MORPHOLOGICAL STUDIES ON THE SEXUAL MATURATION IN THE MALE JAPANESE QUAIL (COTURNIX COTURNIX JAPONICA) II. THE GERM CELL TYPES AND CELLULAR ASSOCIATIONS DURING SPERMATOGENESIS

MORPHOLOGICAL STUDIES ON THE SEXUAL MATURATION IN THE MALE JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*)

II. THE GERM CELL TYPES AND CELLULAR ASSOCIATIONS DURING SPERMATOGENESIS

By

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(*Received, July 1, 1967*)

Introduction

It must be necessary to acquire a thorough knowledge of the normal process of the spermatogensis regarding the testicular function. In this respect, most mammalian species including man were repeatedly investigated by many workers since Roosen-Runge and Giesel (1) restudied the spermatogenesis of the albino rat. The many detailed investigations on mammalian species were recently reviewed by Leblond et al. (2). There was, however, no investigation on avian spermatogenesis, except that of Cavazos and Melampy (3) who investigated the spermiogenesis of the rooster. Nor such study was present on the Japanese quail, the avian species in which the authors were specially interested.

In most mammalian species, the various generations of germ cells make up a limited number of cellular associations (1,4-8). This characteristic arrangement of the germ cells gives rise to the so-called cycle of the seminiferous epithelium. Each cellular association was considered as the stage of the cycle. The associations were classified and identified by the two principal cytological criteria (6, 9). The normal process of the spermatogenesis was easily investigated using these stages as markers.

The histological appearance of the seminiferous epithelium in the Japanese quail (13) as well as in man (10, 11) suggested the irregular arrangement of the germ cells which was called as the heterogenous cellular association (11). The regular arrangement of the germ cells described in most mammalian species was never seen in the Japanese quail. Namely, the various regions of the seminiferous tubules in this bird showed variable cellular associations, and consequently the cycle of the seminiferous epithelium was not easily distinguishable (Fig. 1). Yama-

moto (13) investigated the sexual maturation of the male Japanese quail and described preliminarily the cytology of the germ cells in the seminiferous epithelium, but the stages of the cycle was not established.

The present study was designed to clarify thoroughly the classification of the various cell types of the seminiferous tubules and to identify the stages of the cycle of the seminiferous epithelium in the quail as the second step of the studies on the sexual maturation of the male Japanese quail (*Coturnix coturnix japonica*). In the present study, the cellular associations were identified by the method proposed by Curtis (9) and adapted by Roosen-Runge and Giesel (1) and by Ortavant (12).

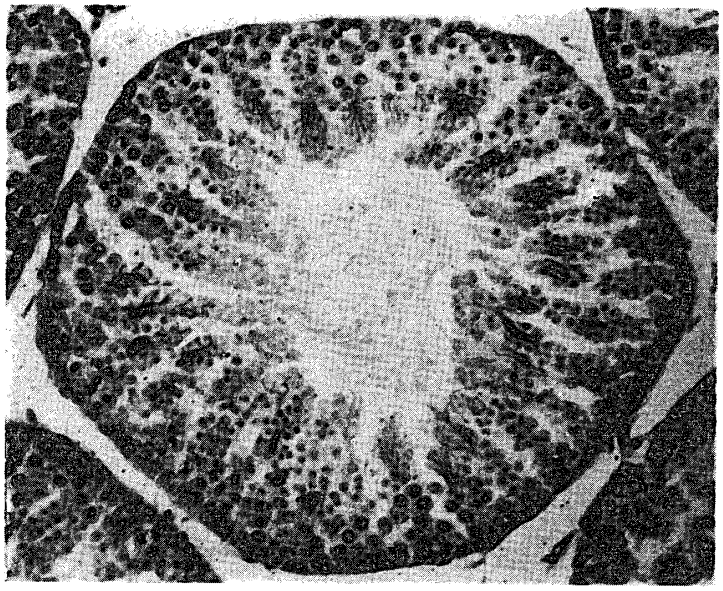


Fig. 1. Cross section of a Japanese quail seminiferous tubule stained with PAS-hematoxylin. ×80.
The cross section showed the various cellular associations. In most mammals, the seminiferous epithelium may consist of any one of limited number of cellular associations.

