COLORIMETRIC DETERMINATION OF CYTOCHROME *C* OXIDASE BY FORMATION OF A QUINONEDIIMONIUM PIGMENT FROM DIMETHYL-*p*-PHENYLENEDIAMINE*

by

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Previously, a colorimetric method for the determination of cytochrome oxidase was described which was based on the formation of indophenol blue from α-naphthol and dimethyl-*p*-phenylenediamine (EHRLICH's Nadi reaction)[1]. In order to determine low concentrations of cytochrome oxidase in small cytoplasmic fractions from rat kidneys, another more sensitive method was adopted. The new method is based on the observation that mitochondrial suspensions transform dimethyl-*p*-phenylenediamine into the red pigment "Wurster's Red" in amounts proportional to the concentration of cytochrome oxidase and to the time of incubation at 37° during the first 1 to 3 minutes. As compared with the formation of indophenol blue under similar conditions[1], "quinonediimonium red"** is developed by mitochondrial suspensions at a considerably faster rate. Since it is soluble in water, it can be measured directly in the test solution. Very small amounts of cytochrome oxidase (1 μg of mitochondrial N) can thus be determined by simply measuring the color intensity of the test solution after short incubation with dimethyl-*p*-phenylenediamine.

REAGENTS

Borate buffer pH 8.5 (Sørensen's borate- HCl mixture 6.5:3.5, diluted with 2 volumes of water) was used to neutralize dimethyl-*p*-phenylenediamine hydrochloride and to bring the pH within the range of 6.7 to 7.2. A commercial preparation of *dimethyl-p-phenylenediamine hydrochloride* (Eastman Organic Chemicals) was used without further purification. On prolonged standing, solutions of dimethyl-*p*-phenylenediamine hydrochloride take on a red color by autoxidation, between pH 5.5 and 7.3. Since the formation of the pigment is strongly decreased in a more alkaline or acid medium as well as at low temperature, 0.4% dimethyl-*p*-phenylenediamine hydrochloride solutions (pH around 4) could be kept on ice for a few hours without appreciable formation of color. The experiments shown in Figs. 2, 4, 6, 7 and 8 and in Table I were carried out with *cytochrome c* prepared from ox heart according to KEILIN AND HARTREE[3]; those in Figs. 3 and 5 with a commercial preparation (Delta Chemical Works). *Ethyl alcohol* to make a final volume of 30 to 40% was added to the blanks before incubation and to the test solutions after incubation in order to destroy cytochrome oxidase and to stop the enzymic formation of

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** For simplification, the term "quinonediimonium red", abbreviated "QDI Red" in the figures, is used in analogy to indophenol blue, instead of meri-dimethyl-quinonediimoniumchloride or "Wurster's Red". "Wurster's Red" is now mostly considered to be a semiquinone[2]. The normal quinonediimine, produced by strong oxidation of dimethyl-*p*-phenylenediamine, is yellow.

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"quinonediimonium red". Fresh solutions of *sodium azide* (Bios Laboratories) were used to test the inhibition of cytochrome oxidase activity. *Sodium-2,6-dichlorobenzenone-indophenol* (Fisher Scientific Company) was used for the estimation of the reducing capacities of the test solutions.

METHOD

To a 100 × 15 mm test tube, graduated at 10 ml, the following reagents were added: 0.4 ml of a 1/60 *M* borate buffer solution, pH 8.5; 0.1 to 0.4 ml of a 0.04% cytochrome *c* solution**; aliquots of a mitochondrial suspension containing 1 to 30 μg of N; distilled water to bring the volume to 1.8 ml. After warming for 2 minutes in a constant temperature water bath at 37°, 0.2 ml of a 0.4% solution of dimethyl-*p*-phenylenediamine hydrochloride was added. The sample was incubated from 1 to 3 minutes at 37° depending on the concentration of enzyme until it had developed a distinctly red color. The formation of the pigment was halted by the quick addition of 1 ml of ethyl alcohol followed by thorough mixing and the incubation time was read from a stop watch. The solution was cooled in ice for about 1 minute, diluted with water to the 10 ml mark, stirred with a glass rod, and the color intensity was measured with the Evelyn photoelectric colorimeter, filter no. 515. A blank was run together with the sample. It was identical in composition to the sample described above except that 1 ml of ethyl alcohol was added before the tube had been warmed at 37° and the amine hydrochloride added. Duplicate samples or those containing different concentrations of enzyme or cytochrome *c* were often run together with the main sample and blank. In such cases, the reactions in each sample were started by adding the amine hydrochloride solution from the same pipette to each tube in the series in intervals of 5 or 10 seconds.

