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## The effect of oxygen tension on tetrazolium reduction by respiratory enzyme systems of tissue culture cells

Iwao Matsuoka\*

Takuzo Oda<sup>†</sup>

Satimaru Seno<sup>‡</sup>

\*Okayama University,

<sup>†</sup>Okayama University,

<sup>‡</sup>Okayama University,

# The effect of oxygen tension on tetrazolium reduction by respiratory enzyme systems of tissue culture cells\*

Iwao Matsuoka, Takuzo Oda, and Satimaru Seno

## Abstract

1. Attempts have been made to confirm how the formazan formation is affected in the presence of oxygen gas when the cells are incubated with neotetrazolium salt and the substrates for the enzymes to be tested. 2. In the cases of succinoxidase formazan formation is minimized under pure O<sub>2</sub> tension, it increases with decrease in O<sub>2</sub> tension, and reaches its maximum value under N<sub>2</sub> gas. 3. This relationship between the oxygen tension and the diformazan formation can likewise be observed even after pretreatment of the system with KCN. 4. In measuring enzyme activity of the DPN-diaphorase system with L-glutamate and DPN as substrate and NT as hydrogen acceptor, the same relationships between the oxygen tension and the NT-reduction can be seen as in succinoxidase system. 5. In the determination of enzyme activity of the cytochrome-c-cytochrome oxidase system with p-phenylene-diamine as substrate and NT as hydrogen acceptor, likewise the diformazan formation is markedly affected by oxygen tension and increased with the reduced oxygen tension but under pure N<sub>2</sub> gas the value is reduced. When the system is pretreated with KCN, however, the diformazan formation reveals its maximum value under pure nitrogen gas, the values of which correspond to those values of endogenous reaction without substrate. 6. The above results show that the neotetrazolium salt can compete with O<sub>2</sub> as hydrogen acceptor, and less values of formazan formation may be obtained under higher oxygen tension and the higher values under lower oxygen tension independently from the true activity of the enzyme.

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**THE EFFECT OF OXYGEN TENSION ON TETRAZOLIUM  
REDUCTION BY RESPIRATORY ENZYME SYSTEMS  
OF TISSUE CULTURE CELLS**

Iwao MATSUOKA, Takuzo ODA and Satimaru SENO

*Department of Pathology, Okayama University Medical School  
(Director: Prof. S. Seno)*

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In the course of the observations on the activities of respiratory enzymes conducted biochemically and histochemically by using neotetrazolium salt as electron acceptor, the authors have noticed that the increased  $O^2$  tension in the gaseous space during the incubation of the cells acts as to decrease the reduction of tetrazolium salt. The papers concerning the relationship between the formazan formation and the  $O^2$  tension appeared in the past but they presented much disagreement with each other, resulting in the confusion of the opinions. The main reason for this seems to be due to the fact that the materials and methods for the observation have been at a variance. e. g. even in the observation of a similar kind of cells the values obtained by using tissue slices, whose thickness cannot be strictly controlled and in which a small difference in thickness results in a big difference in cell number of a given sample, may be less reliable or difficult to compare with each other or with those obtained by cell suspension.

From this viewpoint the authors observed the living cell suspension of L-cells cultured in such a fashion by which the cell number can be controlled strictly and the living activity of the cells can also be controlled by culturing under the same conditions. The results are presented in this paper and show a definite influence of  $O^2$  tension on the reaction of the respiratory enzymes to be demonstrated by using the tetrazolium salt.

