of deficiency symptoms for 2 months. At the same time the daily urinary excretion of riboflavin increased from the preimplantation level of 100 γ-250 γ to a concentration of 1000 γ-3500 γ per liter (Table II).

Discussion. The fact that it is possible to maintain a high riboflavin level within the body of animals and man, and to eliminate deficiency symptoms for a period of 1-1½ months, is encouraging. Cases are frequently encountered where regular oral intake of the required riboflavin is ineffective because of inadequate resorption in patients with gastric, intestinal or hepatic diseases. In such patients the riboflavin is only utilized when injected[6] in repeated daily doses. Since the maintenance of such treatment is often difficult, if not impossible, we believe that the implantation of pellets has a definite practical value. Furthermore, we intend to apply this method to other vitamins.

Summary. 1. A method is described for the preparation of riboflavin pellets that can be implanted in animals and in humans.

2. The effect of these riboflavin pellets (containing cholesterol) in cases of retarded riboflavin resorption lasts from 1-1½ months.

3. The riboflavin pellets implanted in a patient suffering from riboflavin deficiency brought about complete relief of relatively long duration from deficiency symptoms.

4. The method may be of practical value for patients suffering from riboflavin deficiency due to inadequate resorption, and when repeated injections are difficult to carry out.

[6] Bicknell, F., and Prescott, F., *The Vitamins in Medicine*, London, W. Heinemann, Ltd., 2nd edit., 1946.

15792

Action of Sodium Salicylate on Hyaluronidase.

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The purpose of this communication is to report certain experiments on the inhibition of hyaluronidase by sodium salicylate *in vivo* and *in vitro*.

Despite wide therapeutic usefulness little is known concerning the mechanism of action of salicylates. A number of investigators have shown that sodium salicylate at relatively high concentrations (0.05 *M*-0.5 *M*), causes a reversible denaturation of certain biologically active proteins.[1-4] Euler and coworkers[5] found that salicylates inhibit certain enzyme reactions in concentrations as low as 0.01 *M*. Some of these are partially reversible by diphosphopyridine nucleotide. Ivanovics[6] observed that the growth of certain organisms such as *Staphylococcus aureus* was inhibited by concentrations as low as 0.002 *M* while others such as *Proteus morganii* required much larger amounts. He correlated this difference with the ability to synthesize pantothenic acid and found that added pantothenic acid or to a lesser extent β,β-dimethyl-α-hydroxy-γ-butyrolactone was able partially to reverse the salicylate inhibition of *S. aureus*. He concluded that sodium salicylate owes its antibacterial effects to 2 different properties, namely specific inhibition of the synthesis of β,β-dimethyl-α-hydroxy-γ-butyrolactone and nonspecific protein denaturation.

[1] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1934, **17**, 399.

[2] Holden, H. F., *Austral. J. Exp. Biol. Med.*, 1937, **15**, 43.

[3] Bawden, F. C., and Pirie, N. W., *Biochem. J.*, 1940, **34**, 1278.

[4] Best, R. J., *Austral. J. Exp. Biol. Med.*, 1946, **24**, 27.

[5] Euler, H. v. and Ahlstrom, L., *Z. physiol. Chem.*, 1943, **279**, 175.

[6] Ivanovics, G., *Z. physiol. Chem.*, 1942, **276**, 33.

Guerra[7,8] has recently reported that the intravenous injection of sodium salicylate causes an inhibition of the "spreading effect" of testicular extracts (hyaluronidase) in albino rabbits, as well as in patients either suffering from or having recovered from rheumatic fever. He has concluded that hyaluronidase is inhibited by sodium salicylate and that this activity is responsible for the therapeutic efficacy of this drug in the rheumatic state. This observation seems to be of the utmost importance since one of the most definite facts about rheumatic fever is the efficacy of salicylates in relieving certain symptoms of this disease. The further facts that pathologically this disease may be characterized as a disease of connective tissue and that the enzyme hyaluronidase attacks hyaluronic acid, a component of connective tissue, suggest a possible ray of light in the study of this enigmatic disease.

