The Hyaluronidase Content of Semen

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The occurrence in mammalian testes and aqueous extracts of spermatozoa of a substance causing increased spread of fluids injected intradermally was described by Hoffman & Duran-Reynals (1931) and McClean (1930, 1931). These observations have since been confirmed by numerous workers (Chain & Duthie, 1940; McClean, 1943; Humphrey, 1943) and the 'spreading factor' has been shown to be identical with the enzyme hyaluronidase first described by Meyer, Dubos & Smythe (1937). The work of Long (1912), Yamane (1930), and Pincus & Enzmann (1935) had demonstrated that a substance present in semen had the property of dissolving the viscous gel cementing the cumulus cells around freshly ovulated rat ova, and the implication of testicular hyaluronidase in the fertilization process was suggested by McClean & Rowlands (1942) who demonstrated its ability to perform the same function, a finding subsequently confirmed by Fekete & Duran-Reynals (1943) for the mouse and by Leonard & Kurzrok (1945) for the rat. Rowlands (1944) observed an increase in the fertilizing capacity of dilute rabbit sperm suspensions on the addition of an hyaluronidase-containing medium. The existing evidence, therefore, suggests that the role of seminal hyaluronidase in the fertilization process consists in denuding the egg of its surrounding follicle cells so as to enable penetration and syngamy to be effected by a spermatozoon, and that the ineffectiveness of oligospermic semen may be accounted for, at least in part, by its inability to establish, in the vicinity of the egg, a sufficient concentration of the enzyme to dissolve off the cumulus.

In order to investigate this hypothesis further, and also to provide data which might serve as a basis for the possible therapeutic use of hyaluronidase in the treatment of infertility due to oligospermia, it was thought useful to make some quantitative investigations of the hyaluronidase content of the semen from different species and individuals. This paper sets forth some of the results which have been obtained.

MATERIAL AND METHODS

(1) *Human semen.* Fifty-eight human semen samples have been assayed. They were obtained either by coitus interruptus followed by ejaculation into a glass jar or by masturbation; no condom specimens have been used. In order to prevent loss of potency of the contained enzyme they were stored, as soon as possible after being produced, in a refrigerator, and after sperm counts had been made, in the frozen state. For assay, they were thawed, diluted with 0·5% gum arabic in the proportion of 1 ml. semen to 3 ml. gum arabic (or higher dilutions in the case of very dense specimens) and filtered through cotton wool to remove particles which might have blocked the viscosimeter tubes.

(2) *Rabbit semen.* Eleven rabbit semen samples have been assayed. They were obtained by the artificial vagina technique. For assay, 0·1 ml. was diluted with 9·9 ml. water and allowed to stand at 0° for at least 24 hr. in order to allow the release of all the hyaluronidase from the spermatozoa (Swyer, 1947). If some time was to elapse before viscosimetric assay, the suspensions were stored frozen at −10°. Further dilutions were made to the required degree with 0·5% gum arabic.

(3) *Boar semen.* Six boar semen samples, which had been collected by the artificial vagina technique and freeze-dried in bulk at the end of 1944 and beginning of 1945, have been assayed. They were reconstituted to the original volume by addition of the appropriate quantities of water, sperm counts being made on the reconstituted semen. For assay, they were diluted, if necessary, with 0·5% gum arabic.

(4) *Bull semen.* Five samples of bull semen, obtained by the artificial vagina technique, have been assayed. The first two were pooled samples of several ejaculates. They were stored frozen until required when they were thawed and diluted with suitable amounts of 0·5% gum arabic for assay.

(5) *Dog semen.* Seven samples of dog semen, collected by the artificial vagina technique, have been tested, some of them after storage in the frozen state and others within a few hours of ejaculation.

(6) *Fowl semen.* Ten samples of fowl semen, obtained by manual stimulation have been tested. Sperm counts and viscosimetric testing were done on the first two samples while fresh. The remainder were stored frozen, after which treatment it was, unfortunately, found impossible to do sperm counts with any accuracy because of agglutination of many of the spermatozoa; there is, however, no reason to believe that tests for hyaluronidase were vitiated by this procedure.

