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Reduction of a disulphide in relation to the metabolic states of mitochondria

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Reduction of a disulphide in relation to the metabolic states of mitochondria*

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Abstract

The role of -SH groups in mitochondrial energy transfer reaction was studied by observing the reduction of a disulphide, 5, 5'-dithiobis (2-nitrobenzoic acid), DTNB, a specific analytical agent for the estimation of -SH groups in biological materials, by addition of it to the isolated rat liver mitochondria in various respiratory states, as defined by CHANCE and WILLIAMS. 1. In the various respiratory states, states 1 to 5, the reduction of DTNB proceeds most rapidly at state 5, and most slowly at state 3. DTNB reduction at state 5 is suppressed by the partial oxidation of respiratory carriers with oxygen (state 4) and the addition of respiratory substrate does not affect the DTNB reduction. 2. The retardation in the reduction rate at state 3 is relieved partially by a respiratory inhibitor, KCN, and is intensified markedly by oligomycin, an inhibitor of oxidative phosphorylation. An uncoupler for oxidative phosphorylation, DNP, does not affect the reduction rate at state 3. At state 4 the reduction is stimulated by DNP and KCN, but is unaffected by oligomycin. The results suggest that the alteration in the functions of the energy transfer reaction in mitochondria is accompanied by changes in the occurrence and the functioning of -SH groups which can be detected by the reactivity with DTNB. The data suggest also that there are at least two kinds of -SH groups reacting with DTNB: the one is the -SH group which reacts DTNB actively when the respiratory carriers are kept reduced, and the other is the one which reacts actively when the respiratory carriers are kept oxidized, participating in the phosphorylating system and its reactivity with DTNB diminishes in the actively phosphorylating states (states 2 and 3).

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REDUCTION OF A DISULPHIDE IN RELATION TO THE METABOLIC STATES OF MITOCHONDRIA

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Observations of the mitochondrial functions by using sulfhydryl reagents, such as disulphides, SH-compounds and mercurials, have revealed some important roles of -SH groups in mitochondria. The results indicate that mitochondria have two kinds of -SH groups of different function : the one participating in the translocation of ions across the mitochondrial membrane (1—3) and the other in the energy transfer reactions, especially in oxidative phosphorylation (4—6) and its partial reactions (6—9).

In the past observations two different kinds of approach have been made for the study on the role of -SH groups in mitochondria : one is to observe how mitochondrial functions are affected by the sulfhydryl reagents, and the other how the reduction of the reagent varies with the changes in the functions of mitochondria. By the latter approach it has been reported that the mitochondria reduce a number of disulphides of small molecule and that the reduction is stimulated by Pi, AMP, ADP, oxygen and respiratory substrates (10, 11). The reduction of disulphides is brought about by reacting with reduced lipoic acid, since the reduction of disulphide such as cystamine in a high concentration is stimulated by the addition of respiratory substrates or respiratory inhibitors, and it is suppressed by DNP (11). On the other hand, it has been reported that DTNB, a disulphide in a high concentration gives strong inhibitory effects on mitochondrial energy transfer reactions (3), while DTNB in a low concentration does not inhibit mitochondrial respiration, though respiratory control decreases and does inhibit DNP-ATPase without giving any inhibitory effects on ATP-Pi exchange reaction (6). The results suggest the participation of the DTNB sensitive -SH groups in oxidative phosphorylation.

In this paper some experimental data are presented, supporting the participation of -SH groups in oxidative phosphorylation by observing the reduction of DTNB in a low concentration under the varied metabolic states as defined by CHANCE and WILLIAMS (12).

