The Action of Neutral Hypochlorite on Epithelial Mucopolysaccharides

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1. The uptake of dilute neutral hypochlorite by epithelial mucopolysaccharides has been compared with that of proteins, polysaccharides, amino acids and sugars. The uptake has been shown to be related to the protein content of the mucopolysaccharides rather than their polysaccharide content. 2. The destruction of the components of epithelial mucopolysaccharides, certain sugars and polysaccharides after oxidation with dilute neutral hypochlorite at 0–4°C has been studied. Very little destruction of the sugar components occurred and in epithelial mucopolysaccharides the only amino acid destroyed specifically was arginine. 3. Oxidation of bovine submaxillary-gland mucoprotein and ovalbumin caused very little destruction of hexosamine and no detectable liberation of this residue as a free reducing group, indicating that the O-seryl galactosaminide and the N-acyl-glycosylamine linkages reported to be present in these compounds were relatively stable to hypochlorite. 4. Depolymerization of epithelial mucopolysaccharides by neutral hypochlorite has been studied by using gel-filtration columns and compared with the depolymerization of polysaccharides and proteins under similar conditions. The polysaccharides examined were relatively resistant to oxidation whereas the proteins were extensively broken down. It is inferred that the extensive depolymerization of epithelial mucopolysaccharides by hypochlorite is related to their protein content rather than their polysaccharide content. 5. Fractionation of the products of oxidation of epithelial mucopolysaccharides by column procedures has revealed that the relative proportions of components in all fractions were similar to those in the original material. 6. Though this study does not provide unequivocal evidence from which the overall structure of this type of epithelial mucopolysaccharide may be deduced, the balance of probabilities now appears to favour a long polypeptide chain to which a large number of oligosaccharide side chains are attached via O-seryl and O-threonyl glycosidic linkages. The results, however, are also consistent with an alternating sequence of short polysaccharide and polypeptide chains and further evidence is necessary before this structure can be ruled out.

Boyland (1946) found that bovine cervical mucus was rapidly dissolved by sodium hypochlorite at pH values between 7 and 11. Other oxidizing agents such as hydrogen peroxide, potassium permanganate, potassium peroxydisulphate and potassium peroxydicarbonate were also effective in solubilizing oestrous mucus, but hypochlorites and hypobromites were the most satisfactory solvents. These could rapidly solubilize the thicker pregnancy and dioestrous mucus as well as oestrous mucus under very mild conditions. These studies were carried out on the crude mucus secretion and no attempt was made to identify the structures being attacked by this oxidation. The object of the present work was to investigate the effect of neutral hypochlorite on mucopolysaccharides isolated from crude epithelial mucus and to attempt to identify the structures being attacked. In this way it was hoped that some information could be obtained about the overall structure of the mucopolysaccharides.

It became apparent at an early stage that the polypeptide was very reactive with dilute neutral hypochlorite, whereas sugars, even when a free reducing group was present, reacted very slowly. This appeared to open a line of investigation, since
it suggested that the amino acid residues might be destroyed preferentially, allowing isolation of the oligo- or poly-saccharide portion of the molecule. No technique that will permit this with the particular type of epithelial mucopolysaccharide under study has yet been evolved, these substances being characteristically resistant to proteolytic enzymes. Thus Pusztai & Morgan (1961) reported that, although the blood-group substances are attacked by pepsin and ficin to yield smaller fragments, these are still of quite high molecular weight and are not greatly reduced in nitrogen content compared with the starting material. We can confirm that treatment of cervical mucopolysaccharides with ficin or Pronase, even if repeated and prolonged, does not result in the isolation of fragments containing less than 5-5% (w/w) of nitrogen. In the event, however, our expectation of the preferential removal of peptide from epithelial mucopolysaccharides by neutral hypochlorite has not been realized and a possible reason for this failure is discussed.

**EXPERIMENTAL**

**Materials**

*Amino acids, peptides and proteins.* L-Arginine hydrochloride, L-glutamic acid, L-glycine and L-lysine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset. Glycylglycine, glycylglycyglycine and ovalbumin (2x crystallized) were supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Bovine serum albumin and haemoglobin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Gelatin was supplied by a local pharmacist. Poly-L-proline was purchased from Yeda, Rehovoth, Israel. Dr R. Aschaffenburg kindly gave us a sample of casein (total casein) and a-lactalbumin. N-Benzylglucine methyl ester was generously supplied by Dr H. Gutfreund. Dioxopiperazine was prepared according to the method of Schott, Larkin, Rockland & Dunn (1947).

*Carbohydrates.* D-Galactose, D-glucosamine hydrochloride, D-glucose, sucrose and potato starch were obtained from British Drug Houses Ltd. N-Acetyl-d-glucosamine, chondroitin sulphate and larch galactan were supplied by L. Light and Co. Ltd. Dextrins of known molecular-weight range and Sephadex G-25 (medium grade) were obtained from Pharmacia, Upsala, Sweden. For preparation of granulated agar columns Davis Standard Agar from Davis Gelatin Ltd., London, E.C. 3, was used. Samples of methyl α-d-glucopyranoside and methyl N-acetyl-d-glucosaminide were kindly supplied by Professor W. T. J. Morgan.