Sperm counts were made in a Spencer Bright Line haemocytometer, the dilutions, made with haemocytometer pipettes, being adjusted so that in general 200–400 sperms were counted for each sample. Hyaluronidase assays were made by the viscosimetric method described by Swyer & Emmens (1947), in which the unit of hyaluronidase activity is approximately equal to 53·5 viscosity reducing units of McClean & Hale (1941). The standard error of this method is less than 10%.

RESULTS

(1) *Human semen.* The hyaluronidase contents, expressed in units/ml. (Swyer & Emmens, 1947) and in units/total ejaculate, of fifty-eight samples of human semen are shown in Table 1. It can be seen that the four azoöspermic specimens were totally devoid of hyaluronidase, and that with increasing sperm density there is an increasing hyaluronidase concentration. Analysis of these figures for the linear regression of enzyme content on sperm density yields a correlation coefficient of 0·942, and a regression coefficient of 0·003369, indicating that 88·7 % of the hyaluronidase content can be accounted for by the sperm density. The equation for the regression line is $y = 0{\cdot}003369x + 0{\cdot}024545$, where y = hyaluronidase content in units/ml. and x = sperm count in millions/ml. (see Fig. 1).

Table 1. *Sperm count and hyaluronidase content of human semen*

Sperm count (millions/ml.)	Volume of ejaculate (ml.)	Hyaluronidase (units/ml.)	Hyaluronidase units in total ejaculate
0	1·4	0	0
0	3	0	0
0	3·2	0	0
0	1·3	0	0
0·08	2·5	Very faint trace	—
c. 0·5	2·2	Trace	—
1·5	2	0·017	0·025
2·3	5	0	0
2·5	2·5	0·05	0·125
2·7	7	0·038	0·266
3	3·5	0·027	0·095
6·2	0·8	Trace	—
7	3·6	0·093	0·335
9·5	5	0·05	0·25
12	3	0·08	0·24
12	2	0·108	0·22
14	6	0·051	0·306
14	5·5	0·042	0·23
15	2·7	Trace	—
17	6·2	0·14	0·87
23	2·7	0·08	0·27
28	2·7	0·15	0·4
28	2·5	0·116	0·29
33	6·6	0·077	0·51
40	3·5	0·058	0·202
40	2·5	0·32	0·8
45	2	0·28	0·56
48	3·2	0·1	0·32
52	2·4	0·19	0·45
54	1·6	0·36	0·575
61	5·8	0·066	0·38
65	3·5	0·46	1·62
67	1·7	0·365	0·62
80	1·7	0·16	0·27
80	5·6	0·395	2·2
92	1·8	0·425	0·765
97	1·8	0·313	0·536
100	6·0	0·41	2·46
115	3·8	0·364	1·385
120	1·8	0·41	0·74
125	1·2	0·68	0·815
140	5	0·33	1·65
140	2·6	0·534	1·39
145	1·5	0·23	0·34
145	2·5	0·55	1·375
160	2·5	0·29	0·72
160	1·5	0·23	0·32
160	6·3	0·415	2·62
165	4	1·0	4·0
180	6·3	0·99	6·25
185	4	0·49	1·96
200	3·1	0·86	2·67
235	2·5	0·735	1·84
275	2·8	1·015	2·84
280	2·3	1·06	2·44
320	3·8	1·15	4·37
460	2·4	1·62	3·9
490	1·3	1·57	2·04

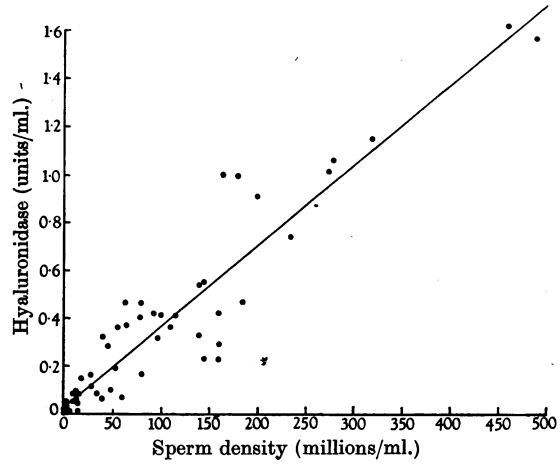

Fig. 1. Relationship between sperm density and hyaluronidase content in human semen.