Duran-Reynals[9] has pointed out that many factors such as nutrition, age, and sex hormones influence the activity of hyaluronidase *in vivo*. It thus seemed of importance to determine whether sodium salicylate was actually an inhibitor of this enzyme or whether the effects observed by Guerra were due indirectly to changes produced in the animal. Consequently, after verifying the results of Guerra *in vivo*, we have conducted a series of experiments on the effect of sodium salicylate on the activity of hyaluronidase *in vitro*.

Testicular hyaluronidase was prepared from bull testis by the method of Hahn,[10] the purification being carried through the first ammonium sulfate fractionation. Our preparations had an activity of 145 viscosity-reducing units per mg N. Hyaluronidase from *Cl. perfringens* (NIH SR-12) was obtained by growing the organism according to the method of Rogers,[11] in a semisynthetic medium containing hyaluronic acid. The bacterial filtrate was dialyzed at 4°C previous to use. Such preparations had an activity of 500 viscosity reducing units per mg N. Hyaluronic acid was prepared from umbilical cords by a modification of the method of Haas,[12] using a much shorter extraction procedure in the Waring blendor.

Spreading activity was determined by injection into the skin of the back of albino rabbits, 0.25 cc of enzyme preparation mixed with 0.25 cc of unfiltered india ink (diluted 1:1). After 24 hours the animal was sacrificed and skinned. The area of spread on the inner surface of the skin was traced on to cellophane and measured with a planimeter. This area was compared to a control area in which india ink mixed with 0.85% saline was injected. This method allows a somewhat more accurate estimation of spreading than has been possible heretofore. Viscosity-reducing activity was measured essentially according to Haas,[12] and was expressed as the reciprocal of the half-life time $\times 10^3$. It was found that when viscosity was plotted against the square root of the time a straight line function was obtained thus making the graphic determination of the half-life time more precise. All assays were done at 37°C. The mucin clot prevention test was found inapplicable to the salicylate experiments since the mixture of sodium salicylate with the horse serum albumin resulted in precipitation. The hydrolysis of hyaluronic acid was followed by the determination of N-acetyl-glucosamine by a modification of the method of Morgan and Elson[13] utilizing the Colman Junior Spectrophotometer at a wave length of 540 mμ. Salicylates were determined by the method of Brodie.[14]

The first experiments conducted were a repetition of those of Guerra using hyaluronidase prepared from *Cl. perfringens* cultures. Sodium salicylate was given both by mouth and intravenously in doses suffi-

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8 Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

9 Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

10 Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1945, **21A**, No. 1.

11 Rogers, H. J., *Biochem. J.*, 1945, **39**, 435.

12 Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

13 Morgan, W. T. J., and Elson, L. A., *Biochem. J.*, 1934, **28**, 988.

14 Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Therap.*, 1944, **80**, 114.

TABLE I.

Reduction of Viscosity of Hyaluronic Acid by Hyaluronidase in the Presence of Sodium Salicylate.

Enzyme incubated with varying concentrations of sodium salicylate for 15 minutes and activity determined by viscosity reducing method. Inhibition is calculated by comparison with control under identical conditions of pH and salt concentration. (pH = 5.8.)

Enzyme	Concentration of sodium salicylate				
	0.20 *M*	0.10 *M*	0.05 *M*	0.025 *M*	0.013 *M*
	% inhibition				
Testicular	100	90	50	42	17
Cl. perfringens	100	75	43	19	0

TABLE II.

Release of N-Acetyl-Glucosamine by Hyaluronidase in Presence of Sodium Salicylate. Enzyme incubated with hyaluronic acid (12 cc) in 0.2 *M* acetate buffer at pH 5.0.

Time (hr)	Control mg	Testicular Hyaluronidase. 0.12 *M*		0.09 *M*		0.06 *M*		0.03 *M*	
		*mg	†%	mg	%	mg	%	mg	%
1	300	55	82	120	60	185	38	235	22
2	330	55	84	130	60	230	30	240	27
4	400	55	86	175	54	260	35	320	20
		Cl. perfringens Hyaluronidase							
1	415	150	65	290	30	390	6	455	0
2	485	155	49	290	40	440	9	480	0
4	490	145	51	375	24	460	6	485	1

* Mg N-acetyl-glucosamine.
† % inhibition.

cient to attain a blood level of 20-30 mg per 100 cc of blood. Definite inhibition of spreading activity of hyaluronidase was obtained in the animals receiving salicylates. These results were similar to those reported by Guerra.

