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Pulmonary Alveolar Macrophage. Oxidative Metabolism of Isolated Cells and Mitochondria and Effect of Cadmium Ion on Electron- and Energy-Transfer Reactions*

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ABSTRACT: Pulmonary alveolar macrophages (PAMs), obtained by endobronchial lavage of sheep lungs, manifest an endogenous respiration of 9 nmoles of O_2/mg of protein per min at 30°. A phenomenon similar to the Crabtree effect is produced if glucose, oligomycin, or Cd^{2+} is added to a cell suspension and the phenomenon is removed in the presence of an uncoupler or Wurster's blue. Isolated PAM mitochondria oxidize a number of substrates. Typical rates are 67 and 91 nmoles of O_2 per mg of protein per min at 30°, respectively, for α-oxoglutarate and succinate as substrates. These mitochondria characteristically utilize α-glycerophosphate (74 nmoles of O_2/mg of protein per min) but not β-hydroxybutyrate (6 nmoles of O_2/mg of protein per min). Substrate oxidation is completely inhibited by 50 μM Cd^{2+} and also by classical inhibitors of respiration. Cd^{2+} inhibits substrate oxidation presumably by interfering with dehydrogenases of mitochondrial respiratory chain. Isolated PAM mitochondria carry out coupled phosphorylation. The ADP:O ratios are >1 (approaching 2) for flavin-linked substrates and >2 (approaching 3) for pyridine nucleotide linked substrates. This oxidative phosphorylation is sensitive to 2,4-dinitrophenol, oligomycin, and Cd^{2+}. A concentration of 5–10 μM Cd^{2+} completely abolishes coupled phosphorylation and respiratory control. Cd^{2+} also inhibits adenosine triphosphatase activity of PAM mitochondria and plasma membrane.

The pulmonary alveolar macrophages (PAMs)[1] have specialized properties in common with other cells of the mononuclear phagocyte system, such as amoeboid motility, endocytic processes, and bactericidal activities. Like other mononuclear phagocytes, they are probably derived from marrow stem cells and circulating monocytes (Virolainen, 1968; Brunstetter *et al.*, 1971). However, PAMs have features which are unique compared to cells of most other organs. For example, they (along with other pulmonary cells) reside in high oxygen tensions of the alveolar gas (90–130 mm; *cf.* West, 1970).

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[1] Abbreviations used are: PAM, pulmonary alveolar macrophage; Wurster's blue, semiquindiamine radical of tetramethyl-*p*-phenylenediamine (*cf.* Mustafa *et al.*, 1968); PCP, pentachlorophenol; DNP, 2,4-dinitrophenol; ATPase, adenosine triphosphatase; ADP:O, a ratio of the number of moles of adenosine diphosphate esterified with inorganic phosphate to the number of atoms of oxygen consumed by the system.

Secondly, PAMs exhibit a high respiratory rate compared with other mononuclear phagocytes residing in much lower oxygen tensions (Oren *et al.*, 1963; Karnovsky *et al.*, 1970).

PAMs play an important role in the lower airway defense mechanism against noxious living and nonliving particulate inhalants (Green, 1968) and in host expression of cellular immunity (Benacerraf and Green, 1969; Pearsall and Weiser, 1970). These cells constitute a significant portion of the lung cellular population (Bertalanffy, 1964). They may be obtained in nearly pure cell line by pulmonary endobronchial lavage (Myrvik *et al.*, 1961). During the past decade renewed interest has yielded new knowledge about the functional biochemistry of these cells (Karnovsky *et al.*, 1970; Pearsall and Weiser, 1970; Van Furth, 1970). Major attention has been given to characterizations of the metabolic and enzymatic apparatus subserving their phagocytic (Dannenberg *et al.*, 1963; Ouchi *et al.*, 1965; Karnovsky *et al.*, 1970; Gee *et al.*, 1970) and lysosomal hydrolytic activities (Pearsall and Weiser, 1970; Van Furth, 1970; Hurst and Coffin, 1971). However, the pathways of mitochondrial substrate utilization and oxidative phosphorylation in PAMs have not been characterized extensively.

PAMs depend on aerobic oxidations as an energy source during phagocytosis whereas the macrophages from peritoneum and other tissues utilize glycolysis for phagocytic energy requirement (Karnovsky *et al.*, 1970; Van Furth, 1970). As PAMs come into an intimate contact with gaseous external environment, perturbations in this environment potentially may have important consequences on the metabolism of these macrophages as well as other lung cells (Green, 1968).

The purpose of the present study was twofold. First, the oxidative metabolism of PAMs has been sparsely explored and no systemic study characterizing their isolated mitochondria has been reported. Secondly, it was thought to be of interest to investigate the effects of cadmium ion[2] on the energy metabolism of PAM. This is because the inhalation of cadmium and its ion has been implicated in certain types of experimental and clinical pulmonary pathology (Johnstone and Miller, 1960; Browning, 1961; Patty, 1962; Bonnell, 1965; Kendrey and Rose, 1969). Cadmium is a component of both urban and industrial air (Carroll, 1966; Molokhia and Smith, 1967; Kendrey and Rose, 1969) as well as cigarette smoke (Schroeder *et al.*, 1961; Manis *et al.*, 1969). Furthermore, Cd^{2+} is known to uncouple oxidative phosphorylation in liver mitochondria (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960). In this communication we present results of our studies on the kinetics of oxygen uptake in PAMs, preparation of PAM mitochondria, and their rates of substrate utilization, and, finally, effects of cadmium ion on the electron- and energy-transfer processes of these mitochondria.

Experimental Section

Isolation of Pulmonary Macrophages. All operations were carried out at 0–4°. PAMs were prepared from sheep according to the lung irrigation technique of Myrvik *et al.* (1961) with some modifications. Lungs from 1- to 2-year-old sheep were removed and kept ice cold soon after their sacrifice at the slaughterhouse and transported to the laboratory within 0.5 hr. Each lung was washed twice with 0.154 M NaCl and the washing was spun at 400*g* for 10 min. Surfactant foam and the supernatant fluid were siphoned off and the pellets of cells obtained were pooled. Cells were washed once with the same medium and resuspended in an appropriate volume. For each lung 1- to 2-ml packed volume of cells was collected. More than 95% of the cells obtained were large mononuclear cells containing abundant cytoplasm with large vacuoles and granular structures (*cf.* Figures 1 and 2). The remainder consisted of small monunuclear cells and occasional polymorphonuclear cells (<2%). Hemorrhagic lungs were discarded and any trace of erythrocytes appearing on the surface of pellets was carefully removed. The viability of cells was determined by the eosin dye exclusion technique (Hanks and Wallace, 1958); 70–80% of the cells in a routine preparation excluded the dye. PAMs were also tested for possible content of bacteria using serum-agar or whole blood-agar medium, but no viable bacteria were observed upon incubation of the culture plate. However, as seen under light microscope, particulate material was often visualized within PAMs. Upon centrifugation of cell homogenate this particulate material sedimented with subcellular fractions.

