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CONTRIBUTIONS TO THE MECHANISM OF SPERMATOGENESIS, ON THE BASIS OF STUDIES OF THE ACID-FAST STAINING, SUCCINIC DEHYDROGENASE ACTIVITY AND HISTOCHEMICAL EXAMINATION OF LIPIDS

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(Received June 6, 1960)

Research into the mechanism of spermatogenesis may be considered to have begun with V. EBNER's (1871) classical studies in rats. Since then, the rat has been the main object in the studies concerned with spermatogenesis, apparently due to the fact that it is in that animal that can best be observed the complicated processes taking place one after the other or simultaneously in the germinal epithelium of the seminiferous tubules. In most mammals the fundamental processes of spermatogenesis are identical and thus the results obtained for rats may be applied to a wide variety of species, with minor restrictions.

The process of spermatogenesis is known now in considerable detail, especially from the histochemical and electronmicroscopic studies of the past few years (LEBLOND and CLERMONT, 1952*a*, CLERMONT and LEBLOND, 1955, DAOUST and CLERMONT, 1955, HANSON et. al., 1952, KAYE, 1957, SOTELO et. al., 1958, BURGOS, 1955, YASUZUMI et. al., 1956, 1958). However, little is known about the mechanism, about the enzymes involved in the process. In some of our more recent studies we have shown that in the course of spermiogenesis, when the spermids are transformed into spermatozoa, an acid-fast component appears in the head and is in a close relationship to the morphological maturation of that past of the spermatozoon (PÓSALAKY and TÖRŐ, 1957, 1958, 1959). The present studies were intended to gain a deeper insight into the mechanism of this phenomenon. We have assumed spermatogenesis to be a high-energy process and as such, its indicator may be the activity of the enzyme dehydrogenase. We have therefore studied the behaviour of succinic dehydrogenase in the course of spermatogenesis.

Methods

Sexually mature Wistar rats weighing about 180 g each were killed by decapitation, the testes were removed, frozen and cut up into sections 10 and 20 μ thick in a cryostate of the Linde type. The sections were dried at room temperature for about 1,5 minutes, incubated without fixation in a neotetrazolium solution (BARKA, 1959), for from 30 minutes to 4 hours.

A period of 2 hours was found the most suitable. Some sections were stained with methyl green. The control sections were incubated for 2 hours in a solution containing 3 mg/ml malonic acid.

The method of acid-fast staining has been described earlier (PÓSALAKY and TÖRŐ, 1957), it consisted in staining with crystal violet, differentiating in acetic acid, then alcohol, and finally staining with Bismarck brown.

Lipids were studied by the acid haematein method of Baker (PEARSE, 1954).

Results

The results will be described according to the stages of spermatogenesis, as determined on the basis of the evidence published by v. EBNER (1871, 1888), CURTIS (1918), ROSEN-RUNGE (1950, 1955), LEBLOND and CLERMONT (1952a, 1955) and observed by us.

Stage 1. Spermatozoa are not visible in, or are just disappearing from, the tubular lumen. Granules, the so-called "residual bodies" of REGAUD (1901) are visible on the surface. The spermids are located usually in 3 to 4 layers: they are round cells with pale, round nuclei. Under them there are usually two layers of spermatocytes: the upper layer is older, and the nuclei are in the meiotic prophase (pachytene). The lower layer on the basal membrane, between the spermatogonia is either in the "resting" phase, with fine, granular nucleus, or has begun the meiotic prophase (leptotene), with the nuclei more intensely staining. Spermatogonia are few, and their nuclei are pale and contain chromatin in the form of dust-like particles (type "A").

In this stage dehydrogenase activity is the strongest in the basal membrane. Spots sometimes as big as half a nucleus indicate activity around the nuclei of the younger layer of spermatocytes. The layer of spermids shows clusters of activity, apparently in the cytoplasm. The cytoplasm of the older primary spermatocytes exhibits a fine, granular, pale reaction.

There is no acid-fast staining in this stage.

The results of the lipid reaction will be described with stage 3.

Stage 2. The nuclei of the spermids have become elongated and are grouped in bundles, forming 3 to 4 layers on one side of the cell. The two layers of spermatocytes are present, both in the meiotic prophase (the upper in pachytene, the lower in leptotene). The number of spermatogonia is somewhat larger, the nuclei are still "dust-like" (type "A").

Dehydrogenase activity continues to increase along the basal membrane, while no major change is noticeable in the rest of the preparation *(Fig. 1, Fig. 7)*.

There is no acid-fast staining in this stage.

The results of the lipid reaction will be described with stage 3.

Stage 3. The nuclei of the spermids are more elongated and the longitudinal, bundle-like pattern has fully developed. The nuclei stain more intensely

