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An analytical study on the reduction of neotetrazolium chloride by the terminal electron transport system

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Abstract

In order to determine the steps with which the reaction of neotetrazolium chloride reduction conjugates in the terminal electron transport system, an analytical study on the neotetrazolium reduction by tiue homogenates was carried out using various substrates such as sodium succinate, p-phenylenediamine, sodium malate, sodium α-glutamate and DPN, and inhibitors such as sodium malonate, potaium cyanide and antimycin A, as the results the following conclusions were drawn. 1. The reaction of neotetrazolium reduction by tiue homogenate using sodium succinate as substrate is mainly the succinoxidase system reaction; and the reaction takes place conjugating about 50 per cent in the step of the succinic dehydrogenase system (succinic dehydrogease, cytochrome b and cytochrome C1), of these about 15 per cent conjugates in the step prior to the antimycin A sensitive step and 35 per cent in the step itself; and about 50 per cent in the step of cytochrome c oxidase. 2. In the case using p-phenylenediamine as substrate the reaction of neotetrazolium reduction is the reaction due to the activity of cytochrome c-cytochrome oxidase system; and when p-phenylenediamine is used with the sufficient amount of cytochrome c, the reaction appears to be dependent on cytochrome c oxidase activity. Neotetrazolium reduction in all these reactions takes place conjugating in the step of cytochrome c oxidase. 3. In the case where DPN and substrates taking DPN as a coenzyme are used, the reaction of neotetrazolium reduction is mainly the reaction conjugating at the step below antimycin A sensitive step in the DPNHcytochrome c reductase system (flavoprotein, cytochrome b and cytochrome c;), probably with the flavoprotein of DPNH-dehydrogenase. 4. Endogenous dehydrogenase reactions are the sum total reactions conjugating at the steps prior to the antimycin A sensitive step in the terminal electron transport system and with other various reduction systems which are not inhibited by antimycin A.

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AN ANALYTICAL STUDY ON THE REDUCTION OF NEOTETRAZOLIUM CHLORIDE BY THE TERMINAL ELECTRON TRANSPORT SYSTEM

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The use of tetrazolium salts for the histochemical demonstration[1] or colorimetric estimation[2] of the activity of dehydrogenase systems has been extended widely in recent years.[3–11] However, there still remain many unsolved problems as regards the mechanism of reduction of tetrazolium salts by living tissues, especially the conjugating step in the oxido-reduction systems and co-factors required for the reaction. Furthermore, for the colorimetric estimation of succinic dehydrogenase activity with the use of neotetrazolium chloride we had encountered various difficulties and discrepancies[12–13] in spite of the exellence in bringing about the reaction. Therefore, for the purpose of solving these problems, we have previously scrutinized the reaction of neotetrazolium chloride reduction by tissue homogenate with sodium succinate as substrate by altering conditions of various factors involved in the reaction and extracting solvents for the reduction product and we have established a method simple and accurate for the estimation of the succinoxidase system activity.[14–16] It is the purpose of this paper to analyze the reaction of neotetrazolium chloride reduction by tissue homogenate by using various substrates and inhibitory agents in order to clarify the conjugating steps of the reaction in the terminal electron transport system.

MATERIALS AND METHODS

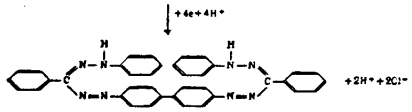
Liver and kidney tissues of the mice were used as the source of enzyme. General method for the analysis of neotetrazolium chloride (NT) reduction by succinoxidase system was as follows.[14,15] Incubation medium contained 0.2 ml. of 0.2 M sodium succinate, 0.2 ml. of 0.2 per cent neotetrazolium chloride, 0.2 ml. of tissue homogenate containing 5 or 10 mg. of liver tissue in 0.1 M phosphate buffer (pH 7.6), and 0.2 ml. of activator or inhibitor, whose conditions will be described in each item. The incubation was carried out at 37°C during 30 minutes. The reaction was stopped by

the addition of 0.4 ml. of 20 per cent formalin solution. The reduction product, diformazan, was extracted by ether-acetone (1 : 1). The optical density was determined at 520 mμ by Beckman's spectrophotometer. As the substrate control, 0.2 ml. of distilled water was used in place of the succinate solution.

