The Role of Testicular Hyaluronidase in Spermatogenesis of Mammals

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(Tables 1-2; Pls. 1-3; Text-fig. 1)

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I. INTRODUCTION

In earlier papers of the senior author on artificial insemination *in vitro* with rabbit ova, it was reported that spermatozoa showed a twofold action consisting in dispersing sticky follicular cell-mass surrounding the ovum and activating the second maturation division of it (YAMANE, 1930; 1935 a & b; 1937). The causative agent for these two phenomena was experimentally deduced to be a proteolytic enzyme presumably of the trypsin group. The steps by which this conclusion was drawn were as follows:

1. The spermatozoon living or killed with toluene contains a substance, the action of which appears in the dispersion of follicular cell-mass surrounding the corona radiata and loosening the cells of the latter.
2. This active substance can be isolated by extraction from the spermatozoa with Tyrode solution. It is active in an alkaline medium, thermolabile and adsorbed by kaolin, showing the nature of tryptic ferment.
3. The formation of the second polar body in the ripe ovum is dependent on the penetration of the spermatozoon into it.
4. The formation of the second polar body can artificially be induced by treating the unfertilized ovum with pancreatin.
5. The above two phenomena can also be caused by heterologous spermatozoa, hence the reaction is of species non-specific nature.

The detachment of the follicular cell-mass from the ovum by the action of spermatozoa reaching it, was observed by LONG (1912) in the mouse, PINCUS (1930), and PINCUS &

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ENZMANN (1932) in the rabbit, but these authors did not make any statement concerning the agent responsible for it. As regards the formation of the second polar body following the penetration of spermatozoon into the ovum, PINCUS & ENZMANN (1932; 1936) and PINCUS (1936) confirmed this with rabbit ova *in vitro*. Nevertheless, no worker in this field has so far shown any particular concern on the problem of the relationship between the polar body formation and the enzyme.

On the other hand, it was discovered by DURAN-REYNALS (1928; 1929) that testicular extract has an enhancing action on the development of vaccine virus, and this enhancing agent, later known as spreading or diffusing factor, was shown by HOFFMAN & DURAN-REYNALS (1931) and MCCLEAN (1931) to be associated with the spermatogenic cells or spermatozoa. A decade had elapsed before MCCLEAN & ROWLANDS (1942), and FEKETE & DURAN-REYNALS (1943) proved with tubal ova of the rat and the mouse respectively that the spreading factor in spermatozoa should be identical with hyaluronidase, suggesting also that the gel enclosing the ovum would be hyaluronic acid. Ever since, testicular hyaluronidase has called forth not only academic interest but also attention of gynecologists and animal breeders in relation to the promotion of fertility.

However, the present knowledge on the role played by this enzyme is exclusively confined to its hydrolysing effects on the hyaluronic acid in the ground substance of the mesenchyme. As far as the reproductive process is concerned, hyaluronidase has been known only as the spreading factor for the follicular cell-mass surrounding the ovum at fertilization. Since it has heen known that in some mammals, the ovum at ovulation is devoid of such a cell-mass (cit. from PARKES, 1952, p. 18), nevertheless, hyaluronidase is present in the testes of the male belonging to the same species, there seems still to be no evidence on the proper action of this enzyme. On the other hand, the intracellular activity of the sperm enzyme as revealed in the aforesaid experiments on the dependence of polar body formation seems to show some relation with the real role of the hyaluronidase. Having been confirmed by YAMANE that the alleged "proteolytic" enzyme released from spermatozoa is identical with hyaluronidase (Pl. 1, figs. 1–3), and based upon the finding of LEONARD & KURZROK (1945) that testicular hyaluronidase can act as an antigen, the present experiments were carried out to find antigenic effects of the hyaluronidase, on the male germ cells by means of antigen-antibody reaction. If this is the case, the bearing of the hyaluronidase upon the physiology of mammalian reproduction will be clarified.

II. MATERIALS AND METHODS

In the present experiments mice of a pure strain interbred for more than ten generations were employed to eliminate familial variation whereas rabbits were of an ordinary strain, both having been raised in our laboratory. The injections were carried out with sexually matured males for which fertility had been proved beforehand. The hyaluronidase used as an antigen was a highly purified bull testis preparation with a commercial name of "Sprase," MOCHIDA[1] which was prepared according to the process of MANDINAVEITIA & QUIBELL (1940) and MANDINAVEITIA (1941) and assayed 5,000 V.U.M. (Viscosity

1) This enzyme preparation was supplied to us by MOCHIDA Pharmaceutical Mfg. Co., Ltd., Tokyo, to whom we express our gratitude.

Reducing Unit of MOCHIDA[2]) in an ampulla content as evaluated by the "half-viscosity-level" method. "Sprase" is an amorphous white substance and soluble in water. In a dried condition it is very stable and preservable for a fairly long period without reducing the enzyme power. An ampulla content of 1.0 mg. of this enzyme preparation approximately equivalent to 5,000 V.U.M., was dissolved in 1.0 ml. of physiological saline solution and directly after dissolution injected subcutaneously, sometimes in a constant dose, sometimes in an ascending dose.

The injection was made to 13 mice and 5 rabbits with intervals of 5 to 7 days to attain 7,500 to 45,000 V.U.M. to a mouse and 30,000 to 60,000 V.U.M. to a rabbit in a total accumulated dose, whereas the duration of treatment varied 23 to 31 days for both the species. Testis removal occurred either bilaterally or unilaterally at a different interval. For microscopical examination, the testicular tissue was fixed in Bouin's picroformol solution, and prepared by the usual paraffin section method, cutting through in thickness of 10 μ and stained with Heidenhain's iron-haematoxylin. Sometimes, stamp preparations of cut surface of the testis were employed with application of Giemsa's dye.

In parallel to the fractional injections the fertility tests on mice and rabbits treated were carried out in order to check the spermatogenic activity of testes.

III. RESULTS OF EXPERIMENTS

A. Observations in Mice

1. Condition of Animals

In conformity with the findings of SEIFTER (1950) and TISLOW & CHASE (1950) most animals treated here showed no toxicity of testicular hyaluronidase inspite of its repeated injections for a long period and in a fairly high dosage; no constitutional symptom was observed and the postmortem results revealed also macroscopically no perceivable damage to the internal organs. The body weight remained unchanged or even slightly increased except a few mice (Nos. 11, 12, 13) on which the highest dose was administered.

