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The Ohio State University, Ph.D., 1972
Biochemistry

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THE EFFECT OF HEAVY METALS ON THE PERMEABILITY
OF THE MITOCHONDRIAL MEMBRANE AND ITS
ENERGY-LINKED REACTIONS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Kou Mau Hwang, M.S.

The Ohio State University
1971

Approved by
Adviser
Department of Physiological Chemistry
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PUBLICATIONS

Merola, A. J., Hwang, K. M., Jurkowitz, M., and Brierley G. P.,
Structural Requirements in the Uncoupling Oxidative Phosphorylation by N, N'-Bis (Dichloroacetyl) Diamine, Biochemical Pharmacology, 20, 1393 (1971).


Scott, K. M., Hwang, K. M., Jurkowitz, M., Brierley, G. P., Ion Transport by Heart Mitochondria XXIII
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate.</td>
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<td>ATPase</td>
<td>ATP phosphohydrolase, E.C. 3.6.1.3. Enzyme which catalyzes the hydrolysis of ATP to ADP and orthophosphate.</td>
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<td>CCP</td>
<td>m-Cl-Carbonyl cyanide phenylhydrazone.</td>
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<tr>
<td>Cd²⁺</td>
<td>Cadmium nitrate.</td>
</tr>
<tr>
<td>CMB</td>
<td>p-hydroxymercuribenzoate.</td>
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<tr>
<td>CMS</td>
<td>p-chloromercuriphenyl sulfonate.</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Cupric nitrate.</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4 Dinitrophenol.</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid.</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(B-aminoethylether)-N-N'-tetracetic acid.</td>
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<td>Hg²⁺</td>
<td>Mercuric chloride.</td>
</tr>
<tr>
<td>La³⁺</td>
<td>Lanthanum nitrate.</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinic adenine dinucleotide (reduced form)</td>
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<tr>
<td>NAD</td>
<td>Nicotinic adenine dinucleotide (oxidized form)</td>
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LITERATURE REVIEW

The Effect of Metals on Mitochondrial Reactions

Rauflaub (1, 2) and Tapley (3) were first to demonstrate that several cations including Ag$^{2+}$, Hg$^{2+}$, Ca$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Fe$^{2+}$, Sn$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Co$^{2+}$, can alter the mitochondrial volume to different degrees. When mitochondria were suspended in 0.3M sucrose and 0.02 M Tris, pH 7.4, the change of optical density was observed for 30 minutes in the presence of 10-100 uM cations as indicated above. The results are indicated as follows:

1. Ag$^{2+}$, Hg$^{2+}$, Ca$^{2+}$, CMB are swelling enhancing agents. In contrast to slow and relatively limited swelling produced by Ca$^{2+}$, the swelling produced by Ag$^{2+}$, Hg$^{2+}$ (or CMB) was rapid and much more pronounced.

2. Pb$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Fe$^{2+}$ are partially active and cause only slight swelling (change of optical density is less than 25% of control in 30 minutes).

3. Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$ have no significant effect in mitochondrial swelling, and Mg$^{2+}$ can prevent mitochondria from swelling induced by Ca$^{2+}$.

On the basis of the finding that 0.01 M iodoacetamide is as effective as Hg$^{2+}$ in inducing mitochondrial swelling, the involvement of sulfhydryl groups in determining mitochondrial structure and
permeability was suggested.

Jacobs et al. (4) have also studied the effect of a number of metals (Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Hg$^{2+}$, Cu$^{2+}$) on respiration and oxidative phosphorylation in 0.25 M sucrose. The data showed that only Cd$^{2+}$ at 5 uM completely uncoupled phosphorylation associated with the oxidation of succinate and of citrate. EDTA, BAL, propane-1,3-dithiol, Mn$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ reverse the effect of Cd$^{2+}$. It was suggested that the uncoupling effect of Cd$^{2+}$ was due to either Cd$^{2+}$ displacing another cation essential for phosphorylation from its active site, or that Cd$^{2+}$ blocks a free active site presumably a thiol or imidazole.

Scott and Gamble (5, 6) studied the effect of mercurial compounds on K$^+$ binding and oxidative phosphorylation. It was concluded that K$^+$ is bound to both liver mitochondria and fragmented mitochondria in tightly and loosely bound forms. The bound K$^+$ is not removed by washing in isotonic sucrose of NaCl solutions, but exchanges with K$^+$ ion in the media and can be removed by phosphate. Respiratory activity enhances the stability of the K$^+$-binding site and stimulates the rate of K$^+$ exchange at the site. High pH (8.7) can stimulate the exchange rate but the quantity of total K$^+$ remained the same. In contrast to high pH, DNP produced a marked lowering of total K$^+$ and little change in the rate of K$^+$ exchange. NaCl and MgCl$_2$ can effect the exchange rate to greater extent than the total quantity of bound K$^+$. Mercurial reagents lower the retained K$^+$ while stimulating a 5 fold increase in the exchange rate. Moreover, a differential effect on oxidative phosphorylation, and K$^+$ binding and exchange can be obtained by
different mercurial agents. The particular sensitivity of phosphorylation to the action of organic mercurial is in contrast to the mitochondrial K\textsuperscript{+} binding that is more sensitive to inorganic mercurials. This led them to conclude that susceptible components of oxidative phosphorylation and K\textsuperscript{+}-sensitive exchange may be located on the surface and matrix side of inner membrane respectively. Therefore, reagents with different polarities can show a different ability to reach those components. More recently Brierley and associates have extensively studied the mercurials effect on mitochondria. They demonstrated that mercurials and other heavy metals can activate energy-linked K\textsuperscript{+} and Mg\textsuperscript{2+} accumulation (7, 8). In addition they reported that mercurials bound to beef heart mitochondria to different extent induce an alteration in permeability to different ions (9, 10), and affect the ATPase activity and energy-linked ion movement (11). Brierley and Scott suggested that under the condition that exterior thiols are modified by CMS the ATPase activity is activated; if interior thiol is altered, both permeabilities to cation and anion are induced and ATPase activity is inhibited. Mercurials were also reported to inhibit phosphate transport (12, 13, 44), to inhibit respiration, uncouple phosphorylation, and interfere with the process of energy transport in submitochondrial particle (14-17).

It has been established early that sulfur containing compounds including insulin, oxytocin, and GSH (18-21) were potential inducing agents for mitochondrial swelling. Their action was suggested as resulted from the interaction of sulfhydryl-disulfide groups each
located in mitochondrial membrane and added reagents (22, 23). A subsequent study of the effect of Fe$^{2+}$ by Hunter et al. (24) indicated a close correlation between lipid peroxidation and swelling and led to an investigation of the action of the metal contaminants in biologically active sulfur containing compounds. Cash et al. (25-27) and Campbell and Mertz (28) observed that metal ion present as contaminants in GSH, insulin, and oxytocin were responsible for the swelling action of these reagents. The principle metal ions involved were Fe$^{2+}$ and Zn$^{2+}$.

With the hope of throwing light upon the brain damage found in patients with Wilson's disease and the mechanism of the specific convulsion due to the accumulation of Cu$^{2+}$, Peter, Walsh, and their associates (29-32) have investigated the effect of Cu$^{2+}$ on brain mitochondria. The results indicated that Cu$^{2+}$ inhibits pyruvate respiration in isolated mitochondria, but shows a slight stimulation and inhibition ($\pm$15%) effect on this respiration in vivo. They have also reported that Cu$^{2+}$ at 6-24 ug atom/g of tissue diminished the tissue ATP, phosphocreatine, and K$^+$ content. Tissue Na$^+$ and Cl$^-$ was increased and the respiration response of tissue to electrical stimulation was diminished. Despite those observations at the mitochondrial level, their later discovery of inhibition of microsomal ATPase by Cu$^{2+}$ (33) (about 25% inhibition) have led them to abandon their initial hypothesis that mitochondria were the locus of the toxic action of Cu$^{2+}$ in brain.

Verity and Gambell (34) have studied the swelling of isolated
liver mitochondria induced by Cu$^{2+}$. They found Cu$^{2+}$-induced swelling is not associated with lipid peroxidation in contrast to Fe$^{2+}$-induced swelling. Cu$^{2+}$ at 22 μmoles/mg of protein inhibits both B-hydroxy butyrate and glutamate respiration. No rapid burst of slow increase in oxygen consumption was coupled to the Cu$^{2+}$-induced swelling. α-ketoglutarate had no significant effect on the kinetics of Cu$^{2+}$-induced swelling and glutamate inhibited Cu$^{2+}$-induced swelling. Succinate shortened time of onset and increase in initial swelling rate with appearance of a biphasic curve. The Cu$^{2+}$-induced swelling was inhibited by EDTA, 8-hydroxy-quinoline, CN$^-$, citrate, ATP, GSH, dithiothreitol, and sucrose that were added prior to Cu$^{2+}$. It has been suggested that Cu$^{2+}$-induced swelling is mediated through interaction of Cu$^{2+}$ with a membrane thiol, probably by formation of a mercaptide (34, 35).

It is well known that the pathological effects of hyperbaric oxygen which are strikingly manifest as convulsion of central origin are related to disturbances of metabolism, especially of energy metabolism in cerebral cells (36-38). At elevated pressure of O$_2$, but not N$_2$, the rate of oxygen uptake is depressed. The oxidation of pyruvate or α-ketoglutarate is found to be particularly susceptible to inhibition by oxygen (39, 40) and net ATP synthesis is also inhibited (41). Hauggard and William (41) also investigated the effect of heavy metals on the toxic action of O$_2$ on cerebral metabolism. They found both Cu$^{2+}$ and Fe$^{2+}$ accentuated oxygen toxicity but a different mechanism Co$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$ exert a protective effect.
Glycolysis in brain was relatively resistant to oxygen toxicity except in the presence of added Cu²⁺. This is attributed to glyceraldehyde phosphate dehydrogenase inhibition in the presence of O₂ and Cu²⁺. The mechanism of inhibition of oxidative phosphorylation by oxygen and Cu²⁺ is speculated to be an oxidation of a sulfhydryl group (42-45). Cu²⁺ was very effective in catalyzing the oxidation of sulfhydryl groups from protein or non-protein sources (46, 47) and Cu²⁺ inhibition can be counteracted by dithiothreitol and reduced glutathione. Fe²⁺ is not as effective as Cu²⁺ in catalyzing oxidation of sulfhydryl groups and dithiothreitol does not protect against Fe²⁺.

Lanthanum, which has a relatively small size and high charge density and thus greater electrostatic attraction toward negative ligands as compared with calcium (48), has been reported to be an inhibitor of calcium flux across various membranes including muscle fiber (49, 50), axon of lobster and squid (51), an artificial membrane with negative group of phospholipids (52) and mitochondria (53, 54). In isolated mitochondria considerable evidence now supports the concept that there is a divalent cation carrier located in the inner membrane (53-58) which is capable of translocating Ca²⁺ and Mg²⁺, but not K⁺, against a concentration gradient in the presence of energy. Chance (59) suggested this carrier is a phospholipid component of the membrane and could be identical with high energy intermediate X-I.

Using murexide (a probe for Ca²⁺ movement) and BTB (an intramitochondrial pH indicator), and measuring the oxidation-reduction change of cytochromes, Mela (54) demonstrated that lanthanides at the concen-
tration about 0.05-0.07 mmoles/mg of protein specifically inhibits the energy-dependent uptake of Ca$^{2+}$ and its concomitant reactions including oxidation of cytochromes, alkalization of intramitochondrial space, activation of State 4 respiration and inhibition of State 6 respiration. La$^{3+}$ does not inhibit any other mitochondrial reaction, like oxidative phosphorylation or monovalent cation accumulation.

Binding studies (54, 56, 60) indicate that there are specific and less specific sites for Ca$^{2+}$. The numbers of specific and of less specific sites are 0.05-0.07 and 40 n moles/mg protein respectively. Both sites are phospholipids in nature. La$^{3+}$ block the specific high-affinity sites (carrier) whose turnover rates of Ca$^{2+}$ are 140 time/sec.; while, local anesthetics block the less specific sites and leave more Ca$^{2+}$ available for the specific site and thus enhance the Ca$^{2+}$ accumulation. More recently Jacobus and Brierley (61) and Scanpa and Azzi (62) have established the binding of divalent and monovalent cations are competitive with each other for a common site. This site appears to be phospholipid in nature and binds the following cations in the sequence of La$^{3+}$ Ca$^{2+}$ Mg$^{2+}$ K$^+$ and Na$^+$. Additionally, it has been shown that Zn$^{2+}$ and other heavy metals preferentially bind to site involved in protein and saturates the lipid site only at high concentrations of cation. The binding of cations to this protein site is inhibited noncompetitively by cations listed above and competitively by Zn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ (61). Those observations were in contrast to those reported by Mela et al. (54) that both high-affinity and low-affinity sites for Ca$^{2+}$ are phospholipids and that Ca$^{2+}$ and La$^{3+}$
(or Pr$^{3+}$) bind only noncompetitively.

Very recently Lehninger and Carofoli (63) have investigated the interaction of La$^{3+}$ with mitochondria in relation to respiration-coupled Ca$^{2+}$ transport and concluded that La$^{3+}$ is bound to high-affinity sites competitively with Ca$^{2+}$, that this binding results in inhibition of the Ca$^{2+}$ carrier, and that La$^{3+}$ is bound by external sites on mitochondria for which Ca$^{2+}$ is not a strong competitor. La$^{3+}$ itself is not transported by the Ca$^{2+}$ carrier in a respiration-dependent process (63).

Mitochondria appear to be a potential site for the toxic effects of lead in lead-intoxicated animals (64-68) and plants (69). Mitochondria in proximal tubular lining cells in the kidney of the lead-intoxicated rat have been shown to be structurally and functionally abnormal (64). The basilar mitochondria of these cells are swollen and have shortened and marginal cristae and few matrical granules. There is an associated aminoaciduria which occur in these rats. In vitro study of these isolated mitochondria shows a decreased respiratory control ratio and partial uncoupling of oxidative phosphorylation in the presence of pyruvatemalate (65). The electron microscopic and osmotic swelling experiments have suggested that lead induces a defect in membrane integrity and thus oxidation and phosphorylation are impaired (65). Goyer (70) has further demonstrated the ultrastructural transformation of lead treated mitochondria during controlled respiration in the absence of phosphate acceptor. He concluded that a large portion of kidney mitochondria from lead-poisoned rats do not change
from condensed to orthodox conformation during State 4 respiration, in contrast to the transformations observed for normal kidney and liver mitochondria by Hackenbrock (71). A portion of the lead-treated mitochondria do transform to the orthodox form, but they rapidly degenerate. The conclusion is that those mitochondria that do not undergo change in ultra-structure have impairment of electron transport, and those that do become orthodox have increased membrane lability and undergo degeneration.

