RESOLUTION OF THE SUBCELLULAR COMPONENTS OF CYANIDE INSENSITIVE AND SENSITIVE RESPIRATION IN A DURUM WHEAT

by

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STATEMENT BY AUTHOR

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Polarographic measurements of mitochondria isolated via differential centrifugation from the germ of durum wheat seedlings showed that the ratio of antimycin-A insensitive to total respiration fell from 0.32 to 0.12 at 12 through 64 hrs. of development. Further purification of the initial mitochondrial pellet on a linear Percoll^{R} density gradient separated the mitochondria into two bands of physiologically distinct activity. Based on the usual respiratory criteria, these organelles were qualitatively similar to those of the crude pellet, however no antimycin-A insensitive component of respiration was observed in either mitochondrial gradient band through 64 hrs. Antimycin-A insensitive respiration could be restored exclusively to the upper band of mitochondria by the addition of linoleic acid and this activity was inhibited either by salicylhydroxamic acid (SHAM) or propyl gallate, a known lipoxygenase inhibitor. Likewise, addition of linoleic acid to the crude pellet elicited a 4 to 5 fold respiration increase. This 0_2 consumption was insensitive to antimycin-A and inhibited by either propyl gallate or SHAM. Electron microscopic examination revealed that only the lower gradient band contained purified mitochondria which in turn lacked ability to oxidize linoleic acid. These data suggest that <u>in vitro</u> antimycin-A insensitive respiration in wheat seedlings is the result of lipoxygenase activity not directly associated with purified mitochondria.

INTRODUCTION

It has become apparent that the distribution of "high energy" metabolites within an actively photosynthesizing cell is under tight intracellular regulation. In particular, experimental evidence accumulating on the existence of numerous and highly specific transport and shuttle systems (Heber, 1974; Wiskich, 1977) indicates that there may be a regulatory system involving chloroplastic, mitochondrial and cytoplasmic components which act to control the fate of labile photosynthate. A necessary prerequisite for studying such a system is the ability to separate the various subcellular components in order to assess their individual functions and capabilities. We have evaluated the utility of Percoll^{R} as a density gradient medium in terms of its effects on several physiological parameters associated with mitochondrial integrity, with the conclusion that Percoll^{R} density gradients enable the separation of purified, active wheat mitochondria.

Cyanide insensitive respiration has often been measured by researchers primarily interested in the mitochondrial energetics of ATP production (Solomos, 1977). However, it now seems clear that lipoxygenase may account for a substantial portion of total O_2 consumption at certain times during development in soybean and wheat (Eskin, Grossman and Pinsky, 1977). The present work reports a series of respiratory studies carried out on conventionally and highly purified mitochondria isolated from germinating wheat seedlings at 12 through

64 hours of development. Data are presented which strongly suggest that all antimycin-A insensitive respiration in germinating wheat is the result of lipoxygenase activity which is developmentally controlled. The experiments reported here serve to reemphasize the importance of a) utilizing purified organelles in plant respiratory measurements and b) assessing all systems which may be involved in oxygen consuming reactions.

LITERATURE REVIEW

Subcellular Components of Respiration

The history of research on aerobic respiration has been reviewed by Lehninger in his excellent book on the mitochondrion (Lehninger, 1964). It is interesting to note that Warburg's original concept, which assumed most if not all utilization of oxygen by cells takes place through the intervention of an iron containing catalyst, is still representative of our qualitative understanding of electron transport after almost 70 years of intensive investigation.

This is not to say that remarkable progress has not been made in the area of bioenergetics,progress that has been comprehensively reviewed by Racker (1965, pp.83-87). Beginning in the 1930's with the studies of Englehardt (cited by Racker, 1965), who recognized the relationship between oxidation and phosphorylation and Kalckar (cited by Racker, 1965), who demonstrated the respiration-dependent formation of ATP in cell free preparations; the experimental puzzle of oxidative phosphorylation has been pieced together to a point where the major components of the respiratory chain and their relative positions in the inner mitochondrial membrane are now known.

Figure 1 shows the postulated electron transport chain for mammals and it is generally accepted that all aerobic organisms have the same type of system with minor modifications (Lehninger, 1975, 477-507). However, in addition to the pathway shown by the full arrows,

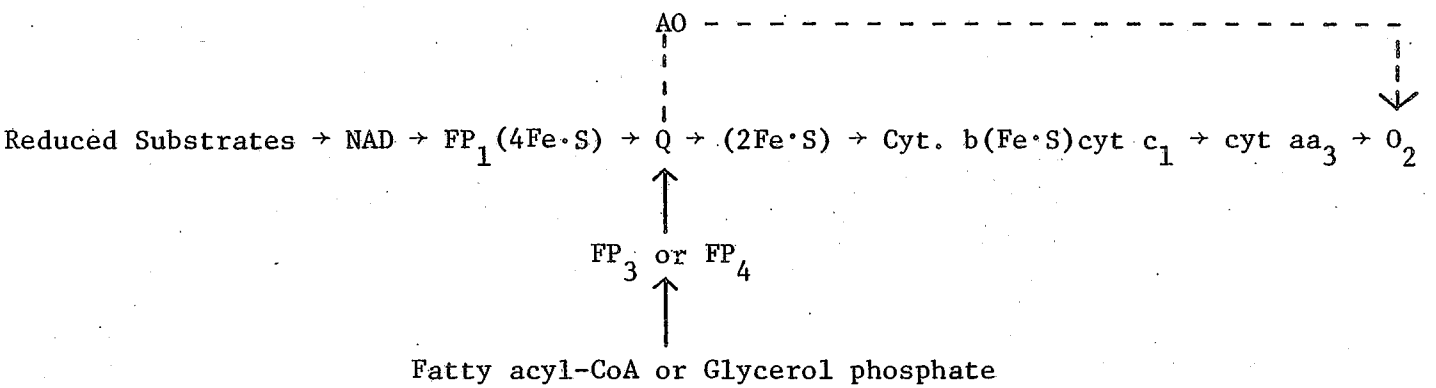


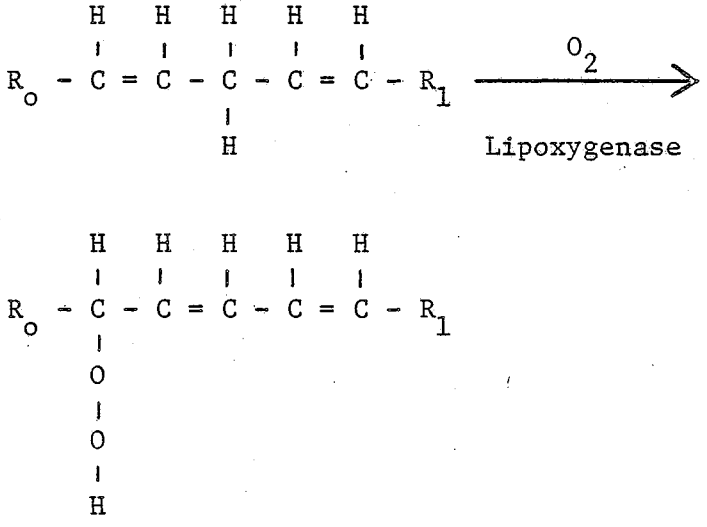
Figure 1. The respiratory chain, labeling scheme after Lehninger (1975, p. 495) except AO = alternate oxidase.

workers (Solomos, 1977) have now proposed the existence of an alternative mitochondrial electron transport system (Figure 1, broken arrows) resulting from a branch point in the ubiquinone region (Q) of the main chain and terminating at an alternative oxidase. The nature of this alternate oxidase is unknown but it could conceivably be a flavoprotein or other non-iron containing enzyme.

Other O_2 Consuming Systems within the Cell

In addition to the oxygen utilization resulting from oxidative phosphorylation and any alternative mitochondrial oxidations, a number of other subcellular oxygen consuming enzyme systems are known to exist. These enzymes include

Lipoxygenase, also called lipoxidase (Eskin et al., 1977), is an enzyme that catalyzes the addition of molecular oxygen to linoleic acid as shown below, resulting in the formation of a hydroperoxide which subsequently breaks down into numerous products:



(1)

Monooxygenases, which catalyze the insertion of one oxygen atom from molecular oxygen into the substrate in the general manner shown below, and exemplified by phenylalanine hydrolase (Lehninger, 1975, p. 501):

$$AH + XH_2 + O_2 \xrightarrow[\text{Monooxygenase}]{} AOH + H_2O + X \qquad (2)$$

Dioxygenases, which catalyze the formation of a dihydroxylated derivative of the substrate:

$$AH_2 + O_2 \xrightarrow[\text{Dioxygenase}]{} A(OH)_2 \qquad (3)$$

An example of this type of enzyme would be tryptophan 2,3-dioxygenase (Lehninger, 1975, p. 501).

