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Effects of Guanidine Inhibitors on Mung Bean Mitochondria[1]

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ABSTRACT

The effects of phenylethylbiguanidide, decamethylenediguanidide, and octylguanidine have been studied with mung bean hypocotyl mitochondria (*Phaseolus aureus* var. Jumbo) supplied with malate, reduced nicotinamide adenine dinucleotide, succinate, or ascorbate-tetramethyl-*p*-phenylenediamine as substrates. The guanidines act as energy transfer inhibitors, all three inhibiting all three phosphorylation sites. Phenylethylbiguanidide causes only partial inhibition even at relatively high concentrations. Decamethylenediguanidide inhibits about 70% of the malate respiration, 55% of the succinate respiration, and 35% of the ascorbate-tetramethyl-*p*-phenylenediamine respiration.

Octylguanidine inhibits all three phosphorylation sites and the cyanide-insensitive respiration, but to differing extents and at different concentrations. Both states 3 and 4 are inhibited by octylguanidine. Inhibition of state 4 is preceded by an uncoupling action at lower concentrations of inhibitor, while inhibition of state 3 is influenced by the state of the mitochondria when the inhibitor is added. Application of the guanidine to state 4 mitochondria is more effective than application to mitochondria already in state 3.

Ikuma and Bonner (6) have described a reliable method for the production of tightly coupled mitochondria from etiolated mung bean hypocotyl tissue. The mitochondria have been characterized (6-8) with respect to a series of substrates, inhibitors, and uncouplers of electron transport and oxidative phosphorylation.

Extensive studies of the guanidine inhibitors have been carried out with rat liver mitochondria, in which a differential sensitivity of the three coupling sites to the various guanidine inhibitors has been observed. Coupling site 1 is most effectively inhibited by the alkylguanidines, the effectiveness of the compounds increasing with increasing chain length. Octylguanidine was found to be the most effective compound acting on site 1 (10). Chappell observed that the alkylguanidines were most effective when supplied to the rat liver mitochondria in states 1 or 4 (3). DBI[2] appears to be specific in its inhibition of the coupling site 2 (11) while synthalin has been reported to be a specific inhibitor of coupling site 3 (4).

The guanidine inhibitors appear to have a site of action closer to the respiratory chain than either oligomycin or uncouplers of oxidative phosphorylation. Pressman has observed a slow release of the inhibition of the alkyguanidine to uncouplers such as DNP (11).

The guanidine inhibitors have not been studied, however, with plant mitochondria. In this paper the effects of octylguanidine, DBI, and synthalin have been examined on mung bean mitochondria. Differential effects of octylguanidine on the three coupling sites and the cyanide-insensitive respiration will be described.

METHODS

Mitochondria were prepared from the hypocotyls of 5-day-old dark-grown mung beans (*Phaseolus aureus* var. Jumbo) as described earlier (6). The mung bean tissue was disrupted by a 5-sec treatment with a Polytron mixer.

Octylguanidine sulphate, DBI, and synthalin were kindly supplied by Dr. B. Pressman of the Johnson Research Foundation and were dissolved in ethanol or distilled water. NADH was obtained from Sigma, and TMPD from Eastman. 1799, a potent uncoupler of oxidative phosphorylation, was kindly supplied by Dr. P. Heytler of Dupont De Nemours, Wilmington, Delaware.

The effects of the guanidines on respiration, with malate, NADH, succinate, or ascorbate-TMPD (12) as substrates, were measured by monitoring the oxygen uptake with a conventional Clark electrode (Yellow Springs Instrument Co.) in a stirred cuvette. Two methods of application were used.

A. Inhibitor Added to Mitochondria in State 4. After addition of substrate, mitochondria were brought into state 4 by the addition of 0.54 mM ATP and incubated for 1.5 min when a steady rate of oxygen uptake was reached. The inhibitor was then added and after 1 min of incubation the mitochondria were brought to state 3 by the addition of 0.54 mM ADP. The steady rate of oxygen uptake reached after this addition was measured.

B. Inhibitor Added to Mitochondria in State 3. Mitochondria were incubated in the presence of substrate, 0.54 mM ATP, and 0.54 mM ADP for 1.5 min when the rate of oxygen uptake was constant. Successive small additions of inhibitor were then added at 1-min intervals, or after oxygen uptake had reached a constan rate following the previous addition.