*Epithelial mucopolysaccharides.* Bovine cervical mucopolysaccharides were prepared by the method of Gibbons (1959) from pooled bovine cervical mucus plugs of pregnant animals and from pooled materials of animals at or near oestrus. The washed gels were dissolved in aqueous saturated CaCl2 soln.-ethanol (9:1, v/v) and precipitated between 48 and 62% (v/v) of ethanol. The precipitate was redissolved and reprecipitated between these ethanol concentrations two further times. The behaviour of the mucopolysaccharides from pregnant animals and animals at oestrus was very similar with regard to hypochlorite oxidisa-

**Methods**

**Analytical methods.** Nitrogen, hexosamine, hexose, fucose and amino acids were estimated as described by Gibbons (1959). Neuraminic acid was estimated by the thiobarbituric acid method of Warren (1959) after prior hydrolysis with 0.1N-H3SO4 at 80° for 1 hr. This hydrolysis period does not liberate the neuraminic acid quantitatively from all the mucopolysaccharides studied (Gibbons, 1963) but was used as the standard condition for comparative purposes. Further, the neuraminic acid in the cervical mucopolysaccharide is entirely N-glycolyl, whereas the standard analytical reference substance was a crystalline laboratory preparation of the N-acetyl compound. The former gives only 60% of the colour yield of the latter in the thiobarbituric acid reaction. To obtain a comparison of the relative amount of nitrogen present that was released as amino nitrogen in samples where only small amounts were available, hydrolysis in 6% HCl for 16 hr. followed by neutralization with 2% NaOH and ninhydrin estimation according to Moore & Stein (1954) were carried out. The N-acetylatedhexosamine reaction was carried out as described by Aminoff, Morgan & Watkins (1952). Glucose was determined with glucose oxidase according to Huggett & Nixon (1957) on samples that had been hydrolysed with 0.5N-HCl for 16 hr. at 100° and subsequently neutralized with N-NaOH.

**Determination of galactose by quantitative paper chromatography.** Solutions of the mucopolysaccharides (3 mg.) and mannose (1 mg.) in 6-2 ml. of 0.5N-HCl were hydrolysed for 16 hr. at 100°. These were then deionized by passing them through columns (10 cm. x 1.1 cm.) of Dowex 50 (200-400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) in the H+ form. The eluates off these were allowed to drop straight on to columns (14 cm. x 1.1 cm.) of Amberlite IR-4B (British Drug Houses Ltd.) in the acetate form. The first 10 ml. off these Amberlite IR-4B columns was discarded and the next 25 ml. was collected and freeze-dried. The residues were dissolved in 0-05 ml. of water, and the galactose was estimated by quantitative paper chromatography according to Wilson (1960) by using mannose as an
internal standard. Standard solutions of galactose and mannose were run simultaneously.

**Preparation and use of gel-filtration columns.** Granulated agar-gel columns (60 cm. x 2-4 cm.) were prepared and used as described by Gibbons & Roberts (1963), 3 ml. fractions being collected. When used for estimating the depolymerization of polysaccharides and mucopolysaccharides these were calibrated with dextran fractions of known molecular-weight ranges with 0-02 m-LiCl as eluting solvent and detecting the dextrans by means of the anthrone reagent (Yemm & Willis, 1954). During these calibration runs a small amount of phenylalanine (1 mg.) was mixed with the dextran solution to correct for slight differences in column performances during different runs. In this way the elution volumes of the different dextrans could be referred to the same elution volume of phenylalanine that was detected by measuring the extinction at 215 mμ in a 1 cm. cell. For the estimation of the depolymerization of proteins, however, calibration with a mixture of sucrose (9 mg.), α-lactalbumin (6 mg.), haemoglobin (3 mg.) and bovine serum albumin (10 mg.) with 0-3 m-NaCl for elution was used. The proteins were detected by measuring the extinction at 280 and 410 mμ in 1 cm. cells, whereas the sucrose was detected with the anthrone reagent.

Gel filtration on Sephadex G-25 columns (100 cm. x 3 cm.) was carried out with water for elution, the column being packed as recommended by Pharmacia in their brochure *Sephadex in Gel Filtration*. In the studies with gel filtration, flow rates corresponding to approx. 25 μl./hr. for a column 60 cm. x 2-4 cm. and approx. 50 μl./hr. for a column 110 cm. x 3-0 cm. were used.

**Paper chromatography.** This was carried out by the usual descending technique in (1) ethyl acetate–pyridine–water (2:1:2, by vol.), (2) butanol-1–pyridine–water (6:4:3, by vol.) or (3) 3-methylbutanol-1–pyridine–water–diethyl-amine (10:7:7:0-3, by vol.). Spray reagents used for detecting sugars were those described for the aniline hydrogen phthalate procedure of Partridge (1949) and the AgNO₃ method of Trevelyan, Procter & Harrison (1950). Amino acids were detected with a 0-2% solution of ninhydrin in butan-1-ol.

**Oxidations with hypochlorite.** The HClO was prepared by distillation of a suspension of bleaching powder (25g.) and boric acid (60g.) in water (750 ml.). The first 250 ml. of distillate was collected and adjusted to pH 7 with NaOH.

**Hypochlorite uptake.** Solutions of 0-06% amino acids and 0-3% sugars, polysaccharides and proteins were made up in 0-03 m-sodium phosphate buffer, pH 7. The uptake of hypochlorite by these materials was measured by mixing 1 vol. of the precooled (4°) solution of material under investigation with 2 vol. of precooled NaClO (approx. 0-05 n) and allowing them to stand at 0–4°. At intervals 1 ml. samples were withdrawn into 3 ml. of 10% (w/v) KI, 2 ml. of acetic acid was added and the I₂ liberated was titrated with 0-01 n-Na₂S₂O₃ with 1% (w/v) soluble starch as an indicator.

