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.

My most sincere thanks are due to Dr C. W. Emmens for his invaluable advice in the design and statistical evaluation of the experiments reported, and to Dr A. A. Miles for his interest and for introducing the pontamine blue technique to me.

REFERENCES

- Guerra, F. (1946*a*). *Science*, **103**, 686.
Guerra, F. (1946*b*). *J. Pharmacol.* **87**, 193.

- Swyer, G. I. M. (1948). *Biochem. J.* **42**, 32.
Swyer, G. I. M. & Emmens, C.W. (1947). *Biochem. J.* **41**, 29.

Failure of *in vitro* Inhibition of Hyaluronidase by Salicylates

By G. I. M. SWYER, *National Institute for Medical Research, Hampstead, London, N.W. 3*

(Received 22 April 1947)

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.

Table 1. *Effect of sodium salicylate on hyaluronidase action*

Na salicylate in reaction mixture (%)	pH	Flow-time index (Hyaluronidase, unit/ml.)			Notes
		(0.125)	(0.25)	(0.5)	
0	—	605	—	872	Control
0.003	—	677	—	834	} No inhibition
0.033	—	624	—	860	
0.066	—	605	—	872	
0.16	—	644	—	872	
0.33	—	733	—	904	Facilitation
0	7.0	560	765	—	Control
0.33	7.0	666	890	—	} Facilitation
0.833	7.0	653	835	—	
1.66	6.6	276	515	—	} Inhibition
2.5	6.4	133	292	—	
3.33	6.2	150	318	—	

The tests were repeated with dilutions of 10 % sodium salicylate, using enzyme solutions containing 0.25 and 0.125 unit/ml. The results (Table 1) confirm the facilitation effect over the range of salicylate concentrations 0.33–0.83 % in the reaction mixture. When the salicylate concentration rose to 1.66 % and higher, increasing inhibition was observed, but it was also found that the pH of the reaction mixture fell progressively with increasing salicylate concentration (see below).

Acetylsalicylic acid was first tested in serial dilutions of a 1 % (w/v) solution against an enzyme solution containing 0.5 unit/ml. Complete inhibition occurred when the concentration in the reaction mixture reached 0.33 %. Further tests with concentrations ranging between 0.066 and 0.33 % against enzyme solutions containing 0.125 and 0.25 unit/ml. revealed increasing inhibition over this range (Table 2). The pH of the reaction mixtures in one of these latter tests was estimated with indicators using the British Drug Houses capillator outfit, and equivalent mixtures were made, using acetic acid in place of acetylsalicylic acid, so as to obtain as near as possible the same pH. The degree of enzyme activity in these mixtures resembled quite closely that found in the former ones, thus indicating that the observed inhibitory effect could be explained simply on the basis of hydrogen ion concentration (Table 2). As can be seen from Table 1, the fall of pH with strong sodium salicylate mixtures was considerably less marked than with acetylsalicylic acid; the greatly increased molarity of the mixture is probably to be held partly responsible for the inhibitory effect with strong sodium salicylate mixtures.

The effect of heparin on hyaluronidase activity was investigated by using serial dilutions of a solution of heparin containing 10 mg. (101 Toronto units/mg.) in 10 ml. water in the same way as previously described, the hyaluronidase solution

Table 2. *Effect of acetylsalicylic acid and acetic acid on hyaluronidase action*

(a) Addendum: acetylsalicylic acid

Acetylsalicylic acid in reaction mixture		Flow-time index (Hyaluronidase, 0.5 unit/ml.)	
(%)	pH		
0	—	872	
0.003	—	898	
0.033	—	840	
0.066	—	848	
0.16	—	645	
0.33	—	0*	
		(Hyaluronidase, 0.125 unit/ml.)	(Hyaluronidase, 0.25 unit/ml.)
0	7.0	560	775
0.066	6.6	433	545
0.133	5.4	388	297
0.2	5.0	174	166
0.266	4.2	66	157
0.33	3.8	0*	111

(b) Addendum: acetic acid

	(Hyaluronidase, 0.25 unit/ml.)
7.0	—
6.6	505
5.4	350
5.0	155
4.2	123
3.8	0*

* Complete inhibition.

containing 0.25 unit/ml. From Table 3 it can be seen that inhibition is complete when the reaction mixture contains 6.6 units of heparin/ml. (i.e. 0.0066 %), while below this concentration the degree of inhibition decreases until, with 0.163 unit/ml. in the reaction mixture, it disappears. In no case was the pH of the reaction mixture altered from its initial value of 7.0.

