Biological effect of high and low oxygen tension–a morphological study

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Abstract

1. Morphological observations were carried out on the strain HeLa cells cultured under various oxygen tension. 2. The growth of the strain HeLa cells in the present experiments was markedly inhibited when they were cultured under high oxygen tension or in nitrogen gas environment. It has been clarified that air offers the most optimal gas environment. 3. Effects of the changed gas environment on the fine structures of HeLa cells were studied by phase contrast microscope and electronmicroscope. As the results it has been found that in these cells cultured under a high oxygen tension there occurs a marked swelling of mitochondria. Under anaerobic condition, however, these cells undergo degeneration, as in oxygen environment revealing no swelling of mitochondria but rather contraction. 4. Endoplasmic reticulum (ER) of HeLa cells cultured under oxygen environment is transformed from its vesicular form to lamellar form. The mechanism of lamellar transformation of ER is obscure but no morphologic connection with nuclear, cytoplasmic and mitochondrial membranes has been detected. 5. A discussion was made on these findings concerning the changes in fine structures of the cells, with a special reference to the swelling of mitochondria and morphological changes of ER under oxygen environment.

BIOLOGICAL EFFECT OF HIGH AND LOW OXYGEN TENSION
— A MORPHOLOGICAL STUDY —

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The biological effect of oxygen tension was observed for the first time by FISCHER and ANDERSON[1] who revealed the suppression of the cell proliferation under high oxygen pressure. Since then this important finding, however, has been left without clarifying the mechanism, though a few works have been reported on this problem. Among the reports those of WARBURG and of BÜCHNER and his associates are worthy of attention. BÜCHNER and his collaborators[2] revealed that hypoxygen tension in some period of the embryonic stage will be responsible for the malformation e. g. malformations in the nervous system and heart.

WARBURG[3] suggested that the hypoxygen tension in tissues will be responsible for cancer formation as described precisely in his articles entitled "In the origin of cancers. " He pointed out several facts suggesting that an irreversible respiratory disturbance will occur in the cells exposed to hypoxygen tension repeatedly and this disturbance will alter normal cell to cancer cell with the changes in energy producing pattern, from respiration type to fermentation type. Thereafter, a spot light has again been placed on the problem concerned with the oxygen tension in tissues. It is a well-known fact that under complete lack of oxygen most of living organisms cannot conduct their metabolic processes, however, it must also be noted that under excess oxygen supply likewise cells cannot maintain their lives and they ultimately all die. These facts seem to show that oxygen tension would affect most primitively the respiratory process and subsequently the cell proliferation, i. e. a moderate oxygen tension will be required for the adequate cell proliferation. Consequently, concerning the regulation for cell proliferation in tissues the oxygen tension might be one of the essential factors.

However, there are few works concerning the cellular response to the varying O_2 tension, especially those dealing with morphologic changes of the cell structure exposed to the abnormal oxygen concentration, which may give some advantages in revealing mechanism of cell proliferation. From this view point the author conducted morphologic observations of the cells cultured and exposed to various oxygen tension, by using usual light microscope, phase contrast

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microscope and electronmicroscope. In this paper, the changes in the fine struc-
tures of the cell under various oxygen tension are presented.

MATERIALS AND METHODS

Strain HeLa cells served as materials. They were cultured in the medium
composed of 10 per cent bovine serum containing 0.4 per cent lactalbumin hy-
drolysate and 0.68 per cent yeast extract in saline D[4]. TD-15 flasks[5] served as
culture-vessels each containing 2 ml. of the medium.

For the first day of culture the cells were incubated under air environment.
Confirming vigorous cell proliferation after one day, air in each vessel was
replaced by the gases prepared by mixing O_2 and N_2 gases in various proportion.
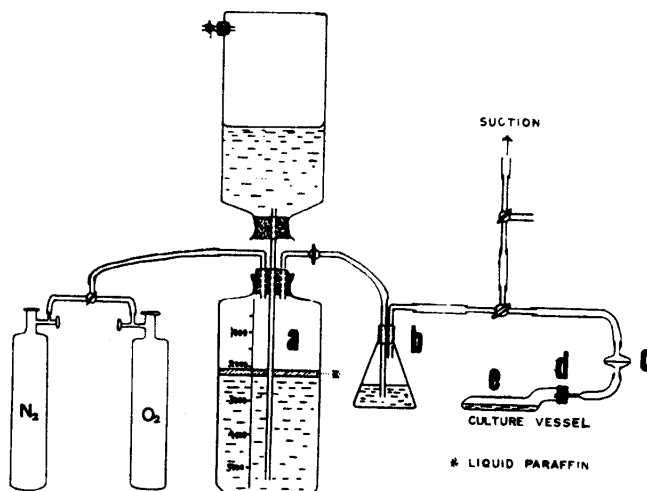

Fig. 1. Gas mixture apparatus: By means of suction of water-flow pump air in the
culture vessel is replaced by a given gas mixture by turning each cock.

To obtain the gases of varied oxygen tension the gas mixture apparatus devised
by the author as shown in Fig. 1 was used. Oxygen and nitrogen gases
were introduced into the bottle a having calibration in the volume ratio desired.
The mixed gases in a is further led into the culture vessels e through the
chamber b and c, filters for bacteria, and finally into the needle inserted in W-gum
stopper d. Prior to the gas-introduction the air in the culture vessel had been

Figs. 2~5.   Strain HeLa cells (36 hours of gas culture, Giemsa stain)
        (O) denotes oxygen; (A) air; (N) nitrogen; ratios 6 : 4 and 2 : 8 stand for O_2 : N_2. The
        extreme right side tube with N, Fig. 2-f is the one where traces of air leaked in
        by mistake. Fig. 3 shows those cultured in pure oxygen and Fig. 4. 6 : 4.. in O_2 : N_2
        Fig. 5 in air.