Scott et al. (72) have recently investigated the interaction of lead with intact mitochondria and submitochondrial particles and reported some lead-induced reactions. First, lead alters the passive permeability of the mitochondrial membrane to cations and to anions differently. Second, lead activates the energy-linked uptake of ions by the mitochondria and lead itself is accumulated by an energy-dependent reaction, this energy-dependent process of Pb\(^{2+}\) uptake is blocked by La\(^{3+}\) and abolished by uncoupler and Pb\(^{2+}\) is probably transported by the Ca\(^{2+}\) carrier. Third, succinoxidase is considerably more sensitive to Pb\(^{2+}\) than is DPNH dehydrogenase in submitochondrial particles in a KCl medium. Pb\(^{2+}\) stimulated the oxidation of exogenous DPNH by 200-600% depending on the reaction medium, whereas lead inhibited succinate oxidation by more than 80% in corn mitochondria (69). Fourth, lead interferes with succinate exchange and retention (72).

The energy-linked accumulation of ions by isolated heart mitochondria induced by metals and mercurials has been intensively studied in Brierley's laboratory (7, 8, 73-75). These studies have established
that Zn$^{2+}$ (or other heavy metals) markedly increases the energy-linked uptake of Mg$^{2+}$ and K$^+$ by isolated heart mitochondria. This reaction is similar to that of parathyroid hormone (76-77) in that increases in both K$^+$ and Mg$^{2+}$ transport are seen, but in contrast to that of valinomycin and gramicidin which is limited to increased transport of monovalent cations (78, 79). The transport of K$^+$ induced by Zn$^{2+}$ closely resembles that reported by other investigators in the presence of valinomycin (78, 79), gramicidin (78, 80) and parathyroid. The energy-linked accumulation of Mg$^{2+}$ induced by Zn$^{2+}$ can be supported either by substrate oxidation or exogenous ATP. The ATP-supported reaction induced by Zn$^{2+}$ is strongly potentiated by CMS; while, Zn$^{2+}$ induced substrate-dependent accumulation is inhibited by addition of CMS (7). When both substrate and ATP are available as an energy source for Mg$^{2+}$ accumulation activated by Zn$^{2+}$, the reaction is sensitive to DNP but not oligomycin. Addition of CMS under these conditions reverses this pattern of inhibition.

The mechanism of action of these ion-transport inducing agents is not established. A carrier-mediated ion transport mechanism (81) and a transmembrane movement in response to a potential gradient (82) have been suggested by several investigators.

**Physiological Role of Cu$^{2+}$**

Cu$^{2+}$, like other trace elements such as Fe$^{2+}$, Zn$^{2+}$ Co$^{2+}$, Mo$^{2+}$, is essential for normal life (83-85). However, the mechanism of Cu$^{2+}$ action has remained elusive in most cases. Most of dietary Cu$^{2+}$ tends to be absorbed under acidic conditions which occur in the stomach or
the upper small intestine. Ingested Cu$^{2+}$ exists in serum in two distinct forms and the total Cu$^{2+}$ in the body is about 80 mg. The major part of serum Cu$^{2+}$ (about 90%) is tightly bound to ceruloplasmin, which catalyzes the oxidation of a variety of substrates including physiological amines (serotonin, epinephrine, tyrosine), polyphenol, steroid. A small portion of the circulating Cu$^{2+}$ (about 7-10%) is loosely bound to serum albumin and this fraction is thought to be associated with dynamic flow of Cu$^{2+}$ from tissue to tissue. This distribution of Cu$^{2+}$ is strictly linked to physiological and pathological state of biological system.

The most important tissue involved in Cu$^{2+}$ metabolism is the liver. The liver receives its Cu$^{2+}$ from the serum albumin and is the major organ for Cu$^{2+}$ storage. Several important Cu$^{2+}$ functions associated with the liver (87) are as followed: (A) storage, (B) ceruloplasmin synthesis, (C) preparation of the metal for biliary excretion. Three functions are operating in three compartments as indicated above. Suppose that the body is operating under normal conditions and all body Cu$^{2+}$ is in the state of equilibrium. Now if Cu$^{2+}$ has been ingested, the storage compartment will begin to fill and this extra Cu$^{2+}$ will subsequently move to the compartments of biliary excretion and of ceruloplasmin synthesis as they come to handle the increased load.

Cu$^{2+}$ is also found in the red blood cell and its concentration is not influenced by either total serum Cu$^{2+}$ or ceruloplasmin level (88). The blood Cu$^{2+}$ exists as erythrocuprein (60%) and non-erythro-
cuperin (40% labile). The former arises from bone marrow from whence it appears as a constituent of the red blood cell, and this is in equilibrium with the non-erythrocuperin fraction and the non-erythrocuperin fraction is in equilibrium with direct reacting serum Cu$^{2+}$ (85).

Cu$^{2+}$-containing enzymes are frequently found in catalytic function. One of the most important function is that Cu$^{2+}$ is a constituent of various enzymes in biological oxidation in animals and plants. These include cytochrome oxidase, the terminal oxidase in the electron transport chain from which ATP is derived; phastocyanin, a photosynthetic electron carrier in chloroplast; ceruloplasmin, an oxygen-transporting serum protein in vertebrate blood plasma. A recent hypothesis for ceruloplasmin function (89) is that it promotes iron saturation of transferrin which in turn stimulates the biosynthesis of iron-containing proteins. However, convincing evidence of this hypothesis is not available and this protein function still remain obscure. Several other enzymes (90, 91) including tyrosinase, ascorbate oxidase, amine oxidase, dopamine B-hydroxylase, laccase, uricase, transulfurase, galactose oxidase, all require Cu$^{2+}$ to catalyze their individual reactions.

Another role of Cu$^{2+}$ in the body is in relation to hemoglobin formation. This is based on the fact that despite an adequate supply of iron, Cu$^{2+}$ is required to prevent anemia. Cu$^{2+}$ has been shown to be involved in the release of iron stores, facilitation of iron absorption, and stimulation of enzymatic activity for heme synthesis (92, 93).
Cu\(^{2+}\) has also been shown to be associated with formation of aortic elastin (94). In a deficiency state, elastin is not synthesized properly and aortic rupture may occur. Recent work has shown that Cu\(^{2+}\) and amine oxidase, may play an analogous role in the catalysis of cross-linking in collagen (95).

Cu\(^{2+}\)-deficient guinea pigs showed a marked under-development of myelin in the brain (96). One possible explanation for this observation is that Cu\(^{2+}\) may be involved in the formation of phosphatidic acid that is a precursor for phospholipid synthesis, and this defect in phospholipid synthesis could result in demyelination. Recently, Gallagher (86) reported Cu\(^{2+}\) deficiency in rat depresses phospholipid synthesis by liver mitochondria, but not by liver microsomes and the effect of Cu\(^{2+}\) deficiency on mitochondrial phospholipid synthesis is eliminated by adding ATP. Therefore, the inhibition of phospholipid synthesis is suggested as a result of the inhibition of energy generation in mitochondria.

The investigation of Colburn (97) reveals that a fraction of pinched-off nerve ending particle (synaptosomes) and synaptic vesicles from brain tissue contained large quantities of Cu\(^{2+}\), Fe\(^{2+}\) and Mg\(^{2+}\). Additional investigations have suggested that Cu\(^{2+}\) might be coordinating with membrane constituents and hence be importantly related to membrane structure and function, such as nerve excitation, ion movement and transport of nerve transmitter.
Toxicity of Cu$^{2+}$ and Other Metals

Excessive Cu$^{2+}$ appears to be toxic. In experimental animals Cu$^{2+}$ injected into the subarachnoid space results in rapid onset of convulsions and death. This convulsive action is believed to be due to a direct effect on the plasma membrane of brain cells. Cu$^{2+}$ inhibits membrane ATPase (30-33, 98) and pyruvate dehydrogenase complexes (99). Wilson's disease, a well-known pathological condition in man manifest by excessive body stores of Cu$^{2+}$ in liver, brain, kidney and eye, is characterized by a form of neurological degeneration, cirrhosis of liver, kidney defect and Cu$^{2+}$ accumulation in cornea and presence of corneal pigment ring. Total body stores of Cu$^{2+}$ in Wilson's disease are much increased, plasma Cu$^{2+}$ levels are reduced due to reduced level of ceruloplasmin-bound Cu$^{2+}$. Much of the Cu$^{2+}$ circulating in the plasma is bound loosely to non-specific protein and is therefore much more labile and free to diffuse into the tissue. In a case of Wilson's disease, a significant portion of the pathological hepatic Cu$^{2+}$ was found in the mitochondrial fraction (100). In brain the largest portion of the pathological Cu$^{2+}$ was found in the subcellular supernatent fraction. Pathological Cu$^{2+}$ in both brain and liver is bound to a number of different types of protein (100). The real pathological mechanism of Wilson's disease is not well known. It has been felt that the primary defect may be failure of the liver to remove albumin-bound Cu$^{2+}$ from plasma and incorporate the Cu$^{2+}$ into ceruloplasmin. This defect is felt to be due to the genetically determined absence of a liver enzyme system (101). It has also been shown (102)
that serum Cu\(^{2+}\) increases in several chronic and acute diseases such as malignant tumor, rheumatoid arthritis, leukemia, and pernicious anemia. In most cases, the rise in serum Cu\(^{2+}\) was accompanied by a rise in ceruloplasmin content without an increase in the Cu\(^{2+}\) content of the erythrocyte or leukocyte. Ninety patients with multiple sclerosis had reduced Cu\(^{2+}\) oxidase (102). Serum Zn\(^{2+}\), on the other hand, is decreased in the disease mentioned above (102). Trace metals are rapidly increasing environmental pollution and have been implicated as industrial health hazard and as the causative factor for cancers, myocardial infarction, renal dysfunction, hypertension, atherosclerosis and several chronic diseases (102-105). Mustafa et al. (106) have recently demonstrated a number of divalent metals (Cd\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Pb\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\), Sn\(^{2+}\), Sr\(^{2+}\)) induced injury of the function of pulmonary alveolar macrophage (PAM) system by inhibition of membrane-ATPase. The specialized bulk flow mechanisms of PAM such as phagocytosis, pinocytosis, and energy-dependent active ion transport would presumably be impaired. Mazaleski et al. (107) showed that animals exposed to currently accepted "no effect" threshold limit value (TLV) of CO (50 ppm) showed shifts and/or accumulation of trace metals. At the subcellular level such shifts could ultimately also manifest themselves in chronic diseases by disrupting a major metabolic pathway.

**Effect of Cu\(^{2+}\) and Other Metals on Membrane ATPase**

The discovery of the Na\(^{+}\)-K\(^{+}\) ATPase system in the microsomal fraction from crab nerve by Skou (108-110) has provided an enzymatic basis for study of active cation transport across cell membranes.
Dunham and Glynn (111) and Post et al. (112) have reported isolation of the enzyme from the erythrocyte membrane. Bonting and associates (113) systematically studied the distribution of the enzyme system in thirty-nine tissues of the cat and man. They found the enzyme present in all but six of the tissues including corneal stroma, lens capsule, lens fiber, adipose tissue, and serum. Those tissues mentioned above have no cell or very low cell density. The main function of this enzyme is to maintain high intracellular K⁺ level and ion gradient in cell. It is characterized as follows (110): The enzyme is located in the membrane. It utilizes only ATP as the energy source and catalyzes ATP-ADP exchange. It requires both Na⁺ and K⁺; Na⁺ activates phosphorylation and K⁺ stimulates dephosphorylation (114). It is inhibited by cardiac glycosides. It has been reported that divalent cations including Ca²⁺, Co²⁺, Zn²⁺, Hg²⁺, Pb²⁺, Cu²⁺, Cd²⁺, Fe²⁺, Ni²⁺, are inhibitory to this enzyme, while Mg²⁺ and Mn²⁺ have an activation effect for Na⁺-K⁺-ATPase isolated from brain (115, 116).

Peter, Shorthouse, Walshe and their colleagues (29-33, 117) investigated Cu²⁺ toxicity in brain tissue by injecting Cu²⁺ into the subarachnoid space. They concluded that the reason for the rather specific convulsive and lethal action of Cu²⁺ is due to an inhibition of membrane transport by inhibition of the microsomal membrane ATPase. This enzyme is surprisingly activated by Mg²⁺ but little by Na⁺ and K⁺. This enzyme is inhibited not only by Cu²⁺, but also by ouabain. The inhibition by those reagents, however, did not run parallel. Ouabain was much less effective than Cu²⁺. The inhibition by Cu²⁺ and
by ouabain are 30% and 8% respectively and these inhibitions are not additive. They postulated that this membrane enzyme has at least 3 components a, b and c, which have a different susceptibility to \( \text{Cu}^{2+} \) and ouabain and that the chemical group inhibited by \( \text{Cu}^{2+} \) is not the same as that inhibited by ouabain. The catalytic site of microsomal ATPase is postulated to be a group that is inhibited by \( \text{Cu}^{2+} \).

In order to understand the role in active ion transport played by \( \text{Na}^{+}-\text{K}^{+}\)-ATPase and by \( \text{Mg}^{2+}\)-ATPase, Bouler and Duncan (118) have studied the differential sensitivity of these two ATPase isolated from rat brain and rat erythrocyte to \( \text{Cu}^{2+} \). They found both \( \text{Mg}^{2+}\)-ATPase and \( \text{N}^{+}-\text{K}^{+}\)-ATPase are inhibited by \( \text{Cu}^{2+} \), but the latter is more sensitive. This result is in contrast with that reported earlier by Epstein (32) who found the \( \text{Mg}^{2+}\)-ATPase to be the more sensitive. It has been concluded that both enzymes are implicated in biological transport of cation across the plasma membrane (110); \( \text{Mg}^{2+}\)-ATPase has a role in the control of passive permeability in excitable cell and erythrocytes (118-120). Both enzymes are capable of undergoing configurational and conformational changes (121, 122) and control the shape of membrane (123-126).