The basic reaction mixture for the oxygen electrode experiments consisted of 0.3 mM mannitol, 10 mM KCl, 10 mM potassium phosphate buffer (pH 7.2), and 5 mM $MgCl_2$; 10 mM succinate, 1 mM NADH, or 40 mM malate was added as substrate. Studies of site 3 were carried out with a freshly prepared mixture of 8 mM ascorbate and 0.8 mM TMPD as substrate (12).

The effects of octylguanidine on site 2 by method A was studied by following the reduction of 3 mM potassium ferricyanide (9) in the presence of 0.06 mM KCN at 420 to 470 nm with a double beam spectrophotometer or at 420 nm in a split beam spectrophotometer (2).

[1] This work was supported by a grant from the National Science Foundation.

[2] Abbreviations: DBI: phenylethylbiguanidide; synthalin: decamethylenediguanidide; 1799: bis(hexafluoroacetonitryl) acetone; TMPD: N,N,N^1,N^1-tetramethyl-*p*-phenylenediamine.

RESULTS

DBI. Only slight inhibition of succinate, malate, or NADH oxidation resulted when DBI was used as inhibitor with method B of application (Fig. 1). The half-maximal inhibition was 1 mM for succinate oxidation, 0.08 mM for malate oxidation, and 0.7 mM for NADH oxidation. Because of the poor response to the inhibitor, DBI was not studied further. A poor response was also obtained when method A was used, the concentrations required being slightly lower than those for method B.

Synthalin. Synthalin was found to inhibit malate, succinate, and ascorbate-TMPD oxidations; the inhibitions, however, were incomplete (Fig. 2). With method B of application the proportion of respiration not inhibited by synthalin is similar to the state 4 respiration; inhibition of state 4 respiration was observed when method A was used. The half-maximal inhibitory concentrations for state 3 were 0.3, 0.6, and 0.6 mM for malate, succinate, and ascorbate + TMPD, respectively, with maximum inhibitions of 70, 55, and 35% for each of the substrates, respectively. Contrary to observations with octylguanidine, synthalin inhibition was slowly released by 1799 (Fig. 3).

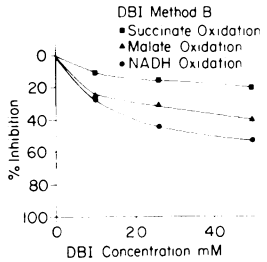

FIG. 1. The effects of DBI on oxygen uptake by mung bean mitochondria supplied with succinate, malate, or NADH as substrate. Method B of application. Experimental conditions as described in the text.

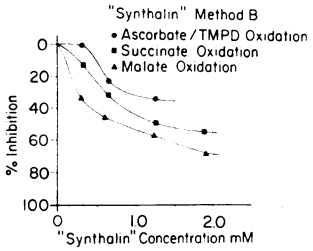

FIG. 2. The effects of synthalin on oxygen uptake by mung bean mitochondria supplied with malate, succinate, or ascorbate-TMPD mixture as substrate. Method B of application. Experimental conditions as described in the text.

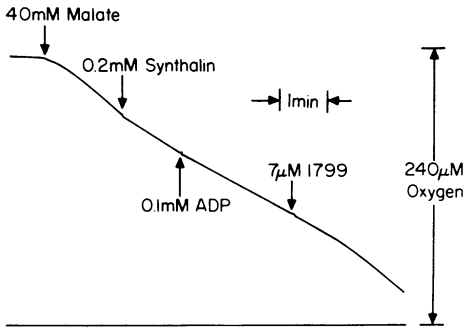

FIG. 3. The release of synthalin inhibition of malate oxidation by 7 μM 1799. Method A of application. Experimental conditions as described in the text.