Preparation of Mitochondria. The procedure for isolation of mitochondria from PAMs was an adaptation of methods published for other systems (Mustafa *et al.*, 1968, 1969). The choice of homogenization procedure was critical. When a loosely fitting (0.25-mm clearance) glass-Teflon homogenizer was used, the extent of cell breakage was poor even after ten strokes. Use of the Lourdes Multi-Mix homogenizer for 15–20 sec at 5000–6000 rpm resulted in a good yield of mitochondria, but they were functionally poor as judged from the rate of substrate oxidation and respiratory control. We then employed a more tightly fitting (0.1-mm clearance) glass-Teflon as well an an all-glass homogenizer and both types proved satisfactory. For routine preparation of mitochondria, PAMs were suspended (approximately 10% w/v) in a medium containing 0.17 M sucrose, 0.15 M mannitol, and 5 mM Tris-chloride (pH 7.4) and homogenized in a tightly fitting glass-Teflon homogenizer allowing three to five strokes. The homogenate was centrifuged at 600*g* for 10 min (Servall RC-2B, rotor SS-34) to remove the nuclei and broken cell debris. A thin layer of white foam which appeared on the supernatant was carefully removed and the supernatant was then spun at 10,000*g* for 10 min to sediment the mitochondrial fraction. The pellet thus obtained often consisted of three different layers: a small amount of residual nuclei, a large zone of packed material, and a deposit of loose material. The pellet was resuspended in the sucrose–mannitol medium and the suspension was spun first at the low speed to remove the remaining nuclei and then at the high speed to sediment the mitochondrial fraction. The pellet this time consisted of a packed layer and an overlying loose layer. For a study of oxidative phosphorylation the loose layer was discarded and the packed layer was resuspended in an appropriate volume to give 10–12 mg of protein/ml. For all other purposes, the loose layer which retained a considerable amount of mitochondria was resuspended and subjected to further centrifugations at 10,000*g* for two additional times to collect more mitochondria as packed layers. Mitochondria from all the packed layers were then pooled.

The mitochodrial preparation obtained as above still contained a significant proportion of cell membrane fragments as viewed in electron micrographs. For further purification the mitochondrial suspension (4.5 ml) was layered over 1.2 M

[2] Although the literature emphasizes the toxic effects of metal fumes and oxides on pulmonary parenchyma, it is presumably the divalent cations that react with the tissues. Both fumes and oxides of these cations may form hydrates and then a hydroxide in the slightly alkaline pH of the physiological milieu. The overall reaction giving this ionic form may be represented by : $M \cdot nH_2O$ or $MO \cdot nH_2O \rightarrow M(OH)_2 \rightleftarrows M^{2+} + 2(OH)^-$, where M is the metal and n is requisite number of water molecules.

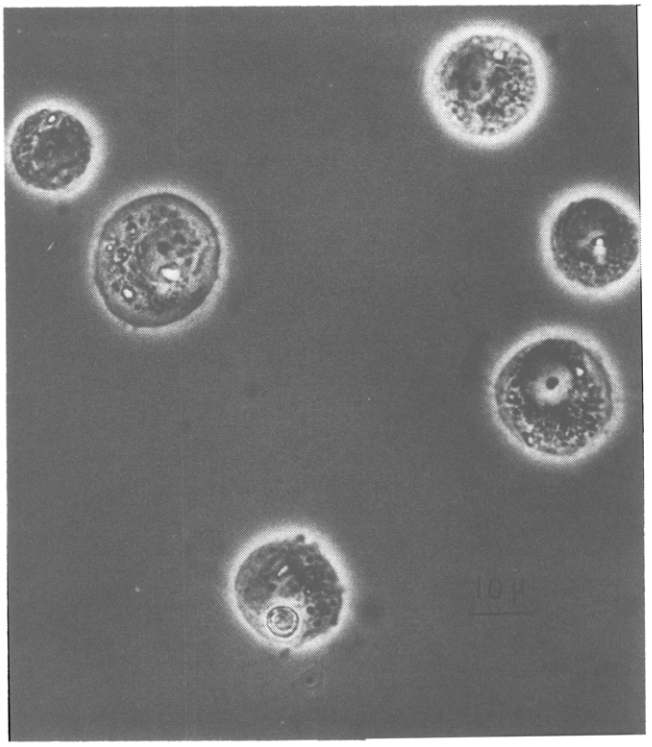

FIGURE 1: Morphology of pulmonary alveolar macrophage. Cells were under a cover slip and viewed through a phase-contrast microscope. A well-developed nucleus and numerous granules in the cytoplasm are noticeable.

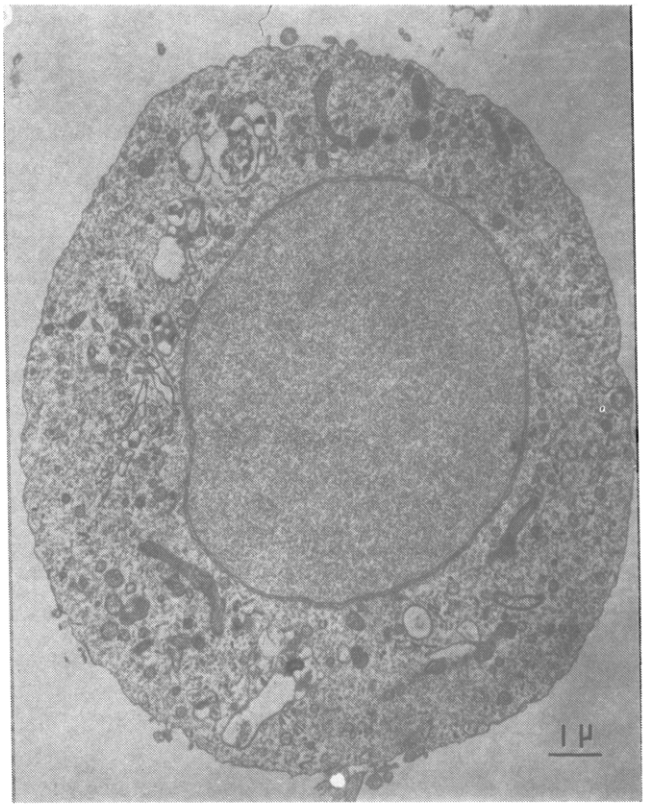

FIGURE 2: Transmission electron micrograph of pulmonary alveolar macrophage. Mitochondria, various osmiophilic granules, and other cytoplasmic inclusions are abundant.

sucrose (34 ml) and spun at 100,000*g* for 90 min (Beckman L-2, rotor SW-27). Three fractions were collected: a top film of fluffy material, a dense middle layer, and a pellet. The top and middle layers were devoid of mitochondria. The materials of the pellet were mitochondria as judged from the enzymatic properties and electron micrographs (*cf.* Figure 3B). The pellet was resuspended in an appropriate volume of sucrose–mannitol medium.

