THE EFFECT OF ANTIMYCIN ON THE b-CYTOCHROMES OF PLANT MITOCHONDRIA. OXIDATION-REDUCTION BEHAVIOR OF CYTOCHROME b-566

Alan M. Lambowitz and Walter D. Bonner, Jr.

Department of Biophysics and Physical Biochemistry, University of Pennsylvania
Philadelphia, Pennsylvania 19174

Received April 3,1973

SUMMARY: Antimycin plus O_2 increases the reducibility of cytochrome b-566 in plant mitochondria to an extent consistent with an increase of over 110 mv in the apparent midpoint potential of b-566. Enhanced reduction of b-566 is relaxed following consumption of O_2, but contrary to previous reports this relaxation does not show a close kinetic correlation with reduction of cytochrome c_1. Experiments with cyanide-inhibited mitochondria demonstrate that neither active cytochrome oxidase nor extensive oxidation of cytochrome c_1 is required for enhancement of b-566 reduction.

Antimycin, in addition to inhibiting electron transport between cytochromes b and c_1, also directly affects the spectra and redox properties of the b-cytochromes (1-7). Particularly striking is the ability of antimycin, acting in both plant and animal mitochondria, to cause enhanced reduction of the long wavelength b-cytochrome, b-566 (1-9). This effect is frequently considered to bear directly on the control of electron transport and the mechanism of energy transduction (10-13). A surprising aspect of the enhanced reduction of b-566 is its further dependence on the presence of an oxidizing agent (O_2 or ferricyanide) (3,4,12-17), and indeed, the role of the oxidant is often emphasized in formulating the mechanism of the effect. Thus, as considered in detail in this paper, Erecińska and coworkers (13,18,19) and Storey (9) proposed that the enhanced reduction of b-566 was a response to the oxidation of cytochrome c_1, the latter cytochrome being assigned a key role in regulating the reducibility of b-566 or of an antimycin/b-566 complex. To account for the behavior of b-566, these authors postulated that oxidation of c_1 -- as occurs in substrate-reduced mitochondria upon addition of antimycin plus oxidant -- causes the midpoint potential (E_m) of b-566 to shift to a more positive value, leading to increased reduction of b-566. Conversely, reduction of c_1 was considered to decrease the E_m of b-566, leading to relaxation of the enhanced reduction. In support of such a mechanism, Storey (9) reported an apparent synchronous reduction of c_1 and relaxation of b-566 reduction upon anaerobiosis in antimycin-treated mung bean mitochondria which had been respiring with succinate. In our opinion, however, the data are not sufficient to warrant the conclusion that the redox state of c_1 controls that of b-566. It is the purpose of this paper to re-evaluate the evidence for the

proposed mechanism in plant mitochondria through more detailed examination of the relationships between the redox states of these two cytochromes.

MATERIALS AND METHODS

Tightly coupled, gradient-purified mitochondria were prepared from mung bean hypocotyls and potato tubers as described by Douce et al. (20). Reactions were carried out in medium A (0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM K-PO_4 buffer, pH 7.2) or medium B (0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, pH 7.2). Prior to each experiment, mitochondria were suspended in reaction medium and soaked in ATP (0.33 mM) for 2 min to activate succinate dehydrogenase. Spectra at room temperature and at 77° K were obtained with a scanning split beam spectrophotometer as described by Chance (21). The time course and extent of oxidation and reduction of the cytochromes were measured with a dual wavelength spectrophotometer (21). Potentiometric titrations were carried out as described by Dutton et al. (22). Mitochondrial protein was assayed by the method of Lowry et al. (23). Antimycin (Cal-Biochem) was added from a 1 mg/ml stock solution in absolute ethanol.

The b-cytochromes are named according to their α-absorption maxima in room temperature difference spectra.

RESULTS

Table I shows the extent of reduction of cytochrome b-566 by 10 mM succinate in coupled and uncoupled plant mitochondria under normal conditions (lines A,B) and in the presence of antimycin plus oxidant (lines D,E). The enhanced reducibility of b-566 under the latter conditions is readily apparent. The extent of reduction of this cytochrome increases from a relatively low initial level (0 to 20%; lines A,B) to at least 85 or 90% in aerobic, antimycin-treated mitochondria (lines D,E). The low level of reduction prior to addition of antimycin seems somewhat surprising at first, but actually it agrees closely with the redox level normally expected for equilibrium between b-566 and succinate based on their measured E_m's (7%; line C). Significantly, the increased reduction of b-566 under the influence of antimycin plus oxidant is seen to result from displacement of this normal redox equilibrium. It is also noteworthy that the effect occurs equally well in coupled and uncoupled mitochondria. Quantitatively, the extra reduction of b-566 is consistent with an increase of over 110 mv in its apparent E_m, in agreement with analogous estimates for animal mitochondria (16,17,24).

