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New colorimetic methods for the estimation of cytochrome c oxidase and of cytochrome c-cyto-chrome oxidase system*

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Abstract

New colorimetric methods for the estimation of the activities of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system in tissue homogenates are described, using neotetrazolium chloride in the presence of p-phenylenediamine with or without additional cytochrome c. Optimal time of incubation, optimal concentration of the incubation medium and amounts of tissue, and simple method for the extraction of the reduced neotetrazolium chloride were determined. The reduction of neotetrazolium chloride was proportional to the amount of enzyme. Using this method, colorimetric estimations of cytochrome c-cytochrome oxidase system activity in the kidney, heart, liver, brains, and skeletal muscle were made. The procedures of these methods are very simple, and they are considered to be feasible in the combination with histochemical demonstrations of these enzyme activities.

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NEW COLORIMETIC METHODS FOR THE ESTIMATION OF CYTOCHROME C OXIDASE AND OF CYTOCHROME C-CYTOCHROME OXIDASE SYSTEM

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For the determination of cytochrome c oxidase activity, manometric method using the Warburg's apparatus or spectrophotometry of the absorption band reduced cytochrome c have generally been employed. However, there appears to be no ideal colorimetric method for the estimation of this enzyme. In this connection, STRAUS (1954)[1] described a method for the colorimetric microdetermination of cytochrome c oxidase using the Nadi reaction with some attendant difficulty, but this reaction, basing on the formation of indophenol blue from α-naphtol and dimethyl-*p*-phenylenediamine, takes place easily by autoxidation or by lipid peroxides even in the absence of the enzyme, and there are many descrepancies of opinions concerning the specificity of the reaction as a test for cytochrome oxidase.

In order to clarify the relationship of morphological structure and biochemical function of cells or tissues, it is desirable to establish an ideal integrating method for histochemical detection and biochemical determination of this enzyme, which is one of the most important enzymes for the respiration of cells. In the previous papers, we have analyzed the reaction of neotetrazolium chloride reduction by tissue homogenates by using various substrates and inhibitory agents, and clarified the conjugating steps of the reaction in the terminal electron transport system[2,3]. At the same time, we found that tissue homogenates reduced neotetrazolium chloride sensitively in the presence of *p*-phenylenediamine with or without additional cytochrome c and the reactions appeared to represent the activities of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system in the tissue, respectively. On the basis of these findings tests were made in order to learn whether these reactions would permit the quantitative microdeterminations of the enzyme and enzyme system.

MATERIALS AND METHODS

Fresh liver tissue homogenates of mice were mainly used as a source of enzyme for the test of methods, and applying these methods colorimetric

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estimation of the enzyme activities was made in the kidney, heart, liver, brain, and skeletal muscle of the same animals. As the details of the method for colorimetric estimation of the activity of succinoxidase system are available in the previous papers[3,4], brief descriptions will be given in the present paper. Incubation medium for cytochrome c-cytochrome oxidase system contained generally 0.2 ml. of 0.2 M *p*-phenylenediamine (*p*-PDA), 0.2 ml. of 0.2% neotetrazolium chloride (NT) (Fig. 1) (these were mixed

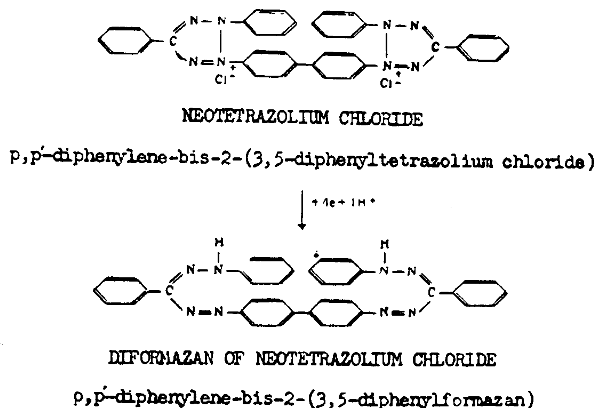

Fig. 1. Structural formulas of neotetrazolium chloride and its reduction product.

immediately before incubation), 0.2 ml. of tissue homogenate containing 20 or 40 mg of liver tissue in 0.1 M phosphate buffer (pH 7.6). For cytochrome c oxidase 0.2ml. of 10^{-4} M cytochrome c was added. The substrate control, i. e. endogenous dehydrogenase, 0.2 ml. of distilled water was used in place of *p*-PDA solution, and for enzyme control, 0.2 ml of 0.1M phosphate buffer was used in place of tissue homogenates.