As the method for cytochrome c-cytochrome oxidase system[15], 0.2M *p*-phenylenediamine (*p*-PDA) was used in place of sodium succinate and 20 mg. of tissue was used, and the reaction was stopped by the addition of 0.4 ml. of 1 N sulfuric acid in place of formalin solution in the previously described procedure.

For the method for diphosphopyridine nucleotide (DPNH) dehydrogenase system[7, 15], incubation medium contained 0.15 ml. of 0.5 M sodium malate, 0, 25 ml. of 0.5 M sodium α-glutamate, 0.10 ml. of 0.45% DPN, 0.25 ml. of 0.03 M potassium cyanide adjusted at pH 7.6 with monobasic sodium phosphate, 0,35 ml. of 0.2% neotetrazolium chloride, 0.35 ml. of tissue homogenate containing 50 mg. of kidney tissue per 1 ml. of 0.1 M phosphate buffer (pH 7.6), and distilled water to make a final volume 1.5 ml. As the control, each or all of malate, α-glutamate, DPN was omitted from the incubating media. The other procedures were the same as those in the succinoxidase reaction. The structural formulas of neotetrazolium chloride and its reduction product are presented in Fig. 1.

NEOTETRAZOLIUM CHLORIDE
p, p'-diphenylene-bis-2- (3, 5-diphenyltetrazolium chloride)



DIFORMAZAN OF NEOTETRAZOLIUM CHLORIDE
p, p'-diphenylene-bis-2 (3, 5-diphenylformazan)

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

EXPERIMENTALS

NT reduction by tissue homogenate with p-phenylenediamine : Tissue homogenate was found to reduce NT sensitively in the presence of *p*-phenylenediamine (*p*-PDA) as substrate, even when kept under the condition where the tissue homogenate received no additional substrate, i. d.

endogenous dehydrogenase, hardly reduced NT. In the absence of tissue, NT reduction has never occurred by *p*-PDA alone or even by the combination of p-PDA and cytochrome c. Therefore, the NT reduction by tissue in the presence of *p*-PDA may be explained as follows : endogenous cytochrome c was reduced by *p*-PDA and the reduced cytochrome c subsequently reduced NT by the action of cytochrome c oxidase in the tissue[17], in other words, the reaction may represent the activity of cytochrome c-cytochrome oxidase system. When the reaction was stopped by adding sulfuric acid, the yellowish brown or dark brown oxidized products of *p*-PDA became unsoluble in ether-acetone while the reduction product of NT, diformazan, could be easily extracted with ether-acetone. The absorption curves of the extract obtained by using Beckman DK type of autorecording spectrophotometer were the same as those in the case where sodium succinate was used as substrate, showing the maximum absorption at 520 mμ of wave length (Fig. 2). Hence, the optical density of the diformazan in ether-acetone extract was determined at 520 mμ with the spectrophotometer. Ascorbic acid also gave similar reaction but the rate of reaction was lower than that in the case of *p*-PDA.

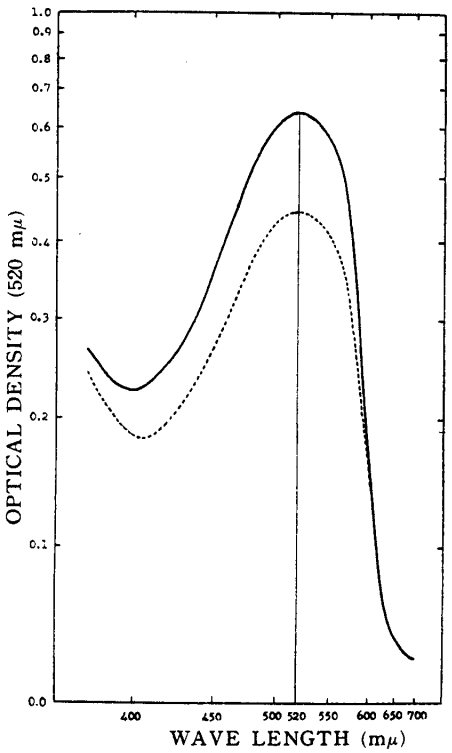


Fig. 2. Absorption spectra of neotetrazolium diformazan obtained by chemical reduction with sodium hydrosulfite, a solid line, and by enzymatic reduction, a dotted line.

