THE WAVE OF THE SEMINIFEROUS EPITHELIUM OF THE RAT

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Sciences.

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August 1960

A C K N O W L E D G M E N T S

It has been a most rewarding experience to work and study in the anatomy department. I express my sincere gratitude to Dr. C.P. Leblond for this privilege and for his guidance and kindness during the last several years.

I extend my very sincere appreciation to my Research Director, Dr. Y. Clermont, for his excellent suggestions and criticisms as well as for allowing me to benefit from his wide experience.

My warmest thanks go to Mrs. T. Troitsky for her impressive contribution to the microscopic work and many other phases of the project.

The work here has been made possible by the excellent quality of the histological preparations provided by Mr. Hans Torunski.

To Mr. A. Graham for his excellent photography, to Mr. J. Poliquin for his art work and to many other members of the department who deserve an individual word, goes my sincere appreciation.

Finally, there is no one I could thank more than my wife Betty- Lou, not only for typing the manuscript but most of all for her kind understanding and devotion.

TABLE OF CONTENTS

INTRODUCTION

The histology of the seminiferous epithelium of the rat has been repeatedly investigated since the early work of Von Ebner (1871). These studies have led to the formulation of widely accepted concepts and have produced an extensive terminology. It is the object of this introduction to review briefly the present knowledge of the morphology and dynamics of the seminiferous epithelium of the rat.

Spermatogenesis is the process by which the male gamete is produced. It consists of a series of changes undergone by the so-called stem spermatogonia as they evolve through the spermatocyte and the spermatid stage to become free spermatozoa. These changes take place within the epithelium of the seminiferous tubules, where spermatogonia and their progeny are kept in close contact with Sertoli cells. While the life history of the cells during spermatogenesis has been known for nearly a century, important advances in our knowledge of the subject have been made during recent years.

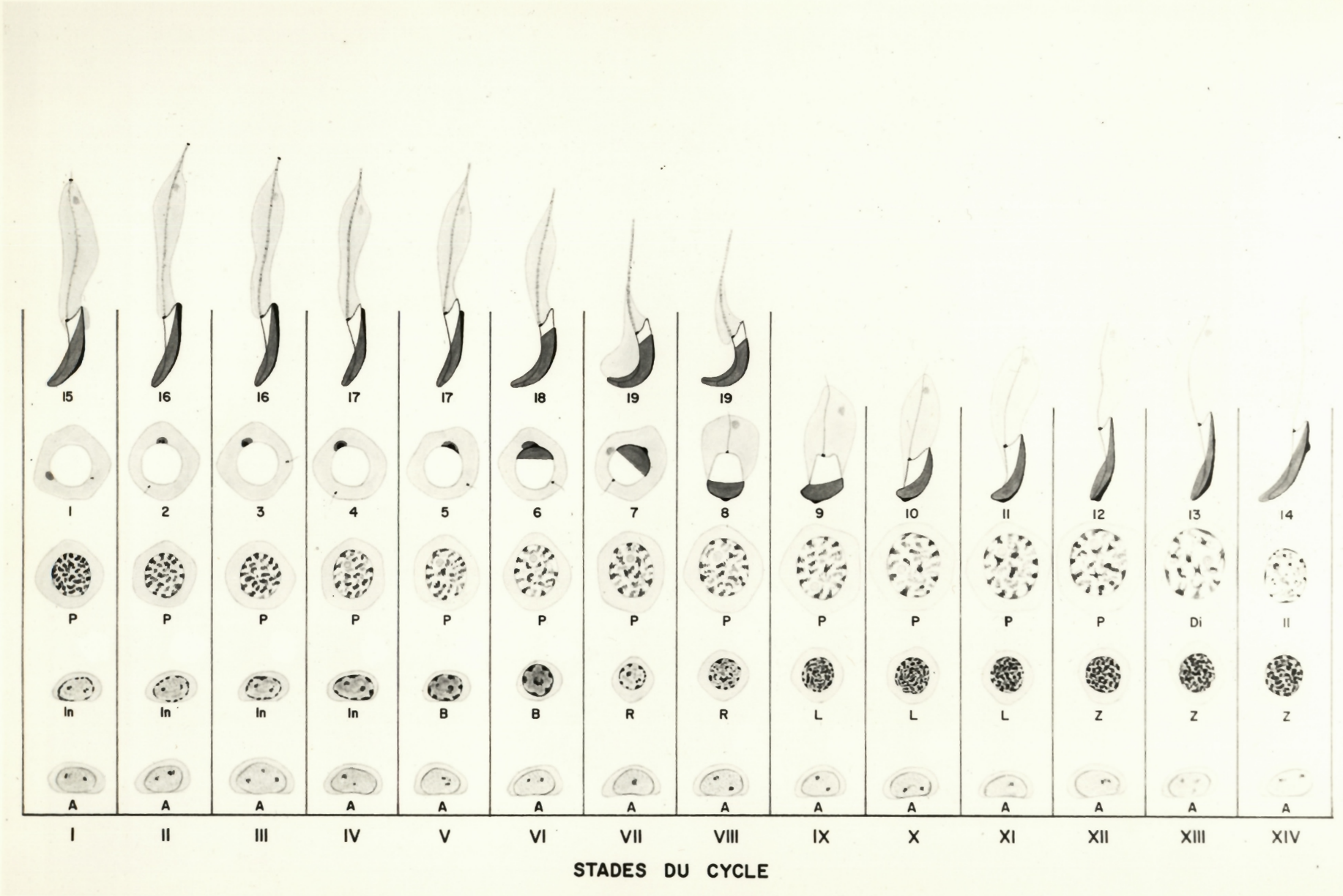
If we examine figure I from left to right row after row (without considering the columns) we can see the changes occurring in the cells with time.

Figure 1 The 14 Types of Cellular Associations

Each column contains the various cell types which make up a type of cellular association. The first 14 steps of spermiogenesis (1 to 14) can be used as the sole criterion whenever one identifies a cellular association. As the various components of any given association mature in time, the next type of association is immediately obtained. Association I arises from the development of association XIV, thus establishing a repetitive cycle which has been described as the "cycle of the seminiferous epithelium".

Cell Types:

A: type A spermatogonium
In: intermediate type spermatogonium
B: type B spermatogonium
R: primary spermatocyte, Resting
L: primary spermatocyte, Leptotene
Z: primary spermatocyte, Zygotene
P: primary spermatocyte, Pachytene
D: primary spermatocyte, Diakenesis
II: secondary spermatocyte
1 to 19: spermatids throughout spermiogenesis (step 1 to 19)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VIII
IX
X
XI
XII
XIII
XIV
STADES DU CYCLE

FIG. 1

Let us start from the bottom row, which shows type A spermatogonia. These do not change in morphological appearance, but they proliferate, increasing their number. At the time period corresponding to the end of the bottom row, most type A spermatogonia become the so-called intermediate spermatogonia (In, second row,left), while a few remain for a repetition of the evolution on the bottom row. The intermediate spermatogonia later divide, to become type B spermatogonia, which in turn divide into resting primary spermatocytes (R). The primary spermatocytes undergo the long evolution known as meiotic prophase, through leptotene (L), zygotene (Z), pachytene (P, third row), diakinesis (D), first maturation division giving rise to secondary spermatocytes (II), and second maturation division giving rise to spermatids (fourth row). When rat spermatids are stained by means of the periodic acid-Schiff technique, they can be classified so as to define 19 successive steps of development, referred to as the 19 steps of spermiogenesis. Steps 1-14 are seen along the fourth row and steps 15-19 along the fifth row. At step 19, spermatids become detached from the Sertoli cytoplasm and become spermatozoa.

Now that the life history of the germinal cells has been reviewed, the next step is to see how they are associated in the seminiferous epithelium at various stages of their evolution.

Examination of sections of seminiferous tubules reveals at a glance that the cells do not evolve at random as in lymphatic tissue for instance, but are arranged into homogeneous groups (fig.7). These groups consist of large numbers of cells which are at the same step of development and may be assumed to develop at the same rate. Each homogeneous cell group forms a layer referred to as a "cell generation". In the rat, as in most mammals, such groups of cells extend around the entire circumference of the tubule, thus making concentric homogeneous layers of cells (fig. 18). Proceeding from the lumen to the limiting membrane of the tubule, one finds I) spermatids (one or two generations) 2) spermatocytes (one or two generations) 3) spermatogonia. Further analysis of the tubular cross-sections shows that spermatids (of the deep layer if two are present) at a given step of development are always associated with the same types of spermatocytes and spermatogonia. As a consequence, a certain number of possible cellular associations arise. For example, at the time when step 19 spermatids are about to be released, the cells present in the epithelium are shown in column VIII of figure I. There are type A spermatogonia, two types of spermatids (step 8 and 19). After release of step 19 spermatids (sperm release) a different cellular association has developed, (fig. I- column IX), which includes leptotene spermatocytes and step 9 spermatids.

With time, successive cellular associations are encountered, which are schematically represented in the columns of figure 1. The number of recognizable cellular associations varies with each author depending on the arbitrary criteria which are chosen. The classification into 14 cellular associations represented on figure 1 was suggested by Leblond and Clermont ('52) and lends itself well to experimental investigation. Readily workable criteria for the definition of these associations were given by Clermont and Perey ('57). The 14 cellular associations are referred to by Roman numerals from I to XIV. It will be noted that evolution of the last cellular association (XIV) will give rise to the first cellular association (I). Nobody has ever observed in vitro the evolution of the cellular associations with time, but it is accepted that in any one area of seminiferous epithelium the 14 associations appear in an orderly sequence which repeats itself time and time again. This phenomenon is known as the cycle of the seminiferous epithelium, each cellular association representing a different stage of the cycle. The duration of the cycle is the time between two successive appearances of the same cellular association in any one area of the tubule. Using radioautography with tritium labelled thymidine, Clermont, Leblond and Messier ('59) showed that the duration of the cycle is 12 days $\pm$ 0.2 day. It is remarkably constant in different areas of tubules as well as in different animals. The duration of each stage can then be determined from the frequency of occurrence of the corresponding cellular association (Leblond and Clermont '52). The

The duration of the whole spermatogenesis may be assessed in relation to that of the cycle. The type A spermatogonia undergo mitoses at stages IX and again at stages XII and XIV. Hence, it is usually considered that spermatogenesis starts with stage IX mitoses. Examination of figure 1 makes it clear that it takes about four cycles for a type A spermatogonium of stage IX to become a free spermatozoon. Thus, the duration of spermatogenesis is about 48 days.

Clermont and Leblond ('53) have proposed a "stem cell renewal" theory to explain how, at every cycle, some of the type A spermatogonia enter spermatogenesis while other are retained to replenish the stock of type A spermatogonia for the following cycle.

The concepts of "spermatogenesis" and "cycle of the seminiferous epithelium" must be clearly distinguished from that of "wave of the seminiferous epithelium". Whereas the concept of cycle referred to the changes taking place in time in any one area of the seminiferous epithelium, the concept of wave refers to the pattern of morphological features seen along the length of the seminiferous tubule.

Credit for the earliest work on the wave must be given to Von Ebner. In 1871, after describing eight stages of the cycle in the rat, he isolated fragments of seminiferous tubules measuring about 10 to 15 mm in length and crushed them under a cover slip. From this material he reported that the various regions of a tubule displayed different types of cellular associations. Furthermore, successive portions of tubule appeared to show a pattern corresponding to chronologically

successive stages of the cycle. For instance, a portion of tubule displaying cellular association III would be followed by successive portions containing respectively cellular associations, IV, V, VI and so on. Each complete series of the known cellular associations, from the first to the eight, was then called a wave (" Art einer Welle"). Waves would follow one another along the tubules. In later experiments (1888), Von Ebner isolated fragments of tubule up to 95 mm in length. He cut them into smaller bits, oriented them, and sectioned them serially. Waves, as he defined them, were found to measure 25 to 38 mm in length with an average of 32 mm. Like most authors who followed him, (Regaud, Curtis, etc...) Von Ebner used the same term to refer to a stage of the cycle and to the portion of tubule occupied by one cellular association (Von Ebner: " stadium " ; Regaud: "stade"; Curtis: "phase"). In the present study, the term "segment" was used to describe the latter entity, each segment being identified by a number, from I to XIV, according to the cellular association which it contained.

The existence of waves was also described by Benda (1887) in several mammalian species (mouse, guinea pig, rabbit, bull, ram, boar, dog, and cat) and by Furst (1887) in marsupials.

It remained for Regaud ('OI, '09) who studied the rat, to emphasize the findings of his predecessors with the

striking statement that "the wave is in space what the cycle is in time". He went on further by explaining that "the length of the wave is proportional to the duration of the cycle. Therefore stages of the cycle are represented by segments of tubule of determined length which is proportional to their duration". In order to explain some irregularities in the wave, Regaud then postulated that the segments making up the waves followed a spiral course around the tubules. This idea was even endorsed by Von Ebner ('02). Finally, Regaud explained the wave by a " mouvement spermatogénétique" which would travel along the tubules like an impulse, initiating spermatogenesis on its way. It should be stressed that Regaud's conclusions were reached from indirect evidence and without the help of measurements. Nevertheless, the intricacy of this reasoning must have discouraged subsequent objective criticism of his views, which are widely accepted at the present time (Roosen-Runge '50: Tobias '56 ; Moree '47).

Alone, during the last 50 years, Curtis ('18) who had made an extensive study of serial sections in the mouse and rabbit, reported the presence of irregularities which strongly refuted Regaud's concept of the wave (Curtis, however appeared to be unaware of Regaud's work at the time of his own publication in 1918). Curtis also reconstructed entire tubules in three dimensions. These were found to be usually " arranged in the form of an arch, the tubule beginning and ending in a tubulus

rectus, both tubule ends being open and having a functional connection with the rete". Other tubules were more complex with branching arches and more than two openings into the rete testis. This author, who divided the cycle of the seminiferous epithelium into eight stages called "phases" saw the previously reported continuity in the order of the cellular associations along the tubule. Previous authors believed that this order was steadily ascending in one direction, but Curtis found many irregularities with the order of the "phases" ascending in some areas and descending in others. In spite of these irregularities, the overall pattern of these "phases" was seen to descend away from the rete testis as far as a " point of reversal" beyond which the order of the "phases" ascended toward the other tubular extremity. The description of this pattern by Curtis is much confuded. He usually called a wave a complete series of his 8 phases, beginning at phase 1 and ending at phase 8. The irregularities in the order of the phases were described in turn as "phases out of order", or "reversed waves", or "descending waves" depending apparently on the number of phases involved in the irregularity. In fact, it is difficult to see exactly what Curtis meant by the term wave.

In summary, various authors have made, at one time or another, important observations on the pattern of the morphological features seen along the seminiferous tubule.

These observations have led to the concept of the wave of the seminiferous epithelium. However, this concept has meant different things to different observers, and all available definitions of the wave are either inaccurate or obscure. One particular aspect deserves clarification: when Von Ebner found an orderly pattern of distribution of the cellular associations he seems to have detected the presence of a dynamic phenomenon explaining this pattern, which he describes as "a kind of a wave" (Art einen Welle). Regaud was more explicit in his interpretation, and stated that the pattern was due to an impulse which actually travelled along the tubules. At any rate, both authors used the term "wave", which has a dynamic connotation, to refer to a series of tubular portions, which is a static entity. This practise was perpetuated by other authors as well as by Curtis, who came closer to the actual facts than any of his predecessors. Consequently, this well established terminology was preserved in the present study in spite of its shortcomings.

The microanatomy of the seminiferous tubules of the rat must now be reviewed. It has been investigated by Clermont et al (in press) mainly on the material used for the present study. About 35 to 40 tubules are usually found in a rat testis. The serially sectioned testis used in the present study contained 37 tubules, 20 of which were reconstructed

from end to end in three dimensions. Sixteen of these had an overall U shape with both ends opening into the rete testis; three other tubules branched off so as to have three openings, while the last one opened into the rete at one extremity only and was blind at the other end (fig. 17). After leaving the rete testis, each seminiferous tubules runs a zig-zag course going alternately cranially and caudally, resulting in a large number of loops (about 100 per tubule). On three dimensional reconstructions, each tubule appears as a palissade which follows a U-shaped course in the testis (fig.3). The whole palissade is itself disposed like a funnel presenting its large opening cranially, with all the loops lying at an angle with the long axis of the organ. This angle is approximately the same for all the loops of the same tubule. The average length of the unbranched tubules is approximately 32 centimeters.

It is the purpose of the present investigation to obtain a more precise understanding of the pattern of the histological features seen along the seminiferous tubule. The better known theories on the nature of the wave will be reexamined with the help of new techniques and quantitative analyses of a comprehensive material.

METHODS

Longitudinal sections of seminiferous tubules

The testes of 5 albino rats weighing about 250-300 gm were fixed in Zenker-formol and the tubules were micro-dissected under water at a magnification of 45 diameters. A fine mounted sewing needle and a small forceps were used to remove the interstitial connective tissue and isolate the seminiferous tubules. Histological processing of the freed portions of tubules was difficult because of their extreme fragility. The problem was simplified by placing each of them into individual flat glass chambers built from ordinary histological slides(fig.2). Free circulation of solutions in and out of the chambers could take place through the slits provided in their walls. The whole chamber could be carried through water, alcohol, dioxane and melted paraffin without handling the tubule directly.

After passing through paraffin the chamber containing the processed tubule was allowed to cool, and was taken apart, leaving the tubule included in a small amount of paraffin over the bottom slide. The tubule was then uncoiled with a hot needle. Meanwhile, a block of paraffin was prepared, one surface of which was made flat with a hot glass slide. Finally, the slide carrying its tubule was reversed over the flat surface of the prepared paraffin block. The tubule was made to penetrate the block by rubbing a warm knife over the back of the slide. Pressure was also applied upon the slide

Figure 2 The Glass Chamber

above: middle slide. An ordinary histological slide has been fragmented as indicated. It will make up the side walls of the chamber.

below: Side view of the completed chamber. Intact histological slides make up the top and the bottom of the chamber. Adhesive tape is used to secure the fragments of the middle slide to the bottom slide. The chamber is closed with thread. Note the various slits provided for the circulation of fluids.

