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Diazoxide, an inhibitor of succinate oxidation

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CONSIDERABLE interest has been paid to the diabetogenic action of diazoxide (3-methyl-7-chloro-1, 2, 4-benzo-thiadiazin-1, 1dioxide), mediated by a suppression of insulin secretion *in vivo*[1] and *in vitro*.[2] Moreover, the hyperglycemic effect has been attributed to activation of glycogenolysis in the liver on the basis of either a direct inhibition of enzymatic degradation of 3',5'-AMP, or liberation of catecholamines from adrenals and extraadrenal reserves.[3a, 3b] The present report, however, presents evidence that diazoxide may function as an inhibitor of citric acid-cycle oxidations by means of a pronounced inhibition of succinate oxidation in isolated liver mitochondria.

Rat liver mitochondria were prepared and incubations were carried out as described previously.[4] Respiration was measured polarographically with a platinum micro electrode,[4] and succinate dehydrogenase has been tested with cytochrome *c* as terminal electron acceptor as described elsewhere.[5] Diazoxide was solubilized in ethanolic 0·1 N NaOH and adjusted to pH 7·6 with HCl. ATP-ase was measured according to Pressman.[6]

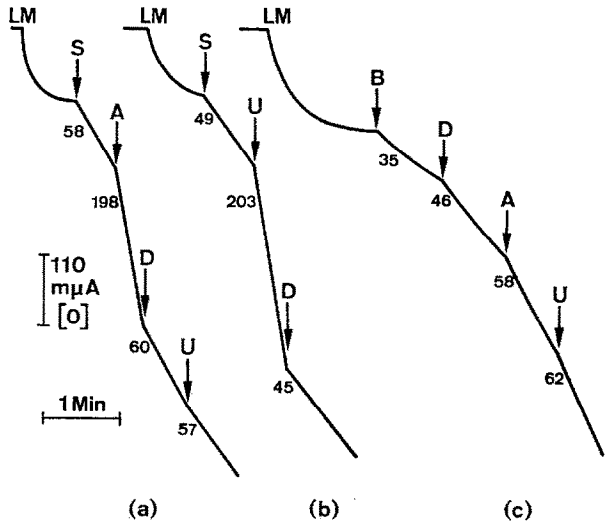


FIG. 1. Polarographic record of respiratory inhibition by diazoxide. At the beginning of each trace 3·6 mg rat liver mitochondria were added; total volume 2·54 ml; buffer: 0·25 M sucrose, 1mM triethanolamine pH 7·2; 4 mM $MgCl_2$, 4 mM phosphate; 25°; other additions: S = 2·5 mM succinate, B = 5 mM β-hydroxybutyrate, A = 0·5 mM ADP, U = 2 μM uncoupler (CCP), D = 0·4 mM diazoxide. The figures signify respiratory rates in nA G/min·mg protein.

As demonstrated by experiment (a) in Fig. 1, the rapid respiration of mitochondria with succinate in presence of ADP was immediately inhibited by addition of diazoxide. This inhibition was not released by subsequent addition of an uncoupler, suggesting that diazoxide does not act on the energy conservating reactions of the respiratory chain. This is supported by experiment (b), making obvious

that also in uncoupled mitochondria a strong inhibition of succinate dependent respiration was obtained with diazoxide. A direct inhibition of electron transport in the respiratory chain is ruled out by the observation that diazoxide is unable to inhibit the respiration with β-hydroxybutyrate which delivers electrons to the respiratory chain at the NADH-level. Experiment (c) shows that despite the presence of diazoxide the oxidation of β-hydroxybutyrate could be accelerated by ADP or by an uncoupler, which was not the case when succinate served as hydrogen donor for the respiratory chain. Thus, it is concluded from these results that the inhibitory effect of diazoxide is highly specific to succinate oxidation.

