Anti-histamine Effect of Sodium Salicylate and its Bearing upon the Skin-diffusing Activity of Hyaluronidase

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It was claimed by Guerra (1946 *a, b*) that the administration of sodium salicylate to rabbits in a dosage of 0.1 g./kg. body weight inhibited the skin-diffusing activity of hyaluronidase to a significant extent. Swyer (1948), on the other hand, failed to find any inhibitory effect of salicylates on the viscosity-reducing activity of hyaluronidase except when the concentration of the former reached such high levels that the accompanying reduction of pH itself caused inhibition of the enzyme action. In an attempt to account for this discrepancy between the *in vivo* and *in vitro* results, it was suggested that the hyaluronidase preparation used by Guerra might have contained histamine, or some substance with a similar action, which, by increasing the capillary permeability, would have enhanced the spreading effect of the hyaluronidase. The further suggestion was made that salicylate might have acted as a histamine antagonist, thereby reducing the spreading action of the hyaluronidase preparation. In order to investigate the above suggestion, a study, the results of which are reported in this paper, was made of the effect of salicylate on the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom, together with its effect on the capillary-damaging action of these substances, and also of histamine alone and when mixed with hyaluronidase.

METHODS

Two experiments, similarly planned, were made: the first tested the spreading action and capillary-damaging effect of bull-testis hyaluronidase and snake venom before and after the administration of salicylate; the second tested the spreading effect of hyaluronidase and of hyaluronidase together with histamine, and the capillary-damaging action of histamine alone, before and after the administration of salicylate. In each experiment three albino rabbits weighing between 2 and 3.3 kg. were used. They were depilated several hours before the commencement of the experiment by means of a barium sulphide paste. Each rabbit received an intravenous injection of pontamine blue, 5% (w/v) in saline, 0.6 ml./kg. body weight, followed 15 min. later by the intradermal injection in 0.2 ml. quantities of the substances to be tested, the injections being confined to the left flank. Two injections of each substance were made in each rabbit, four substances being tested at a time, the arrangement of injections being completely randomized in eight approximately square areas marked out on the

rabbits' flanks. Injections containing India ink in approximately 10% concentration were used to show up the spreading action, while injections devoid of India ink were used to demonstrate the capillary-damaging effect, manifested by the appearance of the blue dye. The average diameters of the areas of blue dye or India ink respectively were measured 30 min. after the intradermal injections. The rabbits were then given intravenous injections of sodium salicylate in 10% (w/v) solution, each receiving 0.1 g./kg. body weight. After 15 min., the intradermal injections were repeated on the right flank, the results being read after a further 30 min. as before.

In Exp. 1 the substances tested were bull-semen hyaluronidase, 10 units (Swyer & Emmens, 1947) in 200 ml. saline (0.1 ml. + 0.1 ml. saline or 0.1 ml. India ink 20% v/v in saline) and rattlesnake venom, 14 mg. in 40 ml. saline, this solution having the same viscosity-reducing activity as that of the bull hyaluronidase (0.1 ml. + 0.1 ml. saline or 0.1 ml. India ink). The arrangement of injections is illustrated in Table 1.

Table 1. *Illustration of arrangement of intradermal injections in Exp. 1*

(a) Mixtures			
Substance		Symbol	
Hyaluronidase + saline		1, 3	
Hyaluronidase + India ink		2, 4	
Snake venom + saline		5, 7	
Snake venom + India ink		6, 8	

(b) Positions			
Head			
Before salicylate		After salicylate	
7	6	8	1
3	2	7	3
5	8	5	6
1	4	2	4

In Exp. 2 the substances tested were bull-semen hyaluronidase as in Exp. 1 containing 20% (v/v) India ink (0.1 ml. + 0.1 ml. saline); histamine diphosphate 0.1 mg./ml. (0.1 ml. + 0.1 ml. saline); hyaluronidase + histamine (0.1 ml. of each); and saline (0.2 ml.).

Reading the results was complicated by the difficulty of separating, in some of the cases, the effect produced by the spread of India ink from that of staining with pontamine

blue caused by increased capillary permeability. It was decided, therefore, to measure in each case the maximum blue area regardless of whether due to India ink or pontamine blue. That this was a satisfactory criterion of the effects under consideration is shown by the analysis of variance (Tables 3 and 7).