Table 3. *Effect of heparin on hyaluronidase action*

Heparin: (units/ml. in reaction mixture)	Flow-time index (Hyaluronidase, 0.25 unit/ml.)	Notes
0	775	Control
0.033	790	No inhibition
0.163	800	
0.33	405	
1.63	375	Increasing inhibition
3.3	152	
6.6	0	Complete inhibition

Table 4. *Combined effect of variation of heparin and hyaluronidase concentrations—factorial scheme showing viscosity index for each of sixteen combinations of hyaluronidase and heparin*

Hyaluronidase (unit/ml.)	Heparin (units/ml.)			
	40	20	10	5
0.125	0	0	0	96
0.25	0	0	72	134
0.5	0	85	102	420
1.0	195	273	495	745

Flow-time indices

Fig. 1. *Effect of concentration of heparin on the viscosity reducing activity of hyaluronidase solutions of different strengths: x—x 0.125 unit/ml.; ●—● 0.25 unit/ml.; ■—■ 0.5 unit/ml.; ▲—▲ 1.0 unit/ml.*

The effect of combined variation of heparin and of enzyme concentration was studied by means of a factorial test involving four concentrations of

heparin and four of enzyme, the sixteen combinations being tested in random order (Table 4). The results are illustrated by Fig. 1, in which the flow-time index is plotted against concentration of heparin for the four concentrations of enzyme; it is clear that a relationship exists between quantity of heparin and amount of enzyme inhibited, whereas in the case of salicylates, the evidence suggested that no such relationship held.

DISCUSSION

Comparison of the effects of salicylates with those of heparin on the viscosity-reducing activity of hyaluronidase shows quite clearly the difference between a true hyaluronidase inhibitor, heparin, which, in a concentration of 0.0066 % in the reaction mixture, completely inhibits the activity of an enzyme solution containing 0.25 unit/ml. without altering the pH, and the salicylates, whose inhibitory action, manifested only in very high concentrations (more than 3 % for sodium salicylate and 0.33 % for acetylsalicylic acid), appears to be directly due to their effect on the pH of the reaction mixture, or, in the case of sodium salicylate, to the combined effect of lowered pH and increased salt concentration. Since, however, a close correlation has been shown to exist between the skin-diffusing and viscosity-reducing activities of hyaluronidases (McClean, 1943; Humphrey, 1943), it is surprising that sodium salicylate should possess the inhibitory effect on skin diffusion as described by Guerra. The recent work of Hechter (1946, 1947) may help to provide an explanation for this discrepancy. This investigator has shown very clearly that the skin-diffusing activity of hyaluronidase depends not only on the concentration of the enzyme, but also on the volume of solution injected, or on the interstitial fluid pressure; if the latter be maintained as a result of increased capillary permeability (such as is produced by histamine and inflammation-inducing agents) the extent of spread is considerably greater than when there is no accompanying capillary change. It is suggested, therefore, that the hyaluronidase preparation used by Guerra contained histamine, or some substance with a similar action, and that sodium salicylate, by inhibiting this substance, reduced the apparent spreading effect of hyaluronidase. The validity of such an explanation is rendered more probable by the observations (Swyer, 1948) that histamine enhances the spreading action of hyaluronidase, as could be predicted from the findings of Hechter (1946, 1947), and that sodium salicylate in the dose employed by Guerra (0.1 g./kg. intravenously in rabbits) inhibits the ability of histamine to increase capillary permeability and thereby reduces the spreading action of a hyaluronidase-histamine mixture.

