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Note

Alkaline borohydride treatment of cartilage keratan sulfate: a comparison of two reaction conditions

FRED J KIERAS
The Rockefeller University, New York, N Y 10021 (U S A)
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Cartilage keratan sulfate is composed[1] of the repeating disaccharide unit (1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate The carbohydrate chains of this glycosaminoglycan are linked to a protein backbone by *O*-glycosyl bonds involving a 2-acetamido-2-deoxy-D-galactose residue of the polysaccharide and threonine or serine residues of the peptide chain[2].

Treatment of cartilage keratan sulfate peptide with dilute alkali causes a facile β-elimination reaction which cleaves the polysaccharide from the peptide chain The 2-acetamido-2-deoxy-D-galactose residue of the carbohydrate–protein linkage is converted to a "chromogen"[3], and 2-aminopropenoic and 2-amino-2-butenoic acid residues are formed in the peptide chain[2] The ease of "chromogen" formation in cartilage keratan sulfate is due to substitution at O-3 of the 2-acetamido-2-deoxy-D-galactose residue by a D-galactosyl residue[4–7] The degradation of the keratan sulfate chain by β-elimination of the 2-acetamido-2-deoxy-D-galactose residue is an example of a "peeling" reaction, and is greatly enhanced by substitution at O-3 of this residue[8]

If the β-elimination reaction is performed in the presence of a reducing agent, such as sodium borohydride, reduction competes with "chromogen" formation by converting the liberated 2-acetamido-2-deoxy-D-galactose residue into the corresponding alditol, thereby suppressing the "peeling" reaction The amounts of 2-amino-2-deoxyalditol produced relative to the reduced "chromogens" depend on the conditions of alkaline borohydride treatment[9–13]. It is advantageous in studying the "linkage region" of cartilage keratan sulfate peptide to minimize "chromogen" formation and degradation of the chain by "peeling"

In the work of Bray *et al* [2], alkaline borohydride treatment of cartilage keratan sulfate was performed with 0.5M sodium hydroxide–M sodium borohydride for 24 h at room temperature, conditions that degrade the "linkage" 2-acetamido-2-deoxy-D-galactose residue A study by Weber and Winzler[12] showed that minimal degradation of the 2-acetamido-2-deoxy-D-galactose residue linked to the peptide chain in various mucins occurs during alkaline borohydride treatment with 0.1–0 2M sodium hydroxide–0 4M sodium borohydride for 16–24 h at 50°. More recently, Mayo and Carlson[9] and Iyer and Carlson[13] have shown that similar conditions,

i e 0 05M sodium hydroxide–M sodium borohydride for 16 h at 50° produced minimal degradation of the 2-acetamido-2-deoxy-D-galactose residues of *N*-acetylchondrosine [*O*-(β-D-glucopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-D-galactose] and of a blood-group substance (for a brief review of the application of alkaline borohydride to the study of glycoproteins and proteoglycans, see Pigman and Moschera[14])

In this study, I have compared two conditions of alkaline borohydride cleavage applied to cartilage keratan sulfate, those of Bray *et al*[2] and those of Mayo and Carlson[9] The conditions of Mayo and Carlson[9] were found to cause minimal degradation of the 2-acetamido-2-deoxy-D-galactose residue linked to the peptide chain

EXPERIMENTAL

Material — Cartilage keratan sulfate peptide was prepared by proteolysis of bovine nasal septum with papain and purified by the use of ethanol fractionation and chromatography on Dowex 1 ion-exchange resin The detailed procedure has been described[5]. In these experiments, the material eluted with 2M sodium chloride from the Dowex-1 column was used and had the following components Hexose 25 2%, hexosamine 21 6%, uronic acid 1 3%, and amino acids 8 8% The sulfate to hexosamine ratio was 1 06 1, and 12% of the total hexosamine was 2-acetamido-2-deoxy-D-galactose Approximately 80% of the amino acids are accounted for by threonine, serine, glutamic (glutamine), proline, and phenylalanine The methods used for analysis of various components have been described[5]

Method — The conditions of alkaline borohydride cleavage used were as follows 0 5M sodium hydroxide–M sodium borohydride for 24 h at 24° (*Method A*)[2], 0 05M sodium hydroxide–M sodium borohydride for 16–24 h at 50° (*Method B*)[9]

Keratan sulfate peptide (2 mg) was treated in 250 μl of solution by following either *Method A* or *B* The reaction was terminated by addition of 4M hydrochloric acid with cooling in an ice-bath and, after hydrolysis, for 2-amino-2-deoxy sugars, 2-amino-2-deoxyalditol, and amino acids were determined as described[5] For control, samples of the keratan sulfate peptide were added after neutralization of the alkaline borohydride solution with 4M hydrochloric acid. For other reasons, analyses were also performed on treated samples after exhaustive dialysis against de-ionized water In these cases, the reaction was terminated by addition of glacial acetic acid