Inhibitory effect of malonate on the reaction of NT reduction : The reaction using sodium succinate as substrate was completely inhibited by the addition of 0.025 M sodium malonate, the value of endogenous dehydrogenase reaction being deducted from the total value (Fig. 3). Therefore, it is clear that the reaction in this case took place through the action of succinic dehydrogenase, irrespective of whether the NT reduction occurs directly conjugating with succinic dehydrogenase itself or not. On the other hand, the reaction using *p*-PDA as substrate was not inhibited by so-

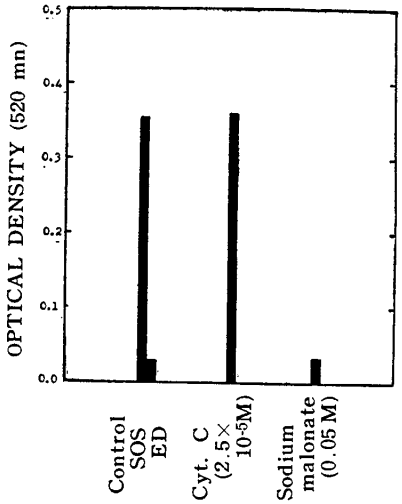


Fig. 3. Effect of sodium malonate and cytochrome c on the succinoxi dase system activity expressed in optical density of reduced neotetrazolium using sodium succinate as substrate. In 5 mg. of liver tissue.

dium malonate, showing no participation of succinic dehydrogenase in the reaction.

The effect of cytochrome c : By the addition of cytochrome c to the concentration of 2.5×10^{-5}M, the NT reduction by endogenous dehydrogenase was slightly inhibited, which might mean the reaction took place conjugating with the reduction systems lower than cytochrome c. The NT reduction using succinate as substrate was hardly effected or slightly accelerated (Fig. 3). However, the NT reduction using *p*-PDA as substrate was accelerated by 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 (Fig. 4). Hence, it is clear that cytochrome c participates in this reaction using *p*-PDA, but in the presence of a sufficient

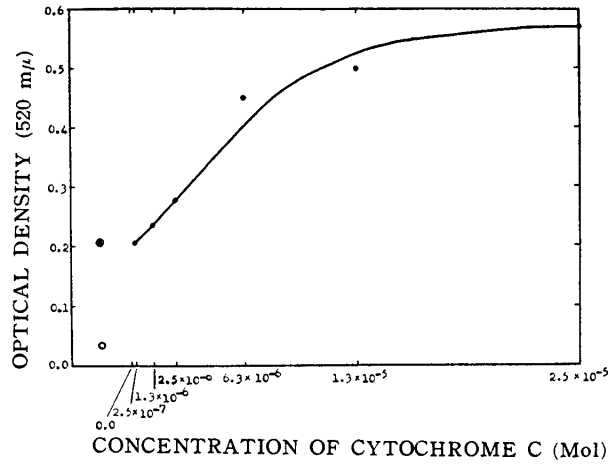


Fig. 4. Effect of cytechrome c concentration on cytochrome c oxidase activity expressed in optical density of reduced neotetrazolium using *p*-phenylenediamine as a substrate.
Amount of tissue : 20 mg. ⊙ : omitted cytochrome c.
○ : omitted *p*-phenylenedianine

amount of cytochrome c the rate of the reaction may be subjected to the activity of cytochrome c oxidase in the tissue[15].

The effect of cyanide inhibition : The NT reduction using sodium succinate as substrate was inhibited about 50 per cent by potassium cyanide at the concentration of 10^{-2}M～10^{-5}M bufferized with monobasic sodium phosphate at pH 7.6 (Fig. 5). Therefore, about one half of the reaction by sodium succinate seems to take place

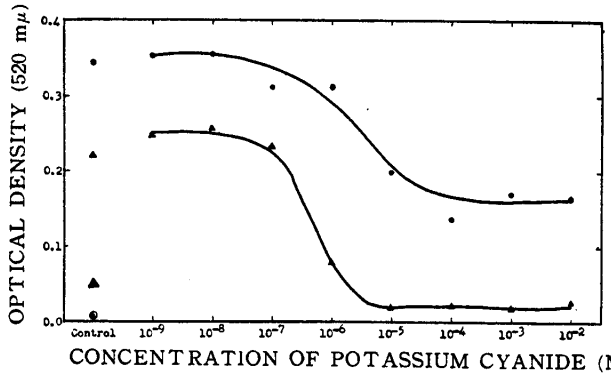


Fig. 5. Effect of potassium cyanide concentration on the reduction of neotetrazolium chloride by the succinoxidase system (A, upper curve) and cytochrome c cytochrome oxidase system (B, lower curve) activities.

