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A Radiochemical Approach to the Determination of Carboxylic Acid Groups in Polysaccharides

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SUMMARY

A method for the routine radiochemical determination of carboxylic acid groups in polysaccharides is described. The procedure is based on sodium borotritide reduction of the product of the reaction between water-soluble carbodiimide derivatives and carboxyl groups on the polysaccharide. Carboxymethyl-cellulose and hyaluronic acid were used as test polysaccharides.

INTRODUCTION

Many different procedures have been described for the determination of carboxylic acid groups contributed by uronic acid residues in polysaccharides but none has been generally accepted. In this paper we report the development of a sensitive, radiochemical procedure suitable for routine use. The method is based on a procedure described by Taylor & Conrad (1972) for the determination of polysaccharide structure involving the reduction of modified carboxylic acid groups to their corresponding alcohols.

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Carboxylic acids are reduced by $LiAlH_4$ and other strong reductants (Brown & Krishnamurthy, 1979), but are not directly reduced by $NaBH_4$, which does, however, readily reduce carboxylic acid esters. Buchala & Wilkie (1973) proposed that carboxylic acid groups on polysaccharides could be quantitatively determined by reduction of their propyl esters with NaB^3H_4. Complete esterification required three, 7-day treatments with propylene oxide, after which the reduced polysaccharide was hydrolysed and the labelled sugars separated by paper chromatography prior to counting. Recently Fazio *et al.* (1982) proposed methyl esterification of uronic acid residues by a 24 h treatment with a methanol : chloroform : conc. HCl (10 : 1 : 1, by vol.) mixture followed by a 24 h reduction with NaB^2H_4. The reduced polysaccharide is then hydrolysed and the deuterated alditols identified and quantitated by g.c.–m.s. This method may not be satisfactory if acid-labile glycosidic linkages are present in the polysaccharide, for example those involving terminal arabinofuranosyl residues. The approach we have used is based on the observation that water-soluble carbodiimides react readily with carboxylic acid groups on proteins (Hoare & Koshland, 1967) and uronic acid-containing polysaccharides (Taylor & Conrad, 1972) to form an intermediate (Hoare & Koshland, 1967) that reacts readily with nucleophiles. Such intermediates, or the products of the reaction of the intermediate and a nucleophilic hydroxyl on a polysaccharide (Taylor & Conrad, 1972), are readily reduced by sodium borohydride. By including borotritide in reaction mixtures the glucuronic and iduronic acid contents of heparin have been determined quantitatively (Taylor *et al.*, 1973). The carbodiimide procedure has been used (Darvill *et al.*, 1978) to prepare deuterohexoses from hexuronic acids in pectic polysaccharides prior to their quantitative determination and identification of their alditol acetates by g.c.–m.s. (Darvill *et al.*, 1978) and to estimate the hexuronic acids in alginic acid after reduction to their corresponding hexoses (Vadas *et al.*, 1981).

The optimum pH for the reaction between carbodiimides and carboxyl groups is 4·75. However, during the reaction, protons are consumed so that the pH must be controlled to allow complete reaction. Taylor and coworkers (Taylor *et al.*, 1973) maintained the pH by titration with HCl in a pH-stat and as a consequence only one sample could be handled at a time. We have adapted the procedure for the simultaneous analysis of several samples by maintaining the pH during the carbodiimide reaction using buffers.

MATERIALS

Carboxylic acid containing polysaccharides

Carboxymethyl cellulose (CM-cellulose; Edifas 'B', a product of ICI Ltd, Stevenston, Ayrshire, Scotland) was used as the test material in developing the method. The degree of substitution was determined to be 0·54[1] carboxymethyl groups per monosaccharide residue by potentiometric back-titration following acid washing (Eyler *et al.*, 1947; Green, 1963). The second test polysaccharide was hyaluronic acid prepared from human umbilical cords (Laurent *et al.*, 1960).