The incubation was conducted at 37°C during 30 minutes. The reaction was stopped by the addition of 0.4 ml. of 1N sulfuric acid. When the reaction was stopped by adding sulfuric acid, the yellowish brown or dark brown oxidized product of *p*-PDA became insoluble in ether-acetone, while the reaction product of NT, diformazan, could be easily extracted with ether-acetone (1 : 1). The extracts were collected and the volume was read. The absorption spectrum of the diformazan in ether-acetone extract (Fig. 2) was written with the Beckman DK style of autorecording photoelectric spectrophotometer and the optical density was determined at the wave length of the maximum absorption, 520 mμ, with the photo-electric spectrophotometer (Shimazu Co. type OD-50).

The values of the optical densities were multiplied by the volumes of the extracts in ml., divided by 5, and thus the optical densities of the diformazan in 5 ml. of solvent were obtained. The results were expressed in the optical densities. If the amounts of neotetrazolium chloride reduced or oxygen consumption was required, the former was obtained from the standard curve of neotetrazolium chloride reduced and the later from the conversion of μM of the formazan to μM of oxygen in proportion of 1 : 1.

EXPERIMENTAL

The absorption spectra and standard curves of neotetrazolium diformazan : Fig. 2 indicates the identity of absorption spectra of NT diformazan obtained by the enzymatic reduction and by chemical reduction with sodium hydrosulfite, showing maximum absorption at 520 mμ. The optical densities of the extracts obtained by gradual dilution of an original extract of diformazan keep parallel relationship with the concentration (Fig. 3). The optical densities of diformazan extract obtained from

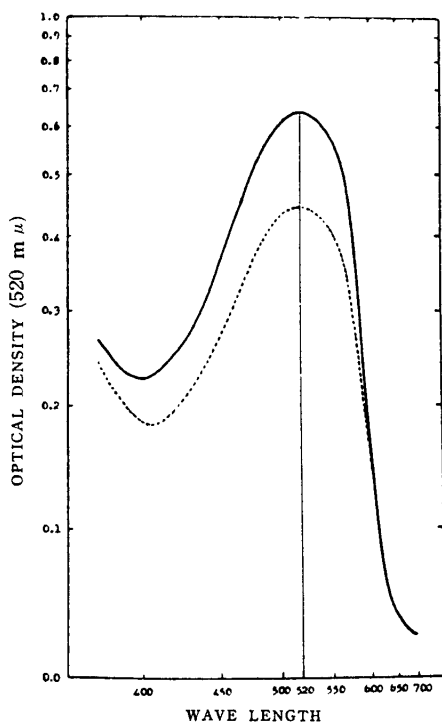

Fig. 2. Absorption spectra of neotetrazolium diformazan obtained by chemical reduction with sodium hydrosulfite, a solid line, and by enzymatic reduction, a dotted line.

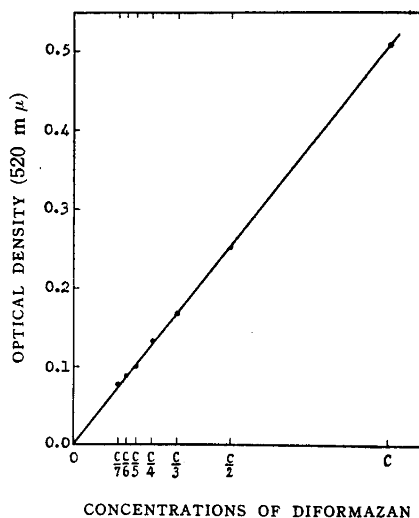

Fig. 3. Straight line relationship between the concentration of diformazan and optical density.

different amounts of NT by chemical reduction with sodium hydrosulfite are also proportional to the amount of NT used (Fig. 4.). From such a standard curve the activity of the enzyme can be expressed in the amount of NT. The extract are stable at least during 12 to 24 hours in room light and in room temperature, but it is preferable to preserve in dark and at a lower temperature.