Preparation of Plasma Membranes. For isolation of plasma membranes from PAMs, a procedure was adapted from various methods of membrane preparation (Neville, 1960; Emmelot *et al.*, 1964). Cells were first subjected to a hypotonic shock in 10 mM imidazole-chloride (pH 7) for 20 min with occasional stirring. The ruptured cells were spun at 2000*g* for 10 min; the supernatant was discarded and the pellet obtained was resuspended and subjected to a second hypotonic shock as above. The material was spun again at 2000*g* and the pellet containing cell nuclei, plasma membranes, and some mitochondria was collected. The pellet was resuspended in sucrose–Tris medium and the suspension (3.5 ml) was layered over a discontinuous density gradient containing sucrose solusitions: 13 ml of 0.677 M (d = 1.094), 12 ml of 1.085 M (d = 1.142), and of 1.50 M (d = 1.192) and spun at 100,000*g* for 90 min. Three different layers and a pellet were obtained. Detailed characterization of these fractions will be reported elsewhere. Of the three layers, the second one that was located at an approximate density of 1.14 (at the interphase of top two gradients) contained most of the plasma membranes (*cf.* Figure 4). For this present study this fraction was collected with a syringe, diluted with four volumes of sucrose–Tris medium (approximately 1 mg of protein/ml).

Assays. All measurements, except those made spectrophotometrically, were carried out at 30° in buffered solutions as detailed in legends to figures and tables. Protein was determined by the method of Lowry *et al.* (1951). Oxygen uptake and oxidative phosphorylation were measured polarographically in an oxygraph (General Medical Electronics, Model KM) using a Clark electrode. Spectra of cytochromes were recorded in a Cary spectrophotometer (Model 14). To determine ATPase activity mitochondria or membranes were incubated with ATP and the amount of inorganic phosphate liberated was measured according to Wahler and Wollenberger (1958). For electron microscopic studies tissue samples were fixed with 1% glutaraldehyde in phosphate buffer and then postfixed with 1% osmium tetroxide. Fixed samples were dehydrated with increased percentages of ethanol in water and embedded in Epon 812 resin. Electron micrographs were taken in thin sections (80–90 nm) using an AEI-801 electron microscope.

Results

Pulmonary Macrophage. MORPHOLOGY. Under a phase-contrast microscope the entire population of PAMs appear strikingly similar, although there is some variation in cell size. Figure 1 presents the morphology of cells from a routine preparation. A well-developed nucleus and numerous granular structures in the cytoplasm are characteristically seen. A thin-section electron micrograph (Figure 2) shows the intracellular structures of a PAM. A large nucleus and abundant cytoplasm are bound by a surface membrane. The Golgi apparatus and a great many cytoplasmic inclusions, mitochondria, and lysosomes are visible.

RESPIRATION. Freshly prepared PAMs manifested endogenous respiration. As shown in Table I, a typical respiratory rate was approximately 9 nmoles of O_2/min per mg of

TABLE I: Oxygen Consumption of Pulmonary Macrophage Cells; Effect of Cadmium Ion.[a]

Addition	O_2 Consumption (nmoles/min per mg of Protein)
None (endogenous)	9.3 ± 0.9
DNP	11.8 ± 1.2
Glucose	3.0 ± 0.6
Glucose + DNP	10.8 ± 1.2
Oligomycin	2.7 ± 0.6
Oligomycin + DNP	11.0 ± 1.3
5 mM Cd^{2+}	3.3 ± 0.9
5 mM Cd^{2+} + DNP	9.7 ± 1.5
Wurster's blue	12.8 ± 1.6
Glucose + Wurster's blue	11.6 ± 1.3
Oligomycin + Wurster's blue	12.0 ± 1.2
5 mM Cd^{2+} + Wurster's blue	11.2 ± 1.5
EDTA	8.5 ± 1.5
3 mM Cd^{2+}	5.3 ± 1.0
3 mM Cd^{2+} + EDTA	8.0 ± 1.5

[a] Basal medium contained 80 mM NaCl, 50 mM KCl, 10 mM imidazole-chloride, 10 mM Tris-chloride, and 3–4 mg of protein equivalent of cells per ml (pH 7.5). Other additions as indicated were 10 mM glucose, 3 mM EDTA, 100 μM Wurster's blue, 50 μM DNP, 1 μg of oligomycin per mg of protein, and $CdCl_2$ as shown. The values represent an average of three to four different experiments.

protein. Oxygen uptake was inhibited approximately 70% in the presence of added glucose demonstrating an occurrence of the Crabtree phenomenon (*cf.* Dallner and Ernster, 1962a,b; Packer and Mustafa, 1966) in PAMs. Similar inhibition also occurred in the presence of oligomycin or Cd^{2+}. In all three cases the inhibition of respiration could be reversed by addition of an uncoupler, *e.g.*, DNP, or Wurster's blue, an artificial electron carrier for the mitochondrial electron-transport chain (Mustafa and King, 1967; Mustafa *et al.*, 1968). For inhibition by Cd^{2+} EDTA was also effective in restoring respiration.

Pulmonary Macrophage Mitochondria. MORPHOLOGY. The overall morphologic appearance of mitochondria was examined both in intact cells and in isolated preparations. Figure 3A shows a thin section of macrophage mitochondria *in situ*. The mitochondria appear either elongated or round with distinct crystal structures. Some of them have vacuoles inside and in some the dense matrix appears compartmentalized within a limited portion of the mitochondrial structure.

The morphological characteristics may be seen more clearly in thin section of isolated mitochondria (Figure 3B). As referred to the terminology of Hackenbrock (1966) one observes a mixed population of mitochondria, *e.g.*, morphological forms having matrices in the orthodox structure in some and condensed structure in most others. In a typical orthodox form, the inner membrane is more regularly folded into organized cristae. In the condensed forms, the inner membrane is variously folded with occasional crystal structures in some, intracristal spaces appearing as vacuoles in some, and dense round structures of matrix in most others. In some mitochondria the matrix is surrounded by an internal compartment indicating a distinct vacuole inside.

TABLE II: Oxygen Consumption of Pulmonary Macrophage Mitochondria; Effect of Cadmium Ion.[a]

Addition	O_2 Consumption (nmoles/min per mg of Protein)	
	Control	Plus Cd^{2+}
α-Oxoglutarate	67.2 ± 5.5	1.2 ± 1.2
α-Oxoglutarate + EDTA	66.0 ± 5.5	62.4 ± 6.8
α-Oxoglutarate + dimercaprol	77.4 ± 4.0	72.0 ± 6.5
α-Oxoglutarate + Wurster's blue	91.2 ± 8.6	68.4 ± 10.2
Glutamate	58.7 ± 4.0	2.4 ± 1.5
Glutamate + EDTA	57.5 ± 4.2	54.0 ± 7.8
Malate	55.2 ± 3.8	4.8 ± 2.4
Malate + EDTA	54.9 ± 4.4	53.4 ± 6.8
Pyruvate + malate	57.0 ± 4.2	7.2 ± 3.5
Pyruvate + malate + EDTA	56.4 ± 5.1	51.6 ± 8.2
β-Hydroxybutyrate	6.0 ± 3.0	0.8 ± 0.8
β-Hydroxybutyrate + EDTA	4.6 ± 2.4	3.6 ± 2.4
NADH[b]	78.0 ± 8.0	21.6 ± 5.4
NADH[b] + EDTA	75.6 ± 7.5	72.0 ± 8.5
Succinate	91.2 ± 6.5	2.4 ± 2.4
Succinate + EDTA	90.4 ± 7.8	88.2 ± 8.4
Succinate + dimercaprol	98.5 ± 8.4	94.8 ± 7.8
Succinate + Wurster's blue	138.0 ± 10.2	95.0 ± 10.2
α-Glycerophosphate	73.8 ± 7.8	7.2 ± 3.5
α-Glycerophosphate + EDTA	71.6 ± 7.8	69.0 ± 9.5
Succinate + α-glycerophosphate	110.6 ± 4.6	7.2 ± 3.8
Succinate + α-glycerophosphate + EDTA	105.0 ± 7.5	97.3 ± 10.2
Ascorbate + Wurster's blue	197.8 ± 12.0	197.8 ± 12.5

