STUDIES OF MITOCHONDRIAL TRANSPORT SYSTEMS

A Thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy at the University of Warwick, Department of Molecular Sciences

By

AZIZ HAMOUD HASAN AL-LAMI, B.Sc., M.Sc.

May, 1977

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURES

ABBREVIATIONS

AA	Agaric acid
ADP	Adenosine-5̄-diphosphate
Amytal	5-Ethyl-5-isoamylbarbituric acid
ATP	Adenosine-5̄-triphosphate
ATPase	Adenosine-5̄-triphosphatase
Atr	Atractyloside
A 20668	Leucinostatin
BKA	Bongkrekic acid
BSA	Bovine serum albumin
CAT	Carboxyatractyloside
Cl-CCP	m-Chlorocarbonyl cyanide phenylhydrazone
CPDS	6,6̄-dithiodinicotinic acid
DBCT	Dibutylchloromethyltin chloride
DCCD	N,N̄-Dicylcohexyl-carbodiimide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetra-acetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)N,N̄-tetraacetic acid
F_1	Soluble mitochondrial ATPase
FCCP	p-Trifluoromethoxy-carbonyl cyanide-phenyl hydrazone
g	gram(s)
HCP	Hexachlorophene
Hepes	N-2-hydroxyethylpiperazine N̄-2-ethanesulfonic acid
K_m	Michaelis constant
L	Litre
m	metre
M	Molar (concentration)
min	minute (time)

μ	micro (10^{-6} x)
NADH	Reduced nicotinamide adenine dinucleotide
n	nano (10^{-9} x)
NEM	N-Ethylmaleimide
OD	Optical density
p-CMBA	p-Chloromercuribenzoic acid
PCP	Pentachlorophenol
Pi	Inorganic phosphate
POPOP	1, 4, di-2(5-phenyloxyazolyl benzene)
PPO	2.5.diphenyloxyazole
RhB	Rhodamine B
Rh3G	Rhodamine 3G
Rh6G	Rhodamine 6G
RhS	Rhodamine S
RLM	Rat liver mitochondria
RNA	Ribonucleic acid
SBP	Sulfobromophthalein
s	second (time)
S.D.	Standard deviation
SMP	Submitochondrial particles
TCC	Tricarboxylic acid cycle
TET	Triethyltin sulphate
Tris	Tris-hydroxymethylaminomethane
w/v	Weight by volume
w/w	Weight by weight
X ~ I	High energy non-phosphorylated intermediate
X ~ P	High energy phosphorylated intermediate
1799	1, 1, 5, 5-trifluoromethyl-1, 5-hydroxy-pentan-3-one

ACKNOWLEDGEMENTS

My sincere thanks to my supervisor, Dr. D.E. Griffiths, in the Department of Molecular Sciences, for his continuing help and advice during this research.

I would like to thank Dr. K. Cai, Dr. J. Mottley and Dr. J. Kabayo for their general help during the writing of this thesis. The loan of certain equipment and chemicals would have been very difficult without the help of Mrs. M. West.

It is a great pleasure to acknowledge Professor K.R. Jennings and Professor V.M. Clark for the availability of research facilities in the Department of Molecular Sciences.

Thanks are due to the Ministry of Higher Education and Scientific Research (Baghdad) for the award of this scholarship.

I should also like to express my gratitude to my wife, Mrs. Elham Al-lami, for her patience and encouragement throughout the course of this work.

SUMMARY

The inhibition of mitochondrial translocation of ions ADP, Pi, Ca^{++} and dicarboxylic acids by sulfobromophthalein (SBP), agaric acid (AA), hexachlorophene (HCP), rhodamine 6G (Rh6G) and other inhibitors, ionophorous antibiotics and uncouplers of oxidative phosphorylation has been examined in rat liver mitochondria. It was found that the exchange of ADP is competitively inhibited by SBP and AA, and uncompetitively by HCP. 2,4-dinitrophenol stimulated ATPase is inhibited also by SBP, AA and HCP. SBP and AA were also found to inhibit the ADP binding to rat liver mitochondria. However, it was shown that Rh6G has no effect on ADP translocase under the present conditions.

SBP, AA and HCP inhibit the ^{3}H-atractyloside binding to mitochondria and submitochondrial particles, while Rh6G has no effect on ^{3}H-atractyloside binding. ^{14}C-HCP has a high affinity binding site ($K_d = 2.2 \times 10^{-8}$ M) and low affinity binding site ($K_d = 1.5 \times 10^{-5}$ M) and ^{14}C-HCP binding was not affected by SBP, AA, atractyloside, Rh6G and other inhibitors used in the present work. On the other hand, Rh6G has one affinity binding site ($K_d = 1.3 \times 10^{-5}$ M). Both ^{14}C-HCP and Rh6G binding depends on mitochondrial phospholipids composition.

The interaction of HCP, Rh6G, DBCT and triethyltin and ionophorous antibiotics with calcium translocation, binding and release from mitochondria shows that the above compounds inhibit the calcium translocation and binding to mitochondria, and they also induced a release of accumulated mitochondrial calcium.

Experiments carried out with rat liver mitochondria show that SBP, HCP, Rh6G, DCCD, DBCT, triethyltin and ionophorous antibiotics were potent inhibitors of the ATP-^{32}Pi exchange reaction. However, agaric acid caused only a slight effect. The ATP-^{32}Pi

exchange reaction in submitochondrial particles was inhibited by HCP, Rh6G, DCCD, triethyltin, venturicidin and uncouplers of oxidative phosphorylation.

The results of the present study demonstrate that SBP and HCP are potent inhibitors of Pi uptake by mitochondria. Furthermore, SBP was found to inhibit the ^{32}Pi-Pi exchange in mitochondria. Agaric acid and Rh6G can inhibit the Pi uptake, which are present at high concentrations. SBP was also shown to inhibit the dicarboxylate uptake and dicarboxylate-dicarboxylate exchange, which are mediated by the dicarboxylate carrier.

CHAPTER 1

INTRODUCTION

1.1 General introduction

The transport of ions by mitochondria has now become one of the most popular topics in the field of bioenergetics, and is perhaps the most important alternative to oxidative phosphorylation as a means of utilizing energy made available by the respiratory chain. Due to the impermeability of the mitochondrial membrane, mitochondria transport substrate anions, inorganic phosphate, and adenine nucleotides in a series of exchange-diffusion processes, catalysed by specific carrier systems. In addition, mitochondria can directly utilize metabolic energy to concentrate a number of monovalent and divalent cations against their chemical gradient, e.g. K^{+} and Ca^{++}.

All of these processes are linked to the energy conservation system in the mitochondrial inner membrane as emphasized in the chemiosmotic hypothesis proposed by Mitchell (1961) as has been discussed in many reviews (Pressman, 1976; Stryer, 1975).

The major feature of mitochondrial membrane structure and energy conservation mechanisms are discussed in detail below.

1.2 Architecture of mitochondria and localization of their functions

The mitochondrion is a subcellular organelle whose dimensions are 1 to 2 microns(Young, Blondin and Green, 1972 ; Sluse and Sluse-Goffart, 1974). As schematically represented in Fig. 1.1. the mitochondria consists of two membranes, an inner and an outer membrane, each of which forms a closed surface. Active transport in the mitochondrion thus involves an energy-linked flux of ions across the inner mitochondrial membrane.

The inner mitochondrial membrane is highly structured. It has been hypothesized to consist of lipoprotein tripartite repeating units as shown in Fig. 1.2. Each tripartite repeating unit consists of a base piece, which contains the components of the mitochondrial electron transfer chain, a head piece which is the site of the mitochondrial ATPase, and a stalk which links the basepiece and headpiece. It is claimed that the primary function of the mitochondrion is to couple electron transfer, which occurs in the "baseplece", to ATP synthesis, which occurs in the "headpiece".

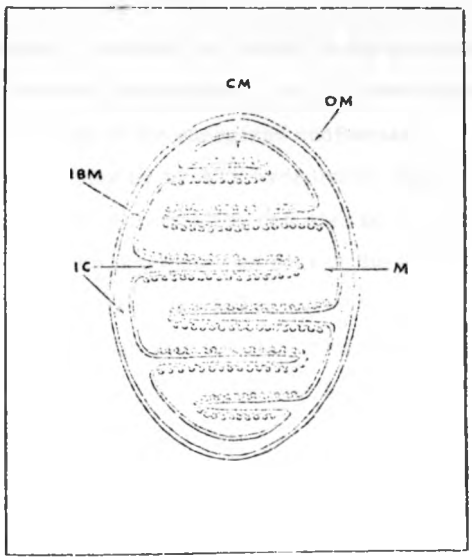


FIGURE 1.1 Schematic representation of the mitochondrion. OM, outer membrane; M, matrix space; CM, cristal membrane; IBM, inner boundary membrane; IC, intracristal space; (from Young et al., 1972).

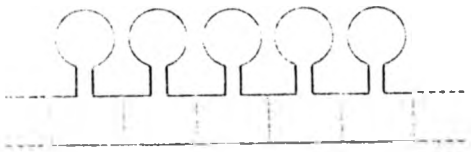


FIGURE 1.2 Schematic representation of inner mitochondrial membrane as made up of tripartite repeating units. Each repeating unit consists of a basepiece, a headpiece, and a connecting stalk; (from Young et al., 1972).

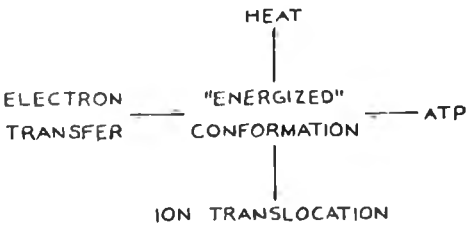


FIGURE 1.3 Schematic representation of coupling scheme in conformational model of oxidative phosphorylation; (from Young et al., 1972).

Electron transfer is reversibly coupled to the generation of the energized or metastable conformation, and ATP synthesis is reversibly coupled to the relaxation of the energized conformation. Once generated, either by electron transfer or by ATP hydrolysis, the energy of the intermediate metastable conformation can also be utilized for ion translocation or it can be dissipated as heat in the presence of uncouplers of oxidative phosphorylation (Fig. 1.3).

In isolated mitochondria, the external membrane does not constitute a barrier for the rapid flux of molecules with molecular weight lower than 5000 (Sluse and Sluse-Goffart, 1974). This is not true of the inner membrane which seems to be impermeable to the free diffusion of a large number of substances and in particular to the diffusion of the substrates and products of intra- and extramitochondrial metabolism. The inner membrane seems to be freely permeable only to a certain number of small neutral molecules of molecular weight lower than 150 that equilibrate with the matrix space in less than one millisecond.

Almost all reactions of the citric acid cycle and substrate-level phosphorylation, as well as pyruvate dehydrogenation, take place in the matrix space (Fonyo et al., 1976). The enzymes of the respiratory chain and of the phosphorylation coupled to it are exclusively located in the inner membrane, and it should be borne in mind that the cristae are extensions of the inner membrane. In addition, one single enzyme of the citric acid cycle, succinate dehydrogenase, is also an integral part of the inner membrane. The functional significance of the outer membrane is poorly understood, although its enzymatic composition is known. Nucleoside diphosphokinase and adenylate kinase are found in the space between the two membranes.

Cytochrome c of the respiratory chain is located loosely bound to the membrane at the surface facing the cytoplasm (called the 'c' face). Succinate dehydrogenase and the enzymes of ATP synthesis are at that surface which faces the matrix ('m' face) (Fig. 1.4). It follows logically that Pi, ADP and ATP have to cross the inner membrane constantly. If any substrate from the cytoplasm enters the citric acid cycle, or, on the other hand, if the cycle furnishes intramitochondrially the carbon skeleton for cytoplasmic synthesis, the corresponding organic ions will again pass through the inner membrane.

Electron transfer is reversibly coupled to the generation of the energized or metastable conformation, and ATP synthesis is reversibly coupled to the relaxation of the energized conformation. Once generated, either by electron transfer or by ATP hydrolysis, the energy of the intermediate metastable conformation can also be utilized for ion translocation or it can be dissipated as heat in the presence of uncouplers of oxidative phosphorylation (Fig. 1.3).

In isolated mitochondria, the external membrane does not constitute a barrier for the rapid flux of molecules with molecular weight lower than 5000 (Sluse and Sluse-Goffart, 1974). This is not true of the inner membrane which seems to be impermeable to the free diffusion of a large number of substances and in particular to the diffusion of the substrates and products of intra- and extramitochondrial metabolism. The inner membrane seems to be freely permeable only to a certain number of small neutral molecules of molecular weight lower than 150 that equilibrate with the matrix space in less than one millisecond.

Almost all reactions of the citric acid cycle and substrate-level phosphorylation, as well as pyruvate dehydrogenation, take place in the matrix space (Fonyo et al. 1976). The enzymes of the respiratory chain and of the phosphorylation coupled to it are exclusively located in the inner membrane, and it should be borne in mind that the cristae are extensions of the inner membrane. In addition, one single enzyme of the citric acid cycle, succinate dehydrogenase, is also an integral part of the inner membrane. The functional significance of the outer membrane is poorly understood, although its enzymatic composition is known. Nucleoside diphosphokinase and adenylate kinase are found in the space between the two membranes.

Cytochrome c of the respiratory chain is located loosely bound to the membrane at the surface facing the cytoplasm (called the 'c' face). Succinate dehydrogenase and the enzymes of ATP synthesis are at that surface which faces the matrix ('m' face) (Fig. 1.4). It follows logically that Pi, ADP and ATP have to cross the inner membrane constantly. If any substrate from the cytoplasm enters the citric acid cycle, or, on the other hand, if the cycle furnishes intramitochondrially the carbon skeleton for cytoplasmic synthesis, the corresponding organic ions will again pass through the inner membrane.

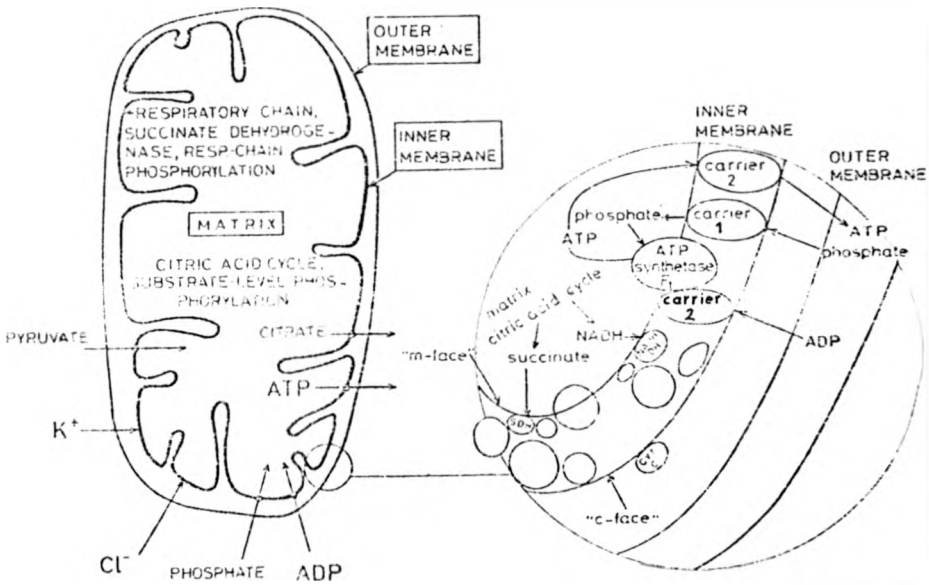


FIGURE 1.4 Scheme of mitochondrial structure and compartmentation. Penetration is indicated by arrows. A part of the inner membrane is shown enlarged in the right half of the figure; (from Fonyo et al., 1976).

1.3 Proposed mechanisms for oxidative phosphorylation

Oxidative phosphorylation is the process by which respiratory chain energy is made available to the ATPase so that it can produce ATP. The mechanism of oxidative phosphorylation is not yet known. Three kinds of mechanisms have been proposed:

1. The chemical-coupling hypothesis (Slater, 1953) was formulated by analogy to glyceraldehyde-3-phosphate oxidation. The demonstration that energy transfer can take place between the individual coupling sites indicates that coupling may proceed by way of non-phosphorylated intermediates. These are postulated to be the electron carriers in the respiratory chain. The reaction pathways leading to the formation of ATP are suggested to be

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \qquad (1)$$

$$A \sim I + X \rightleftharpoons A + X \sim I \qquad (2)$$

$$I \sim X + Pi \rightleftharpoons I + X \sim P \qquad (3)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \qquad (4)$$

The simplest scheme is one in which a high-energy compound denoted as $A \sim I$ is formed when the reduced form of A transfers its electrons to the oxidized form of B (A and B are electron carriers in the respiratory chain, whereas I is some other component). Then, the high-energy bond in $A \sim I$ is split as ADP is phosphorylated to ATP.

2. The chemiosmotic hypothesis (Mitchell, 1961) was postulated to provide an explanation for the requirement of an intact membrane for phosphorylation, since this requirement is not implicit in the chemical-coupling hypothesis. It also sought to explain the failure of all attempts to identify the intermediate X and I. In its simplest form, the chemiosmotic hypothesis consists of the generation of a proton gradient across the proton impermeable inner membrane by the electron transport chain. This proton gradient then drives the synthesis of ATP from ADP and Pi by means of an anisotropic ATPase.

The proposal depends on the possibility that the ATPase may lie in the membrane, isolated from the aqueous phase on both sides, but accessible to OH^- ions on one side and to H^+ on the other. If we consider the hydrolysis of ATP,

$$ATP + H_2O \rightleftharpoons ADP + Pi \qquad (5)$$

This reaction may be written

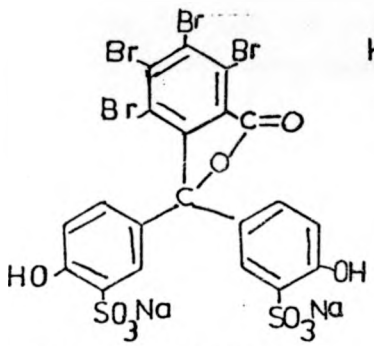
$$ATP + H^+ + OH^- \rightleftharpoons ADP + Pi \qquad (6)$$

given that such an anisotropic ATPase, hydrolysis of ATP would be accompanied by the effective translocation of protons from one side of the membrane to the other. Similarly, the reverse reaction, ATP synthesis, would be effected by a flow of protons across the membrane in the opposite direction.

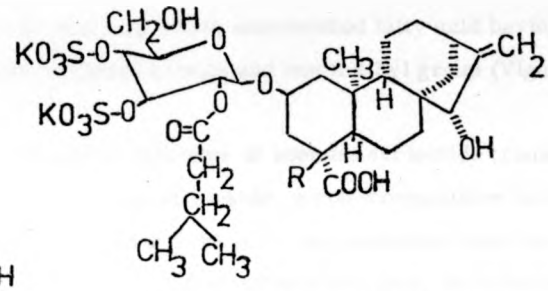
3. The conformational hypothesis (Boyer, 1964) and a new expanded version known as the Electromechanochemical model (Green and Ji, 1972a). The driving force for the synthesis of ATP is a conformational change in the coupling apparatus. The basic unit of mitchondrial energy conservation is suggested to be a 'supermolecule'. The supermolecule consists of two components, one of which catalyses a polarising reaction and one which catalyses a depolarising reaction. The depolarising element, the ATPase, is polarised by separation of electrons and protons in the electron transfer complexes. A conformational strain is set up in the ATPase such that conditions are energetically suitable for a chemical reaction between ADP and Pi. Current evidence tends to favour the chemiosmotic hypothesis although the other hypothesis have not been firmly excluded. Some of the evidence for specific reaction steps in oxidative phosphorylation has come from studies of the mode of action of specific inhibitors and specific information on mitochondrial transport processes can also be gained by use of specific inhibitors of ion transport.

1.4 Inhibitors and uncouplers of oxidative phosphorylation used in the present study

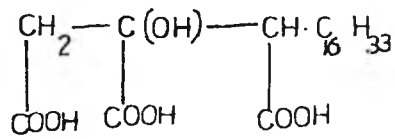
Three specific inhbitors are currently used in experiments on adenine nucleotide transport, namely atractyloside, carboxyatractyloside (for review see ref. Vignais et al, 1973b) and bongkrekic acid (Henderson and Lardy, 1970; Henderson et al., 1970). Atractyloside and carboxyatractyloside (Fig. 1.5) are glucosides whose aglycone is a diterpene. Both molecules contain two sulfate groups and one isovaleric acid residue (Lefrance, 1968: Defaye et al., 1973). Carboxyatractyloside differs from atractyloside by a supplementary carboxyl group at the C-4 of the diterpene nucleus (Defaye et al., 1971).



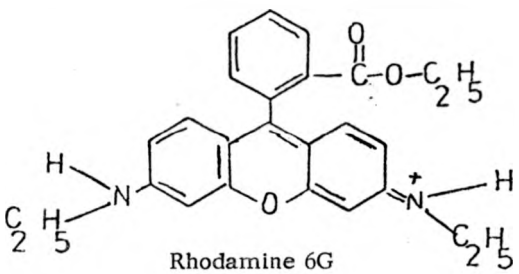
Sulfobromophthalein (SBP)



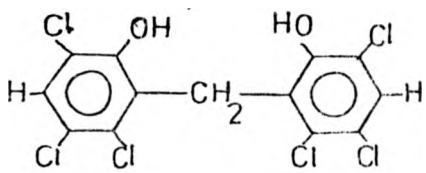
R = H = Atractyloside (Atr)
R = COOH = Carboxyatracyloside (CAT)



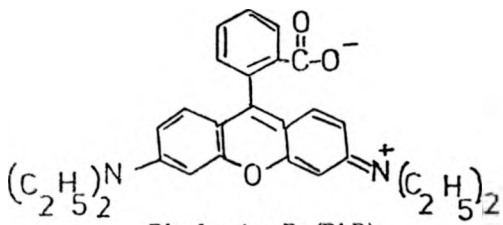
Agaric acid (AA)



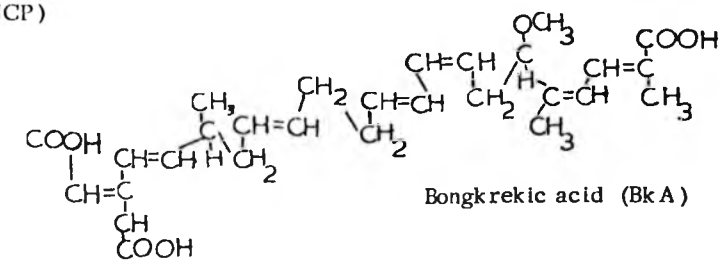
Rhodamine 6G
(Rh6G)



Hexachlorophene (HCP)



Rhodamine B (RhB)



Bongkrekic acid (BkA)

Figure 1.5 Structures of SBP, Atr, CAT, AA, HCP, Rh6G, RhB and BkA

Bongkrekic acid (Fig. 1.5) is a long-chain unsaturated fatty acid having seven double bonds, three carboxyl groups and one methyl group (Vignais, 1976).

Atractyloside is a competitive inhibitor of adenine nucleotide transport (Vignais et al., 1973a), carboxyatractyloside, a non-competitive inhibitor (Vignais et al., 1973a); Bongkrekic acid is an uncompetitive inhibitor (Lauquin et al., 1976). All can be radio-actively labelled, thus facilitating ligand-binding studies. The first binding studies were carried out with biosynthetically labelled inhibitors (Vignais et al., 1971a). Radiochemically labelled atractyloside (Brandolin et al., 1974) and bongkrekic acid (Lauquin and Vignais, 1976) can now be readily made with a high specific radioactivity.

Vignais et al. (1961 and 1962) reported that atractyloside was able to block partial reactions of oxidative phosphorylation in mitochondria, such as the ATP-Pi exchange and the 2, 4-dinitrophenol stimulated ATPase activity. Inhibition of adenine nucleotide transport by bongkrekic acid requires that the pH of the incubation medium be lower than 7, and to be compatible with the effectiveness of adenine nucleotide transport, incubations are routinely carried out at pH 6.5 - 6.8. Studies with ^{3}H-bongkrekic acid show that the protonated acid diffuses readily through the inner mitochondrial membrane and enters the matrix space (Lauquin and Vignais, 1976); bongkrekic acid may, therefore, be considered as a penetrant inhibitor. On the other hand, atractyloside and carboxyatractyloside remain bound to the outer surface of the inner mitochondrial membrane; they are non-penetrant inhibitors (Klingenberg, 1970).

Meisner (1973) reported briefly that sulfobromophthalein (SBP) is an effective inhibitor of the adenine nucleotide exchange, and the Pi/OH as well as Pi/malate uptake in mitochondria. SBP is also called bromosulfophthalein, hepartest, bromthalein, bromsulphalein and brom-tetragnost, ($C_{20}H_8Br_4Na_3O_{10}S_2$). SBP is used in medicine, in liver function tests.

The effect of agaric acid on the transport of ADP and ATP has been examined by Chavez and Klapp (1975) in rat liver mitochondria. Respiration stimulated by ADP is progressively inhibited by agaric acid; maximal inhibition is attained at 40 μM agaric acid. ATPase activity is inhibited 30%

by 20 μM agaric acid. The exchange of adenine nucleotide is competitively inhibited by agaric acid. Agaric acid is also called agaricic acid, laricic acid, agaricin, n-hexadecylcitric acid or α-cetylcitric acid, ($C_{22}H_{40}O_7 \cdot 1\frac{1}{2}H_2O$). It is used in medicine, as an anhydrotic. One optical form of agaric acid (α-hexadecylcitric acid) occurs in the basidiomycete, Polyporus officinalis. It has been synthesised by Evans (1959).

Since the introduction of hexachlorophene, HCP or 2, 2-methylenebis-(3, 4, 6-trichlorophenol) as an antibacterial additive to soaps, detergents and cosmetics, a large number of toxicological studies have been carried out. These studies dealt with the systemic and the oral acute and subacute toxicity in laboratory animals, also with the toxic symptoms and deaths observed in cows and sheep where HCP is used for the control of fascioliasis. Reports on toxic effects in man and poisoning from improper use have also been published (Gump, 1969). The primary lethal action of HCP thus appears to be respiratory inhibition at a site within the membrane-bound part of the electron transport chain (Frederick et al., 1974).

The lipophilic dye, rhodamine 6G, has been shown to be a potent inhibitor of oxidative phosphorylation (Gear, 1974). ATP-supported Ca^{++} accumulation was blocked. Concentrations of rhodamine 6G above 10 μM slowly uncouple respiration and inhibit respiration-dependent Ca^{++} uptake. Neither the dinitrophenol-stimulated ATPase nor uncoupled respiration of intact mitochondria were inhibited by the dye, even at 50 μM. The related compound, rhodamine B, a free acid and uncharged at pH 7, was completely without influence on mitochondrial energy-linked functions. Rh6G blocks adenine nucleotide binding to intact mitochondria, both for ^{14}C-ATP and ^{14}C-ADP (Gear, 1974). Gear concluded that rhodamine 6G blocks the adenine nucleotide translocase apparently being similar to atractyloside and bongkrekic acid. However, in sharp contrast to these inhibitors, rhodamine 6G did not inhibit the 2, 4-dinitrophenol-stimulated ATPase of intact mitochondria.

Triethyltin and other trialkyltins affect the mitochondria in two distinct ways. Firstly, they are potent inhibitors of coupled respiration and of phosphate esterification of respiring mitochondria (Aldridge, 1958; Aldridge and Threlfall, 1961; Sone and Hagihara, 1964). In this respect they appear

by 20 μM agaric acid. The exchange of adenine nucleotide is competitively inhibited by agaric acid. Agaric acid is also called agaricic acid, laricic acid, agaricin, n-hexadecylcitric acid or α-cetylcitric acid, ($C_{22}H_{40}O_7 \cdot 1\frac{1}{2}H_2O$). It is used in medicine, as an anhidrotic. One optical form of agaric acid (α-hexadecylcitric acid) occurs in the basidiomycete, Polyporus officinalis. It has been synthesised by Evans (1959).

Since the introduction of hexachlorophene, HCP or 2, 2-methylenebis-(3, 4, 6-trichlorophenol) as an antibacterial additive to soaps, detergents and cosmetics, a large number of toxicological studies have been carried out. These studies dealt with the systemic and the oral acute and subacute toxicity in laboratory animals, also with the toxic symptoms and deaths observed in cows and sheep where HCP is used for the control of fascioliasis. Reports on toxic effects in man and poisoning from improper use have also been published (Gump, 1969). The primary lethal action of HCP thus appears to be respiratory inhibition at a site within the membrane-bound part of the electron transport chain (Frederick et al., 1974).

The lipophilic dye, rhodamine 6G, has been shown to be a potent inhibitor of oxidative phosphorylation (Gear, 1974). ATP-supported Ca^{++} accumulation was blocked. Concentrations of rhodamine 6G above 10 μM slowly uncouple respiration and inhibit respiration-dependent Ca^{++} uptake. Neither the dinitrophenol-stimulated ATPase nor uncoupled respiration of intact mitochondria were inhibited by the dye, even at 50 μM. The related compound, rhodamine B, a free acid and uncharged at pH 7, was completely without influence on mitochondrial energy-linked functions. Rh6G blocks adenine nucleotide binding to intact mitochondria, both for ^{14}C-ATP and ^{14}C-ADP (Gear, 1974). Gear concluded that rhodamine 6G blocks the adenine nucleotide translocase apparently being similar to atractyloside and bongkrekic acid. However, in sharp contrast to these inhibitors, rhodamine 6G did not inhibit the 2, 4-dinitrophenol-stimulated ATPase of intact mitochondria.

Triethyltin and other trialkyltins affect the mitochondria in two distinct ways. Firstly, they are potent inhibitors of coupled respiration and of phosphate esterification of respiring mitochondria (Aldridge, 1958; Aldridge and Threlfall, 1961; Sone and Hagihara, 1964). In this respect they appear

to behave somewhat like oligomycin, being capable of inhibiting not only oxidative phosphorylation, but also partial reactions such as the ^{32}Pi-ATP exchange reaction and mitochondrial ATPase activity (Aldridge and Street, 1964). Secondly, and in addition to its inhibitory action on mitochondrial phosphorylation, triethyltin has been found to uncouple mitochondrial respiration and induce mitochondrial ATPase activity (Aldridge, 1958). Stockdale et al. (1970) have succeeded in separating the two effects of the trialkyltin compounds. They found that when mitochondria were incubated in sucrose medium in the absence of permeating anions, only the oligomycin-like effects of the trialkyltin compounds were observed; that is, inhibition of coupled respiration. No inhibition of other uncoupler stimulated respiration was observed, and neither was mitochondrial swelling. Incubation of mitochondria with trialkyltins in the presence of permeant anions such as contained in KCl buffers resulted, on the other hand, in an uncoupling effect. Uncoupling by trialkyltin compounds is probably due to the action of the anion-hydroxyl exchange reaction which trialkyltin compounds appear to be able to mediate (Selwyn et al., 1970a) followed by leakage of the anion from the mitochondria; this results in a discharge of both the pH and membrane potentials. Manger has observed that triethyltin chloride inhibits intra-mitochondrial accumulation of substrate anions (Manger, 1969) and this effect is presumably related to the discharge of the pH gradient and accumulation of chloride ion within the mitochondrion caused by the trialkyltin mediated anion-hydroxide exchange.

Valinomycin interferes with oxidative phosphorylation in mitochondria by making them permeable to K^+. The result is that mitchondria use the energy generated by electron transport to accumulate K^+ rather than to make ATP. Binet and Volfin (1975b) reported that the stability of the membrane is regulated by the presence of a low amount of Mg^{++}. If this Mg^{++} is released by the means of ionophores (e.g. A23187), the membrane Ca^{++} becomes labile and this process leads to the loss of the impermeability of the mitochondrial membrane. Leucinostatin (Lardy et al., 1975) and X-537A (Akerman et al., 1974) may have the same effect as A23187.

Many chemicals have been found to exert an uncoupling effect (e.g. DNP, FCCP, Cl-CCP, 1799 and pentachlorophenol) on mitochondrial oxidative phosphorylation. While the mechanisms by which these compounds uncouple are not entirely clear, there are a number of common effects which serve to characterize this group. These include (1) an increase in mitochondrial respiration rate when the incubation medium is deficient in phosphate acceptor or inorganic phosphate, (2) a decrease in orthophosphate uptake, (3) increased mitochondrial ATPase activity,and (4) inhibition of the ATP-^{32}Pi exchange reaction (Parker, 1965). Most uncoupling agents are weakly acidic and lipophilic and are presumed to exert their uncoupling effects through a nonspecific interaction with the mitochondrial membranes.

1.5 Ion carrier systems

A. Adenine nucleotide carrier

The finding by Bruni et al. (1964) that Atr inhibits the binding of external ADP to mitochondria opened the way to further studies that led to the identification of the adenine nucleotide (Adn) translocator in the inner mitochondrial membrane (Klingenberg and Pfaff, 1966) and to the characterization of Atr as an inhibitor of the transmembrane exchange between internal and external Adn (Duee and Vignais, 1965).

The adenine nucleotide translocase is especially important since it performs two interrelated functions (Spencer and Bygrave, 1972). First, it is able to control the relative concentration of ATP and ADP, perhaps the most important chemical species in the cell, in the cytoplasm and mitochondrial matrix. These two compounds are involved in many aspects of cell metabolism, if not as substrates for a reaction, then certainly as chemical effectors or modifiers of numerous enzymic reactions. Secondly, the translocase is responsible for the transport of ADP and ATP into and out of the mitochondria, and in this way it acts as a channel in the energy metabolism of the cell by assisting in the production of ATP (Klingenberg et al., 1976).

ADP or ATP is transported across the inner mitochondrial membrane by an exchange-diffusion mechanism as shown by the fact that when mitochondria are incubated with [^{14}C]ADP or [^{14}C]ATP, the radioactivity is taken up by

mitochondria, but there is no change in the internal Adn pool; for each molecule of ADP or ATP entering the mitochondrion there is one molecule of ADP or ATP leaving the mitochondrion (Klingenberg and Pfaff, 1965; Pfaff et al., 1965).

Another interesting feature is the effect of the Adn transport on the morphology of mitochondria; changes occurring in the conformation of the inner mitochondrial membrane upon addition of ADP or ATP suggest that protein components of this membrane in close proximity to the Adn translocator are able to change their conformation in response to the functioning of the Adn translocator.

A schematic survey of the metabolite fluxes through the mitochondrial membrane is depicted in Fig. 1.6. In principle, in the same manner as in an intact cell, a selected number of substances are taken up or released from the mitochondria which, with their largely impermeable membrane, retain not only enzymes and co-enzymes but also cations and certain metabolites (Klingenberg, 1970).

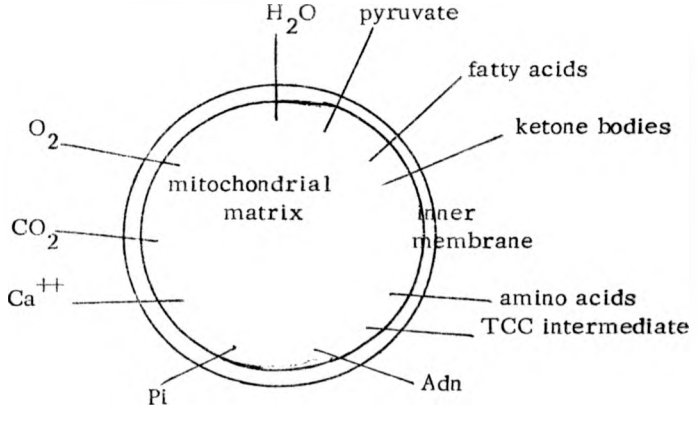
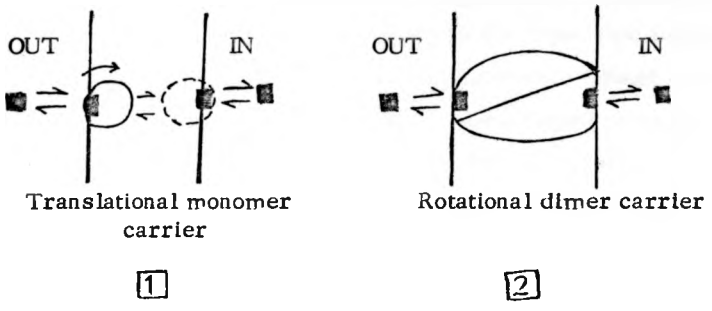


Figure 1.6 The metabolite flux through the mitochondrial membrane (TCC = Tricarboxylic acid cycle)

Mitchell (1961) suggested that the low permeability of the inner membrane to the substrates and products of those reactions that are catalysed by the intramitochondrial enzymes, would be a serious limiting factor for such an activity if the membrane did not contain specific carriers for these metabolites and products. Thus these carriers may regulate intramitochondrial metabolism by permitting transfer of metabolites through the mitochondrial membrane. The evidence available for the existence of anion translocators in the inner membrane is based on three classical criteria of the so-called facilitated transport. The first criterion is a saturation kinetics of the Michaelis type which suggest that the carriers contain specific sites to which the substances undergo reversible binding. The second criterion is the specificity for the substances transported; it has thus been postulated that mediated-transport systems in membranes contain a binding site complementary to the substance transported, resembling in its specificity the active site of enzyme molecules. The third criterion is that it may be inhibited quite specifically. Some carriers are inhibited competitively by substances structurally related to the substrates, and non-competitively by reagents capable of blocking or modifying specific functional groups of proteins, such as sulphydryl group-blocking agents or amino-group-blocking agents.

The different models which have been proposed (Vignais et al., 1973b) to take account of some of the properties of the ADP carrier and in particular of the competitive displacement of ADP by Atr are shown in Fig. 1.7. In all these models, loading of the carrier is a prerequisite to its mobility and therefore to its functioning.

A-MOBILE CARRIER



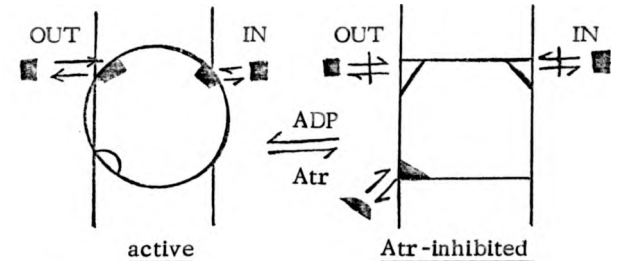
Translational monomer carrier

1

Rotational dimer carrier

2

B- Transconformation



active

Rotational diffusion

or

Atr-inhibited

conformational change

3

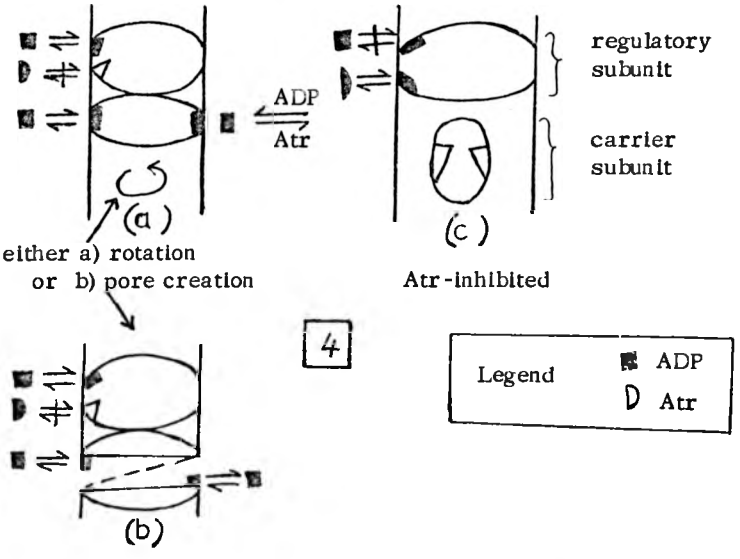


FIGURE 1.7 ADP Carrier Models (from Vignais et al., 1973b).

The first two models (Weidemann et al., 1970) assume no conformational change. Model 1 is a mobile single site carrier, with orientation of its ADP site alternatively to the inner and outer side of the membrane. Once loaded with ADP or ATP of one compartment, when juxtaposed to one side of the membrane, the carrier moves by translation to the other side and exchanges its bound ADP with free ADP of the other compartment. Model 2 is a dimer carrier with 2 sites diametrically opposite. Once loaded on its two faces, the carrier can rotate and bring nucleotides to the other side of the membrane. In these two models, Atr is supposed to bind at the same site as ADP.

The functioning of models 3 and 4 involve allosteric transconformation. Model 3 represents an allosteric interconversion of ADP and Atr binding sites as first suggested by Vignais et al. (1966). Model 4 (Vignais et al., 1971b) includes a regulatory site besides the carrier, to explain the asymmetric behaviour of the translocation process (preferential exchange of external ADP for internal ATP). The regulatory unit is assumed to contain two distinct interacting sites, one for ADP, the other for Atr. In the absence of ADP or when Atr is bound to the regulatory unit, the ADP carrier is in an inactive state corresponding to a definite conformation (4c). Once ADP is added, it binds first to its regulatory site; this results in a conformational change of the membrane propagated to the carrier which takes an active form capable of binding and transferring ADP by rotational diffusion (4a) or by opening of a pore (4b). Model 4b illustrates the principle of a pore opened through the membrane and whose opening and specific functioning is under the control of a regulatory unit.

The carrier is most probably a highly hydrophobic protein embedded in a lipid structure. The fluidity of the lipid part of the membrane determines the rate of the transport process (Fonyo et al., 1976).

Apart from the protein-lipid interaction there are indications that the carrier forms an integrated functional unit with the enzyme which catalyzes the phosphorylation of ADP to ATP, the so-called F_1-ATPase system. When [^{14}C] ADP was incubated with mitochondria under conditions where oxidative phosphorylation occurred, it was not [^{14}C] ADP that was detected in the matrix space of mitochondria, but [^{14}C] ATP. Consequently, [^{14}C] ADP did not mix with the intramitochondrial [^{12}C] ADP pool but was phosphorylated as it entered the inner

compartment during its passage through the membrane. To explain this finding it has been postulated that the ADP carrier is in close spatial proximity to, and eventually forms a functional unit with, the F_1-ATPase system (Vignais et al., 1975).

The "physiological" exchange reaction catalyzed by the carrier is between ADP external and ATP internal. Other exchanges may also occur: between ATP external and ADP internal (as in measuring ATPase activity in mitochondria), between ADP and ADP, and between ATP and ATP. Since the highest pK_a value of ADP is 6.65 and that of ATP is 6.97, at the pH of the cell ADP is almost in the fully ionized form (ADP^{3-}), while ATP is mostly fully ionized (ATP^{4-}) with a fraction present as $HATP^{3-}$ (Fonyo et al., 1976). ADP^{3-} may exchange with $HATP^{3-}$ resulting in an electroneutral exchange; however, the exchange may also occur between ADP^{3-} and ATP^{4-}, and in this case the process is electrogenic, which is charge-compensated by Pi uptake (Fig. 1.8).

Because of the availability of tightly bound specific ligands in radioactive form, the Adn carrier seemed particularly well suited for isolation. [^{35}S] carboxyatractyloside (CAT) was used by one group of investigators to label the carrier in the intact mitochondria, and the labelled protein fraction was then purified (Klingenberg et al., 1974). Another group of workers pretreated the mitochondria with either Atr or CAT and then put the lysed mitochondria on an affinity column in which succinylatractyloside was bound to sepharose. Finally the Atr binding protein was eluted with ^{3}H-labelled Atr, which replaced the unlabelled Atr and became bound to a protein (Brandolin et al., 1974). A third approach was to use [^{14}C]N-ethylmaleimide (NEM) as a marker (Klingenberg et al., 1974). It had been shown earlier that ADP stimulates the alkylation of a mitochondrial component by NEM. An attempt was made to isolate that protein fraction into which [^{14}C]NEM incorporation was stimulated by ADP.

Metabolic significance of ADP and ATP transport

The transport of ADP and ATP together with Pi is the most powerful transport system in eukaryotic cells which relies mainly on respiratory energy. The main function is the exchange of ADP, originating from energy consuming reactions in the cytosol, against ATP, generated in the mitochondria (Fig. 1.9) (Klingenberg,

compartment during its passage through the membrane. To explain this finding it has been postulated that the ADP carrier is in close spatial proximity to, and eventually forms a functional unit with, the F_1-ATPase system (Vignais et al., 1975).

The "physiological" exchange reaction catalyzed by the carrier is between ADP external and ATP internal. Other exchanges may also occur: between ATP external and ADP internal (as in measuring ATPase activity in mitochondria), between ADP and ADP, and between ATP and ATP. Since the highest pK_a value of ADP is 6.65 and that of ATP is 6.97, at the pH of the cell ADP is almost in the fully ionized form (ADP^{3-}), while ATP is mostly fully ionized (ATP^{4-}) with a fraction present as $HATP^{3-}$ (Fonyo et al., 1976). ADP^{3-} may exchange with $HATP^{3-}$ resulting in an electroneutral exchange; however, the exchange may also occur between ADP^{3-} and ATP^{4-}, and in this case the process is electrogenic, which is charge-compensated by Pi uptake (Fig. 1.8).

Because of the availability of tightly bound specific ligands in radioactive form, the Adn carrier seemed particularly well suited for isolation. [^{35}S] carboxyatractyloside (CAT) was used by one group of investigators to label the carrier in the intact mitochondria, and the labelled protein fraction was then purified (Klingenberg et al., 1974). Another group of workers pretreated the mitochondria with either Atr or CAT and then put the lysed mitochondria on an affinity column in which succinylatractyloside was bound to sepharose. Finally the Atr binding protein was eluted with ^{3}H-labelled Atr, which replaced the unlabelled Atr and became bound to a protein (Brandolin et al., 1974). A third approach was to use [^{14}C]N-ethylmaleimide (NEM) as a marker (Klingenberg et al., 1974). It had been shown earlier that ADP stimulates the alkylation of a mitochondrial component by NEM. An attempt was made to isolate that protein fraction into which [^{14}C]NEM incorporation was stimulated by ADP.

Metabolic significance of ADP and ATP transport

The transport of ADP and ATP together with Pi is the most powerful transport system in eukaryotic cells which relies mainly on respiratory energy. The main function is the exchange of ADP, originating from energy consuming reactions in the cytosol, against ATP, generated in the mitochondria (Fig. 1.9) (Klingenberg,

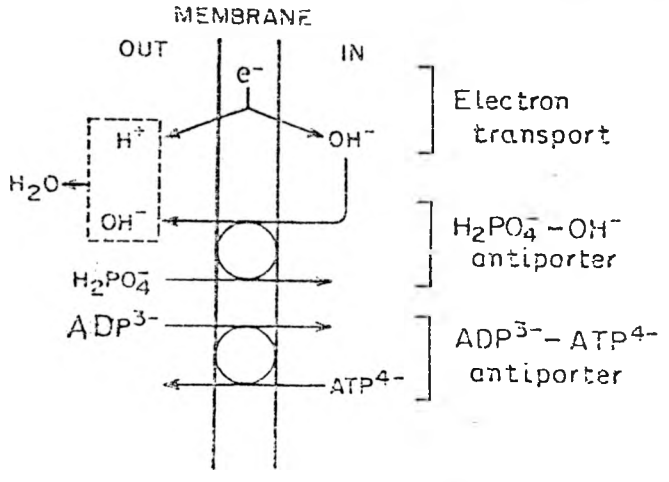


FIGURE 1.8 Sequential coupling of phosphate^{-}, ADP^{3-}, and ATP^{4-} transport to electrochemical gradient generated by electron transport (from Lehninger, 1971).

1976). The ADP, ATP carrier is the exclusive link between intra- and extra-mitochondrial Pi-transfer reactions.

Mitochondria have a striking ability to transport ADP in preference to ATP. This explains the finding that with mitochondria added ADP isnearly completely converted to ATP with a relatively sharp break in the respiratory rate. In other experiments the efflux of ADP versus ATP is shown to be changed by energization in the opposite direction such that the efflux of ATP is strongly preferred to that of ADP.