Calculation of results. The photometric densities corresponding to the colorimeter readings for the sample and blank were read from the table. The photometric density of the blank was deducted from that of the sample. The result was expressed in "quinonediimonium red" units by dividing by 0.75 when the Evelyn colorimeter with filter 515 was used. The "quinonediimonium red" units per minute per mg of N were taken as a basis of comparison for different samples.

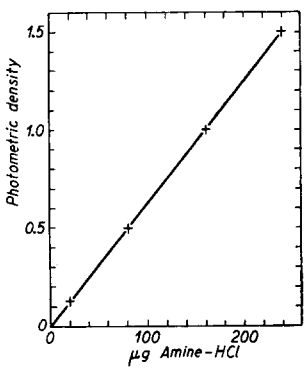

Fig. 1. Photometric densities of "quinonediimonium red", formed from different amounts of dimethyl-*p*-phenylenediamine hydrochloride by oxidation with $K_2Cr_2O_7$. In 10 ml of phosphate buffer pH 6.5; Evelyn photoelectric colorimeter, filter No. 515.

"Quinonediimonium red" units. A "quinonediimonium red" unit was defined as the photometric density of a 10 ml solution of the pigment at pH 6.5, formed by the oxidation of 0.12 mg ($0.69 \cdot 10^{-6}$ *M*) of dimethyl-*p*-phenylenediamine hydrochloride. Fig. 1 shows that the photometric densities of "quinonediimonium red" formed from different amounts of dimethyl-*p*-phenylenediamine hydrochloride by oxidation with $K_2Cr_2O_7$ are proportional to the concentration of the pigment. Fig. 1 also indicates that the photometric density of "quinonediimonium red", formed from 0.12 mg of dimethyl-*p*-phenylenediamine hydrochloride, is 0.75. This factor was used for the calculation of "quinonediimonium red" units as indicated above.

The standard curve was obtained by pipetting 0.2 to 2.4 ml of a 0.01% solution of dimethyl-*p*-phenylenediamine hydrochloride to 1 ml of a 1/15 *M* phosphate buffer solution, pH 6.5, and adding 0.15 to 1.8 ml of a 0.02% solution of $K_2Cr_2O_7$. After 2 to 3 minutes' standing, water was added to the 10 ml mark, and the color intensity was measured in the colorimeter, filter 515. In the presence of low concentrations of $K_2Cr_2O_7$, the red pigment is stable for a few minutes, and is then slowly oxidized to the yellow, normal quinonediimine.

** The amount of added cytochrome *c* was adjusted approximately to the concentration of enzyme as will be indicated later (Fig. 4).

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TEST AND APPLICATION OF METHOD

Time of incubation and concentration of enzyme

As shown in Fig. 2, the amount of "quinonediimonium red" formed by mitochondrial suspensions from rat kidney increased in proportion to the quantity of mitochondrial N and also to the time of incubation during the first few minutes, but decreased rapidly when a certain concentration of the pigment was attained.

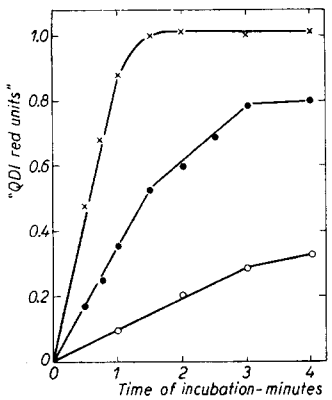

Fig. 2. Formation of "quinonediimonium red" by mitochondrial suspension from rat kidney in relation to incubation time at 37° and concentration of enzyme. Samples containing 2.6 μg of N (○), 10.4 μg of N (●) and 31.2 μg of N (×). pH 7.1.

