STUDIES OF ENERGY TRANSFER PROCESSES

IN MAMMALIAN MITOCHONDRIA

STUDIES OF ENERGY TRANSFER PROCESSES IN MAMMALIAN MITOCHONDRIA

BY

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SCOPE AND CONTENTS: The present investigation was concerned with mitochondrial energy transfer reactions and their relationship to mitochondrial structural integrity. Experiments with azide demonstrated a close relationship between oxidative phosphorylation and large amplitude mitochondrial volume changes. Azide inhibited energy transfer and energy-linked mitochondrial swelling by competing with adenine nucleotide for a site on the terminal phosphorylating enzyme. As a permeant anion azide exerted secondary effects on mitochondrial structure and function.

Experiments with mitochondria treated with phlorizin and phloretin emphasized the importance of Mg^{++} as a contolling factor in maintaining the integrity of mitochondrial energy transfer processes. The results indicated that these compounds interfered directly with oxidative phosphorylation, and that mitochondrial swelling was either a consequence of impaired energy transfer, or a separate phenomenon.

PREFACE

This thesis describes studies carried out in the Research Unit in Biochemistry, Biophysics and Molecular Biology, McMaster University, from September 1966 to June 1970. Except where others are specifically mentioned, it consists entirely of the authors own work. No similar thesis has been submitted at any other University.

The author would like to express his thanks to Dr. F. D. Ziegler for his critical insight and co-operation at all times, and to Dr. K. B. Freeman for his constructive comments. The author also thanks Dr. W. B. Elliott, State University of New York at Buffalo for the use of the Aminco-Chance Spectrophotometer. The reliable assistance of Mrs. Mary Wills, Miss Linda Clark and Mr. Barry Honda in carrying out phosphate and protein assays is gratefully acknowledged. The author is deeply indebted to the Research Unit in Biochemistry, Biophysics and Molecular Biology of McMaster University and the Ontario Government for the award of Scholarships covering the period of research and study. The author wishes also to thank Mrs. Judy Vigers for her skillful typing and forbearance throughout.

Research Unit in Biochemistry,
Biophysics and Molecular Biology,
McMaster University
September, 1970.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
DNP	2,4-dinitrophenol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol Bis (2-aminoethyl ether) tetraacetic acid
ΔE_o	oxidation-reduction potential
Fp	flavoprotein
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate
N.HFe	non-heme iron
nm	nanometer
P_i	inorganic phosphate
Q	coenzyme Q, ubiquinone
Succ.	succinate
Tris	tris (hydroxymethyl)-aminomethane

CHAPTER 1

A. INTRODUCTION

Research workers during the past thirty years have recognized mitochondria as centers for the production of useful energy in the cell. Mitochondria contain enzyme systems which conserve and transfer the energy liberated by the oxidation of foodstuffs in a chemical form as ATP. Oxidative energy can also drive changes in mitochondrial volume and structure. While the membrane systems of mitochondria are known to be intimately involved with the organization and control of energy transfer processes, the relationships of structure to function are not well understood. The techniques of investigation employed to study these relationships have involved isolation of pure enzymes and enzyme complexes, morphological studies of mitochondria and the application of inhibitors to mitochondrial systems. This thesis is limited to studies employing the inhibitors azide, phlorizin and phloretin to investigate interactions between structure and function. The present study demonstrates that impairment of the physiological integrity of mitochondria with these compounds is associated with decreased efficiency of ATP synthesis and large changes in mitochondrial volume. The results indicate one of the physiological controls on mitochondrial membrane integrity, as well as defining energy relationships between mitochondrial volume changes and ATP synthesis.

B. GENERAL CONSIDERATIONS

Biological Oxidations - In the second decade of this century Warburg (1) reported a complex iron-containing substance (Atmungsferment) which participated in biological oxidations. He proposed (2, 3) that the iron complex functions in cellular oxidations to activate and transfer molecular oxygen to metabolite molecules. In opposition, Wieland (4) and Thunberg (5) argued that biological oxidations involved hydrogen activation of the metabolite molecule and removal of the activated hydrogen by dehydrogenases. Szent-Györgyi (6) pointed out that these mechanisms were insufficient in themselves and that both oxygen activation and hydrogen activation are necessary to account for most biological oxidations.

Biological oxidations were related to specific components of the cell by Keilin (7) in a series of spectrophotometric studies. He observed that a variety of tissues contained reversibly oxidizable haemochromagens, which he referred to as cytochromes. Keilin and Hartree (8), using a particulate fraction from beef heart muscle, characterized the oxidation sequence of the cytochromes. By inhibiting the oxidation of succinate, ascorbate and adrenalin with azide and cyanide, they found that cytochrome *c* functioned between cytochrome *b* and the terminal oxidase (cytochrome *a* + a_3). Cytochrome a_3 reacted with oxygen and was characterized as being identical with Warburg's Atmungsferment.

Oxidative Phosphorylation - An interaction between biological oxidations and phosphorylation was first suggested by W. A. Engelhardt (9, 10). Kalckar (11) recognized that the free energy liberated during the oxidation of tricarboxylic acids was adequate to drive the synthesis

of adenosine-5'-triphosphate (ATP)* and observed that only aerobic respiration resulted in phosphorylation of adenosine-5-diphosphate (ADP) to ATP. Oxygen uptake and phosphorylation were stimulated by addition of fumarate or malate. From thermodynamic considerations Belitzer and Tsibakova (12) observed that multiple phosphorylations were associated with the transfer of a pair of reducing equivalents to oxygen via respiratory carriers. Ochoa (13 - 15) used cell-free brain and heart preparations to determine quantitatively the moles of phosphate esterified per g-atom of oxygen consumed which he referred to as the P/O ratio. P/O ratios approaching 3.0 were obtained for the NADH-mediated oxidation of pyruvate and α-ketoglutarate. The difference in the oxidation-reduction potential (ΔE_o) between NADH and oxygen is 1.14 volts which is equivalent to 51.3 kcal/mole of reducing equivalents transferred (17). Since the energy required to synthesize three moles of ATP is about 25 kcal/mole (15) a P/O ratio of 3.0 corresponds to an efficiency of energy conversion of about 50%. Similarly P/O ratios of 2.0 obtained with succinate as substrate approach 50% efficiency since the ΔE_o between succinate and oxygen is about 0.82 volts.

Ochoa's observations have been confirmed in many laboratories (18) and P/O ratios of 3.0 have been generally accepted as the maximum obtainable for oxidative phosphorylation in tightly coupled mitochondria (18). However, Lynn (19) and others (20, 21) have reported P/O ratios

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*The abbreviations used are those recommended by the NAS - NRC Office of Biochemical Nomenclature (16).

approaching 6.0 with NADH-linked substrates and 5.0 in the presence of succinate, which represents net phosphorylation efficiencies approaching 100%. These results may be explained by recent observations (22) that K^+ efflux from mitochondria generates ATP resulting in apparent P/O ratios greater than 3.0.

In 1948 a particulate fraction, isolated from rat liver by differential centrifugation, was identified as a cytological structure, the mitochondrion (23). The finding that the processes of oxidation (24, 25) and phosphorylation (26) were carried out almost exclusively by this organelle permitted correlation of earlier lines of research. Lehninger and coworkers (27, 28), using mitochondrial preparations, demonstrated the respiration-dependent incorporation of $^{32}P_i$ into esterified phosphate. Anaerobiosis, omission of Mg^{++}, ATP, or substrate inhibited phosphate esterification. They concluded that ADP was the specific phosphate acceptor. Lardy and Wellman (29) postulated that the rate of respiration was limited by the rate of transfer or hydrolysis of high energy phosphate compounds whose synthesis is coupled with oxidative electron transport. Chance and Williams (30) found that water and saline treatment of phosphorylating mitochondrial preparations yielded non-phosphorylating electron transport systems similar to those reported earlier by Keilin and Hartree (8), indicating that both enzymatic systems were derived from the same organelle.

Localization of Phosphorylation Sites - In his studies of oxidative phosphorylation Lehninger (28) observed that phosphate esterification accompanied electron transport from NADH to oxygen, but did not accompany the oxidation of β-hydroxybutyrate by NAD^+. Judah (31) reported that

the oxidation of reduced cytochrome c resulted in P/O ratios approching 1.0. P/O ratios approaching 4.0 were obtained with α-ketoglutarate, L-glutamate or pyruvate as substrate; and a P/O ratio of 3.0 was observed in the presence of β-hydroxybutyrate. With $Fe(CN)_6^{3-}$ as electron acceptor, $P/2e^-$ ratios of 0.9 were obtained with β-hydroxybutyrate and α-ketoglutarate as substrates. Using a variety of substrates, Copenhaver and Lardy (32) determined P/O ratios as well as $P/2e^-$ ratios with $Fe(CN)_6^{3-}$ as electron acceptor. A balance sheet was constructed showing that $P/2e^-$ ratios were less than P/O ratios by approximately 1.0. Addition of antimycin A, which interacts in the region of cytochrome b (30, 33), resulted in P/O ratios of zero while $P/2e^-$ ratios with $Fe(CN)_6^{3-}$ were unchanged. Correlation of these results indicated three sites of phosphorylation between NADH and oxygen. Site III was localized between cytochrome c and oxygen; site I was between NADH and the region of $Fe(CN)_6^{3-}$ interaction (possibly cytochrome b (33)); and by elimination, site II was localized between cytochrome b and cytochrome c.

Chance and Williams (30, 34) used a different approach to the same problem. From spectrophotometric studies of the respiratory carriers under various conditions they first defined five steady state levels of oxidation (Table 1). Then, from the postulate of Lardy and Wellman (29), Chance and Williams reasoned that the absence of ADP (state 4) inhibited respiration. Thus substrate, ie. electrons, should accumulate on carriers previous to the block (site of interaction of ADP) while carriers in the sequence after the block should be more oxidized.

TABLE 1. Steady States of Respiratory Pigments in Mitochondria (from Chance and Williams (34) and Chance (35)).

Steady State	Substrate Level	ADP Level	Oxygen	Respiratory Rate	Rate Limiting
1	low	low	aerobic	slow	ADP
2	low	high	aerobic	slow	substrate
3	high	high	aerobic	fast	capacity of system
4	high	low	aerobic	slow	ADP
5	high	high	anaerobic	0	oxygen
6(35)	high	low; Ca^{++}high	aerobic	fast	capacity of system

With the aid of azide, an inhibitor of the terminal respiratory enzyme, Chance and Williams identified three such sites of interaction, or crossover points as they were termed. These were between NADH and flavoprotein, between cytochrome b and cytochrome c, and between cytochrome c and cytochrome oxidase (cytochrome a + a_3).

Wilson and Chance (36) recently presented evidence that the terminal crossover point is between cytochrome a and cytochrome a_3. They found that in tightly coupled mitochondria, state 3 succinate oxidation was ten times more sensitive to azide then respiration uncoupled by DNP. It was concluded that azide inhibited state 3 respiration primarily by interacting with energy transfer processes. This conclusion was supported by observations that azide inhibited DNP-stimulated ATPase in intact mitochondria (37) as well as the ATP-$^{32}P_i$ exchange reaction and $H_2{}^{18}O$-P_i exchange reaction (38).

Steady state difference spectra of systems inhibited by azide in the presence of succinate and ADP revealed that cytochrome *a* was reduced with an α-band at 596 nm while cytochrome $\underline{a}_3$ remained oxidized. Cytochrome $\underline{a}_3$ became more reduced upon addition of uncoupling agents (39) indicating a crossover point between these cytochromes (36, 39).

Keilin (8) and others (40) interpreted similar spectrophotometric observations to indicate that respiratory inhibition resulted from the formation of an oxidized azide-cytochrome $\underline{a}_3$ complex. Supporting this viewpoint were the facts that azide is a strong heme-binding agent and that cytochrome $\underline{a}_3$ is very reactive towards carbon monoxide and cyanide which are also strong heme-binding agents. However this argument is weakened by the findings that the inhibition kinetics of azide and cyanide differ (41, 42) and that cyanide inhibition of oxygen uptake is not relieved by uncoupling agents (36). In support of Keilin, Nicholls and Kimelberg (43) suggested that relief of azide inhibition by uncouplers resulted from changes in electron transfer pathways permitting a direct reduction of cytochrome $\underline{a}_3$ by cytochrome *c*, and bypassing cytochrome *a*. They postulated that spectral shifts in cytochrome *a* were the result of a heme-heme interaction between reduced cytochrome *a* and the classical cytochrome $\underline{a}_3$-azide complex.

Palmieri and Klingenberg (44) recently presented another argument against azide inhibition of energy transfer. They suggested that azide acts on cytochrome *a* only. Interference with energy transfer by lesser concentrations of azide was postulated to be more apparent than real since azide is accumulated as a permeant anion

by mitochondria during state 3 oxidation of substrate.

Knowledge of the relationships of other respiratory carriers to phosphorylation sites has developed from the application of inhibitors in conjunction with elegant spectrophotometric techniques. A method of reading out the quantum efficiency of Flavin fluorescence (45) led Chance et al. (46) to propose two flavoproteins in the NADH-linked respiratory chain with an Amytal and rotenone site of inhibition between them. ESR studies of oxidation-reduction signals from non-heme iron in mitochondria permitted Beinert and Lee (47) to resolve three components with different g-values. A non-heme copper component of mitochondria was reported which underwent reversible oxidations in association with cytochrome oxidase (48). These studies, together with reconstruction of the respiratory chain from purified enzyme components (49 - 51) suggested that the probable sequence of electron transfer carriers and phosphorylation sites from substrate to oxygen is as described by Lardy and Ferguson (52) (Fig. 1).

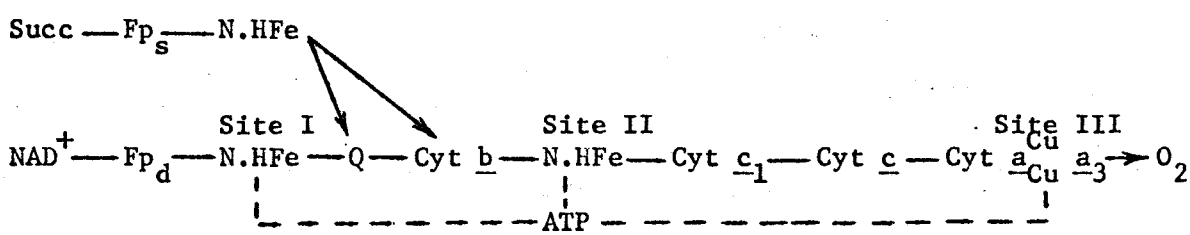


Figure I. Probable sequence of electron carriers and phosphorylation sites.

Mechanisms of oxidative phosphorylation - Control of the rate of electron transfer by phosphorylation raised the question of how the two processes were coupled. The present hypotheses of the coupling

mechanism resulted from work with inhibitors, enzyme isolation and reconstruction studies, and studies of mitochondrial ion uptake and swelling. Early work showed that respiration and phosphorylation could be dissociated or "uncoupled" by DNP, resulting in decreased P/O ratios (53). Judah (31) proposed that the uncoupling action of DNP was a consequence of sparing inorganic phosphate. Supporting this proposal was the observation that systems uncoupled by DNP did not require inorganic phosphate for maximal electron transport (54, 55). DNP at uncoupling concentrations activates a mitochondrial ATPase activity (53). Lardy et al. (56) and Huijing and Slater (57) found that the antibiotic oligomycin inhibited respiration, inhibition of respiration by oligomycin being relieved by DNP at uncoupling concentrations. Moreover, oligomycin inhibited the DNP-activated ATPase indicating that this enzyme represented the terminal phosphorylation reaction. Concurrently, Boyer (58) reported an ATP-$^{32}P_i$ exchange reaction which required the presence of ADP. Other workers (59 - 61) reported an AT^{32}P-ADP exchange in mitochondria which was inhibited by oligomycin. Cohn (62) observed an $H_2{}^{18}O$-P_i exchange in mitochondria which was activated by ATP or respiration. Boyer (63) determined from ^{18}O labelling studies that the bridge oxygen in ATP was derived from ADP rather than inorganic phosphate. These observations enabled Lehninger (64) to formulate the steps in energy transfer from electron carrier to ATP in general terms (Fig. 2). A and B are members of the electron carrier chain and $A_{ox}{\sim}I$ is a hypothetical high-energy chemical intermediate which is regenerated upon formation of ATP or hydrolysis of $A_{ox}{\sim}I$ by DNP. Because of the

uncertain nature of A_{ox}~I, it is preferably described as X~I.

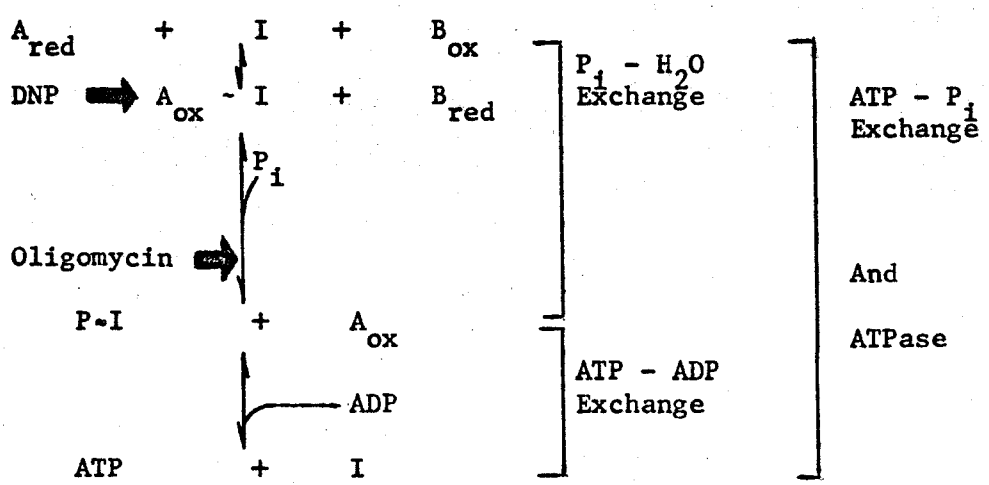


Figure 2. Sequence of energy transfer according to the chemical hypothesis (after Lehninger (64)).

Recently Wang (65) advanced a molecular model of oxidative phosphorylation based on the above mechanism of energy transfer and incorporating the known electron carriers of the respiratory chain. According to this hypothesis, electron transport causes imidazolyl-ferriheme radical formation in close association with phosphodiester groups of phospholipids to form "energy-rich" or oxidized intermediates. These intermediates react with inorganic phosphate to yield an unstable diphospholipid. The terminal phosphate of the diphospholipid is transferred to a phosphate acceptor (ADP). Cytochromal histidine provides an imidazol moiety which functions as an electron sink. Previous observations (66) of high $^{32}P_i$ incorporation into phospho-histidine are thought to result from further transphosphorylations and bear no direct relationship to this mechanism. (cf. refs. 67 and 68 for summaries of the status of possible chemical intermediates).

Racker and coworkers have done notable work in isolating the enzymes involved in energy transfer. In 1960 they reported a purified protein component (F_1) from mechanically disrupted beef heart mitochondria (69, 70) which in the presence of either NADH or succinate, together with the particulate submitochondrial residue, gave small but significant P/O ratios and supported an ATP-$^{32}P_i$ exchange reaction. F_1 exhibited an ATPase activity which was stimulated by DNP but was insensitive to oligomycin. Sonication of F_1 yielded another factor, F_2. F_1 and F_2 were both essential in restoring phosphorylation to the particulate submitochondrial residue (71). A soluble factor (F_3) exhibiting ATP-$^{32}P_i$ exchange activity was obtained by sonicating beef heart mitochondria in phosphate buffer and exposing the sonicated particles to trypsin digestion. This factor, together with F_1, restored phosphorylation to trypsin-treated particles (72). A mixture of F_2, F_3, and a highly purified protein (F_5) were required to restore the ATP-$^{32}P_i$ exchange reaction, oxidative phosphorylation, and the ATP-dependent reduction of $NADP^+$ by NADH, but were not required for the reduction of $NADP^+$ driven by respiratory energy (73). It is probable that these factors are concerned with the formation of a phosphorylated rather than a non-phosphorylated intermediate (73).

To account for experimental observations, chemical hypotheses require a non-phosphorylated high energy intermediate, be it an anhydride bond or a conformational state of the phosphorylation enzymes. Positive identification of an intermediate has not been forthcoming. From the work of Racker's group (69 - 73) and others

(49, 74), it is evident that oxidative phosphorylation is intimately associated with mitochondrial membrane structures. Furthermore the chemical hypothesis incorporates H^+ and ion movements across the mitochondrial membrane as secondary processes, not vital to ATP synthesis. These facts together with unexplained observations of swelling and shrinkage phenomena accompanying phosphorylation prompted Mitchell (75, 76) to propose a chemiosmotic theory of energy coupling. Basic to the chemiosmotic theory is the utilization of H^+ and ion transport to drive oxidative phosphorylation. Therefore, a demonstration of the primary or secondary nature of H^+ transport would support one of the hypothesis. From the following discussion it is evident that the chemiosmotic theory differs radically from previous mechanisms in that the synthesis and hydrolysis of ATP are proposed to be spatially and chemically separate from redox reactions. Components of the respiratory chain are arranged within an osmotically and electrically impermeable coupling membrane (the inner membrane of mitochondria) such that electron transfer from substrate to oxygen drives the translocation of protons across the membrane from the inner phase to the outer phase. The respiratory chain is folded into three oxidoreduction (O/R) loops composed of one hydrogen and one electron carrier. Each loop corresponds functionally to a phosphorylation site (76). Also localized within the coupling membrane is a proton-translocating reversible ATPase which is permeable to H^+ only from the inner phase and to OH^- only from the outer phase. The proton current generated by electron transfer

flows in a closed circuit driving the proton translocating ATPase in reverse to synthesize ATP (Fig. 3). Realizing that free exchange of cations and anions across the coupling membrane could discharge the membrane potential, Mitchell also postulated that ion-specific exchange diffusion systems reside in the coupling membrane which permit stoichiometric exchange of cations for H^+ and anions for OH^-.

Decreased phosphorylation efficiency results from increased permeability of the coupling membrane to H^+ (77, 78). Mitchell (76) demonstrated that when tightly coupled anaerobic mitochondria were pulsed with limited quantities of oxygen, an initially rapid translocation of protons occured prior to the respiratory carriers attaining steady state levels of reduction. Steady state levels resulted from the build up of a proton gradient sufficient to retard respiration. In this respect the proton gradient may be considered equivalent to X~I (79).

Mitchell and Moyle (80) reasoned that in tightly coupled mitochondria, a stoichiometric relationship of H^+: oxygen: P_i should exist such that

$$P_i/O = (\rightarrow H^+/O) \times (P_i/\rightarrow H^+)$$

where $(\rightarrow H^+)$ equals the protons extruded to the outer phase. They measured pH changes in anaerobic suspensions of rat liver mitochondria pulsed with limiting quantities of oxygen or ATP and found that the $P_i/\rightarrow H^+$ of ATPase approached 1/2 and that $\rightarrow H^+/O$ for FAD-linked and NAD^+-linked oxidations was 4 and 6 respectively. These observations conform to the known stoichiometry of phosphorylation and demonstrated that H^+ translocation was associated with redox reactions and ATP hydrolysis. However, the long preincubation

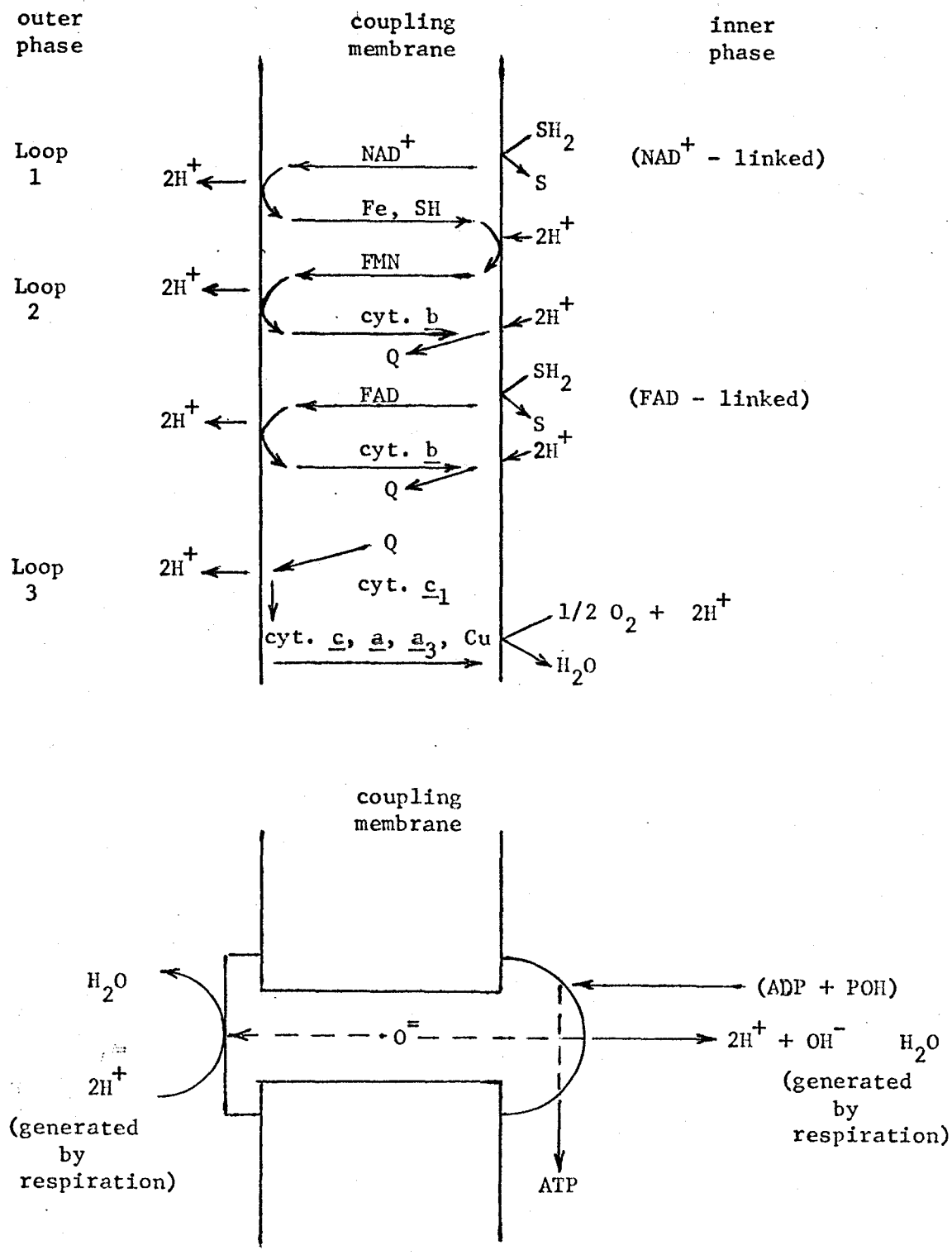


Figure 3. Possible configuration of the respiratory chain and ATPase system, according to Mitchell and Moyle (76).

required to establish anaerobiosis may result in the loss of Ca^{++}, Mg^{++} and K^{+} from mitochondria. Therefore, an oxygen pulse into the anaerobic suspension could cause respiration-dependent uptake of these cations with accompanying proton ejection (81). Mitchell (76) refuted this by pointing out that ferrocyanide oxidation by the cytochrome chain (which involves pure electron transfer) is not accompanied by proton translocation as should be the case if H^{+} ejection were the consequence of respiration-dependent cation uptake.

