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The Reduction of Tetrazolium Salts by Plant Mitochondria

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Summary. Data has been obtained concerning the reduction of tetrazolium salts by mitochondria isolated from Jerusalem artichoke tubers with succinate as the substrate using a direct recording spectrophotometric method of assay. ATP was found to increase the rate of reduction of the tetrazolium salts, this being independent of the effect ATP had on the rate of oxygen uptake. The magnitude of the stimulation by ATP depended on the concentration of tetrazolium salts present and under certain circumstances was suppressed by the addition of azide and cyanide. The sites at which the tetrazolium salts were reduced along the electron transport chain were investigated. The role of ATP has been discussed in relation to the mechanism of tetrazolium reduction.

Tetrazolium salts are readily reduced, by various dehydrogenase systems, to the insoluble highly coloured formazan and have been widely used as reagents for the histochemical localization of enzymes in sections of animal and plant tissue (PEARSE, 1960). The behaviour of the various tetrazolium salts in animal tissue has been widely studied and considerable information exists to show at what point in the electron transport chain the tetrazolium salts are reduced (SKELTON *et al.*, 1957; ODA *et al.*, 1958; KAMIN *et al.*, 1957; SLATER *et al.*, 1963; LESTER and SMITH, 1961; and NACHLAS *et al.*, 1960). No such information is available from studies using plant tissue.

Recently it has been shown that in both animal (SATO and SATO, 1965; CLARK *et al.*, 1965) and plant tissue (PALMER and KALINA, 1966) tetrazolium salts both uncouple phosphorylation from electron transport and block the flow of electrons from NAD^+ linked substrates to cytochrome b. When studying the reduction of tetrazolium salts by mitochondria it is therefore essential to use succinate as the electron donor. The studies concerning the bahaviour of tetrazolium salts in plant mitochondria, described in this paper, can be divided into two groups (1) factors affecting the rate of reduction of the tetrazolium salts and (2) the sites of reduction of the tetrazolium salts by the succinate-tetrazolium reductase system.

Materials and Methods

The tetrazolium salts[1] (TTC, BT, NT, MTT, INT, NBT, TNBT) and ADP, ATP were obtained from Sigma Chemicals.

The Jerusalem artichoke tubers were supplied by the Biological Supply Unit of the University of London and were washed and then stored at 4°C before use.

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[1] *Abbreviations:* TTC = 2,3,5-triphenyl-2,1,3,4-tetrazolium chloride; BT = 5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium chloride; NT = 2,2,5,5′-tetraphenyl-3,3′-(p-diphenylene)-ditetrazolium chloride; MTT = 3-(4,5′-dimethyl thiozolyl-2)-2,5-diphenyl tetrazolium bromide; INT = 2-(p-iodophenyl)-3-p-dinitrophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride; NBT = 2,2′-dinitrophenyl-5,5′-diphenyl-3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium chloride; TNBT = 2,2′-5,5′-tetra-p-nitrophenyl-3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride.

Extraction of Mitochondria. The mitochondria were extracted from Jerusalem artichoke tubers according to the method of WEDDING and BLACK (1962). The washed tubers (250 g) were hand-grated under the surface of 200 ml of 0.6 M sucrose, 0.05 M tris and 5 μM EDTA (pH 8.0). The resulting homogenate was then pressed through a closely woven cotton bag and centrifuged at 3,000 ×g for 10 minutes and the supernatant was recentrifuged at 15,000 ×g for 20 minutes; the sediment obtained was then resuspended in 50 ml of 0.6 M sucrose and 0.05 M tris (pH 7.0) and again centrifuged for 25 minutes at 15,000 ×g. The supernatant was discarded and the mitochondria finally suspended in 4.0 ml of 0.4 M sucrose and 0.05 M tris (pH 7.0). All procedures were carried out between 1° and 4°C. Nitrogen content of the mitochondria was measured according to the method described by JOHNSON (1941).

Measurement of the Rate of Respiration. The rates of oxygen uptake were measured polarographically using a vibrating microplatinum electrode (GME oxygraph) covered with collodion film. The reaction mixture consisted of 1.6 ml of medium containing 500 μmoles sucrose; 40 μmoles tris; 20 μmoles KH_2PO_4; 150 μg mitochondrial nitrogen; 50 μmoles of substrate. The pH of the medium was adjusted to 7.0.