Further experiments centered on the possibility that the enhanced reduction of b-566 reflects control of its E_m by the redox state of cytochrome c_1 (see INTRODUCTION). Fig. 1 shows dual wavelength spectrophotometer recordings which are addressed to the previously reported correlation in the oxida-

TABLE I

Extent of Reduction of b-566 by 10 mM Succinate in Different Steady States in Potato Mitochondria

The table shows levels of reduction of b-566 measured with a dual wavelength spectrophotometer at 566-575 nm and expressed as percent b-566 reduced by dithionite. Correction was made for spectral interference from the neighboring α-peak of b-560 by measuring its contribution to the total absorbance potentiometrically and by making the assumption that b-560 -- which has a relatively high E_m; ca. 80 mv; Lambowitz and Bonner, manuscript in preparation -- is completely reduced in the steady states indicated. Both b-560 and b-566 were assumed to be fully oxidized in aerobic mitochondria prior to addition of succinate. Reactions were carried out at room temperature in 3.0 ml medium A or B. Antimycin was added at a concentration of 0.5 to 1.5 μg/mg protein; the uncoupler was 1799 (6.7 μM). Each value represents two or more determinations. A consistent set of results was obtained from low temperature difference spectra. Line A: refers to the stable level of reduction reached 2 to 5 min after anaerobiosis. Line C: level of reduction calculated on the basis of measured E_m of b-566 (ca. -75 mv; Lambowitz and Bonner, manuscript in preparation) assuming equilibrium between b-566 and succinate. For this calculation, the potential of the medium after anaerobiosis in the presence of 10 mM succinate was estimated to be -8 mv.

	Conditions	Percent reduction b-566
A.	Succinate/anaerobic	5 to 20
B.	Succinate + uncoupler/anaerobic	0 to 5
C.	Succinate -- calculated "normal" equilibrium level	7
D.	Succinate + antimycin/aerobic	90
E.	Succinate + antimycin + uncoupler/aerobic	85 to 90

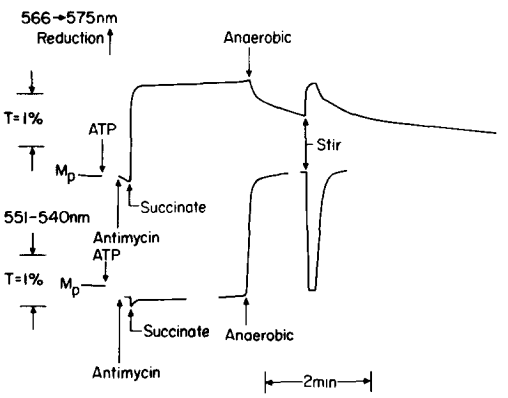
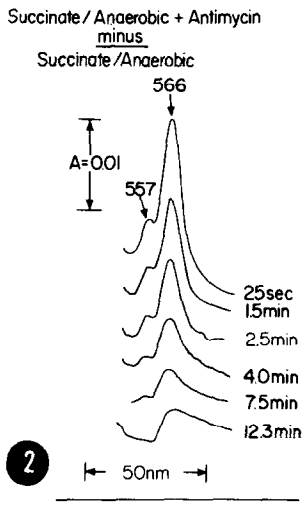


Figure 1: Dual wavelength spectrophotometer recordings of the oxidation-reduction behavior of cytochromes b-566 and c + c_1 in antimycin-treated mung bean mitochondria. Upper trace: cytochrome b-566 monitored at 566-575 nm. Bottom trace: cytochrome c + c_1 monitored at 551-540 nm. The light path was 1 cm. Concentrations were: ATP (0.33 mM), antimycin (0.5 to 1.1 μg/mg mitochondrial protein), succinate (8.3 mM). Mp = 6.0 and 4.6 mg mitochondrial protein in upper and lower traces, respectively. Reactions were carried out in 3.0 ml medium A at room temperature. Similar results -- with comparable fast and slow oxidations -- were obtained in medium B; the extent of the fast oxidation was decreased in uncoupled mitochondria.

