RESEARCH PAPERS

SOME OBSERVATIONS ON THE INHIBITION OF THE ACTION OF HYALURONIDASE ON HYALURONIC ACID BY GENTISIC ACID AND ITS OXIDATION PRODUCTS

BY J. FORREST, B. G. OVERELL, V. PETROW and O. STEPHENSON

*From The Research Laboratories, The British Drug Houses, Ltd., London, N.*1

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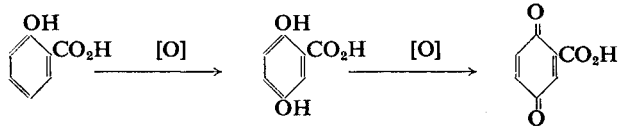
THE spreading factor, hyaluronidase, plays an important part in health and in disease. Its biological significance lies in its ability to depolymerise hyaluronic acid, which is present in many animal tissues and which appears to play a part in binding water in interstitial spaces. In synovial fluid, for example, hyaluronic acid holds cells together in a jelly-like matrix which serves as a lubricant and shock absorber in joints. Balance between hyaluronidase and hyaluronic acid is maintained by an enzyme inhibitor linked with the pseudoglobulin fraction of the serum. Hyaluronidase inhibition is also exercised by heparin, chondroitinsulphuric acid and gastric mucin.

Certain rheumatic conditions are characterised by destruction or disorganisation of the synovial membranes, a process in which hyaluronidase is thought to play a part. Thus Guerra[1] observed that intradermal injection of 1 per cent. hyaluronidase solution containing 1 per cent. of Evans Blue into patients suffering from rheumatic fever gave unique reactions characterised by enormous diffusion of the dye. Guerra interpreted these results as indicating increased hyaluronidase activity during the disease process. He made the further observation that administration of sodium salicylate decreased the severity of these reactions. He concluded that salicylates inhibit the "spreading effect" of hyaluronidase, an action to which he attributed, in part, the beneficial results of salicylate therapy in disorders of this character.

Following these observations attempts were made by a number of workers to substantiate Guerra's claims by *in vitro* studies. The method employed consisted in measurement of the degree of depolymerisation of a hyaluronic acid solution by a standardised enzyme preparation. The results obtained, however, showed clearly that salicylates had no effect upon the hyaluronidase-hyaluronic acid system except in relatively enormous concentrations.[2,3,4,5] The observations recorded by Guerra were, therefore, ascribed to the formation of salicylate metabolites, which were considered to be the true inhibitors of the action of hyaluronidase upon hyaluronic acid.



(I) (II) (III)

The metabolic changes undergone by salicylates (I) *in vivo* were studied by Meyer and Ragan,[6] who isolated gentisic acid (II) and gentisuric acid from the urine of patients on salicylate therapy. In their hands gentisic acid was found to exercise a markedly inhibiting effect on the depolymerisation of hyaluronic acid by hyaluronidase after short incubation with the enzyme. Clinical studies,[7] too, appeared to show that gentisates were equivalent, and in some respects superior, to salicylates in the treatment of rheumatic fever.

The observations recorded by the foregoing authors on the hyaluronidase inhibiting activity of gentisates were not supported by the independent studies of Lowenthal and Gagnon,[8] who found that both (I) and (II) were devoid of activity *in vitro*. Gentisoquinone (III), however, which may be regarded as a simple oxidation product of (II), proved, in contrast, to have very marked activity. No evidence was obtained that the latter compound was formed from (II) *in vivo*.

Studies reported by Roseman, Pearson and Dorfman[9] supplied an explanation for these apparently conflicting results. The hyaluronidase inhibiting activity previously ascribed to gentisic acid was traced to the presence of impurities in the material. Highly purified (II) was devoid of activity, but could be made active by shaking its alkaline solution in air. Roseman *et al.* concluded, on this evidence, that the hyaluronidase inhibiting activity of gentisic acid was due to an impurity which was probably an oxidation product.

