The Respiratory Chain of Plant Mitochondria

XVI. INTERACTION OF CYTOCHROME $b_{max}$ WITH THE RESPIRATORY CHAIN OF COUPLED AND UNCOUPLED MUNG BEAN MITOCHONDRIA: EVIDENCE FOR ITS EXCLUSION FROM THE MAIN SEQUENCE OF THE CHAIN

ABSTRACT

Cytochromes $b_{max}$, $b_{min}$, and $b_{tot}$ of mung bean (Phaseolus aureus) mitochondria become partially reduced with endogenous substrate on addition of antimycin A to the aerobic mitochondrial suspension. Addition of ATP causes partial reoxidation of the three cytochromes. This partial oxidation by ATP is inhibited by oligomycin and reversed by uncoupler. Ubiquinone does not appear to act as electron acceptor for the oxidation reaction, but a nonfluorescent flavoprotein, or possibly iron-sulfur protein, component does appear to act as acceptor. This is consistent with reverse electron transport driven by ATP across the first site of energy conservation of the respiratory chain. Endogenous pyridine nucleotide and the fluorescent flavoprotein with $E_{m2} = -155$ mv (midpoint potential at pH 7.2, referred to normal hydrogen electrode) in uncoupled mitochondria become reduced in anaerobiosis attained by oxidation of succinate in the absence of respiratory inhibitors of the cytochrome chain, provided that Pi and ATP are present. Under these same conditions, cytochrome $b_{max}$ is completely reduced but cytochrome $b_{tot}$ remains nearly completely oxidized. There is no equilibration across the first site of energy conservation between the carriers on the low potential side and cytochrome $b_{max}$ with $E_{m2} = -77$ mv on the high potential side. It is concluded that cytochrome $b_{max}$ is not a part of the main sequence of electron transport carriers of the mitochondrial respiratory chain of plants; it can participate in redox reactions with the respiratory chain in coupled mitochondria but not in uncoupled mitochondria unless antimycin A is present.

Plant mitochondria have long been known to have three $b$ cytochromes (1–3). One of these has, in its reduced form, an $a$-band at 562 nm at $-196$ C and at 565 nm at room temperature; it is designated cytochrome $b_{max}$, the subscript referring to the reduced minus-oxidized difference absorbance maximum at

$-196$ C (7, 27). Cytochrome $b_{max}$ has a midpoint potential of $E_{m2} = -77$ mv which does not change with the state of energization of the mitochondria (15). It is reduced by succinate in energized mitochondria but remains largely oxidized in uncoupled mitochondria in the presence of cyanide with both succinate and exogenous NADH as substrates (29, 32). It is, however, fully reduced by these substrates in the presence of antimycin A when the $c$ cytochromes are fully oxidized, and becomes partially oxidized as the latter cytochromes become reduced on anaerobiosis or on addition of cyanide or sulfide (19, 33). It has been an open question whether cytochrome $b$ is part of the main sequence of the respiratory chain of plant mitochondria, or whether it merely indicates the mitochondrial energy state without participating in the sequential reactions of electron transport through the second site of energy coupling. This question is examined in the study reported in this paper.

MATERIALS AND METHODS

Mitochondria were prepared from the hypocotyls of 5-day etiolated seedlings of mung bean (Phaseolus aureus) as described previously (27). These mitochondrial preparations yield one major band on the discontinuous sucrose gradient described by Douce et al. (12), with a diffuse minor band at lower density. The ratio of the succinate-cytochrome $c$ reductase activity, sensitive to antimycin A, in hypotonically shocked and isotonically maintained mitochondria (12) is about 16 in these preparations.

The mitochondria were assayed for respiratory control in a medium containing 0.3 m mannitol, 10 mM TES, and 5 mM Pi, brought to pH 7.2 with KOH. This medium is designated TP; the same medium without phosphate is designated T. Oxygen consumption by the mitochondrial suspension with succinate or malate as substrate was measured in a closed cuvette with a Clark electrode (Yellow Springs Instrument Co.), as described by Estabrook (18). Mitochondrial protein content was determined by the method of Miller (21).

