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Active Insolubilized Antibiotics Based on Cellulose and Cellulose Carbonate

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The coupling of a number of antibiotics to cellulose and cellulose *trans*-2,3-carbonate under a series of coupling conditions has been investigated, and it has been shown that by such couplings active insoluble derivatives of antibiotics can be produced. It was found that the antibiotics became firmly bound to cellulose itself, whereas use of cellulose carbonate extended the range of antibacterial activity retained. In the case of cellulose, it was considered that physical adsorption phenomena were operating, whereas a covalent bond was more likely in the case of cellulose carbonate where the antibiotic amino groups could attack the electrophilic cyclic carbonate carbon atoms. The release of antibiotic from the matrix during testing of antibacterial activity was likely due to the action of a cellulase or additionally an esterase in the case of cellulose carbonate. The importance and potential of these new types of chemical derivatives are discussed in terms of new outlets for the commercial use of cellulose.

The use of antibiotics in the clinical prevention and treatment of internal and external bacterial and fungal infection is universally accepted, and there are few clinics where these drugs are not employed. However, on account of their general solubility in water, it is necessary to repeat constantly the application until the infection has been eliminated. The thought occurred, however, that if antibiotics could be insolubilized with retention of this antimicrobial activity, then in many cases the repetition of application could be avoided. Furthermore, methods for the insolubilization of antibiotics could also be expected to have a much wider application, e.g., provision of antimicrobial surfaces in industrial reactors. Some work on attachment of streptomycin and gentamicin to specialized polymers, poly(*N*-acryloyl-4 and 5-aminosalicylic acids), has recently been reported (6).

An examination of the degree of physical association of antibiotics with cellulose is now reported. Furthermore, the possibility existed that antibiotics could be reacted with cellulose derivatives with retention of antimicrobial activity with a view to providing covalent, water-insoluble derivatives. For this purpose, cellulose *trans*-2,3-carbonate (3), which has been shown to react with nucleophiles with concomitant opening of the carbonate ring and attachment of the attacking species (5), and which has proved useful for the covalent insolubilization of enzymes (1), was chosen. The reaction of antibiotics containing nucleophilic groups with cellulose *trans*-2,3-carbonate is therefore also now reported.

MATERIALS AND METHODS

Ten antibiotics were obtained as follows: ampercillin (Beecham Research Laboratories), amphotericin B type 2 (E. R. Squibb and Sons Ltd.), chloramphenicol and gentamicin sulfate (Aspro-Nicholas Ltd.), kanamycin sulfate (Winthrop Laboratories), natamycin (pimaricin, Brocades G.B. Ltd.), neomycin sulfate (Boots Pure Drug Co., Ltd.), paromomycin (Parke, Davis & Co.), polymixin B sulfate (Burroughs Welcome & Co.), and streptomycin sulfate (Glaxo Laboratories Ltd.).

The sources of bacteria employed were as follows: *Escherichia coli* NCTC 86, *Pseudomonas aeruginosa* BUC 53 (Department of Chemistry, University of Birmingham), *Streptococcus faecalis* NCTC 370, and *Staphylococcus pyogenes* NCTC 7447. The sources of the fungi employed were as follows: *Aspergillus niger* BUCD 18, *Mucor hiemalis* BUCD 60; *Penicillium chrysogenum* BUCD 58, and *Saccaromyces cerevisiae* BUCD 56. Nutrient agar, prepared by solidifying nutrient broth no. 1 with nutrient agar no. 3 (Oxoid Ltd.), was used for bacterial culture, and malt extract agar (Oxoid) was used for fungal culture.

Cellulose was in microcrystalline form (Sigmacell, particle size 38 μ, Sigma Chemical Co.) and cellulose carbonate was prepared from this using the previously described (3) optimum reaction conditions.

Reaction of antibacterial antibiotics: (i) In phosphate buffer. Cellulose or cellulose carbonate (100 mg) was suspended in 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.8, and antibiotic (100–150 mg) was added. The mixture was stirred at 25 C for 24 h after which the sample was dialyzed against 1.0 M sodium chloride for 24 h and then against running water for 48 h. For certain antibiotics, additional samples were similarly prepared but with the omission of dialysis. All solids were finally washed by stirring and centrifugation with distilled water (three times, 5 ml each time), dry ethanol (three times, 5 ml each time), and diethyl ether (three times, 5 ml each time) at 20 C, and stored in vacuo at 4 C in sterile containers.