Superoxide dismutase and catalase, which form an important free radical scavenging system that acts to prevent damage to cellular components by the O_2^- superoxide anion (Bonner and Varner, 1976, pp. 103-105):

$$2O_2^- + 2H^+ \xrightarrow[\text{dismutase}]{\text{Superoxide}} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow[\text{Catalase}]{} H_2O + 1/2O_2 \qquad (4)$$

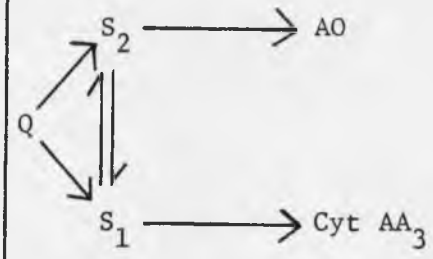
In addition to these oxygen consuming enzyme systems, plants are known to exhibit light induced photorespiration, a metabolic system that is the subject of intense investigation (Zelitch, 1971). All of these systems must be considered and evaluated when attempting to identify the subcellular components of respiration in a given plant system.

Mitochondrial Cyanide Insensitive Respiration

Cyanide insensitive respiration is defined operationally as the O_2 consumption remaining after one has titrated the biological material with cyanide to a point where further addition of the inhibitor does not cause further diminution of respiration. This phenomenon was first demonstrated in plant tissues by Van Herk (1937). A recent extensive review by Henry and Nyns (1975) shows cyanide insensitive respiration to be fairly widespread among plants and microorganisms.

Beginning with the initial observation by James and Elliot (1955) and carrying on to the present, data have been accumulating in the literature which shows that mitochondria isolated from cyanide resistant tissues also exhibit insensitivity to cyanide. The development of the branched mitochondrial electron transport pathway theory as the biochemical basis for cyanide insensitive respiration has been documented in a recent review by Solomos (1977). A detailed kinetic model has been developed, mainly by Bonner and his co-workers which includes a branched electron transport pathway with an alternate terminal oxidase (Bendall and Bonner, 1971; Bahr and Bonner, 1973) and a dynamic equilibrium involving the redox state of the first electron carrier in each pathway after the branch point (Figure 2).

Putative physiological roles for alternate oxidase induced CIR in plants range from thermogenesis in Aroids (Meeuse, 1975) to climacteric respiration in certain fruits and vegetables (Solomos and Laties, 1975). However, the specificity of the criteria used to identify AO induced CIR must now have been called into question.



Q = ubiquinone

S_1 = First electron carrier to cyt. AA_3

S_2 = First electron carrier to alternate oxidase

Cyt AA_3 = Cytochrome AA_3

AO = Alternate oxidase

Figure 2. Proposed branch point for alternate oxidase electron transport.

Mitochondrial Purification and Alternate Oxidase Respiration

Experimental data used in formulating the branched electron transport pathway model have been interpreted using two *a priori* assumptions: 1) Respiration in the subcellular fractions used for the various experiments was attributable to mitochondrial activity and 2) substituted hydroxamic acids acted as specific inhibitors of the alternate terminal oxidase in those same experiments.

In view of recent experimental evidence both of these presuppositions should be re-evaluated. Although Douce, Christensen and Bonner (1972) reported the purification of plant mitochondria to homogeneity (as judged by electron micrographs) in 1972, almost all of the experimental data used to characterize mitochondrial cyanide insensitive respiration was obtained by workers utilizing various modifications of Bonner's original isolation technique (Bonner, 1967) involving a series of differential centrifugations, and most probably contained significant amounts of contaminants.

Use of contaminated subcellular fractions was justified on the basis of the putitative inhibitor specificity of cyanide, antimycin-A and hydroxamic acids for mitochondrial enzymes (Schonbaum et al., 1971; Lehninger, 1975); and the belief that a lack of endogenous respiration prior to the addition of a Kreb's Cycle intermediate implied that no O_2 utilizing systems other than mitochondrial enzymes were present. However, hydroxamic acids have recently been shown to inhibit a wide class of redox enzymes (Rich et al., 1978) including lipoxygenase. This enzyme has been shown to be highly active in some plants during certain stages of growth and development (Axlerod, 1974). Unfortunately, Douce et al. (1972) did not assay AO activity or determine whether or not lipoxygenase was present in the purified mitochondrial fraction. Other workers have also attempted to purify their organelles by density gradient methods, however, the preparations of those who published electron micrographs were far from homogeneous (Douce, Moore and Neuburger, 1977). Others (Day et al., 1978) were satisfied to harvest a 'uniform band' from the gradient and used their mitochondrial fraction without any further investigation into its relative purity. Assays for lipoxygenase activity have not been routinely performed by workers in the field of mitochondrial respiration. Hopefully, the results presented in the body of this thesis will help to correct this oversight.

Present Status of Cyanide Insensitive Respiration in Plants

Parrish and Leopold (1978) were the first to point out that substituted hydroxamic acids inhibited lipoxygenase and that without the criteria of specific inhibition by hydroxamic acids there was a significant possibility for workers to confound alternate oxidase induced respiration with lipoxygenase activity. Therefore, although an impressive array of low temperature difference spectra has been gathered (Bendall and Bonner, 1971) indicating that several components of the electron transport will remain partially oxidized after cyanide titration of all cytochrome AA_3 activity; and other data exists showing some ATP formation with the same types of organelles (Bahr and Bonner, 1973), the branched mitochondrial electron transport chain must be considered as only a theoretical model. In fact, at present, there are no experiments available in the literature demonstrating cyanide insensitive respiration in an electron microscopically homogeneous preparation of plant mitochondria shown to be free of lipoxygenase activity. It is likely, however, that workers in the field will quickly clarify this situation.

MATERIALS AND METHODS

Isolation of the Initial Mitochondrial Fraction

Wheat (Triticum durum L. cv. 'Jori') seed was surface sterilized by soaking for 2 minutes in a 2.0% bleach solution and then rinsed thoroughly in double distilled H_2O. The seed was then placed between layers of moist paper towelling, encased in two plastic meat packing trays and partially sealed with masking tape. The trays were held in an incubator (Stults Scientific Supply Co.) operating at a continuous 25°C. Germinated seed was removed within one hour of the time indicated for a given experiment. All work was carried out on ice with prechilled instruments and all buffers were adjusted to an initial pH of 7.2 with either KOH or HCl at room temperature (approximately 22°C). The entire scutellum including root and shoot primordia was removed by hand and placed into a preweighed volume of grinding (gx.) buffer (0.057 M K_2PHO_4; 0.018 M KH_2PO_4; 0.004 M EDTA; 0.5 M sucrose and 1.0% w/v BSA). Each differential centrifugation pellet (and each density gradient) contained material from 0.6 gm of tissue. Each 0.6 gm sample and approximately 1 ml of gx. buffer were placed in a glass petri plate and minced with a razor blade that had previously been rinsed with acetone and double distilled H_2O. The minced tissue was poured into a test tube containing 9 ml of the gx. buffer, disrupted for 3 sec. in a Polytron (Brinkman Inst.) at setting 4.5, and centrifuged at 1000 xg (Sorvall SM-24 rotor) in a Sorvall RC-5 refrigerated centrifuge for 5

min. The resulting supernatant was filtered through one layer of 50 micron nylon cloth (Tetko, Inc.). The filtered homogenate was centrifuged at 40,000 xg for 5 min. and the resulting buff mitochondrial pellet separated from the accompanying white starch pellet by rotating the centrifuge tube 90° in the direction of rotation and carrying out an additional 2 minute centrifugation at 40,000 xg (Sarkissian and Srivastava, 1969). The gx buffer was decanted and the upper inside portion of the tube wiped with a tissue to remove any adhering lipid. The final mitochondrial pellet was surface rinsed with 5.0 ml of wash buffer (0.0014 M K_2HPO_4, 0.5 M sucrose), carefully removed from the adjacent starch pellet with a thin glass rod and resuspended in 0.5 ml of wash buffer for further use. This isolation procedure averaged 25 to 30 minutes.

