Poly(N-Acryloyl-4- and -5-Aminosalicylic Acids)

II. Antibacterial Properties and Uses for the Preparation of Active, Insoluble Antibiotics

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Received for publication 24 August 1972

The antibacterial properties of poly(N-acryloyl-4- and -5-aminosalicylic acids) and their borate complexes were evaluated. Poly(N-acryloyl-4- and 5-aminosalicylic acids) were used to render the antibiotics streptomycin and gentamicin insoluble. The antibacterial properties of the resultant water-insoluble streptomycin and gentamicin derivatives were evaluated, and the results show that the polymers are suited to the preparation of active insoluble antibiotics. Potential antimicrobial applications of the polymers are discussed.

Salicylic acid has been the topic of many patents for preparations with biological activity. A mixture of salicylic and boric acids was reported to inhibit the growth of organisms causing lactic or acetic fermentations, formation of mannitol, and biological degradations; hence, this mixture has found use as an antiseptic for cleaning casks and vats used in fermentation or in storage of wines, ciders, beers, and milk (G. A. L. C. Denys, French Patent 1,181,869, 1959).

Mixtures of borate complexes, made by treating alkaline tetraborate with organic acids such as salicylic acid, were shown to have disinfectant and antiphlogistic properties (J. Muller, Austrian Patent 174,696, 1953).

Stability and other desirable properties have been conferred upon established therapeutic agents by mixing them with salicylic acid; e.g., a mixture of tetracycline base or its hydrochloride and a metal salt of salicylic acid may be stored in solid form, but the preparation is completely water-soluble to yield an injectable preparation (L. Schoring, L. Middendorf, and G. Rose, German Patent 1,039,707, 1959).

It was also reported by Michel (3) that acryloyl derivatives show substantial biological activity; e.g., a 0.1% aqueous solution of sodium acrylate inhibits the growth of Escherichia coli present in the intestinal flora of pigs. It was later found by Brisou and de Raultrie de la Roy (1) that sodium acrylate was active against Staphylococcus pyogenes. In both of these cases, the activity was found to be only transient, because the aqueous solutions were chemically unstable and the resulting polymers were biologically inactive. The association of biological activity with α,β-unsaturation appears quite widespread. Penicillic acid, patulin, griseofulvin, and novobiocin all contain an α, β-unsaturated carbonyl group. This group is essential for biological activity in a variety of compounds, as was shown by Rinderknecht et al. (4). They found that a replacement of the

\[
\text{O} \quad \text{C} = \text{C} - \text{C} \quad \begin{array}{c} \text{O} \\ \text{C} = \text{CH}_2 - \text{CH}_2 = \text{C} \end{array}
\]

\text{group present in patulin and penicillin acid with the saturated variant}

\[
\text{O} \quad \text{C} = \text{C} = \text{C} \quad \begin{array}{c} \text{O} \\ \text{C} = \text{CH}_2 - \text{CH}_2 = \text{C} \end{array}
\]

resulted in a loss of activity. Of the 80 or more α,β-unsaturated keto compounds investigated by McGowan, Brian, and Hemming (2) for their fungistatic activity, the strongest activity was exhibited by those compounds with a strongly electron-withdrawing group attached to one of the carbon atoms involved in the double bond.

The preparation of N-acryloyl derivatives of 4- and 5-aminosalicylic acids has been reported elsewhere (J. F. Kennedy et al., J. Chem. Soc., \textit{in press}), and in view of the foregoing the possibility existed that these compounds might possess antibacterial properties. Similarly, poly(salicylic acids), namely, poly(N-acryloyl-4- and 5-aminosalicylic acids) and their borate complexes, were candidates. The present work therefore reports an investigation of the antibacterial activities of these compounds, the poly-acids being tested in both soluble and insoluble forms. Furthermore, the ability of the
polymers to form insoluble complexes with antibiotics and the antibacterial properties of such complexes were tested.

**MATERIALS AND METHODS**

Gentamicin sulfate was obtained from Aspro-Nicholas Ltd., and streptomycin sulfate was obtained from Glaxo Laboratories Ltd.