Materials and Methods

Testes used for the cytological study were obtained from the completely matured male Japanese quails. The tissues were fixed in Zenkerformol for 24 hours and embedded in paraffin with the routine procedure. The sections were cut at 5μ and stained with PAS-hematoxylin and Heidenhain's hematoxylin. The sections stained with Feulgen reaction were also used for identifying the spermatogonia and spermatocytes. They were observed under light microscope and the sizes of the nuclei and cytoplasm were measured with an ordinary micrometer.

Results and Discussion

I. Description of the germ cell types.

1. Spermatogonia — Three types of the spermatogonia were found along the basement membrane. The one type had an ovoid or elongated nuclei containing fine chromatin granules and three to four large particles which were lightly stained by hematoxylin. The size of their nuclei varied from 11 to 15μ in longer diameter. The other type had slightly elongated and smaller nuclei containing a large flaskes of coarse chromatins which were adherent to the nuclear membrane. The size of the nucleus of this type was about 10μ in longer diameter. The cells with ovoid or slightly elongated nucleus were identified as type A and type B spermatogonia, respectively. The another type was a group of spermatogonia which had larger nucleus than those of the type B spermatogonia. The nucleus of this cell had coarser chromatins than those of the type A spermatogonia. They were identified as intermediate spermatogonia (Fig. 2).

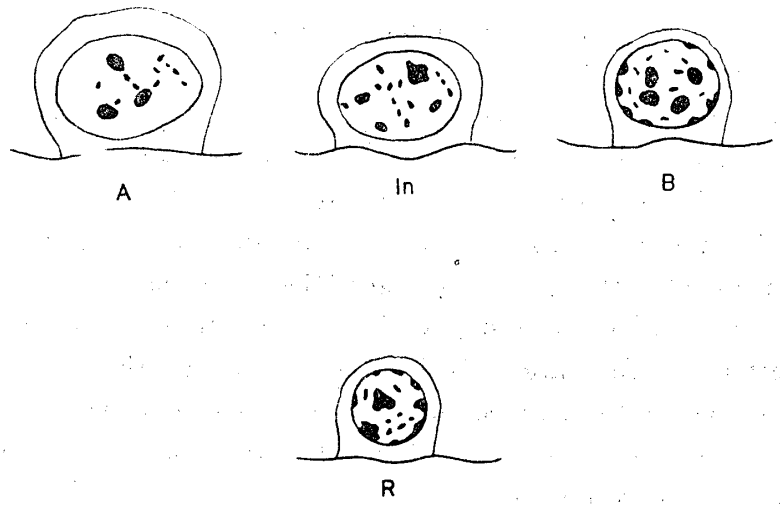


Fig. 2. Types of spermatogonia and resting primary spermatocytes.
The density and granuality of the chromatin and the size of nuclei make it possible to characterize type A (A), type B (B) and intermediate (In) spermatogonia and resting primary spermatocytes (R).

2. Primary spermatocytes — A cell type, which had a much smaller and spherical nucleus than those of the type B spermatogonia, was found along the basement membrane. Its darker nucleus contained more granular chromatin and in this respect was clearly distinguished from the type B spermatogonia (Fig. 2). In mammalian species, such cell type was classified as the resting or preleptotene primary spermatocytes (2, 15, 16). The identification of the resting primary spermatocytes of mammals could be easily done by the application of the definite cellular associations of the seminiferous epithelium (2,4-8). As reported by one of the present authors (13), the pattern of the cellular associations in the

Japanese quail was so variable that the precice identification of this cell type by the same technique was not possible in this study.

The remaining primary spermatocytes were classified into younger (smaller) and older (larger) types. The younger type consisted of the leptotene and zygotene primary spermatocytes. They were characterized by the somewhat increased cell bodies and chromatin condensation. The chromatin, in the form of thin coils and beaded filaments, were located excentrically in the nuclei. These primary spermatocytes were usually found at the second layer of the germ cell associations.

