ULTRACYTOCHEMICAL DEMONSTRATION OF NADPH-FERRICYANIDE REDUCTASE ACTIVITY

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As an extension of our studies on the copper ferrocyanide method for the ultracytochemical demonstration of oxidoreductase activity, the method for the detection of NADPH-ferricyanide reductase activity was developed. The incubation medium consists of 20 mg of NADPH, 13.0 ml of 0.1 M phosphate buffer, pH 7.0, 1.3 ml of 0.1 M sodium citrate, 2.0 ml of 30 mM copper sulfate, 1.7 ml of double distilled water, 2.0 ml of 5 mM potassium ferricyanide and 3.0 g of sucrose. It was observed in the preliminary experiment that potassium sodium tartrate caused more diffusion of the final reaction products than sodium citrate.

Fresh tissues from brain, liver, heart and kidney of normal adult rats were used. Formaldehyde inhibited the enzymatic activity completely. Incubation with medium was carried out for 30 minutes at 37°C under aerobic and anaerobic conditions. After the incubation specimens were fixed in 1% osmium tetroxide and processed for electron microscopy.

The NADPH-ferricyanide reductase activity was extremely low in all tissues examined. In the enzymatically positive tissues the activity was observed solely in mitochondria. No activity was demonstrated in any element of the endoplasmic reticulum. The enzymatic activity in specimens incubated under anaerobic conditions was slightly higher in terms of the number of enzymatically positive mitochondria and the rate of positive reaction in each mitochondrion than that incubated under aerobic conditions.

In mitochondria the reaction product was observed in the membrane system including the outer membrane, the intracristal space and the outer space. In some areas, the activity in the outer membrane was quite evident. No activity was observed in the mitochondrial matrix.

Ogawa, Saito and Mayahara (6) reported the copper ferrocyanide method for the demonstration of the oxidoreductase activity. In this original report, however, the case of using nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) as substrate was not included. In the present investigation attempts were made to demonstrate the NADPH-ferricyanide reductase activity at the ultrastructural level in various tissues of the normal adult rat.

The present investigation was presented before the 11th Annual Meeting of the Japan Society of Histochemistry and Cytochemistry held in Tokyo on November 9-10, 1970 (1).

MATERIALS AND METHOD

Materials: Materials used in the present observation were the liver, kidney, cardiac muscle and brain (cerebrum and cerebellum) from the normal male adult rat weighing 150 to 200 g. Animals were sacrificed under anesthesia and tissues excised. Tissues used for the reaction were in the form of extremely small diced blocks (approximately $0.1\text{ mm} \times 0.1\text{ mm} \times 0.1\text{ mm}$) or non-frozen sections (approximately $40\text{ }\mu$ thick) cut by an automicrochopper (S. Horo Co., Osaka, Japan) or a Vibratome (Oxford Laboratories, San Mateo, Calif., U.S.A.). Since it was found in a preliminary experiment that the enzymatic activity was not detected in tissues fixed in 4% formaldehyde for 1 hour, fresh unfixed specimens were used for reaction with the incubation medium.

Composition of the incubation medium: Basic components of the incubation medium are similar to that used for the demonstration of the succinate-ferricyanide reductase (6); substrate, phosphate buffer, pH 7.0, sodium citrate, copper sulfate, potassium ferricyanide and sucrose. In a series of the preliminary experiment different concentrations of substrate and a chelating agent in the incubation medium were tested in order to obtain the optimal condition; two concentrations, 10 mg (0.6 mM in the final concentration) and 20 mg (1.2 mM) of substrate (tetrasodium salt of nicotinamide adenine dinucleotide phosphate, reduced form, NADPH, type I, Sigma Chemical Co., St. Louis, Mo., U.S.A.) were tried, and as a chelating agent sodium citrate (2.5 to 10 mM) and potassium sodium tartrate (2.5 to 50 mM) were tested.

When potassium sodium tartrate was used as a chelating agent, an almost immediate change of color, *i.e.* browning of the medium, took place even with 50 mM in the test tube and diffusion of final reaction products was evident in sections, when observed under electron microscope, indicating that potassium sodium tartrate ($\log K' = 3.2$, where K' is conditional stability constant) has much weaker chelating activity than sodium citrate ($\log K' = 8.0$) for copper at pH 7.0 (Ringbom, 1963) (7). In the case of the demonstration of the succinate-ferricyanide reductase activity sodium citrate was chosen as a routine chelating agent to be used in the incubation medium, although it was originally reported by Ogawa *et al.* (6) that both sodium citrate and potassium sodium tartrate could be used. An extreme excess amount of tartrate, 300 mM or more, such as used by Kerpel-Fronius and Hajos (3) or Kokko *et al.* (4) (Kokko *et al.* used 5–300 mM of tartrate. Usually 60 mM was used by them, but 60 mM is insufficient to stop diffusion as shown in the present investigation), may help preventing diffusion. However, even with 300 mM of tartrate the localization of the final reaction product seems to be not discrete enough as can be seen in electron micrographs obtained by Kerpel-Fronius and Hajos (3). In addition to the problem of tendency of the obscure localization of the reaction product, an excessive hypertonicity of the incubation medium seems to cause certain degree of tissue damage in some cases. Based on these facts 6.5 mM of sodium citrate was used in the present investigation. As a concentration of substrate, 20 mM was found to be satisfactory.

