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Abstract

New histochemical and cytochemical methods for the demonstration of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system are described, using neotetrazolium chloride in the presence of p phenylenediamine with or without additional cytochrome c. These enable the cytochemical visualization of the sites of enzyme activity at the intracellular level in fresh cell suspensions and in fresh tissue blocks under aerobic conditions, and permit the histochemical visualization of the distribution of the enzyme activity in various tissues in frozen tissue sections. The colorimetric estimation of the enzyme activity is also possible in the combination of the methods previously described.

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NEW METHODS FOR THE HISTOCHEMICAL AND CYTOCHEMICAL DEMONSTRTION OF CYTOCHROME C OXIDASE AND OF CYTOCHROME C-CYTOCHROME OXIDASE SYSTEM

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For the histochemical detection of cytochrome oxidase, the Nadi reaction[1] is frequently employed. This reaction consists in the formation of indophenol blue from dimethyl-*p*-phenylenediamine and α-naphthol, and the enzyme catalyzing the reaction has been called indophenol oxidase[2] in the field of biochemistry, which was identified with cytochrome oxidase by KEILIN and HARTREE (1938)[3]. In the field of histochemistry, however, two kinds of enzymes had been known in the enzymes which could be detected using the Nadi reaction. The one is M-Nadi oxidase (GRÄFF, 1922)[4], syn. stable oxidase (VON GIERKE, 1911)[5], which is detected in formalin fixed myeloid white blood cells[6] and a few kinds of tissue cells[7], and it is now considered as verdo-peroxidase[8,9]. The other is G-Nadi oxidase (GRÄFF, 1922)[4], syn. labile oxidase (VON GIERKE, 1911)[5], which is detected in most of fresh tissue cells. LISON (1936)[10] reported that the G-Nadi oxidase must be identical with indophenol oxidase[11]. BRACHET (1944)[12] stated also that the Nadi reaction would take place by the action of cytochrome oxidase but at the same time the reaction product would be reduced to its leucoderivative by the action of dehydrogenase, accordingly no accurate picture on the enzyme distribution could be obtained by this reaction. Moreover, as is well-known this reaction takes place easily by autoxidation[13] and by lipid peroxides[11,14,15] even in the absence of enzyme, and the reaction product secondarily stains lipids[13,14] and other various elements in the cells[11]. For these reasons, there are many objections to the use of the reaction as a test for the histochemical detection of cytochrome oxidase[13,16].

In our previous communications[17,18], we described the new methods for colorimetric estimation of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system in tissue homogenates, using neotetrazolium chloride in the presence of *p*-phenylenediamine with or without additional cytochrome c. It is the purpose of this study to establish a method for

the histochemical demonstration of the enzyme or enzyme system basing on the theory in the previous communications[17,18,19,20].

MATERIALS AND METHODS

Fresh liver, kidney, heart and bone marrow tissues and cells from decapitated mice were used as materials. Incubation was carried out on fresh tissue blocks of about 1 to 2 mm. thick, and on frozen tissue sections of about 10 μ thick. Incubating medium for cytochrome c oxidase was prepared immediately before incubation by combining equal volumes (mainly 0.2 ml. in each) of phosphate buffer (0.1M, pH 7.6) or sucrose solution (1 M), *p*-phenylenediamine solution (*p*-PDA) (0.2M), neotetrazolium chloride solution (0.2 %) and cytochrome c (10^{-4}M). For cytochrome c-cytochrome oxidase system cytochrome c was omitted and equal volume of phosphate buffer was added. For substrate contols *p*-PDA or both *p*-PDA and cytochrome c were omitted and equal volume of phosphate buffer was added. Endogenous dehydrogenase reaction conjugating at the steps below antimycin A sensitive step[20] could be almost completely eliminated by freezing the tissue under $-20°$ during 5 to 10 minutes before incubation. As described in our previous communications[18,20,21], it is required to use the adequate amount of tissue comparing to the volume and concentration of substrate solution for the quantitative reaction by these methods. Hence, about 40 mg. of tissue sections or a tissue block were directly put into the incubation medium of 0.8 ml.