The older type, the pachytene primary spermatocytes, could be divided into the early and late steps. The early step involved larger cells than the leptotene and zygontene cells, and its nuclei contained thicker and tortuous chromosomes. The late step contained the largest cell type in the germ cell series. Its nuclei were the largest, containing shortened and thickened chromosomes. The stages of diplotene and diakinesis together with the metaphase of the first maturation division (the large division) were also included in this group. The older pachytene primary spermatocytes were found in the third and fourth layer of the germinal epithelium.

3. Secondary spermatocytes — The secondary spermatocytes contained nuclei which were much smaller than those of the preceeding pachytene primary spermatocytes. The nucleus had clumped chromatins with heavily stained nuclear membrane. Usually the cells were found adjacent to the largest type of the primary spermatocytes, though the number of them was relatively few. They were also rather similar to the early stages of the spermatids, but were differentiated from the latter by the larger nuclei. Theoretically, this stage involved the secondary division, of the maturation division. The metaphase of the division, small division, was only occasionally observed in this study, probably due to the unsychronized and rapid process of this step.

4. Spermiogenesis — As reported by Yamamoto (13), the process of the spermiogenesis was only indirectly applicable to the identification of the germ cell types in the Japanese quail. This, in turn, made it difficult to determine the various steps of the spermiogenesis in this bird. However, the four main steps of the spermiogenesis described by many workers (3, 7, 14, 15) in the PAS-stained sections in the mammalian testis were identified in the Japanese quail, as seen in Section II.

The present results were not comparable with those obtained in other birds, because as far as we know, there was no report on the cytology of the germ cells except that of spermatids by Cavazos and Melampy (3). It was noted that the spermatogonia were lighter and rounder than those in mammalian species (2, 5, 11, 12, 15, 16). However, whether this was characteristic, in general, to avian species or not, was not determined in this study. The primary spermatocytes and

secondary spermatocytes in the Japanese quail showed the same general appearance as those in mammals. Namely, the excentric location of chromatins in the leptotene and zygotene primary spermatocytes were commonly observed in mammalian species (2, 7, 4, 8, 12, 16).

II. Steps of spermiogenesis.

The spermiogenesis in the Japanese quail as revealed by PAS technique generally appeared similar to that in the rooster reported by Cavazos and Melampy (3). It was divided into four main phases, Golgi phase, Head cap phase, Acrosome phase, and Maturation phase. The four phases were recognized as follows: In the Golgi phase, the Golgi zone of the newly formed spermatids elaborates the proacrosomic granules, from which a single acrosomic granule arises. In the Head cap phase, the acrosomic granule becomes elongated to form the acrosomic system with the head cap and attached to the cranial part of the nucleus. In the Acrosome phase, the acrosome becomes conical and nucleus is flattened and takes a discoid shape. In the Maturation phase, the spermatids complete their morphological evolution to become spermatozoa.

The aforementioned phases of the spermiogenesis could be further divided into 12 subdivisions, mainly based on the development of the acrosomic system and the change of nucleus of the spermatids (Fig. 3). The Golgi phase includes steps 1 to 3, the Head cap phase, steps 4 to 6, the Acrosome phase, steps 7 to 9, and the Maturation phase, steps 10 to 12. They were preliminarily reported in the previous paper (13). Steps 2 and 3 in the previous paper were included in step 2 in this study, because of the difficulty to distinguish these two steps. In the rodent (2,4-8), steps 1 to 9, excepting the Maturation phase, usually were included in the cellular associations. These steps, however, do not always correspond to the stages in the Japanese quail.

Step 1. The newly formed spermatid by the second maturation division of the secondary spermatocytes. No acrosomic granules positive to PAS staining are observed.

Step 2. The acrosomic granule appears in the cytoplasm somewhat apart from the nucleus and moves towards the nucleus.

Step 3. The acrosomic granule is now attached to the surface of the nucleus and increases in size.

Step 4. The acrosomic granule becomes flattened on the surface of the nucleus and the condensation of the nucleus begins, but the head cap does not develop as well as in the rooster (3).

Step 5. The condensation occupies nearly half of the whole nucleus and flattening of the nucleus begins.