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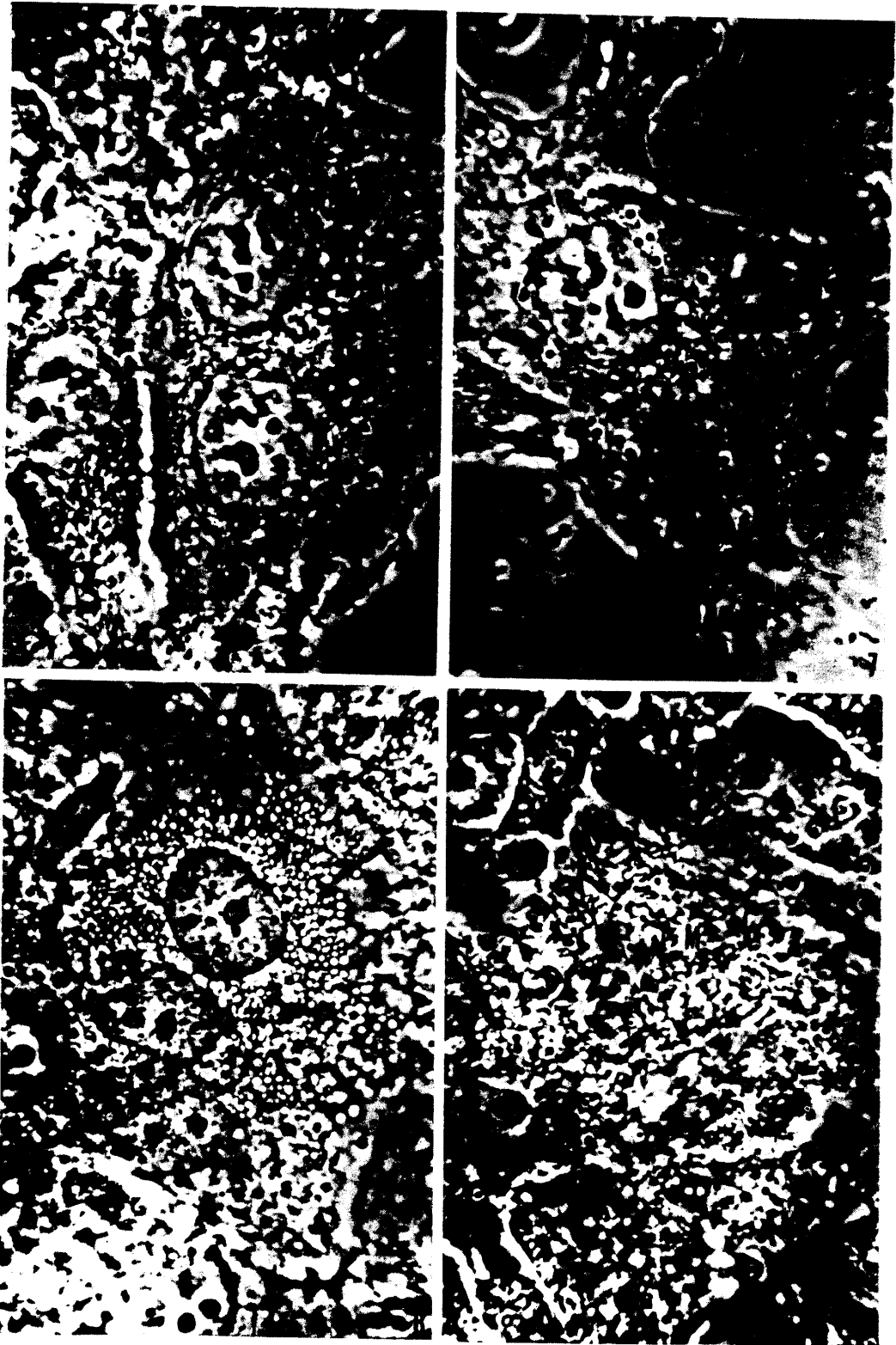
drawn out by water-flow pump and then the gas was introduced. By repeating drawing and introduction 5 times the air was replaced almost completely with the gas. The method also served as to drive out oxygen dissolved in the medium.

The observations by phase contrast microscope were carried out on the cells growing on the slides by using dark contrast DLL × 100 as the objective. The cells were grown on the slide, 8 mm × 40 mm in TD-15 culture vessels. For electronmicroscope observation, the cells cultured in TD-15 without slide were used. The cells of one to two days' culture were washed three time with Hank's solution and fixed with 1 ml. of 1 per cent osmic acid in Hank's solution, which was introduced into the culture vessels after the three washes. The fixation was made for one hour at room temperature. The cells so fixed were harvested gently detaching from the vessel wall by using rubber cleaner and they were taken up into a small glass tube about 0.5 mm. in diameter in tip and centrifuged for 5 minutes at 2,000 r. p. m. The water in the tube was removed as completely as possible by inserting a slender filter paper and the packed cells were obtained as a mass by being blown out on filter paper after breaking the top of the tube. The packed cells were then sliced gently into small blocks by a razor. The blocks were dehydrated through alcohol, embedded in methacrylate, and sectioned by using microtome of Porter-Bloom type.

RESULTS

Gross appearance of the colonies of HeLa cells proliferating under various oxygen tension may be seen in Figs. 2 to 5, which show six representative tubes having growing cells cultured 36 hours under the given conditions. As can be seen in the vessels b, c and d, these vessels contain a moderate amount of oxygen, 60 per cent in b, 20 per cent in c and air in d, which contains about 20 per cent of O_2. But in the vessel a containing pure oxygen the cell growth is severely inhibited. Under pure N_2 gas the cell proliferation is also markedly suppressed as seen in vessel e, but a small amount of O_2 seems to enhance the cell growth markedly as can be seen in the cases where occasionally traces of air leaked in. Under the increased O_2 tension the cell sheets characteristic to HeLa cells are deformed and torn into small fragments and they ultimately fall off from the glass wall of the culture vessel. Microscopic observation reveals the dissociation of intercellular connection and some signs of cell degeneration (Fig. 3). By reducing O_2 tension the inhibition of cell growth is also reduced, but a marked deformation of cell sheets and the dissociation of the cells under 60% O_2 tension

Figs. 6~9. Phase-contrast microscopic photographs of HeLa cells (36—40 hrs. culture) Fig. 6. those cultured in air. Fig. 7 in N_2 and Figs. 8, 9 in oxygen. In Fig. 6 filamentous mitochondria can be seen clearly but not in Fig. 7. Fig. 8. shows numerous vesicles (V) and Fig. 9. swollen mitochondria (M).

(Fig. 4). It is of interest that the cells change their shape to fibroblastic type from epithelial cell type by high oxygen tension. The picture of the cells appearing in Figs. 3 and 4, clearly shows the changes in contrast to those appearing in Fig. 5, which shows the cells of the same strain but growing under air. Under phase contrast microscope the cells growing under higher O_2 tension show a marked morphologic change in their mitochondria. The cells growing under air reveal a number of filamentous mitochondria which are arranged orderly in cytoplasm (Fig. 6), whereas the cells growing in higher oxygen tension the mitochondria can hardly be detected in cytoplasm which contains numerous fat droplets (Fig. 8). Some others do not reveal swollen mitochondria which appear as the neck lace-like bodies arranged irregularly in the cytoplasm (Fig. 9). In the cells growing under nitrogen environment mitochondria are small and indistinct. Even in the cytoplasmic area spread out and appearing thin the filamentous mitochonria as seen in the cells growing under air can hardly be detected.

Under electronmicroscope the cells growing under high O_2 tension that had the swollen mitochondria under phase contrast microscope show the balloon-like mitochondria having distorted or broken crystae, whereas in the cells growing in air the mitochondria appear round or crooked, or straight rod shape bodies in cytoplasm (Figs. 10. 11.). In the cells incubated with pure oxygen for about 36 hours, this characteristic change in mitochonria appeares most marked (Figs. 12, 13). Mitochondrial cristae are shortened and there can be found mitochondria completely devoid of cristae. Another remarkable change found in the cells, which seem to be characteristic to the cells exposed to high oxygen tension, is the appearance of thin filamentous endoplasmic reticulum (ER) (Figs. 14, 15), which appear vesicular in general in the cytoplasm of the cells growing under air. Being exposed to high O_2 environment, they turn to the thin filamentous elongated ERs and appear often as a structure being arranged parallel forming lamellar layer of ER or taking some whirlpool structure with an opaque core in the center (Figs. 16, 17). In normal HeLa cells the Palade's granules free from ER are rich in cytoplasm being scattered throughout the cytoplasmic matrix often forming small clusters gathering together. But in those cells cultured under high O_2 tension the Palade's granules show a tendency to be arranged on the thin filamentous ER which seems to increase in number (Figs. 14, 15).


Figs. 10~25. Electronmicroscope pictures.

