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Cytochemical studies of the hemoglobin synthesis of erythroblasts*

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Abstract

The process of hemoglobin sythesis in erythroid cells have been traced mainly by observing cells under the light of 4,060 Å. To scrutinize the theory of hemoglobin synthesis in the nucleus of erythroblasts, several cytochemical and morphological observations were also carried out. The conclusions derived from them are as follows: 1 The absorption at 4,060 Å of the cell, which indicates the location of heme, appeared in the nucleus as early as in the develpmental stage of basophilic erythroblasts. The absorption of hcme in cytoplasm likewise appeared in this stage showing nearly the same intensity of the absorption. The absorption picture of heme in the nucleus, which is coincidental with that of interchromatin, increased along with the progess of maturation as well as in the cytoplasm. The absorption in the nucleus disappeared at the orthochromatic stage where the picture of interchromatin disappeared, while the intensity of absorption in the cytoplasm continued to increase till the stage of reticulocyte. 2 The pseudoperoxidase reaction of hemoglobin, the appearance of acidophlic protein and masked lipids detectable in the location of hemoglobin gave an exactly identical picture with that of the absorption of heme in the nucleus as well as in the cytoplasm. 3 Permeability test performed by supravital staining with Nile blue revealed that the nucleus of erythroblasts from the basophilic to the orthorchromatic stages has increased its permeability being stained selectively as in the case of dead cells. 4 The mitochondria and the endoplasmic reticulum proved to be retained well in the entire course of hemoglobin synthesis, even after the denucleation, the reticulocyte stage. From these observations the authors believe that the hemoglobin syntheis will take place in the cytoplasm throughout the life cycle of erythroid cells, pointing out that the absorption picturebf heme appearing in the nucleus will be in all likelihood due to the infusion of the hemoglobin from the cytoplasm.

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CYTOCHEMICAL STUDIES OF THE HEMOGLOBIN SYNTHESIS OF ERYTHROBLASTS

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The hemoglobin synthesis of erythroblasts denotes the processes of cell differentiation and maturation, which are closely correlated with the cellular structure and the metabolic cycles. The chemical process of hemoglobin synthesis, which means the synthesis of heme, protoporphyrin synthesis followed by iron incorporation, and globin, and the combination of the two, have been studied profoundly by many aurthors. A bird eye view of this may be made by the mongraph "Porphyrin biosynthesis & metabolism."[1] Concerning the intracellular site of biological synthesis of hemoglobin, however, there are only a few reports and many problems still remain to be solved ; e. g. the problems concerning the shift in the site of heme synthesis according to the maturation of cells. In general it is believed that the hemoglobin is synthesized in cytoplasm of erythroblasts. THORELL[2,3] observed spectrophotometrically the contents of ribonucleic acid, heme and whole protein of erythroblasts and reticulocytes in the various developmental stages showing that hemoglobin synthesis mainly proceeds in the later stages of basophilic erythroblasts and most actively in the polychromatic and orthochromatic stages, and a few in the reticulocyt stage. Concerning the site of hemoglobin synthesis in cells, VANNOTTI[4], CARVALHO[5,6,7], ODA[8], and NAKAO[9] are of the opinion that in the early developmental stages of basophilic erythroblasts the hemoglobin synthesis will occur in the nucleus and in the following developmental stages the site of the hemoglobin synthesis will change from the nucleus to cytoplasm.

These conclusions are led from the cytological studies by observing the cells at near 4000 Å or by using labelled glycine or iron isotope. This ought to be an important finding in the point that the nuclei, which are generally

consdiered to be only the carriers of genes and to have the functions for the cell division, have the ability to synthesize heme, the very element of respiratory enzymes. And yet the nuclei of erythroblasts, as distinguished from other cells, are destined to be degenerated or disintegrated in the course of maturation of erythroblasts. Then it will be strange to suppose that such nuclei have gained the ability of heme synthesis in the initial stages of their degeneration course. In these points the theory of nuclear synthesis of heme must be carefully scrutinized. For this purpose, we traced the processes of the hemoglobin synthesis cytologically as well as cytochemically.

