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NEW SENSITIVE METHOD FOR THE SPECTRAL ANALYSIS OF RESPIRATORY-CHAIN COMPONENTS IN MITOCHONDRIA

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SUMMARY

A new analytical method (two-wavelength/double beam method) for identification of respiratory-chain components in mitochondria was developed using a commercially available two-wavelength spectrophotometer. Cancellation of the scattered light due to the turbidity of the sample and correction of baseline due to the errors in the appartus were achieved using an optical attenuator for the reference cuvette over a wide range of wavelength (λ_x) taking λ_1 as the reference wavelength. Then the same beams were passed through the sample cuvette and the difference between the absorbance of the sample and reference cuvettes at λ_x was obtained.

Difference spectra between reduced and oxidized rat-liver mitochondria were obtained on a full scale of A 0–0.01 or 0.03 in varying wavelengths of 300–650 nm. Using this method the α-bands of cytochrome a ($+ a_3$), b and c, the Soret bands of cytochrome a_3 ($+ a$) and b, and the bands of flavoprotein and pyridine nucleotides were detected in a quite sensitive manner with reasonable accuracy.

A discussion is given on the theory and application of this method.

INTRODUCTION

Special methods are required for identification of the absorption spectra of respiratory-chain components in mitochrondria, since mitochondrial preparations are generally very turbid and the optical changes in respiratory components such as cytochromes which occur during metabolic changes are extremely small. As pointed out by Chance[1,2], commercially available spectrophotometers are usually not suitable for accurate measurement of small absorbance changes. For the cancellation of turbidity, a constant part of the transmitted light may be eliminated in either of two ways: (a) by a double beam method in which a second light beam of the same wavelength passes through a reference cuvette containing material of similar absorbance, such as filter paper, opal glass[3,4] or reference material (*e.g.* oxidized mitochondria), or (b) by a two-wavelength method (dual-wavelength method) in which a second light beam as a reference wavelength (λ_1) passes through the sample cuvette, and a reference cuvette is not used. A Split-beam spectrophotometer[2,5–7], essentially based on (a), has been used for this purpose coupled with an end-on photomultiplier and an electrical device. The two-wavelength spectrophotomater[1,2,7–10] based on (b) has been found to be very

sensitive in the kinetic studies[11,12] on the redox states of respiratory carriers, when a pair of wavelengths (λ_2 and λ_1), suitable for measurment of carriers and for elimination of the effect of light scattering, is chosen. However, it has not been possible to use this method for scanning a wide range of wavelengths, as is discussed later.

This paper reports a new method for identification and characterization of respiratory-chain components of mitochondria, in which the scattered light due to the turbidity of the sample and errors in the apparatus were cancelled out by an optical attenuation for reference material at λ_x taking λ_1 as the reference wavelength. Using a commercially available two-wavelength spectrophotometer, the difference spectrum between the sample and reference cuvettes can be obtained with reasonable accuracy at full scale of A 0–0.01 in wavelengths from 300–650 nm.

The method is tentatively named the two-wavelength/double beam method.

MATERIALS AND METHODS

Experiments were carried out with a Hitachi-Model 356 Two-Wavelength/Double Beam Spectrophotometer, in which two-wavelength and double beam modes are combined in one instrument. The reference cuvette, containing material with the same light scattering as the sample (*e.g.* State 2 mitochondria[11], in which all respiratory carriers were nearly 100 % oxidized) was inserted into the cell compartment in the two-wavelength mode. The cuvette is in close contact with a head-on photomultiplier, as shown in the diagram of the optical system (Fig. 1). Taking λ_1 as the reference wavelength, the signal due to the reference cuvette at λ_x was adjusted to a constant point on the recorder paper (usually adjusted to 20 % of the full scale) using an optical attenuator (O_1 and O_2 in Fig. 1). This means that the light scattered in the reference cuvette and optical errors in the apparatus are cancelled out by the same device as in the two-wavelength method, as mentioned in the Discussion. Next, the sample cuvette (*e.g.* State 5 mitochondria[11], in which all electron carriers were nearly 100 % reduced) was inserted into the same cell compartment manually and the signal due to sample material was recorded on the paper under the same optical conditions