Incubation at 37°C was conducted aerobically or anaerobically for 30 minutes to 1 hours. Then, cell suspensions were smeared and some of the tissue block were stamped on slides. Some of these specimens and tissue sections and blocks were washed with 0.33M sucrose solution or physiologic saline solution. The sections were mounted on slides. The smeared or stamped specimens were fixed with vapored formalin, and the sectioned specimens and the blocks were fixed with 10 per cent formalin solution. The blocks were sectioned by freezing or by carbowax embedding and then mounted on slides. Most of these specimens were examined without counter staining in glycerine immersion. The specimens was conservable for a long time at least during one year. Some of the specimens were counterstained with hematoxylin and eosin weakly or safranin O. For removing absolutely *p*-PDA or its oxidized product, the specimens were washed with 0.1 M sulfuric acid after fixation.

RESULTS AND DISCUSSION

The reactions for the enzyme and enzyme system in cell suspensions or tissue blocks resulted in a rich violet diformazan which was deposited on mitochondria-like organellas in the cytoplasm in the form of fine granules and rods. The reactions were completely negative in the nucleus. The distribution of the enzyme reations in cells was almost the same as that of the succinoxidase system reaction[19,22,23], but the activity was not always parallel between the cytochrome c-cytochrome oxidase system and succinoxidase system in various kinds of cells[18,21,22]. The reaction in frozen tissue sections appeared less intensely and less distinctly than the reaction in cell suspensions or tissue blocks. In this case, too, the reaction was completely negative in the nucleus.

Distribution of enzyme activity in various kinds of tissues or cells will be briefly described in the explanation of figures.

Specificity of the reaction in tissue homogenates was already proved in the previous communications[18,20] but it was also tested in the present histochemical reaction: By the addition of potassium cyanide (10^{-3}M) bufferized at pH 7.6 with monobasic sodium phosphate in the incubation medium, the histochemical reaction of the enzyme and enzyme system seemed to be completely inhibited when weak endogenous reaction in living cells was eliminated. The specimens exposed to 80°C for 5 minutes before incubation were totally inactivated. Sodium malonate and antimycin A caused no inhibition. Anaerobic conditions enhanced the enzyme reaction. *p*-PDA could be replaced by ascorbic acid, but the reaction using *p*-PDA was more intense than the reaction using ascorbic acid. Endogenous dehydrogenase reaction hardly appeared in frozen section but appeared weakly in fresh cell suspensions and tissue blocks. The endogenous reaction takes place conjugating at the step below the antimycin A sensitive step in the terminal electron transport system and at the other reduction systems as presented in the previous communication[20], and most of the reaction could be eliminated by freezing the tissue before incubation. As cytochrome c hardly penetrates into living cells, it was difficult to demonstrate selectively cytochrome c oxidase activity irrespective of the amount of endogenous cytochrome c by the histochemical reaction in fresh tissue blocks and cell suspensions, and therefore the reaction of NT reduction by living cells in the presence of *p*-PDA with or without additional cytochrome c are all considered to be responsible for cytochrome c-cytochrome oxidase system activity in the tissues. In this case, it is considered that *p*-PDA reduces endogenous cytochrome c and the reduced

cytochrome c subsequently reduces NT by the action of cytochrome c oxidase; in other words, this reaction takes place through co-operation of cytochrome c and cytochrome c oxidase in the tissue[3]. In frozen sections the reaction was enhanced by the addition of cytochrome c, therefore in this case the reaction with the addition of cytochrome c is considered to be responsible for cytochrome c oxidase activity, as described in the previous communication[18]. However, as the reaction due to the endogenous cytochrome c could hardly be eliminated even in frozen section, it will be necessary to compare with the control specimens without additional cytochrome c in order to examine the cytochrome c oxidase activity selectively, without any regard to the influence of the endogenous cytochrome c.