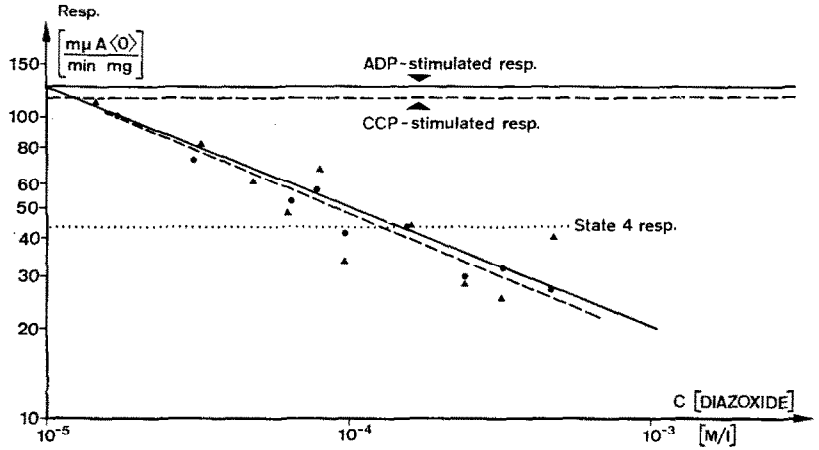


FIG. 2. Correlation of respiratory inhibition by diazoxide and inhibitor concentration. Expt. conditions as in Fig. 1. Maximum respiration in presence of excess ADP or of uncoupler indicated by upper lines. Dotted line: respiration with succinate in absence of ADP and inhibitor. ● CCP stimulated system. ▲ ▲ ▲ ADP stimulated system.

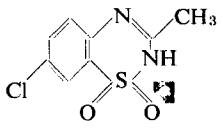
In FIG. 2. the respiratory rate of mitochondria with succinate is plotted against increasing concentrations of diazoxide. In one series of experiments maximum respiration was induced by excess ADP, in the other by the presence of an uncoupler. In both cases oxygen uptake was diminished proportionally to the inhibitor concentration in a double logarithmic plot. The regression curves are practically identical for both types of experiments. A particular inhibition of succinate oxidation is suggested not only by the fact, that oxygen uptake is suppressed below the state 4 rate (respiration in absence of ADP), but also by the finding that the activity of succinate dehydrogenase in disrupted liver mitochondria is gradually inhibited by increasing concentrations of diazoxide, as summarized in Table 1. In addition, the table shows that the rate of NADH oxidation by nonphosphorylating sonic particles from beef heart mitochondria was not influenced by diazoxide, which is in support of the above result that the compound does not inhibit NAD-dependent oxidations or the respiratory chain itself. On the basis of its molecular structure diazoxide has been expected to exert an uncoupling effect,

TABLE 1. ACTION OF DIAZOXIDE ON THE ACTIVITY OF SUCCINATE-DEHYDROGENASE AND ON NADH-OXIDATION BY THE RESPIRATORY CHAIN

Concentration diazoxide × 10^{-6} M/l.	0	16	49	82	115	164	170	350	520
SDH activity mU/mg Prot. (liver mitochondria)	86–93	69	48	39	36	27	—	—	—
Rate of NADH-oxid. nA O/min/mg Prot. (heart sonic particles)*	205	—	—	—	—	—	240	205	202

* Sonic particles were prepared according to Linnane *et al.*[7]

since the molecule shares some common characteristics with a large group of typical uncou



lipophilic properties in the undissociated state of the molecule, and the presence of an acidic group. This concept is realized with compounds as the uncoupling benzimidazoles[10,11] or the tives of CCP (carbonylcyanide phenylhydrazone), which can be completely inactivated by *N* lation as recently shown in our laboratory.[12] However, with diazoxide uncoupling activ essentially absent. Only a negligible increase of mitochondrial ATP-ase could be observed concentrations. The stimulation of ATP-ase was more pronounced when ATP-ase has alrea activated with CCP; in this case diazoxide caused a further increase of ATP-decomposition, as in Table 2.

