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Site-specific uncoupling and inhibition of oxidative phosphorylation by biguanides. II

The inhibitory action of guanidine and its derivatives on mitochondrial respiration, as reported by HOLLUNGER[1], CHANCE AND HOLLUNGER[2], CHAPPELL[3], and PRESSMAN[4,5], shares some common properties with oligomycin inhibition, which is released by uncouplers. The same is true of biguanide derivatives[6]. Whereas oligomycin generally acts on oxidative phosphorylation independent of the source of electrons, a site specificity of certain guanidine derivatives has been observed by PRESSMAN[7] and SCHÄFER[8]. Moreover, diguanidines and biguanides, in contrast to oligomycin, require an energized state of the mitochondria in order to act as inhibitors of energy transfer[3,9]. *n*-Heptylbiguanide inhibits phosphorylation in mitochondria oxidizing glutamate–malate but uncouples oxidative phosphorylation when succinate is the substrate. According to the chemiosmotic theory, these observations have been interpreted by MITCHELL[10] as an increase of ionic permeability of the coupling membrane due to the detergent character of the compound and as a simultaneous blocking action on NADH-linked respiration, as found with low concentrations of Triton X-100. However, site specificity is hardly consistent with the chemiosmotic hypothesis, and other explanations seem possible.

The experiments of the present paper demonstrate a bifunctional mode of action on energy transfer at the site of cytochrome *b* and an inhibition of the energy-dependent transhydrogenase by alkylbiguanides.

Rat liver mitochondria were prepared as described previously[6]. Sonic particles were obtained from liver mitochondria according to the procedure of DANIELSON AND ERNSTER[11]. The protein content of the preparations was estimated by a biuret method[12]. Oxygen uptake was measured polarographically[13], and direct observation of cytochromes were performed with a Phoenix dual-wavelength spectrophotometer. Pyridine nucleotides were measured fluorimetrically, according to ESTABROOK[14].

Alkylbiguanides inhibit the transition of respiration from the "resting" state to the actively respiring state upon addition of ADP to mitochondria oxidizing NAD-linked substrates. Table I gives the average rate of State-3 respiration as observed

TABLE I

INHIBITION OF STATE-3 RESPIRATION OF LIVER MITOCHONDRIA BY BIGUANIDES

Reaction mixture (2.5 ml) contained 250 mM sucrose, 2 mM triethanolamine, 4 mM P_i, 2 mM $MgCl_2$, 0.5 mM ADP, rat liver mitochondria (3.5–7.5 mg protein) and either 5 mM glutamate *plus* 5 mM malate or 5 mM succinate. Final pH, 7.2. Temp., 25°. Each figure is the mean of 12–15 single measurements.

Substrate	*Oxygen uptake (μatoms/min per g protein)*									
	Control	*+ Biguanide Number of C atoms in side chain:*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*12*
Glutamate + malate	46	46	42	41	36	32	29	26	18	8
Succinate	109	110	101	96	94	95	102	119	125	131

after 1 min of preincubation of liver mitochondria with 1 mM of the inhibitor prior to ADP addition. In the glutamate–malate system the inhibitory effect increases with the number of carbon atoms in the side chain. In the succinate system only weak inhibition occurs with short-chain derivatives, whereas uncoupling is observed when the number of C atoms in the side chain exceeds 6.

In liver mitochondria, energized by succinate, ADP causes a partial oxidation of cytochrome *b*. Oligomycin blocks oxidative phosphorylation and leads to a reduction of cytochrome *b* (Fig. 1a). Subsequent addition of *n*-octylbiguanide primarily increased the reduction but subsequently caused an oxidation of cytochrome *b* due to an uncoupling effect, releasing the inhibition by oligomycin. This biphasic response to octylbiguanide was particularly pronounced in Expt. b of Fig. 1 where a reduction–oxidation cycle occurred upon addition of the inhibitor at State 3 in the absence of oligomycin. Two conclusions may be drawn from these results: Firstly, biguanides probably intercept the pathway of energy conservation prior to the locus of oligomycin action. Secondly, uncoupling by biguanides is a secondary process, preceded by intermediate formation of an inhibited state, the latter being documented by the transitory reduction of cytochrome *b*.