The experiments by Mitchell's group using proton pulses of HCl to drive mitochondrial synthesis of ATP gave very low yields (82), such as to be unaccountable if ATP synthesis were driven by a proton gradient. Extending these studies, Lehninger et al. (83) found that there is an apparent ejection of $2H^{+}$ and accumulation of $2OH^{-}$ accompanying Ca^{++} uptake by mitochondria. It was reasoned (84) that an H^{+} gradient thus formed should be discharged by the phosphorylation of ADP. Addition of ADP after the formation of such a gradient resulted in stoichiometric respiratory stimulation, ATP formation, and removal of H^{+} from the medium but the H^{+} concentration in the mitochondrial phase as measured by microtitration techniques, did not change. These results suggested that the titratable H^{+} gradient formed by Ca^{++} uptake was not utilized for ATP formation and emphasized the difficulties in testing Mitchell's hypothesis.

Mitochondrial Ion Transport and Swelling - Mitochondria are relatively impermeable to alkali-metal cations (85, 86) as required

by the chemiosmotic hypothesis (76). The findings of Pressman et al. (87, 88, 89) and Chappell and Crofts (90) that the antibiotics valinomycin and gramicidin increased the permeability of mitochondria towards these ions led to studies which related membrane permeability to phosphorylation efficiency. Valinomycin facilitated the penetration of K^+, Rb^+, and Cs^+. Gramicidin was less specific, inducing the uptake of Li^+, K^+, Na^+, Cs^+, and Rb^+. It was found that mitochondrial ion transport, induced by valinomycin, decreased phosphorylation efficiency (87). From the effects of inhibitors of electron transport, of uncoupling agents and of oligomycin, Chappell and Crofts (91) concluded that in the presence of valinomycin, energy derived from a non-phosphorylated high energy intermediate, or proton gradient, was required for monovalent cation uptake. Valinomycin induced mitochondrial swelling which required energetic conditions identical to those necessary for cation uptake, both being inhibited or reversed by DNP, oligomycin or ADP (87). Swelling occured only when both cation and anion could penetrate the mitochondrial membrane (92). In agreement with Mitchell (76) and Azzone and Azzi (93), Chappell and Crofts (91) proposed that an exchange diffusion system in the mitochondrial membrane permitted $H_2PO_4^-$ to enter in exchange for OH^-. The penetrating species dissociated in the more alkaline mitochondrial matrix giving rise to H^+ and a non-penetrating species. In the presence of valinomycin or gramicidin energy-linked H^+ efflux drove K^+ uptake and swelling was a consequence of increased osmotic pressure within the mitochondria.

More recently, Blondin et al. (94) have observed that certain alkali metal salts cause large amplitude swelling of mitochondria in the absence of either electron transfer or ATP hydrolysis. They proposed that this type of swelling, referred to as pseudo-energetic swelling, was driven by the Donnan membrane effect. The presence of ion-transducing antibiotics facilitated the competence of salts to induce swelling and it was concluded that the ability of a salt to induce swelling was a function of the rate of penetration of the mitochondrial membrane by the salts in question. The rate of ion penetration through the mitochondrial membrane can be greatly increased by hypotonic media or chemical agents resulting in large irreversible increases in mitochondrial volume and decreased phosphorylation efficiency.

Proposals - From these considerations it is evident that the structural intactness of the mitochondrial membrane is essential in maintaining the integrity of energy transfer processes. This poses the problem of defining those physiological controls on mitochondrial structure which are related to interactions between ion transport and ATP synthesis. The nature of these relationships may be indicated by an examination of the effects of compounds which inhibit phosphorylation and induce large changes in mitochondrial volume. Such a study is presented in this thesis using the compounds azide, phlorizin and phloretin.

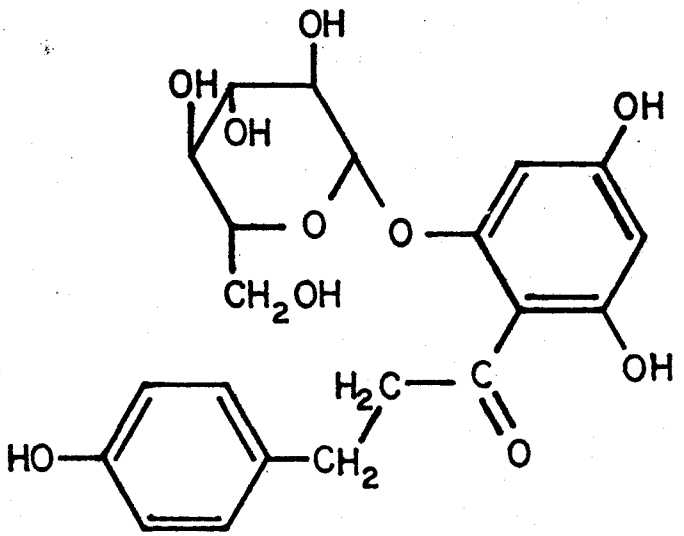
Azide inhibits cytochrome oxidase (8, 41) presumably by interaction with cytochrome a (36, 39) or cytochrome a_3 (8, 43). Azide also inhibits DNP-activated ATPase in intact mitochondria (37, 95) as well as the ATP - $^{32}P_i$ exchange reaction and $H_2{}^{18}O$ - P_i exchange

reaction (38). Like DNP, azide can act as an uncoupling agent (96, 97) but its effect on the $AT^{32}P$ - ADP exchange reaction is similar to oligomycin (56, 57). Palmieri and Klingenberg (44) have suggested that azide acts on cytochrome a only and that interference with energy transfer is a secondary result of azide accumulation as a permeant anion.

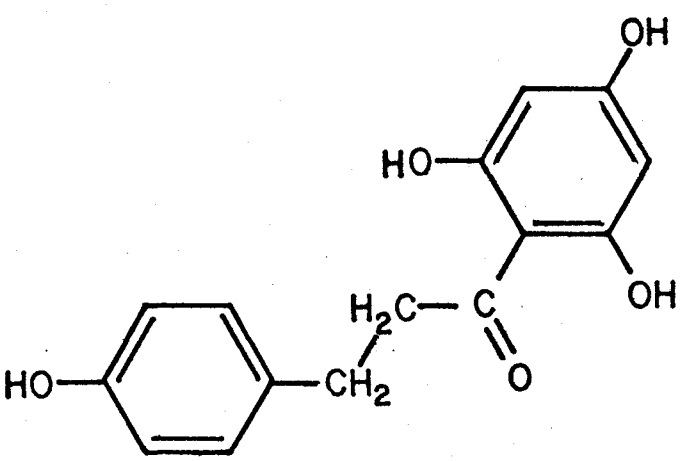
Phlorizin (3,5-dihydroxy-2-(p-hydroxyhydrocinnamoyl) phenyl β-D-glucopyranoside) (Fig. 4) inhibits a number of enzyme activities, particularly those requiring adenine nucleotides as participants or cofactors (98). Keller and Lotspeich (99, 100) observed increased mitochondrial volume following treatment with phlorizin and attributed the associated decrease in electron transport and phosphorylative capacity to secondary effects arising from mitochondrial swelling. Phlorizin also decreases the ATPase activity of mitochondrial membranes extracted with 0.6M KCl (101). Phloretin, the aglucone of phlorizin (Fig. 4) is a more potent inhibitor than phlorizin of this ATPase (101). The effects of phloretin on energy transfer and mitochondria volume have not been otherwise examined.

Figure 4

The molecular structure of phlorizin and phloretin.



PHLORIZIN



PHLORETIN

CHAPTER 2

METHODS AND MATERIALS

Rat Liver Mitochondria - Experimental animals were deprived of food for twenty-four hours before they were killed but were permitted water ad libidum. Each batch of liver mitochondria were prepared from two 150 - 250 g male hooded rats killed by cranial fracture. The livers were rapidly excised and placed in chilled mannitol-sucrose-EDTA isolation medium as outlined by Strickland et al. (102). Livers were minced with surgical scissors and rinsed twice with isolation medium. A Potter-Elvehjem homogenizer with a snugly fitting teflon pestle was used to homogenize the liver mince. The homogenate from each liver was divided into two 50 ml polypropylene centrifuge tubes, diluted to 40 ml with isolation medium and centrifuged at 800 x g for five minutes. The pellet was discarded, the supernatant being transferred to clean tubes and centrifuged at 800 x g for ten minutes. The pellet was again discarded. The supernatant was transferred to clean tubes and centrifuged at 7000 x g for ten minutes. The supernatant was discarded, fatty material being first wiped from the tubes with a Kim-wipe. The mitochondrial pellet in each tube was gently washed with approximately one ml of isolation medium and the wash discarded. The pellets were each suspended in approximately one ml of isolation medium and the suspensions transferred by pipette to a single clean centrifuge tube. The suspension, diluted to 40 ml with isolation medium, was

centrifuged a final time at 7000 x g for ten minutes. The supernatant was discarded. The pellet was washed gently with approximately one ml of isolation medium, the wash being discarded. The mitochondrial pellet was suspended in approximately 1.5 ml isolation medium and was kept on ice ready for use. All operations were carried out at 0 - 4°C.

Beef Heart Preparations - Beef heart mitochondria and beef heart sub-mitochondrial particles were isolated by the methods of Haas and Elliott (103) with the following changes in procedure: smaller batches of heart muscle were used (800 g/batch vs 2.5 kg/batch) and the isolation medium was that used for rat liver mitochondria. Mitochondria were pelleted in 250 ml steel containers at 10,000 x g rather than by continuous flow. This change in procedure proved more efficient with smaller batches and prevented undue exposure of the preparation to room temperatures.

Mitochondrial ATPase Preparations - Purified mitochondrial ATPase was prepared essentially as outlined by Pullman, et al. (69, 70) with the following variations. Mitochondria from rat liver or beef heart were suspended (0.4 g/ml) in a sonication medium containing 0.4 M $(NH_4)_2SO_4$, 250 mM sucrose, 10 mM Tris, and 1.0 mM EDTA, pH 7.4. Sonication was carried out for 10 seconds at one minute intervals for a total of 50 seconds (Branson Sonic Oscillator). During this procedure the sonication vessel was immersed in an ethanol/dry ice bath at -10° maintaining the mitochondrial suspension at 0° (104). The sonicated suspension was centrifuged at 105,000 x g for 35 minutes at room temperature as the enzyme is cold labile (70). The pale yellow supernatant was applied to a Sephadex G-100 column

(1.5 x 90 cm) previously equilibrated in a solution of 100 mM sodium acetate, 100 mM sodium bicarbonate, and 1.0 mM ATP. ATP was included to prevent inactivation of the enzyme (70). Elution was carried out with a solution containing 100 mM sucrose, 5.0 mM Tris, and 4.0 mM ATP. The presence of protein in the eluate was followed spectrophotometrically at 260 nm. The enzyme appeared in the first major protein band (Fig. 5) and passed through the column in 39 - 47 ml. The enzyme was precipitated by addition of two volumes of saturated ammonium sulfate, and collected by centrifugation at 105,000 x g. The supernatant was decanted and the enzyme was suspended in 250 mM sucrose, 50 mM Tris, and 100 mM $MgCl_2$, pH 7.4, when used immediately. Otherwise it was preserved by storage at room temperature in the sonication medium (70). Purified ATPase and the ATPase of intact mitochondria was activated by including 1.0 mM Mg^{++} (Mg^{++}-activated ATPase) in the reaction medium, or 1.0 mM Mg^{++} plus 100 µM DNP (DNP-activated ATPase) (105).

The reaction medium of Myers and Slater (105) was employed to study ATPase activity. ATPase reactions were initiated by the addition of ATP to mitochondria suspended in reaction medium. Reactions were carried out in a final volume of 1.0 ml and were stopped by the addition of 0.2 ml of 20% trichloroacetic acid. As a control for each experiment, ATP was added to reaction medium containing 0.2 ml trichloroacetic acid plus an aliquot of mitochondrial stock suspension. ATPase activity was determined by measuring the release of inorganic phosphate. The release of inorganic phosphate at 2, 4 and 6 incubation periods was plotted and the slope given by a straight line

through the points was used to determine the rates of ATP hydrolysis. Inhibitor concentrations required to depress ATPase activity were determined from the amount of inorganic phosphate released in 15 minutes. In studies of azide inhibition of ATPase activity, inorganic phosphate was determined by the method of Chen et al. (106). In all other instances inorganic phosphate was assayed using the Sumner modification (107) of the Fiske-SubbaRow method (108).

For respiratory studies, mitochondria were suspended in reaction medium containing 250 mM sucrose, 40 mM KCl, 1.0 mM $MgCl_2$ and 20 mM potassium phosphate buffer, pH 7.4.. Oxygen uptake was measured at 25° with a Clark oxygen electrode in a 4.5 ml sealed vessel (102). All additions to the sealed vessel were injected by microliter syringe through an airtight vaccine bottle stopper. Care was taken to ensure that all additions were free of air bubbles.

Ionic calcium was measured at 25° in a 5.0 ml sealed vessel with a Corning calcium electrode attached to an Orion meter and Varian recorder. Additions were made with microliter syringes as in respiratory studies.

Spectral studies of components of the mitochondrial respiratory chain were carried out using a Cary 14 spectrophotometer with a high intensity light source and expanded scale, or an Aminco-Chance dual wavelength spectrophotometer.

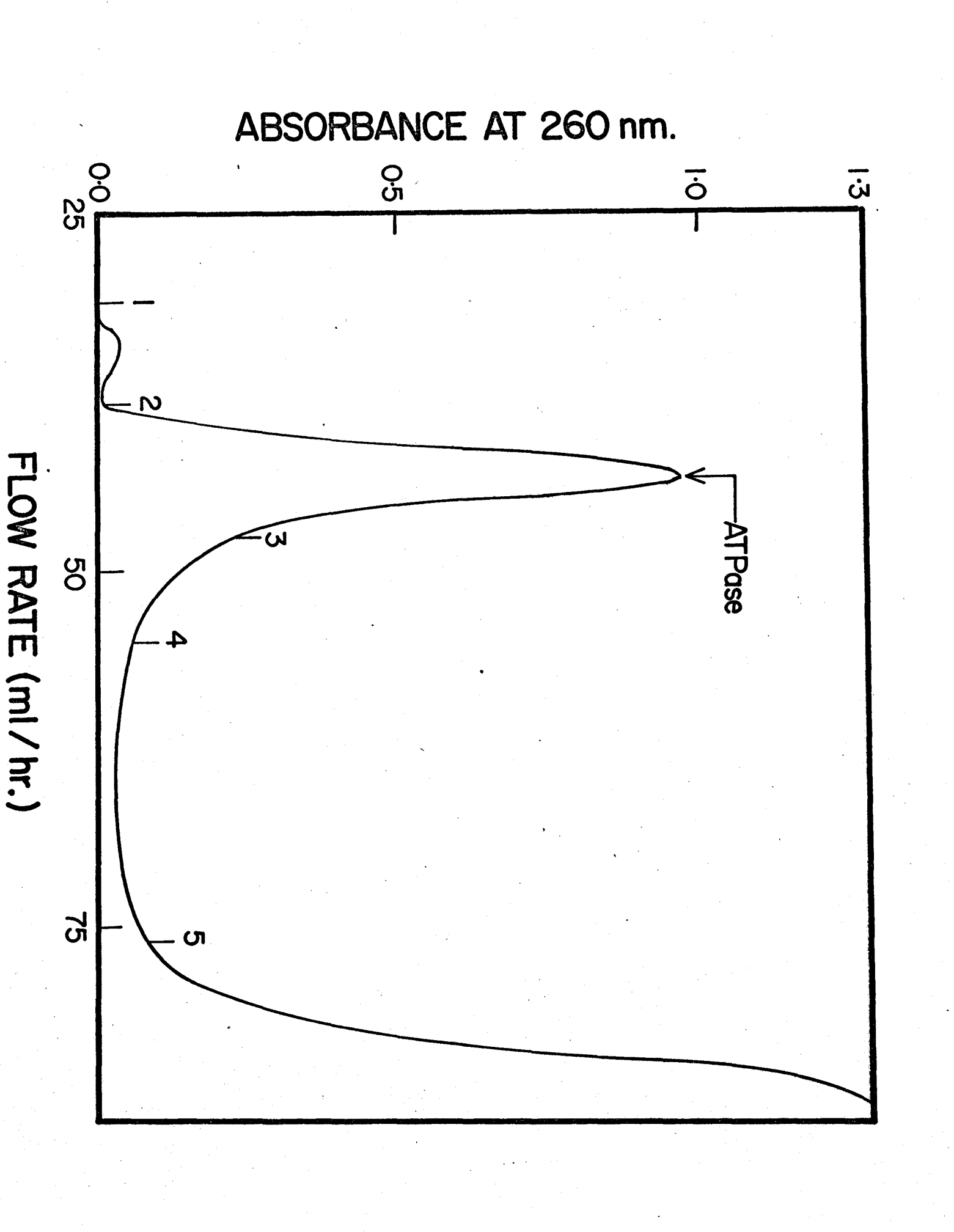
Mitochondrial volume changes were observed qualitatively as changes in optical density at 520 nm with the Cary 14 spectrophotometer (109). A decrease in optical density was interpreted as an increase in mitochondrial volume (110, 111).

In order to rapidly determine the protein concentration of the original mitochondrial suspension, before dilution for ATPase or respiratory studies, an aliquot was solubilized in 2% cholate. An optical density of 1.0 at 280 nm was assumed to be equivalent to a protein concentration of 1 mg/ml. The final protein concentration in each experiment was determined by the Lowry method (112) with bovine serum albumin as standard.

Reagents - Phlorizin, obtained from Aldrich Chemical Co., was recrystallized from 95% ethanol to give a melting point range of 108 - 109° and stored as a 250 mM ethanolic solution. Phloretin, obtained from Sigma Chemical Co., was recrystallized from 95% ethanol to give a melting point range of 274 - 276° and stored as a 100 mM ethanolic solution. Microliter quantities of these solutions were added to the mitochondrial preparation under study to give the desired final concentration. The same microliter amounts of ethanol alone, added to control experiments, did not affect the respiratory rate, ATPase activity, or mitochondrial volume. Sodium azide was obtained from Eastman Chemical Co. and used without any further purification. Oligomycin, consisting of 15% oligomycin A and 85% oligomycin B was obtained from Sigma Chemicals. Rotenone, recrystallized from ethanol-ice water to give a melting point of 157-159°, was a generous gift of Miss Ivy Fettes. Amytal was obtained from the Eli Lilly Co. All other reagents were the purest available commercially and were used without further purification. Deonized glass-distilled water was used throughout.

Figure 5

Elution curve of ATPase from sonicated mitochondria. Flow rate 25 ml per hour, temperature 23-25°. Fractionation was performed on a 1.5 x 90 cm Sephadex G-100 column as outlined in methods. ATPase activity was found in the 39th to 47th ml of eluate (fraction 2).


ABSORBANCE AT 260 nm.
0·0
0·5
1·0
1·3
25
50
75
FLOW RATE (ml / hr.)
1
2
3
4
5
ATPase

CHAPTER 3

EFFECT OF AZIDE ON MITOCHONDRIAL VOLUME, ATPASE AND RESPIRATION

Inhibition of energy-linked mitochondrial swelling by azide - Mitochondria prepared in the presence of EDTA and suspended in potassium acetate buffer swell when supplied with succinate, β-hydroxybutyrate or ATP (Fig. 6). Addition of 330 μM azide inhibited the rate of succinate-induced swelling by 65%; β-hydroxybutyrate-induced swelling by 58%; and ATP-induced swelling by 75%. A second addition of 330 μM azide depressed swelling to a rate comparable to that observed before the addition of an energy source. In the absence of substrate, azide at μMolar concentrations had little if any effect on mitochondrial volume.

Energy-independent mitochondrial swelling induced by azide - Palmieri and Klingenberg (44) observed that azide induced swelling of mitochondria suspended in the presence of ammonium chloride, but not in the presence of potassium chloride or potassium acetate. Fig. 7 extends their observations to show that mitochondrial swelling induced by 30 mM azide was completely arrested by the addition of 10 mM Mg^{++}. Further, the rate of mitochondrial swelling in ammonium chloride medium was dependent on azide concentration, since the rate increased with successive additions of azide (Fig. 8). Interestingly, the inclusion of 3.3 mM succinate in the ammonium chloride medium prevented azide induced swelling of the mitochondria.

Comparison of these activities of azide with those of Fig. 6 differentiated two types of mitochondrial swelling. One type of mitochondrial swelling, driven by respiratory substrate or ATP hydrolysis is inhibited by azide, reflecting an interaction with energy transfer. Figs. 7 and 8 show a second type of swelling where azide at high concentrations enters the mitochondria as a permeant anion in the absence of an added energy source. Swelling was independent of energy transfer when a permeant cation such as NH_4^+ was present.

Mitochondrial ATPase activities inhibited by azide - Fig. 9 shows the inhibitory effect of azide on rat liver mitochondrial ATPase activated by 100 μM DNP. Inhibition increased with inhibitor concentration, 20 mM azide inhibiting 70% of the DNP-activated ATPase activity. The sensitivity of DNP-activated ATPase to azide was enhanced by the presence of 50 μM ADP, 85% inhibition of enzyme activity being obtained with 20 mM azide.

Kinetic analysis showed that azide inhibition of DNP-activated ATPase in intact mitochondria was competetive with a K_i of 33 μM (Fig. 10).

The results depicted in Fig. 11 demonstrates the inhibition of purified ATPase by azide. Some inhibition of the ATPase from beef heart was observed at 1.0 μM azide, while ATPase from rat liver mitochondria, was not affected by azide at this concentration. However, ATPase from both rat liver and beef heart mitochondria were strongly inhibited by azide at concentrations above 10 μM with maximal inhibition being given by about 10 mM azide. Half-maximal inhibition was obtained with 100 μM azide in both rat liver and

beef heart ATPase.

The sensitivity of ATPase to azide was tested in the presence of 50 μM ADP (Fig. 11). Under these conditions, purified rat liver mitochondrial ATPase was more sensitive to azide than when ADP was absent. Maximal inhibition was given between 4.0 - 6.0 mM azide and the azide concentration required for half maximal inhibition in the presence of ADP was 30 μM.

Respiratory Inhibition - Wilson and Chance (36) observed that mitochondria respiring in the presence of substrate and ADP (state 3) are more sensitive to inhibition by azide than mitochondria respiring in the presence of substrate alone (state 4). The results depicted in Fig. 12 confirm their observations. In the presence of succinate and ADP, inhibition of mitochondrial respiration was linear with azide concentration up to 500 μM. Above this concentration, respiratory inhibition approached a maximum. In the presence of succinate alone (state 4), azide at concentrations up to 500 μM caused a linear increase in respiratory rate. Higher concentrations of azide depressed oxygen uptake to the state 4 level.

Figure 6

The inhibition of energy-linked mitochondrial swelling by azide. The mitochondrial suspension was adjusted to an initial optical density of 0.8 at 520 nm in medium containing 120 mM potassium acetate, 2.0 mM EGTA, pH 7.4. Volume 3.0 ml. An upward deflection of the trace indicates swelling. Rate of swelling in OD units/minute are given above each trace. Arrows indicate additions of 330 μM azide. Substrates are indicated opposite each trace.

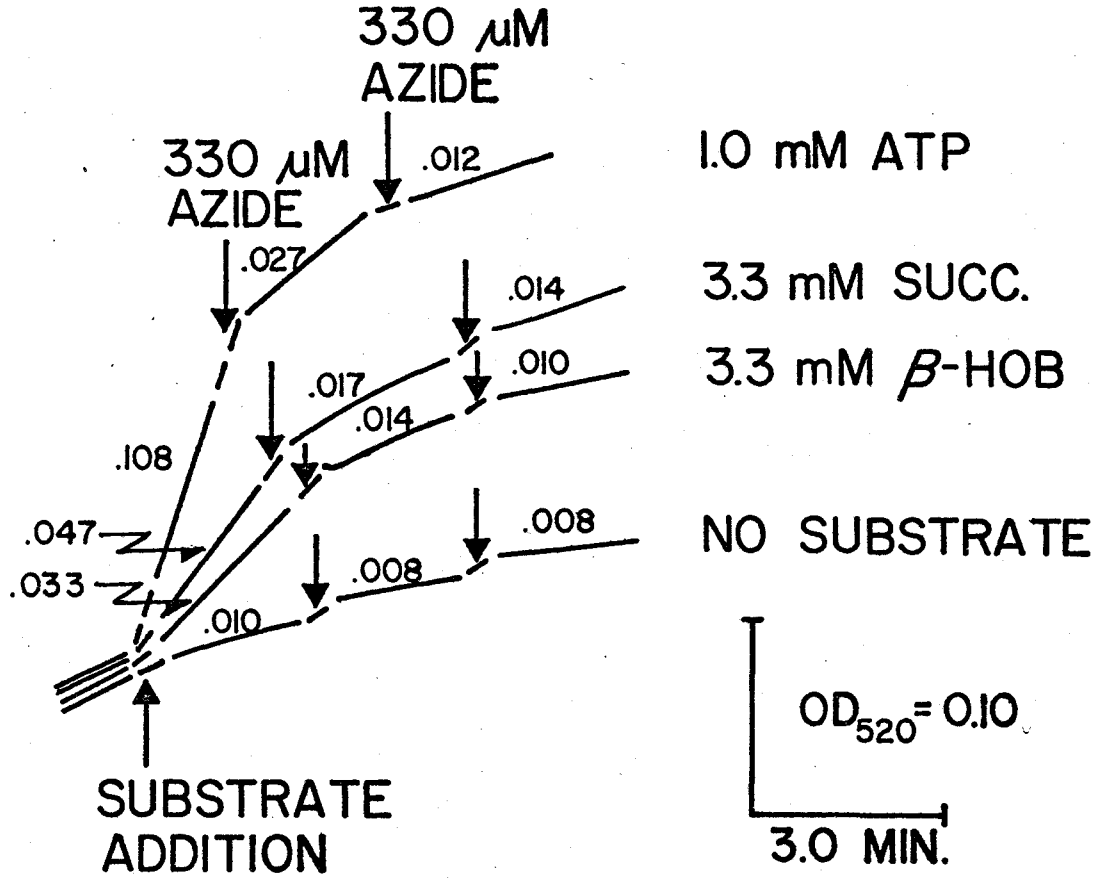

330 µM AZIDE
330 µM AZIDE
.012
.027
.014
.010
.017
.014
.108
.047
.033
.008
.008
.010
1.0 mM ATP
3.3 mM SUCC.
3.3 mM β-HOB
NO SUBSTRATE
SUBSTRATE ADDITION
OD520= 0.10
3.0 MIN.

Figure 7

Effect of Azide and Mg^{++} on mitochondrial volume in isontonic media. The reaction medium contained 2.0 mM EGTA, 6 µg rotenone and 120 mM of the indicated salt pH 7.4. 30 mM azide and 10 mM $MgCl_2$ were added as indicated.

