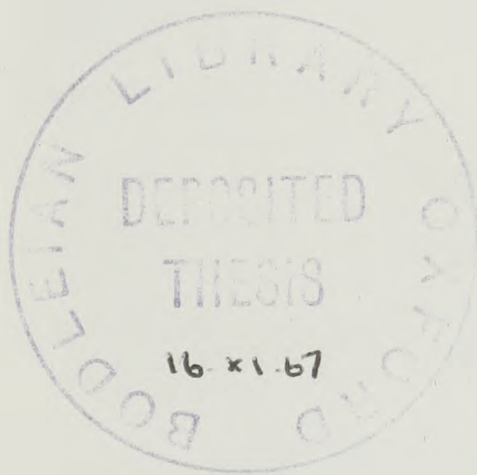


STUDIES ON SOME ASPECTS OF OXIDATIVE PHOSPHORYLATION



A thesis submitted to the University of Oxford
for the degree of Doctor of Philosophy

by

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Trinity Term

1967

Keble College,

Oxford

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. D. E. Griffiths, for introducing me to the subject and for the valuable advice and support he has given me at all stages of the work.

I would also like to thank:

Professor Sir Hans Krebs F.R.S. for the privilege of working in his department, and for suggesting the work described in Chapter 5.

Professor V. M. Clark and the authorities of Warwick University for enabling me to work for the final year in the School of Molecular Sciences, University of Warwick.

Mrs. A. V. Thomas and Mrs. M. Young for their technical assistance.

I am grateful to the Medical Research Council for the award of a Scholarship for Training in Research Methods.

ABBREVIATIONS

AMP	Adenosine-5'-monophosphate
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
CoA	Coenzyme A
CCP	Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol di (aminoethyl) tetra-acetic acid
F6P	Fructose-6-phosphate
G6P	Glucose-6-phosphate
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
HMP	Hexose monophosphate
NAD	Nicotinamide adenine dinucleotide (Oxido-reduction state not specified)
NAD ⁺	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide diphosphate (oxido-reduction state not specified)
NADP ⁺	Oxidised nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAD(P)	= NAD + NADP
NAD(P) ⁺	= NAD ⁺ + NADP ⁺
NAD(P)H	= NADH + NADPH
P _i	Inorganic phosphate
TMFD	2,3,5,6 tetramethyl phenylene diamine
Tricine	Tris (hydroxymethyl) methyl glycine

MATERIALS

Standard laboratory reagents were obtained from British Drug Houses Ltd. in the purest analytical grades available. Norit A charcoal was cleaned by boiling in 40% ethanol.

Enzymes and standard biochemicals were purchased from Boehringer and Soehne, G. m. b. H., Mannheim, Germany, or from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Other compounds were obtained from the following sources:-

Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; rotenone.

Bio-Rad Labs., Richmond, California, U.S.A.; Dowex-1-X8 and Dowex-50-X4 resins.

Eastman Kodak Co., Rochester, New York, U.S.A.; TMPD.

General Biochemicals, Chagrin Falls, Ohio, U.S.A.; tricine.

Koch-Light Labs., Colnbrook, England; EGTA.

Kyowa Fermentation Co., Japan; antimycin A.

Mann Research Labs., New York, U.S.A.; oligomycin A, gramicidin, valinomycin.

Pharmacia, Uppsala, Sweden; G25-, G100-, G200-, CM-, and DEAE-Sephadex.

These resins were washed by the procedure of Mandeles (1960).

Radiochemical Centre Amersham; carrier free $^{32}\text{P}_1$ (3-5 mC/ml.), PBS 1.

Rohm and Haas Co., Philadelphia, U.S.A.; Triton X-100.

Teikoku Chemical Industry Co., Ltd., Osaka, Japan; Nagarse proteinase.

Atractyloside was the gift of Dr. P. Vignais.

Aurovertin and Valinomycin were given by Professor H. A. Lardy.

CCP was a gift from Dr. P. G. Heytler.

Octylguanidine and TTFB were the gift of Dr. R. B. Beechey.

Thin teflon membrane for use in the Clark electrode was the gift of Professor W. B. Elliot.

Solutions of oligomycin A, aureoverdin and rotenone were made up in absolute ethanol. Octylguanidine, valinomycin and gramicidin were dissolved in a mixture of 50% ethanol, 50% water. All other solutions were made up in double-distilled water.

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ABSTRACT

This thesis presents the results of studies on some aspects of oxidative phosphorylation, and of mitochondrial reactions which utilize the energy of oxidative phosphorylation.

The Stoichiometry of Oxidative Phosphorylation

- (1) ADP:O and P:O ratios of rat liver mitochondria (RLM) and heavy beef heart mitochondria (RHBM) were re-examined under a number of conditions, and by independent methods of measuring oxygen consumption and P_i esterification.
- (2) When appropriate precautions were taken ADP:O and P:O ratios obtained by all methods and under each of the conditions closely agreed, and did not exceed 3 for pyruvate plus L-malate, 2 for succinate, and 4 for α -oxoglutarate oxidation.
- (3) The high P:O ratios reported by Smith & Hansen (1964), Gurban & Cristea (1964, 1965) and Lynn & Brown (1965) were shown to be caused by an under-estimation of oxygen consumption.

The Primary Phosphate Acceptor in Oxidative Phosphorylation

- (1) The nature of the nucleotide acceptor, to which P_i is first linked in oxidative phosphorylation, was investigated by measuring the labelling by $^{32}P_i$ of the bound adenine nucleotides of RHBM and phosphorylating sub-mitochondrial particles (STP_H) during the first 2 min. of succinate oxidation.
- (2) In all labelling experiments in the absence of uncouplers $AT^{32}P$ had a higher specific radioactivity than $AD^{32}P$, and it was concluded that ADP is the primary endogenous acceptor of $^{32}P_i$. This disagrees with the results of

Ozawa & MacLennan (1965), who claimed that $AD^{32}P$ contained most of the radioactivity in such experiments. Possible reasons for this discrepancy are discussed.

(3) In the presence of uncouplers, $AD^{32}P$ had a higher specific activity than $AT^{32}P$ in labelling experiments with HBHM. The labelling of $AD^{32}P$ was inhibited by arsenite, showing that it was caused by substrate-level phosphorylation. It is postulated that in substrate-level phosphorylation the sequence of labelling of endogenous nucleotides is $GT^{32}P \rightarrow AD^{32}P \rightarrow AT^{32}P$, catalysed by succinate thiokinase, nucleoside monophosphate kinase and myokinase, as shown by Heldt & Schwalbach (1967) for rat liver mitochondria.

(4) The ability of AMP and ADP to stimulate the respiration of HBHM was investigated, and the phosphorylation of adenine nucleotides in such experiments was measured. It was found that AMP could only stimulate respiration in the presence of added Mg^{2+} . In the absence of Mg^{2+} , or if a concentration of EDTA greater than that of Mg^{2+} was added, AMP was not phosphorylated. Under all the above conditions ADP stimulated the respiration of HBHM, and more than 90% of the ADP was converted to ATP at the State 3 $\rightarrow$ State 4 transition.

(5) It was concluded that ADP is the primary external phosphate acceptor in HBHM, and that AMP is only indirectly phosphorylated in oxidative phosphorylation via the myokinase reaction, which requires added Mg^{2+} . The results of Ozawa (1966), who claimed that AMP was the primary phosphate acceptor, may be explained by wrong assumptions concerning the calculation of AMP:O ratios, and possible errors in the isolation and assay of adenine nucleotides.

The Purification of Coupling Factors, and the Effect of NAD^+ and NADH on the AD^{32}P -ATP and $^{32}\text{P}_i$ -ATP Exchange Reactions Catalysed by such Fractions

- (1) Attempts to purify a soluble factor from mitochondria, which was specific for oxidative phosphorylation at Site 1 in depleted sub-mitochondrial particles, were unsuccessful, but the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction of the supernatant from sonicated HMMH increased the P:O ratios of 'salt-extracted particles' (Green et al., 1963) and WETP_H (Linnane & Titchener, 1960) with NADH or succinate as substrate.
- (2) This crude preparation of coupling factor had a high AD^{32}P -ATP exchange activity, and a small $^{32}\text{P}_i$ -ATP exchange activity which depended in a complex way on the relative concentrations of P_i , ADP and ATP, and was inhibited by uncouplers or inhibitors of oxidative phosphorylation.
- (3) The effect of NAD^+ and NADH on the AD^{32}P -ATP and $^{32}\text{P}_i$ -ATP exchange reactions catalysed by the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction was investigated over a wide range of experimental conditions, but no significant increases or decreases in activity were observed. These results are discussed in relation to the chemical theory of oxidative phosphorylation.
- (4) The P_i -ATP exchange activity of the crude coupling factor was further characterised, and was shown to be associated with a high molecular weight component containing cytochromes a, b and c, which could be spun down at 150,000 g for 1 hr. It was concluded that the P_i -ATP exchange activity is associated with small fragments of the mitochondrial membrane and is not a soluble or homogeneous enzyme.
- (5) It was shown that although much of the AD^{32}P -ATP exchange activity of the crude coupling factor was associated with myokinase, there was an excess

of $AD^{32}P$ -ATP exchange activity which was possibly associated with the coupling factor.

The Mechanism of Penetration of Oxaloacetate and L-malate into Mitochondria

- (1) The penetration of oxaloacetate and L-malate into mitochondria was investigated by measuring the effect of oxaloacetate or L-malate on the concentration of intramitochondrial NADH, using the Aminco-Chance dual-wavelength spectrophotometer at 340-374 m μ .
- (2) NADH oxidase was inhibited by rotenone plus antimycin A, and as the activity of the mitochondrial malate dehydrogenase under the test conditions was very high in relation to the rates of penetration of oxaloacetate and L-malate, the initial rate of oxidation of NADH was a measure of the rate of penetration of oxaloacetate, and the initial rate of reduction of NAD^+ was a measure of the penetration of L-malate.
- (3) The rates of penetration of oxaloacetate and L-malate were greatly stimulated by TMPD plus ascorbate or by preincubation with ATP, and this stimulation was abolished by uncouplers. Oligomycin A, aureoverdin, octylguanidine and atractyloside inhibited the stimulation by ATP, but slightly increased or did not affect the stimulation by TMPD plus ascorbate.
- (4) The penetration of oxaloacetate and L-malate into liver mitochondria obeyed Michaelis-Menten Kinetics, and apparent K_m values were respectively 40 μM and 130 μM at 20°. The K_m values were similar both in the presence or absence of an energy-supply, but V_{max} values were greatly increased by TMPD plus ascorbate or ATP.
- (5) Arrhenius plots of the temperature-dependence of the penetration of oxaloacetate and L-malate above 12° gave activation-energies of +10 and

+8 Kcal/mole respectively, both in the presence or absence of an energy-supply.

(6) The penetration of oxaloacetate and L-malate was competitively inhibited by D-malate, malonate, succinate, mesotartrate, malate and citraconate, both in the presence or absence of an energy-supply. The K_i values of these inhibitors were similar for penetration of both oxaloacetate and L-malate.

(7) Valinomycin or Gramicidin plus KCl had a twofold effect on the penetration of oxaloacetate in the presence of an energy-supply. In the presence of low concentrations of KCl, rates of penetration of oxaloacetate and L-malate were stimulated, but in the presence of higher concentrations of KCl rates of penetration were inhibited.

(8) EDTA and EGTA (up to 5 mM) had no effect on the rates of penetration of oxaloacetate into RIM prepared in the presence of 0.1 mM EDTA. If mitochondria were depleted of bound Ca^{2+} by preparation in the presence of 1 mM EGTA, the stimulation of the penetration of oxaloacetate by TNPD plus ascorbate or by ATP was greatly reduced. The energy-dependence could be restored by addition of 0.1 mM CaCl_2 . It was concluded that bound Ca^{2+} was required for the stimulation of oxaloacetate penetration by an energy-supply.

(9) Preliminary experiments have been performed to investigate the stoichiometry of the energy-requirement for the stimulation of oxaloacetate penetration into RIM. Initial rates of penetration of oxaloacetate were, measured spectrophotometrically, and 'extra' ATPase activity (see Klingenberg 1963), or oxidation of ascorbate was measured under identical conditions. It was found that 1.5-1.7 'extra' molecules of oxaloacetate entered the

mitochondria per high-energy bond consumed, but as this only applies to the initial rate of penetration, it is not known whether the stoichiometry is fixed.

(10) These results suggest that oxaloacetate and L-malate enter mitochondria by a carrier-system which is probably the same for both these anions. The carrier system is energy-dependent and activated by the bound Ca^{2+} of the mitochondria. Possible mechanisms for the carrier-system are discussed.

The Temperature-Dependence of Energy-linked Reactions in Mitochondria and Sub-mitochondrial Particles

(1) The temperature dependence of the following energy-linked reactions were investigated:-

(a) The energy-linked transhydrogenase in sub-mitochondrial particles from rat liver and beef heart.

(b) The ATP-driven reduction of NAD^+ by succinate in sub-mitochondrial particles from rat liver and beef heart.

(c) The stimulation of the respiration of HBM by ADP.

(d) The stimulation of the penetration of oxaloacetate and L-malate into rat liver mitochondria by TMPD plus ascorbate or ATP.

(2) Arrhenius plots of the temperature-dependence of all these reactions were similar in form. Plots of respiration-driven reactions did not show a marked break, but plots of ATP-driven reactions showed a sharp break in the temperature range 8-17°C. Below this 'transition-temperature' the activation-energies of the reactions were greatly increased.

(3) It is postulated that the sharp break in the Arrhenius plots of ATP-driven reactions reflect a configurational change in the enzyme(s) which

I(i) General Introduction

In aerobic organisms most of the energy used to drive endergonic reactions is supplied by the oxidation of fatty acids and the substrates of the tricarboxylic acid cycle to carbon dioxide and water. These reactions are catalysed by multienzyme complexes which in plants and animals are located in the mitochondria, and energy is conserved and converted to suitable forms by the process of oxidative phosphorylation. This process is carried out by a particulate multienzyme complex residing in the membrane of the mitochondrial cristae, and couples the large free energy changes associated with the passage of reducing equivalents along the respiratory chain to the formation of the pyrophosphate bond-energy of ATP.

Detailed accounts of the respiratory chain and oxidative phosphorylation may be found in the reviews by Chance & Williams (1956), Racker (1961, 1965), Lehninger & Wadkins (1962), Massey & Veeger (1963), Lehninger (1964), Ernster & Lee (1964), Griffiths (1965), Slater (1958, 1967a), and Mitchell (1966a,b).

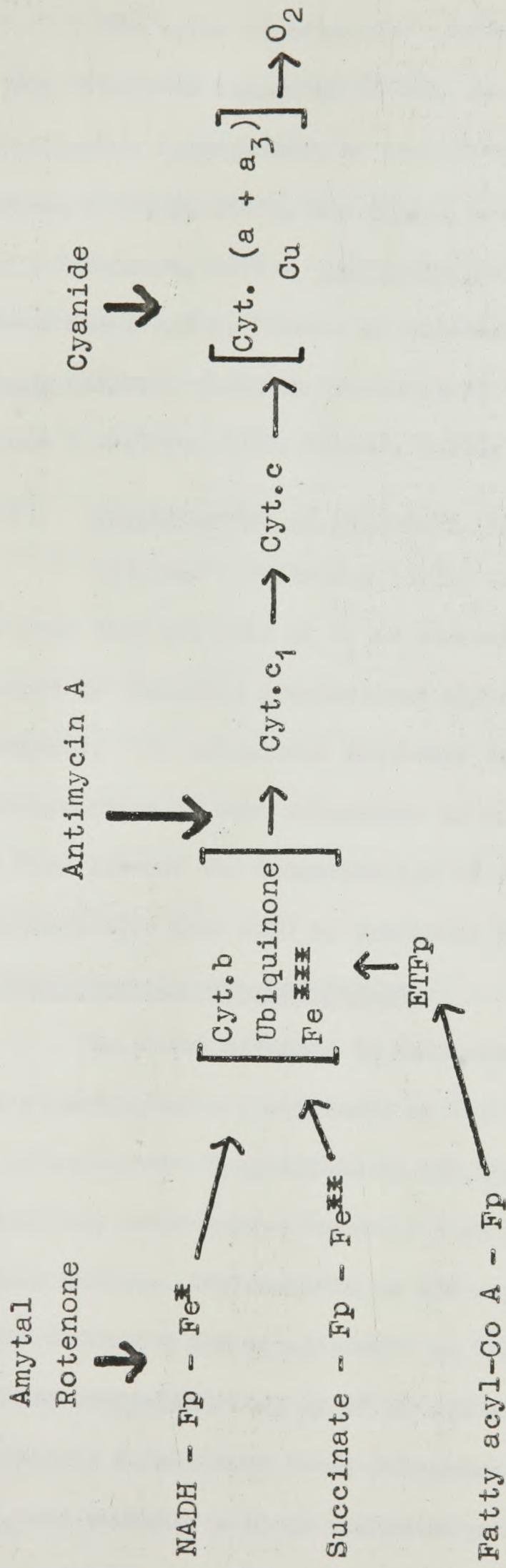
In this chapter those aspects of oxidative phosphorylation which are relevant to the work carried out for this thesis will be discussed.

I(ii) Components of the Respiratory Chain

The current concept of the respiratory chain is summarised in Fig. 1A. Reducing equivalents enter the respiratory chain via a number of specific flavoprotein enzymes, and pass along the chain by a sequence of oxido-reduction reactions between adjacent carriers, finally reacting with oxygen via the cytochrome oxidase complex. The sequence of the respiratory carriers in the cytochrome b-ubiquinone region, and in the cytochrome oxidase area are still the subject of controversy.

Figure 1A. The Respiratory Chain in Animal Tissues, and the Sites of

Action of Inhibitors of Electron Transport.



The figure has been adopted from Griffiths (1965). The heavy arrows represent the sites of action of inhibitors of electron transport. Abbreviations: Cyt., cytochrome; Fp, flavoprotein; ETFp, electron transfer flavoprotein; Fe^{II}, iron metalloprotein associated with NADH dehydrogenase; Fe^{III}, iron metalloprotein associated with succinate dehydrogenase; Fe^{III}, iron metalloprotein associated with ubiquinol-cytochrome c reductase.

The sites of action of inhibitors of electron transport used in this thesis are indicated in Fig. 1A. Electron transport from NADH dehydrogenase flavoprotein to the cytochromes is inhibited by rotenone (Lindahl & Oberg, 1960), and amytal (Ernster, Jalling, Löw & Lindberg, 1955 ; Estabrook, 1957). Antimycin A inhibits electron transport between cytochromes b and c, (Chance & Williams, 1956; Keilin & King, 1960). Cyanide inhibits electron transport at the cytochrome oxidase complex (Keilin & Hartree, 1939; Chance, 1953).

I(III) Stoichiometry of Oxidative Phosphorylation

Belitzer & Tsibakova (1939) and Ochoa (1940, 1941) observed that more than one mole of P_i is esterified for every atom of oxygen consumed by respiring preparations under conditions where there is no glycolysis. The subsequent discovery that the NADH formed by the dehydrogenation of most substrates is oxidised by the respiratory chain (see Fig. 1A) and the demonstration of Friedkin & Lehninger (1948) of phosphorylation with NADH as substrate established the concept of respiratory-chain phosphorylation.

The first attempts to determine the stoichiometry of respiratory-chain phosphorylation, expressed as the P:O ratio, were made by Ochoa (1943). Such attempts were complicated by the high ATPase activity of crude preparations which caused esterified phosphate to be returned as P_i to the reaction medium. Improvements in the experimental technique have since greatly decreased the significance of this source of error. Firstly, cell fractions consisting largely of mitochondria replaced the tissue homogenates (see Kennedy & Lehninger 1948; Schneider & Hogebom 1950; Kielley & Kielley, 1951), and secondly a large excess of purified yeast hexokinase was added to the incubations, and so favoured the reaction of ATP with added glucose,

that losses due to ATPases became negligible (see Loomis & Lipmann, 1948; Hunter, 1949; Cross et al., 1949).

The experimentally found P:O ratios are 3-4 for α oxoglutarate, 2-3 for NAD-linked substrates and 1-2 for succinate. The average values found usually differ significantly from a whole number, which implies that side-reactions are taking-place, if it is assumed that oxidative phosphorylation proceeds in a fixed stoichiometric manner. Such side-reactions may be due to mechanical damage of the mitochondria during isolation, to contamination with natural uncoupling-agents e.g. unsaturated free-fatty acids (Pressman & Lardy, 1956; Wojtczak & Lehninger, 1961), or to the utilisation of the energy of oxidative phosphorylation to maintain the structural and ionic composition of the mitochondria. These considerations have recently led to a re-examination of the efficiency of oxidative phosphorylation, and reports have appeared in which it is claimed that the P:O ratios previously generally accepted may be greatly exceeded. (Smith & Hansen, 1964; Garban & Cristea, 1964, 1965; Lynn & Brown, 1965).

I(iv) The primary phosphate acceptor in oxidative phosphorylation

Many attempts have been made to resolve the identity of the nucleotide acceptor to which P_1 is first linked in a high-energy bond during oxidative phosphorylation. Early studies by Slater and Holton (1953) and Lindberg & Ernster (1952) on heart mitochondria, and Kielley & Kielley (1951) and Barkulis & Lehninger (1951) with rat liver mitochondria suggested that ADP was the primary extramitochondrial acceptor of P_1 . More detailed studies on the separation and estimation of intramitochondrial nucleotides of rat liver mitochondria by Siekevitz & Potter (1955, a, b); Pressman, (1958); and Heldt, Jacobs & Klingenberg, (1965), led these authors to conclude that ADP is the primary phosphate acceptor both inside

and outside mitochondria. Brierley & O'Brien (1965) reached a similar conclusion with beef heart mitochondria.

The work of Heldt, Klingenberg and co-workers (Heldt, 1963; Heldt, Jacobs & Klingenberg, 1965, 1966; Pfaff, Klingenberg & Heldt, 1965; Klingenberg & Pfaff, 1966; and Heldt & Klingenberg, 1965), with rat liver mitochondria led them to conclude that the phosphorylation of added ADP is preceded by phosphorylation of the endogenous ADP of the mitochondria. The ATP thus formed is transferred to the outside of the mitochondrial membrane by a specific atractyloside-sensitive adenine nucleotide transport mechanism and is replaced by external ADP.

Recently, Ozawa & MacLennan (1965) have pointed out that previous results may be inconclusive because of the difficulties imposed by compartmentation of the endogenous adenine nucleotides of mitochondria, and also because myokinase and other phosphoryl transfer systems have the ability to redistribute the phosphoryl group among the adenine nucleotides. Ozawa and MacLennan (1965) claimed that in short-term incubations of beef heart submitochondrial particles with succinate as substrate, endogenous AMP was the primary phosphate acceptor. Ozawa (1966) further claimed that added AMP was also the primary phosphate acceptor during oxidation of succinate or pyruvate plus malate by intact beef heart mitochondria.

I(v) Sites of Oxidative Phosphorylation

One of the phosphorylation steps of α oxo-glutarate oxidation has been shown to be a substrate level phosphorylation catalysed by succinic thiokinase. Measurements of P:O ratios show that there are (at least) three sites of phosphorylation during the oxidation of NAD-linked substrates, and two sites during the oxidation of succinate.

In no case have the sites been exactly defined, but there is good evidence that the three sites lie between NADH and cytochrome b (Smith & Hansen, 1962; Sanadi & Fluharty, 1963); cytochrome b and cytochrome c; and cytochrome c and oxygen (Jacobs, 1960). These proposed locations are based on P:O or P:2e measurements over segments of the respiratory chain, isolated by the use of a combination of inhibitors of electron transport, and artificial electron carriers. e.g. external cytochrome c plus antimycin A isolates the phosphorylation site associated with cytochrome oxidase. Other artificial electron carriers include ferricyanide, quinones, ascorbate and TMPD. These results are complicated by the possibility that these artificial substrates can act at more than one site. e.g. TMPD can by-pass the antimycin A sensitive block by acting as a bridge for electron transport between cytochrome b and cytochrome c (Lee, Nordenbrand & Ernster, 1965).

The phosphorylation sites have been independently identified by experiments identifying the crossover-points in difference spectra between controlled and actively respiring mitochondria (Chance & Williams, 1956).

I(vi) Coupling Factors

Many attempts have been made to resolve the mechanism of oxidative phosphorylation by fractionation of mitochondria using the methods which have proved successful in other biochemical problems. Such studies have involved the resolution of mitochondria into particulate and soluble fractions, and the further purification of specific soluble proteins required for oxidative phosphorylation which have been called coupling factors.

The main problem in this approach has been to obtain submitochondrial test-particles which catalysed maximal oxidation without phosphorylation, but which retained the ability to recombine with coupling factors and regain their phosphorylative ability.

Linnane (1958) and Linnane & Titchener (1960) fractionated phosphorylating sub-mitochondrial particles (ETP_H) into particulate and soluble protein fractions, both of which were required for phosphorylation. Extension of this work in Green's laboratory led to the claim that site-specific coupling factors had been isolated (Green et al., 1963).

Other approaches to the isolation of soluble proteins required for oxidative phosphorylation have been to purify proteins which catalyse partial reactions of oxidative phosphorylation such as the ADP-ATP exchange enzyme of Wadkins & Lehninger (1963b) the ATPase enzyme (F_1) purified by the group of Racker (see Pullman et al., 1960; Penefsky et al., 1960); and the factor of Sanadi which stimulates the energy-linked reduction of NAD^+ by succinate in urea-treated submitochondrial particles (see Andreoli, Lam & Sanadi, 1965).

The results of such fractionation studies have recently been reviewed by Slater (1967d).

I(vii) The Mechanism of Oxidative Phosphorylation

Before considering the problems of exchange reactions in mitochondria, the mode of action of inhibitors and uncouplers of oxidative phosphorylation and the mechanism of energy-linked reactions in mitochondria, current hypotheses concerning the mechanism of oxidative phosphorylation will be briefly discussed. The above problems will then be discussed in relation to these hypotheses.

The mechanism of oxidative phosphorylation is far from being understood, and has been the subject of many hypotheses. These may be divided into two main categories: the chemical, and the chemi-osmotic hypotheses of oxidative phosphorylation.

following postulates:-

(a) The chemical hypothesis of oxidative phosphorylation

This has been adequately reviewed by Slater (1958, 1967d) and Ernster & Lee (1964). The main postulate of the chemical hypothesis is that the primary event of oxidative phosphorylation in the formation of a high-energy chemical bond, designated ($\sim$). The hypothetical reaction sequence used by Ernster & Lee (1964) (see Fig. 1B) will be used in this discussion as it is a general scheme incorporating most of the features of the earlier hypotheses.

It is assumed that similar types of coupling mechanism occur at all three sites of oxidative phosphorylation. It is proposed that the high-energy bond is formed between one of the carriers C and a group or compound I (not P_i). Whether the carrier is in the reduced or the oxidised form is not stipulated. The high-energy bond is transferred via non-phosphorylated ($X\sim I$) and phosphorylated ($X\sim P$) high-energy intermediates, and $X\sim P$ finally reacts with ADP to give ATP. This general scheme is supported by the properties of the exchange-reactions catalysed by mitochondria, and the sites of action of inhibitors of oxidative phosphorylation such as oligomycin A (see Lardy, Connelly & Johnson, 1964).

As yet there is no direct evidence for the existence of any of the intermediates postulated in Fig. 1B.

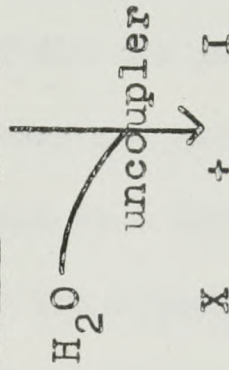
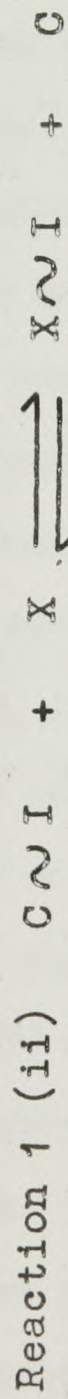
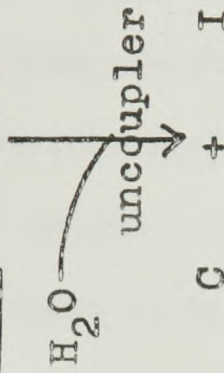
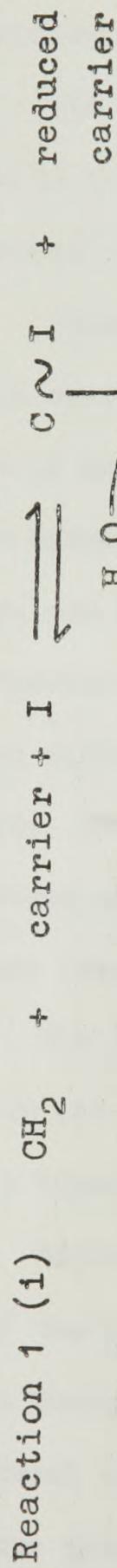
(b) Chemiosmotic Hypothesis

The alternative formulation of oxidative phosphorylation is the chemiosmotic hypothesis, first suggested by Lundegardh (1945) and Davies & Krebs (1952), and recently extended by Mitchell (1962, 1966a, 1966b).

Mitchell's chemiosmotic hypothesis is based on the following postulates:-

Figure 1B. Chemical Hypothesis of Oxidative Phosphorylation, and the Sites

of Action of Inhibitors and Uncouplers of Oxidative Phosphorylation.



Oligomycin A, aurovertin,
octylguanidine, DCCD.

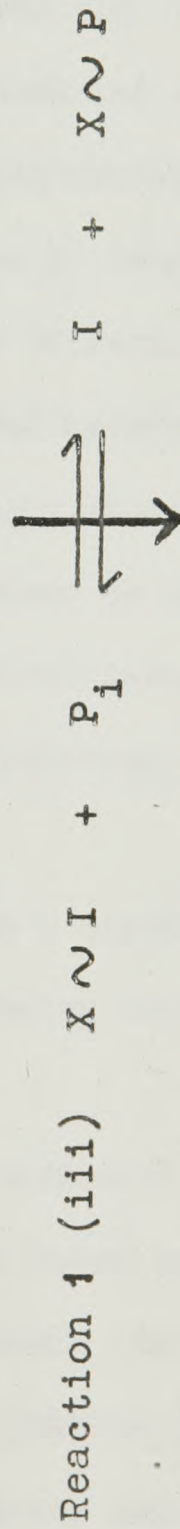


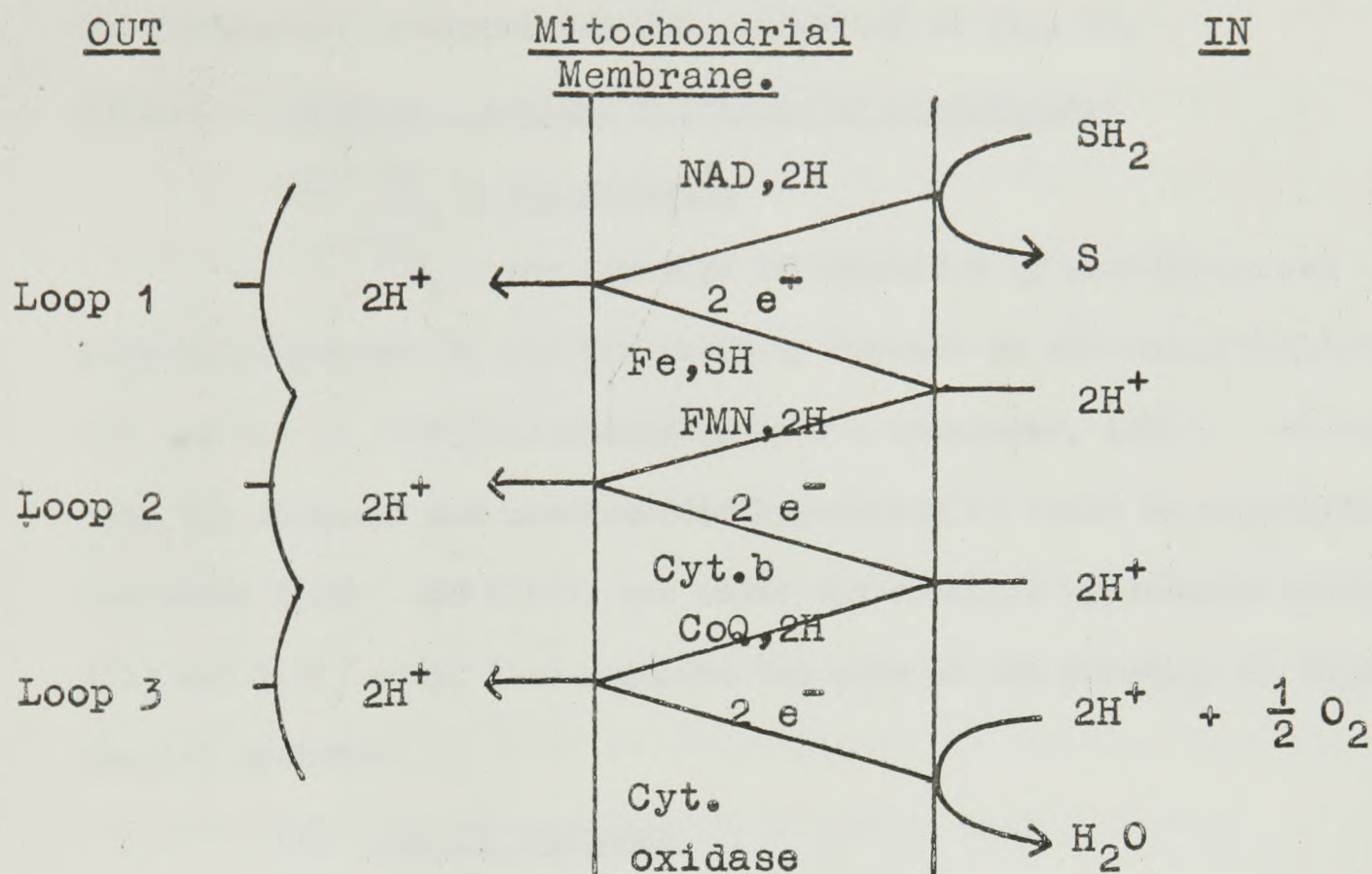
Figure 1C. Possible Proton-translocation

- (1) The respiratory chain is arranged anisotropically in a series of loops across the mitochondrial membrane, such that a flow of reducing equivalents along the respiratory chain is coupled reversibly to a translocation of protons across the membrane (see Fig. 1C).
- (2) The mitochondrial cristae contain an anisotropic ATPase, whose function is to couple reversibly the translocation of protons across the mitochondrial membrane and the formation of ATP (see Fig. 1D).
- (3) There are present in the mitochondrial membrane specific exchange-diffusion carrier-systems that permit the reversible exchange across the membrane of anions for OH^- ions, and of cations for H^+ ions. The function of these systems is to regulate the pH and osmotic differential across the membrane, and permit the entry and exit of essential metabolites. Under normal conditions there would be a sufficiently large electrochemical potential difference across the mitochondrial membrane to drive ATP synthesis. The major component of the electrochemical potential would be the membrane potential, and there need only be a relatively small pH difference (see Mitchell, 1961, 1966b).
- (4) The postulated systems 1-3 are located in a mitochondrial membrane which, with the exception of specific exchange-diffusion carrier-systems, is highly impermeable to ions.

Mitchell (1966b) and Slater (1967a) have discussed the relative merits of the two main hypotheses in explaining the known facts of oxidative phosphorylation. The chemiosmotic hypothesis stresses that the primary event of oxidative phosphorylation is the ejection of protons, whereas the chemical hypothesis states that the primary event is the formation of a chemical high-energy bond. Both hypotheses agree that the terminal step of oxidative phosphorylation is catalysed by a membrane-

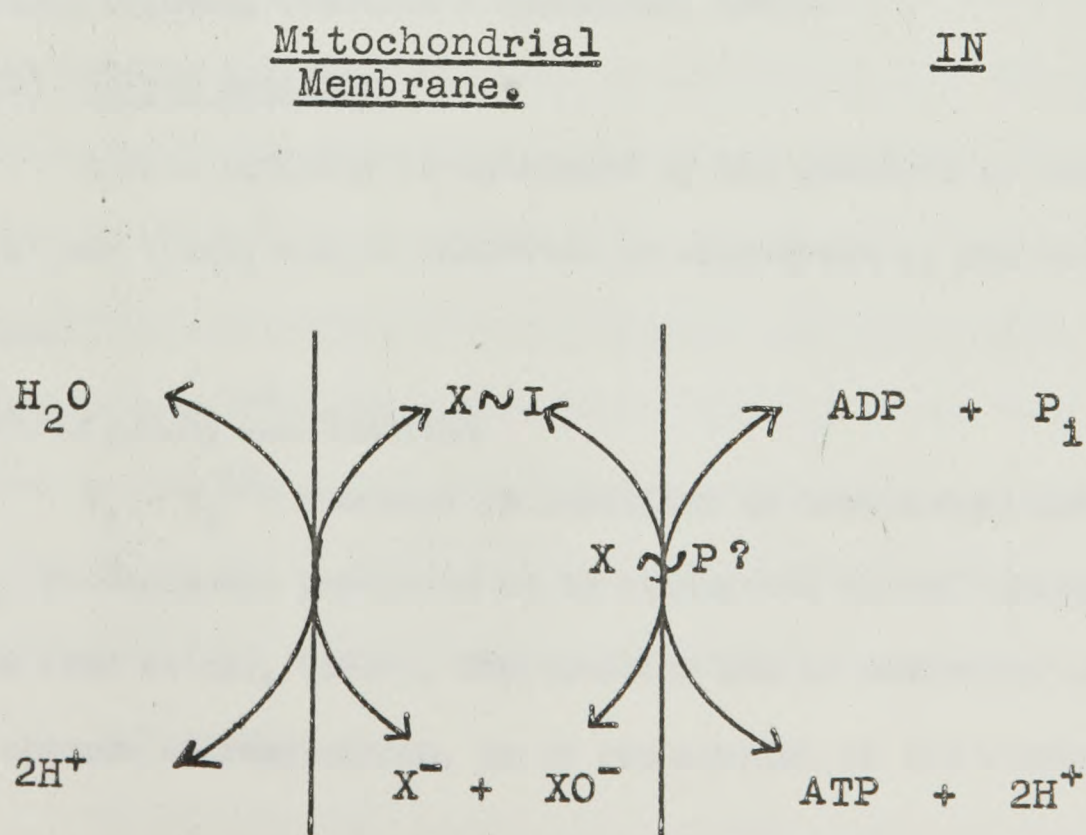
Figure 1C. Possible Proton-translocating Oxido-reduction

Loops of the Respiratory Chain (after Mitchell, 1966b).



Abbreviations: SH_2 , substrate; Fe, SH , non-haem iron or sulphhydryl group of NADH dehydrogenase; Cyt. , cytochrome.

Figure 1D. Proton-translocating ATP-ase (after Mitchell, 1966b).



bound reversible ATPase which is oligomycin-sensitive, and could involve the postulated intermediates $X\sim I$ and/or $X\sim P$ of Fig. 1B.

I(viii) Exchange reactions catalysed by mitochondria

(a) $^{32}P_i$ - ATP exchange

$^{32}P_i$ - ATP exchange is inhibited by uncouplers and inhibitors such as oligomycin A, and its activity depends on the concentrations of P_i , ADP and ATP in a complex manner (Cooper & Lehninger, 1957). According to both the chemical and chemiosmotic hypotheses it would be catalysed by reactions I(iii) and I(iv), and under the chemical hypothesis reactions I(i) and I(ii) could also regulate the rate of the reaction in tightly-coupled systems.

(b) ADP-ATP exchange

ADP-ATP exchange requires reaction I(iv), and can occur in the absence of P_i in loosely-coupled mitochondria. In tightly coupled mitochondria it requires P_i , and is partially inhibited by uncouplers and oligomycin A, presumably due to interactions with reactions I(ii) and I(iii) of the coupling sequence (Wadkins & Lehninger, 1963).

(c) ATPase activity

ATPase activity is catalysed by the reversal of reactions I(ii), I(iii) and I(iv), and is inhibited by oligomycin A, but stimulated by uncouplers.

(d) P_i - $H_2^{18}O$ exchange

P_i - $H_2^{18}O$ exchange is inhibited by both uncouplers and oligomycin. In digitonin particles it is stimulated by ADP (Cooper 1965). According to Chan et al., (1960), the reaction can be activated either by ATP in the absence of respiration, or by respiration in the absence of

ATP, but Boyer et al. (1966) found the reaction to be independent of the oxidation reactions of oxidative phosphorylation. It is important to resolve this point, as the reaction should depend on the partial-reaction which forms the first anhydride-bond. In the chemical hypothesis this is reaction I(i) which would be affected by the redox state of C, whereas in the chemi-osmotic hypothesis there would be no such dependence.

(e) ATP - $H_2^{18}O$ exchange

If Fig. 1B is correct, ATP- $H_2^{18}O$ exchange would be expected to be preceded by $H_2^{18}O - P_i$ exchange. It is inhibited by uncouplers and oligomycin, but proceeds at a much higher rate than $P_i - H_2^{18}O$ exchange, which is not explained by Fig. 1B. An attempt to explain this anomaly has been made by Cooper (1965), in which it is proposed that ATP is the first phosphorylated intermediate of oxidative phosphorylation.

The chemical hypothesis would predict that $P_i - H_2^{18}O$, ATP $^{18} - H_2O$ and $P_i - ATP$ exchange reactions could depend on the redox state of the electron transport carriers, but there is no firm evidence of this. The chemiosmotic hypothesis can be reconciled with the evidence of the exchange reactions provided that the ATPase reaction involves an $X \sim I$ (and possibly an $X \sim P$) intermediate which reacts with P_i and ADP.

I(ix) Inhibitors and Uncouplers of Oxidative Phosphorylation

The formation of ATP by oxidative phosphorylation is prevented by inhibitors and uncouplers of oxidative phosphorylation. The sites of action of uncouplers and of these inhibitors of oxidative phosphorylation used in the experimental work have been included in Fig. 1B.

(a) Uncouplers

Uncouplers such as 2:4 dinitrophenol (DNP) uncouple

oxidative phosphorylation, according to the chemical hypothesis, by hydrolysing non-phosphorylated high-energy intermediates (either $X \sim I$ or $C \sim I$) (Lardy, Johnson & McMurray, 1958; Borst & Slater, 1961; Chance, Williams & Hollunger, 1963). Tetrachloro-trifluoromethyl benzimidazole (TTFB) (Beechey, 1965), and carbonyl cyanide m-chlorophenyl hydrazone (CCP) (Goldsby & Heytler, 1963), are claimed to uncouple in the same way as DNP. According to the chemiosmotic hypothesis, uncouplers act by making the mitochondrial membrane permeable to H^+ , and thus collapsing the pH differential across the membrane (Mitchell, 1966b).

(b) Oligomycin A, Aurovertin and Dicyclohexylcarbodiimide (DCCD)

Oligomycin A and aurovertin inhibit oxidative phosphorylation $^{32}P_i$ - ATP exchange, P_i - $H_2^{18}O$ exchange, ATP - $H_2^{18}O$ exchange and ATPase activity. (Lardy, Johnson & McMurray 1958; Lardy, Connelly & Johnson, 1964). Beechey et al. (1966), claim that DCCD acts in the same way as oligomycin A. Aurovertin is much less effective than oligomycin A in inhibiting ATPase induced by uncoupling agents (Connelly & Lardy, 1964). Both the chemical and chemi-osmotic hypotheses agree that oligomycin A acts by inhibiting the formation of ATP from $X \sim I$ (or $X \sim P$).

In lower concentrations, oligomycin A stimulates oxidative phosphorylation, and ATP-driven reactions in sub-mito-

(d) Streptolydigin

Ornani, Lelander, & Gorman (1964) showed that streptolydigin inhibits mitochondrial particles (Lee & Ernster, 1965). Lee & Ernster, (1966) suggest that in addition to inhibiting ATP formation, oligomycin A inhibits the hydrolysis of non-phosphorylated high-energy intermediates.

(c) Octylguanidine

Guanidine and its alkyl derivatives inhibit oxidative phosphorylation (Pressman, 1963). Octylguanidine differs from oligomycin A in its effects in the following respects:-

(i) It is much more effective in inhibiting phosphorylation at Site 1 than at other sites (Pressman, 1963; Haas, 1964).

(ii) Inhibition sets in slowly compared with that by oligomycin A.

(iii) Reversal of the inhibition of respiration by DNP is not instantaneous, as it is with oligomycin A.

(2) According to the chemical hypothesis, guanidines act at a point closer to the respiratory chain than oligomycin A (Slater, 1967a). For the chemi-osmotic view of the mode of action of guanidines see Mitchell (1966b).

According to the chemical hypothesis of oxidative phosphorylation the energy utilised in the reactions is in the form of non-phosphorylated high energy intermediates, since oligomycin A does not inhibit the reactions when they are supported by respiration. According to the chemi-osmotic

(d) Atractyloside

Bruni, Luciani, & Contessa (1964) showed that atractyloside competitively inhibits the binding of adenine nucleotides to mitochondria, and inhibits respiration in the presence of phosphate acceptor (State 3), but not in the presence of DNP or arsenate, (Bruni & Azzone, 1964).

Atractyloside inhibits the phosphorylation of added ADP, without affecting the phosphorylation of the endogenous ADP of mitochondria (Heldt, Jacobs & Klingenberg, 1965; Chappell & Crofts, 1965). These authors suggested that atractyloside specifically inhibits the adenine nucleotide translocation system in the mitochondrial membrane.

I(x) Energy-Linked Reactions of Mitochondria

It was discovered that a number of endergonic reactions in mitochondria could be driven either by ATP under anaerobic conditions or by coupled respiration even in the presence of oligomycin A. (see Chance, 1963). A list of the well-substantiated energy-linked reactions is given in Table 1A. The properties exhibited by energy-linked reactions include:-

- (1) An energy-requirement; supplied by either ATP under anaerobic conditions, or by coupled respiration.
- (2) The ATP driven reaction is inhibited by oligomycin A, aurovertin, octylguanidine or atractyloside, but these inhibitors have no effect on the respiration-driven reaction or indeed give a slight stimulation.
- (3) Uncouplers inhibit the reaction whether driven by ATP or by respiration, by inhibiting the supply of energy.

According to the chemical hypothesis of oxidative phosphorylation the energy utilised in the reactions is in the form of non-phosphorylated high energy intermediates, since oligomycin A does not inhibit the reactions when they are supported by respiration. According to the chemi-osmotic

Table 1A. Endergonic Mitochondrial Reactions Driven by the Energy of Oxidative Phosphorylation.

1. NAD^+ reduction driven by succinate oxidation. (Chance & Hollunger, 1957).
 NAD^+ reduction by succinate plus ATP. (L^öw, Krueger & Ziegler, 1961).
 NAD^+ reduction by ascorbate plus TMPD and ATP. (L^öw, Vallin & Alm, 1963).
 NAD^+ reduction by ascorbate plus ATP. (Sanadi, Andreoli, Pharo & Vyas, 1963).
2. NADP^+ reduction by NADH in the energy-linked trans-hydrogenase reaction. (Danielson & Ernster, 1963).
3. Translocation of divalent cations into mitochondria. (Chappell, Cohn & Greville, 1963; Brierley, 1963).
4. Translocation of monovalent cations into mitochondria in the presence of valinomycin or gramicidin. (Pressman, 1965; Chappell & Crofts, 1966).
5. Glutamate synthesis from α -oxo-glutarate and NH_3 with L-malate as hydrogen donor. (Tager & Slater, 1963).
6. Swelling and shrinkage of mitochondria. (Packer, Marchant & Mukohata, 1963; Chappell & Crofts, 1966).

hypothesis the reactions are driven by the electrochemical potential across the mitochondrial membrane developed by the proton-translocating respiratory chain or the proton-translocating ATPase.

I(xi) Mitochondrial Ion Transport

(a) Transport of Divalent Cations

Ca^{2+} , Mg^{2+} , Mn^{2+} and Sr^{2+} are accumulated by mitochondria in a reaction which may be driven by substrate oxidation or by ATP (Chappell, Cohn & Greville, 1963; Brierley, 1963). In the absence of P_i there is a decrease in the rate of respiration when the greater part of the divalent metal ion has been accumulated (Rossi & Lehninger, 1964). The $\text{Ca}^{2+}:\text{O}$ ratio is 5.6 with β hydroxybutyrate and 3.8 with succinate as substrate. Thus each energy-conserving site of the respiratory chain can support the uptake of 2Ca^{2+} .

ATP can support the uptake of Ca^{2+} in the presence of P_i with a $\text{P}_i:\text{O}$ ratio of 1.0 and a $\text{Ca}^{2+}:\text{O}$ ratio of 1.85. P_i uptake under these conditions appears to be passive as it occurs after Ca^{2+} uptake (Rossi et al., 1966).

Chappell, Greville & Cohn (1963) showed that mitochondria suspended in the absence of P_i are capable of accumulating Mn^{2+} in a reversible energy-dependent reaction. The subsequent addition of P_i led to the uptake of that ion in a respiration independent reaction. This finding supports the conclusion that the uptake of the divalent cation is the energy-utilising process, and the subsequent uptake of anion is a passive process. P_i is accumulated as $\text{M}_3(\text{PO}_4)_2$ for Mg and Mn^{2+} (Chappell, Greville & Cohn, 1963; Brierley, Bachman & Green, 1962), and at pH 7.2 one H^+ appears in the medium for each divalent metal ion.

(b) The translocation of Monovalent cations in the presence of Valinomycin or Gramicidin

Gramicidin and valinomycin, in the presence of ATP or oxidizable substrate, and an alkali metal ion, cause the uptake of the alkali metal ion by mitochondria and the appearance of H^+ ions in the suspending medium, with a $\Delta H^+ : \Delta K^+$ ratio ≈ 1 . (Chappell & Crofts, 1966; Pressman, 1965; Moore & Pressman, 1964). When P_i is also present respiration is stimulated and mitochondrial swelling occurs. Gramicidin differs from valinomycin in that Na^+ , Li^+ , K^+ , Rb^+ and Cs^+ are translocated, whereas valinomycin does not act with Na^+ and Li^+ .

Chappell & Crofts (1966) explain these results by postulating that:-

- (1) The normal mitochondrial membrane is relatively impermeable to monovalent cations.
- (2) Gramicidin and valinomycin cause an increased rate of penetration of the mitochondrial membrane by monovalent cations.
- (3) There is an energy-dependent H^+ pump which tends to eject H^+ from the mitochondrion.

In the presence of valinomycin or gramicidin, K^+ is translocated into mitochondria in exchange for H^+ , with the expenditure of energy. In the presence of P_i , intramitochondrial OH^- exchanges out of the mitochondrion for P_i , and the net accumulation of soluble potassium phosphate results in swelling of the mitochondria.

The stoichiometry of K^+ uptake was found to be $2K^+$ per high-energy bond, but recently higher values have been reported which vary with conditions (Cockrell, Harris & Pressman, 1966).

(c) Proton Translocation

Mitchell & Moyle (1966a, b) showed that mitochondria can

eject protons with the following stoichiometry:-

$6H^+ : O$ for β hydroxybutyrate oxidation

$4H^+ : O$ for succinate oxidation

$2H^+ : ATP$ for ATPase

Mitchell interprets these results as strong evidence for the chemi-osmotic hypothesis of oxidative phosphorylation, but they could also be explained by the alternative hypothesis that proton ejection is a secondary process driven by high-energy intermediates of oxidative phosphorylation and perhaps largely the result of translocation of divalent cations into the mitochondria (see Slater, 1967b).

(d) Anion Translocation

The work of Chappell and co-workers suggests that there are specific translocation systems in rat liver mitochondria for the entry of P_i (Chappell & Crofts, 1965), for citrate and malate (Chappell, 1964) and other anions (Chappell & Hearhoff, 1967). Chappell has evidence, that the translocation systems are specific for the anionic species, and effectively catalyse exchange diffusion against OH^- ions (Chappell & Crofts, 1966). Chappell considers that anion translocation is a passive process occurring subsequently to an energy-requiring translocation of cations (Chappell, Cohn & Greville, 1963).

The inhibition of the respiration of anionic substrates by high concentrations of uncouplers (see Heaker, 1962, 1964), and the ability of mitochondria to maintain high internal concentrations of L-malate and citrate (Gamble, 1965), suggests that the translocation of anionic substrates into mitochondria may require energy. Similarly, the release by uncouplers of the inhibition of electron transport by the anionic inhibitors saytal (Chance & Hollunger, 1963), and azide

(Wilson & Chance, 1966) could be explained if the mitochondria require energy to maintain high intramitochondrial concentrations of these anionic inhibitors. Recently, Harris, Van Dam & Pressman (1967a, b) have reported an energy-dependence for the oxidation of succinate by liver mitochondria.