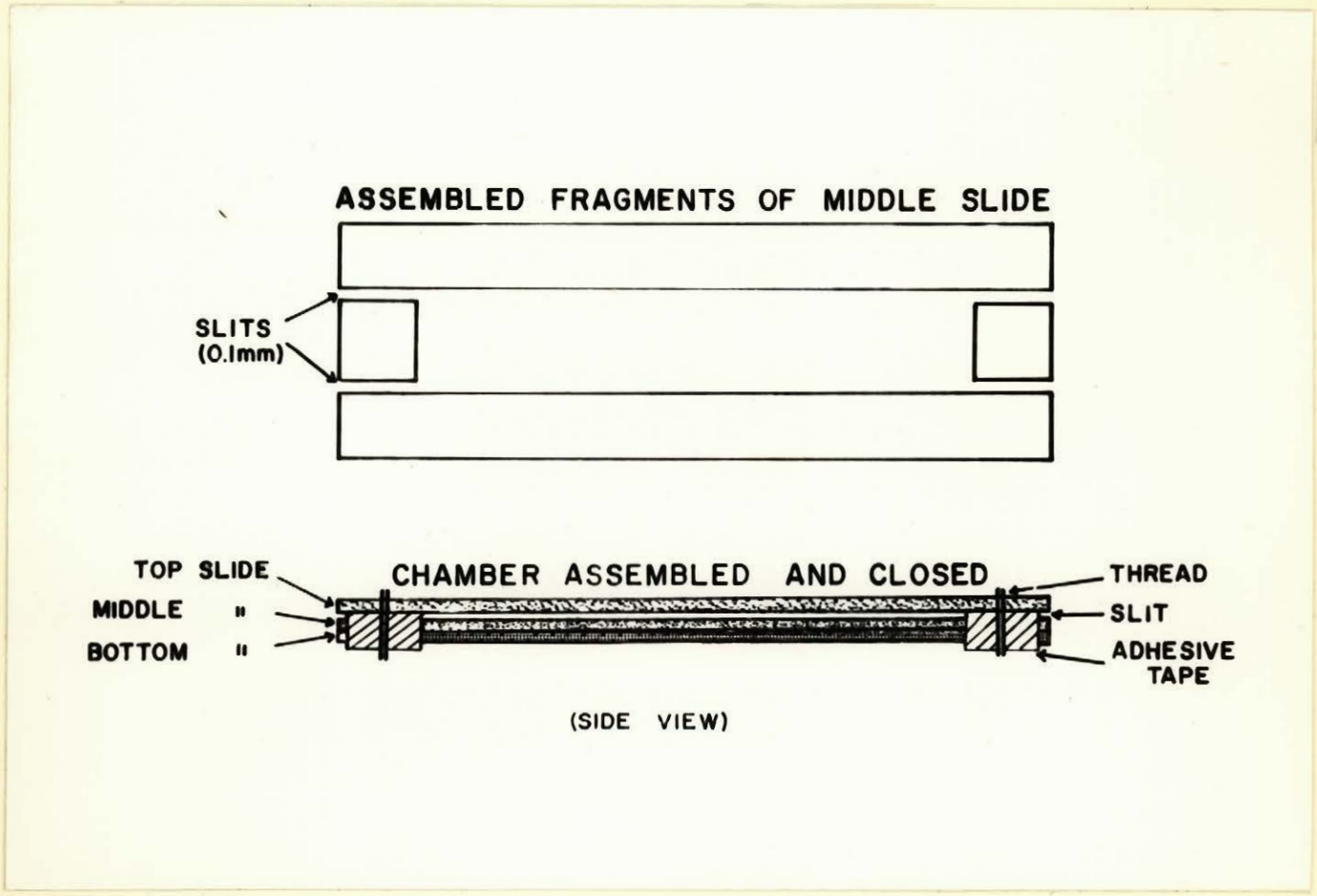
ASSEMBLED FRAGMENTS OF MIDDLE SLIDE
SLITS
(0.1mm)
TOP SLIDE
MIDDLE "
BOTTOM "
CHAMBER ASSEMBLED AND CLOSED
THREAD
SLIT
ADHESIVE TAPE
(SIDE VIEW)

FIG. 2

Figure 3 Morphology of the Seminiferous Tubule

A model of a tubule has been made. It shows the oblique disposition of the tubular loops in a conical plane. All the loops of a tubule lie at approximately the same angle with the axis of the cone which is also the long axis of the testis.

FIG. 3

Figure 4 Longitudinal Section of a Testis

The 20 tubules described in the present study are seen in cross section. Each tubule is seen to lie at a different angle with the long axis of the testis.

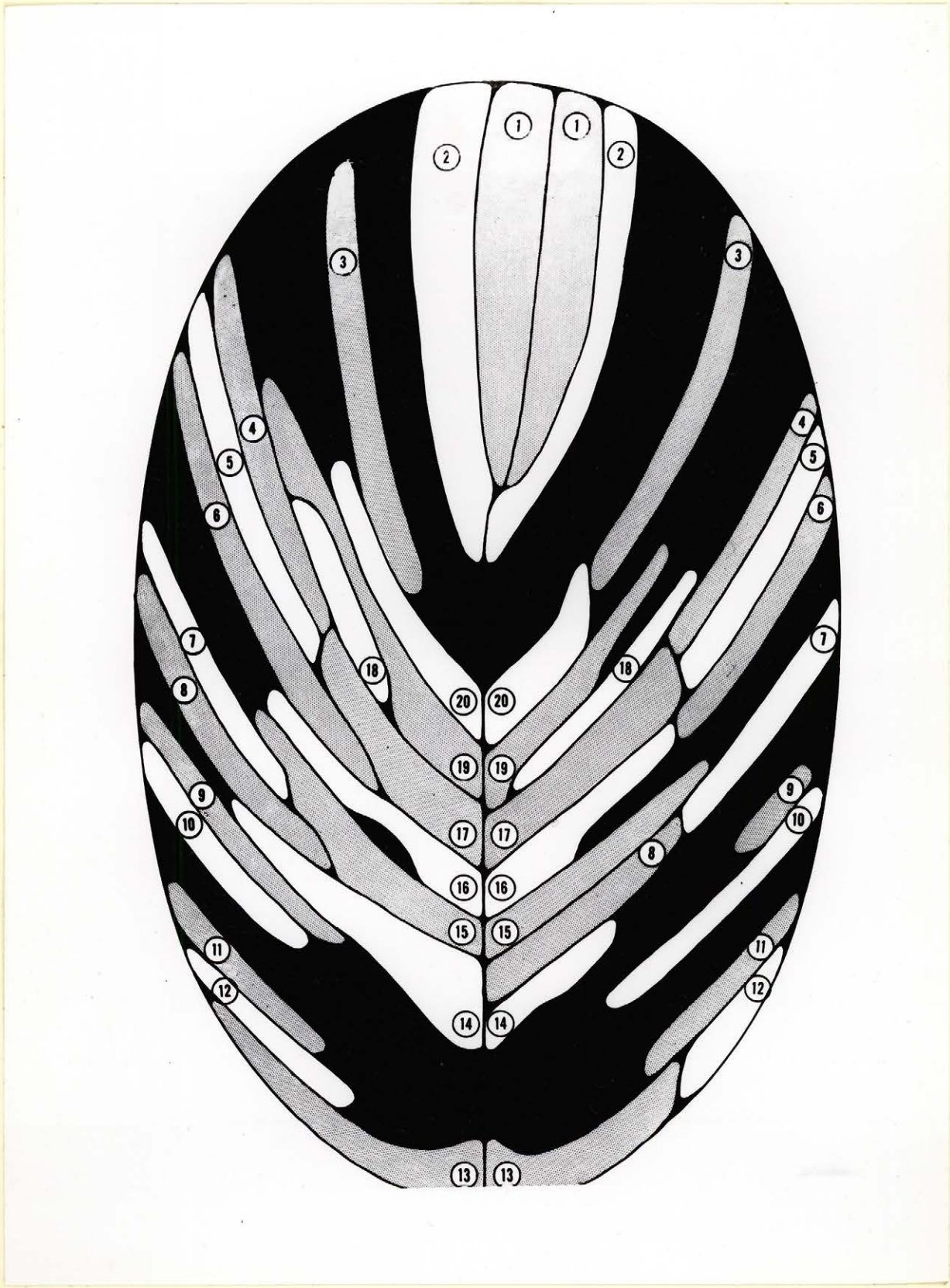
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FIG. 4

in order to flatten out the coils of the tubule. After a few minutes, the slide was removed and the tubule was lying evenly at the surface of the block. Eventually, the block was sectioned serially with the microtome knife kept parallel to its surface. The 7μ thick serial sections were stained with the periodic acid-Schiff method and counterstained with hematoxylin. Measurements and results of microscopic analysis were recorded on low power photographs (x39) of the sections (fig.7A, fig.12).

In this manner, 34 tubular fragments (each from a different tubule) were obtained, 21 of which included the junction of the tubule with the rete testis. Twenty-two of the 34 fragments measuring from 13 to 128 mm each, were used for quantitative data. The combined length of these fragments was about 104 cm.

Serial sections of tubules

One testis from an adult albino rat weighing 340 gm was fixed in Zenker formol. After impregnation in paraffin, serial sections were made of the entire organ going from its cranial to its caudal pole. A total of 3410, 5μ thick, serial sections was obtained. The thickness of the sections was checked against the length of the testis which was 17mm in the fixed state. After staining with the periodic acid-Schiff-hematoxylin method, a low power photograph was taken of every twentieth section (i.e. every 100 μ) giving a total of 170 low power photographs. Subsequently, all tubular cross sections (about 400 per photograph) were examined under micros-

cope. The cellular association encountered in each one of them was identified according to the criteria given by Clermont and Perey ('57), and was recorded on the picture of the corresponding cross-section.

Individual seminiferous tubules were then examined as follows. The site of connection of a tubule to the rete testis was identified and the tubular convolutions were followed from this point in the serial sections as far as the other extremity of the tubule. At the same time, a code number identifying the tubule (1 to 20) was written on the photographs of all its cross-sections, and a list was made of the type of cellular association encountered at the level of each photograph. Knowing that the photographs were taken every 100 μ the length of the tubule occupied by each cellular association could be calculated from the list. These length values then had to be correeted since the tubular convolutions lie at a definite angle with the long axis of the testis (fig.5). This angle which is approximately the same for all convolutions of a tubule was determined from diagrammatic reconstructions of each tubule. It was found to vary from 0 to 60 degrees depending on the location of the tubule within the testis (fig. 4). The correction factor was found to be the secant of that angle (fig. 5) and thus varied from 1 to 1.7. Figure 5 illustrates a portion of tubule lying at a 30 degree angle. Another correction was required for the turns of the tubular loops. The length

Figure 5 Correction of Tubule Length for Obliquity

Diagram illustrates the case of a tubular portion lying at 30 degrees with the long axis of the testis. ℓ represents a distance of 1000 μ between two given histological sections. L is the true or corrected length of the corresponding tubular portion. The correction factor is the secant of the angle.

Figure 6 Correction of Tubule Length for the Turns

Diagram illustrates why each tubular turn was given an estimated length of 500 u.

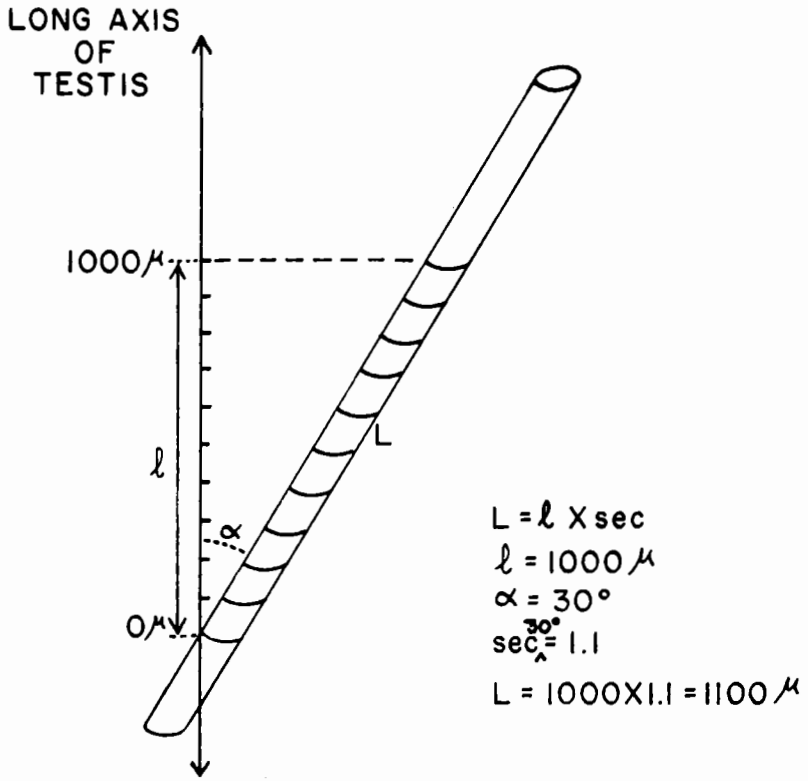

LONG AXIS
OF
TESTIS
1000μ
l
0μ
α
L
L = l X sec
l = 1000 μ
α = 30°
sec 30° = 1.1
L = 1000X1.1 = 1100 μ

FIG. 5

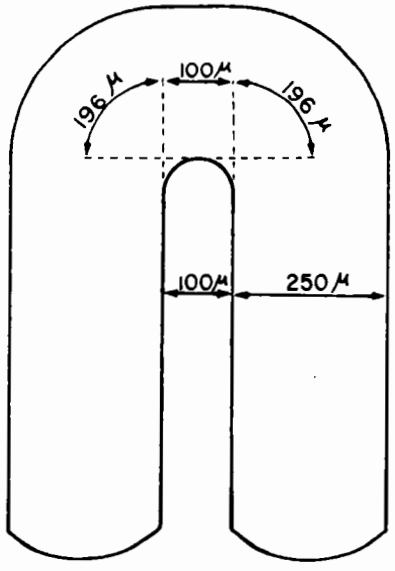

196 μ
100μ
196 μ
100μ
250 μ

FIG. 6

of each turn was estimated to be 500 μ, as shown on figure 6.

Twenty complete seminiferous tubules were examined in the manner just described. Their combined length was 6.95 meters.

Finally, some of the histological features of the seminiferous epithelium were recorded on maps of tubular portions reconstructed from the serial sections (fig. 15).

The cellular associations were classified into 14 types as recommended by Leblond and Clermont ('52). Precise criteria were given for this classification by Clermont and Perey ('57). The cell types making up each association are depicted on figure 1, while microphotograhs of the associations are shown on figure 7B. In this last figure however, nine of the fourteen types of associations have been divided into subtypes which point out some of the progressive transformations taking place. For example, it will be seen that as the seminiferous epithelium passes through stage VII of the cycle it will first have the appearance of association VIIa, then of VIIb, VIIc and VIId in succession. Such subdivision was only used when a more refined study of the epithelium appeared desirable.

Figure 7A (left). A Wave Without Modulation

PAFSA - Hematoxylin Mag X 40

A portion of tubule has been sectioned longitudinally through its long axis. "Proximal" indicates the end which is closest to the rete testis, while "distal" indicates the other end. The boundaries of the segments have been determined with the microscope and are represented in dotted lines. Roman numerals indicate the type of each segment (I to XIV). The small letters point to the sites where the enlarged microphotographs of figures 7B were taken. The continuity of the order of the segments and the descent of the order from right to left will be noted. The arrows delimitate a wave without modulation from a segment I to a segment XIV. Only 20% of the waves are free of modulations

Figure 7B (right) The 14 Types of Cellular Associations and their Subtypes

PAFSA - Hematoxylin Mag X

The 14 basic types of cellular associations are represented according to the criteria given by Leblond and Clermont ('52) and Clermont and Perey ('57). Nine of the 14 types have been subdivided into subtypes corresponding to different degrees of maturation of each cellular association. This refined classification was only used when a more detailed study appeared indicated. The microphotographs were all taken from the tubule on figure 7A (small letters indicate the sites where the photographs of the subtypes were taken). Enlarged spermatids in the insets illustrate some of the important criteria.

Definition of the types and subtypes of cellular associations:

Ia newly formed spermatids with small dark nuclei.
Ib the nuclei of the young spermatids are larger and lighter. Idiosome becomes visible only later.
Ic idiosome becomes apparent in young spermatids.
II appearance of one or several minute acrosomic granules.
III the small acrosomic granules have fused into a larger and spherical one.
IVa early flattening of the acrosomic granule.
IVb pronounced flattening of the acrosomic granule.
Va head cap begins to appear. Often seen only on one side of the acrosomic granule.
Vb head cap is definite but covers less than a quarter of the nuclear circumference.
Vc head cap covers exactly one quarter of the nuclear circumference.
VIa head cap covers between one quarter and one third of the nuclear circumference.
VIb head cap covers exactly one third of the nuclear circumference.
VIIa head cap covers more than one third of the nuclear circumference. Residual bodies have not yet appeared in the mature spermatids.
VIIb appearance of residual bodies in the distal cytoplasm of the mature spermatids.
VIIc the residual bodies are larger. They have migrated among the nuclei of the mature spermatids.
VIId the residual bodies form a dense row just deep to the nuclei of the mature spermatids.
VIIIa and b the large majority of the head caps are facing towards the limiting membrane of the tubule. VIIIa and b are respectively before and after sperm release.
IXa very early assymetry of the spermatid nuclei.
IXb more advanced assymetry of the spermatid nuclei.
X the nuclei of the spermatids begin to elongate. Acrosomic system begins to have a sickle shape.
XI the spermatid nuclei are even more elongated.
XIIa spermatid nuclei begin to lose their curvature. They have not reached maximal darkness and elongation.
XIIb spermatid nuclei are dark, straight and narrow.
XIII the apex of the spermatids becomes curved, pointed and light staining.
XIVa the large division figures of primary spermatocytes (prophase excluded).
XIVb secondary spermatocytes, resting or in prophase.
XIVc the small division figures of secondary spermatocytes (prophase excluded).