Carbodiimides

1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, USA, or Sigma Chemical Co., St Louis, Missouri, USA. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl and 2-(*N*-morpholino)ethane sulphonic acid (MES) were from Sigma Chemical Co. and sodium borotritide (162 mCi/mM) was from New England Nuclear, Massachusetts, USA. Sodium borohydride and Tris buffer were commercial, analytical grades.

METHODS

Sample preparation

All polysaccharides were dried to constant weight prior to dissolution in water. In some cases 8 M urea was used as the solvent where the samples were partially water-soluble or became insoluble during the reaction.

It has been shown that 8 M urea does not interfere with the reaction between water-soluble carbodiimides and the carboxyl groups on proteins (Hoare & Koshland, 1967).

General procedure

The polysaccharide (containing up to 2·5 μmol carboxyl equivalents) in 250 μl of water or 8 M urea was mixed with 100 μl of pyridine–pyridinium chloride buffer (0·05 M, pH 4·75) or MES buffer (0·2 M, pH 4·75) and reacted with 1-cyclohexyl-3-(2-morpholinoethyl)carbo-

diimide (200 μl, 1·18 M) for 1 h at 30°C. The pH was then increased to 7 by the addition of Tris buffer (500 μl, 2 M, pH 7) which also kept the pH at this value during the reduction with labelled sodium borohydride (250 μl of 1·69 M $NaBH_4$ plus approximately 0·4 μmol of NaB^3H_4 (100 mCi/mM) in 0·05 M NaOH. The mixture was held at 30°C for 2 h.

When the reduction was complete the reaction mixture was dried onto four 2·2 cm discs of Whatman GFA glass fibre paper and successively and exhaustively washed with 90% v/v ethanol–0·1 M HCl, 80% ethanol and absolute methanol to remove labelled impurities and side products. The radioactivity on the dry disc was then counted in a scintillation vial using toluene scintillator (5 ml). Blank determinations were made omitting either carbodiimide, or both carbodiimide and polysaccharide.

RESULTS

Tritium incorporation was shown to be directly proportional to CM-cellulose concentration when the polysaccharide was dissolved in either urea or water (Figs 1 and 2) and to the concentration of hyaluronic acid in water (Fig. 3). Each data point represents the average of at least two determinations. Variation between determinations was always less than ± 5%, usually ± 3%, of the mean.

Selection of a suitable buffer for the carbodiimide reaction

Most compounds used as buffer ions in the pH 4·75 region contain carboxyl groups and are consequently unsuitable for use in these determinations. Pyridine–pyridinium chloride buffer ($pK_a = 5·17$) was selected for preliminary experiments. This buffer had been successfully used to maintain the pH at 4·75 during the carbodiimide reaction with the carboxyl groups on an enzymic protein (Moore & Stone, 1972). The extent of the carbodiimide reaction was markedly dependent on the concentration of the pyridine–pyridinium chloride buffer. The optimum buffer concentration was in the range 0·007–0·022 M. The reaction did not go to completion at lower buffer concentrations, presumably due to inadequate buffering capacity, and was inhibited at higher pyridine concentrations.