MATERIALS AND METHODS

Bone marrow cells of normal and blood depleted anemic rabbits were mainly used. For the cytochemical studies of heme, the cells stamped, dried, and fixed with methanol were observed at 4, 060 Å, without staining, or the cells suspended in physiologic saline solution were observed at the same wave length of light in living state. For the light source a high pressure mercury lamp was used, and the apparatus for a microspectrophotometry, that of Olympus Co., was utilized for the observation at various wave lengths. With the purpose to remove the effect by refraction, reflection, or phase contrast effect, wich might interfere with the true absorption of heme, the same cells were photographed at 4,960 Å at which the absorption of heme is entirely diminished, and these were observed comparing to those photographed at 4,060 Å.

For the detection of hemoglobin, the pseudoperoxidase reaction of hemoglobin by LYSON[20] were performed using white zinc. For the detection of globin, the cells were stained with Giemsa solution whose pH was regulated at pH 4～5 using phosphate buffer. Besides these, for the observation of the distribution of desoxyribonucleic acid Feulgen reaction was performed on the cells fixed with Carnoy solution. Pyronine methyl green staining for DNA and RNA was also performed after exposing or not exposing to the ribonuclease which was prepared from ox pancreas by the McDonald's method. For the detection of masked lipids, which are generally detectable in a great amount at the site where hemoglobin exists, the cells stamped, not dried completely and fixed with a formol vapor were stained with Sudan black B in 70 % ethanol for 30 minutes at 37°C after exposing to 0.1 % pepsine solution for a few minutes in an acidic media. For the observation of the changes of fine structure of cells occurring with the process of maturation, an electron microscope, Hitachi

Co. s, type HU-10, was used. According to the conventional manner the small pieces of bone marrow tissues were fixed with 1 % osmic tetraoxide bufferized with 0.1 M phosphate buffer solution (pH 7.4), embedded in methacrylate and sectioned to the thickness of about 0.02-0.03 μ, using an ultramicrotome of Shimazu Co. With a view to know the grade of activity of mitochondria which will give the energy source for the hemoglobin synthesis, the cytochemical reaction of the succinic dehydrogenase was observed. For the precision of the method, refer to "Cytochemical and Biochemical Studies on the Succinic Dehydrogenase System", by ODA[10,11].

For observations of the permeability of the nuclear membrane of erythroblasts, the supravital staining was performed. A drop of bone marrow blood from anemic rabbits was stained supravitally on Nile blue film prepared by smearing of 1 % dye solution in pure ethanol, and observed in wet.

RESULTS

By observing the cells in each developmental stage, from proerythroblasts to reticulocytes, it was revealed that the absorption of heme at 4,060 Å appeared in the basophilic erythroblasts but not in the proerythroblasts, the absorption of that light was recognized in the nucleus as well as in the cytoplasm. There were no such cases where the absorption of heme was seen limited in the nucleus but not in the cytoplasm. The absorption picture of heme in the nucleus is almost identical with that of interchromatin while the heme in cytoplasm appears diffusely. According to the proceedings of maturation of the cells, the absorption picture of heme in the nucleus increases its intensity till the terminal stage of polychromatic erythroblasts (fig. 1) as well as in cytoplasm. In the stage of orthochromatic erythroblasts, the absorption of heme disappears in the nucleus in which the nucleus becomes pycnotic and homogenous and the interchromatic spaces also disappear, while the absorption in cytoplasm increases its intensity to the maximum. Later on after the denucleation the absorption of heme increases in the stages of reticulocytes and stops on reaching the completely matured red cells.

The histochemical reaction of pseudoperoxidase of hemoglobin was also traced through each developmental stage of erythroblasts and reticulocytes as in the case of hemesynthesis. The results was almost the same as in the case of heme. The appearance of acidophilic protein, globin, detectable by acidic Giemsa stain (Fig. 2) and the picture of masked lipids detected by Sudan black B stain (Fig. 3) gave almost identical results

with the picture of heme absorption depending in the site and intensity.

The reaction of DNA observed by Feulgen's reaction or by methyl green pyronine staining after exposing to ribonuclease appeared always limited in the nucleus. The ribonucleic acid detected in the cytoplasm by pyronine staining, which disappears after the digestion with ribonuclease, increases its intensity during the development of proerythroblasts to basophilic erythroblasts, showing its maximum in the latter. From the terminal stage of basophilic erythroblasts, the staining intensity decreases step by step and becomes scarcely visible in the stage of orthochromatic erythroblasts and reticulocytes, though they still contain some quantiy of ribonucleic acid[12,13].