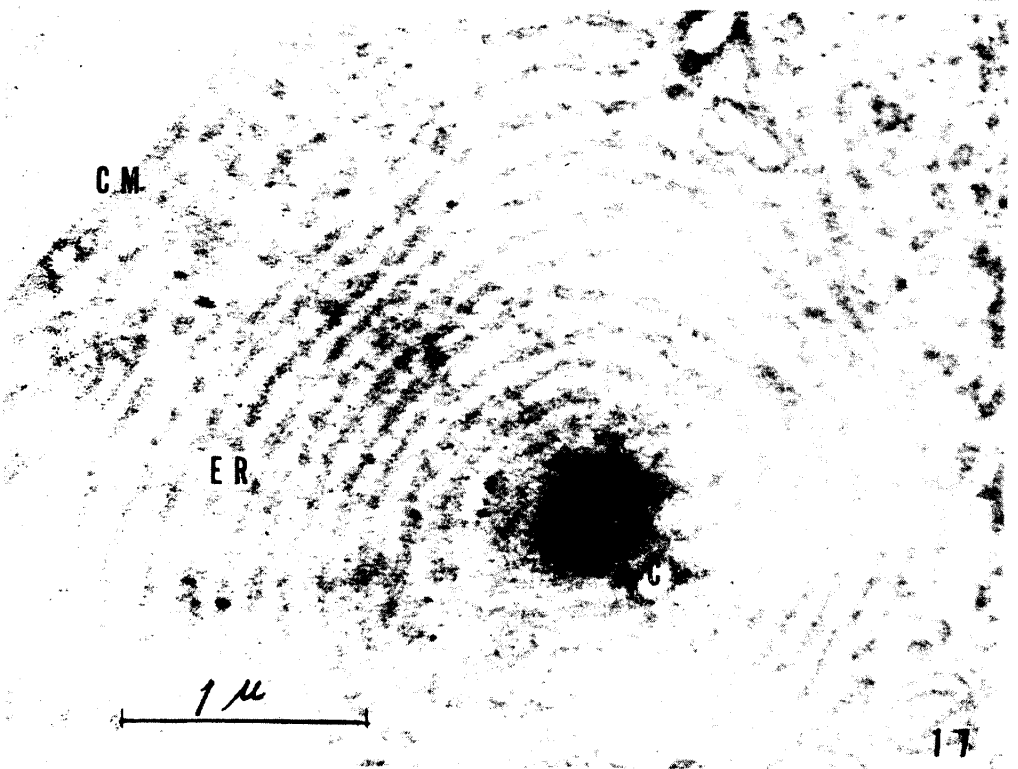Figs. 10~11. HeLa cells cultured in air ERs are in vesicular form.
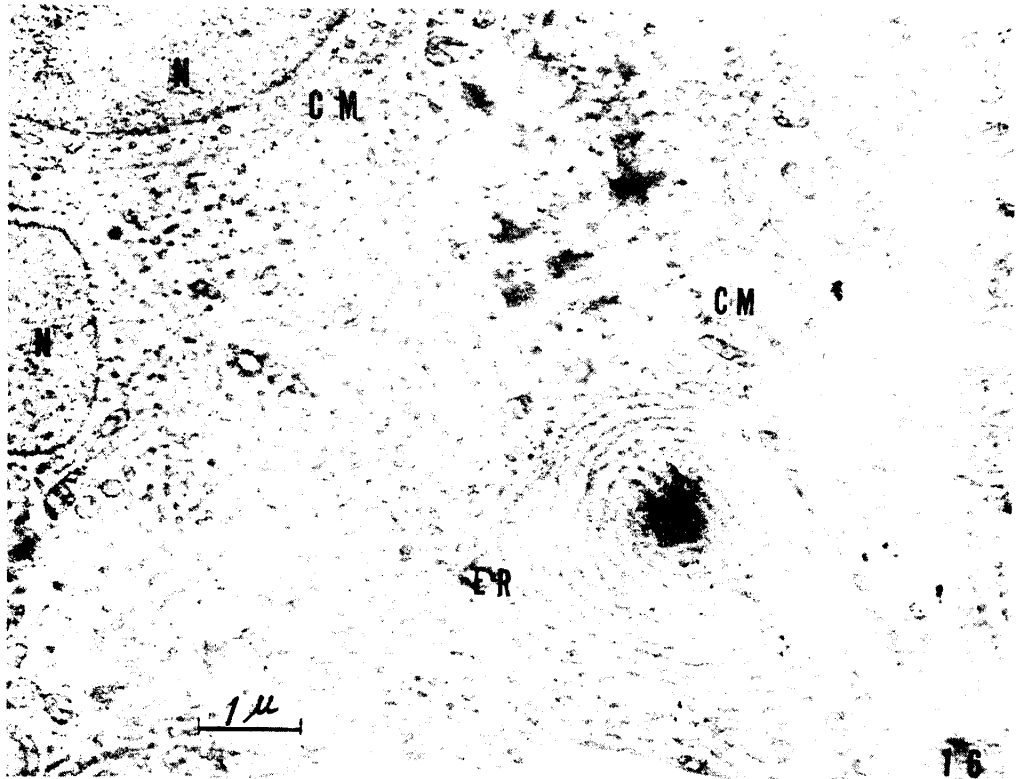
Figs. 12~13. HeLa cells after 36-hours oxygen gas culture : Mitochondria are swollen and there can be seen filamentous ER ($\times 27,500$, $\times 32,000$).