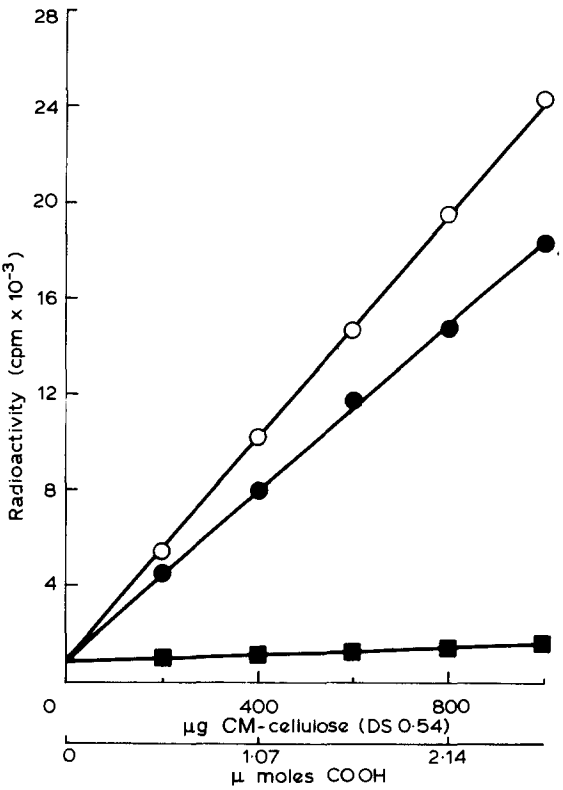

Fig. 1. Effect of MES (○—○) and pyridine-pyridinium chloride (●—●) buffers on the radioactivity incorporated in CM-cellulose by reduction of the carbodiimide complex. CM-cellulose samples (0-1 mg) were dissolved in 8 M urea (0·25 ml). Results of a control experiment in pyridine-pyridinium chloride buffer without carbodiimide are shown (■—■).

MES buffer was tested as an alternative to pyridine. Although the buffering capacity of MES ($pK_a = 6{\cdot}15$ at 20°C) at pH 4·75 is not as high as that of pyridine ($pK_a = 5{\cdot}17$ at 25°C) it could be used in higher concentrations without inhibiting the carbodiimide reaction (Table 1).

A slightly higher tritium incorporation was observed when MES rather than pyridine buffer was used under reaction conditions that were otherwise identical (Fig. 1 and Table 1). The reason for this is not clear.

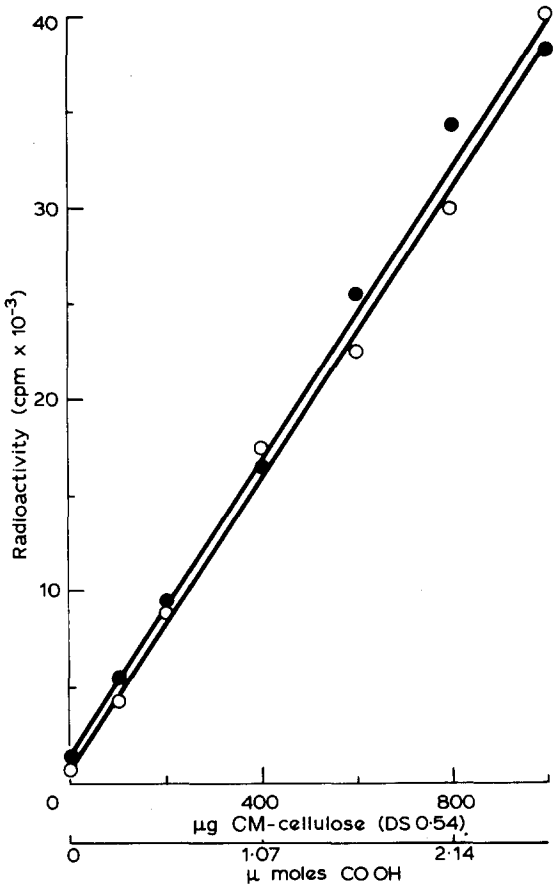

Fig. 2. Effect of sodium borohydride concentration on the incorporation of radioactivity into CM-cellulose by reduction of the carbodiimide complex. The carbodiimide reaction mixture contained CM-cellulose (0–0·25 ml, 4 mg/ml ≡ 2·68 µmol COOH), carbodiimide (CMCarb, 100 µl, 1·18 M) and pyridine–pyridinium chloride buffer (0·12 ml, 0·05 M, pH 4·75). After the carbodiimide reaction, Tris buffer (0·05 ml, 2 M, pH 7·0) was added to the reaction mixture and the products were reduced with either 250 or 500 µl of a sodium borohydride solution (1·69 M $NaBH_4$ plus 1·69 mM NaB^3H_4 in 0·05 M NaOH). All reaction mixtures were adjusted to the same volume by the addition of either water or 0·05 M NaOH. o—o, 0·422 M $NaBH_4$; ●—●, 0·845 M $NaBH_4$.