Step 6. The condensation occupies the whole nucleus and the nucleus takes the shape from the spherical to ovoid. The cytoplasm remains round in shape.

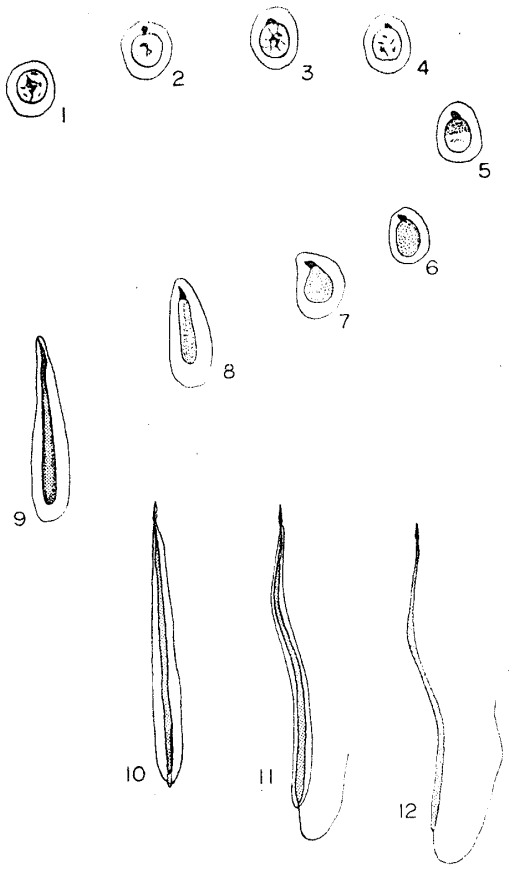


Fig. 3. Spermiogenesis of the Japanese quail as revealed with PAS staining.
Steps. 1–3 Golgi phase Steps. 4– 6 Head cap phase
Steps. 7–9 Acrosome phase Steps. 10–12 Maturation phase

Step 7. The nucleus takes the cylinder-like shape as the result of the elongation. The acrosomic granule becomes oriented in the direction of the basement membrane on the seminiferous tubules. The cytoplasm still remains round in shape.

Step 8. As the result of the advanced elongation of the nucleus the spermatids become rod-like with comcomitant elongation of the cytoplasm. The acrosomic granule of the spermatids becomes in contact with the cytoplasmic membrane.

Step 9. Further elongation of the nucleus and the separation or sloughing of the cytoplasm of the spermatid is noted.

Step 10. A bundle of spermatids with completely flattened and discoid nucleus is seen attached to the cytoplasmic extention of the Sertoli cells, and located in the deeper stratum of the seminiferous epithelium among younger generations of the spermatids.

Step 11. The fibrilar spermatids more or less retract towards the lumen of the seminiferous tubules from the Sertoli cells.

Step 12. The spermatids, now morphologically a mature type of spermatozoa, occasionally lined along the luminal surface of the tubules, are released to the lumen.

The results mentioned above confirmed the findings in the previous study (13). They also agreed with that of Cavazos and Melampy (3) who reported that the spermatids did not form the typical head cap and that the nuclei of the spermatozoa became strikingly elongated in the rooster. However, neither idiosome nor proacrosomic granules were seen in the Golgi phase spermatid in the Japanese quail. In this respect, this phase of spermatid was cytologically different from those in the rooster and also in most mammalian species. The reason why idiosome or proacrosomic granules were not demonstrable in the Japanese quail was not determined in this study. This problem demanded further investigation.

III. The cellular associations of the seminiferous epithelium.

The cellular associations as the expressions of the so-called spermatogenic cycle have been classified by the following two principal methods. The one, proposed by Curtis (9) and adapted by Roosen-Runge and Giesel (1) and by Ortavant (12), was based on the meiotic divisions, variations in shape of spermatid nucleus and the release of spermatozoa into the lumen of the seminiferous tubules. The other one, proposed by the Canadian workers (2, 7, 14, 16), was based on the development of the acrosomic system in PAS stained section (4-7, 10). The former method was employed in this study.