Adenine nucleotides were obtained from Boehringer Mannheim Corp., succinic acid from Aldrich Co., antimycin A and TES from Cal Biochem Co., oligomycin from Sigma Chemical Co., and mannitol from J. T. Baker Chemical Co.; these were used without further purification. The uncoupler 1799 (bis-hexafluoroacetyl acetone) was generously supplied by Dr. Peter Heytler of E. I. du Pont de Nemours Co., and a sample of m-chlorophenylhydrazinic acid was generously provided by Dr. Gregory Schonbaum.

Absorbance changes corresponding to the reduction or oxidation of the respiratory chain carriers were recorded using a dual wavelength spectrophotometer (5) with a compensation

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circuit to reduce noise from light source fluctuations (8). The following wavelength pairs were used for the various carriers: 468 to 493 nm for flavoprotein; 282 to 295 nm for ubiquinone; 552 to 520 nm for cytochrome c\textsubscript{m}; 556 to 540 nm for cytochrome b\textsubscript{m}; 560 to 540 or 570 nm for cytochrome b\textsubscript{m}; and 565 or 566 to 540 or 570 nm for cytochrome b\textsubscript{m}. The subscripts give the reduced-minus-oxidized difference absorbance maxima for the cytochrome in spectra obtained at −196 C (27).

Fluorescence changes corresponding to the oxidation and reduction of endogenous pyridine nucleotide were monitored with an Eppendorf fluorimeter, as described previously (31). Fluorescence changes corresponding to the oxidation and reduction of the low potential, fluorescent flavoprotein F\textsubscript{p} (28) were recorded by two methods. The first utilized the Eppendorf fluorimeter equipped with an Heraeus (Hanau) mercury arc No. ST-75 as described in earlier papers (28, 30). The primary filter for the excitation light was an interference filter with maximum transmittance at 463 nm with 10 nm band width, or the filter transmitting at 436 nm supplied by Eppendorf. The transmittance of the secondary filter for the emitted light lay between 490 and 1000 nm. The second method utilized the compensated dual wavelength spectrophotometer (8), with an additional detector for fluorescence changes to be monitored simultaneously with absorbance changes (9). Fluorescence was excited 468 nm. The fluorescence emission, which has a maximum at 525 nm, was detected from the side face of the cuvette, as compared with detection from the front face in the method utilizing the Eppendorf fluorimeter.

Difference spectra of the mitochondrial suspensions were obtained at liquid nitrogen temperature with the split beam spectrophotometer described by Chance (5), using the technique developed by Estabrook (17), as subsequently modified by Bonner (1); samples were obtained utilizing the "frozen steady state" technique of Chance and Schoener (10).

RESULTS

The response of the cytochromes b of mung bean mitochondria to energization of the mitochondrial membrane by ATP in the near absence of electron transport is shown in Figure 1. Addition of antimycin A to the aerobic mitochondria in the absence of added substrate reduces in reduction of all three cytochromes b; subsequent addition of ATP causes 50% reoxidation of cytochromes b\textsubscript{m} and b\textsubscript{m} (Fig. 1, A and C) and 35% reoxidation of cytochrome b\textsubscript{m} (Fig. 1B). Addition of succinate then gives full reduction of the cytochromes b. These additions have virtually no effect on the redox state of cytochrome c\textsubscript{m} which remains highly oxidized (Fig. 1D). No inhibitor of the alternate terminal oxidase of these mitochondria is present, and the suspensions become anaerobic within 3 min of the addition of succinate. The characteristic reoxidation of some 20% of cytochrome b\textsubscript{m} (33) is observed (Fig. 1B). The apparent rapid reduction of b\textsubscript{m} on anaerobiosis (Fig. 1C) is largely due to spectral interference at this wavelength pair from cytochrome c\textsubscript{m} (15) which becomes rapidly reduced on anaerobiosis (Fig. 1D).

