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Effect of Uterine Environment and Uterine Extract on Guinea Pig Sperm Metabolism

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It was demonstrated that rabbit spermatozoa was acquired the fertilizing capacity in estrous uterus. And this capacitated spermatozoa was inhibited to penetrate into the ova treating with seminal plasma.

In the present report, to clear these phenomena from the biochemical aspect, it was studied that how respiration and anaerobic fructolysis of guinea pig spermatozoa were influenced by the uterine environment and uterine extract.

1) Guinea pig spermatozoa incubated in estrogen dominated uterus shows much greater respiration and anaerobic fructolysis activity than that of incubated in progesterone dominated uterus.

2) Treatment with seminal plasma caused great inhibition of respiratory activity of spermatozoa that was incubated in estrogen dominated uterus.

3) Respiration and anaerobic fructolysis stimulating factor of guinea pig spermatozoa was extracted from the estrogen treated guinea pig uterus. And from bovine uterus the same stimulating factor (a protein of approximately 50000 molecular weight estimated from the behavior on Sephadex G-75 gel filtration).

4) This bovine uterine extract recovered or accelerate the low respiratory activity of the spermatozoa for the existence of seminal plasma.

From these results, it is suggested that the extract from the guinea pig and bovine uterus might play a major role in sperm respiration and anaerobic fructolysis, and further be related to capacitation process in estrous uterus.

Chang[2)] and Austin[3)] independently demonstrated that rabbit spermatozoa normally require a period of residence in the female reproductive tract to develop the capacity to fertilize ova. Subsequent evidence suggested that this phenomenon of sperm capacitation was prerequisite to fertilization in many mammals.[4,5)] Sperm capacitation has been conventionally demonstrated by the incubation in uterus or oviduct of an estrous female. Sperm capacitation did not occur effectively in blood serum or when spermatozoa were placed in a dialysis bag in the uterus.[6)] Meanwhile, Chang[7)] found the decapacitation phenomenon that the fertilizing ability of capacitated rabbit spermatozoa were inhibited by seminal plasma.

The spermatozoa retained their motility, however, and if redeposited into Fallopian tubes for a certain period of time before ovulation, they regained their ability to fertilize ova (recapacitation).[7)] Seminal plasma from bull, boar, stallion and primates as well as rabbit decapacitated the spermatozoa.[8)]

In this report, to clear these phenomena from biochemical aspect, respiration and anaerobic fructolysis of guinea pig spermatozoa incubated in estrogen- or progesterone-dominated uterus were measured, and the effect of seminal plasma on uterine sperm respiration was

1) Location: *6-1-1 Toneyama, Toyonaka, Osaka.*
2) M.C. Chang, *Nature*, **168**, 697 (1951).
3) C.R. Austin, *Australian J. Sci. Res.*, **4**, 581 (1951).
4) R.W. Noyes, *J. Dairy Sci.*, **43**, *Suppl.*, 68 (1960).
5) M.C. Chang, *Nature*, **175**, 1036 (1955).
6) R.W. Noyes, A. Walton, and C.E. Adams, *J. Endocrinol.*, **17**, 374 (1958).
7) M.C. Chang, *Nature*, **179**, 258 (1957).
8) W.R. Dukelow, H.N. Chernoff, and W.L. Williams, *J. Reprod. Fert.*, **14**, 393 (1967).

studied. Moreover, it was attempted to extract the sperm respiration-stimulating factor from the uterus.

Experimental

Preparation of Sperm Suspension——Epididymal spermatozoa was obtained from the male guinea pig weighing about 600 g and suspended in Ca-free Krebs-Ringer phosphate buffer (KRP buffer, pH 7.4) or Ca-free Krebs-Ringer bicarbonate buffer (KRB buffer, pH 7.4) containing penicillin (50 IU/ml) and streptmycin (50 μg/ml) at 37°.

This suspension was washed twice carefully to avoid the mechanical damages to spermatozoa.