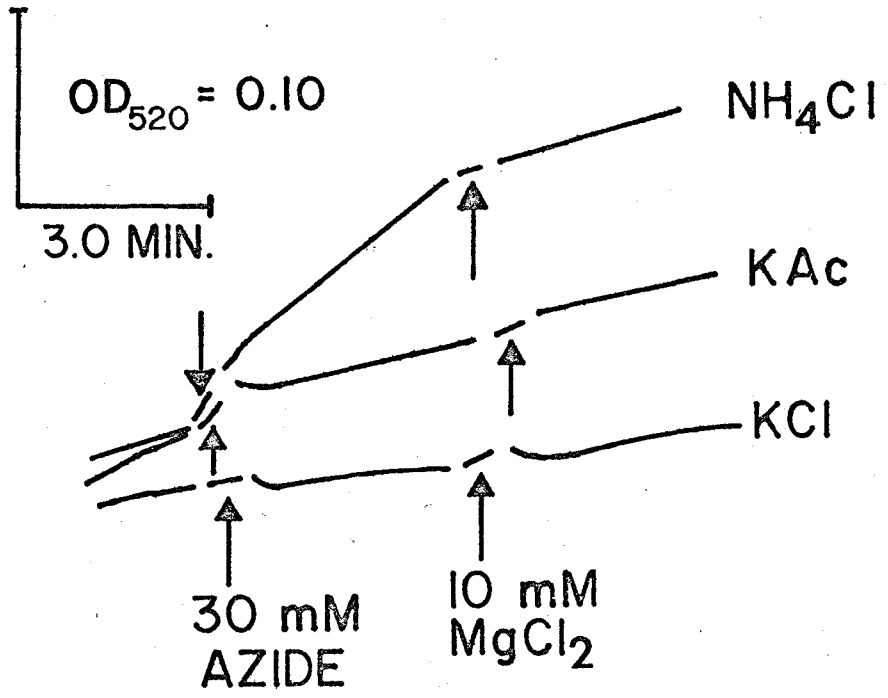

OD_{520} = 0.10
3.0 MIN.
NH_4Cl
KAc
KCl
30 mM
AZIDE
10 mM
$MgCl_2$

Figure 8

Succinate control of azide-induced mitochondrial swelling in isotonic ammonium chloride medium. The reaction medium contained 2.0 mM EGTA and 120 mM ammonium chloride, pH 7.4. An upward deflection in the trace indicates swelling.

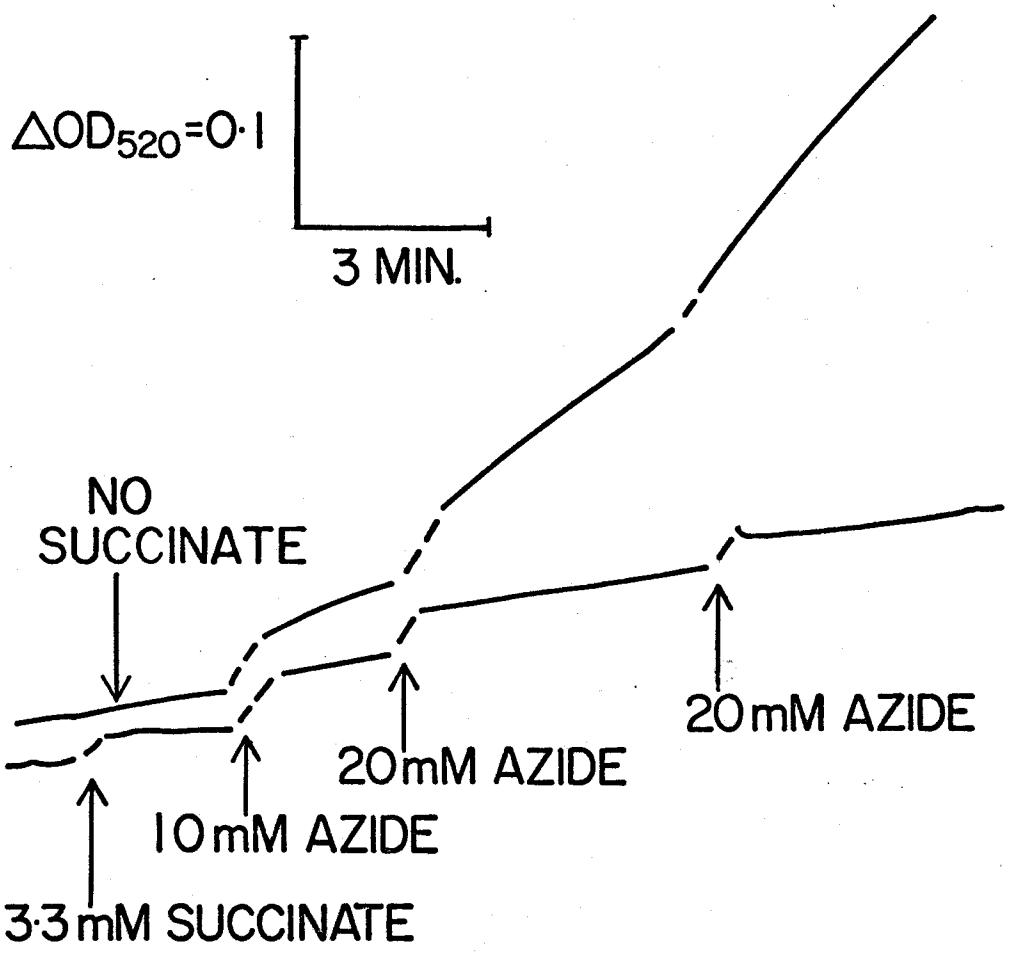

ΔOD520=0·1
3 MIN.
NO SUCCINATE
20mM AZIDE
20mM AZIDE
10mM AZIDE
3·3mM SUCCINATE

Figure 9

Effect of sodium azide on the hydrolysis of ATP in rat liver mitochondria uncoupled with 100 μM 2,4-dinitrophenol in the absence of ADP (●----●) and with 50 μM ADP in the incubation medium (O----O). Incubation medium, 250 mM sucrose, 50 mM Tris, 0.1 mM $MgCl_2$, 1.0 mM ATP, pH 7.4. Incubation time, 15 minutes, temperature 22°.

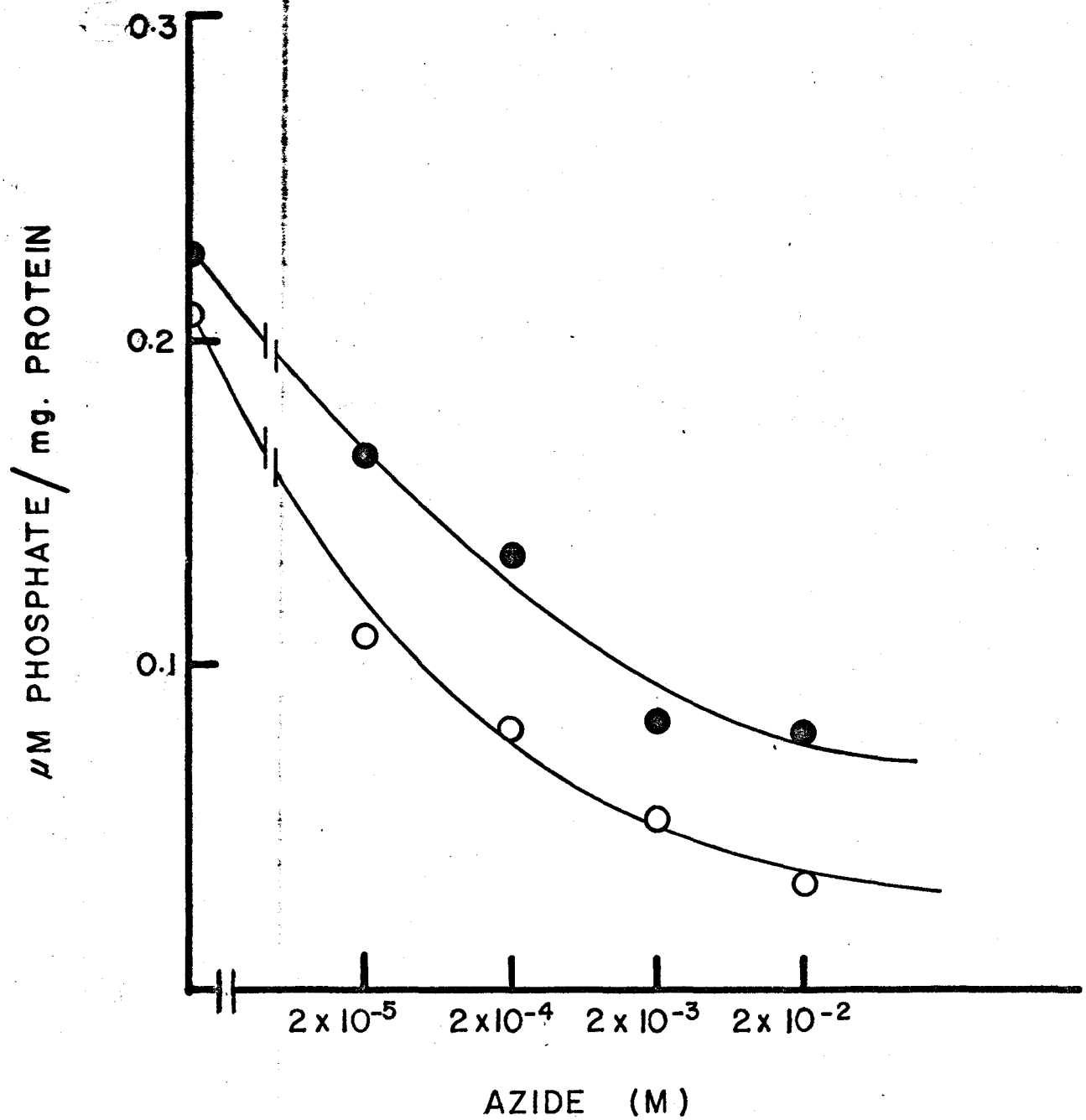

0·3
0·2
0·1
μM PHOSPHATE / mg. PROTEIN
2 x 10⁻⁵
2x10⁻⁴
2 x 10⁻³
2 x 10⁻²
AZIDE (M)

Figure 10

Double reciprocal plot of azide inhibition of DNP-activated ATPase in rat liver mitochondria. The mitochondrial suspension was 0.7 mg protein/ml in medium containing 75 mM KCl, 50 mM sucrose, 1.0 mM $MgCl_2$, 0.6 mM EDTA, 100 μM DNP, 50 mM Tris, pH 7.4. (●----●), no azide; (O----O), 100 μM azide.

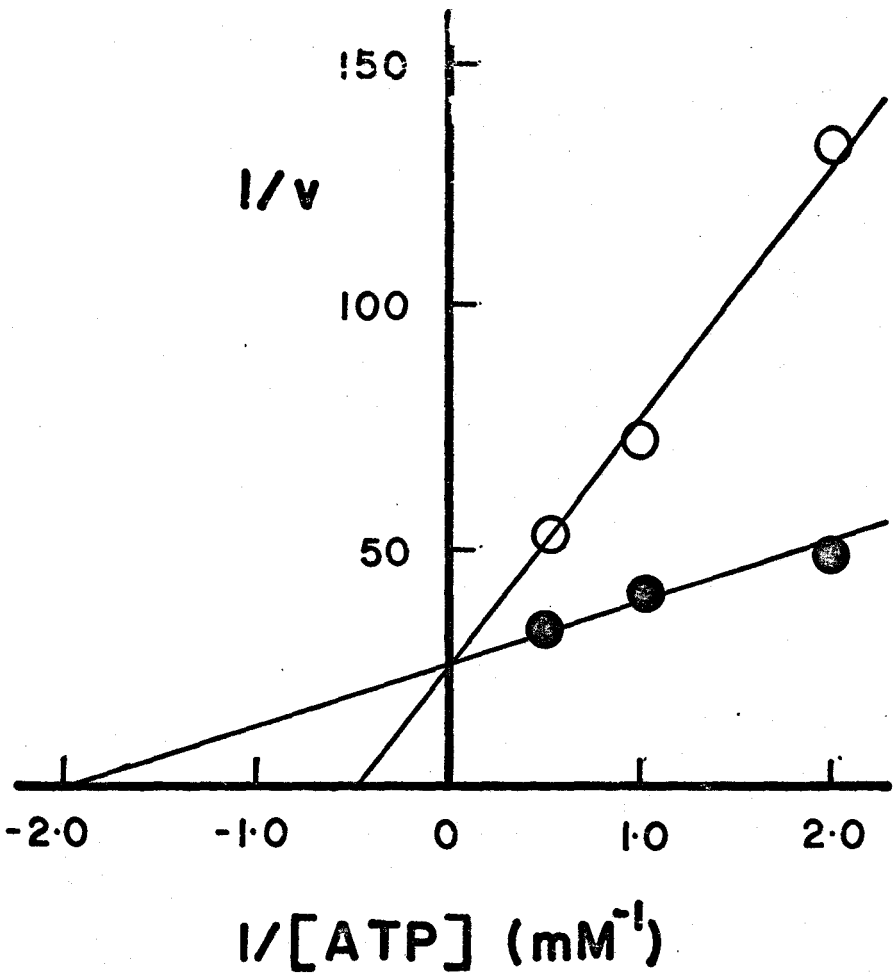

150
100
50
1/v
-2·0
-1·0
0
1·0
2·0
1/[ATP] (mM⁻¹)

Figure 11

Effect of sodium azide on the hydrolysis of ATP by purified ATPase from beef heart and rat liver mitochondria. (●----●) Beef heart ATPase; (O----O) rat liver ATPase; (Δ----Δ) rat liver ATPase in the presence of 50 μM ADP. Conditions of incubation were the same as in Fig. 9.

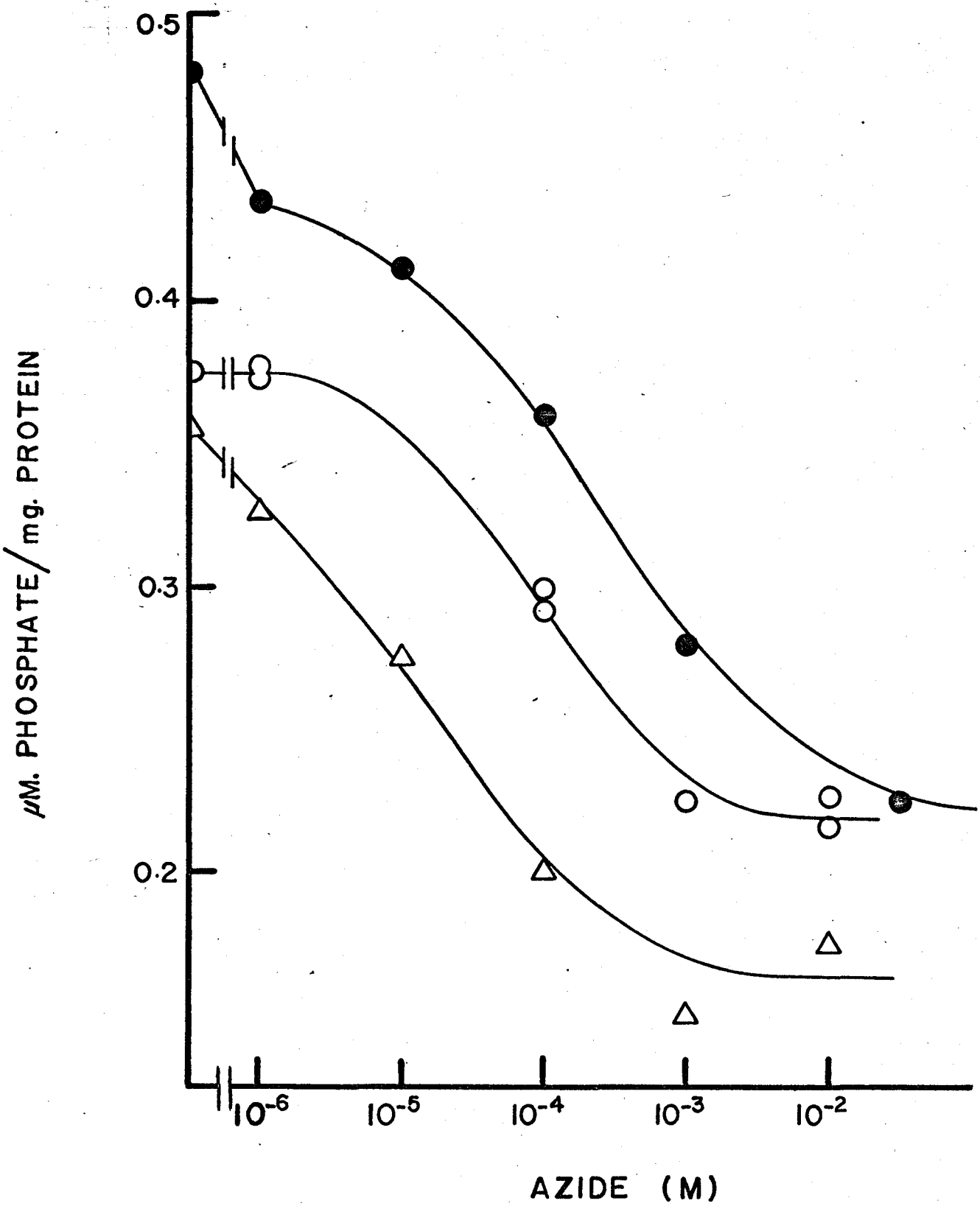
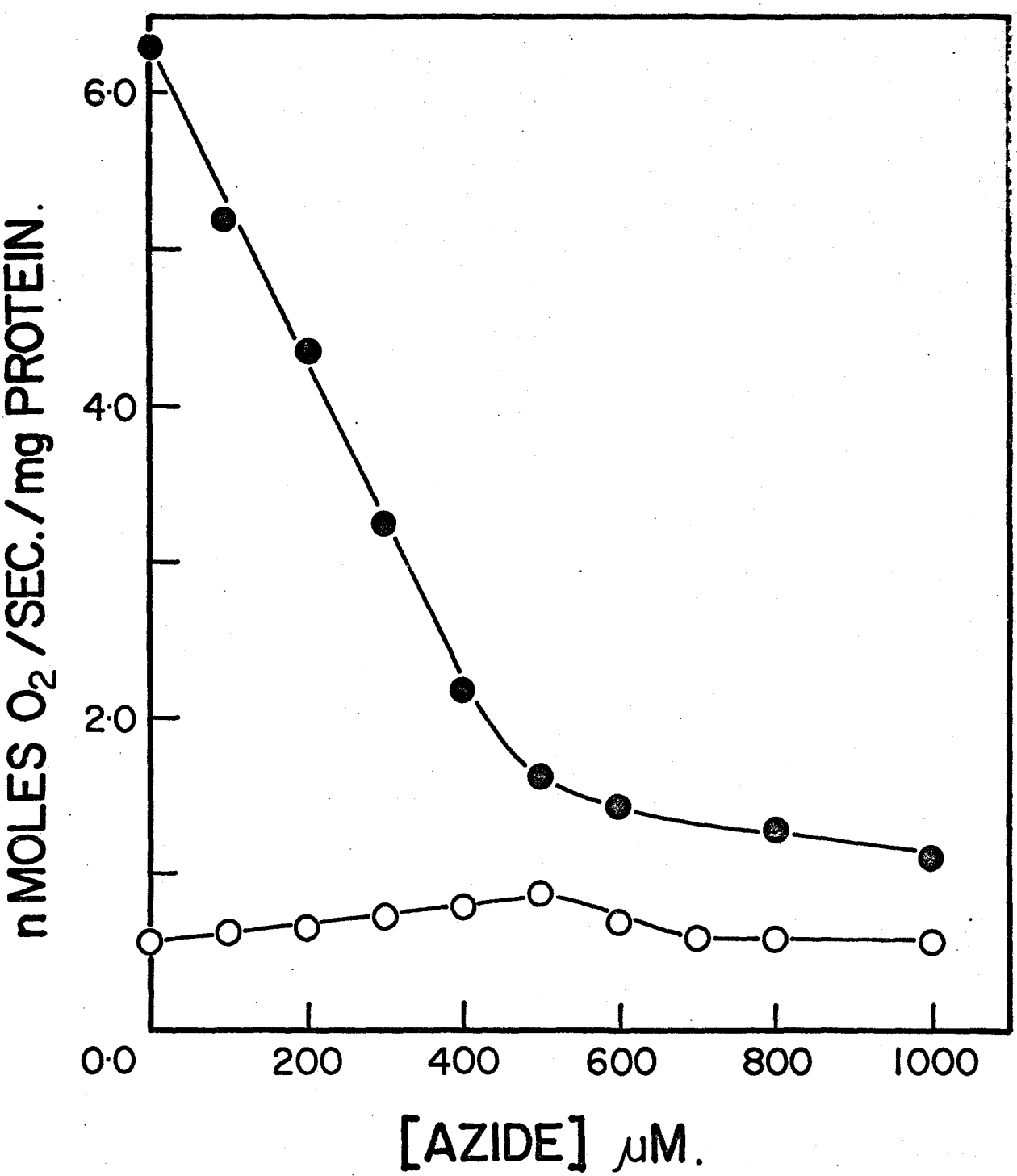

0.5
0.4
0.3
0.2
μM. PHOSPHATE/mg. PROTEIN
10-6
10-5
10-4
10-3
10-2
AZIDE (M)

Figure 12

Azide inhibition of oxygen uptake by rat liver mitochondria. The mitochondrial suspension contained 2.0 mg protein/ml in reaction medium outlined in methods. 6.0 μM rotenone was added prior to the substrate which was 10 mM succinate. (O----O), no ADP; (●----●), 600 μM ADP. Oxygen uptake expressed as nmoles O_2/sec/mg protein.


6·0
4·0
2·0
0·0
200
400
600
800
1000
nMOLES O_2 /SEC./mg PROTEIN.
[AZIDE] μM.

CHAPTER 4

PHLORIZIN INHIBITION OF OXIDATIVE PHOSPHORYLATION

Phosphorylation efficiency and respiratory control - When tightly coupled rat liver mitochondria were treated with phlorizin, there was a decrease in the ability of ADP to stimulate respiration and a decline in the ADP/O ratio (Table 2). With succinate as substrate, phlorizin, at concentrations up to 700 μM, decreased respiratory control ratios by stimulation of state 4 respiration. State 3 respiration, on the other hand, remained unchanged throughout this range. When the phlorizin concentration was increased to 1.0 mM, state 3 respiration was inhibited and both respiratory control and ADP/O ratios were abolished. In a series of experiments with β-hydroxybutyrate as substrate, phlorizin similarly stimulated state 4 respiration. Phlorizin also inhibited the state 3 oxidation of this substrate in contrast to the results obtained in the presence of succinate; 700 μM phlorizin abolishing ADP/O and respiratory control ratios completely.

Results of experiments with phloretin, the aglucone of phlorizin, were similar to effects of phlorizin on phosphorylation efficiency and respiratory control (Table 3). With β-hydroxybutyrate or succinate as substrate, phloretin, at one-third the concentration of phlorizin, abolished respiratory control and ADP/O ratios.

Substrate Oxidation in State 3 and State 4 - When tightly coupled mitochondria were respiring at a maximal rate in the presence

of excess NADH-linked substrates and ADP, the addition of phlorizin caused an immediate inhibition of oxygen uptake. Figure 13, A and B, shows that inhibition increased with inhibitor concentration up to 400 μM phlorizin in the presence of β-hydroxybutyrate or α-ketoglutarate. A plateau region existed between 400 μM and 600 μM, and, above 600 μM, phlorizin strongly inhibited the oxidation of both substrates. In the absence of ADP, phlorizin stimulated the rate of oxygen uptake with maximal stimulation at 200 μM. Phlorizin above this concentration was strongly inhibitory, 600 μM giving almost complete inhibition. Inhibition of respiration uncoupled by DNP in the presence of excess β-hydroxybutyrate was linear with phlorizin concentration up to 700 μM (Fig. 13,B). At a concentration of 1.0 mM phlorizin, the inhibited respiratory rates were the same in the presence or absence of ADP or DNP. The double reciprocal plot of Fig. 14 displayed mixed inhibition (113) weighted towards noncompetative inhibition of state 3 β-hydroxybutyrate oxidation, with a K_i of 1.3 mM.

Similar results were obtained in an identical series of experiments with phloretin. As shown in Fig. 15 the addition of phloretin to tightly coupled mitochondria respiring in the presence of excess β-hydroxybutyrate and ADP resulted in a prompt decrease in oxygen uptake. Inhibition was curvilinear with inhibitor concentration, maximal inhibition being obtained with 170 μM phloretin. As with phlorizin, phloretin had a biphasic effect on the state 4 oxidation of β-hydroxybutyrate. 60 μM phloretin maximally stimulated respiration in the presence of this substrate.

Phloretin at higher concentrations decreased the rate of oxygen uptake, with 120 μM depressing respiratory rates to the initial state 4 level.

Repeating these experiments using succinate as substrate, and phlorizin as inhibitor, no inhibition of state 3 oxidation was observed until the phlorizin concentration reached levels above 700 μM. Phlorizin stimulated state 4 succinate oxidation at concentrations up to 1.2 mM (Fig. 16).

In contrast, phloretin, at concentrations up to 350 μM, inhibited oxygen uptake in the presence of succinate and ADP. Inhibition was linear with inhibitor concentration. The activity of phloretin on state 4 succinate oxidation was qualitatively identical to that observed with phlorizin in that stimulation of oxygen uptake was linearly dependent on phloretin concentration. A three-fold increase in respiratory rate, representing maximal stimulation, was obtained with 260 μM phloretin (Fig. 17).

Effect of DNP, Rotenone and ATP on Inhibition - No release of respiratory inhibition was observed when uncoupling concentrations of DNP were added to a system in which state 3 oxidation was inhibited by phlorizin (Fig. 18, A and B). The addition of 100 μM DNP to mitochondria respiring in the presence of succinate and 1.0 mM phlorizin caused a fourfold increase in the rate of respiration which slowed after one minute to a rate equivalent to that observed prior to the addition of DNP. Subsequent addition of ATP and oligomycin stimulated respiration. Further, in the presence of phlorizin plus rotenone, the maximal respiratory rate obtained by the addition of uncoupling concentrations

of DNP was maintained until anaerobiosis (Fig. 18, C and D).

Effect of Oligomycin on Uncoupling - The addition of 2.5 µg of oligomycin to a mitochondrial preparation respiring in state 3 decreased oxygen uptake to a rate which was the same as that in the presence of β-hydroxybutyrate alone. This inhibition was not relieved by the subsequent addition of phlorizin at concentrations up to 1.0 mM (Fig. 19, A). When the experiment was repeated using succinate as substrate phlorizin stimulated respiration slightly (Fig. 19, B).

The experiment was repeated using phloretin and Trace C of Fig. 19 shows that this compound, at a concentration of 240 µM, induced a two-fold increase in the rate of oxygen uptake.

Inhibition of ATPase activity - Studies were carried out on DNP-activated ATPase, Mg^{++}-activated ATPase, and partially purified ATPase activity isolated by sonication. As shown in Fig. 20, all ATPase activities were sensitive to 10 µM phlorizin. While the relative insolubility of phlorizin in water prevented measurements much above 1.0 mM the linear inhibition plot which was obtained may be extrapolated to give maximal inhibition of DNP-stimulated ATPase at 7.8 mM and of isolated ATPase at about 2.4 mM. Mixed inhibition kinetics were observed intermediate between competetive and non-competative with a K_i of 280 µM as determined from the double-reciprocal plot of Fig. 21.