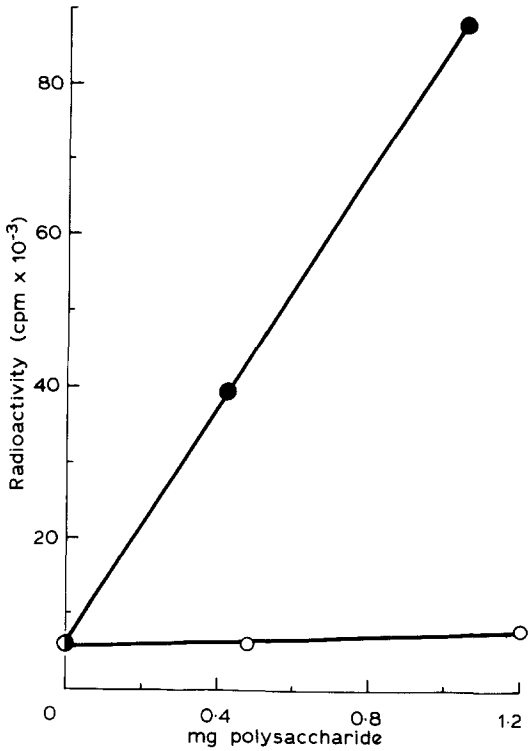

Fig. 3. Incorporation of radioactivity into hyaluronic acid (●—●) and barley β-glucan (○—○). The polysaccharides were dissolved in water and the pH was maintained at 4·75 during the carbodiimide reaction with pyridine–pyridinium chloride buffer.

Preparations of the carbodiimide solution

Taylor & Conrad (1972) found that both 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCarb) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (CMCarb) react quantitatively with carboxyl groups on polysaccharides. CMCarb was used in most of the current experiments and compared with EDCarb in others.

Carbodiimides are slowly hydrolysed in aqueous solutions and as a consequence are normally added to reaction mixtures in solid form

TABLE 1

Radioactivity Incorporated into CM-cellulose after Carbodiimide Reaction in either MES or Pyridine Buffers at Varying Concentrations

CM-cellulose (200 µl, 2 mg/ml) was mixed with 100 µl of each of the buffers to give the final concentration shown in the table and reacted with carbodiimide (CMCarb, 100 µl, 1·18 M).

Final concn of buffer in the carbodiimide reaction (M)	*pH after the carbodiimide reaction*	*Radioactivity incorporated (c.p.m. x 10^{-3})*
MES pH 4·75		
0·011	5·5	251 ± 2·8[b]
0·022	5·5	243 ± 3·3
0·11	5·0	252 ± 3·6
Pyr-PyrCl pH 4·75		
0·011	5·0	223 ± 0·4
0·022[a]	5·0	221 ± 2·3
Controls		
No carbodiimide		28 ± 7
No CM-cellulose		25 ± 4

[a] Above 0·022 M leads to inhibition.
[b] Percentage error.

(Hoare & Koshland, 1967). This is impractical when several samples are being handled at once. The carbodiimide was therefore dissolved in water immediately before use. However, the reaction was significantly reduced if aqueous solutions of the carbodiimide (CMCarb) were allowed to stand at room temperature for 1 h before addition to the polysaccharide, especially when the carbodiimide was present in limiting amounts. This effect was much more pronounced when the carbodiimide was dissolved in pyridine–pyridinium chloride buffer (Fig. 4), indicating that the pyridine either catalyses the hydrolysis of the carbodiimide or reacts with it in some other manner. This may explain the inhibition of the carbodiimide reaction at high pyridine concentrations.