The bacteria employed were *Escherichia coli* NCTC 86, *Pseudomonas aeruginosa* culture no. 53, Department of Chemistry, University of Birmingham, *Streptococcus faecalis* NCTC 370, and *Staphylococcus pyogenes* NCTC 7447.

Nutrient agar was prepared by solidifying Oxoid Nutrient Broth No. 1 with Oxoid Nutrient Agar No. 3 (Oxoid Ltd.).

Preparation of poly(N-acryloyl-4- and -5-aminosalicylic acids). N-acryloyl-4-aminosalicylic acid was polymerized by a method described elsewhere by Kennedy et al. (in press). N-acryloyl-4-aminosalicylic acid (15 g) and sodium tetraborate (9.36 g) were dissolved in distilled water (180 ml). After the pH had been adjusted to 9.0 with 10 N sodium hydroxide, a solution of azobisisobutyronitrile (Biato, 150 mg) in ethanol (50 ml) was added, and the resulting solution was heated at 80°C in a water bath for 48 hr under reflux. The resulting viscous solution was diluted with distilled water (200 ml), and 5 ml hydrochloric acid was added to precipitate the white polymeric solid. The product was washed 10 times with 1-liter portions of distilled water by decantation, and the polymer was rotary-evaporated in vacuo with methanol (four times, 250 ml each) to remove any remaining borate as methyl borate. After a further water wash (250 ml), the poly (N-acryloyl-4-aminosalicylic acid) (yield, 11.4 g; 76%) was stored as a suspension in distilled water.

The method described above for poly(N-acryloyl-4-aminosalicylic acid) was followed with N-acryloyl-5-aminosalicylic acid as monomer to produce the pink poly(N-acryloyl-5-aminosalicylic acid) (yield, 14.2 g; 94%).

Preparation of (N-acryloyl-4- and -5-aminosalicylic acid) borate complexes. (i) Preparation from the borate complex of the monomer was as follows. N-acryloyl-4-aminosalicylic acid was polymerized as described for poly(N-acryloyl-4-aminosalicylic acid) up to the stage where the viscous solution was diluted with distilled water (70 ml). The solution was then dialyzed for 48 hr against 10 changes (5 liters each) of 0.005 M sodium tetraborate buffer, pH 7.0.

(ii) For preparation from poly(N-acryloyl-4-aminosalicylic acid), a sample (2 ml, 100 mg) of the poly(N-acryloyl-4-aminosalicylic acid) suspension was centrifuged and the supernatant fluid was removed. Sodium tetraborate solution (10%, w/v; 2 ml), pre-cooled to 4°C, was added to the cooled residue. The mixture was thoroughly shaken and was centrifuged; the supernatant fluid was removed. The treatment at 4°C was repeated twice, and then the polymer was washed with distilled water to remove any remaining sodium tetraborate solution.

The borate complexes of poly(N-acryloyl-5-aminosalicylic acid) were prepared as described above with (i) N-acryloyl-5-aminosalicylic acid (5 gm) and (ii) (N-acryloyl-5-aminosalicylic acid) (2-ml sample, 100 mg) used in place of the corresponding 4-amino isomers.

Preparation of poly(N-acryloyl-4- and -5-aminosalicylic acid) streptomycin complexes. Poly(N-acryloyl-4- and -5-aminosalicylic acids), 1 g (net weight), were packed into glass columns (bed volume, 20 ml). The gel beds were first washed twice with 5 n hydrochloric acid (20 ml each time) after which they were washed twice with distilled water (20 ml each time). A solution of streptomycin sulfate (1.5 mg/ml, 40 ml) was then passed through each column, and the column eluate was monitored for streptomycin by use of the Scudi colorimetric assay (5). The gel beds were then washed five times with distilled water (20 ml each time), and finally with 0.5 n calcium chloride; the eluates again were assayed for streptomycin.

Preparation of poly (N-acryloyl-4- and -5-aminosalicylic acid) gentamicin complexes. The gentamicin complexes of the polymers were prepared as described above for the streptomycin complexes, but in this case a solution of gentamicin sulfate (1 mg/ml, 40 ml) was used to treat the columns of the poly(N-acryloyl-4- and -5-aminosalicylic acids), 1 g (net weight).