MATERIALS AND METHODS

Mitochondria were isolated from male albino rats of about 50 days old by the modified method of HOGEBOOM and SCHNEIDER (13), suspended in 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4), and stored in ice-water bath for the experiments. The principal reaction system used for the measurement of DTNB reduction by mitochondria was composed of the mitochondria (about 9 mg protein) and a basic medium containing 100 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, 0.02 % H_2O_2 and 5 mM Tris-HCl buffer (pH 7.4). The reduction of DTNB was measured by tracing the increase in absorbance at 412 mμ ($+\Delta A_{412}$) after the addition of 20 μM DTNB to the one cuvette which had been conditioned in various respiratory states as defined by CHANCE and WILLIAMS (12), by using a Beckman-type Hitachi autospectrometer. The final volume of the reaction mixture was adjusted by the basic medium to 3.05 ml. 0.02 % H_2O_2 was added for the generation of about 480 mμ atoms oxygen. 20 μM DTNB used in the experiments gives inhibitions, 50 % in DNP-stimulated ATPase, 7 % in ATP-Pi exchange reaction and 25 % in state 3 respiration (6). Protein was determined by the 10,000 $\times$g, 20 min-centrifuged supernatant of the biuret colored sample of the reaction mixture (14) with bovine serum albumin as standard. The details of the experiments were described in the footnote and the legend of the each result. DTNB, ADP and bovine serum albumin were obtained from Sigma Chemical Co. (U. S. A.) and the other chemicals used were commercial products of the highest purity.

RESULTS

Properties of DTNB reduction by rat liver mitochondria. DTNB at a low concentration inhibited DNP-stimulated ATPase without giving any inhibitory effects on ATP-Pi exchange reaction (6) but at a high concentration it inhibited the ADP-stimulated respiration and the translocation of Pi across the mitochondrial membrane (3). The present experiment indicates that DTNB is reduced by the living rat liver mitochondria. As shown in Fig. 1 the DTNB reduction at state 4 proceeds in a rapid rate at the initial phase within about 30 seconds after the addition of DTNB and thereafter proceeds almost proportionally with the time lapse. But as is obvious from the figure the reduction of DTNB, when added 10 μM in the final concentration, did not proceed proportionally with the time lapse of the reaction. Next as shown in Fig. 2, the reduction with the mitochondria at state 4 5 minutes after DTNB addition takes place proportionally with the increased concentration of DTNB added. But as shown in the figure, the reaction proceeds at a rapid rate without showing any proportionality to the DTNB concentration at the 10 μM DTNB addition. These data indicate that the mode of the DTNB reduction varies with the time

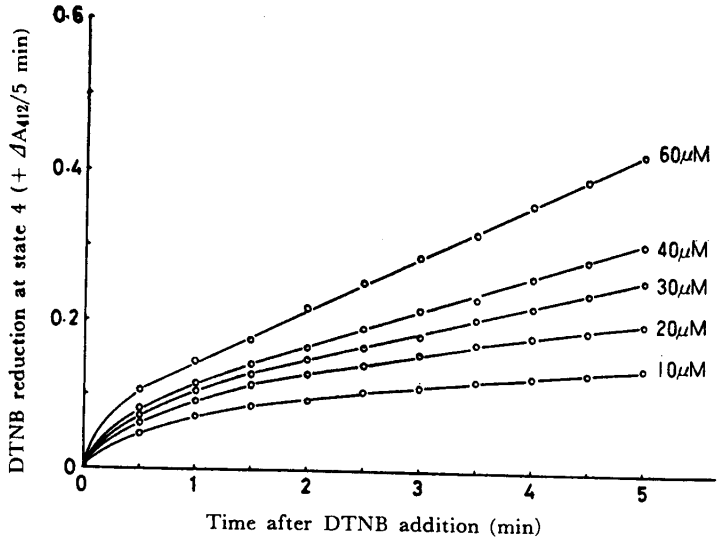

Fig. 1 The mode of DTNB reduction at state 4 in relation to the added DTNB

The principal experimental methods are referred to the text. The reaction was performed as follows in each case: to the principal reaction system 6.6 mM succinate was added and the $+\Delta A_{412}$ was measured after the addition of DTNB in the final concentration as indicated in the figure. DTNB was added 30 seconds after the addition of succinate. The final volume of the reaction mixture was 3.05 ml. The mitochondria were added at the concentration of 2.9 mg mitochondrial protein per ml of the reaction mixture.