**Effect of \( \text{Cu}^{2+} \) and Other Metals on Membrane Permeability and Transport of Various Tissue**

The concept of the membrane acting as a control system is receiving more attention and becomes an increasingly important area of research. The balance of influx and efflux of material through the membrane is one of the determining factors in the regulation of intracellu-
lar composition and its activity. The nature of the membrane and its transport mechanism continue to be central themes of membrane studies. Membranes play a passive role in the control of metabolism by a cell in that they offer a resistance to flow of material. Control is built into the nature of membrane mechanism which linked to metabolic reaction such as carrier and active transport. Control by synthesis of new membrane component in the presence of inducer which is in turn controlled by genetic apparatus such as galactose permease of E Coli (127).

Information concerning specific membrane ligands and their corresponding functional response could be deduced by different chemical modification of the membrane. This has been particularly true for the sulfhydryl group, amino group and phosphoryl group. The involvement of sulfhydryl in maintenance of functional and structural integrity of the cell membrane has been demonstrated in several biological systems including erythrocyte (128), toadfish islet tissue (129), axon (130), sarcoplasmic membrane of skeletal muscle (131). Sulfhydryl groups of membrane have been categorized by their reactivity to a variety of sulfhydryl agents and by the rate of penetration into the "sensitive" site of membrane. Taking advantage of the different reactivity and polarity of various mercurials, the specific permeability and other functions, alterations have been studied by Rothstein and other investigators (132-134). Sugar transport, for example, is affected by a small population of sulfhydryl group located near the outer surface of red blood cell (132), whereas other sulfhydryl groups
deeper within the membrane play a role in cation permeability (135) and active cation transport (134). More recently Knauf and Rothstein (135) investigated the effect of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. They observed that SITS (a amino group reagent, 4-acetamido-4'-isothiocyanostilbene-2.2'-disulfonic acid) decreases the anion permeability of the human red blood cell as measured by sulfate flux, whereas PCMS does not. In contrast PCMS increases cation permeability as measured by K⁺ leakage, whereas SITS does not. SITS does not penetrate into the membrane and its failure to affect cation permeability is attributed to its inability to reach an internal population of amino groups. They concluded that the permeability barrier for both cation and anion are involved in protein. The anion barrier is controlled by amino groups located superficially, whereas the cation barrier is controlled by amino and sulfhydryl groups located deeper within the membrane.

**Effect on the Glycerol, Amino Acid, Sugar**

Interaction of heavy metal with several membranes will result in disturbance of flow of several biological important substances including ions, amino acids, sugars, glycerol, mannitol, etc. Human erythrocytes are readily permeable to glycerol. The temperature coefficient is low and the rate of penetration is very sensitive to changes of pH. The entry of glycerol into the red cells can be inhibited by traces of Cu²⁺ and Hg²⁺ (136, 137). Cu²⁺ was also reported to inhibit facilitated diffusion of glycerol across the Schwann cell and squid axon membrane (138). Only a very small amount of Cu²⁺ attached
to the cell membrane can block this transport, and the inhibition is dependent on time. However, the Hg$^{2+}$ block in glycerol transport is only temporary. After a lag period the cell membrane suddenly regains its original permeability. Therefore Hg$^{2+}$ is presumably temporarily fixed to ligands in the membrane that control glycerol permeability; subsequently, it moves into the interior of the cells and combine with other complexing substances. As long as some Hg$^{2+}$ is bound to the membrane, inhibition of glycerol permeability is observed, but as soon as all the Hg$^{2+}$ has passed through the membrane the inhibition disappears. At very high concentration of Hg$^{2+}$, the complexing ligands of the cell interior become saturated, the excess metal remains attached to the cell membrane and a permanent time-independent reduction of glycerol permeability is produced (139).

Recently, Watkin and associates (140) reported heavy metals (Cd$^{2+}$, Co$^{2+}$, Hg$^{2+}$, AsO$_2^-$) increase islet tissue permeability to D-mannitol-$^1$-C$^{14}$ and concluded that alloxan as well as heavy metals damage the B-cell membrane and induce diabetogenic effect.

The role of surface a polyphosphate groups has been implicated in the enzymatic phosphorylation of a carrier and transmembrane transport of sugar in yeast cell (141). This phosphorylated carrier complex has high affinity for sugar and can maintain the uphill transport. This phosphoryl site are thought to be involved in uphill transport by maintenance of high affinity of a carrier for sugar. This phosphoryl group is located on the outer surface of the cell. Heavy metals (Ni$^{2+}$ and UO$_2^-$) have a high affinity toward either a phosphoryl site or
polyphosphate. It has been shown metal binding to those sites will result in conformational change and the affinity for sugar is considerably low (142, 143). Therefore the rate of uphill influx is reduced, but the downhill movement by carrier mediated facilitated diffusion is not affected (144). Both Cu$^{2+}$ and Hg$^{2+}$ are reported to have inhibitory effects on uptake of glucose by erythrocyte and muscle (145).

Aminoaciduria, a well-known symptom of heavy metal intoxication, has been widely reported. Pb$^{2+}$ poisoning, reported by Goyer (64) is an example. Foulkes (146) reported that the peritubular cell membrane in kidney is damaged by uranium or PCMB in poisoned animals and this poisoning results in the depression of the transmembrane transport of dicarboxylic acids such as glutamate and asparate.

Ion fluxes through membrane is also disturbed by heavy metal. Losses of K$^+$ from red cells (147) and yeast cells (148) and erythrocytes (149) produced by Pb$^{2+}$, Hg$^{2+}$, Au$^{2+}$ were reported. It is always difficult to differentiate between a direct effect on membrane permeability or the ion fluxes driven by energy. Because ion transport is always intimately coupled to energy mechanisms built in the membrane such as membrane ATPase and membrane potential.

Although the Na$^+$ pump seems to be the most common form of active transport in cells, active transport of Cl$^-$ has also been widely reported by several authors from several sources. Such as in non-secreting gastric mucosa of cat (150), squid giant axon (151), smooth muscle (152) and turtle bladder (153) etc. Most of the chloride pump is inhibited by DNP, CN$^-$ and ouabain. The Cl$^-$ pump functions in an
electrogenic manner in some cases (153-155) and operate in an electrically neutral manner, presumably exchanging Cl\(^-\) for HCO\(_3^-\) (156). Ussing (157-159) has shown that Cl\(^-\) movement across the isolated frog skin is purely passive. The addition of Cu\(^{2+}\) will decrease this Cl\(^-\) permeability and increase the transepithelial potential. Dietz (156) study ion transport in larval salamanders and report dilute Cu\(^{2+}\) has an effect on transient period of depression of transepithelial potential difference.
STATEMENT OF PROBLEM

The ion-translocating properties of the inner mitochondrial membrane are of great importance in relation to energy-transduction and membrane phenomena. Investigation of the effects of heavy metals on this membrane function in mitochondria may help us to understand the mechanism of energy coupling and the nature of the toxicity of heavy metals.

Results obtained in this laboratory have established that heavy metals, detergents and organic mercurials will modify the mitochondrial membrane and result in activated energy-dependent ion transport. The binding properties of this membrane for trivalent, divalent and monovalent ions have been studied using submitochondrial particles.

The present investigation was carried out in an effort to relate the possible role of the interaction of membrane components with heavy metals on energy coupling in intact mitochondria. Modification of membrane functions including passive permeability, active ion transport, oxidative phosphorylation and ATPase activity will be observed under different degrees of surface alteration of mitochondrial membrane by different metals.
EXPERIMENTAL METHODS

Preparation of Mitochondria

Beef heart mitochondria were prepared by using the modified Nagarse procedure (160) of Settlemire et al. (161) in which EDTA is replaced by EGTA for the removal of excess Ca$^{2+}$ without affecting the Mg$^{2+}$ level. All protein determinations were made by a biuret method (162).

Preparation of Submitochondrial Particles

Submitochondrial particles were prepared by sonication in the absence of added chelater, nucleotides and Mg$^{2+}$ as described by Jacobus and Brierley (61). Preparations from several days were pooled and kept frozen until used. A sample of about 600 ml of mitochondria (25 mg of protein/ml) was thawed and sonicated for 30 seconds in 30 ml batches in a 50 ml beaker held at 0°C using a Blackstone probe sonicator operated at maximum intensity. The suspension was centrifuged for 10 minutes at 17,000 g in a Sorvall SS-34 rotor to remove the larger particles. The supernatant was centrifuged for 30 minutes at 100,000 x g in a Spinco 40 rotor. The resultant pellets were suspended in 0.25 M sucrose and the protein was adjusted to 15 mg/ml. Lipid-free submitochondrial particles were prepared by extraction with aqueous acetone containing NH$_3$ as described by Fleischer et al. (163).
Binding of Metals

The binding of Cu\(^{2+}\), Pb\(^{2+}\), and Zn\(^{2+}\) was determined by atomic absorption spectroscopy (Perkin Elmer Model 303) of supernatant solutions following the removal of mitochondria (or submitochondrial particles) by either centrifugation or filtration through Millipore filters. The composition and condition of the individual incubations are given in the figure legends. All the points reported are the average of duplicate or triplicate determinations.

Simultaneous Determination of Respiration
Swelling and pH Change

Simultaneous determination of the change in swelling, respiration, and pH was done by using a plexiglass cuvette equipped with a magnetic stirrer, A USI 5331 Clark-type oxygen electrode (203) and a combination glass electrode mounted in the light path of an Eppendorf photometer. Swelling and contraction were monitored at 25°C by the absorbance change at 546 nm. A decrease in absorbance at 546 nm represents swelling whereas an increase correspond to contraction.

Determination of Oxidative Phosphorylation

The determination of P/O ratio and respiratory control were made by the method of Chance and Williams (164).

Determination of ATPase Activity

ATPase activity was estimated by the rate of H\(^+\) production detected by the pH electrode (165) and by the determination of Pi release
as described by Lindberg and Ernster (166). The experimental details are given in each individual legend.
RESULTS

The Potency of Metals in Inducing Mitochondria Swelling

Heavy metals induce osmotic swelling of beef heart mitochondria suspended in 100 mM KCl. The dose-swelling relationship induced by various metals including Cu\(^{2+}\), Pb\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), La\(^{3+}\) is shown in Figure 1. Isolated mitochondria respond to added metals right after their addition and swell to different degrees. The rate and extent of mitochondrial swelling depend on several factors such as species and amount of metal given, suspending media, metabolic states etc. (167). Accumulated titration data indicated that 3 minutes generally represents a proper interval to evaluate the gross swelling of mitochondria because the extent of the passive mitochondrial swelling in KCl (100 mM) is linear or nearly linear with time. However, Cu\(^{2+}\) and La\(^{3+}\) in some concentration ranges (Cu\(^{2+}\) over 20 uM, La\(^{3+}\) over 60 uM) induce a rapid swelling which is followed by contraction. It must be noted that the contraction phase occurs only after the swelling phase has reached a maximal initial rate. Figure 1 shows that Cu\(^{2+}\) and La\(^{3+}\) are very potent in inducing swelling in concentration below 20 uM and 60 uM respectively. Hg\(^{2+}\) and Pb\(^{2+}\) above 20 uM and 150 uM also induce good swelling. Zn\(^{2+}\) and Cd\(^{2+}\) induce little swelling in the suspending medium of KCl (0.1 M) pH 7. Figure 2 shows that at the condition that allow mitochondria to have the same amount of metals bound
Figure 1. Swelling of isolated beef heart mitochondria in the presence of different concentrations of various metals as indicated. Beef heart mitochondria (5 mg of protein) were treated with rotenone (2 ug/mg) and added to 8 ml of medium of KCl (100 mM) and Tris Cl (2 mM) pH 7. The reaction was carried out at 25°C in a stirred plexiglass cuvet mounted on an Eppendorf photometer, or on Beckman spectrophotometer (Model B). Swelling is determined by the change of absorbance at 546 nm 3 minutes after the addition of metals.
Figure 2. The relationship between mitochondrial swelling and bound metal. Mitochondria (5 mg of protein) were treated with rotenone (2 ug/mg) and incubated at 25°C in 8 ml of KCl (100 mM) buffered by Tris Cl (2 mM) pH 7.0. Swelling was determined by decrease of absorbance at 546 mu for 3 minutes using a Beckman (Model B); the mitochondria were removed by centrifugation rapidly at 20,000 rpm in a Sorvall SE-12 rotor for 2 minutes. The binding of metals was determined by atomic absorption spectroscopy of the resulting supernatant.
will reflect the different magnitude of the swelling in KCl suspending medium. For example, if mitochondria are allowed to have 10 n moles/mg of Cu²⁺ bound in membrane, it will swell to such an extent that the change of optical density is 0.2 unit for 3 minutes in KCl medium. To obtain the same magnitude of mitochondrial swelling induced by 10 n moles/mg of Cu²⁺, 85 n moles/mg of Pb²⁺ bound by mitochondria is required. Mitochondria will not swell to such a magnitude even at much higher levels of Zn²⁺ which is bound by membrane. This may tentatively be speculated that different metals have different affinities toward the membrane ligands that possess different abilities to translocate the ions across membrane (also see Figure 7-10); however, other possibilities cannot be excluded.

