STUDIES ON THE SENESCENCE OF THE COCK SPERMATOZOA DURING STORAGE

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INTRODUCTION

It the majority of farm animals the effective length of life of spermatozoa within the body of the female is extremely short. In domestic fowls, the duration of fertility, after the removal of the male, the single mating or insemination, is exceedingly long; the longest interval reported is above 30 days (CREW, 1926; DUNN, 1927; NALBANDOV and CARD, 1943). But several investigators are in agreement that it is about 12 or 14 days following the removal of the males (CURTIS and LAMBERT, 1929; WARREN and KILPATRICK, 1929; WALTON and WHETHAM, 1933; NICOLAIDES, 1934; VAN DRIMMELEN, 1945). The behavior of sperm in the oviduct during such a long fertility has been studied by several investigators. Some investigators have failed to find sperm in oviducts of mated or inseminated hens (WALTON and WHETHAM, 1933). Other investigators have come to assume the intraovarian fertilization owing to failure to abolish fertility by irrigating oviduct with spermicidal solutions (IVANOV, 1924). OLSEN and NEHER (1948) conclusively proved that the sperm normally fertilize the ovum after ovulation and before it reaches the magnum by cross-switching ova from inseminated hens to virgin hens. VAN DRIMMELEN (1945) found the active and morphologically normal sperm in the infundibulum during 8 to 14 days following insemination. He (1946) subsequently found masses of sperm (sperm nests) in deep crypts in the infundibular mucous membrane of a hen 8 days after insemination. He suggested that the release of sperm from sperm nests for fertilization would occur as a result of stretching the oviduct during passage of the yolk. GRIGG (1957) supported this suggestion by his experiment which showed that the intact infundibulum-magnum of an inseminated hen with irrigated and no sperm was found in the washings; and after an artificial ovum was pulled through the lumen, several hundred spermatozoa were dislodged by the subsquent washing.

As stated above, the cock sperm survives and maintains the fertilizing ability in the oviduct of a hen for such a long time. On the other hand, the loss of fertilizing capacity of stored semen in vitro is remarkably rapid, the semen stored for 24 hours is almost unfertile in spite of persistence of motility (SHIBATA, 1938; JASPER, 1950; GARREN and SHAFFNER, 1952; BOGDONOFF and SHAFFNER, 1954; SCHINDLER et al., 1955).

The problem on the behavior of sperm in the oviduct of a hen is atracting the interest of investigators. On the other hand, the fact that the fertilizing capacity of stored semen in vitro is rapidly decreased is a great obstacle on practice of artificial insemination of domestic fowls.

This study was undertaken in an attempt to clear the cause of the rapid decline of the fertilizing capacity of stored cock semen in vitro.

CHAPTER I CHANGES OF THE COCK SPERMATOZOA DURING STORAGE

In order to make clear the cause of the rapid decline of the fertilizing capacity of cock sperm during storage, various changes of cock semen during

storage must be examined. In the present chapter, fertilizing capacity, motility, resistance, oxygen consumption rate, glycolysis and dehydrogenase abilities of cock sperm in some storage durations were determined, and moreover the life duration and deformation of sperm were observed.

Section 1 Decline in the Fertilizing Capacity of Cock Semen during Storage

It has been reported that cock semen was practicuIy difficult to store in vitro for artificial insemination (SHIBATA et al., 1938; JASPER, 1950; GARREN and SHAFFNER, 1952; BOGDONOFF and SHAFFNER, 1954; SCHINDLER et al., 1955.)

Several investigators have observed the fertility of cock semen in relation to the sperm motility (SHAFFNER, 1941; JASPER, 1950; BOGDONOFF and SHAFFNER, 1954; COOPER and ROWELL, 1958; MCDANIEL and CRAIG, 1959; WILCOX, 1959, 1960).

Some investigators have reported that the fertility of fresh cock semen immediately after collection fell with rise of the rate of abnormal sperm (SAMPSON and WARREN, 1939; SAEKI, 1960). On the other hand, no attemp has been made to find out the relation among the fertility, motility and normality (the rate of normal sperm).

The work reported in this section was undertaken to clear the correlation between fertilizing capacity and motility or the rate of abnormal sperm on the stored semen.

Materials and Methods

The semen was collected by the one-man technique descrived by BOGONOFF and SHAFFNER (1954). Semen from 8 White Leghorn cocks were mixed and were used to this study. The densities of mixed semens were 2 to 2.5 million per mm^3. The semen was diluted at the rate of 1:4 with several diluents (Table 1). The samples were stored in refrigerator at 0° to 2°C. and 8° to 10°C. for 4 to 55 hours.

The motility was observed under the microscope at 35°C. and the motility score was calculated by the method of NISHIKAWA (1958). The motility score was as follow.

$$\text{Motility score} = (100\ A + 75\ B + 50\ C + 25\ D) / 100$$

A……Very active movement sperm %
B……Active movement sperm %
C……Moderate movement sperm %
D……Sluggish movement sperm %

To determine the rate of abnormal or normal sperm (abnormality or normality), the stored semen was smeard on slide-glass, and fixed by formaldehyde vapour, then it was stained with carbol-fuchsin for examination.

At the end of the storage period, White Leghorn hens maintained in laying cages were inseminated with 0.2 c.c. of semen (about 1 million of sperm) with tuberculin syringes. Eggs laid during the 2 nd to 8 th day after insemination were collected and examined the fertility on the 4 th day of incubation.

Table 1 Composition of diluents

Diluent	Concentration gm/1. dist. H_2O
Tyrode's solution	
NaCl	8.00
KCl	0.20
$CaCl_2 \cdot 2H_2O$	0.20
$NaHCO_3$	1.00
$MgCl_2 \cdot 6H_2O$	0.10
$NaH_2PO_4 \cdot H_2O$	0.05
Glucose	1.00
Lake's solution	
Sodium glutamate	17.40
Potassium citrate $\cdot H_2O$	1.28
Sodium acetate $\cdot 3H_2O$	8.51
$MgCl_2 \cdot 6H_2O$	0.68
Fructose	10.00
Sodium glutamate solution	
Sodium glutamate	30.00

Results and Discussion

The obtained results are shown in Table 2.

Table 2 Fertility, Motility and Normality of stored semen

Trial	Diluent	Storage temp. (°C.)	Storage hours	Motility	Normality (%)	Total eggs set	Eggs from fert. hens	No. fert. eggs	Fertility all eggs %	Fertility eggs from fert. hens %
1	Tyrode		soon after	85	81.5	30	30	28	93.3	93.3
	Tyrode	0−2	4	60	62.5	31	19	11	35.5	57.9
	Tyrode	0−2	24	50	5.5	31	31	0	0	0
	Lake	0−2	24	60	62.9	31	24	12	38.7	50.0
2	Tyrode	10	8	70	89.6	32	32	29	90.6	90.6
	Tyrode	0−2	8	60	34.5	33	19	0	0	0
3	Undiluted	0−2	24	50	54.9	31	31	4	12.9	66.7
	Lake	0−2	24	70	82.8	28	17	15	53.6	88.2
	Lake	0−2	55	60	70.8	32	25	18	56.3	72.0
4	Sodium glutamate	0−2	8	50	82.8	26	26	20	76.9	95.2
	Sodium glutamate	0−2	24	25	71.2	28	28	0	0	0

Each group contain 5 hens.
No. of eggs and percentage of fertile eggs laid during days 2 to 8 inclusive after insemination.

In the trial 1, semen samples were diluted, at the rate of 1:4, with

Tyrode's solution and Lake's solution and preserved at 0° to 2°C. The samples were stored for 4 and 24 hours. A control sample was kept at 30°C. until insemination after collection.

The fertilities of the semen diluted with Tyrode's solution and stored 4 hours, and those of the semen diluted with Lake's solution and stored for 24 hours were lower than those obtained with fresh semen. No fertile eggs were obtained from the semen diluted writh Tyrode's solution and stored for 24 hours. In the lower fertile semen, the normality declined remarkably but motility did not decline so much.

In the trial 2, semen were diluted 1:4 with Tyride's solution, and these samples were stored for 8 hours at 0° to 2°C. and 10°C. The fertilizing capacity of the semen stored at about 10°C. was considerably high but the other semen stored at 0° to 2° C. lost completely. When these semen samples were observed at the time of insemination, the formar had higher normar normality than the latter.

In the trial 3, undiluted semen was stored for 24 hours at 0° to 2°C. and then was diluted 1:4 with Lake's solution just before infore insemination after storage. The other semen were diluted 1:4 with the same solution before storage and were stored for 4 to 55 hours at the same temperature. As the result, the formar showed lower motility, lower normality and lower fertility.

In the trial 4, semen were diluted 1:4 with sodium glutamate solution and were stored at 0° to 2°C. for 8 and 24 hours. Both semen samples showed high normality. But the semen stored for 24 hours had a marked drop in motility, and no fertile eggs were obtained from this semen.

The correlation between the fertility and the sperm motility, and that between the fertility and the rate of normal sperm were +0.68 and +0.77 respectively.

If the following expression indicates motility of normal sperm, it can be considered to be semen soundness.

(Motility score × Percentage of normal sperm)/100

The correlation between the fertility and the soundness was +0.91.

Schindler et al. (1955) reported that the undiluted semen and the semen diluted with physiological salt solution retained full fertilization capacity after 4 hours' storage at 10°C., but the undiluted semen partially maintained fertility after 24 hours' storage at 4°C., and the diluted semen retained no fertility.

In this data, no fertile eggs were obtained from the semen diluted with Tyrode's solution stored either 8 or 24 hours at 0° to 2°C. Both of them had poor normality, but showed suitable motility.

In the semen diluted with sodium glutaminate solution, the normality was suitable but the motility was poor. Then no fertile eggs were obtained.

Recently, Lake (1960) developed a diluent contained mainly sodium glutamate, sodium acetate and fructose. Cock semen were stored at 0° to 2°C. for 24 or 48 hours, and these stored semen showed good fertility.

The present results show that the semen diluted with this diluent maintained good fertility after storage at 0° to 2°C. for 24 and 55 hours, and

then both motility and normality of these semen were suitable.

It would be appeared that the fertilizing capacity of stored cock semen depended on motility and abnormality of semen. Therefore, the writer consideres that the semen quality must be estimated by both motility and abnormality.

Section 2 Decline in The Motility and the Life Duration of Cock Spermatozoa During Storage

Many investigations have been carried out on the length of life of cock sperm in vitro (MOTOHASHI and MORITOMO, 1927; WARREN and KILPATRICK, 1929; ISHIKAWA, 1930; NIKITINA, 1932; GROZINSKI and MARCHLEWSKI, 1935; SHIBATA et al., 1938; HAYASHI, 1938; SHAFFNER et al., 1941; JASPER, 1950; BOGDONOFF and SHAFFNER, 1954; WILCOX, 1959).

In the undiluted cock semen, the sperm motility retained extending over 8 to 10 hours at 40°C., 56 to 96 hours at 10°C. and 7 to 14 days at 0°C. in vitro.

The diluents used in this experiment were designed by modification of mammalian semen diluents and physiological salines. One investigater reported that the semen diluted with Ringer's, Tyrode's and Locke's solutions had a tendency to survive longer than undiluted controls, and the other indicated that a higher percentage of motility could be retained when diluted with avian blood serum.

Only a few papers have shown the effect of storage period on motility of cock sperm.

This section discribes the length of life and the decline in motility of the undiluted and the diluted cock semen.

Materials and Methods

The ejaculated semen was collected from 8 White Leghorn cocks in the

Table 3 Composition of diluents

Diluent	Concentration (gm./1. dist. H_2O)	Diluent	Concentration (gm./1. dist. H_2O)
Glucose solution	5.00	Lake's solution	see Table 1.
NaCl solution	9.00	Seminan solution	
Ringer-Locke's sol.		Sodium citrate	16.00
NaCl	9.00	Potassium citrate	1.10
KCl	0.42	Na_2HPO_4	1.50
$CaCl_2 \cdot 2H_2O$	0.24	Sulfamethadine	0.50
$NaHCO_3$	0.20	Homosulfamine	1.00
Ringer's solution		Glucose	9.70
NaCl	9.00	Egg yolk	250.00
KCl	0.30	Gluco-Citrate solution	
$CaCl_2 \cdot 2H_2O$	0.25	Sodium citrate	15.0
$NaHCO_3$	0.20	Glucose	25.0
Tyrode's solution	see Table 1.		

manner discribed by BURROWS and QUINN (1939) or BOGDONOFF and SHAFFNER (1954). The densities of semen used in this experiment were 2 to 3 million per mm^3. The Vas deferens semen was collected by the following manner. Namely, several cocks were killed and the Vas deferens were removed immediately. The dense semen in them were sqeezed out into dry sterile watch glasses. The diluents were prepared according to Table 3. All samples were cooled solwly to protect from temperature shock. Small test tubes contained semen samples were covered with thin cotten cloth, and kept in 10 ml. test tubes, and then placed in a refrigerator.

Sperm activity was ovserved under the microscope at 35°C. Sperm motility score was the same as described in Section 1.

Results and Discussion

(1) Life duration of sperm in the undiluted semen

As shown in Table 4, irreversible loss of motility was much more rapid at higher temperature (40°C.). Life duration of the sperm in Vas deferens semen was longer than that of the sperm, in ejaculated semen in storage at 10°C. and 0° to 2°C.

Table 4 Life duration of ejaculated semen and Vas deferens semen

	Stored temp. (°C.)	No. of samples	Life duration (days)		
			Mean	S.D.	Range
Ejaculated semen	40	9	10.22 hs	1.50 hs	13−9 hs
	10	57	4.09	1.28	7−1
	0−2	16	6.50	2.02	12−4
Vas deferens semen	10	3	8.67		10−7
	0−2	3	13.33		22−8

In all animal sperm, generally, the duration of survival increases with lower temperatures. It is probable that the increased survival is due to a reduction in the rate of metabolic processes of sperm; a decrease in the consumption of the source of metabolic energy and in the accumulation the harmful metabolic products. In the semen of high density, such a tendency would be respectively remarkable.

In the present results, there were no diluted semen survived longer than control undiluted semen statistically. It seems reasonable to assume that if the suitable diluent for cock sperm develop, the life duration of sperm in semen diluted with such a diluent will extend more than that of undiluted semen.

The sperm in Vas deferens semen survived more longer than the sperm in the ejaculated semen. MANN (1954) descrived that sperm was gived momentary vitality by seminal plasma, owing to chemical substance in seminal plasma and dilution effect. Vas deferens semen contains the plasma only a

little, so the sperm survive probably for a long time.

(2) Life duration of sperm in the diluted semen

The results are shown in Table 5. The ejaculated semen were diluted to 10 times with several diluents (Table 4,) and were preserved at 10°C. The life

Table 5 Life duration of sperm in diluted semen at 10°C.

Diluent	No. of samples	Life duration (days)	
		Diluted semen	Undiluted semen
		Mean ±S. D.	Mean± S. D.
Glucose sol.	6	0.83±0.43	4.67±0.54 *
NaCl sol.	8	1.50±0.79	4.13±0.68 *
Ringer-Locke's sol.	8	1.75±1.06	4.13±0.68 *
Ringer's sol.	13	1.85±0.80	3.85±1.30 *
Gluco-Citrate sol.	6	2.17±1.40	4.67±0.54 *
Tyrode's sol.	9	3.11±0.33	4.33±1.54 *
Seminan sol.	7	3.84±0.41	4.57±1.64
Lake's sol.	8	4.45±1.53	4.50±1.51

* Statistical significance of differences
Dilution rate: 1 part ejaculated semen to 9 parts diluent.

duration of sperm in each diluent was compared with that in each control undiluted semen.

In the semen diluted with Seminan and Lake's solutions, there was no statistical difference between the diluted semen and the control undiluted semen.

In the semen diluted with other diluents, the life duration of control undiluted semen was longer than that of diluted semen in all cases.

The life duration at lower temperature, at 0° to 2°C., was shown in Table 6. Maintenance of motility at these temperature was better than that at 10°C.

Table 6 Life duration of diluted semen at 0° to 2°C.

Diluent	No. of samples	Life duration (days)
		Mean ± S.D.
Ringer's sol.	5	2.60±0.59
Tyrode's sol.	5	4.20±0.90
Lake's sol.	5	5.60±1.23
Seminan sol.	5	6.00±1.31
Undiluted *	57	6.50±2.02

* Cited from Table 4

Since the density of cock semen is very high, it is probable that lower temperature is more suitable for maintaine sperm activity as the results of this experiment.

In the semen of higher density such as ram, goat and bull semen, it had been considered that survial of sperm in undiluted semen was longer that in diluted semen at low temperature (HATZIOLOS, 1937; BONADONNA, 1939; HERMAN and SWANSON, 1941). The storage of these semen had been carried out in undilution at low temperature practically. But PHILLIPS and LARDY (1940) and SALISBURY et al. (1941) reported that yolk phosphate buffer and yolk citrate buffer were useful in survival of bull sperm, the practical storage of bull semen is curring out in dilution with these diluent widely.

