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OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM
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Studies with whole mitochondria or submitochondrial particles indicated the presence of a soluble nonphosphorylated high energy intermediate of oxidative phosphorylation. The formation of this intermediate was dependent on the operation of the respiratory chain and some disruption of the mitochondrion released the intermediate to the incubating medium.

The ability to form $AT^{32}P$ in the presence of $^{32}P_i$ and ADP or glucose-6-phosphate ^{32}P (G-6- ^{32}P) when a hexokinase trap was used has been used to detect the existence and amount of the high energy intermediate as well as the activity of the transfer reaction in the supernatant fraction. The high energy intermediate released to the supernatant fraction was proteinaceous, resistant to dialysis and survived ammonium sulfate fractionation and acetone treatment.

Catalytic amounts of succinate and CoA added to the supernatant fraction produced a marked stimulation in the formation of ATP (or G-6-P). Other dicarboxylic acids would not substitute for succinate; neither would cysteine substitute for CoA. PCMB completely inhibited the transfer reaction, however, CoA could overcome this effect. Transfer activity was lost during ammonium sulfate fractionation or acetone treatment but succinate and CoA would restore the original level of activity. It was concluded from these observations that succinate and CoA were required for energy transfer activity.

Succinic thiokinase activity was measured by observing $^{32}\text{P}_i$ -ATP exchange in the presence of succinate and CoA. Attempts to separate exchange activity from the transfer activity were unsuccessful yet the difference in response of these two reactions to inhibitors indicated that the two processes were distinct. It was concluded that in the presence of hexokinase the stimulation of transfer activity by succinate and CoA was not due to thiokinase mediated activity.

The succinate-CoA stimulated transfer reaction showed the following characteristics: (i) GDP cannot substitute for ADP (ii) The rate of transfer reaction was dependent on the concentrations of Mg^{++} and P_i (iii) Transfer activity was not inhibited by DNP,

while oligomycin was slightly inhibitory as was hydroxylamine. Arsenate was the most active inhibitor observed.

The high energy nonphosphorylated intermediate has been shown to energize the energy-dependent reduction of TPN^+ by DPNH coupled to the reduction of DPN^+ by succinate. The reaction required soluble fraction, sub-particles, succinate, DPN^+ , TPN^+ . ATP showed little effect. Arsenate inhibited the reaction probably due to the uncoupling of some of the intermediate. The further competitive effect for high energy intermediate has been shown by the addition of P_i and ADP resulting a low reaction rate in energy-dependent reaction.

On the basis of experimental results obtained in this study schematic representation of the energy transfer reaction sequence and the sites of inhibitor action was proposed. It was suggested that the primary nonphosphorylated high energy intermediate $\text{acyl} \sim \text{X}$ was present in the soluble fraction. Addition of CoA would lead to the formation of a secondary high energy intermediate of the enzyme bound $\text{acyl} \sim \text{S}$ type. Succinate probably functioned in promoting of $\text{Acy} \sim \text{S}$ formation.

EVIDENCE FOR A SOLUBLE HIGH ENERGY INTERMEDIATE
OF OXIDATIVE PHOSPHORYLATION IN
MITOCHONDRIA FROM BLOWFLIES

by

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Typed by Ruth Baines

To My Wife

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EVIDENCE FOR A SOLUBLE HIGH ENERGY INTERMEDIATE OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM BLOWFLIES

INTRODUCTION

The process of oxidative phosphorylation occupies a central position in the energy balance of the cell. Energy produced in the oxidation of Krebs cycle intermediates is conserved by the concurrent formation of adenosine triphosphate (ATP)¹, the most important carrier of metabolically available energy in the cell. Electrons produced in the initial oxidative process are transferred to oxygen through a sequence of electron acceptors of decreasing oxidation potential.

¹ The following abbreviations are used in this thesis: ATP, adenosine-5'-triphosphate; ADP, adenosine-5' -diphosphate; AMP, adenosine-5'-monophosphate; P_i , inorganic orthophosphate; $^{32}P_i$, radioactive phosphate-32; P_i - $^{32}P_i$, $^{32}P_i$ in carrier P_i ; ^{32}P , unspecified form of radioactive phosphorus; P/O ratio, esterified P_i per atom of oxygen consumed during oxidative phosphorylation; $AT^{32}P$, ATP terminally labeled with ^{32}P ; AT Pase, adenosine triphosphatase; GDP, guanosine-5'-diphosphate; GTP guanosine-5'-triphosphate; DPN^+ , $DPNH$, diphosphopyridine nucleotide and its reduced form; TPN^+ and $TPNH$ triphosphopyridine nucleotide and its reduced form CoA or CoASH. coenzyme A; CoQ, coenzyme Q; DNP, 2, 4-dinitrophenol; PCMB, p-chloromercuribenzoic acid; EDTA, ethylene diaminetetraacetic acid; cyt., cytochrome; BSA bovine serum albumin; BAL, 2, 3-dimercaptopropanol; FP_s , succinate-linked flavoprotein; FP_d , $DPNH$ -linked flavoprotein; TMPD, NNN'-tetramethyl-p-phenylenediamine; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl) aminomethane; N. E., non-extractable; A. B., successive respiratory carriers; X, I, unknown components of an intermediate of oxidative phosphorylation; cpm, counts per minute; expt., experiment; +, present; -, absent.

The identity of these acceptors and the sequence in which they react is reasonably well defined. The formation of ATP from ADP and P_i is tightly coupled to this electron transport process, but the mechanism by which this coupling is effected is not understood. It is this phase of oxidative phosphorylation which has been the concern of this thesis.

In attacking this rather complex system, research workers have investigated partial reactions of the energy transfer process such as ATP- P_i exchange, ADP-ATP exchange, P_i - H_2O exchange and ATP- H_2O exchange. These studies have led to the postulating of a sequence of reactions involving "high energy" intermediates which culminate with the formation of ATP. Further information concerning the role of these high energy intermediates has been derived from studies of the relation of oxidative phosphorylation to other energy dependent processes of the mitochondrion. Attempts have been made to resolve this energy transfer system into its catalytic components with some limited success, while in other laboratories some effort has been made to isolate the high energy intermediates involved. In all these studies the use of inhibitors has played an important part.

This research has been conducted with mitochondria from the flight muscle of blowflies. Mitochondria derived from this source

are quite large (5 microns or greater in diameter) and oxidize substrate and supply ATP to the muscle fibrils at the high rate needed to maintain the very high energy output of sustained flight. In fact it has been reported that flight muscle exhibits the most highly developed aerobic mechanism for linking carbohydrate catabolism to ATP production (46, p. 50). These qualities are quite significant in consideration of system in which to study oxidative phosphorylation. It should also be noted that the components of the respiratory chain have also been studied in these mitochondria (21, 108). Except for the replacement of cytochrome c_1 by cytochrome 551 little difference from the mammalian system was observed.

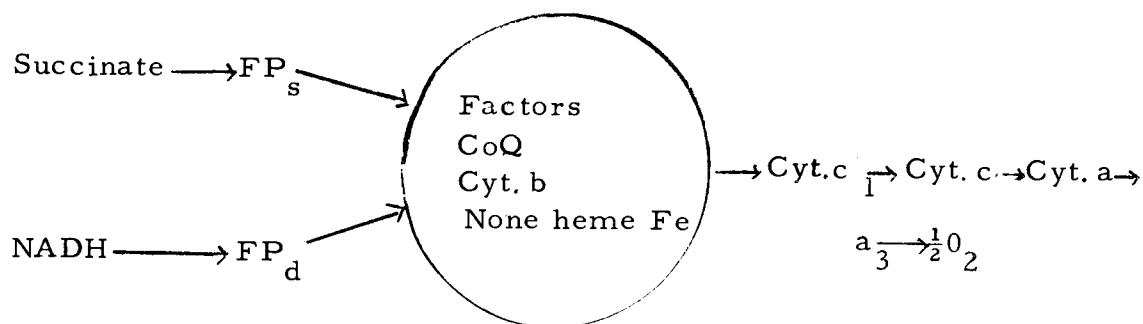
Extensive studies in this laboratory have defined optimum conditions for the preparation of mitochondria from this source. These preparations show good respiratory control and high levels of activity in oxidative phosphorylation. Studies by Meegunwan (89) with these systems had indicated the presence of possibly one or two intermediates of oxidative phosphorylation. The aim of this research program was to extend these observations, defining the nature of the intermediates and the manner in which they are involved in the process of oxidative phosphorylation.

REVIEW OF LITERATURE

General Considerations

The mechanism by which free energy is transferred during mitochondrial oxidative phosphorylation remains poorly understood. Energy is required to produce ATP from ADP and P_i and in the mitochondria this energy is derived primarily from the oxidation of nutrients degraded and channeled into the Krebs cycle. The oxidation of a Krebs cycle intermediate releases electrons which are accepted eventually by oxygen after passage through the respiratory chain. In intact mitochondria, the exogenic electron transport reaction and endogenic oxidative phosphorylation are tightly coupled, that is, in the absence of ADP and P_i little oxygen is consumed even though substrate and oxygen are present in excess (22).

The enzymes of the respiratory chain are proteins which contain three principal coenzymes or prosthetic groups which function as the electron carriers; pyridine nucleotides, flavin nucleotides and iron porphyrins. Although the status of CoQ as a component of this chain has not been firmly established, the logical arrangement of the component enzymes of the respiratory chain can be derived from the relative redox potential of their prosthetic groups as shown in scheme I (66).



Scheme I: The respiratory electron transport chain

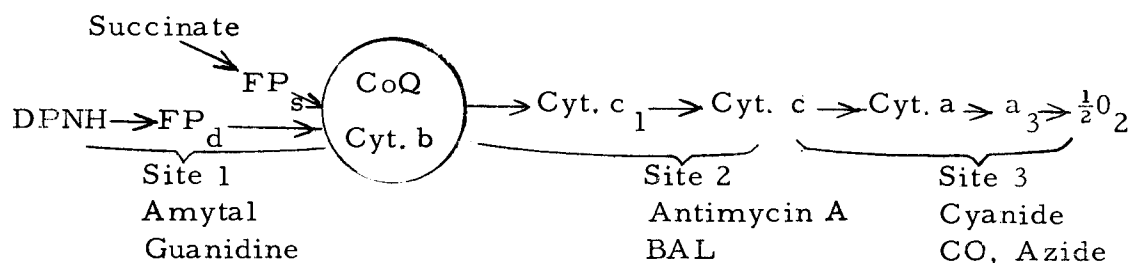
The status of the components in the circle with respect to their sequence or even their qualifications as electron carriers is uncertain at the present time.

Reduced nicotinamide-adenine dinucleotide (DPNH), formed in the oxidation of Krebs cycle intermediates, or succinate can reduce flavoprotein (FP_s or FP_d). The reduced flavoprotein may be reoxidized by the Fe^{+++} of cytochrome. Each cytochrome in its oxidized form (Fe^{+++}) can accept an electron and become reduced to the Fe^{++} form. This Fe^{++} in turn can donate its electron to the next carrier and so on. The cytochromes differ somewhat chemically and in molecular weight. Only the last one, called cytochrome oxidase, can give up its electron to molecular oxygen directly. The resulting $O^{\cdot -}$ ion is not stable and immediately picks up two H^+ ions to form H_2O (81).

If the oxidative process were effected by direct transfer of electrons from the Krebs cycle intermediate to oxygen without the

intervention of the respiratory chain the free energy change would be very large due to the difference in redox potential of the two reactants. The mediation of the respiratory chain enables the cell to utilize this energy by accomplishing the same overall process through a series of steps, such that the free energy changes are comparable to those required for the synthesis of ATP. The electron transport process and phosphorylation are tightly coupled and the efficiency of the overall process is given by the P/O ratio which denotes the amount of inorganic phosphate esterified during the passage of two electrons through the respiratory chain.

It has been established that three moles of ATP are formed for each pair of electrons passed from DPNH to oxygen and two moles ATP for succinate oxidation (102, P. 84-85). Recent publications (53, 118) have reported data which suggest higher coupling efficiency. Brierley (10) and Lenaz and Beyer (84) have attributed this apparent increased efficiency to experimental factors such as short incubation times and small levels of oxygen uptake. Furthermore, the demonstration by spectrophotometric techniques of three "cross-over points" in the electron transport sequence (22) substantiates the hypothesis that there are three sites of energy conservation in oxidative phosphorylation (Scheme II); one mole of ATP is formed at each site with the transfer of each pair of electrons (102, P. 14).



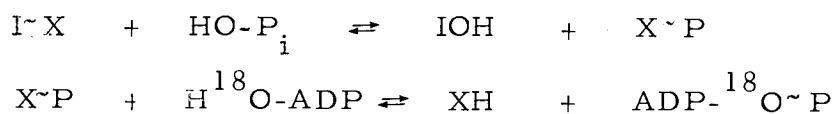
Scheme II. Location of Sites of Phosphorylation and Inhibitors in the Respiratory Chain

The study of the mechanism of mitochondrial oxidative phosphorylation has been hindered by a serious gap in our knowledge of the high energy intermediates. The low concentration and highly labile character of high energy intermediates pose a problem in their isolation, although the existence of high-energy intermediates has been deduced from a large body of experimental evidence in the past (40). The experimental difficulties are characteristic of the study of a complex multienzyme particulate system.

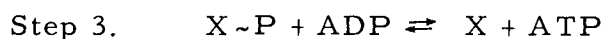
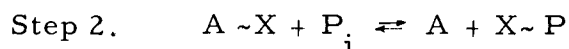
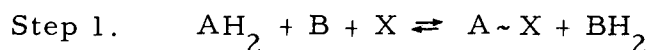
Our knowledge of the mechanism of oxidative phosphorylation stems from the study of several exchange reactions and inhibitor effects. In mitochondrial and submitochondrial particles, the $^{32}P_i$ - ATP exchange represents one step in the high energy phosphate transfer process. This conclusion is based on numerous similarities in response of both the exchange and the overall reaction to uncouplers and inhibitors (8, 30). In addition several soluble protein

factors required for oxidative phosphorylation are also needed for the exchange reaction (102, P. 117). Such studies have demonstrated the reversibility of coupled ATP synthesis and suggest the existence of some high energy phosphate compounds, $X\sim P$, as an intermediate.

Early in 1953, Slater (115) suggested that during the operation of respiratory chain, high energy intermediates were formed before the intervention of inorganic phosphate. This concept that phosphate is not involved in the primary oxidation reaction is supported by the work of Borst and Slater (4) and also by Ernster (38) and Snoswell (121). Experiments with ^{18}O by Boyer (5) have shown that the terminal bridge oxygen in ATP is derived from ADP. This could be explained by the following where I & X represent some unknown entities:

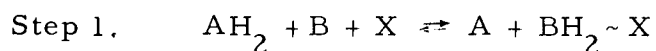


Cooper and Kulda (20) have demonstrated that ADP-ATP exchange is considerably faster than the P_i -ATP exchange. This also indicates that the terminal reaction of oxidative phosphorylation is the reaction of ADP with a high energy phosphate donor to form ATP. From this evidence, the overall process of oxidative phosphorylation may be visualized as involving the sequence of reactions formulated in Scheme III (102, P. 15).



Scheme III. Partial Reactions of Oxidative Phosphorylation

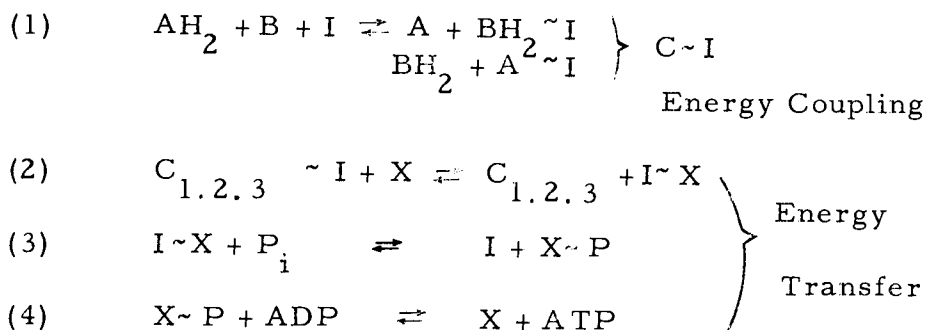
In Scheme III, as a pair of electrons pass from carrier A to carrier B, a high energy derivative of A is formed with some unknown participant X, presumably a protein. In Step 2, carrier A is released as it is displaced by P_i from $\text{A} \sim \text{X}$. The phosphorylated intermediate, $\text{X} \sim \text{P}$, thus formed, transfers the phosphoryl group to ADP in Step 3. This scheme corresponds to that postulated by Lehninger (131) who also suggests that the oxidized form of carrier A is involved in the formation of a high-energy intermediate. On the other hand, Chance and Williams have claimed that the reduced rather than the oxidized carrier is involved in the formation of the intermediate (22) resulting in the following variation in step 1.



The nature of the primary high-energy intermediate is not yet clarified. It is not known whether these intermediates contain the electron carrier in the oxidized or the reduced form. Both alternatives have been considered and debated (116). Nevertheless, Step 2 and Step 3 in Scheme III account for $^{32}\text{P}_i$ -ATP exchange, while ADP-ATP exchange is produced by Step 3 alone.

Studies with agents which inhibit electron or energy transfer, or uncouple the two processes have played a decisive role in the elucidation of the energy transfer pathway. DNP has been shown to uncouple oxidative phosphorylation and to eliminate the requirement of inorganic phosphate for respiration (86). Originally, the action of dinitrophenol was attributed to the cleavage of an $X\sim P$ or $E\sim P$ intermediate (58). More recently, investigators have favored a DNP-catalyzed hydrolysis of a non-phosphorylated "high-energy" intermediate (5, 12, 22). The observation made in Slater's laboratory that inorganic phosphate is not required for the action of DNP would support this conclusion (4).

Studies at a number of laboratories (34, 40, 50, 77) have shown the following effects of DNP on freshly prepared animal mitochondria: it "uncouples" phosphorylation from respiration, that is, it inhibits phosphorylation while leaving the rate of respiration essentially unchanged; it stimulates the hydrolysis of ATP to ADP and P_i ; it inhibits the $ATP - {}^{32}P_i$ exchange reaction; it also inhibits the ADP-ATP exchange reaction; in the absence of a phosphate acceptor it releases respiration, that is, it greatly stimulates respiration as would the addition of ADP; finally, it inhibits the aerobically supported energy-dependent reactions (See page 15). All these effects can be explained on the basis of the reactions given in Scheme IV.



Scheme IV: Proposed reactions of respiratory-chain-linked phosphorylation. A and B are electron carriers of the respiratory chain; C is a common symbol for either A or BH_2 . The subscripts in C, $\text{C}_{1,2,3}$ refer to the three energy-coupling sites of respiratory chain. I and X are hypothetical energy-transfer carriers.

It has been observed by Chance et al. (23) and Lee and Ernster (75) that coupling energy conserved in the form of $\text{C}_{1,2,3} \sim \text{I}$ is dissipated by DNP. Most of the effects of DNP on the process of oxidative phosphorylation can be explained by the breakdown of this intermediate. Both exchange reactions would be inhibited since the breakdown of $\text{C}_{1,2,3} \sim \text{I}$ by DNP would uncouple oxidative phosphorylation since no ATP could be formed, and in the absence of phosphate acceptor (ADP) DNP would release respiration by regenerating C and I. The DNP-stimulated ATPase activity can be visualized by the reversal of reaction 2 to 4. The action of DNP would force reaction 2 to the left since $\text{C}_{1,2,3} \sim \text{I}$ is decomposed by the action of DNP. The role of $\text{I} \sim \text{X}$ in the energy-dependent reactions will be discussed later, but it is apparent that DNP would inhibit the energy-dependent reaction by

uncoupling the formation of $I\sim X$.

Arsenate has been known for some time as an uncoupler of substrate-level phosphorylation as well as electron transport-linked oxidative phosphorylation (31). The uncoupling effect of arsenate has been confirmed in many laboratories (4, 129). In the process of oxidative phosphorylation, it markedly depresses both phosphorylation and the $^{32}\text{P}_i$ - ATP exchange activity probably by competing with phosphate for the high-energy intermediate $I\sim X$ at reaction 3 of Scheme IV

Oligomycin inhibits oxidative phosphorylation and associated respiration apparently by blocking the reaction of phosphate with the nonphosphorylated intermediate at reaction 3 of Scheme IV (13, 132). It inhibits ATPase, net phosphorylation, the $^{32}\text{P}_i$ - ATP exchange (73) and ATP-ADP exchange reactions (15). A most important point for the theoretical analysis of the mechanism of phosphorylation is the finding that oligomycin inhibits the energy-linked reduction of DPN^+ by succinate when ATP is used as the energy donor but not when respiratory energy is used (38, 121). This response could be visualized from Scheme IV by postulating that the high energy intermediate ($I\sim X$) used to energize the reduction of DPN^+ by succinate can be produced either by ATP through the reversal of energy transfer reaction or by the participation of the respiratory chain.

These data are also significant in regard to the sequence of energy transfer and the site of action of the uncouplers and inhibitors.

The use of oligomycin has also been important in differentiations between the stimulation of respiration produced by DNP or arsenate. The stimulation of respiration by arsenate was further studied by Estabrook (41) and it was observed that a small amount of ADP was needed for maximum stimulation and that the stimulation effect was different from the DNP-stimulation of mitochondrial oxidation. Oligomycin inhibited arsenate stimulation but not DNP stimulation of mitochondrial oxidation. Since the reaction of arsenate is competitive with phosphate, it appears that oligomycin likely acts prior to the intervention of P_i on the sequence of energy transfer steps (reaction 3 of Scheme IV).

Recently, Lardy et al. (72) suggested that oligomycin prevents phosphate transfer to acceptor, that is oligomycin prevents reaction 4 of Scheme IV. This conclusion was based on the observation that oligomycin inhibits the arsenate-induced hydrolysis of ATP. Another antibiotic aurovertin shows qualitative effects similar to oligomycin but varies in the degree of activity. Lardy has thus postulated that these two antibiotics act at distinct but related sites.

Hodges and Hanson (55) using corn mitochondria found that the accumulation of Mg^{++} and Ca^{++} is also an energy dependent process

as has been observed with animal mitochondria (23). The energy can be supplied by either substrate oxidation or ATP. Oligomycin inhibits ATP driven Ca^{++} uptake but not substrate - driven uptake. They thus suggested that a high-energy intermediate of oxidative phosphorylation is a common energy source for ATP formation and for ion transport. Transport appeared to be the result of some reaction of Ca^{++} with $\text{X}\sim\text{P}$. The observation that oligomycin blocks ATP formation, but not $\text{Ca}^{++} + \text{P}_i$ transport indicated that the inhibition lies between $\text{X}\sim\text{P}$ and ATP. Furthermore, studies in the same laboratory showed that the contraction of corn mitochondria, as in animal mitochondria, can be maintained or initiated with ATP + Mg^{++} or an oxidizable substrate. Oligomycin inhibited the ATP - powered contraction, however, it did not inhibit the substrate-powered contraction (124). The substrate-powered contraction is strongly inhibited by phosphate probably due to the competition for $\text{I}\sim\text{X}$ which is supposedly associated with contraction. This inhibition by phosphate of the substrate-powered contraction is unaffected by oligomycin. Thus they concluded that the inhibition of oligomycin lies between $\text{X}\sim\text{P}$ and ATP and strongly suggested that Lardy's view is correct.

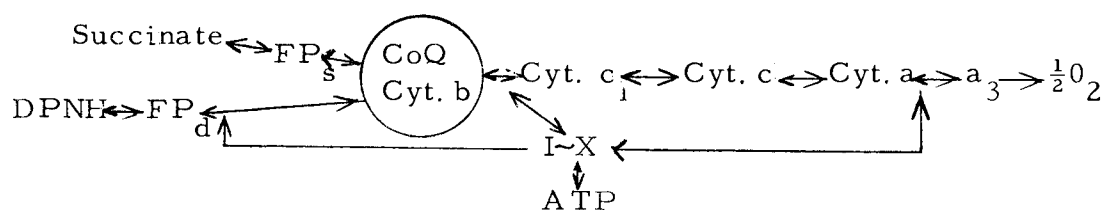
Chance (17) and Fonyo and Bessman (43) have also demonstrated that a small amount of Ca^{++} causes a rapid respiration by "draining" of the high-energy intermediates prior to the site of the oligomycin

effect, that is, oligomycin does not inhibit the Ca^{++} -induced respiratory "jump". Oligomycin appears, therefore, to inhibit the last step of energy transfer reaction.

Energy-linked Reactions of Mitochondria

I. Reduction of DPN^+ coupled with the oxidation of succinate - - - - Reversal of Electron Transport

Chance's original suggestion (19) that the reduction of mitochondrial DPN^+ could be brought about by the addition of succinate to mitochondria under aerobic conditions has been supported by reports from several laboratories (20, 38, 120). This reversal of electron transport requires energy which can be supplied either by the addition of ATP or by high energy intermediates generated by the respiratory chain. It has now been established that the reversal of oxidative phosphorylation resulting in the reversal of electron transport through all the carriers of respiratory chain can occur in mitochondria and sub-mitochondrial particles (40) as illustrated in Scheme V.



Scheme V. Relationship and reversability of oxidative phosphorylation and electron transport

I~X is the nonphosphorylated high-energy intermediate which is common for the three energy coupling sites.

The reversed electron flow can be observed by measuring the changes in redox state of the components of the electron transport chain (18, 56, 87, 112, 116). In addition, when DPNH-oxidizing substrate, such as acetoacetate, oxaloacetate, or α -ketoglutarate + NH_3 (67, 116) were added, the disappearance of these or the appearance of their reduced products, can be employed to demonstrate the activity of the reversal of electron transport.