Figs. 14~15. HeLa cells after 36-hours oxygen culture, showing the appearance of fat droplets and ERs are arranged in a order ($\times 25,600$, $\times 25,600$).
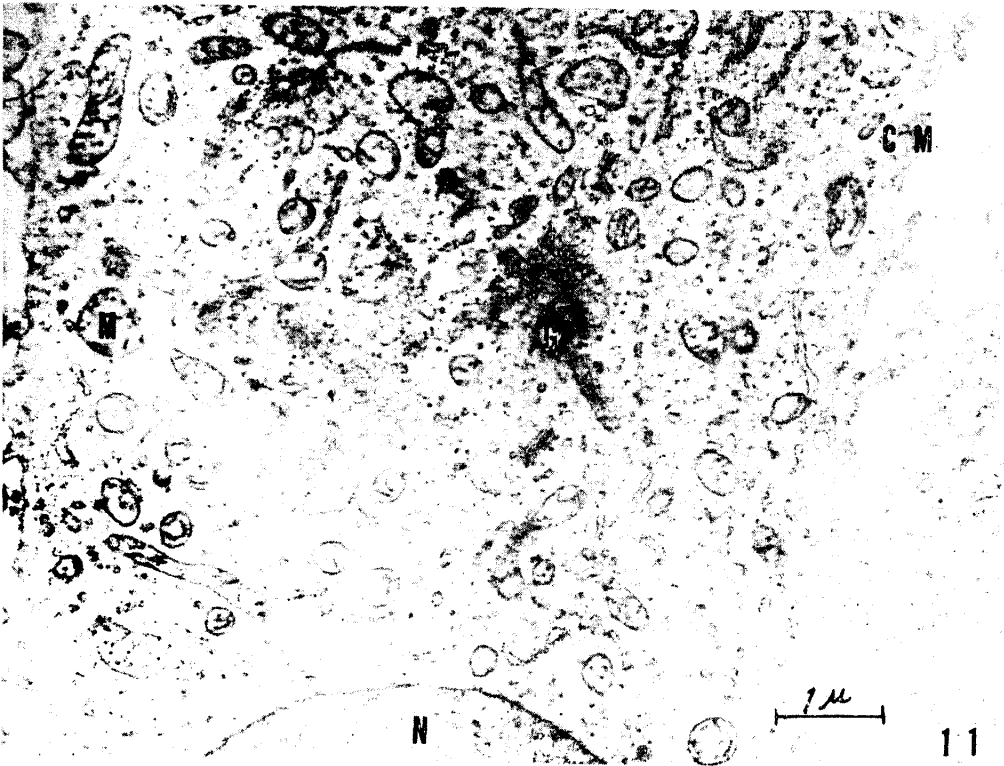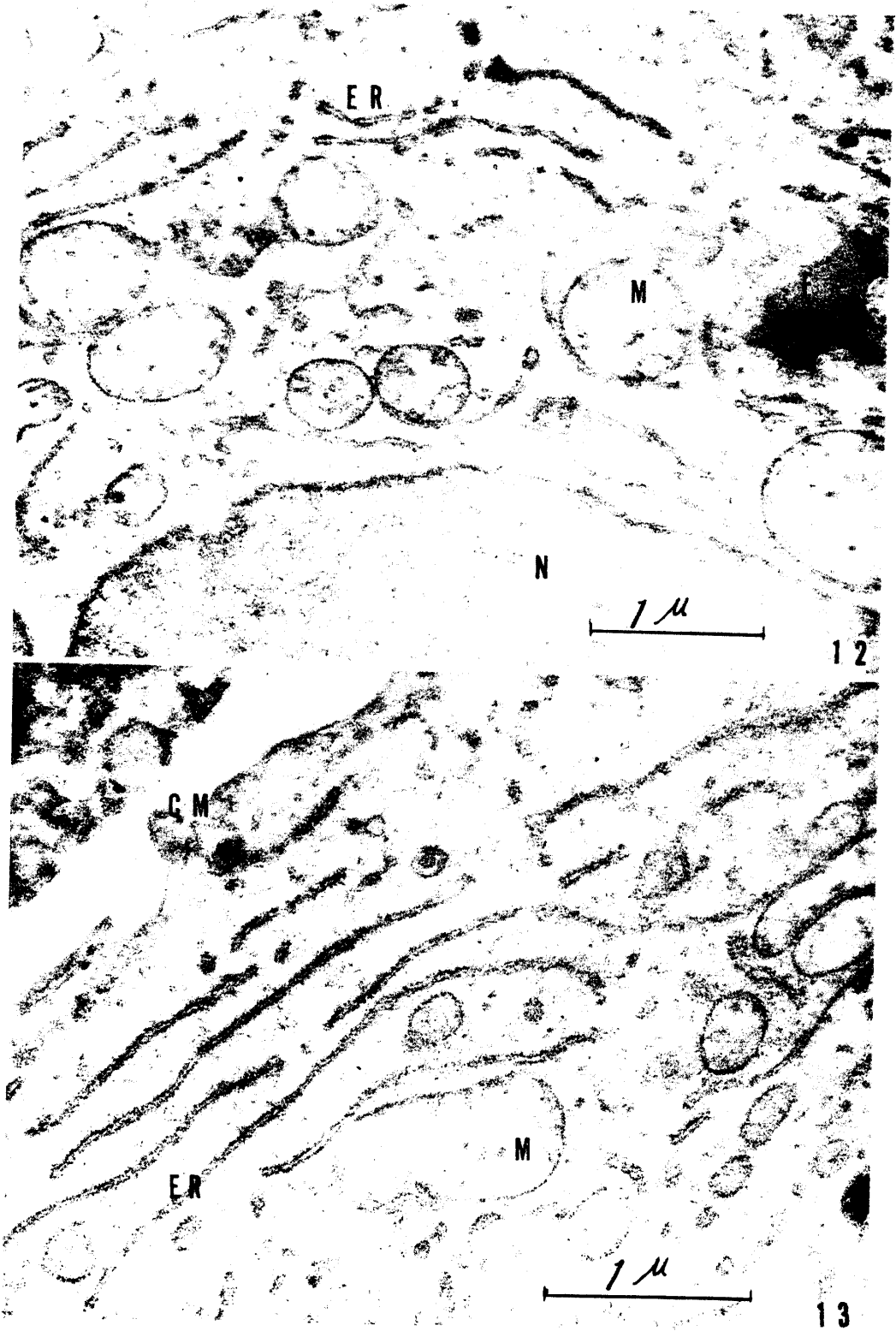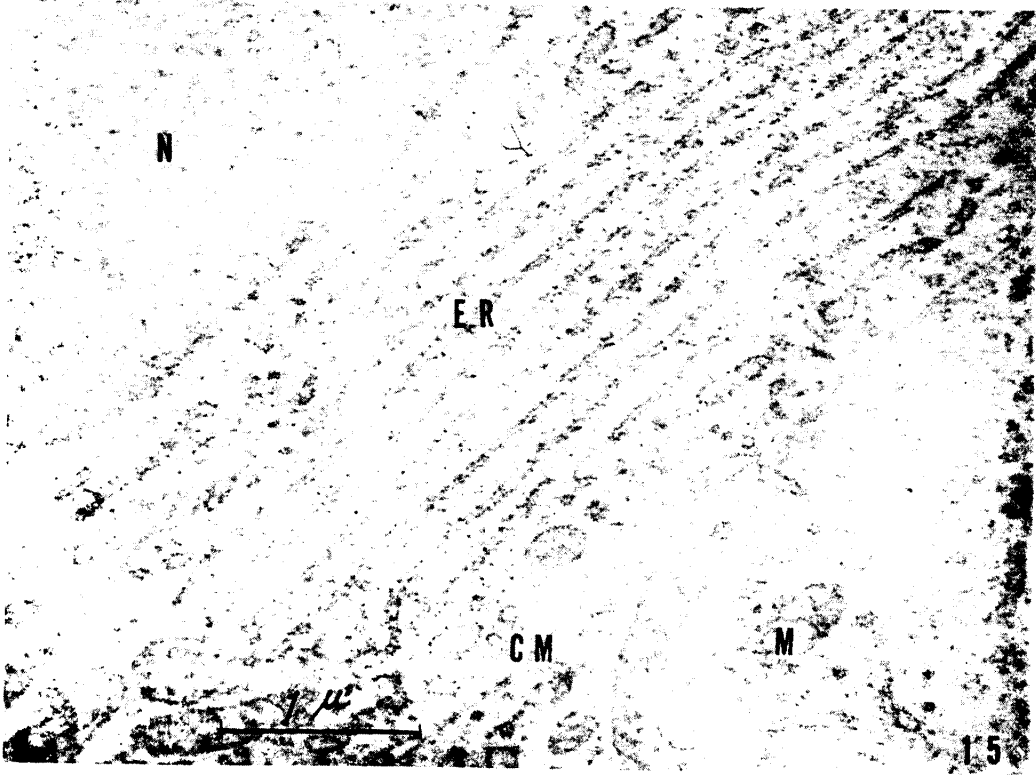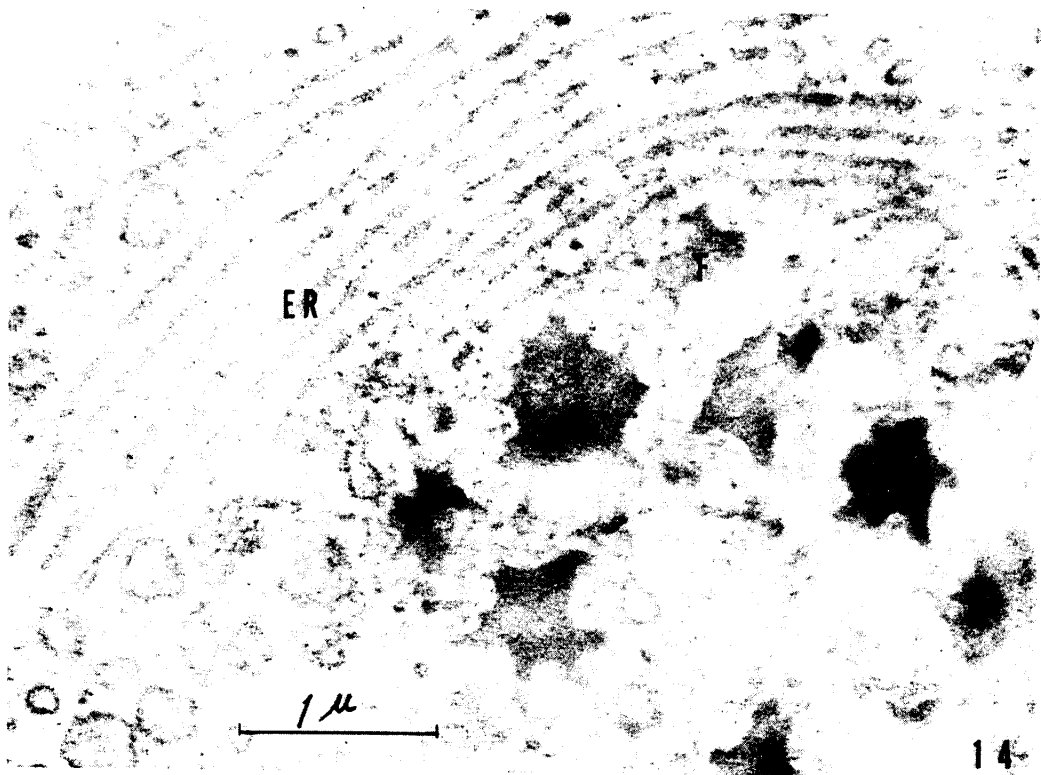
The fat droplets seen under phase contrast microscope, which seem to increase in number under high O_2 tension culture, can also be recognized under electron microscope (Figs. 12, 14). The morphological observations reveal no close relationship between the appearance of these fat droplets and the swollen mitochondria. At first small fat droplets appear in the vesicular ER (Fig. 18), and they seem to grow to bigger ones in the course of time accumulating in some area. However, the accumulation of lipids in mitochondria cannot be completely denied, as some lipid droplets have double membrane (Fig. 19). In the cells severely damaged by the longstanding culture in pure O_2 gas, the chromatin in the nucleus agglomerates mainly adhering to the inner membrane of nuclear envelope and nucleolus or forming a dense mass free in the scanty nuclear sap (Figs. 20, 21). The picture is the general one seen in the degenerating cell. In cytoplasm the swollen mitochondria are completely demolished in their cristae (Fig. 20, M) and the Palade's granules agglomerate with each other forming a dense masses (Figs. 20, 21). The ERs are stripped of Palade's granules and agglomerated with each other forming a mass of smooth surfaced endoplasmic reticulum (Fig. 20).

