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Synthetic Inhibitors of Hyaluronidase

I. Demonstration of the High Inhibitory Power of some Diphenylmethane and Triphenylmethane Derivatives

L. HAHN and J. FEKETE

Research Laboratory of AB Ferrosan, Malmö, Sweden

Some 25 years ago Duran-Reynals [1] demonstrated in rabbits that vaccinial infection is considerably enhanced by a simultaneous injection of testis extract into the skin. Two years later, Hoffman and Duran-Reynals [2] and McClean [3] showed that the testis extract revealed its enhancing effect by promoting the spread of the virus. The occurrence of spreading factors was later demonstrated in certain pathogenic bacteria [4], in poisonous snakes and in insects [5]. In 1939 Chain and Duthie [6] observed that testis extract contains an enzyme capable of hydrolysing hyaluronic acid. They suggested that the hyaluronidase may be responsible for the spreading properties of the extract. These observations stimulated extensive investigation of aspects bearing on the problem complex *spreading factor-hyaluronidase-hyaluronic acid.*

Already before Chain and Duthie's publication Meyer, Dubos and Smyth [7] had demonstrated the occurrence of a hyaluronic acid splitting enzyme in pneumococcal autolysate. Such hyaluronidases have since been recovered from many other species of pathogenic bacteria. This occurrence of bacterial hyaluronidases together with the knowledge of the presence of hyaluronic acid in the connective tissue, where it is believed to form an essential part of the interfibrillar cement substance, gave rise to wide speculation on a possible relationship between the hyaluronidase-hyaluronic acid system and certain pathological changes, particularly those seen in rheumatic diseases.

A possible way of checking whether any imbalance of the hyaluronidase-hyaluronic acid system is involved in the etiology or course of such diseases, is to study the response, if any, to the administration of inhibitors of hyaluronidase.

The inhibition of hyaluronidase by serum or certain serum fractions has been the subject of much research [8]. It is generally accepted that inhibition is ascribable to thermolabile substances of high molecular weight. Some well-defined chemical compounds such as dicoumarol and rutin are also known to exert an inhibitory action *in vitro* [9], and heparin is claimed to inhibit hyaluronidase activity *in vivo*, too [10].

In 1948 an extensive search for inhibitors of hyaluronidase was started at this laboratory. Below a report will be given of an investigation of the inhibitory effect of a number of organic compounds on the splitting of hyaluronic acid by testis hyaluronidase *.

METHOD

Substrate solution. 2 g sodium hyaluronate prepared from human umbilical cord by the method of Blix [12] were dissolved in 1 litre $M/6.5$ phosphate buffer solution of pH 7. The solution thus prepared was stored in the refrigerator.

Enzyme solution. Hyaluronidase was extracted from the bull's testes and purified by the method earlier described [13]. The stock solution was kept in the refrigerator and dilutions were prepared with isotonic saline once a week for laboratory use. The concentration of the diluted enzyme solution was such that on addition of 0.3 ml enzyme to 2.7 ml substrate solution at 37° C, the viscosity value of the latter was reduced by half in about 2 minutes.

Preparation of the test substances. The substances to be tested for inhibition of hyaluronidase activity were dissolved in distilled water or in dilute solutions of hydrochloric acid or sodium hydroxide and the pH was adjusted to 7. The solutions were made isotonic by the addition of saline.

Method of determination of inhibitory power. The initial viscosity of the substrate solution was determined by adding 0.3 ml isotonic saline to 2.7 ml substrate solution in an Ostwald viscosimeter submerged in a water bath at 37° C and noting the flow time. Enzyme activity was then estimated by the following procedure: 1.8 ml enzyme solution was mixed with 0.2 ml isotonic saline and stored at 37° C. A volume of 0.3 ml of this mixture was added to 2.7 ml of the substrate solution provided in the viscosimeter, through which nitrogen was allowed to bubble in order to secure good intermixture. The flow times were recorded in rapid succession, and on the basis of these recordings the time necessary for the viscosity to decrease by half (half-viscosity time) was calculated. This procedure was also used for determining any inhibitory power of the test substances, but here 1.8 ml enzyme solution was mixed with 0.2 ml of a test solution of arbitrary concentration instead of with isotonic saline. Any inhibitory power was reflected by an increase in the halfviscosity time.

