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MICROSPECTROPHOTOMETRIC QUANTITATION OF THE DIAMINOBENZIDINE REACTION FOR HISTOCHEMICAL DEMONSTRATION OF CYTOCHROME OXIDASE ACTIVITY

A. C. C. FRASCH, M. E. ITOIZ AND R. L. CABRINI

Department of Radiobiology, National Atomic Energy Commission, Buenos Aires, Argentina

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Polyacrylamide models in which an extract of cattle heart mitochondria was incorporated, as well as cryostat sections of tongue muscle and epithelium, were used to set up the conditions under which the histochemical reaction for the demonstration of cytochrome oxidase can be quantitated. Using diaminobenzidine in a concentration of 5.5 *mM*, cytochrome C in a fixed concentration of 76 μM and keeping the incubation medium away from direct light action, enzyme activity can be evaluated by means of direct microphotometry on tissue sections. Each biologic model requires previous individual determination of the measurement limits. These limits can be readily established by using a small chamber for the incubation medium, which can be placed in the microphotometer, allowing the reaction rate to be followed using a single section.

The recently developed methods that make possible quantitative determinations in enzyme histochemistry are generally too complex to be used by most laboratories (13). However, one of the feasible procedures is direct microphotometry, which when carried out on tissue sections, yields relative results that allow numerical comparisions between adjacent tissue areas on the same section or between different tissue sections mounted and processed together on the same slide.

This method has proved of great value in the quantitation of formazan reactions (4, 5, 9), of Gomori type reactions with final lead precipitate (2, 8) and of the azo-dye method for unspecific esterases (3).

In this report we delineate the conditions for the quantitation of the histochemical reaction for the demonstration of cytochrome oxidase activity based upon the oxidative polymerization of diaminobenzidine (DAB) (15).

Several authors have discussed the accuracy of this reaction and described conditions that modify it, such as the DAB oxidation by UV light (7) or the the nonenzymatic binding of auto-oxidazed DAB to cytochrome C (7, 10, 11). An increase of the DAB concentration used in the original technique is also reported to enhance the final staining (6, 10). However, since it has been proved that the reaction shows the mitochondrial cytochrome oxidase activity (11, 12, 16), the conditions under which the enzymatic reaction can be quantitated using direct microphotometry were studied.

MATERIALS AND METHODS

Preparing the models: Blair's technique (1) was used to prepare cow heart mitochondria. Sonication was then applied in order to break the mitochondrial membrane. The crude extract served as a source of cytochrome oxidase (EC1.9.3.1). It was not our purpose to achieve absolute quantitation and, therefore, we did not evaluate biochemically the activity of the extract.

Different dilutions of the extract in 0.1 *M* phosphate buffer, pH 7, were mixed with equal parts of a 20% acrylamide solution, prepared according to the method of Van Duijn *et al.* (17). Final concentrations were of 0.025, 0.062, 0.081 and 0.1 ml of extract per milliliter of acrylamide. With the addition of tetramethyl-acethylene diamine and ammonium persulphate, the preparations were polymerized between two glass plates with spacers of 240 ± 10 μ, for 30 min at 40°C.

Models containing the same amount of enzyme (0.081 ml of mitochondria extract per milliliter of acrylamide) were used to study the kinetics of the reaction. They were incubated in darkness at 37°C, in a medium containing 0.13, 0.24, 0.73, 5.0 and 13.0 *mM* DAB tetra-HCl (Sigma Chemical Company, St. Louis, Mo.); 2 μg/ml catalase; 76 μM cytochrome C and 0.22 *M* sucrose in 0.05 *M* phosphate buffer, pH 7.4. After 1, 2, 4, 8, 15 and 30 min incubation periods, models were rinsed in 0.1 *M* phosphate buffer and mounted in glycerol-gelatin.

Microphotometric readings were performed within the following 30 min in a Zeiss Cytoscan. The wave-

length of 480 mμ was set after carrying out an absorption spectrum on one of the models (Fig. 1). The total magnification of the system was of approximately ×50. The homogeneity of the reaction on the films allowed areas of 300 μ^2 to be measured without appreciable distributional error.