Interpretation of this energy-driven exchange comes from considering that ATP has one negative charge in excess to that of ADP. In the exchange of ADP against ATP one negative charge is released from the mitochondria. A membrane potential positive outside would electrophoretically pull this charge outside under the expense of energy. The energy is taken from the pool of energy provided by the respiratory chain which provides positive charges outside (Klingenberg, 1976).

The Adn carrier is extremely specific. It catalyzes the transport of ADP and ATP but not of AMP. The di- and triphosphates of other nucleotide bases are inactive with two possible exceptions: GDP and GTP are transported in mitochondria of brown fat and UDP and UTP in mitochondria of yeast cells, where these nucleotides have an important specific role in metabolism. Some synthetic methylene and hypophosphate analogues are also transported by the carrier (Fonyo et al., 1976). The specificity for nucleotides of adenine is particularly significant in view of the fact that all the different nucleotide triphosphates are present in mitochondria and are necessary for mitochondrial biosynthesis of DNA, RNA and protein. The mitochondrial pool of nucleotides thus is segregated from the cytoplasmic pool (Lehninger, 1971). The AMP is not transported, so that the intra- and extramitochondrial AMP remain separated (Klingenberg, 1970). This has important metabolic consequences in that the intra- and extramotochondrial AMP depots are rephosphorylated to ADP by separate systems, within the mitochondrial matrix by the GTP-AMP-transphosphorylase coupled to the substrate level phosphorylation, and outside by the adenylate kinase which is exclusively located in the intramembrane space.

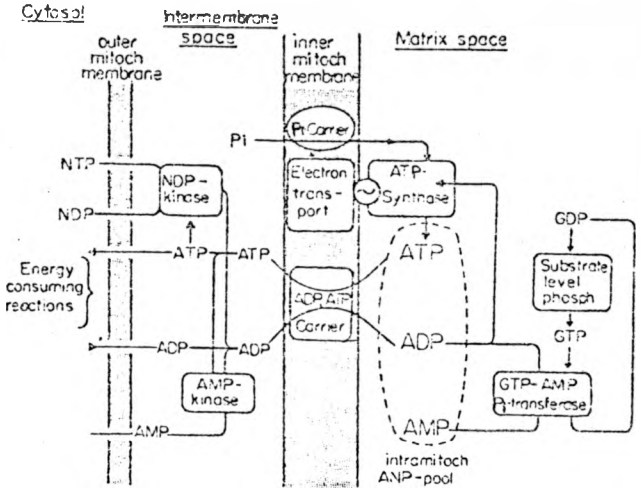


FIGURE 1.9 The role of the ADP, ATP carrier in mitochondrial phosphate transfer reactions.

The function of the intramitochondrial Adn pool as an intermediate in the synthesis of the extramitochondrial ATP. localization of transport systems on the impermeable inner mitochondrial membrane and diffusion through the permeant outer mitochondrial membrane. Exclusion of Adn by the inner mitochondrial membrane and localization of AMP re-utilizing phosphate transferases in the peri-mitochondrial and intramitochondrial space (from Klingenberg, 1976).

B. Calcium carrier

Of the various ions which can be transported across the cell and intracellular membrane systems, of key importance is Ca^{++}. An impressive list of reactions taking place in various cells are known to be influenced by this cation, as seen in Table 1.1 (Carafoli et al., 1976), and it is thus rather obvious that the regulation of the intracellular concentration of Ca^{++} is a phenomenon of physiological significance.

The most interesting properties of the process of active calcium transport by mitochondria can be summarized as follows (Carafoli and Lehninger, 1971): the energy source can be either the respiratory chain or the hydrolysis of ATP, inhibition being induced by respiratory inhibitors in the first case, and by oligomycin in the second. Both ATP and the activity of the respiratory chain energize the uptake by generating a membrane potential across the inner membrane (Rottenberg and Scarpa, 1974), which is probably the immediate pulling force for the uptake of calcium.

Experiments mentioned by Carafoli et al. (1976) indicate that a large fraction of the endogenous pool is in a dynamic steady state, suggesting that calcium, or a large fraction of it, is not maintained in mitochondria as insoluble salt. This is a rather important conclusion. If it is assumed, for instance, that all the endogenous mitochondrial calcium is ionized, its concentration within the organelle would be about 10^{-2} M, that is, five to six orders of magnitude higher than that in the cytosol (10^{-7} - 10^{-8} M). This in turn opens the question of how the balance of calcium between mitochondria and the cytosol is regulated in vivo, given the fact that the amount of calcium present in mitochondria under normal conditions seems to be relatively constant, and rarely becomes much larger than 10 - 15 n moles per mg of protein. This is, perhaps, the most important problem in the physiology of mitochondrial calcium. It clearly requires, however, that the process by which mitochondria assume cytosolic calcium be reversible, otherwise this efficient uptake mechanism would lead to mitochondrial calcification.

When both calcium and phosphate are present in the incubation medium, the maximum level of calcium accumulated by the organelle increases several fold. In this case, however, most of the calcium is accumulated in the form of an insoluble phosphate salt, and can logically be considered of little importance for

TABLE 1.1

Calcium-dependent reactions in cells

1) Activation of enzyme systems:
Glycogenolysis (phosphorylase b kinase); lipases and phospholipases; α-glycerophosphate dehydrogenase; pyruvate dehydrogenase; succinate oxidation; synthesis of some phospholipids; NADH dehydrogenase (plant mitochondria); cytochrome c to mitochondrial membrane.

2) Inhibition of enzyme systems:
Pyruvate kinase; synthesis of some phospholipids; substrate oxidation (NADH leakage) in lung mitochondria.

3) Activation of contractile and motile systems:
Muscle myofibrils; cilia and flagella; microtubules and microfilaments; cytoplasmic streaming; pseudopod formation.

4) Hormonal regulation:
Formation and/or function of cyclic AMP; release of insulin, steroids, vasopressin, oxytocin, catecholamines, thyroxine and progesterone.

5) Membrane-linked functions:
Excitation-secretion coupling at nerve endings; excitation-contraction coupling in muscles; exocrine secretion (pancreas, salivary glands and HCl in the stomach); aggregation of platelets; action potential (nerve and muscle cells); $Na^+ + k^+$-activated ATPase of several membranes; tight junctions; cell contact; binding of prostaglandins to membranes.

From Carafoli et al., (1976).

the rapid regulation of cytosolic calcium (Carafoli et al., 1976). An interesting problem, however, is whether, in the conditions prevailing, the intracellular uptake of calcium uncomplicated by the simultaneous uptake of phosphate is possible. The high concentrations of inorganic phosphate present in the cytosol have recently prompted Lehninger (1974) to suggest that phosphate could play an essential role in the uptake of calcium, as visualized in the scheme of Fig. 1.10.

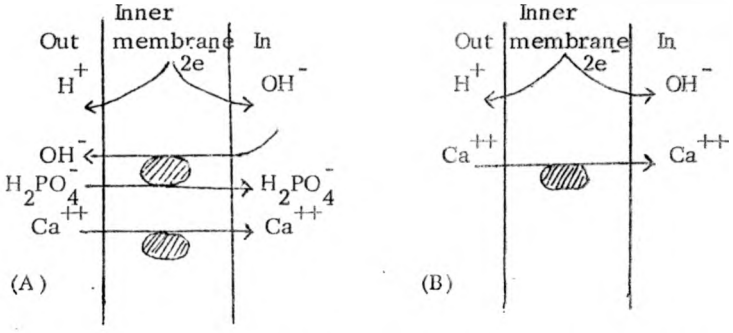


FIGURE 1.10 (A) Uptake of Ca^{++} and phosphate (phosphate first);
(B) Uptake of Ca^{++} alone
(from Carafoli et al., 1976).

Three types of calcium transport have been identified in rat liver mitochondria (Carafoli and Rossi, 1971):

1) The energy-linked transport is usually stoichiometric with electron flow, and is accompanied by alkalinization of the membrane and by ejection of hydrogen ions, and depends on the conservation of energy up to a level prior to the point of action of oligomycin.

2) The metabolism-independent binding most likely reflects the complexing of calcium with the phospholipids of the mitochondrial membranes.

3) The high-affinity binding is carried out by a very small number of sites (sometimes less than one per mg of mitochondrial protein) having a very high affinity for calcium ($K_m = 10^{-7}$ to 10^{-8} M). They are inhibited by uncouplers, by local anaesthetics and by lanthanum at very low concentration. They function in the total absence of metabolism. Some agents that inhibit the high affinity binding also inhibit the energy-linked calcium accumulation. It would seem logical to postulate that high-affinity binding is a necessary preliminary step in the energy-linked translocation of calcium.

The divalent cation composition of the outer membrane is variable, but is particularly high in Mg^{++} content (Bogucka and Wojtczak, 1971). This indicates that Mg^{++} and Ca^{++} do not play the same role in the inner membrane and in the outer membrane. As the principal functions of the mitochondria are located in the inner membrane which represents 85% of the total membranes. Binet and Volfin (1975a)have considered that the movements of the membrane divalent cations studied occur essentially in the inner membrane.

Calcium, through its positive charges, is implicated in a ternary complex between anionic groups of membrane proteins (and possible glycoproteins) and/or phospholipids. Calcium would act as a cement between the components of the membrane and contribute to its stability. In contrast, Mg^{++}, present in much smaller amount in the mitochondrial membrane, exerts essentially a regulatory role in the maintenance of the membrane integrity. The presence of this Mg^{++} on a number of membrane sites is essential for some functions of the mitochondrial membranes and especially for the maintenance of the impermeability of the mitochondrial inner membrane. The displacement of this regulatory Mg^{++} by calcium, by uncouplers of oxidative phosphorylation or by specific ionophores (Binet and Volfin, 1975b), induces severe modifications of the mitochondrial functions including uncoupling of oxidation phosphorylation, and to a lesser extent the impermeability of the membrane for k^{+} and other ions or molecules.

C. Phosphate and dicarboxylate carrier

Mitchell (1953) discovered a phosphate exchange-diffusion carrier in Micrococcus pyogenes that was strongly inhibited by organic mercurials. His theory and observations suggested that a study of the effect of organic mercurials on phosphate metabolism in isolated mitochondria might reveal the existence of a similar carrier in the mitochondrial membrane. Tyler (1968) found that the organic mercurial sodium mersalyl inhibited respiration and swelling dependent on the entry of inorganic phosphate into rat liver mitochondria.

Pi transported by two carriers: either by the Pi carrier in exchange for OH^{-} ions (this being inhibited by NEM), or via the dicarboxylate carrier in exchange for dicarboxylate ions (which is inhibited by the substituted malonates). Therefore the Pi-Pi exchange (that is, ^{32}Pi-^{31}Pi exchange) is catalyzed by both carriers, and neither NEM alone nor substituted malonates alone inhibit the process, but their

combination results in complete inhibition (Meijer et al., 1970). The affinity of both carriers for Pi appear to be similar. Most of the ^{32}Pi-^{31}Pi exchange, however, occurs via the Pi carrier (Coty and Pedersen, 1974). A question which naturally emerges is whether the Pi transporting site of the Pi carrier and that of the dicarboxylate carrier are identical. The specificity of inhibitors argues against the identity of the sites.

Kinetic analysis of the dicarboxylate carrier revealed that all the dicarboxylates and dicarboxylate analogues compete for the same site and that Pi has a binding site distinct from the dicarboxylate site (Palmieri et al., 1971).

D. Tricarboxylate carrier

This carrier transports tricarboxylates (citrate, isocitrate, cis-aconitate) and dicarboxylates (malate, succinate, but not malonate) as well as phosphoenol pyruvate.

E. Oxoglutarate carrier

Exchange characteristics as well as inhbitor studies revealed that 2-oxoglutarate is transported via a specific carrier of its own, which catalyzes an electroneutral exchange between oxoglutarate, malate, succinate, malonate and some other dicarboxylates. As expected, the exchanges malate-malate, malate-malonate, malonate-malonate, etc. may also occur.

1.6 The coupling of metabolite transport to electron transport

The various metabolite-transport systems shown in Fig. 1.11 are passive systems (Lehninger, 1975); in the absence of respiration they transport metabolites down concentration gradients in the direction that will result in thermodynamic equilibrium across the membrane. However, these systems can also transport their substrates against gradients of concentration when they are coupled to electron transport as a source of energy. We have already seen that the energy derived from electron transport can drive the formation of an H^+-ion gradient across the mitochondrial membrane. This gradient in turn may be utilized to transport certain metabolites against gradients, into or out of mitochondria. For example, phosphate can be accumulated from the surrounding medium by respiring mitochondria, against a concentration gradient, through the action

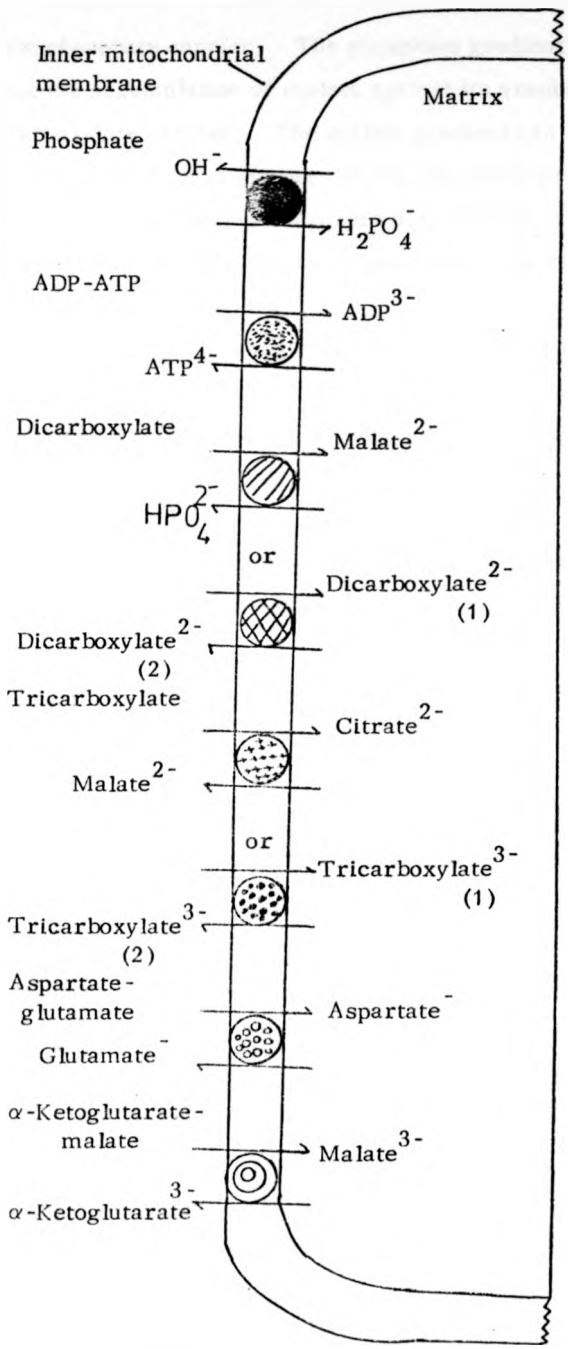


Figure 1.11 Important transport systems of the inner membrane in rat-liver mitochondria (circles). They can function in either direction in response to the concentration gradients of the transported metabolites. (From Lehninger, 1975).

of the phosphate carrier. The phosphate gradient so generated can cause the accumulation of malate against its gradient via the dicarboxylate carrier. The malate gradient can in turn be used to generate a gradient of citrate via the tricarboxylate carrier. In this way, through shared substrate specificities, the various transport systems of the mitochondria can be linked to the electron-transport chain as a source of energy.

1.7 Bindings of inhibitors to mitochondria and submitochondrial particles

Due to the importance of the inhibitors (^{3}H-Atr, ^{14}C-HCP and Rh6G) which were used in the present study, their binding to mitochondria and submitochondrial particles were investigated.

The question which naturally arises is why study binding ? Azzone and Massari (1973) consider the binding process of utmost importance for several reasons: (a) it may represent a step in the translocation process; (b) it is conceivable that the metabolic force is acting at this level and (c) the analysis of the ligand microenvironment provides useful information on the membrane structure.

Binding studies are an increasingly important tool for studying molecular interactions in pharmacology and in chemical and biochemical research. Drug binding is becoming clinically important because pharmacologists now believe that the therapeutic and toxic effects of a drug are directly related to the level of free (unbound) drug in the blood.

Several recent studies have recommended that plasma binding of certain drugs be evaluated routinely to determine the proper dosage for each patient (Cosmides, 1974). Because the extent of binding varies from patient to patient, individuals receiving the same drug dosage may be getting proper therapy, or too little benefit, or an overdose. This problem can be very serious. Anticonvulsants and heart-treatment drugs are among the earliest candidates for routine assay.

1.8 ATP-^{32}Pi exchange reaction

The present work describes another activity of the mitochondria, submitochondrial particles and enzyme complex, namely the exchange reaction between orthophosphate and the terminal phosphate of ATP which occurs in the absence of oxidation. This reaction is designated as the ATP-^{32}Pi exchange.

The reversible exchange of orthophosphate with the phosphate of ATP is considered to be part of the energy-transferring chain in oxidative phosphorylation. This reaction has been demonstrated in mitochondria (Boyer et al., 1954; Boyer et al., 1956; Swanson, 1956; Löw et al., 1957), in particles derived from mitochondria by various means but which still carry out oxidative phosphorylation (Cooper et al., 1955; Cooper and Lehninger, 1957; Wadkins and Lehninger, 1957 and 1959; Bronk and Kielly, 1957), and in enzyme complexes (Hatefi et al., 1975). In view of the association between the ATP-^{32}Pi exchange reaction and oxidative phosphorylation, and the inhibition (by agaric acid, sulfobromophthalein, hexachlorophene and other inhibitors used in the present work) of ADP,-Pi translocations, as well as the inhibition of the 2, 4-dinitrophenol stimulated mitochondrial ATPase, the influence of these inhibitors upon the ATP-^{32}Pi exchange reaction has been examined.

1.9 The aims of this thesis

The aims of the work presented in this thesis were as follows:-

1. To study the nature of the effect of SBP, HCP and agaric acid on ion transport reactions and their binding to rat liver mitochondria.
2. To clarify the binding of ^{14}C-HCP and Rh6G to mitochondria and to study the effect of SBP, HCP and agaric acid on ^{3}H-atractyloside binding to mitochondria and submitochondrial particles.
3. To elucidate the effect of SBP, HCP and agaric acid on ATP-^{32}Pi exchange reaction.
4. To compare the effects of the above inhibitors with the effects of others, such as atractyloside, carboxyatractyloside, bongkrekic acid, triethyltin, DBCT, DCCD, ionophorous antibiotics and uncouplers of oxidative phosphorylation.

CHAPTER 2

ADP TRANSLOCATION IN MITOCHONDRIA

2.1 INTRODUCTION

Studies of the mitochondrial adenine nucleotide (Adn) translocase system have been greatly facilitated by the use of specific high affinity inhibitors such as:

Atractyloside	Winkler et al. (1968).
	Lauquin and Vignais (1973).
	Vignais et al. (1973b).
	Klingenberg (1976).
	Vignais (1976).
Carboxyatractyloside	Vignais et al. (1973a).
Bongkrekic acid	Henderson et al. (1970).
	Henderson and Lardy (1970).
	Kemp et al. (1971).
	Erdelt et al. (1972).
	Klingenberg (1976).
Fatty acyl Co.A	Duszynski and Wojtczak (1974).

The use of these inhibitors has been the major experimental tool for analysis of the function and structure of the translocase system and the development of current theories of the mechanism of the carrier system. The study of other inhibitors of the translocase system may thus be of value, and this chapter describes studies with three other inhibitors which are effective inhibitors of adenine nucleotide translocase in rat liver mitochondria. These inhibitors are

Suflobromophthalein (SBP)
Agaric acid (AA)
Hexachlorophene (HCP)

SBP has been briefly reported by Meisner (1973) to inhibit Adn translocase, and is of interest as it has some general structural features similar to those of atractyloside (Atr), but no detailed investigation of its mode of action has been reported. Agaric acid

has been reported to inhibit translocase activity in a recent study by Chávez and Klapp (1975) who have shown that it is a competitive inhibitor of ADP translocation in contrast to bongkrekic acid which is also a lipophilic tricarboxylic acid, but is an uncompetitive inhibitor.

There is no previous work concerning the effect of hexachlorophene on translocase activity. However, HCP is reportedly an uncoupler of oxidative phosphorylation in mammalian systems (Caldwell et al., 1972; Cammer and Moore, 1972).

This chapter describes studies on the mode of inhibition of rat liver mitochondrial ADP translocase by SBP, AA, HCP, bongkrekic acid (BkA) atractyloside (Atr), uncouplers and ionophorous antibiotics, as well as studies of the effect of Atr, SBP and AA on the binding of radioactive ADP to the mitochondrial membrane and the effect of these inhibitors on the ATPase activity.

The effect of rhodamine 6G (Rh6G), claimed to be an ADP translocase inhibitor by Gear (1974), will also be discussed.

2.2 MATERIALS AND METHODS

MATERIALS

^{14}C-ADP was obtained from the radiochemical centre (Amersham, U.K.). ATP, ADP, adenosine-3-monophosphate, valinomycin, oligomycin, butacaine hemi-sulfate, agaric acid and Tris(hydroxymethyl)aminomethane were obtained from [Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.]. HCP, SBP were obtained from Koch-light Laboratories, Ltd., Colnbrook, Buckinghamshire, England. Cl-CCP was obtained from Calbiochem (Los Angeles, California, U.S.A.). PCP, Rh6G and RhB were obtained from B.D.H., (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.). DNP and EDTA were obtained from Hopkin and Williams, Ltd., England. Rh3G and RhS were obtained from Phase Sepharations Ltd. FCCP was obtained from Pierce Chemicals, X-537A from Hoffman-La Roche Inc., CPDS from New Cell Biochemicals, 631 Hear Avenue, Berkeley, California 94710, U.S.A. 1799 was a gift from Dr. P.G. Heytler (E.I. Du Pont de Nemours and Co. Inc., Wilmington, Delaware, U.S.A.). TET was prepared from triethyltin hydroxide, a gift from Dr. J. Price, Tin Research Institute. Leucinostatin (A20668) was obtained from Eli Lilly and Co., and also as a gift from Dr. R. Hamil. Venturicidin was a gift from

Dr. I.D. Fleming (Glaxo Laboratories, Stoke Poges, U.K.). Bongkrekic acid was a gift from Dr. W. Berends, Technical University, Delft, The Netherlands. PPO was obtained from Nuclear Enterprises, Ltd., Sighthill, Edinburgh 11, Scotland. Atractyloside and carboxyatractyloside (CAT) were purchased from Boehringer, Mannheim, Germany. Cellulose filters, 0.45 μM HAW 02500 were obtained from Millipore. All other chemicals were of analytical reagent grade.

METHODS

Preparation of mitochondria and submitochondrial particles

Liver mitochondria were prepared from Wistar female rats (200-300g) by the method of Johnson & Lardy (1967) utilizing buffered sucrose (0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCL, pH 7.4) as described by Duszynski and Wojtczak (1974). Protein was determined by a biuret method (Gornall, Bardawill and David, 1949). Submitochondrial particles (referred to as particles) were obtained by sonication of rat liver mitochondria (20 mg protein per ml, in sucrose-Tris-EDTA buffer) utilizing a M.SE. 150 W Sonicator at maximal power for four successive periods of 30 seconds at 0^{o}. Submitochondrial particles were isolated by centrifuging at 10000 xg for 10 min to remove large particles and unbroken mitochondria and the turbid supernatant was centrifuged at 80000 xg for 30 min. The pellet was resuspended in buffered sucrose-Tris-EDTA.

Estimation of ADP translocase activity

ADP translocase activity in well coupled mitochondria was estimated either by "Forward Exchange" or "Back Exchange" methods essentially as described by Pfaff and Klingenberg (1968). Mitochondria (1 mg protein) in 1.0 ml of the incubation buffer (either 0.25 M sucrose, 1mM EDTA and 10 mM Tris-HCl, pH 7.4, as described by Duszynski and Wojtczak, 1974; or 110 mM KCl, 20 mM Tris-HCl, pH 7.4 and 0.2 mM EDTA, as described by Vignais et al., 1975; or 15 mM KCl, 1 mM EDTA, 90 mM sucrose, 50 mM Triethanolamine-HCl, pH 6.5 and 5 mM $MgCl_2$, as described by Kemp et al., 1971) with or without inhibitors for 10 min at

4-5° and the reaction initiated by rapid mixing with 0.5 ml of the same incubation buffer containing either ^{14}C-ADP (in Forward Exchange) or ADP (in Back Exchange) so that the final adenine nucleotide concentrations ranged from 2 to 200 μM. The reaction was terminated at 30 sec by rapid filtration (2 sec) through 0.45 μm Millipore filters and washed quickly with 5 ml of ice-cold incubation buffer. The filtration was carried out in special holders which were individually linked to a negative pressure reservoir maintained by a vacuum pump, consequently a large negative pressure could be applied to any filter holder enabling very rapid filtration (2 sec) and reaction termination times to be achieved.

In "Back Exchange" (^{14}C-ADP efflux) data expressed as Δ[^{14}C]ADP was determined by subtracting the amount of ^{14}C-ADP found in the presence of unlabelled adenine nucleotide from that in its absence.

Preloading of mitochondria with ^{14}C-ADP was carried out essentially as described by Vignais et al. (1973a). Rat liver mitochondria (50 mg protein in 10 ml of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) were incubated with 90 μM ^{14}C-ADP for 15 min at 4°; after two successive washings by resuspension and centrifugation in the incubation buffer, they were resuspended in sucrose-EDTA-Tris buffer.

All measurements of ADP translocation were carried out by Millipore filtration (Winkler et al., 1968).

Estimation of ADP binding to mitochondria

Binding of ^{14}C-ADP was carried out by the method of Wiedemann, Erdelt and Klingenberg (1970) using liver mitochondria depleted of endogenous adenine nucleotides by the arsenate method. Depleted mitochondria (1 mg) were incubated in 1 ml suspension buffer (0.25 M sucrose, 20 mM triethanolamine-HCl, pH 7.0, 1 mM EDTA, 0.5 mM adenosine-3-mono-phosphate). Binding was initiated by 0.5 ml suspension buffer containing ^{14}C-ADP. Final volume 1.5 ml, the

temperature of the binding medium was 0^{o}. The reaction was carried out in centrifuge tubes, 1 x 7.5 cm, as follows:

(a) Control reaction without inhibitors; mitochondria and ^{14}C-ADP added to the buffer at zero time and the tubes centrifuged after 2 min.

(b) Inhibitors added before ^{14}C-ADP addition; mitochondria and inhibitors were added at zero time and at 1 min ^{14}C-ADP was added and after a further 2 min incubation time the tubes were centrifuged.

(c) Inhibitors added after ^{14}C-ADP addition; mitochondria and ^{14}C-ADP were added at zero time and inhibitors at 2 min and the tubes centrifuged after a further minute.

Assay tubes were centrifuged for 15 min at 10000 xg in plastic adapter designed to fit SS-34 rotor of the Sorvall RC_2-B.

The supernatants were removed by aspiration and the mitochondrial pellets washed once with ice-cold sucrose medium (1 ml). The washing medium was removed by careful aspiration with a fine plastic tip attached to a water pump. The pellets were resuspended in 2% (w/v) Triton X-100 (1 ml) for scintillation counting of bound ^{14}C-ADP.

From the labelled adenine nucleotide measured in samples (a), (b) and (c) the three single portions can be evaluated as follows: labelled adenine nucleotide bound to the carrier sites = atractyloside-removable binding (a-c), labelled adenine nucleotide exchanged with the endogenous adenine nucleotide in the mitochondria (c-b) and the unspecifically bound labelled adenine nucleotide (b).

Measurement of radioactivity

In translocase experiments, the Millipore filter was dried and the amount of radioactivity in the filter that contained the mitochondria was measured in a Packard Tricarb liquid scintillation counter in a POPOP/PPO system, using 10 ml of a scintillation mixture containing 4.0 g PPO (2.5 diphenyloxyazole), 0.2 g dimethyl POPOP [1,4,di-2(5-phenyloxyazolyl benzene)] in 1 L of toluene (Patterson and Green, 1965).

In the binding experiments, the pellets were dissolved in 1.0 ml of 2% (w/v) Triton X-100 for scintillation counting of bound ^{14}C-ADP, and

added to 10 ml of a scintillation mixture containing 500 ml Triton X-100, 1 L of toluene and 7.0 g of butyl BPD.

ATPase assay

The ATPase assay was carried out by the method of Myer and Slater (1957) in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 75 mM KCl, 1 mM EDTA, 0.1 M sucrose, 0.003 M $MgCl_2$, 10 mM ATP, in final volume of 1.0 ml. In all cases 1.0 mg of mitochondrial or submitochondrial particles protein was used for each assay and this was preincubated in the reaction mixture for 10 min with inhibitor and with 2, 4-dinitrophenol (DNP) when necessary. Atractyloside, agaric acid and HCP were dissolved in ethanol. SBP and DNP were dissolved in aqueous solution. The assays were started by the addition of ATP and were run for 5 min at 30^{o}; 0.5 ml of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation, to sediment the protein, 0.5 ml samples of the supernatant were used for the determination of phosphate according to the method of Fiske and Subbarow (1925). In a control assay, addition of ethanol alone has no effect on ATPase activity.

1.3 RESULTS

Time course of ADP uptake

The time course of ^{14}C-ADP uptake and efflux (in exchange with external ADP) of ^{14}C-ADP by intact rat liver mitochondria at 5^{o} C are shown in Figs. 2.1 and 2.2 respectively. There is a rapid phase which is linear for 30 sec, followed by a much slower phase extending over 5 min. Consequently, a time period of 30 sec was chosen for all subsequent experiments. These results are in agreement with the very rapid uptake followed by a slow increase observed in rat liver mitochondria by Duszynski and Wojtczak (1974) and Winkler et al., (1968).

The amount of ^{14}C-ADP taken up or effluxed by rat liver mitochondria in the rapid phase, measured after 30 sec is 4-5 n moles of ADP per mg or protein; longer incubations gave increasingly higher values, approaching

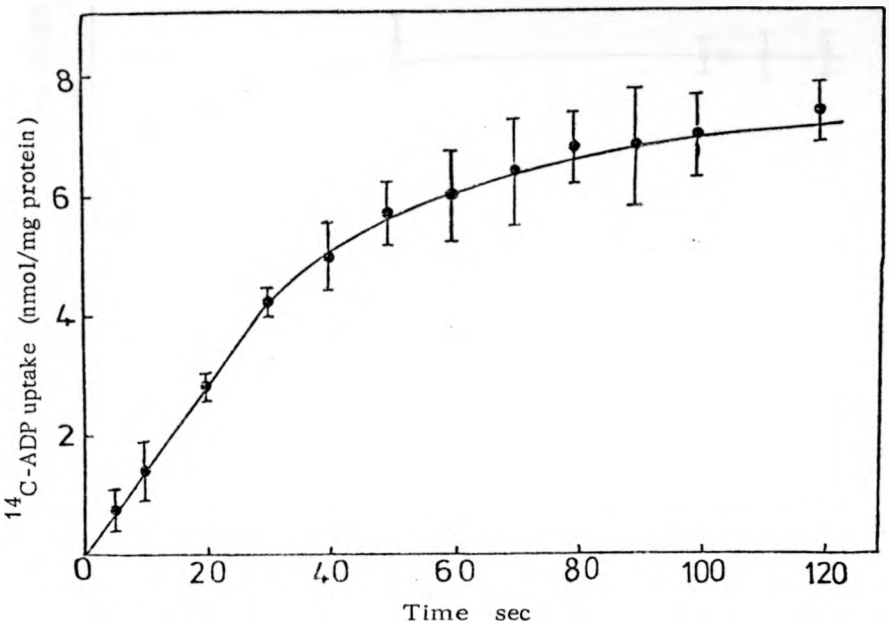


Figure 2.1 Time course of the uptake of ADP

Rat liver mitochondria (1 mg protein) were incubated at 5° in 1.5 ml buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) containing 40 μM ^{14}C-ADP for the indicated time before sampling. Other details were described in "Method". Results are mean ± S.D. of five experiments.

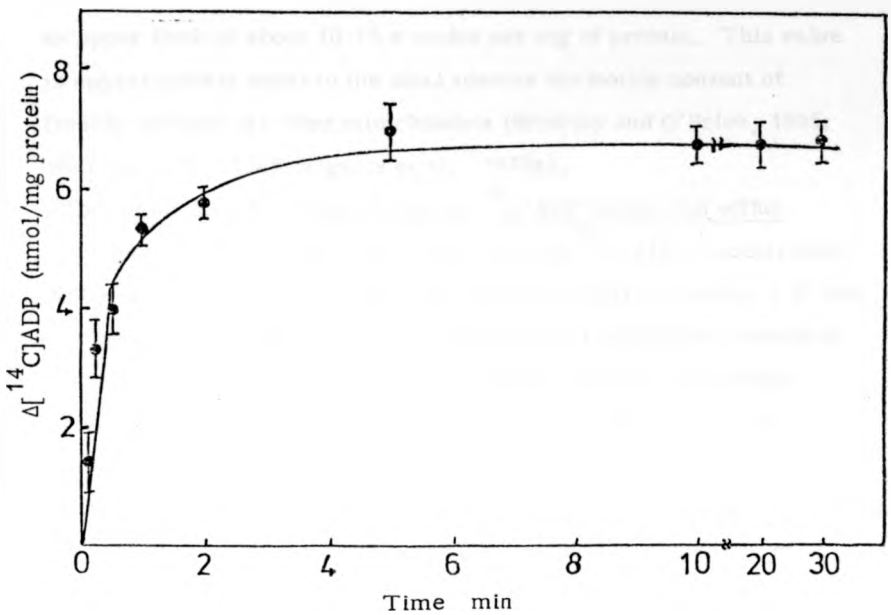


Figure 2.2 Time course of the ^{14}C-ADP efflux in exchange with external ADP

^{14}C-ADP-labelled mitochondria (0.5 mg protein) were incubated in 1.0 ml of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4 at 5°. ADP in 0.5 ml was added to a final concentration of 50 μM. The incubation lasted for indicated time before termination by Millipore filtration. Other details are described in "Method". Results are mean ± S.D. of five experiments.

an upper limit of about 10-15 n moles per mg of protein. This value is approximately equal to the total adenine nucleotide content of freshly isolated rat liver mitochondria (Brierley and O'Brien, 1965; Winkler et al., 1968; Vignais et al., 1973a).

Effect of nucleotide concentration on ^{14}C-ADP uptake and efflux

Figure 2.3 shows the effect of increasing ^{14}C-ADP concentration on the amount of ^{14}C-ADP taken up by mitochondria following a 30 sec incubation at 5°. It is seen that the amount of labelled nucleotide in the mitochondria increases with the concentration in the medium. From the data, a K_m (ADP) value of approximately 7.4 μM was calculated. This value is lower than the values of 12 to 17 μM found by (Klingenberg, 1970; Spencer and Bygrave, 1972 ; Shrago et al., 1974) and higher than the values of 1 to 3 μM reported by (Souverijn et al., 1973; Weidemann et al., 1970; Winkler and Lehninger, 1968).

In the case of the ^{14}C-ADP efflux from mitochondria in exchange with externally added ADP (Fig. 2.4) a K_m (ADP) value of approximately 6.7 μM was obtained which is similar to the K_m value obtained in the forward exchange assay of ^{14}C-ADP.

Effect of atractyloside and carboxyatractyloside on the mitochondrial adenine nucleotide translocation

Three specific inhibitors of adenine nucleotide translocation are known: Atr, CAT (Gummiferin) and BkA (Vignais et al., 1973b). Bruni, Luciani and Contessa first described the ability of Atr to prevent uptake of isotopically labelled nucleotides into mitochondria (Bruni et al., 1964; Bruni et al., 1966). Since that time, a number of techniques have been developed for studying the kinetics and specificity of the nucleotide translocase and its susceptibility to atractyloside (Winkler et al., 1968; Duee and Vignais, 1969; Klingenberg and Pfaff, 1968).

Results obtained with Atr and CAT by utilizing the technique described under "Methods and Materials" are shown in Fig. 2.4 and Table 2.1. Atr (0.94 μM) increased the K_m value of ADP from

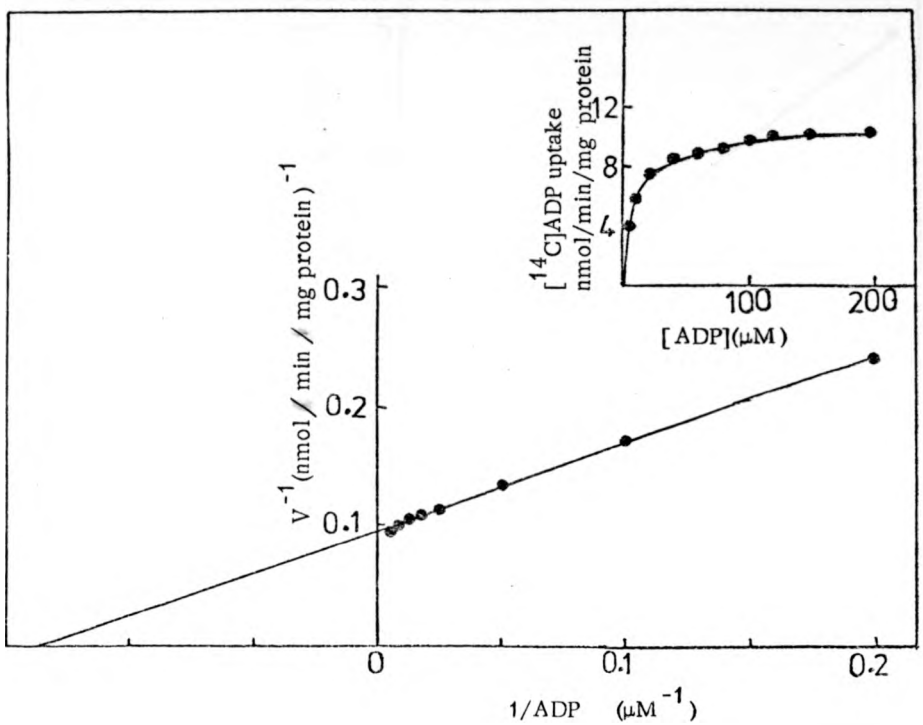


Figure 2.3 Double reciprocal plot of the exchange of ADP

ADP exchange was measured as described in methods; 1 mg of mitochondrial protein was incubated in media that contained 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 at 5° and the indicated concentrations of ^{14}C-ADP. After 30 sec of incubation, the reaction was stopped by Millipore filtration. Each result is the mean of four experiments. K_m(ADP) = 7.4 μM.

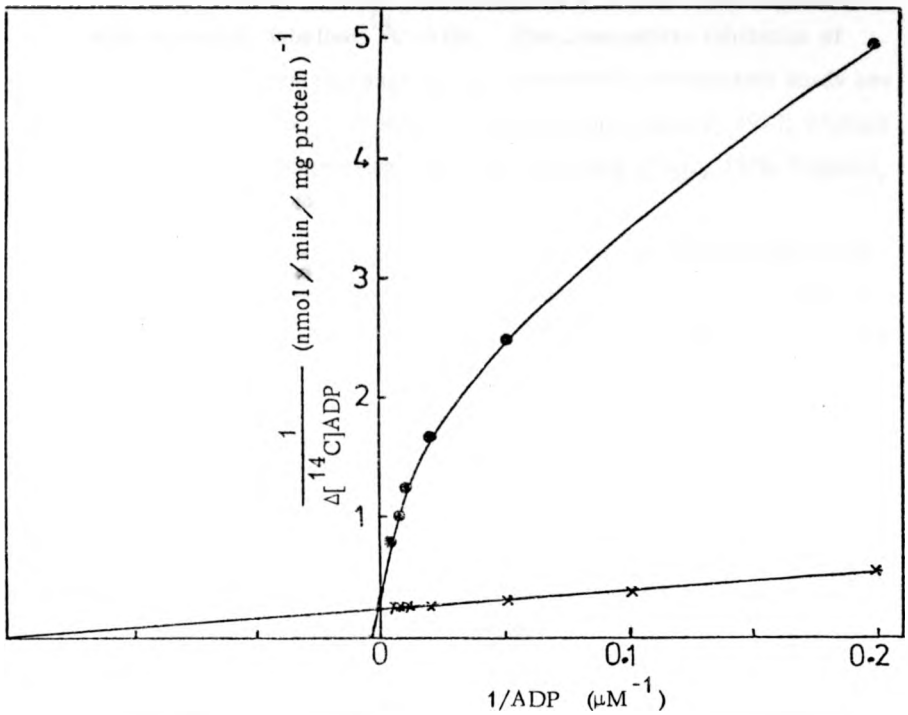


Figure 2.4 Effect of Atr on the kinetics of ^{14}C-ADP efflux in exchange with external ADP

^{14}C-ADP-loaded mitochondria (1.0 mg protein) were incubated in 1.0 ml of 0.25 M sucrose, 1 mM EDTA and 10 mM Tirs-HCl, pH 7.4, for 10 min at 5° with Atr. The efflux of ^{14}C-ADP was initiated by the addition of ADP(5-200 μM). At 30 sec, the reaction ended by Millipore filtration. The results are mean of four experiments.

X —— X, NO, Atr, K_m(ADP) = 6.7 μM

● ——●, + 0.94 μM, Atr, K_m(ADP) = 200 μM

6.7 μM to 200 μM. Thus, Atr competes with ADP when exchanged with internally labelled ^{14}C-ADP. The competitive inhibition of adenine nucleotide exchange by Atr observed in the present study are in agreement with the results of (Lauquin and Vignais, 1973; Vignais et al., 1973b; Klingenberg, 1974; Klingenberg et al., 1976; Vignais, 1976).

Carboxyatractyloside (0.2 μg/mg protein) caused 94% inhibition of ^{14}C-ADP uptake from rat liver mitochondria, thus confirming the previous findings that CAT is a potent inhibitor of adenine nucleotide translocation from mitochondria (Vignais et al., 1973a; Vignais et al., 1973b).

Inhibition by uncouplers and ionophores of ADP translocation

Table 2.1 shows that HCP, Cl-CCP, FCCP, 1799, PCP and DNP inhibit ADP uptake in agreement with Meisner (1971) who found that the ADP exchange in the presence of m-chlorocarbonyl cyanide phenylhydrazone is reduced from 2.7 to 1.5 nmoles/min/mg protein. However, in disagreement with Kemp, Jr. (1970) and Babior et al., (1973) who found that Cl-CCP, FCCP, DNP stimulate the ADP exchange. Moreover, it was shown by Pfaff and Klingenberg (1968) and Spencer et al. (1976) that uncouplers strongly stimulate only the ATP exchange and do not greatly influence the ADP exchange. Vignais et al. (1973b) reported that FCCP has only a slight effect on ADP translocation.

On the other hand, the results of the present study show that Ca^{++} has no effect on ADP uptake, confirming previous studies (Spencer and Bygrave, 1971) who mentioned that Ca^{++} stimulates the translocation of ATP but not of ADP. It was proposed (Thorne and Bygrave, 1974) that ADP translocation by tumor mitochondria is particularly sensitive to the added Ca^{++}. In the presence of 100 nmoles Ca^{++} per mg protein the rate of translocation of 50 μM ADP is inhibited some 75%. In other experiments, Thorne and Bygrave have found that Ca^{++} also inhbits the translocation of both ADP and ATP in mitochondria isolated from Yoshida

hepatoma ascites cells but not in mitochondria isolated from the minimally-deviated Morris 5123C hepatoma cells.

TABLE 2.1

Effect of inhibitors, uncouplers and ionophores on ADP uptake

Mitochondria (1.0 mg protein) were incubated in 1.0 ml of 110 mM KCl, 20 mM Tris-HCl, pH 7.4 and 0.2 mM EDTA for 10 min at 5° with inhibitors, uncouplers and ionophores. ^{14}C-ADP in 0.5 ml was added to a final concentration of 100 μM. The incubation lasted for 30 sec and was stopped by Millipore filtration. The results are of the mean ± S.D. of four experiments.

Additions	Inhibitor concentration μg/mg protein	Inhibition (%)
Carboxyatractyloside	0.2	94 ± 5.6
Atractyloside	0.2	90 ± 5.5
HCP	1.0	65 ± 2.0
Cl-CCP	3.0	65 ± 5.0
FCCP	3.0	70 ± 3.0
1799	3.0	55 ± 1.0
PCP	5.0	51 ± 3.0
DNP	3.0	35 ± 3.1
TET	1-10	0
$CaCl_2$	2-5	0
Leucinostatin	5.0	18 ± 1.7
Valinomycin	2.0	76 ± 5.2
Venturicidin	5.0	25 ± 2.0
X-537A	2-5	0
Oligomycin	2-5	0
Butacaine	0.42	14 ± 0.7
CPDS	2-5	0
DCCD	10-20	0

In the case of the effect of ionophorous antibiotics on ADP translocation, it was found that X-537A has no effect, leucinostatin has a slight effect (Table 2.1). Valinomycin (2 μg/mg protein) caused 76% inhibition of the ADP uptake, confirming Shertzer and Racker (1976) who showed that in the presence of K_2SO_4 valinomycin inhibited both initial rate and extent of ADP uptake.

Oligomycin has no effect on ADP uptake; the same findings were also reported for ATP translocation by (Bruni et al., 1966; Spencer and Bygrave, 1972). Butacaine at 0.42 μg/mg protein has a very slight inhibitory effect. While Spencer and Bygrave (1974) showed that butacaine was the most effective inhibitor with 50% inhibition occurring at 10 μM for 200 μM ADP. The results of Table 2.1 clearly show that TET has no effect on ADP uptake, which is in agreement with Cain (1976) who showed that there is no inhibition of ADP uptake by TET in yeast mitochondria.

Effect of rhodamine compounds on ADP translocation

The results of the present work, Table 2.2, show that Rh6G, Rh3G, RhB and RhS have no effect on ADP translocation. The results with RhB, confirming the finding of Gear (1974) that this compound, a free acid and uncharged at pH 7, was completely ineffective on mitochondrial energy-linked functions. However, the results with Rh6G on translocase are in contrast with the finding of Gear, who found that Rh6G blocked adenine nucleotide binding to intact mitochondria, both for ^{14}C-ATP and ^{14}C-ADP. Inhibition by 10 μM Rh6G at low levels of added nucleotide was identical with that caused by 10 μM atractyloside, but inhibition was only very slight at higher nucleotide concentrations (0.2 mM). From the above findings, he concluded that Rh6G blocks adenine nucleotide translocator. However, the present study (Table 2.2) shows that Rh6G (7-13 μg/mg protein) has no effect on ADP translocase, with 40 μM ADP. When 2 μM ADP was used, it was found that Rh6G (7 μg/mg protein) also had no effect on the ADP uptake (1.34 nmol/min/mg protein, no Rh6G; and 1.3 nmol/min/mg protein with Rh6G).

TABLE 2.2

Effect of rhodamine compounds on ADP uptake

Experimental conditions as in Fig. 2.3. Inhibitor incubated with mitochondria for 10 min at 5°. ^{14}C-ADP in 0.5 ml was added to a final concentration of 40 μM.

	^{14}C-ADP uptake (nmol/min/mg protein)	
	Rhodamine concentration	
Rhodamine	7 μg/mg protein	13 μg/mg protein
None	8.8 ± 0.11	
Rh6G	8.9 ± 0.07	9.2 ± 0.15
Rh3G	9.1 ± 0.08	8.2 ± 0.45
RhB	9.2 ± 0.06	9.3 ± 0.07
RhS	9.3 ± 0.50	9.2 ± 0.30
*None	1.34 ± 0.05	
*Rh6G	1.30 ± 0.04	

*using 2 μM ^{14}C-ADP

Influence of incubation period on inhibition of ADP uptake by agaric acid, SBP and HCP

Table 2.3 shows the action of the three inhibitors on ADP translocase is not time dependent and maximal inhibitory activity is observed within 30 sec. Thus these inhibitors are unlike bongkrekic acid, which was shown by Henderson et al. (1970) to inhibit a number of ADP-ADP, ATP-ATP and ADP-ATP exchange reactions at 23°, 0.7 - 1.1 nmoles of bongkrekic acid/mg of protein was sufficient for complete inhibition with a 10 min incubation before nucleotide addition, whereas 50 - 70 nmoles/mg was required without the prior incubation period. These inhibitors (agaric acid, SBP, HCP) are similar to atractyloside as the extent of inhibition by a fixed concentration of atractyloside was not augmented by prior incubation with the mitochondria, and atractyloside inhibition of adenine nucleotide exchange appeared to be instantaneous (Heldt, 1969 and Henderson et al. 1970).

