Brit. J. Pharmacol. (1953), 8, 30.

INVESTIGATION *IN VIVO* OF NEW INHIBITORS OF HYALURONIDASE

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(RECEIVED JULY 15, 1952)

New polymeric condensation products containing diphenylmethane and triphenylmethane units and possessing the power to inhibit hyaluronidase *in vitro* were recently described by one of us (Hahn, 1952). The present report is concerned with the inhibitory effect of some substances of this type *in vivo* as well as their anti-inflammatory action, as measured by the reduction of oedema produced by injection of egg-white. The compounds were also studied for toxicity.

MATERIALS AND METHODS

We selected for test four *triphenylmethane derivatives,* polycondensed trihydroxytricarboxytriphenylmethane (" Tri-*para*hydroxybenzoic acid," Compound 20 P), polycondensed hexahydroxytricarboxytriphenylmethane (" Trigentisic acid," Compound 21 P), and two polycondensed heptahydroxytricarboxytriphenylmethanes (" Di-β-resorcylic-gallic acid," Compound 16 P, and " Digentisic-gallic acid," Compound 30), as well as two *diphenylmethane derivatives,* polycondensed dihydroxydicarboxydiphenylmethane (" Di-*para*hydroxybenzoic acid," Compound 2 P) and polycondensed tetrahydroxydicarboxydiphenylmethane (" Digentisic acid," Compound 7 P). For purposes of comparison two simple hydroxybenzoic acids, *p*-hydroxybenzoic acid and gentisic acid, were also tested.

The diphenylmethane derivatives were prepared by condensing hydroxybenzoic acids with formaldehyde under conditions favouring the formation of products of fairly high molecular weight. The triphenylmethane derivatives were obtained by substitution in the methylene groups of polycondensed diphenylmethane derivatives by hydroxycarboxyphenyl radicals.

Measurement of Inhibitory Power in Vitro.—The *in vitro* activity was determined viscosimetrically with resorcinol as standard. A solution of testes hyaluronidase, extracted and purified by the method of Hahn (1943), was mixed with a neutral solution of the substance to be tested. After 30 minutes' incubation the mixture was added to a 0.2% solution of hyaluronic acid from umbilical cord, which was adjusted to *p*H 7 by means of phosphate. Readings were taken at 37° C. after different intervals. That concentration of the compound that was necesssary to reduce the rate of hydrolysis of hyaluronic acid by hyaluronidase to one-fifth was determined. This concentration was found to be 0.2% for resorcinol. A corresponding effect can be obtained with a 1% solution of sodium salicylate.

Measurement of Inhibitory Power in Vivo.—The inhibitory effect was estimated by a method based on the action of hyaluronidase on the rate of absorption of drugs injected subcutaneously. It permitted quantitative determination of the minimum dose capable of producing practically complete inhibition.

Female mice weighing 20–22 g. were used. The animals were of the same breed, they had been brought up on a uniform diet under identical conditions, and the environmental temperature was maintained at 21–22° C. All experiments were performed on groups of 5 mice between noon and 6 p.m. Urethane in a dose of 1.2 mg./g. body weight was injected subcutaneously as a 10% aqueous solution, and the interval was noted between the injection and the production of anaesthesia, which was said to be complete when the animal offered no resistance to being placed on its side. This interval will be referred to as the " absorption time of urethane." (It also includes the time necessary for urethane to exert its effect on the brain cells.)

A dose of 0.01 ml./g. body weight of a hyaluronidase solution containing 380 TRU/ml. was injected into one of the tail veins, the intravenous route being chosen in order to obtain a generalized effect of the hyaluronidase. Two minutes later the animals received urethane subcutaneously. The dose of hyaluronidase given was sufficient markedly to decrease the absorption time of urethane.

The inhibitory activity of the substances tested was assessed by their power to counteract the increase in the rate of absorption of urethane produced by hyaluronidase. They were dissolved in a dilute solution of NaOH and the *p*H was adjusted to 7. Forty to fifty minutes before the injection of the hyaluronidase a varying dose of these solutions was administered subcutaneously to some mice and by mouth to others.

The minimum dose necessary to inhibit completely the reduction by hyaluronidase of the absorption time of urethane is expressed as M.I.D.$_{100}$. Every dose level used was tried on two groups of mice. As the M.I.D.$_{100}$ was approached the number of groups was increased to four. Control tests using either urethane only or hyaluronidase and urethane were performed simultaneously.

The activity of the inhibitors was also checked by the skin-spreading method on rabbits (Duran-Reynals, 1942).