Reactions on tissue sections: Unfixed tongue of Wistar rat was cut in transverse sections of 10–12 μ thickness in a thermoelectrically-cooled cryostat. Sections were mounted on coverslips and immediately frozen. In order to perform microspectrophotometric measurements during the reaction, the coverslips were placed with the section facing downward covering partially a hole of 2 cm diameter drilled in a lucite plate of 2-mm thickness, which was previously affixed to a slide. The system was placed in the stage of the Cytoscan, the lens was focused on the section and immediately afterward the incubation solution was added by pouring it with a syringe on the uncovered part of the hole in the lucite plate. The incubation medium was prepared in the same way as for the polyacrylamide models in a DAB concentration of 5.5 *mM*.

The optical densities in a fixed area of 1 μ diameter of tongue muscle tissue were automatically registered during 60 min.

In order to gather data on the reaction kinetics in a tissue with different enzyme activities, a scan of the tongue's ventral epithelium in the basal-horny direction was registered starting at 0 min of incubation, when the solution was poured into the chamber, and repeated at 10, 20, 30, 40 and 50 min of the incubation. Blank measurements were performed through the incubation medium in an area of the coverslip adjacent to the sections.

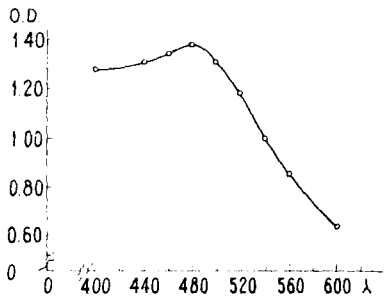

FIG. 1. Absorption spectrum from a polyacrylamide model containing 0.081 ml of mitochondrial extract per milliliter of acrylamide, incubated in Seligman's medium with 5.5 *mM* of DAB. O.D., optical density.

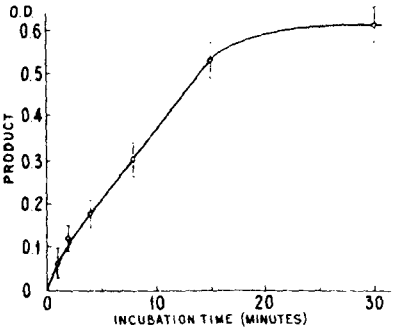

FIG. 3. Variation of the reaction product (O.D., optical density) plotted against incubation time. The bars indicate standard deviations of 10 measured areas of each polyacrylamide film containing 0.081 ml of extract per milliliter. The incubation medium contained 5.5 *mM* of DAB. These conditions rendered a linear response within 2 to 15 min.

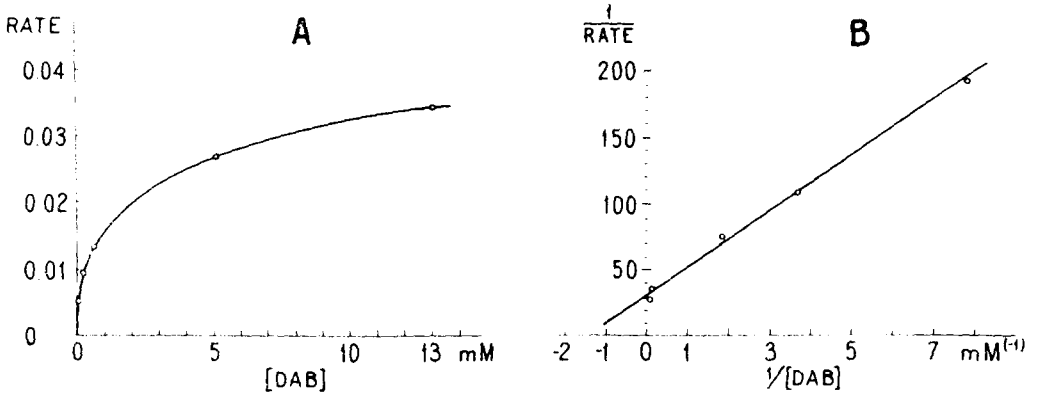

FIG. 2. A, Reaction rate obtained as ratio between the reaction product (in arbitrary units of optical density) and the incubation period (in minutes) as function of different DAB concentrations in the incubation medium. The microspectrophotometric measurements were carried out in areas of 300 μ^2 of polyacrylamide films containing 0.081 ml of mitochondrial extract per milliliter. The reaction rate was calculated within 2 to 15 min. B, Lineweaver-Burk double reciprocal plot of the data of Figure 2A, from which an apparent Km of 0.71 *mM* is obtained for the DAB concentration.