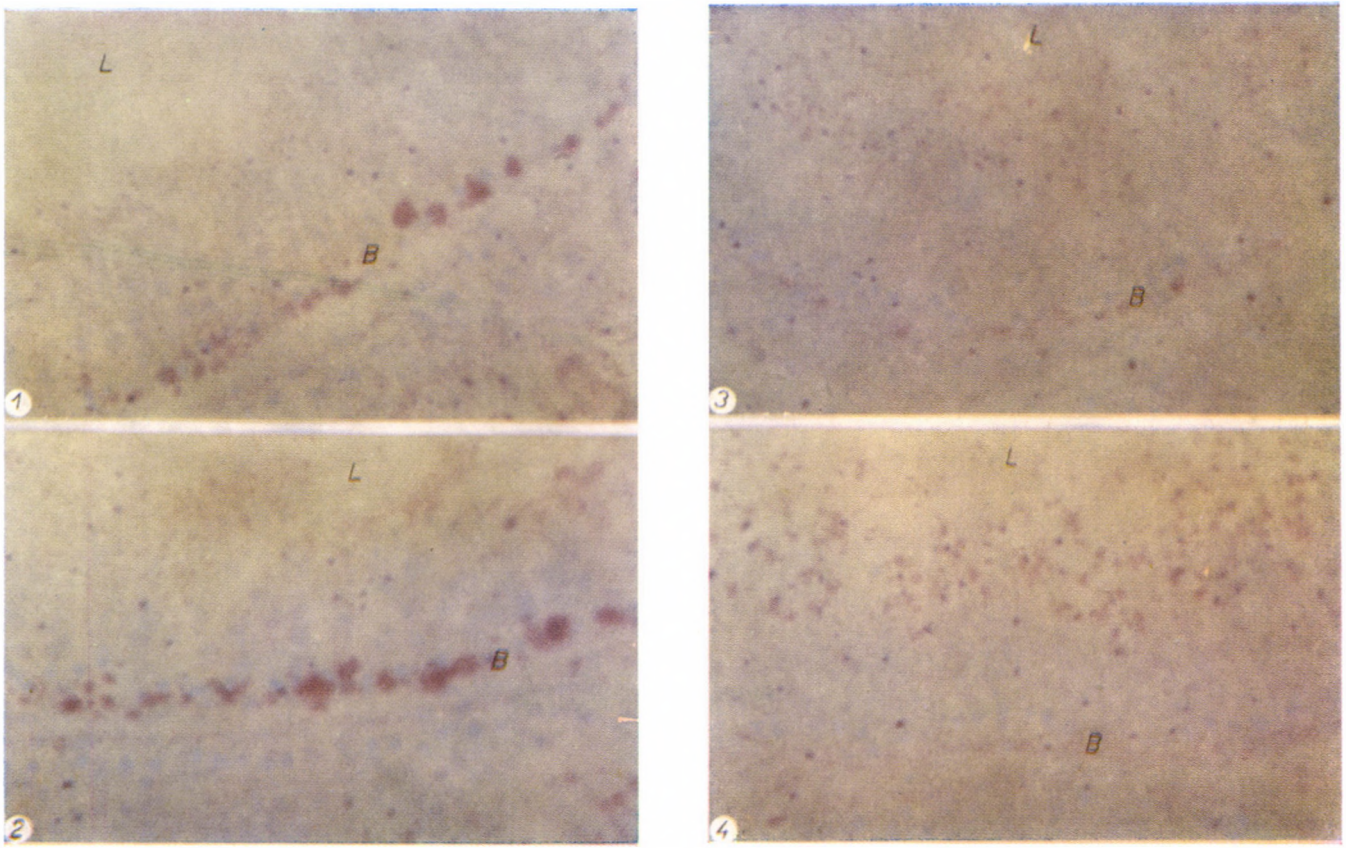

Fig. 1 to 4. Seminiferous tubule. Succinic dehydrogenase reaction, methyl green contrast staining. Cryostate sections
Fig 1 = Stage 2 Fig 3 = Stage 4
Fig 2 = Stage 3 Fig 4 = Stage 6.
B = basal part of tubule. L = luminar part of tubule

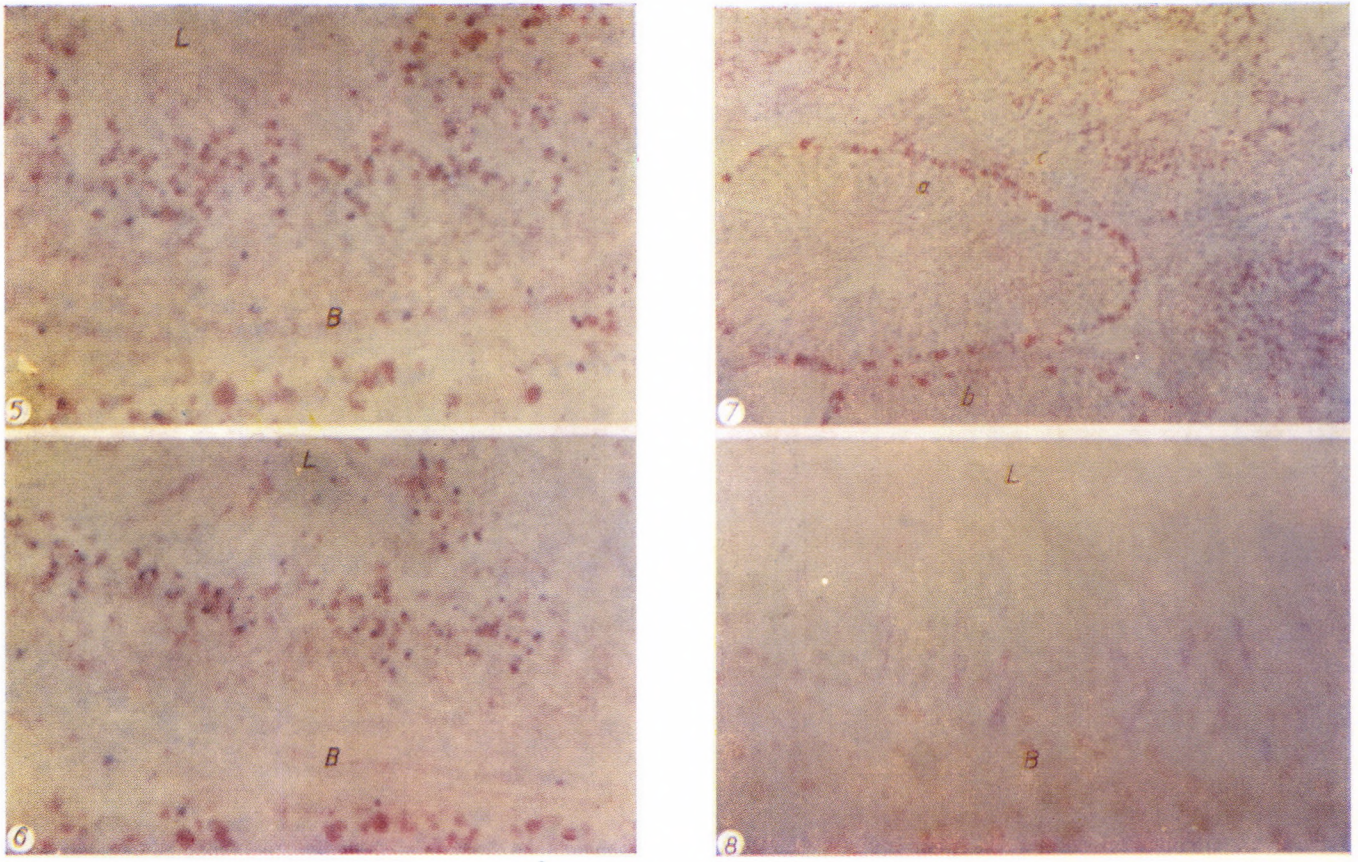

Fig. 5 to 7. Seminiferous tubule. Succinic dehydrogenase reaction, methyl green contrast staining. Cryostate sections
Fig. 5 = Stage 7 Fig. 7 = *a)* Stage 2
Fig. 6 = Stage 8 *b)* Stage 4
c) Stage 7

Fig. 8. Seminiferous tubule. Fixation in Carnoy, embedding in paraffine, crystal violet — Bismarck brown acid-fast staining. Acid-fast staining of the head of the spermatozoon is visible

than in stage 2. Among the spermatocytes in the meiotic prophase those nearer the lumen (the older generation) are in the diplotene or eventually the diakinetic phase, while the younger generation is in the strongly staining zygotene phase. The spermatogonia of type "A" occur in about the same numbers as in stage 2.

The dehydrogenase reaction along the basal membrane appears in the form of big, confluent structures, so-to-say embracing the nuclei of the young spermatocytes. In and around the spermids it appears in the form of clusters while a weaker reaction showing a pattern of fine granules is visible in the older spermatocytes *(Fig. 2)*.

There is no acid-fast staining.

In the first 3 stages the lipid reaction is very strong along the basal membrane, where the younger layer of spermatocytes is to be found *(Fig. 10)*. There it forms structures reaching sometimes the size of the nuclei. In the luminar areas microgranular reaction is visible, located apparently to the cytoplasm of transforming spermids.

Stage 4. The spermids have already assumed the head-shape characteristic of spermatozoa. Early in this stage the nucleus is reduced to about $^2/_3$ of its former size and stains more intensely. The spermid-Sertoli cell complexes are clearly visible and the spermids begin to migrate toward the basal part of the tubule. Both division of the spermatocytes take place in this stage. The younger layer of the primary spermatocytes, nearer the base, is in the meiotic prophase (zygotene, or pachytene), while the older generation shows division resulting in secondary spermatocytes. The secondary spermatocytes are smaller than the primary ones, have round reticular nuclei containing round chromatin granules. The number and shape of spermatogonia are similar to those in the former stages.