(ii) In phosphate buffer-dimethyl sulfoxide. Cellulose or cellulose carbonate (100 mg) was suspended in a mixture of 0.1 M sodium phosphate buffer (pH 7.8; 2.0 ml) and dimethyl sulfoxide (4.0 ml), and antibiotic (240–360 mg) was added. Stirring, dialysis, and washing were carried out as in (i).

(iii) In water-triethylamine. Cellulose or cellulose carbonate (100 mg) was suspended in a mixture of water (5.0 ml) and triethylamine (150 μliters) and antibiotic (200–300 mg) was added. Stirring, dialysis, and washing were carried out as in (i).

Treatment of a solution of polymixin B with the water-triethylamine system gave a gelatinous precipitate which was soluble in 0.25 N hydrochloric acid. Aliquots of the solid-phase polymixin B samples were further treated with 0.25 N hydrochloride acid (three times, 5 ml each time) by stirring and centrifuging, after which the final washing process as in (i) was repeated.

Reaction of antifungal antibiotics. Cellulose or cellulose carbonate was suspended in a saturated solution of antibiotic in a mixture of 0.1 M sodium phosphate buffer (pH 7.8; 2.0 ml) and dimethyl sulfoxide (4.0 ml) and stirred at 25 C for 24 h. The solids were washed by stirring and centrifuging with dimethyl sulfoxide (20 times, 5 ml each time), water (three times, 5 ml each time), dry ethanol (three times, 5 ml each time), and diethyl ether (three times, 5 ml each time) at 20 C, and stored in vacuo at 4 C in sterile containers.

Antibacterial testing of insolubilized antibiotics. Samples of insolubilized antibiotics (15 mg) were suspended in nutrient agar (about 3 ml) and samples of the free antibiotics were dissolved/suspended in water, and treated with an equal volume of double-strength (1.6%) nutrient agar.

"Ditch plates" were prepared by allowing nutrient agar to solidify in a petri dish. A strip of the nutrient agar, approximately 1 cm in width, was removed to form the ditch. The sample suspension/solution (1 ml) was placed in the ditch, and the plates were inoculated with cultures of *E. coli*, *P. aeruginosa*, *S. faecalis*, and *S. pyogenes* perpendicular to the ditch. (The organisms, two gram positive and two gram negative, were selected as being representative of bacteria.) The plates were incubated at 37 C for 18 to 24 h. The degree of inhibition of growth of each organism across the ditch was observed, and the extent of inhibition from the edge of the ditch was determined. All parent antibiotics were strongly active against all four organisms, inhibition occurring across the ditch and 5 to 25 mm from the ditch edge, with the exception of ampercillin which was inactive against *P. aeruginosa*. The results for the insolubilized antibiotics are shown in Tables 1 (cellulose) and 2 (cellulose carbonate).

Antifungal testing of insolubilized antibiotics. Samples of insolubilized antibiotics and free antibiotics were prepared for testing as above by using malt extract agar. Ditch plates, also prepared as above by using malt extract agar, were loaded with the sample suspension/solution (1 ml). Four such plates were prepared for each sample and cultures of *A. niger*, *M. hiemalis*, *P. chrysogenum* or *S. cerevisiae* were applied to each plate. (These organisms were selected as being representative of fungi.) The plates were incubated at 30 C for 48 h after which it was found that free natamycin had inhibited the growth of all four fungi, whereas the solid-phase sample showed no such inhibition. Similarly, whereas free amphotericin B, type 2 inhibited the growth of *P. chrysogenum* and *S. cerevisiae*, the insoluble products were inactive.

RESULTS AND DISCUSSION

Three systems were employed for the coupling of antibiotic to cellulose, but the spectrum of antibacterial activity of the product from each antibiotic was largely independent of the mode of coupling (Table 1). The most important outcome of this part of the work is that cellulose has the ability to bind a number of antibiotics with retention of some of their antibacterial activities. The nature of the binding is presumably physical and, although the details are not understood, the guanido group of streptomycin and the highly basic natures of kanamycin, neomycin, and paromomycin, for example, may be involved. However, chloroamphenicol failed to give an active derivative, suggesting that either in spite of its amido and aromatic residues it did not become bound to the cellulose or that, if it did become bound, then the mode of binding inhibited manifestation of antibacterial activity. It was disappointing, however, that none of the antibiotic derivatives exhibited actively against *P. aeruginosa*, an organism known to frequently infect the human eye.