It may therefore be concluded that the hyaluronidase content of human semen is almost entirely dependent on the sperm count, since the combined errors of assaying and counting would easily account for the remaining 11·3 % of variation. A further corollary is that the output of hyaluronidase/sperm in different individuals must be remarkably constant. It may be pointed out that in this series, which is not a random sample from a normally distributed population, the correlation coefficient is scarcely permissible; the use of the regression function, on the other hand, is not affected by the nature of the series and so is preferable.

From Fig. 1 it can be seen that the expected hyaluronidase content of a human semen sample containing 100 million sperms/ml. is about 0·38 unit. With an average volume of 3 ml. this would give 1·08 units in the total ejaculate.

(2) *Rabbit semen.* The hyaluronidase contents of eleven samples, the densities of which ranged from 120 to 1150 million sperms/ml. are shown in Fig. 2 and range from 39 to 254 units/ml. The close correlation between sperm count and enzyme content is evident.

The hyaluronidase contents of a few rabbit testes were also estimated by grinding with sand, extracting with water in the cold for 24 hr., filtering and assaying the filtrate. One buck, whose ejaculates obtained weekly for 15 weeks were found to contain from 115 to 820 million sperms/ml., had a unilateral congenitally undescended testicle. The normal testis had a moist weight of 1·94 g. and contained 62·5 units of hyaluronidase (31·7 units/g. moist tissue). The undescended testis weighed 0·23 g. and contained no demonstrable hyaluronidase. The two testes of a 27-day-old male (they were as yet undescended) weighed 45 mg. each and also contained no demonstrable hyaluronidase.

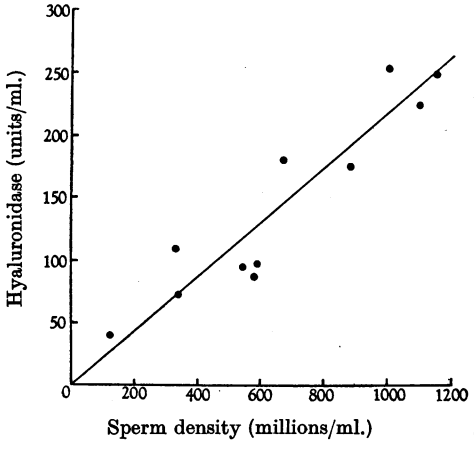

Fig. 2. Relationship between sperm density and hyaluronidase content in rabbit semen. (In this and subsequent figures, the straight line drawn through the origin is not a fitted regression line.)

(3) *Boar semen.* The hyaluronidase contents of six samples, the densities of which ranged from 120 to 390 million sperms/ml., are shown in Fig. 3, and are seen to range from 0·24 to 0·77 unit/ml. With the exception of one specimen, a very close correlation between sperm count and hyaluronidase content can be seen.

(4) *Bull semen.* The hyaluronidase contents of five samples, the densities of which ranged from 300 to 1320 million sperms/ml., are shown in Fig. 4, and range from 22 to 94 units/ml. Again, a close correlation between sperm count and hyaluronidase content is evident.

(5) *Dog semen.* Of the seven samples of dog semen tested for hyaluronidase activity, sperm counts were made on only four. In three of these, the sperm counts were 4, 155 and 300 millions/ml. and no hyaluronidase activity could be detected. In the fourth, there were 130 million sperms/ml. and 0·28 unit of hyaluronidase/ml. Of the remaining three samples whose densities were not estimated, one contained a trace of hyaluronidase and the others 0·015 and 0·025 unit respectively. (Both the latter specimens were able to effect the disintegration of the cumulus surrounding freshly ovulated rabbit ova in the course of about 1 hr. at room temperature.) The variability in results cannot be ascribed to the treatment which the semen samples had received

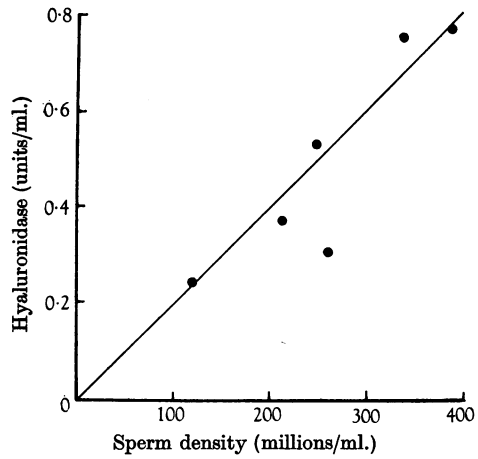

Fig. 3. Relationship between sperm density and hyaluronidase content in boar semen.

