EFFECTS OF STREPTONIGRIN DERIVATIVES AND SAKYOMICIN A ON THE RESPIRATION OF ISOLATED RAT LIVER MITOCHONDRIA

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Though all the streptonigrin derivatives with modifications in the carboxyl group on C2′ were active as electron acceptors in the oxidation of NADH by *Clostridium kluyveri* diaphorase as well as streptonigrin, the glycine derivative did not show any marked effect on the KCN-insensitive oxidation of glutamate by rat liver mitochondria, suggesting a poor membrane transport of the glycine derivative. Sakyomicin A also induced the KCN-insensitive oxidation of glutamate by mitochondria, while a trace activity was observed by mitomycin C and the effect of doxorubicin was negligible. Like streptonigrin, the autoxidation of a reduced form (hydroquinone) of sakyomicin A to a quinone accompanied the generation of H_2O_2. However, exogenous NADH was oxidized by mitochondria in the presence of sakyomicin A but not streptonigrin.

Streptonigrin (**1**), an aminoquinolinequinone antitumor antibiotic, was first isolated from *Streptomyces floculus* in 1960[1]. Since then, some clinical trials in malignant diseases had been attempted[2,3], but were not continued mainly due to its marked side effect on the gastrointestinal tract and especially on the bone marrow[4].

Based on the improved chemotherapeutic index of streptonigrin methyl ester (**2**)[5,6], several novel derivatives with modifications in the carboxyl group on C2′ such as streptonigrin amide (**3**), the glycine derivative (**4**) and streptonigrin hydrazide (**5**) were synthesized to study their biological properties[7~9]. Although the antibacterial activity of **1** against Gram-positive and Gram-negative bacteria was almost lost in all the derivatives, they showed cytocidal activities against murine lymphosarcoma L5178Y cells to various extents[8]. While ID_{50} of **1** against a parental line of L5178Y cells (L5178Y/S) was 0.0043 μg/ml, those of its derivatives (**2**~**5**) ranged from 0.014 μg/ml (**5**) to >4.0 μg/ml (**4**).

LASZLO and his colleagues[10~12] suggested the importance of a quinone ring of **1** to its cytocidal activity; this is attributable to H_2O_2 generation coupled with the catalytic oxidation of reduced nicotinamide adenine dinucleotide by mitochondrial DT-diaphorase. The deprival of NADH from the oxidative phosphorylation system results in decreased ATP synthesis, adversely affecting the synthesis of high molecular substances.

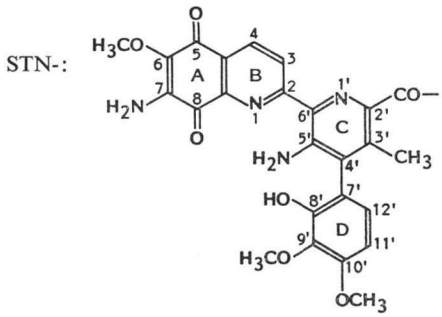
It is of our major interest to elucidate whether this property of **1** is shared by its derivatives and, furthermore, whether this is a common property of antibiotics with a quinone ring.

Materials and Methods

Materials

1 and its derivatives (**2**~**5**) were prepared as described previously[7,13]. Sakyomicin A[14,15] and

Fig. 1. Structures of streptonigrin derivatives.



1	STN-OH
2	STN-OCH_3
3	STN-NH_2
4	STN-$NHCH_2COOH$
5	STN-$NHNH_2$

adriamycin were kindly donated by Prof. N. TANAKA, Institute of Applied Microbiology, University of Tokyo, and Kyowa Hakko Kogyo Co., Ltd., Tokyo, respectively. Diaphorase from *Clostridium kluyveri* was purchased from Oriental Yeast Co., Ltd., Tokyo. All other materials were commercial products of the analytical grade.

Determination of H_2O_2

H_2O_2 was measured by the oxidative coupling of 4-aminoantipyrine with phenol by horseradish peroxidase to produce a quinoneimine dye with an absorption maximum at 500 nm[16]. A reaction mixture (2.3 ml) consisting of 10 mM Tris-HCl (pH 8.0), 0.54 mM 4-aminoantipyrine, 0.006% phenol and 6 units/ml peroxidase was incubated with a test solution (0.2 ml) at 37°C for 20 minutes.

