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Hyaluronidase in the Fertilization of Mammalian Ova.

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The effect of hyaluronidase and other spreading factors is essentially intercellular.[1] Hyaluronic acid and its components present in the fundamental substance of some mesodermal structures are rapidly and specifically hydrolyzed by these enzymes, leading to a pronounced increase in the permeability of the tissue. Similar effects exerted by certain toxic secretions result in such important phenomena as the invasion of the host by bacteria and by poisonous secretions from animals.[1] In addition, hyaluronidase occurs in large amounts in the testes and sperm of mammals, a fact which has heretofore been inexplicable.

Mammalian ova which have migrated into the oviduct are surrounded by a sphere of follicular cells. The mechanism whereby the ovum is freed from these cells to promote fertilization is quite obscure, although it is known that the phenomenon is easily brought about *in vitro* by suspensions of sperm from the same species and also by sperm from species other than that supplying the ova, and that the responsible agent is heat labile.[2] Manipulation of the follicular cells around the ovum reveals the sticky nature of the intercellular cement, the *liquor folliculi* responsible for the marked cohesion which maintains the *cumulus oöphorus*.

This led us to suspect that the effect of spermatozoa on follicular cells could be of an enzymatic nature comparable to the hydrolysis of the fundamental substance of the connective tissue which results in spreading. Accordingly, experiments were devised in which we studied the effect of several materials rich in hyaluronidase on the follicular cells surrounding the ova.

The materials tested were extracts, in Locke's solution, of: (a) fresh testicular tissue from rats and mice; (b) heads of leeches (supplied by Dr. A. Claude); (c) powdered, highly purified preparations of hyaluronidase from sheep testes (supplied by Dr. K. Meyer); and (d) desiccated venom from the rattlesnake. All of these preparations were very active in both hydrolysing hyaluronic acid and in causing spreading in the rabbit skin, the preparation from sheep testes being the most effective.

Female mice were mated overnight to males whose deferent ducts had been cut. The following morning the females were examined, and those having a vaginal plug were killed. The ova were secured from the oviducts, following the method of Lewis and Wright.[3] They were collected by suction in a fine glass pipette and discharged into 1 cc of Locke's solution placed in a watch-glass. Each glass contained one or two ova, surrounded by the *cumulus oöphorus*. Then, amounts of each of the test substances were added to and mixed with the Locke's solution, and changes in the follicular cells were followed under the dissecting microscope. A test was considered completed when total dispersion of the follicular cells was obtained so that the ova appeared completely naked. Data concerning the dilution of the materials employed and their power in dispersing follicular cells are summarized in Table I, and show that all materials tested were active in this respect. When concentrated solutions were employed an effect of dispersion on the ova was noticeable almost instantly after exposure. With more diluted solutions several minutes elapsed before an effect could be clearly observed.

Additional tests carried out as those above described showed that undiluted blood serum

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

2 Pincus, G., *The Eggs of Mammals*, New York, The Macmillan Company, 1935.

3 Lewis, W. H., and Wright, E. S., *On the Early Development of the Mouse Egg*, Carnegie Institution of Washington, Contributions to Embryology, No. 148, 113-142, 1935.

TABLE I.

Substances tested showing positive reaction	Dilution in Locke solution	Complete dispersion of follicular cells (in min)	
Mouse testicular extract	1:20	10	
" " "	1:200	180	
Rat " "	1:20	60	
" " "	1:200	230	
Rattlesnake venom	1:300	3	
" "	1:600	4	
" "	1:1,000	8	
" "	1:2,000	8	
" "	1:12,000	14	
" "	1:70,000	50	
" "	1:120,000	164	(almost complete)
Leech extract	1:400	15	
" "	1:600	27	
" "	1:1,000	35	
Purified hyaluronidase prep. from sheep testicle	1:1,000	1	
" " " " " "	1:6,000	1	
" " " " " "	1:40,000	15	
" " " " " "	1:120,000	35	

and concentrated extracts (1:20) of kidney and liver from mice and rats were entirely devoid of any dispersing effect on follicular cells, the observations lasting for 8 hours after exposure. However, spleen extracts at dilutions of 1:20 were found to exert a definite although slight effect.

The effect of heating was studied with the following results: Purified sheep testicle extract diluted 1:12,000 and heated at 60° for 30 minutes brought about complete dispersion of the ova in 12 minutes, and in a dilution of 1:65,000 in 230 minutes. Crude mouse testicle extract diluted 1:30 and heated at the same temperature effected complete dispersion in 300 minutes, but only slight dispersion when diluted at 1:180. Heating the same preparations at 70° and 100° completely destroyed the dispersing effect.