Measurement of the Rate of Tetrazolium Salt Reduction. The rate of tetrazolium salt reduction was measured directly in a recording spectrophotometer. The measurements were carried out at 30°C in silica cuvettes (light path 1.0 cm) which were placed in a Unicam SP500 spectrophotometer linked to a Hilger-Gilford absorbance converter and recorder. The reaction mixture consisted of 2.5 ml of medium containing 0.4 M sucrose; 0.05 M Tris; 0.02 M KH_2PO_4; 0.02 M succinate, pH 7.0, the experiment was initiated by adding mitochondria containing 100 μg nitrogen (200 μg when using TTC, BT or NT as electron acceptors). Additions of the various cofactors, inhibitors and tetrazolium salts were as described in the results section. Controls were carried out to estimate possible complications due to the swelling and contracting of the mitochondria used. It was also found that this method of assay gave results comparable with those obtained previously (PALMER and KALINA, 1966) when the formazan was extracted from the mitochondria before its optical density was determined. The rates obtained in this study were those from the linear portion of the recording.

Results

Throughout this study it was essential to measure the rate of reduction of tetrazolium salts over a period of time since preliminary experiments had indicated that certain changes in the rate occurred in the first few minutes of the reaction (Fig. 1). By following the rates of formazan production, using a continuously recording spectrophotometer, it was possible to show a definite lag phase in the rate of reduction of tetrazolium salts when succinate was used as the substrate, and that the duration of this lag period was markedly influenced by the components included in the reaction mixture. Especially interesting in this respect were the effects of ATP, ADP and AMP, summarised in Fig. 1.

It can be seen that the addition of ATP completely abolished the lag phase and enhanced the linear rate of reduction of tetrazolium salts. ADP shortened the lag phase and, to a lesser extent than ATP, stimulated the subsequent linear rate of reduction. AMP, on the other hand, had no apparent effect on the duration of the lag period or on the subsequent rate of reduction. When AMP was added to the reaction mixture together with ADP it completely suppressed the effect of ADP; however, when added together with ATP, AMP had no suppressing effect. This antagonism between ADP and AMP would be expected if the activity of ADP in influencing the rate of tetrazolium reduction was due to its conversion to ATP by the enzyme myokinase; this enzyme is known to be inhibited by AMP (COLOWICK and KALCKAR, 1943).

The rate of oxygen uptake by mitochondria, when measured polarographically, also showed a lag period when succinate was supplied as the substrate (Fig. 2).

Addition of ATP prior to succinate, or preincubation of the mitochondria with succinate, removed or shortened the lag period without affecting the final rate of oxygen uptake. The lag in the rate of oxygen uptake when using succinate as the substrate is apparently due to a low activity of succinate dehydrogenase possibly caused by a deficiency of substrate. Additional evidence supporting this suggestion was obtained using the succinate-tetrazolium reductase system, in which it is possible to remove the lag phase in the rate of reduction by either adding ATP or by preincubating the mitochondria with succinate in the absence of ATP before adding the tetrazolium salt (Fig. 3).

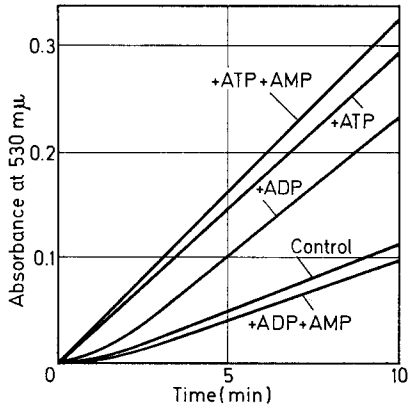

Fig. 1. The influence of added nucleotides on the rate of tetrazolium reduction. The rate of reduction of NBT (0.1 mg/ml) was measured spectrophotometrically at 530 mμ. The final concentration of the nucleotides was 10^{-3} M

It is, however, apparent from these data that preincubation with succinate removed the lag phase but had no effect on the subsequent linear rate of reduction compared with the control without preincubation, whereas ATP both removed the lag phase and increased the linear rate of reduction. Thus ATP appears to have a dual effect on the rate of tetrazolium reduction; (i) it removed the lag phase which is common to both the reduction of tetrazolium salts and the uptake of oxygen, and (ii) it enhanced the linear rate of tetrazolium reduction without having a similar effect on the rate of oxygen uptake.

