carboxyl groups.

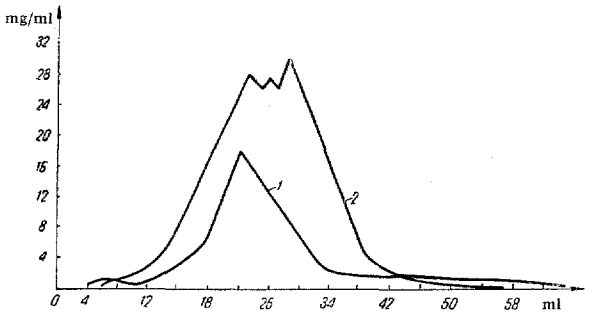

Fig. 3. Fractionation curves on Sephadex G-50 of carboxymethyldextran (CMD) and the methyl ester of (N-glycyl) carboxymethyldextran: 1) CMD; 2) methyl ester of (N-glycyl)-CMD. Fractionation in water.

EXPERIMENTAL

Dry "Polyglucin" "clinical" dextran with a weight-average mol. wt. of 55 ± 15 thousand was used to obtain the CMD. The aminoacyl derivatives were synthesized from the CMD that we obtained, and also from the Na salt of CMD.

Electrophoresis was carried out (by N. D. Papush) on a Karl Zeiss (GDR) EF-35 instrument by the mobile boundary method at 100 V and 11.2 mA, in veronal buffer, pH 8.6. The evaporation and drying of the substances, gel filtration, titration, ultracentrifuging, and the spectral investigations were carried out as described previously [1] [the IR spectra were recorded by M. M. Ushakova and interpreted by O. S. Chizhov, and the ultracentrifuge investigations were performed by V. M. Shlimak].

Highly-substituted carboxymethyldextran (CMD) (with the participation of T. V. Polushina). To a solution of 10 g of dextran in 80 ml of water at 18–20° C was gradually added 12.3 g of NaOH in the form of a 50% aqueous solution, and this was followed, dropwise over 30 min, by a freshly-prepared solution of Namonochloroacetate (from 29 g of monochloroacetic acid in 50 ml of water and 15 g of solid anhydrous sodium carbonate). The resulting solution was heated at 60–65° C for 1.5 hr, cooled to 20° C, and neutralized with conc HCl to pH 7. The high-molecular-weight reaction product was precipitated with 1.3 volumes of ethanol with vigorous shaking, and after 1.5 hr the solution was decanted off and the residue was dissolved in 100 ml of water and again precipitated with an equal volume of ethanol. After 1.5 hr the solution was decanted off and the residue was dissolved in 100 ml of water and freeze-dried (freezing onto the walls of the vessel at −40 to −45° C, vacuum sublimation to 20° C, and final drying at 40–50° C). The yield of the Na salt of CMD was 14.7 g. White amorphous powder readily soluble in water and insoluble in organic solvents.

100 ml of a 10% solution of the Na salt of CMD was passed through a column containing 30 g of KU-2 cation-exchange resin in the H-form, and from the eluate, with pH 2.1–2.2, the polymer was precipitated with ~4.5 volumes of ethanol with vigorous shaking. After 1 hr the liquid was decanted off and the residue was triturated with 96% and then with absolute ethanol and was dried in vacuum. Yield 9.5 g. The Na content (0.038%) was determined on a GP-21A flame photometer. The degree of substitution of the dextran with free carboxymethyl groups (44%) was found by titration with 0.1N NaOH to phenolphthalein after gel filtration through Sephadex G-50 (coarse). The acid number was 2.8; $[\alpha]_D^{20}$ + 151.9° (c 1; water). The sedimentation coefficient of the CMD at pH 2.1 and $\mu \sim 0.1$ was $S_{19} = 2.71 \times 10^{-13}$; for dextran under the same conditions, $S_{19} = 2.71 \times 10^{-13}$.

Carboxymethyldextran with a low degree of substitution. To a solution of 7 g of dextran in 50 ml of water at 5° C was gradually added 8.6 g of NaOH in the form of a 50% aqueous solution, and then, dropwise, a solution of Na monochloroacetate (from 18 g of monochloroacetic acid in 32 ml of water and 10 g of anhydrous sodium carbonate). The solution was stirred at 20° C, 17-ml samples being taken after 1, 3, and 26 hr. With cooling, the samples were brought to pH 3 with concentrated HCl and the CMD was precipitated from the solution with two volumes of ethanol and was triturated with 96% and absolute ethanols and dried. The product was desalted by gel filtration through Sephadex G-50 (coarse) and was passed twice through KU-2 ion-exchange resin (2 × 15 g). The degrees of substitution of the dextran with free carboxymethyl groups for the CMD fractions obtained (titration to phenolphthalein) were, respectively, 3, 8, and 140.