Phloretin exerted a similar effect on DNP-activated ATPase. Fig. 22 shows that the inhibitor at concentrations up to 50 µM had no effect on this ATPase activity. Minimal inhibition of DNP-activated-ATPase was obtained with 100 µM phloretin. Maximum inhibition of DNP activated ATPase was obtained with 1.0 mM phloretin. In contrast with

phlorizin, phloretin, at concentrations of 10 μM to 500 μM, stimulated the Mg^{++}-activated ATPase. The maximum concentration of phloretin used (1.0 mM) depressed Mg^{++}-activated ATPase and DNP-activated ATPase activity to about the same level.

A comparison was made of the inhibitory effects of phlorizin and Amytal on mitochondrial ATPase activated by DNP at various concentrations (Fig. 23). In the absence of inhibitors, DNP at a concentration of 5.0 μM stimulated ATPase activity ten-fold to maximum values. In the presence of 10 μM to 16 μM DNP, DNP-activated activity was minimal. DNP at higher concentrations enhanced ATPase activity slightly above minimal values. Maximum ATPase activity, obtained with 5.0 μM DNP, was decreased 34% in the presence of 300 μM Amytal. Amytal was much less inhibitory towards ATPase activity stimulated by DNP at higher concentrations. On the other hand about 50% inhibition of ATPase activated by all concentrations of DNP was observed in the presence of 500 μM phlorizin.

Site of Respiratory Inhibition - Difference spectra were obtained using digitonin particles reduced with NADH vs particles reduced with NADH in the presence of an inhibitory concentration of phlorizin (Fig. 24). With phlorizin in the sample cuvette and no inhibitor in the reference beam, troughs appeared at 605 nm, 560-562 nm and 550 nm when NADH was simultaneously added to both compartments. These results demonstrated that the sample containing the inhibitor had a slower anaerobiosis time than the uninhibited sample and that the site of inhibition lay previous to cytochrome b. In this experiment no peak corresponding to flavoprotein was observed. An experiment to test the response of flavoprotein to phlorizin inhibition was carried

out in the dual wavelength spectrophotometer. As is seen in Fig. 25, the addition of phlorizin to a mitochondrial sample respiring at a maximal rate in the presence of β-hydroxybutyrate and ADP resulted in the oxidation of this carrier. Similarly, oxidation of cytochrome b was observed upon the addition of phlorizin to a mitochondrial suspension respiring at a maximal rate in the presence of succinate and ADP. Thus, the tentative site of interaction may be placed at, or previous to the level of flavoprotein. Because phlorizin absorbs strongly in the region of 340 nm, its effect on the oxidation-reduction state of pyridine nucleotide could not be determined.

TABLE 2

Effect on Phlorizin on respiratory control and ADP/O

Respiratory rates expressed as n moles O_2/sec/mg protein. Rat liver mitochondria were suspended in reaction medium described in Methods, Protein concentration was 1.8 mg/ml with succinate as substrate and 1.6 mg/ml with β-hydroxybutyrate. Substrate concentration was 10 mM.

Substrate	Phlorizin (μM) concentration	Respiratory rate -ADP	Respiratory rate +ADP	Respiratory Control Ratio	ADP/O
Succinate	0	1.50	6.40	4.4	2.0
	100	1.55	5.55	3.6	1.8
	300	2.00	6.65	3.3	1.7
	500	2.50	7.40	3.0	1.5
	700	2.95	6.25	2.1	1.4
	1000	4.30	4.65	---	---
β-HOB	0	0.56	2.50	4.5	2.9
	50	0.72	2.06	3.0	2.8
	200	0.69	1.80	2.6	2.4
	300	0.82	1.75	2.1	2.1
	500	1.00	1.45	1.5	1.8
	700	1.06	1.06	---	---

TABLE 3

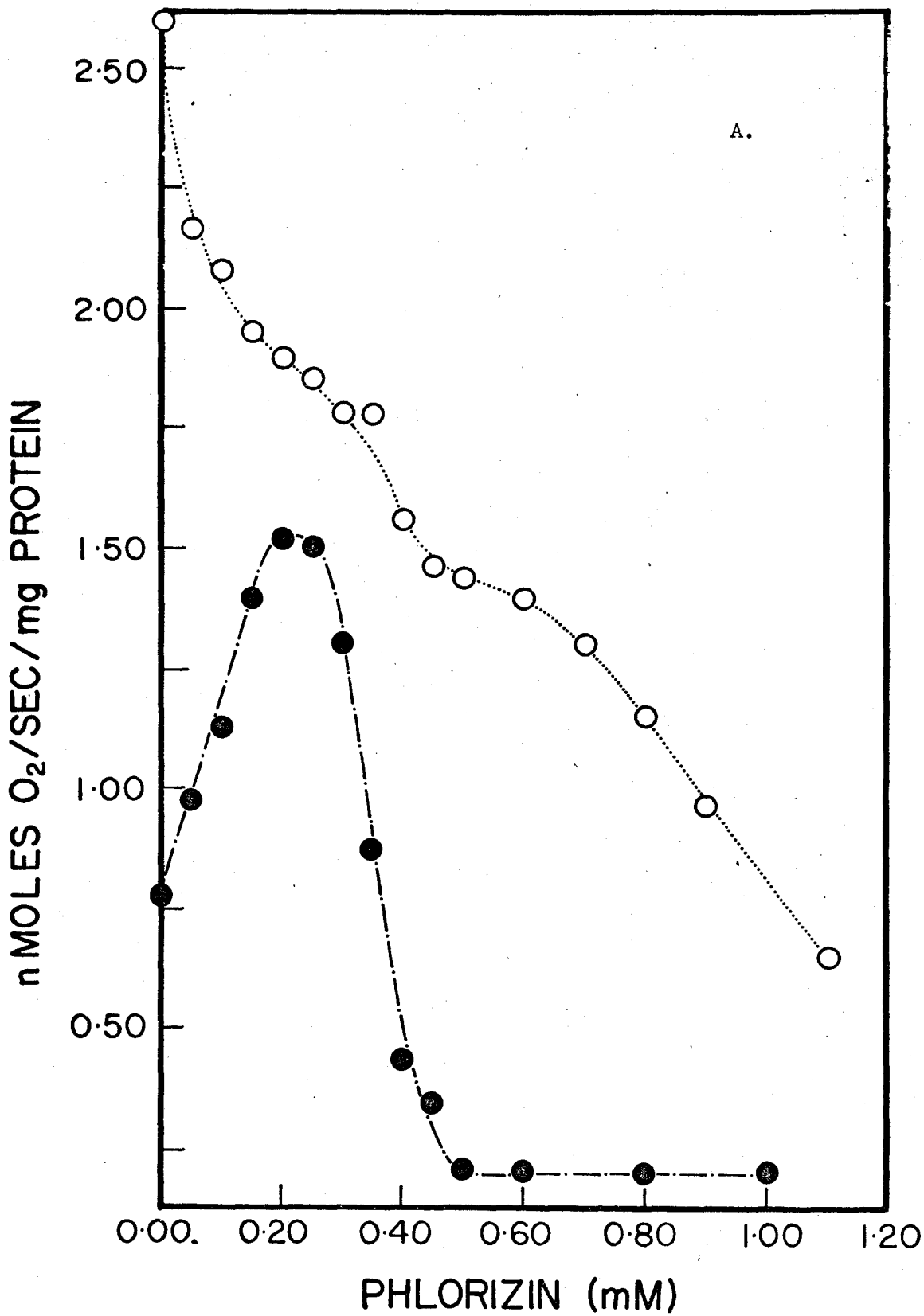
Effect of Phloretin on respiratory control and ADP/O

Protein concentration was 2.0 mg/ml with succinate as substrate and 1.5 mg/ml with β-hydroxybutyrate. Respiratory rates and experimental conditions as described in Table 2.

Substrate	Phloretin (μM) concentration	Respiratory rate -ADP	Respiratory rate +ADP	Respiratory Control ratio	ADP/O
Succinate	0	0.88	5.05	5.8	1.9
	100	1.5	4.5	3.0	1.5
	200	2.0	3.3	1.6	1.1
	300	2.4	2.9	1.2	0.88
	350	2.2	2.3	---	----
β-hydroxy-butyrate	0	0.64	2.7	4.2	2.75
	20	0.65	2.1	3.2	2.15
	40	1.3	2.1	1.6	1.9
	100	1.3	1.9	1.5	1.4
	140	1.0	1.2	1.2	1.2
	180	0.9	0.9	---	---

Figure 13

Effect of phlorizin on the oxidation of α-ketoglutarate (A) and β-hydroxybutyrate, (B). (O----O) 590 μM ADP present; (●----●), ADP absent; (Δ----Δ), 33 μM DNP present. Respiratory rates are given as nmoles of oxygen/sec/mg protein. The reaction conditions are described in Methods. Mitochondrial protein was 1.8 mg/ml.


A.
2·50
2·00
1·50
1·00
0·50
nMOLES O2/SEC/mg PROTEIN
0·00
0·20
0·40
0·60
0·80
1·00
1·20
PHLORIZIN (mM)

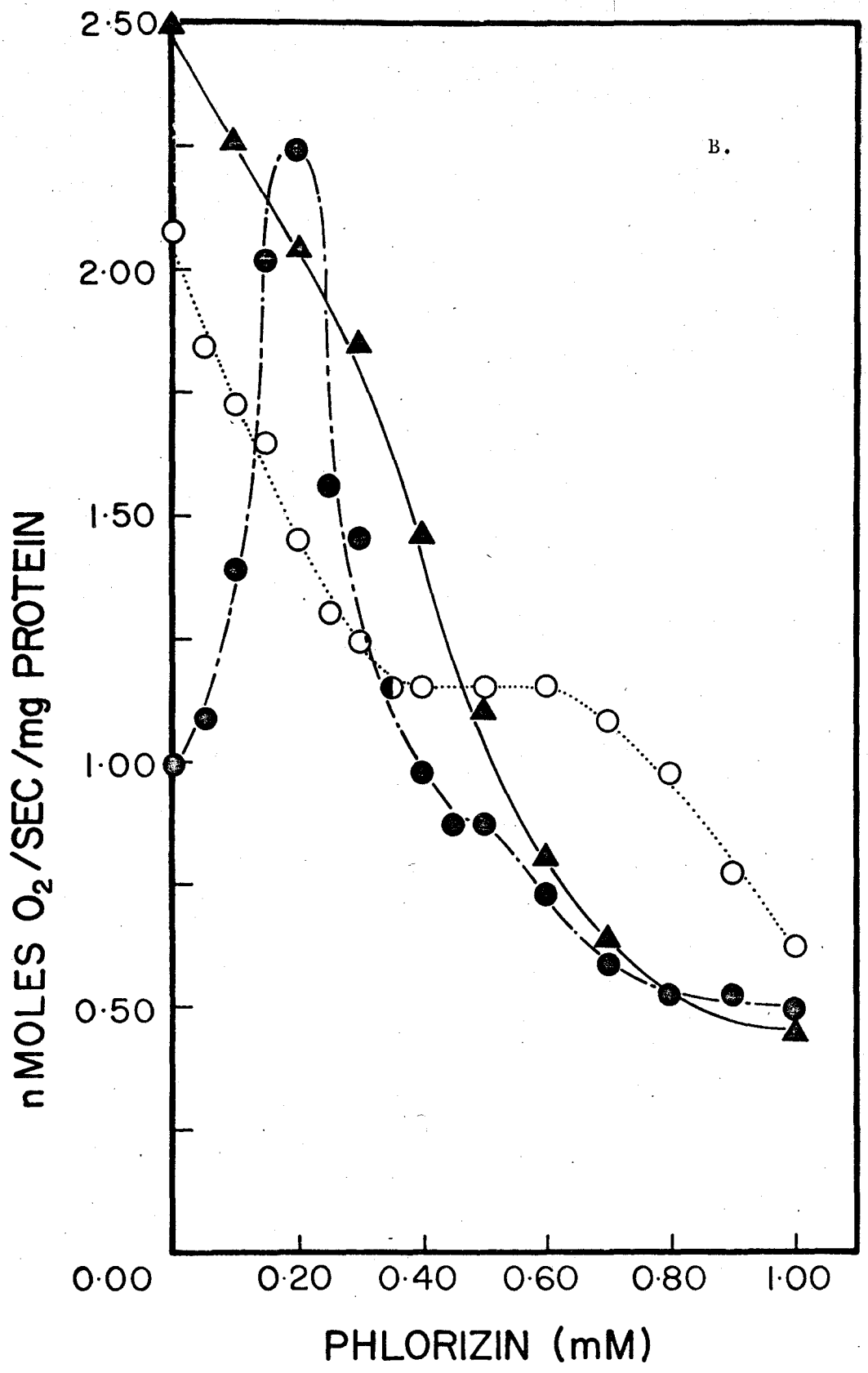

B.
2·50
2·00
1·50
1·00
0·50
0·00
0·20
0·40
0·60
0·80
1·00
n MOLES O_2 /SEC /mg PROTEIN
PHLORIZIN (mM)

Figure 14

Double reciprocal plot of phlorizin inhibition of β-hydroxybutyrate oxidation in state 3. Experimental conditions were as described in Methods with 2.2 mg protein/ml. Phlorizin was added after maximal respiration was attained with 590 μM ADP. (0----0), phlorizin absent; (●----●), 1.0 mM phlorizin.

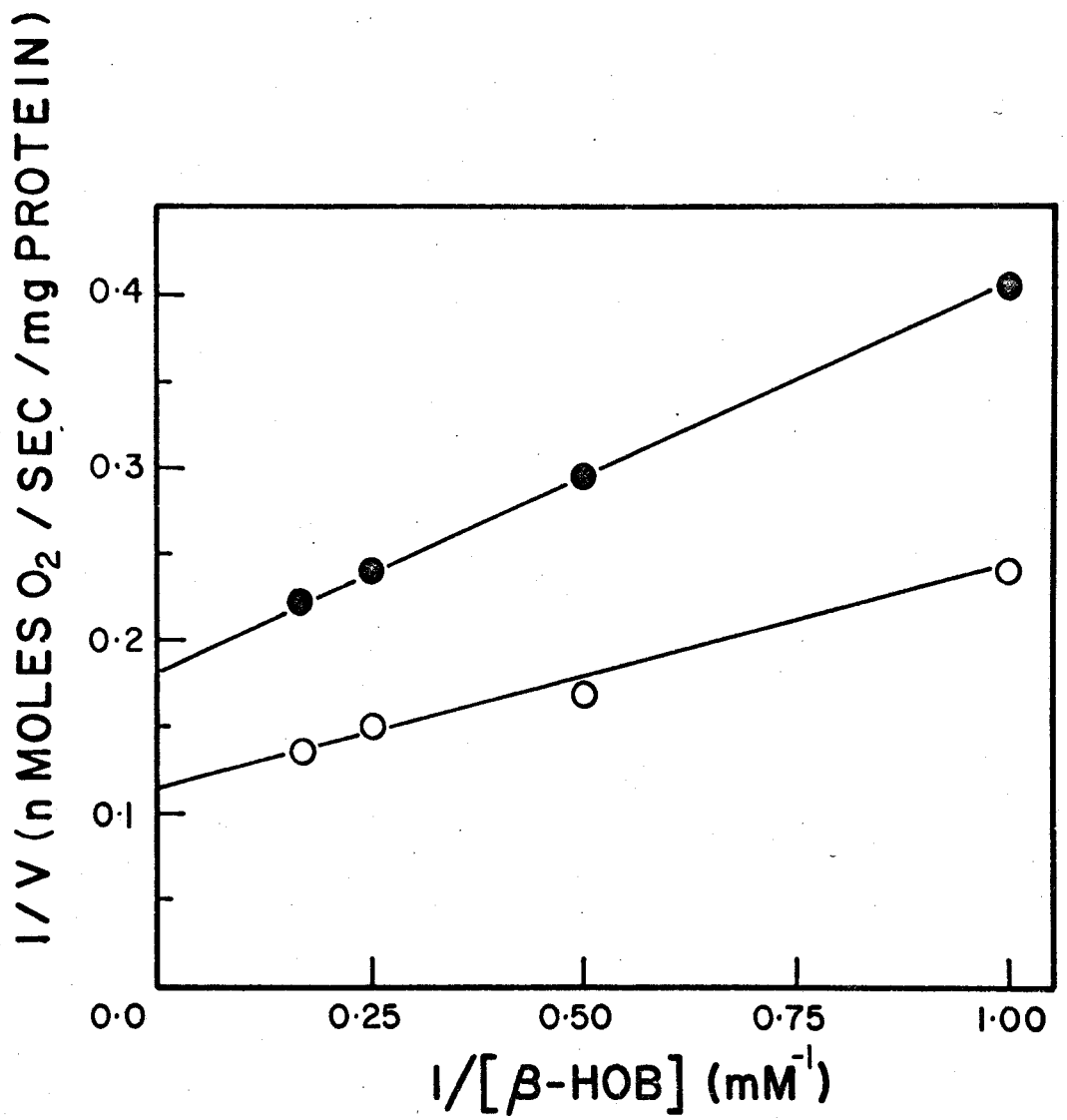

1/V (n MOLES O_2 / SEC / mg PROTEIN)
0·4
0·3
0·2
0·1
0·0
0·25
0·50
0·75
1·00
1/[β-HOB] (mM^{-1})

Figure 15

The activity of phloretin on the state 3 and state 4 oxidation of β-hydroxybutyrate in the presence of ADP (O-----O) and in the absence of ADP (●-----●). Experimental conditions are as described in Methods. Mitochondria were suspended at a concentration of 1.8 mg/ml.

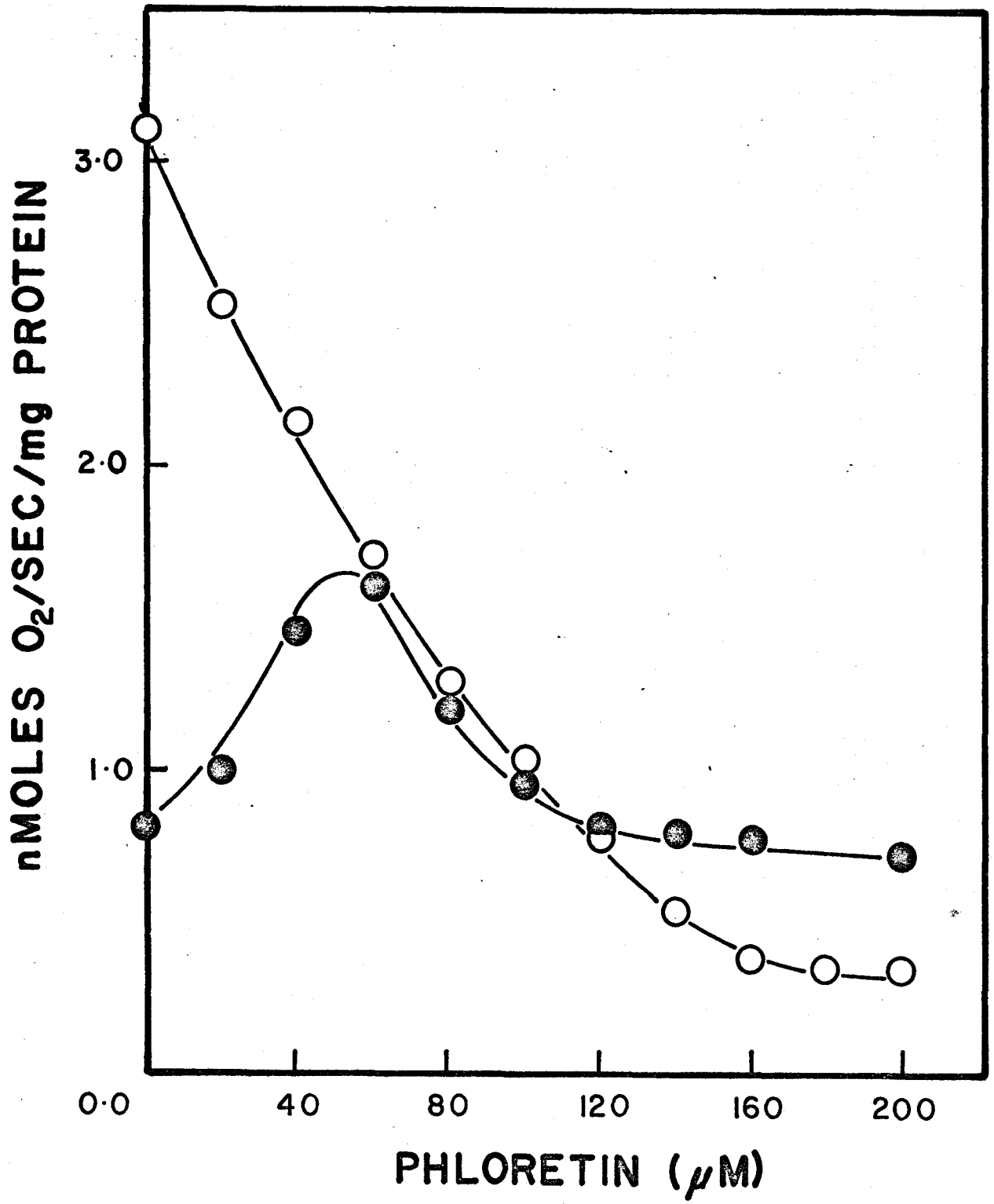

nMOLES O_2/SEC/mg PROTEIN
3·0
2·0
1·0
0·0
40
80
120
160
200
PHLORETIN (μM)

Figure 16

Effect of phlorizin on succinate oxidation. (0----0), state 3; (●----●), state 4. Protein was 1.9 mg/ml. Experimental conditions as in Methods.

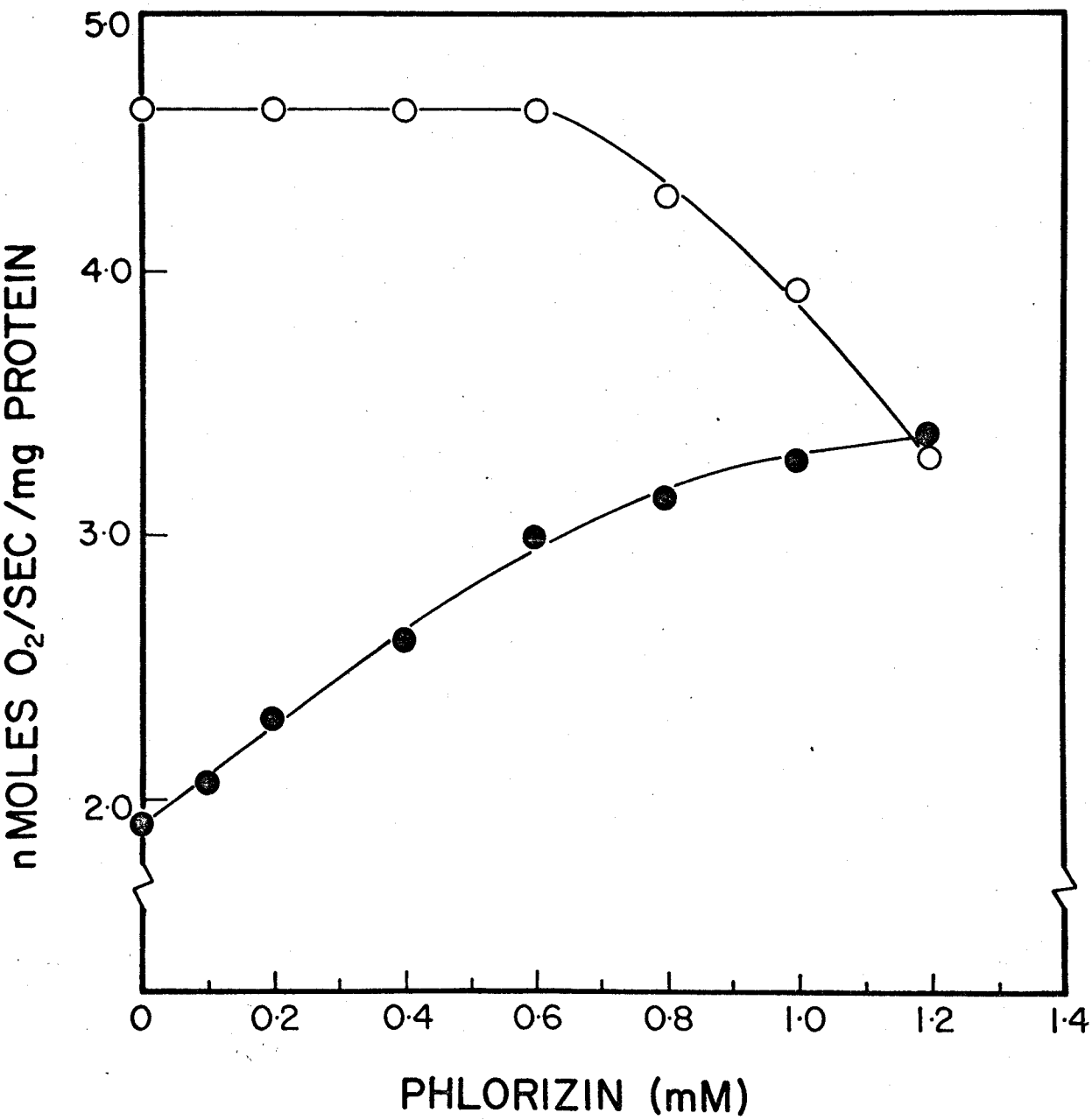

5·0
4·0
3·0
2·0
n MOLES O_2/SEC /mg PROTEIN
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
PHLORIZIN (mM)

Figure 17

The effect of phloretin on succinate oxidation. (●----●), 530 μM ADP present; (O----O), ADP absent. The mitochondrial suspension contained 2.0 mg protein/ml. Other conditions as in Methods.