Our own interest in derivatives of salicylates and gentisates having hyaluronidase inhibiting activity arose from the view that such compounds might prove of value in certain rheumatic disorders in which disorganisation of the hyaluronic acid matrix of synovial fluid by hyaluronidase is thought to occur. Indirect evidence supporting this viewpoint has recently been provided by a number of workers, who have shown that the increased permeability of the synovial membrane to dyestuffs resulting from hyaluronidase action may be antagonised by both cortisone and adrenocorticotrophic hormone.[10,11] We, therefore, undertook a study of certain oxidation products of (I) and (II), hoping thereby to obtain a product worthy of biological study *in vivo* (cf. the publication by Hetzel and Hins[12] which appeared after completion of our studies).

Our own work has fully confirmed the earlier views expressed by Roseman *et al.* (*loc. cit.*). Crude gentisic acid does, in fact, contain a small quantity of a highly active inhibitor, the identification of which formed our first objective. For this purpose we required an adequate supply of the crude acid, which was obtained by oxidising 10 kg. of salicylic acid with ammonium persulphate.[13] Purification of this material gave 2 kg. of "sludge" which showed marked activity and was, therefore, submitted to exhaustive fractionation, the progress of which was followed by hyaluronidase inhibiting activity evaluation at every stage.

A small fraction (1·5 per cent; *Compound A*) was ultimately obtained which had an inhibitory index of 10,000 units/g. (cf. pure gentisic acid; 58 units/g.) (see *Experimental* for definition of units employed). The material was nearly black. It was almost insoluble in water, chloroform,

and ether, but was somewhat more soluble in ethyl acetate and in the lower alcohols. Its acidic character was indicated by its solubility in alkalis, in which it readily dissolved to give a dark brown solution. Combustion analysis indicated a formula roughly approximating to $(C_6H_4O_3)_n$. The physical and chemical properties of *Compound A* were thus identical with those attributed by Eller and Koch[14] to the "humic acids" which are formed by oxidation of phenols with persulphate or with air in alkaline solution. Its formation from salicylic acid must, therefore, proceed through an hydroxyquinone type of intermediate (*vide infra*) by a mechanism involving polymerisation.[15] Some evidence supporting this view was obtained by parallel experiments in which humic acids were obtained from gentisic acid by the aerial oxidation procedure already referred to (Eller and Koch, *loc. cit.*).

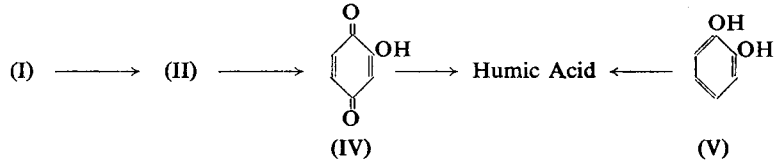
Aerial oxidation of a solution of gentisic acid containing 3 equivalents of alkali for a period of 40 hours gave a black solution which liberated carbon dioxide on acidification. Extraction with ether removed unchanged material (*ca.* 50 per cent.). Further extraction with ethyl acetate and with *n*-butanol gave a series of fractions ranging from dark semi-solids to black tars which showed low activity (300 to 900 units/g.) and thus differed sharply from *Compound A*. The results were nevertheless sufficiently encouraging to warrant further extension.

The facility with which decarboxylation apparently occurred during the aerial oxidation of gentisic acid appeared to be highly significant in view of the proposed formulation of *Compound A* as a humic acid. We were, therefore, led to examine the alkaline aeration of an equimolar mixture of gentisic acid and hydroquinone. Although in this instance results similar to the foregoing oxidation were obtained, a trace of a black compound of inhibitory index *ca.* 3000 units was nevertheless isolated. Pursuing this line of reasoning further we next studied the oxidation of an equimolar mixture of gentisic acid and benzoquinone in a solution containing 3 equivalents of alkali. The result proved satisfactory. A nearly black sparingly soluble humic acid, *Compound B*, was obtained, which strongly resembled *Compound A* in appearance, in physical and chemical properties, and in inhibitory index. Variation in the proportions of gentisic acid to benzoquinone caused only slight change in the activity of the product, although the analytical figures of the humic acids thus obtained varied somewhat over a range of values.

In view of these results the oxidation of benzoquinone itself was studied. Aeration of its solution in the presence of one equivalent of alkali gave high yields of black insoluble products which, after washing well with water and drying at 95° C., had inhibitory indices of *ca.* 10,000 units, i.e., were only slightly less active than *Compounds A* or *B*. Benzoquinone itself, it should be added, reacted exothermically with aqueous alkali, to yield, without aeration, a product having activity *ca.* 3000 units/g.