***In Utero* Insemination of Guinea Pig Spermatozoa**——Female guinea pig weighing about 400 g was treated with estradiol-17β (150 μg/day) or progesterone (150 μg/day) for 2 weeks. Under ether anesthesia, uterus was ligated at utero-tubal and utero-cervical junction. Then the washed sperm suspention was inseminated into the uterus (0.2 ml volume) through a 22-gauge needle. Several hours after the insemination, the spermatozoa were recovered from the tracts and washed with buffer and resuspended in sufficient volume to give a concentration of 1.0×10^8 spermatozoa per ml for the following experiments.

Measurement of Respiration and Anaerobic Fructolysis——Oxygen uptake and CO_2 output were measured by means of Warburg apparatus. As the medium Ca-free KRP buffer was used for the assay of respiration in air phase, and Ca-free KRB buffer was used for anaerobic fructolysis in N_2 gas phase.

Fructose was added into the apparatus as the substrate (2.5 mg/apparatus). To measure the sperm respiration-stimulating factor, uterine extract (5 mg) was also put into the chamber. The sperm number were counted for each experimental flask and all values were expressed as μl of O_2 uptake and CO_2 output per 10^8 sperm cells.

Extraction of Sperm Respiration-stimulating Factor——Estrogen- or progesterone-dominated guinea pig uteri and bovine uteri were homogenized with equal amount (w/v) of 0.15 M NaCl and centrifuged. Ammonium sulfate was added into the supernatant to make 50% saturation and pH was adjusted to 6.5 with 1 N NaOH under constant stirring. The precipitate which appeared to 50% saturation was removed by centrifugation.

The supernatant was made to 100% saturation by further addition of ammonium sulfate at pH 6.5 and the precipitate was obtained. These fractions (50% ppt, 100% ppt) were dialyzed against water, lyophilized and designated as Fr. Es-50, Fr. Es-100 (Es; estrogen-dominated uteri), Fr. Pro-50, Fr. Pro-100 (Pro; progesterone-dominated uteri) from guinea pig and Fr. A, Fr. B from bovine uteri respectively.

Gel Filtration on Sephadex G-75——Gel filtration on Sephadex G-75 was carried out, using a gel column (2.8×85 cm) equilibrated with 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. The eluates were examined at 280 mμ for protein. Fr. B (about 50 mg/2 ml) extracted from bovine uteri was applied to the column, which was developed with the same buffer.

Result

Effect of Uterine Environment and Seminal Plasma on Anaerobic Fructolysis and Respiration of Spermatozoa

The anaerobic fructolysis of guinea pig spermatozoa incubated in progesterone-dominated (Pro) uterus for 6 hours was resulted in 45 μl CO_2 output after 2.5 hours as shown in Fig. 1.

When incubated in estrogen-dominated (Es) uterus, CO_2 output increased 2-fold over at 2.5 hours period, compared with that in Pro uterus. The CO_2 output of epididymal (Ep) spermatozoa incubated in KRB buffer *in vitro* was slightly lower than that of spermatozoa incubated in Pro uterus.

The ratio of anaerobic fructolysis of spermatozoa incubated in Es (or Pro) uterus to that of Ep spermatozoa incubated in KRB buffer for 1, 3, 6 and 9 hours respectively was illustrated in Fig. 2.

Although there were no difference in the ratio of Es or Pro uterine spermatozoa to Ep spermatozoa at 1 and 3 hours incubation, the Es/Ep ratio slightly increased for 6 hours, and remarkably increased ($p<0.05$) for 9 hours compared with that of Pro/Ep.

In the cases of experiments in sperm respiration, the spermatozoa incubated in Es uterus for 6 hours caused a marked increase. There were, however, little differences between Pro uterine spermatozoa and Ep spermatozoa as shown in Fig. 3.

Chang reported that the spermatozoa which had been capacitated *in utero* could be decapacitated by an addition of 5% seminal plasma.[7] Therefore Es uterine spermatozoa which obtained stimulating abilities in respiration was used as the capacitated spermatozoa for the following experiments.