Respiration of Rat Liver Mitochondria

Rat liver mitochondria were isolated by the method of JOHNSON and LARDY[17] and protein concentrations were determined by the method of LOWRY *et al.*[18]. The respiration of mitochondria was measur-

Fig. 2. Effects of various antibiotics on the oxidation of glutamate by rat liver mitochondria.

The respiration of mitochondria was measured in 3 ml of a basal medium (described in the text) supplemented with 15 mM glutamate at 30°C.

The antibiotics were dissolved in DMSO at 5 mg/ml and used at the doses indicated in the figure. The other additions were as follows (stock solution): Mitochondria (M), 1.5 mg as protein; rotenone (1.5 mM in MeOH), 0.015 μmol; ADP (37 mM in H_2O), 0.37 μmol; KCN (100 mM in H_2O), 1.0 μmol.

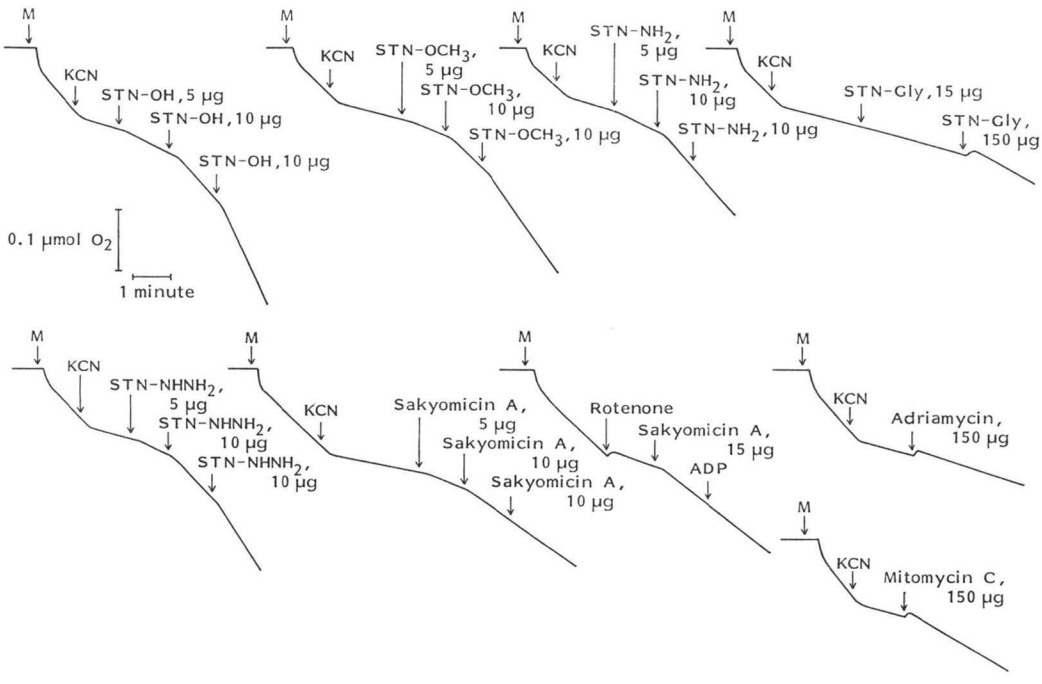
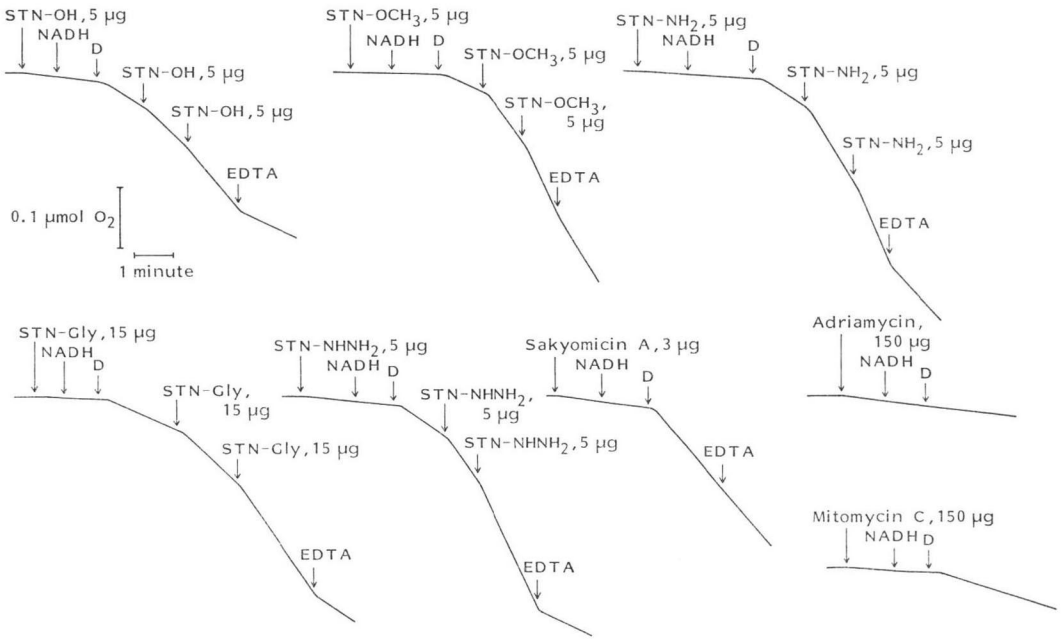


