Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STRUCTURAL AND IMMUNOLOGICAL STUDIES OF CHICK, ALLANTOIC ANTIGEN

PART IV. REACTION WITH SODIUM METAPERIODATE

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(Received February 26th, 1970; accepted for publication, May 5th, 1970)

ABSTRACT

The structure of a sulphated, antigenic glycopeptide from chick, allantoic fluid has been investigated by using the techniques of periodate oxidation and Smith degradation. Most of the fucose and 50% of the D-galactose residues in the antigen are oxidized by sodium metaperiodate; other monosaccharide components are not oxidized. The fucose residues are present preponderantly in terminal, non-reducing positions, and most of the oxidized D-galactose residues are substituted at O-6 and/or O-2. The presence of D-galactofuranose residues (4.7% of the antigen) is confirmed. General structural features of the antigen, which are consistent with these and earlier findings, are discussed.

INTRODUCTION

Previous work has shown[1] that a sulphated, antigenic glycopeptide from chick, allantoic fluid contains acetyl (6.5%) and sulphate (12%) residues, together with peptide (13%), D-galactose (26%), 6-deoxygalactose (fucose, 4.3%), 2-amino-2-deoxyglucose (17.9%), and 2-amino-2-deoxygalactose (7.7%). Part of the D-galactose is present as D-galactofuranose residues, both in terminal, non-reducing positions and as internal residues in carbohydrate chains[2]. Most of the fucose is present as terminal, non-reducing residues, and the carbohydrate–peptide linkage regions in the antigen comprise alkali-labile, glycosidic linkages between serine and threonine residues and 2-acetamido-2-deoxygalactose residues[3], with the latter also substituted by oligosaccharides at O-3 and O-6. The present paper reports further structural studies of the antigen by using the techniques of periodate oxidation and Smith degradation. In view of the relatively small amounts of antigen that were available for structural studies, these techniques were evaluated by using 50 μg of methyl α-L-fucopyranoside as a model compound. This was chosen by virtue of the presence in the antigen of terminal, non-reducing residues of fucose.

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MATERIALS AND METHODS

An apparently homogeneous preparation of chick, allantoic antigen was a generous gift from Dr. A. Harboe.

Smith degradation of methyl α-L-fucopyranoside. — In a typical experiment, methyl α-L-fucopyranoside (50 μg) was treated with 10mM sodium metaperiodate (150 μl) at 4° in the dark. The consumption of periodate was measured spectrophotometrically[4] at intervals during 26 h, the reaction was then terminated by the addition of excess of ethylene glycol, and aliquots of the solution were assayed[5] for formic acid. In an essentially similar experiment, the consumption of periodate and production of formic acid were determined titrimetrically[6]. The solution that was obtained after reaction of methyl α-L-fucopyranoside (50 μg) with 10mM sodium metaperiodate for 10 h as described above was treated with barium carbonate until free from periodate and iodate ions[4]. The solution was centrifuged, and sodium borohydride (2 mg) was added to the supernatant during 3 h. The pH of the solution was maintained at 7–7.5 (pH meter) by using carbon dioxide. After 18 h at pH 7 and 4°, AG-50W resin (H^+ form) was added, and the solution was filtered and freeze-dried. Boric acid was removed by repeated distillation of methanol from the residue. An aliquot of an aqueous solution of the residue was tested for reducing groups[7]. Other aliquots were made 0.1M and 0.5M with respect to hydrochloric acid and were analysed for glycolaldehyde[8] and propane-1,2-diol[9] at intervals during 24 h at room temperature.

Separation of Smith degradation products. — (*a*) A column (105 × 0.8 cm) of Sephadex G-10 in water was calibrated by elution of aqueous solutions of chick, allantoic antigen, maltose, D-galactose, threitol, D-glyceraldehyde, glycerol, glycolaldehyde, and propane-1,2-diol. Eluates were scanned for carbohydrate[10], for reducing sugar[7], and, after periodate oxidation, for formaldehyde[8] and acetaldehyde[9].