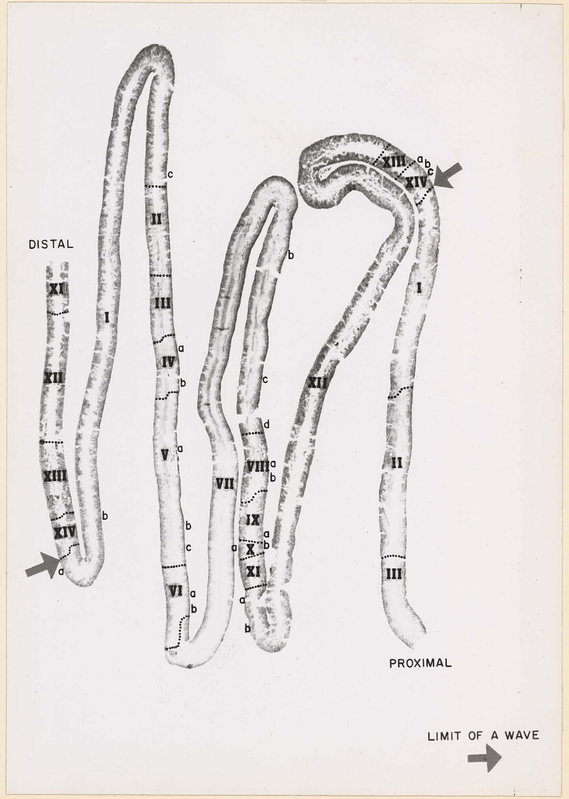
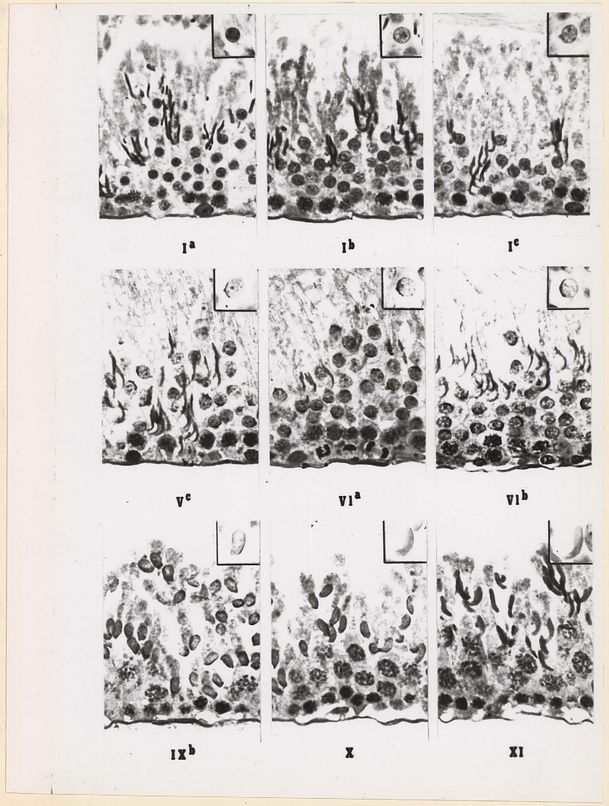

DISTAL
PROXIMAL
LIMIT OF A WAVE

FIG. 7A


Ia
Ib
Ic
Vc
VIa
VIb
IXb
X
XI

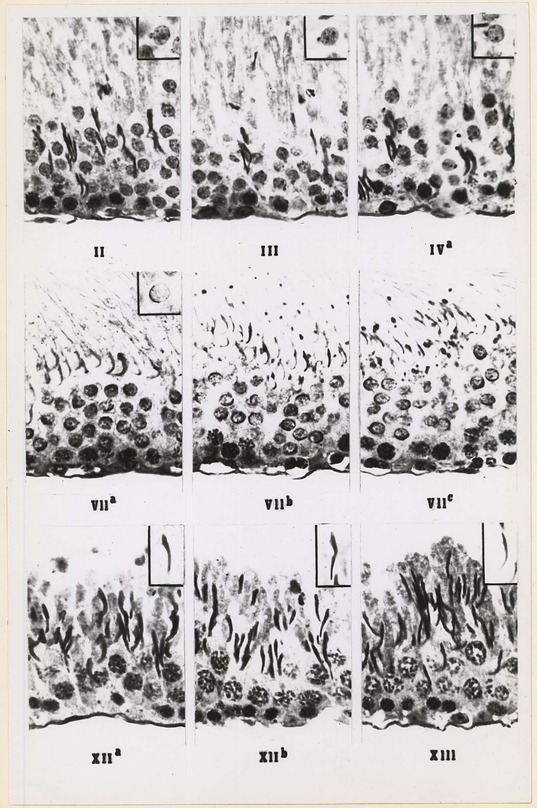
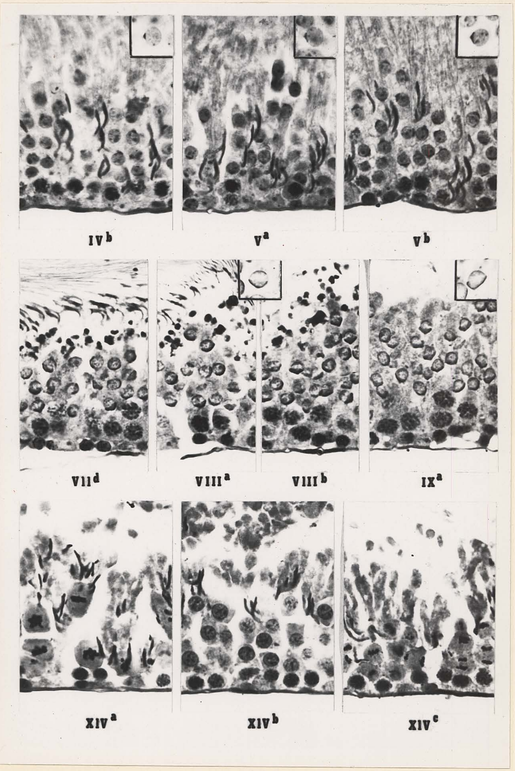

II
III
IVa
VIIa
VIIb
VIIc
XIIa
XIIb
XIII

FIG. 7B


IVb
Va
Vb
VIId
VIIIa
VIIIb
IXa
XIVa
XIVb
XIVc

RESULTS

A. OBSERVATIONS MADE ON LONGITUDINALLY CUT TUBULES

1. Histology of the seminiferous tubules at the junction with the rete testis.

The seminiferous tubule communicates with the rete testis by a small canal of variable length called tubulus rectus (fig. 8). This tubule is lined by a low cuboidal epithelium similar to that of the rete testis itself. At the junction of the tubulus rectus with the seminiferous epithelium the low cuboidal epithelium is abruptly replaced by an epithelium made exclusively of tall, flame-like Sertoli cells (fig. 8). They show a fibrous, elongated cytoplasm extending in the direction of the rete testis. Together, the cell bodies form a little papilla which projects into the tubulus rectus. This structure may conceivably function as a valvule allowing the exit but not the entry of cells and fluids. After a progressive increase in thickness the Sertoli epithelium is abruptly replaced by typical seminiferous epithelium. This epithelium may be at any one of the 14 stages of the cycle (stage XII in fig. 8).

2. The "segments"

The cellular association shown by the seminiferous epithelium near the tubulus rectus was seen around the circumference and over a certain length of the tubule.

Figure 8 Connection of a Tubule With the Rete Testis

The seminiferous epithelium occupying the end of this tubule is at stage XII, but any stage may be seen in this position (table 2). The area of pure sertoli epithelium and the valvule which it projects into the tubulus rectus will be noted.

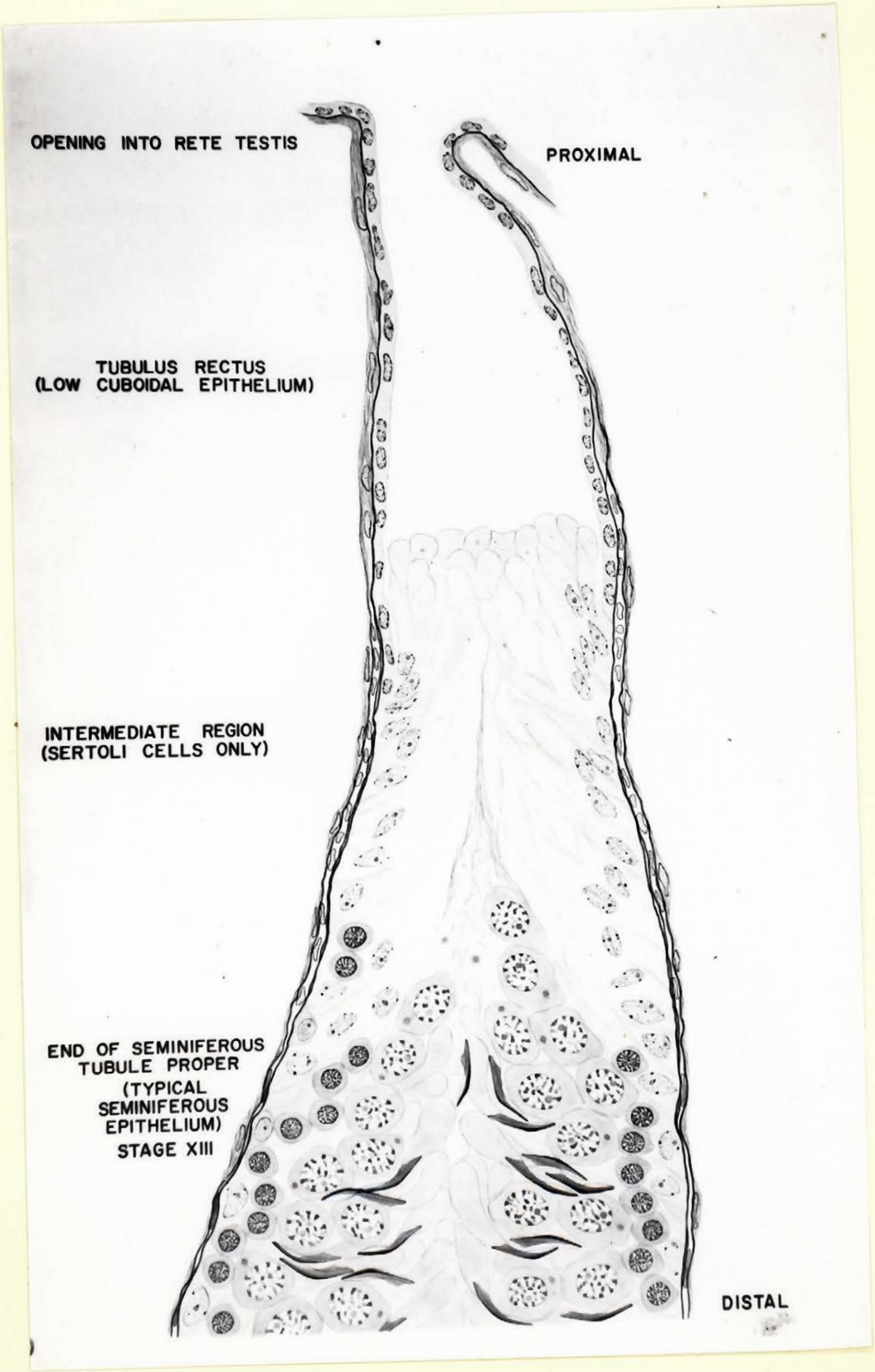

OPENING INTO RETE TESTIS
PROXIMAL
TUBULUS RECTUS
(LOW CUBOIDAL EPITHELIUM)
INTERMEDIATE REGION
(SERTOLI CELLS ONLY)
END OF SEMINIFEROUS
TUBULE PROPER
(TYPICAL
SEMINIFEROUS
EPITHELIUM)
STAGE XIII
DISTAL

FIG. 8

TABLE 1

LENGTHS OF THE SEGMENTS (MEASURED ON LONGITUDINALLY CUT TUBULES)

Segmental type	Number of segments measured	Length of longest segment (mm)	Length of shortest segment (mm)	Average length of segment (mm)	Percent of total tubular length occupied by each segmental type
I	56	7.2	0.10	2.6	15.2
II	53	4.5	0.17	1.2	6.8
III	59	5.0	0.06	0.6	3.5
IV	60	3.9	0.03	0.9	5.6
V	53	5.7	0.22	1.4	7.6
VI	50	6.2	0.10	1.5	7.9
VII	48	9.1	0.31	3.2	15.8
VIII	58	6.2	0.20	1.4	8.3
IX	65	3.1	0.06	0.8	5.2
X	68	1.9	0.02	0.4	2.5
XI	65	6.0	0.03	0.6	3.9
XII	54	6.8	0.07	1.5	8.2
XIII	56	6.1	0.07	0.8	4.4
XIV	56	3.3	0.07	0.8	4.9

At a variable distance, another type of cellular association was seen, to be followed farther along the tubule by a third type of association, and so on (fig. 9). The portion of tubule occupied by one type of cellular association was called a "segment", hence there were 14 types of segments which were referred to by the number (I to XIV) of their cellular association. Table 1 gives the lengths of the segments according to type, as measured on the longitudinally cut tubules. The wide variations in the length of segments of the same type will be noted, in rare instances a segment was extremely short and only appeared on one side of the circumference of the tubule.

Attention was then focused on the inner structure of the segments. By careful histological examination, it was possible to determine the distribution of the subtypes of cellular associations. (These were defined on figure 7 for 9 of the 14 types of associations.) Segments could thus be divided into two or more subsegments. It was found that transitions always took place from one end of a segment to the other (fig. 10). When a segment comprised more than two subsegments, these usually followed one another in an evenly progressive pattern (fig. 10A), while in other cases they followed instead a fluctuating pattern such as illustrated on fig. 10B.

Figure 9 Three Ends of Tubules

Mag X 23

The tapered ends on the left are the connections of the tubules with the rete testis. Note that the order of the segments descends away from the rete testis.

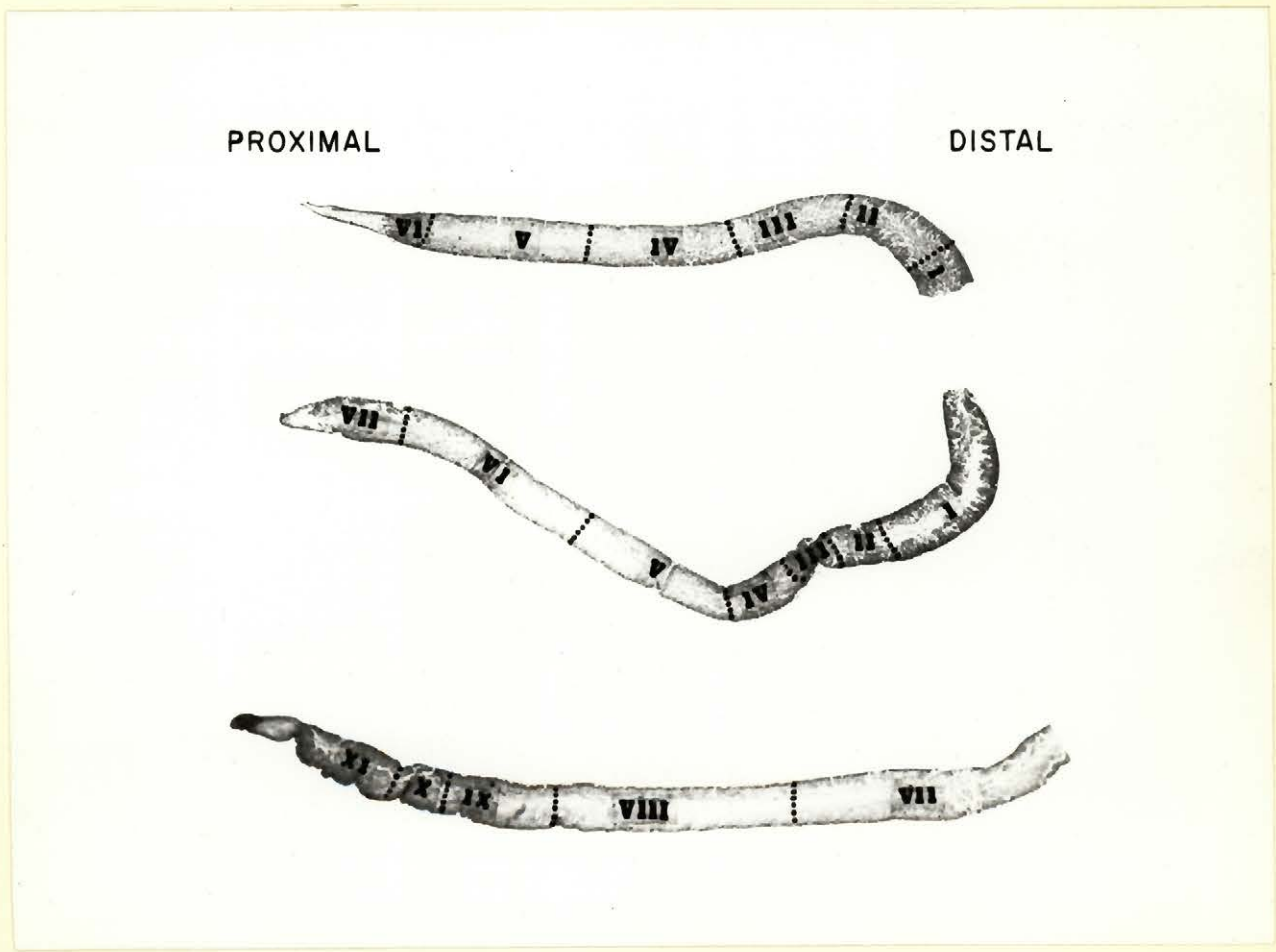

PROXIMAL
DISTAL
VI
V
IV
III
II
I
VII
VI
V
IV
III
II
I
XI
X
IX
VIII
VII

FIG. 9

Figure 10 The order of the Subsegments

Mag X 23 and X 32

A. Subsegmental descent.
In this fragment of tubule the subsegments follow one another in an evenly descending order from the proximal to the distal end.

B. Subsegmental modulation.
In this fragment of tubule, the order of the subsegments first descends from the proximal end as far as VIIa, then it ascends again to VIIb, and finally descends again. It is this kind of fluctuation in the order of the subsegments (or segments) which is referred to as a "modulation".