In A, used 5 mg. of tissues and sodium succinate as a substrate. In B, used 20 mg. of tissues and *p*-phenylenediamine as a substrate. The marks ⊙ and △ are the values in the absence of succinate and *p*-PDA, respectively.

conjugating at the step below cytochrome c, namely, the succinic dehydrogenase system[18,19], and the other half conjugating at cytochrome oxidase level. On the other hand, the NT reduction by *p*-PDA was inhibited about 76 per cent by cyanide at the concentration of 10^{-6}M, and 84 per cent at 10^{-5}M～10^{-3}M (Fig. 5). When the value of endogenous dehydrogenase reaction is subtracted from the value of the residual reaction, most of the NT reduction by *p*-PDA is considered to be abolished, showing the reaction to take place conjugating at cytochrome oxidase level. The NT reduction by DPNH-dehydrogenase system took place markedly when we used the combination of DPN and substrates, such as sodium malate and sodium *a*-glutamate, and slightly in the absence of each one of them as shown in Fig. 6. In all of these cases, the reaction was accelerated markedly by the addition of cyanide ; the DPNH-dehydrogenase system reaction using the combination of DPN, *Na*-malate and

Na-α-glutamate was accelerated 32 per cent and endogenous dehydrogenase system reaction was accelerated 66 per cent.

Inhibitory effect of antimycin A : Purified crystalline antimycin A donated by Dr. D. E. Green was used as ethanol solutions containing 0.1γ, 1.0γ, 10γ, and 100γ per 0.02 ml. of 95% alcohol. The solution was added to tissue homogenate at such a concentration of alcohol as it would not excess 5 per cent of homogenate. After mingling the homogenate sufficiently, pre-incubation was carried out for 10 minutes. In the control homogenate, an equivalent amount of alcohol or water was added, and it was confirmed that there was no inhibitory effect of alcohol on the enzyme activity. As shown in Fig. 7, the NT reduction by liver tissue homogenate in the presence of succinate as substrate was inhibited about 50 per cent by 0.1 γ of antimycin A and 83 per cent by 1 γ, and 87 per cent by 100 γ. Thus, about 15 per cent of this reaction are considered to take place conjugating at the step below antimycin A sensitive step, and about 85 per cent conjugating at the step above antimycin A sensitive step inclusive of this step. The NT reduction by tissue homogenate in the presence of *p*-PDA as substrate was

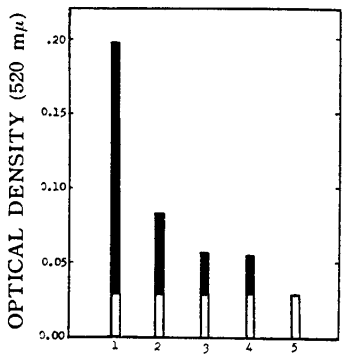


Fig. 6, Colorimetric estimations of "DPN diaphorase activity" of kidney tissue by the method presented in the text.
1. DPN diaphorase activity in the presnce of all compositions presented in the text. 2. (1)-DPN. 3. (1)-glutamate. 4. (1)-malate. 5. endogenous dehydrogenase.

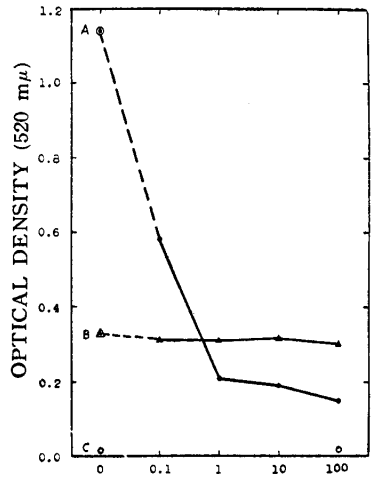


Fig. 7. Inhibitory effect of antimycin A on the reduction of neotetrazolium chloride by tissue homogenates using sodium succinate (A) and *p*-phenylenediamine (B) as substrates and no additional substrate (C), respectively.
Weight of tissue 20mg, 37°C, 30min.

hardly inhibited by antimycin A. The NT reduction by endogenous dehydrogenase systems or by the DPNH-dehydrogenase system mentioned above has never been inhibited but accelerated slightly by antimycin A.