MATERIALS AND METHODS

The L-strain and HeLa cells in culture served as materials. After 3-day culture they were washed twice with Hank's solution and suspended in an adequate amount of Hank's solution by being detached mechanically from the culture vessel wall. The cell suspension thus obtained contained 500,000 to 1,500,000 cells per ml. Each one ml of the suspension was taken into the test tube of 1.5 mm in diameter and 10 cm in length, total of 10 tubes for one observation. The culture vessel should be kept shaking during the transfer. The cell suspension in each test tube was added with 2 ml of the mixture of equal

volume of substrate solution, 0.2 per cent neotetrazolium chloride aqueous solution (NT) and 0.2M phosphate buffer solution (pH 7.6). After adding the solution each tube was tightly stoppered with rubber W stopper and the gaseous space of the tube was immediately filled with the oxygen-nitrogen mixture in a certain proportion by the method described in the previous paper<sup>1,2</sup>. Thus 10 test tubes contained pure oxygen, 80%, 70%, 50%, 30%, and 20% oxygen, air, and pure nitrogen gas. Then the cells were incubated at 37°C, being gently moved by the roller tube method. After one hour incubation 2 ml of neutralized 10% formalin was added in each tube to stop the reaction. Then the packed cells were obtained by centrifugation (3,000 r. p. m. for 10 min). To the packed cells were added exactly 4 ml of ether-aceton mixture in an equal volume and the amount of diformazan extracted by ether-aceton was measured by spectrophotometer at the wave length of 520 m $\mu$ <sup>3,4</sup>. For the measurement of succinoxidase activity 0.2M sodium succinate was used as the substrate solution, for the DPN-diaphorase the mixture of 0.45% DPN, 0.5 M sodium malate, pH 7.6, and 0.5 M sodium-L-glutamate in the volume ratio of 1.0: 1.5: 2.5 and for the cytochrome c cytochrome oxidase system 0.2 per cent *p*-phenylene diamine was used. In the case of cytochrome-c-cytochrome oxidase 2 ml of 1N sulfuric acid was added to stop the reaction, and in case of DPN diaphorase reaction was stopped after 30 min of incubation.

KCN, which was used for the purpose to inhibit the activity of cytochrome oxidase, was added to the medium so as to make the final concentration of KCN to be 0.005 M. In the control series endogenous substrate reaction was observed by adding an equal volume of buffer solution in place of substrate solution.

## RESULTS

The amount of diformazan produced by the L-cells incubated for 60 minutes with NT and succinate was minimum in the tube containing pure oxygen and increased with the decrease in oxygen tension, reaching the maximum value under pure nitrogen. Exactly the same tendency as in L cells was observed in the cases of HeLa cells (Figs. 1, 2). Under air the diformazan formation was almost the same as that under the gas having 20 per cent oxygen. Endogenous reaction in the medium containing no succinate showed the same tendency though the amount of formazan was much less than that in the cases having substrate.

The reaction curve in the case of air proceeded in a straight line with time until 90 min. After that it declined slowly reaching plateau after 3 hours (Fig. 3). Consequently, the following observations were carried out with the incubation time of 30 to 90 minutes, and 180 minutes at longest.

In the reaction series, preincubated with pure oxygen for various periods

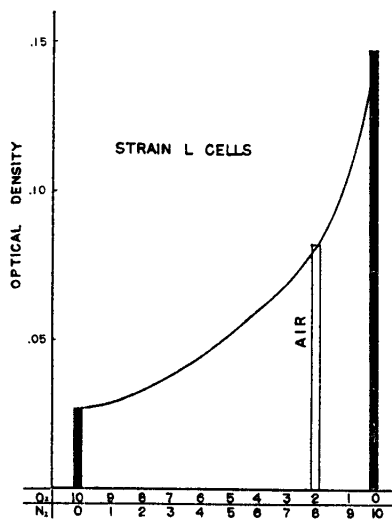


Fig. 1 Effect of oxygen tension on the NT reduction by succinoxidase system of strain L cells. Columns show the amount of NT reduced, in optical density at 520  $m\mu$ , after one hour incubation in the reaction medium with succinate as substrate under pure oxygen, air, and pure nitrogen gases, respectively.