RESULTS

Experiment 1. Bull-testis hyaluronidase and snake venom, before and after salicylate

The mean diameters of stained areas produced by these substances, and read as described above, are shown in Table 2. The analysis of variance for this experiment is set out in Table 3, from which it may be concluded that (1) there are significant differences in the reactions of different rabbits; (2) the responses to hyaluronidase and to snake venom show highly significant differences, and (3) the overall effect of salicylate is highly significant. In addition, the differential response with India ink and with pontamine blue to hyaluronidase is significantly different from that to snake venom, as is the differential

response before and after salicylate to the two substances.

In order further to analyze these differential responses, the results were split so as to furnish analyses of variance for hyaluronidase and for snake venom separately; these are set out in Tables 4 and 5 respectively. From Table 4 it can be seen that the difference in response of pontamine blue and India ink to hyaluronidase is highly significant; in other words, as appears from Table 2, the hyaluronidase preparation had no significant effect on the pontamine blue reaction. The difference in response to hyaluronidase before and after the administration of salicylate is seen not to be significant. In the case of the response to snake venom, Table 5 shows that there was again a significant difference of response of pontamine blue and of India ink, though reference to Table 2 shows that snake venom, in contrast to the hyaluronidase preparation, produced a marked pontamine blue reaction. There was, moreover, a highly significant reduction of response to snake venom after the administration of salicylate.

Table 2. Mean diameters in millimetres of stained skin areas in Exp. 1

Rabbit	Hyaluronidase + saline		Hyaluronidase + India ink		Snake venom + saline		Snake venom + India ink	
	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	0	5	12	7	24	20	20	17
	7	8	9	8	27	18	15	13
2	4	0	7	5	20	(14)	11	7
	0	0	9	5	22	12	10	9
3	0	0	10	6	24	22	15	10
	0	0	6	9	22	9	15	7
Totals	11	13	53	40	139	95	86	63

The value (14), missing from the data, was replaced by the method of least squares and one degree of freedom was dropped in the analysis of variance.

Table 3. Analysis of variance for Exp. 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	188.5417	94.2709	11.98	<0.001*
Hyaluronidase v. snake venom (B)	1	1474.0834	1474.0834	187.31	<0.001*
Pontamine blue v. India ink (C)	1	5.3334	5.3334	—	—
Before salicylate v. after salicylate (D)	1	126.7501	126.7501	16.11	<0.001*
Interactions					
A/B	2	18.2916	9.1458	1.16	>0.05
A/C	2	3.7916	1.8958	—	—
A/D	2	6.1249	3.0625	—	—
B/C	1	494.0832	494.0832	62.78	<0.001*
B/D	1	65.3332	65.3332	8.30	>0.001, * <0.01
A/B/C	2	14.0414	7.0207	—	—
A/B/D	2	8.0418	4.0209	—	—
A/C/D	2	5.3751	2.6876	—	—
B/C/D	1	27.0002	27.0002	3.43	>0.05
A/B/C/D	2	9.1252	4.5626	—	—
Error	23	181.0000	7.8696	—	—
Total	46	2627.6667	—	—	—

* Highly significant.

Table 4. *Analysis of variance for responses to hyaluronidase in Exp. 1*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	54.25	27.125	5.76	>0.01, <0.05
Pontamine blue v. India ink (C)	1	198.375	198.375	42.13	<0.001*
Before salicylate v. after salicylate (D)	1	5.0416	5.0416	1.07	>0.05
Interactions					
A/C	2	14.25	7.125	1.51	>0.05
A/D	2	7.5834	3.7917	—	—
C/D	1	9.3751	9.3751	1.99	>0.05
A/C/D	2	9.2499	4.6250	—	—
Error	12	56.5	4.7083	—	—
Total	23	354.625	—	—	—

* Highly significant.

Table 5. *Analysis of variance for responses to snake venom in Exp. 1*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	152.5833	76.2917	6.74	>0.01, <0.05
Pontamine blue v. India ink (C)	1	301.0416	301.0416	26.60	<0.001*
Before salicylate v. after salicylate (D)	1	187.0416	187.0416	16.53	>0.001, <0.01*
Interactions					
A/C	2	3.5834	1.7917	—	—
A/D	2	6.5834	3.2917	—	—
C/D	1	18.3751	18.3751	1.62	>0.05
A/C/D	2	5.2499	2.6250	—	—
Error	11	124.5000	11.3182	—	—
Total	22	798.9583	—	—	—

* Highly significant.