Acid chloride of carboxymethyldextran (II). A solution of 0.4 ml of freshly-distilled thionyl chloride in 1.5 ml of absolute benzene was added to a suspension of 800 mg of the Na salt of CMD [1] (γ 56) in 3.5 ml of absolute benzene, and the mixture was heated at 72° C for 4 hr. After two days at 5° C, the clear benzene layer was decanted off and the gel-like residue was washed repeatedly with absolute ether until the reaction for Cl^- was negative and was then dried. Yield 680 g (84.4%). Found, %: Cl 2.5, which corresponds to the substitution of 26.4% of the carboxymethyl groups of the CMD.

Ethyl ester of (N-glycyl)-CMD (III). A solution of 620 mg of the ethyl ester of glycine in 5 ml of absolute ether was added to 500 mg of II. The mixture was stirred at 20° C for 24 hr, and the precipitate was filtered off, washed with methanol (3 × 5 ml) and with ether (2 × 5 ml), and dried. Desalting was carried out by gel filtration through Sephadex G-50 (coarse). Yield 470 mg, 71.6%; $[\alpha]_D^{20}$ + 132.9°. Found, %: N 0.19, which corresponds to the substitution of 5.22% of the carboxylmethyl groups of the CMD.

Methyl ester of (N-glycyl)-CMD (Va). A solution of 1.2 g of the hydrochloride of the methyl ester of glycine and 1.34 ml of triethylamine in 2 ml of water cooled to 5° C was gradually added to a solution of 21 g of CMD (γ 44) in 8 ml of water cooled to the same temperature, after which the mixture was immediately treated with 5 ml of pyridine. Then a solution of 5 g of DCHC in 10 ml of pyridine was gradually added, with stirring, to the resulting solution. After 48 hr at 20° C the reaction mixture was diluted with three volumes of water and the precipitated dicyclohexylurea was filtered off. The filtrate was extracted with ether (3 × 20 ml) to eliminate the unchanged DCHC, and the aqueous layer was concentrated in vacuum to 8–10 ml and subjected to gel filtration through Sephadex G-50 (coarse). The fractions containing the polymer were analyzed by the method described previously [1], combined, and concentrated in vacuum to

5–8 ml, and the polymer was precipitated with ethanol. The precipitate was triturated successively with 96% and absolute ethanols and dried. Yield 2.3 g. White hygroscopic powder soluble in water and insoluble in organic solvents. The degree of substitution of the COOH groups with aminoester residues was 100%. Found, %: N 3.46; $[\alpha]_D^{20}$ + 138.3° (c 1; water).

Methyl ester of (N-histidyl)-CMD (Vb). As in the preceding experiment, a solution of 93 mg of the dihydrochloride of the methyl ester of histidine and 0.11 ml of triethylamine in 3.4 ml of pyridine, followed by a solution of 158 mg of DCHC in 4 ml of pyridine were added to a solution of 1 g of CMD (γ 44) in 4 ml of water. After 48 hr at 20° C, the reaction mixture was worked up. This gave 930 mg of a white hygroscopic powder soluble in water and insoluble in organic solvents. The degree of substitution of the carboxyl groups with amino ester residues was 11.4%. Found, %: N 1.08.