**Antibacterial testing.** Samples (2 ml) of suspensions of the test substance were adjusted to pH 7.0 with dilute acid or alkali and were then mixed with double-strength agar (15 to 17 g/liter, 2 ml) at 55 to 60°C. When the test substance was in solution, the liquid was mixed with an equal volume of double-strength nutrient agar (15 to 17 mg/ml) at 55 to 60°C.

"Ditch plates" were prepared by allowing single strength nutrient agar (7.5 to 8.5 mg/ml) to solidify in a petri dish. A strip of the nutrient agar, approximately 1 cm in width, was removed to form the ditch. The solution containing the test substance (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 plates then were incubated at 37°C for 18 to 24 hr.

Poly(N-acryloyl-4- and -5-aminosalicylic acids) were tested in aqueous solution at concentrations in the range of 0 to 250 μg/ml. Streptomycin and gentamicin were similarly tested.

The poly(N-acryloyl-4- and -5-aminosalicylic acids) were prepared for anti-bacterial testing by suspension in water (17 and 50 mg of net weight/ml, respectively). The poly(N-acryloyl-4-aminosalicylic acid) and poly(N-acryloyl-5-aminosalicylic acid) were also tested after dissolution in 96% (w/v) aqueous sorbitol and 100% (w/v) aqueous glycerol, respectively, to give concentrations of 10 mg/ml.

The borate complexes of the poly(N-acryloyl-4- and -5-aminosalicylic acids) as prepared from the borate complexes of the monomers were tested by use of solutions in 0.005 M borate at a concentration of 80 mg/ml. The borate complexes of the polymers as prepared from the poly(salicylic acids) themselves were prepared for testing by dissolution in water, by warming to 50°C and cooling, to give a concentration of 17 mg/ml.

Poly(N-acryloyl-4- and -5-aminosalicylic acid) streptomycin and gentamicin complexes were pre-
pared for testing by suspension in distilled water to give a concentration of 50 mg/ml.

RESULTS

N-acryloyl-4- and -5-aminosalicylic acids and antibiotics. Antibacterial testing of N-acryloyl-4- and -5-aminosalicylic acids in solution showed that the compounds were inactive against the four organisms employed at concentrations up to 250 μg/ml. Streptomycin and gentamicin were active against all four organisms.

Poly(N-acryloyl-4- and -5-aminosalicylic acids) and their borate complexes. Assay of the column eluate from treatment of the poly(N-acryloyl-4- and -5-aminosalicylic acids) with streptomycin showed that all of the antibiotic had been removed from solution by the columns. The subsequent washing with water did not elute streptomycin, but the wash with 0.5 N calcium chloride effected elution of the antibiotic. The results of antibacterial testing of the antibiotic complexes are shown in Table 1.

DISCUSSION

Since the N-acryloyl-4- and -5-aminosalicylic acids possessed antibacterial properties only when in polymeric and insoluble form, it is clear that the acryloyl side chain of the aromatic nucleus is not responsible for the activity. Because it was demonstrated previously by Kennedy et al. (in press) that these polymers are water-insoluble but are soluble in the presence of polyhydroalcohols, the polymers could be tested in solution only in the presence of glycerol or sorbitol. In these instances, no antibacterial activity attributable to the polymers was observed. The results thus indicate that the polymers possess antibacterial activities as surfaces against certain bacteria, and not in solution. The way in which the activity is mediated is uncertain, but the possibility exists that this is an ionic effect provided by the carboxyl and phenolic groups of the salicyl residues. This is supported in part by the observation that the borate complexes, independent of their mode of production, were inactive. It has already been shown that borate ion complexes with the carboxyl and phenolic groups of salicyl residues (Kennedy et al., in press).