Density Gradient Purification

Percoll^R (PVP-coated silica sol, Pharmacia) and sucrose were employed in this study as density gradient materials. In both cases linear gradients were formed using a gravity feed type gradient maker with both chambers initially containing the same weight of material. The sucrose gradient ran from 0.8 to 1.8 molar sucrose in the wash buffer. The Percoll^R gradient ran from 2 to 60% (v:v) in the wash buffer. The pure Percoll^R was made 0.5 M in sucrose and 0.0014 M in K_2HPO_4 before mixing to insure uniform osmotic composition. All initial densities were obtained by the difference between replicate weights of empty and filled 10 µl pipettes, with linearity verified

using a Bausch and Lomb model ABBE-3L refractometer. The gradients were formed in 5 ml cellulose nitrate tubes and held at 2°C until used.

For density gradient purification the suspension of the initial mitochondrial fraction (0.5 ml) was carefully layered onto the Percoll^{R} (sucrose) gradient with a widemouth pasteur pipette and centrifuged at 37,000 (84,000) xg in a SW.50.1 rotor for 20 (50) min. in a Sorvall OTD-2 ultracentrifuge. Gradient bands were harvested with a widemouth pasteur pipette. To increase the efficiency of organelle recovery identical bands from 4 to 6 gradients were pooled, slowly resuspended dropwise in 2 volumes of wash buffer followed by 3 more volumes of wash buffer. After gentle inversion of the test tube, the organelles were repelleted at 28,000 xg for 7 min. The supernatant was removed with a pipette to a point just above the loose pellet and the mitochondria were then resuspended to the appropriate final volume of wash buffer after transfer to a volumetrically calibrated test tube. For comparative studies, final volumes were such that 0.5 ml contained the material from one gradient band; which in turn had been obtained from one differential centrifugation pellet.

Respiratory Studies

Oxygen uptake was measured with a Clark type oxygen electrode (Yellow Springs Instrument Co.) in a magnetically stirred glass cuvette of 1.5 ml capacity. For each experiment 0.5 ml of either initial or gradient purified material was added to 1 ml of reaction medium (0.3 M mannitol; 0.007 M TES; 0.15 M KH_2PO_4; 0.0002 M thiamine pyrophosphate; 0.0163 M $MgCl_2$). Results were calculated on the basis of 260 µM oxygen

in air saturated medium at 25°C with constant reaction temperature being maintained via a water circulator. Q_{O_2} is defined as nanomoles of O_2 consumed per minute per mg protein, in state 3; except in the case of LA and the Tween-20/αKG/ADP control where Q_{O_2} is taken to be the initial rate of induced respiration.

All substrate and inhibitor concentrations except Tween-20 are given on the basis of the final concentration of the compound in the total 1.5 ml reaction volume. Alpha-ketoglutarate, 12 mM, (Sigma) and ADP, 150 μM (Sigma) were prepared and kept at -20°C until used; all other substrates and inhibitors were prepared daily. Linoleic acid, 1.0 mM or 0.1 mM, (Eastman practical grade) was mixed 1:1 with Tween-20, 0.66% v/v, (Sigma) to form a stable emulsion and was then diluted with vigorous stirring to 10X volume with wash buffer. Care was taken to agitate the emulsion before each injection. Antimycin-A, 2.0 μM or 0.2 μM, (Calbiochem.), SHAM, 1.0 mM, (Aldrich) and PG, 1.0 mM, (Eastman practical grade) were dissolved in abs. EtOH and stirred until a homogeneous solution was obtained. Antimycin-A was used in all inhibitor experiments since it has been shown to affect respiratory systems in a manner similar to cyanide (Bendall and Bonner, 1971) with ADP/O and R.C. values calculated according to Estabrook (1967). Nomenclature of respiratory states is according to Chance and Williams (1955) except steady state 2, which in the present study is taken to mean initial presence of substrate, absence of acceptor. Protein values were obtained by a modification of the method of Lowry et al. (1951).

Reaction vessels and stirrers were washed with Alconox^{R}/hot water and rinsed thoroughly with EtOH/followed by DD H_2O after each inhibitor experiment. Likewise, the oxygen electrode was dismantled and cleaned after each experiment with the lucite plunger being treated in the same manner as the reaction vessel. The electrode tip was swabbed repeatedly with a 1:1 NH_4OH/H_2O solution, thoroughly rinsed and reassembled with fresh electrolyte solution and membrane. Each time the membrane was changed the electrode was allowed to equilibrate for at least one hour in a stirred 2:1 solution of reaction buffer/wash buffer and then calibrated with a freshly oxygenated solution before use. Three Clark electrodes and two Oxygen monitors were used so that mitochondria could be assayed immediately following isolation.

Electron Microscopy

Mitochondrial preparations; crude pellets, and fractions from sucrose and Percoll^{R} density gradients were fixed for 12 hrs. with 3% (v/v) gluteraldehyde in "wash" buffer pH 7.2 at 4^{o}C. The fixed mitochondrial samples were then stained with 1% (w/v) osmium tetraoxide in "wash" buffer for 1 hr., dehydrated in a graded acetone series and embedded in Spurr's low viscosity resin using the firm formulation (Polysciences). The mitochondrial samples were sectioned on a Sorvall MT-2B ultramicrotome using glass knives and floated onto 200 mesh copper grids coated with 0.3% Formvar. The ultrathin sections were then counter-stained for 15 min. with 1% (w/v) uranyl acetate (in 1:1 water/ethanol) and 5 min. with Reynold's lead citrate. Samples were

evaluated using a Hitachi H-500 electron microscope at 50 or 75 kV accelerating voltage.

RESULTS

Developmental Studies

The characteristic developmental pattern for respiration is shown in Figure 3, where antimycin-A insensitive respiration in wheat mitochondria isolated by differential centrifugation comprised 32% (CIR/TR = 0.32) of total respiration at 12 hr of development and then declined rapidly to 21% where it remained for the next 24 hrs., falling to 12% at 64 hrs. Except for the initial developmental study, all experiments were carried out on scutellar tissue one day after imbibition. The 24 hr. material was chosen as a matter of convenience since all isolated mitochondrial and lipoxygenase fractions through 64 hrs. showed the same qualitative behavior towards the various inhibitors. Figure 4 compares the behavior of the mitochondrial fraction during the two successive stages of purification. A final concentration of 2 μM AA was necessary to completely block the AA-sensitive respiratory component, with 1.0 mM SHAM being sufficient to inhibit all remaining respiration in the DC pellet. By comparison, a 1.4 μM AA concentration was sufficient to totally inhibit respiration in the highly purified mitochondrial fraction.

This same qualitative behavior was observed in all gradient fractions containing mitochondrial activity; i.e., in both the sucrose and Percoll^{R} gradients (Figures 5 and 6) all respiration induced by ADP in the presence of αKG was inhibited by AA, as shown in Table 1 where

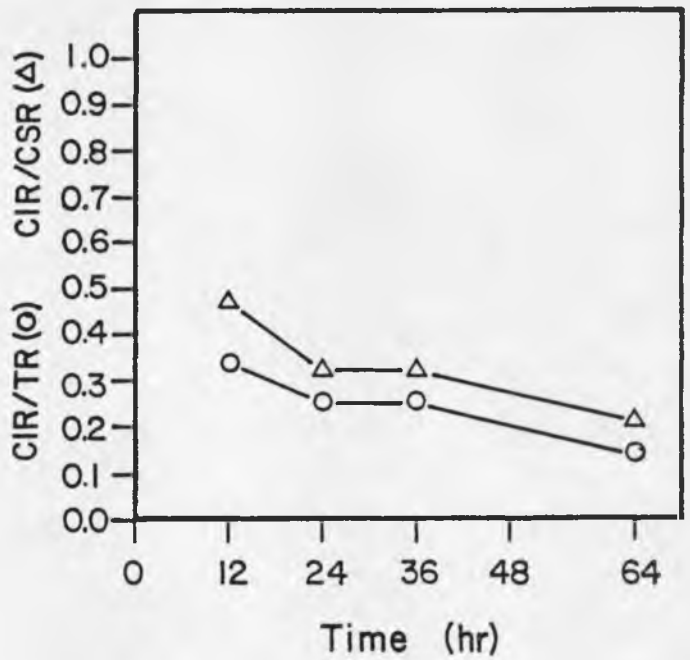


Figure 3. Development of antimycin-A insensitive respiration in germinating wheat mitochondria, isolated by differential centrifugation.