(*b*) The trimethylsilyl (TMS) derivatives[11] of certain sugars and alditols were separated and determined quantitatively by using a temperature-programmed, Pye 104 gas-chromatograph and a column packing of 10% SE-30 on siliconised Celite (100–200 mesh, Pye Unicam Ltd.) at 70° for 20 min and then heated to 200° at 4°/min and maintained at that temperature for 30 min.

(*c*) AG-1 resin (200–400 mesh, sulphate form)[12] was equilibrated in 86% v/v ethanol and packed into a jacketed column (80 × 0.6 cm) that was maintained at 50°. The resin was eluted with degassed ethanol (86% v/v) at 0.3 ml/min for 3 h prior to use and was calibrated by elution (with that solvent at 0.3 ml/min) of a solution containing propane-1,2-diol (97 μg), glycerol (59 μg), and threitol (64 μg) in 86% v/v ethanol (0.7 ml). The eluate was split so that 60% was analysed by an automated version of the Nash[8] assay for formaldehyde, and the remainder was collected automatically in fractions (0.9 ml). In some experiments, a spectrofluorimetric assay[13] for formaldehyde was used.

Smith degradation of chick, allantoic antigen. — In a typical experiment, the antigen (25.3 mg) was treated with 10mM sodium metaperiodate (20 ml) at 4° in the dark, and the consumption of periodate and liberation of formic acid were determined

titrimetrically at intervals during 17 h. Aliquots were also assayed[8] for formaldehyde. After 17 h, excess of ethylene glycol was added to the solution which was dialysed successively against 0.1M sodium chloride and water at 4°. The non-diffusible product was treated with excess of sodium borohydride at neutral pH (carbon dioxide), and the product ("oxidized and reduced" antigen, 24 mg) was isolated by using the methods described for methyl α-L-fucopyranoside. The product was analysed[1] for component monosaccharides and amino acids, and for heterogeneity by elution from the calibrated column of Sephadex G-10.

Mild hydrolysis with acid of "oxidized and reduced" antigen. — "Oxidized and reduced" antigen (24 mg) was treated with 0.5M hydrochloric acid (0.5 ml) at room temperature for 20 h, and the hydrolysate was eluted with water from a column of Sephadex G-50 (136 × 1.3 cm). Fractions (2 ml) were collected automatically, and aliquots were analysed for carbohydrate[10] and for formaldehyde[8] and acetaldehyde[9] liberated on periodate oxidation. Fractions that contained the components of small molecular weight were combined, and an aliquot was analysed by chromatography on AG-1 resin (sulphate form). A subsequent preparation of "oxidized and reduced" antigen (760 μg) was hydrolysed with 0.5M hydrochloric acid (0.5 ml) as described above, and the hydrolysate was eluted with water from the calibrated column of Sephadex G-10. Fractions that were eluted in the region calibrated for glycolaldehyde and propane-1,2-diol were combined, and the components of an aliquot were converted into TMS derivatives and were analysed by g.l.c. The components of large molecular weight from the Sephadex G-50 column were refractionated by elution from Sephadex G-50 and Sephadex G-75. The three fractions (Fractions 1, 2, and 3) thus obtained, which were homogeneous on gel chromatography, were analysed for component monosaccharides and amino acids. An aliquot (150 μg) of Fraction 2 was investigated for heterogeneity by elution from a column (20 × 0.6 cm) of DEAE-Sephadex (chloride form) with Tris hydrochloride buffer (pH 7.0), followed by increasing concentrations of sodium chloride (0 to saturated) in Tris buffer. Fractions 1 and 2 were separately treated with 10mM sodium metaperiodate, and the consumption of periodate during 22 h was measured titrimetrically.