tion-reduction behavior of b-566 and c_1 (9). After addition of antimycin and succinate, the mitochondria enter a steady state during which O_2 is consumed by non-cytochrome pathways (25), and during which, in accord with the proposed mechanism, cytochrome c + c_1 (bottom trace) is almost completely oxidized and cytochrome b-566 (top trace) almost completely reduced. Upon anaerobiosis (following the aerobic steady state, or following the transient aerobic steady state induced by stirring), one observes a rapid reduction of cytochrome c + c_1 and an oxidation of cytochrome b-566, the latter reflecting relaxation of the enhanced reduction. This relaxation is biphasic, however. Only the relatively small initial fast phase is closely correlated with reduction of cytochrome c + c_1, and control experiments -- appropriate difference spectra and potentiometric titrations -- showed that at least half of this apparent fast oxidation is actually spectral interference from reduction of cytochrome c + c_1 (which appears as a decrease in absorbance at this wavelength pair). More importantly, the greater part of the relaxation is a previously over-looked slow oxidation, and this occurs much later than reduction of cytochrome c + c_1 (shown in Fig. 1, and confirmed by repetitive difference spectra; Fig. 2). A very similar picture of the redox behavior of b-566 was obtained from spectra of the corresponding steady states frozen in liquid nitrogen (Fig. 3). Comparison of the spectrum of the aerobic steady state (Fig. 3B) with that at 30 sec after anaerobiosis suggests that very little oxidation of b-566 occurs concomitantly with reduction of cytochrome c + c_1. Instead, as shown in Fig. 3D, the spectrum at 45 min after anaerobiosis, oxidation of b-566 to the normal redox level (Fig. 3A) occurs only after an extended period of anaerobiosis corresponding to the slow oxidative phase in Fig. 1. In further experiments, primarily with the dual wavelength instrument, the same behavior of b-566 was observed in coupled and uncoupled mitochondria from both mung beans and potatos. However, the rate and extent of the slow oxidative phase were found to vary for different preparations with 30 to 100% of the extra reduction of b-566 relaxed in 5 to 15 min after anaerobiosis. In all cases as previously demonstrated for rat liver mitochondria by Wikström (26), the relaxation could be accelerated to completion (ca. 10% reduction of b-566; cf. Table I) by addition of low concentrations of redox mediators (e.g. 10 μM phenazine methosulfate) to establish oxidation-reduction equilibrium. From the above data considered together, we conclude that relaxation of b-566 reduction upon anaerobiosis does not show a close kinetic correlation with reduction of cytochrome c_1, as had been reported previously (9). There is no basis from this more complete picture for the unqualified conclusion that the redox state of cytochrome c_1 controls that of b-566.

The above data are not sufficient to rule out such control, however,



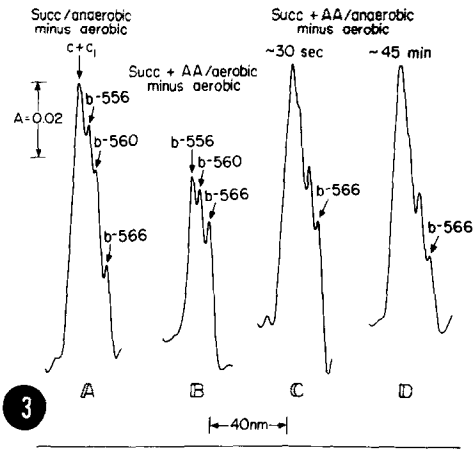
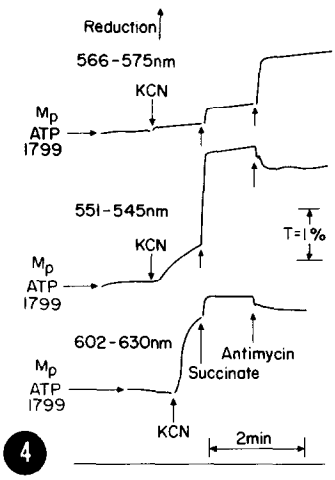


Figure 2: Repetitive difference spectra showing relaxation of enhanced reduction of b-566 (and of b-558, a second component which responds to antimycin; ref. 30) after anaerobiosis in antimycin-treated mung bean mitochondria. Mitochondria (9.9 mg protein) were suspended in 3.0 ml medium A, soaked in ATP (0.33 mM), and made anaerobic by addition of succinate (8.3 mM). The spectra show the extra reduction in a sample treated with antimycin (0.51 μg/mg protein) at various times following partial aeration by vigorous stirring. Relaxation of enhanced reduction is complete at 12.3 min (bottom spectrum); at this point, the spectrum shows only an antimycin-induced red shift in the α-peak of b-560 (a high potential b-cytochrome; see ref. 30). 1 cm light path, room temperature.