[a] Basal medium contained 200 mM sucrose, 30 mM Tris-chloride, 10 mM KCl, 1 mM phosphate, 10 μM cytochrome *c*, and 0.3–0.5 mg of protein equivalent of mitochondria (pH 7.5). Other additions as shown were 15 mM each of α-oxoglutarate, β-hydroxybutyrate, pyruvate, malate, glutamate, succinate, α-glycerophosphate, and ascorbate, 0.1 mM Wurster's blue, 50 μM $CdCl_2$, 0.2 mM EDTA, and 0.2 mM dimercaprol. The values represent an average of three to four different determinations from three experiments. [b] Mitochondria were made permeable to external NADH by omitting 200 mM sucrose from the basal medium.

RESPIRATION. Mitochondria prepared by the commonly used differential centrifugation techniques contained other cell fragments and membranes. Mitochondria employed for respiration studies were, therefore, purified by density gradient centrifugations. The specific activity of purified mitochondria was at least twofold higher than that of the original crude mitochondrial fraction as measurd for succinate oxidation.

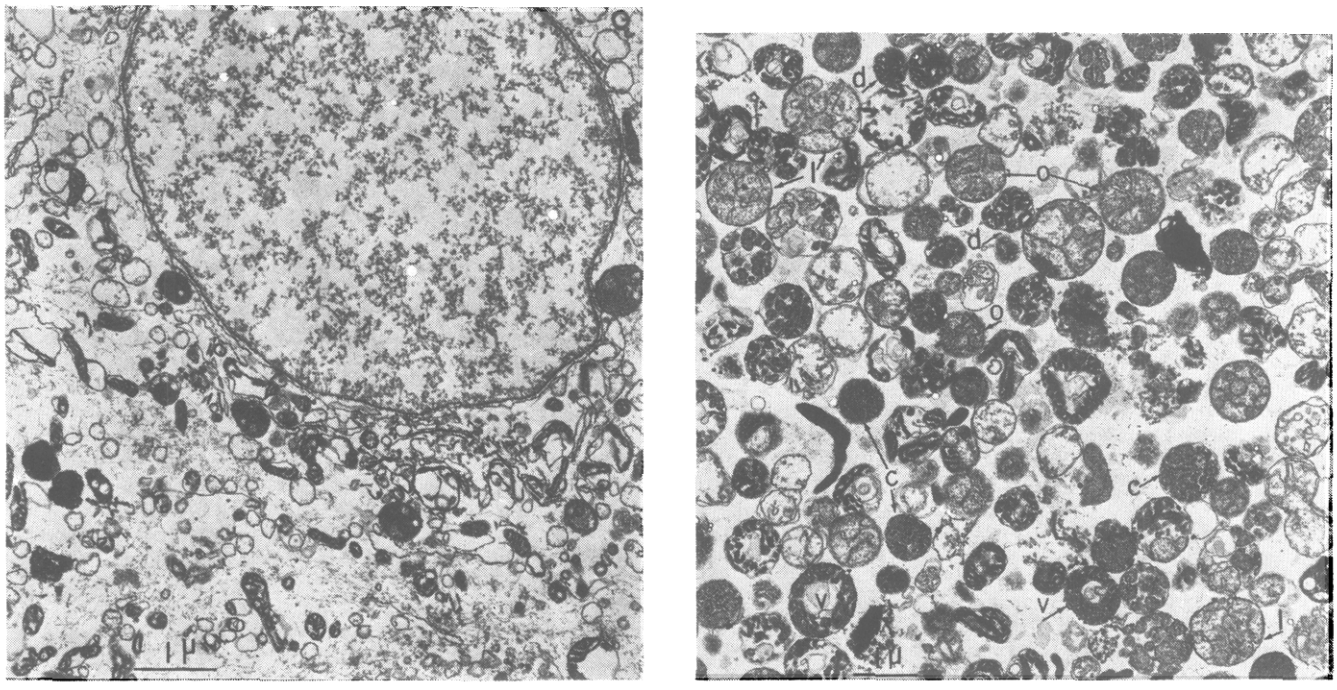

FIGURE 3: (A, left) Transmission electron micrograph of pulmonary macrophage mitochondria *in situ*. Mitochondria appear either elongated (cylindrical) or round (spheriodal) in shape. Distinct crystal structures with dense matrix are noticeable. Some of the mitochondria are vacuolated. (B, right) Transmission electron micrograph of isolated mitochondria from pulmonary alveolar macrophage. A mixed population of mitochondria: orthodox (o), condensed (c), lightly stained matrix (l), densely stained matrix (d), and vacuolated (v) forms are noticeable. Occasional damaged or swollen mitochondria may be seen.

Table II presents a list of substrates that were oxidized by PAM mitochondria. Of the pyridine nucleotide linked substrates, α-oxoglutarate and glutamate were oxidized more effectively than malate, pyruvate plus malate, β-hydroxybutyrate, and citrate. A rate of 67 nmoles of O_2/min per mg of protein was obtained for oxidation of α-oxoglutarate, whereas significantly lower rates were obtained for other substrates. β-Hydroxybutyrate utilization was practically absent in PAM mitochondria, an observation appeared to be true for pulmonary mitochondria in general (Reiss, 1966; Mustafa *et al.*, 1971). External NADH was oxidized at a sluggish rate presumably due to lack of penetration (Chance and Williams, 1955; Greville, 1966; Mustafa and King, 1967; Mustafa *et al.*, 1968). However, in this case mitochondria were suspended in a relatively hypotonic medium rendering them permeable to added NADH. The rate of oxygen uptake was high compared to other pyridine nucleotide linked substrates.

Of the flavin-linked substrates, succinate and α-glycerophosphate were tested and both initiated rapid rates of oxygen consumption. It may be noted that when these two substrates were added in combination, somewhat additive effect on respiration resulted. For example, the rates were approximately 91 and 74 nmoles of O_2 per min/mg of protein for succinate and α-glycerophosphate, respectively; their simultaneous oxidation gave a rate of approximately 110 nmoles of O_2/min per mg of protein.

Ascorbate itself was poorly oxidized by PAM mitochondria (*cf.* Packer and Mustafa (1966), Mustafa and King (1967), and Mustafa *et al.* (1968) for hepatic mitochondria), but in the presence of Wurster's blue a rapid rate was obtained (approximately 198 nmoles of O_2/min per mg of protein). Similarly, NADH in the presence of Wurster's blue gave a high rate of respiration (approximately 100 nmoles of O_2/min per mg of protein using 0.1 mM Wurster's blue) (*cf.* Mustafa and King (1967) and Mustafa *et al.* (1968) for hepatic mitochondria).

