ULTRACYTOCHEMICAL DEMONSTRATION OF INTRACELLULAR AMINO GROUPS BY THE HYDROQUINONE-FERRICYANIDE SYSTEM*

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Ultracytochemical methods for the demonstration of reactive amino groups in the cell were presented. The method utilizes either the hydroquinone-ferricyanide system or the hydroquinone-tetrazolium system.

The reactive amino groups were observed in the mitochondrial matrix and probably in substances in Z in the cardiac muscle of the normal adult albino mice.

Main aims in our laboratory for the past several years have been the elucidation of the topographic localization of various enzymes and related chemical activities at the level of fine structures with correlation of cellular functions. As a part of studies on reactions with ferricyanides (3, 7), the system containing hydroquinone and ferricyanide was studied ultracytochemically in the present investigation, since it has been reported that the hydroquinone-tetrazolium system could demonstrate the tissue amino groups light histochemically (1), and that ferricyanide could be used as electron acceptors in lieu of tetrazolium salts (6). For the sake of comparison, the hydroquinone-tetrazolium system was also investigated.

MATERIAL AND METHODS

a. General procedures

The cardiac muscles (left ventricular walls) of the normal adult mice were used. Unfixed diced specimens or nonfrozen sections (ca. 50 μ) of tissues fixed in 8% buffered formaldehyde, pH 7.2-7.4, or 2-4% buffered glutaraldehyde (10), pH 7.2-7.4, for 2 hr at 0-4°C were incubated in various incubation media described below for 30-60 min at 37°C. Following incubation, specimens were refixed in 2-4% buffered glutaraldehyde for 1-2 hr at 0-4°C, dehydrated through graded ethanols, processed with alcoholic phosphotungstic acid (PTA) solution by the method of Ogawa et al. (8) and embedded in epon routinely. Refixation of specimens in osmium tetroxide was found to be unsatisfactory in terms of the preservation of final reaction products in the preliminary experiments. Thin sections made by a LKB ultratome were

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All specimens are from unfixed cardiac muscles of normal adult mice.

Fig. 1. Specimens (unfixed dices) incubated in the medium of the hydroquinone-ferricyanide system. Note the electron dense-reaction product in the mitochondrial matrix of all mitochondria in the field. Substances in Z line show some electron density. $\times 30,000$

Fig. 2. Specimens (unfixed dices) incubated in the substrate-free medium of the hydroquinone-ferricyanide system. No reaction product can be seen in the mitochondrial matrix. $\times 44,000$

observed under JEM-7 electron microscope with or without poststaining with uranyl acetate and/or lead acetate.

b. Incubation medium

1. Incubation medium for the hydroquinone-ferricyanide system.

Based on the preliminary experiments, the following medium was found to be satisfactory for the ultracytochemistry; 6.5 ml of 0.1 M phosphate buffer, pH 6.0, 0.1 ml of 0.1 M hydroquinone, 0.5 ml of 300 mM sodium citrate, 1.0 ml of 30 mM copper sulfate, 1.0 ml of 5 mM potassium ferricyanide and 0.8-1.6 g sucrose.

2. Incubation medium for the hydroquinone-tetrazolium system.

Tetranitroblue tetrazolium chloride (TNBT) was used instead of MTT used by Carmichael et al. (1), since it has been shown that TNBT is more suitable for the ultracytochemistry than MTT (5). The incubation medium is composed of 1.4 ml of 250 mg% TNBT, 5.0 ml of 0.1 M phosphate buffer, pH 6.0, 8.6 ml of double distilled water, 0.125 ml of 0.1 M hydroquinone and 1.2-2.4 g of sucrose.

3. Control media.

a. Substrate-free medium.

Hydroquinone was replaced by the buffer in the medium 1 and the medium

2.

b. Medium containing SH inhibitor.

As a SH inhibitor, N-ethylmaleimide (N-EM) was incorporated in the medium 1 or 2 in the final concentration of 1 mM or 5 mM.

c. Pretreatment with 2,4-dinitro-1-fluorobenzene (DNFB).

The blocking effect of DNFB on tissue amino group was tested. The specimens were pretreated with DNFB by the method of Zerlotti and Engel (11) prior to the incubation with the incubation medium 1 or 2.

RESULTS

Results obtained by the hydroquinone-ferricyanide system (Figs. 1-4) were entirely similar to those obtained by the hydroquinone-tetrazolium system (Figs. 5-7). The reaction product, copper ferrocyanide in the case of the hydroquinone-ferricyanide system and TNBT-formazans in the case of the hydroquinone-tetrazolium system, was found almost exclusively in mitochondria (Figs. 1 and 5). Substances in Z line seemed to show some density indicating the site of reaction product. Within mitochondria the reaction product was confined to the matrix, leaving mitochondrial membranes in negative contrast. The intracrystal space and the outer space depicted some density in some areas.

