Use of Some New Poly-phenolic Resins for Fractionation of Carbohydrates and Immobilisation of Carbohydrate Hydrolases and Isomerases

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New matrices, exhibiting selective adsorption of polymeric carbohydrates, have been synthesised by copolymerisation of hydroquinone and other phenolic compounds with formaldehyde or glutaraldehyde. These gel-type resins are capable of separating high molecular weight polysaccharides from low molecular weight oligosaccharides and monosaccharides, and branched from unbranched polysaccharides. The formaldehyde–hydroquinone resin is also an effective matrix for the simple and rapid immobilisation of various enzymes and (other) proteins. It is therefore additionally suitable for the separation of proteins from low molecular weight peptides and amino-acids. α-Amylase, glucoamylase, and D-glucose isomerase immobilised on the resins retain their activity in continuous use. Since the resin matrices are highly porous and hydrophilic, they allow high flow rates to be achieved when used in packed-bed reactors. Both the formaldehyde and glutaraldehyde types of resin are cheap and easy to produce, relatively non-toxic in preparation and use, porous, hydrophilic, and extremely stable to changes in temperature and pH.

ENZYMES have been immobilised covalently on a number of solid supports [1] including cellulose *trans*-2,3-carbonate,[2-5] poly(allyl cyclic carbonate),[6,7] metal chelates of polysaccharides and Celite,[8] and aminobenzoic acid–formaldehyde resins on metals [9] and on glass.[10-12] All these methods involve the creation of specially reactive matrices prior to immobilisation of the protein. Simpler methods involving the direct adsorption of the protein on inorganic [10,13,14] and organic [15] supports have also been described. Hydrophilic supports have been shown to be most useful owing to the increased stability imparted to the enzyme on immobilisation.[1]

The affinity of aromatic compounds for carbohydrates has been widely discussed,[16-20] and aromatic resins have been used to adsorb proteins [21,22] and carbohydrates.[23] So far, however, little work has been reported on the concurrent immobilisation of both the enzyme and the substrate on the same support, but this would seem to be possible, with an amylase as enzyme and starch as substrate, on such aromatic resins. This dual adsorption in a packed-bed reactor should give rise to high conversion rates in the reactor eluate, provided that the reaction product is not adsorbed.

In general, the aromatic resins reported previously and used for enzyme and carbohydrate adsorption [22,23] are best used in continuous flow stirred-tank or fluidised bed reactors, since they show poor flow-rate characteristics in packed-bed (plug-flow) reactors owing to rapid reduction in flow rate with time. We were concerned with finding a porous hydrophilic aromatic gel that had good selective and direct adsorption characteristics for both proteins and polysaccharides but with no affinity for low molecular weight oligosaccharides or monosaccharides. The gel was also required to be capable of undergoing high flow rates in a packed-bed reactor, in order to reduce the bulk diffusion effect of transport of the substrate without the necessity of an inert support. In the choice of enzymes for this work, α-amylase, glucoamylase, and D-glucose isomerase were selected not only because they are amenable to immobilisation [11,24,25] but because these enzymes are very important in the preparation, from corn starch, of carbohydrate sweeteners as substitutes for sucrose. D-Glucose can be produced from the corn starch by the use of α-amylase and glucoamylase and, for increased sweetness,[26] converted into D-fructose by the D-glucose isomerase. Syrup production with the soluble enzymes uses large quantities of the enzymes and entails a complex ion-exchange and carbon refining system. The incubation times in batch isomerisations are greater than 24 h and this gives rise to high colour formation and poor product quality. If the enzymes could be immobilised by a low-cost process and used in a packed-bed reactor having a low pressure drop across the bed and high retention of the enzymic activity, the corn syrup could be converted directly into a mixture of largely D-glucose and D-fructose, conveniently, economically, and continuously in a purer form. Immobilisation of these enzymes, therefore, represents an important industrial application of a protein-immobilising resin.

This paper describes the preparation and use of resins which adsorb protein and macromolecular carbohydrate and which therefore have analytical, separative, and enzyme immobilisation properties. The characteristics of the above enzymes immobilised on these resins are also described.

EXPERIMENTAL AND RESULTS

D-Glucose and maltose were obtained from Hopkin and Williams Ltd., D-fructose, maltotriose, soluble starch, and bovine serum albumin from B.D.H. Ltd., dextran T40 ($\bar{M}_w$ 40 000) from Pharmacia Fine Chemicals AB, Uppsala, glycogen (from shellfish, type II) from Sigma Chemical Co. Ltd., amylose and amylopectin from potato (blue values 1.20 and 0.243, respectively), L-tyrosine, D-glucose 6-phosphate, and β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21, *ex* sweet almonds) from Koch–Light Ltd., glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3, Agidex liquid concentrate) from Glaxo Ltd., D-glucose isomerase (D-glucose ketol-isomerase, EC 5.3.1.18) from Novo Industri A/S, Copenhagen, and α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1, *ex Bacillus subtilis*) from Sigma Chemical Co., Ltd. Human pituitary follicle stimulating hormone (type CP1) was kindly donated by Dr. W. R. Butt of The Birmingham and Midland Hospital for Women.

Preparation of the Phenolic Resins.—Hydroquinone (11 g) was dissolved in boiling hydrochloric acid (35% w/v; 300 ml), and aqueous 38% w/v formaldehyde solution (40 ml) was added dropwise to the refluxing solution. The resultant brown gelatinous suspension was refluxed for 3 h before addition of cold distilled water (3 l). The product was filtered off and washed free from hydrochloric acid with cold distilled water. The gel (settled volume *ca.* 300 ml) could be dried by lyophilisation and would swell to its original volume on reconstitution with distilled water. In another preparation aqueous glutaraldehyde (25% w/v; 40 ml) replaced the aqueous formaldehyde solution.

The formaldehyde–hydroquinone and glutaraldehyde–hydroquinone gels, dried by means of a filter pump, contained 96 and 93% water, respectively. Their elemental analyses gave $C_{8.0}H_{7.3}Cl_{0.1}O_{3.0}$ and $C_{11.0}H_{11.1}Cl_{0.1}O_{3.0}$ for the formaldehyde and glutaraldehyde gels respectively. The i.r. spectra (KBr disc) of the resins are shown in Figure 1.

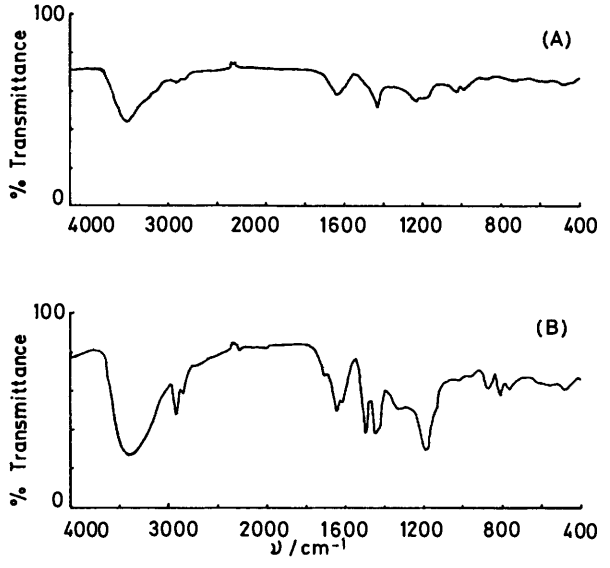


FIGURE 1 I.r. spectra of the formaldehyde–hydroquinone resin (A) and the glutaraldehyde–hydroquinone resin (B)

Various physical properties of the formaldehyde–hydroquinone resin were also noted or investigated. The resin was light brown/dark brown depending on the temperature at which the reaction was carried out (higher temperature ⟶ darker brown) and the time of the final refluxing stage (longer time ⟶ darker brown). On drying the colour becomes lighter. The resin had m.p. >150 °C. Its solubility in water, methanol, ethanol, benzene, and acetone was zero. In chromic acid there was slow oxidation but the compound was not appreciably soluble. The gel has a wet settled water content of *ca.* 96% and a water content of *ca.* 85—95% after drying at a filter pump. It could be further dried either by washing with acetone or alcohol and heating in an oven (*ca.* 140 °C), or by just drying in an oven (140 °C) where there is a fairly rapid loss of 80% of this water followed by a slower loss of the remainder. Drying in the oven, however, sometimes resulted in oxidation.

