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ELECTRON MICROSCOPIC STUDY OF THE BULL SPERMATOZOON*¹ I

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INTRODUCTION

Three hundred years ago, LEEUWENHOEK using a simple light microscope described the sperm as a kind of animalcule. Since this time, great advancements in light microscopy, provided the impetus for continued study of sperm morphology. The morphological studies of sperm from various species by RETZIUS⁸⁶⁾ are especially noteworthy and are considered classical today. Early studies on sperm have been reviewed by JÖEL and BELOMOSCHKIN.

In 1940, the electron microscope, a new apparatus having a high resolving power, was introduced into the fields of medicine and biology. Whole cell preparations such as bacteria and sperm were studied. At first SEYMOUR & BENMOSCHE tried the electron microscopic study of human sperm. In the veterinary field, since BAYLOR et al. made first a comparative study between human sperm and bull sperm, many reports have been published concerning sperm morphology by electron microscope about bull^{25~29)}, ram⁸¹⁾, boar¹⁸⁾ and other domestic animals^{53,63)}. Since 1950, a new technique, ultramicrotomy, has been employed in this field, but the number of reports^{3,14,23,87,95)} about this method are not so many up to now. In addition, a few reviews about sperm morphology and spermatogenesis on the basis of the electron microscopic study have been published^{11,38,116)}.

In the field of animal reproduction, semen examination has become more important and a great deal of knowledge on the morphology and physiology of the sperm has been obtained in accordance with a rapid development of artificial insemination. However, the detailed structure of sperm still remains to be investigated.

The present author has carried out the morphological study of bull sperm by means of electron microscopy from April, 1959 to December, 1961, and obtained

*¹ This report is an originally full paper of the summary reported formerly on this journal **10**, 72 (1962).

The list of references and plates will be appeared at the end of this series.

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some interesting, epochal results on the fine structure of the bovine spermatozoon.

NOMENCLATURE AND SYNONYMS

The nomenclature of most structure of the sperm differs according to investigators, thus communication of research on this subject is quite difficult. To simplify terminology and integrate the current findings with the existing literature the words shown in the text figures 1, 2, 4, 6 and 8 will be used in this paper and their synonyms presented in the following paragraphs.

The anterior half of head is that portion covered by the head cap and the posterior half of head is that portion covered by the post nuclear sheath. The basal part is the area adjacent to the fossula-like depression, respectively.

Note When immotile sperms are suspended in a proper medium, each sperm tends to stop in a situation in which the surface having the acrosome corpuscle turns above and the opposite surface, i. e., the surface without the acrosome corpuscle is on the bottom. Therefore, the former will be referred to as the dorsal surface and the latter, as the ventral surface.

spermatozoon: sperm, animalcule; Spermazellen, Spermium, Spermafaden, Spermatozoon

cell membrane: cytoplasmic membrane, manchette, capsule; Manschette

outer layer of cell membrane: outer cell membrane

inner layer of cell membrane: inner cell membrane

head: sperm head; Chromosom, Kopf, Spermienkopf; caput

anterior half of head: Vorderstück des Kopfes; pars anterior capitis

head cap: acrosome, anterior head cap, cytoplasmic cap, acrosome cap, perforatorium, spermatie veil, inner acrosome cap, cephalic cap, protoplasmic cap, acrosomic system;

Kopfkappe, Kappe, Plasmahülle; spermioalcythrotheca, acrosoma; galea capitis

acrosome membrane: acrosome components

external acrosome membrane: outer acrosomal membrane, cap outer membrane, outer cap, head cap

outer layer of external acrosome membrane

inner layer of external acrosome membrane

internal acrosome membrane: inner acrosomal membrane, cap inner membrane

outer layer of internal acrosome membrane

inner layer of internal acrosome membrane

acrosome: acrosomal substance, acrosomic substance, acrosome material; Acrosom, Akrosoma

acrosome corpuscle: acrosome, acrosomal body, apical vacuole, apical body, fold, arc-like thickening; Acrosom, Akrosom

equatorial zone: horizontal zone, equatorial line, equatorial segment, central segment, distinct girdle, horizontal line, circular band; äquatoriale Zone, Ringband des Kopfes, gehüllte Zone, dunkle Linie, Innenkörper; pars centralis capitis, pars intermedia