The dehydrogenase reaction along the basal membrane appears in the form of a few dispersed large clusters, while the cytoplasm of the spermatocytes shows a pale, granular reaction. Likewise, the new spermids exhibit small, pale granular reaction. Around the transforming spermatozoa the reaction is more intense *(Fig. 3, Fig. 7)*.

By the end of this stage the spermatozoal head begins to show a pale acid-fast staining (*Fig. 9*).

The lipid reaction appears in the form of clusters along the base; in the lumen the granules of finer distribution are larger and stain more intensely than in the earlier stages. The reaction is almost negative in the layer of spermatocytes *(Fig. 11)*.

Stage 5. The developing spermatozoa penetrate in bundles corresponding to the cells of Sertoli toward the basal part of the tubule and reach in this stage about the half thickness of the germinal epithelium. Among them there are 3 or 4 layers of newly formed spermids, with pale, lightly staining cyst-like

nuclei. Under them the layer of the growing primary spermatocytes is visible, in the meiotic prophase (pachytene). In this stage the spermatogonia are dividing and a different type of cell, with oval nuclei parallel with the base is formed (type "B").

In the basal area the dehydrogenase reaction is seen in the form of occasional, large clusters, apparently localized to the cells of Sertoli, though this could not be reliably ascertained. The spermatocytes and spermids show

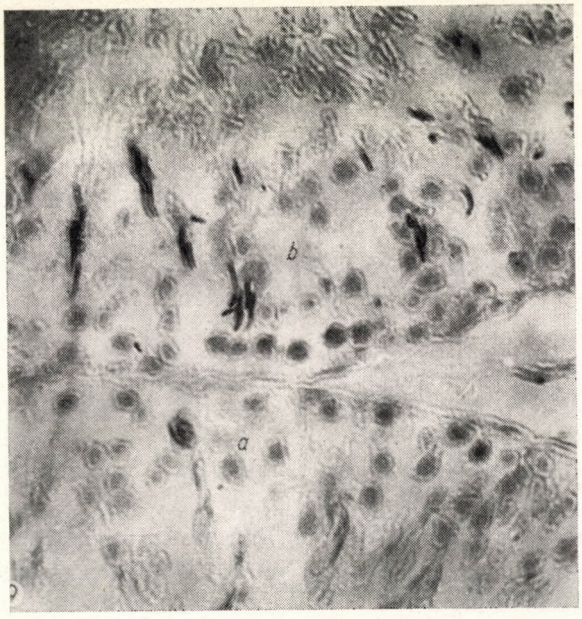

Fig. 9. Two seminiferous tubule sections, fixed in Carnoy, embedded in paraffine, crystal violet-Bismarck brown acid-fast staining. *a)* Stage 4, some of the spermatozoon heads beginning to show acid-fast staining. *b)* Stage 5, marked acid-fast staining of every spermatozoon head

the same pale, fine granular reaction. The reaction is stronger around the spermatozoa undergoing transformation.

In this stage the whole of the head has become acid-fast, especially in the spermatozoa which have penetrated deeper into the cells of Sertoli *(Fig. 8, Fig. 9)*.

The results of the lipid reaction are presented with stage 8.

Stage 6. The developing spermatozoa penetrate in bundles the cells of Sertoli, reaching their nuclei and after passing through the germinal epithelium appear in the spermatogonium layer. Like in the former stage, there are

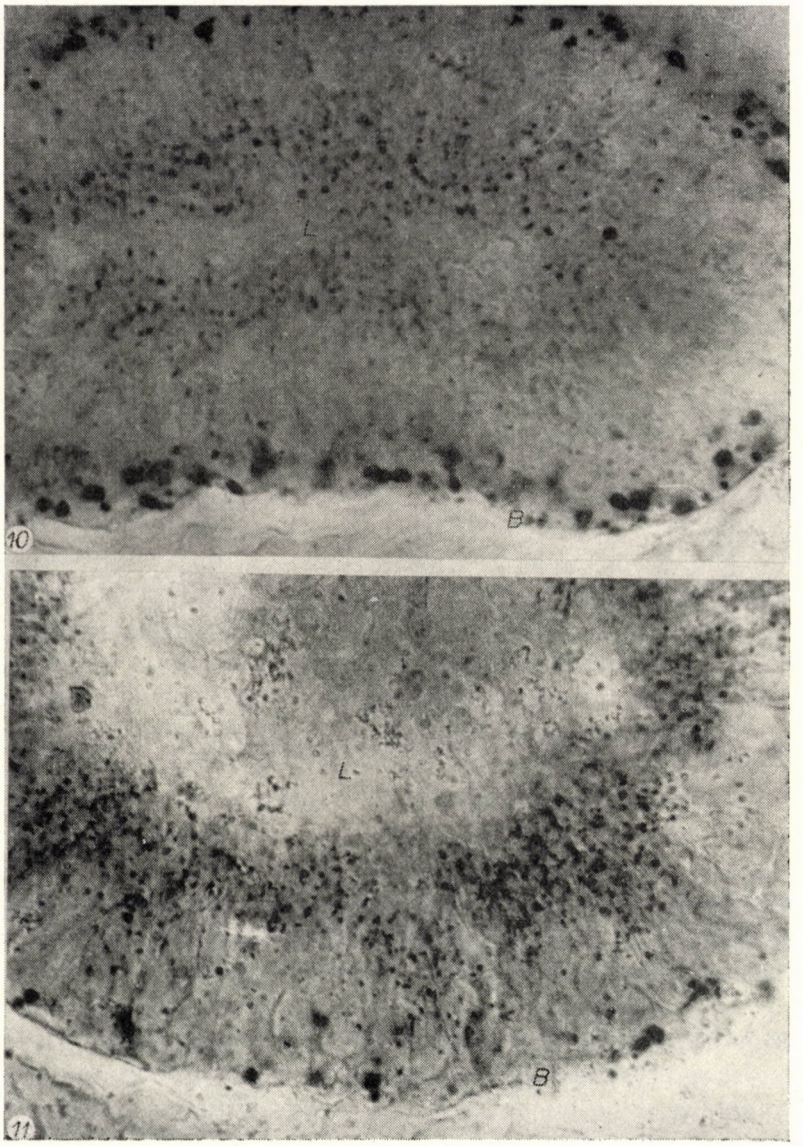

Fig. 10. Cross section of seminiferous tubule. Baker's lipid reaction. Stage 2

Fig. 11. Seminiferous tubule. Baker's lipid reaction. Stage 4

3 or 4 layers of spermids. There is no change in the primary spermatocytes. The spermatogonia are dividing and develop into the type B form.

In this stage the dehydrogenase reaction does not appear any longer in the form of large clusters near the basal part of the tubule. In some areas

a circular reaction in the form of intense by staining but small granules is visible around the nuclei of the cells of Sertoli. The same appears in a few spermatogonia, in the form of pale granules in the spermatocytes, and as rougher, but even smaller granules in the spermids. Larger spots of activity of irregular shape are seen near the cytoplasm of the developing spermatozoa, on the surface of the germinal epithelium *(Fig. 4)*.