High-energy intermediate generated by oxidation at one coupling site can provide energy for the reversal of electron transport at another coupling site. Aerobic oxidation of succinate generates high energy intermediates at coupling sites 2 and 3 which can result in reversed electron transport at site 1. Studies in several laboratories have reported the reduction of DPN^+ coupled to aerobic oxidation of succinate (2, 120, 127). The generation of high energy intermediates at coupling site 2 and their utilization for reversing coupling site 1 has been illustrated by Ernster (38). In this study it was shown that the reduction of DPN^+ could be coupled to the oxidation of succinate by ferricyanide. Slater and his collaborators (125, 126) have also studied the generation of high energy intermediates at coupling site 3 and their utilization for reversing of electron flow at site 1

by coupling the reduction of DPN^+ by succinate to the aerobic oxidation of ascorbate + TMPD. Both dinitrophenol and oligomycin inhibit when ATP is used as energy source, but when a high energy intermediate of respiration provides the energy, the reduction is still sensitive to dinitrophenol but insensitive to oligomycin (38). These are important clues in locating the site of action of the inhibitors.

II. Energy-dependent pyridine nucleotide transhydrogenation

Klingenberg and Slencka (68) found that the incubation of rat-liver mitochondria with DPN^+ -specific substrate or succinate in the absence of phosphate acceptor resulted in a rapid and almost complete reduction of mitochondrial TPN^+ . This observation was confirmed by Danielson and Ernster (32) who demonstrated this energy-dependent conversion of DPNH and TPN^+ into DPN^+ and TPNH in phosphorylating sub-mitochondrial particles from rat liver and beef heart. The energy for the reaction could be supplied either by added ATP or by the aerobic oxidation of DPNH or succinate. Oligomycin inhibited the ATP supported reaction whereas it did not affect the reaction proceeding at the expense of aerobically generated high energy intermediates and in fact oligomycin provided a slight stimulation under these conditions. DNP inhibits the reaction in both cases but was more efficient with aerobically generated high energy intermediates than with ATP as the source of energy. The same particles also

catalyzed the energy dependent reaction of DPN^+ by succinate.

Hommes and his collaborators (56) have investigated the relationship between the energy-dependent TPN^+ reduction and the non-energy-dependent transhydrogenase reaction as well as succinate-linked DPN^+ reduction. The energy- and non-energy-dependent transhydrogenase reactions were found to be catalyzed by the same enzyme, and the high energy-dependent transhydrogenation and succinate-linked reduction of DPN^+ involved a common high energy intermediate. More recently, Lee and Ernester (76) showed that energy-linked reduction of TPN^+ by DPNH competes efficiently with oxidative phosphorylation coupled to the aerobic oxidation of succinate in sub-mitochondrial particles from beef heart. This is in good accordance with the concept that the energy linked transhydrogenase reaction derives energy from an intermediate of the phosphorylating system which is coupled to the respiratory chain.

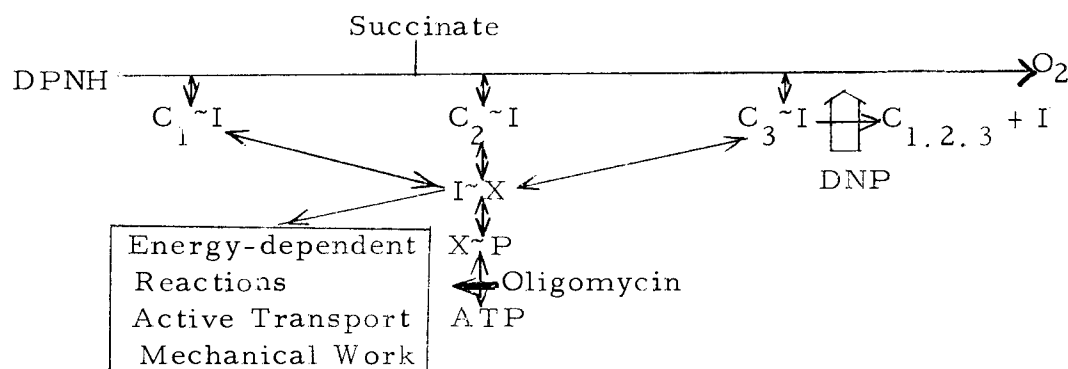
III. Other energy-dependent events

Lehninger (78) has pointed out that three different modalities of respiratory energy transduction are visualized: (a) the synthesis of ATP from ADP and P_i , (b) active transport of certain ions and (c) the mechano-chemical changes in permeability and in conformation of membranes of mitochondria. These three processes are related in that each requires the high energy intermediates of respiration. The active accumulation of ions, such as K^+ , requires

respiration and is inhibited by agents such as DNP. Uptake of Ca^{++} , Mg^{++} and Mn^{++} into mitochondria has also been demonstrated to require high energy intermediates generated by the respiratory chain (11, 25, 83, 109). The three energy-coupling sites of the respiratory chain appear to be equally efficient in supporting active ion uptake (106) and the process is insensitive to oligomycin.

The mechanical work of energy coupling which brings about the swelling and contraction of mitochondria was studied thoroughly by Lehninger's laboratory and others (79). Respiration-dependent swelling of mitochondria is oligomycin insensitive and the ATP- induced mitochondrial contraction is oligomycin sensitive (80). These observations suggested a close relationship of the active uptake and extrusion of water to respiratory chain-linked high energy intermediates.

From these and other evidence a schematic presentation of the polymodalities of respiratory energy transduction can be shown in Scheme VI as modified from Lee and Ernster (75).



Scheme VI. The polymodalities of respiratory energy transduction

Soluble Factors and Intermediates of Oxidative Phosphorylation

I. Soluble factors

Considerable attention has been directed to the study of soluble factors and high energy intermediates with the hope of shedding some light on the mechanism of oxidative phosphorylation. A series of coupling factors have been reported by Racker's laboratory (103). Factor F_1 was obtained by shaking mitochondria in a Nossal disintegrator and was shown to catalyze the hydrolysis of ATP. Also, it was required for oxidative phosphorylation at site 1 and 2 and for other ATP-dependent processes in mitochondria, such as the reduction of DPN^+ by succinate (28), and the reduction of TPN^+ by DPNH (103). It is not required in the generation of high energy intermediate formed during the respiratory oxidation (102, P. 169). F_1 most probably catalyzed the transphosphorylation step from $X\sim P$ to ADP.

Another factor, F_2 was obtained from the sonic oscillation of the Nossal disintegrated particle. This factor was required for phosphorylation associated with the oxidation of succinate with a submitochondrial particle which had been exposed to sonic treatment. F_3 appeared to stimulate phosphorylation associated with site 2. P-particles, the sub-particles of beef heart mitochondria obtained after sonic oscillation in the presence of 2 percent phosphatides, required F_1 and another factor F_4 , a protein factor extracted from

mitochondria with 0.4N ammonia. Purified F_4 was required for oxidative phosphorylation at all three sites, and the ATP dependent reduction of DPN^+ by succinate and TPN^+ by DPNH (103). F_4 is not required for energy-dependent reactions driven by the operation of respiratory chain. It appeared that F_4 was involved with the formation of the phosphorylated intermediate. Recently, Fessenden et al. (42) have demonstrated that F_4 can be replaced by two soluble coupling factors F_2 and F_3 . Contamination of F_4 with F_2 and F_3 may account for its stimulatory effect on oxidative phosphorylation.

Watkins and Lehninger have also described a coupling factor, which appears to be specific for site 3 (132). This purified protein factor also catalyzes ADP-ATP exchange activity. Other soluble coupling factors required for oxidative phosphorylation have been demonstrated by Linnane and Titchener (85) and Green et al. (49). The relationship among these coupling factors and their role in oxidative phosphorylation is poorly understood.

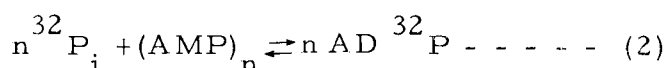
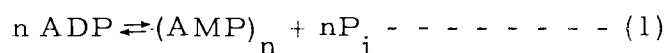
Lee et al. (74) have demonstrated that non-phosphorylating particles could utilize aerobically generated high energy intermediates, but not ATP, as the source of energy for energy-dependent pyridine nucleotide transhydrogenation with oligomycin having no effect. One might presume that non-phosphorylating particles which are commonly employed by other laboratories should be capable of

the generation of nonphosphorylated high energy intermediates. Thus most of the coupling factors described so far probably play a role only in energy transfer rather than energy coupling, i. e. they are probably involved in step 3 and 4 in Scheme IV.

II. Intermediates

The evidence is quite conclusive that inorganic phosphate does not participate in the formation of the primary high energy intermediate generated by the respiratory chain. Although the evidence for the existence of nonphosphorylated intermediates has been accumulated from several laboratories (38, 40, 102, P. 159-177), only one possible nonphosphorylated intermediate of oxidative phosphorylation has been reported (97, 98). Working with the bacterium Alcaligenes faecalis, Pinchot and his coworker reconstructed an enzyme system which catalyzed oxidative phosphorylation by combining a particulate DPNH oxidase with a heat labile soluble component and a heat stable factor. Upon incubation of the system with DPNH in the absence of ADP, P_i and Mg^{++} , a soluble protein complex was released which incorporated $^{32}P_i$ into $AT^{32}P$ in the presence of ADP and Mg^{++} . They have also shown that the protein contained DPN^+ which was stoichiometrically displaced by P_i upon incubation with P_i and ADP and yielded an equivalent amount of ATP. This high energy complex of the heat labile factor has been

isolated and demonstrated to be an enzyme~ compound which can also be formed in the back reaction by incubating soluble factor DPN^+ and ATP. The validity of these observations has been questioned by Pandit-Hovenkamp (94) who found that incorporation of $^{32}\text{P}_i$ into organic phosphate is mainly by $\text{ADP}-^{32}\text{P}_i$ exchange, possibly catalyzed by polynucleotide phosphorylase as illustrated:



Definite conclusions will have to await experiments demonstrating the absence of $\text{ADP}-^{32}\text{P}_i$ exchange in the purified preparation.

Griffith (52) has reported the occurrence of a radioactive phosphorylated derivative derived from DPN^+ and $^{32}\text{P}_i$ when mitochondria were exposed to succinate DPN^+ and $^{32}\text{P}_i$. This phosphorylated form of DPN^+ , differed from TPN^+ and when added to suitable particles together with ADP and Mg^{++} , yielded ATP and DPNH. DNP, antimycin A and malonate inhibited the formation of the phosphorylated DPN^+ , but not its subsequent conversion into DPNH and ATP.

From this evidence, Griffiths claimed that the phosphorylated DPN^+ had the properties of a high-energy intermediate of oxidative phosphorylation. Yet Griffith failed to demonstrate the formation of the phosphorylated derivative on incubation with DPN^+ linked substrates. This phosphorylated DPN^+ could be involved in another pathway such as the energy-dependent reduction of TPN^+ by DPNH

as suggested by Ernster (39).

Incubating mammalian mitochondria with $^{32}\text{P}_i$ or AT^{32}P produced a radioactive phosphoprotein. The label was shown to appear in a bound phosphohistidine group of the protein (7, 95, 96). Oxidative phosphorylation was then considered to involve the formation of bound phosphohistidine. Further studies showed that the phosphoprotein was actually involved in the substrate-level phosphorylation mediated by succinate thiokinase (91). A similar association has also been found with highly purified E. coli succinate thiokinase (70). The study of soluble coupling factors and high energy intermediates of oxidative phosphorylation has provided some leads, but the real nature of the intermediates and the mechanism of oxidative phosphorylation remains obscure.

MATERIALS AND METHODS

I. Materials

The blowflies, Phormia regina, used in this study were from a laboratory colony maintained by the Department of Entomology at Oregon State University. The diets of the breeding colony and of the adult flies were the same as described previously (50, P. 15).

The following chemicals were obtained from the Sigma Chemical Company: ATP, ADP, hexokinase (Type II, or III), BSA, DPN^+ , TPN^+ , CoA, GDP, PCMB, glycylglycine, Tris (Sigma 121), sodium pyruvate, malic acid, α -ketoglutaric acid, alcohol dehydrogenase and antimycin A. Sucrose, EDTA, magnesium chloride, potassium phosphate, potassium dihydrogen arsenate, hydroxylamine hydrochloride, ammonium molybdate and benzene were the reagent grade products from Baker Chemical Company. 2,4-dinitrophenol, citric acid, sodium azide, succinic acid, and isobutyl alcohol were purchased from Eastman Organic Chemicals. The other reagents were obtained as follows: fumaric acid, Delta Chemical Company; $^{32}\text{P}_i$ (carrier-free) was supplied by Oak Ridge National Laboratory earlier and for the last two batches, it was purchased from International Nuclear Corporation. Oligomycin was purchased from the Wisconsin Alumni Research Foundation.

Double distilled water was used in preparing all solution used in the reaction mixture and enzyme preparation media.

II. Methods

Isolation of mitochondria from blowfly muscle

The mitochondria were isolated using a modification of the procedure described by Gregg et al. (51). Adult flies, about six days old, were anesthetized by chilling at 1°C for about one-half hour, then 40-150 ml of the whole flies were transferred to a tray standing on ice and covered by another tray containing ice. The thoraces were removed by dissection and collected in a cold beaker and then gently ground in a glass mortar with 120-360 ml of homogenizing medium. The homogenate was filtered through eight layers of cheesecloth and then through a glass wool filter to remove pieces of chitin and other debris. Additional debris, muscle fibers and whole cells were removed by centrifuging at 460 xg for three minutes in a refrigerated Sorvall centrifuge. The supernatant fluid was decanted through a glass wool plug, and centrifuged at 7,000xg for eight minutes to sediment the mitochondria. The supernatant was discarded and the walls of the centrifuge tubes were carefully wiped with Kleenex tissue to remove fatty and incompletely sedimented materials streaked along the sides. Mitochondrial pellets were washed by suspending in 40-80 ml

washing medium using a loosely fitting, glass, Potter Elvehjem homogenizer, operated by hand, followed by centrifuging. This washing process was repeated. Finally, the mitochondrial pellets were suspended to the desired volume in the suspending medium.

The procedure given above was used throughout this study. However, the composition of the media used for homogenizing, washing and suspending was varied as outlined below. The pH was adjusted to 7.4 with KOH or HCl.

Method A:

The homogenizing medium consisted of 0.3M Sucrose, 0.05M Tris, 0.003 M EDTA, 1×10^{-4} M MgCl_2 , 0.005M pyruvate, 3.3×10^{-5} M fumarate and 1 mg albumin per ml of medium. The washing and suspending media were of identical composition.

Method B:

This method was identical to Method A except that pyruvate and fumarate were omitted from the washing and suspending medium.

Method C:

This method was the same as Method A except that the washing and suspending medium contained only 0.3M Sucrose, 0.05M Tris, 1×10^{-4} M MgCl_2 , 1×10^{-4} M EDTA, and 0.3 mg BSA/ml.

Preparation of submitochondrial particles.

Sonicated particles were isolated by a modification of the procedure of Kielley and Bronk (65). The normal practice was to suspend twice-washed mitochondria, derived from the thoraces of 80 ml of blowflies in the appropriate suspending medium to a volume of 50 ml. The suspension was then sonicated in the cavity of a 10 KC Raytheon sonic oscillator adjusted to produce maximum power output; the cavity was maintained at approximately 0°C by circulating ice water. After sonication, intact mitochondria and heavy particles were removed by centrifuging the suspension at 14,000xg for 10 minutes. The supernatant fluid was decanted and then centrifuged at 105,000xg for 20 minutes to sediment the submitochondrial particles. The dark reddish-brown pellets were suspended in the appropriate medium with the aid of a hand operated glass, Potter Elvehjem homogenizer. The suspension of sonic particles was either used immediately, or stored at -20°C for later use.

Treatment of stock $^{32}\text{P}_i$

It was found necessary to treat the $^{32}\text{P}_i$ to remove heavy metal ions present as impurities. $^{32}\text{P}_i$ shipments were adjusted to pH 8.0 with KOH, boiled for one hour, cooled and filtered through a fine sintered glass funnel. The filtrate was then adjusted to pH 1.0

with HCl and boiled for two hours to hydrolyze any pyrophosphate. This solution was stored at -20°C and the pH adjusted to 7.4 immediately before the experiment.

Preparation of a soluble mitochondrial enzyme fraction

The mitochondrial suspension was sonicated twice as described previously, after which intact mitochondria and heavy particles were removed by centrifuging at 14,000 xg for 10 minutes. The supernatant fluid was decanted and then centrifuged at 105,000xg for 90 minutes to sediment all particulate material. The clear supernatant was either used immediately or stored at -20°C for later use.

Preparation of the acetone-treated fraction

A freshly prepared soluble enzyme fraction was dialyzed against 0.05M Tris buffered double distilled water, pH 7.4, for 1-2 days, and then was poured directly into two volumes of acetone at -20°C with constant stirring. After standing for 30-60 minutes in the freezer, the acetone suspension was centrifuged for 10 minutes at 12,000xg and the acetone solution decanted and discarded. The bottles containing a pale white residue were allowed to drain for two or three minutes to remove as much acetone as possible and the pale white precipitate was dissolved in the suspension medium.

Preparation of ammonium sulfate fractions

The soluble enzyme preparation was fractionated with ammonium sulphate at 4°C using the method described by Dixon and Webb (33). The enzyme preparation was placed in a beaker and the weighed amount of ammonium sulfate to achieve 50 percent saturation was added with stirring over a 10 minute period. Following a further 15 minute equilibration the mixture was centrifuged for five minutes at 23,500xg to collect the <50 percent fraction. The resulting supernatant was brought to higher $(\text{NH}_4)_2\text{SO}_4$ concentrations and fractions were collected in the same manner. Each fraction was redissolved in the appropriate medium and dialyzed against 10 volumes of Tris buffered solution, pH 7.4, at 4°C.

Incubation procedures

Usually, two incubation steps have been used in this study. In the first incubation the whole mitochondria were incubated with pyruvate or other oxidizable substrate in a phosphate free medium to yield more intermediate or in the presence of inhibitors to prevent further production of soluble intermediate. In method A, the soluble intermediate presumably leaked out of whole mitochondrial particles. The supernatant fraction containing the soluble intermediate was obtained by centrifuging out the whole mitochondria.

In method B, breaking of the whole mitochondria by sonication after the first incubation released more soluble high energy intermediates. All the particulate fraction can be removed by high speed centrifugation. Most of the experiments have used this procedure. The soluble intermediate can be detected in the second incubation where all the components, such as P_i - $^{32}P_i$, ADP (or ADP + hexokinase + glucose) and Mg^{++} , which are necessary for the energy transfer reaction are present. The formation of ATP (or glucose-6-phosphate) was estimated by the amount of radioactive $^{32}P_i$ fixed as esterified phosphate or nonextractable phosphate (see the determination of esterified ^{32}P).

All incubations were carried out at $25^{\circ}C$ in 25 ml Erlenmeyer flasks. The total volume (including the enzyme preparation) was 3.0 ml except where noted otherwise. The composition of the incubation medium used in the different experiments is given in Method A, B and C.

Method A:

In the early experiments, whole mitochondria were incubated in the first incubation medium for 5 or 10 minutes at $25^{\circ}C$ in a Dubnoff shaking incubator. The components of this incubation medium are listed in the legend of each table of figure. Following the first incubation, the incubation mixture was chilled in an ice bath, then

the mitochondria were centrifuged down in a Sorvall centrifuge at 20,000xg for 10 minutes. A 2 ml aliquot of each supernatant was then used in the second incubation where the acceptor system has been added.

The amount of $^{32}\text{P}_i$ employed, where indicated, was equivalent to 5.0×10^5 to 4.0×10^6 counts per minute per flask. The second incubation was conducted in a Dubnoff constant temperature bath, and the reactions were initiated by the addition of the enzyme preparation. The reactions were stopped with 1.0 ml of 0.8M TCA and the precipitated protein was removed by filtration through Whatman No. 2 filter paper. Aliquots from the filtrates (TCA-filtrate) were used for $^{32}\text{P}_i$ analysis.

Method B:

This method was identical in procedure to that described in method A except that, after the first incubation, the mitochondria were broken down by sonication (see preparation of submitochondrial particles). The suspension was first centrifuged in a Sorvall centrifuge at 20,000 x g for 10 minutes then in a Spinco ultracentrifuge at 105,000 x g for 90 minutes. The clear supernatant (soluble mitochondrial enzyme fraction) so obtained was used in the second incubation.

Method C:

This method differed from A and B by having no first incubation procedure. The mitochondrial suspension obtained as described previously was subjected to sonication. The particulate fractions were centrifuged down first in a Sorvall centrifuge at 20,000 x g for 10 minutes then in a Spinco ultracentrifuge at 105,000 x g for 90 minutes and discarded. The supernatant (soluble mitochondrial enzyme) fraction so obtained was used in the incubation procedure.

Determination of esterified ^{32}P

Method A:

The esterified phosphate (ATP or G-6-P) was estimated using a modification of the method of Nielsen and Lehninger (92). The free inorganic phosphate forms a molybdate complex which will be extracted by the organic solvent (isobutanol benzene reagent). The esterified ^{32}P or nonextractable phosphate-32 (NE^{32}P) remains in the aqueous fraction in the sample.

A one ml aliquot of each TCA filtrate was transferred to a separate glass-stoppered centrifuge tube containing the following: 4 ml isobutyl alcohol-benzene reagent; one ml ammonium molybdate reagent, and two ml of distilled water. Each tube was stoppered and shaken vigorously for 30 seconds. After the organic and aqueous phases had separated, the aqueous layer, which contained the

esterified ^{32}P compounds, was transferred to a second set of centrifuge tubes containing 4 ml of isobutyl alcohol-benzene reagent. The tube was stoppered and again shaken vigorously for 30 seconds. The aqueous layer was filtered into clean culture tubes through Whatman No. 2 paper. The culture tubes were kept stoppered until one ml aliquots could be counted with a gas flow Geiger counter.

The isobutyl alcohol-benzene reagent was prepared by mixing 200 ml isobutyl alcohol, 200 ml of benzene and 40 ml of distilled water, and shaking the mixture for 30 minutes. The ammonium molybdate reagent was prepared as described by Martin and Doty (88).

Method A, employed here, can only estimate the total amount of esterified phosphate or nonextractable phosphate. It was presumed that AT^{32}P was formed when ADP and $^{32}\text{P}_i$ was added and G-6- ^{32}P when the hexokinase trap was employed. In addition, a small amount of acid labile phosphorylated intermediate might be present in the form of nonextractable phosphate. In the light of this possibility a modified method (Method B) was used to test the stability of the final products. G-6- ^{32}P would be more stable and more resistant to acid hydrolysis than ATP or other intermediary phosphorylated compounds. This can be shown from the result in Table IV. However, Method A was routinely used to estimate the amount of esterified phosphate or NE^{32}P throughout this investigation.

Method B:

The esterified phosphate (NE^{32}P) was determined as follows:

A one ml aliquot of each TCA filtrate was mixed with one ml of 2N H_2SO_4 in a glass-stoppered centrifuge tube. The tube was covered with aluminium foil and immersed in boiling water bath for two minutes. After cooling the tube to room temperature, one ml of distilled water, one ml of special molybdate reagent (containing only 2N H_2SO_4) and 4 ml of isobutyl alcohol-benzene reagent was added. After this the procedure was the same as described in Method A.

Protein determination

Protein determinations were carried out by the Biuret method as described by Gornall et al. (47) using crystallized bovine serum albumin as standard. Protein in the whole mitochondria or sub-particles was estimated by the method of Jacobs et al. (59).

Assay of succinic thiokinase activity

The activity of this enzyme in the soluble fraction, acetone treated fraction and ammonium sulfate fraction was assayed by a modification of the $^{32}\text{P}_i$ -ATP exchange procedure of Kaufman (62). The experimental conditions were identical to those of the second incubation system except that ATP replaced ADP, and potassium

succinate and CoA were added. The formation of $AT^{32}P$ was determined as $NE^{32}P$.

Assay for energy-dependent pyridine nucleotide trans-hydrogenase reaction

The composition of the reaction mixture was described with the figures. All incubations were performed at 25°C. Pyridine nucleotide reduction was recorded at 340 m μ with a Beckman DU spectrophotometer with a Gilford Automatic recorder. DPNH was generated by alcohol dehydrogenase and ethanol or by succinate as described by Lee et al. (74).

RESULTS AND DISCUSSION

I. Oxidative Phosphorylation in the Whole Mitochondria

Active mitochondrial preparations have been prepared using procedures developed by Gregg et al (51), Meegunwan (89) and Steele (123). The oxidation of pyruvate by mitochondria is rapid and tightly coupled when the incubation medium is supplemented with ADP (or ATP), P_i , glucose, Mg^{++} , and hexokinase (123). Studies with whole mitochondria suggested that it might be possible to isolate a soluble intermediate of oxidative phosphorylation (89). The existence of one or possibly two intermediates of oxidative phosphorylation was indicated. Typical results are exemplified in Table I. Using mitochondria prepared by method A it was observed that in the presence of inorganic phosphate alone, a small amount of P_i was esterified probably due to the presence of endogenous P_i -acceptor or phosphorylated intermediate formed during the incubation. Addition of acceptor system into the incubation medium substantially increased the amount of phosphate esterified. This increase was curtailed by antimycin A, which is an effective inhibitor of the electron transport chain, as well as DNP, which is an effective uncoupler of energy transfer in oxidative phosphorylation. These data would indicate that oxidative phosphorylation linked to

Table I. Oxidative phosphorylation in whole mitochondria

| Acceptor system | Inhibitor | Pi-esterified (m μ moles) x 10 |
|-----------------|-------------|---------------------------------------|
| - | - | 1.80 |
| + | - | 8.16 |
| + | Antimycin A | 6.78 |
| + | DNP | 5.46 |

Incubation medium contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris, 6 μ moles EDTA, 20.2 μ moles MgCl_2 , 0.06 μ moles cold P_i , 2×10^6 cpm $^{32}\text{P}_i$, 10 μ moles Pyruvate and 0.06 μ moles Fumarate. The acceptor system was composed of 2 μ moles ADP, 25 μ moles glucose and 50 KG units hexokinase. The amount of inhibitors added was, antimycin A 10 μg , DNP 0.6 μ moles. Two ml of mitochondrial suspension containing 1.08 mg protein per ml was added to initiate the reaction and the incubation was carried out at 25° for 12 minutes.

electron transport chain was operative in the particles.