The point of greatest interest in the present

<table>
<thead>
<tr>
<th>Sample under test*</th>
<th>Conc of solution tested (mg/ml)</th>
<th>Growth of organism*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>P4A I</td>
<td>17</td>
<td>–</td>
</tr>
<tr>
<td>P4A II</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>P4B I</td>
<td>80</td>
<td>–</td>
</tr>
<tr>
<td>P4AB II</td>
<td>17</td>
<td>–</td>
</tr>
<tr>
<td>P4AS</td>
<td>50</td>
<td>+ d</td>
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<tr>
<td>P4AG</td>
<td>50</td>
<td>+ d</td>
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<tr>
<td>P5A I</td>
<td>50</td>
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<td>P5A II</td>
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<td>P5AB I</td>
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<td>P5AB II</td>
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<tr>
<td>P5AG</td>
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</tbody>
</table>

*P4A I, poly(N-acryloyl-4-aminosalicylic acid) in insoluble form; P4A II, poly(N-acryloyl-4-aminosalicylic acid) dissolved in glycerol; P4AB I, poly(N-acryloyl-4-aminosalicylic acid) borate complex made from monomer; P4AB II, poly(N-acryloyl-4-aminosalicylic acid) borate complex made from the polymer; P4AG, poly(N-acryloyl-4-aminosalicylic acid) streptomycin salt; P4AS, poly(N-acryloyl-4-aminosalicylic acid) gentamicin salt; P5A I, poly(N-acryloyl-5-aminosalicylic acid) in insoluble form; P5A II, poly(N-acryloyl-5-aminosalicylic acid) dissolved in sorbitol; P5AB I, poly(N-acryloyl-5-aminosalicylic acid) borate complex made from the monomer; P5AB II, poly(N-acryloyl-5-aminosalicylic acid) borate complex made from the polymer; P5AS, poly(N-acryloyl-5-aminosalicylic acid) streptomycin salt; P5AG, poly(N-acryloyl-5-aminosalicylic acid) gentamicin salt.

*Symbols: –, no inhibition of growth of organism; +, inhibition of growth of organism across ditch; d, inhibition of growth of organism up to at least 5 mm from ditch.
The work is the ability of the poly(N-acryloyl-4- and 5-aminosalicylic acids) to form complexes with the antibiotics streptomycin and gentamicin. It is envisaged that the complexes are in fact due to salt formation between the salicylic acid carboxyl groups and the guanido groups of streptomycin and the amino group of the galosamine unit of gentamicin. The antibacterial activity of these complexes was fairly widespread among the organisms used (Table 1). In certain cases, the growth of the organisms was inhibited some distance from the ditch, indicating that diffusion of the antibacterial species had occurred. The theory of complex formation is compatible with this diffusion, which would arise either from slow, spontaneous release of the antibiotics or from the action of bacteria initially viable and in contact with the complex.

Recently, considerable interest has been shown in the preparation of water-insoluble derivatives of enzymes, antibodies, and antigens in which the biological activities of the free molecules are retained. The present paper presents a means of preparing water-insoluble derivatives of certain antibiotics. Thus, in view of their properties and characteristics, the poly(N-acryloyl-4- and 5-aminosalicylic acids) hold potential for the preparation of bacteriostatic surfaces, membranes to which antibiotics can be attached, and slow-release forms of antibiotics for use in, for example, surgical dressings. On account of the hydrophilic and gel-like nature of the polymers, as shown by Kennedy et al. (in press), they could also find application in providing selective protection against microbial attack on chromatographic fillings and in the preparation of antibacterial columns. Since the polymers themselves were able to remove the antibiotics from solution very efficiently, and the antibiotics could apparently be subsequently desorbed, the polymers are suitable as recovery agents for such antibiotics, and materials of similar chemical constitution, from dilute solution. This use would be applicable in the industrial processes where small losses may be significant. The poly(N-acryloyl-4- and 5-aminosalicylic acids) are also proving to be useful matrices in the preparation of insoluble enzymes (Kennedy and Epton, Carbohydr Res., in press) and in metal ion extraction (Kennedy et al., J. Chem. Soc., in press).

ACKNOWLEDGMENTS

We thank M. Stacey and S. A. Barker for their interest in this work, and Aspro-Nicholas Ltd. for a research scholarship to J. E. E. T. J. Chelton of the Department of Chemistry, University of Birmingham is thanked for his cooperation in carrying out biological tests.

LITERATURE CITED