The head of the spermatozoon shows a definitely acid-fast staining.

The results of the lipid reaction are presented with stage 8.

Stage 7. The developing *spermatozoa* have withdrawn from the inside of the cells of Sertoli and are to be found in the inner one-third of the germinal epithelium. The luminar layers of the epithelium are formed by 3 to 4 layers of young spermids. The primary spermatocytes have increased in size, but continue to be in the prophase (pachytene). The type B spermatogonia are dividing and form a new, low layer of the primary spermatocytes, that can be found among the fewer type A spermatogonia, with rougher chromatin clustered onto the nuclear membrane. These are called resting, non-dividing spermatocytes. Their nuclei are similar to those of the type B spermatogonia, only smaller.

An increase of dehydrogenase activity is visible corresponding to the layer of newly formed spermatocytes, in the form of small granules in the cytoplasm of these cells and in an occasional cell of Sertoli. The older spermatocytes exhibit pale, granular reaction and the spermids show more intense, cluster-shaped activity. In the detached cytoplasmic parts of the spermatozoa moving toward the lumen strong activity is visible in the form of clusters. In the middle piece of the spermatozoa enzyme activity appears in the form of small granules, corresponding to the distribution of mitochondria *(Fig. 5, Fig. 7)*.

The head of the spermatozoa shows definitely acid-fast staining.

The results of the lipid reaction are presented with stage 8.

Stage 8. The newly formed spermatozoa are grouped in regular rows on the luminar surface of the germinal epithelium and have completely lost the excess cytoplasm. In the row of the heads there are several granules of irregular shape (the "residual bodies" of Regaud). By the end of this stage the spermatozoa detach themselves from the germinal epithelium and disappear in the tubular lumen. The upper, older generation of the primary spermatocytes is in the prophase (pachytene). Under them on the basal membrane are the younger, resting spermatocytes, which have increased in size and show now a finer distribution of chromatin. There are few spermatogonia (which are of the A type).

Dehydrogenase activity is very strong in the large granules of irregular shape that can be found between the spermatozoa and that apparently correspond to detached excess cytoplasm. In the middle piece of spermatozoa protruding into the lumen there are regular rows of reaction in the mito-

chondria. Likewise, large granules of strong activity are visible immediately above the basal membrane, especially in young spermatocytes. This reaction increases in intensity with the disappearance of spermatozoa and appears in the form of confluent large granules by the end of this stage. Fine granular activity of even distribution appears around the prophasic nucleus of older spermatocytes. In the layer of spermids the reaction is intense and takes the form of larger granules *(Fig. 6)*.

The head of the spermatozoa shows marked acid-fast staining.

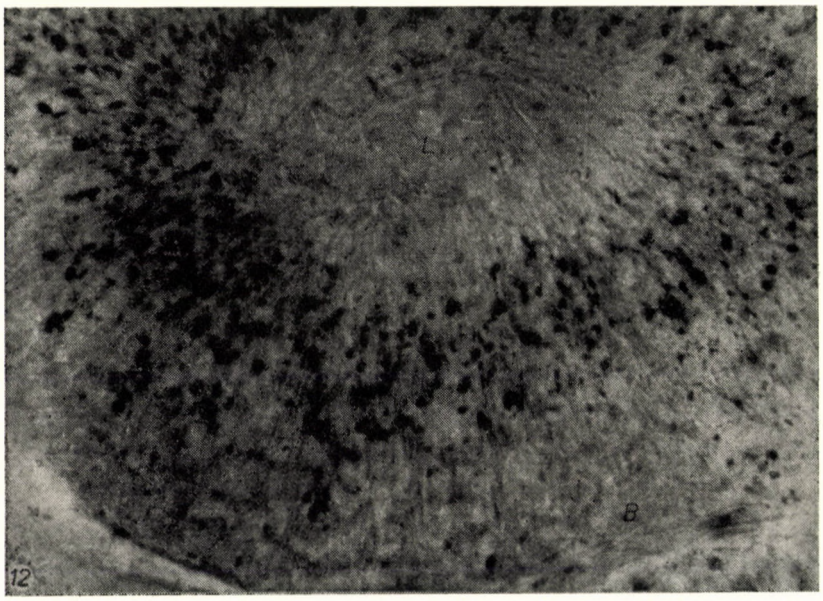

Fig. 12. Seminiferous tubule. Baker's lipid reaction. Stage 7

The lipid reaction disappears almost completely in stages 5 and 6 from the basal part of the germinal epithelium, but at the same time it becomes more intense in the luminar area, along the bundles of spermatozoa penetrating into the epithelium. The latter is especially marked in stage 6. In stages 7 and 8 the reaction tends to appear in the form of large granules chiefly in the luminar area, corresponding to the row of spermatozoon heads, while in the rest of the epithelium there are only a few granules. In a few tubules, along the basal membrane and apparently in the newly formed spermatocytes, the granular form of the reaction is intensified in stages 7 and 8 *(Fig. 12)*.

In the control sections treated with malonic acid, succinic dehydrogenase activity is absent.

Discussion

Research into the process of spermatogenesis dates back to the earliest phase of histology. As early as 1841—1854 KÖLLIKER showed active cells in the wall of the seminiferous tubule. SERTOLI described the cells named after him in 1865. In 1866 HENLE differentiated the two types of cell in the tubule, emphasizing the difficulties of studying the cytogenesis of spermatozoa. The eight distinct stages in the spermatogenesis of the rat were described in 1871 by v. EBNER. The various stages of development can be found side by side in the seminiferous tubule and therefore the cross sections of the tubules show the different stages of development. Measurement of the length of one stage in the tubule showed that it averaged 32 mm (v. EBNER, 1888). This development by stages in the rat and other animal species has been observed by several authors (BENDA, 1887, FÜRST, 1887, v. EBNER, 1888, HERMANN, 1889, MOORE, 1893, LENHOSSÉK, 1898, REGAUD, 1900, 1901, v. EBNER, 1902, CURTIS, 1918, BINDER, 1927). ROOSEN-RUNGE (1950) reinvestigated the genesis of spermatozoa and its division into eight stages, with the purpose of using it in quantitative studies of testicular function. According to him the first stage begins with the transformation of spermid nuclei and the eighth stage ends with the disappearance of the mature spermatozoa; the single stages are judged on the basis of the histologic pattern, taking into account the single forms of development of spermatozoa and the meiotic division. LEBLOND and CLERMONT (1952) introduced a new classification. They studied the PAS reaction of the acrosome system and divided spermatogenesis almost exclusively on the basis of the transformation of spermids into spermatozoa. The beginning is marked by the appearance of the PAS-positive granule in the Golgi substance of the spermid and the end with the development of new spermids. They divided the period between these two events into 14 stages, characterizing each stage with one form of acrosome development. This method undoubtedly makes it possible accurately to define the phases of spermatogenesis and thus helps to understand not only the development of the acrosome, but also the events in spermatogenesis. The method, however, has the disadvantage that special fixation (HELLY) and embedding are required and even then a proper judgement of the slight differences between the single stages is rather difficult. The advantages of the two methods have been united by ROOSEN-RUNGE (1955), introducing the PAS reaction for the determination of stages 5 and 6.