Further studies revealed that a soluble non-phosphorylated high-energy intermediate was released to the incubation medium. After the preincubation of whole mitochondria with substrate in the absence of added P_i and acceptor system, the particulate fraction was centrifuged off and the supernatant fraction was used for the assay of energy transfer activity by adding P_i - $^{32}P_i$, acceptor system together with supernatant fraction in the second incubation. Typical results are shown in Method A of Table III. As will be demonstrated later in this thesis, there exists a low steady state concentration of the soluble intermediate inside the mitochondria and this soluble intermediate can also be released to the soluble fraction during the incubation period. This could probably explain the low sensitivity of whole mitochondria to antimycin A inhibition.

II. Oxidative Phosphorylation Activity in the Submitochondrial Particles

The ability of submitochondrial particles to esterify phosphate was impaired even in the presence of the acceptor system. Addition of the supernatant fraction, which was separated from submitochondrial particles earlier in the preparation greatly enhanced the activity. Either some essential components for the coupling of

phosphorylation were lost after sonic treatment as shown by Racker and Conover (103), or a low, steady-state concentration of high energy intermediate which could produce ATP (or G-6-P) was released by the sonic treatment. Further investigations of this soluble fraction have revealed the existence of a soluble high energy intermediate of oxidative phosphorylation.

III. The Isolation of Transfer Activity in the Mitochondrial Soluble Fraction

A non-phosphorylated high energy intermediate was apparently released to the incubation medium when mitochondria were incubated with substrate in the absence of added P_i and acceptor system. This nonphosphorylated high energy intermediate was detected by the formation of $AT^{32}P$ when the soluble fraction was incubated with $^{32}P_i$ and ADP or G-6- ^{32}P when $^{32}P_i$ and the hexokinase trap was employed. The amount of $AT^{32}P$ (or G-6- ^{32}P) formed in a certain period of incubation could reflect the activity of energy transfer from nonphosphorylated high energy intermediate to the ATP terminal phosphate bond formation. In an effort to generate more high energy intermediate mitochondria were first incubated with pyruvate and other oxidizable substrate in the absence of added P_i and acceptor system. Sonic treatment was used to disintegrate the particles and thus released more high energy intermediate. The

Table II. The activity of oxidative phosphorylation in submitochondrial system

| Enzyme System | Acceptor | Phosphate Esterified (mμmoles) x 10 |
|---------------------------|----------|--|
| Sub-particles alone | - | 0.39 |
| Sub-particles alone | + | 4.36 |
| Sub-particles + supernate | - | 1.62 |
| Sub-particles + supernate | + | 16.23 |

The incubation procedure was the same as stated in Table I. Two ml of sub-particle suspension containing 1.65 mg of protein or 2 ml of sub-particle suspended supernatant containing 2.25 mg of protein was used to initiate the reaction. Incubation was carried out in Dubnoff shaking bath for 12 minutes at 25°C.

activity of energy transfer in the soluble fraction was then evaluated in a second incubation.

A first incubation with whole mitochondria was carried out at 25°C as described (Table III), after which the mixture was chilled in an ice bath. The mitochondria were then centrifuged down and the supernatant fraction used in the second incubation in which inorganic phosphate and acceptor system were added. The measurement of nonextractable ^{32}P (NE ^{32}P) (Table III, Method A) showed that definite transfer activity was present. Addition of ADP increased the amount of NE ^{32}P . A soluble high-energy intermediate was apparently released into the soluble fraction. In Method B the soluble fraction was obtained by rupturing the whole mitochondria by sonication following the first incubation. This sonic treatment resulted in more phosphate being esterified due to the release of more intermediate. A significant amount of intermediate was detected in the soluble fraction obtained by simply rupturing the mitochondria without previous incubation. These results show that (i) energy transfer activity can be demonstrated in this soluble fraction, (ii) a nonphosphorylated high energy intermediate exists in the soluble fraction and, (iii) the intermediate exists in a low steady-state concentration inside the mitochondria. More experiments have been conducted by using the soluble fraction of Method B to define the

Table III. The release of soluble high-energy intermediate from mitochondria

| Incubation | Procedure | Second Incubation | | % NE 32 P |
|------------|-----------|-------------------|------------|----------------|
| | | Acceptor | Time(min.) | |
| Method A | | - | 10 | 0.61 |
| Method A | | ADP | 10 | 4.40 |
| Method B | | - | 10 | 0.53 |
| Method B | | ADP | 10 | 18.40 |
| Method C | | - | 12 | 1.57 |
| Method C | | ADP | 12 | 6.78 |

The reaction mixture for the first incubation contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris, pH 7.4, 20 μ moles MgCl_2 , 30 μ moles Pyruvate and 0.2 μ moles fumarate, 6 μ moles EDTA. Two ml of mitochondrial suspension was added to initiate the reaction. The first incubation was carried out in a Dubnoff shaking bath at 25°C for 5 min. The second incubation medium contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris pH 7.4, 4 μ moles EDTA, 34 μ moles MgCl_2 , 1×10^6 cpm of $^{32}\text{P}_i$. Two μ moles of ADP was used where indicated. Two ml of supernatant fraction from method A, B, and C contain 0.42 mg, 0.55 mg, 0.60 mg protein per ml respectively, was added to initiate the reaction. The reaction was carried out in a Dubnoff incubator at 25°C for the period indicated.

properties of this intermediate and these will be discussed further in the thesis.

Two different systems have been employed as phosphate acceptors in these investigations: ADP alone, and the hexokinase system. The latter yields a more stable product (mainly G-6-P) than the former (mainly ATP), as demonstrated in Table IV in which NE^{32}P was determined after acid hydrolysis (Method B). The estimation of NE^{32}P was carried out after the filtrate from the second incubation medium was heated to 100°C for two minutes in $1\text{N H}_2\text{SO}_4$ as described in Method. G-6-P formed in the system would be more resistant to such treatment. Only a small amount of acid labile phosphorylated compounds were hydrolyzed. If ADP alone was employed, ATP formed would be expected to be more labile to acid hydrolysis, besides, more acid labile phosphorylated intermediates could exist in equilibrium with ATP and these could account for the greater loss after acid hydrolysis in this case.

IV. Possibility of Substrate Level Phosphorylation

Racker (102, P. 7) has pointed out that cells use four types of reactions to generate ATP. They are: substrate level oxidative phosphorylation; cleavage of C-S, C-C, and C-N bonds; dehydration of 2-phosphoglycerate; and phosphorylation linked to electron

Table IV. Stability of final products in different acceptor systems*

| Second Incubation | | % NE ^{32}P | |
|-------------------|-------------|----------------------|------------|
| Acceptor System | Time (min.) | A - Method | B - Method |
| - | 0 | 0.09 | 0.11 |
| ADP | 20 | 3.05 | 2.60 |
| Hexokinase System | 20 | 5.15 | 4.93 |

* The experimental procedure was the same as Method B in Table III. Supernatant fraction containing 0.69 mg/ml of protein was employed to initiate the second incubation. In second incubation medium, 1.3×10^6 cpm of ^{32}P was used. 50 μmoles glucose, 50 KG units of hexokinase, and 2 μmoles ADP constituted the hexokinase system. B-method of determining NE ^{32}P was carried out after that the filtrate from second incubation medium was heated to 100° for two minutes in 1 N H_2SO_4 .

transport (oxidative phosphorylation and photophosphorylation).

The first three types are catalyzed by soluble enzymes; the fourth type occurs in complex structures such as mitochondria and chloroplasts. One might thus question whether the increase in NE^{32}P observed on addition of ADP to soluble fraction is due to the first three types of phosphorylation, or to the electron linked oxidative phosphorylation. An enzyme-bound high energy intermediate can be produced during substrate level phosphorylation, such as the thioester proposed by several laboratories in the reaction mechanism for glyceraldehyde-3-phosphate dehydrogenase (9, 102, P. 17-54). The presence of such intermediates could result in transfer activity.

Since the mitochondria have been washed thoroughly during preparation, the possibility of these systems being contaminated with nonmitochondrial enzymes is remote. Furthermore, as demonstrated in Table III, the breakage of mitochondria releases the energy transfer activity. This ensures that the energy transfer activity comes from the mitochondria, thus ruling out many possibilities involving substrate level phosphorylation.

The existence of intermediates resulting from processes other than oxidative phosphorylation is not impossible. The oxidation of α -ketoglutarate and pyruvate gives compounds of the type

$$\begin{array}{c} \text{S} \sim \text{C} - \text{R} \\ \diagup \quad \parallel \\ \text{E} \quad \text{O} \\ \diagdown \\ \text{SH} \end{array}$$

formed in the oxidation of the acyl semialdehyde thiamine pyrophosphate intermediate by enzyme-bound lipoate (110), oxidation of fatty

acids yield enzyme-bound acyl~CoA (133) and the thiokinase reaction can form compound such as E~CoA (16). A series of experiments have been conducted to evaluate the possible involvement of these intermediates. Specific inhibitors and uncouplers of oxidative phosphorylation become the best tool in these investigations.

The formation of the high energy intermediate depends on the operation of electron transport chain. Mitochondria prepared from blowfly muscle are capable of oxidative phosphorylation with a P/O ratio of 2.6, using pyruvate as substrate and 2.2 for the mixture containing citrate, malate, succinate, fumarate and pyruvate as substrate (123). The use of pyruvate or Krebs cycle intermediates in the first incubation increased the amount of intermediate (Table V). The large increase in transfer activity observed when succinate was used as substrate in the first incubation was rather surprising. It was later shown that succinate itself plays a role in energy transfer reactions which effect will be discussed in detail later. Antimycin A, azide, cyanide have been employed as the inhibitors to block the electron transport chain (See Scheme II). Antimycin inhibited the production of high energy intermediates. Azide had no effect on the amount of phosphate esterified; in fact, it enhanced the transfer activity, probably due to the inhibition of ATPase activity (104). The formation of high energy intermediate was further

Table V. Effect of different substrates and inhibitors in the first incubation upon transfer activity in the second incubation

| First Incubation | | Second Incubation | | |
|-------------------------------|---------------------------------|-------------------|------------|----------------|
| Substrate | Inhibitor | Acceptor | Time (min) | % NE 32 P |
| <u>Experiment No. A</u> | | | | |
| Control | - | - | 10 | 0.32 |
| Control | - | ADP | 10 | 8.55 |
| Control + Pyruvate | - | - | 10 | 0.69 |
| Control + Pyruvate | - | ADP | 10 | 9.85 |
| Control + Succinate | - | - | 10 | 6.25* |
| Control + Succinate | - | ADP | 10 | 30.85* |
| <u>Experiment No. B</u> | | | | |
| Control + pyruvate + fumarate | - | - | 12 | 7.30 |
| Control + pyruvate + fumarate | - | Hex. Syst. | 12 | 12.85 |
| Control + pyruvate + fumarate | Antimycin A | - | 12 | 3.45 |
| Control + pyruvate + fumarate | Antimycin A | Hex. Syst. | 12 | 5.98 |
| Control + Pyruvate + fumarate | Azide | - | 12 | 7.43 |
| Control + pyruvate + fumarate | Azide | Hex. Syst. | 12 | 15.20 |
| Control + pyruvate + fumarate | Azide + Anti. A + CN^- | - | 12 | 1.10 |
| Control + pyruvate + fumarate | Azide + Anti. A + CN^- | Hex. Syst. | 12 | 2.03 |

The incubation procedure is that of method B. The reaction mixture of the control system for the first incubation was the same as stated in Table III except only 1.0 μmole pyruvate and 0.06 μmole fumarate were present. The amount of substrates or inhibitors, where indicated, 30 μmoles pyruvate, 20 μmoles succinate, 0.2 μmoles fumarate, 6 μg antimycin A, 9 μmoles azide, 9 μmoles cyanide. The first incubation was carried out at 25°C for 5 minutes. In the second incubation 2 ml of supernatant fraction was used to

start the reaction. The incubation mixture contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris pH 7.4 4 μ moles EDTA 34 μ moles MgCl_2 , 1×10^6 cpm of $^{32}\text{P}_i$. 2 μ moles of ADP alone or 2 μ moles ADP plus 50 μ moles glucose and 50 KG units of hexokinase was used as acceptor. The protein contents for each experiment were: Expt A, 0.60 mg/ml, Expt B, 0.56 mg/ml. In the cases of Expt B, 0.03 μ moles of carrier P_i was also added into second incubation mixture.

* The sudden jump in NE^{32}P was due to the involvement of succinate in transfer reaction to be discussed in detail later.

inhibited by the combined effect of antimycin A, azide and cyanide. These results suggest that the formation of high energy intermediate depends on the oxidation of substrate through the electron transport chain.

DNP is a true uncoupler of oxidative phosphorylation, in that with intact mitochondria it can isolate oxidation from energy transfer. Experiments have been designed to study the effect of DNP on the formation of the high energy intermediate itself. The effect of DNP upon the formation of high energy intermediate is given in Table VI. When the supernatant fraction was incubated with sub-mitochondrial particles, an increase in the amount of esterified phosphate was observed, suggesting that more intermediate was formed during the incubation. This increase in the amount of phosphate esterified was not observed in the presence of DNP. In fact, addition of DNP resulted in a decrease in the amount of phosphate esterified which was lower than that observed with supernatant fraction alone. It was assumed that DNP destroyed some of the intermediate.

Attempts have been made to exhaust the preformed high energy intermediates. Cold P_i and acceptor system, composed of ADP and hexokinase system, was used in the pre-incubation so that all the preformed intermediate could be converted into unlabeled glucose-

Table VI. The effect of DNP upon the formation of high energy intermediate

| Expt. No. | Enzyme System | Preincu- bation | Acceptor | Inhibitor | Pi-esterified ($\mu\text{moles} \times 10$) |
|--------------|--------------------------------|--------------------|----------|-----------|--|
| A | Supernatant + fraction | - | + | - | 10.56 |
| | Supernatant + sub-particles | - | - | - | 0.54 |
| | Supernatant + sub-particles | - | + | - | 13.45 |
| | Supernatant + sub-particles | - | + | DNP | 8.40 |
| | | | | | |
| B | Supernatant fraction* | + | + | - | 0.24 |
| | Supernatant + sub-particles | + | + | - | 9.42 |
| | Supernatant + sub-particles | + | + | DNP | 1.71 |

The incubation procedure of Experiment A was the same as stated in Table I. Protein content of supernatant fraction was 0.28 mg/ml; that of supernatant + sub-particles was 0.78 mg/ml. 0.06 μmoles DNP was added where indicated. The incubation procedure of Expt B was essentially the same as that of Expt B in Table V except that the supernate fraction has been deprived of high energy intermediate by adding cold P_i and acceptor system into the first incubation medium. The protein content for supernate was 0.60 mg/ml. 0.2 ml of sub-particle containing 8.0 mg protein/ml was also added where indicated.

6-phosphate. Little transfer activity remained after exhausting the high energy intermediate during the preincubation (Table VI). The addition of submitochondrial particles resulted in the production of new intermediate, greatly increasing the amount of phosphate esterified in the presence of phosphate and acceptor system. The effect of the submitochondrial particles was again counteracted by the addition of DNP. These results showed that the uncoupling effect of DNP is due either to interference in the formation of the high energy intermediate, as suggested by Eisenhardt and Rosenthal (35), or to the breaking of the intermediate itself (40).

In other experiments, the effect of DNP was studied using the preformed intermediate in the supernatant fraction. Increasing the concentration of DNP resulted in decreasing amounts of phosphate esterified (Figure 1). One might assume that the more DNP added to the soluble system, the more high energy intermediate will be destroyed, resulting in less phosphate being esterified. DNP could react with one of the intermediates of oxidative phosphorylation in a competitive manner, probably through the formation of a readily hydrolyzable enzyme-bound DNP derivative.

If DNP was allowed to react with the intermediate alone for twelve or thirteen minutes before the addition of P_i and acceptor system, a significant decrease was observed in the amount of

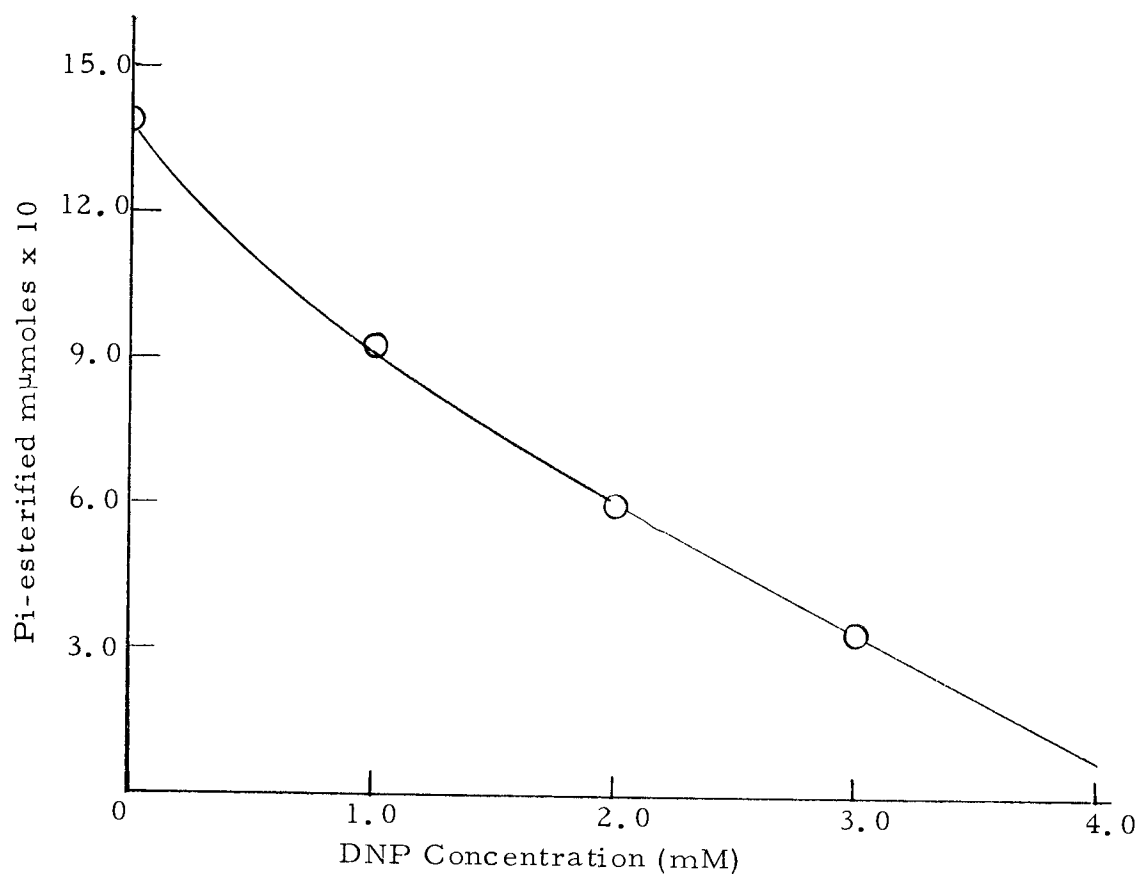


Fig. 1. The uncoupling effect of DNP in the energy-transfer reaction

The incubation flask contained (in 3 ml) 600 μmoles sucrose, 100 μmoles Tris. pH 7.4, 0.2 μmoles EDTA, 20.2 μmoles MgCl_2 , 0.06 μmoles cold P_i , 1.0×10^6 cpm of $^{32}\text{P}_i$, and 2 μmoles of ADP. 2.0 ml of supernatant fraction containing 0.3 mg protein/ml was added to initiate the reaction. The incubation was terminated after 12 minutes.

phosphate esterified (Table VII, Experiment A). In the presence of P_i , the DNP effect was minimized. This would suggest that the P_i and DNP are reacting with the same component. It is also interesting to note that DNP does not influence the marked stimulation of the transfer activity obtained with succinate and CoA. The effect of DNP could be summarized as follows: (i) Increasing concentration of DNP resulted in a decreasing amount of phosphate esterified; presumably some high energy intermediate was destroyed by forming a readily hydrolyzable enzyme-bound DNP derivative. (ii) DNP and P_i appear to react with the same component in a competitive manner. (iii) DNP does not influence the marked stimulation of the transfer activity obtained with succinate and CoA. As will be discussed later, in the presence of succinate and CoA it is assumed that the intermediate is used so efficiently that the action of DNP is minimized. Based on this evidence, one might suggest that the action of DNP is due to its participation in the destruction of the nonphosphorylated high energy intermediate. Further discussion of the action of DNP will be presented along with other results in this thesis.

Borst and Slater (4), and Azzone and Ernster (1) have pointed out that oxidative phosphorylation is sensitive to DNP uncoupling while the substrate level phosphorylation is known to be resistant to this inhibitor. The effect of DNP in the energy transfer activity

Table VII. The uncoupling effect of DNP on the soluble high-energy intermediate

| Expt No * | 32 Pi+ Pi | Acceptor | DPN | Other Variables | Incubation Time (min) | Pi-esterified m μ moles x 10 |
|--------------|-------------------|----------|-------|----------------------|--------------------------|-------------------------------------|
| A | + (0) | -- | -- | -- | 12 | 21.90 |
| | + (0) | + (0) | -- | -- | 12 | 38.55 |
| | + (0) | + (12) | + (0) | -- | 24 | 41.01 |
| | + (13) | + (12) | + (0) | -- | 24 | 14.88 |
| B | + (0) | -- | -- | -- | 12 | 0.78 |
| | + (0) | + (0) | -- | -- | 12 | 19.20 |
| | + (0) | + (0) | + (0) | -- | 12 | 11.42 |
| | + (0) | + (0) | -- | (Succinate + CoA)(o) | 12 | 296.58 |
| | + (0) | + (0) | + (0) | (Succinate + CoA)(o) | 12 | 302.58 |

* The experimental procedure of Expt A was that of Method B, using 30 μ moles pyruvate and 0.2 μ moles fumarate as substrate; the other components are the same as the control system of Table V in the first incubation. Experiment B was carried out as stated in the legend of Figure 1. Protein content of supernate fraction for each experiment: Expt. A, 0.56 mg/ml, Expt. B, 0.47 mg/ml. 2ml of supernate fraction was added to each flask at the beginning of the incubation (0 time) the number in the parenthesis indicates the minutes thereafter when that reagent was added. The concentration of other variables where indicated: Pi, $6.0 \times 10^{-2} \mu$ moles. DNP, 0.3 μ moles, Succinate: 0.2 μ moles, CoA 0.025 μ moles, about 1.0×10^6 cpm 32 Pi was added.

in our system is strong evidence for the respiratory coupled phosphorylation rather than substrate level phosphorylation.

Oligomycin is a very potent inhibitor of oxidative phosphorylation and the associated respiration. It has been suggested by some that its action is due to the blocking of the reaction of phosphate with a nonphosphorylated intermediate, (13, 102, P. 157-158, 132). On the other hand, Lardy and his collaborators (72) indicated that oligomycin prevented phosphate transfer to acceptor. Lardy's view has been supported by Kenefick and Hanson (64) in that oligomycin blocks ATP formation and utilization but not $\text{Ca}^{++} + \text{P}_i$ transport. They concluded that the inhibition lies between $\text{X}\sim\text{P}$ and ATP and does not affect the Ca^{++} -activated discharge of $\text{X}\sim\text{P}$ in ion transport. In any event, oligomycin inhibition has been found to be very specific for respiratory chain-linked phosphorylation, and it fails to inhibit substrate level phosphorylation (24). The results presented in Table VIII show that oligomycin does inhibit the energy transfer activity. When added with the acceptor system oligomycin reduced the amount of phosphate esterified by some 25%. A similar response was obtained when oligomycin was incubated with the soluble system prior to the addition of the acceptor.

The evidence presented virtually eliminates the possibility of substrate level phosphorylation. It is thus assumed that coupled

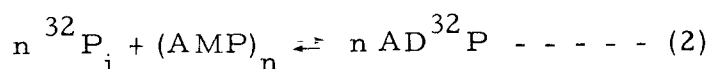
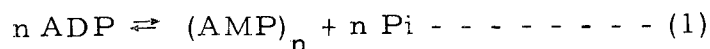
Table VIII. The inhibitory effect of oligomycin on energy-transfer activity

| Acceptor | Inhibitor | Incubation Time (min.) | % NE ^{32}P |
|----------|-----------------|-------------------------|----------------------|
| ADP (0) | - | 10 | 42.30 |
| ADP (0) | - | 20 | 55.90 |
| ADP (0) | Oligomycin (0) | 10 | 33.50 |
| ADP (0) | Oligomycin (10) | 20 | 51.00 |
| ADP (10) | Oligomycin (0) | 20 | 25.60 |

The incubation procedure was that of method B as stated for Experiment A in Table VII except that an additional 20 μ moles of succinate has been added to the first incubation medium as substrate. 2 ml of supernatant fraction containing 0.64 mg protein/ml was added to each flask at the beginning of the incubation, the number in parenthesis indicates the time when that reagent was added. Other variables in the second incubation medium where indicated: 2 μ moles ADP and 6 μ g oligomycin.

electron transport produces a small amount of a nonphosphorylated high energy intermediate, which exists in the supernatant fraction. This intermediate provides the energy required for the formation of ATP in the absence of the particulate fraction of the mitochondria. In the presence of hexokinase and glucose the esterified phosphate existed primarily as glucose-6-phosphate.