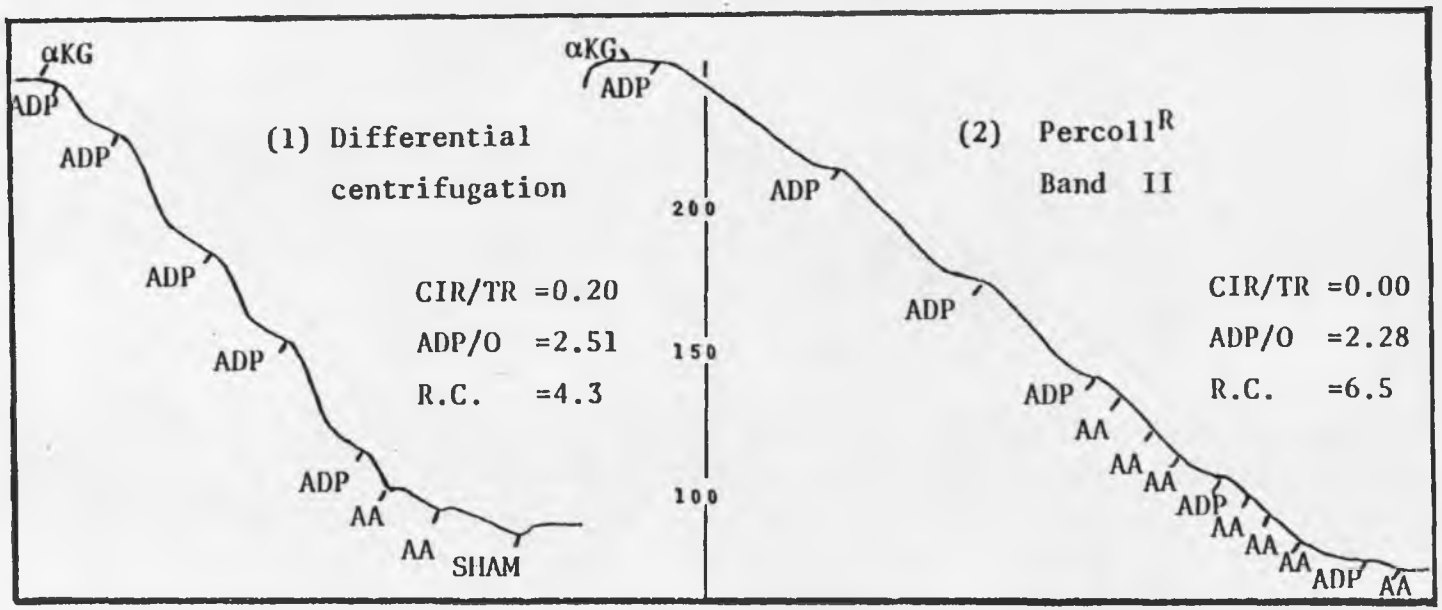


Figure 4. Differential centrifugation (1) and Percoll Band II (2) mitochondria at 24 hrs. of development. -- Note the lack of cyanide insensitive respiration in the gradient purified organelles. Each injection of AA in (1) made the final reaction volume 2.0 µM whereas each injection in (2) made the final volume 0.2 µM.

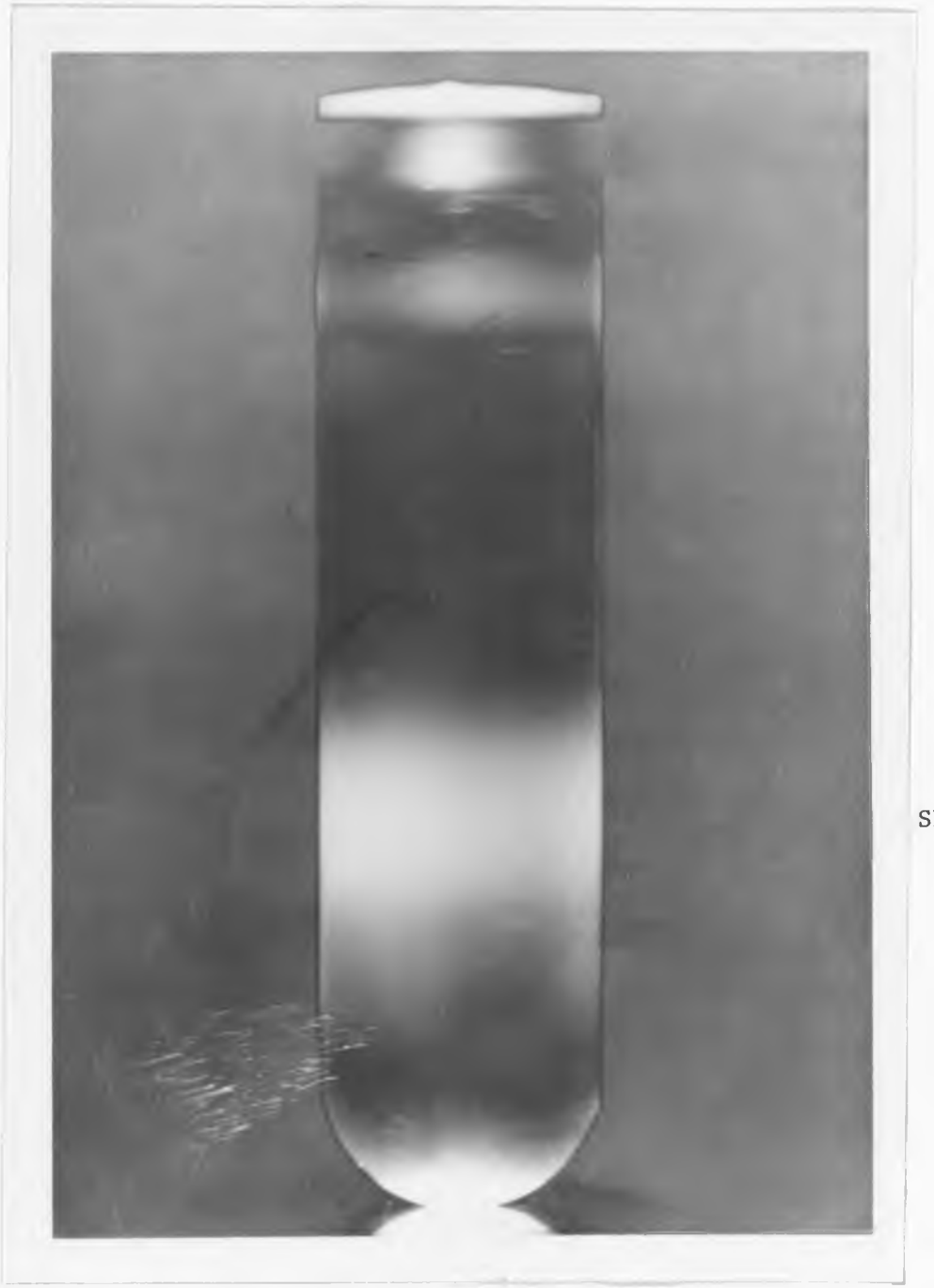


Figure 5. Photo of a sucrose density gradient with the band of mitochondrial activity indicated.