Other experiments were carried out using the malate-tetrazolium reductase system (Fig. 4) where it was found that the addition of ATP decreased the rate of oxygen uptake whilst it increased the rate of reduction of the tetrazolium salts. No lag phase was observed in either oxygen uptake or tetrazolium reduction when malate was supplied as the substrate.

These results support the suggestion that the effect of ATP in removing the lag phase of tetrazolium reduction may be the result of its ability to change the permeability of the mitochondria to succinate; however its effect on the linear rate of reduction is independent of any effect it may have on the rate of oxygen uptake.

The stimulation of tetrazolium reduction by ATP was not removed by disrupting the mitochondria using either ultrasonic oscillations, freezing and thawing procedures or osmotic shock.

The rate of formazan production was also influenced by the concentration of the salt present in the reacting medium. In most experiments the percentage stimulation, by ATP, of the rate of formazan production was greater when low concentrations (0.1 mg/ml) of tetrazolium salts were used, and lower when the concentration of tetrazolium was increased to 0.5 mg/ml, as shown in Fig. 5.

Addition of 10^{-3} M azide or cyanide almost completely prevented the stimulation caused by ATP when low concentrations of NT were used (Fig. 5a); however,

ATP still removed the lag period in the presence of azide or cyanide. The same inhibitors when added to a system containing a higher concentration of NBT (0.5 mg/ml) did not cause any decrease in the ATP dependent stimulation (Fig. 5 b). Similar results were obtained with high and low concentrations of MTT, INT and TNBT as the electron acceptors.

Since it is apparent that some of the respiratory inhibitors affect the stimulation of tetrazolium reduction caused by addition of ATP it was decided to omit

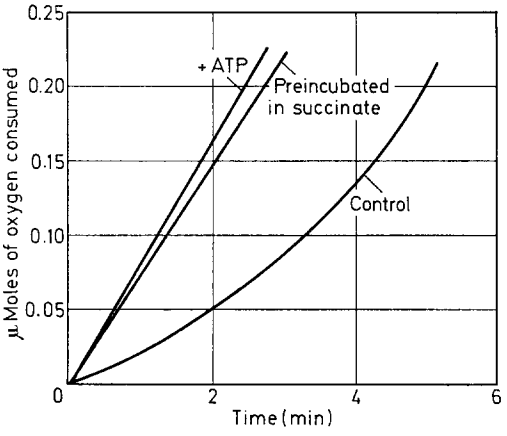

Fig. 2

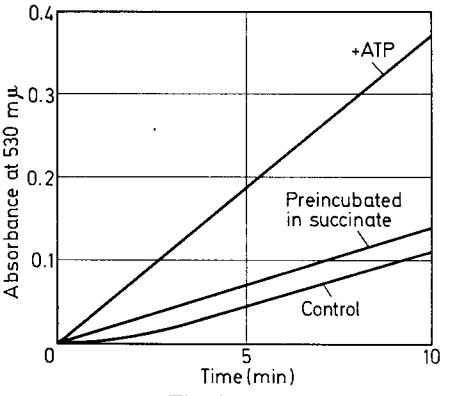

Fig. 3

Fig. 2. The rate of oxygen uptake by isolated mitochondria using succinate as the substrate. Rate of oxygen uptake was measured polarographically. The ATP (final concentration 10^{-3} M) was added 2 minutes before the succinate. Preincubation was carried out for 10 minutes at 0° C in 0.3 M succinate (pH 7.0)

Fig. 3. The rate of NBT reduction by isolated mitochondria using succinate as the substrate. Rate of reduction was measured spectrophotometrically at 530 mμ. The concentration of ATP was 10^{-3} M. Preincubation was carried out for 5 minutes prior to the addition of NBT

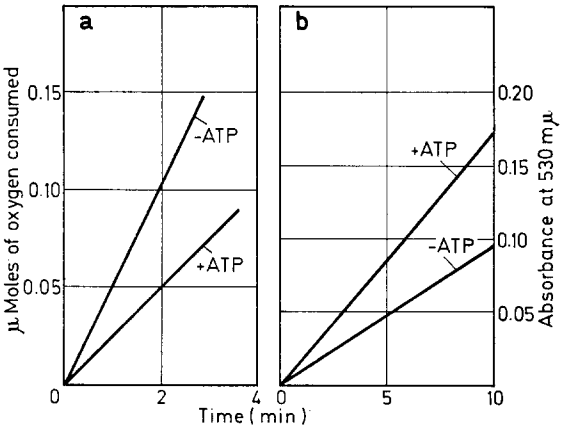

Fig. 4 a and b. The effect of ATP on the reduction of NBT and the rate of respiration with malate as the substrate. a The rate of oxygen consumption was measured polarographically in the absence or presence of 10^{-3} M ATP. b The rate of NBT reduction measured spectrophotometrically at 530 mμ in the absence or presence of 10^{-3} M ATP