If a substance tested produced such a prolongation, it was retested, but in concentrations as close as possible to that estimated to produce a fivefold increase in the half-viscosity time. The results of these trial and error tests were plotted and a curve was

* Recently, Forrest, Overell, Petrow and Stephenson [11] published an interesting paper on the inhibitory power *in vitro* of oxidation products of gentisic acid.

Table 1.

Compound	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)	Compound	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
Methyl alcohol	< 0.1	4,4′-Dihydroxy-3,3′-dicarboxy-D,L-benzoin	3.8
Propyl alcohol	< 0.1	2,5-Dihydroxyterephtalic acid	1
Sodium formiate	0.1	3,4-Dimethoxy-*a*-carboxystilbene	0.1
Sodium oxalate	< 0.1	2-Iodo-4-amino benzoic acid	0.1
Sodium succinate	< 0.1	4-Methyl-1-(*p*-carboxy-*m*-hydroxy-phenyl)-thiosemi-carbazide	0.2
Acetone	0.4	4-Phenyl-1-(*p*-carboxy-*m*-hydroxyphenyl)-thiosemi-carbazide	2.5
Acetonylacetone	< 0.1	4,4′-Dihydroxy-diphenylsulfone	0.1
Furfurole	0.8	4,4′-Dihydroxy-diphenylsulfone disulfonic acid	0.1
Glucosamine-HCl	< 0.1	5-Formylsalicylic acid	1.8
N-acetylglucosamine	< 0.1	Stearylaminosalicylic acid	2
Pentaacetylglucose	< 0.1	Fluoresceine	8
Ethyl acetate	< 0.1	*β*-Dimethylaminoethyl-benz-hydryleter-hydrochlorid	0.1
2-Aminoethanol	0.5	Inosite	0.5
Urea	< 0.1	Pyridine	0.4
Urethane	< 0.1	Sodium-diethyl-barbiturate	0.4
Sodium diethyl-dithio-carbamate	< 0.1	Alloxan	0.2
Acetoxime	< 0.1	Triamino-hydroxy-pyrimidine sulfate	0.2
Aconitic acid	< 0.1	3-Hydroxy pyridine	0.2
m-Aminophenol	< 0.1	Piperidine	0.2
Pyrocatechol	< 0.1	Isonicotinic acid	0.1
Hydroquinone	0.5	Succinylamino-benzene-sulfonamide	0.2
Phloroglucinol	0.2	Quinoxaline	1
1,2,4-Benzenetriol	< 0.1	Quinoline-HCl	1.2
Salicylic acid	0.2	Quinhydrone	1
3-Hydroxy benzoic acid	0.4	N-iodoethyl-quinoline	< 0.1
4-Hydroxy benzoic acid	< 0.1	Sodium taurocholate	< 0.1
4-Aminosalicylic acid	0.5	D-Gluconic acid	< 0.1
4-Methylaminosalicylic acid	< 0.1	Protocatechualdehyde	1.3
Hippuric acid	0.5	Bis-(4-hydroxy-coumarinyl)-acetic acid	1.2
Gentisic acid	0.8	Methylene-bis-(4-hydroxy-7-carboxycoumarin)	2.5
a-Resorcylic acid	0.4	N,N′-Dianthranilomethane	1.5
β-Resorcylic acid	1.5		
γ-Resorcylic acid	1.9		
2,4,6-Trihydroxy benzoic acid	1.5		
Gallic acid	1.0		
Mandelic acid	0.5		
Saccharin	0.4		
Benzenesulfonic acid	< 0.1		
4-Benzoylaminosalicylic acid	0.1		
3-Carboxy-4-hydroxy-succinic acid monoanilide	0.9		

fitted to the plottings. The concentration necessary to produce a fivefold increase in the half-viscosity time was read off the diagram.

The reciprocal values of these concentrations of the test substances were taken as a measure of their inhibitory effect on hyaluronidase activity. As the absolute values thus obtained vary with the degree of polymerization of the hyaluronic acid and the age of the enzyme solution, all the recordings were compared with those of a standard solution and recalculated as relative units.