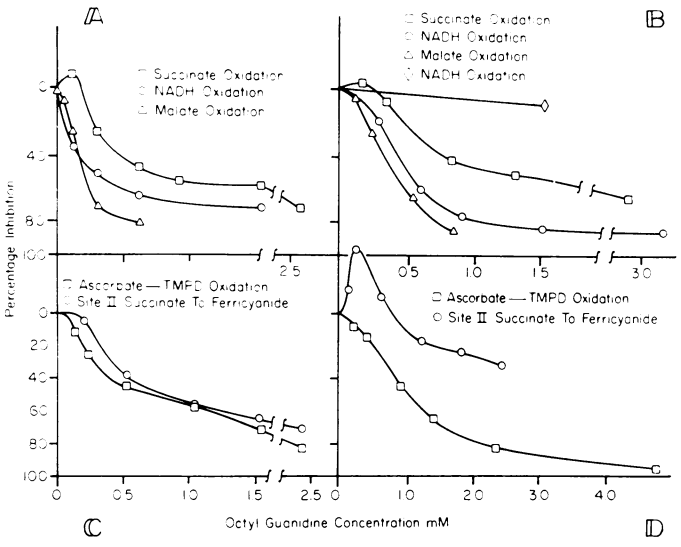

FIG. 4. The effects of octyl guanidine on oxygen uptake by mung bean mitochondria. Assay of coupling sites II and III with succinate, malate, or NADH as substrate. Experimental conditions as described in the text. Curves A and C: Method A of application; B and D: method B.

Table I. *Octyl Guanidine Half-maximal Inhibitory Concentrations for State 3 Mung Bean Mitochondrial Oxidation*

Substrate	Method A	Method B
Malate	0.16	0.38
NADH	0.14	0.36
Succinate	0.42	0.68
Ascorbate-TMPD	0.46	0.88
Site II succinate-cytochrome *c*	0.46	0.78
KCN-insensitive succinate	1.12[1]	2.68[1]

[1] Mitochondrial concentration-dependent, values shown are for 0.8 and 1.2 mg of mitochondrial protein per ml of reaction medium for methods A and B, respectively.

Octylguanidine. Unlike the observations with rat liver mitochondria, octylguanidine inhibited all substrates and coupling sites in both states 3 and 4. The state 3 inhibition in mung bean mitochondria was similar to that of rat liver mitochondria in that the inhibitor was most effective when added to the mitochondria in state 4. Figure 4 shows the effects of octylguanidine on state 3 mung bean mitochondria with both methods of application and Table I lists the half-maximal inhibitory concentration of octylguanidine for each of the substrates and coupling sites examined.

With the mung bean mitochondria it was not possible to release the octylguanidine inhibition by uncouplers. However, treatment of the mitochondria with 1799 for 10 min or freezing them overnight resulted in a loss of sensitivity to octylguanidine. Thus, the inhibition of malate oxidation by 1.25 mM octylguanidine was reduced from 100 to 39% after uncoupling for 10 min; there was no significant change in the oxidation rate in the absence of inhibitor. Overnight freezing of the mitochondria reduced the inhibition of NADH oxidation from 60 to 10%, but here there was some loss in the mitochondrial oxidative capacity.

The inhibition of the cyanide-insensitive respiration by the two methods of application is illustrated in Figures 5A and 6A. Inhibition of the cyanide-insensitive oxidation of succinate (8)

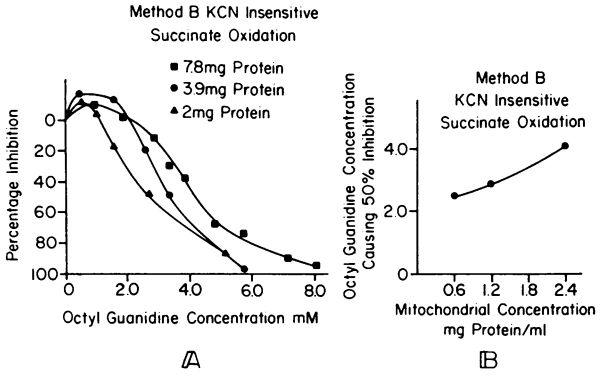

FIG. 5. Inhibition of oxygen uptake by mung bean mitochondria supplied with succinate as substrate in the presence of 70 μM KCN. Method A of application. Experimental conditions as described in the text. A: Comparison of percentage inhibition with octyl guanidine concentration; B: comparison of concentration of octyl guanidine causing 50% inhibition with mitochondrial protein concentration.