(3) Decline of motility in the stored cock semen

Semen was diluted at the rate of 1:9 with Lake's, Tyrode's or Seminan solution. The diluted and the undiluted semen were stored at 0° to 2°C. or 10°C.

As shown in Table 7, irreversible loss of motility was much rapied except the semen diluted with Lake's solution and stored at 0° to 2°C. After 72 hours in storage at both 0° to 2°C. and 10°C., both motility of the undiluted

Table 7 Motility of sperm in stored cock semen

Storage temp. (°C)	Diluent	No. of samples	Days stored 0 Mean±S.D.	1 Mean±S.D.	2 Mean±S.D.	3 Mean±S.D.	4 Mean±S.
0−2	Undiluted	10	83.80± 7.45	50.00±12.48	33.50± 9.44	21.60± 5.78	11.60± 5.78
	Lake's sol.	26	80.73± 9.08	70.92±10.00	60.19±13.38	40.00± 5.48	17.70±10.53
	Tyrode's sol.	13	82.76± 8.31	58.33±12.67	38.33±16.94	15.00± 3.53	3.60± 5.40
	Seminan sol.	5	82.60±11.70	43.40± 5.78	28.40± 7.64	20.00± 4.47	6.60± 2.88
10	Undiluted	15	80.26±10.77	49.60±13.71	23.33± 7.48	9.22± 7.39	3.22± 4.00
	Lake's sol.	20	81.65± 7.57	41.05±28.07	28.40±30.77	17.50±20.17	6.00±13.82
	Tyrode's sol.	21	82.85± 5.04	63.24±16.76	32.95±28.07	9.58±12.28	0.41± 0.51
	Seminen sol.	18	79.33± 6.67	58.05± 9.87	24.72±19.92	11.85±15.10	0.71± 0.15

Dilution rate: 1 part semen to 9 part diluent
Motility score: Described in Secsion 1.

semen and that of the diluted semen almost fell down below the range of practical use for artifical insemination.

DAVIS et al. (1940), NISHIKAWA and SUGIE (1949) reported the duration of sperm motility of stored semen at various temperatures in bull and stallion. In general, the tendency of decline in motility of cock sperm during storage was similar to that of bull sperm, but decline in cock sperm was much more rapid than that of bull semen.

Section 3 Deformation of the Cock Spermatozoa During Storage

It seems that the fertilizing capacity of sperm is influenced by the sperm numbers of samples injected, motility or viability and the rage of abnormal sperm in semen.

In bull semen, HERMAN and SWANSON (1941), TRIMBERGER and DAVIS (1942)

reported that fertility fell remarkably in accordance with the raise of the rate of abnormal sperm above 50 percent. CUPPS et al. (1953) stated that the coefficient of correlation between fertility and the rate of abnormal sperm was −0.83.

In cock semen, SAPSON and WARREN (1939) reported that one sterile male was found to have a very large number of defective sperm in his seminal fluid. SAEKI (1960) stated that the coefficient of correlation between fertility and incidence of the abnormal sperm was −0.77.

The main purpose of the present investigation was to study the morphology of the abnormal sperm during storage and increasing tendencies of them with lapse of time.

Materials and Methods

The collection, dilution and storage of semen were the same as descrived in Section 1.

The densities of semen used in this experiment range from 2.5 to 3.5 million per mm^3. The examination for abnormal sperm was done by the method of WAKELY and KOSIN (1951): a semen sample was smeard on a slide-glass, and was fixed by formaldehyde vapour. Care must be taken to avoid the mechanical sperm damage or artifacts. The semen smears were then stained with Derafield's, Heidenhain's iron heamatoxylin or carbor-fuchsin.

The rate of abnormal sperm or normal sperm (abnormality or normality) was calculated from the observation of 3,000 to 5,000 sperm per one slid in 5 or more microscopic fields, and the rate was shown by percentage.

In the post-vital staining for discrimination of life or dead sperm, the modified method of SWANSON and BEORDEN (1951) was used. This involved mixing the semen samples with phosphate buffer solution contained 1% eosin and 5% nigrosin, drying at 20° to 30°C.

The electron microscopic studies were followed the method stated by GRIGG and HODGE (1949). Namely, semen samples were diluted 10 to 20 times with Tyrode's solution, and the sperm were freed from colloidal material by centrifugal force at 3000 r. p. m. This process was repeated 3 times. The sperm were then fixed by addition of a small volume of 10% formaldehyde solution, at least 24 hours at 0°C. The sperm were washed 2 times by centrifugation with distilled water. The final suspension in distilled water was ready for mounting on the electron microscope specimen screen. The mounting were prepared by allowing dry a drop of the suspension on a collodion film supported by the screen.

Results and Discussion

(1) Increase in the rate of abnormal sperm in cock semen during storage

Decrease in the rate of normal sperm (i. e. increase in the rate of abnormal sperm) of cock semen undiluted and in various diluents during storage at low

temperature (0° to 2°C.) and at moderate temperature (8° to 12°C.) was shown in Table 8.

Table 8 Increase in the percentage of normal sperm of cock semen during storage

Diluent	Storage temp. (°C.)	No. of samples	Storage times in days 0 Mean±S.D.	1 Mean±S.D.	2 Mean±S.D.
Undiluted	0–2	14	90.3± 8.2	46.7±29.1	44.3± 9.4
Tyrode's		22	88.5± 8.7	6.0± 3.6	5.3± 0.3
Ringer's		11	90.8± 6.2	12.4±11.0	6.8± 5.7
Lake's		13	87.9± 7.7	73.9±14.0	74.2±16.0
Seminan		4	89.1± 8.0	62.1±20.8	51.6±29.8
Undiluted	8–12	37	91.2± 6.2	61.9±17.9	42.1±18.4
Tyrode's		11	89.6± 7.8	34.7±22.7	24.3±14.0
Ringer's		13	87.9±14.5	45.2±19.8	22.8±11.9
Lake's		40	89.1± 8.4	81.9± 8.8	51.2±22.8
Seminan		16	93.7± 2.2	82.9±31.1	52.7±22.3
Cock serum *		7	65.8±20.2	10.5± 3.8	4.5± 1.8

Dilution rate: 1 part semen to 9 parts diluent.
* It was prevented sperm-agglutination by incubation at 50° to 55°C.

Decrease of the rate of normal sperm was markedly high in physiological saline solution (i. e. Tyrode's or Ringer's solution) and cock blood serum, on the contrary, they were remarcably low in Lake's or Seminan solution.

In general, decrease of the rate of normal sperm in the semen stored at lower temperature was higher than the semen stored at moderate temperature. The rate of normal sperm in the semen diluted with Tyrode's or Ringer's solution was about 90%, and that in the diluted semen stored 0° to 2°C. was about 10% after 24 hours, and about 5% after 48 hours. Whereas in storage at 8° to 12°C., these were about 40 and 20% after 24 and 48 hours respectively.

In the semen diluted with Lake's or Seminan solution, the rate of normal sperm was more than 50 % after 48 hours. In undiluted semen, that was about 40 % after 48 hours.

In the present study, the writer observed the high proportion of abnormal sperm in stored semen after 24 hours. Moreover, this tendency was remarkable in the semen diluted with physiological saline solutions or cock blood serum and store at low temperature.

The similar facts have been observed by only a few investigaters. Zayat and Tienhoven (1959) obseved a high percentage of abnormal sperm in the cock semen diluted with Tyrode's solution.

Saeki (1960) reported that percentages of the crooked-necked sperm in cock semen increased during storage, the percentage of them in semen diluted with fructose solution was especially high, and incidence of them was greater in the semen stored at 6°C. than that stored at 15°C., and the coefficient of correlation between fertility and incidence of the crooked-necked sperm was −0.77. It was probable that this phenomenon was due to the peculiar character

in the cock sperm.

(2) **Morphology of abnormal sperm**

The abnormality obserbed in this study was clasified as follows.

Head abnormal sperm:

This abnormal type included the sperm with hooked, swollen or lacking acrosomes, and the sperm with broken, swollen, bent, coiled or psinal head.

Midpiece abnormal sperm:

This abnormal type included the sperm with bare axial filament, broken or bent midpieces.

Tail abnormal sperm:

This abnormal type included the sperm with bent, coiled or lacking tail.

Abnormal sperm appeared in the semen were shown in Plate 1 and 2.

The rate of abnormal sperm in fresh cock semen was low. And the rate of midpiece abnormal sperm was higher than that of the head and tail abnormal sperm (Table 9). The percentage of classified abnormal sperm in fresh semen and in the semen stored for 24 hours at 0° to 10° C. was shown in Table 10. These results showed that the most prevalent abnormal sperm in cock

Table 9 The percentage of classified abnormal sperm in fresh cock semen

	Type of abnormal sperm			
	Total	Head	Midpiece	Tail
Range	10.1−1.7	2.2−0	8.2−0.9	1.7−0
Mean	6.1	1.1	4.5	0.5
S.D.	2.5	0.6	2.3	0.5

Semen was collected from 8 cocks in April to June.
Number of samples was 15

Table 10 Increase in the percentage of classified abnormal sperm accompanying storage

Abnormal type	Fresh semen	Semen stored for 24 hours			
		0°C.		10°C.	
		Undiluted	Diluted with Tyrode's	Undiluted	Diluted with Tyrode's
Total	5.1	59.0	95.7	51.5	55.4
Midpiece	4.3	51.9	79.3	42.6	48.1
Head and Tail	0.9	7.1	16.3	8.9	7.3

Dilution rate: 1 part semen to 4 parts Tyrode's solution.
Number of samples was 5.

semen was midpiece type. The most general midpiece abnormal type was the bend of head backwards. This shape involved the bend not only at midpiece but at head-midpiece junction or midpiece-tail junction. These sperm were perfectly motile. And they moved vigorously with midpiece foremost. And so, the observation need to be care, when living sperm were observed under the low power microscope.

The bent sperm shown in Plate 1 (5) looked like a normal sperm. These bent sperm was classifed in normal type by PARKER et al. (1942). WAKELY and KOSIN (1951) observed that the abnormalities of turkey sperm included the sperm with bent at midpiece. LAKE (1954) stated that in fowl sperm, under enveronmental conditions where the cytoplasmic bulb was disrupted, the regular mitochondrial arrangement became granulated and the sperm head bent backwards. SAEKI (1960) described that many crookednecked sperm were found by microscopic examinations of chicken semen as stated above.

In the light or electron microscopic observations, the writer found a number of sperm with destroyed midpiece but with normal head and tail [Plate 2 (11, 12 and 13)].

These observations showed that the midpiece of cock sperm would be easily destroyed. Addititonally, from that the bent midpiece sperm was perfectly motile, it was presumed that the external power was set up at broken midpiece and the sperm head was bent backward by the pressure of the pressure of the fluid.

(3) The rate of abnormal sperm in living sperm

The rate of normal living sperm was shown in Table 11. The rate of normal sperm in eosinstaining sperm was approximately similar to that in whole sperm (or eosin-staining and eosin-unstaining sperm).

Table 11 The percentage of normal sperm on whole sperm and living sperm

	Fresh semen	Semen stored for 48 hours	
		Uudiluted	Diluted with Tyrode's
In all sperm	95.9	70.6	11.6
In living sperm (Eosin nonstaining sperm)	96.3	67.4	12.3

Figures show the mean percentage in 5 samples.

From both the result of this experiment and the fact that the sperm with abnormal midpiece were perfectly motile, it may be no much difference if the rate of abnormal sperm in whole sperm is used as that in living sperm.

In this premiss, "(Percentage of living sperm × Percentage of normal sperm) / 100" shows the rate of living and normal sperm in semen sample.

As the abnormal sperm grow rapidly in the cock semen, it seems more probable that this score was the better evaluation of the quality of the semen. If the motility of abnormal sperm is alike that of normal sperm, the culculation exchanged the percentage of living sperm for the motility of sperm will bring the more excellent evaluation score. The soundness of semen discribed in Section 1 shows this score.

Section 4 Decline in the Resistance and in Certain Metabolic Activities of Cock Spermatozoa during Storage

The physiological change should be investigated to clear the decline in

fertilizing capacity of cock semen during storage.

In the cock semen, however, the decline in sperm resistance, oxygen consumption, glycolysis and dehydrogenase abilities during storage were scarcely observed. Therefore, this investigation was attempted.

Materials and Methods

The collection and the storage of semen samples, the estimation of motility and the rate of normal sperm were used the method menthiond in the preceding sections. The diluents used for storage were contained 90 thousand unit of penicillin G potassium and 90mg. of dihydrostreptomycin per 1 dl.

Sperm resistance, oxygen consumption rate, glycolysis and dehydrogenase activity were estimated as follow:

Sperm resistance:

Sperm resistance was determined according to method of MILOVANOV (1934) and NAGORNYI and SMIRNOV (1939), 0.02 ml. of semen sample was placed in a 200 ml. Erlenmeyer flask and added 1% NaCl solution slowly until all sperm lost their motility. Sperm resistance was calculated from the formula $R=V/v$, where V was the volume of the NaCl solution and v was the volume of the semen sample (i.e. 0.02 ml.). The addition was done at 17° to 24°C. In the diluted semen, V was multiplied by 4 as the semen was diluted 4 times with diluents.

Oxygen consumption rate:

Fresh and stored semen samples were used to determination of oxygen consumption. The whole semen (undiluted semen) was diluted 10 times with Ringer's solution, the diluted semen was diluted further 2.5 times with Ringer's solution before determination. The determination carried out at 37°C.

The undiluted semen and the diluted semen were diluted 10 and 2.5 times respectively with 0.02 M glucose Ca free Krebe's Ringer Phosphate, and the sperm were freed from the seminal plasma by the centrifugation at 2000 to 2500 r.p.m. for 10 minutes. This process was repeated 2 times, and then the sperm were resuspended with 0.02M Ca free Krebe's Ringer Phosphate to 4 times volume of the undiluted semen. These sperm and the rate of normal sperm were observed on the semen sample befor washing.

Oxygen consumption of the sperm suspensions was determined by the direct method of Warburg under air at 37°C. The CO_2 was absorbed by 0.2ml. of 20% KOH, which was placed in the central cup of the vessel together with a piece of filter paper.

The evaluation of the oxygen consumption was used ZO_2.

$$ZO_2 = \frac{O_2 \text{ ul.}}{100 \text{ million sperm/hour}}$$

Glycolysis:

Evaluation of aerobic glycolysis was used $Z_L^{O_2}$.

$$Z_L^{O_2} = \frac{\text{Lactic acid mg.}}{100 \text{ million sperm/hour}}$$

Lactic acid produced by 100 million sperm for one hour at 37°C. was

determined by the method of Baker-Summerson.

Dehydrogenase activity:

For evaluation of dehydrogenase activity the methylen blue reduction test was used. Methylen blue reduction times (MBRT) was determined according to the modified method of Beck and Salisbry (1943); 0.2 ml. of the semen sample was mixed with 0.1 ml. methylen blue solution (10 mg. methylen blue in 100 ml. of 0.02 M glucose Ca free krebe's Ringer Phosphate) in a 5× 30 mm. small test tube. The mixture was covered with 5mm. of liquid paraffin and the tube was placed in a water bath at 37°C. The time required for disappearance of the blue color was recorded. And the requisite minutes per 100 million sperm was calculated.

Results and Discussion

(1) Decline in resistance of cock sperm during storage

The obtained results are shown in Table 12. The strong resistance of sperm in the semen diluted with Lake's solution was probably due to protective effect of this solution (substances contained in this solution) against the injury of NaCl. Further R shown in this case included the rate of dilution done for the purpose of preservation (R=4V/v: the rate of dilution was 4 times).

Table 12 Decline in resistance of cock sperm during storage

Diluent	Storage temp. (°C.)	No. of samples	Storage time in hours		
			0	24	48
			Mean±S.D.	Mean±S.D.	Mean±S.D.
Undiluted	0–2	9	43.0± 3.1 (100)	40.6± 4.0 (94.3)	38.3± 4.6 (89.1)
Ringer's	0–2	10	40.8± 6.8 (100)	25.6± 4.4 (63.1)	17.2± 2.7 (42.4)
Ringer's	8–10	10	58.0±13.6 (100)	42.4±11.2 (72.8)	21.6± 7.2 (37.2)
Lake's	0–2	10	79.6±18.4 (100)	68.0±15.2 (85.6)	55.2±17.2 (69.5)

Semen was diluted 1:3 with each diluent.
Figure in ( ) show percentage of the value in stored semen against the value in fresh semen.
Value of resistance is thousand unit.

But the decline in resistance was rapid in semen diluted with Ringer's solution. The decline in resistance in undiluted semen was most slow. This data show probably that the dilution of semen was harmfull to hold the resistance of sperm. The value obtained by this method did not indicate the mean value of all sperm but the highest value of the most tolerant sperm, then we must take consideration in case of the evaluation of semen.

