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Title: CERTAIN ASPECTS OF ELECTRON AND COUPLED ENERGY TRANSFER IN CARDIAC MUSCLE

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The first area of investigation involves the electron transport of sub-mitochondrial particles and their components. The second area is the synthesis of ATP and other energy linked reactions and components of the energy conservation and transfer apparatus.

Investigations on the nature of the tetramethyl-p-phenylenediamine (TMPD) shunt indicated that tetrachlorohydroquinone (TCHQ) as well as TMPD was able to overcome inhibition of succinate oxidase by antimycin A and quinoline-N-oxide but not thenoyltrifluoroacetone (TTA). TCHQ and TMPD also overcame inhibition of NADH oxidase by rotenone, antimycin A and quinoline-N-oxide. Kinetic studies indicated that exogenous cytochrome c greatly facilitated interaction of TMPD and TCHQ possibly by generating new pathways of electron transfer. Determination of kinetic constants for the process of restoration indicated that one site of oxidation and one site of reduction existed for TMPD with succinate oxidase. The TCHQ restoration was

complicated by an inhibition of succinate oxidase that was greatly potentiated by exogenous cytochrome c. TMPD seems to interact at two sites of reduction with NADH oxidase while TCHQ has only one site of reduction. Both have single sites of oxidation. The effects of TCHQ and TMPD were also examined with NADH oxidase, succinate oxidase and cytochrome c oxidase.

The site of action of TTA was investigated using the catalytic activities of the Keilin-Hartree heart muscle preparation (HMP) and soluble succinate dehydrogenases. The TTA apparently acts at a site which is closely related with the binding of the succinate dehydrogenase flavoprotein to the remainder of the chain. In addition, a new catalytic property of reconstitutively active succinate dehydrogenase was found: A "low K_m" succinate ferricyanide reductase.

The interaction of cationic and neutral, stable free radicals was examined with the respiratory chain and a variety of soluble oxidation-reduction enzymes. The catalytic reduction of the stable free radical, Wurster's blue, seems to depend on flavoprotein oxidation-reduction enzymes.

The properties of the adenosine triphosphatase of HMP were investigated in situ and in solution. The soluble enzyme from HMP was purified and, using nossal particles, its ability to participate in oxidative phosphorylation was demonstrated.

A factor was isolated (alkali extractable soluble factor, AESF)

which was found to be capable of restoring oxidative phosphorylation to several non-phosphorylating sub-mitochondrial particles. The relation of the AESF and oligomycin stimulation of the energy linked processes was investigated with HMP. The AESF was also able to stimulate the energy linked transhydrogenase of HMP. The properties of the factors were investigated as well as the interaction between the soluble AESF and the particulate respiratory assembly.

Certain Aspects of Electron and Coupled Energy Transfer in Cardiac Muscle

by

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Typed by Clover Redfern for John Rutherford Kettman, Jr.

DEDICATION:

for Marjorie Simila Kettman

Wife and mother, 1 April 1965

"She did not see what she caused to be"

ACKNOWLEDGMENT

This thesis is written because of my wife. Her constant love and help wrote this thesis. Jacqueline Louise Kettman predicated its completion.

In the course of the last five years, many have given unstintingly their time and effort for my education. No acknowledgment can express proper gratitude for their efforts. Of special note is the patience and understanding of Professor Tsoo E. King who advised this thesis.

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To my friends and colleagues in the Department of Chemistry and the Science Research Institute goes my everlasting gratitude. Their friendship and love gave meaning to my last years. Without these gifts, this thesis is but paper and this life and Margie's were wasted.

NOMENCLATURE

AA	Antimycin A
AESF	Alkali extractable soluble factor
aHMP	Alkaline treated heart muscle preparation
ASP	Ammonia sonic particles
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHM	Beef heart mitochondria
BSA	Bovine serum albumin
cHMP	Cold heart muscle preparation
DCIP	Dichlorophenolindophenol
DNP	2, 4-dinitrophenol
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EmM	Millimolor extinction coefficient
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HLBHM	Heavy layer beef heart mitochondria
HMP	Heart muscle preparation
K_m	Michaelis-Menten constant
LLBHM	Light layer beef heart mitochondria
METPH	Modified phosphorylating electron transport particle

NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nictoinamide adenine dinucleotide
NADP	Nictoinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NP	Nossal particles
PCMP	P-Chloromercuribenzoate
PMS	Phenazine methosulfate
QNO	Quinoline-N-oxide
SDH	Succinate dehydrogenase
SHMP	Sucrose heart muscle preparation
TCHQ	2, 3, 5, 6-Tetrachlorohydroquinone
TMPD	N, N, N' N'-Tetramethyl-p-phenylenediamine
Tris	Tris (Hydroxymethylamino) methane
TTA	Thenoyltrifluoroacetone
USHMP	Urea treated sucrose heart muscle preparation
USP	Urea sonic particles
V_{max}	Maximum velocity
WP	Wurster's blue or Wurster's blue perchlorate

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CERTAIN ASPECTS OF ELECTRON AND COUPLED ENERGY TRANSFER IN CARDIAC MUSCLE

INTRODUCTION

Oxidative phosphorylation is the process by which certain metabolites are oxidized and part of the resulting energy is conserved in the form of adenosine triphosphate (ATP) or other high energy compounds. This thesis investigates, in part, both aspects of this process and also the chemical inter-relationships between the oxidative and energy conservation apparatus.

Many reviews exist which detail recent developments in specific areas of this field. More recently monographs reviewing the entire field have appeared. Fine historical and other reviews such as those by Keilin (32), Racker (66) and Lehninger (49) as well as recent comprehensive symposia edited by B. Chance (7), King, Morrison and Mason (42), Tager et al. (79), E.C. Slater et al. (76), make extensive review of oxidative phosphorylation unnecessary. A brief review of the recent literature relating to each of the topics as they arise will be presented. Such reviews, due to the scope of this thesis, must be limited.

The topics arising in this thesis developed during a general study of the process of oxidative phosphorylation using the Keilin-

Hartree heart muscle preparation (HMP) as prepared by King (37) with bovine heart muscle as the specific system under investigation. Since this investigation was conducted during a period of great development in oxidative phosphorylation, the work of other laboratories necessarily altered the direction and influenced the design of some experiments.

If it were not for the slowness of scientific communication, many of the experiments in this thesis would not have been attempted. Hence, there exists in oxidative phosphorylation a great deal of redundency which is the result of several laboratories working at the same time on the same or similar problems.

Because of their variety, the principal subjects of this thesis are introduced below and treated separately in the Results and Discussion.

The TMPD Shunt

The observation was made recently (48), that tetramethy-p-phenylenediamine (TMPD) could restore oxidation in a system containing substrate, respiratory particle and a respiratory inhibitor such as Antimycin A (AA) or quinoline-N-oxide (QNO). The phenomena was named the "TMPD Shunt." This work was extended and amplified in a chapter in "Oxidases and Related Redox Reactions" by the same authors (42, p. 960). The data presented in this thesis was

reported at a Northwest Regional Meeting of the American Chemical Society (34). Since that report, Packer and Mustafa (58) have also communicated information about the "shunt" phenomena. There exists in the discussion of "Oxidases and Related Redox Reactions," several significant comments concerning the shunt mechanisms. Kaniuga (31) has reported the use of the TMPD shunt in analysis of damage to the respiratory chain.

Parks et al. (59) noted an uncoupling effect that TMPD exhibited when added to respiring rat liver mitochondria which could be reversed with a number of agents. They attributed the uncoupling as due to the free radical of TMPD, Wurster's Blue (WB). Substituted p-phenylenediamines were introduced as donors for cytochrome oxidase by Borei and Bjorklund (4). These authors noted the deliterious effects of the oxidation products of TMPD. The use of TMPD in conjunction with ascorbate was introduced by Jacobs (26). The TMPD system was used to donate electrons in the terminal region of the respiratory chain with the endogenous cyotchrome c or c_1 presumably reduced by the TMPD. The concept developed is that the TMPD acted as a catalyst with the ascorbate being the principal source of reductant. With the TMPD-ascorbate system, Packer and Jacobs were able to demonstrate respiratory control with rat liver mitochondria in the terminal phosphorylation region (57). The work of Howland (23) introduced the possibility that the TMPD might act at more than

one site, this helped to support the earlier results of Ramirez and Mujica (68) who made anomalous observations concerning the phosphorylation between cytochrome c and oxygen.

The tetrachlorohydroquinone (TCHQ) pathway, using ascorbate as the ultimate reductant, introduced by Jacobs and Crane (27) and elaborated by Machinist and others (51), was found to be nonphosphorylating. Many anomalies remain with the TCHQ oxidase activity which will be commented upon later. Tyler et al. (84) have also studied the interaction of the TMPD-ascorbate system with phosphorylating preparations.

Results in this laboratory with the ascorbate-TMPD system and beef heart mitochondria led to the belief that TMPD was acting at more than one site in the respiratory chain. A more systematic study of the nature of the TMPD shunt using HMP as the respiratory chain preparation was undertaken. Besides being readily available, the HMP does not suffer from the problem of mitochondrial permeability barriers. Several inconsistenties between the results presented in this thesis and the results of others have become apparent. The results reported here greatly extend the concept and scope of shunt mechanisms and suggest further experimentation which may give insignt into the nature of enzymic electron transfer reactions and the non-enzymic redox reactions. Understanding of much of this data awaits a greater understanding of the chemistry of the enzymes and

compounds used in these studies.

Succinate-Wurster's Blue Reductase

It follows from the nature of a shunt that shunt compounds which are oxidized at one point in the respiratory chain must be reduced at another point. It was observed that systems in which the TMPD shunt was operating became intensely blue. This blue color could also be generated by oxidation of TMPD with ferricyanide. The blue color so generated was found to require only one equivalent of ferricyanide for maximal formation. These observations indicated that the color was characteristic of the well known, stable, cation radical of TMPD, known in the literature as one of Wurster's salts or more specifically as Wurster's Blue. This compound can be prepared as a stable perchlorate salt (53). Other methods of preparation have now been worked out in this laboratory (22). Other properties and aspects of this compound are presented in part III of Results and Discussion.

It was observed that when the blue color was generated in a cuvet and when HMP, a terminal inhibitor, and succinate were added the WB rapidly disappeared. This reaction was found to be thenoyltriflouroacetone (TTA) sensitive (82). This will be elaborated later. The perchlorate salt of Wurster's blue (WB) has been used successfully as an electron acceptor in HMP.

Since WB seemed to interact at a basic point in the respiratory

chain, various forms of succinate dehydrogenase were examined. Succinate-WB reductase activity was found in the two forms examined. The activity was much higher in reconstitutively active succinate dehydrogenase (SDH) than phenozine methosulphate reductase (PMS reductase). The activity seemed to decay to a great extent very rapidly in SDH but not in PMS reductase. On examination, part of this decay was only apparent.

As mentioned before, the succinate-WB reductase of HMP was TTA sensitive but the succinate-WB reductase of SDH was not TTA sensitive. When SDH is rebound to the deficient particle, alkaline treated HMP (aHMP), the TTA sensitivity is restored. The implications are discussed with respect to the site of action of WB as well as TTA.

Kinetic studies of Succinate Ferricyanide Reductase

After using TTA as a diagnostic reagent in the previous section of the thesis, it was realized that there is a paucity of information on the subject of TTA inhibition. Since the announcement of the specific inhibition of succinate oxidase by TTA (82), little information has been published. No information exists in the literature which fits the pattern seen with the new artifical acceptor, WB. No published record exists of the titration of activity with TTA concentration. Section II of Results and Discussion was undertaken to obtain data with other

artifical acceptors to present to contrast to data obtained with WB. Dichlorophenolindophenol (DCIP) was chosen because it is an acceptor whose reduction by succinate depends on reconstitution of SDH with aHMP (36). After analysis of eight separate HMPs, the results indicate that succinate-DCIP reductase is inhibited by TTA much like succinate-WB reductase. The TTA sensitivity of succinate-ferricyanide reductase activity of HMP was investigated. Redfearn (90) indicates that there are at least two sites of ferricyanide interaction with the succinate oxidase chain. Further, he indicates that the TTA inhibition is variable, depending on the concentration of ferricyanide used in the assay. Reinvestigation of these experiments indicated no variation in TTA inhibition with ferricyanide concentration. Since these results were so dramatically different from those of Redfearn, TTA inhibition was investigated in greater detail by using Antimycin A inhibited preparations. According to Estabrook one of the main sites of ferricyanide interaction is in the cytochrome c region of the respiratory chain (12). In AA inhibited preparations this site is eliminated. This would present, it was hoped, a clearer kinetic picture for TTA inhibition. Also this technique might serve to address Redfearns contention that there are several sites of TTA inhibition.

The AA inhibited preparation exhibited variable TTA inhibition depending on ferricyanide concentration but the dependence was in the opposite direction from that observed by Redfearn. The titer and

maximal inhibition by TTA varied with ferricyanide concentration when AA inhibited preparations were used. This was studied in greater detail using Lineweaver-Burke analysis (12), which indicated that AA acted as a competitive inhibitor, TTA as a non-competitive inhibitor and TTA+AA exhibited mixed inhibition. Attempts at explaining the data are made but suffice it to say that TTA is interpreted to act at one site, primarily in the succinate dehydrogenase region. The kinetics of this inhibition are not simple, as might be expected considering the complexity of the respiratory chain. Redfearn's observations were not reproduced but extension of information concerning TTA inhibition of succinate-[acceptor] reductase activity was accomplished.

Low K_m (Ferricyanide) Site of Soluble Succinate Dehydrogenase

In the course of the investigation reported in section II of Results and Discussion, it was desired to measure the succinate-ferricyanide reductase of reconstituitively active and inactive forms of succinate dehydrogenase. The method related by King (39) was employed and the change of activity with aging of the enzyme was measured. Anomalous effects were observed with SDH at low concentrations of ferricyanide (less than 1 mM). The effects disappeared with aging of the enzyme. These effects were not observed with PMS-reductase or with aged SDH or when fresh SDH was assayed with

ferricyanide concentrations higher than 1 mM.

These experiments can be intrepreted as a "second site" of ferricyanide reduction in SDH. The second site has a very low K_m. The "second site" is not present in PMS-reductase and is perhaps related to the reconstitutive activity of SDH. These preliminary experiments merely open the door to a new aspect of SDH which must be yet explored.

Reduction of Organic Free Radicals in Biological Systems

As noted before, it was found that WB was reduced by succinate in a TTA sensitive reaction. This was used as a tool in investigation with SDH. It was similarly found that NADH reduced WB both enzymatically and nonenzymatically. Dr. Hegdekar examined various soluble and particulate NADH dehydrogenases for WB reductase activity. This has been partially reported (41). These studies have been extended to many other redox systems. It has been found that only flavoenzymes are active with WB.

Since a cation radical interacts with biological systems and by analogy, the anion radical of TCHQ reacts with such systems, the question was posed as to whether a neutral radical could also be reduced. The commercially available organic free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was examined. As is indicated in the results section, this radical is also reduced in biological systems

although the chemistry is not as clear as the WB experiments.

The ATPase of HMP

Part of what is presented in this section has been reported (35). The enzymological properties of the ATPase of submitochondrial particles has been much studied. Much early work exists on the ATPase of HMP (66). Myers and Slater early studied the pH optima and other characteristics (56). Oligomycin sensitivity of the ATPase of HMP was also early noted (25). The mitochondrial enzyme was isolated by Racker's group and studied (66, 61). This enzyme, prepared from mitochondria, seemingly takes part in the synthesis of ATP. Selwyn (71, 72) has reported on the preparation of the ATPase from the acetone powder of beef heart mitochondria and HMP. The details of their preparation have never been published.

Two methods for the preparation and partial purification of the soluble ATPase of HMP were developed. The aim has been slightly different from that of other investigators. The approach taken was that of attempting to remove the enzyme, study its properties and reconstitute the original structure. The first aspect contains no conceptual difficulties. However, the term reconstitution must be defined within the parameters of the experimental system (36). Many workers have used the technique of reconstitution as the final test of the "nativity" of the enzyme preparation (41). The rationale is that

if the enzyme were changed during isolation, it would not resume its original properties when restored to its original position in the biological system. This is of particular use when the properties of the enzymic activity seem to change on isolation or removal from its biological environment. Restoration of the original properties on reconstitution have assured the investigator that the isolated enzyme is related to the enzyme *in situ*.

Two kinds of reconstitution criteria were examined. The first is the reparticulization of the ATPase. The second is the restoration of oxidative phosphorylation in Nossal particles. An ancillary tool for reconstitution studies was the resensitization of the ATPase activity to oligomycin inhibition. In order to do the reconstitution experiments, an ATPase deficient particle is necessary. This could be prepared using a technique drawn from Racker's work: Urea depletion of HMP's ATPase activity (67). The soluble enzyme isolated from mitochondria was found to be a cofactor for oxidative phosphorylation. The identity of the ATPase from mitochondria and that from HMP was established even as far as participation in ATP synthesis.

Alkali Extractable Soluble Factor

One of the principal aims of investigators in the last several years has been the elucidation of the scheme of oxidative phosphorylation. The technique of reconstitution has been one of the few to

yield much information. Elucidation of oxidative phosphorylation has been refractory to the usual techniques applied to soluble systems. Some aspects of investigations into reconstitution of oxidative phosphorylation systems reported here, have been published (33).

The work reported here has centered in two areas, first is stimulation of ATP synthesis, the second is the stimulation of the "energy-linked" reactions (7). These experiments in many ways, paralleled the lines of thought of two other laboratories. As a result, some of the data presented here has been also reported from other laboratories. In particular, the work with HMP has been essentially repeated by Slater's lab but without the investigations with AESF (20). The work with ammonia sonic particles (ASP) has been essentially reported by Racker's group with other coupling factors.

The work with coupling factors for the synthesis of ATP was started several years ago and proceeded with little success. When the procedure for AESF was developed, a survey of several types of non-phosphorylating submitochondrial particles was undertaken. This survey gave indications that other types of preparations were somewhat more facile than HMP as far as restoration of ATP synthesis with AESF is concerned. HMP has been well suited for the investigation of energy-linked transhydrogenase. Similar work from other laboratories with HMP and other preparations has recently been published (85, 20). These experiments, in part, were done parallel to

investigations reported here. Results presented here differ somewhat from those of other authors. Suffice it to say that the experiments of some laboratories has been reproduced and the main arguments seem to be concerned with intrepretation.

MATERIALS AND METHODS

Materials

Chemicals were obtained from commercial sources in the highest available purity. TMPD was purified by the methods of Cox and Smith (9). Antimycin A was a product of the Wisconsin Alumni Research Foundation. Cytochrome *c* was type III obtained from Sigma. Wurster's Blue perchlorate was prepared by the method of Michaelis and Granick (53). Thenoyltriflouroacetone was a product of Aldrich Chemical and added as an acetone solution. The L-amino acid oxidase was crude *Crotalus adamanteus* venom obtained from the Ross Allen Reptile Institute. Ammonium sulfate was Mann "enzyme grade". Reduced coenzyme Q_{10} was prepared by the method of Green and Burkhard (19).

Particulate Enzymes

HMP. HMP was prepared by the method described by King (37).

Sucrose-HMP (SHMP). SHMP was used for the preparation of acetone powders. A suitable quantity (usually 200 ml) of the normal HMP was diluted with an equal volume of 0.25 M sucrose and centrifuged for 90 minutes in the #30 rotor of a Spinco model L preparative ultracentrifuge at 30,000 rpm. The supernatant was decanted and

discarded. The pellet was resuspended to the original volume with 0.25 M sucrose and recentrifuged for 45 minutes. The pellet was resuspended to the desired volume with 0.25 M sucrose.

Urea Treated HMP (USHMP). SHMP (20 mg/ml) was diluted with one volume of six M urea, pH 7.4. After standing on ice for five minutes, the mixture was centrifuged for 30 minutes at 50,000 rpm in the Spinco. The supernatant was decanted and discarded. The pellet was resuspended in the same volume of 0.25 M sucrose and recentrifuged. This pellet was finally resuspended in the desired volume of 0.25 M sucrose.

Nossal Particles (NP). This preparation was carried out essentially as described in the literature (60). Of considerable importance in the successful preparation was the ratio of the volume of glass beads to the total volume of the nossal capsule and the establishment of good vacuum when the capsule was sealed. During the course of the several preparations, the EDTA level was varied between one and four mM with little change in the resulting particles. In this study the yield of NP, once the above variables were adjusted, was two to three fold higher than reported in the literature. The residual P/O value was always higher and more stable than reported in the literature.

Beef Heart Mitochondria (BHM). BHM were prepared by the method of Crane *et al.* (10). The light and heavy layer mitochondria

(LLBHM and HLBHM) were separated by the method of Hateti et al. (21).

Cold HMP (cHMP). cHMP was prepared by a modification of the procedure previously described by King (37). Fresh heart tissue was obtained on the day of preparation. The tissue was treated as usual except that all washings and buffer extractions were carried out with chilled solutions in the cold room. The washed mince was ground in a chilled mechanical mortar in the cold room. During the grinding step the temperature of the preparation rose to 9°. The submitochondrial fraction was isolated as usual except that the final suspension was in 0.25 M sucrose. The product was frozen at either -15° or liquid nitrogen temperatures.

Modified Phosphorylating Electron Transport Particle (METPH). These preparations are modifications of the procedure of Linnane and Titchner (50).

Twenty-five ml of frozen heavy layer beef heart mitochondria (25 mg/ml, five days old, submitted to three freeze-thaw cycles) were thawed and EDTA was added to a concentration of one millimolar, pH 7.4. This mixture was sonicated with the Branson Sonic Probe for two, thirty-second periods with cooling between sonications. Temperature was maintained by immersing the glass vessel in an ice-water mixture during sonication. Sonication was at seven amps output of the sonic probe. The sonicate was diluted with one volume

of 0.25 M sucrose and centrifuged at 19,000 rpm for 15 minutes in a # 50 rotor of the Spinco. The turbid supernatant was decanted and the pellet discarded. The supernatant was then centrifuged for 25 minutes at 50,000 rpm. The clear yellow supernatant was then discarded. The red, gelatinous pellet was resuspended with the aid of a glass and Teflon homogenizer in 20 ml of a one mM EDTA-0.25 M sucrose solution and centrifuged for 25 minutes at 50,000 rpm. The supernatant was again discarded and the pellet resuspended in 20 ml of 0.25 M sucrose. The suspension was finally centrifuged for 25 minutes at 50,000 rpm, the supernatant discarded and the pellet resuspended in the following preservation solution: five mM $MgCl_2$, two mM reduced glutathione, two mM ATP, six mM sodium succinate and 0.25 M sucrose at pH 7.4. The yield of particulate protein was 22% and the product was used on the same day as prepared.

Urea Sonic Particles (USP). The procedure was modified from that of Lee, Azzone and Ernster (45).

Twenty-five ml of frozen heavy layer beef heart mitochondria was thawed. To this was added 12.5 ml of a solution which was 3.6 M urea and 0.25 M sucrose (21.6 g urea dissolved in 100 ml of 0.25 M sucrose). Nitrogen was passed through this mixture for five minutes. The suspension was then sonicated for four, thirty second periods with cooling between sonications. The Branson sonic probe was used at seven amps output with cooling accomplished by sonicating in

a glass vessel immersed in an ice-salt bath. During sonication, the temperature rose to 10° but was lowered to 3° before the next period of sonication.

To the sonicate was added 30 ml of 0.25 M sucrose and the mixture centrifuged for ten minutes at 12,000 g in a refrigerated centrifuge. The turbid supernatant was carefully decanted from the small mitochondrial pellet to give about 60 ml of turbid suspension. This was then centrifuged for 25 minutes at 50,000 rpm. The supernatant was decanted and discarded and the pellet resuspended to a volume of 40 ml with 0.25 M sucrose and recentrifuged. The supernatant was again discarded and the pellet finally resuspended in 0.25 M sucrose. The protein recovery in the sub-mitochondrial fraction was 55%.

Ammonia Sonic Particles (ASP). These were prepared after a modification of the methods of Lee *et al.* (45).

Twenty-five ml of heavy layer beef heart mitochondria were thawed (at least three days frozen) and to this was added 2.5 mls of 0.25 M ammonium hydroxide. Nitrogen was then passed through the mixture for five minutes and the mixture sonicated for three or four, thirty-second periods with the Branson sonic probe at a power output of seven amps. The sonication was carried out in a glass container in an ice bath. Cooling was allowed between sonications. To the sonicate was added 25 ml of 0.25 M sucrose. The combined material was centrifuged at 13,000 rpm for ten minutes in a # 50 Spinco rotor.

The turbid supernatant was decanted from the small mitochondrial pellet and centrifuged for 25 minutes at 50,000 rpm. The clear supernatant was decanted and discarded. The pellet was resuspended in 0.25 M sucrose and recentrifuged. The clear supernatant was again discarded and the pellet was finally resuspended in 0.25 M sucrose. This preparation gave a particulate protein yield of at least 40%.

Soluble Enzymes

Reconstitutively active succinate dehydrogenase (SDH) was prepared by method IIIA of King (38). The preparation was carried through the gel elution stage and used for assay or frozen in liquid nitrogen. Succinate-PMS reductase (reconstitutively inactive succinate dehydrogenase) was prepared by a modification of the above method. The preparation was carried out as described except the enzyme was prepared in the absence of succinate or other reducing agents.

The "37° NADH dehydrogenase" was prepared by the method of King and Howard (40). The "30° NADH dehydrogenase" was prepared by the method of Cremona and Kearney (11). The D-amino acid oxidase was partly purified by the method of Burton (6). The NADPH-cytochrome *c* reductase was partially purified by the method of Huennekens and Felton (24).

Acetone Powders for ATPase Preparation. One hundred ml of SHMP (about 40-50 mg/ml) was slowly added to about one liter of cold

(-15°) acetone which was rapidly stirred. Then 2.5 liters of cold acetone was added. After stirring for five minutes, the precipitate was allowed to settle and most of the acetone was decanted. An additional three liters of cold acetone were then added to the residue and stirred for five minutes followed by settling and decantation. The residue was collected on a Buchner funnel and washed with cold acetone. The damp powder was collected and the remaining acetone removed *in vacuo*. The product was a fluffy, tan powder, about 1/3 to 1/2 protein by weight (biuret assay). The powder was stored at -20° until used.

Preparation of the soluble ATPase. The early preparations of the soluble ATPase were by the method of Lardy and Wellman (43). These preparations were largely particulate and few experiments were done with this preparation.

Method I: The "Nosslation" Technique. This procedure is already presented in the literature (35). The yield of ATPase activity was low but the preparations did show the same characteristics as the later sonic oscillation preparations. The purification of the ATPase is described later. Using the crude extract from Method I, only moderate purification could be accomplished.

Method II: Sonic Oscillation. Three grams of acetone powder were homogenized in a solution that was one mM EDTA, one mM ATP and pH 7.4. This was diluted to a final volume of 50 ml. Room

temperature was maintained in this and subsequent steps.

The dark brown turbid suspension was added to a polypropylene beaker with 20 ml of washed glass beads (Superbrite # 091, 3M Co.). This mixture was sonicated for about 30 minutes using a Branson sonic probe at a constant output of seven amps. The temperature was maintained at about 28° by suspending the beaker in an ice-salt bath. During the sonication, the temperature was not allowed to go below 20° or rise above 30°. The sonicated mixture was decanted and the glass beads washed a few times with the EDTA-ATP solution. The washings were added to the decantate and loaded into a # 50 rotor which was at room temperature. The material was centrifuged for 40 minutes at 50,000 rpm in a Spinco where refrigeration was turned off. It has been observed that the temperature rise during the centrifugation was not significant.

Usually a clear separation of pellet and supernatant is obtained. The light yellow supernatant was decanted as the "crude extract". The supernatant could be stored at room temperature for short periods of time, or, the enzyme precipitated with room temperature saturated ammonium sulfate to give 50% saturation and the precipitate stored in the cold. The latter treatment was normally reserved for purified preparations. All operations were carried out so that the soluble enzyme is not cooled. As is common experience, higher ionic strengths greatly influence the cold lability of the enzyme and should

be avoided.

Ammonium Sulfate Fractionation. The following procedure has been generally successful. Variable, however, is the exact ammonium sulfate concentration at which the ATPase begins precipitating. Generally the 35-45% room temperature saturated fraction contained most (80%) of the recovered activity. Rarely the 25-35% fraction contained significant activity. The purified fraction besides having a specific activity varying between 20 and 30 was also free of any color.

Step One. 0-11% Room Temperature Saturated Ammonium Sulfate Fraction. Solid ammonium sulfate was added until 11% room temperature saturation was obtained, if no precipitate was observed, step two followed immediately. If a precipitate was observed after ten minutes, the solution was centrifuged at 10,000 g for five minutes centrifuge at room temperature. The pellet was a dark brown material that was no longer soluble and contained little activity; the supernatant, yellow and clear, was decanted for step two.

Step Two. 11-60 % Room Temperature Saturated Ammonium Sulfate Fraction. Solid ammonium sulfate was added to 60% saturation and the turbid suspension centrifuged after standing for ten minutes. The supernatant, yellow and clear, was discarded and the pellet resuspended in about 1/10 the original volume with ATP-EDTA solution. This gave a yellow, turbid suspension.

Step Three. The Suspension from step two was centrifuged for

about ten minutes at 10,000 g. This yielded a yellow, clear supernatant and a pellet that was not soluble. The pellet rarely carried activity.

This solution was fractionated stepwise with room temperature saturated ammonium sulfate. Usually the ATPase activity is found in the 35-45% saturation fraction. This fraction was always a clear, uncolored solution. This purified fraction was stored at 0° as a 50% ammonium sulfate precipitate.

The yield in the crude supernatant has been up to 75% of the activity in the sonicated suspension. The ammonium sulfate purification does proceed with some loss of activity but the yield is about 60%. Failure to remove any dark brown material that precipitates at 11% saturation leads to coprecipitation with the ATPase at 25% ammonium sulfate saturation.

Acetone Powder for AESF. To one hundred ml of beef heart mitochondria was added ATP, EDTA and succinate to give: one mM ATP, six mM succinate and one mM EDTA. An acetone powder is made from this mixture directly, or, after the mixture had been stored frozen for several days. The acetone powder was made in the previously described manner and stored at -15° with no special precautions. AESF could be made from such powders up to several weeks after preparation of the powder.

Extraction of the Crude AESF. One gram of the acetone powder

was suspended in 25 ml of the following chilled mixture with the aid of a glass-Teflon homogenizer: 50 mM Tris-acetate, pH 8.9, two mM ATP, 20 mM succinate and one mM EDTA. The suspension was allowed to stand in the cold for 15 minutes. The incubated suspension was centrifuged for 20 minutes at 12,000 g in a refrigerated centrifuge. The yellow supernatant was carefully decanted and the pH adjusted to 7.4 with one N acetic acid. The neutralized extract was quickly used as AESF. The yield was always 20 ml of 4.8 $\pm$ 0.2 mg/ml protein solution.

Analytical Methods

Protein Determination

Protein determinations were carried out in the following manners: for clear, colorless proteins either the Warburg and Christian method (44), the TCA turbidometric method (44) or the biuret method (44) were used. For clear colored samples, biuret method in the presence of hydrogen peroxide or employing a color blank was used. For turbid protein samples, either biuret employing a color-scatter blank containing deoxycholate or "total solids" method was used (75). For all methods except Warburg-Christian and total solids, bovine serum albumin was used as the standard.

Oxygraph Methods for Section I of Results and Discussion

All experiments reported were performed on at least two different HMPs and the results were essentially the same. Brackets around K_m values denote some variability among preparations. In some cases the data presented is representative of several experiments but only a protocol was chosen for presentation.

All the rates of oxygen uptake were measured in a Gilson Oxygraph at room temperature with the apparatus standardized with 0.1 M Sørenson phosphate buffer, pH 7.4.

The first type of experiment is illustrated in Figure 1. Five mM ascorbate is the substrate and a convenient amount of HMP is added. The blank rate was determined, and then small quantities of the other compounds were added and the rates measured. The rates were almost immediately linear and were followed for a minute or two before additional aliquots of the compound were added. The rates were corrected for the increased volume. Reagent blank controls (no HMP) were found to be negligible for almost all calculations. For the cytochrome c oxidase measurements, TMPD was added as a 6.1 mM solution, TCHQ as ten mM and cytochrome c as 20 mg/ml. In some cases the high ethanol concentrations at the terminus of the experiment were uncorrected.

Figure 2 illustrates the second procedure. This is used for the

shunt experiments. Enzyme and substrate (either succinate at 30 mM or NADH at 0.2 mM) are added and a basal rate established. If exogenous cytochrome c is a parameter, it is present in the initial mixture so that the basal rate reflects stimulation of the activity by the cytochrome c. Inhibitor is then added and the inhibited rate determined. Then small aliquots of restorant are added with the rate determined between additions.

The third method is described in Figure 3 and is like the second except that no inhibitor is added during the course of the experiment.

Units

Units are in terms of μM (or mM) substrate turned over per minute at defined temperature and assay conditions. Specific activities, unless otherwise stated, are in terms of μM (or mM) substrate acted upon per minute at an enzyme concentration of one mg/ml in the assay mixture. For spectrophotometric assays, the temperature is ambient, usually 25°. The temperature is maintained at 30° for oxidative phosphorylation and ATPase assays.

Succinate - WB Reductase in HMP. This assay is carried out in 0.1 M Sørensen phosphate buffer, pH 7.4. Succinate is added to 15 mM and KCN to two mM. WB concentration was then varied. When low concentrations of WB were used, the rate was measured at 612 mμ where the millimolar extinction coefficient (EmM) was found

to be 11. When V_{max} determinations were made, the rate was followed at 650 mμ where the millimolar extinction coefficient (EmM) was 1.1.

Succinate - WB Reductase With Soluble Succinate Dehydrogenating Enzymes. The reaction was run as above but no KCN was added. The reaction was started by the addition of the enzyme. Some assays of SDH's succinate-WB reductase activity were run in 0.1 M Tris-acetate buffer, pH 7.4. The initial rates were the same as when phosphate buffer was used. The addition of bovine serum albumin did not affect the rates.

Succinate - DCIP Reductase in HMP. This was assayed as outlined by Takemori and King (81) in the presence of two mM KCN.

Succinate-ferricyanide Reductase in HMP. This was assayed as outlined by King (39) with the addition of two mM KCN.

Succinate-ferricyanide Reductase in SDH. This reaction was assayed in the Cary model 11 spectrophotometer equipped with a 0.0-0.1 A slide wire. In a final volume of three ml was 33 mM sodium succinate, 67 mM Sørensen's phosphate buffer, pH 7.4, and potassium ferricyanide between 0.1 and 0.5 mM. No KCN was used and the reaction was started by the addition of the enzyme. The initial rate was observed about five seconds after mixing.

ATPase Assay. Assay method I was designed by Myers and Slater(56), whereas assay method II was used by Gatt and Racker (16)

as modified by Pullman et al. (64). Method II differs from I in that an ATP regenerating system is incorporated into the assay. Method II gives accurate, linear results. The phospho(enol)pyruvate was prepared by the method of Clark and Kirby (8) and used as the monocyclohexylammonium salt.

Reconstitution of the ATPase. The first method of reconstitution (method I) was taken from the description by Penefesky et al. (60). In this method NP and the ATPase are incubated together for ten minutes at 30° with 25 mM tris-acetate buffer, pH 7.4 and 13 mM $MgCl_2$. The sucrose concentration was not strictly controlled. After incubation, an aliquot of the mixture was added directly to the chilled Warburg flasks and the assay commenced.

Method II. The incubation mixtures used in reconstitution method II were similar to those in method I. After the incubation the sample was diluted with 0.25 M sucrose containing ten mM $MgCl_2$ to a final volume of ten ml. Each sample was loaded into a Spinco # 50 rotor tube and centrifuged for 25 minutes at 50,000 rpm. The supernatant was decanted and the surface of the small pellet rinsed with an aliquot of the sucrose-$MgCl_2$ solution. The pellet was finally resuspended in one or two ml of the sucrose-$MgCl_2$ mixture and an aliquot added to the chilled Warburg flasks and the assay commenced.

Assay of Oxidative Phosphorylation. The method, modified in the sequencing of events, is the one outlined by Penetsky et al. (60).

The incubated sample was added to the chilled Warburg vessel containing the same reagents as described by Penetsky et al. The chilled vessels were attached to the manometers. Each sample was run in duplicate with a single zero time control. The sidearms of the Warburg flasks contained the hexokinase. The substrate was added directly to the main compartment of the Warburg vessel. The assembled manometers were placed in the Warburg bath for five minutes to allow thermal equilibration. The taps were turned and the sidearms tipped and the measurement of oxygen uptake commenced. The control flasks were removed at this time, and 0.2 ml of 40% TCA was added to stop the reaction. After the TCA addition, the sidearms of the control flasks were tipped. The reaction was allowed to proceed for 20 to 40 minutes. The assay was stopped by removing the manometers from the bath and the flasks from the manometers. The flasks were quickly chilled and TCA added. The contents of the flasks were decanted and centrifuged to remove the precipitate. Two, 0.1 ml samples were removed from each flask for inorganic phosphate analysis (15). The phosphate uptake was defined as the difference between the control flasks and the sample flasks. They oxygen uptake was measured directly. The manometers were read just before removal from the Warburg bath, hence any error in the oxygen uptake would be slight. The assay method was satisfactory for most cases. The assay system was modified if the P/O measured was greater than one.

At very small P/O's, the variation encountered was so great as to preclude significant observations.

Reconstitution with AESF and Submitochondrial Particles. The samples were prepared as follows: Tubes were made up and chilled so that when all the contents have been added, the final concentrations will be ten mM $MgCl_2$, six mM sodium succinate, 30 mM Tris-acetate, pH 7.4. Usually ten to 15 mg of particulate material was added to each tube, and AESF was added in varying amounts, the difference to ten ml being made up by 0.25 M sucrose. The coupling factor and particle are added last and the tubes inverted for mixing. Then each sample was transferred to the # 50 rotor tube and centrifuged for 25 minutes at 50,000 rpm.

The supernatants were usually decanted and discarded. Then 0.25 M sucrose was used to wash the sides of the tube and surface of the pellet. Each pellet is suspended and homogenized in two ml of a suspension medium, and 0.2 ml aliquots of this are added to each Warburg vessel. This procedure is varied by leaving out $MgCl_2$ in incubation media when metal requirement is being tested, however, the same suspension medium is used. Suspension medium: 0.2 M sucrose, ten mM $MgCl_2$, six mM sodium succinate, 30 mM Tris-acetate, pH 7.4.

Purification of AESF used for the Stimulation of Energy-linked Reactions. Figure 4 describes the purification of AESF which was

active in stimulating the energy-linked transhydrogenase. The factor was purified using stimulation of succinate driven transhydrogenase.

Purification of AESF Used for Reconstitution of Oxidative Phosphorylation. This is a purification of the same crude extract but now using stimulation of oxidative phosphorylation as the assay. This purification is described in the text of section V of Results and Discussion.

Energy-linked Reactions

The following energy-linked reactions were those most often utilized and appeared to be most reproducable and reliable.

Transhydrogenase Supported by Succinate Oxidation

The assay mixture contained: 20 μg rotenone, 50 mM Tris-acetate, pH 7.4, 15 μg crystalline lactate dehydrogenase (Sigma), 200 mM lactate, 200 μM NAD, 200 μM NADP, ten mM succinate and 0.5 to 1.0 mg of the HMP or other sub-mitochondrial particle in a total volume of three ml.

Ten mM $MgCl_2$ was added with AESF. The increase in absorbance at 340 mμ was measured on a Cary spectrophotometer, model 11, at about 25° using a 0 - 0.1 slide wire. Succinate or NADP was used to start the reaction. The reading was made against the blank in the spectrophotometer which contained all constituents except

succinate.

The substitution of one mg of alcohol dehydrogenase and 0.05 ml of ethanol could be made for the lactate-lactate dehydrogenase system. In accordance with other laboratories, activity is expressed as--A 340 mμ per minute per g protein.

Transhydrogenase supported by ATP.

The assay mixture contained: two mM sodium sulfide, 50 mM Tris-acetate, pH 7.4, 200 μM NAD, 200 μM NADP, 15 μg lactate dehydrogenase, 200 mM lactate, ten mM $MgCl_2$, 1.5 mM ATP and 0.5 to 1.0 mg HMP or other sub-mitochondrial particle. The reaction was started by NADP when the enzyme had been preincubated with ATP for about five minutes at room temperature, or by ATP when the enzyme had been preincubated with NADP. A lag period was observed when ATP was used to start the reaction. Again, the ethanol-alcohol dehydrogenase system could be substituted for the lactate system.

Reducation of NAD by Succinate Supported by ATP.

The assay mixture contained: 50 mM Tris-acetate, pH 7.4, two mM sodium sulfide, 200 μM NAD, ten mM succinate, ten mM $MgCl_2$, 1.5 mM ATP and one mg of HMP or other sub-mitochondrial particle. The reaction was started by succinate when the enzyme had been preincubated with ATP for approximately five minutes at room

temperature, or by ATP when the enzyme had been preincubated with succinate. In addition, two other energy-linked assays were examined. These were: Transhydrogenase driven by the TMPD-ascorbate terminal oxidation system and the succinate reduction of NAD driven by the same terminal oxidation system. Although some activity could be demonstrated with these systems, the stimulation with AESF was somewhat equivocal, and this data is not presented.

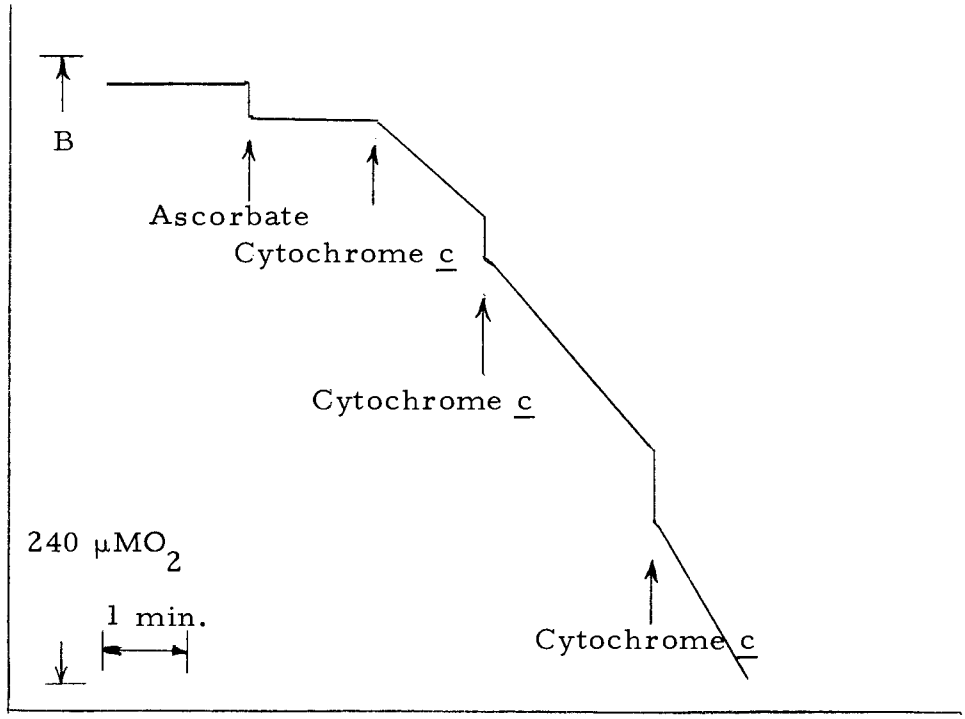


Figure 1. Oxygraph recording of cytochrome oxidase assay: effect of concentration of cytochrome c.