We did not use the method of LEBLOND and CLERMONT (1952), because in the cryostate sections the development of the acrosome could not be followed by means of the PAS reaction, obviously because of the destructive action of ice crystals during freezing (NEUMANN, 1958). We therefore used the Roosen-Runge method based on the principles laid down by v. EBNER (1871), taking into account also the evidence published by other authors (CURTIS 1918,

LEBLOND and CLERMONT 1952, CLERMONT and LEBLOND 1955, DAOUST and CLERMONT 1955, ROOSEN-RUNGE 1955). Our results indicate that the single stages of spermatogenesis may readily be distinguished by taking into account the condition of every layer of the germinal epithelium, in the cryostate sections, with the succinic dehydrogenase reaction (with methyl green contrast staining) and the acid-fast staining alike.

In previous investigations we studied in detail the acid-fast staining of the spermatozoon head (PÓSALAKY and TÖRŐ 1957, 1958, 1959), pointing out that it seems to develop in the course of spermatogenesis, presumably in connection with the synbiosis with the cells of Sertoli. This has been proved by the present results. The acid-fast staining appears in the form of a pale reaction by the end of stage 4 *(Fig. 9)*, while in stage 5 it takes its stronger, final form *(Fig. 8, Fig. 9)*. In stage 4 the bundles of spermatozoa begin to penetrate toward the base of the cells of Sertoli and this process continues in stages 5 and 6. It is in the latter phase that the processes required for the morphological maturation take place. One sign of this is the acid-fast staining of the head and as it appears in stage 5 (in stage 4 the acid-fast staining is still vague), this stage may be considered to be the most important one in the head's differentiation. We are unable to put forward an ultimate explanation of the development of the acid-fast staining; its relationship with the testicular lipids (TÖRŐ, PÓSALAKY 1958) and with the structural changes in the nucleic acid and other substances of the head (PÓSALAKY, TÖRŐ 1959; TÖRŐ, PÓSALAKY 1959) had been discussed in previous papers.

There are several reasons why we know so little about the mechanism of spermatogenesis. One of them is that in the testis various tissues occur side-by-side and interwoven, and the processes take place so close to one another that they can be examined almost exclusively by histochemical methods, being hardly accessible for biochemical tests. This explains why so much is known about the composition of the semen and about the metabolism of the spermatozoa in it (MANN 1954) and why evidence is lacking as to the metabolic processes involved in spermatogenesis. A sequence of events, such as spermatogenesis, in which cells divide, migrate, then become transformed, requires a great amount of energy and the function of every enzyme system involved in its creation. However, the known enzyme histochemical methods do not suffice to study the question in all its details. Of the energy-producing processes in the cell, oxidation is the most significant. Succinic dehydrogenase, one of the enzymes of the Szent-Györgyi—Krebs cycle, can be studied by more or less reliable histochemical techniques. The other enzymes of the cycle are not directly amenable to histochemical study, because they are taking place in the soluble phase of the cell. For the demonstration of succinic dehydrogenase the methods based on the reduction of tetrazolium salts are the most suitable. The valuable properties of the tetrazolium salts had been pointed

out by KUHN and JERCHEL (1941) and were discussed in detail by REMMELE (1958). KUN and ABOOD (1949) were the first to use tetrazolium salt for the demonstration of succinic dehydrogenase in tissue homogenates. Blue tetrazolium was introduced into histochemical studies by SELIGMAN and RUTHENBURG (1951). Later, some authors (PADYKULA, 1952, ROSA and VELARDO, 1954) preferred neotetrazolium. These salts take over electrons from the enzyme after it has dehydrogenated succinate, and are reduced to an insoluble formazan dye. But both tetrazolium salts have the disadvantage that the formazan formed is thick, often needle-shaped, crystal-like, highly lipid-soluble and appears in the sections in two colours, red and blue. It is generally accepted that the colour depends on the intensity of the reaction and the red is the monoformazan and the blue is the diformazan. Recently, the use of both blue tetrazolium and neotetrazolium has been attacked by several authors, because of the lipid-solubility and the presence of the two kinds of formazan. According to BURTNER et al. (1957) blue formazan and red formazan (mono- and diformazan) resulting from the reduction of neotetrazolium would originate from different tetrazoliums, thus they would not be products depending on the intensity of the reaction and appearing one after the other. FARBER, STERNBERG, DUNLAP (1956) kept sections in formazan and found that monoformazan was non-specifically deposited from the solution into the section, while the diformazan was not. However, it was also found that in the area of enzyme activity first a red, then a blue colour develops when incubation is continued. These results make it doubtful whether these substances are actually suitable for the demonstration and quantitative study of succinic dehydrogenase activity at the cytochemical level.

We, too, used neotetrazolium and the problems outlined above are therefore to be taken into account in our work as well. It is first of all the lipid content of the testis that interferes with evaluation. The tests *(Figs. 10, 11* and *12)* have shown the lipid content to be considerable and changing in the course of spermatogenesis. The pictures showing the changes in the lipid content of the basal and luminar parts of the seminiferous tubule are extremely similar to those obtained by studying succinic dehydrogenase activity by the use of neotetrazolium *(Figs. 1* to *7)*.

Taking into consideration the data in the literature (BURTNER 1957, FARBER et al. 1956, NACHLASS 1957, PEARSE 1957, 1958), it might be suggested that what we deal with is a non-specific dissolution of monoformazan in lipids and thus it cannot be brought into correlation with enzyme activity. The negative results obtained following inhibition with malonic acid absolutely contradict this suggestion, indicating that the staining obtained was a result of enzyme activity, as the lipid reaction is unchanged after treatment with malonic acid. Malonic acid being a competitive inhibitor of enzyme activity, a negative reaction proves also that the lipids in the testis could not by them-

selves reduce the neotetrazolium even to monoformazan. Had this taken place, it would have been dissolved in the lipids and staining would have resulted even after inhibition with malonic acid. It had therefore to be concluded that formazan formation resulted from enzyme activity, but the monoformazan part of it was dissolved in the cellular lipids. In some areas diffuse staining occurred over practically the entire cytoplasm, so that an exact location of the reaction within the cell could not be determined and quantitative conclusions must be drawn with caution.