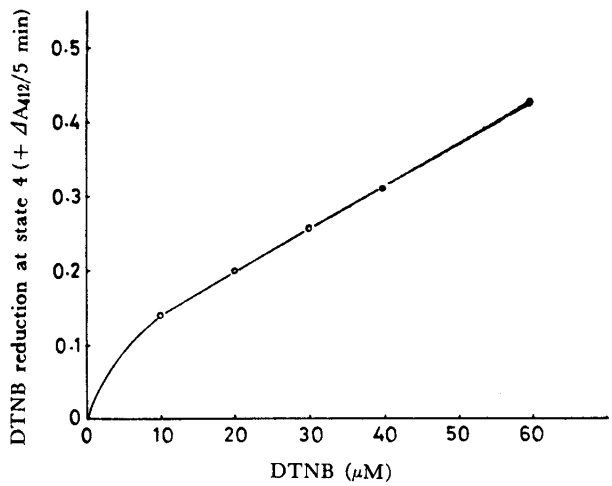

Fig. 2 The proportion in DTNB reduction at state 4 in relation to the concentrancion of DTNB

The experimental methods are shown in the footnote in Fig. 1. The amount of DTNB reduced at 5 min in Fig. 1 was plotted.

lapse of the reaction and DTNB concentration used, suggesting the occurrence of some different properties in -SH groups, sensitive to DTNB, in the mitochondria at state 4.

Fig. 3 shows the ratio of the DTNB reduction to the amount of mitochondria. As shown in the figure, the DTNB reduction at state 4 proceeded proportionally with increase in the amount of mitochondria. The results and the findings indicate the occurrence of some kinds of -SH groups in its

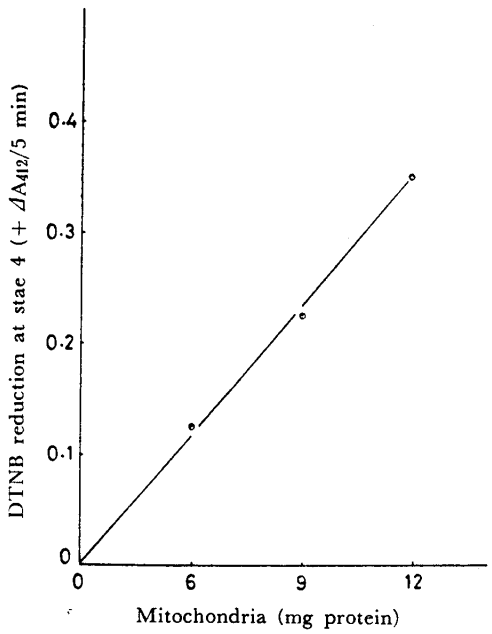

Fig. 3 The proportion in DTNB reduction in relation to the mitochondria In each case 20 μM DTNB was added to the reaction mixture and the amount of DTNB reduced during 5 min were plotted. For the experimental procedures refer to the footnote in Fig. 1.

reactivity to DTNB (Figs. 1 and 2). It is important to estimate the changes in reactivity of DTNB reduction by the mitochondria in the various respiratory phases. Thus the changes in the activity of DTNB reduction by mitochondria stored for various length of time at 0 to 4°C were examined. The data indicated that both of the reactivities in DTNB reduction remained constant at state 3 and state 4, and that the reduction rate with the mitochondria at state 3 was strongly suppressed, compared with that at state 4 (Table 1). It was also shown that the ratios of DTNB reduction at state 3 to that at state 4 remained almost constant, irrespective of the storage time of mitochondria. However, the DTNB reduction was raised

Table 1 Stationary activity of DTNB reduction in relation to the storage
At a giving time indicated the reduction of DTNB was measured by adding 20 μM DTNB to the principal mitochondrial suspension (2.5 mg mitochondrial protein/ml reaction mixture) 30 seconds after the addition of 6.6 mM succinate for state 4, and the addition of 6.6 mM succinate, 6.6 mM Pi and 2 mM ADP for state 3. The total reaction mixture was 3.05 ml.

	$+\Delta A_{412}$/5 min							
respiratory condition	time stored at 0—4°C (min)							
	0	20	30	45	60	75	90	105
state 3	0.115		0.110		0.110		0.110	
state 4		0.202		0.207		0.200		

by a long stored mitochondria at all respiratory states.