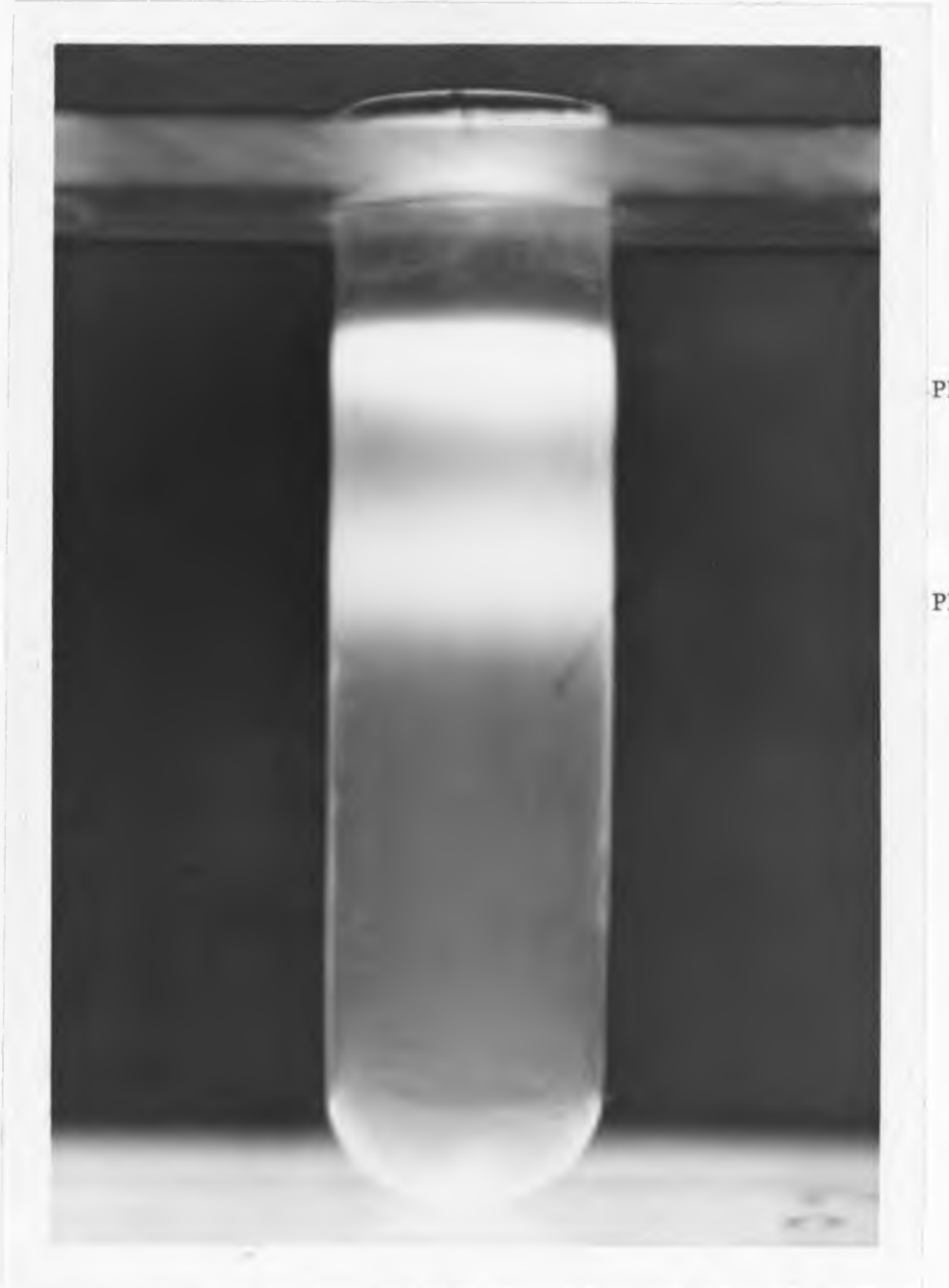


Figure 6. Photo of a Percoll^{R} density gradient with the two bands of mitochondrial activity indicated.

Table 1. Physiological characterization of mitochondria isolated* from embryonic (24 hrs) wheat tissue.

Treatment	ADP/O	R.C.	Q_{O_2}	CIR/TR	Protein
1. DC Pellet	2.31 ± 0.10	5.13 ± 0.90	22.26 ± 2.25	0.20	2.72 mg/ml
2. PDG I	1.93 ± 0.70	2.00 ± 0.50	23.14 ± 0.93	0.0	0.54 mg/ml
3. PDG II	2.52 ± 0.20	5.43 ± 0.90	44.73 ± 0.87	0.0	0.57 mg/ml
4. SDG	2.42 ± 0.12	4.70 ± 1.80	35.83 ± 2.20	0.0	0.60 mg/ml

* Average of three experiments.

relative protein values are also presented. Only the DC pellet and PDG I responded to the addition of LA by initiating AA-insensitive respiration and this oxygen consumption was inhibited by both SHAM and PG. Appropriate complementary inhibitor experiments performed are summarized in Table 2 with the specific substrate-induced respiration rates shown in Table 3. In addition, the upper gradient loading zone and pellet materials from the Percoll^{R} gradient were assayed but no respiration was observed in the presence or absence of LA.

Physiological Characterization

Quantitative alterations in the various parameters used to characterize mitochondria are shown in Table 1. Mitochondrial activity was resolved as two physiologically distinct fractions on the Percoll^{R} density gradient. The mitochondria present in PDG I exhibited a lowering of ADP/O and R.C. ratios when compared with the DC pellet, whereas both PDG II and the SDG band show the enhanced specific activity that one would expect from an increase in organelle purity. Examination with the electron microscope (see Figures 7, 8, 9, 10) revealed intact double membranes and highly condensed cristae in all mitochondrial fractions. Mitochondria essentially free of contamination with other organelles or fragments were observed only in the PDG II. All other preparations included greater or lesser amounts of contaminants.

Additional control experiments (Table 4) demonstrated that SHAM apparently had a detrimental effect on mitochondrial function as exhibited by a lowering of the ADP/O and R.C. ratios. However, this

Table 2. Effects on oxidation by the mitochondrial fraction resulting from the sequential addition (left to right) of substrates and inhibitors. -- Initial induced respiration rate = 100%.

Mitochondrial* Source	Additions	Conclusions
1. DC Pellet	αKG/ADP(100%)→ PG(63.6%)→ AA(0%) αKG/ADP(100%)→ AA(20.8%)→ PG(0%) αKG/ADP(100%)→ AA(19.6%)→ SHAM(0%) LA(100%)→ AA(100%)→ PG(3.6%) Tween-20(0%)→ αKG/ADP(100%)→ AA(5.6%)→ LA(71.5%)→ PG(0%)	In the DC pellet cyanide insensitive respiration = 20%. No component of respiration sensitive to AA with LA as substrate
2. PDG I	αKG/ADP(100%)→ AA(0%)→ LA(96.0%)→ AA(96.0%)→ SHAM(0%) LA(100%)→ AA(100%)→ PG(9.1%)→ αKG/ADP→ (90.9%)→ PG or SHAM(90.9%)→ AA(0%)	In PDG I no CIR with αKG/ADP as substrates, LA restores CIR that is inhibited by PG or SHAM
3. PDG II	αKG/ADP(100%)→ AA(0%) LA(0%)→ αKG/ADP(100%)→ AA(0%)	In PDG II or SDG Band no CIR with αKG/ADP or LA as substrates
4. SDG Band	αKG/ADP(100%)→ AA(0%)→ LA(0%)	

* 24 hr of development.

Table 3. Substrate induced Q_{O_2} in the various mitochondrial fractions.

Mitochondrial Source	Substrate	Final Concentration	Q_{O_2}
Differential Centrifugation	None	-	2.21
Differential Centrifugation	αKG/ADP	12.0mM/150.0μM	20.22
Differential Centrifugation	LA	1.0mM	10.29
Differential Centrifugation	Tween-20/αKG/ADP	0.66%*/12.0mM/150.0μM	12.86
PDG Centrifugation Band I	None	-	0.0
PDG Centrifugation Band I	αKG/ADP	12.0mM/150.0μM	23.14
PDG Centrifugation Band I	LA	0.1mM	20.37
PDG Centrifugation Band II	None	-	0.0
PDG Centrifugation Band II	αKG/ADP	12.0mM/150μM	44.74
PDG Centrifugation Band II	LA	0.1mM	0.0
SDG Centrifugation	LA	0.1mM	0.0
SDG Centrifugation	αKG/ADP	12.0mM/150.0μM	37.50

* Expressed as injected volume/final volume x 100.