Time of incubation : As Fig. 5 shows, the rate of NT reduction by

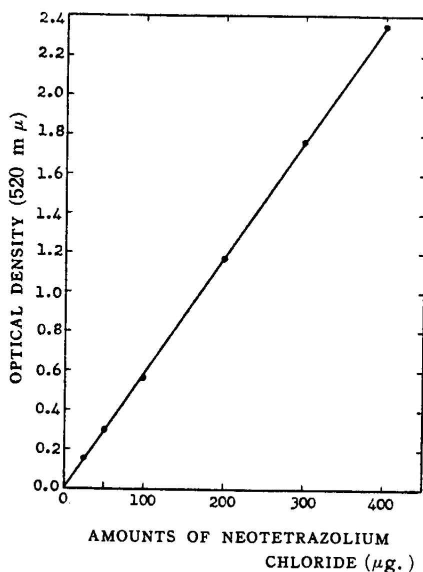

Fig. 4. Optical density of diformazan obtained by the reduction of various amounts of neotetrazolium chloride with sodium hydrosulfite.

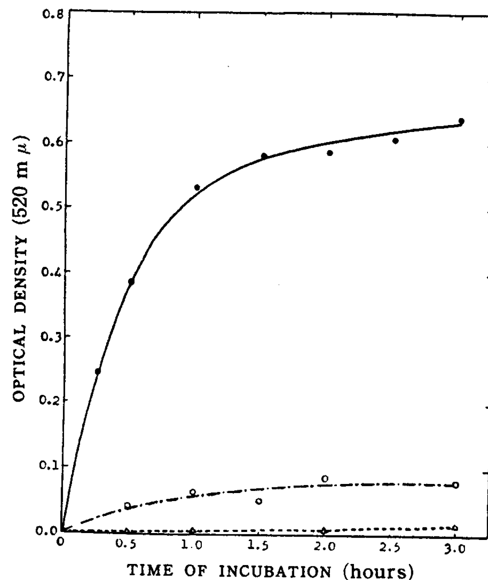

Fig. 5. Time curve for the rate of reduction of neotetrazolium by the liver tissue homogenates using *p*-phenylenediamine as substrate.
●——● ; cytochrome c-cytochrome oxidase system activity, in the presence of *p*-phenylenediamine. ○——○ ; endogenous dehydrogenase activity, in the absence of *p*-phenylenediamine. △——△ ; combinations of neoteterazolium chloride and *p*-phenylenediamine without tissue homogenate.

tissue homogenate using *p*-PDA as substrate increased almost proportionally with the time of incubation during the first 30 minutes, then the rate declined gradually. The rate of NT reduction by tissue homogenates without additional substrates, i. e. endogenous dehydrogenase, was very low and did not show parallel relationship with time of incubation. In the absence of tissue, the reduction of NT hardly occurred during 30 minutes

and imperceptively after 1 to 3 hours. From these data 30 minutes was decided as the optimal time of incubation.

The influence of variation of p-phenylenediamine concentration : When *p*-PDA was used at the concentration of 0.05 to 0.1 M, the optical densities of reduced NT were in the range of 0.50 to 0.55, and at the concentration below 0.01 M the densities decreased markedly.

The influences of variation in NT conentration, phosphate buffer concentration, and in pH: As they were scrutinized in the succinoxidase reaction in the previous papers[3,4], this point is omitted in the present paper.

The influences of cytochrome c added : The NT reduction using *p*-PDA as substrate was accelerated by the addition of cytochrome c in parallel with its concentration from 2.5×10^{-7} M to 10^{-5} M, reaching the plateau at the concentration of 2.5×10^{-5} M as presented in the previous paper[2].