Another possibility is that the acceptor effect (the increase in the percentage of nonextractable phosphate, i. e. the amount of esterified phosphate after addition of acceptor) could be due to the incorporation of $^{32}\text{P}_i$ by ADP- $^{32}\text{P}_i$ exchange, possibly catalyzed by polynucleotide phosphorylase (44). This exchange reaction can be described by the following equations:



The data summarized in Table IV suggest that a more stable organic phosphate compound was produced with the hexokinase system as the $^{32}\text{P}_i$ acceptor than with ADP alone as $^{32}\text{P}_i$ acceptor. This would indicate that the terminal ^{32}P had been transferred to a more stable compound (G-6-P) while ADP- ^{32}P (AT- ^{32}P) and the other phosphorylated intermediate formed were more labile to the acid hydrolysis, resulting in less organic phosphate ester after such treatment. This in turn showed that $^{32}\text{P}_i$ incorporation actually involves the terminal phosphate of ATP which is formed through

energy transfer reaction. Thus the ADP- P_i exchange does not appear to be a significant factor. The other alternative that the acceptor effect is due to ATP- P_i exchange due to ATPase or thiokinase will be considered in the following section.

V. The Role of Succinate and CoA in Oxidative Phosphorylation

It has been mentioned that the addition of succinate and CoA to the soluble fraction, which contained at least one of the intermediates of oxidative phosphorylation, brought about a marked increase in the energy transferring activity. This observation has focussed attention on the possible role of succinate or CoA in the energy transfer reaction. Initial studies with the supernatant fraction showed that either succinate or CoA when added in the second incubation stimulated the acceptor effect (Table IX). The response when both succinate and CoA were added was greater than when either component was added alone. A similar effect could be obtained by adding one component in the first incubation and the other in the second incubation.

Other dicarboxylic acids failed to give the same response (Table X). Malonate and fumarate even inhibited the acceptor effect. Fumarate in higher concentration further suppressed the succinate effect. Only α -ketoglutarate produced an increase in the

Table IX. Stimulating effect of succinate and/or CoA upon energy transfer activity

| First Incubation | Second Incubation | | | NE 32 P |
|---------------------|-------------------|-----------------|-----------|--------------|
| | Acceptor | Additions | Time(min) | |
| Control | - | - | 0 | 0.33 |
| " | - | - | 6 | 0.49 |
| " | + | - | 6 | 2.25 |
| " | - | Succinate | 6 | 0.46 |
| " | + | Succinate | 6 | 12.80 |
| " | - | CoA | 6 | 0.36 |
| " | + | CoA | 6 | 4.40 |
| " | - | Succinate + CoA | 6 | 0.35 |
| " | + | Succinate + CoA | 6 | 24.00 |
| Control + Succinate | - | - | 6 | 0.42 |
| " " | + | - | 6 | 14.50 |
| " " | + | CoA | 6 | 25.00 |
| " " | - | CoA | 6 | 0.33 |
| Control + CoA | - | - | 6 | 0.64 |
| " " | + | - | 6 | 4.20 |
| " " | + | Succinate | 6 | 29.30 |
| " " | - | Succinate | 6 | 0.41 |

The incubation procedure was the same as that of method B. The control system for the first incubation contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris pH 7.4, 20 μ moles MgCl_2 , 31 μ moles pyruvate, 0.26 μ moles fumarate, 6 μ moles EDTA. Two ml of mitochondrial suspension was added to initiate the reaction. The first incubation was carried out at 25°C for 5 minutes. The supernatant fractions were obtained by sonicating the incubation mixture after the first incubation; centrifuging out the particulate fractions as described. In the second incubation 2 ml of the supernatant fraction (containing 0.84 mg protein/ml) was used to initiate the reaction. The incubation medium contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris, pH 7.4, 4 μ moles EDTA, 34 μ moles MgCl_2 , 1×10^6 cpm of $^{32}\text{P}_i$, 2 μ moles of ADP was used as acceptor. Other variables as indicated 2 μ moles succinate, 0.15 μ moles CoA.

Table X. Substrate specificity of succinate-stimulated energy transfer reaction

| Expt No. | First Incubation | Second Incubation Addition | Time | % NE ³² P |
|----------|------------------|-------------------------------|------|----------------------|
| A | Control | - | 20 | 3.05 |
| | " | Succinate | 20 | 39.50 |
| | " | Malate | 20 | 4.70 |
| B | Control | - | 10 | 2.10 |
| | " | Succinate | 10 | 18.30 |
| | " | Malonate | 10 | 2.53 |
| | " + Malonate | - | 10 | 4.15 |
| | " + " | Succinate | 10 | 14.87 |
| | " + Succinate | - | 10 | 22.87 |
| | " + " | Malonate | 10 | 21.05 |
| C | Control | - | 10 | 3.48 |
| | " | Fumarate (2) | 10 | 3.44 |
| | " | Succinate | 10 | 42.90 |
| | " | Succinate + Fumarate (2) | 10 | 39.00 |
| | " | Succinate + Fumarate (20) | 10 | 20.45 |
| | " | α -Ketoglutarate | 10 | 14.50 |

The incubation procedure and the control system of the first incubation were the same as described in Table IX except only 1.0 μ moles pyruvate and 0.06 μ moles fumarate were used as substrate. The amount of other variables added were succinate, 20 μ moles, malate, 20 μ moles, malonate, 20 μ moles, α -ketoglutarate, 5 μ moles. In case of Expt. C, only 2 μ moles of succinate were added where indicated. The number after fumarate represents the amount of fumarate used in μ moles. Expt A, B, and C contained 0.69 mg, 0.74 mg, and 0.86 mg protein per ml respectively.

amount of esterified phosphate compared to the control which had acceptor alone in the medium. The effect observed with this was only 25% of that observed with both succinate and CoA in the same experiment (Experiment C).

Either some structural relationship exists between succinate and α -ketoglutarate with succinate being the more efficient or α -ketoglutarate is converted to succinyl CoA. These data pose the question whether succinate and CoA are involved in transfer activity or stimulate phosphate esterification through the thiokinase reaction (54). This question will be discussed further in this section.

A. Definite requirement for succinate and CoA in transfer reaction

Studies with fractions of the supernatant fraction obtained by ammonium sulfate fractionation or acetone treatment indicated a definite requirement of succinate and CoA in the energy transfer reaction. In addition, since continuous flow dialysis of the supernatant fraction did not lead to the loss of intermediary activity, it appeared that this intermediate in the supernatant fraction was proteinaceous (Table XI). This view was further supported by the results of experiments using salt fractionation and the acetone powder process.

Table XI. Illustration of the protein nature of the intermediate and the requirement of succinate and CoA in energy transfer reaction

| Expt No | Enzyme System | Treatment after First Incubation | Second Incubation | | Time (min) | % NE ³² P |
|---------|------------------|----------------------------------|-------------------|-------------------|------------|----------------------|
| | | | Acceptor | Additions | | |
| A | Soluble Fraction | -- | -- | -- | 10 | 3.24 |
| | " | -- | ADP | -- | 10 | 6.70 |
| | " | -- | ADP | Succ. | 10 | 20.40 |
| | " | Dialysis | -- | -- | 10 | 0.23 |
| | " | " | ADP | -- | 10 | 2.96 |
| | " | " | ADP | Succ. | 10 | 14.65 |
| B | 0-50% Fraction | Salt Fractionation | Hex. Syst. | -- | 6 | 0.17 |
| | " | " | Hex. Syst. | Succ. + CoA | 6 | 0.08 |
| | " | " | -- | Succ. + CoA + ATP | 6 | 0.11 |
| | 50-75% Fraction | " | Hex. Syst. | -- | 6 | 0.13 |
| | " | " | Hex. Syst. | Succ. + CoA | 6 | 42.80 |
| | " | " | -- | Succ. + CoA + ATP | 6 | 37.70 |
| | 75-100% Fraction | " | Hex. Syst. | -- | 6 | 0.054 |
| | " | " | Hex. Syst. | Succ. + CoA | 6 | 11.12 |
| | " | " | -- | Succ. + CoA + ATP | 6 | 15.20 |
| | 100% Fraction | " | Hex. Syst. | -- | 6 | 0.049 |
| C | Soluble Protein | Acetone powder | Hex. Syst. | -- | 12 | 0 |
| | " | " | " | Succ. | 12 | 0.32 |
| | " | " | " | CoA | 12 | 0.59 |
| | " | " | " | Succ. + CoA | 12 | 10.06 |
| | " | " | " | Succ. + CoA | 12 | 10.06 |

The incubation procedure of Expt. A was the same as stated in Table IX. 2 μ moles ADP and 20 μ moles of succinate were added where indicated. The soluble fraction contained 0.77 mg/ml. In Expt. B the incubation procedures for the first and second incubation were the same as above except 0.003 μ moles cold Pi and a hexokinase system consisting of 2 μ moles ADP, 50 μ moles of glucose and 50 KG units of hexokinase was used as acceptor and 2 μ moles succinate, 0.15 μ mole CoA and 2 μ moles ATP, were used where indicated. The protein content of each fraction was: 0-50%, 0.52 mg/ml; 50-75%, 1.80 mg/ml; 75-100%, 1.12 mg/ml; > 100%, 1.12 mg/ml. Incubation procedure of Expt. C was the same as that of Method C. Incubation flask contained (in 3 ml) 600 μ moles sucrose, 100 μ moles Tris, 0.2 μ moles EDTA, 20.2 μ moles MgCl_2 , 0.06 μ moles cold Pi, 10^6 cpm of ^{32}Pi , 0.1 ml of 3% albumin. The other variables, where indicated, 0.2 μ moles succinate, 0.075 μ moles CoA, hexokinase system composed of 2 μ moles ADP, 25 μ moles glucose, 50 KG unit hexokinase, protein content, 0.45 mg/ml.

In Experiment B (Table XI) the supernatant fraction was obtained after the 10 minute preincubation of the whole mitochondria with pyruvate and fumarate as substrate and then subjected to ammonium sulfate salt fractionation as described in Methods. The protein precipitates of each fraction were collected after centrifugation, redissolved in suspension medium and dialyzed with the buffered solution (pH 7.4) for 20 hours. The resultant clear, dialyzed solution was then tested for transfer activity. The addition of acceptor alone failed to show any transfer activity, suggesting that some essential factor must have been lost during the process. Addition of succinate and CoA together with the acceptor system restored activity and indicated that the intermediate was retained in the fractions of 50-75% and 75-100% of salt concentrations with about 75% of the activity residing in the fraction of 50-75% saturation. In this experiment ATP was used to test for thiokinase activity as will be discussed later.

Convincing evidence for the involvement of succinate and CoA in the transfer process has been obtained by acetone treatment of the supernatant fraction. The supernatant fraction was dialyzed against 0.05 M Tris buffer, pH 7.4, for 36 hours and the dialyzed supernatant was poured into 2 volumes of precooled acetone with constant stirring. The protein precipitated was centrifuged and reconstituted

with the same volume of suspension medium. The reconstituted protein solution was then tested for transfer activity. No transfer activity was evident even in the presence of acceptor system (Table XI). Addition of either succinate or CoA restored some activity but addition of both components gave a marked increase in activity. These results show that the high energy intermediate survived such treatment and that the essential factor or factors for the transfer activity were lost during the process. The requirement of CoA and succinate is thus clearly demonstrated.

In other experiments, it has been shown that only a catalytic amount of both succinate and CoA are required for the transfer activity (Figure 2 and Figure 3). The inhibitory effect of high concentrations of succinate and CoA is possibly due to product inhibition. This type of inhibition is very common, especially for readily reversible reaction, such as the inhibition of ATPase by ADP (3) and inhibition of glycine transacylase by CoA (114). If succinate and/or CoA are involved in the transfer reaction in some intermediary step by combining with enzyme or one of the components, they will be released eventually at some later step. Inhibition in the presence of excess succinate or CoA could result from the reversal of one of the steps in the reaction sequence or from the combination with the enzyme making it unavailable to the substrates. This type of

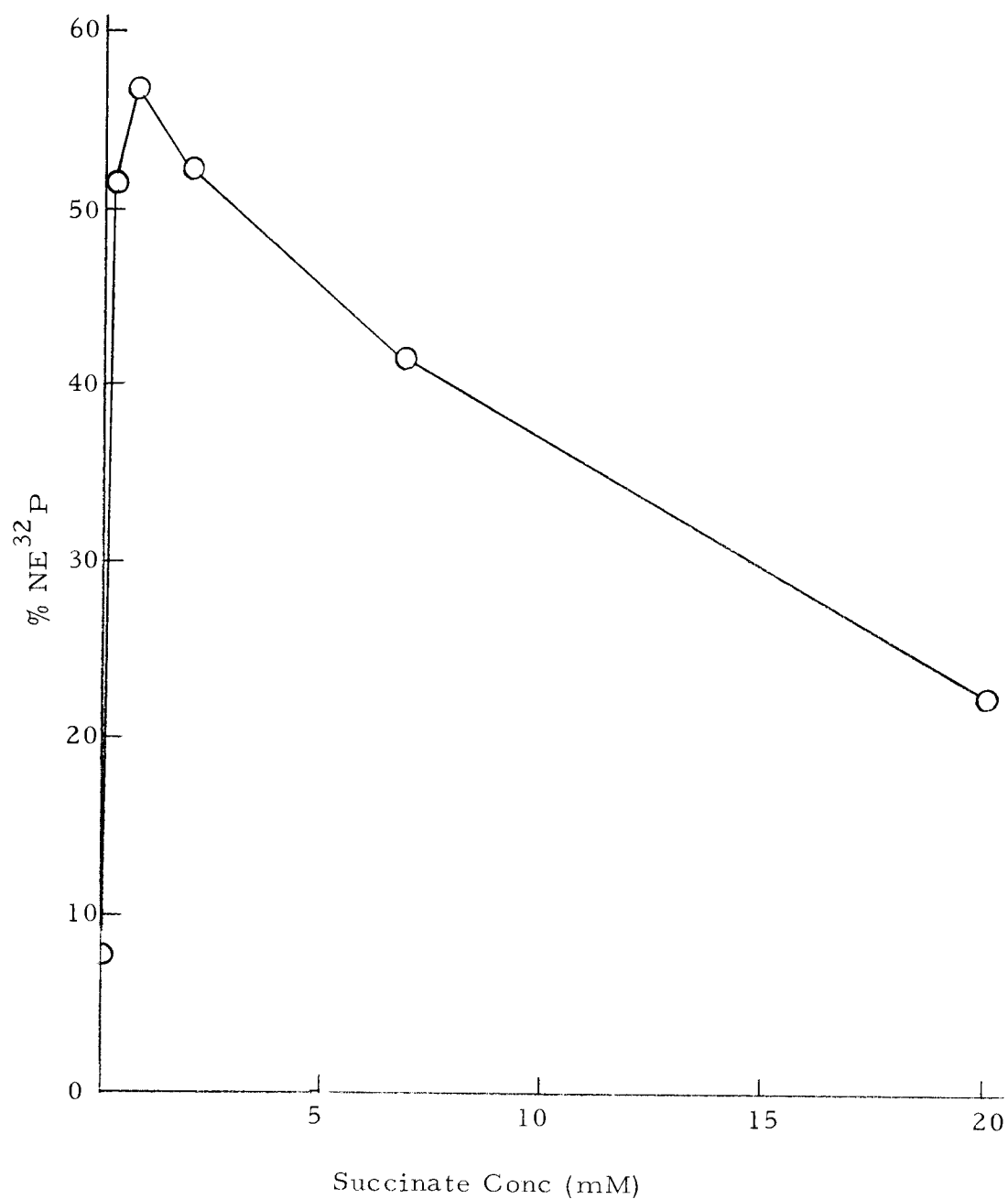


Fig. 2. Effect of succinate concentration on energy transfer activity

The incubation procedure was the same as stated in Table IX. Two moles ADP was used as acceptor. Protein content of supernatant fraction 0.7 mg/ml

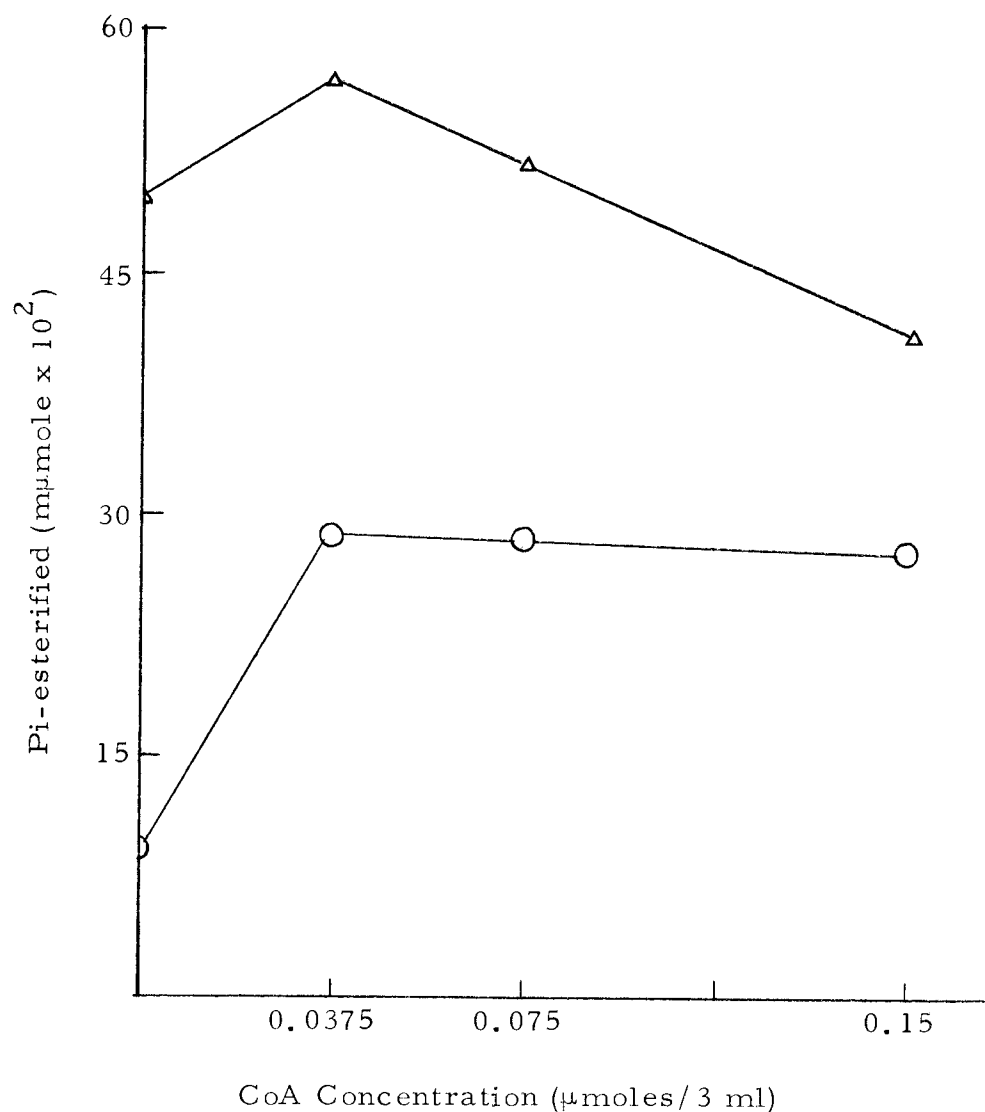
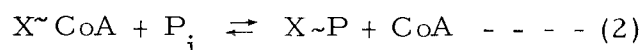


Fig. 3. Effect of CoA concentration on energy transfer activity

The incubation procedure was the same as stated in Table IX except 0.003 μmoles of cold phosphate was added as carrier. 2 μmoles ADP, 50 μmoles glucose and 50 KG units constituted the acceptor system. Protein content of the supernatant fraction was 0.54 mg/ml. control \circ — \circ , control + added succinate (2 μmoles) Δ — Δ .

effect can be illustrated in the following two equations:



Excess CoA forces Eq. (2) in reverse direction or binds with X making it unavailable to P_i .

In the absence of added CoA the system still showed some transfer activity especially when catalytic amounts of succinate were added. This is probably due to endogenous CoA within the mitochondria released during the sonication process. The action of SH specific reagents, such as p-chloromercuribenzoate (PCMB), in the second incubation medium where the transfer reaction takes place would support this assumption. PCMB inhibits the transfer activity completely in the presence or absence of added succinate. Addition of CoA restored the activity in both cases as shown in Figure 4.

PCMB could react with SH groups on the enzyme molecules, however our data would suggest that the involvement of SH group on the enzyme surface in the transfer reaction is not likely. It is generally understood that aging would result in the oxidation of the SH group of protein molecules and hence lead to the loss of enzyme activity if SH groups are involved at the active centers. Experimental evidence showed that aging did not affect the energy transfer

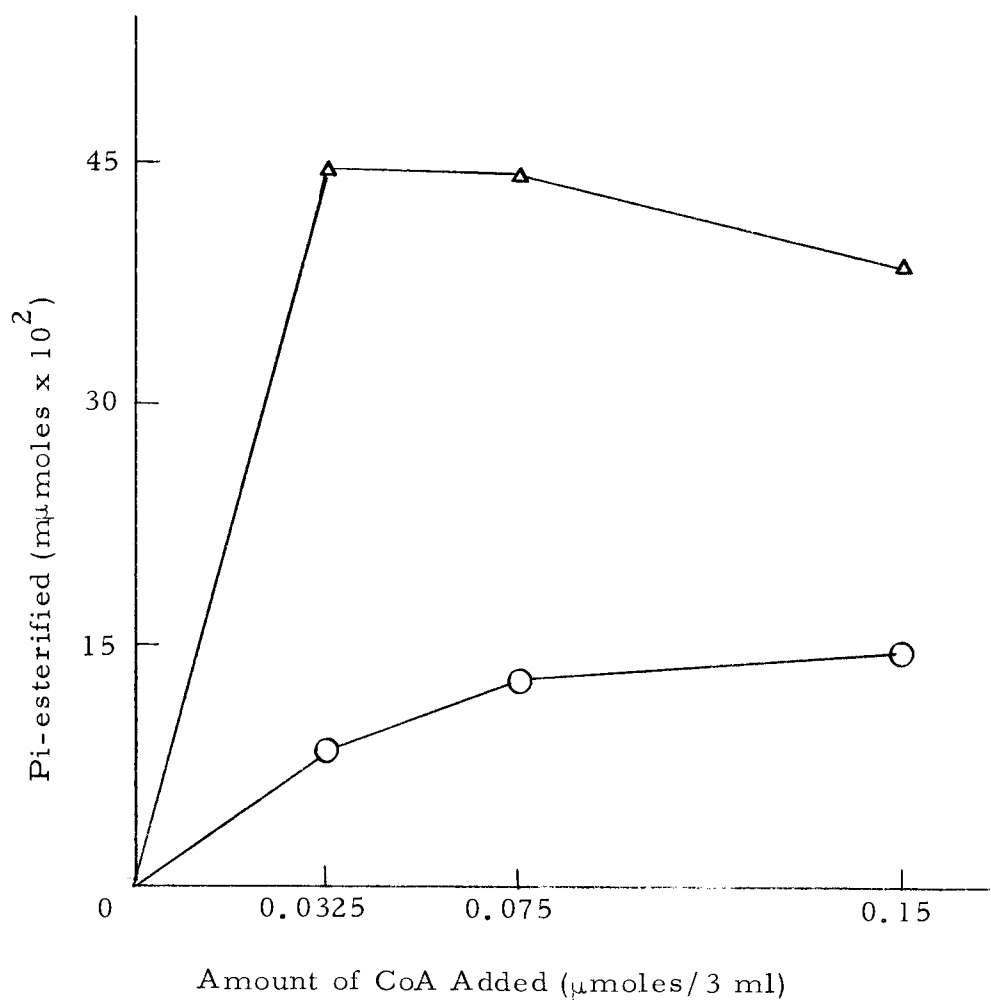


Fig. 4. PCMB and CoA interaction in transfer activity

The incubation procedure and conditions are the same as stated in Fig. 3 except $2 \times 10^{-5} \text{M}$ of PCMB was present in the second incubation mixture. Control ○—○, control + succinate ($2 \mu\text{moles}$) Δ—Δ. Second incubation was carried out at 25°C for 6 min.

activity. This might explain why little transfer activity was lost during salt fractionation and acetone treatment. Furthermore, cysteine should show the same "protective effect" as CoASH if SH groups attached to the enzyme were involved in energy transfer activity. The addition of cysteine had no effect on transfer activity (Table XII). This result again shows that CoA is very specific for the energy transfer reaction. Summarizing the evidence to date we observe that:

1. PCMB inhibits completely the transfer reaction; addition of CoA restores the activity (Figure 4).
2. Ammonium sulfate fractionation and acetone treatment led to the loss of transfer activity; addition of succinate and CoA restored transfer activity in some fractions obtained from salt-fractionation and in the acetone powder preparation.
3. Dialysis led to the loss of some transfer activity.
4. Aging did not affect the activity and thus the oxidation of the enzyme would not appear to be a factor in accounting for the loss of transfer capacity during various analytical processes.
5. Cysteine cannot replace CoA in the stimulating of transfer activity.