According to our results, during spermatogenesis enzyme activity changes in the following way. The spermatogonia show no appreciable activity. The spermatocytes arising after their division exhibit in the resting and growing phases (stages 7 and 8, *Fig. 5*, *Fig. 6)* increasing activity. The intensity of the reaction continues to increase in the early meiotic prophase of these young spermatocytes (stages 1, 2, 3 and in part 4, *Figs. 1, 2, 3)*. Owing to the high lipid content of the cells the staining is diffuse. Late in the meiotic prophase the intensity of the reaction decreases above the basal membrane, in the so-called older spermatocytes near the lumen and is seen in the form of evenly distributed pale granules (stages 5, 6, 7, 8, 1, 2, 3, *Figs. 4, 5, 6, 1, 2)*. The same pattern is exhibited following meiotic division by the young spermids (in part stages 4 and 5, *Figs. 3* and *7)*. This corresponds to the period called the Golgi phase by LEBLOND and CLERMONT (1952*a*), when the acrosoma granule develops. Subsequently the reaction of the spermids becomes somewhat more intensive and this form of the reaction is visible until the so-called maturation phase of transformation to spermatozoon (*i. e.* during the "cap" and "acrosome" phases; LEBLOND and CLERMONT 1952*a*; stages 6, 7, 8, 1, 2, 3; *Figs. 4, 5, 6, 1, 2)*. During the maturation phase (stages 4 to 8, *Figs. 3, 4, 5, 6)* the reaction continues to increase rapidly in intensity together with the lipid reaction and is seen in the form of large confluent clusters. The detaching cytoplasmic parts are also giving the reaction (stages 7 and 8, *Figs. 5* and *6)*, but this ceases when the spermatozoa have disappeared into the lumen. Almost simultaneously with the detachment of excess cytoplasm (approximately in stage 7, *Fig. 7)* the fine granular form of the reaction appears in the mitochondria of the middle-piece of the spermatozoa. The Sertoli cells show marked reaction in stages 5 and 6, and sometimes in stage 7 as well, when the spermatozoa are burying themselves in the cytoplasm of the Sertoli cells.

As an important enzyme of the Szent-Györgyi—Krebs cycle, succinic dehydrogenase plays a significant role in the oxidation of several metabolites. It has close connections with the cytochrome system, with the terminal oxidation responsible for the production of high-energy phosphate compounds, thus its activity may be considered to be proportionate to oxygen consumption. No activity, or a low activity suggests that in that area the Szent-Györgyi—Krebs cycle functions poorly or not at all. A high activity permits one to

assume that the cycle plays an important role in the metabolism of the cells of that region. The activity of the enzyme may on this way reflect the state of function and metabolism in tissues. The cycle is mitochondrium-bound, though it has been suggested (NOVIKOFF, 1957) that some of its enzymes may take place also in the soluble fractions.

The activity of the Szent-Györgyi—Krebs cycle may be found in virtually every stage of spermatogenesis, though it varies in intensity and is in general less marked than in other organs, for example in the kidney. Two phases are outstanding with their marked activity. One is the layer of young spermatocytes, in the resting and early prophase, the other is the maturing phase of spermatozoa. Both are important in spermatogenesis. The first is the condition preparing for meiotic division, the other is the final step in spermatozoon maturation, as a sign of which the acid-fast staining of the head appears. The undisturbed course of these two phases seems to require very lively metabolic activity. Later in the meiotic phase and during divisions the low, almost negative reaction is in agreement with the observation that during mitosis succinic dehydrogenase activity is considerably lower than in the interphase (OGAWA and ZIMMERMAN, 1959). The increasing intensity of the reaction observed in the spermids during transformation obviously reflects the high-energy requirement of the metabolic process involved.

The importance of the stages with increased activity is shown also by the increased lipid metabolism. The precise chemical composition of the lipids in question is not known. Our histochemical data suggest the presence of phosphatide-containing lipids, but this requires further confirmation. SCOTT (1952) claimed that the testicular lipids may eventually have certain ties with hormonal activity. We, too, think that the said lipid reaction is an expression not of an increase in ballast lipids, but of an increase associated with certain functional activity, such as that observable in the adrenals. Just as the adrenal lipid reactions are not specific for ketosteroids, and yet we interpret this battery of reactions as a certain reflection of function, we may also in the case of the testis accept the lipid reaction as a more or less reliable indicator of functional activity. This is the more justified since in the adrenals, too, the lipid reaction presents itself together with a strong succinic dehydrogenase activity.

As already mentioned, owing to the dissolution of formazan the intracellular site of enzyme activity cannot be precisely located in the areas showing strong lipid activity. In recent years attempts have been made to produce substrates superior to neotetrazolium; promising results have been obtained with nitro blue tetrazolium (NACHLASS, 1957) and MTT [3-(4,5-dimethylthiazol-2)-2,5-*diphenyl* tetrazolium bromide (MTT)] (PEARSE, 1957). In these compounds the lipid-solubility of formazan was eliminated and this localization improved in the lipid-rich areas. However, dissolution of nitro blue tetra-

zolium formazan has been described to occur in the adrenals (PEARSE, 1957) and it is likely that this occurs also in the testis. NACHLASS does not mention it (1957), though he studied the testis and described it to show slight activity. Likewise, BRUNO and GERMINO (1958) mention the testis just briefly, as an organ not showing major activity with neotetrazolium. As they did not study the changes in enzyme activity during the various stages, no conclusions may be drawn from their studies as to the process of spermatogenesis.

At any rate, the MTT method of PEARSE (1957) seems to hold some promise of being suitable for use in studying the dehydrogenase activity during spermatogenesis.

Summary

Changes in acid-fast staining, lipid content and succinic dehydrogenase activity have been studied in the course of spermatogenesis. The development of spermatozoa has been divided into 8 stages. The acid-fast staining of the head becomes marked in stage 5 and at the same time changes occur in succinic dehydrogenase activity and the lipid reaction of the germinal epithelium. During spermatogenesis enzyme activity is more marked in the young spermatocytes and in the maturing spermatozoa, indicating an increased metabolic activity during these phases. According to the evidence obtained the changes of the testicular lipids may to some extent be considered as an indicator of functional activity.

The methodological problems involved in the evaluation of succinic dehydrogenase activity are discussed.

REFERENCES

1. ARGYRIS, T. S. (1956): The Distribution of Succinic Dehydrogenase and Non-specific Esterase in Mouse Skin throughout the Hair Growth Cycle. Anat. Rec. *125*, 105. — 2. BAKER, J. R. cit. A. G. E. PEARSE (1954): Histochemistry. Churchill, London. — 3. BANGHAM, A. D., HANCOAK, I. L. (1955): A New Method for Counting Live and Dead Bull Spermatozoa. Nature (Lond.) *176*, 656. — 4. BARKA, T., DALLNER, G. (1958): The Reduction of Tetrazolium Salts by Ascites Tumor Cells in the Presence of Glucose. J. Histochem. Cytochem. *6*, 174—180. — 5. BARKA, T. (1959): in KOVÁCH, A. A kísérleti orvostudomány vizsgáló módszerei, Akadémiai Kiadó, Budapest, Vol. 5, 231—487. — 6. BARNETT, R. J., PALADE, G. E. (1957): Histochemical Demonstration of the Sites of Activity of Dehydrogenase Systems with the Electron Microscope. J. biophys. biochem. Cytol. *3*, 577—588. — 7. BENDA, C. (1887): Untersuchungen über den Bau des funktionierenden Samenkanälchens einiger Säugethiere und Folgerungen für die Spermatogenese dieser Wirbelthierklasse. Arch. mikr. Anat. *30*, 49—110. — 8. BINDER, S. (1927): Spermatogenese von Macropus giganteus mit Berücksichtigung einiger allgemeiner Fragen der Säugerspermatogenese. Z. Zellforsch. *5*, 293—346. — 9. BRUNO, W., GERMINO, N. I. (1958): Distribution of Succinic Dehydrogenase in the Organs of the Adult Rat. Acta Anat. (Basel) *33*, 161—174. — 10. BURGOS, M. H., FAWCETT, D. W. (1955): Studies on the Fine Structure of the Mammalian Testis. I. Differentiation of the Spermatids in the Cat. J. Biophys. biochem. Cytol. *1*, 287—300. — 11. BUTNER, H. I., BAHN, R. C., LONGLEY, J. B. (1957): Observations on the Reduction and Quantitation on Neotetrazolium. J. Histochem. Citochem. *5*, 127—134. — 12. CLERMONT, Y., LEBLOND, C. P. (1955): Spermiogenesis of Man, Monkey, Rat and other Mammals as shown by the Periodic Acid-Schiff Technique. Amer. J. Anat. *96*, 229—253. — 13. CURTIS, G. M. (1918): The Morphology of the Mammalian Seminiferous Tubule. Amer. J. Anat. *24*, 339—394. — 14. DAOUST, R., CLERMONT, Y. (1955): Distribution of Nucleic Acids in Germ Cells during the Cycle of the Seminiferous Epithelium in the Rat. Amer. J. Anat. *96*, 255—283. — 15. EBNER, V. V. (1871): Untersuchungen über den Bau der Samenkanälchen und die Entwicklung der Spermatozoiden bei den Säugethieren und beim Menschen. Rollet's Untersuchungen aus dem Institut f. Physiol. u. Histol. in Graz. Engelmann, Leipzig, S. 200. — 16. EBNER, V. V. (1888): Zur Spermatogenese bei den Säugethieren. Arch. mikr. Anat. *31*, 236—292. — 17. EBNER, V. V. (1902): Die Geschlechtsorgane in A. Kölliker,