Milovanov (1934) reported that the sperm resistances of bull, ram and stallion were 300 to 20000, 100 to 5000 and 100 to 1500 respectively. The mean resistance of the sperm in the fresh cock sperm was 43000 in the present data. This result showed that the resistance of sperm of cock was very

higher than that of the other farm animals.

(2) Decline in oxygen consumption rate, aerobic glycolysis and methylen blue reduction time of cock sperm during storage

The obtained results were shown in Table 13 and 14. After 24 hours storage the decline in oxygen consumption rate of sperm without washing was more remakable than the decline in washing sperm. This result suggested that the decline in oxygen consumption rate of sperm with storage was due to rather accumulation of harmful substances in sperm suspension than decline of function in sperm oneselves (Table 13 and 14). And the decline in motility was more rapid than that in oxygen consumption (Table 14).

The rate of lactic acid production (aerobic glycolysis) decreased in the sperm stored for 24 hours, but the rate increased again after 48 hour storage. And the value exceeded that in fresh semen except the sperm diluted with Ringer's solution and presurved at 10°C. From the result in the plate culture method, the increase of bacteria in these semen samples except the undiluted semen was not found. Therefore, it is probable that the increase of this rate did not owing to the increase of bacteria in the samples.

The reason for the high lactic acid production ($Z_L^{O_2}$) in the sperm stored for 48 hours was not clear in the present experiment.

SHETTLES (1940) and LARDY and PHILLIPS (1941) reported that in bull and ram sperm the rate of oxygen consumption decreased in accordance with the ratio of live sperm after storage.

In the present data, the decrease in the rate of exgen consumption after storage in washing sperm was less than the decline in motility.

The prolongation of MBRT (methylen blue reduction time) with lapse of storage time showed that the decline of dehydrogenase activity of sperm. Therefore, the determination of this activity may be important for evaluation of fertilizing capacity of stored semen. The relation between fertilizing capacity and MBRT will be investigated further in future.

SHAFFNER and ANDRFWS (1948) found that fertility in the fowl was correlated significantly with MBRT.

COOPER and ROWELL (1957) reported that by measuring resazurin reduction time (dehydrogenase activity), was possible to identify cocks whose semen has an abnormally low fertilizing capacity. Their investigations were carried out in fresh semen.

Changes in MBRT, motility and the incidence of dead sperm during storage were studied by VAN TIENHOVEN and STEEL (1957). But they did not observe the fertilizing capacity in their semen samples.

In the present studies, as the decline of motility was rapid rather than that of resistance and some metabolic abilities, it could not be considered that these abilities were more important than motility for rapid decrease of fertilizing capacity in stored cock sperm in vitro.

Table 13 Decline in oxygen consumption of cock semen during storage

Diluent	Storage temp. (°C.)	No. of samples	Storage time in hours						Rate of decrease after 24 hours (%)		
			0			24					
			Motility	Normality	ZO_2	Motility	Normality	ZO_2	Motility	Normality	ZO_2
			Mean	Mean	Mean±S.D.	Mean	Mean	Mean±S.D.			
Undiluted	10	9	83.3	87.4	6.8±2.4	50.2	46.2	2.8±0.5	60.3	52.9	41.1
Ringer's	10	8	77.5	88.9	8.6±2.2	50.0	26.4	3.2±0.5	63.0	29.7	37.2
Seminan	10	5	80.0	92.0	5.6±0.6	59.0	85.6	3.4±0.9	73.8	93.0	60.7

Semen was diluted 1:3 with each solution.

Sperm was suspended with Ringer's solution, and the suspension was measured at 37°C.

Table 14 Decline oxygen consumption, aerobic glycolysis and methylen blue reduction time of cock semen during storage.

Diluent	Storage temp. (°C.)	Storage time (hours)	No. of samples	Motility Mean	Normality Mean	ZO_2 Mean±S.D.	$Z_L^{O_2}$ Mean±S.D.	MBRT Mean±S.D.	Rate of decrease (%) Motility	Normality	ZO_2	$Z_L^{O_2}$	MBRT
Undiluted	2	0	3	86.6	91.5	3.7±0.4	12.7±0.9	19.5± 1.6					
Ringer's	2		7	86.7	89.9	5.5±1.0	19.7±4.5	17.6± 8.1					
Ringer's	10		12	83.3	94.2	3.9±1.0	18.5±7.0	18.9± 4.6					
Lake's	2		10	79.5	88.2	4.0±1.0	17.7±3.5	18.6± 4.2					
Undiluted	2	24	3	60.0	64.6	3.4±0.2	9.3±1.4	20.8± 2.1	69.3	70.6	91.8	73.1	106.7
Ringer's	2		7	61.5	27.0	3.4±0.8	8.0±3.6	34.0± 8.4	70.9	30.0	61.8	40.6	193.2
Ringer's	10		12	54.8	54.6	3.3±0.8	8.4±2.4	44.7±16.9	65.8	58.0	84.6	45.4	241.7
Lake's	2		10	76.0	86.1	4.8±1.1	22.2±2.8	14.5± 4.6	95.6	97.6	120.0	125.4	77.9
Undiluted	2	48	3	44.9	50.0	2.5±0.1	13.6±1.9	22.9± 3.9	53.1	54.6	67.5	107.0	117.4
Ringer's	2		7	34.0	14.9	4.0±1.6	9.6±1.6	47.9± 5.6	39.3	16.5	72.7	48.7	272.1
Ringer's	10		12	35.8	36.1	2.5±0.4	25.0±2.8	69.7±16.3	42.9	38.3	64.1	135.1	368.7
Lake's	2		10	72.0	84.6	4.3±1.4	21.3±3.0	16.4± 7.4	90.5	95.9	107.5	120.3	88.1

Semen was diluted 1:3.

Sperm were suspended with 0.02 M glucose Ca free KRP solution after washing.

Suspension was measured at 37°C.

Section 5 Summary and Conclusion

The correlation between the fertility and the sperm motility, and that between the fertility and the rate of normal sperm in stored cock semen were considerably high (+0.68 and +0.77). But the correlation between the soundness that probably indicates the motility of normal sperm in semen and the the fertility is higher (+0.91) than the above-mentioneds correlations. The excessive decreae of either motility or normality and the decrease below some extent in both of them brought low fertility. The degrees of decrease in motility and normality were not always of the same tendency. Therefor, it is to be considered that the semen quality must be estimated by both motility and normality.

The average life duration of sperm in the ejaculated cock semen was 4.1 days in storage at 10°C. and was 6.5 days at 0° to 2°C. In the other hand, that of the Vas deferens semen was 8.7 days at 10°C. and was 13.3 days at 0° to 2°C. The dilution with several diluents that was reported to be considerably suitable for the cock semen could not extend the life duration of sperm more than control undiluted semen. The tendency of decline in motility of cock sperm during storage was similar to that of bull sperm, but the degree of decline in cock sperm was much more rapid. After 72 hours in storage at both 0° to 2°C. and 10°C., both motility of the undiluted and that of the semen dilute ten times with several diluents almost fell down below the range of practical use for artifical insemination.

Incidence of abnormal sperm in cock semen was excessively rapid when the semen was diluted with physiological saline solution and cock blood serum, and when the semen was stored at a low temperature. The most prevalent abnormal sperm were of midpiece type, and the majority of midpiece abnormal type were the sperm whose head bent backwards. These spermatozoa were perfectly motile, and moved vigourously with midpiece foremost. From the results of the microscopical observation and the examination on the fertility it is expected that they were certainly almost infertile.

The resistance of cock sperm was higher than that of the other farm animals. The decline of sperm resistance in undiluted semen during storage was slower than that in diluted semen, and the degree of sperm resistance in diluted semen differs according to difference of diluents. But it is to be considered that the sperm resistance determined by Milovanov method was not suitable for the evaluation of semen, as the resistance did not show the mean value of all sperm but the highest value of the most tolerant sperm. The decline of motility was rapid rather than that of sperm resistance (R) and that of ability of oxygen consumption (ZO_2), aerobic glycolysis ($Z_L^{O_2}$) or dehydrogenase (MBRT). It cannot be considered that these abilities were more important than motility for rapid decrease of fertilizing capacity in stored cock sperm in vitro.

It is to be considered from the investgations in this chapter that an important factor which causes the rapid decline of fertilizing capacity of cock

sperm during storage is not only the decline of motility but the rapid and excessive incidence of abnormal sperm.

CHAPTER II EFFECTS OF SEVERAL FACTORS ON THE SENESCENCE OF COCK SPERMATOZOA DURING STORAGE

It has been considered from the results obtained in Chapter I that the rapid decline of fertilizing capacity of the stored cock semen in vitro depends chiefly on the rapid and excessive incidence of specific abnormal shape sperm (midpiece bent sperm) as well as on the decline of motility. Therefor, in this chapter the effects of several environmental factors on the incidence of abnormal sperm and the decline of motility of the stored cock semen in vitro were investgated.

Section 1 Effect of Storage Temperature on the Decline of Motility and on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

The facts that the motility, life duration of sperm and incidence of abnormal sperm of cock semen was extremely affected by storage temperatures of semen were already described in Chapter I. The present section examined in detail the effect of storage temperatures within the range from 0° to 30°C. on the decline of motility and the incidence of abnormal sperm.

Materials and Methods

Semen collected by the one-man technique from 6 White Leghorn males were mixed and were used in this investigation. The densities of semen samples used were in the range of 1 to 1.5 million per mm^3. It seems that these lower densities are owing to the collection of semen in molting season. The diluents used in the present study were Tyrode's, Wilcox's and Lake's solutions contained both penicillin G potassium (900,000 units/1.) and dihydrostreptomysin (900mg./1.). The composition of these solutions are shown in Table 15. The semen samples were diluted at the rate of 1:9 with each diluent.

Table 15 Composition of diluents

Diluent	Concentration (gm./1. dist. H_2O)
Tyrode's solution	see Table 1
Wilcox's solution	
Na_2HPO	16.34
$NaH_2PO \cdot 2H_2O$	5.82
Lake's solution	see Table 1

Each semen sample divide into four equal parts was stored at 0°, 10°, 20° and 30°C. respectively.

The scoring system of motility and the rate of normal sperm (or abnormal sperm) were the same as descrived previously (Chapter 1).

Results and Discussion

(1) Effect on motility

The results are shown in Table 16. In the undiluted semen, it seems that 10°C. was most suitable to the motility of sperm. The mean motility score of the semen stored for 48 hours was about 40. Semen samples store at 0°C. showed least decline of the motility, but the motility of semen stored for 48 hours was significantly lower than the motility of semen stored at 10°C. The motility of sperm stored at 30°C. decrease rapidly; after 24 hours the motility almost disappeared.

Table 16 Effect of storage temperature on motility in cock semen during storage

Diluent	Storage time (hours)	Storage temp. (°C.) 0	10	20	30
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Undiluted control (7 samples)	0	87.1± 2.7			
	6	67.9±11.9	72.9± 4.9	64.3± 7.3	62.9±20.4
	24	47.9±10.7	51.4±13.5	33.6±10.3	0.3± 0.2
	48	20.7± 7.9	37.1± 9.9	2.0± 2.2	0
Tyrode's solution (7 samples)	0	87.9± 2.7			
	6	53.6±17.7	72.9± 5.7	78.6± 5.5	79.3± 6.7
	24	18.1±25.8	51.4±24.6	57.1±19.3	9.7±15.7
	48	0	17.3± 5.6	0	0
Wilcox's solution (5 samples)	0	80.0±12.3			
	6	62.0± 8.4	52.0±21.7	26.0±13.4	16.6± 8.8
	24	44.0±15.2	32.0± 5.7	5.8± 8.0	0.2± 0.5
	48	20.6± 8.2	8.2± 6.5	0.2± 0.5	0
Lake's solution (5 samples)	0	80.0±12.3			
	6	78.0±13.0	77.0± 6.7	54.0±20.7	33.0±17.2
	24	76.0± 9.5	54.0±20.4	13.4±14.1	0.6± 0.6
	48	60.0± 9.4	24.0±14.3	0.8± 1.8	0

Dilution rate····· semen 1: diluent 9

In the semen diluted with Tyrode's solution, the optimum temperature for storage was 10°C. The storage at 20°C. was suitable until 24 hours, but the motility of semen stored at 0 and 30°C. decreased rapidly.

In the semen diluted with Wilcox's or Lake's solution, the best result was obtained at 0°C., and the storage at 10°C. was significantly inferior than at 0°C. In the storage at 20 and 30°C., the decline of motility was very rapid.

In general, it seems that the optimum temperature in maintenance of motility of cock semen is 0° to 10°C. But in the semen diluted with Tyrode's solution, the storage at 0°C. was harmful. It seems that the difference

between this result and the result in Chapter I (Section 2) is owing to the difference of densities in semen.

Several investigators reported that the optimum storage temperature for maintenance of motility in semen of bull, stalion and goat was 1° to 4°C., and sperm motility was decreased rapidely at above 20°C. (HATZIOLOS, 1937; MOECKEL, 1937; DAVIS et al., 1940; NISHIKAWA and SUGIE, 1949).

In the present results, the undiluted semen and the semen diluted with Wilcox's or Lake's solution showed almost same tendencies, and slower decline was accompanied with lower temperature. But only the semen diluted with Ringer's solution showed rapid decline of motility in storage at 0°C.

(2) Effect on incidence of abnormal sperm

The decline of normal sperm rate in the semen stored at several temperatures was shown in Table 17. In the undiluted semen, the optimum

Table 17 Effect of storage temperature on the percentage of normal sperm in cock semen during storage

Diluent	Storage time (hours)	Storage temp. (°C.) 0	10	20	30
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Undiluted control (7 samples)	0	84.9± 4.7			
	6	26.9± 9.4	70.0± 9.0	68.7± 4.6	75.2± 7.8
	24	7.8± 5.7	22.3± 8.5	34.1± 7.6	14.0± 6.8
	48	6.3± 4.1	16.0± 4.7	13.2± 5.7	3.4± 3.3
Tyrode's solution (7 samples)	0	83.5± 8.7			
	6	18.1± 9.8	59.2±14.9	71.6± 8.6	75.7± 6.7
	24	2.9± 2.4	13.6± 8.8	45.0±15.6	10.9±10.1
	48	1.0± 0.4	3.3± 4.5	3.2± 1.4	2.6± 1.7
Wilcox's solution (5 samples)	0	84.5± 7.9			
	6	65.2± 4.6	75.6± 8.8	79.8±10.8	83.9± 7.7
	24	54.0±10.3	71.1±14.7	80.2±10.7	85.2±6.1
	48	42.4±13.5	68.2±16.1	73.1±13.6	71.3±10.2
Lake's solution (5 samples)	0	87.4± 7.1			
	6	61.2±11.2	76.9± 6.6	75.8± 6.9	68.4±11.0
	24	52.1±14.9	67.0±14.7	70.8±11.6	54.6±18.0
	48	39.6±20.1	64.9±15.1	53.6±12.3	34.8±25.2

Dilution rate····· semen 1: diluent 9

temperatue of storage was 10° to 20°C., and the rate of normal sperm in the semen stored at 0° and 30°C. decreased less than 15% after 24 hours; after 48 hours, the rate was only 10%.

In the semen diluted with Tyrode's solution, the storage temperature of 20°C. gave better result until 24 hours, but the normal sperm scarecely remained in the semen stored for 48 hours at all storage temperatures.

In the semen diluted with Wilcox's solution, the rate of normal sperm decreased in the order of storage temperature of 20°, 30°, 10° and 0°C. The

differences between the mean of semen stored at 0°C. and the means of semen stored at another temperatures after 48 hours were all significant.

In the semen diluted with Lake's solution, the rates of normal sperm in the semen stored at 10° and 20°C. were higher than those stored at 0° and 30°C.

In general, the abnormal sperm arose easily in the semen stored at 0° and 30°C. than at 10° and 20° C. There was statistical difference between the semen stored at 30°C. and the semen stored at 10° or 20°C. after 48 hours.