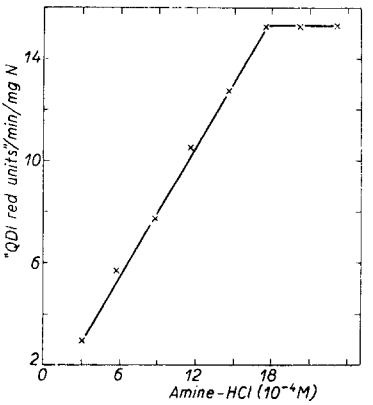

Fig. 3. Formation of "quinonediimonium red" by mitochondrial suspension (32.2 μg of N) from rat kidney in relation to concentration of dimethyl-*p*-phenylenediamine hydrochloride. Incubation for 1 minute at 37°; pH 6.8 to 7.2.

Concentration of dimethyl-p-phenylenediamine hydrochloride

Fig. 3 shows that maximal formation of "quinonediimonium red" required a $1.7 \cdot 10^{-3}$ molar concentration of the amine hydrochloride when the test solution contained 32.2 μg of mitochondrial N. The concentration used for the routine tests, as indicated under METHOD, was $2.3 \cdot 10^{-3}$.

Added cytochrome c

Fig. 4 shows the influence of different concentrations of added cytochrome *c* on three samples of a mitochondrial suspension from the kidney of the rat containing 3.6, 26.6 and 53.2 μg of total N, respectively. When very small amounts of enzyme (less than 5 μg of mitochondrial N) were

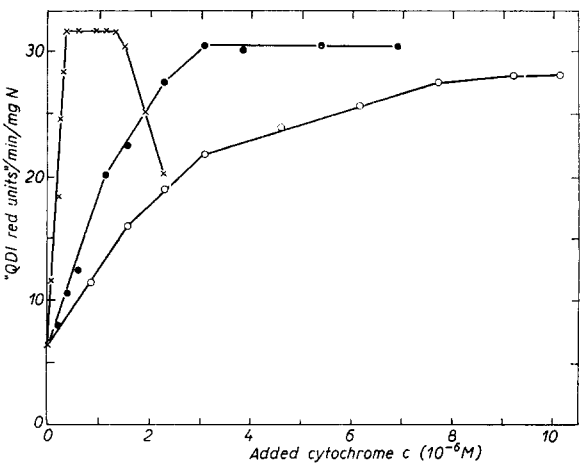

Fig. 4. Influence of added cytochrome *c* on formation of "quinonediimonium red" by rat kidney mitochondria resuspended in distilled water. Samples containing 3.6 μg of mitochondrial N (×), 26.2 μg of mitochondrial N (●) and 53.2 μg of mitochondrial N (○). Incubation for 2 (×), 1 (●) and 0.5 (○) minutes at 37°; pH 7.1.

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tested, a great excess of added cytochrome *c* inhibited the formation of the pigment,* whereas with larger amounts of enzyme, a 3 to 5 fold excess of cytochrome *c* had little effect (Fig. 4).

Influence of the pH

Fig. 5 shows that the formation of the pigment by a mitochondrial suspension from rat kidney was optimal from pH 6.7 to 7.3. The decrease in color development above pH 7.3 is not related to the enzyme, since a similar decrease of the pigment formation was also observed during autoxidation of dimethyl-*p*-phenylenediamine hydrochloride in buffer solutions above pH 7.3.

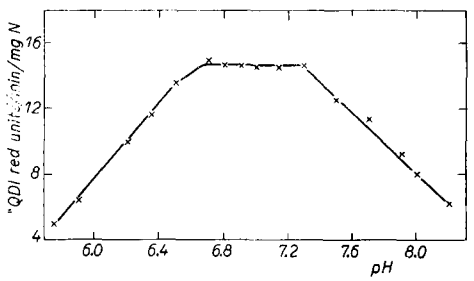

Fig. 5. Formation of "quinonediimonium red" by mitochondrial suspension (30.0 μg of N) from rat kidney in relation to pH. Incubation for 1 minute at 37°.