Control reactions at electron microscopic level: Nonfrozen sections of the tongue's epithelium, previously fixed in 3% glutaraldehyde in phosphate buffer during 30 min and rinsed during 1 hr in phosphate buffer, were incubated for 2 hr in the aforementioned medium, treated with 2% Os04 in phosphate buffer during 1 hr, and embedded in Epon 812. The sections without contrast staining were observed in an electron microscope Philips EM 300.

Inhibition controls: In all experiments, the polyacrylamide models and the tissue sections were simultaneously incubated with KCN 0.001 *M* for the inhibition of cytochrome oxidase.

All experiments were repeated three to five times. Different areas of each film were measured at least five times.

RESULTS

The reaction rate, obtained as a ratio between the reaction product (in arbitrary units of optical density) and the incubation period (in minutes) varies as function of the DAB concentration used, (Fig. 2A). When the double reciprocal plot of Lineweaver-Burk was made, an apparent Km = 0.71 *mM* was obtained for the DAB (Fig. 2B). This concentration can be easily increased up to approximately 5 *mM*. At a higher concentration, it is difficult to prepare the solution at room temperature, and at 13 *mM* the solution becomes cloudy and precipitates. For this reason, the following experiments were carried out with a DAB concentration of 5.5 *mM*.

Under these conditions, the reaction proved to be linear within 2 to 15 min (Fig. 3). The reaction rate is faster in the first 2 min. This fact could be due to the action of endogenous cytochrome C. Figure 4 shows the variation in the reaction rate per minute, measured between the 2nd and 15th min, as function of the concentration of the extract incorporated into the models. This graph also indicated that no variation took place when the models were incubated with 0.001 *M* KCN in the medium.

The activity of unfixed frozen sections of tongue muscle measured under the same conditions proved to be linear within 4 to 18 min. Longer periods cause saturation of the final product (Fig. 5A). The lingual epithelium having a smaller amount of mitochondria than the muscle, could be measured even 40 min after the reaction started (Fig. 5B,C). The scannings through the epithelial thickness revealed different reaction rates in zones of diverse enzyme content. As has already been subjectively described, cytochrome oxidase activity decreases from the basal layers to the top layers (14).

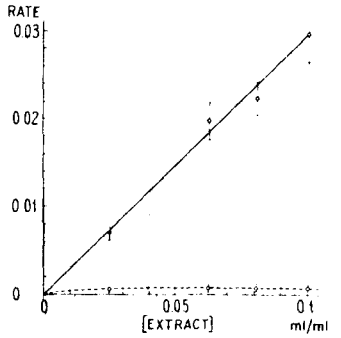

FIG. 4. Reaction rate (variation of optical density per minute within 2 to 15 min) as function of the amount of extract incorporated in the polyacrylamide films. The bars indicate standard deviations of 10 measured areas of each film. Incubation in 5.5 *mM* DAB medium. Dotted line: values from portions of the same models incubated in medium containing 0.001 *M* KCN.

The control sections observed with the electron microscope showed that the DAB concentration of 5.5 *mM* produced neither diffusion of the final product nor unspecific precipitation, and the mitochondrial localization is as accurate as described by Seligman *et al.* (15) in his original description (Fig. 6).

DISCUSSION

The results show that the microspectrophotometric quantitation of cytochrome oxidase can be easily achieved, once conditions for its applicability have been established. Some of these conditions can be generally defined, whereas others must be tested separately for each model.