ATP from the reaction mixture when attempts were made to locate the various points on the electron transport chain, at which the different tetrazolium salts accept electrons. The omission of ATP resulted in the reappearance of the lag period, therefore the reaction rate quoted in all the subsequent results is the linear rate occurring after the initial lag phase.

Malonate, 4,5-dichloro-2-trifluoromethylbenzimidazole (DCTFB), antimycin A, cyanide and azide were tested as inhibitors of the succinate-tetrazolium reductase system. DCTFB is an inhibitor of electron flow from succinate to cytochrome b (JONES and WATSON, 1967) but had no effect on the electron flow from succinate

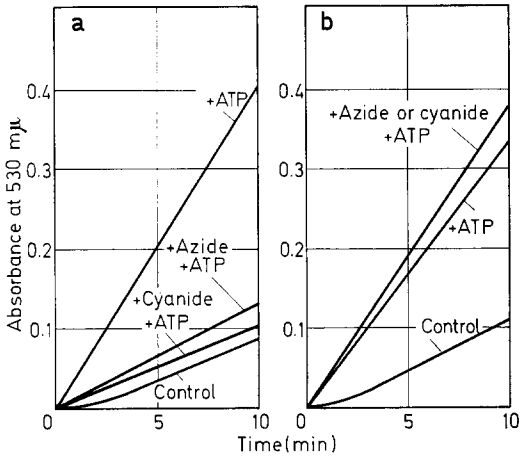

Fig. 5a and b. The effect of cyanide and azide on the ATP enhancement of the rate of NBT reduction. The concentrations of cyanide and azide and ATP were 10^{-3} M. a Results obtained using low concentrations of NBT (0.1 mg/ml). b Results obtained using high concentrations of NBT (0.5 mg/ml)

to PMS. This indicates that it probably inhibits electron transport between the flavoprotein of succinate dehydrogenase and cytochrome b. As the concentration of tetrazolium salts had some significance in the reaction both high (0.5 mg/ml) and low (0.1 mg/ml) concentrations were employed in these experiments. The results in Table 1 show that the reduction of both TTC and BT at low and high concentrations was completely inhibited by all of the inhibitors tested suggesting that both of these tetrazolium salts accept electrons from the cytochrome oxidase region of the electron transport chain. The reaction of succinate-NT reductase was partially inhibited by cyanide, azide or antimycin A when the low concentration of NT was used (Table 1). However no inhibition by cyanide or azide occurred when the higher concentration of NT was used; under these conditions antimycin A again caused a partial inhibition suggesting sites of NT reduction located on both sides of the antimycin sensitive point. The reduction of MTT, INT, NBT and TNBT at both high and low concentrations was stimulated when either cyanide, azide or antimycin A was added to the reaction medium (Table 2). DCTFB caused a partial inhibition of the reduction of MTT and INT but had no effect on the rate of reduction of NBT or TNBT which accept electrons from the flavoprotein of succinate dehydrogenase while MTT and INT accept electrons between the flavoprotein and the antimycin A sensitive point.

Table 1. *The effect of certain inhibitors on the reduction of TTC, BT and NT, catalysed by succinate-tetrazolium reductase in mitochondria isolated from Jerusalem artichoke (without added nucleotide)*

Additions	Final concentration (mM)	ΔOD/min/mg mitochondrial nitrogen		
		TTC	BT	NT
Low concentration of tetrazolium salt (0.1 mg/ml)				
Succinate	50	0.025	0.100	0.095
Succinate + malonate	100	0	0.005	0.005
Succinate + azide	4	0	0	0.035
Succinate + cyanide	0.4	0	0	0.045
Succinate + antimycin A	1 γ/ml	0	0	0.040
High concentration of tetrazolium salt (0.5 mg/ml)				
Succinate	50	0.080	0.050	0.150
Succinate + malonate	100	0	0	0.005
Succinate + azide	4	0.015	0	0.150
Succinate + cyanide	0.4	0.005	0	0.155
Succinate + antimycin A	1 γ/ml	0	0	0.058

The initial linear rate of formazan production was measured spectroscopically at 530 mμ; assay conditions as specified in the text.