Changes in the rate of DTNB reduction by the mitochondria under varied respiratory states. The reduction of some disulphides, such as cystamine and its derivatives, by mitochondria are stimulated by Pi and ADP (10) and the stimulation by these agents may be the cause of the increased formation of thiol (11). The data in Fig. 4 indicate the time course of DTNB reduction by the mitochondria which had been conditioned in various respiratory phases, such as states 1, 2, 3, 4 and 5 as defined by CHANCE and WILLIAMS (12). As shown in the figure it is noted at first that the modes of reduction at states 5, 4 and 1 are quite different from those at states 2 and 3, and that the different modes in the reduction rate in these two groups occurred from the initial phases of the time lapse after DTNB addition. It is also noted that the reduction in all cases proceeded in more rapid rate within 60 seconds after DTNB addition and became slower with the time lapse thereafter. The data indicate that two kinds of -SH groups different in the sensitivity to DTNB are present in all respirtatory phases: the one is the -SH group rapidly reacting with DTNB and the other more slowly reacting one. In comparing the reduction rate at various respiratory states of mitochondria, the reduction rate was the highest at the respiratory condition in the absence of Pi, ADP and oxygen (state 5). The presence of oxygen (state 4) suppressed the reduction rate at state 5 and the rate was little affected in the absence of respiratory substrate (state 1). The result is quite different from the observation that the reduction of a disulphide, cystamine, is stimulated by the addition of respiratory substrate (10). In the presence of substrate (state 4) both additions of Pi and ADP (at state 3) gave about 50 % inhibition on the reduction rate at state 4. In the absence of substrate (state 1) both additions Pi and ADP (state 2) also strongly inhibited the reduction rate at state 1. The reduction rate at state

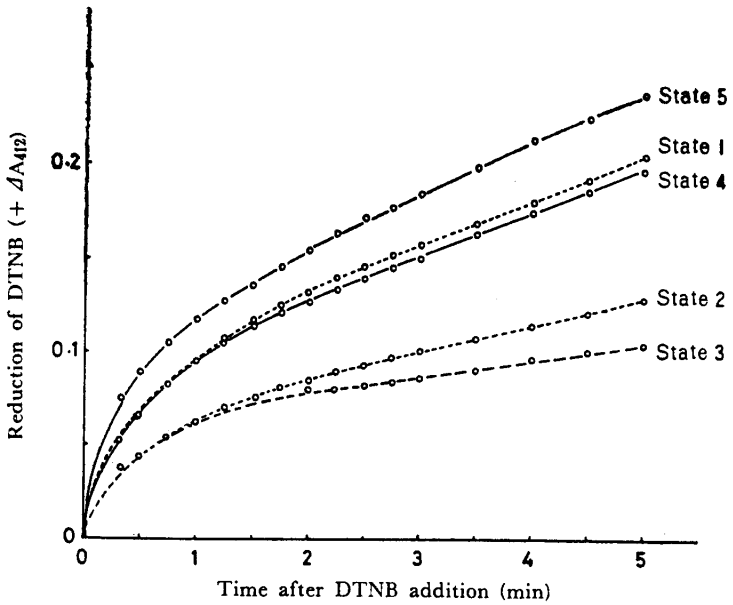

Fig. 4 Changes of DTNB reduction in relation to the various respiratory states of mitochondria

The experimental procedures are principally the same as in Fig. 1. The mitochondria were 2.8 mg protein/ml of the reaction mixture. The addition and the order of the agents were as follows. 20 μM DTNB was added 30 seconds after the final addition in each case. State 1: the basic medium, 0.02 % H_2O_2 and mitochondria were mixed. State 2: the basic medium, 0.02 % H_2O_2, 6.6 mM Pi and mitochondria were mixed, and 2 mM ADP was added. State 3: the basic medium, 0.02 % H_2O_2, 6.6 mM succinate and mitochondria were mixed, and 6.6 mM Pi and 2 mM ADP were added. State 4: the basic medium, 0.02 % H_2O_2 and mitochondria were mixed, and 6.6 mM succinate was added. State 5: the basic medium, 6.6 mM succinate and mitochondria were mixed. N_2 gass was bubbled to the full anaerobic condition before the addition of DTNB.