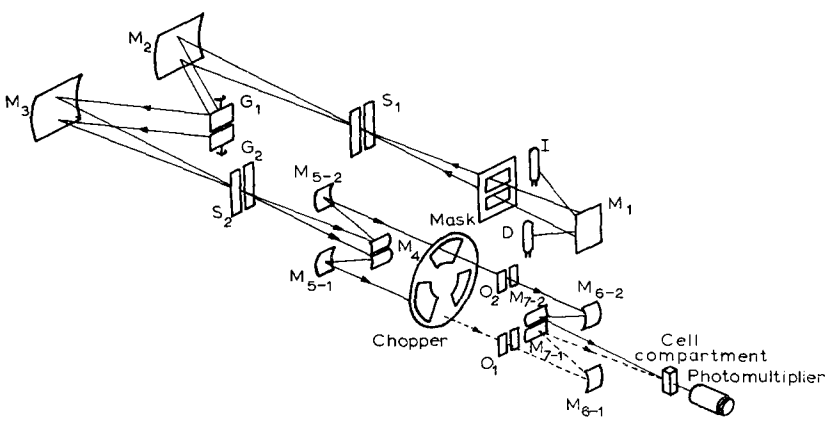

Fig. 1. Optical arrangement in the two-wavelength spectrophotometer used. G_1, G_2, gratings; M_2, collimating mirror; M_3, camera mirror; M_1–M_{7-2}, concave mirrors; O_1, O_2, attenuators; S_1, S_2, slits; D, deuterium discharge lamp; I, tungsten–iodide lamp.

used for the reference cuvette. The difference value between the two points was read on the recorder paper and this procedure was repeated under the varying wavelengths (λ_x), usually at intervals of 2 nm. By plotting the difference values at λ_x a continuous spectrum was obtained.

Sigma Type III cytochrome *c* from horse heart was used. Rat-liver mitochondria were isolated by the method of Hogeboom[13] as described by Myers and Slater[14]. Protein was determined by the biuret method as described by Cleland and Slater[15]. All reactions were carried out in the reaction mixture containing 25 mM Tris–chloride buffer, 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl. The other components used are indicated in the legends to figures. The final volume of the mixture was 3 ml and the pH was 7.4.

The extinction coefficients (ΔE_{mM}) used for determination of respiratory-chain components were those reported by Chance[2], and Sato and Hagihara[16], based on the absorption differences between the reduced and oxidized carriers in two-wavelength pairs. Their values were cytochrome a ($+ a_3$) at $\Delta E_{605-630\,nm} = 16.5$; cytochrome b at $\Delta E_{562-575\,nm} = 17.9$; cytochrome c ($+ c_1$) at $\Delta E_{550-540\,nm} = 19.0$; flavoprotein at $\Delta E_{460-500\,nm} = 11.0$ and nicotinamide nucleotides at $\Delta E_{340-375\,nm} = 6.0\ mM^{-1} \cdot cm^{-1}$.

RESULTS

First the new method was employed for determination of the reduced form of cytochrome *c*, since an aqueous solution of cytochrome *c* is transparent. Fig. 2 illustrates the absorption spectrum of $1.67 \cdot 10^{-7}$ M reduced cytochrome *c* taking 650 nm as the reference wavelength (λ_1) with a scale range of A 0–0.01. The absorption peak lays exactly at 550 nm, and 101 % recovery was obtained based on the observed value $\Delta A_{550-540nm} = 0.0032$. Thus this method seems sufficiently accurate and sensitive for identification and characterization of the respiratory-chain components in mitochondria.

Next the method was tested on a turbid suspension of rat-liver mitochondria containing various cytochromes and other hydrogen carriers. Fig. 3 shows the difference spectra between the reduced (State 5) and oxidized (State 2) mitochondria using various concentrations of mitochondrial protein in the range from 650–500 nm ($\lambda_1 = 650$ nm) with a scale of A 0–0.03. On addition of 0.2, 0.1, 0.05 or 0.02 ml of mitochondrial suspension in a total volume of 3 ml, the α-bands of cytochrome a ($+ a_3$), b and c were observed with great sensitivity. For example, the characteristic absorption peaks of cytochrome a ($+ a_3$) at 605 nm, cytochrome b at 562 nm and cytochrome c at 550 nm could be seen in a cuvette containing 0.02 ml of mitochondrial suspension (final concentration, 0.44 mg protein per ml). A value $\Delta A_{550-540\,nm} = 0.0036$ was obtained for cytochrome c ($+ c_1$) and this value agreed with the value reported[17] for rat-liver mitochondria. The complicating effect from the turbidity of the mitochondrial preparation seems to be completely overcome with this method, since a straight line through the origin was obtained for the relationship between the cytochrome c content ($\Delta A_{550-650\,nm}$) and protein concentration over a wide range as shown in Fig. 4.