The product was an amorphous powder which could be ground very fine. A preparation in a granular form (*ca.* 1 mm diam.) could be formed by the use of 50% acetone–hydrochloric acid in place of hydrochloric acid solution. In general, the finer the particles the more efficient is the adsorption of starch but the higher the back-pressure in the column.

Variation of molar ratio of hydroquinone to formaldehyde was also investigated. When the reaction was performed with an initial excess of formaldehyde (>4 mol. equiv.) before the addition of hydroquinone, a generally lighter coloured (less polymerisation; more benzyl alcohol type product) material was obtained. When an excess of hydroquinone (>1 mol equiv.) was used a pale brown powder was formed which did not have great water retention power; some hydroquinone remained unchanged. A variety of other analogous polymers were prepared by replacement of the hydroquinone and/or formaldehyde/glutaraldehyde with other phenolic and aldehydic compounds respectively (as listed in Table 1).

Methods of Assay—(1) *Free* D-*glucose in* D-*glucose–starch mixtures.* D-Glucose was separated from the starch by molecular filtration chromatography on a column (30 × 1.4 cm i.d.) of AG-50W × 2 ion-exchange resin (BioRad; 200—400 mesh; Li^+ form) [27] at a flow rate of 1.36 ml min^{-1}. The resultant column eluates were analysed for carbohydrate by the automated L-cysteine–sulphuric acid assay [28] and the ratio of D-glucose to starch was calculated.

(2) *Reducing sugar.* Reducing sugar was assayed by a modification of the automated procedure [29] based on the method of Bernfeld,[30] measuring the chromophore absorption at 500 nm.

(3) D-*Fructose.* D-Fructose in D-fructose–D-glucose mixtures was determined by a new automated procedure based on the ease with which D-fructose forms a chromogen on dehydration in hydrochloric acid solution.[31]

(4) *Protein and amino-acids.* Protein and L-tyrosine were determined by u.v. absorption at 280 nm and reference to appropriate standard calibration curves. N^{α}-benzoyl-L-arginine ethyl ester was determined by u.v. absorption at 253 nm and reference to an appropriate standard calibration curve. L-Histidine and L-arginine were determined by a ninhydrin method as follows. Ninhydrin (60 g) and titanium(III) chloride solution (14.5% w/v; 13.0 ml) were dissolved in a mixture of 2-methoxyethanol (3 l) and 4M-sodium acetate buffer (pH 5.5; 2 l). Samples to be analysed (1 ml) were added to the reagent (5 ml), air being displaced with nitrogen. After heating at 100 °C for 10 min, absorbances were determined at 570 nm, quantitation being achieved by reference to a standard calibration curve.

(5) *Separation of oligosaccharides.* Starch solutions and partially hydrolysed starch solutions were analysed by gel chromatography on a BioGel P-2 column [32] (—400 mesh; BioRad; 1.5 m × 2.0 cm i.d.; 65 °C) with degassed distilled water as eluant (0.27 ml min^{-1}), the eluate being pumped and analysed on a modified JEOL JLC-6AH sugar analyser operating at a slower eluate flow rate (16 ml h^{-1}), with an orcinol–sulphuric acid assay for carbohydrate determination.[33]

(6) *Glucoamylase activity.* This was determined by hydrolysis of a starch solution [1% in 0.1M sodium acetate buffer (pH 4.8)] at 45 °C to D-glucose, which was determined by method (1).

(7) α-*Amylase activity.* This was determined from the increase in reducing sugar content of a starch solution [0.1% in 0.2M sodium acetate buffer (pH 5.8) containing 10^{-2}M-calcium chloride] at 40 °C, by method (2).

(8) D-*Glucose isomerase activity.* This was determined from the increase in D-fructose content of a 50% w/v D-glucose solution [0.1M sodium phosphate buffer (pH 6.8)

TABLE 1

Preparation and properties of phenol aldehyde resins

Phenol, *etc.*	Aldehyde, *etc.*	Acid	Properties *
Acetylated hydroquinone	Formaldehyde	35% HCl	1
2-Aminobenzoic acid	Formaldehyde	35% HCl	7, 8
3-Aminobenzoic acid	Formaldehyde	35% HCl	7, 8
4-Aminobenzoic acid	Formaldehyde	35% HCl	7, 8
2-Aminophenol	Formaldehyde	35% HCl	7
3-Aminophenol	Formaldehyde	35% HCl	7
4-Aminophenol	Formaldehyde	35% HCl	7
Anthracene	Formaldehyde	35% HCl	6
Anthracene	Formaldehyde	90% H_3PO_4	4
Benzaldehyde	Formaldehyde	35% HCl	4, 8
Catechol	Formaldehyde	35% HCl	1
Catechol	Formaldehyde	90% H_3PO_4	1
Catechol	Formaldehyde	50% H_2SO_4	1
Catechol	Formaldehyde	98% H_2SO_4	1
2-Cresol	Formaldehyde	35% HCl	4, 8
3-Cresol	Formaldehyde	35% HCl	4, 8
4-Cresol	Formaldehyde	35% HCl	4, 8
Hydroquinone	Acetaldehyde	35% HCl	3
Hydroquinone	Formaldehyde	Glacial AcOH	6
Hydroquinone	Formaldehyde	50% Me_2CO–50% HCl	1
Hydroquinone	Formaldehyde	50% HCl–50% H_3PO_4	1
Hydroquinone	Formaldehyde	90% H_3PO_4	1
Hydroquinone	Formaldehyde	50% H_2SO_4	1
Hydroquinone	Formaldehyde	98% H_2SO_4	1
Hydroquinone	Formic acid	35% HCl	6
Hydroquinone	Formaldehyde + urea	35% HCl	1
Hydroquinone	Furfuraldehyde	35% HCl	1
Hydroquinone	Glutaraldehyde	35% HCl	1
Hydroquinone	Glyoxal	35% HCl	1
Hydroquinone	Methanol	35% HCl	6
4-Hydroxybenzoic acid	Formaldehyde	50% Me_2CO–50% HCl	4
4-Hydroxybenzoic acid	Formaldehyde	35% HCl	6
4-Hydroxybenzoic acid	Formaldehyde	90% H_3PO_4	4
2-Naphthol	Formaldehyde	50% Me_2CO–50% HCl	5
2-Naphthol	Formaldehyde	35% HCl	6
2-Naphthol	Formaldehyde	90% H_3PO_4	3, 8
2-Naphthol	Formaldehyde	98% H_2SO_4	7
4-Nitrophenol	Formaldehyde	35% HCl	6
Phenol	Formaldehyde	35% HCl	4
Phenolphthalein	Formaldehyde	35% HCl	6
Phenolphthalein	Formaldehyde	98% H_2SO_4	4
Pyrogallol	Formaldehyde	35% HCl	2, 8
Resorcinol	Formaldehyde	35% HCl	2, 8
Resorcinol	Formaldehyde	90% H_3PO_4	1, 8
Resorcinol	Formaldehyde	50% H_2SO_4	1
Resorcinol	Formaldehyde	98% H_2SO_4	1
Thymol	Formaldehyde	35% HCl	3
Toluene	Formaldehyde	98% H_2SO_4	4
1,3,5-Trihydroxybenzene	Formaldehyde	35% HCl	2
Tyrosine	Formaldehyde	35% HCl	6
Tyrosine	Formaldehyde	90% H_3PO_4	6
Tyrosine	Formaldehyde	98% H_2SO_4	6
Vanillin	Formaldehyde	35% HCl	3
4-Xylene	Formaldehyde	98% H_2SO_4	4