TABLE 2.3

Influence of incubation period on inhibition of ADP uptake by agaric acid, sulfobromophthalein and hexachlorophene

Experimental conditions as in Fig. 2.3 with 40 μM ^{14}C-ADP. Results are mean ± S.D. of three experiments.

Addition of inhibitors nmol/mg of protein	^{14}C-ADP uptake nmol/min/mg of protein
1. Agaric acid (12·8)	
No agaric acid	7.93 ± 0.43
Added after ADP	7.08 ± 0.25
Added with ADP	3.34 ± 0.31
Added 0.5 min before ADP	1.80 ± 0.16
Added 1.0 min before ADP	2.03 ± 0.07
Added 4.0 min before ADP	1.36 ± 0.10
Added 10.0 min before ADP	1.55 ± 0.21
2. Hexachlorophene (2·8)	
No HCP	10.00 ± 0.50
Added 5 sec after ADP	8.61 ± 0.35
Added 0.5 min before ADP	5.42 ± 0.43
Added 1.0 min before ADP	5.40 ± 0.20
Added 5.0 min before ADP	5.61 ± 0.24
Added 10.0 min before ADP	5.62 ± 0.10
3. Sulfobromophthalein (8.7)	
No SBP	8.81 ± 0.30
Added 5 sec after ADP	3.20 ± 0.20
Added with ADP	0.80 ± 0.10
Added 1.0 min before ADP	0.80 ± 0.07
Added 4.0 min before ADP	0.82 ± 0.06
Added 10.0 min before ADP	0.72 ± 0.11

Note The inhibitor concentrations for half maximal inhibition are SBP (1.5 nmol/mg protein); AA (3 nmol/mg protein); HCP (3 nmol/mg protein) (Fig. 2.5).

Effect of different concentrations of inhibitors on ADP translocase

The action of agaric acid, SBP and HCP on ADP uptake is shown in Fig. 2.5, which clearly shows that the half-maximal inhibition of ADP uptake when ADP concentration was 40 μM is as follows: 1.5 nmol SBP/mg protein; 3 nmol AA/mg protein; 3 nmol HCP/mg protein. Thus, according to these results, these inhibitors can be arranged according to their potency of inhibition of ADP translocase as follows: SBP > agaric acid ≥ HCP. These inhibitors are not as potent as atractyloside as Winkler et al. (1968) has reported a value of 0.8 μM for half-maximal inhibition with 200 μM ADP.

Effect of SBP, agaric acid and HCP on translocation of various concentrations of ADP

Study of the effect of SBP and agaric acid on the ADP translocase assay shows that they are potent inhibitors of ADP translocase. A double reciprocal plot of this data (Fig. 2.6) indicates that both inhibitors are competitive inhibitors of ADP uptake. Dixon plots (Dixon, 1953) gave values for K_i for SBP of 1.5 μM and for agaric acid 1.9 μM (Fig. 2.8) as compared with the K_i of atractyloside of 0.1 μM (Klingenberg, 1970). WhileFig. 2.7 shows that HCP is an uncompetitive inhibitor, since both the apparent K_m and the apparent V_{max} are altered (Gut freund, 1975).

Double reciprocal plots of the effect of SBP and agaric acid (Fig. 2.6) and HCP (Fig. 2.7) on the exchange of ADP in mitochondria shows the following K_m (ADP) values:

Inhibitor concentrations (μM)		apparent K_m (μM)
No inhibitor		7.4
SBP	2.9	40.0
	5.7	66.0
	11.3	308.0
Agaric acid	1.7	20.0
	3.4	29.0
	8.5	133.0
HCP	1.85	17.0
	9.25	29.0
	18.50	77.0

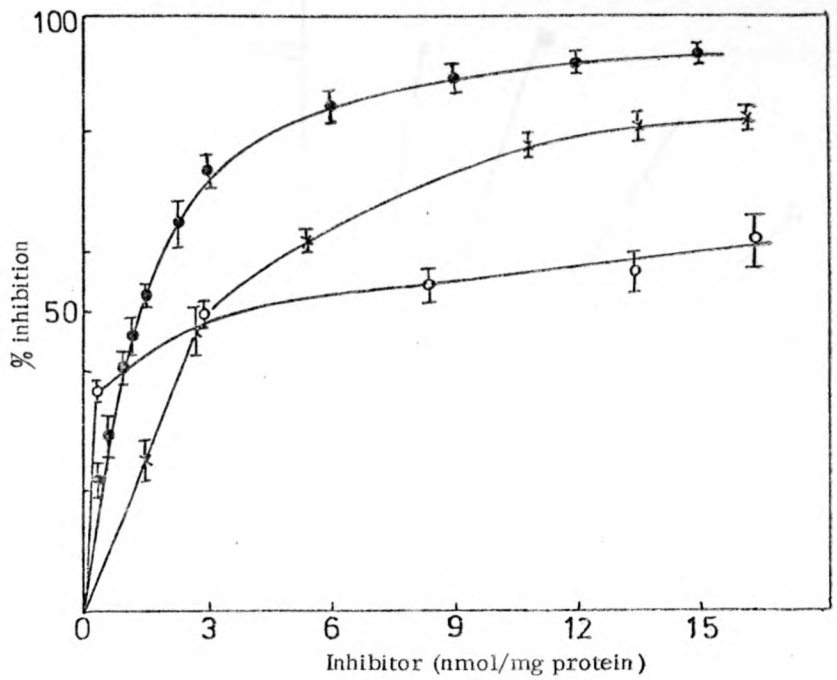


Fig. 2.5 Inhibition of ADP translocase by SBP, agaric acid and HCP

Experimental conditions as in Fig. 2.3 with 40 μM ^{14}C-ADP. Results are mean ± S.D. of three experiments.

● ——— ●, + SBP

X ——— X, + agaric acid

O ——— O, + HCP

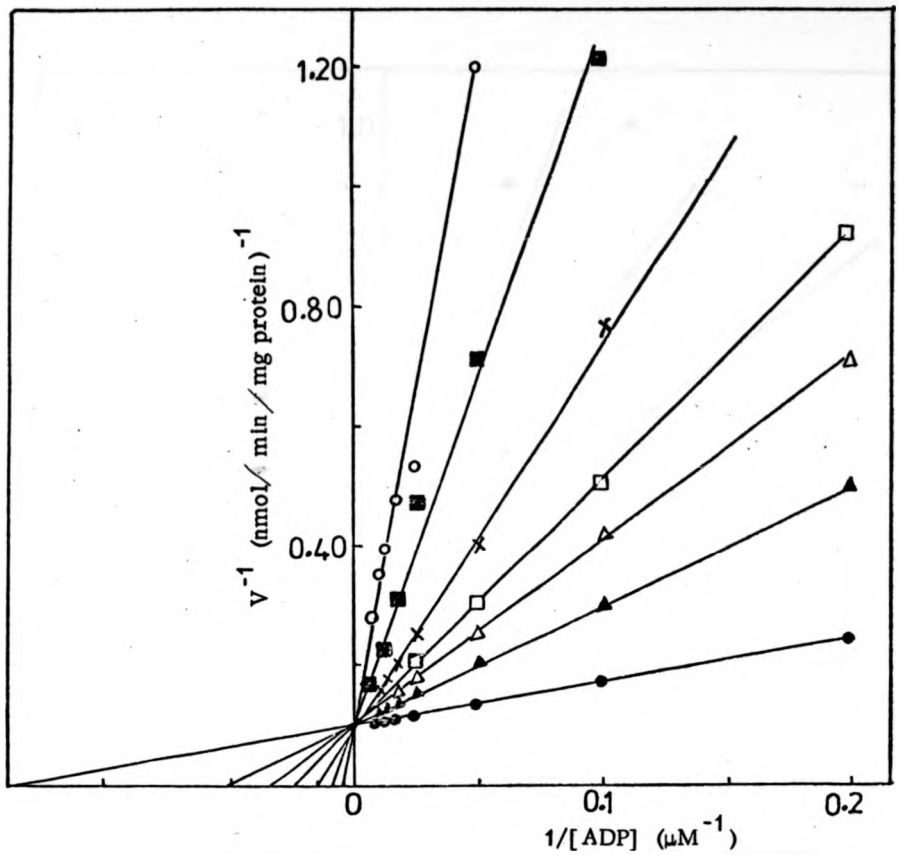


Figure 2.6 Double reciprocal plot of the exchange of ADP in the presence of SBP and agaric acid

Experimental conditions as in Fig. 2.3

● ——— ●, no inhibitor, K_m(ADP) = 7.4 μM

▲ ——— ▲, + 1.7 μM AA, K_m(ADP) = 20 μM

△ ——— △, + 3.8 μM AA, K_m(ADP) = 29 μM

■ ——— ■, + 8.5 μM AA, K_m(ADP) = 133 μM

□ ——— □, + 2.9 μM SBP, K_m(ADP) = 40 μM

X ——— X, +5.7 μM SBP, K_m(ADP) = 66 μM

O ——— O, + 11.3 μM SBP, K_m(ADP) = 308 μM

Results are mean of five experiments.

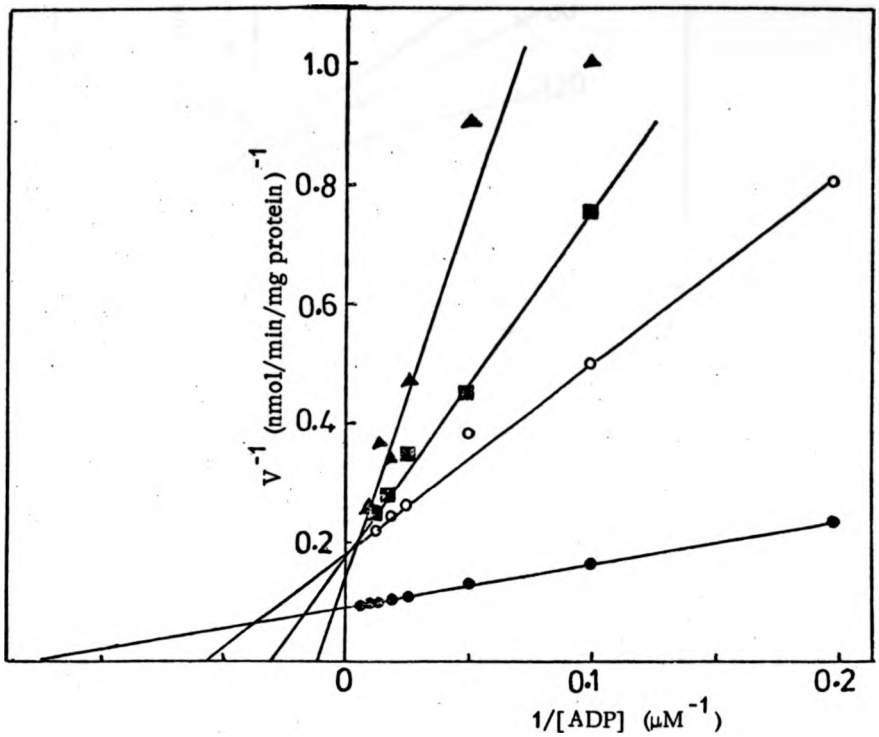


Figure 2.7 Double reciprocal plot of the exchange of ADP in the presence of HCP

Experimental conditions as in Fig. 2.3

● ——● , no inhibitor, K_m(ADP) = 7.4 μM

O —— O, + 1.8 μM HCP, K_m(ADP) = 17 μM

■ ——■, + 9.28 μM HCP, K_m(ADP) = 29 μM

▲——▲, + 18.50 μM HCP, K_m(ADP) = 77 μM

The results are mean of six experiments.

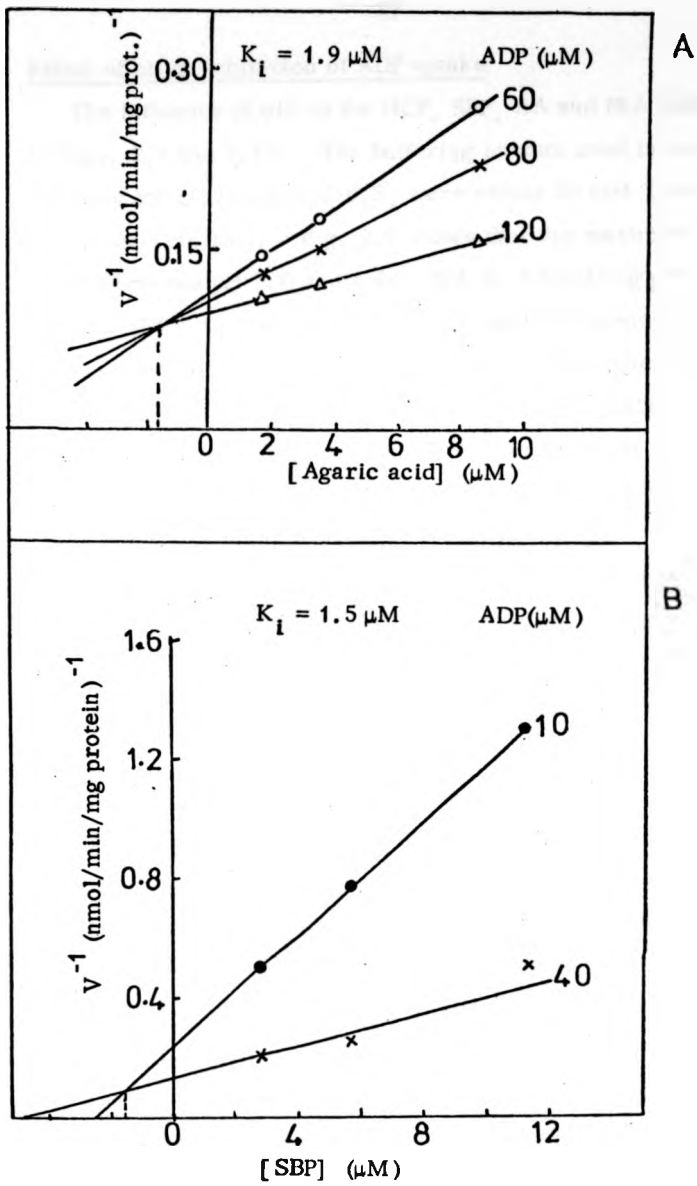


Figure 2.8 Inhibition of the ADP uptake by agaric acid (A) and SBP (B) presented in the form of a Dixon plot. The same conditions as those in Fig. 2.3. ^{14}C-ADP in 0.5 ml was added to a final concentration of (10-120 μM). The results are mean of five experiments.

Effect of pH on inhibition of ADP uptake

The influence of pH on the HCP, SBP, AA and BkA inhibition is shown in Figs. 2.9 and 2.10. The buffering system used in these experiments, covering the pH range 6.0-8.5, were either 50 mM Triethanolamine-HCl or 10 mM Tris-HCl. Fig. 2.9 shows that the maximum ADP uptake occurs between pH 7.0 and 8.0. BkA (0.7 nmol/mg protein)greatly inhibits ADP uptake at pH 6.0 and 6.5, and its potency of inhibition tends to decrease after pH 6.5 and loose its effect after pH 7.5. In the case of HCP, there is only a slight change in the inhibition of the exchange of ADP in the range between pH 6.0 - 7.5; above pH 7.5 there is little inhibition. Thus BkA and HCP have nearly similar effects on ADP translocase in basic medium.

As shown in Fig. 2.9A, the SBP curve is similar to the control curve. So, the inhibitory action of SBP is not pH dependent within the pH range 7.0 - 8.5, and the SBP effect is markedly different from BkA which exhibits a marked dependency on pH (Kemp et al., 1971; Klingenberg et al., 1973).

It is supposed that agaric acid is partly undissociated at pH 6-6.5 and thus affects translocase activity in a similar way to BkA, as both compounds are tricarboxylic acids. However, the results of Fig. 2.9 show that agaric acid has little effect on translocase in the pH range 6.0-6.5, and the maximal inhibition by agaric acid on ADP uptake is in the pH range 7-7.5 which is in agreement with the results of Chávez and Clapp (1975).

It was of particular interest to examine the effect of various concentrations of BkA on ADP translocase at two different pH as shown in Fig. 2.10. BkA (1.2 nmol/mg protein) at pH 6.5 caused 90% inhibition of ADP translocation, while the same concentration of BkA at pH 7.4 caused only 10% inhibition. This is further support for the previous finding that BkA is a pH dependent inhibitor.

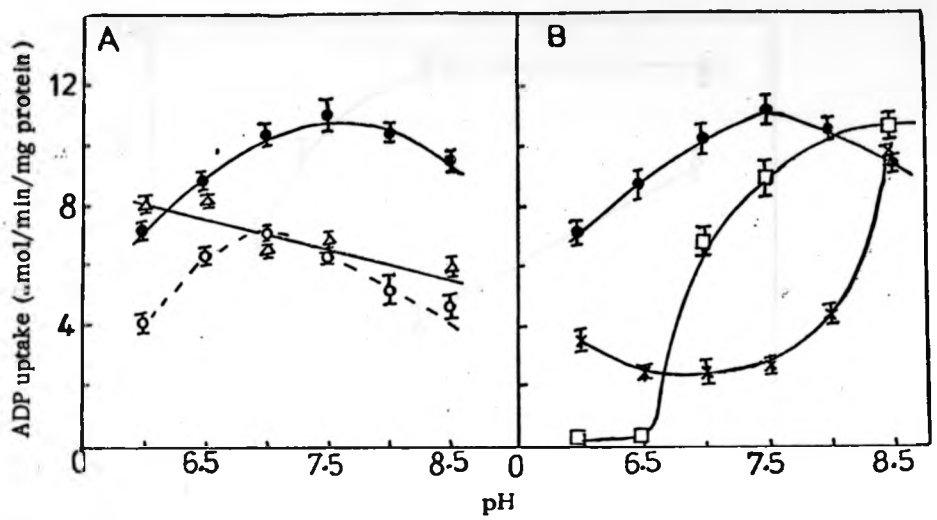


Figure 2.9 The effect of pH on the inhibition by BkA, HCP, SBP and agaric acid of ADP uptake

Mitochondria (2.0 mg protein) were incubated in 1.45 ml of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, covering the pH range 6.0 to 8.0, for 10 min (except for BkA, 20 min) at 5° with these inhibitors. [^{14}C] ADP in 50 μl was added to a final concentration of 100 μM. The incubation lasted for 30 sec and was stopped by Millipore filtration. Results expressed as mean of three experiments ± S.D.

●——●, no inhibitors.

□——□, + 0.7 nmoles BkA/mg protein] B

X——X, + 5.6 nmoles HCP/mg protein]

△——△, + 2.6 nmoles AA/mg protein] A

O- - - -O, + 1.4 nmoles SBP/mg protein]

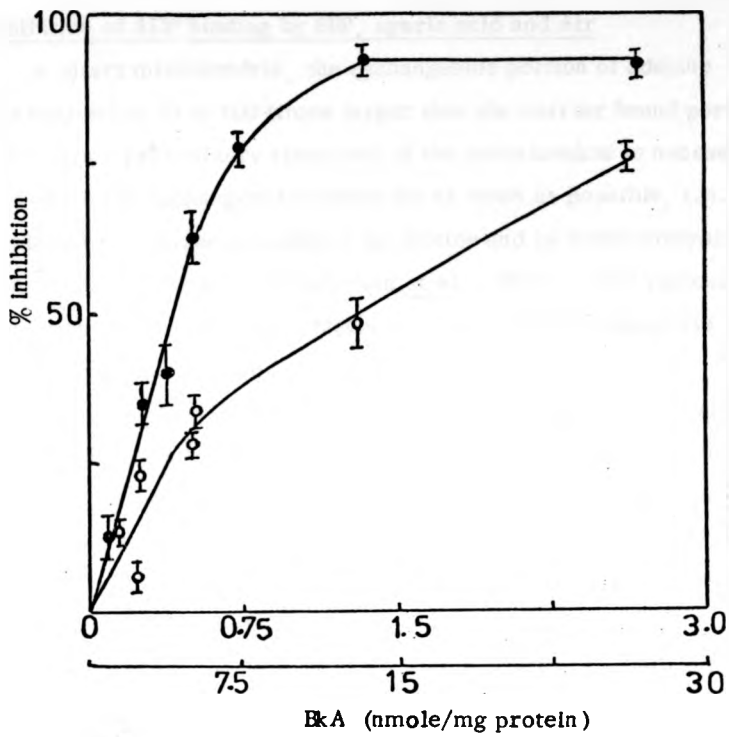


Figure 2.10 Influence of pH on inhibition of ADP uptake by bongkrekic acid

●——●, mitochondria (1.0 mg protein) were incubated in 1.0 ml of 15 mM KCl, 1 mM EDTA, 90 mM sucrose, 50 mM Triethanolamine-HCl, pH 6.5 and 5 mM $MgCl_2$ for 20 min at 5° with 0.14-2.7 nmoles BkA/mg. [^{14}C] ADP in 0.5 ml was added to a final concentration of 100 μM. The incubation lasted for 30 sec and was stopped by Millipore filtration.

O——O, conditions similar to ●——● except that mitochondria were incubated in 1.0 ml of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4 for 20 min at 5° with 2.7 - 26.6 nmoles BkA/mg protein. [^{14}C] ADP in 0.5 ml was added to a final concentration of 40 μM. Results are mean ± S.D. of three experiments.

Inhibition of ADP binding by SBP, agaric acid and Atr

In intact mitochondria, the exchangeable portion of adenine nucleotides is 10 to 100 times larger than the carrier bound portion, therefore a preliminary treatment of the mitochondria is necessary to reduce the exchangeable portion by as much as possible, i.e. by depletion of endogenous adenine nucleotide and by interconversion to non-exchangeable AMP (Weidemann et al., 1970). The various depletion methods used are freezing, treatment with phosphate and treatment with arsenate.

Erdelt et al. (1972) defined the ADP bound to the carrier sites as that portion of total ADP bound to mitchondrial membranes, which is removed by subsequent addition of atractyloside. The addition of atractyloside at different times before and after ADP has served to differentiate the carrier-bound or 'specific' portion from the exchanged portion of adenine nucleotide.

Results of the present study showed that SBP and agaric acid were competitive inhibitors of ADP translocation; thus it is of interest to study the effect of these inhibitors on the binding of ADP from rat liver mitochondria (Fig. 2.11). A lineweaver-Burk plot of the effect of SBP and agaric acid on the specific ADP binding by mitochondria (Fig. 2.12) shows that SBP (13.5 μM) and agaric acid (17 μM) increases the Km for ADP binding from 4.2 to 10 μM and 5.7 μM respectively, with no change in the V_{max}, which suggests that there is a competition between SBP, agaric acid and the ADP binding.

DNP-stimulated mitochondrial ATPase and submitochondrial ATPase activities

Slekevitz et al. (1958) described the existence of two distinct types of ATPase in liver mitochondria, one activated by DNP and only present in intact mitochondria capable of oxidative phosphorylation, and another, activated by Mg^{++} and characteristic of structurally disorganized mitochondria devoid of phosphorylative ability.

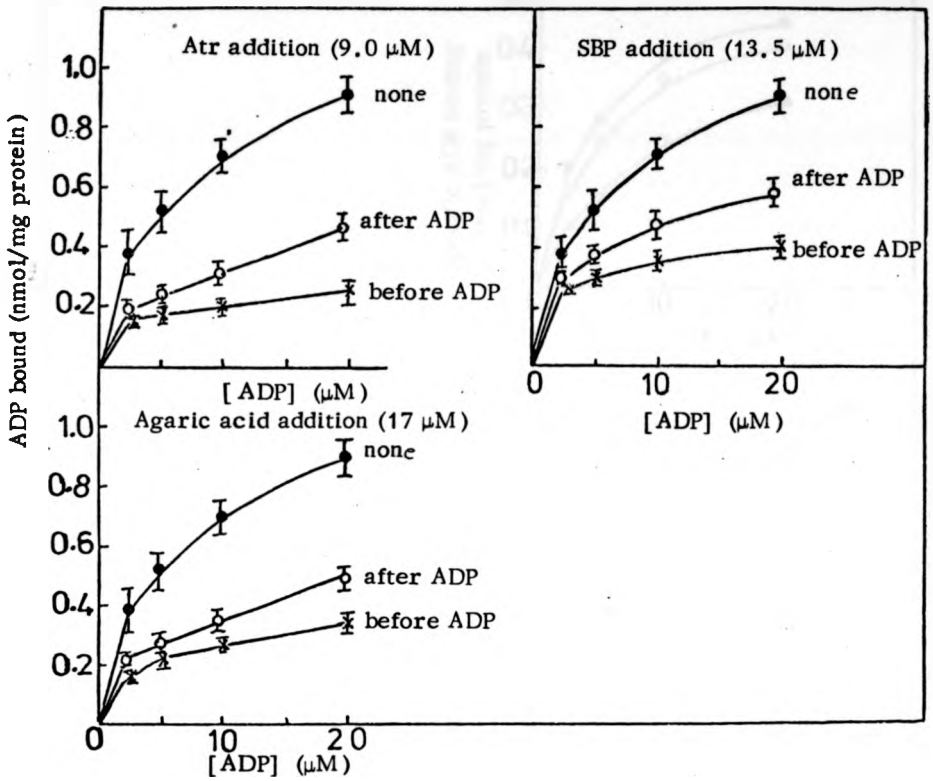


Figure 2.11 Effect of Atr, SBP and AA on ^{14}C-ADP binding

AS_1-depleted mitochondria (1.0 mg protein) were incubated in 1.0 ml (0.25 M sucrose; 20 mM Triethanolamine-HCl, pH 7.0; 1.0 mM EDTA; 0.5 mM adenasine-3-monophosphate) at 0° with inhibitor. ^{14}C-ADP in 0.5 ml was added to a final concentrations of (2.5-20 μM). At each concentration of ^{14}C-ADP; Atr (9.0 μM) or SBP (13.5 μM) or AA (17 μM) were either omitted from the medium (●) or added after (O) or 2 min before ADP (X). The mitochondria were centrifuged after 2 min incubation. Results are mean of four experiments ± S.D. For experimental detail, see Method.

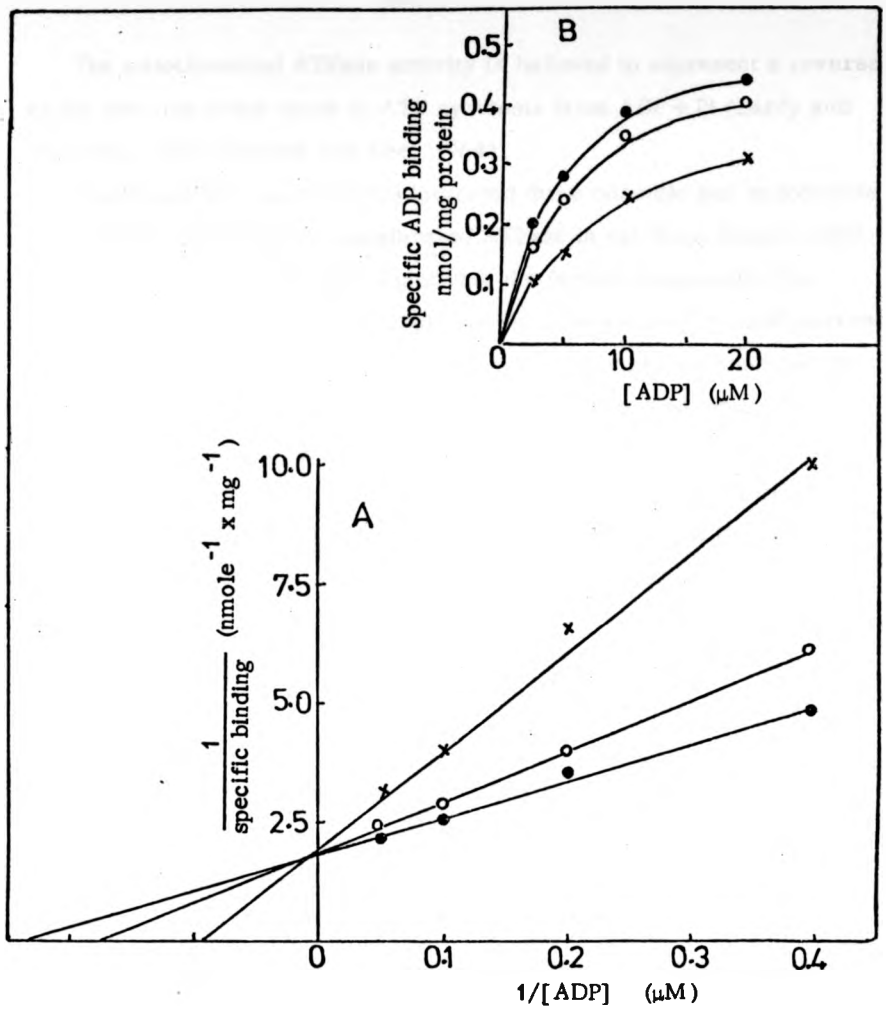


Figure 2.12 Inhibition of specific ADP binding by SBP and agaric acid

(A) double reciprocal plot. (B) concentration dependence of the atractyloside-removable binding.

Specific binding = labelled ADP bound to carrier sites = atractyloside-removable binding (binding without inhibitor - binding where inhibitors added after ADP); data calculated from Fig. 2.11.

●——● , no inhibitors, Km (ADP) = 4.2 μM

O —— O, +agaric acid (17 μM), Km (ADP) = 5.2 μM

X —— X, +SBP (13.5 μM), Km (ADP) = 10.0 μM

The mitochondrial ATPase activity is believed to represent a reversal of the reaction which leads to ATP synthesis from ADP + Pi (Lardy and Wellman, 1953; Ernster and Lee, 1964).

Henderson and Lardy (1970) indicated three possible loci susceptible to inhibition of uncoupler-accelerated ATPase in rat liver mitochondria by bongkrekic acid. It could inactivate the factors responsible for coupling electron transport to oxidative phosphorylation; it could prevent access of adenine nucleotides to mitchondrial enzymes contained within the cristae; or it could prevent the uptake of Pi required for oxidative phosphorylation, thus inhibiting the ATPase via a mass action effect.

The results of the present study, Fig. 2.13, confirms the previous findings by (Vignais et al., 1961; Vignais et al., 1962 and Henderson and Lardy, 1970) that Atr inhibits the DNP-stimulated ATPase activity in intact mitochondria, and that Atr is, in fact, inactive on ATPase activity of submitochondrial particles, confirming the finding of other workers (Luciani and Varotto, 1975; Vignais et al., 1973a; Henderson and Lardy, 1970).

The effect of agaric acid on DNP-stimulated mitochondrial ATPase activity is shown in Fig. 2.13; the present results therefore confirm the work of Chávez and Klapp (1975) who found that in rat liver mitochondria, the ATPase activity is inhibited 30% by 20 μM agaric acid. This result substantiates the findings showing the inhibition of ADP uptake by agaric acid. Furthermore, agaric acid has only a slight effect on the ATPase in submitochondrial particles. Moreover, SBP, as shown in Fig. 2.13, also inhibits the DNP-stimulated mitochondrial ATPase. The ATPase activity is inhibited 45% by 20 μM SBP, as compared with 65% inhibition caused by 20 μM Atr under the same conditions, and that SBP has very slight effect on submitochondrial particles ATPase. However, SBP, as will be shown in Chapter 6, also inhibits the Pi uptake by mitochondria; thus, the inhibition of mitochondrial ATPase activity by SBP is not merely due to the inhibition of adenine nucleotide uptake, a result which indicates that SBP is an unspecific inhibitor of ADP uptake.

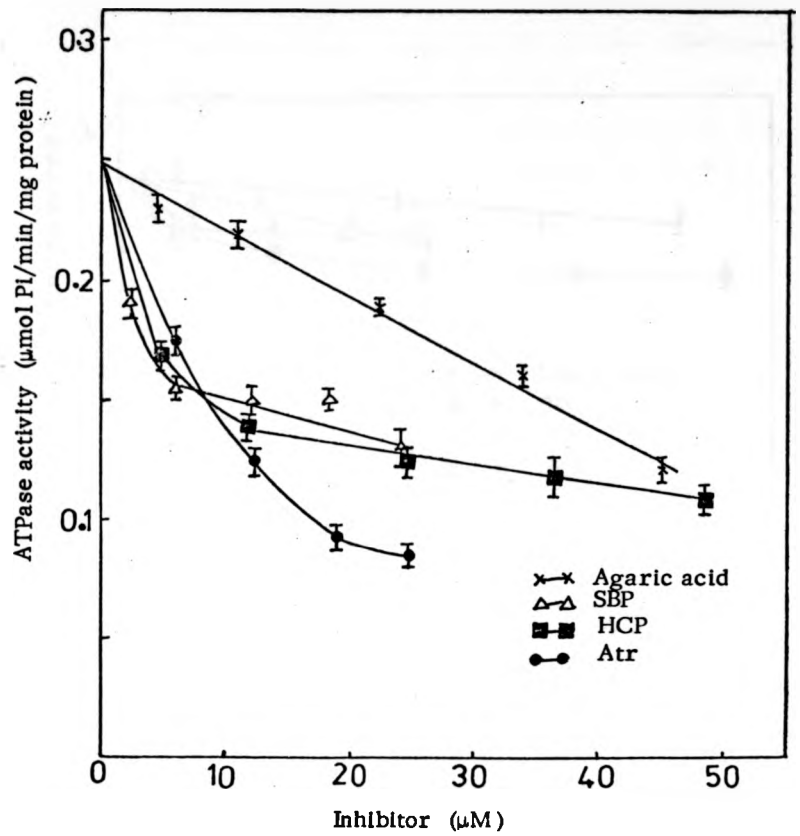


Figure 2.13 Effect of agaric acid, SBP, HCP and Atr on the DNP stimulated ATPase activity

The mixture (final volume 1.0 ml) contained 50 mM Tris-HCl, pH 7.4, 75 mM KCl, 1 mM EDTA, 0.1 M sucrose, 0.003 M $MgCl_2$, 10 mM ATP, 1.0 mg of mitochondrial protein, 0.1 mM DNP. After an incubation period of 5.0 min, the reaction was stopped. Temperature 30^{o}. Results expressed as mean of four experiments ± S.D.

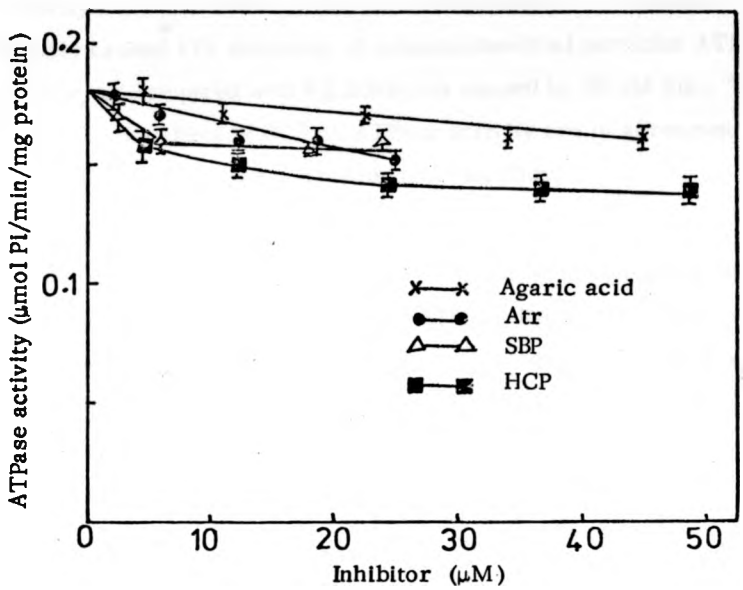


Figure 2.14 Effect of agaric acid, Atr, SBP and HCP on submitochondrial particles ATPase

The mixture (final volume 1.0 ml) contained 50 mM Tris-HCl, pH 7.4, 75 mM KCl, 1 mM EDTA, 0.1 M sucrose, 0.003 M $MgCl_2$, 10 mM ATP, 1.0 mg of submitochondrial particles protein. After an incubation period of 10 min, the reaction was stopped. Temperature 30^{o}. Results expressed as mean of four experiments ± S.D.

The effect of HCP on DNP-stimulated mitochondrial ATPase activity was also [illegible] as shown in Fig. 2.13. HCP has virtually the same effect as SBP on ATPase activity. However, HCP (20 μM) caused 17% inhibition of submitochondrial particles ATPase activity, as compared with 9% inhibition caused by 20 μM Atr. These results on the effect of HCP on ATPase activity are in agreement with the inhibition of adenine nucleotide translocation.

2.4 DISCUSSION

Atractyloside, a potent inhibitor of mitochondrial adenine nucleotide translocator (Pfaff et al., 1965) and ADP binding (Bruni et al., 1964) was also shown to inhibit Dinitro-phene -stimulated mitochondrial ATPase and has no effect on the ATPase of submitochondrial particles (Henderson and Lardy, 1970). Due to the importance of atractyloside and bongkrekic acid, they were used in the present work for comparison the results of the inhibitors SBP, HCP and agaric acid with their effects.

The results of the present work suggest that SBP, AA and HCP at the concentrations mentioned under the "Results" section, are powerful inhibitors of oxidative phosphorylation. The effects of SBP, AA and HCP on ADP translocase were substantiated when it was found that these compounds inhibit the mitochondrial ATPase activity and is inactive on ATPase activity of submitochondrial particles. Thus, they are similar to the potent inhibitors of adenine nucleotide translocation, namely Atr, CAT and BkA (Leuciani et al., 1971; Vignais et al., 1973a).

SBP and AA, which compete with the ADP translocase, were also found to compete with the ADP binding in rat liver mitochondria; thus SBP and AA are similar to Atr which competes with ADP translocation and binding (Weidemann et al., 1970; Vignais et al., 1973a; Klingenberg, 1976).

The influence of SBP on the rate of adenine nucleotide translocation could involve an effect on (a) the binding of Adn to the Atr-sensitive binding sites on the translocase protein, (b) the translocation of Adn

across the mitochondrial inner membrane or (c) an effect on a combination of those processes. The present results show that, in depleted mitochondria (from endogenous Adn), SBP inhibits the binding of ADP to the Atr-sensitive binding sites, and also it inhibits the rate of ADP translocation in native rat liver mitochondria.

It was suggested by Chávez and Klapp (1975) that the mechanism by which agaric acid inhibits adenine nucleotide exchange was that the citrate moiety of agaric acid interacts with the carrier. This interaction would be stabilized by hydrophobic interactions between the alkyl chain of AA and the hydrophobic phase of the membrane.

The mechanism by which HCP inhibits Adn translocation in the rat liver mitochondria is not clear at this time. Several possibilities are currently under examination, one of which involves interaction of the HCP with the lipoprotein translocator. Results presented in Chapter 3 indicate the dependence of HCP binding on mitochondrial membrane phospholipid. Other work (by Thorne and Bygrave, 1974; Klingenberg et al., 1975; Spencer and Bygrave, 1972 and 1974) provides strong evidence for the direct involvement of phospholipid in Adn translocation. The second possibility of inhibition of ADP uptake by HCP may be due to its uncoupling effect.

The inhibition of ADP uptake by uncouplers in RLM observed in the present work, may be explained according to the suggestion of Klingenberg (1975) that in the uncoupled state the exchange with ADP is strongly decreased, first of all since less ATP is available for the exchange. The comparison with the unenergized state with a high ADP content suggests that energy even increases the rate of influx of ADP. The apparent specificity for the entry depends strongly on the energization of the membrane which appears to increase the rate of entry of ADP but strongly inhibits the entry of ATP. This is in line with the assumption that the membrane potential is the main factor controlling the active transport by preventing the entrance of an excess of negative charges during the ATP-ADP exchange. The abolishment of membrane potential and abolishment of the asymmetric exchange by uncouplers or valinomycin plus K^+ was also confirmed by Vignais (1976).

It can be concluded from this chapter that, SBP and AA are potent inhibitors of oxidative phosphorylation. They compete with ADP translocase, while HCP is uncompetitive with ADP translocase. 2, 4-dinitrophenol stimulated mitochondrial ATPase was also inhibited by SBP, AA and HCP. It is more likely that SBP and AA are similar to Atr, while HCP has some properties similar to BkA.

CHAPTER 3

^{3}H - ATRACTYLOSIDE, ^{14}C-HEXACHLOROPHENE AND RHODAMINE 6G BINDING TO MITOCHONDRIA

3.1 INTRODUCTION

Atractyloside (Atr), one of the toxic compounds of the Mediterranean thistle, Atractylis Gummifera, exerts a powerful inhibitory effect on oxidative phosphorylation in mitochondria (for reviews, see Bruni et al., 1966; Chappell, 1968; Heldt, 1969). The history of Atr research spans a period of more than one century. It began in 1868 when Lefrance (1868) succeeded in crystallizing Atr, an important achievement which opened the way to chemical, toxicological and biochemical studies. The molecule of Atr is formed of one glucose moiety, a diterpene nucleus, two sulfate groups and one residue of isovaleric acid (Fig. 1.4). Its injection into mammals leads to an acute hypoglycemia, causing convulsions as shown especially by the Italian School of Santi and by Stainslas in Algiers in the period 1955 to 1960. Atractyloside toxic effects were identified to an inhibition of mitochondrial respiration and ATP synthesis (Bruni et al., 1962). Studies undertaken in the same period by Vignais et al. (1961 and 1962) showed that Atr was able to block partial reactions of oxidative phosphorylation in mitochondria, such as the ATP-Pi exchange and the 2,4-dinitrophenol-stimulated ATPase activity.

(^{35}S)-labelled Atr was recently obtained from Atractylis Gummifera grown in the presence of (^{35}S)-sulfate. By using these radioactive compounds, it was possible to show that (^{35}S)-Atr binds selectively and with very high affinity to the inner mitochondrial membrane (Klingenberg et al., 1975,; Vignais and Vignais, 1970; Vignais et al., 1970). These features qualify Atr not only as a probe to analyse at the molecular level the mechanism of the ADP transport in mitochondria, but also as a tracer to follow the course of the purification of the ADP carrier, an important objective for the ultimate elucidation of transport mechanism.

Thus it is of use to study the effect of the new inhibitors of adenine nucleotide translocase, especially sulfobromophthalein, agaric acid and hexachlorophene on ^{3}H-Atr binding to mitochondria.

It has been shown that HCP is a potent uncoupler of oxidative phosphorylation in liver mitochondria in vitro (Caldwell et al., 1972; Cammer and Moore, 1972) as well as in liver mitochondria isolated from rats dosed with HCP (Caldwell et al., 1972).

HCP is known to hydrogen-bond strongly to proteins (Hague and Buhler, 1972). Therefore a variety of factors may be active in the production of the toxic effects of HCP, including both direct actions, such as binding by HCP to protein, and/or indirect actions, such as uncoupling.

Due to the importance of HCP in inhibition of mitochondrial ions transport systems and other related mitochondrial functions, the present work was carried out on HCP binding in an attempt to correlate its inhibition effects and its binding properties.

The binding of rhodamine 6G (Rh6G), a lipophilic fluorescence dye, was shown by Huang et al. (1969), who studied the binding of Rh6G to phosphatidyl choline vesicles. Gear (1974) showed that this dye is positively charged at pH 7, compared to its close relative, rhodamine B, which is not. Some studies on Rh6G by Huang et al. (1969) pointed to specific binding with well characterized liposome preparations.

It was found that Rh6G strongly inhibited both oxidative phosphorylation and ATP-supported ion transport, while having little action on respiration driven cation uptake by mitochondria (Gear, 1974). Interestingly, 2, 4-dinitrophenol-stimulated ATPase was not inhibited at all. Concentrations above 10 μM began to uncouple respiration and inhibit respiration-driven cation transport.

Results of the present study show that Rh6G inhibits some mitochondrial functions, e.g. ATP-^{32}Pi exchange reaction and ATP-supported calcium transport. Thus it is of use to study Rh6G binding to mitochondria in order to correlate between its inhibition effects and its binding properties.

3.2 MATERIALS AND METHODS

Materials

^{3}H-Atractyloside (^{3}H-Atr) was synthesised by decarboxylation of carboxyatractyloside in tritiated water (K. Cain and D.E. Griffiths, unpublished results). Methylene-^{14}C-hexachlorophene (^{14}C-HCP) was obtained from New England Nuclear Industries, Ltd. Amytal (5-ethyl -5-isoamyl barbituric acid) and procaine hydrochloride were obtained from Sigma Chemical Co., Kingston-upon-Thames, U.K. All other inhibitors, uncouplers, ionophorous antibiotics and local anaesthetics were obtained from the same sources that were mentioned in Chapter 2. All other chemicals were of A.R. grade. The Eppendorf fluorimeter apparatus was obtained from Netheler and Hinz GmbH, Hamburg.

Methods

Preparation of mitochondria

Rat liver mitochondria and submitochondrial particles were prepared as described in Chapter 2.

Estimation of ^{3}H-Atr and ^{14}C-HCP binding

Liver mitochondria or submitochondrial particles (1 mg protein) were incubated for 10 min in 2 ml buffered sucrose (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) in cellulose nitrate tubes at 0^{o} with varying amounts of ^{3}H-Atr or ^{14}C-HCP in the presence or absence of inhibitors. After an incubation period of 5 min, the tubes were centrifuged at 5^{o} at 10000 x g for 15 min (for mitochondria) and 80000 x g for 30 min (for particles). The supernatant and pellet were separated and the tubes drained and wiped free from supernatant. The pellet was dissolved in 1 ml of 2% w/v Triton X-100 for scintillation counting of bound atractyloside or HCP and a 1 ml sample of the supernatant was added to 9 ml of scintillation mixture (Triton X-100, 500 ml; toluene, 1 litre and butyl BPD, 7 g) to determine free ^{3}H-Atr. The results were plotted by the procedure of Scatchard (1949). B = bound; F = free; B/F = bound/free; n = number of binding sites.

Estimation of rhodamine binding to mitochondria

Liver mitochondria (1 mg protein) were incubated for 5 min in 2 ml of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2, 10 mM KCl, 5 mM potassium phosphate, 5 mM $MgCl_2$, 0.2 mM EDTA at 0^o at varying concentrations of rhodamine in the presence or absence of inhibitors. After incubating for 5 min the tubes were centrifuged at 10000 x g for 15 min. The amount of bound dye was determined from the fluorescence of the dye in the supernatant, using an Eppendorf fluorimeter, with excitation filter 366 nm and an emission band pass of 530-3000 nm.

Extraction and rebinding of phospholipid to mitochondria

Phospholipid-depleted mitochondria were prepared according to the method of Fleischer et al. (1967), using 1 volume mitochondrial suspension and 24 volumes of a mixture of 90% acetone + 10% water + 12 µl NH_3 at 0^o. Mitochondria collected by centrifugation at 10000 x g followed by washing twice with buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4). The restoration of the phospholipid content was obtained, using the individual phospholipids, according to the method of Scarpa and Azzone (1969). Phospholipid-depleted mitochondria corresponding to 20 mg mitochondrial protein were incubated at 0^o, in a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and either 50 mg (lecithin) or 25 mg (cardiolipin), or 50 mg (asolectin), or 25 mg (phosphatidylethanolamine), or 25 mg (phosphatidylinositol) in a total volume of 2 ml. After an incubation time of 10 min, the mitochondria were sedimented at 10000 x g and washed twice with the sucrose-Tris medium at 0^o, in order to remove the unbound phospholipid present in the supernatant.

Measurement of radioactivity

^{14}C and ^{3}H were counted in a Packard Tricarb Liquid Scintillation counter in a X-100/toluene/butyl/PBD scintillation mixture (Patterson and Green, 1965).

3.3 RESULTS

Actractyloside binds to the inner mitochondrial membrane which specifically contains high affinity atractyloside binding sites; only low affinity binding sites are found in the outer membrane. The Atr binding characteristics are in good agreement with the fact that the mitochondrial transport system of ADP and ATP is located in the inner membrane (Vignais et al., 1971b).