- anterior margin of equatorial zone: anterior border of equatorial zone, anterior line, anterior limit, posterior border of inner acrosome cap, margin of head cap
- posterior margin of equatorial zone: posterior border of equatorial zone, posterior line, posterior limit, posterior border of outer acrosome cap, margin of post-nuclear cap, nuclear ring
- posterior half of head: Hinterstück des Kopfes; pars posterior capitis
- nuclear sheath: post-nuclear sheath, posterior nuclear cap, post-nuclear cap, post-nuclear cup, nuclear shell, nuclear cup, basic small cup, head tunica, head fibrillar sheath, post nuclear cap; Becherhülse
- basal part: base, basal portion; Basal-Teil
- basal granule: nuclear ring, upper centriole, basal ring, anterior end knob, upper centriole, neck granule, ring-shaped membrane, kern, fascicle; Proximalkern, Nebenkern, Zentralkörperkorn
- basal plate: collar base of nucleus, plate, lip
- fossula-like depression: basal cavity, joint cavity, implantation cavity, insergion base, implantation socket, head recess
- nuclear membrane: surface membrane of nucleus, nuclear shell, nuclear sheath, cytoplasmic sheath, fibrillar network covering, net-work structure, microsome; Kopfhülle, gekreuzte Fibrille
- outer layer of nuclear membrane: outer nuclear membrane, outer membrane of nucleus
- inner layer of nuclear membrane: inner nuclear membrane, inner membrane of nucleus
- nucleus: sperm nucleus, chromosomes, karyoplasm; Nukleus, Kern
- vacuole: nuclear vacuole, opening
- neck: neck region, implantation region, centriolar region; Hals, Halsteil, Scheibe, Gelenkscheibe; collum
- mitochondrial sheath: cuff, band coil round neck, bread band coil round neck, coil-effect round fibril bundle, mitochondrial helix, mitochondria, mitochondrion, mitochondrial strand; Halsspirale, Gelenkkapsel
- large radix: main stem; Centrosomfäden
- medium radix
- small radix
- neck platelet: implantation plate, funnel shaped structure, osmophilic body
- centriole: proximal centriole, anterior end knob, anterior proximal centriole, neck granule, centrosome, head centriole, basal granule; proximales Centrankörperkorn, Artikulationsknopf der Fibrille, Kern, Basalkelch; centrosoma anterius, centrosoma posterius
- central radix: Zwischenmasse des Halses, Zwischensubstanz, Centrosom, doppeltes Knöpfchen; massa intermedia
- middle piece: middle-piece, mid-piece, midpiece, connecting piece, connecting body, body, main tract; Verbindungsstück, Mittelstück
- mitochondrial helix: mitochondrial thread, spiral sheath, mitochondrial spiral, spiral filament, thick coil, cytoplasmic wrapper, spiral remnant, splitting up into thinner thread,

- bead helix, double helix, spiral body, Jensen's spiral body, Golgi-body, mitochondrion; Spiralband, Spiralfaden, zartgewundene Spirale, Doppelspiral des Verbindungsstückes, äussere Scheide des Verbindungsstückes, Verbindungsstückspirale; filum spirale
- mitochondrial membrane
- outer layer of mitochondrial membrane: mitochondrial membrane, mitochondrial outer membrane, outer wall of mitochondrial sheath
- inner layer of mitochondrial membrane: lamella, mitochondrial inner membrane
- mitochondrial lamella: mitochondrial matrix, mitochondrial substance
- mitochondrial matrix: mitochondrial substance, mitochondrial material, Golgi-material; Zwischensubstanz; substantia intermedia
- Jensen's ring: Jensen's terminal ring, terminal ring, thick end ring, annules, distal centriole; Schlussring von JENSEN, distales Centriolkörperkorn, Schlussring, Zentralkörper, Jensen'scher Schlussring
- axial fibril bundle: axial fibre, axial bundle, axial core of filaments, axial filament, axial fiber bundle, fibrils, central filament, central fibril, articular strand, axial filament complex, fascicle; Axialfibrille, Subfibrille, axiales Filament, Protofibrille, Fibrille, Axenfaden
- outer ring of axial fibril bundle
- peripheral fibril: outer ring's fibril, peripheral coarse fibril, thinner fibril, fibril, fibre, intermediate fiber
- thicker peripheral fibril: main fiber, thicker fibril, large fiber
- inner ring of axial fibril bundle: axial ring of fibrils, filaments
- peripheral fine fibril: inner ring's fibril, thinner fibril, axial filament, filament, thin fiber
- central fine fibril: central pair filament, central fibril, central pair of fibrils; filament
- matrix: interconnecting substance, cortical plasm, cytoplasma
- tail piece: tail, tail-piece, main piece, main tail-piece, tailpiece, flagellum, tail proper, principal piece; Schwanz, Hauptstück, Geissel, Achsenfaden; pars principalis
- fibrillar coil sheath: tail sheath, coil sheath, cytoplasmic covering, cytoplasmic sheath, fibrillar sheath, cortical helix, microsome, periodical formation, fibrillar band, spiral sheath, fibrous helix; Spiralfibrille, Scheide, Spirale; involucreum
- fibrillar coil: spiral, spiral coil, helix, coil, tail helix
- longitudinal fibrillar tail strip: fibrillar band, ridge, membrane thickening, rib, longitudinal element
- end piece: tail end, tail-end, tail flagellum, terminal filament, terminal piece, terminal fibril, flagellum, protofibril, brush-like structure; Endstück, Spitze, Terminalstück, Endfibrille; pars terminalis