Since alcohol caused partial fading of the pigment in a medium more alkaline than pH 7.4, alcohol could not be used to stop the enzyme reaction above pH 7.4. By chilling the test solution in ice after incubation and diluting with ice-cooled water to the 10 ml mark, the enzymic color formation was slowed down sufficiently to allow for a quick reading in the colorimeter.

Borate buffer, phosphate buffer, added salts and sucrose

Inhibition of cytochrome oxidase activity by phosphate buffer, NaCl and KCl, which was previously observed in the indophenol blue method[1], was also found in the present method. Increasing concentrations of borate buffer also inhibited the enzyme though less than phosphate buffer.

Fig. 6 indicates that higher than 0.001 *M* concentrations of $CaCl_2$ and 0.01 *M* concentrations of NaCl caused strong inhibition of cytochrome oxidase activity when the mitochondria had been resuspended in water and fully activated by addition of cytochrome *c* whereas the same concentrations of the salts caused an enhancement of activity when no cytochrome had been added. In the latter case, in which only the endogenous cytochrome *c* took part in the reaction, the activity of the mitochondrial suspension was only about 25% of that attained after addition of cytochrome *c*. The effects of $Mg(NO_3)_2$ on the activity of the enzyme were similar to those of $CaCl_2$ while those of KCl were similar to those of NaCl.

Fig. 7 shows that the effects of the salts were different when the mitochondria were resuspended in 0.88 *M* sucrose solution

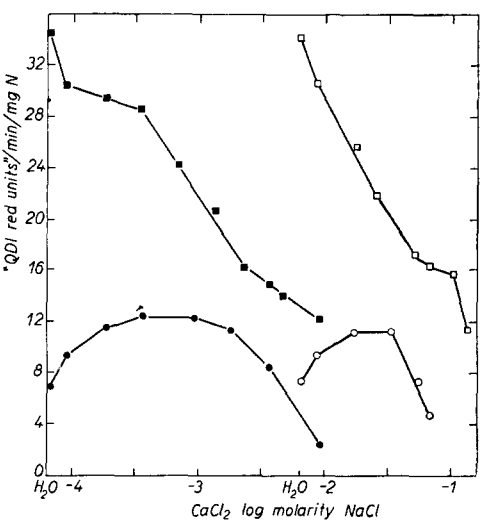

Fig. 6. Influence of $CaCl_2$ and NaCl, in presence and absence of added cytochrome *c*, on formation of "quinonediimonium red" by rat kidney mitochondria (18.3 μg N), resuspended in distilled water. Effect of $CaCl_2$ after addition of cytochrome *c* (■), without addition of cytochrome *c* (●). Effect of NaCl after addition of cytochrome *c* (□), without addition of cytochrome *c* (○). Incubation for 1 minute at 37°; pH 7.1.

* After completion of the manuscript, a cytochrome *c* preparation from the Sigma Chemical Company, St. Louis, was tested. This preparation was purer than the ones employed and did not show any inhibition.

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instead of distilled water. The inhibition of the enzyme by salts was only slight with mitochondria in hypertonic sucrose solution whereas the enhancing effect of the salts (in absence of added cytochrome *c*) was the same as with disintegrated mitochondria in distilled water.

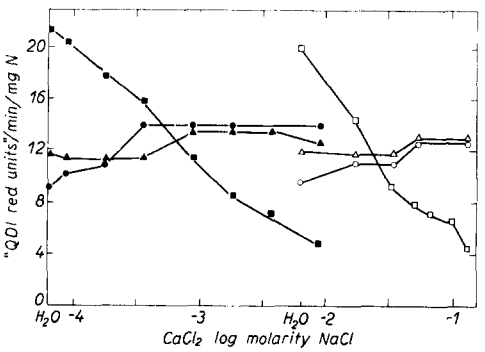

Fig. 7. Influence of $CaCl_2$ and NaCl, in presence and absence of added cytochrome *c*, on formation of "quinonediimonium red" by rat kidney mitochondria (21.6 μg of N), resuspended in 0.88 *M* sucrose solution, and by mitochondria first disintegrated in water and then brought to 0.88 *M* sucrose. Effect of $CaCl_2$ after addition of cytochrome *c* (▲), without addition of cytochrome *c* (●). Effect of NaCl after addition of cytochrome *c* (△), without addition of cytochrome *c* (○). Effects of $CaCl_2$ (■) and NaCl (□) after addition of cytochrome *c* to mitochondria disintegrated in distilled water and then brought to 0.88 *M* sucrose. Incubation for 1 minute at 37°; pH 7.1.