SUMMARY

New histochemical and cytochemical methods for the demonstration of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system are described, using neotetrazolium chloride in the presence of *p*-phenylenediamine with or without additional cytochrome c. These enable the cytochemical visualization of the sites of enzyme activity at the intracellular level in fresh cell suspensions and in fresh tissue blocks under aerobic conditions, and permit the histochemical visualization of the distribution of the enzyme activity in various tissues in frozen tissue sections. The colorimetric estimation of the enzyme activity is also possible in the combination of the methods previously described.

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EXPLANATION OF FIGURES

All figures show the reaction of cytochrome c-cytochrome oxidase system in various tissues of the mouse. In Figs. 1—6, the reaction was conducted for one hour in tissue blocks, and then the blocks were fixed with 10 per cent formalin solution, embedded in carbowax, washed briefly with 0.1 M sulfuric acid, immersed in glycerin without counterstaining.

Fig. 1. Cardiac muscle. Strong reaction appears as pigment granules deposited on mitochondria-like organellae between muscle fibrils in the cardiac muscle. Nuclei are negative.

Fig. 2. Skeletal muscle (*m. soleus*). Red muscle fibers are slender and strongly active, while white muscle fibers are thick and weakly active. Between red and white muscle fibers, one or two kinds of muscle fibers of intermediate activity are

demonstrated. Pigments are deposited on mitochondria in rows between muscle fibrils. The intensity of the reaction in muscle fibers is dependent on the number and the activity of mitochondria contained in them, which differ distinctly among red and white muscle fibers.

Fig. 3. Liver. Pigment depositon on the organellae of the hepatic and Kupffer cells showing the oval or rod-like shapes which the mitochondria may take. Nuclei are completely negative.

Fig. 4. Kidney. Convoluted tubular epithelial cells are strongly active. Glomeruli are very weakly active. In medulla, though they are not presented in this picture, the Henle's tubules are strongly active but the collecting tubules are weakly active.

Fig. 5. Small brain. Right half of this picture is the molecular layer and left half is the granular layer. Two Purkinje cells are presented between two layers. Pigment granules are deposited in the cytoplasm and fibers of the cells but the nuclei are inactive. Unfavorable crystal formations are seen scatteringly in the molecular layer. In granular layer, two kinds of cells, which differ distinctly in the activity, are presented.

Fig. 6. Spleen. Reticulum cells are moderately active, while lymphocytes are weakly active.

Fig. 7. Bone marrow cells of a mouse. The reaction was conducted in very small pieces of and cell suspensions of bone marrow tissues. After the reaction stamped or smeared specimen was prepared, fixed with formalin vapor, washed briefly with 0.1 N sulfuric acid, washed with water, and counterstained weakly with hematoxylin-eosin. All blood cells with a single exception of matured red cells are active, though the activity varies according to the kind of cells and grade of maturation. Two granulocytes, a reticulocyte and a red cell are presented. The reaction appears on mitochondria in the cytoplasm of white blood cells. Reticulocytes also contain mitochondria and the enzyme activity is demonstrated.

Fig. 8. Ascitic hepatoma cells of a rat. The reaction was conducted in cell suspensions, and then smeared specimen was prepared, immersed in glycerin without counterstain. The reaction appears on mitochondria in the cytoplasm. The activity of cancer cells is very irregular and differs according to the origin and the grade of maturation. The reactions even in one cell being also irregular, strongly and weakly active mitochondria are mingled.