* 1, Gel with high water content and with starch adsorption properties; 2, gel with low water content and with starch adsorption properties; 3, amorphous powder with low water content; 4, oily product; 5, gel with high water content; 6, no apparent reaction; 7, soluble product; 8, gave a product with high water content and starch adsorption properties if hydroquinone (1 mol. equiv.)was added to form a copolymer.

containing 10^{-2}M-calcium chloride and 10^{-3}M-cobalt(II) chloride] at 72 °C,[31] by method (3).

(9) β-D-*Glucosidase activity*. This was determined from the increase in 2-nitrophenol released from 2-nitrophenyl β-D-glucopyranoside solution [0.1% in 0.1M-sodium acetate buffer (pH 4.8)] at 37 °C.

General Adsorption/Fractionation Properties of the Phenolic Resins.—Columns (30 × 1.2 cm i.d.) containing a packed bed of the phenolic resin were washed with at least 30 column volumes of distilled water (0.614—1.36 ml min^{-1} at 20 °C) and then eluted with the buffer (or water) in which a sample was to be applied (>5 column volumes). The samples in their appropriate buffers were applied, elution with the buffer (or water) was re-commenced (0.614 ml min^{-1}), and the eluate was monitored continuously by the appropriate automated assay. The elution volumes and yields of various solutions on the columns are shown in Table 2.

Adsorption of Macromolecular Carbohydrates by the Resins.—The adsorption of starch by the formaldehyde–hydroquinone resin was studied in three ways. The dry resin (20 mg) was suspended in a solution of soluble starch (1% w/v; 10 ml) for 2 h at 20 °C. The gel was washed (10 × 10 ml of distilled water) and hydrolysed (2N trichloracetic acid; 3 h; 100 °C), and the resultant D-glucose was determined by the L-cysteine–sulphuric acid assay (38.0 mg ≡ 1.707 g per g of resin). To another solution of starch (1% w/v; 100 ml), dry resin was gradually added with stirring. The

TABLE 2

Elution volumes and yields of various materials from columns of aldehyde–hydroquinone resins

	Sample [a]	Amount applied (mg)	Elution volume (ml)	Yield (%)
Formaldehyde–hydroquinone resin (30 ml)	α-Amylase	10	∞	0 [b]
	β-Amylase	2	∞	0 [b]
	Amylopectin	1	∞	0
	Amylose	1	∞	0
	Bovine serum albumin	1	40	3
	Dextran T40	1	∞	0
	Follicle-stimulating hormone	1	∞	0
	D-Fructose	50	36	99
	Glucoamylase	3	36	15 [b]
	D-Glucose	1	36	102
	D-Glucose isomerase	100	∞	0 [b]
	D-Glucose 6-phosphate	1	34	104
	β-D-glucosidase	1	∞	0
	Glycogen	1	∞	0
	Maltose	1	36	95
	Maltotetraose through maltodecaose mixture [c]	1	36	100
	Maltotriose	1	36	103
	Partially hydrolysed starch	1	35	70
	Soluble starch	20	∞	0
	L-Tyrosine	1	35	94
Glutaraldehyde–hydroquinone resin (30 ml)	Amylopectin	1	33	1
	Amylose	1	33	85
	D-Glucose	1	33	101
	Maltose	1	33	98
	Maltotriose	1	33	100
	Soluble starch	1	33	17

[a] All samples were dissolved in, and eluted with, distilled water except the enzymes, which were used in their assay buffers. [b] Protein content; in all cases there was zero enzymic activity in the eluate. [c] Fractionated partial hydrolysate of starch.

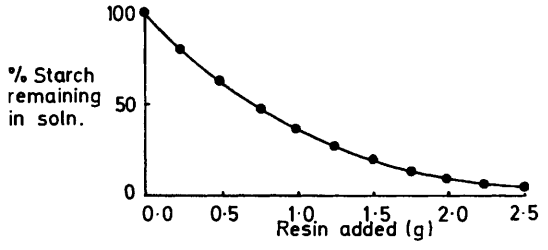


FIGURE 2 Adsorption profile of soluble starch on formaldehyde–hydroquinone resin

starch content of the supernatant was continuously monitored by the L-cysteine–sulphuric acid assay (Figure 2). A third starch solution (0.5 mg ml^{-1}) was pumped continuously (0.7 ml min^{-1}) through a column of the resin (25 ml) and the eluate was monitored in order to determine the maximum amount of starch that can be adsorbed on the resin without any appearing in the supernatant (0.154 g of starch per g of dry resin).

Once attached to the resin, the starch was very difficult to remove. Sustained elution with water, buffer solutions [0.1M-sodium acetate (pH 4.8 and 5.8) and 0.1M-sodium phosphate (pH 6.8)], acid solutions (1M hydrochloric acid), acidic and basic sugar-complexing solutions (2% w/v sodium borate, 20% w/v potassium borate, and 2.5% w/v sodium molybdate acidified to pH 3 with hydrochloric acid), phenol solution (1% w/v), and salt solutions (saturated sodium sulphate), all at 20 °C, did not liberate the immobilised starch. Hot water (50 °C) and 5N-hydrochloric acid gradually leached carbohydrate from the gel (<0.1 and <0.7% per ml of eluate, respectively) but all the carbohydrate could be removed (99.2% yield) as D-glucose by elution with a solution of glucoamylase [30 mg ml^{-1} in distilled water (0.1 ml)] at 20 °C followed by distilled water.

The adsorption by the resin of high molecular weight D-glucose oligomers from partially acid-hydrolysed starch solutions was investigated as follows. Partially hydrolysed starch (1 mg in 0.2 ml of water) was applied to a short column (1 × 1.2 cm i.d.) of formaldehyde–hydroquinone resin, and after a contact time of 1 min the column was eluted with water (4 ml). The combined eluate was collected and a sample (equivalent to 250 μg of original hydrolysate) was chromatographed on a column (50 × 1.2 cm i.d.) of AG-50W × 2 ion-exchange resin (BioRad; 200—400 mesh; Li^+ form) at a flow rate of 0.4 ml min^{-1}. The eluate was continuously monitored by the L-cysteine–sulphuric acid assay (Figure 3). The fractionation was repeated with a sample of partially hydrolysed starch (0.25 mg in 1 ml of water) which had not been treated with formaldehyde–hydroquinone resin (Figure 3).