The characteristics of ^{3}H-Atr binding to mitochondria are shown in Fig. 3.1; the binding data can be resolved by Scatchard analysis into two binding sites. The binding site with the highest affinity ($K_d = 5.4 \times 10^{-8}$ M) is present at a concentration of approximately 0.175 nmol/mg protein, the lower affinity site ($K_d = 7.7 \times 10^{-6}$ M) is present at approximately 0.28 nmol/mg protein concentration. The concentration of high affinity sites has been shown by other workers to be 0.15-0.16 nmol/mg protein (Bruni et al., 1965; Klingenberg et al., 1972) in agreement with the results obtained in the present work. Figure 3.1 shows that ADP (200 μM) completely abolished the high affinity site, a result which confirms the previous findings by Vignais et al. (1971b, 1973a, 1973b). Moreover, the high affinity site of ^{3}H-Atr was also completely abolished by SBP (Fig. 3.2) and by agaric acid (Fig. 3.3). However, these competitive inhibitors have no effect on ^{3}H-Atr low affinity site.

On the other hand, HCP in concentration of 24.5 nmol/mg protein or 49 nmol/mg protein did not affect the binding affinity of ^{3}H-Atr to mitochondria (Fig. 3.4). However, the concentration of high affinity sites is diminished; the influence of HCP therefore appears to be non-competitive, a finding which is similar to the effect of bongkrekic acid on Atr binding shown by Vignais et al (1971a), which suggests that the binding site of ^{14}C-HCP and ^{3}H-Atr are distinct but nevertheless can interact with one another.

Atractyloside also binds to submitochondrial particles, which only contain the right-sided configuration, and the concentration of binding

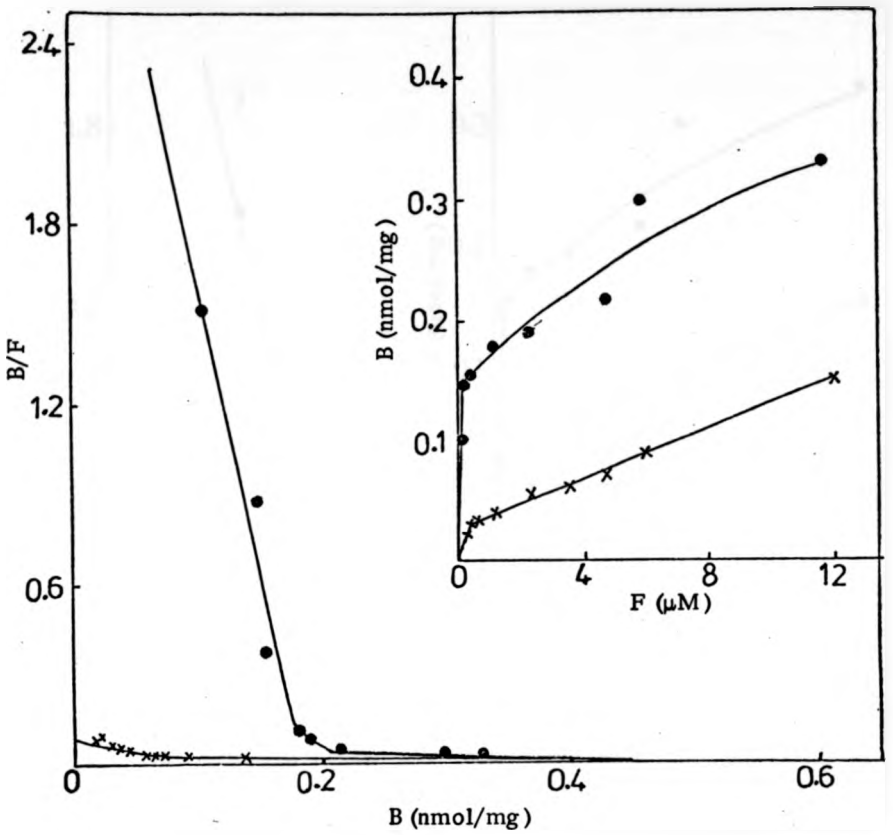


Figure 3.1 Effect of ADP on ^{3}H-atractyloside binding to mitochondria

Experimental conditions as indicated under methods. The results are the mean of four experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
●——● , no ADP	5.4×10^{-8}	0.175	7.7×10^{-6}	0.28
X——X, + 200 μM ADP	–	–	"	"

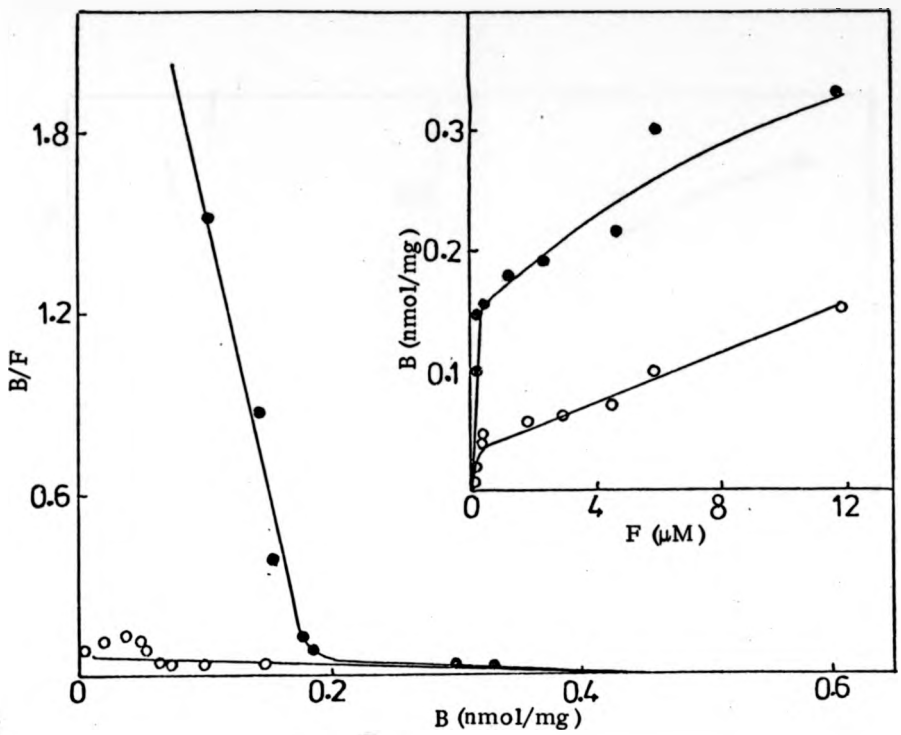


Figure 3.2 Effect of SBP on ^{3}H-atractyloside binding to mitochondria

Experimental conditions employed were as indicated under Methods.

The results are the mean of four experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
●——●, no SBP	5.4×10^{-8}	0.175	7.7×10^{-6}	0.28
O——O, + 24 nmol SBP/mg protein	–	–	"	"

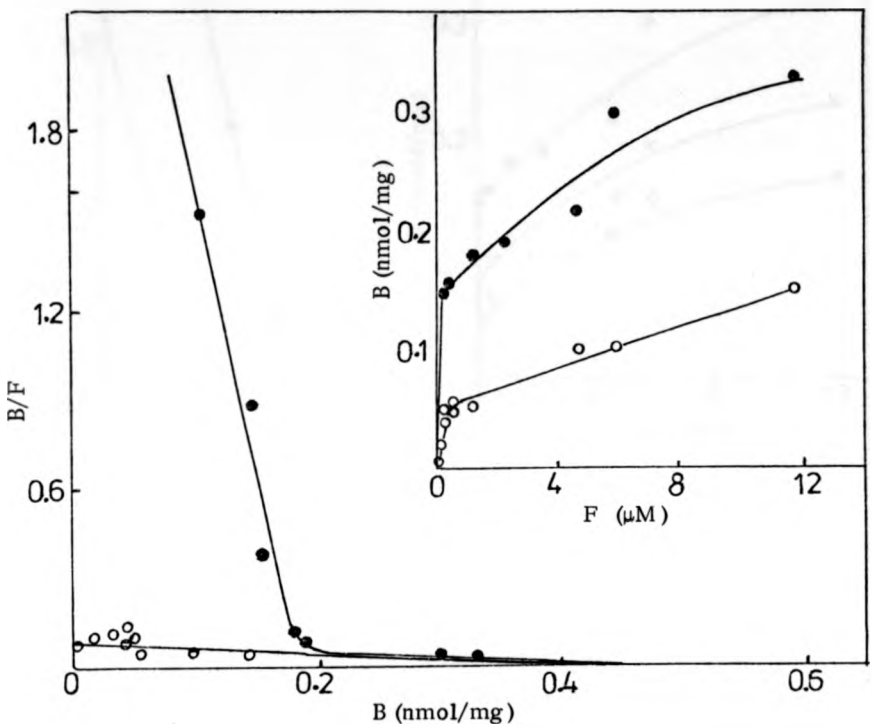


Figure 3.3 Effect of agaric acid on ^{3}H-atractyloside binding to mitochondria

Experimental conditions employed were as indicated under Methods. The results are mean of four experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
● ——● , no agaric acid	5.4×10^{-8}	0.175	7.7×10^{-6}	0.28
O ——O, + 49 nmol agaric acid/mg protein	–	–	"	"

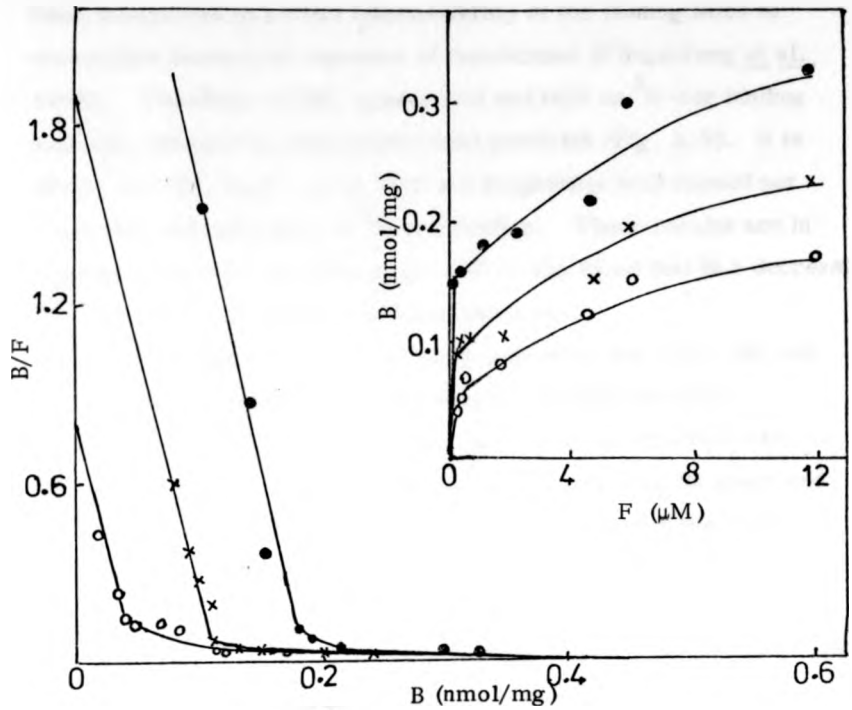


Figure 3.4 Effect of HCP on ^{3}H-atractyloside binding to mitochondria. Experimental conditions as indicated under Methods. The results are mean of five experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
●——●, no HCP	5.4×10^{-8}	0.175	7.7×10^{-6}	0.28
X —— X, + 24.5 nmol HCP/mg protein	5.7×10^{-8}	0.110	"	"
O —— O, + 49 nmol HCP/mg protein	5.6×10^{-8}	0.045	"	"

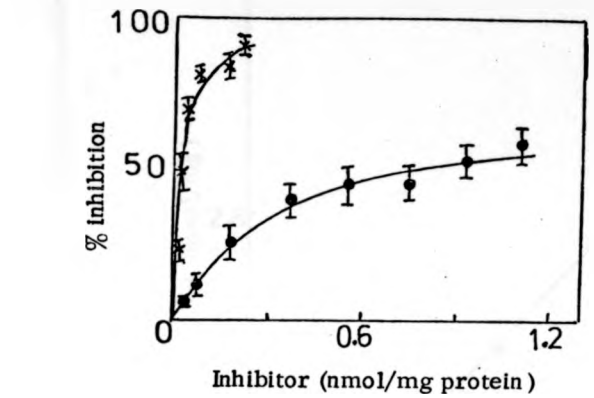
Note It was found that the addition of 2, 4-DNP (56 nmol/mg) or Rh6G (2-100 nmol/mg) or FCCP (40 nmol/mg) had no effect on the binding of ^{3}H-Atr (0.3 nmol/mg protein) to mitochondria.

sites are less than those in intact mitochondria, and these have been interpreted to reflect inaccessibility of the binding sites to atractylate because of inversion of membranes (Klingenberg et al., 1975). The effect of SBP, agaric acid and HCP on ^{3}H-Atr binding was also extended to submitochondrial particles (Fig. 3.5). It is shown that SBP, agaric acid, HCP and bongkrekic acid caused not more than 80% inhibition of ^{3}H-Atr binding. These results are in agreement with Klingenberg et al. (1974) who found that BkA decreases ^{35}S-Atr binding in submitochondrial particles.

From the above results, it can be concluded that ADP, SBP and agaric acid antagonize ^{3}H-Atr binding to its inhibitory site.

The characteristics of ^{14}C-HCP binding to mitochondria are shown in Fig. 3.6. The binding data can be resolved by Scatchard analysis into two binding sites. The binding site with the highest affinity ($K_d = 2.2 \times 10^{-8}$ M) is present at a concentration of approximately 0.25 nmol/mg protein, the lower affinity site ($K_d = 1.5 \times 10^{-5}$ M) is present at approximately 40 nmol/mg protein. The characteristics of ^{14}C-HCP binding to submitochondrial particles are shown in Fig. 3.7. The binding site with the highest affinity ($K_d = 2.5 \times 10^{-8}$ M) which is similar to that in mitochondria and is present at a concentration of 0.4 nmol/mg protein, which is nearly two times more than that of intact mitochondria. The lower affinity site in submitochondrial particles ($K_d = 0.9 \times 10^{-5}$ M) is more or less the same as that in the intact mitochondria and is present at a concentration of approximately 34 nmol/mg protein, which is slightly less than the value found in intact mitochondria.

Tables 3.1, 3.2 and 3.3 show that the binding of ^{14}C-HCP to mitochondria is not affected by ADP, inhibitors, uncouplers of oxidative phosphorylation, ions, local anaesthetics and temperature. The results obtained in the experiments carried out on the relationship between ^{14}C-HCP binding and phospholipid composition of mitochondria are shown in Fig. 3.8, and clearly show the importance of phospholipids



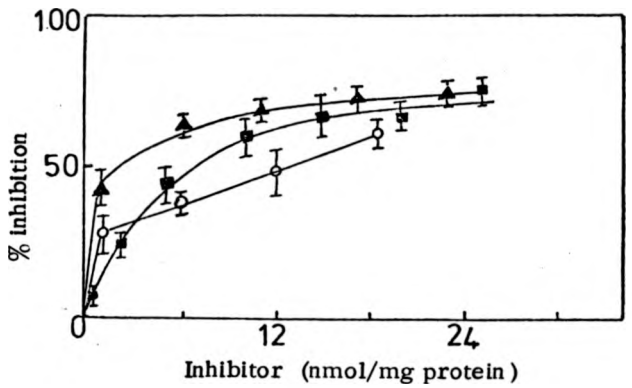


Figure 3.5 Inhibition of ^{3}H-Atractyloside binding to submitochondrial particles

Experimental conditions as indicated under Methods. ^{3}H-Atractyloside concentration used was 1 nmole/mg protein.

X ——— X, C-Atr; ●———●, BkA; ▲———▲, agaric acid; ■———■, SBP; O ——— O, HCP.

The results are expressed as mean of four experiments ± S.D.

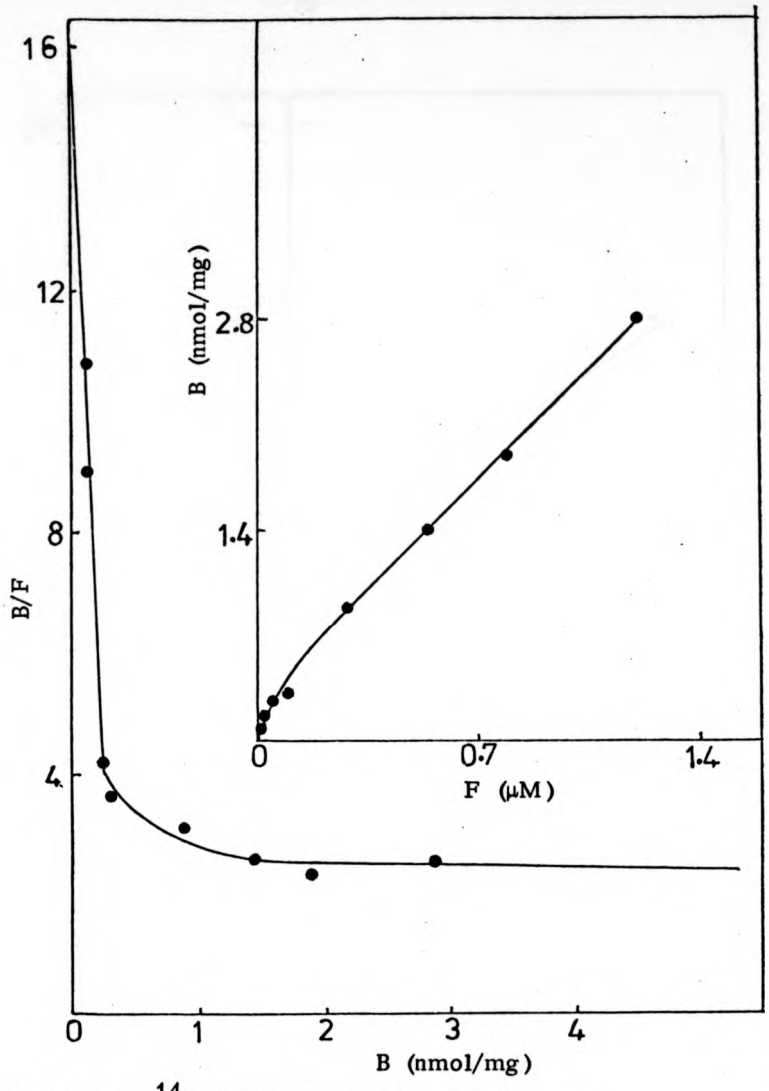


Figure 3.6 ^{14}C-HCP binding to mitochondria

Binding assays were carried out as described under Method. ^{14}C-HCP was added at concentrations ranging from 0.15 to 5 nmole/mg. The results are expressed as mean of four experiments.

	High affinity site	Low affinity site
K_d	2.2×10^{-8} M	1.5×10^{-5} M
n	0.25 (nmol/mg)	40 (nmol/mg)

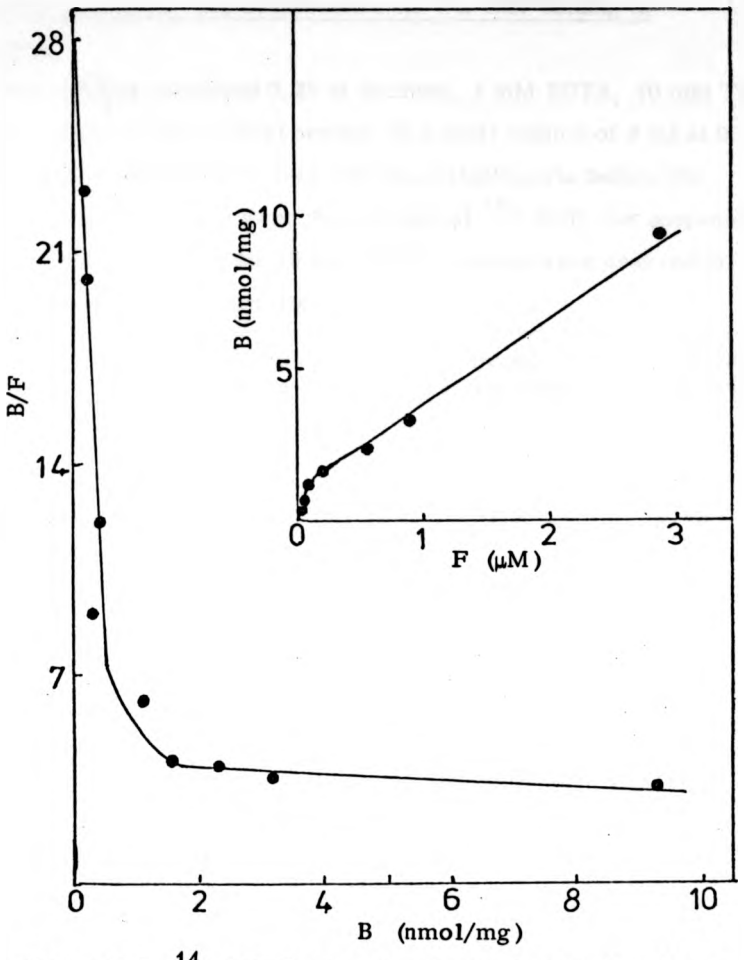


Figure 3.7 ^{14}C-HCP binding to submitochondrial particles

Binding assays were carried out as described under Methods. ^{14}C-HCP was added at concentrations ranging from 0.15 to 15 nmole/mg protein. The results are expressed as mean of four experiments.

	High affinity site	Low affinity site
K_d	2.5×10^{-8} M	0.9×10^{-5} M
n	0.4 (nmol/mg)	34 (nmol/mg)

TABLE 3.1

Effect of ADP, inhibitors and anaesthetics on ^{14}C-HCP binding to mitochondria

The reaction medium contained 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 1 mg of mitochondrial protein (in a total volume of 2 ml at 0°). Inhibitors were incubated for 10 min with the mitochondria before the addition of ^{14}C-HCP. 5 min after the addition of ^{14}C-HCP, the suspension was centrifuged at 10000 x g for 15 min and the pellets were assayed for radioactivity as previously described.

Inhibitor	Concentration (nmol/mg protein)	^{14}C-HCP concentration (nmoles)	^{14}C-HCP bound (nmol/mg protein)
No inhibitor	—	2.5	1.27 - 1.63
ADP	200-1600	2.5	1.48 - 1.80
Atractyloside	5-45	2.5	1.42 - 1.52
Carboxyatractyloside	26-52	2.5	1.42 - 1.45
Bongkrekic acid	24-48	2.5	1.27 - 1.37
SBP	2-48	2.5	1.39 - 1.52
No inhibitor	—	0.5	0.29 - 0.37
ADP + 2 - 4 mM $MgCl_2$	200	0.5	0.35
ADP + 2 - 4 mM $CaCl_2$	200	0.5	0.36
Agaric acid	12-226	0.5	0.27 - 0.28
Rh6G	10-200	0.5	0.31 - 0.37
Amytal	110-220	0.5	0.34 - 0.37
Procaine hydrochloride	90-180	0.5	0.33 - 0.35
Butacaine	70-140	0.5	0.35 - 0.36
Valinomycin	10-20	0.5	0.34 - 0.35

TABLE 3.2

Effect of uncouplers on ^{14}C-HPC binding to mitochondria

The protocol for this experiment is the same as in Table 3.1. The concentrations of DNP and FCCP were 10 μg/mg protein, and pcp was 5 - 50 μg/mg protein.

^{14}C-HCP concentration (nmol/mg)	^{14}C-HCP bound (nmol/mg protein)				
	0.25	0.5	2.5	25	2.50
Control	0.16-1.17	0.30-0.32	1.18-1.42	9.43-9.00	45.38-55.45
DNP	0.15-0.17		1.27-1.32	9.53-9.70	45.4-47.2
FCCP	0.17-0.18		1.17-1.22	8.91-9.50	49.8-50.3
pcp	—	0.29-0.32	—	—	—

TABLE 3.3

Effect of incubation period and temperature on ^{14}C-HCP binding to mitochondria

Binding assays were carried out as described in Table 3.1. ^{14}C-HCP was added at concentrations of 0.5 nmol/mg protein. The results are expressed as mean of three experiments.

Period of incubation of ^{14}C-HCP with mitochondria (sec)	^{14}C-HCP bound (nmol/mg)	Temperature of incubation (^{o}C)	^{14}C-HCP bound (nmol/mg)
0	0.29	0	0.31
15	0.33	10	0.34
30	0.35	20	0.34
45	0.33	30	0.34
60	0.34	40	0.33
110	0.32	50	0.34
130	0.32	60	0.33
150	0.32	70	0.33

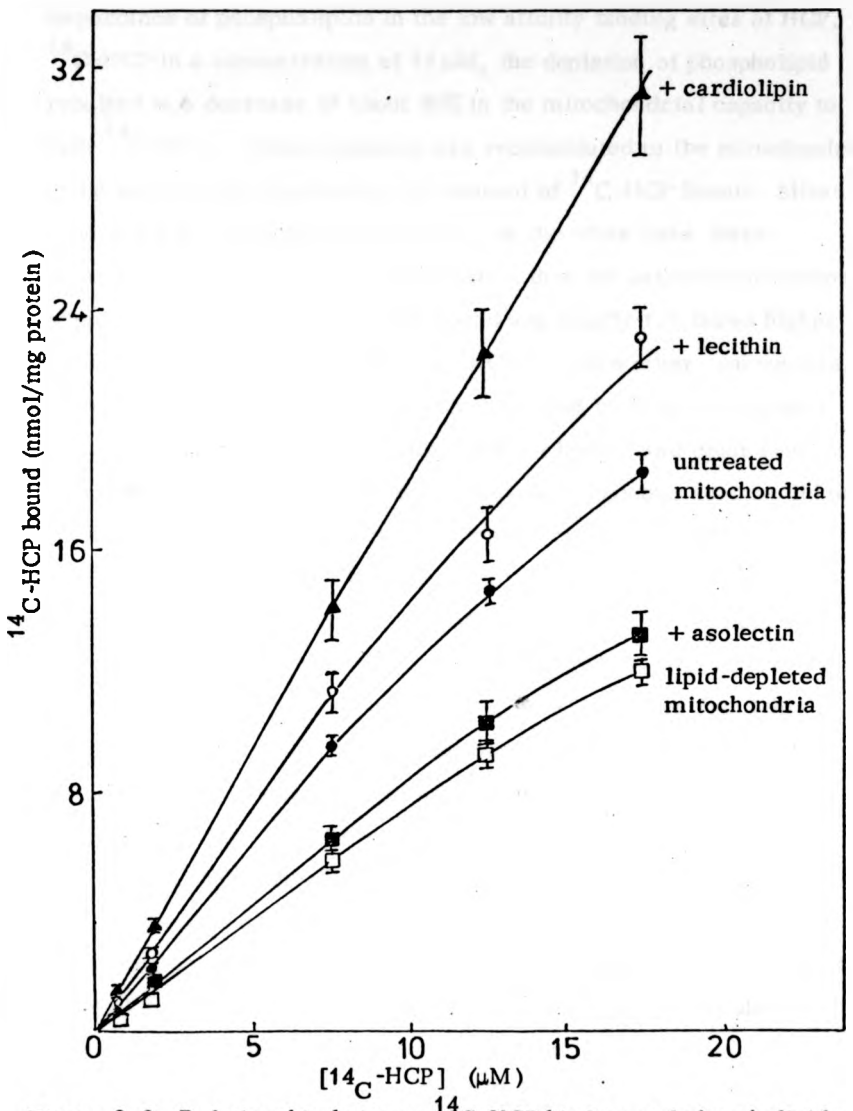


Figure 3.8 Relationship between ^{14}C-HCP binding and phospholipids Composition of mitochondria

Extraction and rebinding of phospholipids was carried out as described under Methods. Binding assays were carried out as described in Table 3.1. The results are expressed as mean of three experiments ± S.D.

^{14}C-HCP binding to mitochondria. This may be related to the importance of phospholipids in the low affinity binding sites of HCP. ^{14}C-HCP in a concentration of 18 μM, the depletion of phospholipid resulted in a decrease of about 40% in the mitochondrial capacity to bind ^{14}C-HCP. When asolectin was reconstituted to the mitochondria, there was a slight increase in the amount of ^{14}C-HCP bound. Mitochondria reconstituted with lecithin, on the other hand were able to bind ^{14}C-HCP to a greater extent than the untreated mitochondria, whereas the amount of ^{14}C-HCP bound was nearly 1.7 times higher in the case of cardiolipin. However, HCP is unlike other uncouplers such as 2-azido-4-nitrophenol (NPA) since NPA binding site appears not to involve the bulk of the mitochondrial lipids, and prior removal of more than 80% of mitochondrial lipids with aqueous acetone did not change the extent of NPA binding (Hanstein and Hatefi, 1974).

Since HCP is known to bind strongly to bovine serum albumen (BSA) (Flores and Buhler, 1971), it was of interest to study the removal of ^{14}C-HCP from mitochondria by consecutive washes with buffered 0.25 M sucrose with or without added BSA (Fig. 3.9). It was found that, after three washes with 0.25 M sucrose buffer, more than 74% of the original ^{14}C-HCP was retained with the mitochondrial pellets. After a single wash with the BSA-containing medium, only 18% of the original ^{14}C-HCP remained bound. These results are in agreement with Caldwell et al. (1972) who carried out similar experiments on rat liver mitochondria after intraperitoneal injection of the rats with a lethal dose of ^{14}C-HCP (30 mg/kg). The above results show that HCP is not covalently bound to mitochondria.

TABLE 3.4

Comparison between the binding of rhodamine compounds to mitochondria

The experimental conditions as described under Methods. The results are expressed as mean of five experiments ± S.D.

	Rhodamine concentration (μM)	Rhodamine bound (nmol/mg protein)
Rh6G	50	36.0 ± 2.2
Rh3G	50	6.5 ± 1.0
RhB	50	0.0
RhS	50	0.0

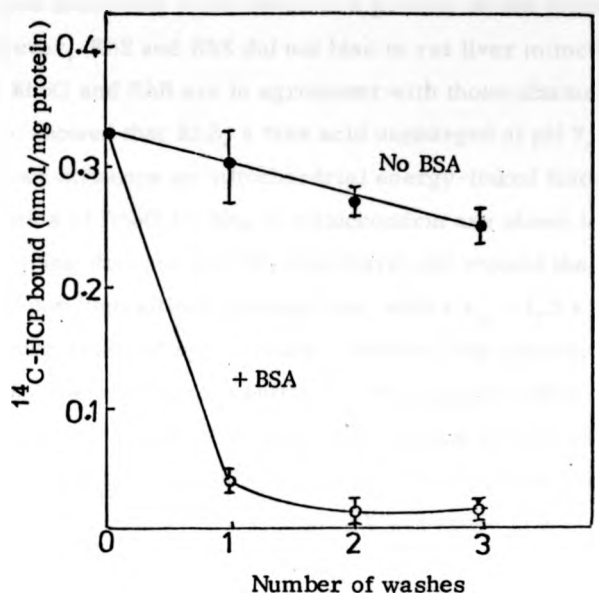


Figure 3.9 Effect of washing on ^{14}C-HCP binding to mitochondria

The wash medium represented by (●———●) contained 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, while that represented by (O——— O) contained 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 30 mg bovine serum albumen. 2 sets of four assay tubes containing mitchondria (10 mg protein/7 ml washing medium) and 0.5 nmol/mg ^{14}C-HCP were incubated for 10 min at 0°. After centrifugation the supernatant were decanted. Aliquots of pellets were examined for protein and ^{14}C-HCP content after each wash. The pellets were resuspended in 7 ml of washing buffer and recentrifuged. This procedure was repeated a further three times.

The zero wash does not contain BSA.

The results expressed as mean of four experiments ± S.D.

In studies of the binding of rhodamine compounds by mitochondria (Table 3.4) it was found that Rh6G bound to a greater extent followed by Rh3G. However, RhB and RhS did not bind to rat liver mitochondria. The results on Rh6G and RhB are in agreement with those obtained by Gear (1974) who showed that RhB, a free acid uncharged at pH 7, was completely without influence on mitochondrial energy-linked functions. The characteristics of Rh6G binding to mitochondria are shown in Fig. 3.10, and the binding data analysed by Scatchard plot showed the presence of only one high affinity binding site, with a $K_d = 1.3 \times 10^{-5}$ M present at a concentration of approximately 50 nmol/mg protein.

Leucinostatin, the ionophorous antibiotic, in a concentration of 1.28 to 3.2 nmol/mg protein, slightly inhibits Rh6G binding affinity and also decreases the number of binding sites from 50 to 32 nmol/mg protein (Fig. 3.10). The same figure shows that calcium added in a concentration of 90 nmol/mg did not affect the affinity of Rh6G to mitochondria . However, the number of high affinity sites is decreased to 16 nmol/mg protein; the influence of calcium appears to be non-competitive . Similar effects to calcium on Rh6G binding were found with TET and DBCT (Fig. 3.11). Valinomycin and 1799 (Fig. 3.12) caused a slight decrease of both the affinity and the number of binding sites. It was also found that, anaesthetics, such as butacaine and amytal also inhibit the binding of Rh6G to mitochondria (Fig. 3.13).

The results of the present study show that with 50 μM Rh6G the amount of Rh6G bound is 35-40 nmol/mg protein, which is similar to the finding of Yaginuma et al. (1973).

On the other hand, it was found that (Table 3.5) the period of incubation of Rh6G with mitochondria, the temperature of incubation, pH and the inhibitors of oxidative phosphorylation, have no effect on Rh6G binding.

An experiment carried out on the relationship between Rh6G binding and phospholipid composition of mitochondria is shown in Fig. 3.14.

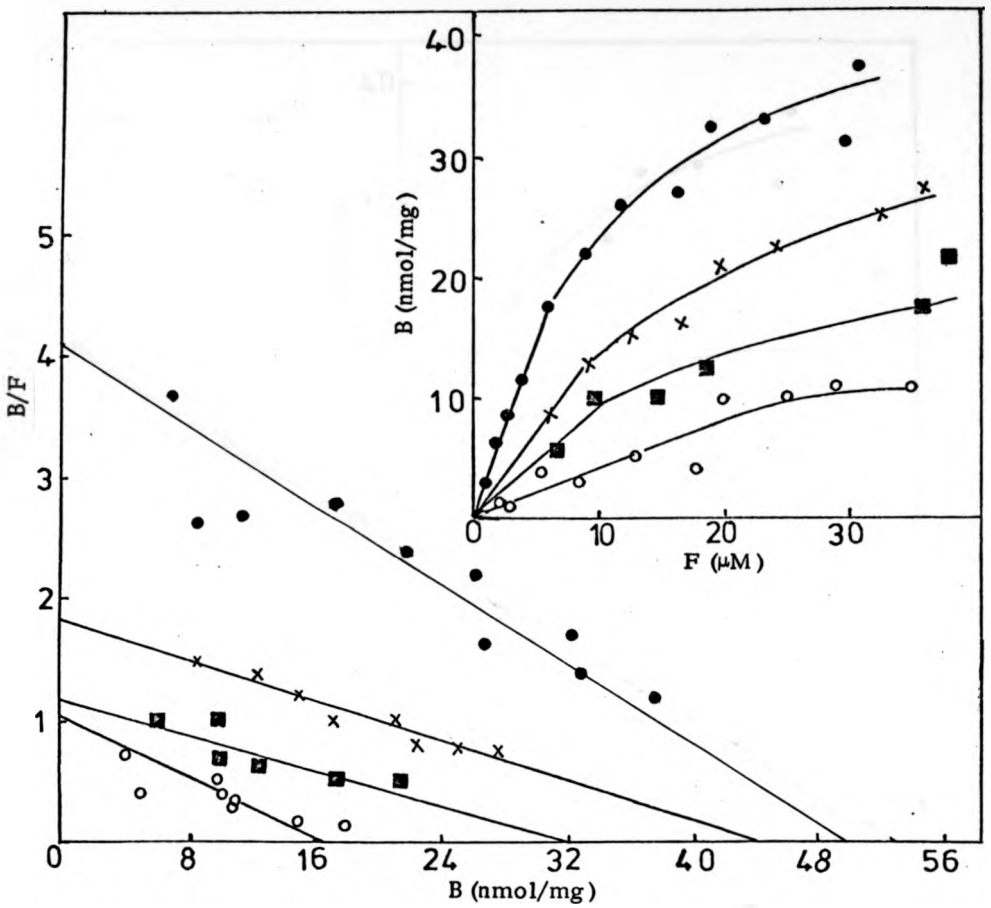


Figure 3.10 Effect of leucinostatin and calcium on Rh6G binding to mitochondria

The experimental conditions as described under Methods. The results are mean of four experiments.

	K_d(M)	n(nmol/mg protein)
●——●, no inhibitor	1.3×10^{-5}	50
X——X, + 1.28 nmol/mg, leucinostatin	2.5×10^{-5}	44
■——■, + 3.2 nmol/mg, leucinostatin	2.8×10^{-5}	32
O——O, + 90 nmol/mg, $CaCl_2$	1.6×10^{-5}	16

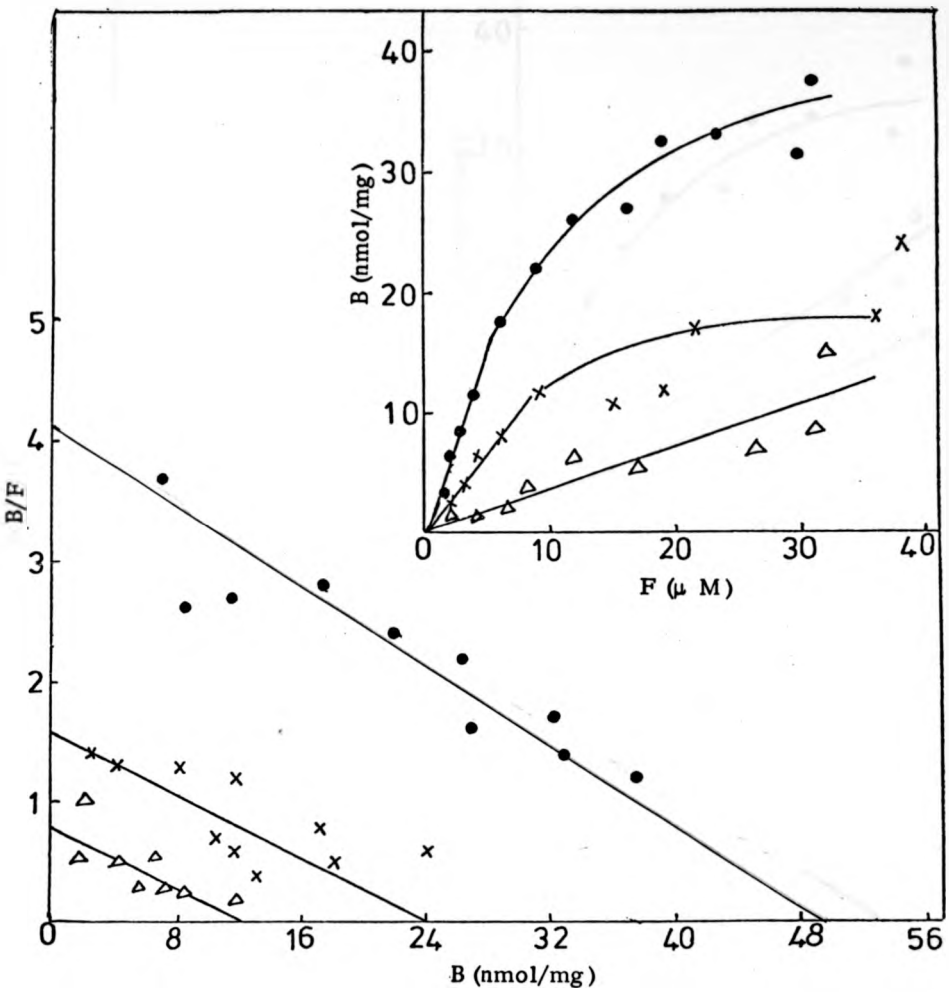


Figure 3.11 Effect of TET and DBCT on Rh6G binding to mitochondria

The experimental conditions as described in the Methods. Results are mean of three experiments.

	K_d(M)	n(nmol/mg protein)
●——●, no inhibitor	1.3×10^{-5}	50
X——X, + 40 nmol/mg TET	1.5×10^{-5}	24
△——△, + 31 nmol/mg DBCT	1.6×10^{-5}	12

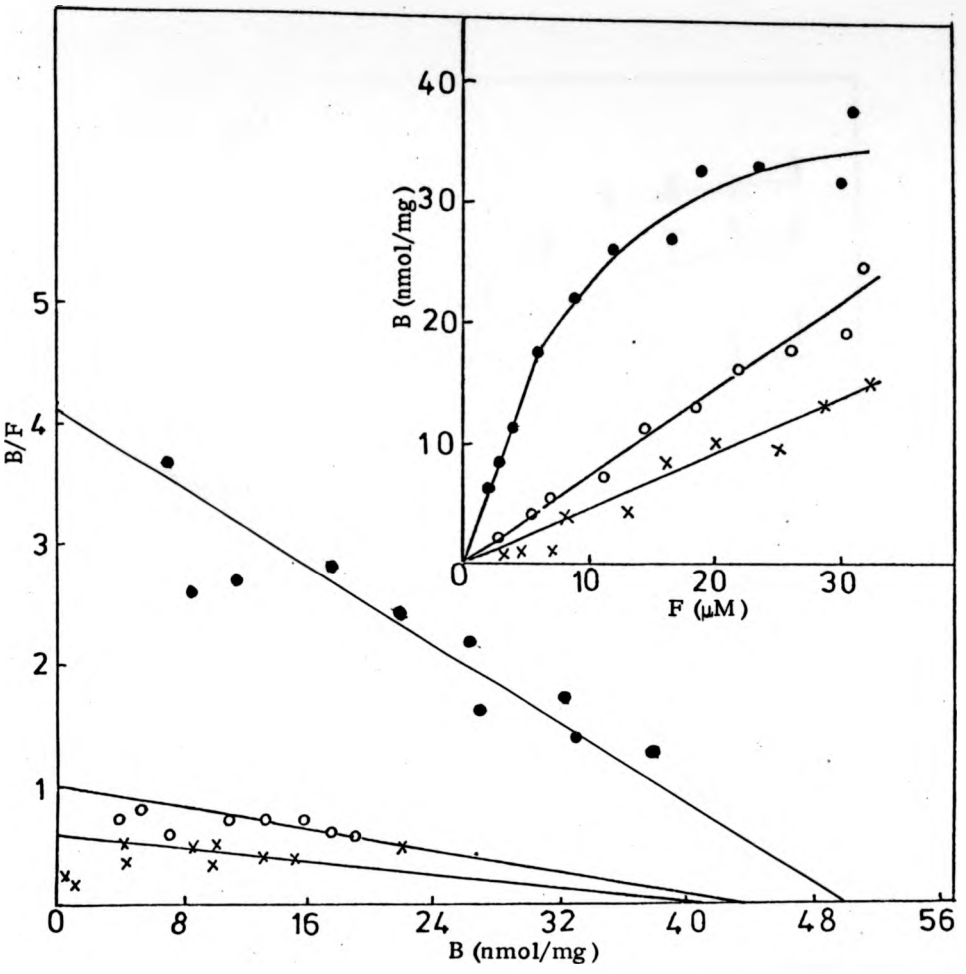


Figure 3.12 Effect of valinomycin and 1799 on Rh6G binding to mitochondria

The experimental conditions as described in the Methods. Results are mean of three experiments.

	K_d(M)	n(nmol/mg protein)
●——●, no inhibitor	1.3×10^{-5}	50
O —— O, + 9 nmol/mg, valinomycin	4.4×10^{-5}	44
X —— X, + 25 nmol/mg, 1799	6.6×10^{-5}	40

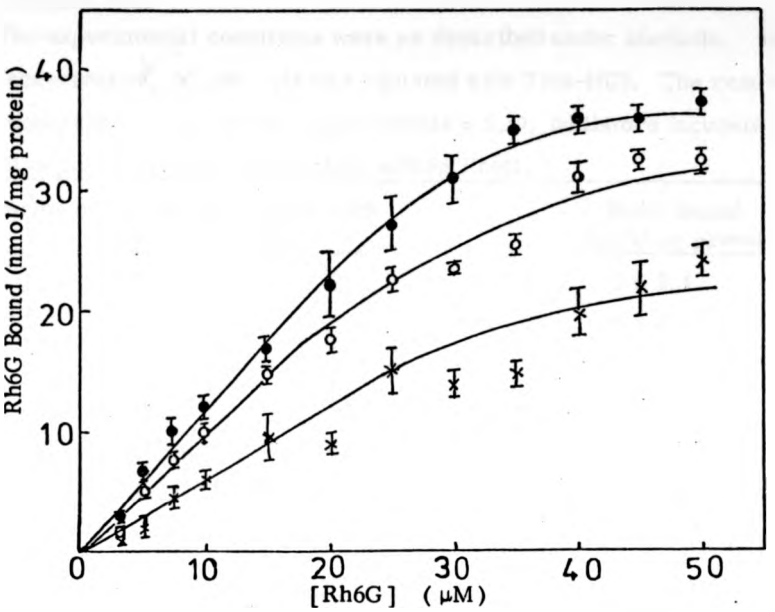


Figure 3.13 Effect of butacaine and amytal on Rh6G binding to mitochondria

The experimental conditions as described in the Methods. The results are expressed as mean ± S.D. of three experiments.

●——●, no inhibitor

O——O, + 4 μmol/mg butacaine

X——X, + 1 μmol/mg amytal

TABLE 3.5

Effect of incubation periods, temperature and pH on Rh6G binding to mitochondria

The experimental conditions were as described under Methods. Rh6G concentration, 50 μM. pH was adjusted with Tris-HCl. The results are expressed as mean of four experiments ± S.D. Inhibitors incubated for 10 min with mitochondria before adding Rh6G.

Period of incubation of Rh6G with the mitochondria	Rh6G bound nmol/mg protein
0 sec	33 ± 3.1
7 sec	38 ± 3.3
15 sec	34 ± 2.5
60 sec	33 ± 1.9
3 min	36 ± 2.0
5 min	34 ± 2.6
10 min	34 ± 1.8
Temperature of incubation medium (^{o} C)	
0	34 ± 2.9
5	36 ± 3.0
10	33 ± 2.4
15	37 ± 1.8
20	35 ± 2.0
25	36 ± 3.1
30	33 ± 2.7
pH of incubation medium	
6.0	36 ± 3.3
6.5	35 ± 2.8
7.0	37 ± 2.9
7.5	34 ± 2.1
8.0	33 ± 1.9
8.5	35 ± 2.4
9.0	36 ± 3.2

/continued

TABLE 3.5 (continued)

Inhibitors	Concentrations (nmol/mg)	Rh6G bound nmol/mg protein
No inhibitor	—	35 ± 2.4
Atractyloside	1-100	35 ± 2.4
Carboxyatractyloside	1-100	35 ± 2.4
Bongkrekic acid	0.5-46	35 ± 2.4
Sulfobromophthalein	6-660	35 ± 2.4
Agaric acid	6-660	35 ± 2.4

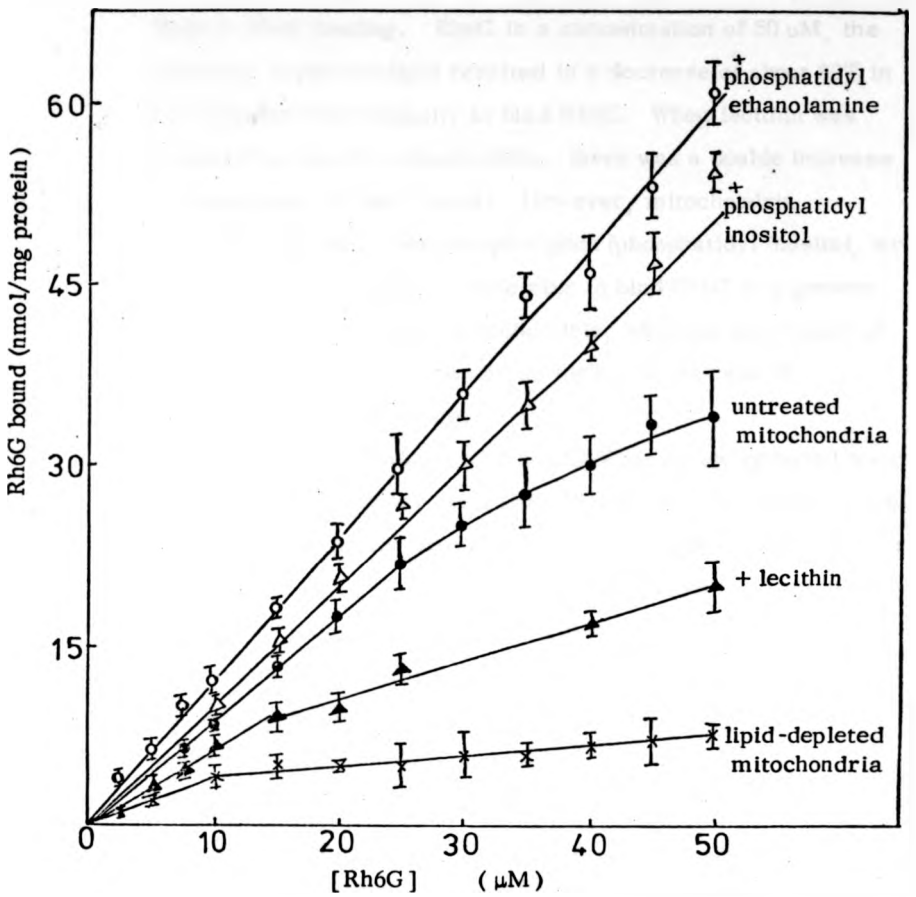


Figure 3.14 Relationship between Rh6G binding and phospholipid composition of mitochondria

Extraction and rebinding of phospholipids and binding assays were carried out as described under Methods. Asolectin and cardiolipin have similar effects to phosphatidylinositol. The results are expressed as mean of three experiments ± S.D.