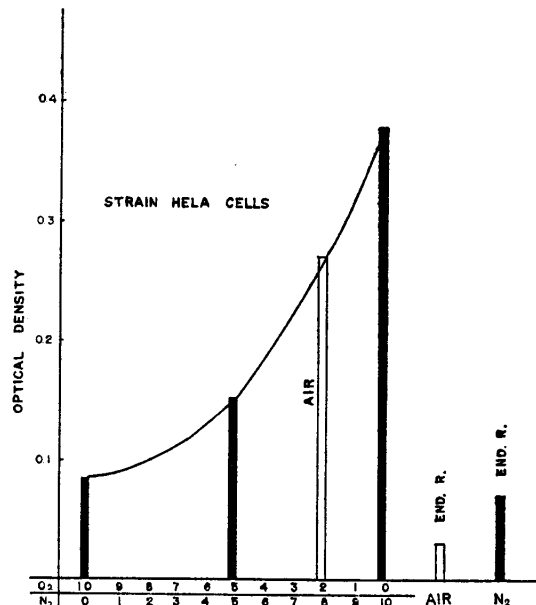


Fig. 2 The same as Fig. 1. but for strain HeLa cells.

of time (30 min to 120 min) and then replaced with pure nitrogen the formation of diformazan was accelerated under nitrogen (Fig. 4). As demonstrated in Fig. 4, formazan was formed more under N<sub>2</sub> gas than under O<sub>2</sub>, about 4 times as much as that of the latter (A and B in Fig. 4). This relation had been clearly recognized in the cases previously incubated under O<sub>2</sub> gas for the various periods of time and placed under N<sub>2</sub> gas. The difference in the rate of formazan formation was also observed in the case incubated previously with N<sub>2</sub> gas followed by replacing with O<sub>2</sub> gas (Fig. 5).

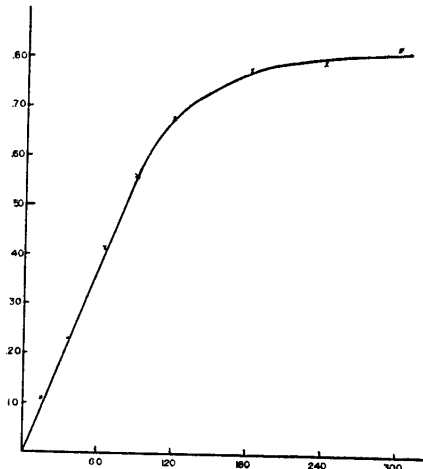


Fig. 3 The relationship between reaction time and the amount of NT reduced, expressed in optical density at 520  $m\mu$ .

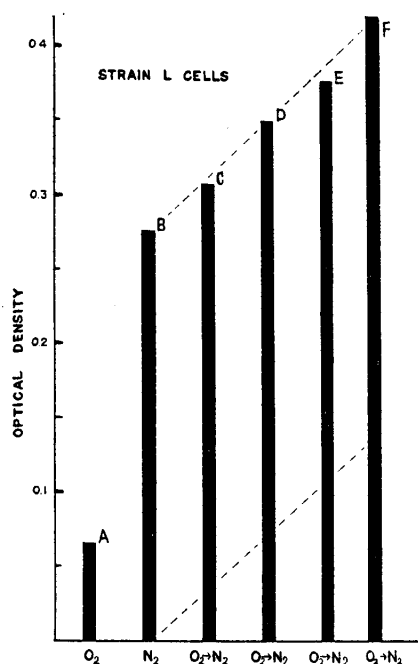


Fig. 4 Effect of preincubation under pure oxygen during various periods of time on the NT reduction by succinoxidase system of strain L cells. (A) was incubated in the reaction medium under pure oxygen from the beginning to end for 60 min. (B) was incubated under pure nitrogen from beginning to end for 60 min. (C), (D), (E), and (F) were incubated under pure oxygen for 30 min., 60 min., 90 min., and 120 min., respectively, and then the gasses were replaced with pure nitrogen and further incubated for 60 min.