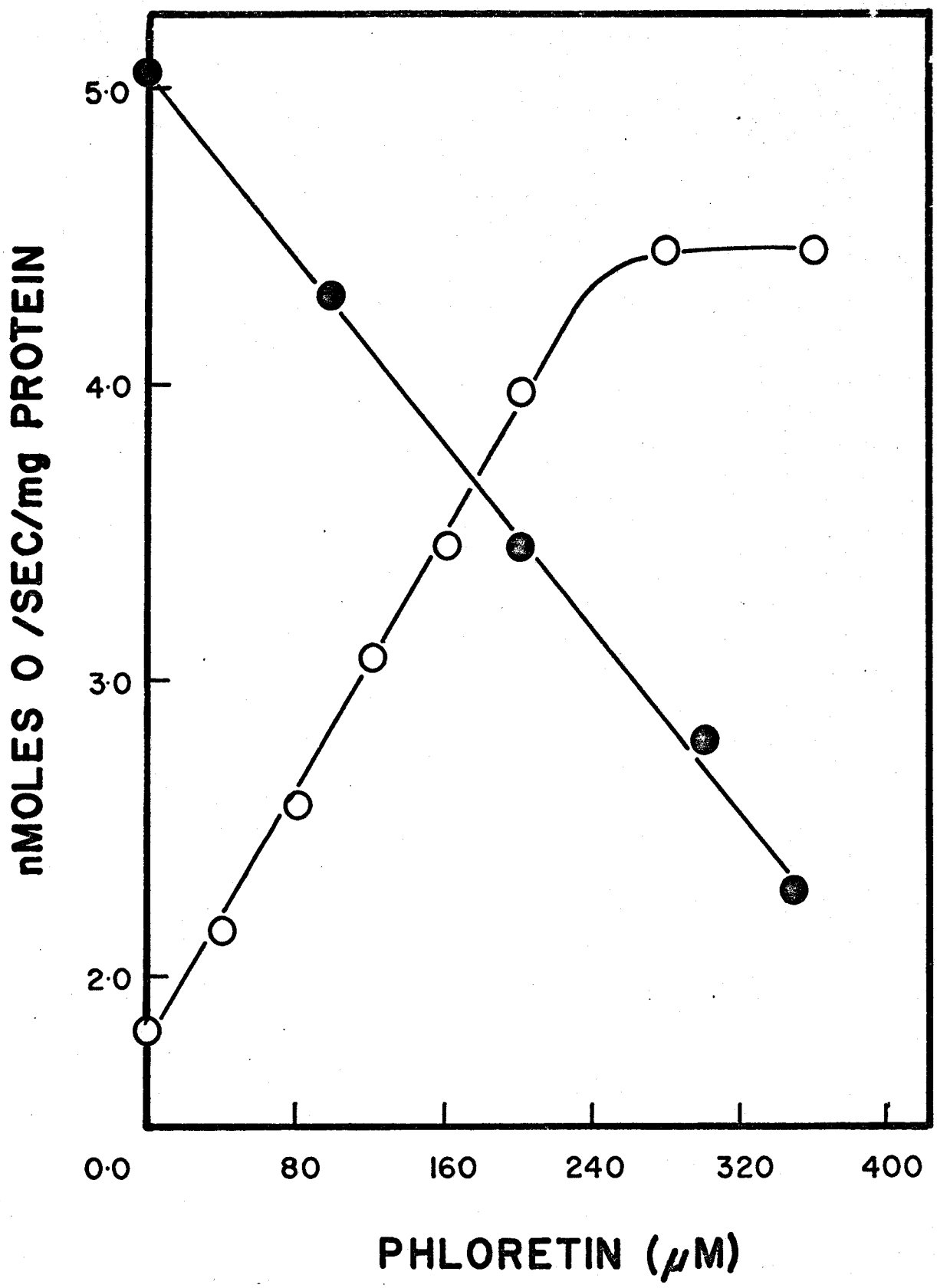

5·0
4·0
3·0
2·0
0·0
80
160
240
320
400
nMOLES O /SEC/mg PROTEIN
PHLORETIN (μM)

Figure 18

Effect of DNP, rotenone, and ATP on respiration in the presence of phlorizin. Respiratory rates are shown above the corresponding curve in nmoles O_2/sec/mg protein. Experimental conditions were as outlined in Methods.

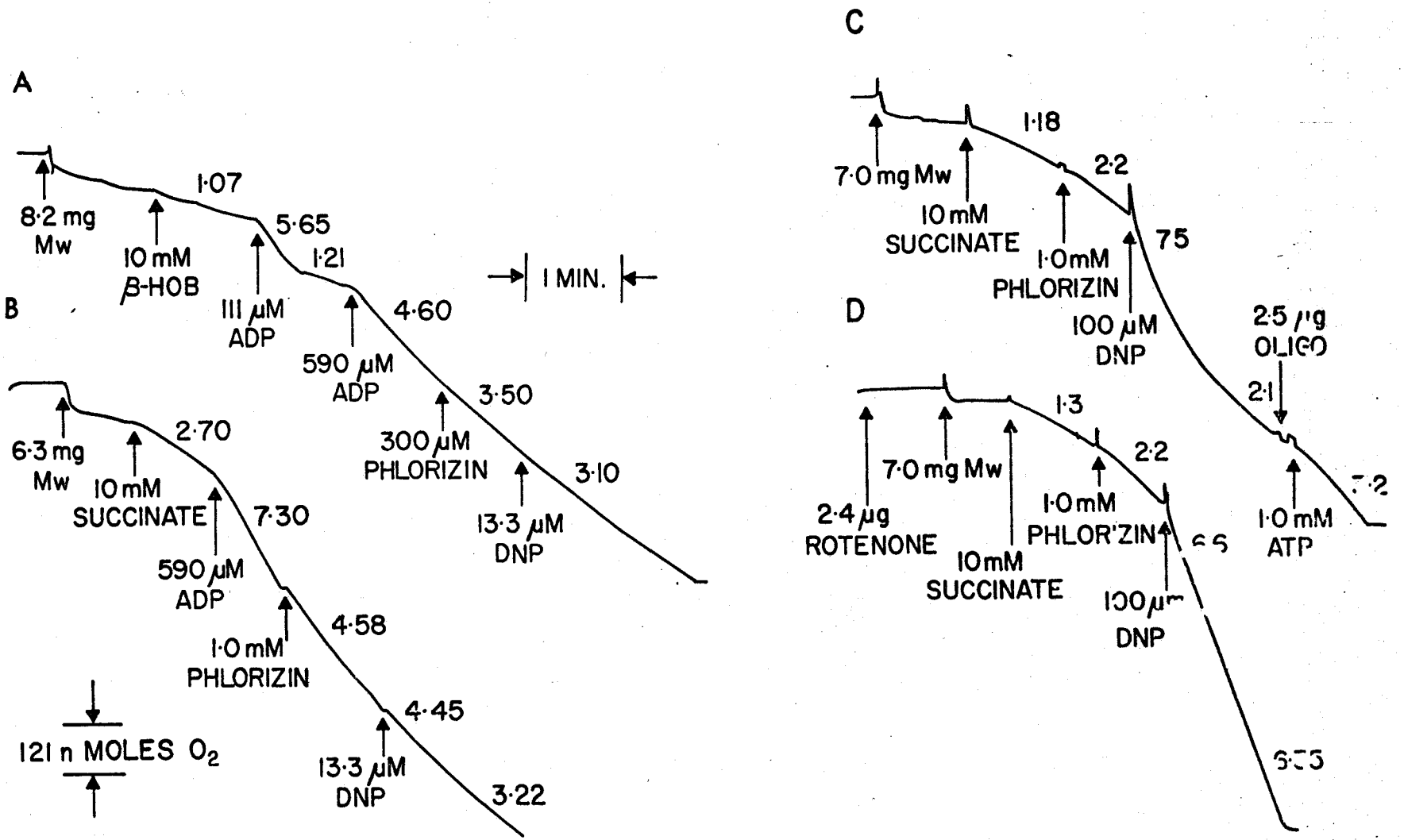
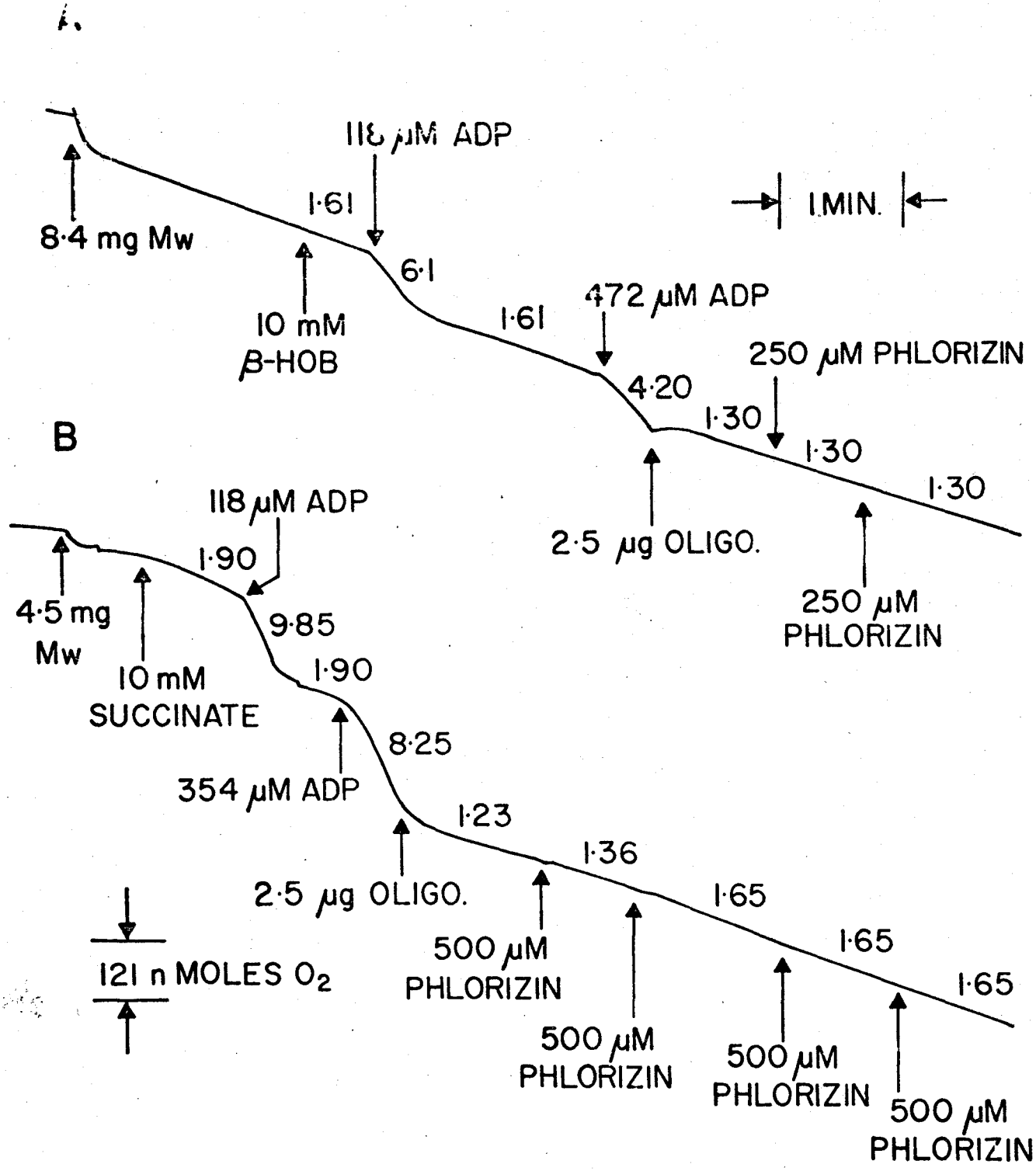

A
8·2 mg Mw
1·07
10 mM β-HOB
5·65
1·21
111 μM ADP
4·60
590 μM ADP
3·50
300 μM PHLORIZIN
3·10
13·3 μM DNP
1 MIN.
B
6·3 mg Mw
2·70
10 mM SUCCINATE
7·30
590 μM ADP
4·58
1·0 mM PHLORIZIN
4·45
13·3 μM DNP
3·22
121 n MOLES O2
C
7·0 mg Mw
1·18
10 mM SUCCINATE
2·2
1·0 mM PHLORIZIN
75
100 μM DNP
D
2·4 μg ROTENONE
7·0 mg Mw
1·3
10 mM SUCCINATE
2·2
1·0 mM PHLORIZIN
100 μM DNP
2·5 μg OLIGO
2·1
1·0 mM ATP

Figure 19

The release of oligomycin inhibition of respiration by phlorizin and phloretin. Numbers above each trace indicate respiratory rates in nmoles O_2/sec/mg protein. Reaction conditions as outlined in Methods.


A
118 μM ADP
1·61
8·4 mg Mw
1 MIN.
6·1
10 mM
β-HOB
1·61
472 μM ADP
250 μM PHLORIZIN
4·20
1·30
1·30
B
2·5 μg OLIGO.
250 μM
PHLORIZIN
118 μM ADP
1·90
4·5 mg
Mw
9·85
10 mM
SUCCINATE
1·90
8·25
354 μM ADP
1·23
1·36
2·5 μg OLIGO.
1·65
121 n MOLES O2
500 μM
PHLORIZIN
1·65
500 μM
PHLORIZIN
500 μM
PHLORIZIN
1·65
500 μM
PHLORIZIN

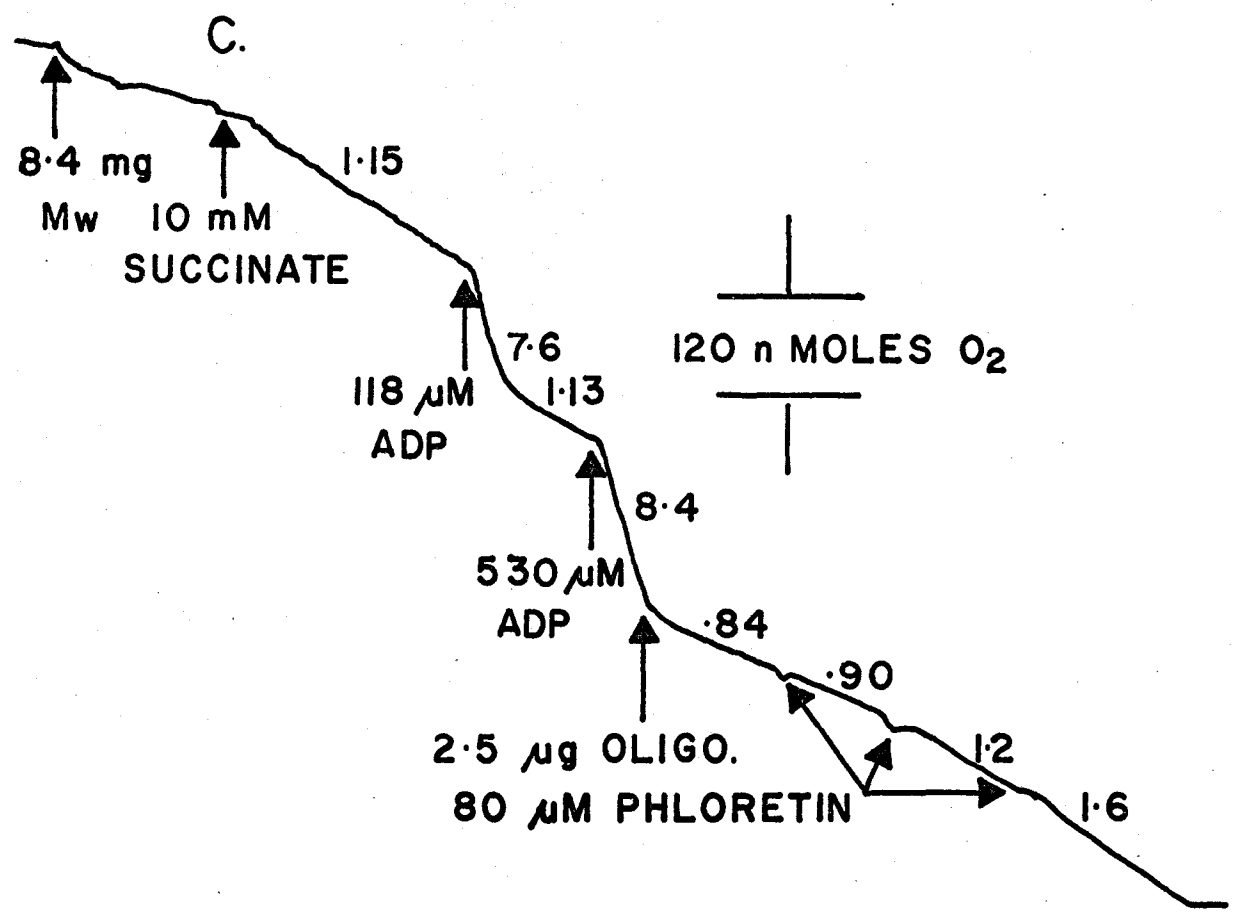


Figure 20

Comparison of phlorizin inhibition of DNP-stimulated ATPase (●----●), Mg^{++}-activated ATPase (Δ----Δ), and partially purified (O----O) ATPase. Incubation was carried out in the presence of 1.0 mM ATP at 25° for 15 minutes. ATPase reaction medium is described in Methods.

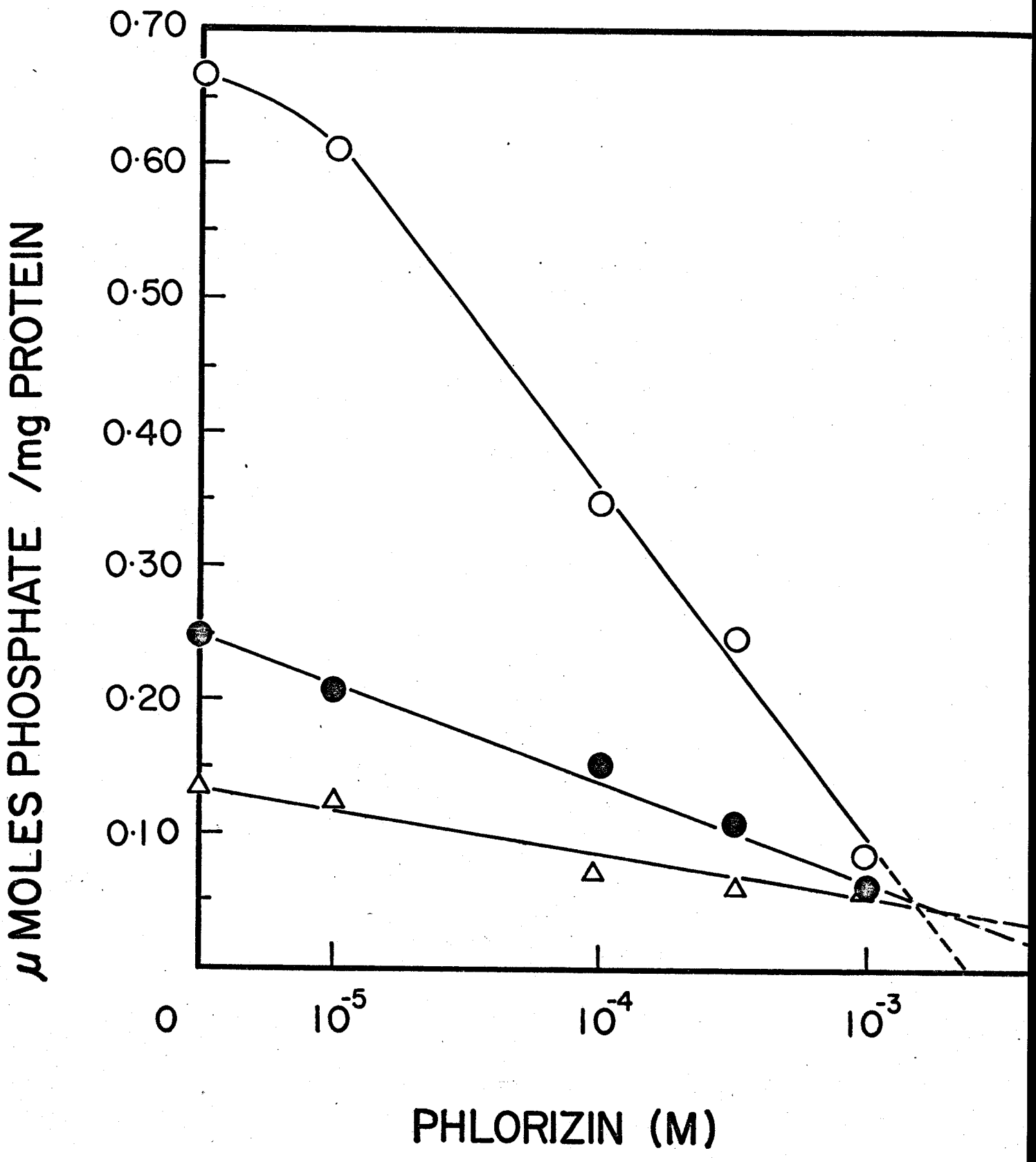

0·70
0·60
0·50
0·40
0·30
0·20
0·10
μ MOLES PHOSPHATE /mg PROTEIN
0
10-5
10-4
10-3
PHLORIZIN (M)

Figure 21

Double-reciprocal plot of phlorizin inhibition of DNP-stimulated ATPase. (O----O), no phlorizin; (●----●), 500 μM phlorizin. Reaction conditions as described in Methods.

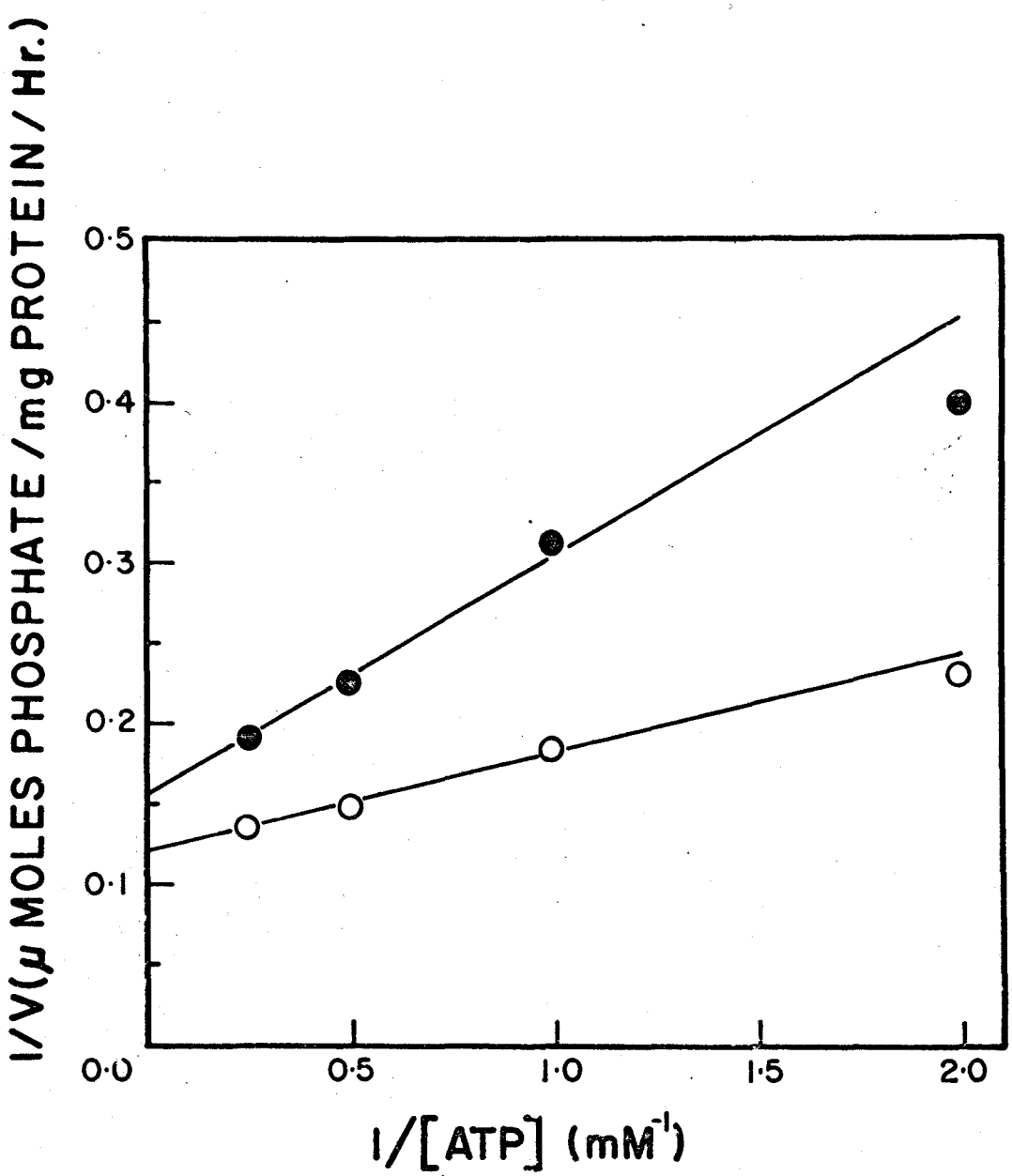

1/V(μ MOLES PHOSPHATE/mg PROTEIN/Hr.)
0·5
0·4
0·3
0·2
0·1
0·0
0·5
1·0
1·5
2·0
1/[ATP] (mM⁻¹)

Figure 22

The effect of phloretin on ATPase activated by DNP (0----0) or Mg^{++} (●----●). Incubation was carried out in the presence of 1.0 mM ATP at 25° for 15 minutes. Reaction conditions are described in Methods. The mitochondrial suspension contained 0.48 mg protein/ml.

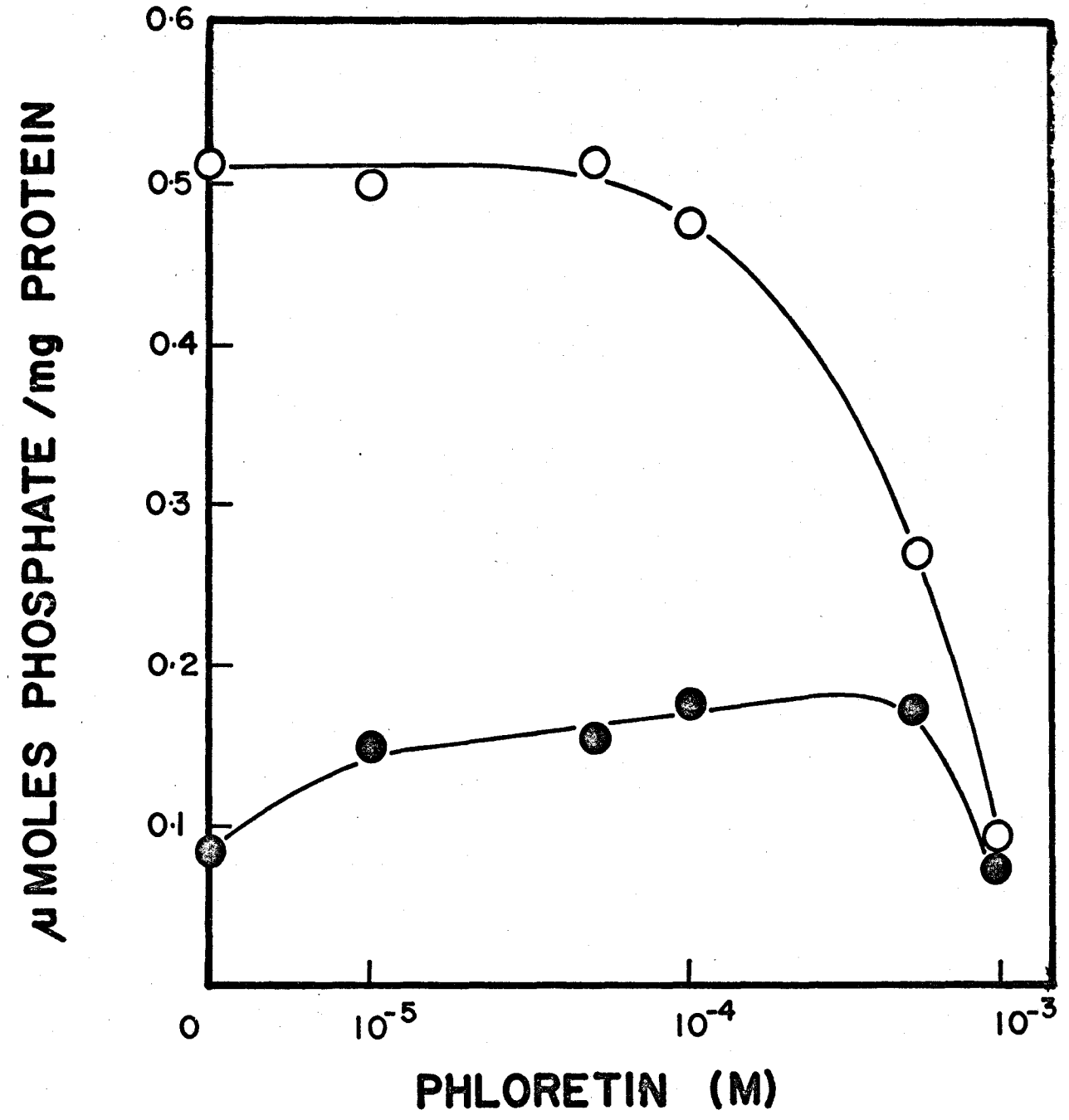

0·6
0·5
0·4
0·3
0·2
0·1
0
10^{-5}
10^{-4}
10^{-3}
μMOLES PHOSPHATE /mg PROTEIN
PHLORETIN (M)

Figure 23

Comparison of Amytal and phlorizin inhibition of mitochondrial ATPase activated with different concentrations of DNP. Incubation was carried out in the presence of 1.0 mM ATP at 25° for 15 minutes. The mitochondrial suspension contained 0.45 mg protein/ml. Experimental conditions were as in Methods except that the concentration of DNP was varied as indicated. (O----O), DNP-activated ATPase activity; (●----●), plus 300 μM amytal; (Δ----Δ), plus 500 μM phlorizin.