The electron-microscopic observation revealed that the erythroblasts have fairly bright cytoplasm and round nuclei whose surface is rather smooth and has no special groovings and the structure of the chromatic nets appears homogenous differently from the picture seen by phase-contrast microscope on living cells or those seen on the cells stained with Giemsa. (Fig. 5, 6, 7). The cytoplasm has a reasonable number of mitochondria having cristae. They appear as an oval or rod-like structure 0.2-0.23 μ in breadth. These decrease in number according to the process of maturation. After the denucleation, however, they can still be seen in the stage of reticulocytes (Fig. 8) though some of them show the picture of degeneration in reticulocytes, demolished cristae or scanty in content[14,15]. Besides mitochondria, in the cytoplasm of erythroblasts and reticulocytes, there are some vesicular structures, and these appear sometimes as small holes in the cut section (Fig. 5, 6, 7) and also decrease in number according to the proceedings of maturation, and some of them are still remaining in the reticulocytes (Fig. 8), and they have been poved to be the cut profiles of endoplasmic reticulum[14,15].

Supravital staining of erythroblasts showed a markedly increased permeability of the nuclear membrane. The nucleus of erythroblasts was stained selectively by Nile blue, while the nuclei of myelogenous cells including polynuclear leucocytes, and lymphocytes were left unstained (Fig. 4). Cytochemical study on succinic dehydrogenase activity proved that the cytoplasm of proerythroblasts, erythroblasts, and reticulocytes have the acitivity. The most intense reaction was observed on young erythroblasts and slight reaction in reticulocytes. The reaction appeared to coincide with the sites of the mitochondria[10,11].

COMMENTS

As early as in the initial stage of basophilic erythroblasts a slight absorption of heme appears in the nucleus of erythroblasts and its intensity increases till the final stage of polychromatic erythroblasts. The spaces of interchromatin become distinct and decrease in number, and disappear in the orthochromatic stage. The heme absorption appears in the spaces of interchromatin and disappears spontaneously by pycnosis of the nucleus at the orthochromatic stage. These pictures seem to show the provalbe intranuclear synthesis of heme.

As described above, CARVALHO, NAKANO and others claimed the heme synthesis will be conducted by nucleus in the early developmental stages of erythroblasts. However, from the observations on erythroblasts at 4,060 Å only we could not draw any decisive conclusion on the nuclear synthesis of heme, because the absorption of heme in the nucleus was always accompanied by the absorption of cytoplasm. The absorption intensity, too, was almost the same both in the nucleus and in the cytplasm. Any cells in basophilic erythroblasts we could not find out in which the absorption of heme was limited in the nucleus. On the other hand, globin was always found in the regions where the absorption of heme was recognized both in the nucleus and cytoplasm. The pseudoperoxidase reaction of hemoglobin also appeared in the same regions. The masked lipids,which are generally seen in the site of hemoglobin, were also proven to exist in the same sites. These facts will show that the hemoglobin actually exists in the regions showing heme absorption, suggesting that hemoglobin synthesis might occur in the nucleus as in the cytoplasm.

However, before drawing this conclusion, the following must be definitely solved. The first one is that the hemoglobin recognized in the region of the nucleus is actually intranuclear but not existing in the grooves of the nuclear surface. The second one is that the transfer of hemoglobin never takes place from the cytoplasm into the nucleus *in vivo*. The first problem may be overlooked by electronmicroscopic observations as the nuclear membrane of erythroblasts has no such grooves as to coincide with the picture of the hemoglobin absorption in the nucleus. The second problem has been left uncertain, however, our experiment leaves many possibilities that the absorption of heme in the nucleus appearing in basophilic erythroblasts is effected by the imbibition of hemoglobin from the cytoplasm into the nucleus. The supravital staining of erythroblasts proved a markedly increased permeability of the nuclear membrane. The nuclei of erythroblasts from basophilic to orthochromatic stages were

stained selectively by supravital staining with Nile blue. HOLTFRETER observed the penetration of hemoglobin into the isotated nuclei of frog oöcyte.[21]