Anti-inflammatory Effect.—This was assessed by the power to depress artificial oedema produced by the injection of egg-white into female rats weighing 60–90 g. (Selye, 1937; Halpern, 1949; Gross, 1950; Wilhelmi and Domenjoz, 1950). Fresh egg-white (0.1 ml.) was injected subcutaneously into the dorsal side of one hind paw and an equal quantity of saline into the other. Ninety minutes later the animals were killed with ether. Both hind legs were removed at the knee joint and weighed. The difference in weight was taken as a measure of the oedema produced by the egg-white.

An arbitrary dose of a solution of the inhibitor to be tested was injected subcutaneously, and again 30 minutes later. Immediately after the second injection 0.1 ml. egg-white was injected into the hind paw. The smallest dose of the inhibitor needed to diminish the oedema produced by egg-white was determined by trial and error. As the inflammatory action of egg-white varies from one egg to another, simultaneous control tests were always made with the white of the same egg. Every dose was tried in two or three groups of 5 rats each.

Toxicity.—The substance to be tested was emulsified in an aqueous solution of gum arabic and sugar. The concentration of the emulsion varied from 2 to 30% according to the dose used. Single doses of 0.01–0.05 ml./g. body weight of the emulsion were given by stomach tube to female mice weighing 20–24 g. As a rule six dosage levels were studied, each on six mice. The animals were then carefully observed for one week. When deaths occurred, they usually did so within 48 hours. The LD50 values were determined by the method of Behrens-Kärber.

In chronic toxicity studies the substances were given by mouth to groups of 10 animals. Female mice weighing 20–24 g., rats weighing 80–140 g., guinea-pigs weighing 250–300 g., and rabbits weighing 1.8–2.5 kg. were used. The substances were administered daily for 30 days. The volume of the daily dose of the emulsion was kept within 0.01–0.02 ml./g. body weight.

The substances were given to man in the form of tablets. Groups of 5 apparently healthy volunteers received two daily doses of 0.5 g. the first week, four daily doses of 0.5 g. the second week, four daily doses of 1 g. the third week, four daily doses of 1.5 g. the fourth week, and four daily doses of 2 g. the fifth week.

TABLE I
INHIBITION OF HYALURONIDASE AND TOXICITY

Substances	Inhibitory Power *in vitro* in Relative Units (Resorcinol =1)	Hyaluronidase Inhibition *in vivo* M.I.D.$_{100}$		Acute Toxicity LD50 Per os μg./g.
		s.c. μg./g.	Per os μg./g.	
No. 21 P "Trigentisic acid"	2,485	10	50	>20.000
No. 20 P "Tri-*para*hydroxybenzoic acid"	1,275	25	75	2.700
No. 30 "Digentisic-gallic acid"	2,275	25	75	12.500
No. 16 P "Di-β-resorcylic-gallic acid"	1,300	50	200	5.400
No. 7 P "Digentisic acid"	2,000	50	200	10.000
No. 2 P "Di-*para*hydroxybenzoic acid"	400	150	750	4.500
Gentisic acid	0.8	—*	—*	4.500
p-Hydroxybenzoic acid	0.1	—*	—*	4.800

* No effect in tolerated doses.

RESULTS

The *in vitro* activity† of the compounds investigated is given in Table I.

Inhibition of the Increase by Hyaluronidase of the Rate of Absorption of Urethane

In control experiments on 265 mice the absorption time of urethane administered subcutaneously was found to be 20.4 ± 4.17 minutes. The corresponding values for 240 mice pretreated with hyaluronidase were 8.7 ± 1.64. The hyaluronidase thus decreased the absorption time by about half. This difference was highly significant ($P<0.01$).

All the polycondensed substances tested reduced the action of hyaluronidase (Table I). Inhibition was said to be complete when the absorption time for the hyaluronidase treated animals exceeded 16 minutes. The triphenylmethane derivative, "Trigentisic acid," showed greatest activity. A dose of 10 μg./g. injected subcutaneously or 50 μg./g. administered by mouth was sufficient to abolish the effect of hyaluronidase injected intravenously. Of the two diphenylmethane derivatives the more active was "Digentisic acid." The inhibitory power of "Di-*para*hydroxybenzoic acid" was much weaker and only slightly stronger than that of heparin. The inhibitory action of heparin on hyaluronidase has been described by earlier workers (McClean, 1942; Rogers, 1946) and was included here only for purposes of comparison. The M.I.D.$_{100}$ of heparin, when administered subcutaneously, was found to be 200 μg./g. Tolerable doses (max. 1,000 μg./g.) of gentisic acid and *p*-hydroxybenzoic acid produced no signs of inhibition.

† The *in vitro* experiments were performed in co-operation with J. Fekete and E. Frank. A detailed report is in preparation.