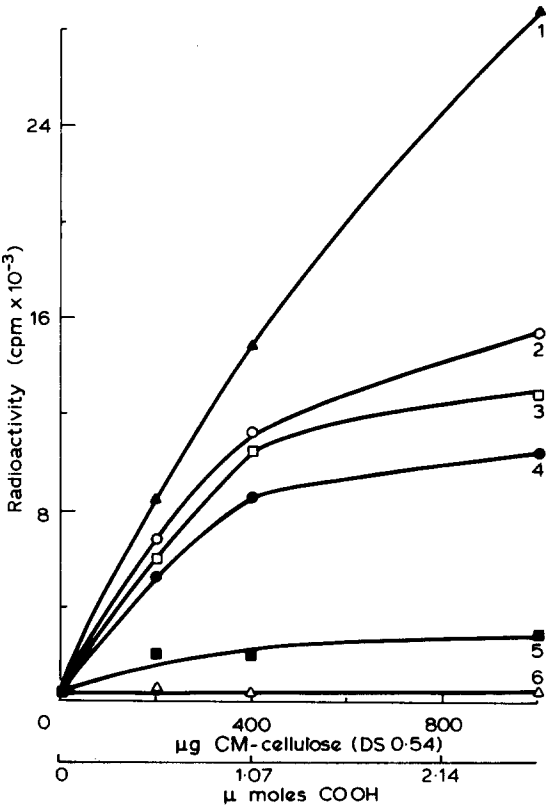

Fig. 4. Effect of mode of addition of carbodiimide on the incorporation of radioactivity into CM-cellulose. CM-cellulose (0–0·25 ml, 4 mg/ml ≡ 2·68 mol COOH) was mixed with pyridine-pyridinium chloride (0·1 M, 0·12 ml) followed by limiting amounts of carbodiimide (CMCarb) in one of the following ways: 1, 20 mg (47·3 µmol) solid carbodiimide (▲—▲); 2, 10 mg solid carbodiimide (○—○); 3, 10 mg carbodiimide in water (0·12 ml) used immediately after dissolution (□—□); 4, 10 mg carbodiimide in water (0·12 ml), stored 1 h, room temperature, before use (●—●); 5, 10 mg carbodiimide in Pyr-PyrCl buffer (0·12 ml, 0·1 M), stored 1 h, room temperature, before use (■—■); 6, no carbodiimide (△—△).

Optimum carbodiimide concentration

The effect of concentration of two carbodiimide derivatives on the completeness of the reaction is shown in Fig. 5. Addition of 200 µmol (85 mg) CMCarb allowed the reaction with 1 mg carboxymethyl cellulose (DS 0·54; equivalent to 2·68 µmol COOH) to go to completion in

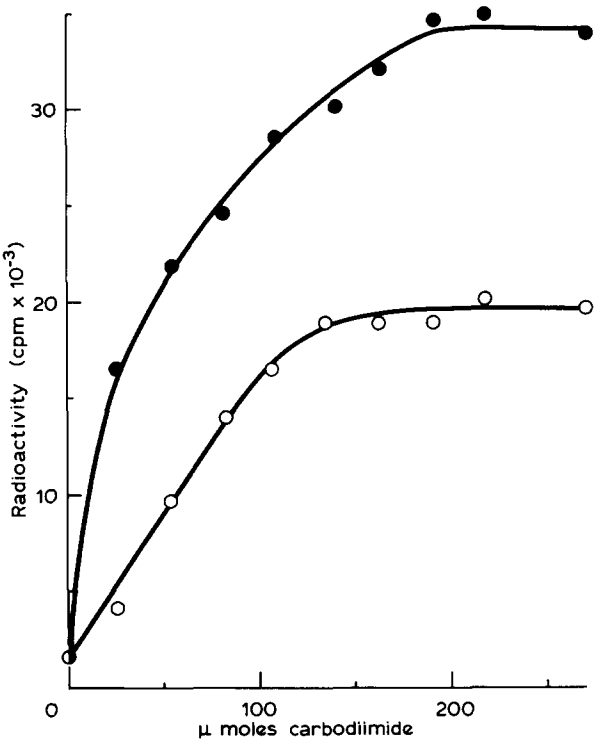

Fig. 5. Radioactivity incorporated into carboxymethyl-cellulose after reaction with differing amounts of water-soluble carbodiimide. Carboxymethyl-cellulose (250 μl, 4 mg/ml of H_2O) was mixed with pyridine-pyridinium chloride buffer (100 μl, 0·1 M, pH 4·75) and reacted for 1 h at 30°C with 0–270 μmol of either 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (0–114 mg; ●—●) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0–52 mg; ○—○) in 0·12 ml water.

a pyridine-pyridinium chloride buffer (0·05 M, pH 4·65) whereas 170 μmol (33 mg) of EDCarb was required for complete reaction under the same conditions although tritium incorporation was lower in this case. The reason for this difference is not clear.