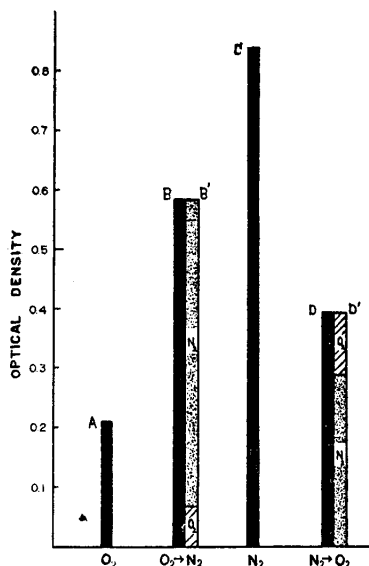


Fig. 5 Effect of alternative changing of oxygen tension on the NT reduction. (A) was incubated under pure oxygen from beginning to end for 90 min. (B) was incubated under pure oxygen for 30 min and then under nitrogen for 60 min. (C) was incubated under nitrogen for 90 min from beginning to end. (D) was incubated under nitrogen for 30 min and then under oxygen for 60 min.

Observation on the DPN-diaphorase revealed identically the same relation to oxygen tension as in the case with the succinic oxidase system (Fig. 6). But in this case the amount of the formazan formed under N<sub>2</sub> gas was about two times that under O<sub>2</sub> gas.

Observations on the cytochrome-c-oxidase system conducted with para-phenylene diamine (p-PDA) as the substrate gave some different data from those in the succinoxidase system and in the DPN-diaphorase system. In this case, too, the quantity of diformazan formation was greatly suppressed under pure oxygen and increased with the reduced oxygen tension, reaching a very high value under air as high as 10 times that under pure oxygen but it decreased again

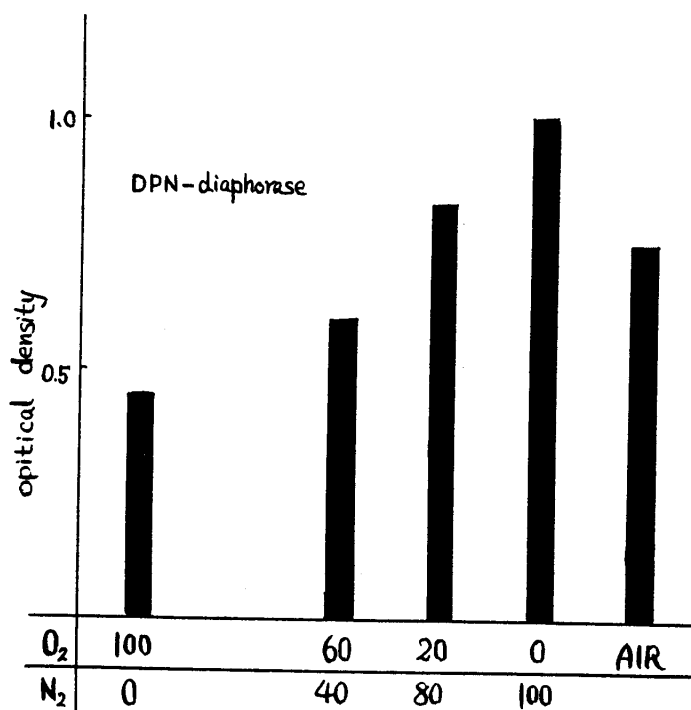


Fig. 6 Effect of oxygen tension on the NT reduction by DPN-diaphorase of strain L cells. Columns represent the amount of NT reduced, expressed in optical density at 520  $m\mu$ , after 30 min incubation under various oxygen tension at indicated ratio.

under nitrogen gas (Fig. 7).

Observation of the cells previously treated with 0.005 M KCN for 10 minutes proved that the rate of NT reduction by succinoxidase system under various oxygen tensions decreased, though it showed the same pattern as those of no pretreatment with KCN (Fig. 8). Control experiment done under air without pretreatment of KCN proved that only a part of the activity (about 3/7) of this enzyme was suppressed by KCN. On the other hand, the cytochrome-c-oxidase system showed a marked change in the formazan production by the pretreatment with KCN, losing the characteristic pattern seen in the cases without pretreatment with KCN (Fig. 9). The NT reduction under nitrogen gas reached the maximum value as in the case of succinoxidase system though the amount of the formazan formed was much less.

