

A Revised Method for Histochemical Demonstration of Cytochrome Oxidase

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Introduction

A mixture of solutions of alpha-naththol and dimethylparaphenylenediamine will form a blue indophenol dye when oxidized. This mixture is named nadi reagent. This reaction was introduced in biology by P. Ehrlich in 1885, who showed that by the injection of this reagent into animals the formation of indophenol blue by living tissues could be observed. Afterward Winkler demonstrated that this reagent will stain pus cells in fixed smears at an alkaline reaction. Schultz tried this reaction on tissue sections and emphasized the diagnostic value of this reaction in distinguishing myeloid from lymphoid cells. The distribution of this nadi reaction in tissue sections is differ by treatments. In fresh tissue sections treated with an unalkalized solution of the reagent the reaction is widespread throught all tissues and not partiquarly intense in myeloid leucocytes. On the other hand the reaction of fixed tissue sections is almost limited to the granules of myeloid leucocytes. These differences were noticed by von Gierke in 1922. In the former case, the activity of the sections is very labile and readily destroyed by moderate heating and by many fixative reagents. This reaction is designated the labile nadi reaction or tissue nadi reaction (G=Gewebe indophenol oxidase). In the latter case, the reaction factor is relatively stable to heating and insensitive to formalin and other fixatives or alkali. This myeloid granule type is called stable oxidase or M (Myeloid) nadi reaction. More recently Keilin has proved that the tissue nadi reaction is due to the activity of cytochrome oxidase and his theory has become generally accepted.

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As is already well known, the cytochrome-oxidase system plays an important role in the respiration of the cells. The technique of the histochemical demonstration of this cytochrome oxidase has nevertheless remained essentially unchanged since the time of Graeff and Schultz. Recently Takamatsu and Ijima tried a modification of the method of demonstrating this enzyme using the indophenol purple reaction, but this method still seems to have some defects in its practical application. So the present authors revised it again as in the following description.

Revised method

Fresh tissue must be used. We removed tissues from animals after sacrificing by bleeding or by surgical operation and cut the tissues about 1 mm in thickness by hand using a razor blade. Then tissues were immediately immersed in the following reagent solution and incubated at room temperature over night (12 to 15 hours).

Preparation of the reagent solution. The following three reagents were prepared first. (1) 3 g of α naphthol was dissolved in 100 cc of 40 per cent alcohol. (2) 1/15 M phosphate buffer solution of pH 7.4. (3) 0.04 per cent water solution of paraphenylenediamine. To about 50 cc of phosphate buffer solution (2) was added 1 cc of α naphthol alcohol solution (1). Before use, this buffered α naphthol solution was mixed with an equal amount of paraphenylenediamine solution (3). The paraphenylenediamine solution must be freshly prepared.

Glass wool was spread in a dish, and the mixed reagent solution containing tissue slices was poured into this dish. During the incubation tissue pieces were turned with a glass stick occasionally. The reagent solution penetrates with difficulty into the midpart of the tissue pieces, so it is difficult to accomplish the reaction in the innerpart of the pieces without using glass wool or some means of turning.

After incubation, the tissue pieces were washed with running water thoroughly for about 2 hours, then immersed in dilute Lugol's solution for 2 to 5 hours and then rewashed in running water for 2 hours or more. After washing the tissue pieces were fixed in a formalin solution saturated with lithium carbonate. Tissue sections were prepared from these tissue pieces by frozen sectioning and mounted as usual in gum syrup.

Results obtained by this method

During incubation, the tissue pieces are stained a dark violet. Without turning the tissues or using glass wool, the back surface of the tissues did not stain. If incubation is not sufficient, the midpart of each piece is not stained. After incubation, the tissue pieces must be washed thoroughly, otherwise nonspecific colouring may occur with the following procedures. Immersing in dilute Lugol's solution converts the colour of tissue pieces to brown. During this process also care

must be taken to give sufficient time for the reagent to penetrate into the midpart of each piece. After immersing in Lugol's solution, it is necessary to wash sufficiently. In the course of fixing in the lithium carbonate saturated formalin solution, a dark violet colouring of the tissues pieces will reappear.