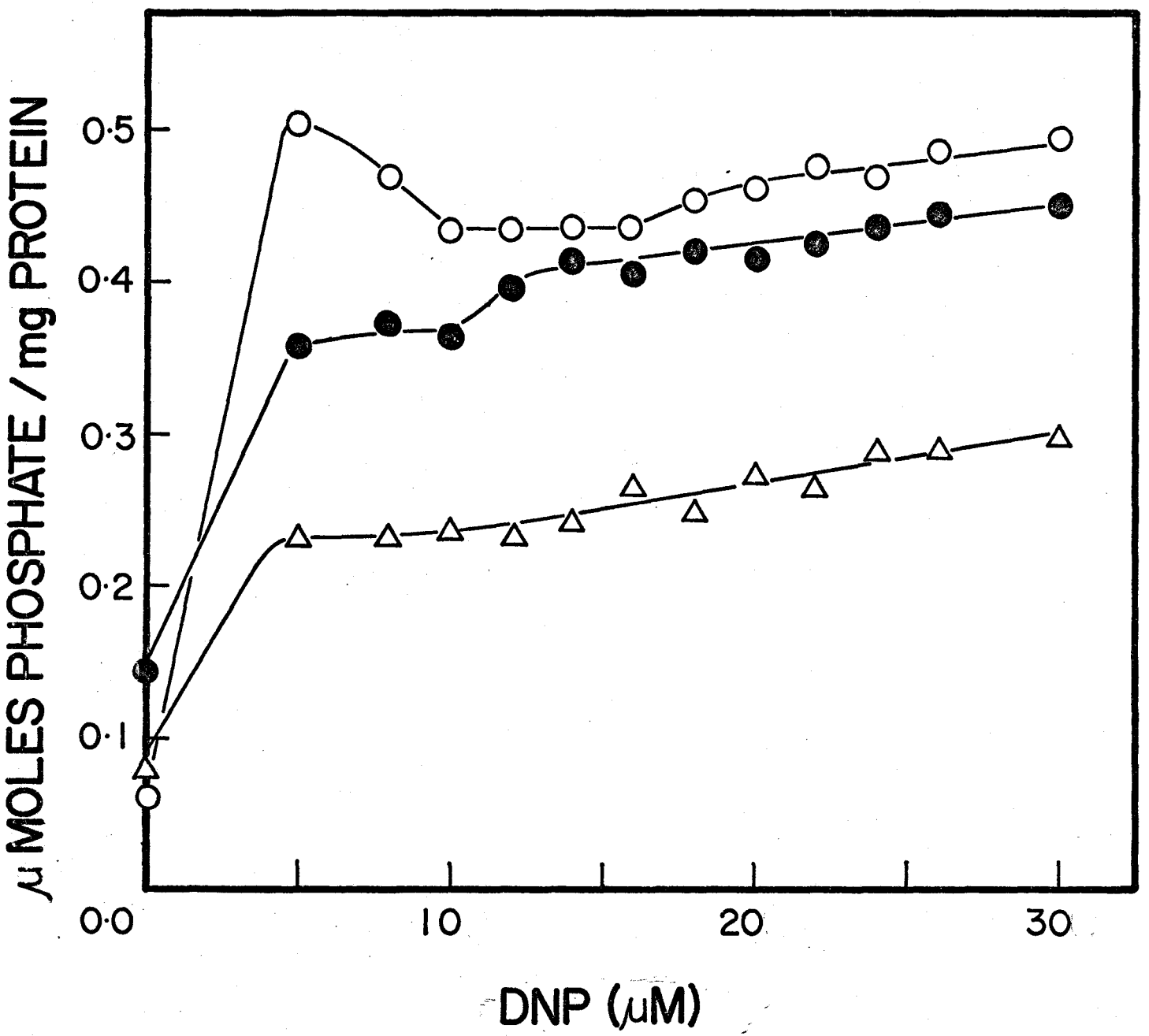
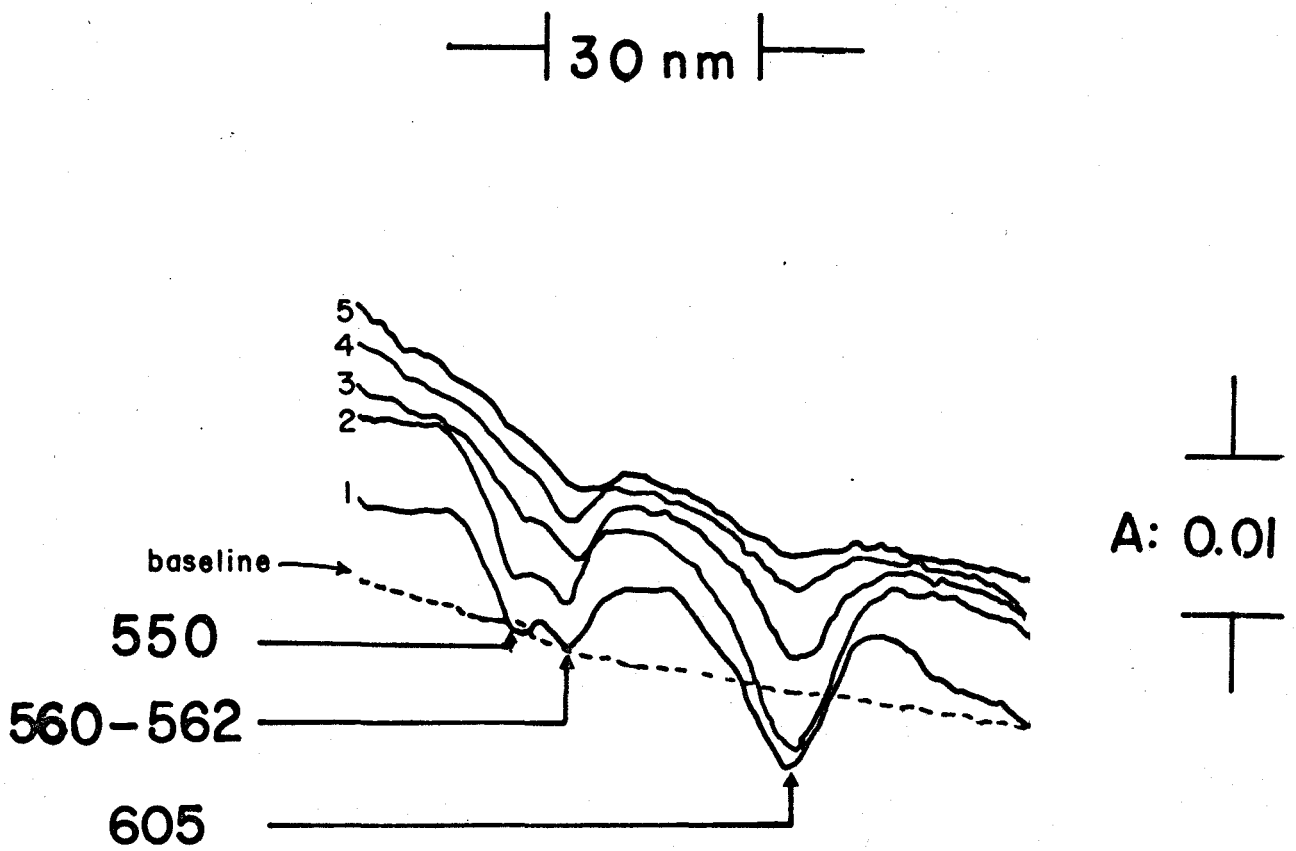

μ MOLES PHOSPHATE / mg PROTEIN
0·5
0·4
0·3
0·2
0·1
0·0
10
20
30
DNP (μM)

Figure 24

Difference spectra of digitonin particles reduced with NADH (reference beam) vs particles reduced with NADH in the presence of 3.0 mM phlorizin (sample beam). Particles were suspended in sucrose-phosphate medium (see Methods) containing 800 µM ADP. 500 µM NADH was added at time 0.



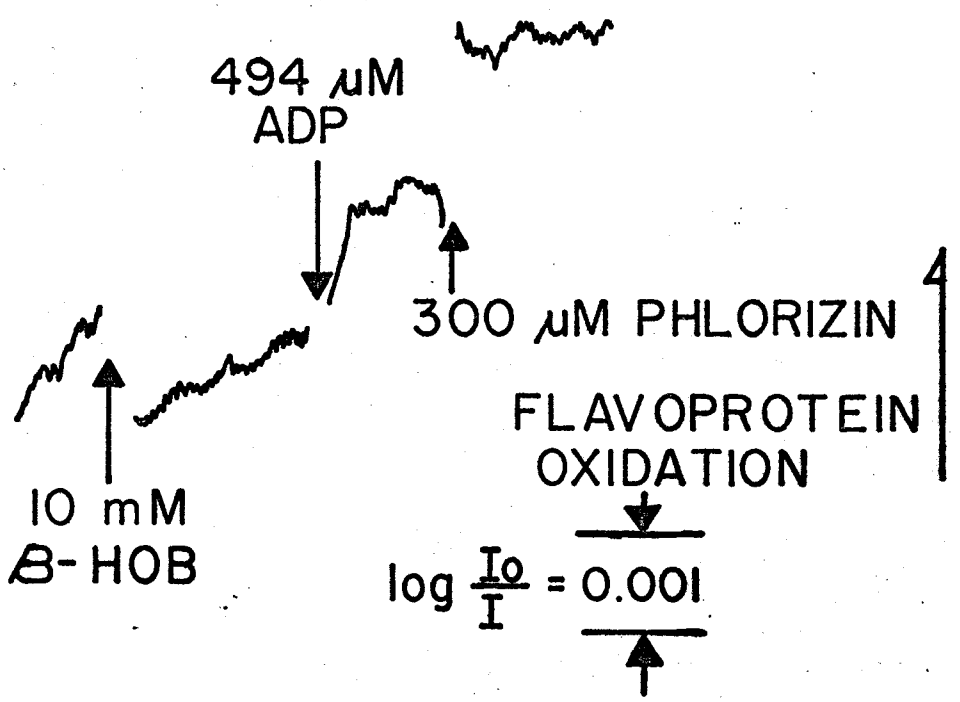
SPECTRUM	TIME (MIN)
1	1.5
2	4
3	6
4	8.5
5	15

Figure 25

Effect of phlorizin on the oxidation-reduction state of (A), mitochondrial flavoprotein in the presence of β-hydroxybutyrate and ADP and (B) cytochrome b in the presence of succinate and ADP. Mitochondria were suspended in the sucrose-phosphate medium described in Methods. Protein concentration was 3.6 mg/ml, in a total volume of 3.0 ml. Temperature was 23°.

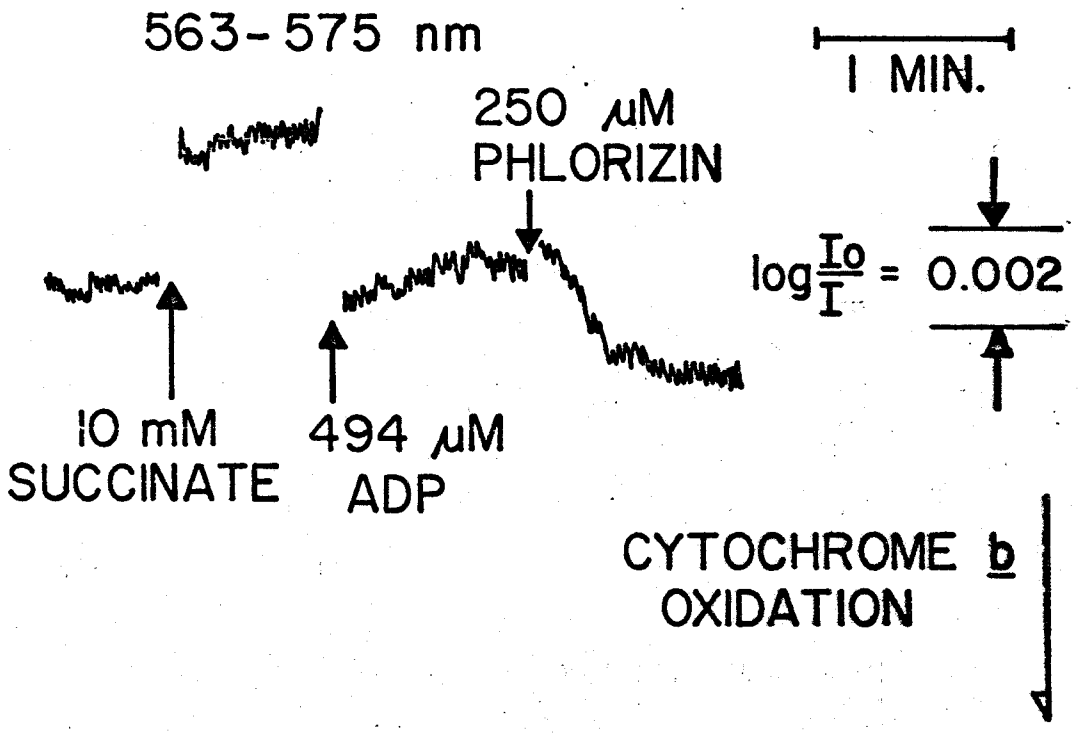
A.

460 - 500 nm



B.

563 - 575 nm



CHAPTER 5

PHLORIZIN-INDUCED MITOCHONDRIAL SWELLING

Mitochondrial swelling in the presence of succinate - Suspension of tightly coupled rat liver mitochondria in reaction medium in the presence of 3.0 mM succinate resulted in immediate large amplitude swelling (Fig. 26). Addition of 1.0 mM phlorizin facilitated the rate and extent of mitochondrial swelling. Exposing an aliquot of the mitochondrial preparation to reaction medium in the presence of 3.0 mM succinate and 133 μM ADP resulted in a two minute lag period after which the rate of swelling was comparable to that in the absence of ADP. The addition of 1.0 mM phlorizin during the lag period induced a prompt increase in mitochondrial volume of a magnitude comparable to that observed in the presence of reaction medium plus succinate alone.

Ion specificity - Mitochondria suspended in various isotonic salt solutions were studied to determine the ionic requirements of phlorizin-induced mitochondrial swelling. Fig. 27 shows that in the presence of rotenone and EGTA, 1.0 mM phlorizin, added to mitochondria suspended in isotonic potassium chloride or ammonium chloride, did not induce significant changes in mitochondrial volume. In contrast, mitochondria suspended in isotonic potassium acetate underwent a large increase in volume upon addition of 1.0 mM phlorizin. Exposure

of mitochondria to potassium phosphate or ammonium phosphate resulted in instantaneous swelling in the absence of phlorizin similar to that obtained in reaction medium containing succinate (cf Fig. 26).

In order to delineate the functional groups of phlorizin which may be responsible for this specificity, a similar series of experiments were carried out with phloretin. At a concentration of 100 μM, phloretin induced relatively slow mitochondrial swelling in isotonic ammonium chloride. However, phloretin at a concentration of 1.0 mM induced instantaneous maximal swelling of mitochondria in the presence of potassium chloride, ammonium chloride and potassium acetate (Fig. 28).

Energy requirement for swelling - Fig. 29 depicts the results of experiments carried out to determine whether energy is required for phlorizin-induced mitochondrial swelling in isotonic potassium acetate. To obtain an energy depleted state, mitochondria were incubated with inhibitory concentrations of rotenone, oligomycin and malonate. Neither these conditions, nor the addition of DNP at uncoupling concentrations prevented phlorizin-induced mitochondrial swelling.

Control of swelling by Mg^{++} - Rat liver mitochondria, prepared in medium which contained EDTA, swell upon addition of succinate in the presence of a permeant anion and impermeant cation (Fig. 30). Swelling was inhibited by DNP or oligomycin which indicated that the increase in mitochondrial volume was energy dependent. Fig. 30 further shows that succinate-induced swelling was prevented by incubating the mitochondrial suspension with 1.0 mM $MgCl_2$.

Experiments were therefore conducted to determine the effect of Mg^{++} on phlorizin-induced swelling. Mitochondria were depleted of energy as before, and swelling was initiated by exposing the mitochondrial suspension to 1.0 mM phlorizin. As can be seen in Fig. 31, 4.0 mM Mg^{++} retarded the swelling process. Incubation of mitochondria with 8.0 mM Mg^{++} completely inhibited the ability of phlorizin to cause mitochondrial swelling. Fig. 32 shows that Mg^{++} inhibited the rate of phlorizin-induced swelling linearly at concentrations up to 6.0 mM.

Studies with phloretin demonstrated that mitochondrial swelling caused by the analogue was relatively insensitive to Mg^{++}. In potassium acetate solution, Mg^{++} at concentrations up to 8.0 mM decreased the magnitude, but not the initial rate of swelling resulting from the addition of 200 μM phloretin to the mitochondrial suspension (Fig. 33).

Chelation - The above results, showing that Mg^{++} prevents swelling of mitochondria treated with EDTA or phlorizin, prompted an examination of the ability of phlorizin to chelate divalent cations. Using the calcium electrode, it was found that phlorizin readily removes Ca^{++} from the medium in the presence or absence of mitochondria (Fig. 34). Ultraviolet spectra of phlorizin exhibited maxima at 223 nm and 285 nm (Fig. 35). The intensity of the spectra were varied slightly by sample dilution to avoid confusing the absorption peaks. The shoulder at 320 nm may be identified with the most extended resonance structure of the compound, a phenolic ring with an α-keto group (114). The presence of Ca^{++} or Mg^{++} effectively removed the shoulder at 320 nm, suggesting an interaction of divalent cation with this group.

Figure 26

The effect of phlorizin on mitochondrial volume changes in the presence of succinate, ADP and inorganic phosphate. The mitochondrial suspension was adjusted to an initial optical density at 520 nm of 0.8 in medium containing 250 mM sucrose, 40 mM KCl, 1.0 mM $MgCl_2$. 5.0 mM KH_2PO_4 (P_i), pH 7.4, 3.0 mM succinate, and 133 μM ADP were added as indicated.

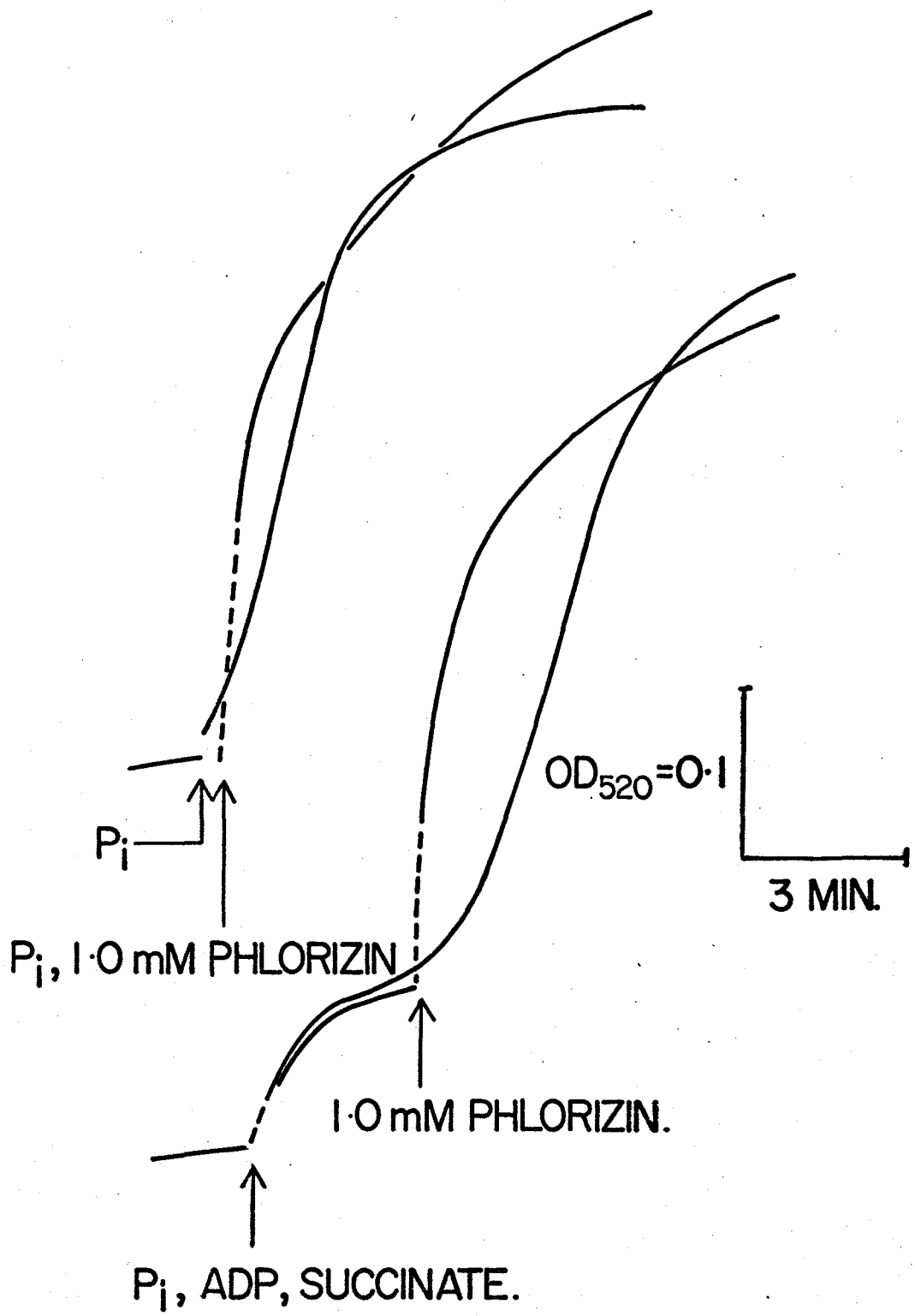

OD_{520}=0·1
3 MIN.
P_i
P_i, 1·0 mM PHLORIZIN
1·0 mM PHLORIZIN.
P_i, ADP, SUCCINATE.

Figure 27

The induction of mitochondrial swelling by phlorizin in the presence of various salts. The mitochondrial suspension was adjusted to an initial optical density of 0.8 at 520 nm in medium containing 120 mM of the indicated salt, 2.0 mM EDTA, 6 µg rotenone, pH 7.4. Volume, 3.0 ml.

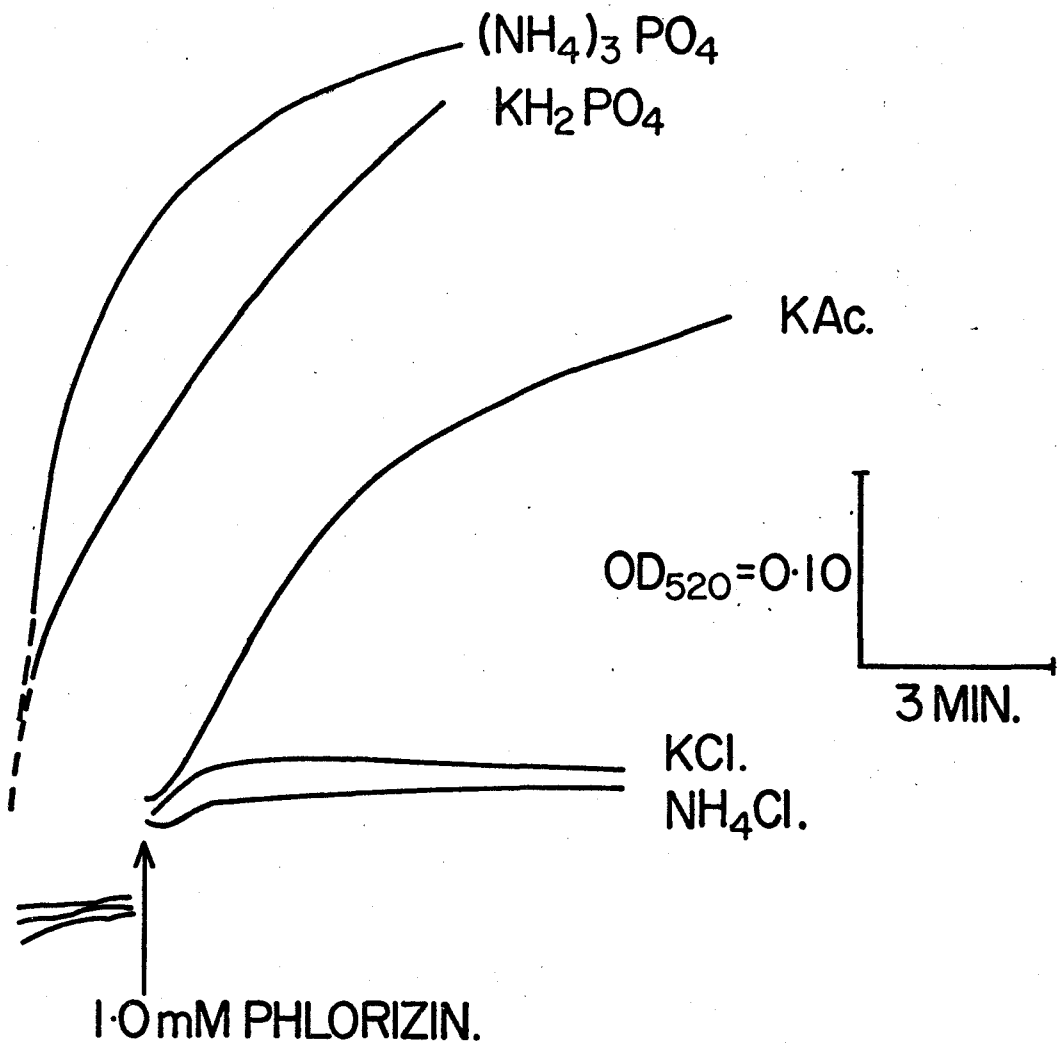

$(NH_4)_3 PO_4$
KH_2PO_4
KAc.
OD_{520}=0·10
3 MIN.
KCl.
NH_4Cl.
1·0 mM PHLORIZIN.