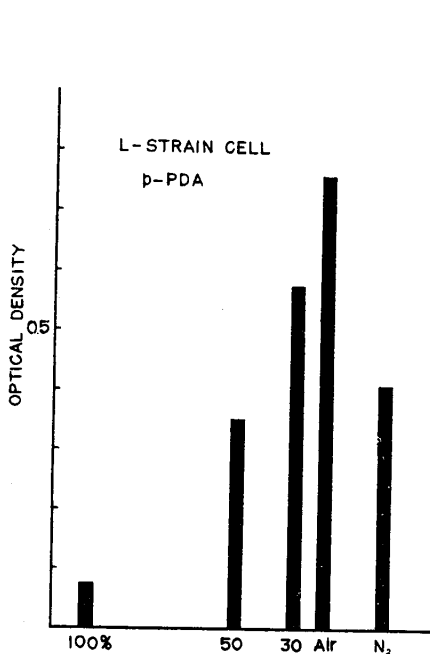


Fig. 7 Effect of oxygen tension on the NT reduction by cytochrome-c-cytochrome oxidase system of strain L cells. Columns represent the amount of NT reduced, expressed in optical density at 520 m $\mu$ , after 60 min incubation under various oxygen tension at indicated ratio.

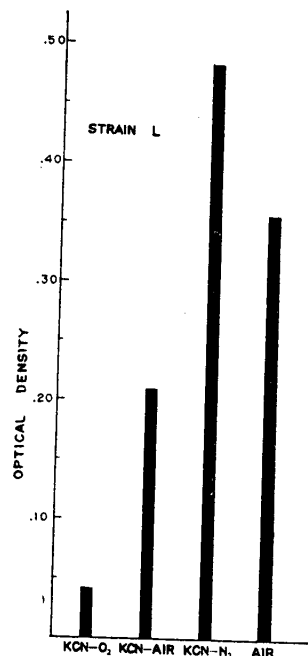


Fig. 8 Effect of potassium cyanide pretreatment on the NT reduction by succinoxidase system of strain L cells. The cell suspension was pretreated with neutralized 0.05 M potassium cyanide in final concentration for 10 min and then the reaction was made to proceed for 60 min with succinate as substrate under various gas tension at indicated ratio.

#### DISCUSSION

Up to date many attempts have been made to estimate enzyme activities in succinoxidase system, DPN, or TPN-diaphorase system, etc. with the use of tetrazolium salts, and the majority of investigators have found an increase in the formazan formation under anaerobic conditions<sup>9-11</sup> though some investigators insist that anaerobic conditions have no appreciable influence on the diformazan formation<sup>12,13</sup>.

SELIGMAN, NACHLAS *et al*<sup>9</sup>. found an increased reduction rate of tetrazolium salts (TPT, BT, NT, excepting nitro-BT) in the determination of succinoxidase system in an anaerobic condition, especially in the reactions by using tissue sections instead of homogenates.

ICHIKAWA<sup>12</sup> also observed the increased formazan formation in the reaction