All the antibiotics presently studied possess structures which contrain primary amino groups, with the exception of chloroamphenicol which contains an amido group. These antibiotics were therefore amenable to attachment to cellulose *trans*-2,3-carbonate by nucleophilic attack of their amino groups on the cyclic carbonate groups. Comparison of the results obtained for cellulose with those obtained for coupling of antibiotics to cellulose carbonate (Table 2) shows that it is advantageous to use

TABLE 1. *Antibacterial activities of antibiotics insolubilized on cellulose*

Antibiotic	Solvent system for coupling[a]	Growth of organism[b]			
		E. coli	*P. aeruginosa*	*S. pyogenes*	*S. faecalis*
Ampercillin	P	−	−	+++	−
	P/DMSO	−	−	+++	−
	HOH/TE	−	−	+++	−
Chloroamphenicol	P	−	−	−	−
	P/DMSO	−	−	−	−
	HOH/TE	−	−	−	−
	P/DMSO[c]	+++ (7)	−	+++	+++
Gentamicin	P	+++	−	+++ (4)	+++
	P/DMSO	+++ (5)	−	+++ (5)	+++
	HOH/TE	+++ (6)	−	+++ (5)	+
Kanamycin	P	+++ (2)	−	+++ (4)	+++
	P/DMSO	+++	−	+++ (3)	+
	HOH/TE	+++	−	+++ (3)	+
Neomycin	P	+++	−	+++	−
	P/DMSO	+++	−	+++	−
	HOH/TE	+++	−	+++	−
Paromomycin	P	+++ (5)	−	+++ (5)	+++ (5)
	P/DMSO	+++	−	+++	+++ (5)
	HOH/TE	+++ (3)	−	+++ (3)	+++ (3)
Polymixin B	P	+++	−	−	−
	P/DMSO	+++	−	−	+
	HOH/TE	+++ (10)	+++ (5)	+++ (5)	+++ (5)
Polymixin B (HCl treatment)	P	−	−	−	−
	P/DMSO	+	−	−	−
	HOH/TE	+	−	−	−
Streptomycin	P	+++	−	−	+
	P/DMSO	+++	−	+++	−
	HOH/TE	+++	−	+++	−

[a] Abbreviations: P, phosphate buffer; DMSO, dimethyl sulfoxide; HOH, water; TE, triethylamine.

[b] Symbols: −, no inhibition of growth of organism; +, slight inhibition of growth of organism across ditch; +++, complete inhibition of growth of organism across ditch, numbers refer to maximal distance (millimeters) of inhibition of growth of organism from the ditch.

[c] Dialysis omitted.

cellulose, there is no general rule concerning the wider spectra of antibacterial activities can be obtained. This, therefore, suggests that covalent attachment had occurred. As in the case of cellulose, there is no general rule concerning the efficacy of any particular coupling system, although the use of triethylamine to facilitate the covalent reaction of neomycin with cellulose carbonate did permit formation of a product which was active against *P. aeruginosa*. Trials were also carried out with an aqueous dimethyl formamide system for the coupling, but it was found that this rapidly destroyed the cyclic carbonate groups.

From the point of view of producing products in which the antibiotic was firmly bound, the importance of dialysis against aqueous sodium chloride in the work-up procedure for derivatives of both cellulose and cellulose carbonate was established. Antibacterial activities of samples which had not been dialyzed were much greater (e.g., chloroamphenicol, Table 1). The general occurrence of diffusion from the ditch, as indicated by the inhibition of growth for some distance from the ditch as in the case of the free antibiotics, suggested that appreciable amounts of loosely bound antibiotic remained. However, the occurrence of diffusion may also be due to the action of a cellulase or an esterase pre-existent, or induced, in the organisms or their secretion. Whereas a cellulase would, of course, degrade the insoluble matrix to soluble antibiotic-cellulose oligosaccharide products, an esterase could liberate the antibiotic by cleavage of the N-substituted carbonate ester group formed by covalent reaction of an amino-antibiotic with a cyclic carbonate group. Such a theory could be tested by using the analogous but non-biodegradable poly (allyl cyclic carbonate) (2 and 4) as the matrix. However, the likelihood of the action of a cellulase is strengthened by the fact that diffusion effects were

TABLE 2. *Antibacterial activities of antibiotics insolubilized on cellulose carbonate*[a]