Translocation of Oxaloacetate

Added oxaloacetate strongly inhibits succinate dehydrogenase (Das, 1937; Pardee & Potter, 1948), but its effect on aerobic succinate oxidation in intact mitochondria is not great unless Ca^{2+} is present (Gutfreund & Jones, 1964), or oxaloacetate is added before the succinate (Chappell, 1961). This suggests that special factors may regulate the translocation of oxaloacetate into mitochondria.

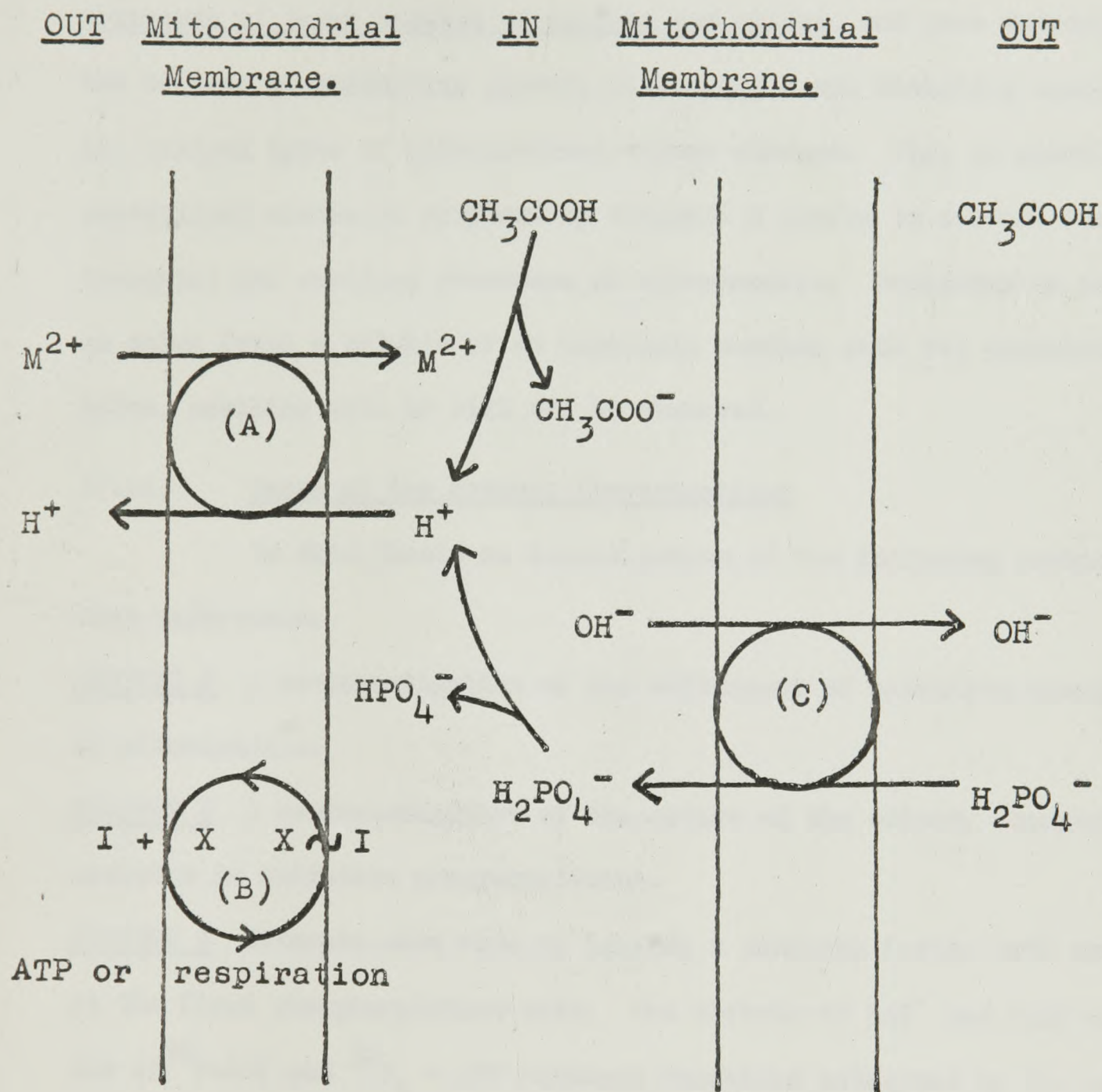
Glutamate Synthesis with L-malate as hydrogen donor

Hydrogen transfer from L-malate to α -oxo-glutarate plus NH_3 or to acetoacetate requires a supply of energy (Tager 1963, 1964, 1966; Tager & Slater, 1963). The stoichiometry of the requirement for ATP has been measured by extra ATPase activity (see Klingenberg, 1963), on addition of L-malate in the presence of α -oxo-glutarate plus NH_3 and has found to be in the range (0.25 - 1.0) ATP per glutamate synthesised. It is difficult to explain this small and variable energy-requirement, but it possibly reflects an energy-requirement for the translocation of the anionic substrates of the reaction. Tager (1966) has suggested that the energy-requirement for glutamate synthesis may depend on the energy-controlled movement of oxaloacetate from one compartment of the mitochondrion to another.

(a) Volume changes in Mitochondria

Lehninger (1962), and Chappell & Crofts (1966) have

Figure 1E. Generalised Mechanism to Account for Ion Transport and Swelling Phenomena in Mitochondria (after Chappell & Crofts, 1966).



The accumulation of divalent cations by carrier-system (A) is driven by ATP or respiration (system B). Permeant anions such as $H_2PO_4^-$ enter the mitochondrion in exchange for OH^- ions in carrier-system (C).

reviewed the factors effecting volume changes in mitochondria. Chappell & Crofts (1966) consider that the respiration of ATP driven swelling of mitochondria is associated with the osmotic changes produced by movements of large amounts of cations and anions, and have summarised the effects of uncoupling agents, oligomycin A and chelating agents on the various types of mitochondrial volume changes. Fig. 1E gives the generalised mechanism proposed by Chappell & Crofts to account for ion-transport and swelling phenomena in mitochondria. Depending on whether an anion forms a soluble or an insoluble complex with the accumulated anion, swelling will or will not be observed.

I(xii) Scope of the Present Investigations

In this thesis an investigation of the following problems has been undertaken.

CHAPTER 2 A re-investigation of the efficiency of oxidative phosphorylation in mitochondria.

CHAPTER 3 A re-investigation of the nature of the primary phosphate acceptor in oxidative phosphorylation.

CHAPTER 4 Attempts were made to isolate a coupling factor with activity at the first phosphorylation site. The effects of NAD^+ and NADH on the $\text{AD}^{32}\text{P-ATP}$ and $^{32}\text{P}_i - \text{ATP}$ exchange reactions catalysed by the partially-purified coupling factor were investigated.

CHAPTER 5 A direct assay of the rates of penetration of oxaloacetate and L-malate into mitochondria was developed.

CHAPTER 6 The mechanism of penetration of oxaloacetate and L-malate into mitochondria was investigated, with particular reference to the energy-dependence of this process.

CHAPTER 7 The temperature-dependence of a number of energy-linked reactions of mitochondria and sub-mitochondrial particles was investigated.

With a number of substrates, reported values of Q_{10} for respiration were 4-5 with pyruvate and 2.5 - 3.5 with succinate for 10°C values of most substrates. Burton & Wilson (1964, 1965) reported Q_{10} values of 4.5 to 5 with succinate, and up to 5.5 with pyruvate as substrate in experiments with rat liver mitochondria. (see Burton (1965) also reported Q_{10} values of higher than 7 with α -oxoglutarate, more than 5 for succinate and more than 4 for succinate oxidation by rat liver mitochondria. All these groups of workers measured oxygen uptake by the indirect, manometric technique.

This chapter reports an investigation of the experimental procedures which appear to give high Q_{10} values. It was observed that the methods used to measure oxygen consumption could give rise to systematic errors if used uncritically, and the procedures used to obtain accurate results will be discussed in detail.

2.11. Materials

PREPARATION OF RAT LIVER MITOCHONDRIA

(a) RAT LIVER MITOCHONDRIA

Rat liver mitochondria (RIM) were prepared from the livers of 200g. female rats using the method outlined by Segall (1955).

Rats were starved, anaesthetized and their livers quickly removed and cooled in ice-cold homogenization medium (0.25M sucrose; 0.1M Tris-HCl, pH 7.4; 0.1M α -methyl-2-thiouracil, pH 7.4). The livers were passed through a 150 mesh sieve, and then homogenized in 5-6 volumes of medium using a glass-tissue homogenizer. The homogenates were spun down at

CHAPTER 2

P:O RATIOS OF ISOLATED MITOCHONDRIA

2(i) Introduction

Smith & Hansen, (1964), reported values of 4.4 - 5.7 with pyruvate, 4-5 with glutamate and 2.7 - 3.9 with succinate for P:O ratios of beef heart mitochondria. Gurban & Cristea (1964, 1965) reported P:O ratios of up to 5 with succinate, and up to 6.8 with glutamate as substrate in experiments with rat liver mitochondria. Lynn & Brown (1965) also reported P:O ratios of higher than 7 with α oxo-glutarate, more than 6 for L-malate and more than 4 for succinate oxidation by rat liver mitochondria. All three groups of workers measured oxygen uptake by the Warburg manometric technique.

This chapter reports an investigation of the experimental procedures which appear to give high P:O ratios. It was discovered that the methods used to measure oxygen consumption could give rise to considerable sources of error if used uncritically, and the precautions taken to obtain unambiguous results will be discussed in detail.

2(ii) Methods

Preparation of Intact Mitochondria

(a) Rat Liver Mitochondria

Rat liver mitochondria (RLM) were prepared from the livers of 250g. female rats using the methods outlined by Hogeboom (1955).

Rats were stunned, decapitated and their livers rapidly removed and washed in ice-cold homogenization medium: 0.25M sucrose; 10 mM tris-HCl, pH 7.6; 0.1 mM tris-EDTA, pH 7.6. The livers were passed through a Fischer mincer, and then homogenised in 5-6 volumes of medium using a glass-teflon homogeniser. The homogenates were spun twice at

500g for 8 min. in the (6 x 100 ml) angle head of the M.S.E. Mistral centrifuge, and the pellets were discarded. The supernatant was spun at 5400g for 15 min. and the high-speed supernatant discarded. The 'fluffy' layer of the mitochondrial pellet was sloughed off, and the 'heavy' mitochondria were resuspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6. The high speed spin was repeated a second time, and the mitochondria were finally suspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6 at a protein concentration of 15-30 mg/ml.

(b) Beef Heart Mitochondria

Heavy beef heart mitochondria (HBHM) were prepared by the method of Hatefi et al. (1961) in a medium containing 0.25M sucrose; 10 mM tris-HCl, pH 7.6; 0.1 mM tris-EDTA.

The lean meat from fresh beef hearts was minced, and then softened by preincubation at $0-4^{\circ}$ with the bacterial proteinase, Nagarse, after which it could be broken up in a glass-teflon homogeniser. The mitochondria were then isolated by differential centrifugation as described for rat liver, and light mitochondria were discarded by sloughing off in 0.25M sucrose. The heavy mitochondria were suspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6; 0.1 mM tris-EDTA at a protein concentration of 10-20 mg/ml.

Preparation of Non-Phosphorylating Sub-mitochondrial particles (ETP)

Non-phosphorylating sub-mitochondrial particles, capable of oxidizing only NADH and succinate, were prepared by a modification of the method of Crane et al. (1956).

Light beef heart mitochondria were subjected to sonic oscillation for two periods of 30 sec. at 0° using the precooled probe of the M.S.E.

ultrasonic power-unit, in a medium containing 50 mM KCl; 50 mM KPO_4 , pH 7.4; 0.25M sucrose. Unbroken mitochondria were spun down at 25,000g for 7 min. in the 30 head of the Spinco Model L ultra centrifuge. The supernatant was spun at 105,000g for 40 min., and the pellet resuspended in the same medium. After spinning down for a second time for 40 min. at 105,000g, the pellet was finally resuspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6.

Determination of P:O ratios

The determination of P:O ratios involves two measurements: the amount of P_i esterified, and the amount of substrate oxidised. The latter is conveniently measured by determining oxygen consumption, which may be measured either manometrically using the Warburg apparatus, or polarographically using the oxygen electrode.

Measurement of Oxygen Consumption

(a) Manometric estimation of Oxygen consumption

Oxygen consumption of respiring mitochondria was measured manometrically by conventional Warburg techniques (see Umbreit, Burris & Stauffer, 1964), using a rotary Warburg apparatus (Braun, Melsungen). 15 ml double side-arm manometer flasks were used in all experiments, and were shaken at 120 oscillations per minute. After an initial period of thermal equilibration, incubations were initiated by tipping the first side-arm, and at the end of the incubation, reactions were stopped by tipping perchloric acid from the second side-arm.

(b) Polarographic estimation of Oxygen Consumption

Three types of oxygen electrode were used to measure polarographically the oxygen consumption of respiring mitochondria.

(i) The vibrating platinum electrode of the Oxygraph
(Gilson Medical Electronics)

Initially measurements of oxygen consumption were made using the vibrating platinum electrode of the Oxygraph (Gilson Medical Electronics). This method suffered two disadvantages.

(1) The vibrating platinum electrode necessitated the use of an open vessel, and oxygen was stirred in during the course of an incubation. Corrections had to be applied for the stirring in of oxygen, which were relatively large when the incubation medium was more than 50% depleted in oxygen.

(2) It was not possible to stir the contents of the incubation vessel adequately. Accordingly this method was abandoned in favour of methods (ii) and (iii).

(ii) The Clark electrode cell described by Chappell (1964)

The Clark electrode cell described by Chappell (1964), and obtained from Rank Bros., Bottisham, Cambridge had the following advantages.

(1) The electrode was at the base of the cell and protected by a Teflon membrane.

(2) Incubations could be well stirred by a magnetic flea without causing noise on the recorder.

(3) The cell was adequately sealed from the atmosphere by a tightly-fitting lid, and additions of reagents could be made through a small hole in the lid by means of a micro-syringe without introducing air bubbles.

Media were equilibrated for 1 hour at the temperature of the incubation bath, which also acted as the thermostat to the water jacket of the apparatus. When the cell had been filled with the incubation medium the lid was carefully lowered so as to force out air bubbles through the hole in the lid.

Figure 2A. Calibration of the Oxygen Concentration in

(iii) Syringe repette with Clark electrode attached (Garland et al. (1965))

An apparatus which allows sampling of incubations and continuous recording of oxygen consumption was described by Garland et al. (1965). This apparatus was obtained from Jencons Scientific Co. Ltd., Hemel Hempstead and consisted of a syringe-repette, into which was inserted an E.I.L. Clark electrode. This apparatus was used to follow the phosphorylation of AMP and ADP during State 3 and State 4.

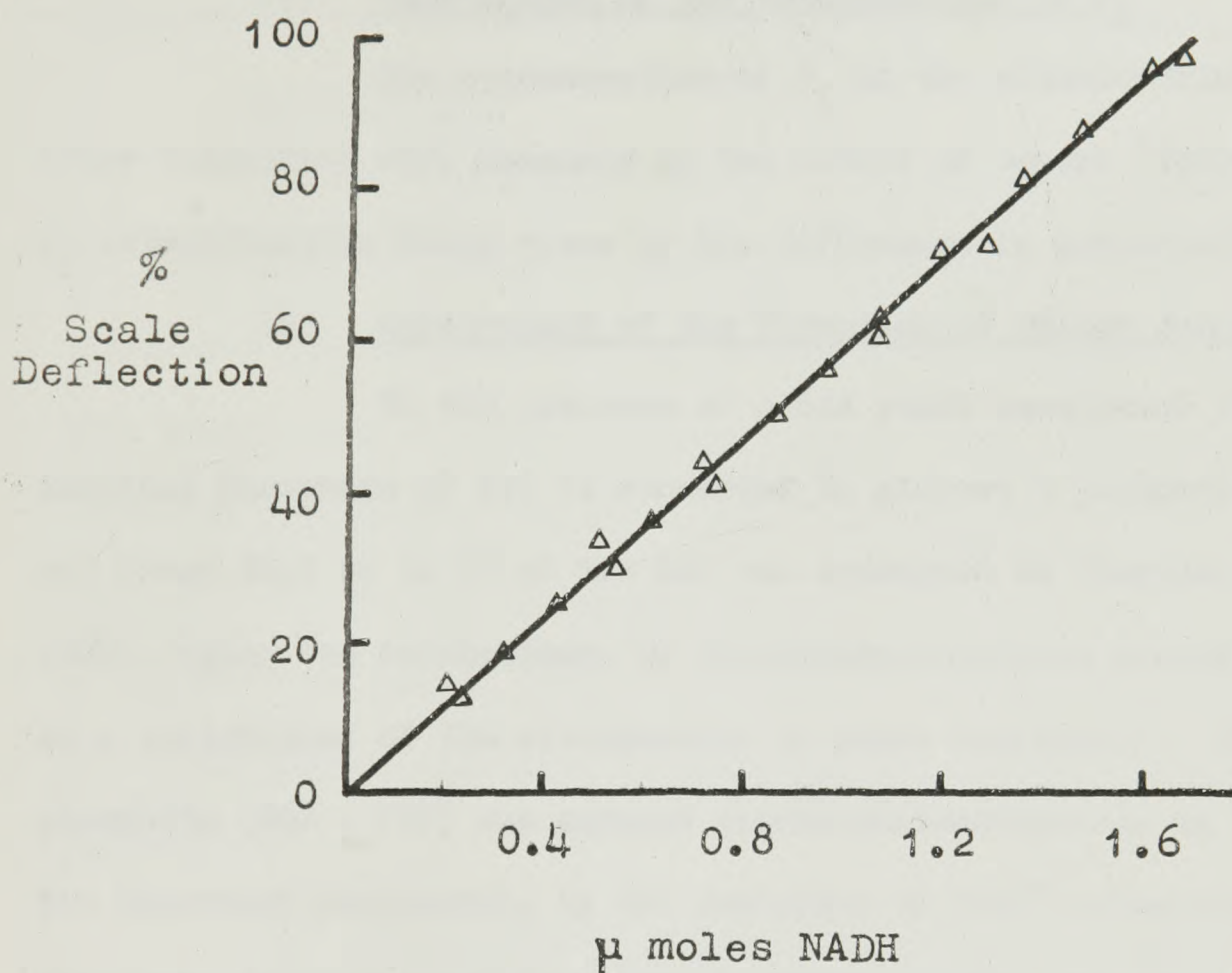
Calibration of the oxygen content of incubation media

The oxygen content of water, at different temperatures and in equilibrium with air at 1 atmosphere is given in the International Critical Tables (1928), and at 30° is $0.505 \mu\text{g atoms/ml}$. However, the solubility of oxygen in aqueous solution is affected by the ionic strength of the medium and also the concentrations of non-electrolytes, (Seidell, 1919).

The oxygen content at 30° of the medium used in mitochondrial incubations was determined by the method of Chappell (1964). A solution of NADH was first accurately assayed by the method described by Klingenberg (1965), and measured samples of it were oxidized in the Clark electrode cell by ETP prepared by the modification of the method of Crane et al. (1956) described above. Fig. 2A shows the results of such a calibration. A medium containing 0.25M sucrose; 50 mM tris-HCl, pH 7.6; 5 mM Mg Cl_2 ; 5 mM KPO_4 ; 2 mg/ml bovine serum albumen (BSA), was found to contain $0.465 \mu\text{g atoms oxygen/ml}$ at 30° , and the response of the electrode was proportional to oxygen concentration over the whole of the range measured. The value obtained by Chappell (1964) for a mitochondrial incubation medium based on KCl was $0.445 \mu\text{g atoms/ml}$ at 30° and is in reasonable agreement.

The experimentally determined value of $0.465 \mu\text{g atoms oxygen/ml}$

Figure 2A. Calibration of the Oxygen Concentration in
Sucrose Medium at 30°.



Medium: 3.60 ml. containing:- 0.25 M sucrose; 50 mM tris-HCl, pH 7.6; 5 mM KPO_4 ; 5 mM MgCl_2 ; BSA (2 mg./ml.); ETP (1 mg. protein); 2-20 μl . 100 mM NADH added from a calibrated Hamilton micro-syringe.

At 30° 1.00 ml. of medium contains 0.465 μg . atoms of oxygen.

at 30° is 92% of the oxygen content of distilled water. For measurements at other temperatures the oxygen content of distilled water given in the International Critical Tables (1928) were multiplied by 0.92.

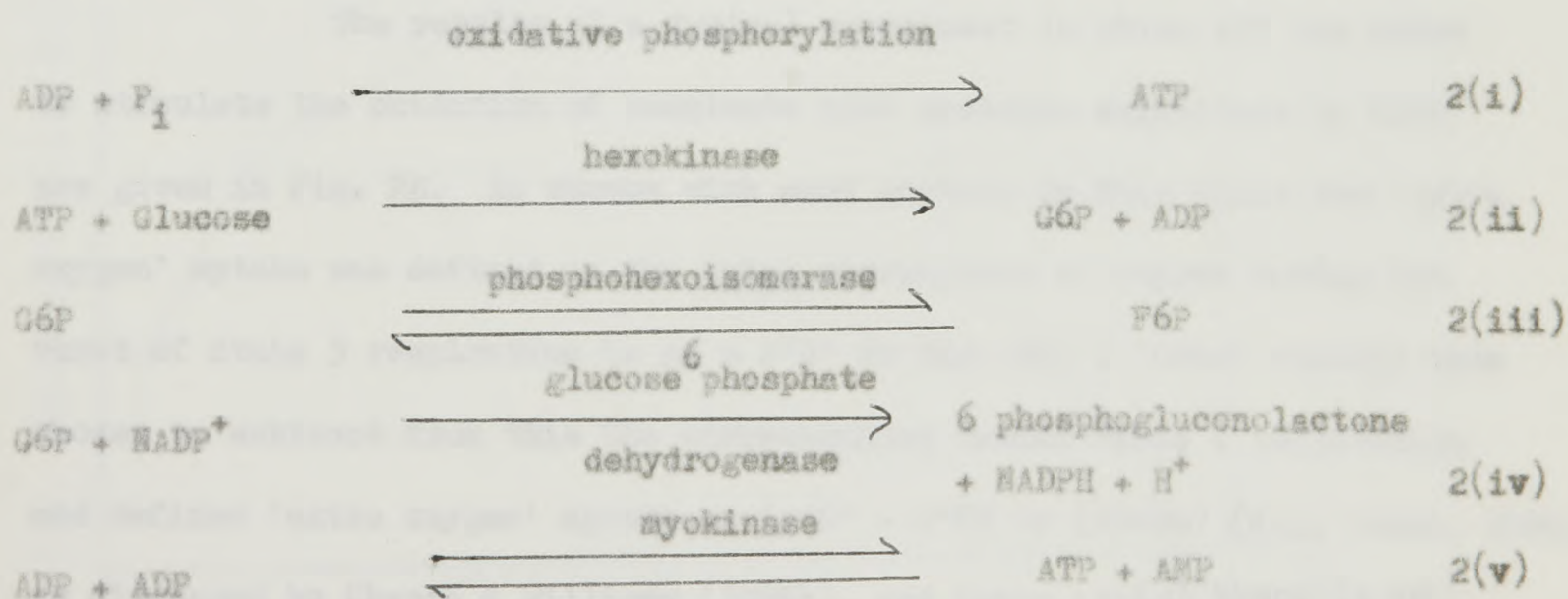
Measurement of P_i Esterification

(a) Measurement of the disappearance of P_i

The concentration of P_i in the mitochondrial medium before and after incubation were measured by the method of Gomori (1942), the amount of P_i esterification being given by the difference in concentration.

(b) Measurement of the Formation of Hexose Monophosphate (HMP)

In the presence of added yeast hexokinase and glucose, the terminal phosphate of ATP is converted to glucose 6 phosphate (G6P). It was found that up to 5% of the G6P was converted to Fructose 6 phosphate (F6P) during the incubations, by phosphohexoisomerase present in the medium as a contaminant of the mitochondria or yeast hexokinase. Total hexose monophosphate (G6P + F6P) was assayed spectrophotometrically at $334\text{ m}\mu$, using the Eppendorf photometer, by the reduction of NADP^+ catalysed by glucose 6 phosphate dehydrogenase plus phosphohexoisomerase (see Hohorst, 1965a)



The amount of hexose monophosphate synthesized by the combined action of adenylate kinase and hexokinase (Reactions 2(v) and 2(ii)) was

determined in a control experiment in which 1 mM KCN was added to inhibit respiration. This amount was subtracted from the amount of HMP found in the experimental flasks.

Both P_i disappearance and HMP formation were determined in many experiments, and gave similar values for P_i esterification.

ADP:O Ratios

Chance & Williams (1955 a, b) introduced a method of measuring the efficiency of oxidative phosphorylation based on the discovery by Engelhardt (1932) and Lardy (1952) that in the absence of enzymes hydrolysing ATP, respiration is inhibited when all the ADP is phosphorylated. Since oxidative phosphorylation has a very high affinity for ADP, there is a sharp 'cut-off' in the rate of oxygen uptake at this point (State 3 $\rightarrow$ 4 transition of Chance & Hollunger, 1963). By means of an oxygen polarograph the amount of 'extra oxygen' necessary to phosphorylate a known amount of ADP was determined.

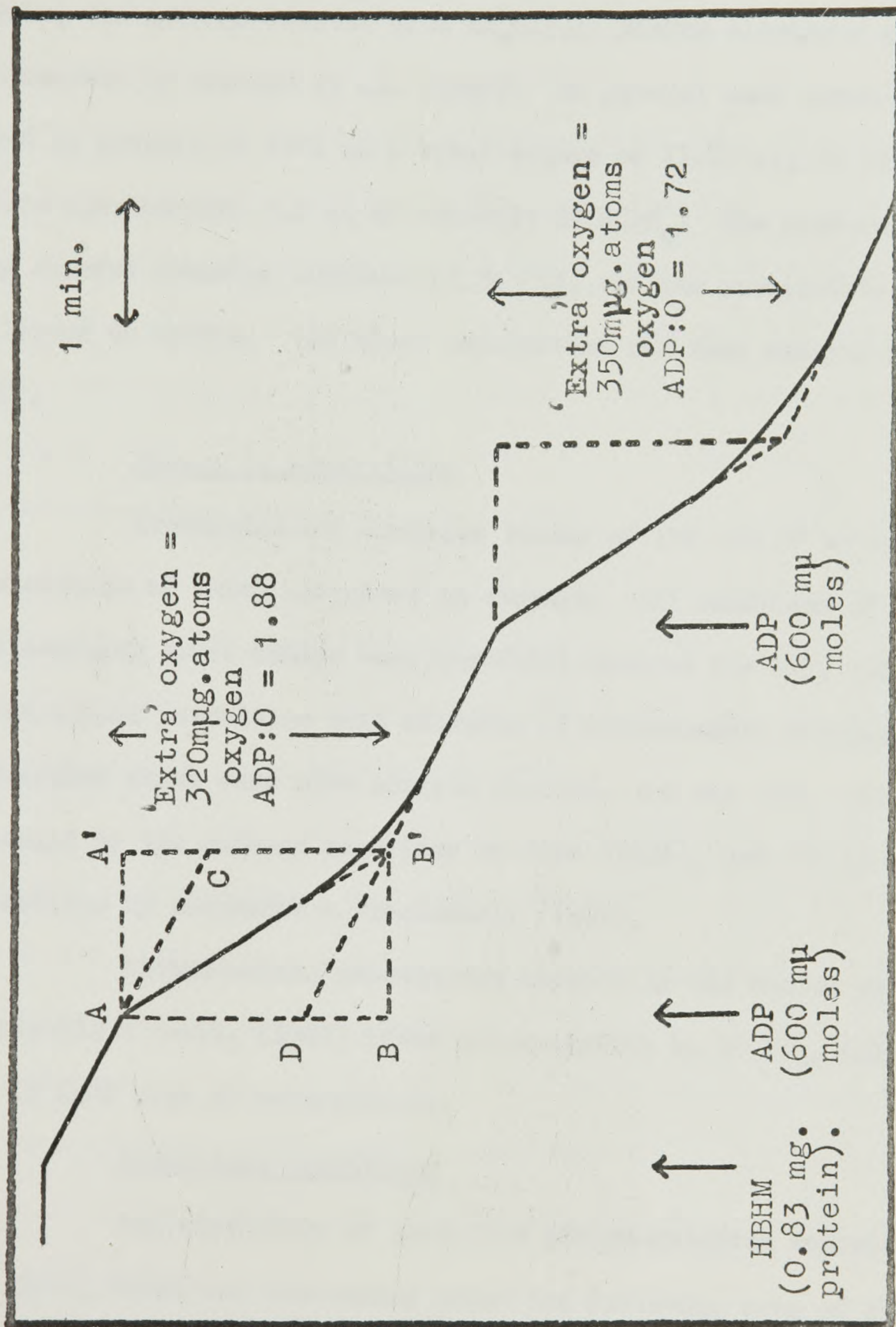
(a) Definition of 'extra oxygen' in ADP:O the determination of ratios

The results of a typical experiment in which ADP was added to stimulate the oxidation of succinate plus cysteine sulphinic acid by HBHM are given in Fig. 2B. In common with most workers in this field the 'extra oxygen' uptake was defined as the total consumption of oxygen during the burst of State 3 respiration ($= AB = A'B'$ in Fig. 2B). Other workers have chosen to subtract from this the corresponding amount State 4 respiration and defined 'extra oxygen' uptake as $(A'B' - A'C')$ or $(AB - DB)$ (e.g. Ozawa, 1966). As discussed by Chance & Williams (1955b), and Ochoa (1943) there is no justification for this correction, which would overestimate ADP:O ratios.

(b) The time course of phosphorylation of ADP

As stressed by Hagihara (1961), it is important to validate

Figure 2B. Polarographic Determination of ADP:O Ratios at 30°.



Medium: 3.60 ml. containing:- 0.25M sucrose; 50 mM tris-HCl, pH 7.6; 5 mM KPO_4 ; 5 mM MgCl_2 ; 1 mM disodium succinate; 0.5 mM tris-cysteine sulphinate, pH 7.6; 0.3 μ moles rotenone.

the method of ADP:O ratios as a measure of the efficiency of oxidative phosphorylation by assaying the net phosphorylation of added adenine nucleotides. The time course of phosphorylation of added ADP was studied using the syringe-repette with an E.I.L. Bishop electrode attachment described by Garland et al. (1965). In general each incubation contained 6-8 mg protein of HBM in a total volume of 17-18 ml, at 25°. 1.0 ml samples were ejected into 0.2 ml of ice-cold 3N HClO₄. The mixture was neutralized by careful dropwise addition of 3N KOH, and the precipitate of KClO₄ and allowed to settle. The clear supernatant was then assayed for AMP, ADP and ATP.

Assays of metabolites

Commercial ADP contains traces of ADP and 5% or more AMP, the percentage of which increases on storage. All solutions of ADP used in determining ADP:O ratios were carefully assayed for AMP, ADP and ATP. Neutralized perchloric acid extracts of mitochondria obtained by the methods described above were also assayed for AMP, ADP and ATP. AMP and ADP were assayed by the methods described by Adam (1965), and ATP by the method described by Lamprecht & Trautschold (1965).

Mitochondrial protein was assayed by the biuret method of Gornall, Bardawill & David, (1949) after precipitation by 5% TCA, and solubilization in 1N NaOH plus 1% deoxycholate.

Incubation Conditions

The efficiency of oxidative phosphorylation expressed by the P:O or ADP:O, ratio was determined under the following sets of experimental conditions:-

(a) After Smith & Hansen (1964)

Oxygen consumption was measured by the manometric technique

and P_i esterification by both P_i disappearance and HMP formation under the conditions of Smith & Hansen (1964).

The main compartment of the Warburg flask contained in 2.00 ml of 0.25M sucrose:- 25 μ moles tris-HCl, pH 7.4; 25 μ moles KPO_4 , pH 7.4; 10 μ moles $MgCl_2$; 5 mg BSA; RLM (4-15 mg. protein) or HHHM (1-5 mg. protein), and the substrates:- 10 μ moles tris α -oxo-glutarate plus 5 μ moles tris-malonate, pH 7.4; 10 μ moles sodium pyruvate plus 10 μ moles tris-L malate, pH 7.4; or 10 μ moles tris-succinate, 5 μ moles tris-cysteine sulphinate, pH 7.4 and 0.5 μ mole rotenone/mg. protein. Side-arm 1 contained in 0.2 ml:- 60 μ moles glucose, 100 μ g. dialysed Boehringer hexokinase and 5 μ moles ADP, pH 7.4. Side-arm 2 contained 0.3 ml of 0.9M $HClO_4$, 0.6M Na_2SO_4 . The centre-well contained 0.2 ml of 10% KOH. After an eight minute preincubation to obtain full thermal equilibration, the incubations were started by tipping side-arm 1. The incubations were stopped after a further 5-10 min. by tipping side-arm 2. The manometer flasks were then replaced in the bath to equilibrate for a further 5-10 min.

Controls were run in which both side-arms were tipped after the initial preincubation. The small volume change which resulted was subtracted from the final volume changes of the other incubations. This control was also assayed for initial levels of P_i and HMP. Other controls were, incubations in the presence of 0.1 mM DNP to determine substrate level phosphorylation, and a control containing 0.3 mM KCN to determine HMP formed by the combined action of hexokinase and myokinase in the absence of respiration.

(b) After Lynn & Brown (1965)

Oxygen consumption was determined by the manometric technique, and P_i esterification by both P_i disappearance and HMP formation under the

conditions of Lynn & Brown (1965).

The main compartment of the Warburg flask contained in 1.90 ml 200 μ moles sucrose; 10 μ moles tris-HCl, pH 7.4; 1 μ mole tris-EDTA, pH 7.4; 0.5 μ moles ATP; 40 μ moles glucose; 10 μ moles MgCl_2 ; 25 μ moles KF; 2.5 mg. BSA and RLM (4-15 mg. protein), or HBHM (1-5 mg. protein). Side-arm protein 1 contained in 0.3 ml 25 μ moles K-PO_4 , pH 7.4; 100 μ g. dialysed Boehringer hexokinase and the substrates described under (a). Side-arm 2 contained 0.3 ml of 0.9M HClO_4 ; 0.6M Na_2SO_4 . The centre-well contained 0.2 ml of 10% KOH. The incubation procedure was as in (a). ATP, MgCl_2 , EDTA, KF and tris-HCl were mixed to form a stable emulsion of 'fluoromagnesium ATP' as described by Lynn & Brown (1965).

(c) After Smith & Hansen (1964)

Oxygen consumption was measured polarographically in the Clark electrode cell described by Chappell (1964). ADP:O ratios were determined by the method of Chance & Williams (1955b) under the conditions of Smith & Hansen (1964). RLM (1-5 mg. protein) or HBHM (0.5 - 2 mg. protein), were incubated at 30° in 3.60 ml containing 0.25M sucrose; 5 mM MgCl_2 ; 5 mM KPO_4 ; pH 7.4; 5 mg. BSA and substrates 3 mM tris α -oxo-glutarate plus 1.5 mM tris-malonate, pH 7.4; 3 mM sodium pyruvate plus 3 mM tris-malate, pH 7.4; 3 mM tris succinate; 1.5 mM tris cysteine-sulphate, pH 7.4, and 0.5 μ mole rotenone/mg. protein. Mitochondria were added last and allowed to respire in State 4 for 2-3 min. 1-2 μ moles of ADP were then added from a calibrated Hamilton micro-syringe, and the oxygen uptake during the burst of State 3 respiration calculated as described above.

(d) After Lynn & Brown (1965)

Oxygen consumption was measured polarographically at 30° in the Clark electrode cell described by Chappell (1966) and P_i esterification

determined by HMP formation under the conditions of Lynn & Brown (1965).

RLM (1-5 mg. protein) or HBHM (0.5 - 2 mg. protein) were pre-incubated in the polarograph cell at 30° for 3-5 min. in 2.2 ml containing 400 μ moles sucrose; 20 μ moles tris-HCl, pH 7.4; 2 μ moles tris-EDTA; 1.0 μ mole sodium ATP; 80 μ moles glucose; 20 μ moles $MgCl_2$; 50 μ moles KF and 5 mg. BSA. The incubation was started by adding 2.20 ml containing 50 μ moles KPO_4 , pH 7.4; 100 μ g. dialysed Boehringer hexokinase and the same concentrations of substrates described under (a).

Incubation times of 4-8 min. were used. A 2.0 ml sample was then withdrawn and quickly mixed with 0.5 ml of ice-cold 0.9 M $HClO_4$, 0.6M Na_2SO_4 . The sample was carefully neutralized by dropwise addition of 3N KOH at 0° , and the crystals of $KClO_4$ allowed to settle. The supernatant was assayed for HMP. DNP and KCN controls were run as described above.

(e) After Smith & Hansen (1964)

Oxygen consumption was measured polarographically at 30° in the Clark electrode cell, and P_i esterification determined by HMP formation under the conditions of Smith & Hansen (1964). RLM (1.5 mg. protein) or HBHM (0.5 - 2 mg. protein) were incubated in 3.60 ml containing the same components as in (c) plus the hexokinase trap: 100 μ g. dialysed Boehringer hexokinase; 5 μ moles ADP; 40 μ moles glucose. The incubation was started by adding the mitochondria in 0.1 or 0.2 ml of 0.25M sucrose. After an incubation of 4-8 min. a 2.0 ml aliquot was removed, mixed with 0.5 ml of ice-cold 0.9M $HClO_4$ 0.6M Na_2SO_4 , and assayed for HMP.

(f) Timecourse of phosphorylation of ADP

HBHM (5-10 mg. protein) were incubated at 25° in the syringe-repette polarograph described by Garland et al. (1965) in a medium containing in 17-19 ml, 0.25M sucrose; 50 mM tris-HCl, pH 7.6; 5 mM $MgCl_2$; 5 mM KPO_4

Table 2A. Sample P:O ratios of RLM at 30° in the Warburg Manometer.

<u>Substrate</u>	<u>µg. atoms oxygen</u>		<u>µmoles P_i esterified</u>		<u>P:O ratio</u>	
	<u>AB-AD</u>	<u>AC-AD</u>	<u>HMP</u>	$\frac{\Delta P_i}{AB-AD}$	$\frac{\Delta P_i}{AC-AD}$	
Succinate	1.91	3.24	6.2	6.1	3.19	1.88
	3.36	4.54	8.05	8.2	2.44	1.81
Succinate + 0.1 mM DNP	1.82	2.63	0.18	0.18	0.10	0.07
α oxo-glutarate	1.92	3.88	14.9	15.8	8.23	4.07
	2.03	4.16	15.8	16.0	7.89	3.84
	2.89	4.92	15.1	18.8	6.48	3.80
α oxo-glutarate + 0.1 mM DNP	2.46	4.47	1.6	2.9	1.18	0.65

RLM (10 mg. protein) were incubated in Warburg manometers using conditions (b). AB, AC, AD, refer to the notation of Figs. 2C, 2D. HMP was corrected for HMP formed in a control incubation, poisoned with 0.3 mM KCN.

Table 2B. The Efficiency of Oxidative Phosphorylation in Mitochondria.

<u>Mitochondria.</u>	<u>Method of</u> <u>Determination.</u>	<u>Substrate.</u>	<u>Succinate.</u>	<u>Pyruvate +</u> <u>L-malate.</u>	<u>α oxo-glutarate</u>
Rat Liver (RLM).	ΔP_i /AB-AD	a,b	1.8-3.5	2.8-5.6	3.7-8.3
	ΔP_i /AC-AD	a,b	1.7-1.95	2.5-2.9	3.5-4.0
	ADP:O	c	1.7-1.95	2.5-2.9	3.2-3.5
	HMP/O	d,e	1.7-1.95	2.4-2.8	3.4-3.8
Ox Heart (HBHM).	ΔP_i /AB-AD	a,b	1.8-3.5	2.8-5.3	3.7-7.0
	ΔP_i /AC-AD	a,b	1.8-1.95	2.7-3.0	3.5-3.9
	ADP:O	c	1.6-1.95	2.7-3.0	3.2-3.5
	HMP/O	d,e	1.8-1.95	2.7-3.0	3.4-3.7

Each value represents the range of 5-10 experiments. AB, AC, AD, refer to the notation of Figs. 2C and 2D.

and the substrates described in (a).

The reaction was initiated by adding 6-10 μ moles of ADP.

1.00 ml samples of the medium were taken at intervals, and rapidly mixed with 0.20 ml of ice-cold 3N HClO_4 . The samples were neutralized to pH 7.6 by dropwise addition of 3N KOH with stirring. The precipitate of KClO_4 was allowed to settle, and the clear supernatant was assayed for AMP, ADP and ATP.

2(iii) Results and Discussion

P:O ratios of freshly prepared HHHH and RLM were examined using the manometric methods described by Smith & Hansen (1964). Method (a) and of Lynn & Brown (Method (b)) and similar results to these authors were obtained (see Tables 2A, 2B). However, parallel determinations of ADP:O or G6P:O ratios in identical media (Methods (c) (d) and (e)), using the polarographic measurement of oxygen consumption resulted in much lower ADP:O or HMP:O ratios (see Tables 2B).

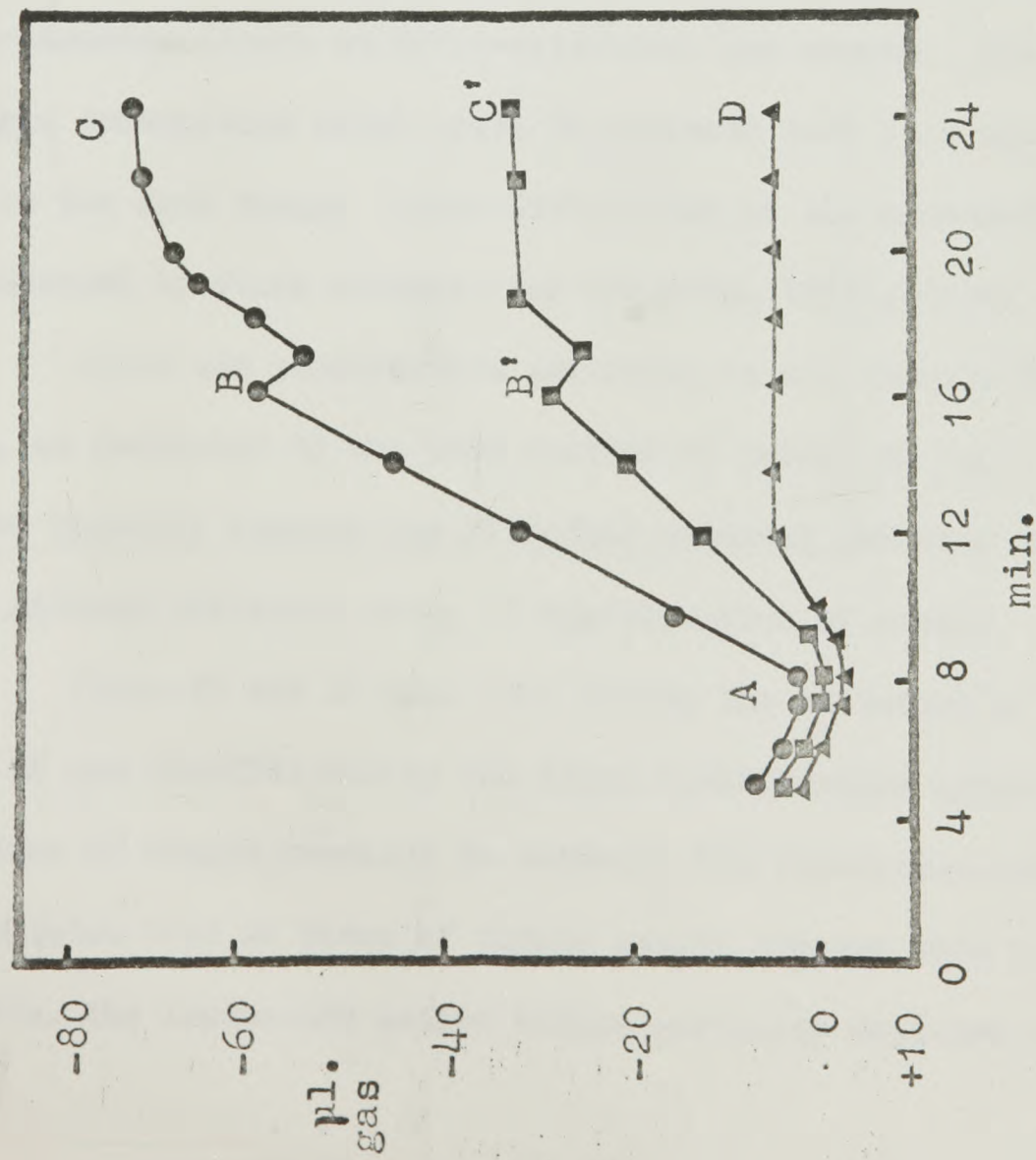
Accordingly (1) the validity of the manometric measurement of oxygen consumption was investigated (2) the validity of the ADP:O method of Chance & Williams (1955b) was investigated using incubation method (f).

Manometric Measurements of Oxygen Uptake

The time course of gas uptake in manometric incubations was investigated (see Figs. 2C, 2D). It was found that if the oxygen consumption of the respiring incubations exceeded 0.2 to 0.3 μ g atoms/min., there was a further uptake of gas during the final equilibration period. In Figs. 2C and 2D the volume change during the incubation was AB-AD, and the further uptake of gas during the final equilibration was BC. If AB-AD was taken to represent oxygen uptake high P:O ratios were obtained (see Tables 2A, 2B).

If AC-AD was taken to represent oxygen uptake, normal P:O ratios

Figure 2C. Manometric Measurement of Succinate oxidation.

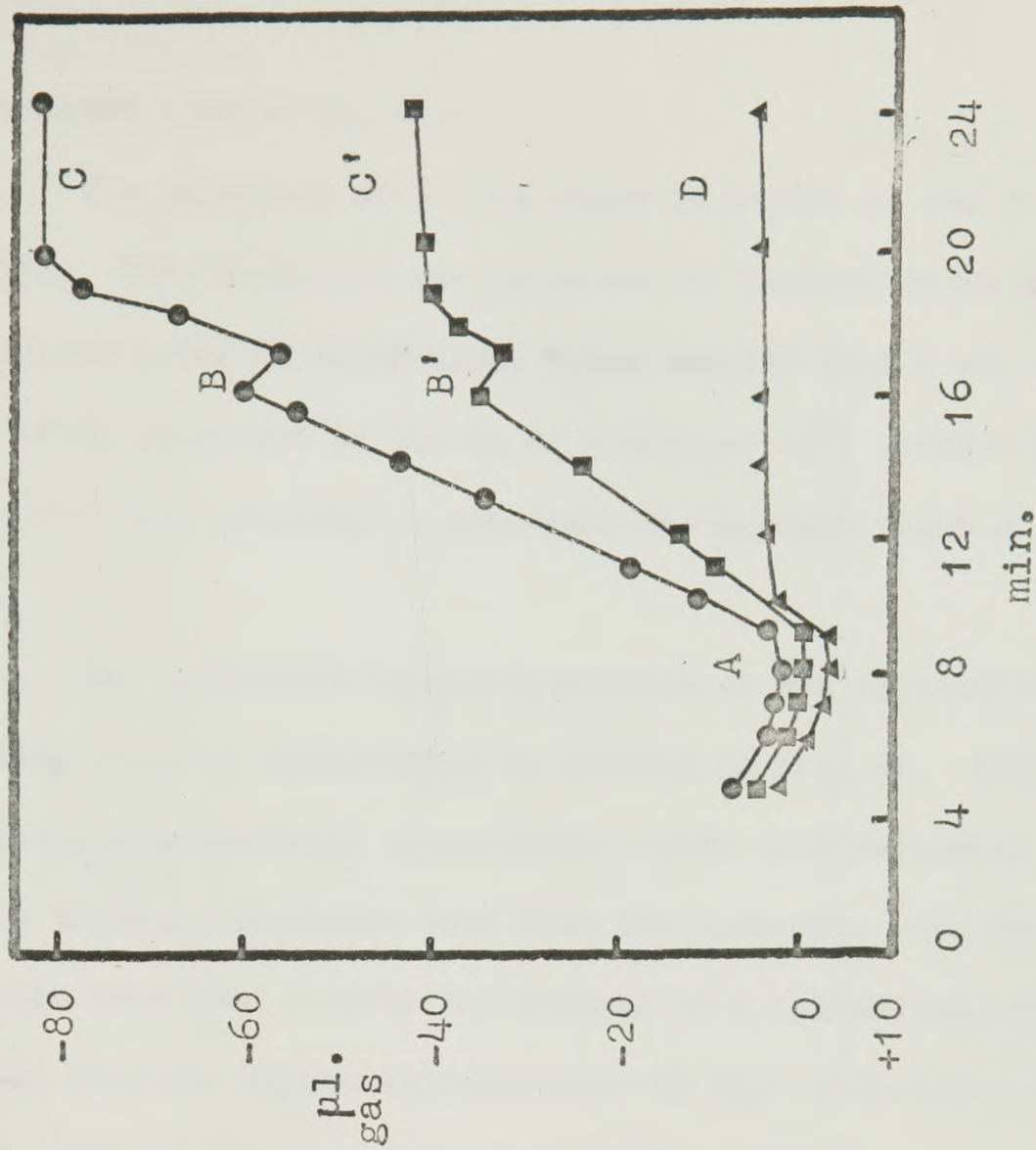


Respiration of HBHM under conditions (b).

- Oxygen uptake = 0.69 $\mu\text{g. atoms/min}$;
BC = 11.9 pl.
- Oxygen uptake = 0.31 $\mu\text{g. atoms/min}$;
BC' = 3.5 pl.
- ▲ Control.

Side-arm 1 was tipped at 8 min. (side-arms 1 and 2 in control); side-arm 2 was tipped at 16 min.

Figure 2D. Manometric Measurement of α -oxo-glutarate oxidation.



Respiration of HBHM under conditions (b).

- Oxygen uptake = 0.69 $\mu\text{g. atoms/min}$;
BC = 22.5 pl.
- Oxygen uptake = 0.34 $\mu\text{g. atoms/min}$;
BC' = 9.5 pl.
- ▲ Control.

were obtained (Tables 2A, 2B).

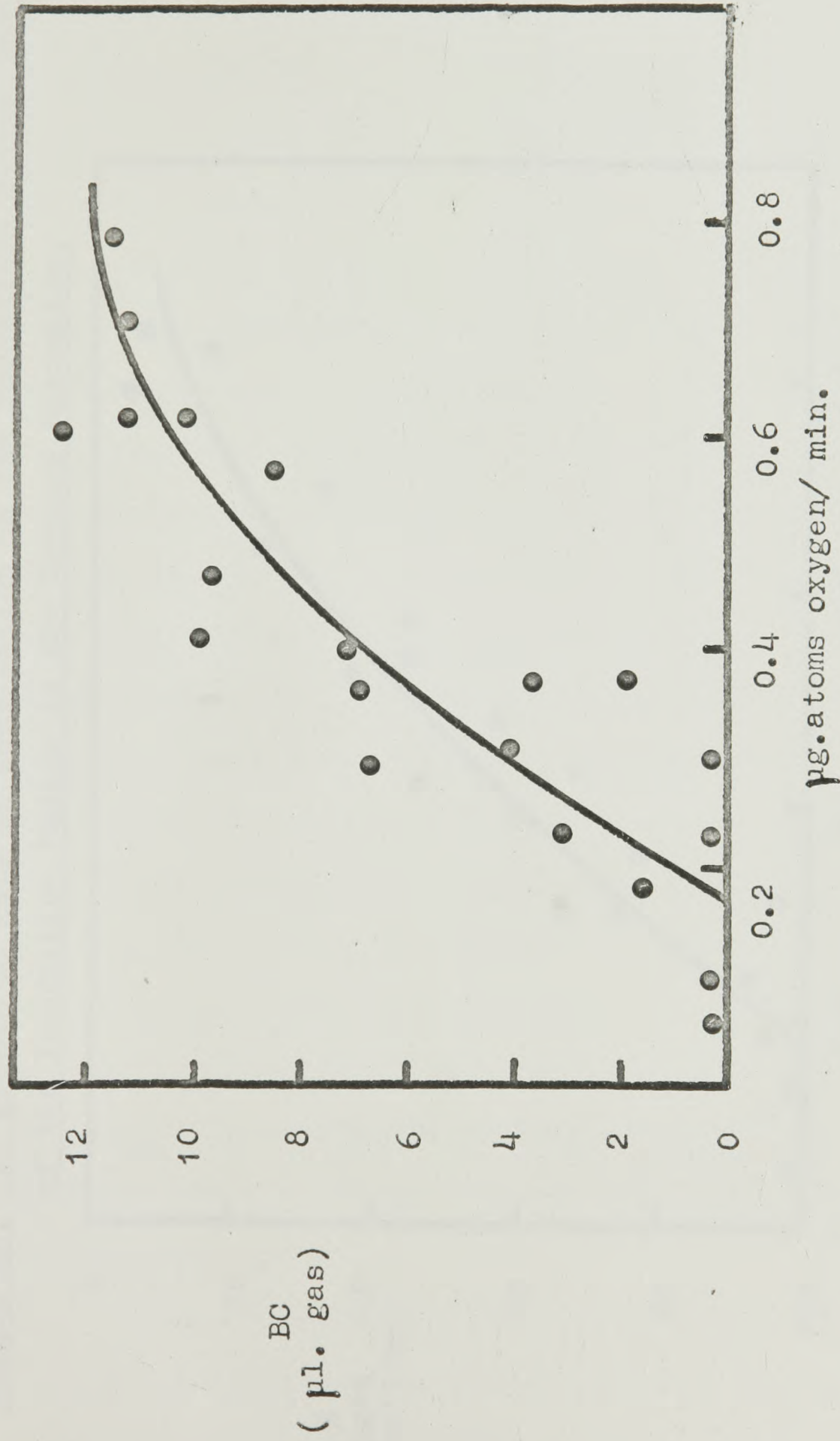
The magnitude of BC was shown to depend on the rate of respiration (Fig. 2E). With succinate as substrate the maximum value of BC was 11.5 μ l. for a 2.2 ml incubation, which exactly equals the volume of oxygen which dissolves in 2.2 ml of medium at 30°. With α oxo-glutarate or pyruvate plus L-malate as substrate the maximum value of BC was 15 - 23 μ l.

