Enzymes That Destroy Blood Group Specificity

V. THE OLIGOSACCHARASE OF CLOSTRIDIUM PERFRINGENS*

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SUMMARY

Evidence is presented for the presence of an enzyme, α-N-acetylgalactosaminy trial glycosidase, in filtrates of C. perfringens. Action of this enzyme on hog submaxillary glycoprotein results in the release of a disaccharide, characterized as 3-O-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-galactose. The possibility that this oligosaccharide has resulted from transgalactosylation has been eliminated. Enzymatic treatment of several glycoproteins and blood group substances from miscellaneous sources also resulted in the production of this oligosaccharide. Since in the blood group substances N-acetylgalactosamine at the reducing end of the oligosaccharide chain is α-linked to the hydroxy amino acids of the protein core, we have here a very useful α-N-acetyl-D-galactosaminyl oligosaccharase for structural studies of the carbohydrate-protein linkage in the blood group substances and in glycoproteins.

In our investigations on the action of a crude preparation of α-N-acetylgalactosaminidase from C. perfringens on hog submaxillary glycoprotein with blood group A specificity (1), we noted the anticipated release of free N-acetylgalactosamine. The amount of the acyl amino sugar released was determined by a modification (2) of the Morgan-Elson reaction (3). Prolonged incubation of the enzyme with substrate resulted in the complete release of the N-acetylgalactosamine. However, the amount of color obtained in the colorimetric test was far in excess of the total amount of free N-acetylgalactosamine present in the molecule, as determined by the hexosamine content after complete acid hydrolysis (4). Indeed, the color obtained was even more than could be accounted for by the simultaneous release of N-glycolylmannosamine from the sialic acid, as a consequence of the action of sialidase and N-acetylneuraminic acid-aldolase. Both of these enzymes are known to be present in crude extracta of C. perfringens (5, 6).

Similar results were obtained when the A-H− and H-specific submaxillary glycoproteins from hogs were used (1). On the other hand, prolonged incubation of the enzyme with ovine submaxillary glycoprotein, or the neutral tetrasaccharide, 2-acetamido-α-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-D-galactose, gave almost the theoretically expected amount of N-acetylgalactosamine.

Studies to determine the nature of the “excess” Morgan-Elson positive-reacting material resulted in the identification in C. perfringens of an oligosaccharase that is capable of hydrolyzing the linkage of oligosaccharide chains to the polypeptide backbone of the glycoprotein. This report will present our data in support of the belief that the enzyme involved is an oligosaccharase, and not a galactosyltransferase, and that the reaction product formed is 3-O-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-galactose.

EXPERIMENTAL PROCEDURE

Materials

The sugars used as standards in this investigation were obtained from the following sources: N-acetylgalactosamine, N-acetylmannosamine, galactosamine hydrochloride, and N-glycolyl neuraminic acid (Sigma Chemical Co.), N-acetyl neuraminic acid (Koch-Light Laboratories), and L-fucose, lactose, and N-acetylgalactosamine (Pfanstiehl Laboratories, Inc.). N-Acetylglucosaminidase and galactosaminidase were prepared according to the procedure of Crimmin (8).

Hog submaxillary glycoproteins with A, H, or In (A-H−) reactivity were prepared as already described (9). Partially purified ovine submaxillary glycoprotein was also prepared as previously described (9). The other glycoproteins were prepared in this laboratory from the following sources: ovarian cyst fluid of known phenotype (4), hog stomach mucin (commercial pool), and individual hog stomachs of individual rhesus monkey intestine (10, 11). Glycoproteins isolated from Coccoidea (12) and bovine colostrum (13) were also used. The neutral tetrasaccharide, 2-acetamido-α-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-D-galactose was also used.

1 The abbreviations used are: In, hog submaxillary glycoprotein showing neither A nor H activity, that is, A-H−; ME+, Morgan-Elson positive-reacting material.