It seems reasonable to conclude from the above evidence that the development of hyaluronidase-inhibiting properties by solutions of salicylates and gentisates (cf. Roseman *et al.*[9]) is due to the production of compounds of the humic acid type. This view is further strengthened

by the observation that the naturally-occurring humic acids of soil and peat show definite hyaluronidase-inhibiting properties (cf. Table II). On this basis the formation of humic acids from salicylates (I) and gentisates (II) would presumably occur through intermediate formation of hydroxyquinones (cf. IV[15]), which would then take part in a polymeric process in which other suitably constituted phenolic components could participate.

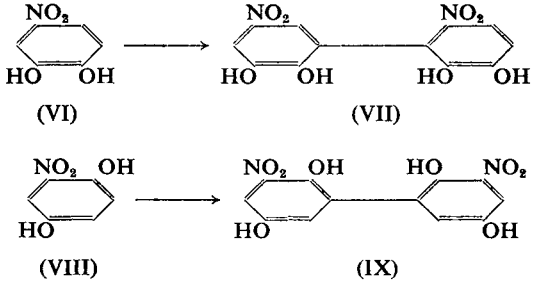


(IV) (V)

The formation of humic acids from (I) or (II), however, is accompanied by complex side reactions, as gentisic acid, for example, absorbs up to *ca.* 2 moles. oxygen on shaking in alkaline solutions (see Experimental, Table I), a value greatly in excess of that required to convert (II) into (IV). The extent of oxidation of phenols by air in alkaline solution appears, in fact, to depend largely upon the amount of alkali present (cf. Table I), which likewise determines the degree of oxidation of the resulting humic acids. Expressions purporting to represent the empirical formulae of humic acids are thus meaningless, agreement between analytical figures and formulae such as $(C_6H_4O_3)_n$ proposed by Eller and Koch[14] being purely fortuitous.

Some evidence supporting the view that the hydroxyquinone (IV) forms an important link in the chain of reactions which lead to the humic acids is also furnished by observations on the oxidation of catechol (V). This compound contains preformed an ortho-dihydroxy-group corresponding to that present in (IV), and gives a product (*Compound C*) of high activity (13,800 units/g.) on alkaline oxidation. 1:2:4-Trihydroxybenzene, the reduction product of (IV), likewise gives material of high activity, though in somewhat lower yield.

The marked activity exhibited by the catechol oxidation product (*Compound C*) led us to examine the aerial oxidation of a number of compounds containing the *ortho*-dihydroxybenzene residue. These are listed in Table II, from which it can be seen that products of activity 10,000 to 20,000 units/g. may be obtained from several different starting materials.



(VI) (VII)

(VIII) (IX)

The oxidation of 4-nitrocatechol (VI) and nitrohydroquinone (VIII), it will be noted, led to the formation of crystalline tetrahydroxy-dinitrodiphenyl derivatives, which we tentatively formulate as (VII) and (IX), respectively. Of these, the latter showed pronounced activity (8000 units/g.). All the other oxidation products listed in Table II formed dark infusible solids of the typical humic acid type.

Biological evaluation of *Compounds A*, *B* and *C* was kindly undertaken by Dr. S. W. F. Underhill and his staff (Physiological Research Laboratories). All three compounds exercised inhibition on the spread of hæmoglobin co-injected with hyaluronidase intradermally into the backs of rabbits, but the effects were accompanied by physiological actions of undesirable character.

Experimental

Melting points are uncorrected.

Hyaluronidase was prepared from bull testes by the method of Madinaveita.[16] Hyaluronic acid was prepared from umbilical cords by ethanol precipitation as described by McClean,[17] or by the ammonium sulphate fractionation procedure of Hadidian and Pirie.[18]

Hyaluronidase assay. The method employed closely followed the viscometric assay developed by Swyer and Emmens[13] in which the reduction in viscosity of a standard hyaluronic acid solution is measured after 20 minutes incubation with the enzyme. An Ostwald type viscometer of 5 ml. capacity and flow time 10 seconds was employed throughout the investigation. The concentration of the hyaluronic acid solution was adjusted to give a flow time of *ca.* 30 seconds at 25° C.