Figure 3: Low temperature difference spectra of mung bean mitochondria. Mitochondria (8.25 mg protein) were suspended in 1.1 ml medium A and soaked in ATP (0. 3 mM). Spectrum A: succinate (10 mM)/anaerobic minus aerobic. Spectrum B: succinate (10 mM) + antimycin (AA; 0.13 μg/mg protein)/aerobic minus aerobic. The positive sample was treated with antimycin for 2 min, succinate was added, and 30 sec later, the sample was frozen in liquid nitrogen. Spectra C,D: succinate (10 mM) + antimycin (AA; 0.13 μg/mg protein)/anaerobic minus aerobic. The positive sample was made anaerobic by addition of succinate, and antimycin was added. 2 min later, the sample was partially aerated. The sample was frozen 45 sec after aeration in spectrum C and 45 min after aeration in spectrum D. The spectra show α-peaks of cytochromes c + c_1, b-556, b-560, and b-566; the actual locations of these peaks are 549, 553-554, 557-558, and 563 nm, respectively. 2 mm light path, 77°K.

since it remains possible that reduction of c_1 upon anaerobiosis does cause a concomitant decrease in the E_m of b-566 and that the slow oxidation observed in Fig. 1 is relaxation of disproportionately reduced b-566. For this reason, to determine whether oxidation of c_1 is a prerequisite for enhanced reduction of b-566, we examined the effect of antimycin in aerobic, succinate-reduced mitochondria in which oxidation of c_1 was prevented by blocking cytochrome oxidase with cyanide. The upper trace in Fig. 4 shows that antimycin does cause enhanced reduction of b-566 (b-566 becomes 55 to 80% reduced; cf. Table I)



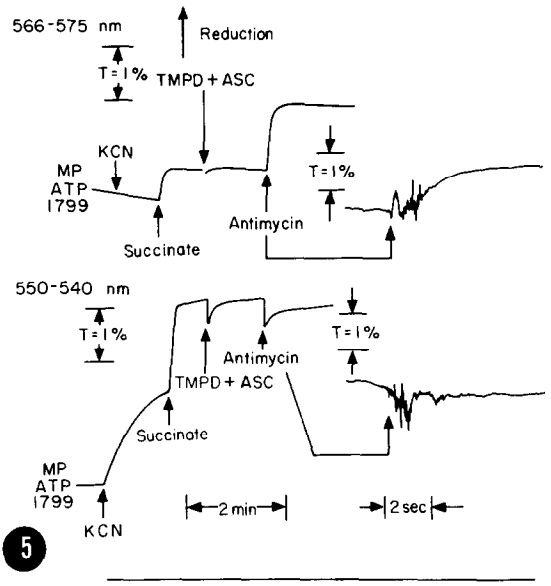


Figure 4: Dual wavelength spectrophotometer recordings showing the effect of antimycin on the oxidation-reduction levels of cytochromes b-566, c + c_1, and aa_3 in aerobic, cyanide-treated potato mitochondria. Upper trace: cytochrome b-566 monitored at 566-575 nm. Middle trace: cytochrome c + c_1 monitored at 551-545 nm. Bottom trace: cytochrome aa_3 monitored at 602-630 nm. The light path was 1 cm. Concentrations were: ATP (0.33 mM), 1799 (an uncoupler; 6.7 µM), KCN (0.83 mM), succinate (8.3 mM), antimycin (0.89 µg/mg protein). Mp = 5.6 mg mitochondrial protein. Reactions were carried out in 3.0 ml medium A at room temperature. Assignment of redox changes to b-566 were confirmed by difference spectra (not shown).