If one accepts the pontamine blue reaction as a reliable criterion of increased capillary permeability, the conclusion to be reached from the above results is (1) that a satisfactory comparison has been made of the action on capillaries and the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom; (2) that salicylate had no effect on the skin-diffusing activity of hyaluronidase but did significantly reduce both the capillary-damaging and the skin-diffusing activities of snake venom.

Experiment 2. Hyaluronidase alone and with histamine, before and after salicylate

In view of the results obtained in Exp. 1, it was considered worth while to compare the spreading action of hyaluronidase with a hyaluronidase-histamine mixture, both before and after the administration of salicylate, since it seemed probable that part at least of the responses to snake venom could be attributable to the presence of histamine in the venom. By means of the technique previously described and used in Exp. 1, it was possible to test at one and the same time the effect of histamine, alone and in combination with hyaluronidase, on

capillary permeability before and after the administration of salicylate.

The results of this experiment, given as mean diameters of stained areas, are shown in Table 6. The analysis of variance is set out in Table 7, from which it may be concluded (1) that differences between rabbits were significant, and (2) that the overall effects of hyaluronidase, histamine, and salicylate, were all highly significant. Furthermore, there was no significant interaction between histamine and hyaluronidase, which implies that their effects were strictly additive; the interaction between hyaluronidase and salicylate was significant but not great, while, in contrast, that between histamine and salicylate was highly significant. The somewhat artificial reduction in the sum of squares used as the error term by the inclusion of the six pairs of zeros contributed by saline alone accounts for the significance of the hyaluronidase and salicylate interaction. The other significant effects would, of course, remain significant with a much larger error term.

It is therefore to be concluded that the spreading action of hyaluronidase is increased in the predicted manner by the presence of histamine, and that, as in Exp. 1, salicylate scarcely influenced the spreading action of hyaluronidase, while, as can be seen by

Table 6. Mean diameters in millimetres of stained skin areas in Exp. 2

Rabbit	Hyaluronidase + India ink		Hyaluronidase + histamine + India ink		Histamine + saline		Saline	
	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	9	12	17	6	17	0	0	0
	7	9	30	11	19	0	0	0
2	9	9	25	12	19	0	0	0
	9	9	22	14	19	0	0	0
3	6	7	17	6	15	0	0	0
	8	10	20	7	14	0	0	0
Totals	48	56	131	56	103	0	0	0

Table 7. Analysis of variance for Exp. 2

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	45.7917	22.8959	4.43	>0.01, <0.05
Hyaluronidase (B)	1	736.3334	736.3334	142.52	<0.001*
Histamine (C)	1	720.7501	720.7501	139.50	<0.001*
Salicylate (D)	1	602.0834	602.0834	116.53	<0.001*
Interactions					
A/B	2	11.7916	5.8958	1.14	>0.05
A/C	2	22.8749	11.4375	2.21	>0.05
A/D	2	4.2916	2.1458	—	—
B/C	1	8.3332	8.3332	1.61	>0.05
B/D	1	26.9999	26.9999	5.23	>0.01, <0.05
C/D	1	720.7499	720.7499	139.50	<0.001*
A/B/C	2	3.0418	1.5209	—	—
A/B/D	2	2.6251	1.3126	—	—
A/C/D	2	7.8751	3.9376	—	—
B/C/D	1	8.3335	8.3335	1.61	>0.05
A/B/C/D	2	10.0415	5.0208	—	—
Error	24	124.0000	5.1667	—	—
Total	47	3055.9167	—	—	—

* Highly significant.

reference to Table 6, salicylate in the dosage used completely inhibited the capillary-damaging action of histamine diphosphate injected in 0.01 mg. amounts. The skin-diffusing action, moreover, of a hyaluronidase-histamine mixture, was reduced, by means of salicylate, to that of the hyaluronidase component alone.

DISCUSSION

Allusion has already been made to the discrepancy between the *in vivo* inhibitory effect of salicylate on the skin-diffusing activity of hyaluronidase described by Guerra (1946*a, b*) and the failure of *in vitro* inhibition of viscosity-reducing activity reported by Swyer (1948), as well as to the tentative explanation offered. The evidence presented in this paper regarding the potentiation of hyaluronidase skin-diffusing activity by histamine and its inhibition by salicylate lends substantial support to such an explanation. At the same time, the effect of salicylate in preventing increase of capillary permeability

by histamine is in itself a finding of considerable interest.