Hydrolysis of "oxidized and reduced" antigen with M sulphuric acid. — "Oxidized and reduced" antigen (1.14 mg) was hydrolysed with M sulphuric acid (1.5 ml) for 4 h at 100° under nitrogen in a sealed tube, and the neutralized [$Ba(OH)_2$] hydrolysate was eluted with water from the calibrated column of Sephadex G-10. In a control experiment, aliquots of a solution that contained erythritol (139 μg), threitol (156 μg), and propane-1,2-diol (212 μg) were eluted from the column before and after hydrolysis. The hydrolysate of "oxidized and reduced" antigen was recovered from the Sephadex G-10 eluate and was concentrated to dryness by rotary evaporation. One aliquot of the product was analysed by chromatography on AG-1 resin, and the components in a second aliquot were converted into TMS derivatives and analysed by g.l.c.

RESULTS

Smith degradation of methyl α-L-fucopyranoside. — Certain quantitative data

were obtained from a Smith degradation sequence with 50 μg of the model compound methyl α-L-fucopyranoside. Thus, the theoretical amount of periodate (2 moles/mole) was consumed during 3 h with the production of formic acid (0.95 mole/mole as measured by the method of Barker and Somers[5]). Slightly lower values were obtained from titrimetric measurement of formic acid. The "dialdehyde" that was obtained on periodate oxidation of methyl α-L-fucopyranoside was reduced with sodium borohydride at neutral pH, and the product alcohol gave a negative reaction for reducing groups with alkaline ferricyanide. This reaction could not be used, however, to follow the reduction of the product that was obtained by periodate oxidation of the antigen. Previous studies have shown that the antigen gives high values for reducing sugar, as measured by the reaction with alkaline ferricyanide, due to alkali-mediated cleavage of 2-acetamido-2-deoxygalactosyl-serine and -threonine linkages with the concomitant exposure of reducing sugar residues. The maximal amounts of propane-1,2-diol and glycolaldehyde that were detected in acid hydrolysates of the Smith alcohol obtained from methyl α-L-fucopyranoside were 47.5 and 24%, respectively, of the theoretical yields.

Smith degradation of chick, allantoic antigen. — The antigen consumed a maximum of 1.5 μmoles of periodate per mg after 6 h with the concomitant release of formic acid (0.24 μmole/mg) and formaldehyde (0.26 μmole/mg). Analytical data (Table I) for the "oxidized and reduced" antigen show that *ca.* 50% of the D-galactose

TABLE I

CHEMICAL ANALYSES OF CHICK, ALLANTOIC ANTIGEN BEFORE (*B*) AND AFTER (*A*) OXIDATION WITH PERIODATE

Component (%)	*B*	*A*	*Extent of oxidation by periodate (μmoles/mg of antigen)*
Fucose	4.3	0.7	0.22
D-Galactose	26.0	13.5	0.70
2-Amino-2-deoxyhexose	25.0	25.0	0
Peptide	13.0	13.0	0

residues and most of the fucose residues were destroyed on oxidation with periodate. The product that was obtained on mild hydrolysis of the "oxidized and reduced" antigen with acid was separated, by elution from Sephadex G-10, into products of small molecular weight, which liberated formaldehyde and acetaldehyde on periodate oxidation, and a fraction of large molecular weight, which had a similar component analysis to that of the intact antigen. The presence, in the components of small molecular weight, of propane-1,2-diol, corresponding to 62% of the fucose residues that were oxidised by periodate, and glycerol was confirmed by using g.l.c. and ion-exchange methods. Tetritols and monosaccharides were not detected, and glycolaldehyde was only tentatively identified. Elution of the hydrolysate from Sephadex G-50 indicated (Fig. 1) the presence of at least three products having relatively large molecular weights. These were subsequently isolated by an extensive series of refrac-