EFFECTS OF INHIBITORS AND Cd^{2+} ON RESPIRATION. The utilization of substrates by PAM mitochondria was blocked in the presence of conventional respiratory inhibitors. The inhibition studies were carried out in both states 3 and 4 conditions of respiration. For example, α-oxoglutarate oxidation was completely inhibited by 1 μM rotenone or 3 mM Amytal, compounds which inhibit the electron-transport chain between NADH dehydrogenase and cytochrome *b* (Lehninger, 1965; Mustafa *et al.*, 1968). Oxidation of α-oxoglutarate, succinate, and α-glycerophosphate was inhibited practically 100% in the presence of antimycin A (1 μg/mg of protein), an antibiotic known to inhibit the chain between cytochromes *b* and c_1 (Lehninger, 1965). Finally, oxidation of these substrates and also that of ascorbate plus Wurster's blue were completely abolished in the presence of 0.1 mM cyanide or 1 mM azide, compounds which block the terminal oxidase (Lehninger, 1965; Wilson and Chance, 1967).

The oxidation of both pyridine nucleotide linked and flavin-linked substrates was sensitive to Cd^{2+} (Table II). A 50 μM concentration of Cd^{2+} for half the maximal inhibition was approximately 15 μM. At low (<50 μM) concentrations of Cd^{2+} an initial lag phase was observed in the inhibition, which disappeared at higher (>50 μM) concentrations. It may be noted that oxidation of NADH was only partially (72%) inhibited by Cd^{2+}; possibly a part of added NADH reacted directly with cytochrome *c* thus circumventing the sites of Cd^{2+} inhibition (*cf.* Mustafa and King, 1967; Mustafa *et al.*, 1968). This observation is further substantiated by the findings that oxidation of ascorbate plus Wurster's blue or NADH plus Wurster's blue, which feeds electrons at the terminal region of the respiratory chain (Mustafa and King, 1967; Mustafa *et al.*, 1968) was resistant to this metal ion.

It was of interest to determine whether the inhibition of respiration by Cd^{2+} was reversible. The addition of an

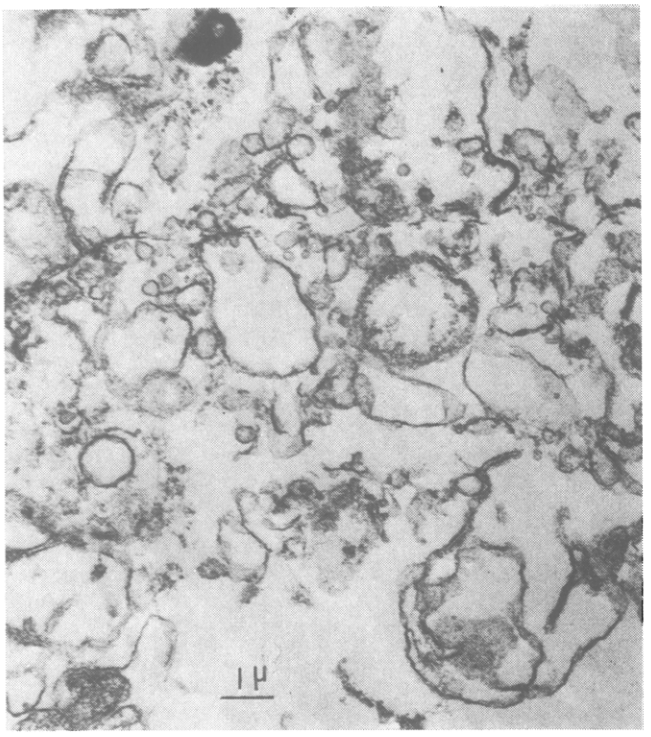

FIGURE 4: Transmission electron micrograph of cytoplasmic membrane of pulmonary alveolar macrophage. The original cell ghosts are fragmented, but the membrane pieces have assumed vesicular structures presumably by resealing.

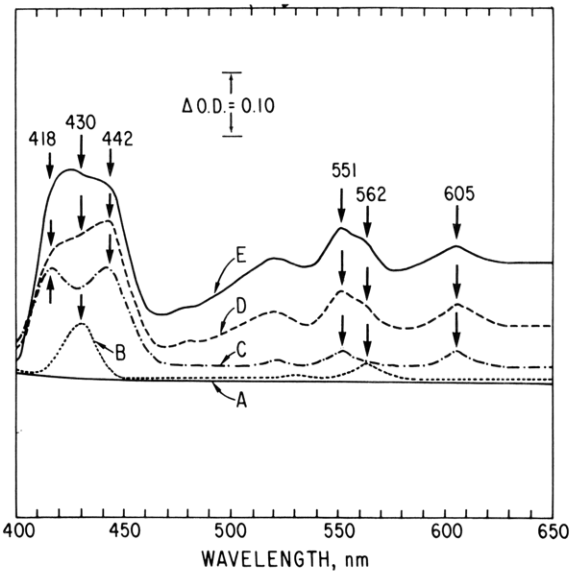

FIGURE 5: Differential absorption spectra of cytochromes in pulmonary macrophage mitochondria. The reaction mixture contained 100 mM NaCl, 20 mM Tris-chloride (pH 7.5), and 4.5 mg of protein equivalent of mitochondria. Other additions as indicated were 20 mM succinate, 10 mM ascorbate, 100 μM Wurster's blue, 1 μg of antimycin A per mg of protein, and a few grains of dithionite. Difference spectra are presented as follows.

Curve	System	Minus Reference
A	Oxidized	Oxidized
B	Succinate + antimycin A	Oxidized
C	Succinate + antimycin A + ascorbate + Wurster's blue	Succinate + antimycin A
D	Succinate	Oxidized
E	Succinate + dithionite	Oxidized

uncoupler is known to remove the inhibition of mitochondrial respiration by antimycin A (Howland, 1968). The presence of DNP, however, did not alter the inhibition of PAM mitochondria by Cd^{2+}. EDTA, a chelating agent for divalent cations, is known to reverse the inhibition of electron-transport chain by Zn^{2+} (Nicholls and Malviya, 1968). Dimercaprol, a dithiol compound, has been shown to reverse the inhibition of α-ketoglutarate dehydrogenase by Cd^{2+} (Searls *et al.*, 1961) and to prevent the uncoupling effect of Cd^{2+} on the oxidative phosphorylation in liver mitochondria (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960). As shown in Table II, both EDTA and dimercaprol were effective in almost complete reversal of the inhibitory effect of Cd^{2+} on PAM mitochondrial respiration. The alleviating effect of these compounds was presumably due to their binding with Cd^{2+} (*cf.* Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960; Searls *et al.*, 1961). A slight augmentation of respiration observed in the presence of dimercaprol was due to its oxidation by mitochondria.