**Depolymerization of material.** The oxidations were carried out as described under 'Hypochlorite uptake' except that in some cases unbuffered solutions were used. In these cases it was found that, when dealing with substances that showed a low uptake of hypochlorite, the pH of the solution did not fall more than 0-5 pH unit during a 9 hr. oxidation. The destruction of components was determined by withdrawing samples at intervals, reducing the excess of hypochlorite by passing SO₂ through the solution until no colour was given with a starch–KI paper and then purging the solution with N₂ to remove excess of SO₂. Analyses were carried out on these solutions without further isolation.

Measurement of the extent of depolymerization was carried out on solutions in which the excess of hypochlorite had been reduced with SO₂ as above, then neutralized and freeze-dried. The residues (containing about 10 mg. of polysaccharide or mucopolysaccharide) were made up to 3-5 ml. water and examined on agar columns, elution being carried out with 0-02 m-LiCl. The eluates were analysed by measuring the extinction at 215 mμ and by the anthrone reaction (Yemm & Willis, 1954) on 1 ml. samples. Uronic acid determinations (Dische, 1947) were used to assay depolymerization of chondroitin sulphate, and the cocrino reaction (Syngle & Wood, 1958) was used for detecting the carbohydrate material in eluates containing ovalbumin. When the depolymerization of proteins was examined less material (about 5 mg.) was put on the columns and elution was carried out with 0-3 m-NaCl instead of 0-02 m-LiCl. The position of the peaks was determined by measuring the extinction at 280 mμ or at 215 mμ.

**Fractionation of hypochlorite oxidation products.** Epithelial mucopolysaccharides were oxidized with neutral hypochlorite under similar conditions to those described above. Reduction of the excess of hypochlorite at the termination of the oxidation was carried out in a number of different ways in an attempt to remove the inorganic ions from the low-molecular-weight material. The two most frequently used are given below.

(a) Oxidation was carried out with Ca(CIO)₃; to terminate the reaction the solution was acidified to pH 3 with acetic acid, freeze-dried, dissolved in water and treated with a few drops of 50% (w/v) CaI₂. The I₂ liberated was extracted with carbon tetrachloride; the aqueous layer was freeze-dried again and extracted with ethanol, and the ethanol-insoluble material was dissolved in water and fractionated on a 12% (w/w) agar column with 10% (v/v) ethanol for elution. This method was rather lengthy and gave large amounts of u.v.-absorbing material.

(b) It was finally concluded that the best procedure was reduction with SO₂ as described above in the section on depolymerization. The solution after freeze-drying and dissolving in water was fractionated on a 12% (w/w) agar column with 0-02 m-LiCl for elution. After analysis of the eluates at 215 mμ and by the anthrone reaction, tubes constituting part of the same peak were combined and freeze-dried. The fractions that were free of inorganic ions except for LiCl were then extracted with ethanol, dissolved in a small amount of water and freeze-dried again. The residues finally obtained were dried, weighed and analysed. Where other inorganic ions were present the residues were made up to a known volume in a standard flask and samples of this were taken for analyses. In this way the absolute amounts of each component present in the fraction could be obtained.

To fractionate further the smaller fragments that contained inorganic ions the material was put on a Sephadex G-25 column (110 cm. x 3 cm.) and eluted with water saturated with chloroform.

**Deionization of material on ion-exchange columns.** Before paper chromatography, solutions of galactose (2 ml.) that had been oxidized with hypochlorite and reduced with SO₂,
as described above in the section on destruction of materials, were deionized as described above in the section on determination of galactose by quantitative paper chromatography.

Reduction of the oxidation products of bovine submaxillary-gland mucoprotein and ovalbumin with sodium borohydride. Solutions of 0.1% bovine submaxillary-gland mucoprotein and 0.2% ovalbumin in 0.07M-sodium phosphate buffer, pH7, were oxidized with 0.44N-NaClO at 0–4°. After 9 hr. 4 ml. samples were withdrawn, the excess of NaClO was reduced with SO2 and the samples were then purged with N2 to remove SO2. These solutions were treated with 0.25 ml. of NaHCO3 (40 mg./ml.) and 0.25 ml. of NaBH4 (40 mg./ml.) at room temperature for 16 hr. The excess of NaBH4 was then destroyed with 0.5 ml. of 8N-HCl and analyses were carried out on these solutions. As controls, solutions of bovine submaxillary-gland mucoprotein and ovalbumin were mixed with a suitable amount of NaClO that had been previously reduced with SO2 and purged with N2. The control solutions were then reduced with NaBH4 as above.