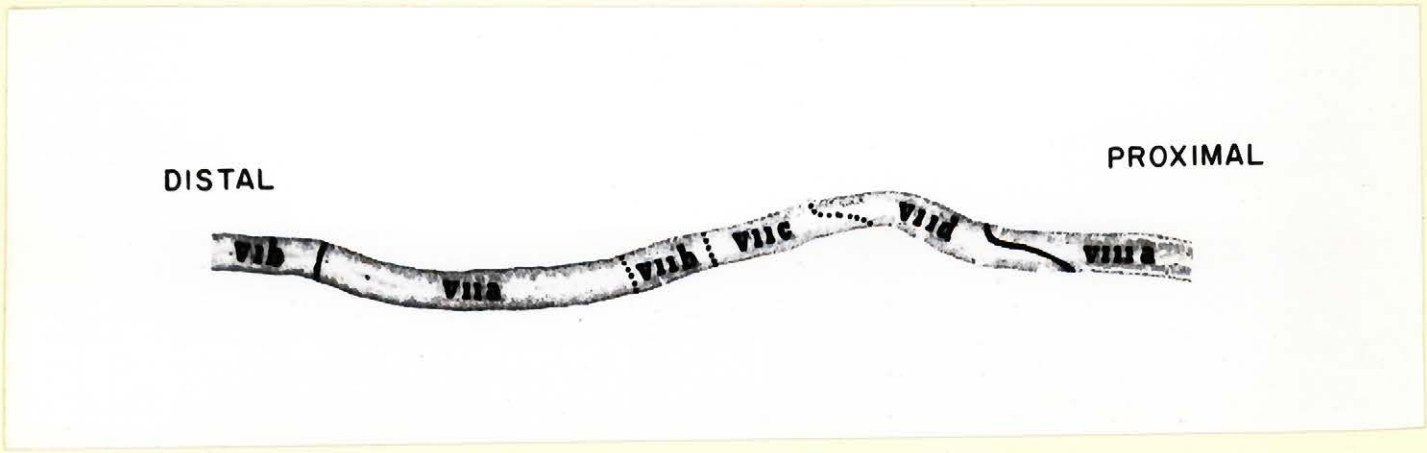


FIG. 10 A

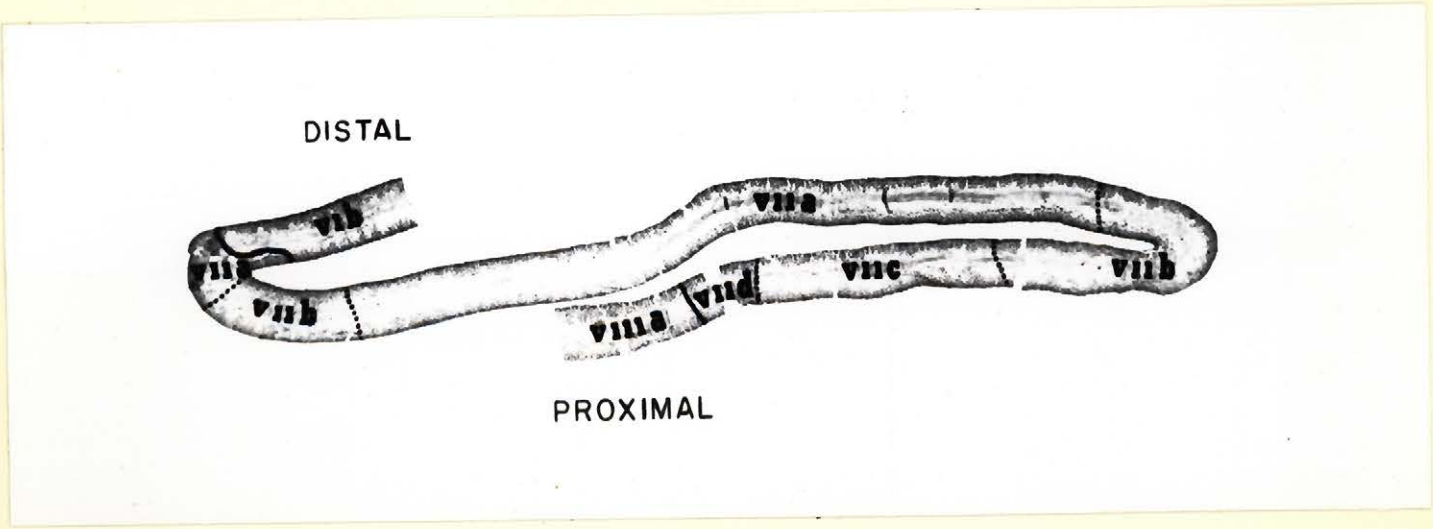


FIG. 10 B

3. The segmental order

When considering a portion of tubule in which each segment had been labelled with its proper number (fig. 7A, fig. 11, fig. 12), it became evident that any two adjacent segments carried consecutive numbers, with the only exception of segments XIV and I which often followed each other along the tubule (fig. 12). This striking feature of the arrangement of the segments was referred to as the "continuity of the segmental order". In the present study, segments XIV and I were considered as logical adjacent segments in spite of the fact that numbers 14 and 1 are not arithmetically consecutive. Indeed, cellular associations XIV and I are as closely related as, for example, associations XIII and XIV, since type I arises directly from type XIV during the cycle of the seminiferous epithelium. Therefore, although there was a breach in the numerical continuity at each XIV-I junction, there was no breach in the histological continuity of the segmental order.

While each segment was usually located between one with a less advanced and one with a more advanced type of cellular association (e.g. every segment in figure 7A) an occasional segment was found between two segments of the same type. Thus, in figure 11, a segment II was located between two segments III. However, this arrangement did not alter the continuity of the segmental order which was present in every portion of tubule examined.

Figure 11 A Segmental Modulation

Mag X 32

The order of the segments usually descends from the proximal to the distal end.

Occasionally however, as in the fragment shown here, the order first descends (IV-III-II), then ascends (II-III) and finally descends again. Such fluctuations in the segmental order are referred to as "modulations".

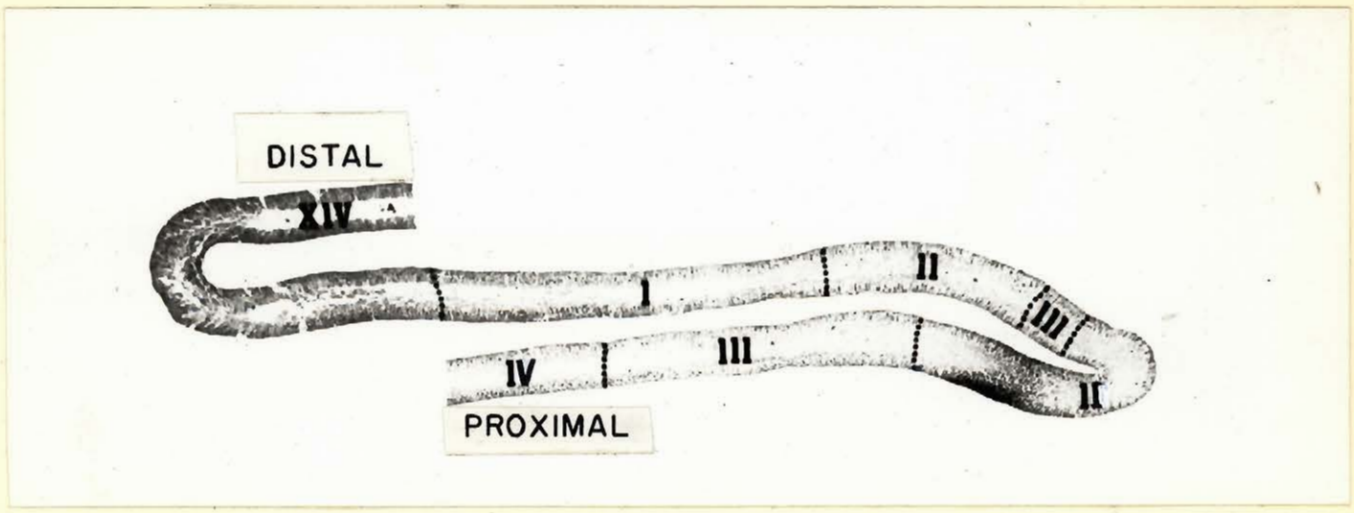


FIG. II

A study of the area of contact of adjacent segments revealed in most cases a smoothly progressive histological transition between one segment and the next. As a result, the process of delineating accurately the segmental boundaries was often difficult. Smooth intersegmental transitions are shown in figure 10A and B, where subsegments have also been delineated. Thus, in figure 10B, the closely resembling subsegments VIb and VIIa were found at the VI-VII boundary, while the closely resembling subsegments VIId and VIIIa appeared at the VII-VIII boundary.

4. Pattern of the segmental order

When the sequence of the segmental numbers was considered from the rete testis onwards (fig.9), it was found that each segment was usually followed by a segment with the next lower number (with the addition of segment I usually following segment XIV). As a result, the overall pattern of the segmental order was descending away from the rete testis. This pattern was present in each of the terminal portions of tubules examined and was referred to as the "descent" of the segmental order.

5. Variations in the "descent" of the segmental order: the "modulations"

While the overall pattern of the segmental order was always that of an obvious descent from the rete testis, local variations were seen throughout this pattern. Namely, in

some areas, a segment was followed by one with the next higher instead of the next lower number, so that a group of two or more segments would be arranged according to an ascending order. However, the descending pattern was then resumed without breach in the continuity of segmental order. For instance, in figure 12 the usual descending order is seen at top left as far as the shadowed area, where a segment IX is followed by a segment X instead of the expected segment VIII. Farther on, ascent continues with segments XI andXII, but reversion to a descending pattern takes place with segments XI-X-IX-VIII and so on. The shadowed segments in figure 12 constitute what was called a "modulation" of the segmental order. In the course of the present study, the limits of the modulations were arbitrarily determined as follows. The tubules were followed in the direction of overall descending segmental order (i.e. away from the rete);whenever the order became ascending a modulation was said to begin; the order soon became descending again; when a segment with the same number as that preceding the modulation was encountered, the modulation was taken to end (fig. 12). Hence, a modulation was preceded and followed by segments with the same rank, and thus always had an odd number of segments. (The limits of the modulations might just as well have been determined by following the segmental order in the ascending -i.e. towards the rete- instead of the descending direction; in which case however, the modulations would have different limits. The modulation in figure 12, for instance, would

Figure 12 A Wave With A Modulation

Mag X 23

The usual descending order is seen at top left as fas as the shadowed area where a segment IX is followed by a segment X instead of the expected segment VIII. Farther on, ascent continues with segments XI and XII, but reversion to a descending pattern takes place with segments XI- X- IX- VIII and so on. The whadowed segments constitute a "modulation" of the segmental order.

The arrows delimitate a wave. A wave is defined as "the shortest series of adjacent segments in which the 14 possible types of segments are seen in addition to those making up the modulations". A wave may be considered to start at any segment providing that it is not in a modulation.

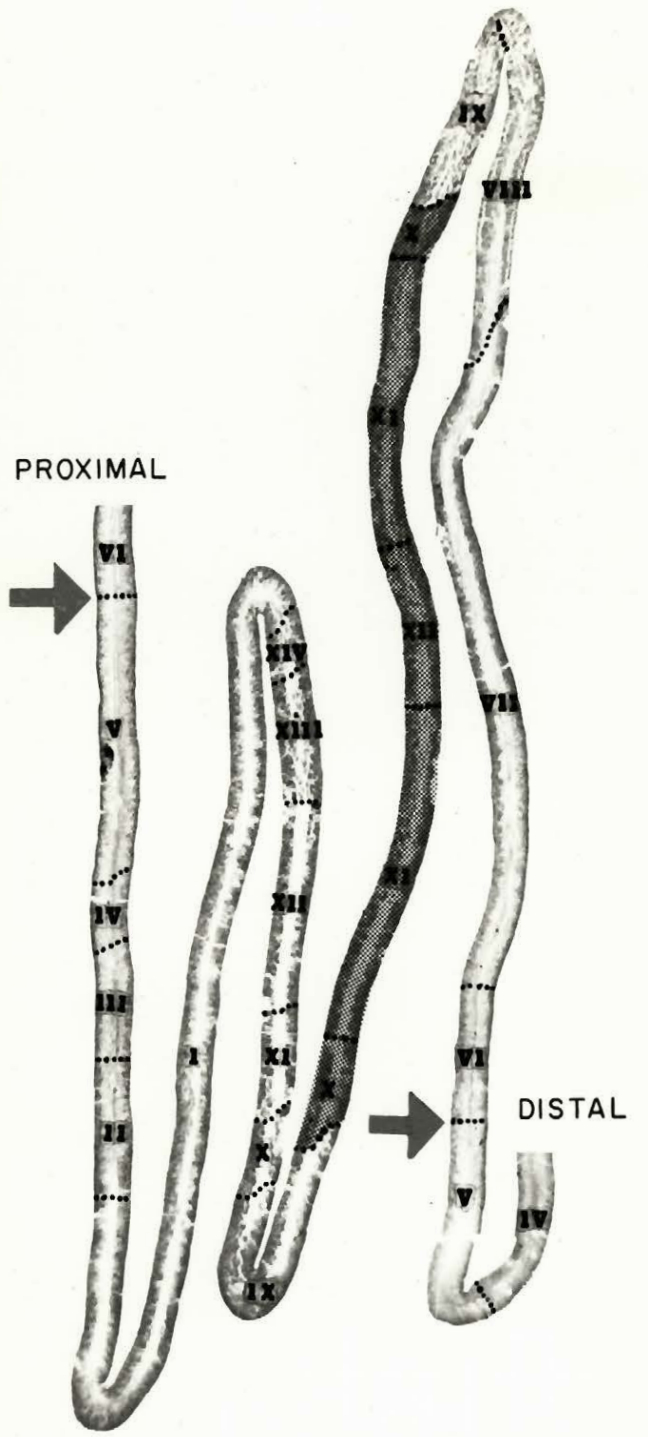


FIG. 12

begin between the shadowed segments XII and XI and would end between the unshadowed segments XI and XIII).

6. The "wave" of the seminiferous epithelium

At this time, a decision had to be made as to what, if anything, should be called a "wave". It will be recalled that previous authors had used the term to refer to a series of consecutive segments seen along the tubules. The existence of modulations was certainly not compatible with Regaud's concept of the wave as an undisturbed and complete series of segmental types. Such complete series of 14 segments without modulations did occur indeed (figure 7A), but they only accounted for a small fraction (about 20%) of the total tubular length. On the other hand, in spite of the modulations, the overall descent of the segmental order was always obvious, and an attempt could still be made at dividing it into definable units. The manner in which this was accomplished is illustrated in the upper part of figure 13, where a portion of tubule is represented semi-diagrammatically. As a preliminary step, the modulations were delimited as previously indicated. If the segments involved in the modulations were then ignored temporarily, the tubule appeared as a succession of series of the 14 known types of segments, as delimited by the arrows on figure 13. Finally, it was decided to call "waves" the entire series of segments between the arrows. These waves, which include the modulations, stand out well in

Figure 13 Delimitation of the Waves Along a Tubule

Above: a portion of tubule is represented diagrammatically with the order of the segments shown as observed in the histological sections. The number and length of the tubular loops are also shown. The modulations are shaded in green.

Below: the same tubule is represented on a curve with the length of each segment shown as measured from the histological sections.

Successive waves are delimited with arrows. They have been defined as "the shortest series of adjacent segments in which the 14 possible types of segments are seen in addition to those making up the modulations". Here, waves arbitrarily begin with a segment XIV, but a wave may begin with any segment providing that it is not in a modulation. An incomplete wave is seen near the rete testis and is followed by four consecutive complete waves. It will be noted that the first two complete waves are shorter than the third and fourth ones. (This was found to be statistically true- table 5-, but did not necessarily apply to each individual tubule as in the one shown here).

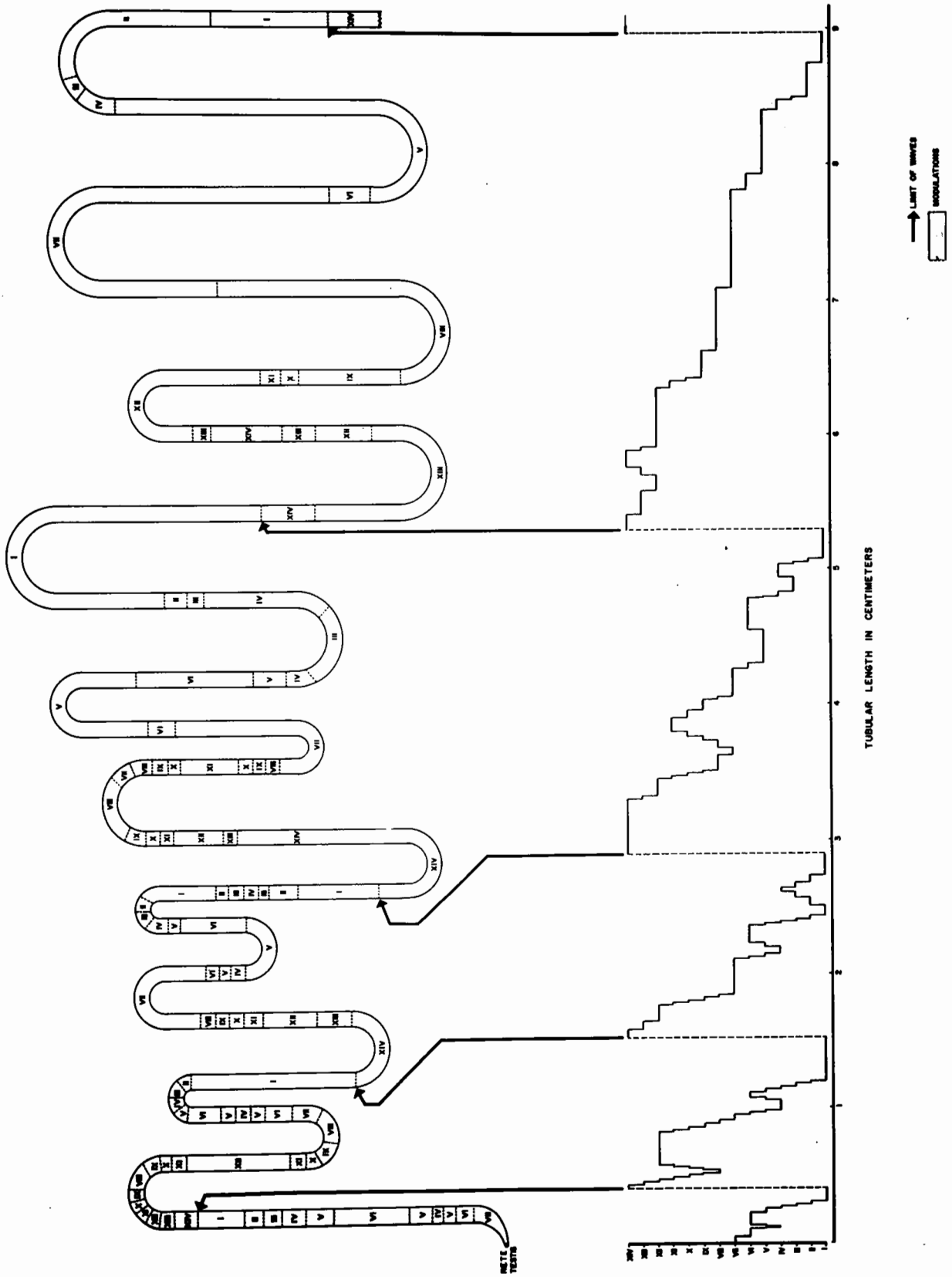

LIMIT OF WAVES
MODULATIONS
TUBULAR LENGTH IN CENTIMETERS
0
1
2
3
4
5
6
7
8
9
METE TESTS

FIG. 13

the curve at the bottom of figure 13. In the present example the waves were considered to start at segment XIV. However, a wave may be considered to start at any convenient segment providing that it is not in a modulation (fig. 12). Modulations appear on the curve as spikes (fig. 13, bottom).

In summary, a definition of the wave which takes the modulations into account may be given; "a wave is the shortest series of adjacent segments in which the 14 possible types of segments are seen in addition to those making up the modulations".

There were 31 such complete waves in the longitudinally cut tubules. Their average length was 23.2 mm (5.5 to 42.5) and they presented an average of 1.35 modulations per wave (0 to 4).

7. Orientation of the tails of spermatids in the tubular lumen.

The delicate tails of the spermatids which had not reached step 17 of spermiogenesis were seen to point either toward the rete testis or in the opposite direction, without any apparent preference.

But the maturing spermatids (steps 17-18-19 of spermiogenesis) seen in segments V-VI-VII and VIII had thick tails which always pointed away from the rete testis (fig. 14). This pattern was remarkably constant.

Figure 14 Orientation of the Tails in the Tubular Lumen

PAFSA- Hematoxylin Mag X 270

This longitudinal section of a tubule shows a portion of seminiferous epithelium at early stage VII (VIIa). The tails of the implanted maturing spermatids always pointed away from the rete testis (i.e. toward the reversal site) regardless of their location along the tubules.