RESULTS

A number of organic compounds of various types were tested for any inhibitory action on hyaluronidase activity (Table 1). The inhibitory power of the compounds investigated was expressed in relative units, the inhibitory activity of resorcinol being taken as unity. The inhibitory effect of this substance has been described earlier by Calesnick and Beutner [12]. Under the conditions used in the present investigation resorcinol in a concentration of 0.2 per cent produces a fivefold increase in the half-viscosity time and complete inhibition occurs in a concentration of 0.8 per cent. None of the compounds first tested showed an inhibitory power of more than 8 times that of resorcinol.

In 1949 it was found in collaboration with K.-G. Rosdahl at this laboratory that the inhibitory power of methylene-disalicylic acid is about 40 times as high as that of resorcinol. This finding prompted us to prepare a number of related

Table 2. Hyaluronidase-inhibiting power of condensation products of diphenylmethane type.

Compound condensed with formaldehyde	Condensation product	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
4-Hydroxy benzoic acid	Compound 2	380
Gentisic acid	Compound 7	1 200
α-Resorcylic acid	Compound 8	760
β-Resorcylic acid	Compound 9	400
γ-Resorcylic acid	Compound 19	980
2,5-Dihydroxy-terephtalic acid	Compound 6	2
2,4,6-Trihydroxy benzoic acid	Compound 3	780
Gallic acid	Compound 1	250
4-Methylamino salicylic acid	Compound 11	19
Acetylsalicylic acid	Compound 4	25
o-Thymotic acid	Compound 5	30
Anthranilic acid	Compound 13 [2]	2
Phloroglucinol	Compound 12	— [1]

[1] Saturated solution at pH 7 gives no inhibition.

[2] Prepared from N,N′-dianthranilomethane by rearrangement with hydrochloric acid.

Table 3. Hyaluronidase-inhibiting power of condensation products of triphenylmethane type obtained on reaction of diphenylmethane derivatives with phenol derivatives in the presence of an oxidizing agent.

Diphenylmethane derivative	Phenol derivative	Condensation product	Hyaluronidase-inhibiting power in relative units (Resorcinol = 1)
Compound 2	4-Hydroxy benzoic acid	Compound 20	1 100
Compound 7	Gentisic acid	Compound 21	1 450
Compound 1	Gallic acid	Compound 18	200
Compound 4	β-Resorcylic acid	Compound 10	32
Compound 9	Gallic acid	Compound 16	1 070
Compound 9	Salicylic acid	Compound 15	900
Compound 12	Salicylic acid	Compound 14	750
Compound 12	4-Hydroxy benzoic acid	Compound 17	640

diphenylmethane derivatives by condensation of hydroxy benzoic acids and other benzoic acid derivatives with formaldehyde.

0.2 mole of the substituted benzoic acid was suspended in 90 g 50 per cent sulfuric acid. 0.1 mole of formaldehyde as a 40 per cent solution was added and the mixture was heated on a water bath for 5 hours. Vigorous stirring throughout the reaction insured a thorough intermixture. The reaction mixture was cooled and the precipitate was filtered off and washed with hot water until free from sulfuric acid. The product was then dissolved in a slight excess of diluted sodium hydroxide solution and reprecipitated with diluted sulfuric acid. The precipitate was washed free from mineral acid and dried *in vacuo*.

Most of the condensation products prepared showed pronounced inhibitory activity (Table 2). The condensation product of gentisic acid with formaldehyde proved the most powerful inhibitor of this group. The corresponding derivatives of 2,4,6-trihydroxy benzoic acid, α-, β- and γ-resorcylic acid and 4-hydroxy benzoic acid were also found to exert a strong inhibitory effect.

Further investigation showed that the inhibitory power of most of the condensation products described above is potentiated by substitution of a hydrogen atom of the methylene bridge with a carboxy-hydroxy phenyl group (Table 3).

5 g of sodium nitrite were ground and thoroughly mixed with 10 g of a condensation product of diphenylmethane type and 5 g of a hydroxy benzoic acid. 105 g of concentrated sulfuric acid was then gradually added and the mixture agitated until it gave off red nitrogen oxide gas. The temperature of the mixture was kept below 10° C throughout the reaction. The solution

Table 4.