Handbuch der Gewebelehre des Menschen. Engelmann, Leipzig, VI/3. — 18. FARBER, E., STERNBERG, W. H., DUNLAP, CH. E. (1956): Histochemical Localisation of Specific Oxidative Enzymes. I. Tetrazolium Stains for Diphosphorpyridine Nucleotide Diaphorase and Triphosphopyridine Nucleotide Diaphorase. J. Histochem. Cytochem. *4*, 254—265. — 19. FARBER, E., STERNBERG, W. H., DUNLAP, CH. E. (1956): Histochemical Localisation of Specific Oxidative Enzymes. III. Evaluation Studies of Tetrazolium Staining Methods for Diphosphopyridine Nucleotide Diaphorase, Triphosphopyridine Nucleotide Diaphorase and the Succindehydrogenase System. J. Histochem. Cytochem. *4*, 284—294. — 20. FARBER, E., BUEDING, E. (1956): Histochemical Localisation of Succinic Dehydrogenase from Carriers by Lipase and the Specific Histochemical Localisation of the Dehydrogenase with Phenazine Methosulfate and Tetrazolium Salts. J. Histochem. Cytochem. *4*, 357—362. — 21. FARBER, E., LOUVIERE, C. D. (1956): Histochemical Localisation of Specific Oxidative Enzymes. IV. Soluble Oxidation-Reduction Dyes as Aids in the Histochemical Localisation of Oxidative Enzymes with Tetrazolium Salts. J. Histochem. Cytochem. *4*, 374—356. — 22. FÜRST, C. (1887): Über die Entwicklung der Samenkörperchen bei den Beutelthieren. Arch. mikr. Anat. *30*, 336. — 23. HANSON, J., RANDELL, J. T., BAYLEY, S. T. (1952): The Microstructure of the Spermatozoa of the Snail Viviparus. Exp. Cell Res. *3*, 65—78. — 24. HENLE, J. (1873): Handbuch der systematischen Anatomie des Menschen, Braunschweig. (cit. Curtis, 1918.) — 25. HERMAN, F. (1889): Beiträge zur Histologie des Hodens. Arch. mikr. Anat. *34*, 58—106. — 26. KAYE, J. S. (1957): Changes in the Fine Structure of Mitochondria During Spermatogenesis. J. Morph. *102*, 347—400. — 27. KÖLLIKER, A. (1841): Beiträge zur Kenntnis der Geschlechtsverhältnisse und der Samenflüssigkeit wirbelloser Tiere nebst einem Versuche über das Wesen und die Bedeutung der sogenannten Samentiere. Berlin. (cit. Curtis. 1918.) — 28. KÖLLIKER, A. (1847): Die Bildung der Samenfäden im Bläschen als allgemeines Entwicklungsgesetzt. Neue Denksch. der Schweizerischen Gesellschaft für die Naturwissenschaften. — 29. KÖLLIKER A. (1854): Manual of Human Anatomy, Translated by Busk and T. H. Huxley, Philadelphia (cit. Curtis 1918.) — 30. KUHN, R., JERCHEL, D. (1941): Über Invertseifen. VII. Mitt.: Tetrazolium Salze. Ber. dtsch. chem. Ges. *74*, 941—948. — 31. KUHN, R., JERCHEL, D. (1941): Über Invertseifen. VIII. Mitt.: Reduktion von Tetrazolium Salzen durch Bakterien, gärende Hefe, und keimende Samen. Ber. dtsch. chem Ges. *74*, 949—952. — 32. KUN, E., ABOOD, L. G. (1949): Colorimetric Estimation of Succinic Dehydrogenase by Triphenyltetrazolium Chloride. Science, *109*, 144—146. — 33. LEBLOND, C. P., CLERMONT, Y. (1952): Spermiogenesis of Rat Mouse, Hamster and Guinea Pig as revealed by the Periodic Acid-Fuchsin Sulfurous Acid Technique. Amer. J. Anat. *90*, 167—216. — 34. LENHOSSÉK, M. (1898): Untersuchungen über Spermatogenese. Arch. mikr. Anat. *51*, 215—318. — 35. MANN, T. (1954): The Biochemistry of Semen. Methuen. London. — 36. MOORE, J. E. S. (1893): Mammalian Spermatogenesis. Anat. Anz. *8*, 683—688. — 37. NACHLASS, M. M., TSOU K. C., DE SOUSA, E., CHANG S. S., SELIGMAN, A. M. (1957): Cytochemical Demonstration of Succinic Dehydrogenase by the Use of a New p-nitrophenyl Substituted Ditetrazole. J. Histochem. Cytochem. *5*, 420—436. — 38. NOVIKOFF, A. B., PODLER, E. (1957): The Contributions of Differential Centrifugation to the Intracellular Localisation of Enzymes. J. Histochem. Cytochem. *5*, 552—564. — 39. OGAWA, K., ZIMERMANN, A. M. (1959): The Activity of Succinic Dehydrogenase in the Experimental Ependymona of C_3H Mice. J. Histochem. Cytochem. *7*, 342—349. — 40. PADYKULA, H. A. (1952): The Localisation of Succinic Dehydrogenase in Tissue Sections of the Rat. Amer. J. Anat. *91*, 107—145. — 41. PEARSE, A. G. E. (1957): Intracellular Localisation of Dehydrogenase Systems using Monotetrazolium Salts and Metal Chelation of their Formazans. J. Histochem. Cytochem. *5*, 515—527. — 42. PEARSE, A. G. E. (1958): Extension of the Limits of Cellular Pathology: the Role of Enzyme Histochemistry. J. clin. Path. *11*, 520—534. — 43. PEARSON, B., DEFENDI, V. (1954): Histochemical Demonstration of Succinic Dehydrogenase in Thin Tissue Sections by means of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride under Aerobic Conditions. J. Histochem. Cytochem. *2*, 248—257. — 44. PEARSON, B., DEFENDI, V. (1955): Histocheminal Studies of Succinic Dehydrogenase by means of Tetrazolium in Rat Liver Tumors Induced by p-dimethylaminoazobenzene. Cancer Res. *15*, 593—596. — 45. PEARSON, B. (1958): Improvement in the Histochemical Localisation of Succinic Dehydrogenase by use of Nitroneotetrazolium Chloride. J. Histochem. Cytochem. *6*, 112—121. — 46. PÓSALAKY, Z. (1958): Spermiumok szerkezetének hisztokémiai vizsgálata. MTA. Orv. Oszt. Közl. (Budapest) *9*, 301—310. — 47. PÓSALAKY, Z., TÖRŐ, I. (1959): Investigations into the Fate of Intravenously Administered Spermatozoa, Based on their Acidfastness. Folia Biol. *5*, 243—250. — 48. PÓSALAKY, Z., TÖRŐ, I. (1957): Fate of Spermatozoa not Participating in the Fertilization Process. Nature (Lond.) 179, 150—151. — 49. PÓSALAKY, Z., TÖRŐ, I. (1957): Experimental Investigation into the Destiny of Spermatozoa not Participating in Fertilization. Acta Biol. Hung. *8*, 1—10. — 50. REGAUD, C. (1900): Direction hélicoidale du mouvement spermatogénetique dans les tubes seminifères