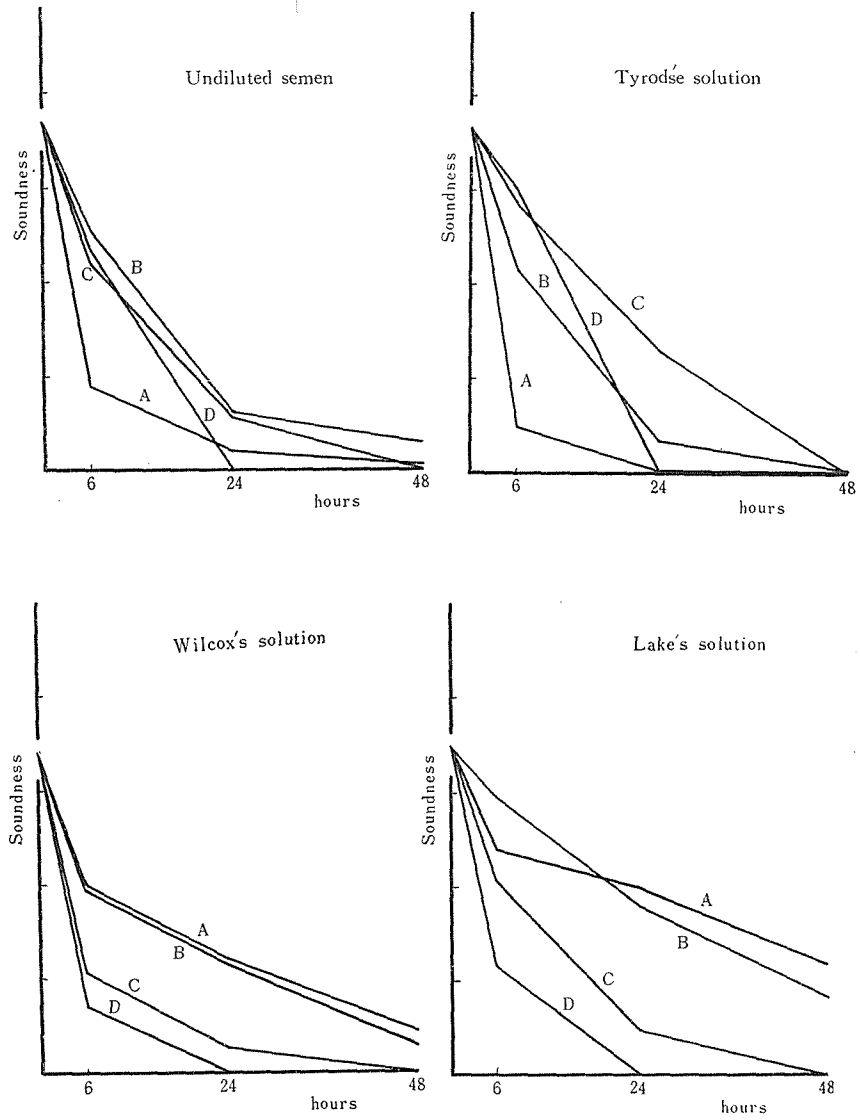

Fig. 1 Effect of storage temperature on soundness in cock semen during storage
Dilution rate……semen 1 : diluent 9
Storage temperature…… A−0°C., B−10°C., C−20°C., D−30°C.

(3) Effect on soundness

Soundness was already explained in Section 1 of the previous chapter. The decline of soundness in the semen stored several temperatures was shown in Fig. 1. The decline of soundness of semen stored at 30°C. was remarkable in all examples becoming almost zero after 24 hours. In the semen diluted with Wilcox's or Lake's solution, the decline of soundness of sample stored at 20°C. was similar to that of the sample stored at 30°C. It was clear that this decline of soundness was due to mainly the decline of motility. In the semen diluted with Tyrode's solution and the undiluted semen, the decline of the soundness was more rapid when the semen was stored at 0°C., at that time, the decline of the soundness was to the decline of the rate of normal sperm. It was considered that the suitable condition was at 10° or 20°C. in the undiluted semen, at 20°C. in the semen diluted with Tyrode's solution, at 0° or 10°C. in the semen diluted with Wilcox's or Lake's solution. But after 48 hours, no soundness of the undiluted semen and the semen diluted with Tyrode's solution were found.

Several investigaters have stated that the optimum temperature for storage of cock semen was 10° to 20°C., even in which range at these temperatures fertilizing capacity was rapidly lost, and lower temperatures as well as higher temperatures were harmful for the preservation (BURROWS and QUINN, 1939; WARREN and GISH, 1943; GARREN and SHAFFNER, 1949; HARPER, 1955; CARTER et al., 1955; HUNSAKER et al., 1956).

Recently, LAKE (1960) and WILCOX (1960) reported that the semen stored in the diluent prescribed by them (Lake's solution and Wilcox's solution) at 2°C. maintained good fertility after 48 hours and 50 hours.

The decline of the soundness showed in the present investigation almost agreed with the results of these investigaters.

Section 2 Effect of PH of Medium on the Decline of Motility and on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

It was a well-kown fact that PH influenced motility, metabolism and survival of sperm. But only a few studies of effect of PH on the motility of cock semen was reported. The effect of PH on the incidence of abnormal sperm of cock semen was scarcely known. Therefor, the present investigation was attempted.

Materials and Methods

Semen samples collected by the preceding method were diluted at the range of 1:9 with the following diluents. Diluents used in these investigation were glucose phosphate buffer solution and modified Lake's solution. The composition of glucose phosphate buffer solution was shown in Table 18. Modified Lake's solution was adjusted to PH of 5.0, 6.0, 7.0, 8.0 and 9.0 by addition of HCl or NaOH and was tested with a glass electrone PH meter.

Table 18 Composition of glucose phosphate buffer solution

PH of solution	Composition (gm./1. dist. water)		
	Na_2HPO_4	$NaHPO_4 \cdot 2H_2O$	Glucose
4.1	13.51	0.76	23
5.0	13.06	1.25	23
6.0	10.37	4.21	23
7.0	4.40	10.76	23
8.0	1.70	13.73	23
8.9	0	15.60	23

The depression of freezing point of these diluents were ranged from −0.60° to −0.66°C. in glucose phosphate buffer solution, and −0.65° to −0.67°C. in modified Lake's solution.

The diluted semen samples were stored at 0°C. The determinations of motility and abnormal sperm rate of stored semen were carried out after 6, 24 and 48 hours of storage. PH of the diluted semen was determinated immediately after dilution and after 48 hours storage.

Results and Discussion

The mean PH value of fresh semen used in this investgation was 7.7. But the mean PH value of these semen stored for 48 hours at 0°C. rose to 8.2. On the other hand, the PH of sperm suspension (diluted semen) scarcely changed after 48 hours (Table 19).

(1) Effect on motility

The results are shown in Table 19. In PH less than 5.3 and above 8.8. sperm motility declined rapidly. And then the spermatozoa in these suspen-

Table 19 Effect of PH on motility in cock semen during storage at 0°C.

Diluent	Storage time (hours)	Acrual average PH of suspension				
		5.3(5.4)	6.1(6.1)	7.1(7.1)	8.0(8.0)	8.8(8.7)
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Glucose phosphate buffer solution (5 samples)	0			87.0± 6.7		
	6	7.6±11.5	79.0± 5.5	76.0± 2.2	42.0±7.5	2.0±0
	24	0.2± 0.4	39.0±25.1	61.0±26.1	26.0±4.2	0.4±0.5
	48	0	1.2± 1.1	39.0±17.8	11.0±5.5	0
		Actual average PH of suspension				
		5.1(5.1)	6.2(6.3)	7.3(7.6)	8.0(7.9)	9.0(8.9)
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D	Mean±S.D.
Modified Lake's solution (5 sample)	0			87.0± 2.7		
	6	0	1.4± 0.9	76.0± 4.2	65.0±9.1	0.2±0.4
	24	0	0.4± 0.9	69.0± 6.5	58.0±7.6	0
	48	0	0.2± 0.4	42.0±13.0	39.0±3.6	0

( )……Averdge PH of suspension after 48 hrs. Dilution rate……semen 1: diluent 9

sions were almost immotile after 6 hours. The highest motility was maintained in PH 7.1 to 7.3. In PH 8.0, the semen diluted with modified Lake's solution showed a good result, while the decline of motility in the semen diluted with glucose phosphate buffer solution was fairly rapid. On the other hand, in PH 6.0, the semen diluted with the former was scarecely motile at 6 hours, while the semen diluted with the latter fairly maintained the motility until 24 hours. The cause of difference in the tendendencies of decline was not clear in this experiment.

(2) Effect on incidence of abnormal sperm

The results are shown in Table 20. In semen diluted with glucose phosphate buffer solution, the PH values of these diluted semen samples were

Table 20 Effect of PH on the rate of normal sperm in cock semen during storage at 0°C.

	Storage time (hours)	Actual average PH of suspension				
		5.3(5.4)	6.1(6.1)	7.1(7.1)	8.0(8.0)	8.8(8.7)
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Glucose phosphate buffer solution (5 samples)	0			94.8± 3.8		
	6	83.7± 6.3	82.7± 8.0	78.4± 7.2	53.1±11.2	31.8± 6.4
	24	80.8± 6.5	70.1±13.1	60.5±13.2	44.3±18.2	21.5± 9.4
	48	52.8± 7.5	54.9±13.1	50.0±12.2	40.3± 9.8	19.9±10.8
		Actual average PH of suspension				
		5.1(5.1)	6.2(6.3)	7.3(7.6)	8.0(7.9)	9.0(8.9)
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Modified Lake's solution (5 samples)	0			94.8± 3.8		
	6	18.9± 4.0	85.0±18.8	78.7±13.8	67.7±11.3	42.6±10.4
	24	11.2± 8.0	53.9±14.9	52.2±15.2	43.0±13.9	35.3±18.6
	48	6.5± 3.0	44.8±13.4	42.5±19.0	35.8±17.4	30.0±20.1

( )……Average PH of suspension after 48 hours.
Dilution rate……semen 1: diluent 9

arranged in the following descending order of the normal sperm rate throughout the storage duration—PH 5.3, 6.1, 7.1, 8.0 and 8.8. The normal sperm rate in PH 8.8 decreased excessively, but among others, there were no statistical differences between each other except between PH 5.3 and 8.0 at 48 hours.

In the semen diluted with modified Lake's solution, the PH values of these diluted semen samples were arranged in the following descending order of the normal sperm rate—PH 6.2, 7.3, 8.0, 9.0 and 5.1. The decrease of normal sperm rate in PH 5.1 was especialy rapied, but among others, there were no statistical differences between each other at 48 hours.

(3) Effect on soundness

As shown in Fig. 2, in the semen diluted with glucose phosphate buffer solution, the best soundness was maintained when the PH was close to 7.0, and a slight acid suspension (PH 6.1) had a tendency to survive longer than a

slight alkaline one (PH 8.0). While in the semen diluted with modified Lake's solution, the best soundness was preserved too when the PH was close to 7.0, but the slight alkaline environment (PH 8.0) was much better than the slight acid one (PH 6.2).

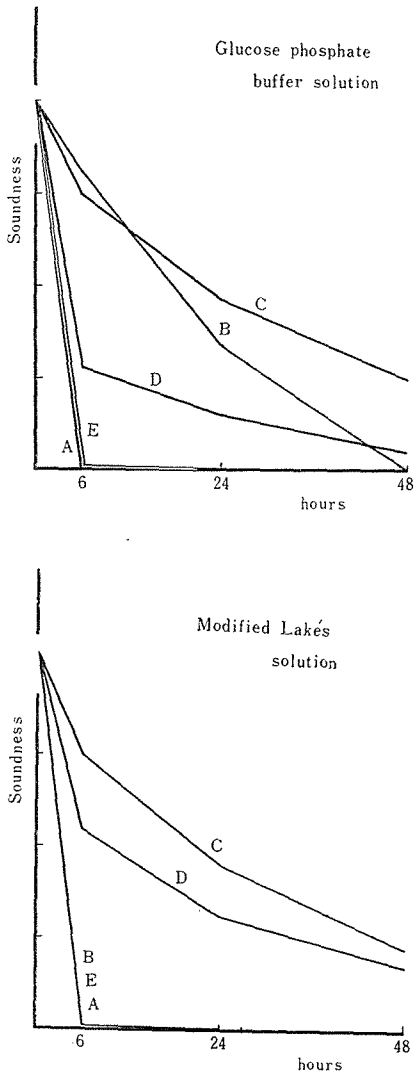

Fig. 2 Effect of PH on soundness in cock semen during storage 0°C.
Dilution rate····· semen 1: diluent 9
PH of diluent·····
Glucose phosphate buffer solution A−5.3, B−6.1, C−7.1 D−8.0, E−8.8
Modified Lake's solution A−5.1, B−6.2, C−7.3 D−8.0, E−9.0

Bogdonoff and Shaffner (1954) stated that when diluted cock semen was stored at 7 to 9° C. in media adjusted to PH 6.0, 7.0 and 8.0 by adding HCl or NaOH, maintenance of motility was best at PH 6.0 where sperm activity was reduced. No fertile eggs were obtained from every treated semen stored for 4 to 4.5 hours at 10° to 12°C.

Wilcox (1959) reported that sodium phosphate buffers of PH 6.5, 7.2 and 7.8 were used for dilution and storage of the cock semen, there was no apparent effect of PH within the range employed.

Bernstein and Beschlebnov (1937) described that the optimum PH for storage of bull semen varied according to different diluent.

The present reslut showed different tendencies between the semen diluted with glucose phosphate buffer solution and the one diluted with modified Lake's solution on the decline of motility and the incidence of abnormal sperm of stored cock semen. It will be due to different characters of these diluents. In the range of PH 6, 7 and 8, there was no statistical difference among three suspensions diluted with both glucose phophate buffer solution and modified Lake's solution on normal sperm rate within 48 hours storage: but the motility of sperm in suspension with PH 7.1 was statisticaly higher than that of PH 6.1 and 8.0 in glucose phosphate buffer solution, and the motility in suspensions with PH 7.3 and 8.0 were statisticaly higher than that of PH 6.2.

Section 3 Effect of Osmotic Pressure of Medium on the Decline of Motility and on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

The effect of hypertonic or hypotonic solution on activity, metabolism and morphology of sperm have been observed by several investigators. But these observations have been carried out in mammalian sperm.

The investigation described in this section was carried out on the effects of osmotic pressure on the decline of motility and the incidence of abnormal sperm of cock semen during storage.

Materials and Methods

Semen samples collected by the preceding method were diluted at the rate of 1:9 with NaCl, glucose, sodium citrate or sodium glutamate solution with several concentrations, and the sperm was separated from liquid (seminal plasm and diluted solution) by centrifugal force at 2200 to 2400 r. p. m. for 10 minutes, and then the sperm was resuspended to the previous volume by addition of the same solution.

Each concentration of solutions was shown by mean of the freezing point depression (Δ).

The densities of semen used in this study were 3 to 5 million per mm^3. Each suspension was stored at 0° or 10°C. The motility was observed at 35°C. after the suspension was diluted about 10 times with 6% glucose solution.

Results and Discussion

(1) Effect on motility

The decline of motility of spermatozoa which were suspended with NaCl and glucose solutions adjusted from Δ 0.3 to 0.8 and were stored at 10°C. were shown in Table 21.

Table 21 Effect of osmotic pressure on motility in cock semen during storage at 10°C.

Diluent	Storage time (hours)	Freezing point depression of solution (°C.) 0.3 Mean ±S.D.	0.4 Mean ±S.D.	0.5 Mean ±S.D.	0.6 Mean ±S.D.	0.7 Mean ±S.D.	0.8 Mean ±S.D.
NaCl solution (8 samples)	0				90.0±5.2		
	6	32.5±12.2	41.9±11.9	62.3±5.3	66.9±8.0	70.6±5.6	68.1±6.4
	24	10.6± 6.2	14.0± 8.8	1.8±3.4	1.5± 1.4	1.3±3.5	1.3± 3.5
	48	0	0	0	0	0	0
Glucose solution (4 samples)	0				95.3± 3.8		
	6	18.8±13.2	10.3± 7.8	7.7±9.6	0.3± 0.5	1.0±0	1.0±0
	24	0	0	0	0	0	0
	48	0	0	0	0	0	0

Dilution rate……semen 1: diluent 9

In NaCl solution, the motility of sperm in every suspension above Δ 0.5 was higher than that with Δ 0.4 or 0.3 after 6 hours. There were statistical defferences between the mean of suspensions in each density of higher group and each one of lower group. But after that, the motility of sperm in suspensions above Δ 0.5 decreased rapidely, while the motility of suspensions with Δ 0.4 and 0.3 decreased slowly. Consequently, at 24 hours storage, the motility of the formers fell off below the motility of the latters. All suspensions contained no motile sperm after 48 hours.

In glucose solution, the motility of sperm in all suspensions decreased exceedingly, the sperm in suspensions above Δ 0.6 were scarcely motile at 6 hours. At 24 hours, there was nothing sperm maintained motility. In this case, PH of suspension decreased rapidly during storage; namely the PH of suspension with Δ 0.3 fell from 6.8 to 5.9; that of suspension with Δ 0.4 or 0.5 fell to 5.3; and that of suspension with 0.6, 0.7 or 0.8 fell to 5.1 after 6 hours. It appears that such a rapid decline of motility is due to the fall of PH in these suspensions.

The decline of motility of sperm suspended with NaCl, glucose, sodium citrate or sodium glutamate solution of eachy concentration at 0°C. during storage was shown in Table 22. In NaCl solution, each suspension with Δ 0.41, 0.52 or 0.64 showed statistically higher motility than each suspension below Δ 0.29 or above Δ 0.75 after 6 hours, but after 24 hours, the motilities of all suspensions decreased rapidly.