The percentage oxygen-saturation of the incubation medium at increasing rates of respiration is plotted in Fig. 2F. This was obtained by stopping mitochondrial incubations, which were respiring at a steady rate, by tipping perchloric acid from the side-arm. The contents of the flasks were then rapidly transferred to a calibrated polarograph. It was found that the oxygen concentration of the incubation medium approached zero at rates of oxygen consumption greater than 0.8 μ g atoms/min. for 15 ml. manometer flasks at 120 oscillations per minute. The maximum rates of oxygen consumption which could be achieved were also measured, and found to be in the same range. These limitations of the manometric method have been stressed by other workers (see Roughton, 1941; Myers & Matsen, 1955).

There was considerable variation in the results for different flasks, as indicated by the wide scatter of points in Figs. 2E and 2F and this was probably because the different internal geometry of individual flasks allowed different rates of equilibration of gases.

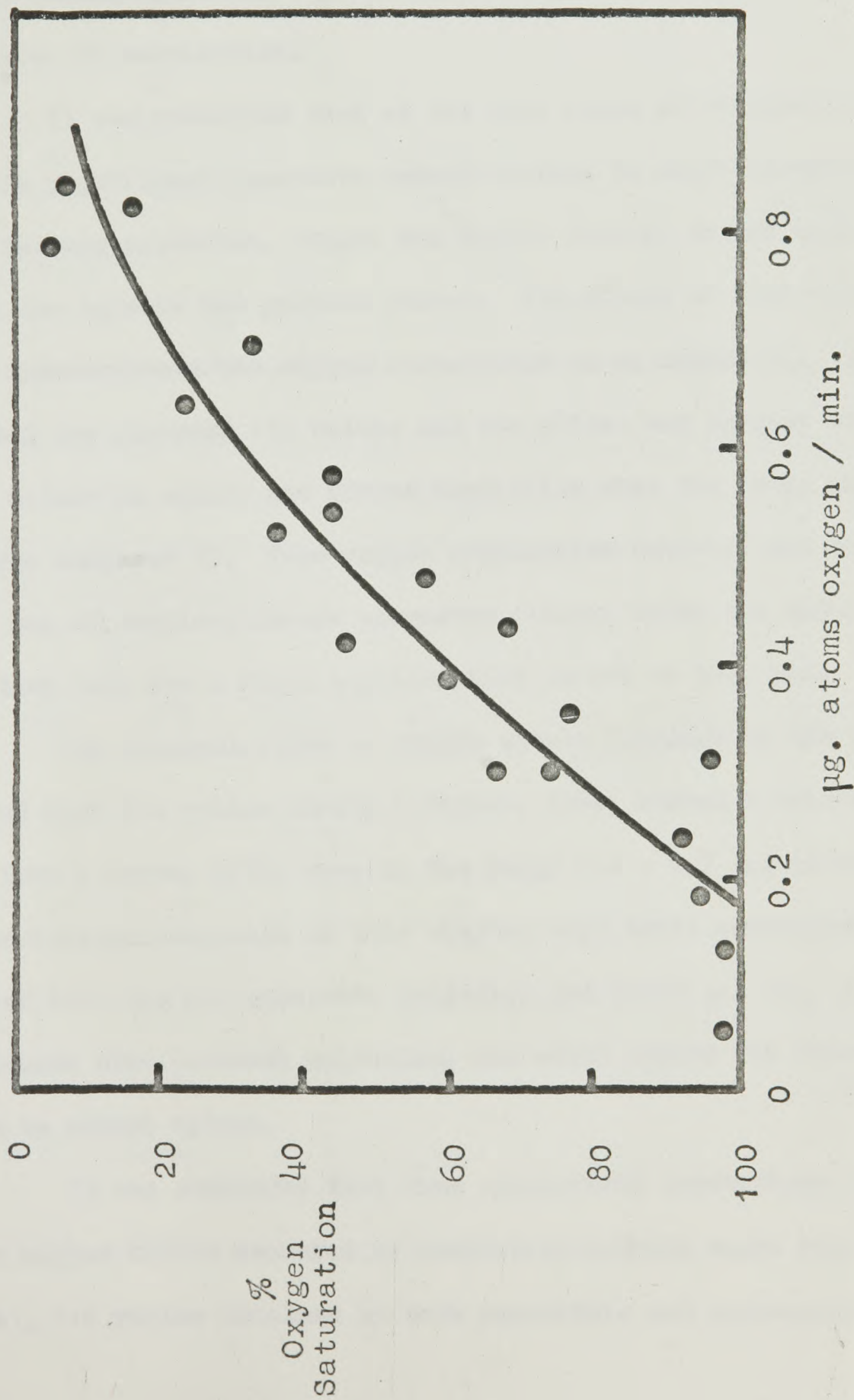
Figs. 2E and 2F show that during the oxidation of succinate the volume of gas absorbed during the final equilibration agreed closely with the volume of oxygen required to saturate the incubation medium, and it was concluded that at rates of oxygen uptake greater than 0.2 - 0.3 μ g atoms/min. the incubation medium became partially depleted of oxygen.

Figure 2E. Dependence of BC on the Rate of Respiration.



BC, the uptake of gas during the final equilibration period, was plotted against the rate of oxidation of succinate by RLM in the Warburg manometer under conditions (b).

Figure 2F. The Effect of Rate of Respiration on the Percentage Oxygen Saturation of the Incubation Medium in the Warburg Manometer.



Mitochondrial incubations, respiring at a steady rate, were stopped by tipping perchloric acid from the side-arm, and transferred to a calibrated polarograph cell to determine the percentage oxygen saturation.

Figure 20. The Time Course of Phosphorylation of ADP

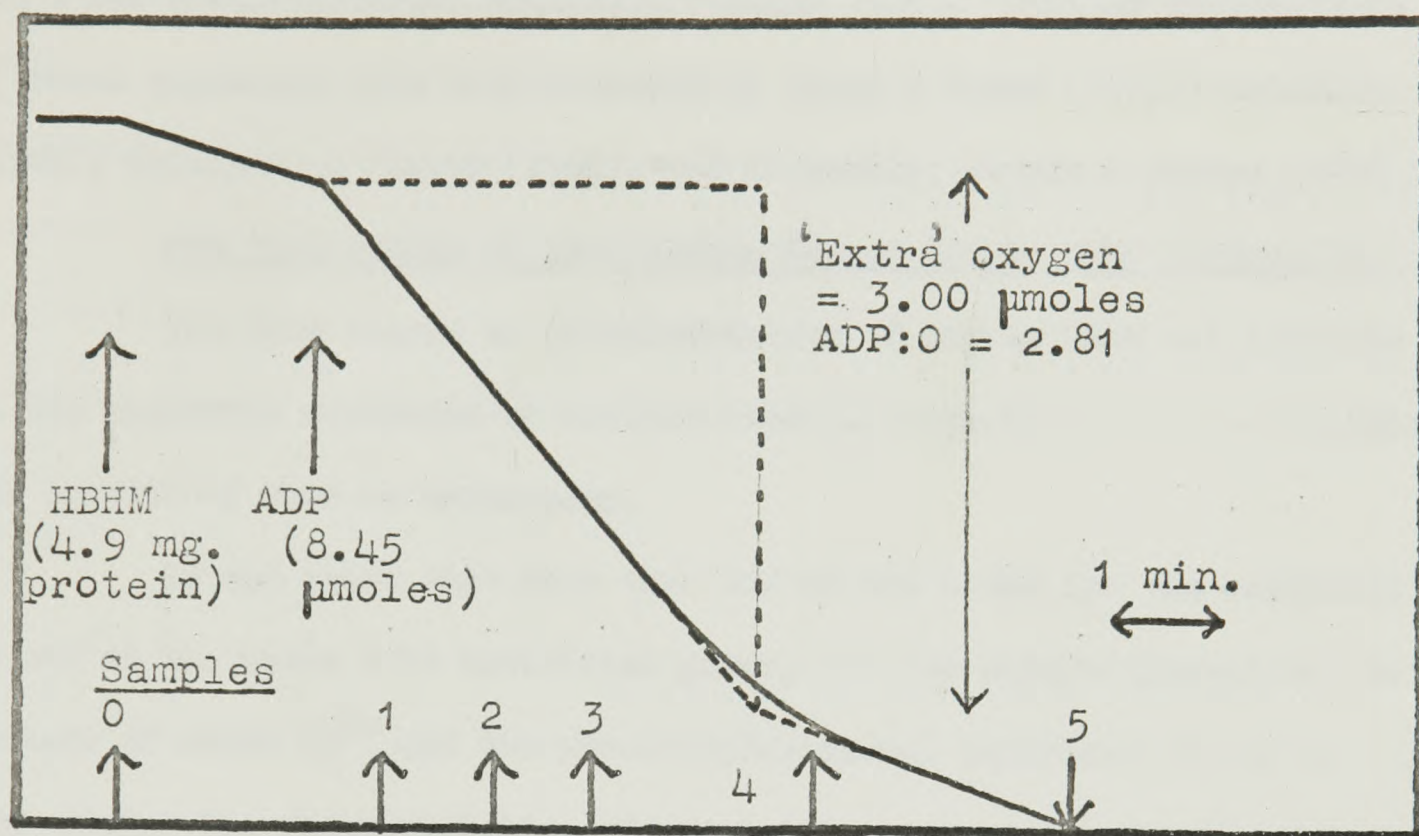
With α -oxo-glutarate or pyruvate plus L-malate as substrate the values of BC were larger than could be accounted for by the absorption of oxygen alone, and it is postulated that part of BC represents the absorption of CO_2 by KOH in the centre-well during the final equilibration period. It has previously been shown (Dixon, 1951; Myers & Matsen, 1955) that there is a finite concentration of CO_2 in the gas phase of the manometer flasks during rapid rates of respiration.

It was concluded that at the high rates of respiration required to obtain significant phosphate esterification in short incubation periods in the Warburg apparatus, oxygen and carbon dioxide do not equilibrate between the aqueous and gaseous phases. The effect of such a non-equilibration was to underestimate the oxygen consumption by an amount BC. Such errors increased the observed P:O ratios and the effect was largest when the total oxygen uptake is small, and almost negligible when the total oxygen uptake was large compared BC. True oxygen consumption ($=\text{AC}-\text{AD}$) can be obtained by the use of double-side-arm manometer flasks, which are returned to the incubation bath for a final equilibration period of 5-10 min.

The apparent rates of oxygen uptake obtained by the workers who reported high P:O ratios (Smith & Hansen, 1964; Gurban & Cristea, 1965, 1966; Lynn & Brown, 1965) were in the range 0.4 - 0.7 $\mu\text{g atoms/min}$. In the incubations described in this chapter this would correspond to values of BC of 5-11 μl . for succinate oxidation and 10-23 μl . for α -oxo-glutarate or pyruvate plus L-malate oxidation, and would reduce the observed P:O ratios to normal values.

It was concluded that when appropriate corrections were applied to the oxygen uptake measured by manometric methods under conditions (a) and (b), P:O ratios obtained by both manometric and polarographic techniques

Figure 2G. The Time Course of Phosphorylation of ADP-
by HBHM at 25°.



The incubation contained in 17.2 ml.:— 0.25M sucrose;
 50 mM tris-HCl, pH 7.6; 5mM KPO₄; 1 mM tris-EDTA, pH 7.6;
 5.8 mM sodium pyruvate; 4.8 mM tris-L-malate, pH 7.6;
 HBHM (4.9 mg. protein).

<u>Sample</u>	<u>ADP</u>	<u>ATP</u>	<u>Total</u>	<u>~P</u>	<u>μg.atoms oxygen</u>	<u>~P:O</u>
0	8.45	0	8.45	0	0	—
1	7.29	1.12	8.41	1.12	0.39	2.88
2	6.07	3.21	9.28	3.21	1.13	2.84
3	3.85	4.84	8.69	4.84	1.88	2.57
4	0.53	8.54	8.57	8.54	3.20	—
5	0.02	8.45	8.47	8.45	4.10	—

agreed, and did not exceed 3 for pyruvate plus L-malate 2 for succinate and 4 for α oxo-glutarate oxidation (Haslam 1965). Similar explanations of these phenomena have been advanced by Lenaz & Beyer (1965); Brierley (1965); Koivasalo & Slater (1965); and Koivasalo, Currie & Slater (1966).

The time course of phosphorylation of ADP by HHM (Method (f))

The time course of phosphorylation of ADP by HHM was measured in the apparatus described by Garland (1965). Fig. 20 gives the results of such an experiment.

It was found that more than 92% of the added ADP was converted to ATP at the State 3 $\rightarrow$ 4 transition point. ADP was phosphorylated in the absence of added Mg^{2+} , and the phosphorylation was unaffected by up to 5 mM EDTA. The phosphorylation of added AMP required added Mg^{2+} and was inhibited by EDTA, and will be discussed in detail in Chapter 3.

The methods used to assay ATP at the State 3 $\rightarrow$ 4 transition involved stopping the reaction with perchloric acid, followed by a careful neutralisation with KOH. During these procedures some of the ATP could become degraded to ADP and AMP, and the figure of 92% conversion to ATP at the State 3 $\rightarrow$ 4 transition point could be an underestimate. These experiments clearly validate the method of Chance & Williams (1955b) as a measure of the efficiency of oxidative phosphorylation.

2(iv) Summary and Conclusions

- (1) P:O ratios of HHM and HHM have been re-examined under a number of conditions and by independent methods of measuring oxygen consumption and P_i esterification.
- (2) When appropriate precautions were taken the P:O ratios obtained by all methods and under all conditions described in this chapter agreed,

and did not exceed 3 for pyruvate plus malate 2 for succinate and 4 for α -oxo-glutarate oxidation.

(3) The high P:O ratios reported by Smith & Hansen (1964), Gurban & Cristea (1964, 1965) and Lynn & Brown (1965) can be explained as being caused by an underestimation of oxygen consumption. This was due to non-equilibration of oxygen and carbon dioxide between the gaseous and aqueous phases of the Warburg manometer at the high rates of respiration used in these experiments.

(4) The method of Chance & Williams (1955b) for the estimation of the efficiency of oxidative phosphorylation in NBHM has been validated by measurement of the phosphorylation of ADP during State 3 respiration and at the State 3 $\rightarrow$ 4 transition point.

Materials

Preparation of State 3 and State 4 NBHM and measurement of intramitochondrial pH.

NBHM were prepared on a small scale as described by Chance & Williams (1955), or on a large scale as described by Chance & Williams (1955). The layer of light mitochondria was stirred off as described by Chance & Williams (1955), and the heavy-layer mitochondria were described by Chance & Williams (1955). NBHM were stirred at -2°C for 10 min before use in a medium containing 0.25 M sucrose; 1 M KCl; 1 M Tris; 1 M ascorbate; 25 mM Tris-HCl, pH 7.4.

CHAPTER 3

THE NATURE OF THE PRIMARY PHOSPHATE ACCEPTOR IN OXIDATIVE PHOSPHORYLATION

3(i) Introduction

An important problem in the field of oxidative phosphorylation is the nature of the nucleotide acceptor to which phosphate is first linked in a high-energy bond. It has been generally assumed that ADP is the primary acceptor, but recently Ozawa & MacLennan (1965), and Ozawa (1966) have presented evidence that AMP is the primary acceptor.

This problem has been investigated by two methods:- (a) The labelling of the bound adenine nucleotides of HBHM and ETP_H by $^{32}\text{P}_i$ was measured in short (20-120 sec.) incubations with succinate as substrate, as described by Ozawa & MacLennan (1965). (b) The ability of AMP to release the controlled respiration of HBHM was investigated, and AMP:O ratios determined by the method of Chance & Williams (1955b). The phosphorylation of adenine nucleotides in such incubations was also assayed enzymically.

3(ii) Methods

Preparation of Heavy Beef Heart Mitochondria and phosphorylating sub-mitochondrial particles

HBHM were prepared on a small scale by the method of Hatefi et al. (1961), or on a large scale as described by Sanadi & Fluharty (1963). The layer of light mitochondria was sloughed off as described by Hatefi & Lester (1958), and the heavy-layer mitochondria were suspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6. HBHM were stored at -15° for up to two weeks before use in a medium containing 0.25M sucrose; 4 mM MgCl_2 ; 1 mM ATP; 1 mM succinate; 25 mM tris-HCl, pH 7.6.

Phosphorylating sub-mitochondrial particles (ETP_H) were prepared as described by Hansen & Smith (1964), except that the medium contained 15 mM HgCl_2 and no KCl_2 . The pellet from the low speed centrifugation (25,000g for 7 min.), described as 'large ETP_H ', was washed by spinning down in 0.25M sucrose; 10 mM tris-HCl, pH 7.6, and resuspended in the same medium. The pellet from the high speed centrifugation (105,000g for 40 min.), described as 'small ETP_H ' was washed as above and resuspended in 0.25M sucrose; 10 mM tris-HCl, pH 7.6.

Purification of $^{32}\text{P}_i$. Carrier-free $^{32}\text{P}_i$ was obtained from Amersham, England, and purified by the method of Conover, Prairie & Racker (1963). An abbreviated form of this procedure was also employed. 2-10 mCi of carrier-free $^{32}\text{P}_i$ was added to 5-10 μ moles of P_i , and HCl added to 3N. This was heated at 100° for 30 min. to hydrolyse pyrophosphate and polyphosphate, diluted to 0.02 N HCl, and passed through a column of Dowex 50-X4- H^+ resin. The eluate was neutralised and adsorbed to a column of Dowex 1-X8- Cl^- , which was then washed with water, and the $^{32}\text{P}_i$ eluted with 0.05 N HCl. The eluate was neutralised to pH 7.6 with 1N KOH.

Separation of Mitochondrial Adenine Nucleotides

Perchloric acid extracts of mitochondria and of ETP_H were chromatographed as described by Pressman (1958), using charcoal-celite or charcoal-G25 Sephadex columns, followed by ion-exchange chromatography on Dowex 1-X8-Formate columns.

(a) Preparation of Columns

(4 x 2) cm. charcoal-celite columns were prepared from acid-washed celite plus 100 mg. Norit A charcoal (capacity = 2 μ moles adenine nucleotide). The celite was washed 6-8 times with 3N HCl prior to use, but because of the difficulty of removing all ultra-violet absorbing material

Figure 3A. Separation of ^{32}P -labelled adenine nucleotides.

in this way, it was subsequently replaced by G25-Sephadex. The columns were washed prior to use with 40 ml. of a 50% ethanol, 2% NH_3 mixture, and then 100 ml. 0.1 N HCl. (1 x 3) cm. columns of Dowex 1-X8-Formate were prepared, and washed with water until neutral before use.

(b) Separation Procedures

The separation procedures used to determine the specific-activities of ^{32}P -labelled adenine nucleotides are given in Fig. 3A and Table 3A. Radioactivity was counted at Stage 2, and inorganic phosphate determined by the method of Chen et al. (1956). AMP, ADP and ATP were separated at Stage 4, and eluted from the Dowex 1-X8-Formate column in fractions 3, 6 and 9 respectively (see Table 3A). The radioactivity of these fractions was counted, but the adenine nucleotide concentrations could not be accurately determined by the absorbance at 260 $\text{m}\mu$ because the high concentrations of ammonium formate absorbed at this wavelength. Accordingly, fractions 3, 6 and 9 were de-salted by adsorbing the adenine nucleotides to a second series of charcoal columns. Adenine nucleotides were then eluted with 40 ml. of a 50% ethanol, 2% NH_3 mixture, and reduced to a volume of 5-10 ml. by rotary evaporation (= Stage 7). Radioactivity was again counted, and adenine nucleotide concentration determined by the absorbance at 260 $\text{m}\mu$ ($E_{\text{mM}} = 14.5$).

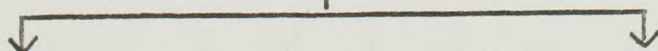
In certain experiments, adenine nucleotide concentrations were also determined enzymically at Stage 7. AMP and ADP were determined by the method of Adam (1965), and ATP by the method of Lamprecht & Trautshold (1965). The results of such an experiment are included in Table 3A. It was found that, although the elution pattern at Stage 4 closely corresponded to that obtained by Freneman, (1958), at Stage 7 the ADP and ATP (fractions 6 and 9)

Figure 3A. Separation and Determination of the Specific Activities of ^{32}P -labelled Mitochondrial Adenine Nucleotides.

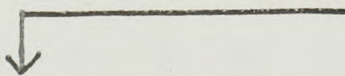
Perchloric acid extracts of mitochondria spun (500g for 10 min.) to remove denatured protein.



Supernatant loaded on to charcoal-Celite or charcoal-G25 Sephadex columns (Stage 1).



- (a) Washed with (3 x 40) ml. H_2O . Aqueous eluate assayed for $^{32}\text{P}_i$, and radioactivity counted. (Stage 2).
- (b) Eluted with 40 ml. of a 50% ethanol, 2% NH_3 mixture, and rotary-evaporated to remove ethanol and NH_3 . (Stage 3).



Loaded on to Dowex 1-X8-Formate column. Stepwise elution with $\text{HCOOH}/\text{HCOONH}_4$ (see Table 3A). (Stage 4).



Fractions 3, 6, and 9 neutralised with NH_3 , and radioactivity counted. (Stage 5).



Readsorbed to charcoal-Celite or charcoal-G25 Sephadex columns and washed to remove HCOONH_4 . (Stage 6).



Eluted with 40 ml. of a 50% ethanol, 2% NH_3 mixture, and rotary-evaporated to remove ethanol and NH_3 . Radioactivity counted, and adenine nucleotide concentration measured by absorbance at 260 mμ. (Stage 7).

Specific activity of adenine nucleotide =

Radioactivity at Stage 5

Adenine nucleotide concentration at Stage 7

Table 3A. Elution of Mitochondrial Nucleotides from Dowex 1-X8-Formate Columns (after Pressman, 1958).

<u>Fraction</u>	<u>Tubes</u> <u>(10 ml.)</u>	<u>Eluant</u>	<u>Eluate</u> <u>(Pressman, 1958).</u>	<u>Counts/100 sec.</u>		<u>μmoles Nucleotide (Stage 7)</u>		
				<u>Stage 5</u>	<u>Stage 7</u>	<u>AMP</u>	<u>ADP</u>	<u>ATP</u>
1	1-4	0.04M HCOONH ₄	NAD ⁺	2000	-	-	-	-
2	5-12	0.15M HCOONH ₄	P _i	45000	-	-	-	-
3	13-16	0.20M HCOOH	AMP	5000	0	266	0	0
4	17-20	0.90M HCOOH	NADP ⁺	1000	-	-	-	-
5	21-24	1.50M HCOOH	'AD'	2000	-	-	-	-
6	25-28	2.20M HCOOH	ADP	117000	67000	185	136	0
7	29-32	4.00M HCOOH	Flavin	2000	-	-	-	-
8	33-36	4.00M HCOOH + 0.15M HCOONH ₄	'ADX'	3000	-	-	-	-
9	37-40	4.00M HCOOH + 0.27M HCOONH ₄	ATP	225000	110000	5	90	80

HBHM (140 mg. protein) were incubated for 2 min. at 30° in 10ml. containing:- 0.25M sucrose; 20 mM tris-HCl, pH 7.6; 4 mM MgCl₂; 1mM cysteine sulphinate; 3mM disodium succinate; and 0.02 mM KPO₄ (containing 2.0 x 10⁶ counts/100sec. of ³²P). The reaction was stopped by addition of 1.0 ml. of 0.9M HClO₄, 0.6M Na₂SO₄, and mitochondrial adenine nucleotides were separated according to Fig. 3A. Adenine nucleotides were assayed enzymically after Stage 7.

had lost 40-60% of their terminal phosphate groups, and also 40-60% of the radioactivity that they possessed at Stage 5. It was concluded that although fractions 6 and 9 were respectively ADP and ATP at Stage 4 (Pressman, 1958; Brierley & O'Brien, 1965), a high percentage of the terminal phosphate groups, and hence of the ^{32}P , was lost during Stages 5-7. This hydrolysis of the terminal phosphate groups of ADP and ATP was probably caused by the high concentrations of formic acid at Stage 4, and the heat generated during the neutralisation by NH_3 at Stage 5.

The $^{32}\text{P}_i$ thus formed would be counted at Stage 5, but would be lost when the adenine nucleotides were reabsorbed to the charcoal columns (Stage 6). The correct radioactivity of the adenine nucleotides is given by radioactivity at Stage 5, and since control experiments showed that adenine nucleotides were quantitatively recovered from the charcoal columns, the specific activity of the adenine nucleotides =

$$\frac{\text{Radioactivity at Stage 5}}{\text{Adenine nucleotide concentration at Stage 7}}$$

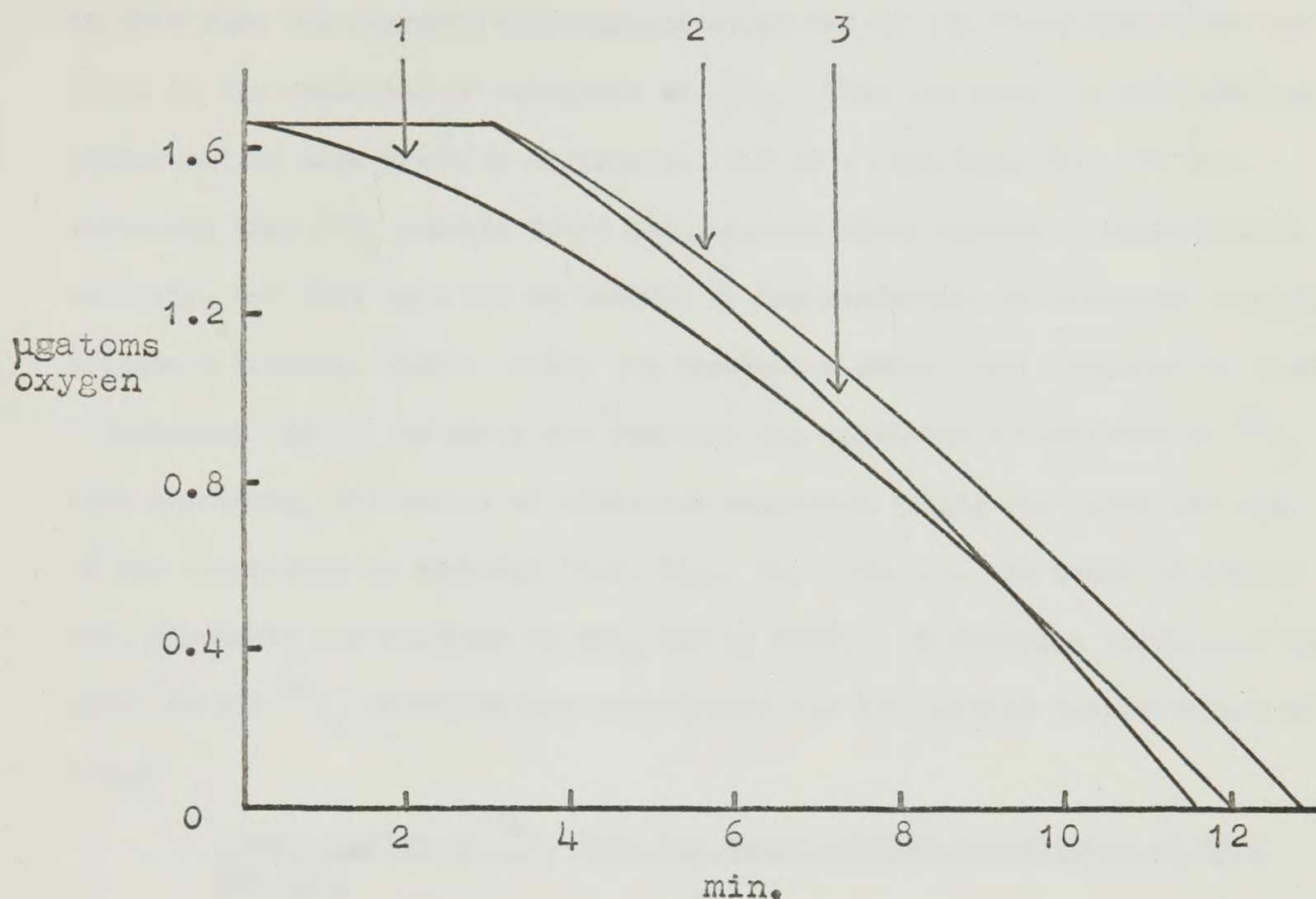
Determination of AMP:O ratios. Under certain conditions AMP released the controlled respiration of HEHM. This was studied by the methods described in Chapter 2, and AMP:O ratios were determined polarographically by the method of Chance & Williams (1955b). The phosphorylation of AMP was also measured in the syringe-repette polarograph cell (Garland et al., 1965), as described in Chapter 2.

3(iii) Results

Oxidation of Succinate by ETP_H

Ozawa & MacLennan (1965) claimed that AMP was the primary phosphate

Figure 3B. The Effect of Cysteine Sulphinate on the Oxidation of Succinate by ETP_H.



Oxygen consumption was measured polarographically at 30°. Incubations contained in 3.60 ml.:— 0.25M sucrose; 20 mM tris-HCl, pH 7.6; 4 mM MgCl₂; 3mM disodium succinate; and ETP_H (0.85 mg. protein), added to initiate the reaction.

Expt.	Conditions. of Preincubation.	Oxygen Consumption (μgatoms/min./ mg. protein)	
		First 60 sec.	Final Rate.
1	No additions	24	202
2	0.9 mM cysteine sulphinate, 3 min.	102	208
3	1.8 mM cysteine sulphinate, 3 min.	148	214

acceptor in oxidative phosphorylation on the basis of experiments involving short incubations (20-120 sec.) with succinate as substrate. Oxygen uptake in this time was measured polarographically, and it was found that there was a lag in the oxidation of succinate by ETP_H . This lag could be abolished by preincubation with cysteine sulphinate (1-2 mM), (see Fig. 3B). It was concluded that ETP_H contain bound oxaloacetate which inhibits succinoxidase activity, but that this can be removed by transamination by cysteine sulphinate (Singer & Kearney, 1956). Under the incubation conditions described by Ozawa & MacLennan (1965), in which the reaction was initiated by addition of $^{32}\text{P}_i$ plus succinate, the extent of succinate oxidation during the first 120 sec. of the incubation is doubtful (c.f. Fig. 3B, Expt. 1). In order to ensure that succinate was oxidised by ETP_H and by HHBM, 1 mM cysteine sulphinate was added to all $^{32}\text{P}_i$ incorporation experiments for the initial preincubation of 5 min.

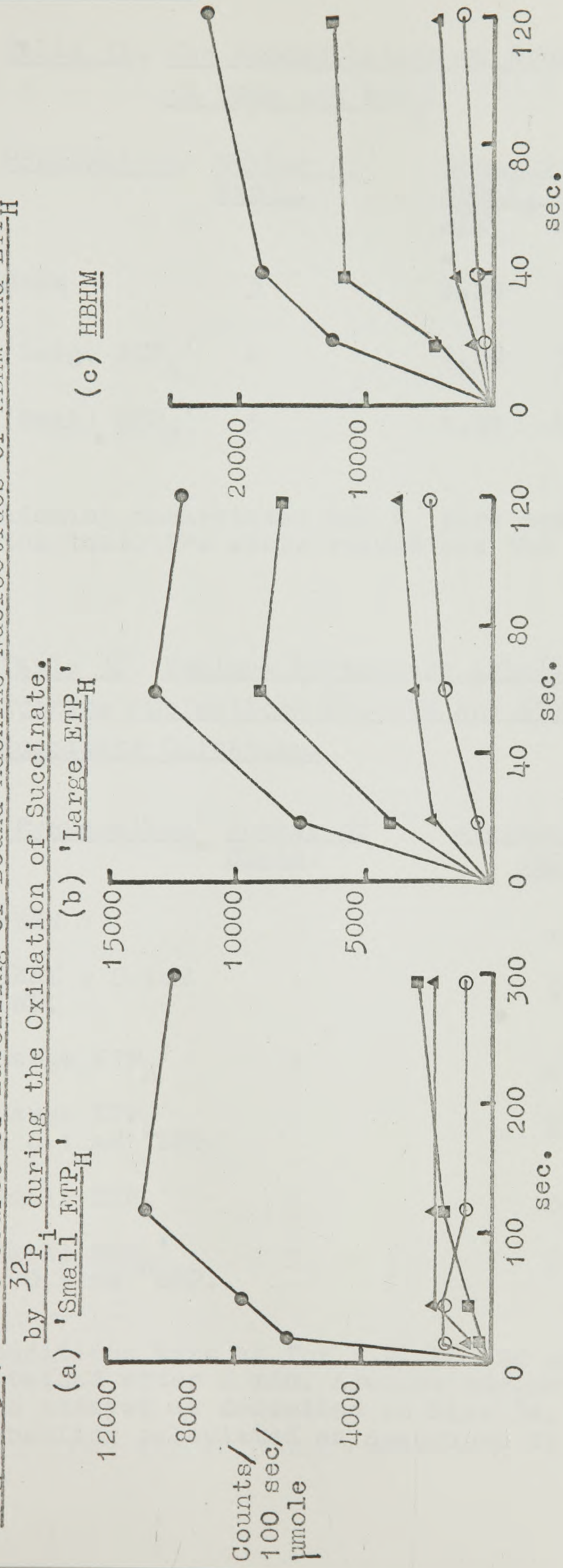
Incorporation of $^{32}\text{P}_i$ into the bound adenine nucleotides of HHBM and ETP_H

Fig. 3C shows the incorporation of $^{32}\text{P}_i$ into the bound adenine nucleotides of 'small ETP_H ', 'large ETP_H ', and HHBM during the first 120 sec. of succinate oxidation. In the absence of uncouplers, ATP had a higher specific activity than ADP at all stages of the incubation. This supports the conclusion that bound ADP is the primary acceptor of high-energy phosphate in oxidative phosphorylation.

The procedures used to separate and assay the specific radioactivities of AMP, ADP and ATP (see Fig. 3A) attempted a quantitative recovery of the adenine nucleotides and P_i , and the concentrations of these are given in Table 3B.

The percentage labelling of bound adenine nucleotides was calculated

Figure 3C. Time-course of Labelling of Bound Adenine Nucleotides of HBHM and ETP_H



'Small ETP_H' (50-100 mg. protein), 'Large ETP_H' (50-100 mg. protein), or HBHM (100-200 mg. protein), were preincubated in 8.70 ml. containing: - 2500 μmoles sucrose; 200 μmoles tris-HCl, pH 7.6; 40 μmoles MgCl₂; 10 μmoles cysteine sulphinate, pH 7.6. 0.30 ml. of 0.1M disodium succinate was then added, and after a further 2 min. 1.00ml. $^{32}\text{P}_i$ (0.1-0.2 μmoles KPO₄; 1-10 million counts/100sec.) was added to initiate the reaction. Incubations were at 30° in conical flasks in a shaker-incubator (120 oscillations/min.). Identical incubations were stopped at 20, 40, 60 or 120 sec. by addition of 1.0 ml. of 0.9M HClO₄, 0.6M Na₂SO₄, and the specific activities of bound adenine nucleotides were determined as described in Fig. 3A. Control incubations were performed in the presence of uncoupler, either 0.1 mM DNP or 50 μM TTFB.

●—● ATP; ○—○ ATP in the presence of uncoupler; ■—■ ADP; ▲—▲ ADP in the presence of uncoupler.

Table 3B. The Concentration of Bound Adenine Nucleotides in HBHM and ETP_H.

<u>Preparation</u>	<u>Number of Expts.</u>	<u>Concentration of Bound Nucleotides and P_i (μmoles/mg. protein).</u>			
		<u>AMP</u>	<u>ADP</u>	<u>ATP</u>	<u>P_i</u>
HBHM	3	1.94	3.58	2.41	40
'Large ETP _H '	4	2.08	3.00	2.04	25
'Small ETP _H '	5	1.21	2.16	1.92	5.34

Adenine nucleotides and P_i were assayed as described in the text. The above values are the average concentrations.

Table 3C. Maximum Percentage Labelling of the Bound Adenine Nucleotides of HBHM and ETP_H by ³²P_i during Succinate Oxidation.

<u>Preparation</u>	<u>Number of Expts.</u>	<u>Average Maximum %age Labelling</u>		
		<u>AMP</u>	<u>ADP</u>	<u>ATP</u>
HBHM	3	0	52	102
HBHM + 0.1mM DNP.	3	0	31	13
'Large ETP _H '	4	0	6.4	13.2
'Large ETP _H ' + 0.1 mM DNP.	4	0	4.6	1.9
'Small ETP _H '	5	0	0.45	2.1
'Small ETP _H ' + 0.1 mM DNP.	5	0	0.12	0.35

Conditions were as for Fig. 3C and maximum labelling was obtained after 2 min. Adenine nucleotides were separated and assayed as described in Fig. 3A, and the percentage labelling calculated as described in the text.

Figure 3D. The Effects of Arsenite and DNP on the Labelling

from the specific activity of the $^{32}\text{P}_i$ added, and the specific activity of the adenine nucleotides isolated:

$$= 100 \times \frac{\text{specific activity of adenine nucleotide}}{\text{specific activity of } ^{32}\text{P}_i \text{ added}}$$

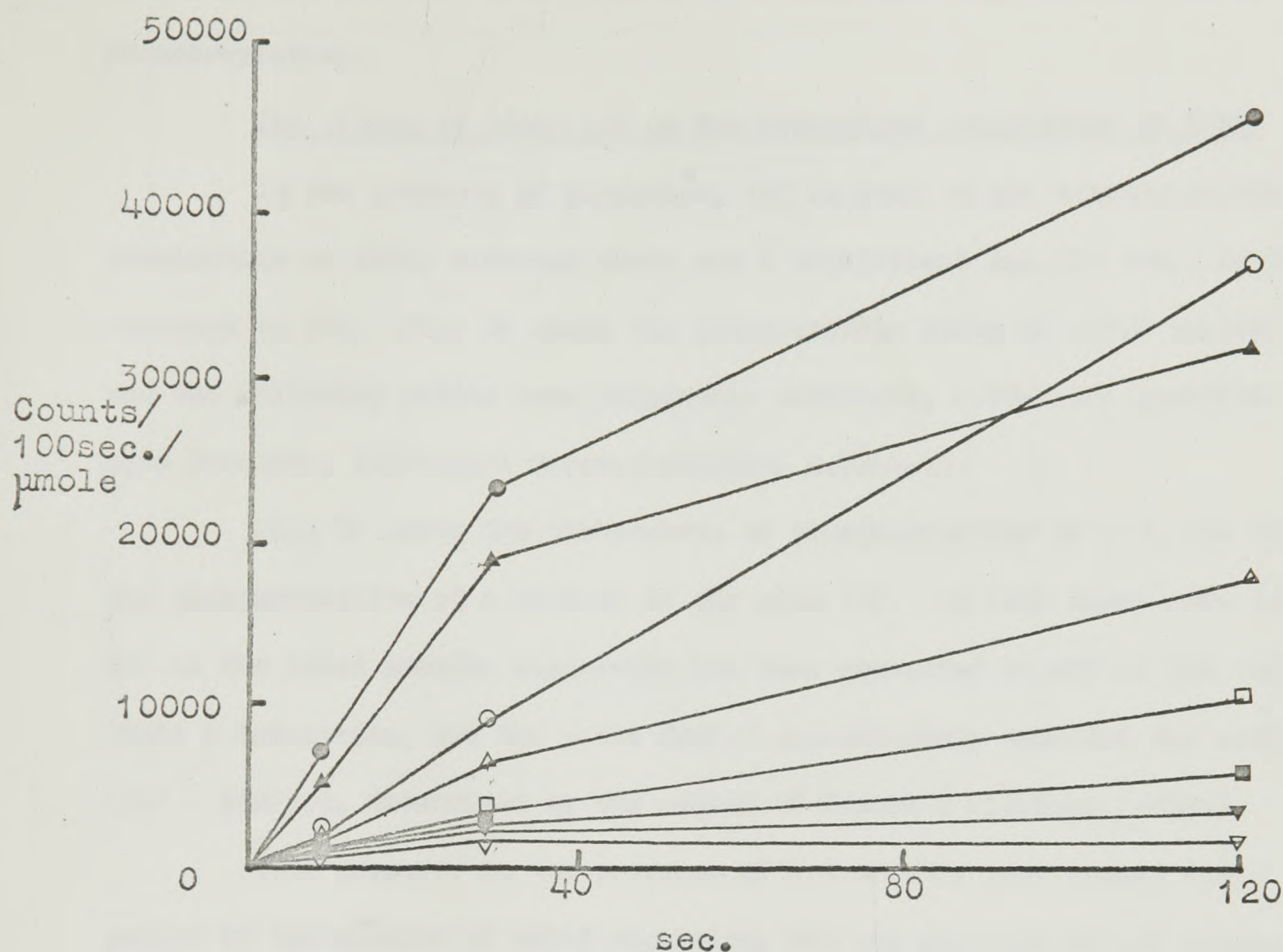
Maximum labelling of ADP and ATP occurred after 30-60 sec., and is given in Table 3C. The maximum percentage labelling of ADP and ATP was high in HMM, moderate in 'heavy ETP_H ' and low in 'light ETP_H '. 80-90% of the labelling of ATP was inhibited by uncouplers, but the labelling of ADP was inhibited much less, and depended on the preparation. 80-90% of the labelling of bound ADP of 'small ETP_H ' was inhibited by uncouplers, and the inhibition was greater at 20 sec. than at 120 sec. In 'large ETP_H ' and HMM the inhibition was only 50-80%, and was less at 20 sec. than at 120 sec, and the specific activity of AD^{32}P exceeded the specific activity of AT^{32}P at all stages of the 120 sec. incubation.

The effect of arsenite on the labelling of bound adenine nucleotides by $^{32}\text{P}_i$

The main source of incorporation of $^{32}\text{P}_i$ into adenine nucleotides in the presence of uncouplers, would be expected to be via substrate-level phosphorylation. To test this hypothesis experiments were performed in the presence of 5 mM arsenite, which specifically inhibits α -oxo-glutarate dehydrogenase, and hence the substrate-level phosphorylation catalysed by succinic thiokinase.

Fig. 3D shows that arsenite inhibits the labelling of ADP more than the labelling of ATP. In the presence of arsenite, the specific activity of AT^{32}P was higher than that of AD^{32}P , even in the presence of uncoupler, and the ratio $\frac{\text{specific activity } \text{AT}^{32}\text{P}}{\text{specific activity } \text{AD}^{32}\text{P}}$ was increased, especially in the initial stages (10 sec. and 30 sec.). It was concluded that the labelling

Figure 3D. The Effects of Arsenite and DNP on the Labelling of Bound Adenine Nucleotides of HBHM by $^{32}\text{P}_i$.



Incubation conditions were as for Fig. 3C.

<u>Symbols</u>	<u>Additions</u>	<u>Specific Activity AT^{32}P</u> <u>Specific Activity AD^{32}P</u> <u>(at 30 sec).</u>
○—○ ADP; ●—● ATP.	None.	2.45
□—□ ADP; ■—■ ATP.	0.1mM DNP	0.59
△—△ ADP; ▲—▲ ATP.	5mM arsenite.	3.10
▽—▽ ADP; ▼—▼ ATP.	5mM arsenite + 0.1mM DNP.	2.80

of ADP in the presence of uncoupler was associated with substrate-level phosphorylation.

The Effect of Added AMP on the Controlled Respiration of HBHM

In the presence of magnesium, AMP as well as ADP stimulated the respiration of HBHM, although there was a significant lag (20 sec.) in the response to AMP. Fig. 3E shows the polarographic assay of AMP:O ratios, and the following ratios were obtained:- succinate, 0.83-0.94; pyruvate plus L-malate, 1.33-1.48; α -oxo-glutarate, 1.79-1.90.

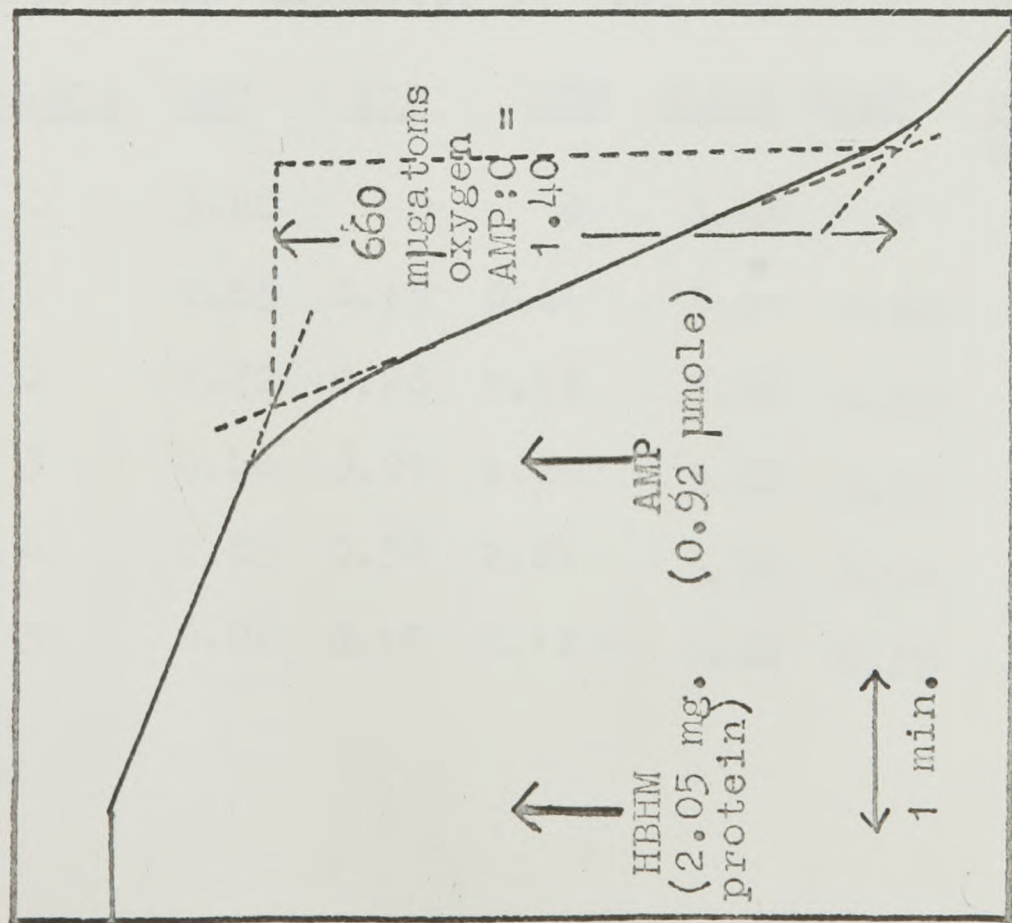
Fig. 3F shows the time-course of phosphorylation of AMP, and Fig. 3G the phosphorylation of a mixture of AMP plus ADP. In both cases more than 90% of the added adenine nucleotide had been converted to ATP at the State 3 $\rightarrow$ State 4 transition, and the ratio Δ NP:O approximately equalled the ratio (ADP + 2AMP):O, determined by the method of Chance & Williams (1955b).

HBHM prepared in the presence of 0.1 mM EDTA gave normal ADP:O ratios in the absence of added magnesium, but the addition of AMP produced only a slight stimulation of respiration. In the presence of 1 mM EDTA, or 5 mM $MgCl_2$ + 6 mM EDTA, AMP did not stimulate respiration, although normal ADP:O ratios were still obtained. Enzymic assays of adenine nucleotides showed that in the absence of added magnesium AMP was not phosphorylated.

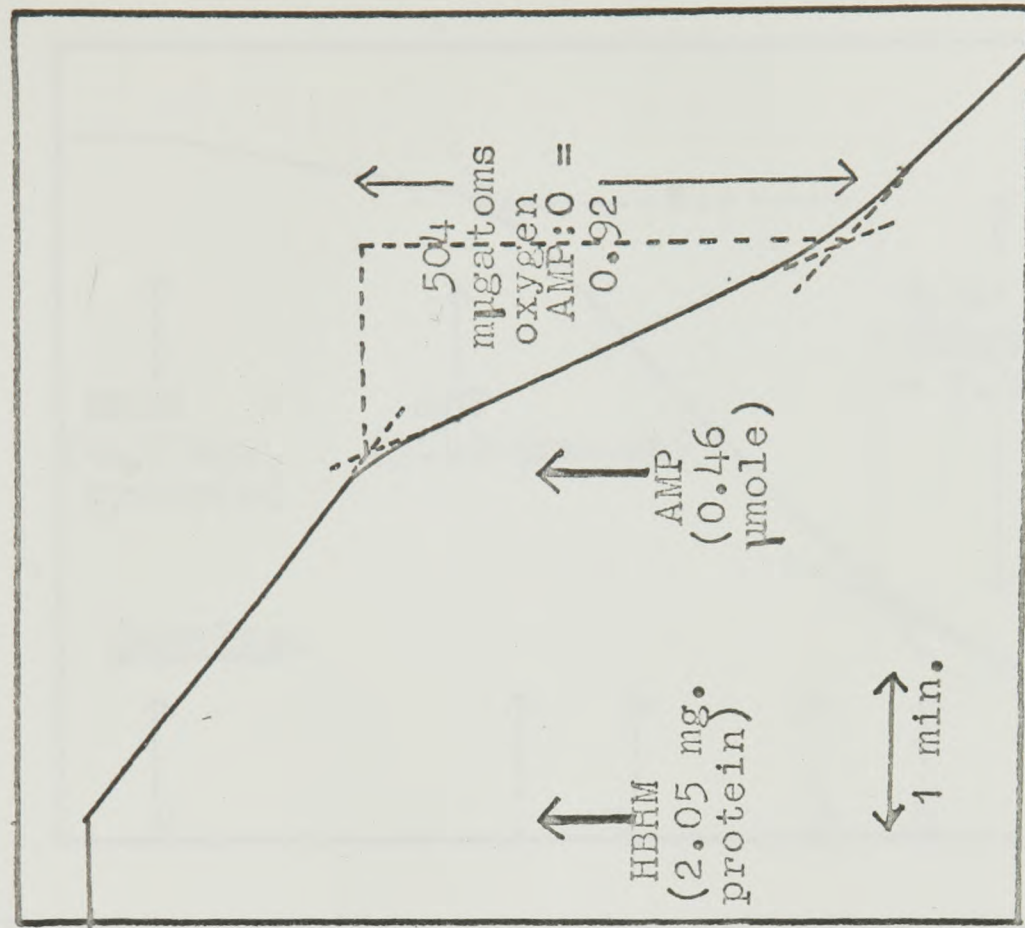
Succinate Oxidation by HBHM in the Absence of Cysteine Sulphinate

High AMP:O ratios (2.6-2.8) were obtained in certain experiments in which succinate was oxidised in the absence of cysteine sulphinate. Adenine nucleotides were assayed enzymically in the inhibited state of respiration (apparent State 4). It was found that only part of the added AMP had been converted to ATP, and that the Δ NP:O ratio was 1.90-1.95. Thus the inhibition of respiration in these experiments was not due to the State 3 $\rightarrow$ State 4

Figure 3E. The Effect of AMP on the Controlled Respiration of HBHM.



