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SYNTHETIC POLYANIONIC INHIBITORS OF HYALURONIDASE

by

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In recent years several different types of high molecular weight polyanions have been shown to be inhibitors of hyaluronidase and certain other enzymes. Thus heparin[1], cellulose trisulphate and chitin disulphate[2], nitrated and acetylated hyaluronic acid[3], phosphate polymers derived from a number of aromatic hydroxy and amino compounds[4,5,6], and "humic acid" (an undialysable polyacid formed from benzoquinone)[7,8] are active in this respect.

Gentisic acid when pure is inactive but when allowed to oxidize in alkaline solution yields a hyaluronidase inhibitor, possibly of structure similar to "humic acid"[7,8,9]. Gentisic acid may also be polymerized with formaldehyde and in this way HAHN[10,11,12] obtained the highly potent anti-hyaluronidase "rehibin" which was the best of a series of active formaldehyde polymers produced from various mono-, di- and tri-hydroxybenzoic acids. The initial condensation was carried out by vigorous stirring of a suspension of gentisic acid in 50% (v/v) sulphuric acid with formaldehyde at 100° C for 5 hours. We find that equally active material can be more simply obtained by adding formaldehyde to a solution of gentisic acid in 25% sulphuric acid and collecting the insoluble product after 1 hour's heating under reflux. Exclusion of air has little or no effect. Active material can also be obtained by heating equimolecular proportions of sodium gentisate and formaldehyde in aqueous solution under reflux for 5 hours, acidifying and collecting the precipitate.

In view of the existence of sulphate groups in heparin and chondroitin sulphate and because of the rather poor solubility of the substituted benzoic acid-formaldehyde polymers at physiological pH's attention was directed to polymers based on substituted benzenesulphonic acids. Such of these substances as have been examined, condense with formaldehyde under acid conditions to give water and alkali insoluble materials due presumably to elimination of sulphonic acid groups; this is probably a general reaction. Under alkaline conditions condensation takes a different course and, providing a slight excess over 1 molecular equivalent of alkali has been employed, a fair proportion of high molecular weight, highly water soluble polymer is obtained. Thus if equimolecular quantities of hydroquinone sulphonic acid and formaldehyde and 1.2 moles of sodium hydroxide are heated together in aqueous solution under reflux for 5 hours and the resulting dark brown solution neutralized and evaporated to dryness a very dark resin (PS 53) remains. This has a similar order of activity as an inhibitor of testicular hyaluronidase to "rehibin" but is very soluble in water over a wide range of pH's; a neutral, homogeneous syrup containing 50% of the material can be produced. Its toxicity is low, the LD/50 in mice being greater than 1250 mg/kg given subcutaneously and greater than 250 mg/kg intravenously. Mice fed 400 mg/kg/day for 21 days were without toxic symptoms. This, in our experience, is even less toxic than the gentisic acid-formaldehyde polymers.

On dialysis of PS 53 in cellophane approximately a quarter (PS53D) is retained. This latter is the most active anti-hyaluronidase so far examined. A small proportion of the original activity also passed through the cellophane.

The method used for estimating inhibitor potency was to incubate buffered solutions of the polymers at pH 6.0 together with partially purified testicular hyaluronidase (Rondase, Evans Medical Supplies Ltd. England) for 30 min at 37° C. The enzyme was first diluted in a 0.2 g/100 ml solution of gelatin containing $2 \cdot 10^{-4}$ *M* sodium pyrophosphate; this diluent protects the enzyme against inactivation by incubation and heavy metal ion contamination[13]. After incubation enzyme activity was estimated by the turbidity reduction technique[14]. Four concentrations of each polymer

in buffered aqueous solution were assayed against one turbidity reducing unit of enzyme. The ues of gelatin-pyrophosphate diluent helps to distinguish between specific enzyme inhibition and non-specific inactivation. If any of these hyaluronidase inhibitors are to be used for *in vivo* studies their effects on the enzyme must not be reversed by other proteins. The presence of gelatin in our test may account for the finding that some of the polymers were less inhibitory than had previously been reported[6,12].

The table shows the results of testing several materials by the above procedure, the figures given for activity being the inverse of the concentration of inhibitor in mg/ml necessary to produce 50% inhibition of 1 turbidity reducing unit of the enzyme. Of these compounds, PARKES[15] has shown that "rehibin" inhibits fertilization in rabbits when mixed with the semen at the rate of about 5 mg/ml and the same test carried out with PS 53 and PS 53D showed a similar action to a degree concordant with the *in vitro* tests. No effect on fertility could be detected when PS 53 was administered by mouth.

TABLE I

	Compound No.	*Activity*
Gentisic acid-formaldehyde polymer produced in 50% sulphuric acid (HAHN method)	—	42
Gentisic acid-formaldehyde polymer produced in 25% sulphuric acid	PS 55	42
Gentisic acid-formaldehyde polymer produced in 1 mol of alkali	PS 51	30
"Rehibin" ("trigentisic acid")	—	45
Hydroquinone sulphonic acid-formaldehyde polymer	PS 53	28
Hydroquinone sulphonic acid-formaldehyde polymer, undialysable portion	PS 53D	60
Catechol sulphonic acid-formaldehyde polymer, undialysable portion	PS 58D	28
Resorcinol sulphonic acid-formaldehyde polymer, undialysable portion	PS 60D	20
p-Phenolsulphonic acid-formaldehyde polymer, undialysable portion	PS 59D	20
Polyresorcinol phosphate (FERNÖ *et al.* method)	—	<10

For the preparation of the sulphonic acids of hydroquinone and catechol used in this work the method described by PETERSON[16] for synthesizing 2:4-dihydroxybenzenesulphonic acid from resorcinol was found applicable.

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