Now, coming to those cells incubated under nitrogen gas as just pointed out, the cell growth is markedly suppressed by pure N_2 gas. The cells fall off from the glass wall, showing a characteristic appearance. Microscopically, mitochondria are not swollen but they appear thin and opaque in comparing to those growing in air (Figs. 22, 23). Cellular components other than mitochondria show hardly any differences from those of the control group incubated in air. As the cell degeneration proceeds, the cells detach from the glass wall but they are destroyed without swelling of mitochondria in contrast to the cells incubated in oxygen (Fig. 24). ER does not show a marked change in its form in any way from that of the case under oxygen gas. Agglomeration of chromatin in the nucleus and the granules in cytoplasm occur in the cells whose degeneration proceeds further, as in the cases of culture in oxygen.

DISCUSSION

From the data just presented it is clear that an excess of oxygen is very toxic to the cell. It causes the suppressed growth of the cells *in vitro* followed by degeneration and cell death, as can be seen on HeLa cells cultured under a high O_2 tension. The specificity of the "oxygen-intoxication" of the cell may be presented on the morphologic changes of those exposed to O_2 environment for

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Figs. 16~17. HeLa cells after 36-hours oxygen culture
    ERs are arranged in a concentric formation and in the center of this concentric figure an electron dense core can be recognized ($\times 13,000$, $\times 32,000$).

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a certain period of time. And this change may give some clues for the solution of the mechanism of the cell damage by oxygen, which is the essential substance for cell respiration, metabolism, growth and division.

Under a high O_2 tension the general polygonal type of HeLa cells in air, turns to the spindle form taking mesenchymal cell type with the swelling of mitochondria and changes in their arrangement in cytoplasm. Electron microscope observation reveals the demolition of cristae of mitochondria and the appearance of filamentous ER, which has Palade's granules and forms layer in early stage. Later these filamentous ERs turn to the agglomerated mass of smooth surfaced ER by stripping off Palade's granules.

Of these, the change occurring in mitochondria may be essential, because it is supposed that high O_2 tension will act as to disturb the cell respiration. And yet the similar changes of mitochondria like swelling and demolition of cristae are known to be induced by CCl_4[26], KCN or 2, 4-DNP, which will act as to disturb the cell respiration or to induce the uncoupling of respiration to phosphorylation. Much more data should be accumulated before the essential mechanism of the mitochondrial swelling can be explained. However, our present knowledge about the molecular structure of mitochondrial membrane as revealed by GREEN and ODA etc. [6,16] and the mechanism of swelling of mitochondria as thoroughly studied by UTSUMI[10], TAPLEY[11], and LEHNINGER[13] will serve as a great aid in discussing the mechanism.

According to the work of GREEN and ODA, the membrane of mitochondrial cristae is constructed of molecular layer of lipoprotein in which repeating functioning units are assembled in an orderly manner. The functioning units called as the "elementary particles" by them execute a smooth conduction of TCA cycle, electron transfer and oxidative phosphorylation in the state being embedded in the structural proteins and lipid network. The elementary particles seem to be closely associated with some other energy consuming systems to make it possible to use the produced energy rich P-bonds for the needed reaction. To keep the general size as a whole the mitochondria seem to regulate actively the transport of hydrophilic ions and subsequently water. The energy required for the regulation of this active transport is given by ATP and other triphosphates. So it is

Figs. 18~19. HeLa cells after 36-hour oxygen culture
    There can already be seen marked fat droplets in some of the cells.
Figs. 20~21. Pictures of osmium fixed HeLa cells that fell off completely from the glass wall, the same material in Fig. 12 but 12 hours later. Lamellar filamentous structures (f) are thought to be the vestiges of ER. Nucleoles appear in a radial form and nuclear chromatins are congregated on the periphery of nucleus. In cytoplasm are scattered numerous granules as dense as chromatin ($\times$17,000, $\times$16,000).
Figs. 22~23. Electron microscopic pictures of HeLa cells after 36-hour N_2 culture
    Mitochondria are not swollen and more electron dense ($\times$17,000, $\times$16,000).