Fig. 3. Specimens (unfixed dices) incubated in the medium of the hydroquinone-ferricyanide system containing N-EM (5 mM). The reaction in the mitochondrial matrix is retained. $\times 55,000$

Fig. 4. Specimens (unfixed nonfrozen sections made by an automicrochopper) pretreated in DNFB and subsequently incubated in the medium of the hydroquinone-ferricyanide system. Mitochondria with no reaction product as well as mitochondria with patchy electron dense areas can be seen. Substances in Z line and glycogen particles show relatively higher electron density also. $\times 43,000$

The reaction product was completely absent in mitochondria from specimens incubated in the substrate-free medium (Figs. 2 and 6). The reaction was not inhibited by N-EM, a SH inhibitor (Figs. 3 and 7). In specimens pretreated with DNFB mitochondria having no reaction product were observed (Fig. 4). However, in some mitochondria the patchy areas of relatively higher electron density were also observed. These patchy electron dense areas seemed to correspond roughly to the mitochondrial matrix. Substances in Z line and nearby glycogen particles also showed higher electron density (Fig. 4).

It should be added here that the reaction was much weaker in aldehyde-fixed specimens than in unfixed ones. In some case almost no reaction was obtained in aldehyde-fixed specimens.

DISCUSSION

It has been reported that ferricyanide can be used as electron acceptors in the ultracytochemistry and in fact the copper ferrocyanide method has been applied for the demonstration of the activity of oxidoreductases (3, 4, 7, 8). On the other hand, Carmichael et al. (1) has shown light histochemically that the hydroquinone-tetrazolium system demonstrates the site of tissue amino groups. In the present investigation it was shown that both the hydroquinone-ferricyanide system and the hydroquinone-tetrazolium system revealed entirely the similar results, and that these methods can be used for the ultracytochemical demonstration of tissue amino groups. The amino groups demonstrated by the methods seemed to be confined mainly to the mitochondrial matrix in the cell. This finding may correspond to the diffuse cytoplasmic staining obtained by Carmichael et al. (1) at the level of light microscope. It should be aware of the fact, however, that the present method may not demonstrate all tissue amino groups present; by these methods only certain metabolically active amino groups might have been picked up. Substances in Z line may have the active amino groups, but the increased electron density of glycogen particles observed may be due to the affinity of glycogen particles to lead used in poststaining.

The decreased or no reaction observed in aldehyde-fixed specimens can be explained by the chemical reactivity of aldehyde with tissue amino groups (2), resulting in the loss of reactive amino groups in the cell by fixation.

The electron dense reaction product observed in patchy distribution in some mitochondria in specimens pretreated with DNFB (Fig. 4) does not seem to represent copper ferrocyanide, since it shows some tendency of diffusion from the original site of reaction corresponding to the mitochondrial matrix. The reaction product in the hydroquinone-ferricyanide system never diffuses out of the matrix (compare with Fig. 1). It is not unlikely that the electron dense material showing patchy

Fig. 5. Specimens (unfixed dices) incubated in the medium of the hydroquinone-tetrazolium system. Note the reaction product, TNBT formazans, observed in the mitochondrial matrix. $\times 49,000$

Fig. 6. Specimens (unfixed dices) incubated in the substrate-free medium of the hydroquinone-tetrazolium system. Unpoststained. No reaction product is seen. $\times 21,000$

Fig. 7. Specimens (unfixed dices) incubated in the medium of the hydroquinone-tetrazolium method containing N-EM (5mM). The mitochondrial reaction was not inhibited. $\times 27,000$

distribution in some mitochondria may be DNFB chemically bound with amino groups. In reality DNFB *per se* has been used to demonstrate the site of amino groups in the cell, although incompletely (9). Thus, mitochondria with patchy electron dense areas in or near the matrix observed in Fig. 4 may represent the loci of chemical combination of DNFB with amino groups. The mitochondria without reaction products may be explained in such a way that DNFB which blocked the reaction of the hydroquinone-ferricyanide system with amino groups once during incubation might have been subsequently washed out in the later stage of preparation. At any rate, if the patchy electron dense materials corresponded to DNFB chemically bound with amino groups, it can be deduced that the distribution pattern of amino groups is entirely similar to that observed by the present methods, supporting the present connotation that the systems reported in the present investigation can demonstrate reactive amino groups in the cell. DNFB *per se*, however, may not be a choice of chemical substance for the ultracytochemical demonstration of amino groups because of its diffusibility.

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