The increase of the DAB concentration up to 5.5 *mM* does not affect the precision of the final precipitate's localization, while increasing the reaction rate. This allows one to obtain optical densities measurable after relatively short incubation periods. Although these periods are shorter than those described as necessary to produce a nonenzymatic oxidation of DAB (7), it is advisable to prepare and maintain the incubation medium in a dark place until use.

Another general condition that calls for special care is the use of a fixed cytochrome C concentration, since it has been demonstrated that the incorporation of cytochrome C during incubation increases the amount of final product (7, 10, 11). In our models containing a fixed

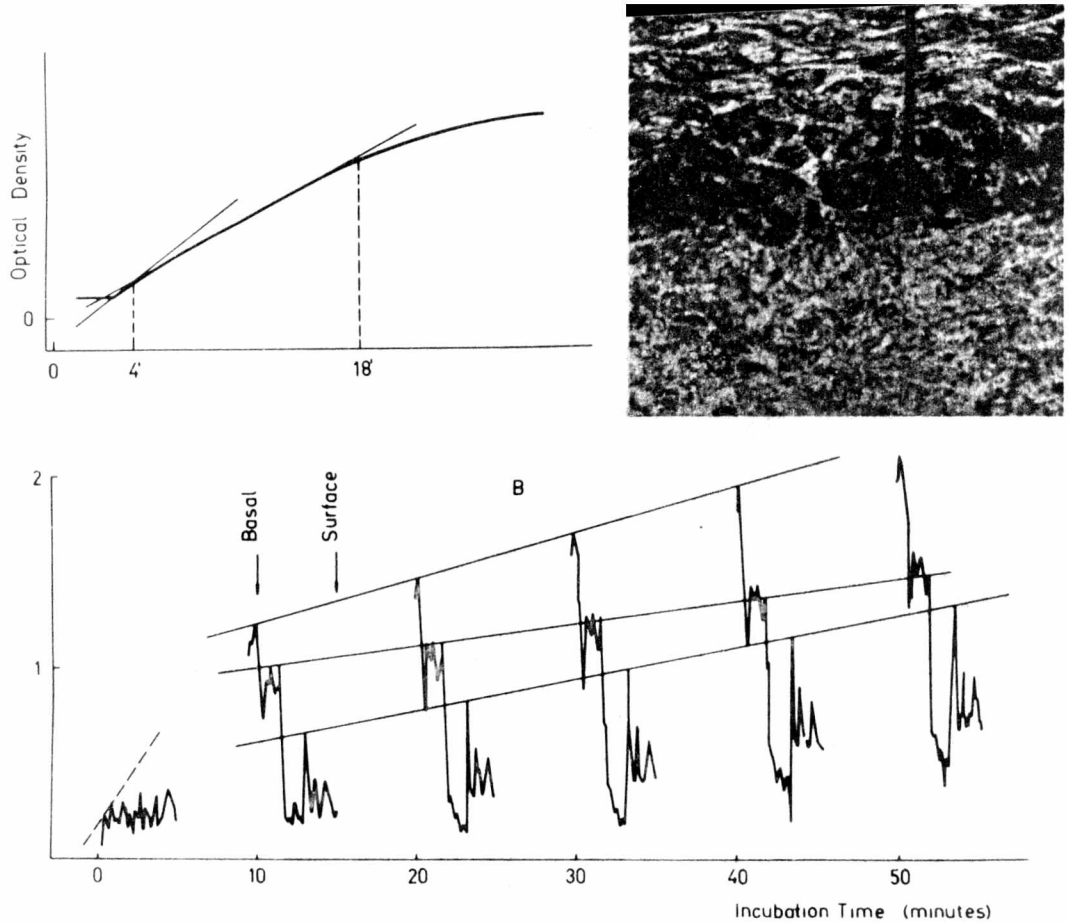

FIG. 5. A, Graphic record of enzyme reaction in a fixed 3 μ diameter area of tongue muscle cryostat sections. Recording was started immediately after the incubation medium containing 5.5 *mM* DAB was added, using a small chamber placed under the objective of the microspectrophotometer. The reaction rate was greater during the first 3 min. The linearity was maintained during 18 min. B, Automatic scanning graphs carried out from the basal to the horny layer of the ventral tongue epithelium, during incubation under similar conditions as described for Figure 1A. The basal layer exhibited the greatest activity, which decreased towards the epithelial surface. The reaction linearity was maintained for 40 min at each point of the scan. Three of them are marked on the graph. C, Microphotograph of the scanned epithelium after 30 min of incubation. The 1 μ diameter spot and its trajectory are indicated. Horny layer was not measured.