Blanks

As may be seen from Fig. 8, only small amounts of "quinonediimonium red" are formed by autoxidation of dimethyl-*p*-phenylenediamine during 3 minutes, the maximal incubation time used in this test. The autoxidation of the amine in most blanks or alcohol-inactivated samples was further decreased by traces of reducing substances contained in the cell material (see below). The reducing agents present in the cell extracts often caused a slight fading of the color on prolonged standing. This effect, as may be seen from the upper curve in Fig. 8, was negligible when the colorimeter readings were taken in the first few minutes after incubation. Stabilization against autoxidation was also attained by cooling the samples and blanks in ice after incubation and by diluting with ice-cooled water.

The slight cloudiness of mitochondrial suspensions caused only a small increase in the photometric densities. The turbidities of the samples and blanks after incubation were measured in the colorimeter after decolorizing the suspensions by one drop of ascorbic acid (1% solution). With mitochondrial suspensions, only insignificant differences in the degree of cloudiness between samples and blanks were observed. With some total homogenates in which the turbidity was considerable and the concentration of cytochrome oxidase relatively low (Table I), differences in the degree of turbidity of blanks and samples were corrected by using, for the calculation of the results, the turbidity of the sample (instead of the turbidity of the blank) as found after decolorization of the sample with ascorbic acid, together with the color of the blank as found from the colorimeter readings of the blank before and after decolorisation by ascorbic acid. A similar procedure was adopted when the test solutions contained natural pigments such as chlorophylls or carotinoids (Table I).

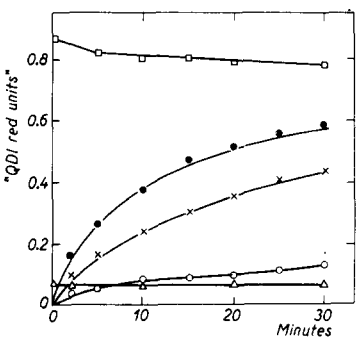

Fig. 8. Formation of "quinonediimonium red" by autoxidation in blanks, in presence and absence of cell extract. Reagents alone, diluted to 10 ml, at 37° (●), 22° (×) and 0° (○). Blank, diluted to 10 ml, containing alcohol-inactivated cell extract (26 μg mitochondrial N), at room temperature (△). Mitochondrial suspension (26 μg N), incubated for 1 minute at 37° and then inactivated by alcohol, diluted to 10 ml, at room temperature (□).

The routinely used, alcohol-inactivated blanks were often compared with blanks in which the enzyme was destroyed by 2 minutes heating at 70°. No significant differences were observed between both types of blanks.

Test of interference by oxidizing and reducing agents

Experiments were made in order to estimate the degree of interference by oxidizing agents other than cytochrome oxidase or reducing agents which might be present in test solutions.

Measurement of color formation in blanks in which cytochrome oxidase has been inhibited by NaN_3 or the enzymes have been destroyed by boiling permits differentiation between oxidizing enzymes not inhibited by NaN_3 and oxidizing agents of non-enzymic nature. Among several tissues which were tested for cytochrome oxidase (Table I), color formation

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in the blank by an unknown agent was observed only in the case of total homogenates from wheat embryos. The activity, amounting to 0.35 "quinonediimonium red" unit per minute per mg N, was not inhibited by NaN_3 or 33% alcohol but was destroyed by boiling.