On the contrary it is obvious that the cytoplasm of erythroblasts can actually synthesize heme, because in the denucleated cells, reticulocytes, the hemoglobin synthesis proceeds as was reported by THORELL[2,3] and recently by SENO and his coworkers[16] from their precise quantitative estimation. It will not be unreasonable to assume that the cytoplasm will be responsible for the hemoglobin synthesis in basophilic erythroblasts, too. In the cytopasm of erythroblasts, a number of mitochondria which have a marked activity of succinic dehydrogenase and yet in the stage of basophilic erythroblasts and polychromatic erythroblasts, the cytoplasm contains a quantity of ribonucleic acid and many of endoplasmic reticulum which is now actually proven to have an ability of protein synthesis[17] and yet the content of ribonucleic acid is at the maximum in the cytoplasm of basophilic erythroblasts as was precisely estimated by THORELL. This fact will show the active globin synthesis in these cells, because the rate of the reproduction, the mitosis, is rather low. Actually a marked acidophilicity can be seen in the cytoplasm of basophilic erythrobalsts by acidic Giemsa stain. The energy for protein synthesis will be given by the mitochondria. It will be true in the case of reticulocytes too, as the reticulocytes consume oxygen[12] and the mitochondira of reticulocytes also have a marked succinic dehydrogenase activity.

From the morphologic stand point, too, it seems rather curious to assume that the nucleus of erythroblast, which is in the course of degeneration, acquires again the active ability of hemoglobin synthesis. CARVALHO showed a mass of incorporation of glycine labelled with C^{14} into the nuclei of erythroblasts, however, it must be borne in mind that glycine is actively used for the synthesis of nucleo-protein[18] and globin[3], as well as for the protoporphyrin[7]. NAKAO proved that the iron Fe^{59} is incorporated into the nuclei of erythroblasts and he claimed the sites of hemoglobin synthesis in the early developmental stages of erythroblasts should be the nucleus from observations on the nuclear fraction obtained by centrifugation. But this method is, of course, inadequate to decide the site of heme synthesis, because of the contamination of cytoplasmic elements or of transfer of iron in the course of the preparation. The radioautographes of erythroblasts, which they showed, seem not to be accepted as the evidence for the nuclear heme synthesis.

Only with these observations mentimed so far we can not deny that the hemoglobin sythesis takes place in the nucleus in the early develop-

mental stages of erythroblasts, but it may be said that there is a possibility of migration of the hemoglobin sythesized in the cytoplasm into the nucleus.

The hemoglobin found in the nucleus of fowl erythrocytes[19] may also be the one that is transferred into nucleus from cytoplasm, because it is proven by STERN *et al.*[19] that the nuclei of muscle cells has no heme, when these cells have an ability to synthesize heme and have the lively nuclei.

KONNO observed the heme synthesis of the isolated nuclei of erythroblasts in vitro, but the nuclear fraction, which he obtained by Schneider-Hoogeborn's method, contained about 10 % living erythroblasts, 35 % of nucleus adhering cytoplasm and a number of reticulocytes which still have an active ability of hemoglobin synthesis[22].

SUMMARY

The process of hemoglobin sythesis in erythroid cells have been traced mainly by observing cells under the light of 4,060 Å. To scrutinize the theory of hemoglobin synthesis in the nucleus of erythroblasts, several cytochemical and morphological observations were also carried out. The conclusions derived from them are as follows :

1 The absorption at 4,060 Å of the cell, which indicates the location of heme, appeared in the nucleus as early as in the develpmental stage of basophilic erythroblasts. The absorption of heme in cytoplasm likewise appeared in this stage showing nearly the same intensity of the absorption. The absorption picture of heme in the nucleus, which is coincidental with that of interchromatin, increased along with the progess of maturation as well as in the cytoplasm. The absorption in the nucleus disappeared at the orthochromatic stage where the picture of interchromatin disappeared, while the intensity of absorption in the cytoplasm continued to increase till the stage of reticulocyte.

2 The pseudoperoxidase reaction of hemoglobin, the appearance of acidophlic protein and masked lipids detectable in the location of hemoglobin gave an exactly identical picture with that of the absorption of heme in the nucleus as well as in the cytoplasm.