Table 2. *The effect of certain inhibitors on the reduction of MTT, INT, NBT and TNBT, catalysed by succinate-tetrazolium reductase in mitochondria isolated from Jerusalem artichoke (without added nucleotide)*

Additions	Final concentration (mM)	ΔOD/min/mg/mitochondrial nitrogen			
		MTT	INT	NBT	TNBT
Low concentration of tetrazolium salt (0.1 mg/ml)					
Succinate	50	0.155	0.185	0.140	0.115
Succinate + malonate	100	0.010	0.025	0.005	0.005
Succinate + azide	4	0.160	0.207	0.150	0.135
Succinate + cyanide	0.4	0.230	0.210	0.210	0.180
Succinate + antimycin A	1 γ/ml	0.215	0.285	0.190	0.185
High concentration of tetrazolium salt (0.5 mg/ml)					
Succinate	50	0.275	0.265	0.180	0.120
Succinate + malonate	100	0.015	0.025	0.005	0.005
Succinate + azide	4	0.285	0.270	0.220	0.165
Succinate + cyanide	0.4	0.290	0.265	0.260	0.220
Succinate + antimycin A	1 γ/ml	0.520	0.495	0.260	0.240
Succinate + DCTFB	1	0.045	0.130	0.180	0.120

The initial linear rate of formazan production was measured spectrophotometrically at 530 mμ; assay conditions as specified in the text.

Discussion

Nucleotides such as ATP and ADP are known to have no effect on the activity of succinate or NAD(P)H-tetrazolium reductase in plant or mammalian tissue sections (Hess *et al.*, 1958). However a considerable stimulation of succinate-tetrazolium reductase activity was obtained after the addition of ATP or ADP

to mitochondria isolated from Jerusalem artichoke tubers. ADP was probably effective only after its enzymatic conversion to ATP. The stimulatory effect of ATP was not apparently connected with its role in oxidative phosphorylation, since all the tetrazolium salts tested were powerful uncouplers of oxidative phosphorylation. Moreover, compounds such as dinitrophenol, an uncoupler of oxidative phosphorylation, and dicyclohexylcarbodiimide, an inhibitor of oxidative phosphorylation (BEECHEY *et al.*, 1966), had no effect on the stimulation caused by ATP or ADP. ATP had two distinct effects; (i) it removed the lag period in the reduction of tetrazolium salts by succinate, possibly by increasing the amount of succinate entering the mitochondria; (ii) it markedly enhanced the rate of tetrazolium reduction by both succinate and malate. The reason for this stimulation is open to at least two different interpretations.

One possible interpretation is that ATP is associated with the transport of electrons from the main electron transport system to the various tetrazolium salts. It has been found that no direct transfer of electrons occurred between the components of the electron transport chain such as cytochrome c and the various tetrazolium salts (LESTER and SMITH, 1961). Therefore it is possible to suggest that an intermediary system may be responsible for the transfer of electrons to the tetrazolium salts and that ATP may activate this system. Such an interpretation however, offers no explanation for the different degrees of ATP stimulation observed with high or low dye concentrations nor for the inhibition of the ATP stimulation by cyanide and azide (see Fig. 5), when using low dye concentrations. A second possible interpretation is that ATP affects the penetration of the tetrazolium salts into the sites of reduction in the mitochondria. Two different mechanisms may be involved in the uptake of tetrazolium salts into the mitochondria. The first mechanism could be a passive diffusion, into the mitochondria. The second mechanism could be an active process dependent upon the utilization of energy to cause the tetrazolium salts to enter the mitochondria. If these two processes proceded simultaneously then it is possible that at low concentrations of tetrazolium salts the active process may be more important than the passive penetration; this would then explain the greater stimulation by ATP at low concentrations of the tetrazolium salts and the removal of this stimulation by cyanide and azide. However, when high concentrations of tetrazolium salts were used, the passive uptake might be more significant and the response to added ATP would be less. This high passive uptake may also be the explanation of the lack of inhibition by azide and cyanide. In such a case the effect of cyanide and azide is mainly to block the electron transport chain at the cytochrome oxidase region with a consequent increase in the transfer of electrons to the tetrazolium salt. The effect at low concentrations of the tetrazolium salt may be much less, if its concentration is not sufficient to saturate the site of reduction. As all attempts to disrupt the mitochondria failed to remove the stimulation of the rate of reduction caused by ATP it must be assumed that ATP assists the penetration of the tetrazolium salts through an internal barrier.