2. Histological

In the majority of cases, the size of testes in mice injected with hyaluronidase was apparently not different from those of untreated individuals. Histological observations revealed also generally neither hypertrophy nor hypotrophy of the testicular tissue even after three months following the last injection. In some cases (Nos. 2, 3, 12), however, a slight shrinkage of seminiferous tubules occurred, their caliber measuring 160 – 180 μ against 200 – 220 μ of that of the normal tubules. On the other hand, the spermatogenic epithelia of tubules showed strikingly degenerative changes although the degree of damage varied greatly at different doses and different post-treatment periods. It was noteworthy that in two mice (Nos. 2, 10), which received a total dose of 7,500 V.U.M. and 40,000 V.U.M. of "Sprase" respectively, a remarkable thinning or even disappearance of the basement membrane of tubules occurred in places. The interstitial cells exhibited as a rule very few

2) V.U.M. is a unit for the hyaluronidase commonly used in Japan. In the viscosity assay, 500 v.r.u. of American preparations "Wydase" (WYETH Inc.) and "Alidase" (SEARLE Co.) are approximately equivalent to 2,000 V.U.M. of "Sprase".

alterations at any dose or interval but exceptional cases showed their increase more or less in number. In one example (No. 7) a cyst formation accompanying the pronounced slackiness of seminiferous tubules was observed.

On the whole, however, it may be said that the effect of hyaluronidase injection on the testicular tissue was not so conspicuous as cytological changes within the spermatogenic epithelia of tubules.

3. Cytological

It was found in a number of cases that a remarkable disorganization in spermatogenic epithelia occurred (Pl. 1, fig. 4). In the degree of damage, however, a considerable variation was found depending on the dosage and the post-treatment interval until the testis removal. It was noted in the first place that the interval between the last injection and the testis removal played a great role even in one and the same individual. In the mouse No. 4, for example, on the 5th day following the last injection the left testis showed yet no abnormality in spermatogenesis except appearance of some karyorrhexis in spermatids, whereas a remarkable disorder manifested themselves in spermatogenic layers of the right testis which was examined at 11 days after the last injection (Pl. 2, figs. 10, 11).

Generally speaking, the conspicuous disorganization of tubules was found approximately during 10 days covering the 10th to 20th day after the the last injection, and thereafter the damaged tubules were gradually in restitution. There was a direct evidence for recovery in the mouse No. 8 which showed partially normal spermatogenesis after a span of 6 weeks following the last injection, despite the fact that this animal had received hyaluronidase injection of the highest dose administered in this experiment totalling 45,000 V.U.M. Thus the span from the 10th to 20th day after the cessation of injections seemed to be the most sensitive period for the spermatogenic cells. During this period, lack of meiotic divisions or meiotic irregularities as pycnosis, tripolar divisions, chromosome bridging etc. in the spermatocytes (Pl. 1, figs. 5, 6, 7), pycnotic changes together with nuclear fragmentation of spermatids resulting further in debris formation, karyorrhexis and karyolysis of the spermatids (Pl. 1, fig. 8; Pl. 2, fig. 12) were prevailing phenomena throughout the damaged part of the testis. As a natural consequence, a remarkable decrease in number of intratubular spermatozoa and in extreme cases their entire disappearance followed; also the agglutination of sperm heads was not unusual (Pl. 2, fig. 15). It was noteworthy that two-, three- or more nucleated cells, sometimes giant cells containing more than ten, not exactly countable, nuclei were observed (Pl. 2, fig. 14). Since these multinucleated or giant cells contained nuclei very similar to the nucleus of the spermatid in size, form and staining, they can be taken for multinucleated or giant spermatids. Also spermatids with a giant sperm head could be sporadically met with (Pl. 1, fig. 9). Karyorrhexis accompanying the hyperchromasy of the cytoplasm was another type of degeneration in the multinucleated spermatids. A peculiar feature was sporadic occurrence of giant prophase in the primary spermatocytes which made a strong support for the unicellular origin of the said multinucleated or giant spermatid (Pl. 2, fig. 16). Thus, the meiotic irregularities so far found resembled more or less closely those caused by various artificial means as application of heat and radiomimetic drugs, experimental cryptorchism etc.

The spermatogonia proved to be the most resistant among spermatogenic cells aganist

the hyaluronidase injection, because in the majority of cases they appeared practically unaffected, except a case (No. 12) where a remarkable hydropic degeneration took place throughout the whole epithelia (Pl. 3, fig. 17), showing very similar figures observed by LAGERLÖF (1934) in sterile bulls with hypoplastic testes. More resistant was the Sertoli cell; it showed no response to the injection at any dose.

At last, it may not be out of place to state here, that the inactivated hyaluronidase solution kept at 60°C for half an hour is likewise effective, if not so extensive as a fresh one. This showed that the meiotic abnormalities caused by the hyaluronidase injection are not merely due to the enzyme-substrate reaction.

B. Observations in Rabbits

Within a short period of the 10th to 20th day after the last injection, disorganization of seminiferous tubules was observed if not so extensively as in mice above described. This is undoubtedly due to the relatively small dose of hyaluronidase administered; to give this enzyme preparation corresponding to the body weight of the rabbit in the same proportion as in the mouse was not possible for us merely because of economic reason. In principle, however, injuries as abnormal divisions of spermatocytes, multinucleation and pycnosis of spermatids, strikingly diminishing of intratubular spermatozoa etc. did not differ from those found in the mouse (Pl. 3, figs. 18, 19)

A more striking and prevalent feature was the agglutination of spermatozoa within the tubules showing the production of agglutinins which were detectable in the serum obtained after three months following the final injection (Pl. 3, fig. 20).