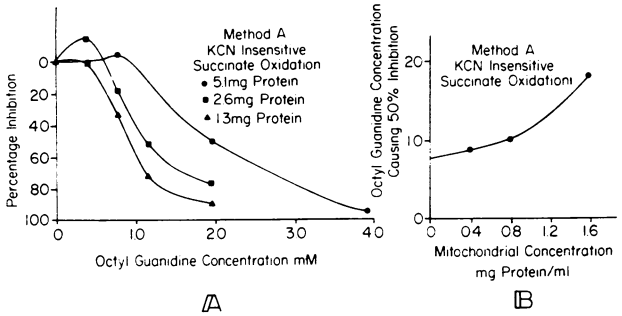

FIG. 6. Inhibition of oxygen uptake by mung bean mitochondria supplied with succinate as substrate in the presence of 70 μM KCN. Method B of application. A: comparison of percentage inhibition with octyl guanidine concentration; B: comparison of concentration of octyl guanidine causing 50% inhibition with mitochondrial protein concentration.

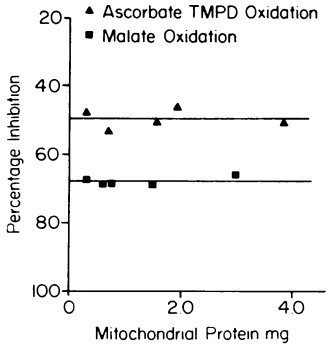

FIG. 7. Effects of octyl guanidine of state 3 malate and ascorbate-TMPD oxidation by mung bean mitochondria at various mitochondrial protein concentrations. Ascorbate-TMPD oxidation measured in 3-ml cuvette with 0.5 mM octyl guanidine, malate oxidation in 1-ml cuvette with 0.12 mM octyl guanidine. Method A of application. Experimental conditions as described in the text.

was sensitive to changes in the mitochondrial protein concentration (Figs. 5B and 6B). No comparable effect was observed in the remaining sites, the inhibition of malate, succinate, and ascorbate-TMPD oxidation being independent of mitochondria protein concentration (Fig. 7).

Figure 8 shows the effects of octylguanidine on state 4 respiration of mung bean mitochondria. Low concentrations of octylguanidine stimulated respiration, and increasing octylguanidine concentrations led to inhibition. A similar but less marked stimulation was observed with succinate oxidation in state 3. Maximal stimulation of state 4 was observed at concentrations similar to the half-maximal inhibition of state 3 by method B. When a stimulatory level of octylguanidine was supplied to mitochondria oxidizing malate or succinate in the presence of cyanide, the stimulation was independent of protein concentration. However,

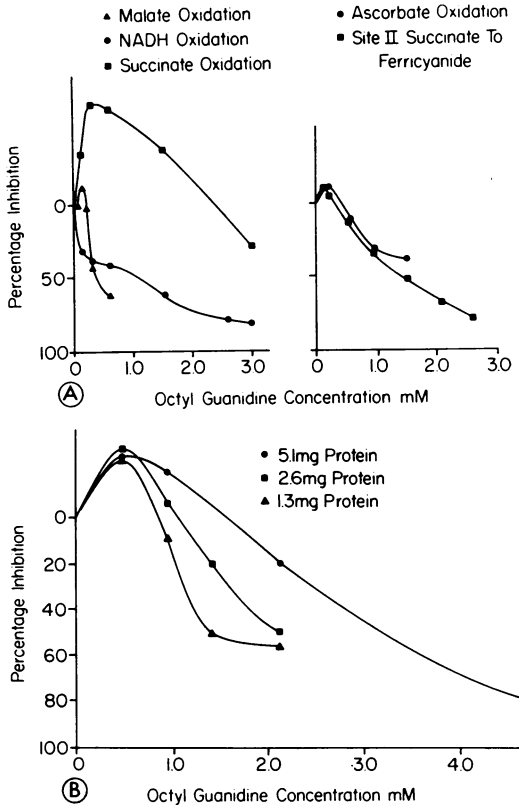

FIG. 8. The effects of octyl guanidine on state 4 respiration of mung bean mitochondria. Method A of application. Experimental conditions as described in the text. A: Malate NADH or succinate oxidation, site II and site III (ascorbate-TMPD oxidation); B: KCN-insensitive respiration with succinate as substrate.

