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Action of Semen Inducing Rupture of Yolk Membrane in Chicken Egg. (25047)

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Pincus(1) and Yamane(2) simultaneously reported that cumulus cells which surround the eggs of rabbits were diffused by adding their semen. The latter concluded that 2nd maturation division of the ovum is dependent on penetration of spermatozoon and that it also can be artificially induced by treating with pancreatin. Recently in experiments on fertilization we found that the yolk membrane of hen's eggs was ruptured on addition of semen. These tests were made *in vitro* where artificially ovulated eggs were used. The present study was made to analyze above-mentioned phenomenon.

Methods. Newly laid eggs and ova artificially ovulated *in vitro* according to methods described by Neher, Olsen and Fraps(3) and Saeki and Katsuragi(4) were used. Semen collected from the animal was placed in contact with the yolk membrane of eggs kept in porcelain dishes and incubated at 38°C. Lengths of time required for semen to rupture the vitelline membrane and release of yolk material were recorded. Hyaluronidase activity of fowl semen was estimated by decreasing the viscosity of hyaluronic acid solutions (a modification of McClean's method) (5) and by increasing the area of spread of intradermally injected substances. Purified hyaluronidase "Sprase" of Mochida Pharma. Mfg. Co., Tokyo, was used.

Results. Effect of Semen of Different Animals on Yolk Membrane. Rupture of yolk membrane of hen's eggs is brought about by semen from cocks and from other domestic animals. Length of time required for rupturing the membrane after adding semen is shown in Table I. Vitelline membrane of eggs serving as controls where semen was not added, was kept intact during first 5 days in the following experiment (Table I). The vitelline membrane of artificially ovulated ova was ruptured within 2 hours by cock's semen. The same semen required 6.3 hours to rupture the yolk membrane of normally laid egg. It seemed strange that semen of rabbits, although unrelated to Aves, was more effective than semen from the duck, which is in the same class with the domestic fowl. Boar's semen was least effective.

Action of spermatozoa and semen serum. Spermatozoa were separated from seminal fluid by centrifuging the semen 20 minutes at 20,000 RPM, then both fractions were placed separately on the vitelline membrane of egg yolks. As shown in Table II, the destructive action of spermatozoa was higher than that of semen serum. This suggests that the former contains more effective substance(s) than the latter.

TABLE I. Effect of Adding Animals' Semen on Rupture of Yolk Membrane of Chicken Egg.

Exp.						Control
Semen collected from	No. of eggs used	cc semen added	Times required for rupturing Hr	Min.	Range	
Cock	10*	.2	1	53	1:00– 3:30	Untreated 35 eggs did not rupture during first 5 days
"	68	.1	6	18	2:00–22:10	
Drake	15	.1	9	28	7:10–20:00	
Rabbit	25	.1	4	37	0:30– 9:15	
Goat	13	.5	20	47	10:45–48:00	
	8	.1	Not rupt.†			
Bull	17	.5	18	38	10:20–46:20	
	8	.2	Not rupt.†			
Boar	25	.5	39	00		
	8	.1	Not rupt.†			

* These are ovulated ova *in vitro* from excised follicles.
† These did not rupture during first 5 days.

Effect of heat on semen. Semen was heated at 65, 70, 75, 80, 97 and 100°C for 10-20 minutes to test its heat resistance. Semen heated above 70°C took longer to rupture the vitelline membrane. In 11 of 15 samples heated at 100°C for 20 minutes, the destructivity was retained for as long as 28 hrs, indicating that the destructive principle(s) present in semen possess(es) to some extent thermal resistance. When rabbit's semen was heated at 80°C and 97°C for 10 minutes, it took 33.75 and 41.7 hours respectively for rupture of yolk membrane of eggs. On the basis of the last experiments, it is suspected that the active principle is a hyaluronidase-like substance.