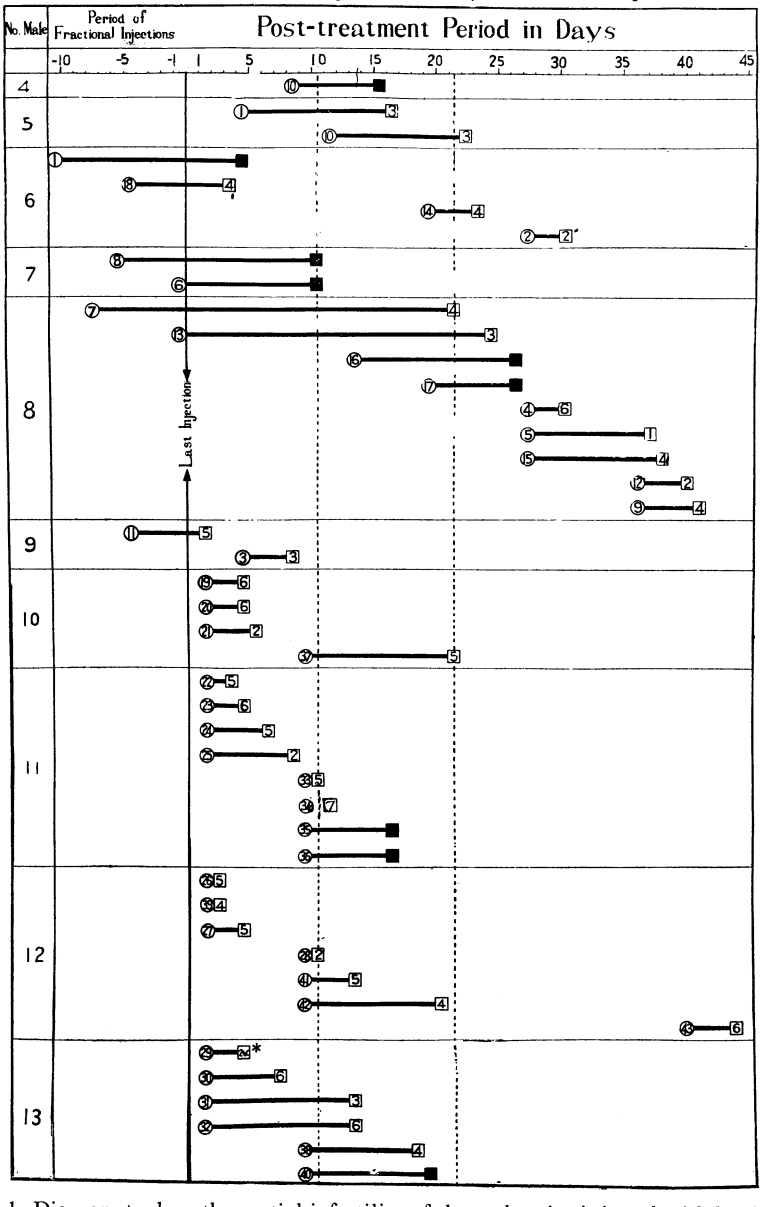
C. Fertility Tests on the Male Mice Treated with Hyaluronidase

In order to test the fertility of male mice subjected to injections of testicular hyaluronidase, one or more females of known fertility were added to each cage of 10 males at varying times before and after the termination of injection and allowed to stay there continuously. In this test, when a female had passed a span of time (4–5 days) including at least one estrous cycle (12–28 hours) with a male without following parturition, this male was judged to have been infertile during the period of cohabitation, although it may have been due either to the incapability of mating or to the sterility of the male. In those females which bore the young, the date of fertilization was estimated by counting backward from that of parturition, assuming that the duration of pregnancy was 20 days. The results of mating experiments are shown in the form of graphs in Text-fig. 1.

At an inspection of this figure, if not conclusive by itself, it would seem that the injection of testicular hyaluronidase into male mice remarkably delayed fertility without completely stopping it for a certain period, i. e. about 10 to 20 days after the cessation of treatment. Before this period, on the contrary, the treatment had exerted but little or practically no effect on the fertility of the male. On the whole, this finding seems to be consistent with the results of microscopical examinations of the testes of treated animals from which we learned that disintegrative changes of the seminiferous tubules were most noticeable during the aforesaid period. With regard to the number of the young produced, the effect of hyaluronidase injections was not perceivable until about 10 days after the last injection, whereas in a span from 10 to 20 days following this period the decrease of the size of the

litter was conspicuous (Nos. 5, 12, 13) and this continued still later (Nos. 5, 6, 8). Thus, in view of the litter size, the injection effect of hyaluronidase was again clearly shown.

Fertility Test for Male Mice injected with Hyaluronidase Preparations



Text-fig. 1. Diagram to show the partial infertility of the male mice injected with hyaluronidase preparations.

Notes. A circle represents a female mate with its serial number. A white square denotes the date of fertilization estimated by counting backward from that of parturition; the figure shows a size of litter. A black square indicates the infertility judged for the female in which the cohabitation extended over more than one oestrous cycle without subsequent pregnancy. A heavy transversal line denotes a duration of cohabitation. A span of infertility period is shown by two vertical dotted lines.

* The figure $2+\alpha$ means that the youngs excepting two were devoured by their mother before inspection.

D. Demonstration of Antibodies Produced by Hyaluronidase Injections

In the previous article it has been shown that male germ cells of both the animals, mouse and rabbit, react to the "Sprase", a testicular hyaluronidase of bovine origin, repeatedly injected, resulting in a meiotic abnormalities of germ cells and even fragmentation of nuclear material. These findings suggest that it might concern with antigen-antibody reaction, inasmuch as it has been known that hyaluronidase and the proteins associated with it are antigenic to some extent (LEONARD & KURZROK, 1945) despite the observations of DURAN-REYNALS (1932), MCCLEAN (1936) and MCCLEAN & HALE (1941). To test this possibility two series of experiments were performed with blood sera prepared from three rabbits subjected to injections (Nos. 1, 2, 3) on the 10th day after the last injection. The detection of antibody of hyaluronidase was carried out as follows: Every 0.5ml. serum

Table 1. Inhibitory effect of the antihyaluronidase-sera on the follicle cell-dispersing capacity *in vitro* of hyaluronidase (500 V. U. M.)

Mixtures of serum and "Sprase" (500 V.U.M.)	0.5 cc. of serum of immunized buck No. 1 + 0.05 cc. of "Sprase"	0.5 cc. of serum of immunized buck No.2 + 0.05 cc. of "Sprase"	0.5 cc. of serum of immunized buck No.3 + 0.05 cc. of "Sprase"	0.5 cc. of serum of a non-immunized buck (control) + 0.05 cc. of "Sprase"
Tubal eggs	TE 592	TE 594	TE 595	TE 593
15 min.	No change	No change	No change	Disintegration of FCM beginning
20 min.	〃	〃	〃	FCM dispersed completely, the egg being released from it; contour of the egg cell indistinct (Pl. 3, fig. 23)
1 hr. 15 min.	〃	FCM slightly loosened but not yet dispersed	FCM fairly loosened; its peripheral parts broken down in numerous pieces	———
1 hr. 50 min.	〃	〃	A great part of FCM dispersed, thence the egg liberated perfectly	———
4 hrs.	〃	〃	FCM completely dispersed but corona radiata adhered to the egg in a more thicker layer than that of TE 593 in the normal serum; the egg showed a zig-zag outline	———
6 hrs.	FCM underwent no disintegration still now but more or less transparent; the egg shrank markedly (Pl.3, fig. 21)	FCM completely dispersed but a thick layer of str. granulosum and corona radiata adhered to the egg; precipitin reaction occurred but vitelline membrane distinctly visible (Pl.3, fig.22)	———	———

N.B. FCM=Follicular cell-mass. TE=Tubal eggs.

taken from three immunized rabbits and from a non-immunized rabbit was mixed with 0.05 ml. "Sprase" solution of 1,000 and 500 V.U.M. in a hollow slide with a depression of 23 mm in diameter and 3 mm in depth. Each four mixtures in two series of experiments were at first incubated for 30 minutes at 37°C and then the capacity of antisera and normal serum to inhibit the activity of "Sprase" was compared. Fresh rabbit ova recovered from oviducts at $10\frac{1}{2}$ to $13\frac{1}{2}$ hours after sterile matings were used for this purpose. Since unfertilized ova thus obtained are without exception surrounded by a follicular cell-mass which can easily be dispersed by "Sprase", the length of time for complete dispersion at a

Table 2. Inhibitory effect of the antihyaluronidase-sera on the follicle-cell dispersing capacity *in vitro* of hyaluronidase (1,000 V. U. M.)