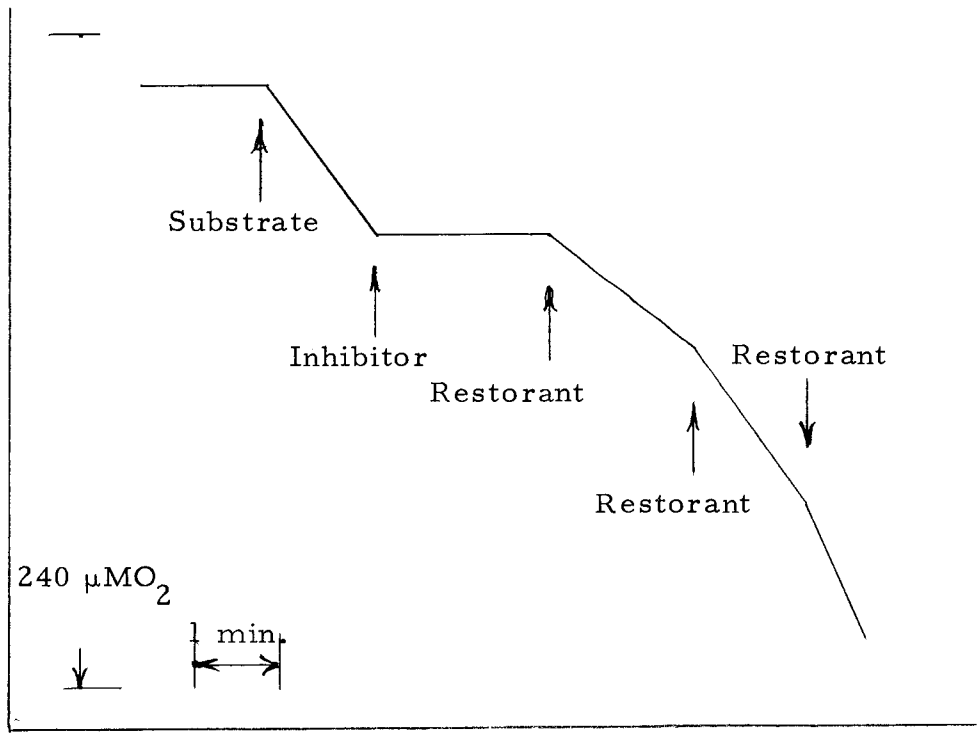


Figure 2. Oxygraph recording of typical shunt type of experiment.

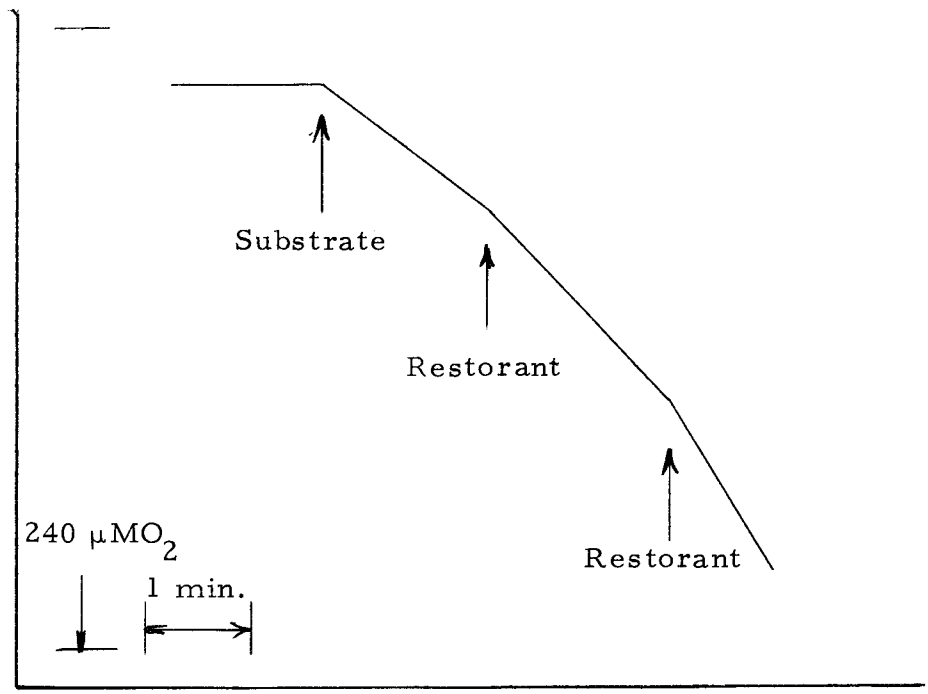


Figure 3. Oxygraph recording of typical experiment where effect of TMPD or TCHQ on substrate oxidation is determined.

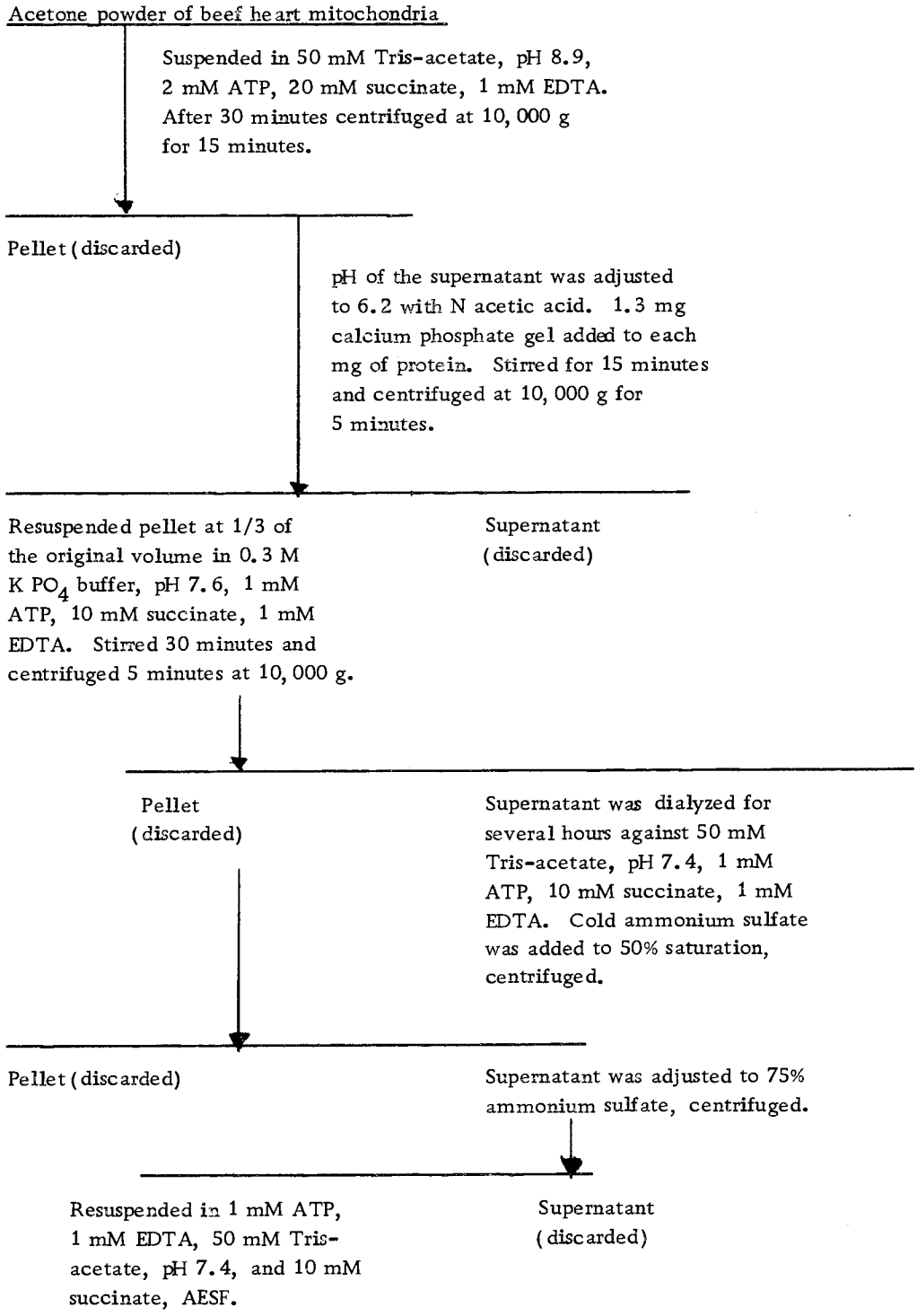


Figure 4. Purification scheme for alkali extractable soluble factor.

RESULTS AND DISCUSSION

Part I. Investigations with Shunt Systems

A. The Effects of TCHQ, TMPD and Exogenous Cytochrome c on NADH Oxidase

Before examining the effect of TCHQ and TMPD on the activity of inhibited systems, the effect of the compounds on the uninhibited system was investigated. The effects of exogenous cytochrome c were also considered as a parameter.

The NADH oxidase activities of HMP 469 and 472 behaved the same in these experiments, only the results of experiments using HMP 472 will be presented. Exogenous cytochrome c stimulated NADH oxidase only slightly and the activity was fully stimulated at less than 30 μM exogenous cytochrome c. Figure 5 shows the NADH oxidase activity as the TCHQ and TMPD concentrations were varied. Exogenous cytochrome c has slight effect on NADH oxidase, whereas TMPD or TCHQ had no effect on NADH oxidase activity. The presence of 32 μM cytochrome c allows a stimulation of the oxidase activity by TMPD or TCHQ. From inspection, the TCHQ seems to be the more efficient stimulant at this level of exogenous cytochrome c. In any event, with exogenous cytochrome c, TMPD and TCHQ do have an effect, in the absence of cytochrome c, they have little or no effect.

B. The Effect of TMPD on Succinate Oxidase

The effect of TMPD and exogenous cytochrome c on succinate oxidase activity is shown in Figure 6 for two different HMPs. Note that the exogenous cytochrome c level is 20 μM not 32 μM. The results indicate practically no effect of TMPD on succinate oxidase activity. Exogenous cytochrome c may potentiate a slight stimulation. This behavior contrasts strongly with that seen in Figure 5.

C. Effect of TCHQ on Succinate Oxidase

When TCHQ was added to HMP actively oxidizing succinate, there was sometimes inhibition, sometimes not, but when exogenous cytochrome c was added, TCHQ always inhibited at a very low concentration. It was decided to look a little closer at this phenomena with two different HMPs. The results of both were qualitatively the same and the results of one experiment are shown in Figure 7. As is seen, TCHQ in this preparation had little affect. However, as little as five μM exogenous cytochrome c potentiated a powerful inhibition by TCHQ. Higher concentrations of exogenous cytochrome c gave even greater potentiation.

Figure 5. Effect of TCHQ, TMPD and exogenous cytochrome c on the NADH oxidase activity of HMP 472.

Line A. No addition except TCHQ

Line B. TCHQ added in presence of 32 μM cytochrome c

Line C. TMPD added

Line D. TMPD added in presence of 32 μM cytochrome c

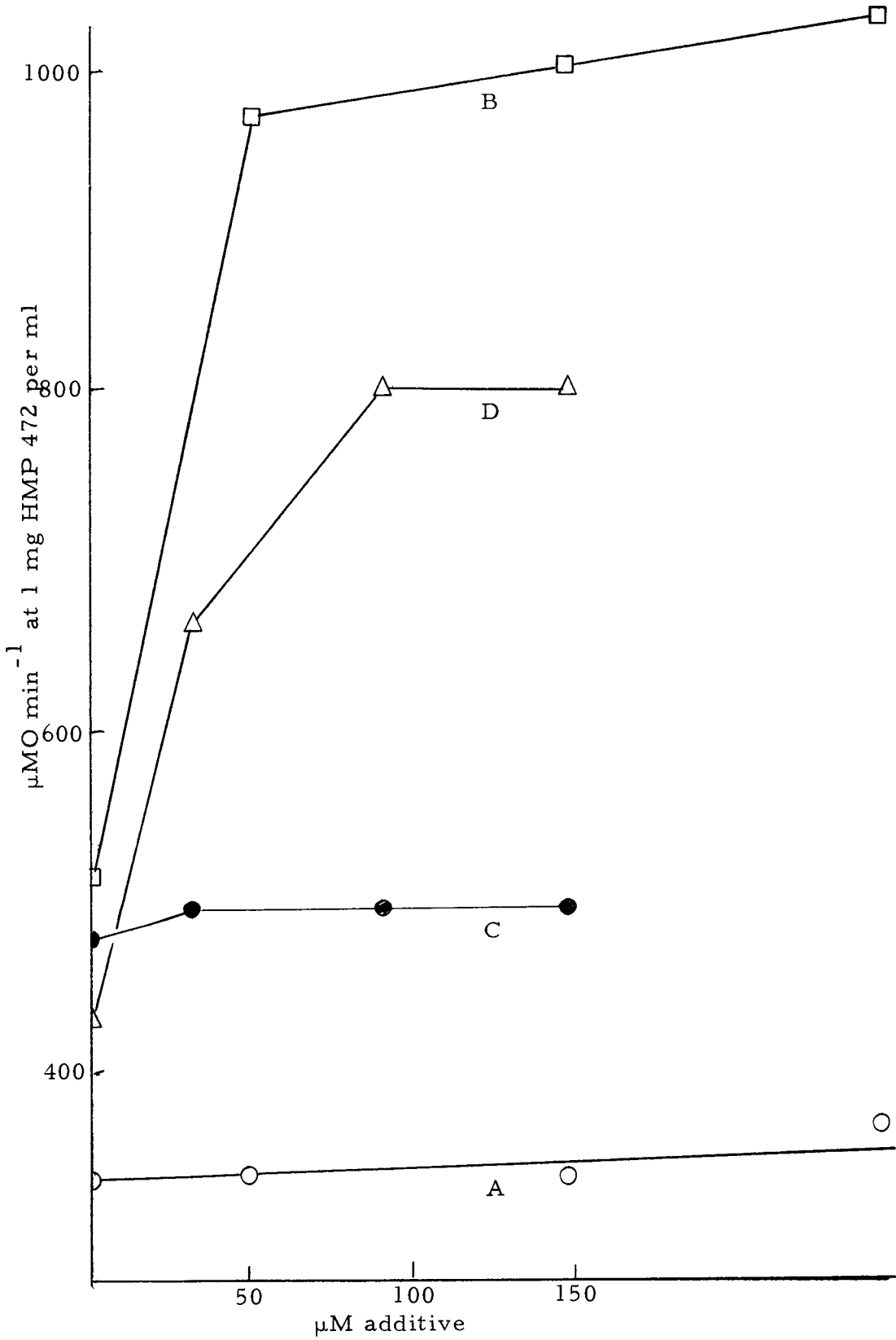

1000
800
600
400
μMO min^{-1} at 1 mg HMP 472 per ml
B
D
C
A
50
100
150
μM additive

Figure 6. Effect of TMPD, exogenous cytochrome c on the succinate oxidase activity of HMP.

Line A. HMP 468, addition of TMPD

Line B. as in A but in presence of 20 μM cytochrome c

Line C. HMP 469, addition of TMPD

Line D. as in C but in the presence of 20 μM cytochrome c

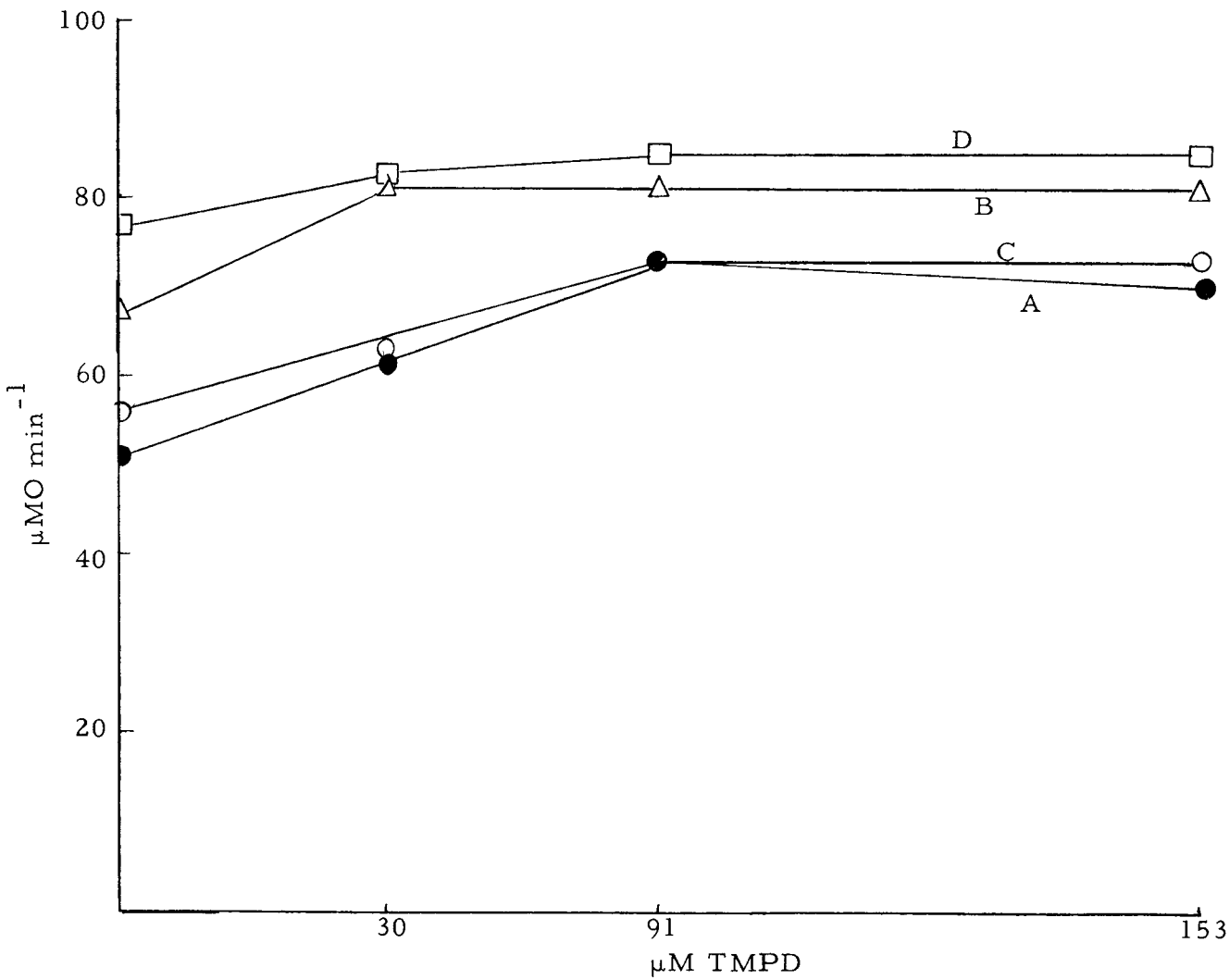

100
80
60
40
20
μMO min^{-1}
D
B
C
A
30
91
153
μM TMPD

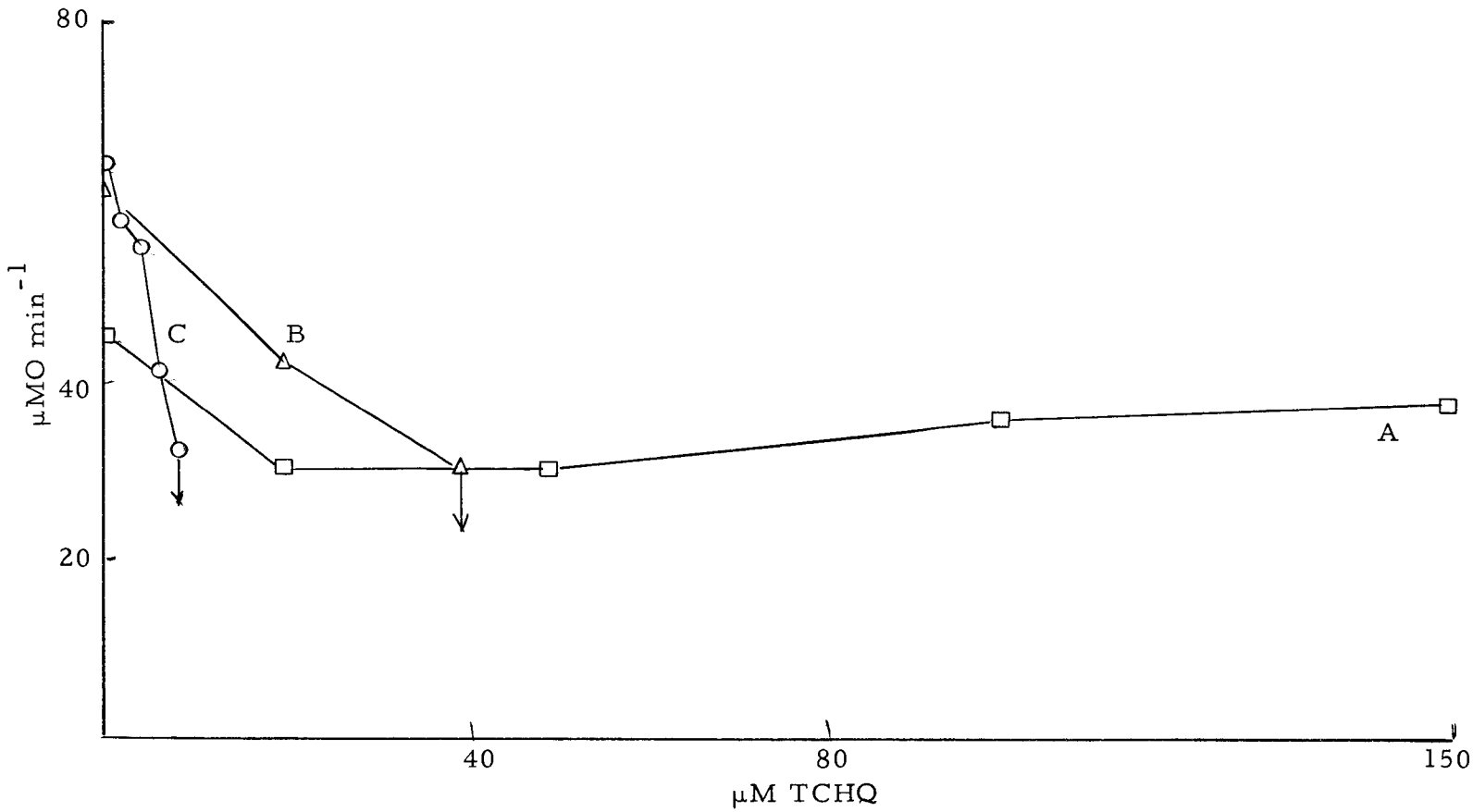
Figure 7. Effect of TCHQ on the succinate oxidase inhibition potentiated by exogenous cytochrome c.

System contained: 20 μl of HMP 524, 15 mM succinate. TCHQ added as either 2.4 mM or 0.24 mM ethanolic solutions. Where the arrow is drawn indicates that after that addition, the initial rate was linear but the rate declined to zero with time, usually after a few minutes.

Line A. No additions

Line B. Five μM exogenous cytochrome c

Line C. 20 μM exogenous cytochrome c


80
40
20
μMO min^{-1}
C
B
A
40
80
150
μM TCHQ

Three kinds of effects are seen in these experiments. The first type is reflected by TMPD with succinate oxidase. There is little or no effect on velocity with TMPD or TMPD and exogenous cytochrome c. A second effect is seen with TMPD or TCHQ added to NADH oxidase. Here the components alone do not affect the oxidation rate with exogenous cytochrome c, they greatly stimulate the rate. The third effect is specific for TCHQ and succinate oxidase. In the absence of exogenous cytochrome c, no effect is noted but when exogenous cytochrome c is added, inhibition is found.

The first effect indicates that the TMPD is not shunting over a rate limiting step.

The effects with NADH oxidase are less satisfactorily explained. The lack of effect of TMPD and TCHQ alone indicates no shunting over a rate limiting step. In the presence of exogenous cytochrome c, a clear explanation is lacking for the great stimulation of the rates. Possible explanations include exogenous cytochrome c generation of external pathways, or, direct chemical reduction of oxidation products of TCHQ and TMPD. Neither explanation is alone satisfactory.

The third effect is dramatic. Again no significant increase in the rate is seen with TCHQ. Although not surprising, this is perhaps contrary to the statements of Jacobs (42, p. 979). When exogenous cytochrome c is added, powerful inhibition results. A possible

explanation is as follows: TCHQ cannot donate effectively to endogenous cytochrome c, however, a known external pathway of TCHQ oxidation exists (51). This pathway requires exogenous cytochrome c or polylysine or some other polycation. When exogenous cytochrome c is added, the new pathway is possible. The product of TCHQ oxidation is probably the anion radical $[\cdot TCHQ]^-$. This radical can react with R-SH or dismutate to tetrachloroquinone and TCHQ. Now quinone can react with R-SH to oxidize the SH or form addition compounds. This R-SH destruction is exhibited by inhibition of SDH, bringing about the onset of inhibition. SDH is known to be sensitive to benzoquinones (2, 77). Note that a steady state of some oxidation product of TCHQ is necessary, not TCHQ itself.

D. Shunt Experiments

The inhibitors and restorants as well as the respiratory chain are depicted in Figure 8. In strictly qualitative tests, the ability of each restorant to overcome each inhibitor with two different substrates is shown in Table 1. None of the restorants tried accomplished restoration over the TTA inhibition of succinate oxidase. On the other hand, all restorants were effective with all inhibitors used when NADH was used as the substrate. Cytochrome c was not added during these tests.

Table 1. Shunt effectiveness.

Substrate	Inhibitor	Restorant		
		TCHQ	TMPD	DCIP
succinate	QNO	+	+	+
succinate	AA	+	+	+
succinate	TTA	-	-	-
NADH	QNO	+	+	+
NADH	AA	+	+	+
NADH	Rotenone	+	+	+

+ indicates a restoration, even partial, of the inhibited activity.

The rates measured were plotted reciprocally versus the reciprocal of the accumulated concentration of the restorant. The "Lineweaver-Burke" plot would then give the amount of restorant necessary to half maximally restore the inhibited preparation. The V_{max} or maximal rate would be the rate were an infinite concentration of the restorant present. Typical reciprocal plots for different restorants with inhibitors are presented in Figure 9.

Figure 10 indicates the effects of various restorants on the HMP system containing NADH as substrate and antimycin A as the inhibitor. Exogenous cytochrome c is added as 20 μM. TMPD and TCHQ are of about equal effectiveness in restoration except when cytochrome c is added. Cytochrome c greatly increases the stimulatory efficiency but the stimulation is much greater in the case of TCHQ than TMPD. Only a few μM TCHQ is required to restore the oxidation rates to above the uninhibited levels in the presence of cytochrome c.

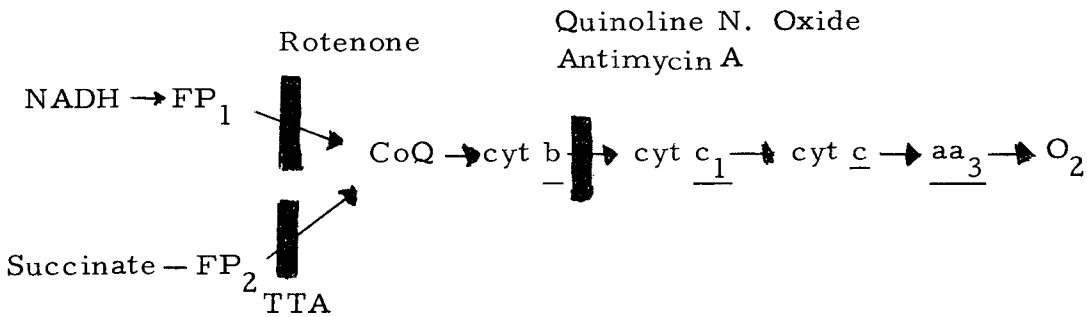
Figure 8. The respiratory chain and shunt compounds.

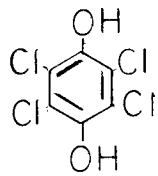
Figure 9. Determination of apparent K_m and V_{max} for shunt systems.

System A: Ten μl HMP 472, inhibited with QNO, NADH oxidase activity, TMPD added.

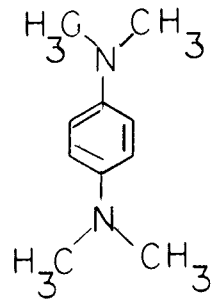
System B: Twenty μl HMP 469, inhibited with QNO, NADH oxidase, TCHQ added

System C: As in system B but including 20μm cytochrome c

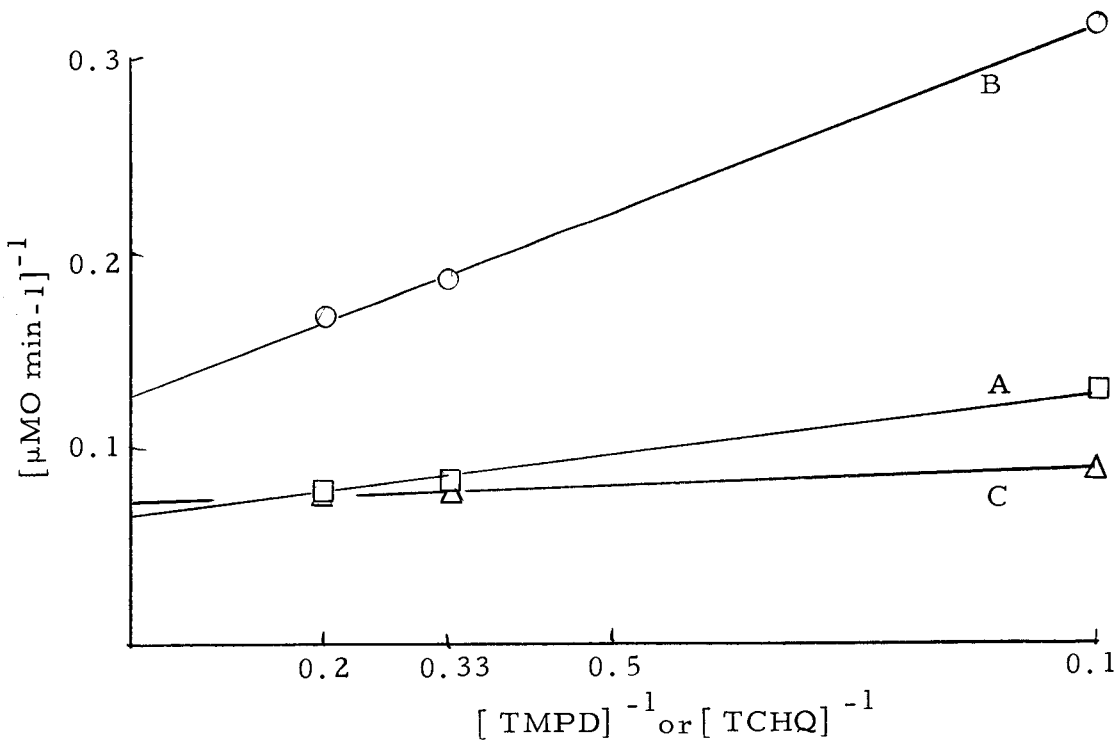

Rotenone
Quinoline N. Oxide
Antimycin A
NADH → FP1
CoQ → cyt b → cyt c1 → cyt c → aa3 → O2
Succinate — FP2
TTA


OH
Cl
Cl
Cl
Cl
OH

TCHQ


H3C
CH3
N
N
H3C
CH3

TMPD


0.3
0.2
0.1
[μMO min-1]-1
B
A
C
0.2
0.33
0.5
0.1
[TMPD]-1 or [TCHQ]-1

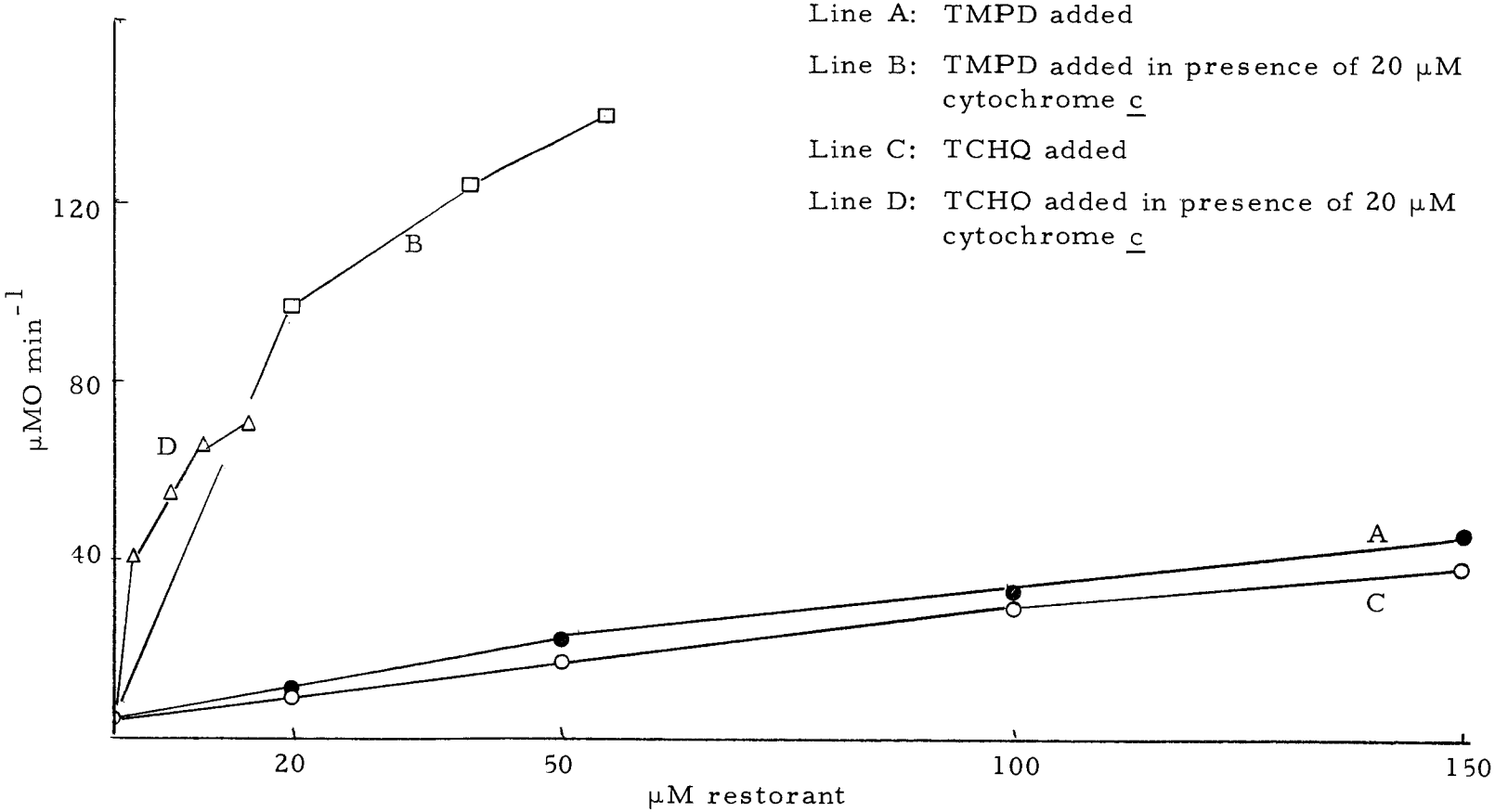


Figure 10. Relative effectiveness of TMPD and TCHQ in overcoming the antimycin A inhibition of NADH oxidase: Effect of exogenous cytochrome c.

Table 2 summarizes a treatment of the data in Figure 10 and also analagous data where the NADH oxidase was inhibited by great excess of rotenone. Again, cytochrome c was added to 20 μM. Both restorants effectively shunted over the two inhibitor sites. When TCHQ is used, the K_m and V_{max} are about the same, e.g. they were independent of the site of inhibition. Cytochrome c does have a profound effect on the effectiveness of the restorant with little change in maximal activity. The TMPD data shows considerable differences in both K_m and V_{max}. With antimycin A, exogenous cytochrome c does little to increase the already high V_{max} while greatly changing the K_m. When shunting over the rotenone site, there is cytochrome c stimulation of V_{max} and again a dramatic decrease in K_m. In both cases, the decreases in K_m are very similar. Although the V_{max}'s may differ between systems, they are relatively little changed by the addition of cytochrome c. It is apparent that cytochrome c is acting in the same manner regardless of the site of inhibition. This implies that cytochrome c is acting in the terminal region of the respiratory chain.

Using HMP inhibited with antimycin A, the effects of two restorants were checked with both NADH and succinate as substrates. In addition, the presence of 20 μM cytochrome c was a parameter. The results are presented in Table 3. With TCHQ the results were quite reproducable. The K_m for the different substrates were very

different but were again greatly affected by the addition of exogenous cytochrome c. With NADH, the V_{max} was not greatly affected but with the succinate as substrate, some V_{max} increase was observed. When TMPD was the restorant, the K_m's were variable except in the case of NADH oxidase when a clear lowering of the K_m by cytochrome c was consistently observed. With TMPD, the V_{max}'s were not stimulated by cytochrome c although there was a great difference between V_{max}'s depending on substrate.

Table 2. Shunt of NADH oxidase by TMPD and TCHQ studied with two inhibitors: kinetic constants.

Restorant added	Inhibitor added: Antimycin A		Inhibitor added: Rotenone	
	K_m (μM)	V_{max}*	K_m (μM)	V_{max}
TCHQ	200	600	150	500
TCHQ in 20 μM cyt. c	2	500	2.6	500
TMPD	500	1400	200	700
TMPD in 20 μM cyt. c	36	1600	14	1000

*Expressed as μM 0 min^{-1} at 1 mg HMP ml^{-1}.
All of K_m's and V_{max}'s are apparent as described in text.

Figure 11 indicates the effect of adding TCHQ to a system supplemented with succinate and inhibited with QNO. When no cytochrome c is present, the rate increases in a monotonic manner, however, when cytochrome c is added at different concentrations, the effectiveness of TCHQ in increasing the rate increases but an inhibition is also

seen. Using the ascending portions of the data from Figure 11, and the Lineweaver-Burke method outlined previously, the apparent K_m's are tabulated vs. the exogenous cytochrome c concentration in Table 4. Two different HMPs are listed here to show striking similarity in the data. The subsequent inhibition at higher levels of TCHQ and its relation to cytochroms c level will be later explored.

Table 3. Shunt of TCHQ and TMPD over succinate oxidase and NADH oxidase inhibited by antimycin A.

Restorant added	Substrate			
	Succinate		NADH	
	K_m (μM)	V_{max}*	K_m (μM)	V_{max}
TCHQ	23	300	200	600
TCHQ with 20 μM cytochrome c	0.5	500	2	500
TMPD	(150)	300	(120)	1400
TMPD with 20 μM cytochrome c	(127)	300	36	1600

*μM 0 min^{-1} at 1 mg HMP ml^{-1}.

Table 4. Effect of exogenous cytochrome c on K_m for restoration by TCHQ of succinate oxidase inhibited with QNO.

Exogenous cytochrome c (μM)	Apparent K_m (μM) for TCHQ HMP 525	HMP 524
0	27.8	22.7
5	1.8	1.2
20	----	0.5
40	1.0	0.2

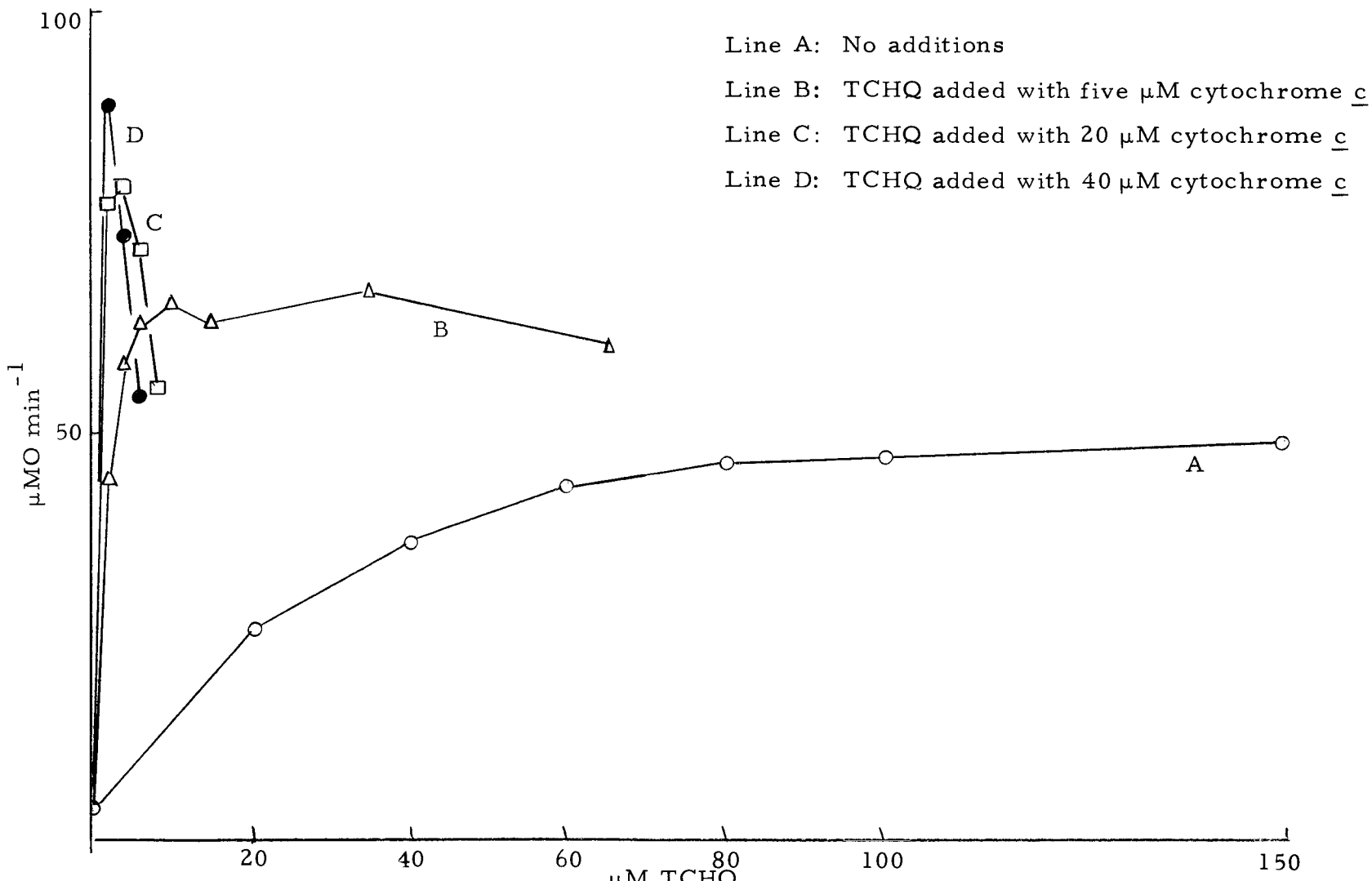


Figure 11. Effect of TCHQ and exogenous cytochrome c on restoration of QNO inhibited succinate oxidase.