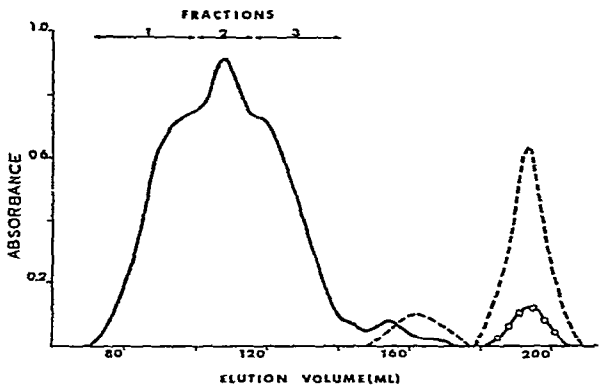

Fig. 1. Elution from Sephadex G-50 of "oxidized and reduced" antigen after hydrolysis with 0.5M hydrochloric acid at room temperature for 20 h. Fractions were assayed with cysteine and sulphuric acid (————), and for formaldehyde (– – – – –) and acetaldehyde (—o—o—) after periodate oxidation.

tionations with Sephadex G-50 and G-75 and were designated as Fractions 1, 2, and 3 which were homogeneous on gel chromatography on Sephadex G-50 and G-75. Fraction 2 was strongly adsorbed onto DEAE-Sephadex; it was not eluted from the ion-exchanger even with saturated salt solution. Component analyses indicated that Fraction 1 was similar to the "oxidized and reduced" antigen, with a high proportion of D-galactose and small proportions of 2-amino-2-deoxyhexose and peptide. Fraction 2, however, contained a high proportion of peptide and a relatively high proportion of 2-amino-2-deoxygalactose. Fraction 3 had a high proportion of D-galactose and relatively small proportions of peptide and 2-amino-2-deoxyhexose, especially 2-amino-2-deoxygalactose. No detectable consumption of periodate by Fractions 1 and 2 was noted, and insufficient amounts of Fractions 3 were available for treatment with periodate.

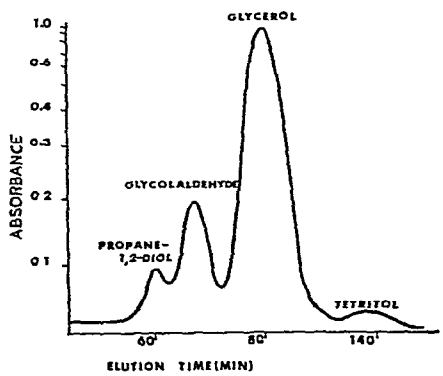

Fig. 2. Fractionation of hydrolysate (M sulphuric acid, 4 h, 100°) of "oxidized and reduced" antigen on AG-1 resin (sulphate form). Eluate analysed by an automated version of the Nash assay for formaldehyde liberated on periodate oxidation.

No material that was excluded on elution from Sephadex G-10 was obtained following hydrolysis of the "oxidized and reduced" antigen with M sulphuric acid at 100° for 4 h. Application of this sequence of hydrolysis and fractionation to a standard solution of propane-1,2-diol, threitol, and erythritol showed that 98% of the propane-1,2-diol and 76% of the tetritols were recovered. Fractionation and analysis by the g.l.c. and ion-exchange resin methods showed (Fig. 2) the presence of glycerol (0.49 μmole/mg of "oxidized and reduced" antigen), propane-1,2-diol, glycolaldehyde, and trace amounts of threitol in the M acid hydrolysate of the "oxidized and reduced" antigen.

DISCUSSION

The preliminary experiments showed that it was possible to measure quantitatively the consumption of sodium metaperiodate and the production of formic acid that occur during the periodate oxidation of a 50-μg sample of methyl α-L-fucopyranoside. Propane-1,2-diol was detected, but not in quantitative yield, on hydrolysis of the "oxidized and reduced" glycoside. Manipulative losses are probably the main cause of the low yield of propane-1,2-diol.