Thus, the final incubation medium used for the demonstration of the NADPH-ferricyanide reductase activity in the present study consists of 20 mM of NADPH (1.2 mM), 13.0 ml of 0.1 M phosphate buffer, pH 7.0 (65 mM), 1.3 ml of 0.1 M

sodium citrate (6.5 mM), 2.0 ml of 30 mM copper sulfate (3 mM), 1.7 ml of double distilled water, 20 ml of 5 mM potassium ferricyanide (0.5 mM) and 3.0 g of sucrose (440 mM).

Procedure: Unfixed specimens (small diced blocks, automicrochopper sections or Vibratome sections) were incubated in the incubation medium described above for 30 minutes at 37°C, under aerobic or anaerobic conditions, fixed in buffered 1% osmium tetroxide for 1 hour at 0–4°C, dehydrated through graded ethanols and embedded in epon. Thin sections made by a LKB microtome were doubly stained by uranyl and lead by conventional methods, and examined with JEM-7 electron microscope.

As a control the specimens were incubated in the substrate-free incubation medium.

RESULTS AND DISCUSSION

By and large the NADPH-ferricyanide reductase activity was extremely low in all tissues investigated. In particular no detectable enzymatic activity was observed in the brain (cerebrum and cerebellum). When the enzymatic activity was observed in the tissue, the activity was solely confined to mitochondria (Figs. 1–4). No activity was observed in the endoplasmic reticulum in spite of the biochemical report indicating the presence of the NADPH-cytochrome c reductase activity in the small vesicle fraction from the rat liver microsome (Dallman *et al.*, 1969) (2).

Among liver, kidney and cardiac muscle, the cardiac muscle seemed to reveal the highest mitochondrial activity, but in all tissues the mitochondrial activity was much higher in specimens incubated anaerobically (compare Fig. 3 with Fig. 4). The activity was higher in terms of the number of enzymatically positive mitochondria and the rate of positive reaction in each mitochondrion.

Not all mitochondria were positive for the reaction, indicating the presence of functional heterogeneity among mitochondria as reported previously (5, 6).

In enzymatically positive mitochondria, the activity was observed in the membrane system including the outer membrane, the intracristal space and the outer space. It should be noted that in some areas the activity in the outer membrane was quite evident as shown in Fig. 4 (arrow).

FIG. 1–4. The NADPH-ferricyanide reductase activity demonstrated by the copper ferrocyanide method. Materials are from the normal male adult rat.

FIG. 1. A part of a liver parenchymal cell. Unfixed nonfrozen Vibratome section (40 μ in thickness) incubated aerobically. The enzymatic activity is confined within mitochondria. $\times 42,000$

FIG. 2. A part of a renal tubular epithelium. From unfixed small diced block incubated aerobically. The low activity can be seen in mitochondria. $\times 42,000$

FIG. 3. A mitochondrion showing low activity from the cardiac muscle. Small diced specimen incubated aerobically. $\times 75,000$

FIG. 4. Mitochondria from the cardiac muscle incubated anaerobically. Specimen was in the form of small diced block. Note the increased NADPH-ferricyanide reductase activity. The outer membrane shows the distinct activity (arrow). The functional heterogeneity among mitochondria in terms of the enzymatic activity is also present. $\times 38,000$

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REFERENCES

1. Ago, Y. and Ogawa, K.: The development of the method for the demonstration of NADPH-ferricyanide reductase activity and its application in several tissues of rats. *Acta histochem. cytochem.*, 3; 205-206, 1970.
2. Dallman, P. R., Dallner, G., Bergstrand, A. and Ernster, L.: Heterogeneous distribution of enzymes in submicrosomal membrane fragments. *J. Cell Biol.* 41; 357-377, 1969.
3. Kerpel-Fronius, S. and Hajos, F.: An attempt to evaluate the specificity of the fine structural localization of oxidoreductase activity with a simultaneous coupling method. *J. Histochem. Cytochem.* 18; 219-220, 1970.
4. Kokko, A., Mautner, H. G. and Barnett, R. J.: Fine structural localization of acetylcholinesterase using acetyl- β -methylthiocholine and acetylselenocholine as substrates. *J. Histochem. Cytochem.* 17; 625-640, 1969.
5. Ogawa, K. and Barnett, R. J.: Electron cytochemical studies of succinic dehydrogenase and dihydronicotinamide-adenine dinucleotide diaphorase activities. *J. Ultrastr. Res.* 12; 488-508, 1965.
6. Ogawa, K., Saito, T. and Mayahara, H.: The site of ferricyanide reduction by reductases within mitochondria as studied by electron microscopy. *J. Histochem. Cytochem.* 16; 49-57, 1968.
7. Ringbom, A.: Complexation in Analytical Chemistry, Interscience Publishers, John Wiley and Sons, New York, 1963.