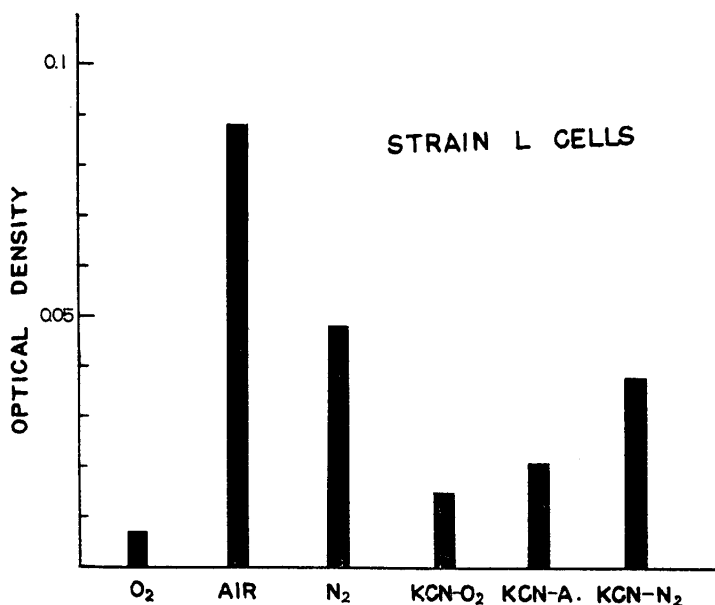


Fig. 9 Effect of potassium cyanide pretreatment on the NT reduction by cytochrome-c-cytochrome oxidase system of strain L cells. The pretreatment was done with neutralized 0.005 M potassium cyanide in final concentration for 10 min and then the reaction was made to proceed for 60 min with paraphenylenediamine as substrate under various gas tension at indicated ratio.

of the dehydrogenase in living cells under anaerobic condition when glutamic acid was used as substrate. The intensity of NT reduction, however, was the same both under anaerobic and aerobic conditions when succinate was used as substrate. Finally, he contends the reaction generally does not require anaerobic conditions. FARBER, STENBERG *et al.*<sup>18</sup> likewise claim that there is no need of expelling oxygen by boiling nor is it necessary to change the reaction atmosphere to nitrogen gas, in their studies on DPN and TPN-diaphorase systems by histochemical staining of tissue sections 20  $\mu$  thick, in which they used blue tetrazolium (BT) and NT as hydrogen acceptors.

Judging from the authors' own experiences, it seems that the selection of the enzyme systems, tetrazolium salts, and the methods of analysis are responsible for these conflicting findings. It appears that in the preparation of homogenate, mechanical damage may bring about injury to the intracellular membranous structures and hence the changes in enzymic activities resulting in losing the reacting ability to aerobic or anaerobic conditions. In the experiments by using tissue sections the difference in the thickness will act as a definite factor as compared to that of gaseous atmosphere, as the cell number contained in the

slice is greatly changed by a slight change in thickness. Furthermore, ability for oxidative phosphorylation and active transport is largely damaged in the frozen tissue sections. Consequently, it is to be expected that the reasonable result will be given by observing the living cells suspended in an adequate medium where the cell number can be controlled fairly exactly and the vital activity is well retained. As just presented in the authors' experiments an unexpectedly tremendous effect of oxygen tension can be seen on the formazan formation. The highest value under  $N_2$  gas in succinoxidase and DPN-diaphorase systems clearly show that  $O_2$  competes with neotetrazolium salts as hydrogen acceptor.

The decrease in the formazan formation in  $N_2$  gas in the case of the cytochrome-c-cytochrome oxidase system shows that the tetrazolium salt is not so effective hydrogen acceptor as  $O_2$  gas for this enzyme system. The inhibition of the activity of the cytochrome oxidase system by KCN resulted in the formazan formation in the type of succinoxidase system, the highest value under  $N_2$ . This coincides with the endogenous reaction without substrate. In the succinoxidase system the pretreatment with KCN inhibits about 3/7 of the reaction and a part of electrons can directly flow to the dye without intermediation of cytochrome system.

Thus, it is clear that the tetrazolium salt can compete with  $O_2$  in a varying degree according to the kinds of enzymes to be tested. In the case of succinoxidase system the tetrazolium salt can be used as hydrogen acceptor efficiently as well as oxygen, and under  $N_2$  gas the highest value can be obtained independently from the true activity of the enzyme, while in the case of cytochrome c-cytochrome oxidase system,  $O_2$  is essential for keeping the unchanged activity of the enzyme, and formazan formation is reduced under anaerobic conditions. The finding supports the opinion of OKUNUKI<sup>14</sup> who claims the cytochrome a- $O_2$  complex for cytochrome oxidase. According to this theory  $O_2$  is necessary for the formation of cytochrome a- $O_2$  complex, which will act as the terminal oxidase of the cytochrome system.