In glucose solution, the suspension with Δ 0.80 shown highest motility, and there statistical diferences between the average motility of suspension with Δ 0.80 and each average motility of suspension below Δ 0.70, but in all suspensions, spermatozoa completely lost their motility after 24 hours. And the PH values of all suspensions were 6.2 to 6.4 at 6 hours storage and they were 6.3 to 6.5 at 24 hours storage.

In sodium citrate solution, the motility decreased rapidely until 6 hours, then it decreased slowly. The motility of sperm in suspension with Δ 0.52 or 0.70 was higher statistically than each one of suspensions below Δ 0.35 and above Δ 0.89. In general, the motility of this suspension was decreased with advancing of hyper or hypotonic concentration.

In sodium glutamate solution, the suspensions with Δ 0.56 and 0.74 showed the best result and these suspensions maintained highly motility after 24 hours. The motility of the suspensions decreased with advancing of hyper or hypotonic concentration, viz., the concentrations of suspensions were arranged in the following descending order of the motility Δ 0.56, 0.74, 0.93. 0.38, 1.11, 1.30, 0.20 and 1.48. At 24 hours, the average motility of suspension with Δ 0.56 or 0.74 was higher than each one of suspensions with Δ 0.38 and 0.93 statistically, and there was statistical difference between each average motility of suspension with Δ 0.38 or 0.93 and every one of suspensions below Δ 0.20 and above 1.11. In general, throughout the storage duration, the sperm in isotonic solution maintained the highest motility, and the sperm in hyper or hypotonic solution decreased motility with advancing of hyper or hypotonic concentration. But in NaCl solution, the motilities in hypertonic solutions

Table 22 Effect of osmotic pressure on motility in cock semen during storage at 0°C.

Diluent	Storage time (hours)	Freezing point depression of solution (°C.)							
		0.17	0.29	0.41	0.52	0.64	0.75	0.87	0.98
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D
NaCl solution (4 samples)	0					90.0±0			
	6	10.0±7.1	35.0±5.8	60.0±8.2	60.0±0	55.0±5.8	31.3±20.1	28.8±21.7	20.0±26.8
	24	1.3±0.5	6.5±3.0	2.5±1.7	1.3±0.5	0.3±0.5	0	0	0
	48	0.5±0.5	1.0±0.8	0.5±0.5	0.3±0.5	0	0	0	0
		Freezing point depression of solution (°C.)							
		0.3		0.4	0.5	0.6		0.7	0.8
Glucose solution (6 samples)	0				94.7±6.8				
	6	13.3±9.8		18.3±7.5	16.7±8.2	11.7±6.8		11.7±6.8	45.0±12.2
	24	0		0	0	0		0	0
	48	0		0	0	0		0	0
		Freezing point depression of solution (°C.)							
		0.18	0.35	0.52	0.70	0.87	1.05	1.22	1.39
Sodium citrate solution (4 samples)	0			87.5± 2.9					
	6	1.0±0	20.0±7.1	40.0±21.2	35.0± 4.1	6.5±9.3	0.3±0.5	0	0
	24	0.3±0.5	2.0±2.2	23.8±13.2	20.0±10.8	0.3±0.5	0	0	0
	48	0	1.8±2.2	10.0± 8.2	5.5± 9.7	0	0	0	0
		Freezing point depression of solution (°C.)							
		0.20	0.38	0.56	0.74	0.93	1.11	1.30	1.48
Sodium glutamate solution (4 samples)	0			92.5±2.9					
	6	3.0±4.4	35.0±4.1	75.0±9.1	68.7±4.8	47.5±15.0	21.5±17.1	6.0±4.8	2.5±2.4
	24	0	20.0±0	60.0±4.1	60.0±8.2	23.7± 7.4	2.0± 2.0	0.2±0.5	0
	48	0	3.5±4.4	11.2±2.5	19.5±9.9	2.5± 1.7	1.0± 0.8	0.2±0.5	0

Dilution rate……semen 1: diluent 9

were rather higher than these in hypotonic solutions after 6 hours storage at 10°C., however after 24 hours the order of motility in those suspensions became inverse, viz., the motilities in hypotonic solutions were rather higher than those in hypertonic solutions after 6 hours, and the spermatozoa in all suspensions were scarecely motile after 24 hours (Table 22).

From these results, it was probable that the ionic action of NaCl was harmful to motility of sperm during storage, and this injury was heavier in storage at 0°C. than 10°C.

In glucose solution, the more solution was hypertonic, the more motility decreased at 10°C. storage. And the motilities in these solutions declined with fall of PH as has been previously mentioned. In the storage at 0°C. the solutions with Δ 0.80 maintained fairly good motility until 6 hours later, but after 24 hours storage, the spermatozoa in all suspensions showed complete absence of motility (Table 22), and the PH values of these suspensions had fallen to about 6.2 to 6.4. From these results, it is considered that the rapid decline of motility of sperm in glucose solution is due to rapid fall of PH of this suspension, the optimum concentration in glucose solution is comparatively high.

(2) Effect incidence of abnormal sperm

The decline of the rate of normal sperm in NaCl, glucose, sodium citrate or sodium glutamate soution during storage at 0° or 10°C. was shown in Table 23 and 24.

In NaCl solution, when the suspensions were stored at 10°C., the spermatozoa in isotonic solution maintained the highest normal sperm rate. The normal sperm rates in hypertonic solutions were lower than in isotonic solution and the rates in hypotonic solutions were lower than those in hypertonic solutions. When the suspension was stored at 0°C., there was also the similar tendency but the decline of the normal sperm rate was more rapid at 0°C.

In glucose solution, the highest concentration (Δ 0.8=7.4 %) maintained the best appearance of sperm during storage at 0° to 10°C. The more concentration of solution decrease, the more normal sperm rate decrease. The normal sperm rate in suspension less than Δ 0.4 showed excessive decline.

In sodium citrate and sodium glutamate solutions, all the hypertonic solutions over Δ 0.7 maintained high rate of normal sperm during storage for 48 hours, while the rate in hypotonic solution under Δ 0.38 decreased exceedingly.

In general, the hypertonic solution gave a good result on the maintenance of normal sperm rate during storage, while the hypotonic solution promoted the incidence of abnormal sperm during storage. NaCl solution was an only exception in which the hypertonic solution was as harmful as the hypotonic solution, but the injury in hypotonic solution was heavier than that in hypertonic solution. In storage at 0°C., however, the incidence of abnormal sperm was very rapid in all concentrations including the isotonic solution. From these results, it is considered that NaCl gives the morphological injury to sperm during storage and the injury is especially heavy at 0°C.

Table 23 Effect of osmotic pressure on the rate of normal sperm in cock semen during storage at 10°C.

Diluent	Storage time (hours)	Freezing point depression of solution (°C.) 0.3 Mean ±S.D.	0.4 Mean ±S.D.	0.5 Mean ±S.D.	0.6 Mean ±S.D.	0.7 Mean ±S.D.	0.8 Mean ±S.D.
NaCl solution (8 samples)	0				93.0± 8.3		
	6	5.2±3.6	20.1±10.1	58.6±20.0	83.3± 9.2	84.9± 3.1	85.6± 1.6
	24	1.8±1.7	8.4± 5.4	10.8± 7.0	32.0±16.5	19.9±12.0	26.8±14.3
	48	2.1±1.2	2.3± 2.0	2.3± 2.0	3.2± 2.2	3.7± 1.6	3.1± 1.0
Glucose solution (4 samples)	0				93.6± 3.1		
	6	6.2±2.2	12.7± 4.2	55.4±14.0	81.7± 6.4	81.6± 5.7	88.9± 2.2
	24	4.0±1.8	10.5± 2.9	50.8±14.6	64.0± 3.1	69.9± 6.1	74.4± 5.9
	48	1.4±0.7	8.3± 2.6	55.0±20.7	63.9± 3.6	71.2±15.7	75.9± 9.9

Dilution rate……semen 1: diluent 9

(3) Effect on soundness

The obtained results were shown in Fig. 3 and 4. The sperm in sodium glutamate solution with Δ 0.56 (3%) and Δ 0.74 (4%) maintained the best soundness in this experiment throughout the storage durations of 48 hours.

In sodium citrate solution better soundness was maintained in Δ 0.52 (3%) to Δ 0.70 (4%), but the degree of soundness was not fairly high. This fact

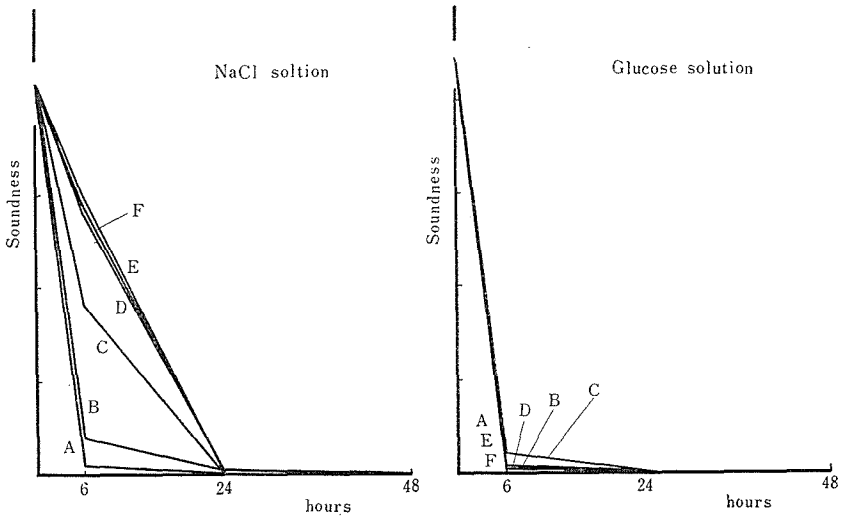

Fig. 3 Effect of osmotic pressure on soundness in cock semen during storage at 10°C.

Washing sperm were suspended with the diluent of 10 times volume of the undiuted semen.
Freezing point depression of diluent (°C.)……
A−0.3, B−0.4, C−0.5, D−0.6, E−0.7, F−0.8

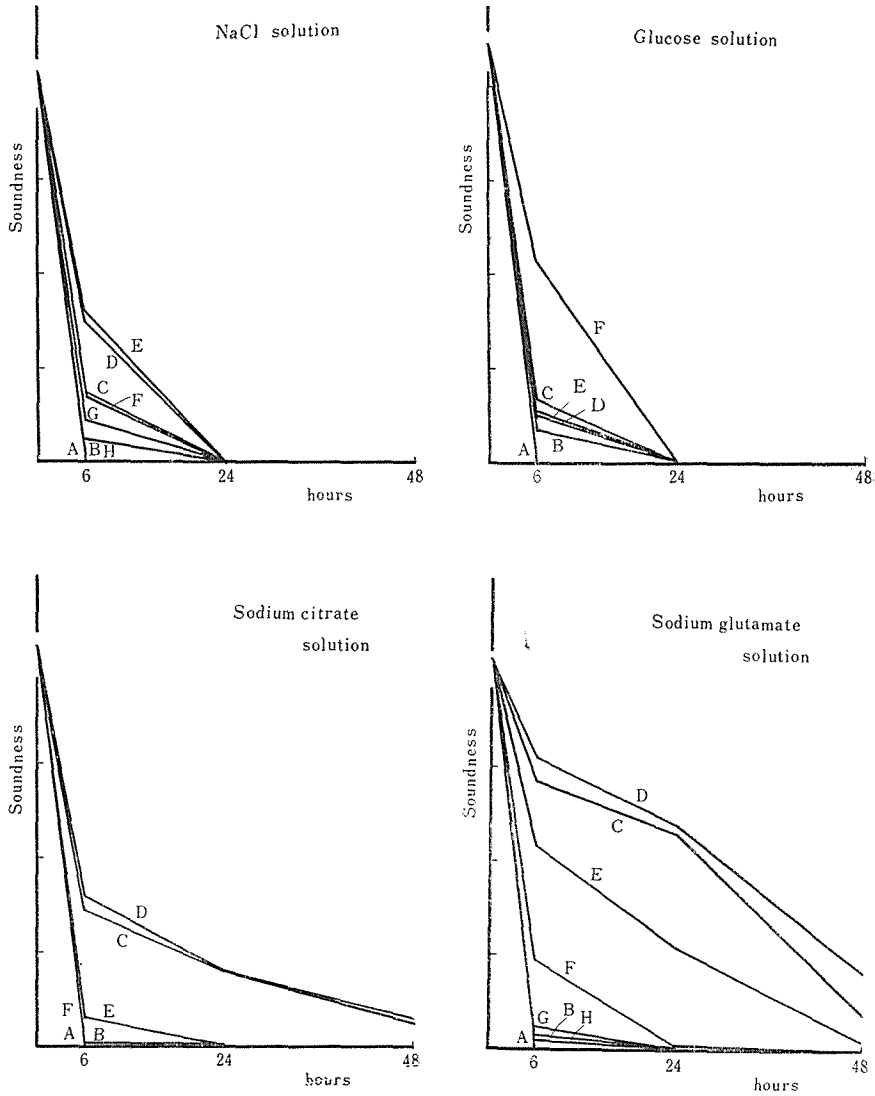

Fig. 4 Effect of osmotic prossure on soundness in cock semen during storage at 0°C.

Washing sperm were suspended with the diluent of 10 times volume of the undiluted semen.
Freezing point depression of diluent (°C.)……

NaCl solution	A−0.17, B−0.29, C−0.41, D−0.52
	E−0.64, F−0.75, G−0.87, H−0.98
Glucose solution	A−0.3, B−0.4, C−0.5
	D−0.6, E−0.7, F−0.8
Sodium citrate solution	A−0.18, B−0.35, C−0.52
	D−0.70, E−0.87, F−1.05
Sodium glutamate solution	A−0.20, B−0.29, C−0.56, D−0.74
	E−0.93, F−1.11, G−1.30, H−1.48

Table 24 Effect of osmotic pressure on the rate of normal sperm in cock semen during storage at 0°C.

Diluent	Storage time (houre)	Freezing point depression of solution (°C.)							
		0.17	0.29	0.41	0.52	0.64	0.75	0.87	0.98
		Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
NaCl solution (4 samples)	0				91.7± 4.8				
	6	4.0±4.5	14.5±13.0	25.7±12.9	49.4±11.4	57.2±15.6	43.4±16.4	32.7±14.6	26.2±11.9
	24	4.4±3.3	0.7± 0.7	4.0± 2.6	3.8± 1.6	8.4± 4.3	6.4± 3.5	7.5± 5.3	6.9± 1.2
	48	3.1±3.3	2.9± 2.8	2.9± 1.3	3.7± 2.5	5.6± 2.6	2.6± 1.3	6.3± 5.4	2.1± 1.5
		Freezing point depression of solution (°C.)							
		0.3	0.4		0.5	0.6		0.7	0.8
Glucose solution (6 samples)	0					94.3± 1.5			
	6	4.1±2.5	36.0±20.8		79.6± 7.1	88.1± 7.8		91.8± 7.2	95.3±1.7
	24	1.8±1.5	31.6±19.9		79.2± 9.6	85.9± 8.5		90.0±10.5	92.3±9.1
	48	1.3±1.3	32.3±22.1		78.8±10.3	83.9±11.8		85.5±12.9	90.8±7.2
		Freezing point depression of solution (°C.)							
		0.18	0.35	0.52	0.70	0.87	1.05	1.22	1.39
Sodium citrate solution (4 samples)	0			90.1± 6.3					
	6	2.1±0.6	3.4± 2.1	72.8±12.2	90.6± 6.1	87.9±6.3	89.5±4.9	86.9±2.6	90.5±3.6
	24	3.1±2.2	3.9± 3.6	68.0± 5.4	81.2±17.2	86.1±4.1	89.4±3.8	85.3±2.7	87.6±2.2
	48	0.4±0.3	2.9± 2.1	62.1±10.3	73.8± 7.0	78.8±4.7	82.4±5.4	79.7±3.8	82.0±7.3
		Freezing point depression of solution (°C.)							
		0.20	0.38	0.56	0.74	0.93	1.11	1.30	1.48
Sodium glutamate solution (4 samples)	0			90.2±13.1					
	6	2.8±2.4	7.4±6.0	75.6±10.4	89.9±4.3	89.5±8.5	89.7±4.5	88.2±2.0	86.8±5.6
	24	1.3±1.4	5.9±5.9	75.9±11.1	88.9±2.5	84.5±3.6	86.3±3.4	83.6±5.2	84.8±3.4
	48	2.0±2.7	3.4±3.9	70.7± 0.7	84.9±3.3	82.3±5.6	85.8±2.7	85.1±1.6	85.5±1.9

Dilution rate……semen 1:diluent 9

was due to the rapid decline of the motility which was one factor of the soundness.

In NaCl solution, the sperm in solution with arange of from Δ 0.6 (1.04%) to Δ 0.8 (1.39%) maintained better soundness until 6 hours at 10° C., but in storage at 0° C. better soundness was maintained in the solution with Δ 0.52 (0.9%) to Δ 0.64 (1.1%), but after 24 hours the soundness was almost lost.