21 D. Aminoff, unpublished studies.
deoxy-n-galactitol, was isolated from the alkaline borohydride
degradation products (7) of the hog A-specific submaxillary
glycoprotein (13). A*-Oligosaccharide alditols were obtained
from A* hog submaxillary glycoprotein by alkaline borohydride
degradative cleavage (7). This represents a mixture of oligosac-
charides with the reducing N-acetylgalactosaminyl residue con-
vected to the corresponding alditol.

A commercial preparation of hyaluronidase (Wortington Bio-
chemical Co.) (14) was used as the source of β-D-galactosidase.
N-Glycolylmannosamine was obtained from Boehringer and
Soehne, Mannheim, Germany. The crude enzyme of enzymes
from C. perfringens was obtained as described elsewhere
(15). This preparation was further fractionated by the use of
ammonium sulfate at concentrations of between 50 and 52% saturat-
ation which was essentially free of N-acetylneuraminic acid-
aldolase (6), but still contained sialidase (5) and fucosidase (15).

Methods

Galactose was determined by the anthrone method (16), in
which both galactose and fucose have the same molecular extinc-
tion coefficients, and corrected for the total fucose content of the
specimen. Total fucose was determined after a 10-min heating
period (17). Total sialic acid was assayed by a modification (5)
of the Svennerholm procedure (18). The nitrogen contents of the
oligosaccharides were determined by a ninhydrin method after
digestion with sulfuric acid (19).

The enzymatic activity of filtrates was tested by previously
published procedures, as follows: fucosidase (15), sialidase (5),
N-acetylneuraminic acid-aldolase (6), β-galactosidase by a two-
step reaction involving the use of galactose dehydrogenase (20).

Oligosaccharides or glycopeptides were hydrolyzed with 0.5 N
HCl for 4 hours at 100° in sealed ampules (4) for qualitative
chromatographic and electrophoretic analysis as well as for quan-
titative analyses. Chromatography and electrophoresis of the
oligosaccharides and sugars were performed on Schleicher and
Schuell SS No. 559 green ribbon paper. Solvents used for ir-
frared chromatography were: Solvent 1, 1-butanol-
pyridine-water (6:4:3 v/v); Solvent 2, phenol saturated with
water. Paper electrophoresis was performed in 1% sodium
borate buffer, pH 9. The oligosaccharides and free sugars were
detected by the use of AgNO3-NaOH, while the hexosamines and
N-acetylgalactosamines were detected with Ehrlich reagents (21).

The total hexosamine content was determined by the Rondle
and Morgan modification (22) of the Elson-Morgan (23) reaction
on a neutralized hydrolysate of the glycoprotein or oligosac-
charide. N-Acetylhexosamines were determined by the Morgan-
Eison reaction (3) using the modification of Hansen (2) adapted
to a micro scale. Under these conditions for the determination,
N-acetylgalactosamine gives 224%, N-acetylglucosamine 123%,
and N-acetylgalactosamine 139% of the color given by an
equimolar amount of N-acetylgalactosamine.

Structural studies involved determination of the amount of
periodate reduced (24) at room temperature over a period of time
at pH 4.5, 0.1 M sodium acetate, in the dark. The amount of
formaldehyde released was determined by a chromotropic acid
procedure (25) and the amount of formic acid by the thiorbarbi-
tric acid method (26); the determinations were carried out simul-
taneously with the studies on periodate consumption. Values
reported represent the maximal amounts.

| Table I |Mobilities of products of incubation mixture and standard sugars in n-butanol-pyridine-water (6:4:3) relative to mobility of N-acetylgalactosamine (Kf) |
|---------|-------------------------------------------------------------------------------------------------|
| Substrate | Morgan Elson Kf |
|---------|-----------------|
| Hog A- and H-specific submaxillary glycopeptides |
| A | 0.36 |
| B | 0.40 |
| C | 1.00 |
| Sheep submaxillary glycoprotein |
| A | 0.38 |
| C | 1.00 |
| N-Acetylgalactosamine | 1.00 |
| N-Acetylmannosamine | 1.11 |
| N-Glycolylmannosamine | 1.10 |
| N-Acetylgalactosamine | 0.38 |
| N-Acetylneuraminic acid | 0.36 |
| Galactose | 0.26 |
| Fucose | 1.13 |

* Detectable with AgNO3 spray.