The results obtained concerning the sites on the electron transport chain at which the various tetrazolium salts are reduced are summarised in Fig. 6.

TTC and BT are shown accepting electrons from the cytochrome oxidase region. This is in agreement with earlier findings using mammalian tissue (ODA

and OKAZAKI, 1958; NACHLAS *et al.*, 1960). Reduction of MTT and INT is shown to occur before the antimycin A sensitive point. In previous work using animal tissue the site of reduction was found to be on the oxygen side of the antimycin A block (NACHLAS *et al.*, 1960; LESTER and SMITH, 1961; and SLATER *et al.*, 1963). Reduction of both NBT and TNBT also occurs before the point at which antimycin A inhibits electron transport. Similar results are available from studies on animal tissue for NBT (no data is available for TNBT) (NACHLAS *et al.*, 1960; LESTER and SMITH, 1961). The use of DCTFB made it possible to locate the sites

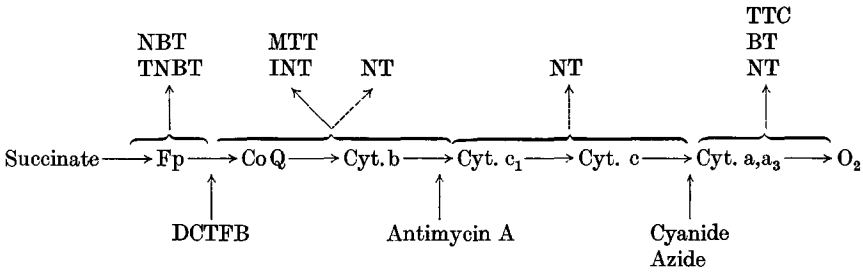

Fig. 6. Sites of reduction of various tetrazolium salts along the succinate oxidase electron transport chain in mitochondria isolated from Jerusalem artichoke tubers. ——— Sites of high affinity towards tetrazolium salts (see text). — — — Sites of low affinity towards tetrazolium salts (see text)

of MTT and INT reduction between the flavoprotein and the antimycin A block, and of NBT and TNBT in the region of the flavoprotein itself.

The reduction of NT is more complicated and the apparent site of reduction varies with the concentration of NT used. At the low concentration of NT either cyanide, azide or antimycin A cause a partial decrease in the rate of reduction indicating two sites of reduction, one in the cytochrome oxidase region and another prior to the antimycin sensitive region. However, at high concentrations of NT antimycin A caused a partial inhibition and cyanide and azide were without effect, indicating that the sites of reduction were again on either side of the antimycin A block, but not at the cytochrome oxidase region as before. These results are consistent with three sites of reduction for NT, one before and two after the antimycin sensitive region. The latter two sites can be demonstrated using different concentrations of NT in the medium, indicating differences in the affinities of the sites for the tetrazolium salt; thus the site in the cytochrome oxidase region has a high affinity and operates efficiently at the low concentration of NT while the site in the region of cytochrome c has a lower affinity and can only operate at the higher NT concentration. ODA and OKAZAKI (1958) and NACHLAS *et al.* (1960) reported that NT was reduced in the cytochrome oxidase region while LESTER and SMITH (1961), and SLATER *et al.* (1963) found NT to be reduced in the region of cytochrome c. The possible differences in affinities described in this paper may explain some of these apparent discrepancies.

It is interesting to observe that tetrazolium salts such as NBT and TNBT with redox potentials in the region of −0.05 volts (PEARSE, 1960) accept electrons from the flavoprotein (redox potential −0.06 v) while TTC and BT with redox

potentials of −0.49 v and −0.160 v respectively accept electrons from the cytochrome oxidase region of the electron transport chain (redox potential +0.29 v). If the redox potentials published for the tetrazolium salts are those applicable under the experimental conditions used it is necessary to assume that expenditure of energy must be involved in reducing the tetrazolium salts, since in general their redox potentials are more negative than those for the components of the electron transport chain from which they appear to accept electrons. The role of added ATP in increasing the reduction of the tetrazolium salts may be to supply this energy.

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