RESULTS

Hypochlorite uptake. The hypochlorite uptake by various amino acids and their derivatives at neutral pH and 0–4° is shown in Fig. 1. Although this is termed hypochlorite uptake, any chlorine bound up in chloramide formation would be released by acid and would be determined as hypochlorite. However, the conditions of the assay are such that chlorate and chlorite would not be determined (Chernyshev & Semenova, 1954), stronger acidic conditions being required to assay these. In this way hypochlorite used for oxidation rather than N-chlorination is determined. Fig. 1 demonstrates that the uptake of hypochlorite by free amino acids is very rapid, whereas amino acid derivatives with substituted amino and carboxyl groups such as dioxopiperazine and N-benzyglycine methyl ester do not show this initial rapid uptake. There is, however, with these substituted amino acids a substantial increase in hypochlorite uptake with time of oxidation. By comparison, uptake of hypochlorite by sugars under similar conditions, shown in Fig. 2, is very much less than that of amino

![Fig. 1. Amount of NaClO consumed (moles/mole) at pH 7 and 0–4° by dioxopiperazine (○), N-benzyglycine methyl ester (●), glycine (△), glycylglycine (●), arginine (□) and glycylglycylglycine (■) plotted against time.](image1)

![Fig. 2. Amount of NaClO consumed (moles/mole) at pH 7 and 0–4° by methyl α-N-acetyl-D-glucosaminide (△), galactose (○), N-acetylgalactosamine (●) and glucosamine hydrochloride (▲) plotted against time.](image2)

![Fig. 3. Amount of NaClO consumed (μmoles/mg.) at pH 7 and 0–4° by poly-L-proline (○), ovalbumin (●), bovine serum albumin (△) and gelatin (▲) plotted against time.](image3)
acids, galactose, N-acetylglucosamine and methyl α-N-acetyl-d-glucosaminide taking up negligible amounts even after 9 hr. Glucosamine hydrochloride does show a rapid uptake of hypochlorite, due, presumably, to oxidation of the free amino groups.

In Figs. 3 and 4 a comparison is made of the hypochlorite uptake at pH 7 by proteins, polysaccharides and mucopolysaccharides. Proteins such as bovine serum albumin, casein, gelatin and ovalbumin show a rapid large uptake of hypochlorite that increases with time of oxidation. Polysaccharides such as dextran 500 and potato starch, however, show very little consumption of hypochlorite under the conditions used. Chondroitin sulphate takes up slightly more hypochlorite, but this is still very much less than the amount taken up by the epithelial mucopolysaccharides such as bovine cervical mucopolysaccharide and hog gastric mucopolysaccharide. These show an uptake intermediate between that of proteins and the small uptake of polysaccharides. The amount of hypochlorite consumed appears to depend on their protein content, since bovine submaxillary-gland mucoprotein with its protein content of about 60% takes up a greater amount than the epithelial mucopolysaccharides of the blood-group-substance type, which contain 15–25% of amino acid residues.

Poly-L-proline takes up relatively small amounts of hypochlorite even after 9 hr. of oxidation.

**Table 1. Destruction of components of mucopolysaccharides and related materials by neutral sodium hypochlorite**

<table>
<thead>
<tr>
<th>Material under investigation (μg./ml.)</th>
<th>Time of oxidation (hr.)</th>
<th>Hexose by anthrone reaction</th>
<th>Hexosamine</th>
<th>Reducing sugar</th>
<th>Sialic acid</th>
<th>Amino nitrogen</th>
<th>Fucose</th>
<th>N-Acetylhexosamine</th>
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</thead>
<tbody>
<tr>
<td>Bovine cervical mucopolysaccharide (1000)</td>
<td>0</td>
<td>304</td>
<td>245</td>
<td>464</td>
<td>83</td>
<td>67</td>
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<td></td>
<td>9</td>
<td>298</td>
<td>237</td>
<td>450</td>
<td>78</td>
<td>65</td>
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<tr>
<td>Hog gastric mucopolysaccharide (1000)</td>
<td>0</td>
<td>304</td>
<td>222</td>
<td>287</td>
<td>518</td>
<td>50</td>
<td>32</td>
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<tr>
<td></td>
<td>9</td>
<td>293</td>
<td>226</td>
<td>249</td>
<td>540</td>
<td>48</td>
<td>92</td>
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<td>Galactose (113)</td>
<td>0</td>
<td>113</td>
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<td></td>
<td>3</td>
<td>109</td>
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<td></td>
<td>9</td>
<td>103</td>
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<tr>
<td>N-Acetylglucosamine (164)</td>
<td>0</td>
<td>104</td>
<td>----</td>
<td>----</td>
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<td>----</td>
<td>----</td>
<td>164</td>
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<td></td>
<td>1</td>
<td>101</td>
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<td>----</td>
<td>155</td>
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<tr>
<td></td>
<td>18</td>
<td>100</td>
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<td>----</td>
<td>140</td>
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<tr>
<td>Methyl α-N-acetyl-d-glucosaminide (186)</td>
<td>0</td>
<td>104</td>
<td>----</td>
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<tr>
<td></td>
<td>0-5</td>
<td>101</td>
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<tr>
<td></td>
<td>43</td>
<td>100</td>
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</tbody>
</table>
As determined by the anthrone reaction, oxidation of galactose for 9 hr at 0-4° caused only about 10% destruction of galactose. Deionization of a solution of galactose oxidized for 9 hr. followed by paper chromatography showed the presence of a strong galactose spot with only a faint trail of material behind the main spot. No other major component could be detected by the aniline hydrogen phthalate spray. Oxidation of N-acetyl-glucosamine with hypochlorite for 18 hr at 0° caused the destruction of about 15% of the material as determined by the N-acetylhexosamine reaction. Methyl \(\alpha\)-N-acetyl-\(\beta\)-glucosaminide is even more resistant to neutral hypochlorite, less than 5% of hexosamine being destroyed in up to 43 hr. oxidation. Similarly, oxidation of methyl \(\alpha\)-D-glucopyranoside, starch and dextran 500 for 9 hr. followed by estimation of the glucose content after hydrolysis with glucose oxidase showed no detectable destruction. When glucose was treated similarly and the amount remaining after 9 hr. of oxidation measured with glucose oxidase it was found that only 5% of the glucose had been destroyed. The destruction of components of hog gastric mucopolysaccharide and bovine cervical mucopolysaccharide also shown in Table 1 indicate that there is a general non-specific loss of small amounts of all the carbohydrate material. In hog gastric mucopolysaccharide there appears to be a greater destruction of hexosamine than galactose but in bovine cervical mucopolysaccharide the reverse occurs. However, it is doubtful whether this result is significant since these values are dependent on the difference in two analytical values and as such the small destruction observed is probably subject to an appreciable error. That the difference in anthrone values did give a true indication of the amount of galactose destroyed and that oxidation products were not causing any serious error was shown by quantitative paper chromatography. In the cervical mucopolysaccharide very little destruction of sialic acid had occurred, which was rather surprising in view of the labile nature of this component. The amino nitrogen content was lowered slightly after hypochlorite oxidation, but with bovine cervical mucopolysaccharide this decrease was only marginally greater than the experimental error. Hog gastric mucopolysaccharide after a 9 hr. hypochlorite oxidation did, however, show a significant decrease in amino nitrogen content. Comparison of amino acid analyses of the oxidized materials with those of the degraded substances by using the resin-column technique of Moore & Stein (1951, 1954) showed that only arginine was completely lost during the oxidation; further, this amino acid was completely destroyed after only 0.5 hr. oxidation. A general non-specific destruction of the amino acids must therefore have occurred since the relative amounts of the other amino acids was unchanged even after oxidation for 9 hr.