This experiment shows the necessity of mitochondrial phospholipid in Rh6G binding. Rh6G in a concentration of 50 μM, the depletion of phospholipid resulted in a decrease of about 80% in the mitochondrial capacity to bind Rh6G. When lecithin was reconstituted to the mitochondria, there was a double increase in the amount of Rh6G bound. However, mitochondria reconstituted with other phospholipids (phosphatidyl inositol, or asolectin, or cardiolipin) were able to bind Rh6G to a greater extent than the untreated mitochondria, whereas the amount of Rh6G bound was nearly two times higher in the case of phosphatidyl-ethanolamine.

In order to find whether or not Rh6G is easily removed from mitochondria, an experiment (Fig. 3.15) was carried out in which Rh6G removed by consecutive washes with buffered 0.25 M sucrose. It was found that Rh6G was completely removed from its binding sites after four washes, with or without BSA. These results show that Rh6G is not covalently bound to rat liver mitochondria, and also that it is more easily removed from mitochondria as compared with ^{14}C-HPC.

3.4 DISCUSSION

The results of the present study, Fig. 3.1, show that ADP completely abolished the high affinity site of ^{3}H-Atr in agreement with the findings of Vignais et al. (1971b; 1973a; 1973b). SBP and agaric acid were also found to abolish the high affinity site of ^{3}H-Atr, which indicates clearly the interaction between ^{3}H-Atr binding and these inhibitors. However, HCP did not affect the binding affinity, but it did decrease the number of high affinity binding site per mg protein, indicating the noncompetitive type of inhibition. Other uncouplers such as DNP and FCCP did not affect ^{3}H-Atr binding to mitochondria (with Fig. 3.4). These findings differentiate HCP from other uncouplers.

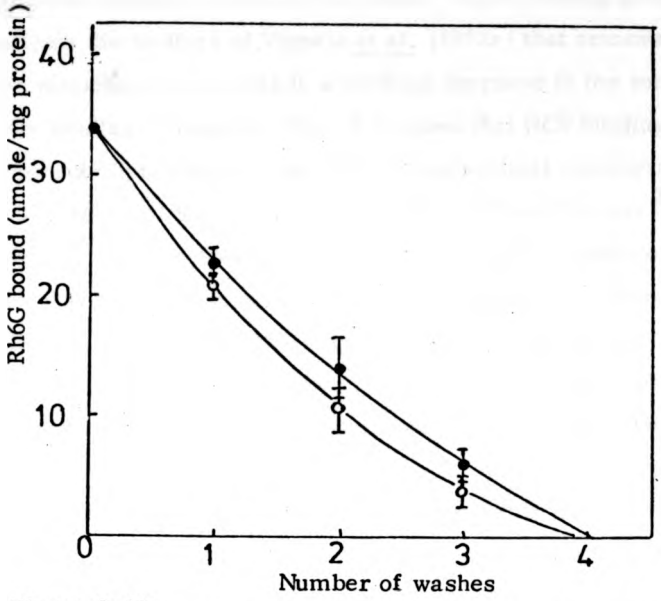


Figure 3.15 Effect of washing on Rh6G binding to mitochondria

The wash medium represented by (●——●) contained the same buffer mentioned in Methods, while that represented by (O —— O) contained the same buffer + 50 mg bovine serum albumen. 2 sets of 4 assay tubes containing mitochondria (10 mg protein/10 ml washing medium) and100 nmol/mg Rh6G were incubated for 5 min at 0^{o}; after centrifugation, the amount of bound dye was determined from the abosrbance of the dye in the supernatant. The pellets were resuspended in 10 ml of washing buffer and recentrifuged. This procedure was repeated a further four times. After each wash the protein concentration and Rh6G bound was examined. The zero wash does not contain BSA. The results are expressed as mean of four experiments ± S.D.

Binding of HCP to membrane phospholipids could exclude the normal membrane binding sites and thus affect ^{3}H-Atr binding sites. This confirms the findings of Vignais et al. (1973a) that removal of lipids from mitochondria results in a striking decrease in the total amount of Atr binding. Further, Fig. 3.8 shows that HCP binding depends on the phospholipid composition of the mitochondrial membrane.

The effect of SBP, agaric acid, HCP, CAT and BkA on ^{3}H-Atr binding to submitochondrial particles (right-sided particles) (Fig. 3.5) show that SBP, agaric acid, HCP and BkA caused less than 80% inhibition. However, CAT caused nearly 90% inhibition. This great inhibition of ^{3}H-Atr binding by CAT may be due to the high affinity of CAT which is about 25-fold higher than that of Atr (Scherer et al., 1973).

The results presented in Table 3.1 show that the binding of ^{14}C-HCP to mitochondria is not affected by a variety of inhibitors, and also that it is not affected by certain uncouplers (Table 3.2). However, the binding of other uncouplers such as NPA is competitively inhibited by some uncouplers, e.g. DNP, PCP, Cl-CCP (Hanstein and Hatefi, 1974).

It was shown that the amount of HCP bound to mitochondria (after injection) is directly proportional to the degree of uncoupling (Caldwell et al., 1972) and the chlorinated bisphenols have also been shown to bind strongly to serum protein (Flores and Buhler, 1971), and to various polypeptides (Hague and Buhler, 1972). Moreover, it is shown that in Fig. 3.8 the binding of ^{14}C-HCP to mitochondria depends on the mitochondrial phospholipids, especially lecithin and cardiolipin; thus HCP differs from other uncouplers such as NPA whose binding site appears not to involve the bulk of the mitochondrial lipids (Hanstein and Hatefi, 1974). In this respect HCP binding is similar to atractyloside and ADP binding (Klingenberg et al., 1974; Vignais et al., 1973a). Thus, ^{14}C-HCP have properties of both uncouplers (e.g. FCCP) and inhibitors (e.g. Atr and BkA) of oxidative phosphorylation.

The inhibition of Rh6G binding to mitochondria by leucinostatin, valinomycin, DBCT, TET, calcium and 1799 (Figs. 3.10, 3.11 and 3.12) may be due to the disturbance of proton gradient in mitochondrial membranes.. According to the chemiosmotic hypothesis of oxidative phosphorylation, uncouplers act by rendering the mitochondrial membrane freely permeable to protons, (Mitchell, 1961), since a proton is ejected into the medium (in exchange for Rh6G), in order to maintain electroneutrality (Yaginuma et al., 1973; Gear, 1974).

On the other hand, the inhibition of Rh6G binding to mitochondria by anaesthetics (butacaine and amytal) may be due to the disorder in membrane structure caused by anaesthetics (Spencer and Bygrave, 1974). These changes in membrane 'fluidity' probably reflect interaction of the anaesthetics with membrane phospholipid (Seeman, 1972; Fayle et al., 1975). Thus, the inhibition of Rh6G binding by butacaine and amytal may be due to the interaction of these anaesthetics with phospholipids, which form the phospholipid environment of the binding, leading to an alteration in Rh6G binding. The mechanisms by which anaesthetics interact with these phospholipids is at present unknown, although it is likely (in the case of butacaine) to involve both electrostatic and hydrophobic interactions (Seeman, 1972).

The results of the present work (Fig. 3.14) indicate the importance of mitochondrial phospholipids in Rh6G binding, and also support the suggestion that a lipid binding step is involved in the action of Rh6G (Gear, 1974).

The work in this chapter supports the findings shown in Chapter 2, that SBP and agaric acid are competitive inhibitors with both ADP translocase and ^{3}H-Atr binding. However, HCP competes with neither ADP translocase nor ^{3}H-Atr binding. On the other hand, the present results show that the inhibitors of ADP translocase (e.g. Atr, EkA, CAT, SBP, agaric acid) have no effect on Rh6G binding, in agreement with the previous findings (Table 2.2) that Rh6G has no effect on ADP translocase, and in contrast to Gear (1974) who showed that Rh6G competes with adenine nucleotide binding to mitochondria.

CHAPTER 4

CALCIUM TRANSPORT IN MITOCHONDRIA

4.1 INTRODUCTION

The reason for studying calcium transport and binding by mitochondria in the present work is related to the following facts: (a) calcium is important in the regulation of a number of mitochondrial functions, and the calcium pumped in or out of mitochondria may be an essential factor in the control of several reactions taking place in the cell (Table 1.1), (b) in biological membranes, the divalent cations, chiefly Ca^{++} and Mg^{++}, seem to control the stability of the membrane by formation of a ternary complex between the anionic groups of a protein and a phospholipid molecule (Binet and Volfin, 1975b), (c) calcium is needed to couple the electrical events of the membrane to the physiological response of the cell, and intracellular Ca^{++} may regulate the permeability of the cell membrane for both ions and neutral solutes (Seeman, 1972).

In the present study (Fig. 4.1) it was found that hexachlorophene (HCP) inhibits calcium-stimulated mitochondrial respiration. This inhibition of respiration may be due to prevention of calcium uptake by mitochondria, and this is the main reason which prompted us to carry out studies on the effect of HCP on calcium transport.

In view of the association between HCP binding and mitochondrial phospholipid composition (Fig. 3.8) and the inhibition of calcium uptake by HCP, the relationship between calcium binding and phospholipid composition of mitochondria can be examined. Figure 4.7 shows that DBCT and TET cause a release of mitochondrial calcium in a way similar to that of HCP. The toxicological effects of TET and HCP are similar. In the present work, the effects of TET and DBCT on calcium transport and binding were studied. The effect of other uncouplers of oxidative phosphorylation and inhibitors, such as ruthenium red and Rh6G, and ionophorous antibiotics were also studied in comparison.

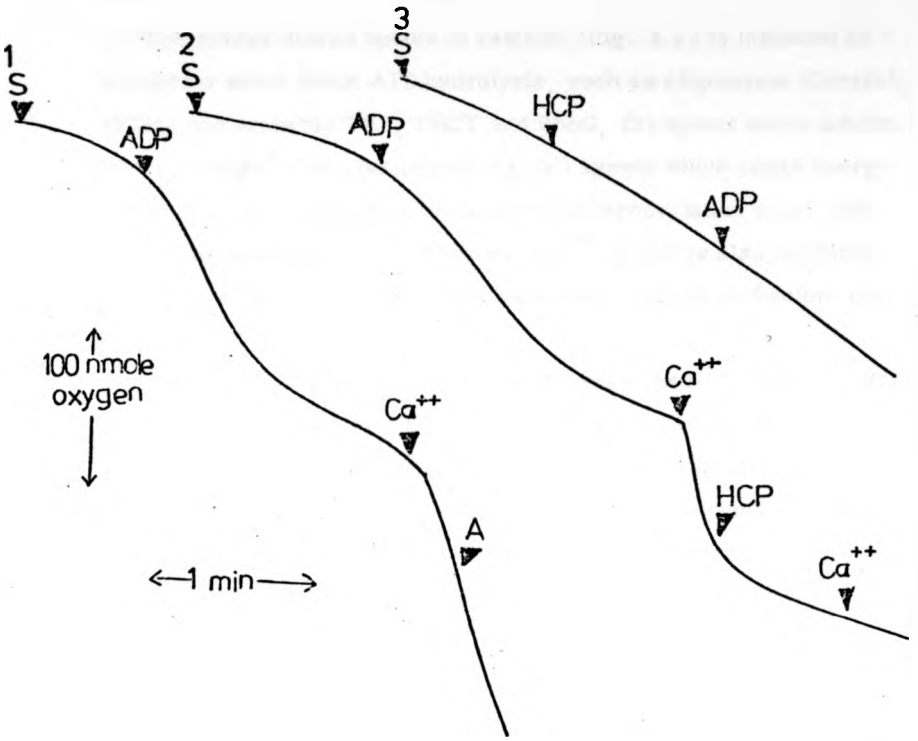


Figure 4.1 Inhibition of calcium stimulated respiration in rat liver mitochondria by HCP.

Respiration was measured with a Clarke oxygen electrode in 3 ml of 0.25 M sucrose, 20 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM KCl, 5 mM KH_2PO_4, pH 7.4 buffer at a temperature of 30°. Mitochondria (6.0 mg protein) were preincubated in the respiration buffer for 1 min before adding 10 µl of 1.0 M succinate (S), 5 µl of 50 mM of ADP and 10 µl of 50 mM $CaCl_2$ (Ca^{++}) as indicated on the traces.
Trace 1 was a control experiment with 10 µl of ethanol added at A.

In trace 2, HCP in 10 µl of ethanol was added (25 nmoles).

Trace 3 shows the inhibition of ADP stimulated respiration by HCP.

The energy-linked uptake of calcium (Fig. 4.2) is inhibited by (a) agents which block ATP hydrolysis, such as oligomycin (Carafoli, 1975a) and probably TET, DBCT and Rh6G, (b) agents which inhibit the respiratory chain (antimycin A), (c) agents which cause energy dissipation like uncouplers of oxidative phosphorylation (e.g. DNP, 1799) and probably HCP. However, Ca^{++} uptake is also inhibited by agents which inhibit the calcium carrier, such as ruthenium red (Moore, 1971).

In this chapter, the work done on the effect of HCP, Rh6G, DBCT and TET on calcium transport, binding and release by mitochondria, is reported with a view to assessing whether these inhibitors act on the calcium carrier or as an uncoupler of oxidative phosphorylation or block ATP hydrolysis or by combination of these effects.

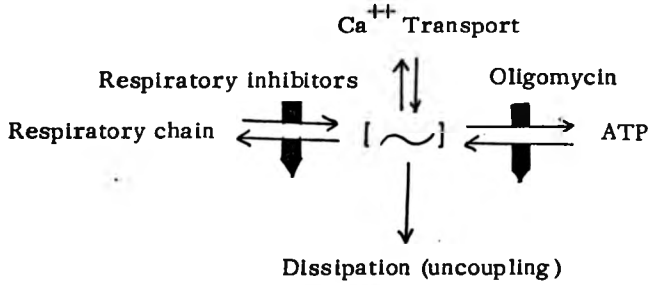


Figure 4.2 Energy-linked Ca^{++} transport in mitochondria (from Carafoli et al., 1975a).

4.2 MATERIALS AND METHODS

Materials

$^{45}CaCl_2$ was obtained from the Radiochemical Centre (Amersham, U.K.). Hepes, ruthenium red, gramicidin D, EGTA, succinate, rotenone, and antimycin A were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. DBCT was a gift from Dr. K. Cain (Department of Molecular Sciences, University of Warwick, England). Asolectin (soybean phosphatides) was purchased from Associated Concentrates (Woodside, N.Y.). Lecithin (egg lecithin) was obtained

from Lipid Products, (South Nutfield NR, Redhill, Sy). Cardiolipin (ex bovine heart), DL-3-phosphatidylethanolamine and phosphatidyl-inositol were obtained from Koch-Light Laboratories, Ltd., (Colnbrook, Buckinghamshire, England). The other chemicals were obtained from the sources mentioned in Chapter 2. All other chemicals were of A.R. grade.

Methods

Preparation of mitochondria

Preparation of rat liver mitochondria and protein determination were carried out as described in Chapter 2.

Estimation of $^{45}Ca^{++}$ uptake by mitochondria

The experimental conditions are essentially those described by Carafoli and Rossi (1971): the reaction medium (25°, 2 ml final volume) contained 80 mM NaCl, 10 mM sodium succinate, 10 mM Tris-HCl, pH 7.2, and 1.0 mg of mitochondrial protein. Inhibitors were incubated for 30 sec with the mitochondria before the addition of labelled calcium. At 20 sec after the addition of labelled calcium, the suspension was filtered by rapid Millipore filtration (as described in Chapter 2) followed by washing with 4 ml of incubation medium. The radioactivity on the filters was used to calculate the amount of $^{45}Ca^{++}$ uptake.

Estimation of $^{45}Ca^{++}$ binding to mitochondria.

The experimental conditions are those described above by Carafoli and Rossi (1971). Mitochondria were preincubated with 1 μM rotenone and 0.5 μg antimycin A for 5 min followed by incubation with inhibitors for 1 min before adding labelled calcium. 30 sec after the addition of labelled calcium (0.4 - 80 nmol/mg protein) the suspension was centrifuged at 10000 x g for 15 min, and the distribution of radioactivity between the pellet and the supernatant was measured. Results expressed as B=bound Ca^{++} (nmol/mg protein); F = free Ca^{++} (μM); B/F= bound/free and n = number of binding sites, as described by Scatchard (1949).

Extraction and rebinding of phospholipid to mitochondria

Phospholipid-depleted mitochondria were prepared according to the method of Fleischer et al. (1967), using 1 volume mitochondrial suspension and 24 volumes of a mixture of 90% acetone + 10% water + 12 µl NH_3 at 0^o. Mitochondria collected by centrifugation at 10000 x g, followed by washing twice with buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4). The restoration of the phospholipid content was obtained using the individual phospholipids, according to the method of Scarpa and Azzone (1969). Phospholipid-depleted mitochondria corresponding to 20 mg mitochondrial protein, were incubated at 0^o in a medium containing, in 2 ml: 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 50 mg (lecithin), or 25 mg (cardiolipin), or 50 mg (asolectin), or 25 mg (phosphatidylethanolamine), or 25 mg (phosphatidyl-inositol); after an incubation time of 10 min, the mitochondria were sedimented at 10000 x g and washed twice with the sucrose-Tris medium at 0^o in order to remove the unbound phospholipid present in the supernatant.

The binding experiments were carried out essentially as described by Scarpa and Azzone (1969), in 2 ml of medium (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 at 0^o). The added mitochondria (1 mg protein) were preincubated for 5 min in the presence of 5 µM rotenone and 1 µg antimycin A, and incubated for 5 min in the presence of labelled calcium; the mitochondria were sedimented at 10000 x g for 15 min. The pellets were washed twice in cold sucrose and the radioactivity in the pellets was measured.

Measurement of radioactivity

^{45}Ca in a Triton X-100/toluene/butyl PBD scintillation mixture was counted using a Packard Tricarb Scintillation counter, as described in Chapter 2 (Patterson and Green, 1965).

4.3 RESULTS

Effect of pH on calcium transport

As is shown in Fig. 4.3, it is clear that the calcium transport increases as the pH increases from pH 6.5 to 7.5. The results indicate that there is little significant variation in V (nmol/min/mg protein) with pH values between 7.5 and 9.5. The results of the present study are in agreement with the findings of Reed and Bygrave (1975a), who showed that the rate constant for calcium binding is largest at high pH. Conversely, the rate constant for calcium release is highest at low pH.

ATP-supported calcium accumulation

The effect of ATP is known to promote the accumulation of calcium by isolated mitochondria (Carafoli and Rossi, 1971; Spencer and Bygrave, 1973; Brand and Lehninger, 1975; Jacobus et al., 1975; Lazarewicz and Hamberger, 1975; Sul et al., 1976). This is in agreement with the present work, shown in Fig. 4.4. It is clear from this figure that Rh6G (12.3 μM) abolishes the ATP-stimulated calcium accumulating. Jacobus et al. (1975) showed that mitochondria have the ability to transport calcium and that the process could be energized by either the activity of the respiratory chain or the hydrolysis of ATP.

Factors affecting respiration driven calcium uptake by mitochondria

The K_m value of calcium uptake (Fig. 4.5) was found to be 13 μM which is slightly more than the range (1–9 μM) determined by previous workers (Carafoli and Azzi, 1972; Spencer and Bygrave, 1973; Reynafarje and Lehninger, 1973; Carafoli et al., 1975b). The difference may be due to the variations in the experimental methods.

The results shown in Fig. 4.6 indicate that ruthenium red is a powerful inhibitor of mitochondrial calcium uptake, in agreement with the findings of several workers (Binet and Volfin, 1975a; Carafoli, 1975a; Sordal and Silver, 1975). Ruthenium red at a concentration of 9 nmol/mg caused an increase of the K_m value of calcium uptake nearly sixty times, as shown by double reciprocal plots (Fig. 4.5).

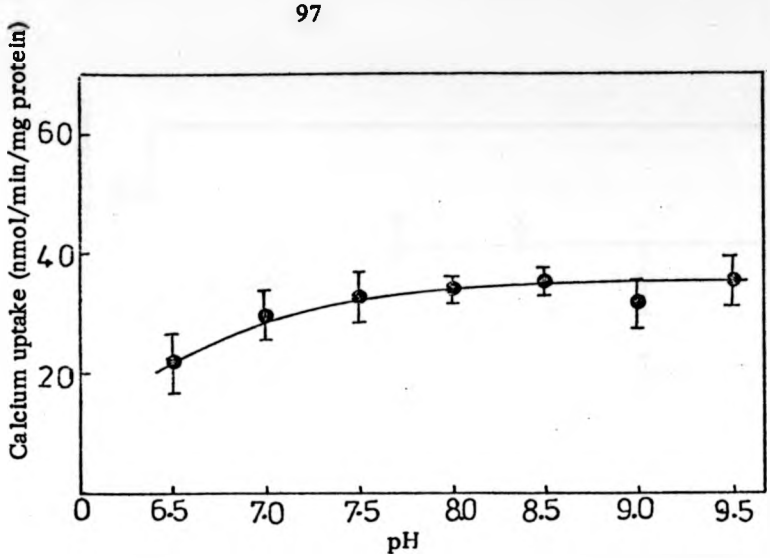


Figure 4.3 Effect of pH on calcium uptake

The experimental conditions are essentially those described by Reed and Bygrave (1975b). Mitochondria (1 mg protein) were incubated in 2 ml of 0.25 M sucrose, 2 mM Hepes, 2 mM succinate. The pH was adjusted with Tris. Temperature, 0^{o}. $^{45}CaCl_2$ was added to a final concentration of 200 μM. The incubation lasted for 15 sec and was stopped by 1 ml of incubation medium supplemented with 2 mM EGTA and 6 μM ruthenium red; after 5 min the mitochondria separated by Millipore filtration (as described in Chapter 2), followed by washing with 3 ml of incubation medium. The results shown are the mean ± S.D. of three experiments.

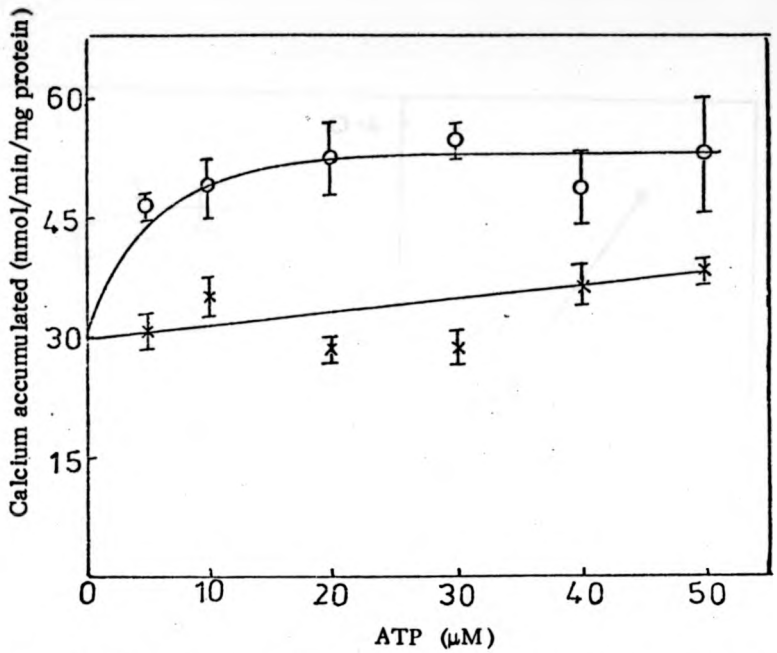


Figure 4.4 Rh6G inhibition of ATP stimulation of calcium accumulation

Experimental conditions are essentially those described by Spencer and Bygrave (1973). Mitochondria (1 mg protein) were incubated in 2 ml of 0.25 M sucrose, 0.5 μg antimycin A, 2 mM Hepes-Tris, pH 7.4 for 30 sec at 25° with or without Rh6G, followed by addition of ATP and $^{45}Ca^{++}$. $^{45}Ca^{++}$ added to a final concentration of 200 μM. The incubation lasted for 30 sec and was stopped by rapid Millipore filtration, followed by washing with 2 ml of incubation medium.

Results expressed as mean of four experiments ± S.D.

O——O , no Rh6G

X—— X, + 12.3 μM Rh6G

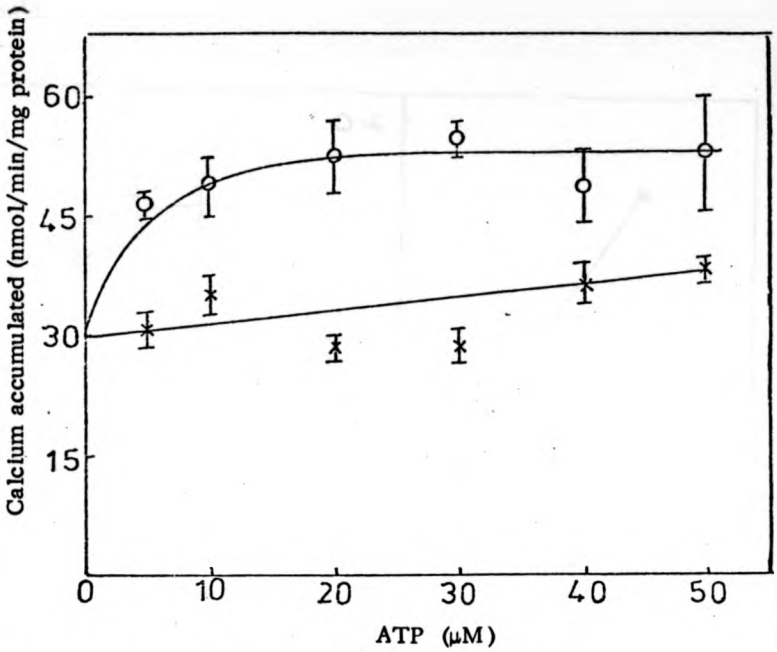


Figure 4.4 Rh6G inhibition of ATP stimulation of calcium accumulation

Experimental conditions are essentially those described by Spencer and Bygrave (1973). Mitochondria (1 mg protein) were incubated in 2 ml of 0.25 M sucrose, 0.5 μg antimycin A, 2 mM Hepes-Tris, pH 7.4 for 30 sec at 25° with or without Rh6G, followed by addition of ATP and $^{45}Ca^{++}$. $^{45}Ca^{++}$ added to a final concentration of 200 μM. The incubation lasted for 30 sec and was stopped by rapid Millipore filtration, followed by washing with 2 ml of incubation medium.

Results expressed as mean of four experiments ± S.D.

O——O, no Rh6G

X——X, + 12.3 μM Rh6G

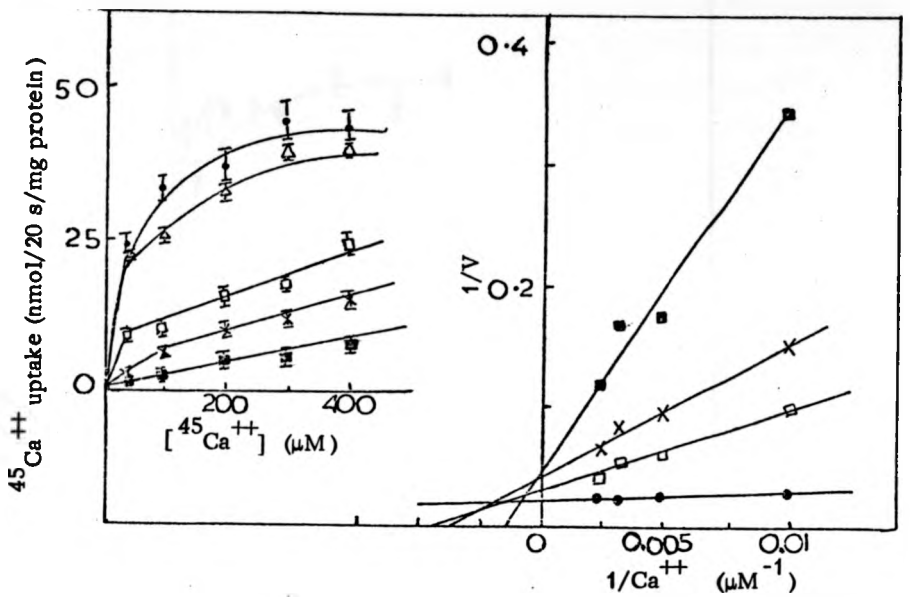


Figure 4.5 Effect of ruthenium red, DNP and HCP on calcium uptake by mitochondria

The experimental conditions as described in Methods. Results are mean ± S.D. of four experiments.

● ——● , no inhibitor, K_m (Ca^{++}) = 13 μM

■ ——■ , +9 nmol/mg, ruthenium red, K_m (Ca^{++}) = 800 μM

X —— X, +0.2 μmol/mg, DNP, K_m (Ca^{++}) = 285 μM

□ ——□, +0.74 nmol/mg, HCP, K_m (Ca^{++}) = 250 μM

△ ——△, +0.24 nmol/mg, HCP

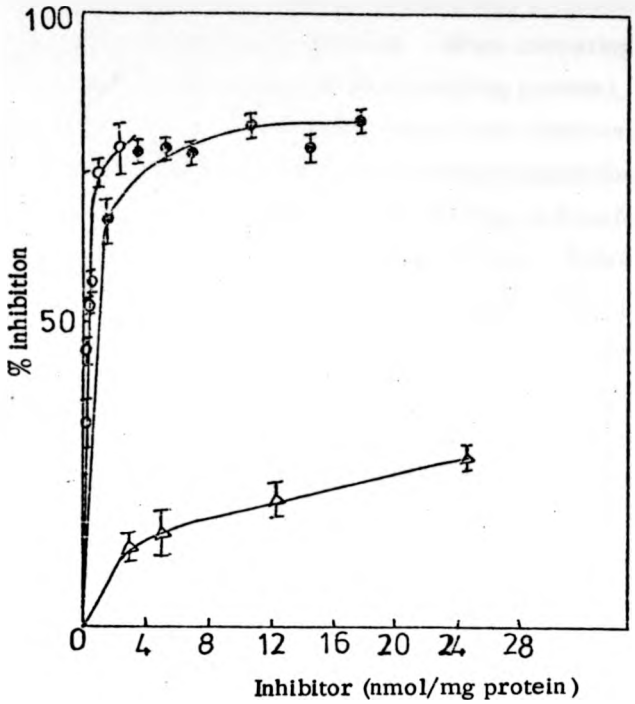


Figure 4.6 Inhibition of $^{45}Ca^{++}$ uptake by mitochondria

The same conditions as in Fig. 4.3.

The inhibitors incubated for 5 min with mitochondria at 0^{o} before the addition of $^{45}Ca^{++}$. pH 7.4.

O ——— O, + HCP; ● ——— ●, + ruthenium red; △ ——— △, + Rh6G.

In addition, Rh6G (48 nmol/mg protein) caused 65.1 ± 6.7% inhibition of calcium uptake.

The results are expressed as mean ± S.D. of four experiments.

Figures 4.5 and 4.6 show that HCP is a potent inhibitor of calcium uptake in mitochondria. HCP (0.74 nmol/mg) increased the K_m value of calcium uptake by nearly 20 times. When comparing the effect of HCP (0.74 nmol) with that of DNP (0.2 μmol/mg protein), (Fig. 4.5), it is apparent that HCP is nearly 300 times more effective than DNP. The inhibitory effect of uncouplers on calcium uptake, found in the present study, confirmed the previous findings of Renafarje and Lehninger (1974) and Reed and Bygrave (1974b). Rh6G (12 nmol/mg protein) caused 20% inhibition of calcium uptake (Fig. 4.6). However, with higher concentration (48 nmol/mg protein) it caused 65% inhibition. The inhibition of Ca^{++} uptake by higher concentrations of Rh6G may be due to a Rh6G uncoupling effect, since Rh6G in higher concentrations uncouples oxidative phosphorylation (Gear, 1974).

Figure 4.7 shows that both DBCT and TET are similar to ruthenium red; DNP and HCP are uncompetitive inhibitors of calcium uptake since they affect both V_{max} (nmol/20 sec/mg protein) and K_m. DBCT (31 nmol/mg) and TET (40 nmol/mg) increased the K_m value of calcium uptake 13 times.

Maintenance of accumulated calcium in mitochondria

Table 4.1 shows that after 3 min of addition of 10 mM Pi and calcium, there is a great release of mitochondrial calcium in agreement with the results of Carafoli and Rossi (1971). Table 4.1 also shows that the addition of HCP, 1799, Rh6G, DBCT, leucinostatin or X-537A to mitochondria 3 min after the addition of calcium, caused a release of mitochondrial calcium. A similar finding has been reported by Carafoli and Rossi (1971), Vallieres et al. (1975) and Luthra and Olson, (1976). The release of mitochondrial calcium by leucinostatin and X-537A, may be due to the release of mitochondrial Mg^{++}, as was shown by Binet and Volfin (1975b) that the addition of an ionophorous antibiotic (A 23187) to Ca^{++}-loaded mitochondria induces a fast release of Mg^{++} followed by the efflux of Ca^{++}.

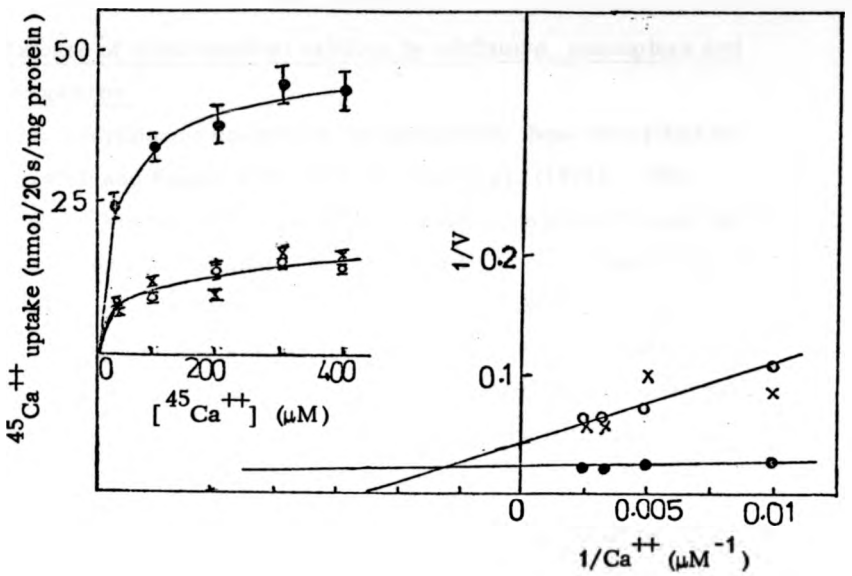


Figure 4.7 Effect of DBCT and TET on calcium uptake

The same conditions as described in Methods.

● ——● , no inhibitor, K_m (Ca^{++}) = 13 μM

O —— O, + 31 nmol/mg, DBCT, K_m (Ca^{++}) = 158

X —— X, + 40 nmol/mg, TET, K_m (Ca^{++}) = 158

The results are expressed as mean ± S.D. of four experiments.

TABLE 4.1

Release of mitochondrial calcium by inhibitors, uncouplers and ionophores

The experimental conditions are essentially those described by Carafoli and Rossi (1971) and Carafoli et al. (1976). The reaction medium (25°, 2 ml final volume) contained 80 mM NaCl, 10 mM sodium succinate, 10 mM Tris-HCl, pH 7.2 and 1 mg of mitochondrial protein. At zero time, 300 μM $^{45}Ca^{++}$ was added, and the uptake was allowed to proceed for 3 min. 15 sec later, the compounds listed below were added. After a further 15 sec, the mitochondria were separated by Millipore filtration. The results are mean ± S.D. of four experiments.

Inhibitor	Concentrations of inhibitors	$^{45}Ca^{++}$ in mitochondria nmole/mg/protein
–	–	60.0 ± 5.2
* Pi	20 μmole/mg protein	22.3 ± 2.4
HCP	2.46 nmole/mg protein	23.0 ± 3.0
Rh6G	12.4 nmole/mg protein	31.0 ± 2.7
DBCT	15.4 nmole/mg protein	11.0 ± 1.0
1799	12.8 nmole/mg protein	11.0 ± 1.2
Leucinostatin	3.2 nmole/mg protein	20.0 ± 1.7
X-537A	8.5 nmole/mg protein	14.0 ± 2.1

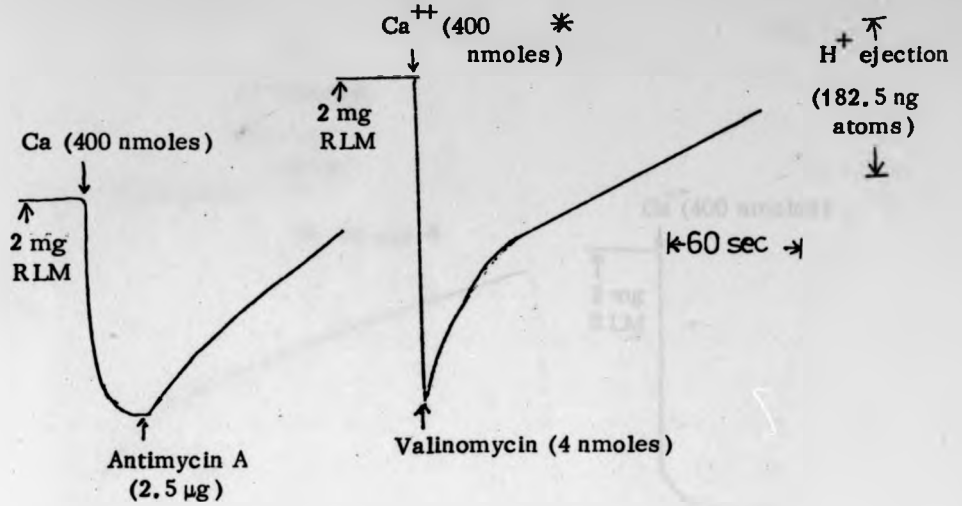
* Pi incubated at zero time with calcium.

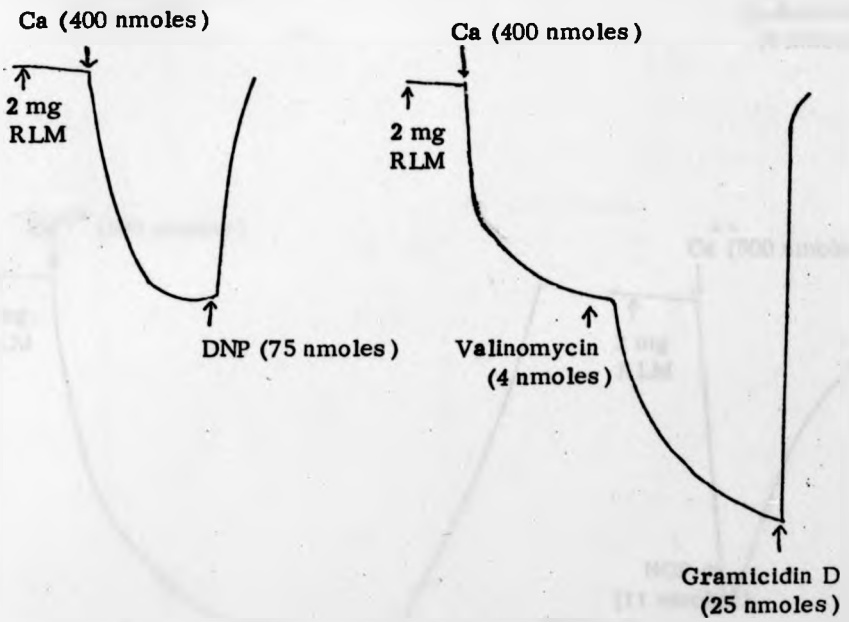
Figure 4.8 shows the results obtained by measuring the movement of protons in and out of mitochondria, with an expanded scale pH meter, to further elucidate the effect of HCP, ruthenium red, DBCT, TET, uncouplers, ionophorous antibiotics and respiratory inhibitors on calcium release. It was shown that the ejection of calcium induced by uncouplers is complete, and is at least as rapid as the uptake after membrane loading. HCP, DBCT and TET caused a release of calcium in a way similar to DNP. Drahota et al. (1965) and Carafoli (1974a) showed that, if the energy flow is interrupted by uncouplers, the accumulated calcium leaves mitochondria down its concentration gradient. In addition to uncouplers, a number of agents capable of inducing rapid release of calcium from mitochondria have been described, among them respiratory inhibitors (antimycin), ionophorous antibiotics (valinomycin), and, under some circumstances, ruthenium red. It is noteworthy that ruthenium red induces release only if added while calcium is being transported to its binding sites in the inner membrane (Rossi et al., 1973; Carafoli, 1974a). No release is observed if ruthenium red is added after the energy-linked accumulation of calcium has been completed (Fig. 4.8). The present results on the effect of ruthenium red are in agreement with those of Carafoli (1974a).

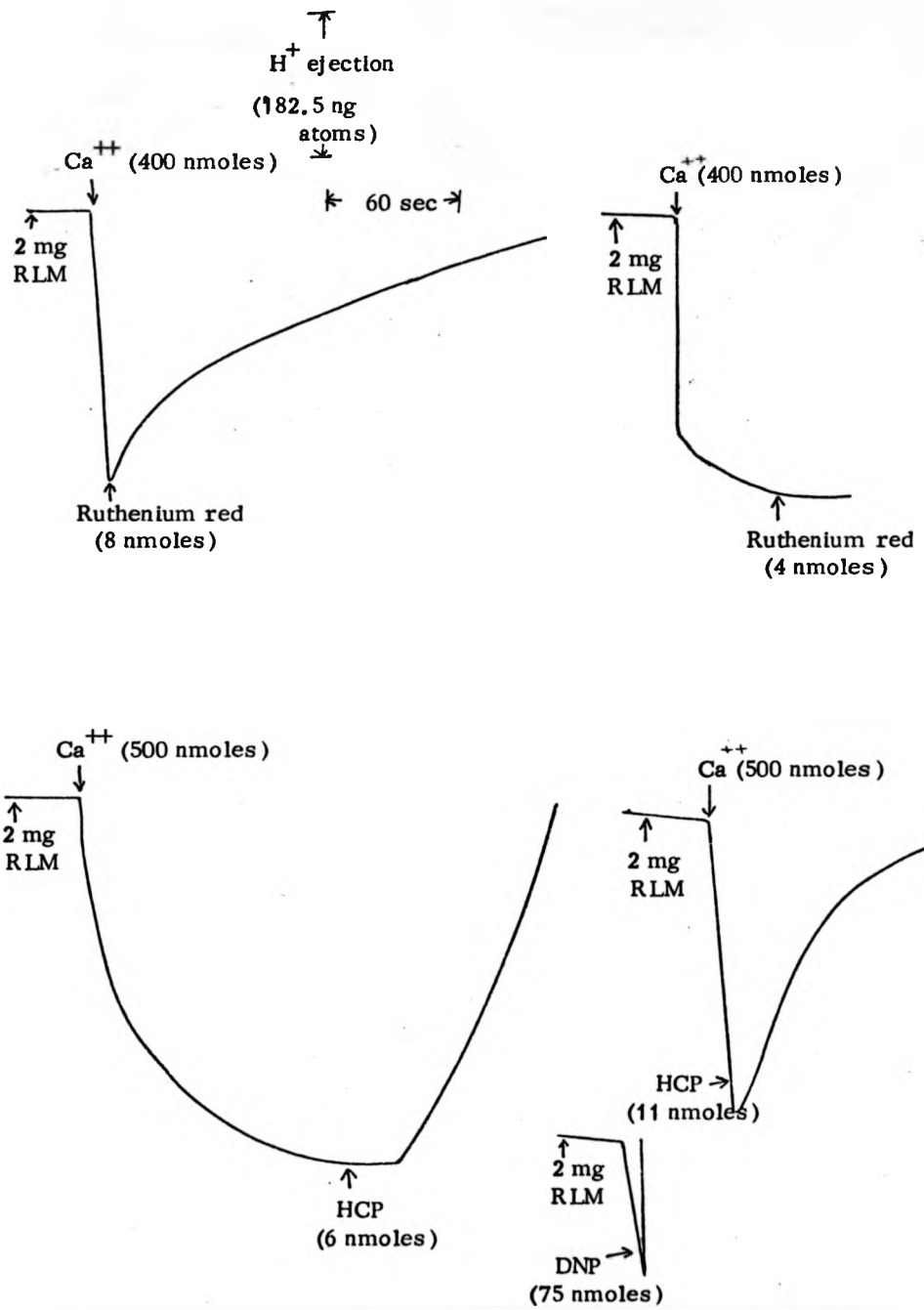
Figure 4.8 shows the results obtained by measuring the movement of protons in and out of mitochondria, with an expanded scale pH meter, to further elucidate the effect of HCP, ruthenium red, DBCT, TET, uncouplers, ionophorous antibiotics and respiratory inhibitors on calcium release. It was shown that the ejection of calcium induced by uncouplers is complete, and is at least as rapid as the uptake after membrane loading. HCP, DBCT and TET caused a release of calcium in a way similar to DNP. Drahota et al. (1965) and Carafoli (1974a) showed that, if the energy flow is interrupted by uncouplers, the accumulated calcium leaves mitochondria down its concentration gradient. In addition to uncouplers, a number of agents capable of inducing rapid release of calcium from mitochondria have been described, among them respiratory inhibitors (antimycin), ionophorous antibiotics (valinomycin), and, under some circumstances, ruthenium red. It is noteworthy that ruthenium red induces release only if added while calcium is being transported to its binding sites in the inner membrane (Rossi et al., 1973; Carafoli, 1974a). No release is observed if ruthenium red is added after the energy-linked accumulation of calcium has been completed (Fig. 4.8). The present results on the effect of ruthenium red are in agreement with those of Carafoli (1974a).