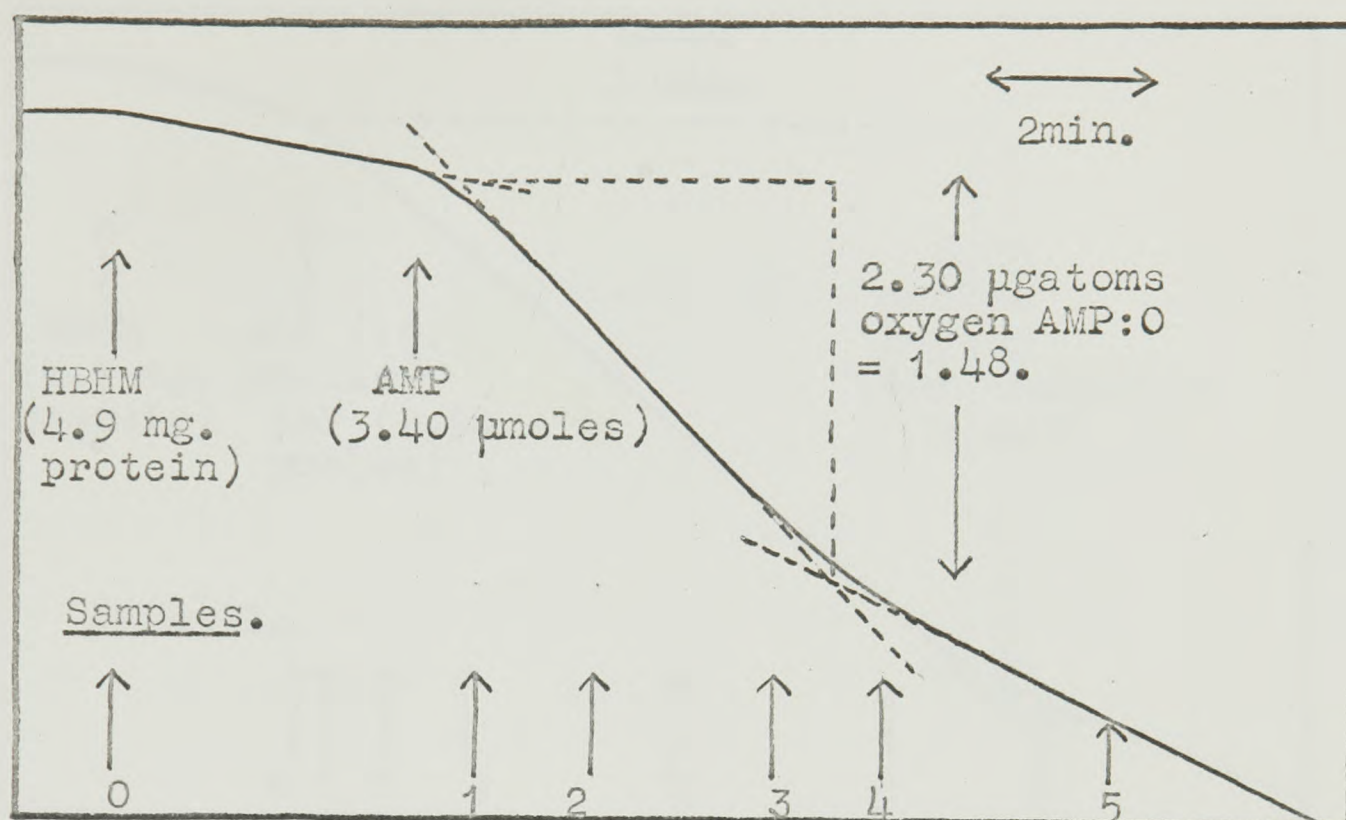
(a) Pyruvate plus L-malate.



(b) Succinate plus cysteine sulphinate.

Oxygen consumption was measured polarographically in the Clark electrode cell. The incubations contained in 3.60 ml.:— 0.25M sucrose; 50mM tris-HCl, pH 7.6; 5mM MgCl_2 ; 5 mM KPO_4 ; and 2.8mM pyruvate plus 2.8mM L-malate, pH 7.6 or 2.8 mM succinate plus 1.0 mM cysteine sulphinate, pH 7.6.

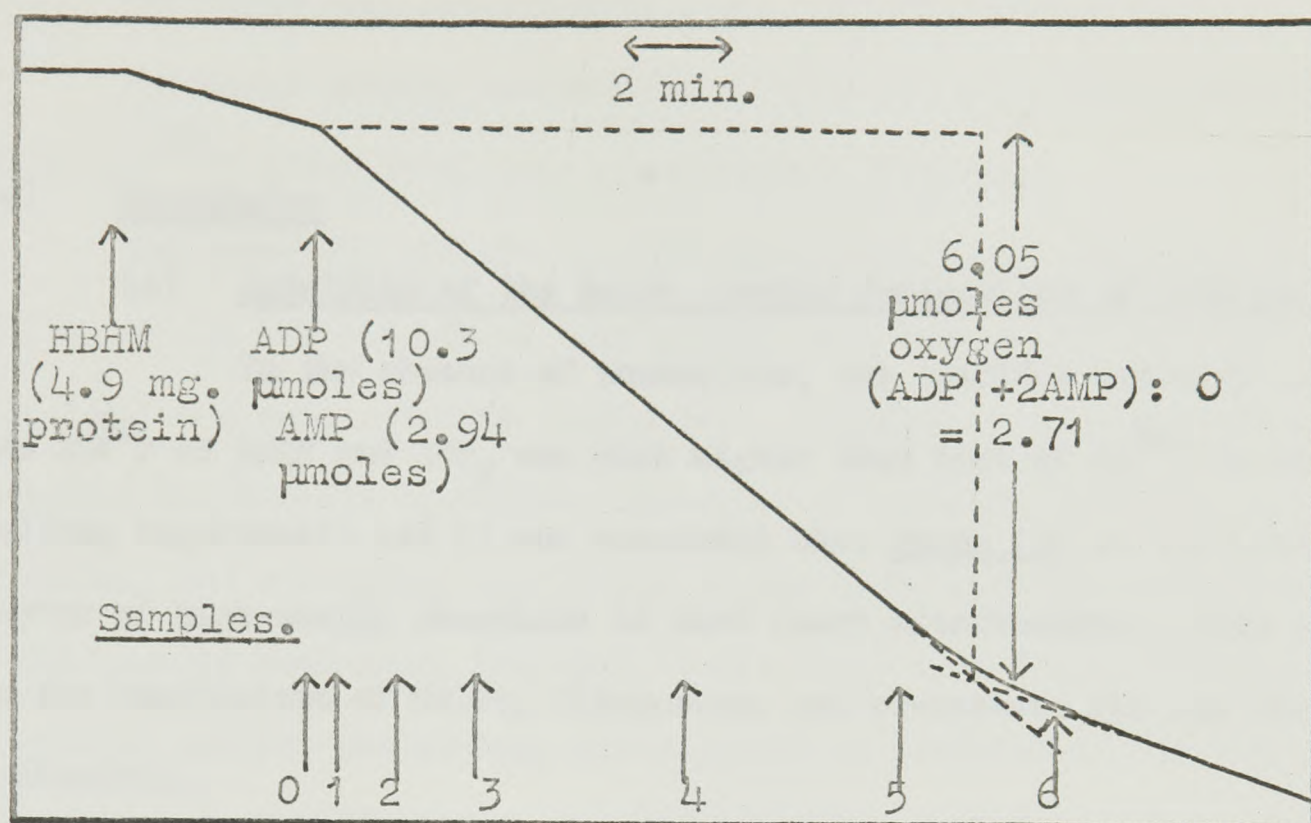
Figure 3F. The Time-course of Phosphorylation of AMP by HBHM with Pyruvate plus L-malate as Substrate.



Oxygen consumption was measured polarographically in the syringe-repette described by Garland et al. (1965). The incubation contained in 17.4 ml.:— 0.25M sucrose; 50mM tris-HCl, pH 7.6; 5mM $MgCl_2$; 5mM KPO_4 ; 2.8 mM pyruvate plus 2.8mM L-malate, pH 7.6; and HBHM (4.9 mg. protein).

<u>Sample</u>	<u>AMP</u>	<u>ADP</u>	<u>ATP</u>	<u>Total</u>	<u>ΔNP</u>	<u>μgatoms oxygen</u>	<u>ΔNP:O</u>
0	3.40	0	0	3.40	0	0	-
1	2.65	0.15	0.41	3.21	0.82	0.30	2.73
2	1.72	0.28	1.16	3.16	2.60	0.93	2.80
3	0.46	0.97	2.20	3.63	5.30	2.02	2.62
4	0.05	0.38	2.81	3.24	6.00	2.49	-
5	0.02	0.15	3.12	3.29	6.39	3.19	-

Figure 3G. The Time-course of Phosphorylation of (AMP + ADP) by HBHM with Pyruvate plus L-malate as Substrate.



Conditions were as for Fig. 3F.

Sample	AMP	ADP	ATP	Total	$\Delta \sim P$	<u>μg atoms oxygen</u>	$\Delta \sim P:O$
0	2.94	10.34	-	13.3	0	0	-
1	6.87	3.77	3.75	14.4	0.92	0.35	2.63
2	6.13	3.50	4.49	14.1	2.13	0.83	2.57
3	6.27	3.42	5.44	15.1	4.30	1.63	2.64
4	4.86	3.13	6.77	14.8	6.37	3.37	1.90
5	1.14	0.87	10.1	12.1	10.8	5.34	2.04
6	1.02	0.20	10.6	11.8	11.1	6.30	-

transition, and the AMP:O ratios were spuriously high.

3(iv) Discussion

(a) Labelling of the Bound Adenine Nucleotides of HBHM and ETP_H

In the absence of uncouplers, the specific activity of the bound AT^{32}P of HBHM and ETP_H was much higher than that of AD^{32}P in all labelling experiments and it was concluded that bound ADP is the primary acceptor of high-energy phosphate in beef heart mitochondria. This agrees with the conclusions of Heldt, Klingenberg and co-workers for rat liver mitochondria.

The results of Ozawa & MacLennan who claimed that up to 92% of the $^{32}\text{P}_\text{i}$ was incorporated into ADP in 2 min. incubations under identical conditions were not reproduced, but the following observations can be made.

(1) Carrier-free $^{32}\text{P}_\text{i}$ was used in their experiments without purification, and as the percentage incorporation of counts was very small, the counts found in the ADP fraction could represent a small contaminant of the carrier-free $^{32}\text{P}_\text{i}$ which co-chromatographs with ADP.

(2) No attempts were made to record P_i and adenine nucleotide concentrations, and hence calculate the specific activity of ADP and ATP, which is essential in establishing a precursor relationship.

(3) ETP_H show a lag in succinate oxidation which may be relieved by preincubation with cysteine sulphinic acid (Fig. 3B). Ozawa & MacLennan initiated the reaction by addition of $^{32}\text{P}_\text{i}$ plus succinate, and it is doubtful to what extent succinate was oxidised in the 2 min. incubation.

Under these conditions exchange-reactions e.g. associated with lower concentrations, indicating that P_i is being used for other purposes.

substrate-level phosphorylation, could account for most of the incorporation.

The Percentage Labelling of Bound Adenine Nucleotides

The bound adenine nucleotides of HBHM and ETP_H were maximally labelled by $^{32}\text{P}_i$ after 30-60 sec. of succinate oxidation.

The bound ADP and ATP of HBHM were extensively labelled, but the labelling of the ADP and ATP of 'small ETP_H ' was very small and that of 'large ETP_H ' was only moderate (Table 3C). Brierley & O'Brien (1965) used ^{14}C adenine nucleotides to measure the rates of exchange of bound adenine nucleotides, and concluded that 65-70% of the total adenine nucleotide of HBHM was rapidly exchanged, but that 'small ETP_H ' contained relatively little exchangeable adenine nucleotide, and appeared to concentrate the non-exchangeable bound nucleotide. It is, therefore, postulated that most of the bound adenine nucleotide of ETP_H is very tightly bound, and does not participate in oxidative phosphorylation or exchange with added adenine nucleotides.

The Adenine Nucleotide and P_i Concentrations of HBHM and ETP_H

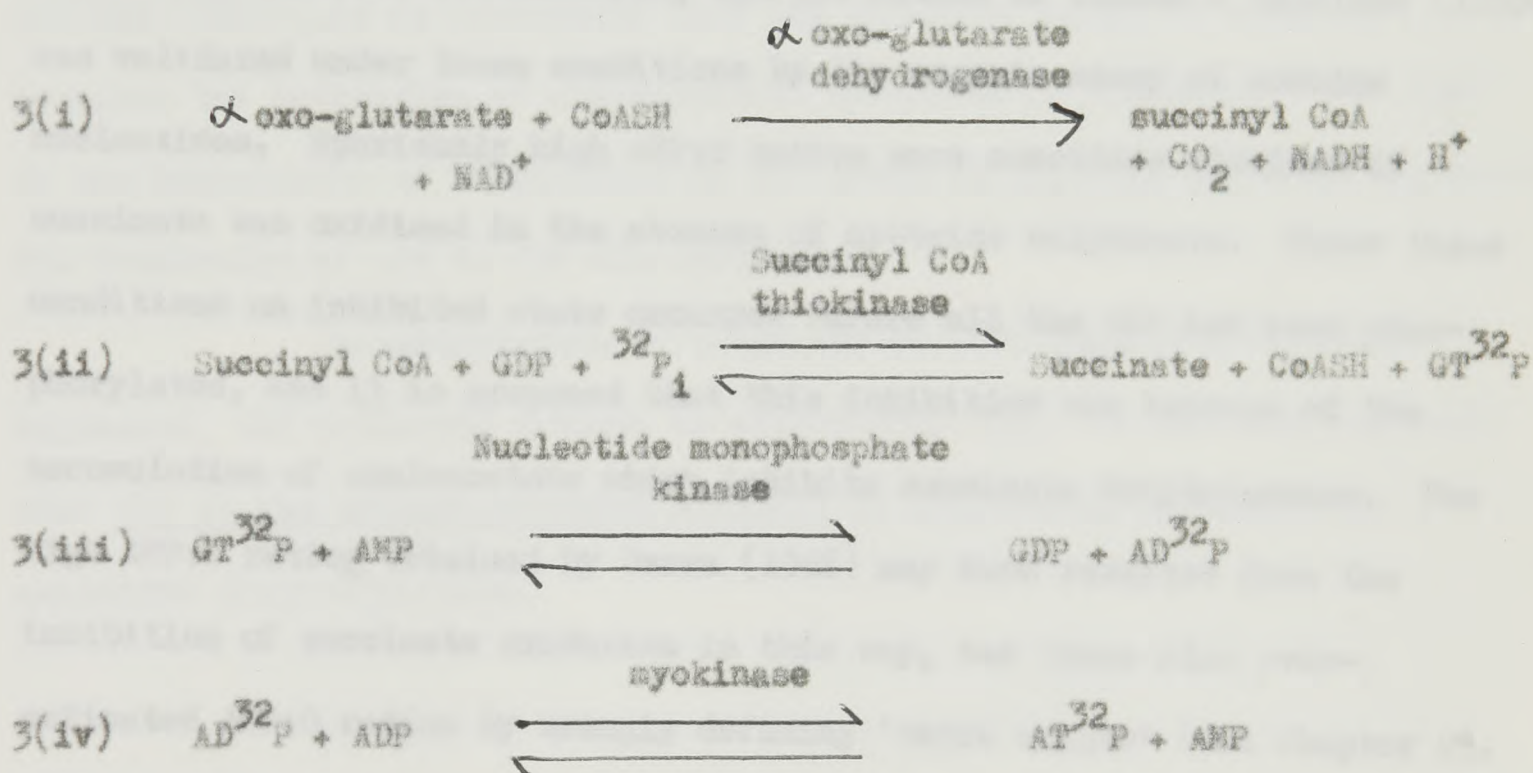
The bound adenine nucleotide concentrations of HBHM and ETP_H given in Table 3B are 50-80% higher than those found by Brierley & O'Brien (1965). A possible explanation of this is that, since HBHM were stored in a medium containing 1 mM ATP, and ETP_H were prepared in a medium containing 1 mM ATP, these preparations would contain maximal concentrations of bound adenine nucleotides. It is unlikely that significant soluble adenine nucleotide contaminated the preparations, as HBHM and ETP_H were washed with a large volume of 0.25M sucrose prior to use.

HBHM and 'large ETP_H ' (i.e. partially broken mitochondria) contained high concentrations of P_i , but small ETP_H contained much lower concentrations, indicating that P_i is largely located in solution

in the mitochondrial matrix, and is lost on disruption of the mitochondria by sonic oscillation.

Labelling of Adenine Nucleotides in the Presence of Uncouplers

In the presence of uncouplers, the specific activity of $AD^{32}P$ was higher than that of $AT^{32}P$ in HEHM. Similar results have been described for rat liver mitochondria by Heldt & Schwalbach (1967), who showed that the incorporation of $^{32}P_1$ into ADP occurs by the following sequence of reactions:-



During substrate-level phosphorylation associated with the oxidation of α oxo-glutarate, ADP is the first adenine nucleotide to be labelled, and ATP is subsequently labelled via myokinase.

The data of Fig. 3D supports the same hypothesis for heart mitochondria. Arsenite, which inhibits substrate-level phosphorylation by inhibiting the α oxo-glutarate dehydrogenase complex (Reaction 3(i)), had little effect on the labelling of ATP, but inhibited the labelling of ADP by 50%, and increased the ratio $\frac{\text{specific activity } AT^{32}P}{\text{specific activity } AD^{32}P}$. 0.1 mM DNP plus

5 mM arsenite abolished 90% of the labelling of ATP and ADP but the ratio $\frac{\text{specific activity AT}^{32}\text{P}}{\text{specific activity AD}^{32}\text{P}}$ was again high. It was thus concluded that substrate level phosphorylation was responsible for the rapid labelling of ADP in the presence of uncouplers.

(b) Release of the Controlled Respiration of HBHM by AMP

In the presence of 5 mM MgCl_2 , AMP released the controlled respiration of tightly coupled HBHM. AMP:O ratios were 50% of the ADP:O ratios for the same mitochondria, and the method of Chance & Williams (1955b) was validated under these conditions by the enzymic assay of adenine nucleotides. Spuriously high AMP:O ratios were sometimes obtained if succinate was oxidised in the absence of cysteine sulphinic acid. Under these conditions an inhibited state occurred before all the AMP had been phosphorylated, and it is proposed that this inhibition was because of the accumulation of oxaloacetate which inhibits succinate dehydrogenase. The high AMP:O ratios obtained by Ozawa (1966) may have resulted from the inhibition of succinate oxidation in this way, but Ozawa also overestimated AMP:O ratios by wrongly defining 'extra oxygen' (see Chapter 2).

Ozawa found that at the State 3 $\rightarrow$ State 4 transition-point the composition of the adenine nucleotides was 25% AMP, 30-35% ADP and 40-45% ATP. This disagrees with the data presented above but can be explained by the techniques used by Ozawa to separate and assay the adenine nucleotides. He separated the adenine nucleotides by the method of Pressman (1958), and assayed AMP, ADP and ATP enzymically after the samples had been exposed to concentrated formic acid. As already described in this Chapter ADP and ATP lose a high percentage of their terminal phosphate groups under these conditions. If samples were rapidly neutralised, and assayed enzymically as described above, it was found that over 90% of added AMP and ADP had been

converted to ATP at the State 3 $\rightarrow$ State 4 transition.

In the absence of added MgCl_2 , or if a concentration of EDTA greater than that of the MgCl_2 was added, AMP produced only a small increase in the rate of respiration. This agrees with the results of Slater & Holton (1953) and Lindberg & Ernster (1952) for heart mitochondria, and of Barkulis & Lehninger (1951) and Kielley & Kielley (1951) for rat liver mitochondria.

As suggested by Barkulis & Lehninger (1951) and Kielley and Kielley (1951), it is postulated that the phosphorylation of added AMP requires the activation of mitochondrial myokinase by magnesium. The lag in the stimulation of respiration by AMP shown in Fig. 3E, probably represents the conversion of AMP to ADP via myokinase.

Phosphorylation of added ADP was not dependent on added magnesium, and proceeded without an appreciable lag. It was thus concluded that ADP is the primary external acceptor of high-energy phosphate in oxidative phosphorylation.

3(v) Summary

- (1) The primary phosphate acceptor in oxidative phosphorylation both inside and outside beef heart mitochondria is ADP.
- (2) In the presence of uncouplers, ADP is labelled more rapidly than ATP by $^{32}\text{P}_i$. This labelling is caused by substrate-level phosphorylation, and $^{32}\text{P}_i$ is incorporated via succinic thiokinase, nucleotide monophosphate kinase and myokinase. In this sequence of reactions ADP is the first adenine nucleotide to be labelled.
- (3) In HBBM, AMP is phosphorylated indirectly via myokinase, and this requires the presence of added magnesium.

CHAPTER 4

THE EFFECTS OF NAD^+ AND NADH ON THE $\text{AD}^{32}\text{P-ATP}$ AND $^{32}\text{P}_i\text{-ATP}$ EXCHANGE
 REACTIONS CATALYSED BY SUPERNATANT FRACTIONS REQUIRED FOR
 OXIDATIVE PHOSPHORYLATION IN SUB-MITOCHONDRIAL PARTICLES

4(i) Introduction

The work of Green et al. (1963) suggested that site-specific coupling factors catalyse the terminal reactions of oxidative phosphorylation, corresponding to Reactions 1(ii), 1(iii), and 1(iii) of the scheme summarised in Figure 1B, and involving high-energy intermediates of the respiratory carriers. Smith & Hansen (1962) claimed to have isolated a soluble coupling factor which was specific for Site 1 of oxidative phosphorylation (NADH to cytochrome b), and further details of this work were given by Green et al. (1963). Evidence for the existence of high-energy intermediates of oxidative phosphorylation at Site 1 was provided by Pinchot (1960), who claimed to have isolated a protein-bound high-energy derivative of NAD , and Griffiths (1963), who isolated a compound of NAD containing high-energy phosphate.

If high-energy intermediates of NAD are involved in oxidative phosphorylation at Site 1, and if the Site 1 coupling factor catalyses Reactions 1(ii), 1(iii) and 1(iv) of Figure 1B, it is predicted that high concentrations of NAD^+ or NADH would affect the $^{32}\text{P}_i\text{-ATP}$ or $\text{AD}^{32}\text{P-ATP}$ exchange reactions catalysed by the coupling factor.

Using the methods described by Smith & Hansen, (1962) and Green et al. (1963), and further information given in a personal communication (Green, 1964), attempts were made to isolate a soluble coupling factor specific for Site 1 of oxidative phosphorylation. Supernatant fractions with coupling activity were obtained, but the activity was not specific

for Site 1.

Recently much of the evidence for site-specific coupling factors and high-energy intermediates of oxidative phosphorylation containing respiratory carriers has been shown to be unsatisfactory (see Slater 1967a), and thus the original basis for the investigations described in this chapter is no longer tenable. However, supernatant fractions isolated from sonicated HBHM were shown to have coupling activity for the oxidation of NADH or succinate by depleted sub-mitochondrial particles, and also contained high $\text{AD}^{32}\text{P-ATP}$ exchange activity and low $^{32}\text{P}_i\text{-ATP}$ exchange activity. This chapter describes attempts to characterise the $\text{AD}^{32}\text{P-ATP}$ exchange, $^{32}\text{P}_i\text{-ATP}$ exchange and coupling activities of these fractions, and an investigation of the effects of NAD^+ and NADH on the exchange activities.

4(11) Methods

Preparation of a Soluble Mitochondrial Factor with Coupling Activity (after Green et al., 1963; Green 1964)

50-100 ml. of HBHM (20 mg. protein/ml.) were suspended in 0.15 M KCl; 10 mM tris-HCl, pH 7.5; 5 mM tris-EDTA, pH 7.5. 25 ml. volumes were sonicated for (2 x 30) sec. using the pre-cooled probe of the M.S.E. sonic oscillation power unit, and the pH was re-adjusted to 7.5 with 1 M tris base. Unbroken mitochondria were removed by centrifuging for 6 min. at 20,000 g. The supernatant was then spun at 80,000 g for 60 min. The pellet (=salt-extracted particles) was suspended in 0.25 M sucrose; 10 mM tris-HCl, pH 7.6, spun down for a second time at 80,000 g for 60 min., and finally resuspended in the same medium. The supernatant was brought to 45% $(\text{NH}_4)_2\text{SO}_4$ saturation by careful addition of 25.6 g./100 ml. of solid $(\text{NH}_4)_2\text{SO}_4$ with stirring, and allowed to stand at 4° for 2 hr.

The 0-45% $(\text{NH}_4)_2\text{SO}_4$ precipitate was spun down at 20,000 g for 10 min.

The supernatant was decanted, and the residue was dissolved in 50 mM tris-acetate, 0.2 mM ATP, pH 7.6 (ATP-buffer) at a protein concentration of 10-15 mg./ml. The solution was dialysed against 100 volumes of ATP-buffer for 2 hr., and then overnight against a further 100 volumes of ATP-buffer. Any precipitate was removed by centrifuging at 80,000 g for 10 min. This supernatant fraction was shown to contain coupling activity for the oxidation of NADH or succinate by salt-depleted sub-mitochondrial particles, high $AD^{32}P$ -ATP exchange and myokinase activities, ATPase activity, and low $^{32}P_i$ -ATP exchange activity.

Preparation of Sub-mitochondrial Particles

Four different preparations of sub-mitochondrial particles were used as test-particles in the assay of the coupling activity of supernatant fractions.

(a) Salt-extracted particles (SEP) were prepared as described above by the method of Green et al. (1963).

(b) ETP_H were prepared according to the method of Linnane & Ziegler (1958).

(c) METP_H were prepared according to the method of Linnane & Titchener (1960).

(d) ETP_H (M_g, M_n) were prepared according to the method of Hansen & Smith (1964).

Preparation of ^{32}P -Labelled ADP

$AD^{32}P$ was prepared by reacting 2-5 mC of carrier-free $^{32}P_i$ with 15 μ moles AMP in the presence of N,N'-dicyclohexylcarbodiimide as described by Lowenstein (1960).

The $AD^{32}P$ was mixed with 2-5 μ moles of carrier ADP and separated from the other products of the reaction by ion-exchange chromatography on a (1 x 3)cm. column of Dowex 1-X8 Cl⁻, using the elution procedure described

by Lowenstein (1960). $AD^{32}P$ was freed from salt by adsorption to a (1 x 3) cm. column of Norit A-carlite, and eluted with a 50% ethanol, 50% water mixture containing 0.15N NH_3 . Ethanol and NH_3 were removed from the frozen eluate by evaporation under reduced pressure.

Assay of $AD^{32}P$ -ATP Exchange Activity

$AD^{32}P$ -ATP exchange activity was measured by a modification of the procedure of Watkins & Lehninger (1963), and described in Figures 4A and 4B.

Assay of $^{32}P_i$ -ATP Exchange Activity

$^{32}P_i$ -ATP exchange activity was assayed by a modification of the method of Hagihara & Lardy (1960), described in Figure 4C. It was desirable to separate more than 99.9% of the $^{32}P_i$ from the $AT^{32}P$ formed in the incubation, as many of the activities measured were very low. NAD^+ and NADH also reduced the efficiency of the extraction procedure, and the precautions taken to ensure the extraction of more than 99.9% of the $^{32}P_i$ are described in Appendix 1.

Assay of ATPase and Myokinase Activities

ATPase and myokinase activities were assayed spectrophotometrically in the same cuvette at 334 m μ using the Eppendorf photometer with a Sargent recorder. This method was an adaptation of the ATPase assay of Pullman et al. (1960), and is described in Table 4D.

Assay of the P:O Ratios of Sub-mitochondrial Particles

The P:O ratios of sub-mitochondrial particles were determined by the methods outlined in Chapter 2. Oxygen consumption was measured either manometrically in the Warburg apparatus or polarographically in the Clark oxygen electrode cell. Esterification of P_i was measured by the formation of BHP in the presence of glucose and yeast hexokinase.

4(iii) Results(a) Characterisation of the Coupling Activity of Mitochondrial SupernatantsFurther Purification of the Crude Coupling Factor

The dialysed 0.45% $(\text{NH}_4)_2\text{SO}_4$ fraction was passed through a (6 x 2) cm. column of CM-Sephadex which had been equilibrated with 50 mM tris-acetate, 0.2 mM ATP, pH 7.6. The fraction which failed to be adsorbed to the CM-Sephadex column (= 50 mM tris-acetate CM-eluate) had increased coupling activity for depleted sub-mitochondrial particles with NADH or succinate as substrate. The 50 mM tris-acetate CM-eluate also had increased AD³²P-ATP exchange, ³²P_i-ATP exchange and ATPase activities.

All the ATPase, ³²P_i-ATP exchange and coupling activity was adsorbed by a (6 x 2) cm. column of DEAE-Sephadex, which had been equilibrated with 50 mM tris-acetate, pH 7.6. ATPase and ³²P_i-ATP exchange activities were eluted from the column by 400 mM tris-acetate, pH 7.6, but although the specific activities were increased, the recovery of activity was very low. Coupling activity could not be found in any of the fractions eluted by a linear 50-600 mM gradient of tris-acetate, pH 7.6.

ATPase and ³²P_i-ATP exchange activities were stabilised by 0.2 mM ATP. Fractionation of the 50 mM tris-acetate CM-eluate by gel-filtration on a (40 x 2) cm. column of G200 Sephadex in a medium containing 50 mM tris-acetate, 0.2 mM ATP, pH 7.6, resulted in higher recoveries of ATPase and ³²P_i-ATP exchange activities in a fraction which was only slightly retarded by the G200 Sephadex, but coupling activity could not be found in any of the fractions.

It was subsequently shown that the fraction containing all the

$^{32}\text{P}_i$ -ATP exchange activity and most of the ATPase activity of the crude supernatant, consisted of small sub-mitochondrial particles containing cytochromes a, b and c.

Since attempts to obtain a purified coupling factor were unsuccessful, because of the instability of the coupling activity, the properties of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction were studied. This crude preparation contained coupling activity for the oxidation of NADH or succinate by depleted particles and also catalysed the AD^{32}P -ATP and $^{32}\text{P}_i$ -ATP exchange reactions.

The Effect of Coupling Factors on the P:O Ratios of Depleted Sub-mitochondrial Particles

Table 4A gives the P:O ratios of sub-mitochondrial particles obtained by four different methods. Table 4B shows the effect of the dialysed 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction on the P:O ratios of these particles. The supernatant fraction only increased the P:O ratios of SEP and METP_H. P:O ratios were very low, but 0.5 mg. supernatant protein/mg. particle protein increased the P:O ratios by 100%. P:O ratios were increased with either NADH or succinate as substrate.

Table 4C shows the coupling activity of the 50 mM tris-acetate eluate from CM-Sephadex. Similar results were obtained to those of Table 4B, but less supernatant protein was required to increase the P:O ratios to their optimal values.

(b) Effects of NAD^+ and NADH on Exchange Reactions Catalysed by the Crude Coupling Factor

Effects of NAD^+ and NADH on the AD^{32}P -ATP Exchange-activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction

Figure 4B shows the effects of NAD^+ and NADH on the AD^{32}P -ATP exchange activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction. Maximal rates of

Table 4A. P:O Ratios of Sub-mitochondrial Particles.

<u>Sub-mitochondrial Particles.</u>	<u>Number of Preps.</u>	<u>Average P:O Ratios.</u>	
		<u>NADH</u>	<u>Succinate</u>
Salt-extracted particles (SEP), (Green et al., 1963).	4	0.05	0.06
ME _{TP} _H , (Linnane & Titchener, 1959).	6	0.09	0.15
ET _P _H , (Linnane & Ziegler, 1958).	4	0.71	0.66
ET _P _H (Mg, Mn), (Hansen & Smith, 1964).	10	0.38	0.36

Oxygen consumption was measured manometrically in the Warburg apparatus or polarographically in the Clark electrode cell. P_i esterification was measured by the formation of HMP in the presence of glucose and hexokinase, and was corrected for HMP formed by the combined action of myokinase and hexokinase by performing control incubations in the presence of 1mM KCN. Details of these methods are given in Chapter 2.

The incubations contained sub-mitochondrial particles (0.5-2.0 mg. protein); 0.25M sucrose; 50mM tris-HCl, pH 7.6; 5mM MgCl₂; 40 μ moles glucose; 100 μ g. dialysed Boehringer hexokinase; and 0.1mM ADP. The substrates were 3mM disodium succinate or an NADH regenerating system containing:- 100 μ g. dialysed Boehringer yeast alcohol dehydrogenase; 0.28M ethanol; 0.25mM NAD⁺; and 5mM semicarbazide-HCl, pH 7.6.

Table 4B. The Coupling Activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction of the Mitochondrial Supernatant.

<u>Particles</u>	<u>P:O Ratios</u>			
	<u>NADH</u>		<u>Succinate</u>	
	<u>Alone</u>	<u>+ CF</u>	<u>Alone</u>	<u>+ CF</u>
1. SEP	0.05	0.12	0.06	0.09
2. METP_H	0.09	0.17	0.15	0.20
3. ETP_H	0.71	0.71	0.66	0.56
4. $\text{ETP}_\text{H}(\text{Mg}, \text{Mn})$	0.38	0.41	0.36	0.43

P:O ratios were measured as described under Table 4A.
 CF = 0.5mg. protein of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction per
 mg. of particle protein.

Table 4C. The Coupling Activity of the 50mM tris-acetate Eluate from CM- Sephadex.

<u>Particles</u>	<u>P:O Ratio</u>		<u>$\mu\text{g. CM-eluate protein/}$ <u>mg. particle protein.</u></u>
	<u>NADH</u>	<u>Succinate</u>	
SEP	0.067	0.070	0
	0.089	0.141	200
	0.104	0.150	600
METP_H	0.050	0.069	0
	0.115	0.199	250

P:O ratios were measured as described under Table 4A.

exchange were obtained where the ADP:ATP ratio exceeded 0.8, but neither NAD^+ nor NADH (up to 3.3 mM) had any significant effect on the rate of the exchange reaction.

The Effects of NAD^+ and NADH on the $^{32}\text{P}_1$ -ATP Exchange Activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction

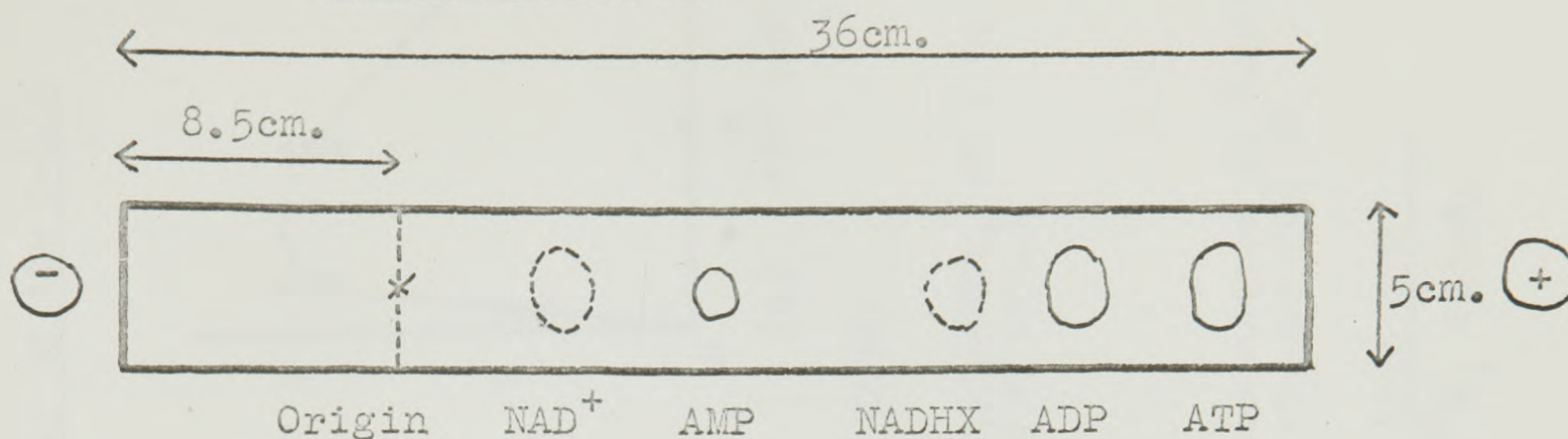
The $^{32}\text{P}_1$ -ATP exchange activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction depended in a complex manner on the relative concentrations of P_i , ADP and ATP. The effects of NAD^+ and NADH on the $^{32}\text{P}_1$ -ATP exchange reaction are shown in Figure 4C, and were investigated over a wide range of concentration of P_i , ADP and ATP. Under all conditions investigated, NAD^+ (up to 10 mM) and NADH (up to 5 mM) had no significant effect on the rate of the $^{32}\text{P}_1$ -ATP exchange reaction. Optimal exchange activity at pH 7.6 was obtained in a medium which contained 1 mM ATP; 1.2 mM ADP; and 5 mM KPO_4 .

(c) The Characterisation of the AD^{32}P -ATP Exchange Activity of Supernatant Fractions

All supernatant fractions contained both myokinase and AD^{32}P -ATP exchange activities and AD^{32}P -ATP exchange activity was highest in those fractions which contained the highest myokinase activity (see Table 4D). Purified Boehringer rabbit muscle myokinase also had a high AD^{32}P -ATP exchange activity. This indicated that much of the AD^{32}P -ATP exchange activity of mitochondrial supernatants could be associated with myokinase activity.

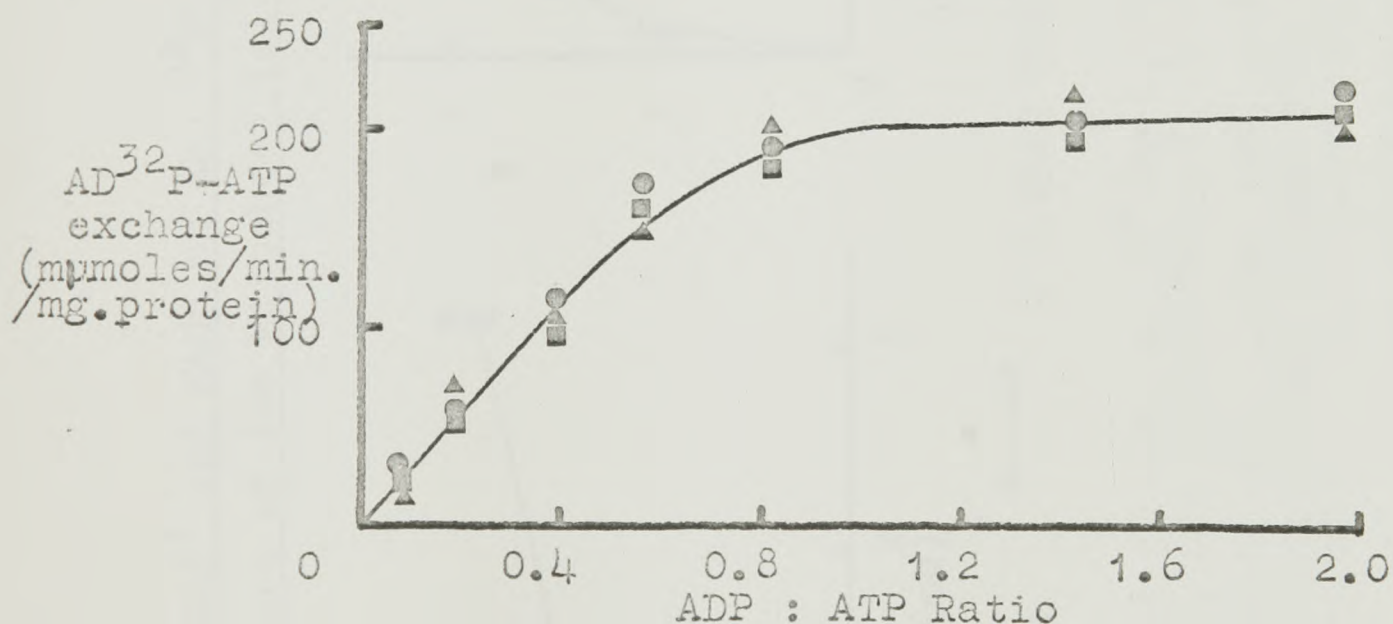
A comparison of the AD^{32}P -ATP exchange and myokinase activities of supernatant fractions is given in Table 4D. The ratio $\frac{\text{AD}^{32}\text{P-ATP exchange activity}}{\text{Myokinase activity}}$ was used as a criterion to distinguish between AD^{32}P -ATP exchange activity associated with myokinase and that associated with other enzymes. A high ratio denoted the presence of AD^{32}P -ATP exchange activity other than that of myokinase.

Figure 4A. Separation of Adenine Nucleotides by Paper Electrophoresis.



Nucleotides were separated by electrophoresis on Whatman 3MM paper at a potential difference of 10V/cm. in 50mM citrate buffer, pH 4.5. Nucleotide spots were located under an ultra-violet lamp, and quantitatively eluted with (2 x 5)ml. of 10mM HCl.

Figure 4B. The Effects of NAD^+ and NADH on the AD^{32}P -ATP Exchange Activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction of the Mitochondrial Supernatant.

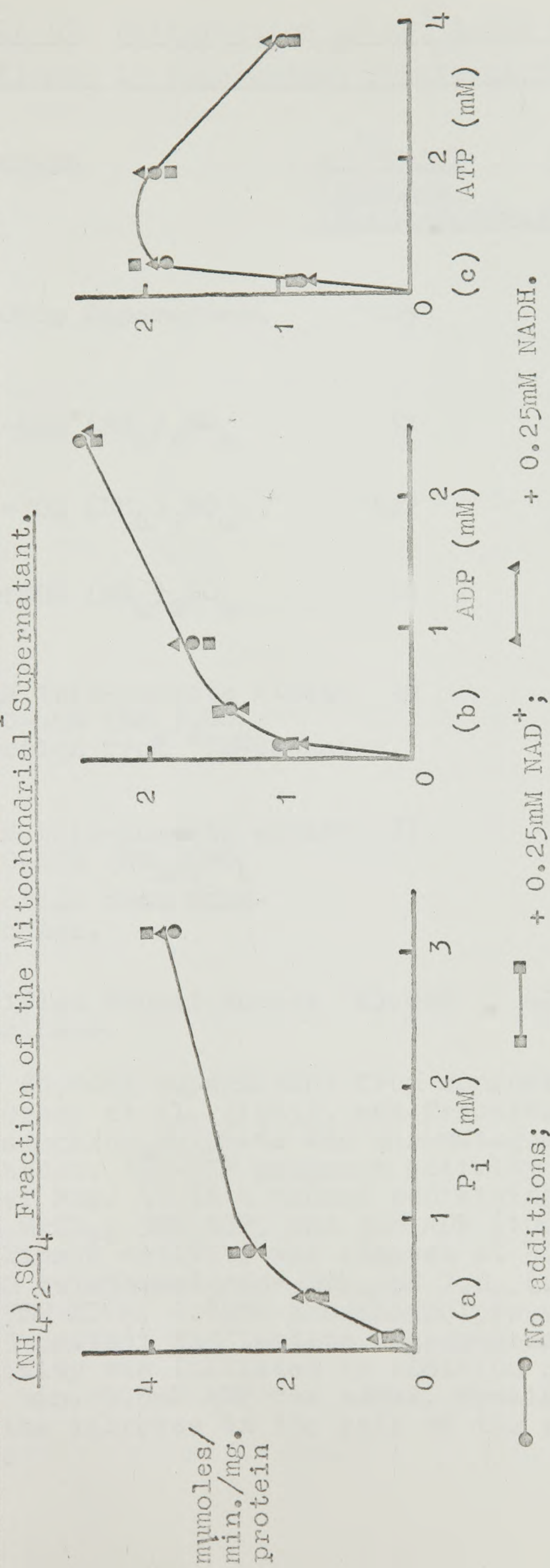


●—● No additions; ■—■ + 3.3mM NAD^+ ; ▲—▲ + 3.3mM NADH.

Incubations were for 10min. at 30° in 1.00ml. containing : 50mM tris-HCl, pH 7.6; 5mM MgCl_2 ; 5mM ATP; and 0.5-10mM AD^{32}P (100,000-300,000 counts/100 sec.). The reaction was stopped by addition of 0.1ml. of 30% trichloroacetic acid. 25 μ l samples were subjected to paper electrophoresis (see Fig. 4A). The radioactivity of the ADP and ATP spots was counted, and adenine nucleotides were assayed by absorbance at 260mp. Provided that exchange did not exceed 20% of radioactive equilibrium, exchange activity =

$$\frac{\text{Radioactivity of ATP isolated}}{\text{Specific radioactivity of ADP added}}$$

Figure 4C. The Effects of NAD^+ and NADH on the $^{32}\text{P}_i$ -ATP Exchange Activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction of the Mitochondrial Supernatant.



Incubations were for 10 min. at 30° in 1.00ml. containing 100mM tris-HCl, pH 7.6; 5mM MgCl_2 ; ADP, ATP, and radioactive P_i (0.5-2.0 million counts/100 sec.).

(a) contained:— 0.73mM ADP; 0.95mM ATP; and 0.1-3.2mM KPO_4 .
 (b) contained:— 0.1-2.4mM ADP; 0.95mM ATP; and 0.83mM KPO_4 .
 (c) contained:— 0.73 mM ADP; 0.1-3.8mM ATP; and 0.83mM KPO_4 .

$^{32}\text{P}_i$ was separated from organic phosphate by a modification of the method of Hagihara & Lardy (1960), (see Appendix 1), and if the exchange did not exceed 20% of radioactive equilibrium,

$$^{32}\text{P}_i\text{-ATP exchange activity} = \frac{\text{Radioactivity incorporated into ATP}}{\text{Specific Radioactivity of } ^{32}\text{P}_i \text{ added.}}$$

Table 4D. Distribution of AD³²P-ATP Exchange Activity and Myokinase in Supernatant Fractions from Mitochondria.

<u>Fraction</u>	<u>AD³²P-ATP exchange (μmoles/min./mg.protein)</u>	<u>Myokinase</u>	<u>AD³²P-ATP exchange: Myokinase</u>
80,000g Supernatant	105	673	0.156
0-45% (NH ₄) ₂ SO ₄	32	315	0.102
45-70% (NH ₄) ₂ SO ₄	197	3330	0.059
70-100% (NH ₄) ₂ SO ₄	60	619	0.096
50mM tris-acetate eluate of 0-45% (NH ₄) ₂ SO ₄ fraction from CM-Sephadex.	48	500	0.096
50mM tris-acetate eluate of 0-45% (NH ₄) ₂ SO ₄ fraction from DEAE- Sephadex.	77	1520	0.051
Purified Rabbit Muscle Myokinase.	23,000	425,000	0.055

The 80,000g supernatant from sonicated HBHM, prepared according to Green et al. (1963), was fractionated by precipitation by ammonium sulphate and chromatography on CM- and DEAE-Sephadex. ADP-ATP exchange activity was assayed as described under Fig. 4B in a medium containing:- 100mM tris-HCl, pH 7.6; 5mM MgCl₂; 5mM ATP; and 5mM ADP (100,000-300,000 counts/100sec.) Myokinase activity was assayed at 30° in 1.00ml. containing:- 50mM triethanolamine-HCl, pH 7.6; 6.3mM MgCl₂; 50mM KCl; 0.35mM EDTA; 0.85mM phosphoenolpyruvate; pyruvate kinase (1.3 units); and lactate dehydrogenase (2 units). ATPase activity was initiated by addition of the sample, and after 2-3 min. 0.5mM AMP was added. Myokinase activity was given by the increase in the rate of the reaction on addition of AMP.

The 0-45% and 70-100% $(\text{NH}_4)_2\text{SO}_4$ fractions had high AD^{32}P -ATP exchange:myokinase ratios, whereas the 45-70% $(\text{NH}_4)_2\text{SO}_4$ fraction had a low ratio and contained most of the myokinase activity. Only the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction had coupling activity, and was therefore of interest. If this fraction was passed through a CM-Sephadex column, the 50 mM tris-acetate eluate still had a high AD^{32}P -ATP exchange: myokinase ratio, but passage through a DEAE-Sephadex column decreased the AD^{32}P -ATP exchange: myokinase ratio of the 50 mM tris-acetate eluate.

It was concluded that much of the AD^{32}P -ATP exchange activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction and of the 50 mM tris-acetate CM-eluate was associated with enzymes other than myokinase and as these fractions also had coupling activity, part of the AD^{32}P -ATP exchange activity was possibly associated with the enzyme(s) which catalyse the terminal step of oxidative phosphorylation (Reaction 1(iv) of Fig. 1B).

(d) Characterisation of the $^{32}\text{P}_i$ -ATP Exchange Activity of Supernatant Fractions

As there were no previous reports of a soluble enzyme associated with oxidative phosphorylation which catalysed $^{32}\text{P}_i$ -ATP exchange, attempts were made to characterise the $^{32}\text{P}_i$ -ATP exchange activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction of supernatants from sonicated mitochondria.

Effect of Inhibitors

Table 4B shows the effects of inhibitors and uncouplers of oxidative phosphorylation on the $^{32}\text{P}_i$ -ATP exchange activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction. The exchange reaction was inhibited by the same concentrations of uncouplers and inhibitors of oxidative phosphorylation that inhibit the $^{32}\text{P}_i$ -ATP exchange activity of ETP_H , but was unaffected by inhibitors of electron transport or by atractyloside.

Table 4E. The Effect of Inhibitors on the $^{32}\text{P}_i$ -ATP Exchange Activity of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction of Mitochondrial Supernatant.

<u>Inhibitor.</u>	<u>Concentration.</u>	<u>% Inhibition.</u>
2,4- dinitrophenol	100 μM	55
	200 μM	78
4-octyl, 2,4- dinitro-phenol.	25 μM	64
	50 μM	84
Carbonyl cyanide m- chlorophenyl hydrazone	0.1 μM	31
	0.2 μM	77
4,5,6,7- tetrachloro- 2- trifluoromethyl- benzimidazole.	0.025 μM	41
	0.050 μM	68
Oligomycin A	0.18 $\mu\text{g.}/\text{mg. protein}$	59
	0.36 $\mu\text{g.}/\text{mg. protein}$	85
Dicyclohexyl- carbodiimide	5 μM	87
Octylguanidine	400 μM	52
	600 μM	86
Antimycin A	2 $\mu\text{g.}/\text{mg. protein}$	0
Rotenone	0.5 $\mu\text{moles}/\text{mg. protein}$	0
Amytal	3000 μM	0
Atractyloside	500 μM	0

Assays were at 30° in 1.00ml. containing:- 100mM tris-HCl, pH, 7.6; 5mM MgCl_2 ; 5mM KPO_4 ; 1.0mM ATP; 1.2mM ADP; 0.4mg. supernatant protein; and carrier-free $^{32}\text{P}_i$ (0.5-1.0 million counts/100 sec.). $^{32}\text{P}_i$ -ATP exchange activity was calculated as for Figure 4C.

Table 4F. Purification of the $^{32}\text{P}_i$ -ATP Exchange Activity

Purification of the $^{32}\text{P}_i$ -ATP Exchange Activity

The purification of $^{32}\text{P}_i$ -ATP exchange activity is summarised in Table 4F, and consisted of ion-exchange chromatography on CM-Sephadex, followed by ion-exchange chromatography on DEAE-Sephadex or gel-filtration through G100 or G200 Sephadex.

The exchange activity was markedly stabilised by 0.2 mM ATP, which was added at all stages of the fractionation, except for the fractionation on DEAE-Sephadex which strongly binds ATP. The exchange activity passed through a column of CM-Sephadex, which had been equilibrated with 50 mM tris-acetate, 0.2 mM ATP, pH 7.6, but was adsorbed to DEAE-Sephadex, which had been equilibrated with 50 mM tris-acetate, pH 7.6. The activity was eluted from DEAE-Sephadex by 400 mM tris-acetate, pH 7.6, but most of the activity was lost unless 0.2 mM ATP was added to the fractions as soon as they left the column.

A more successful purification of $^{32}\text{P}_i$ -ATP exchange activity was obtained by fractionating by gel-filtration through columns of G100 or G200 Sephadex, which had been equilibrated with 50 mM tris-acetate, 0.2 mM ATP, pH 7.6. The activity was not retarded by G100 Sephadex, and only slightly retarded by G200 Sephadex, but the specific activity was increased 4-5 fold.