4 ml. of hyaluronic acid solution was pipetted into a test-tube containing 1 ml. of M/60 *p*H7 buffer (McIlvaine's buffer containing disodium hydrogen phosphate, citric acid, with added sodium chloride[20]), and the mixture equilibrated in a water bath thermostatically maintained at $25 \pm 0{\cdot}1°$ C. 1 ml. of hyaluronidase solution in 0·5 per cent. gum arabic (added to preserve potency), which had also been equilibrated, was then run in, a stop-watch was started, and the two solutions mixed with a warm pipette. 5 ml. of the mixture was then pipetted into the temperature-equilibrated viscometer and, after exactly 20 minutes incubation, the flow time (f_e) measured. The flow times of the substrate and solvents without enzyme (f_s) and solvents alone (f_o) were measured in the same way. A measure of the enzyme activity was then given by calculation of the flow-time index (F.T.I.):

$$\text{F.T.I.} = 1000 \left(\frac{f_s - f_e}{f_s - f_o} \right)$$

Inhibitor Assay. Most of the inhibitors studied were soluble in McIlvaine's buffer. The inhibitors could, therefore, be introduced directly into the system. 0·06 g. of the inhibitor was weighed out, dissolved in buffer by warming on the steam bath and made up to 10 ml. with buffer. 1 ml. of this 0·6 per cent. solution was added to 4 ml. of hyaluronic acid solution and the assay procedure followed as before. The final

concentration of the inhibitor in the viscometer under these conditions was 0·1 per cent. w/v. If complete inhibition of the enzyme occurred at this concentration, the inhibitor solution was diluted 1 in 10 with buffer and again assayed. The dilution procedure was continued until a reading (f_i) was obtained which showed between 25 and 75 per cent. inhibition of the enzyme. Outside these limits of inhibition the experimental error became too large, for reasons which are discussed below.

The percentage inhibition of the enzyme was calculated from the flow-time index:

$$\text{Per cent. Inhibition} = \frac{\text{F.T.I.}_{\text{enzyme}} - \text{F.T.I.}_{\text{inhibitor}}}{\text{F.T.I.}_{\text{enzyme}}} \times 100$$

$$= \left(\frac{f_i - f_e}{f_s - f_e}\right) \times 100$$

Scale of Units. The expression of inhibitory activity in terms of the concentration of inhibitor required to produce a certain percentage of enzyme inhibition has been employed by, *inter alia*, Meyer and Ragan[6] and Lowenthal and Gagnon.[8] It is not entirely satisfactory, however, as percentage inhibition does not vary with concentration of inhibitor in linear fashion (*vide infra*). Thus, the concentration of inhibitor which causes partial inhibition varies with concentration of the enzyme and the temperature and may, in addition, be affected by extraneous protein or by decomposed enzyme. Furthermore, the activity of a solid enzyme preparation decreases on storage over a period of months at a somewhat greater rate than does its inhibitor-binding power. Finally, the preparation of successive batches of enzyme possessing reasonably constant activity is difficult, whilst the maintenance of a single standard enzyme preparation of constant activity over the period of time occupied by the investigation was clearly impossible. In these circumstances it was necessary to evolve some method whereby the results obtained with successive enzyme preparations could be correlated and compared.

By plotting (percentage inhibition) against — log [inhibitor] (where [inhibitor] represent the concentration of inhibitor in g./100 ml.), sigmoid curves of the type shown in Figure 1 are obtained for different enzyme preparations but with the same inhibitor.

Examination of these curves reveals (i) that the relationship between the two functions varies approximately in linear fashion over the inhibition range of 25 to 75 per cent. and (ii) that various enzyme preparations (A, B, C) give approximately parallel curves in this region of inhibition. It is thus possible to adopt a single typical curve AA′ to express the relationship in question and to equate the values for a particular enzyme preparation with those of the "standard" curve AA′ by introducing a simple arithmetical correction into the value of — log [inhibitor] which will correspond to $\frac{XA''}{XB''}$ for enzyme B, $\frac{XA''}{XC''}$ for enzyme C, etc. Calibration of successive enzyme preparations in terms of the standard preparation and inhibitor may thus be effected.