In the Japanese, quail, the cellular associations of the seminiferous epithelium was quite variable. For instance, the associations in a given cross section of one tubule never appeared uniform, and any two adjacent cellular groups in one locus of the tubule frequently appeared dissimilar.

The following eight cellular associations of the seminiferous epithelium in the Japanese quail were classified basically on the oldest generations of the germ cells (Table 1 and Figs. 4–11).

Stage 1. The mature spermatozoa are completely released from the seminiferous epithelium, and the bundle formation of the oldest Acrosome phase spermatids begins.

Stage 2. The definite bundle formation of the younger Mature phase spermatids is noted. The second generation contains the oldest Golgi phase spermatids. The larger type of the pach tene primary spermatocytes are commonly seen in this stage.

Stage 3. The beginning of the dusty appearance of the spermatids nucleus (early Head cap phase) with regular bundles of the Mature phase spermatozoa is noted. The smaller type of the pach tene primary spermatocytes are also common.

Stage 4. The half darkening and beginning of elongation of the Head cap phase spermatid is noted. The development of tail in the immature spermatozoa is also noted. The first meiotic division of the full-grown primary spermatocytes is common.

Stage 5. The definitely ovoid nucleus of the older Head cap phase spermatid,

Table 1. The 8 stages of the cycle of the seminiferous epithelium in the Japanese quail

Stage of cycle	1	2	3	4	5	6	7	8
The arrangement of the germ cells	M_1	M_2	M_2	M_2	M_2	M_3	M_3	M_3
	G_2 G_3	G_3	H_1	H_2	H_3	AC_1	AC_2	AC_3
	P_2	P_2	II P_2 G_1	II P_2 G_1	II (P_2) G_1	II (P_2) G_1	G_1 (II) G_2	G_1 (II) G_2
	(P_1) Z	(P_1) Z	P_1	P_1	P_1	P_1	P_2	P_2
	B (R)	(B) R	(R) L	L (Z)	L (Z)	L (Z)	Z (P_1)	Z (P_1)
	A	A	A	In B	In B	(In) B	B (In)	B (In)
	A	A	A	A	A	A	A	A

Abbreviation: A, type A spermatogonia; In, intermediate type spermatogonia; B, type B spermatogonia; R, resting primary spermatocytes; L, leptotene primary spermatocytes; Z, zygotene primary spermatocytes; P_1, early pachytene primary spermatocytes; P_2, late pachytene primary spermatocytes; II, secondary spermatocytes; G_1, early Golgi phase spermatids; G_2, mid Golgi phase spermatids; G_3, late Golgi phase spermatids; H_1, early Head cap phase spermatids; H_2, mid Head cap phase spermatids; H_3, late Head cap phase spermatids; Ac_1, early Acrosome phase spermatids; Ac_2, mid Acrosome phase spermatids; Ac_3, late Acrosome phase spermatids; M_1, early Maturation phase spermatids; M_2, immature spermatozoa; M_3, mature spermatozoa;

Note: (P_2) included first meiotic division.
(II) included secondary meiotic division.

with almost mature spermatozoa as bundles. The secondary spermatocytes may be often seen.

Stage 6. The spermatids with the cylinder-like nuclei of the Acrosome phase is noted. The almost mature spermatozoa are located in the deeper stratum.

Stage 7. The beginning of the release of the mature spermatozoa into the tubular lumen. The extensive elongation of the cell bodies of the Acrosome phase spermatids is noted.

Stage 8. The mature spermatozoa are released into the lumen of the seminiferous tubules. A few secondary meiotic division is still seen. The older fully elongated spermatids of the Acrosome phase start to form loose bundles, their nuclei move towards the basement membrane of the tubules.

It appeared that in the Japanese quail as well as in mammalian species there was a definite cycle of the seminiferous epithelium, though the cellular associations described in the Japanese quail were not so clear-cut as those in the mammalian species (2,4-8,10,12). It is still not certain that the characteristic heterogenous associations observed in the Japanese quail were common in the avian species. The problem should be clarified by investigating the other avian species.