Reduction of the carbodiimide polysaccharide derivative

Selection of a suitable buffer

When estimating the levels of iduronic acid and glucuronic acid in

heparin, Taylor *et al.* (1973) did not buffer the reaction mixture during the sodium borohydride reduction. However, in earlier experiments (Taylor & Conrad, 1972) it was stressed that a pH of 7 should be maintained during reduction to prevent alkaline hydrolysis of the carbodiimide-carboxyl product. The instability of this compound in alkali led to the suggestion that the product was an ester or lactone formed by the reaction of the *O*-acylisourea intermediate with a hydroxyl group on the polysaccharide (Taylor & Conrad, 1972).

In the current experiments the reaction mixture was buffered at pH 7. Either sodium phosphate (1 M, pH 7·0) or Tris buffers (2 M, pH 7·0) were used to buffer reaction mixtures. Tris was more suitable since, unlike the phosphate, it remained soluble during the reaction procedure and it was also readily soluble in the solvents used to wash the labelled side products from the discs.

The sodium borohydride–borotritide solution

Sodium borohydride (1·69 mmol) and sodium borotritide (1·6 μmol, 100 mCi/mM) were dissolved in a solution of sodium hydroxide (0·05 M, 1 ml). For the complete reduction of 1 mg of carbodiimide-modified carboxymethyl-cellulose (DS 0·54, equivalent to 2·68 μmol COOH), 0·42 mmol sodium borohydride was used. Doubling the amount of $NaBH_4$ did not lead to a higher tritium incorporation (Fig. 2); lower amounts were not tested. Frothing was controlled by the addition of a drop of *n*-octanol.

Removal of labelled side products

The carbodiimide, or the carbodiimide reaction products, were labelled substantially during the sodium borohydride reduction step and consequently had to be separated from the labelled polysaccharide prior to counting.

These labelled side products were charged and of low molecular weight and were removed by ion exchange on Bio-Rad AG501-X8 (H^+, HCO_3^- form) or by dialysis. The most convenient method for their removal, however, involved drying the reaction mixture onto glass fibre discs and selectively solubilising them by washing with suitable solvents.

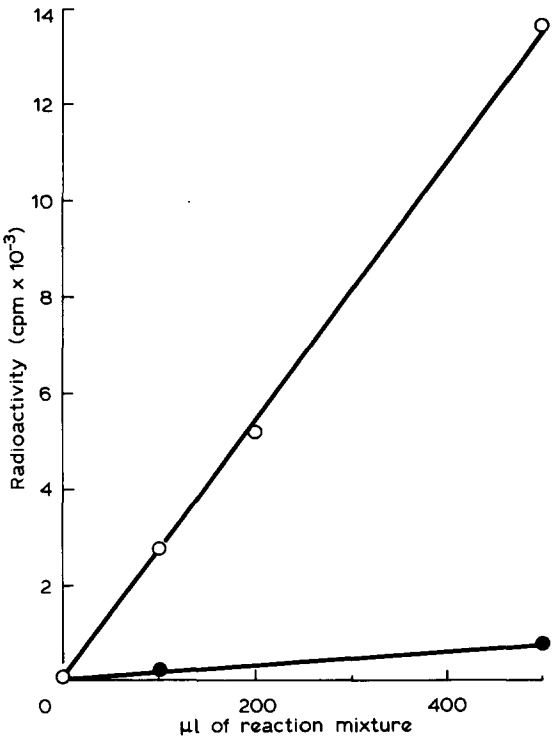

Fig. 6. Relationship between disc loading and the radioactivity retained on the discs after washing. CM-cellulose (0·25 ml, 4 mg/ml of H_2O 2·68 µmol COOH) was reacted with carbodiimide (CMCarb) and reduced according to the procedure described in the text. Aliquots of 100, 200 and 500 µl were then taken and loaded onto glass fibre discs (one disc per sample). The discs were washed and the incorporated radioactivity estimated as described in the text (○—○). A solution in which the CM-cellulose was replaced by water was treated in the same manner (●—●).