Relationship between the amounts of tissue and the rate of reduction of NT: As Fig. 6 shows, the rate of reduction of NT was linear

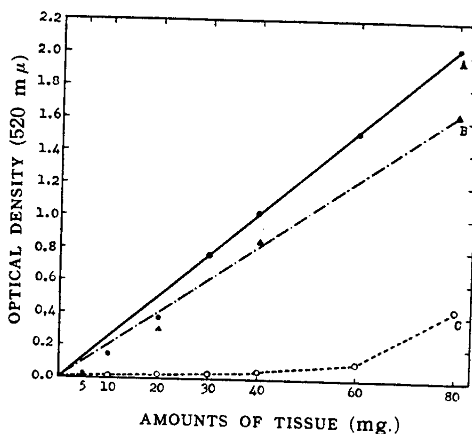

Fig. 6. Relationship between the amounts of tissue and the activities of cytochrome c oxidase, cytochrome c-cytochrome oxidase system and endogenous dehydrogenase expressed in optical density of reduced neotetrazolium using *p*-phenylenediamine and cytochrome c (A), *p*-phenylene diamine (B) as substrates and no additional substrate (C), respectively.

with the amounts of liver tissue between 30 and 80 mg. in the presence of *p*-PDA with or whithout additional cytochrome c, and both curves passed through the origin, although the rate was not propotional to the amounts of tissue below 20 mg. The rate of NT reduction by tissue homogenates in the absence of *p*-PDA, i. e. endogenous dehydrogenase, was very low when the tissue was used in the amount below 60 mg., and

then it increased rather markedly without parallel relationship with the amount of tissue. Therefore, the adequate amount of liver tissue of the mice in this reaction using *p*-PDA will be about 40 mg. In addition, the rate of endogenous dehydrogenase activity showed marked variation according to the conditions of mice used.

The effects of malonate, cyanide, antimycin A, formalin, and heat: As presented in the previous paper[2], the reduction of NT by tissue homogenates using *p*-PDA as substrate was neither inhibited by the addition of 0.025 M sodium malonate nor by 0.1 r to 100 r of antimycin A. On the other hand, it was abolished almost completely by cyanide at the concentration of 10^{-5}M to 10^{-3}M when the value of endogenous dehydrogenase reaction were deducted from those of the residual reaction. The NT reduction by tissue homogenates was also completely abolished by heating the homogenates for 5 minutes at 80 C and by the addition of formalin solution to the concentration of 5 or 10 per cent. In the absence of tissue, NT reduction has hardly occurred by *p*-PDA only or by the combination of *p*-PDA and cytochrome c.

Comparison of cytochrome c-cytochrome oxidase sytsem activity in various tissues: Fig. 7 shows the rate of reduction of NT by five kinds of

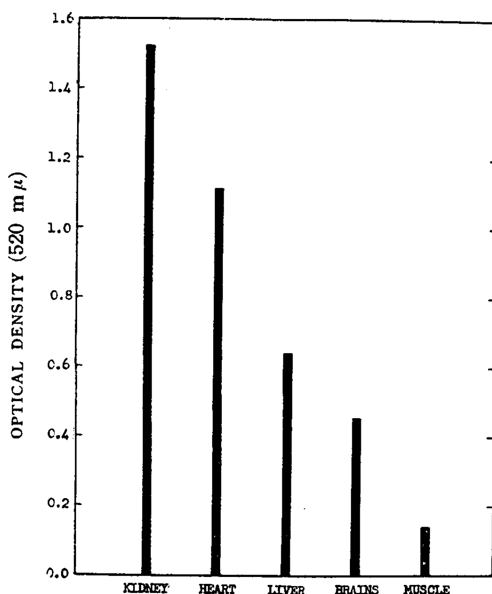

Fig. 7. Comparison of the activities of cytochrome c-cytochrome oxidase system in various tissues expressed in optical densities of reduced neotetrazolium.

Forty mg. of tissue was used in each. Endogenous dehydrogenase activities are deducted.

tissue of mice in the presence of *p*-PDA, deducted the value of those in the absence of *p*-PDA, respectively. The rate stands in order of the kidney (in the combinations of the cortex and medulla), heart, liver, brains (in the combinations of the cortex and medulla of the large and small brains), and skeletal muscle (*m. quadriceps femoris*). It is interesting that succinic dehydrogenase system activity is higher in the heart than in the kidney while cytochrome c-cytochrome oxidase system and DPNH dehydrogenase activities are higher in the kidney than in the heart[8].