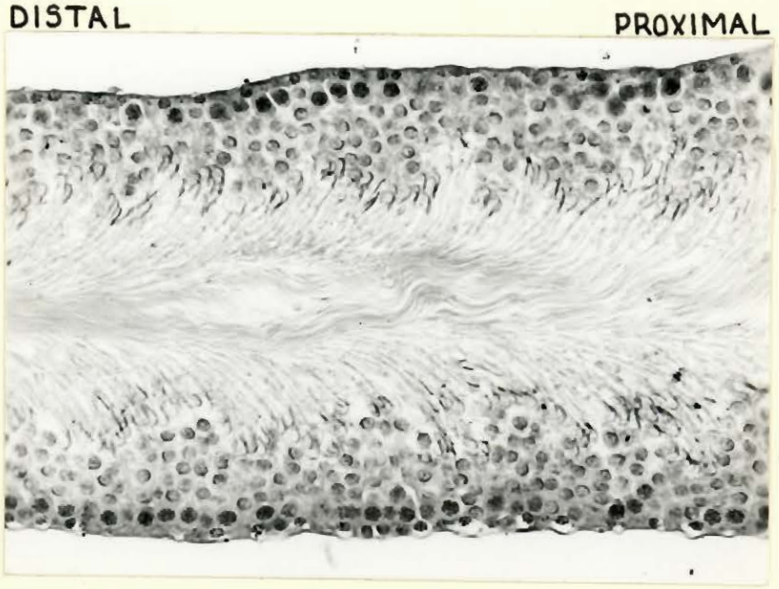


FIG. 14

B. OBSERVATIONS MADE ON TUBULES RECONSTRUCTED FROM SERIAL SECTIONS.

Since only one testis was examined in serial sections, it is a possibility that all the observations reported below do not apply to rats of different species or different ages. On the other hand, none of these observations were found to conflict with the ones reported above from animals of different ages and origin.

The findings reported so far will now be amplified with observations made in the serial sections. The microanatomy of the tubules under study has already been described in the introduction.

1. Junction of the tubule with rete testis

Table 2 shows that each type of cellular association occurred about as frequently at the junction with the rete testis as anywhere else along the tubule.

2. The segments

In view of Regaud's assumption that the segments were distributed along the tubules in a spiral fashion, the contours of many intersegmental boundaries were carefully reconstructed from serial sections and mapped out. A few examples are given in figure 15 A,B and C. These various maps showed that the boundaries assumed a wide variety of shapes, frequently sending finger-like projections into the segments. No constant pattern

TABLE 2

FREQUENCY OF OCCURRENCE (%)

OF THE 14 TYPES OF CELLULAR ASSOCIATIONS (I-XIV)

IN TUBULAR CROSS SECTIONS

Type of cellular association	Frequency (%) anywhere along the tubules. (3600 sections examined)	Frequency (%) at the junction with tubulus rectus (70 cases examined)
I	12.1	15.7
II	8.0	8.6
III	2.0	1.4
IV	4.5	7.1
V	5.1	8.6
VI	9.2	10.0
VII	21.8	21.4
VIII	7.3	1.4
IX	2,5	2.9
X	2.5	1.4
XI	2.5	1.4
XII	11.2	11.4
XIII	6.0	1.4
XIV	4.9	5.7

Figure 15A Map of a portion of tubule showing segments VII- VIII- IX- X- XI

The outline of the segments has been reconstructed on a flat plane from landmarks taken in serial cross sections, so that the entire circumference of the tubular portion is represented. The upper end of the map (proximal) corresponds to the end of the tubular portion closest to the rete testis. An area with very short segments has been selected here. It will be noted that segment IX does not quite cover the entire circumference of the tubule (arrow). This only occured with segments measuring less than 100 u in length, usually segments III, IV, IX, X, XI or even XIV. The irregular outline of the segments is evident, as well as the lack of a spiral pattern to their distribution.

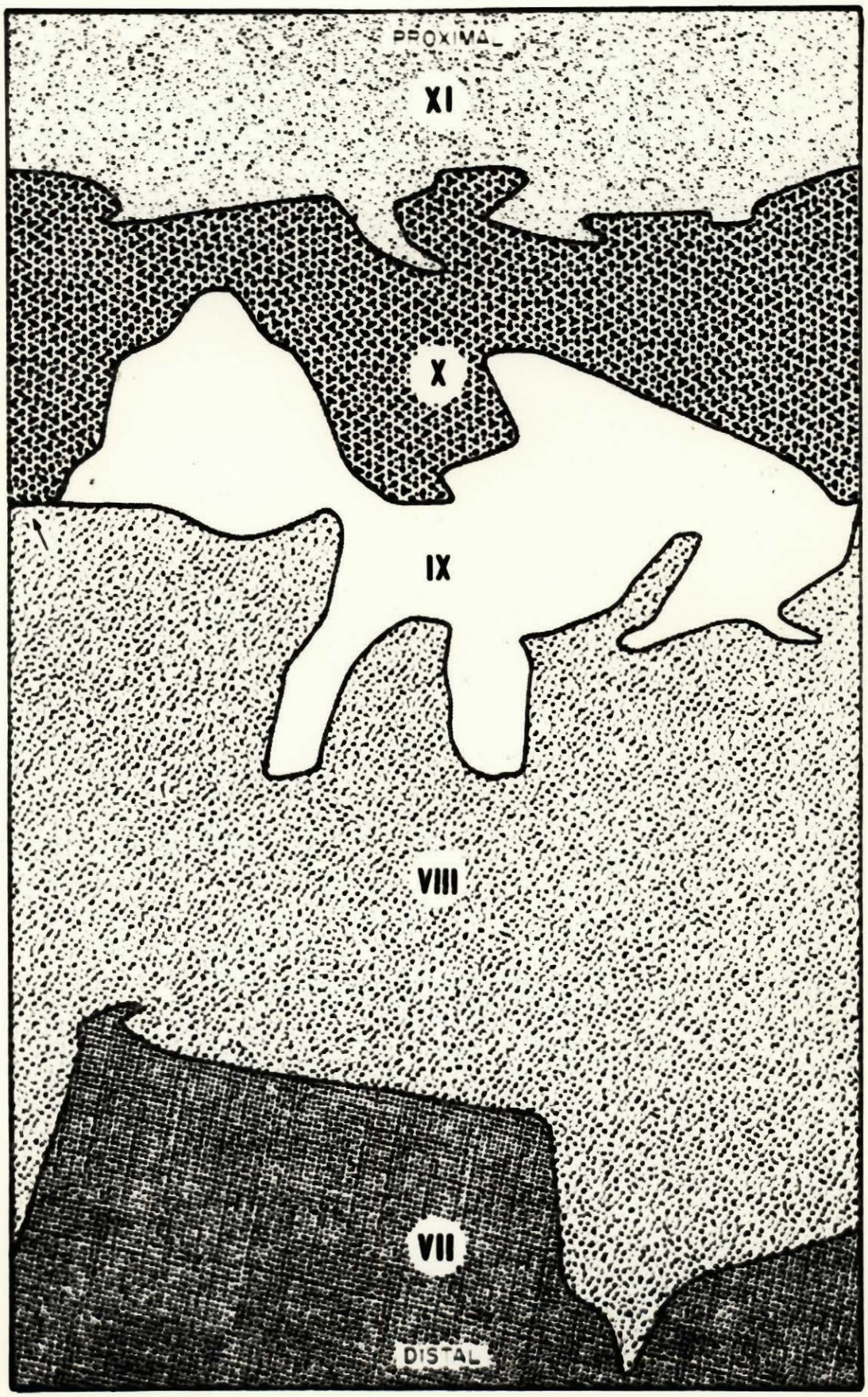


FIG. 15A

suggestive of a spiral could be detected. The continuity of the segmental order was again noted, even at the level of the bifurcations of the branched tubules. As already mentioned, an extremely short segment could be occasionally found to occupy only part of the circumference of the tubule. In figure 15A for instance, there is a small area marked by an arrow, which is not covered by segment IX, thus bringing segments X and VIII in contact. This failure of a segment to occupy the entire tubular circumference was found to occur in segments measuring less than 100 μ in length, (usually segments III,IV, IX,X,XI, or XIV; see table 1).

The histological transition between adjacent segments was found to be smoothly progressive in the majority of cases, while in other instances the transition was abrupt due to the absence of an intermediate type of subsegment, or of part of a segment (arrow on fig. 15A).

Finally, while precise delineation of the segments was always easy for some types of segments (XIV), it was more difficult for others (V,VI,VII, VIII). This depended on whether the histological criteria defining the segmental type could be identified with great precision. Thus, the presence of step 1 spermatids in segment I always permitted pin pointing of the XIV-I boundary. On the other hand, the degree of rotation of the young spermatids, which is difficult to assess accurately always made location of the VII-VIII boundary less precise.

Everything which was just said about intersegmental transitions was found to apply to transitions between subsegments. However, since subsegments were shorter than segments they failed more often to occupy the entire tubular circumference. As a result, whereas continuity of the segmental order was always present, the continuity of the subsegmental order was sometimes broken by the lack of one or even two subsegments. Subsegmental discontinuity was always more easily recognized at the level of segments XIV (fig. 15B), for the above mentioned reasons.

3. <u>The" descent" of the segmental order over the entire tubules. The "site of reversal"</u>.

The general pattern of the segmental order was again found to descend away from the rete testis in all tubules. This was also true at a distance from the rete. However, since most tubules have two extremities which both open into the rete testis, one would expect to find along the tubules a point where the two descending segmental orders meet. This was indeed found to be the case. The area, which could always be sharply localized, consisted of an abrupt reversal of the segmental order. It was thus called the "site of reversal". The segmental order of an entire tubule was plotted on a curve in figure 16. The same principle as in figure 13 was used, except that the consecutive XIV to I waves (marked with arrows) were placed end to end for better visualization of the general curve.

Figure 15 B Map of a Segment XIV with Outline of its Subsegments

This map has been constructed as in figure 15A. A very short segment has again been selected. The definition of the subsegments is as follows:
XIVa: the large division figures of primary spermatocytes (meiotic prophase excluded)
XIVb: secondary spermatocytes, resting or in prophase
XIVc: the small division figures of secondary spermatocytes (prophase excluded)

Whereas the segments almost always extended around the complete circumference of the tubule, the subsegments often did not, as shown here. As a result, the continuity of the order of the subsegments was not always as evident at that of the order of the segments. Nevertheless, it will be noted here that XIVa tends to be near XIII, and XIVc tends to be near I.

Figure 15C Map of a Segment VI

The line of small circles represents the line of contact of the type B spermatogonia (below) with the newly formed primary spermatocytes (above). This landmark is taken from a peripheral layer of cells while the outline of the segments is taken from a central layer (i.e. the spermatids). The discrepancy between the two layers is evident : e.g. primary spermatocytes are seen in segment V, while type B spermatogonia are seen in segment VII.

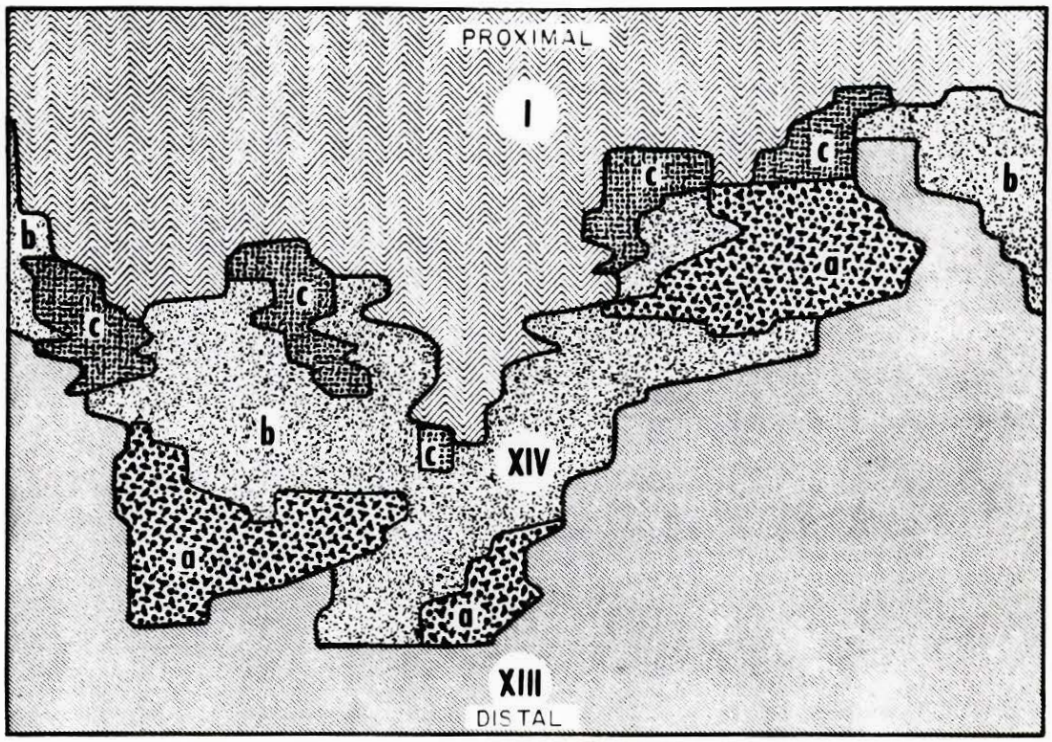


FIG. 15 B

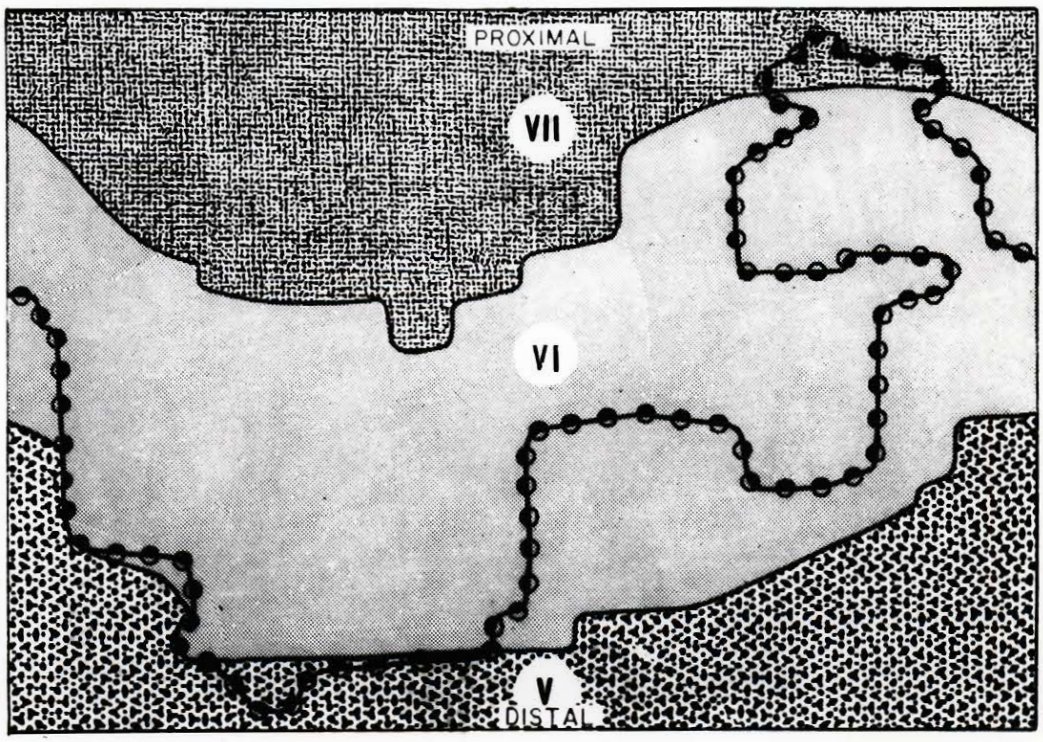


FIG. 15 C

Figure 16 Waves along a Complete Tubule

The same principle was used as for the curve in figure 13, except that the consecutive waves (marked with arrows) were placed end to end for better visualization of the general curve. The reversal site is seen at the lowermost point of the curve with the segmental order descending to it from both tubular ends. The site of reversal divides this tubule in two halves of almost equal lengths but of unequal number of waves.

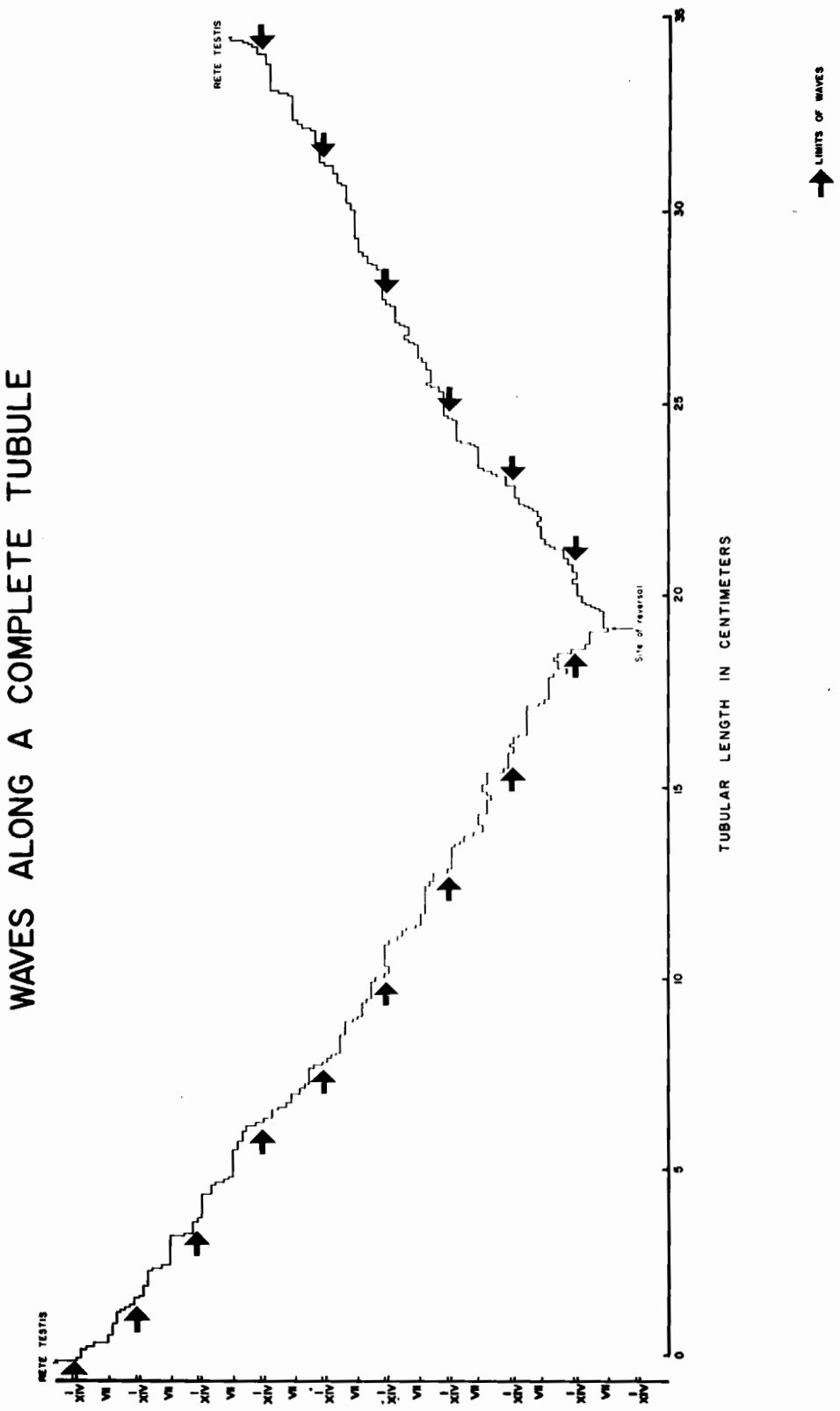
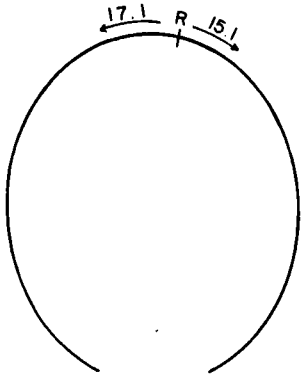

WAVES ALONG A COMPLETE TUBULE
RETE TESTIS
RETE TESTIS
Site of reversal
TUBULAR LENGTH IN CENTIMETERS
0
5
10
15
20
25
30
35
LIMITS OF WAVES

FIG. 16

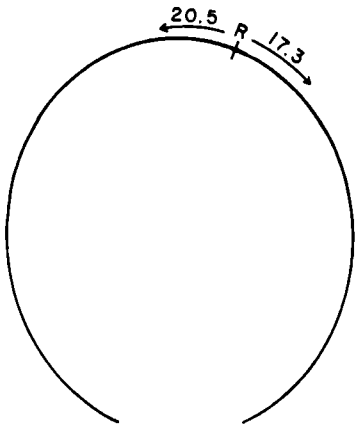
Figure 17 Diagrammatic Representation of the Various Types of Tubules

Of the 20 tubules reconstructed in three dimensions, 16 had an overall U shape with both ends opening into the rete testis (as typified by No 4 and No 5); one opened into the rete at one end only (No I); and the three remaining ones (No 2, No 3 and No 14) branched off so as to have three openings.