This method can be used at wavelengths of 300–650 nm in the visible region and of 200–300 nm in the ultraviolet region (not shown), though it takes rather a long time in its present form. The difference spectrum shown in Fig. 5 was obtained under essen-

tially the same conditions as in Fig. 3. The Soret bands of cytochrome a_3 (+ a) at 445 nm and of cytochrome b at 430 nm as well as α-bands of cytochromes a (+ a_3), b and c can be seen. The bands of reduced form of the flavoprotein at 460 nm, and of pyridine nucleotides at approximately 340 nm are also seen in this spectrum. From this spectrum the amounts of the respiratory components (in μmoles per g protein) were calculated: cytochrome a (+ a_3), 0.21; cytochrome c (+ c_1), 0.35; cytochrome b, 0.20; flavoprotein, 0.66; and nicotinamide nucleotides, 3.4. These values agree well those reported previously (*cf.* ref. 17).

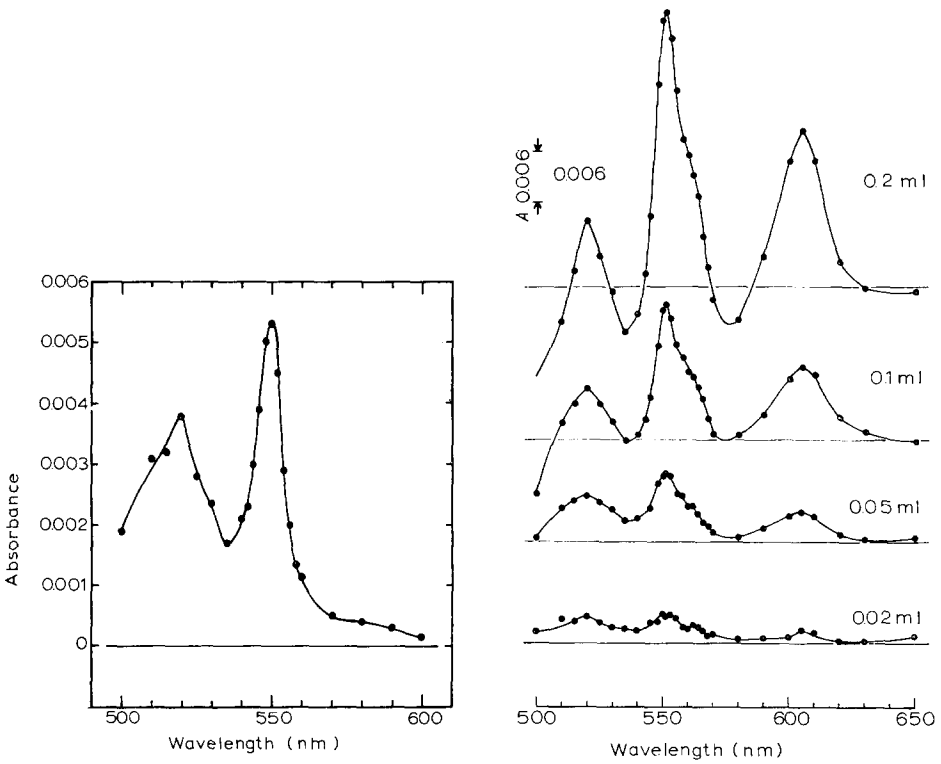

Fig. 2. Absorption spectrum of reduced cytochrome c. Cuvettes of 1-cm light path were used. The sample cuvette contained $1.67 \cdot 10^{-7}$M cytochrome c reduced by approx. 2 mg of $Na_2S_2O_4$ in a total volume of 3 ml. The reference cuvette contained all the same constituents except cytochrome c. Reference wavelength, $\lambda_1 = 650$ nm; band pass, 1 nm; full scale. A 0–0.01.