Figure 5: Dual wavelength spectrophotometer recordings similar to those of Fig. 4 but with TMPD plus ascorbate present. Upper trace: cytochrome b-566 monitored at 566-575 nm. Lower trace: cytochrome c + c_1 monitored at 550-540 nm. Oscilloscope traces to the right show addition of antimycin on expanded time scale. The light path was 1 cm. Concentrations were: N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.2 mM), ascorbate (10 mM), Mp = 5.8 mg mitochondrial protein. All other concentrations were as in Fig. 4. Reactions were carried out in 3.0 ml medium A at room temperature. Similar results were also obtained with coupled mitochondria in medium B.

under these conditions. Concomitantly, as shown in the lower traces, there is only a small -- ca. 15% -- oxidation of cytochromes c + c_1 and aa_3, which may be attributed to inhibition of a slow residual flux through the cyanide-inhibited chain. In other experiments, shown in Fig. 5, under the same conditions, but with TMPD plus ascorbate present as an additional clamp on the redox state of cytochrome c + c_1, antimycin has the same effect on b-566, and concomitantly, c + c_1 remains at least 95% reduced. The possibility that a transient oxidation of c_1 occurs in conjunction with the enhanced reduction is ruled out in the expanded time scale oscilloscope traces to the right in Fig. 5. We conclude

that neither active cytochrome oxidase nor more than 5% oxidation of cytochrome $\underline{c} + \underline{c}_1$ is required for the enhanced reduction of $\underline{b}$-566. (However, that O_2 continues to play a crucial role, even under these conditions, was confirmed by subsequent experiments showing that the enhanced reduction in cyanide-inhibited mitochondria was relaxed upon anaerobiosis.)

DISCUSSION

The results presented in this paper show (a) that the reducibility of cytochrome $\underline{b}$-566 in plant mitochondria is increased by antimycin plus O_2, (b) that relaxation of the enhanced reduction following consumption of O_2 does not show a close kinetic correlation with reduction of cytochrome $\underline{c}_1$, and (c) that enhancement of $\underline{b}$-566 reduction does not require oxidation of cytochrome $\underline{c}_1$.

The lack of kinetic correlation between reduction of cytochrome $\underline{c}_1$ upon anaerobiosis and relaxation of $\underline{b}$-566 reduction is in agreement with the recent results of Wikström and Berden (17) who reported apparent fast and slow oxidations of $\underline{b}$-566 following anaerobiosis in antimycin-treated beef heart mitochondria. Here too, under comparable experimental conditions, the fast oxidation was synchronous with reduction of cytochrome $\underline{c} + \underline{c}_1$ and was mainly attributed to spectral interference, while the slow oxidation was assigned to $\underline{b}$-566. Wikström and Berden also pointed out that the antimycin plus O_2-enhanced reduction of $\underline{b}$-566 is stable for some time in the presence of reduced cytochrome $\underline{c}_1$ in both anaerobic and cyanide-inhibited mitochondria. Our results extend theirs by demonstrating that an initial oxidation of cytochrome $\underline{c}_1$ is not required to produce this effect.

That section of our results describing the relaxation of the enhanced reduction upon anaerobiosis (Fig. 1) is also in substantial agreement with results reported for plant mitochondria by Storey (9), even though the latter author considered the relaxation to be closely correlated with reduction of $\underline{c}_1$. This discrepancy is explained by the fact that Storey was concerned only with the fast oxidation and did not attribute any slow oxidation to $\underline{b}$-566. In light of other results presented in our report, it appears that the importance of the fast oxidation was over-emphasized.

In general terms, the enhanced reduction of $\underline{b}$-566 in plant and animal mitochondria can be attributed equally well to a decrease in the effective redox potential of the substrate, or to an increase in the E_m of $\underline{b}$-566. The fact that the effect occurs only under non-equilibrium conditions, in the presence of an oxidant, makes it extremely difficult to distinguish between these possibilities. It should be emphasized, in view of proposals made in the literature (9,10,27,28) that the enhanced reduction does not necessarily require formation of an antimycin/$\underline{b}$-566 complex. The detailed mechanism of the effect

remains of great interest. Although our results indicate that oxidation of cytochrome c_1 is not a key factor, they are compatible with three other suggested mechanisms. (i) The effect could reflect the response of b-566 to oxidation of some other regulatory component -- designated X -- perhaps an iron sulfur protein (16,29). X would have to behave as an electron transport component similar to cytochrome c_1, but should also possess the unique ability to remain oxidized in the presence of substrate and cyanide (since enhanced reduction of b-566 occurs under these conditions; Fig. 4). The latter consideration could imply that X, if it exists, is reduced slowly and is not on the main electron transport pathway. (ii) The enhanced reduction of b-566 could reflect an increase in its E_m resulting from accumulation of a high energy form of b-566, generated by electron flow through the terminal segment of the chain and stabilized by antimycin (12). However, the occurrence of the effect in cyanide-inhibited mitochondria (see Fig. 4) means that even very slow electron flow should be sufficient to maintain the high energy form. (iii) Finally, the enhanced reduction could be caused by a decrease in the redox potential expressed at b-566, resulting from an oxidant-induced shift in an equilibrium below the antimycin block of the type proposed by Wikström and Berden (17). The data available are not sufficient to determine which, if any, of these mechanisms is correct.