The Es and Pro uterine spermatozoa were treated with 5% seminal plasma for 30 min or incubated in KRP buffer as control, and then they were tested for respiration (Fig. 4).

The data revealed that the Es uterine spermatozoa treated with 5% seminal plasma was extremely inhibited the respiration ability to about half of the initial level. However, Pro uterine spermatozoa was only slightly inhibited.

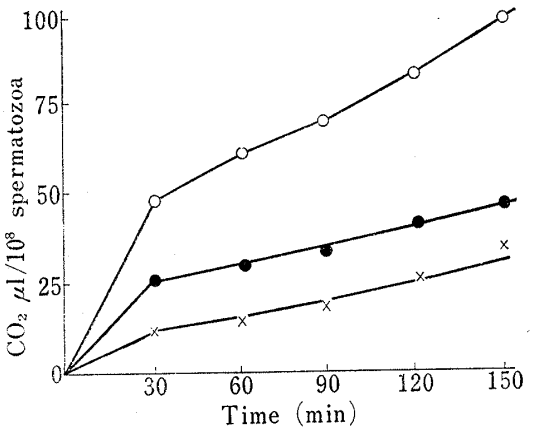

Fig. 1. Fructolysis of Guinea Pig Spermatozoa incubated *in utero* for 6 Hours

Fructolysis was determined with Warburg apparatus in Ca-free KRB buffer at 37°. gas phase; N_2, substrate; fructose (2.5 mg)
—×—: (Ep) spermatozoa
—○—: (Es) uterine spermatozoa
—●—: (Pro) uterine spermatozoa

Effect of Uterine Extract on Sperm Respiration

The fact that the respiration and anaerobic fructolysis of epididymal spermatozoa were stimulated during the incubation in estrogen-dominated uterus, but not in progesterone-dominated uterus, suggests that there are some stimulating factors for respiration and anaerobic fructolysis of spermatozoa in estrogen-dominated uterus.

Effect of the extracts from estrogen- or progesterone-treated guinea pig uterus on epididymal sperm respiration is illustrated in Fig. 5.

Only Fr.Es-100 increased the O_2 uptake of spermatozoa, but stimulating effects were not found in other uterine extracts (Fr.Es-50, Fr.Pro-50 and Fr.Pro-100).

From Fig. 6 the spermatozoa incubated with uterine extract (Fr. B) from bovine showed the promoting effect in O_2 uptake at the rate similar to Fr.Es-100 in Fig. 5. Consequently,

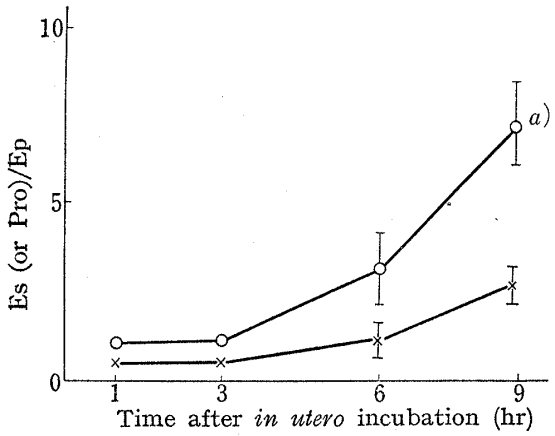

Fig. 2. Effect of Uterine Incubation on Guinea Pig Fructolysis

Es or Pro; CO_2 evolution of the spermatozoa incubated in estrogen- or progesterone-dominated uterus for 150 minutes
Ep; CO_2 evolution of epididymal spermatozoa for 150 minutes
a) $p < 0.05$; significant difference between the ratio of Es/Ep and Pro/Ep for 9 hours incubation
—○—: Es/Ep, —×—: Pro/Ep