Oxidation of bovine submaxillary-gland mucoprotein and ovalbumin with hypochlorite followed by reduction of the oxidation products with sodium borohydride did not destroy any appreciable amount of hexosamine. This indicated that the linkage involving the hexosamine in these compounds was stable to hypochlorite.

**Depolymerization of macromolecules by hypochlorite.** Gel filtration on granulated agar columns was used to follow the depolymerization of a number of proteins, polysaccharides and mucopolysaccharides. To estimate the average molecular weight of the oxidized polysaccharides and mucopolysaccharides the agar columns were first calibrated with dextrans of known molecular-weight ranges. In this way it was found that a 12% (w/w) agar column completely excluded dextrans molecules with mol.wt. greater than 40000 but not those with mol.wt. smaller than 10000. A 7% (w/w) column excluded molecules with mol.wt. greater than 250000 but not those with mol.wt. 40000, whereas on a 4% (w/w) column even molecules with mol.wt. as large as 500000 were not excluded and molecules with mol.wt. as large as 10000 were completely included. As an illustration of the usefulness of these columns for approximate molecular-weight determination Fig. 5 shows a plot of peak volume against log(mol.wt.) for a series of dextrans on a 4% (w/w) agar column. When the depolymerization of proteins was studied the columns were calibrated with a set of proteins of known molecular weight, namely bovine serum albumin, haemoglobin, \(\alpha\)-lactalbumin; sucrose was put in to estimate the complete inclusion volume. Large errors could

![Plot of peak volume (ml.) against log(mol.wt.) for dextrans on a 4% (w/w) agar column (60 cm. x 2.4 cm.) with 0.02 M LiCl for elution.](image-url)
arise by attempting to estimate the depolymerization of proteins on a column calibrated with dextrans because of the difference in shape of the molecules, the proteins being more compact than the rather extended structures of the polysaccharides.

The progressive depolymerization of bovine cervical mucopolysaccharide by oxidation with neutral hypochlorite is shown by elution diagrams on a 7% (w/w) agar column in Fig. 6. Although the molecular weight of the starting material was about $4 \times 10^6$ (Gibbons & Glover, 1959), after oxidation for 9 hr. a large amount of the material had mol.wt. less than 40,000. Even after only 0·5 hr. of oxidation the average molecular weight had dropped appreciably. There is obviously a very rapid and extensive depolymerization of the molecule.

The depolymerization of dextran 500, chondroitin sulphate, ovalbumin, and serum albumin after oxidation for 9 hr. is shown in Fig. 7. Although dextran 500 and chondroitin sulphate have been

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**Fig. 6.** Gel filtration of bovine cervical mucopolysaccharide on a 7% (w/w) agar column. (a) Unoxidized material; (b) after oxidation with NaClO at pH 7 and 0–4° for 0·5 hr.; (c) after oxidation for 9 hr.

**Fig. 7.** Gel filtration of ovalbumin, bovine serum albumin, chondroitin sulphate, and dextran 500 before (○) and after (●) oxidation with neutral NaClO at 0–4° for 9 hr. (a) Ovalbumin on a 12% (w/w) agar column; (b) bovine serum albumin on a 12% (w/w) agar column; (c) chondroitin sulphate on a 4% (w/w) agar column; (d) dextran 500 on a 4% (w/w) agar column.
depolymerized to a small extent, the observed depolymerization is not comparable with the extensive depolymerization of ovalbumin and serum albumin under the same conditions.