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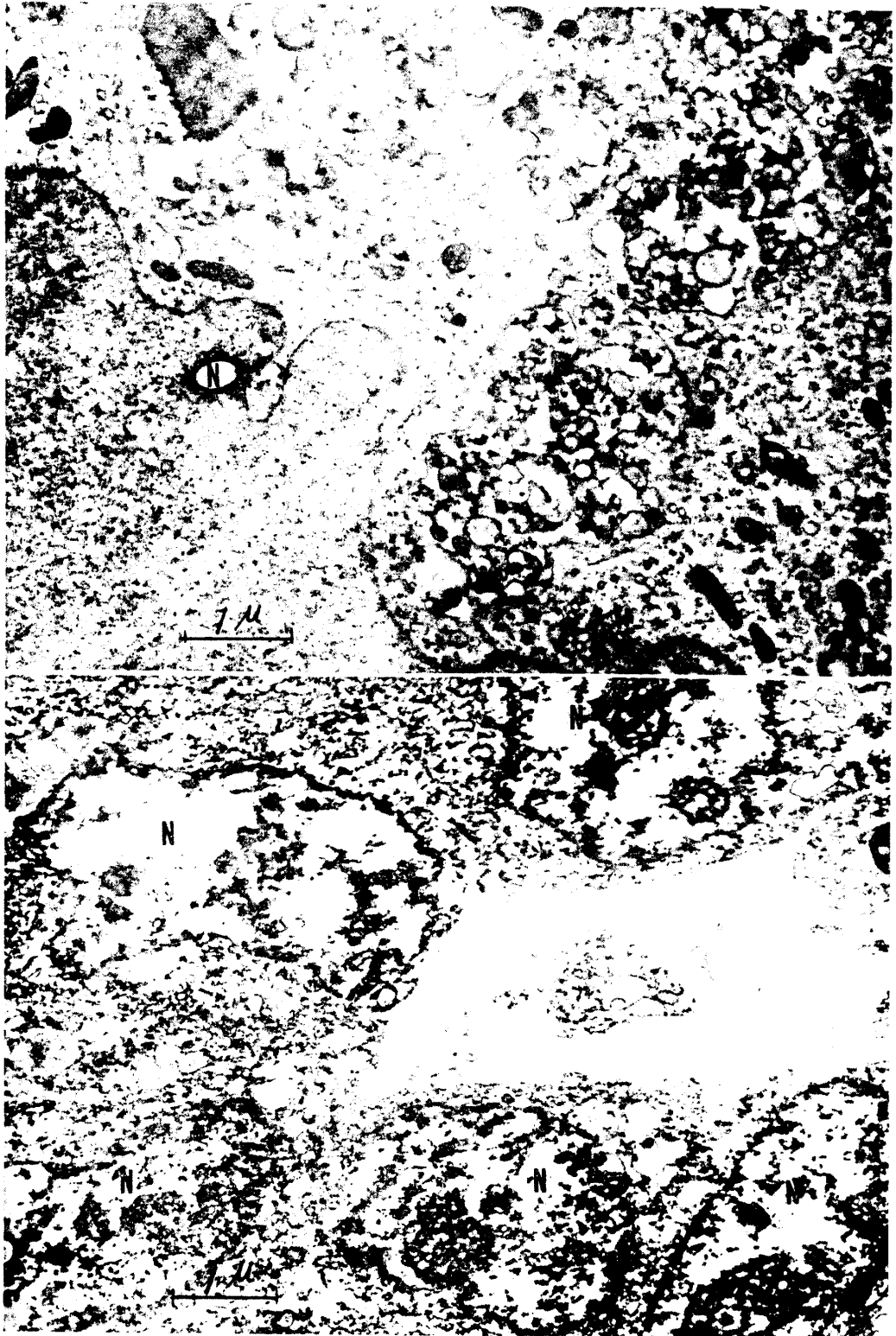
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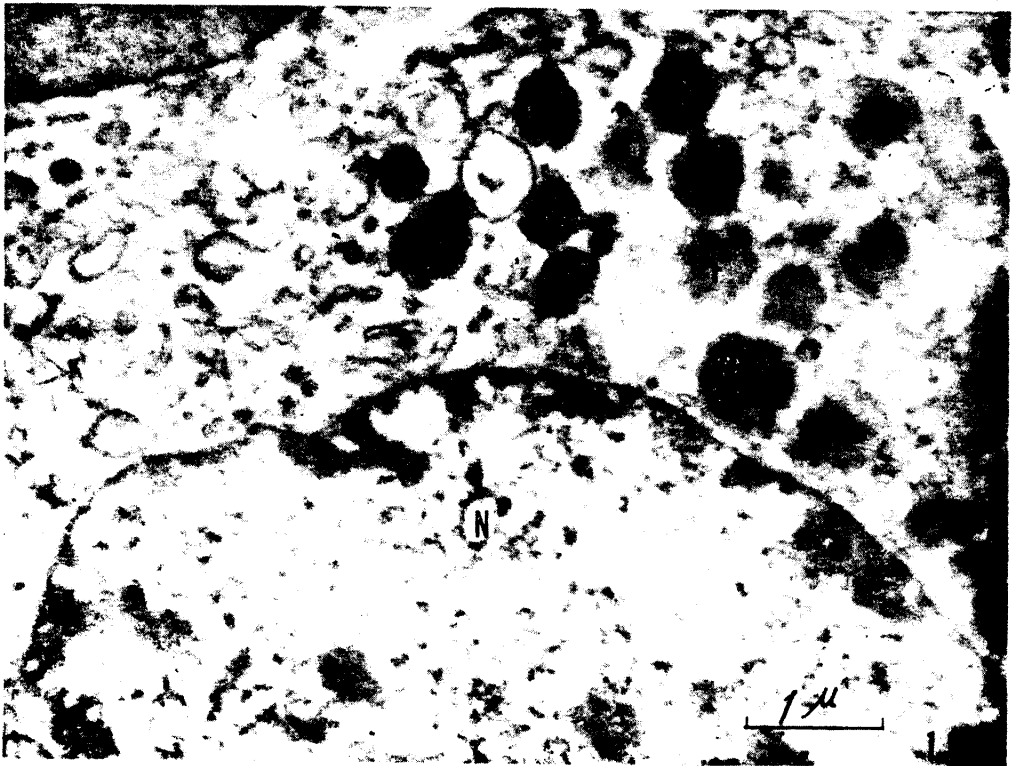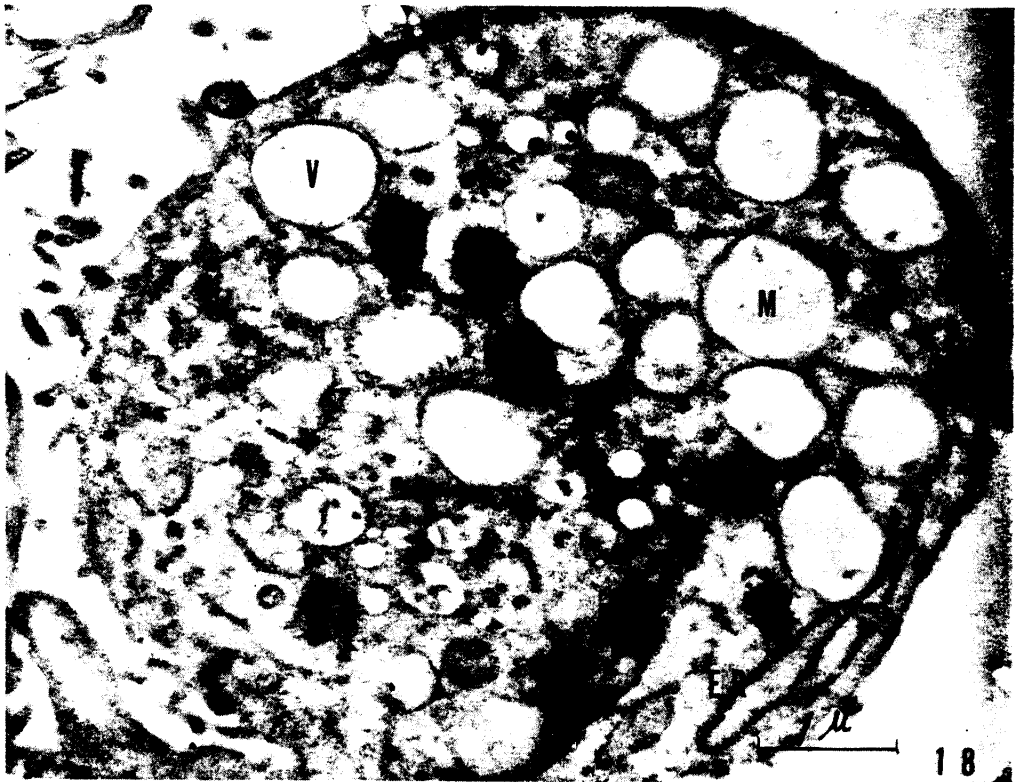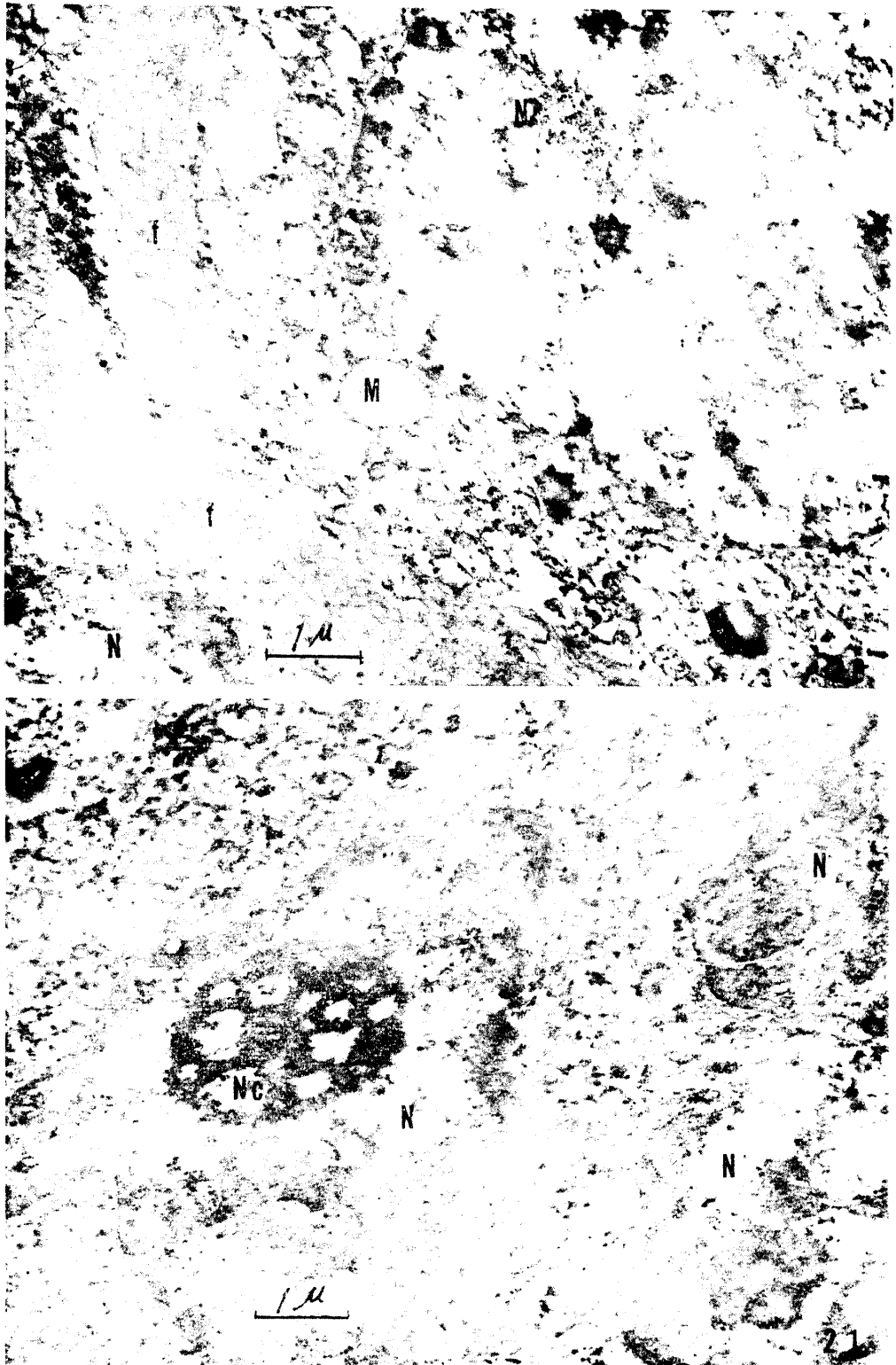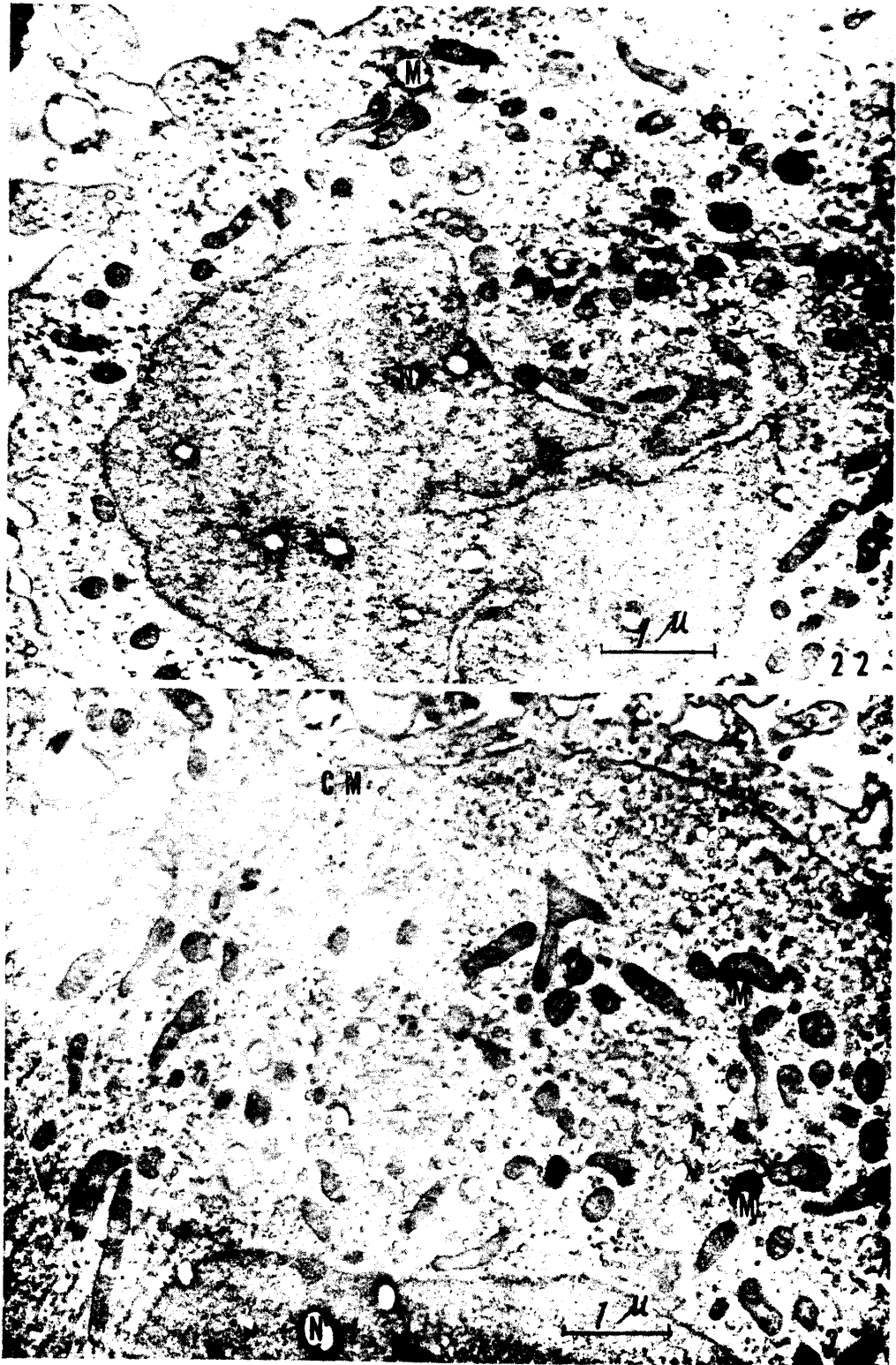
well understood that the inhibition of respiration by KCN or of oxidative phosphorylation by 2, 4-DNP results in the swelling of mitochondria, and the swelling can be prevented by adenine nucleotide[7,9], activated-phosphorylation mechanism[8,15], various metal ions like Mg^{++}, Mn^{++}, known as the activator for ATPase, etc. though there is some inconsistency in the interpretation of the respective action mechanism of these factors, e. g. CHAPPEL et al.[12] assert the accelerated swelling by the increased oxidative phosphorylation, and other authors state that 2, 4-dinitrophenol can prevent or accelerate the mitochondrial swelling depending upon the conditions[15]. On the basis of morphologic changes alone it is impossible to suppose the mechanism of mitochondrial swelling induced by excessive O_2. However, it may be most plausible that the disturbances in electron transport system is induced which will result in the deficiency of ATP, and then the invasion of ions and water into mitochondria, because oxygen is essential for the cell respiration.   But against this supposition the author's experiment proved that deficiency in O_2, which will lead to the deficiency of ATP, does not result in the swelling of mitochondria but a marked shrinkage, as seen on HeLa cells in culture under pure N_2 gas. The tumor cells, which gain energy for living mainly from the anaerobic glycolysis, may not result in the ATP deficiency under an insufficient O_2 supply, but HUNTER's work[14] shows that oxygen is indispensable for the swelling of mitochondria, as demonstrated on the isolated mitochondria from normal rat liver. This suggests that some oxidation or oxygenation mechanism will be required for the swelling of mitochondria, the increased transport of ions and water, and the excess of O_2 will accelerate the mechanism. The mechanism may be due to a rapid electron flow without linking to oxidative phosphorylation. This is the problem to be looked later by the author.