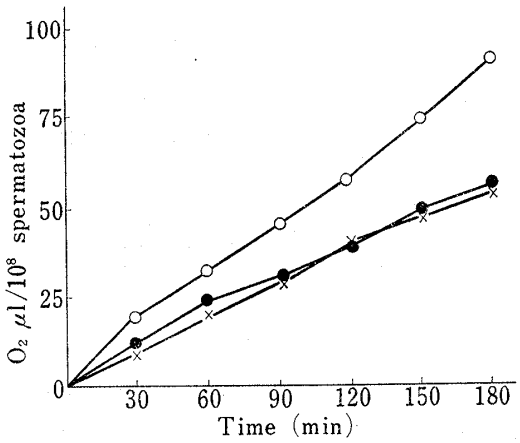

Fig. 3. Respiration of Guinea Pig Spermatozoa incubated *in utero* for 6 Hours

Respiration was determined with Warburg apparatus in Ca-free KRP buffer at 37°. gas phase; air, substrate; fructose (2.5 mg)
—×—: (Ep) spermatozoa
—○—: (Es) uterine spermatozoa
—●—: (Pro) uterine spermatozoa

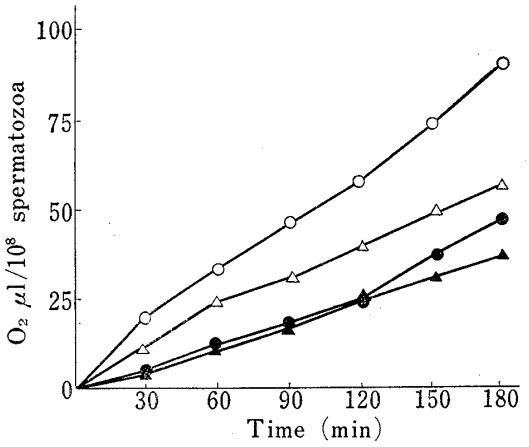

Fig. 4. Effect of Seminal Plasma on Uterine Sperm Respiration

Respiration was determined with Warburg apparatus in Ca-free KRP buffer at 37°
—○—: (Es) uterine spermatozoa
—●—: (Es) uterine spermatozoa treated with seminal plasma
—△—: (Pro) uterine spermatozoa
—▲—: (Pro) uterine spermatozoa treated with seminal plasma

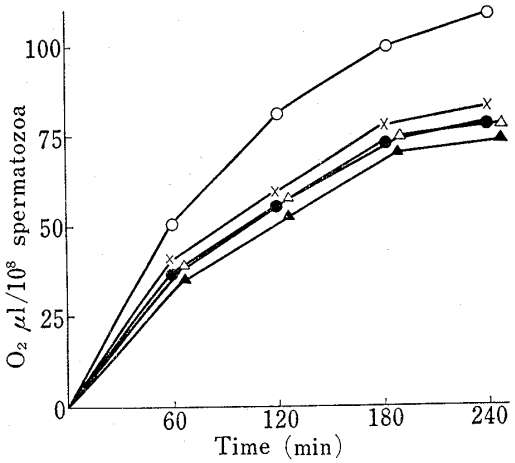

Fig. 5. Effect of Guinea Pig Uterine Extract on Guinea Pig Sperm Respiration

Respiration was determined with Warburg apparatus in Ca-free KRP buffer at 37°.
—×—: control (bovine serum γ-globulin, 2.5 mg +bovine serum albumin, 2.5 mg)
—○—: Fr.Es-100 (5 mg)
—●—: Fr.Es-50 (5 mg)
—△—: Fr.Pro-100 (5 mg)
—▲—: Fr.Pro-50 (5 mg)