2 was quite similar to the rate at state 3 within 120 seconds of the reaction time, but the inhibition rate was gradually relieved with the time lapse after the DTNB addition. These difference in the rate of DTNB reduction suggest changes in the occurrence and the functioning of -SH groups reacting with DTNB in the energy transfer reactions of mitochondria under the varied respiratory phases. The data indicate that the presence of Pi and ADP, the phase of active oxidative phosphorylation, suppresses the reaction of DTNB to the -SH groups in mitochondria. Furthermore, at state 4 the DTNB reduction is strongly inhibited by adding 2 mM ADP (state 4 +ADP) and also by adding 10 mM Pi (state 4+Pi), and the extent of the inhibition was stronger with ADP than with Pi as shown in Fig. 5. The

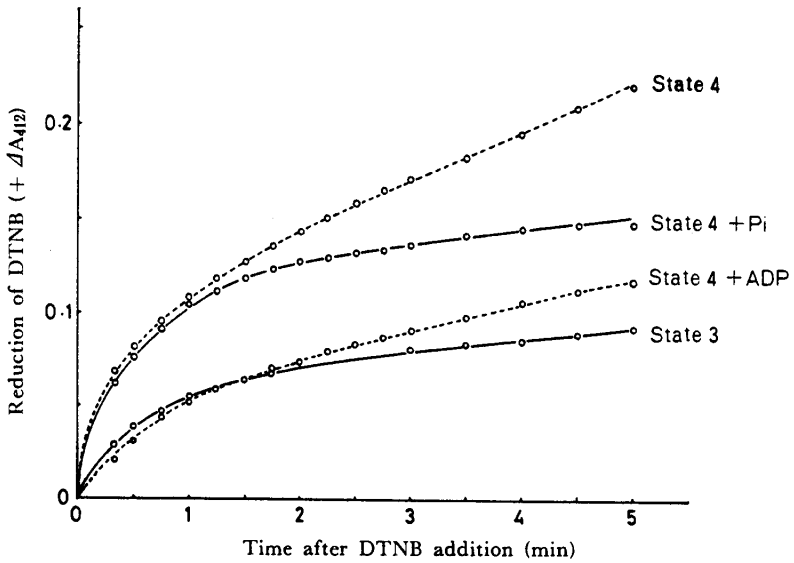

Fig. 5 The effects of Pi and ADP on the DTNB reduction at state 4

The experimental procedures were principally the same as in Fig. 4, and the mitochondria were 2.8 mg protein/ml of the reaction mixture. The addition and the order of the agents were as follows. 20 μM DTNB was added 30 second after the final addition of the agent at each time. The procedures at state 3 and state 4 were the same as in Fig. 4. State 4+Pi: the basic medium, 0.02 % H_2O_2, 6.6 mM succinate and mitochondria were mixed, and 6.6 mM Pi was added. State 4+ADP: the basic medium, 0.02 % H_2O_2, 6.6 mM succinate and mitochondria were mixed, and 2 mM ADP was added.

mode of the inhibition by ADP was quite similar to that of inhibition at state 3 within about 120 seconds after DTNB addition.

The effects of energy transfer inhibitors on the DTNB reduction at states 3 and 4. As the data shown in figures 4 and 5 indicate that the proceeding of oxidative phosphorylation suppresses the reduction of DTNB, it may be of importance how the energy transfer inhibitors affect the DTNB reduction at the varied respiratory phases, especially at states 3 and 4. As shown in Fig. 6, the DTNB reduction by the mitochondria at state 4 is strongly suppressed at state 3, and the suppressed reduction at state 3 was relieved partially by a respiratory inhibitor, KCM (5×10^{-5}M), but the relieved rate was far slow from the reduction rate at state 4 (state 3+KCN). On the other hand, the inhibition of the reduction at state 3 was further intensified by oligomycin (1.2 μg/mg protein), an inhibitor of oxidative phosphorylation (state 3+oligomycin). In this instance, the reduction proceeded as much as the one at state 3 within the initial phase of the time