Measurement of hyaluronidase level and effect of its substance. As far as we know, the hyaluronidase level is very low or absent in cock's semen. Both samples of "semen (0.2 cc) Indian-ink (0.1 cc)" and "Indian-ink (0.3 cc)" were injected into rabbit's derma respectively. Average area of sample containing semen was larger than that of the control, and the level in both samples during 1 hr. was linear. Hyaluronidase level as determined by the viscosimetric method is shown in Table III. Although individual differences exist, average level in cock's semen was remarkably low as compared to that reported in other animals, and it averaged 87.2 viscosity reducing units.

To further ascertain this effect, hyaluronidase (Sprase) extracted from bull testicles was used. The result fell short of our initial assumption, and by using diluted Sprase solution in 525 V.R.U., it took 38.8 hours to rupture the yolk membrane.

Effects of (1) homogenized fluids of various organs and (2) trypsin. The effect of homogenized fluids of various organs of a laying hen and a male chick are shown in Table IV. The solutions prepared from kidney liver and pancreas of laying hens showed nearly the

TABLE II. The Action of Spermatozoa and Semen Serum on the Rupture of the Yolk Membrane.

	No. of eggs	Added cc	Times required for rupturing Hr	Min.	Remarks
Spermatozoa	15	.1	2	52	Semen separated by ultra-centrifuge at 20,000 r.p.m. for 20 min.
Semen serum	15	.1	5	58	
Spermatozoa	15	.1	7	30	Semen separated using centrifuge at 3,000 r.p.m. for 20 min.
Semen serum	12	.1	17	22 (2)	
			Not rupt.	(10)	
	9	.5	12	16	

Note: Ten untreated eggs did not rupture during first 5 days.

TABLE III. Hyaluronidase Level of Cock's Semen Determined by Viscosimetric Technic.

Sperm conc./cc (million)	% surv. sp. and motility	H-ase level/cc (V.R.U.)	Remarks
2.85	100	86.7	Estimated using 0.2% potassium hyaluronate as substrate.
2.98	100	34.9	
2.75	90	131.4	
2.98	100	92.3	
2.95	100	78.0	
2.84	90	150.8	
2.70	100	66.6	
1.97	90	57.1	
Rabbit		3833.5	mean of 4 times

same effect as did their semen. Trypsin, a product of Merck, was strongly effective only in very high concentration, but less effective in 1% solution.

Discussion. The mechanism of fertilization in lower animals, especially in the *sea urchin,* is well known. We thought that the destruction of the yolk membrane in chicken egg on addition of semen might be related to fertilization, and that determination of the chemical substance(s) effecting it might contribute to a higher level of fertility in artificial insemination.

Yamane(1) reported that the proteolytic action of mammalian spermazoa was due probably to a tryptase active in an alkaline medium. However, Huggins and Neal(6) found a little "trypsin" in the semen. Lundquist(7) described a casein-hydrolysing enzyme like "trypsin." Recently, Lundquist *et al.*(8) accomplished the separation of 2 proteolytic enzymes: proteinase and aminopeptidase in human semen.

Furthermore, it is said that the vitelline membrane is composed of keratin and mucin (Morgan and Hall(9)) and collagenous membrane (McNally(10)). Considering these reports, including our data where trypsin was used, there is no denying the fact that proteolytic action participates in the rupture of vitelline membrane.

TABLE IV. Effects of Adding Homogenized Fluid of Various Organs on Rupture of the Yolk Membrane.

	Laying hen		Chick (♂, 2 mo)		
Organs	Hr	Min.	Hr	Min.	Control
Liver	7	44	25	16	10 eggs on which normal semen was added, ruptured during first 7½ hr.
Breast muscle	25	53	25	41	
Kidney	6	35	12	20	
Oviduct	12	25			
Comb	11	36	13	43	
Pancreas	7	41	5	20	
Testis			15	36	

Although it is recognized that differences exist in the action of a purified substance, our experiment using "trypsin" showed unsatisfactory results.