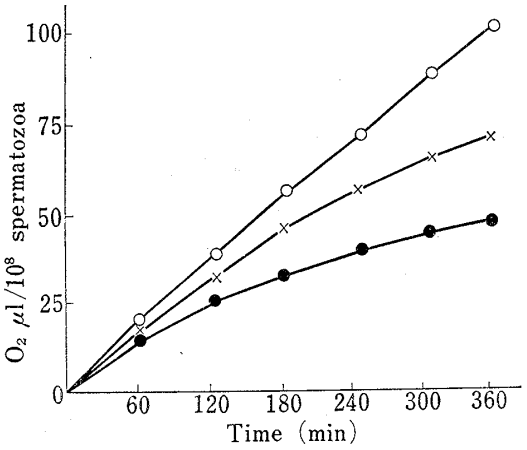

Fig. 6. Effect of Bovine Uterine Extract on Guinea-Pig Sperm Respiration

Respiration was determined with Warburg apparatus in Cafree KRP buffer at 37°.
—×—: control (bovine serum γ-globulin, 2.5 mg +bovine serum albumin 2.5 mg)
—●—: Fr.A; $(NH_4)_2SO_4$, 50% Ppt (5 mg)
—○—: Fr.B; $(NH_4)_2SO_4$, 100% Ppt (5 mg)

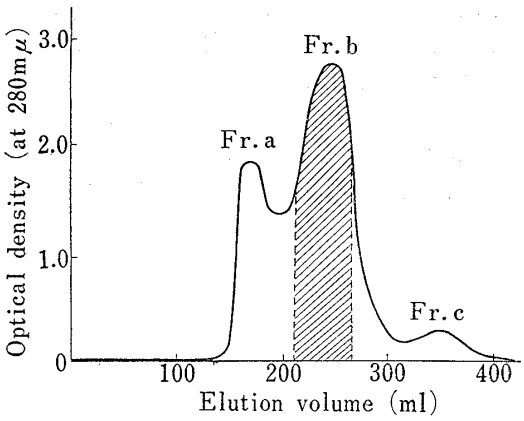

Fig. 7. Chromatography of Fr. B on Sephadex G-75

The column scale was 2.8×85 cm. The eluate was 0.1M phosphate buffer (pH 7.4) containing 0.15M NaCl, and the flow rate was about 20 ml/hour.

the substance in Fr.Es-100 or Fr.B which was extracted from uterus might be a cause of the stimulating effect on respiration and anaerobic fructolysis of guinea pig spermatozoa incubated in estrogen-dominated uterus.

As an atempt to pursue the principle of this stimulating effect Fr.B was fractionated with gel filtration on Sephadex G-75 column chromatography and separated into three peaks; Fr.a, Fr.b and Fr.c (Fig. 7).

As clearly seen in Fig. 8 showing the effect on sperm respiration *in vitro*, Fr.b was the only fraction markedly increasing the O_2 uptake. This stimulating effect on sperm respiration

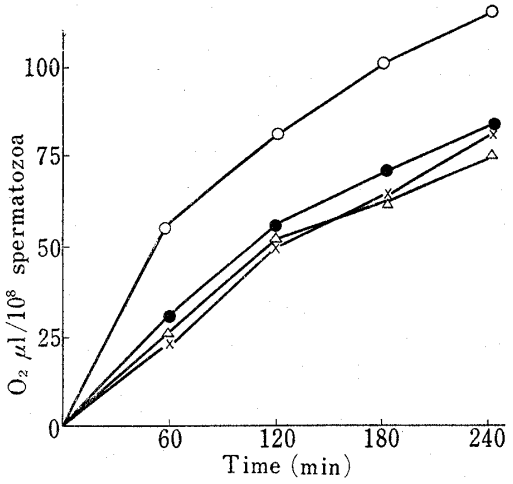

Fig. 8. Effect of Bovine Uterine Extract on Guinea Pig Sperm Respiration

Respiration was determined with Warburg apparatus in Ca-free KRP buffer at 37°.
—×—: control (bovine serum γ-globulin, 2.5 mg +bovine serum albumin, 2.5 mg)
—●—: Fr.a (5 mg)
—○—: Fr.b (5 mg)
—△—: Fr.c (5 mg)