** Enzyme Assay

Incubation mixtures contained the following components: 50 μl of
substrate (containing 0.25 μmole of bound N-acetylgalac-
tosamine), 20 μl of the N-acetylgalactosamine acid-aldolase-free prepara-
tion from C. perfringens and 10 μl of 0.5 M sodium phosphate
buffer, pH 6.3, and 0.02% NaN3 (as preservative) in a final
volume of 125 μl. Aliquots of the incubation mixture were
removed at intervals and assayed for Morgan-Elson-positive
material. The amount of color produced is expressed as a per-
centage of that theoretically expected on the basis of the N-ac-
tetylgalactosamine content of the material. After 24 hours of
incubation at 37°, both hog A-* and H*-specific submaxillary
glycoproteins released "excess" ME+ material (180% and 205%,
respectively), whereas ovine submaxillary glycoprotein released
126% and the neutral tetrasaccharide from hog A*-specific
glycoprotein, only 88.5%.

In order to determine the nature of the components giving this
"excess" color in the Morgan-Elson reaction, 24-hour incubation
mixtures were treated with ethanol to a final concentration of
80% to stop the reaction and to precipitate the salts and de-
natured proteins. The supernatant solutions were concentrated
and, along with standard sugars, chromatographed in duplicate
for 6 hours in Solvent 1. The chromatograms were then de-
veloped for reducing sugars and ME+ material, respectively
(Table I). Both A* and H*-specific hog submaxillary glycope-
proteins gave rise to three ME+ reacting components which also
reacted as reducing sugars with the silver nitrate reagent. The
mobilities of Components A and C corresponded to N-glycolyl-
neuraminic acid and N-acetylgalactosamine. Sheep submaxil-
ary glycoprotein gave only two ME+ spots, corresponding to
N-acetylgalactosamine and N-acetylgalactosamine. Free galactose and fucose were also detected in these enzymatic
hydrolysates, when incubation mixtures containing hog submaxil-
ary glycopeptides were used. The unknown ME+ reacting material obtained from the hog submaxillary glycopeptides,
Component B, also reduced alkaline silver nitrate and had an
Rf (mobility of spot relative to the mobility of N-acetylgalactos-
mine taken as unity) of 0.52 to 0.59.

Isolation—For further characterization of Component B, 1 g of
the A*-specific hog submaxillary glycoprotein was enzymat-
ically hydrolyzed with 125 μl of 0.5 M sodium phosphate,
buffer, pH 6.3, and 0.02% NaN3 in a final volume of 125 μl.
The resulting mixture was incubated at 37°, both hog A* and
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3 A donation from Dr. M. M. Baig (see Reference 13).
4 J. Dietler, to be published.
cally hydrolyzed under the above conditions in a final volume of 116 ml for 24 hours at 37°. The reaction was stopped by heating in a boiling water bath, and the cooled solution was treated with absolute ethanol to a final concentration of 80%, cooled to -20° overnight, and centrifuged at 18,000 × g for 15 min. The supernatant was concentrated by vacuum distillation and applied to eight sheets (24 × 38 cm) of SS 889 green paper. The papers were irrigated with Solvent 1 for 6 hours.

Appropriate guide strips were cut on either side of the sheets and sprayed with Ehrlich reagent to locate the ME+ reacting material. The unknown component, with an Rf of 0.55 to 0.57, was then eluted from the appropriate area of the unstained sheets. The eluates from the eight sheets were pooled, concentrated in vacuo to a syrup, and further purified by passage through a column (2.5 × 38 cm) of Sephadex G-15 to remove a faintly yellow contaminant. The final yield of product was 170 μmoles.