The Presence of Cytochromes in Purified Preparations

Containing $^{32}\text{P}_i$ -ATP Exchange Activity

The $^{32}\text{P}_i$ -ATP exchange activity of the most purified fractions from G200 Sephadex columns was 20-40 $\mu\text{moles/min./mg. protein}$. These preparations had a yellowish colour, and spectra showed absorption in the region of 400-600 $\text{m}\mu$. Difference spectra (oxidised-dithionite reduced) were run on the Cary Model 14 Recording Spectrophotometer, with 0-0.1

Table 4F. Purification of the $^{32}\text{P}_i$ -ATP Exchange Activity from the High-speed Supernatant of Sonicated Mitochondria.

<u>Stage of Purification.</u>	<u>$^{32}\text{P}_i$-ATP exchange ($\mu\text{moles/min./mg.protein}$)</u>
1. 80,000g supernatant.	1 - 2
2. 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction.	3 - 4
3. 50mM tris-acetate eluate from CM- Sephadex.	6 - 8
4a. 400mM tris-acetate eluate from DEAE- Sephadex.	8 - 12
4b. Most active fraction from G-100 Sephadex.	15 - 20
4c. Most active fraction from G-200 Sephadex.	20 - 40

HBHM (1-2g.protein) were sonicated as described by Green et al. (1963), and the supernatant from a 1 hr. spin at 80,000g brought to 45% saturation with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate was spun down, dialysed, and passed through a (6 x 2)cm. column of CM- Sephadex, equilibrated with 50mM tris-acetate, 0.2mM ATP, pH 7.6. The eluate was then fractionated by procedures 4a, 4b, or 4c. Procedure 4a involved adsorption to a (6 x 2)cm. column of DEAE- Sephadex, which had been equilibrated with 50mM tris- acetate, pH 7.6, followed by elution with a linear gradient of 50-600mM tris-acetate, pH 7.6. $^{32}\text{P}_i$ -ATP exchange activity was eluted by 400mM tris-acetate. Procedures 4b and 4c involved separation on (40 x 2)cm. columns of G-100 or G-200 Sephadex which had been equilibrated with 50mM tris-acetate, 0.2mM ATP, pH 7.6. $^{32}\text{P}_i$ -ATP exchange activity was assayed as described for Table 4E.

absorbance scale. Peaks were observed at 605, 565, 450, 430 and 415 $m\mu$, and a shoulder at 530-540 $m\mu$. These peaks were identified as follows:-
 605 $m\mu$ = α band of cytochrome a; 565 $m\mu$ = α band of cytochrome b; 450 $m\mu$ = γ band of cytochrome a; 430 $m\mu$ = γ band of cytochrome b; 415 $m\mu$ = δ band of cytochrome (C + C₁); the shoulder at 530-550 $m\mu$ = β band of cytochrome b plus the α band of cytochrome (C + C₁). The peaks corresponding to cytochromes a and b were large, but those corresponding to cytochrome (C + C₁) were small. The highest peak was the Soret band of cytochrome b at 430 $m\mu$, and the relative concentrations of cytochrome b in preparations were compared by the absorbance of the 430 $m\mu$ peak per mg. protein.

Ultracentrifuge Studies on the $^{32}P_1$ -ATP Exchange Activity of Supernatant Fractions

Table 40 shows the stepwise centrifugation of a preparation of sonicated HMM. The $^{32}P_1$ -ATP exchange activity and the relative cytochrome b concentrations of each pellet and supernatant were measured. Both $^{32}P_1$ -ATP exchange activity and cytochromes were progressively pelleted by prolonged centrifugation. All pellets had similar $^{32}P_1$ -ATP exchange activities, and the ratio of $^{32}P_1$ -ATP exchange activity to cytochrome b concentration, determined from the absorbance at 430 $m\mu$ of the (oxidized-dithionite reduced) difference spectrum, was similar for both pellets and supernatants.

It was concluded that all the $^{32}P_1$ -ATP exchange activity of the mitochondrial supernatant was associated with sub-mitochondrial particles containing all the cytochromes. The smallest sub-mitochondrial particles (150,000 g pellet of Table 40) had the same specific $^{32}P_1$ -ATP exchange activity as the larger particles.

The $^{32}P_1$ -ATP exchange activity of the 80,000 g supernatant was purified by passing through a column of CM-Sephadex, and by gel-filtration

Table 4G. Ultracentrifuge Studies on the $^{32}\text{P}_i$ -ATP Exchange Activity of the Supernatant from Sonicated Mitochondria.

<u>entrifugation.</u>	<u>Fraction.</u>	$^{32}\text{P}_i$ -ATP exchange ($\mu\text{moles/min.}/\text{mg. protein}$)	<u>Relative cyt.b concentration.</u>	$^{32}\text{P}_i$ -ATP exchange : <u>Cyt.b concentration.</u>
1 6 min. at 20,000g	Pellet	13.2	0.60	22
2. 60 min. at 80,000g	Pellet	21.6	1.00	22
	Supernatant	2.5	0.15	17
3. 60 min. at 108,000g	Pellet	16.6	0.80	21
	Supernatant	0.7	0.05	14
4. 60 min. at 150,000g	Pellet	15.6	0.60	26
	Supernatant	0.15	Trace	-

Mitochondria were sonicated according to Green et al. (1963), and sub-mitochondrial particles spun down in a stepwise manner, the supernatant from each spin being transferred to the next stage. $^{32}\text{P}_i$ -ATP exchange activity was assayed as in Table 4E, and the relative concentration of cytochrome b was determined by measuring dithionite difference spectra on the Cary Model 14 recording spectrophotometer, and comparing the height of the peak at 430m μ (Soret band of cyt.b) with that of the 80,000g pellet on a protein basis.

through G200 Sephadex as described in Table 4F. The most active fraction, with a $^{32}\text{P}_i$ -ATP exchange activity of 40 μ moles/min./mg. protein was run in the Spinco analytical ultracentrifuge, and a major peak was observed with an S_{20}^{20} value of 26.

Oxidase Activities of the Small Sub-mitochondrial Particles

The small sub-mitochondrial particles isolated by centrifugation (150,000 g pellet of Table 4G) had 40-50% of the NADH oxidase activities and succinate oxidase of the 80,000 g particles. The oxidase activities of the small sub-mitochondrial particles isolated from the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction of the 80,000 g supernatant by ion-exchange chromatography on CM-Sephadex and gel-filtration through G200 Sephadex, were very low (10-20% of the activity of the 80,000 g pellet). This was to be expected as these particles were shown by difference spectra to be depleted in cytochrome c. The cytochrome c was probably dissociated from the particles during the ammonium sulphate step, and would be retained by the CM-Sephadex column. A better assay of the electron-transport activity of these particles would have been to have measured NADH-ferricyanide reductase, succinate-ferricyanide reductase, and succinate-cytochrome c reductase activities, or to measure NADH oxidase and succinate oxidase activities in the presence of added cytochrome c.

4(iv) Discussion

The purpose of the work described in this chapter was to purify a coupling factor specific for Site 1 of oxidative phosphorylation and, if this factor catalysed the AD^{32}P -ATP exchange or Pi^{32} -ATP exchange reactions, to investigate the effects of NAD^+ and NADH on these exchange activities.

Purification of Coupling Factors

The purification of coupling factors was made difficult by the instability of the coupling activity, and the inability to obtain depleted sub-mitochondrial test-particles which could be reactivated to give normal P:O ratios. High-speed supernatants from sonicated mitochondria were shown to increase the P:O ratios of salt-extracted particles (Green et al., 1963), or of MEMPH (Linnane & Titchener, 1960). The 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction of these supernatants contained all the coupling activity, and this activity was increased by passage through CM-Sephadex. Both these fractions increased P:O ratios with either NADH or succinate as substrate, and were not specific for Site 1 as claimed by Green et al. (1963). Attempts to purify coupling activity beyond this stage resulted in the complete loss of the activity, and the further purification was therefore abandoned.

The difficulties of purifying coupling factors are well-recognised (see Racker, 1965), and evidence has accumulated which throws doubt on the existence of site-specific coupling factors (see Slater, 1967a). The most clearly characterised coupling factor is the ATPase (F_1) purified by Racker and co-workers, which has coupling activity at all three sites of oxidative phosphorylation (Racker 1965).

Characterisation of $^{32}\text{ADP-ATP}$ and $^{32}\text{P}_i\text{-ATP}$ Exchange Activities

The crude preparations of coupling factor described in this chapter i.e. the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction, and the 50 mM tris-acetate CM-eluate, catalysed both the $\text{AD}^{32}\text{P-ATP}$ and $^{32}\text{P}_i\text{-ATP}$ exchange reactions. Attempts were made to characterise these activities, and to decide whether they represented partial reactions of oxidative phosphorylation, or were catalysed by enzymes unconnected with oxidative phosphorylation.

It was shown that myokinase catalysed an active $\text{AD}^{32}\text{P-ATP}$ exchange reaction, and that the crude preparations were contaminated with myokinase. However, the $\text{AD}^{32}\text{P-ATP}$ exchange activity of the supernatants which contained coupling activity was greater than could be accounted for by myokinase alone, and it is possible that the excess $\text{AD}^{32}\text{P-ATP}$ exchange activity is associated with the coupling factor which catalyses the terminal step of oxidative phosphorylation (c.f. Wadkins & Lehninger (1963)b.).

The $^{32}\text{P}_i\text{-ATP}$ exchange activity of the supernatant fractions was inhibited by concentrations of uncouplers and inhibitors of oxidative phosphorylation which also inhibit oxidative phosphorylation and $^{32}\text{P}_i\text{-ATP}$ exchange in HRHM and ETP_H , and was thus clearly identified as a partial reaction of oxidative phosphorylation. However, this activity was shown to be associated with small sub-mitochondrial particles, and was distinct from the coupling activity of the preparation.

Effects of NAD^+ and NADH on the $\text{AD}^{32}\text{P-ATP}$ and $^{32}\text{P}_i\text{-ATP}$ Exchange Activities of the 0-45% $(\text{NH}_4)_2\text{SO}_4$ Fraction

The effects of NAD^+ and NADH on the $\text{AD}^{32}\text{P-ATP}$ and $^{32}\text{P}_i\text{-ATP}$ exchange reactions catalysed by the 0-45% $(\text{NH}_4)_2\text{SO}_4$ fraction were investigated over a wide range of experimental conditions, and no significant changes in the activity of either reaction were found. Possible reasons for these negative results are the following:-

- (1) There is now very little evidence for the idea of site-specific coupling factors catalysing reactions 1(ii), 1(iii) and 1(iv) of Figure 1B (see Slater 1967a), and the crude coupling factor used in these studies was not specific for Site 1. The $\text{AD}^{32}\text{P-ATP}$ exchange activity of the crude coupling factor was largely associated with myokinase, and even if it is assumed that the remaining activity was associated with a coupling factor,

AD³²P-ATP exchange activity would not be affected by high concentrations of the carrier C, unless the coupling factor catalysed reactions 1(ii), 1(iii) and 1(iv).

(2) The ³²P_i-ATP exchange activity of the 0-45% (NH₄)₂SO₄ fraction was shown to be associated with small sub-mitochondrial particles containing all the cytochromes, and to be distinct from the coupling activity of the preparation. Such sub-mitochondrial particles would be expected to catalyse ³²P_i-ATP exchange by reactions 1(iii) and 1(iv), and also retain reactions 1(i) and 1(ii). ³²P_i-ATP exchange could result from all three sites of oxidative phosphorylation, and the proportion catalysed by Site 1 might be small, and hence unaffected by NAD.

(3) ³²P_i-ATP exchange depended in a complex way on the relative concentrations of P_i, ADP and ATP (c.f. Cooper & Lehninger, 1957), showing that the optimal activity of the reaction depends on the kinetics of Reactions 1(iii) and 1(iv) in both directions. The concentration of C will only affect the activity of the ³²P_i-ATP exchange reaction if C reacts rapidly with X~I, so that the steady-state concentration of X~I becomes rate-limiting for ³²P_i-ATP exchange.

Thus the failure of NAD⁺ and NADH to affect the AD³²P-ATP and ³²P_i-ATP exchange reactions of the 0-45% (NH₄)₂SO₄ fraction does not rule out the possibility that compounds of NAD⁺ and NADH are involved in the intermediate reactions of oxidative phosphorylation at Site 1.

Properties of the Small Sub-mitochondrial Particles

The sub-mitochondrial particles which were responsible for the ³²P_i-ATP exchange activity of the 0-45% (NH₄)₂SO₄ fraction exhibited a number of interesting properties:-

- (1) The particles in the 80,000 g supernatant had an S_{20}^0 value of 26. This sedimentation constant indicates a molecular weight in the range 1-3 million, which is surprisingly low for sub-mitochondrial particles containing all the cytochromes, and retaining a high $^{32}\text{P}_i$ -ATP exchange activity. This $^{32}\text{P}_i$ -ATP exchange activity was inhibited by concentrations of uncouplers and inhibitors of oxidative phosphorylation, and was greatly stabilised by 0.2 mM ATP.
- (2) The particles could be spun down by prolonged centrifugation at 150,000 g, but could also be purified by ion-exchange chromatography on DEAE-Sephadex or by gel-filtration through G200 Sephadex. $^{32}\text{P}_i$ -ATP exchange activity was recovered in high yield provided that 0.2 mM ATP was added to the medium.
- (3) The particles contained all the cytochromes, but were depleted in cytochrome c. The loss of cytochrome c is explained by their treatment with ammonium sulphate and fractionation on CM-Sephadex. The loss of cytochrome c also explains the low NADH-oxidase and succinate-oxidase activities of the particles but further work is required to characterise the various electron-transport activities of the particles.

The extremely small size of these particles, and the fact that they retain a high $^{32}\text{P}_i$ -ATP exchange activity and some oxidative activity suggests that they may represent the smallest functional sub-units of the inner mitochondrial membrane, consisting of a multi-enzyme complex of the enzymes for both electron-transport and energy-conservation. However, it is doubtful that such a non-specific process as sonication would yield a homogeneous preparation of small membrane subunits, unless the subunits are arranged in an ordered array which can be split along certain cleavage planes. The

particles with $^{32}\text{P}_i$ -ATP exchange activity were homogeneous with respect to ion-exchange chromatography on DEAE-Sephadex, but it is very likely that these particles are heterogeneous with respect to other properties, and consist of a mixture of small fragments of both inner and outer mitochondrial membranes. A large percentage of the inner membrane fragments must have retained intact complexes of the enzymes which catalyse $^{32}\text{P}_i$ -ATP exchange to account for the high activity of this reaction, and this may be explained by the stabilising effect of ATP on the $^{32}\text{P}_i$ -ATP exchange activity of the preparation.

An approach to the characterisation of the small sub-mitochondrial particles would be to fractionate them by centrifugation on a sucrose density-gradient which contained 0.2 mM ATP to stabilise the $^{32}\text{P}_i$ -ATP exchange activity. The concentrations of oxide-reduction carriers, enzymic activities, molecular weight, and possibly the appearance in electron-micrographs of the separate fractions could then be examined.

Previous approaches to the study of the structure of inner and outer mitochondrial membranes by Green and Co-workers (see Green, 1967) have involved the use of detergents which completely destroy all the reactions associated with oxidative phosphorylation, and structural studies based on the methods suggested above may overcome this difficulty.

CHAPTER 5

THE RATES OF PENETRATION OF OXALOACETATE AND L-MALATE INTO MITOCHONDRIA

5(i) Introduction

Mitochondria can maintain high internal concentrations of L-malate and citrate (Gamble, 1965), and the inhibition of respiration by anionic inhibitors such as amytal (Chance & Hollunger, 1963) and azide (Wilson & Chance, 1966) can be reversed by uncouplers. A possible explanation of these facts is that mitochondria can maintain high internal concentrations of anions by the energy-dependent accumulation of such ions, and recently, evidence for an energy-dependence of succinate oxidation by liver mitochondria has been reported (Harris, Van Dam & Pressman, 1967 a, b).

The measurement of the rates of penetration of anions into mitochondria has previously relied on indirect methods:-

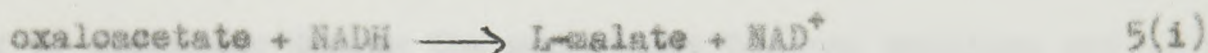
- (1) Measurement of the concentration of anions which have been separated from the incubation medium by centrifugation (e.g. Gamble, 1965).
- (2) Measurement of the rates of oxidation of substrate anions (e.g. Harris, Van Dam & Pressman, 1967 a).
- (3) Measurement of the rate of swelling of mitochondria in isotonic solutions of the ammonium salt of the acid (Chappell & Haarheff, 1967).

In order to investigate whether anions enter mitochondria by an energy-dependent mechanism, it was desirable to develop a direct method of measuring the rates of penetration of anions into mitochondria. A model system in which direct measurements of the rates of penetration of anions can be made is provided by the substrate anions oxaloacetate and L-malate.

The rates of penetration of oxaloacetate and L-malate into mitochondria were measured by their effects on the concentration of intra-

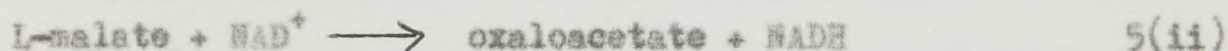
mitochondrial NADH. As the activity of the mitochondrial malate dehydrogenase under the test conditions was very high in relation to the rates of penetration of oxaloacetate and L-malate, the initial rate of oxidation of intramitochondrial NADH was a measure of the rate of penetration of oxaloacetate, and the initial rate of reduction of intramitochondrial NAD^+ was a measure of the penetration of L-malate.

The measurement of the rate of the reaction:



depends on the presence of a relatively high concentration of intramitochondrial NADH. A high initial concentration of intramitochondrial NADH was generated from endogenous substrate by inhibiting NADH oxidase with rotenone plus antimycin A. The continual reduction of NAD^+ by endogenous substrate was slow ($< 5\%$) compared with the initial rate of oxidation of NADH by oxaloacetate ($> 2 \mu\text{M}$), and could be neglected. Under these conditions the NAD(P)H concentration of fresh mitochondria was up to 4 $\mu\text{moles NAD(P)H/mg. protein}$, but gradually fell on incubation at 0° in 0.25 M sucrose, and was negligible after 8-18 hours.

The measurement of the reaction:



requires a very high $\text{NAD}^+:\text{NADH}$ ratio because of the unfavourable equilibrium constant of the reaction. The penetration of L-malate was measured with mitochondria whose NAD(P)H concentrations were depleted by preincubation at 0° for 8-18 hours.

5(ii) Methods

Methods of Measuring the Kinetics of NAD(P) Reduction

Three methods of measuring changes in NAD(P) reduction were used.

(1) Direct Spectrophotometric Measurements

The main method used was the spectrophotometric method developed by Klingenberg & Slenczka (1959) employing the Aminco-Chance dual-wavelength spectrophotometer (Chance 1951, 1957). Changes in NAD(P) reduction were measured at (340-374) $m\mu$ using $E_{m\mu}$ at (340-374) $m\mu = 4.4 \text{ cm}^{-1} \cdot m\mu^{-1}$ (Chance & Hollunger, 1961).

This technique is inaccurate if the mitochondria undergo extensive swelling or shrinkage during the experiment because swelling and shrinkage affect the optical density especially at lower wavelengths (see Lehninger, 1962). Swelling can be detected by measuring the absorption at 340 $m\mu$ and 374 $m\mu$ independently and alternately. Fig. 5A Expt. (a) shows such a test with rat liver mitochondria. For NAD(P)H oxidation $E_{m\mu}$ at 340 $m\mu = 6.2$ and $E_{m\mu}$ at 374 $m\mu = 1.8$. Thus if the mitochondria did not undergo volume changes

$$\frac{\Delta E_{340 \text{ } m\mu}}{\Delta E_{374 \text{ } m\mu}} = 3.4$$

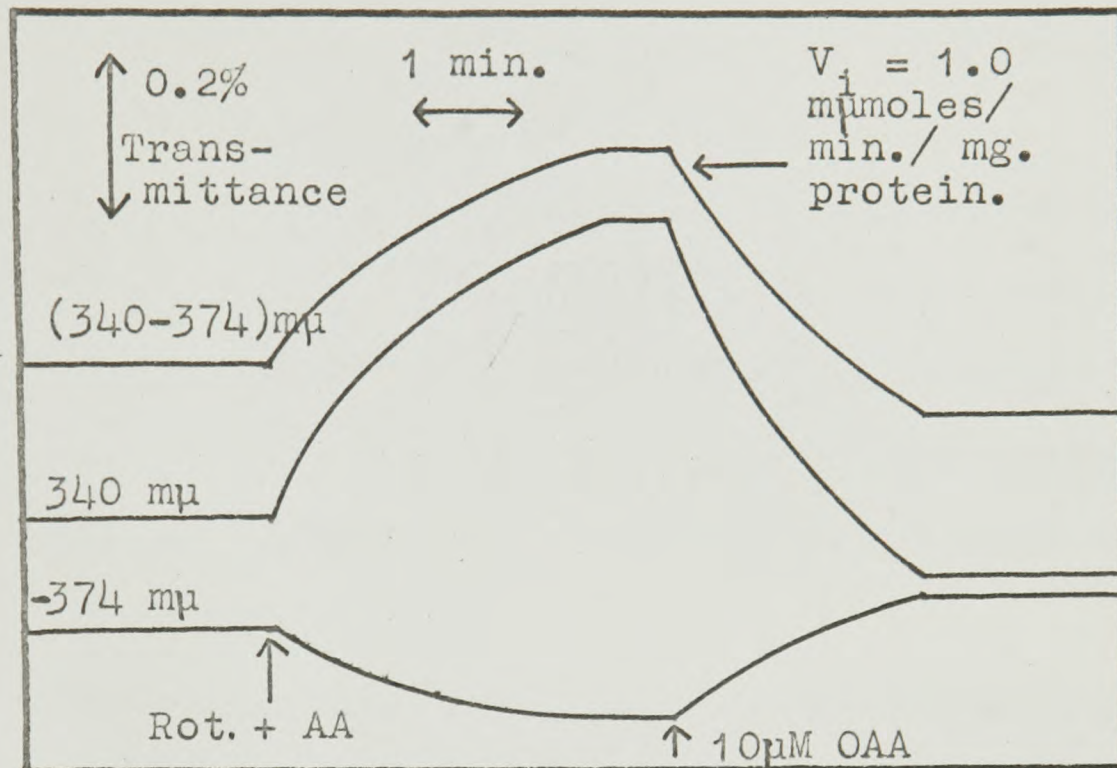
If swelling or shrinkage occurred during the experiment, the ratio would decrease. Tests showed that liver and heart mitochondria did not swell or shrink in tris-HCl or tricine-KOH buffered media under any of the normal conditions employed in Chapters 5 and 6. Kidney mitochondria swelled in tris-HCl buffered media in the presence of TMPD plus ascorbate, but not in tricine-KOH buffered media, and hence the latter medium was employed.

(2) Fluorimetric Measurements

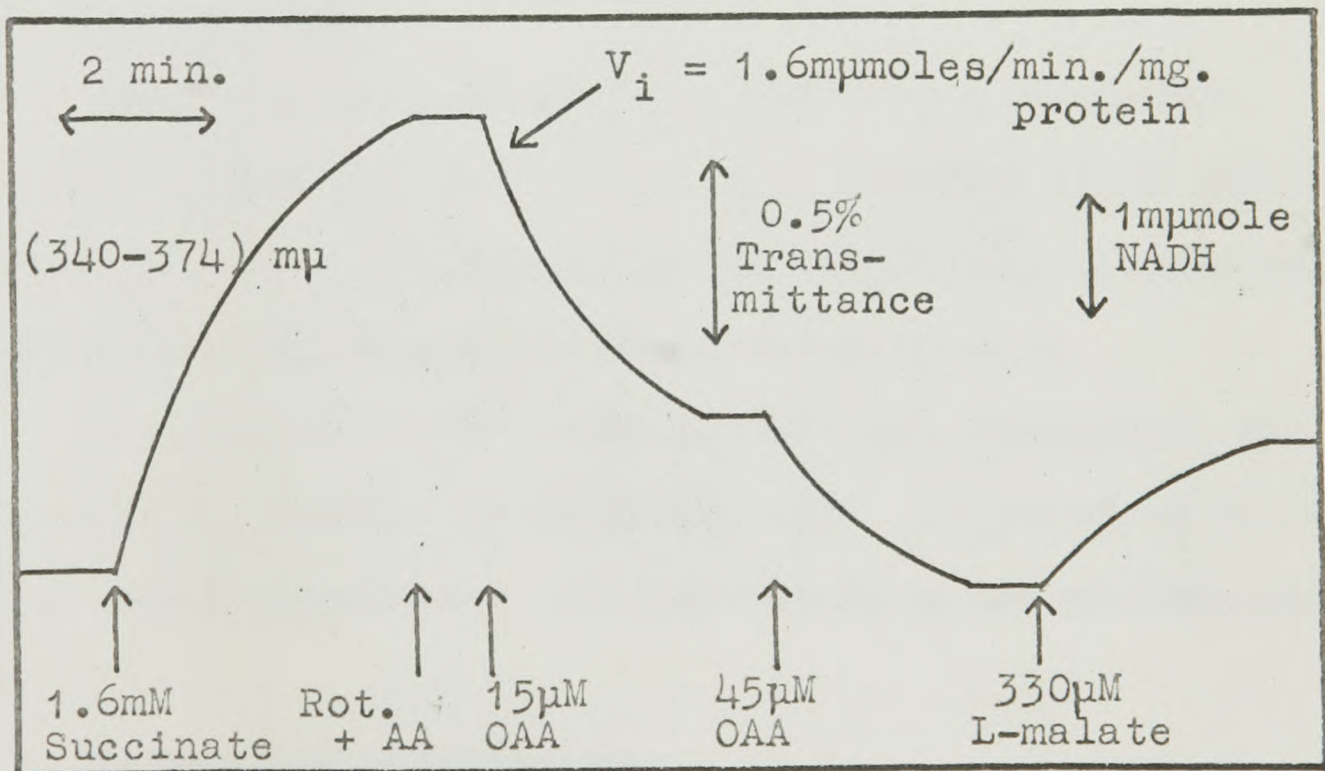
Changes in intramitochondrial NAD(P)H concentration were measured by the fluorimetric method of Estabrook & Maitra (1962), using the Sppendorf photometer with fluorimetric attachment. The (313 + 366) $m\mu$ mercury vapour lines were used as activating light, and emitted light was selected by the 400-3000 $m\mu$ filter. The fluorimeter was calibrated by adding accurately

Figure 5A. The Oxidation of Intramitochondrial NAD(P)H by Oxaloacetate, Measured Spectrophotometrically.

(a) NAD(P)H Generated from Endogenous Substrate in the Presence of Rotenone and Antimycin A.



(b) NAD(P)H Generated from Succinate.



Incubations were at 30° in 3.00ml. containing :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 0.3 μM rotenone; 0.5 μg. antimycin A; and rat liver mitochondria (1.05 mg. protein). Abbreviations :- Rot, rotenone; AA, antimycin A; OAA, oxaloacetate.

assayed amounts of NADH to the cuvette. This calibration gives only apparent changes in NAD(P)H concentration, as intramitochondrial NAD(P)H exhibits an enhancement of fluorescence, and the enhancement factor depends on the metabolic state of the mitochondria (Estabrook, 1962).

The fluorescence enhancement factor was obtained by comparing the apparent rates of oxidation of NAD(P)H obtained by the fluorimetric method with the direct rates obtained by the spectrophotometric technique. Fig. 5B shows a typical fluorimetric trace, and compares fluorimetric and spectrophotometric data. The fluorescence enhancement factor of 5 agrees with the value obtained by Estabrook (1962).

Fluorimetric measurements were performed only to confirm qualitatively data obtained by the spectrophotometric technique, and in all cases parallel results were obtained.

(3) Enzymic Assays

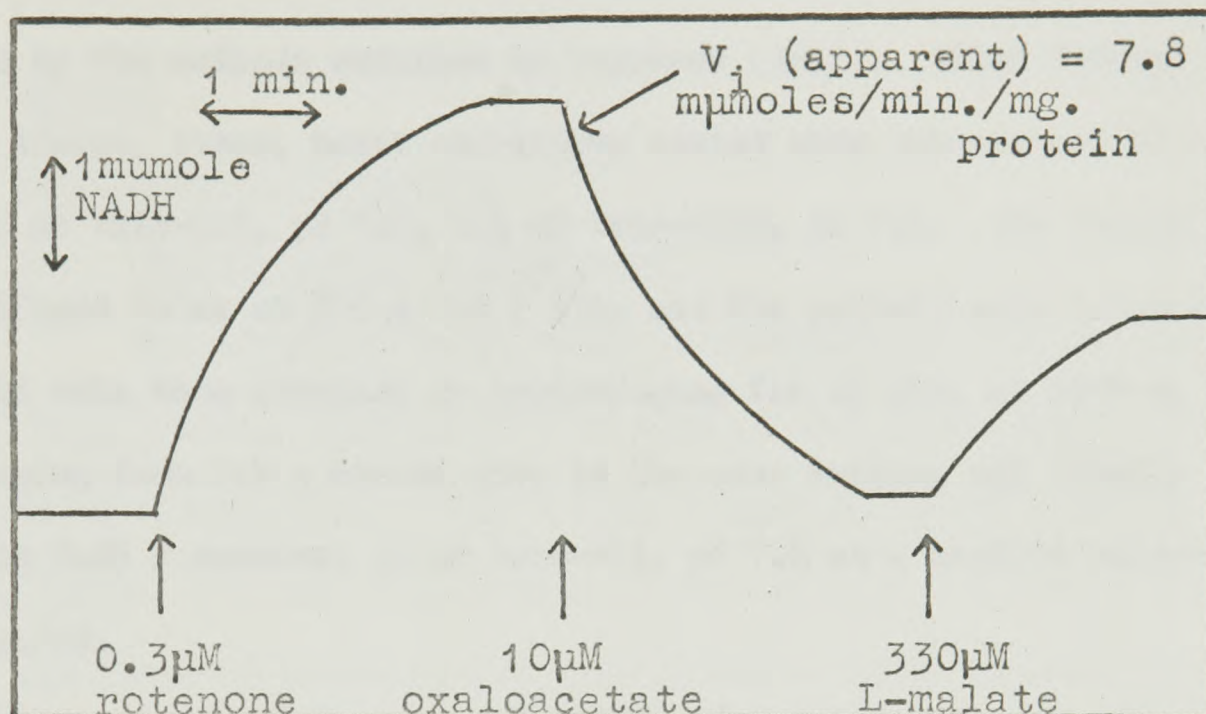
Neither the spectrophotometric nor the fluorimetric method distinguishes between NADH and NADPH, but as mitochondrial malate dehydrogenase is specific for NADH, it was assumed that the initial rates of change in the reduction of NAD(P) corresponded to changes in NAD reduction.

As a check NAD^+ , NADP^+ , NADH and NADPH were determined by the enzymic procedures described by Klingenberg (1965), in incubations in which changes in NAD(P)H concentration were also measured by the spectrophotometric method.

Determination of Metabolites

Oxaloacetate was assayed by the method of Hohorst & Reim (1965), and L-malate by the method of Hohorst (1965b). Mitochondrial protein was measured by the method of Cornall, Bardawill & David (1949).

Figure 5B. The Oxidation of Intramitochondrial NAD(P)H by Oxaloacetate, Measured by Fluorimetry.



Incubations at 30° contained in 3.00ml. :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; and rat liver mitochondria (1.05 mg. protein. Further additions were :- 0.3μM rotenone; 10μM oxaloacetate; and 330μM L-malate. Incident light was (313 + 366)mμ, and emitted light was measured at (420 - 3000)mμ. Fluorescence was calibrated against a standard solution of NADH. Rates have to be corrected for the enhancement of fluorescence of intramitochondrial NAD(P)H, and correction factors were obtained by comparing the rates of oxidation of intramitochondrial NAD(P)H by oxaloacetate, measured spectrophotometrically and fluorimetrically under identical conditions, e.g. in the presence of rotenone :-

<u>Mitochondria.</u>	<u>Oxidation of NAD(P)H by 10μM Oxaloacetate (μmoles/min./mg. protein).</u>		<u>Fluorescence Enhancement Ratio.</u>
	<u>Spectrophotometry.</u>	<u>Fluorimetry.</u>	
Liver	1.5	7.8	5.2
Kidney	1.0	5.0	5.0
Heart	2.4	12.6	5.3

Preparation of Mitochondria

Twice-washed mitochondria were prepared from the organs of 250 g. female rats by the methods outlined by Hogeboom (1955). After removal of connective tissue, liver, heart and kidney cortex were homogenized in 0.25 M sucrose; 10 mM tris-HCl, pH 7.6; 0.1 mM tris-EDTA, pH 7.6. The homogenates were centrifuged twice at 500 g for 7 min. and the pellets were discarded. Mitochondria were then obtained by centrifuging for 15 min. at 5400 g, washed by centrifuging down for a second time in the same medium, and finally suspended in 0.25 M sucrose; 10 mM tris-HCl, pH 7.6 at a protein concentration of 15-30 mg./ml.

These mitochondria gave P:O ratios of 2.6-2.8 with pyruvate plus L-malate as substrate, and exhibited good respiratory control. There was no significant leakage of NAD^+ as tested by the addition of ethanol plus yeast alcohol dehydrogenase, or of NADH as tested by the addition of pyruvate plus lactate dehydrogenase.

Preparation of Disrupted Mitochondria

Disrupted mitochondria required for the assay of mitochondrial malate dehydrogenase were prepared either by incubation with 0.1% Triton X-100 for 30 min. at 0° , or by ultrasonic oscillation for 2 min. using the W.B.E. ultrasonic power unit in a medium containing 0.25 M sucrose; 10 mM tris-HCl, pH 7.6.

Assay of Malate Dehydrogenase

The malate dehydrogenase activity of disrupted mitochondria was measured spectrophotometrically at 334 $\text{m}\mu$ using the Eppendorf photometer or at (340 - 374) $\text{m}\mu$ using the Aminco-Chance dual-wavelength spectrophotometer.

The basic medium contained 0.25 M sucrose; 20 mM tris-HCl, pH 7.6. Reaction 5(1) was measured with 0.1 mM NADH; 0.5 - 1 μg . mitochondrial

protein and varying amounts of oxaloacetate. Reaction 5(ii) was measured with 0.26 mM NAD^+ ; 2-10 μg . mitochondrial protein and varying amounts of L-malate.

5(iii) Results

Conditions of Incubation

(a) Penetration of Oxaloacetate

The effective measurement of reaction 5(i) depends on a relatively high initial concentration of NADH in the mitochondria. Addition of succinate to mitochondria causes a rapid reduction of NAD^+ (Chance & Hollunger, 1957). To test whether this system was suitable, 0.5 - 2 mM succinate was added to mitochondria. This caused a rapid reduction of intramitochondrial NAD(P) , and when the maximum level of reduction had been reached, antimycin A and rotenone were added to prevent further interaction of succinate with the respiratory chain. Addition of oxaloacetate ($>2\mu\text{M}$) now produced a rapid oxidation of mitochondrial NAD(P)H . The results of such an experiment are shown in Fig. 5A, Expt. (b).

The quantitative details of such experiments were difficult to reproduce as the initial rates of NADH oxidation by oxaloacetate, and the final steady-state concentrations of NAD(P)H both depended on the concentration of succinate added and the length of the succinate oxidation period. A high concentration of succinate or a long period of succinate oxidation decreased the rate and extent of the subsequent oxidation of NAD(P)H by oxaloacetate. These difficulties arose because of the following reasons:-

- (1) The further metabolism of the fumarate, produced by the oxidation of succinate, would cause the regeneration of NADH.
- (2) It was later shown that succinate is a competitive inhibitor of oxaloacetate penetration into mitochondria. (see Chapter 6).

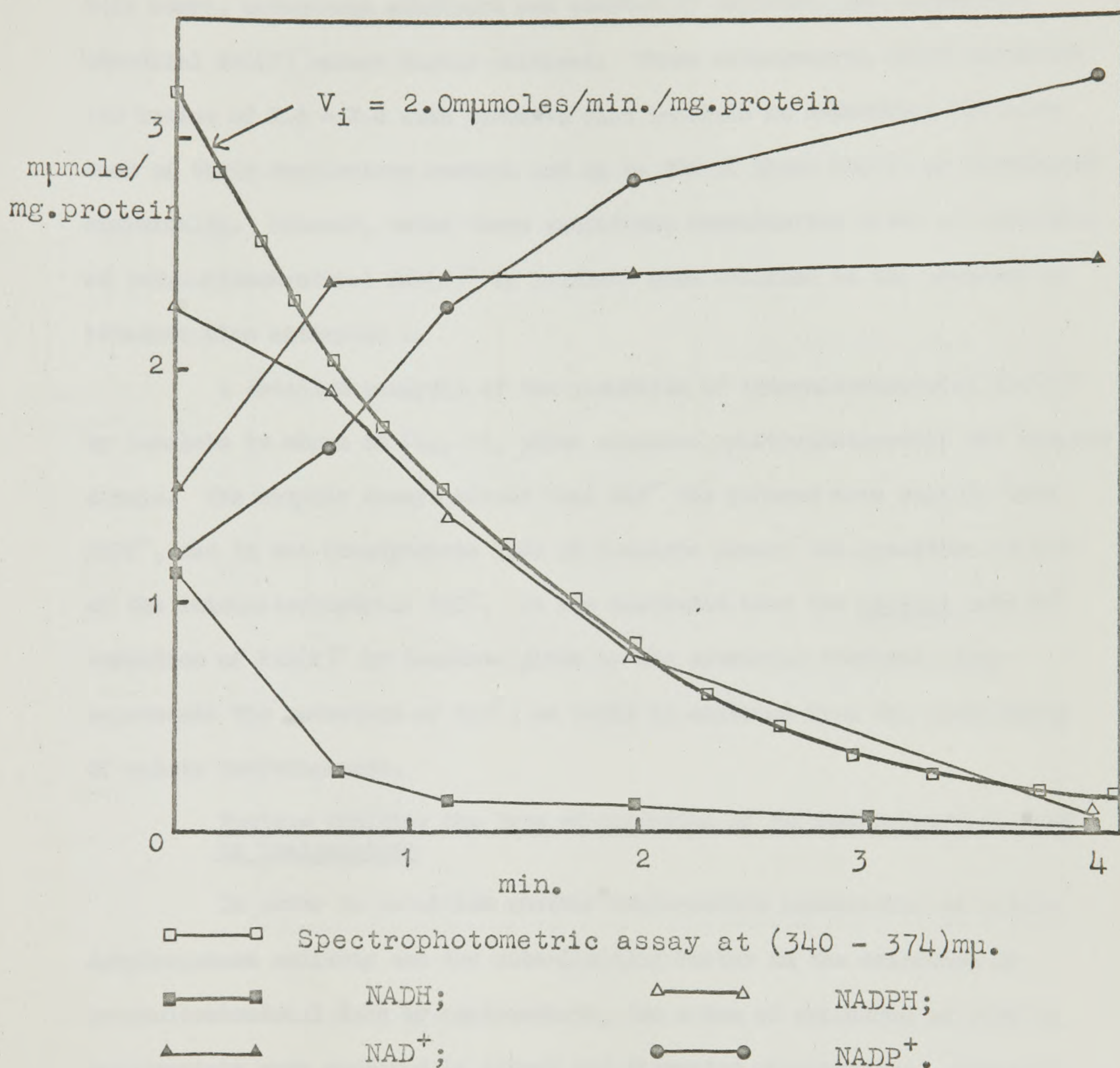
The above method was, therefore, abandoned in favour of the following procedure. Mitochondria were preincubated with rotenone ($0.5 \text{ } \mu\text{M}$ moles/mg. protein) and antimycin A ($0.5 \text{ } \mu\text{g./mg. protein}$). This resulted in a reduction of intramitochondrial NAD(P) to a new steady-state concentration. The increase in the concentration of NAD(P)H was produced by the oxidation of endogenous substrates, and could only be obtained in freshly isolated mitochondria. Addition of $2\text{--}20 \text{ } \mu\text{M}$ oxaloacetate caused the rapid oxidation of up to $4 \text{ } \mu\text{moles NAD(P)H/mg. protein}$. The initial rates of oxidation of intramitochondrial NAD(P)H by oxaloacetate in such experiments varied little from preparation to preparation.

A detailed analysis of the oxidation of intramitochondrial NAD(P)H by oxaloacetate is shown in Fig. 5C, which compares spectrophotometric and enzymic assays. On addition of $20 \text{ } \mu\text{M}$ oxaloacetate, NADH was oxidised much more rapidly than NADPH, but at equilibrium both were completely oxidised. The spectrophotometric assay of NAD(P)H oxidation closely corresponded to the enzymic assay of the oxidation of NADH plus NADPH at all stages. It was concluded that the initial rate of oxidation of NAD(P)H in the spectrophotometric assay represents NADH oxidation, as would be expected from the specificity of malate dehydrogenase, and that NADPH is subsequently oxidised indirectly via transhydrogenase reactions which regenerate NADH.

(b) Penetration of L-malate

The effective measurement of reaction 5(ii) depends on a high $\text{NAD}^+:\text{NADH}$ ratio in the mitochondria as the equilibrium of the reaction favours L-malate formation. A high $\text{NAD}^+:\text{NADH}$ ratio can be obtained by adding uncouplers to the mitochondria, but as this also affects the rate of L-malate penetration into mitochondria by inhibiting the supply of energy (see Chapter 6) an alternative method was sought.

Figure 5C. Spectrophotometric and Enzymic Assays of the Oxidation of Intramitochondrial NAD(P)H by Oxaloacetate in Rat Liver Mitochondria.



Rat liver mitochondria (2.5mg. protein) were incubated at 30° in 3.00ml. containing :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 0.5μM rotenone; 2μg. antimycin A. The reaction was initiated by adding 20μM oxaloacetate. The oxidation of intramitochondrial NAD(P)H was measured spectrophotometrically at (340-374)mμ in a series of identical incubations. The reaction was stopped at 0, 40, 70, 120 or 240 sec. by adding to 0.6ml. of 3M HClO₄ or 1.5ml. of 1N KOH-ethanol (50:50) at 0°, and NAD⁺, NADP⁺, NADH and NADPH determined enzymically as described by Klingenberg (1965).

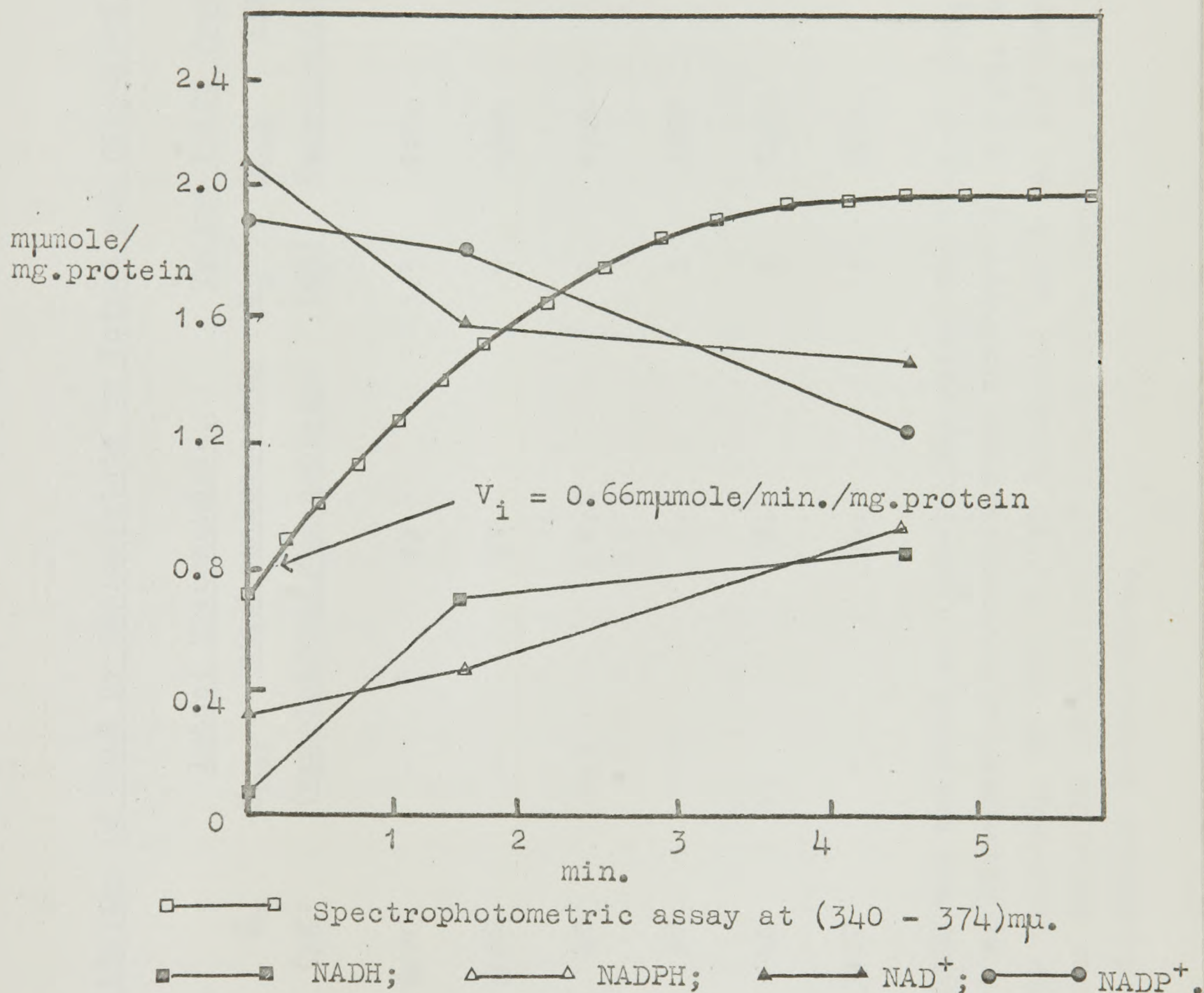
It was found that if mitochondria were preincubated at 0° for 8-18 hours, endogenous substrate was eventually depleted, and intramitochondrial NAD(P) became highly oxidised. These mitochondria still exhibited P:O ratios of 2.4 - 2.6 with pyruvate plus L-malate as substrate, but lost much of their respiratory control and up to 20% of their NAD(P) as determined enzymically. However, under these conditions reproducible rates of reduction of intra-mitochondrial NAD(P) $^{+}$ by L-malate were obtained in the presence of rotenone plus antimycin A.

A detailed analysis of the reduction of intramitochondrial NAD(P) $^{+}$ by L-malate is shown in Fig. 5D, which compares spectrophotometric and enzymic assays. The enzymic assays showed that NAD $^{+}$ was reduced more rapidly than NADP $^{+}$, and in the steady-state 1.67 mM L-malate caused the reduction of 40% of the intramitochondrial NAD $^{+}$. It was concluded that the initial rate of reduction of NAD(P) $^{+}$ by L-malate given by the spectrophotometric assay represents the reduction of NAD $^{+}$, as would be expected from the specificity of malate dehydrogenase.

Factors Limiting the Rate of Oxidation of Intramitochondrial NADH by Oxaloacetate

In order to establish whether oxaloacetate penetration or malate dehydrogenase activity was the rate-limiting factor in the oxidation of intramitochondrial NADH by oxaloacetate, the rates of oxidation of NADH by oxaloacetate were compared in intact and disrupted mitochondria. Table 5A shows that in kidney, heart and liver mitochondria the rate of oxidation of NADH by $10\mu\text{M}$ oxaloacetate is more than 1000 times faster than it is in intact mitochondria even under optimal conditions (i.e. in the presence of TMPD plus ascorbate - see Chapter 6). Table 5A also compares the apparent K_m and V_{max} values for the two systems. It was not possible to obtain these values for

Figure 5D. Spectrophotometric and Enzymic Assays of the Reduction of Intramitochondrial NAD(P)⁺ by L-malate in Rat Liver Mitochondria.



Incubation conditions and the spectrophotometric and enzymic assays were as for Figure 5C. The reactions were initiated by addition of 1.67mM L-malate; temperature = 30°.

Table 5A. The Oxidation of NADH by Oxaloacetate in Intact and Disrupted Mitochondria.

<u>Mitochondria.</u>	<u>Temp.</u>	<u>Intact Mitochondria.</u>		<u>Triton X-100 Treated Mitochondria</u>	
		K_m (μM)	V_{max} ($\mu moles/min./mg.protein$)	K_m (μM)	V_{max} ($\mu moles/min./mg.protein$)
Kidney	30	$\gg 20$	-	3.5	2200
			12		1550
Heart	30	$\gg 20$	-	6	6900
			27		5050
Liver	5	40	5.1	2	1700
			1.2		1500
	13	30	7.6	3	2600
			1.5		2300
	22	40	15	3	3400
			2.7		2900
	30	$\gg 20$	-	3	5000
			4.0		4600

The oxidation of NADH was measured at (340-374)m μ using the dual-wavelength spectrophotometer. Intact or disrupted mitochondria were incubated in 3.00ml. containing :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 0.5 μM rotenone; and 2 μg . antimycin A. 2.8mM ascorbate plus 0.13mM TMPD was added to incubations of intact mitochondria, and 0.1mM NADH to incubations of disrupted mitochondria.

intact mitochondria at 30° as the rates of reaction were too fast to be accurately measured, but values were obtained for liver mitochondria at lower temperatures. The apparent K_m for the oxidation of NADH by oxaloacetate was much higher in intact mitochondria, and the apparent V_{max} more than 200 times faster in disrupted mitochondria. It was concluded that the rate-limiting step in the reaction is the penetration of oxaloacetate, and that the initial rates of oxidation of intramitochondrial NADH by oxaloacetate approximate to the rates of penetration of oxaloacetate.

Factors Limiting the Rate of Reduction of Intramitochondrial NAD^+ by L-malate

Table 5B compares the rates of reduction of NAD^+ by $50 \mu M$ L-malate, and the apparent K_m and V_{max} values for this process in intact and disrupted mitochondria. The rates of reduction of NAD^+ were 100-200 times faster in disrupted mitochondria than in intact mitochondria even under optimal conditions (i.e. in the presence of TMPD plus ascorbate - see Chapter 6). The apparent V_{max} for the reduction of NAD^+ by L-malate was 100 times faster in disrupted heart and liver mitochondria and 10 times faster in disrupted kidney mitochondria than in the intact mitochondria. The apparent K_m values were similar for intact and disrupted heart and liver mitochondria, but the apparent K_m was higher in intact than in disrupted kidney mitochondria.

These data show that the rate-limiting step in the reduction of intramitochondrial NAD^+ by L-malate is the penetration of L-malate, and that the initial rates of reduction of intramitochondrial NAD^+ by L-malate approximate to the rates of penetration of L-malate.

The Respiration of Mitochondria in the Presence of L-malate

As a further check on the validity of the spectrophotometric method of measuring the rates of penetration of L-malate into mitochondria the rate

Table 5B. The Reduction of NAD⁺ by L-malate in Intact and Disrupted Mitochondria.

	<u>Intact Mitochondria.</u>			<u>Triton X-100 Treated Mitochondria.</u>		
	$\frac{K_m}{(mM)}$	$\frac{V_{max}}{(m\mu moles/min./mg. protein)}$	$\frac{50\mu M L-malate}{(m\mu moles/min./mg. protein)}$	$\frac{K_m}{(mM)}$	$\frac{V_{max}}{(m\mu moles/min./mg. protein)}$	$\frac{50\mu M L-malate}{(m\mu moles/min./mg. protein)}$
Kidney	0.80	14.3	0.6	0.16	230	50
Heart	0.40	4.9	0.4	0.12	475	114
Liver	0.13	1.8	0.6	0.21	310	60

The reduction of NAD⁺ was measured at (340-374)m μ using the dual-wavelength spectrophotometer. Intact or disrupted mitochondria were incubated in 3.00ml. containing :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 0.5 μ M rotenone; and 2 μ g. antimycin A. 2.8mM ascorbate plus 0.13mM TMPD was added to incubations of intact mitochondria, and 0.1mM NADH to incubations of disrupted mitochondria, temperature = 30°.

of penetration of L-malate into mitochondria was also measured indirectly by rate of oxidation of L-malate by mitochondria in State 3.

Both kidney and liver mitochondria oxidised L-malate in a medium containing 0.25 M sucrose; 20 mM tris-HCl, pH 7.6; 2.8 mM KPO_4 ; 2.8 mM MgCl_2 and 2 mM ADP. Oxygen uptake was non-linear, increasing with time to 5-6 times the initial rate (see Fig. 5E, Expt. (a)). Fig. 5E(b) shows the apparent K_m and V_{max} values obtained from Lineweaver-Burk plots of the initial and final rates of respiration of kidney mitochondria with L-malate as substrate.

Table 5C compares the K_m and V_{max} values for L-malate respiration with the K_m and V_{max} values for L-malate penetration into respiring mitochondria (TMPD plus ascorbate as substrate).

