A UNIQUE, DICOUMAROL-SENSITIVE, NON-PHOSPHORYLATING OXIDATION OF DPNH AND TPNH CATALYZED BY STREPTONIGRIN

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Streptonigrin (SN) is a quinoid antibiotic which has been isolated from Streptomyces flocculus (Rao, et al., 1963). This agent inhibits net DNA synthesis in bacterial cells, initiates bacterial DNA degradation, and induces phage production in lysogenic bacteria (Levine and Borthwick, 1963). In addition, SN causes chromosome breakage in human leukocytes cultured in vitro (Cohen, et al., 1963), and it is under investigation as a potential anti-tumor agent in man (Harris, et al., 1964).

In this paper, we report a unique effect of SN which may well represent the basis for its action on biosynthetic processes and its cytotoxicity in the systems described above. This effect involves the catalytic oxidation of intra- and extra-mitochondrial DPNH and of extra-mitochondrial TPNH. The oxidation is sensitive to dicoumarol and thus is presumably mediated by the enzyme DT diaphorase (Ernster, et al., 1962a). However, unlike the oxidation of DPNH and TPNH mediated by DT diaphorase through vitamin K_3 (Conover and Ernster, 1962), that induced by SN is characterized by insensitivity to antimycin A and cyanide, and by a complete lack of respiratory control. However, SN-dependent respiration is sensitive to EDTA and is accompanied by the formation of H_2O_2.

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Methods: Mitochondria were prepared from rat liver in 0.25 M sucrose as described by Ernster, et al. (1962b). DT diaphorase was purified from the soluble supernatant fraction of rat liver homogenates by the method of Ernster, et al. (1962a). Glutathione peroxidase was prepared from rat liver homogenates and from sheep erythrocytes (Hochstein and Cohen, 1964). Incubations were carried out in Tris buffer, 0.025 M, pH 7.4 at 22°-24° C. The exact compositions of the reaction media are described in the legends to the figures and table. DPNH and TPNH disappearance were measured spectrophotometrically at 340 mu. Reduced glutathione was determined by the DTNB method (Beutler, et al., 1963). Inorganic phosphate was analyzed by the procedure of Martin and Doty (1949). Oxygen consumption was measured polarographically with a Gilson Oxygraph fitted with a Kahn vibrating platinum electrode (Kahn, 1964).

Results: A suspension of rat liver mitochondria incubated with glutamate consumed low amounts of oxygen until ADP was added to the system (Figure 1). With ADP good respiratory control was established with the P/O approaching 3. Amytal blocked this respiration. In the presence of vitamin K_3 an amytal-insensitive bypass of respiration was observed as reported by Conover and Ernster (1962). This K_3 dependent respiration was sensitive to cyanide. However, the addition of catalytic amounts of SN established a new amytal- and cyanide-insensitive respiration which, in turn, was blocked by dicoumarol.

SN-induced respiration in the presence of glutamate was not dependent on phosphate acceptor (ADP), as shown in Figure 2. The P/O was zero, and the respiration was completely uncoupled. Antimycin A had no effect on SN-catalyzed respiration, although this agent inhibits

respiration mediated by K_3. In addition, EDTA, which had no effect on respiration with K_3, completely blocked SN-induced oxygen consumption.

The sensitivity of the SN-induced respiration to dicoumarol indicated the involvement of endogenous mitochondrial DT diaphorase in mediating the transfer of electrons from intra-mitochondrial DPNH to SN.

Figure 3 shows how in the presence of purified DT diaphorase SN also catalyzed an oxidation of extra-mitochondrial DPNH. Again, this respiration was not dependent upon the presence of ADP, but was inhibited by EDTA (as well as by dicoumarol). Vitamin K_3 re-established