IV. The spermatogonia in the various stages of the seminiferous epithelium.

Generally the size of the type A spermatogonia is smaller in Stages 1, 2, and 3. and the largest in Stage 8. Type B spermatogonia is present at all stages but very few in Stage 3. An intermediate type spermatogonia is noted in Stages 5 and 8. The above data suggested that, generally, the transition of type A spermatogonia into intermediate type spermatogonia is likely to occur between Stages 4 and 5, and that of the type B spermatogonia into resting primary spermatocytes in Stages 8 and 1, because the latter type of the germ cell is present in these two stages. The resting primary spermatocytes probably transform into the leptotene primary spermatocytes in Stage 3.

It appeared that the very regular periodic division of spermatogonia existed in the rodent (4–7, 15, 17) and also in the man (10, 18). Namely, type A spermatogonia were present in all stages in mammals and type B spermatogonia in the limited stages successive to the stages in which the intermediate type spermatogonia were present. In the man (10, 18), type Ap and type Ad, corresponding to the types A and intermediate spermatogonia in the Japanese quail respectively, were present in all stages, but type B spermatogonia were present in only stages 1 and 2. In this respect, man is not fundamentally different from the other mammalian species, as mentioned by Clermont (10). However, in the Japanese quail, type B spermatogonia were present in almost all stages. This suggested a possibility of the differentiation of type B spermatogonia into primary spermatocytes at different times and in small groups as stated by Roosen-Runge (17), resulting in the heterogenous associations of the germ cells. It is likely that the spermatogenseis in the Japanese quail were fundamentally different from that of mammalian species, even from that in the man.

V. Variation in the cellular components of the eight stages.

The above description of the cellular associations in the germinal epithelium of the Japanese quail, and the schematic illustration of them in Table 1 should be regarded as averages. While the identification of these stages was done by the oldest two generations of the spermatids (only one generation in Stages 1 and 2), the cellular components of the successive generations were quite variable. For instance, they included the cells of first meiotic division, secondary spermatocytes and the younger type of the Golgi phase spermatids in Stage 4, and various ages of the Golgi phase spermatids and the younger type of the Head cap phase spermatids in Stage 2.

In the rodents, the spermatogenic cycle of the seminiferous epitheliun was strikingly clear cut due to the strictly fixed cellular components present in the epithelium. In the man, however, Roosen-Runge and Barlow (11) and Roosen-Runge (17) could not identify the exact stages of the spermatogenic cycle. They

suggested that, in the man, the heterogenous associations of the germ cells were produced by the differentiation of type B spermatogonia into primary spermatocytes at different time and in small groups (17). In spite of presence of the irregularity in the man, Clermont (10) reported recently that there is a clearcut spermatogenic cycle. The author stated that man is not fundamentally different from other mammals in opposition to Roosen-Runge.

In the Japanese quail, it was noted that the germ cells formed as many as six layers in the seminiferous epithelium, and that when the uppermost two generations were taken as markers of the associations, the lower generations extremely varied. This could not be explained by the hypothesis of Roosen-Runge (17), because in the case of the Japanese quail, the irregularity were seen only in one given layer. Together with the temporal unsynchronicity of the spermatogensis, it seemed that there was a spatial factors characteristic to the Japanese quail in comparison with rodents. In the former, the cross section of the seminiferous tubules were less frequently observed than the longitudinal sections. Therefore, in the quail, the irregularity appeared to result from the lack of the spatial factors as well as temporal ones regulating the spermatogenesis. The present authors agree with Roosen-Runge (17), who stated that a well-regulated pattern of spermtogenesis is theoretically not necessary for a continuous sperm supply.

Summary

The normal process of the spermatognesis in the nature Japanese quail was investigated with the PAS-hematoxylin or Feulgen stained sections of Zneker-formol fixed testes, as the second step of the studies on the sexual maturation in the male Japanese quail (*Coturnix coturnix japonica*).