Table XII. Effect of aging and SH protecting reagent on transfer activity

| Expt. No. | Treatment after First Incubation | Second Incubation Addition | % NE ³² P |
|--------------|-------------------------------------|-------------------------------|----------------------|
| A | - | - | 3.57 |
| | Aging* | - | 3.50 |
| | - | Succinate + CoA | 15.10 |
| | Aging | " " | 18.25 |
| B | - | - | 5.33 |
| | - | CoA | 13.35 |
| | - | Succinate | 17.65 |
| | - | Succinate + CoA | 28.50 |
| | - | Cysteine (0.45) | 4.12 |
| | - | Succinate + Cysteine (0.15) | 16.50 |
| | - | Succinate + Cysteine (0.45) | 15.35 |

(1) The incubation procedure was stated in Fig. 3. Other components were added in the following amounts where indicated. 4 μ moles succinate, 0.5 μ moles CoA in Expt. A, 2 μ moles succinate, 0.039 μ moles CoA. The amount of cysteine was stated in μ moles in parenthesis. Protein content: 0.64 mg/ml for Expt. A; 0.54 mg/ml for Expt. B. The incubation was carried out at 25°C for 6 minutes.

*(2) Aging of the soluble fraction was carried out in a shaking incubator at 25°C for 15 minutes before the second incubation.

It appeared that the external or loosely bound SH reagent (CoA) is involved rather than the SH group attached to the enzyme itself for the transfer activity.

Other studies with the acetone powder showed that malonate had no effect on transfer activity stimulated by succinate and CoA. Oligomycin at a concentration of 3.3 $\mu\text{g/ml}$ inhibits the succinate and CoA stimulated transfer activity by about 40%. Arsenate severely inhibits the reaction, while DNP has no effect at all (Table XIII). These results are very consistent with those observed using the supernatant fraction. Antimycin A was generally regarded as an uncoupler of the high energy intermediate $\text{DPN}^{\sim}\text{I}$ or A^{\sim}I according to Hulsmann's theory (117). From the experiments with the acetone-treated protein fraction, the inhibitory effect on transfer activity observed with antimycin A is due to the absolute alcohol in which it was dissolved. GDP cannot substitute for ADP in the transfer reaction. In fact GDP added to the succinate and CoA stimulated transfer system inhibited the transfer activity, probably due to the inhibition by coenzyme analogs (135). The system in which ATP, succinate and CoA are added is used in the study of $^{32}\text{P}_i$ -ATP exchange reaction to investigate the possible involvement of succinate and CoA in the succinyl thiokinase reaction. This will be discussed in detail in the next section.

Table XIII. Some properties of the acetone treated protein fraction

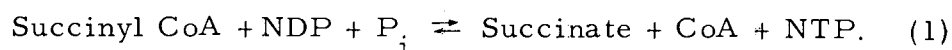
| Expt. No. | Acceptor | Additions | Inhibitor | Esterified Phosphate μmoles x 10 |
|--------------|----------|------------------|-------------|--|
| A | + | - | - | 0 |
| | + | Succ | - | 1.92 |
| | + | Succ | Arsenate | 0 |
| | + | CoA | - | 3.54 |
| | + | CoA | Arsenate | 0.42 |
| | + | Succ + CoA | - | 60.36 |
| | + | Succ + CoA | Arsenate | 0.48 |
| | - | Succ + CoA + ATP | - | 44.02 |
| | - | Succ + CoA + ATP | Arsenate | 29.28 |
| | + | Succ + CoA | Malonate | 59.64 |
| | - | Succ + CoA + ATP | Malonate | 21.24 |
| | + | Succ + CoA + GDP | - | 14.40 |
| | - | Succ + CoA + GDP | - | 0.18 |
| B | + | - | - | 1.02 |
| | + | Succ + CoA | - | 69.06 |
| | + | Succ + CoA | Antimycin A | 25.68 |
| | + | Succ + CoA | Alcohol | 24.12 |
| | + | Succ + CoA | DNP | 67.02 |
| | + | Succ + CoA | Oligomycin | 39.18 |

The experimental procedure and conditions are the same, as stated in Table XI. Other components were added in the following amounts where indicated: 0.2 μmoles succinate, 0.075 μmoles CoA, 2 μmoles ATP, 1.0 μmoles malonate, 2 μmoles GDP, 0.6 μmoles DNP, 10 μg antimycin A in 0.1 absolute alcohol, 10 μg oligomycin, 0.1 ml alcohol. Twenty-five μmoles glucose and 50 KG units of hexokinase were used. Protein content of both experiments: Expt. A, 0.45 mg/ml; Expt. B, 0.40 mg/ml.

B. Succinate thiokinase activity

In a series of studies in Boyer's group on the possible role of bound phosphohistidine in mitochondrial phosphorylation, it was demonstrated that the soluble protein from mitochondria capable of forming bound phosphohistidine was succinate thiokinase (91). It was also observed that highly purified succinate thiokinase from *E. coli* contained bound phosphohistidine (70). The question arose in this study whether the influence of succinate and CoA was also due to succinate thiokinase.

Succinate thiokinase has been shown to catalyze the following reaction:



The NDP and NTP in the equation may be either guanosine or inosine derivatives with the mammalian enzyme preparations (111) or adenosine di- and triphosphates with enzyme preparations from spinach and *E. coli* (63, 119). An exchange reaction between ATP and $^{32}\text{P}_i$ as demonstrated by Kaufman (62) has been attempted and it has been shown that ATP may be used as the source, nucleoside diphosphokinase, which catalyzes the equilibration of GTP (or ITP) with ATP according to Equation 2, may be present in the system.



Table XIV summarizes the $^{32}\text{P}_i$ -ATP exchange activity in different

Table XIV. $^{32}\text{P}_i$ -ATP exchange activity in various enzyme preparations

| Expt No * | Enzyme Preparation | Protein Content (mg/ml) | Substrate | ATP Added | Inhibitor or Activator | Incubation Time (min) | % NEP |
|-----------|--------------------|-------------------------|-----------------------|-----------|------------------------|-----------------------|-------|
| A | Supernate fraction | 0.52 | -- | + | -- | 6 | 0.12 |
| | " | | Succ (2)+CoA(0.15) | - | -- | 6 | 0.14 |
| | " | | " + " | + | -- | 6 | 26.50 |
| B | " | 0.96 | Succ (20) | - | -- | 10 | 0.27 |
| | " | | " | + | -- | 10 | 0.36 |
| C | " | 0.63 | Succ (2)+CoA(0.15) | + | Mg^{++} (34) | 6 | 7.95 |
| | " | | " + " | + | Mg^{++} (14) | 6 | 4.30 |
| | " | | " + " | + | EDTA (20) | 6 | 0.17 |
| D | " | 0.94 | " + " | + | -- | 12 | 12.15 |
| | " | | " + " | + | GDP (0.2) | 12 | 14.65 |
| | " | | " + " | + | Hydroxylamine (400) | 12 | 0.47 |
| E | " | 0.51 | " + " | + | -- | 6 | 20.90 |
| | " | | " + " | + | Oligomycin (6) | 6 | 19.05 |
| F | " | 0.63 | " + " | + | -- | 6 | 7.95 |
| | " | | " + " | + | PCMB (0.12) | 6 | 3.88 |
| G | " | 0.47 | Succ (0.2)+CoA(0.075) | + | -- | 12 | 22.30 |
| | " | | " + " | + | DNP (0.6) | 12 | 18.30 |
| H | " | 0.08 | " + " | + | -- | 12 | 19.80 |
| | " | | " + " | + | Arsenate (1) | 12 | 11.05 |
| I | Acetone treated | 0.40 | " + " | + | -- | 12 | 9.52 |
| | " | | " + " | + | Antimycin A (10) | 12 | 8.84 |

* The incubation procedure was stated in Table IX except cold P_i has been added as ^{32}P -carrier throughout the experiments except Expt. B and E. The amounts of other components added in μ moles in parenthesis following that reagent except antimycin A and oligomycin which were expressed in μg .

enzyme preparations in addition to those shown in Table XI and Table XIII. The percentage of $^{32}\text{P}_i$ incorporated into ATP is measured as $\% \text{NE}^{32}\text{P}$. The results have shown that the exchange activity depends on the presence of succinate, CoA, ATP and Mg^{++} ion. GDP enhances the activity a little. Hydroxylamine at 400 $\mu\text{moles}/3\text{ ml}$ completely inhibits the reaction. PCMB at $4 \times 10^{-5}\text{ mM}$ inhibits the exchange activity to about 50 percent. DNP and antimycin A have little effect while arsenate inhibits to about 40 percent. The substrate specificity and inhibitor studies appear to be consistent with observations from other laboratories studying succinate thio-kinase (16, 54). The small response observed with oligomycin, DNP and antimycin was probably due to transferring activity which was also present in the system.

It is of interest to observe the difference in kinetics of the transfer and exchange reactions (Figure 5). There is a lag phase for the first six minutes for exchange activity which is probably due to a lack of ADP. After accumulating sufficient ADP then both the reversal of succinate thiokinase activity and the transfer reaction can occur simultaneously, resulting in a linear increase in the amount of phosphate esterified. The following equations might indicate the possible reactions when ATP, succinate and CoA are added to the incubation system.

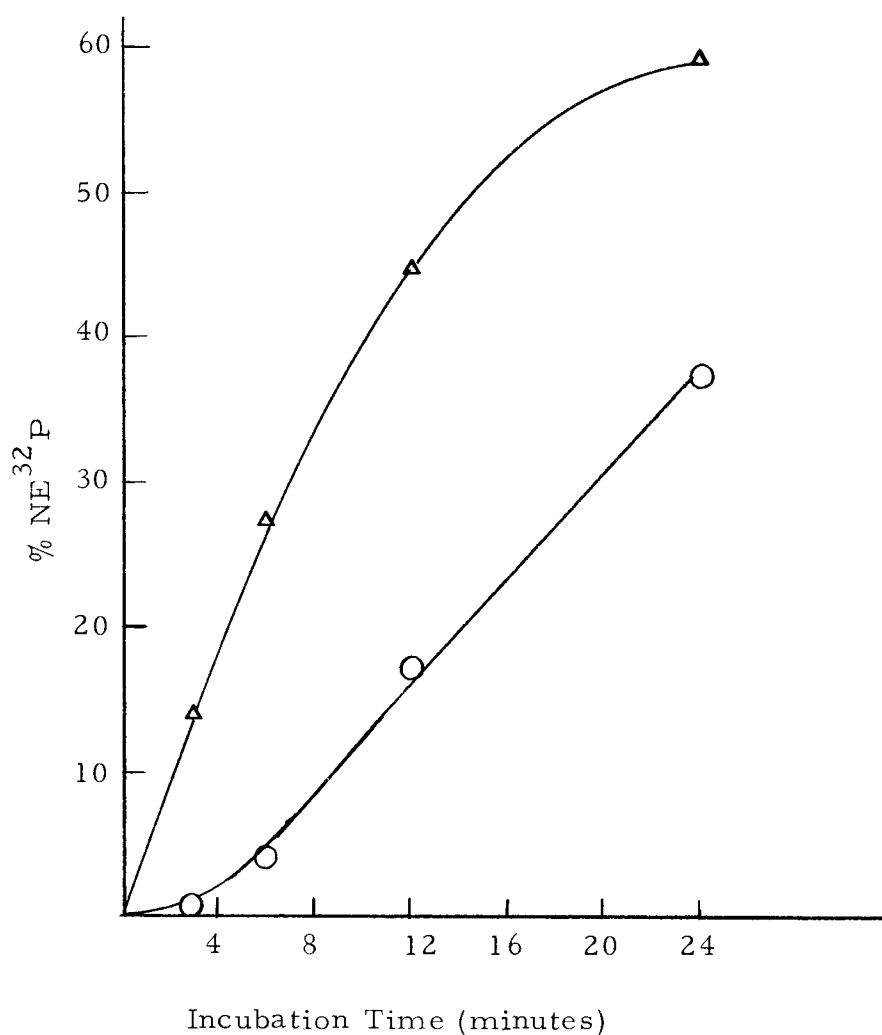
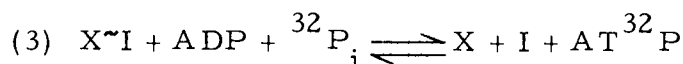
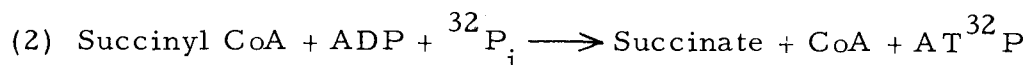


Fig. 5. Time course of $^{32}\text{P}_i$ -ATP exchange activity and transfer activity

The incubation procedure was the same as stated in Fig. 3, except 2 μmole ATP was added instead of hexokinase system for exchange activity (curve I), Curve II represents the succinate and CoA stimulated transfer activity. In both cases, 2 μmoles succinate and 0.15 μmoles CoA was added. Protein content of supernatant fraction : 0.66 mg/ml.



Equations (1) and (2) represent reversible thiokinase reaction resulting in ATP- ${}^{32}\text{P}_i$ exchange. Equation (3) represents the direct energy transfer. On the other hand, in the presence of hexokinase acceptor system the transfer reaction occurred immediately and flattened off at a later stage of incubation, indicating a limited amount of intermediate present in the incubation medium.

The stimulation of ATP- ${}^{32}\text{P}_i$ exchange by succinate and CoA demonstrated that our system contained thiokinase activity. The next question which had to be answered was to what extent the thiokinase activity accounts for the succinate-CoA effect on transfer activity. Since it was not possible to isolate the thiokinase activity from the transfer activity, our approach has been to study the influence of inhibitors on both the exchange and the transfer system.

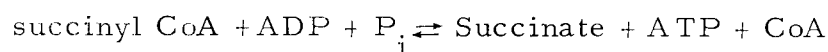
C. Comparison of exchange and transfer systems

Addition of ATP to the incubation medium containing succinate and CoA has resulted in an increase in %NE ${}^{32}\text{P}$ which is probably due both to exchange activity due to thiokinase and transfer activity (Table XIV and Figure 5). It could be argued that the stimulating

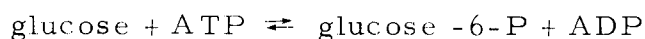
effect of succinate and CoA in transfer activity could be a result of $^{32}\text{P}_i$ -ATP exchange through succinate thiokinase activity, especially since this activity is associated with the transfer activity even after salt fractionation and acetone treatment (see Tables XI, XIII). Although the attempt to remove succinate thiokinase activity has failed, the following considerations indicate that the possibility of the succinate and CoA stimulating effect on transfer activity resulting from $^{32}\text{P}_i$ -ATP exchange is remote.

1. From thermodynamic considerations in the presence of hexokinase, any ATP formed would appear to form predominantly glucose-6-phosphate rather than participate in an exchange reaction. This may be understood better by calculating the ratio of G-6-P and succinyl CoA from the same source (ATP) at equilibrium condition. The equilibrium constants of succinate thiokinase and hexokinase are $K_1 = 3.7$ at pH 7.0 (54) and $K_2 = 2,460$ at pH 7.0 (105) respectively.

At equilibrium



$$K_1 = \frac{(\text{Succinate})(\text{CoA})(\text{ATP})}{(\text{Succinyl CoA})(\text{ADP})(\text{P}_i)} = 3.7 \quad (1)$$



$$K_2 = \frac{(\text{Glucose 6-P})(\text{ADP})}{(\text{Glucose})(\text{ATP})} = 2,460 \quad (2)$$

Under most of the experimental conditions

$$(\text{Succinate}) = 0.2 \text{ } \mu\text{moles} / 3 \text{ ml or } 0.067 \text{ mM}$$

$$(\text{CoA}) = 0.075 \text{ } \mu\text{moles} / 3\text{ml or } 0.025 \text{ mM}$$

$$(\text{ADP}) = 2 \text{ } \mu\text{moles} / 3 \text{ ml or } 0.67 \text{ mM}$$

$$(\text{P}_i) = 0.06 \text{ } \mu\text{moles} / 3 \text{ ml or } 0.020 \text{ mM}$$

$$(\text{Glucose}) = 25 \text{ } \mu\text{moles} / 3\text{ml or } 8.34 \text{ mM}$$

$$\text{From equation (1) } (\text{ATP})_1 = \frac{3.7(\text{P}_i)(\text{ADP})(\text{Succinyl CoA})}{(\text{Succinate})(\text{CoA})}$$

$$(2) (\text{ATP})_2 = \frac{(\text{G-6-P})(\text{ADP})}{(\text{Glucose})(2,460)}$$

At equilibrium

$$(\text{ATP})_1 = (\text{ATP})_2$$

Thus

$$\frac{3.7 (\text{P}_i) (\text{ADP}) (\text{Succinyl CoA})}{(\text{Succinate}) (\text{CoA})} = \frac{(\text{G-6-P}) (\text{ADP})}{(\text{Glucose})(2,460)}$$

$$\text{i. e. } \frac{(\text{G-6-P})}{(\text{Succinyl CoA})} = \frac{3.7 (\text{P}_i)(\text{Glucose})(2,460)}{(\text{Succinate}) (\text{CoASH})} =$$

$$\frac{(3.7)(0.02)(8.34)(2,460)}{(0.067) (0.025)}$$

$$\frac{(\text{G-6-P})}{(\text{Succinyl CoA})} = 8.7 \times 10^5$$

Higher pH gives higher values of K_2 , increasing the magnitude of this ratio. Thus in the presence of hexokinase it is most unlikely that the incorporation of P_i into NEP is due to a simple exchange reaction -- any ATP formed will be converted to G-6-P. Besides,

it is significant that only catalytic amounts of ADP were needed for transfer activity (Figure 6). If the ATP formed did not produce G-6-P, from Law of Mass Action, increasing ADP concentration should increase the amount of esterified $^{32}\text{P}_i$. In fact, at high concentration of ADP less $^{32}\text{P}_i$ was esterified (Fig. 6). This might be explained by product inhibition of the hexokinase reaction (14, 44, 134).

The additional evidence which further supports the conclusion that the stimulating effect of succinate and CoA on transfer activity is not due to thiokinase mediated $^{32}\text{P}_i$ -ATP exchange is as follows:

2. Malonate did not inhibit the transfer reaction, while at equivalent concentrations exchange activity was inhibited by about 50 percent (Table XIII).
3. GDP enhances exchange activity (Table XIV) while inhibiting transfer (Table XIII).
4. Arsenate completely inhibits the succinate and/or CoA stimulated transfer activity, while the exchange activity is only decreased by about 40 percent at comparable concentrations (see Table XIII)
5. The different response of exchange and transfer reaction toward PCMB is illustrated in Figure 7. The transfer activity in the absence of succinate and CoA was very

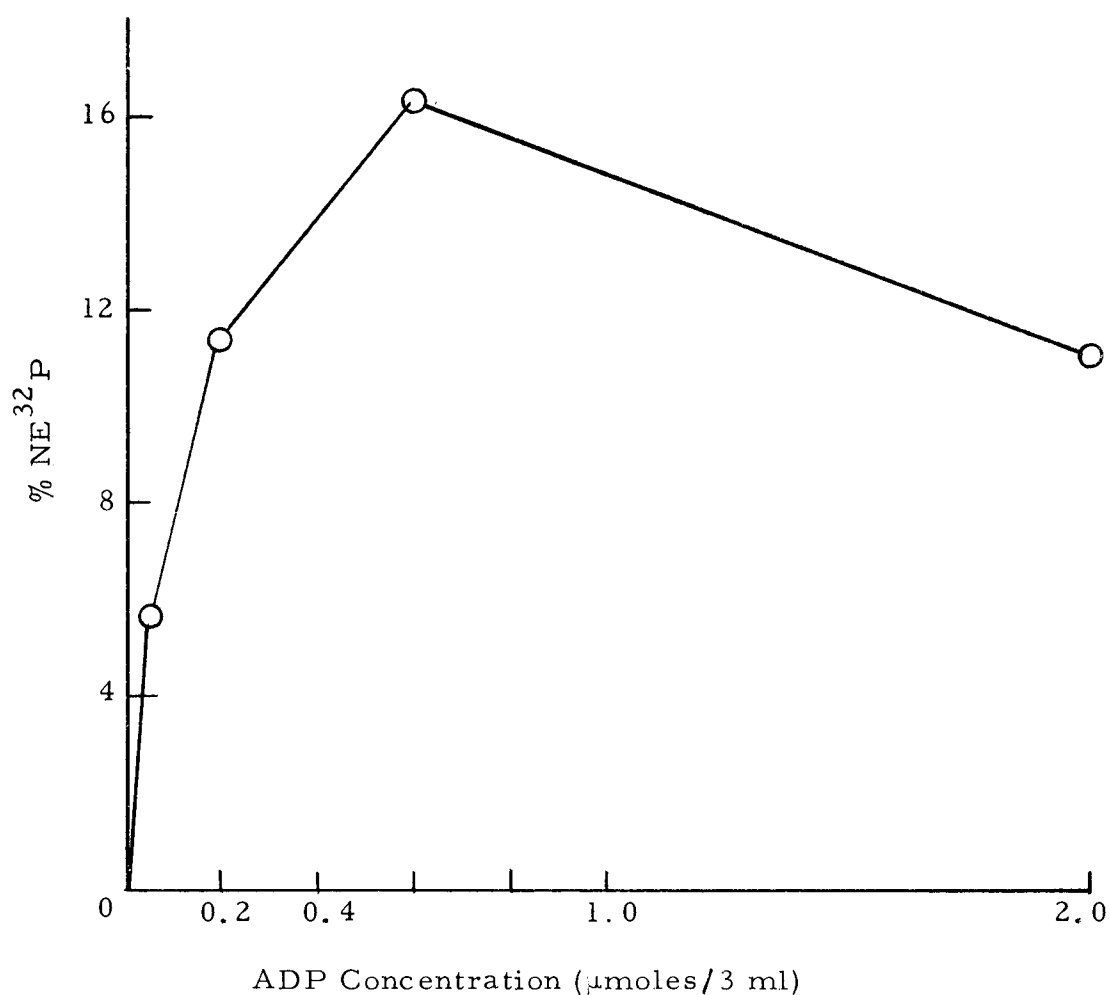


Fig. 6. Effect of ADP concentration on energy transfer activity using hexokinase system as acceptor

The incubation procedure and conditions were the same as stated in Table XIII, except different concentration of ADP was added to the control medium which contained 0.2 μmoles succinate and 0.075 μmoles CoA. The reaction was carried out at 25°C for 12 minutes.

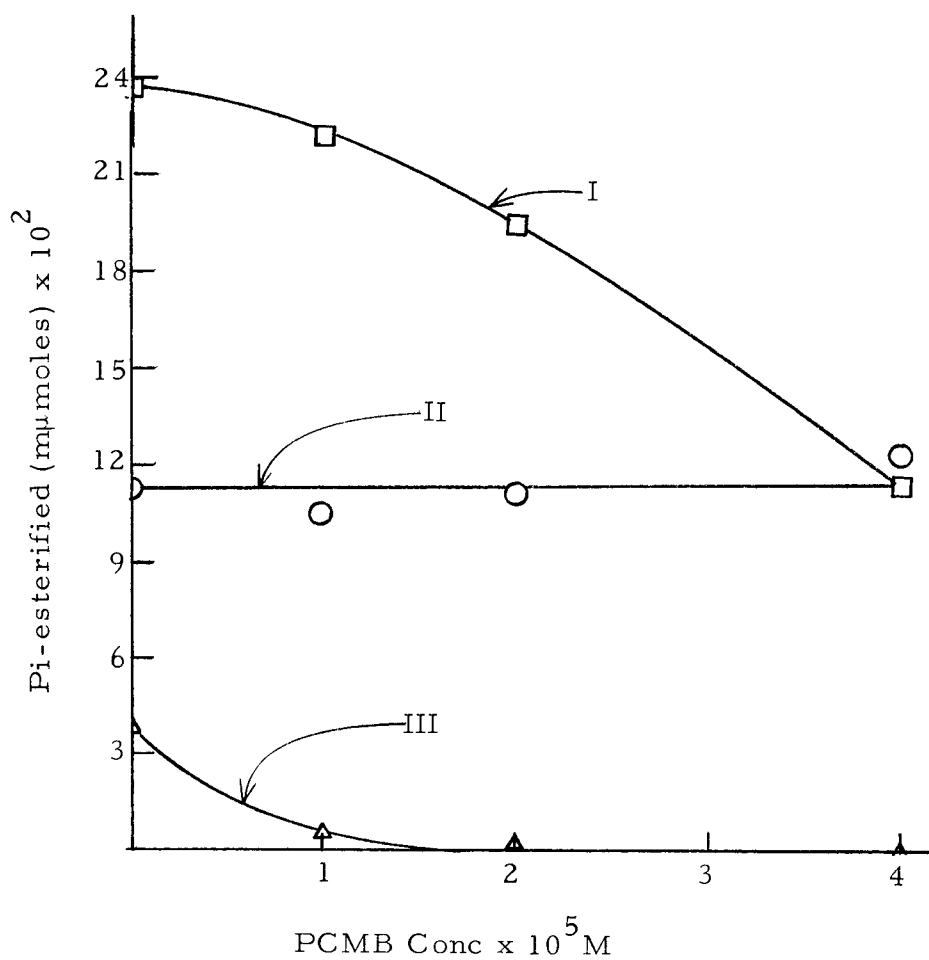


Fig. 7. The inhibitory effect of PCMB on transfer and exchange reaction

The incubation procedure and conditions were the same as stated in Fig. 5. I ATP- $^{32}\text{P}_i$ exchange activity, II succinate and CoA stimulated transfer activity, III transfer activity without added succinate and CoA, Protein content: 0.63 mg/ml. Reaction was carried out at 25°C for 6 min.

sensitive to PCMB. Addition of succinate and CoA produced the usual increase in the amount of phosphate esterified (G-6-P), and PCMB at concentrations as high as 4×10^{-5} mM did not appear to have any effect under these conditions. In contrast, ATP- $^{32}\text{P}_i$ exchange activity dropped gradually to more than 50 percent of the control with increasing levels of PCMB. This difference in response could be accounted for by a difference in the role of CoA in these two reactions. For the transfer reaction, CoA acted catalytically, and only small amounts were needed for activity, while for the exchange reaction CoA acted as a substrate. Thus for equivalent concentrations of CoA, PCMB would have a greater influence on exchange activity.