The next group of experiments was performed to determine the effect of sodium salicylate, *in vitro*, on both hyaluronidase prepared from *Cl. perfringens* filtrates and from bull testis. Graded amounts of salicylates were incubated with enzyme and at indicated times samples were withdrawn and assayed. Salicylates were added to the assay mixture in sufficient concentration to maintain the salicylate concentration constant. Total salt and hydrogen ion concentrations were kept constant.

Table I shows the composite results of a group of experiments in which activity was determined by the viscosity reduction method. It is evident that marked inhibition is obtained with a concentration of 0.05 *M* with both types of enzyme, but at lower concentrations only testicular enzyme was inhibited.

The inhibition of hyaluronidase by salicylates is reversible since removal of salicylate by dialysis produced almost complete restoration of activity.

The effect of salicylates on hyaluronidase was studied further by determining the effect of sodium salicylate on the liberation of N-acetyl-glucosamine. Enzyme was mixed with varying concentrations of sodium salicylate and hyaluronic acid and at definite intervals samples were withdrawn and N-acetyl-glucosamine was determined. Table II illustrates the results obtained under these conditions. It will be noted that a definite inhibition of release of N-acetyl-glucosamine from hyaluronic acid by both testicular and perfringens hyaluronidase is obtained. The degree of inhibition is apparently independent of time. Similar results were obtained with the viscosity reduction method. The inhibition of testicular enzyme has consistently been more complete and occurs at lower concentrations. This, it will be noted, is in conformity with the results obtained when the viscosity reduction assay has been used.

This observation is of interest in view of the recent reports of Haas[16] showing that hyaluronidase from different sources shows different sensitivity to antiinvasin, the hyaluronidase inhibitor present in normal blood. He has attributed these differences to the presence of other substances which he has termed proinvasin I and antiinvasin II. It is possible that our results depend on differences in purity of the various enzymes or merely chemical differences in the hyaluronidases from different sources. That such chemical differences exist is indicated by the work of Hahn.[10,15]

It is apparent from these data that sodium salicylate does exert an inhibitory effect on the activity of partially purified testicular and perfringens hyaluronidase. The specificity of this effect may be open to doubt in that the concentrations necessary are in the range in which sodium salicylate produces denaturation of other biologically active proteins. This concentration (0.01 *M*-0.1 *M*) is considerably above that obtained therapeutically (0.001 *M*-0.005 *M*). Our work and that of Guerra indicates that sodium salicylate at therapeutic levels does however affect the spreading activity of hyaluronidase. The direct inhibition of hyaluronidase at these concentrations would seem unlikely on the basis of the data presented above but cannot be ruled out in view of the impurity of the preparations used and the variability of sensitivity of hyaluronidases from different sources. The complex mechanism proposed by Haas[12] as well as the demonstration of the role of salicylates in inhibiting antibody formation[16] raise other possibilities as to the locus of action of salicylates on the hyaluronidase system.

Summary. Sodium salicylate has been found to inhibit the spreading effect of hyaluronidase *in vivo*. Hyaluronidase derived from *Cl. perfringens* and bull testis was also inhibited by sodium salicylate *in vitro*. The concentrations necessary for *in vitro* inhibition are considerably higher than those obtained *in vivo*.

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16 Homburger, F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 101.

15793

Recovery of Herpes Simplex Virus from Rabbit Brain Nine Months After Inoculation.*

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We reported the precipitating effect of anaphylactic shock on latent Herpetic encephalomyelitis.[1] Further progress has been made in substantiating our original observations.[2] Although at that time we did not demonstrate the existence of the Herpes simplex virus in the central nervous tissues of the rabbits showing the encephalitic symptoms following anaphylactic shock, we pointed out the symptomatological and pathological identity of the disease produced with that of Herpes encephalomyelitis. We further reported the finding of intranuclear inclusion bodies in the brain of the rabbit. Experiments pointing out the production of encephalitic symptomatology and central nervous system pathology by various noninfective mechanisms[3-6] made it of considera-

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2 Campbell, B., and Good, R. A., unpublished MS.

3 Rivers, T. M., and Schwenkter, F. F., *J. Exp. Med.*, 1935, **61**, 689.

4 Ferraro, A., *Arch. Neurol, and Psychiat.*, 1944, **52**, 443.