A new microspectrophotometric method for quantifying cytochrome oxidase activity has been developed based on a color-modified diaminobenzidine (DAB) reaction. The reaction consists of pretreating tissue sections or cell smears with 10 mM cobalt acetate dissolved in 0.1 M tris-HCl buffer of pH 7.4 for 10 min at 37°C, washing and then treating with the DAB reagent for cytochrome oxidase for 15 min at 37°C. Mitochondria turn reddish blue in the stained sections or smears. This reaction is enzymatic and conforms to Michaelis-Menten’s formula. The microspectrophotometric quantification is effected by scanning the selected cytoplasmic area at 575 nm or by measuring the cytoplasmic plug at two wavelengths, 575 and 665 nm. As an example, the monomodal distribution in cytochrome oxidase activity was demonstrated among isolated rat liver cells by the two wavelength method.

Since diaminobenzidine (DAB) has been introduced into histochemical use by Graham et al. (7), many efforts have been devoted to applying the DAB reaction as a means of locating the activity of cytochrome oxidase, peroxidase and catalase in a variety of cells and tissues (4, 11, 13, 21, 23). In contrast, fewer efforts have been exerted towards using the reaction as a means of quantifying the activity concerned microspectrophotometrically. Frasch et al. (5) and Geerts et al. (6) have done pioneering work along this line by utilizing the broad absorption peak of 450–480 nm of the DAB oxidation product formed by cytochrome oxidase and catalase, respectively. Recently, a DAB oxidation product of blue color tone has been obtained using cobalt or nickel ions as a modifier (1, 2, 9). Taking advantage of the fact that this color-modified product exhibits a sharp absorption peak, a new microspectrophotometric method for quantifying cytochrome oxidase activity has been developed, and this will be reported below.

MATERIALS AND METHODS

Liver sections: Adult rats of Wistar strain were anaesthetized, and the liver
was perfused with physiological saline solution, then with 2.5% glutaraldehyde dissolved in 0.1 M cacodylate-HCl buffer of pH 7.4 for 5 min, and finally with the saline solution thoroughly. Sections, 15–60 µm in thickness, were obtained with the aid of a freezing microtome (Yamato Koki Co., Tokyo). They were used as the specimens for checking the reaction conditions and for studying the reaction mechanism.

Isolated liver cell smears: About 200 liver sections, 15 µm thick, were incubated at 37°C for an hour in a maceration medium. It consisted of 0.25% trypsin (Difco Lab., Detroit) and 0.05% collagenase (Sigma Chem. Co., St. Louis) dissolved in Dulbecco’s medium containing no calcium ions. The resulting turbid suspension was filtrated through four layers of gauze, and the filtrate was subjected to centrifugation at 330 g for 5 min. The pellet, which contained single cells or groups of several cells, was washed several times with 0.1 M tris-HCl buffer of pH 7.4, and finally smeared on a cover glass. The smears were air-dried at 4°C and used as the specimens for microspectrophotometric quantification of cytochrome oxidase activity.

Reagents: Ten mM cobaltous acetate dissolved in 0.1 M tris-HCl buffer of pH 7.4 was used as a color-modifying reagent. The DAB reagent for cytochrome oxidase consisted of 2.5 mM DAB tetrahydrochloride (E. Merck, Darmstadt), 0.1 mg/ml catalase (Sigma) and 0.5 mg/ml cytochrome c (Type III, Sigma) dissolved in the same buffer unless otherwise stated (22). Modified DAB reagents of different DAB concentrations, those dissolved in 0.1 M tris-maleate buffer at different pH values, and those supplemented with KCN were prepared. Blank reagents, in which DAB was omitted, were also prepared for each of the DAB reagents mentioned above.

Color-modified reaction for cytochrome oxidase: The specimens were pretreated with the color-modifying reagent for 10 min at 37°C, washed three times with tris-HCl buffer of pH 7.4, and then incubated in the DAB reagent for cytochrome oxidase for 15 min at 37°C. After being washed thoroughly with the buffer, they were dehydrated with a graduated ethanol series, infiltrated with xylol and mounted in diatex (Becker A. B., Sweden). Besides this standard procedure, the reaction was carried out at different temperatures, at different pH values and for different durations. In some cases, the pretreatment was omitted for comparison’s sake. Concurrently with these procedures, control runs were made in two ways; 1) specimens were pretreated with the color-modifying reagent and then treated with the blank reagent in succession, or 2) specimens which had been heated at 80°C for 10 min were subjected to the color-modified reaction.