If a shunt is operating, it presumes that the "restoring agent" is being oxidized on the oxygen side of the site(s) of inhibitor and the oxidized restorant is reduced at a site(s) on the substrate side of inhibitor. At any one time, during constant rate of oxidation (or catalytic activity), there should be a steady state of reduced restorant and oxidized restorant. As is presented elsewhere, it is believed that the redox system consists of a one electron cycle between the reduced compound and its one electron oxidation product. In the case of TMPD, the cation radical is the oxidized restorant and TCHQ, the anion radical. The radical states of TMPD and TCHQ have visible spectra and they can be seen when shunt systems are operating.

It is possible to obtain apparent kinetic constants if one step, either reduction or oxidation of restorant, is rate limiting. Cytochrome c was added in the hope of affecting only the oxidation step of the shunt.

So far, for the NADH side of the chain, only shunts involving NADH-linked substrates have been reported in the literature (48, 58). This may be due to the nature of the respiratory chain preparation used, e.g. mitochondria have permeability barriers. HMP avoids that problem. Shunts are observed over most inhibitors, the exception being TTA inhibited succinate oxidase. These results are contrary to work done with mitochondria and NADH-linked substrates (48, 58). But, as perhaps related to the literature results, V_{max}'s

found with rotenone inhibited systems are considerably lower (see Table 2) than with other inhibitors.

TMPD and TCHQ allow a shunt over all inhibitors when NADH oxidase is examined. With TCHQ, the K_m is high and does not vary with inhibitor site, hence there is probably only one site of interaction of TCHQ and that is close to the dehydrogenase. Since the V_{max}'s do not vary this site is probably rate limiting. Cytochrome c greatly affects restorant effectiveness but not the V_{max}. Possibly the exogenous cytochrome c can create a new pathway of electron transfer requiring less TCHQ but the V_{max} is constant. This result is difficult to reconcile with the effect of cytochrome c and TCHQ on NADH oxidase activity. This observation may eliminate the explanation based on non-enzymic reduction of tetrachlorohydroquinone [or ·TCHQ] with NADH.

With TMPD and NADH oxidase the K_m and V_{max} vary with the inhibitor. The V_{max} with rotenone and a TMPD shunt is comparable with results obtained with a TCHQ shunt. This may indicate similar sites or action. Again exogenous cytochrome c greatly increases the effectiveness of the restorant while little changing the V_{max}. This implicates the reducing side as the rate limiting portion of the chain.

The affect of substrate was examined and tabulated in Table 3. Some trouble was found in reproducing those values which are

bracketed. Interestingly, cytochrome c does not affect the K_m or V_{max} of TMPD shunting over succinate oxidase with TCHQ it increases V_{max} slightly and greatly changes the K_m.

Figures 12 and 13 attempt to correlate the above data and tentative conclusions.

Previously the effect of cytochrome c in the inhibition of succinate oxidase supplemented with TCHQ was noted. TCHQ can restore succinate oxidase activity to QNO inhibited preparations, again a profound effect of cytochrome c was found. Cytochrome c greatly increases the effectiveness of TCHQ while again promoting inhibition at higher levels of TCHQ. Correlation of K_m for TCHQ (Table 4) with cytochrome c level has not yielded results or obvious information. The same mechanism for inhibition by TCHQ can be called into explanation for the inhibition by TCHQ that was discussed earlier.

E. The Effect of TCHQ, TMPD and Exogenous Cytochrome c on the Cytochrome c Oxidase Activity of HMP

The rates found on addition of TMPD, exogenous cytochrome c, TMPD in the presence of 8 μM cytochrome c and cytochrome c in the presence of 30 μM TMPD are shown in Figures 14 and 15. Each Figure presents a different HMP. Figures 16 and 17 show the effect of TCHQ, TCHQ in the presence of 8 μM cytochrome c and cytochrome c in the presence of 50 μM TCHQ. The points taken from this data are

plotted in the double reciprocal manner with some modification [noted in the legends of the figures] to give an apparent K_m and apparent V_{max}. Representative Lineweaver-Burke plots are shown in Figure 18 and 19 for cytochrome c concentration and TMPD concentration in the presence and absence of 8 μM cytochrome c. Legends of these figures will illsutrate any problems found in data treatment. Table 5, 6 and 7 list the K_m's and V_{max}'s found.

Table 5. Effect of various compounds on the K_m and V_{max} of the cytochrome c oxidase activity of HMP.

Conditions	HMP 468		HMP 469	
	K_m (cytochrome c)	V_{max} ($\mu M\ 0\ min^{-1}\ mg^{-1}\ HMP\ ml^{-1}$)	K_m (cytochrome c)	V_{max}
no additions	32	1000	36	1130
30 μM TMPD	27	2080	32	3850
50 μM TCHQ	28	2320	28	2970

Table 6. The effect of TMPD on cytochrome c oxidase activity of HMP.

Additions	HMP 468		HMP 469		Average	
	K_m (μM TMPD)	V_{max}** as before	K_m	V_{max}	K_m	V_{max}
none	153	805	169	735	161	770
8 μM cyt. c	160*	1430	214	800	187	1120

*This K_m was done by a difference method as outlined in Figure 19, otherwise the apparent K_m is about 30 percent the listed value.

**These V_{max}'s are total V_{max} e.g., the activity in the presence of 8 μM cyt. c and infinite amount TMPD.

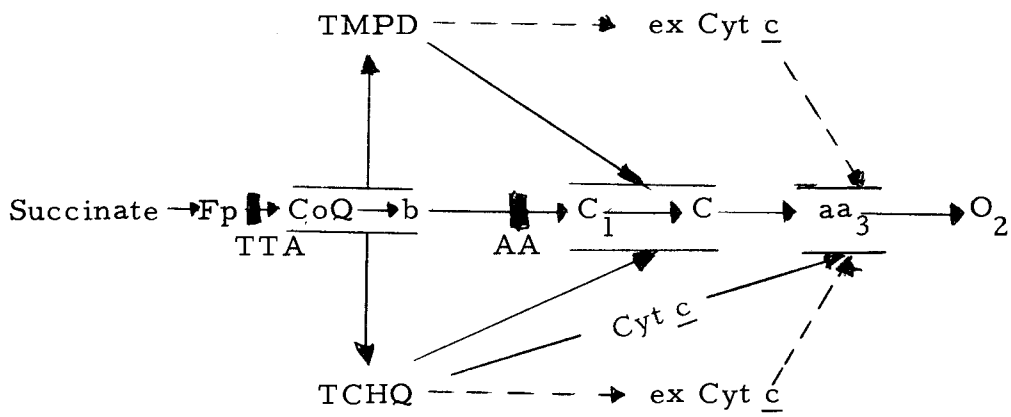


Figure 12. Interactions of TMPD and TCHQ with the succinate oxidase chain.

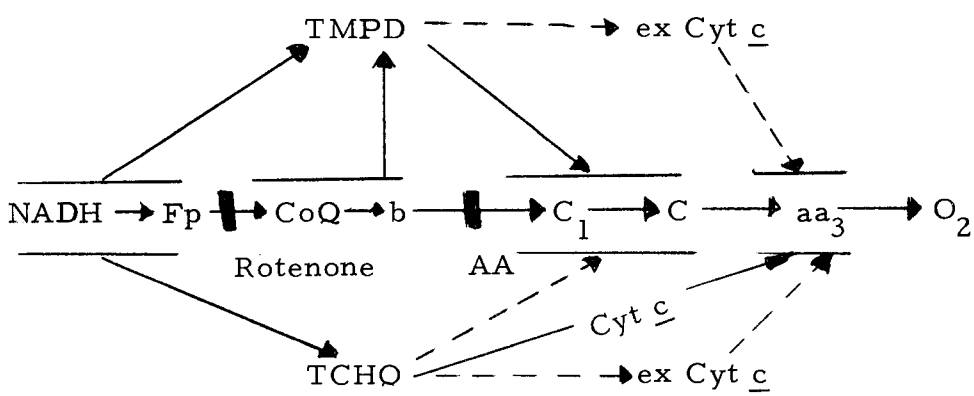


Figure 13. Interactions of TMPD and TCHQ with the NADH oxidase chain.

Figure 14. HMP 468. Effect of TMPD, cytochrome c on cytochrome oxidase activity.

Line A: Cytochrome c added as 20 mg ml^{-1}

Line B: TMPD added as 6.1 mM

Line C: TMPD added in presence of 8 μM cytochrome c

Line D: Cytochrome c added in presence of 30 μM TMPD

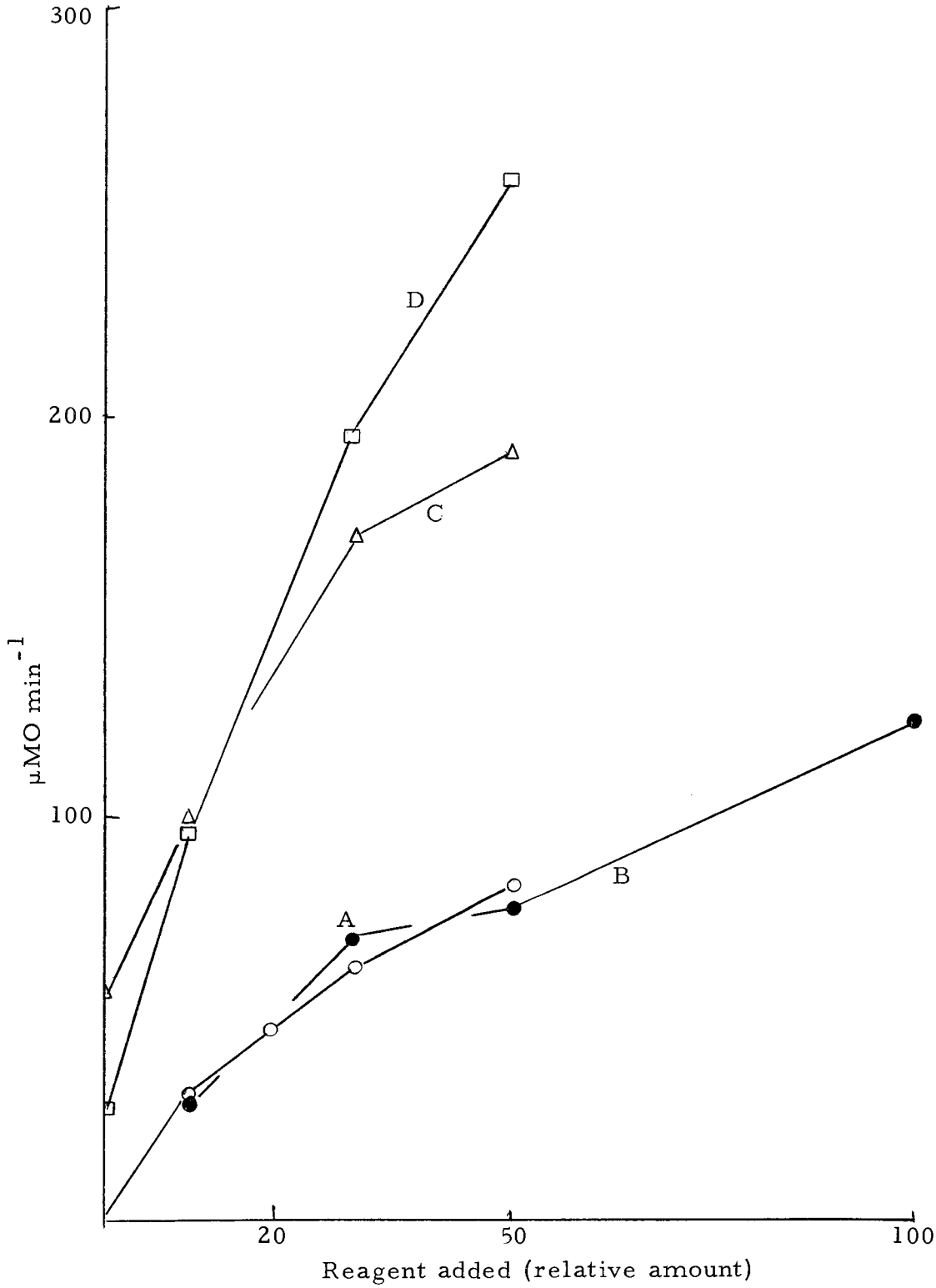

300
200
100
μMO min^{-1}
D
C
A
B
20
50
100
Reagent added (relative amount)

Figure 15. HMP 469. Effect of TMPD and cytochrome c on the cytochrome oxidase.

Line A: Cytochrome c added as 20 mg ml^{-1}

Line B: TMPD added as 6.1 mM

Line C: TMPD added in presence of 8 μM cytochrome c

Line D: Cytochrome c added in presence of 30 μM TMPD

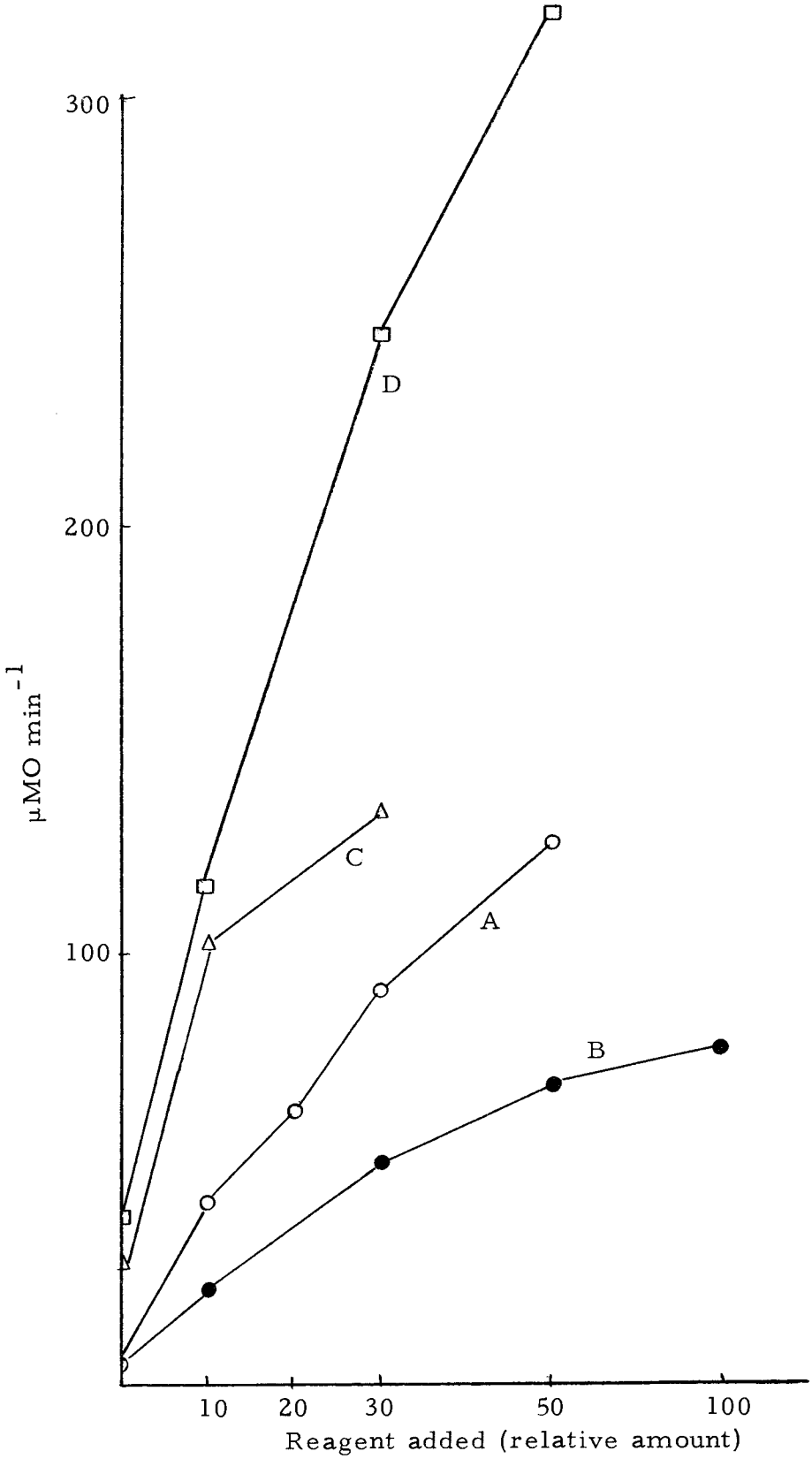

300
200
100
μMO min^{-1}
D
C
A
B
10
20
30
50
100
Reagent added (relative amount)

Figure 16. Effect of TCHQ and cytochrome c on cytochrome oxidase activity of HMP 468.

Line A: TCHQ added as 10 mM solution

Line B: TCHQ added in presence of 8 μM cytochrome c

Line C: Cytochrome c added in presence of 50 μM TCHQ

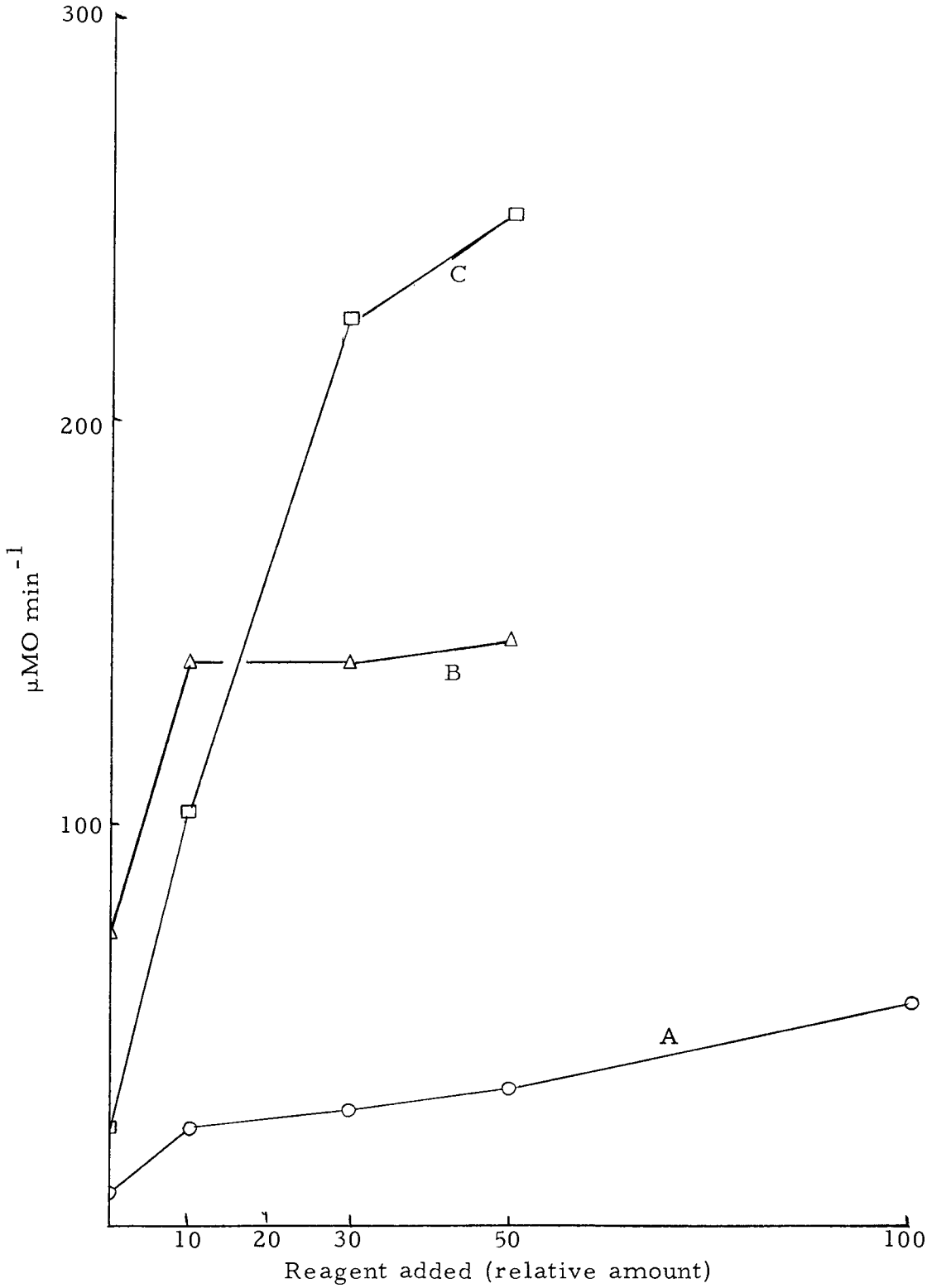

300
200
100
μMO min-1
C
B
A
10
20
30
50
100
Reagent added (relative amount)

Figure 17. Effect of TCHQ and cytochrome c on the cytochrome oxidase activity of HMP 469.

Line A: TCHQ added as 10 mM solution

Line B: TCHQ added in presence of 8 μM cytochrome c

Line C: Cytochrome c added in presence of 50 μM TCHQ

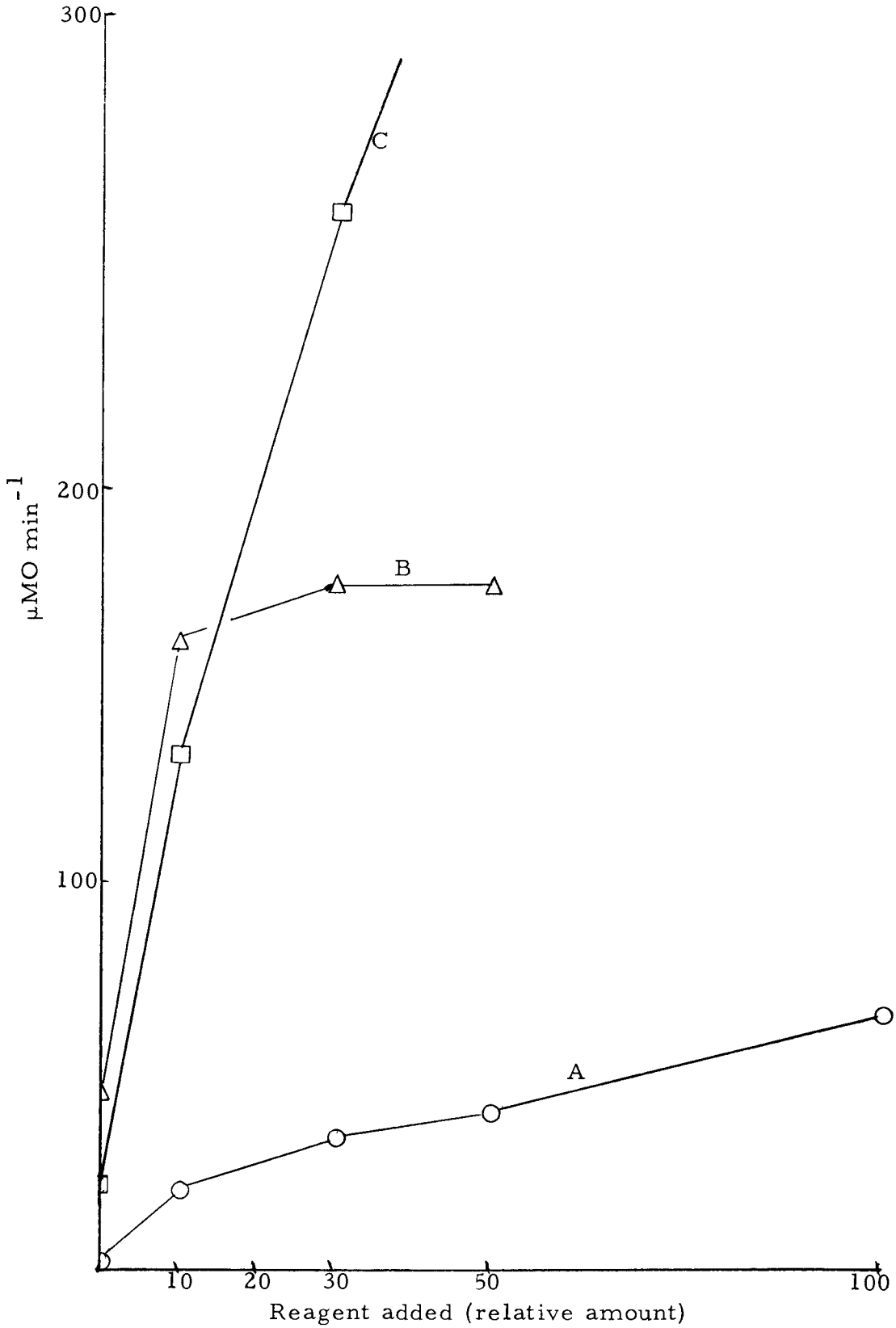

300
200
100
μMO min^{-1}
C
B
A
10
20
30
50
100
Reagent added (relative amount)

Figure 18. Lineweaver-Burke analysis of cytochrome oxidase activity: HMP 468 and 469.

Line A: HMP 468

Line B: HMP 469

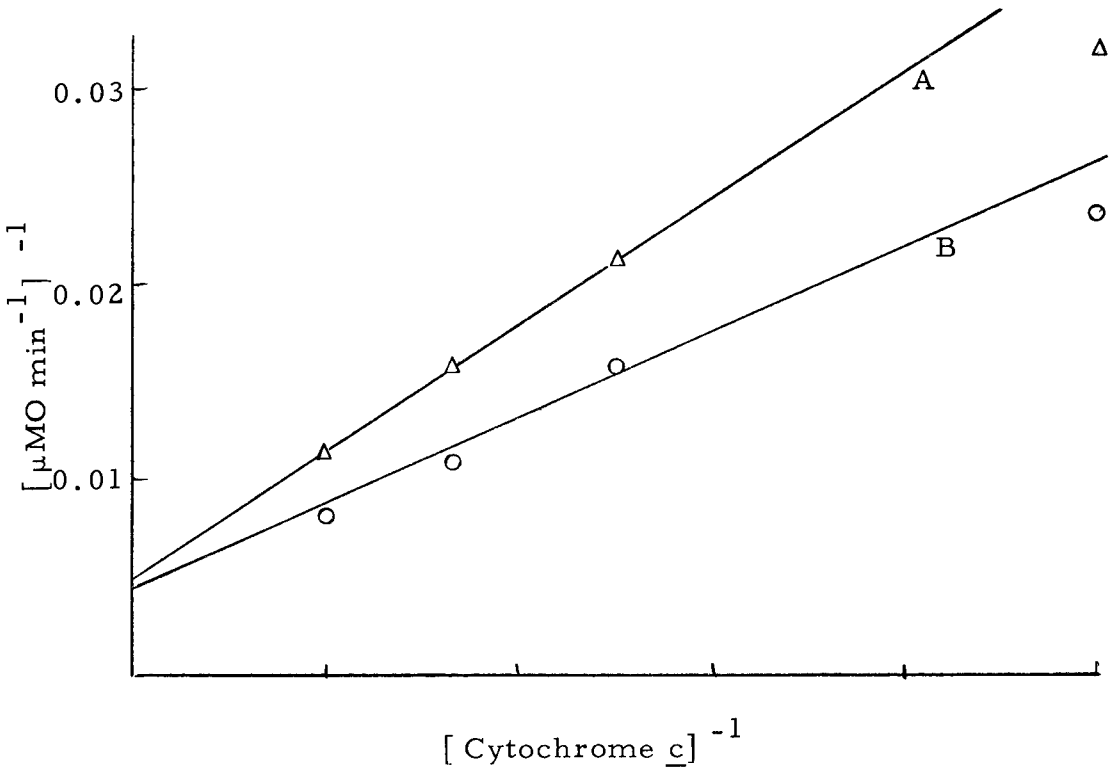
Figure 19. Modified Lineweaver-Burke analysis of cytochrome oxidase activity: TMPD in the presence and absence of exogenous cytochrome c.

Line A: This line is V^{-1} as recorded in presence of 8 μM cytochrome c

Line B: This line is [V(in presence of 8 μM cytochrome c + TMPD)] - [V(in presence of 8 μM cytochrome c)] $^{-1}$.

Line C: This is V^{-1} in absence of any other additions.

In line A, we are looking at the influence of TMPD and cytochrome c on the rate of each TMPD concentration examined. In line B, looking at the influence of TMPD on the rate measured in the presence of exogenous cytochrome c.


0.03
0.02
0.01
[μMO min^{-1}]$^{-1}$
A
B
[Cytochrome c] $^{-1}$

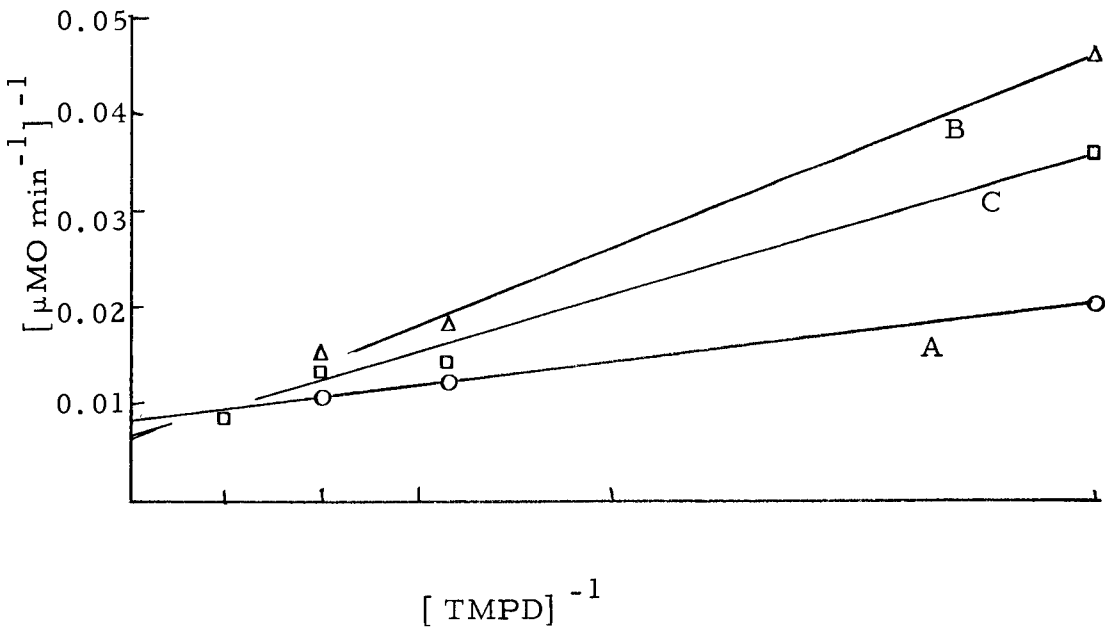

0.05
0.04
0.03
0.02
0.01
[μMO min^{-1}]$^{-1}$
B
C
A
[TMPD] $^{-1}$

Table 7. The effect of TCHQ on the cytochrome c oxidase activity of HMP.

Additions	HMP 468		HMP 469	
	K_m (μM TCHQ)	V_{max} as usual	K_m	V_{max}
none	*	*	410	625
8 μM cyt. c	less than 50**	2500	less than 50	880

*Lineweaver-Burke plot not acceptable but by inspection the K_m is apparently high.

**By inspection.

The cytochrome oxidase activity measured with exogenous cytochrome c-ascorbate reflects a different pathway of electron transfer since exogenous reduced cytochrome c does not interact with endogenous cytochrome c (78). The K_m measured for cytochrome c (about 30 μM) is for the half maximal stimulation of the external cytochrome c oxidase pathway. The addition of TMPD or TCHQ does not alter that K_m. TMPD at a level far below its own K_m (added at 30 μM and K_m is 160 μM) will stimulate the rate of oxidation at least one fold. TMPD is believed to react with endogenous cytochrome c (c_1). Table 2 shows that TMPD has a V_{max} like the one found with exogenous cytochrome c but with a K_m of 160 μM. When exogenous cytochrome c is added to the TMPD ascorbate system, the V_{max} is not stimulated much nor is the K_m changed. The level of exogenous cytochrome c added is well below its K_m so that in the presence of exogenous cytochrome c, the pathway of electrons in the presence of

TMPD does not change. TMPD does seem to interact with a separate population of cytochrome c under these conditions. In the reverse case however, TMPD seems to facilitate the exogenous cytochrome c oxidase activity, perhaps by interceeding in ascorbate reduction of the oxidized cytochrome c to remove a rate limiting step.

Similarly, TCHQ does not affect the K_m (cytochrome c) but greatly influences the V_{max}. TCHQ concentration used was much above the observed K_m (TCHQ) so that the system depends on cytochrome c for activity. The increase in rate is probably the creation of a new pathway requiring cytochrome c but as an alkaline protein. This hypothesis is amenable to experimental verification.

TCHQ does not interact well with endogenous cytochrome c as is clearly shown in Table 7 and requires exogenous cytochrome c to establish the new pathway which has rapid rate and a high affinity for TCHQ.

TMPD is interacting with the endogenous cytochrome $c + c_1$ pathway and can interact with an exogenous cytochrome c oxidase pathway probably by facilitation of the exogenous cytochrome c oxidase. TCHQ interacts outside the endogenous cytochrome c pathway and is dependent on cytochrome c for the TCHQ exogenous pathway.

The patterns seen here recall the effects seen in the shunt experiments. The results found here can help to sort out the complex reactions occurring in the shunted systems.

Part II. Succinate Dehydrogenase and Related Studies

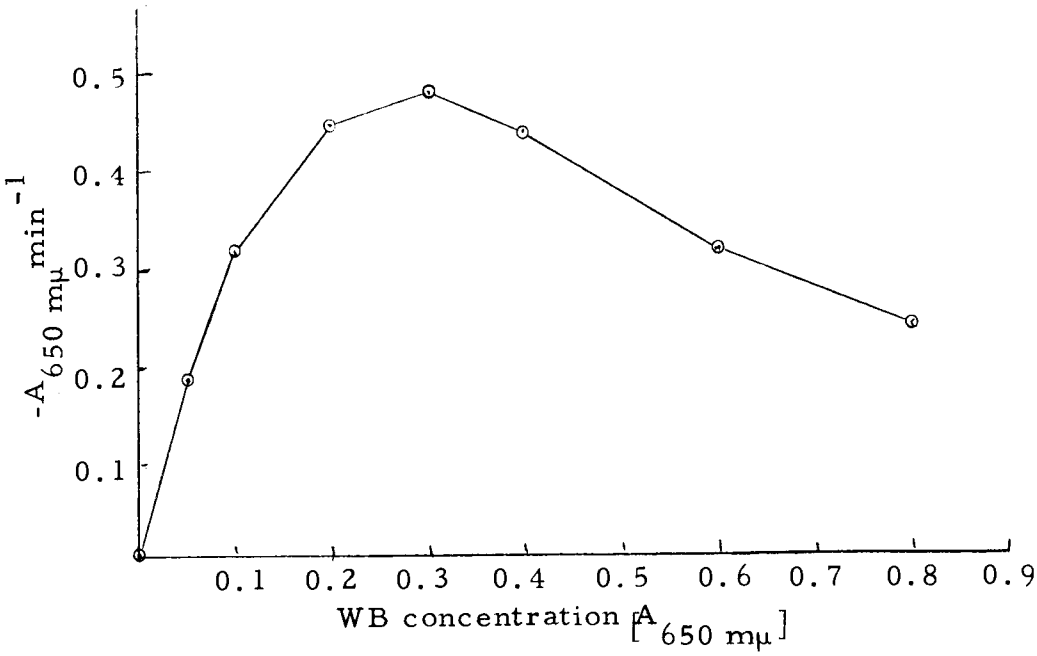
A. Succinate-WB Reductase

1. In HMP. HMP exhibits a succinate dependent reduction of WB which is TTA sensitive (see A.4). The time course of reduction of WB was investigated. There is an initial lag period, followed by a decline in the rate of reduction of WB until all of the WB is gone. When lesser amounts of WB were reduced, the initial lag period was no longer present, hence, the lag seemed to be a reversible inhibition of activity by high levels of WB. Slopes tangent to the time course curve were taken and plotted in Figure 20. Then the reciprocal of the rate was plotted versus the reciprocal of the WB concentration where the tangent was drawn. A Lineweaver-Burke plot of this is shown in Figure 21. From this type of data manipulation, a V_{max} and K_m (apparent) for WB could be obtained. In addition, a Lineweaver-Burke plot could be obtained from initial rates at varying WB concentrations to yield a similar Lineweaver-Burke plot which is shown in Figure 22. Table 8 lists data for some HMPs obtained by both methods. The sensitivity to excess WB was variable.

Figure 20. Variation of rate of WB reduction with WB concentration: Succinate-WB reductase of HMP 484.

Data taken from a time course curve. Assayed as indicated in Materials and Methods with reduction followed spectrophotometrically at 650 mμ.

Figure 21. Lineweaver-Burke plot taken from Figure 20.


0.5
0.4
0.3
0.2
0.1
$-A_{650\ m\mu}\ min^{-1}$
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
WB concentration [$A_{650\ m\mu}$]

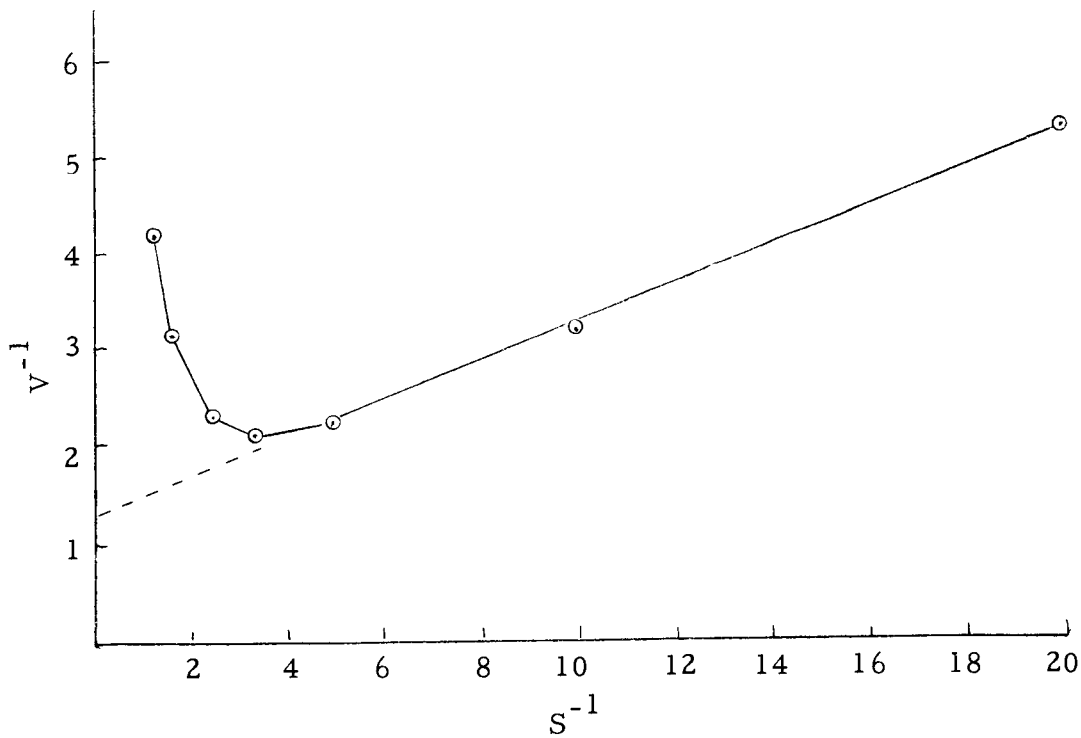

6
5
4
3
2
1
V^{-1}
2 4 6 8 10 12 14 16 18 20
S^{-1}

Table 8. Activity of succinate-WB reductase in HMP.

Preparation#	Method of analysis	K_m (μM) (WB)	V_{max} (mM succinate min-1 at 1 mg HMP/ml)
480	Time course	87	1.56
480	Time course	100	2.07
480	Time course	93	1.49
497	Initial slope	94	0.86
498	Initial slope	65	1.02
Average		88	1.15

Method of assay described in Materials and Methods.

Some inhibitor studies were carried out at a fixed WB concentration of 145 μM. It was found that Antimycin A, rotenone or amytal do not inhibit the activity. Exogenous cytochrome c or coenzyme Q_{10} did not affect the activity. The only compound besides TTA found to inhibit was pCMB. Complete inhibition was observed at millimolar levels of pCMB.

2. In Soluble Forms of SDH. In addition to the particulate enzymes, some soluble forms of succinate dehydrogenase were examined. Examined were the reconstitutively active SDH of King (36) and the four atoms iron per mole of flavin form of SDH (PMS reductase) of Singer (36, p. 170). Not examined was the two atoms iron per mole of flavin form of PMS reductase (36, p. 169). Table 9 summarized the data obtained for the enzymes freshly prepared. Succinate-ferricyanide reductaseactivity was not available for the SDH preparation reported here, but other experiments have noted about the

same activities reported by King (36, p. 170), hence that data is inserted. In subsequent discussion, each of the soluble enzymes is examined in greater detail.

Table 9. Kinetic constants for WB and ferricyanide in succinate: (acceptor) reductases.

Enzyme	WB as acceptor		Ferricyanide as acceptor	
	K_m (mM)	V_{max}*	K_m (mM)	V_{max}*
HMP	0.088	1.15	0.2	0.4
PMS reductase	0.45	4.6	2.9	3.0
SDH	0.26	16.4	11.0	4.1

*Units of mM succinate $min.^{-1}$ at 1 mg enzyme/ml. Assayed as indicated in Materials and Methods.