Be it as it may, it can safely be said that such a swelling of mitochondria would impair the respiratory system and thus inhibit energy production, leading the cells to death.   The second problem to be commented is the transformation of the type of ER by a high oxygen tension. The membrane system of cancer cell generally does not possess an organized lamellar system and consequently RNA granules are usually scattered in the ground substance of cytoplasm[17,18]. HeLa cells are by no means the exception. ER is very poor and vesicular in form, and those lamellar form are very rare. But after being exposed to pure oxygen for 36 hours they have rich lamellar ergastoplasmic structure

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Figs. 24~25.   The same HeLa cells of Fig. 22 cultured in nitrogen gas (cells fallen off)

Fig. 26   shows the cells with less changes and Fig. 25 those with greater changes.
The changes of nucleoles and nuclei differ not any markedly from those cultured in oxygen, and there can be observed no swelling of mitochondria until the cells die ($\times$ 16,000, $\times$5,000).

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attached with Palade's granules. Whirl-like structure of ER also can be seen. The latter has a core, an electron dense spherical body in the center of the lamellar membraneous whirl. It is worthwhile to notice that no vesicular form ER can be recognized in the region where lamellar ergastoplasm is located. This finding resembles quite closely to the regeneration picture of ER as demonstrated by Weiss[21] in pancreatic exocrine cells of Swiss albino mouse. This whirl structure of ER may develop from the repeated engulfing of the membraneous ER, as supposed from the mechanism revealed by Kimoto[22] on the mouse pancreas treated with RNase injection, but such a process could not be observed. Furthermore, no morphological relationship has been revealed between whirl-structure of ER and the nuclear membrane[19, 24, 25] nor mitochondrial membrane[20]. Anyway it is of interest that such changes of ER occur in the cells cultured under oxygen environment. The mechanism of morphogenesis of lamellar ER is obscure, but Hodge's work on chlorplasts of Nitella and Zea[23] may be suggestive. The lamellae of chlorplast are formed rapidly by fusion or coalescence of minute vesicles on exposure of the plant to sunlight. The fusion step seems to require chlorophyll. As is already known, chlorophyll is oxidized in the presence of sunlight. The significance of this oxidation is not so well known but it seems that the lamellar formation of ER under a high oxygen conentration may be a phenomenon somehow related to the oxidation phenomenon, and it may show some functioning phase of ER, as can be seen in some well differentiated secretory cells, e. g. pancreas exocrine cells, plasma cells, etc. The problems require further studies but the data seem to suggest that continuous oxygen supply in adequate amount may have a possibility to make the tumor cells mature in some way.

SUMMARY

　　1.　Morphological observations were carried out on the strain HeLa cells cultured under various oxygen tension.

　　2.　The growth of the strain HeLa cells in the present experiments was markedly inhibited when they were cultured under high oxygen tension or in nitrogen gas environment. It has been clarified that air offers the most optimal gas environment.

　　3.　Effects of the changed gas environment on the fine structures of HeLa cells were studied by phase contrast microscope and electronmicroscope. As the results it has been found that in these cells cultured under a high oxygen tension there occurs a marked swelling of mitochondria. Under anaerobic condition, however, these cells undergo degeneration, as in oxygen environment revealing no swelling of mitochondria but rather contraction.

　　4.　Endoplasmic reticulum (ER) of HeLa cells cultured under oxygen en-

vironment is transformed from its vesicular form to lamellar form. The mechanism of lamellar transformation of ER is obscure but no morphologic connection with nuclear, cytoplasmic and mitochondrial membranes has been detected.

5. A discussion was made on these findings concerning the changes in fine structures of the cells, with a special reference to the swelling of mitochondria and morphological changes of ER under oxygen environment.

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