OXIDATION–REDUCTION OF CYTOCHROMES AND Cd^{2+} INHIBITION. It was of interest to study the oxidation reduction behavior of the respiratory chain of PAM mitochondria and, especially, to determine if Cd^{2+} was involved in binding any specific component of this chain. Figure 5 presents the characteristic absorption peaks for reduced cytochromes of PAM mitochondria under different conditions. As shown in curve B, the addition of succinate and antimycin A to an aerobic suspension of mitochondria resulted in a reduction of cytochrome *b* as characterized by α peak at 562 nm and γ peak at 430 nm. Other cytochromes remained in the oxidized state as antimycin A is known to inhibit the respiratory chain between cytochromes *b* and c_1 (Lehninger, 1965). As shown in curve C, the addition of ascorbate plus Wurster's blue (succinate plus antimycin A was present in both cuvets to cancel cytochrome *b* absorption) caused a reduction of cytochromes $c_1 + c$ and $a + a_3$ as seen from the absorption maxima: for $c_1 + c$, α peak at 551 nm and γ peak at 418 nm; and for $a + a_3$, α peak at 605 nm and γ peak at 442 nm. Curve D shows the simultaneous reduction of all cytochromes in the presence of succinate. The absorption peaks were somewhat more developed when dithionite was also added (curve E).

Figure 6 presents the oxidation–reduction behavior of cytochromes in the presence and the absence of Cd^{2+}. As shown in curve A, the addition of succinate plus Cd^{2+} to a suspension of mitochondria caused no reduction of cytochromes (*cf.* curve D of Figure 5 where Cd^{2+} was absent). This shows that the electron transport through the chain was blocked by Cd^{2+}. However, when EDTA was added to the succinate plus Cd^{2+}-treated system, the electron transport was restored as seen from the reduction of cytochromes (curve B). Similar observations were made using α-oxoglutarate as a substrate. However, if ascorbate plus Wurster's blue was added to the mitochondrial suspension, either in the presence or the absence of Cd^{2+}, all the cytochromes were found to be reduced (same as curve B). These latter two experiments, particularly, demonstrate that Cd^{2+} was not directly involved in blocking the cytochrome reactions. The inhibition of entry of reducing equivalents from succinate and α-oxoglutarate occurred presumably due to interference of Cd^{2+} with the dehydrogenases of the respiratory chain.

In the foregoing experiments it was observed that upon addition of ascorbate plus Wurster's blue all the cytochromes, including cytochrome *b*, were reduced both in the absence or

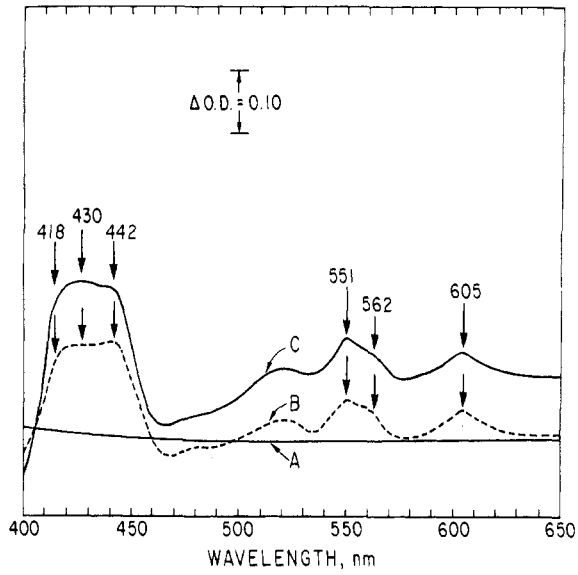

FIGURE 6: Reduction of cytochromes in presence and absence of Cd^{2+}. Basic conditions were as in Figure 5. Other additions as indicated were 20 mM succinate, 10 mM ascorbate, 100 μM Wurster's blue, 200 μM $CdCl_2$, 1 mM EDTA, and a few grains of dithionite. Difference spectra are presented as:

Curve	System	Minus Reference
A	Oxidized	Oxidized
	Or succinate + Cd^{2+}	Cd^{2+}
B	Succinate	Oxidized
	Or succinate + Cd^{2+} + EDTA	Cd^{2+} + EDTA
	Or ascorbate + Wurster's blue	Oxidized
	Or ascorbate + Wurster's blue + Cd^{2+}	Cd^{2+}
C	As in B + dithionite	As in B

the presence of Cd^{2+}. The reducing equivalents of ascorbate plus Wurster's blue enter the respiratory chain at the cytochrome c_1 level thus reducing cytochromes $c_1 + c$ and $a + a_3$. The reduction of cytochrome *b* observed under these conditions might be due to a reversed electron flow (Packer and Mustafa, 1966; Mustafa and King, 1967; Mustafa *et al.*, 1968). Since the reversed electron transport is an energy-requiring process, the energy for cytochrome *b* reduction was likely to arise from the oxidation of ascorbate plus Wurster's blue. Although Cd^{2+}-uncoupled oxidative phosphorylation (*cf.* Table III), the generation of high-energy state to drive the reversed electron flow was still possible. Such characteristics of ascorbate plus Wurster's blue and also of NADH plus Wurster's blue have been documented in hepatic and cardiac mitochondria (Mustafa and King, 1967; Mustafa *et al.*, 1968). As presented in Figure 7, we have further substantiated this observation in PAM mitochondria. To a suspension of mitochondria Cd^{2+} and cyanide were added, which isolated the respiratory chain from interacting with oxygen as well as with pyridine nucleotide or flavin-linked substrates. Under these conditions only a small reduction of cytochromes $c_1 + c$ and $a + a_3$ was noticed (curve B). Upon addition of ascorbate plus Wurster's blue a large reduction of both *c*- and *a*-type cytochromes occurred (curve C). As expected, no reduction of cytochrome *b* took place which was due to lack of generation of energy and the subsequent reversed electron flow. Addition of ATP to this system provided energy for the reversed electron transport leading to reduction of cytochrome *b* (curve D). If oligomycin, an inhibitor of energy transfer (Lehninger, 1965), was present in the system, ATP addition was ineffective for driving this reduction reaction. As in curve E, a full reduction of all the cytochromes occurred when dithionite was added.

TABLE III: Oxidative Phosphorylation of Pulmonary Macrophage Mitochondria; Effect of Cadmium Ion.[a]

Addition	nmoles of O_2/min −ADP	+ADP	ADP:O
α-Oxoglutarate	11.0	27.5	2.24
α-Oxoglutarate + BSA	10.7	32.7	2.40
α-Oxoglutarate + $CdCl_2$	10.4	10.4	0
α-Oxoglutarate + dimercaprol	13.8	30.6	2.10
α-Oxoglutarate + $CdCl_2$ + dimercaprol	12.6	24.6	1.80
Succinate	20.4	44.8	1.36
Succinate + BSA	20.4	66.3	1.48
Succinate + $CdCl_2$	19.2	19.2	0
Succinate + dimercaprol	29.4	61.9	1.26
Succinate + $CdCl_2$ + dimercaprol	28.2	49.4	1.10
Succinate + EDTA	19.8	39.0	1.20
Succinate + $CdCl_2$ + EDTA	19.2	34.8	0.72
Ascorbate + Wurster's blue	34.8	66.5	0.90
Ascorbate + Wurster's blue + BSA	34.8	76.6	0.98
Ascorbate + Wurster's blue + $CdCl_2$	34.2	34.8	0
Ascorbate + Wurster's blue + dimercaprol	41.2	74.2	0.80
Ascorbate + Wurster's blue + $CdCl_2$ + dimercaprol	40.6	60.9	0.70

[a] Basal reaction mixture contained 180 mM sucrose, 30 mM Tris-chloride, 20 mM KCl, 5 mM $MgCl_2$, 5 mM potassium phosphate, and 2 mg of protein equivalent of mitochondria per ml, pH 7.5. Other additions as shown were 20 mM each of α-oxoglutarate, succinate, and ascorbate, 50 μM Wurster's blue, 165 μM ADP, 100 μM EDTA, 100 μM dimercaprol, 10 μM $CdCl_2$, and 1 mg of bovine serum albumin per ml.