On microscopic examination, the sites of cytochrome oxidase are stained blue violet in granules and in somewhat diffuse shapes. Positive reactions are observed in many tissues, especially strong reactions occurring in heart muscle. Preparations of subcutis on spread and smeared glass slide also produce good figures by following similar procedures. We tried this method on the thyroid glands of many cases of Basedow's disease and always obtained good results.

Comment

The principle of the revised method of the demonstration of cytochrome oxidase is based upon indophenol synthesis, using alpha naphthol and paraphenylenediamine. Nadi reagent, dimethylparaphenylenediamine, has been used in the classical method. The two methods are almost identical, but this revised method synthesizes a violet dye called indophenol purple instead of the indophenol blue of the classical method. Keilins theory is common to both reactions. Our experiments indicated that the indophenol purple reaction is superior to the others in its staining figures.

A special feature of this revised method is the use of paraphenylenediamine instead of dimethylparaphenylenediamine. When paraphenylenediamine is used for this reaction it is observed sometimes that crystals of this reagent are crowded over the surface of tissues in a thick solution. To avoid this crystalization, we used a dilute solution as mentioned above. For the same reason and also to avoid destroying cytochrome oxidase, our method incubated the tissue pieces in reagent solution before sectioning by microtome.

Recovery of the indophenol dye in the lithium carbonate solution after immersing in Lugol's solution has been noticed for many years (1916) and this process has been adopted by many authors to keep the colour. The same phenomena can apply to the indophenol purple reaction. To get thin sections without destroying tissues, we tried embedding in gelatine, but heating to dissolve the gelatine caused the indophenol purple dye to fade gradually. In the former description, Takamatsu and Iijima had tried embedding in paraffin in the usual way but this process also damaged the colouring. The usual method of frozen sectioning is applicable. As a mounting media gum syrup is useful. In this media the reaction product will keep for a long time.

We also tried many other modifications of this technique besides those mentioned above, but the method described in this paper is convenient and satisfactory for the usual studies.

Summary

The authors revised the method of demonstration of cytochrome oxidase using an indophenol purple reagent, namely alpha naphthol and paraphenylenediamine. This revised method makes the staining figure of cytochrome oxidase clearer and more distinct and more stable than the classical methods.

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References

- 1) GIERKE, E.V.; Zbl. f. allg. Path. **27**. 318. 1916.
- 2) GRAEF, S.; Zbl. f. allg. Path. **27** 313. 1916.
- 3) EHRLICH, P.; Das Sauerstoffbedurfnis des Organismus. Eine farbenanalytische Studie. Berlin. 1885.
- 4) WINKLER, F.; Folia haemat., **4** 323. 1907.
- 5) SCHULTZ, W.H.; Beitr. path. . Anat. **45** 1909.
- 6) KEILIN, D.; Ergebn. d. Enzymforsch. **2** 239. 1933.
- 7) TAKAMATSU, H. and K. IJIMA: Transac. Soc. Path. Jap. I ber. **42** Editio Reg. **253** 1953.

Explanation of the plates.

Fig. 1) Cytochrome oxidase reaction in the thyroid gland of a case of Basedow's disease, stained by the revised method. Granular intense reactions are observed in the follicular epithelium.



Fig. 2) Cytochrome oxidase reaction in the thyroid gland of an other case of Basedow's disease. The reaction is identical to the case in Fig. 1.



Fig. 3) Cytochrome oxidase reaction in the thyroid gland of a third case of Basedow's disease. The findings are essentially the same as those in Figs. 1 and 2. Intense reactions are observed especially in the proliferating epithelium.



Fig. 4) Cytochrome oxidase reaction in a fibrocyte in a spread and smeared preparation of the connective tissues of the subcutis of a rabbit.