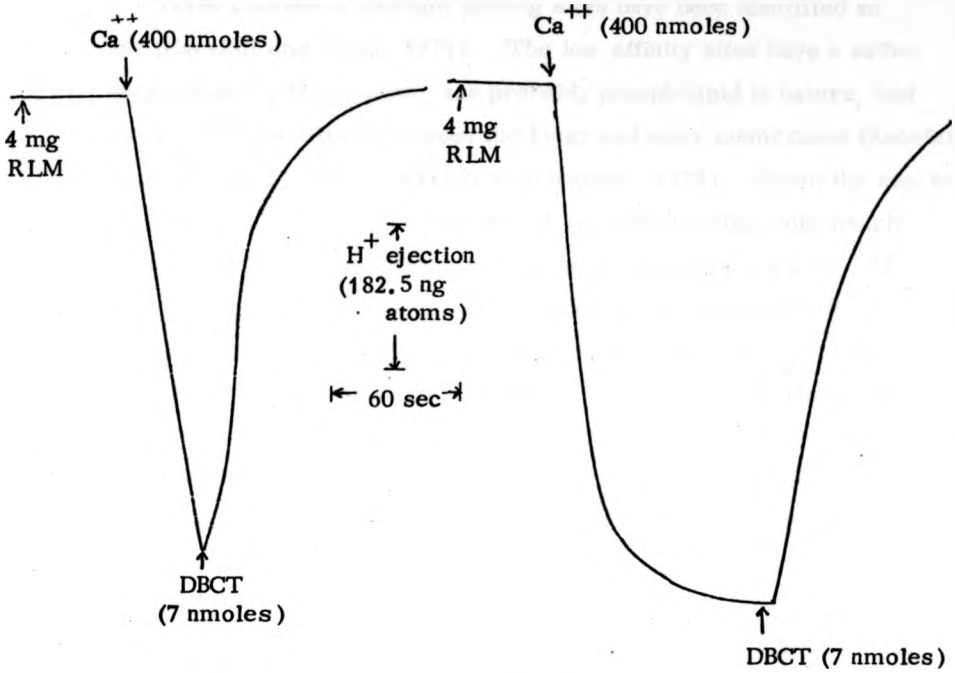
Figure 4.8 Maintenance of accumulated Ca^{++} in mitochondria.

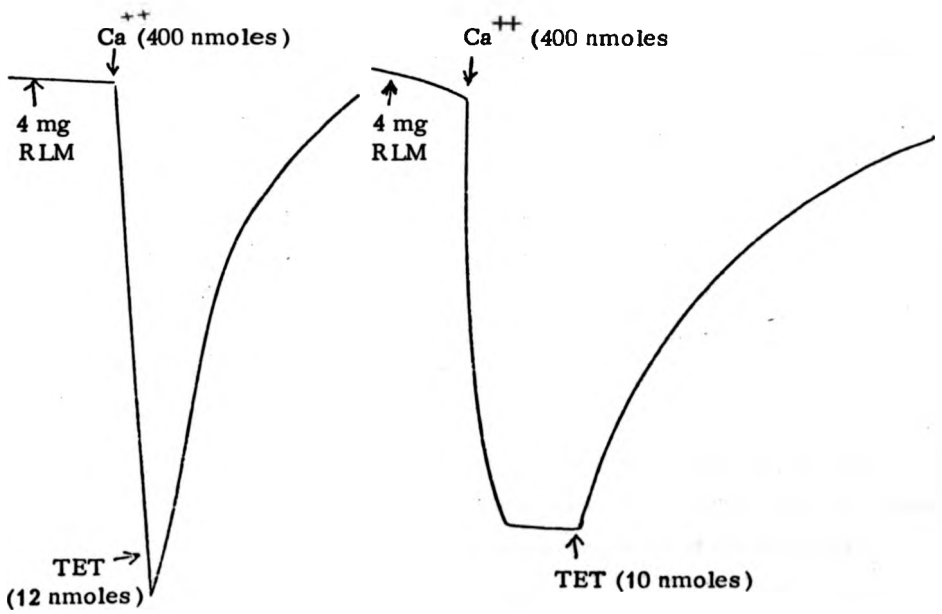
The experimental conditions are essentially those described by Carafoli (1975b). Effect of respiratory inhibitors, uncouplers, ionophorous antibiotics, DBCT, TET, ruthenium red and HCP. The movements of Ca^{++} were followed indirectly by measuring the opposite movements of protons in and out of mitochondria with an expanded scale pH meter. The reaction medium for experiments contained 80 mM NaCl, 2 mM Tris-HCl, pH 7.4, 5 mM sodium succinate and 2-4 mg of mitochondrial protein in a final volume of 1.8 ml, at 25°. In the valinomycin experiment (*), NaCl and sodium succinate were replaced by KCl and potassium succinate respectively. The K^+-specific ionophore valinomycin induced release only in a K^+-containing medium, whereas the ionophore gramicidin was equally active in media containing either Na^+ or K^+.


Ca (400 nmoles)
2 mg RLM
Antimycin A (2.5 µg)
Ca++ (400 nmoles)
2 mg RLM
Valinomycin (4 nmoles)
H+ ejection (182.5 ng atoms)
60 sec


Ca (400 nmoles)
2 mg RLM
DNP (75 nmoles)
Ca (400 nmoles)
2 mg RLM
Valinomycin (4 nmoles)
Gramicidin D (25 nmoles)


H+ ejection
(182.5 ng atoms)
Ca++ (400 nmoles)
60 sec
2 mg RLM
Ruthenium red (8 nmoles)
Ca++ (400 nmoles)
2 mg RLM
Ruthenium red (4 nmoles)
Ca++ (500 nmoles)
2 mg RLM
HCP (6 nmoles)
Ca++ (500 nmoles)
2 mg RLM
HCP (11 nmoles)
2 mg RLM
DNP (75 nmoles)


Ca++ (400 nmoles)
4 mg RLM
DBCT (7 nmoles)
H+ ejection (182.5 ng atoms)
60 sec
Ca++ (400 nmoles)
4 mg RLM
DBCT (7 nmoles)


Ca++ (400 nmoles)
4 mg RLM
TET (12 nmoles)
Ca++ (400 nmoles
4 mg RLM
TET (10 nmoles)

Calcium binding by mitochondria

Three classes of calcium binding sites have been identified so far (Carafoli and Rossi, 1971). The low affinity sites have a rather large capacity for calcium, are probably phospholipid in nature, and are equally distributed between the inner and outer membranes (Renafarje and Lehninger, 1969; Carafoli and Gazzotti, 1973). From the results expressed in Fig. 4.9, the number of low affinity sites were nearly equal to 30 nmole/mg protein and the value of K_d was 4.4×10^{-5} M, this confirming the previous results found by Rossi et al (1967), Scarpa and Azzi (1968) and Carafoli and Lehninger (1971). The results of the present study show that the number of high affinity site are 1.6 nmole/mg protein and that $K_d = 3.5 \times 10^{-6}$ M, within the range reported previously (0.5-2 nmoles of Ca^{2+}/mg protein; K_d = 0.1-1 μM), (Lehninger, 1969; Renafarje and Lehninger, 1969; Carafoli and Sottocasa, 1974). A third class of inner membrane sites, specifically involved in the oxidation of α-glycerophosphate by the mitochondrial α-glycerophosphate dehydrogenase (Carafoli and Sacktor, 1972) is probably not involved in the process of transmembrane calcium translocation.

Figure 4.9 shows that both ruthenium red and HCP abolished the high affinity site of calcium binding, and that ruthenium red (9 nmol/mg) has a slight effect on the low affinity site. However, HCP (1.28 nmol/mg) decreased the number of low affinity site but had no effect on the K_d. The inhibition of calcium binding affinity by ruthenium red found in the present study are in agreement with the findings of Carafoli (1974b), Carafoli et al. (1974) and Carafoli and Sottocasa (1974). The effect of DBCT and TET on the calcium binding is shown in Fig. 4.10. It is clear that both DBCT (62 nmol/mg) and TET (78 nmol/mg) have no effect on the K_d; however, both inhibitors caused a decrease in the number of high and low affinity sites. The number of high affinity site decreased from 1.6 to 0.8 nmol/mg protein in the presence of the inhibitors.

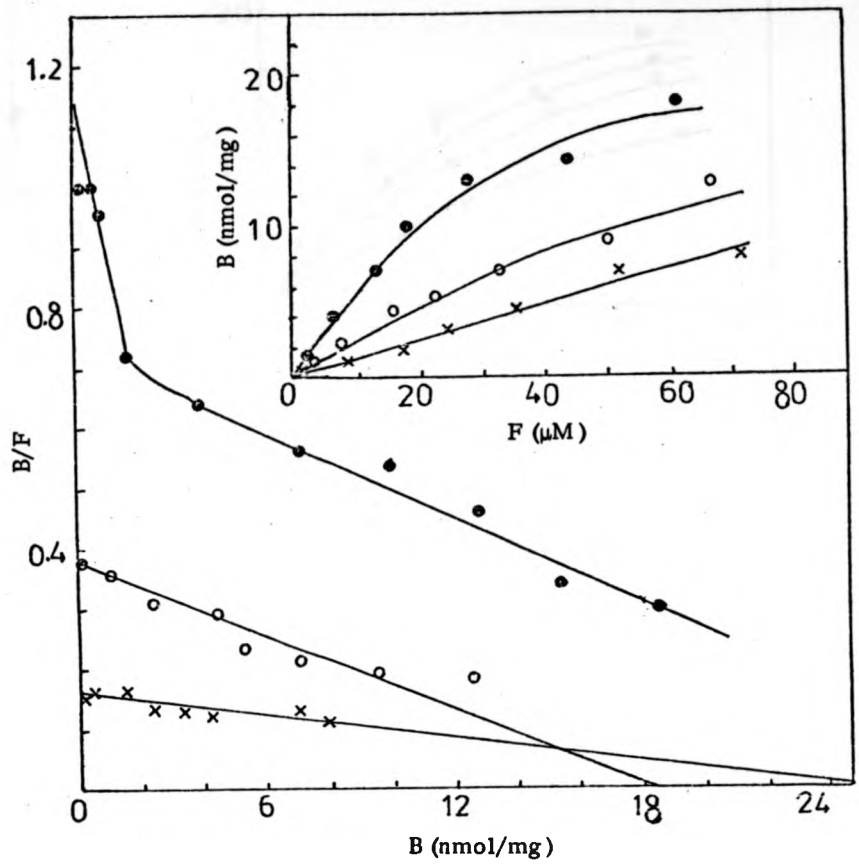


Figure 4.9 Effect of ruthenium red and HCP on $^{45}Ca^{++}$ binding.

The experimental conditions as described in Methods. The results are mean of five experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
●——●, no inhibitor	3.5×10^{-6}	1.6	4.4×10^{-5}	30.0
x——x, + 9 nmol/mg, ruthenium red	–	–	1.5×10^{-4}	25.0
o——o, + 1.28 nmol/mg HCP	–	–	5×10^{-5}	18.6

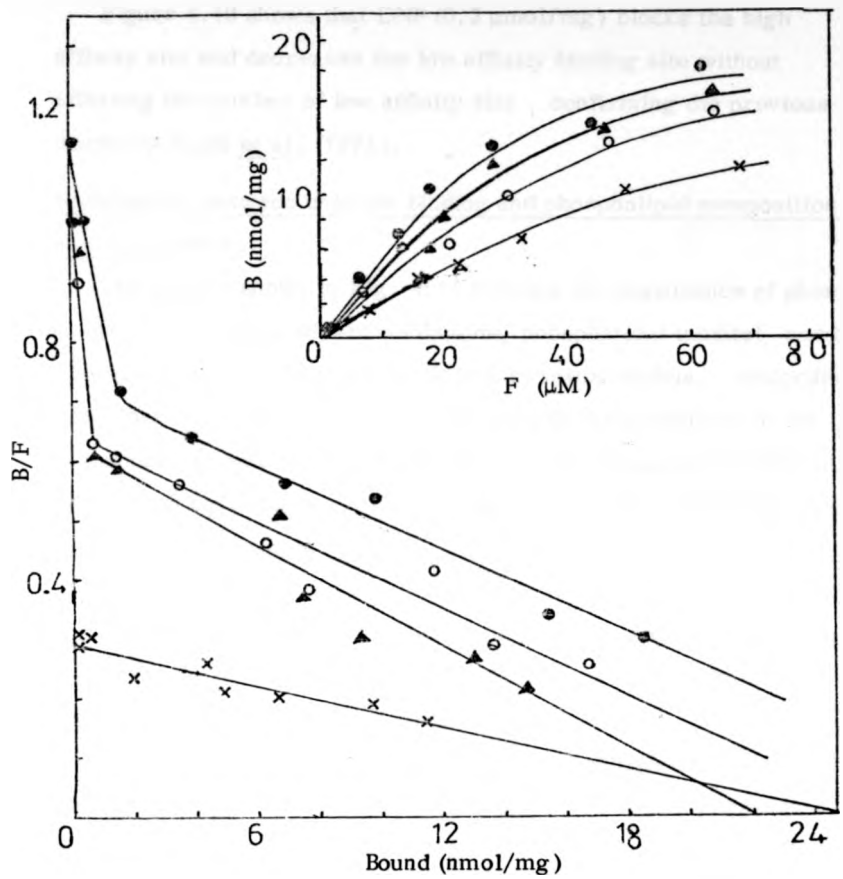


Figure 4.10 The effect of DBCT, TET and DNP on $^{45}Ca^{++}$ binding. The experimental conditions as described in Methods. The results are mean of five experiments.

	High affinity site		Low affinity site	
	K_d(M)	n(nmol/mg)	K_d(M)	n(nmol/mg)
●——●, no inhibitors	3.5×10^{-6}	1.6	4.4×10^{-5}	30.4
O——O, + 62 nmol/mg, DBCT	2×10^{-6}	0.8	4.0×10^{-5}	25.2
▲——▲, +78 nmol/mg, TET	2×10^{-6}	0.8	3.5×10^{-5}	21.2
X——X, +0.2 μmol/mg, DNP	–	–	1.25×10^{-4}	32.0

Figure 4.10 shows that DNP (0.2 μmol/mg) blocks the high affinity site and decreases the low affinity binding site without affecting the number of low affinity site , confirming the previous works by Rossi et al. (1974).

Relationship between calcium binding and phospholipid composition of mitochondria.

The results shown in Fig. 4.11 indicate the importance of phospholipids (e.g. phosphatidyl-ethanolamine, phosphatidyl-inositol, cardiolipin and asolectin) in calcium binding by mitochondria. According to Fleischer et al. (1967), the main phospholipid constituents of the mitochondrial membrane are lecithin, 43.3%; phosphatidyl-ethanolamine, 34.5% and cardiolipin, 17.2%. Minor components, including phosphatidyl-inositol, make up 4.9%.

Another essential feature of artificial and natural membranes is the capacity to bind cations. According to previous reports, this property appears to be mainly dependent on the presence of phospholipids (Rojas and Tobias, 1965; Slater and Cleland, 1953).

The results of experiments on the role of phospholipids in calcium binding are shown in Fig. 4.11. The depletion of phospholipids resulted in a decrease of about 66% in the mitochondrial capacity to bind calcium. When lecithin was added to the phospholipid-depleted mitochondria, there was a slight increase in the amount of calcium bound. Mitochondria reconstituted with other phospholipids, on the other hand, were able to bind more calcium than the untreated mitochondria, approximately 35% with asolectin, 42% with cardiolipin, 49% with phosphatidyl-inositol, whereas the amount of calcium bound was nearly 3 times higher in the case of phosphatidyl-ethanolamine than the untreated mitchondria. The calcium binding analysed above was independent of metabolism and occurred in the presence of rotenone and antimycin A. The above findings confirm those of Scarpa and Azzone (1969) except that cardiolipin caused a lot more increase in calcium binding in the present work, than reported in the latter paper.

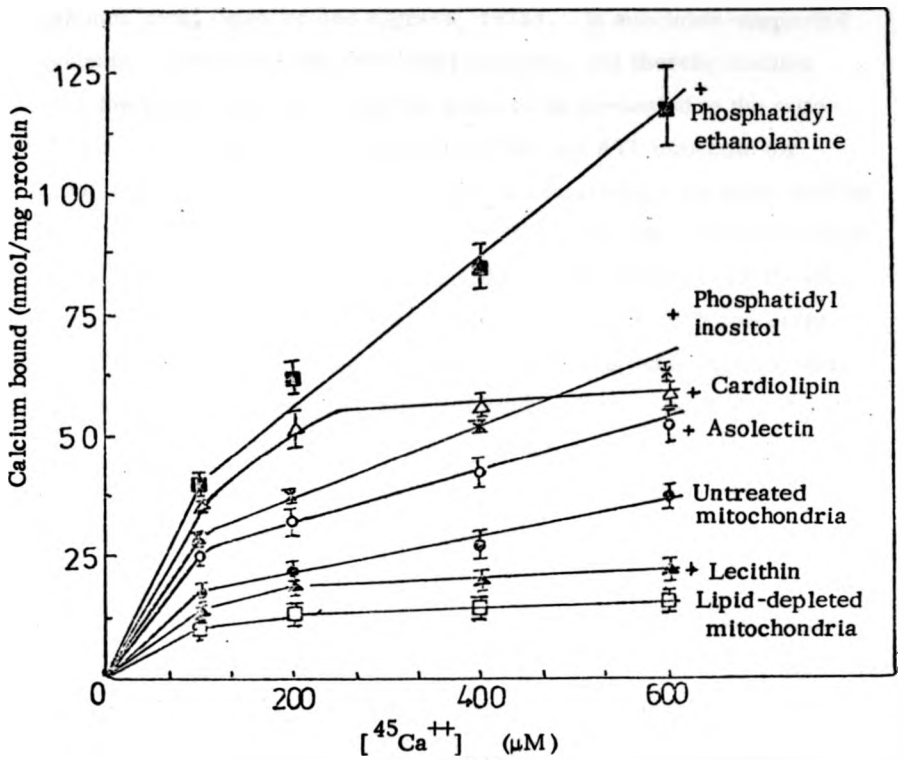


Figure 4.11 Relationship between Ca^{++} binding and phospholipid composition of mitochondria

The experimental conditions as described in Methods. The results are mean ± S.D. of three experiments.

4.4 DISCUSSION

ATP serves a dual function, that of being an ENERGY source and, by virtue of its strong ability to CHELATE calcium, that of creating a calcium 'sink'. (Spencer and Bygrave, 1973). In succinate-supported systems, ATP serves only the latter function, and thereby enables very low concentration of 'free' calcium to be presented to the mitochondria at any one time. It is known that one ATP molecule (or hypothetical high energy intermediate) is required for the accumulation of 2 Ca^{++} ions (Rossi and Lehninger, 1963). The inhibition of calcium accumulation caused by Rh6G (Fig. 4.4) is in agreement with the data of Gear (1974). The inhibition of calcium uptake by high concentration of Rh6G as well as its release when it is accumulated in mitochondria may be due to the uncoupling effect of Rh6G. It was shown that the uncoupler (1 μM, FCCP) caused rapid disappearance of ATP (Brand and Lehninger, 1975) and that when DNP was present, no stimulation of calcium accumulation by ATP was observed (Bielawski and Lehninger, 1966; Spencer and Bygrave, 1973).

In the opinion of the present worker, the inhibition of calcium uptake by HCP, DBCT and TET (Figs. 4.5, 4.6 and 4.7)may be due either to (a) the uncoupling effect of these uncompetitive inhibitors, it was shown that HCP (Caldwell et al., 1972) and TET in the chloride medium (Selwyn et al., 1970a and b) are uncouplers of oxidative phosphorylation, and it was known that the energy-linked uptake of calcium is inhibited by agents which dissipate the membrane potential generated by respiration, like the uncouplers of oxidative phosphorylation); or (b) to the probable effect of these inhibitors on mitochondrial phospholipids necessary for calcium binding, a preliminary and necessary step in the sequence of events that lead to its translocation across the inner mitochondrial membrane.

From the data presented (Fig. 4.11) it appears that the phospholipids play an important role in determining some physical properties of the mitochondria, e.g. the rate of ion diffusion and the binding of cations.

Among the effects of the various phospholipids, those of phosphatidyl-ethanolamine, which is a major component of mitochondrial phospho-lipid, were the most efficient in causing the restoration of osmotic and ion-binding properties of the mitochondria. The addition of phospho-lipids to phospholipid depleted mitochondria made it bind calcium more than untreated mitochondria, and this may be due to an increase in the low affinity binding site of calcium.

The information provided by the experiments (Fig. 4.8) with the release-inducing agents, e.g. uncouplers, ionophores, DBCT, TET, ruthenium red and HCP, has been important for the demonstration of the reversibility of the calcium-transport process, which is perhaps the most important parameter to be considered when discussing a role ot mitochondria in the biological regulation of calcium. In regard to the effect of leucinostatin and X-537A on calcium release, it was shown by Binet and Volfin (1975b) that the stability of the membrane is regulated by the presence of a low amount of a Mg^{++} bound in or on the surface of the membrane. If this Mg^{++} is released, by the means of the ionophore, the membranar Ca^{++} becomes labile and this process leads to the loss of the impermeability of the mitochondrial membrane.

It can be concluded from this work that HCP, TET and DBCT inhibit the calcium uptake in a way similar to that of the potent inhibitor of calcium transport (ruthenium red); however, HCP, TET and DBCT caused a release of accumulated mitochondrial calcium in a way similar to dinitrophenol; since the uncoupler can inhibit calcium uptake and binding. Thus, the effect of these compounds may be of the uncoupler type. The inhibition of ATP-supporting calcium accumulation by Rh6G may be due to inhibition of ATP hydrolysis which is required to energize the mitochondria for accumulation of calcium. However, the inhibition of calcium uptake by higher concentrations of Rh6G may be related to the Rh6G uncoupling effect

CHAPTER 5

THE ATP-^{32}Pi EXCHANGE REACTION

5.1 INTRODUCTION

This chapter describes another reaction catalyzed by the mitochondrion, which is a partial reaction of oxidative phosphorylation, namely the exchange reaction between orthophosphate and the terminal phosphate of ATP which occurs in the absence of oxidation (Cooper and Lehninger, 1957). This reaction is designated as the ATP-^{32}Pi exchange. The ATP-^{32}Pi exchange was first observed in liver mitochondria (Boyer et al., 1956 and Swanson, 1956). Since the reaction is inhibited by 2, 4-dinitrophenol at concentrations which uncouple oxidative phosphorylation, it was concluded (Cooper and Lehninger, 1957) that the ATP-^{32}Pi exchange reaction is a reflection of the activity of a terminal transphosphorylation reaction involved in oxidative phosphorylation. The exchange reaction requires the preservation of an energized intermediate ($X \sim I$) and the liberation of Pi in the medium so that when the reaction is reversed, ^{32}Pi is incorporated into ATP. Breakdown of an intermediate in the energy transfer sequence leads to ATPase activity (Sanadi et al., 1968).

The chemical intermediate theory of oxidative phosphorylation originally proposed by Slater (1953) and subsequently extended is represented by Eq. 1.3.

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \qquad (1)$$

$$A \sim I + X \rightleftharpoons X \sim I + A \qquad (2)$$

$$X \sim I + ADP + P \rightleftharpoons X \sim I + ATP \qquad (3)$$

Eq. 3 has been further broken down into two steps

$$X \sim I + P \rightleftharpoons X \sim P + I \qquad (4)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \qquad (5)$$

where AH_2 and B are adjactent members of the respiratory chain and I is a hypothetical coupling factor.

The chemiosmotic hypothesis of Mitchell (1961) considers separation of H^+ and OH^- charges across the mitochondrial membrane, with a resulting development of membrane potential as the primary energy-conserving reaction. This potential then leads to the formation of $X \sim I$, the nonphosphorylated energy-rich intermediate. The conformation-coupling bypothesis, a variation of the chemical theory, also considers a nonphosphorylated energized state of the redox protein or membrane as an intermediate step (Mitchell et al., 1967; Green et al., 1968) in ATP formation.

The purpose of this chapter is to describe the effect of sulfobromophthalein (SBP), agaric acid (AA), hexachlorophene (HCP) and rhodamine 6G (Rh6G) on ATP-^{32}Pi exchange reaction and to compare the effect of these inhibitors with the effects of potent inhibitors and uncouplers of oxidative phosphorylation (Atr, oligomycin, venturicidin, TET, DCCD, FCCP, 1799, PCP or DNP) in both mitochondria and submitochondrial particles (SMP).

5.2 MATERIALS AND METHODS

Materials

^{32}Pi was obtained from the Radiochemical Centre, (Amersham, U.K.). Dithiothreitol (DTT) and oligomycin (mixture of oligomycin A, 15% and B, 85%) were obtained from Sigma Chemical Co. Ltd. Dicyclohexylcarbodiimide (DCCD) was obtained from the British Drug Houses, Ltd. Naphthalene (scintillation grade) was obtained from Fisons Scientific Apparatus, England. All other inhibitors and uncouplers of oxidative phosphorylation were obtained from the sources mentioned in Chapter 2. All other chemicals were of A.R. grade.

Methods

Preparation of mitochondria and submitochondrial particles

Preparation of mitochondria and submitochondrial particles (SMP) and the protein determination were carried out as described in Chapter 2.

Measurements of ATP-^{32}Pi exchange

The ATP-^{32}Pi exchange reaction was carried out according to the method of Fisher et al. (1971). 0.1 ml of mitochondrial or submitochondrial particles suspension containing 1 mg protein were added to 0.9 ml of buffer containing 0.25 M sucrose, 50 mM Tris-H_2SO_4 (pH 7.5), 10 mM potassium phosphate (pH 7.5) containing ^{32}Pi with 10^6 cpm, 10 mM $MgCl_2$, 1 mM dithiothreitol. The reaction was started by the addition of 10 mM ATP (pH 7.5) and the mixture was incubated at 30° for 10 min unless otherwise indicated. The reaction was terminated by the addition of 0.5 ml of 20% trichloroacetic acid. ^{32}Pi incorporated and Pi in the medium were determined as described by Andreoli et al. (1965). After centrifugation, to 1 ml of the supernatant fluid was added 0.1 ml bromine water, 1.5 ml of 2-5% ammonium-molybdate in 2.5 N H_2SO_4, 1 ml acetone, 10 ml isobutanol-benzene (1:1), water saturated, and the mixture shaken for 10 sec. After allowing the liquid phases to separate by standing for 5 min, the upper isobutanol layer was removed by aspiration and discarded ; the lower phase being re-extracted twice with isobutanol (10 ml). Traces of isobutanol were removed by shaking the aqueous phase with 2 ml of ethyl ether, and removing this by aspiration. An aliquot (0.5 ml) of the aqueous phase was taken for determination of the radioactivity of the organically bound phosphate. The amount of phosphate esterified was calculated as the quotient of the total radioactivity of the organically bound phosphate (CPM) over the initial specific radioactivity (counts/min/μmole) of the orthophosphate. Phosphate was determined by the Fiske and Subbarow method (1925).

Counting

^{32}Pi was counted in a Packard Tricarb Scintillation counter in 5 ml of a dioxan based scintillation mixture, 60 g scintillation grade naphthalene, 4 g PPO, 0.1 g dimethyl POPOP and 1 litre 1,4-dioxan (Williams and Orr, 1976).

5.3 RESULTS

Rat liver mitochondria catalyze an extensive exchange of ^{32}Pi with the phosphate of ATP. The time course of this exchange reaction is shown in Fig. 5.1. The results show that the ATP-^{32}Pi exchange reaction continues in a nearly linear manner over the 15 min reaction period, which is in good agreement with the data on rat liver mitochondria Boyer et al. (1956) and on beef heart submitochondrial particles Conover et al. (1963).

The ATP-Pi exchange reaction occurs at significant and easily observable rates over a wide range of pH and concentrations of ATP and phosphate. Figure 5.2 demonstrates the effect of varying pH; it is seen that activity is shown over the whole range tested, with a peak at pH 7.5. Figures 5.3 and 5.4 demonstrate the effect of varying the concentration of ATP and phosphate. The K_m value for ATP in the exchange reaction is approximately 2.9 mM and that for phosphate approximately 2 mM.

The inhibition of the ATP-^{32}Pi exchange reaction , found in the present work, can be classified as follows:

(a) Inhibition of ATP-^{32}Pi exchange reaction in intact mitochondria. It is shown in Figs. 5.5 and 5.6 that Atr, SBP, HCP and agaric acid inhibit the exchange reaction in intact mitochondria, and this may be due to their property to inhibit adenine nucleotide translocase, as shown in Chapter 2. The results of the effect of Atr on the exchange reaction is in agreement with the findings of Vignais et al., (1961 and 1962), Groot et al., (1971), and Verdow and Bertina, (1973). The inhibition caused by Rh6G may be due to inhibition of oxidative phosphorylation in a way similar to oligomycin, venturicidin, TET or DCCD (Figs. 5.6 and 5.7). The present results on the inhibition of the exchange reaction by oligomycin are in agreement with the findings of Lardy et al. (1964), Kovac et al. (1968) and Boyer et al. (1975).

5.3 RESULTS

Rat liver mitochondria catalyze an extensive exchange of ^{32}Pi with the phosphate of ATP. The time course of this exchange reaction is shown in Fig. 5.1. The results show that the ATP-^{32}Pi exchange reaction continues in a nearly linear manner over the 15 min reaction period, which is in good agreement with the data on rat liver mitochondria Boyer et al. (1956) and on beef heart submitochondrial particles Conover et al. (1963).

The ATP-Pi exchange reaction occurs at significant and easily observable rates over a wide range of pH and concentrations of ATP and phosphate. Figure 5.2 demonstrates the effect of varying pH; it is seen that activity is shown over the whole range tested, with a peak at pH 7.5. Figures 5.3 and 5.4 demonstrate the effect of varying the concentration of ATP and phosphate. The K_m value for ATP in the exchange reaction is approximately 2.9 mM and that for phosphate approximately 2 mM.

The inhibition of the ATP-^{32}Pi exchange reaction, found in the present work, can be classified as follows:

(a) Inhibition of ATP-^{32}Pi exchange reaction in intact mitochondria. It is shown in Figs. 5.5 and 5.6 that Atr, SBP, HCP and agaric acid inhibit the exchange reaction in intact mitochondria, and this may be due to their property to inhibit adenine nucleotide translocase, as shown in Chapter 2. The results of the effect of Atr on the exchange reaction is in agreement with the findings of Vignais et al., (1961 and 1962), Groot et al., (1971), and Verdow and Bertina, (1973). The inhibition caused by Rh6G may be due to inhibition of oxidative phosphorylation in a way similar to oligomycin, venturicidin, TET or DCCD (Figs. 5.6 and 5.7). The present results on the inhibition of the exchange reaction by oligomycin are in agreement with the findings of Lardy et al. (1964), Kovac et al. (1968) and Boyer et al. (1975).

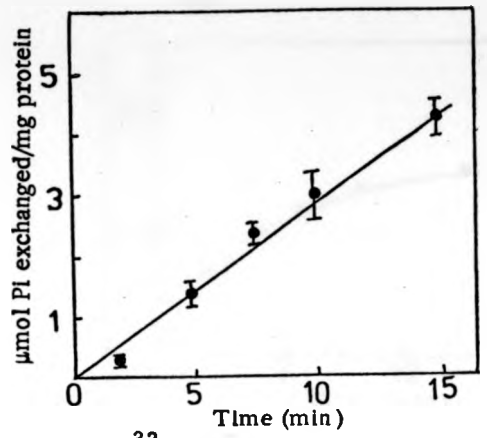


Figure 5.1 Rate of ATP-^{32}Pi exchange reaction

The test system contained 10 mM ATP, 10 mM ^{32}Pi, 1 mg mitochondrial protein, in a total volume of 1 ml; incubated at pH 7.5 at 30°. The results are expressed as mean of four experiments ± S.D. For detail of the experiment, see Method.

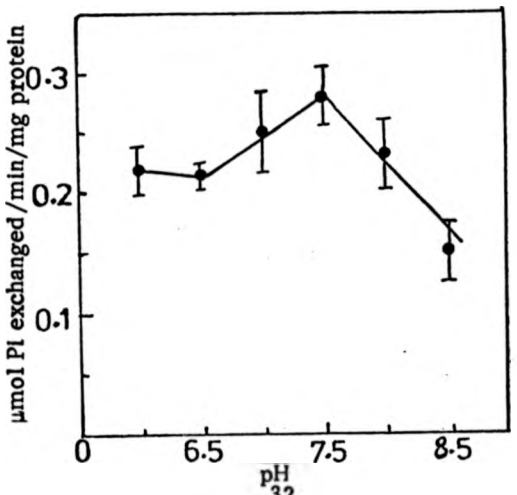


Figure 5.2 Effect of pH on ATP-^{32}Pi exchange reaction.

The test system contained in a final volume of 1 ml, 0.25 M sucrose, 50 mM Tris-H_2SO_4, 10 mM ^{32}Pi, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP and 1 mg of mitochondrial protein. pH adjusted with Tris-H_2SO_4. Incubated for 10 min at 30°. The results are expressed as mean of four experiments ± S.D.

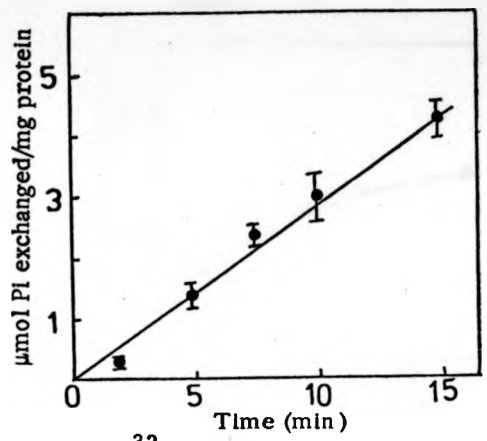


Figure 5.1 Rate of ATP-^{32}Pi exchange reaction

The test system contained 10 mM ATP, 10 mM ^{32}Pi, 1 mg mitochondrial protein, in a total volume of 1 ml, incubated at pH 7.5 at 30°. The results are expressed as mean of four experiments ± S.D. For detail of the experiment , see Method.

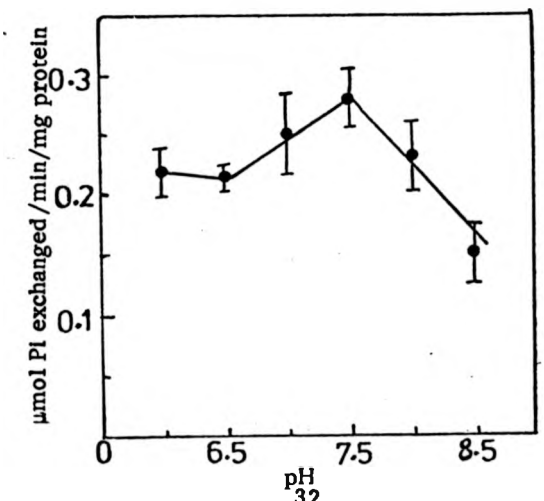


Figure 5.2 Effect of pH on ATP-^{32}Pi exchange reaction.

The test system contained in a final volume of 1 ml, 0.25 M sucrose, 50 mM Tris-H_2SO_4, 10 mM ^{32}Pi, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP and 1 mg of mitochondrial protein. pH adjusted with Tris-H_2SO_4. Incubated for 10 min at 30°. The results are expressed as mean of four experiments ± S.D.

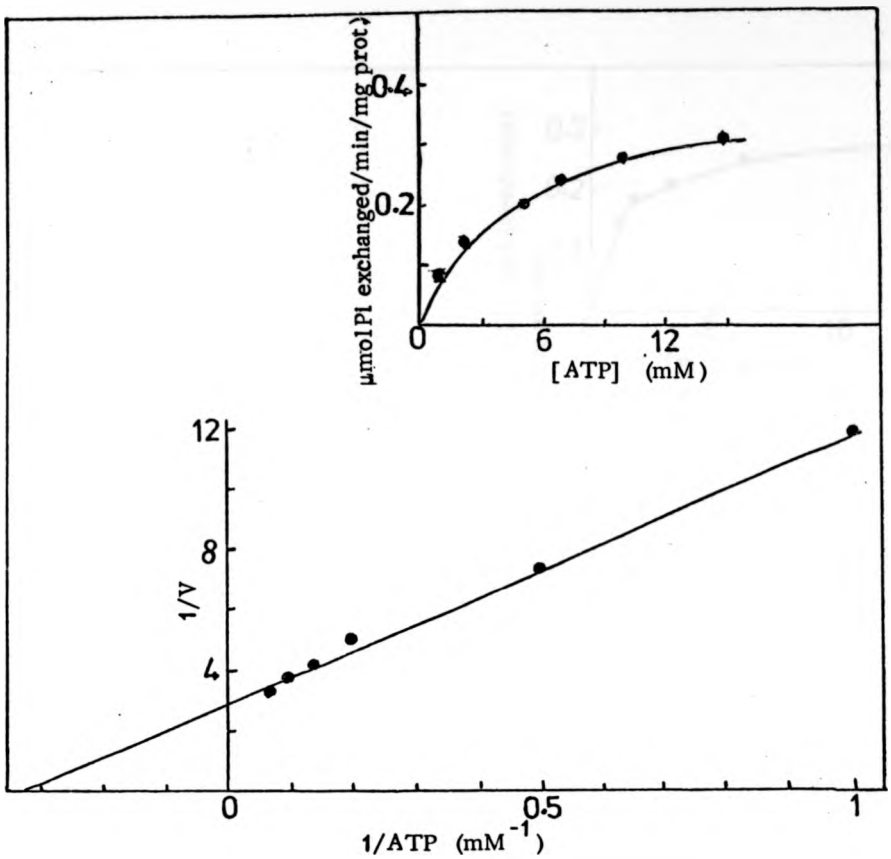


Figure 5.3 Effect of ATP concentrations on exchange reaction.
The system contained 10 mM ^{32}Pi, ATP at the concentrations shown and 1 mg mitochondrial protein in a total volume of 1 ml and was incubated at pH 7.5 at 30^{o} for 10 min. The results are expressed as a mean of four experiments. For detail, see Method.

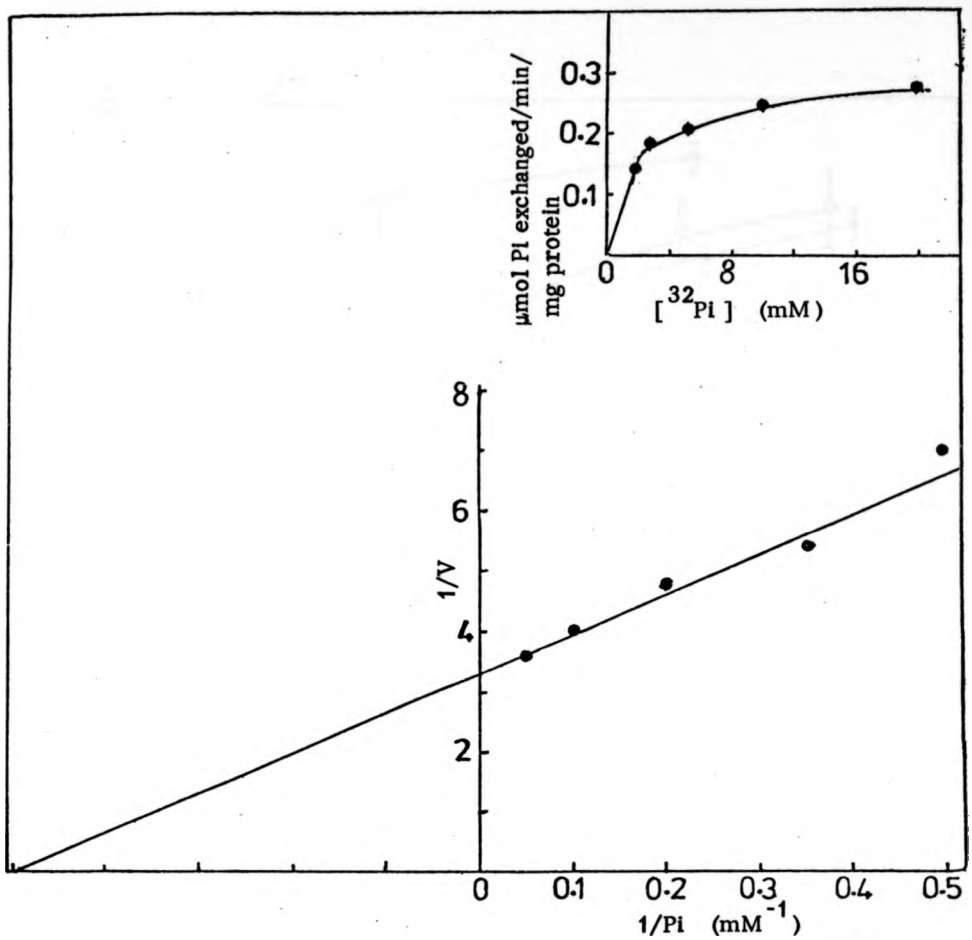


Figure 5.4 Effect of ^{32}Pi concentration on ATP-^{32}Pi exchange reaction

The test system contained 1 mg of mitochondrial protein, 10 mM ATP, phosphate in the concentration shown, final volume was 1 ml, and the incubation time 10 min at 30°; pH 7.5. The results are expressed as mean of four experiments.

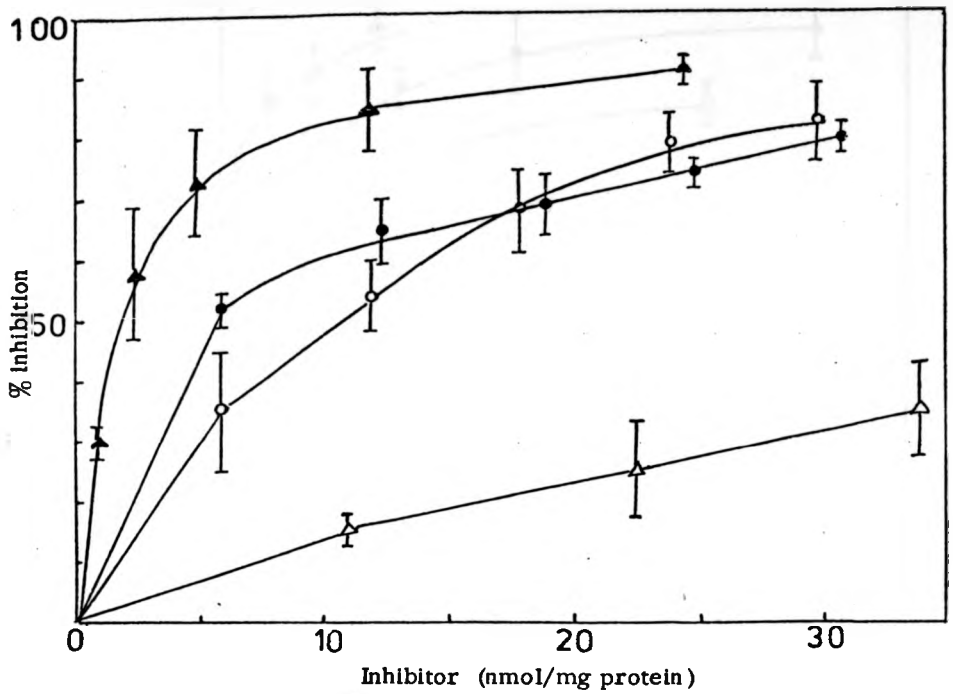


Figure 5.5 Effect of Atr, SBP, agaric acid and Rh6G on ATP-^{32}Pi exchange reaction.

The same conditions as in Fig. 5.2. The inhibitors incubated for 5 min with mitochondria on ice before initiating the reaction, with ^{32}Pi and ATP, pH 7.5. The results are expressed as a mean of four experiments ± S.D. The uninhibited results are in the range of 0.25-0.30 µmol Pi exchange/min/mg of mitochondrial protein.

▲——▲, + Rh6G

●——●, + Atractyloside

O——O, + SBP

△——△, + agaric acid

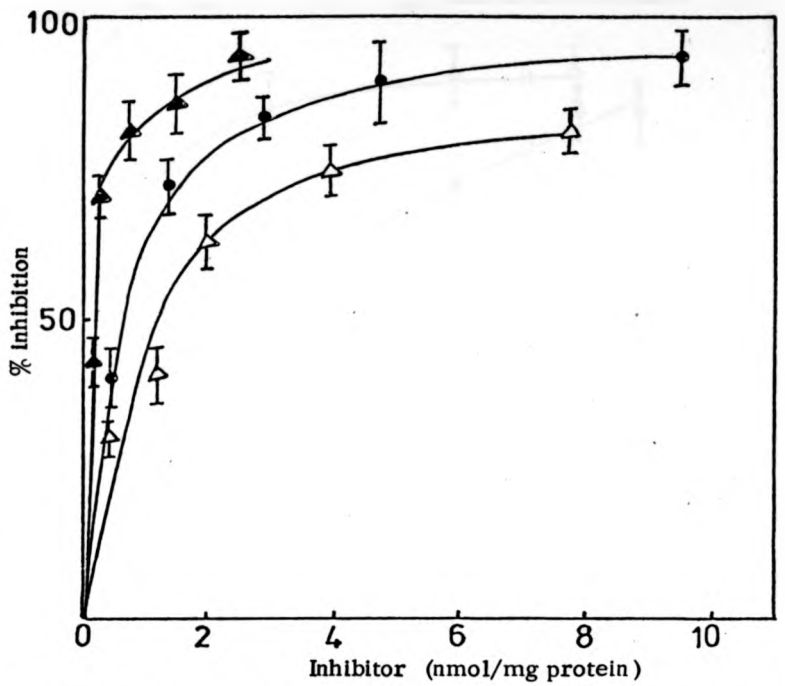


Figure 5.6 Effect of HCP, DCCD and TET on ATP-^{32}Pi exchange reaction

The same conditions as in Fig. 5.2. The inhibitors were incubated with 1 mg mitochondrial protein for 5 min on ice before initiating the reaction with ^{32}Pi and ATP, pH 7.5. The results expressed as mean of four experiments ± S.D.

The uninhibited results are in the range of 0.30-0.35 µmol Pi exchanged/min/mg of mitochondrial protein.

▲——▲, + HCP

●——●, + DCCD

△——△, + TET

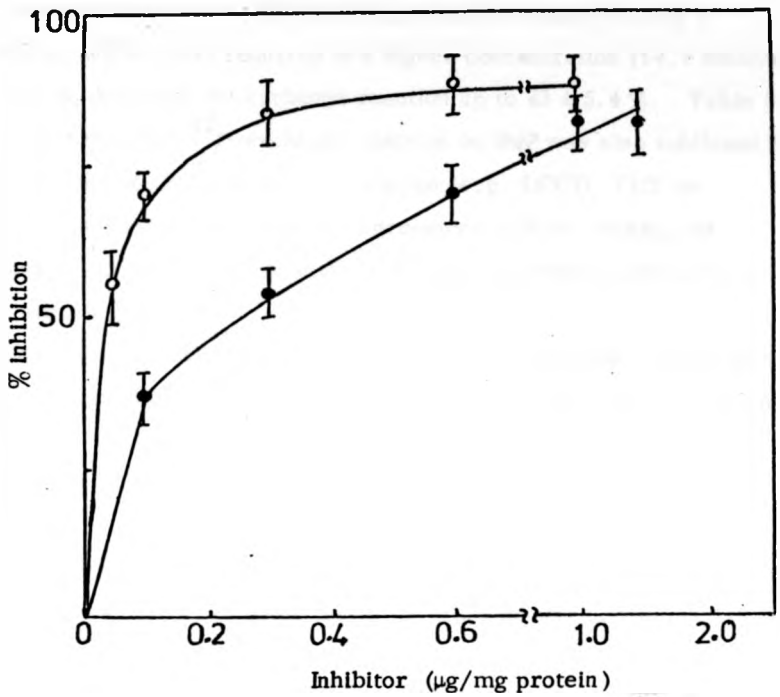


Figure 5.7 Effect of oligomycin and venturicidin on ATP-^{32}Pi exchange reaction

The same condition as in Fig. 5.2. The inhibitors incubated for 5 min with mitochondria before initiating the reaction with ^{32}Pi and ATP, pH 7.5. The results are expressed as mean of three experiments ± S.D. The uninhibited results are in the range of 0.25-0.30 μmol Pi exchanged/min/mg of mitochondrial protein.

O —— O, + oligomycin (mixture of A and B)

● —— ●, + venturicidin (mixture of A and B)

(b) Inhibition of ATP-^{32}Pi exchange reaction in submitochondrial particles. The effect of HCP and Rh6G are shown in Table 5.1. HCP with low concentration (0.25 nmol/mg protein) caused 54 ± 8% inhibition, while Rh6G required in a higher concentration (14.8 nmol/mg protein) to decrease the exchange reaction up to 43 ± 5.4 %. Table 5.1 shows that the ATP-^{32}Pi exchange reaction in SMP was also inhibited by the inhibitors of oxidative phosphorylation (e.g. DCCD, TET or venturicidin); these results are in agreement with the findings of Serrano et al. (1976) for reconstituted vesicles prepared from beef heart mitochondria.