The dose-response curves for the various inhibitors are given in Fig. 1, from which it is clear that the effect increased with increasing dosage only up to the level of the M.I.D.$_{100}$. Every substance was also tested in animals not treated with hyaluronidase. In these control experiments we used a dose of the inhibitors five times as large as the M.I.D.$_{100}$. The purpose of these experiments was to determine whether the substances influence the rate of absorption of urethane and whether they exert any analeptic or anaesthetic action. None of the compounds tested showed any such effects.

Inhibition of the Skin-spreading Effect of Hyaluronidase

Although the skin-spreading technique is less suitable for quantitative determinations, the inhibitory effect of these new polycondensed products was checked by the skin-spreading technique, and it was found that an oral dose of 500 μg./g. of any of them was sufficient markedly to inhibit the skin-spreading effect of hyaluronidase.

Anti-inflammatory Action

Various doses of the following substances were tested for their anti-inflammatory action, as measured by the reduction of oedema produced by the injection of egg-white into rats: "Trigentisic acid," "Tri-*para*hydroxybenzoic acid," "Digentisic acid," "Di-*para*hydroxybenzoic acid," gentisic acid, and *p*-hydroxybenzoic acid. The results are given in Table II.

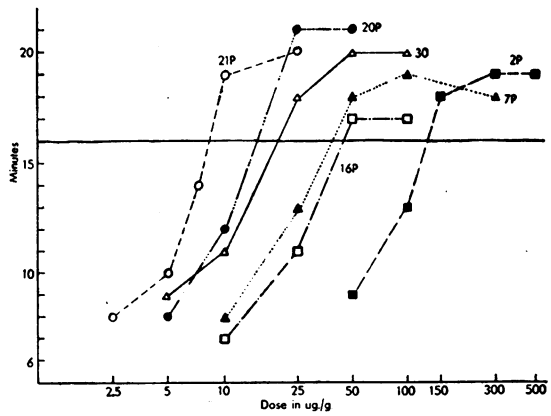

FIG. 1.—Dose-response curves for polycondensed diphenylmethane and triphenylmethane derivatives injected subcutaneously. Ordinate: rate of absorption of urethane (1.2 mg./g. s.c.) given two minutes after hyaluronidase (3.8 TRU/g. i.v.), as judged by the onset of anaesthesia. Abscissa: dose of inhibitor (μg./g.). No. 21 P "Trigentisic acid." No. 20 P "Tri-*para*hydroxybenzoic acid." No. 30 "Digentisic-gallic acid." No. 16 P "Di-β-resorcylic-gallic acid." No. 7 P "Digentisic acid." No. 2 P "Di-*para*hydroxybenzoic acid."

TABLE II

EFFECT ON THE OEDEMA PRODUCED BY INJECTION OF EGG-WHITE INTO THE HIND PAWS OF RATS
Each inhibitor was given subcutaneously in two doses, the first dose half an hour before, and the second dose simultaneously with, the injection of 0.1 ml. egg-white. Neither gentisic acid nor *p*-hydroxybenzoic acid had any action in these doses.

Dose s.c. μg./g.	% Depression of Oedema			
	No. 21 P "Trigentisic Acid"	No. 20 P "Tri-*para*hydroxybenzoic Acid"	No. 7 P "Digentisic Acid"	No. 2 P "Di-*para*hydroxybenzoic Acid"
2×5	0	0	0	0
2×12.5	25	0	0	0
2×25	32	16	14	0
2×50	36	30	29	0
2×100	35	36	35	33
2×250	41	32	33	35
2×500	37	40	38	33

Doses of about the same size as those which just inhibited hyaluronidase *in vivo* reduced the oedema by 25–45%. Larger doses produced no further reduction. The minimum effective dose of "Trigentisic acid," was 2×12.5 μg./g.; the corresponding doses for "Tri-*para*hydroxybenzoic acid" and for "Digentisic acid" were 2×50 μg./g. and for "Di-*para*hydroxybenzoic acid" 2×100 μg./g. "Di-β-resorcylic-gallic acid" and "Digentisic-gallic acid" were only tried in doses of 2×250 μg./g. and more, and produced a pronounced anti-inflammatory effect. Gentisic acid and *p*-hydroxybenzoic acid had no obvious effect on the inflammatory reaction, even in doses of 2×500 μg./g.

Toxicity

The acute oral toxicity values are given in Table I. "Trigentisic acid" was least toxic. Daily doses of 200 μg./g. for 30 days of "Trigentisic acid," "Digentisic-gallic acid," and "Digentisic acid" produced no manifest signs of intoxication. "Trigentisic acid" was also administered daily in a dose of 400 μg./g. for 30 days without toxic effects. A daily dose of 200 μg./g. "Di-β-resorcylic-gallic acid," "Tri-*para*hydroxybenzoic acid," and "Di-*para*hydroxybenzoic acid" caused diarrhoea and moderate loss of weight.