amount of cytochrome C in the incubation medium, it seems obvious that during the period in which the response is linear, the reaction rate depends on cytochrome oxidase activity. This was also proved by the inhibition obtained with KCN, which in a concentration as low as 0.001 *M* is a specific inhibitor of this enzyme.

The relative microspectrophotometric evaluation of a determined experimental problem requires one to previously establish the limits of the incubation period and of the optical density between which the response is proportional to the amount of enzyme. A small incubation chamber as the one described above is very

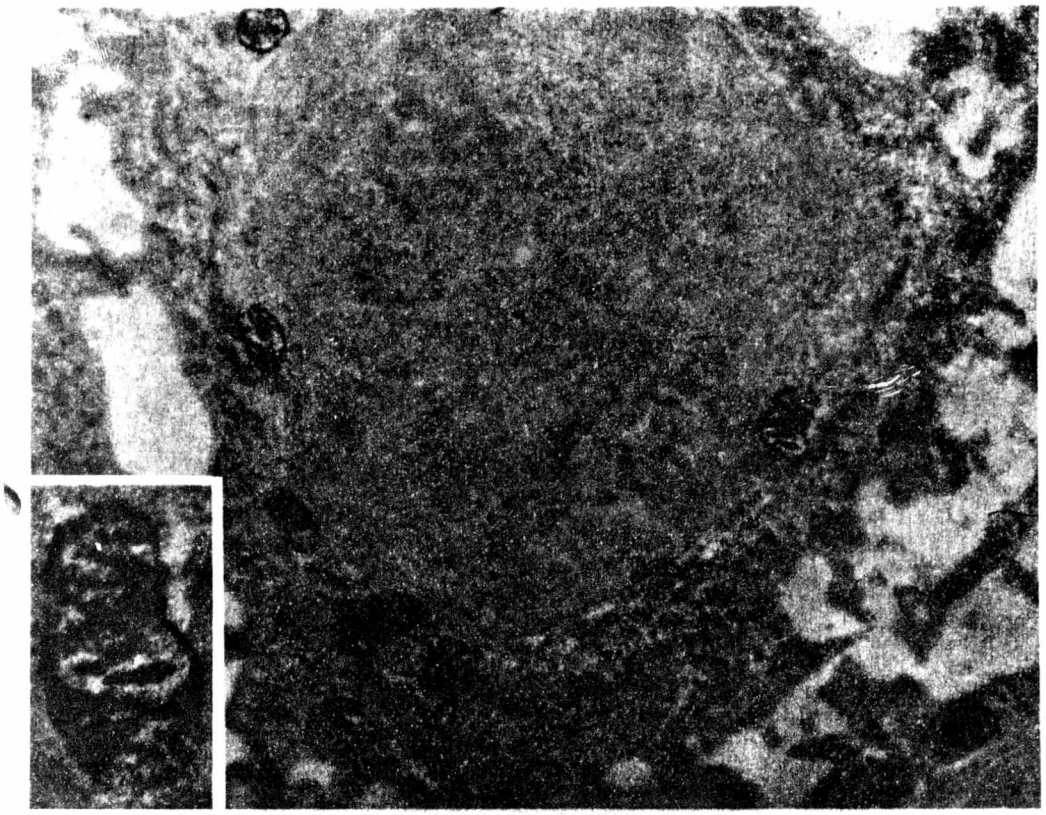

FIG. 6. Electron micrograph showing mitochondrial reaction in a basal cell of the ventral tongue epithelium. The tissue was incubated with DAB 5.5 *mM*. The reaction product is localized in the space between inner and outer limiting mitochondrial membrane and in the intracristate spaces (inset).

useful for this purpose since it allows one to follow the reaction rate, using a single tissue section.

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