Analysis of reaction mechanism: A parafilm frame of 0.125 × 10 × 10 mm inside dimensions was fixed on a slide glass by heat. A rat liver section, 15 µm thick, which had been pretreated with the color-modifying reagent, was stuck on the slide in the middle of the frame. Immediately after the addition of the DAB reagent or modified ones containing 1 × 10⁻³ and 1 × 10⁻⁴ M KCN, the color development due to the reaction product was followed at 575 nm at 23°C. A similar color development was also followed using modified DAB reagents of different DAB concentrations. The reaction rate, that is, the increase in absorbance at 575 nm per min during the linear period of reaction, was calculated against the external DAB concentration of the reagents used. Control runs were carried out using heat-injured
sections as the specimens to check the possible effect of autooxidation of the reagents.

Model wedges: A crude mitochondrial fraction was obtained from fresh rat liver after Johnson et al. (10). The fraction was sonicated and its protein concentration was estimated (19). One part of this fraction was mixed with five parts of 1% agarose sol, and the mixture was kept warm. One hundredth ml volume of this mixture was quickly poured onto a heat-fixed parafilm frame of $0.125 \times 10 \times 10$ mm inside dimensions, and was spread evenly over the whole frame area. After being dried under an infra red lamp, the resulting thin film attained a uniform thickness except at its periphery. The film was trimmed to remove its margin, isolated from the slide by immersion in distilled water, subjected to the color-modified reaction for cytochrome oxidase, and cut into small pieces. The deviation in absorbance at 575 nm among them remained within 5-6 per cent. Several of them were assembled, one over another, to form a step wedge. A control wedge was also obtained from similar agarose-mitochondrial fraction film treated with the color-modifying and the blank reagent in succession. These wedges were air-dried, immersed in xylol and mounted in diatex.

Measurements: A spectrophotometer equipped with a microscope attachment (MPS-5000, Shimadzu Corp., Kyoto) was used to establish absorption spectra of the reaction products and to measure absorbances by the two wavelength method. The spectra were obtained by measuring selected circular plugs of $4 \mu m$ diameter through specimens of $15 \mu m$ thick using a $25 \times$ objective lens. The two wavelength measurements were carried out by estimating absorbances of similar plugs through the liver cell cytoplasm at 575 and 665 nm (18, 20), and the chromophore amount was calculated after Patau (14, 20). A microspectrophotometer equipped with an automatic scanning stage and an integration unit (MMSP-TU, Olympus Optical Industry Co., Tokyo) was used to quantify the amount by the scanning method. This equipment was characterized by the use of a pair of similar objective lenses to eliminate the Schwarzchild-Villiger's effect (17). The integrated absorbances were obtained by scanning the liver cell cytoplasm with a monochromatic spot of $2 \mu m$ diameter at 575 nm for $6 \mu m$ distance; that is, over a 15 square micra area. All the spectra and the absorbances of the cytoplasmic plugs and scanning areas in the stained specimens were obtained by subtracting the values of the corresponding plugs and areas in the control specimens concerned.

RESULTS

The color-modified reaction: Mitochondria were stained reddish blue in rat liver cells subjected to the color-modified DAB reaction for cytochrome oxidase, while they remained colorless in the corresponding cells of control specimens. Those mitochondria which were stained reddish blue looked brighter than those stained yellowish brown by the color-unmodified DAB reaction. They were the only sites of positive reaction for cytochrome oxidase in liver cells.

The conditions suitable for the color-modified reaction: The relationship between the yield of reaction by cytochrome oxidase in single liver cells and the temperature at which the reaction was carried out, that between the yield and the pH value at which the reaction was performed, and that between the yield and the section thickness were examined. The color-modified reaction was optimal at 37°C (Fig. 1,
solid circles) and at pH 7.4 (hollow circles). The section thickness which assured homogeneous penetration of the reagent was 40 µm at the maximum (squares).

Absorption spectra of color-modified and color-unmodified reaction products: The absorption spectra of the color-modified and the color-unmodified DAB reaction products are given in Figs. 2 and 3 respectively. A comparison of these figures revealed that the color-modification by cobalt ions made the absorption peak of the product shift towards longer wave lengths, and at the same time, enhanced it. It was a common characteristic of these two kinds of product that their absorption peak shifted to shorter wave lengths as the staining was lighter (Figs. 2, 3). Based on the spectral curves of the color-modified product (Fig. 2), microspectrophotometry by scanning was carried out at 575 nm, and that by the two wavelength measurement at 575 and 665 nm (18, 20).

The establishment of Beer's law in microspectrophotometry: A step wedge, which contained the color-modified DAB reaction product as the chromophore, was used as a model system for microspectrophotometry. A linear relationship was obtained between the absorbance at 575 nm and the step number of the wedge (Fig. 4).

The stability of the color-modified reaction product: The color-modified reaction product decayed rapidly at high temperature, especially in the presence of light, but was stable if kept at low temperature in the dark (Fig. 5).

