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"Spreading" Properties and Mucolytic Activity of Leech Extracts.

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In addition to their well known anticoagulating properties, leech extracts have the power to increase skin permeability.[1] Chemical studies from this laboratory have shown that the so-called spreading or "Reynals" factors, either from testicle,[2] or from the leech, were proteins.[3, 1] Recently, Chain and Duthrie[4] found that testicular extract would destroy the viscosity of the synovial fluid, and they have shown that this effect was due to the action of an enzyme on a polysaccharide. These authors suggested that this "mucinase" and the testicular spreading factor might be identical. If this is correct, one may expect leech extracts to have a high mucolytic activity, since their spreading power is 50 to 100 times greater than that of testicular extracts. In preliminary tests it was found that leech extract would destroy the viscosity of chicken tumor I extracts[5] with a remarkable speed, even at room temperature.

In the following experiments, the spreading power of various leech extracts was compared with their mucolytic activity, as measured by their action on chicken tumor I mucin.

Effect of Leech Extracts on Chicken Tumor I mucin. Previous work has shown that the spreading factor is located in the anterior part of the leech (head).[1] It is assumed that the substance is produced by the pharyngeal epithelium and plays a rôle in the process of feeding. On the other hand, a similar extract from the rest of the body is found to contain very little of the same factor.

In the present experiments, separated heads and bodies were extracted, in the usual way,[1] with 10 to 20 times their own weight of distilled water or phosphate buffer at pH 7.0, and centrifuged at 18,000 times gravity for 2 hours. These extracts were used as the source of enzyme. The substrates for mucolytic activity were concentrated extracts of chicken tumor I desiccates,[5] purified by high

1 Claude, A., *J. Exp. Med.*, 1937, **66**, 353.

2 Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327; Hoffman, D. C., and Duran-Reynals, F., *Science*, 1930, **72**, 508; *J. Exp. Med.*, 1931, **53**, 387; McClean, D., *J. Path. and Bact.*, 1930, **33**, 1045.

3 Claude, A., and Duran-Reynals, F., *J. Exp. Med.*, 1937, **65**, 661.

4 Chain, E., and Duthrie, E. S., *Nature*, 1939, **144**, 977.

5 Claude, A., *J. Exp. Med.*, 1935, **61**, 27.

speed centrifugation. The speed of the enzymatic reaction was studied at a temperature of 25°C.

Under these conditions the leech head extract, in the concentration of 1%, by volume, was found to have a powerful effect on the chicken tumor mucin, bringing the relative viscosity of the extract from 7.7 to 1.5 in 5 to 10 minutes. At that concentration, 30 minutes of contact with the leech factor brought the viscosity of the fluid close to that of water, the reduction in viscosity corresponding to 99.25% of the original value. Leech head extract, in a final concentration of 0.01% reduced the original viscosity by 73.6% whereas the leech body extract, in a final concentration of 1%, brought about a reduction in viscosity of 73.9%. This indicates that a 0.01% head extract and a 1% body extract have about equal strength, the former being then about 100 times more active than the latter.

The spreading power of the leech extracts was tested by the injection of 10-fold dilutions of the solutions, mixed with India ink, in the rabbit skin.[2, 1] The head extract, at a dilution of 10^{-3}, gave an area of spread equal to 20.3 sq cm, as compared with 22.6 sq cm for the body extract, at 10^{-1} dilution. This would indicate that the head extract is about 100 times more active than the body extract. The above results are illustrated in Table I.

The close parallelism between the rate of action of the mucinase and the spreading power of different extracts is strong evidence that the two factors, in the leech, are identical.*

This view is also supported by the fact that bull testicular extract was about 100 times less active than the leech extract, as regard both spreading power and mucolytic activity.

Mechanism of Spread. No satisfactory explanation has been found to account for the phenomenon of spread.[1] The spread, considered as the result of the mucolytic activity of the spreading factor would assume the presence, in the skin, of a chemically suitable substrate for the mucinase to act upon. From the histochemical studies of Bensley[6] and Sylvén[7] it appears that normal or pathological tissues contain a viscid ground substance which, from its staining properties, resembles mucin.

In the present work freshly removed rabbit skin was passed through a meat grinder and extracted by contact with 2 volumes of a 10% NaCl solution at 2°C for 24 hours. This extract was filtered

* The present work has been confined to the study of changes in viscosity under the effect of leech extracts assuming that the spreading phenomenon is brought about by similar changes in the skin without requiring necessarily complete hydrolysis of the substrate.

6 Bensley, S. H., *Anat. Rec.*, 1934, **60**, 93.

7 Sylvén, B., *Virchows Arch. Path. Anat.*, 1938, **303**, 280.

TABLE I.
"Spreading" Property and Mucolytic Activity of Leech Extracts.

Leech head extract					Leech body extract				
Spread in rabbit skin			Mucolytic activity		Spread in rabbit skin			Mucolytic activity	
Amt solids injected, g	Area of spread, cm^2	Ratio active spread to spread of control	Conc. leech extr. in mucin sol. (by vol.), %	Reduction viscosity after 30 min. at 25°C, %	Amt solids injected, g	Area of spread, cm^2	Ratio active spread to spread of control	Conc. leech extr. in mucin sol. (by vol.), %	Reduction viscosity after 30 min. at 25°C, %
3.6×10^{-3}	91.0	16.0	1.00	99.3	2.35×10^{-3}	66.9	11.7	1.0	73.9
3.6×10^{-4}	38.0	6.6	0.10	97.9	2.35×10^{-4}	22.6	4.0	0.1	35.2
3.6×10^{-5}	31.0	5.4	0.01	73.6	2.35×10^{-5}	8.0	1.4		
3.6×10^{-6}	20.3	3.2			2.35×10^{-6}	6.3	1.0		
3.6×10^{-7}	6.8	1.2			2.35×10^{-7}	7.0	1.0		
3.6×10^{-8}	8.0	1.4			2.35×10^{-8}	7.5	1.3		
3.6×10^{-9}	5.5	1.0			2.35×10^{-9}	6.2	1.0		

TABLE II.
Mucolytic Activity of Leech Extract on Normal Rabbit Skin Mucoprotein.