Heart mitochondria showed only a brief burst of respiration on addition of L-malate unless cysteine sulphinic acid was added to the medium. This was probably because heart mitochondria cannot utilise the oxaloacetate formed by the action of malate dehydrogenase, and the accumulation of oxaloacetate then inhibited malate dehydrogenase.

The Possible Role of Glutamate-Oxaloacetate Transaminase in the Penetration of Oxaloacetate into Mitochondria

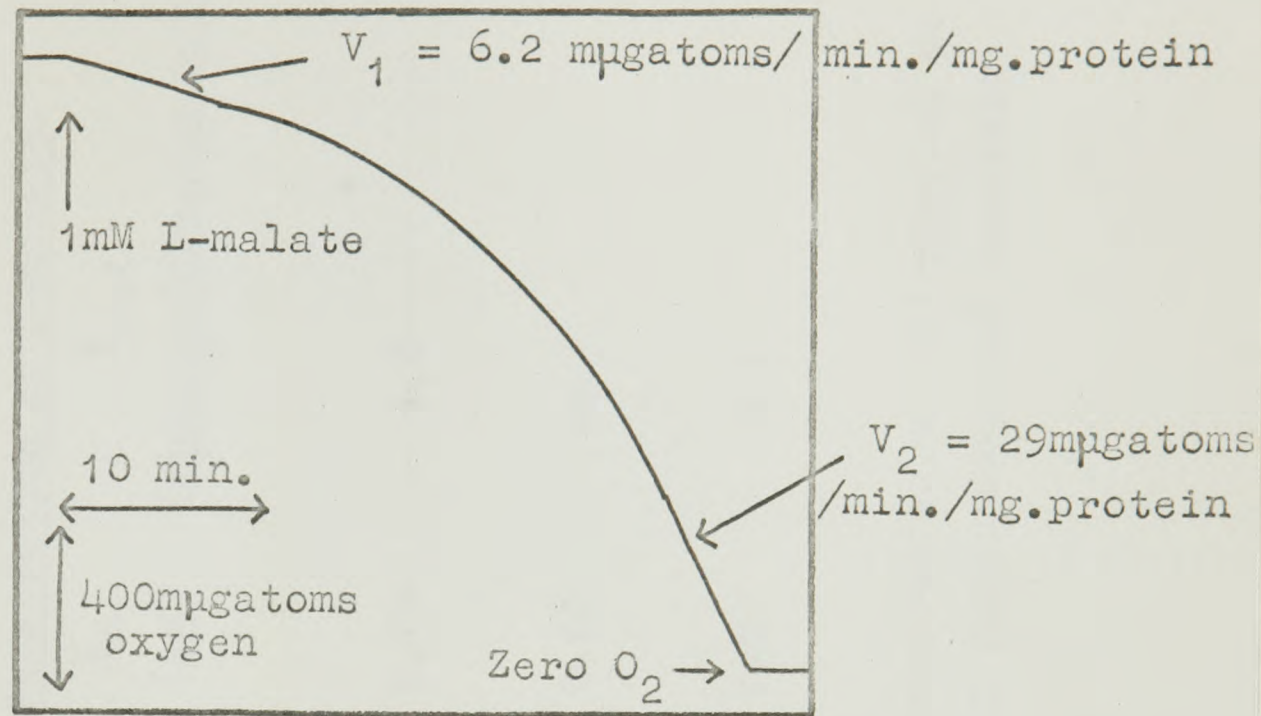
Lardy, Paetkau & Walter (1965), and Chappell (1967) have postulated that the mitochondrial membrane is impermeable to oxaloacetate, which can only enter mitochondria by first being converted to aspartate by an extramitochondrial glutamate-oxaloacetate transaminase (GOT). This hypothesis is summarised in Figure 5F.

This hypothesis was tested as follows:-

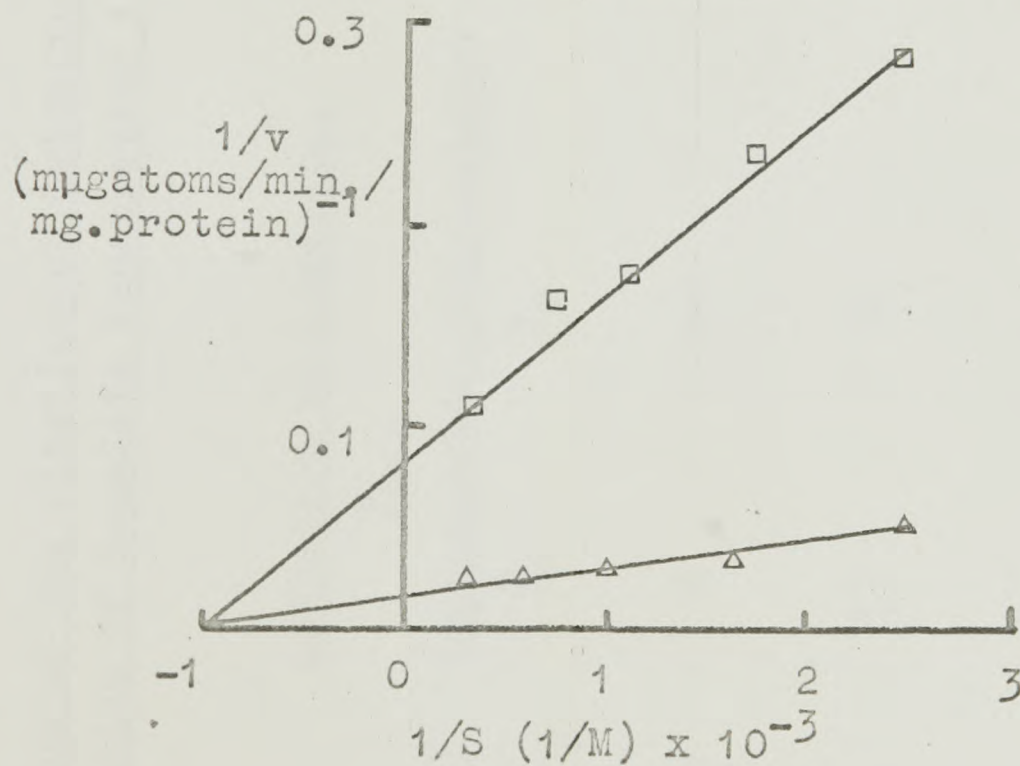
- (1) The extramitochondrial GOT activity of liver mitochondria was shown to be 5-10 $\mu\text{moles/min/mg. protein}$ at 30° in the presence of 1.5 mM aspartate, 1.5 mM α -oxo-glutarate, 0.05 mM NADH and rotenone plus antimycin A. This

Figure 5E. Oxidation of L-malate by Rat Kidney Mitochondria.

(a) Polarographic Measurement of Oxygen Consumption.



(b) Lineweaver-Burk Plot of Oxygen Consumption.



- $\square$ — $\square$ V_1 : $K_m = 1.1\text{mM}$; $V_{\max} = 15$ mpigatoms/min./mg.protein.
 $\triangle$ — $\triangle$ V_2 : $K_m = 1.1\text{mM}$; $V_{\max} = 73$ mpigatoms/min./mg.protein.

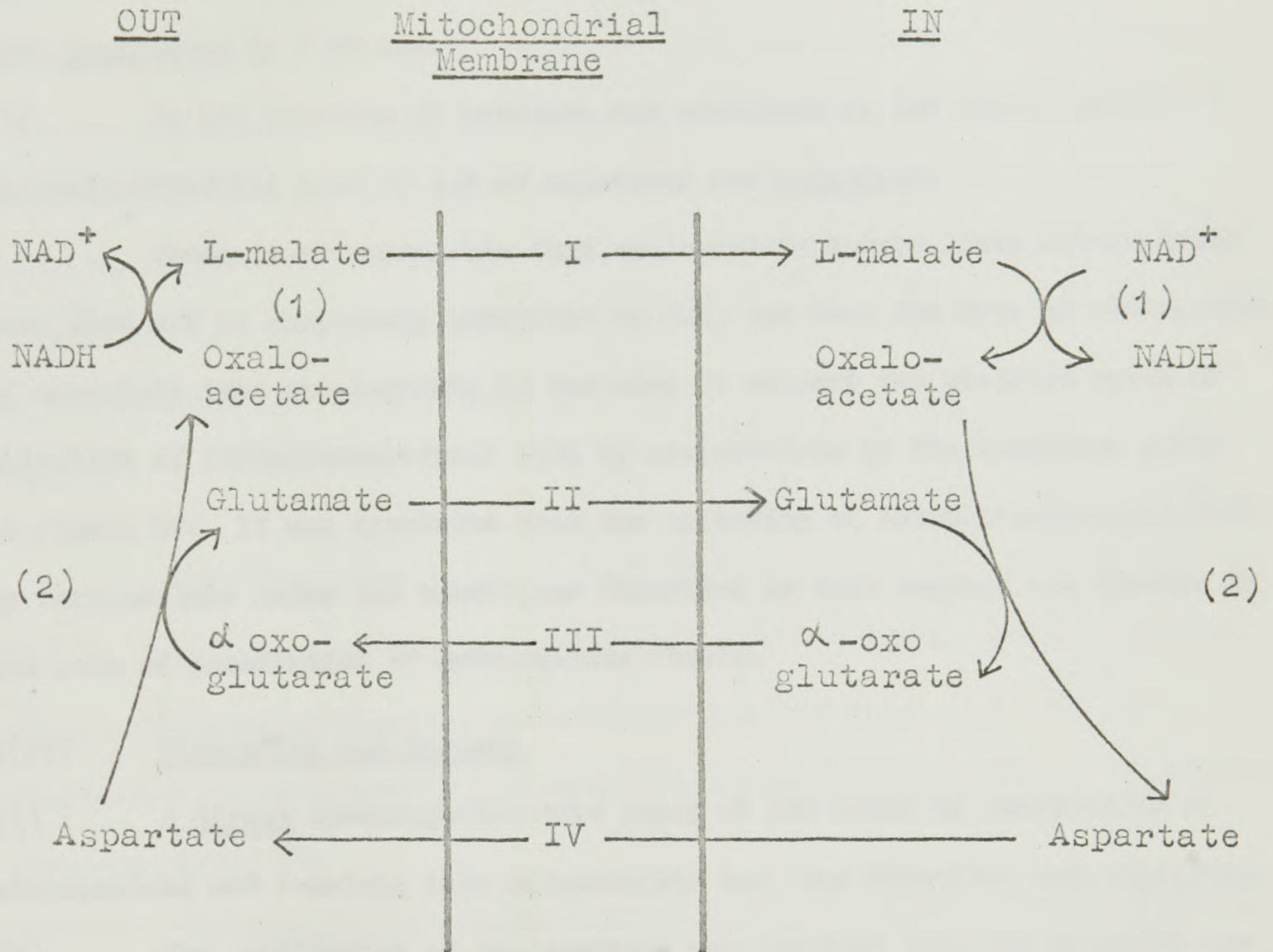
Oxygen consumption was measured polarographically at 30° in the Clark electrode cell. Incubations contained in 3.60ml. :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 2.8mM KPO_4 ; 2.8mM MgCl_2 ; 2mM ADP; and rat kidney mitochondria (3.5 mg. protein).

Table 5C. Comparison of the Kinetics of Oxidation of L-malate by Mitochondria and the Kinetics of L-malate Penetration into Mitochondria.

<u>Mitochondria.</u>	<u>Oxidation of L-malate.</u>			<u>Penetration of L-malate.</u>	
	K_m (mM)	V_{max} (μ moles/min./mg.protein)		K_m (mM)	V_{max} (μ moles/min./mg.protein)
Liver	0.15	2	13	0.13	1.8
Kidney	1.1	15	73	0.80	14.3
Heart	-	-	-	0.40	4.9

Rates of oxidation of L-malate by mitochondria were measured and plotted as described for Figure 5E. The data on the rates of penetration of L-malate into mitochondria are taken from Table 5B.

Figure 5F. The Transfer of Reducing Equivalents into Mitochondria (after Chappell, 1967).



- (1) Malate dehydrogenase.
- (2) Glutamate - oxaloacetate dehydrogenase.
- I Carrier-system for L-malate.
- II Carrier-system for glutamate.
- III Carrier-system for α oxo-glutarate.
- IV Carrier-system for aspartate.

activity was completely inhibited by 2 mM KCN, if the KCN was added before the aspartate.

(2) The rates of oxidation of intramitochondrial NADH by oxaloacetate in the presence of rotenone and antimycin, or rotenone, antimycin A and ATP, were unaffected by 2 mM KCN.

(3) In the presence of rotenone and antimycin A, the oxidation of intramitochondrial NADH by 1.5 mM aspartate was very slow.

These experiments show that oxaloacetate enters liver mitochondria even when GOT is completely inhibited by KCN, and that the rate of penetration of aspartate into mitochondria is too slow to support the observed rates of oxidation of intramitochondrial NADH by oxaloacetate by the mechanism given in Figure 5F. It was concluded that the oxidation of intramitochondrial NADH by oxaloacetate under the conditions described in this chapter was limited by the rate of penetration of oxaloacetate itself.

5(iv) Discussion and Summary

(1) A direct spectrophotometric assay of the rates of penetration of oxaloacetate and L-malate into mitochondria has been described and validated.

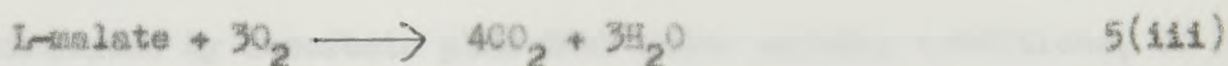
(2) The penetration of oxaloacetate and L-malate into mitochondria was measured by the effect of oxaloacetate or L-malate on the concentration of intramitochondrial NAD(P)H using the Aminco-Chance dual-wavelength spectrophotometer.

(3) Enzymic assays of NAD and NADP showed that the initial rate of oxidation of NAD(P)H by oxaloacetate, and the initial rate of reduction of NAD(P)⁺ by L-malate represented changes in NADH concentration.

(4) As the activity of the mitochondrial malate dehydrogenase under the test conditions was very high in relation to the rates of penetration of

oxaloacetate and L-malate, the initial rate of oxidation of NADH was a measure of the rate of penetration of oxaloacetate, and the initial rate of reduction of NAD^+ was a measure of the penetration of L-malate.

(5) It was shown that the rates of penetration of L-malate into respiring liver and kidney mitochondria (TMPD plus ascorbate as substrate) closely corresponded to the initial rates of oxidation of L-malate by these mitochondria in State 3. The final steady-state rates of oxidation of L-malate by liver and kidney mitochondria were 5-6 times greater than the initial rates, which is the value expected if malate is completely oxidised by mitochondria (Reaction 5(iii) shows that L-malate $\equiv$ 6 equivalents of oxygen.)



(6) It was shown that under the conditions of the spectrophotometric assay glutamate-oxaloacetate transaminase does not play a role in the penetration of oxaloacetate into mitochondria as postulated by the hypothesis given in Figure 5F.

(7) The direct spectrophotometric assay of the rates of penetration of oxaloacetate and L-malate into mitochondria provides a model system for studying the mechanism of penetration of substrate anions into mitochondria.

(8) L-malate has been postulated as a carrier of reducing equivalents into mitochondria (see Krebs, 1966), and this hypothesis requires that the mitochondrial membrane be freely permeable to both L-malate and oxaloacetate. A discussion of this hypothesis in relation to the results given in this chapter is outside the scope of the title of this thesis, but is briefly summarised in Appendix 2.

CHAPTER 6

FACTORS AFFECTING THE PENETRATION OF OXALOACETATE AND L-MALATE INTO MITOCHONDRIA

6(i) Introduction

The previous Chapter describes a spectrophotometric method for the direct measurement of the rates of penetration of oxaloacetate and L-malate into mitochondria. This technique was used to investigate the mechanism of penetration of oxaloacetate and L-malate into mitochondria, and the results of these studies are described in this Chapter.

It was shown that the rates of penetration of oxaloacetate and L-malate into mitochondria in the presence of rotenone and antimycin A were greatly stimulated by ascorbate plus TNPD under aerobic conditions, or by ATP under anaerobic conditions. The effects of inhibitors of energy-transfer (see Chance, 1963) were consistent with the hypothesis that the penetration of oxaloacetate and L-malate into mitochondria is an energy-dependent process.

A more detailed investigation of the factors affecting the penetration of oxaloacetate and L-malate into liver mitochondria, showed that penetration was probably mediated by a specific carrier-system in the mitochondrial membrane which is activated by an energy-requiring process involving bound calcium.

6(ii) Methods

All methods were as described in Chapter 5 with the addition of the following:-

Assay of ATPase Activity

The ATPase activity of mitochondria was measured by three methods:-

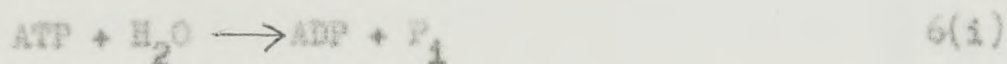
(1) Formation of P_i

ATPase activity was assayed by the formation of P_i from ATP as

described in Figure 6G.

(2) pH-stat Titrations

ATPase activity was measured by a continuous-recording titrimetric pH-stat (see Jacobsen et al., 1957), comprising the Radiometer titrator, TTT1c; Autoburette, ABU1b; Titrigraph, SBR2c; and stirring-motor assembly, M22. The H^+ formed by ATPase activity was titrated with 2.50 or 5.00 mM NaOH. At pH 7.6 Reaction 6(i) liberates 0.92 μ moles H^+ / μ mole ATP in the absence of Mg^{2+} (Nishimura et al., 1962).



Incubation conditions are described in Figure 6H.

(3) pH Titrations

ATPase activity was determined by continuously recording pH at 7.60 ± 0.17 . The pH-measuring equipment consisted of a Pye-Ingold type electrode (No. 401 E07), connected to a Vibron model 33B-2 electrometer, and a Servoscribe recorder (see Gutfreund, 1965). The medium was calibrated by adding known amounts of standard 0.1N HCl or 0.1N KOH from an Agla micro-syringe. All reagents were neutralised to pH 7.6 with tris base, and the incubation medium was recalibrated after additions which affected the buffering-power of the medium. At pH 7.6 H^+ : ATP = 0.92 (Nishimura et al., 1962). Incubation conditions are given in Table 6I.

6(iii) Results

Stimulation of the Penetration of Oxalosuccinate and L-malate into Mitochondria by TMPD plus Ascorbate or by ATP

Table 6A shows the effects of ATP and TMPD plus ascorbate on the initial rates of penetration of 10 μ M oxalosuccinate and 1.7 mM L-malate into liver, kidney and heart mitochondria preincubated in a medium containing

Table 6A. The Stimulation of the Penetration of Oxaloacetate and L-malate into Mitochondria by ATP or TMPD plus Ascorbate.

<u>Mitochondria</u> <u>Conditions</u>	<u>Penetration of Oxaloacetate.</u> (<u>mpmoles/min./mg.protein</u>)			<u>Penetration of L-malate.</u> (<u>mpmoles/min./mg.protein</u>)		
	<u>Liver</u>	<u>Kidney</u>	<u>Heart</u>	<u>Liver</u>	<u>Kidney</u>	<u>Heart</u>
Rotenone.	1.54	1.05	2.4	1.48	3.6	1.3
Rotenone, antimycin A.	1.08	0.22	1.7	0.71	2.2	0.7
Rotenone, antimycin A, TMPD plus ascorbate.	3.40	10.2	26.7	2.41	14.3	4.9
Rotenone, antimycin A, ATP.	2.92	2.72	3.3	1.82	6.0	2.3

The penetration of oxaloacetate was measured using the dual-wavelength spectrophotometer at (340-374)m μ as described in Chapter 5. Incubations were at 30° in 3.00ml. containing:- 0.25M sucrose; 20mM tris-HCl, pH 7.6; and mitochondria (1-3 mg. protein). Other additions during the preincubation were :- 0.5 μ M rotenone; 2 μ g. antimycin A; 0.13mM TMPD plus 3mM ascorbate; and 2.8mM ATP. Mitochondria were preincubated for 2-3 min. (or 5 min. with ATP), and the reaction initiated by adding 10 μ M oxaloacetate or 1.7mM L-malate.

0.25 M sucrose.

Rates of penetration were minimal in the presence of antimycin A plus rotenone (Table 6A, Line 2), but slightly higher in the presence of rotenone alone (Table 6A, Line 1). Parallel experiments in the oxygen electrode showed that 80-90% of the respiration of endogenous substrates was blocked by rotenone, and that the further addition of antimycin A caused 100% inhibition of respiration. Addition of 2.8 mM ascorbate plus 0.13 mM TMPD (see Packer & Jacobs, 1962) initiated respiration (100 m μ g atoms/min./mg. protein in liver mitochondria), and greatly stimulated the rates of penetration of oxaloacetate and L-malate (Table 6A, Line 3).

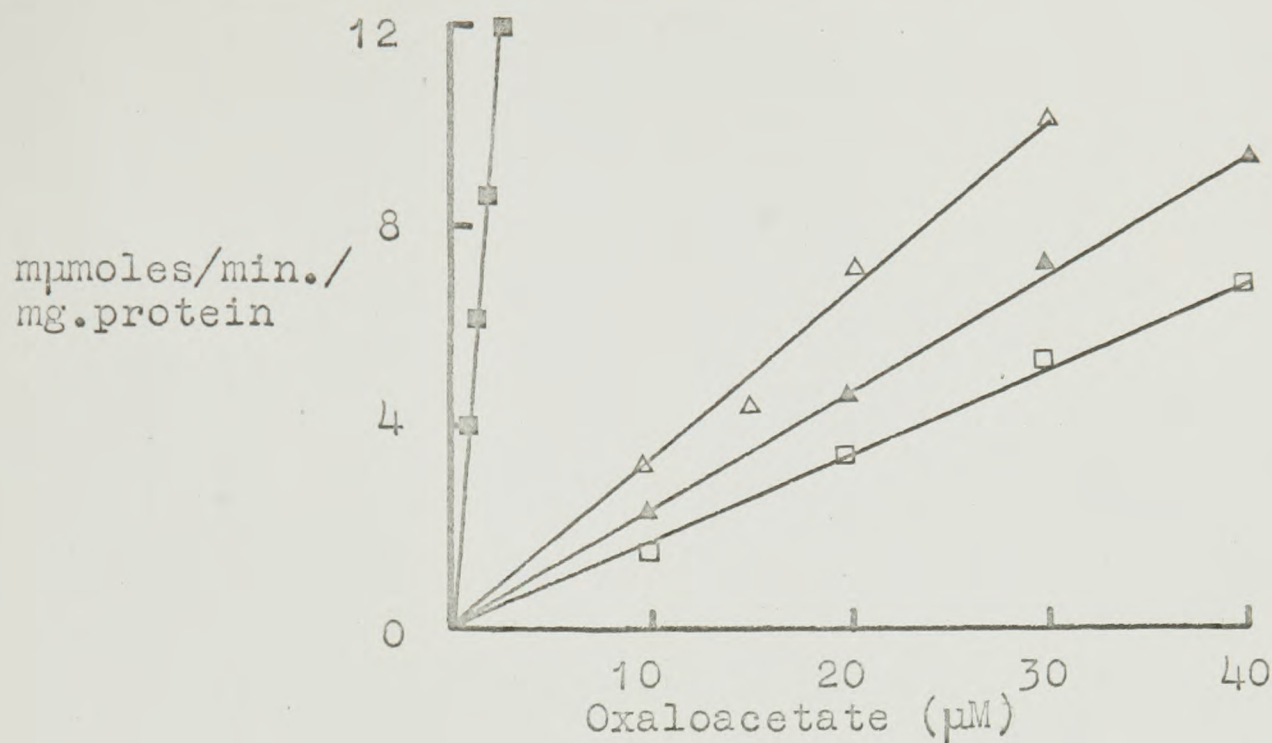
If mitochondria were preincubated with 2.8 mM ATP for 3-5 min. at 30° under anaerobic conditions (rotenone plus antimycin A), the penetration of subsequently added oxaloacetate or L-malate was greatly stimulated (Table 6A, Line 4).

In liver mitochondria the stimulation was as large as that caused by TMPD plus ascorbate, but in kidney and heart mitochondria the stimulation was smaller.

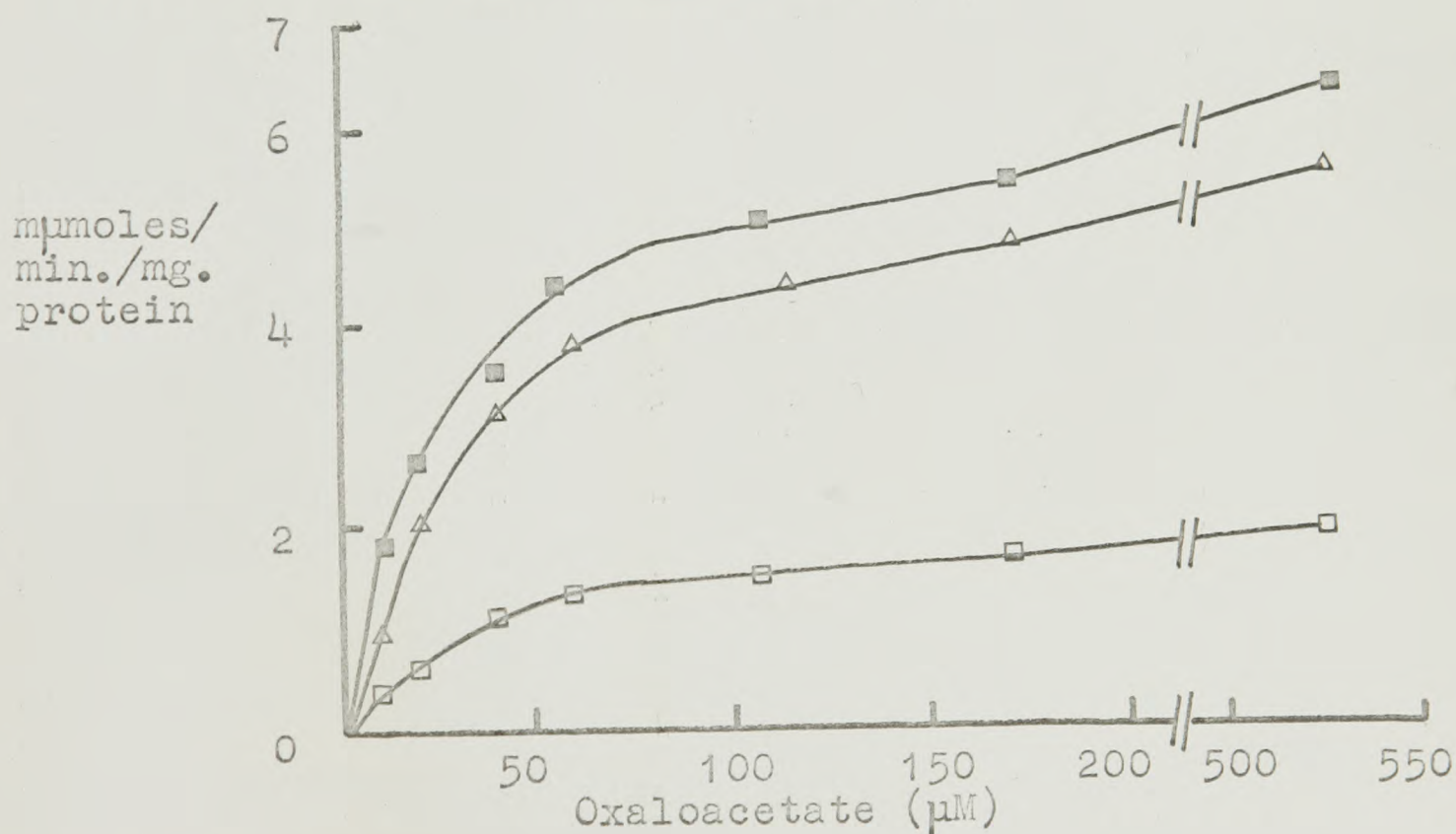
Figure 6A Expt. (a) shows that at 30° the rates of penetration of oxaloacetate into heart mitochondria, up to the highest rates measurable by the spectrophotometric method were proportional to the concentration of oxaloacetate. Figure 6A Expt. (b) shows that at 15° the rates of penetration of oxaloacetate into liver mitochondria, reach a plateau at higher concentrations of oxaloacetate. Lineweaver-Burk plots of these data gave straight lines, from which apparent K_m and V_{max} values could be calculated for the penetration of oxaloacetate (see Figure 6B, Expt. (a)). Figure 6B, Expt. (b) shows Lineweaver-Burk plots of the penetration of L-malate into rat kidney mitochondria at 30°. Penetration again obeyed Michaelis-Menten Kinetics, and

Figure 6A. The Rates of Penetration of Oxaloacetate into Mitochondria.

(a) Rat Heart Mitochondria at 30°.



(b) Rat Liver Mitochondria at 15°.

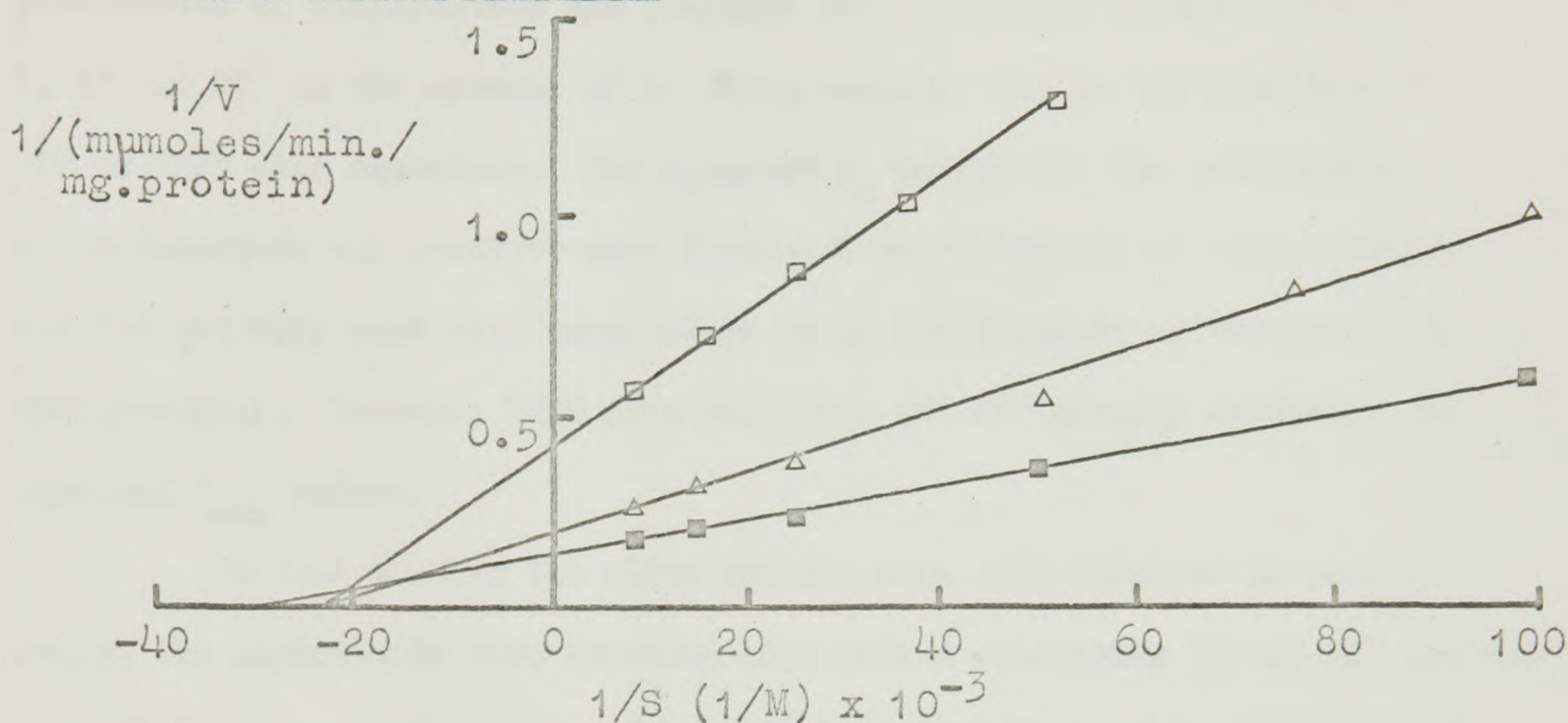


▲—▲	Rotenone;	■—■	Rotenone, antimycin A, TMPD plus ascorbate;
□—□	Rotenone, antimycin A;	△—△	Rotenone, antimycin A, ATP.

The rates of penetration of oxaloacetate were measured spectrophotometrically as described under Table 6A.

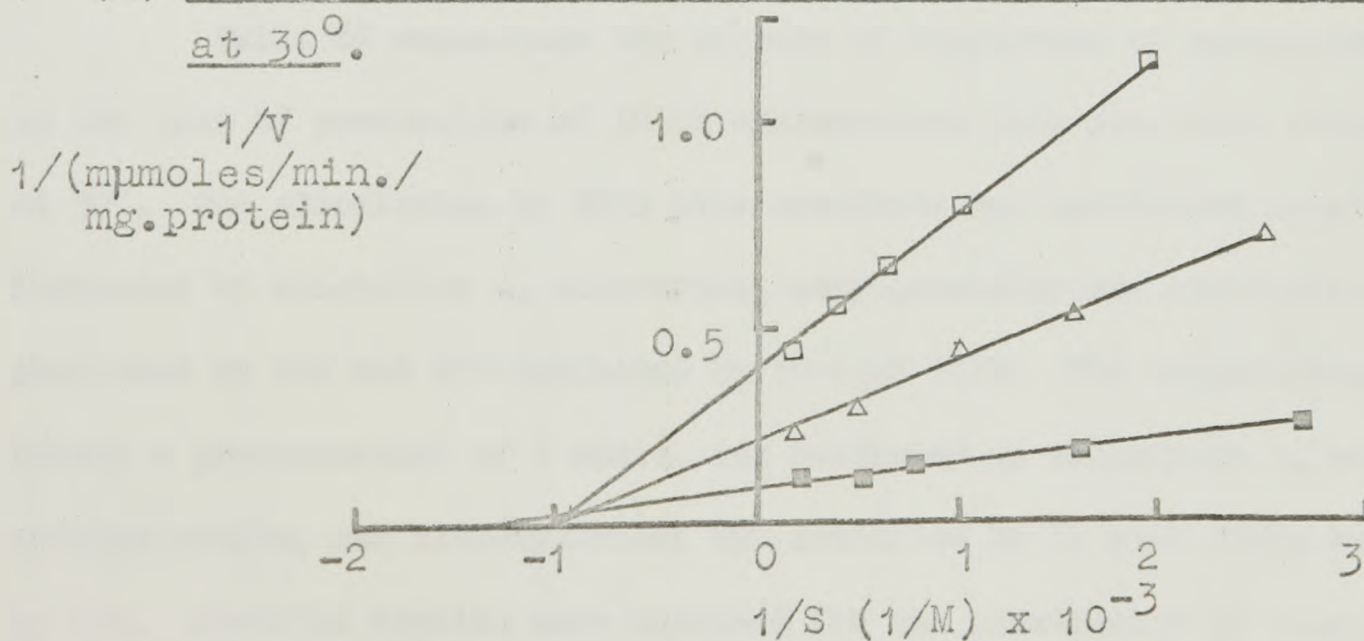
Figure 6B Lineweaver-Burk Plots of the Penetration
of Oxaloacetate and L-malate into Mitochondria.

(a) Penetration of Oxaloacetate into Rat Liver Mitochondria at 13°.



- Rotenone, antimycin A: $K_m = 40\mu\text{M}$; $V_{\text{max}} = 2.5$
 Δ—Δ Rotenone, antimycin A: $K_m = 40\mu\text{M}$; $V_{\text{max}} = 4.6$ μmoles/
 ATP. min./mg.
 ■—■ Rotenone, antimycin A: $K_m = 30\mu\text{M}$; $V_{\text{max}} = 7.6$ protein.
 TMPD plus ascorbate.

(b) Penetration of L-malate into Rat Kidney Mitochondria at 30°.



- Rotenone, antimycin A: $K_m = 1.0\text{mM}$; $V_{\text{max}} = 2.9$
 Δ—Δ Rotenone, antimycin A: $K_m = 1.0\text{mM}$; $V_{\text{max}} = 5.2$ μmoles/
 ATP. min./mg.
 ■—■ Rotenone, antimycin A: $K_m = 0.8\text{mM}$; $V_{\text{max}} = 14.3$ protein.
 TMPD plus ascorbate.

Rates of penetration were measured as for Table 6A.

apparent K_m and V_{max} values were calculated.

Table 6B summarises the apparent K_m and V_{max} values for the penetration of oxaloacetate and L-malate into rat liver mitochondria at 5, 13 and 22° in the absence of an energy-supply, and in the presence of ATP or TMPD plus ascorbate. The apparent K_m values for the penetration of oxaloacetate and L-malate were 30-40 μ M and 0.13-0.21 mM respectively, and did not vary much with temperature or in the presence or absence of an energy-supply. However, TMPD plus ascorbate and ATP greatly increased the apparent V_{max} values.

To test whether the above results were only obtained in sucrose media, the experiments were repeated in a medium containing 150 mM KCl instead of 0.25 M sucrose. The rates of penetration of oxaloacetate were 30-40% less in KCl medium, but TMPD plus ascorbate or ATP again caused a 3-4 fold stimulation of the rate of penetration into liver mitochondria.

Effects of Inhibitors of Energy-transfer on the Penetration of Oxaloacetate into Liver Mitochondria

Table 6C summarises the effects of inhibitors of energy-transfer on the rate of penetration of 10 μ M oxaloacetate into rat liver mitochondria at 30°. The stimulation by TMPD plus ascorbate was unaffected or slightly increased by oligomycin A, aurovertin, octylguanidine and atractyloside, but abolished by KCN and 90% inhibited by 50 μ M TTFB. The stimulation by ATP (after a preincubation of 5 min.), was abolished by oligomycin A, aurovertin, octylguanidine, and atractyloside; 65% inhibited by 50 μ M TTFB; but unaffected by KCN. Parallel results were obtained for the penetration of L-malate into liver mitochondria.

Experiments were performed to determine the time-dependence of the effects of inhibitors which interrupted the supply of energy from ATP or

Table 6B. The Effect of ATP and TMPD plus Ascorbate on the Kinetics of the Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria.

<u>Temp.</u>	<u>Conditions.</u>	<u>Penetration of Oxaloacetate.</u>		<u>Penetration of L-malate.</u>	
		K_m (μM)	V_{max} ($\frac{mpmoles}{min/mg.}$ protein).	K_m (mM)	V_{max} ($\frac{mpmoles}{min./mg.}$ protein).
5	Rotenone, antimycin A.	40	2.0	0.20	0.24
	Rotenone, antimycin A, ATP.	40	2.05	0.20	0.25
	Rotenone, antimycin A, TMPD plus ascorbate.	30	5.1	0.20	0.75
13	Rotenone, antimycin A.	40	2.5	0.20	0.31
	Rotenone, antimycin A, ATP.	40	4.6	0.21	0.49
	Rotenone, antimycin A, TMPD plus ascorbate.	30	7.6	0.13	0.98
22	Rotenone, antimycin A.	40	3.7	0.20	0.65
	Rotenone, antimycin A, ATP.	45	10	0.20	1.46
	Rotenone, antimycin A, TMPD plus ascorbate.	40	15	0.15	1.83

Rates of penetration were measured as for Table 6A.

Table 6C. The Effect of Inhibitors of Energy-transfer on the Rate of Penetration of Oxaloacetate into Rat Liver Mitochondria.

<u>Expt.</u>	<u>Conditions.</u>	<u>Penetration of Oxaloacetate (mmoles/min./mg. protein).</u>
1	Rotenone, antimycin A.	1.08
2	"	1.06
3	" + TMPD, ascorbate.	3.49
4	" + TMPD, ascorbate, oligomycin A.	3.69
5	" + TMPD, ascorbate, aurovertin.	3.50
6	" + TMPD, ascorbate, octylguanidine.	3.40
7	" + TMPD, ascorbate, atractyloside.	3.40
8	" + TMPD, ascorbate, TTFB.	1.46
9	" + TMPD, ascorbate, KCN.	0.95
10	" + ATP.	2.92
11	" + ATP, oligomycin A.	1.08
12	" + ATP, aurovertin.	1.02
13	" + ATP, octylguanidine.	1.00
14	" + ATP, atractyloside.	1.04
15	" + ATP, TTFB.	1.75
16	" + ATP, KCN.	2.93

Rates of penetration were measured as for Table 6A.

Incubations contained in 3.00ml. :- 0.25M sucrose; 20mM tris-HCl, pH 7.6; and rat liver mitochondria (1-2mg. protein). Other additions were :- TMPD, 0.13mM; ascorbate, 2.8mM; ATP, 2.8mM; oligomycin A, 1µg./mg. protein; aurovertin, 0.2µg./mg. protein; octylguanidine, 0.66mM; atractyloside, 0.05mM; TTFB, 50µM; and KCN, 0.33mM. Reactions were initiated by addition of 10µM oxaloacetate.

TMPD plus ascorbate. Oligomycin A or atractyloside were used to cut off the supply of energy from ATP, and KCN was used to inhibit the energy supplied by TMPD plus ascorbate. In all three types of experiment there was a lag of approximately 20-25 sec. at 30° before these inhibitors abolished the stimulation of the initial rates of penetration of subsequently added oxaloacetate.

Effects of Valinomycin and Gramicidin on the Penetration of Oxaloacetate and L-malate into Liver Mitochondria

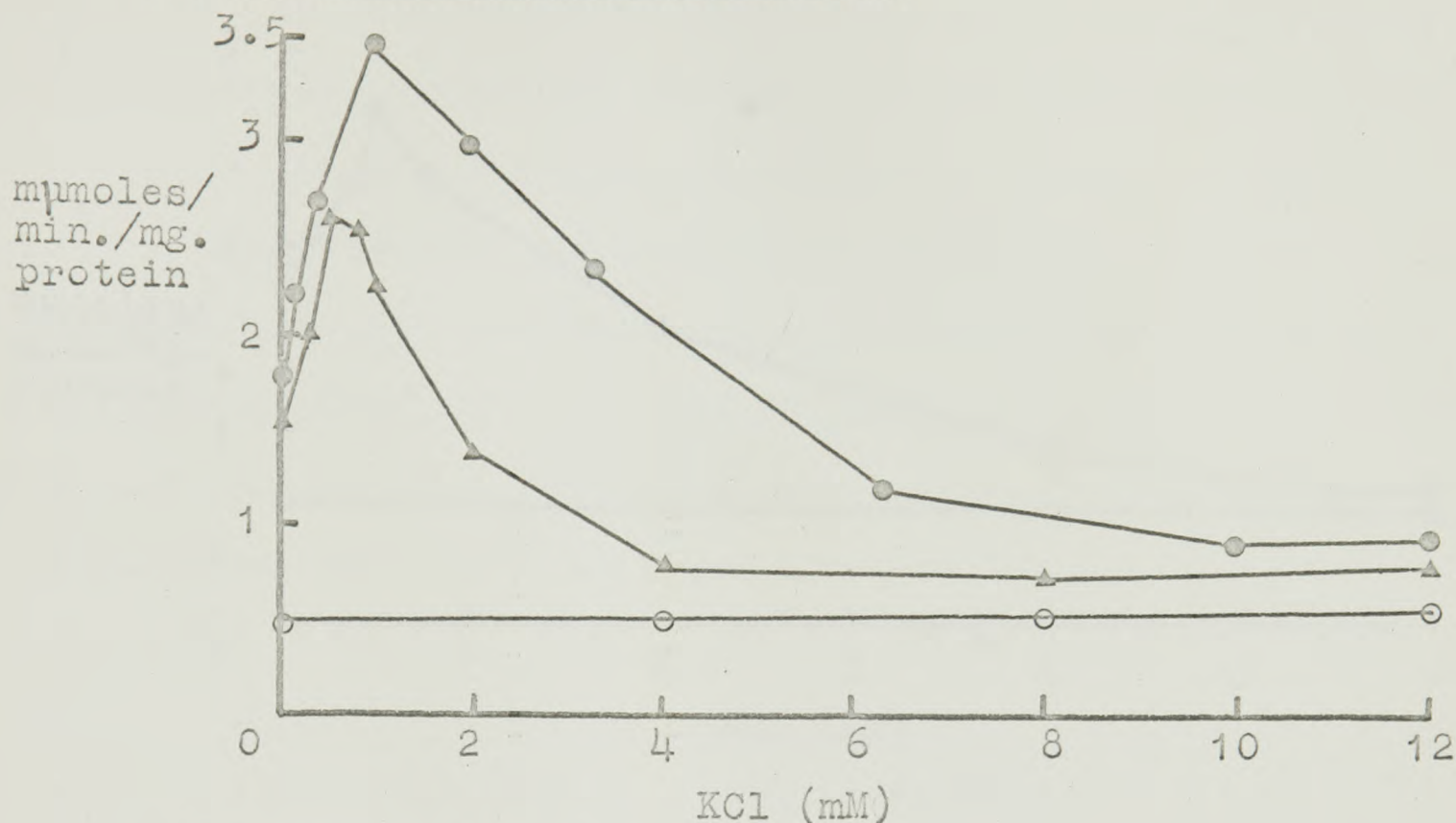
Figures 6C and 6D show the effects of valinomycin and gramicidin on the penetration of oxaloacetate and L-malate into rat liver mitochondria in the presence of increasing concentrations of KCl. Valinomycin or gramicidin plus KCl had no effect on the rates of penetration of oxaloacetate and L-malate in the absence of an energy-supply. In the presence of ATP or TMPD plus ascorbate, valinomycin plus low concentrations of KCl enhanced the rates of penetration of oxaloacetate and L-malate, but at concentrations of KCl $> 2-4$ times those which gave maximal rates of penetration, the rates of penetration of oxaloacetate and L-malate were inhibited. In the presence of valinomycin or gramicidin and > 10 mM KCl, the rates of penetration of oxaloacetate and L-malate were only 20% greater than the rates of penetration in the absence of an energy-supply.

The concentration of KCl which gave maximal rates of penetration of $10 \mu\text{M}$ oxaloacetate, and the maximal percentage stimulation, both increased with increasing concentrations of valinomycin:— 0.014 μg . valinomycin/mg. protein gave a maximal stimulation of 60% with 0.3 mM KCl; 0.056 μg . valinomycin/mg. protein gave a maximal stimulation of 160% with 1.6 mM KCl; and 0.280 μg . valinomycin/mg. protein gave a maximal stimulation of 150% with 2.0 mM KCl.

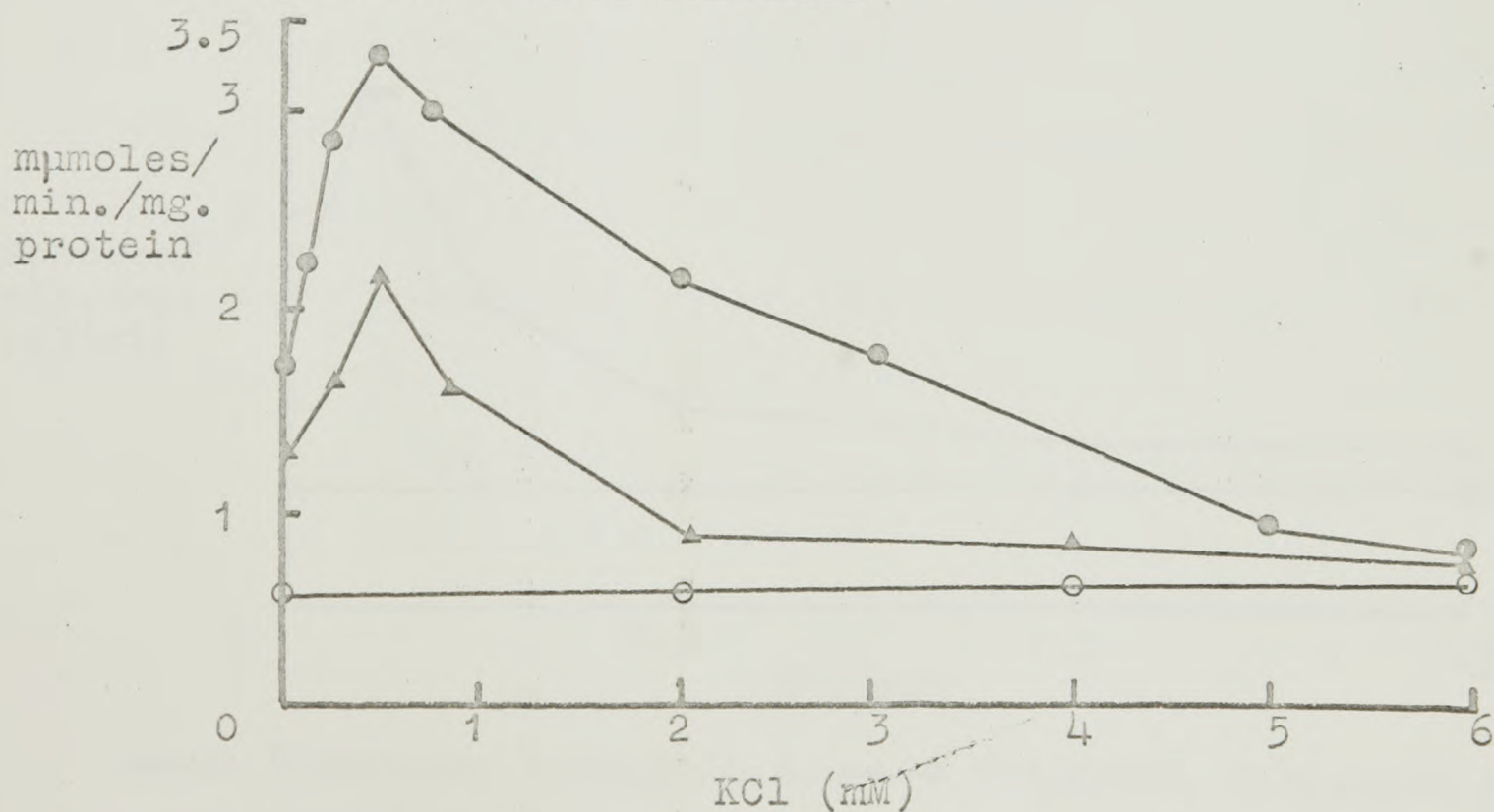
Rates of penetration of $10 \mu\text{M}$ oxaloacetate were measured as for Table 6A. In the presence of 0.056 μg . valinomycin/mg. protein, the

Figure 6C. The Effect of Valinomycin plus KCl on the Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria.

(a) Penetration of Oxaloacetate.