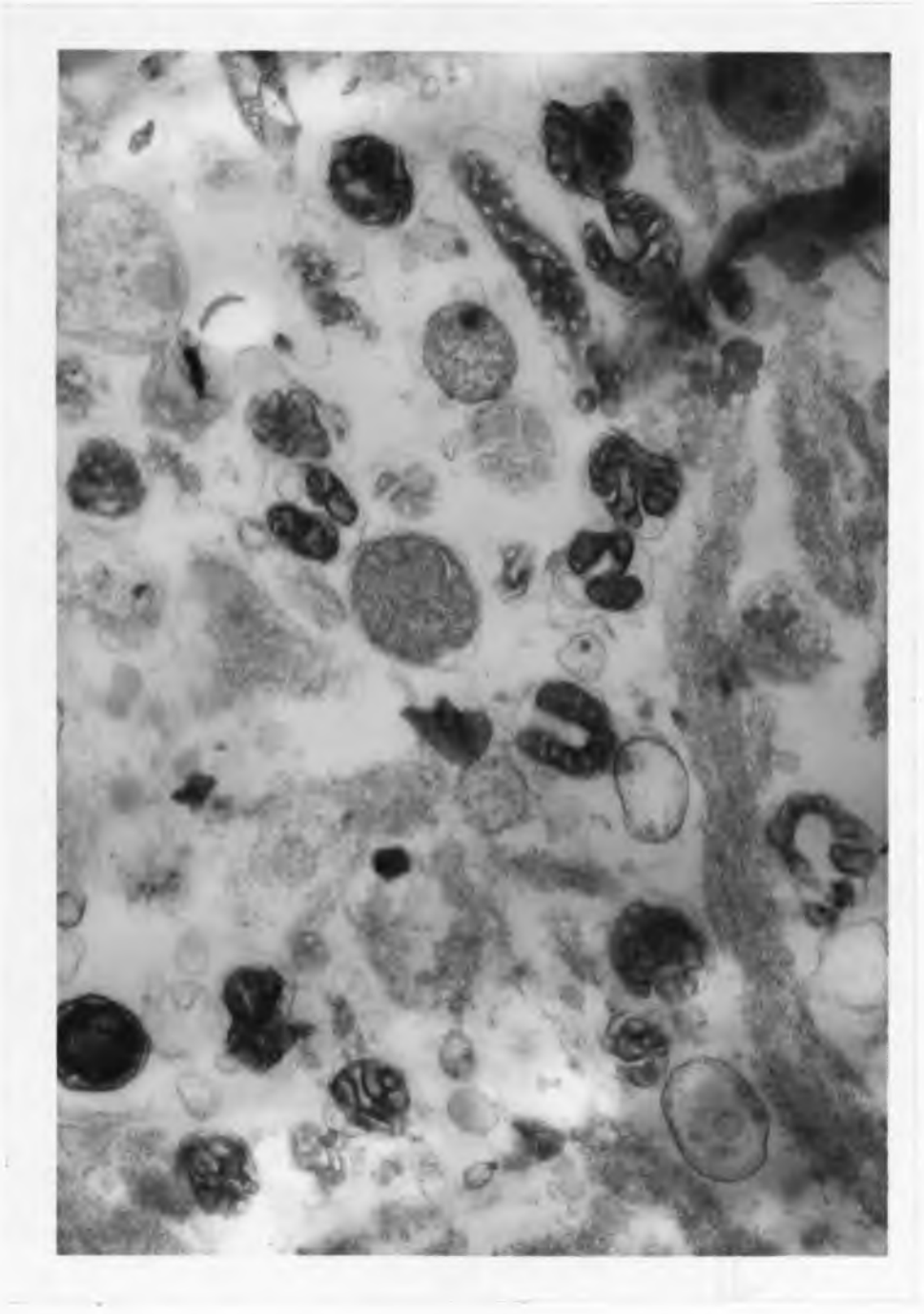


Figure 7. Transmission electron micrograph of the DC pellet. X12,000.

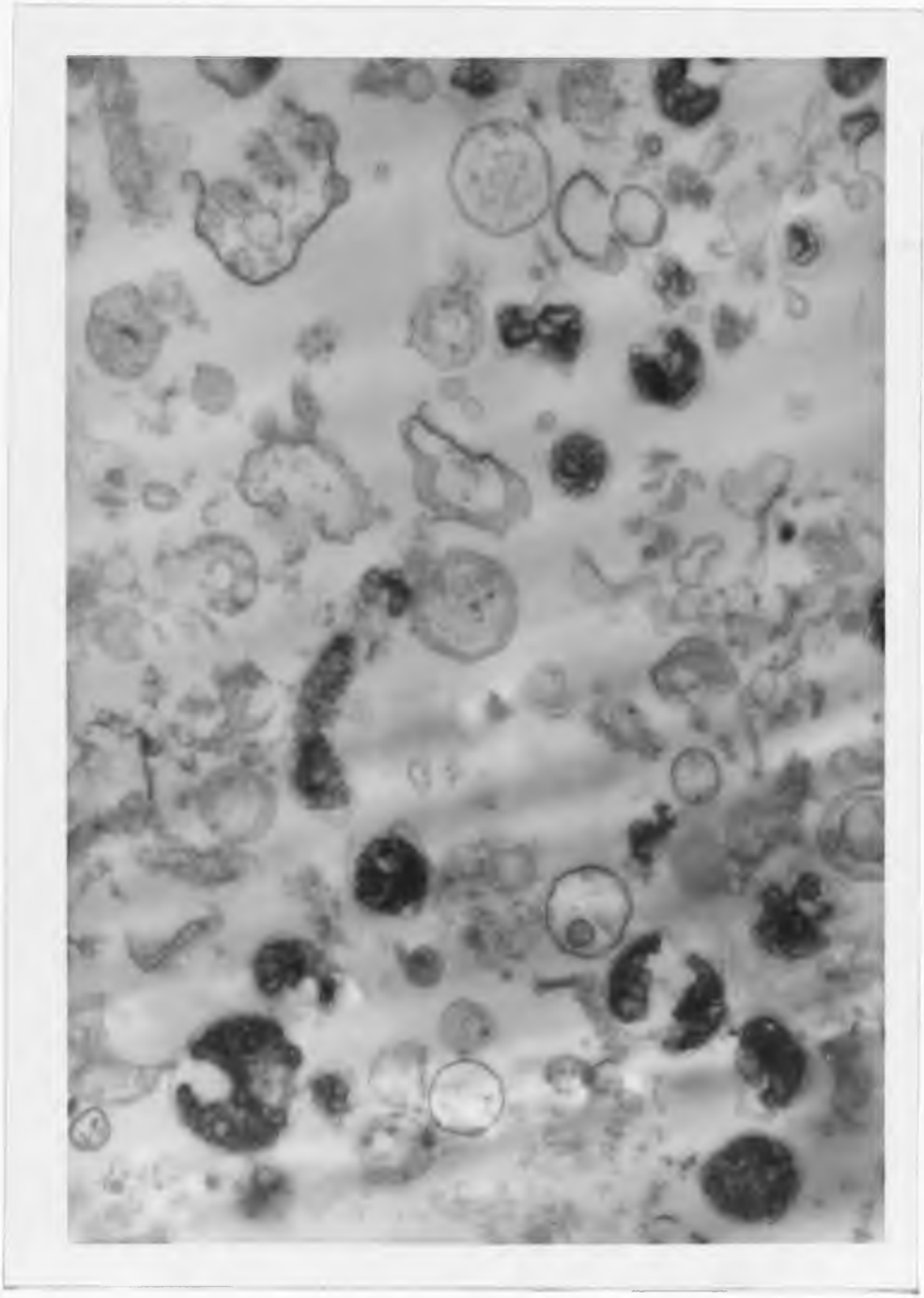


Figure 8. TEM of the SDG band of mitochondrial activity. X10,000.