Differential Passive Permeabilities of Ions Induced by Metals

Ions show different tendencies to be translocated across the mitochondrial membrane. K⁺, H⁺, NH₄⁺, Cl⁻ etc., for example, are passively impermeable; however, Na⁺, phosphate, acetate and nitrate can go into the mitochondria by simple diffusion or by exchange diffusion. Therefore, using the same experimental criteria taking advantage of some diffusion-limiting ion species will help evaluate the ion permeabilities induced by different metals. Increases in K⁺ permeability can be shown by suspending mitochondria in KN0₃. In this medium nitrate diffuses freely into the mitochondria and the permeability of K⁺ will limit swelling. Alterations in the membrane which increase the permeability to K⁺ will therefore result in more rapid swelling in
KNO₃. Changes in Cl⁻ permeability can be estimated, in the presence of uncoupler, by suspending mitochondria in NH₄Cl. Since uncoupler can carry H⁺ and NH₃ can diffuse into mitochondria by itself in this system, the chloride becomes the swelling-limiting ion. Followed the same argument, NH₄NO₃ can be used as a medium to estimate the NH₄⁺ or H⁺ permeabilities and KCl is used to estimate both K⁺ and Cl⁻ permeabilities. Although the above experimental criteria are not rigorous enough to measure absolute permeabilities of each ion, the identical conditions kept through the experiments will shed some light on the relative permeabilities of ions induced by the metals. The results shown in Figures 3-6 indicated that metals can induce both cation and anion permeabilities disproportionally passively; Cd²⁺ stimulates H⁺ permeability greater than that of other ions. Pb²⁺ (above 75 uM) enhances more the permeability of K⁺ to a greater extent than that of anions. La³⁺ (above 10 uM) induces Cl⁻ permeability to a greater extent as compared with cation. Cu²⁺ activates both cation and anion more evenly as compared with other metals and Zn²⁺ fail to induce permeabilities to ion.

**pH Dependent Swelling Induced by Metals**

One of the classic and fundamentally important methods to locate a ligand and to study ligand-metal interactions is to study the pH profile. pH, as well as other variables, will affect the nature of ligand, charge distribution, stability and binding capacity of membrane to metals. It is therefore desirable to study the osmotic response of
Figure 3. Mitochondrial swelling induced by Pb$^{2+}$ in the presence of various suspending media. The experimental conditions for swelling were identical with those in Figure 1 except that in trace D CCP ($6 \times 10^{-7} M$) is added before metal.
Figure 4. Mitochondrial swelling induced by La$^{3+}$ in the presence of various suspending media. The experimental conditions were identical with those described in Figure 3.
Figure 5. Mitochondrial swelling induced by Cd\textsuperscript{2+} in the presence of various suspending media. The experimental conditions were identical with those described in Figure 3.
Figure 6. Mitochondrial swelling induced by Cu$^{2+}$ in the presence of various suspending media. The experimental conditions were identical with those described in Figure 3.
mitochondria induced by metals at various pH in order to understand the regulatory effect of membrane ligands on the ion permeabilities. Figures 7-10 show that in salt solutions the swellings of mitochondria induced by metals are dependent on pH. Their pH profiles of the swelling developed by various metals are quite different. Pb²⁺ is a strong swelling inducer in alkaline media (above pH 8). Binding study of Pb²⁺ by intact mitochondria at various pH revealed that mitochondria have higher capacity to bind Pb²⁺ at an alkaline condition (see Figure 12, 13); however, the efficiency of bound Pb²⁺ by mitochondria at alkaline pH is considerably higher than that of Pb²⁺ at neutral pH. La³⁺ can induce swelling most effectively at neutral pH around 7-7.5 and has little swelling-inducing effect at other pH. Hg²⁺-induced swelling seems to be less sensitive to the change of pH. Cu²⁺ can induce mitochondrial swelling in both neutral and alkaline pH when KCl is used as a suspending medium, but this swelling is linear to pH when suspended in KOAc. These results establish that metals may interact or bind to different ligands, and that each ligand plays a different role in the regulation of ion permeabilities.

Cu²⁺ and Pb²⁺ Binding to Intact Mitochondria, Submitochondrial Particles and Lipid-Depleted Particles

Mitochondria can bind metals avidly and the degree of binding depends on the composition of the suspending medium, pH, and the metabolic states of mitochondria. Figure 11 indicates that the amount of Cu²⁺ bound passively is influenced by the anionic composition of the suspending medium. The presence of phosphate results in less Cu²⁺
Figure 7 & 8. pH-dependent swelling of mitochondria induced by Pb^{2+} and by La^{3+} in the presence of several suspending media. Beef heart mitochondria (5 mg of protein) were treated with rotenone (2 ug/mg) and added to 8 ml of medium of KCl (100 mM) + Tris Cl (2 mM), or KNO_3 (100 mM) + Tris NO_3 (2 mM), or KOAc (100 mM) + Tris OAc (2 mM) at various pH that are adjusted by their conjugated acids and bases. The swelling measurements have been corrected for blank swellings. Both swelling are determined by the change of absorbance at 546 mu for 3 minutes in a stirred plexiglass cuvet mounted on a Beckman spectrophotometer (Model B).
Figure 9 & 10. pH-dependent swelling of mitochondria induced by Cu²⁺ and by Hg²⁺ in the presence of different suspending media. The experimental conditions were identical with those described in Figure 7 & 8.
Figure 11. Binding of Cu$^{2+}$ by mitochondria in salt solutions. Beef heart mitochondria (2.5 mg protein) were treated with rotenone (2 μg/mg) and was added to 4 ml of medium of slightly buffered salt solutions by Tris (2 mM) as indicated in Figure. After the mitochondria have reacted with metal in salt solution for 7 minutes at 25°C, the reaction mixture is spun down by centrifugation at 17,000 rpm in Sorvall SE 12 for 3 minutes. The Cu$^{2+}$ binding was determined by atomic absorption spectroscopy of the supernatant.
being bound by the membrane as compared to the binding in a chloride medium. Table 1 shows Cu\(^{2+}\) binding to intact mitochondria at different conditions. At the low level of Cu\(^{2+}\) (4 n moles/mg available), 80% can be bound for 4 minutes incubation. The bound Cu\(^{2+}\) can be partially removed by EDTA but surprisingly not by cysteine and thiocarbamate at the same concentration as EDTA. Energy and organic mercurials (CMS and CMB) do not significantly alter the Cu\(^{2+}\) binding to mitochondria at this low level of Cu\(^{2+}\). CMS (100 uM) decreases the high-level of Cu\(^{2+}\) binding to mitochondria which is pretreated with mercurial. The passive binding of Pb\(^{2+}\) to mitochondria and anion effect on binding were shown to be similar to that of Cu\(^{2+}\); moreover, Pb\(^{2+}\) itself was accumulated by an energy-dependent reaction that can be followed either by murexide probe using the Aminco-Chance dual wavelength spectrophotometer or by direct Pb\(^{2+}\) determination using the atomic absorption method as previously described (72). Figure 12 shows that both energy, pH, and the anionic composition of the media will profoundly affect the binding of Pb\(^{2+}\). In a relatively alkaline condition mitochondria can either offer more binding sites to Pb\(^{2+}\) or take up more Pb\(^{2+}\) in the matrix compartment. Lipid-depleted submitochondrial particles can also be demonstrated to bind more Pb\(^{2+}\) under alkaline conditions (see Figure 13). The remarkable increment in binding of Pb\(^{2+}\) by lipid-depleted particles, which consists of the major portion of protein, occurs in the pH range from 7 to 8. The bindings of metals (Cu\(^{2+}\), Pb\(^{2+}\)) by intact mitochondria, submitochondrial particles and lipid-depleted particles were studied and the results are shown in
Cu\textsuperscript{++} binding by mitochondria at different conditions. Mitochondria (2.5 mg protein) were treated with rotenone (2.8 µg/mg) and added to 4 ml of a medium consisting of KCl (0.1 M), Tris Cl (2 mM), and Cu\textsuperscript{++} (4 n moles/mg) and allowed to incubate for 4 minutes at 25°C (experiment 1); in the experiments 2 to 7 add EDTA (3 uM), cysteine (3 uM), thiocarbamate (3 uM), CMS (6 uM), CMB (6 uM), K Succinate pH 7 (2 mM) individually to 2 minutes' incubated-reaction mixtures as indicated in experiment 1, and allow to incubate 2 more minutes after the addition of the reagents; in the experiment 8, add rotenone treated mitochondria (2.5 mg) to 4 ml of medium of KCl (0.1 M), Tris Cl (2 mM), Cu\textsuperscript{++} (4 n moles/mg) and K Succinate (2 mM) and is allowed to incubate for 4 minutes; in experiments 9-13 add EDTA, cysteine, thiocarbamate, CMS, CMB individually to 2 minutes' incubated reaction mixtures as indicated in experiment 8 and let incubate 2 more minutes after the addition. After each reaction has been completed (total incubation time is 4 minutes for each experiment), the reaction mixtures were rapidly spun down by centrifugation at 20,000 rpm for 2 minutes in Sorvall SE-12 rotor. The Cu\textsuperscript{++} bindings were determined by atomic absorption spectroscopy of the resulting supernatant.
# TABLE 1

Cu$^{++}$ BINDING BY MITOCHONDRIA AT DIFFERENT CONDITIONS

<table>
<thead>
<tr>
<th>Condition of Incubation</th>
<th>Cu$^{++}$ Bound (n moles/mg) When 4 n moles Cu$^{++}$/mg Avail.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rot. (25 ug/mg Protein) 4 Min.</td>
<td>3.3</td>
</tr>
<tr>
<td>2. Rot. 2 Min. + EDTA (3uM) 2 Min.</td>
<td>1.3</td>
</tr>
<tr>
<td>3. Rot. 2 Min. + Cysteine (3uM) 2 Min.</td>
<td>3.05</td>
</tr>
<tr>
<td>4. Rot. 2 Min. + Thiocarbamate (3uM) 2 Min.</td>
<td>3.4</td>
</tr>
<tr>
<td>5. Rot. 2 Min. + CMS (6uM) 2 Min.</td>
<td>3.05</td>
</tr>
<tr>
<td>6. Rot. 2 Min. + CMB (6uM) 2 Min.</td>
<td>2.5</td>
</tr>
<tr>
<td>7. Rot. 2 Min. + K Suc. (2mM) 2 Min.</td>
<td>3.05</td>
</tr>
<tr>
<td>8. Rot. + K Suc. (2mM) 4 Min.</td>
<td>2.9</td>
</tr>
<tr>
<td>9. Rot. + K Suc. 2 Min. + EDTA (3uM) 2 Min.</td>
<td>1.84</td>
</tr>
<tr>
<td>10. Rot. + K Suc. 2 Min. + Cysteine (3uM)</td>
<td>2.9</td>
</tr>
<tr>
<td>11. Rot. + K Suc. 2 Min. + Thiocarbamate</td>
<td>3.65</td>
</tr>
<tr>
<td>12. Rot. + K Suc. 2 Min. + CMS (6uM)</td>
<td>2.9</td>
</tr>
<tr>
<td>13. Rot. + K Suc. 2 Min. + CMB (6uM)</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Figure 12. Active, passive and uncoupled binding of Pb^{2+} to mitochondria in several media with different pHs. Mitochondria (2.5 mg of protein) were incubated for 6 minutes at 25°C in 4 ml of medium of KCl (100 mM), Tris Cl (2 mM) or KOAc (100 mM), Tris OAc (2 mM) at various pH. The conditions of the incubation are as follows: (1) Rotenone 2 ug/mg is added (passive condition), (2) Tris-Succinate (2.5 mM) and rotenone are added (active condition), (3) CCP (8 x 10^{-7} M at acidic media, 10 x 10^{-7}M at neutral media, 15 x 10^{-7}M at alkaline media) is added to condition (2) (uncoupled condition). After reactions have completed, the mixture is spun down by centrifugation (17,000 rpm Sovall SE-20, 3 min.). The Pb^{2+} bindings are determined by atomic absorption spectroscopy of supernatents.

A: Passive in KOAc
B: Active in KOAc
C: Uncoupled in KOAc
D: Passive in KCl
E: Active in KCl
F: Uncoupled in KCl
Pb\textsuperscript{++} Bound (nmoles/mg)
Figure 13. pH effect on Pb$^{2+}$ binding to lipid-depleted submitochondrial particle. Lipid-depleted submitochondrial particle (2.5 mg of protein) were incubated for 6 minutes at 25°C in 4 ml of a medium of KCl (100 mM), Tris Cl (2 mM) pH 7. The Pb$^{2+}$ binding was determined by atomic absorption spectroscopy of supernatent following the removal of the particle by centrifugation (3 min. at 17,000 rpm, Sorvall Se 12).
Figures 14 and 15. The affinity of Cu$^{2+}$ for these preparations is in the order of lipid-depleted ETP > intact mitochondria > ETP on the basis of n moles of metal bound by per mg of protein. In the case of Pb$^{2+}$, the order of affinity is intact mitochondria > ETP > lipid-depleted ETP. It seems to be clear from these experiments that lipid (especially phospholipid) plays an important role in metal binding and affects membrane permeability. When phospholipid is removed by the aqueous acetone extraction, the binding of Cu$^{2+}$ is much increased, in contrast the binding of Pb$^{2+}$ is much decreased, especially at large amounts of Pb$^{2+}$ available. This leads to the conclusion that Cu$^{2+}$ has a higher affinity to protein than to phospholipid and thus mainly bound by protein in the range of concentration studied; Pb$^{2+}$ is distributed to both protein and lipid. The different affinity of metals to bind either protein or lipid may be an important factor in determining the membrane permeability to various ions.

**Relationship between Passive Binding and Swelling of Cu$^{2+}$ and of Pb$^{2+}$**

When metals are allowed to react with mitochondria, both chemical and physical events associated with the membrane such as surface tension, ion flux, ligand catalysis, membrane conformation, and membrane potential will be altered. The membrane phenomena are certainly related to the degree of membrane alteration. Figures 16 and 17 show that mitochondrial swelling is a function of the amount of metal bound. When a given amount of Pb$^{2+}$ is bound by mitochondria, K$^+$ and NO$_3^-$ diffuse across the membrane more rapidly than K$^+$ and Cl$^-$. Figure 17
Figure 14. Binding of Cu$^{2+}$ to intact mitochondria, submitochondrial particles and lipid-depleted submitochondrial particles. The experiment conditions of binding of Cu$^{2+}$ to three preparations were identical except using the different centrifugational forces to isolate pellets and rotenone (2 ug/mg) is added to intact mitochondria. All incubations were carried out at 25°C for 5 minutes and started by the addition of the intact mitochondria (2.5 mg protein), or submitochondrial particles, or its lipid-depleted particles to a medium of 4 ml of KCl (100 mM), Tris Cl (2 mM) pH 7. The suspensions were rapidly centrifuged for 5 minutes at 17,000 rpm in a Sorvall SE-12 rotor for intact mitochondria and for lipid-depleted submitochondrial particles; at 40,000 rpm in spinco 40 rotor for submitochondrial particles. The Cu$^{2+}$ bindings were determined by atomic absorption spectroscopy of the resulting supernatents. The points shown in Figure are an average of three determinations. The preparations of submitochondrial particles and lipid-extracted particles were described in the section of method. The total phosphate content of particles before and after extraction were 595 n moles/mg and 52 n moles/mg respectively.
Cu²⁺ Bound (nmol/mg)

100 mM KCl
2 mM Tris Cl
pH 7

ETP (Lipid Depleted)

ETP

Intact Mito.