Although it is not a specific response, esters of polysaccharides of high molecular weight stained with toluidine blue give a purple metachromatic reaction.[1] Application of this staining to sections of mouse ovary and oviduct after fixation with any of the following reagents—formol-alcohol, formol, and Carnoy's fluid, showed that the *liquor folliculi*, in which follicular cells surrounding the ova are embedded, stains a purplish blue.

There is thus good reason to assume that hyaluronidase secreted by or liberated from the spermatozoa may be largely or entirely responsible for freeing the ova from the surrounding follicular cells. This view is supported by the fact that the substance active in this respect shares with hyaluronidase the following properties—(a) it is present in large amounts in testicle and sperm, leech tissues, and snake venom; in small amounts in spleen[1], and is absent in other organs; (b) it is found in large amounts in the same testicle fraction freed from much inert material and endowed with a very high spreading and enzymatic activity; (c) it is much impaired by heating at 60° and totally destroyed at higher temperatures; and (d) it acts on intercellular viscid cements which respond to staining methods as do compounds of hyaluronic acid. To this, one must add that with the preparations here studied there is a good quantitative correlation between their spreading and enzymatic effects and their power to disperse follicular cells.

Indirect evidence can also be found in the fact that in reptiles, amphibia, and birds no follicular cells surround the ova in the oviduct, and in line with this the testes from the species so far tested belonging to these zoological groups were practically devoid of hyaluronidase.[1]

Experiments by Yamane (quoted by Pincus[2]) showed that trypsin is effective in freeing the ova from follicular cells. However, on repeating these experiments Pincus

and Enzmann[2] found that the enzyme exerted a strong damaging effect on the ova themselves. The effect would seem to be one of destruction rather than disaggregation, and the data available do not warrant the conclusion that proteolytic enzymes play any part in the phenomenon here studied as it occurs in the oviduct. On the other hand, it seems quite plausible that motion of the cilia of the oviduct epithelium has a complementary action.

Summary. Crude or highly purified preparations known to be very rich in hyaluronidase, such as extracts from rattlesnake venom, leech tissues, and testicle, have a very pronounced effect in dispersing the follicular cells surrounding the ova of mice. It appears very probable that hyaluronidase itself, which plays such an important part in infection, is also responsible for this effect, an indispensable step in fertilization. Final conclusions cannot be reached until the enzyme is obtained in pure state.

P.S. When the manuscript of this paper was ready for publication McClean and Rowlands[4] reported the results of experiments similar to ours but carried out on rat ova and reached the same conclusions as we have.

[4] McClean, D., and Rowlands, L. W., *Nature*, 1942, **150**, 627.

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Complement Fixation with Dissimilar Antigens in Primary Atypical Pneumonia.

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In complement-fixation tests with sera from patients with primary atypical pneumonia of unknown etiology, it was found unexpectedly that the convalescent serum from a number of patients reacted positively in high dilution with various and apparently unrelated antigens. Since this peculiar and as yet unexplained property can lead to difficulty in the interpretation of the results of complement-fixation tests with sera obtained from patients with this clinical syndrome the phenomenon has been studied.

Materials and Methods. Specimens of serum were obtained from patients acutely ill with primary atypical pneumonia in the Rockefeller Hospital. Additional specimens of serum were obtained from these patients throughout the course of the illness and during convalescence. The sera were stored at 4°C.

Mouse lung antigens. Antigens were prepared from the lungs of normal albino Swiss mice and from the lungs of similar mice which had been infected with one or another of the following viruses: a. pneumonia virus of mice, Horsfall and Hahn;[1] b. influenza A virus, PR8 strain; c. cat pneumonitis virus, Baker;[2] and d. meningo-pneumonitis virus.[3] Mice infected with agents a, b, and d were killed usually on the fifth day after intranasal inoculation, while mice infected with agent c were killed on the second day. The lungs were removed aseptically, ground with abrasive and suspended in a final concentration of 2% by wet weight in 0.85% NaCl buffered at pH 7.2. The suspension was centrifuged at 1500 RPM for 10 minutes and the supernate withdrawn and used as antigen. When parallel tests were done with different antigens they were all

Note: The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

[1] Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[2] Baker, J. A., *Science*, 1942, **96**, 455.

[3] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.