Fucose and D-galactose were the only monosaccharides in the antigen that were oxidized by sodium metaperiodate. The presence of propane-1,2-diol in acid hydrolysates of the "oxidized and reduced" antigen is diagnostic of (1→2)-linked and/or terminal, non-reducing fucose residues. Previous studies, however, showed that approximately 80% of the fucose residues in the antigen are very labile to acid, and the consumption of periodate, liberation of formic acid, and destruction of *ca.* 80% of the fucose residues in the antigen support the view that these are terminal, non-reducing, rather than (1→2)-linked, residues.

The production of 1 mole of formic acid per mole of fucose that was oxidized would account for *ca.* 92% of the formic acid that was measured and thus excludes the presence of significant proportions of unsubstituted, terminal, non-reducing or (1→6)-linked D-galactopyranose residues in the antigen. The production of formaldehyde, equivalent to 4.7% of D-galactofuranose residues in the antigen, is consistent with our earlier report that D-galactose (4.5% of the antigen) is liberated on mild hydrolysis with acid. The majority of the remainder of the D-galactose residues that are oxidized by periodate would consume one mole of periodate per mole of sugar. Assuming that the fucose and D-galactofuranose residues consumed 2 moles of periodate per mole, the total consumption of periodate by the antigen would be 1.4 μmoles per mg. The presence of 0.9% of unsubstituted, non-reducing, terminal D-galactopyranose residues would increase this figure to the observed 1.5 μmoles per mg with a corresponding increase in the amount of formic acid that was produced.

Approximately 50% of the D-galactose residues in the antigen must be substituted at O-3 or at O-2 and O-4, or are physically inaccessible to the periodate ion. The absence of significant proportions of threitol in hydrolysates of the "oxidised and reduced" antigen indicates that few of the oxidized D-galactose residues are (1→4)-linked. The formation of at least three products of relatively high molecular

weight on mild hydrolysis of the "oxidized and reduced" antigen with acid shows, however, that some of the D-galactose residues must be substituted at O-6 and/or O-2. One such product of hydrolysis (Fraction 2) contains the periodate-resistant, carbohydrate–peptide linkage regions in which serine and threonine are linked glycosidically to 2-acetamido-2-deoxygalactose residues which are substituted at O-3 and O-6. Fraction 3 which contains a high proportion of D-galactose and relatively small proportions of 2-amino-2-deoxyhexose (especially 2-amino-2-deoxygalactose) and peptide comprises primarily an oligosaccharide(s) from the periodate-resistant portion of the carbohydrate regions of the antigen. The product of apparently highest molecular weight (Fraction 1) probably represents "oxidized and reduced" antigen that is undegraded or only partially hydrolysed.

This work, together with acid and alkaline degradation studies of the antigen, indicate that the antigen contains a peptide "backbone" in which the β-hydroxyl groups of most of the serine and threonine residues are linked to 2-acetamido-2-deoxygalactose residues that are substituted at O-3 and O-6 by oligosaccharides. The latter, in which all the 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose residues and some of the D-galactose residues resist oxidation by periodate, are terminated mainly or exclusively by fucose and D-galactofuranose residues and possibly, in a few cases, by D-galactopyranose residues. The carbohydrate chains also contain D-galactopyranose residues that are susceptible to oxidation by periodate, but are not (1→4)-linked, as well as a small proportion of D-galactofuranose residues. Spectroscopic evidence indicates the presence of axial, rather than equatorial, sulphate residues, but their precise location in the antigen is unknown. The relationship between the structure of the antigen and its immunological activity will be reported elsewhere.

ACKNOWLEDGMENTS

We thank Dr. A. Harboe and his colleagues of the National Institute of Public Health, Virus Department B, Oslo, Norway, for valuable discussions and provision of purified, chick, allantoic antigen; Professor M. Stacey, C.B.E, F.R.S., and Professor S. A. Barker for their interest; and the Science Research Council for financial support.

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