Fig. 3. Effect on the oxidation of NADH by *C. kluyveri* diaphorase.

The oxidation of NADH by bacterial diaphorase was measured by the same method as the respiration of mitochondria in 3 ml of a basal medium.

Additions: Diaphorase (D) (900 units/ml in H_2O), 9.0 units; NADH (100 mM in H_2O), 1.0 μmol; EDTA (150 mM in H_2O), 7.5 μmol.



ed at 30°C by oxygen consumption as determined with a Clark type electrode (Yellow Spring Instrument Co., Yellow Spring, Ohio)[10]. The vessel of the electrode held 3 ml of a basal medium (225 mM sucrose, 5 mM potassium phosphate and 10 mM Tris-HCl, pH 7.4).

Antibiotic-dependent Oxygen Consumption Coupled with the Oxidation of NADH by Bacterial Diaphorase

Oxygen consumption catalyzed by an antibiotic in conjugation with the oxidation of NADH by *C. kluyveri* diaphorase was measured by the same method as the respiration of rat liver mitochondria.

Results

The effects of antibiotics on the oxidation of glutamate by rat liver mitochondria were measured in the presence of KCN to prevent oxygen from being consumed by the electron transfer complex in mitochondria (Fig. 2). **1** and its derivatives **2**, **3** and **5** accelerated mitochondrial oxygen consumption, which proceeded in a continuous fashion at the dosage as low as 5 μg without showing a stoichiometric relationship to the added amount of antibiotic. However, no marked effect was observed by **4** even at 150 μg. Although sakyomicin A increased oxygen consumption at the same dosage as **1** but to a lesser extent, the progressive increase in oxygen consumption was not observed over 15 μg of accumulated dose. In addition, sakyomicin A-dependent oxygen consumption was observed in the presence of rotenone without being coupled with the phosphorylation of ADP, resembling a streptonigrin-dependent one. At the dosage of 150 μg, no enhancement in oxygen consumption was observed by adriamycin and there was only a slight enhancement by mitomycin C.

Though **4** failed to enhance the KCN-insensitive respiration of mitochondria, it was shown to be

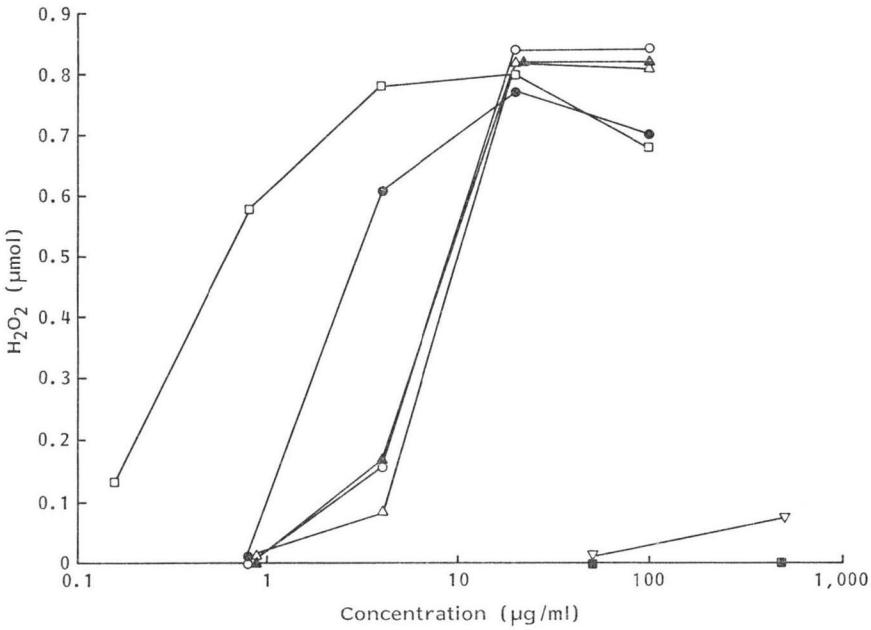
Fig. 4. Generation of H_2O_2 coupled with the oxidation of NADH by *C. kluyveri* diaphorase.