Fig. 3. Difference spectra of cytochrome components in State 5 and State 2 with various concentrations of mitochondria. Sample cuvette, State 5 mitochondria induced from State 2→3→4→5 by adding 10 mM succinate to State 2 mitochondria; reference cuvette, State 2 mitochondria containing 1 mM ADP and 10 mM P_i. The concentrations of mitochondria (ml) are indicated in the figure. 2 μg/ml or rotenone were present. A suspension of 66.5 mg/ml of rat-liver mitochondria was used. Reference wavelength, $\lambda_1 = 650$ nm; full scale, A 0–0.03. Other conditions were as for Fig. 2.

DISCUSSION

This paper describes a new type of analytical method for the identification of respiratory-chain components in mitochondria. Results clearly showed that the influence of light scattering from the sample could be overcome and a fine correction

of the baseline could be achieved simply by adjustment of the optical attenuator. In this way difference spectra between reduced and oxidized mitochondria were obtained over a wide range of wavelengths with high sensitivity and reasonable accuracy.

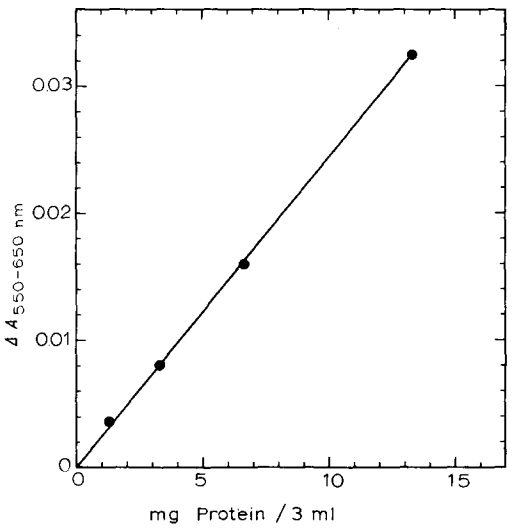

Fig. 4. Relationship between $\Delta A_{550-650\ nm}$ and protein content of mitochondria. Values were taken from the results in Fig. 3.

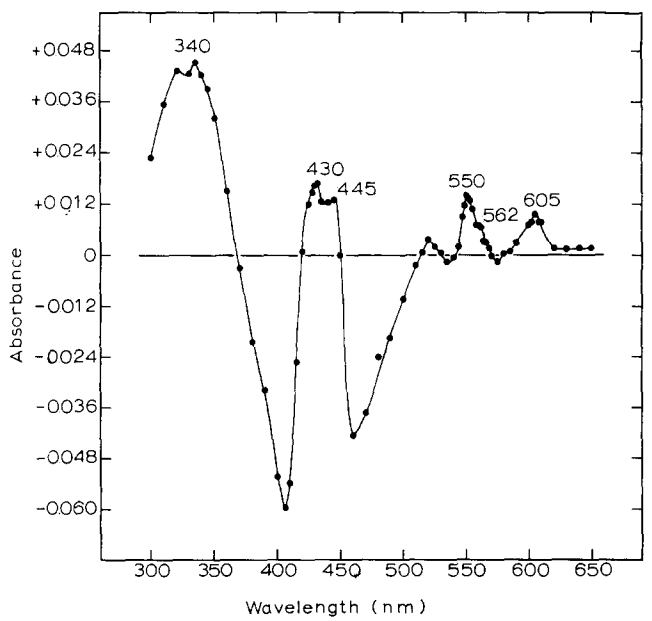

Fig. 5. Difference spectrum between State 5 and State 2 mitochondria at 300–650 nm. Sample cuvette, State 5 mitochondria; reference cuvette, State 2 mitochondria induced as described in Fig. 3. Rat-liver mitochondria, 2.22 mg/ml. Other conditions were as for Fig. 3.

In the two-wavelength method, an optical attenuator or a dynode feedback loop[2,7-9] is used for elimination of high background at λ_2 taking λ_1 as a reference wavelength. Some workers (*e.g.* refs 18–20) have used this principle to obtain spectra, since the two-wavelength method has a higher sensitivity than other conventional methods including the split-beam method. However, scanning over a wide range of wavelengths is impossible with this method, because the exact cancellation of light scattering and errors in the apparatus can be achieved only at λ_2. To avoid this disadvantage, we have introduced a reference cuvette containing a reference material with the same light scattering as the sample. Since the reference cuvette is used in the double beam method, we tentatively named the method the two-wavelength/double beam method.