DISCUSSION

It is the established fact that antimycin A blocks selectively the linkage between cytochrome b and cytochrome c[20], probably by binding with heme of cytochrome b or cytochrome c_1[21], resulting in a complete break-down of the succinoxidase system[20,21]. Therefore, putting all the data presented above together, in the liver tissue homogenate in phosphate buffer in the presence of succinate as substrate, NT appears to be reduced about 15 per cent conjugating at the step of succinic dehydrogenase, about 35 per cent at the step of cytochrome b or c_1, and 50 per cent at cytochrome oxidase level of the terminal electron transport system. The reaction of NT reduction in the presence of *p*-PDA as substrate is the reaction conjugating at the step of cytochrome oxidase (Fig. 8). In the case of the combination of DPN and substrates mentioned previously,

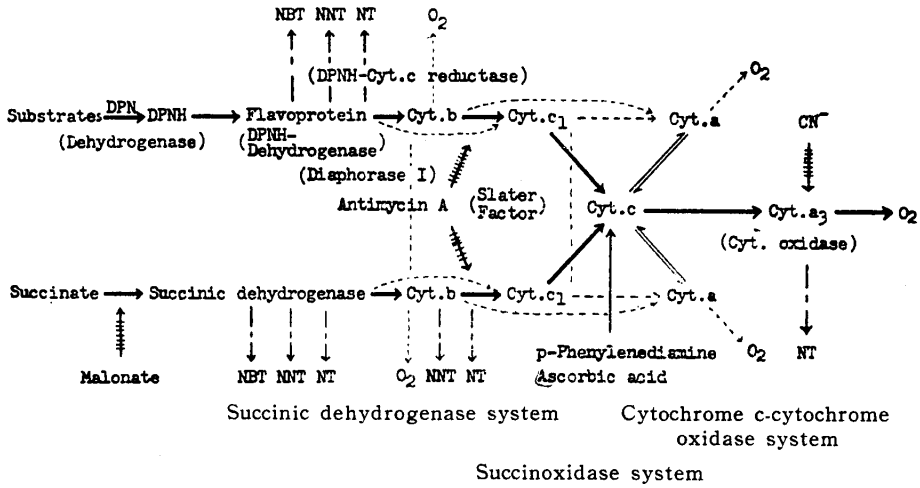


Fig. 8. The reduction of neotetrazolium chloride (NT), nitro-neotetrazolium chloride (NNT), and nitro-blue tetrazolium chloride (NBT) by the terminal electron transport system.

the reaction of NT reduction is the reaction conjugating at the step below antimycin A sensitive step, in all likelihood mainly with flavoprotein in DPNH-cytochrome c reductase system. Endogenous dehydrogenase reactions are not inhibited by antimycin A, and therefore they are the sum

total reactions coupling with the step prior to antimycin A-sensitive step in the terminal electron transport system and with other various reduction systems which are not inhibited by antimycin A. (DPNH-cytochrome c reductase has been confirmed to be concentrated both in mitochondria and in microsomes[22-24] and there are indications that there exist two forms of the mitochondrial DPNH-cytochrome c reductase : an "external" one acting on exogenous DPNH and an "internal" one acting on the DPNH generated by the oxidations of substrates whose enzymes require DPN as the coenzyme[25,26]. The "internal" enzyme is inhibited by antimycin A but the microsomal and "external" mitochondrial enzymes are not inhibited by antimycin A.[24,26,27,28])

Concerning cytochrome c oxidase, there has been descrepancy of opinions whether it is responsible for cytochrome a_3 or for the combinations of oxidized cytochrome a and cytochrome c[29]. Apart from this problem, (on the basis of the findings mentioned previously) we could establish new methods for histochemical demonstration and colorimetric estimation of the activities of cytochrome c oxidase and cytochrome c-cytochrome oxidase system by using NT. The details will be reported in separated papers.[15,16]