I MATERIALS AND METHODS

MATERIALS

Semen used in this investigation was obtained from 4 healthy Holstein bulls having normal fertility. Samples of semen were collected by artificial vagina and examined macroscopically and with light microscope. Except for frozen semen used in the preliminary experiment, each sample was kept in a refrigerator at 4°C until used within 24 hours.

METHODS

1) Suspension preparations

The suspension method was first employed in this study. The procedure is summarized in table 1.

TABLE 1 Schedule for suspension preparation

| METHOD | WASHING | | | | FIXATION* ³ (vapor) | WASHING | |
|--------|----------------------|------|-----------------------|-----------------|-----------------------------------|-----------|------|
| | Medium | Time | Centrifugation rpm | Duration min | | Medium | Time |
| S-1 | aq. dest. | 1 | 2000 | 5 | min hr 5~12 | — | — |
| S-2 | " | 2 | " | " | | — | — |
| S-3 | " | 5 | 3000 | " | | — | — |
| S-4 | saline | 2 | 2000 | 3 | min hr 10~12 | aq. dest. | 1~3 |
| S-5 | buffer* ¹ | " | " | " | | | |
| S-6 | P G S* ² | 2 | 2000 | 3 | min min 10~60 | | |
| S-7 | " | 3 | " | " | | | |

Notes: Materials are collected from Bull "WGL".

*¹ Sørensen's phosphate buffer solution (pH 6.89)

*² Mixture of one part of buffer, one part of 5% glucose solution and three parts of saline

*³ 2% osmium tetroxide solution

About 1 or 2 ml of raw semen was poured into a small centrifuge tube (diameter: ca. 1 cm, capacity: 10 ml) and centrifuged from 2 to 5 times. Two or four ml of one of the following mediums was added at equal temperature to semen: distilled water, physiological saline, phosphate buffer solution and P G S solution.

After washing, the same medium used in centrifuging was used to suspend the sperm at a suitable concentration (1~5 sperms per one square of the 200 mesh grid). One drop of the suspension was put on a collodion-coated grid dried by a chip of filter paper or in an incubator at 37°C, fixed with osmium tetroxide vapor, and finally shadowed with palladium.

When using saline or P G S solution in the processes mentioned above, crystals often appeared, and they were removed by repeated washing with distilled water after fixation.

2) Replica preparations

The same suspension mentioned above was used for the replica method. Several drops of the suspension were poured on a slide glass, dried at room temperature and fixed with osmium tetroxide vapor. Then 2% collodion solution was mounted quickly on the glass slide. After drying, the covering collodion was cut to the size of the grid. Then each collodion sheetlet was floated in distilled water, picked up on a grid, dried in an incubator and finally shadowed with palladium.

3) Section preparations

The procedures for section preparations are summarized in tables 2 and 3. Table 2 shows the method used in the preliminary experiment. Section preparations made from blocks in the preliminary experiment were used only in the cases where good microphotographs could not be obtained in the principal examination. However, the procedures outlined in tables 2 and 3 do not differ essentially. The advantages and disadvantages of the procedure in the preliminary experiment will be described later. In this chapter procedures for making blocks and section preparations in the principal examination shown in table 3, are stated.