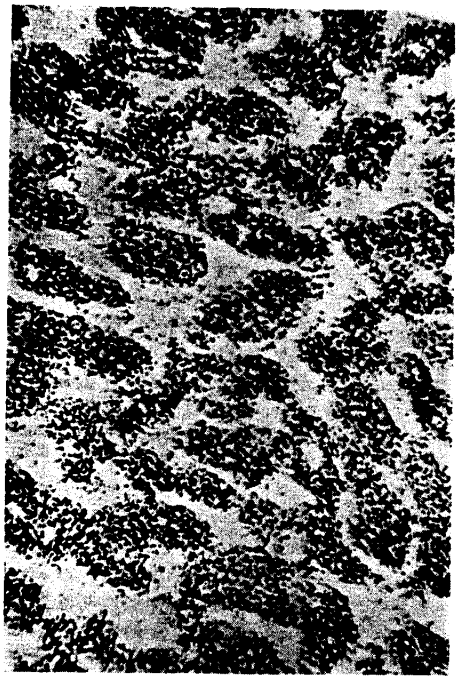

Fig. 1.

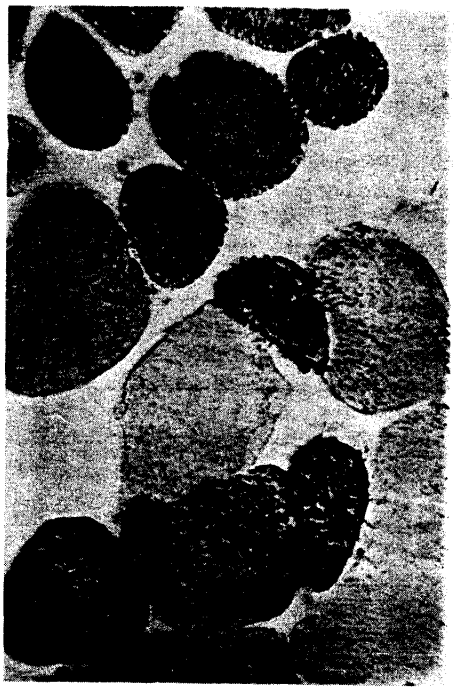

Fig. 2.

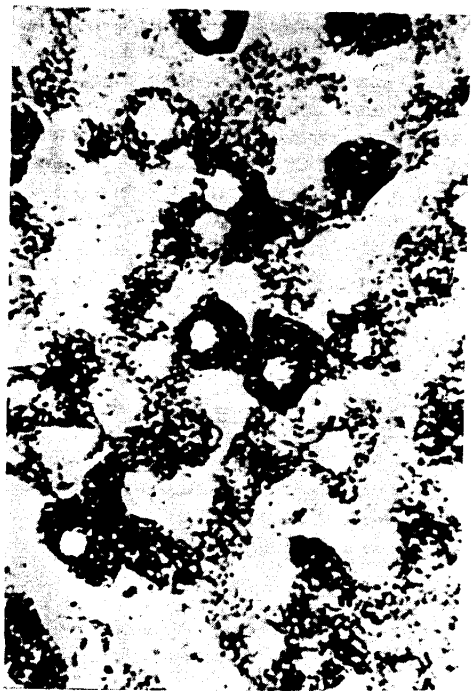

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

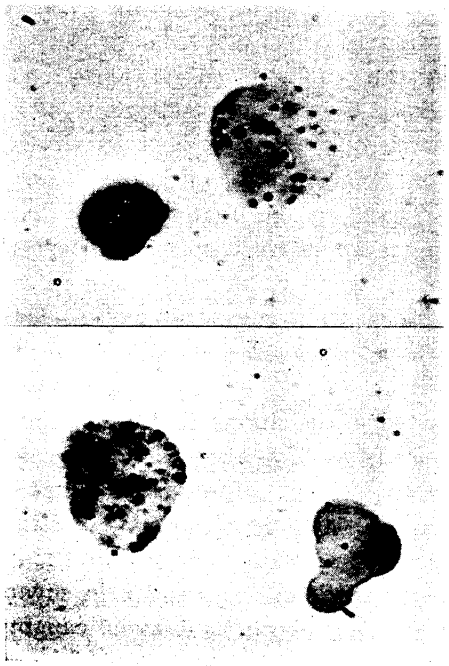

Fig. 7.

Fig. 8.