6. These two reactions responded differently to hydroxylamine (Figure 8). Hydroxylamine severely inhibited ATP- ^{32}P exchange at low concentration, while the succinate-CoA stimulated transfer activity was inhibited by only 50 percent at a concentration as high as 400 $\mu\text{moles } \text{NH}_2\text{OH} / 3 \text{ ml.}$

This evidence suggests that the succinate-CoA stimulatory effect on transfer activity is not due to $^{32}\text{P}_i$ -ATP exchange through

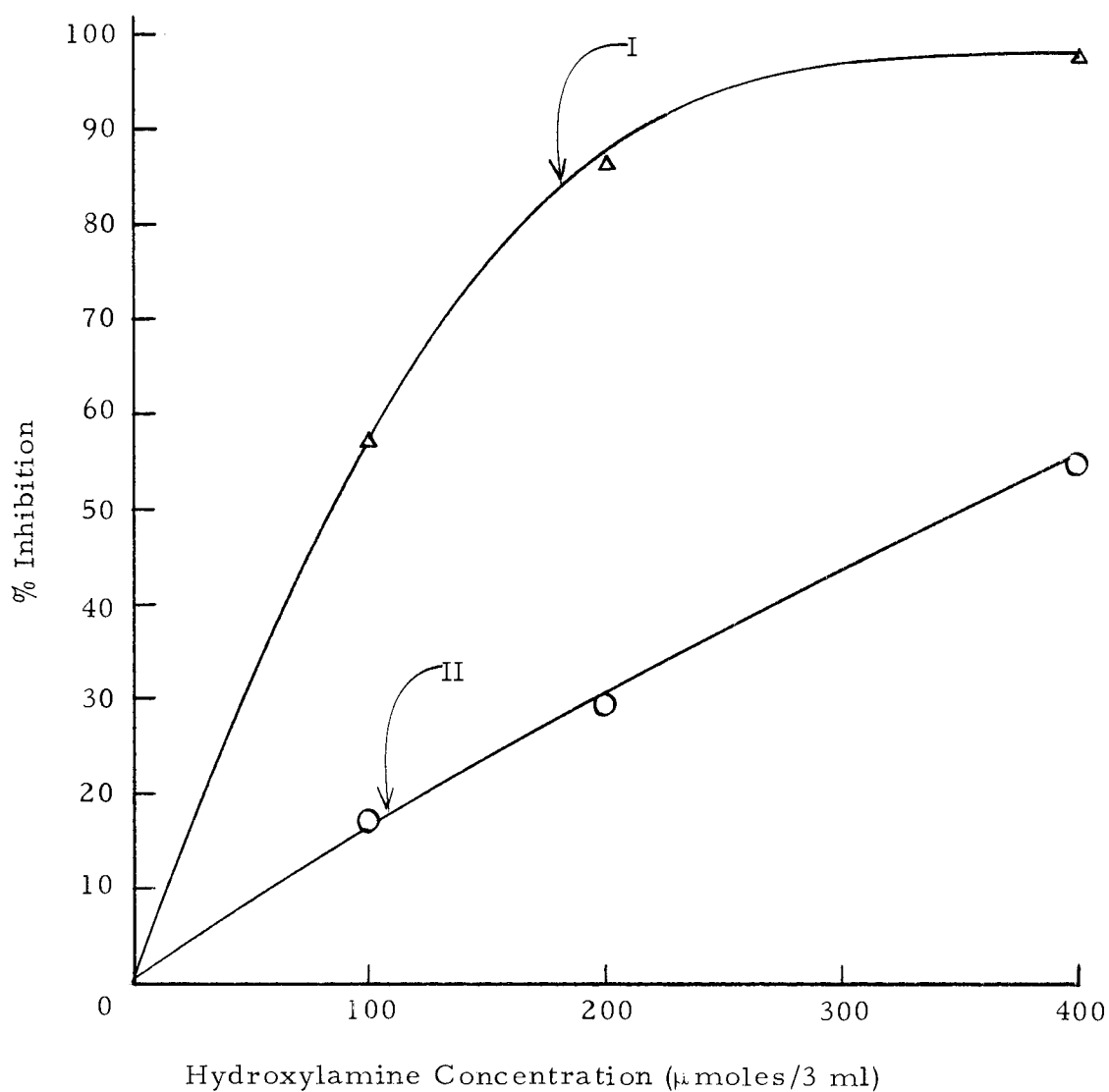


Fig. 8. The inhibitory effect of hydroxylamine toward transfer and exchange activity.

The experimental procedure and conditions were the same as stated in Fig. 5. I. ATP-³²P_i exchange. II. Succinate and CoA stimulated transfer activity. Protein content: 0.94 mg/ml. Reaction was carried out at 25°C for 6 minutes

the succinate thiokinase reaction, nor is it the result of a by-pass through succinate thiokinase reaction via the formation of succinyl CoA.

VI. Characterization of Succinate and CoA Stimulated Transfer Reaction

ADP specificity

The highest level of transfer activity has been demonstrated in the presence of succinate, CoA, $^{32}\text{P}_i\text{-P}_i$, and ADP (or ADP + hexokinase and glucose). Little phosphate is esterified if ADP is omitted from the system (Table IX). Also, substitution of GDP for ADP resulted in loss of transfer activity (Table XIII). ADP is therefore specific for ATP formation in the transfer reaction, and in the presence of hexokinase and glucose only a catalytic amount is required (Figure 6). In the presence of hexokinase, high concentrations of ADP showed an inhibitory effect. This is consistent with the observation of Gamble and Najjar (44) and Bueding and MacKinnon (14) that ADP inhibits hexokinase activity. From the study of the free energy change of the hexokinase reaction and the absence of G-6-P inhibition of the yeast hexokinase, they were able to show that the inhibition by ADP is not due to the reverse reaction but is due to the combination of ADP with enzyme so that the forward

rate is inhibited (134).

Effect of orthophosphate concentration

Changing the concentration of orthophosphate increased the rate of phosphate esterification in a normal manner (Figure 9). The reaction shows first order kinetics for phosphate at low concentrations of orthophosphate. A level of maximal activity is attained at a concentration of 0.5 mM.

Mg⁺⁺ dependency

The role of Mg⁺⁺ on transfer reaction is difficult to define since it is required for hexokinase activity (71) on the one hand, and also is involved in the association of soluble fraction with the particles (85) on the other hand. The need of Mg⁺⁺ for oxidative phosphorylation was demonstrated, though indirectly, in Racker's Laboratory (1963). They showed the complete dependency of the reduction of DPN⁺ by succinate on Mg⁺⁺ when the energy required for this process was supplied by ATP. The dependency of Mg⁺⁺ in energy transfer reaction was also demonstrated in this study; the most striking result was shown by the addition of EDTA which completely inhibited the transfer activity. Increasing concentration results in an increase of transfer activity until a maximal level is

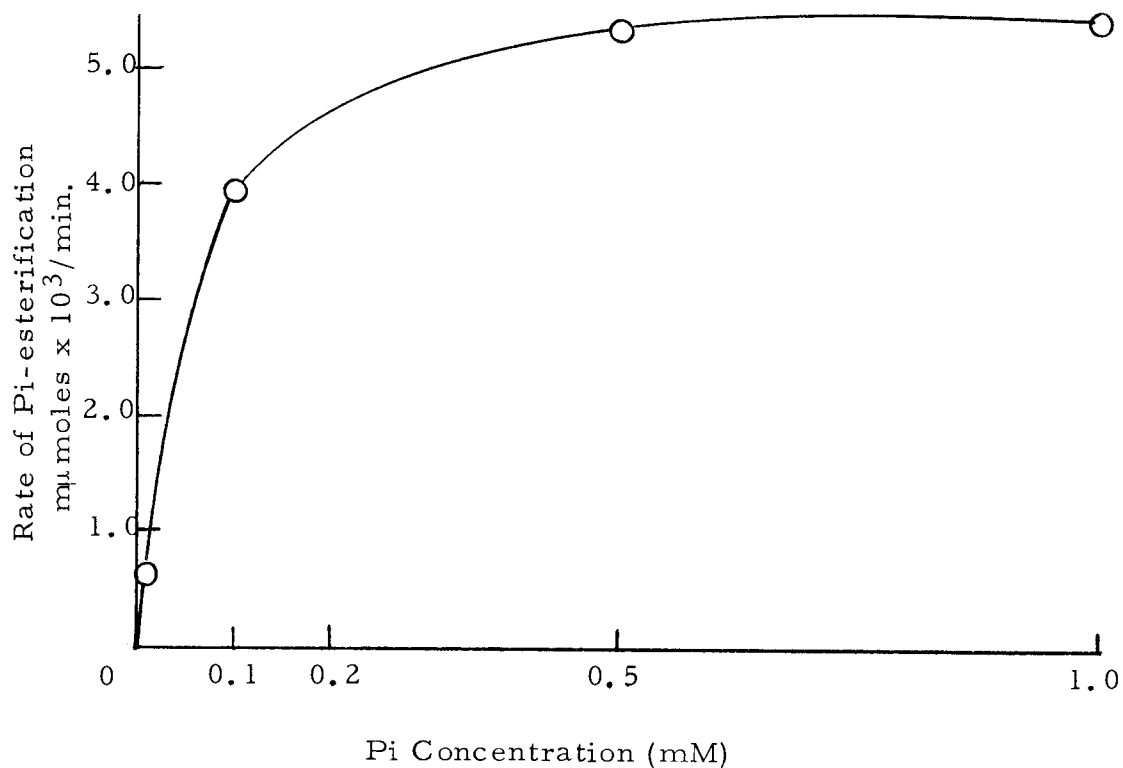


Fig. 9. Effect of orthophosphate concentration on the rate of energy transfer reaction.

The incubation procedure and conditions were the same as stated in Fig. 3 except different concentrations of orthophosphate were added in the second incubation medium. 2 μ moles succinate and 0.15 μ moles CoA were also added to the second incubation mixture. Reaction was carried out at 25°C for 12 minutes.

reached as shown in Figure 10.

Inhibitor effect

The inhibitory effect of different reagents on the succinate and CoA stimulated transfer reaction has been shown in Table XIII, Figure 6 and Figure 7. As already mentioned, the inhibition of antimycin A was probably due to the absolute alcohol. DNP has little or no effect on the transfer activity; it appears to act on the nonphosphorylated high energy intermediate formed in the soluble fraction. Addition of succinate and/or CoA stabilized or transformed the pre-existed intermediate to a new intermediate or final product which is resistant to DNP attack. Oligomycin in a concentration of more than $3\text{ }\mu\text{g}$ per ml only inhibits the succinate and CoA stimulated transfer reaction by about 40 percent. This is in contrast to earlier finding that oligomycin in low concentration ($1\text{ }\mu\text{g}/3\text{ ml}$) completely inhibits oxidative phosphorylation (73). However, Slater and his collaborator (115) have demonstrated that a much higher concentration of oligomycin is needed to inhibit solubilized ATPase. Racker (101) also has shown that ATPase in mitochondria is very sensitive to oligomycin, but the soluble ATPase obtained from mitochondria is resistant to oligomycin. A structural factor must be responsible for oligomycin sensitivity and must be modified or missing

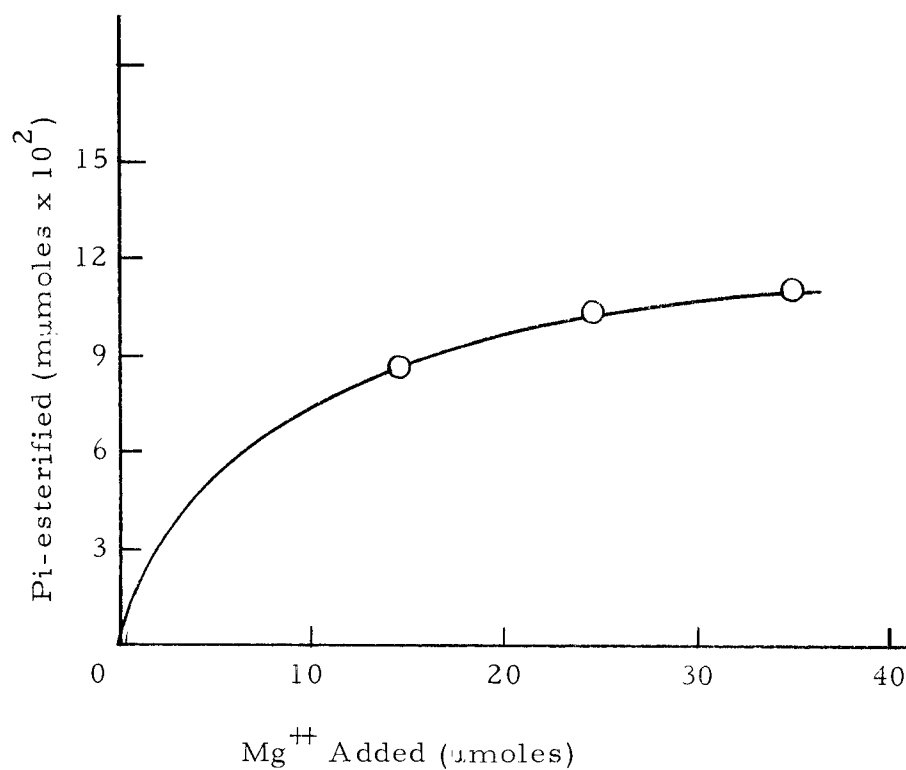


Fig. 10. Influence of Mg^{++} concentration on energy transfer

The incubation procedure and conditions were the same as stated in Fig. 3 except different concentrations of $MgCl_2$ were added in second incubation medium. 20 μ moles EDTA was added to arrest the endogeneous Mg^{++} . 2 μ moles succinate and 0.15 μ moles CoA were added to the second incubation mixture. Reaction was carried out at 25^o C for 6 minutes.

in the soluble fraction. A striking result was obtained in the study of arsenate effect on transfer activity. As shown in Figure 11, arsenate severely inhibits the formation of esterified phosphate at very low concentration and completely uncouples the reaction at a concentration of 1.0 μ moles per 3 mls. Arsenate probably competes with phosphate for the high energy intermediate.

Rate of reaction

Initially the energy transfer reaction proceeds rapidly and then levels off after 24 minutes of incubation due to the exhausting of the high energy intermediate (Figure 5). The supernatant fraction obtained after centrifugation at 105,000 x g for 90 min. contained no particulate fraction and thus no more high energy intermediate would be synthesized in the second incubation. Curve II in Figure 12 shows the same general form for the rate of transfer activity in the supernatant fraction. Addition of submitochondrial particles to this supernatant fraction gives an initial, rapid phase followed by a slower, steady state phosphorylation. The initial rapid reaction reflects the exhaustion of preformed high energy intermediate similar to that observed with the supernatant fraction. This kinetic study can very well explain the results of the "ATP jump" observed in Chance's Laboratory (35). Chance and his collaborators showed

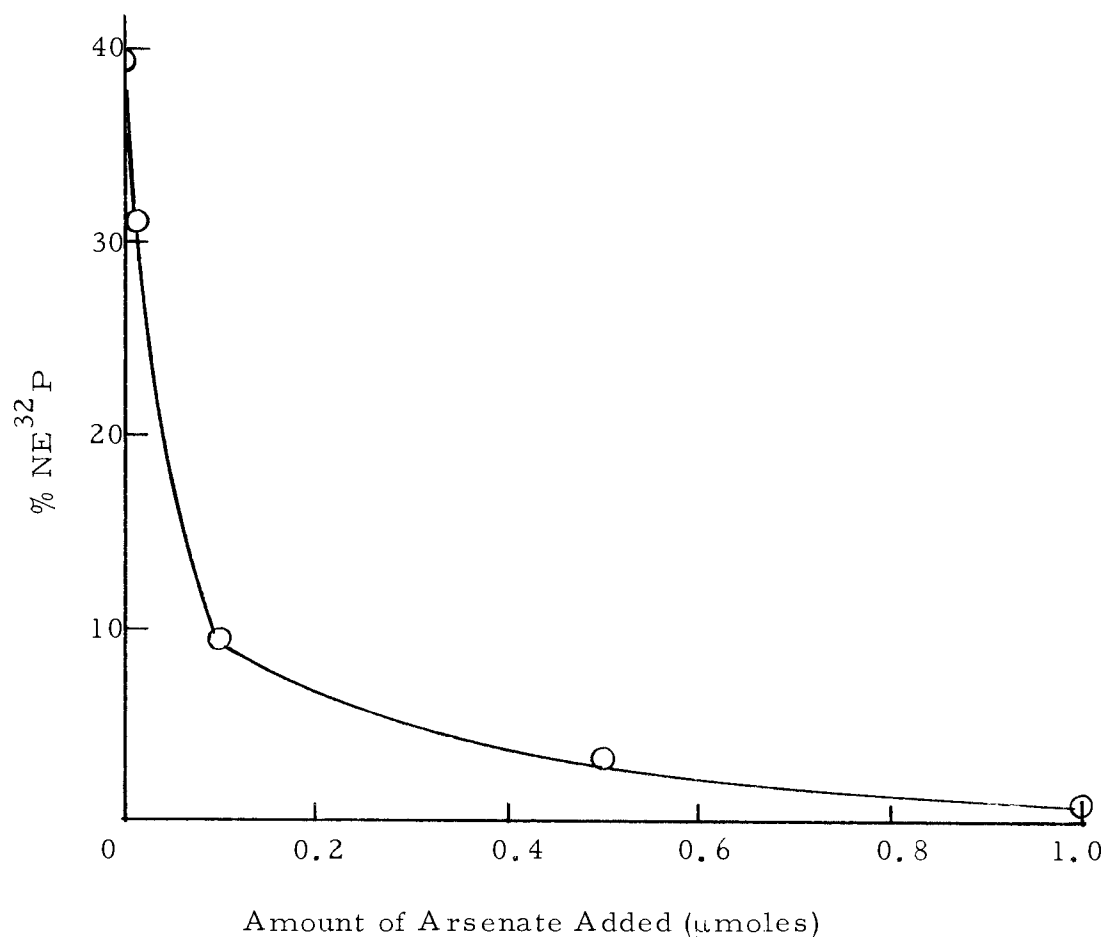


Fig. 11. Effect of arsenate on energy transfer reaction

The incubation procedure and condition were the same as in Table XI for Expt. except that the supernatant fraction was used instead of acetone powder. Different amounts of arsenate were then added to the second incubation mixture. 0.2 μmoles succinate and 0.075 μmoles CoA were also added. The reaction was carried out at 25°C for 12 minutes.

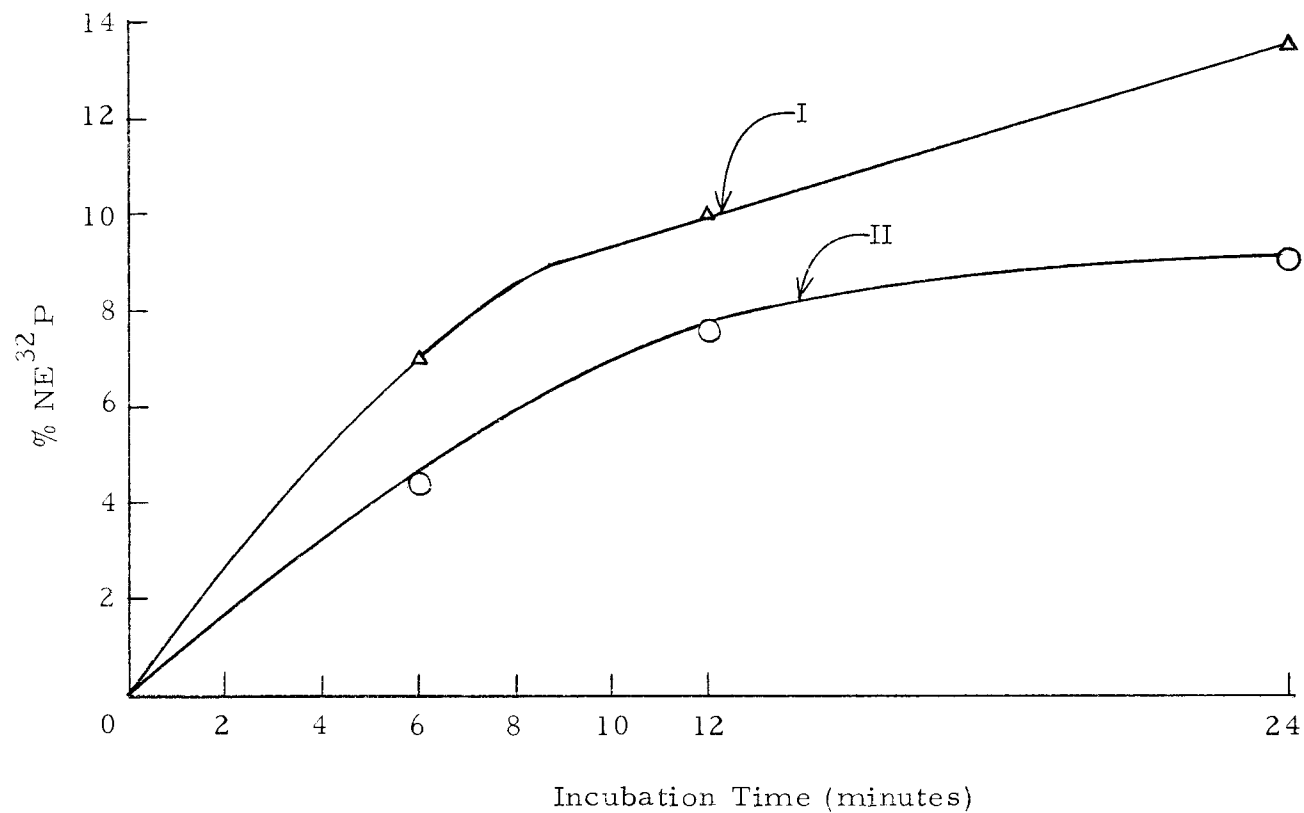


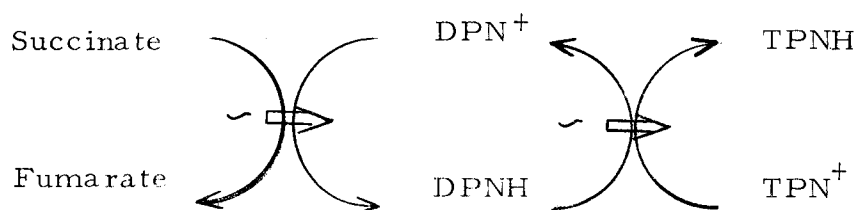
Fig. 12. Kinetic study of the energy transfer activities on both supernatant fraction alone and together with the submitochondrial particles.

The incubation procedure and condition were the same as stated in Figure 11 except 0.2 ml of submitochondrial particles containing 3.8 mg protein per ml were added to the supernatant fraction for the result showed in Curve I. Curve II represents supernatant fraction alone.

that the addition of ADP to mitochondria preincubated with substrate and P_i leads to two consecutive stages of ATP formation. They proposed that the preformed energy rich intermediate could account for the brief period of more rapid ATP formation. This experiment definitely demonstrated the "ATP jump" is really due to the preformed nonphosphorylated high energy intermediate.

VII. The Participation of High Energy Intermediate in Energy-Dependent Reactions

The energy dependent reactions such as energy-linked nucleotide transhydrogenation and reverse electron transport (reduction DPN^+ by succinate) have been studied in several laboratories (20, 40, 68, 103). It has been established that either added ATP or an intermediate of the respiratory chain-linked phosphorylation system is able to supply the energy for these reactions and the transhydrogenation and reversed electron transport involve a common high energy intermediate. Attempts have been made to determine whether the high energy intermediate in this system could support these energy-dependent reactions. Experiments were carried out observing the reduction of TPN^+ by $DPNH$; while $DPNH$ was generated in these systems by the reduction of DPN^+ with succinate through reverse electron transport as shown in the following scheme:



Both of these reactions require energy (~) which was supplied by the high energy intermediate preformed in the soluble fraction.

Procedures for the study of this system have been described by Danielson and Ernster (32). The reaction mixture consisted of Mg^{++} , DPN^+ , TPN^+ and succinate in Tris-buffer, pH 7.4. KCN was added to stop the electron flow. The supernatant and sub-mitochondrial suspension was prepared as described earlier, mixed immediately before the incubation, and poured into the reaction mixture to initiate the reaction. Danielson and Ernster (32) have established that under these conditions TPNH was formed. The amount of TPN^+ reduced was measured by observing the increase in optical density at 340 m μ (A_{340}).