Compound	Hyaluronidase-inhibiting power in relative units ($resorcinol_{30\ min.} = 1$)	
	after 30 minutes' incubation	after 24 hours' incubation
2	380	900
7	1 200	2 200
8	760	1 200
9	400	920
3	780	1 200
20	1 100	2 100
21	1 450	2 700
16	1 070	2 000
15	900	1 750
14	750	1 520

obtained was poured into cold water. The precipitate thus obtained was filtered off and washed with cold water until free from sulfuric acid. It was then dissolved in a slight excess of diluted sodium hydroxide solution and reprecipitated with diluted sulfuric acid. The precipitate was washed with cold water until free from sulfuric acid and finally with hot water. It was dried in a vacuum desiccator.

In this group, too, the condensation product prepared from gentisic acid was the most active inhibitor.

In order to study any influence of the incubation period on the inhibition of hyaluronidase the mixture of enzyme and inhibitor was sometimes incubated for 24 hours, instead of for 30 minutes, before being added to the substrate. This prolonged incubation was found to produce a varying increase in the inhibition of hyaluronidase by the test substance (Table 4).

The most active compounds were tested for chronic toxicity in rats. Daily oral administration of compounds 3, 7, 14, 19 and 21 in doses of 200 mg/kg bodyweight produced no manifest toxic side effects. A similar daily dose of compounds 15, 16 and 20 caused toxic symptoms in the form of diarrhoea and loss of weight.

COMMENTS

The investigation of various groups of organic compounds for any inhibitory effect on testis hyaluronidase activity showed that certain diphenylmethane and triphenylmethane derivatives built up of nuclei substituted with 1—3 hydroxy groups are powerful inhibitors of hyaluronidase. On the other hand, corresponding uninuclear compounds such as phenols, mono-, di- and tri-

hydroxy benzoic acids inhibited hyaluronidase only when they were used in such concentrations as would cause denaturation of proteins.

Knowledge of new diphenylmethane and triphenylmethane derivatives with a strong inhibitory effect on hyaluronidase and only slight toxicity opens up a new approach to the treatment of diseases characterized by pathological changes in the ground substance of the mesenchymal tissues. It seems reasonable to suppose that these inhibitors may be able to control any imbalances of the hyaluronidase-hylauronic acid system of the body due to (a) hyaluronidase produced by infective agents, particularly bacteria (b) disturbances of the hyaluronic acid metabolism or (c) insufficient production of hyaluronidase inhibitors by the body.

In infections with hyaluronidase-producing bacteria, the spread of these micro-organisms in the body is facilitated by the hydrolytic action of the bacterial hyaluronidase on the hyaluronic acid in the interfibrillar substance of the connective tissue. Furthermore, the resultant increase in the permeability of the connective tissue invites invasion by other nonhyaluronidase-producing micro-organisms. This suggests that the inhibitors may also be of value in the prophylaxis and therapy of certain bacterial and viral infections.

SUMMARY

More than 90 organic compounds were investigated for any inhibitory effect on testis hyaluronidase *in vitro*. As standard use was made of resorcinol, whose inhibitory power was taken as unity. Resorcinol produced practically complete inhibition when employed in a concentration of 0.8 per cent.

Salicylic acid, gentisic acid, γ-resorcylic acid and other hydroxy benzoic acids showed an inhibitory power of less than 2. Diphenylmethane derivatives obtained by condensation of dihydroxy and trihydroxy benzoic acids with formaldehyde proved strong inhibitors. Of these, the condensation product of gentisic acid (compound 7) possessed the strongest inhibitory power (1 200 rel. units). The corresponding derivative of 4-hydroxy benzoic acid showed also a pronounced inhibitory activity.

Triphenylmethane derivatives were prepared by substitution of condensation products of diphenylmethane type in the methylene bridge with hydroxy-carboxy phenyl radicals. The inhibitory capacity of three of these compounds exceeds 1 000 units. The most active derivative of this group was the one made up of gentisic acid (compound 21), which showed an inhibitory power of 1 450. Compound 7 and compound 21 produced no demonstrable toxic effects in rats on oral administration for 30 days in total daily doses of 200 mg/kg bodyweight.

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