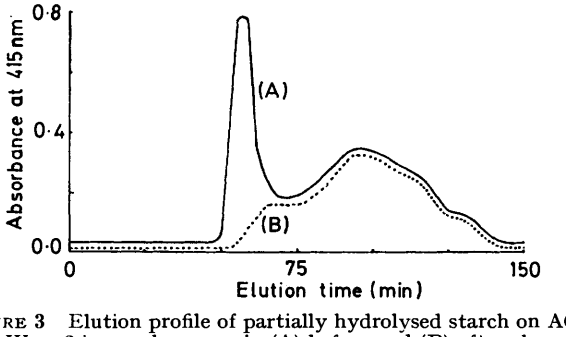


FIGURE 3 Elution profile of partially hydrolysed starch on AG–50 W × 2 ion exchange resin (A) before and (B) after chromatography on formaldehyde–hydroquinone resin; for clarity the baselines, *etc.*, have been separated

Partially hydrolysed starch (30 mg in 3 ml of water) was also applied to a column (25 × 1.2 cm i.d.) of formaldehyde–hydroquinone resin and after a contact time of 40 min the column was eluted with water. A sample (0.25 ml) of the combined eluate (first 30 ml) was chromatographed on a column (1.15 m × 2.0 cm i.d.) of BioGel P-2 (Figure 4). The yield of oligosaccharides (DP $\gtrsim 12 \equiv M$ 2 000) from the formaldehyde–hydroquinone resin, as determined from the BioGel P-2 column was 95%. Partially hydrolysed starch (0.25 mg in 1 ml of water) was also fractionated

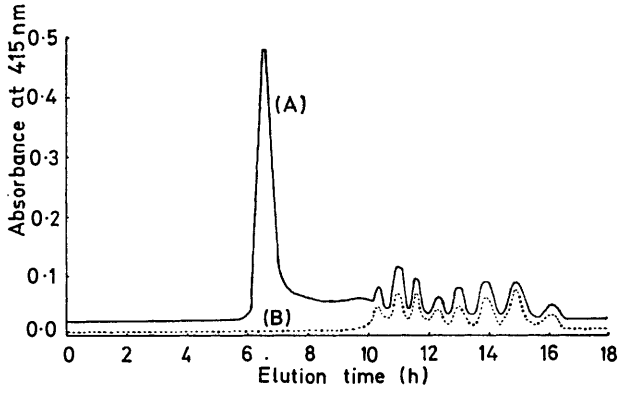


FIGURE 4 Elution profile of partially hydrolysed starch on BioGel P-2 (A) before and (B) after chromatography on formaldehyde–hydroquinone resin; for clarity the baselines, *etc.*, have been separated

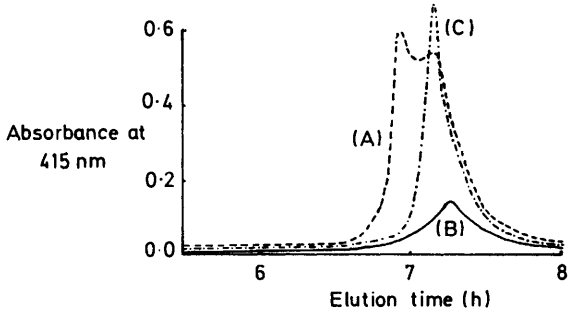


FIGURE 5 Elution profile of soluble starch on BioGel P-2 (A) before and (B) after chromatography on glutaradehyde–hydroquinone resin; the elution profile of amylose (C) is included for reference

directly (Figure 4). The experiment was repeated with soluble starch.

Soluble starch (30 mg in 30 ml of water) was applied to a column (25 × 1.2 cm i.d.) of glutaraldehyde–hydroquinone resin and after a contact time of 40 min the column was eluted with water. A sample (0.25 mol of the combined eluate (first 30 ml) was chromatographed on Bio-Gel P-2 as above (Figure 5). Soluble starch (0.25 mg in 1 mol of water) was also fractionated directly (Figure 5). A solution of amylose (1 mg in 1 ml of water) was similarly chromatographed (Figure 5).

Adsorption of Enzymes by the Resins.—A solution of glucoamylase (30 mg ml^{-1}; 1 ml) was applied to a column of formaldehyde–hydroquinone resin (30 × 1.2 cm i.d.) and the column was eluted continuously with unbuffered 0.2% w/v

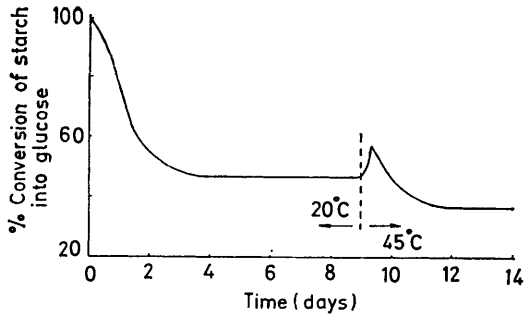


FIGURE 6 Variation with time in activity of a constantly operated column of glucoamylase, immobilised on formaldehyde–hydroquinone resin, at two temperatures

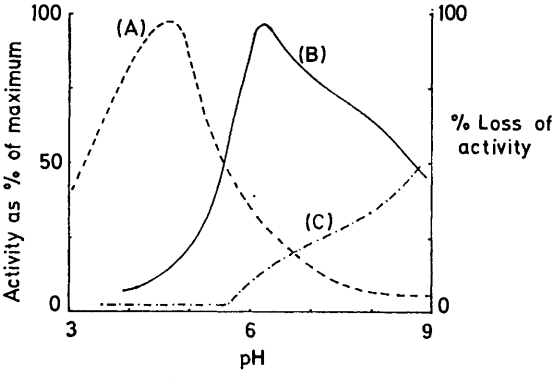


FIGURE 7 pH–Activity profiles of (A) free glucoamylase and (B) glucoamylase immobilised on formaldehyde–hydroquinone resin; the loss of activity on incubation on the immobilised glucoamylase at 37 °C for 24 h is also shown (C)

starch solution (0.614 ml min^{-1}) at 20 °C. After *ca.* 9 days the temperature was raised to 45 °C. The eluate was analysed for starch conversion into D-glucose (by measurement of the amount of free D-glucose liberated), for protein (Figure 6), and for enzyme activity content (Table 2). The variation in activity of the free and immobilised glucoamylase with solution pH was investigated by using a series of starch solutions [1% w/v in 0.1M-sodium phosphate buffers (pH 3—10)], again by measuring the release of free D-glucose (Figure 7).

The loss of activity of the immobilised glucoamylase on incubation for 24 h at 37 °C at various pH values [0.1M-sodium phosphate buffers (pH 4.00—8.75)] was also determined by incubation with starch solution [1% w/v in 0.1M-sodium acetate buffer (pH 4.8)] at 45° and measuring the rate of production of D-glucose (Figure 7).