Figure 28

Mitochondrial swelling induced by phloretin in the presence of various salts. Experimental conditions as in Fig. 27.

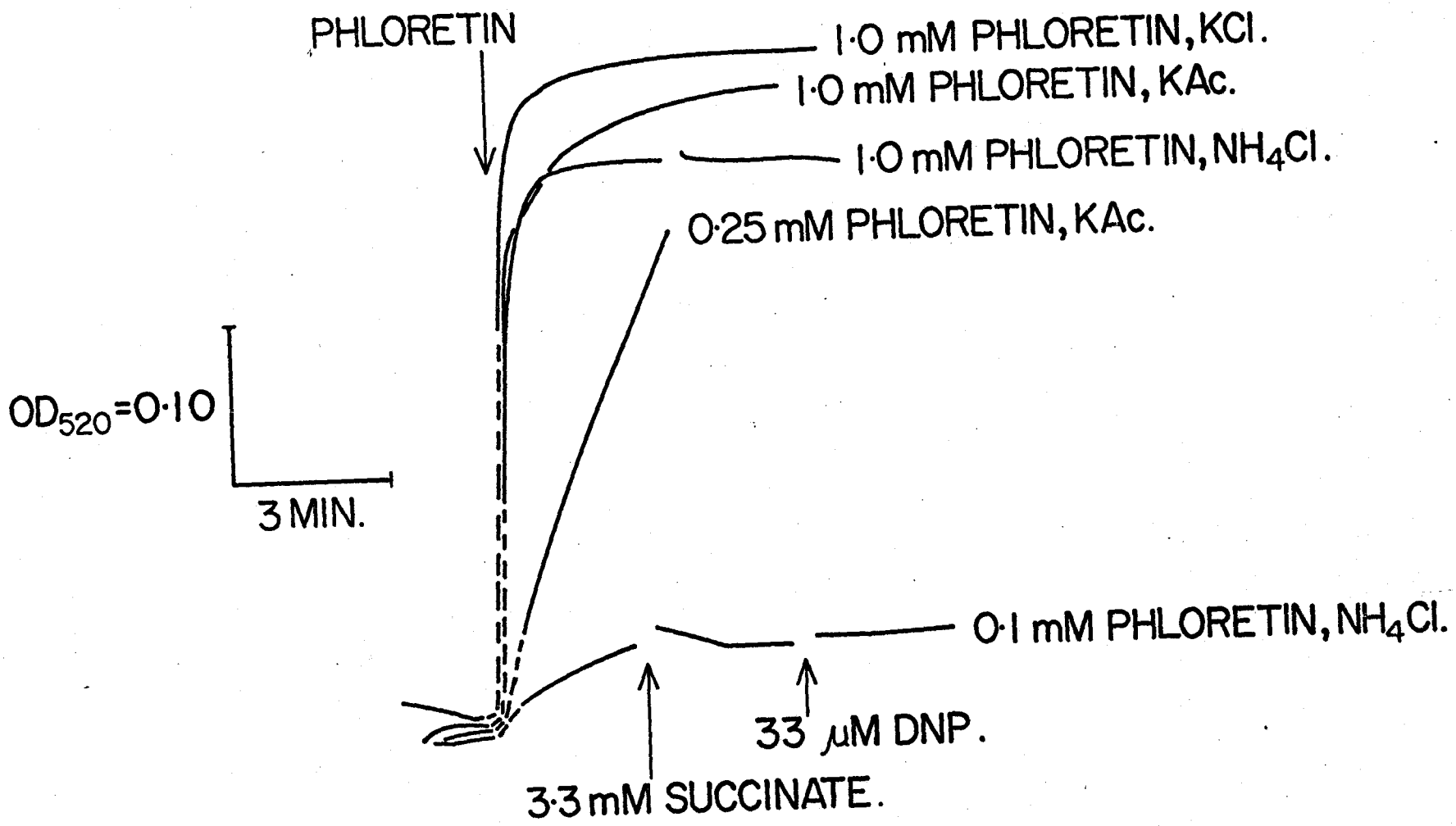

PHLORETIN
1·0 mM PHLORETIN, KCl.
1·0 mM PHLORETIN, KAc.
1·0 mM PHLORETIN, NH4Cl.
0·25 mM PHLORETIN, KAc.
OD520=0·10
3 MIN.
0·1 mM PHLORETIN, NH4Cl.
33 μM DNP.
3·3 mM SUCCINATE.

Figure 29

The effect of oxidative phosphyloration inhibitors on phlorizin-induced swelling of mitochondria. The reaction medium contained 2.0 mM EGTA, 6 µg rotonone, 10 µg oligomycin, 5.0 mM malonate, 120 mM potassium acetate, pH 7.4. Phlorizin and DNP added as indicated.

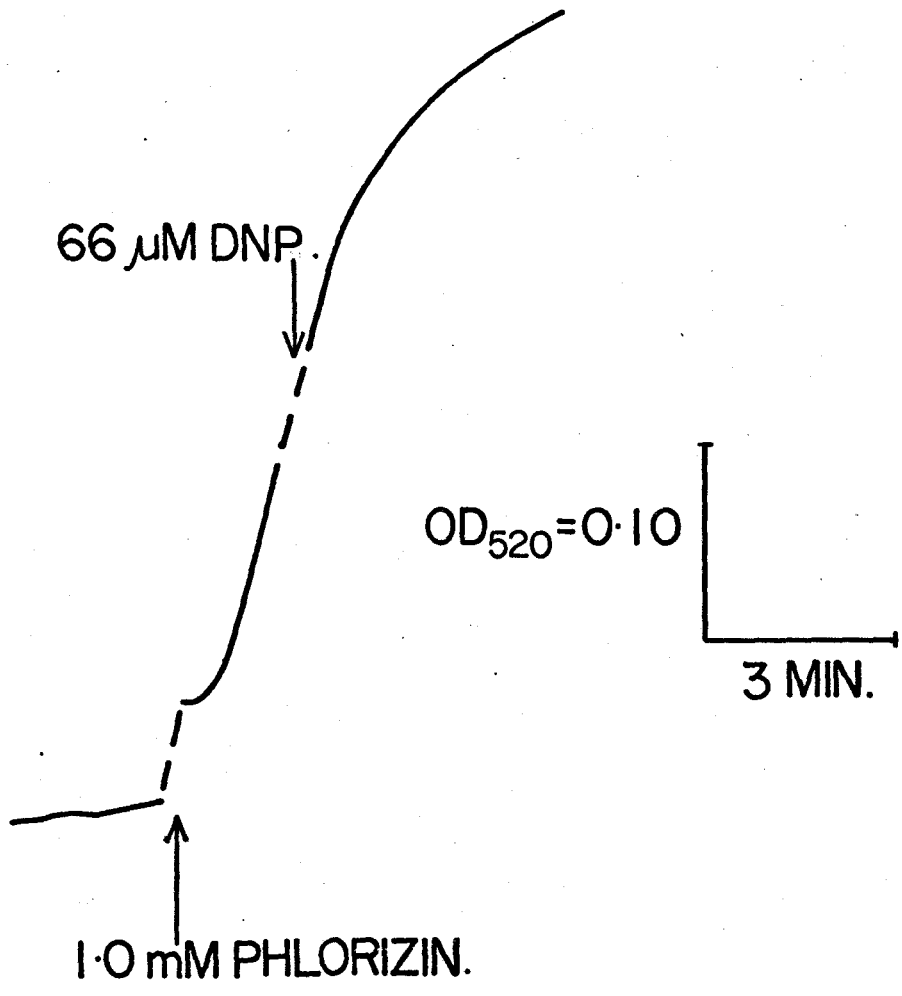

66 μM DNP.
OD520=0·10
3 MIN.
1·0 mM PHLORIZIN.

Figure 30

Control by Mg^{++} of energy-linked swelling induced by succinate. Mitochondria were suspended in medium containing 120 mM potassium acetate, 2.0 mM EGTA, 6 µg rotenone, pH 7.4, at a final volume of 3.0 ml. Other reagents added as indicated.

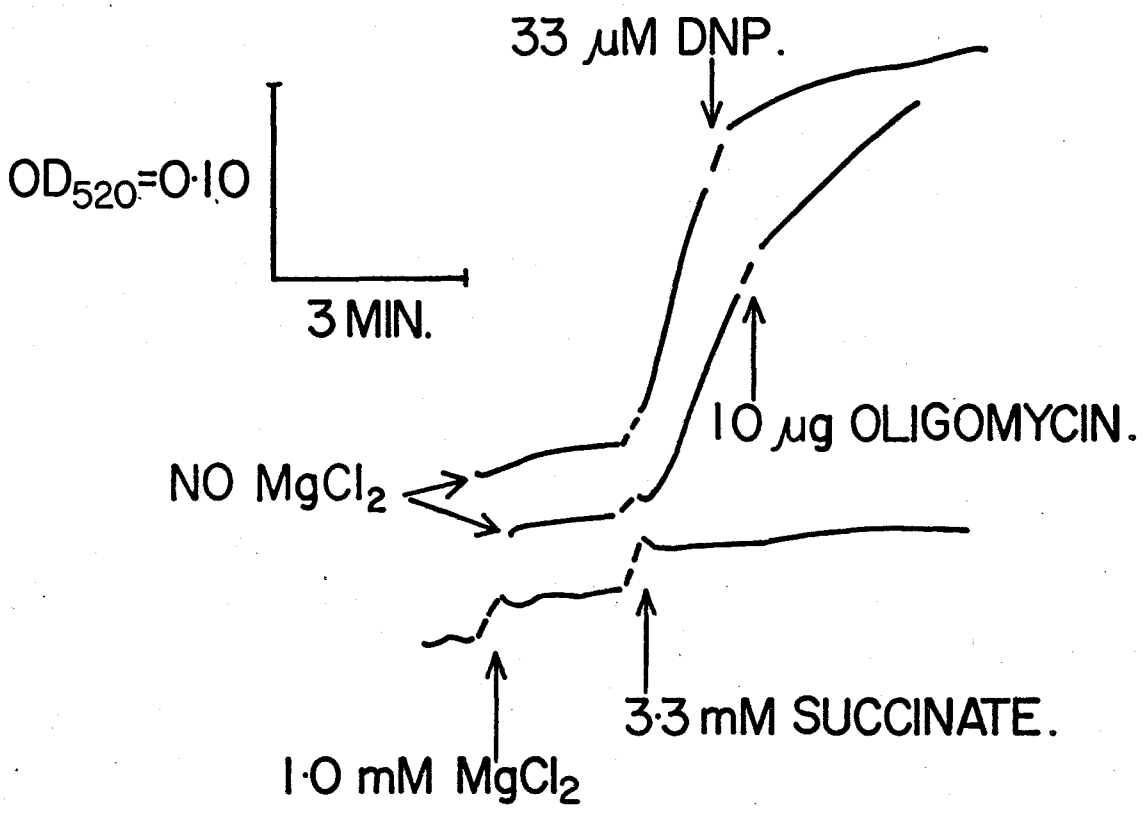

33 μM DNP.
OD520=0·10
3 MIN.
10 μg OLIGOMYCIN.
NO MgCl2
3·3 mM SUCCINATE.
1·0 mM MgCl2

Figure 31

Mg^{++} inhibition of energy-independent swelling of mitochondria treated with phlorizin. The reaction medium contained 2.0 mM EGTA, 6 µg rotenone, 10 µg oligomycin, 5.0 mM malonate, 120 mM potassium acetate, pH 7.4.

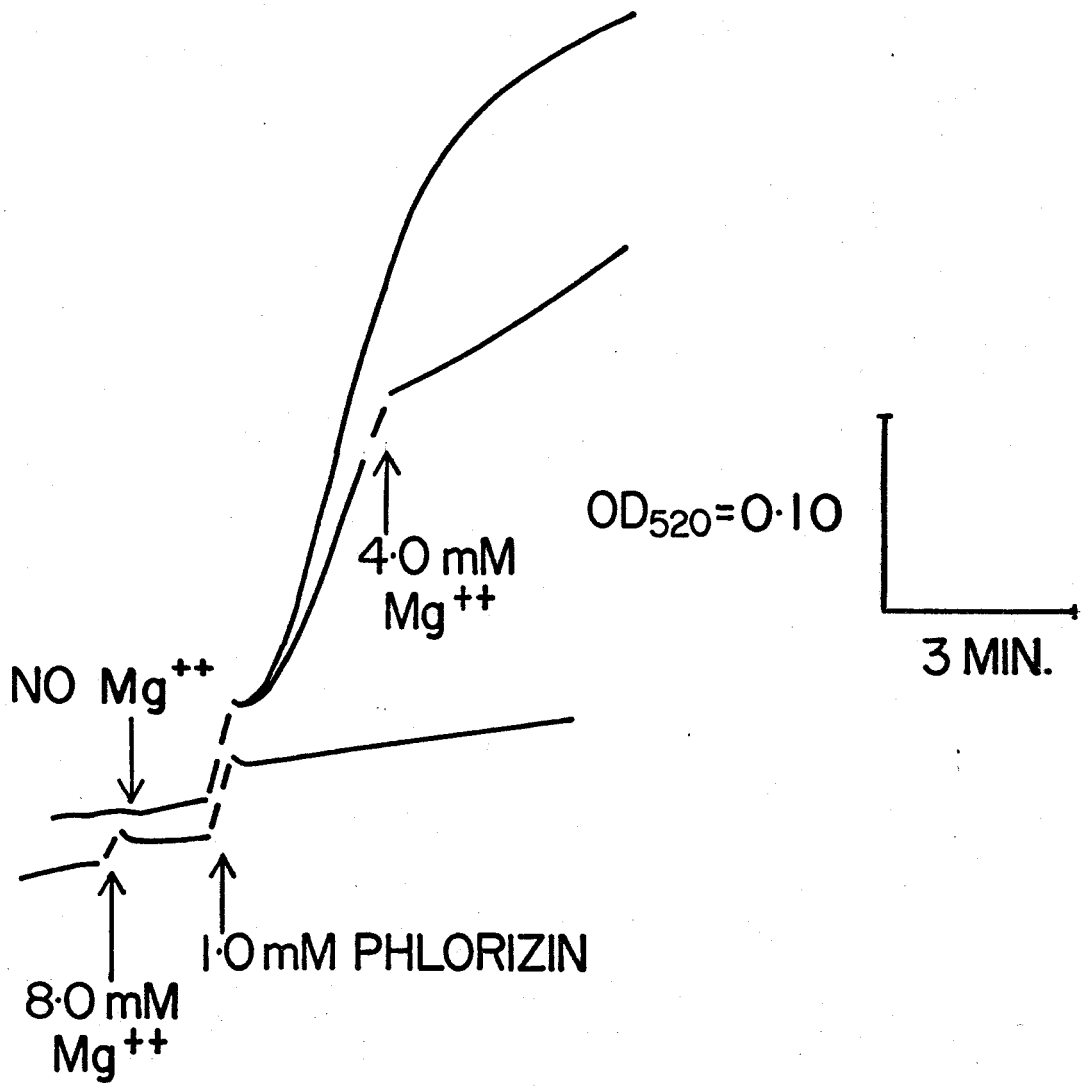

NO Mg++
4·0 mM Mg++
OD520=0·10
3 MIN.
8·0 mM Mg++
1·0 mM PHLORIZIN

Figure 32

Plot of Mg^{++} inhibition of mitochondrial swelling induced by 1.0 mM phlorizin. Experimental conditions as in Fig. 31.

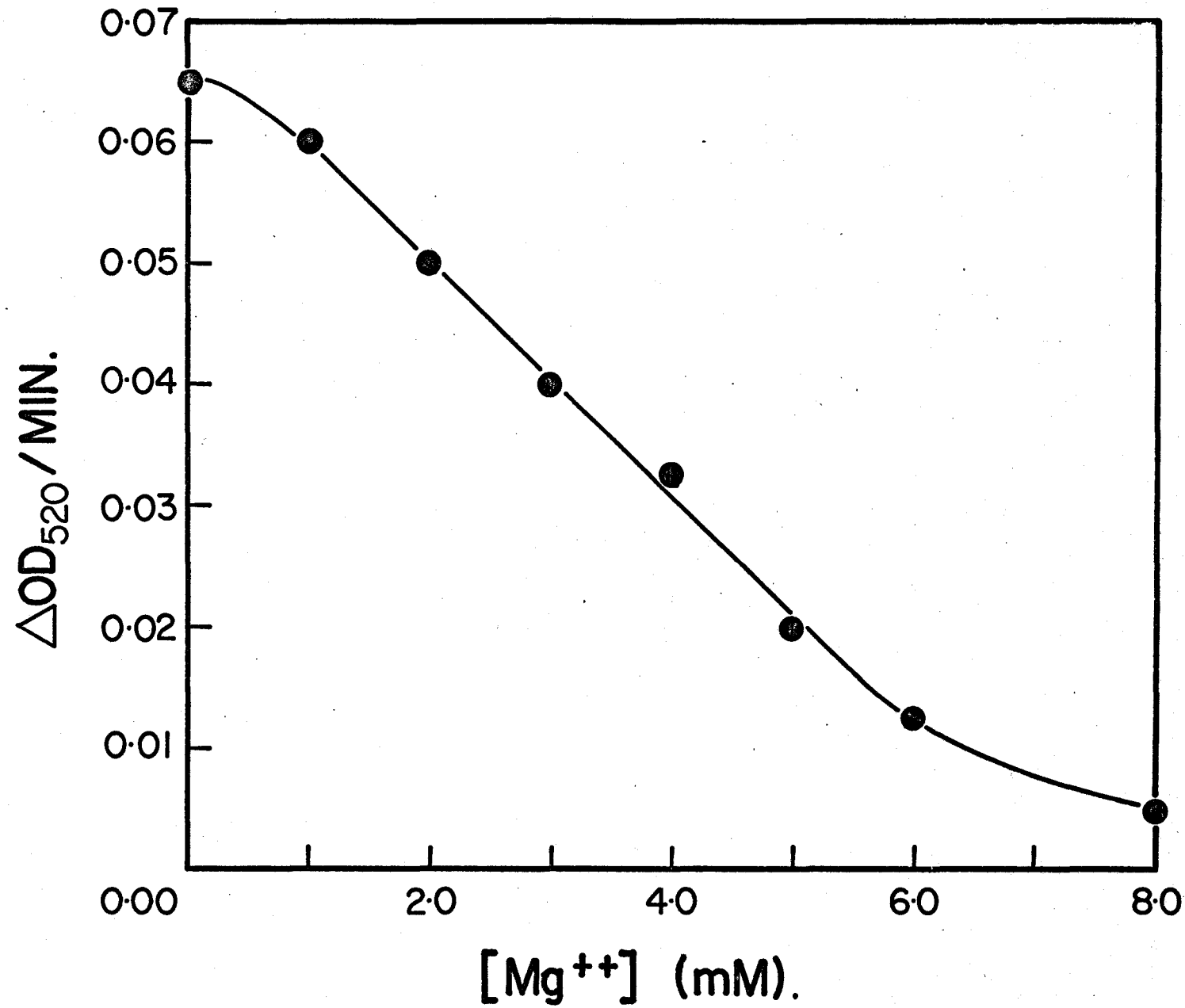

0·07
0·06
0·05
0·04
0·03
0·02
0·01
0·00
ΔOD520 / MIN.
2·0
4·0
6·0
8·0
[Mg++] (mM).

Figure 33

The effect of Mg^{++} on swelling of mitochondria treated with 200 μM phloretin. Experimental conditions as described in Fig. 31.

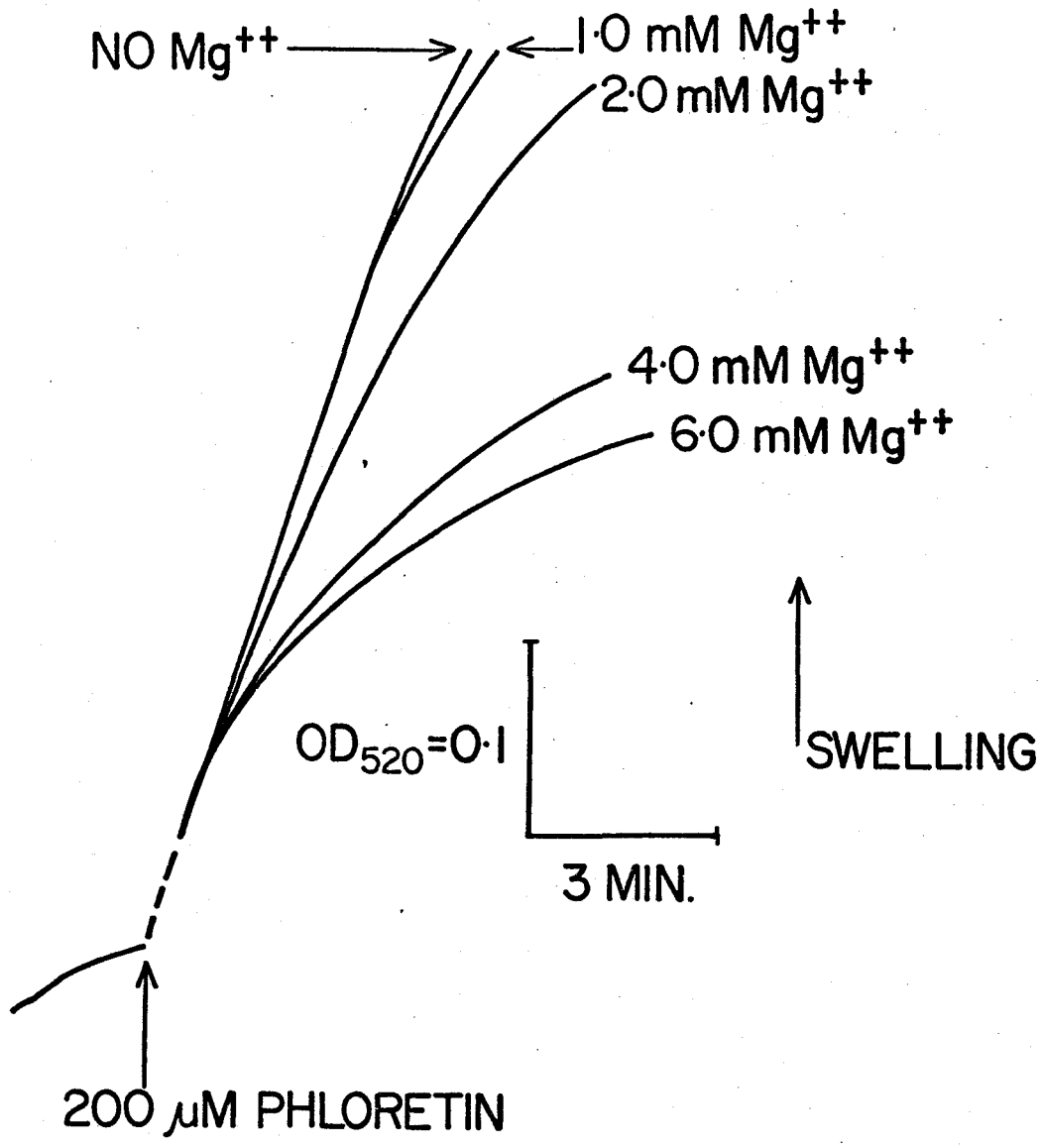

NO Mg++
1·0 mM Mg++
2·0 mM Mg++
4·0 mM Mg++
6·0 mM Mg++
OD520=0·1
SWELLING
3 MIN.
200 μM PHLORETIN

Figure 34

A calcium electrode trace demonstrating the ability of phlorizin to remove Ca^{++} from solution. (A), mitochondria (2.0 mg/ml) suspended in the sucrose-phosphate medium described in Methods; (B) sucrose-phosphate medium alone.