**Purity**—The purity of the component with an Rf of 0.56 was established by obtaining only one spot on chromatography in Solvent 1, Rf 0.55, and in Solvent 2, Rf 0.855, and on elecrophoresis in 1% sodium borate buffer, pH 9. In all cases only one spot was obtained with the same Rf value with both developing agents, alkaline silver nitrate and Ehrlich reagent for N-acetylhexosamine.

**Composition**—Quantitative analysis of the purified unknown compound for component sugars indicated the absence of any sialic acid or fucose. Galactose and Morgan-Elson-positive material were present equivalent to 15.3 and 20.6 μmoles per ml, respectively. The total nitrogen content was 17 μmoles per ml. Acid hydrolysis followed by paper chromatography revealed the presence of galactose and galactosamine only. Quantitative analysis of the hydrolysis products indicated the presence of 15.3 μmoles per ml of galactose as determined by galactose dehydrogenase and by gas liquid chromatography (27), and 16.0 μmoles per ml of galactosamine as determined by the Elson-Morgan reaction and by the use of an amino acid analyzer (28); the total nitrogen content was 16.4 μmoles per ml. On the basis of these analytical data, we can conclude that the unknown compound is a disaccharide of galactose and galactosamine.

Hydrolysis of the oligosaccharide with β-galactosidase from bovine testes and with the β-galactosidase in commercial hyaluronidase preparations resulted in the release of galactose, as determined by galactose dehydrogenase, and in the release of N-acetylgalactosamine. The presence of these two component sugars was further established by chromatography; both the Rf values and reactivity with alkaline silver nitrate and Ehrlich reagents identified the sugars with the standards run in parallel.

**Structure of Oligosaccharides**

**Reduction of Oligosaccharide with NaBH₄**—The oligosaccharide (16 μmoles) was reduced with excess sodium borohydride (2700 μmoles) in a final volume of 2 ml in an ice bath for 18 hours and in the dark (25). The excess borohydride was then destroyed by titration with 4 N HCl to pH 6. The salt was removed by passage through a column (2.5 × 38 cm) of Dowex 50 (H⁺, 200 to 400 mesh) and eluted with 500 ml of water. The eluate was evaporated to dryness and the bovine acid removed as its methyl ester (29).

**Oxidation of Oligosaccharide with NaOCl**—The oligosaccharide (23 μmoles) was oxidized with NaOCl in an incubation mixture containing 2.5 ml of 0.1 M I₂ in 0.2 M KI and 0.9 ml of 5% Na₂CO₃ in a final volume of 10 ml, according to the analytical procedure of Macleod and Robison (30). The mixture was set aside in the dark at room temperature for 2 hours to completely oxidize the oligosaccharide. The solution was then acidified with 4 N acetic acid and the resulting I₂ was successively extracted with several aliquots of chloroform. The aqueous layer was then concentrated in vacuo and passed through a column (2.5 × 38 cm) of Sephadex G-15 to remove low molecular weight contaminants.

**Composition of Reduced and Oxidized Oligosaccharides**—The ability of the original oligosaccharide to give a positive Morgan-Elson reaction was completely abolished in the reduced and oxidized derivatives respectively, whereas the reactivity of galactose in the anthrone reaction was unimpaired. Hydrolysis of the reduced and oxidized oligosaccharides followed by chromatography revealed the presence of free galactose as determined by Rf and, in each case, a second compound that reacted with the alkaline silver nitrate but gave no reaction with the Ehrlich reagents, and corresponded to galactosaminomalt and galactosamnosidase, respectively.

**Oxidation with Periodate**—Oxidation of 1 μmole of oligosaccharide with excess periodate for 24 hours resulted in the consumption of 4.6 μmoles of periodate and the release of 1.08 μmoles of formaldehyde and 2.05 μmoles of formic acid.

**Tests for Possible Transglycosylation**

The disaccharide could arise as a consequence of transglycosylation since both galactose and N-acetylgalactosamine were detected in a 24-hour incubation mixture. The following experiments were therefore set up to eliminate that possibility.