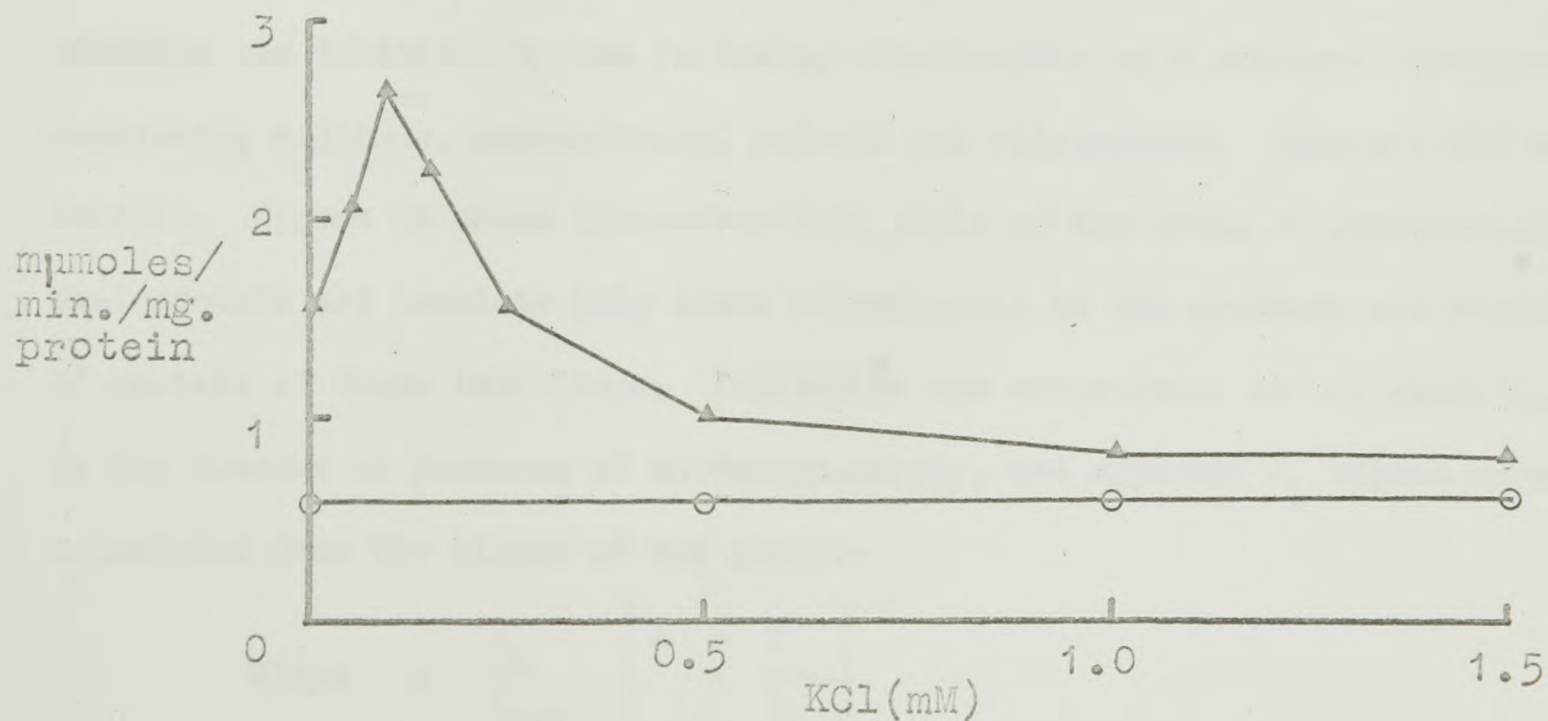
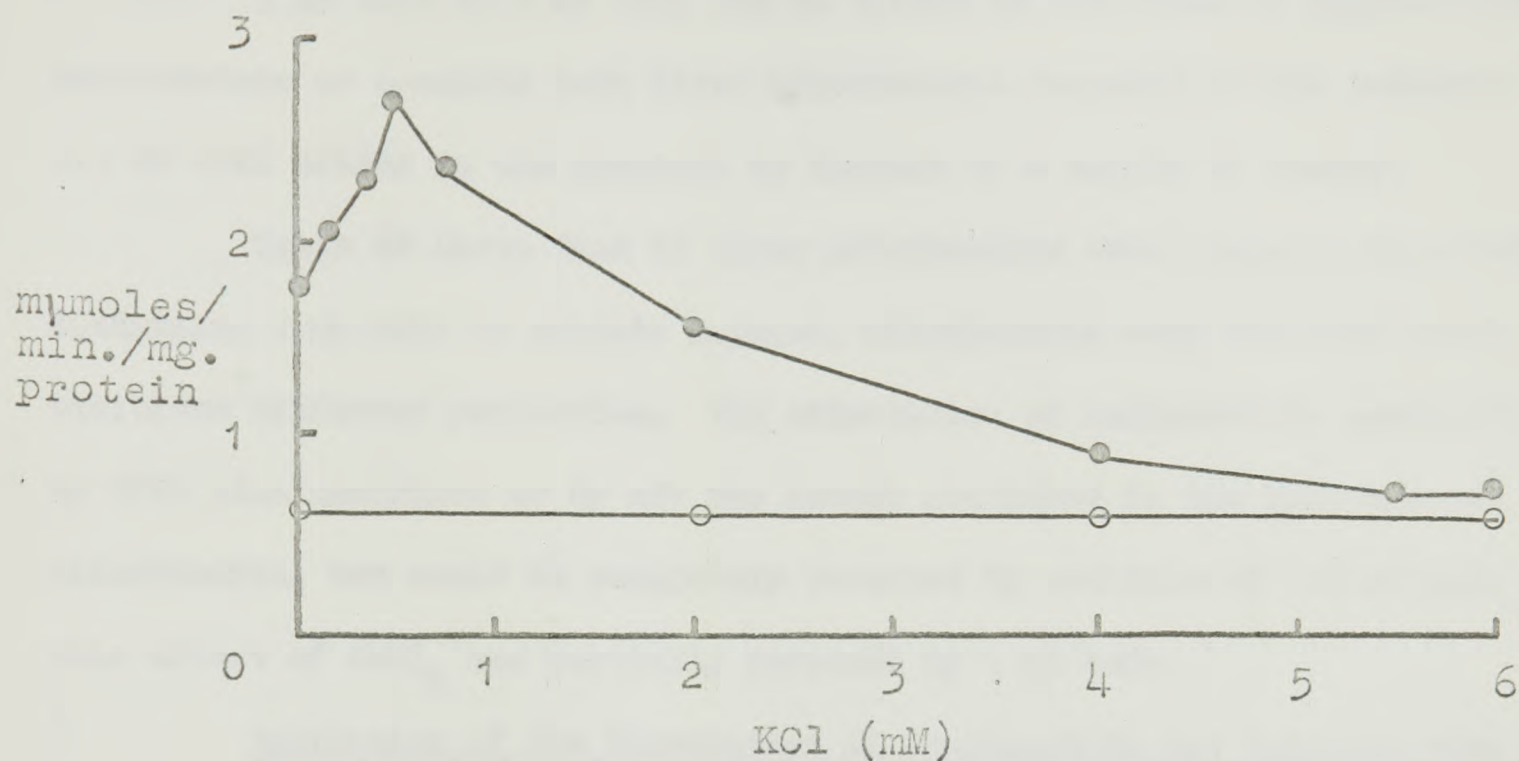
(b) Penetration of L-malate.



○—○ Rotenone, antimycin A; ▲—▲ Rotenone, antimycin A, ATP;
 ●—● Rotenone, antimycin A, TMPD plus ascorbate.

Rates of penetration of 10μM oxaloacetate and 0.14mM L-malate were measured as for Table 6A. Incubations contained 0.06μg. valinomycin/mg. protein, and were at 20°.

Figure 6D. The Effect of Gramicidin plus KCl on the Penetration of Oxaloacetate into Rat Liver Mitochondria.



○—○ Rotenone, antimycin A; ●—● Rotenone, antimycin A, ATP.
 ▲—▲ Rotenone, antimycin A, TMPD plus ascorbate.

Rates of penetration of $10\mu\text{M}$ oxaloacetate were measured as for Table 6A. Incubations were at 20° , and contained $0.08\mu\text{g.}$ gramicidin/mg.protein.

Effects of EDTA and EGTA on the Penetration of Oxaloacetate and L-malate into Liver Mitochondria

5 mM EDTA or 5 mM EGTA had no effect on the rates of penetration of oxaloacetate or L-malate into liver mitochondria prepared in the presence of 0.1 mM EDTA either in the presence or absence of a supply of energy.

Table 6E shows that if liver mitochondria were prepared in a medium containing 1 mM EGTA to chelate calcium, mitochondria were obtained which exhibited different properties. The stimulation of oxaloacetate penetration by TMPD plus ascorbate or by ATP was almost abolished in the EGTA-treated mitochondria, but could be completely restored by addition of 0.1 mM CaCl_2 . This effect of CaCl_2 was partially reversed by 1 mM EGTA.

Inhibition of the Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria by Dicarboxylic Acid Anions

The penetration of oxaloacetate and L-malate into rat liver mitochondria was inhibited by the following dicarboxylic acid anions:- D-malate, succinate, malonate, mesotartrate, maleate and citraconate. Fumarate did not inhibit. Figure 6E shows Lineweaver-Burk plots of the rates of penetration of oxaloacetate and L-malate into liver mitochondria in the presence and absence of certain of these inhibitors. Inhibition was competitive in all cases both in the absence or presence of an energy-supply, and apparent K_i values were calculated from the slopes of the plots:-

$$\text{Slope} = \frac{K_m}{V_{\max}} \left[1 + \frac{I}{K_i} \right]$$

K_i values were similar both in the presence or absence of an energy-supply (see Figure 6E, Expt. (a)), and are given in Table 6D. Similar K_i values for each of the inhibitors were obtained for the penetration of both oxaloacetate and L-malate.

Table 6D. Inhibitor Constants of Anions which Inhibit the Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria.

<u>Inhibitor.</u>	<u>K_i (μM) for penetration of:-</u>	
	<u>Oxaloacetate.</u>	<u>L-malate.</u>
D(+) Malate	290	450
Succinate	130	210
Malonate	280	310
Mesotartrate	430	460
Maleate	55	40
Citraconate	55	55

Rates of penetration of oxaloacetate and L-malate were measured at 20° as described under Table 6A. K_i values were calculated from double reciprocal plots (see Fig.6E).

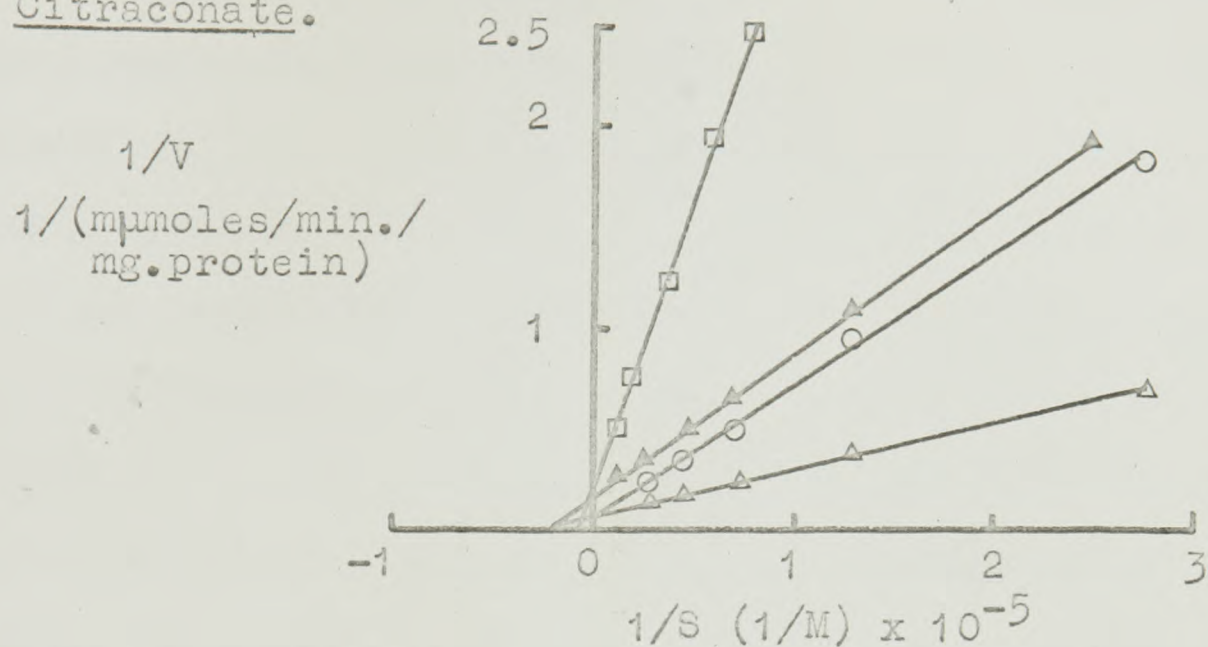
Table 6E. Rates of Penetration of Oxaloacetate into Rat Liver Mitochondria Prepared in the Presence of 1mM EGTA.

<u>Conditions.</u>		<u>Rate of Penetration of</u> <u>10μM oxaloacetate.</u> <u>(μmoles/min./mg.protein).</u>
1	Rotenone, antimycin A.	0.53
2	" + TMPD, ascorbate.	0.80
3	" + ATP.	0.79
4	" + TMPD, ascorbate, 0.1mM CaCl ₂ .	2.21
5	" + TMPD, ascorbate, 0.1mM CaCl ₂ , 1.0mM EGTA.	1.30
6	" + ATP, 0.1mM CaCl ₂ .	1.27
7	" + ATP, 0.1mM CaCl ₂ , 1.0mM EGTA.	1.04

The rates of penetration of 10μM oxaloacetate at 30° were measured as described under Table 6A.

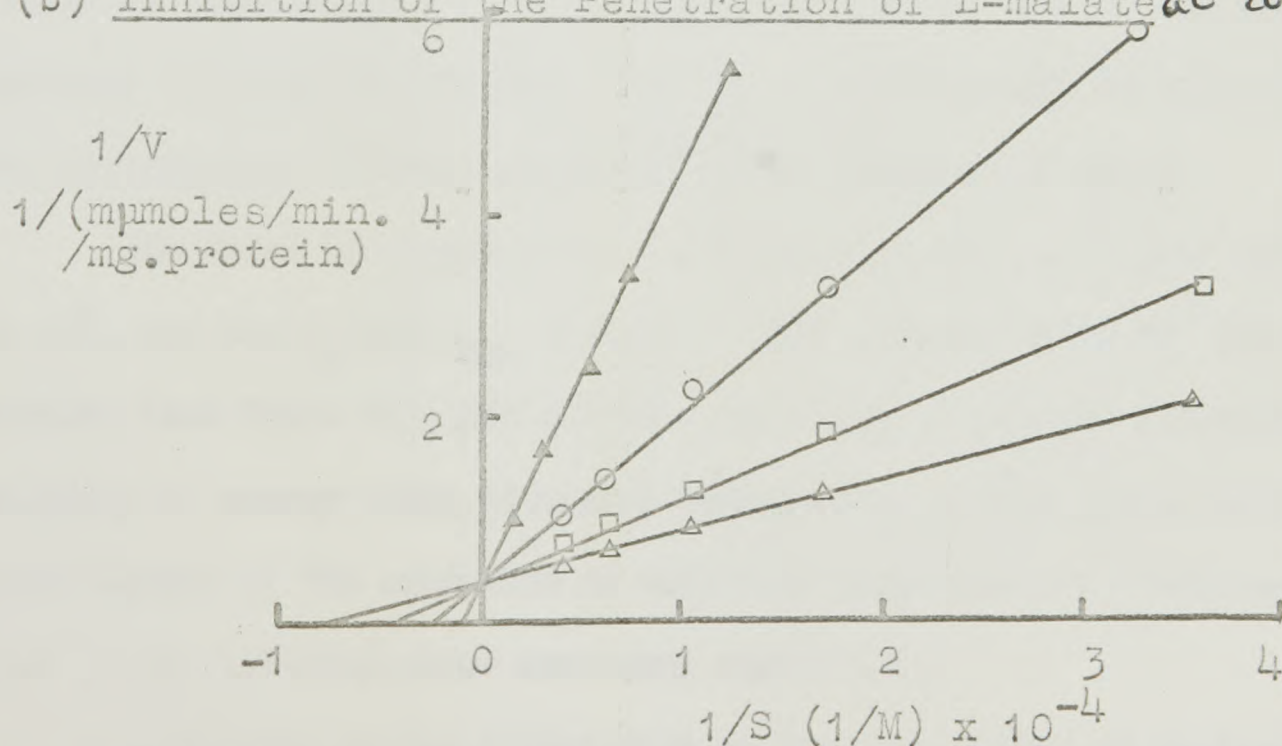
Figure 6E. Inhibition of the Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria by Dicarboxylic Acid Anions. (Methods- see Table 6A.)

(a) Inhibition of the Penetration of Oxaloacetate at 25° by Citraconate.



- ▲—▲ Rotenone, antimycin A; $K_m = 40\mu\text{M}$; $V_{\text{max}} = 5.6\mu\text{moles/min./mg. protein.}$
- Rotenone, antimycin A, $167\mu\text{M}$ citraconate; $K_i = 50\mu\text{M}$.
- △—△ Rotenone, antimycin A, TMPD $K_m = 40\mu\text{M}$; $V_{\text{max}} = 20\mu\text{moles/min./mg.protein.}$ plus ascorbate;
- Rotenone, antimycin A, TMPD plus ascorbate, $167\mu\text{M}$ citraconate; $K_i = 55\mu\text{M}$.

(b) Inhibition of the Penetration of L-malate at 20°



- △—△ Rotenone, antimycin A, $K_m = 140\mu\text{M}$; $V_{\text{max}} = 2.2\mu\text{moles/min./mg.protein.}$ TMPD plus ascorbate;
- " + $165\mu\text{M}$ citraconate; $K_i = 55\mu\text{M}$;
- " + $667\mu\text{M}$ mesotartarate; $K_i = 460\mu\text{M}$.
- ▲—▲ " + $500\mu\text{M}$ succinate; $K_i = 210\mu\text{M}$.

Temperature-dependence of the Penetration of Oxaloacetate and L-malate into Liver Mitochondria

Figure 6F shows Arrhenius plots of the temperature-dependence of the rates of penetration of oxaloacetate and L-malate into liver mitochondria between 5° and 30°. Linear plots were obtained in the absence of an energy-supply or in the presence of TMPD plus ascorbate, from which activation energies of +10 and +8 Kcal/mole for oxaloacetate and L-malate respectively were calculated. Similar activation energies were obtained for the penetration of oxaloacetate and L-malate at higher temperatures in the presence of ATP, but there were sharp breaks in the Arrhenius plots at 15° for oxaloacetate and at 12° for L-malate. Below these 'transition-temperatures' the activation energies for the penetration of oxaloacetate and L-malate were +48 and +42 Kcal/mole respectively.

Preliminary Experiments on the Stoichiometry of the Energy-requirement for the Penetration of Oxaloacetate into Liver Mitochondria

As it had been shown that TMPD plus ascorbate or ATP greatly stimulated the rates of penetration of oxaloacetate and L-malate into mitochondria, it was of interest to determine whether there was a stoichiometry between the stimulation of the penetration of these anions and the consumption of energy.

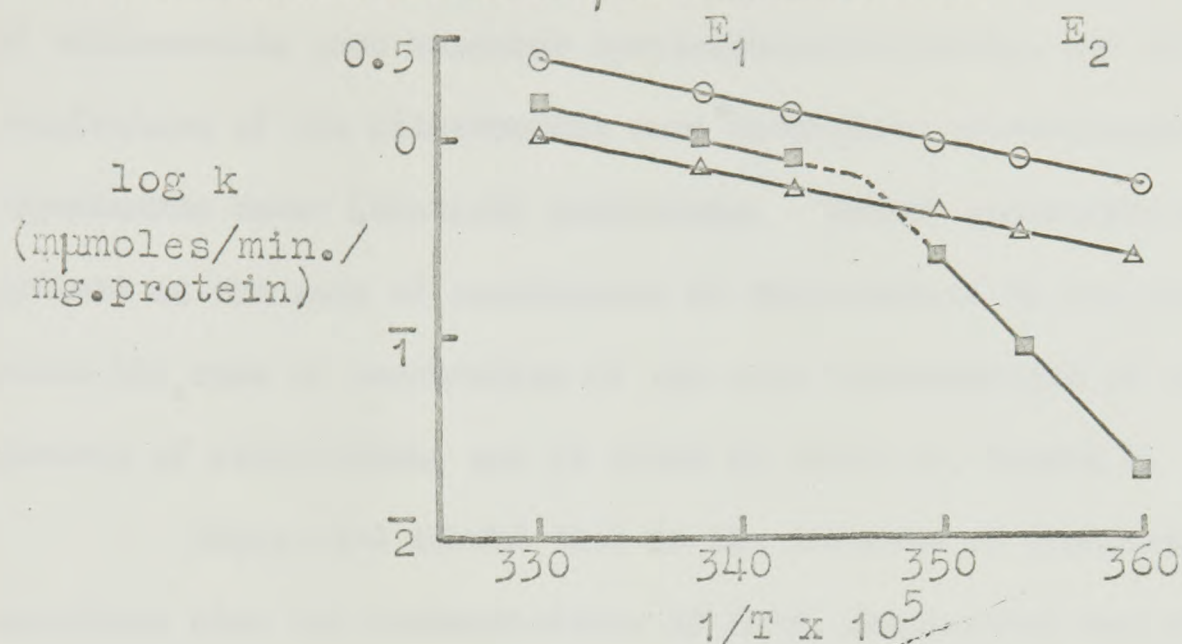
The rates of penetration of oxaloacetate into rat liver mitochondria up to 30°, and the K_m and V_{max} values for this process below 25° had been determined (see Table 6B), and attempts were made to measure the rates of consumption of energy under identical conditions. Energy consumption was measured either by the oxidation of ascorbate under aerobic conditions, or by 'extra' ATPase activity under anaerobic conditions.

(1) Dependence of the Stimulation of the Penetration of Oxaloacetate into Liver Mitochondria on the Rate of Oxidation of Ascorbate.

Table 6F shows a preliminary experiment on the stimulation of the

Figure 6F Arrhenius Plots of the Rate of Penetration of Oxaloacetate and L-malate into Rat Liver Mitochondria.

(a) Penetration of 10 μ M Oxaloacetate.

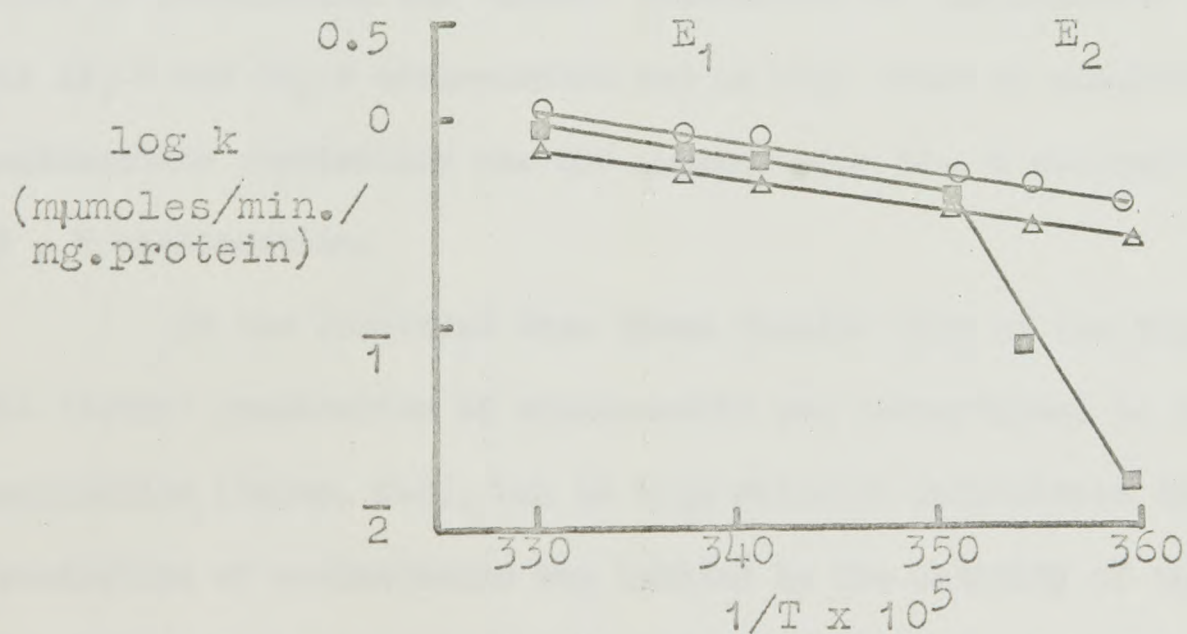


Δ — Δ Rotenone, antimycin A; $\Delta E = +10$ Kcal/mole.

$\circ$ — $\circ$ (Rotenone, antimycin A, TMPD plus ascorbate) minus rate with (rotenone, antimycin A); $\Delta E = +9.5$ Kcal/mole.

$\blacksquare$ — $\blacksquare$ (Rotenone, antimycin A, ATP) minus rate with (rotenone, antimycin A); $\Delta E_1 = +9.6$, $\Delta E_2 = +48$ Kcal/mole.

(b) Penetration of 1.60mM L-malate.



Δ — Δ Rotenone, antimycin A; $\Delta E = +8.1$ Kcal/mole.

$\circ$ — $\circ$ (Rotenone, antimycin A, TMPD plus ascorbate) minus rate with (rotenone, antimycin A); $\Delta E = +8.0$ Kcal/mole.

$\blacksquare$ — $\blacksquare$ (Rotenone, antimycin A, ATP) minus rate with (rotenone, antimycin A); $\Delta E_1 = +8.2$, $\Delta E_2 = +42$ Kcal/mole.

Rates of penetration were measured as for Table 6A.

penetration of oxaloacetate into liver mitochondria by the oxidation of ascorbate or of ascorbate plus low concentrations of TMPD. Rates of penetration of oxaloacetate were measured spectrophotometrically, and the rates of respiration of the mitochondria were determined polarographically in parallel experiments under identical conditions. 'Extra' oxaloacetate penetration was defined as the rate of penetration of oxaloacetate in the presence of respiration minus the rate of penetration of the same concentration of oxaloacetate in the absence of respiration, and is given in Table 6F, Column 4.

Expts. 1-4 showed that in the presence of ascorbate alone, or ascorbate plus low concentrations of TMPD, respiration was slow but the ratio of 'extra' oxaloacetate penetration to oxygen consumption (Column 5) was constant, and in the range 1.6-1.9. Expts. 4 and 5 showed that increasing the concentration of TMPD from 0.6 to 66 μ M caused a 20 fold increase in the rate of respiration, but had no effect on the 'extra' penetration of oxaloacetate. A comparison of Expts. 2 and 7 with 5 and 8, shows that at low rates of respiration the 'extra' penetration of oxaloacetate was the same for 15 μ M and 30 μ M oxaloacetate, but at high rates of respiration 'extra' oxaloacetate penetration was 65% greater with 30 μ M oxaloacetate than with 15 μ M oxaloacetate.

It was concluded from these results that at low rates of respiration the 'extra' penetration of oxaloacetate was proportional to the rate of respiration (Expts. 2-4), but at high rates of respiration the 'extra' penetration of oxaloacetate was limited by the activity of the carrier-system for oxaloacetate, and most of the energy generated by respiration was wasted. The stoichiometry of the energy-requirement for 'extra' oxaloacetate penetration was thus given by Expts. 2-4, in which the supply of energy limited the rate of penetration of oxaloacetate.

Table 6F. The Stimulation of the Penetration of Oxaloacetate into Rat Liver Mitochondria by Ascorbate and the Rate of Oxidation of Ascorbate.

<u>Expt.</u>	<u>Conditions.</u>	<u>Respiration</u> <u>($\frac{\text{mg.atoms}}{\text{mg.protein}}/\text{min.}$)</u>	<u>OAA</u> <u>(μM)</u>	<u>OAA</u> <u>Penetration</u> <u>($\frac{\text{mpmoles}}{\text{min.}}/\text{mg.protein}$)</u>	<u>'Extra' OAA</u> <u>Penetration</u> <u>($\frac{\text{mg.protein}}$)</u>	<u>'Extra' OAA</u> <u>: oxygen</u> <u>consumption</u>
1	Rotenone, antimycin A.	0	15	1.09	0	-
2	" + 2.8mM ascorbate.	1.27	45	3.11	2.02	1.59
3	" + 2.8mM ascorbate, 0.3 μM TMPD.	2.03	15	5.00	3.91	1.93
4	" + 2.8mM ascorbate, 0.6 μM TMPD.	2.79	15	6.00	4.91	1.76
5	" + 2.8mM ascorbate, 66 μM TMPD.	55	15	6.00	4.91	0.09
6	Rotenone, antimycin A.	0	30	1.90	0	-
7	" + 2.8mM ascorbate.	1.27	30	3.80	1.90	1.50
8	" + 2.8mM ascorbate, 66 μM TMPD.	55	30	12.0	8.2	0.15

Oxygen consumption was measured polarographically in the Clark electrode cell at 30°. The penetration of oxaloacetate was measured as described under Table 6A. Incubations contained:-- 0.25M sucrose; 20mM tris-HCl, pH 7.6; 0.5mpmoles/mg.protein, rotenone; 0.5 $\mu\text{g.}/\text{mg.protein}$, antimycin A; and rat liver mitochondria (1-3 mg.protein in penetration measurements, or 10-20 mg. protein in polarographic incubations).

(2) The Effect of Oxaloacetate on the ATPase Activity of Rat Liver Mitochondria

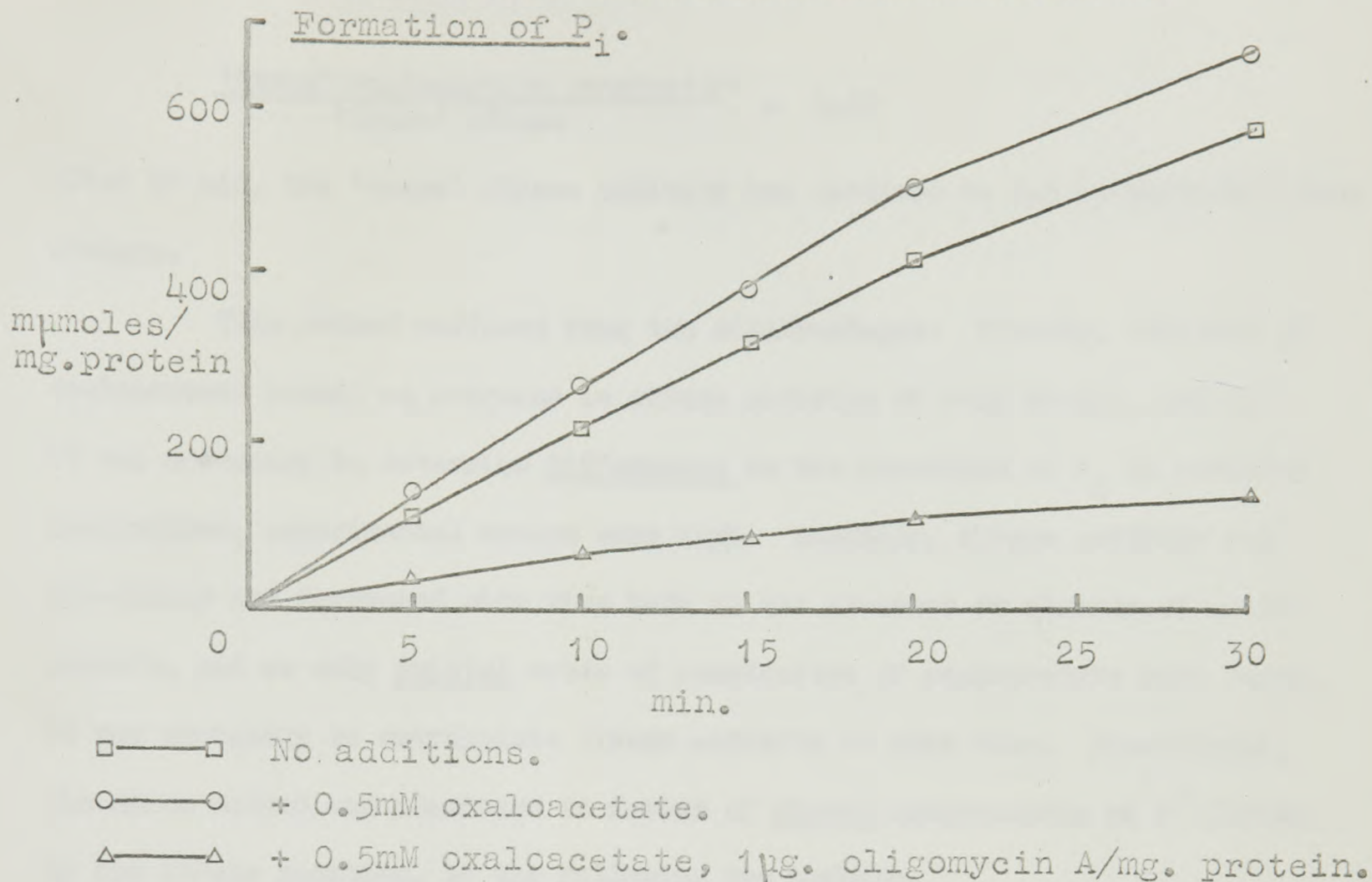
The ATPase activity of freshly isolated rat liver mitochondria at 22° in a medium containing:- 0.25 M sucrose; 20 mM tris-HCl, pH 7.6; 0.5 μ moles rotenone plus 0.5 μ g. antimycin A/mg. protein, was 10-30 μ moles/min./mg. protein. In the presence of ATP, the $V_{\max}$ for penetration of oxaloacetate was 10 μ moles/min./mg. protein, and was 3.7 μ moles/min./mg. protein in the absence of an energy-supply (see Table 6B). If we define 'extra' oxaloacetate penetration as the difference between these two rates, $V_{\max}$ for 'extra' oxaloacetate penetration at 22° = 6.3 μ moles/min./mg. protein, and the corresponding value for 25° = 9.6 μ moles/min./mg. protein. These values are sufficiently large in comparison with the endogenous ATPase activity of the mitochondria to detect 'extra' ATPase activity on addition of oxaloacetate, if ATP is consumed stoichiometrically during the penetration of oxaloacetate into mitochondria.

A concentration of oxaloacetate at least 5 times greater than the K_m for oxaloacetate penetration (> 0.2 mM) was added to mitochondria, and the ratio of 'extra' ATPase activity to the $V_{\max}$ for 'extra' oxaloacetate penetration (defined above) under identical incubation conditions was determined. Preliminary experiments have been performed using three different methods of determining ATPase activity.

(a) Measurement of the Formation of P_i from ATP

Figure 6G shows the effect of 0.5 mM oxaloacetate on the formation of P_i from ATP by liver mitochondria at 22° . Samples were taken at 5 min. intervals and assayed for P_i . 'Extra' ATPase activity in the presence of oxaloacetate was 4 μ moles/min./mg. protein for the first 10 min, corresponding to a ratio :-

Figure 6G. The Effect of Oxaloacetate on the ATPase Activity of Rat Liver Mitochondria, Measured by the Formation of P_i .



The formation of P_i from ATP was measured at 22° in 20ml. containing:- 0.25Msucrose; 50mM tris-HCl, pH 7.6; 2mM ATP; 0.5mmpmoles rotenone plus 0.5µg. antimycin A/ mg. protein; and rat liver mitochondria (1-2 mg.protein/ml.). The reaction was initiated by addition of the mitochondria, and 3.0ml. samples were taken at 5, 10, 15, 20, and 30 min. and added to 0.3ml. 30% trichloroacetic acid. Denatured protein was spun down, and the supernatant assayed for P_i by the method of Gomori (1942).

<u>Conditions.</u>	<u>Initial ATPase activity</u> (mmpmoles/min./mg. protein)
No additions	22
+ 0.5mM oxaloacetate	26
<u>'Extra' ATPase</u>	<u>4</u>

Table 6 shows that addition of ATP at 22° increases the rate of penetration of oxaloacetate from 3.7 to 10 mmpmoles/min./mg.protein. Under these conditions 'extra' oxaloacetate penetration = 6.3 mmpmoles/min./mg. protein, and the ratio 'Extra' oxaloacetate penetration : 'Extra' ATPase = 1.57.

$$\frac{\text{'Extra' oxaloacetate penetration}}{\text{'Extra' ATPase}} = 1.57$$

After 20 min. the 'extra' ATPase activity had declined to 1.5 μ moles/min./mg. protein.

This method suffered from two disadvantages. Firstly, addition of oxaloacetate caused an increase in ATPase activity of only 20-30%, and as it was necessary to determine differences in the formation of P_i in parallel incubations, experimental errors were high. Secondly, ATPase activity was non-linear and decreased with time both in the presence or absence of oxaloacetate, and as only initial rates of penetration of oxaloacetate were known, it was necessary to extrapolate ATPase activity to zero time. Accordingly, the above method was abandoned in favour of direct measurements of H^+ formed by the ATPase reaction, by the following two methods.

(b) pH-stat Titrations

Figure 6H shows a pH-stat titration of the ATPase activity of liver mitochondria at 25°C, and the effects of adding oxaloacetate and oligomycin A. The ratio

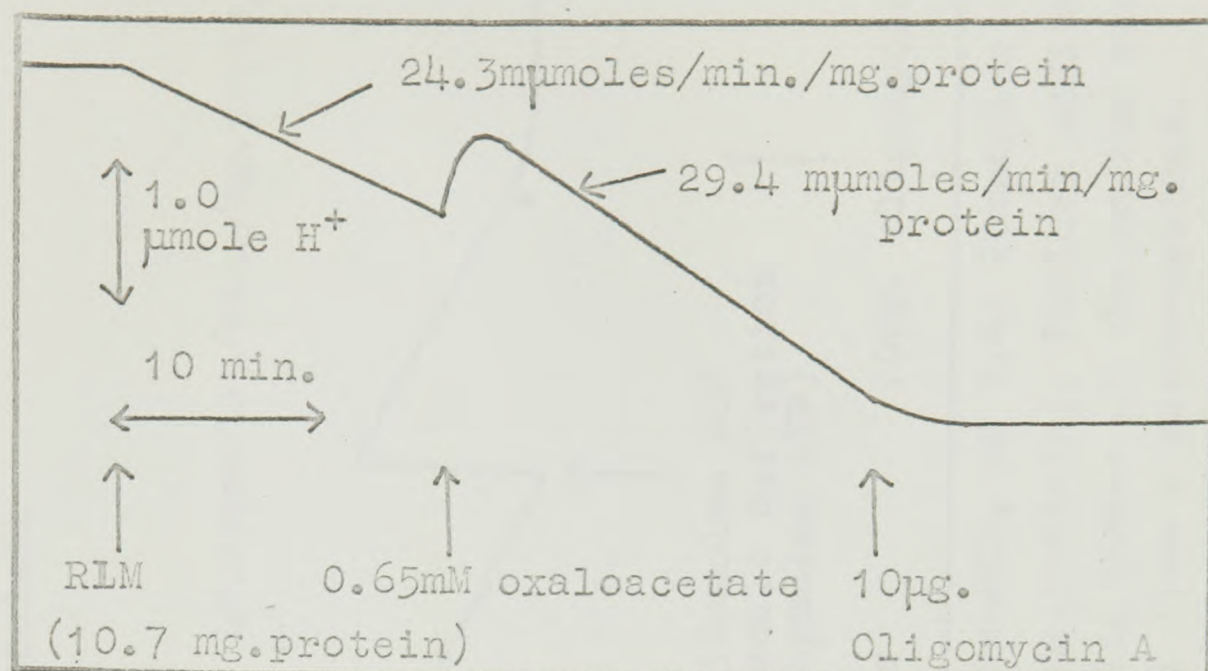
$\frac{\text{'Extra' oxaloacetate penetration}}{\text{'Extra' ATPase}}$ was calculated as described above, and ratios of 1.2-1.9 μ moles 'extra' oxaloacetate penetration/ μ mole 'extra' ATPase were obtained.

(c) Δ pH Titrations

Figure 6I shows the effect of oxaloacetate on the ATPase activity of liver mitochondria at 25°C, measured by changes in pH, and calibrated against standard KOH. Preliminary experiments gave ratios of 1.2-2.0 μ moles 'extra' oxaloacetate penetration/ μ mole 'extra' ATPase.

Table 6G summarises preliminary data obtained on the

Figure 6H. pH-stat Titration of the ATPase Activity of Rat Liver Mitochondria.

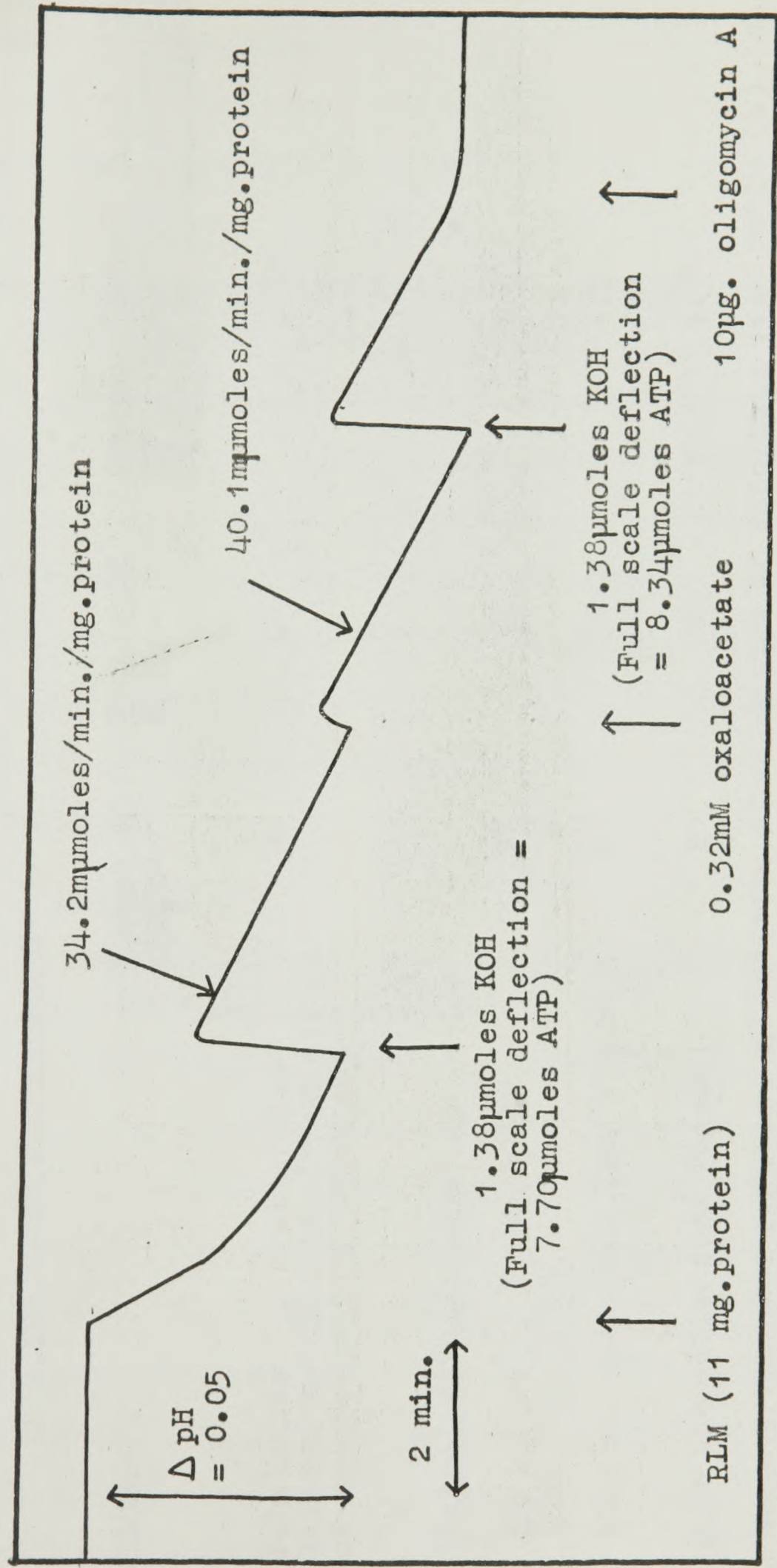


Rat liver mitochondria (10-20 mg.protein) were incubated at 25° in 6.60ml. containing:- 0.25M sucrose; 5mM tris-ATP, pH 7.6; and 0.5μmoles rotenone plus 0.5μg. antimycin A /mg. protein or 0.15mM KCN. Other additions were:- 0.65mM oxaloacetate, pH 7.6; 1μg. oligomycin A /mg.protein; 5mM tris-maleate, pH 7.6; and 1mM tris-citraconate, pH 7.6. The pH was maintained at 7.60 by automatic titration against 2.50mM NaOH. Under these conditions 1.00μmole H⁺ = 1.08μmoles ATP (Nishimura et al.,1962).

<u>Expt.</u>	<u>Additions.</u>	<u>ATPase</u> (μmoles/min./mg.protein)	<u>Δ ATPase</u>
1	(a) Mitochondria	24.3	-
	(b) 0.65mM oxaloacetate	29.4	+5.1
	(c) 10μg. oligomycin A	0	-29.4
2	(a) Mitochondria	27.4	-
	(b) 5.0mM maleate	18.3	-9.1
	(c) 0.65mM oxaloacetate	18.3	0
3	(a) Mitochondria	27.0	-
	(b) 1.0mM citraconate	17.3	-9.7
	(c) 0.65mM oxaloacetate	19.1	+1.8

'Extra' oxaloacetate penetration at 25° in the presence of ATP = 9.6μmoles/min./mg.protein. Thus the ratio 'Extra' oxaloacetate penetration : 'Extra' ATPase = 1.88.

Figure 6I. Δ pH Titration of the ATPase Activity of Rat Liver Mitochondria at 25°.



The incubation contained in 6.60ml.:- 0.25M sucrose; 5mM tris-ATP, pH 7.6; 20 μmoles rotenone; 20 $\mu\text{g. antimycin A}$; and rat liver mitochondria (11mg.protein). Further additions were 0.32mM oxaloacetate and 10 $\mu\text{g. oligomycin A}$. The buffering power of the medium was calibrated after each addition by adding standard KOH or HCl from a micro-syringe. 'Extra' ATPase on addition of oxaloacetate = 5.9 $\mu\text{moles/min.}/\text{mg.protein}$. 'Extra' penetration of oxaloacetate under these conditions = 9.6 $\mu\text{moles/min.}/\text{mg.protein}$. Thus the ratio 'Extra' penetration of oxaloacetate : 'Extra' ATPase = 1.63.

Table 6G. The Stoichiometry of the Energy-requirement for the Penetration of Oxaloacetate into Rat Liver Mitochondria.

<u>Method.</u>	<u>Number of Preps.</u>	<u>Number of Expts.</u>	<u>Average 'Extra' Penetration of Oxaloacetate : Ascorbate Oxidised or 'Extra' ATPase.</u>
(1) Penetration of oxaloacetate limited by the rate of oxidation of ascorbate (see Table 6F).	2	8	1.69
(2) <u>'Extra' ATPase activity.</u>			
(a) Formation of P_i from ATP (see Fig. 6G).	2	6	1.63
(b) pH-stat titration (see Fig. 6H).	6	20	1.59
(c) ΔpH titration (see Fig. 6I).	2	8	1.74

Energy consumption was measured by the rate of oxidation of ascorbate (see Table 6F), or by the increase in ATPase activity on addition of 0.3mM oxaloacetate (= 'extra' ATPase). The stimulation of the penetration of oxaloacetate was measured under identical conditions, as described for Table 6A. 'Extra' penetration = the increase in the rate of penetration in the presence of the energy-supply.

stoichiometry of the energy-requirement for the stimulation of the penetration of oxaloacetate into rat liver mitochondria. Average values of 1.5-1.8 molecules of 'extra' oxaloacetate transported per 'extra' high energy-bond consumed were obtained.

The Effects of Inhibitors of Oxaloacetate Penetration into Liver Mitochondria on 'Extra' ATPase Activity

Figure 6H shows that oligomycin A completely inhibited the endogenous ATPase activity of liver mitochondria both in the presence or absence of oxaloacetate. Maleate and citraconate, which are potent competitive inhibitors of the penetration of oxaloacetate into mitochondria (see Table 6D), but which unlike the other inhibitors in this group are not transported into mitochondria (Chappell & Hearhoff, 1967), inhibited 30-40% of the endogenous ATPase activity of the mitochondria and almost abolished the 'extra' ATPase activity produced by oxaloacetate.

6(iv) Discussion

Evidence for a Carrier-system for the Penetration of Oxaloacetate and L-malate into Mitochondria

Several lines of evidence support the hypothesis that the penetration of oxaloacetate and L-malate into mitochondria is mediated by carrier-systems similar to those postulated by Chappell & Hearhoff (1967) for other anions.

(1) The penetration of oxaloacetate and L-malate into mitochondria obeyed Michaelis-Menten Kinetics with K_m values of $40 \mu M$ and $130 \mu M$ respectively for liver mitochondria (see Figure 6B). The K_m values did not vary greatly with temperature, and were similar both in the presence or absence of an energy-supply (Table 6B), suggesting that the same carrier-systems were used under both these conditions.

(2) The penetration of oxaloacetate and L-malate was competitively

inhibited by a number of dicarboxylic acids of similar structure: D-malate, succinate, malonate, mesotartrate, maleate and citraconate (Figure 6E, Table 6D). These inhibitors had similar K_i values both in the presence of absence of an energy-supply, suggesting that the same carrier-systems were used under both these conditions. The K_i values of each of the inhibitors were also similar for the penetration of both oxaloacetate and L-malate (Table 6D), and this suggests that oxaloacetate and L-malate enter liver mitochondria by the same carrier-system.

(3) Arrhenius plots of the temperature-dependence of the penetration of oxaloacetate and L-malate into liver mitochondria gave activation-energies of +10 and +8 Kcal/mole respectively, both in the presence or absence of an energy-supply. These activation-energies are much higher than would be expected for a process of simple diffusion.

Chappell & Haarhoff (1967) have presented evidence that D-malate; L-malate, succinate, malonate and mesotartrate enter liver mitochondria by the same carrier-system, but that maleate and citraconate are non-penetrants. This data and the evidence presented in this Chapter support the hypothesis that the above-mentioned anions and oxaloacetate can compete with one another for the same carrier-system. It is postulated that the carrier-system facilitates the transport of D-malate, L-malate, succinate, malonate, mesotartrate and oxaloacetate, but not maleate and citraconate.

The Energy-dependence of the Penetration of Oxaloacetate and L-malate into Mitochondria

(1) General Properties of the Energy-dependence and the Effects of Inhibitors of Energy-transfer

The rates of penetration of oxaloacetate and L-malate into liver, kidney and heart mitochondria were greatly stimulated by TMPD plus ascorbate or by ATP (Table 6A, Figure 6A). TMPD plus ascorbate or ATP did not affect

apparent K_m values, but V_{max} values were greatly increased (Figure 6B, Table 6B).

Table 6C shows that the stimulation by TMPD plus ascorbate was inhibited by uncouplers or KCN, but unaffected or slightly increased by oligomycin A, aureoverdin, octylguanidine and atractyloside. The stimulation by ATP was inhibited by uncouplers, oligomycin A, aureoverdin, octylguanidine and atractyloside, but unaffected by KCN. These effects (see Chance, 1963) are consistent with the hypothesis that the penetration of oxaloacetate and L-malate into mitochondria is an energy-dependent process of mitochondria.

The rates of penetration of oxaloacetate and L-malate were greater in the presence of rotenone alone than in the presence of rotenone plus antimycin A. Since rotenone alone did not completely inhibit the endogenous respiration of mitochondria, it is postulated that under these conditions the slow oxidation of endogenous flavin-linked substrates stimulated the penetration of oxaloacetate and L-malate by supplying energy at a slow rate.

ATP was less effective than TMPD plus ascorbate in stimulating the penetration of oxaloacetate and L-malate, particularly in kidney and heart mitochondria. A possible explanation of this is that ATP reacts more slowly than TMPD plus ascorbate to produce the high-energy state which is the immediate cause of the stimulation. That mitochondria must be preincubated with ATP in order to observe the stimulation of the penetration of oxaloacetate and L-malate, also supports the view that ATP reacts slowly in this reaction. A similar lag is observed in sub-mitochondrial particles in the ATP-driven reduction of NAD^+ by succinate (Low, Krueger & Ziegler, 1961) and in the ATP-driven reduction of $NADP^+$ by NADH (Danielson & Ernster, 1963).

Experiments to determine the time-dependence of the effects of inhibitors which blocked the supply of energy from ATP or TMPD plus ascorbate showed that, if oligomycin A or atractyloside were used to inhibit the supply

of energy from ATP, or KCN was used to inhibit the oxidation of TMPD plus ascorbate, there was a lag of 20-25 sec. at 30° before the energy-linked stimulation of the rates of penetration of oxaloacetate was abolished. Since KCN and atractyloside can be shown to inhibit the respiration of intact mitochondria virtually instantaneously, and oligomycin A requires less than 10 sec. to do so, the lag was not caused by a delay in the inhibitors reaching their sites of action. It is suggested that the lag of 20-25 sec. represents the period for which a high-energy state can persist in the mitochondria after the supply of energy has been interrupted, and during which mitochondria are capable of enhanced initial rates of penetration of oxaloacetate.

(2) The Role of Bound Calcium in the Energy-dependent Stimulation of the Penetration of Oxaloacetate and L-malate into Mitochondria

5 mM EDTA or 5 mM EGTA had no effect on the energy-dependence or rates of penetration of oxaloacetate and L-malate into mitochondria. These metal-chelators would be expected to bind all extra-mitochondrial Mg^{2+} and Ca^{2+} , and it was concluded that free Mg^{2+} and Ca^{2+} were not required for the energy-dependent stimulation of the penetration of oxaloacetate and L-malate into mitochondria. However, if mitochondria were depleted of bound Ca^{2+} by preparing them in the presence of 1 mM EGTA, the stimulation of oxaloacetate penetration by TMPD plus ascorbate or ATP was almost abolished (Table 6E). The energy-dependence could be restored by addition of 0.1 mM $CaCl_2$, and this was only partially reversed by 1 mM EGTA.

It is postulated that the energy-requirement for the penetration of oxaloacetate and L-malate into mitochondria is caused by an energy-requirement for the activation of the carrier system by bound Ca^{2+} , and possible mechanisms for this will be discussed later. The Ca^{2+} required for this process must be tightly-bound to the mitochondrial membrane as it can only be removed with

difficulty even in the presence of a large excess of EGTA.