Cells were washed twice in PGS solution, and centrifuged for three minutes at 2000 rpm except for the last centrifugation before embedding. After washing, sperms were homogeneously suspended in PGS solution and fixed by adding an equal volume of 2% buffered osmium tetroxide solution. Excess fixative was removed by washing with distilled water. Dehydration was accomplished using a graded ethyl-alcohol series. After dehydration, the material was placed in a 50:50 mixture of 100% ethyl-alcohol and monomer (8 parts of normal-butyl-methacrylate and 2 parts of methyl-methacrylate) for 60 minutes at 4 °C and then through two changes of pure monomer. The material was then placed in No. 00 gelatin capsules containing monomer with 2% benzol peroxide (the catalyzer). Polymerization of monomer was carried out at 37°C or 50°C for 24 hours. Sections were cut on a JEM-5 ultramicrotome using a glass knife. Each section was spread in hot water at 40°~60°C. Section was examined using a JEM-4 CHD electron microscope. Electron micrographs were taken at initial magnifications of 3,000~10,000 ×.

DISCUSSION

Since the suspension preparation was first employed in the electron microscopic study of the sperm in 1941, many procedures for this purpose have been reported. However, it is difficult to decide the most superior method, because each procedure has both merits and demerits.

Hitherto, in order to remove semen substances other than sperm from semen, washing method has been widely used and many washing mediums have been reported such as distilled water^{7,17,27,53,62)}, physiological saline^{47,53,98)}, Ringer's solution⁸¹⁾, Tween 80³⁾, Tyrode's solution⁹⁵⁾, phosphate buffer solution^{26,81)}, alcohol¹¹⁰⁾, etc. Among them, distilled water is most effective for this purpose, but it is not suitable for living of sperm. Likewise, other mediums are lack of substances to keep vitality of sperm. Especially, repeated washing by distilled

TABLE 2 Schedule for preparing block (preliminary experiment)

| BLOCK NO. | DATE | BULL NAME | WASHING | | FIXATION | WASHING | | DEHYDRATION | | | | | | MONOMER*2 | EMBEDDING | | |
|-----------|-----------|-----------|---------|------|------------|-----------|------|-------------------------|------|----|----|----|----|-----------|-----------|----------|----|
| | | | Medium | Time | (solution) | Medium | Time | Duration in Alc. (min) | | | | | | | Temp. | Duration | |
| | | | | | | | | % 50 70 90 95 99 100 | | | | | | | °C | hr | |
| E-1 | 1959-8-25 | WGL | buffer | 4 | 4hr | — | — | — | 12hr | 60 | 60 | 60 | 60 | 9 | : 1 | 37 | 16 |
| E-2 | 9-3 | " | " | 2 | 14hr | aq. dest. | 4 | — | 12hr | 60 | 60 | 60 | 60 | 9 | : 1 | 58 | 18 |
| E-3 | 1960-1-28 | KCG | PGS | 4 | 60min | " | 3 | — | 60 | — | 60 | 60 | 60 | 9 | : 1 | 45 | 24 |
| E-4 | 3-30 | RJA (F)*1 | " | 3 | 90min | " | 3 | — | 15 | — | 15 | 15 | 15 | 8.5 | : 2.0 | 46 | 10 |
| E-6 | 5-17 | PRM (F) | — | — | 60min | " | 3 | — | 15 | — | 15 | 15 | 15 | 8 | : 2 | 50 | 9 |
| E-10 | 11-15 | WGL | PGS | 3 | 60min | " | 1 | < 5 | 30 | — | 30 | 30 | 30 | 8 | : 2 | 50 | 12 |

Notes: *1 Frozen material *2 Ratio of normal-butyl-methacrylate to methyl-methacrylate

TABLE 3 Schedule for preparing block (principal experiment)

| BLOCK NO. | DATE | WASHING | | FIXATION | WASHING | | DEHYDRATION | | | | | | MONOMER | EMBEDDING | | | |
|-----------|-----------|---------|------|------------|-----------|------|------------------------|----|----|----|----|---|---------|-----------|----------|--|--|
| | | Medium | Time | (solution) | Medium | Time | Duration in Alc. (min) | | | | | | | Temp. | Duration | | |
| | | | | | | | % 50 70 95 99 100 | | | | | | | °C | hr | | |
| L-1 | 1960-1-13 | | | | | | | | | | | | | | | | |
| L-5 | 2-1 | PGS | 2 | 60min | | | | | | | | | | | | | |
| L-6 | 2-6 | | | | | | | | | | | | | | | | |
| L-7 | 2-10 | PGS | 2 | 15hr | aq. dest. | 1 | 5 | 30 | 30 | 30 | 60 | 8 | : 2 | 50 | 15 | | |
| L-9 | 2-20 | | | | | | | | | | | | | | | | |
| L-10 | 2-24 | PGS | 2 | 10min | | | | | | | | | | | | | |
| L-10' | 2-24 | — | — | 60min | | | | | | | | | | | | | |

Notes: cf. table 2 Materials are collected from Bull "WGL".

water or saline has a tendency to injure sperm.