Small amounts of reducing agents are present in all cell materials. In order to evaluate the potential error, the reducing capacities of the test solutions were estimated with dichlorobenzenone-indophenol. The fading of the blue dye was measured in the colorimeter for the first minute of incubation at 37° at pH 7.0 in the presence of added cytochrome c*. In the last column of Table I, the reducing capacities for different samples are indicated. By comparing the photometric density of the pigment produced by cytochrome oxidase to that decolorized by the reducing agents, it can be estimated that the potential errors were relatively small with the method but larger with the indophenol blue method. This is due to the fact that the formation of "quinonediimonium red" takes place at a much faster rate than that of indophenol blue.

The following experiment showed that interference by an excess of reducing agents such as ascorbic acid may be prevented by preliminary enzymic oxidation. If 20 to 100 μg of ascorbic acid was added to a mitochondrial suspension from rat kidney containing 18.3 μg of total N, the formation of the red pigment was delayed for 0.5 to 2.5 minutes and the delay was approximately proportional to the amount of added ascorbic acid. After oxidation of the ascorbic acid by the mitochondrial suspension, the color developed to the same extent as in the untreated sample. A similar lag in color formation was observed during determination of cytochrome oxidase in total homogenates from rat adrenals (Table I) known to be rich in ascorbic acid.

TABLE I

CYTOCHROME OXIDASE ACTIVITIES OF SOME ANIMAL AND PLANT TISSUES

	Cytochrome oxidase activity		*Reducing capacity*
	*"Quinonediimonium red" Units/min/mg N**	*Indophenol blue Units/min/mg N***	*"Chlorindophenol blue" Units/min/mg N****
Mitochondria, rat kidney	32.4	5.5	0.06
Mitochondria, rat liver	20.4	3.6	0.06
Heart muscle, rat, total homogenate	12.5	2.0	0.08
Kidney, rat, total homogenate	9.2	1.5	0.18
Brain, rat, total homogenate	8.3	1.4	0.20
Liver, rat, total homogenate	6.0	0.9	0.17
Adrenals, rat, total homogenate	5.8		0.95
Pancreas, rat, total homogenate	3.5		0.16
Wheat embryo, total homogenate	1.7		0.01
Carrot root, total homogenate§	1.3		0.05
Spinach leaf, total homogenate§	1.0		0.20
Potato tuber, total homogenate§	0.6		0.02

* 1 unit defined as photometric density of pigment from 0.12 mg of dimethyl-*p*-phenylenediamine hydrochloride; this density equals 0.75 under the conditions employed.

** 1 unit defined as photometric density of pigment from 0.10 mg of α-naphthol[1]. This density equals 1.5 under the conditions employed.

*** 1 unit defined as photometric density of 0.24 mg of sodium-2,6-dichlorobenzenone-indophenol; this density equals 1.5 under the conditions employed.

§ Press juice after grinding or grating. Most total homogenates were centrifuged shortly at low speed to separate tissue fragments, whole cells, starch grains, *etc.*

* 1 ml of 1/15 *M* phosphate buffer solution pH 7.0, 1 ml of a 0.04% cytochrome *c* solution, the sample containing 0.2 to 1.0 mg of N, and distilled water to make a final volume of 10 ml, were warmed to 37° directly in the colorimeter tube and held at 37° in a beaker close to the colorimeter. After addition of 0.4 ml of a 0.05% solution of sodium-2,6-dichlorobenzenone-indophenol and mixing, the change of the photometric density during 1 minute was observed in the Evelyn colorimeter, filter 540. One "chlorindophenol blue" unit was defined as the photometric density of a solution of 0.24 mg of sodium-2,6-dichlorobenzenone-indophenol in 10 ml of phosphate buffer pH 7.0. This density was 1.5 when measured with the Evelyn colorimeter, filter 540.

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Application of method to various tissues

The method was applied to fractions and total homogenates from diverse organs of the rat, and also from some plant tissues. Assays were also performed by the indophenol blue method and the results of both determinations compared.