Reduction of TPN^+ was observed when both the submitochondrial particles and the supernatant fraction were added to the system (Figure 13). The increase in absorption in the presence of the submitochondrial particles alone is due to some components other than TPNH. The soluble fraction could be contributing some limiting enzyme, high energy intermediate or both. The fact that the rate of reduction of TPN^+ leveled off suggests that some component became limiting, probably the high energy intermediate. Addition

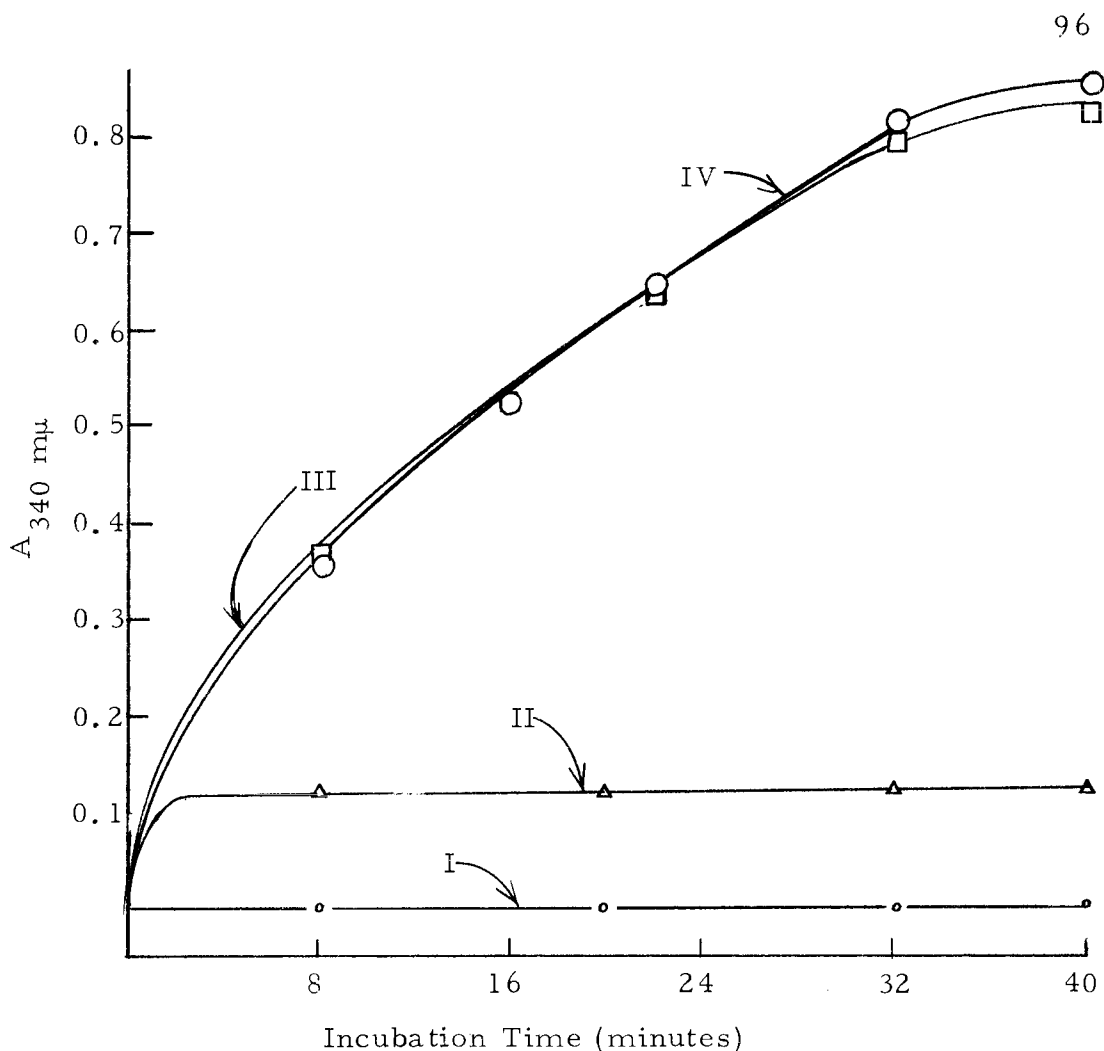


Fig. 13. High energy dependent reduction of TPN^+ by DPNH , coupled to the reduction of DPN^+ with succinate.

The reaction mixture of control consisted of 2 ml of supernatant fraction (0.60 mg protein per ml), 0.2 ml sub-particle suspension (contained 4.8 mg protein per ml), 2mM KCN, 6.67 mM MgCl_2 . Further additions were: DPN^+ , 0.067 mM; TPN^+ , 0.667 mM; succinate, 0.667mM; ATP, 0.333 mM. Final volume was 3 ml. Reaction was initiated by the addition of enzyme preparation at 25°C and followed by the measuring of the increase of optical density at 340 $\text{m}\mu$. Curve I, control; Curve II, complete system less supernatant fraction; Curve III, complete system; Curve IV, complete system + ATP. The complete system included DPN^+ , TPN^+ , and succinate together with the control system.

of ATP as a potential energy source did not provide any additional effect. This was consistent with the observations of Lee and Ernster (75) who have reported that the submitochondrial preparations can carry out this energy-dependent transhydrogenation more efficiently in the presence of aerobically generated high energy intermediates than in the presence of ATP.

That this reduction of TPN^+ was due to the reverse electron transport is confirmed by the demonstration of a succinate requirement (Figure 14). The rate of reduction of TPN^+ in these systems is reduced both by arsenate (Figure 15) and phosphate + ADP (Figure 16). ADP appeared to enhance the uncoupling effect of arsenate (Figure 15). This could be explained by a competition of these components for the high energy intermediate. The competition for the high energy intermediate between oxidative phosphorylation and energy-dependent reactions in submitochondrial particle has been demonstrated recently by Lee and Ernster (76). This is again very good evidence to support the concept that the energy-linked transhydrogenase reaction derived energy from an intermediate of the respiratory chain-linked phosphorylation system. Since the presence of KCN in the medium inhibits electron flow in the respiratory chain, it is considered unlikely that the submitochondrial particles together with the supernatant fraction could generate high energy intermediate.

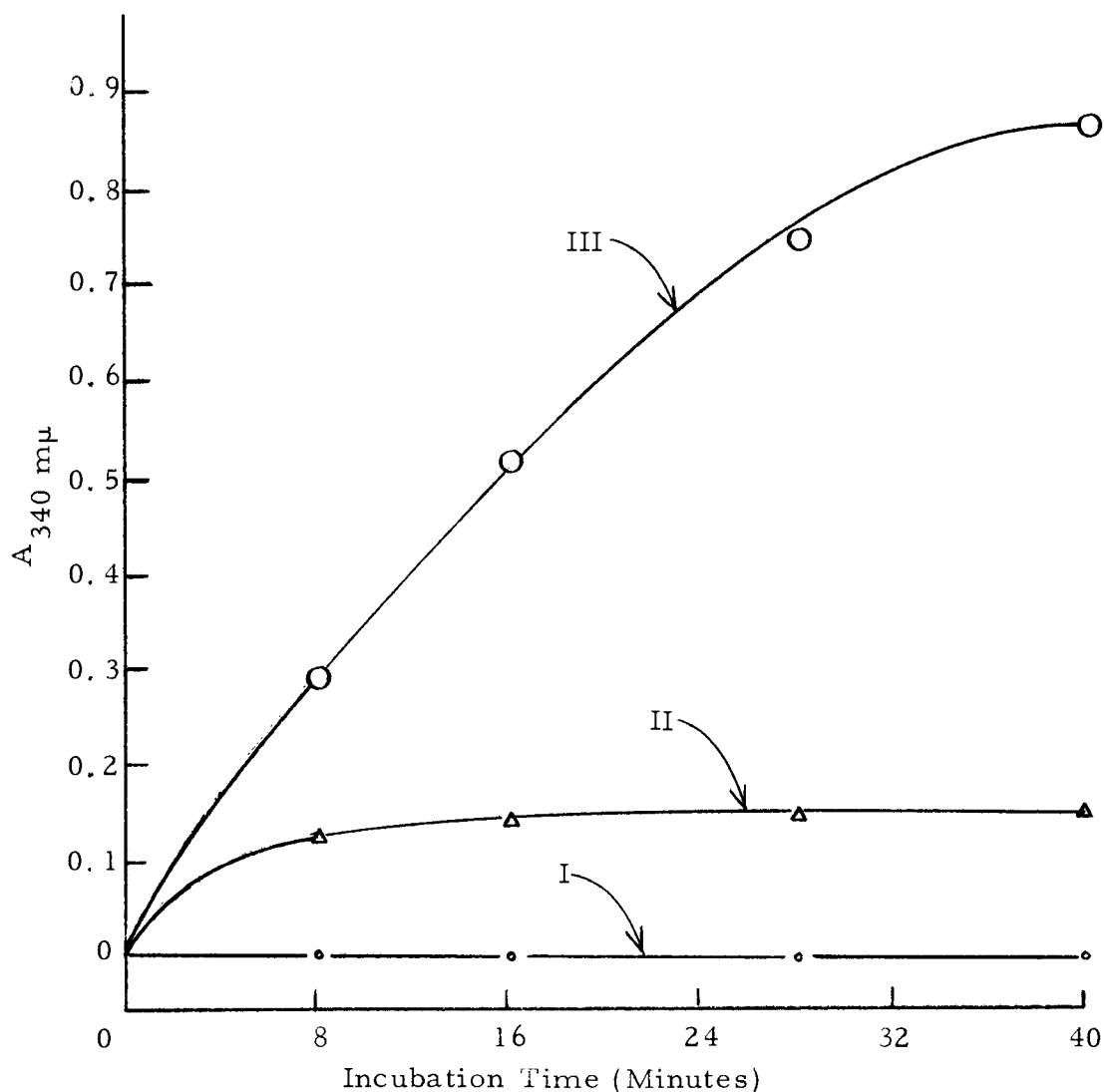


Fig. 14. Succinate requirement for the energy-dependent pyridine nucleotide transhydrogenation coupled to the reduction of DPN^+ with succinate.

The procedure and condition were the same as stated in Fig. 13 except 0.333 mM of arsenate and 0.667 mM ADP were added in control mixture. Curve I, control; Curve II, Complete less succinate; Curve III, Complete (control + DPN^+ + TPN^+ + succinate).

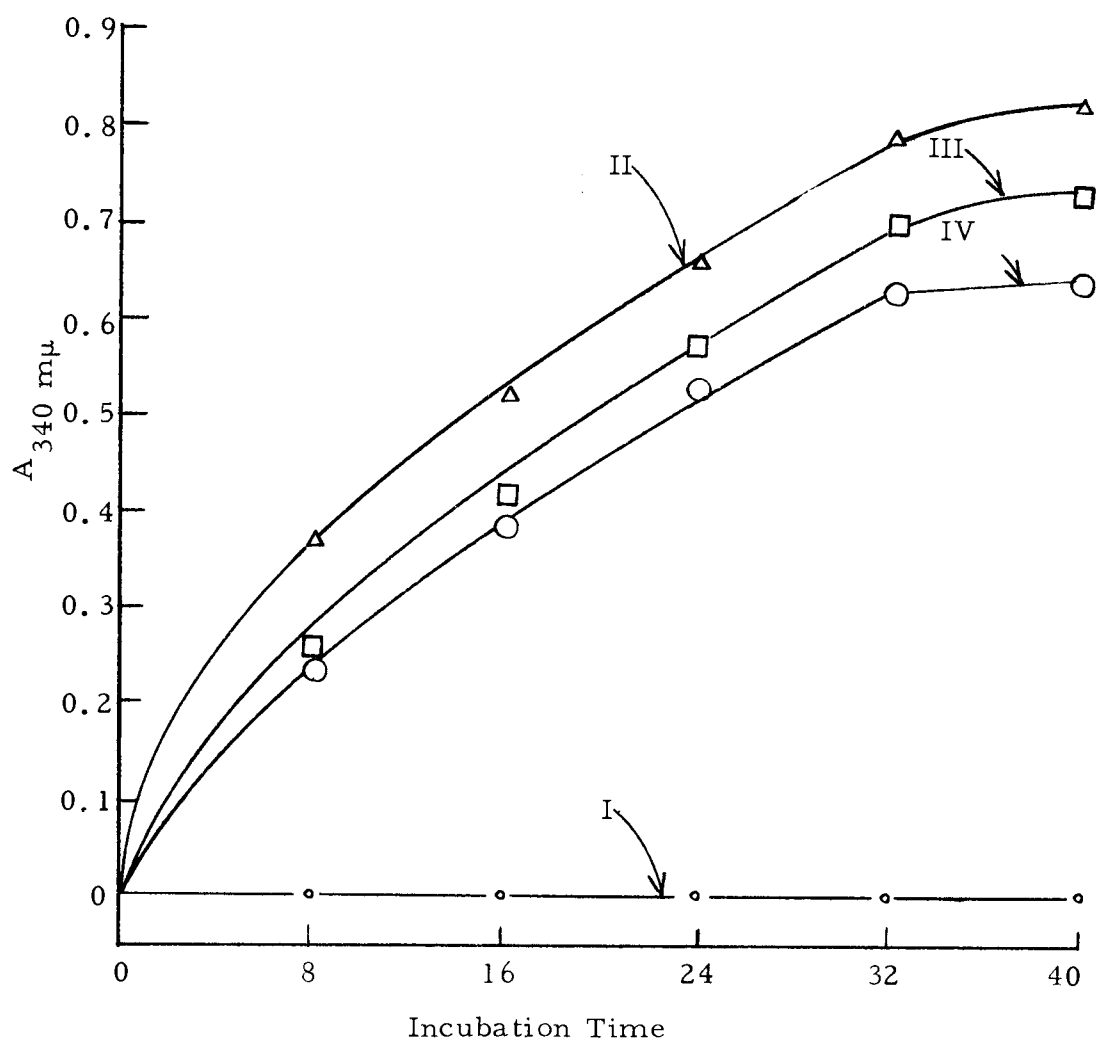


Fig. 15. Effect of arsenate on the high energy dependent reduction of TPN^+ by DPNH coupled to the reduction of DPN^+ with succinate.

The procedure and condition were the same as stated in Fig. 13 except in Curve II, 0.333mM arsenate and 0.667 mM ADP were added, where indicated. Curve I, control; Curve II, complete system; Curve II, Complete + arsenate; Curve IV, Complete + ADP + arsenate.

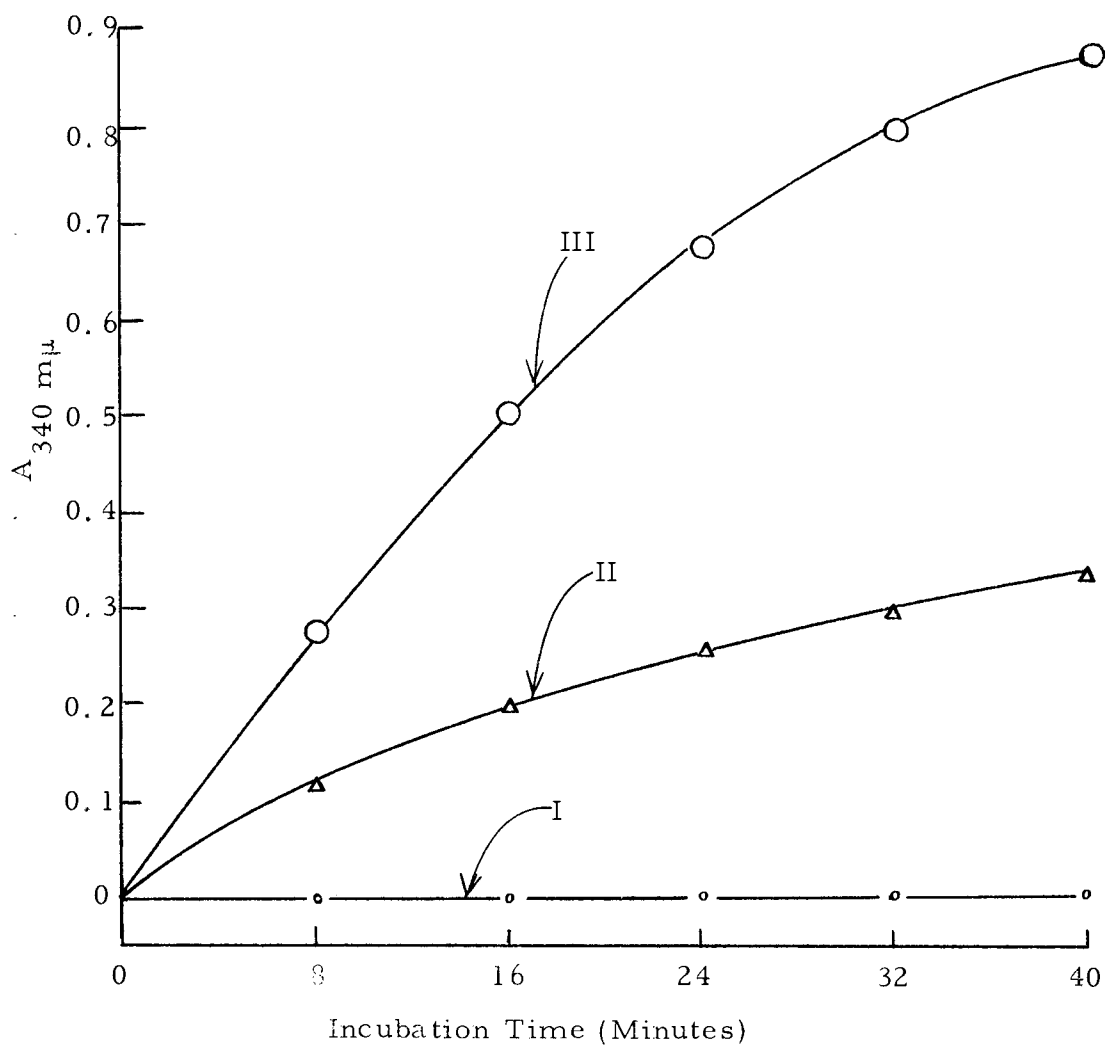


Fig. 16. Competitive effect of oxidative phosphorylation on energy dependent reactions.

The procedure and condition were the same as stated in Fig. 14 except 0.01 mM phosphate was present in addition to arsenate and ADP in Curve II. Curve I, control; Curve II, Complete + arsenate + ADP + P_i; Curve III, complete + arsenate + ADP

It is thus concluded that the supernatant fraction is contributing high energy intermediate which is used in these energy dependent reactions.

VIII. Mechanistic Consideration

We have concluded from these studies that a soluble, non-phosphorylated high-energy intermediate was present in the soluble fraction in a protein bound form. This conclusion is significant in that this is the first time that the existence of a nonphosphorylated high-energy intermediate has been demonstrated in an animal system. In addition, the transduction of energy with the formation of ATP has been accomplished in a soluble system without the participation of the respiratory chain.

From the evidence we have, it is proposed that the soluble nonphosphorylated high-energy intermediate is in the form of enzyme bound acyl~X, where X is some unknown entity -- possibly a thiol or an imidazol group as will be discussed later. Since the intermediate is fairly stable and can survive acetone precipitation or salt fractionation, both the acyl and X group appear to belong to the same enzyme molecule or to two tightly bound enzyme molecules. The fact that CoASH is an absolute requirement for the energy transfer reaction has led us to postulate the involvement of an acyl group.

The existence of acyl~S intermediate in the energy transfer process has been proposed recently by Boyer (6) and Fonyo and Bessman (43) due to the fact that PCMB inhibits mitochondrial respiration, and that the inhibition can be partially overcome by DNP. Other laboratories have also shown that PCMB inhibits oxidative phosphorylation (30, 43), ADP-ATP exchange (61, 130), P_i -ATP exchange (26, 30, 82), and $H_2O^{18}-P_i$ exchange reactions (6). These data would suggest the involvement of -SH groups in the phosphate transfer process. Furthermore, the action of PCMB is distinct from that of oligomycin. Chance (7) has shown that Ca^{++} in small amounts causes a rapid respiration by "draining" the high-energy intermediate prior to the site of oligomycin effect, since oligomycin does not inhibit this Ca^{++} induced respiratory "jump". PCMB, on the other hand, prevents the Ca^{++} -induced respiratory "jump". Fonyo and Bessman (43) thus proposed that the site of PCMB interaction with the energy transfer reaction (preventing the formation of acyl-S) is between the DNP and the oligomycin sensitive sites. Acyl-SCoA is the most probable intermediate in the catalytic transfer reaction. In fact, Upper and Gunsalus (6) have reported some evidence for an enzyme bound form of CoA which could be an acyl~SCoA.

We have observed that acetone treatment or salt fractionation of the mitochondrial soluble fraction gives preparations which show

no transfer activity in the absence of CoA. Addition of CoA greatly increases the transfer activity, especially in the presence of catalytic amounts of succinate. This increase in transfer activity is inhibited by hydroxylamine, arsenate and oligomycin, but is unaffected by DNP. One might predict that acyl~SCoA is formed rapidly from its precursor, acyl~X, while not allowing the interaction of DNP with acyl~X. The formation of acyl~SCoA can explain the inhibition by hydroxylamine. As pointed out by Jaenick and Lynin (60), hydroxylamine reacts with acyl~CoA to form hydroxamate. Since it is enzyme bound, the rate of reaction could be slower than with a free acyl~CoA, such as succinyl CoA. This could account for the different response to hydroxylamine of the energy transfer and succinic thiokinase reactions (Figure 8).

The stimulatory effect of succinate is probably due to its participation in the promotion of acyl~CoA formation by a concerted mechanism which bears a resemblance to other enzymatic displacement reactions, described by Koshland (69). It is proposed that the enzyme is a polyfunctional catalyst which facilitates the simultaneous electrophilic and nucleophilic interaction of the substrates. A possible transition complex in the formation of acyl~CoA is depicted in Figure 17. According to this proposal, CoASH initiates the nucleophilic attack on the carboxyl carbon, but the reaction is not achieved

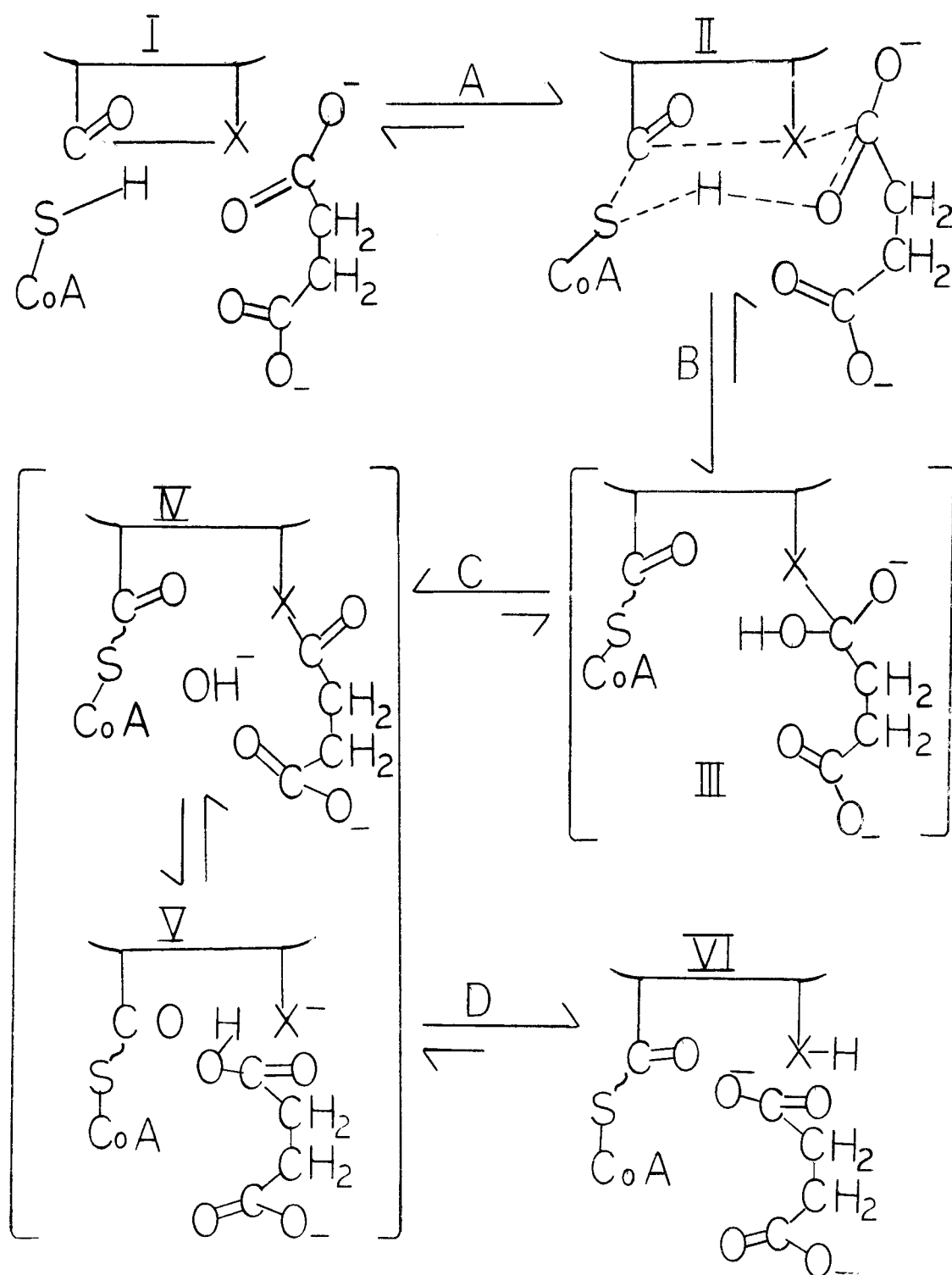
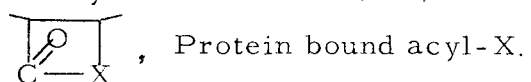


Fig. 17. Illustration of concerted mechanism for the formation of acyl-CoA intermediate.

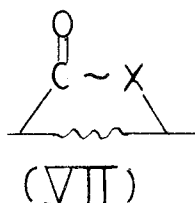


unless a simultaneous electrophilic attack takes place in the proximity of the X group. The nucleophilic effect makes the X group more amenable to the electrophilic attack by carboxyl carbon of succinate. In Figure 17 the dotted line represents the partial interactions. The formation of any new covalent bonds in the products of the breakdown of this transition complex (II) requires a concerted participation of both the nucleophilic (CoASH) and electrophilic (succinate) reactants. This simultaneous push and pull of electrons by the catalysts could probably reduce the activation energy and in turn could account for the acceleration of this transfer reaction. Two products in equilibrium could be found in step (c). If -OH^- is a much stronger base than X^- , intermediary species IV will predominate and the reaction will proceed directly to the final product VI. If X^- and OH^- are of equal strength, IV and V will be in equilibrium (45). Under the physiological or assay condition (the pH value is about 7.0), the ionization of succinic acid is able to displace the equilibrium in the forward direction.