ACKNOWLEDGMENTS

The authors thank Drs. C.-P. Lee, D. C. Lee, C. A. Mannella, and V. I. Pye for many helpful discussions, and Miss Eva L. Christensen for preparations of purified mitochondria and general inspiration. This work was supported by grants from the National Science Foundation and the Herman Frasch Foundation.

REFERENCES

1. Chance, B., J. Biol. Chem. 233, 1223 (1958).
2. Slater, E.C. and Colpa-Boonstra, J.P., in Haematin Enzymes (J.E. Falk, R. Lemberg and R.K. Morton, eds.) p. 575, Pergamon Press, New York (1961).
3. Pumphrey, A.M., J. Biol. Chem. 237, 2384 (1962).
4. Wikström, M.K.F., in Energy Transduction in Respiration and Photosynthesis (E. Quagliariello, S. Papa and C.S. Rossi, eds.) p. 693, Adriatica Editrice, Bari (1971).
5. Sato, N., Wilson, D.F., and Chance, B., Biochim. Biophys. Acta 253, 88 (1971).
6. Brandon, J.R., Brocklehurst, J.R., and Lee, C.P., Biochemistry 11, 1150 (1972).
7. Berden, J.A. and Opperdoes, F.R., Biochim. Biophys. Acta 267, 7 (1972).
8. Lance, C. and Bonner, W.D., Jr., Plant Physiol. 43, 756 (1968).
9. Storey, B.T., Biochim. Biophys. Acta 267, 48 (1972).
10. Slater, E.C., Lee, C.P., Berden, J.A., and Wegdam, H.J., Nature 226, 1248 (1970).
11. Bonner, W.D., Jr. and Slater, E.C., Biochim. Biophys. Acta 223, 349 (1970).
12. Wilson, D.F., Koppelman, M., Erecińska, M., and Dutton, P.L., Biochem. Biophys. Res. Commun. 44, 759 (1971).

13. Erecińska, M., Chance, B., Wilson, D.F., and Dutton, P.L., Proc. Nat. Acad. Sci., U.S.A. 69, 50 (1972).
14. Kováč, L., Šmigáň, P., Hrušovská, E., and Hess, B., Arch. Biochem. Biophys. 139, 370 (1970).
15. Baum, H. and Rieske, J.S., Biochem. Biophys. Res. Commun. 24, 1 (1966).
16. Rieske, J.S., Arch. Biochem. Biophys. 145, 179 (1971).
17. Wikström, M.K.F. and Berden, J.A., Biochim. Biophys. Acta 283, 403 (1972).
18. Chance, B., FEBS Lett. 23, 3 (1972).
19. Chance, B. and Erecińska, M., Biophys. Soc. Abstracts 12, 221a (1972).
20. Douce, R., Christensen, E.L., and Bonner, W.D., Jr., Biochim. Biophys. Acta 275, 148 (1972).
21. Chance, B., Methods Enzymol. 4, 273 (1957).
22. Dutton, P.L., Wilson, D.F., and Lee, C.P., Biochemistry 9, 5077 (1970).
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem. 193, 265 (1951).
24. Wilson, D.F., Erecińska, M., Leigh, J.S., Jr., and Koppelman, M., Arch. Biochem. Biophys. 151, 112 (1972).
25. Bendall, D.S. and Bonner, W.D., Jr., Plant Physiol. 47, 236 (1971).
26. Wikström, M.K.F., Biochim. Biophys. Acta 253, 332 (1971).
27. Boveris, A., Oshino, R., Erecińska, M., and Chance, B., Biochim. Biophys. Acta 245, 1 (1971).
28. von Jagow, G. and Klingenberg, M., FEBS Lett. 24, 278 (1972).
29. Baum, H., Rieske, J.S., Silman, H.I., and Lipton, S.H., Proc. Nat. Acad. Sci., U.S.A. 57, 798 (1967).
30. Lambowitz, A.M. and Bonner, W.D.,Jr., Biophys. Soc. Abstracts 13, 51a (1973).