A mixture of 9.0 units/ml diaphorase, 0.9 mM NADH, 0.05 mM EDTA and 10 mM Tris-HCl (pH 8.0) was incubated with an antibiotic at 37°C for 5 minutes in a final volume of 1 ml.

H_2O_2 formed was measured by the method described in the text.

The antibiotics were dissolved in and diluted with DMSO to adjust the final concentration of DMSO in a reaction mixture at 1%.

1 ○, **2** ●, **4** △, **5** ▲, sakyomicin A □, adriamycin ■, mitomycin C ▽.



active as an electron acceptor for *C. kluyveri* diaphorase (refer Figs. 3 and 4). The effect of sakyomicin A in enhancing the oxygen consumption and the generation of H_2O_2 which accompanied the oxidation of NADH by bacterial diaphorase was greater than for any streptonigrin derivatives. Even at 150 μg (Fig. 3), there was no significant effect of doxorubicin and mitomycin C.

Since the autoxidation of reduced streptonigrin had been reported to be inhibited by EDTA[10], the effect of EDTA on the oxidation of NADH by mitochondria was investigated. As shown in Fig. 3, EDTA (approximately 250-fold, on a molar basis) did not completely inhibit antibiotic-dependent oxygen consumption for all the compounds tested. This was further confirmed by the experiment in which H_2O_2 was determined (Fig. 5). In the range of 0.05~0.5 mM EDTA, the streptonigrin-dependent generation of H_2O_2 showed a maximum value, but no marked effect of EDTA in the sakyomicin A-dependent case was observed with concentrations up to 5 mM.

For both **1** and sakyomicin A, the oxygen consumption by mitochondria using succinate as a substrate was not observed in the presence of KCN (Fig. 6). Although the streptonigrin-dependent oxygen consumption by mitochondria using glutamate was abolished by the addition of dicumarol, an inhibitor of DT-diaphorase, sakyomicin A-dependent reaction was not affected by dicumarol (Fig. 7). In agreement with the previous reports[10,11], the oxygen consumption by mitochondria using extramitochondrial NADH as a substrate was not observed by **1**; sakyomicin A initiated the oxygen consumption by mitochondria using NADH but not NADPH (Fig. 8).

Fig. 5. Effect of EDTA on the antibiotic-dependent H_2O_2 generation catalyzed by *C. kluyveri* diaphorase.

A mixture of 9.0 units/ml diaphorase, 0.9 mM NADH, 50 μg/ml either **1** or sakyomicin A, EDTA in the range of 0.005~50 mM and 10 mM Tris-HCl (pH 8.0) was incubated at 37°C for 5 minutes in a final volume of 1 ml.

1 ○, sakyomicin A □.