In glucose solution, good soundness was maintained in only the solution with Δ 0.8 (7.4%) until 6 hours. The soundness in glucose solution was almost lost after 24 hours. The cause of loss of the soundness was due to loss of motility in glucose solution and of both motility and normal sperm rate in NaCl solution.

Nishikawa and Sasaki (1946) stated that the motility and the viability of sperm of horse in the abnormal solution (hyper and hypotonic solutions) were rapidly lost, and the abnormal figure, such as "tail coilling", was always found. Furter more they reported that NaCl exerted a harmful effect on sperm by agglutination wihch increased accordance with raising of the concentration of the solution. The tails in hypertonic solution showed irregular zig-zag bends, while in hypotonic solution the tails were curled in ring.

Pursley and Herman (1950) reported the similar observation on abnorality of bull sperm.

In the range of hypertonic solution of this experment, the abnormal sperm such as zig-zag shape, scarcely appeared, and agglutination of sperm was found in only glucose solution.

Section 4 Effect of Salts on the Decline of Motility and on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

The effect of ions on activity or viability of mammalian sperm have been stated by several investigators (Sato, 1916; Yamane, 1921; Roemmele, 1927; Dubincik, 1934; White, 1956). A few observations have been carried out in cock semen, and the effect of salts on the incidence of abnormal sperm have been scarcely reported. Therefore the present experiment was attempted.

Materials and Methods

The semen was collected by the proceding method. The density used in this experiment was 3 to 5 million per mm^3. The semen was diluted at the rate of 1:9 with each solution. The motility and the rate of normal sperm of semen stored at 0°C. were determined by the preceding method after 24 and 48 hours.

Three trials were contained in this experiment.

Trial 1. Effect of unwashing sperm (sperm suspension contained seminal plasma).

The semen was diluted with each isotonic solution (Δ 0.6) of several inorganic and organic salts.

Trial 2. Effect on washing sperm (sperm suspension was free from seminal

plasma).

(1) Each isotonic solution of several inorganic salts was mixed with equal volume of isotonic solution of sodium glutamate (3.42 %). And then, PH of mixed solution was adjusted to 7.0 by adding a small quantity of 0.17 N NaOH solution.

(2) Isotonic solution of organic salt was adjusted to PH 7.2 by adding 0.17 N HCl solution. Cl ion quantity of each solution was corrected by adding 0.97 % NaCl solution.

Semen was diluted 10 times with each solution, and then sperm in the diluted semen were separated from liquid by centrifugal force. The separated sperm were resuspeuded to previous volume with the same solution.

Trial 3. Effect of heavy metals.

The semen was diluted with Lake's solution contained a heavy metal. The concentration of a heavy metal in Lake's solution was 0.0015M.

Results and Discussion

(1) Effect on motilty

The results obtained in three trials are shown in Table 25.

In Trial 1, the semen diluted with the isotonic solution of sodium acetate, Na_2SO_4, sodium glutamate, sodium tartrate or sodium oxalate maintained high motility after 48 hours at 0°C., and there was no statistical difference between each other in four salts solutions except sodium oxalate solution, but the motility of semen in sodium oxalate solution was lower than that in sodium acetate or Na_2SO_4 solution statistically.

The motility in sodium citrate, KCl or NaCl solution decreased considerably during storage, and there was no statistical difference between each other. The decline of motility in $MgCl_2$, NH_4Cl, $NaNO_3$ or $CaCl_2$ solution was greatly rapid, and the sperm stopped their motility after 48 hours. This result showed that the injurious effect was ordered as following: $NO_3 > Cl = \text{citrate} \gg \text{oxalate} \geqq \text{tartrate} = \text{glutamate} = SO_4 = \text{acetate}$, $Ca \geqq NH_4 = Mg \gg K = Na$.

In Trial 2 (1), the washing sperm suspended in the isotonic sodium glutamate solution contained Na_2SO_4 maintained the highest motility during storage. The motility of sperm suspended in the solutions containing NaCl, KCl or $NaNO_3$ was more declined than that in the solution containing Na_2SO_4, and the difference between the formers and the latter was significant statistically. The motility of sperm in the suspinsions containing NH_4Cl, $MgCl_2$ or $CaCl_2$ decreased rapidely. This resut showed that the injurious effect was arranged in the following order: $NO_3 = Cl \gg SO_4$, $Ca = Mg = NH_4 \gg K = Na$.

In Trial 2 (2), the washed sperm suspended in the isotonic solution of sodium glutamate maintained the highest motility, the motility of sperm suspended in sodium oxalate, sodium tartrate or potassium oxalate solution decreased more rapidly than that in sodium glutamate solution, and there was statistical difference between the formers and the latter. The decline of motility in sodium citrate or sodium acetate solution was further more rapid than that in sodium oxalate, sodium tartrate or potassium oxalate solution.

Table 25 Effect of salts on motility in cock semen during storage at 0°C.

Trial 1 Motility of unwashing sperm in isotonic salt solution

Storage time (hours)	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$	$NaNO_3$	Na_2SO_4	Na oxalate	Na acetate	Na citrate	Na tartrate	Na glutamate
0	86.2 ± 7.4											
24	34.0 ±11.9	28.6 ±12.1	4.8 ±6.0	0.2 ±0.4	6.4 ±3.4	1.0 ±1.2	69.0 ±2.2	59.0 ±17.5	79.0 ±2.2	36.0 ±9.6	66.0 ±19.8	69.0 ±13.4
48	8.0 ± 6.0	8.6 ±6.9	0	0	0	0	67.0 ±10.3	50.0 ±12.2	74.0 ±8.9	15.0 ±5.0	59.0 ±16.7	63.0 ± 8.4

Trial 2 (1) Motility of washing sperm in isotonic sodium glutamate solution contained inorganic salt

Storage time (hours)	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$	$NaNO_3$	Na_2SO_4
0	95.3± 3.8						
24	51.3±16.5	45.0±17.3	4.5± 4.9	0	1.0±0	50.0±20.0	63.8±11.1
48	18.8±11.1	20.0± 4.0	1.5± 2.4	0	0	18.3± 9.3	36.3± 9.5

Trial 2 (2) Motility of washing sperm in isotonic organic salt solution

Storage time (hours)	Sodium oxalate	Sodium acetate	Sodium citrate	Sodium tartrate	Sodium glutamate	Potassium oxalate	Ammonium oxalate
0			94.5±3.3				
24	36.3±11.1	17.5±9.6	11.3±2.5	32.5±12.6	71.3±15.5	35.0±12.9	0
48	15.8± 9.9	0.3±0.5	4.0±4.5	15.5± 5.0	42.5±18.5	14.0±13.7	0

Trial 3 Motility of unwashing sperm in Lake's solution contained heavy metal*

Storage time (hours)	Control	$FeSO_4$	$Fe_2(SO_4)_3$	$CuSO_4$	$ZnSO_4$	$COSO_4$
0	80.7±7.6					
24	75.0±5.8	51.3±9.0	52.5±9.6	67.5±12.6	80.0±7.8	77.5±9.6
48	57.0±5.0	2.8±1.5	2.0±2.9	7.3± 6.4	55.0±5.8	50.0±8.2

In Trial 1 and 3, semen was diluted 10 times with the solution.
In Trial 2, sperm were suspended with the solution of 10 times volume of the semen.
* Heavy metal concentration wae 0.0015M.
Motility was average score of 4 samples.

The decline of motility in ammonium oxalate solution was the most rapid, and the motility in this solution was was lost completely after 24 hours. This result showed that the injurious effect was arranged in the following order: acetate=citrate≫ tartrate=oxalate≫glutamate, NH_4>K=Na.

It appears that the disparity of result between Trial 1 and 2 is due to the existence of seminal plasma. A cause for marked difference in effect on sodium acetate between Trial 1 and 2 is not clear.

In Trial 3, the semen diluted with Lake's solution contained $CoSO_4$ or $ZnSO_4$ maintained as high motility as the semen diluted with Lake's solution contained $CuSO_4$, $FeSO_4$ or $Fe_2(SO_4)_3$ decreased rapidly after 48 hours. There was statistical difference between the formers and the latters. This result showed that injurious effect was arranged in the follwing order: $Fe^{\cdot\cdot}$=$Fe^{\cdot\cdot\cdot}$= Cu≫Zn=Co.

It is to be considered that citrate which used generally in diluent of mammalian semen gives an injurious effect on maintenance of the motility to cock semen. It is assumed by these results that an injurious effect on maintenance of the motility during storage can be arranged in the following order: citrate≫ tartrate=oxalate> glutamate, NO_3≧Cl≫SO_4, Ca≧Mg=NH_4≫ K=Na, $Fe^{\cdot\cdot}$=$Fe^{\cdot\cdot\cdot}$=Cu≫ Zn=Co.

It is important fact that sodium sulphate showes the best result among inorganic salts.

(2) Effect on incidence of abnormal sperm

The results obtained in three trials are shown in Table 26. In Trial 1, the semen diluted with isotonic solution of Na_2SO_4, sodium citrate, sodium glutamate, sodium tartrate or sodium oxalate maintained high percentage of normal sperm during 48 hours at 0°C., and there was no statistical difference between these solutions. The normal sperm rate in $CaCl_2$, $MgCl_2$ or sodium acetate solution showed considerable decrease. The decrease of normal sperm rate in NaCl, KCl, $NaNO_3$or NH_4Cl solution was especially rapid. This result showed that the injurious effect was arranged in the following order: NO_3= Cl> acetate≫oxalate=tartrate=glutamate=citrate=SO_4, NH_4=K=Na>Mg= Ca.

In Trial 2 (1), the normal sperm rate in isotonic sodium glutamate solution contained Na_2SO_4 decrease very slowly during storage at 0°C. But the normal sperm rate in the solution contained NH_4Cl, NaCl, KCl, $CaCl_2$, $NaNO_3$ or $MgCl_2$ decrease gradually during storage, and there was no statistical difference between the rates in these diluted semen samples. This result showed that the injurious effect was arranged in the following order: NO_3=Cl≫SO_4, Ca=Mg≧NH_4=K=Na.

In Trial 2 (2), the normal sperm rate in isotonic sodium citrate solution showed the highest percentage during storage, but after 48 hours, the difference between this solution and the solutions of sodium tartrate, sodium oxalate and sodium glutamate had not statistical significant. Only the rate in sodium acetate solution decrease rapidly. The rate in potassium or ammonium oxalate decreased considerably, but there was no statistical diffe-

Table 26 Effect of salts on the rate of normal sperm in cock semen during storage at 0°C.

Trial 1 Normality of unwashing sperm in isotonic salt solution

Storage time (hours)	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$	$NaNO_3$	Na_2SO_4	Sodium oxalate	Sodium acetate	Sodium citrate	Sodium tartrte	Sodium glutamate
0	94.0 ±1.5											
24	7.8 ±2.7	4.8 ±1.7	2.2 ±1.1	36.2 ±9.5	42.6 ±9.1	2.8 ±2.6	78.8 ±3.9	75.0 ±5.4	32.6 ±10.1	78.1 ±6.8	75.7 ±6.2	77.6 ±2.5
48	1.9 ±1.5	2.1 ±2.0	1.7 ±0.8	11.3 ±4.0	11.6 ±1.6	1.8 ±1.6	73.6 ±5.5	65.9 ±6.3	11.3 ±4.3	73.1 ±4.5	67.4 ±6.4	66.7 ±5.9

Trial 2 (1) Normality of washing sperm in isotonic sodium glutamate solution contained inorganic salt

Storage time (hours)	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$	$NaNO_3$	Na_2SO_4
0	89.2±10.0						
24	51.8± 6.5	50.1±11.7	51.7±9.3	56.6± 6.9	46.3±4.8	41.9±12.9	75.9±5.9
48	43.8± 9.5	44.1± 9.0	46.5±7.9	37.2±11.1	34.5±4.3	34.6±11.7	68.2±9.8

Trial 2 (2) Normality of washing sperm in isotonic organic salt solution

Storage time (hours)	Sodium oxalate	Sodium acetate	Sodium citrate	Sodium tartrate	Sodium glutamate	Potassium oxalate	Ammonium oxalate
0			93.5± 6.1				
24	74.9± 6.4	10.3±4.2	78.3± 4.5	69.9± 6.7	65.3±11.9	52.6±11.7	52.6±15.6
48	60.3±16.5	4.2±1.9	72.0±10.5	61.6±11.0	57.1± 7.3	46.2±15.7	34.6±10.0

Trial 3 Normality of unwashing sperm in Lake's solution contained heavy metal*

Storage time (hours)	Control	$FeSO_4$	$Fe_2(SO_4)_3$	$CuSO_4$	$ZnSO_4$	$CoSO_4$
0	80.7±9.1					
24	76.1±6.1	77.3±11.3	75.1±11.8	75.5± 9.7	70.7± 6.5	77.2± 6.5
48	68.3±3.6	69.8±13.0	64.5±14.0	60.5±13.1	60.0±14.5	67.4± 9.4

In Trial 1 and 3, semen was diluted 10 times with the solution.
In Trial 2, sperm were suspended with the solution of 10 times volume of the semen.
* Heavy metal concentration was 0.0015 M.
Motility was average percentage of 4 samples.

rence after 48 hours between the rates in these solutions and in sodium oxalate solution. From these results, the injurious effect was arranged in the following order: acetate$\gg$glutamate=oxalate=tartrate=citrate, NH_4=K=Na. It was a considerable fact that Na_2SO_4 solution maintained the highest normal sperm rate during storage in inorganic salt solutions examined in this investigation, and sodium acetate solution decreased the normal sperm rate most rapidly in organic salt solutions examined in this investigation. It is to be considered by these results that an pormotive action on incidence of abnormal sperm could be arranged in the following order: acetate$\gg$ tartrate =oxalate=glutamate=citrate, NO_3=Cl$\gg SO_4$, Na=K=$NH_4\geqq$Mg=Ca.

In Trial 3, the statistical difference of protective action to incidence of abnormal sperm in solution contained $Fe^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$, Co, Zn or Cu was not found in this experiment.

(3) Effect on soundness

As shown in Fig. 5, in Trial 1, the semen diluted with the isotonic solutions of Na_2SO_4, sodium glutamate, sodium tartrate, sodium oxalate, maintained good soundness. The soundness of semen diluted with sodium citrate or sodium acetate solution decreased comparatively, and the decrease in soundness of semen diluted with inorganic salt solutions was excessively rapid. This result showed that the injurious effect was arranged in the following order: NO_3=Cl$\gg$ acetate=citrate$\gg$ oxalate$>$ tartrate$\geqq$ glutamate$>$ SO_4.

In Trial 2 (1), the soundness in sodium glutamate solution contained Na_2SO_4 was higher than that contained KCl, NaCl or $NaNO_3$, and the solution contained NH_4Cl, $MgCl_2$ or $CaCl_2$ decreased rapidly. The injurious effect was, therefore, as the following order: $NO_3\geqq$Cl$\gg SO_4$, Ca=Mg=$NH_4\gg$ K=Na.

In Trial 2 (2), it was observed that the injurious effect was as the following order: acetate$>$ citrate$\gg$ tartrate=oxalate$\gg$ glutamate, $NH_4>K\geqq$Na.

In Trial 3, it was found that the injurious effect was as following order: $Fe^{\cdot\cdot}$=$Fe^{\cdot\cdot\cdot}$=Cu$\gg$Zn=Co.

On account of these results, in general, the harmfull action to maintenance of the soundness could be arranged in the following order:

acetate$\geqq$citrate$\gg$tartrate=oxalate$>$glutamate,
$NO_3\geqq$Cl$\gg SO_4$, Ca=Mg=$NH_4>$K=Na,
$Fe^{\cdot\cdot}$=$Fe^{\cdot\cdot\cdot}$=Cu$\gg$Zn=Co.

The order of available or harmful action of various ions on activity or viability of mammalian sperm was not necessarily agreed in several investigators (SATO, 1916; YAMANE, 1921; ROEMMELE, 1927; DUBINCIK, 1934).
MIROVANOV (1934) stated that chlorate and nitrate aided swelling of colloids and by destroying the lipoid capsule caused rapid death of sperm, therefore physiological saline solution had a destructive action of the capsule, on the other hand, sulphate prevent the swelling and the destruction of the lipoid capsule which delayed the transition of sperm into an irreversible state. Actually, sulphate diluent had been emploied for mammalian semen.