A solution of α-amylase (10 mg ml^{-1}; 1 ml) in 0.2M-sodium acetate buffer (pH 5.8) containing 10^{-2}M-calcium chloride was applied to another column of formaldehyde–hydroquinone resin (30 × 1.2 cm i.d.) and the column was eluted with the buffer. Samples of a solution of starch (1% w/v; 2.0 ml) in the buffer were applied consecutively to the gel and the column was eluted with buffer at 40 °C. The eluate was analysed continuously for reducing group content (Figure 8), protein content, and enzyme activity (Table 2). A sample (1 ml *ca.* 0.25 mg) of the eluate was fractionated

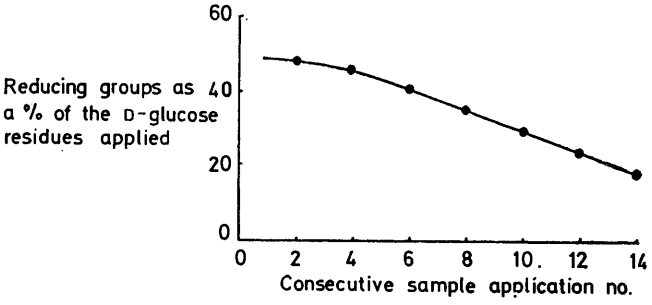


FIGURE 8 Variation in activity with re-use of a constantly operated column of α-amylase immobilised on formaldehyde–hydroquinone resin

on BioGel P-2 (1.5 m × 2.0 cm i.d., as before) to give the distribution of oligosaccharides (Table 3) which differed from that of commercial hydrolysates produced by acid plus enzymic hydrolyses. The variation in activity of the

TABLE 3

Comparison of the carbohydrate components of starch solutions before and after treatment with α-amylase immobilised on formaldehyde–hydroquinore resin

Carbohydrate (degree of polymerisation)	Elution position (V_e/V_0 from BioGel P2 column)	Composition of starch (%) Untreated	Treated with immobilsed α-amylase [b]	Commercial starch hydrolysate A [c]	Commercial starch hydrolysate B [c]
D-Glucose (1)	2.72	0	11.6	3.4	3.9
Maltose (2) [a]	2.52	0	26.0	6.3	23.0
Maltotriose (3)	2.35	0	20.7	7.6	13.7
Maltotetraose (4)	2.18	0	6.5	6.2	12.3
Maltopentaose (5)	2.06	0	16.3	6.1	5.3
Maltohexaose (6)	1.94	0	2.8	11.0	3.9
Maltoheptaose (7)	1.84	0	2.4	10.2	4.9
Malto-octaose (8)	1.75	0	2.1	4.5	2.2
Maltononaose (9)	1.67	0	1.6	2.8	1.9
Maltodecaose (10)	1.61	0	1.4	2.2	1.7
Maltoundecaose (11)	1.55	0	1.4	1.8	1.6
Maltododecaose (12)	1.50	0	1.2	1.7	1.4
Maltotridecaose (13)	1.45	0	0.9	1.4	1.1
Higher oligosaccharides (14—22)	1.13—1.43	0	4.4	8.1	} 12.0
Higher oligosaccharides (22—25)	1.04—1.13	0	0.5	3.0	
Amylose fraction (>25)	1.03	17	0.1	} 23.7	} 11.0
Amylopectin fraction (>25)	1.00—1.03	83	0.0		

[a] Based on the assumption that all oligosaccharides are linear and linked α(1 → 4). [b] Run 8 (see Figure 7). [c] The columns are included for comparison with the oligosaccharide distribution pattern produced by the immobilised α-amylase.

free and immobilised α-amylase with solution pH was investigated by using a series of starch solutions [0.5%; 0.1M-sodium acetate or sodium borate (pH 4—9)].

The observed shift in the profile to alkaline pH on immobilisation of the enzyme was similar to that observed for glucoamylase. D-Glucose isomerase (100 μg) was applied to a third similar column and the column was eluted continuously with degassed 50% w/v D-glucose solution in sodium phosphate buffer [pH 6.8; containing 10^{-2}M-calcium chloride and 10^{-3}M-cobalt(II) chloride] at 72 °C (0.17 ml min^{-1}). The eluate was analysed continuously for D-fructose (Figure 9), protein content, and enzymic activity (Table 2).

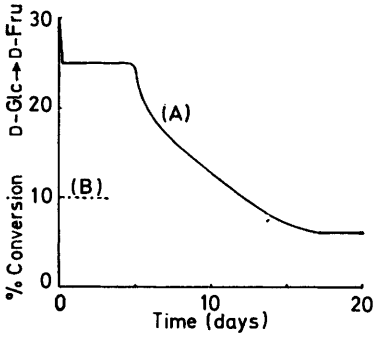


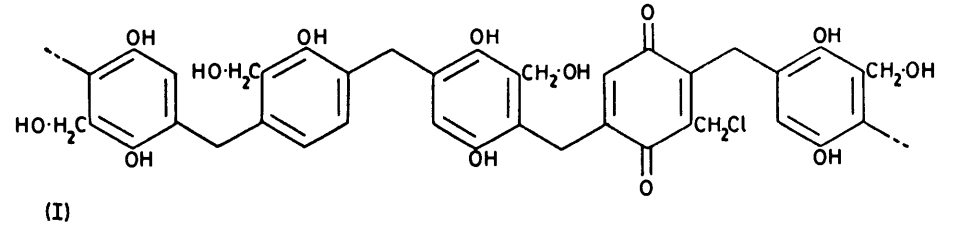
FIGURE 9 Variation with time of the activity of a constantly operated column of D-glucose isomerase immobilised on formaldehyde–hydroquinone resin; (A) conversion of 50% w/v D-glucose at 72 °C; (B) conversion of 10% w/v D-glucose at 40 °C

Adsorption of Amino-acids and Derivatives by the Resins.—250μM-N^{α}-Benzoyl-L-arginine ethyl ester solutions (25 ml) were diluted with 0.02M-sodium phosphate buffer (pH 6.8; 25 ml). A blank was prepared with water in place of the ester solution. Formaldehyde–hydroquinone resin (10 mg) was added to the solutions, and after 1 h at 20 °C the amount of N^{α}-benzoyl-L-arginine ethyl ester in the supernatants was determined. Solutions of L-arginine and L-histidine hydrochlorides were similarly treated. The amounts of the compounds taken up by the resin were as follows: N^{α}-benzoyl-L-arginine ethyl ester hydrochloride, 38.0; L-arginine hydrochloride, 28.4; L-histidine hydrochloride, 4.8 μmol g^{-1}.