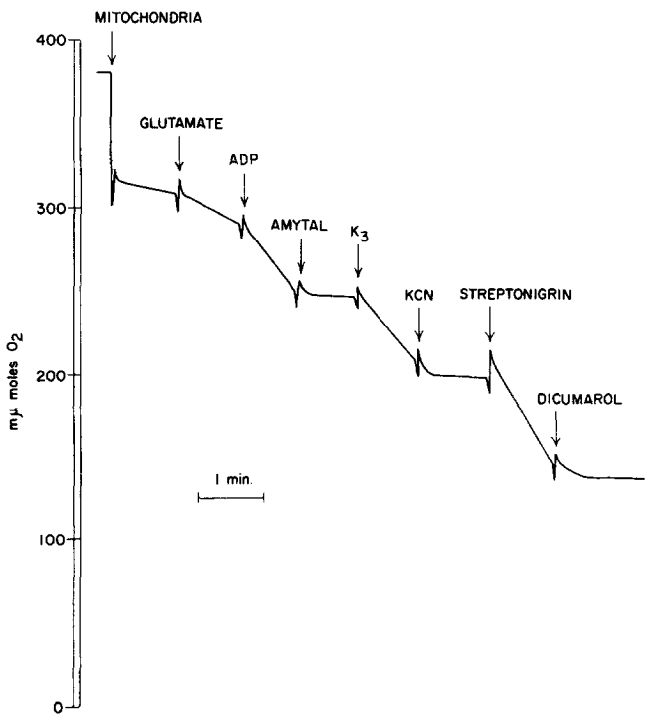

Fig. 1. Dicoumarol-sensitive respiration catalyzed by Streptonigrin. Basal medium contained in the following final concentrations: Tris, 25 mM; $MgCl_2$, 5 mM; KPO_4, 10 mM. Additions: mitochondria, 4.5 mg. protein; Glutamate, 10 mM; ADP, 1.0 mM; Amytal, 1.5 mM; K_3, 0.01 mM; KCN, 1.0 mM; SN, 0.01 mM; Dicoumarol, 0.015 mM. Final Volume: 1.5 ml.

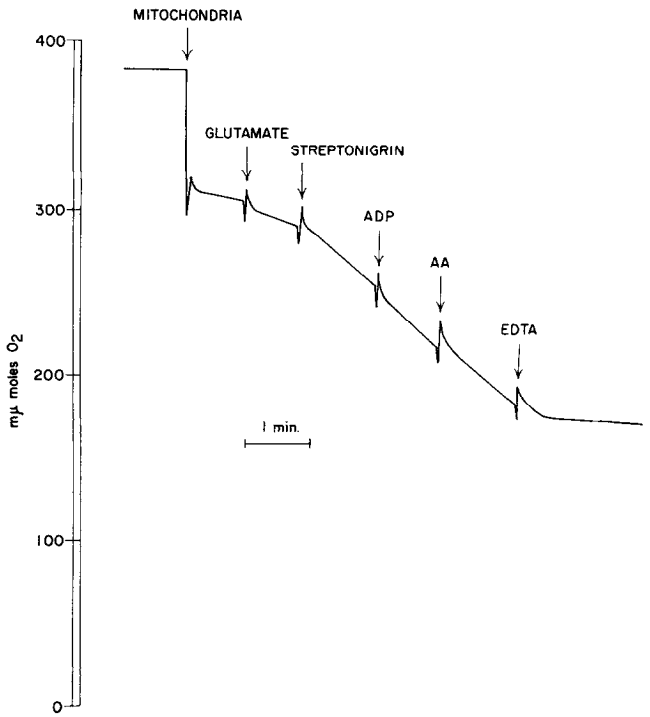

Fig. 2. EDTA-sensitive respiration catalyzed by Streptonigrin. The basal medium was as described in Fig. 1. Additions were as follows: mitochondria, containing 4.5 mg. protein; Glutamate, 10 mM; SN, 0.01 mM; Antimycin A, 5 ug; EDTA 1.5 mM. Final Volume: 1.5 ml.

the EDTA-sensitive respiration, and this respiration then became sensitive to cyanide (as well as to antimycin A). Identical results were obtained when TPNH was substituted for DPNH in these experiments.

Experiments not presented in this preliminary report have demonstrated that purified DT diaphorase - with no acceptor other than oxygen - will mediate the oxidation of DPNH and TPNH (as measured spectrophotometrically and polarographically) in the presence of catalytic amounts of SN in a dicoumarol- and EDTA sensitive fashion.

The catalytic action of SN in mediating DPNH and TPNH oxidation and its sensitivity to EDTA, suggested that SN was alternating between reduced and oxidized forms with the resultant generation of hydrogen peroxide. That this was indeed the case in shown in Table 1. In the presence of catalytic amounts of SN, and a highly purified preparation of glutathione peroxidase, the peroxide formed through the oxidation of DPNH or TPNH could be coupled to the oxidation of reduced glutathione.