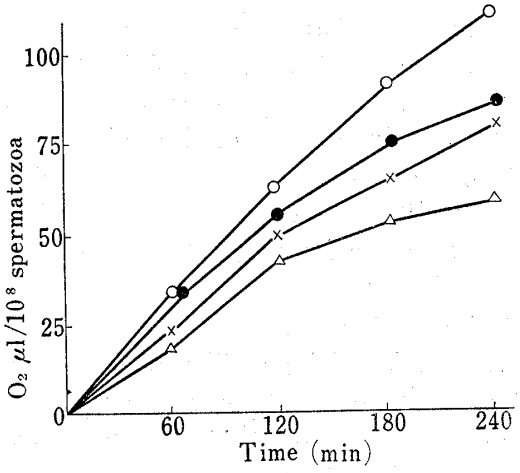

Fig. 9. Effect of Bovine Uterine Extract on Respiration of Guinea Pig Spermatozoa treated with Seminal Plasma

Respiration was determined with Warburg apparatus in Ca-free KRP buffer at 37°.
—×—: control (bovine serum γ-globulin, 2.5 mg +bovine serum albumin, 2.5 mg)
—○—: seminal plasma+Fr.B (5 mg)
—●—: seminal plasma+Fr.b (5 mg)
—△—: seminal plasma

of Fr.b was almost the same extent as that of the incubation in estrogen-dominated uterus in Fig. 3.

Since Chang has been reported the recapacitation phenomenon, the effect of Fr. B and Fr. b on sperm respiration in the presence of seminal plasma was examined (Fig. 9).

The O_2 uptake of the spermatozoa treated with seminal plasma diminished to the extent of 70% of control level. However, the O_2 uptake decreased by seminal plasma was recovered to the control level or higher by the coexistence of Fr. B or Fr. b.

Discussion

Hamner, *et al.*[9] and other investigators[10] have informed that the estrous condition was ideal for capacitation of rabbit spermatozoa in the uterus of the doe. Conventionally, capacitation has been demonstrated by the incubation in estrogen-dominated uterus.

In the anaerobic fructolysis and respiration, the spermatozoa incubated in estrogen-dominated uterus revealed the active metabolism compared with that in progesterone-dominated uterus. These results represented the reproducible biochemical changes in the spermatozoa while residing in the female genital tract. These are also strongly suggestive of the possibility of the essential changes in the sperm cell itself, because the spermatozoa are throughly out of the reproductive tract secretions and suspended in the same physiological media as freshly washed spermatozoa.

Concerning the changes in spermatozoa founded on capacitation, Austin and Bishop[11] observed that the acrosome caps were removed from the head of spermatozoa before entry into the ova and suggested this phenomenon might be the mechanism of capacitation.

9) C.E. Hamner, J.P. Jones, and N.J. Sojka, *Fert. Steril.*, **19**, 137 (1968).
10) *a*) M.C Change, *Endocrinol*, **63**, 619 (1958); *b*) P. Soupart, *J. Reprod. Fert. Suppl.*, **2**, 49 (1967).
11) C.R. Austin and M.W.H. Bishop, *Nature*, **181**, 851 (1958).

Chang and Slechta,[12] and Aonuma, *et al.*[13] have shown that the guinea pig and hamster spermatozoa incubated in estrous uterus for several hours were lacking in much acrosome caps compared with that of epididymal spermatozoa. As to rabbit spermatozoa, however, they could not find any differences by the ordinary visual examinations between the spermatozoa obtained from epididymis and those recovered from the uterus.

As one of the mechanisms of capacitation, Aonuma, *et al.*[14] recently demonstrated that the decapacitation factor (DF) was combined with guinea pig spermatozoa in the epididymis and that capacitation might be derived from the removal of DF from the spermatozoa in the female genital tract.

While seminal plasma from male rabbit, bull and man could bring about a reversible inhibition of fertilizing ability of capacitated rabbit spermatozoa, our findings showing that treatment with 5% seminal plasma of the spermatozoa preincubated in estrogen-dominated uterus remarkably inhibited the respiration (Fig. 4) suggest the sperm cells are biochemically influenced by the treatment in utero and semen. In the present data, a possible relationship between the increase (or decrease) of O_2 uptake and capacitation (or decapacitation) is reasonably indicated.