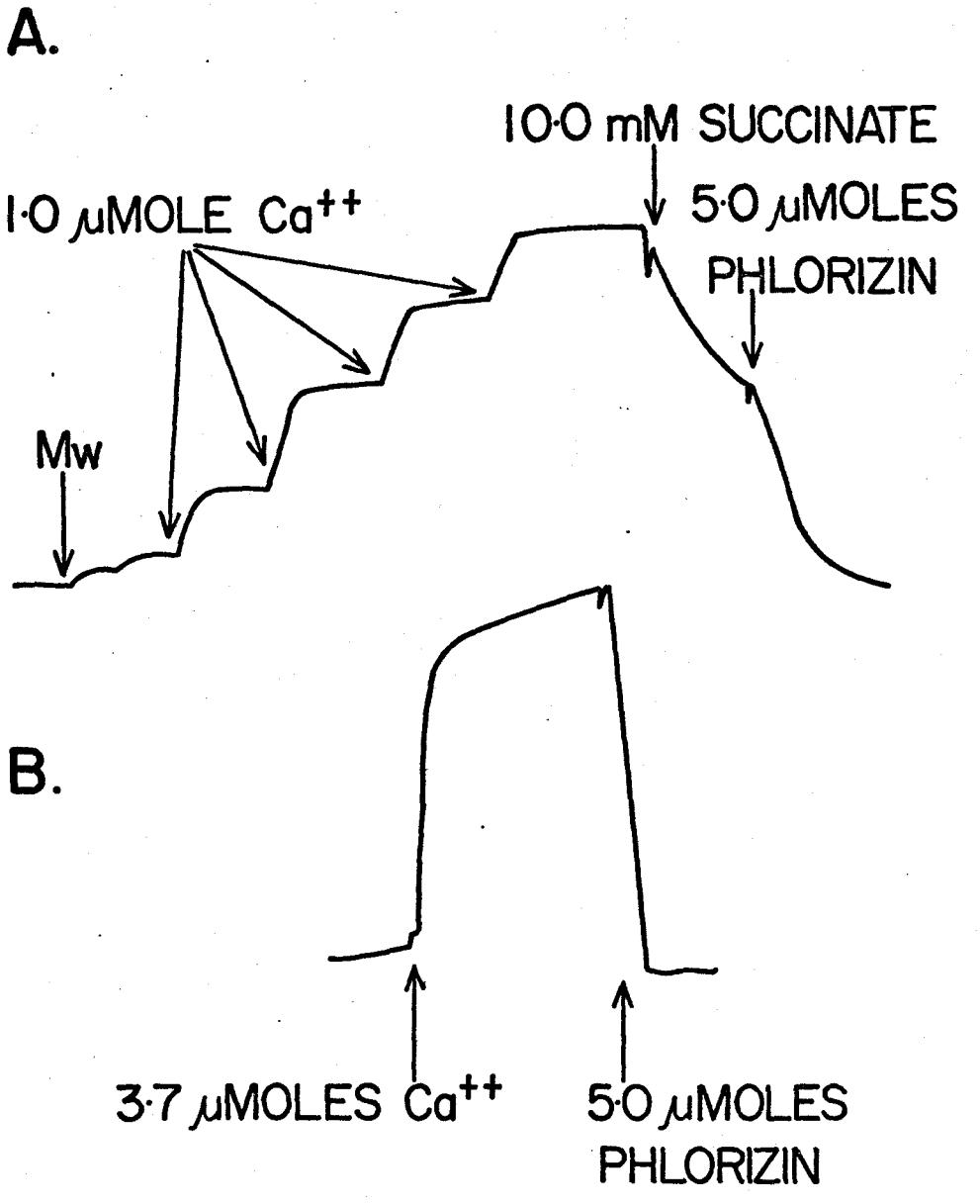
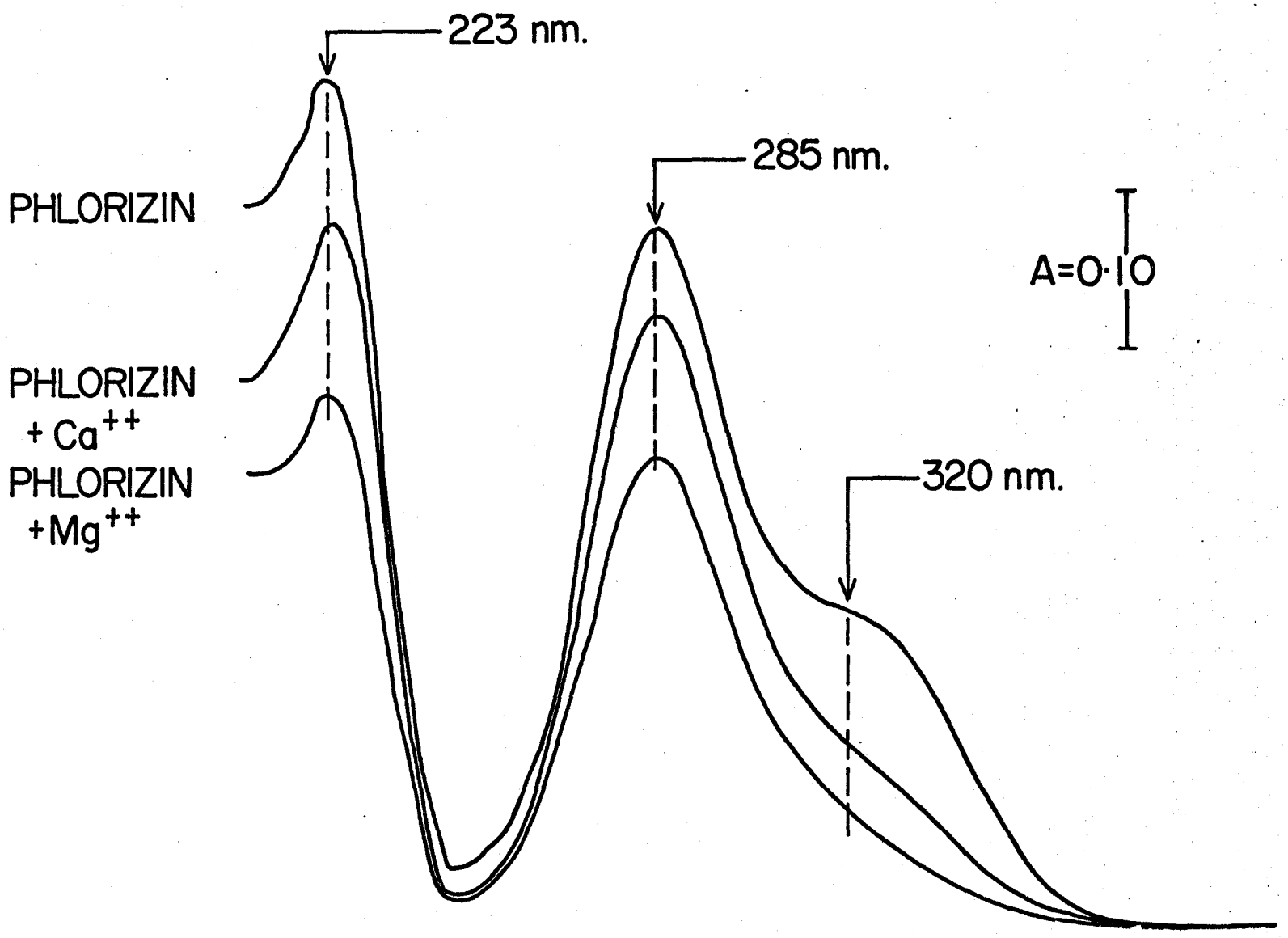

A.
10·0 mM SUCCINATE
1·0 μMOLE Ca++
5·0 μMOLES
PHLORIZIN
Mw
B.
3·7 μMOLES Ca++
5·0 μMOLES
PHLORIZIN

Figure 35

Ultraviolet spectra of phlorizin in the presence and absence of Ca^{++} or Mg^{++}. Spectra were obtained by mixing equimolar amounts of phlorizin and the chloride salts of divalent cations in aqueous solutions.


223 nm.
285 nm.
320 nm.
PHLORIZIN
PHLORIZIN
+ Ca++
PHLORIZIN
+Mg++
A=0·10

CHAPTER VI

DISCUSSION AND CONCLUSIONS

The results of the preceding chapters characterized the activities of azide, phlorizin, and phloretin on mitochondrial volume, energy transfer, and electron transport to determine relationships between these processes. Studies on respiration and ATPase activity showed that azide, phlorizin and phloretin were inhibitory uncouplers of oxidative phosphorylation. Studies of mitochondrial volume changes demonstrated that azide inhibited energy-dependent mitochondrial swelling at concentrations that affected energy transfer, but at higher concentrations azide induced energy-independent (pseudo-energetic) mitochondrial swelling. It is postulated that azide specifically inhibits the terminal phosphorylating enzyme to prevent mitochondrial swelling supported by the generation of energy transfer intermediates. Azide-induced mitochondrial swelling was pseudo-energetic and may be a secondary effect of azide accumulation as a permeant anion.

Phlorizin and phloretin caused mitochondrial swelling at all concentrations tested. Present evidence suggests that the activity of these agents on mitochondrial volume and respiratory processes may be a consequence of chelation of mitochondrial Mg^{++}.

These points are discussed and collated in the succeeding paragraphs.

Azide Inhibition of Energy Transfer - Results presented here show that the amount of azide required to inhibit succinate oxidation in tightly coupled mitochondria respiring in state 3 is

also sufficient to inhibit purified ATPase. The fact that azide at low concentrations did not stimulate purified ATPase confirms the earlier conclusions of Pullman et al. (70) that this enzyme is the DNP-activated ATPase of intact mitochondria rather than Mg^{++}-activated ATPase which is stimulated by azide (37). It is probable that DNP-activated ATPase and Mg^{++}-activated ATPase are the same enzyme but isolation or treatment with DNP reduces the stimulatory effect of azide (115). Therefore, a dual activity of azide on energy transfer as suggested by Bogucka and Wojtzak (37) is compatible with the present results.

Negative control of respiration by ADP has been observed under conditions of inhibited phosphorylation (36, 116, 117). It has been suggested (117) that ADP acts to make the sensitive site on the phosphorylation enzyme(s) in intact mitochondria more accessible to inhibitors. The results presented here show that the presence of ADP enhanced azide inhibition of ATPase activity to an extent unaccountable on the basis of mass action alone. It is, therefore, possible that ADP facilitates the entry of azide to a sensitive site on the isolated ATPase as well. Competitive inhibition of ATPase activity by azide reported here and elsewhere (95) suggests that the azide-sensitive site may be the site of adenine nucleotide interaction. These observations support the conclusion that azide is an inhibitor of energy transfer. This effect is distinct from the binding of azide to a component of cytochrome oxidase reported by Wilson and Chance (36) and Nicholls and Kimelberg (43).

Competitive inhibition of the terminal phosphorylating enzyme may be consistent with reports (36, 44) that azide inhibition is

uncompetitive with succinate oxidation. According to Cleland (118), uncompetitive inhibition of a multi-substrate enzyme system occurs when an inhibitor-enzyme complex forms in a manner that cannot be overcome by saturation with one of the substrates. Competitive inhibition results when inhibitor and substrate compete for the same form of enzyme so that each component affects the concentration of enzyme available to the other. By increasing the substrate concentration, the effects of the inhibitor are overcome. If respiratory inhibition by azide is due to energy transfer inhibition, then, by Cleland's definitions, the substrate X~P generated by succinate oxidation cannot overcome the effects of azide, whereas adenine nucleotide can. This may be explained on the basis of a single interaction of azide with the terminal phosphorylating enzyme. In Fig. 36, for the sake of visualizing the type of interaction which may occur, azide is depicted competing with adenine nucleotide for a site on the enzyme, possibly in place of purine nitrogen. The site of X~P binding is shown distal to the pyrimidine nitrogen site and no direct interaction of bound azide and X~P occurs. Thus, azide does not compete with X~P for an enzyme site, but in the presence of azide, X~P cannot be utilized to generate products plus free enzyme. ie. inhibition is uncompetitive with succinate oxidation, but competitive with ATP hydrolysis.

Effect of Azide on Mitochondrial Volume - Palmieri and Klingenberg (44) have observed that azide is taken up in the mitochondria as a permeant anion. They concluded that azide uptake was dependent on energy transfer intermediates and that mitochondrial swelling and impaired energy transfer were secondary effects of accumulated azide.

Results presented here showing that azide caused pseudo-energetic swelling of mitochondria only in the presence of the permeant cation NH_4^+ confirm that azide may act as a permeant anion. The finding that azide-induced swelling was inhibited by succinate indicates a possible mechanism by which azide may enter the mitochondria. Van Dam et al. (119, 120) have observed that DNP was competitive with substrate for entry into the mitochondria. They proposed that uncoupler anions are tranported into the mitochondria via the same carriers that are responsible for the transport of substrate. It is, therefore, possible that azide may also penetrate the mitochondria via the dicarboxylate transporter to induce pseudo-energetic swelling.

Pseudo-energetic swelling required a 100-fold greater concentration of azide than that required for energy-transfer inhibition. Because of the large difference in azide concentrations, it is doubtful that azide-induced mitochondrial swelling is related to the effects of the inhibitor on energy transfer.

Studies on the effects of azide on energy-linked mitochondrial swelling lead to the conclusion that because of interference with energy transfer, energy-linked accumulation of azide is limited according to the supply of energy transfer intermediates present before the addition of inhibitor. This conclusion is based on observations that the rate of energy-linked mitochondrial swelling induced by ATP, β-hydroxybutyrate or succinate was inhibited more than 50% by azide. It is important to note that these results were obtained employing azide at concentrations which gave sub-maximal inhibition of ATPase activity and state 3 substrate oxidation. Supporting evidence has been provided by the work of Zvyagilskaya

et al. (121). They observed a two-fold increase in azide concentration inside the mitochondria under conditions of state 3 and state 4 substrate oxidation. This increase is very small compared to that observed with energy-linked uptake of other ions (eg. 40 - 1000 with K^+ (122)). Taken together, these results favour the interpretation that inhibition of energy transfer is the primary effect of azide.

The present report and other available data lead to the following summary of azide action. First, azide has a direct effect on cytochrome oxidase (8, 36, 43); second, azide has a specific inhibitory effect on the terminal phosphorylating enzyme system; third, energy transfer inhibition limits the energy-linked accumulation of azide; fourth, as concluded by Palmieri and Klingenberg (44), azide probably has secondary effects due to its accumulation as a permeant anion. These secondary effects may include mitochondrial swelling, uncoupling activity and stimulation of Mg^{++}-activated ATPase in intact mitochondria.

Phlorizin Inhibition of Respiration and Phosphorylation - The effect of phlorizin on mitochondrial respiratory processes is perhaps best understood by considering those activities which lead to decreased respiratory control. With NADH-linked substrates, phlorizin exerted a dual activity of uncoupling and inhibition of electron transport, evidenced by stimulated respiratory rates in state 4, decreased maximal respiratory rates in the presence of ADP or DNP and decreased ADP/O ratios. These activities of phlorizin on NADH-linked oxidations appear to resemble the effects of azide (37) and thiopental (123) which, in addition to inhibiting electron transport directly, uncouple and inhibit respiration by interacting

with phosphorylation reactions.

The fact that the maximal rate of oxygen uptake with NADH-linked substrate was equally sensitive to the inhibitory effects of phlorizin, whether in the presence of ADP or DNP, and that DNP failed to relieve phlorizin inhibition of respiration indicates a direct interaction with some component of the respiratory chain. In the presence of succinate, the uncoupling activity of phlorizin decreased respiratory control as demonstrated by stimulated respiratory rates in state 4 and decreased ADP/O ratio. A secondary activity of phlorizin, inhibition of succinate oxidation in state 3, is probably the result of accumulated oxaloacetate, a potent inhibitor of succinate dehydrogenase (124, 125), since in the presence of rotenone and DNP (conditions which block oxaloacetate production), no respiratory inhibition was observed at the highest possible phlorizin concentration. It therefore seems likely that uncoupling at sites II and III is the major effect of phlorizin on the oxygen side of cytochrome b and respiratory inhibition is confined to the NADH region of the chain. The stimulatory effect of phlorizin on NADH-linked substrate oxidation in the absence of ADP may be due to uncoupling at low concentrations (less than 200 μM) while phlorizin at higher concentrations inhibits electron transport and masks uncoupling activity. Similar effects have been reported for the nitrophenols which inhibit NADH-linked oxidations at concentrations which are stimulatory towards succinate oxidation (126).

According to the crossover theorem (34), NAD^+ reduction is typical of respiratory inhibition between pyridine nucleotide and

flavoprotein, but, because phlorizin absorbs strongly in the region of 340 nm, NAD^+ reduction could not be determined. Nevertheless, the spectrophotometric data show that, with β-hydroxybutyrate as substrate, phlorizin promotes oxidation of flavoprotein and the cytochromes. These data are consistent with an interference with the transfer of reducing equivalents at some point between NADH and flavoprotein. It has been observed by Chance and Hollunger (123) that thiopental promotes oxidation of pyridine nucleotide while exerting an Amytal-like inhibition of oxygen uptake. Flavoprotein oxidation was attributed by these authors to uncoupling activity which prevented flavoprotein from remaining in the reduced state. Similar effects by phlorizin could account for the observed flavoprotein oxidation in the presence of concentrations of this compound which are inhibitory to respiration. Phlorizin inhibition of β-hydroxybutyrate oxidation displayed mixed inhibition kinetics supporting the interpretation that inhibited respiration is the result of more than one interaction with the respiratory chain.

In view of its uncoupling activity, the inhibition of ATPase by phlorizin is difficult to explain, since uncouplers generally promote ATPase. But Hemker (127) has shown that about 60% of DNP-stimulated ATPase is associated with phosphorylation site I and is inhibited by Amytal. Results presented here showed that this ATPase was also inhibited by phlorizin. Thus an Amytal-like interaction by phlorizin at site I could account for inhibition of both oxygen uptake and ATPase.

Lardy et al. (56, 128) have shown that oligomycin inhibition of tightly coupled respiration can be counteracted by uncouplers, but phlorizin did not relieve oligomycin inhibition in the presence of β-hydroxybutyrate and only slightly with succinate as substrate. It may, therefore, be inferred that phlorizin is active at a site close to the oligomycin site. Support for this conclusion is drawn from the data showing that phlorizin inhibits ATPase activity. If uncoupling is associated with ATPase inhibition, as may be the case with azide (37), then a single site of interaction could be responsible for both effects. In view of the work of Fessenden and Racker (25) and Lee and Ernster (129) showing that oligomycin may prevent hydrolysis of non-phosphorylated intermediates, the observation that uncoupling concentrations of phlorizin produced a slight release of oligomycin-inhibited succinate oxidation may reflect alteration of the oligomycin binding site and the molecular conformation necessary to maintain phosphorylation at that level of the chain.

It is generally held that DNP-activated ATPase represents terminal energy transfer (70). DNP-activated ATPase and ATPase isolated by sonication are similar in sensitivity to azide (cf. Figs. 9 and 11) and the observation that both ATPase activities are inhibited by phlorizin leads to the conclusion that this compound is an inhibitor of energy transfer.

The schematic diagram of the activities of phlorizin (Fig. 37) show two interactions of phlorizin at each phosphorylation site. It may also be compatible with a dual activity of phlorizin arising from a single interaction with each phosphorylation site. A site of electron transport inhibition is tentatively placed between NADH

and flavoprotein.

Phloretin was a more potent inhibitor of mitochondrial respiratory processes than phlorizin. Apparently the glucose moiety restricts the action of phlorizin. None-the-less, the activities of phloretin and phlorizin on respiration and phosphorylation were essentially identical, thus providing supporting data for the interactions of phlorizin described above.

It should be noted that the ability of phlorizin to chelate Mg^{++} might well account for inhibition of ATPase activity reported here since DNP-activated ATPase and purified ATPase both require Mg^{++} for full activity (115). Ultraviolet spectra of phlorizin indicated that the ketone function may be involved in Mg^{++} chelation. A possible chelated configuration of phlorizin is depicted in Fig. 38.

Phlorizin inhibits most enzyme systems requiring adenine nucleotide as participant or cofactor (98). In view of the present report one might speculate that phlorizin inhibition of adenine nucleotide-linked enzyme systems is a consequence of Mg^{++} chelation.

Mitochondrial Swelling Induced By Phlorizin - Keller and Lotspeich (99,100) postulated that alteration of phosphorylation efficiency and mitochondrial volume by phlorizin was due to a general effect of this agent exerted most probably at the membrane. Observations of phlorizin-induced swelling of rat liver mitochondria presented here confirm and extend earlier reports (100) that phlorizin at concentrations of 1.0 mM caused prompt swelling of mitochondria. The data demonstrated that phlorizin caused mitochondrial swelling when acetate or phosphate, which are permeant anions (92), were present in the medium. When chloride was the only anion in the medium, swelling of phlorizin-

treated mitochondria did not occur even when a permeant cation (NH^+) was present. The inability of phlorizin to cause swelling in the absence of permeant anions is apparently due to the presence of the glucose moiety in the molecule, since, in the absence of permeant anions, the aglucone induced prompt mitochondrial swelling.

Mitochondrial swelling requires the uptake of both cation and anion (92) and it is therefore concluded that phlorizin induced swelling by facilitating the penetration of cations into mitochondria. In this regard phlorizin appears similar to the ion-transducing antibiotics, valinomycin and gramicidin (91). However, basic differences in activity exist. The ion-transducing antibiotics stimulate mitochondrial ATPase activity (88, 89) whereas phlorizin inhibited all ATPase activities tested. Further, phlorizin-induced mitochondrial swelling did not exhibit a requirement for high energy intermediates, as evidenced by the inability of DNP, rotenone, malonate and oligomycin to prevent swelling. Mitochondrial swelling in the presence of these inhibitors must be pseudo-energetic, of the type induced by divalent cations of the transition metals (94). It has been postulated that energy for pseudo-energetic swelling is derived from the Donnan membrane effect (94).

These findings contradict the observations of Keller and Lotspeich (89) that energy transfer inhibitors prevent phlorizin-induced swelling of kidney mitochondria suspended in isotonic sucrose. Their swelling studies employed kidney mitochondria suspended in isotonic sucrose, whereas the present study utilized liver mitochondria suspended in isotonic salt solutions. Considering the well-documented

fact that sucrose does not penetrate the inner mitochondrial membrane (132-134) and the different sources of mitochondria, their observations may reflect a different mechanism of mitochondrial swelling.

It is likely that phlorizin potentiates the competence of monovalent cation to cause swelling by altering the physiological integrity of the mitochondrial membrane. Azzone and Azzi (93) and Settlemire et al. (133) have suggested that Mg^{++} may be a controlling factor in cation uptake, a suggestion supported by present observations that mitochondria prepared in the presence of EDTA swell in isotonic potassium acetate upon addition of an energy source, and that swelling was prevented by the addition of Mg^{++}. The results presented here, indicating that phlorizin chelates Mg^{++}, lead to the conclusion that this agent increases the permeability of the mitochondrial membrane to monovalent cation by removing Mg^{++} from the mitochondrial environment. A critical role for Mg^{++} in mitochondrial membrane intactness is compatible with the view that the mitochondrial membrane is maintained by hydrophobic and electrostatic bonding (134) since it is possible that Mg^{++} is reversibly associated with the membrane by ionic (electrostatic) forces.

Ultrastructural studies (135, 136) have established that changes in mitochondrial volume accompany changes in respiratory states. The data presented here and elsewhere (93) show that mitochondria which suffer large increases in volume resulting from suspension in reaction medium, exhibit P/O ratios approaching 2.0 with succinate as substrate. This suggests that mitochondrial swelling of a similar magnitude resulting from the addition of phlorizin to a mitochondrial suspension is separate from the activity of this agent on phosphorylation

efficiency, although both effects may be related to chelation of Mg^{++} by phlorizin.

The present results and other available data may be summarized as follows. First, phlorizin interacts in the region of flavoprotein to inhibit mitochondrial respiration; second, phlorizin inhibits energy transfer by uncoupling and inhibiting the terminal phosphorylation enzyme; third, phlorizin induces mitochondrial swelling by increasing the permeability of the mitochondrial membrane to monovalent cations; these activities were confirmed with phloretin, the aglucone analogue of phlorizin; finally, the activities of phlorizin on mitochondrial structure and function may be the consequence of removal of Mg^{++} from the mitochondrial environment.

Figure 36

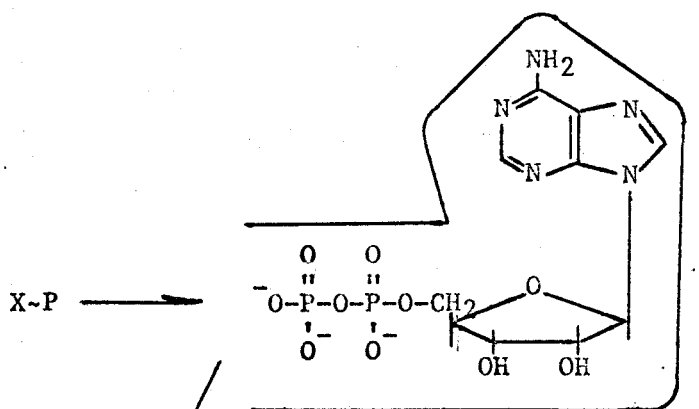
Diagrammatic representation of possible interaction of azide with the terminal phosphorylation enzyme.

ADENINE NUCLEOTIDE SITE ON

TERMINAL PHOSPHORYLATING ENZYME

ADENINE NUCLEOTIDE - ENZYME COMPLEX

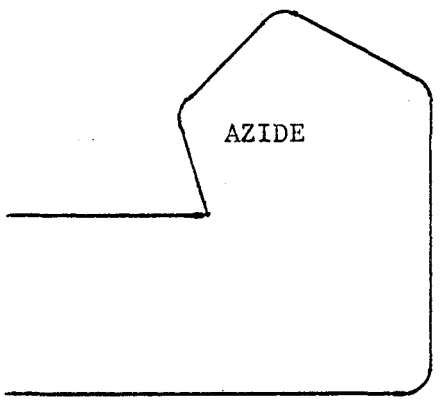
AZIDE -ENZYME COMPLEX



ATP

(product)

Azide competative with adenine nucleotide



X~P

(no reaction)

Azide uncompetative with X~P

(no product)

Figure 37

Schematic representation of the activities of phlorizin on electron transport and oxidative phosphorylation.  ,  indicate energy transfer action;  , possible site of interaction with electron transport.

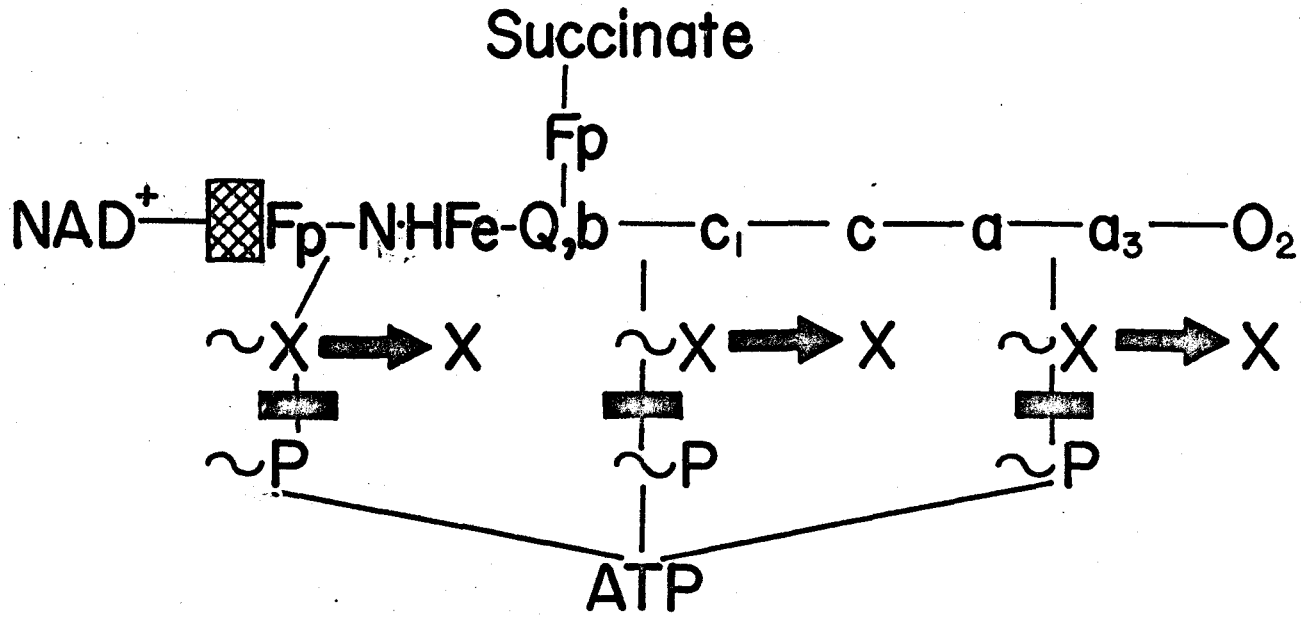
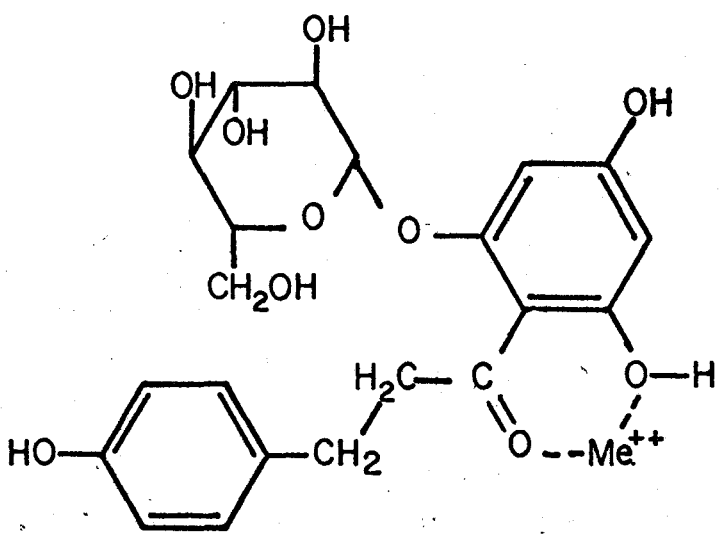

Succinate
Fp
NAD+
Fp-NHFe-Q,b
c1
c
a
a3
O2
~X
X
~P
ATP

Figure 38

The structural conformation of phlorizin showing the possible involvement of a ketone function of chelation of divalent cations (Me^{++}).


OH
OH
OH
O
CH2OH
O
OH
H2C
C
O
O--Me++
O—H
HO
CH2

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