**Fractionation and analysis of hypochlorite-oxidized fragments.** Attempted fractionation of hypochlorite-oxidized epithelial mucopolysaccharides by adsorption chromatography on charcoal and ion-exchange chromatography on DEAE-cellulose was unsuccessful, a large amount of the material becoming irreversibly adsorbed on the column media. However, fractionation according to size of the fragments on gel-filtration columns was possible, almost quantitative recoveries being obtained off agar columns. An elution diagram on 12% (w/w) agar of hog gastric mucopolysaccharide (455 mg.) oxidized for 9 hr. is shown in Fig. 8. The eluates were assayed by extinction at 215 mg. and the fractions combined as shown in Table 2. The first two combined fractions were freeze-dried, deionized by extracting the lithium chloride with ethanol and analysed. The third combined fraction, which contained large amounts of salt other than lithium chloride, was made up to a known volume in a standard flask and analyses were carried out on this. In this way the total amount of components in the fraction could be obtained. The analyses of these fractions are shown in Table 2. There is little difference in the ratios of the components in each fraction. The third fraction shows a higher anthrone/hexosamine ratio than the first two but this is probably due to the chloride present in this fraction interfering slightly with the anthrone reaction, causing it to give high results. There is no significant difference in the hexosamine/amino nitrogen ratio in the three fractions, indicating that separation of a protein-rich or carbohydrate-rich fragment has not occurred in this fractionation. The remainder of the third fraction was freeze-dried and fractionated on a Sephadex G-25 column with water for elution; the elution diagram obtained is shown in Fig. 9. Over half of the material was excluded from the Sephadex column, indicating that it was greater than 3000–4000 in mol. wt. Analyses of the fractions

![Fig. 8. Fractionation of hog gastric mucopolysaccharide (455 mg.) oxidized with neutral NaClO at 0–4°C for 9 hr. on a 12% (w/w) agar column (60 cm. x 2.4 cm.) with 0.02 M LiCl for elution.](image)

![Fig. 9. Fractionation of pooled fractions 54–77 off a 12% (w/w) agar column (see Fig. 8) of hog gastric mucopolysaccharide oxidized with neutral NaClO on a Sephadex G-25 column (110 cm. x 3 cm.) with water for elution.](image)

**Table 2. Analyses of fractions obtained by fractionating sodium hypochlorite-oxidized hog gastric mucopolysaccharide (455 mg.) on a 12% (w/w) agar column (60 cm. x 2.4 cm.) (see Fig. 8)**

<table>
<thead>
<tr>
<th>Amount of component (mg.)</th>
<th>Hexose by anthrone reaction</th>
<th>Hexosamine</th>
<th>Reducing sugar</th>
<th>Fuose</th>
<th>Amino nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>20–44</td>
<td>48.8 (0.82)</td>
<td>59.7 (1)</td>
<td>91-6 (1.50)</td>
<td>14.7 (0.27)</td>
</tr>
<tr>
<td></td>
<td>45–53</td>
<td>16.9 (0.98)</td>
<td>17.1 (1)</td>
<td>20-6 (1.67)</td>
<td>4.94 (0.31)</td>
</tr>
<tr>
<td></td>
<td>54–77</td>
<td>51.8 (1.45)</td>
<td>35.6 (1)</td>
<td>70-9 (1.95)</td>
<td>9-68 (0.29)</td>
</tr>
</tbody>
</table>
Table 3. Analyses of fractions of sodium hypochlorite-oxidized hog gastric mucopolysaccharide off a Sephadex G-25 column (see Fig. 9)

The values in parentheses are molar ratios, hexosamine being taken as unity.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Hexose by anthrone reaction</th>
<th>Hexosamine</th>
<th>Reducing sugar</th>
<th>Fucose</th>
<th>Amino nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>58-73</td>
<td>17.2 (0.96)</td>
<td>18.3 (1)</td>
<td>33.1 (1-8)</td>
<td>4.48  (0.27)</td>
<td>2.61 (1-9)</td>
</tr>
<tr>
<td>74-88</td>
<td>7.81 (0.98)</td>
<td>7.66 (1)</td>
<td>14.7 (1-9)</td>
<td>1.97  (0.28)</td>
<td>1.13 (1-9)</td>
</tr>
<tr>
<td>89-100</td>
<td>3.71 (1.1)</td>
<td>3.41 (1)</td>
<td>6.54 (1-9)</td>
<td>1.10  (0.35)</td>
<td>0.81 (3-1)</td>
</tr>
<tr>
<td>101-110</td>
<td>1.92 (1-2)</td>
<td>1.62 (1)</td>
<td>3.22 (2-0)</td>
<td>0.62  (0.42)</td>
<td>0.63 (5-0)</td>
</tr>
</tbody>
</table>

obtained are shown in Table 3. Here again there is little difference in anthrone/hexosamine ratio in the various fractions, but the amino nitrogen/hexosamine ratio does increase appreciably as the fragments become smaller. However, the total amount of these small fragments is very small, being less than 5% of the starting material. A partial separation of SO₄²⁻ and Cl⁻ ions occurred on this Sephadex column. There is some ionic material with a high u.v.-absorption travelling slower than the Cl⁻ ion, but this did not contain any sugar or nitrogenous material and was not examined any further.

Fractionation of bovine submaxillary-gland mucoprotein and bovine cervical mucopolysaccharide after hypochlorite oxidation on 12% (w/w) agar columns gave elution patterns similar to that in Fig. 9, but in these cases the second peak was smaller, probably owing to the starting material being a larger molecule.