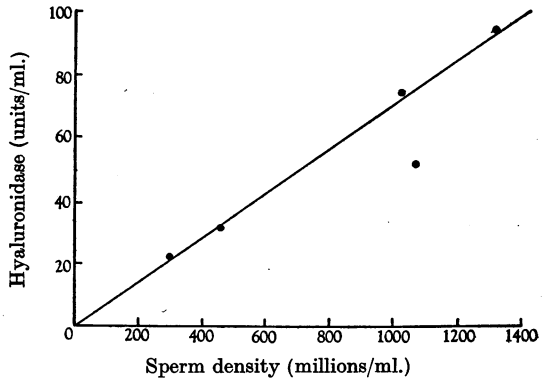

Fig. 4. Relationship between sperm density and hyaluronidase content in bull semen.

since hyaluronidase activity and inactivity were found among the fresh as well as the frozen specimens. It is evident, therefore, that the hyaluronidase content of dog semen is variable independently of the sperm density, is of a low order, and in some cases at least, is too small to be detected by the method of assay used.

(6) *Fowl semen.* Of the ten samples tested for hyaluronidase activity, six were found to show none at all. In the remaining four it was impossible to make any attempt at accurate assay since the degree

of activity and the quantity of semen available in each case was insufficient to perform the necessary procedures. A very rough estimate of activity would assign to the four samples hyaluronidase contents of 1, 0·5, 0·3 and 0·05 unit/ml. respectively. The only two samples on which sperm counts were made contained 4600 and 2500 million sperms/ml., and both showed no hyaluronidase activity (they were not frozen before testing, but since four of the frozen samples also showed no hyaluronidase activity, it is not likely that freezing would have led to a different result). The densities of those specimens possessing viscosity-reducing activity were in no case greater and in some far less (probably as low as 10 million sperms/ml. in one) than these. It appears, therefore, that fowl semen contains little or no hyaluronidase and that no correlation between hyaluronidase activity and sperm density exists in this species.

DISCUSSION

The number of sperms necessary to fertilize a mammalian ovum is apparently very great, and is considerably larger than would appear to be sufficient merely to ensure the chance contact of at least one sperm with the egg. The suggestion of McClean & Rowlands (1942) that the presence of a large number of sperms in the vicinity of the egg may be necessary to ensure the production of concentration of hyaluronidase sufficient to liquefy the hyaluronic acid gel cementing the follicle cells around the egg, assumes that the hyaluronidase content of semen is related to its sperm density. The evidence presented in this paper justifies this assumption and thereby lends considerable support to the above-mentioned theory. Pincus (1930) and Pincus & Enzmann (1932) have shown that while spontaneous dissolution of the cumulus takes place as the unfertilized ovum descends the uterine tube, this process requires some 6–8 hr. to reach completion in the rabbit, by which time the egg has acquired a layer of protein sufficiently thick to prevent penetration by spermatozoa. The hyaluronidase mechanism, therefore, enables fertilization to occur within 2 or 3 hr. of ovulation, while the deposition of protein around the unfertilized ovum prevents fertilization of ageing eggs.

With further regard to the direct proportionality between sperm density and hyaluronidase content in mammalian semen, some evidence to this effect, in the case of man, had already been provided by Joël & Eichenberger (1945) and by Werthessen, Berman, Greenberg & Gargill (1945), though at the time the work reported here was done these papers were unknown to the author. Since azoöspermic human semen is devoid of hyaluronidase, while the regression line of enzyme content on sperm density passes through zero, it follows that no hyaluronidase is supplied by the accessory gland secretions. The intimate association of the enzyme with the spermatozoa, which is further borne out by the results reported in an accompanying paper (Swyer, 1947), and the evidence relating to the hyaluronidase contents of active and inactive rabbit testes, may therefore be regarded as establishing beyond reasonable doubt the origin of seminal hyaluronidase.