In order to calculate the activity of a particular inhibitor, the value

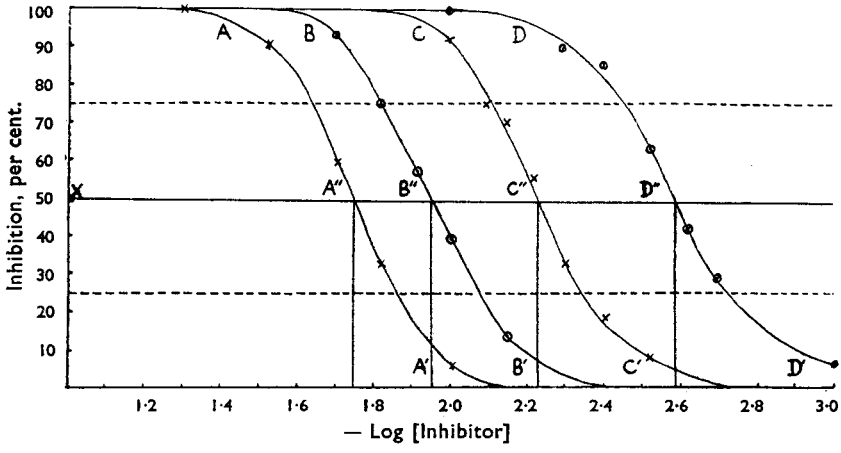


FIG. 1.

Variation of percentage hyaluronidase inhibition with the negative logarithm of the concentration of inhibitor.

Curve AA′; enzyme preparation (2) with inhibitor (a).
Curve BB′; enzyme preparation (2) with inhibitor (b).
Curve CC′; enzyme preparation (1) with inhibitor (a).
Curve DD′; enzyme preparation (1) with inhibitor (b).

Enzyme preparation (1) contained 100 mg./100 ml. organic solids.
Enzyme preparation (2) contained 400 mg./100 ml. organic solids.

$$\frac{XA''}{XB''} = 0{\cdot}89; \qquad \frac{XC''}{XD''} = 0{\cdot}86$$

of — log [inhibitor] required to bring about an inhibition in the region 25 to 75 per cent. is determined. This value is corrected for the enzyme preparation which is being used, and the corrected value employed to determine the — log [inhibitor] required to produce 50 per cent. inhibition by reference to curve AA′. The antilog of this value gives the reciprocal of the concentration producing 50 per cent. inhibition, which is defined as the hyaluronidase inhibiting activity. This in units/g. thus becomes $\frac{1}{x}$, where x = [inhibitor] required to produce 50 per cent. inhibition. The scale of units is, of course, purely arbitrary, and is related only to the original activity of the enzyme preparation used for developing the technique.

The success of the method clearly depends upon accurate calibration of each enzyme sample for, whereas the reproducibility of values with the same enzyme is to within ± 5 per cent., the reproduction of results obtained with different enzyme preparations may only be to within ± 20 per cent. Nevertheless, the values obtained for the different inhibitors over a period of 8 months would appear to be comparable.

Isolation of Compound A. Oxidation of 10 kg. of salicylic acid with persulphate as described by Forrest and Petrow[13] gave 2 kg. of sludge. The dried material was finely ground to a sternutatory powder in a ball mill in a draught chamber, and the resulting product extracted with

chloroform (10 l.) under a reflux condenser for 1 hour, followed by immediate filtration of the boiling solution. The extraction process was repeated a further 9 times, the amount of material extracted decreasing with successive operations from >350 g. on the first extraction to less than 40 g. at the 10th extraction. The material recovered from the chloroform (*ca.* 1·6 kg.) had very low activity and was largely salicylic acid admixed with a small quantity of gentisic acid. The chloroform-insoluble dark brown residue (*ca.* 120 g., m.pt. 170° to 190° C.) was extracted 3 times with 1 l. quantities of ether to give:—

fraction	(i):	66 g., m.pt. 188° to 190° C.; activity	< 200
„	(ii):	12·3 g., m.pt. 177° to 183° C.; „	= 1200
„	(iii):	1·5 g., m.pt. 166° to 178° C.; „	= 7500