Difference spectra corresponding to the aerobic redox state transitions seen in the records of Figure 1 are shown in Figure 2A. Spectrum 1 is the difference spectrum of antimycin-treated minus fully oxidized mitochondria; it shows a broad absorbance maximum at 559 nm. This is resolved into three separate maxima in the difference spectrum obtained at −196 C for the same conditions (Fig. 2B, spectrum 1). Spectrum 2 in Figure 2A is the difference spectrum of antimycin-treated minus ATP plus antimycin-treated mitochondria; the absorbance maximum is now at 558 nm, reflecting the smaller contribution of cytochrome b\textsubscript{m} caused by its lower degree of reoxidation on addition of ATP. This is also evident from the low temperature difference spectrum for the same conditions (Fig. 2B, spectrum 2). Spectrum 3 in Figure 2A is the difference spectrum of mitochondria treated with succinate plus ATP plus antimycin minus mitochondria treated only with antimycin, both samples being aerobic. The contribution of cytochrome b\textsubscript{m} to this difference spectrum is dominant, as one would infer by comparison of Figure 1B with Figure 1, A and C; the absorbance maximum at 565 nm is clearly seen.

It is evident from these experiments that all three cytochromes b of plant mitochondria, reduced by endogenous substrate in the presence of antimycin A, can be partially reoxidized by energization of the membrane with added ATP. Further, it was shown in control experiments that the reoxidation is sensitive to inhibition by oligomycin and reversed by uncoupler.

The reoxidation reaction could be redistribution of electrons within the region of the respiratory chain comprising the low potential, or substrate, side of the second site of energy conservation. Alternatively, it could be ATP-driven reverse electron transport across the first site of energy conservation, which would be readily seen under these conditions of near absence of forward electron transport. In the first instance, ubiquinone would be the mostly likely acceptor for the reac-

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Fig. 1. Response of cytochrome b\textsubscript{m} (A), cytochrome b\textsubscript{m} (B), cytochrome b\textsubscript{m} (C), and cytochrome c\textsubscript{m} (D) in mung bean mitochondria (M.B.M.) to sequential addition of antimycin A (AA), ATP, and succinate. The mitochondria are suspended in medium T (see "Materials and Methods") at 2.1 mg protein/ml. Upward deflection of the trace corresponds to cytochrome reduction.

Fig. 2. Difference spectra corresponding to the aerobic redox state transitions seen in the records of Figure 1 are shown in Figure 2A. Spectrum 1 is the difference spectrum of antimycin-treated minus fully oxidized mitochondria; it shows a broad absorbance maximum at 559 nm. This is resolved into three separate maxima in the difference spectrum obtained at −196 C for the same conditions (Fig. 2B, spectrum 1). Spectrum 2 in Figure 2A is the difference spectrum of antimycin-treated minus ATP plus antimycin-treated mitochondria; the absorbance maximum is now at 558 nm, reflecting the smaller contribution of cytochrome b\textsubscript{m} caused by its lower degree of reoxidation on addition of ATP. This is also evident from the low temperature difference spectrum for the same conditions (Fig. 2B, spectrum 2). Spectrum 3 in Figure 2A is the difference spectrum of mitochondria treated with succinate plus ATP plus antimycin minus mitochondria treated only with antimycin, both samples being aerobic. The contribution of cytochrome b\textsubscript{m} to this difference spectrum is dominant, as one would infer by comparison of Figure 1B with Figure 1, A and C; the absorbance maximum at 565 nm is clearly seen.
Fig. 2. Difference spectra of mung bean mitochondria corresponding to the conditions of Fig. 1 obtained at room temperature (A) and at liquid nitrogen temperature (B). For the room temperature spectra (A), the mitochondria are suspended in medium T at 3.6 mg protein/ml and treated with 0.72 mm mCLAM to inhibit the alternate oxidase. For the spectra obtained at liquid nitrogen temperature (B), the mitochondria are suspended at 9.5 mg protein/ml with 1.4 mm mCLAM. Spectrum 1 in both A and B is the difference spectrum of aerobic mitochondria treated with 3 nmoles/mg antimycin A minus untreated aerobic mitochondria. Spectrum 2 in both A and B is the difference spectrum of antimycin-treated aerobic mitochondria minus antimycin-treated aerobic mitochondria plus 1.1 mM ATP. Spectrum 3 in A is the difference spectrum of aerobic mitochondria treated with antimycin plus ATP plus succinate minus aerobic mitochondria treated with antimycin alone.