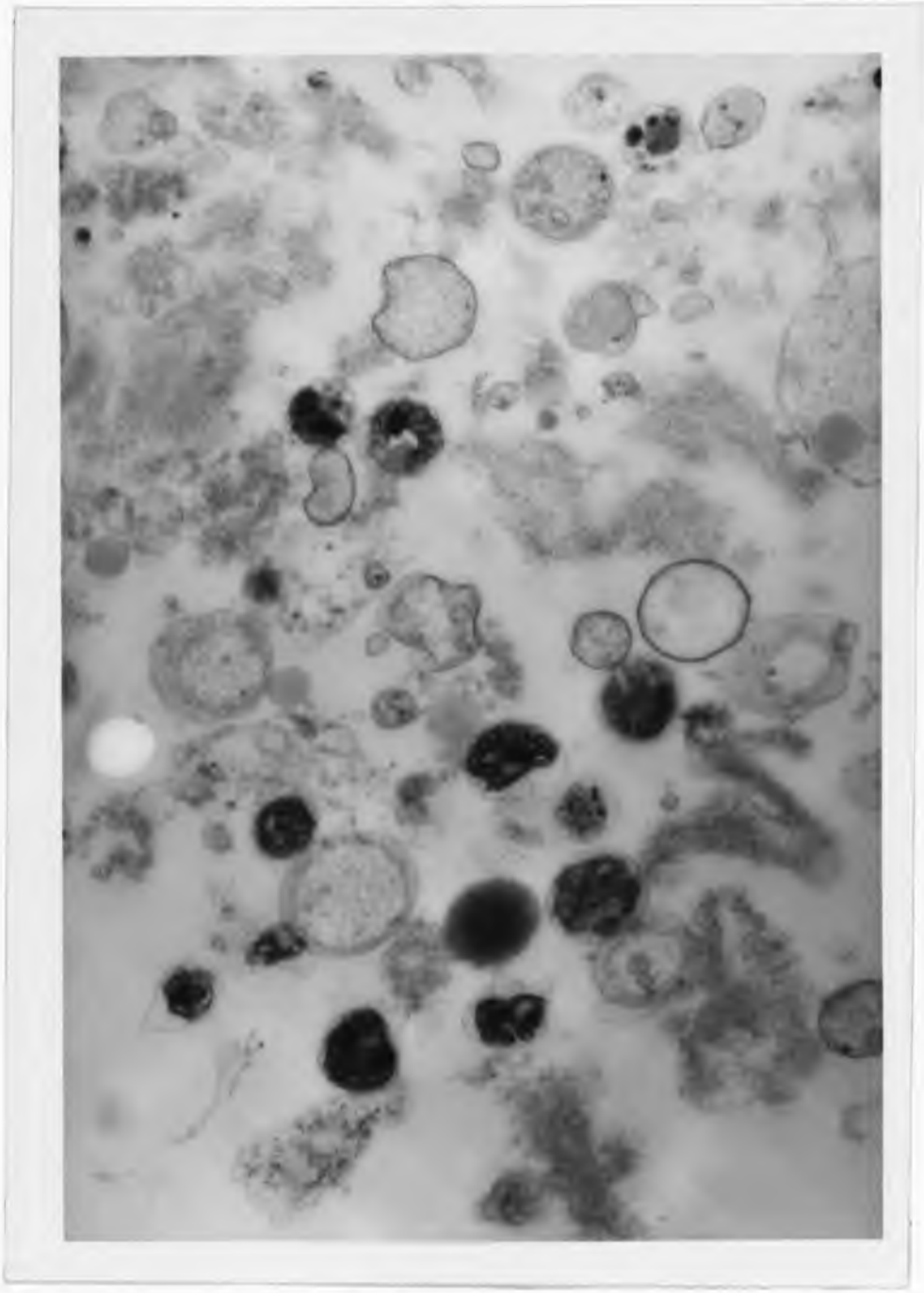


Figure 9. TEM of PDG I. X10,000.

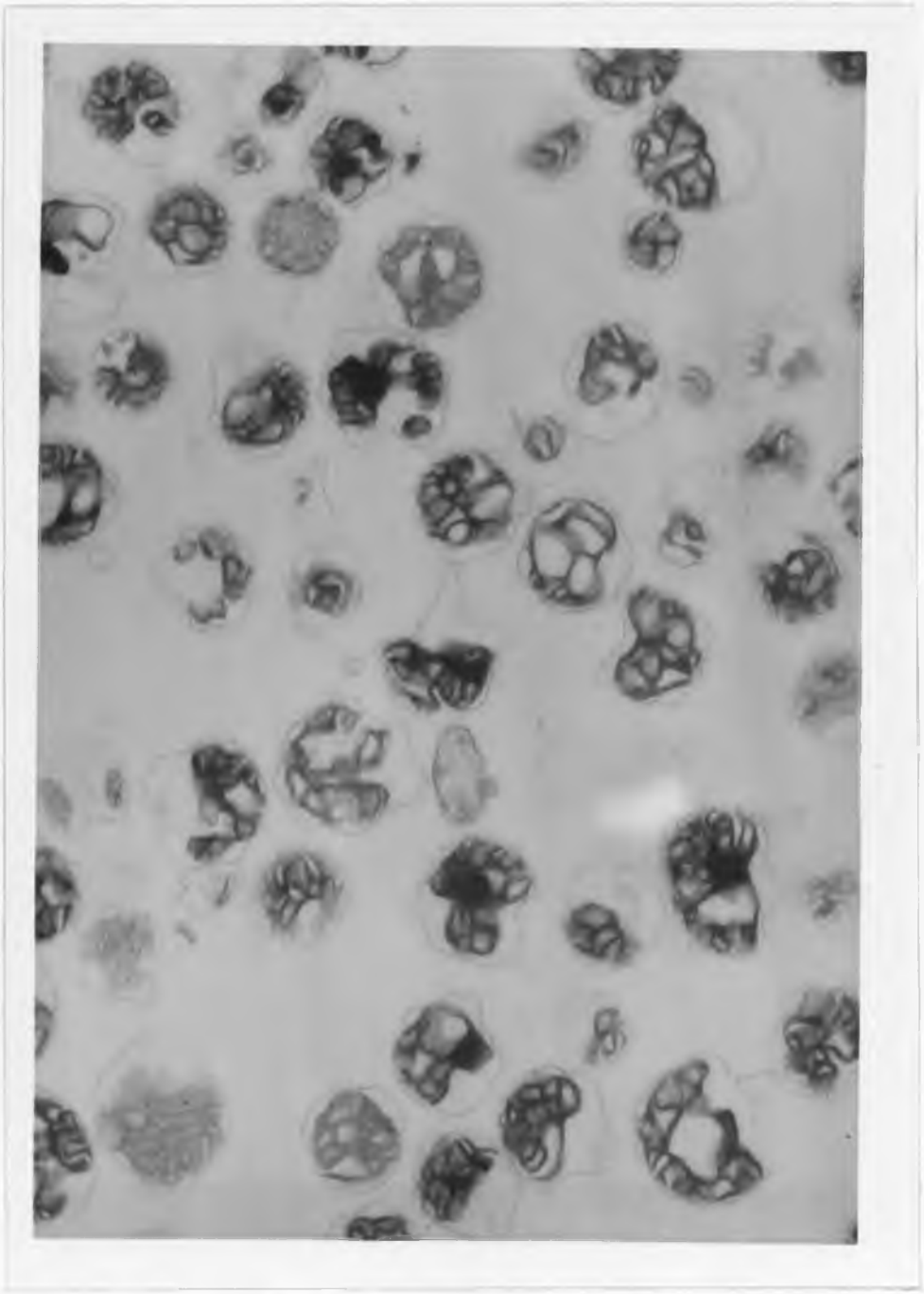


Figure 10. TEM of PDG II, containing highly purified mitochondria. X12,000.

Table 4. Additional controls carried out on 24 hr. mitochondria.

Mitochondrial Fraction/ Treatment	Pretreatment	Post-treatment			Post-antimycin	
	Q_{O_2}	ADP/O	R.C.	Q_{O_2}	Q_{O_2}	CIR/TR
1. PDG II titrated with 8 µl EtOH.	43.86	2.42	4.0	43.86	-	-
2. PDG II titrated with 8 µl EtOH/SHAM.	43.86	2.08	2.6	43.86	-	-
3. DC Pellet incubated for 20 min. with 30% Percoll and subsequently treated as a PDG Band.	-	1.98	3.5	18.01	3.68	0.20
4. As in 3, but washed and repelleted twice.	-	2.24	5.0	17.28	3.67	0.21

effect was not associated with any inhibition of respiration since the Q_{O_2} of Percoll band II mitochondria was the same irregardless of whether this fraction was titrated with 8 µℓ of EtOH/SHAM or with the control buffer. It would appear that SHAM was acting as a quasi-uncoupler of both oxidative phosphorylation and respiratory control in this system. While the biochemical basis for this uncoupling activity is unclear, hydroxamic acids are now known to interact with a wide range of redox enzymes including at least one cytochrome enzyme (Rich et al., 1978).