DISCUSSION

From the results described previously, the reduction of NT by tissue homogenates in the presence of *p*-PDA without additional cytochrome c will be explained as follows : *p*-PDA reduces endogenous cytochrome c and the reduced cytochrome c subsequently reduces NT by the action of cytochrome c oxidase, in other words the reaction is responsible for the activity of cytochrome c-cytochrome oxidase system (Fig. 8). The NT

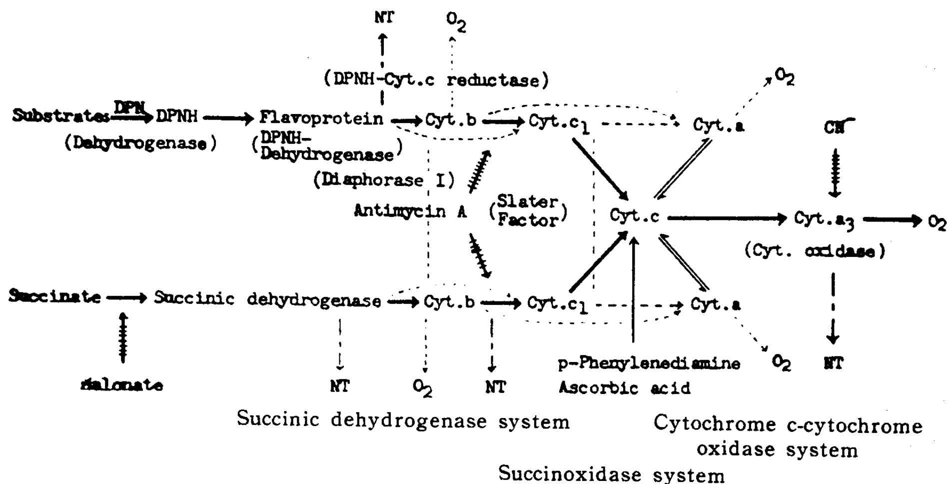

Fig. 8. The reduction of neotetrazolium chloride (NT) by the terminal electron transport system.

reduction in the presence of *p*-PDA was accelerated by the addition of cytochrome c in parallel with its concentration from 2.5×10^{-7} M to 10^{-5} M, reaching the plateau at the concentration of 2.5×10^{-5} M. Therefore, in the presence of or by the addition of sufficient amount (over 2.5×10^{-5} M) of cytochrome c, the reaction is dependent on cytochrome c oxidase activity. The NT reduction in both reactions are considered to take place

conjugating at cytochrome c oxidase level. Furthermore, as the rate of reduction of NT is linear with the amounts of tissue in the presence of *p*-PDA both with and without additional cytochrome c, and as the curves pass through the origin, it is considered that the present methods will permit the quantitative micro-determinations of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system, respectively. In these methods NT may be substituted by other tetrazolium salts, i. e. TPT, INT or BT, though the rates of reaction conjugating to various steps in the terminal electron transport system may be different. Moreover, applying these reactions we could establish a method for histochemical and cytochemical demostration of these enzyme activities, which will be reported in the separate paper[5]. In these reactions, *p*-PDA could be replaced by ascorbic acid but the rate of reaction using ascorbic acid was lower than that using *p*-PDA. Anaerobic condition accelerated the rate of NT reduction. When the amount of tissue was decreased markedly comparing with the volume of incubating medium, NT reduction decreased markedly keeping no parallel relationship with the amount of tissue. For the reason of this phenomenon, many factors must be considered, especially endogenous cytocrome c and some other co-factors participating in the reaction may be dissociated or diluted in such condition as discussed in the previous paper[2]. Therefore, it is necessary to use adequate amount of tissue according to the kind of and enzyme activity of each tissue for the estimations of these enzyme activities. Concerning these problems, detailed descriptions are available in our previous papers[3,4].

SUMMARY

New colorimetric methods for the estimation of the activities of cytochrome c oxidase and of cytochrome c-cytochrome oxidase system in tissue homogenates are described, using neotetrazolium chloride in the presence of *p*-phenylenediamine with or without additional cytochrome c. Optimal time of incubation, optimal concentration of the incubation medium and amounts of tissue, and simple method for the extraction of the reduced neotetrazolium chloride were determined. The reduction of neotetrazolium chloride was proportional to the amount of enzyme. Using this method, colorimetric estimations of cytochrome c-cytochrome oxidase system activity in the kidney, heart, liver, brains, and skeletal muscle were made. The procedures of these methods are very simple, and they are considered to be feasible in the combination with histochemical demonstrations of these enzyme activities.

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