The soluble forms of SDH gave reasonably good Lineweaver-Burke plots and WB was not found to be inhibitory at high concentrations. A typical Lineweaver-Burke plot for SDH is shown in Figure 23.

The PMS reductase was prepared by modification of method III A of King (38). The change in activity with aging is presented in Table 10. As expected, the ferricyanide activity was stable, but the WB reductase activity underwent a slow decay.

3. Studies of the Decay of Activity in SDH. In addition to studying PMS reductase, several experiments were performed with succinate dehydrogenase, prepared by the III A method of King to the gel

eluate stage of purification. When this SDH was assayed at a fixed WB concentration (150 μM) at various intervals after preparation, one observed the pattern seen in Figure 24. It appears that the activity is rapidly lost, like reconstitutive activity. In addition, like reconstitutive activity, the succinate-WB reductase is preserved by storage *in vacuo*. However, when Lineweaver-Burke determinations were made at each time interval, the result was somewhat different. To be sure, there was a change in the V_{max} of the enzyme, this is shown in Figure 25. However, this accounted for only part of the loss of activity, the remaining loss was seen if one examined the K_m (WB) vs. time as is shown in Figure 26. Here, it is indicated that the K_m remains constant for about three hours, then it changes to a higher value. This is very similar to the change of K_m exhibited by the succinate-ferricyanide reductase activity of SDH, except that in that case, the change in K_m is in the opposite direction. This data from King (36, p. 183) is superimposed on Figure 26. Attention should also be called to the V_{max} and K_m (WB) of the SDH. It is noted that the K_m is different at first, but after the aforementioned K_m transformation, the K_m of the aged SDH and the PMS reductase are very similar (see Table 11). The V_{max} of the SDH is almost three fold higher than that of the PMS reductase. It may be that the only difference between these enzymes is the presence of succinate during the preparation of the SDH. It is not known if the non-heme

iron concentrations of the two enzymes are the same. The preparation of King does contain about twice as much non-heme iron as that enzyme prepared by Singer's laboratory (36, p. 168, 171).

Table 10. Effect of aging on activities of PMS reductase.

Age of enzyme (hours)	WB as acceptor		Ferricyanide as acceptor	
	K_m (mM) WB	V_{max}*	K_m (mM) Ferricyanide	V_{max}*
0	0.45	4.6	2.9	3.0
3	0.61	3.4	3.3	3.0
6	0.48	2.7	5.0	3.5

*Units expressed as mM succinate min^{-1} at 1 mg enzyme ml^{-1}.

Table 11. Effect of aging on catalytic properties of SDH (succinate-WB activity).

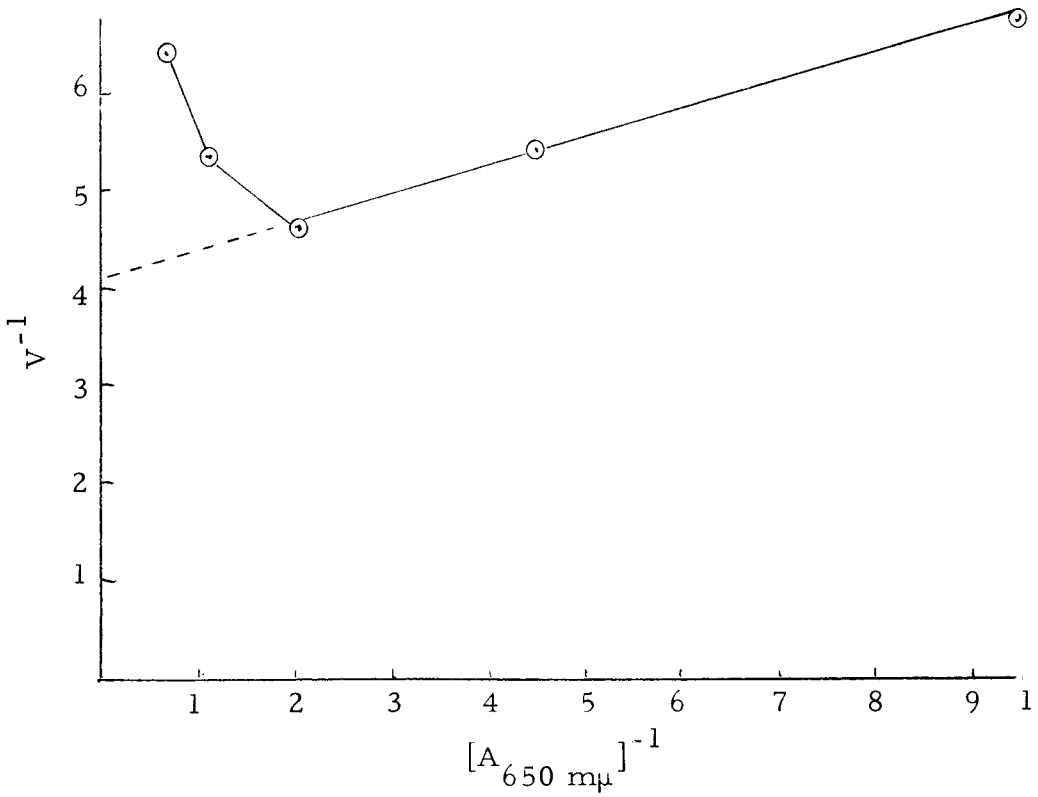
Age SDH hours	V_{max} mM succinate min^{-1} at 1 mg SDH/ml	%	K_m [μM WB]
(1) 0	10.0	100	207
1	8.0	80	175
2	6.9	69	193
3	6.1	61	222
5.5	6.1	61	480
8	5.2	52	465
11	4.3	43	455
(2) 0	16.4	100	260
1	9.8	60	160
2	9.6	58	194
3	10.0	61	283
4	8.3	51	276
5.5	10.7	63	535
7	7.0	43	391

Figure 22. Succinate-WB reductase: Lineweaver-Burke study of succinate-WB reductase of HMP 498.

Data obtained from initial rates.

Figure 23. Lineweaver-Burke study of succinate-WB reductase activity of SDH.

SDH was assayed at early age, assayed as indicated in Materials and Methods.


6
5
4
3
2
1
V^{-1}
1
2
3
4
5
6
7
8
9
10
$[A_{650\ m\mu}]^{-1}$

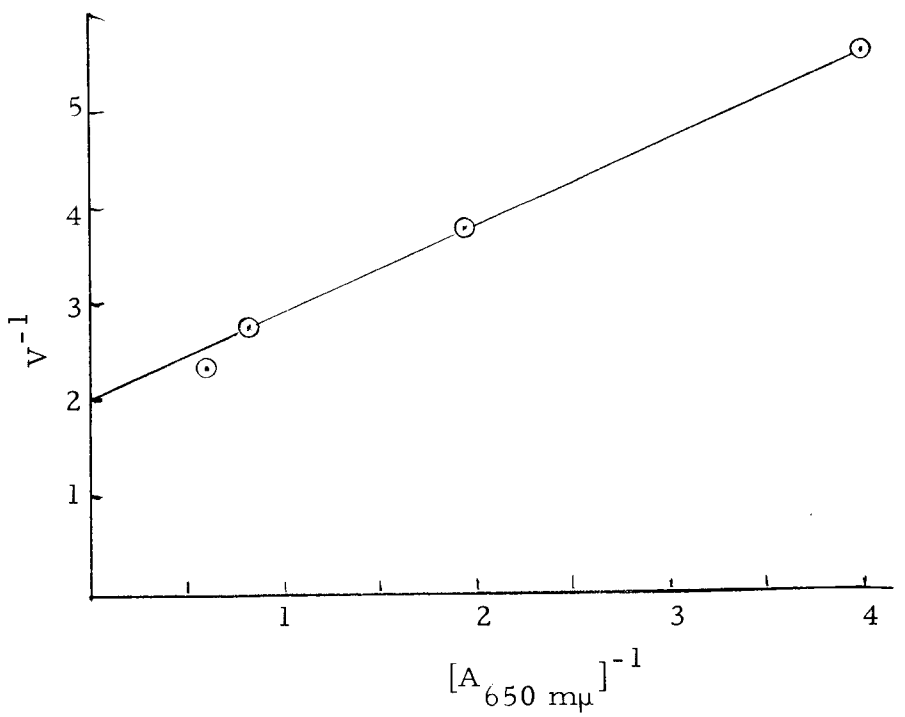
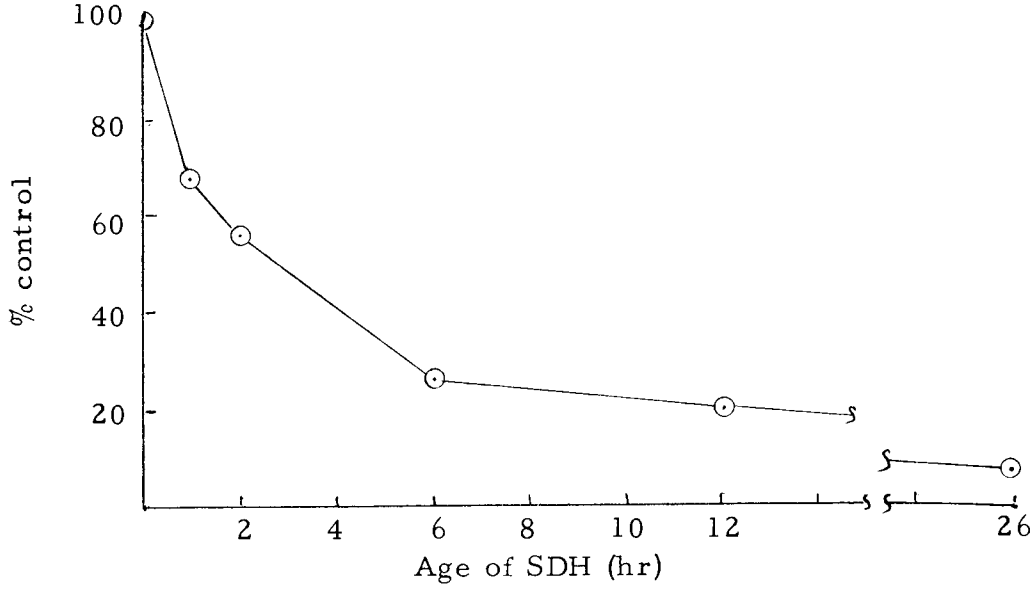

5
4
3
2
1
V^{-1}
1
2
3
4
$[A_{650\ m\mu}]^{-1}$

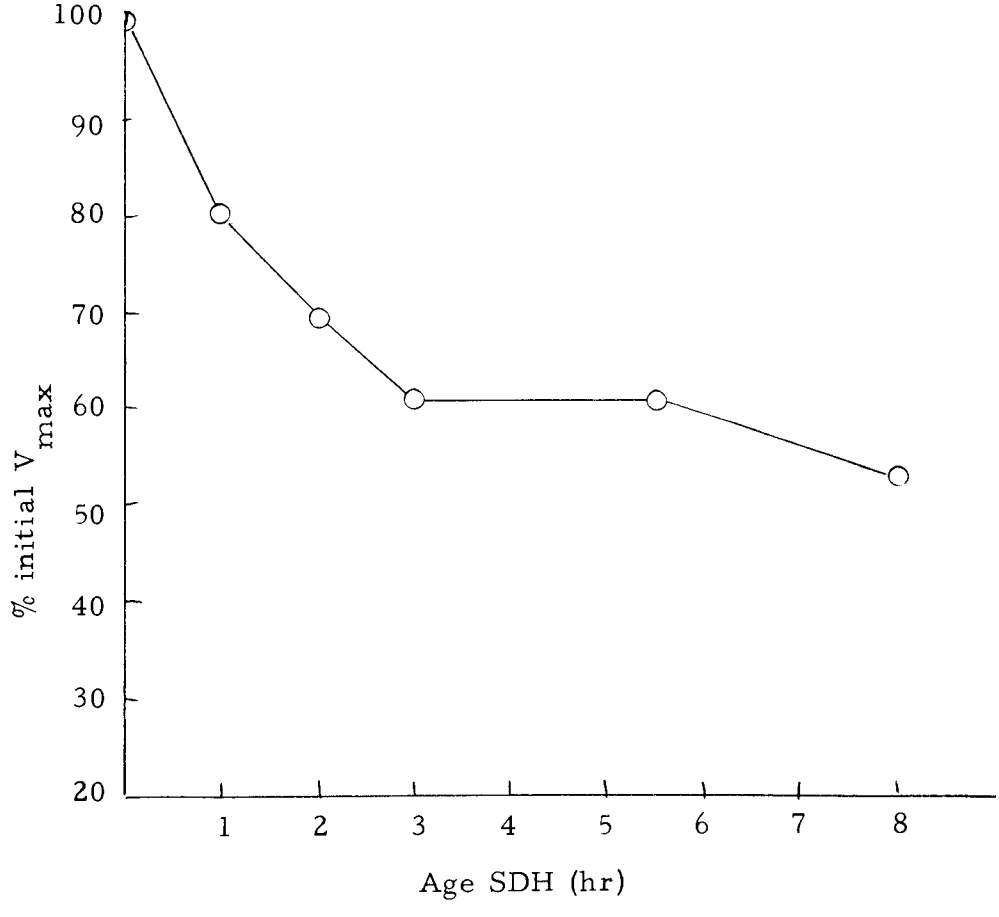
Figure 24. Decay of succinate-WB reductase activity in SDH.

The activity was assayed at a single WB concentration of 150 μM. The enzyme was assayed as soon as prepared. This was called apparent zero time activity. The preparation was then allowed to stand in the air at 0° and samples withdrawn and assayed. Protein concentration during the aging process was 1 mg/ml.

Figure 25. Variation of V_{max} of succinate-WB reductase activity of SDH with age of SDH.

Aged as described above. The V_{max} and K_m were obtained from extrapolation of the linear portion of Lineweaver-Burke plots of data obtained by variation of WB concentration, using the initial rates of reaction.


100
80
60
40
20
% control
2
4
6
8
10
12
26
Age of SDH (hr)


100
90
80
70
60
50
40
30
20
% initial V max
1
2
3
4
5
6
7
8
Age SDH (hr)

In addition to the above observation, it is to be noted that the succinate-WB reductase activity of SDH is quite sensitive to heat. Table 12 indicates other inhibitor studies. The effect of the last inhibitor studied is unusual. Figure 27 indicates the effect of $MgCl_2$ on succinate-WB reductase of SDH. This unusual effect was sometimes also found in the succinate-WB reductase activity of HMP. In HMP, the effect was variable among the several preparations examined.

Table 12. Effect of various compound on succinate-WB reductase activity of SDH.

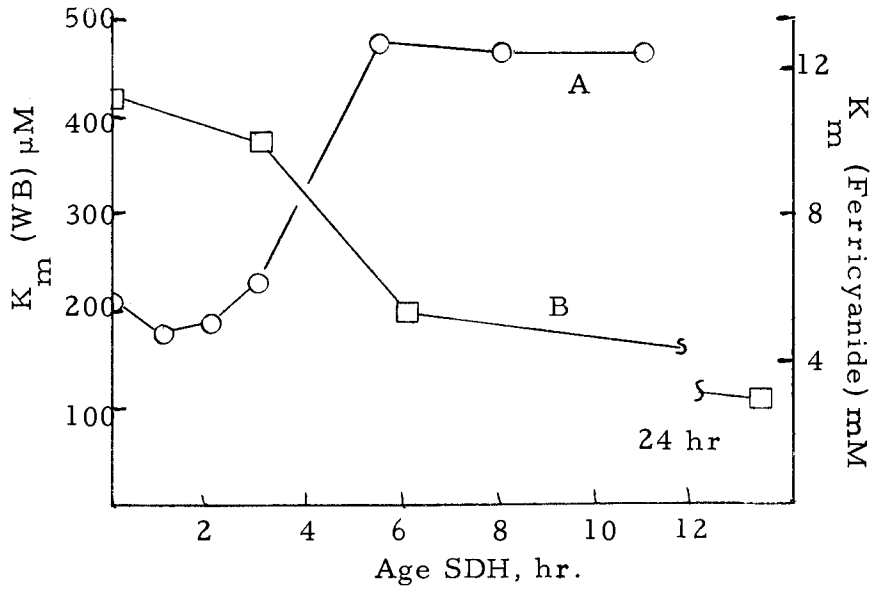
Compound	Concentration	% control (corrected for added organic solvents)
KCN	6.7 mM	116
	13.3	127
	26.7	170
TMPD	67.0 μM	89
	167.0	80
	667.0	51
EDTA	14.0 mM	116
Tiron	1.0 mM	99
	2.0	95
Rotenone	50.0 μg/ml	95
pCMB	0.9 μM	33
	0.9	72 succinate preincubation
		19 no succinate preincubation

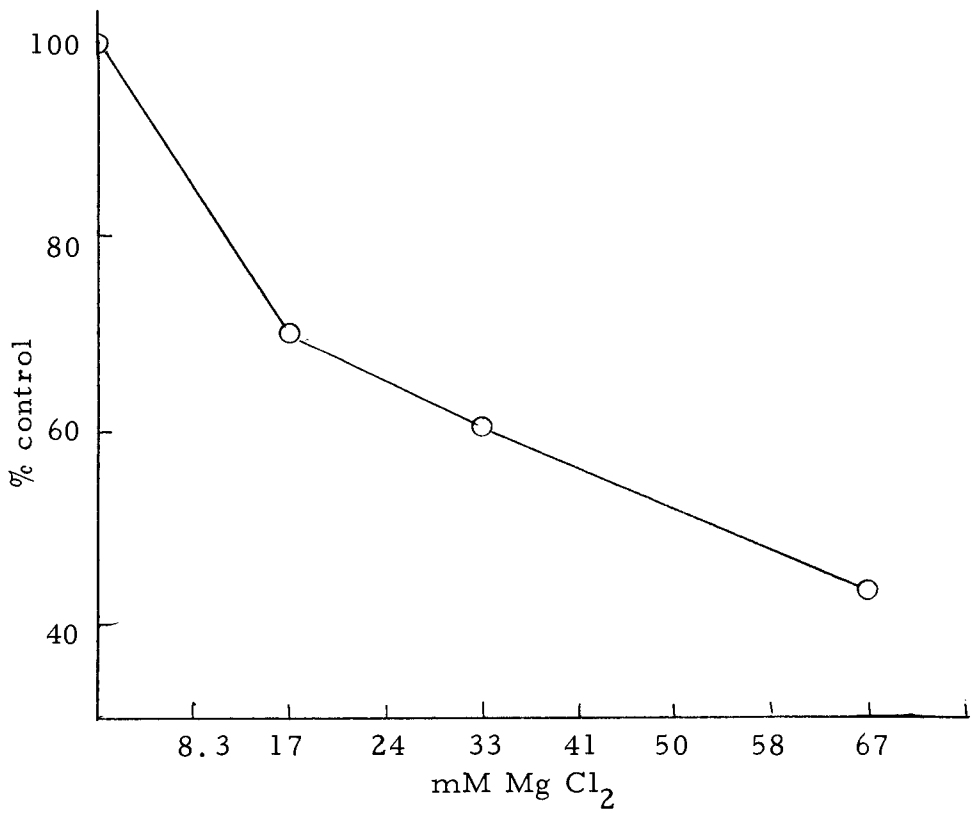
Figure 26. Variation of K_m (WB) of succinate-WB reductase activity of SDH with age of SDH.

K_m determined as described in Figure 25. Line A is K_m for succinate-WB reductase activity. Line B is K_m for succinate-ferricyanide reductase activity taken from King (36, p. 183)

Figure 27. Inhibition of succinate-WB reductase activity by $MgCl_2$.

The enzyme used was at an apparent age of about 4 hours. The SDH was assayed at a fixed WB concentration of 120 μM.


500
400
300
200
100
K_m (WB) μM
12
8
4
K_m (Ferricyanide) mM
A
B
24 hr
2
4
6
8
10
12
Age SDH, hr.


100
80
60
40
% control
8.3
17
24
33
41
50
58
67
mM Mg Cl_2

4. The TTA Sensitivity in Reconstituted Systems. Figure 29 indicates the effect of TTA on succinate-WB reductase activity in HMP. The data is of two different preparations. Also presented in Figure 29 is the effect of TTA on the succinate-WB reductase activity of SDH. As can be seen, the inhibition in the particulate preparation is only about 80% but with a 1/2 maximal inhibition at about 10 μM TTA. This agrees well with data presented in following sections of this thesis for succinate-DCIP reductase and succinate-ferricyanide reductase. On the other hand, the SDH activity requires more than a thousand times as much inhibitor with a 1/2 maximal inhibition at about 15 mM TTA.

Since the soluble enzyme is insensitive to TTA while the particulate enzyme is very sensitive to TTA, it would seem as though TTA sensitivity is characteristic of the succinate-WB reductase (and SDH) *in situ*.

Towards that end, the following experiments were performed. The repetitions of the experiment were reasonably reproducable. Only one of the characteristic experiments will be detailed.

The method was simple; SDH was incubated with aHMP, the mixtures were centrifuged, and supernatants decanted for assay of soluble succinate-WB reductase activity which was not absorbed on the particles. The pellet was resuspended in buffer, recentrifuged and finally resuspended in buffer. The samples were then assayed for

succinate-WB reductase activity and TTA inhibition. The controls were samples of SDH, aHMP and HMP carried through the entire process. Alkaline HMP (aHMP) was prepared by a slight variation of a procedure of King (36, p. 173). SDH was freshly prepared and immediately added to incubation mixtures containing the particle. A preparation in the gel eluate stage of procedure III A by King (38) was used for these experiments.

Figure 28 shows the soluble activity (succinate-WB reductase) remaining in the sample supernatants after centrifugation. The data indicate that a binding of soluble SDH would have to be the mechanism of reconstitution, not a "reactivation" as some authors have put forward (73). This also strengthens the arguments of King and closely replicates the observations of Wang and Wang (86).

The activity recovered in the final pellet is indicated in Figure 30, also shown is the activity of the HMP taken through the same procedure. As is evident, by comparison, about 50% reconstitution has been achieved.

The TTA sensitivity of the reconstituted samples is examined in Figure 31 and 32. Figure 31 plots the TTA concentration versus the % of the control activity. As can be seen, with increasing amounts of bound SDH (succinate-WB reductase activity), the apparent effectiveness of TTA decreases, that is effectiveness as far as maximal inhibition is concerned. In terms of at what concentration TTA exhibits

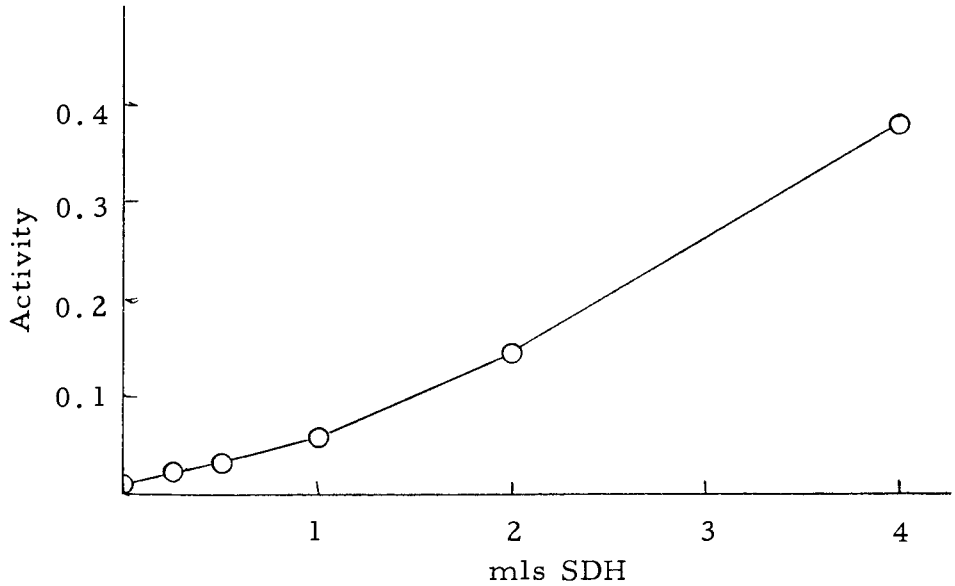
its 1/2 maximal effect, the samples are much more similar. Perhaps a more meaningful plot is that in Figure 32. Here the particle specific activity is plotted versus the TTA concentration. As reported above, TTA little affects the soluble activity, but it does inhibit the particle activity. This comparison can be made because each sample contains, for all practical purposes, the same amount of protein. As can be seen in Figure 32, the amount of activity actually inhibited by TTA is apparently constant, but as the activity is bound about the amount inhibited to a 70% level, that activity remains uninhibitable by TTA. King has reported (35, p. 177) the lowest ratio of SDH:aHMP sample in this series (line c, Figure 31). By including the remainder of the data, it seems that two kinds of binding of SDH are taking place, the first is TTA sensitive and occurs preferentially; the second is TTA insensitive. Both kinds of binding are tight, that is, each sample has withstood the washing to remove extraneous SDH. What remains to be answered is the question of reconstitution of other activities, such as succinate-DCIP reductase or succinate-cytochrome c reductase in these samples. It may be that the second binding is not related to reconstitution of other succinate: (acceptor) reductase activities.

Figure 28. Succinate-WB reductase activity remaining in solution after incubation with a fixed amount of aHMP.

After the amounts of SDH shown on abcissa were incubated for 1 hour with 16 mg of aHMP in the presence of succinate, the mixtures were centrifuged for 25 min. at 50,000 rpm in Spinco # 50 rotor. The pellets were further treated but the supernatants were decanted and a fixed volume assayed for succinate-WB reductase activity.

Figure 29. Effect of TTA on succinate-WB reductase activity of HMP and SDH.

Enzymes were assayed as described in Materials and Methods. SDH had an apparent age of 2 hours.


Activity
0.4
0.3
0.2
0.1
1
2
3
4
mls SDH

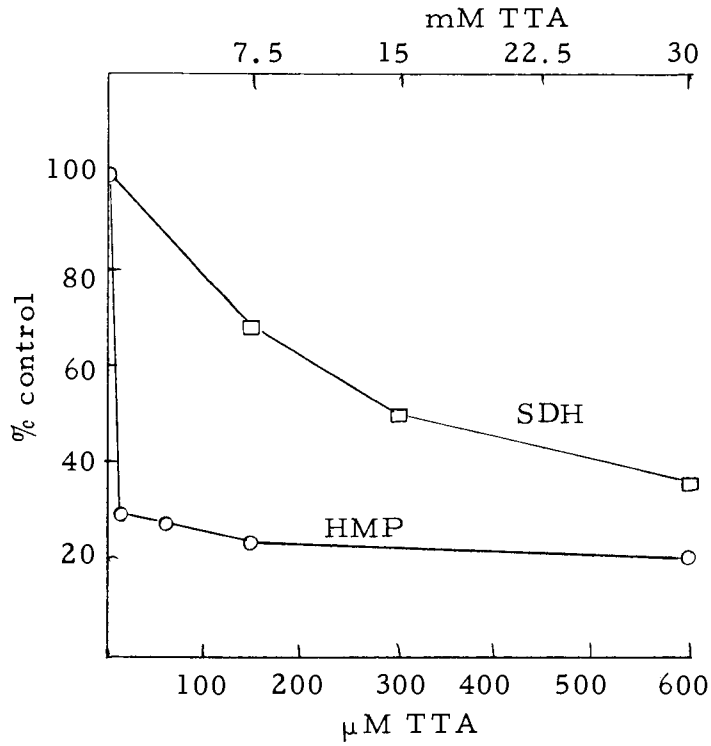
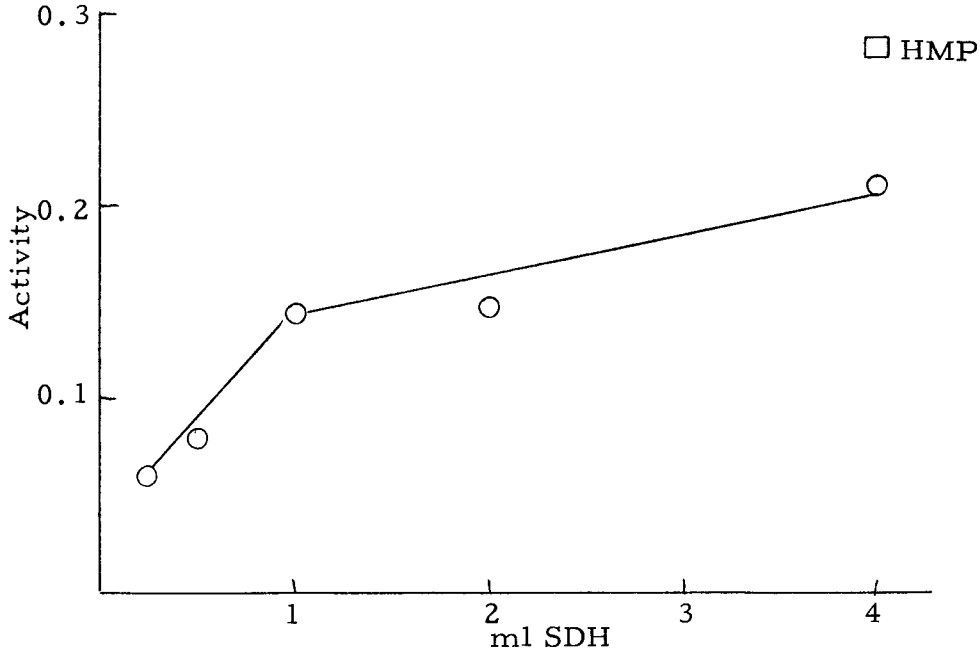

mM TTA
7.5
15
22.5
30
% control
100
80
60
40
20
SDH
HMP
100
200
300
400
500
600
μM TTA

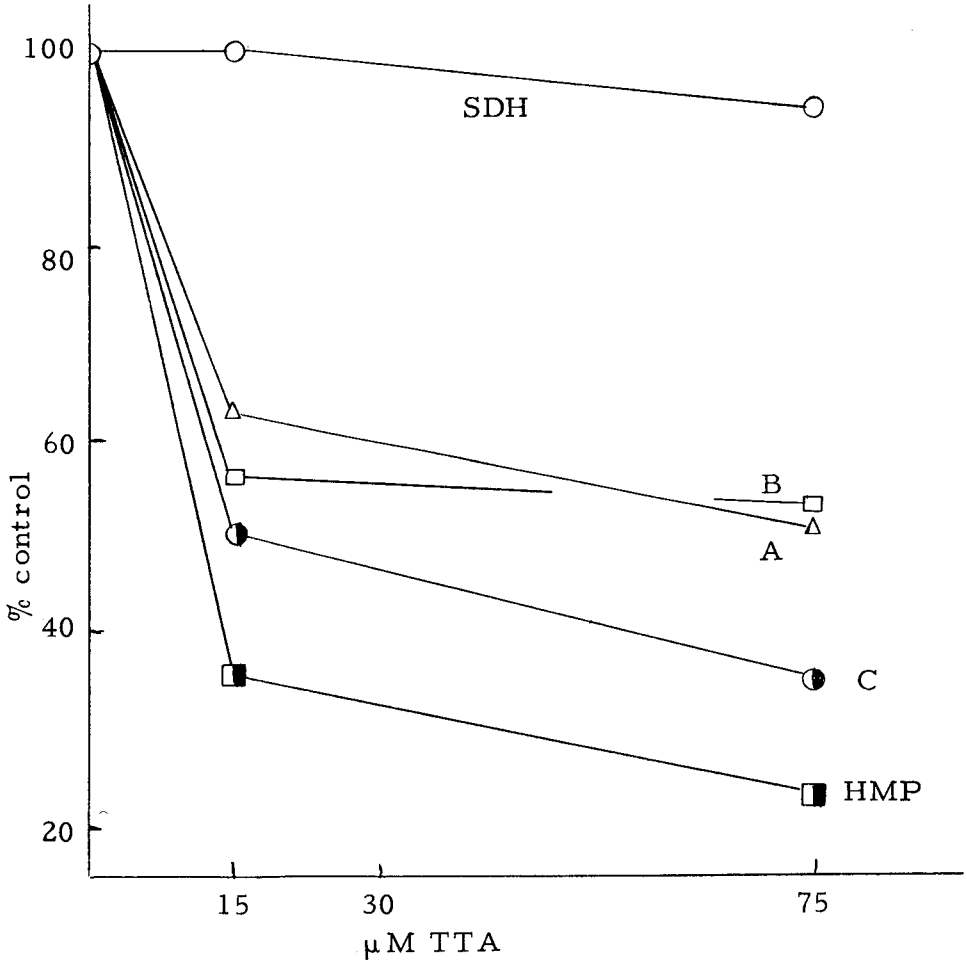
Figure 30. Reconstitution of succinate-WB reductase activity.

The pellets from the above procedure were resuspended in 0.1 M Tris-acetate, pH 7.4 to a volume of 10 ml and recentrifuged as above. The supernatants were discarded and the pellet resuspended in 0.1 M tris buffer. The assays were carried out as usual except that preincubation at room temperature with succinate was necessary to eliminate a lag period (83). For these assays, the reaction was initiated by addition of the WB. The point marked "HMP" is a sample which was treated like the other samples except not incubated with SDH. Proper dilutions were made so that the sample could be directly compared with aHMP. The same HMP was used for the control and making the aHMP. Activity is expressed in arbitrary units. Assays done with a fixed amount of WB (150 μM).

Figure 31. Inhibition of succinate-WB reductase in original and reconstituted systems by TTA.

In an experiment similar to the one outlined in figures 29 and 30, pellets were collected and assayed in the presence of TTA. "SDH" refers to a sample of SDH carried through the procedure, hence is aged. "HMP" refers to the control activity. Line A is a sample where 3 mg of SDH was incubated with 5 mg of aHMP and the resultant washed pellet assayed. Line B is for 1 mg SDH and Line C for 0.2 mg SDH with the same amount of aHMP. Inhibition is expressed as % of the sample with organic solvent, but no TTA.


0.3
0.2
0.1
Activity
HMP
1
2
3
4
ml SDH


100
80
60
40
20
% control
SDH
B
A
C
HMP
15
30
75
μM TTA

B. TTA Inhibition of Succinate-DCIP Reductase Activity of HMP[1]

As part of the gathering of information concerning the site of action of TTA, it was decided to investigate the TTA inhibition of succinate-DCIP reductase activity. The assays were carried out as described in the Materials and Methods section. Eight batches of HMP were examined, all gave the same extent of inhibition and titration of activity curves with TTA. Table 13 gives the batches examined, their specific activity under the conditions of assay, the maximal inhibition of activity observed and the level of TTA necessary for 1/2 maximal inhibition. A typical titration curve is presented in Figure 33.

Table 13. Inhibition of succinate-DCIP reductase in HMP by TTA.

HMP#	Specific activity*	Maximal inhibition [200 μM TTA]	TTA necessary for 1/2 maximal inhibition μM
525	0.191	85.2 %	13
526	0.189	84.0	10
527	0.155	84.4	10
528	0.164	82.9	14
529	0.190	83.9	11
530	0.167	86.1	14
531	0.147	83.3	11
532	0.164	82.9	13

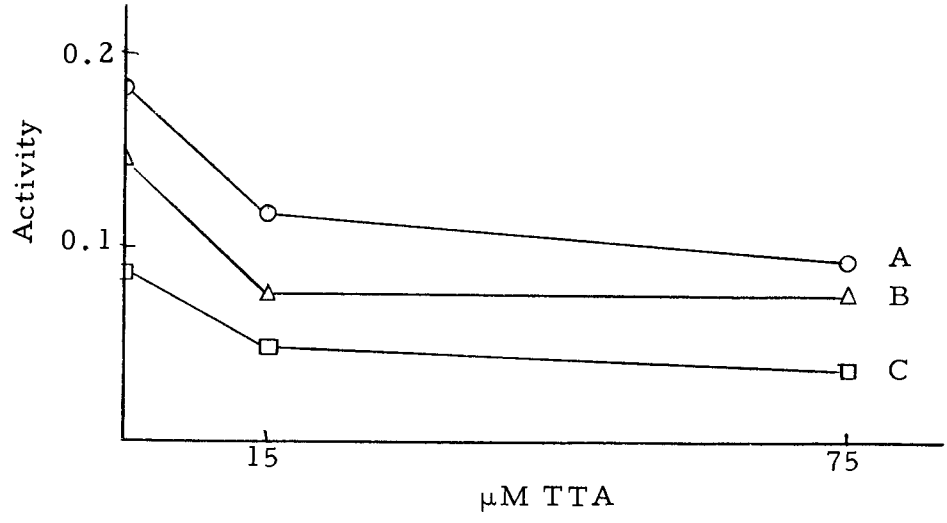
*mM succinate min^{-1} at 1 mg HMP/ml.

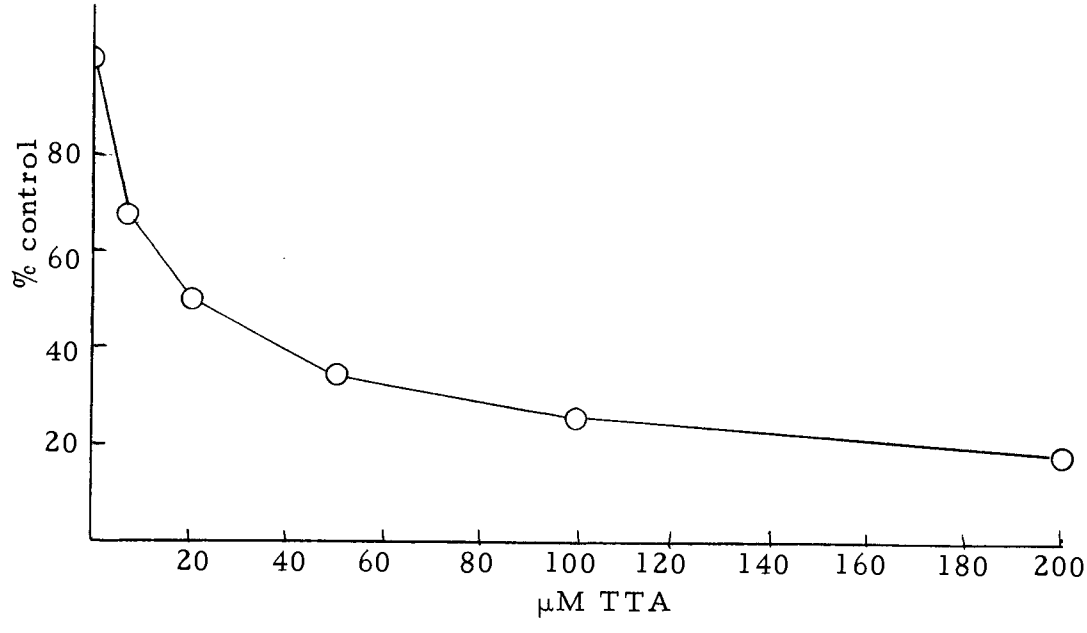
[1] I wish to acknowledge Mrs. JoAnn Potter who most excellently performed most of the assays of experiments detailed in the next two topics.

Figure 32. Activity of reconstituted systems: Activity in the presence of TTA.

This is the same data as is plotted in Figure 31 only here relative activity is plotted instead of % control. Lines A, B, and C have the same meaning as in Figure 31.

Figure 33. Inhibition of succinate - DCIP reductase of HMP by TTA.


0.2
0.1
Activity
A
B
C
15
75
μM TTA


% control
80
60
40
20
20
40
60
80
100
120
140
160
180
200
μM TTA

The data was very consistent from one batch of HMP to the other as long as they were of the same age. In the above cases, the HMP was assayed directly after preparation. However, it has been observed in the course of this study that the level of TTA necessary for 1/2 maximal inhibition increases with the age of the preparation, from about 10-14 μM to as much as 20 μM or more. A similar observation with the inhibition of succinate oxidase has been made in this laboratory, hence it is possible that the site of TTA inhibition is affected by aging. Perhaps experiments involving the effect of fatty acids or other surface active agents on the titration of activity by TTA should be performed.

The regularity of inhibition and the similarity of the inhibition curve to that observed by succinate-WB reductase or succinate-ferricyanide reductase, point to a general mechanism for all of these inhibitions of artifical acceptor activities. Since TTA is similar in its effect with a variety of acceptors which are believed to react in a variety of loci, the TTA sensitive site is probably not multiple.

In general, the data seems to fit the observation of the Beinert school; indeed, their observations present evidence for a reasonable interpretation of the data obtained here. Working with succinate-Q (10) reductase, Ziegler has found a TTA inhibition of succinate-Q_{10} reductase activity (92). It was also found that a non-heme iron electron spin resonance signal was generated when succinate was added to

a TTA treated preparation, but the signal did not change when Q_{10} was added (3). This was interpreted to mean that TTA acted between non-heme iron and Q_{10}. Now Q_{10} reductase is particulate but contains the same amount of non-heme iron per mole of flavin as King's SDH (36, p. 210). The structural difference between the two is apparently related to the presence of lipid and other components. SDH has succinate-WB reductase activity which is not sensitive to TTA; but when rejoined to aHMP, the TTA sensitivity is regained. In addition, TTA seems to prevent the cleavage of SDH from its particulate environment (91). It is concluded that TTA acts very near or at the SDH level in particulate systems. Sanadi concludes that TTA has another activity, namely uncoupling (87). Perhaps these observations will aid in resolving the difference of findings between this laboratory and Redfearn's.

C. Antimycin A and TTA Inhibition of Succinate-ferricyanide Reductase

Figure 34 indicates the titration of succinate-ferricyanide reductase activity in HMP by TTA at three different levels of ferricyanide. In direct contrast to the report of Redfearn, no variation is seen in the titration of activity or ultimate level of inhibition with ferricyanide concentration. The data is plotted versus a control. The control as did all samples, contained three % (v/v) acetone, which

decreased the activity by about five %. The figure legend lists control specific activities. Table 14 lists data from Figure 34, e.g. TTA concentration necessary for 1/2 maximal inhibition with inhibition at 200 μM taken as maximal observed inhibition. These results directly contradict those of Redfearn (70, 89).

Table 14. TTA inhibition of succinate-ferricyanide reductase of HMP.

Ferricyanide conc. mM	TTA for 1/2 maximal inhibition* μM	TTA for 50% inhibition μM
Experiment I, HMP 544		
0.20	11	31
0.60	16	32
2.25	18	31
Experiment II, HMP 545		
0.60	9	21
2.25	7	18

Assayed as described in Materials and Methods.
*20% of control activity.