In cock semen, physiological saline solution had been pressume an excellent

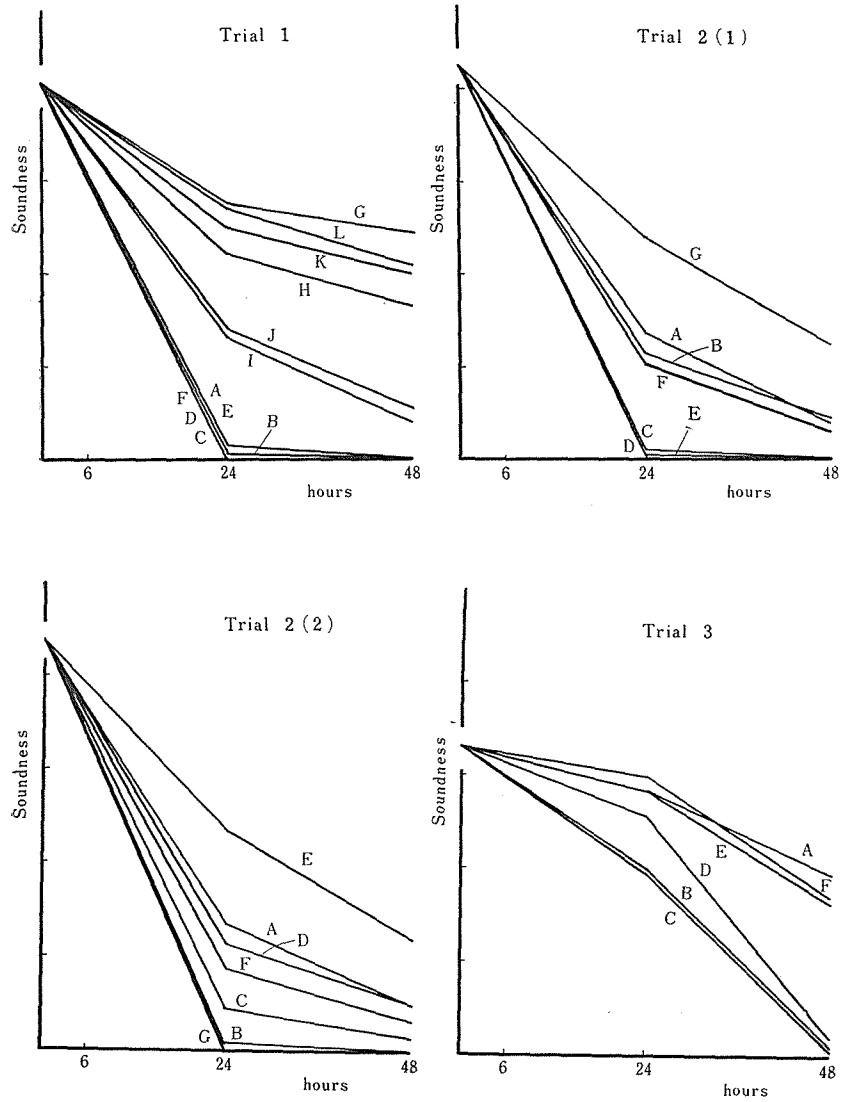

Fig. 5 Effect of salts on soundness in cock semen during storage at 0°C.

Trial 1 Soundness of sperm diluted 10 times with isotonic salt solution.
Salt in solution……
A−NaCl, B−KCl, C−NH_4Cl, D−$CaCl_2$, E−$MgCl_2$, F−$NaNO_3$, G−Na_2SO_4, H−Na oxalate, I−Na acetate, J−Na citrate, K−Na tartrate, L−Na glutamate
Trial 2 (1) Soundness of washing sperm suspended in isotonic sodium glutamate solution contained inorganic salt of 10 times volume of the semen.
Salt in solution……
A−NaCl, B−KCl, C−NH_4Cl, D−$CaCl_2$, E−$MgCl_2$, F−$NaNO_3$, G−Na_2SO_4
Trial 2 (2) Soundness of washing sperm suspended in isotonic organic salt solution of 10 times volume of the semen.
Salt in solution……
A−Na oxalate, B−Na acetate, C−Na citrate, D−Na tartrate, E−Na glutamate, F−K oxalate, G−NH_4 oxalate
Trial 3 Soundness of sperm diluted 10 times with Lake's solution contained heavy metal.
Heavy metal in solution……
A−nothing, B−$Fe^{\cdot\cdot}$, C−$F^{\cdots}$, D−Cu, E−Zn, F−Co

diluent, recently LAKE (1960) was used a diluent which contained sodium glutamate in major part, according to investigation on the content of cock seminal plasma, and he obtained good results.

The excellent result which was obtained by Na_2SO_4 or sodium glutamate in the present experiment will be useful to making the suitable diluent for storage of cock semen.

Section 5 Effect of the Addition of Non-Electrolytes on the Decline of Motility and on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

Several investigators have been reported that the addition non-electrolytes or nutrients prolong the motile life span of sperm (ISHIKAWA, 1930; MILOVANOV, 1934; GRODZINSKI and MARCHLEWSKI, 1935; HAYASHI, 1938; KNOOP, 1941; SALISBURY, 1941; KOCK and ROVILLARD, 1945; LORENZ and TYLER, 1951; MORAVEC et al., 1954). These additional substances probably act primarily to protect the sperm from traces of toxic ions.

It seemed reasonable to assume that the addition of abnormal cock sperm during storage. But such a investigation had been scarcely reported. Therefore, the present experiment was attempted.

Materials and Methods

For the aim of this experiment, the semen was stored in such a condition that the incidence of abnormal sperm might be enhanced. The semen was diluted at the rate of 1:4 with Ringer's solution, and then the sperm were separated from liquid by centrifugal force. The separated sperm were resuspended in Ringer's solution contained a non-electrolyte to the previous volume. The suspension was stored for 48 hours at 0°C. Non-electrolytes used in this experiment were gelatin white powder, fructose, egg yolk and egg albumen (outer watery albumen). The concentrations of these substances in the diluents were 1, 2 and 3% in gelatin; 1, 2.5 and 5% in fructose; and 20, 40 and 60% in yolk or albumen.

Results and Discussion

(1) Effect on motility

The results are shown in Table 27. The motility of sperm in gelatin diluent decreased as rapidly as that in the control diluent (non-gelatin diluent). Therefore, it appears that the addition of gelatin does not obstruct the decline of motility during storage.

The sperm in the fructose diluent showed higher motility than that in control after 24 hours, but after 48 hours the motility decreased extremely. Only the sperm in 2.5% fructose diluent maintained the motility partly after 48 hours, but the statistical difference between the motility of the sperm in this diluent and in 1 or 5% diluent was not found.

In yolk diluent, the high motility was kept in every concentration thro-

Table 27 Effect of non-electrolytes on motility in cock semen during storage at 0°C.

Storage time (hours)	Control diluted semen	Additional substance											
		Gelatin			Fructose			Egg yolk			Egg albumen		
		1 %	2 %	3 %	1 %	2.5%	5%	20%	40%	60%	20%	40%	60%
0	86.3												
6	46.3	46.3	39.6	22.1	63.3	64.6	57.5	75.0	73.3	77.3	70.4	71.3	64.2
24	5.4	0.4	1.4	0.2	47.5	43.3	33.3	46.7	60.8	52.1	41.7	53.8	39.6
48	0	0	0	0	0.8	12.4	0.4	22.5	33.3	32.5	35.0	34.2	22.9

Washing sperm were suspended with Ringer's solution of 5 times volume of the semen. Figure indicated average motility of 3 samples.

ughout the storage duration. In the albumen diluent, the similar high motility was maintained for 48 hours.

The results of this experiment showed that the maintenance of motility of cock sperm stored at 0°C. was enhanced by the addition of egg yolk or egg albumen.

MILOVANOV (1934) investigated the effect of protective colloids on sperm. The colloids were arranged in the following ascending order of their protective effect—(1) gum, (2) gelatin, (3) egg albumen, (4) alkaline egg albumen, (5) alkaline albumen from meat or blood serum, and (6) mucin from boar semen.

KNOOP (1941) stated that the motility of bull sperm stored in diluent was enhanced by gelatin.

Addition of glucose (KOCK and ROBILLARD, 1945) or fructose (MORAVEC et al., 1954) to diluent extended the survival of fowl sperm in suspension. Glucose or fructose are commonly used as a source of energy, but they also might serve as a protective agent.

SALISBURY et al. (1941) described that diluents using egg yolk greatly increased the survival of bull sperm.

ISHIKAWA (1930) and HAYASHI (1938) stated that egg albumen served prolongation of the life span of cock sperm. GRODZINSKI and MARCHLEWSKI (1935) reported that the diluting solutions of cock semen might be graded according to their influence following order: blood serum, embryonic extract, albumen, Tyrode's fluid. LORENZ and TYLER (1951) found that addition of glycin or egg white protein to saline diluents prolonged the motile ilfe span of sperm.

MILOVANOV (1934) considered that the effect on addition of non-electrolyte was to protect the sperm the harmful action of toxic ions to lipoid capsule.

The present results showed that addition of egg yolk or egg albumen to the physiological salt solution greatly obstructed decline of the motility of cock sperm during storage, and addition of fructose partly served mainteance of the motility.

(2) Effect on incidence of abnormal sperm

The results are shown in Table 28. The rate of normal sperm in 3% gelatin diluent was higher than that in the control diluent throughout the storage duration. But the rate of normal sperm in 1 or 2% gelatin diluent decreased in the same degree as the control diluent.

In fructose diluent, the high rate of normal sperm was kept, and that in 5% fructose diluent was lower than in 1 or 2.5% fructose diluent.

The normal sperm rate in the yolk diluent was higher than that in the control diluent, and the rate in 60% yolk diluent was the highest.

The decrease of the normal sperm rate in the albumen diluent was as rapid as that in the control diluent.

These results showed that the addition of fructose and egg yolk obstructed the transformation of sperm and gelatin also possessed this effect in certain concentration.

Table 28 Effect of non-electrolytes on the rate of abnormal sperm in cock semen during storage at 0°C.

Storage time (hours)	Control diluted semen	Additional substance											
		Gelatin			Fructose			Egg yolk			Egg albumen		
		1%	2%	3%	1%	2.5%	5%	20%	40%	60%	20%	40%	60%
0	97.3												
6	47.6	41.0	44.6	73.4	82.0	76.3	61.4	68.9	77.9	84.4	38.2	30.6	30.7
24	13.7	10.4	13.1	33.6	41.4	47.3	34.2	21.6	27.0	43.0	13.9	15.5	12.9
48	5.3	7.3	8.0	19.8	35.8	32.2	21.5	10.4	19.0	28.4	8.9	10.0	9.0

Washing sperm were suspended with Ringer's solution of 5 times volume of the semen.
Figure indicated average percentage of 3 samples.

(3) Effect on soundness

As shown in Fig. 6, addition of gelatin or egg albumen to saline diluent was scarcely useful for maintenance of the soundness during storage. Addition of fructose slightly enhanced maintenance of the soundness until 24 hours, but after 48 hours this effect was almost lost. Egg yolk, when added to saline solution, was useful for maintenance of the soundness, but this effect was not so great. The degree of effect increased with the concentration of yolk, and 60% solution showed the highest soundness.

Section 6 Effect of Degree of Dilution on the Decline of Motility on the Incidence of Abnormal Spermatozoa in Cock Semen during Storage

It has been reported by several investigators that the effect of dilution on motility, survival and fertility of sperm. These results did not always agree in detail. These differences were due to probably the difference of animals, diluents, degrees of dilution and storage temperatures in these experiments.

The effect of dilution on the incidence of abnormal sperm in cock semen was scarcely known. The experiment in the present section was attempted by reason of these fact.

Materials and Method

The semen samples from 6 White Leghorn cocks were mixed and divided

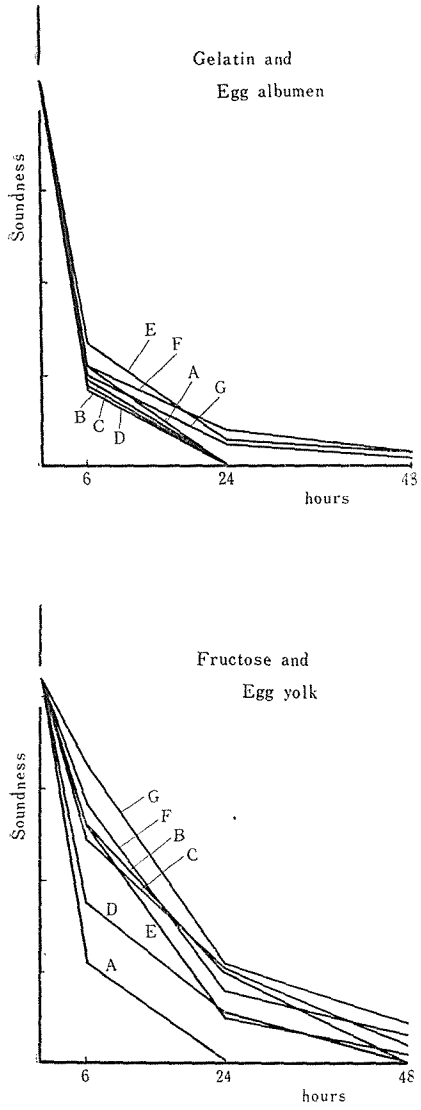

Fig. 6 Effect of non-electrolytes on soundness in cock semen during storage at 0°C.

Washing sperm were suspended with Ringer's solution of 5 times volume of the semen.
Gelatin and Egg albumen……

A—Control	B—Gelatin 1%
C—Gelatin 2%	D—Gelatin 3%
E—Albumen 20%	F—Albumen 40%
G—Albumen 60%	

Fructose and Egg yolk……

A—Control	B—Fructose 1%
C—Fructose 2.5%	D—Fructose 5%
E—Yolk 20%	F—Yolk 40%
G—Yolk 60%	

into 7 parts, one of which was used as the control or undiluted semen and 6 of which were diluted 2, 10 and 20 times with Lake's and Tyrode's solutions. These samples were stored at 10°C., and the determinations of motility and normal sperm rate were carried out by the previous methods. The densities of semen samples used in the present experiment were in the range of 3 to 4 million per mm^3.

Results and Discussion

(1) Effect on motiliy

The obtained resuts were shown in Table 29. In the dilution of semen with Lake's solution, the semen diluted ×10 or ×20 maintained high motility throughout storage duration, and the motility of these diluted semen samples at 48 hours were higher than that of ×2 statistically. The decline in motility of semen diluted ×2 was rapid and was lower than that of undiluted control semen statistically.

On the other hand, in the dilution with Tyrode's solution, the decline of motility of semen diluted ×2 was slow, and the semen diluted ×10 showed more rapid depresion in motility than the semen diluted×2. The motility of semen diluted ×20 was most rapid among these examples. There were statistical differences in motility among each others and between the semen diluted×20 and the undiluted control semen. Namely, in the range of dilution used in the present experiment, the motility in Lake's solution during storage declined with the decrease of dilution rate. On the other hand, the motility in Tyrode's solution declined with the increase of dilution rate. As stated in Chapter I, in high density semen such as bull, ram and goat, it had been reported that the survival of

Table 29 Effect of dilution on motility in cock semen during storage at 10°C.

Storage time (hours)	Control undiluted	Lake's sol.			Tyrode's sol.		
		×2	×10	×20	×2	×10	×20
	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.
0	84.0± 4.1						
6	59.4±10.9	57.0± 7.2	73.5±4.7	73.2± 8.1	59.4±5.8	74.7± 2.8	61.3±2.5
24	50.0±12.8	16.0±11.3	54.7±5.3	54.7±23.6	53.8±2.7	61.9± 4.3	49.1±8.1
48	38.2±25.7	0.7± 0.8	37.9±6.4	44.7± 5.4	51.1±5.1	23.0±17.8	2.7±3.1

sperm in undiluted semen was longer than that in diluted semen at low temperature. Recently, it is admitted that the dilution of semen with favourable diluent prolongs the survival of sperm in vitro beyond that of those in undiluted controls, however, in any diluent, the survival time and fertility of sperm decrease when the extent of dilution exceeds a certain optimum level, and this adverse effect is clearly shown in diluents with a high content of electrolytes (SALISBURY et al., 1943; CHANG, 1946; EMMENS, 1948; ROTHSCHILD, 1948; CHENG et al., 1949; LORENZ and TYLER, 1951; WILLETT, 1953).

In cock semen, and in the limited dilution rate, the diluents for example glucose and fructose solutions, blood serum and physiological saline solutions, showed favorable effect to sperm motility in comparison with undiluted semen (SHAFFNER et al., 1941; JASPER, 1950; BOGDONOFF and SHAFFNER, 1954). The harmful effect of high dilution was reported by LORENZ and TYLER (1951) in cock semen too.

The present result in Tyrode's solution showed the injurious effect of extensive dilution. The high dilution (×20) with Lake's solution did not give injurious effect to stored sperm. These results would be due to the difference of suitability of Lake's and Tyrode's solutions for survival of cock sperm in vitro.

The motility of semen diluted ×2 with Lake's solution decreased exceedingly. This phenomenon can be explained probably the following reasons. When semen was diluted with Lake's solution and was stored at a higher temperature than 10°C., PH of the diluted semen decreased rapidly, namely in storage at 0°, 10° and 20°C. its PH fell from 7.0 to 6.8, 6.0 and 5.4 respectively after 24 hours. From this fact, it was considered that in the diluted semen metabolic products easily accumulated. And so, these harmful metabolic products would not be able to dilute in the case of a low rate dilution.