The marked decrease in the quantity of material extracted, combined with the decrease in m.pt. and increase in activity in passing from fraction (i) to (iii) appeared to indicate the presence, in the insoluble residues of a chloroform/ether insoluble or sparingly soluble material of high activity. The residues from the ether extractions were, therefore, dissolved in absolute ethanol (250 ml.) at 50° C., filtered from *ca.* 5 g. of inorganic matter, the solvent removed under reduced pressure, and the residue (32 g.; m.pt. 230° to 240° C. (decomp.); activity (*ca.* 11,000 units/g.) extracted with hot water (150 ml., at 80° to 85° C.) for 30 minutes. Collection of the insoluble material, followed by washing with water and drying, gave *Compound A* as a black amorphous solid (16 g.), m.pt. *ca.* 240° C. (decomp.). Found: C, 57·1; H, 3·9; $C_6H_4O_3$ requires C, 58·1; H, 3·2 per cent.; hyaluronidase inhibiting activity 11,500 units/g., apparently unchanged by further extraction or precipitation procedures.

The red-brown aqueous extracts from *Compound A* gave a permanent violet colour with ferric chloride. Evaporation yielded a red-brown hygroscopic solid of negligible activity.

Aerial Oxidation of Gentisic Acid in Alkaline Solution. Methyl gentisate is readily accessible in a state of high purity and was, therefore, employed in many of the experiments in preference to the free acid. It undergoes facile and rapid hydrolysis in the presence of caustic alkalis. Numerous oxidation experiments were performed in the course of the investigation. Only typical data for each group of reactants and conditions has been recorded for reasons of brevity.

Methyl gentisate (100 g.) in water (400 ml.) was treated with potassium hydroxide (100 g.) and the mixture cooled to room temperature. A rapid stream of carbon dioxide free air was then aspirated through the solution for 40 hours. The black mixture was acidified with concentrated hydrochloric acid (150 ml.) when evolution of carbon dioxide occurred. The dark crystalline material which separated (38 g., m.pt. 197° to 202° C. (decomp.), activity 76 units/g.) was collected and identified as unchanged gentisic acid. The aqueous liquors were then extracted 4 times with ether (300 ml. per extraction) yielding a further quantity of crude gentisic acid (34 g., m.pt. 195° to 200° C. (decomp.), activity 74 units/g.). The aqueous mother liquors were then extracted with ethyl acetate

(3 × 300 ml.) to yield a pitch-like black solid (4 g., activity 933 units/g.) and finally with *n*-butanol to give a black solid of activity 501 units/g.

Aerial Oxidation of Gentisic Acid + Hydroquinone in Alkaline Solution. Methyl gentisate (50·4 g.) and hydroquinone (33 g., 1 equiv.) in water (250 ml.) and potassium hydroxide (3 equivs.) was oxidised by rapid aspiration of air for 16 hours. Acidification with concentrated hydrochloric acid (100 ml.) gave a small quantity of a black solid (2 g., activity 3,150 units/g.) which was collected. Ether extraction of the mother liquors gave crude gentisic acid (56 g., activity 102 units/g.). Subsequent extractions with (i) ethyl acetate gave nearly black material (10 g.) of activity 379 units/g.; (ii) *n*-butanol gave a similar product (10 g.) activity 83 units/g.

Aerial Oxidation of Gentisic Acid + Benzoquinone in Alkaline Solution. Methyl gentisate (33·4 g.) and benzoquinone (21·6 g., 1 equiv.) in water (200 ml.) containing 3 equivalents of potassium hydroxide were oxidised as above for 24 hours. Addition of concentrated hydrochloric acid precipitated a black, infusible solid, *Compound B* (26·5 g.) of activity 11,000 units/g. Ether extraction of the mother liquors gave crude gentisic acid (19 g. activity 65 units/g.). Subsequent butanol extraction gave a smaller quantity (4 g.) of less active black material (activity 1075 units/g.). Variation in the gentisic acid/benzoquinone ratio gave materials of comparable activity.

Aerial Oxidation of Catechol. Aerial oxidation of catechol (55 g.) in aqueous solution in the presence of 3 equivalents of alkali for 30 hours, followed by acidification as before, gave a black infusible solid (30 g.), *Compound C.* Found: C, 52·5; H, 3·5 per cent., activity 13,800 units/g.