1. **Incubation of Free Sugars with Enzymes from C. perfringens**—An artificial mixture of sugars was prepared. The proportions of the sugars in this mixture were as present in the A+ active hog submaxillary glycoprotein (4.4 μmoles of galactose, 2.2 μmoles of fucose, 4.1 μmoles of N-glucosieuraminic acid, and 7.75 μmoles of N-acetylgalactosamine). The mixture was incubated with the C. perfringens preparation under the conditions described for the preparation of the oligosaccharide. A similar incubation mixture was set up containing the same sugars and in the same proportions, with the exception of the replacement of N-acetylgalactosamine by β-p-nitrophenyl-N-acetylgalactosaminide. The two incubation mixtures were maintained at 37° for 24 hours. Aliquots taken at the beginning and end of the incubation period showed no change in the intensity of color obtained in the Morgan-Elson test. The reactions were stopped by the addition of alcohol. Chromatographic analysis of the 80% ethanol supernatants of both incubation mixtures in both solvents, 1 and 2, likewise gave no evidence of the presence of the oligosaccharide.

2. **Possible Transfer of Galactose from Oligosaccharides to N-Acetylglactosamine and β-p-Nitrophenyl-N-acetylglycosaminide**—Incubations were set up as in Section 1 above with (a) N-acetylgalactosamine, 2.5 μmoles, or (b) β-p-nitrophenyl-N-acetylgalactosaminide, 2.5 μmoles, as acceptors, and the following galactosylglycosides as potential donors of galactose: (a) lactose, 1.2 μmoles, (b) melibiose, 1.25 μmoles, (c) A⁺ hog submaxillary glycoprotein, 2.25 μmoles of bound galactose plus fucose, (d) A⁺ oligosaccharide alditol, 2.27 μmoles of bound galactose plus fucose.

The incubation products were chromatographed as in Section 1 above for the detection of disaccharide. No galactosyl-N-acetylgalactosaminide disaccharide was detected with lactose or melibiose as galactosyl donor even though both these oligosaccharides were hydrolyzed by the crude enzyme preparation. Some galactosyl-N-acetylgalactosaminide was detected with the oligosaccharide alditol mixture (d) but the intensity was less than that obtained with the intact A⁺ glycoprotein (c) (cf. Table III). Moreover, the amount of disaccharide formed was about the same for the detection of disaccharide. No galactosyl-N-acetylgalactosaminide was detected with lactose or melibiose as galactosyl donor even though both these oligosaccharides were hydrolyzed by the crude enzyme preparation. Some galactosyl-N-acetylgalactosaminide was detected with the oligosaccharide alditol mixture (d) but the intensity was less than that obtained with the intact A⁺ glycoprotein (c) (cf. Table III). Moreover, the amount of disaccharide formed was about the same for the detection of disaccharide. No galactosyl-N-acetylgalactosaminide was detected with lactose or melibiose as galactosyl donor even though both these oligosaccharides were hydrolyzed by the crude enzyme preparation. Some galactosyl-N-acetylgalactosaminide was detected with the oligosaccharide alditol mixture (d) but the intensity was less than that obtained with the intact A⁺ glycoprotein (c) (cf. Table III). Moreover, the amount of disaccharide formed was about the same for the detection of disaccharide.
when the A+ oligosaccharide alditols were incubated alone with the enzyme and without the addition of excess N-acetylglactosamine or p-nitrophenyl-N-acetylglactosamidase. These results would suggest an incomplete alkaline borohydride degradative cleavage of the A+ glycoprotein, rather than the possible synthesis of disaccharide by transglycosylation. In order, therefore, to further substantiate that there is no net synthesis of the galactosyl-N-acetylglactosaminidase by transglycosylation, the following experiments were carried out using radioactively labeled galactose and N-acetylglactosaminidase.