Cu²⁺ (nmol/mg Avail.)
Figure 15. Binding of Pb²⁺ to intact mitochondria, submitochondrial particles and lipid-depleted submitochondrial particle. The experiments were carried out as described for Figure 14. The preparations are identical as that used in Figure 14.
Swelling (ΔA₄₅₄ in 3 Min.)

Pb²⁺ Bound (nmoles/mg)

Figure 16. Passive swelling as a function of bound Pb²⁺, in KNO₃ and KCl. The experimental conditions are identical to that described in Figure 2 except that the change of optical density is determined by Eppendorf photometer.
Figure 17. Kinetic relationship between passive binding and swelling. The reactions were carried out as described in Figure 2 or Figure 16. The swelling and binding were determined at 3, 5, 8 minutes after the addition of Cu²⁺. A, B, C, D stand for different media used.

A: NH₄Pi (100 mM), Tris Pi (2 mM), pH = 7.
B: KP₁ (100 mM), Tris Pi (2 mM), pH = 7.
C: NH₄Cl (100 mM), Tris Cl (2 mM), pH = 7.
D: KCl (100 mM), Tris Cl (2 mM), pH = 7.
shows that if mitochondria are reacted with Cu$^{2+}$ at the level indicated, passive permeability to several cations such as K$^+$, NH$_4^+$, H$^+$ and anion Cl$^-$ will be developed, but phosphate entrance is retarded. Kinetic data indicate that the sluggish influx of phosphate induced by Cu$^{2+}$ is only temporarily. In contrast the inhibition of Pi transport induced by mercurial is time-independent and not reversible by itself (12). The cause of reversible inhibition of Pi transport by Cu$^{2+}$ is not known but several possibilities could be involved. First, Cu$^{2+}$ induced permeabilities to other ions such as H$^+$, NH$_4^+$, or OH$^-$ which may be linked to the function of carrier in such a way that facilitate the Pi entrance. This suggests that the other ion movement across membrane may have an important role in the operation of Pi/OH exchange indirectly. Second, Cu$^{2+}$ is translocated to other ligands or removed from membrane in response to swelling or during the passage of other ions.

Effect of Cu$^{2+}$ on Respiratory Activity of Intact Mitochondria and of Submitochondrial Particles

The effect of Cu$^{2+}$ on respiratory activities of intact mitochondria and of submitochondrial particles is different. Cu$^{2+}$ inhibits the oxidations of both succinate and of NADH but slightly activates TMPD + ascorbate respiration in submitochondrial particles. Figure 18 shows that the concentration of Cu$^{2+}$ required to inhibit the oxidation of succinate and of DPNH by 50 percent is 13 and 32 n moles/mg of Cu$^{2+}$ available,respectively. This is quite consistent with the inhibition of respiration by Pb$^{2+}$ reported previously (72). In the
Figure 18. Cu$^{2+}$ effect on succinate, DPNH and TMPD + ascorbate oxidation in submitochondrial particle. Particles (10 mg of protein) were added to 16 ml of a medium of KCl (100 mM) and Tris Cl (2 mM) pH 7, treated with the indicated amount of Cu$^{2+}$ for 2 minutes and respiration then initiated by the addition of either K Succinate (2.5 mM) or DPNH (1 mM) or TMPD (60 uM) + ascorbate (2 mM). The oxygen uptake was measured polarographically by using a Yellow Spring Instrument Co. electrode fitted to a water jacketed reaction vessel kept at 25°C. The initial uninhibited rates were 0.13 uatom O$_2$/mg, 0.25 uatom O$_2$/mg and 0.21 uatom O$_2$/mg for succinate, DPNH and TMPD + ascorbate respectively.
case of intact mitochondria (see Figure 19) Cu$^{2+}$ can both inhibit and activate respiration depending on the Cu$^{2+}$ concentration available. Up to about 16 n moles/mg of Cu$^{2+}$ available, respiration supported by either succinate or DPNH-linked substrates is activated to different degrees. Succinate respiration is activated 5 fold (maximally) at 16-20 n moles/mg of Cu$^{2+}$ available as compared with the control rate. \(\beta\)-hydroxy butyrate and malate + glutamate respirations are activated about two fold (maximally) at 10 n moles/mg. The respirations of succinate and of malate + glutamate is a biphasic response at higher levels of Cu$^{2+}$; respiration rate supported by either substrate is first activated (first phase) and then abruptly slowed down and becomes an inhibition phase compared with the first phase. Respiration supported by \(\beta\)-hydroxy butyrate was inhibited at high levels of Cu$^{2+}$.

\[\text{Cu}^{2+} \text{ Effect on Respiratory Control and Oxidative Phosphorylation}\]

Although the presence of phosphate at 100 mM results in much less of Cu$^{2+}$ being bound by membrane as compared to the binding in a chloride medium, a small amount of the phosphate (2 mM) in the presence of KCl (100 mM) does not affect the passive binding of Cu$^{2+}$ to mitochondria and the results of the titration of Cu$^{2+}$-induced swelling in either KCl (100 mM) or KCl (100 mM) mixed with 2 mM phosphate show identical swelling pattern. Therefore, the effect of Cu$^{2+}$ on oxidative phosphorylation and respiratory control can be studied in the presence of phosphate acceptor and their results are shown in Table 2. Cu$^{2+}$ at the level of 6 n moles/mg available completely uncouples
Figure 19. Cu²⁺ effect on succinate, DPNH-linked substrates and TMPD + ascorbate respirations in intact mitochondria. Experiments were done as described in Figure 18 except that 1) particles were replaced by intact mitochondria, 2) rotenone was used in succinate and TMPD + ascorbate respirations. Solid lines: initial phase of respiration. Dash lines: later phase of respiration.


### TABLE 2

**EFFECT OF Cu^{++} ON PHOSPHORYLATION AND RESPIRATORY CONTROL**

<table>
<thead>
<tr>
<th>Cu^{++} Conc. (n moles/mg)</th>
<th>Respiratory Substrate</th>
<th>Respiratory Rate (uatoms/min/mg) State 3</th>
<th>State 4</th>
<th>Control Ratio State 3/4</th>
<th>P/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>K Succinate</td>
<td>0.176</td>
<td>0.068</td>
<td>2.6</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td>K Succinate</td>
<td>0.184</td>
<td>0.104</td>
<td>1.8</td>
<td>1.35</td>
</tr>
<tr>
<td>5</td>
<td>K Succinate</td>
<td>0.196</td>
<td>0.176</td>
<td>1.1</td>
<td>0.75</td>
</tr>
<tr>
<td>6</td>
<td>K Succinate</td>
<td>0.204</td>
<td>0.204</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>β-OH Butyrate</td>
<td></td>
<td></td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>β-OH Butyrate</td>
<td></td>
<td></td>
<td></td>
<td>1.85</td>
</tr>
<tr>
<td>6</td>
<td>β-OH Butyrate</td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
</tbody>
</table>

Mitochondria (10 mg protein) were treated with rotenone (2 ug/mg protein) and added to 16 ml of a medium containing KCl (100 mM), Tris Cl (2 mM), K Suc. (2 mM) or β-OH butyrate (2 mM) and KPi (2 mM), pH 7.15. The respiratory rate at 25 C was recorded by a Clark-type electrode, respiratory control and P/O ratios were estimated by adding a pulse of ADP as described by Chance & Williams and the order of addition is K Suc., Cu^{2+} and ADP.
oxidative phosphorylation if K\(^+\)-succinate is used as substrate and two-thirds is uncoupled if β-hydroxy butyrate is used. It must be noted that Cu\(^{2+}\) effect on respiratory control and oxidative phosphorylation depends on the order of addition of reagents including Cu\(^{2+}\), ADP and substrates. If substrate is added first, followed by Cu\(^{2+}\) and finally ADP, the result is that both State 3 and State 4 are activated. If Cu\(^{2+}\) is added first, then ADP and finally K\(^+\) succinate, the State 4 is activated and the State 3 is inhibited as shown in trace A of Figure 20. If K\(^+\)-succinate is added first followed by ADP and finally Cu\(^{2+}\), the State 4 is also activated, but State 3 is less susceptible to inhibition as shown in trace B of Figure 20. The Cu\(^{2+}\) effect on the uncoupled rate of respiration is shown in Table 3, the uncoupled rate of respiration associated with succinate oxidation is antagonized by the addition of Cu\(^{2+}\).

**Activation of Energy-Linked Ion Transport by Cu\(^{2+}\) and Other Metals**

In conjunction with the previous report by Brierley, et al. (7, 8), the present study establishes that under proper treatment of mitochondria with various metals, the membrane will be altered and active ion transport induced. The order of activity is Cu\(^{2+}\) > Hg\(^{2+}\) > Pb\(^{2+}\) > La\(^{3+}\) > Zn\(^{2+}\) in a medium of sucrose (250 mM) and K\(^+\)-acetate (10 mM). The effects of Cu\(^{2+}\) shown in Figure 21 indicate a low concentration of Cu\(^{2+}\) (below 10 uM) activates the succinate supported ion accumulation and results in moderate swelling of mitochondria in a medium of KCl (100 mM). This active ion uptake is coupled to activa-
Figure 20. Cu$^{2+}$ effect on state 3 respiration. Mitochondria (6.25 mg of protein) were treated with rotenone (2 ug/mg protein) and added to 10 ml of a medium of KCl (10 uM), Tris Cl (2 mM), KPi (2 mM), and the order of addition of Cu$^{2+}$, ADP and K Suc. is as follows:

(A). Add Cu$^{2+}$ and evaluate the initial rate as state 3 respiratory rate.

(B). Add K Suc. first and followed by ADP (0.1 mM) and finally Cu$^{2+}$.
TABLE 3

Cu⁺⁺ EFFECT ON THE UNCOUPLED RATE OF RESPIRATION

<table>
<thead>
<tr>
<th>Cu⁺⁺ Added (uM)</th>
<th>Rate of State 4 Respiration (uatoms/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-CCP</td>
</tr>
<tr>
<td>0</td>
<td>0.068</td>
</tr>
<tr>
<td>1.25</td>
<td>0.126</td>
</tr>
<tr>
<td>2.5</td>
<td>0.180</td>
</tr>
<tr>
<td>5.0</td>
<td>0.260</td>
</tr>
<tr>
<td>8.0</td>
<td>0.280</td>
</tr>
<tr>
<td>15</td>
<td>0.207</td>
</tr>
</tbody>
</table>

Mitochondria (10 mg protein) were treated with rotenone (2 ug/mg protein) and added to 16 ml of a medium of KCl (100 M), Tris Cl (2 mM), pH 7.1 and K Suc. (2 mM). Respiratory rate at 25°C was recorded by a Clark-type electrode. The order of addition is Cu²⁺ and followed by CCP (5.3 x 10⁻⁷M).
Figure 21. Simultaneous changes in swelling, pH and respiration of isolated beef heart mitochondria induced by Cu²⁺. Mitochondria (10 mg of protein) were treated with rotenone and added to 16 ml of a medium of KCl (100 mM), Tris Cl (2 mM) and K Succinate (2.5 mM) pH 7. The measurement of O₂, pH and swelling are described in section of method.
tion of respiration and cyclic pH change. These phenomenon are similar to that induced by valinomycin (1). The Cu$^{2+}$-activated ion uptake shows a marked specificity for K$^+$. Other cations, such as Na$^+$ and tetramethylammonium, result in only slight activation (see Figure 22 and Table 4, and Figure 23). In contrast, the Cu$^{2+}$-treated membrane fails to differentiate cation permeabilities such as K$^+$, Na$^+$, Li$^+$, H$^+$ in the presence of passive and uncoupled conditions. The Cu$^{2+}$-induced active ion uptake is greatly enhanced by the addition of acetate as shown in Figure 23, 24 and Table 4.

