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Note

The reaction of cellulose carbonate with amino and mercapto compounds

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The ring-opening reactions of *trans*-cyclic carbonate groups upon nucleophilic attack are well known for monosaccharide carbonates, and in this way methanol, benzyl alcohol, α-toluenethiol, ammonia, piperidine, glycine, and starch have been coupled to methyl 4,6-*O*-benzylidene-α-D-glucopyranoside[1–3]. We have reported the preparation of water-insoluble *trans*-carbonates of cellulose[4] and other polysaccharides[5], and have shown that cellulose carbonate is suitable for the insolubilisation of β-D-glucosidase with retention of enzyme activity[6]. The attack of a free-amino group in the enzyme on the *trans*-cyclic carbonate group was considered to be the basis of formation of the covalent bond between the cellulose and enzyme. We now report on reactions of cellulose *trans*-2,3-carbonate with amino and mercapto compounds, which were investigated to determine further the potential of the reaction. The reactions were carried out in aqueous solution and under mild conditions in order to simulate those suitable for biological molecules.

That the presence of triethylamine[3] is not necessary for ring opening of cyclic carbonates has already been shown by the work with β-D-glucosidase[6]. When ammonia and amino acids were allowed to react competitively with cellulose carbonate at the optimal pH found for reaction of the enzyme, the ammonia reacted most strongly (Table I), presumably on account of its nucleophilicity. The competitive reaction by the amino acids appears to be complex; for example, the extent of reaction of lysine (a basic amino acid) was not appreciably greater than that for some less-basic amino acids. The extensive occurrence of non-specific opening of carbonate rings by water molecules has already been discussed[6,7]; in the present case, in addition to the fact that the attack by the amino group is a minor reaction, it is arguable that the low extents of reaction are due to reduced nucleophilicity of the amino groups on account of the pH employed and to the ionic nature of the cellulose once some amino acid molecules have become attached.

The protein contents of β-D-glucosidase attached to cellulose carbonate could not be measured by the ninhydrin method[6], although the level of enzymic activity was acceptable. The present work with albumin, in which the amount of reacted protein was determined by radio-labelling, shows that this protein reacts to a smaller

extent than amino acids (taking into account relative molecular weights). However, in the insolubilisation of macromolecules for subsequent biological use, this is not a very serious disadvantage since high protein-loading gives rise to overcrowding and reduced accessibility, particularly to large molecules.

TABLE I

REACTION OF AMINO ACIDS WITH CELLULOSE CARBONATE

Amino acid	*Content of amino acid in cellulose carbonate*	
	(μmoles per 100 mg)	*(μg per 100 mg)*
Aspartic acid	0.0289	3.31
Threonine	0.0303	3.05
Serine	0 0658	5.75
Glutamic acid	0.0417	5.38
Proline	0.0739	7.17
Glycine	0 1287	7.33
Alanine	0.0299	2.13
Valine	0 0208	2.32
Cysteine	0.0149	2 07
Methionine	0.0354	4.64
Isoleucine	0.0269	3.03
Leucine	0.0325	3.67
Tyrosine	0.0755	12 32
Phenylalanine	0.0755	11.11
Ammonia	1.8961	32.22
Lysine	0.0552	6 84
Histidine	0.2468	33.85
Arginine	0.0666	10.37
Total	1 9454	156.52

Although higher concentrations were employed in the reaction of sodium 4-aminosalicylate with cellulose carbonate, the highest degree of coupling (Table II) cannot be regarded as extensive, and presumably the same factors are involved as with the amino acids. This result also indicates that the use of higher concentrations of protein, which could be quite costly, could be expected to achieve little.