On the other hand, when the amount of tissue was decreased markedly comparing with the volume of incubating medium or washed mitochondria in hypotonic solution were used for quantitative estimation of the activity of succinoxidase system, cytochrome c oxidase, and DPNH dehydrogenase system, NT reduction decreased markedly keeping no parallel relationship with the amount of tissue or mitochondria[14-15]. In connection with these phenomena, MARTIN et al[30]. and SUGIMURA[31] reported the requirement of co-factors, coenzyme A or a certain protein component and unknown coenzyme, for triphenyl tetrazolium chloride (TTC) reduction by succinoxidase complex from tuberculous guinea-pig kidney or from pigeon breast muscle mitochondria. WALDSCHMIDT-LEITZ[32] reported that diluted mouse liver homogenate could not reduce TTC with succinate but the reducing activity was restored with addition of boiled extract of liver or DPN, and he suggested the dissociation of enzyme-coenzyme system. BRIL[33] also considered DPN as the co-factor, and HOCH et al[34]. described that TTC reduction of rat liver mitochondria with succinate was enhanced by the addition of ATP. Recently KAMIN et al. (1957)[35] stated that a heat-labile factor ("KS") in guinea-pig or beef kidney water homogenates, sedimentable between 4,000 and 12,000 $\times$ g, which did not itself catalyze NT reduction, stimulated NT reduction by succinate in dialyzed whole homogenates of normal guinea-pig kidney,

and that the factor could be replaced by cytochrome c. They stated also that NT reduction by succinate, and KS or cytochrome c stimulation, was abolished by antimycin A and cyanide, and suggested that NT appeared to be reduced at the cytochrome oxidase level in guinea-pig kidney water homogenates in the presence of succinate. We used mouse liver homogenate in phosphate buffer, and our data agree, in some part, with their data. However, the NT reduction by fresh tissue or tissue homogenates appears to be complicated, and it seems many co-factors to participate in the reaction. Furthermore, the conjugating step of NT reduction with succinoxidase system by tissue homogenates in phosphate buffer is not single, and the steps and rates of conjugation with each step in the terminal electron transport system are considered to be determined by the present study. Moreover, we confirmed that the mechanisms and conjugating steps of various tetrazolium salt reductions are not uniform but differ according to each compound[16]. For example, nitro-blue tetrazolium chloride reduction takes place coupling to succinic dehydrogenase level[8,16] and nitro-neotetrazolium chloride to the succinic dehydrogenase system, of which about half is coupled to succinic dehydrogenase and another half to cytochrome b or c_1 level[16], and NT to various levels in the succinoxidase system mentioned previously. Although most of ditetrazolium salts including NT contain monotetrazolium salts in some proportions[13,14] and the conjugating steps of the mono- and di-tetrazolium might differ to some extent, the absorption curves of reduced NT showed the identical curves both by using succinate and by using *p*-PDA as substrates. Therefore, the multiplicity of conjugating steps is considered to be due not to the impurity[13] of the compound but rather manly to the specificity and variety of electron transportation in the terminal electron transport system.

SUMMARY

In order to determine the steps with which the reaction of neotetrazolium chloride reduction conjugates in the terminal electron transport system, an analytical study on the neotetrazolium reduction by tissue homogenates was carried out using various substrates such as sodium succinate, *p*-phenylenediamine, sodium malate, sodium *a*-glutamate and DPN, and inhibitors such as sodium malonate, potassium cyanide and antimycin A, as the results the following conclusions were drawn.

1. The reaction of neotetrazolium reduction by tissue homogenate using sodium succinate as substrate is mainly the succinoxidase system

reaction ; and the reaction takes place conjugating about 50 per cent in the step of the succinic dehydrogenase system (succinic dehydrogease, cytochrome b and cytochrome c_1), of thcse about 15 per cent conjugates in the step prior to the antimycin A sensitive step and 35 per cent in the step itself ; and about 50 per cent in the step of cytochrome c oxidase.

2. In the case using *p*-phenylenediamine as substrate the reaction of neotetrazolium reduction is the reaction due to the activity of cytochrome c-cytochrome oxidase system; and when *p*-phenylenediamine is used with the sufficient amount of cytochrome c, the reaction appears to be dependent on cytochrome c oxidase activity. Neotetrazolium reduction in all these reactions takes place conjugating in the step of cytochrome c oxidase.

3. In the case where DPN and substrates taking DPN as a coenzyme are used, the reaction of neotetrazolium reduction is mainly the reaction conjugating at the step below antimycin A sensitive step in the DPNH-cytochrome c reductase system (flavoprotein, cytochrome b and cytochrome c_1), probably with the flavoprotein of DPNH-dehydrogenase.

4. Endogenous dehydrogenase reactions are the sum total reactions conjugating at the steps prior to the antimycin A sensitive step in the terminal electron transport system and with other various reduction systems which are not inhibited by antimycin A.

Acknowledgement is due to Prof. D. E. Green of University of Wisconsin for kindly supplying antimycin A.

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