In view of the evidence which has been presented that hyaluronidase is not inhibited by salicylates in therapeutic concentrations, it would seem that Guerra's explanation of the anti-rheumatic action of these drugs in terms of hyaluronidase inhibition must be abandoned. The question naturally arises as to whether or no their apparent anti-histamine action can be invoked in this connexion, as well as whether the effect of hyaluronidase on the skin of rheumatic fever patients described by Guerra and its diminution after administration of salicylate can be explained by the same mechanism. A voluminous literature exists regarding the effects of salicylates on immunological reactions, but the evidence, though some is suggestive, seems to be scarcely sufficient to provide a complete answer to these questions. The response to hyaluronidase described by Guerra in rheumatic fever, however, appears to be of the nature of a hypersensitivity reaction, such as was described by Jones & Mote (1934) as occurring in rheumatic fever patients following repeated injections of very small amounts of rabbit serum. It is true that only a single injection of hyaluronidase appeared to have been given (in Guerra's paper no details are provided, nor is any mention made of control tests in normal subjects), but since streptococci of the type believed to be responsible for predisposing to rheumatic fever are hyaluronidase

producers, sensitivity to hyaluronidase might well be of general occurrence in rheumatic fever. If such be the case, inhibition of histamine, which is believed to play a part in such hypersensitivity reactions, would account for the reduced response to intradermal injections of hyaluronidase after treatment with salicylate.

SUMMARY

1. Investigation of the effects of sodium salicylate and acetylsalicylic acid on the viscosity-reducing activity of hyaluronidase has shown that they are inhibitory only in relatively enormous concentrations (more than 3% for sodium salicylate and 0.33% for acetylsalicylic acid).

2. Such inhibition is apparently due to lowering of pH and, in the case of sodium salicylate, to increased salt concentration.

3. Heparin, on the other hand, is completely inhibitory in a concentration of 0.0066%.

4. The significance of these findings in relation to the claims made by Guerra (1946*a*) of the association of hyaluronidase with rheumatic fever, and the anti-rheumatic action of salicylates is discussed.

My sincere thanks are due to Drs C. W. Emmens and J. H. Humphrey for advice generously given.

REFERENCES

- Guerra, F. (1946*a*). *Science*, **103**, 686.
 Guerra, F. (1946*b*). *J. Pharmacol.* **87**, 193.
 Hechter, O. (1946). *Science*, **104**, 409.
 Hechter, O. (1947). *J. exp. Med.* **85**, 77.
 Humphrey, J. H. (1943). *Biochem. J.* **37**, 177.
 Jones, T. D. & Mote, J. R. (1934). *New Engl. J. Med.* **210**, 120.
 McClean, D. (1942). *J. Path. Bact.* **54**, 284.
 McClean, D. (1943). *Biochem. J.* **37**, 169.
 Rogers, H. J. (1946). *Biochem. J.* **40**, 583.
 Swyer, G. I. M. (1948). *Biochem. J.* **42**, 18.
 Swyer, G. I. M. & Emmens, C. W. (1947). *Biochem. J.* **41**, 29.

The Influence *in vitro* of Deoxycorticosterone on Glycogen Formation in Muscle

BY F. VERZÁR AND V. WENNER, *Physiological Laboratory, University of Basle*

(Received 6 January 1947)

The main action of the adrenal cortical hormone is generally believed to be the regulation of glycogen metabolism. In a series of experiments from this Institute the phosphorylation of glycogen was investigated in normal and adrenalectomized animals. This reaction, by which glucose-1-phosphate is formed from glycogen, is the first phase in the breakdown of glycogen. It was shown that the phosphorylation of glycogen *in vitro* was greatly reduced in tissue from adrenalectomized animals (Verzár & Montigel, 1942; Doetsch, 1945).

In order to study directly the synthesis of glycogen, we chose the technique of Gemmill (1940, 1941) and Gemmill & Hamman (1941) by which the synthesis of glycogen from glucose can be observed in the surviving diaphragm of the rat. The rat's diaphragm is suitable for this experiment because, owing to its thin structure, it can be kept alive in Ringer solution for a few hours if saturated with oxygen. If glucose is added to this solution, the diaphragm synthesizes glycogen. Gemmill (Gemmill, 1940, 1941; Gemmill & Hamman, 1941) and also