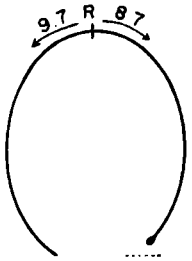
The sites of reversal are indicated (R) as well as their distance from the tubular ends in centimeters.



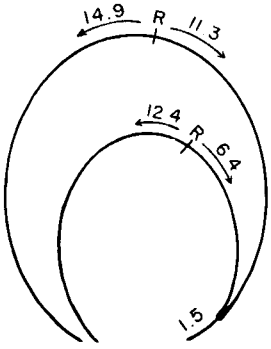
No 4



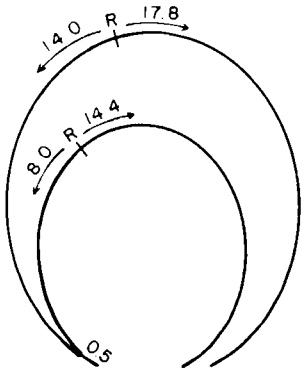
No 5



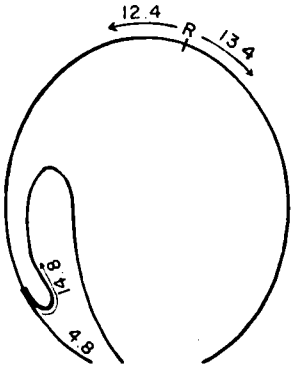
No 1



No 2



No 3



No 14

FIG. 17

The reversal site is seen at the lowermost point of the curve. Continuity of the segmental order was preserved in all of 22 sites of reversal examined. Furthermore, no histological peculiarity seemed to differentiate these areas from any other tubular regions. The distance from the site of reversal to the two extremities of the simple U-shaped tubules (table 3) was found to be approximately the same. Indeed, the site of reversal was separated from the mid-point of the tubule by only 1.6 cm (0.4 to 3.1) on the average, which is about 5% of the average tubular length. The findings for the three branched tubules are shown in fig. 17. Two of these tubules had two sites of reversal while the third tubule only had one. A site of reversal was also found in the blindly ending tubule, as shown on fig. 17.

The difference in the number of waves on each side of the reversal varied from 0 to 3 with an average of one (table 4).

4. Number of waves per tubule

The unbranched tubules had an average of twelve waves, but this varied widely from tubule to tubule (table 4).

5. Quantitative data on the wave lengths.

Systematic measurements of the length of the waves were carried out in twenty entire tubules (table 5). The mean length was 2.6 cm with extreme values of 0.2 and 6 cm. The average wave length per tubule varied from 1.9 to 3.2 cm.

A correlation between the length of a wave and its location within the tubule was obtained from table 5. In this table,

TABLE 3

POSITION OF THE SITE OF REVERSAL (IN 17 UNBRANCHED) SEMINIFEROUS TUBULES

Tubule number	Length of the tubule (cm)	Distance of the site of reversal to the rete testis (cm)		Distance of the site of reversal to the mid-point of tubule (cm)
		Longest side (L)	Shortest side (l)	$\frac{L-l}{2}$
1	18.7	9.7	8.7*	0.4
4	32.5	17.1	15.1	1.0
5	38.0	20.5	17.3	1.6
6	37.9	19.8	18.1	0.8
7	34.3	19.6	14.2	2.7
8	38.2	21.1	17.0	2.0
9	38.0	20.6	17.2	1.7
10	36.8	19.3	17.4	0.9
11	39.6	22.9	16.6	3.1
12	25.7	14.6	10.9	1.8
13	18.4	11.7	6.5	2.6
15	47.4	24.8	22.7	1.0
16	29.8	15.3	14.5	0.4
17	30.6	15.9	14.6	0.6
18	29.6	16.3	13.1	1.6
19	28.7	16.8	11.5	2.6
20	23.9	14.5	9.3	2.6
Averages	32.2			1.6 ± S.E.

* This end is blind but is considered here as if opening into the rete.

TABLE 4

NUMBER OF "WAVES" PER TUBULE

AND ON EACH SIDE OF THE SITE OF REVERSAL

IN 17 UNBRANCHED TUBULES

Tubule number	Total number of waves	Number of waves on each side of site of reversal	
		Longest side	Shortest side
1	9.9	5.5	4.4
4	13.1	7.8	5.3
5	15.4	7.5	7.9
6	15.3	7.7	7.6
7	14.9	8.8	6.0
8	13.0	7.4	5.6
9	10.7	5.1	5.6
10	13.2	7.1	7.1
11	13.8	8.4	5.4
12	8.1	4.0	4.1
13	7.9	4.3	3.6
15	15.0	8.4	6.6
16	10.6	5.7	4.9
17	10.5	5.9	4.6
18	12.6	6.9	5.6
19	11.9	6.4	5.6
20	9.8	5.3	4.4

Average 12.1

the wave lengths were recorded for each tubule on two (or more) lines, each line representing one side of the site of reversal. Each wave was placed in a different column according to its proximity to the rete (A,B,C,D) or to the site of reversal (a,b,c,d) whichever of the two was closer. Statistical analysis by the student method revealed that the first wave after the rete (A) was significantly shorter than the second one (B) with a p value < 0.001, whereas both of them were significantly shorter than all other waves ($p < 0.001$). No other differences were noted.

6. Quantitative data on the modulations.

The number of segments making up a modulation was called its amplitude. Examination of 510 modulations revealed an average amplitude of 3 segments per modulation; 56% of modulations were composed of one segment, 20% had three segments, 12% had more than five (up to 39). The average frequency of the modulations as expressed in number of modulations per unit of length of tubule was 0.7 modulation per centimeter.

Twenty percent of the waves did not show a modulation while 28% had one modulation, 20% had two, 15% had three and only 17% had more than three (up to 9).

The average amplitude and frequency (per cm) of the modulations is shown for each tubule in table 6. It was found that the frequency was highest in tubules that occupy the cranial (1 and 2) and caudal (12 and 13) poles of the testis. The latter two tubules also had the highest

Table 5 Legend

Wave lengths are shown for all complete consecutive waves seen in 20 tubules.

The wave lengths are recorded for each tubule on two (or more) lines, each line representing one side of the site of reversal. Each wave is placed in a different vertical column according to its proximity to the rete (A-B-C-D) or to the site of reversal (a,b,c,d) whichever of the two was closer.

A correlation between the length of a wave and its location within the tubule may be obtained from this table, It was found that the first wave after the rete (A) was significantly shorter than the second one (B) whereas both of them were significantly shorter than all other waves.

TABLE 5

LENGTH OF ALL WAVES (cm) SEEN IN 20 TUBULES

Tubules \ RETE	A	B	C	D	d	e	REVERSAL SITE b	a	Average wave length per tubule
1	1.8 1.4	2.3 1.6	 2.2				2.2 2.3	1.9 2.0	1.9
2 2B	1.1 1.0 1.9	1.7 1.8 2.2	1.8 3.2 2.3	2.4		2.5 2.6 2.1	3.2 2.4 3.1 1.9	2.3 1.9 1.6 2.1	2.2
3 3B	2.7 1.4 1.5	3.1 2.7 4.5 1.7	1.8 2.6 2.9	 3.8		2.1 3.7	2.9 2.1 3.6 1.0	1.4 2.4 2.9 2.2	2.5
4	1.7 1.4	2.3 1.8	2.4 2.3	 2.0		 1.6	3.4 2.7	4.4 2.3	2.6
5	0.7 1.9	0.9 3.5	1.9 3.1	3.5 3.0		2.5 2.4	2.3 3.3	2.6 2.7	2.4
6	1.2 1.7	1.1 3.7	2.1 1.6	3.1 2.0		5.5 2.2	1.6 2.8	2.1 3.0	2.4
7	1.2 1.5	2.2 2.9	3.0 3.3	1.2	2.3	3.2 2.7	2.3 2.0	3.2 2.0	2.3
8	1.6 2.2	2.5 2.3	2.2 2.4	 3.6		 4.0	5.9 3.3	3.6 2.3	3.0
9	2.8 2.1	2.8 3.3	4.7 5.9				3.5 4.6	2.4 4.0	3.6
10	0.2 2.5	1.5 2.1	3.0 2.6	3.1		4.1 3.3	4.3 3.2	2.8 3.6	2.8
11	2.4 1.4	1.9 1.5	3.8 2.1	 1.8	 3.5	 3.9	4.5 3.6	2.7 4.2	2.9
12	4.3 1.5	3.0 2.2					4.5 6.0	2.8 1.2	3.2
13	1.3 1.6	1.9 1.5					4.5	3.5 3.1	2.5
14 14B	1.0 0.7 0.9	1.5 1.6 1.1	3.1 3.3 2.2	 4.9	 5.8	 3.2	3.4 2.5	3.6 2.8	2.6
15	1.6 1.3	1.7 3.4	1.8 3.9	3.4	4.2	4.2 3.5	3.5 4.2	3.6 4.9	3.2
16	0.8 1.7	2.4 2.4	 2.9				3.5 2.5	5.4 3.2	2.7
17	1.2 1.1	2.3 2.8	3.1				2.5 3.4	3.3 5.0	2.7
18	2.0 0.9	1.8 1.7	3.7 2.6			 3.5	2.4 1.7	2.6 3.0	2.4
19	2.2 1.1	2.8 2.3	3.6 2.1			2.8	2.1 2.0	2.6 2.6	2.4
20	1.2 0.8	2.5 1.0	2.9				4.0 3.1	2.5 3.8	2.4
Average Wave Length	1.5	2.2	2.8	2.9	4.0	3.1	3.1	2.9	mean length for all waves 2.6

TABLE 6

FREQUENCY AND AMPLITUDE OF THE MODULATIONS

Tubule number	Average number of modulations per cm (frequency)	Average number of segments per modulation (amplitude)
1	1.2	3.0
2 + 2B	1.5	2.1
3 + 3B	0.8	3.1
4	0.9	1.6
5	0.9	1.5
6	0.8	3.3
7	0.7	1.8
8	0.9	2.4
9	0.6	2.2
10	0.5	2.2
11	0.7	3.7
12	1.4	6.9
13	1.3	7.8
14 + 14B	0.7	2.7
15	0.6	2.2
16	0.3	1.9
17	0.4	2.8
18	0.7	3.0
19	0.6	2.8
20	0.5	2.5
Average	0.7	3.0

amplitudes.

A study was made of the possible relation between the location of modulations and their amplitude (table 7) and frequency (table 8). Modulations near the rete showed no statistically significant differences from those near the reversal site in either amplitude or frequency.

The existence of large modulations containing smaller ones was noted. About 30% of the modulations (with an amplitude of 5 or more segments) contained one or more small modulations. Such modulations, inside larger ones, usually had a small amplitude (3 segments or less), but were found to have as many as 9 segments.

7. Orientation of the tails in the tubular lumen.

It has already been observed in the longitudinal sections that the tails of maturing spermatids seen in segments V-VI-VII and VIII (steps 17-18-19- of spermiogenesis) always pointed away from the rete testis (fig. 14). In the serial sections, the direction of the tails could only be observed in the hairpin turns taken by the tubules. Of all such locations to be examined, 125 occurred at the site of a segment V,VI, VII or VIII. In everyone of these cases, the tails were pointing toward the reversal site (i.e. away from the rete) regardless of their distance from, or proximity to, the reversal site. (One such case was 0.4 mm from the reversal site while 11 cases were less than 5 mm away from it).

TABLE 7

AVERAGE NUMBER OF SEGMENTS PER MODULATION
AT THE LEVEL OF THE FIRST TWO WAVES
(AMPLITUDE)

Tubules	Starting from the reversal	Starting from the rete
1	3.00	2.75
2	1.36	3.20
3	2.23	4.38
4	1.67	1.25
5	1.33	1.44
6	3.36	3.80
7	2.25	1.00
8	2.82	2.00
9	2.78	1.67
10	2.40	1.67
11	3.67	7.67
12	6.77	5.00
13	1.03	2.43
14	1.00	2.50
15	1.91	2.33
16	1.00	3.00
17	1.67	2.33
18	2.71	2.33
19	1.33	4.00
20	1.00	4.20
Average	2.26	2.95

TABLE 8

NUMBER OF MODULATIONS PER CM
AT THE LEVEL OF THE FIRST TWO WAVES
(FREQUENCY)

Tubules	Starting from reversal	Starting from rete
1	1.57	1.27
2	1.19	1.03
3	0.70	0.99
4	0.94	1.13
5	1.11	1.30
6	1.15	0.65
7	0.85	0.78
8	0.73	0.94
9	0.62	0.27
10	0.72	0.47
11	1.01	0.42
12	1.79	0.82
13	1.36	1.14
14	0.49	0.60
15	0.70	0.38
16	0.21	0.55
17	0.42	0.41
18	0.72	0.47
19	0.65	0.72
20	0.30	0.92
Average	0.86	0.76

Therefore, the tails were usually pointing towards segments of descending order, except at the level of the modulations (37 cases examined) where they pointed towards segments of ascending order.

The tails of maturing spermatids were also oriented within the tubular lumen in a helicordal or whorl-like fashion (fig. 18). Moreover, they always went in a clockwise direction after leaving their respective nuclei.

Figure 18 Cross Section of a Tubule Showing the Helicoidal Direction of the Tails

PAFSA - Hematoxylin Mag X 340

The seminiferous epithelium shown here is at stage VIII. The tails of maturing spermatids were always oriented within the tubular lumen in a spiral fashion. Moreover, they always went in a clock-wise direction after leaving their respective nuclei. This is believed to be due to the inner structure of the tails.

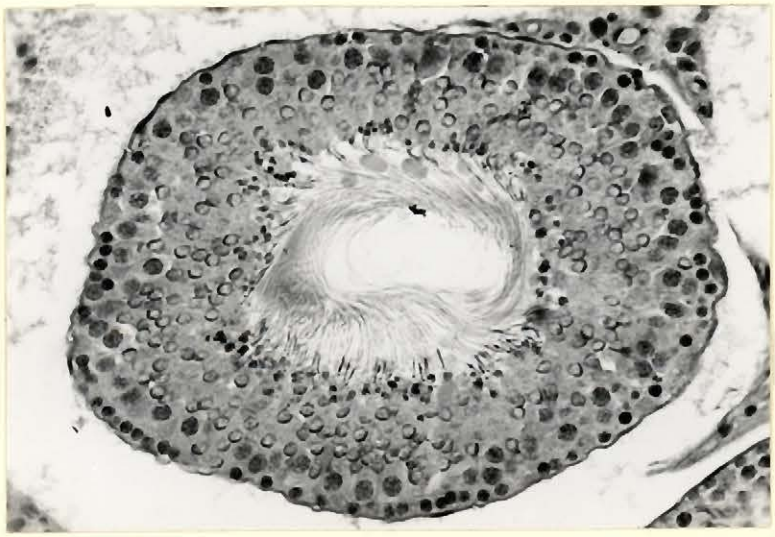


FIG. 18

DISCUSSION

A. Criticism of Von Ebner's work

Von Ebner was the first one to recognize the existence of complete series of cellular associations along the tubules (1871). He was also the first one to compare this arrangement to a wave. However, since he failed to recognize the modulations, it is difficult to interpret his data on the wave lengths (average of 3.2 cm with extremes of 2.5 and 3.8 cm).

B. Criticism of Regaud's theories

It is a popular misconception that Regaud made a thorough study of the wave. The fact is that he actually made few direct observations pertaining to the wave. Like Von Ebner, he was impressed by the perfect continuity of the segmental order, but like him, he did not recognize the modulations. Regaud offered no evidence to support his contention that "the wave is in space what the cycle is in time". As for the theory that the wave follows a spiral course around the tubule, he only offered indirect evidence. Both theories will now be refuted:

1. It becomes obvious that the wave is <u>not</u> in space what the cycle is in time:

Whereas the sequence of the stages of the cycle is irreversible, the existence of modulations points to the reversibility of the order of the segments.

Whereas the duration of the cycle is remarkably constant, namely 12.0 ± 0.2 days (Clermont et al '59), the wave length varies considerably (table 5).

Whereas each stage of the cycle has its own constant duration (Clermont et al '59), all segments of the same type vary considerably in length (table 1). Furthermore, the length of a given segment gives no precise indication of the length of the wave in which it is located. (For example, at the top of figure 13, a long segment XIV is seen in the short second complete wave while a shorter segment XIV is found in the long fourth complete wave.

2. There is no spiral wave:

No evidence of a spiral wave was found on the maps of figure 15 as well as on many other maps not presented here. Regaud created the idea of a spiral wave in order to reconciliate his concept of a perfect wave with the presence of abrupt changes in the seminiferous epithelium of the type shown in map 15 A (arrow). He also saw in the spiral wave an explanation for the corkscrew orientation of the tails of maturing spermatids. No correlation could be found here between the "wave" and this orientation of the tails. Another explanation for the corkscrew orientation of the tails will be offered later.