Similar fractions were combined and analysed as for hog gastric mucopolysaccharide. With bovine cervical mucopolysaccharide there was little difference in the ratios of the components present in the first two fractions, which were similar to the starting material. In the third fraction the high salt concentration relative to the material being analysed made reliable analyses difficult and an apparent increase in anthrone and amino nitrogen was obtained. This was probably due to interference by salt, since the same fraction did not show such an increase when the products were isolated by a procedure designed to give very little salt, namely method (a) in the Methods section. When the products of oxidation of bovine submaxillary-gland mucoprotein were isolated by method (a) and fractionated on a 12% (w/w) agar column the hexosamine/amino nitrogen ratios did not vary very much in the three fractions, indicating that the hypochlorite had not attacked the linkage joining the disaccharide prosthetic groups to the main polypeptide chain, nor had it liberated appreciable amounts of an oligosaccharide-rich fragment.

DISCUSSION

It is rather surprising that sugars with free reducing groups are relatively stable to hypochlorite under the conditions used in this study, since one of the accepted methods of oxidizing a free reducing group to the acid is by means of hypohalites. However, these oxidations are usually carried out at a more extreme pH value, at a higher temperature and with hypobromite or hypoiode. The amount of ClO⁻ ion, dissolved chlorine and undissociated hypochlorous acid in hypochlorite solutions depends very markedly on the pH (Green, 1948), and these three oxidizing agents react differently. The stability of the glucosaminide linkage to neutral hypochlorite is evident from the lack of chlorine uptake by methyl α-N-acetyl-D-glucosaminide and the negligible destruction of glucosamine linked in this way. Free glucosamine, however, is rapidly oxidized, as might be expected, the attack presumably being at the free amino group, since N-acetylglucosamine is relatively stable. Whistler & Schweiger (1957) investigated the oxidation of amylopectin with hypochlorite at various pH values and found that attack was most rapid at pH 7. The main products of oxidation were glyoxylic acid and erythronic acid, indicating that cleavage of the carbon–carbon bond had occurred between C-2 and C-3. However, these experiments were carried out at 25° for long periods of time, and examination of the oxidation of glucose, methyl α-D-glucopyranoside, dextran and starch with neutral hypochlorite at 0–4° showed that very little destruction of glucose had occurred in 9 hr.

An examination of the hypochlorite uptake by amino acids and peptides has shown that, whereas a rapid uptake is observed in the presence of a free amino group, attack of a peptide bond is also possible. This is illustrated by the hypochlorite reduction by dioxopiperazine and N-benzyglycine methyl ester. Although papers have appeared on the oxidation of amino acids at various pH values,
little is known about the effect of neutral hypochlorite on the peptide bond. It is known that, under acidic conditions, amino acids and peptides give \( N \)-chloro derivatives, which are then further oxidized (Wright, 1936), and a number of authors have described the reaction of hypohalite with proteins, peptides and amino acids (Goldschmidt, Wiberg, Nagel & Martin, 1927; Herken & Schunk, 1949; Stankovic & Vasátko, 1960; McGregor & Carpenter, 1962). McGregor & Carpenter (1962) showed that at a weakly alkaline \( \text{pH} \) (9-4) the free amino group of peptides is oxidatively deaminated to give \( N-(\alpha\text{-oxoacyl}) \)-peptides, and that these are very readily cleaved on further oxidation with hydrogen peroxide.

Although a small non-specific destruction of the sugar components of epithelial mucopolysaccharides occurred this would be insufficient to explain the large uptake of hypochlorite. There was a small decrease in nitrogen released as amino nitrogen on hydrolysis and an amino acid analysis revealed that arginine had been completely destroyed. Arginine forms rather a small proportion of the total amino acids in the molecule, but this does rule out the possibility of an oligosaccharide side chain being attached to the guanidyl residue of arginine. Such a linkage has been suggested by Michela & Hulsmann (1960). The rapid disappearance of arginine is most probably due to the oxidation of the guanidine group and this need not imply scission of any peptide bond.

The hexosamine content of bovine submaxillary-gland mucoprotein is not substantially decreased by oxidation with neutral hypochlorite even after reducing the oxidation products with sodium borohydride. This indicates that sugars linked glycosidically to serine and threonine are not preferentially attacked by hypochlorite since most of the galactosamine in bovine submaxillary-gland mucoprotein has been shown to be linked in this way (Hashimoto, Tsuki, Nisizawa & Pigman, 1963; Anderson et al., 1964; Tanaka, Bertolini & Pigman, 1964; Tanaka & Pigman, 1965). The destruction of serine and threonine in bovine cervic al mucopolysaccharide under mild alkaline conditions (R. A. Gibbons & G. P. Roberts, unpublished work) suggests that similar linkages are present but here again little decrease in the hexosamine content was observed, and no sugar component was preferentially destroyed. The \( N \)-acylglucosylamine linkage present in ovalbumin (Johansen, Marshall & Neuberger, 1961; Nueneke & Cunningham, 1961) also appears to be resistant to attack by neutral hypochlorite since no liberation of oligosaccharide units from ovalbumin was observed. Further evidence of the stability of this linkage is provided by the lack of destruction of any appreciable amount of the hexosamine residues on reducing the oxidized products with sodium borohydride. The loss of viscous properties of cervical mucus during hypochlorite oxidation observed by Boyland (1946) may be attributed to the depolymerization of the mucopolysaccharide component present in the mucus. A very rapid and extensive depolymerization of this mucopolysaccharide occurs, the mol.wt. being decreased from \( 4 \times 10^5 \) to below 250000 in 30 min., whereas after 9 hr. the average mol.wt. is well below 40000 and a large amount of the material has mol.wt. less than 10000. Although polysaccharides and connective-tissue mucopolysaccharides such as dextran and chondroitin sulphate are depolymerized to a small extent by hypochlorite, it is clear that glycosidic, glycosiduronic and \( N \)-acyethylhexosaminidic linkages are attacked at a considerably lower rate than peptide bonds; in fact, the elution diagrams from suitably calibrated agar columns indicate that ovalbumin and serum albumin fragment approximately 5 and 6 times as rapidly respectively as dextran. Our gel-filtration data for chondroitin sulphate and cervical mucopolysaccharide do not allow deduction of the molecular weights of the depolymerized products of these samples since our only reference substances for these two materials were fractionated dextran samples. Nevertheless, it is justifiable to state that chondroitin sulphate breaks up slowly and cervical mucopolysaccharide quite rapidly in this respect the latter resembles protein rather more than polysaccharide. From this it can be inferred that the peptide part of the mucopolysaccharide is concerned with the integrity of the molecule, possibly as a long peptide chain with oligosaccharide prosthetic groups.