The germ cell types and the cellular associations were described in detail. Spermatogonia were divided into three types, type A, intermediate, and type B. Primary spermatocytes were divided into resting, leptotene, zygotene and pachytene phases. The pachytene primary spermatocytes were subdivided into early and late. The diplotene and diakinesis of the first meiotic division were not identified, though secondary spermatocytes were so. The steps of the spermiogenesis were divided into the four phases, Golgi phase, Head cap phase, Acrosome phase, and Maturation phase. These were divided into 12 subdivisions.

The eight cellular associations were described on the basis of the meiotic division, variation in the shape of the nucleus of the spermatids and the release of the spermatozoa into the tubular lumen. In the Japanese quail, the cellular associations were quite variable, so that the several cellular associations could be seen in a given cross-section of the tubules. The heterogeneous pattern of the cellular associations in this bird, as compared with the strictly regulated ones in mammals, was discussed briefly.

Acknowledgements

The authors wish to express sincere thanks to Dr. Ikuo Ushigoshi for his help and encouragement during this work, and to Mr. Sadamitsu Yoneya and Mr. Takashi Sasahara for their technical assistance.

References

1) Roosen-Runge, E.C. and Y. Giesel, Jr. (1950). Am. J. Anat., **87**, 1.
2) Leblond, C.P., E. Steinberger and E.C. Roosen-Runge (1963). Mechanisms Concerned with Conception. p. 1, Pergamon Press, N.Y.
3) Cavazos, L.F. and R.M. Melampy (1954). Am. J. Anat., **95**, 467.
4) Clermont, Y. (1960). Fertil. and Steril., **11**, 563.
5) Leblond, C.P. and Y. Clermont (1952). Am. J. Anat., **90**, 167.
6) Leblond, C.P. and Y. Clermont (1952). Ann. N.Y. Acad. Sci., **55**, 548.
7) Perey, B., Y. Clermont and C.P. Leblond (1961). Am. J. Anat., **108**, 47.
8) Oakberg, E.F. (1956). Am. J. Anat., **99**, 507.
9) Curtis, G.M. (1918). Am. J. Anat., **24**, 339. (Cited from Ortavant)
10) Clermont, Y. (1963). Am. J. Anat., **112**, 35.
11) Roosen-Runge, E.C. and F.D. Barlow (1953). Am. J. Anat., **93**, 170.
12) Ortavant, R. (1959). Reproduction in Domestic Animals. p. 1, Acad. Press, N.Y.
13) Yamamoto, S. (1964). Tohoku J. Agr. Res., **14**, 241.
14) Clermont, Y. and C.P. Leblond (1955). Am. J. Anat., **96**, 229.
15) Oakberg, E.F. (1956). Am. J. Anat., **99**, 391.
16) Clermont, Y. and C.P. Leblond (1959). Am. J. Anat., **104**, 237.
17) Roosen-Runge, E.C. (1952). Ann. N.Y. Acad. Sci., **55**, 574.
18) Clermont, Y. (1966). Am. J. Anat., **118**, 509.

Plate I

Explanation of the Figures

The seminiferous epithelium of the Japanese quail at different stages of the cycle. All microphotographs were taken from the PAS-hematoxylin staining preparation.

Fig. 4. Stage I: A bundle of immature spermatozoa in the bottom. Spermatid nuclei are round with no acrosomic granule (early stage of Golgi phase). ×1200.

Fig. 5. Stage II: Immature spermatozoa toward the basement membrane. Spermatid nuclei are still round (mid or late stage of Golgi phase). ×1200.

Fig. 6. Stage III: Immature spermatozoa penetrate to the basement membrane. Spermatid nuclei are beginning to darken (early stage of head cap phase). ×1200.