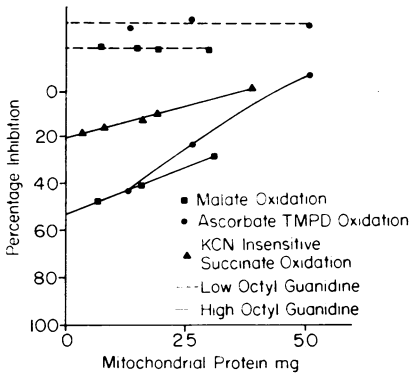

FIG. 9. Effects of mitochondrial protein concentration on inhibition or stimulation of state 4 oxidation with high or low octyl guanidine, respectively. Substrates: 40 mM malate, high octyl guanidine = 0.5 mM, low = 0.2 mM; 10 mM succinate plus 70 μM KCN, high octyl guanidine = 1.7 mM, low = 0.5 mM; 8 mM ascorbate plus 0.8 mM TMPDl octyl guanidine = 0.5 mM. Method A of application. Experimenta, conditions as described in the text.

Table II. *Effectiveness of Guanidine Inhibitors on Rat Liver Mitochondria compared to Mung Bean Mitochondria*

Type of Mitochondria	Substrate	Half-maximal Inhibition	Maximal Inhibitor Concentration	Maximal Inhibition	Reference
		mM	mM	%	
Rat liver mitochondria					
Octyl guanidine	Glutamate-malate	0.02	0.10	100	10
	Succinate	0.06	0.20	50	10
DBI	Succinate	0.3	3	→100	11
Synthalin	β-Hydroxybutyrate	0.03	0.3	→100	4
	Glutamate-malate	0.029	...	→100	4
Mung bean mitochondria					
Octyl guanidine	Malate	0.16	0.7	90–100	This paper
	Succinate	0.42	2.5	70	This paper
DBI	Succinate	1	5	20	This paper
Synthalin	Malate	0.3	2	70	This paper
	Succinate	0.6	1.5	55	This paper

an inhibitory level of octylguanidine resulted in an inhibition which had some dependence on protein concentration for malate, ascorbate-TMPD, and cyanide-insensitive succinate oxidation (Fig. 9).

DISCUSSION

The results presented in this paper clearly show that the guanidine inhibitors act on mung bean mitochondria in a manner differing slightly from that observed with rat liver mitochondria. The differential selectivity of the three inhibitors toward the phosphorylation sites is not observed in mung bean mitochondria, although the various phosphorylation sites differ in sensitivity toward the inhibitors. Mung bean mitochondria are less sensitive to the guanidines than rat liver mitochondria, as has been summarized in Table II. DBI, which has been shown in this paper to be an ineffective inhibitor of mung bean mitochondria, is also ineffective on avian liver and heart mitochondria; DBI appears to be effective only on mammalian mitochondria.

Two alternative sites of action of the guanidines can be considered, either acting on the respiratory chain *per se*, or acting on the energy transfer chain. Our results confirm the findings of Pressman (11) that the guanidines act close to but not on the respiratory chain, since destruction of the energy transfer system prevents the guanidine inhibition while it is not possible to release the inhibition with uncouplers. This conclusion is supported by observations on mitochondria from a variety of plant tissues where levels of octylguanidine resulting in 12 and 26% inhibition of succinate oxidation reduced the ADP:O ratio by 26 and 38%, respectively.

The results presented in this paper agree with those of Chappell (3), who deduced that the alkylguanidines were most effective when applied to mitochondria in the high energy states 1 or 4. Thus, method A of application results in a lower half-maximal inhibitory concentration than does method B. It has been concluded elsewhere that the alkylguanidines probably act on a high energy intermediate which is present only in low concentrations in the low energy mitochondrial states.

Contrary to the conclusion of Hackett *et al.* (4), the guanidine inhibition of cyanide-insensitive respiration suggests that this respiratory pathway is capable of energy conservation. The latter conclusion is supported strongly by the experiments of Bonner and Bendall (1).

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