To the same preparation was added one microgram of antimycin A per mg of HMP and this was similarly assayed for activity. Here the results were considerably different as is seen in Figure 35. The comparison is to samples which have grossly different specific activities. These are listed in the figure legends. As is evident here and will be more evident in a few paragraphs, the antimycin A inhibition varies with ferricyanide concentration. Most striking feature in Figure 35 is that at lower ferricyanide concentrations (0.2 mM), the TTA does not inhibit while at higher ferricyanide concentrations the

maximal inhibition is much less than in AA uninhibited systems and the amount necessary for 1/2 maximal inhibition has increased (here 30 μM compared to 18 μM in untreated preparation). Another experiment, essentially in duplication of the above gave the same general result, but for this HMP, the amount of TTA for 1/2 maximal inhibition was only 7-9 μM while in the AA treated sample, at the same ferricyanide concentration, 50 μM TTA was necessary for 1/2 maximal inhibition. Again, maximal inhibition was chosen as the 200 μM TTA level activity.

It was decided to investigate the inhibition by TTA and AA of succinate-ferricyanide reductase using conventional kinetic parameters. The results reported in this study will be essentially the average of the same experiment carried out on two separate HMPs. AA was used at one microgram per mg of HMP and TTA at a fixed concentration of 200 μM. The samples of HMP were preincubated with AA and preincubation with a like amount of ethanol was used as a control. It was found that preincubation with 30 mM succinate was necessary to obtain linear kinetics at high ferricyanide concentrations. If succinate preincubation was not performed, lag periods were observed above a ferricyanide concentration of two mM.

A control, control + ethanol, and AA treated HMP were then assayed for succinate-ferricyanide reductase activity in the presence of 200 μM TTA or three % (v/v) acetone or with no additions at

several ferricyanide levels. Previously in control experiments, the amount of enzyme to use for correctly determining the rate was determined by extensive enzyme response curve evaluation. The data, specific activity versus ferricyanide concentration, is plotted in Figure 37, from which several parameters can be calculated. First is the percent inhibition at each acceptor concentration, this is presented in Figure 36. Next the amount that TTA inhibits the AA-insensitive activity is presented in Figure 38. Finally, typical reciporal plots according to the Lineweaver-Burke method are presented in Figure 39. The data is collected and averages or representative values are presented in Table 15. Finally Figure 40 attempts to indicate the general pattern of kinetic constants.

Table 15. Kinetic constants from Lineweaver-Burke studies of HMP succinate-ferricyanide reductase: studies with Antimycin A and TTA.

Conditions of assay	Apparent K_m mM	Apparent V_{max}*
No additions**	0.24	0.41
Incubated with ethanol	0.24	0.41
Incubated with AA	4.0	0.4
Incubated with ethanol, assayed with acetone	0.2	0.36
Incubated with ethanol, assayed with TTA	0.2	0.08
Incubated with AA, assayed with acetone	2.5	0.3
Incubated with AA, assayed with TTA	0.7	0.09

*Units of mM succinate min^{-1} at one mg HMP ml^{-1}.

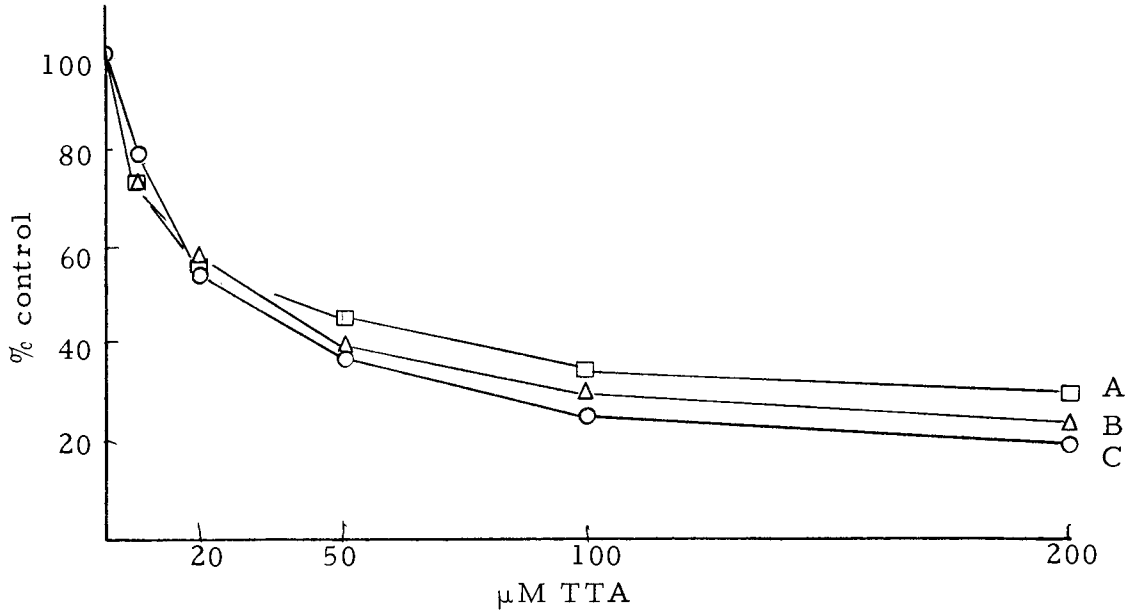
**Other than incubation with 30 mM succinate as were all samples.

Figure 34. Effect of TTA on the succinate-ferricyanide reductase of HMP.

Assays carried out as described in Materials and Methods. Line A: assayed at 0.2 mM ferricyanide, specific activity of control is 0.268 mM succinate min^{-1} at one mg HMP ml^{-1}. Line B: ferricyanide concentration is 0.6 mM and specific activity of 0.323. Line C: 2.25 mM ferricyanide and specific activity of 0.278.

Figure 35. Effect of TTA on the succinate-ferricyanide reductase of Antimycin A inhibited HMP.

HMP treated at one μg of AA/mg of HMP. Assayed as described in Materials and Methods. Line A: Assayed at 0.2 mM ferricyanide with a specific activity of 0.036 mM succinate min^{-1} at one mg of HMP ml^{-1}. Line B: ferricyanide concentration of 0.6 mM and specific activity of 0.070. Line C: 2.25 mM ferricyanide and specific activity of 0.156.


100
80
60
40
20
% control
20
50
100
200
μM TTA
A
B
C

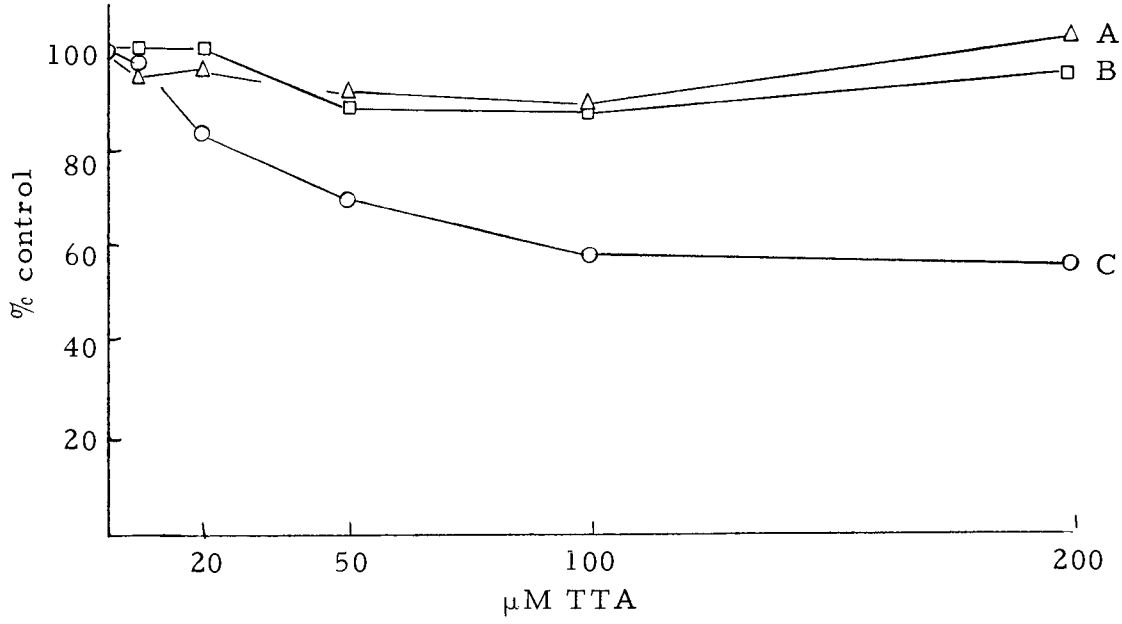

100
80
60
40
20
% control
20
50
100
200
μM TTA
A
B
C

Figure 36. Variation of the inhibition of succinate-ferricyanide reductase activity with ferricyanide concentration: TTA and AA.

Assayed as according to Materials and Methods. Incubations detailed there also. Data expressed as % of the control activity at each ferricyanide concentration. Data taken from Figure 36.

Line A: Assayed after incubation with antimycin A in the presence of acetone.

Line B: Assayed after incubation with Antimycin A in the presence of 200 μM TTA.

Line C: Assayed after incubation in the presence of ethanol in the presence of 200 μM TTA.

Figure 37. Variation of the succinate-ferricyanide reductase activity with the ferricyanide concentration in normal and inhibited HMP.

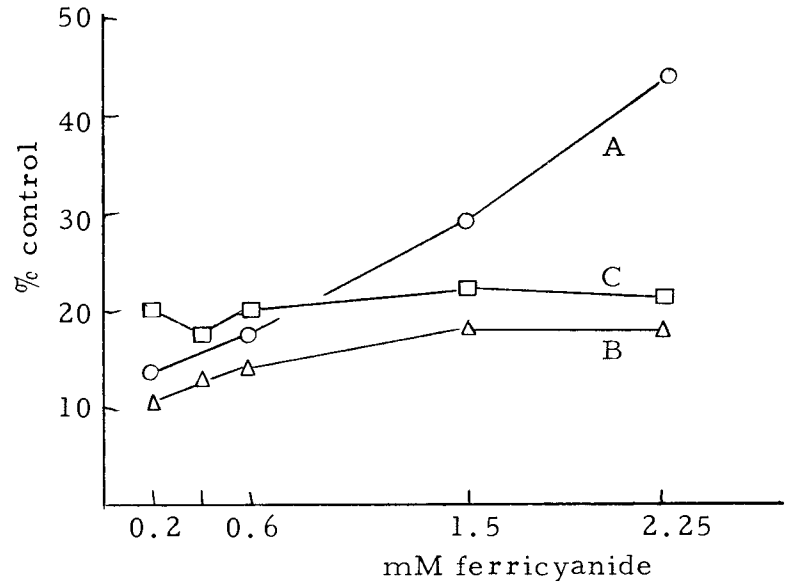
Specific activities are expressed as mM succinate min^{-1} at one mg of HMP per ml.

Line A: After incubation, assayed with 3% v/v acetone.

Line B: After incubation with ethanol, assayed with 200 μM TTA.

Line C: After incubation with AA, assayed with acetone.

Line D: After incubation with AA, assayed with 200 μM TTA.


50
40
30
20
10
% control
A
C
B
0.2
0.6
1.5
2.25
mM ferricyanide

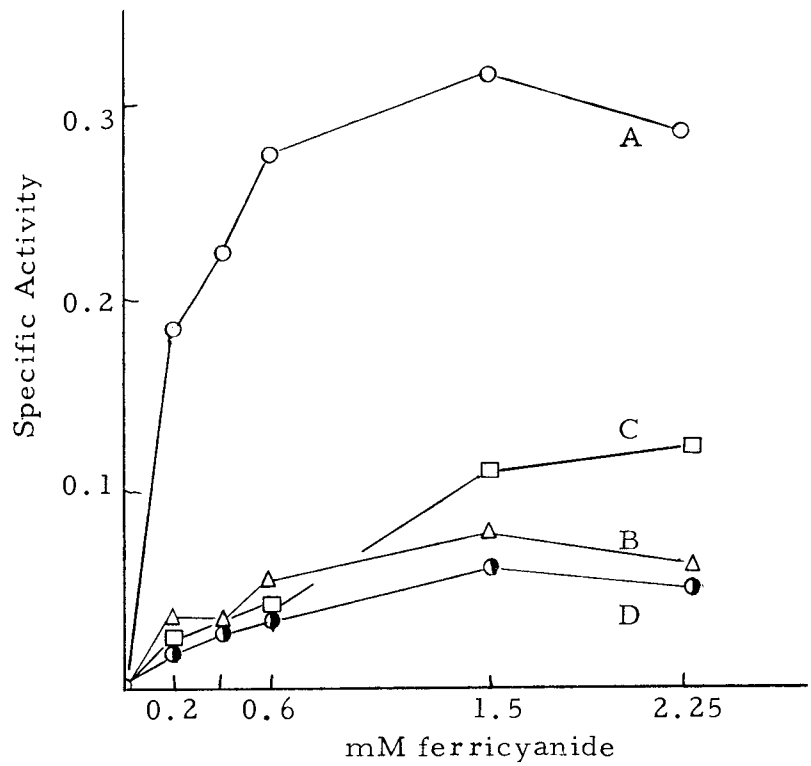

0.3
0.2
0.1
Specific Activity
A
C
B
D
0.2
0.6
1.5
2.25
mM ferricyanide

Figure 38. Effect of ferricyanide concentration on the TTA inhibition of AA-insensitive succinate-ferricyanide reductase activity and the AA inhibition of the TTA-insensitive succinate-ferricyanide reductase activity.

Data is expressed as the ratio of the TTA + AA inhibited sample of the singly inhibited sample. Line A: The ratio of activity of TTA + AA sample to TTA sample at each ferricyanide concentration. Line B: The ratio of TTA + AA sample to the AA inhibited sample at each ferricyanide concentration.

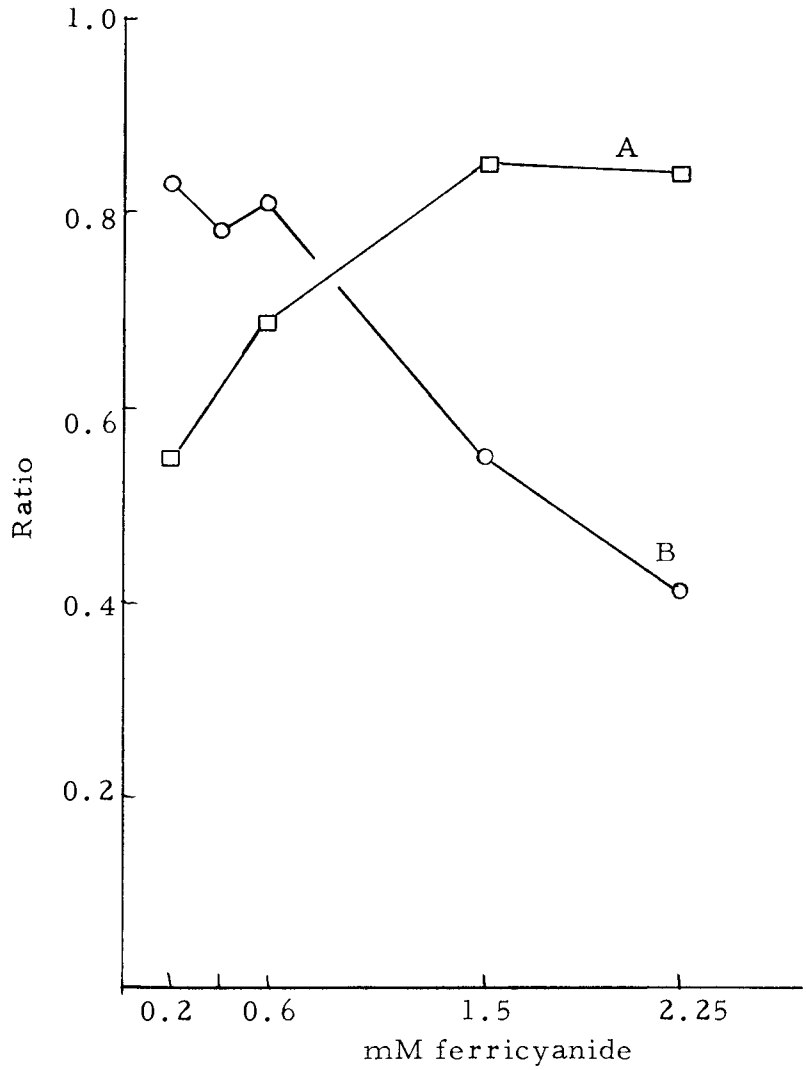

1.0
0.8
0.6
0.4
0.2
Ratio
A
B
0.2
0.6
1.5
2.25
mM ferricyanide

Figure 39. Lineweaver-Burke plots of the effect of ferricyanide concentration of succinate ferricyanide reductase activity: Studies with AA and TTA.

Line A: After incubation, assayed with 3% v/v acetone. Line B: After incubation with ethanol, assayed with 200 μM TTA. Line C: After incubation with AA, assayed with acetone. Line D: After incubation with AA, assayed with 200 μM TTA.

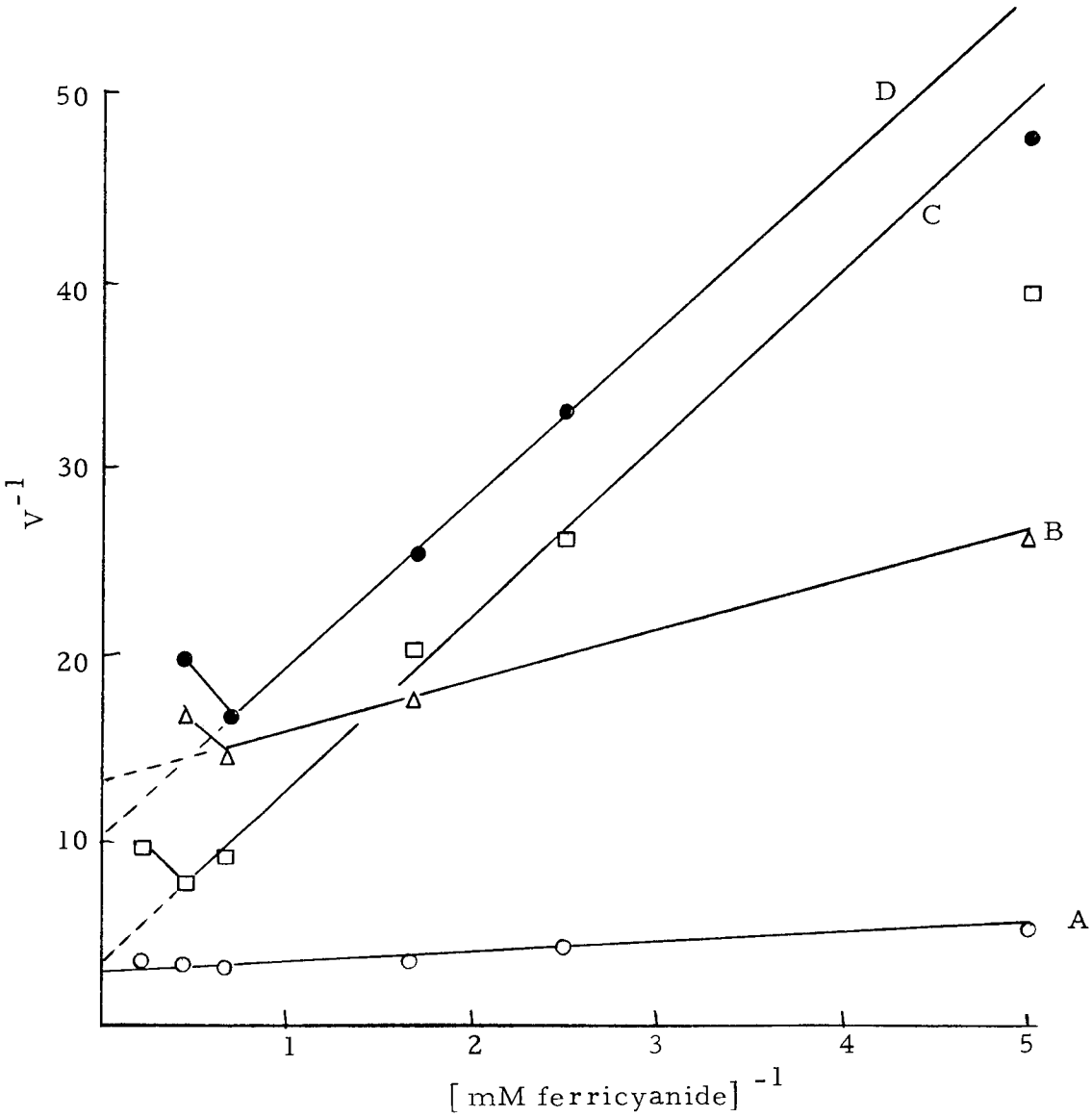
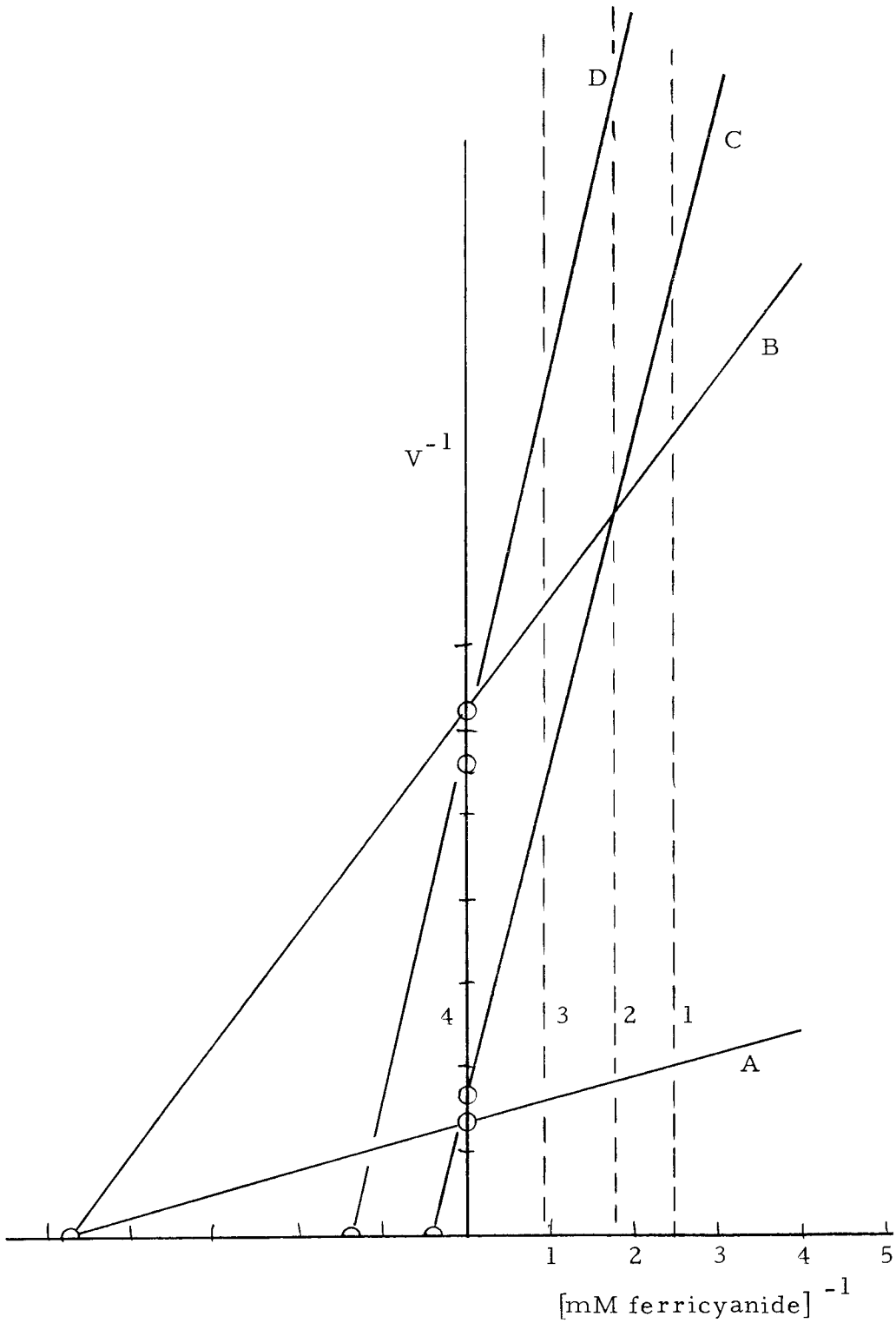

50
40
30
20
10
V^{-1}
D
C
B
A
1
2
3
4
5
[mM ferricyanide] $^{-1}$

Figure 40. Idealized Lineweaver-Burke plots from Figure 39.

Line A: After incubation, assayed with 3% v/v acetone. Line B: After incubation with ethanol, assayed with 200 μM TTA. Line C: After incubation with AA, assayed with acetone. Line D: After incubation with AA, assayed with 200 μM TTA. See text for meaning of lines 1-4. Lines are drawn from data presented in Table 15 which is in turn drawn from Figure 39.


D
C
B
A
V^{-1}
4
3
2
1
1
2
3
4
5
[mM ferricyanide] $^{-1}$

As can be seen from Figure 36, the TTA inhibition is relatively constant at about 80%. On the other hand, the AA inhibition varies from 90% at low ferricyanide to 55% at high ferricyanide. The basic behavior is different. Naturally, TTA + AA reflects both trends and at low ferricyanide is inhibited slightly more than AA alone and at higher ferricyanide concentrations, the inhibition is slightly more than TTA alone.

In Figure 38, examination of the effect of one inhibitor on the activity resistant to the other inhibitor is assessed. Here, at low ferricyanide, TTA only slightly inhibits AA-insensitive activity, but as the acceptor concentration increases, the inhibition becomes greater until TTA inhibits more than half of the AA-insensitive activity. From the other viewpoint, at low acceptor concentrations, AA inhibits half of the TTA insensitive activity, but as acceptor concentration increases, the inhibition caused by AA decreases to about 15%.

The Lineweaver-Burke plots are most illustrative. Very high ferricyanide concentration uniformly inhibits the activity so that the Lineweaver-Burke plot is non-linear over a portion of its course. Also noted is a slight tendency for higher activities at lower ferricyanide concentrations than can be accounted for by a simple Lineweaver-Burke system, this is the tendency for the 0.2 mM ferricyanide point to fall below the Lineweaver-Burke line. The presence of TTA does not change the K_m ferricyanide but drastically changes the V_{max}, or

in classical terms, acts as a non-competitive inhibitor. Antimycin A on the other hand only slightly affects the V_{max} and drastically changes the K_m, or surprisingly acts as a competitive inhibitor. The combination of AA and TTA changes the V_{max} to that found in the presence of TTA alone, and changes the K_m but not as drastically as AA. In other words, it acts as a mixed inhibitor with respect to the control. With respect to the TTA inhibited case, TTA + AA acts as a competitive inhibitor. Lastly and most interestingly, TTA + AA acts with respect to the AA case as a classical un-competitive inhibitor. Hence this example provides several examples of classical kinetics.

Figure 40 has four additional lines. The effects of the inhibitors can be displayed by examining what is happening in the region of acceptor concentrations indicated. At line 1 (0.4 mM ferricyanide) and lower concentrations of ferricyanide, TTA inhibits less than AA, but both inhibit extensively. At line 2, (0.55 mM ferricyanide) TTA and AA inhibit the same amount, but the combination of both inhibits more. At line 3 (1 mM ferricyanide), TTA inhibits more than AA but AA + TAA still inhibits extensively. Finally at line 4, or infinite acceptor concentration, AA alone does not inhibit at all, TTA still inhibits but the combination of TTA and AA inhibits only as much as TTA.

This in interpreted to mean that there are two sites of

ferricyanide reduction in HMP. One is inhibited by AA and the other by TTA. This implies a single site of TTA inhibition. Because these studies with artifical acceptors of several types have not given evidence of more than one site of TTA inhibition, the recent work of Sanadi's laboratory has special importance (87). It is now believed that TTA has two kinds of effects, one is uncoupling, the other is inhibitory. Because the levels at which each effect is demonstrated are so different, it can be concluded, in opposition to Sanadi's opinion, that TTA retains a useful diagnostic role.

There remains in these studies, a residual activity which is inhibited neither by AA or TTA or both. Since AA was present in great excess and TTA at somewhat high concentration, (20 fold higher than that necessary for 1/2 maximal inhibition), this residual activity may be direct reduction of ferricyanide by the SDH flavoprotein. This could possible be considered a third site, the rate is low and the site is a minor contributor to the activity under the conditions of the experiments.

D. Low Ferricyanide Site of SDH

1. Kinetics of the Site. The results reported here are to be considered preliminary. An anomalous observation found at lower ferricyanide concentration when assaying succinate-ferricyanide reductase activity in SDH is that the rate, for the first 30 seconds or

more is much higher than expected but rapidly decays to an almost linear rate. The final linear rate fits the data that King has reported for succinate-ferricyanide reductase in SDH. At low ferricyanide concentrations and high enzyme concentration, this decay of the rapid rate becomes less important until at 200 μM ferricyanide or less, the first, rapid phase becomes the entire reaction course. This initial rapid phase is lost in assays with higher than one mM ferricyanide or with aging of the SDH, further, this initial rapid reaction is not present in PMS reductase.

Time course of the enzyme reaction at low ferricyanide and high enzyme concentration (the most favorable conditions) was analyzed using a Niemann plot method (12, p. 114). The results were somewhat equivocal. Under the most favorable conditions, a $K_{m(ferricyanide)}$ of about 60 μM was found with a V_{max} of 6 mM succinate min^{-1} at one mg of SDH ml^{-1}. Using initial rates and ferricyanide concentrations between 100 and 500 μM, initial rates as high as 5.2 mM succinate min^{-1} at one mg SDH ml^{-1} were observed. This is many fold greater activity than expected using the K_m and V_{max} reported by King.

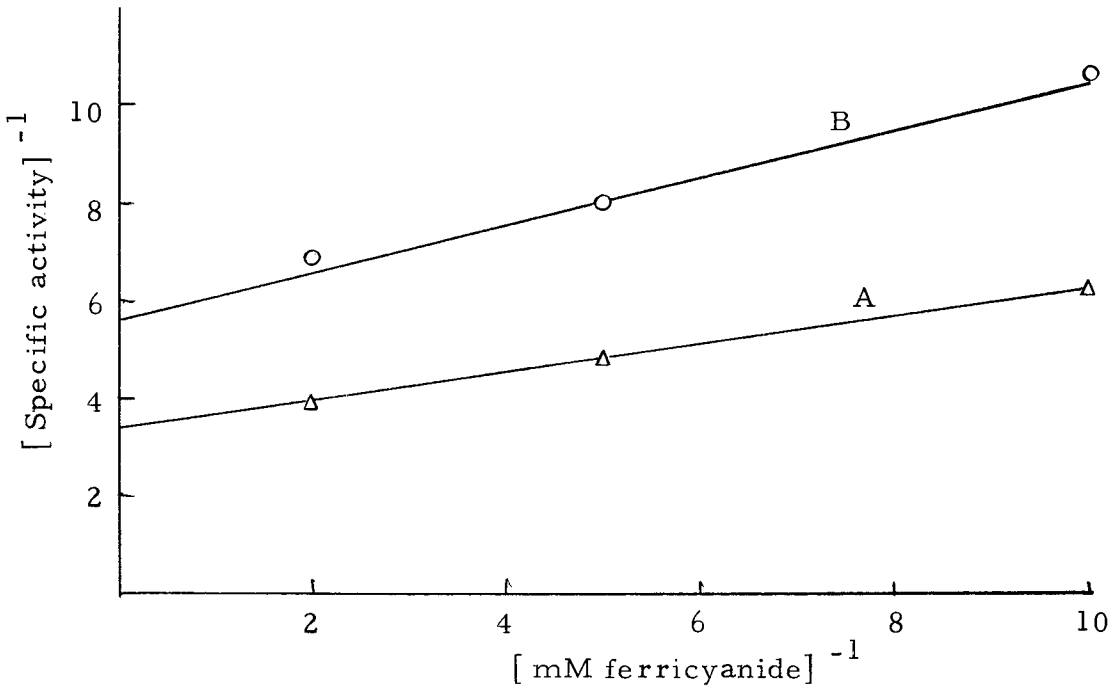
The $K_{m\ ferricyanide}$ and V_{max} at infinite ferricyanide concentrations were determined at two different enzyme ages (see D. 2) from three levels of ferricyanide using initial rates. The Lineweaver-Burke plots were satisfactory and are shown in Figure 41. The K_m

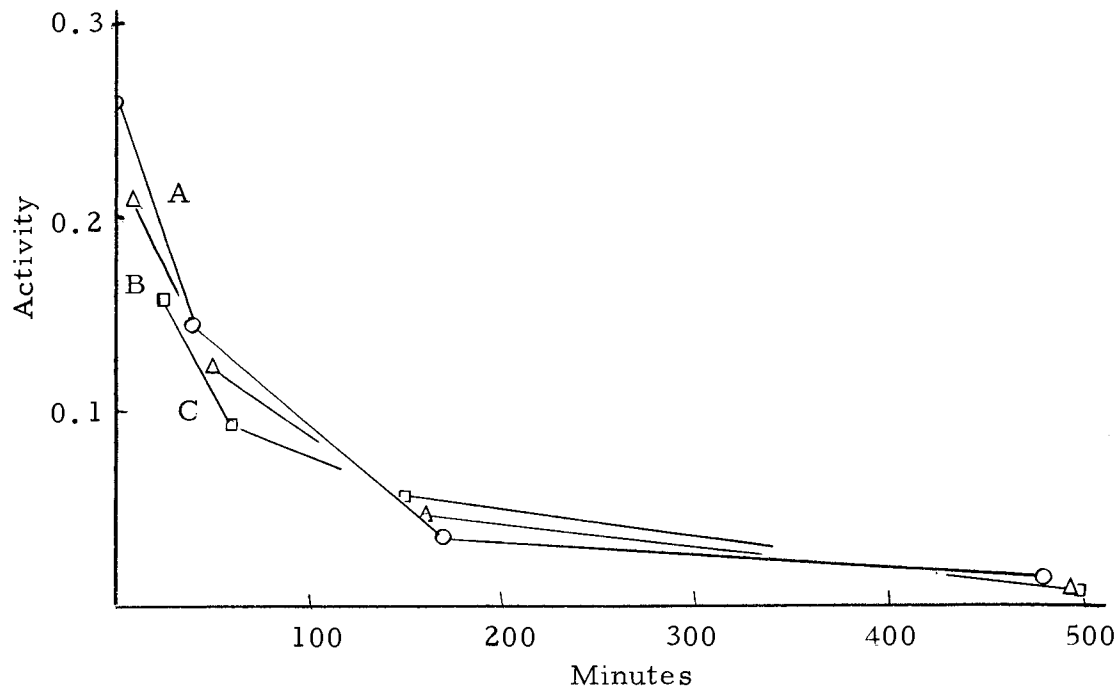
Figure 41. Lineweaver-Burke plot of succinate-ferricyanide reductase activity of SDH.

Assayed as described in Materials and Methods. Line A: enzyme assayed at about zero apparent age. K_m found to be 95 μM ferricyanide and V_{max} was 4.75 mM succinate min^{-1} at one mg SDH ml^{-1}. Line B: Activity at an apparent enzyme age of about one hour. K_m found to be 90 μM. V_{max} found to be 2.65 mM succinate min^{-1} at one mg SDH ml^{-1}.

Figure 42. Variation of succinate-ferricyanide reductase of SDH with time.

Activity expressed in $-A_{520\ m\mu}\ min^{-1}$ observed. Line A: enzyme assayed at 500 μM ferricyanide. Line B: enzyme assayed at 200 μM ferricyanide. Line C: enzyme assayed at 100 μM ferricyanide.


[Specific activity] -1
10
8
6
4
2
B
A
2
4
6
8
10
[mM ferricyanide] -1


0.3
0.2
0.1
Activity
A
B
C
100
200
300
400
500
Minutes

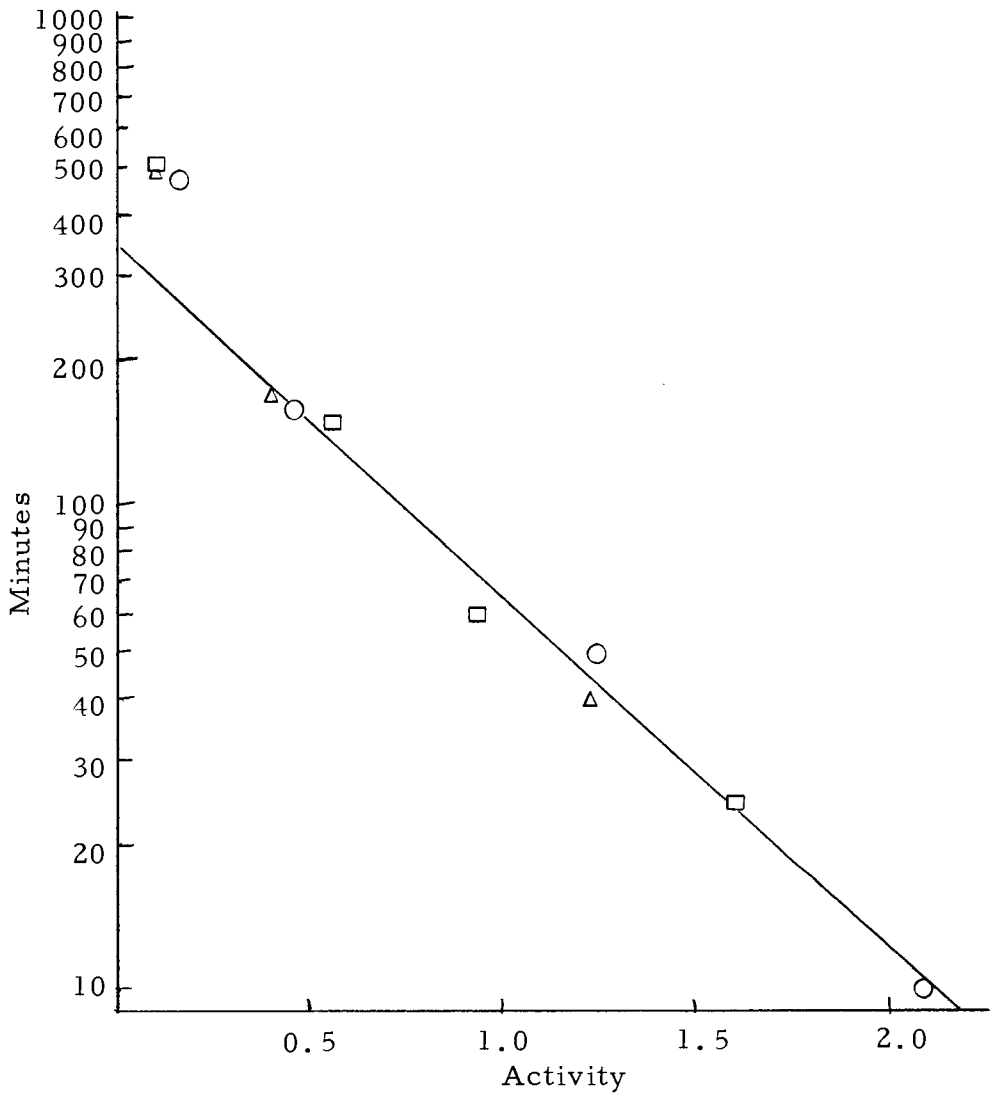


Figure 43. Variation of succinate-ferricyanide reductase activity of SDH with log time.

Activity expressed in $-A_{520\ m\mu}\ min^{-1}$ observed.
Line A: enzyme assayed at 500 μM ferricyanide.
Line B: enzyme assayed at 200 μM ferricyanide.
Line C: enzyme assayed at 100 μM ferricyanide.

Figure 41 is obtained from the same data. This result indicates that the decay for at least 1/2 of the activity is reflected in decay of the V_{max} rather than a K_m change.

These results differ somewhat from those obtained from decay of the succinate-WB reductase activity of SDH in that the decay is probably V_{max} decay and the site is abolished in about two to three hours. The high K_m site of succinate-ferricyanide reductase activity of SDH does not disappear during this period of time but the apparent K_m does change. The relation of the K_m change to the above data is obscure. Further, the relationship between the two sites of ferricyanide reductase in SDH has yet to be investigated.

Part III. Wurster's Blue as Electron Acceptor for Flavoenzymes

In this section some of the properties of aqueous solutions of WB will be examined. Also developed is a survey of the catalytic activity several varieties of redox enzymes. Some of the data reported here has appeared also in the literature (36, 41).

Figure 44 shows the spectra of a 129 μM solution of WB. There is a distinct peak at 612 mμ, a peak at 562 mμ and a distinct shoulder at 520 mμ. The millimolar extinction coefficient (E_{mM}) at 612 mμ was found to be 11. Figures for E_{mM} of 12 have been reported in the literature (1). The oxidation potential of TMPD to WB is

+0.26 volts. The oxidation to higher oxidation states give a non-thermodynamically reversible result, perhaps due to polymerization of the quindiimine (54).

WB is a highly active compound and reacts with many compounds of biological importance. For instance, both ascorbate and glutathione have been used to keep TMPD reduced as donor systems for cytochrome c oxidase assays(52). WB reacts with NADH and NADPH to give unknown products. Preliminary studies have failed to yield obvious kinetics. The non-enzymic oxidation of NADH can be controlled to tolerable levels by keeping both NADH (P) and WB concentrations as low as practical. It was found that NADPH reacted faster than NADH, this is not considered unreasonable since WB is a cation and NADPH carries more negative charge than NADH. When assaying enzymes with NADH and WB the initial non-enzymic rate was subtracted from the initial enzymic rate to yield a net enzymic activity. This does yield activities that are proportional to enzyme concentration and even Lineweaver-Burke plots have been attempted.

WB also reacts with thiols such as Na_2S, mercaptoethanol, cysteine and reduced glutathione. The stoichiometry is about 2 RSH + 4 WB to yield 4 TMPD. The sulfhydryl oxidation was not further characterized. WB is also highly reactive with $Q(10)H_2$, the rate being too rapid to measure. Since WB is an obligate one electron transfer compound, it implies a free radical intermediate of $RS^{\bullet}$, $QH^{\bullet}$, or

ascorbate H•.

WB seems to react with itself. It would seem that a dismutation would seem to be the most likely reaction, but experiments contraindicate a simple reaction. When the products of decay are examined, much more TMPD is found than could be accounted for by a simple dismutation. The decay of WB is much faster at higher pH's than lower, with optimum stability about pH 4-5 (53).

Table 16 lists the enzymes assayed, the source from which the enzymes were obtained, the flavin prosthetic group, and metal ions present. The rates determined at a single level of WB or in some cases, the V_{max} was determined.