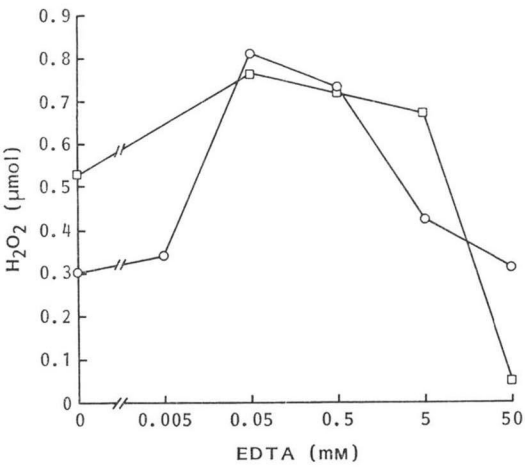
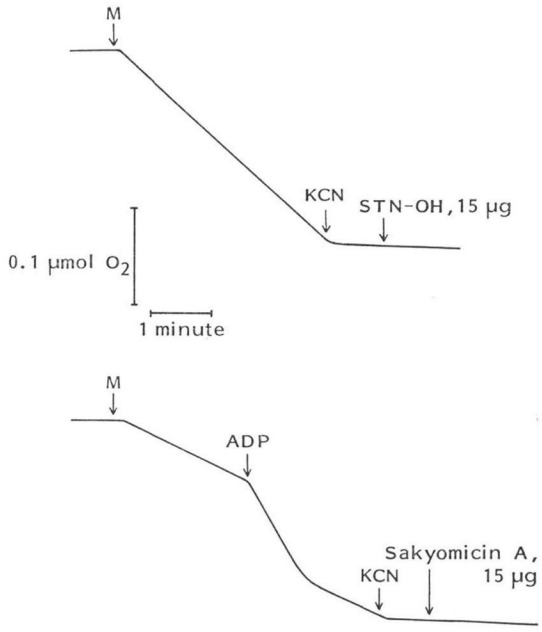


Fig. 6. Effects of streptonigrin and sakyomicin A on the oxidation of glutamate by rat liver mitochondria.

The respiration of mitochondria was measured in 3 ml of a basal medium supplemented with 15 mM succinate.

Additions: Mitochondria (M), 1.5 mg as protein; ADP, 0.37 μmol; KCN, 1.0 μmol.



Discussion

As for the streptonigrin derivatives, the effects of **2**, **3** and **5** on the oxidation of glutamate by rat liver mitochondria were almost comparable to that of **1** (Fig. 2). In contrast, **4** did not show any marked effect even at a higher concentration. All the streptonigrin derivatives, including **4**, were active as electron acceptors in the oxidation of NADH by bacterial diaphorase, but the dosage of **4** necessary to obtain a corresponding activity was approximately 3 times that of **1** (Fig. 3). The electron-acceptor activity of **4** was further confirmed by the generation of H_2O_2 coupled with the oxidation of NADH by the same enzyme (Fig. 4). From these results, it appeared that the extremely low cytotoxicity of **4** reported in the previous paper[8] was mainly due to its reduced membrane transport. The effects of **1** and its derivatives on mitochondrial respiration on glutamate were somewhat related to their cytocidal activities (Fig. 2). These activities were not connected with electron-acceptor properties (Figs. 3 and 4). HOCHSTEIN *et al.*[10] had suggested that since the oxygen consumption by either rat liver mitochondria or DT-diaphorase was completely lost in the presence of EDTA, the autoxidation of a reduced form (hydroquinone) of **1** was facilitated with metals. However, our results were contrary to their findings: The complete inhibition of streptonigrin-dependent oxygen consumption was not observed using even 250-fold EDTA on a molar basis. Moreover, the generation of H_2O_2 catalyzed by bacterial diaphorase showed a maximum value in the presence of an approximately equimolar amount of EDTA (Fig. 5).

Among the other quinone antibiotics tested, only sakyomicin A initiated the KCN-insensitive mitochondrial oxygen consumption; this effect was also insensitive to rotenone, when glutamate was used as a substrate (Fig. 2). Sakyomicin A was found to be more active than any other streptonigrin derivatives as an electron acceptor in the oxidation of NADH by bacterial diaphorase (Figs. 3 and 4). Furthermore, only a catalytic amount of sakyomicin A was necessary for the complete oxidation of NADH by bacterial diaphorase, (Fig. 4). As in the case of **1**, the generation of H_2O_2 was coupled with the autoxidation of sakyomicin A. The KCN-insensitive oxidation of succinate by mitochondria was

Fig. 7. Sensitivity to dicumarol of the antibiotic-dependent oxidation of glutamate by rat liver mitochondria.
The respiration of mitochondria was measured as described in the legend to Fig. 2.
Additions: Mitochondria (M), 1.5 mg as protein; dicumarol (15 mM in H_2O), 0.15 μmol; KCN, 1.0 μmol; ADP, 0.37 μmol.