Fig. 3. Response of cytochrome b₅₅₃ (A), cytochrome b₇₅₃ (B), and cytochrome b₅₅₆ (C) in mung bean mitochondria, suspended in medium T at 2.4 mg protein/ml and treated with 0.8 mm mCLAM plus 0.7 mm ATP, to sequential addition of antimycin A, uncoupler 1799, and succinate. Upward deflection of the trace corresponds to cytochrome reduction. The responses of flavoprotein Fp as measured by absorbance (D) and fluorescence (E) in the same mitochondrial preparation are also shown for the same sequence of additions under the same conditions; the response of ubiquinone (UQ) is shown in F. In these three traces, downward deflection of the trace corresponds to reduction.

In the second instance, a flavoprotein on the low potential side of the first coupling site would be a likely acceptor. An attempt to decide between these alternatives is shown in the experiment of Figure 3. The mitochondria are treated with mCLAM so that the pathway of electron transport to oxygen through the alternate oxidase is effectively blocked. ATP is added before the spectrophotometric recording is begun, so that one can monitor the redox changes of ubiquinone by differential spectrophotometry in the UV region without optical interference from this compound.

Addition of antimycin A to the mitochondrial suspensions results in that degree of reduction of the cytochromes b (Fig. 3, A, B, and C) expected for the antimycin plus ATP steady state seen in Figure 1. Addition of uncoupler abolishes the energized state, thereby increasing the degree of reduction; full reduction is attained with addition of succinate. The responses of the flavoprotein component to these perturbations are shown in Figure 3D (absorbance) and 3E (fluorescence). There is essentially no response from the fluorescent flavoprotein component Fpₐ (29, 30) which remains oxidized under these conditions (Fig. 3E). The absorbing flavoprotein components show a small degree of reduction on addition of antimycin A (Fig. 3D); subsequent addition of uncoupler causes a small rapid reduction followed by a slower oxidation. The slower part of the response to uncoupler under these conditions is in the opposite redox sense to that of the cytochromes b, and thus it is a candidate for the acceptor. The response is near the limit of detection, but has been consistently observed. The degree of reduction of the flavoprotein component on addition of succinate in this particular aerobic steady state, in which electron transport is inhibited with antimycin A and mCLAM, is about half that attained in anaerobiosis under these conditions. Ubiquinone remains highly oxidized until succinate is added (Fig. 3F). The slight apparent reduction on addition of antimycin A was shown in a control experiment to be caused by the intrinsic absorbance of this inhibitor. There is a slight apparent reduction, less than 5% of the total, observed on adding uncoupler. Succinate completes the reduction; the same absorbance change is obtained in the presence or absence of uncoupler. From these results, it is evident that the only absorbance change which can be interpreted as proceeding towards oxidation as the cytochromes b become reduced on addition of uncoupler, is that observed at 468 to 493 nm, indicating involvement of a flavoprotein component, or possibly of an iron-sulfur protein component, which would give an absorbance change at 468 to 493 nm less than, but similar to, flavoprotein (24). Both carriers expected to be involved if the reaction observed is ATP-driven reverse electron transport across the first energy conservation site.

In order that cytochrome b₅₅₃ be reduced in the absence of antimycin A, it is necessary to provide reducing equivalents at a potential more negative than its midpoint potential of ≈−77 mV at pH 7.2. The complications of the dehydrogenase reactions for pyruvate and malate in plant mitochondria (13) make it useful to use succinate as substrate, but, in order to assess the participation of cytochrome b₅₅₃ in electron transport independent of energy state, it is necessary to uncouple the system. Fortunately, for the purposes of this study, the respiratory chain carriers on the low potential side of the first energy conservation site in plant mitochondria can be reduced with succinate in the uncoupled state in anaerobiosis when phosphate and ATP are present (31). The reaction involves removal of

*Abbreviations: mCLAM: m-chlorobenzyloxyacetic acid; Eₘₜ: midpoint potential at pH 7.2, referred to normal hydrogen electrode.
oxaloacetate in such a manner that malate formed from succinate oxidation can cause nearly complete reduction of endogenous pyridine nucleotide. An example of this reaction is shown in Figure 4, where the absorbance (Fig. 4A) and the fluorescence (Fig. 4, B and C) of the flavoprotein components, and the fluorescence of endogenous pyridine nucleotide (Fig. 4D) are monitored during the oxidation of succinate by oligomycin-treated mung bean mitochondria in the presence of 5 mM Pi and 0.7 mM ATP. Both the endogenous pyridine nucleotide and fluorescent flavoprotein are over 90% reduced in the coupled aerobic steady state under these conditions, but become completely reoxidized on addition of uncoupler (Fig. 4, B and D). Upon anaerobiosis, there is a short lag during which these components remain oxidized, after which they then reach a high degree of reduction in 2 to 3 min. The fluorescent flavoprotein Fp, with Eₘₐₓ = -155 mV (30) and endogenous pyridine nucleotide become reduced with the same time course, and thus Fpₜ, can be used to monitor this reaction.