RESULTS AND DISCUSSION

Comparison of the results reported in Tables I and II shows that, under both sets of conditions of β-elimination and reduction, almost equivalent amounts of 2-acetamido-2-deoxy-D-galactose, threonine, and serine were destroyed, *i e* ~75% of each component, in agreement with Bray *et al*[2] All other amino acids show recoveries of 90% or greater Reduction of the 2-aminopropenoic and 2-amino-2-butenoic acid residues would be expected to yield alanine and 2-aminobutyric acid, respectively Under both sets of conditions, ~45% of the destroyed serine was

converted to alanıne. The absence of 2-amınobutyrıc acıd ıs not surprısıng, as quantıtatıve reduction of the unsaturated resıdues requıres a catalyst[15]

TABLE I

COMPONENTS OF CARTILAGE KERATAN SULFATE BEFORE AND AFTER TREATMENT BY METHOD A[a]

Component	*Before*[b]	*After*[b]	*Recovery* (%)
2-Amıno-2-deoxy-D-galactose	0.13	0 034	26
2-Amıno-2-deoxy-D-galactıtol	0 00	0 046	
2-Amıno-2-deoxy-D-glucose	0 92	0 72	78
2-Amıno-2-deoxy-D-glucıtol	0 00	0 00	
Threonıne	0 070	0 020	29
Serıne	0 077	0 018	23
Alanıne	0 027	0 055	204

[a]0 5M Sodıum hydroxıde–M sodıum borohydrıde for 24 h at 24°. [b]μMol per mg

TABLE II

COMPONENTS OF CARTILAGE KERATAN SULFATE BEFORE AND AFTER TREATMENT BY METHOD B[a]

Component	*Before*[b]	*After*[b]	*Recovery* (%)
2-Amıno-2-deoxy-D-galactose	0 14	0 031	22
2-Amıno-2-deoxy-D-galactıtol	0 00	0 090	
2-Amıno-2-deoxy-D-glucose	0 89	0 53	60
2-Amıno-2-deoxy-D-glucıtol	0 00	0 00	
Threonıne	0 059	0 015	25
Serıne	0 065	0 014	22
Alanıne	0 026	0 048	185

[a]0 05M Sodıum hydroxıde–M sodıum borohydrıde for 20 h at 50° [b]μMol per mg

Under both reactıon condıtıons (*Methods A* and *B*), the extent of elımınatıon of *O*-substıtuted serıne and threonıne resıdues was essentıally the same. as determıned by the recoverıes of these amıno acıds after alkalıne borohydrıde treatment Lıkewıse, under both sets of condıtıons, the elımınatıon of 2-acetamıdo-2-deoxy-D-galactose was very sımılar It appears, therefore, that for cartılage keratan sulfate both sets of condıtıons of cleavage produce essentıally complete elımınatıon of *O*-substıtuted serıne and threonine resıdues.

The ımportant dıfference between the two sets of condıtıons of alkalıne borohydrıde treatment ıs the amount of 2-acetamıdo-2-deoxy-D-galactıtol produced, determıned as 2-amıno-2-deoxygalactıtol *Method A* converted (Table I), 48% of the destroyed 2-acetamıdo-2-deoxy-D-galactose ınto the correspondıng aldıtol, whereas *Method B* (Table II) converted 83% ınto the aldıtol The remaınder of the carbohydrate resıdues ınvolved ın the lınkage wıth the peptıde chaın and not converted ınto the aldıtol was presumably converted ınto reduced "chromogen".

The destruction of 2-acetamido-2-deoxy-D-glucose residues under both sets of conditions, with no concomitant production of the corresponding alditol, is not readily explained. Perhaps, it represents alkali cleavage of a yet unknown linkage of these sugar residues to the peptide chain, or a lability due to a special position in the polysaccharide chain. As yet, no definitive explanation for this phenomenon has been found.

When these experiments were performed under identical conditions, except that the samples were dialyzed before hydrolysis and analysis, almost identical results were obtained Under the conditions of *Method A*, 47% of the destroyed 2-acetamido-2-deoxy-D-galactose was converted into the corresponding alditol, whereas under the conditions of *Method B*, 85% conversion occurred In addition, more than 90% of the products derived from the linkage 2-acetamido-2-deoxy-D-galactose residues were retained after dialysis, in agreement with the hypothesis that these products remain attached to the keratan sulfate chains, the linkage sugar residues being substituted at the 6-position[2].

Under both sets of conditions of alkaline borohydride cleavage, the molar ratio of 2-acetamido-2-deoxy-D-galactose destroyed to the total of threonine and serine destroyed was near unity, an indication that the destruction of these components by alkali is specific for an *O*-glycosyl linkage.

The results obtained here are in agreement with studies[9–11] on model disaccharides composed of 3-substituted 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-glucose residues Under conditions similar, or equivalent, to those of *Method B*, reduction of the disaccharide occurred to a much greater extent than degradation by "chromogen" formation In addition, the results obtained here are similar to those obtained by Oates *et al* [16], who applied 0 1M sodium hydroxide–3% sodium borohydride for 48 h at 65° or 0 1M trisodium phosphate–0 05M sodium borohydride for 7 days at 37° to human gastric mucin.

Conditions that produce high yields of alditol after cleavage of the polysaccharide from the peptide chain will prove especially useful in the isolation of "linkage region" oligosaccharides from cartilage keratan sulfate and from other substances containing similar structures[5]

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