The ability of coupling membrane to transport various cations and anions depends on the amount of Cu$^{2+}$ bound by the mitochondrion. At low level of Cu$^{2+}$ binding (below 6 ~ 7 n moles/mg), phosphate transport is blocked transiently, and energy-linked K$^+$ and permeant anion uptake is activated. At higher levels of Cu$^{2+}$ (ab. 7-14 n moles/mg), passive permeability to K$^+$, Cl$^-$, and other ions is gradually developed, energy-linked volume change of the mitochondria depends on the time of addition of Cu$^{2+}$. If Cu$^{2+}$ is added before mitochondria that have been swollen the energy-linked ion uptake is activated and the rate of initial swelling is greatly enhanced, but the magnitude of the total swelling is smaller than that observed in the passive condition. If Cu$^{2+}$ is added to a swollen mitochondrion, an energy-linked contraction with a small magnitude is observed. Cu$^{2+}$ in this concentration range seems to be able to catalyze osmotic equilibrium according to ion gradient across the membrane. At higher levels than 15 n moles/mg of Cu$^{2+}$ bound, chloride permeability is greatly enhanced.
Figure 22. The comparison of Cu$^{2+}$-activated respiration in the presence of NaCl and KCl. Mitochondria (10 mg of protein) were treated with rotenone and added to 16 ml of a medium 0.1 M KCl, 2 mM K Succinate or 0.1 M NaCl, 2 mM Tris Cl and 2 mM Na succinate. The reactions were started by addition of Cu$^{2+}$ whose concentrations are indicated in Figure. Oxygen uptake was measured polarographically by using a Clark-type oxygen electrode fitted to a water jacketed reaction vessel at 25°C. Valinomycin used is 2.5 x 10$^{-7}$M.
O2 uptake, uatoms/min per mg

100 mM KCl or NaCl
2 mM Succinate
PH 7
A: Valinomycin in KCl
B: Valinomycin in NaCl
### TABLE 4

**IONIC SPECIFICITY OF Cu\(^{2+}\)-INDUCED ACTIVE ION TRANSPORT SUPPORTED BY RESPIRATION**

<table>
<thead>
<tr>
<th>Cu(^{2+}) Added (n moles/mg)</th>
<th>Initial Rate Of Active Swelling Supported By K Succinate In Salt Solution (Δ OD/Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KCl (100 mM)</td>
</tr>
<tr>
<td>0</td>
<td>0.006</td>
</tr>
<tr>
<td>4</td>
<td>0.02</td>
</tr>
<tr>
<td>8</td>
<td>0.03</td>
</tr>
<tr>
<td>16</td>
<td>0.13</td>
</tr>
<tr>
<td>24</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>KCl (100 mM)</th>
<th>NaCl (100 mM)</th>
<th>TMACl (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KOAC (2 mM)</td>
<td>NaOAC (2 mM)</td>
<td>TMAOAc (2 mM)</td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>0.015</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>0.020</td>
<td>0.025</td>
</tr>
<tr>
<td>8</td>
<td>0.25</td>
<td>0.027</td>
<td>0.053</td>
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<tr>
<td>16</td>
<td>0.35</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>24</td>
<td>0.37</td>
<td>0.26</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Mitochondria (5 mg protein) were treated with rotenone (2 ug/mg) and suspended in the media as indicated above, 2 mM Tris Cl is also added. Reaction is started by mixing solution with 2 mM K Succinate, Na Succinate, TMA Succinate respectively at 25°C in a plexiglass cuvet mounted on Beckman Model B photometer, the initial rate of swelling was measured from the point of onset of swelling.
Figure 23. The comparison of energy-linked swelling supported by KOAc, NaOAc, KCl and NaCl induced by Cu++. Mitochondria (5 mg protein) were treated with rotenone and added to 8 ml of a medium of KCl (100 mM), Tris Cl (2 mM) and K⁺ Succinate (2 mM) pH 7, or NaCl (100 mM), Tris Cl (2 mM) and Na⁺ Succinate (2 mM). At the indicated points, KOAc (2.5 mM) or NaOAc (2.5 mM) were added. In one of the traces CCP (6 x 10⁻⁷M) is added right before Cu²⁺ (not shown). Absorbance at 546 mu was recorded with an Eppendorf photometer and reaction is carried out at 25°C.
100 mM KCl

K Suc. → KOAc → Cu²⁺ (8 uM) → +KOAc & CCP

A₅₄₆

0.4

1 Min.

100 mM NaCl

Na Suc. → NaOAc → Cu²⁺ (8 uM) → +NaOAc & CCP

A₅₄₆

0.4

1 Min.
Figure 24. The comparison of the swelling induced by Cu$^{2+}$ in the presence and absence of energy in KCl + KOAc or NaCl + NaOAc. The experimental conditions were identical as described in Figure 23.

A: K succinate (2.5 mM) + CCP (6 x 10^{-7}M) in KCl (0.1 M), Tris Cl (2 mM) pH 7.
B: K succinate in suspending medium indicated in A.
C: K succinate (2.5 mM) in KCl (0.1 M), Tris Cl (2 mM) and KOAc (2.5 mM) pH 7.
D: K succinate (2.5 mM + CCP (6 x 10^{-7}M) in the suspending medium as indicated in C.
Swelling (A546 in 15 min)

100 mM KCl
±2.5 mM KOAc

100 mM NaCl
±2.5 mM NaOAc

Cu²⁺ (µM)

Swelling

Cu²⁺ (µM)
Cu$^{2+}$-induced swelling is strictly dependent on metabolic states of mitochondria as shown in Table 5 and Figure 24. The initial rate of swelling in a KCl medium induced by Cu$^{2+}$ in respiring mitochondria is greater than that in non-respiring particles and still greater than uncoupled mitochondria. In the presence of a permeant anion, active swelling is greatly enhanced while passive swelling and uncoupled swelling are not affected as far as both rate and extent are concerned. In the later phase of swelling in KCl, the magnitude of swelling induced by Cu$^{2+}$ is passive > uncoupled > active. The energy-linked cation and permeant anion uptake is blocked by uncoupler, respiratory inhibitor, and by oligomycin if supported by ATP. The energy-linked ion uptake induced by Cu$^{2+}$ can also be abolished by an equimolar concentration of EDTA, antabuse and diethyldithiocarbamic acid but not by iodoacetamide, N-ethylmaleimide, I$_2$, or cysteine at 4 folds the Cu$^{2+}$ concentration.

Comparison of the Ion Movement and of Respiratory Activity Induced by Cu$^{2+}$, Valinomycin, and Uncouplers

It has been well established that valinomycin and uncouplers can conduct K$^+$ and H$^+$ respectively through the inner membrane of mitochondria. Although Cu$^{2+}$ can also alter the membrane to H$^+$ permeability, it closely resembles valinomycin in many of its responses.

Figure 25 shows the effect of Cu$^{2+}$ on respiration, H$^+$ movements, and swelling as compared with valinomycin and CCP. The results indicate that Cu$^{2+}$, valinomycin, and CCP all activate respiration. Valin-
Mitochondria (5 mg protein) were treated with (1) Rotenone (2 ug/mg) and CCP (6 x 10^{-7}M) (2) Rotenone (ug/mg) (3) Rotenone (2 ug/mg) and K Succinate (2 mM) and suspend in the media as indicated in Table. The swelling was carried out at 25°C in a stirred plexi-glass cuvet mounted on a Beckman Model B photometer, the initial rate of swelling was measured from the point of onset of swelling.
## TABLE 5

**Cu$^{2+}$ INDUCED SWELLING AT VARIOUS METABOLIC STATES**

<table>
<thead>
<tr>
<th>Suspending Media</th>
<th>Cu$^{2+}$ Added (n moles/mg)</th>
<th>Initial Rate of Swelling ($\Delta$ OD/Min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM)</td>
<td>0</td>
<td>0.005</td>
<td>0.005</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM)</td>
<td>4</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM)</td>
<td>8</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2mM)</td>
<td>16</td>
<td>0.04</td>
<td>0.08</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>24</td>
<td>0.05</td>
<td>0.12</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>0</td>
<td>0.004</td>
<td>0.007</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>4</td>
<td>0.01</td>
<td>0.02</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>8</td>
<td>0.01</td>
<td>0.02</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>16</td>
<td>0.03</td>
<td>0.11</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>KCl (0.1M) + Tris Cl (2 mM) + KOAc (2 mM)</td>
<td>24</td>
<td>0.05</td>
<td>0.17</td>
<td>0.37</td>
<td></td>
</tr>
</tbody>
</table>
Figure 25. Comparison of the change of respiration, pH and swelling induced by Cu$^{2+}$, valinomycin and CCP. Experiments were carried out as described in Figure 21. Valinomycin $2 \times 10^{-7}$M, CCP $6 \times 10^{-7}$M were used.
Mito

Cu++, Val.
or CCP.

100 mM KCl, pH 7
2 mM Succinate
(A) Cu++ 3 uM
(B) Cu++ 5.5 uM
(C) Cu++ 10 uM

4 uatom

O₂

Mito.

Cu++, Val.
or CCP.

Val.

H⁺

300 nM

Mito.

Cu++, Val.
or CCP.

CCP

CCP

A₅₄₆

0.25

Val.

2 Min.
omycin induces an oscillatory volume change (168). Cu²⁺ cause only swelling but not contraction and CCP does not induce any swelling in KCl (100 mM). An acidification of the medium due to the ionization of Cu(NO₃)₂ itself is observed when Cu²⁺ is added to the mitochondria suspended in a slightly buffered KCl solution. However, a characteristic, respiration-dependent H⁺ movement associated with oxidation of substrates (succinate, β-hydroxy butyrate, malate + glutamate, or endogenous substrates) is also produced by Cu²⁺ (see Figure 25 and Table 6). Cu²⁺-catalyzed H⁺ movement depends on the concentration of Cu²⁺ used. At low levels of Cu²⁺ (about up to 6 - 7 n moles/mg) mitochondria can eject 10-20 n moles of H⁺/mg of protein within 1-2 minutes and no consecutive H⁺ uptake is allowed until anaerobic point where 20-30 n moles/mg of H⁺ is absorbed. At higher levels of Cu²⁺ (above 8 uM), mitochondria rapidly eject H⁺ and this H⁺ efflux is followed by a rapid uptake (trace of Figure 25). The maximum H⁺ ejection is about 40-45 n moles/mg. At still higher levels of Cu²⁺, mitochondria can take up more H⁺ after the initial H⁺ ejection, and absorption of H⁺ during the anaerobic is abolished.

In the presence of CCP (3.4 x 10⁻⁷M) which is added before Cu²⁺, the respiratory-linked H⁺ movement induced by Cu²⁺ or valinomycin will also be inhibited (Figure 26, trace A); however, if CCP is added right after the H⁺ cycle (that is in the reuptake H⁺ phase), CCP can conduct 3 times the amount of H⁺ movement (Figure 26, trace B). These phenomena are completely identical to that caused by valinomycin (Figure 26, trace E).
**TABLE 6**

**TRANSIENT RESPIRATORY PROTON MOVEMENT**
**INDUCED BY Cu^{++}**

<table>
<thead>
<tr>
<th>Cu^{++} Added (uM)</th>
<th>Transient Cyclic pH Change (n moles/10 mg/cycle)</th>
<th>H^{+} Uptake (Anaerobiosis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H^{+} Ejected</td>
<td>H^{+} Reabsorbed</td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>5.5</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td>440</td>
<td>440</td>
</tr>
<tr>
<td>10</td>
<td>435</td>
<td>530</td>
</tr>
<tr>
<td>15</td>
<td>440</td>
<td>620</td>
</tr>
<tr>
<td>20</td>
<td>440</td>
<td>665</td>
</tr>
<tr>
<td>30</td>
<td>435</td>
<td>680</td>
</tr>
</tbody>
</table>

Transient respiratory H^{+} movement induced by Cu^{2+}. Mitochondria (10 mg protein) and added to a medium of KCl (0.1 M), Tris Cl (2 mM), K Succinate (2 mM) pH 7. The Cu^{2+} was added in different concentration as shown in Table. The H^{+} ejected (or OH^- absorbed) is taken as the maximal acidification (at the peak of pH trace see Figure 25 example) occurred in the span of pH cycle (1 - 2 minutes). H^{+} re-absorbed (or OH^- ejected) is taken as the maximal alkalization during the pH change (at the trout of pH trace).
Figure 26. Interrelationships of the respiration dependent-pH changes induced by Cu$^{2+}$, valinomycin and CCP. Mitochondria (10 mg of protein) were treated with rotenone and added to 16 ml of a medium of KCl (0.1 M), Tris Cl (2 mM), K succinate (2 mM). The order of addition of Cu$^{2+}$ (8 uM), valinomycin (2 x 10$^{-7}$M) and CCP (6 x 10$^{-7}$M) were indicated in this figure. The reactions were carried out at 25°C in a stirred plexiglass cuvet equipped with a Clark-type oxygen electrode and glass electrode. The traces of oxygen uptake were not shown in the Figure.
100 mM KCl
2 mM Succinate
PH 7

**A)** CCP

**B)** Cu++ or Val.

**C)** O2=0

**D)** Cu++

**E)** Val.

**Mito.**
If valinomycin (1 - 2 x 10^-7 M) is given before Cu^{2+}, the respiratory-linked pH change induced by Cu^{2+} is also inhibited (Figure 26, trace C), and activation of respiration by valinomycin affected by addition of Cu^{2+}. The reversed order of additions show valinomycin also fail to induce cyclic pH change, respiratory rate activated by Cu^{2+} is enhanced at this time (Figure 26, trace D).

In the presence of CaCl2 (50-100 uM) mitochondria can eject a great amount of H^+. This ejected H^+ can be completely reabsorbed by addition of either Cu^{2+}, valinomycin, or CCP (Figure 27). In those cases the respiratory activations by either valinomycin, Cu^{2+}, or CCP are slowed down and swelling are also decreased.

It must be noted that Ca^{2+} fail to cause ejection of H^+ if mitochondria is first given by either Cu^{2+}, valinomycin or CCP (Figure 28 and compare 27).

In the presence of permeant ions including Na^+, OAc^-, H_2PO_4^-, the magnitude of cyclic pH change is decreased to range from half to one third of the original level (Table 7).