In the two-wavelength method, the output signals through the sample material (S_1 at λ_1; S_2 at λ_2) can be adjusted by multiplying S_2 by a constant (k) with optical or electrical device, when λ_1 is chosen as a reference wavelength.

$$k \cdot S_2 = S_1 \tag{1}$$

The same principle was applied to the reference cuvette (R) under varying wavelengths (λ_x) in the present method.

$$k(x) \cdot R_x = R_1 \tag{2}$$

where $k(x)$ represents the attenuation coefficient at λ_x. R_x and R_1 may be expressed as,

$$R_x = I_x \cdot D_x \cdot \exp\{(-\varepsilon_x \cdot c_r \cdot l) + T_x\} \tag{3}$$

$$R_1 = I_1 \cdot D_1 \cdot \exp\{(-\varepsilon_1 \cdot c_r \cdot l) + T_1\} \tag{4}$$

where I_x and I_1 are the energy of the incident light at λ_x and λ_1; D_x and D_1 are functions of the apparatus at λ_x and λ_1; ε_x and ε_1 represent the molar extinction coefficients at λ_x and λ_1; c_r represents the concentration of component in reference material; l is the thickness of the component layer and T_x or T_1 is the absorbance due to turbidity at λ_x or λ_1. From Eqn 2, the apparent output signal of the reference cuvette (A_r) will be given by the following equation,

$$A_r = -\log \frac{R_x}{R_1} \cdot k(x) = 0 \tag{5}$$

or

$$\log k(x) = -\log \frac{R_x}{R_1} \tag{6}$$

The meaning of Eqn 5 seems to become clearer if we consider the true output signal due to the reference cuvette without the optical attenuation where $k(x) = 1$. From Eqns 3, 4 and 5 the true output signal may be expressed as

$$-\log \frac{R_x}{R_1} = -\left\{\log \frac{I_x \cdot D_x}{I_1 \cdot D_1} - (\varepsilon_x - \varepsilon_1) \cdot c_r \cdot l + (T_x - T_1)\right\} \tag{7}$$

The first term of the right hand of Eqn 7 was already reported by Honkawa *et al.*[21] as a change in the baseline caused by the errors in the apparatus in the absence

of the reference or sample cuvette. Since we can set the conditions where the signal due to Eqn 7 is zero by multiplying $k(x)$, it is evident that the scattered light through the reference cuvette ($T_x - T_1$) and the errors in the apparatus as well as an absorption of the component before biochemical reaction are cancelled by the optical attenuation as indicated in Eqn 5. However, the true signal through the reference cuvette could be fixed as a memory in the attenuation curve as expressed in Eqn 6 (Curve b in Fig. 6).

When we insert a sample cuvette having the same turbidity as the reference material under the same conditions and where the value of $k(x)$ is derived from the reference cuvette by Eqn 5, the output signal through sample cuvette (A_s) will be given as follows

$$A_s = -\log \frac{S_x}{S_1} \cdot k(x) \tag{8}$$

where

$$S_x = I_x \cdot D_x \cdot \exp\{(-\varepsilon_x \cdot c_s \cdot l) + T_x\} \tag{9}$$

$$S_1 = I_1 \cdot D_1 \cdot \exp\{(-\varepsilon_1 \cdot c_s \cdot l) + T_1\} \tag{10}$$

in which c_s represents a concentration of component in sample material. When we read the difference value (ΔA) between the signals due to A_s and A_r from Eqns 8 and 5

$$\Delta A = A_s - A_r = -\log \frac{S_x}{S_1} \cdot k(x) \tag{11}$$

Substituting equation (6) for $k(x)$ we obtain,

$$\Delta A = -\log \frac{S_x \cdot R_1}{S_1 \cdot R_x} \tag{12}$$

Since we have chosen the experimental conditions where $S_1 = R_1$, we get,

$$\Delta A = -\log \frac{S_x}{R_x} \tag{13}$$

From Eqns 3 and 9,

$$\Delta A = \varepsilon_x (c_s - c_r) \cdot l \tag{14}$$

This equation indicates that the absorbance difference obtained by the present method is proportional to the concentration of a component and satisfies Beer's law. Fig. 6 illustrates schematically the relationships between absorbance and the various equations given here.This illustration was supported by experimental results recently obtained in our laboratory (S. Muraoka and K. Takahashi, unpublished). For example, the relationship between values of the attenuation and signal due to Eqn 6 was confirmed using cytochrome *c* as a reference material. When we cancelled out signals at 550 nm due to various concentrations of cytochrome *c* by the attenuator, we obtained a linear relationship between the concentrations of cytochrome *c* and the reading values of the attenuator scale. When we cancelled out signals due to cytochrome *c* under varying wavelengths, we could obtain an attenuation curve having