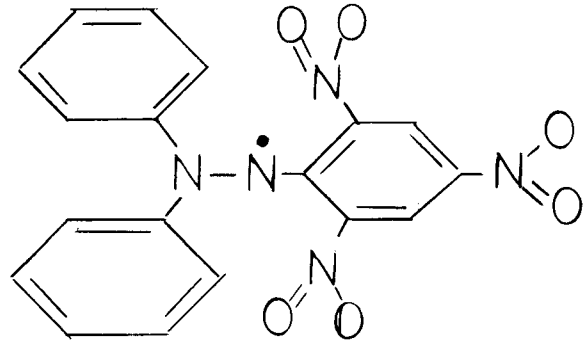
Of the 19 enzymes tested, 15 were flavoproteins. All of the latter reacted to some extent with WB. The presence of metal had no clear cut effect, and the nature of the prosthetic group (FMN, FAD, peptide-bound FAD) was not a ruling factor. On the other hand, the pyridine-nucleotide linked dehydrogenases were inactive towards WB.

A. Reactivity of Other Free Radicals in Biological Systems

Cation-radicals such as WB, may be reduced in biological systems at rates similar to other biological substrates. From the physical appearance of experiments conducted in Part I of the Results and Discussion, it can be inferred that the semi-quinone anion of tetrachloroquinone will also react with biological systems. It was decided

to extend this study to a nonionic radical, and the much studied DPPH was chosen because of its commercial availability.

DPPH was not soluble in water as would be expected, from its structure but it was soluble in many organic solvents. Water miscible solvents were investigated in the hope that a system could be obtained which would allow sufficient concentration of the DPPH and not inactivate the HMP.



2, 2-diphenyl-1-picrylhydrazyl (DPPH)

Acetone-water-phosphate buffer systems were found to allow at least qualitative estimation of radical interaction with the enzyme. Figure 45 indicates a satisfactory solvent system arrived at by trial and error.

Figure 44. Spectrum of Wurster's blue perchlorate.

Solution contained, by weight, 129 μM Wurster's blue perchlorate dissolved in 0.2 M Sørensens phosphate buffer, pH 7.4. The apparent millimolar extinction coefficient at 612 mμ is 11.05.

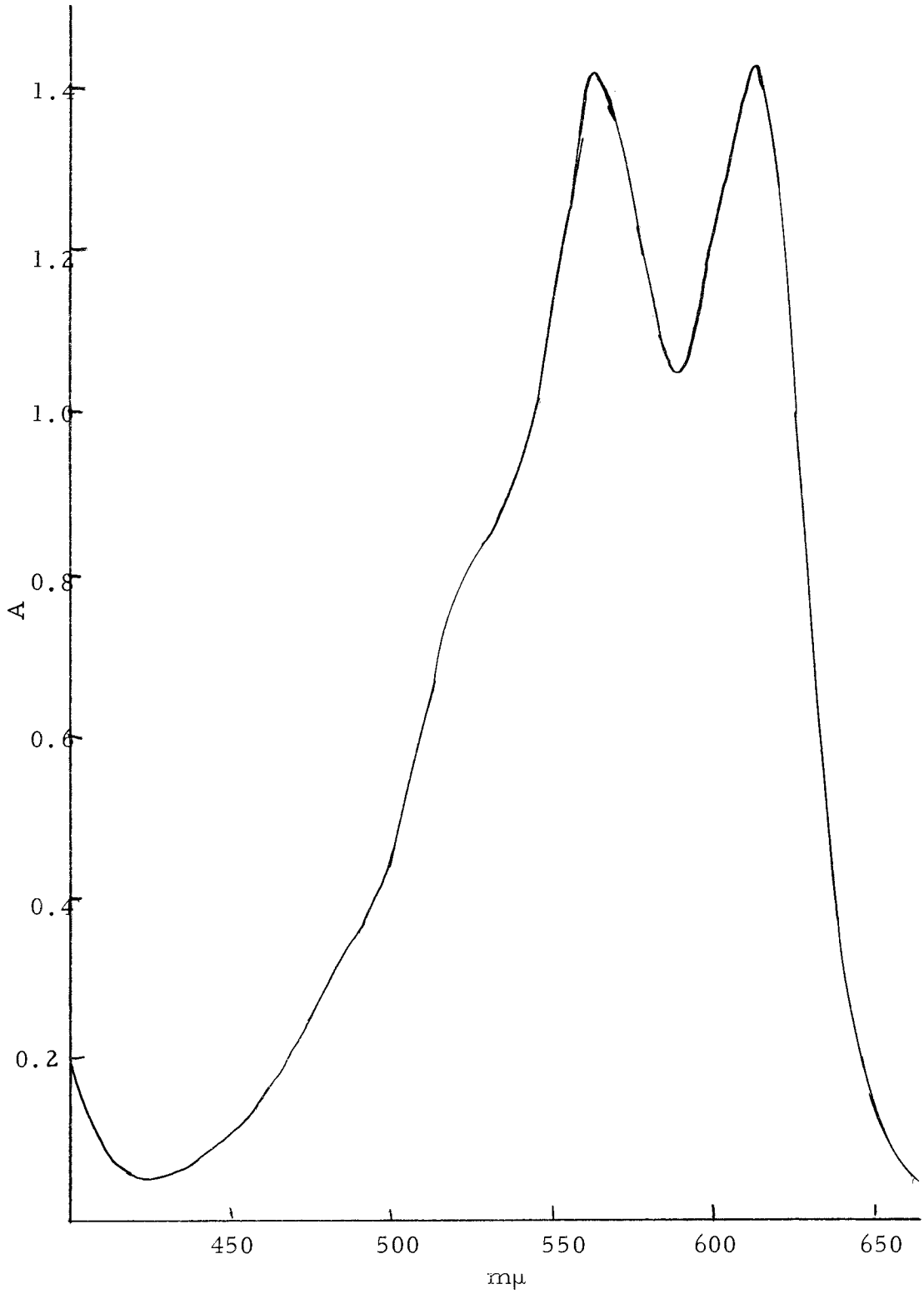

1.4
1.2
1.0
0.8
A
0.6
0.4
0.2
450
500
550
600
650
mμ

Figure 45. Spectra of some solvent systems investigated for enzymic assay with DPPH.

Spectra were run at room temperature on Cary 11 with 0-1 slide wire. The stock DPPH solution was 2.54 mM dissolved in acetone and all solutions were 81.7 μM DPPH.

Curve A contains: 1.0 ml acetone; 0.1 ml DPPH solution; 1.0 ml water; 1.0 ml 0.1 M Sorensen phosphate buffer, pH 7.4. This optically clear solution was eventually used for all enzymatic assays. The observed EmM(526 mμ) is 12.7 and the solution was about 35 percent acetone.

Curve B: 0.5 ml acetone, 0.1 ml DPPH solution. 1.4 ml water, 1.0 ml phosphate buffer. This mixture shows considerable turbidity.

Curve C: 3.0 ml acetone, 0.1 ml DPPH solution. This solution was optically clear.

From curve C, EmM (518 mμ) was 13.7. After standing for 10 minutes, system A was stable to the extent that - delta $A_{526m\mu}$ was less than 0.001 min^{-1}.

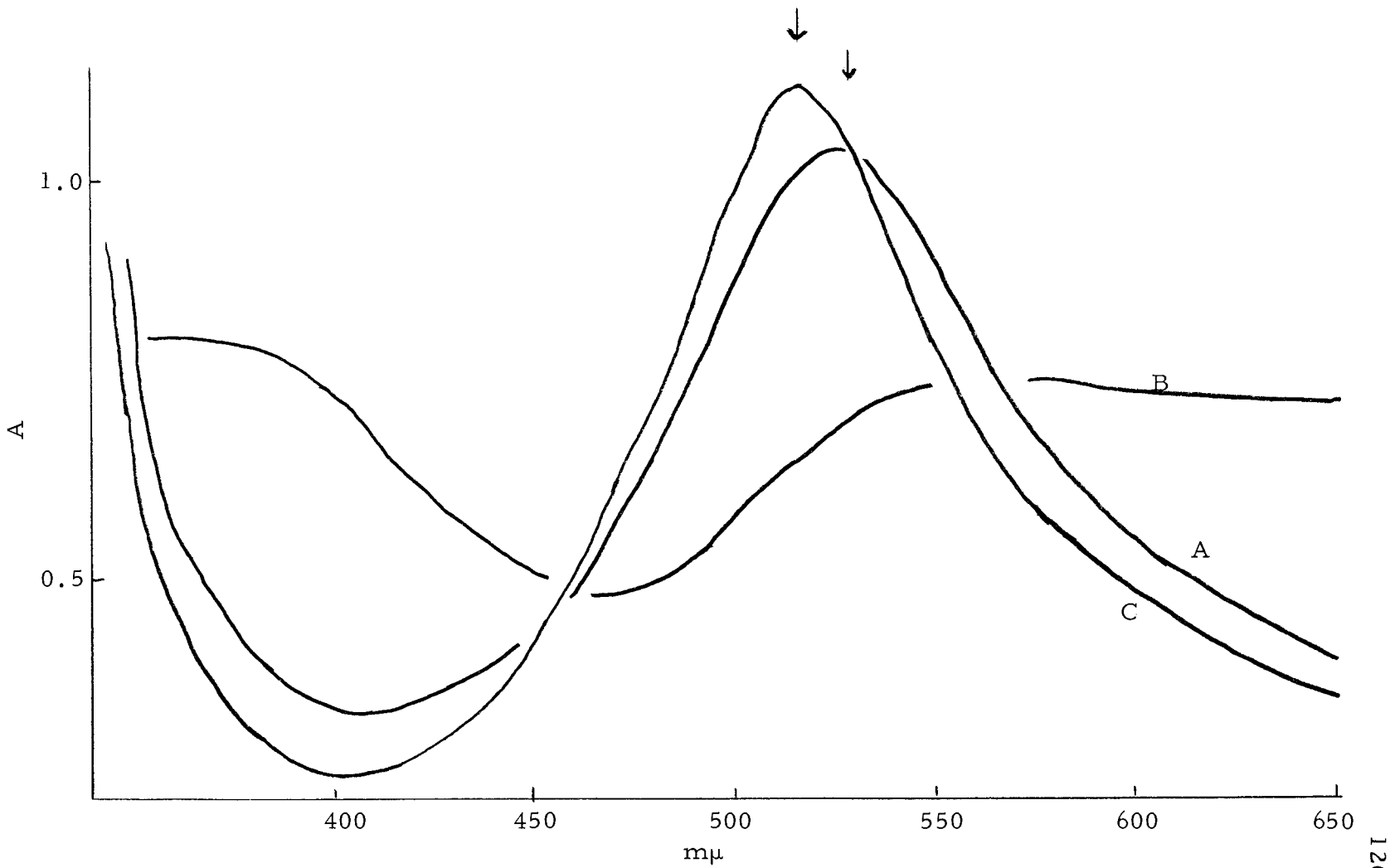

1.0
0.5
A
400
450
500
550
600
650
mμ
A
B
C

Table 16. Activity of various enzymes with Wurster's blue perchlorate.

Enzyme	Source or method of preparation	Constitunent of enzyme		WB conc. employed in assay	Rate*	Remark
		Flavin	Metal			
(1) Beef heart Lactate DH EC 1.1.1.27	Sigma Type III	----	Zn	163	none	(A)
(2) Yeast lactate DH EC 1.1.2.4	Worthington	FMN	heme	137	0.41	(B)
(3) Succinate DH, HMP EC 1.3.99.1	This Lab.	FAD modified	Fe	V_{max}	2.0	(C)
(4) Succinate DH, reconst. active EC 1.3.99.1	This Lab.	FAD modified	Fe	V_{max}	32.0	(C)
(5) Succinate DH, PMS reductase EC 1.3.99.1	This Lab.	FAD modified	Fe	V_{max}	9.2	(C)
(6) NADH DH, HMP EC 1.6.2.1	This Lab	FMN	Fe	V_{max}	20.0	(D)
(7) NADH DH, 30° type	This Lab.	FMN	Fe	V_{max}	28.0	(D)
(8) NADH DH, 37° type	This Lab.	FMN	Fe	V_{max}	27.0	(D)
(9) Glucose oxidase EC 1.1.3.4	Sigma Type II	FAD	none	77	0.22	(E)
(10) Galactose oxidase EC 1.1.3.c	Sigma Type I	none	Cu	125	none	(F)
(11) D-amino acid oxidase EC 1.4.3.3	See text	FAD	none	100	0.014	(G)

Table 16 continued.

(12) L-amino acid oxidase EC 1.4.3.2	See text	FAD	none	128	0.067	(H)
(13) Uricase EC 1.7.3.3	Sigma	none	Cu	123	none	(I)
(14) Yeast alcohol DH EC 1.1.1.1	Sigma	none	Zn	125	none	(J)
(15) Diaphorase (*Clostridium kluyveri*) EC 1.6.99.1	Worthington	FAD	none	107	150	(K)
(16) NADPH-Cytochrome *c* reductase EC 1.6.2.3	See text	FMN	none	98	0.029	(L)
(17) Liver aldehyde oxidase EC 1.2.3.1	(M)	FAD	Mo	-	+	
(18) Milk xanthine oxidase EC 1.2.3.2	(M)	FAD	Mo, Fe	-	+	
(19) Chicken liver xanthine oxidase EC 1.2.3.2	(M)	FAD	Mo, Fe	-	+	

* Expressed as mM WB reduced $min.^{-1}$ at 1 mg ml^{-1} at 23°.

(A) The assay mixture consisted of 0.36 mg lactate dehydrogenase in 3 ml which was: 0.1 M Sorensen phosphate buffer, pH 7.4, 50 mM lactate, 163 μM WB. Decrease in A_{612} mμ was followed with Cary model 11.

(B) Enzyme was diluted with 67 mM phosphate buffer, pH 7.4, 1 mM in EDTA. The assay mixture contained: 60 mM D, L-lactate, 67 mM pyrophosphate buffer, pH 8.4, 1 mM EDTA, and 137 μM WB.

(C) Section II, Results and Discussion.

(D) Personal communication, Dr. B.M. Hegdekar.

Table 16 continued.

(E) Assay method. In a Thunberg cuvet containing 2.8 ml is the following: 7 mM acetate buffer, pH 5.0, 1.2% glucose, and 77 μM WB. In the hollow stopper is 0.2 ml containing the enzyme. After evacuation, the cuvet is tipped and the reaction followed. When assayed in the presence of oxygen, the initial rate was decreased by 32%.

(F) Procedure as in (E). The cuvet contained: 0.1 M phosphate buffer, pH 7.4, 1.2% galactose, 125 μM WB, and galactose oxidase in the stopper (50 μg in 50 μl).

(G) Procedure as in (E). In 2.8 ml in a Thunberg cuvet was 17 mM pyrophosphate buffer, pH 8.3; 50 mM D, L-alanine; and 100 μM WB. The enzyme was placed in the stopper. The cuvet was tipped to start the reaction. The rate fell off rapidly. When assayed in air, the initial rate was inhibited 60%. Excess FAD increased the rate 27% (in vacuo).

(H) Procedure as in (E). The cuvet contained: 1.2 ml, 0.1 M KCl; 1.0 ml, 0.3 Tris-acetate buffer, pH 7.4; 0.5 ml, 0.1 M L(+)-histidine and WB to a final concentrations of 128 μM. The stopper contained crude venom in 0.1 M KCl. In the presence of air the rate is decreased 50% and the apparent K_m is shifted from 4 μM to about 40 μM.

(I) Procedure as in (E). The Thunberg cuvet contained: 0.9 ml, water; 2.0 ml of uric acid-borate buffer solution (equal volumes of 2 mg/ml uric acid and 0.2 M borate buffer, pH 8.5); and WB to 123 μM. The stopper contained enough enzyme to give a rate of 0.174 $A_{290\ m\mu}$/min. when assayed in air without WB.

(J) The assay mixture contained in 3 ml: 10 mM pyrophosphate buffer, pH 8.4; 6% ethanol; and 125 μM WB. The enzyme added to start the reaction was 0.1 ml of freshly reconstituted alcohol dehydrogenase diluted 1;2000 with cold 10 mM phosphate buffer, pH 7.4.

(K) The assay mixture contained in 3ml: 30 mM Tris-acetate buffer, pH 7.4; 107 μM WB; and 50 μM NADH. The non-enzymic rate was subtracted from the overall rate.

(L) The assay mixture contained: 0.10 M Sørenson phosphate buffer, pH 7.4, 98 μM WB, 15 μM NADPH. The non-enzymic rate was subtracted from the overall rate. Under similar conditions, the enzymed showed an activity of 0.02 mM cytochrome c min^{-1} at one mg of enzyme per ml. The cytochrome c concentration was 27 μM.

(M) The results of Dr. K.V. Rajagopalan. "All experiments were carried out in 0.05 M phosphate buffer, pH 7.8. In the case of milk xanthine oxidase, the anaerobic reduction of WB (on the basis

Table 16 continued.

of one-electron equivalent), was as efficient as the aerobic reduction of oxygen. For the chicken liver enzyme, which is a poor oxidase, WB was as efficient as NAD. The results with aldehyde oxidase were somewhat complex since quinones in general are potent inhibitors of the enzyme. However, it would seem that the initial rate of anaerobic reduction of WB by aldehyde oxidase is as efficient as its oxidase activity."

Before examining the enzymatic reduction of the radical, a solution of type A in Figure 45 was reduced with a few grains of dithionite. This caused a marked change of color, e.g. the purple became yellow. A spectrum of the radical and dithionite reduced forms is shown in Figure 46. When allowed to stand for several minutes in air at room temperature, the color of the reduced system became orange, addition of more dithionite caused the recurrence of yellow color. Since dithionite removes oxygen from the solution as well as other reductions, the orange color could well be related to the presence of the hydrazine (reduced form) and oxygen. Since in Figure 46, the presumably reduced form has practically no absorbtion at the absorbtion maximum, it will be assumed that the delta redox E_{mM} is 12.7

B. Reactions of DPPH With Substrates

1. Succinate. When succinate is added to DPPH solution, there is no change in A(526). When the enzyme is added, the reaction ensues. The reaction can be assumed to be catalyzed by the enzyme.

2. NADH. As with WB, DPPH reacts with NADH. The reaction product is orange not yellow and a spectrum of the orange product is shown in Figure 47. Using the system described in Figure 47, the initial non-linear rate was delta A (526 mμ) of -0.275 min^{-1} which slowed to only -0.032 mμ three minutes after NADH addition. If the reaction was run with 84 μM DPPH and 65 μM NADH added, the

initial rate was only -0.072 min^{-1}. Again the product was orange. Note that a change of concentration of NADH of ten fold leads to only a 3.5 decrease in the observed initial rate of reaction.

As Figure 47 indicates, the product of the reaction has an absorption maximum at 421 mμ and a millimolar extinction coefficient of 11.4. At 526 mμ, the millimolar extinction coefficient is only 6.0. Then for the NADH-DPPH non-enzymic reaction, an millimolar extinction coefficient of 6.7 can be calculated. This will hold true for any enzymic reactions assuming the products of enzymic reaction are the same as those of non-enzymic reaction.

C. Succinate-DPPH Reductase

The aqueous acetone solvent system was set up as indicated in Figure 45 with one mg of HMP (final volume of 3.1 ml) and monitered at 525 mμ. The succinate was added to a final concentration of five mM and DPPH to 76 μM. A rapid nonlinear decrease in A ensued with an initial rate of -0.86 A min^{-1}. This proceeded for a total delta A of -0.28 when an abrupt increase in A was observed, again non linear, but the initial rate was 0.134 A min^{-1}. This reaction was not followed to completion. This behavior was quite reproducible in the same cuvet by starting the reaction with repeated additions of DPPH. The initial rate of loss of DPPH was calculated to be 0.21 mM DPPH min^{-1} at 1 mg HMP/ml. This is in the absence of terminal oxidase

inhibitor and in presence or 35 percent acetone. That the reaction took place was surprising.

The color of the resultant cuvet was orange, not yellow, but dithionite could produce the yellow color when added. In an attempt to see if the orange color could be the same product as observed in Figure 47, an experiment was run as described in Figure 48. This experiment illustrates that the orange spectra produced enzymatically has similar characterisitics to the non enzymatically produced color, e.g. broad absorbtion with a maximum in the 400 mμ range. The following reaction is believed to be the case.

```
                succinate [HMP]          Rate 2
DPPH ------------------- Yellow -------- Orange
                Rate 1   compound         compound
```

The first reaction is the fastest at this enzyme level and is responsible for most of the first decrease in absorbtion e.g. the yellow compound does not absorb at 525 mμ. The orange compound is more slowly formed, this is the increase in A (525 mμ) e.g. the orange product absorbs at 525 mμ but not as much as DPPH. When lower amounts of HMP are used, the biphasic kinetics are not seen since now the rate at which the yellow compound is transformed into the orange compound is commensurate with the rate at which the yellow compound is formed. Clearly then there is an enzymatic, succinate-dependent removal of DPPH and formation of another product(s).

Figure 46. Spectra of oxidized and "reduced" DPPH.

Curve A is DPPH in acetone solution as described in curve A of Figure 45.

Curve B is the above sample after addition of a few milligrams of dithionite.

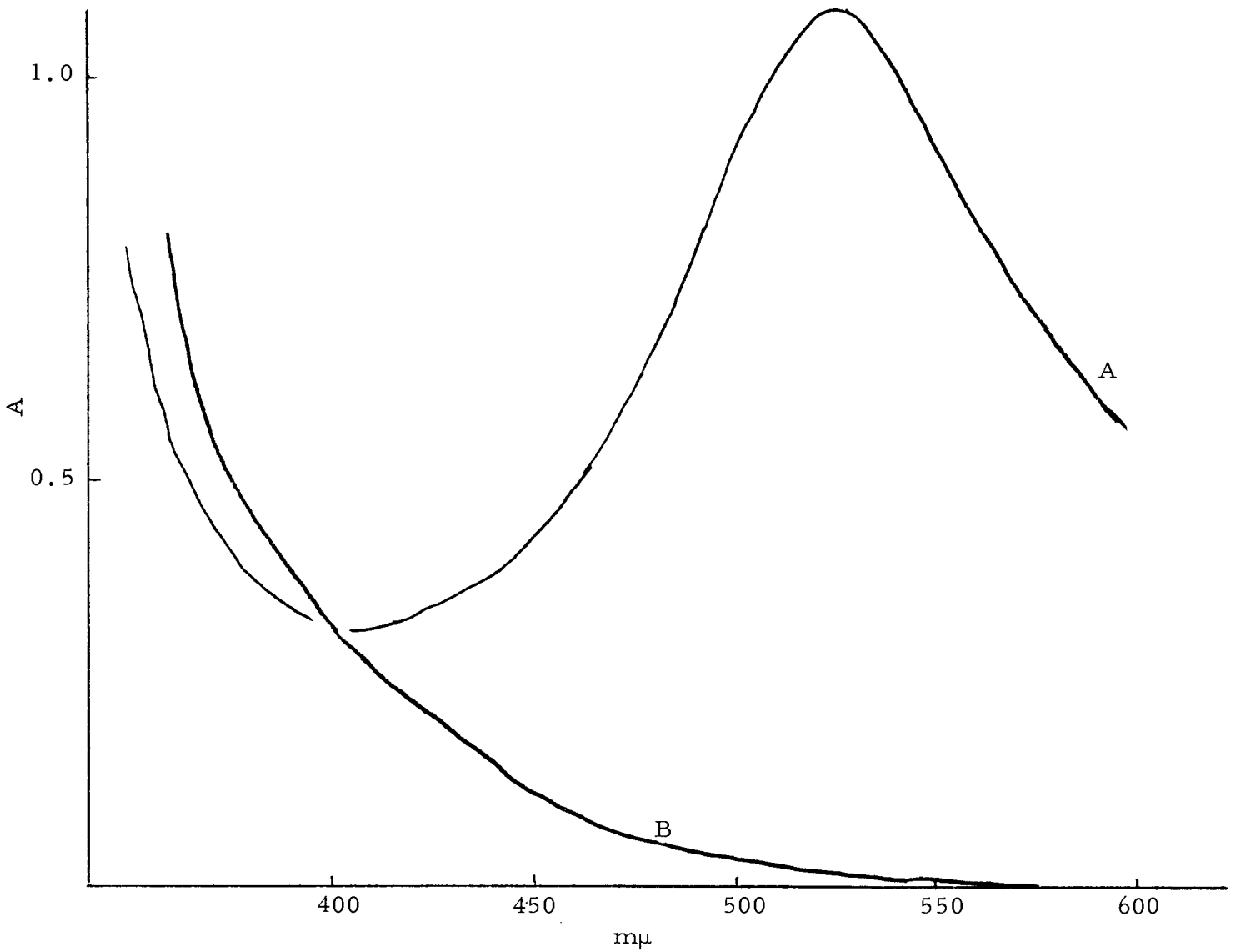
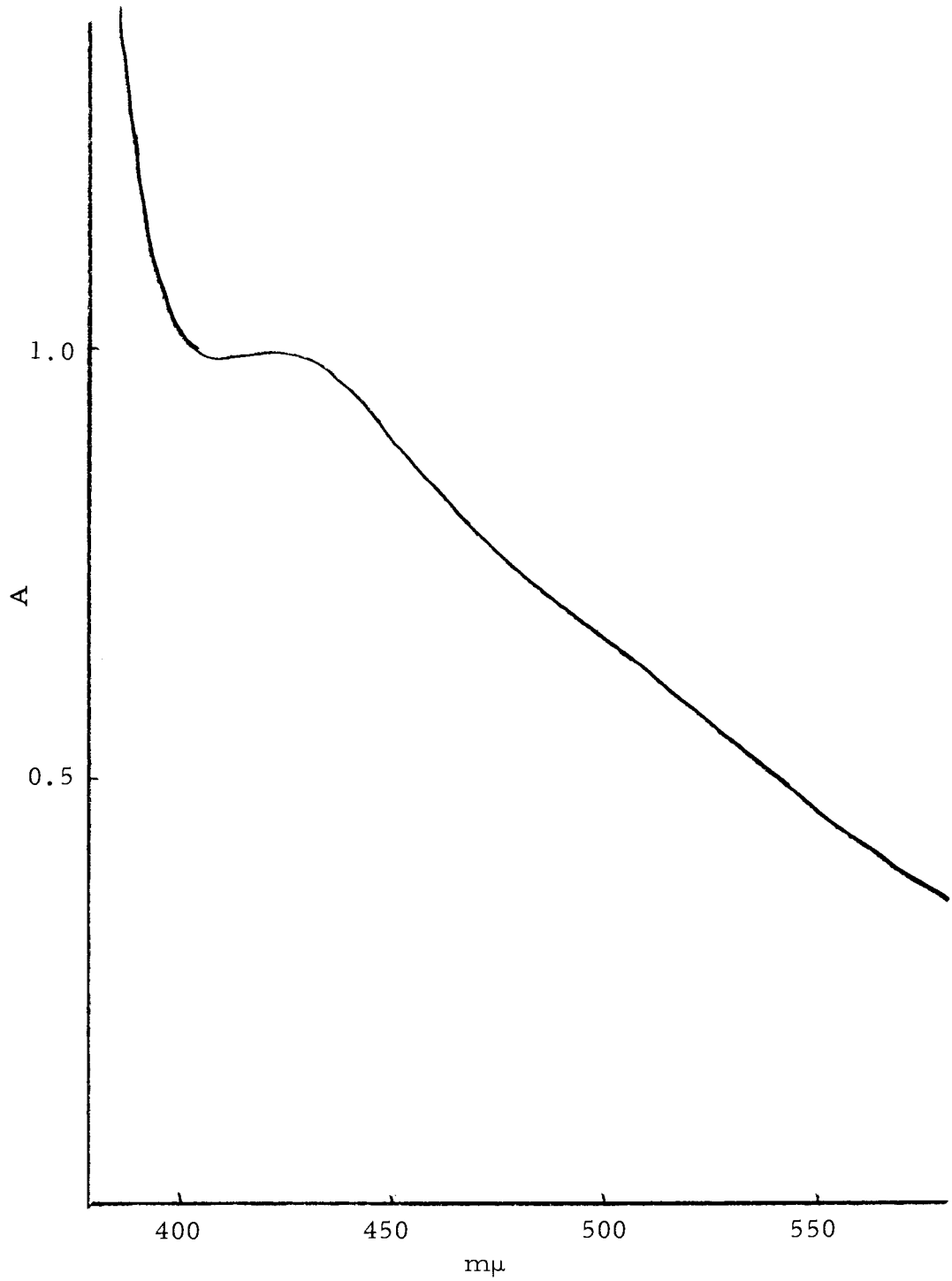

1.0
0.5
A
A
B
400
450
500
550
600
mμ

Figure 47. Spectra of reaction products of DPPH and NADH.

Solvent system A of Figure 45 was used with 87 μM NADH, 0.1 ml of about 20 mM NADH was added to give 650 μM NADH final concentration. A(526 mμ) was monitered. A rapid nonlinear change occurred until A(526 mμ) was 0.5, then additional 650 μM NADH was added and no further change occurred. The spectrum was run vs the solvent blank.


1.0
0.5
A
400
450
500
550
mμ

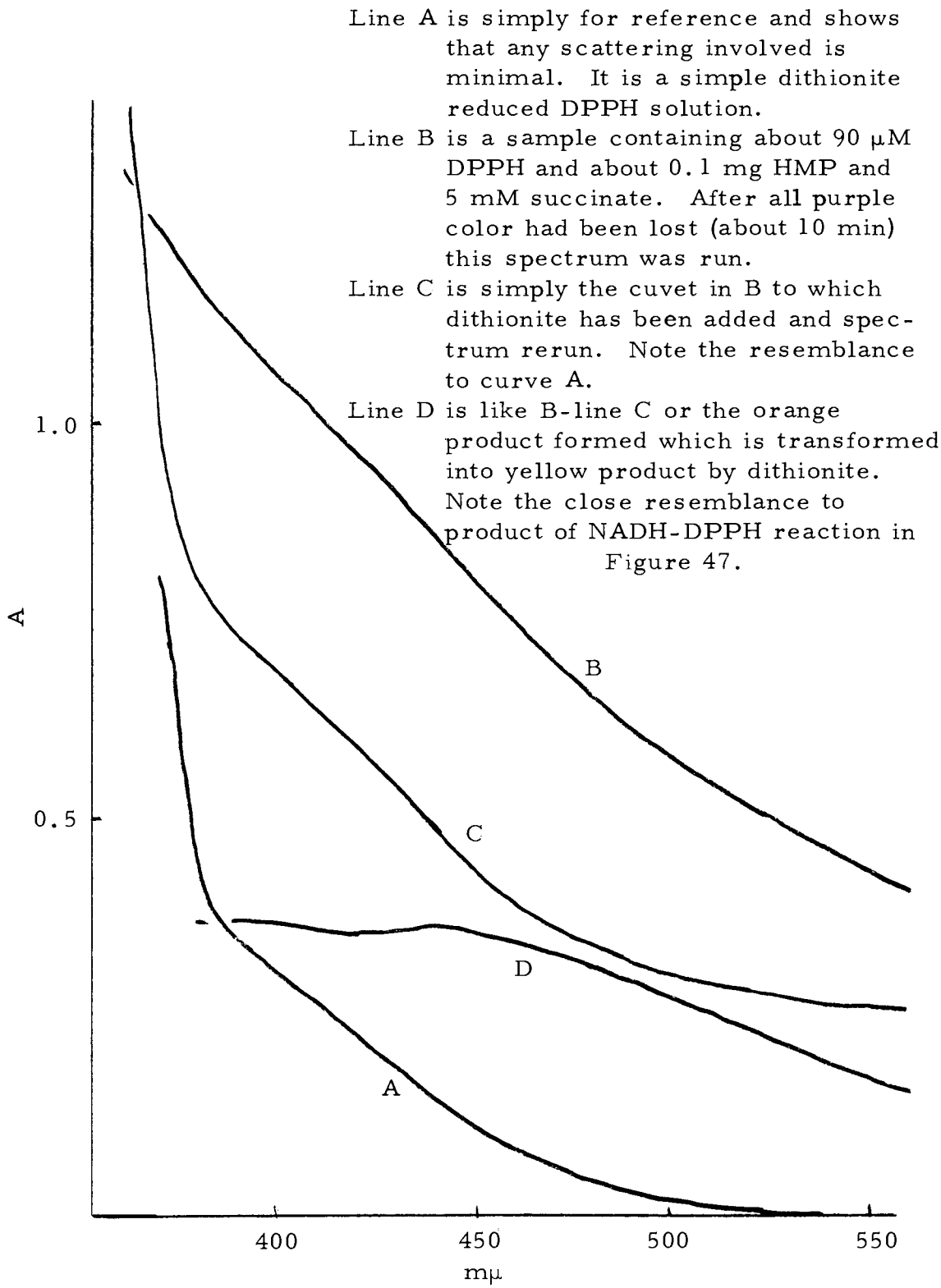


Figure 48. Products of succinate-DPPH activity.

D. NADH-DPPH Reductase

These experiments were run without the usual oxidase inhibitor, and using 35 percent acetone solvent as per Figure 45. The reaction was started by addition of 65 μM NADH to 75 μM DPPH and the initial rate measured. This rate was compared to a system not containing enzyme. The enzymatic system had an initial rate of -0.175 A (525 mμ) min^{-1} while the control was -0.072 min^{-1}. Using the E_{mM} (redox) for NADH discussed before, the rate of 0.116 mM DPPH min^{-1} at 1 mg HMP/ml was found. A smooth non-linear time course was found. The product was orange. If NADH and HMP were incubated and DPPH added to start the reaction, the results were very complicated and not further investigated.

It has been demonstrated that neutral radicals of suitable potential react with redox enzymes. The chemistry of the systems is complex. Certainly these results can only be considered preliminary but promising since many other stable free radicals exist. Some of these are water soluble and may serve as useful substrates in probing the mechanisms of biological redox systems.

Part IV. The Adenosinetriphosphatase Activity of HMP

The first approach to the investigation of oxidative phosphorylation was concerned with determination of those things missing from HMP which were necessary for full biological activity i.e. mitochondrial function. A second, but related approach, was the determination of the activities and enzymes present in HMP and concominant properties contrasted to the analogous enzymes and functions in mitochondria. The last section of the Results and Discussion deals with the first approach and the second approach is elaborated here.

The ATP dephosphorylation to ADP has been long recognized as the reverse of the phosphorylation of ADP to ATP. Such an ATPase is associated with most sub-mitochondrial particles including HMP. This approach was strengthened on the observation by Racker's group some years ago of a protein fraction which was necessary for oxidative phosphorylation and exhibited ATPase activity similar to that found in sub-mitochondrial particles. The focus of attention in this study was to examine the ATPase of HMP *in situ* and in solution. The examination of the relationship of the HMP enzyme to the mitochondrial enzyme was conducted with the overall aim of establishing the functionality of the ATPase in HMP. Since a complex of enzymes was being examined, the tools used in part were reconstitution as well as more conventional techniques.

Since these studies were completed, Racker's laboratory has increased the information available about the molecular properties of the soluble and particulate ATPase. A recent series of articles (29, 30) examined the morphology of the ATPase in mitochondrial structure. Chemical evidence was also presented on the nature and consequences of structurally fixed ATPase.

A. Properties of the Particulate ATPase

Two different kinds of HMP were used in these experiments. Both are described in the Materials and Methods section. Most of the experiments reported in this section were performed with HMP not cHMP, hence the specific activities tend to be a little lower. The published reports of some of this data refer to two assay methods for the ATPase activity. In the later phase of this work, only the ATP regenerating system (method II) was used for determinations of activity.

The specific activity of the ATPase of HMP and cHMP is usually found to be between one and two. When acetone powders are made as described in the materials and methods, the specific activity is slightly less than one. As described by Huijing and Slater (25), the ATPase of HMP is inhibited by oligomycin. Other effects of oligomycin on HMP will be treated in the next section of this thesis (V).

Since the specific activity of the ATPase is somewhat higher than

in mitochondria, and since there is a report of a physiological ATPase inhibitor which is combined in the native state with ATPase to "mask" the activity, experiments were done to investigate the "state of masking" of the ATPase in HMP. Advantage is taken of the lability of the inhibitor to tryptic digestion but the resistance of particulate ATPase to tryptic digestion (67). Figure 49 describes an experiment where HMP and HMP treated with urea (USHMP) were digested with trypsin as described by Racker (67). As can be seen, neither the untreated HMP or the urea treated HMP underwent any ATPase activation. This would indicate that the ATPase of HMP was present in the unmasked state e.g. not bound to the ATPase inhibitor. To extend this observation a little more, a crude preparation of the ATPase inhibitor of Racker's laboratory (63) was prepared and reacted with HMP. A progressive inhibition of activity up to 50% inhibition was observed. This indicates that at least part of the ATPase of HMP is capable of being remasked. These experiments were performed before detailed information on the preparation of the inhibitor were available and probably do not reflect the optimal experimental design.

In order to demonstrate the functional binding of the soluble ATPase to the HMP, it was necessary to prepare a suitable test particle, i.e., one that would be deficient in ATPase activity and capable of rebinding ATPase. Racker has reported the treatment of submitochondrial particles with low concentrations of urea in the cold to

dissociate and inactivate the particle-bound ATPase (29, 30). He was able to reconfer oligomycin sensitivity to soluble ATPase which was incubated with the urea treated, ATPase free, particle.

The fraction of the particulate material responsible for the conferring of oligomycin activity has been isolated free of cytochromes and lipid. The necessity for phospholipid for binding and oligomycin sensitivity was established (29, 30). The experiments reported here were performed before this recent information was published.

The effect of cold urea on the ATPase of HMP is given in Table 17. Over a period of about two hours, the ATPase activity is practically eliminated. It was feared however, that the urea might also affect other activities of the respiratory chain. Towards elucidating that problem, experiments were done as described in Figure 50. The result, in brief, is that low concentrations of urea over a long period of time, or relatively high concentrations for a short period of time, were not harmful to succinate oxidase. Redfearn and King (69) essentially repeated these results and extended these studies to NADH oxidase. They concluded that damage could occur, but more to NADH oxidase than to succinate oxidase. As a simple matter of convience, the method eventually used for the preparation of USHMP (see Materials and Methods) involved limited time treatment of HMP at a high (3 M) urea concentration followed by extensive washing.

Figure 49. Effect of trypsin digestion on the ATPase activity of SHMP and USHMP.

The samples were digested as described by Racker (67). The line labeled A is the sample containing trypsin, the other sample is simply an aging control B. Each sample was incubated at 30° for the time indicated, then the digestion stopped by the addition of a five fold excess of soybean trypsin inhibitor and stored in the cold until assayed by Method II. Similarly for C and D samples. There was a drastic change in the appearance of the samples which is not reflected in any variation in enzymatic activity.

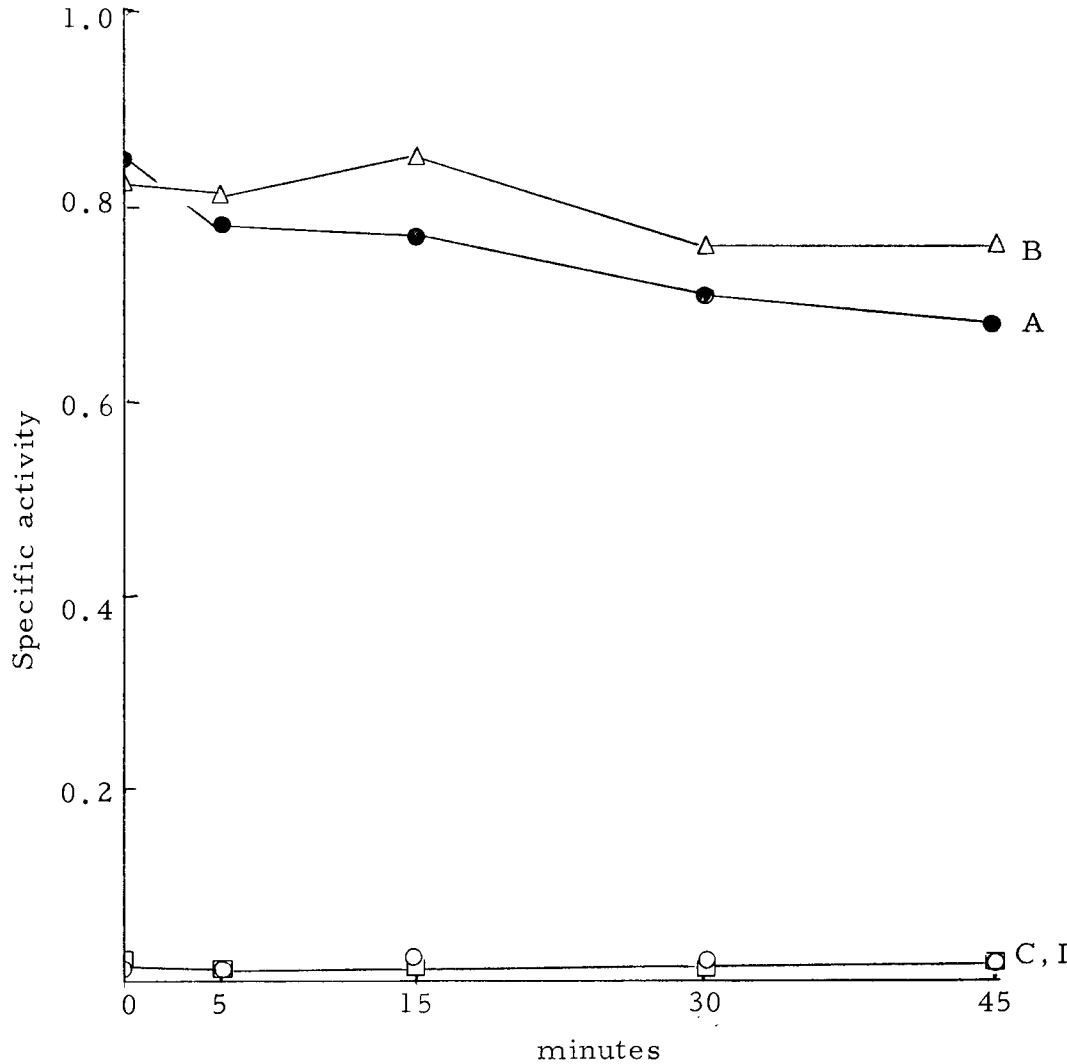

1.0
0.8
0.6
0.4
0.2
Specific activity
B
A
C, D
0
5
15
30
45
minutes

Figure 50. Effect of urea on succinate oxidase.

The samples indicated were made up in phosphate buffer and incubated for the indicated time and temperature. At each time indicated, an aliquot was removed and assayed in a oxygraph for succinate oxidase activity. No exogenous cytochrome c was added. Urea did not affect the oxygraph assay. Samples were diluted 20 to 1 in the assay mixture.