(c) Inhibition of ATP-^{32}Pi exchange reaction in both intact mitochondria and submitochondrial particles. It is shown in Figs. 5.5, 5.6, 5.7 and 5.8, and in Table 5.1 that HCP, Rh6G, TET, DCCD and venturicidin inhibit the exchange reaction in both intact mitochondria and in submitochondrial particles.

The ATP-^{32}Pi exchange reaction is also inhibited by many reagents which uncouple oxidative phosphorylation (Cooper and Lehninger, 1956; Boyer et al., 1975; Hatefi et al., 1975). The data shown in Fig 5.8 and in Table 5.1 demonstrates that the exchange reaction in both intact mitochondria and in SMP was inhibited by low concentration of FCCP, 1799 and PCP. These findings are in agreement with the previous results reported in yeast mitochondria (Kovac et al., 1968; Groat et al., 1971) and in beef heart submitochondrial particles (Conover et al., 1963; Kagawa and Racker, 1971; Serrano et al., 1976).

TABLE 5.1

Inhibition of ATP-^{32}Pi exchange reaction in submitochondrial particles

Submitochondrial particles (1 mg protein) were incubated in 1 ml of 0.25 M sucrose, 50 mM Tris-H_2SO_4, pH 7.5, 10 mM ^{32}Pi, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP for 5 min on ice with inhibitors, before initiating the reaction with ^{32}Pi and ATP, at 30° for 10 min. The results are expressed as mean of three experiments ± S.D. The uninhibited results are in the range of 0.08- 0.09 µmol Pi exchanged/min/mg of submitochondrial particles protein.

Inhibitor	Concentration nmol/mg protein	% Inhibition
SBP	5	0
Agaric acid	5	0
Rh6G	4.9	26 ± 2.6
	14.8	43 ± 5.4
HCP	0.12	37 ± 5.8
	0.25	54 ± 8.0
DCCD	2.40	52 ± 6.0
	14.40	63 ± 6.4
TET	0.98	35 ± 7.4
	3.92	46 ± 3.3
*Venturicidin	0.50	46 ± 2.5
	3.00	58 ± 4.6
DNP	10.00	27 ± 4.0
	30.00	40 ± 4.1
FCCP	0.80	26 ± 4.5
	1.60	49 ± 4.0
1799	1.28	29 ± 2.2
	5.12	52 ± 3.0
PCP	1.00	35 ± 1.0
	5.00	43 ± 5.5

* Venturicidin concentration (µg/mg protein)

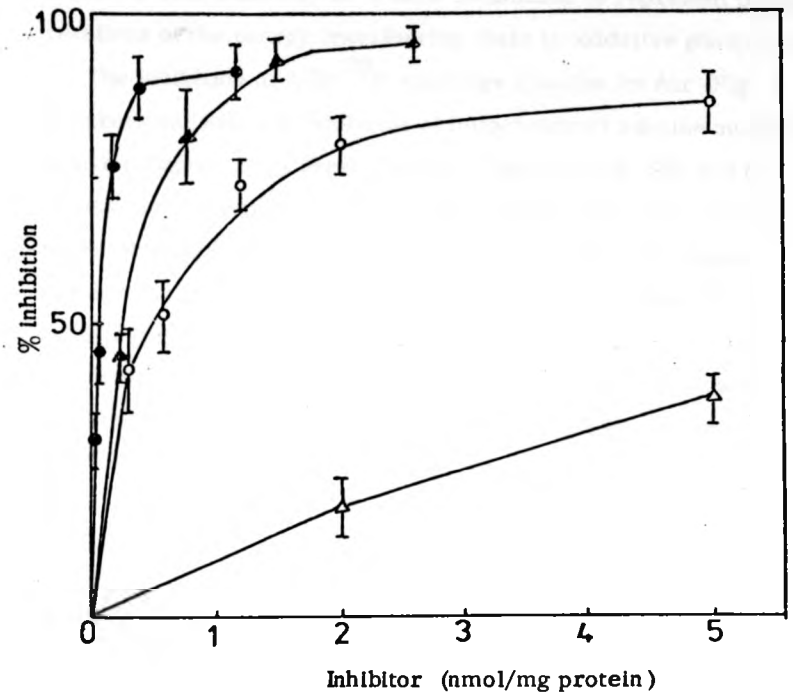


Figure 5.8 Effect of FCCP, 1799, PCP and DNP on ATP-^{32}Pi exchange reaction

The same conditions as in Fig. 5.2. pH 7.5. The results are expressed as mean of three experiments ± S.D. The uninhibited results are in the range of 0.25-0.31 μmol Pi exchanged/min/mg of mitochondrial protein.

●——●, + FCCP

▲——▲, + 1799

O——O, + PCP

△——△, + 2, 4-DNP

5.4 DISCUSSION

The ATP-^{32}Pi exchange reaction and the 2,4-dinitrophenol-induced ATPase activity have been considered to represent partial reactions of the energy transferring chain in oxidative phosphorylation.

The inhibition of ATP-^{32}Pi exchange reaction by Atr (Fig. 5.5) is in agreement with the inhibition of mitochondrial adenine nucleotide uptake (Chapter 2) by atractyloside. Agaric acid, SBP and HCP effects may be explained in the same manner, like Atr, since the three inhibitors, SBP, HCP and agaric acid inhibit the uptake of adenine nucleotide by mitochondria (Chapter 2). However, HCP also inhibits the ATP-^{32}Pi exchange reaction in submitochondrial particles and this may be due to the uncoupling effects of HCP (Caldwell et al., 1972; Cammer and Moore, 1972). On the other hand, the effect of Rh6G on the ATP-^{32}Pi exchange reaction (Fig. 5.5) may either be due to Rh6G uncoupling effect or to Rh6G behaving like the inhibitors of oxidative phosphorylation. The results of Gear (1974) demonstrate that low concentrations of Rh6G markedly reduce the rate of ATP formation, but at higher concentrations (> 10 μM), Rh6G uncouples oxidative phosphorylation.

According to the chemical hypothesis (Slater, 1953), oligomycin probably acts by inhibiting equation 2.

$$A \sim I + X \rightleftharpoons X \sim I + A$$

thus preventing the formation of $X \sim P$ from $X \sim I$. DCCD, TET and venturicidin, the inhibitors of oxidative phosphorylation, apparently have the same inhibitory effect as oligomycin (Beechey et al., 1967; Hatefi et al., 1975). On the other hand, uncouplers are thought to act at $A \sim I$.

It can be concluded from this work that the ATP-^{32}Pi exchange reaction catalysed by intact mitochondria was inhibited by SBP, HCP, agaric acid and Rh6G. Since SBP and agaric acid did not affect the exchange reaction in SMP, it can be concluded that the inhibition

caused by SBP and agaric acid may be due merely to the inhibition of the adenine nucleotide and Pi translocase systems. The inhibition by HCP of the exchange reaction may be related either to the inhibition of the adenine nucleotide and Pi transport systems, or to the HCP uncoupling effect. However, the inhibition by Rh6G of the ATP-^{32}Pi exchange reaction by mitochondria and submitochondrial particles may be either due to its uncoupling effect, or it may be that Rh6G has the same inhibitory effect as oligomycin.

The possibility of inhibition of phosphate transport system by SBP, HCP or agaric acid, suggested above, prompted us to test the effect of these inhibitors on ^{32}Pi transport, as will be shown in Chapter 6.

CHAPTER 6

THE MECHANISM OF PHOSPHATE AND DICARBOXYLATE TRANSPORT IN MITOCHONDRIA

6.1 INTRODUCTION

There is ample evidence that mitochondrial substrate transport involves specific translocators (Mitchell, 1953). Thus phosphate exchanges with hydroxyl via a phosphate translocator (McGivan and Klingenberg, 1971; Hoek et al., 1971), dicarboxylate with phosphate via a dicarboxylate translocator (Chappell, 1968; Palmieri et al., 1971; Robinson and Williams, 1970), tricarboxylate with malate via a tricarboxylate translocator (Palmieri et al., 1972), α-oxoglutarate with certain dicarboxylate ions via an α-oxoglutarate translocator (Chappell et al., 1968) and aspartate with glutamate via an aspartate translocator (Azzi et al., 1967). The Pi-OH exchange serves to neutralize the charge imbalance occurring from uptake of ADP and efflux of ATP during oxidative phosphorylation. While the Pi-dicarboxylate exchange acts catalytically to allow net uptake of anionic substrates at the expense of the mitochondrial pH gradient (McGivan and Klingenberg, 1971).

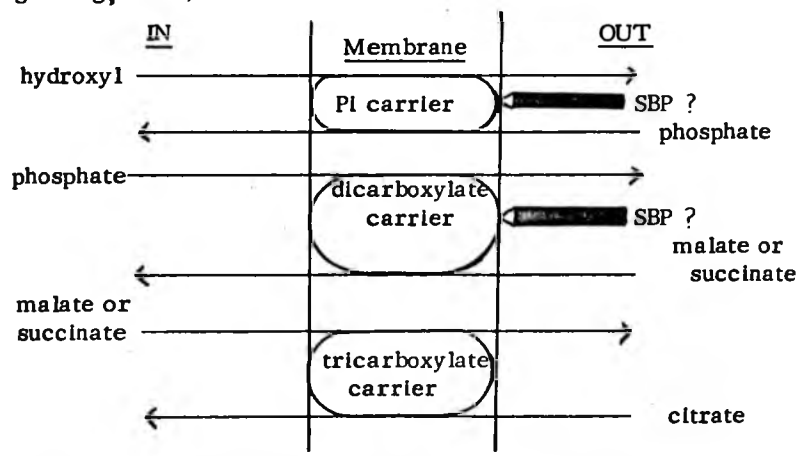


Figure 6.1 Scheme of sequential action of metabolite carriers

SBP = sulfobromophthalein

The substrate-binding site of the tricarboxylate carrier (Palmieri et al., 1972) will accept molecules with at least two carboxyl groups such as malate, or with three neighbouring carboxyl groups such as citrate, or with one carboxyl group and a phosphate group such as phosphoenol pyruvate.

Fonyo and Bessman (1968) and Tyler (1969) have shown that some sulphydryl-binding reagents specifically interfere with the transport of phosphate across the mitochondrial membrane. Mersalyl (Tyler, 1969; Papa et al., 1973), p-hydroxymercuribenzoate (Fonyo, 1968) inhibit both translocation systems of phosphate. While N-ethylmaleimide (Coty and Pedersen, 1974) only inhibits the phosphate-hydroxyl exchange, n-butylmalonate (Johnson and Chappell, 1973) which does not react with thiols, has been used to inhibit the phosphate-dicarboxylate exchange. 6,6'-dithiodinicotinic acid (CPDS), a non-penetrating thiol agent, blocks only a few -SH groups on the outer surface of the inner mitochondrial membrane and affects several mitochondrial functions in a way different from that of mersalyl (Abou-Khalil et al., 1975a).

Meisner (1973) mentioned the possibility that SBP is a potent inhibitor of the Pi/OH as well as Pi/malate uptake of mitochondria, but no experimental verification has so far been reported. It was, therefore, interesting :

(a) to reinvestigate in detail the effects of SBP, HCP, agaric acid and Rh6G on ^{32}Pi transport and on ^{32}Pi-Pi exchanges. Studies on the effects of mersalyl, p-hydroxymercuribenzoate, triethyltin, ionophorous antibiotics and other uncouplers and inhibitors of oxidative phosphorylation on ^{32}Pi transport and on ^{32}Pi-Pi exchanges were performed for comparison;

(b) to reinvestigate the effect of SBP on dicarboxylate transport system by studying the effect of SBP on dicarboxylate uptake, dicarboxylate-dicarboxylate exchange and dicarboxylate-phosphate exchange, which are mediated by the dicarboxylate carrier.

The substrate-binding site of the tricarboxylate carrier (Palmieri et al., 1972) will accept molecules with at least two carboxyl groups such as malate, or with three neighbouring carboxyl groups such as citrate, or with one carboxyl group and a phosphate group such as phosphoenol pyruvate.

Fonyo and Bessman (1968) and Tyler (1969) have shown that some sulphydryl-binding reagents specifically interfere with the transport of phosphate across the mitochondrial membrane. Mersalyl (Tyler, 1969; Papa et al., 1973), p-hydroxymercuribenzoate (Fonyo, 1968) inhibit both translocation systems of phosphate. While N-ethylmaleimide (Coty and Pedersen, 1974) only inhibits the phosphate-hydroxyl exchange, n-butylmalonate (Johnson and Chappell, 1973) which does not react with thiols, has been used to inhibit the phosphate-dicarboxylate exchange. 6,6̄-dithiodinicotinic acid (CPDS), a non-penetrating thiol agent, blocks only a few -SH groups on the outer surface of the inner mitochondrial membrane and affects several mitochondrial functions in a way different from that of mersalyl (Abou-Khalil et al., 1975a).

Meisner (1973) mentioned the possibility that SBP is a potent inhibitor of the Pi/OH as well as Pi/malate uptake of mitochondria, but no experimental verification has so far been reported. It was, therefore, interesting :

(a) to reinvestigate in detail the effects of SBP, HCP, agaric acid and Rh6G on ^{32}Pi transport and on ^{32}Pi-Pi exchanges. Studies on the effects of mersalyl, p-hydroxymercuribenzoate, triethyltin, ionophorous antibiotics and other uncouplers and inhibitors of oxidative phosphorylation on ^{32}Pi transport and on ^{32}Pi-Pi exchanges were performed for comparison;

(b) to reinvestigate the effect of SBP on dicarboxylate transport system by studying the effect of SBP on dicarboxylate uptake, dicarboxylate-dicarboxylate exchange and dicarboxylate-phosphate exchange, which are mediated by the dicarboxylate carrier.

6.2 MATERIALS AND METHODS

Materials

^{32}Pi, L[U-^{14}C] malic acid, [1,4-^{14}C] succinic acid (sodium salt) were obtained from the Radiochemical Centre, (Amersham, U.K.). DCCD, sodium malate, sodium succinate and sodium malonate were obtained from British Drug Houses, Ltd. The following compounds were obtained from the Sigma Chemical Co.:- antimycin A, oligomycin, sodium mersalyl, amytal, p-chloromercuribenzoic acid (p-CMBA) and N-ethylmaleimide (NEM). All other chemicals were obtained from the same sources mentioned in Chapter 2. All other chemicals were of A.R. grade. p-CMBA was dissolved in distilled water and Tris base was added to the final concentration of 5 mM (Fonyo and Bessman, 1968).

Methods

Preparation of mitochondria

Preparation of rat liver mitochondria and the protein determination were carried out as described in Chapter 2.

Measurements of uptake of radioactive phosphate (^{32}Pi)

All ^{32}Pi uptake measurements were made essentially as described by Williams and Orr (1976) as follows, unless indicated otherwise in individual experiments. Assays were carried out in a standard medium of 37 mM sucrose, 37 mM KCl, 37 mM Tris-HCl, 0.2 mM $MgSO_4$ and oligomycin (1.5 μg/ml), pH 7.4 at 0^{o}. All assays were carried out at 0^{o}, in tubes surrounded by ice-cold water. Mitochondria (1 mg protein) were incubated in the assay medium (final volume, 1 ml) with or without inhibitors for 10 min at 0^{o}, after which phosphate uptake was initiated by the rapid addition of ^{32}Pi (final concentration, 2.0 mM); the tubes were mixed briefly, using a Vortex mixer, and after incubation for 1 min uptake was stopped by rapid Millipore filtration (as described in Chapter 2) using 0.45 μm Millipore filter, followed by washing of the mitochondria with 4 ml of the assay medium, and the amount of radioactivity remaining in the filter that contained the mitochondrial pellets was estimated. Correction was made for the sucrose permeable space by subtraction of values obtained with 50 nmoles mersalyl/mg protein added before ^{32}Pi (Abou-Khalil et al., 1975b).

Estimation of ^{32}Pi-Pi exchange

The ^{32}Pi exit from preloaded rat liver mitochondria in exchange for external Pi were carried out essentially as described by Abou-Khalil et al. (1975b). Liver mitochondria (70 mg protein) were incubated with 20 µmole ^{32}Pi at 0°, in ^{32}Pi-uptake assay medium (shown above); after 10 min the mitochondria were separated by centrifugation at 10000 x g, washed with the same incubation medium and suspended at 10 mg protein/ml. The exchange reaction was started by incubation of mitochondria (1 mg protein) with 2 mM unlabelled Pi, following the same steps for ^{32}Pi uptake shown above. The radioactivity found in 1 mg of mitochondrial protein before and after addition of unlabelled Pi were estimated.

Technique for measuring the kinetics of dicarboxylate uptake.

A. Estimation of ^{14}C-malate uptake

Estimation of ^{14}C-malate uptake were made essentially as described by Palmieri et al. (1971) as follows, unless indicated otherwise in individual experiments. Assays were carried out in a medium containing 100 mM KCl, 25 mM imidazol buffer, pH 6.3, 1 mM EGTA, 0.5 µg rotenone, 0.5 µg antimycin A, 5 µg oligomycin, 1 mg mitochondrial protein, and ^{14}C-malate at the concentration indicated below each figure (Figs. 6.8 and 6.10). Reactions were carried out at 9° C, in a final volume of 1 ml. 5 sec after the addition of labelled malate, the reaction was stopped by rapid Millipore filtration, followed by washing of the mitochondria with 4 ml incubation medium. The amount of radioactivity in the filter that contained the mitochondrial pellets was estimated.

B. Estimation of ^{14}C-succinate uptake

Estimation of ^{14}C-succinate uptake was as described above, in a medium containing 80 mM KCl, 50 mM Tris-HCl, pH 6.3, 1 mM EDTA, 1 µg rotenone, 1 µg antimycin A, 10 µg oligomycin, 1 mg mitochondrial protein and ^{14}C-succinate at the concentration shown below each figure (Figs. 6.9 and 6.12). Temperature 11°. Final volume, 1 ml.

Technique for measuring the kinetics of dicarboxylate efflux

Estimations of ^{14}C-malate or ^{14}C-succinate efflux from mitochondria were made essentially as described by Lofrumento et al. (1974). Mitochondria (50 mg protein) were loaded with labelled malate or succinate by incubation for 10 min at 4^{o} in the presence of 2 ml of 0.25 M sucrose, 5 µg oligomycin, 0.34 µg rotenone, 0.17 µg antimycin A, 2 mM of ^{14}C-malate or 6 mM ^{14}C-succinate. The mitochondria were centrifuged at 10000 x g and washed twice. Loaded mitochondria (1 mg protein) were incubated in a medium containing 200 mM sucrose; 20 mM Tris-HCl, pH 7.2; oligomycin, rotenone and antimycin A at the concentrations indicated above. Reactions were carried out at 9^{o} C in a final volume of 1 ml. 5 sec after the addition of unlabelled substrate, the reaction was stopped by rapid Millipore filtration, as described above.

Counting

^{32}Pi was counted in a Packard Tricarb liquid scintiallation counter, in a Triton X-100/toluene/butyl PBD scintillation mixture. ^{14}C was counted in a POPOP/PPO system, as described in Chapter 2 (Patterson and Green, 1965).

6.3 RESULTS

A. Characters of ^{32}Pi uptake

Figure 6.2 demonstrates the kinetics of ^{32}Pi uptake by rat liver mitochondria. The experimental conditions are similar to those employed by Williams and Orr (1976) and the results obtained are in good agreement. Figure 6.2 shows that the maximum amount of phosphate uptake occurs after 1 min incubation period. In the majority of the present experiments, incubation was carried out for 1 min. The present results also show a secondary slow increase which does not appear to be complete even after 10 min.

The measured uptake of 17.0 ± 4.1 nmoles per min per milligramme of mitochondrial protein is also in agreement with the results of Williams and Orr (1976). Fig. 6.3 demonstrates that the K_m value of ^{32}Pi uptake is approximately equal to 0.22 mM, which is nearly 28 times more than the K_m for ADP and 17 times more than the K_m for Ca^{++} uptake, as shown in Chapters 2 and 4 respectively.

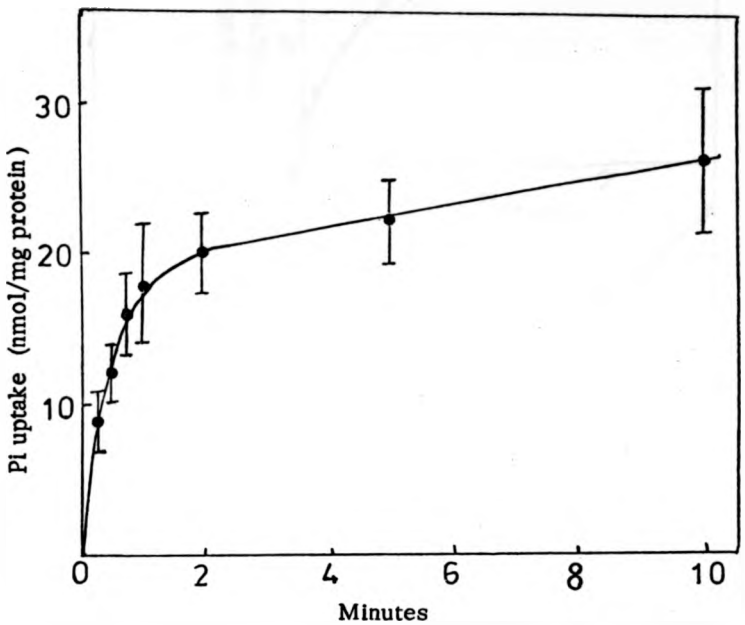


Figure 6.2 Time dependence of ^{32}Pi exchange from medium to mitochondria

The assay conditions and the technique for measuring the exchange are as described under "Methods". ^{32}Pi concentration was 2 mM, pH 7.4, temperature 0°. The results are expressed as a mean of five determinations ± S.D.

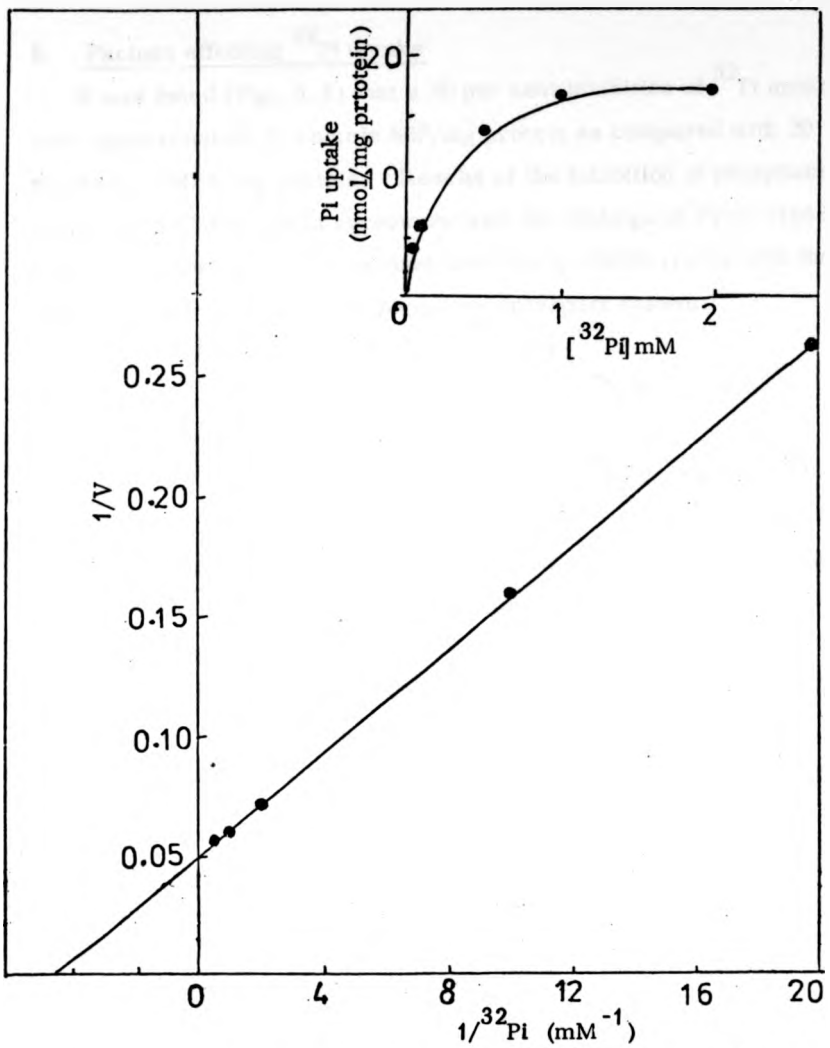


Figure 6.3 Lineweaver-Burk plot of dependence of rates of ^{32}Pi exchange on medium phosphate concentration

The experimental conditions are described under "Methods". ^{32}Pi incubated for 1 min with mitochondria. The values plotted are the mean of four determinations.

K_m (^{32}Pi) = 0·22 mM.

B. Factors affecting ^{32}Pi uptake

It was found (Fig. 6.4) that a 50 per cent inhibition of ^{32}Pi uptake with approximately 2.5 nmole SBP/mg protein as compared with 20 nmoles p-CMBA/mg protein. Results of the inhibition of phosphate uptake by p-CMBA are in agreement with the findings of Tyler (1968). Coty and Pedersen (1975) demonstrated that p-CMBA reacts with the same sulfhydryl group of the phosphate transport system.

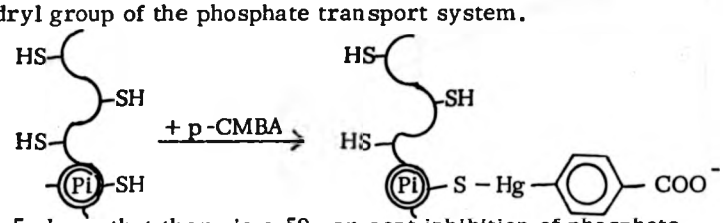


Figure 6.5 shows that there is a 50 per cent inhibition of phosphate entry into mitochondria with 10 nmoles mersalyl/mg protein, 2 nmoles X-537 A/mg protein, 5 nmoles leucinostatin/mg protein and 11 nmoles valinomycin/mg protein. These results confirm the previous findings of Tyler (1968), Coty and Pedersen (1974) and Lardy et al. (1975). Mersalyl and formaldehyde can inhibit reactions requiring phosphate, and may act by preventing the entry of phosphate into the mitochondria (Tyler, 1968). On the other hand, Lardy et al (1975) showed that leucinostatin (A 20668) inhibits Pi uptake in rat liver mitochondria (80 % inhibition with 0.2 μg leucinostatin/mg protein) and that leucinostatin uncouples oxidative phosphorylation at a concentration of 0.11 μM (Reed and Lardy, 1975).

As shown in Fig. 6.6, which demonstrates the comparison between FCCP, the classical uncoupler of oxidative phosphrylation and HCP, the two compounds have nearly similar effects on phosphate uptake, (50 per cent inhibition of phosphate entry into mitochondria, with 1 nmol FCCP or HCP/mg protein). The results of Parker (1965) indicate that a 50 per cent inhibition of phosphate uptake from rat liver mitochondria was caused by 0.06 μM, FCCP; 0.6 μM, Cl-CCP; 9 μM, PCP; and 30 μM, DNP.

Intact rat liver mitochondria contains 10 nmoles of Pi/mg protein. When placed in an isotonic medium at 0°, phosphate added to the medium is taken up into the mitochondria in amounts which exceed these endogenous levels about 1 to 3 fold (Coty and Pedersen, 1974).

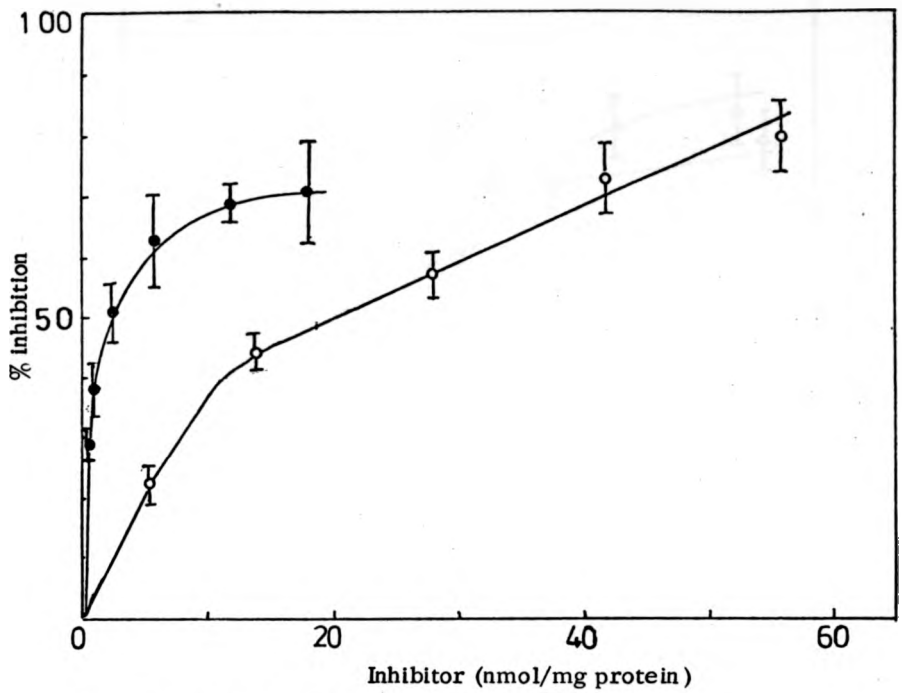


Figure 6.4 Effect of SBP, p-CMBA on ^{32}Pi uptake

The experimental conditions are described under "Methods". The data points are mean values ± S.D. of four experiments. Inhibitors were incubated with mitochondria for 10 min at 0^{o} before initiating the exchange with 2 mM ^{32}Pi. One min after the addition of ^{32}Pi, the reaction was stopped by rapid, Millipore filtration.

●——●, SBP

O——O, p-CMBA

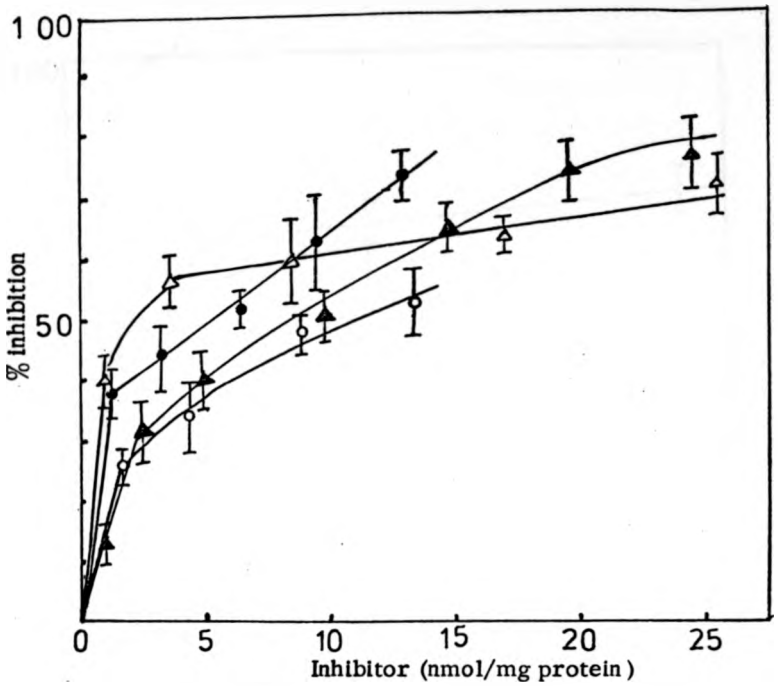


Figure 6.5 Effect of mersalyl and ionophores on ^{32}Pi uptake

The experimental conditions are described under "Methods".

The data points are a mean ± S.D. of four experiments.

▲——▲, mersalyl

●——●, leucinostatin

△——△, X-537A

O——O, valinomycin

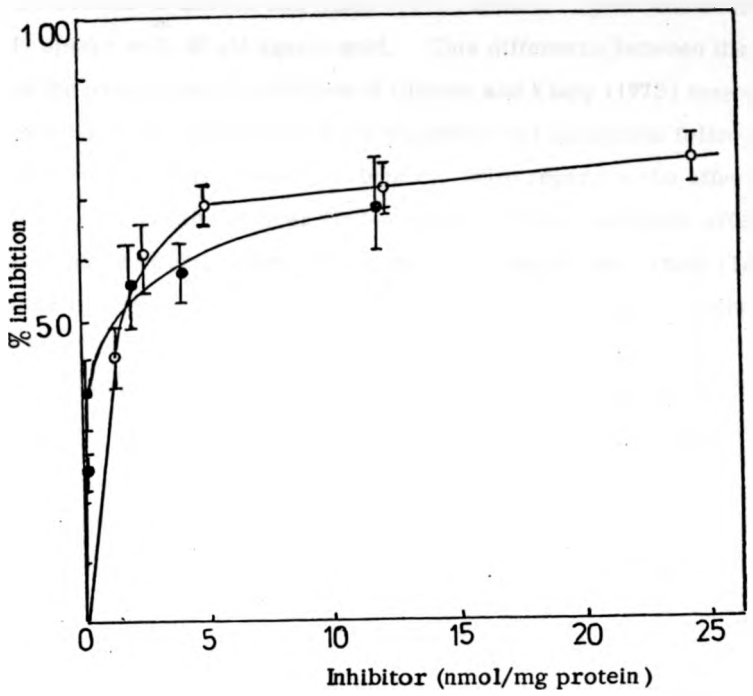


Figure 6.6 Effect of HCP and FCCP on ^{32}PI uptake

The experimental conditions are described under "Methods".

The data points are mean ± S.D. of three experiments.

O——O , HCP

●——● , FCCP

Uptake is slightly inhibited by agaric acid (35.6 ± 8.1 per cent inhibition with 22 nmoles agaric acid/mg protein (Table 6.1). However, the results of Chávez and Klapp (1975) show a 12 per cent inhibition of Pi uptake with 40 μM agaric acid. This difference between the results of the present work and those of Chávez and Klapp (1975) may be related to the differences in the experimental conditions followed in the present study and the last paper. With regard to the effect of Rh6G, the results of Gear (1974) show that Rh6G had little effect on the uptake of phosphate, but the results of the present study (Table 6.1) demonstrate a 26.5 ± 7.1 per cent inhibition with higher concentration of Rh6G (25 nmol/mg protein).

The results of the present work (Table 6.1) show that 20.2 ± 4.4 per cent inhibition of phosphate uptake caused by 12 nmoles atractyloside/mg protein, in agreement with the findings of Vignais et al. (1962). However, Coty and Pedersen (1974) showed that atractyloside has no effect on phosphate uptake.

Phosphate uptake was also inhibited slightly by higher concentrations of DCCD (48 nmol/mg protein) and TET (8 nmol/mg protein) which caused approximately 21% and 28% inhibition of ^{32}Pi uptake respectively (Table 6.1). It was shown by Tyler (1969) that the concentrations of DCCD and tributyltin, sufficient to block the coupled oxidation of respiratory-chain-linked substrates (40 nmoles DCCD/mg protein; 0.9 nmoles tributyltin/mg protein), caused about 10% inhibition of respiration stimulated by phosphate, and had no effect on the substrate-level phosphorylation reaction, indicating that these reagents have little effect on the entry of phosphate into mitochondria.

Table 6.1 also shows 38.4 ± 4.5 per cent inhibition of of phosphate uptake with 16 nmoles CPDS/mg protein, a result which is in agreement with the findings of Abou-Khalil et al. (1975a).

C. Inhibition of ^{32}Pi-Pi exchange reaction

^{32}Pi-Pi exchange was catalyzed by both phosphate and dicarboxylate carriers (Fig. 6.1), and in order to find the effect of SBP on the

TABLE 6.1

Inhibition of phosphate (^{32}Pi) transport into mitochondria

The experimental conditions are described under "Methods". The inhibitors were incubated for 10 min with mitochondria before initiating the exchange with 2 mM ^{32}Pi. After 1 min, the reaction was stopped by Millipore filtration. Temperature, 0°, pH 7.4. The data points are mean values ± S.D. of three experiments.

Inhibitors	Concentrations	Inhibition %
Agaric acid	22.o nmol/mg protein	35.6 ± 8.1
Rh6G	24.6 nmol/mg protein	26.5 ± 7.1
Atractyloside	12.5 nmol/mg protein	20.2 ± 4.4
DCCD	48.0 nmol/mg protein	20.9 ± 3.1
TET	8.0 nmol/mg protein	28.3 ± 3.3
CPDS	16.0 nmol/mg protein	38.4 ± 4.5
Venturicidin	5.0 µg/mg protein	20.1 ± 4.6
Formaldehyde	5.0 µmole/mg protein	51.0 ± 6.8
DNP	20.0 nmol/mg protein	17.3 ± 2.7
1799	0.09 nmol/mg protein	30.4 ± 7.4
PCP	0.8 nmol/mg protein	20.7 ± 4.1
	2.0 nmol/mg protein	40.6 ± 8.3

dicarboxylate carrier, the effect of SBP as well as mersalyl and p-CMBA were carried out on the ^{32}Pi-Pi exchange. Fig. 6.7 shows a titration of the action of SBP, mersalyl and p-CMBA on the exchange-diffusion between mitochondrial ^{32}Pi and external unlabelled Pi. This was followed by exposing for 1 min ^{32}Pi loaded mitochondria to a second incubation with unlabelled Pi. Inhibitors were added to ^{32}Pi-loaded mitochondria, 10 min before the exchange started. A 50 per cent inhibition of exchange caused with 5 nmoles SBP/mg protein, 12 nmoles mersalyl/mg protein, 16 nmoles p-CMBA/mg protein. The present results of the effect of mersalyl and p-CMBA are concordant with the findings of (Meijer et al., 1970; Papa et al., 1973; Johnson and Chappell, 1973; Quagliarello et al., 1974).

D. Inhibition of dicarboxylates transport

On comparing the dependence of substrate concentration on the rate of malate uptake (Fig. 6.8) and succinate uptake (Fig. 6.9) it is found that the K_m (malate) = 0.50 mM and K_m (succinate) = 1.11 mM. These results are in agreement with the results of Palmieri et al., 1971, and Fonyo et al., 1976). The inhibition of the rate of malate uptake by SBP is shown in Fig. 6.8; SBP (12 nmol/mg protein) caused an increase in the K_m (malate) from 0.5 mM to 1.6 mM, with an uncompetitive type of inhibition. Moreover, SBP (12 nmol/mg protein) inhibited uncompetitively both the malate/^{14}C-malate exchange (Fig. 6.10) and Pi/^{14}C-malate exchange (Fig. 6.11), with an increase in K_m from 0.42 to 5.0 mM and from 0.33 to 1.51 mM respectively. Thus the present results are in agreement with Meisner (1973) who briefly reported that SBP inhibits Pi/malate uptake in mitochondria. On the other hand, SBP inhibits noncompetitively the ^{14}C-succinate uptake (Fig. 6.9) and succinate/^{14}C-succinate exchange (Fig. 6.12).

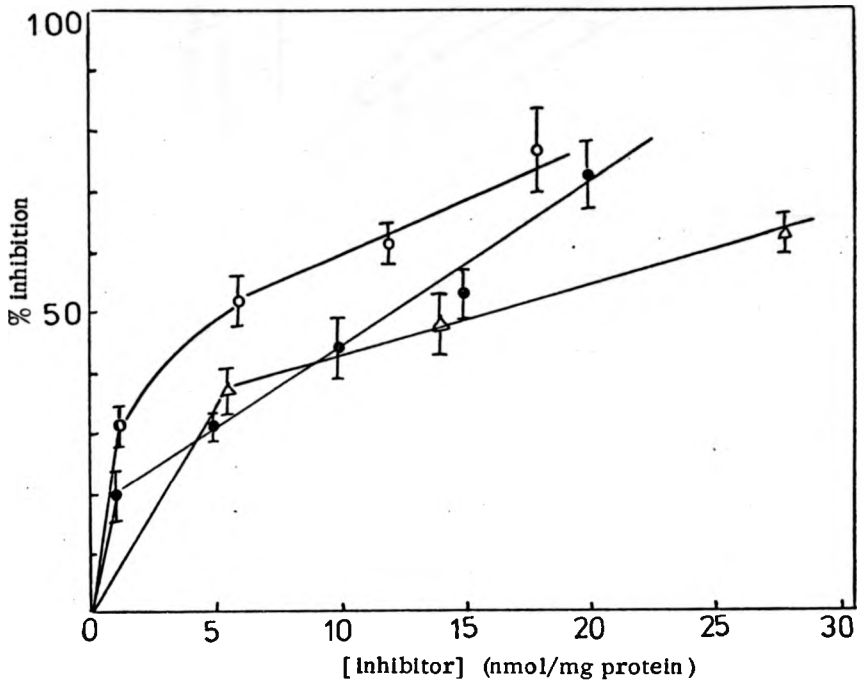


Figure 6.7 Effect of SBP, mersalyl and p-CMBA on the ^{32}Pi-Pi exchange

The assay conditions and techniques for measuring ^{32}Pi-Pi exchange were as described under "Methods", using mitochondria (1 mg protein) labelled with ^{32}Pi, and the inhibitors were incubated with labelled mitochondria for 10 min before initiating the reaction with 2 mM of unlabelled Pi. After 1 min, the reaction was stopped by filtration. The results are the mean ± S.D. of four observations.

O ——O, SBP

●——●, mersalyl

△——△, p-CMBA

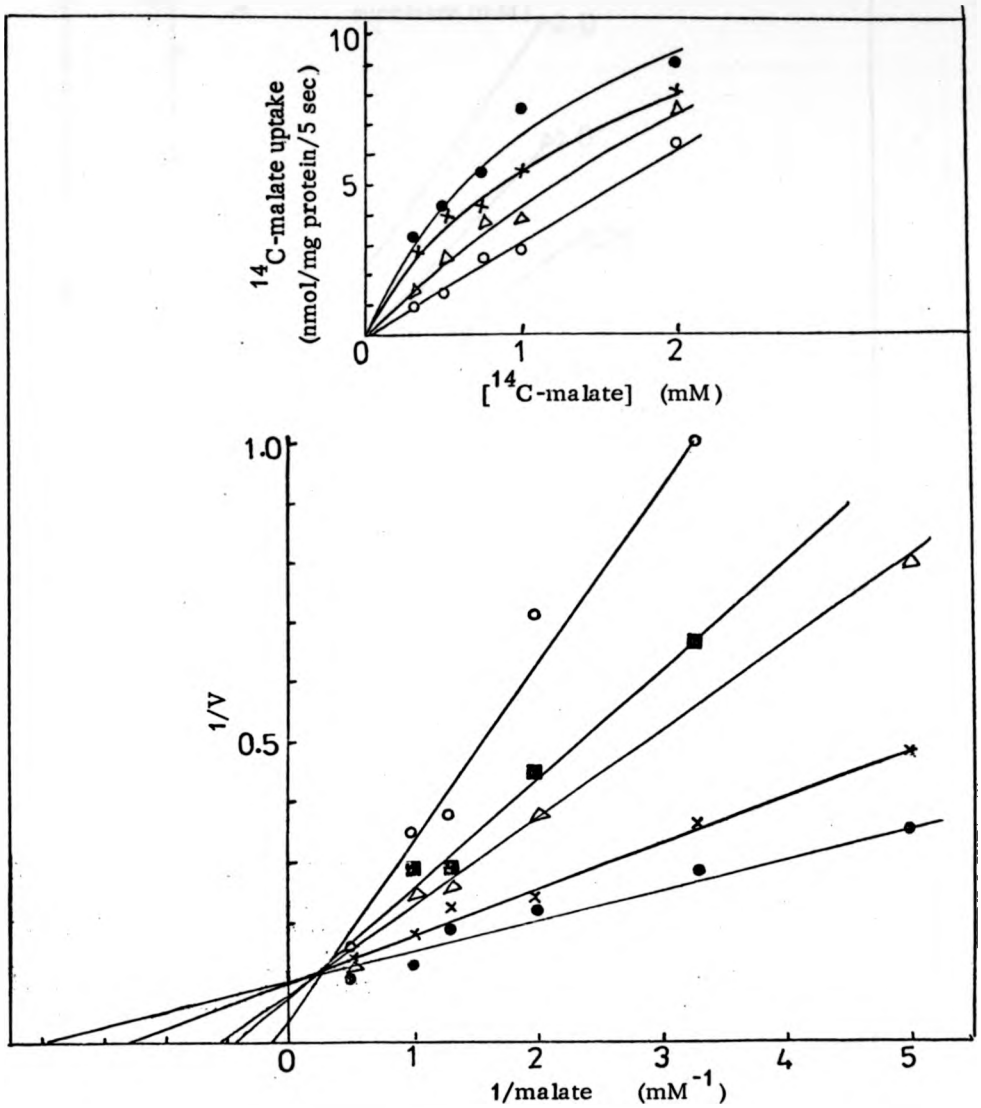


Figure 6.8 Kinetic analysis of the inhibition of ^{14}C-malate uptake by SBP, using the double reciprocal plot

The experimental conditions are described under "Methods". SBP added to mitochondria 10 min before ^{14}C-malate. The data points are mean of five experiments.

●——●, no inhibitor, K_m (malate) = 0.5 mM

X——X, +6 nmol/mg SBP, K_m (malate) = 0.8 mM

△——△, +12 nmol/mg SBP, K_m (malate) = 1.6 mM

■——■, +18 nmol/mg SBP, K_m (malate) = 2.5 mM

O——O, +24 nmol/mg SBP, K_m (malate) = 6.6 mM

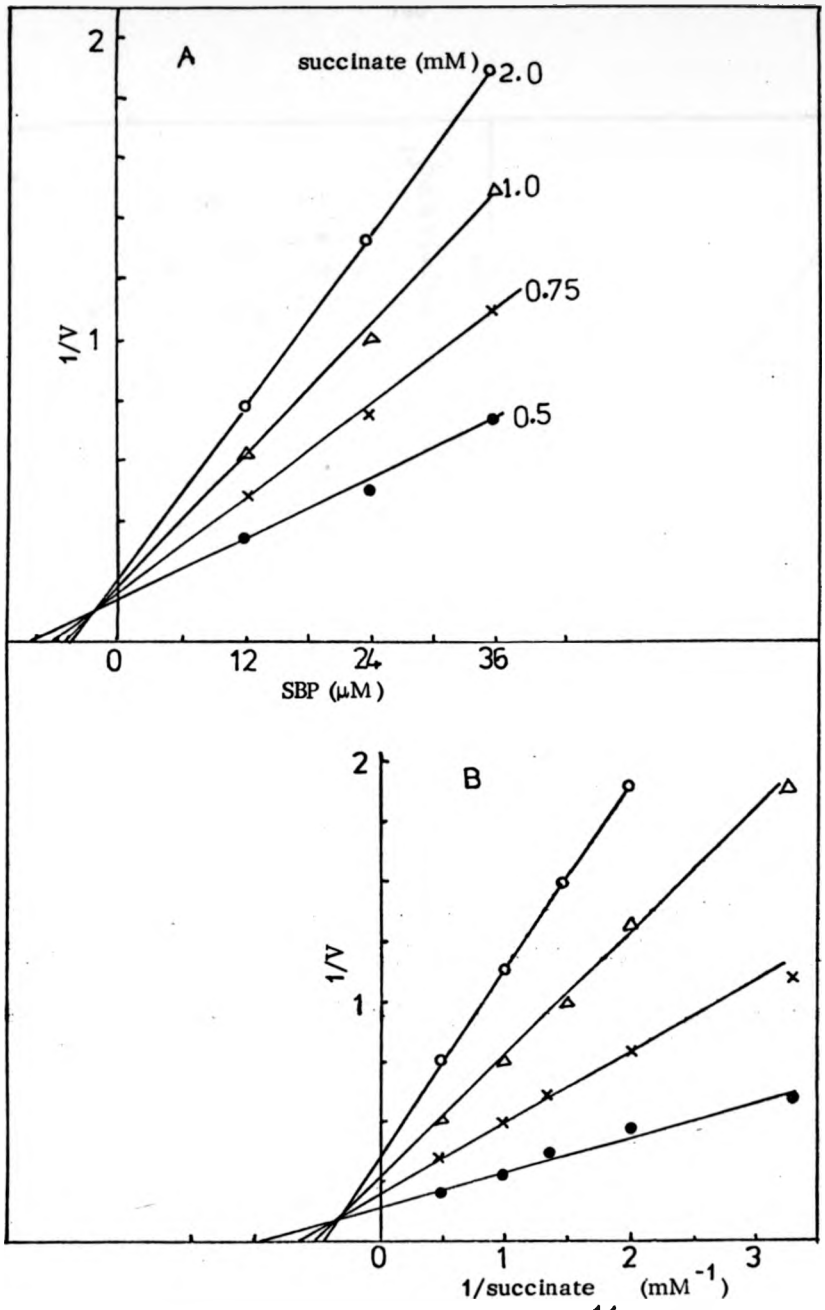


Figure 6.9 Kinetic analysis of the inhibition of ^{14}C-succinate uptake by SBP, using:- A. Dixon plot, and B. double reciprocal plot.