About the changes of uterus which vary with their reproductive state, Daniel has reported that he separated electrophoretically uterine fluid proteins.[15] It is well established that estrogen promotes the biosynthesis of specific proteins in the uterus. Then we pursued the extraction of respiration-stimulating factor from the uterus and revealed the existence of it. The result that the extract, Fr. b, from bovine uterus stimulated the sperm respiration (Fig. 8) suggests that this extract affects to their energy metabolism. From its behavior on Sephadex G-75 column chromatography, its principle would be a protein of approximately 50000 molecular weight.

On the other hand, it has been reported that the stimulating effect of rabbit oviduct fluid on the sperm respiration is due to a bicarbonate in the fluid.[16] The data of Foley and Williams,[17] however, does not support the contention of Hamner and Williams that bicarbonate cannot explain all of the stimulating effects of rabbit oviduct fluids. Furthermore, rabbit spermatozoa incubated in the oviduct fluid consumed 12% more oxygen than that incubated in the medium dissolved an equivalent amount of bicarbonate.[18]

The data in the present study showed that the sperm respiration-stimulating factor extracted from bovine uterus significantly promotes the respiration even after the complete dialysis removing all the small molecular substances containing bicarbonate.

Moreover, the results from Fig. 5 showed that the sperm respiration-stimulating factor should be extracted from only estrogen-dominated uterus but not from progesterone-dominated uterus. The result that the respiratory inhibited spermatozoa by the treatment with seminal plasma was recovered to the control level in the presence of uterine extract suggest that this effect of the uterine extract is related to the increase of O_2 uptake of the spermatozoa incubated in estrogen-dominated uterus.

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12) M.C. Chang and R.F. Slechta, "Recent Progress in the Endocrinology of Reproduction," ed. by C.W. Lioyd, Academic Press, New York, N.Y., 1959, p. 151.

13) S. Aonuma, T. Mayumi, K. Suzuki, M. Okabe, and H. Kawano, *Nippon Naibumpi Gakkai Zasshi*, **49**, 799 (1973).

14) S. Aonuma, T. Mayumi, K. Suzuki, T. Noguchi, M. Iwai, and M. Okabe, *J. Reprod. Fert.*, **35**, 425 (1973).

15) J.C. Daniel, *Comp. Biochem. Physiol.*, **24**, 297 (1968).

16) *a*) C.E. Hamner and W.L. Williams, *Proc. Soc. Exptl. Biol. Med.*, **117**, 240 (1964); *b*) C.E. Hamner and W.L. Williams, *Federation Proc.*, **24**, 430 (1964).

17) C.W. Foley and W.L. Williams, *Proc. Soc. Exptl. Biol. Med.*, **126**, 634 (1967).

18) R.N. Murdoch and I.G. White, *J. Reprod. Fert.*, **14**, 213 (1967).

About the stimulating factor for capacitation of spermatozoa, many experiments were reported.[19] Recently, Aonuma, *et al.*[20] succeeded in the induction of rabbit sperm *in vitro* capacitation in the modified KRB buffer. Although the fertilization percent of the spermatozoa capacitated in estrous uterus extend to about 80%, the spermatozoa incubated *in vitro* in defined medium reached only 27% fertilization. These results strongly suggest the existence of capacitation factor.

One could thus speculate that Fr. b might play a major role in sperm respiration and further capacitation process in estrous uterus.

Acknowledgement We must express our thanks to the fact that this work was partly supported by a grant from the TAKEDA Science Foundation.

19) *a*) A. Iritani, W.R. Gomes, and N.L. Vandemark, *Biol. Reprod.*, **1**, 72 (1969); *b*) A. Iritani, Y. Tsunoda, M. Satoh, and Y. Nishikawa, *Jap. J. Zootech. Sci. Suppl.*, **43**, 41 (1972).

20) S. Aonuma, T. Mayumi, K. Suzuki, and M. Okabe, *Nippon Naibumpi Gakkai Zasshi*, **49**, 1161 (1973).