du rat. C. R. Soc. Biol. (Paris) (cit.: Curtis, 1918.) — 51. Regaud, C. (1901): Études sur la structure des tubes seminifères et sur la spermatogenèse chez les mammifères Arch. Anat. micr. *4*, 101—156, 231—380. — 52. Remmele, W. (1958): Tetrazoliumsalze und Zellfunktion. Frankfurt. Z. Path. *69*, 206—230. — 53. Roosen-Runge, E. C., Giesel, L. O. Jr. (1950): Quantitative Studies on Spermatogenesis in the Albino Rat. Amer. J. Anat. *47*, 1—30. — 54. Roosen-Runge, E. C. (1955): Quantitative Studies on Spermatogenesis in the Albino Rat. III. Volume Changes in the Cells of Seminiferous Tubules. Anat. Rec. *123*, 385—398. — 55. Rosa, C. I., Velardo, J. T. (1954): Histochemical Demonstration of Succinic Dehydrogenase Activity in Tissue Sections by a Modified Technique. J. Histochem. Cytochem. *2*, 110—114. — 56. Scott, W. W., Lynch, K. M. (1952): The Sertoli Cell. In Studies on Testis and Ovary Eggs and Sperm, Ed. E. T. Engle. Thomas, Springfield, Pp. 37—60. — 57. Seligman, A. M., Rutenburg, A. M. (1951): The Histochemical Demonstration of Succinic Dehydrogenase. Science *113*, 317—320. — 58. Sertoli, E. (1865): Morgagni 7, 31. (cit.: Bremer, 1948). — 59. Sotelo, J. R., Trujillo-Cenóz, O. (1958): Electron Microscope Study of the Kinetic Apparatus in Animal Sperm Cells. Z. Zellforsch. *48*, 565—601. — 60. Sternberg, W. A., Farber, E., Dunlap, Ch. E. (1956): Histochemical Localisation of Specific Oxidative Enzymes: II. Localisation of Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide Diaphorases and the Succindehydrogenase System in the Kidney. J. Histochem. Cytochem. *4*, 266—283. — 61. Törő, I., Pósalaky, Z. (1958): Beiträge zur Struktur des Spermiumkopfes. Verh. Anat. Ges. 55. Versammlung in Frankfurt Main, 9—13. April. Fischer, Jena. — 62. Törő, I., Pósalaky, Z. (1959): Histochemische Untersuchung der Spermiogenese, Acta Histochem. *8*, 393—405. — 63. Yasuzumi, G. (1956): Spermatogenesis in Animals as Revealed by Electronmicroscopy. I. Formation and Submicroscopic Structure of the Middle-Piece of the Albino Rat. J. biophys. biochem. Cytol. *2*, 445—450. — 64. Yasuzumi, G., Fujimura, W., Tanaka, A., Ishida, H., Masuda, T. (1956): Submicroscopic Structure of the Sperm-Head as Revealed by Electronmicroscopy. Folia anat. jap. B. *29*, 133—138. — 65. Yasuzumi, G., Fujimura, W., Ishida, H. (1958): Spermatogenesis in Animals as Revealed by Electronmicroscopy. Exp. Cell. Res. *14*, 268—285.

ДАННЫЕ К МЕХАНИЗМУ ПРОЦЕССА СПЕРМИОГЕНЕЗА, НА ОСНОВАНИИ ИССЛЕДОВАНИЯ КИСЛОТОУПОРНОГО ОКРАШИВАНИЯ, АКТИВНОСТИ СУКЦИНОДЕГИДРАЗЫ, КАК И ГИСТОХИМИЧЕСКОГО ИССЛЕДОВАНИЯ ЛИПИДОВ

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Исследовались изменения кислотоупорного окрашивания а также активности сукцинодегидразы и липидов в процессе спермиогенеза. При исследованиях использовалось распределение развития живчиков на 8 стадий. Кислотоупорное окрашивание головок живчиков становится более выраженным в 5. стадии, и в связи с этим меняется также реакция сукцинодегидразы зародышевого эпителия и реакция липидов. В процессе спермиогенеза в молодых спермиоцитах и в фазе созревания живчиков наблюдается более значительная энзиматическая акивность, что указывает на повышенную активность обмена веществ в этих стадиях. Согласно исследованиям и изменение липидов семенников можно рассматривать как выражение определенной функциональной активности.

Обсуждаются связанные с оценкой дегидрогеназной активности сукцина методологические вопросы.

BEITRÄGE ZUM MECHANISMUS DER SPERMIOGENESE AUF GRUND DER SÄUREFESTEN FÄRBUNG, SOWIE DER SUCCINODEHYDROGENASEAKTIVITÄT UND DER HISTOCHEMISCHEN UNTERSUCHUNG DER LIPOIDEN

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Die im Laufe der Spermiogenese erfolgende Veränderungen der säurefesten Färbung, sowie der Aktivität der Lipoiden und der Succinodehydrogenase wurden untersucht. Die Untersuchungen erfolgten auf Grund der 8 Stadien der Spermienentwicklung. Die säurefeste Färbung des Spermiumkopfes wird im 5. Stadium signifikant, und im Zusammenhang damit

verändert sich auch die Succinodehydrogenase- und Lipoidreaktion des Keimepithels. Im Verlauf der Spermiogenese kann in den jungen Spermiozyten und in der Reifungsphase der Spermien eine bedeutendere Enzymaktivität wahrgenommen werden, was auf eine erhöhte Stoffwechselaktivität in diesen Phasen hinweist. Auf Grund der Befunde kann die Veränderung der Hodenlipoiden als der Ausdruck einer gewissen funktionellen Aktivität aufgefaßt werden.

Die mit der Auswertung der Succinodehydrogenaseaktivität verbundenen methodologischen Fragen werden besprochen.

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