C. Criticism of the work of Curtis and others

Curtis was the first author to identify the modulations. However, as previously mentioned, his description of the wave and modulations is confusing. He also described the descent

of the segmental order, the reversal site and the short waves near the rete testis. His average of the wave length in the mouse is 1.83 cm with extremes of 1.2 cm and 3 cm.

Several authors (Benda 1887, Furst 1887....) have reported the presence of waves in many other mammalian species (guinea pig, rabbit, bull, ram, boar, dog, cat, marsupials.....). Cleland ('51) however, did not find any evidence of a wave in the guinea pig.

D. Changes taking place along the tubules with time

The events taking place in time in any one area of a seminiferous tubule are well known: The seminiferous epithelium undergoes the rigid sequence of changes which characterize the cycle. It thus becomes possible to visualize what will happen to long portions of tubules containing several segments.

Let us follow for instance the fate of three consecutive segments seen along a fragment of tubule (figure 19). The duration of each of the three corresponding stages (IX-X-XI) is about 3% of the cycle (Leblond and Clermont '52), that is, about 7 hours (Clermont et al '59). Stage XII on the other hand lasts about 30 hours. It results (figure 19) that after 7 hours, the portions of tubule previously occupied by segments IX-X and XI will be respectively replaced by segments X-XI- and XII. Another 7 hours later the segmental types occupying the same portions of tubules will have changed

Figure 19 Fate of the Segments with Time

Three consecutive segments (IX- X- XI) seen along a tubule are followed as cycle goes on in every part of the seminiferous epithelium. The duration of each of the three corresponding stages (IX- X- XI) is about 7 hours while stage XII lasts about 30 hours. After 7 hours the portions of tubule previously occupied by segments IX-X and XI will be respectively replaced by segments X-XI- and XII. Another 7 hours later the segmental types occupying the same portions of tubules will have changed again, and so on. If the boundary between adjacent segments (such as the XI-XII boundaries) is followed, it is seen to change location with time. Indeed, segmental boundaries are transferred along the tubule in the direction of the descent of the segmental order.

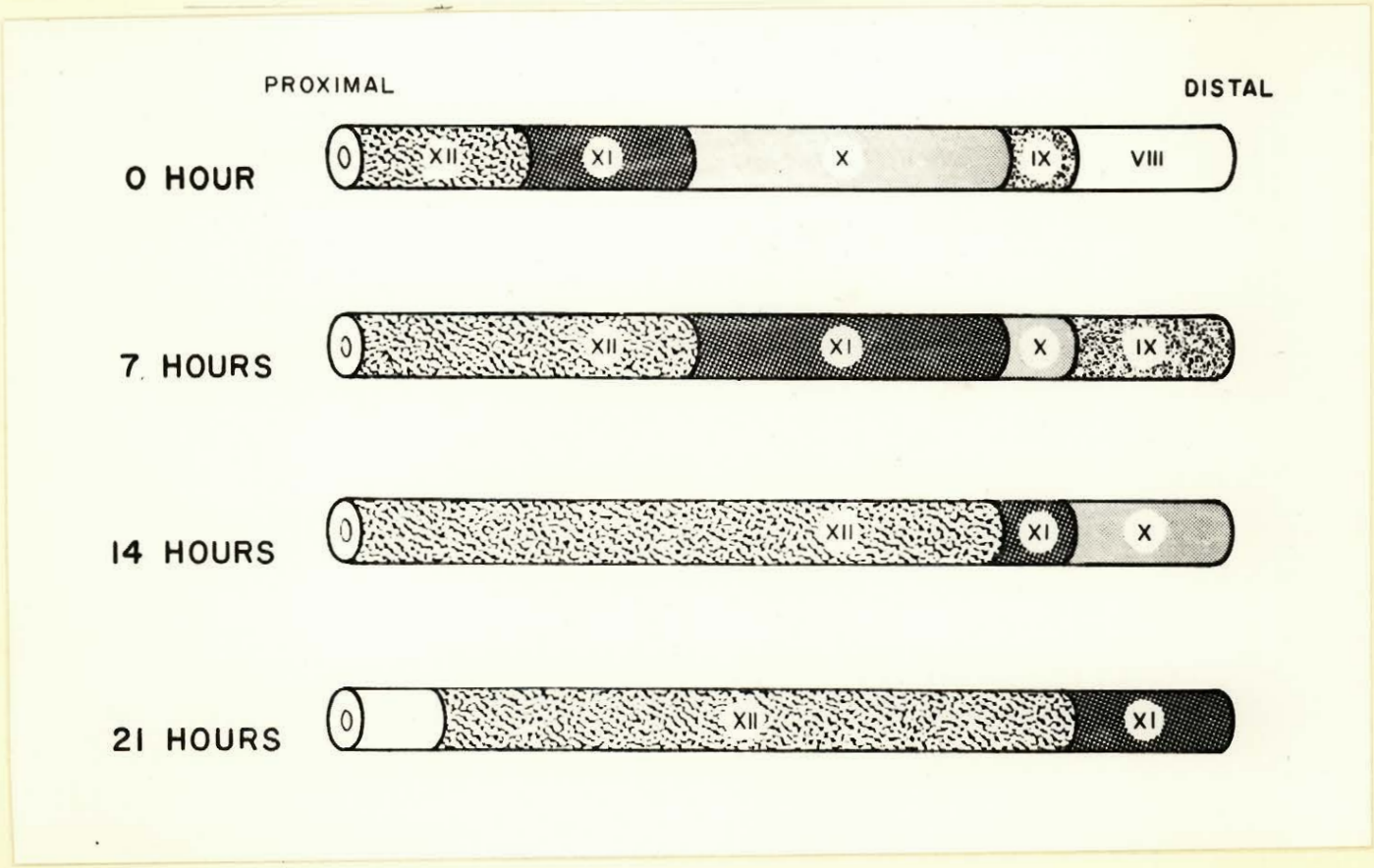

PROXIMAL
DISTAL
0 HOUR
XII
XI
X
IX
VIII
7 HOURS
XII
XI
X
IX
14 HOURS
XII
XI
X
21 HOURS
XII
XI

FIG. 19

again, and so on. If the boundary between adjacent segments (such as the XI-XII boundaries) is followed, it is seen to change location with time. Indeed, segmental boundaries are transferred along the tubule in the direction of the descent of the segmental order. The transfer of the segmental boundaries is not, however, as abrupt as figure 19 might suggest. It has been shown (figure 10) that pregressive histological changes always existed between both ends of each segment. It results that the transfer of the segmental boundaries proceeds relatively smoothly along the tubules. In figure 20 for instance, the VII-VIII boundary moves progressively along the portion of tubule originally occupied by segment VII.

It will be noted on figure 19 that the part of the tubule which originally displayed three segments (IX-X-XI) is occupied 21 hours later by one segment only (XII), or more exactly, by part of this segment. Conversely, the same part of tubule will again display several segments during the course of the following cycle. This is entirely due to the fact that stage XII lasts longer than IX, X and XI combined.

Another observation to be made is that any given segment followed in time in its travel along the tubule will be found to vary greatly in length. In figure 19, for instance, segment XI becomes longer then shorter depending on the part of the tubule which it happens to occupy.

Hence, it is now clear that a segment is a labile entity changing with time in size and location.

Figure 20 The Smooth Transfer of the Segmental Boundaries Along the Tubule

The transfer of the segmental boundaries is not as abrupt as suggested by figure 19. The presence of a continuous subsegmental order across each segment will cause the boundaries to move in a smooth manner rather than by abrupt jumps. A segment VII is illustrated here with its subsegments. The duration of the corresponding stage (VII) is about 64 hours.

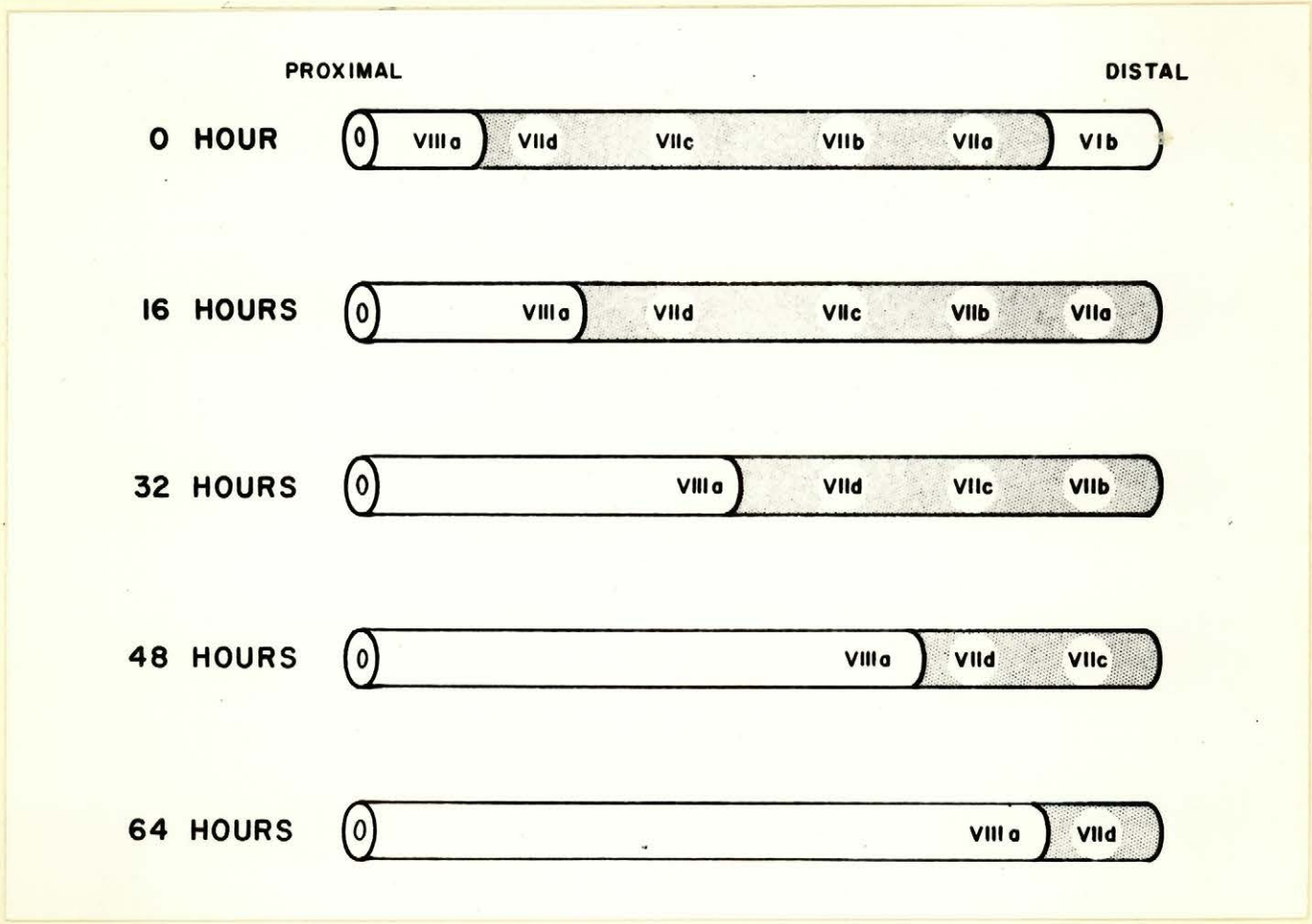

PROXIMAL
DISTAL
0 HOUR
0
VIIIa
VIId
VIIc
VIIb
VIIa
VIb
16 HOURS
0
VIIIa
VIId
VIIc
VIIb
VIIa
32 HOURS
0
VIIIa
VIId
VIIc
VIIb
48 HOURS
0
VIIIa
VIId
VIIc
64 HOURS
0
VIIIa
VIId

FIG. 21

The changes taking place in time at the level of each modulation will now ve considered. Modulations are made of two halves, the first half is an ascent and the second half is a descent of the segmental order (figure 12). Since segments have just been said to travel in the direction of descent of the segmental order, there will be a site of convergence at the beginning and a site of divergence at the mid point of each modulation. A site of convergence and a site of divergence will also exist at the level of the subsegmental modulations.

We have seen how the number of segments occupying a given portion of tubule will vary in time (figure 19). It results that modulations will vary in amplitude (i.e. the number of segments) as time goes on. Indeed some will disappear completely at certain time intervals (e.g. a modulation involving only segments IX-X and XI at one time will later be entirely "masked" by a segment XII or VII). Such "masked" modulations will only be detectable at the subsegmental level (figure 10B). This partial or complete masking of the modulations is again entirely due to the fact that some stages of the cycle last longer than others. Also, more modulations would be detected if more stages were used to divide the cycle, and consequently if more segments were used to divide the wave.

It has been said previously that the segments travelled along the tubules. It must be made clear that this travel which results from the continuity of the segmental order, only refers to the <u>appearances</u> of the <u>types</u> of the cells. The cells

themselves remain in the same area as their ancester cells and therefore do not move along the tubule until they are released in the tubular lumen as spermatozoa. In other words, if a live tubule could be examined in vitro for a period of time, such histological events as sperm release, or mitotic activity would be seen to spread along the tubules like a ripple at the surface of the water (similarly, the molecules of water move up and down but are not carried along the surface of the water by the ripple). This phenomenon could be most suitably described as the "wave" of the seminiferous tubule, Unfortunately, the term has already been used in previous studies as well as in the present one to refer to a series of segments. The dynamic phenomenon just described will instead be referred to as the "motion of the wave". Thus, over most of the tubule the motion of the wave spreads toward the reversal site except at the level of each modulation where it goes in the opposite direction.

Let us now discuss the velocity of the motion of the wave. If we consider for instance (figure 20) a portion of the tubule occupied at one time by a given segment (VII), we know that it will always take the same time (64hours) for the impulse of the wave to travel across this portion of tubule, regardless of the type of segment by which it is occupied. Since we also know that all segments VII seen at the same time on this particular tubule vary greatly in length, it results that each portion of tubule has its own "motion" velocity. This feature of the tubule was called the slope of the wave. A curve of this

slope for a tubule would be similar to the curve on figure 16. On this curve, regions with a steep slope would indicate a slowly travelling motion of the wave while a small slope gradient would indicate a fast travelling motion. The mean slope for the 20 tubules studied here in cross sections can be estimated, Knowing that it takes 12 days for the wave motion to travel from one end of a wave to the other (average wave length - 26mm), the mean slope will be about 2.1 mm / day. This value should actually be reduced by 12% to allow for the modulations which were found to occupy an average of about 12% of the wave length. One of the slowest velocities to be recorded was of the order of 56 μ / day in the case of a segment XII measuring only 70 μ in length (the duration of stage XII is about 30 hours).

E. <u>The continuity of the segmental order</u>

The continuity of the segmental order is one of the most remarkable features of the seminiferous epithelium. We have seen that it remains unbroken at the level of the modulations, the reversal sites or the bifurcation of the branched tubules. However, as shown on figure 15B, the <u>sub</u>segmental order was not found to present such a perfect continuity as the segmental order. Nevertheless, contiguous areas of seminiferous epithelium were never too different in their respective degrees of maturation. Indeed, when focussing attention of the first layer of spermatids (which was used for the identification of the cellular associations) it was found that adjacent cells of this layer were usually

almost identical in their degree of maturation, the greatest difference recorded being equivalent to about 14 hours of spermiogenesis. Meanwhile, it must be realized that adjacent spermatids of the same layer must often descend from separate stem cells. It is remarkable that after 15 cycles (6 months) or more, two neighbouring stem cells have actually given rise to the same number of cycles ± 0.022 cycle (7 hours). Such extreme precision (1/700) would require either an unbelievably constant duration of the cycle, or an unknown factor coordinating the division of neighbouring stem cells. This writer would tend to favour the second possibility although there is no definite evidence to support this view.

F. Speculations on the establishment of the wave

Whatever the method of maintenance of the segmental continuity, the mechanism of its establishment remains problematic. Many questions come to one's mind: Should we only see in the wave a sheer consequence of the growth pattern of the tubule? Or does it become established through the action of a "spermatogenesis-initiating factor" spreading gradually throughout the growing tubule?

Other questions arise concerning the modulations. For instance, do the modulations also reflect the growth pattern of the tubule? Or do they develop progressively on waves which were previously free of modulations?

The wave as we know it may be explained by two kinds of hypotheses, which although largely speculative, may help in guiding future experimental work: 1) The wave pattern would be set once and for all during early development, that is, in fetal life or soon after birth (embryological theory). 2) The wave pattern would appear and be maintained as a result of interactions between neighbouring segments (coordination theory).

1. The embryological theory. Clermont and Perey ('57) found that at birth the seminiferous tubules have a uniform content of gonocytes and supporting cells; and soon thereafter, they observed what may be the first sign of the initiation of the wave pattern, that is, scattered groups of type A cells arising from gonocyte mitoses. From these cells, spermatogenesis starts. The embryological theory proposes that each area of the tubule occupied by these elements or their progeny constitutes the oldest segment of a future wave; that on their distal side, a new type A cells in turn appear, which constitute the next oldest segment and that in the same manner younger and younger segments be added gradually. This principle is illustrated on figure 21 where such a theoretical growth center is adding new portions of epithelium toward the rete. The newly formed epithelium would immediately enter spermatogenesis, thus producing a continuous and descending segmental order. It would appear from preliminary observations in 12 and 15 day-old rats (Clermont and Huckins, unpublished) that the waves, although not clearly distinguishable, may already be established at that

Figure 21 The Embryological Theory: Creation of a Wave by a "Growth Center"

Shortly after birth, a theoretical growth center is every day adding new portions of epithelium toward the rete. Each portion enters spermatogenesis shortly after being formed. Assuming that the duration of the cycle is 12 days as in the adult, a wave is formed after 12 days. From then on, the stem cells follow their cyclic evolution.