TABLE II

REACTION OF SODIUM 4-AMINOSALICYLATE WITH CELLULOSE CARBONATE

Sample	*Sodium 4-aminosalicylate*	
	Concentration in coupling (mg/ml)	*Content of product (mmoles/D-glucose residue)*
Cellulose	1 6	0.7
Cellulose carbonate	1.6	4 8
Cellulose carbonate	3.2	7.6
Cellulose carbonate	6 4	13.7

A further interest in the derivatisation of cellulose carbonate with 4-aminosalicylate was the preparation of an insoluble matrix which could be used to couple with other molecules by complexing with borate, but in which complex formation would not be sterically hindered by having to occur directly on the matrix. Success was demonstrated by the finding that the borate complex of salicylic acid attached to cellulose carbonate was considerably more effective in taking up the carbohydrate antibiotic streptomycin than was the un-complexed matrix (Table III). The controls show that streptomycin is not adsorbed by celluloses treated with 4-aminosalicylic acid, even when treated with borate. The uptake of streptomycin by the un-complexed salicylate derivative of cellulose carbonate is probably caused by salt formation between the guanidino groups of streptomycin and the aromatic carboxyl groups.

TABLE III

ABILITY OF SODIUM 4-AMINOSALICYLATE-TREATED CELLULOSE CARBONATE TO REACT WITH STREPTOMYCIN

Sample	*Borate present*	*Streptomycin content of product (mmoles/D-glucose residue)*
Cellulose	−	0.02
Cellulose	+	0 07
Cellulose carbonate	−	0.92
Cellulose carbonate	+	2 95

The reaction of 2-aminoethanethiol with cellulose carbonate was apparently much more extensive, the validity of the results obtained by measurement of free thiol in the supernatant being ensured by the prevention of oxidation, and being supported by the sulphur analysis. That the reaction occurred *via* the mercapto groups in preference to the amino groups is suggested both from the fact that the product contained few detectable free mercapto groups and from the similar, extensive reaction of *N*-acetylcysteine in which, of course, the amino group is not free to react. However, at first sight these results appear to be at variance with those reported by Stout *et al.*[1] who found that methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate does not react with α-toluenethiol alone, but that 2- and 3-*O*-(benzylthio)carbonyl products are formed when triethylamine is added to the reaction mixture. Because of the need for added base in the non-aqueous system, it is arguable that a basic catalyst is necessary for reaction in the aqueous system. Such a catalyst may be considered to be provided by the buffer system of pH 7.8 or by the presence of the amino groups of the other 2-aminoethanethiol or *N*-acetylcysteine molecules present. Alternative explanations for the case of 2-aminoethanethiol can be offered in terms of reaction of some amino groups followed by cross-linking, failure of the thiol-detecting reagents to react with the solid phase, or inaccuracies in the determination of free thiol in spite of the precautions taken.

In conclusion, it is clear that, whilst the rate of any nucleophilic reaction with cellulose carbonate will be decreased by the insolubility of the polysaccharide deriva-

tive, the reactions that can be achieved by attack with amino compounds under mild pH conditions and in aqueous solution are significant but not extensive. However, cellulose carbonate will react with thiols under similar conditions and much greater degrees of reaction can apparently be achieved. In any further application of cellulose carbonate in the field of insolubilisation, it would be worthwhile investigating the possible conversion of the incoming molecule such that it contained a free and accessible mercapto group. This modification could allow coupling of the protein at a different point in the polypeptide chain, although the addition of catalyst to the coupling system might prove essential.

EXPERIMENTAL

Cellulose *trans*-2,3-carbonate was prepared as described previously[4], using the optimal reaction conditions and a reaction time of 10 min.

Reactions of cellulose carbonate. — (*a*) *With amino acids.* Cellulose carbonate (100 mg) was suspended in 0.1M sodium phosphate buffer (pH 7.8, 5 ml), a standard mixture of amino acids (Bio-Rad Labs., 2.5 μmoles of each of 17 amino acids and ammonia/ml, 1 ml) was added, and the mixture was stirred at 20° for 1.5 h. The product was dialysed in suspension and washed with 0.1M sodium phosphate buffer (pH 7.8). The solid was hydrolysed at 110° for 22 h in 6M hydrochloric acid, and the supernatant was analysed for amino acid content by using a Technicon Autoanalyser (Table I).