Aerial Oxidation of 1:2:4-*Triacetoxy Benzene.* Aerial oxidation for 24 hours of 1:2:4-triacetoxybenzene (50·4 g.) in aqueous solution in the presence of 4 equivalents of potassium hydroxide, followed by acidification of the mixture, gave a black infusible humic acid (8 g.). Found: C, 50·0; H, 3·5 per cent., activity 9550 units/g.

Experiments with Benzoquinone. Benzoquinone (130 g. of moist, freshly steam-distilled material) in water (600 ml.) and sodium hydroxide (60 g.) was heated on the steam bath with shaking for 1 hour, when solution was complete. A portion (260 ml.) of the resulting solution was acidified with concentrated hydrochloric acid (40 ml.) and the black, infusible precipitate (20 g.) collected, washed thoroughly, and dried. Found: C, 60·1; H, 4·1 per cent. activity 4920 units/g.

The remainder of the solution (460 ml.) was aspirated with air for 24 hours. Acidification (150 ml. of hydrochloric acid) was accompanied by liberation of carbon dioxide and precipitation of a dark brown infusible humic acid (60 g.). Found: C, 55·9; H, 2·8 per cent. activity 10,470 units/g.

Absorption of Oxygen by Alkaline Solutions of Gentisic Acid. 0·25 Molar solutions of gentisic acid containing sodium hydroxide were shaken in the presence of oxygen in a Warburg type apparatus at 22° C. until equilibrium had been reached. The results obtained are shown in Table I.

TABLE I

Sodium hydroxide mols./mol. of gentisic acid	Total oxygen absorbed (mols.)
1·00	No absorption
1·25	0·507
1·50	0·859
1·75	1·216
2·00	1·387
2·25	1·662
2·5	1·810
2·75	1·947
3·00	2·092
3·5	2·157

Miscellaneous Experiments. The results obtained by the aerial oxidation of a variety of compounds, in aqueous alkaline solution, are summarised in Table II. Unless otherwise stated, the products obtained formed black infusible solids.

TABLE II

Compound	Equivalents of of potassium hydroxide	Hours Aeration	Hyaluronidase inhibiting activity of product units/g.
Toluquinone	1	22	4600
2:5-Dihydroxybenzoquinone	—	—	70
2:5-Dihydroxybenzoquinone	4	25	5030
2:5-Di*tert.*-butylbenzoquinone (*a*)	2	5·5	210
2:4:5:2′:4′:5′-Hexahydroxydiphenyl	3	24	16,250
Chlorohydroquinone	0·5	24	3730
Nitrohydroquinone	2	11	7800 (*b*)
Purpurogallin	3	30	3200
1:2:4:1′:2′:4′-Hexahydroxydiphenyl methane	6	10	17,020
Protocatechuic acid	4	18	12,070
9-Phenyl-2:3:7:4′-tetrahydroxyfluorone (*c*)	9	20	430
Tetrahydroxyxanthylium chloride	5	20	19,000
Alizarin	2	20	370
β-Methylæsculetin	2	20	360
4-Nitrocatechol	—	—	<10
4-Nitrocatechol	2	24	870 (*b*)
Dihydroxyphenyl-hydroxybenzoquinone (*d*)	—	—	38,000
Soil humic acid	—	—	3100; 2800 (*e*)
Peat humic acid	—	—	3800; 3200 (*e*)

(*a*) Calesnick and Beutner,[21] report that 2:5-di*tert.*-butyl-benzohydroquinone shows very high hyaluronidase inhibiting activity. This observation could not be confirmed as the material proved to be too insoluble for examination by the technique employed in this work.
(*b*) Oxidation product obtained crystalline, see Experimental.
(*c*) Prepared by the method of Liebermann and Lindenbaum.[22]
(*d*) Prepared in very low yield by allowing *o*-benzoquinone to decompose spontaneously in chloroform solution (Jackson and Koch).[23]
(*e*) Determined on sample dried at 90° C.