No explanation can at present be advanced to account for the anomalous results obtained in the case of dog semen.

Of considerable interest is the finding that hyaluronidase was absent from six out of ten samples of fowl semen and present in the remaining samples in amounts which, considering their sperm densities, must be regarded as trivial. In view of the fact that the ova of birds are not surrounded by cumuli (see, for example, Brambell, 1925), this observation accords with the hypothesis concerning the presence and function of seminal hyaluronidase. The observation reported by Hamilton & Laing (1946), that cumulus cells persist around the unfertilized ovum of the cow for 9–14 hr. after ovulation clears up the discrepancy which had appeared to exist in this species by reason of the belief that the ovum of the cow was devoid of a cumulus at the time of ovulation.

Attention may be drawn to the striking differences in the absolute values for the hyaluronidase contents of semen in different species, when expressed in terms of standard sperm densities, in contrast to the uniformity among individuals of the same species. Thus, the hyaluronidase contents/100 million spermatozoa/ml., of the semen of rabbit, bull, man and boar are approximately 20, 7, 0·4 and 0·2 units, respectively. It seems probable that a fairly close relationship exists between the numbers of spermatozoa necessary for fertility in different species and the hyaluronidase contents of the spermatozoa.

From the standpoint of the therapeutic aspects of the problem of infertility, the function of hyaluronidase in the fertilization process suggests the possibility of treating infertility in humans due to oligospermia by supplementing the deficiency in enzyme of the semen with added hyaluronidase. On the other hand, it may be pointed out that the existence of a form of sterility due to quantitative deficiency of hyaluronidase in semen which is otherwise normal in all respects (sperm density, morphology, motility and viability) is rendered very unlikely by the close correlation which has been shown to exist in human semen between hyaluronidase content and sperm density.

SUMMARY

1. Assays of the hyaluronidase content of the semen of men, rabbits, bulls, boars, dogs and fowls have been made by the method described by Swyer & Emmens (1947). These have shown that in the

first four species there is a close correlation between hyaluronidase content and sperm density. In the case of dogs and fowls no such correlation could be found.

2. The absence of hyaluronidase in a cryptorchid and in prepubertal rabbit testes is demonstrated. The conclusion is reached that seminal hyaluronidase originates in active seminiferous epithelium and is not secreted by the accessory glands.

3. The bearing of these results on the role of hyaluronidase in fertilization and its implication in the possibility of treatment of infertility due to oligospermia is discussed.

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The Release of Hyaluronidase from Spermatozoa

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The belief that seminal hyaluronidase is associated with spermatozoa rather than with the seminal plasma is suggested by the close correlation between hyaluronidase content and sperm density in several species of mammals; by the absence of hyaluronidase from azoöspermic semen; and by its presence in testes in which active spermatogenesis is taking place and its absence in inactive testes (prepubertal and cryptorchid), as shown in an accompanying paper (Swyer, 1947). The present report is concerned with some of the factors which appear to be involved in the liberation of the hyaluronidase contained in spermatozoa.

EXPERIMENTAL

Material and methods. All the experiments to be described were carried out with rabbit semen obtained by the artificial vagina technique. Hyaluronidase assays were made by the method of Swyer & Emmens (1947), the unit of hyaluronidase activity being that described by these authors. The standard error of this method is less than 10 %.

Extraction of hyaluronidase with water

McClean (1931) showed that extracts of spermatozoa made with distilled water contain large amounts of the 'spreading factor' now known to be hyaluronidase. It was observed, however, that if an aqueous suspension of spermatozoa were assayed within a short time of being made up, the hyaluronidase value obtained was less than if the suspension had been allowed to stand for some hours before assaying.

The following experiment, therefore, was performed to investigate this point. Three pooled ejaculates were used; four suspensions were made by adding 0·2 ml. semen to 9·8 ml. distilled water. A fifth portion of 0·2 ml. semen was freeze-dried in an ampoule. The five samples of semen were then subjected to the treatment shown in Table 1 and assayed for hyaluronidase. The values, expressed in units/ml. of semen show that a short period of freezing at $-10°$, or a considerable period of standing at 0° enabled an apparently maximal amount of