3. Incorporation Studies with Radioactive Galactose—Incubation mixtures of A+ hog submaxillary glycoprotein were set up with a *Clostridium perfringens* preparation in the following three ways: (a) 1.21 μmoles of galactose-bound A+ glycoprotein alone, (b) the same amount of glycoprotein with the addition of 0.15 μmole of uniformly labeled radioactive [14C]galactose, with a total count of 4 × 10⁴ cpm, (c) same as in b but with a further addition of 1.21 μmoles of nonradioactive galactose. After 24 hours of incubation at 37° the incubation mixtures were heated in a boiling water bath for 1 min and alcohol was added to 80% (v/v). The resulting supernatants were concentrated, aliquots were chromatographed beside suitable standards in Solvent 1 for 6 hours, and papers were well dried and cut into horizontal strips and counted in the Nuclear Chicago liquid scintillation counter using the toluene scintillation mixture (31). The results obtained are shown in Table II and indicate quite clearly that all of the radioactivity is recovered at the RF of galactose, and less than 1% of the total radioactivity was found trailing in the oligosaccharide region. The same was true if the chromatograms were stained with alkaline silver nitrate or Ehrlich reagents (Table IV).

Results with Other Glycoproteins

A number of glycoproteins from miscellaneous sources were tested for their reactivity with the enzyme. The glycoproteins were analyzed for their total hexosamine content after acid hydrolysis. Incubation mixtures were set up to contain the same amount of total hexosamine, 6.5 μmoles. After 24 hours at 37°, an aliquot was removed for the colorimetric determination of total N-acetylhexosamine released, a second aliquot was treated with ethanol (80%), and the supernatant concentrated and chromatographed in duplicate in Solvent 1 for 6 hours. The two chromatograms were developed with alkaline silver nitrate and Ehrlich reagents (Table IV).

**Table II**

| Distance from origin | a | b | c |
|----------------------|---|---|---|
| Oligosaccharide      |   |   |   |
| 10-11 cm             | 0 | 87 | 86 |
| 11-12 cm             | 0 | 136| 113|
| 12-13 cm             | 0 | 150| 137|
| 13-14 cm             | 0 | 127| 115|
| Galactose            |   |   |   |
| 14-15 cm             | 0 | 421| 226|
| 15-16 cm             | 0 | 4,908| 2,330|
| 16-17 cm             | 0 | 22,586| 15,968|
| 17-18 cm             | 0 | 25,835| 23,754|
| 18-19 cm             | 0 | 9,110| 12,127|
| 19-20 cm             | 0 | 820 | 1,504|
| Paper blank          | 0 | 17 | 18 |

**Table III**

| Distance from origin | Distance from origin |
|----------------------|----------------------|
| 10-11 cm (Oligo-     | 19-26 cm (N-Acetyl-   |
| saccharide)          | galactosamine)       |
| A+ hog submaxillary  | A+ hog submaxillary  |
| glycoprotein          | glycoprotein          |
| N-[1-14C]Acetylgalactosamine | [14C]GalNAc |
| 0 | 516 | 0 | 51,746|
| 2.53 | 2.25 |
| 2.43 | 412 |
| 2.18 | 50,229|
| Oligosaccharide alditols | A+ oligosaccharides |
| 0.61 | 1.30 |
| A+ oligosaccharides + [14C]GalNAc | 0.59 | 514 | 1.08 | 52,000 |

**Discussion**

It has been previously reported that *Clostridium welchii* excretes enzymes capable of destroying A, B, and O(H) activity (32). We have already purified and characterized the enzyme capable of destroying O(H) activity and shown it to be an α(1 → 2)-L-fucosidase (15). Turning our attention to the enzyme capable of destroying A activity, we had ventured to use the Morgan-Elson reaction to determine the free N-acetylglactosamine released. As has been discussed previously (33), it is important to develop a suitable chemical assay for the purification of these enzymes rather than to depend on the simple loss of blood group activity. For, as has been shown in the case of *Clostridium tertium*, the enzyme that destroys A activity turned out to be a deacetylase rather than an α-N-acetylglactosaminidase (34).