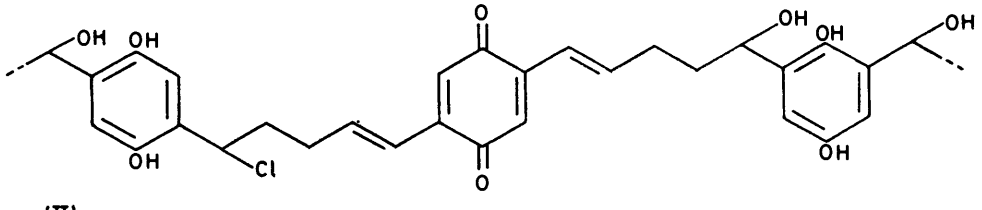
DISCUSSION

The treatment of hydroquinone in concentrated hydrochloric acid solution with either formaldehyde or glutaraldehyde yields a highly hydrophilic brown gel which, on filtration, retains about 30 times its dry weight of water. Formaldehyde, in such a reaction, usually gives methylene bridges [9,33,34] in the 2 or 4 positions of the phenolic residues. The i.r. spectrum of the formaldehyde–hydroquinone resin (Figure 1) shows distinct differences from that of hydroquinone. The O–H stretching vibration (3 226 cm^{-1}) of hydroquinone phenolic groups has shifted to 3 430 cm^{-1}, indicating a decrease in hydrogen bonding ascribable to intermolecular association. Changes in the C–H stretching (2 800—3 000 cm^{-1}) and C–H deformation (1 340—1 470 cm^{-1}) regions indicate the formation of methylene bridges. The aromatic substitution pattern, determined by the C–H in-plane deformations (1 000—1 300 cm^{-1}), also shows change, and there may be some quinone C=O stretching (1 645 cm^{-1}). Glutaraldehyde would be expected to react in much the same manner as formaldehyde with the hydroquinone, but it could also react intramolecularly to give, initially, a pyran-type structure which would then polymerise. There is no indication in the i.r. spectrum of the C–O stretching vibration which would arise from cyclic ether formation. If this by-product is formed it must be removed in the initial brown washings. There is strong indication of methylene groups in the C–H stretching (2 900 cm^{-1}) and CH_2 scissor (1 480 cm^{-1}) deformation regions. Changes are also noticed in the C–H in-plane (1 000—1 300 cm^{-1}) and out-of-plane (700—900 cm^{-1}) deformation regions, indicating changes in the aromatic substitution pattern. A small number of free aldehyde groups is indicated by a peak (1 710 cm^{-1}) in the C=O stretching region. This region also shows some quinone C=O stretching (1 645 cm^{-1}) and aryl C=O stretching (1 625 cm^{-1}). From these i.r. analyses, together with the elemental analyses and reaction mechanism, structures (I) and (II) are postulated for the resins.

In the reaction which generates the formaldehyde–hydroquinone resin, the hydroquinone is completely consumed; none is found in the filtrate. It might be



(I)



(II)

possible to increase the degree of formaldehyde substitution and the degree of polymerisation by increasing the reaction time and using a less polar solvent (like 50% acetone–hydrochloric acid), but this appears to be a slow process, as would be expected as a result of steric hindrance.

A variety of analogous materials were produced by using other phenolic and/or aldehydic monomers (Table 1). However only some of the polymers produced exhibited carbohydrate adsorption characteristics.

The adsorption of starch from solution by formaldehyde–hydroquinone resin varied with the conditions used. This can be rationalised by considering the structure of the starch. Apart from the obvious presence of two species, amylose and amylopectin, there will be a degree of heterogeneity of chain length and branching within these species. These differences in the structures of the polysaccharides would be expected to give rise to differences in their affinity for the resin and this was found to be the case. Under conditions which permitted all molecular types present in the starch solution to be adsorbed, 0.154 g of starch was adsorbed per g of resin. However, it was possible to adsorb, to a much greater extent (1.707 g per g), a fraction of the molecular types present in the starch solution by using conditions which apparently favoured the binding/adsorption of those species which possessed the greater affinity for the resin. The adsorption profile of soluble starch on the resin (Figure 2) shows a total adsorption (about 0.2 g per g of resin) close to the former conditions and an initial rate of adsorption (1 g per g of resin) close to the latter. The specific removal by formaldehyde–hydroquinone resin of high molecular weight carbohydrate from partially acid-hydrolysed starch solution, as proved by gel chromatography (Figures 3 and 4), gave an approximate minimum degree of polymerisation (DP) for adsorption of *ca.* 15 ($\equiv M$ 2 500) although the actual value may be higher. The adsorbed starch could not be removed, except by hydrolysis, even by solutions known to have great affinity for carbohydrates, such as alkaline borate,[35] acid molybdate,[36] and phenol.[37] The immobilised starch could be quantitatively released by glucoamylase, which itself was then adsorbed and could then be used to hydrolyse additional starch samples. The glutaraldehyde–hydroquinone resin separated amylose from amylopectin (Table 2, Figure 5).

The formaldehyde–hydroquinone resin is most effective for immobilising starch when it has maximum polymerisation and minimum oxidation. This is best achieved by adding formaldehyde (which itself inhibits oxidation) fairly rapidly (20—30 min) in excess to a refluxing shaken solution of the quinol under nitrogen, but care must be taken to guard against possible foaming. The nitrogen is not necessary for an adequate product. If only 1 : 1 formaldehyde–quinol is added and refluxing is continued with free access of air, a greenish brown product is formed which, although basically similar in texture to the normal product, has little affinity to starch (owing possibly to oxidation to quinone).

The adsorption of aromatic compounds on polysaccharide gels and carbohydrates on aromatic resins has been explained[23] on the basis of hydrogen-bonding between the hydroxy-groups on the carbohydrate to either the phenolic hydroxy-groups[17] or the π-electrons of the aromatic nuclei.[19,20] Further factors in the present case seem to be the size and three-dimensional structure of the carbohydrate and the possibility of hydrogen-bonding to the primary alcohol (hydroxymethyl) groups on the aromatic resin. Short oligosaccharides and unbranched polysaccharide show weaker adsorption than do highly branched high molecular weight polysaccharides. A physical filtering effect probably enhances the hydrogen-bonding to enable stronger adsorption. The highly branched polysaccharides would make slower progress down the column owing to the physical impedance of the gel compared with short unbranched oligosaccharides and coiled high molecular weight chains, such as amylose. This would allow more time for hydrogen-bond formation in any one area of the resin matrix. The glutaraldehyde–hydroquinone polymer (II) would have a wider spaced gel structure and, therefore, allow larger molecules to pass through with less impedance; this affords a possible explanation for the separation of the components of starch. Clearly, on the basis of the foregoing, these aldehyde–hydroquinone resins have characteristics which render them applicable to both analytical and recovery aspects in the field of carbohydrate chemistry.

Other methods for the collection of polysaccharides by insolubilisation have been reported. Insolubilisation by specific interaction with Concanavalin A[38] permits fractionations of monomeric and polymeric carbohydrates. Concanavalin A in insolubilised form[39] fractionates polysaccharide types according to the degree of branching. However although the latter method has considerable analytical application, the use of the lectin has certain drawbacks for large-scale use in that it is easily biodegradable and expensive. A polyaromatic matrix produced from 1,3-diaminobenzene,[23] although relatively cheap and stable, is of little relevance to the present work since its adsorptive powers are non-specific. The glutaraldehyde–hydroquinone matrix is cheap and easy to produce, inert, and stable and gave good separations of amylose and amylopectin. The formaldehyde–hydroquinone resin is also very cheap and easy to produce, is stable to extremes of pH and temperature, and gives high adsorption of high molecular weight polysaccharides at high flow rates (flow rates up to 5 ml cm^{-2} min^{-1} have been used without high back pressure). It can be used, therefore, in the field of pollution control, to remove polysaccharides (starch and cellulose in particular) from solution. Furthermore, the adsorbed material could be hydrolysed by acid and removed from the matrix, allowing the gel to be re-used, as the gel is highly stable to acid hydrolysis.

Preparations of a range of resins (Table 1) revealed that analogous properties of carbohydrate adsorption could be achieved in a number of instances. Additional

possibilities exist in the use of compounds which yield formaldehyde upon treatment with acid. However in no case were the overall properties of any of these polymers superior to those of the formaldehyde- and glutaraldehyde–hydroquinone resins examined in detail. It was also clear that certain phenol–aldehyde combinations are unsuitable. However the amino-group-containing gels in diazotized form and the aldehyde group-containing gels provide additional means of coupling proteins, including enzymes, according to well established routes.[1] For the same purposes the formaldehyde–hydroquinone gel could be derivatised with epichlorohydrin in base to give a material containing reactive epoxide groups. Furthermore the gels could be prepared in the presence of nickel powder to give magnetic products, the excess of formaldehyde protecting the metal powder from attack by the acid.