TABLE 2. INCREASE OF CCP-STIMULATED ATP-ase ACTIVITY OF LIVER MITOCHONDRIA BY DIAZOXIDE

Diazoxide concentration x10^{-4}M	0	1·0	2·0	3·9	5·9	9·9
P/min liberated x10^{-7}M	2·0	2·4	2·8	3·1	4·2	5·1

In contrast to the weak ATP-ase activation, the inhibitory effect of diazoxide on succinate o may be considered to be related to the metabolic action of the compound, and the quest to be asked, which of the observed effects of diazoxide on intact tissues is the primary c inhibition of insulin release, the inhibition of 3', 5'-AMP phosphodiesterase, or the inhib succinate dehydrogenase. The latter should be reflected by a decreased rate of the citric aci due to a diminished rate of oxaloacetate formation. It is under investigation whether the in of insulin secretion from Langerhans islets by diazoxide—as reported by Löffler[13]—is relate inhibition of aerobic metabolism described here.

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Inhibition of drug demethylation by disulfiram *in vivo* and *in vitro*

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RAM is known as an inhibitor of liver aldehyde dehydrogenase[1, 2] and dopamine-ß-hydroxy- Recent clinical observation showed that disulfiram, when given to patients receiving other imultaneously, increased the serum levels of these drugs, e.g. that of diphenylhydantoin.[5] iphenylhydantoin is metabolized chiefly by *p*-hydroxylation to 5-(*p*-hydroxyphenyl)-5-phenyl-)in,[6] it was to be expected that disulfiram increases the serum levels of diphenylhydantoin by ing with its hydroxylation. In our respective experiments, however, disulfiram and its metabo-ıhyldithiocarbamate interfered with the determination of 5-(*p*-hydroxyphenyl)-5-phenyl)in according to the method of Folin–Ciocalteu[7] presumably by reducing the reagent and ıulating the presence of phenolic hydroxyl groups. Therefore, we studied the effects of disulfiram ; hydroxylation with amidopyrine and *p*-nitroanisole (*p*NA) as substrates. Amidopyrie is ely metabolized to 4-amidoantipyrine (4-AAP) part of which is acetylated to *N*-acetyl-4- N-demethylation). *p*NA is metabolized to *p*-nitrophenol[9] (*O*-demethylation).

ition of amidopyrine demethylation in vivo. In male Sprague–Dawley rats of 200–250 g body the left carotid artery was cannulated with polyethylene tubing according to[10] for repeated rawing. Disulfiram (1 g/kg) in an aqueous 1 % suspension of tragacanth was administered) these animals 15 hr prior to the i.p. application of amidopyrine (50 mg/kg). At 1, 3 and 6 hr ıidopyrine about 1·5 ml of blood was obtained from the cannula. 0·6 ml of plasma was used determination of total 4-APP. To minimize blood loss the sedimented erythrocytes were ed in the same volume of isotonic saline and reinjected through the catheter. Total 4-APP ermined according to[8] after hydrolysis of the acetylated 4-APP (0·5 N HCl, 30 min boiling ath) with slight modifications (12 ml $CHCl_3$, filtration through siliconized filter paper).

e 1 shows the concentration of total 4-APP in plasma of the controls and disulfiram treated normal rats total 4-APP levels increase to reach a maximum at about 3 hr and then decrease ly. In the disulfiram pretreated animals the 4-APP reaches a plateau at about 1 hr after amido- ınd does not change after that for further 5 hr. Apparently this inhibition of amidopyrine lation occurs only at certain dosage and time conditions. Oral application of 1 g/kg of m only 2 hr before amidopyrine did not lead to an *in vivo* inhibition of amidopyrine lation. This finding agrees with the observation that full effects of disulfiram on the disposi- thanol were not seen until 12 to 18 hr after its application to rabbits, at which time acetalde- els are greatly increased.[11] This is perhaps due to slow intestinal absorption or to the high fat y of disulfiram which prevents it from attaining sufficiently high concentrations in the liver