**Cu^{2+}-Induced ATPase Activity**

Elevated rates of ATPase activity have been observed in KCl medium at extremely low levels of Cu^{2+} (about 2 n moles/mg available). The activity reaches a maximum at about 6 - 7 n moles/mg of Cu^{2+}, at higher levels of Cu^{2+} the ATPase activity is greatly inhibited (Figure 29). Cu^{2+}-induced ATPase shows marked specificity for K^+ ion (Table 8 and Figure 29), Na^+ can support only about 20-40% of the value obtained
Figure 27. Alkalinization of Ca$^{2+}$-dependent H$^+$ ejection by Cu$^{2+}$, valinomycin or CCP. Mitochondria (10 mg of protein) were treated with rotenone and were added to a medium of KCl (0.1 M), Tris Cl (2 mM) and K$_2$ succinate pH 7. Ca$^{2+}$ (50 uM) was added at the point indicated. Cu$^{2+}$ (10 uM), valinomycin (2 x 10$^{-7}$M), or CCP (6 x 10$^{-7}$M) were added at A point as shown respectively.
Fig. 28.--Inhibition of Ca\textsuperscript{2+}-dependent H\textsuperscript{+} production by Cu\textsuperscript{2+}, valinomycin and CCP. Experiments were carried out at the same condition as described in Fig. 27 except that the order of addition of Ca\textsuperscript{2+} and Cu\textsuperscript{2+} (or valinomycin or CCP) was used.
### TABLE 7
RESPIRATORY CYCLIC pH CHANGE INDUCED BY Cu^{2+} IN THE PRESENCE OF THE DIFFERENT IONS

<table>
<thead>
<tr>
<th>Media In The Presence Of 10 μM Cu^{2+}</th>
<th>Cyclic pH Change (n moles/10 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H^+ Ejected</td>
</tr>
<tr>
<td>KCl (100 mM)</td>
<td>400</td>
</tr>
<tr>
<td>KCl (100 mM) + KPi (1.25 mM)</td>
<td>270</td>
</tr>
<tr>
<td>KCl (100 mM) + KPi (2.5 mM)</td>
<td>300</td>
</tr>
<tr>
<td>KCl (100 mM) + KPi (5 mM)</td>
<td>240</td>
</tr>
<tr>
<td>KCl (100 mM) + KOAc (1.25 mM)</td>
<td>220</td>
</tr>
<tr>
<td>KCl (100 mM) + KOAc (2.5 mM)</td>
<td>150</td>
</tr>
<tr>
<td>KCl (100 mM) + KOAc (5 mM)</td>
<td>110</td>
</tr>
<tr>
<td>KCl (100 mM) + MgCl_2 (30 μM)</td>
<td>225</td>
</tr>
<tr>
<td>KCl (100 mM) + MgCl_2 (60 μM)</td>
<td>180</td>
</tr>
<tr>
<td>KCl (100 mM) + CaCl_2 (50 μM)</td>
<td>315</td>
</tr>
<tr>
<td>KCl (100 mM) + CaCl_2 (100 μM)</td>
<td>200</td>
</tr>
<tr>
<td>NaCl (100 mM)</td>
<td>220</td>
</tr>
</tbody>
</table>

Respiratory cyclic pH change induced by Cu^{2+} in the presence of the different ions. Experiments were carried out as described in Table 6 except several different suspending media were used. H^+ ejected as absorbed have been corrected for buffer capacities in each system.
Fig. 29.—The ATPase activities induced by Cu$^{2+}$, CMS and DNP in KCl or NaCl solution in the presence of rotenone. Mitochondria (0.5 mg of protein) were treated with rotenone (1 ug/mg) and added to a final volume of 0.8 ml of 100 mM KCl or NaCl containing Tris Cl (2 mM) and the indicated amount of Cu$^{2+}$, DNP (100 mM) and CMS (75 mM). The reaction mixture was allowed to incubate for 2.5 minutes, and the reaction was started by addition of 2.5 mM di-NaATP neutralized with 0.1 M KOH or NaOH at 0°C and terminated after 4 minutes at 25°C by addition of 4.5 ml of silicotungstate-sulfuric acid. Pi was determined by the method of Linberg and Ernster.
TABLE 8
CHARACTERISTIC OF Cu++-INDUCED ATPase ACTIVITY

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATPase Activity In</th>
<th>KCl (100 mM)</th>
<th>NaCl (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>u moles Pi/mg/4 Min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Rot. (2 ug/mg)</td>
<td>0.16</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>(2) Cu++ (6 n moles/mg) + Rot.</td>
<td>1.67</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>(3) DNP (100 n moles/mg) + (2)</td>
<td>0.80</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>(4) Oligomycin (2 ug/mg) + (2)</td>
<td>0.08</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>(5) CMS (120 n moles/mg) + (2)</td>
<td>1.83</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>(6) DNP (100 n moles/mg) + (1)</td>
<td>0.71</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>(7) CMS (120 n moles/mg) + (1)</td>
<td>0.75</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>(8) CMS (120 n moles/mg)</td>
<td>1.25</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

Mitochondria (0.5 mg of protein) were treated with rotenone (2 ug/mg) and added to a final volume of 0.8 ml of 100 mM KCl or NaCl containing Tris Cl (2 mM) and the indicated amounts of Cu^{2+}, DNP and CMS (runs 2, 6, 7). The reaction mixtures were allowed to incubate for 2.5 minutes runs (3) (4) (5), let mitochondria react with Cu^{2+} first for 1 minute and then add reagents as indicated and total incubation time is also 2.5 minutes. The reaction was started by addition of 2.5 mM di-NaATP neutralized with 0.1 M KOH & NaOH at 0°C and terminated after 4 minutes at 25°C by addition of 4.5 ml of silicotungstate-sulfuric acid, Pi was determined by the method of Linberg and Ernstner.
in the presence of K⁺. The activity is sensitive to both oligomycin and uncouplers (Table 8). Oligomycin can completely inhibit Cu²⁺-induced ATPase activity, whereas uncouplers only partially diminish this activity. Mercurial reagents such as CMS potentiate Cu²⁺-induced ATPase. Elevated rate of ATPase activity induced by Cu²⁺ accompanied by mitochondrial swelling. If higher levels of Cu²⁺ are available, the mitochondria swell extensively in KCl and ATPase activity is inhibited.

ATPase activity is enhanced by respiration and consequently is decreased by the presence of inhibitors. This can be seen in the kinetic study of ATPase activity using pH traces in the presence and absence of the respiration (see Figure 31-33).

This study established that (a) Cu²⁺-induced ATPase activity is quite similar to the CMS-induced ATPase reported by Brierley et al. (11), and (b) Cu²⁺ can activate ATPase at lower levels than CMS does, as the former required only 6 n moles/mg available (about 4-5 n moles/mg bound) to induce ATPase maximally, while CMS requires about 4 times as much to induce the same activity.
Fig. 30.--The ATP-supported swelling induced by Cu$^{2+}$ in KCl solution. Mitochondria (5 mg protein) were treated with rotenone (2 ug/mg) and added to a medium consist of KCl (100 mM), Tris Cl (2 mM), pH 7. The orders of additions were indicated in figure.
Figure 31. Inhibition of Cu$^{2+}$-dependent and of CMS-dependent ATPase by rotenone. Mitochondria (10 mg of protein) were suspended in a medium consisted of KCl (0.1 M) and Tris Cl (2 mM) pH 7. The orders of additions of rotenone (2 ug/mg), CMS (70 uM), Cu$^{2+}$ (4 uM), ATP (1.5 mM) were shown in this figure. The reactions were carried out at 25°C in a stirred plexiglass cuvet equipped with a glass electrode. The ATPase activity was followed by the pH trace where an acidification represents the hydrolysis of ATP by ATPase.
Mito. +Rot.  CMS (70 μM)

Mito. ±Rot. Cu (4 μM)

2 Min.

ATP (1.5 mM)

+Rot.  200 nmoles/mg

−Rot.  200 nmoles/mg

H^+  200 nmoles/mg
Figure 32. Respiration-dependent, Cu$^{2+}$-induced ATPase activity. Experiments were carried out in a similar condition as described in Figure 31 except that at A point where three conditions were varied and indicated in Figure. K succinate (2 mM), antimycin (2 ug/mg), rotenone (2 ug/mg), ATP (1.5 mM), Cu$^{2+}$ (2.5 uM) were used in these experiments.
Mito. + Rot. A Cu^{++} (2.5 \mu M)

ATP (1.5 \text{ mM})

+ Antimycin At A

No Addn At A

+ KSuc. At A

200 nmoles/mg

200 nmoles/mg

200 nmoles/mg

H^+

H^+

H^+

2 Min.

H^+
Figure 33. Inhibition of Cu$^{2+}$-induced ATPase by CCP. Experimental conditions were similar to Figure 31 except that 1) rotenone was omitted at the beginning, 2) CCP (6 x 10$^{-7}$M) was added or omitted at the point indicated.
DISCUSSION

The ion-translocating properties of the inner mitochondrial membrane are of great importance in relation to energy coupling and membrane phenomena. Therefore, the permeability of the mitochondrial membrane to various ions has become a subject of great interest, especially since the chemiosmotic hypothesis was formulated (82, 169, 170). A number of different chemical modifications of the mitochondrial membrane result in activation of energy-linked ion transport and its related reactions (7, 8, 11, 13, 78, 81, 171-175). The present study, an extension of previous investigations, establishes that metals have high affinity to interact with the mitochondrial membrane and result in the alteration of several membrane-linked functions including passive permeability, active ion transport, oxidative phosphorylation, electron transport and ATPase activity.

Passive Permeability Induced by Heavy Metals

Ions including $H^+$, $K^+$, $NH_4^+$, and $Cl^-$ are impermeable to the inner mitochondrial membrane. This diffusion barrier of the inner mitochondrial membrane to those ions, however, will be altered and reduced in the presence of metals. The permeability of cations and anions can be differentially induced by different metals including $Cu^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $La^{3+}$, $Hg^{2+}$ and $Cd^{2+}$ as shown in Figure 3-6. For
example, a given amount of Pb\textsuperscript{2+} interaction with mitochondria results in increased K\textsuperscript{+} permeability as compared with other ions. Likewise, a given amount of La\textsuperscript{3+} stimulates a greater amount of Cl\textsuperscript{-} permeability compared with other ions. Cd\textsuperscript{2+} enhances a greater H\textsuperscript{+} permeability than other ions, Cu\textsuperscript{2+} induces cation and anion permeabilities to nearly the same extent, and Zn\textsuperscript{2+} fails to induce permeability to either cations or anions passively. Heavy metals were also reported to disturb the ion fluxes through various membranes including erythrocytes (149), red cell (147), yeast cells (148), and frog skin (157).

The mechanism of the selective permeation of ions into the mitochondrial membrane is not well established. The results of the experiments of pH profiles of osmotic swelling patterns induced by metals (Figures 2, 7-10) and binding studies of metals including Cu\textsuperscript{2+}, Pb\textsuperscript{2+} and Zn\textsuperscript{2+} (61) to intact mitochondria and to its fractional preparations as shown in Figures 14 and 15 suggested that this selective transmembrane transport of those ions could conceivably arise from the interaction of metals with different membrane ligands that play different roles in maintaining the membrane integrity. After a selective alteration of this highly organized membrane by different metals, the affinity and interfacial tension between protein and lipid interaction could be reduced and may result in the exposure of different lipids to the bulk phase of medium. The polar heads of phospholipid which may confer different turn-over rates to each ion would result in different rates of translocation for each ion. The work of artificial membrane system reported by Lehninger (176), Bangham and Papahadjopoulos
and their co-workers (177-180) and Andreoli and Tosteson (181) have established that cations will be translocated more rapidly through a membrane made of basic phospholipids as compared with those made of other phospholipids; this membrane will also show relative specificity to cations. In contrast anions will pass through membrane made by acidic phospholipids more rapidly, and the uncharged or amphitatic lipids will not discriminate between cations and anions. Danielli (182) has pointed out that anions will only pass freely on the acidic side of the isoelectric point of the protein film, cations only on the basic side and a film without sufficient ionizable groups or at its isoelectric point will not be able to raise the electrical potential and will tend to leak out the wrong ions. Based on the above observations, one might say that those heavy metals which can induce increased cation permeability as compared with anion such as Pb\(^{2+}\) will either expose the basic phospholipid or block acid phospholipid by complexation of its polar head. Likewise, those metals interact with protein in such a way that can expose acid phospholipid and block basic phospholipid by complexation will preferably induce anion permeability such as La\(^{3+}\). The metal which reduces both cation and anion to the same extent such as Cu\(^{2+}\) will either possess no selectivity to attack lipids or can interact with protein resulting in exposure to neutral lipid. Those suggestions are quite compatible with the observation of McGivan and Chappell (183), and Chappell and co-worker (184) that the incorporation of cardiolipin into phospholipid micelles reduced anion penetration.
Active Ion Transport Induced by Heavy Metal

Interest in ion transport by mitochondria was aroused by the observation that massive amounts of calcium could be accumulated by the unique energy relationships which prevail for support of the uptake (187, 188). A variety of agents (11, 13, 72, 78, 189, 190) are capable of inducing rapid uptake and release of monovalent and divalent cation by mitochondria, and recently the carrier-mediated transport of di- and tricarboxylic acids into mitochondria has come to light (191-192). A number of reports dealt with cation and anion transport in mitochondria have been reviewed by Lehninger et al. (201) and by Lardy et al. (202).

The present study establishes that when the mitochondrial membrane is altered by metals to a certain critical extent, active ion transport will be activated. The amounts of metals required to induce active ion transport is dependent on the metal used, and is always less than that required to induce passive permeability. Cu$^{2+}$, Pb$^{2+}$, La$^{3+}$, Zn$^{2+}$ can induce active ion transport at different concentration levels. The order of potency for activation of active ion transport supported by TMPD and ascorbate is Cu$^{2+}$ > Hg$^{2+}$ > Pb$^{2+}$ > La$^{3+}$ > Zn$^{2+}$ when a medium of sucrose (0.25 M) and K acetate (10 mu) is used.

The fact that active ion transport can be induced by using smaller amounts of each metal as compared with passive permeability suggests that the external site (or sites) of the inner mitochondrial membrane is involved in inducing active ion transport. This is consistent with the studies of mercurials-induced ion transport reported by Brierley,
Scott and co-workers (9, 11). Additionally, the observation that most of the metal can induce active ion transport at the different levels of binding suggests that there is a specific site presumably located on the external surface of the inner membrane that is most intimately linked to the active ion uptake. This site could be commonly reached by metals at different levels. The most active metal that is capable of inducing active ion transport must possess good chemical affinity to react with this site without interfering with the energy generation mechanism built in the membrane. The less potent metal would either saturate the non-specific site before being able to gain access to the active site or attack the energy system itself. Cu$^{2+}$, for example, at only 5 n moles/mg bound by the membrane can induce active ion uptake. Organic mercurials require about 15-20 n moles/mg for the same reaction (11). Zn$^{2+}$ and Pb$^{2+}$ also require much higher levels than that of Cu$^{2+}$ to induce this effect in a medium of KCl (100 mM) (72, 73). The differential affinity of metals toward this active site and other membrane components associate with its special biological responses will gain information and stimulate reassessment of many mitochondrial phenomena. The properties of active ion transport induced by Zn$^{2+}$ (173), mercurials (11, 13) and Pb$^{2+}$ (72) have been previously reported by Brierley and co-workers.