The formaldehyde–hydroquinone resin showed 100% retention of active β-D-glucosidase, α-amylase, D-glucose isomerase, and glucoamylase molecules. Although in the last case a small amount of non-enzymic material was eluted in the dead volume this probably arose from the crude nature of commercial glucoamylase. The columns containing immobilised enzyme showed retention of activity for long periods. Immobilised glucoamylase exhibited a 54% h^{-1} conversion rate of starch into D-glucose at 20 °C after 9 days (Figure 6), immobilised D-glucose isomerase converted 7.34 g of D-glucose into D-fructose per day at 72 °C after 20 days continuous operation (Figure 9), and immobilised α-amylase (Table 3) still produced a hydrolysed starch solution containing 38% reducing groups, on a dry weight basis, at 40 °C after the eighth sample application (Figure 8).

The pH–activity profiles of the bound glucoamylase (Figure 7) and α-amylase showed pronounced shifts to alkaline pH. This shift of pH–activity optima has been noted before [9] with a negatively charged solid support [40] which raised the local hydrogen-ion concentration of the solution above that measured for the bulk of the solution. At low pH there is a greater retention in the immobilised glucoamylase activity than at high pH (1% loss h^{-1}; 37 °C; pH 8) (Figure 7), indicating that the resin, with this enzyme immobilised, is best used at slightly acid pH. The consecutive immobilisation of glucoamylase and then starch, or starch and then glucoamylase, both resulted in total hydrolysis of the starch to D-glucose. This indicates the existence of two different environments within the gel, one of which preferentially adsorbs the polysaccharide and the other the protein, with flexibility for interaction between the two.

The formaldehyde–hydroquinone resin might be most effective (for immobilising protein) when it has maximum benzyl alcohol-type structure and minimum oxidation with only moderate polymerisation. This structure is achieved by use of cold or cool hydrochloric acid–hydroquinone solution, in place of refluxing solution, and use of a rapidly added excess of formaldehyde (>4 mol. equiv.). This gave a lighter coloured material, which presumably is of a lower degree of polymerisation.

A possible mechanism by which the adsorption and retention of proteinaceous materials on the resin occurred was investigated by using single amino-acids and their derivatives. Since the resin contained reactive groups such as carboxy (including those from oxidation of the hydroquinone structure) and hydroxy, it may be considered amenable to reaction with amino-groups, *etc.*, in proteins. Furthermore since the resin is aromatic in character, it was possible that aromatic amino-acids such as tyrosine, phenylalanine, and histidine would bind readily. However the fact that L-tyrosine was not retained by the resin (Table 2) suggests that under the conditions employed neither amino- nor aromatic groups are responsible for the binding. L-Histidine was only adsorbed to a small extent. However L-arginine was appreciably taken up by the resin, even when the free amino- and carboxy-groups were blocked, thus indicating that a primary mode by which proteins could be adsorbed by the resin is one involving the guanidino-groups. In view of the structures involved it is possible that the binding may be stronger than physical adsorption, *i.e.* covalent reaction.

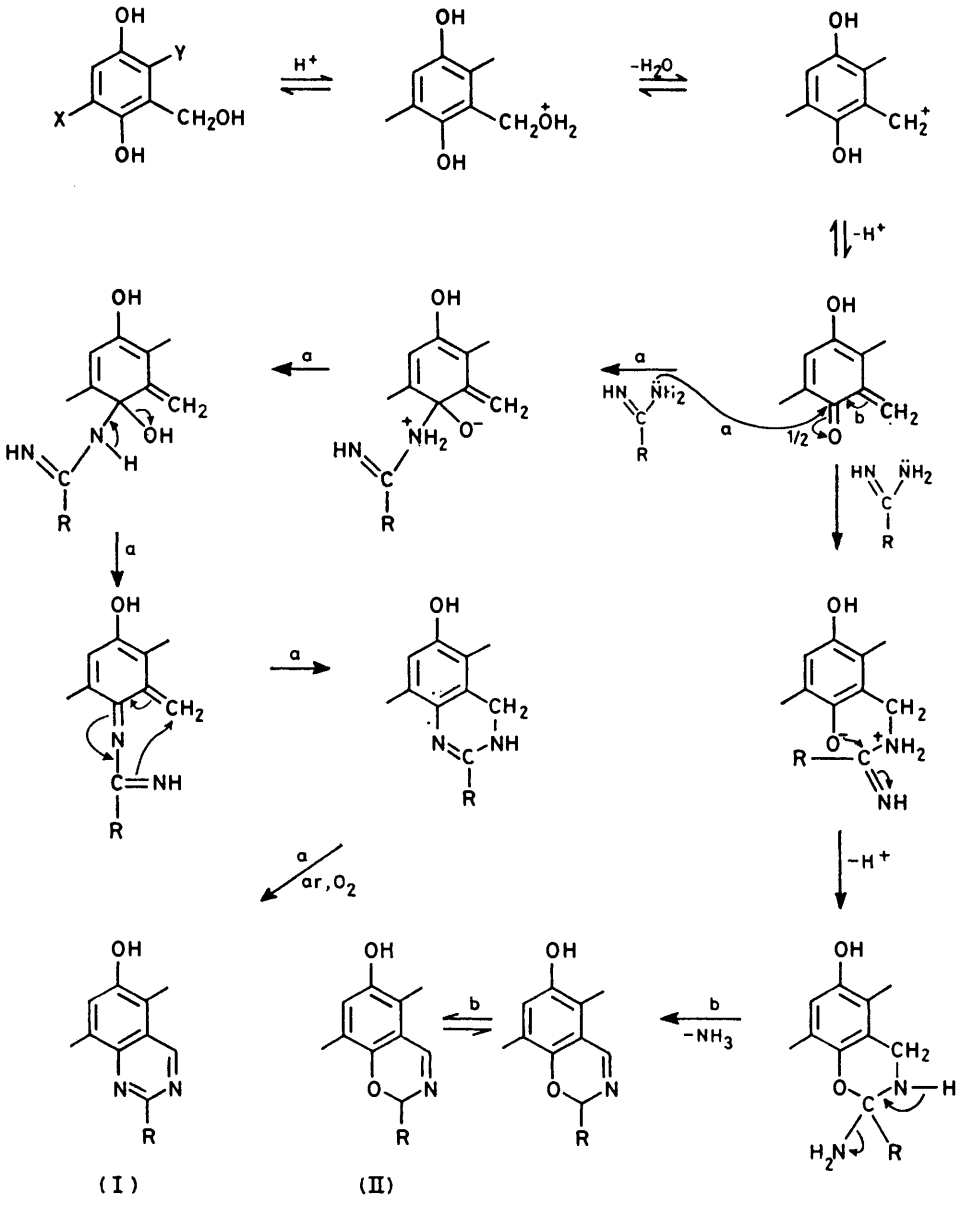
Possible mechanisms for the covalent binding of L-arginyl residues in proteins to the resin are shown in the Scheme. L-Arginine is well known to form cyclic products with diketones and related compounds.[41,42] The driving forces for the reactions involving the poorly nucleophilic guanidino-groups (partly protonated at most pH values) lie in the cyclisation processes. The reaction of L-arginine under similar circumstances has been reported as a Maillard reaction,[43] also occurring with hexoses such as glucose and (particularly) fructose, and therefore it is not unreasonable to predict a similar interaction with the formaldehyde–hydroquinone resin.