3 Permeability test performed by supravital staining with Nile blue revealed that the nucleus of erythroblasts from the basophilic to the orthorchromatic stages has increased its permeability being stained selectively as in the case of dead cells.

4 The mitochondria and the endoplasmic reticulum proved to be retained well in the entire course of hemoglobin synthesis, even after the

denucleation, the reticulocyte stage.

From these observations the authors believe that the hemoglobin syntheis will take place in the cytoplasm throughout the life cycle of erythroid cells, pointing out that the absorption picture of heme appearing in the nucleus will be in all likelihood due to the infusion of the hemoglobin from the cytoplasm.

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LEGENDS FOR FIGURES

Fig. 1. Two basophilic and two polychromatic erythroblasts from the bone marrow of a normal rabbit, smeared and fixed in methanol, and photographed at the wave-length of 4,060 Å.
A strong absorption of that light by heme can be observed in the cytoplasm of erythrocytes and erythroblasts as well as in their nuclei.

Fig. 2. Erythroblasts of rabbit, smeared and fixed with methanol and stained with acidic Giemsa (pH 5.0) for 30 minutes.
Basophilic protein can be observed in the cytoplasm and nuclei of erythroblasts as well as in erythrocytes, coexisting in the region where heme is located.

Fig. 3. Bone-marrow cells smeared and fixed with formol vapor and treated with 0.1 % pepsin at 37°C for two minutes and stained with Sudan black B. Masked lipids are observable at the same location where heme exists in the cytoplasm and the nuclei of erythroblasts and in erythrocytes.

Fig. 4. The picture of supravital staing of bone-marrow cells of a mouse. The nuclei of erythroblasts are stained selectively and spontaneously, while the nuclei of myelogenous cells are devoid of staining.

Figs. 5—8. Sections of erythroid cells in the various ripening stages, electron microscopic photographs. Fig. 5. Basophilic erythroblasts. Fig. 6. Polychromatic erythroblasts. Fig. 7. Orthochromatic erythroblasts. Fig. 8. A Reticulocyte. The cut profiles of mitochondria and endoplasmic reticulam can be seen in the cytoplasm through out the all developemental stages. Both of them decrease in number in the process of maturation. Reticulocytes also has mitochondria and endoplasmic reticulum.