(2) Effect on incidence of abnormal sperm

As shown in Table 30, the semen diluted with Lake's solution maintained comparatively high value of normal sperm rate throughout storage duration at 10°C.

The normal sperm rates of semen diluted with ×2, ×10 and ×20 respectively were shown no statistical differences among each other. On the other hand, the semen diluted with Tyrode's solution decreased the normal

sperm rate remarkably. The decrease of normal sperm rate in the semen diluted ×10 or ×20 was especially rapid. And there was statistical difference between ×10 or ×20 and ×2. Moreover, every diluted semen showed lower value in the normal sperm rate than the undiluted control semen statistically.

It is worthy of notice that the low dilution with Tyrode's solution was favourable for maintenance of motility but was harmful for prevention of incidence of abnormal sperm.

Table 30 Effect of dilution on the rate of normal sperm in cock semen during at 10°C.

Storage time (hours)	Control undiluted	Lake's sol.			Tyrode's sol.		
		×2	×10	×20	×2	×10	×20
	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.
0	92.8± 2.0						
6	84.9± 3.5	86.9± 3.9	83.7± 8.4	87.4± 3.7	81.3±4.3	80.1± 7.0	73.1±8.0
24	71.0±14.6	83.0± 5.2	85.1± 3.3	78.9±11.6	60.3±7.7	41.8±12.0	12.0±5.3
48	62.8±13.4	63.2±12.0	71.0±14.3	63.6±15.3	35.2±5.8	11.8± 6.9	5.9±4.4

(3) Effect on soundness

As shown in Fig. 7, the results of the present experiment was shown that when semen was diluted with Lake's solution, the maintenance of soundness was obstructed in the dilution less than a certain degree. On the other hand,

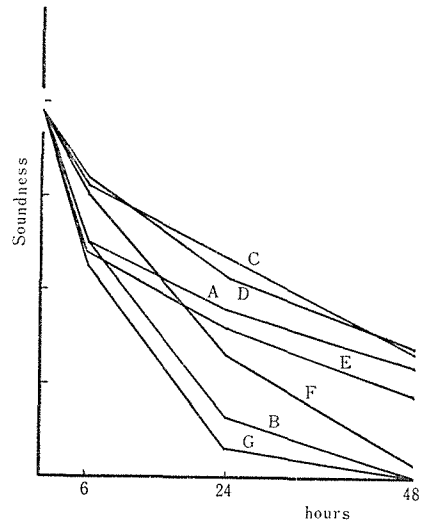

Fig. 7 Effect of dilution on sonudness in cock semen during storage at 10°C.
A—Undiluted, B—Lake(×2), C—Lake(×10), D—Lake(×20)
E—Tyrode(×2), F—Tyrode(×10), G—Tyrode(×20)

when the semen was diluted with Tyrode's solution, the harmful effect to the maintenance of soundness increased in accordance with the extension of dilution rate. This phenomenon appeared in the semen diluted with Lake's solution is probably due to the storage at a high temperature (10°C.) as has been stated above.

Section 7 Summary and Conclusion

The sperm motility in cock semen during storage decreased rapidly above 20°C. The optimum temperature in maintenance of motility was 0° to 10°C., but in the semen diluted with Tyrode's solution it appears that storage at a lower temperature (0°C.) was not always suitable. The abnormal sperm, generally, arose easily in the storage at a lower (0°C.) or a higher (30°C.) temperature than at a moderate temperature (10° to 20°C.). The suitable storage temperature for maintenance of soundness was 10° to 20°C., but in the semen diluted with Wilcox's or Lake's solution a lower temperature (0° to 10°C.) was rather favorable.

The highest motility was maintained by neutral PH (7.0) of suspension through the storage period. The motility decreased in the acid or basic suspension, and the degree of the decline increased with the distance from PH 7.0, and so at PH 5.3 or 8.8 the motility ceased almost after 6 hours. But the motility in modified Lake's solution was maintained comparatively high at PH 8.0. The rate of normal sperm in the suspension with the range of from PH 6.1 to 8.0 showed no statistical differences throughout the storage duration. When the PH of suspension was close to 7.0, the best soundness was maintained. The semen diluted with glucose phosphate solution had a tendency to maintaine fairly good soundness in a slight acidity (PH 6.1), while the semen diluted with modified Lake's solution, had a tendency to maintain fairly good soundness in a slight alkalescence (PH 8.0).

In isotonic solutions (Δ 0.52 to 0.74) near the semen, in general, the stored sperm maintained the highest motility. But in NaCl solution, the sperm in hypotonic solutions maintained rather higher motility in storage at 0°C. It is probably due to the harmful effect of NaCl ion in a low temperature. In a glucose solution, the sperm in the solution of 0.8 maintained higher motility than the sperm in the solutions less than 0.7 for short storage at 0°C. But at 10°C., the sperm in solutions of low concentration maintained higher motility than in these of high concentration. It seems that such a rapid decline of motility in the latter is probably due to the especial rapid fall of PH in sperm suspension. After 24 hours at both 0° and 10°C., the sperm in glucose solution of any concentration completely lost its motility. This fact showed that the glucose solution was not a suitable diluter for the cock sperm. In general, the hypotonic solution excessively promoted the incidence of the abnormal sperm during storage, while the isotonic or hypertonic solution gave a good result on maintenance of the normal sperm rate. NaCl solution was an only exception in which the hypertonic solution was also harmful. In all concentrations including the isotonic solution the

incidence of abnormal sperm in NaCl solution was vary rapid, especially in storage at 0°C. It was found that NaCl gave also a morphological injury with harmful effect to maintenance of motility in storage at a low temperature especially. The best soundness in this experiment was shown in sodium glutamate solution Δ 0.56 (3%) to Δ 0.74 (4%). In sodium citrate solution, better soundness was maintained in Δ 0.52 (3%) to Δ 0.70 (4%), but the degree of soundness was not fairly high. In NaCl or glucose solution, soundness was almost lost after 24 hours. Until 6 hours, the sperm stored at 0° and 10°C. maintained fairly high soundness in NaCl solution of Δ 0.6 (1.04%) to Δ 0.8 (1.39%) and Δ 0.52 (0.9%) to Δ 0.64 (1.1%) respectively, and the sperm stored in glucose solution maintained high soundness in Δ 0.8 (7.4%) at 0°C.

The storage experiments was carried out in the semen which was biluted with the solution of isotonic salt, and in washing the sperm which was suspended in isotonic sodium glutamate solution contained an inorganic salt or in the isotonic solution contained an organic salt. From these experimental results, it was assumed that an injurious effect on maintenance of sperm motility during storage could be arranged in the following order: citrate$\gg$ tartrate=oxalate>glutamate, $NO_3 \geqq Cl \gg SO_4$, $Ca > Mg = NH_4 > K = Na$, and a promotive effect on the incidence of abnormal sperm could be arranged in the following order: acetate$\gg$tartrate=oxalate=glutamate=citrate, $NO_3 = Cl \gg SO_4$, $Na = K = NH_4 \geqq Mg = Ca$.

From the storage experiment, in the semen which was diluted with Lake's solution contained a heavy metal, the injurious effect on maintenance of motility could be arranged in the following order: $Fe^{\cdot\cdot} = Fe^{\cdot\cdot\cdot} = Cu \gg Zn = Co$, and the difference of protective action to the incidence of abnormal sperm in these solutions was not found. The harmfull action to maintenance of the soundness could be arranged in the following order: acetate$\geqq$ citrate$\gg$ tartrate=oxalate>glutamate, $NO_3 \geqq Cl \gg SO_4$, $Ca = Mg = NH_4 > K = Na$, $Fe^{\cdot\cdot} = Fe^{\cdot\cdot\cdot} = Cu \gg Zn = Co$.

An addition of egg yolk or egg albumen to the physiological saline solution greatly obstructed the decline of the motility of sperm, and an addition of fructose partly served maintenance of the motility. Fructose or egg yolk obstructed the transformation of sperm and in a certain concentration gelatin also possessed probably this effect. An addition of gelatin or egg albumen to the saline diluent was scarcely useful for maintenance of the soundness during storage. Fructose partly served maintenance of the soundness. Egg yolk added to the saline solution was useful for maintenance of the soundness, but this effect was not so great.

In the range of dilution used in this experiment ($\times 2$, $\times 10$ and $\times 20$), the sperm motility in Lake's solution decline during storage in accordance with the decrease of dilution rate, and the motility in Tyrode's solution declined with the increase of dilution rate. The result in Tyrode's solution showed the injurious effect of extensive dilution. The result in Lake's solution can be explained probably by the following reasons. When the semen was diluted with Lake's solution and was stored at a higher temperature than

about 10°C., PH of the diluted semen decreased rapidly. From this fact, it was considered that in the diluted semen metabolic products easily accoumulated. And so, these harmful metabolic products would not be able to dilute in the case of a low rate dilution. In Lake's solution, the harmful effect of dilution to transformation of sperm could not be found to the range of 20 times dilution in this experiment. In Tyrode's solution, any dilution rate enhanced the incidence of abnormal sperm, and the harmful effect increased with the extension of dilution rate. The dilution with Tyrode's solution was harmful for the maintenance of soundness, and the harmful effect increased in accordance with the extension of dilution rate. In Lake's solution, the maintenance of soundness was obstructed in the dilution less than a certain degree. It is probably due to the storage at a high temperature (10°C.) as has been stated above.

As has been previously mentioned, the effects of several environmental factors during storage to the decline of motility and transformation of sperm have been made clear from the investigation in this chapter. Moreover it has been found that the effects of several factors to these two main factors which connected closely with the fertility of cock semen have not always the same tendency, and these effects infuluence the sperm themselves derectly or indirectly through the media. If, further, the cooperative effect of these emvirommental factors is made clear, the suitable preserving fluids and methods for the cock sperm will be able to be developed in the near future.

In conclusion, the writer's hearty thanks are due to Prof. S. OKAMOTO under whose direction this work has been done. Acknowledgment is due to Prof. H. NISHIYAMA, to whom the writer is indebted for valuable suggestions in accomplishing this work. Thanks are also due to Prof. H. MIMURA for his kind criticism and helpful suggestion for this study.

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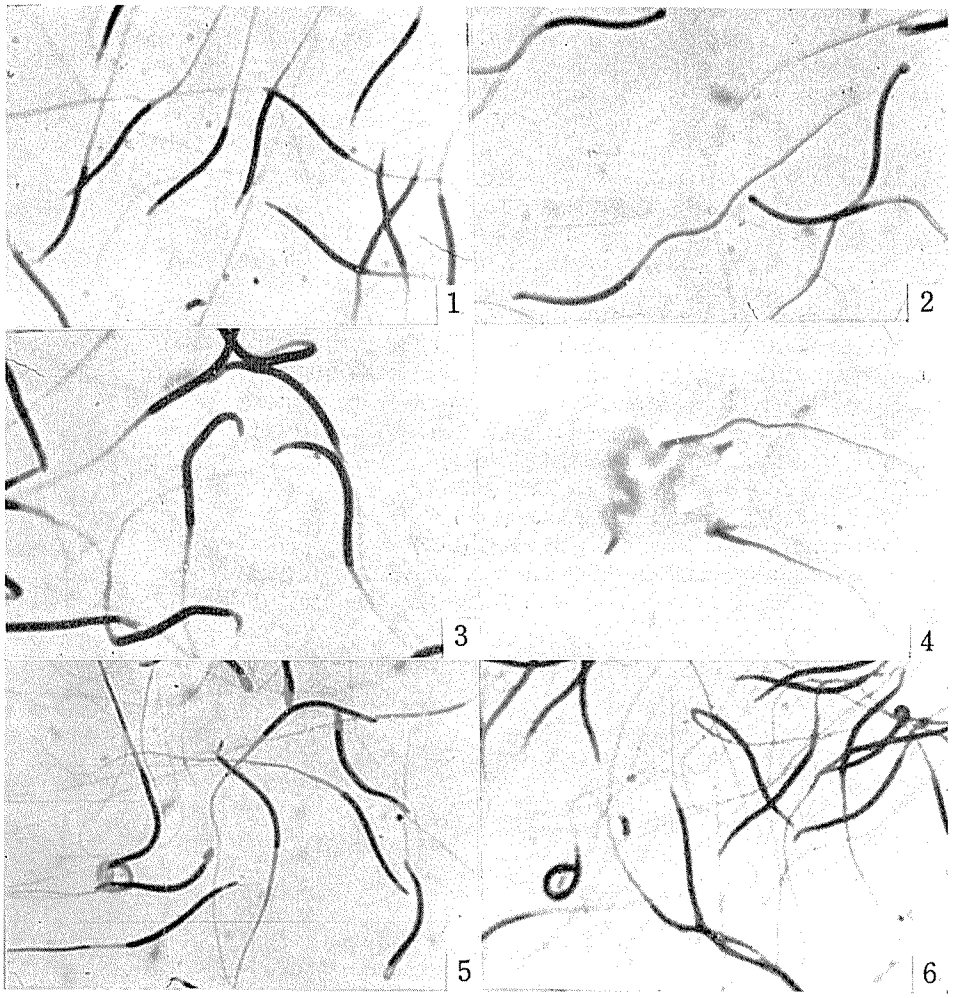

Plate 1 Abnormal spermatozoa appeared in storage cock semen

1 Normal spermatozoa
2 Spermatozoa with spherular acrosome
3 Spermatozoa with hooked acrosome and with bent head
4 Spermatozoa with swollen head
5 Spermatozoa with bent midpiece and with hooked acrosome
6 Spermatozoa with coiled head and with bent midpiece

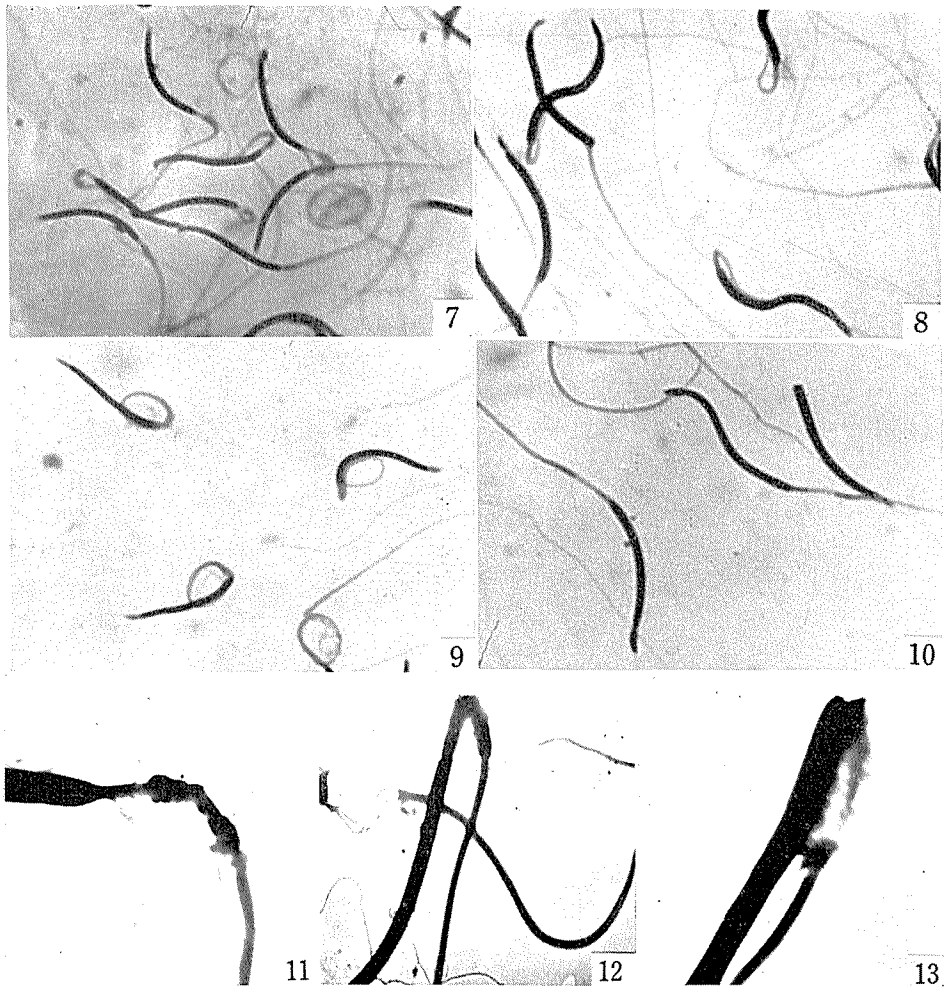

Plate 2 Abnormal spermatozoa appeared in storage cock semen

7 and 8
Spermatozoa with bent midpiece

9 Spermatozoa with coiled tail

10 Spermatozoa with lacking midpiece and tail

11, 12 and 13
Disruption and bend of midpiece observed under electron microscope in sperm stored in Tyrode's solution for 6 hours at 0°C.