L-Arginine seems the most likely amino-acid to react in such a reaction, owing to the possible formation of a (partly) conjugated six-membered ring. The Maillard reaction also occurs with most free amino-acids, utilising the α-NH_2. However, as seen from the reaction of the derivatised arginine, which is the protected form occurring in a protein, the α-amino-group is not necessary for compound formation, and as is seen from the reactions with L-tyrosine and L-histidine the amino-group does not become extensively involved in the present case. There are many possible transitions occurring in a Maillard reaction, but under the pH and temperature conditions used, it seems likely that the initial step is formation of the Schiff base between a guanidino-NH_2 and the carbonyl (Scheme, a); product (I) would be formed thereby. Alternatively reaction could occur *via* a Michael condensation type mechanism (Scheme, b) to give product (II). Other possible, but less likely routes are salt formation and charge-transfer complexation.

The formaldehyde–hydroquinone resin possesses favourable characteristics of easy, selective adsorption of enzymes, with retention of activity. The resin is porous and hydrophilic as is required for the satisfactory im-

mobilisation of enzymes, and so is ideally suited to the immobilisation of proteins for use in packed-bed reactors. The resin has shown itself to be particularly useful for the immobilisation of active enzymes applicable to the conversion of corn starch into D-glucose–D-fructose mixtures, which are used as a substitute for sucrose in carbohydrate sweetener applications. By use of the molecular weight material from the syrup, removing them from the product.

In conclusion, the phenol–aldehyde type resins described in this paper are suitable for the analysis of macromolecular carbohydrates, the selective removal of macromolecular carbohydrates from solution, the immobilization of enzymes with retention of activity, and



SCHEME

enzymes immobilised on the resin, expensive ion-exchange and carbon purification of the products of starch hydrolysis and D-glucose isomerisation is avoided. Because of the low pressure-drop across the resin, several columns with different, or mixed, enzymes could be used in series, such that a continuous process might be operated and individual resin beds replaced as necessary. In particular, the resin would filter out insoluble and high the selective removal of protein from solution. Similar aromatic resins with other reactive groups (for example, borate, quinone, amino, and aldehyde introduced before or after the copolymerisation step) might be a further source of analogous, useful chromatographic materials.

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REFERENCES

[1] J. F. Kennedy, *Adv. Carbohydrate Chem. Biochem.*, 1974, **29**, 305.
[2] S. A. Barker, S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacey, and T. H. Yeo, *Carbohydrate Res.*, 1971, **20**, 1.
[3] J. F. Kennedy and A. Zamir, *Carbohydrate Res.*, 1973, **29**, 497.
[4] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1973, 2293.
[5] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, 1974, 757.
[6] S. A. Barker, J. F. Kennedy, and A. Rosevear, *J. Chem. Soc.* (*C*), 1971, 2726.
[7] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1972, 2568.
[8] J. F. Kennedy and C. E. Doyle, *Carbohydrate Res.*, 1973, **28**, 89.
[9] M. F. Chaplin and J. F. Kennedy, *Carbohydrate Res.*, 1976, **50**, 267.
[10] J. F. Kennedy and P. M. Watts, *Carbohydrate Res.*, 1974, **32**, 155.
[11] J. P. Cardoso, M. F. Chaplin, A. N. Emery, J. F. Kennedy, and L. P. Revel-Chion, *J. Appl. Chem. Biotechnol.*, 1979, **28**, 775.
[12] R. D. Mason and H. H. Weetall, *Biotechnol. Bioeng.*, 1972, **14**, 637.
[13] J. F. Kennedy and I. M. Kay, *J.C.S. Perkin I*, 1976, 329.
[14] J. F. Kennedy, S. A. Barker, and J. D. Humphries, *J.C.S. Perkin I*, 1976, 962.
[15] M. A. Mitz and R. J. Schlueter, *J. Amer. Chem. Soc.*, 1959, **81**, 4024.
[16] B. Gelotte, *J. Chromatog.*, 1960, **3**, 330.
[17] A. J. W. Brook and K. C. Munday, *J. Chromatog.*, 1970, **47**, 1.
[18] H. Determann and I. Walter, *Nature*. 1968, **219**, 604.
[19] L. Sweetman and W. L. Nyhan, *J. Chromatog.*, 1971, **59**, 349.
[20] J. F. Kennedy, *J. Chromatog.*, 1972, **69**, 325.
[21] H. Negoro, *J. Ferment. Technol.*, 1972, **50**, 136.
[22] W. L. Stanley and R. Palter, *Biotechnol. Bioeng.*, 1973, **15**, 597.
[23] J. F. Kennedy, S. A. Barker, and C. A. White, *Carbohydrate Res.*, 1974, **38**, 13.
[24] S. A. Barker, A. N. Emery, and T. M. Novais, *Process Biochem.*, 1971, **6** (10), 11.
[25] W. R. Vieth, S. S. Wang, and R. Saini, *Biotechnol. Bioeng.*, 1973, **15**, 565.
[26] A. T. Cameron, ' The Taste Sense and the Relative Sweetness of Sugars and Other Sweet Substances,' Sugar Research Foundation Inc., New York, Sci. Rept. Ser., No. 9, 1947.
[27] S. A. Barker, B. W. Hatt, J. F. Kennedy, and P. J. Somers, *Carbohydrate Res.*, 1969, **9**, 327.
[28] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, 1974, 757.
[29] S. A. Barker, B. W. Hatt, and P. J. Somers, *Carbohydrate Res.*, 1969, **11**, 355.
[30] P. Bernfeld, *Methods Enzymol.*, 1955, **1**, 149.
[31] J. F. Kennedy and M. F. Chaplin, *Carbohydrate Res.*, 1974, **40**, 227.
[32] M. John, G. Trénel, and H. Dellweg, *J. Chromatog.*, 1969, **42**, 476.
[33] J. F. Kennedy and J. E. Fox, *Carbohydrate Res.*, 1977, **54**, 13.
[34] E. C. Wagner, *J. Amer. Chem. Soc.*, 1933, **55**, 724.
[35] S. A. Barker, M. Stacey, and G. Zweifel, *Chem. and Ind.*, 1957, 330.
[36] S. A. Barker, E. J. Bourne, A. B. Foster, and R. B. Ward, *Nature*, 1957, **179**, 262.
[37] P. R. Carngie, *Biochem. J.*, 1965, **95**, 9P.
[38] I. J. Goldstein, C. E. Hollerman, and J. M. Merrick, *Biochim. Biophys. Acta*, 1965, **97**, 68.
[39] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, 1973, 2041.
[40] L. Goldstein, Y. Levin, E. Katchalski, *Biochemistry*, 1964, **3**, 1913.
[41] E. Wittgenstein and H. K. Berry, *J. Invest. Dermatol.*, 1961, **36**, 283.
[42] M. F. Chaplin, *Biochem. J.*, 1976, **155**, 457.
[43] L. Maillard, *Ann. Chim.* (France), 1916, **5**, 258.