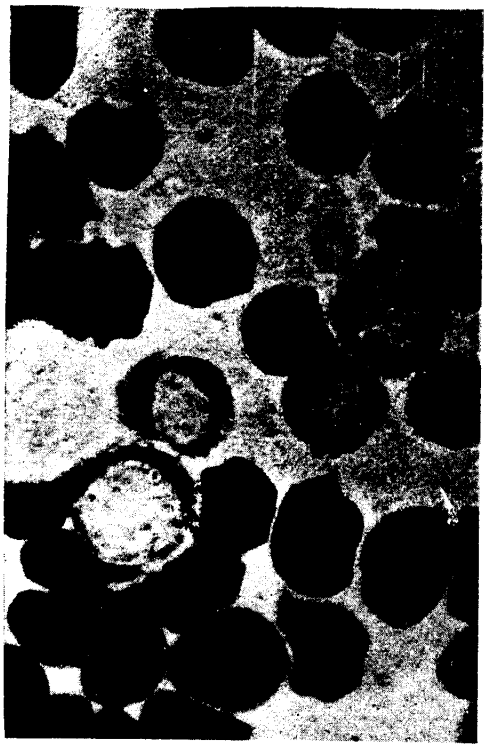

Fig. 1

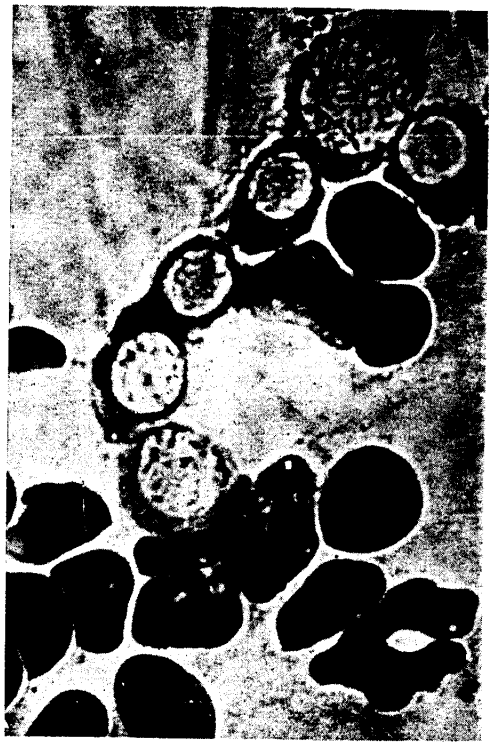

Fig. 2

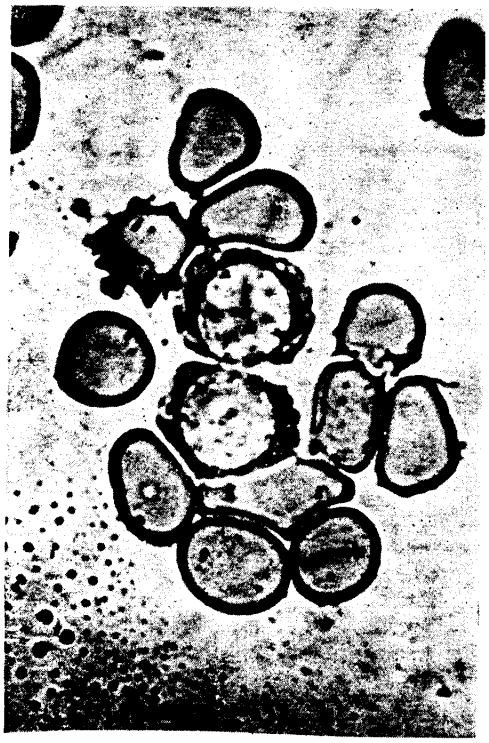

Fig. 3

Fig. 4

Fig. 5

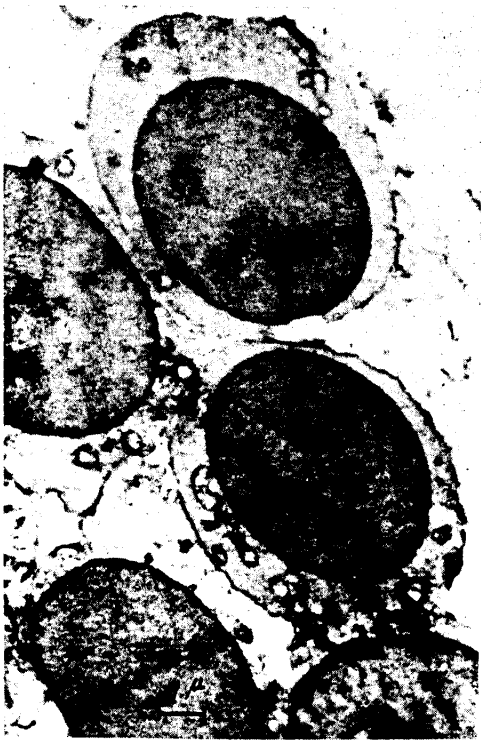

Fig. 6

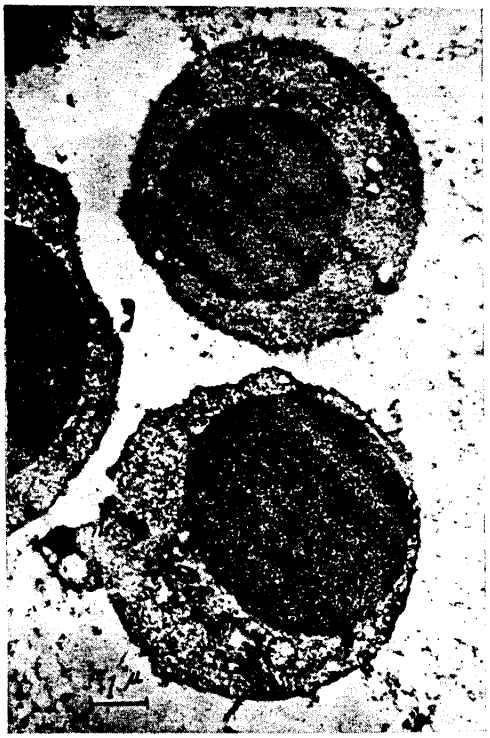

Fig. 7

Fig. 8