Line A: 1 M urea at 25°

Line B: 0.5 M urea at 25°

Line C: 3 M urea at 25°

Line D: 1 M urea at 0°

Line E: 0.5 M urea at 0°

Line F: 3 M urea at 0°

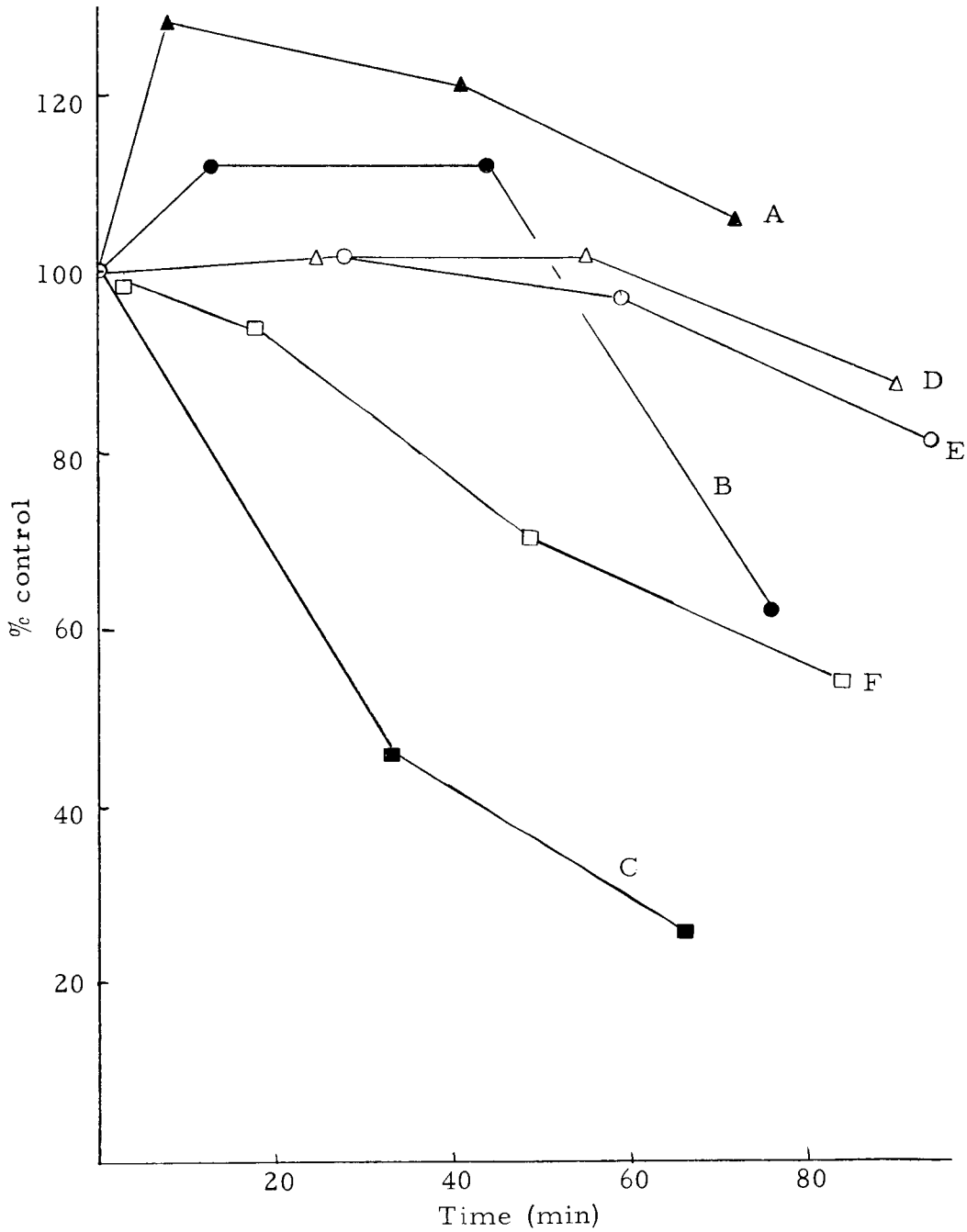

120
100
80
60
40
20
% control
20
40
60
80
Time (min)
A
B
C
D
E
F

Table 17. The effect of cold urea on ATPase of HMP.*

Time (hours)	Activity (mM min^{-1})
0.0	0.45 (0.48)
0.5	0.18
1.0	0.11
1.5	0.07
2.0	0.07 (0.07)

*HMP in 0.25 M sucrose containing approximately 8 mg protein per ml, 1.33 M urea, pH 7.4, was treated a 0-4° for the indicated time. The ATPase activity was determined by method I and expressed in mM min^{-1} ATP reacted at 1 mg HMP protein per ml. The figures in parenthesis are reported by Racker (67) for ATPase activity (determined by method I with a slight modification) in the sub-mitochondrial particles prepared by sonic treatment.

In addition to the above particulate ATPase, the only other form of HMP ATPase investigated was the ATPase activity in an acetone powder preparation. As mentioned before, the activity was found to be decreased. In addition, the activity largely retained its oligomycin sensitivity until after sonication, then the remaining activity was largely oligomycin insensitive. This is expected if solublization was achieved.

B. Properties and Preparation of the Soluble ATPase

The original technique for isolation of the ATPase employed use of the Nossal shaker. Selwyn and Chappell reported the solublization of ATPase from acetone powders of HMP and beef heart mitochondria. Solublization from acetone powders of beef heart mitochondria was

early employed to prepare "soluble" ATPase (43). In this laboratory, this ATPase was largely sedimentable. The exact technique that Selwyn and Chappell used is not known. After several techniques were investigated, Nossalation of the acetone powder was found to be valuble. This technique solublized much more ATPase than other techniques of agitation examined. HMP acetone powder was found to be best for starting material. Nossalation of SHMP yielded only one tenth the solublized activity as the acetone powders.

The medium for the nossalation procedure was also found to be critical. Employed in some cases was EDTA at one or two mM. High buffer concentrations repressed solublization of activity. A pH around seven was found to be satisfactory. The exact technique employed is described in the Materials and Methods. By use of repeated extractions, it was found that up to 40% of the activity could be solubilized. In addition, this preparation did yield purification by ammonium sulfate fractionation. Some samples were examined for reconstitutive activity in the Nossal particle (NP) system and were found to be active.

The drawback to the Nossal technique for solubilization is that it was limited in the amount of material that could be easily processed. At this point, other techniques were again examined for solubilization. It was found that sonic oscillation could be used to give a high degree of solubilization with relatively large amounts of starting material.

As an added benefit, the specific activity of the crude extracted ATPase was about four fold that obtained by the nossalation technique.

To arrive at optimal conditions for solubilization of the sonic oscillation technique, the time course of solubilization was determined. The results are indicated in Figure 51. This experiment aided design of standard procedure for isolation of the ATPase that is included in the Materials and Methods.

The subsequent purification steps finally adopted employ only ammonium sulfate fractionation. Some other purification steps were attempted with concentrated crude extract. The only step of consequence was heat treatment at 60° for two minutes in the presence of four mM ATP at neutral pH. This step gave significant purification. The results showed another interesting observation. Penefsky et al., using a similar step with soluble nossal extract of mitochondria, observed a 2-3 fold increase in total activity with heat treatment. The explanation later developed for this observation (63) was that the ATPase was extracted in a "masked" form and heat treatment "unmasked" the soluble ATPase. Since the crude extract from HMP used here was not activated, it was not "unmasked". This argument agrees with the results of the trypsin digestion experiments which led to the same conclusion.

The properties of the soluble, partially purified (by Racker's criteria), ATPase are extremely similar if not identical to those of

the soluble ATPase isolated by Racker's group. The activity was truly soluble and activatable by 0.5 mM dinitrophenol (DNP). The activation was not always 50% as indicated by Pullman (64). The effect of temperature on the stability of the activity is indicated in Table 18. It was found that salt (KCl) greatly destabilized the ATPase towards cold inactivation. This is indicated in Table 19. Time course of the same information is given in Figure 52.

Table 18. The effect of temperature on the stability of soluble ATPase from HMP.*

Treatment of samples	% of initial activity remaining
3.0 hours at 30°	97
0.5 hour at 0° then 2.5 hours at 30°	76
1.0 hour at 0° then 2.0 hours at 30°	44
1.5 hour at 0° then 1.5 hours at 30°	35
2.0 hour at 0° then 1.0 hours at 30°	41
3.0 hours at 0°	44

*The ATPase activity was determined by Method I in the presence of 0.5 mM DNP. The initial activity was 0.3 mM x min^{-1} at 1 mg protein per ml.

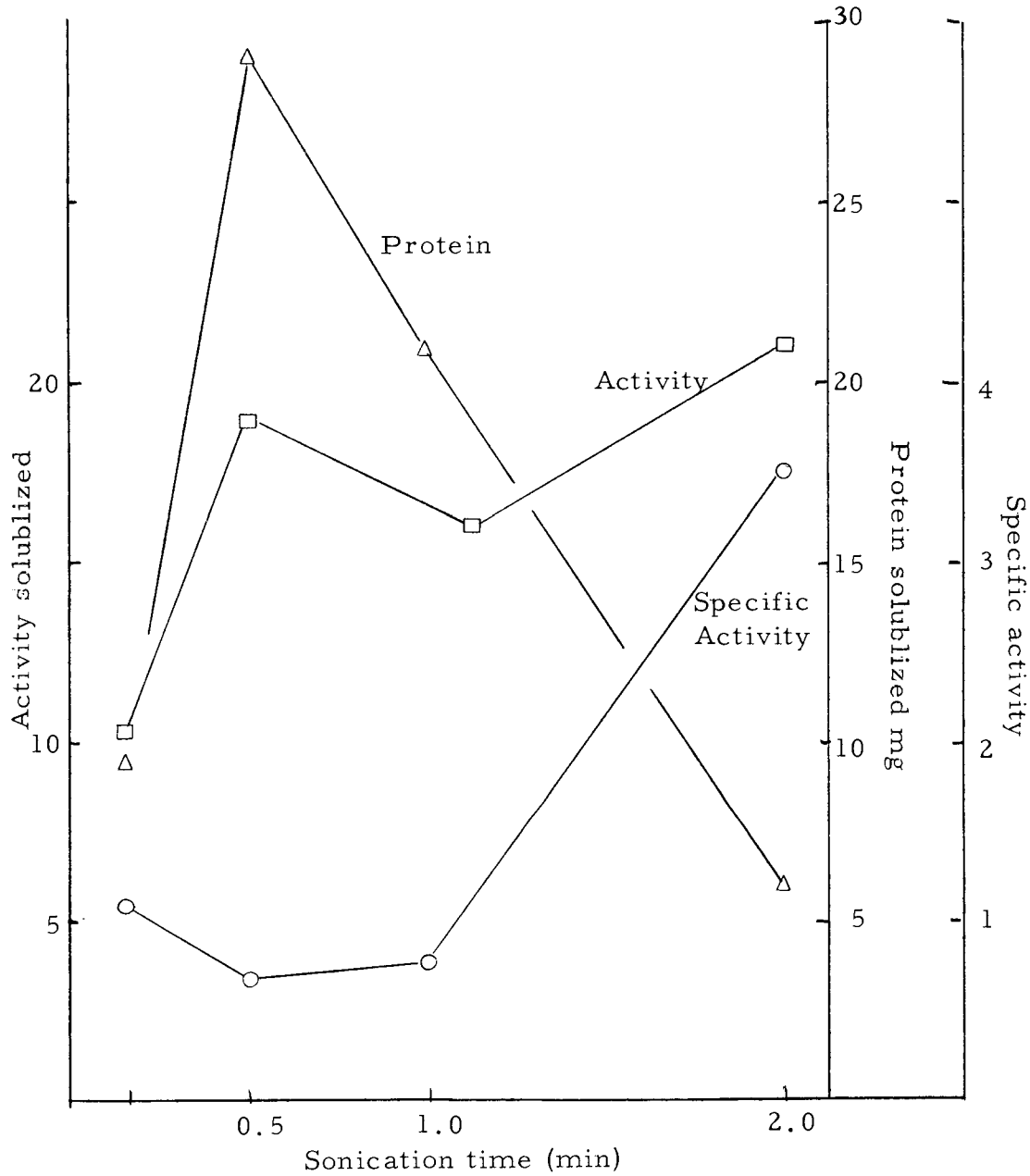
Table 19. The effect of salt on the stability of soluble ATPase from HMP.*

Treatment	% of activity remaining after 2 hours		$t_{30\%}$ (minutes)	
	-DNP	+DNP	-DNP	+DNP
30°	80	65	220	170
30°, 0.26 M KCl	60	55	160	145
0°	26	22	103	(53)
0°, 0.26 M KCl	0	0	8	7

*The ATPase activity was determined by Method I. The initial activity was 0.31 and 0.21 mM x min^{-1} at 1 mg protein per ml in the presence and absence of 0.5 mM DNP, respectively. "$t_{30\%}$" is the time required for 30% diminution of the initial activity.

Figure 51. Effect of sonication time on yield and specific activity of soluble ATPase from HMP acetone powder.

One gram of acetone powder was suspended in 20 ml of water at room temperature. This was divided into 4 fractions. To each fraction was added 2 ml of glass beads (Superbrite #091). Each sample was sonicated for a different length of time with the Branson Sonifier. The power output was about 6.5 amps and the samples were maintained at about room temperature by periodic sonication with cooling between. The four samples were then centrifuged in a room temperature Spinco and the supernatant used for the protein and ATPase assays. Assayed by method II.


Protein
Activity
Specific
Activity
Activity solublized
Protein solublized mg
Specific activity
Sonication time (min)
0.5
1.0
2.0
5
10
20
30
25
15
1
2
3
4

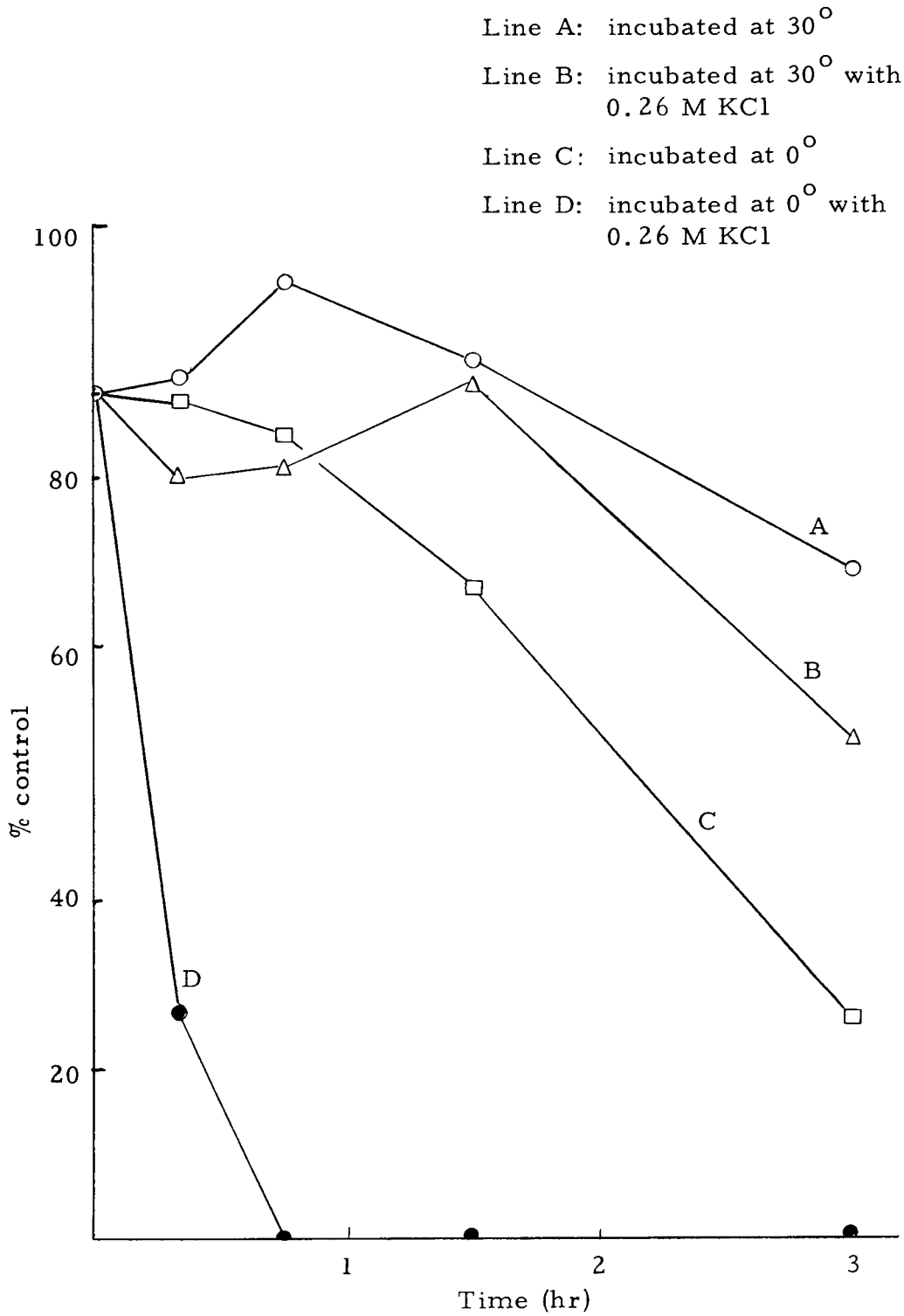


Figure 52. Cold inactivation of the soluble ATPase from HMP.

C. Reconstitution of Oligomycin Sensitivity

As previously noted, the soluble enzyme is insensitive to inhibition by oligomycin, but the HMP ATPase is oligomycin sensitive. The following experiment was designed to examine the binding between the soluble ATPase and the ATPase deficient USHMP (Table 20). This experiment demonstrates that physical binding accompanies restoration of the oligomycin sensitivity. It is to be noted that the binding is dependent on the presence of $MgCl_2$.

Table 20. The effect of oligomycin on ATPase activity and the reincorporation of soluble ATPase into HMP.*

Sample (final volume - ml)	ATPase activity
	mM x min^{-1}
1. HMP, 0.5 mg in 0.25 M sucrose	0.26
2. (1) + 3 μg oligomycin	0.036
3. Urea-treated HMP, 0.5 mg	0.026
4. (3) + 3 μg oligomycin	0.026
5. Soluble ATPase (from HMP) 0.1 mg	0.25
6. (5) + 6 μg oligomycin	0.27
7. (5) + (3)	0.135
8. (7) + 1 μg oligomycin	0.061
9. Pellet from centrifugation of (3)	0.164
10. (9) - ATPase in incubation	0.018
11. (9) - $MgCl_2$ in incubation	0.033
12. (9) + 5 μg oligomycin	0.041

*The ATPase activity was determined by Method II in the absence of DNP. The urea-treated HMP was made by incubating HMP in 0.25 M sucrose and 3.3 M urea at 0° for 5 minutes. The mixture was then centrifuged at 140,000 x g for 20 minutes at 0°, and the pellet was dispersed in 0.25 M sucrose. In the reincorporation sample (No. 9), a mixture of urea-treated HMP and soluble ATPase in the presence of 12 mM $MgCl_2$ was incubated at 30° for 10 minutes. The pellet from centrifugation at 0° was washed once with 0.25 M sucrose and finally suspended in sucrose. Water instead of soluble ATPase was used in the control (No. 10), and no $MgCl_2$ was present in incubation for No. 11.

D. Reconstitution of Oxidative Phosphorylation

It has been noted that the soluble ATPase from HMP behaves like Racker's ATPase. The question of identity could be solved by using the ATPase of HMP to restore ATP synthesis in ATPase deficient Nossal particles (NP). This avenue is explored here.

The NP were prepared as outlined in Materials and Methods. The higher residual P/O presented a real difficulty which could be partially solved only by storing the NP for several days. The stability of the residual P/O observed was also greater than indicated by Pullman. The storage also lowered the response of the particles to the ATPase. The particles after reconstitution were assayed in a method to that described by Penefsky (60) (see Materials and Methods). The experiments were of two general types. The ATPase was incubated with the NP and an aliquot then transferred to the Warburg vessels for assay, or after incubation, the samples were centrifuged, and the particles resuspended and an aliquot of this mixture assayed.

The attempts at reconstituting oxidative phosphorylation started when the nossal shaker solubilized ATPase was available. However, this preparation was not satisfactory. This is indicated in Table 21. As can be seen, the P/O of the preparation rose at first with added ATPase but higher concentrations would uncouple the preparation, hence obscuring any further increases due to added ATPase. When

the more highly purified preparation from the sonic extraction technique was available, data was obtained which is presented in part in Table 22. In this case, the P/O steadily increased but did not increase further beyond the last line of Table 22 when higher amounts of ATPase were added.

Table 21. Effect of ATPase on P/O of Nossal particles.*

Relative ratio ATPase activity/mg NP	μatoms O uptake	μatoms Pi uptake	P/O
0.0	3.5	0.6	0.18
0.5	5.6	1.4	0.25
1.0	6.5	1.8	0.28
1.5	6.0	1.4	0.23
2.0	6.1	1.0	0.16
3.4	7.2	0.7	0.10

*Reconstitution was by Method I. The ATPase was a crude extract from Method I of preparing the ATPase. The column "relative ratio ATPase activity/mg NP" simply states that the ATPase used in this experiment was not assayed. Other preparations prepared in the same manner would give roughly the same ratio of ATPase activity (in units, assayed by Method II)/mg NP.

Table 22. Effect of ATPase on P/O of Nossal particles.*

Units ATPase/mg NP	μations O uptake	μatoms Pi uptake	P/O
0.0	6.1	1.8	0.29
0.36	5.6	1.7	0.30
0.72	6.4	2.4	0.37
2.2	4.6	2.7	0.59

*ATPase measured by Method II. This ATPase had a specific activity of about 25 units/mg. Reconstitution in this experiment was by Method I.

Since the reconstitution was supposedly a physical process, the effect of centrifuging the incubation mixture and assaying the resultant

particle was examined. This data is presented in Table 23. The first experiment was performed with a sample of ATPase prepared by the Nossal technique. The effect is rather dramatic. The second experiment was run with a preparation obtained through the sonic oscillation extraction. Note that the residual P/O values were rather different. The preparation used in the first experiment was the only preparation obtained with a really suitable residual activity. The usual preparations had values closer to that seen in the second experiment.

Table 23. Binding of ATPase to Nossal Particles and stimulation of P/O.*

	Units ATPase/mg NP	μatoms O uptake	μatoms Pi uptake	P/O
(1)	0.0	6.1	0.48	0.06
	2.2	5.9	1.8	0.30
(2)	0.0	6.5	1.2	0.19
	4.0	5.0	2.6	0.51

*ATPase assayed by Method II. Reconstitution by Method II. Experiment (1) was performed with a sample of ATPase derived from preparation Method I which had been purified to a specific activity of 10. Experiment (2) used a sample of ATPase prepared by Method II of specific activity 20. This assay was carried out in the presence of 3 mg Bovine serum albumin, crystalline from Sigma.

Bovine serum albumin has been used classically to protect mitochondria and sub-mitochondrial particles against many uncoupling substances such as fatty acids. BSA acts through binding the undesirable compounds. In fact, mitochondria uncoupled by pentachlorophenol

can be recoupled through incubation with BSA (88). Since the reconstitution with ATPase was accomplished only with some difficulty, the effect of BSA on the reconstitution was examined. Preliminary experiments showed that a consistent BSA effect is found only when BSA is included in the assay mixture. Table 24 indicates the sort of data obtained. A constant but minor stimulation of the residual P/O was seen. This is depicted in the first and third lines of Table 24. An effect on the reconstituted system was irregular. Table 24 indicates the largest effect found. By comparing the second and last lines, it is evident that significant stimulation of phosphorylation has occurred. The possible mechanism for such a stimulation is that the BSA is binding with a substance present which is interferring with the assay. Since these experiments were done, information has come forward which indicates that the preparations examined here were probably deficient in another coupling factor (14). This factor is called F_2.

Although there may be some reservations due to incomplete experimentation , it is believed that the ATPase of HMP is indeed functional. Further, HMP retains at least in part, some aspects of phosphorylating mitochondrial preparations. This is indicated by the identification of the ATPase activity present with a protein factor which is necessary for oxidative phosphorylation. Not only is the ATPase similar to the mitochondrial ATPase, but it can be incorporated into a

system which resumes oxidative ATP synthesis.

Many experiments remain to be done in this system. For example, the mechanism or mode of binding of the ATPase to the mitochondrial membrane remains obscure. The binding may be loosened by urea according to Racker, but the reconstitution requires the presence of $MgCl_2$. The role of the metal ion is as yet unknown.

Table 24. Effect of bovine serum albumin on the reconstitution of oxidative phosphorylation.*

Units ATPase/mg NP	BSA	μatoms O uptake	μatoms Pi uptake	P/O
0	-	8.4	0.9	0.10
1.47	-	8.1	2.4	0.30
0	+	7.7	1.1	0.14
0.74	+	7.5	2.9	0.38
1.47	+	7.1	3.8	0.53

*ATPase measured by assay Method II. Reconstitution by Method I, ATPase prepared by Method II with a specific activity of about 20. Bovine serum albumin, when present, was 3 mg. of a "fatty acid poor" preparation from Pentex Incorp.

Part V. The Alkali Extractable Soluble Factor

The mechanism of the conservation of energy as electrons are passed along the respiratory pathway remains the key problem of oxidative phosphorylation. Currently there are two hypothesis for the conservation of energy in the form of chemical bonds. There is the more conventional and historic chemical intermediate hypothesis and the more recent chemiosmotic coupling hypothesis of P. Mitchell (55).

The approach and experiments developed here do not allow a differentiation between these two mechanisms of energy conservation. The approach has been to examine various degraded forms of mitochondria and ascertain what aspects of energy transfer and energy conservation are present. In addition, mitochondria were degraded and attempts made to reconstruct the original properties of the mitochondrial system. The approach is not new and now may even be considered classical even though it is not common enough to be conventional.

The first system investigated was HMP or HMP like preparations. Table 25 summarizes a series of unsuccessful experiments before the Alkali Extractable Soluble Factor was developed. In addition to the unsuccessful experiments listed in Table 25 and successful ones listed later, E. Racker has asserted in the literature (65) that oxidative phosphorylation in HMP can be stimulated by adding the proper coupling factors. However, no detailed information has been presented.

Among the particles first used in this study for the restoration of oxidative phosphorylation was HMP. When it was found to be not particularly useful, attention was turned to cHMP prepared as is outlined in Materials and Methods. Some small success was found with this particle. Lee and Ernster (47) demonstrated a "restoration" of oxidative phosphorylation in deficient sub-mitochondrial particles

through the addition of small amounts of oligomycin. Example of the interpretation of their results is important. They concluded that oligomycin and the soluble coupling factors may be acting in a similar manner. Fessenden and Racker (14) later answered this assertion with their system. However, Lee and Ernster commented that HMP did not respond to low amounts of oligomycin. It was desirable to confirm Lee and Ernster's observation in the anticipation that the reconstitution observed in HMP would be more firmly established as requiring a coupling factor.

As is seen shortly, Lee and Ernster's observation was not correct. It is here reported as well as elsewhere by others (85), that HMP is indeed stimulated by oligomycin but the stimulation is at a very much lower level of oligomycin than observed by Lee and Ernster. Two cHMPs were examined in detail. Both gave stimulations considerably greater than those reported by ter Welle (85).

Before the oxidative phosphorylation-oligomycin effects were examined, optimal conditions for oxidative phosphorylation were determined. This is indicated in Figure 53. The optimal amount of HMP was used in subsequent experiments. The effect of relatively large amounts of oligomycin on ATPase and oxidative phosphorylation is shown in Figure 54. For oxidative phosphorylation there was a slight stimulation followed by inhibition. For the ATPase only inhibition was observed. The system was then examined at lower oligomycin

levels in an assay that was modified by utilizing lower Mg^{++} levels (2 mM). This was in accordance with later information related by Lee and Ernster (46). The experiment with two different cHMPs is shown in Figure 55. As can be seen, there is a clear and significant stimulation of oxidative phosphorylation.

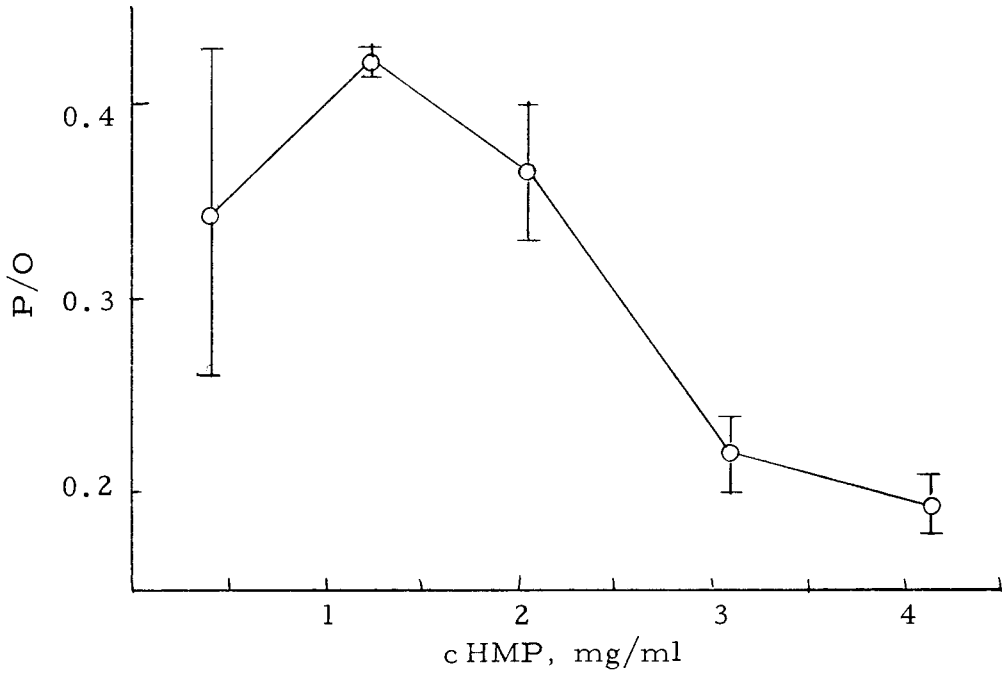
There was now a problem, both oligomycin and AESF stimulated oxidative phosphorylation, were these additive or exclusive stimulations? An experiment was performed to perhaps elucidate the effects. Figure 56 shows a reconstituted system where cHMP was treated with AESF, isolated and assayed. As can be seen, there is an increase in the P/O, and as Table 26 demonstrates, this increase in the P/O with increased AESF is related to an increase in phosphorylation. These same samples were then treated with 63 mμg of oligomycin per mg of cHMP, this is a treatment that would be expected to stimulate oxidative phosphorylation in cHMP alone, and in fact, it does slightly. However, the reconstituted samples, again seen in Figure 56, are inhibited to the same level of oxidative phosphorylation. The action of oligomycin and AESF are not additive and are, in fact, antagonistic. This may be rationalized in terms used by Lee and Ernster, but at this stage of investigation, such philosophizing is unimportant.

Figure 53. P/O versus cHMP concentration during assay.

Samples of cHMP 561 were added directly to chilled Warburg flasks and assayed in usual manner. Reaction was terminated at appropriate times so that the amount of oxygen uptake was about the same for each sample irrespective of amount of enzyme present.

Figure 54. Effect of oligomycin on the ATPase and oxidative phosphorylation of HMP.

Aliquots of cHMP 558 were treated with oligomycin in such a manner that the ethanol concentration in each sample was 5%. During the assay, ethanol concentration was 0.2%. Immediately after mixing the samples, they were diluted for assay so that contact at high ethanol concentrations was minimized. Aliquots of the same mixtures were used for ATPase assay by method II. The control specific activity was 0.7 units.


P/O
0.4
0.3
0.2
1
2
3
4
c HMP, mg/ml

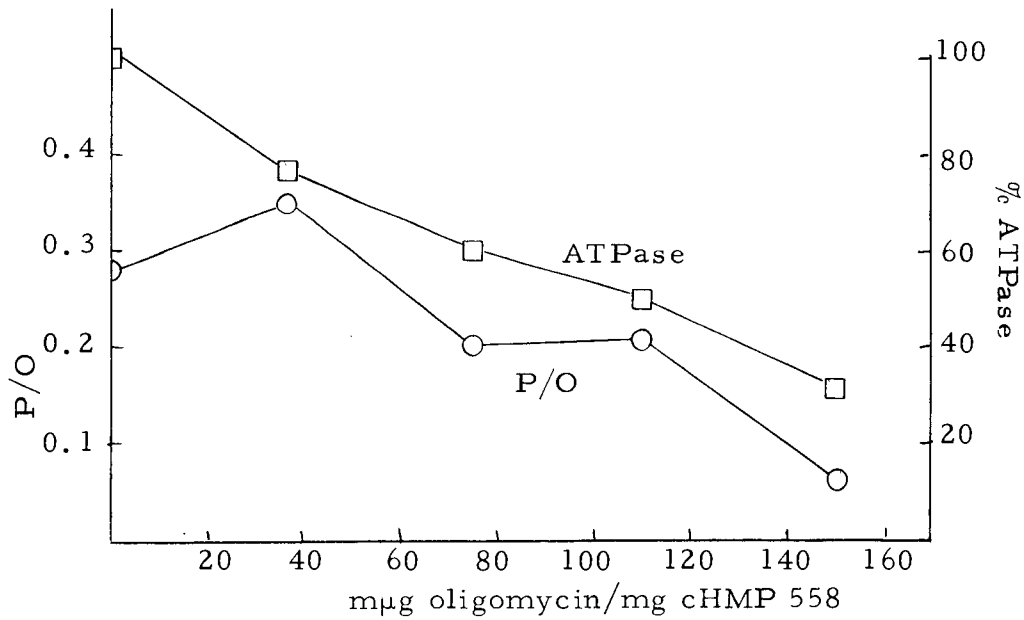
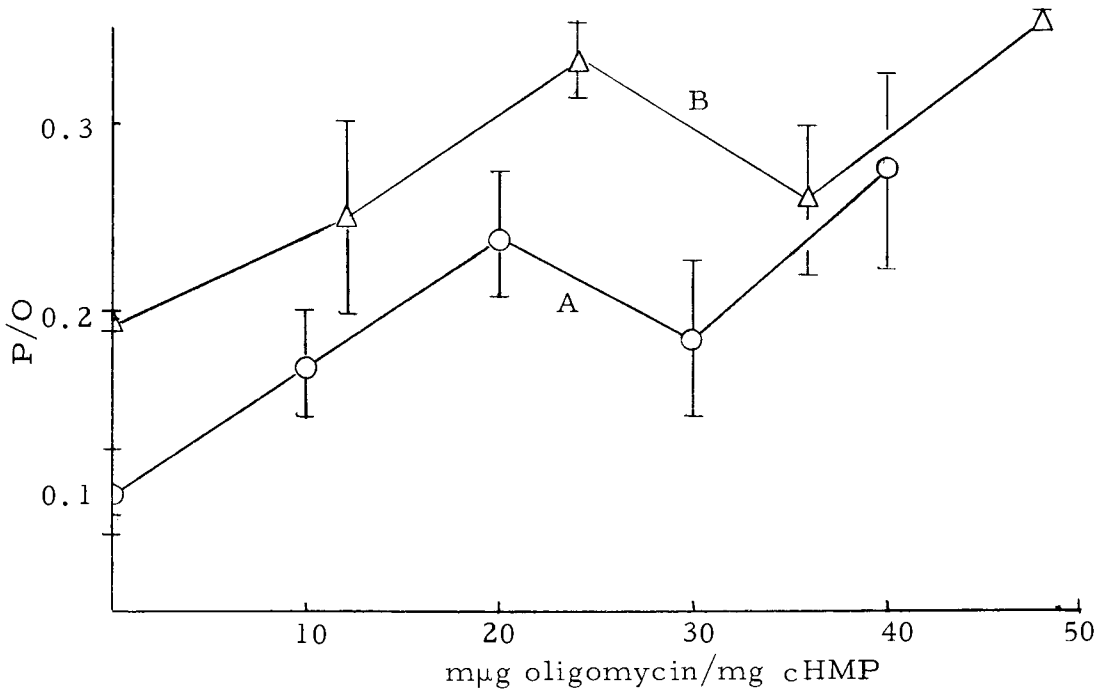

P/O
0.4
0.3
0.2
0.1
100
80
60
40
20
% ATPase
ATPase
P/O
20
40
60
80
100
120
140
160
mμg oligomycin/mg cHMP 558

Figure 55. Effect of low levels of oligomycin on oxidative phosphorylation in cHMP.

Line A represents cHMP 558 and line B cHMP 561. Assays performed as indicated in Figure -54;ethanol lowered to 2% during oligomycin treatment and 0.1% during assay. Different batches of oligomycin used for each assay. The assay media was modified by leaving out bovine serum albumin and lowering the Mg^{++} level to 2 mM.

Figure 56. Reconstitution of oxidative phosphorylation in cHMP by AESF: Effect of oligomycin treatment.

Eleven mg samples of cHMP 561 were incubated with varying amounts of freshly prepared crude AESF (see Materials and Methods) and centrifuged. The pellets were resuspended to 5.2 mg/ml in a medium which was 0.2 M sucrose, 10 mM $MgCl_2$, 6 mM succinate and 30 mM Tris-acetate, pH 7.4. Aliquots were removed for the first assay which was carried out with the assay mixture indicated in Materials and Methods. The results are indicated by line A. The remainder of the samples were treated with oligomycin immediately before assay. Oligomycin was added to give 63 mμg/mg cHMP and an ethanol content of 2%. Samples were then removed and assayed to give result in line B. Five hours elapsed between the assays. Other experiments demonstrated that the reconstituted system does not lose appreciable activity during that period of time.


0.3
0.2
0.1
P/O
B
A
10
20
30
40
50
mμg oligomycin/mg cHMP

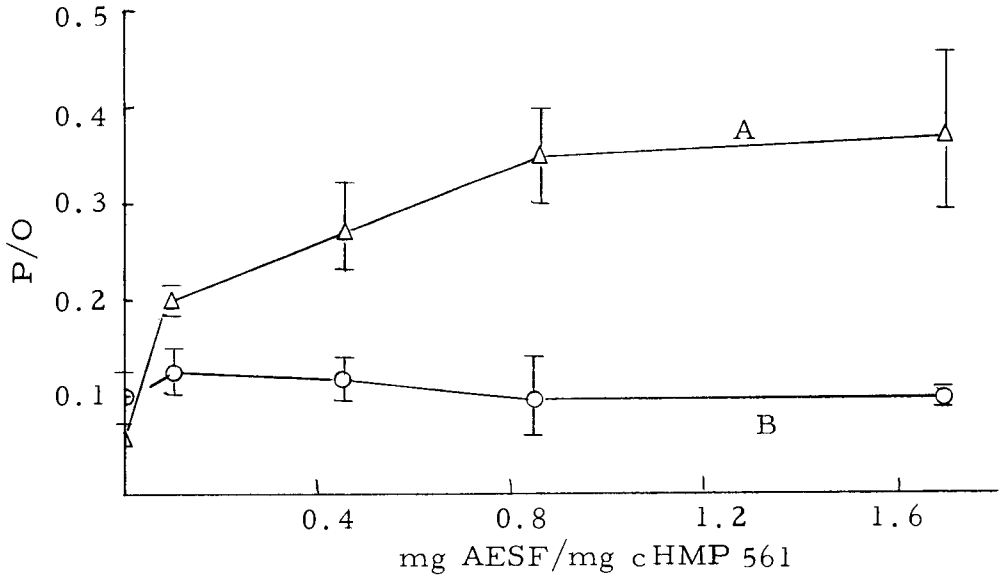

0.5
0.4
0.3
0.2
0.1
P/O
A
B
0.4
0.8
1.2
1.6
mg AESF/mg cHMP 561

Table 25. Unsuccessful attempts at restoring oxidative phosphorylation with HMP or HMP-like sub-mitochondrial particles.

Particle	Soluble factor
1) SHMP 352 SHMP 354	Soluble sonic-EDTA extract of beef heart mitochondria; delta P/O; 0.03, 0.0
2) SHMP 352	(1) purified by ammonium sulfate fractionation, delta P/O with 50-85% saturated fraction: 0.05
3) SHMP 352 SHMP 443	Bovine serum albumin: delta P/O; 0.0, 0.05
4) SHMP 354 SHMP 357	1.0 M KCl extract of beef heart mitochondria delta P/O: 0.0, 0.0
5) SHMP 388 SHMP 295 SHMP 350 SHMP 372	Soluble fraction obtained after grinding beef heart mitochondria by hand in mortor and pestle with sand. delta P/O observed: 0.0, 0.0, 0.05, 0.0
6) mHMP 1-20 (prepared by hand grinding mitochondria in presence of sand and separating sub-mitocondrial particles by differential centrifugation)	as in (5), delta P/O observed: 0.0 for 1-20
7) SHMP 302	Soluble ATPase derived from HMP acetone powders, delta P/O: 0.0
8) bovine serum albumin washed SHMP 399	See (5)
9) SHMP 295 SHMP 350	Soluble extract of beef heart mitochondria after a freeze-thaw cycle. delta P/O: 0.0, 0.0
10) bovine serum albumin washed SHMP 399	Soluble extract of nossalated beef heart mitochondria. delta P/O: 0.0

A preparation was developed that seemed to stimulate oxidative phosphorylation with HMP and cHMP. The source of the material was

the same starting fraction as used for the restoration of the energy-linked reactions. However, realizing the possibility of a multiplicity of coupling factors, the active material was repurified using oxidative phosphorylation as the test assay instead of the energy-linked transhydrogenase. The starting preparation has many resemblances to that of Racker's F_2 (14), but as is developed, this may be only superficial resemblance. A description of the preparation of the crude AESF is detailed in Materials and Methods.

The problem of purification was a complex one. The particle with which the original observation of restoration was made of oxidative phosphorylation (HMP) gave a very limited response. For purposes of purification, a reliable assay was needed. In addition, solution to the problems of stabilization of the soluble factor and particle were necessary. During the course of the purification, it was found that storage and stabilization of the factor and particles was most comfortably accomplished by storage at liquid nitrogen temperatures in a Linde L-25 container.

Recently Lee _et al_. (45) reported on the preparation of a series of sub-mitochondrial particles which had varying degrees of intactness with respect to the energy-linked reactions. There are also many other well known sub-mitochondrial particles which have been prepared which are deficient in oxidative phosphorylation. The preparation of these particles is detailed in Materials and Methods. The

particles were examined for restoration of oxidative phosphorylation with the crude AESF. The particles were additionally examined for the ability to bind the active fraction as well as the requirement of metal ion for the physical reconstitution.

The interaction of the crude factor with cHMP is indicated in Table 26 and Figure 56. Table 27 indicates the effect of not including the metal ion in the incubation mixture. Although this particular example and its repetition do not give striking data, it is nontheless apparent that some metal is necessary for maximal restoration of the activity observed. As can be seen with Figure 56, the restoration was not very great, but this reconstitution was consistent over several preparations. As is seen later, the highly purified preparation may in fact not restore reconstitution with cHMP while retaining great activity with ASP.