OXIDATIVE PHOSPHORYLATION AND EFFECTS OF INHIBITOR, UNCOUPLERS, AND Cd^{2+}. Freshly prepared PAM mitochondria were able to carry out oxidative phosphorylation. Table III shows that ADP:O ratios of 2.24 for α-oxoglutarate, 1.36 for succinate, and 0.9 for ascorbate plus Wurster's blue were obtained. The respiratory control indices were between 2 and 3. Figure 8 shows a recording trace of states 3 and 4 respiration and respiratory control of PAM mitochondria. Supplementation of the mitochondrial suspension with bovine serum albumin seemed to improve, to some extent, both ADP:O ratio and respiratory control. However, for studying the specific effects of Cd^{2+} albumin was excluded from other experiments.

The coupled phosphorylation of PAM mitochondria was sensitive to oligomycin, an inhibitor of energy transfer (Lehninger, 1965), or to PCP and DNP, the classical uncouplers (Lehninger, 1965; Racker, 1965). For example, with succinate as a substrate, the active (state 3) respiration was suppressed in the presence of oligomycin (1 μg/mg of protein) to the level of resting (state 4) respiration. The suppressed res-

TABLE IV: Adenosine Triphosphatase Activity of Macrophage Mitochondria and Plasma Membrane; Effect of Cadmium Ion.[a]

Addition	Activity	% Inhibn
A. Mitochondria		
None	3.50 ± 0.41	
1 mM $CdCl_2$	2.42 ± 0.38	30.9
2 mM $CdCl_2$	1.78 ± 0.20	49.2
B. Plasma Membrane		
None	18.55 ± 2.30	
1 mM $CdCl_2$	13.04 ± 1.60	29.8
2 mM $CdCl_2$	8.75 ± 1.33	52.6

[a] Basal reaction mixture contained 100 mM sucrose, 30 mM glycylclycine, 30 mM imidazole, 50 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM ATP, and tissue preparations as shown, pH 7.5. Other addition was $CdCl_2$ as shown. Activity was expressed as micromoles of inorganic phosphate liberated per milligram of protein per hour at 30°.

piration was released upon addition of 20 μM PCP or 50 μM DNP. These uncouplers at the concentrations indicated would also abolish the respiratory control.

As shown in Table III, mitochondria were uncoupled in the presence of Cd^{2+}. This metal ion at 5–10 μM concentrations completely abolished the oxidative phosphorylation. Unlike DNP, however, Cd^{2+} did not stimulate the state 4 respiration of PAM mitochondria. It is possible that the respiratory stimulation due to uncoupling was masked by the simultaneous respiratory inhibition caused by Cd^{2+}. The uncoupling of phosphorylation was reversible; both dimercaprol and EDTA were effective in preventing as well as restoring the Cd^{2+}-induced uncoupling.

ADENOSINE TRIPHOSPHATASE ACTIVITY AND EFFECT OF Cd^{2+}. Since Cd^{2+} is an inhibitor of respiration and uncoupler of oxidative phosphorylation, it was of interest to test its effect on the ATP-hydrolyzing system. This study was carried out at two levels of organization: mitochondria and plasma membranes of PAM. It should be mentioned that PAMs possess a Na^+–K^+,Mg^{2+}-ATPase activity, which is predominantly located at the plasma membrane and may be related to cellular active transports and endocytic processes (Mustafa *et al.*, 1970; Cross *et al.*, 1971). Table IV shows that the ATPase activities were 3.5 and 18.5 μmoles of P_i per mg of protein per hr for isolated mitochondria and plasma membrane, respectively. The activities were inhibited approximately 30% by 1 mM Cd^{2+} and 50% by 2 mM Cd^{2+} in both mitochondria and plasma membrane.

Discussion

Electron Transport and Cd^{2+} Effect. The overall behavior of PAM mitochondria toward substrate utilization does not differ greatly from that of mitochondria from other mammalian sources, particularly, the mitochondria of pulmonary tissue (Reiss, 1966; Mustafa *et al.*, 1971) or hepatic tissue (Lehninger, 1965; Racker, 1965). The occurrence of α-glycerophosphate oxidation and the lack of β-hydroxybutyrate utilization are somewhat unique for PAM mitochondria, but α-glycerophosphate is also rapidly oxidized by cerebral

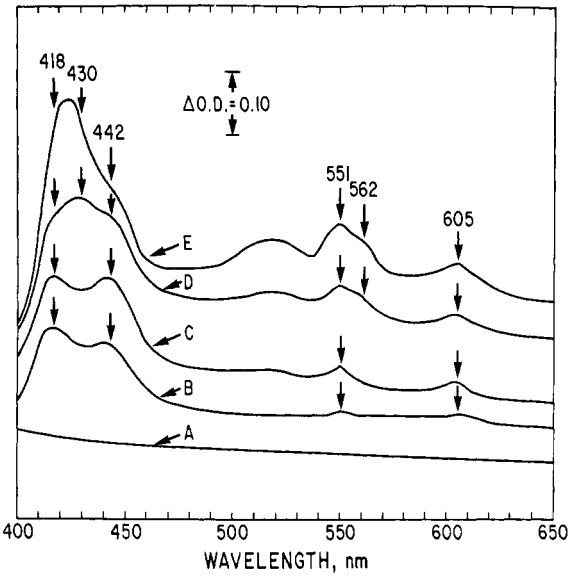

FIGURE 7: Effect of Cd^{2+} on energy-dependent cytochrome *b* reduction in pulmonary macrophage mitochondria. Basic conditions were as in Figure 5. Other conditions as indicated were 10 mM ascorbate, 100 μM Wurster's blue, 200 μM $CdCl_2$, 0.1 mM cyanide, 2 mM ATP, and a few grains of dithionite. Difference spectra are presented as:

Curve	System	Minus Reference
A	Oxidized	Oxidized
B	Cd^{2+} + cyanide	Oxidized
C	As in B + ascorbate + Wurster's blue	Oxidized
D	As in C + ATP	Oxidized
E	As in D + dithionite	Oxidized

mitochondria (Sacktor and Packer, 1962). The sensitivity of mitochondrial electron transport to classical inhibitors was similar to that commonly observed in other mammalian cell mitochondria.