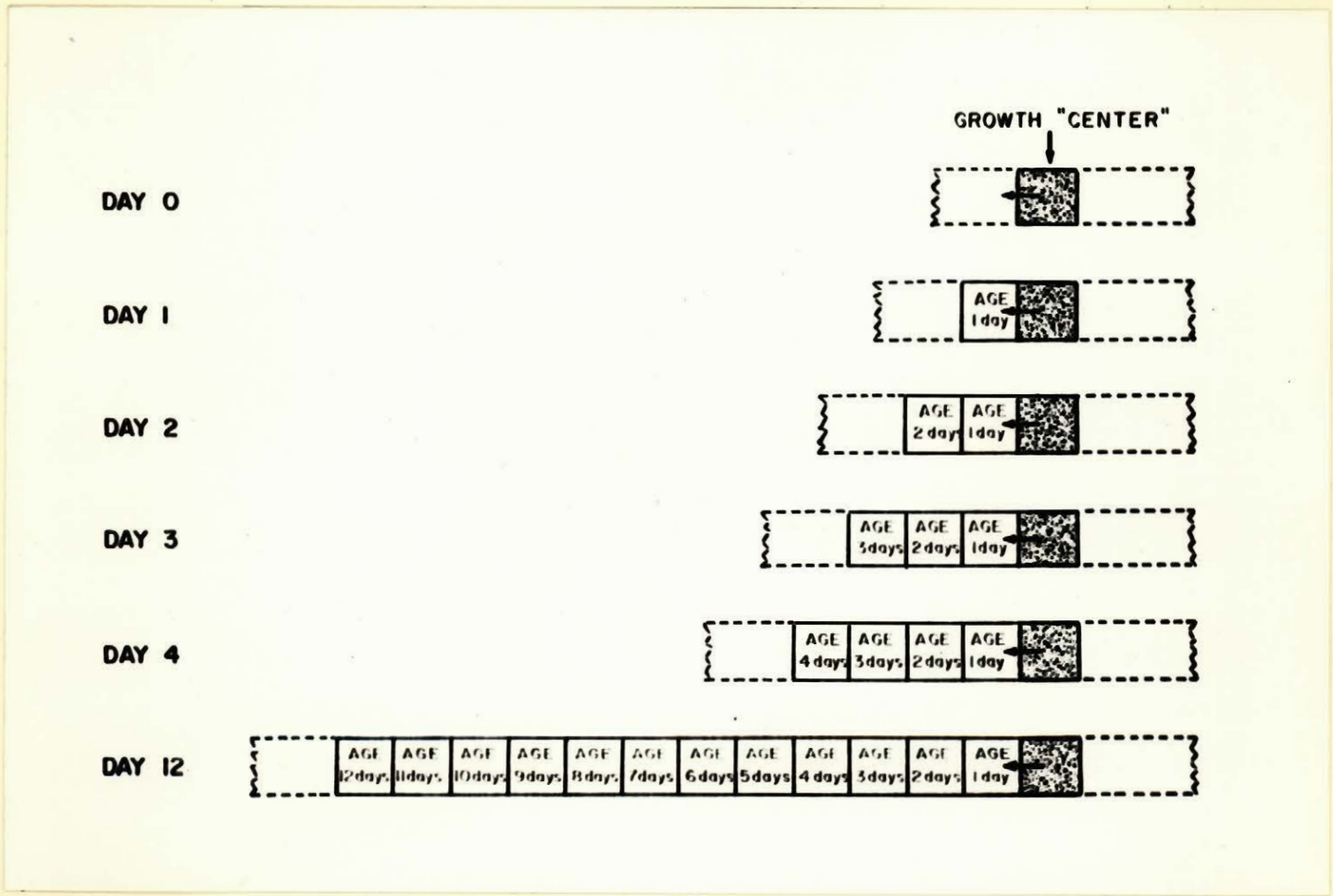

GROWTH "CENTER"
DAY 0
DAY 1
AGE 1 day
DAY 2
AGE 2 days
AGE 1 day
DAY 3
AGE 3 days
AGE 2 days
AGE 1 day
DAY 4
AGE 4 days
AGE 3 days
AGE 2 days
AGE 1 day
DAY 12
AGE 12 days
AGE 11 days
AGE 10 days
AGE 9 days
AGE 8 days
AGE 7 days
AGE 6 days
AGE 5 days
AGE 4 days
AGE 3 days
AGE 2 days
AGE 1 day

FIG. 21

age. In fact, each tubule would contain approximately as many waves as in the adult. Consequently, the embryological theory will have to portulate as many growth centres in each tubule as there are waves (figure 22). The stem cells would follow their cyclic evolution as in the adult by issuing a new generation every 12 days. In order to obtain a continuity of the segmental order throughout the tubule one would have to suppose that the growth centers stop to produce after 12 days of activity. According to the above mentioned authors, even modulations may exist at 12 days . If so, modulations would arise just as the wave pattern is set, some segments of epithelium presumably entering spermatogenesis with some delay.

It is difficult to account for the maintenance of the wave pattern throughout life on the basis of the embryological theory. As previously mentioned, it would suppose an extremely constant duration of the cycle ($\pm$ 1/700= $\pm$ 25 minutes).

2. <u>The coordination theory</u>. A hypothetical factor, probably chemical in nature, would spread along the growing tubule, initiating spermatogenesis on its way. This factor could be released by certain cells such as the type A spermatogonia at a given stage of the cycle. This mechanism would persist throughout life and be responsible for the maintenance of the continuity of the segmental order.

Figure 22 The Embryological Theory: Creation of a Continuity of the Segmental Order Throughout the Tubule

As many growth centers are postulated as there are waves. After 12 days of activity they stop producing, thus giving rise to continuity of the segmental order from one wave to the next.

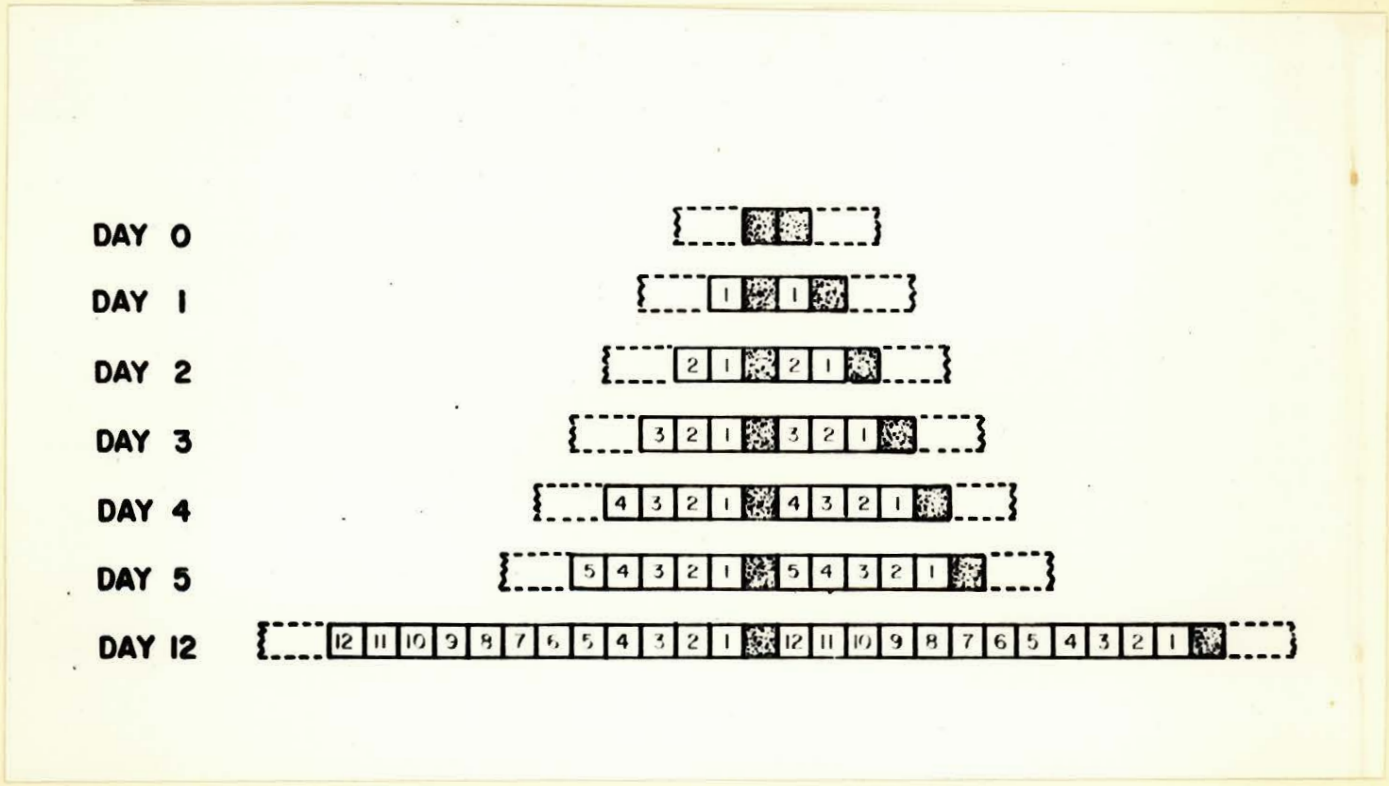


FIG. 22

G. Variations in the Composition of the Cellular Associations

It will be recalled that the criteria used in the identification of the types of cellular associations were the steps of spermiogenesis (except for stage XIV). The maps of figure 15A and C are therefore essentially maps of spermatid distribution. But figure 15C also shows the boundary line between type B spermatogonia, and resting primary spermatocytes (line of circles). This line is a landmark taken from the peripheral layer of the seminiferous epithelium and affords a comparison with the state of maturation of a central layer. We know that two full cycles are required for the newly formed primary spermatocytes to become step 6 spermatids. A significant discrepancy between these two layers is obvious since a few primary spermatocytes are already present at the end of segment V (i.e. in the presence of step 5 spermatids) while a few type B spermatogonia are still seen at the beginning of segment VII (i.e. in the presence of step 7 spermatids). The make-up of the fourteen different types of cellular associations is thus not rigidly constant and allows for some overlap between types. One possible explanation for this finding is that the duration of the spermatogenesis may vary from one generation of cells to the next. Another possibility is that the stem cells (type A spermatogonia at stage IX) do not divide at extremely constant intervals. A third and more likely possibility is that the concentric cellular layers of the epithelium may slide slightly over one another during spermatogenesis.

H. The Travel of Spermatozoa in the Tubular Lumen

Free spermatozoa seen in the tubular lumen in longitudinal sections did not appear to have any definite orientation, their heads pointing either toward the rete testis or away from it. Sometimes they were seen in disorganized bundles. Although these observations may represent an artefact, they are definitely in favour of a passive method of transport of the spermatozoa.

It should be mentioned that the only blind end of tubule found in this study presented a terminal fusiform dilatation which was filled with a large number of degenerating spermatozoa (figure 23). These spermatozoa must have migrated in the wrong direction after release and become trapped there. In this tubule, the site of reversal was also present (tubule number 1 in figure 17) with a segmental order descending towards the blind end. This last observation argues against the possibility that spermatozoa are passively carried along the tubular lumen by a flow of fluid. It is possible that the point of reversal which was shown to determine the orientation of the tails of mature spermatids may also in some way determine the direction followed by the spermatozoa after their release.

I. The Orientation of the Tails of Implanted Spermatids

We have seen that the tails of spermatids point toward the site of reversal from the time they reach step 17 of spermiogenesis (stage V). This pattern is unaffected by the presence of modulations.

Figure 23 The Blind End of a Tubule

The only blind end found among 20 tubules is shown here. As shown on figure 17 (tubule No I) this tubule presented a site of reversal. The hyperchromic mass represents degenerated spermatozoa which have accumulated after travelling in the wrong direction.

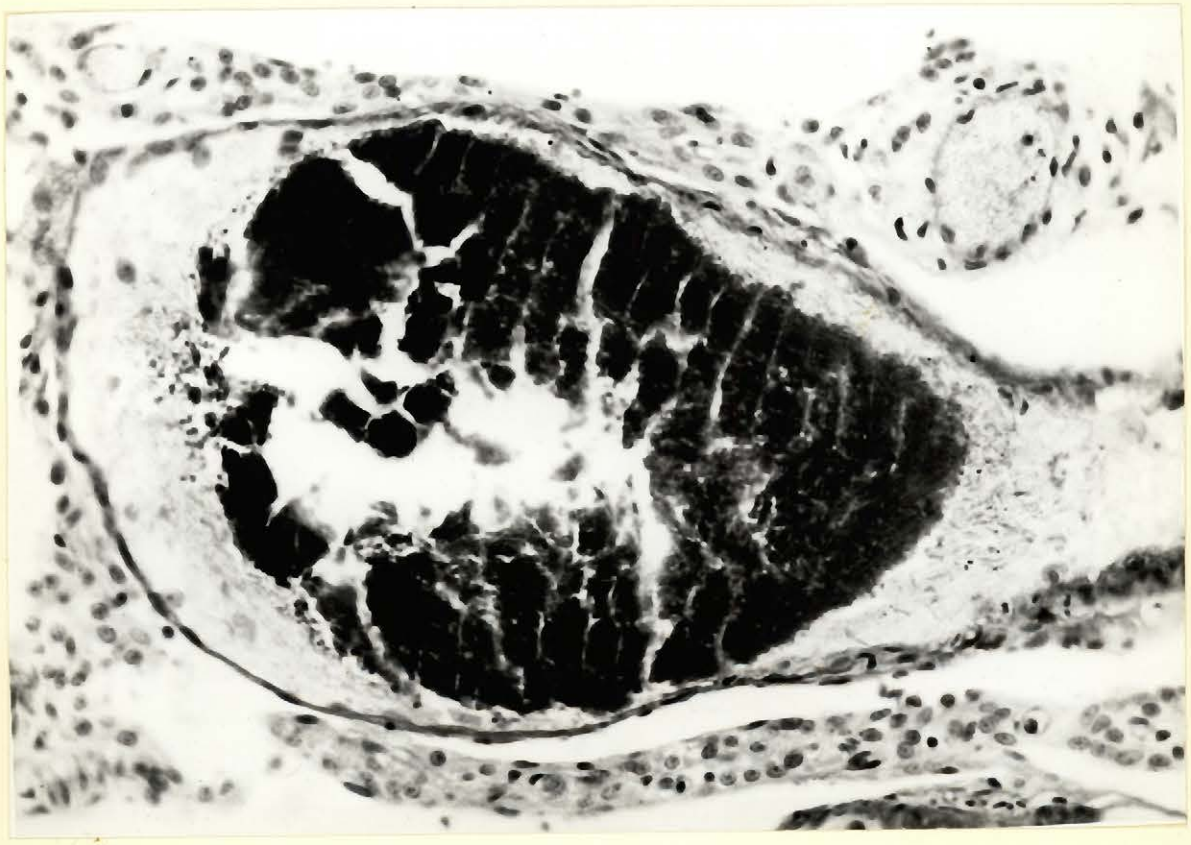


FIG. 23

Two different mechanisms could explain this orientation, namely, an active or a passive one. In an active mechanism, the spermatids would be able to detect in what direction the site of reversal lies and would bend accordingly. This ability would develop at step 17. Free spermatozoa are known to advance head first through the whipping action of their tail. The position taken by the spermatids could well indicate that they are already trying to move out of the tubule on their own with their head facing the rete and their tail toward the site of reversal.

A passive mechanism would require the existence of a continuous flow of fluid inside the tubular lumen. However, this theory is unlikely because the tails of the younger spermatids are not oriented in any constant direction. Also, a flow of fluid strong enough to bend the tails of spermatids would be more likely to be directed toward the rete rather than away from it.

J. Lack of Synchronization of the Cycles in Adjacent Tubules

This question is always raised and therefore should be answered. A given stage (XIV) was selected for this study which was carried out on cross sections of entire testes. The stages of all tubules touching this particular stage were recorded,- statistical analysis of the results showed no tendency for contiguous portions of adjoining tubules to be at the same stage of the cycle.

K. Spiral Arrangement of the Tails of Implanted Maturing Spermatids

It has previously been mentioned that the tails of maturing spermatids were always oriented within the tubular lumen in a helicoidal or whorl-like fashion (figure 18). Moreover, they always went in a clock-wise direction after leaving their respective nuclei. Regaud used this finding to support his theory of a spiral wave. However, no spiral wave could be demonstrated in the present study and it appears more likely that the peculiar helicoidal disposition of the tails is due to their intrinsic structure. It is well known indeed from electron microscopy studies that the tails contain themselves several spirally arranged filaments.

SUMMARY

Various authors have made, at one time or another, important observations on the pattern of the morphological features seen along the seminiferous tubule. These observations have led to the concept of the "wave" of the seminiferous epithelium. However, this consept has been interpreted differently by each observer, and available definitions of the wave are contradictory or obscure. The common feature to all previous studies is the lack of extensive data. It has been the purpose of the present study to reexamine the pattern of the histological features seen along the tubules in the light of new techniques and comprehensive quantitative data.

Two methods were used: one method made use of longitudinal sections of dissected portions of tubules obtained from five different animals, while in the other method serial cross sections of a whole testis were prepared. All sections were stained with PAFSA and hematoxylin. The cellular associations were classified into 14 types as originally defined by Leblond and Clermont. Whenever indicated a more extensive classification into 28 types of cellular associations was used. This more detailed classification was obtained by subdividing 9 of the 14 original types into subtypes.

Each portion of tubule occupied by one type of cellular association was called a segment and was identified by the number (I to XIV) of its cellular association. e.g. "segment VIII". Segments of each type were found to vary widely in length.

It was found that any two adjacent segments always carried consecutive numbers (with the only exception of XIV and I which often followed each other along the tubules). This feature was referred to as the "continuity of the segmental order".

As one followed the tubule away from the rete testis, each segment was usually succeeded by one with a less advanced type of cellular association (i.e. witha smaller number). As a result, the overall pattern of the segmental order was descending away from the rete testis. This pattern was thus called the "descent of the segmental order".

Most tubules have been found by Clermont and Huckins to have a U shape with both ends opening into the rete testis. Accordingly, the segmental order was found to descend from each end of the tubule as far as a meeting point called the "site of reversal". This site was usually located at approximately equal distance from each tubular end.

Throughout the descending pattern there were areas where the order was ascending over a short

distance. These variations in the pattern were called "modulations of the segmental order". Whenever the order was ascending away from the rete a modulation was said to begin. The modulation was ended in such a way that it contained an equal number of ascending and descending segments. There was an average of 3 segments per modulation with extremes of 1 and 39.

The overall descent of the segmental order was always obvious in spite of the modulations. It was divided into units called "waves" which were defined as "the shortest series of adjacent segments in which the 14 possible types of segments are seen in addition to those making up the modulations".

There was an average of 12 waves per tubule with extremes of 8 and 15. The average wave length was 2.6 cm with extremes of 0.2 and 6 cm. The wave length showed wide variations within the same tubule. Furthermore analysis showed that the wave closest to the rete testis was statistically shorter than the second closest wave, while both of them were statistically shorter than all other waves.

Twenty percent of the waves did not show a modulation while only 17% had more than 3 (up to 9).

The shape of the segmental boundaries was studied on maps reconstructed from serial sections. The boundaries assumed a wide variety of shapes, frequently sending finger like prejections into the segments, but

there was no constant pattern and no indication of a spiral wave as suggested by Regaud.

The other well known theory of Regaud that "the wave is in space what the cycle is in time" was also refuted.

The changes taking place in time along the tubule were discussed. The conclusion was drawn that segments are labile entities which change in length and location as time goes on. Indeed, the advent of any particular histological phenomenon or cell type would be seen to travel along the tubule in the direction of the descending segmental order. This travel of the histological landmarks was called the "motion of the wave".

Hypotheses are presented regarding the establishment of the wave in the young rat, as well as its maintenance throughout life.

Finally, the tails of the maturing spermatids were consistantly found to point toward the site of reversal.

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