The relative unreactivity of methyl \( \alpha \)-\( N \)-acyl-D-glucosaminide and \( N \)-acytelyl- or \( N \)-glycolylneuraminic acid residues indicates that, although they readily form \( N \)-chloro derivatives, \( N \)-substituted amides in general do not react further with hypochlorite under the conditions we have used. It is suggested that, in an \( N \)-chlorinated protein, the presence of a peptide carbonyl in a \( \beta \)-position to the chloroamide nitrogen atom facilitates the elimination of hydrogen chloride with the formation of a labile acylimino group. This then splits with the formation of an \( \alpha \)-\( \text{oxoacetyl} \) structure and an amide, and the latter is then rapidly oxidized to nitrogen and a carboxylic residue. The former may also split to give a carboxylic acid and a formyl or carboxamino polypeptide. In either case, the amino end of the resultant split protein is protected against further rapid oxidation. This reaction scheme shown in Scheme 1 is an extension of that of McGregor & Carpenter (1962). The resistance of \( N \)-aspartoamidoglycoside to oxidation is explained by this reaction scheme. It was realized that, if this reaction scheme were correct, poly-\( \text{L} \)-proline
Scheme 1. Possible reaction scheme for the oxidation of the peptide linkage by neutral hypochlorite.

should be unattacked by neutral hypochlorite. When this was tested with synthetic poly-L-proline the hypochlorite uptake was found to be very much less than that of other peptides and proteins, thus providing further evidence in favour of this reaction sequence.

The destruction of one amino acid residue is probably therefore accompanied by the splitting of the peptide bond and the reduction of 2 or 2.5 mol. of chlorine/mol., whereas it seems likely that 0.5-1 mol./mol. would suffice for a polysaccharide (Whistler & Schweiger, 1957). This difference, together with the rapid oxidation of free amide and guanidine groups that occur in proteins, explains why the vast difference in hypochlorite uptake between proteins and polysaccharides is accompanied by a rather moderate difference in the rates of destruction of the component residues (Table 1).

The ready fragmentation of epithelial mucopolysaccharides by hypochlorite is consistent with a structure composed of a long peptide chain with oligosaccharide side chains. On hypochlorite oxidation the peptide chain would be attacked, producing small fragments that would be analytically similar to the intact mucopolysaccharide. The objection to the presence of a long polypeptide chain raised by Gibbons & Roberts (1963) has now been removed (G. P. Roberts, unpublished work), since it has been shown that proteins containing substituted hydroxyamino acids are extremely labile to alkali and are rapidly depolymerized by alkali. The presence of serine and threonine glycosides in epithelial mucopolysaccharides has now been established by several workers (Hashimoto et al. 1963; Anderson et al. 1964; Bhavanandan, Buddecke, Carubelli & Gotschalk, 1964; Harbon, Herman, Rossignol, Jollès & Clauser, 1964; Tanaka et al. 1964).

A structure composed of a long peptide chain with oligosaccharide prosthetic groups is not the only structure that would explain the observations in this paper. If it is accepted that peptide is concerned with the integrity of the molecule then a structure composed of alternating chains of peptide and sugars would also explain the experimental results presented above. Although short oligosaccharide prosthetic groups are considered possible, short polysaccharide chains alternating between short peptide chains are considered less likely because of the extremely large number of sugar-peptide links that would be required. In view of the stability of the glucosidic and glucosaminidic linkage to neutral hypochlorite, if a long polysaccharide chain existed in the molecule it would be relatively resistant to oxidation and it would be expected that such a chain could be isolated on prolonged oxidation. However, there was no increase in the polysaccharide content of the largest fragments isolated by gel filtration after prolonged oxidation, thus indicating that no long polysaccharide chain existed in the molecule. There is, however, a possibility that a small number of the relatively short oligosaccharide prosthetic groups may be linked to more than one peptide chain, thus giving a large network, but there is no evidence for or against this. In view of these possibilities and uncertainties, although a continuous polypeptide chain with numerous O-glycosidically linked oligosaccharide side chains seems likely, further investigation is required before an overall structure can be ascribed to the epithelial mucopolysaccharides with any degree of certainty.

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REFERENCES