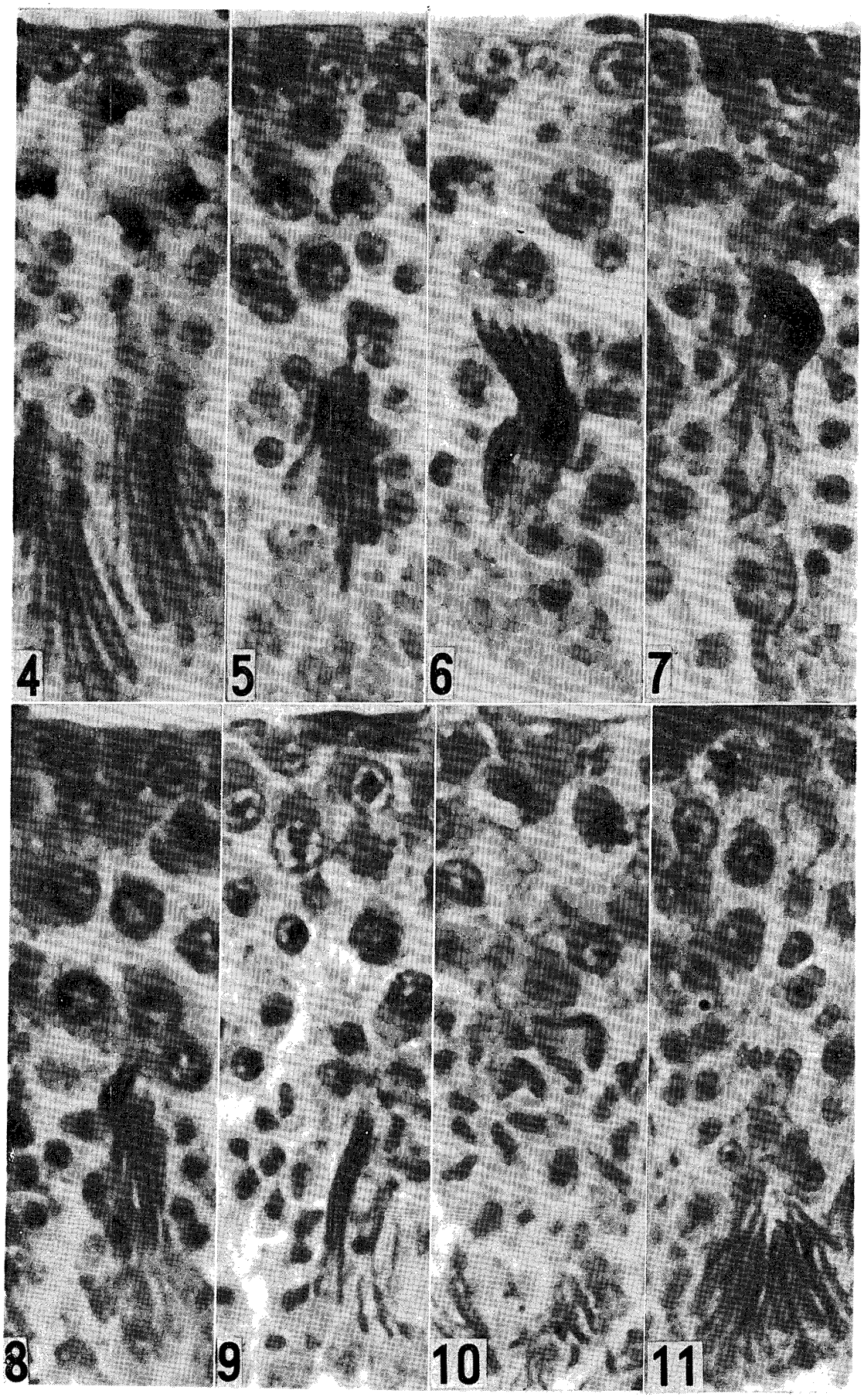
Fig. 7. Stage IV: Nearly mature spermatozoa bundle deep in the semiferous epithelium. Spermatids are mid stage of head cap phase. ×1200.

Fig. 8. Stage V: Spermatozoa toward the lumen. Spermatid nuclei are completely darkened (late stage of head cap phase). ×1200.

Fig. 9. Stage VI: Few spermatozoa present in the bottom. Spermatid nuclei are beginning to elongate (early stage of acrosome phase). ×1200.

Fig. 10. Stage VII: Spermatozoa are released into the lumen. Spermatid nuclei are rod-like (mid stage of acrosome phase). ×1200.

Fig. 11. Stage VIII: No spermatozoa present. Elongated spermatids (late stage of acrosome phase) are beginning to bundle. ×1200.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