Mixtures of serum and "Sprase" (1,000 V.U.M.)	1.0 cc. of serum of immunized buck No.1 + 0.05 cc. of "Sprase"	1.0 cc. of serum of immunized buck No.2 + 0.05 cc. of "Sprase"	1.0 cc. of serum of immunized buck No. 3 + 0.05 cc. of "Sprase"	1.0 cc. of serum of a non-immunized buck (control) + 0.05 cc. of "Sprase"
Tubal eggs	TE 596	TE 597	TE 598	TE 599
30 min.	FCM was perfectly in a gel condition; a strong precipitin reaction occurred thence the egg hardly visible	FCM slightly loosened; no precipitin reaction occurred	FCM fairly loosened; no precipitin reaction	Complete dispersion of FCM; the egg liberated; no precipitin reaction
45 min.	FCM very viscous still now	FCM dispersed but imperfectly; no precipitin reaction	Dispersion of FCM perfect; no precipitin reaction	———
1 hr. 15 min.	FCM turned yellowish in color without changing its initial form whereas the precipitates appeared whitish; a brownish mass within the egg clearly visible	———	———	———

NB. FCM = Follicular cell-mass. TE = Tubal eggs.

certain enzyme concentration could be taken as a criterion for finding out inhibitors by incubating ova introduced into the above mentioned mixtures at 37°C, kept damp and observing under the phase-contrast microscope from time to time. The observations were repeated with consistent results and it will be sufficient to show Tables 1 and 2 giving the results of two typical experiments in detail.

It will be seen that there is a definite suppression of the immediate dispersion of the follicular cell-mass due to "Sprase" in all three immunized rabbits of both the series, although the degree of suppression varies according to the enzyme potency employed (compare Tables 1 and 2). At no time the normal serum could exert any inhibitory influence on "Sprase" action (Pl. 3, fig.23). In their inhibitory effects three antisera differed somewhat as Table 1 shows; the follicular cell-mass in antiserum No. 1 remained undispersed more than 6 hours after incubation whereas in antisera Nos. 2 and 3 it was completely dispersed within 6 and 4 hours respectively (Pl. 3, figs. 21, 22). This may be probably attributed to constitutional factors of different individuals. Further, it is remarkable that in some antisera,

if not always, so strong precipitin reaction could be demonstrated under the microscope, that the whitish precipitates made the egg hardly visible.

The evidence that antiserum produced in the rabbit as a result of repeated injections of the bovine testicular hyaluronidase inhibits the dispersal activity of this enzyme on follicular cell-mass is quite revealing that the said enzyme acts as an antigen and its antigenicity is species non-specific.

According to HOBBY *et al.* (1941) the hyaluronidase of bacterial origin has antigenic properties but its antigenicity is strictly specific. In this point of view, there is a marked difference between the testicular and the bacterial hyaluronidase.

IV. DISCUSSION

It was found that the repeated injections of bovine testicular hyaluronidase to the male mouse and rabbit resulted in a pronounced testicular degeneration involving the disintegration of germ cells. Among these cells the spermatocytes and the spermatids were specially subjected to damage whereas the Sertoli cells and the spermatogonia remained unaffected. The intratubular spermatozoa showed morphologically no damage; their decrease in number or their entire lack was to be seen as a result of affection of their antecessors. As regards the intertubular tissue, the interstitial cells seemed to be most resistent to the treatment.

It is remarkable that the results of cytological observations in the present experiments, except some particular figures, resemble many traits observed by NAKAMURA & MAKINO (1950) on the artificially produced cryptorchid rats. Since, however, in all animals experimented upon here the testes were found in normal scrotal position, these spermatogenic disturbances cannot be attributed to the cryptorchid condition caused by the injection of foreign materials, contrary to the opinion of QUICK (1926), inasmuch as such cytological damages, consistent with temporary infertility, took place only for a short period ranging approximately from 10th to 20th day following the last injection. Further, a parallel occurrence of the same phenomenon in the mouse and the rabbit treated with the hyaluronidase makes also any familial disposition unjustifiable. It is, therefore, legitimate to infer that there should have been a causative relationship between the activity of hyaluronidase injected with and the degenerative changes of germ cells. With regards to limited susceptibility of spermatogenic cells to hyaluronidase injection, there are two possibilities to be considered, either an enzyme-substrate reaction or an antigen-antibody reaction.

When the dose of hyaluronidase was exceedingly high, the enzyme acted to a certain degree upon the intratubular connective tissue and the basement memberane, the fibrous tissue being dissolved. As far as meiotic irregularities concerned, however, the hyaluronidase solution which had been inactivated by heating to 60°C for half an hour was found to be likewise effective to a certain extent. Therefore, it does not seem likely that the meiotic abnormalities above described were caused by a direct enzymatic action of the hyaluronidase injected. On the other hand, the antigenic effects of bovine testicular hyaluronidase upon the spermatogenic cells were so much the more likely as its antigenicity could be definitely demonstrated. It was proved that the sera of rabbits, that had been injected with bovine testicular hyaluronidase, inhibited the dispersal action on follicular cells of

the enzyme used for immunization. The species non-specificity of testicular hyaluronidase in its antigenicity was thus fully established, confirming the results found by LEONARD & KURZROK (1945) on rats.

Needless to say, the fact that the antigenic effect of testicular hyaluronidase on the spermatogenesis is not species-specific but cell-specific or, strictly speaking, phase-specific and confined exclusively to spermatocytes and spermatids, reveals the presence of the homologous antigen i.e. hyaluronidase in these cells. However, the problem whether the inhibition of activity of this enzyme can be a single agent responsible for all degenerative changes of male germ cells observed should be in the first place examined; there can be numerous antigens to be considered because of incompleteness of purification of the enzyme preparation employed. HENLE *et al.* (1938) succeeded, by means of sonic vibration followed by centrifugation, to break the spermatozoa of several mammals into heads and tails and they found by using the two components separately as antigens that there are head-specific and tail-specific antigens, both of which are thermolabile and that there is a species-specific, thermostable antigen which seems to be common to both heads and tails. However highly a testicular extract may be purified, it is not possible at the present condition of our technique to isolate each antigen separately; we have to content ourselves with indirect deduction from the experimental data obtained.