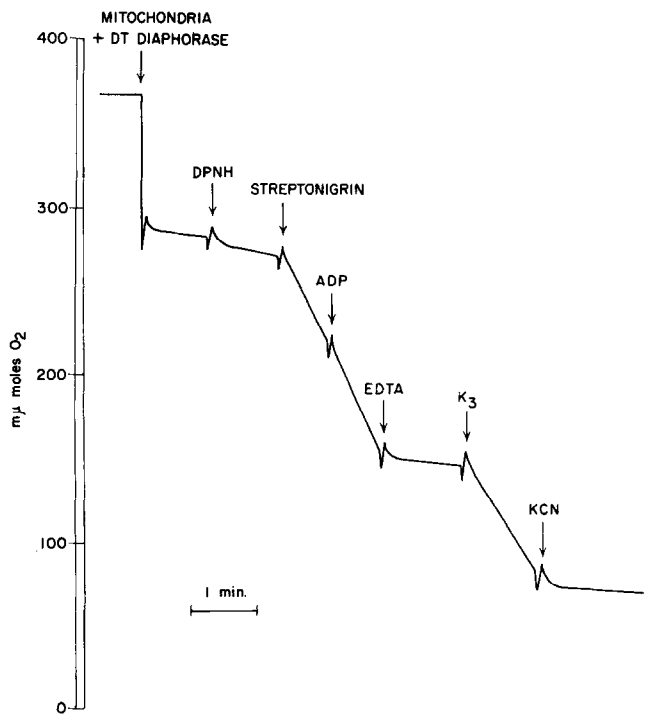

Fig. 3. SN-catalyzed oxidation of extra-mitochondrial DPNH. The basal medium was as described in Fig. 1. Additions were made as follows: Mitochondria containing 4.5 mg. protein; DT diaphorase, 300-fold purified and capable of reducing 5 u-moles DCPIP/min., DPNH, 0.6 mM; SN, 0.01 mM; ADP, 1.0 mM; KCN, 1.0 mM. The final volume was 1.5 ml.

Table 1. The oxidation of reduced glutathione (GSH) by catalytic amounts of SN. The basal medium was as described in Figure 1 and, in addition, contained the following: Mitochondria, 4.5 mg. protein; DT diaphorase, (as described in Figure 3); DPNH, 0.6 mM; GSH, 0.6 mM. Where indicated the following were also added: SN, 0.01 mM; EDTA, 1.0 mM; GSH peroxidase, a preparation purified 600-fold and essentially free of catalase. The final volume was 2.0 ml. and the reactions were run for 20 minutes.

Additions	Δ GSH (umoles)
1. None	0.05
2. Streptonigrin	0.05
3. Streptonigrin, GSH Peroxidase	1.05
4. Streptonigrin, GSH Peroxidase, EDTA	0.03

Discussion: From the results described above it is concluded that SN catalyzes an oxidation of intramitochondrial DPNH via mitochondrial DT diaphorase. SN may also catalyze a similar oxidation of extramitochondrial DPNH or TPNH with DT diaphorase purified from the soluble fraction of the cell. Unlike vitamin K_3-dependent oxidation of DPNH or TPNH mediated by the same enzyme, SN-induced oxidation is not coupled to phosphorylation but leads instead to the generation of hydrogen peroxide. We visualize that SN is reduced by DPNH or TPNH and undergoes a rapid, metal-catalyzed (EDTA-sensitive) autoxidation resulting in the formation of hydrogen peroxide and the regeneration of the quinoid form of SN which is again available for enzymatic reduction. It should be mentioned that we have observed a dicoumarol-insensitive reduction of SN to take place in other tissues which is presumably not mediated by DT diaphorase.

It should also be noted that, whereas in a tissue such as liver the reduction of SN may lead to its conjugation and detoxication, in other tissues and cells SN may be an extremely cytotoxic compound. We view its toxicity as resulting from the operation of one or more of the following effects: 1) depletion of cellular DPNH and TPNH, 2) uncoupling of phos-

phorylation and depletion of cellular ATP, and 3) formation of peroxide.

In this last connection, it is pertinent to note that we have previously shown that glutathione peroxidase represents a major pathway for hydrogen peroxide detoxication, even in catalase-rich cells (Cohen and Hochstein, 1963, 1964). Thus cells exposed to peroxide either directly or through the autoxidation of phenolic compounds such as reduced SN, may undergo extensive damage under conditions in which reduced glutathione is depleted, e. g. in the absence of DPNH or TPNH required for glutathione reductase activity.

We suggest that the effects of SN on cellular biosynthetic processes are consequent to its profound action on electron transport and the associated formation of hydrogen peroxide.

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