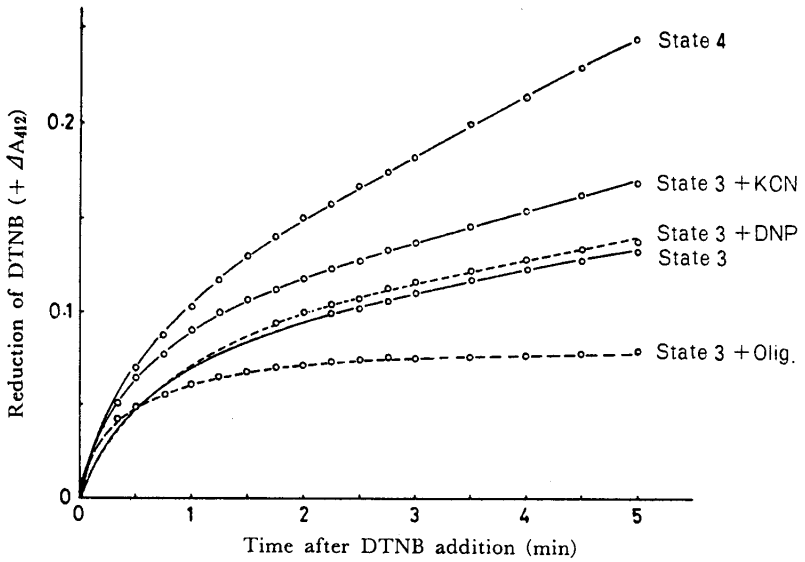

Fig. 6 The effects of energy transfer inhibtors on the DTNB reduction at state 3

The experimental procedures are as in Fig. 4. The mitochondria were 2.7 mg protein/ml of the reaction mixture. The addition and the order of the agent were as follows. 20 μM DTNB was added 30 seconds after the final addition of the agent at each time. The procedures at states 3 and 4 were as in Fig. 4. State 3+KCN: the basic medium, 0.02% H_2O_2, 6.6 mM succinate, 5×10^{-5} M KCN and mitochondria were mixed, and 6.6 mM Pi and 2 mM ADP were added. State 3+DNP: the basic medium, 0.02% H_2O_2, 6.6 mM succinate, 1.6×10^{-5} M DNP and mitochondria were mixed, and 6.6 mM Pi and 2 mM ADP were added. State 3+Olig: the basic medium, 0.02% H_2O_2, 6.6 mM succinate, 3 μg/ml oligomycin and mitochondria were mixed, and 6.6 mM Pi and 2 mM ADP were added.

lapse after DTNB addition and then the DTNB reduction was largely arested within the subsequent reaction time. The effect of its intensified inhibition by oligomycin on the DTNB reduition at state 3 increased with lowering the DTNB concentration. On the other hand, the effects of similar inhibitors on DTNB reduction at state 4 of the no phosphorylating state, were observed. As shown in Fig. 7, at state 4, the rate of DTNB reduction in all the cases tested was similar in the initial stage of the reaction. But thereafter KCN relieved the reduction as did at state 3. A significant difference of these energy transfer inhibitors on the DTNB reduction between states 3 and 4 is the effects of DNP and oligomycin, showing that oligomycin does not give any suppressive effects on DTNB reduction and DNP stimulates the reduction at state 4.