As a result of injections of bovine testicular hyaluronidase, there occurred agglutinins, presumably of species non-specific nature, in the antisera of both the mouse and the rabbit; this was definitely demonstrated not only *in vitro* but also *in vivo*, i.e. within the seminiferous tubules showing the agglutinated masses of spermatozoa of varying sizes according to their potency. Since the normal spermatogenesis returned in about 3 to 6 weeks after the cessation of injections whereas agglutinins were still detectable in the antiserum for over three months, it is reasonable to assume that there is no correlation between sperm agglutinins and spermatogenic irregularities. In one case (Table 1, TE 594 ; Pl. 3, Fig. 22), there occurred a strong precipitin reaction exhibiting the turbidity of the mixture detectable in a depression slide with the phase-contrast microscopy, yet the inhibitory effect was seen in all mixtures quite independently from the degree of turbidness. The precipitin reaction of this kind, therefore, does not appear to be related particularly to any of the spermatogenic abnormalities. Formation of lysins was not able to demonstrate directly.

Since the publication of pioneer works of LANDSTEINER (1899) and METSCHINIKOFF (1899), numerous investigators have contributed to the knowledge of sperm antibodies or spermatotoxins, which can be produced by injecting spermatozoa, testis material or testicular extract and act more or less toxic for the homologous and heterologous germ cells, or male or female gonads, showing sometimes immobilization or agglutination of spermatozoa, sometimes partial or complete sterility of male or female, although the results obtained are not always unanimous (MOXTER, 1900; DE LESLIE, 1901; TAYLOR, 1908; DUNBAR, 1910; DITTLER, 1920; DERVIUX, 1921; GUYER, 1922; TSUKAHARA, 1922; MCCARTNEY, 1923; HEKTOEN & MANLY, 1923; KENNEDY, 1924; FOGELSON, 1926; BASKIN, 1932; GUYER & CLAUS, 1933; HENLE *et al.*, 1938; LEONARD & KURZROK, 1945; FEWSON, 1955).

For producing spermatotoxins against testicular extract, nucleoprotein is the most plausible constituent to be considered as antigen because of its high content in the bull testis.

However, degenerative changes of spermatogenic cells cannot be ascribed to its antigenicity, though traceably present, in view of the fact that GUYER & CLAUS (1933) could not find any abnormal spermatogenesis in a male rat subjected to injections of purified testicular nucleoprotein.

Viewed in these ways, it leads naturally to the conclusion that the disintegrations of spermatogenic epithelia particularly meiotic irregularities are results of toxic action of antibody which is formed against the testicular hyaluronidase administered; hence anti-hyaluronidase might have played a part of heretofore known spermatotoxins.

We have now to interpret the mechanism involved in such a relationship between spermatogenic abnormalities and antigenic effects of hyaluronidase. It is well known that hyaluronidase is an enzyme which hydrolyses the mucopolysaccharide, i.e. hyaluronic acid, a highly polymerized and strikingly viscous compound. Strictly selective reaction of antihyaluronidase to spermatocytes and spermatids is an evidence that the enzymatic activity of hyaluronidase, presumably released from nucleus or chromosome of these cells, must be first initiated at the period of meiotic divisions; thus the *locus in quo*, wherefrom testicular hyaluronidase is produced, has been pursued and confirmed, whereas the spermatozoon seems to be transporter of a gene producing this enzyme. At present, we know very little of the chemical properties of the cytoplasm of mammalian germ cell, much less of the underlying metabolic process associated with the cell division, but in view of its specific biological function the incomparable complexity of chemical composition of the cytoplasm at the maturation stages is highly probable, and the hyaluronic acid may play a part as a constituent of it, presumably serving for anaerobic glycolysis of the host cell which is subjected to successive divisions and sooner or later to be isolated from the germinal epithelium and hence necessitates the means of its independent subsistence for a while. Through accumulation of the hyaluronic acid, the viscosity of the cytoplasm would naturally become so high that the meiotic divisions might be troubled or stopped completely if this compound of high molecules could not be broken down in advance. This breakdown activity prior to glycolysis is the just mission of corresponding enzyme, hyaluronidase. Results of our cytological observations reflect indeed the effects of suppressing the activity of this enzyme due to antibody; frequent occurrences of multinucleated and giant cells of unicellular origin are undoubtedly the consequences of nuclear divisions without accompanying cytoplasmic divisions, figures of arresting at metaphase or of multipolar divisions (most commonly tripolar divisions) or of chromosome bridging are manifestations in aggravated karyokinesis, and several nuclear degenerations as pycnosis, karyorrhexis, karyolysis and debris formations exhibited in spermatocytes and spermatids indicate the cytocidal effects of anti-hyaluronidase.

In this view, any inhibitor acting on hyaluronidase within the testis, be it antibody, be it physical or chemical agent, may be responsible for inducing meiotic irregularities or some types of sterility. For example, sterility or spermatogenic disorder caused by experimental cryptorchism (MOORE, 1924; NAKAMURA & MAKINO, 1950), exposures of testis to the infra-red rays (CUNNINGHAM & OSBORN, 1929; GUYER & CLAUS, 1933) or to high temperatures (MOORE & OSLAND, 1924; MOORE, 1926; LAGERLÖF, 1934; PHILIPPS & MCKENZIE, 1934; KNUDSEN, 1954) may be explained by the inhibitory effect of heat on this enzyme because of its lower optimum than abdominal temperature, coupled with the

fact that there are many similarities in cytological aspects between the results of these experiments and that of ours. In contrast to such an inhibitor, some of viscosity reducing agents for the cytoplasm of germ cells, so far as it is not toxic, can be an accelerator for meiotic divisions; previous finding on the action of the proteolytic pancreatin activating the second polar body formation in the unfertilized rabbit ovum, bears out this assumption (YAMANE, 1930).

