ABSTRACT

The effects of phenylethylbiguanidide, decamethylene
guanidine, and octylguanidine have been studied with
mung bean hypocotyl mitochondria (Phaseolus aureus var.
Jumbo) supplied with malate, reduced nicotinamide ade-
ine dinucleotide, succinate, or ascorbate-tetramethyl-β-
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.
Decamethylene
guanidine inhibits about 70% of the mal-
ate respiration, 55% of the succinate respiration, and 35%
of the ascorbate-tetramethyl-β-phenylenediamine respi-
ration.

Octylguanidine inhibits all three phosphorylation sites
and the cyanide-insensitive respiration, but to differing
extents 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. Ap-
plication of the guanidine to state 4 mitochondria is more
effective than application to mitochondria already in state
3.

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 alkylguanidines 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 mito-
chondria. 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 de-
scribed 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 Founda-
tion 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 conven-
tional 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 addi-
tion 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 consta-
rate following the previous addition.

The basic reaction mixture for the oxygen electrode experi-
ments consisted of 0.3 mm mannitol, 10 mm KCl, 10 mm po-
tassium phosphate buffer (pH 7.2), and 5 mm MgCl₂; 10 mm
succinate, 1 mm NADH, or 40 mm malate was added as sub-
strate. 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 spectro-
photometer (2).
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.](image1)

![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.](image2)

![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.](image3)

![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.](image4)

**Table 1. Octyl Guanidine Half-maximal Inhibitory Concentrations for State 3 Mung Bean Mitochondrial Oxidation**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>0.16</td>
<td>0.38</td>
</tr>
<tr>
<td>NADH</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.42</td>
<td>0.68</td>
</tr>
<tr>
<td>Ascorbate-TMPD</td>
<td>0.46</td>
<td>0.88</td>
</tr>
<tr>
<td>Site II succinate-cytochrome c</td>
<td>0.46</td>
<td>0.78</td>
</tr>
<tr>
<td>KCN-insensitive succinate</td>
<td>1.12*</td>
<td>2.68*</td>
</tr>
</tbody>
</table>

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 1 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)
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 mitochondrial 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,
Table II. Effectiveness of Guanidine Inhibitors on Rat Liver Mitochondria compared to Mung Bean Mitochondria

<table>
<thead>
<tr>
<th>Type of Mitochondria</th>
<th>Substrate</th>
<th>Half-maximal Inhibition</th>
<th>Maximal Inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver mitochondria</td>
<td>Glutamate</td>
<td>0.02 mM</td>
<td>0.10 mM</td>
<td>100 %</td>
</tr>
<tr>
<td>Octyl guanidine</td>
<td>Succinate</td>
<td>0.06 mM</td>
<td>0.20 mM</td>
<td>50 %</td>
</tr>
<tr>
<td>DBI</td>
<td>Succinate</td>
<td>0.3 mM</td>
<td>3 mM</td>
<td>→100</td>
</tr>
<tr>
<td>Synthalin</td>
<td>β-Hydroxybutyrate</td>
<td>0.03 mM</td>
<td>0.3 mM</td>
<td>→100</td>
</tr>
<tr>
<td>Mung bean mitochondria</td>
<td>Malate</td>
<td>0.16 mM</td>
<td>0.7 mM</td>
<td>90-100</td>
</tr>
<tr>
<td>Octyl guanidine</td>
<td>Succinate</td>
<td>0.42 mM</td>
<td>2.5 mM</td>
<td>70</td>
</tr>
<tr>
<td>DBI</td>
<td>Succinate</td>
<td>1 mM</td>
<td>5 mM</td>
<td>20</td>
</tr>
<tr>
<td>Synthalin</td>
<td>Malate</td>
<td>0.3 mM</td>
<td>2 mM</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>0.6 mM</td>
<td>1.5 mM</td>
<td>55</td>
</tr>
</tbody>
</table>

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).

**LITERATURE CITED**