In man "Trigentisic acid" and "Digentisic acid" were given daily in doses increasing up to 8 g. and were well tolerated. In corresponding trials with "Di-β-resorcylic-gallic acid" a daily dose of 6 g. caused nausea and diarrhoea in some volunteers. These side-effects were also produced by a daily dose of 4 g. "Di-*para*hydroxybenzoic acid."

DISCUSSION

All the polycondensed diphenylmethane and triphenylmethane derivatives possessing the power to inhibit hyaluronidase *in vitro* and studied in the

present investigation were found to be highly inhibitory *in vivo*, too. On the other hand, gentisic acid and *p*-hydroxybenzoic acid, which were only one-thousandth to one-hundredth as active *in vitro* as the polycondensed products, appeared to exert no inhibitory effect *in vivo*. A question that naturally presents itself is whether the inhibitory activity of the polycondensed compounds *in vivo* bears any quantitative relationship to their activity *in vitro*.

" Trigentisic acid " showed greatest activity both *in vitro* (2,485 rel. units) and *in vivo* ($M.I.D._{100}$, 10 μg./g. s.c.). " Di-*para*hydroxybenzoic acid " showed least activity *in vitro* (400 rel. units) and *in vivo* ($M.I.D._{100}$, 150 μg./g. s.c.). The *in vitro* activity of the remaining compounds varied between 1,275 and 2,275 relative units and the $M.I.D._{100}$ *in vivo* between 25 and 50 μg./g. s.c. Within these limits no quantitative relationships were observed between the *in vitro* and the *in vivo* activities of the compounds. In evaluating these observations the possible influence of variation in the rate of absorption, breakdown, and excretion, and other factors liable to blur the picture, must be borne in mind.

As to the anti-inflammatory effect it should be stressed that the polycondensed compounds tested reduced artificial oedema in rats and that the smallest dose capable of producing maximum reduction of the oedema was approximately equal to that required completely to abolish hyaluronidase activity *in vivo*. This minimum dose reduced the inflammatory reaction by 30 to 40%, and a ten- to twenty-fold increase of the dose caused no further depression. These observations suggest that the anti-inflammatory action of the substances is related to their inhibitory effect on hyaluronidase and that hyaluronidase activity is involved in the mechanism of inflammation, at least of the type described here.

Since these compounds can affect hyaluronidase activity and inflammation *in vivo*, it may be that they can exert the same effect in those pathological conditions in which hyaluronidase is assumed to be involved. So far nothing is known of the action of inhibitors on the hyaluronidase normally present in the body. The fact that the synthetic inhibitors did not retard the absorption of urethane in normal animals that had not received hyaluronidase does not necessarily mean that they do not inhibit the hyaluronidase normally present in the body ; it is possible that body hyaluronidase plays so small a rôle in absorption that any elimination of its effect does not produce demonstrable changes in the rate of absorption of urethane, for instance. It is also possible that normally the body hyaluronidase is practically completely inhibited, or that the subcutis, capillaries, and the blood-brain barrier contain no hyaluronidase.

As some of the substances described possessed low toxicity and a marked power to inhibit hyaluronidase *in vivo*, with an anti-inflammatory effect, they may open a new approach to the therapy of pathological conditions in which the hyaluronic acid-hyaluronidase system is involved. From a therapeutic point of view " Trigentisic acid " seems to be the most promising compound. It showed not only greatest inhibitory power *in vitro* and *in vivo*, but also the lowest toxicity in animals and in man. In animal experiments doses 400 times larger than the $M.I.D._{100}$ were well tolerated.

SUMMARY

1. Six new polycondensed diphenylmethane and triphenylmethane derivatives possessing a strong inhibitory effect *in vitro* on hyaluronidase were tested for their inhibitory effect *in vivo*, for their anti-inflammatory action, and for their toxicity.

2. All of the substances were found to possess marked *in vivo* activity as judged by their inhibition of the effect of hyaluronidase on absorption of urethane injected subcutaneously into mice. The most active substance, Compound 21 P (" Trigentisic acid "), practically completely inhibited hyaluronidase *in vivo* in a subcutaneous dose of 10 μg./g. and in an oral dose of 50 μg./g. body weight.

3. All the six polycondensed substances reduced the artificial oedema caused by the injection of egg-white in rats. A certain parallelism was found between the intensity of this anti-inflammatory action and the inhibition of hyaluronidase.

4. Toxicity tests showed Compound 21 P (" Trigentisic acid ") to be least toxic to animals. LD50 by mouth in mice was more than 20 mg./g. Daily oral doses of 0.4 mg./g. for 30 days produced no signs of intoxication in mice, rats, guinea-pigs, and rabbits. In human beings a daily oral dose of 8 g. produced no side-effects.

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