The present author used in the processes of washing P G S solution, a special mixture containing neither protein nor lipid. Because even after repeated washing of 3 times (2000 rpm 3 minutes) by P G S solution, many sperms had their moving activity, the solution was considered as the most excellent one among many other mediums reported. This mixture is rather resemble in its composition to semen diluters used in the early stage of artificial insemination in the ox⁷⁴⁾. However, as the P G S solution is not always suitable for the washing technique in other animals' semen, other mediums must probably be prepared for these animals. Washing should be limited within 2 times, and also centrifugation should be minimized (2000 rpm 3 minutes). Further treatment should be avoided, so that sperms are not injured or killed.

If washing procedure is omitted before fixation, protein and other components of semen are also fixed, so this would be inconvenient to obtain a good photograph. At the same time, much more fixative would be needed and more repeated washing after fixation would be required to remove excess fixative.

Physical destruction of sperm by ultrasonic³⁾ or trituration⁸¹⁾ and also chemical resolution with digestive enzyme⁸¹⁾ or chemical drugs^{27,28,62,110)} are very convenient to analyse chemical components of sperm. However, these procedures are likely to produce various kinds of artifacts⁹⁵⁾ in morphological structures. For this reason, if these procedures are used in an electron microscopic study, control preparations without these treatments should be needed.

Junctions of each part of sperm structures are seemed to be not so fragile as was expected. This is suggested from the fact that in the preliminary experiment sperms treated by repeated washing and centrifugation of 7 times with P G S solution possessed almost entirely their fundamental constructions. It has been said that the sperm has a high sensibility for temperature shock, however, it has comparatively a high resistance to centrifugation. The result mentions above would support this conception.

A drop of suspension of sperms is located on a grid, and water component is removed with a small piece of filter paper. The distribution of cells is controlled by volume of diluter (P G S solution). In order to dry materials on the grid without giving any damage of sperm morphology, natural drying at room temperature with an aid of removing excess solution by a filter paper is superior than heating.

For making suspension preparations osmium tetroxide vapor has been used for fixation^{41,47,81,95,118)}. In the present author's experiment, no essential difference was observed in fixative effect between gas and solution of osmium tetroxide. The effect of fixation time in vapor fixation on sperm morphology was compared with from 3 minutes up to 24 hours. The results showed almost no difference,

except contraction of sperm was comparatively severe in the preparations of long time fixation. Through sperm without fixation showed a slight shrinkage by electron beam, such shrinkage was not observed in samples fixed with osmium tetroxide gas in about 10 minutes. Thus, about 10 minutes fixation with osmium tetroxide gas (using 2% solution), seem to be advisable. If there appears any debris or crystal on the grid after fixation, washing with distilled water is recommended to remove them.

A collodion supporting film devised by SEYMOUR & BENMOSCHE was used in the present experiment. This collodion film is convenient to adjust, despite of a rather fragility against electron beam. Carbon reinforcement on a collodion film is also suitable for preparing suspension materials. On the other hand, a formvar film which is widely used because of toughness should not be recommendable, since it is disadvantageous in respect of difficulty in adjustment or control of the thickness.

The shadowing technique was first reported by WILLIAMS & WYCKOFF, and then its modification has been employed both in studies of surface and cubic structures. In the present author's experiment palladion was employed for this purpose. As the shadowing apparatus used in this work had no protractor, the thickness of the material could not be measured. However, shadowing was highly effective to make a sharp contrast in photographs and to increase toughness of the supporting film. As for the study by means of the replica method on domestic animals' sperms, there is only one report of KESSLER, but this method is very useful for studying surface structures of materials. It is convenient to use a glass knife to cut collodion film on a slide glass, and also it is very advantageous to put several sperms on the replica, so that a comparative study is possible between a sperm and its replica.