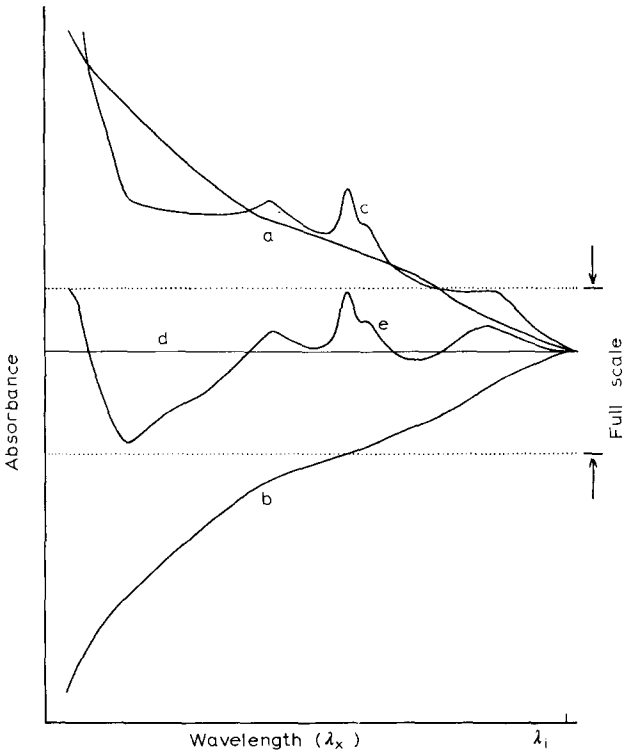

Fig. 6. Schematic representation of the two-wavelength/double beam method. Curve a, $-\log R_x/R_1$, expressed by Eqn 5, when $k(x) = 1$; Curve b, $-\log k(x)$, in Eqn 6; Curve c, $-\log S_x/S_1$, expressed by Eqn 8 when $k(x) = 1$; Curve d, $-\log (R_x/R_1) \cdot k(x) = 0$. expressed by Eqn 5; Curve e, $-\log (S_x/S_1) \cdot k(x)$, in Eqn 8. Full scale: full scale used in the experiment.

a symmetrical shape with an absorption spectrum of cytochrome *c*. In this way it was also demonstrated that a change in the baseline that appeared in Eqn 7 was retained as a memory in its attenuation curve.

The value of the attenuation coefficient $k(x)$ has to be $1 \geqslant k(x) > 0$ and a value of nearly 0 was experimentally obtained in the present work. This may be the main reason why a ratio of R_x to R_1 of more than 10^7, which is mainly caused by the difference of light scattering between T_x and T_1 in the mitochondrial suspension, is easily cancelled by the present method. When we draw an absorption spectrum for turbid biological material, a minimal value of multiplying factors such as $k(x)$ which are performed by an optical attenuator or by a dynode feedback loop directly influences the range of wavelengths, because of an exponential increase in the light scattering due to turbidity at shorter wavelengths. It may be noted that the spectrum shown in Fig. 5 was obtained in a wide range of wavelengths from 650 nm to 300 nm under a flat baseline.

Since an absorption spectrum (Curve e, Fig. 6) is made under a flat baseline (Curve d), the full scale of absorbance can be set at an extremely smaller range in the present method. Moreover the noise level of the instrument used was less than $2 \cdot 10^{-4}$ absorbance unit so that the full scale may be reduced to less than *A* 0–0.01 if a good pair of cuvettes is used with increased sensitivity of the recorder.

The disadvantage of the new method in its present form is that it cannot be used to trace the absorbance changes in a fast reaction, such as steady state changes of respiratory-chain components in mitochondria. Mechanical improvement of the instrument is now in progress.

NOTE ADDED IN PROOF (Received April 4th, 1972)

In a paper which appeared after submission of this manuscript, Wikström[22] used a similar method for the identification of *b*-type cytochromes in rat-liver mitochondria using Aminco-Chance dual wavelength spectrophotometer. Although there was no description on the theoretical background, it seems that his method is essentially based on the same principle, in which $k(x)$ values were obtained with an electrical device instead of an optical attenuation in the present method.

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