Besides oxidative phosphorylation, other energy-linked reactions are inhibited

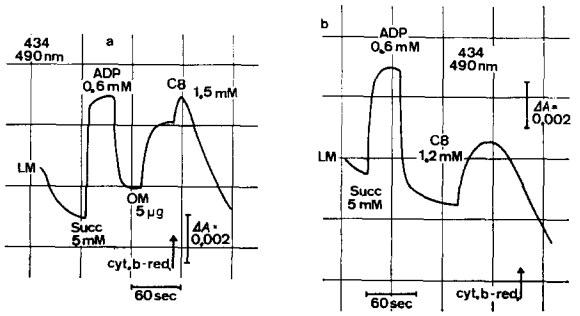

Fig. 1. Redox changes of cytochrome *b* in rat liver mitochondria. Reaction mixture (final vol. 1.5 ml) contained 250 mM sucrose, 2 mM triethanolamine, 2 mM $MgCl_2$, 4 mM P_i, rat liver mitochondria (5.1 mg protein) and the additions shown in the figure. Temp., 25°. *d* = 5 mm. LM = liver mitochondria; Succ = succinate; OM = oligomycin; C8 = octylbiguanide.

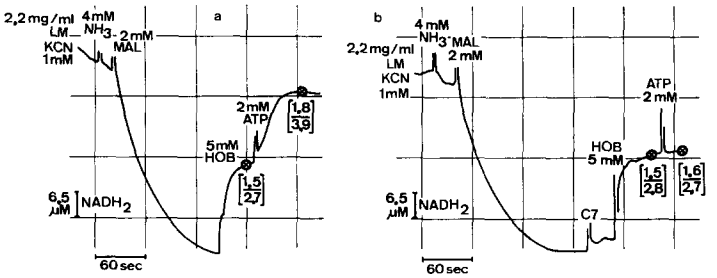

Fig. 2. Fluorimetric measurement of mitochondrial pyridine nucleotides. The reaction medium (final vol., 3.0 ml) contained 250 mM sucrose, 2 mM triethanolamine, rat liver mitochondria (6.5 mg protein) and the additions shown in the figure. Final pH, 7.2. Temp., 25°. The numbers below the trace give the concentration of NAD(P)H in deproteinized samples, the upper figure representing µmoles NADH per g protein and the lower µmoles NADPH per g protein. LM = liver mitochondria; MAL = malate; HOB = hydroxybutyrate; C7 = 1.2 mM *n*-heptylbiguanide.

by alkylbiguanides in liver mitochondria and sonic particles. According to KLINGENBERG[15], in anaerobic mitochondria supplemented with malate and NH_4^+, pyridine nucleotides are mainly oxidized. Addition of hydroxybutyrate causes reduction of NAD^+. ATP addition yields further reduction, mainly due to NADPH formation (Fig. 2a). As shown in Fig. 2b, this energy-dependent reduction is abolished in the presence of *n*-heptylbiguanide. In Fig. 2, the distribution of hydrogens between NADH and NADPH is also shown.

The effect of alkylbiguanides could be distinguished from that of oligomycin by direct observation of the energy-driven hydrogen transfer to $NADP^+$ catalysed by sonic particles in the experimental system described by ERNSTER[11]. Expts. a and b in Fig. 3 illustrate that in the presence of KCN, the ATP-supported $NADP^+$ reduction by NADH is inhibited to various degrees by oligomycin and biguanides as well. However, when the energy is generated by aerobic oxidation of succinate, oligomycin

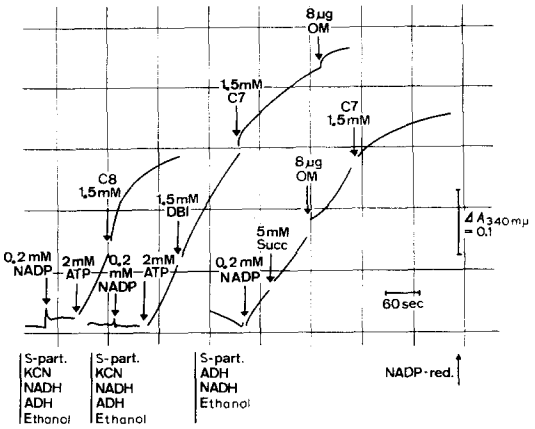

Fig. 3. Energy-dependent reduction of $NADP^+$ by NADH in sonic particles. The reaction mixture (final vol., 3.0 ml) contained 6.1 mg S-particles, 160 mM triethanolamine (pH 7.2), 200 µg alcohol dehydrogenase (ADH) (Boehringer und Soehne, Mannheim), 20 mM ethanol, 5 mM NADH and (in Expts. a and b) 2 mM KCN. Temp., 25°. Registration in a Beckman DK spectrophotometer at 340 nm. OM = oligomycin; C7 = heptylbiguanide; C8 = octylbiguanide; Succ = succinate; DBI = phenethylbiguanide.