A comparison of the degree of reduction achieved by cytochromes bₜ and bₛₑₜ compared to the flavoproteins in anaerobiosis, in uncoupled mung bean mitochondria respiring with succinate in the presence of ATP and Pi, is shown in Figure 5. Flavoprotein absorbance (Fig. 5A) and fluorescence (Fig. 5B) changes have been recorded simultaneously. This is no reduction of Fpₜ in the uncoupled aerobic steady state; after a lag of about 2 min on attainment of anaerobiosis, reduction of this flavoprotein begins and is complete within 5 min. The absorbance due to flavoprotein components at 468 to 493 nm shows rapid attainment of the degree of reduction characteristic of the uncoupled aerobic steady state; there is a rapid further change on anaerobiosis, followed by a slower change with the same time course as Fpₜ reduction. The absorbance changes at 560 to 570 nm (Fig. 5C) and at 565 to 570 nm (Fig. 5D) follow a course qualitatively similar to those in 468 to 493, in that the redox level characteristic of the aerobic steady state is rapidly attained, and there is a rapid reduction in anaerobiosis, followed by a slower one. These wavelength pairs were used for cytochromes bₜ and bₛₑₜ since it has been determined potentiometrically that there is little absorbance change due to other cytochromes and that the mutual interference between these two cytochromes is such that cytochrome bₛₑₜ contributes 78% of the absorbance change at 560 to 570 nm while cytochrome bₜ contributes 57% of the change at 565 to 570 nm (15).

In Figure 5C, the total absorbance change is 97% of that observed with antimycin and succinate in aerobic mitochondria; but in Figure 5D, it is only 50%, which is only slightly greater than the contribution to the absorbance change expected from cytochrome bₛₑₜ alone at this wavelength pair. Further, the two traces have a remarkably similar shape. The percentage of the total absorbance change in the aerobic steady state is 20% in both Figure 5, C and D. The percentage of the change corresponding to rapid reduction in anaerobiosis with succinate from the oxidized state without succinate is 78% in both traces with the slower absorbance change accounting for the remaining 22%. From these results, one concludes that most of the absorbance change at both sets of wavelength pairs is due to cytochrome bₛₑₜ; only 10% at most of cytochrome bₜ can be reduced under these conditions.

DISCUSSION

It is evident from the results presented in Figures 1 and 2 that cytochrome bₑₜ does respond to added ATP in antimycin-treated mitochondria in the same way as the other two cytochromes b, namely, oxidation from partial reduction with endogenous substrate. The experiment shown in Figure 3 indicates that this oxidation is the result of ATP-driven reverse electron transport through the first energy conservation site. Since the rate of forward electron transport from endogenous substrate through this site is very low, its reversal is readily detectable in this system. But one consequence of the low rate of forward electron transport is that antimycin A is required to achieve a degree of reduction of the cytochromes b sufficiently extensive for the ATP-linked reoxidation to be observed. The question then arises whether these energy-linked reactions of cytochrome bₑₜ occur only in the presence of antimycin A. Three observations indicate that this is not the case. (a) Cytochrome bₑₜ can be partially reduced by ATP-driven reverse electron transport through the second coupling site from cytochrome c, which has been reduced by endogenous substrate in the presence of sulfide (32). (b) ATP can maintain cytochrome bₑₜ highly reduced in mung bean mitochondria which have become anaerobic through oxidation of succinate in state 4 in the absence of respiratory or ATPase inhibitors (33). (c) If the mitochondria are first treated with oligomycin and ATP, then given succinate as substrate, the high energy intermediates generated by succinate oxidation can maintain...
as high a degree of reduction of cytochrome \( b_{m} \) in the aerobic steady state as ATP can alone. In anaerobiosis, however, cytochrome \( b_{m} \) is partially reduced then rapidly reoxidized back to the steady state level, whereas further reduction is observed with ATP alone (33). These three observations show that cytochrome \( b_{m} \) can be reduced by succinate through an energy-linked process with ATP or the high energy intermediates generated in coupled succinate oxidation as energy sources, but that this process is slow and requires constant input of free energy: reoxidation of the cytochrome is rapid when the generation of high energy intermediates ceases.