Using the Morgan-Elson test to follow the hydrolysis of the A substance, it soon became apparent that it did not run parallel...
with the extent of inactivation of blood group substance. Moreover, A- glycoprotein from hog submaxillary glands likewise gave rise to Morgan-Elson-positive material. Since C. perfringens is known to contain all of the glycosidases capable of splitting the oligosaccharide chains to the protein core, it was presumed that the ME+ material released from the A- hog submaxillary glycoproteins could arise as the last step in the sequential hydrolysis by the exoglycosidases.

Quantitative estimation of the total amount of ME+ material released on prolonged incubation indicated the production of excess color, calculated on the basis of the total galactosamine content of the glycoprotein. The excess color could be attributed to a number of causes: (a) possible presence of an epimerase in the enzyme preparation capable of converting N-acetylgalactosamine to another, more reactive, N-acetylgalactosamine, e.g. N-acetylgalactosamine, (b) formation of N-glycolylneuraminic acid from the N-glycolylnueraminic acid present in the glycoprotein; and/or (c) the formation of an unsaturated oligosaccharide by a dehydrase action, as has been reported in the degradation of hyaluronic acid by C. welchii extracts (35-37).

Use of an enzyme preparation free of sialidase or N-acetylgalactosaminidase-alcoholase, or the use of a substrate initially freed of sialic acid by treatment with pure sialidase still gave rise to excess ME+ material. The origin of the excess ME color could not therefore be attributed to the presence of sialic acid in the substrates.

Exploration of the other possibilities necessitated the separation of N-acetyl galactosamine from other ME+ reacting materials. This was readily achieved by paper chromatography. The successful separation of Component II (Table I) from free galactose and ME+ reacting material by paper chromatography enabled us to purify it readily. The product obtained was shown to be pure on rechromatography in Solvents 1 and 2 and on electrophoresis. Qualitative and quantitative analysis identified the material as a disaccharide composed of equimolar amounts of galactose and N-acetylgalactosamine.

The reactivity of the oligosaccharide in the ME test and the abolishment of that reactivity after reduction with sodium borohydride or oxidation with sodium hypoiodite identified the position of the N-acetylgalactosamine at the reducing end of the disaccharide. This was further confirmed by (a) the inability of the galactose to react with galactose dehydrogenase, (until it was released by acid or enzymatic hydrolysis) and (b) the stability of the galactose to reduction with borohydride and oxidation with hypoiodite.

The purified oligosaccharide, moreover, gave an excess color in the ME test, which is characteristic of three substituted N-acetylhexosamines (38, 39). The periodate consumed, formaldehyde and formic acid released are consonant with the structure galactosyl-(1→3)-N-acetylgalactosamine (found, 4.6:1.1:2.0, expected, 4:1:2). The nature of the anomeric linkage as β was established by its hydrolysis by β-galactosidase. Thus the structure of the ME+ reacting material is established as the disaccharide: 3-O-β-d-galactopyranosyl-2-acetamido-2-deoxy-d-galactose. This structure is uniquely found at the reducing end of the many oligosaccharide chains in hog A, H, and In submaxillary glycoproteins (7, 13).

The oligosaccharide could, of course, arise also as a result of transgalactosylation activity (40) of the galactosidases known to be present in the C. perfringens extracts (15, 41). The data provided readily exclude this possibility.

The isolation of this disaccharide from a number of glycoproteins that are both ABO blood group active and inactive, e.g. the glycoprotein from bovine colostrum and Collacalia mucin, indicates that the reducing end of many glycoproteins consists of this type of disaccharide. Indeed this disaccharide has been isolated from acid hydrolysates of A, B, H, and Le* (42, 43). Since it has been shown that the anoteric linkage between the reducing N-acetylgalactosamine and serine or threonine of the protein core is α (44), we can presume that this enzyme is an α-β-N-acetylgalactosaminyl oligosaccharase.

This is the first description of an oligosaccharide capable of breaking the protein-carbohydrate linkage in the undergraded glycoprotein to release a complete oligosaccharide. As such it should serve as a valuable tool in the elucidation of the structure of many glycoproteins of the blood group active type. The purification of the enzyme will be reported in a subsequent publication.

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