(*b*) *With albumin.* Human serum albumin (Koch–Light Labs. Ltd.) was labelled with ^{125}I, using chloramine-T as oxidant[12], and the product was purified by gel filtration on Sephadex G-25. Cellulose carbonate (25 mg) was treated with a solution of ^{125}I-albumin in 0.1M phosphate buffer (pH 7.8) (0.9 mg/ml, 1.7 ml) at 20° for 24 h, and the product was washed alternately with 0.1M carbonate buffer (pH 8.3; 5 × 5 ml) and M sodium chloride (5 × 5 ml), and finally with carbonate buffer (3 × 5 ml). Gamma-counting of the final washes showed that no radioactivity was being eluted, whereas counting of the solid showed that the amount of albumin coupled was 58 μg/100 mg of cellulose carbonate.

(*c*) *With sodium 4-aminosalicylate.* Preliminary tests in which cellulose carbonate was treated with solutions of sodium 4-aminosalicylic acid in 0.1M sodium phosphate buffer (pH 7.8), 0.1M sodium hydrogen carbonate buffer (pH 8.3), and aqueous *N*,*N*-dimethylformamide–triethylamine, followed by testing of the washed product with ferric chloride solution, showed that only the products from the inorganic buffer systems contained phenolic groups.

Samples of cellulose carbonate (100 mg) were suspended in solutions of sodium 4-aminosalicylate in 0.1M phosphate buffer (pH 7.8) (1.6, 3.2, and 6.4 mg/ml; 1.25 ml), and the mixtures were stirred at 20° for 24 h; cellulose (100 mg) was similarly treated. The amounts of 4-aminosalicylate remaining in the supernatants were determined from their absorbances at 265 nm and a calibration curve (Table II). The solid products were washed with water (5 × 5 ml), 0.5M hydrochloric acid (5 ml), and water (5 × 10 ml).

Titration with 10 mM hydrochloric acid gave values similar to those shown for the salicylate contents of the products. Use of much higher concentrations of sodium 4-aminosalicylate in the coupling process did not significantly increase the salicylate contents of the products; those prepared at lower concentrations possessed an infrared absorption at 1810 cm^{-1} corresponding to the *trans*-2,3-carbonate group.

Samples of the sodium 4-aminosalicylate adduct (coupled in phosphate; 8.0 mg/ml, 1.25 ml) of cellulose carbonate (50 mg) and cellulose controls which had been subjected to coupling (50 mg) were treated with M sodium tetraborate (adjusted to pH 7.0 with M hydrochloric acid) or with water (2 ml). The solids were then treated with streptomycin sulphate solution (1 mg/ml, 2 ml), and the supernatants were assayed for unreacted streptomycin by the Scudi[8] assay (Table III).

(*d*) *With 2-aminoethanethiol.* Cellulose carbonate (4 g) was suspended in a solution of 2-aminoethanethiol in 0.1M sodium phosphate buffer (pH 7.8; 40 mg/ml, 80 ml) under oxygen-free nitrogen, and the mixture was stirred at 20° for 24 h. The supernatant was assayed by the nitroprusside[9] method, using decay curves to deal with the instability of the chromophore, and a value of 284 mmoles/mole of D-glucose was obtained for the amount of thiol reacted. An elemental analysis of the washed product gave S = 3.9%, corresponding to 224 mmoles of thiol/mole. Estimation of free thiol groups in the product by attempted reaction with cystine[10] gave a value of 3.4 mmoles/mole, and a low value was also obtained when a modified assay[11] using 5,5′-dithiobis(2-nitrobenzoic acid) was employed. Cellulose itself gave an uptake of 10% of the amount of 2-aminoethanethiol taken up by cellulose carbonate.

(*e*) *With* N-*acetylcysteine.* Cellulose carbonate (100 mg) was suspended in a solution of *N*-acetyl-L-cysteine in 0.1M phosphate buffer (pH 7.8; 50 mg/ml, 2.5 ml) and stirred under nitrogen at 20° for 24 h. The supernatant was assayed for *N*-acetylcysteine by the nitroprusside method[9]; the uptake by the cellulose carbonate was 330 mmoles/mole, whereas a similarly processed cellulose control showed no uptake.

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