TABLE II

INHIBITION OF NADH OXIDATION IN SONIC PARTICLES OF RAT LIVER MITOCHONDRIA BY BIGUANIDES

The reaction mixture (2.5 ml) contained 250 mM sucrose, 2 mM triethanolamine, 4 mM P_i, 2 mM $MgCl_2$, 0.4 mM NADH, sonic particles (1.56 mg protein) and (where present) 1.2 mM biguanide. Final pH, 7.2. Temp., 25°.

Prep. No.	*NADH oxidation (µmoles/min per mg protein)*		
	Control	*+ n-Heptyl-biguanide*	*+ n-Octyl-biguanide*
1	1.037	0.355	0.247
2	1.158	0.357	0.252
3	1.079	0.495	0.237
4	1.000	0.355	0.257

is ineffective, whereas *n*-heptylbiguanide again acts as a potent inhibitor. Thus, additional evidence accumulates that inhibition by guanidine derivatives originates from interaction with energy conservation at a site closer to electron transfer than the oligomycin-sensitive step. However, the question arises of whether biguanides inhibit formation or utilization of high-energy bonds. As shown in Table II the rate of aerobic oxidation of NADH by sonic particles is inhibited by heptyl- and octylbiguanide. This inhibition is not released by uncouplers and may be interpreted rather as an interference with an early energized state or its formation during coupled electron transfer.

In conclusion, the above results suggest that biguanides primarily act as blocking agents at the first and second phosphorylating sites. Site specificity may be understood as interaction of the inhibitor with a primary high-energy compound at a particular coupling site. The secondary uncoupling at Site II may be explained by subsequent decay of an intermediate complex of the inhibitor with primary energy conservation, due to its molecular properties.

In this context it should be mentioned that alkylguanidines, like uncouplers, are lipid-soluble compounds when in the form of uncharged molecules. Most uncouplers are capable of anion formation by dissociation of a proton; guanidines, however, with pK values in the range of 10–11 (ref. 16) exhibit proton-capturing properties and form cations. Therefore, a direct interaction with a proton transfer in a hydrophobic reaction space may be considered.

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1 G. HOLLUNGER, *Acta Pharmacol. Toxicol.*, 11, *Suppl.*, 1 (1955) 84.
2 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 238 (1963) 432.
3 J. B. CHAPPELL, *J. Biol. Chem.*, 238 (1963) 410.
4 B. PRESSMAN, *Federation Proc.*, 20 (1961) 51.
5 B. PRESSMAN, *J. Biol. Chem.*, 238 (1963) 401.
6 G. SCHÄFER, *Biochem. Z.*, 339 (1963) 46.
7 B. PRESSMAN, in B. CHANCE, *Energy Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 181.
8 G. SCHÄFER, *Biochim. Biophys. Acta*, 93 (1964) 279.
9 G. SCHÄFER, *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, p. 708.
10 P. MITCHELL, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, B.B.A. Library, Vol. 7, Elsevier, Amsterdam, 1965, p. 65.
11 L. DANIELSON AND L. ERNSTER, *Biochem. Z.*, 338 (1963) 188.
12 G. BEISENHERZ, H. J. BOLTZE, TH. BÜCHER, R. CZOK, E. MEYER-ARENDT AND G. PFLEIDERER, *Z. Naturforsch.*, 8b (1953) 555.
13 B. CHANCE AND R. WILLIAMS, *Nature*, 175 (1955) 1120.
14 R. W. ESTABROOK, *Anal. Biochem.*, 4 (1962) 231.
15 M. KLINGENBERG, in B. CHANCE, *Energy Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 121.
16 F. RAY, *Chem. Rev.*, 61 (1961) 313.

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