If, as seems to be clear, the anti-rheumatic action of salicylates is not due, as Guerra proposed, to their alleged inhibitory effect on hyaluronidase, the question naturally arises as to whether it can be ascribed to their anti-histamine properties. This problem is briefly discussed by Swyer (1948), but the conclusion is reached that the evidence available in the literature on the effects of salicylate on immunological reactions, though fairly extensive, is scarcely sufficient to afford a solution. At the same time, however, such an interesting possibility cannot be excluded.

SUMMARY

1. Using factorial tests on rabbits, comparisons have been made of the skin-diffusing activities of hyaluronidase prepared from bull semen and rattlesnake venom, and their effects in increasing capillary permeability as evidenced by the pontamine blue reaction, both before and after the administration of

sodium salicylate. In the same way, the effect of salicylate on the skin-diffusing and capillary-damaging actions of hyaluronidase, histamine and a hyaluronidase-histamine mixture have been studied.

2. The hyaluronidase preparation had no significant effect on capillary permeability, and its spreading action was not affected by salicylate. Snake venom, on the other hand, had a marked capillary-damaging effect which, along with its spreading action, was significantly reduced by salicylate. Histamine increased the spreading action of

hyaluronidase in a strictly additive fashion, and its capillary-damaging action was inhibited by salicylate to a highly significant extent; salicylate reduced the spreading action of a hyaluronidase-histamine mixture to that of the hyaluronidase component alone.

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Failure of *in vitro* Inhibition of Hyaluronidase by Salicylates

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Guerra (1946 *a, b*) claimed that sodium salicylate in doses of 0.07 and 0.10 g./kg. caused 57 and 66% reduction of the skin diffusion effect of hyaluronidase in rabbits. Moreover, he reported that in humans who had active rheumatic fever, or had suffered it, intradermal injections of hyaluronidase with Evans blue gave 'unique reactions with enormous diffusion of the dye and local oedema that sometimes occupies the arm injected with hyaluronidase'. Salicylates in these cases inhibited the enzyme and reduced its spreading effect in connective tissues. On the basis of these findings he believed that hyaluronidase played an important part in the pathogenesis of rheumatic fever and that the anti-rheumatic action of salicylates could be explained by their inhibition of the enzyme.

Since considerable and increasing attention is being paid to these reports, it seems proper to record the results of some experiments on the *in vitro* effect of salicylates on hyaluronidase action, in which, using a viscosimetric technique, the accuracy of which is far greater than that employing skin diffusion, no direct inhibitory effect was observed except with relatively enormous salicylate concentrations. At the same time, the action of salicylates will be compared with that of heparin, a true hyaluronidase inhibitor (McClean, 1942; Rogers, 1946).

METHODS

The effects of sodium salicylate, acetylsalicylic acid and heparin on the activity of hyaluronidase in decreasing the viscosity of buffered solutions of potassium hyaluronate were studied by a modification of the hyaluronidase assay technique described by Swyer & Emmens (1947). The enzyme preparation was the standard freeze-dried bull-semen filtrate described by those authors. Activity of the enzyme is expressed by the flow-time index, which has been shown to be proportional over a fairly wide range to the logarithm of the concentration of the enzyme. In all cases, the reaction mixture consisted of 0.2% potassium hyaluronate, 1 ml.; McIlvaine's citric acid phosphate buffer, M/60, pH 7.0, containing NaCl (3.5 g./l.), 0.5 ml.; appropriate dilution of the substance to be tested, 1 ml.; enzyme solution, 0.5 ml. The reaction took place, and the flow-time through the viscosimeter tube was read (20 min. after adding enzyme to substrate) at 34° (see Swyer & Emmens (1947) for details).

RESULTS

In the first test, serial dilutions of 1% (w/v) sodium salicylate solution were tested against enzyme solutions containing 0.5 unit (Swyer & Emmens, 1947) and 0.125 unit/ml. The results, shown in Table 1, indicate that over this range of concentration of salicylate no inhibition occurs, while with 0.33% salicylate in the reaction mixture there appeared to be facilitation.