Antimycin does change the properties of cytochrome \( b_{m} \), however. In aerobic mitochondria treated with antimycin \( A \), under which condition the cytochromes \( c \) are highly oxidized, cytochrome \( b_{m} \) appears to form a tight complex with antimycin whose \( E_{m/r} \) is at least 100 mV more positive than that of the uncomplexed cytochrome (19, 33). In this condition, where the oxidation rate is, in effect, inhibited relative to the reduction rate, one observes reduction of the cytochrome by endogenous substrate. This complex formation also seems to promote redox interaction between cytochrome \( b_{m} \) and the other cytochromes \( b \).

With ATP alone, or with ATP plus oligomycin, the full degree of reduction of cytochrome \( b_{m} \) in the aerobic steady state is attained slowly (33), whereas that of endogenous pyridine nucleotide—corresponding to over 90% reduction—is attained quickly and reaches full reduction in anaerobiosis (31). Thus, with oligomycin present, cytochrome \( b_{m} \) remains partially oxidized in coupled mung bean mitochondria in anaerobiosis, under which condition the endogenous pyridine nucleotide and low potential flavoprotein remain totally reduced. In this paper, it is shown that a similar situation is observed in uncoupled mitochondria, with an important difference being that oxidation of the cytochrome is nearly complete. One has the anomalous situation of a cytochrome with \( E_{m/r} \) \(-77 \text{ mV}\) remaining oxidized when the intramitochondrial potential (34) estimated at 90% reduction of endogenous pyridine nucleotide (Fig. 4D), is about \(-350 \text{ mV}\), and this in uncoupled mitochondria in which the rates of electron transport between carriers should be maximal. If cytochrome \( b_{m} \) is an electron transport carrier in the main sequence of the plant respiratory chain, then the ATP-induced reverse electron transport from this cytochrome across the first energy conservation site, seen under the energy-linked conditions of the experiment in Figure 1, should be reversed under the uncoupled conditions of the experiment in Figure 5, and the cytochrome should become reduced. Since it remains highly oxidized, one must conclude that cytochrome \( b_{m} \) is not a carrier in the main sequence of the respiratory chain and, in uncoupled mitochondria, seems functionally capable of interacting with the respiratory chain only in the presence of antimycin \( A \). It can indicate the mitochondrial energized state but cannot participate in its formation.

There arises the question of what biological function is served by cytochrome \( b_{m} \) in the inner membrane of plant mitochondria. One possible function is biosynthetic. Momose and Rudney (22) have shown that the ubiquinone precursor, 3-polyrenyl-4-hydroxybenzoate, is synthesized in the inner membrane of liver and kidney mitochondria, and that the degree of polymerization of the polyprenyl side chain is the same as that of the ubiquinone in the membrane. This implies that the sequence of reactions which convert this precursor to ubiquinone also occurs in the membrane. The conversion requires one decarboxylation, three hydroxylations, and three methylations. Of this set, it is the hydroxylation reactions which would require enzymes capable of participating in redox reactions. It is tentatively suggested here that cytochrome \( b_{m} \) is part of the enzyme complex which carries out these hydroxylations in the plant mitochondrial membrane.

The multiplicity of cytochromes \( b \) in animal mitochondria is now as well established as it is in plant mitochondria (25). While there is still some disagreement concerning the exact
number of these cytochromes in heart or liver mitochondria (4, 20, 25, 26, 36, 37), two have been shown unequivocally to be separate enzymes, designated b-561 or br and b-566 or bl (11, 14, 35, 38). Papa et al. (23) have recently concluded from kinetic measurements of redox state changes and ion movements in mitochondria treated with antimycin A when the cytochromes b-566 in the mammalian system is on a side path rather than one of the main sequence respiratory chain carriers. It is possible that the function suggested above for cytochrome b-562 in plant mitochondria is applicable to cytochrome b-566 in animal mitochondria.

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LITERATURE CITED