The results presented here demonstrate that Cd^{2+} is a potent inhibitor of macrophage mitochondrial electron transport. A complete inhibition of respiration in mitochondria occurs in the presence of 50 μM Cd^{2+}. The inhibition is presumably due to binding of Cd^{2+} to certain components of

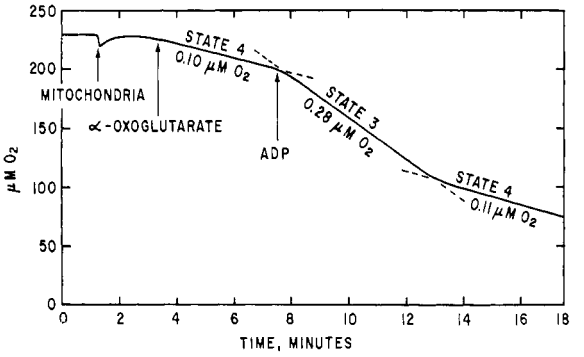

FIGURE 8: Oxygraph trace of respiration and respiratory control. Basic conditions were as in Table III. Other additions as indicated were 20 mM α-oxoglutarate and 430 μM ADP. As obtained from the trace, oxygen consumption in state 3 was 94 μM or 188 μatoms/l. The computed ADP:O ratio, therefore, was 2.29. Respiratory control index, as calculated by dividing state 3 rate (0.28 μM O_2/sec) with the subsequent state 4 rate (0.11 μM O_2/sec), was 2.5.

the respiratory chain. Our data indicate that cytochromes are not involved in Cd^{2+} binding. This is concluded from the ability of ascorbate plus Wurster's blue to reduce all the cytochromes in the presence of Cd^{2+}. On the other hand, the ability of flavin-linked and pyridine nucleotide linked substrates to reduce cytochromes in the presence of Cd^{2+} suggests that the flavorproteins and other dehydrogenases of the respiratory chain may be the targets of Cd^{2+} binding. This latter conclusion is further supported by the work of Searls *et al.* (1961). They have demonstrated that Cd^{2+} is a dithiol binding reagent and that this metal ion inhibits α-ketoglutarate dehydrogenase and flavoproteins by interacting specifically with the functional dithiol groups. The reversal of Cd^{2+} inhibition by dimercaprol and EDTA suggests that these chelators remove bound Cd^{2+} from the reactive sites and that binding of this metal ion does not cause any irreversible damage of the enzymes.

Energy Transfer and Cd^{2+} Effect. PAM mitochondria are able to carry out coupled phosphorylation, but these mitochondria are not as "tightly coupled" as liver mitochondria prepared under similar conditions. Nonetheless, the ADP:O ratios are higher than 1 (approaching 2) for flavin-linked substrates and higher than 2 (approaching 3) for pyridine nucleotide linked substrates.

The oxidative phosphorylation is more sensitive to Cd^{2+} than the electron-transfer process. Both phosphorylation and respiratory control of PAM mitochondria are abolished in the presence of 5–10 μM Cd^{2+}, which are in agreement with the previous findings of Jacobs *et al.* (1956) and Fluharty and Sanadi (1960). The uncoupling action has been reported to involve Cd^{2+} binding to dithiol groups of coupling enzymes (Searls *et al.*, 1961). Cadmium ion also affects the ATPase system of PAM mitochondria and plasma membrane. A 50% inhibition of ATP-splitting reaction occurred in the presence of 2 mM Cd^{2+}.

Cellular Respiration and Cd^{2+} Effect. Endogenous respiration is a sensitive index of cellular integrity and viability. The endogenous uptake of oxygen by PAM is practically abolished if the cells are damaged. The inhibition of PAM respiration in the presence of glucose is a Crabtree-like effect observed in other cell types (Dallner and Ernster, 1962a,b; Packer and Mustafa, 1966). Oligomycin, an inhibitor of energy transfer (Lehninger, 1965), is known to produce a similar effect in ascites tumor cells (Dallner and Ernster, 1962b; Packer and Mustafa, 1966). Although mechanisms of this phenomenon have not been revealed conclusively, the alteration of mitochondria–cytoplasmic relationship is presumably the major cause. The reversal of glucose- or oligomycin-induced inhibition of PAM cellular respiration by DNP or Wurster's blue indicates an uncoupling or bypassing of an obligatory relationship between mitochondria and cytoplasm (*cf.* Dallner and Ernster, 1962a,b; Packer and Mustafa, 1966; Mustafa and King, 1967; Mustafa *et al.*, 1968).

Besides the specific inhibition of electron- and energy-transfer reactions, *e.g.*, by binding to vicinal dithiols of dehydrogenases and coupling enzymes (Jacobs *et al.*, 1956; Searls *et al.*, 1961), Cd^{2+} may also cause nonspecific alterations of cellular functions. Such alterations may result from binding of Cd^{2+} to cellular components involved in cellular compartmental organization. In fact, the inhibition of PAM cellular respiration by Cd^{2+} and its reversal by DNP or Wurster's blue indicate that effect of Cd^{2+} is similar to that of oligomycin.

Biological Significance of Cd^{2+} Effect. The effects of Cd^{2+} on the PAM system observed have implications for the field of environmental toxicology, since cadmium fumes and oxides are hazardous to health (Johnstone and Miller, 1960; Browning, 1961; Patty, 1962; Bonnell, 1965; Kendrey and Rose, 1969). A major intake of this metal in humans occurs through the respiratory tract, but mechanisms of cadmium-induced pulmonary pathology are not known. The interactions of Cd^{2+} with PAM cells and mitochondria might be of relevance in that pulmonary pathology is probably the ultimate results of deleterious effects of toxic metals on lung cellular metabolism. Moreover, the impairment of PAM cellular functions may be detrimental to the vital host defense mechanism in lung. Therefore, studies of this sort may offer a more significant meaning in terms of biochemical mechanisms of metal toxicity and also provide a correlation between lung cellular malfunction and development of pulmonary pathology.

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Microdetermination of Nucleic Acid Phosphorus by Neutron Activation Analysis*

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ABSTRACT: Neutron activation analysis was standardized for the determination of DNA phosphorus down to 0.1 ng. The method was based upon the transformation of stable polynucleotide phosphorus into ^{32}P by capture of thermal neutrons and it included four steps: (1) irradiation of DNA in a nuclear reactor; (2) mineralization of the organic material in Teflon tubes by a HNO_3–$HCOOH$–H_2O_2 mixture changing all ^{32}P species into inorganic radiophosphate; (3) isolation of the $[^{32}P]PO_4^{3-}$ from contaminating radionuclides; (4) assay of ^{32}P by counting its Cerenkov effect in 6 M HF with the aid of a conventional apparatus for liquid scintillation spectrometry. For specimens with more than 100 μg of total phosphorus, oxidative acid digestion of the nucleotides, and separation of the ^{32}P from other radioelements were not always necessary. When such samples were prepared and activated under controlled conditions, the β^- emission of ^{32}P was measured directly in irradiated DNA without interferences after 1 week of decay. Except for neutron activation, all manipulations can be carried out in a normal biochemical laboratory. Some of the auxiliary techniques developed for the neutron activation analysis method may be applied in chemical assays of nucleic acid phosphorus for improving accuracy and sensitivity.

The biochemical characterization of nucleic acids rests primarily on the quantitative measurement of their phosphorus content (Fiske and Subbarow, 1925). The established methods involve mineralization of the sample and colorimetric assay of the released inorganic phosphate. At very low phosphorus concentrations, the overall procedure embodies several sources of error. As a matter of fact, the 1.0 or 2.0 μg of total phosphorus recommended as the smallest amounts for reliable

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