(3) The Effects of Valinomycin and Gramicidin on the Stimulation of the Penetration of Oxaloacetate and L-malate by TMPD plus Ascorbate or ATP

Figures 6C and 6D show that valinomycin and gramicidin have two effects on the rates of penetration of oxaloacetate and L-malate into liver mitochondria in the presence of an energy-supply:-

- (a) A stimulation in the presence of low concentrations of KCl.
- (b) An inhibition in the presence of higher concentrations of KCl.

In the absence of permeant anions, valinomycin or gramicidin cause an energy-dependent uptake of K^+ from the medium accompanied by the extrusion of H^+ (Pressman, 1963). In the presence of permeant anions such as P_i , K^+ uptake is accompanied by the uptake of permeant anions (see Chappell & Crofts, 1966). It is postulated that the stimulation of the penetration of oxaloacetate and L-malate in the presence of valinomycin or gramicidin plus low concentrations of KCl occurs by a similar mechanism, but the inhibition of penetration at higher KCl concentrations requires an additional explanation, which will be discussed later in relation to possible mechanisms for the energy-dependence of the penetration of oxaloacetate and L-malate into mitochondria.

(4) The Stoichiometry of the Energy-requirement for the Penetration of Oxaloacetate into Liver Mitochondria

The energy-dependent stimulation of the rates of penetration of oxaloacetate and L-malate into mitochondria could occur by one of the following mechanisms:-

- (a) An energy-supply could catalytically cause an increased turnover of the carrier-system perhaps by causing structural changes in the mitochondrial membrane. This would increase the rates of penetration of

oxaloacetate and L-malate into mitochondria, but only in the direction of the concentration gradient.

(b) If the transport of oxaloacetate and L-malate by the carrier-system were linked to the expenditure of stoichiometric amounts of energy, an increased rate of anion transport could occur even against a concentration gradient, and would result in the accumulation of oxaloacetate or L-malate within the mitochondria. In relation to this Gamble (1965) has reported that mitochondria can maintain high internal concentrations of L-malate and citrate.

(c) A third possibility is that the initiation of anion transport into mitochondria depleted in energy, requires the expenditure of a small amount of energy, but that the subsequent penetration of the anions could occur without further expenditure of energy e.g. this would occur if product anions formed inside the mitochondria could exchange for substrate anions outside the mitochondria. In this mechanism the stoichiometry of the energy-requirement would be low, and concentration gradients of substrate anions and product anions would tend to equilibrate by a process of exchange-diffusion. The synthesis of glutamate from α oxo-glutarate plus NH_3 and L-malate requires energy (Slater & Tager, 1963), but the stoichiometry of the energy-requirement is low and varies with conditions (0.25-1.0 ATP per glutamate synthesised). It is possible that the energy-requirement for this reaction is an example in which energy is required to initiate anion transport into mitochondria.

In the experiments described in this Chapter, the penetration of oxaloacetate and L-malate into mitochondria was measured in the direction of the concentration gradient, and penetration occurred even in the absence of an energy-supply. The rates of penetration of oxaloacetate were greatly stimulated by TMPD plus ascorbate or ATP, but the K_m values and activation energies of the penetration process were unaffected. This suggests that there is a single

carrier-system, the rate of which is stimulated by an energy-supply, and this could occur by any one of the three mechanisms postulated above.

In order to determine which mechanism is most likely, preliminary experiments have been performed on the stoichiometry of the energy-requirement for oxaloacetate penetration into liver mitochondria. This was done by measuring the initial rates of oxidation of TMPD plus ascorbate or of 'extra' ATPase; and comparing these with the initial 'extra' rates of penetration of oxaloacetate under identical conditions, as described in Table 6F and Figures 6G, 6H and 6I, and summarised in Table 6G. The average value obtained by four independent methods was 1.6-1.7 'extra' molecules of oxaloacetate transported per high-energy bond utilised.

'Extra' ATPase activity was inhibited by oligomycin A and by the non-penetrant anions, maleate and citraconate (Table 6F) under conditions where these also inhibited the penetration of oxaloacetate, and this strongly supports the conclusion that the 'extra' ATPase activity was a consequence of the penetration of oxaloacetate. Table 6F also shows that 35% of the endogenous ATPase activity of the mitochondria was inhibited by 5 mM maleate or 1 mM citraconate, and this suggests the possibility that some of the endogenous ATPase activity of mitochondria may represent the expenditure of energy in driving anion carrier-systems which maintain the ionic composition of the mitochondria.

The above results rule out mechanism (a), but cannot distinguish between mechanisms (b) and (c), as they only apply to the initial rates of penetration of oxaloacetate into mitochondria. 'Extra' ATPase activity decreases with time (see Figure 6G), and if the rate of penetration of oxaloacetate penetration also decreases with time mechanism (b) would be supported.

If the rate of penetration of oxaloacetate is linear with time or increases, mechanism (c) would be supported. However, the decrease in the rate of the 'extra' ATPase activity is not rapid, and if the concentration of oxaloacetate was still much greater than the K_m , the rate of penetration would be expected to be linear with time. It is thus considered that mechanism (b) is the more likely, and that the stoichiometry of the energy-requirement for the stimulation of the penetration of oxaloacetate into mitochondria is approximately 2 molecules of oxaloacetate transported per high-energy bond consumed. This stoichiometry is similar to that of the energy-requirement for the translocation of divalent cations into mitochondria (Rossi & Lehninger, 1964), and supports the hypothesis that the two processes may be linked.

The fact that energy is consumed in the translocation of substrate molecules such as L-malate into mitochondria, may explain why P:O ratios approach but rarely equal whole numbers. If one molecule of L-malate requires $\frac{1}{2}$ ATP molecule to enter the mitochondrion, and its complete oxidation via the Krebs cycle and electron transport chain yields 18 molecules of ATP as predicted by our present knowledge of oxidative phosphorylation, the observed P:O ratio would be 2.92. The effect of the energy-requirement for the translocation of substrates into mitochondria would also be small if the substrate molecules yielded anionic products inside the mitochondria which could leave in exchange for further molecules of substrate e.g. one of the major products of the oxidation of succinate is L-malate, and this could possibly leave the mitochondria in exchange for further molecules of succinate.

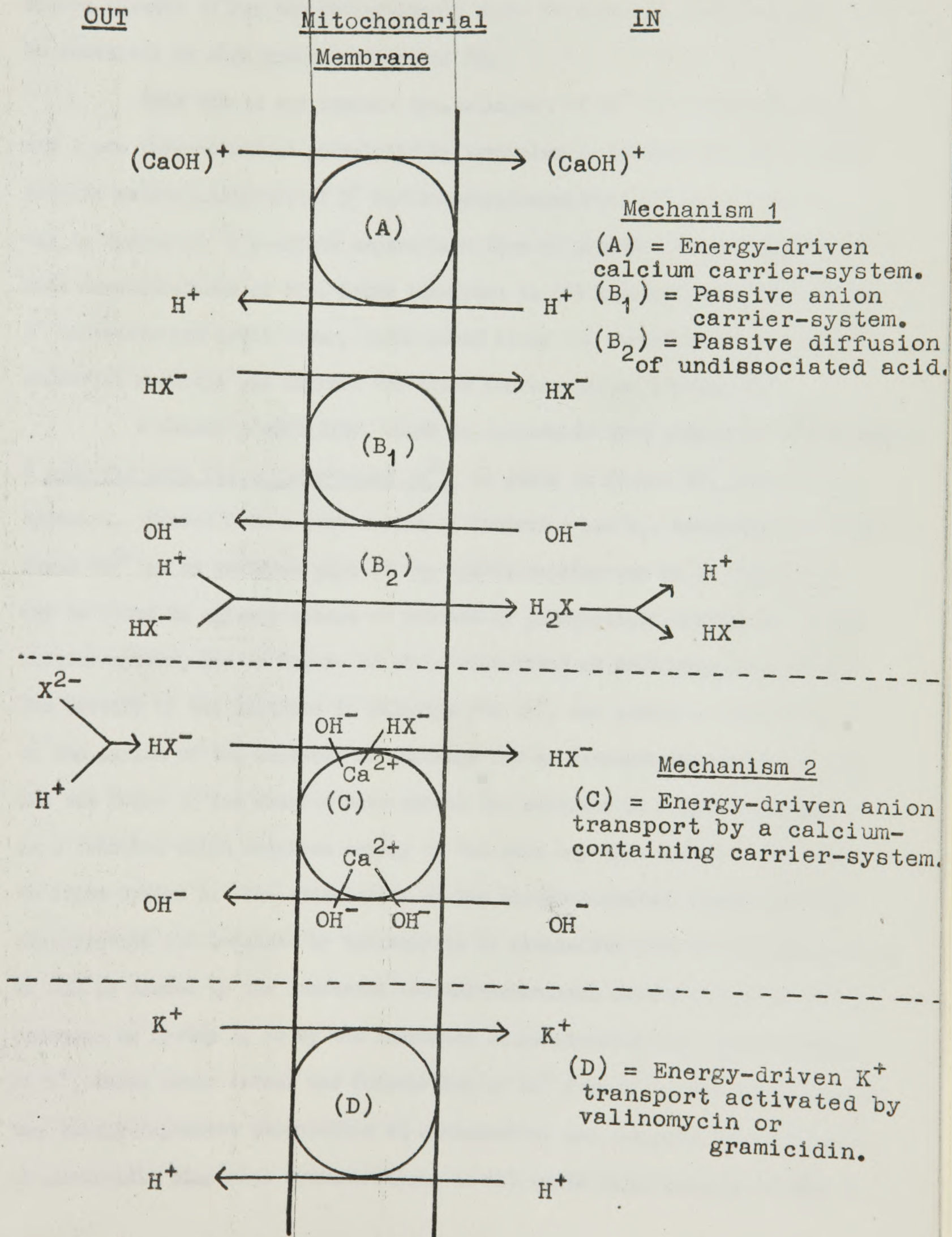
(5) Possible Mechanisms for the Energy-dependent Penetration of Oxaloacetate and L-malate into Mitochondria

Chappell, Cohn & Greville (1963) and Rossi et al. (1966) provided evidence that divalent cations are accumulated by mitochondria in an energy-

dependent process, and that permeant anions subsequently penetrate the mitochondria by a passive process. Chappell & Crofts (1966) proposed that anions enter mitochondria in exchange for OH^- ions, or that the anion HX^- combines with H^+ outside the mitochondria, and enters the mitochondria as the undissociated acid. The mechanism of Chappell & Crofts (1966) for the penetration of permeant anions in the presence of Ca^{2+} is summarised in Fig. 6J, Mechanism 1. This mechanism predicts that one HX^- anion will be transported with each Ca^{2+} cation. The stoichiometry of the energy-requirement for the translocation of Ca^{2+} into liver mitochondria is approximately 2 Ca^{2+} per high-energy bond (Rossi & Lehninger, 1964), and this agrees with the stoichiometry of the energy-requirement for the penetration of oxaloacetate reported in this Chapter. Free Ca^{2+} cannot be involved in the energy-dependent penetration of oxaloacetate and L-malate into mitochondria because the reaction is not inhibited by high concentrations of EGTA which would chelate any Ca^{2+} which was not tightly-bound to the mitochondria. However, if mitochondria were depleted of bound Ca^{2+} by preparation in the presence of 1 mM EGTA, the energy-dependence of the penetration of oxaloacetate was almost completely lost (Table 6E), but could be restored by adding 0.1 mM CaCl_2 to the medium. It is thus postulated that the energy-dependent penetration of oxaloacetate requires bound Ca^{2+} .

Valinomycin or gramicidin plus KCl would be expected to stimulate anion transport in an energy-dependent reaction in the same way as Ca^{2+} (by Systems D, E_1 and E_2 of Figure 6J), and this is true at low concentrations of KCl. At higher concentrations of KCl, valinomycin or gramicidin inhibit the energy-dependent penetration of oxaloacetate and L-malate (Figures 6C, 6D). This is difficult to explain by Mechanism 1 of Figure 6J, and the widely-accepted mode of action of valinomycin and gramicidin (System D, of Figure 6J).

Figure 6J. Possible Mechanisms of Energy-Driven Transport of Anions into Mitochondria.



Competition between the K^+ carrier-system (System D) and the Ca^{2+} carrier-system (System A) for the energy-supply would be expected, and System A would be inhibited at high concentrations of KCl.

This should not inhibit the transport of HX^- if Systems B_1 or B_2 and A are independent as postulated by Mechanism 1, because System D would provide extra-mitochondrial H^+ and intra-mitochondrial OH^- in a similar way to System A. A possible explanation that if system D is very active at high concentrations of KCl, large increases in the intramitochondrial pH and K^+ concentration would occur, which would alter the structure of the mitochondrial membrane and inhibit the anion carrier-system (System B_1).

A second possibility, which is considered more likely as it provides a specific role for tightly-bound Ca^{2+} , is given in Figure 6J, Mechanism 2, System C. System C is a combination of Systems A and B_1 , containing tightly bound Ca^{2+} as an integral part of the carrier-system for HX^- . This Ca^{2+} may be bound to anionic groups of protein or phospholipid components of the carrier-system, and in Figure 6J it is visualised as combining with HX^- on the outside of the membrane in exchange for OH^- , and giving up the HX^- ion on the inside of the membrane in exchange for an intramitochondrial OH^- ion. The two forms of the carrier move across the membrane in a cyclic fashion. in a reaction which requires energy in the same way that energy is required to drive System A. The stimulation of the energy-dependent penetration of oxaloacetate and L-malate by valinomycin or gramicidin plus low concentrations of KCl is caused by the increased intramitochondrial concentration of OH^- produced by System D, or by the increased extramitochondrial concentration of H^+ , which would favour the formulation of HX^- from X^{2-} . The inhibition of the energy-dependent stimulation of oxaloacetate and L-malate by valinomycin or gramicidin plus high concentrations of KCl would occur because of the

competition of Systems C and D for the supply of energy.

Both the above mechanisms could be accommodated by either the chemical or the chemi-osmotic theories of oxidative phosphorylation.

6(v) Summary

- (1) The penetration of oxaloacetate and L-malate into rat liver mitochondria obeyed Michaelis-Menten Kinetics.
- (2) The penetration of both oxaloacetate and L-malate was competitively inhibited by D-malate, succinate, malonate, mesotartrate, maleate and citraconate. The K_i values of each of these inhibitors were similar for the penetration of both oxaloacetate and L-malate.
- (3) Arrhenius plots showed that the activation-energies for the penetration of oxaloacetate and L-malate were respectively +10 and +8 Kcal/mole.
- (4) The rates of penetration of oxaloacetate and L-malate were stimulated by TMPD plus ascorbate under aerobic conditions or by ATP under anaerobic conditions, and the effects of inhibitors of energy-transfer were consistent with the hypothesis that the stimulation of the penetration of oxaloacetate on L-malate is an energy-dependent reaction.
- (5) Calcium-depleted mitochondria did not show the energy-dependent stimulation of the penetration of oxaloacetate, but this could be restored by addition of 0.1 mM CaCl_2 .
- (6) The effects of EGTA were consistent with the hypothesis that the Ca^{2+} involved in the energy-dependent stimulation of the penetration of oxaloacetate and L-malate into mitochondria is tightly-bound to the mitochondria.
- (7) Preliminary experiments showed that the stoichiometry of the energy-requirement for the initial penetration of oxaloacetate was 1.6-1.7 molecules of oxaloacetate per high-energy bond.
- (8) Valinomycin or gramicidin plus low concentrations of KCl stimulated

the energy-dependent penetration of oxaloacetate and L-malate into mitochondria, but penetration was inhibited at higher concentrations of KCl.

(9) It was concluded that oxaloacetate and L-malate penetrate mitochondria by an energy-dependent carrier-system which is probably the same for both anions, and is activated by bound Ca^{2+} .

(10) Possible mechanisms for the penetration of oxaloacetate and L-malate into mitochondria are discussed in relation to the above results.

CHAPTER 7

THE TEMPERATURE-DEPENDENCE OF ENERGY-LINKED REACTIONS IN MITOCHONDRIA AND

SUB-MITOCHONDRIAL PARTICLES

7(1) Introduction

Energy-linked reactions in mitochondria or sub-mitochondrial particles may be driven by ATP under anaerobic conditions, or by respiration even in the presence of oligomycin A, which prevents the formation of ATP. It was postulated that such energy-linked reactions are driven by a common energy source (see Chance, 1963) which can be generated either by respiration, or from ATP by a reversal of the terminal steps of oxidative phosphorylation, and which corresponds to $X \sim I$ or $C \sim I$ of the chemical hypothesis (Figure 1B).

In Chapter 6 it was shown that the penetration of oxaloacetate or L-malate into mitochondria exhibited the properties of an energy-dependent reaction. However, there were differences in the temperature-dependence of the stimulation by TMPD plus ascorbate and that by ATP. (Figure 6F). Linear Arrhenius plots were obtained between 5° and 30° in the absence of an energy-supply or in the presence of TMPD plus ascorbate.

A similar plot was obtained with ATP above 10°, but below 10° there was a sharp break in the plot with large increases in the activation energies for the stimulation of the penetration of L-malate or oxaloacetate.

It was of interest to compare these results with the temperature-dependence of well-established energy-linked reactions driven by respiration or by ATP. The reactions investigated in this Chapter were:-

(1) The energy-linked transhydrogenase in sub-mitochondrial particles from beef heart and rat liver mitochondria, driven by ATP or by succinate oxidation.

(2) The energy-linked reduction of NAD^+ by succinate in sub-mitochondrial particles from beef heart and rat liver mitochondria, driven by ATP.

(3) The stimulation of the respiration of HBHM by ADP (State 4 $\rightarrow$ State 3 transition of Chance & Hollunger (1963)). This can be considered as an energy-linked reaction in which the energy of oxidative phosphorylation is utilized to form ATP.

7(ii) Methods

Preparation of Mitochondria and Sub-mitochondrial Particles

Rat liver mitochondria were prepared by the methods outlined by Hogeboom (1955), and HBHM by the method of Hatefi et al. (1961) as described in Chapter 2. Phosphorylating sub-mitochondrial particles (ETP_H) were prepared from HBHM by the method of Hansen & Smith (1964) except that the sonication medium contained 15 mM MgCl_2 and no MnCl_2 . Phosphorylating sub-mitochondrial particles were prepared from rat liver mitochondria by the method of McMurray, Maley & Lardy (1958).

Assay of the Energy-linked Reduction of NADP^+ by NADH (Energy-linked Transhydrogenase)

The transhydrogenase activity of sub-mitochondrial particles was measured as described by Danielson & Ernster (1963) and Lee, Azzone & Ernster (1964). The formation of NADPH in the presence of an NADH regenerating system was measured spectrophotometrically either at 334 $\text{m}\mu$ using the Eppendorf photometer with Sargent recorder, or at (340-374) $\text{m}\mu$ using the Aminco-Chance dual-wavelength spectrophotometer. The latter apparatus was used to measure slow reaction rates at the lower temperatures. Incubation conditions are given in Figure 7A.

Assay of the ATP-dependent Reduction of NAD^+ by Succinate

The ATP-dependant reduction of NAD^+ by succinate was measured as

described by Low & Vallin (1963). NADH formation was measured at 334 $m\mu$ using the Eppendorf photometer with Sargent recorder, or at (340-374) $m\mu$ using the Aminco-Chance dual-wavelength spectrophotometer. Incubation conditions are given in Figure 7C.

log k
(μ moles/min.
/mg.protein)

Measurement of the Stimulation of the Respiration of HBM by ADP
(State 4 $\rightarrow$ State 3 Transition)

The rates of State 4 and State 3 respiration of HBM were measured in the Clark electrode cell described by Chappell (1964). Incubation conditions are given in Figure 7D.

7(111) Results

Transhydrogenase Activity of Sub-mitochondrial Particles

Figure 7A shows the temperature-dependence of the transhydrogenase activity of rat liver sub-mitochondrial particles and Arrhenius plots of this data. Figure 7B shows similar data for the transhydrogenase activity of beef heart sub-mitochondrial particles.

The Arrhenius plots of the non-energy linked transhydrogenase were linear over the range of temperature measured, and activation-energies were +20 and +15 Kcal/mole for rat liver and beef heart particles respectively. Plots of the energy-linked transhydrogenase driven by ATP or by succinate oxidation were linear above 15°, and gave similar activation-energies to those of the non-energy linked transhydrogenase. Below 10-12° in rat liver particles, and below 13-15° in beef heart particles there were small increases in the activation energies of the succinate-driven reaction, but very large increases in the activation-energies of the ATP-driven reaction.

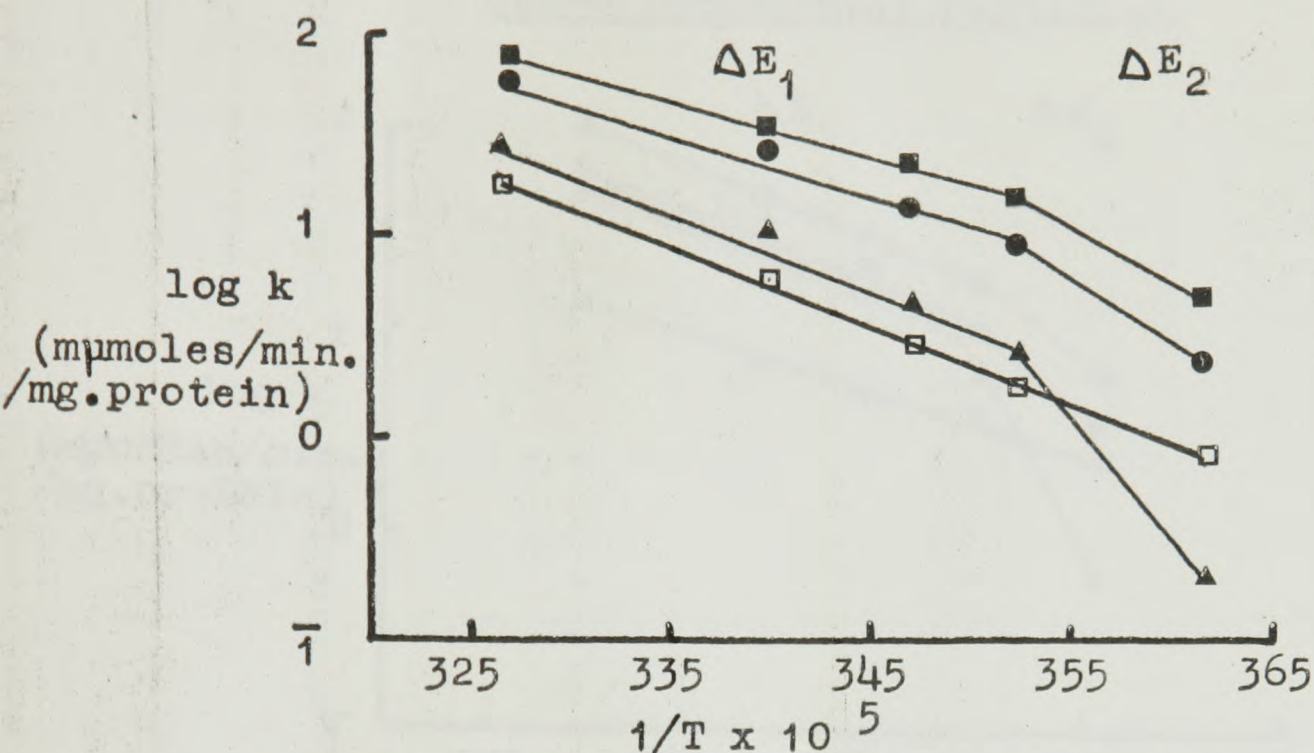
0.20M
dehydro
initiat
protein

The ATP-driven Reduction of NAD⁺ by Succinate in Sub-mitochondrial Particles

initiated by adding
1 μ g. oligomycin.

Figure 7C shows Arrhenius plots of the temperature-dependence of the

Figure 7A. Transhydrogenase Activity of Rat Liver Sub-mitochondrial Particles.



□—□ Non-energy linked;
 $\Delta E = +20$ Kcal/mole.

●—● Succinate driven; $\Delta E_1 = +17$, $\Delta E_2 = +25$ Kcal/mole.

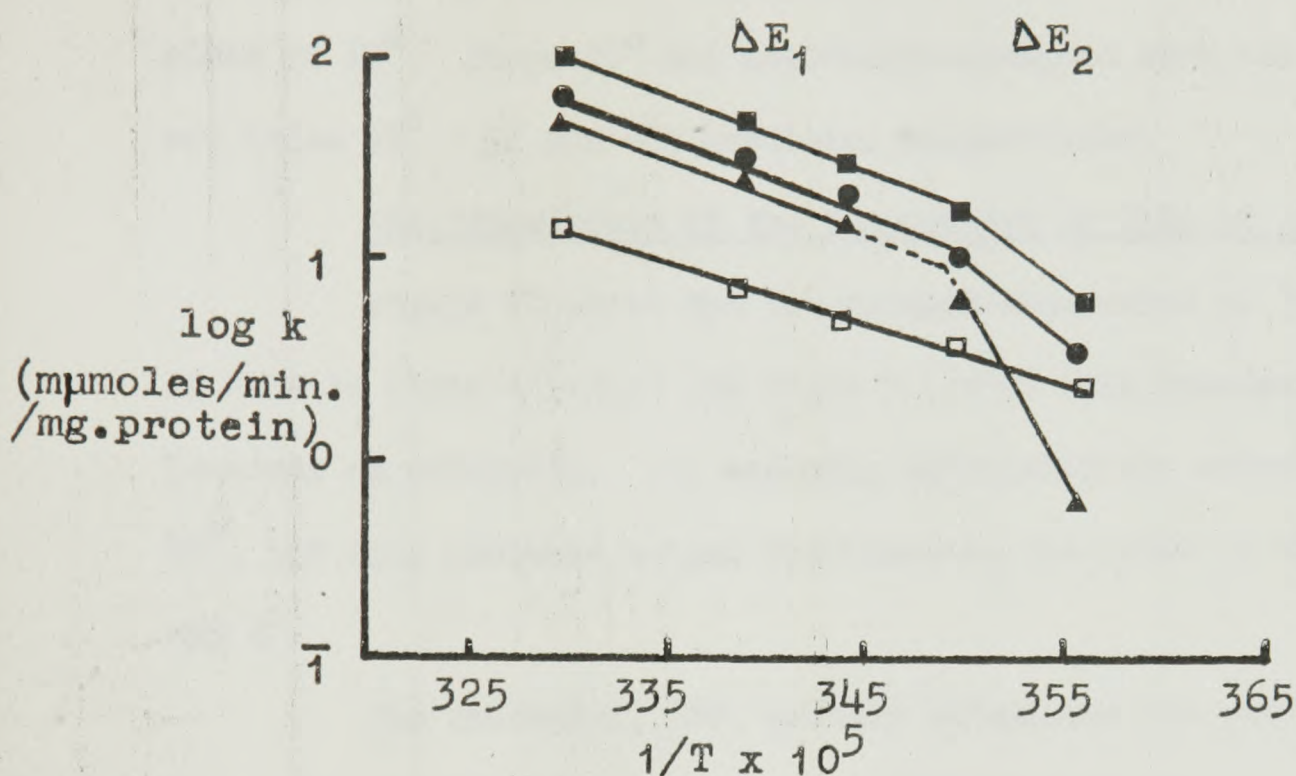
▲—▲ ATP driven; $\Delta E_1 = +17$, $\Delta E_2 = +65$ Kcal/mole.

■—■ Succinate plus oligomycin
 $\Delta E_1 = +16$, $\Delta E_2 = +24$ Kcal/mole.

Temp.°	Transhydrogenase (mpmoles/min./mg. protein).			
	No energy	+ ATP	+Succinate	+ Succinate and Oligomycin A
4	0.7	0.9	3.7	6.1
10	1.9	4.1	11.4	17
15	3.2	10.5	17.5	29
21	6.5	17.5	41	47
32	20.5	43	99	102

Transhydrogenase activity was measured at 334 or (340-374) mμ as described in the text. Incubations contained 0.25M sucrose; 50mM tris-HCl, pH 7.6; 6mM MgCl₂; 0.33mM NADP⁺; 0.5mpmoles rotenone; and an NADH regenerating system :- 0.03mM NAD⁺; 0.28M ethanol; and 0.1-0.4 mg. Sigma salt-free yeast alcohol dehydrogenase. The non-energy linked transhydrogenase was initiated by adding sub-mitochondrial particles (0.1-1.0 mg. protein). The energy linked transhydrogenase reaction was initiated by adding 2.8mM ATP or 3.3mM disodium succinate + 1μg. oligomycinA.

Figure 7B. The Transhydrogenase Activity of Beef Heart Sub-mitochondrial Particles.



- Non-energy linked; $\Delta E = +15$ Kcal/mole.
- ▲—▲ + ATP; $\Delta E_1 = +14$, $\Delta E_2 = +73$ Kcal/mole.
- + Succinate; $\Delta E_1 = +15$, $\Delta E_2 = +32$ Kcal/mole.
- + Succinate and Oligomycin A ; $\Delta E_1 = +15$, $\Delta E_2 = +33$ Kcal/mole.

<u>Temp^o.</u>	<u>Transhydrogenase (mpmoles/min./mg. protein).</u>			
	<u>No energy</u>	<u>+ ATP</u>	<u>+ Succinate</u>	<u>+ Succinate and Oligomycin A.</u>
8	2.4	3.0	5.6	8.8
13	3.4	9.7	14.3	24
18	6.2	27	28	36
23	7.8	37	39	53
30	15	75	94	125

Transhydrogenase activity was assayed as described under Figure 7A.

ATP-driven reduction of NAD^+ by succinate in rat liver particles and beef-heart particles. In both experiments there was a sharp break in the Arrhenius plots at 17° . Above 17° the activation-energies were +24 and +22 Kcal/mole, and below 17° , +51 and +59 particles respectively.

The Stimulation of the Respiration of HBHM by ADP

Figure 7D shows the temperature-dependence of the rates of respiration of HBHM in State 4 (-ADP) and State 3 (+ADP) with succinate or pyruvate plus L-malate as substrate. ADP markedly stimulated the respiration of HBHM above 20° , but only produced slight increases in the rates of respiration at 10.5° and 4° .

The uncoupler, DNP, greatly stimulated the oxidation of pyruvate plus L-malate at all temperatures. Oxidation of succinate in the presence of DNP was non-linear, and rates are not given in Figure 7D. DNP caused an initial stimulation of succinate oxidation followed by a progressive inhibition. This was presumably because oxaloacetate accumulated in the absence of a system for its removal and caused a progressive inhibition of succinate dehydrogenase (see Chapter 3).

Arrhenius plots of these data showed a sharp break at 11° . Above 11° the activation energies were +21 and +23 Kcal/mole, and below 11° , +64 and +63 Kcal/mole for the oxidation of succinate and pyruvate plus L-malate respectively.

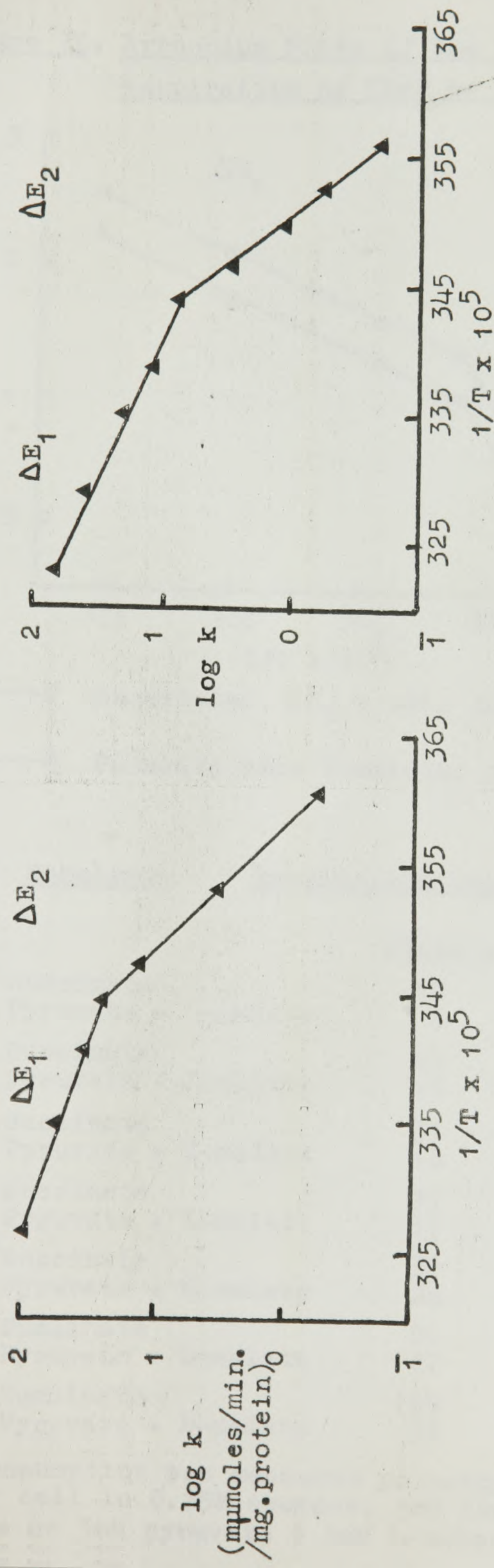
7(iv) Discussion

Table 7A summarises the activation-energies obtained from Arrhenius plots of the temperature-dependence of the reactions studied in this Chapter, and the results obtained in Chapter 6 for the temperature-dependence of the penetration of oxaloacetate and L-malate into rat liver mitochondria.

Differences between the Temperature-dependence of ATP- and Respiration- driven Energy-linked Reactions

The Arrhenius plots of reactions driven by the energy of respiration

Figure 7C. Arrhenius Plots of the ATP-driven Reduction of NAD^+ by Succinate.



(a) Rat liver sub-mitochondrial particles:

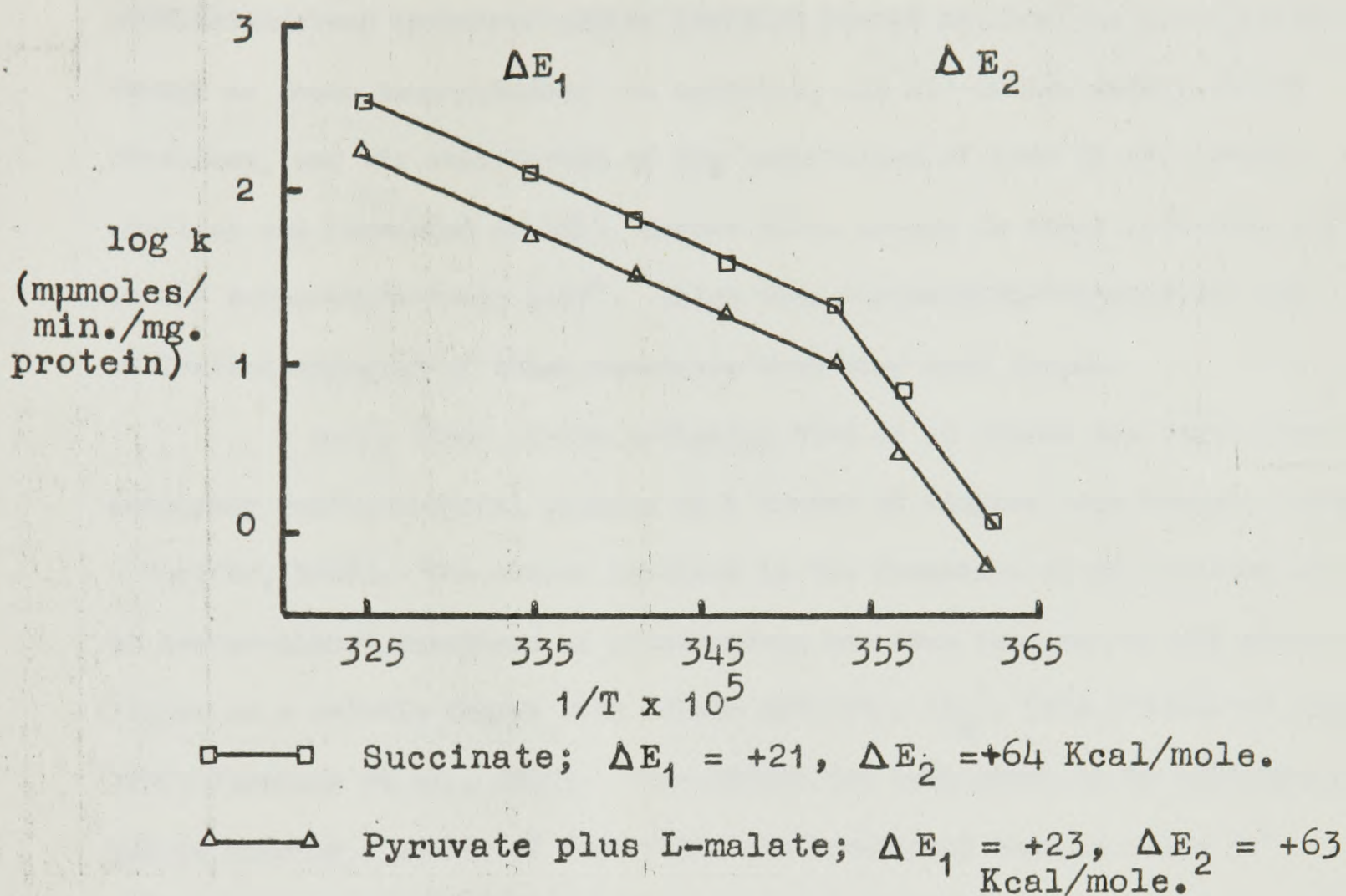
$\Delta E_1 = +24$, $\Delta E_2 = +51$ Kcal/mole,
Transition temperature = 17° .

(b) Beef heart sub-mitochondrial particles:

$\Delta E_1 = +22$, $\Delta E_2 = +59$ Kcal/mole,
Transition temperature = 17° .

NADH formation was measured at 334 or (340-374) mμ as described in the text. Incubations contained :- 0.25M sucrose; 50mM tris-HCl, pH 7.6; 6mM MgCl_2 ; 4mM disodium succinate; 2mM NAD^+ ; 1mM KCN; and sub-mitochondrial particles (0.1-0.4 mg. protein). The reaction was initiated by addition of 2.8mM ATP.

Figure 7D. Arrhenius Plots of the Stimulation of the Respiration of HBHM by ADP.



Temp.	Substrate	Respiration (μgatoms oxygen/min./mg. protein).		
		State 4	State 3	+0.1mM DNP
4	Succinate	10	11	-
	Pyruvate + L-malate	6	6.6	15
7.5	Succinate	25	32	-
	Pyruvate + L-malate	10	12.5	25
10.5	Succinate	31	50	-
	Pyruvate + L-malate	14	24	35
15	Succinate	37	76	-
	Pyruvate + L-malate	17	35	62
20	Succinate	38	107	-
	Pyruvate + L-malate	24	64	73
27	Succinate	81	250	-
	Pyruvate + L-malate	27	107	107
35	Succinate	159	497	-
	Pyruvate + L-malate	32	232	230

Oxygen consumption was measured polarographically in the Clark electrode cell in 0.25M sucrose; 5mM KPO_4 ; 5mM $MgCl_2$; 3mM succinate or 3mM pyruvate + 3mM L-malate, HBHM (0.5-4 mg.protein)

did not show marked breaks in the range 4° - 30° . In this category only the succinate-driven transhydrogenase reaction showed an increase in activation-energy at lower temperatures. In contrast, all ATP-driven energy-linked reactions, and the stimulation of the respiration of BBHM by ADP (which involves the formation of ATP), showed sharp breaks in their Arrhenius plots in the temperature-range 8 - 17° . Below this 'transition-temperature' the activation-energies of these reactions were very much larger.

A sharp break in the Arrhenius plot of an enzyme has been shown to accompany configurational changes in a number of enzymes (see Massey, Curti & Gaather, 1966). The enzyme involved in the formation or utilisation of ATP in energy-linked reactions of mitochondria has been isolated by the group of Backer as a soluble enzyme with ATPase activity, (F_1), (see Pullman et al., 1960; Penefsky et al., 1960). This ATPase has been shown to be cold-labile and to undergo a number of reversible conformational changes below 20° (Penefsky & Warner, 1965). It is postulated that the sharp breaks in the Arrhenius plots of ATP-driven energy-linked reactions and of the stimulation of respiration by ADP, result from conformational changes in the mitochondrial enzyme(s) which catalyse the terminal step(s) of oxidative phosphorylation.

Comparison of the Penetration of Oxaloacetate and L-malate into Mitochondria with Well-established Energy-linked Reactions

The temperature-dependence of the stimulation of oxaloacetate and L-malate penetration into liver mitochondria show the following similarities to the temperature-dependence of well-established energy-linked reactions.

- (1) Respiration-driven penetration does not show a break in the Arrhenius plot.
- (2) Arrhenius plots of ATP-driven penetration show sharp breaks at 10° for oxaloacetate and at 8° for L-malate.

Table 7A. Data Obtained from Arrhenius Plots of Energy-linked Reactions in Mitochondria.

<u>Reaction</u>	<u>Mitochondria</u>	$\frac{\Delta E_1}{+ (\text{Kcal}^1/\text{mole})} \frac{\Delta E_2}{2}$		<u>Transition Temp.</u>
<u>Transhydrogenase</u>				
(a) No energy	Liver	20	-	-
(b) + ATP		17	65	10
(c) + Succinate		17	25	10
(d) + Succinate and oligomycin A		16	24	10
(a) No energy	Heart	15	-	-
(b) + ATP		14	73	15
(c) + Succinate		15	32	14
(d) + Succinate and oligomycin A		15	33	13
<u>ATP driven reduction of NAD⁺ by succinate.</u>				
	Liver	24	51	17
	Heart	22	59	17
<u>Stimulation of Respiration by ADP.</u>				
(a) Succinate	Heart	21	64	11
(b) Pyruvate + L-malate		23	63	11
<u>Oxaloacetate Penetration.</u>				
(a) No energy	Liver	10	-	-
(b) + ATP		10	48	15
(c) TMPD + ascorbate		10	-	-
<u>L-malate Penetration.</u>				
(a) No energy	Liver	8	-	-
(b) + ATP		8	42	12
(c) TMPD + ascorbate		8	-	-

(3) The activation energy of the ATP-driven penetration is greatly increased at lower temperatures.

(4) The activation-energies of the penetration of oxaloacetate and L-malate are of the same order of magnitude as those of well-established energy-linked reactions.

It was concluded that the above evidence is additional confirmation that the penetration of oxaloacetate and L-malate into mitochondria is an energy-dependent reaction.

The Mechanism of the Non Energy-linked Transhydrogenase

The activation energies of the non energy-linked transhydrogenase and of the energy-linked transhydrogenase driven by ATP or succinate oxidation, are identical above 15°. This suggests that the two reactions are catalysed by the same enzyme, and this hypothesis is supported by the identical stereospecificity of the transhydrogenase reaction under both sets of conditions (Griffiths & Robertson, 1966).

The Mechanism of the Non Energy-linked Penetration of Oxaloacetate and L-malate into Mitochondria

The activation-energies of the penetration of oxaloacetate and L-malate into mitochondria above 12° are similar both in the absence or presence of an energy-supply. The K_i values of dicarboxylic acid anions which inhibit the penetration of oxaloacetate and L-malate into mitochondria are also identical both in the absence or presence of energy-supply (Figure 6E). These results suggest that, although the rates of penetration of oxaloacetate and L-malate are greatly increased by a supply of energy, oxaloacetate and L-malate still enter mitochondria by the same carrier-system.

As the activation-energies of the transhydrogenase reaction, and of the penetration of oxaloacetate and L-malate into liver mitochondria are

unaffected by the supply of energy, these activation-energies are not associated with that part of the reaction-mechanism which involves energy-transfer.

7(v) Summary

(1) The temperature-dependence of the following energy-linked reactions were investigated:-

(a) The energy-linked transhydrogenase in sub-mitochondrial particles from rat liver and beef heart.

(b) The ATP-driven reduction of NAD^+ by succinate in sub-mitochondrial particles from rat liver and beef heart.

(c) The stimulation of the respiration of BBHM by ADP.

(d) The stimulation of the penetration of oxaloacetate and L-malate into rat liver mitochondria by TMPD plus ascorbate or ATP.

(2) Arrhenius plots of the temperature-dependence of all these reactions were similar in form. Plots of respiration-driven reactions did not show a marked break, but plots of ATP-driven reactions showed a sharp break in the temperature-range $8-17^\circ$. Below this 'transition temperature' the activation-energies of the reactions were greatly increased.

(3) The above evidence helps to establish the penetration of oxaloacetate and L-malate into mitochondria as a typical energy-linked reaction.

(4) It is postulated that the sharp break in the Arrhenius plots of ATP-driven reactions reflect a configurational change in the enzyme(s) which catalyse the reversible formation of ATP in the terminal step of oxidative phosphorylation.

APPENDIX 1 - ASSAY OF $^{32}\text{P}_i$ -ATP EXCHANGE

Walters & Cooper (1965) showed that relatively low concentrations of adenine nucleotides, NAD^+ , NADH or reducing agents can decrease the efficiency of the extraction of P_i by the isobutanol-benzene extraction method of Berenblum & Chain (1938).

A more satisfactory separation of inorganic and organic phosphate is obtained when hexanol is used as the organic extracting agent for phosphomolybdic acid (Hagihara & Lardy, 1960), and a modification of this procedure was used in the assays of $^{32}\text{P}_i$ -ATP exchange used in Chapter 4.

The incubations were in 1.00 ml., and were stopped by addition of 2.0 ml. of reagent A (0.9 M HClO_4 , 0.6 M Na_2SO_4). P_i was converted to the phosphomolybdate complex by shaking with 3.0 ml. of reagent B (a mixture of 0.5 volume of 4% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$; 0.4 volume methanol; 0.012 volume hexanol; and water to 1.00-volume). One drop of bromine per 100 ml. was added to reagent B immediately prior to use. This destroys any reducing agents present in the incubation mixture e.g. NADH, which decrease the efficiency of extraction of P_i by causing the formation of the blue reduced phosphomolybdate complex, which is more water-soluble.

This mixture was then extracted five times with an equal volume of hexanol. To prevent volume changes occurring between aqueous and organic phases, the hexanol was first equilibrated with a mixture similar in composition to the incubation mixture plus reagents A and B. Hexanol was shaken with an equal volume of 30% aqueous methanol, and after separation of the two layers, the upper layer was shaken again with two volumes of a mixture containing 1 part water, 2 parts of reagent A, and 3 parts of reagent B.

It was found that NAD^+ and NADH ($> 2 \text{ mM}$ in the initial incubation)

APPENDIX 2THE TRANSFER OF REDUCING-POWER BETWEEN MITOCHONDRIA AND CYTOPLASM

Intact mitochondria are virtually impermeable to the four adenine nicotinamide nucleotides (Lehninger, 1951; Lehninger et.al., 1960). However, there are physiological situations in which NADH is generated in, or removed from the cytoplasm e.g. in gluconeogenesis from pyruvate in liver or kidney, NADH must be rapidly generated in the cytoplasm; and in respiring heart muscle, NADH must be rapidly transported from the cytoplasm to the mitochondria.

L-malate has been considered as a carrier for reducing equivalents between mitochondria and cytoplasm (Krebs, 1967; Krebs, Gascoyne & Motton, 1967). A flow of reducing equivalents across the mitochondrial membrane could be mediated if the mitochondrial membrane were freely permeable to both L-malate and oxaloacetate. Lardy, Paetkau & Walter (1965) have presented evidence that the rate of diffusion of oxaloacetate out of mitochondria is slow and requires special transport mechanisms. Chappell (1967) has proposed a mechanism for the transfer of reducing power between mitochondria and cytoplasm based on the proposal by Borst (1963), and Lardy, Paetkau & Walter (1965) that oxaloacetate is first transaminated to aspartate which can then enter or leave the mitochondrion. This mechanism is summarised in Figure 5F. The flow of reducing equivalents is maintained by L-malate and glutamate moving in one direction across the mitochondrial membrane, and α oxo-glutarate and aspartate moving in the opposite direction, and requires intra- and extra-mitochondrial malate dehydrogenases and glutamate-oxaloacetate transaminases. Chappell (1967) further proposes that there are specific carrier-systems in the mitochondrial membrane for L-malate, glutamate, α oxo-glutarate and aspartate.

The data of Chapters 5 and 6 show that the penetration of oxaloacetate

into isolated liver, kidney and heart mitochondrial is rapid at physiological concentrations of oxaloacetate ($3-10 \mu M$). The rate of penetration of oxaloacetate was unaffected by concentrations of KCN which completely inhibited glutamate-oxaloacetate transaminase, thus eliminating the possibility that oxaloacetate was transaminated to aspartate before entering the mitochondria. The oxidation of intramitochondrial NAD(P)H by aspartate was also very slow, and it was concluded that under the experimental conditions investigated, oxaloacetate entered the mitochondria intact. Evidence was also presented in Chapter 6 which suggested that oxaloacetate entered liver mitochondria by a carrier-system similar to that postulated by Chappell & Haarhoff (1967) for L-malate.

The rates of penetration of physiological concentrations of oxaloacetate and L-malate into isolated mitochondria were sufficiently high to support the hypothesis that they can mediate the transfer of reducing power between mitochondria and cytoplasm. The rates of penetration of $10 \mu M$ oxaloacetate into liver, kidney and heart mitochondria at 30° were respectively 4, 12 and 27 $\mu moles/min./mg.$ protein and the rates of penetration of 1 mM L-malate were respectively 2, 10 and 5 $\mu moles/min./mg.$ protein. Krebs and co-workers (see Krebs, 1967) have shown that gluconeogenesis in kidney at 40° requires the generation of 1000 $\mu moles$ NADH/hour/g. dry weight in the cytoplasm. If it is assumed that 1 g. dry weight of kidney contains 250 mg. mitochondrial protein, the corresponding rates of penetration of $10 \mu M$ oxaloacetate and 1 mM L-malate are respectively 180 and 150 $\mu moles/hour/g.$ dry weight at 30° , and are of the same order of magnitude. It is possible that under physiological conditions, the mechanism outlined in Figure 5F may also operate, but this mechanism is unnecessary if oxaloacetate itself can pass through the mitochondrial membrane at high rates.

The transport of oxaloacetate across the mitochondrial membrane cannot be rapid in both directions or $\text{NAD}^+:\text{NADH}$ ratios in the mitochondrial and cytoplasmic compartments would be equilibrated. This does not occur in vivo, and the ratio of $\text{NAD}^+:\text{NADH}$ is always much higher in the cytoplasmic compartment than in the intramitochondrial space.

A possible primitive control mechanism which could regulate the rate of transfer of reducing-power into mitochondria would be competition between oxaloacetate and L-malate (and possibly other dicarboxylic acid anions) for the same carrier-system, for which evidence is described in Chapter 6.

Another factor which could control the penetration of L-malate and oxaloacetate into mitochondria is the competition between the many energy-linked reactions of mitochondria for the supply of energy. It was shown in Chapter 6 that the rate of penetration of oxaloacetate and L-malate into mitochondria is energy-dependent, although it is not yet possible to decide whether the stoichiometry of the energy-requirement is fixed.

A full assessment of the hypothesis that L-malate and oxaloacetate transfer reducing-power between mitochondria and cytoplasm, and of the way in which the direction of the flow of this reducing-power is regulated between two compartments with widely different $\text{NAD}:\text{NADH}$ ratios, requires the measurement of the rates of extrusion of L-malate and oxaloacetate from mitochondria. However, unambiguous methods of measuring these rates have not yet been devised. It would be necessary to devise methods of generating L-malate and oxaloacetate within the mitochondria, and then measure the rates at which these anions appear in the extramitochondrial space. Possible systems for investigating this are:-

(1) Intramitochondrial oxaloacetate could be generated from added α -oxoglutarate

plus aspartate by the action of intramitochondrial glutamate-oxaloacetate transaminase, and oxaloacetate leaving the mitochondria then titrated against NADH in the presence of malate dehydrogenase. However, this system is complicated by the fact that mitochondria are always contaminated with extramitochondrial glutamate-oxaloacetate transaminase which generates oxaloacetate without the need for the substrates to cross the mitochondrial membrane.

(2) Intramitochondrial L-malate could be generated by the oxidation of added succinate. However, because of the unfavourable equilibrium constant of the malate dehydrogenase reaction there is no satisfactory direct method of measuring the appearance of L-malate outside the mitochondria, and the extrusion of L-malate would have to be measured after the separation of the mitochondria from the incubation medium. This could be done by centrifugation or filtration through a Millipore filter, but these methods may result in release of intramitochondrial L-malate, due to anaerobiosis or mechanical damage of the mitochondria.

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