Ultramicrotomy of free cells is rather cumbersome than that of tissue cells⁷⁸⁾, especially in the process for preparing block. Since the success of ultramicrotomy of bacteria by HIGASHI (1956), this technique has been widely used in the electron microscopic study of free cells and the details of the procedure has been developed; for example, the double embedding method with agar devised. However, in spite of convenience in collecting samples, ultramicrotomy is scarcely employed in the study of sperms, excepting ÅNBERG and SCHULTZ-LARSEN on the human sperm, BRADFIELD and NICANDER & BANE on the domestic animals' sperm, and BLOM & BIRCH-ANDERSEN¹⁴⁾, and RAHLMANN on the bull sperm. The reasons may be due to some specific characters of the sperm, that is, the sperm floats in seminal fluid as a free cell, having a motile activity and a comparatively weak resistance against various conditions *in vitro*.

In the preliminary experiment, about 40 blocks of the bull sperm were made

by various methods and the most suitable method for preparing blocks of the bull sperm was decided. An adequate volume of raw semen for preparing blocks was about 1 ml; a smaller volume was disadvantageous because of decrease in number of sperms in a block, likewise, a larger volume was also inadequate because of a tendency of insufficiency in the process of preparing blocks. Making 2 to 4 blocks from about 1 ml of raw semen is most recommendable.

As described above, washing should be carefully treated, and the washing medium and the material should be actually regulated at an equal temperature.

Up to now, osmium tetroxide has been accepted as an excellent fixative for the electron microscopic study⁷⁸⁾, and there are many reports about this fixative^{54,77,79)}, including a review of PORTER & KALLMAN. In the preliminary experiment, the present author has tried other fixatives, such as 10% formaline solution and 2% potassium permanganate solution, but the results were not so good. Two per cent osmium tetroxide solution was always used in the principal experiment. Its final concentration was regulated at 1 per cent by adding an equal volume of medium including sperms. To adjust the pH, PALADE recommended the neutral Michaelis' buffer (pH 7.2~7.4) for tissue materials, however, the present author used Sørensen's phosphate buffer (pH 6.89) contained in the PGS solution.

About fixation time, previous reports recommend 5~10 minutes³⁾ or 4 hours⁹⁵⁾ in sperm materials and 12~24 hours⁵⁴⁾ in bacterial suspensions. In the present work fixation time was varied in 5, 10, 30 minutes, 1, 2, 4 and 24 hours. Among them, one hour fixation gave the best constant result. As it is reported that the water content of the sperm is about 50 per cent⁵⁾, sperms treated by fixation may not reveal the morphological features as same as alive, that is, shrinkage may be unavoidable. Therefore, a comparative study is desirable between materials with a long time fixation and with a short time treatment, if possible. In addition, to examine fine structures of the sperm, especially to count numbers of coarse or fine fibrils which constitute the sperm tail, an artificial destruction by a long time fixation may also be valuable. The temperature during fixation does not seem to give an unfavorable influence, unless the fixation time is so long⁸⁰⁾.

As for procedures for dehydration of tissue materials, some researchers recommend the method in which the materials are put into 70% alcohol after fixation and without washing^{78,116)}. HIGASHI also recommends this method for free cell materials. However, as this method did not give good results for sperm materials in the present author's experiment, a gradual dehydration method with an ascendant series of 50, 70, 95, 99 and 100% of ethyl-alcohol were used after fixation and washing with distilled water. The times for hydration were kept in every 30 minutes in each concentration of alcohol, except 60 minutes in 100%. The present author's method has scarcely concern with making artifacts.

For expecting constant polymerization, a procedure for removing hydroquinone out of n-butyl-methacrylate to be used is always needed, even in the case where some stabilizer is not added in the methacrylate. Substitution and permeation processes must be carried out at low temperature (4°C), as polymerization of monomer proceeds gradually at room temperature⁷⁸⁾. The mix ratio of buthyl- and methyl-methacrylate is important; the ratio higher the embedded block softer. A mixture of 8 parts of n-butyl-methacrylate and 2 parts of methyl-methacrylate will be recommended for sperm materials. If one wants long time storage of a block, the part of n-butyl-methacrylate should be increased, because the monomer is not yet completed in polymerization under the condition of 50°C, 15 hours, and then the block will become gradually hard at room temperature.

Though some workers who tried to make a section of sperm reported that it had a comparative long time for permeation^{54,95)}, ÅNBERG treated the human sperm 3 times only 5 minutes in each. The results of the present work showed that monomer should be changed more than 2 times every 60~90 minutes. If these times are prolonged or shortened, collapse or rupture with various degrees tended to occur at the portions of the head cap and the middle piece.