Disc loading

Best results were obtained when each reaction mixture was evenly distributed onto four discs of glass fibre paper (Whatman GFA 2·2 cm). The high concentrations of buffer salts led to difficulties when loading all the solution onto one disc.

In cases where the reaction mixtures were homogeneous and the final reaction volume was known accurately, smaller samples were taken and placed on one disc. In these cases the radioactivity due to tritium

incorporated into the polysaccharide was directly related to disc loading (Fig. 6).

Disc washing

Discs were placed in small plastic trays and washed by gentle shaking in 90% (v/v) ethanol–0·1 M HCl followed by 80% (v/v) ethanol and absolute methanol. Alternatively the discs could be washed on a Millipore manifold. The acid was added to the first wash to hydrolyse any undegraded borotritide (Richards & Whelan, 1973).

DISCUSSION

The direct relationship between tritium incorporation and polysaccharide concentration (Figs 1–3) indicates that this method is suitable for the quantitative determination of carboxyl groups in polysaccharides. A polymer with a known carboxylic acid content, such as CM-cellulose, should be used as a standard and all other samples compared with this. The method has great potential since it could be readily modified to detect picomoles of carboxyl groups by increasing the specific activity of the sodium borotritide reductant, providing that alcohol-insoluble contaminants in the borotritide were removed.

The carbodiimide reaction between carboxyl groups on soluble polysaccharides appears to be a general one applying equally to carboxyl groups not only on hyaluronic acid and CM-cellulose but also on heparin (Taylor & Conrad, 1972; Taylor *et al.*, 1973), pneumococcal S3 polysaccharide (Anderson & Stone, 1978), rhizobial polysaccharides (Anderson & Stone, 1978), and pectic polymers (Aspinall & Jiang, 1974; Darvill *et al.*, 1978).

Carboxyl groups on other polymers, e.g. proteins, will also be reduced so that knowledge of the purity of samples to be analysed is important. Where purity is unknown the reduced sample could be hydrolysed and the radioactivity in neutral monosaccharides determined after suitable fractionation.

In addition to reacting with the carbodiimide-carboxyl derivative and the carbodiimide itself, sodium borohydride will also react directly with aldehydes and ketones (including those at the reducing terminus of polysaccharides) and with carboxyl esters, e.g. pectin and methyl esters. If necessary, incorporation of tritium due to these reactions could be

avoided by treating the sample with unlabelled borohydride prior to the carbodiimide reaction. If the relative proportion of free uronic acids to esters or lactone forms is to be determined, the sample could be divided into two parts. Free uronic acids could be determined by treating one part with unlabelled $NaBH_4$ at pH 7 to reduce esters, lactones and reducing end groups prior to reaction with carbodiimide and sodium borotritide. Both free and esterified uronic acids would be determined by treatment of a second part with sodium hydroxide (0·5 M) for 30 min at 60°C to completely hydrolyse esters and lactones (Whistler & Feather, 1962); reducing end groups could then be selectively reduced with unlabelled $NaBH_4$ prior to the carbodiimide reaction.

Carbodiimide–borotritide reaction can be used not only to determine uronic acid carboxyls but also to identify the carboxyl-containing residues (Taylor & Conrad, 1972). The simplification of the carbodiimide method using buffered reagents in place of pH-stat control described in this paper allows the processing of large numbers of samples. By using sodium borodeuteride in place of sodium borotritide the method has been successfully used in conjunction with the improved alditol acetate (Blakeney *et al.*, 1983) and methylation procedures (Harris *et al.*, 1984) and g.c.–m.s. (Darvill *et al.*, 1978) to identify and quantify uronic acid residues in plant polysaccharides.

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