DISCUSSION

Lipoxygenase as a Respiratory Component

The differential centrifugation fraction isolated from germinating durum wheat seedlings exhibits a high level of CIR that declines rapidly during the first few days of development. A similar pattern has been noted in soybean (Leopold, 1979) and in both cases the phenomenon may now be attributed to LOX activity. A recent review by Axlerod (1974) indicates that LOX is present in a great number of plants. Also, information concerning the role of alternate oxidase in CIR has been increasing in the literature. Most of the essential information concerning alternate oxidase induced CIR in plants has been summarized in two recent reviews (Henry and Nyns, 1975; Solomos, 1977). The status of our knowledge about plant lipoxygenases has also been the subject of two extensive articles (Axlerod, 1974; Eskin et al., 1977). However, when one attempts to resolve the relative contributions by various metabolic pathways at different stages of plant growth and development, it is essential that CIR resulting from LOX activity be distinguished from AO activity. Parrish and Leopold (1978) have quite correctly pointed out the ease with which these two components of CIR may be confounded. The present work demonstrates that the problem is more complex than originally thought. In addition, the presence of significant LOX activity may provide an explanation

for cases where mitochondrial activity *in vitro* is not adequate to account for observed rates of *in vivo* respiration.

Effects of Organelle Purification on Respiration

In wheat seedlings, the mitochondrial fraction isolated by differential centrifugation is rich in LOX activity and hence exhibits a high level of CIR. In some instances, the lack of low levels of endogenous respiration in the absence of a mitochondrial substrate has been taken as a criterion for mitochondrial purity in a differential centrifugation fraction (cf. Solomos and Laties, 1976). As can be seen (Figure 4) by comparing State 1 vs. State 4 respiration in the DC pellet, the initial low LOX activity is stimulated 4 to 5 fold by the addition of αKG and ADP or by the addition of LA to the DC pellet in the absence of Krebs cycle substrate (Table 3). A high level of state 4 respiration resulting from LOX activity without exogenous LA would imply that some lipid substrate is available. Although the biochemical basis for this phenomenon must await further clarification of the LOX reaction mechanism, the apparent synergistic interaction between LOX and mitochondria makes the aforementioned criteria of mitochondria purity less acceptable. Furthermore, appearance of a single band on a linear sucrose gradient (cf. Day et al., 1978) does not guarantee organelle purity in wheat (Figure 8).

This work serves to confirm the finding that LOX is inhibited by hydroxamic acids (Parrish and Leopold, 1978). The problems inherent in any inhibitor study are well known (Wright, 1972) and in the case of wheat and soybean it has not been possible to distinguish AO

respiration from LOX respiration based on inhibition by hydroxamic acids. The non-specific effect of SHAM on PDG II (Table 4) mitochondria serves to further emphasize this point.

Attempts to resolve LOX from the mitochondrial activity by linear density gradient centrifugation were only partially successful. SDG and PDG II mitochondria showed no response to linoleic acid, in contrast, PDG I retained LOX activity while apparently losing all endogenous lipid substrate. In the SDG and PDG II fractions loss of CIR results in a substantial reduction in State 4 respiration (Figure 4) while the ADP/O and RC ratios remain relatively unchanged from those of the DC pellet. PDG I also exhibits minimal State 4 respiration. At this point, it is uncertain whether both PDG Bands of mitochondrial activity are present in the SDG Band or if some type of mitochondrial inactivation or disruption has taken place during centrifugation through the sucrose gradient. Therefore, one cannot unequivocally exclude the possible association of LOX with PDG I wheat mitochondria either as a true organelle function or as a contaminant possibly adsorbed directly onto the outer membrane, or in a complex with BSA. In light of the preceeding discussion it is clear that in plant systems where LOX activity is demonstrated, only mitochondria purified to homogeneity and that show no induction of respiration by linoleic acid may be used to rigorously measure AO respiration. Furthermore, since at this time there are no studies to determine the effect of PG on the alternate oxidase, pretreatment of tissue or organelles with PG cannot be regarded as a useful approach.

Linear Percoll^{R} or sucrose gradients loaded with identical mitochondrial preparations exhibit a great difference in the final distribution and resolution of subcellular components. Based on the data presented here one may conclude that wheat mitochondria, when properly washed and harvested, show no adverse effects from contact with PVP-coated silica-sol material. Likewise the behavior of LOX in the DC pellet does not appear to be affected by an incubation in 30% Percoll^{R} when the pellet is subsequently washed and harvested in a manner consistent with that reported here. Although a second wash was apparently beneficial to the DC pellet (Table 4), additional washes had a noticeably detrimental effect on the gradient mitochondria (data not shown). There appears to be a tradeoff between the severity of isolation procedure and total isolation time as judged by organelle integrity. Physiologically, the PDG mitochondria compared favorably with the DC organelles, indicating that a single wash was sufficient. Also, due to the difference in total protein between the two fractions (Table 1), the effective wash volume was much greater for the gradient material. Extensive work in animal systems (Pertoft et al., 1977) has also shown that Percoll^{R} is non-toxic to biological membranes.

SUMMARY

In conclusion, these experiments indicate that purified mitochondria from tetraploid wheat lack AO respiration through 64 hrs. of development. Since metabolism is ultimately subject to control by gene expression one cannot conclude that AO respiration is absent in all varieties and species of wheat or at all stages of development. However, a reevaluation of AO respiration in wheat and other systems where LOX activity is shown may be appropriate in view of our results. Work is now underway to separate, if possible, the mitochondrial and LOX activities present in PDG I in order to ascertain the subcellular location of this component of CIR. Since up to four isozymes of LOX have been reported for wheat and soybean (Guss, Richardson and Stahmann, 1967, 1968; Guss, Macko et al., 1968; Wallace and Wheeler, 1975) it is entirely possible that LOX may be found in more than one location within the cell.

APPENDIX A

ABBREVIATIONS

TR-total respiration; CIR-cyanide (antimycin-A) insensitive respiration; CSR-cyanide (antimycin-A) sensitive respiration; AO-alternate oxidase; LOX-lipoxygenase; DC-differential centrifugation; SDG-sucrose density gradient; PDG-Percoll^{R} density gradient; AA-antimycin-A; LA-linoleic acid; PG-propyl gallate; SHAM-salicylhydroxamic acid; αKG-alpha-ketoglutarate; ADP-adenosine 5' diphosphate; TES-(N-tris (hydroxy-methyl) methyl-2-aminoethane sulfonic acid); BSA-bovine serum albumin, fraction V.

APPENDIX B

EXTRACTING MITOCHONDRIA FROM TISSUE WITHOUT BSA IN THE GRINDING BUFFER

Substitution of Polyvinylpyrrolidone (PVP) (M.W. 40,000 D) or Polyethyleneglycol (PEG) (M.W. 20,000 D) for BSA in the grinding buffer resulted in the apparent loss of ½ of a phosphorylation site based on the reduction of ADP/O values from an average of 2.5 to an average of 1.9 (Table B-1). Simultaneously, there was a reduction in the R.C. index from an average of 5.1 to averages around 3.0.

The addition of 1 to 10% powdered Amberlite XAD-2 nonionic polymeric adsorbent (Mallincrodt) along with the PEG restored approximately 1 unit to the R.C. index without raising the P/O ratio above 2. Therefore, it would appear that BSA has at least two separate functions in protecting plant mitochondria. It is also apparent from Table B-1 that grinding in 3% PEG and 1% BSA resulted in organelles intermediate to those obtained when these same agents were used alone indicating either that PEG had a detrimental effect on the mitochondria or that it reacted directly with BSA to reduce its well known protective capacity.

Table B-1. Mitochondria isolated by differential centrifugation after grinding in buffers containing different protective chemicals.

Chemical(s)	P/O	R/C
1% BSA	2.5	5.1
3% PVP	1.7	2.3
3% PEG	1.9	3.4
1% BSA and 3% PEG	2.0	4.3
1% BSA and 1% XAD-2	2.4	4.6
3% PEG and 1% XAD-2	1.9	3.8
3% PEG and 10% XAD-2	1.9	4.1
1% XAD-2	1.9	3.3
None	1.9	2.3

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