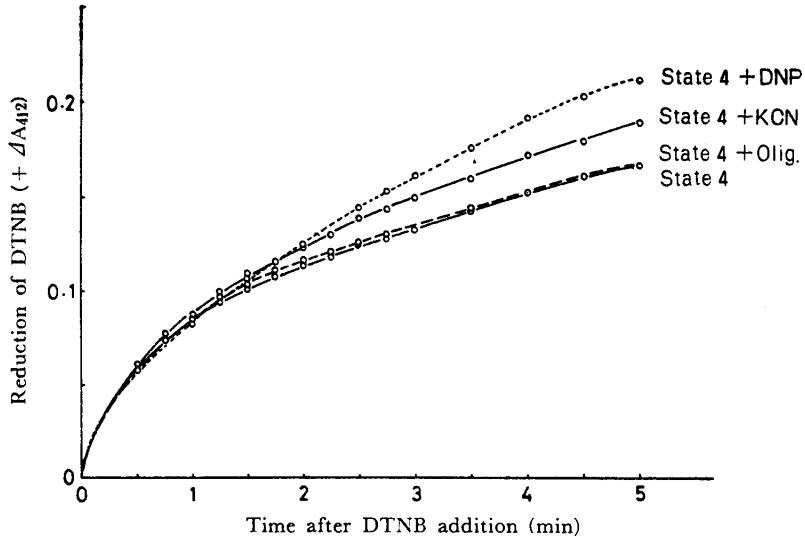

Fig. 7 The effects of energy transfer inhibitors on the DTNB reduction at state 4
For details in the experimental procedures refer to the footnote in Fig. 6. The mitochondria used were 2.9 mg protein/ml. The procedures for state 4 were as in Fig. 4. State 4+KCN: the basic medium, 0.02% H_2O_2, 5×10^{-4}M KCN and mitochondria were mixed, and 6.6 mM succinate was added. State 4+DNP: the basic medium, 0.02% H_2O_2, 1.6×10^{-5}M DNP and mitochondria were mixed, and 6.6 mM succinate was added. State 4+Olig: the basic medium, 0.02% H_2O_2, 3 μg/ml oligomycin and mitochondria were mixed, and 6.6 mM succinate was added.

DISCUSSION

The similar approach to the present work on the study of -SH groups in mitochondria has been made by using a disulphide, cystamine, by several workers, and observations reveal that the reduction of cystamine by mitochondria is stimulated by respiratory substrates, Pi and ADP (10, 11). The present results obtained by using a same kind of disulphide, DTNB, did not agree with the observation and some another results were obtained.

It is shown in the present paper that the reduction of DTNB by rat liver mitochondria varies with the functional respiratory states by CHANCE and WILLIAMS (12). As shown in Figs. 4 and 5, the reduction of DTNB proceeds most rapidly at state 5 in which no ATP synthesis occurs in the absence of Pi, ADP and oxygen, and the reduction is suppressed with the proceeding of the ATP synthesis on addition of oxygen, substrate, Pi, ADP or all of these. A single addition of substrate does not affect the reduction rate at the aerobic condition. The reduction at state 3, the

phase of the most active ATP synthesis, is slowest in all the respiratory phases, suggesting an inverse relationship between the oxidative phosphorylation and the reduction mechanism of DTNB. It is also indicated that oligomycin suppresses the reduction at state 3 as shown in Fig. 6, but it does not give any inhibitory effects on the reduction rate at state 4 (Fig. 7). The stimulatory effect of KCN on the reduction was observed at the both respiratory phases of states 3 and 4. The stimulation was also observed at state 5, the anaerobic condition. DNP did not relieve the suppressed reduction at state 3, but it stimulated the reduction at state 4. These differences in the rate of DTNB reduction suggest that the alterations in the energy transfer function of mitochondria cause changes in the occurrence and in its function of -SH groups in the energy transfer reactions of mitochondria. The data also suggest that these are at least two different kinds of -SH groups which react with DTNB: the first is the -SH group which reduces DTNB at a rapid rate in the intial phase within about 60 seconds after DTNB addition, and the second is the group which react proportionally with DTNB at the less rapid rate than the rate of the first. RILEY and LEHNINGER previously reported that the total mitochondrial -SH groups (95 mμmoles/mg mitochondrial protein) consisted of rapidly reacting groups (12 % of the total), ones reactive within 40 seconds, and the others slowly reacting (88 % of the total) (15). This may be in the case. The second one is characterized by the fact that its reactivity to DTNB varies with the metabolic states of mitochondria (Figs. 2, 4, 5, 6 and 7). The second one is defined into further two subgroups. As summarized in Table 2, a subgroup is the one whose reactivity to DTNB is increased when the respiratory chain carries are kept reduced by KCN or by the anaerobic condition (state 5) in which all of the partial reactions of oxidative phosphorylation are suppressed (16). Another subgroup is the one whose reactivity to DTNB is increased when the respiratory carriers are kept oxidized by DNP which stimulates the respiration and activates the ATPase with reversal reaction of the phosphorylation. It has been recognized that the oxidative phosphorylation is equilibrated by ATPase reaction, the reversal latent reaction in highly coupled mitochondria (7). It is also reported that DTNB does not inhibit significantly DNP-stimulated respiration and ATP-Pi exchange reaction, but does markedly inhibit the DNP-stimulated ATPase (6). In view of the fact that DNP-stimulated ATPase acts to discharge a high energy intermediate at a point between the respiratory carrier and the point blocked by oligomycin (7), these results suggest that some of -SH groups reacting with DTNB occurs in the reaction between the high energy intermediate and its DNP-sensitive site. For