The reaction mechanism of the color-modified reaction: The color-modified reaction product developed continuously with addition of the DAB reagent to the liver section, which had been pretreated with the color-modifying reagent. The color development was practically null in the presence of KCN at 1 × 10⁻³ M in the reagent, while it was depressed at 1 × 10⁻⁴ M. As shown in Fig. 6, the reaction proceeded linearly from 3 to 30 min in the absence, and from 3 to 45 min in the presence of KCN at 1 × 10⁻⁴ M. The rates of reaction during the linear period, from
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15 to 30 min of reaction, were estimated against the different final concentrations of DAB in the reagents used. Fig. 7 shows the Lineweaver-Burk's double reciprocal plots of the results obtained (12). Linear relationships of different inclination angles were established with respect to the color-modified DAB reaction in the absence (hollow circles) and in the presence (solid circles) of $1 \times 10^{-4}$ M KCN, with the Michaelis' constant of 1.04 and 1.02, and the maximum reaction rates of 0.0091 and 0.0045 respectively.

**Microspectrophotometric determination of cytochrome oxidase activity:** The amount of the color-modified reaction product was estimated with respect to the stained and identified liver cells in a smear specimen by means of the two wavelength method and the scanning one in comparison. A fairly good correlation existed between the values obtained by these two methods; its coefficient being $+0.94$ (Fig. 8). The distribution of cytochrome oxidase activity among individual liver cells is given in

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**Figures:**

Figs. 2 and 3. Absorption spectra of the color-modified (Fig. 2) and the color-unmodified (Fig. 3) DAB reaction product produced by cytochrome oxidase in rat liver sections. Curve 1 deals with an intensely stained, curve 2, a moderately stained and curve 3, a lightly stained region. Black circles denote the absorption peaks.
the histogram shown in Fig. 9. This demonstrates the presence of a monomodal distribution activity among them. The activity, as expressed by the chromophore amount formed by the color-modified reaction during 15 min of reaction, was found to be $9.9 \pm 2.8$ in arbitrary units.
DISCUSSION

Use of cobalt acetate as a color-modifier: The color-modification of the DAB reaction product has been reported to be effected by the presence of cobalt or nickel ions during the oxidation reaction of DAB (1, 2, 9). As cobalt ions gave a brighter...
blue color with a sharper absorption peak than nickel ions, the former ions were selected for use. Cobalt acetate, cobalt chloride and cobalt ammonium sulfate had a similar color-modifying effect. The addition of cobalt acetate to the DAB reagent brought about a gradual or a rapid color change from pink to blue depending on the final cobalt concentrations. Preliminary incubations of liver sections with a modified DAB reagent supplemented with cobalt acetate revealed that the color-modifying effect was unsatisfactory at $1 \times 10^{-4}$ M, while satisfactory at $1 \times 10^{-3}$ and $1 \times 10^{-2}$ M. To the authors' regret, blue precipitates stuck onto the sections in these latter two cases. For this reason, a two step procedure of color-modification was selected for use to obtain a sufficient effect without changing the original color of the DAB reagent.

The spectral curves of the color-modified product: It seems worth-while to discuss some spectral features of the color-modified DAB reaction product. The absorption peak of the product shifted to shorter wavelengths as the absorbance decreased (Fig. 2), which makes the microspectrophotometric quantification inaccurate. This inaccuracy seems practically negligible when measuring at the selected wave length of 575 nm, since the absorption peak becomes broader as its value decreases. This presumption is proven to be true by the fact that the color-modified reaction conforms to Beer's law (Fig. 4).

Mechanism of the color-modified reaction: The result given in Fig. 7 implies that
the color-modified DAB reaction is enzymatic, and conforms to Michaelis-Menten's formula (16). This situation is similar to the case of the color-unmodified DAB reaction in a model film containing cat heart mitochondrial extract (5).

**Microspectrophotometric use of the color-modified reaction**: The color-modified DAB reaction has some advantages over the color-unmodified one as the cytochemical reaction for microspectrophotometric use. First, the former reaction is more sensitive and gives a reaction product with a sharper absorption peak than the latter one (Figs. 2, 3). Therefore, the color-modified reaction is superior to the DAB reaction now in use (5, 6). Secondly, the color-modified reaction reduces the distributional error of the chromophore by the two wavelength method (18, 20). It is true that this error can be reduced by the scanning method, but this procedure requires an elaborate instrument. Instead, the two wavelength method can be performed with a simple instrument, and yet allows us to obtain an accurate estimation of cytochrome oxidase activity. This is demonstrated by the fact that the chromophore amounts estimated by these two methods had a satisfactory correlation (Fig. 8). This situation seems similar to the case of nuclear stain with gallocyanin chrome alum in mouse ascite cells (15).

Based on the fact that the activity of cytochrome oxidase, peroxidase and catalase can be selectively detected by DAB reagents of different compositions (3, 8), the color-modified DAB reaction seems to be applicable for determining the activity of those enzymes, besides cytochrome oxidase, which are capable of oxidizing DAB, provided that they are insensitive to the presence of cobalt ions.

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