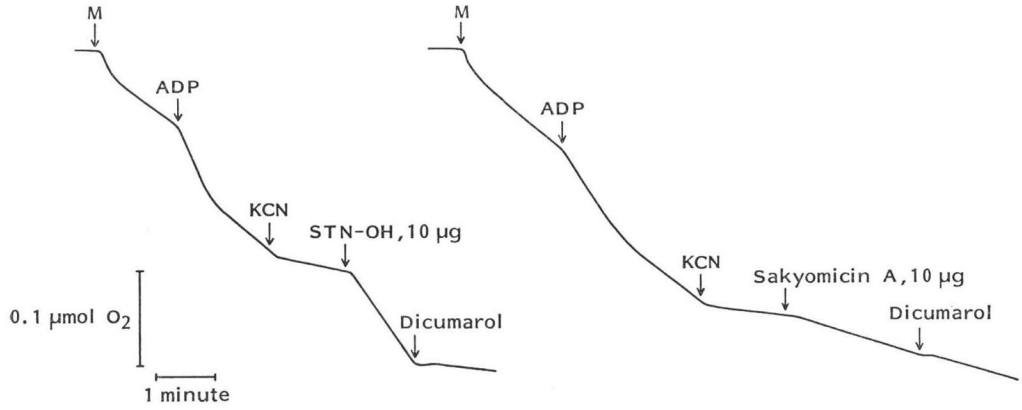
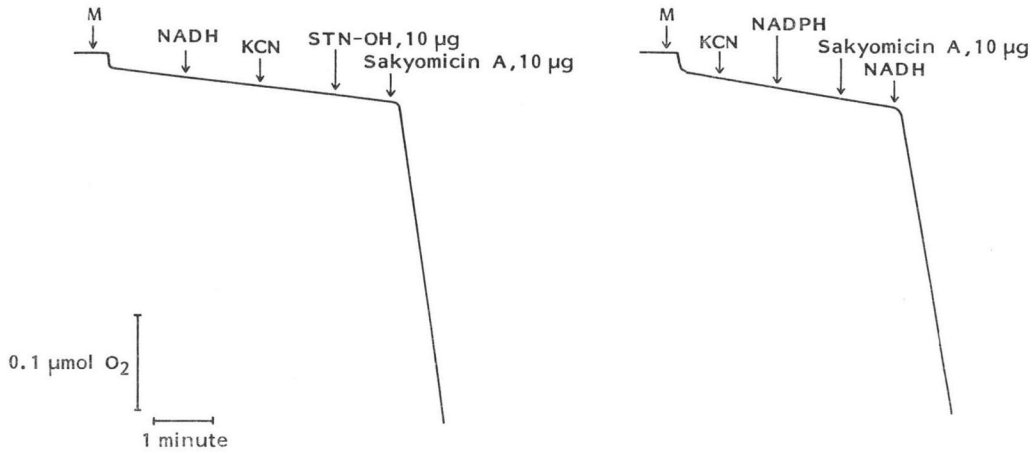


Fig. 8. Effects of streptonigrin and sakyomicin A on the oxidation of extramitochondrial NADH or NADPH by rat liver mitochondria.
The respiration of mitochondria was measured as described in the legend to Fig. 3.
Additions: Mitochondria (M), 1.5 mg as protein; NADH, 1.0 μmol; NADPH (100 mM in H_2O), 1.0 μmol; KCN, 1.0 μmol.



not observed in the presence of either **1** or sakyomicin A. In contrast to **1**, the sakyomicin A-dependent oxidation of glutamate by mitochondria was not affected by dicumarol, an inhibitor of DT-diaphorase. Externally added NADH was consumed by mitochondria in the presence of sakyomicin A but not **1** (Fig. 8); this indicates that the enzyme(s) involved in this reaction (NADH: quinone oxidoreductase) are distant from DT-diaphorase [NAD(P)H: quinone oxidoreductase, EC 1.6.99.2] and the oxidative phosphorylation complex in mitochondria. The difference between the more potent activity of sakyomicin A as an electron acceptor and the reduced effect on the KCN-insensitive oxidation of glutamate by mitochondria in comparison with **1** might result from its poor membrane transport. It remains to be elucidated whether endogenous and exogenous NADHs are oxidized by the same enzyme(s) or different enzymes distinguished from DT-diaphorase.

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