Up-to-date there have been two opposite opinions among investigators on the role of the testicular hyaluronidase. The dispersal activity of this enzyme has led MCCLEAN & ROWLANDS (1942), FEKETE & DURAN-REYNALS (1943) and SALMAN & BIRKELAND (1950) to postulate a theory that denudation of the ovum is a necessary antecedent to the penetration of spermatozoon into it. And based upon it, ROWLANDS (1944), KURZROK *et al.* (1946), SWYER (1946), and SOKOLOVSKAJA (1947) and others reported that either direct or indirect addition of hyaluronidase preparation to the semen to be effective for promoting the fertility in artificial insemination. Not a few of subsequent investigators, however, have produced counter-evidences to this concept (GREENBERG *et al.*, 1945; CHANG, 1947; AUSTIN 1948; JOHNSTON & MIXNER 1950; WADA *et al.*, 1955). It has been known that, though in the cow, tubal ova are found denuded soon after ovulation (HARTMAN *et al.* 1931; EVANS & MILLER, 1935, and HAMILTON & LAING, 1946), the bull testes contain a great deal of hyaluronidase. Aside from the problem whether or not the hyaluronidase is a promoter of fertility for artificial insemination, it is logical to seek its substrate among other sources because there can be no enzyme without substrate. As the most probable substrate the cervical mucus may be taken into consideration from the point ofview that the presence of mucopolysaccharide in it has been repeatedly observed (WOODMAN & HAMMOND, 1925; BOYLAND 1946; SHETTLES, 1951) and KURZROK & MILLER (1928) have found in the human semen an enzyme which dissolves the cervical mucus. This possibility, however, has been denied by ABRABANEL (1946), KURZROK (cit. in ENGLE, 1946) and POMMEREKE & VIERGIVER (1947). Now, our present study seems to throw light on this problem hitherto veiled in obscurity: The leading part played by the testicular hyaluronidase is its catalytic action which enables the reduction of cytoplasmic viscosity associated with an important morphogenetic activity, i. e. meiotic divisions of germ cells.

V. SUMMARY

1. Although the chemical action of testicular hyaluronidase peculiar to the mammal has been fairly well defined by the elaboration of numerous biochemists, its physiological significance cannot be seen as fully established.

2. The generalized concept postulated with the idea of "spreading factor", that its dispersal activity on the follicular cell-mass enclosing the ovum is a necesssary antecedent to ensure the fertilization, seems to be unjustifiable because of lack of such a cell-mass at ovulation in a number of mammalian species.

3. The object of this paper is to present the evidence of intracelullar activity of the testicular hyaluronidase indispensable for normal spermatogenesis.

4. A highly purified bovine testicular hyaluronidase was repeatedly injected to the male

mouse and rabbit.

5. A remarkable disorganization was observed for a while in spermatogenic epithelia.

6. Lack of meiotic divisions or meiotic irregularities in the spermatocytes and pycnosis or nuclear fragmentation resulting further in debris formation, karyorrhexis and karyolysis of the spermatids were prevailing phenomena.

7. As a consequence, a striking decrease in number of intratubular spermatozoa and a partial infertility occurred.

8. In the sera of rabbits subjected to the injections of hyaluronidase, an antibody which inhibits the dispersal action of homologous enzyme on the follicular cell-mass of rabbit ovum was definitely demonstrated.

9. It follows, therefore, that the disintegration of spermatogenic epithelia particularly meiotic irregularities are results of inhibitory action of antibody which is formed agaist the testicular hyaluronidase administered.

10. The mechanism involved in such a relationship between spermatogenic abnormalities and antigenic effects of hyaluronidase was discussed.

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VII. EXPLANATIONS OF PLATES

All photomicrographs are taken by an ordinary camera of Leica size and originals have been magnified discretionally, hence it is difficult to give an exact degree of magnification for each figure.

Pl. 1.

Fig. 1. Follicular cell-mass enclosing an ovum recoverd from the tube of a doe at 13½ hours after a sterile mating; its stickiness is shown by drawing it with a glass needle left upwards.

Fig. 2. Dispersion of the follicular cell-mass by a supernatant fluid of centrifuged rabbit spermatozoa in Tyrode solution. The ovum with corona radiata is completely isolated.

Fig. 3. Dispersion of the follicular cell-mass in a solution of 1,000 V.U.M. "Sprase". At the bottom of a hollw slide numberless denuded nuclei of follicular cells are precipitated; a released ovum lying in the center of the optical field is out of focus. Phase-contrast microscopy.

Fig. 4. Degeneration of the epithelia of seminiferous tubules on the 19th day after the last injection in the mouse No. 10 treated with 40,000 V.U.M. "Sprase".

Fig. 5. Metaphase of a three-nucleated cell observed in the mouse No. 10 above described. Stamp preparation.

Fig. 6. Tripolar metaphase of the same animal.

Fig. 7. Chromosome-bridging in a secondary spermatocyte observed on the 12th day after the last injection in the mouse No. 3 treated with 7,500 V.U.M. "Sprase".

Fig. 8. Pycnosis and karyolysis of spermatids in the mouse No. 10 above described.

Fig. 9. A giant sperm head in spermioteleosis observed in the mouse No. 3 above described; compare its size with that of normal sperm heads in the underlying epithelium.

Pl. 2.

Fig. 10. Seminiferous tubules in the left testis on the 5th day after the last injection in the mouse No. 4 treated with 22,500 V.U.M. "Sprase". Degenerative changes are not yet seen.

Fig. 11. Seminiferous tubules in the right testis on the 11th day after the last injection in the same mouse. Degeneration of spermatogenic epithelia is clearly shown.

Fig. 12. Coalescence of chromosomes of spermatids resulting in debris formation observed in the mouse No. 4 above described.

Fig. 13. A four-nucleated spermatid presumably originated from a two-nucleated secondary spermatocyte; chromosomes are arrested at hollow metaphase. Stamp preparation on the 21st day after the last injection in the mouse No. 5 treated with 22,500 V.U.M. "Sprase".

Fig. 14. A giant spermatid with 15 or 16 nuclei, one of which is undergoing pycnosis, occurred in the mouse No. 3 above described. Stamp preparation.

Fig. 15. Agglutination of sperm heads in a seminiferous tubule on the 12th day after the last injection in the mouse No. 2 treated with 7,500 V.U.M. "Sprase".

Fig. 16. Giant prophase of a primary spermatocyte observed in the mouse No. 3 above described.

Pl. 3.

Fig. 17. Hydropic degeneration with vacuole formation in a spermatogonium observed in the mouse No. 12 treated with 40,000 V.U.M. "Sprase" on the 106th day after the last injection.

Fig. 18. A three-nucleated cell at metaphase found on the 20th day after the cessation of injection in the rabbit No. 5 administered with 30,000 V.U.M. "Sprase". Stamp preparation.

Fig. 19. A five- nucleated spermatid from the testis of the same rabbit. Stamp preparation.