Benzyl ester of (N-glycyl)-CMD (Vc). Under the same conditions, 1.28 g of CMD (γ 44) in 8 ml of water, 1.38 g of the hydrochloride of the benzyl ester of glycine, and 0.7 ml of triethylamine in 3 ml of water, 5 ml of pyridine, and a solution of 2.6 g of DCHC in 11 ml of pyridine were brought to reaction. After 48 hr at 20° C, the mixture was diluted with 80 ml of water and the dicyclohexylurea that had deposited, with mp 231–234° C, was filtered off. The turbid filtrate was centrifuged for 20 min at 2000 rpm and an additional small precipitate of dicyclohexylurea was separated off. The supernatent liquid, also turbid, was decanted off and extracted with ether (3 × 60 ml) to eliminate the unchanged DCHC, and the aqueous layer was evaporated to dryness. The residue, which was insoluble in water and acetone, partially soluble in ethanol, methanol, ethyl acetate, dioxane, and dimethylsulfoxide, and swelled in formamide and pyridine, was triturated with ethanol cooled to 0° C (4 × 30 ml). The extract was evaporated, giving 1 g of low-molecular-weight products. The fraction of the residue insoluble in ethanol was triturated with dimethyl sulfoxide (3 × 30 ml) and the gel-like mass formed was centrifuged at 2000 rpm for 30 min. The supernatant liquid was separated off and the residue was washed with dimethyl sulfoxide (2 × 70 ml). The solution obtained was concentrated to 10 ml in vacuum at 58–61° C, the polymer was precipitated with three volumes of ethanol, and the precipitate was triturated with ethanol (2 × 30 ml) and dried. Samples for analysis were triturated with 0.1 N HCl (2 × 1.5 ml) to eliminate basic substances, washed with water to neutrality, and dried. The yield of product soluble in dimethyl sulfoxide was 0.87 g. Found, %: N 2.64, which corresponds to 100% of the carboxymethyl groups of the CMD. The gel-like transparent product insoluble in dimethyl sulfoxide was treated with 50 ml of ethanol and the white flocs formed were separated off, triturated with ethanol (3 × 30ml), treated with 20 ml of absolute ethanol, and evaporated, and the residue was dried. Yield 0.7 g. Found, %: N 2.62, i. e. 100% substitution of the carboxymethyl groups of the CMD.

Under the conditions of the preceding experiment, 2 g of CMD (γ 44), 88.8 mg of the hydrochloride of the benzyl ester of glycine, 3.66 g of DCHC, and 0.61 ml of triethylamine in 33 ml of pyridine and 22 ml of water gave a product soluble in water. After gel filtration through Sephadex G-50 (coarse) the fractions containing the polymer were combined and concentrated, and the polymer was precipitated with three volumes of dioxane (no precipitate was formed with ethanol). The supernatant liquid was decanted off and the gel-like residue was treated with 30 ml of ether and the solvent was evaporated to dryness. This treatment was repeated twice. The yield of white amorphous powder soluble in water was 1.8 g. Found, %: N 0.80.

(N-Glycyl)-CMD (VI) by the saponification of the ester Va. A solution of 250 mg of the methyl ester of (N-glycyl)-CMD (Va) in 5 ml of water was treated with 5 ml of 2 N NaOH, and after 1 hr at 20° C it was neutralized to pH 7 with 1 N HCl, concentrated to 5 ml, and subjected to gel filtration through Sephadex G-50. The reaction product was precipitated from the fractions containing the polymer with three volumes of ethanol, and the precipitate was triturated with 96% and absolute ethanols, and dried. Yield 191.4 mg. 100 mg of this product was passed through 3 g of KU-2 cation-exchange resin in the H-form. The acid number of this product was 2.7. For a polymer with all the ester groups completely hydrolyzed, calc. 2.8. Found, %: N 2.92. Sedimentation coefficient, $S_{19} = 2.19 \times 10^{-13}$; IR spectrum: 1742 and 1668 cm^{-1}.

(N-Glycyl)-CMD (VI) by the hydrogenolysis of the ester Vc. A solution of 520 mg of the benzyl ester of (N-glycyl)-CMD (Vc) in 30 ml of water was hydrogenated over 600 mg of 5% Pd/C until the absorption of hydrogen ceased. The catalyst was filtered off and washed with water, and the solution was repeatedly filtered to remove the carbon through a layer of Sephadex G-50 (coarse) and an SF asbestos plate. The colorless solution was subjected to gel filtration through Sephadex G-50. The fractions containing the polymer were combined and treated in the usual way. Yield 292 mg. White amorphous powder soluble in water. Acid number: found, 2. 7; calculated for the complete removal of the benzyl group: 2.8. Found, %: N 0.30. Sedimentation coefficient, $S_{19} = 2.17 \times 10^{-13}$; IR spectrum: 1740 and 1640 cm^{-1}.

CONCLUSIONS

1. Acid chloride and carbodiimide methods for the synthesis of N-aminoacyl derivatives of carboxymethyldextran have been developed.
2. Using these methods, derivatives of carboxymethyldextran containing residues of glycine esters (with different degrees of substitution) and residues of the methyl ester of L-histidine have been synthesized.
3. A method for obtaining carboxymethyldextrans with different degrees of substitution has been developed.

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Central Institute of Hematology and Blood Transfusion