The present study established that the alteration of Cu$^{2+}$ bound to the membrane. At low level of Cu$^{2+}$ (below 6 n moles/mg available), energy-linked cation and permeant anion uptake were activated. This energy-linked ion accumulation can be supported by either ATP or
several substrates including succinate, TMPD + ascorbate, malate + 
glutamate and β-hydroxy butyrate (Figures 21, 23, 25, 30). In con-
trast the active ion uptake induced by other metals such as Zn$^{2+}$, 
mercurials can use only TMPD + ascorbate or ATP as energy sources; 
while Pb$^{2+}$-induced active ion uptake is favorably supported by TMPD 
+ ascorbate and DPNH-linked substrates. It must be noted that Cu$^{2+}$ 
+ induced active ion uptake show specificity on K$. This active ion 
uptake induced by Cu$^{2+}$ at low level results is the uncoupling of 
oxidative phosphorylation activation of ATPase activity, and ejection 
of H$^+$ into the medium; however, the active ion uptake induced by 
higher level of Cu$^{2+}$ (7 - 14 n moles/mg) is accompanied by cyclic pH 
change and inhibited the activated-ATPase.

The observed effect at low levels of Cu$^{2+}$ might suggest that 
Cu$^{2+}$ triggers an ATPase linked H$^+/K^+$ exchange. The component responsible for the catalysis of this exchange can only be activated at the 
expense of a pH gradient. The component activated by Cu$^{2+}$ and OH$^-$ 
gradient is sensitive to uncoupler but insensitive to oligomycin in 
the absence of ATP. This component could be the candidate of polar 
channel that functions as a transporter of both Ca$^{++}$ and K$^+$. 
This polar channel will be discussed in detail in the following para-
graphs. The observed effect at higher levels of Cu$^{2+}$ might possibly 
be due to an additional binding of Cu$^{2+}$ to membrane besides the first 
site attacked by low level of Cu$^{2+}$. This additional binding would 
induce not only ejection of H$^+$ as observed at low level of Cu$^{2+}$ but 
also an alkalization of medium. This is conceivably due to a
reversible $H^+/K^+$ exchange, or due to a $OH^-$ leakage in the high level of $Cu^{2+}$ bound. It must be noted that $Cu^{2+}$-induced cation uptake is quite similar to the valinomycin-induced reaction in terms of ion specificity, pH change, and modes of action toward uncoupler and $Ca^{2+}$ as shown in Figures 21, 22, 25, 28.

The mechanism of active ion transport in terms of the chemiosmotic hypothesis and chemical hypothesis have been reviewed by Greville (185) and Moore (186). The mode of action of inducing active cation uptake by various agents remained to be established. The carrier-mediated (81, 193) and transmembrane movement of $K^+$ in response to potential gradient (82) have been suggested by several investigators.

It is reasonable to assume that if an ion will be translocated rapidly by membrane, this ion must not only have a high intrinsic mobility but also have a good initial process being absorbed by ligands followed easy desorption from ligands and escaped from back absorption. This simple ligand catalysis in the membrane based on the coupling between the process of diffusion and chemical reaction in the response to membra potential seems to play a regulatory effect on the process of solute permeation. The work of artificial membranes including lipid-bilayer and liposome have clearly demonstrated that the polar head of membrane lipid are involved in ion translocation. Protein will also be able in some way to facilitate or retard this process.

Based on the mass action, Coulombic law, differential mobility and several other chemical and physical factors, the roles of polar groups involved in the ligand catalysis could be predicted and have
been briefly discussed in the section of passive permeability. However, it must be emphasized that those rules will be complicated by the interaction occurring in the boundary of protein and lipid layer such as charge neutralization, hydrogen bonding, hydrophobic interaction, complexation of metal (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}) and participation of cholesterol etc.

As in Mitchell's hypothesis, the respiration-driven pH gradient will be formed across the coupling membrane. If this imposed alkaline gradient can be partly transmitted to an array of ionogenic ligands located in the inner side of coupling membrane, and result in vectorial arrangement of ligands with a scalar basicity, that is, the most basic site is arranged inward, the weakest basic site outward, and flanked by a medium basic site. Now, if a cation going through this permeation channel from outside to inside, the diffusion rate will be greatly accelerated due to the cooperative ligand catalysis and the assistance of the electrochemical gradient located inside the inner membrane provided that the initial barrier can be reduced by several reagents such as Cu\textsuperscript{2+}, CMS, Zn\textsuperscript{2+} and detergent. In this case the energy-linked cation transport will be induced. The diffusion of cation from the opposite direction in this channel, however, will be largely retarded unless the polarity of this polar channel have been reversed or diminished. The reversal or elimination of the original negative polarity of negative channel could conceivably be obtained by the addition of H\textsuperscript{+} conducted by uncouplers. The channel with positive polarity could also be developed in response to a proton
ejection through the coupling membrane, since the differential protonation of membrane ligands will be expected. This positive channel may function as a anion-facilitated carrier and enhance anion translocation. In short the mitochondrial membrane is organized in an anisotropic loops (82), the asymmetric scalar distribution of $H^+$ and $OH^-$ to membrane could be obtained. As a result of this distribution the membrane ligands located on a cooperative entity will be developed as a channel or carrier with a different polarity. This could be obtained by the aid of either conformational or configurational change of membrane ligands such as transition of lipid-bilayer to liposome.

**Oxidative Phosphorylation Affected by Cu$^{2+}$**

The question of how oxidation-reduction energy is coupled to phosphorylation of ADP, active ion transport and other energy-linked reactions has remained a subject of debate and controversy. It seems clear that oxidative phosphorylation results from several concerted actions including the $F_1$ ATPase located on the matrix side of the inner membrane (194, 195, 196), the mercurial-sensitive phosphate transporter (12, 44), the atractyloside-sensitive adenine nucleotide transporter system (197), substrate transporter (192), and respiration-driven pH gradient (82). The perturbation of any actions mentioned above will result in the impairment of oxidative phosphorylation. The present study established that extremely low levels of Cu$^{2+}$ bound by the membrane will uncouple oxidative phosphorylation. The simultaneous studies of respiration, phosphorylation and swelling indicated
that Cu\(^{2+}\), similar to valinomycin, will activate energy-linked K\(^+\) uptake. Therefore, the energy generated by the oxidation of substrate is primarily used in ion transport and the phosphorylation is uncoupled in the presence of Cu\(^{2+}\). This observation may explain the Cu\(^{2+}\) action in mitochondria reported by Verity and Gambell (34). They showed that Cu\(^{2+}\)-induced swelling is coupled by the alteration of the respiratory activity.

Effect of Cu\(^{2+}\) on respiration supported by succinate, or other substrates is characterized by the increase of the rate of State 4 respiration, but the State 3 respiration remained insensitive at the low level of Cu\(^{2+}\). At higher levels of Cu\(^{2+}\), the State 3 respiration is inhibited and State 4 respiration is still activated, but this activated State 4 respiration is then inhibited in the later phase of respiration. Therefore, the whole process of State 4 respiration is a biphasic response (Figure 19). The prevalent activation of State 4 respiration in response to Cu\(^{2+}\)-induced cation uptake may suggest that State 4 is the condition of maximal conservation of energy and is associated with the K\(^+\) movement predominately.

**Cu\(^{2+}\) Effect on Mitochondrial ATPase**

It has been known that DNP, mercurials and other thiol group reagents have profound effects on mitochondrial ATPase (11, 198-199). The present study establishes that ATPase activity of beef heart mitochondria were first stimulated then inhibited as a result of Cu\(^{2+}\) titration and a similar response to CMS and to DNP were seen. A
different affinity of those reagents toward the mitochondrial ATPase, however, can be observed. \(\text{Cu}^{2+}\) at 4-5 n moles/mg bound by membrane can maximally activate ATPase, while CMS required four times as much to induce the same activity and DNP required even higher concentration to do so. \(\text{Cu}^{2+}\)-induced ATPase activity is quite similar to the CMS-induced ATPase but different from DNP-induced ATPase (11). \(\text{Cu}^{2+}\)-induced ATPase activity is potentiated by mercurial reagents such as CMS but is antagonized by DNP as shown in Table 8. The CMS-induced ATPase, as well as \(\text{Cu}^{2+}\)-induced ATPase is linked to the ion movement across the coupling membrane and shows specificity to \(K^+\); while DNP-induced ATPase does not.

When mitochondria are exposed to metals such as \(\text{Cu}^{2+}\), a sequence of interaction can be expected starting from the outside of the membrane proceeding inward. That is, the first reaction between the \(\text{Cu}^{2+}\) and the mitochondria showed to take place with those external or peripheral ligands of membrane for which \(\text{Cu}^{2+}\) possess a chemical affinity. Low level of \(\text{Cu}^{2+}\) (4-5 n moles/mg bound) can interact with a specific fraction of ligands which responsible for ATPase activity. These ligands are presumably a few of the external thiol groups based on the fact that the similarity of ATPase activity induced by either \(\text{Cu}^{2+}\) or CMS. However, it is interesting to know that \(\text{Cu}^{2+}\) seems only to react to a fraction of the thiol groups that CMS can attack and yet both reflect a similar ATPase activity. CMS at the level which activates ATPase activity also inhibit both succinate and DPNH-linked substrates respiration. In contrast \(\text{Cu}^{2+}\) at the level to induce
ATPase activity does not inhibit the respiration as mercurial does. This leads to the suggestion that CMS preferentially attack some of the thiol which responsible for respiration and then proceed to interact with other thiols and result in an elevation of ATPase activity; Cu^{2+} appears to be able to attack the thiol classified as the latter category. Cu^{2+} does not seem to saturate the thiol groups at the external side with that CMS can react. Cu^{2+}, therefore, can pass through part of the external thiol and then proceed to react with the internal thiol which CMS can reach. A study of CMS effect on Cu^{2+}-binding to mitochondria indicated that CMS fails to inhibit Cu^{2+} high affinity site but CMS can block Cu^{2+} low affinity site. The facts that CMS can potentiate Cu^{2+}-induced ATPase and that CMS fails to inhibit Cu^{2+} high affinity site suggest that both Cu^{2+} and CMS at the level studied could be allosteric effectors; both attack the different ligands which allosterically affect the active site involving in ion transport or oxidative phosphorylation.

The Mode of Action of Heavy Metals

Metal ions have a high affinity for the polar groups of the membrane, and can interact with those polar ligands in several ways. First, metal ions interact with membrane thiols by mercaptide formation (35) or catalyze the thioldisulfide exchange that are important to maintain the mitochondrial membrane structure and function. Second, metal ions can form a complex with nucleophilic ligands with a different stability. It has been demonstrated by Gurd and co-workers
(204-207) that Cu\(^{2+}\) can form a far-ranged complex among the ligands located on several consecutive peptides and introduces a strain into the metal-ligand complex. Thus, the complexation may finally result in an entatic membrane (208), a state of tension, distortion, or stretch. Third, metal ions can open the pore in the membrane by changing the solubility of solvation of membrane. For example, the interaction or binding between the phosphate of the membrane and Pb\(^{2+}\) ion, or between the carboxylic group of membrane and calcium ion may result in either the decrease of the hydrophilicity of the membrane in the polar environment or the precipitation of ligand groups as a metal salts. Therefore, the size of polar pore will be increased as the result of this interaction. Fourth, metal ions can be absorbed on surface and catalyze the chemical reaction including oxidation-reduction, hydrolysis, addition, acid-base equilibrium. Fe\(^{2+}\), for example, catalyzes the peroxidation of unsaturated fatty acid, La\(^{+++}\) hydrolyzes the anhydride bond of polyphosphate. Data present here are insufficient to specify the mode of action for each metal with certainty. However, it seems clear that the mode of action of the metal is dependent on the level of metals used, the reactivity and polarity of the metal, the order of the affinity of ligands for the metal and the location of those ligands on the membrane.
REFERENCES


104. 2nd. Annual Conference on Trace Substances in Environmental Health Proceeding, Univ. of Missouri, Columbia, Missouri (1968).


122. Robinson, J. D., Biochemistry, 6, 3250 (1967).

152. Casteels, R., Amer. J. Digest Disease NS, 12, 231 (1967).


Metal ions have a high affinity for the mitochondrial membrane and the interaction of certain heavy metals with the membrane results in alteration of several membrane associated functions. These include passive permeability to monovalent ions, active ion transport, oxidative phosphorylation, electron transport and ATPase activity.

The diffusion barrier of the coupling membrane to ions such as $H^+$, $K^+$, $Cl^-$, $NH_4^+$ is reduced in the presence of metals, and the passive permeability of cations and anions can be differentially induced by some of the different metals studied. A given amount of $Pb^{2+}$ interaction with the mitochondrion, for example, results in increased $K^+$ permeability as compared with $Cl^-$ and other ions. $La^{3+}$ induces a greater amount of $Cl^-$ permeability compared with other $K^+$ and other ions, whereas a given amount of $Cd^{2+}$ enhances a greater $H^+$ permeability as compared to other ions. $Cu^{2+}$ induces cation and anion permeabilities to nearly the same extent and $Zn^{2+}$ fails to induce permeability
to either cations or anions in the absence of energy. The selective solute permeation appears to arise from a selective alteration of highly organized membrane by different metals.

Active ion transport can be markedly increased by different metal ions, and smaller amounts of each metal are required for this activation as to those necessary for the induction of passive permeability. This suggests that there is a common site located on the external surface of the coupling membrane that is most intimately liked to active ion uptake. This common site could be the initial barrier which is linked to a polar channel that can carry ions in response to an electrochemical gradient.

Low level of Cu$^{2+}$ uncouple oxidative phosphorylation associated with the oxidation of several different substrates. This is attributed to the fact that Cu$^{2+}$, like valinomycin, activates K$^+$ transport. Cu$^{2+}$-induced cation uptake shows a number of similarities to valinomycin including specificity for K$^+$, pH changes, and similar responses to uncouplers and Ca$^{2+}$. Cu$^{2+}$ activates State 4 respiration; State 3 respiration is either insensitive or inhibited depending on the level of Cu$^{2+}$. The ATPase activity of heart mitochondria is first stimulated than inhibited as a result of titration with Cu$^{2+}$. Cu$^{2+}$-induced ATPase activity is quite similar to the ATPase induced by p-chloromercuriphenyl sulphonate, but differs from uncoupler-induced ATPase in that it is rather specific for K$^+$. Possible modes of action of heavy metal ions on the mitochondrial membrane are discussed.