Preparation of 2:4:5:2′:4′:5′-*Hexahydroxydiphenyl.*[24] Triacetoxybenzene (50·4 g.) was hydrolysed by heating for 15 minutes with 10 per cent. sulphuric acid (300 ml.). The mixture was then cooled and added to a suspension of benzoquinone (10·8 g.) in 10 per cent. sulphuric acid (200 ml.). After stirring for 6 hours the light blue-grey solid was collected and dried, m.pt. 277° to 280° C. Found: C, 57·2; H, 4·0. $C_{12}H_{10}O_6$ requires C, 57·6; H, 4·0 per cent. Yield 21 g.

Oxidation of Nitrohydroquinone. Nitrohydroquinone (15·5 g.) in water (100 ml.) containing sodium hydroxide (8 g.) was aspirated with

air for 11 hours. The mixture was acidified with concentrated hydrochloric acid (13 ml.) when marked frothing occurred, together with evolution of some nitrous fumes. The precipitated solid (3·5 g.; m.pt. 250° to 260° C. was collected and crystallised from ethyl acetate-light petroleum to give 2:5:2′:5′-tetrahydroxy-3:3′-dinitrodiphenyl, small bright red needles, m.pt. 240° C. (decomp.). Found: N, 9·1; $C_{12}H_8O_8N_2$ requires N, 9·1 per cent.

Acetylation in pyridine solution with acetic anhydride for 1 hour on the water-bath gave *tetra-acetoxydinitro-diphenyl*, yellow shining plates from acetic acid, m.pt. 191° C. Found: C, 50·2; H, 3·4; N, 6·2; $C_{25}H_{16}O_{12}N_2$ requires C, 50·4; H, 3·4; N, 5·9 per cent.

Oxidation of Nitrocatechol. 4-Nitrocatechol (10 g.) in water (150 ml.) containing sodium hydroxide (5·6 g., 2 equivs.) was aspirated with air for 24 hours. Acidification precipitated a brown microcrystalline material (6 g.; m.pt. > 300° C.) which was purified by recrystallisation from ethyl acetate-light petroleum. 2:3:2′:3′-*Tetrahydroxy*-5:5′-*dinitrodiphenyl* formed brown microcrystals, m.pt. > 300° C. (decomp.). Found: C, 46·7; H, 2·5; N, 9·5; $C_{12}H_8O_8N_2$ requires C, 46·8; H, 2·6; N, 9·1 per cent.).

Acetylation with acetic anhydride under reflux for 2 hours gave 2:3:2′:3′-*tetraacetoxy*-5:5′-*dinitrodiphenyl*, small brown needles from aqueous acetic acid, m.pt. 170° C. Found: C, 50·0; H, 3·7; N, 5·8; $C_{20}H_{16}O_{12}N_2$ requires C, 50·4; H, 3·4; N, 5·9 per cent.

Preparation of soil humic acid.[25] The soil was shaken with cold, dilute hydrochloric acid to decompose "humates" and carbonates and then washed free from acid. The residue was extracted with cold 2 per cent. sodium hydroxide in a closed bottle for several days, and the dark extract decanted off and centrifuged free from suspended particles. The "bright" aqueous extract was acidified with hydrochloric acid and the precipitate centrifuged off and shaken with successive portions of cold, 96 per cent. ethanol, until no further coloured material dissolved. The residual humic acid was divided into two portions, which were dried at room temperature and at 90° C. respectively.

Preparation of peat humic acid. Peat was dissolved in cold, 20 per cent. sodium hydroxide and the solution centrifuged free from insoluble matter. The "bright" extract was acidified with hydrochloric acid and the humic acid centrifuged off and washed with water. It was collected and divided into two portions, which were dried at room temperature and at 90° C. respectively.

The humic acids from soil and peat were only partly soluble in the buffer solutions employed for assay.

SUMMARY

1. Evidence is presented to show that the appearance, in solutions of salicylates and gentisates, of inhibiting properties on the depolymerisation of hyaluronic acid by hyaluronidase, may be due to production of compounds of the humic acid type.

2. This view is strengthened by the observation that the naturally-occurring humic acids of soil and peat inhibit the action of the enzyme upon the mucopolysaccharide acid.

3. The humic acids derived from salicylic acid, gentisic acid, and catechol, show high *in vitro* activity as inhibitors.

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