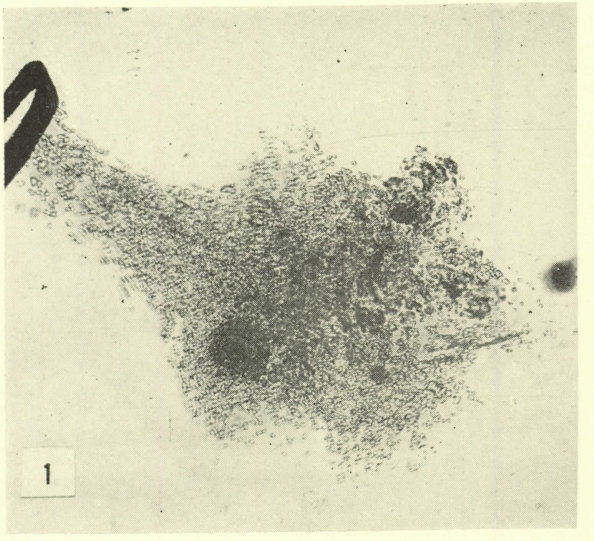
Fig. 20. Agglutination of sperm heads in a seminiferous tubule of the same rabbit.

Fig. 21. Inhibitory effect of the antiserum obtained from the rabbit No. 1 injected with "Sprase" on the follicle cell-dispersing capacity *in vitro* of "Sprase". See Table 1, column 2. No dispersion of the cell-mass took place even 6 hours after incubation of the mixture. Phase-contrast microscopy.

Fig. 22. Inhibitory effect of the antiserum obtained from the rabbit No. 2 treated with in the same way as No. 1. No change in the follicular cell-mass took place until 4 hours after incubation of the mixture though later dispersed; the mixture became turbid due to the precipitin reaction.
See Table 1, column 3. Phase-contrast microscopy.

Fig. 23. Liberation of an ovum from the follicular cell-mass which was dispersed completely in the mixture of nomal blood serum and "Sprase" solution. Complete dispersion took place in 20 minutes after incubation of the mixture. See Table 1, last column. Phase-contrast microscopy.

Fig. 24. A strong agglutination of spermatozoa in the antiserum obtained from the mice No. 8 on the 41st day when the spermatogenic epithelia began to recover from the damage; hence sperm agglutinin is not responsible to the meiotic irregularities. Phase-contrast microscopy.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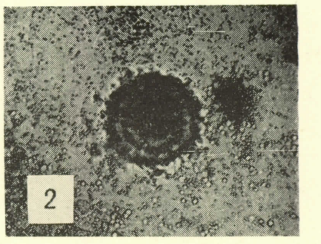
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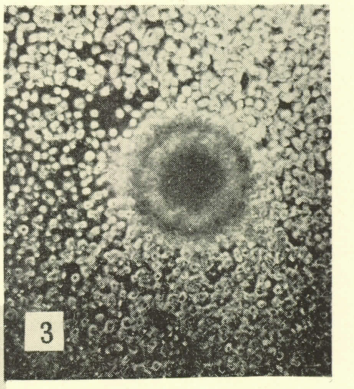
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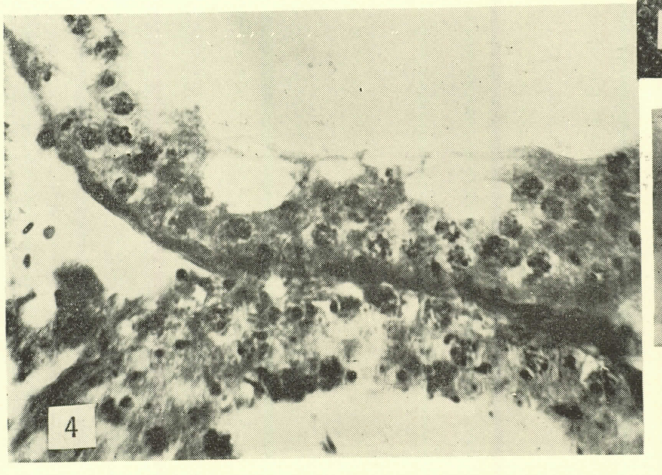
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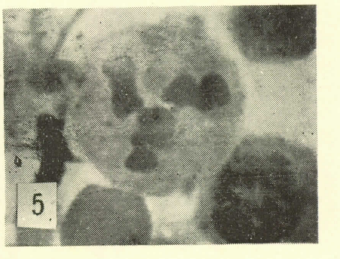
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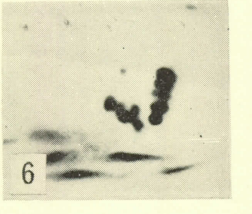
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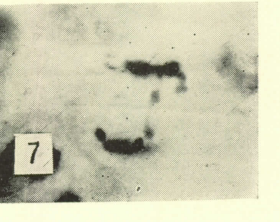
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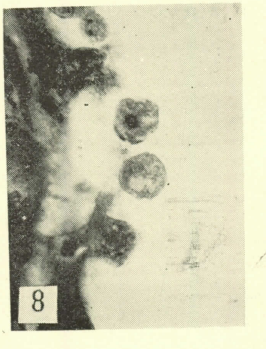
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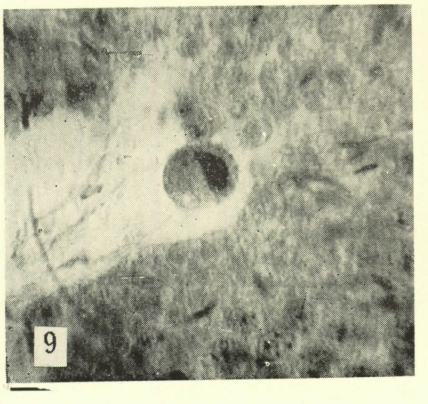
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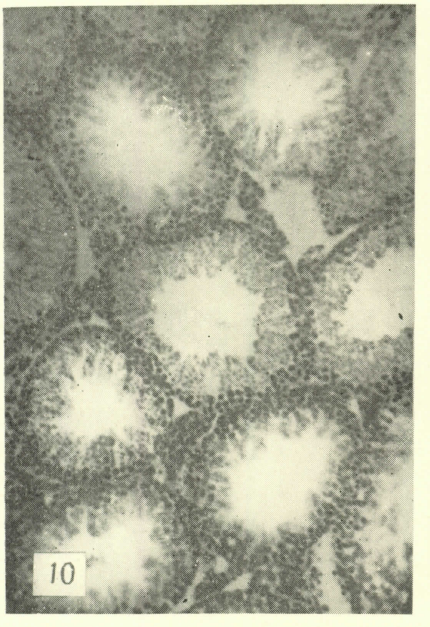