Antibiotic	Solvent system for coupling	Growth of organism			
		E. coli	*P. aeruginosa*	*S. pyogenes*	*S. faecalis*
Ampercillin	P	+++ (5)	–	+++ (10)	+++
	P/DMSO	+++ (5)	–	+++ (10)	+
	HOH/TE	–	–	+++	–
Chloroamphenicol	P	+++ (4)	–	+++ (4)	+++ (4)
	P/DMSO	+++ (4)	–	+++ (4)	+++ (4)
	HOH/TE	–	–	+++	–
Gentamicin	P	+++ (3)	–	+++ (4)	+
	P/DMSO	+	–	+	+
	HOH/TE	+++ (3)	–	+++ (5)	+++
Kanamycin	P	+++ (2)	–	+++ (3)	–
	P/DMSO	+++ (2)	–	+++ (4)	+++ (2)
	HOH/TE	+++ (7)	–	+++ (8)	+
Neomycin	P	+++	–	+++	–
	P/DMSO	+++	–	+++	–
	HOH/TE	+++	+++	+++	+
Paromomycin	P	+++	–	+++	+++
	P/DMSO	+++	–	+++	+++
	HOH/TE	+++ (5)	–	+++ (5)	+++ (4)
Polymixin B	P	+++	–	–	–
	P/DMSO	–	–	–	–
	HOH/TE	+++ (10)	+++ (5)	+++ (5)	+++ (5)
Polymixin B (HCl treatment)	P	–	–	–	–
	P/DMSO	–	–	–	–
	HOH/TE	+	–	–	–
Streptomycin	P	+++ (7)	–	+++ (4)	–
	P/DMSO	+++ (5)	–	+++ (5)	–
	HOH/TE	+++	–	+++	–

[a] For abbreviations and symbols see Table 1.

found for the case of cellulose carbonate as well as for cellulose.

In connection with the phenomenon of diffusion, a further question arises for products which were inactive against some but not all organisms, that is in cases where diffusion was absent and there was no inhibition of growth across the ditch. Clearly, it cannot be argued that antibiotic had not been coupled, nor can it be claimed, in the light of controls with the free antibiotics, that the antibiotic diffused poorly where activity was only observed across the ditch. Thus, it appears in many cases that the antibiotic must be released to manifest activity, but the possibility of activity of the true insolubilized form cannot be ruled out.

The case of polymixin B is unique since it was found that an aqueous solution of this antibiotic formed a gel on treatment with triethylamine (and other bases). Thus it was likely that the antibiotic derivatives produced in the normal way contained the antibiotic in its gel form as well as the insolubilized form arising from attachment to the carbohydrate. Apart from the possibility of physical adsorption, this proved to be the case since treatment of the carbohydrate-antibiotic derivatives with hydrochloric acid, an agent in which the gel was found to be soluble, largely abolished the antibacterial activity of the derivatives.

It is noteworthy that an insolubilized antibiotic which is successfully active against one organism is not necessarily active against all the organisms, the growth of which is inhibited by the antibiotic in its free state. This different response of different organisms to the insolubilized antibodies may well reflect different modes of action of the antibiotics against different organisms. The complete inactivity of the antifungal derivatives suggests that in both cases either the modes of attachment inhibited activity, or that the antibiotics failed to couple to the matrices although the latter seems less likely particularly in the case of cellulose *trans*-2,3-carbonate since both antibiotics contain an amino group.

New applications in the chemistry of cellulose are constantly being sought (7), and the results of the present investigation open up a new field in the use of cellulose and cellulose *trans*-2,3-carbonate. This production of water-insoluble antibacterial antibiotics may be applied in a

number of ways according to the intended use of such derivatives. Thus, where it is required to have a slow continual release of antibiotics but a higher initial release (e.g., bandages and surgeons' thread), cellulose-antibiotic prepared without dialysis would be the choice. The results suggest that cotton bandage/antibiotic applications have hitherto been used unwittingly as slow-release formulations. Similarly, for use in hospitals, cellulose-based wall-paper brushed with aqueous solutions of antibiotics could be expected to retain antibacterial properties for considerable periods. Where, however, an antibacterial surface is required (e.g., water storage tanks, industrial membranes, chromatographic columns), such surfaces could be realized by using cellulose-based paints, membranes, etc., and insolubilization of the antibiotic by covalent attachment. In such cases, loss of the antibiotic would be minimal, and other polysaccharide carbonates (5) and poly (allyl cyclic carbonate) (2 and 4) might be of greater applicability. Since cellulose and other polysaccharides and their derivatives are used extensively in a number of forms as accessories to life, active insolubilized antibiotics could well be of great use in a number of other areas (e.g., food packaging materials).

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