During these processes mentioned above, several times of centrifugation should be done under controlled temperature. For this purpose use of a cooling centrifuge is ideal. If one wants to avoid centrifugation, material has to be kept for a long time in monomer⁹⁵⁾, and this may be unsuitable to obtain good results. As strong centrifugation may injure materials³⁾, a procedure of centrifugation should be done carefully. In order to obtain most suitable sperm suspension for making a block, centrifugation should be limited at 2000 rpm during 3 minutes. BLOM & BIRCH-ANDERSEN¹⁴⁾ tried a double embedding method with agar by only once collecting centrifugation of 2500 rpm during 20~40 minutes. The present author also reexamined this method several times in the preliminary experiment, but the results were unsatisfactory. The reason may be due to the facts that spermatozoa suffer from a strong temperature shock at the melting point of agar and that during every process of fixation, dehydration and permeation, fine structures of spermatozoa embedded in the central portion of an agar pellet are easily damageable. From these points of view, the repeat centrifugation method which was chosen by the present author is superior to the agar method, as is undoubtedly proven from the fact that the cell membrane of the sperm is completely maintained. The methacrylate embedding method was at first introduced by NEWMAN et al. into biological and medical fields. This acryl-monomer can be most easily handled among various embedding substances. Recently, however, it is pointed out that methacrylate has some defects as an embedding substance, such as incomplete polymerization and irregular adhesion to material, etc. To cover these weak points

of methacrylate, other embedding substances, epoxy resin or araldite were introduced by GLAUERT & GLAUERT and KUSHIDA, isolatedly. But, as this substance is too stiff to make a proper sperm suspension, it could not be used in isolate material, except a double embedding method.

When monomer is directly in contact with air in a capsule, the speed of polymerization is delayed and formation of bubbles tends to occur. In order to prevent such trouble, air should entirely be excluded from the inside of the capsule by filling it with monomer and be sealing it with painting adhesive. For the purpose of saving material, pointing the bottom of capsule⁵⁰⁾ should not be recommended because of tendency to crush the capsule or form bubbles. As for the last centrifugation, the preliminary investigation showed that treatment with 3000 rpm 10 minutes is most suitable for a good distribution of spermatozoa in a capsule. Further strong centrifugation^{3,14)} is in danger of deforming the original morphologic character of spermatozoa. The condition of polymerization used in the present investigation (50°C, 15 hours) is based on those of forerunners^{54,95)} and of the present author's experience. Recently, ultraviolet rays were used by WEINREB to accelerate polymerization, and good results were obtained. In the present study, the same results are obtained with ultraviolet rays as heating on polymerization.

Distribution of sperm cells in a block is of three dimensions, their directions and forms showing a great variety. It is almost impossible to control their arrangement or directions in a block. When a block was sectioned in its bottom portion, most of the sectioned preparations tended to show lateral views of sperm cells, and appearance of plane surface views was very rare. Likewise, in the case in which a cutting direction against a block was turned by 90 degrees, this tendency scarcely varied. Eventually, expectation to obtain a plane surface view of a sperm cell may be said to be an only chance problem, because, up to now, none have reached the way how to control arrangement of sperms in embedding processes and also how to make section preparations from desirable portions of a sperm cell in a course of ultramicrotomic technique. Recently, GIBBONS discussed about situation of a spermatozoon in a block, but he did not illustrated any flatten-viewed figure in his paper. For this purpose, a micro-manipulator may be possible to help the technique for arrangement of sperms.

Each block was sectioned with a glass knife introduced by LATTA & HARTMANN. To make to adhere a glass knife with a metal trough, dental wax was very convenient. As a medium to fill a metal trough and to float a section, acetone, alcohol and chloroform solutions have been reported. However, these solutions tended to melt the embedding substance and to lose the fitted meniscus by vaporization. In the present work, distilled water was always used and it gave

constant results. In the case of sectioning of free cells, creasing or folding means a decisive damage, so, in the present work, extension was carried out with hot water (50°~60°C). But the extension method is not recommendable in the case of serial sectioning, because the order of sections is easily confused by the convection of hot water.

Through the present work, the sizes of every part of the spermatozoon were not measured, because various factors, such as unevenness of a supporting grid and instability of voltage may have a great effect on the magnification of specimens. When a size is discussed on Å level, such figures seem to be almost no value. Much more work will be required before these problems can be explained.