The X could be a thiol of cysteine, an imidazole of histidine, an amidine of arginine, an α -amino group of lysine or hydroxyl group of serine on the enzyme molecule. Thiol, imidazole or amidine

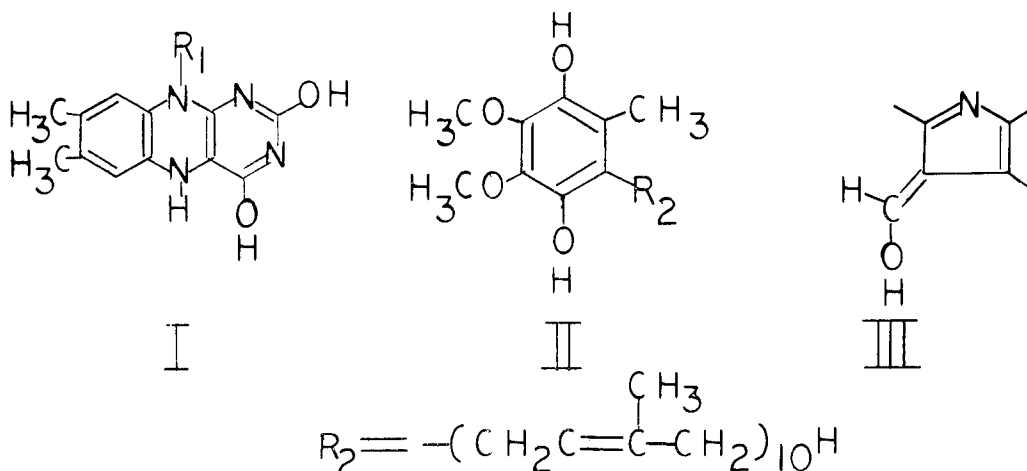
groups are favored because of their ability to form high energy bonds with the carbonyl group; e. g., acyl~S (6), acyl~imidazole (57, 122) or acyl~amidine (57). It does not appear that acyl~S is the primary soluble, nonphosphorylated high-energy intermediate because the primary intermediate is sensitive to DNP uncoupling, while acyl-SCoA is stable to DNP attack. Acyl~imidazole is more likely, not only because it fulfills the criteria of an "energy rich" compound but because acetyl imidazole has been discovered by Stadtman and his collaborator (122), and many of its properties have been reviewed by Haennekins and Whiteley (57) indicating its qualification. Acyl~amidine is also a possibility as a high-energy intermediate (57). Thus it is proposed that imidazole or amidine moieties would be the most likely groups involved at the active site.

It is difficult to visualize, as depicted in Formula IV of Figure 17, that breaking of one high-energy bond could result in the formation of two energy-rich intermediates. The bond between X and carboxylic carbon of succinate might not be formed at that instant unless the bond energy could be conserved in the strain of ring structure as depicted from the modification of Boyer's (6) such as in Formula VII.



In this later case, X could be amino or hydroxyl group, even though the bonds linking acyl-N and acyl-O are not "energy rich" in nature, the extra energy conserved in ring structure will be sufficient to generate acyl~SCoA in the following step. The definition of the real nature of X requires further investigation.

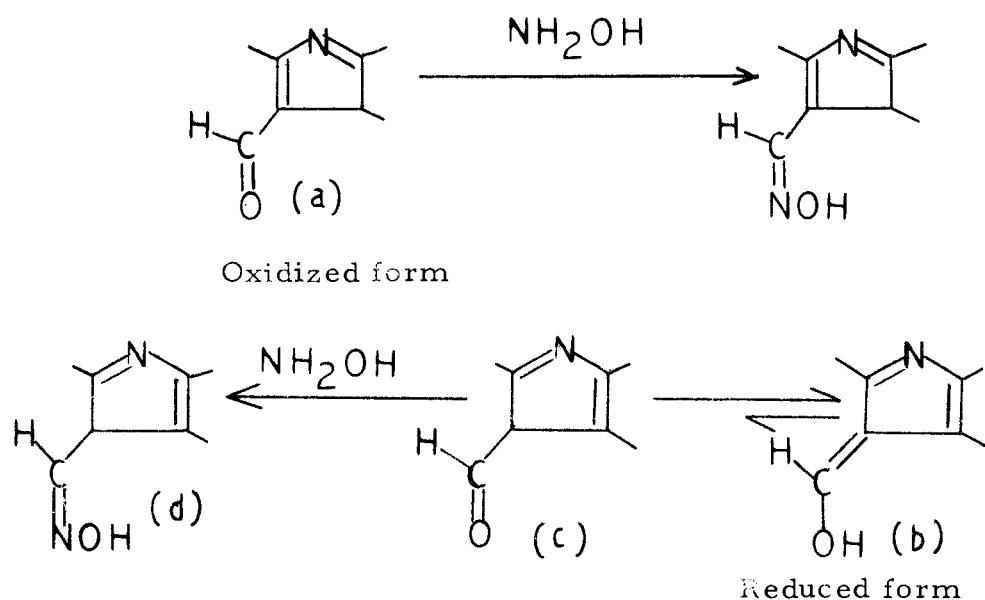
A careful examination of the structure of electron transport carriers and their role in oxidative phosphorylation as demonstrated by other laboratories might give further insight into the mechanism of the energy conservation reaction. From the studies of Grabe (48), Clark et al. (27), and Takemori et al. (128). It has been suggested that the carbonyl group in flavin nucleotide, coenzyme Q and cyt. a, directly or indirectly, participate in electron transport, and play an important role in the terminal coupling reaction. In the reduced state, the carbonyl group would exist predominantly as a phenolic hydroxyl group or an OH group linked to a conjugated system, as shown in Formulas I, II, and III.



Formulas I and II are the reduced form of flavin and coenzyme Q_{10} , while III is the partial formula of the prosthetic group of cyt. a.

Formula III has not been definitely established as yet; it is deduced from the experimental evidence from Okunuki's Laboratory (128).

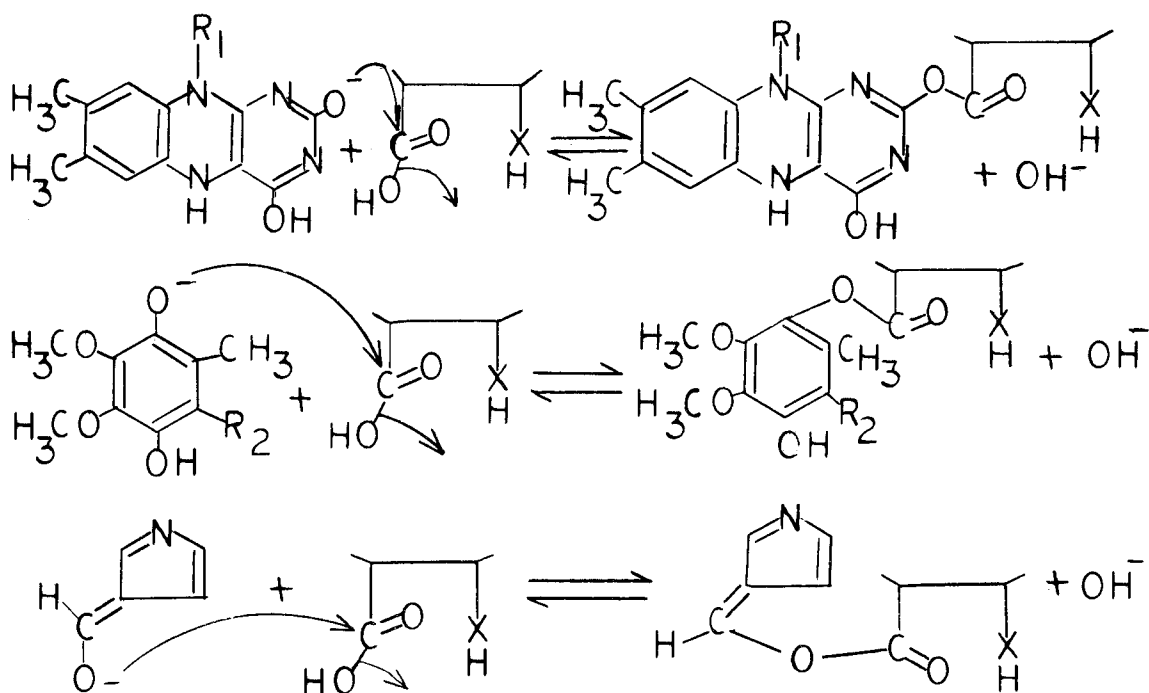
They studied the effect of aldehyde reagents on the spectra of cytochrome a and found that the spectrum of the oxidized form was not changed when incubating the purified cytochrome a with hydroxylamine, but that of the reduced form shifted markedly; The α peak shifted from 605 $m\mu$ to 603 $m\mu$, and the γ peak from 444 $m\mu$ to 433 $m\mu$. A shift in the absorption bands toward the shorter wavelength was also observed when isolated porphyrin a was treated with hydroxylamine. This shift in wavelength of absorption peaks could be interpreted as being due to the loss of a conjugated double bond in the formation of an oxime with hydroxylamine.



In its oxidized form (a) the reaction of hydroxylamine did not change the conjugated system. In the reduced form, which probably exists predominantly in the form of (b) treatment of hydroxylamine shifts the tautomerism equilibrium to (c) and finally forms oxime (d).

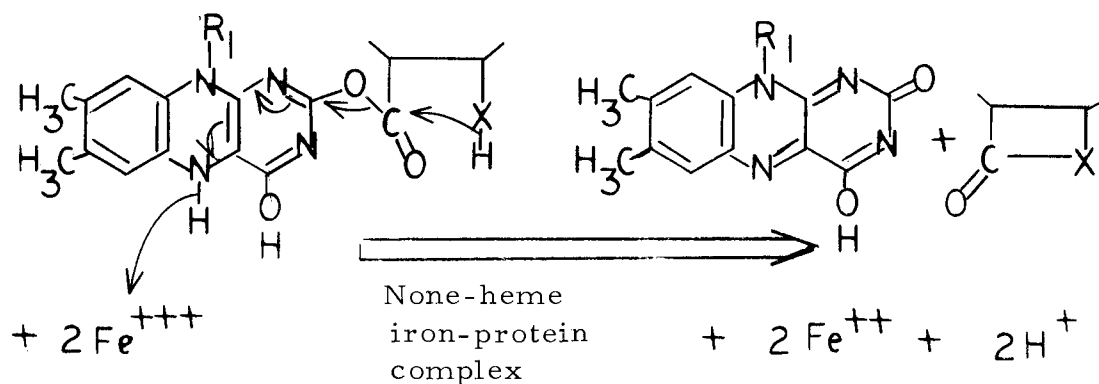
Therefore the proposed formula III might be in its reduced form.

The hydrogen on the OH group could be readily dissociated and the resulting anion, which is in a relatively nonpolar region, could be highly nucleophilic and could initiate a nucleophilic attack on carboxyl carbon to form an ester, as illustrated in the following equations.



The essential feature of the reactions followed is the oxidative withdrawal of electrons from the atoms bearing the acyl group with a concomitant formation of acyl~X high-energy intermediate.

For example,



The same principle could also be applied to the other two sites. Thus the energy coupling and the following energy transfer sequence are proposed as in Fig. 18.

In the proposed scheme, another unsolved problem is the nature of phosphorylated intermediate, I~P. It could be an acyl~phosphate by simple replacement of CoA from its precursor; it could be a phosphoryl CoA, or even another enzyme bound phosphorylated intermediate in which phosphohistidine or bound DPNH~P might be the prospective candidates. With further investigation of the soluble fraction containing high-energy intermediates the possibility of elucidating the nature of the phosphorylated intermediate appears to be very promising.

With this proposed scheme it is possible to explain most of the experimental results observed thus far.

- (i) Oxidative phosphorylation takes place at three different sites with the production of a common intermediate acyl~X by reaction (II). This could explain why the

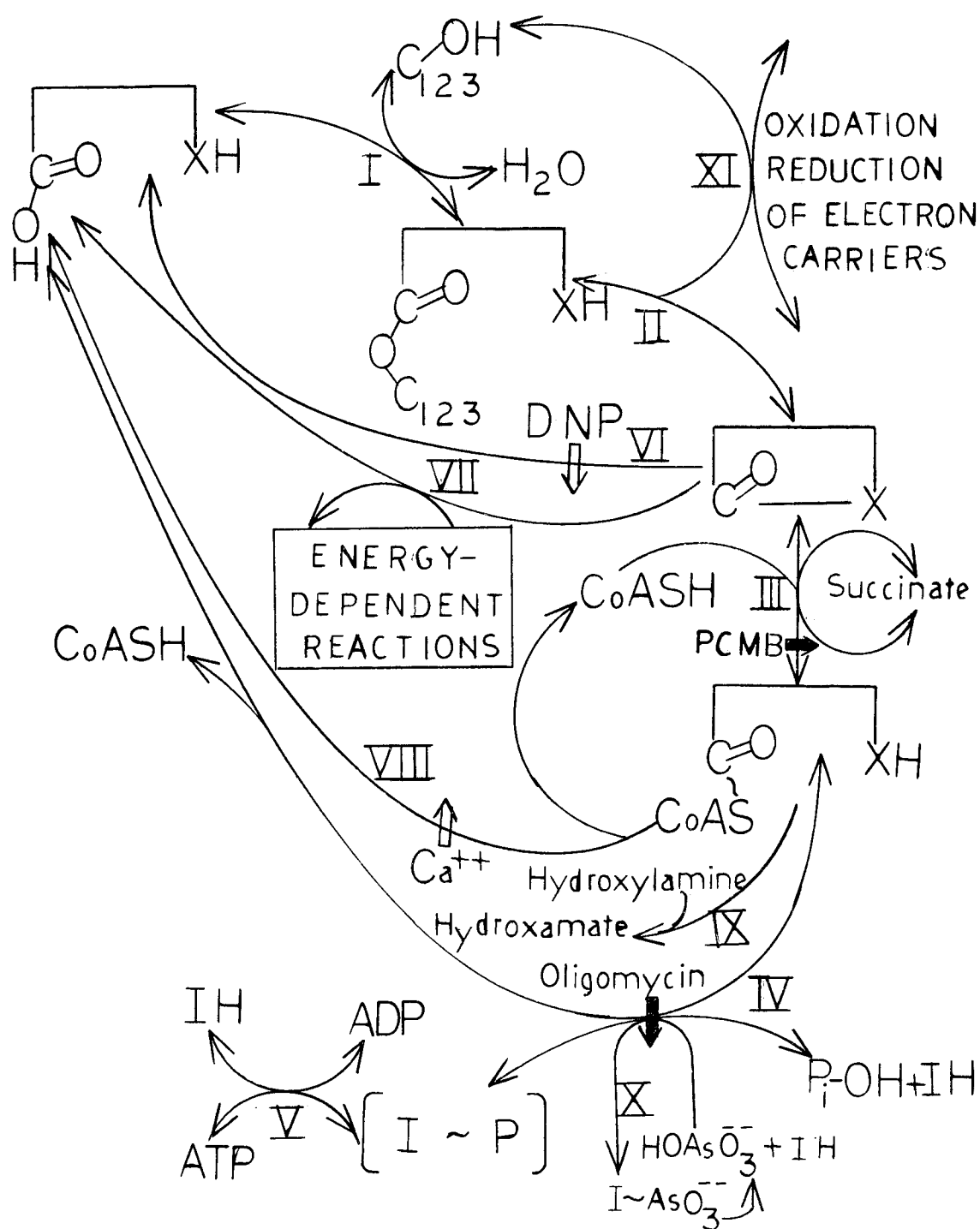


Fig. 18. Proposed scheme of energy-coupling, energy transfer and energy-dependent reactions.

C is the common symbol for electron carriers; the subscripts in $C_{1,2,3}$ refer to the 3 energy coupling sites of respiratory chain. \downarrow inhibition, \Downarrow promotion

mechanism of the conversion of the high-energy intermediate into ATP is essentially the same in each of the three phosphorylation sites as claimed earlier. The transfer reactions from each of the three sites show the same sensitivity to DNP uncoupling and oligomycin inhibition, and all of them require the same coupling factors for full activity (103).

(ii) Step (V) accounts for ATP-ADP exchange while ATP- P_i exchange requires both steps IV and V. Both of these exchange reactions have been demonstrated in soluble enzyme system (102, P. 112-128). An H_2O^{18} - P_i exchange has been demonstrated by several laboratories (5, 34, 136). It was observed that the exchange reaction requires submitochondrial particles and coupling factors (102, P. 127). This could be easily understood with the aid of the proposed scheme. The exchange reaction would include steps IV, V, and I when ATP was present.

(iii). PCMB inhibits the formation of acyl~SCoA, while oligomycin inhibits the formation of phosphorylated intermediate. One could predict that oligomycin interacts with the active center where I~P was formed.

If this is the case, oligomycin will inhibit the formation of $I\sim P$ in either direction. This would explain the observations of Lardy and other investigators (72) that oligomycin inhibits the phosphoryl transfer to ATP.

- (iv) The uncoupling effect of DNP at step VI, Ca^{++} at step VIII and arsenate at step X give more free coupling enzyme to promote the electron transport capacity. This can explain the stimulatory effect of DNP and arsenate on phosphate-deficient respiration and the " Ca^{++} -burst" mentioned earlier. DNP attacks the primary soluble nonphosphorylated high-energy intermediate $Acyl\sim X$. Ca^{++} uncouples the secondary non-phosphorylated intermediate $Acyl\sim ScoA$. This would explain why oligomycin does not inhibit the Ca^{++} -induced stimulation of respiration, while PCMB does (113).

- (v) It appears that the soluble primary nonphosphorylated high-energy intermediate $Acyl-X$ participates in the energy dependent reactions. It is clear that the generation of this intermediate will be inhibited by various uncouplers and inhibitors, which in turn will inhibit the energy dependent reaction. The scheme is very consistent with the findings from various laboratories that oligomycin inhibits the ATP supported

reaction, whereas it does not affect, or very slightly enhances the reaction proceeding at the expense of aerobically generated Acyl~X. DNP, on the other hand, inhibits the reaction in both cases but more efficiently with aerobically generated Acyl~X than with ATP as source of energy (40).

- (vi) As has been discussed, step II and XI involve the oxidation reduction of electron carriers. It appears to be in the reduced state when the carrier forms the phenolic type ester with the carboxylic group of soluble enzyme. Oxidation of the carrier transmits the potential energy to the chemical bond energy stored in the form of Acyl~X and the phenolic OH is oxidized to the carbonyl group. Reduction of the oxidized carrier regenerates $\begin{array}{c} \text{OH} \\ \diagup \\ \text{C}_{1,2,3} \end{array}$ which will be ready for the next energy coupling reaction.

- (vii) Hydroxylamine inhibits energy transfer reaction by the formation of hydroxamate with Acyl~SCoA [step (IX)].

The results of these investigations are significant with respect to a recent hypothesis proposed by Mitchell to account for oxidative phosphorylation (90). This hypothesis proposes a chem-osmotic mechanism for the oxidative phosphorylation in which an

ion gradient across a membrane is generated by respiratory chain activity. This gradient provides the potential energy for the production of ATP from ADP. There is some experimental support for this mechanism. However, the implication of a high energy intermediate in these studies and the observation at high energy transfer in a soluble system would throw some doubt on this chemiosmotic mechanism.

The soluble fraction appears to provide a convenient system to study, at least, the energy transfer reaction from the primary nonphosphorylated high energy intermediate to ATP formation. The successful demonstration of this primary high energy intermediate and the striking evidence of the participation of CoA in transfer reaction have led to the development of the scheme as shown in Figure 18. This scheme shows some possibility of explaining most of the experimental evidence in energy coupling and energy transfer reactions. There are many questions remaining to be answered such as the identity of X and I, the mode of action of DNP and oligomycin, number of enzymes involved, and so on. Further research effort with the supernatant fraction appears to be very promising, especially when purification of intermediate itself or enzymes catalyzing the transfer activity have been attempted.

SUMMARY

Studies with whole mitochondria and submitochondrial particles demonstrated that a soluble nonphosphorylated high energy intermediate of oxidative phosphorylation was released to the soluble fraction after incubating the particles in phosphate-free medium with pyruvate or other oxidizable substrate. The formation of this intermediate was inhibited by antimycin A and uncoupled by DNP.

Mitochondria were incubated in the absence of phosphate and then broken by sonic oscillation to release the high energy intermediate. Centrifuging removed the particulate matter leaving the soluble fraction with which the following observations were made.

- (i) A nonphosphorylated high energy intermediate existed in a soluble form and was detected by its ability to form ATP^{32}P (or $\text{G-6-}^{32}\text{P}$) when the soluble fraction was incubated with $^{32}\text{P}_i$ and ADP (or ADP, hexokinase and glucose).
- (ii) The energy transfer activity from nonphosphorylated intermediate to final product, ATP (or G-6-P), can be demonstrated in the soluble fraction without the participation of particulate fraction of mitochondria.
- (iii) The intermediate exists in a low steady state concentration inside the mitochondria.

- (iv) DNP destroyed the primary nonphosphorylated soluble high energy intermediate probably through the formation of a readily hydrolyzable enzyme-bound DNP derivative. uncoupling effect of DNP depends on its concentration. P_i and ADP might compete with the same intermediate by transforming it into a more stable compound.
- (v) Oligomycin appears to inhibit the formation of phosphorylated intermediate.
- (vi) Addition of succinate and CoA to soluble fraction which contained high energy intermediate brought about a marked increase in energy transfer activity. Other dicarboxylic acids or analogues of succinic acid with the exception of α -ketoglutaric acid cannot substitute for succinic acid. The stimulatory effect of CoA is also very specific. Cysteine has no effect, in fact, higher concentration of cysteine inhibit the succinate - CoA stimulation. Only catalytic amounts of succinate and CoA are needed for the maximal stimulatory effect.
- (vii) PCMB at a concentration of 2×10^{-5} M completely inhibits the transfer activity; addition of CoA restored the energy transfer reaction.
- (viii) Arsenate severely inhibits the transfer activity at a

concentration of 3.3×10^{-4} M probably due to the competition for nonphosphorylated intermediate.

- (ix) The intermediate is proteinaceous surviving dialysis, ammonium sulfate fractionation and acetone treatment.

The definite requirement of succinate and CoA in energy transfer reaction was demonstrated by the studies of ammonium sulfate fractionation and acetone treatment of soluble fraction. Ammonium sulfate fractionation and acetone treatment led to the loss of transfer activity; which can be restored by the addition of succinate and CoA. Transfer activity was observed in the 50-75% and 75-100% salt fractions.

Attempts to remove succinic thiokinase activity from the energy transfer system has not succeeded. ATP- $^{32}\text{P}_i$ exchange activity was used to detect succinic thiokinase. The succinate - CoA stimulation of transfer activity cannot be explained by $^{32}\text{P}_i$ -ATP exchange or reaction resulting from reversal of the succinic thiokinase reaction. The following observations would suggest that it is highly improbable that the thiokinase function is involved in any way with the transfer activity.

- (i) Malonate did not inhibit transfer reaction while it inhibited exchange activity to about 50% at the same concentration.

- (ii) GDP enhanced exchange activity while inhibiting transfer activity.
- (iii) Arsenate completely inhibits transfer while it only inhibits exchange activity to about 40%.
- (iv) PCMB at a concentration as high as 4×10^{-5} M did not inhibit succinate - CoA stimulated transfer activity in the presence of CoA while the exchange activity dropped to more than 50% under comparable conditions.
- (v) Hydroxylamine severely inhibits exchange activity while only about 50% inhibition of transfer activity was observed at a concentration as high as 0.133 M.
- (vi) Thermodynamic considerations would suggest that in the presence of hexokinase ATP formed from the transfer reaction would be utilized predominantly for the formation of glucose-6-phosphate and leave little chance for participating in the exchange reaction.

The succinate and CoA stimulated transfer reaction shows the following characteristics (i). GDP cannot substitute for ADP. (ii). The rate of transfer activity depends on P_i concentration and reaches a maximum at a concentration of 0.5 mM. (iii). M_g^{++} was also required for transfer activity with a concentration of 1.1 mM M_g^{++} giving maximum effect. (iv). DNP has no effect; the preformed intermediate is probably stabilized or transformed

to a new product by succinate and CoA. Oligomycin inhibits the transfer reaction but not to a great extent. Arsenate, on the other hand, severely inhibits the transfer activity. Hydroxylamine also inhibits the transfer reaction. (v). Kinetic studies showed that only a limited amount of high energy intermediate was present and this pre-existent high energy intermediate explained the initial rapid formation of ATP (ATP jump) followed by steady state phosphorylation (35) when incubation was performed in the presence of sub-particles and soluble fraction.

The soluble nonphosphorylated high energy intermediate has been shown to energize the energy-dependent reduction of TPN^+ by DPNH coupled to the reduction of DPN^+ by succinate. Reaction requires soluble fraction, sub-particles, succinate, DPN^+ and TPN^+ . ATP had little effect. Arsenate lowers the reaction activity probably due to the uncoupling of some of intermediate. Further competition for the high energy intermediate has been shown by the addition of P_i and ADP with a resultant lowering of reaction rate for the energy dependent reaction.

A schematic representation of the sequence of energy transfer reactions and the site of inhibition by various reagents was proposed, based on these experimental results. It was suggested that the primary nonphosphorylated high energy intermediate of the

type Acyl~X was presented in soluble fraction. Addition of CoA would lead to the formation of a secondary high energy intermediate of the enzyme bound Acyl~S CoA type. Succinate was probably functioned in promoting Acyl~S CoA formation through some concerted mechanism.

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