The experimental conditions are described under "Methods". SBP added to mitochondria 10 min before ^{14}C-malate. The data points are mean of five experiments.

●——●, no inhibitor; X——X, +12 nmol/mg SBP; △——△, + 24 nmol/mg SBP; O——O, + 36 nmol/mg SBP.

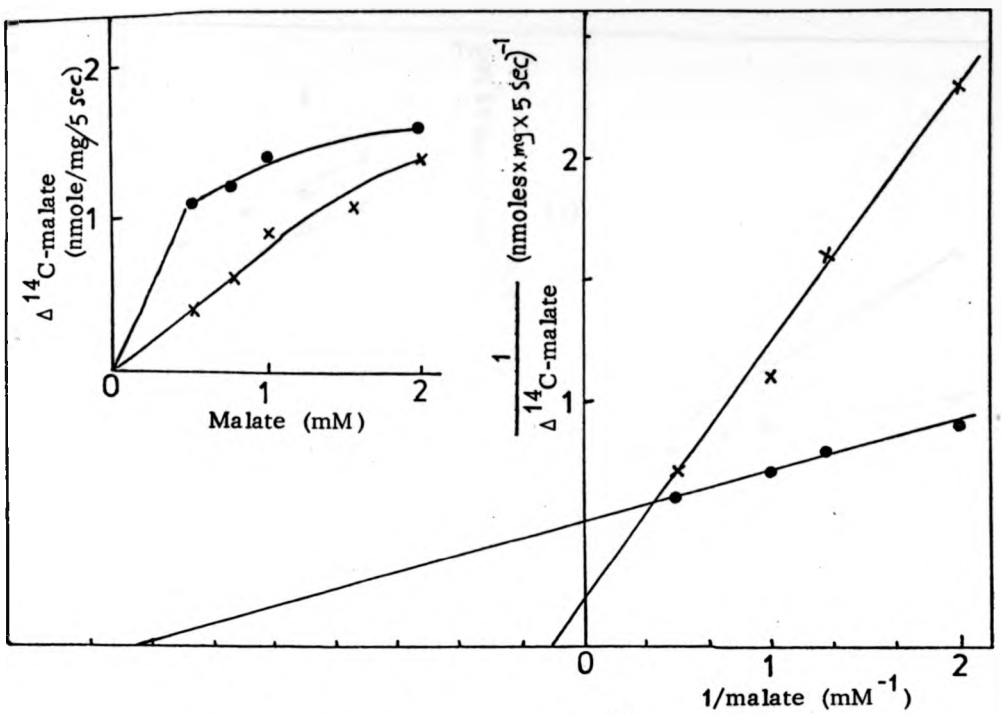


Figure 6.10 Effect of SBP on the kinetics of ^{14}C-malate efflux in exchange with external malate

The efflux of ^{14}C-malate from preloaded mitochondria was followed as described under "Methods". SBP (12 nmol/mg protein) was incubated for 10 min with mitochondria before addition of malate. 5 sec after addition of malate, the reaction was stopped by Millipore filtration. In the absence of unlabelled malate, the ^{14}C-malate content of mitochondria relative to 1 mg protein was 3·8 nmoles. Δ ^{14}C-malate was determined by subtracting the amount found in the presence of unlabelled malate from that in its absence. The data points are mean of four experiments.

●——●, no inhibitor, K_m (malate) = 0.42 mM

X——X, + 12 nmol SBP/mg protein, K_m (malate) = 5.00 mM

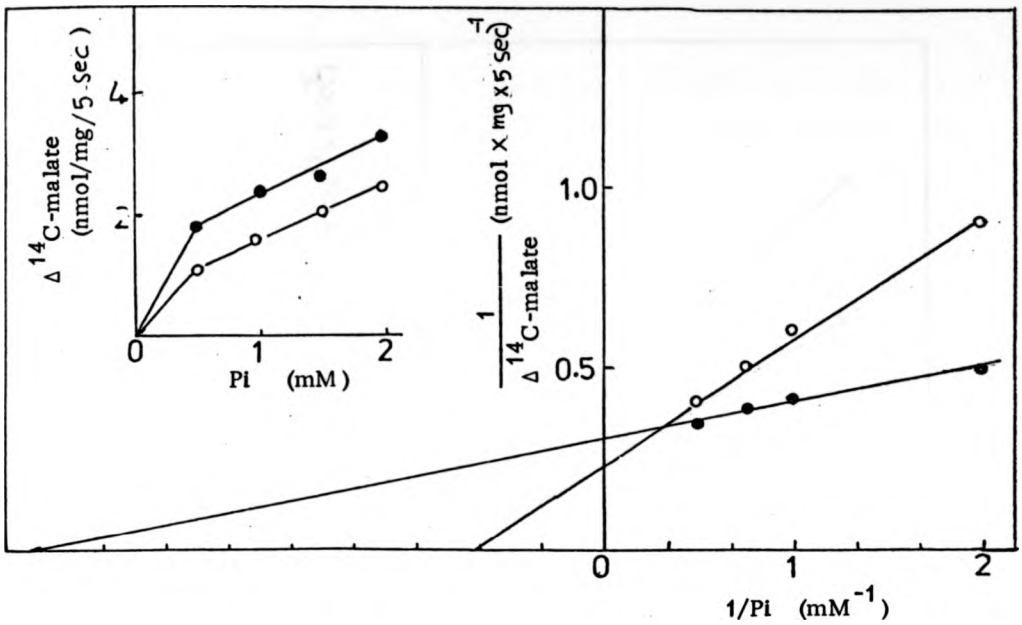


Figure 6.11 Effect of SBP on the kinetics of ^{14}C-malate efflux in exchange with external Pi

Experimental conditions as in Fig. 6.10 .

The results are the mean of four experiments.

●——●, no inhibitor, K_m (Pi) = 0.33 mM

O ——O, + 10 nmol/mg, SBP, K_m (Pi) = 1.51 mM

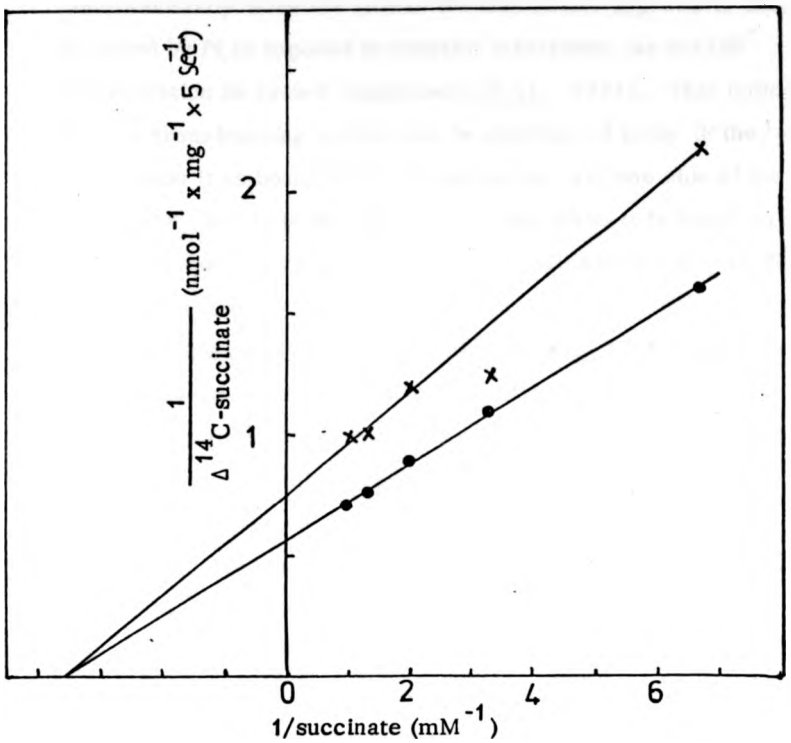


Figure 6.12 Effect of SBP on the kinetics of ^{14}C-succinate efflux in exchange with external succinate

The efflux of ^{14}C-succinate from preloaded mitochondria was followed as described under "Methods". SBP (12 nmol/mg protein) incubated for 10 min with mitochondria before addition of unlabelled succinate. 5 sec after the addition of succinate, the reaction was stopped by Millipore filtration. In the absence of unlabelled succinate, the ^{14}C-succinate content of mitochondria relative to 1 mg protein was 2.8 nmoles. Δ^{14}C-malate was determined by subtracting the amount found in the presence of unlabelled malate from that in its absence. The results are mean of four experiments.

●——●, no SBP, K_m (succinate) = 0.28 mM

X——X, + 12 nmol SBP/mg protein, K_m (succinate) = 0.28 mM

6.4 DISCUSSION

It is well established that Pi moves across the mitochondrial membrane only when the side of the membrane opposite to that occupied by Pi, is exposed to specific substrates, or the OH^- concentration is raised (Lofrumento et al., 1974). This indicates that the translocating system can be considered to be in the 'resting state' when it is bound to its substrates on only one side of the membrane, and is in the 'functional state' when it is bound on both sides to a specific couple of substrates, namely Pi-OH^-, Pi-Pi or Pi-dicarboxylate.

Mitchell's chemiosmotic theory (Mitchell, 1961) postulates that the free energy released during the activity of the respiratory chain leads to ejection of protons in the extramitochondrial space, thus creating a transmembrane pH gradient. There is indeed a pH difference of approximately 0.4 to 1.0 pH units between outside and inside, and this leads to the influx of phosphate, pyruvate and glutamate as a result of an exchange for internal hydroxyl ions that are more concentrated inside (Sluse and Sluse-Goffart, 1974).

In Scheme 6.1, ^{32}Pi can enter the mitochondria in exchange for OH^- ion via the phosphate carrier. In the presence of inhibitors of phosphate transport, such as SBP, mersalyl and p-CMBA, the uptake of ^{32}Pi was prevented (Figs. 6.4 and 6.5). This may be due to inhibition of phosphate carrier. In ^{32}Pi-loaded mitochondria, the ^{32}Pi can leave mitochondria against externally added unlabelled Pi or dicarboxylate ions, via a dicarboxylate carrier. The last carrier may be sensitive to SBP, mersalyl or p-CMBA (Fig. 6.7). Moreover, the phosphate transport was also inhibited by a variety of inhibitors and uncouplers, as shown in Table 6.1.

Treatment with uncouplers (FCCP, DNP, 1799 and PCP), however, greatly decreases phosphate uptake by dissipation of the pH gradient. Two major classes of uncouplers are nitrophenols and halophenols, which are typified by 2, 4-dinitrophenol and pentachlorophenol respectively. The chlorinated bisphenols, such as HCP, have many physical and chemical similarities to these simple phenols. In addition, Caldwell

et al. (1972) have shown that HCP acts as an effective uncoupler of mitochondrial oxidative phosphorylation, and this can be considered as the reason of inhibition of phosphate uptake by HCP.

It was proposed that the concentrations of Rh6G above 10 μM uncouple mitochondrial respiration (Gear, 1974), and this may be the reason for inhibition of ^{32}Pi transport by high concentrations of Rh6G. On the other hand, A 20668 is both a unique uncoupler and inhibitor of phosphoryl transfer reactions in mitochondria (Reed and Lardy, 1975).

The slight inhibition of ^{32}Pi uptake by TET, found in the present study, are in agreement with Aldridge and Street (1971) and this inhibition of ^{32}Pi uptake may be related to the changes in intramitochondrial pH values (Dawson and Selwyn, 1974) since it was shown by Skilleter (1975) that the primary event in KCl media is that the TET-mediated Cl^-/OH^- exchange, one consequence of which is a fall in the intramitochondrial pH.

The inhibition of phosphate transporter by mersalyl or formaldehyde is presumably due to the formation of complexes, between mersalyl or formaldehyde and the thiol and amino groups of an inner-membrane protein or lipoprotein, resulting in the modification of groups catalysing ion-exchange processes, during phosphate transport or in a less specific effect on the conformation of the protein concerned in the transporter mechanism (Tyler, 1969). Moreover, the finding of Papa et al. (1973) suggested that mersalyl inhibits the transport of Pi in mitochondria by fixing the anion on binding site in the mitochondria.

Fonyo (1968) proposed that p-CMBA blocks those reactions of mitochondria in which permeation of inorganic phosphate into the mitochondria was involved. Oxidative phosphorylation is absolutely dependent on inorganic phosphate. The mercurial sensitivity of phosphate permeation may seem to give SH-groups a specific role in the process. However, p-CMBA might act without any specific involvement of SH-groups in translocation. One of the alternative possibilities is that p-CMBA is

bound to an SH-group not directly involved in phosphate permeation, but after combination with the mercurial a change in protein conformation occurs, and thus the carrier function of the membrane protein is lost.

Results of the present study (Table 6.1) show that CPDS inhibits phosphate entry into mitochondria, and this may be due to inhibition of Pi-OH^- exchange (Abou-Khalil et al., 1975b). The residual phosphate which enters into mitochondria in the presence of CPDS must proceed by Pi-dicarboxylate exchange.

SBP inhibits the ^{14}C-malate uptake (Fig. 6.8); with 12 nmol/mg SBP, the K_m (malate) increased nearly three times, while the inhibition of ^{14}C-succinate uptake (Fig. 6.9) requires greater concentration of SBP.

The results obtained from studies on the inhibition of the dicarboxylate/dicarboxylate exchange (Figs. 6.10 and 6.12) and the Pi/dicarboxylate exchange (Fig. 6.11) by SBP substantiate the suggestion (of the present worker) that SBP may interfere with the dicarboxylate translocator.

The exchange of dicarboxylates are not affected greatly by SBP as much as the phosphate exchange is. This may be due to the fact that a system other than the dicarboxylate transporting system can catalyse dicarboxylate exchanges. The most likely candidates for this role are the 2-oxoglutarate and citrate transporting system, both of which have affinities for L-malate and other dicarboxylate anions (Chappell et al., 1968; Chappell and Robinson, 1968).

Ultimately it can be concluded that the action of SBP, mersalyl, as well as other thiol reagents on phosphate transport through the inner mitochondrial membrane may be interpreted as a consequence of an inhibition of a more or less specific "phosphate translocator". Moreover, due to the inhibition of Pi-Pi, dicarboxylate-dicarboxylate, and Pi-dicarboxylate exchanges by SBP, it can also be concluded that SBP may inhibit the dicarboxylate carrier. It remains unknown whether SBP can or cannot inhibit the tricarboxylate and 2-oxoglutarate transporting system.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The results presented in this thesis show that sulfobromophthalein (SBP), agaric acid (AA) and hexachlorophene (HCP) compounds are potent inhibitors of mitochondrial transport systems (e.g. ADP, calcium, phosphate and dicarboxylate). The effect of SBP, AA or HCP on ADP translocase was further supported when it was found that these compounds inhibit the 2,4-di-nitrophenol-stimulated mitochondrial ATPase activity but are inactive on submitochondrial particles ATPase. Thus, the above inhibitors are similar to the potent inhibitors of adenine nucleotide translocase, namely atractyloside, carboxyatractyloside or bongkrekic acid. The effects of SBP and AA were also carried out on the ADP and atractyloside binding to rat liver mitochondria and the observation of inhibition of ADP and atractyloside binding by SBP, AA or HCP, substantiate the conclusion that they are inhibitors of ADP translocase. It can be concluded that the mode of action of SBP and AA on ADP translocase is similar to that of atractyloside. The mechanism by which HCP inhibits ADP translocase is not clear at this time. Several possibilities are currently under examination, one of which involves interaction of the HCP with the lipoprotein translocator. Results presented in Fig. 3.8 illustrated the dependence of HCP binding on mitochondrial membrane phospholipids. Other works by Klingenberg (1975) and Thorne and Bygrave (1974) provide strong evidence for the direct involvement of phospholipids in adenine nucleotide translocation. A second possibility of inhibition of ADP uptake by HCP may be due to its uncoupling effect.

Due to the importance of atractyloside, HCP and Rh6G in the present study, the binding properties of these compounds to mitochondria was investigated. It was found that ^{14}C-HCP has a high affinity binding site in mitochondria (K_d = 2·2 x 10^{-8} M) which is present at a concentration of 0.25 nmol/mg protein, while ^{3}H-Atr high affinity site (K_d = 5·4 x 10^{-8} M)

was present at a concentration of 0.18 nmol/mg protein. However, Rh6G has one affinity site of relatively low affinity ($K_d = 1 \cdot 3 \times 10^{-5}$ M) and present at a concentration of 50 nmol/mg protein. The points which may differentiate the mode of action of HCP from other uncouplers such as DNP or FCCP are:- (a) HCP inhibits the dinitrophenol-stimulated mitochondrial ATPase; (b) HCP inhibits the ^{3}H-Atr binding to both intact mitochondria and submitochondrial particles, while DNP or FCCP did not affect the ^{3}H-Atr binding; (c) the importance of phospholipids in ^{14}C-HCP binding to mitochondria, unlike the other uncouplers such as 2-azido-4-nitrophenol, which binding site appears not to involve the bulk of the mitochondrial lipids.

In the opinion of the present worker, the inhibition of mitochondrial calcium uptake by HCP, DBCT, TET and Rh6G, may be either due to the, (a) uncoupling effect of these compounds, since it was known, that the energy-linked uptake of calcium is inhibited by agents which dissipate the membrane potential generated by respiration, like the uncouplers of oxidative phosphorylation, (b) or maybe these compounds affect mitochondrial phospholipids, which are necessary for calcium binding, the preliminary and necessary step in the sequence of events that leads to passage of cations across the mitochondrial membrane. The last point was substantiated when it was found that HCP or Rh6G binding to mitochondria depends on mitochondrial-phospholipid composition.

The inhibition of ATP-^{32}Pi exchange reaction in mitochondria by SBP, HCP or AA found in the present study, may be related to the inhibition of ATP and Pi transport by mitochondria. However, the inhibition caused by Rh6G may be either due to inhibition of oxidative phosphorylation or to a Rh6G uncoupling effect. Gear (1974) demonstrated that low concentration of Rh6G markedly reduced the rate of ATP formation, but at higher concentrations > 10 μM, Rh6G uncouples oxidative phosphorylation.

It is apparent from this thesis that SBP and HCP are potent inhibitors of Pi transport system, while AA and Rh6G are weak inhibitors. It can be

concluded that the action of SBP and the remaining inhibitors on Pi transport through the inner mitochondrial membrane may be interpreted as a consequence of an inhibition of a more or less specific phosphate translocator. Moreover, due to the inhibition of ^{32}Pi-Pi exchange by SBP, it can also be concluded that SBP may inhibit the dicarboxylate carrier. This was further substantiated when it was found that SBP inhibits dicarboxylate transport and dicarboxylate-dicarboxylate exchange through mitochondrial membrane.

REFERENCES

Abou-Khalil, S., Sabadie-Pialoux, N. and Gautheron, D., (1975a), Biochem. Pharmacol., 24, 49-56.

Abou-Khalil, S., Sabadie-Pialoux, N. and Gautheron, D., (1975b), Biochimie, 57, 1087-1094.

Akerman, K.E., Saris, N.E.L. and Jarvisalo, J.O., (1974), Biochem. Biophys. Res. Commun., 58, 801-807.

Aldridge, W.N., (1958), Biochem. J., 69, 367-376.

Aldridge, W.N. and Street, B.W., (1964), Biochem. J., 91, 287-297.

Aldridge, W.N. and Street, B.W., (1971), Biochem. J., 124, 221-234.

Aldridge, W.N. and Threlfall, C.J., (1961), Biochem. J., 79, 214-219.

Andreoli, T.E., Lam, K.W. and Sanadi, D.R., (1965), J. Biol. Chem., 240, 2644-2653.

Azzi, A., Chappell, J.B. and Robinson, B.H., (1967), Biochem. Biophys. Res. Commun., 29, 148-152.

Azzone, G.F. and Massari, S., (1971), Eur. J. Biochem., 19, 79-107.

Azzone, G.F. and Massari, S., (1973), Biochim. Biophys. Acta, 301, 195-226.

Babior, B.M., Creagan, S., Ingbar, S.H. and Kipnes, R.S., (1973), Proc. Nat. Acad. Sci.USA., 70, 98-102.

Beechey, R.B., Roberton, A.M., Holloway, C.T. and Knight, I.G., (1967), Biochemistry, 6, 3867-3879.

Bielawski, J. and Lehninger, A.L., (1966), J. Biol. Chem., 241, 4316-4322.

Binet, A. and Volfin, P., (1975a), Arch. Biochem. Biophys., 170, 576-586.

Binet, A. and Volfin, P., (1975b), FEBS Lett., 49, 400-403.

Bogucka, K. and Wojtczak, L., (1971), Biochem. Biophys. Res. Commun., 44, 1330-1337.

Boyer, P.D., (1964), "Oxidases and Related Redox System ", 2, 994-1007.

Boyer, P.D., Falcone, A.B. and Harrison, W.H., (1954), Nature, 174, 401-402.

Boyer, P.D., Luchsinger, W.W. and Falcone, A.B., (1956), J. Biol. Chem., 223, 405-421.

Boyer, P.D., Stokes, B.D., Wolcott, R.G. and Degani, C., (1975), Fed. Proc., 34, 1711-1717.

Brand, M.D. and Lehninger, A.L., (1975), J. Biol. Chem., 250, 7958-7960.

Brandolin, G., Meyer, C., Defaye, G., Vignais, P.M. and Vignais, P.V., (1974), FEBS Lett., 46, 149-153.

Brierley, G. and O'Brien, R.L. (1965), J. Biol. Chem., 240, 4532-4539.

Bronk, J.R. and Kielly, W.W., (1957), Biochim. Biophys. Acta, 24, 440-441.

Bruni, A., Contessa, A.R. and Luciani, S., (1962), Biochim. Biophys. Acta, 60, 301-311.

Bruni, A., Contessa, A.R. and Scalella, P., (1965), Biochem. Biophys. Acta, 100, 1-12.

Bruni, A., Luciani, S. and Contessa, A.R., (1964), Nature, 201, 1219-1220.

Bruni, A., Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., (1966), in "Regulation of Metabolic Process in Mitochondria", 7, 275-291. BBA Library, American Elsevier Publishing Company, New York.

Cain, K., (1976), Ph.D. thesis, (Warwick University).

Caldwell, R.S., Nakaue, H.S. and Buhler, D.R., (1972), Biochem. Pharmacol., 21, 2425-2441.

Cammer, W. and Moore, C.L., (1972), Biochem. Biophys. Res. Commun., 46, 1887-1894.

Carafoli, E., (1973), Biochimie, 55, 755-762.

Carafoli, E., (1974a), Biochem. Soc. Symp., 39, 89-109.

Carafoli, E., (1974b), Biomembranes, 221-230.

Carafoli, E., (1975a), Mol. and Cell Biochem., 8, 133-140.

Carafoli, E., (1975b), "Recent Advances in Studies on Cardiac Structure and Metabolism", 5, 151-163.

Carafoli, E. and Azzi, A., (1972), Experientia, 27, 906-908.

Carafoli, E. and Gazzotti, P., (1973), Experientia, 29, 408-409.

Carafoli, E. and Lehninger, A.L., (1971), Biochem. J., 122, 681-690.

Carafoli, E. Malmström, K., Capano, M., Sigel, E. and Crompton, M., (1975a), "Calcium Transport in Contraction and Secretion, (E. Carafoli, ed.), 53-64. North Holland Publishing Company, Amsterdam.

Carafoli, E., Dabrowska, R., Crovetti, F., Tiozzo, R. and Drabikowski, W., (1975b), Biochem. Biophys. Res. Commun., 62, 908-912.

Carafoli, E., Malmström, K., Sigel, E. and Crompton, M., (1976), Clin. Endocrinol., 5, 495-595.

Carafoli, E., Prestipino, G.F., Ceccarelli, D. and Conti, F., (1974), "Membrane Proteins in Transport and Phosphorylation (eds. Azzone, G.F., Klingenberg, M.E., Quagliariello, E. and Siliprandi, N.), p. 85-90. North Holland Publishing Company, Amsterdam.

Carafoli, E. and Rossi, C.S., (1971), Advan. Cytopharmacol., 1, 209-227.

Carafoli, E. and Sacktor, B., (1972), Biochem. Biophys. Res. Commun., 49, 1498-1503.

Carafoli, E. and Sottocasa, G., (1974), "Dynamics of Energy-Transducing Membranes, (ed. L. Ernster, R. Estabrook and E.C. Slater), p.455-469. Elsevier, Amsterdam.

Chappell, J.B., (1968), Br. Med. Bull., 24, 150-157.

Chappell, J.B. and Crofts, A.R., (1965), Biochem. J., 95, 393-402.

Chappell, J.B. and Crofts, A.R., (1966), "Regulation of Metabolic Processes in Mitochondria", (ed. J.M. Tager, S. Papa, E. Quagliariello and E.C. Slater), p. 293-314. Elsevier, Amsterdam.

Chappell, J.B. and Haarhoff, K., (1967), "Biochemistry of Mitochondria", (ed. E.C. Slater, Z. Kaniuga and L. Wojtczak), p. 75-95. Academic Press, London.

Chappell, J.B., Henderson, P.J.F., McGivan, J.D. and Robinson, B.H., (1968), "The Interaction of Drugs and Subcellular Components in Animal Cells", (ed. P.N. Campbell), p. 71-95. Churchill, London.

Chappell, J.B. and Robinson, B.H., (1968), Biochem. Soc. Symp., (Cambridge, England), 27, 123-133.

Chávez, E. and Klapp, M., (1975), Biochem. Biophys. Res. Commun., 67, 272-278.

Conover, T.E., Prairie, R.L. and Racker, E., (1963), J. Biol. Chem., 238, 2831-2837.

Cooper, C., Davlin, T.M. and Lehninger, A.L., (1955), Biochim. Biophys. Acta, 18, 159-160.

Cooper, C. and Lehninger, A.L., (1956), J. Biol. Chem., 219, 519-529.

Cooper, C. and Lehninger, A.L., (1957), J. Biol. Chem., 224, 561-578.

Cosmides, G.J., (1974), Science, 183, 1219-1221.

Coty, W.A. and Pedersen, P.L., (1974), J. Biol. Chem., 249, 2593-2598.

Coty, W.A. and Pedersen, P.L., (1975), J. Biol. Chem., 250, 3515-3521.

Dawson, A.P. and Selwyn, M.J., (1974), Biochem. J., 138, 349-357.

Debise, R. and Durand, R., (1974), Biochimie, 56, 161-170.

Defaye, G., Horton, D. and Wander, D., (1973), Bull. Soc. Chim., P.T.2, 615-617.

Defaye, G., Vignais, P.M. and Vignais, P.V., (1971), C.R. Acad. Sci. Paris, 273, 2671-2673.

Dell'Antone, P., Colonna, R. and Azzone, G.F., (1972), Eur. J. Biochem., 24, 553-565.

Dixon, M., (1953), Biochem. J., 55, 170-171.

Drahota, Z., Carafoli, E., Rossi, C.S., Gamble, R.L. and Lehninger, A.L., (1965), J. Biol. Chem., 240, 2712-2720.

Duee, E.D. and Vignais, P.V., (1965), Biochem. Biophys. Acta, 107, 184-188.

Duee, E.D. and Vignais, P.V., (1969), J. Biol. Chem., 244, 3920-3931.

Duee, E.D. and Vignais, P.V., (1969), J. Biol. Chem., 244, 3932-3940.

Duszynski, J. and Wojtczak, L., (1974), FEBS Lett., 40, 72-76.

Erdelt, H., Weidemann, M.J., Buchholz, M. and Klingenberg, M., (1972), Eur. J. Biochem., 30, 107-122.

Ernster, L. and Lee, C.P., (1964), Ann. Rev. Biochem., 33, 729-779.

Evans, D.W.S., (1959), J. Chem. Soc., 1, 1313-1314.

Fayle, D.R.H., Barritt, G.J. and Bygrave, F.L., (1975), Biochem. J., 148, 527-531.

Fisher, R.J., Chen, J.C., Sani, B.P., Kaplay, S.S. and Sanadi, D.R., (1971), Proc. Nat. Acad. Sci. USA., 68, 2181-2184.

Fiske, C.H. and Subbarow, Y., (1925), J. Biol. Chem., 66, 375-400.

Fleischer, S., Fleischer, B. and Stoeckenius, W., (1967), J. Cell Biol., 32, 193-208.

Flores, G. and Buhler, D.R., (1971), Fed. Proc., 30, 1199.

Fonyo, A., (1968), Biochem. Biophys. Res. Commun., 32, 624-628.

Fonyo, A. and Bessman, S.P., (1968), Biochem. Med., 2, 145-163.

Fonyo, A., Palmieri, F., Quagliariello, E., (1976), "Biochemisty and Biophysics", 2, 60-105, (ed. Quagliariello, E., Palmieri, F., Singer, T.P.). Addison-Wesley Publishing Company, London.

Frederick, J.J., Corner, T.R., Gerhardt, P., (1974), Antimicrobial Agents and Chemotherapy, 16, 712-721.

Gear, A.R.L., (1974), J. Biol. Chem., 249, 3628-3637.

Gornall, A.G., Bardawill, C.J. and David, M.M., (1949), J. Biol. Chem., 177, 751-766.

Green, D.E., Asai, J., Harris, R.A. and Pennistan, J.T., (1968), Arch. Biochem. Biophys., 125, 684-705.

Green, D.E. and Ji, S., (1972a), Proc. Nat. Acad. Sci.USA., 69, 726-729.

Green, D.E. and Ji, S., (1972b), J. Bioenerg., 3, 159-202.

Groot, G.S.P., Kovac, L. and Schatz, G., (1971), Proc. Nat. Acad. Sci. USA, 68, 308-311.

Gump, W.S., (1969), J. Soc. Consmetic. Chemists, 20, 173-184.

Gutfreund, H., (1975), "Enzymes: Physical Principles", John Wiley and Sons, London.

Hanstein, W.G. and Hatefi, Y., (1974), J. Biol. Chem., 249, 1356-1362.

Hague, R. and Buhler, D.R., (1972), J. Am. Chem. Soc., 94, 1824-1828.

Hatefi, Y, Hanstein, W.G., Galante, Y.and Stiggal, D.L., (1975), Fed. Proc., 34, 1699-1706.

Heldt, H.W., (1969), "Inhibitors Tools in Cell Research", (ed. T. Bücher and H. Sies), 301-317. Springer Verlog., Berlin.

Henderson, P.J.F. and Lardy, H.A., (1970), J. Biol. Chem., 245, 1319-1326.

Henderson, P.J.F., Lardy, H.A. and Dorschner, E., (1970), Biochemistry, 9, 3453-3457.

Hock, J.B., Lofrumento, N.E., Meyer, A.J. and Tager, J.M., (1971), Biochem. Biophys. Acta, 226, 297-308.

Huang, C., Charlton, J.P. and Litman, B.J., (1969), Biophys. J., 9, A-37.

Jacobus, W.E., Tiozzo, R., Lugli, G., Lehninger, A.L. and Carafoli, E., (1975), J. Biol. Chem., 250, 7863-7870.

Johnson, R.N. and Chappell, J.B., (1973), Biochem. J., 134, 769-774.

Johnson, D. and Lardy, H.A., (1967), "Methods in Enzymology", (ed. R.W. Estabrook and M.E. Pullman), 10, 94-96.

Kagawa, Y. and Racker, E., (1971), J. Biol. Chem., 246, 5477-5487.

Kemp, Jr. A., (1970), Biochem. J., 116, 10p.

Kemp, Jr. A., Souverijn, J.H. and Out, T.A., (1971), Energy Transduction in Respiration and Photosynthesis, 43, 959-969.

Klingenberg, M., (1970), "Essays in Biochemistry", (ed. P.N. Campbell and F. Dickens), 6, 119-159. Academic Press, London.

Klingenberg, M., (1974), "Dynamics of Energy-Transducing Membrane", (ed. L. Ernster, R. Estabrook and E. Slater), p. 511-528. Elsevier Publishing Company, Amsterdam.

Klingenberg, M., (1975), CIBA Found. Symp., 31, 105-124.

Klingenberg, M., (1976), in "Mitochondria, Bioenergetics, Biogenesis and Membrane Structure", p. 127-149. Academic Press, New York.

Klingenberg, M., Buchholz, M., Erdelt, H., Falkner, G., Grebe, K., Kadner, H., Scherer, B., Stengel-Rutkowski, L. and Weidemann, M.J., (1972), in "Biochemistry and Biophysics of Mitochondrial Membranes", (ed. G.F. Azzone et al.,), p. 465-486. Academic Press, New York/London.

Klingenberg, M., Grebe, K. and Scherer, B., (1975), Eur. J. Biochem., 52, 351-363.

Klingenberg, M. and Pfaff, E., (1965), in "Regulation of Metabolic Processes in Mitochondria, (ed. Tager, J.M., Papa, S., Quagliariello, E., Slater, E.C.), p. 180-201. Elsevier Publishing Company, Amsterdam.

Klingenberg, M. and Pfaff, E., (1966), in "Regulation of Metabolic Processes in Mitochondria" (ed. Tager, J.M., Papa, S., Quagliariello, E., Slater, E.C.), 7, 190. Elsevier Publishing Company, Amsterdam.

Klingenberg, M. and Pfaff, E., (1968), in "The Metabolic Roles of Citrate", (ed. T.W. Goodwin), Biochem. Soc. Symp., 27, 105-122, Academic Press, New York/London.

Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B.B. and Grebe, K., (1976), in "The Structural Basis of Membrane Function", p. 293-311, Academic Press, London.

Klingenberg, M., Riccio, P., Aquila, H., Schmiedt, B., Grebe, K. and Topitsch, P., (1974), in "Membrane Proteins in Transport and Phosphorylation", (ed. G.F. Azzone, M. Klingenberg, E. Quagliariello and N. Siliprandis), p. 229-243. North Holland Publishing Company, Amsterdam.

Klingenberg, M., Scherer, B., Stengel-Rutowski, L., Buchholz, M. and Greb, K., (1973), in "Mechanism in Bioenergetics", (eds. G.F. Azzone, L. Ernster, S. Papa, E. Quagliariello and N. Siliprandi), p. 257-287, Academic Press, New York.

Kovac, L., Bednarova, H. and Greksak, M., (1968), Biochim. Biophys. Acta, 153, 32-42.

Lardy, H.A., Connelly, T.L. and Johnson, D., (1964), Biochemistry, 3, 1961-1968.

Lardy, H.A., Reed, P. and Lin, C.C., (1975), Fed. Proc., 34, 1707-1710.

Lardy, H.A. and Wellman, H., (1953), J. Biol. Chem., 201, 357-370.

Lauquin, G.J.M., Duplaa, A., Klein, G., Rousseau, A. and Vignais, P.V., (1976), Biochemistry, 15, 2323-2327.

Lauquin, G.J.M. and Vignais, P.V., (1973), Biochim. Biophys. Acta, 305, 534-556.

Lauquin, G.J.M. and Vignais, P.V., (1976), Biochemistry, 15, 2316-2322.

Lazarewicz, J.W. and Hamberger, A., (1975), Bulletin de L'Academie Polonaise des Sceiences Serie des Sciences Biologiques, 23, 143-151.

Lefranc, M., (1968), C.R. Acad. Sci. Paris, 67, 954-956.

Lehninger, A.L., (1969), Ann. N.Y. Acad. Sci., 147, 816-823.

Lehninger, A.L., (1971), Biomembrane, 2, 147-164.

Lehninger, A.L., (1974), Proc. Nat. Acad. Sci. USA., 71, 1520-1524.

Lehninger, A.L. (1975), Biochemistry, 2nd edn., Worth Inc., New York (publishers).

Lofrumento, N.E., Zanotti, F. and Papa, S., (1974), FEBS Lett., 48, 188-191.

Löw, H., Siekevitz, P., Ernster, L. and Lindberg, O., (1957), Biochim. Biophys. Acta, 26, 224-225.

Luciani, S., Martini, N. and Santi, R., (1971), Life Sci., 10, 961-968.

Luciani, S. and Varotto, R., (1975), FEBS Lett., 56, 194-197.

Luthra, R. and Olson, M.S., (1976), Biochim. Biophys. Acta, 440, 744-758.

Manger, J.R., (1969), FEBS Lett., 5, 331-334.

Massari, S. and Azzone, G.F., (1970), Eur. J. Biochem., 12, 300-319.

McGivan, J.D. and Klingenberg, M., (1971), Eur. J. Biochem., 20, 392-399.

Meijer, A.J., Groot, G.S. and Tager, J.M., (1970), FEBS Lett., 8, 41-44.

Meijer, A.J. and Tager, J.M., (1969), Biochim. Biophys. Acta, 189, 136-139.

Meisner, H., (1971), Biochemistry, 10, 3485-3491.

Meisner, H., (1973), Biochim. Biophys. Acta, 318, 383-389.

Mitchell, P., (1953), J. Gen. Microbiol., 9, 273-278.

Mitchell, P., (1961), Nature, 191, 144-148.

Mitchell, P., (1966), Biol. Rev., 41, 445.

Mitchell, P., (1969), in "Theoretical and Experimental Biophysics", (ed. A.C. Cole), 2, 159-216, Marcel Dekker, New York.

Mitchell, P., (1973), FEBS Lett., 33, 267-274.

Mitchell, R.A., Hill, R.D. and Boyer, P.D., (1967), J. Biol. Chem., 242, 1793-1801.

Moore, C.L., (1971), Biochem. Biophys. Res. Commun., 42, 298-305.

Myers, D.K. and Slater, E.C., (1957), Biochem. J., 67, 558-572.

Nakaue, H.S., Caldwell, R.S. and Buhler, D.R., (1972), Biochem. Pharmacol., 21, 2273-2277.

Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M., (1971), Eur. J. Biochem., 22, 66-74.

Palmieri, F., Stipani, I., Quallariello, E. and Klingenberg, M., (1972), Eur. J. Biochem., 26, 587-594.

Papa, S., Guerrieri, F., Lorusso, M. and Quagliariello, E., (1970), FEBS Lett., 10, 295-298.

Papa, S., Kanduc, D. and Lofrumento, N.E., (1973), FEBS Lett., 36, 9-11.

Parker, V.H., (1965), Biochem. J., 97, 658-662.

Patterson, M.S. and Green, R.C., (1965), Anal. Chem., 37, 854-856.

Pfaff, E. and Klingenberg, M., (1968), Eur. J. Biochem., 6, 66-79.

Pfaff, E., Klingenberg, M. and Heldt, H.W., (1965), Biochim. Biophys. Acta, 104, 312-315.

Pressman, B.C., (1976), Ann. Rev. Biochem., 45, 501-530.

Quagliariello, E., Passarella, S. and Palmieri, F., (1974), in "Dynamics of Energy Transducing Membranes", (ed. L. Ernster, R.W. Estabrook and E.C. Slater), p. 483-495, Elsevier Scientific Publishing Company, Amsterdam.

Reed, K.C. and Bygrave, F.L., (1974a), Biochem. J., 140, 143-155.

Reed, K.C. and Bygrave, F.L., (1974b), Biochem. J., 142, 555-566.

Reed, K.C. and Bygrave, F.L., (1975a), Eur. J. Biochem., 55, 497-504.

Reed, K.C. and Bygrave, F.L., (1975b), Anal. Chem., 67, 44-54.

Reed, K.C. and Lardy, H.A., (1975), J. Biol. Chem., 250, 3704-3708.

Reynafarje, B. and Lehninger, A.L., (1969), J. Biol. Chem., 244, 584-593.

Reynafarje, B. and Lehninger, A.L., (1973), Proc. Nat. Acad. Sci.USA., 70, 1744.

Reynafarje, B. and Lehninger, A.L., (1974), in "Dynamics of Energy Transducing Membranes", (ed. L. Ernster, R.W. Estabrook and E.C. Slater), p. 447-454, Elsevier Scientific Publishing Company, Amsterdam.

Robinosn, B.H. and Williams, G.R., (1970), Biochim. Biophys. Acta, 216, 63-70.

Rojas, E. and Tobias, J.M., (1965), Biochim. Biophys. Acta, 94, 394-404.

Rossi, C.S., Alexandre, A. and Rossi, C.R., (1974), FEBS Lett., 43, 349-352.

Rossi, C.S., Azzi, A. and Azzone, G.F., (1967), J. Biol. Chem., 242, 951-957.

Rossi, C.S. and Lehninger, A.L., (1963), Biochem. Biophys. Res. Commun., 11, 441-446.

Rossi, C.S., Vasington, F.D. and Carafoli, E., (1973), Biochem. Biophys., Res. Commun., 50, 846-852.

Rottenberg, H., (1970), Eur. J. Biochem., 15, 22-28.

Rottenberg, H. and Scarpa, A., (1974), Biochemistry, 13, 4811-4817.

Senadi, D.R., Lam, K.W. Ramakrishu, A. and Kurup, C.K., (1968), Proc. Nat. Acad. Sci. USA., 61, 277-283.

Scarpa, A. and Azzi, A., (1968), Biochem. Biophys. Acta, 150, 473-481.

Scarpa, A. and Azzone, G.F., (1969), Biochem. Biophys. Acta, 173, 78-85.

Scatchard, G., (1949), Ann. N.Y. Acad. Sci., 51, 660-672.

Scherer, B., Grebe, K., Riccio, P. and Klingenberg, M., (1973), FEBS Lett., 31, 15-19.

Seeman, P., (1972), Pharmacol. Rev., 24, 583-655.

Selwyn, M.J., Dawson, A.P., Stockdale, M. and Gains, N., (1970a), Eur. J. Biochem., 14, 120-126.

Selwyn, M.J., Stockdale, M. and Dawson, A.P., (1970b), Biochem. J., 116, 15P.

Serrano, R., Kanner, B.I. and Racker, E., (1976), J. Biol. Chem., 251, 2453-2461.

Shertzer, H.G. and Racker, E., (1974), J. Biol. Chem., 249, 1320-1321.

Shertzer, H.G. and Racker, E., (1976), J. Biol. Chem., 251, 2446-2452.

Shrago, E., Shug, A., Elson, C., Spennetta, T. and Crosby, C., (1974), J. Biol. Chem., 249, 5269-5274.

Siekevitz, P., Löw, H., Ernster, L. and Lindberg, O., (1958), Biochim. Biophys. Acta, 29, 378-391.

Skilleter, D.N., (1975), Biochem. J., 146, 465-471.

Slater, E.C., (1953), Nature, 172, 975-978.

Slater, E.C. and Cleland, K.W., (1953), Biochem. J., 55, 566-580.

Sluse, F.E. and Sluse-Goffart, C.M., (1974), FEBS Advanced Course on Mathematical Models of Metabolic Regulations, p. 149, Dobogoko, Hungary.

Sone, N. and Hagihara, B., (1964), J. Biochem., 56, 151-156.

Sordal, L.A. and Silver, B.B., (1975), "Recent Advances in Studies on Cardiac Structure and Metabolism", (ed. A. Fleckenstein and G. Rona), 6, 85-93, University Park Press, Baltimore, U.S.A.

Souverijn, J.H.M., Huisman, L.A., Rosing, J. and Kemp, Jr. A., (1973), Biochem. Biophys. Acta, 305, 185-198.

Spencer, T. and Bygrave, F.L., (1971), Proc. Aust. Biochem. Soc., 4, 97-101.

Spencer, T. and Bygrave, F.L., (1972), Biochem. J., 129, 355-365.

Spencer, T. and Bygrave, F.L., (1973), J. Bioenerg., 4, 347-362.

Spencer, T. and Bygrave, F.L., (1974), Biochem. J., 140, 413-422.

Spencer, T.L., See, J.K. and Bygrave, F.L., (1976), Biochem. Biophys. Acta, 423, 365-373.

Stockdale, M., Dawson, A.P. and Selwyn, M.J., (1970), Eur. J. Biochem., 15, 342-351.

Stryer, L., (1975), Biochemistry, Chapter 14, pp. 331-355, W.H. Freman and Company, San Francisco, U.S.A.

Sul, H.S., Shrago, E. and Shug, A.L., (1976), Arch. Biochem. Biophys., 172, 230-237.

Swanson, M.A., (1956), Biochem. Biophys. Acta, 20, 85-91.

Tsuchiya, T. and Rossen, B.P., (1976), J. Biol. Chem., 251, 962-967.

Thorne, R.F.W. and Bygrave, F.L., (1974), FEBS Lett., 41, 118-121.

Tyler, D.D., (1968), Biochem. J., 107, 121-123.

Tyler, D.D., (1969), Biochem. J., 111, 665-678.

Vallieres, J., Scarpa, A. and Somlyo, A.P., (1975), Arch. Biochem. Biophys. , 170, 659-669.

Verdouw, H. and Bertina, M., (1973), Biochem. Biophys. Acta, 325, 385-396.

Vignais, P.V., (1976), Biochem. Biophys. Acta, 456, 1-38.

Vignais, P.V., Duee, E.D., Vignais, P.M. and Huet, J., (1966), Biochem. Biophys. Acta, 118, 465-483.

Vignais, P.V. and Vignais, P.M., (1970), Biochem. Biophys. Res. Commun., 38, 843-847.

Vignais, P.V., Vignais, P.M. and Colomb, M.G., (1970), FEBS Lett., 8, 328-332.

Vignais, P.V., Vignais, P.M. and Defaye, G., (1971a), FEBS Lett., 17, 281-288.

Vignais, P.V., Vignais, P.M., Colomb, M.G., Chabert, J. and Doussiere, J., (1971b), in "Energy Transduction in Respiration and Photosynethesis", 43, 675-690.

Vignais, P.V., Vignais, P.M. and Defaye, G., (1973a), Biochemistry, 12, 1508-1515.

Vignais, P.V., Vignais, P.M., Lauquin, G. and Morel, F., (1973b), Biochimie, 55, 763-778.

Vignais, P.V., Vignais, P.M. and Doussiere, J., (1975), Biochem. Biophys. Acta, 376, 219-230.

Vignais, P.V., Vignais, P.M. and Stanislas, E., (1961), Biochem. Biophys. Acta, 51, 394-396.

Vignais, P.V., Vignais, P.M. and Stanislas, E., (1962), Biochem. Biophys. Acta, 60, 284-300.

Wadkins, C.L. and Lehninger, A.L., (1957), J. Amer. Chem. Soc., 79, 1010-1011.

Wadkins, C.L. and Lehninger, A.L., (1959), J. Biol. Chem., 234, 681-687.

Weidemann, M.J., Erdelt, H. and Klingenberg, M., (1970), Eur. J. Biochem., 16, 313-335.

Werkheiser, W.C. and Bartley, W., (1957), Biochem. J., 66, 79-91.

Williams, G.R. and Orr, J.L., (1976), Can. J. Biochem., 54, 165-170.

Winkler, H.H., Bygrave, F.L. and Lehninger, A.L., (1968), J. Biol. Chem., 243, 20-28.

Winkler, H.H. and Lehninger, A.L., (1968), J. Biol. Chem., 243, 3000-3008.

Yaginuma, N., Hirose, S. and Inuda, Y., (1973), J. Biol. Chem., 74, 811-815.

Young, J.H., Blondin, G.A. and Green, D.E., (1972), Int. J. Neurosci., 3, 207-213.