Tests	Mucoprotein solution		Leech extract (head)		Mucolytic activity			
			Amt added to mucoprotein solution		30 min at 25°C		30 hr at 25°C	
	Solids in sol. mg per cc	Relative viscosity of sol.	By volume, %	Final conc. in mixture, %	Relative viscosity of mixtures	Reduction in viscosity %	Relative viscosity of mixtures	Reduction in viscosity %
I	0.28	1.44	0.74	0.003	1.12	72.7		
II	0.70	2.2	1.0	0.004	1.38	68.4		
"	0.70	2.2	0.1	0.0004	1.43	64.0		
III	0.70	2.2	1.0	0.004	1.46	61.7	1.32	73.4
IV	1.40	3.46	1.0	0.004	2.05	57.3	1.80	67.5

through paper and the filtrate treated with chloroform, with occasional shaking, for another 24 hours. Denatured proteins were discarded by centrifugation. The clear solution was then dialyzed in cellophane bags until free of NaCl. A protein fraction, different from the mucoprotein, separated out on dialysis, and was discarded by filtration through paper. All the operations were conducted in the cold, to avoid the action of a mucolytic enzyme which might have been extracted from the skin, together with the mucoprotein.

The skin mucoprotein is readily soluble in water, giving clear, slightly yellow solutions. The biuret reaction is positive. Solutions containing 0.7 mg solids per cc gave a strongly positive orcinol test. Like chicken tumor I mucin, it gives a stringy precipitate with neutral red. The behavior of the substance in acidic solution is noteworthy. Its solubility decreases progressively down to pH 4.0, giving a flocculent precipitate. At pH 4.0 there is a sudden change in the appearance of the precipitate which is then mucoid, and contracts. Below pH 4.0 the protein becomes more soluble but the precipitate retains its mucoid character. Below pH 2.0 the substance is completely soluble. On account of the physical change taking place at that point, it is uncertain whether pH 4.0 is the point of minimum solubility of the protein.†

A solution containing 1.4 mg skin mucoprotein per cc had a relative viscosity of 3.46 at 25°C. Addition of leech extract to a final concentration of 0.004% produced a sudden drop in the viscosity of the solution. As a result of 5 different tests, it appears that leech extracts, at the above concentration, and acting for 30 minutes at 25°C, will reduce the original viscosity of the solution by 68.4%. A contact of 30 hours at the same temperature will show but a slight additional effect, the total reduction in viscosity amounting to 70.4% of the original value. These results are in agreement with the observations of Meyer and coworkers,[8] who showed that only 69% of the synovial mucin was hydrolyzed by 45 hours' incubation with a pneumococcus enzyme, in contrast with 96% hydrolysis for the free polysaccharide, under the same conditions.

Summary and Conclusions. 1. Leech extracts contain a powerful mucolytic enzyme, as shown by its effect on the viscosity of chicken tumor I extracts. 2. Leech head extracts exhibit a mucolytic activity considerably greater than that of similar extracts obtained

† The name "mucoprotein" refers especially to the physical properties of the material since the solution may contain different soluble components of the skin.

[8] Meyers, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, 1940, **71**, 137.

from the rest of the leech body. A comparable quantitative relationship is found to exist between head and body extracts when tested for another property, *e. g.*, their power to spread in the rabbit skin. 3. The parallelism in the strength of various extracts, as regards both mucolytic activity and spreading power, supports the view that the "mucinase" and the leech spreading factor may be identical. 4. A mucoprotein has been prepared from normal rabbit skin. 5. The viscosity of a skin mucoprotein solution is rapidly and considerably reduced by the action of leech extracts. 6. The effect of leech extracts on the skin mucoprotein *in vitro* suggests that their ability to spread through the skin may be due, at least in part, to their power to cause hydrolysis or depolymerisation of the same or a similar compound *in vivo*.

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Effect of Synthetic Vitamin K Compounds on Prothrombin Concentration in Man.

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The effectiveness of preparations of vitamin K in correcting hypoprothrombinemia has been demonstrated by numerous investigators.[1] Following the discovery of the antihemorrhagic activity of certain naphthoquinones,[2] several synthetic crystalline compounds were made available for clinical use in man. Butt, *et al.*,[3, 4] administered 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol) in doses of 25 to 50 mg intravenously to 9 patients with obstructive jaundice or disease of the liver; and 1,4-dihydroxy-2-methyl-naphthaldehyde in doses of 5 to 10 mg intravenously to 10 patients with obstructive jaundice. These preparations were effective in shortening the prolonged prothrombin time as calculated by the method of Quick. No untoward reactions were observed.

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1 Quick, A. J., *Am. J. Med. Sci.*, 1940, **199**, 118.

2 Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1293.

3 Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc. Staff Meet. Mayo Clin.*, 1939, **14**, 497.

4 Snell, A. M., and Butt, H. R., *J. A. M. A.*, 1939, **113**, 2056, footnote 41.