Since we suspected a stronger acting substance, we attempted to measure the hyaluronidase level in cock's semen.

Sweyer(11) and Yasuda and Takahashi (12) reported that fowl semen did not contain hyaluronidase. In our experiment its level was very low when compared with that of other animals. We wish to emphasize however that the destructive action of spermatozoa was stronger than that of seminal fluid and the hyaluronidase extracted from testicles of bulls was rather weak in causing the rupture of vitelline membrane.

The results mentioned above indicate that both trypsin-like substance(3) and hyaluronidase are not very effective in causing rupture of vitelline membrane. Further study to determine the active substance(s) is necessary.

Summary. We found that the vitelline membrane of hen's egg was ruptured on contact with semen. Also that the destructive action was stronger in spermatozoa than in seminal fluid. Experiments were conducted to determine the substance(s) effecting destruction of the membrane. Trypsin and hyaluronidase are responsible for this phenomenon, but another major factor may exist which determines the destruction.

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Labile Phosphate Changes in Ileum of the Rat. (25048)

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Mass movement of large intestine related to eating is a well-known phenomenon. Grossman(1) states that the small intestine also shows increase in motility within a few minutes after eating and suggested the possibility of hormonal involvement. Picchioni and Edwards(2) noted that isolated intestinal strips had greater activity if animals were fed just prior to sacrifice. Kroeger and Edwards(3) reported when rats were fasted to 48 hours and fed just before sacrificing, this treatment caused not only changes in activity but changes in acid-soluble phosphate fractions related to intermediate metabolism and muscle contraction. Whereas changes in phosphate compounds reported by these authors occurred in combined samples of mucosal and muscularis tissue, our object was to determine the extent to which both tissues separately undergo these changes. The data indicate that each tissue shows significant chemical changes with regard to fasting and feeding treatment but that there is no real difference between the 2 tissues.

Methods. Sixty Sprague-Dawley albino rats of either sex and weighing 170 to 275 g, were divided randomly into 6 equal groups. Rats were fed a standard ration of laboratory pellets† except as noted for different groups. Group I was fed *ad lib* until time of sacrifice. Groups II, III and IV were fasted 24, 48 and 72 hours respectively. Animals in Groups V and VI were fasted 48 hours, then allowed to nibble on food pellets for 10 and 15 minutes prior to sacrifice. All groups were given water *ad lib*. The last 24 cm of ileum was removed, washed with ice-cold saline and divided into 4 segments. These segments were opened longitudinally, laid serosa-side down on absorbent toweling and frozen with liquid nitrogen. After mucosa was cut off with razor blade, both mucosal and muscularis samples were refrozen and pulverized in stainless steel mortar. Extraction and assay methods previously reported(3,4) were used and phosphate fractions determined were: T.A.S.P.-P. representing total acid-soluble phosphates, I.P.-P representing ortho phosphate present in tissue as such, "Cr.P.-P." phosphate hydrolyzed at 25°C for 30 minutes in 1 N.H_2SO_4 and "A.T.P.-P.", the phosphate hydrolyzed at 100°C for 20 minutes in 1 N.H_2SO_4.

Results. Concentrations of various phosphate fractions expressed in mg% phosphorus/wet weight tissue are shown in Fig. 1, 2, 3, and 4, each value representing 40 assays. Analysis of variance and the Mann-Whitney "U" test(5) for ranked data indicates that treatments are significant at least to 0.05 level of confidence. Variation between mucosal and muscularis samples are not significant. All phosphate fractions show a decline in median concentration as period of fasting is extended except A.T.P.P. in mucosal segments. This increases slightly during first 24 hours but is comparable with the muscularis tissue at 72-hour period. This rise was previously noted(3) using combined samples of mucosa and muscularis.

Most concentrations for different fractions decrease gradually, reaching 50 to 60% of control values by 72-hour period. As fasting period is prolonged all phosphate fractions become more uniform in range of concentra-

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