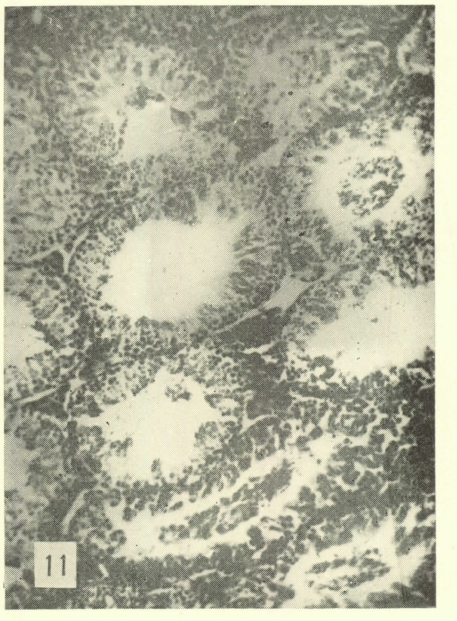

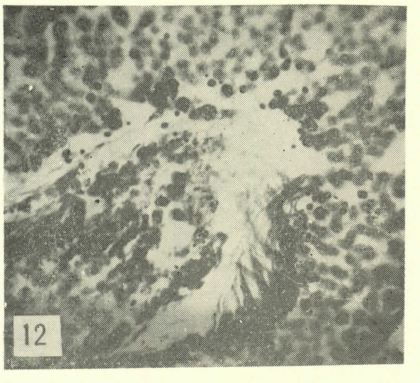

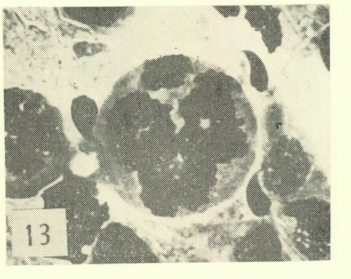

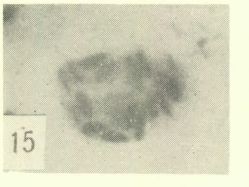

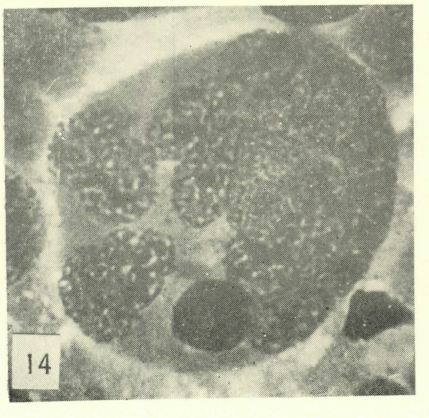

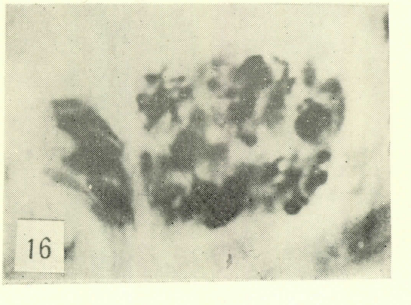

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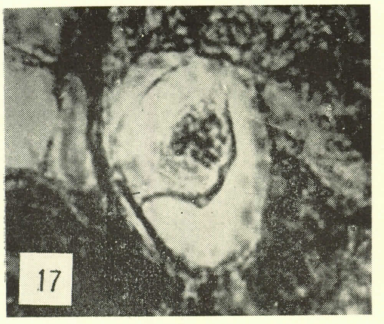
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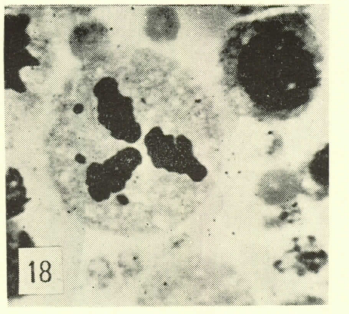
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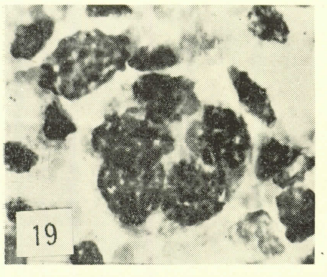
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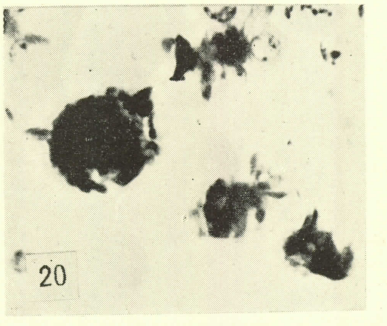
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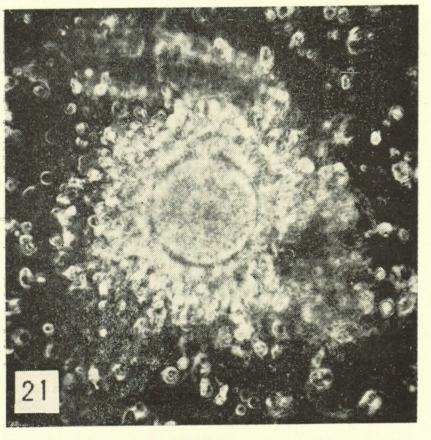
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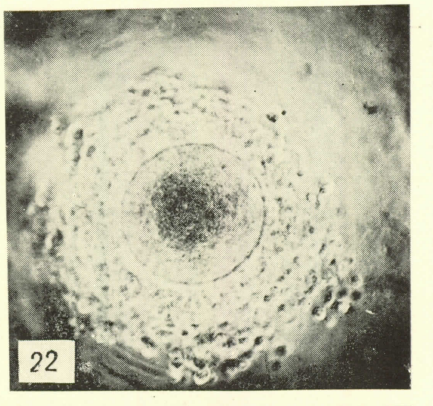
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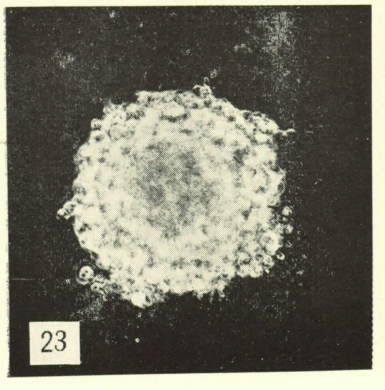
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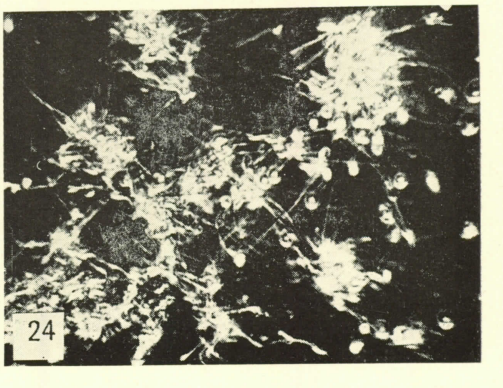
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