The specificity of the enzymic color formation was evaluated by comparing the development of the pigment in the presence and absence of added cytochrome *c*, and also by the inhibition of the color formation by NaN_3. Since all preparations were resuspended or diluted with distilled water, most of the endogenous cytochrome *c* was washed out, resulting in weak activity. The enhancement by addition of cytochrome *c* was taken as an indication that the enzyme responsible for the development of the pigment was predominantly cytochrome oxidase. With the exception of total homogenates from wheat embryos, the color development of all samples was completely inhibited by $2 \cdot 10^{-3}$ *M* NaN_3. Since other oxidative enzymes such as polyphenoloxidase, present for example in potato tubers, are also inhibited by NaN_3, a potato juice was tested by substituting 3,4-dihydroxyphenyl-alanine ("dopa") for cytochrome *c* in the routine procedure. The formation of "quinonediimonium red" was proportional to the N content of the potato juice, and the activity was 2.4 "quinonediimonium red" units per minute per mg N, 4 times more than with cytochrome *c* as substrate (Table I). It may be concluded that this method can be applied to the determination of certain other oxidative enzymes by employing the appropriate substrates.

DISCUSSION

In the conventional method for cytochrome oxidase, the O_2-uptake during the oxidation of a reducing agent such as ascorbic acid by cytochrome *c* -cytochrome oxidase is measured in the Warburg apparatus. SLATER[4] has shown that the accuracy of the manometric analysis is much influenced by the state of the enzyme, the nature of the reducing agent and the concentration of added cytochrome *c* and buffer. The importance of these factors was also noted in the present colorimetric investigation. Cytochrome oxidase can also be determined by measuring the rate of oxidation of cytochrome *c* spectrophotometrically as described by COOPERSTEIN AND LAZAROW[5]. It may be expected that colorimetric methods, first introduced by SMITH AND STOTZ[6], will make the determination of cytochrome oxidase more simple and independent of a costly apparatus.

Errors may arise in all colorimetric methods for cytochrome oxidase through the interference by reducing agents from the tissues. Due to the rapid rate of formation of the quinonediimonium pigment by cytochrome oxidase, there seemed to be relatively little interference using this method (though more with the indophenol blue method) when the tissue samples were kept small (1 to 40 μg of N) and when cytochrome *c* was added in excess (Table I). The absence or the degree of interference by reducing agents can be readily determined by testing two or three different concentrations of the tissue suspensions and verifying that the formation of the pigment is proportional to the concentration of the samples.

The method may be modified by directly following the formation of the red pigment in the colorimeter and by recording the position of the shifting galvanometer needle every 10 or 15 seconds during a 1 to 3 minutes' period. The use of the spectrophotometer may increase the sensitivity.

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SUMMARY

A simple colorimetric method for the micro-determination of cytochrome oxidase, based on the formation of a water-soluble red pigment from dimethyl-*p*-phenylenediamine, is described. The formation of "quinonediimonium red" is proportional to the concentration of cytochrome oxidase (mitochondrial suspensions from the kidney of the rat containing 1 to 30 μg of N) and to the time of incubation during the first 1 to 3 minutes. The method was tried out with various animal and plant tissues.

RÉSUMÉ

Une méthode simple de microdosage colorimétrique de la cytochrome oxydase, fondée sur la formation d'un pigment rouge hydrosoluble à partir de diméthyl-*p*-phénylènediamine, est décrite. La formation de "rouge de quinonediimonium" est proportionnelle à la concentration de cytochrome oxydase (suspensions de mitochondries de rein du rat renfermant 1 à 30 μg de N) et au temps d'incubation au cours des 1 à 3 premières minutes. La méthode a été essayée sur divers tissus animaux et végétaux.

ZUSAMMENFASSUNG

Eine einfache kolorimetrische Methode zur Mikrobestimmung von Cytochrom-Oxydase, auf Grund der Bildung eines in Wasser löslichen roten Farbstoffes aus Dimethyl-*p*-Phenylendiamin, wird beschrieben. Die Bildung von "Quinondiimonium-Rot" geht proportional mit der Cytochrom-Oxydase-Konzentration (Mitochondrienaufschlemmung aus Rattenniere, mit 1–30 μg N-Gehalt) und mit der Inkubationszeit während der ersten drei Minuten. Die Methode wurde mit verschiedenen tierischen und pflanzlichen Geweben ausprobiert.

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