Table 2 The relationship in the activity of DTNB reduction, ATPase activity and the oxidation-reduction state of respiratory chain carriers

The data of the relative activity in DTNB reduction were inreferred from the data in Figs. 4, 5, 6 and 7. ATPase activity and the oxidation-reduction state of respiratory carriers were inreferred from the previous reports cited in the table.

respiratory phase	oxidoreduction of respiratory chain carriers (12, 19, 20)	ATPase activity (16, 21, 22)	DTNB reduced per 5 min(%)
state 1	oxidized		104
2	ox	−	65
3	ox	−	53
4	reduced	±	100
5	red	−	120
state 4	red	±	100
4+Pi	ox	−	68
4+ADP	ox	−	53
3	ox	−	42
state 3	ox	−	54
3+olig	red	−	32
3+KCN	red	−	69
3+DNP	ox	−	56
4	red	±	100
state 4	red	±	100
4+olig	red	−	100
4+KCN	red	−	114
4+DNP	ox	+	127

this reason it may be expected that the DTNB reduction is suppressed when the oxidative phosphorylation takes place by using actively the intermediate, x∼y, for the active ATP synthesis in the presence of respiratory substrate, Pi and ADP, and hence the activity of DNP-stimulated ATPase is low. On the other hand, it is also likely that the reduction of DTNB proceeds actively when the high energy intermediate, x∼y, is discharged actively and hence the activity of DNP-stimulated ATPase is stimulated. This is in good accord with the data summarized in Table 2. From these tentative assumption it is also probable that oligomycin suppresses the DTNB reduction and DNP does relieve the retarded reduction at state 3, since oligomycin is a true inhibitor of oxidative phosphorylation, or of the DNP-stimulated ATPase (17), and ADP at a high concertration is a strong inhibitor of DNP-stimulated ATPase (7, 18). Therefore, it may be assumed that oligomycin does not affect the DTNB reduction and DNP stimulates the reduction at state 4.

SUMMARY

The role of -SH groups in mitochondrial energy transfer reaction was studied by observing the reduction of a disulphide, 5, 5′-dithiobis (2-nitrobenzoic acid), DTNB, a specific analytical agent for the estimation of -SH groups in biological materials, by addition of it to the isolated rat liver mitochondria in various respiratory states, as defined by CHANCE and WILLIAMS.

1. In the various respiratory states, states 1 to 5, the reduction of DTNB proceeds most rapidly at state 5, and most slowly at state 3. DTNB reduction at state 5 is suppressed by the partial oxidation of respiratory carriers with oxygen (state 4) and the addition of respiratory substrate does not affect the DTNB reduction.

2. The retardation in the reduction rate at state 3 is relieved partially by a respiratory inhibitor, KCN, and is intensified markedly by oligomycin, an inhibitor of oxidative phosphorylation. An uncoupler for oxidative phosphorylation, DNP, does not affect the reduction rate at state 3. At state 4 the reduction is stimulated by DNP and KCN, but is unaffected by oligomycin.

The results suggest that the alteration in the functions of the energy transfer reaction in mitochondria is accompanied by changes in the occurrence and the functioning of -SH groups which can be detected by the reactivity with DTNB. The data suggest also that there are at least two kinds of -SH groups reacting with DTNB: the one is the -SH group which reacts DTNB actively when the respiratory carriers are kept reduced, and the other is the one which reacts actively when the respiratory carriers are kept oxidized, participating in the phosphorylating system and its reactivity with DTNB diminishes in the actively phosphorylating states (states 2 and 3).

ACKNOWLEDGEMENT

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Abbreviation; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid). AMP, adenosine 5′-monophosphate. ADP, adenosine 5′-diphosphate. Pi, inorganic phosphate, olig, oligomycin. DNP, 2,4-dinitrophenol. ATPase, adenosine triphosphatase.

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