#### CONCLUSION

1. Attempts have been made to confirm how the formazan formation is affected in the presence of oxygen gas when the cells are incubated with neotetrazolium salt and the substrates for the enzymes to be tested.
2. In the cases of succinoxidase formazan formation is minimized under pure  $O_2$  tension, it increases with decrease in  $O_2$  tension, and reaches its maximum value under  $N_2$  gas.
3. This relationship between the oxygen tension and the diformazan formation can likewise be observed even after pretreatment of the system with KCN.
4. In measuring enzyme activity of the DPN-diaphorase system with L-

glutamate and DPN as substrate and NT as hydrogen acceptor, the same relationships between the oxygen tension and the NT-reduction can be seen as in succinoxidase system.

5. In the determination of enzyme activity of the cytochrome-c-cytochrome oxidase system with p-phenylene-diamine as substrate and NT as hydrogen acceptor, likewise the diformazan formation is markedly affected by oxygen tension and increased with the reduced oxygen tension but under pure N<sub>2</sub> gas the value is reduced. When the system is pretreated with KCN, however, the diformazan formation reveals its maximum value under pure nitrogen gas, the values of which correspond to those values of endogenous reaction without substrate.

6. The above results show that the neotetrazolium salt can compete with O<sub>2</sub> as hydrogen acceptor, and less values of formazan formation may be obtained under higher oxygen tension and the higher values under lower oxygen tension independently from the true activity of the enzyme.

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#### REFERENCES

1. MATSUOKA, I.: Biological effect of oxygen tension on culture cells. I. A morphological study. *Acta Med. Okayama* 16, 205, 1962
2. Matsuoka, I.: Biological effect of oxygen tension on culture cells. II. A biochemical study. *Acta Med. Okayama* 17, 193, 1963
3. ODA, T.: Cytochemical and biochemical studies on the terminal electron transport system. I. Analytical studies on the colorimetric estimation of the activities of the succinic dehydrogenase system and cytochrome oxidase. *Symposia Cell. Chem.* 8, 157, 1958
4. ODA, T., and OKAZAKI, H.: An analytical study on the reduction of neotetrazolium chloride by the terminal electron transport system. *Acta Med. Okayama* 12, 193, 1958
5. KUN, E., and ABOOD, L.G.: Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. *Science* 109, 144, 1949
6. RUTENBURG, A.M., GOFSTEIN, K., and SELIGMAN, A.M.: Preparation of a new tetrazolium salt which yields a blue pigment on reduction and its use in the demonstration of enzymes in normal and neoplastic tissues. *Cancer Res.* 10, 113, 1950
7. GLOCKE, E., and JENSEN, C.O.: The colorimetric determination of plant succinic dehydrogenase. *J. Biol. Chem.* 201, 271, 1953
8. RUTENBURG, A.M., WOLMAN, M., SELIGMAN, A.M.: Comparative distribution of succinic dehydrogenase in six mammals and modification in the histochemical technics. *J. Histochem. and Cytochem.* 1, 66, 1953
9. NACHLAS, M.M., TSOU, K.C., De SOUZA, E., CHENG, C.S., and SELIGMAN, A.M., Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J. Histochem. and Cytochem.* 5, 420, 1957
10. NACKLAS, M.M., MARGULIES, S.I., and SELIGMAN, A.M.: Sites of electron transport to tetrazolium salts in the succinoxidase system. *J. Biol. Chem.* 235, 2739, 1960

11. PEARSE, A.G.E.: Intracellular localization of dehydrogenase systems using monotetrazolium salts and metal chelation of their formazans. *J. Histochem. and Cytochem.* 5, 515, 1957
12. ITIKAWA, O.: Mechanism of dehydrogenating reaction in living cells. *Symposia for the Soc. of Cell. Chem.* 4, 113, 1956 (in Japanese)
13. FARBER, E., STERNBERG, W.H., and DUNLAP, C.E.: Histochemical localization of specific oxidative enzymes. I. Tetrazolium stains for DPN diaphorase and TPN diaphorase. *J. Histochem. and Cytochem.* 4, 254, 1956
14. OKUNUKI, K., HAGIHARA, B., *et al.*: Studies on cytochrome system. Proc. of the Int. Symposium on Enzyme Chem. pp.264—272, Tokyo and Kyoto, 1957