Table 26. Stimulation of oxidative phosphorylation in AESF treated cHMP before and after treatment with oligomycin.

mg AESF/mg cHMP 561	before			after oligomycin		
	delta O	delta Pi	P/O	delta O	delta Pi	P/O
0.0	2.89	0.20	0.07	3.90	0.41	0.10
0.10	3.31	0.68	0.21	3.97	0.50	0.13
0.43	2.73	0.76	0.28	3.31	0.38	0.12
0.85	2.84	1.00	0.35	3.92	0.38	0.10
1.7	3.12	1.14	0.37	4.20	0.43	0.10

Table 27. Stimulation of oxidative phosphorylation in cHMP by AESF: metal ion requirement.

Additions	delta O	delta Pi	P/O
	Experiment 1		
Mg^{++}	4.06	0.67	0.16
$Mg^{++} + 1.02 \frac{mg\ AESF}{mg\ cHMP}$	4.16	1.15	0.27
$\frac{1.02\ mg\ AESF}{mg\ cHMP}$	3.88	0.75	0.19
	Experiment 2		
Mg^{++}	3.41	0.71	0.21
$Mg^{++} + \frac{1\ mg\ AESF}{mg\ cHMP}$	3.61	1.21	0.34
$\frac{1\ mg\ AESF}{mg\ cHMP}$	3.31	0.84	0.25

The next particle examined in the survey was that derived from the nossalation of mitochondria. Table 28 and Figure 57 illustrate the lack of reaction of AESF with the nossal particles (NP). Repeated preparation of NP duplicated the lack of reconstitution. This particle was not examined for interaction of AESF for stimulation of the energy-linked reactions. Lee *et al.* (45) did not examine a particle of this type so it is difficult to say how "degraded" the preparation might be. Since the preparation is deficient supposedly only in ATPase and perhaps F_2 (66), the conclusion is that the AESF does not contain the combination of these factors. Although the AESF was never directly assayed for ATPase activity after being purified, it is known that the AESF used for the energy-linked reaction could not contain the ATPase because of the manner of purification.

Table 28. Effect of AESF on Nossal particles: oxidative phosphorylation.

mg AESF / mg NP	delta O	delta Pi	P/O	ave. P/O
0.0	3.87	0.76	0.19	0.17
	4.19	0.67	0.16	
0.12	3.58	0.19	0.05	0.03
	4.02	0.06	0.02	
0.4	4.00	0.63	0.16	0.16
	4.45	0.71	0.16	
0.8	3.85	0.55	0.14	0.15
	4.18	0.65	0.16	
1.6	4.33	0.92	0.21	0.17
	5.12	0.71	0.14	

The next particle examined was that originally used by Linnane and Titchener (50). This system was later further refined by the Green school (18). The essential aspects of Linnane and Titchener's work was found reproducable in this laboratory when their protocol was carefully followed. Some trouble was obtained with this particle as earlier experienced with the Nossal particle, e.g. it was found difficult to prepare particles which were satisfactorily non-phosphorylating. After several attempts, the data found in Table 29 was obtained. Since the coupling factor was bound to the particle, a suitable reconstitution was indicated. In this investigation, the presence and absence of metal ion for binding of the soluble component to the particulate was not investigated. However, since the particle was exposed to the presence of EDTA, it may be presumed that the metal

ion was necessary for the reconstitution found. This data is represented graphically in Figure 58. It is concluded that the AESF does contain some coupling factors that cross react with the factors reported by Green's school. Since these have been carefully characterized (18), it should be possible to decide if AESF does in fact contain such elements as well as others.

Table 29. Stimulation of oxidative phosphorylation in mETPH by AESF.

mg AESF / mg mETPH	delta O	delta Pi	P/O	ave. P/O
0.0	5.62	1.49	0.27	0.26
	5.16	1.28	0.25	
0.12	4.86	1.89	0.39	0.35
	5.87	1.85	0.32	
0.38	4.95	2.44	0.49	0.47
	5.73	2.56	0.45	
0.77	4.94	3.09	0.63	0.60
	5.52	3.13	0.57	
1.54	4.95	3.36	0.68	0.65
	5.67	3.44	0.61	

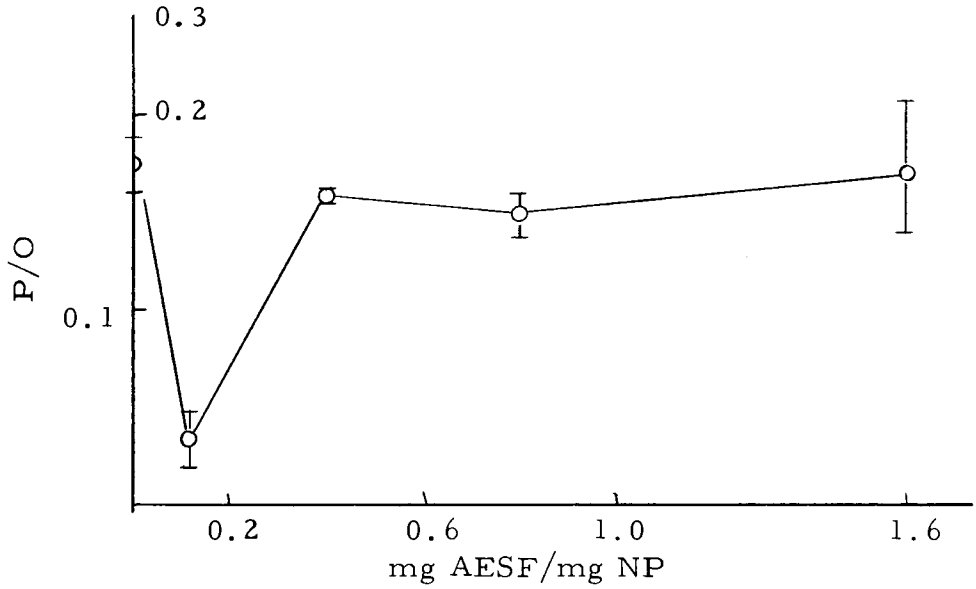
The USP used in the next investigation was one of the partly degraded, non-phosphorylating particles described by Lee et al. (45). Since the particle was exposed to violent conditions in the presence of urea in the cold, a deficiency in ATPase is expected. The particle was not exposed to EDTA, hence is not expected to be metal ion deficient. It is evident that the basic energy conservation apparatus was not destroyed.

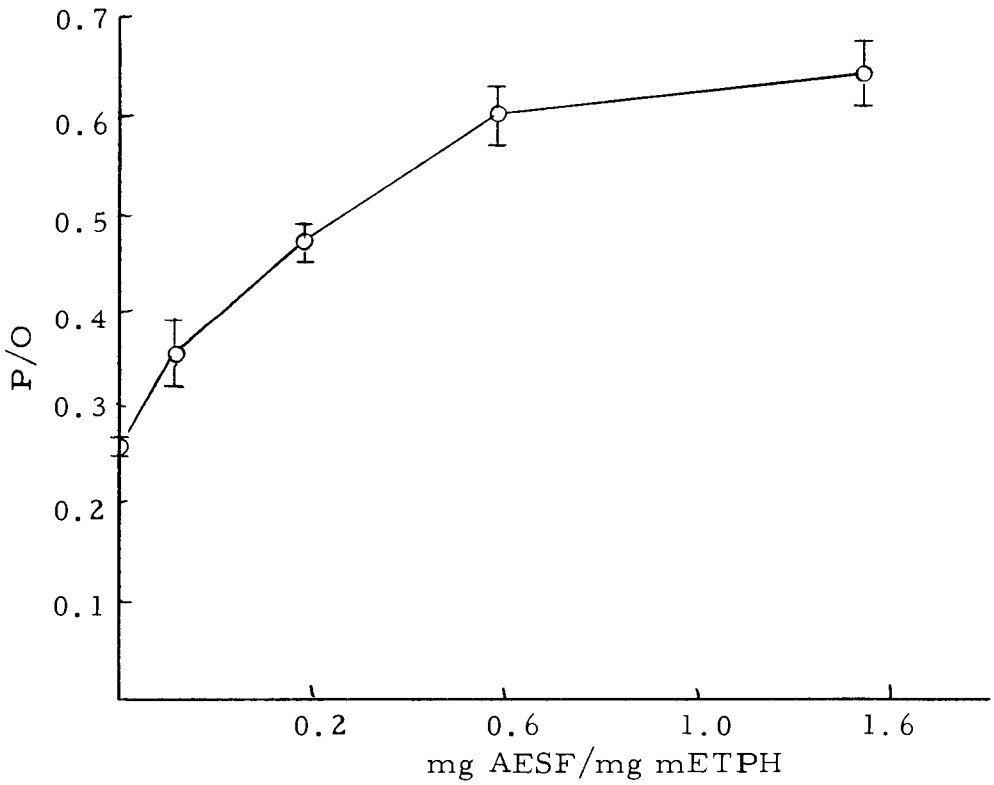
Figure 57. Effect of AESF on Nossal particles: Oxidative phosphorylation.

For this and remaining Figures 58, 59, 60, the incubation mixtures were set up as indicated in Materials and Methods and centrifuged, pellets re-suspended and assayed as usual with succinate as the substrate.

Figure 58. Stimulation of oxidative phosphorylation in mETPH by AESF.

See legend Figure 57.


0.3
0.2
P/O
0.1
0.2
0.6
1.0
1.6
mg AESF/mg NP


0.7
0.6
0.5
0.4
P/O
0.3
0.2
0.1
0.2
0.6
1.0
1.6
mg AESF/mg mETPH

The interaction with AESF is again indicated in Figure 59 and Tables 30 and 31. As is easily evident, a significant restoration of oxidative phosphorylation has been achieved. The coupling factor was again bound to the particle, hence a real reconstitution is evident. This is the first time that phosphorylation has been restored in this type of particle. Besides the Green type of coupling factors, another must be present. Clearly demonstrated is the lack of necessity of Mg^{++} for the restoration of the oxidative phosphorylation. The system does appear to be stable, at least the particle part. The role of this demonstration in the general scheme of phosphorylation factors is obscure. Its characteristics do not seem to fit either the Racker school or the Green school. The Sanadi school of coupling factors remains a possibility, or this may be the demonstration of yet another factor. Certainly the multiplicity of factors is not be be out done by the multiplicity of responding particles.

When considering the kinds of particles they prepared, Lee _et al_. (45) decided that the ammonia sonic particles (ASP) were the most degraded and damaged. In reality, this is simply the most deficient particle that they prepared. Sometime after the experiments reported here were performed, Fessenden and Racker (14) reported an extensive study with a similar particle. The results they reported are not the same as those reported here although similar.

Table 30. Stimulation of oxidative phosphorylation in USP by AESF.

$\frac{\text{mg AESF}}{\text{mg USP}}$	delta O	delta Pi	P/O	ave. P/O
0.0	6.72	1.72	0.26	0.27
	5.52	1.53	0.28	
0.10	4.86	2.83	0.58	0.49
	6.25	2.54	0.41	
0.33	6.31	3.05	0.48	0.46
	8.12	3.57	0.44	
0.66	5.45	3.57	0.66	0.54
	6.60	2.79	0.42	
1.32	6.80	4.12	0.61	0.59
	7.37	4.28	0.58	

Table 31. Stimulation of oxidative phosphorylation in USP by AESF: Metal ion requirement.

additions	delta O	delta Pi	ave. P/O
Mg^{++}	7.50	1.55	0.21
	7.17	1.47	
$Mg^{++} + 1.75 \frac{\text{mgAESF}}{\text{mgUSF}}$	6.99	3.78	0.52
	7.47	3.68	
1.75 mg AESF/mg USP	6.63	3.47	0.50
	7.23	3.44	

The striking response of the ASP to AESF incubation is indicated in Tables 32 and 33. Figure 60 presents the reconstitution in graphic terms. The response is characterized by being rather large and quite linear. This is in contrast to the responses of the other particles examined. The system is not dependent on Mg^{++} for the reconstitution and the method of assay necessitates the reconstitution

being a physical event.

Table 32. Stimulation of oxidative phosphorylation in ASP by AESF.

mg AESF / mg ASP	delta O	delta Pi	P/O	ave. P/O
0.0	5.70	1.74	0.30	0.29
	5.45	1.57	0.29	
0.13	5.41	2.12	0.39	0.35
	5.50	1.74	0.32	
0.43	6.39	3.32	0.52	0.52
	6.33	3.32	0.52	
0.86	5.95	3.42	0.57	0.59
	5.93	3.57	0.60	
1.72	5.21	4.85	0.93	0.87
	6.29	5.17	0.82	

Table 33. Stimulation of oxidative phosphorylation in ASP by AESF: Metal ion requirement.

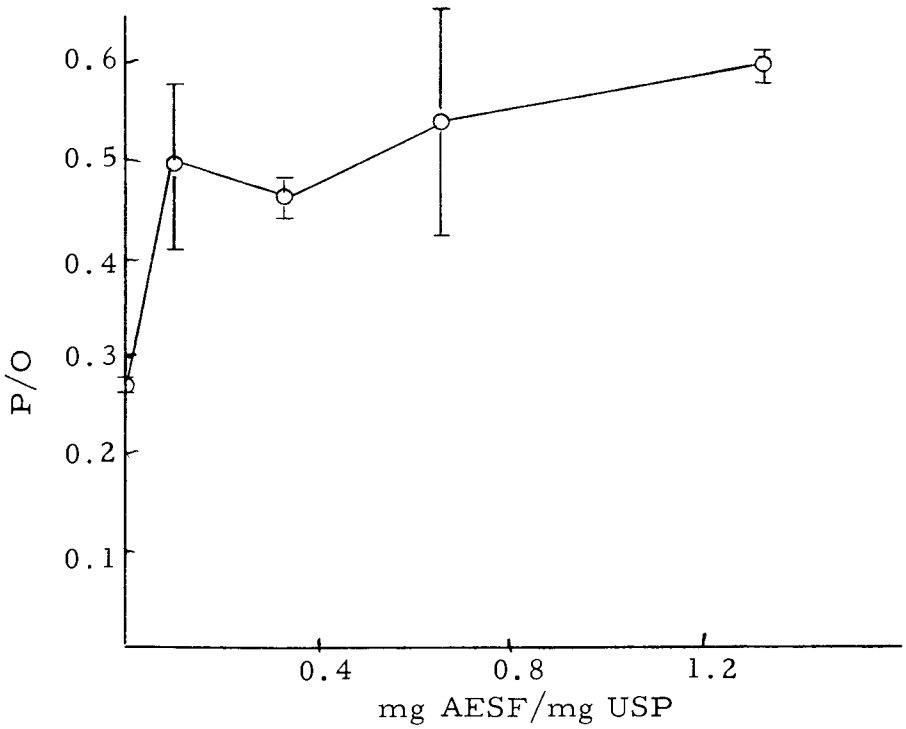
Additions	delta O	delta Pi	ave. P/O
Mg^{++}	4.03	1.05	0.27
	4.03	1.15	
Mg^{++}, 1.2 mg AESF/ mg ASP	4.46	2.16	0.45
	4.98	2.02	
Mg^{++} 3.6 mg AESF/ mg ASP	4.35	3.23	0.71
	5.30	3.57	
1.2 mg AESF/mg ASP	4.94	1.91	0.39
	5.35	2.06	

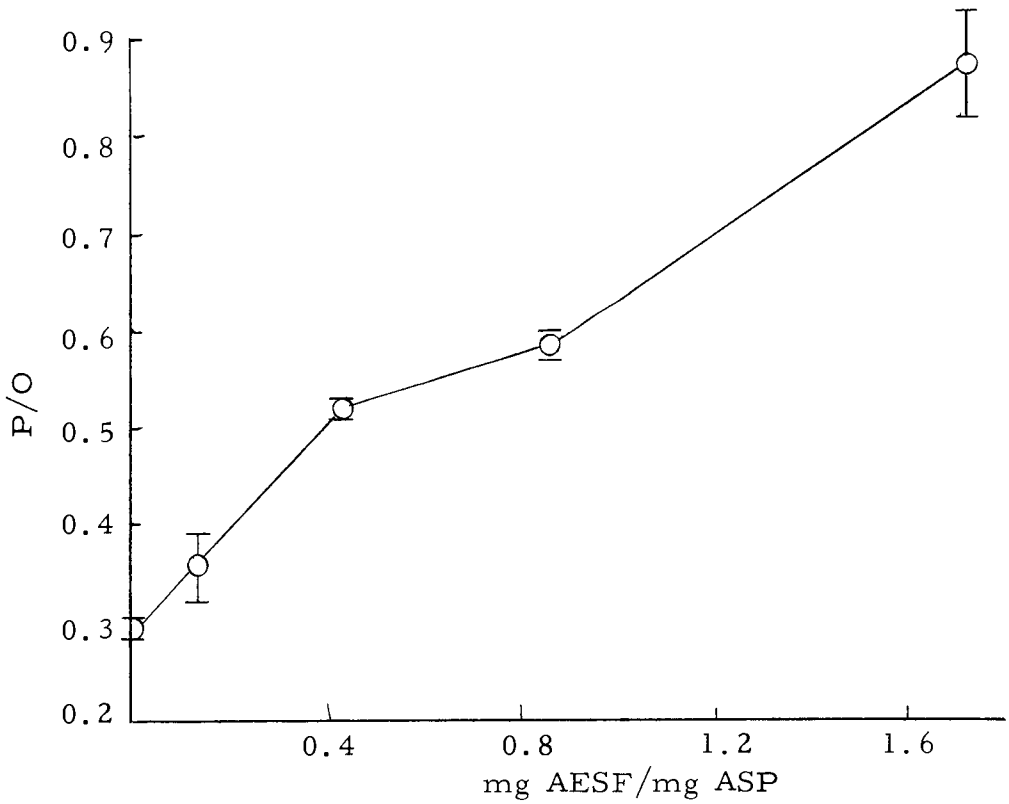
Figure 59. Stimulation of oxidative phosphorylation in USP by AESF.

See legend Figure 57.

Figure 60. Stimulation of oxidative phosphorylation in ASP by AESF.

See legend of Figure 57.


0.6
0.5
0.4
0.3
0.2
0.1
P/O
0.4
0.8
1.2
mg AESF/mg USP


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
P/O
0.4
0.8
1.2
1.6
mg AESF/mg ASP

The system was found to be unstable. After storage overnight, the samples lost 65% of the reconstitution ability. The stability of the reconstituted particles is moderate. Since the magnitude of the response was rather large, and the relation between added factor linear with reconstitution, it was decided to use this particle for the purification study of AESF. In addition, the particle was easily prepared and the factor did seem to be stable under liquid nitrogen conditions.

A. Purification of Alkali Extractable Soluble Factor

The AESF was first prepared and purified using the stimulation of succinate supported transhydrogenase as the assay. It was desired to purify the factor(s) with oxidative phosphorylation as the assay. HMP gave marginal response to AESF and cHMP, the latter although better was not really sufficient. After surveying a series of non-phosphorylating sub-mitochondrial particles, ASP was chosen for the purification assay. As already mentioned, ASP gave responses of large magnitude and the response seemed mostly linear.

As purification progressed, it became quickly evident that AESF was unstable unless stored at liquid nitrogen temperatures, similarly, the magnitude of the ASP response was also sensitive to aging of the ASP. As a result, freshly prepared ASP was used, or a preparation which had been stored in liquid nitrogen for short periods of time. Although the extent of response seemed variable, the specific activity

of the AESF (as defined later) was much more stable and reproducible from preparation to preparation. The purification procedure listed below was quite reproducible.

The extent that a fixed amount of AESF stimulated the P/O of a fixed amount of ASP was chosen as the unit of activity. This was determined from the slope of the titration curve by AESF with a fixed amount of ASP. The activity is expressed as delta P/O at a ratio of one mg of AESF with one mg ASP. The total activity of a given preparation is expressed as the total delta P/O if that preparation were to stimulate one mg of ASP. This is a functional definition since the total delta P/O for any amount of ASP is two.

An attempt was made to see if ion exchange chromatography could be profitably used for the purification of AESF. Crude AESF, as prepared in Material and Methods, was passed over a carboxymethyl cellulose column in a medium with which the column was equilibrated with 50 mM Tris-acetate, pH 7.4, one mM ATP and one mM $MgCl_2$. The effluent was collected and assayed with a sample of the crude AESF. Both samples showed the same specific activity and about the same extent of response. All of the protein applied to the column was recovered from the eluant. There was no net purification and the procedure not pursued further.

The next attempt took advantage of the greater extractability of the active material at high pH. Figure 61 shows the response of an

ASP preparation to crude AESF. A sample of acetone powder of beef heart mitochondria was then treated with a neutral extraction media. The soluble fraction was set aside and the remaining particulate material re-extracted with the usual alkaline extraction medium. Both were then assayed with the ASP preparation previously described, Figure 61. The results are shown in Figure 62. As is evident, the alkaline extract has a higher specific activity (0.22) than the neutral extract (0.07) even though the extent of stimulation is less. A question arises as to whether or not the extracts have the same active material. This might be settled by adding the neutral extract to a sample of ASP containing a saturating amount of the alkaline extract. This experiment is recorded in Table 34. It appears from this experiment as though the factors are not the same since they appear additive. This result implies a multiplicity of coupling factors present in the crude extract. This is perhaps also implied from the variety of particles which respond to AESF.

Table 34. The effect of neutral and alkaline extracts on ASP: oxidative phosphorylation.

Additions	delta O	delta Pi	ave. P/O
None	5.77	0.23	0.02
	4.51	0.0	
2.03 mg NE/mg ASP	4.55	1.26	0.22
	5.22	0.82	
2.03 mg NE + 0.96 mg AE/	5.33	1.66	0.36
mg ASP	5.11	2.04	
0.96 mg AE/mg ASP	4.62	0.97	0.21
	4.95	0.99	

NE, Neutral extract; AE, Alkaline extract.

Figure 61. The effect of crude AESF on ASP.

The AESF used in this experiment and the ASP prepared for this experiment were used in the next experiment (Figure 62). AESF and ASP prepared as outlined in Material and Methods.

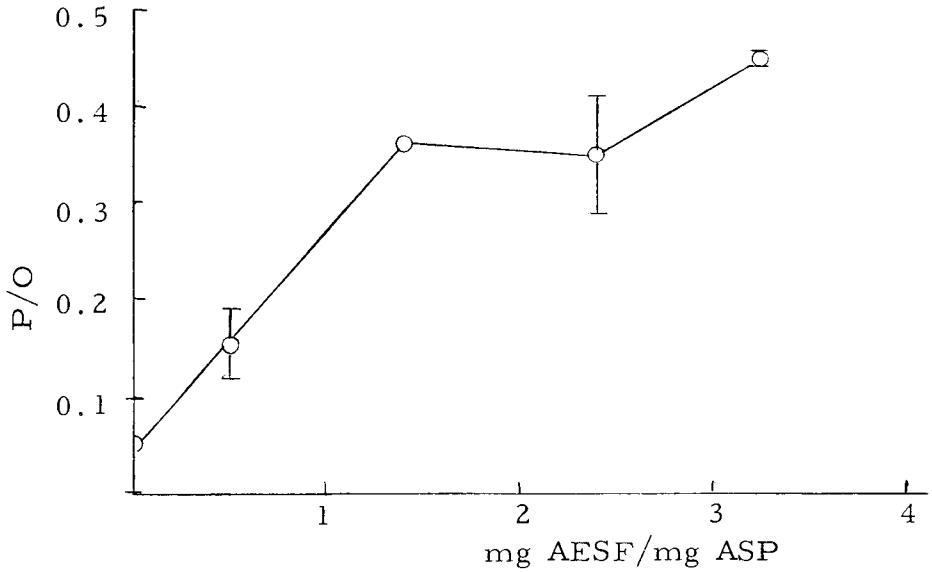
Figure 62. Effect of neutral and alkaline extracts on ASP.

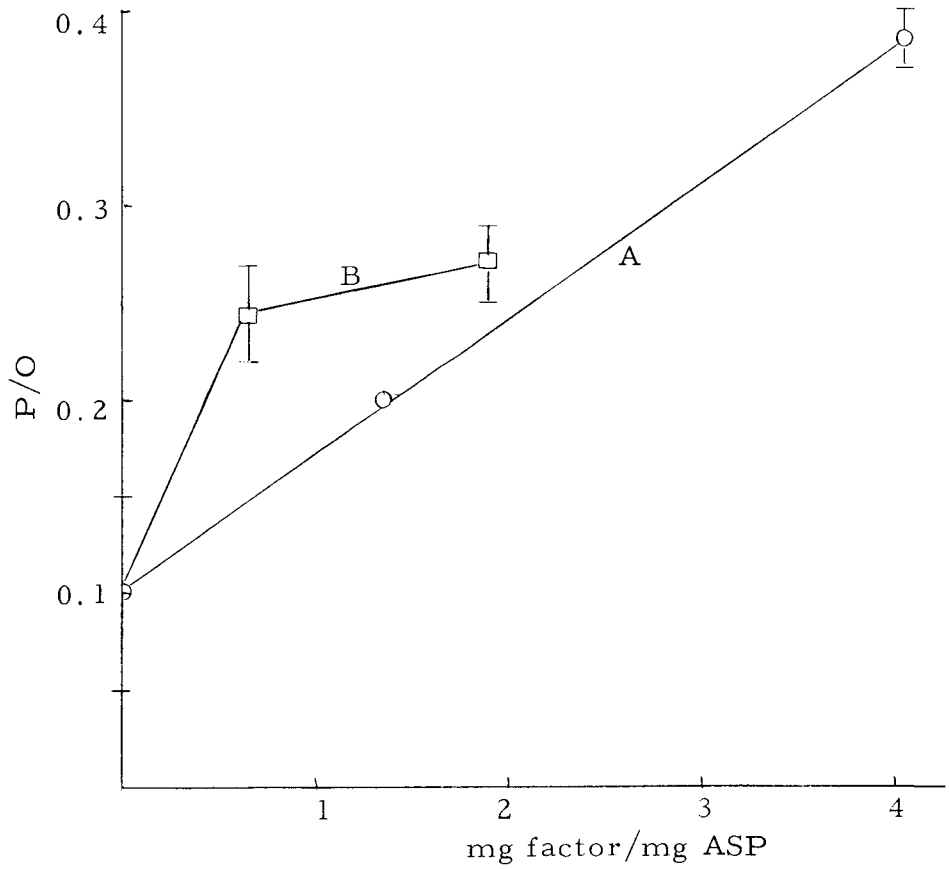
The ASP was same as in Figure 61. Neutral and alkaline extracts prepared as indicated below.

Neutral Extract: One and a half grams of beef heart mitochondria acetone powder (see Material and Methods) was suspended in 25 mls of neutral chilled extraction media. Media: 50 mM Tris-acetate, pH 7.4, 2mM ATP, 12 mM succinate and 1 mM EDTA. After suspension, the mixture was allowed to stand on ice for 10 minutes and then centrifuged in a refrigerated centrifuge for 10 minutes at 12,000 g. The supernatant was carefully decanted and set aside as the neutral extract.

Alkaline Extract: The pellet from the above extraction was resuspended in the AESF extraction media (see Material and Methods) and allowed to stand on ice for 10 minutes and centrifuged at 12,000 g. The yellow supernatant was carefully decanted and adjusted to pH 7.4 with 1 N acetic acid. This was set aside as alkaline extract.

The neutral extract yielded 150 mg of protein and alkaline 70 mgs by biuret assay. The reconstitution incubation medium contained 11 mg ASP and appropriate extract to give ratios found in the figure. Initial slopes of curves were taken to obtain specific activity.


0.5
0.4
0.3
0.2
0.1
P/O
1
2
3
4
mg AESF/mg ASP


0.4
0.3
0.2
0.1
P/O
B
A
1
2
3
4
mg factor/mg ASP

It was observed in the first double extraction experiment that a small quantity of particulate material was not removed during the preparation. To remove doubts, the experiment was repeated and the results are indicated in Figure 63. In this experiment the response of the system was clear. The specific activity of the alkaline extract was 0.42 while the neutral extract had an activity of 0.14. The extent of response was also suitable, here neither extract saturated the particle response. It is further indicated that the response is related to a soluble protein fraction since particulate material was carefully removed from the extracts.

The next purification technique used was absorption of the active fraction onto calcium phosphate gel and elution with 0.3 M phosphate buffer at a neutral pH. The results and protocol of such a purification is indicated in Figure 64. The overall purification achieved was about 40 fold. The yield of units through the procedure was 40%. This purification was repeated several times with larger amounts of acetone powder and gave consistent results. It was found that the purified AESF gave rather different values of yield depending on the method of protein analysis. Table 35 compares three methods of protein analysis on the same preparation of AESF.

As is described below, purified AESF failed to interact strongly with cHMP. Since a multiplicity of coupling factors seems possible, it was decided to repeat the purification with cHMP as test particle.

It was found that the alkaline extract (crude AESF) retained a response to cHMP, even though small, while the neutral extract exhibited no effect. When a double extraction as shown above was attempted (Fig-63), neither extract or the combination of both extracts showed any significant response. The investigation stopped at that point.

Table 35. Determination of protein content of AESF.

Method of Assay	mg/ml found	ratio
TCA turbidometric	1.82	(1.00)
Lowry	2.38	1.25
Biuret	4.65	2.55

The necessity of Mg^{++} for the physical incorporation of coupling proteins onto deficient particles has been often noted. ASP and cHMP are not completely excluded from this class. Indeed, USP is the only particle examined here that absolutely does not require Mg^{++}. The role of the metal is not easy to ascertain and has not been investigated here.

Some investigators have found very important effects on deficient mitochondrial systems by bovine serum albumin (62). Many of these effects involve an explanation requiring the binding of a toxic substance by the serum albumin. The effect of serum albumin was examined on both the reconstitutive step and the assay of the AESF-ASP system. The response of the ASP preparation to crude AESF is shown in Figure 65. Table 36 indicated the effect of one mg of bovine

serum albumin present during the assay of the particle and reconstituted system. It is noted that the albumin give substantially higher P/Os with the reconstituted sample while not affecting the particle. A similar effect was noted with the ATPase-nossal particle system.

Table 36. Effect of bovine serum albumin on the activity of ASP and reconstituted ASP.*

Sample	BSA	delta O	delta Pi	ave. P/O
ASP	-	4.59	0.59	0.14
	-	3.73	0.61	
ASP	+	4.14	0.63	0.12
	+	4.09	0.40	
ASP reconstituted with 2.3 mg AESF/ mg ASP	-	4.09	1.78	0.45
	-	4.70	2.12	
ASP reconstituted with 2.3 mg AESF/ mg ASP	+	3.92	2.33	0.65
	+	4.00	2.77	

*BSA (bovine serum albumin) was added to a final concentration of 2 mg/ml in the assay system. Pentex "Fatty Acid Poor" BSA was used throughout

To eliminate the possibility that the bovine serum albumin is replacing AESF, the effect of serum albumin on the reconstitution was examined. The results are summarized in Table 37. The serum albumin does not greatly affect the reconstitution. The experiment was assayed in the presence of serum albumin as were all assays after this experiment.

Figure 63. Effect of neutral and alkaline extracts on ASP: Elimination of advantageous particulate material.

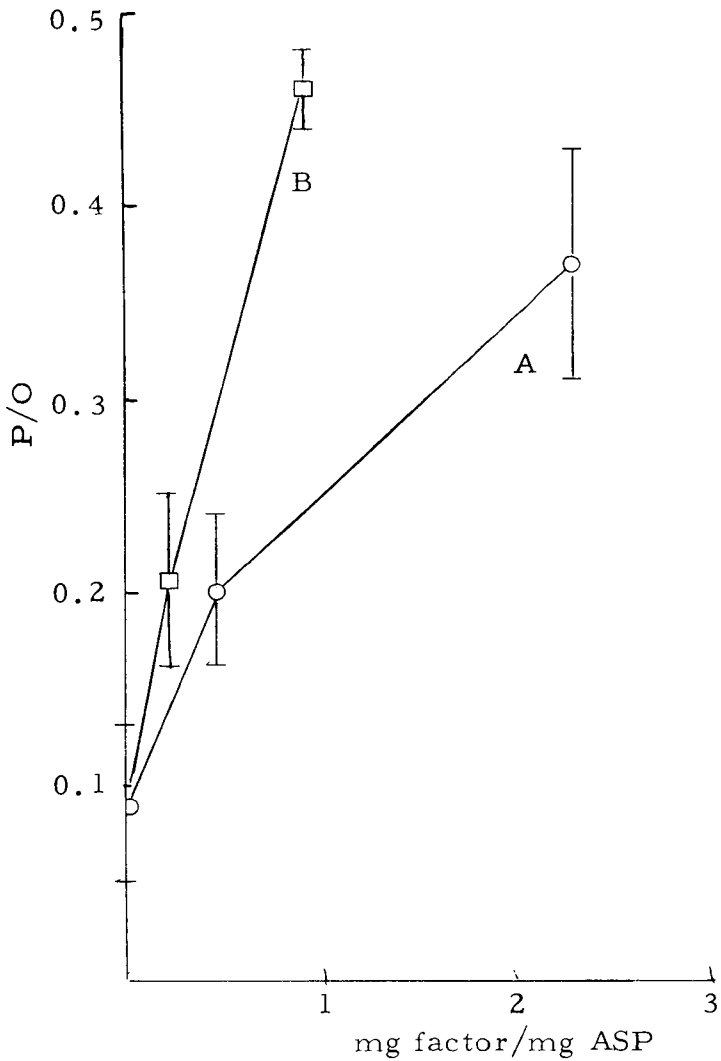
The ASP and acetone powder of beef heart mitochondria was freshly prepared according to Material and Methods. The neutral and alkaline extracts were prepared as described in the legend for Figure 62. Both extracts were centrifuged for 30 minutes at 50,000 rpm in Spinco previous to use in reconstitution with ASP. Initial rates were not used for specific activity, instead a "best line" was visually determined for the response curves.

Figure 64. Effect of purified AESF on ASP.

The ASP was prepared fresh. The AESF was purified by the following procedure.

Purification of AESF: The alkaline extract was prepared as outlined in Figure 62 but at larger scale. To 40 mls of neutralized alkaline extract containing 132 mg protein (biuret assay) was added 146 mg of calcium phosphate gel in 6 ml of water. This gave about 1.1 mg gel/mg protein. The gel was centrifuged after sitting for 5 min. The gel supernatant was removed and later found to have no AESF activity. The gel was suspended in 20 ml of chilled water and centrifuged after 10 minutes. The supernatant was discarded. Finally the gel was resuspended in 15 ml of a solution that was 0.3 M potassium phosphate, pH 7.4, 1 mM ATP and 1 mM EDTA. After standing for 10 minutes, the suspension was centrifuged and the supernatant removed for dialysis.

The sample was dialyzed overnight with several changes of exterior buffer which was 30 mM Tris-acetate pH 7.4, 0.5 mM ATP and 0.5 mM EDTA. The turbid product was centrifuged for 25 minutes in #50 rotor of the Spinco L at 50,000 rpm.


0.5
0.4
0.3
0.2
0.1
P/O
B
A
1
2
3
mg factor/mg ASP

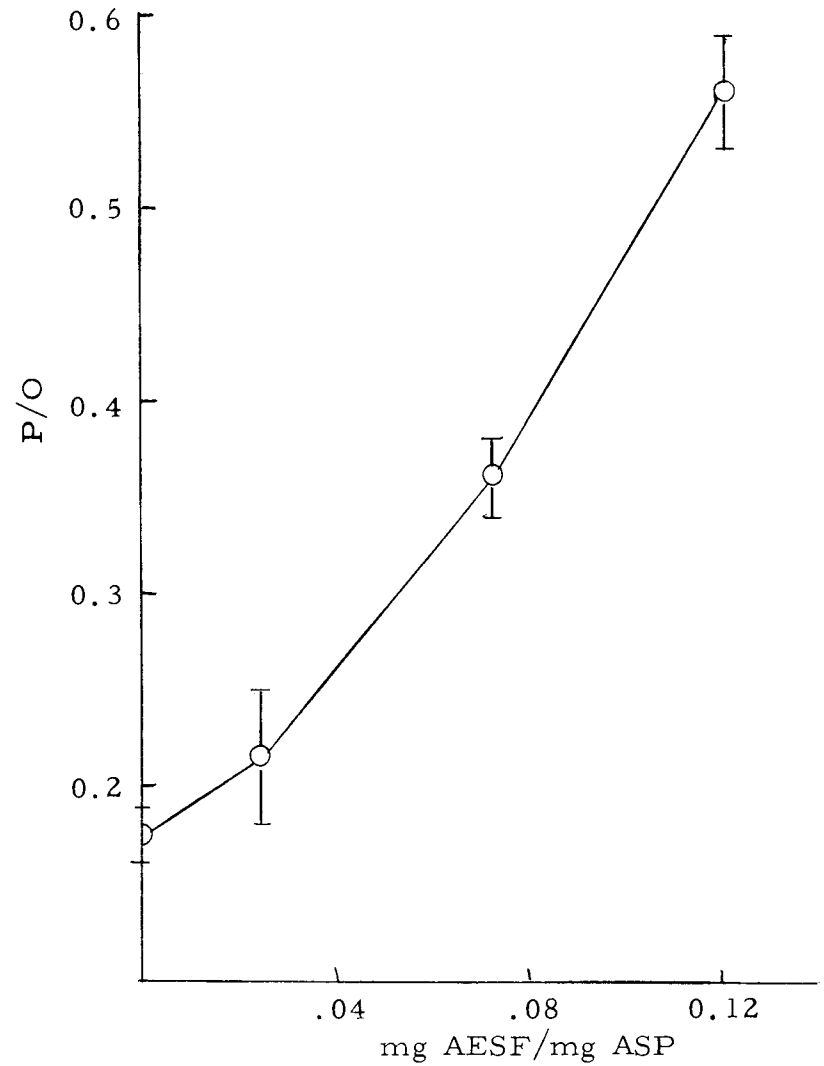

0.6
0.5
0.4
0.3
0.2
P/O
.04
.08
0.12
mg AESF/mg ASP

Table 37. The effect of bovine serum albumin on interaction of ASP and AESF.*

Reconstitution system	delta O	delta Pi	ave. P/O
ASP	4.23	0.52	0.09
	4.10	0.75	
4.4 mg BSA/mg ASP	3.62	0.75	0.12
	4.74	0.13	
4.4 mg AESF/mg ASP	4.45	2.23	0.55
	4.08	2.39	
4.4 mg BSA and 4.4 mg AESF/mg ASP	3.15	2.20	0.63
	4.07	2.29	

*All assays in presence of one mg of BSA.

Following the lines of Jacobs and Sanadi, (28) experiments were designed to ascertain the effect of exogenous cytochrome c on the reconstitutive properties of AESF. Samples of the enzyme were incubated with and without cytochrome c and with and without AESF. The reconstituted system were then assayed with and without cytochrome c, keeping the cytochrome concentration always at the same level. The results are summarized in Table 38. The result, in short, was that there was no detectable effect of cytochrome c on the ASP or reconstituted ASP. Not shown is the lack of effect of cytochrome c on the oxidation rate of ASP.

Since the AESF was now in a somewhat more highly purified state, it was desirable to see if binding of the factor to the particle could be detected. The method chosen was to detect unbound AESF by reincubating ASP in reconstitution systems from which the ASP had

been removed by centrifugation. It was presumed that the factor would be tightly bound. Figure 66 illustrates the results of a double incubation experiment. Sufficient AESF (purified) was present to saturate the ASP present. On reincubation, significant activity remained soluble. From the initial slopes of the response, only 40% of the activity had been removed by a single incubation. This indicates that there is a slow binding of AESF to ASP, or, the equilibrium for reconstituted system is not favorable at these concentrations of particle and soluble factor.

Table 38. Effect of cytochrome c on assay and reconstitution of ASP and AESF.

System	P/O (average)	
	no cytochrome c*	0.4 μ moles* cytochrome c / g ASP
ASP	0.17	0.08
ASP preincubated with cytochrome c**	0.13	0.09
Reconstituted ASP	0.47	0.49
Reconstituted ASP preincubated with cytochrome c**	0.39	0.51

*Present during assay of oxidative phosphorylation. ASP was reconstituted with 2.3 mg crude AESF per mg ASP.

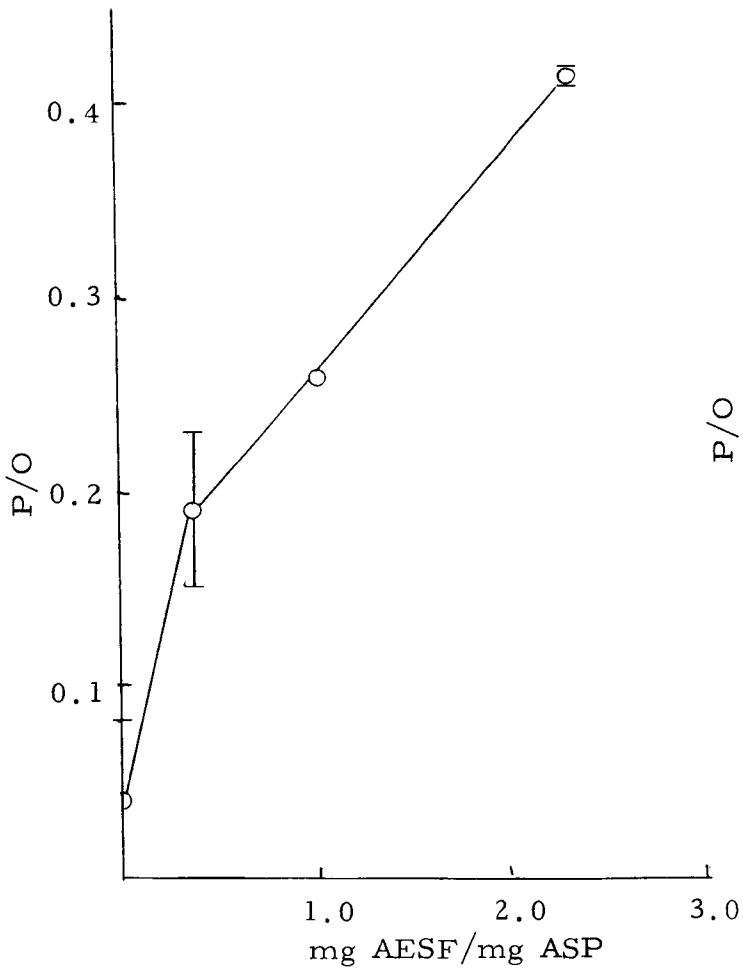
**Cytochrome c was added to reconstitution incubation mixtures to a concentration of 0.4 μ moles/g ASP.

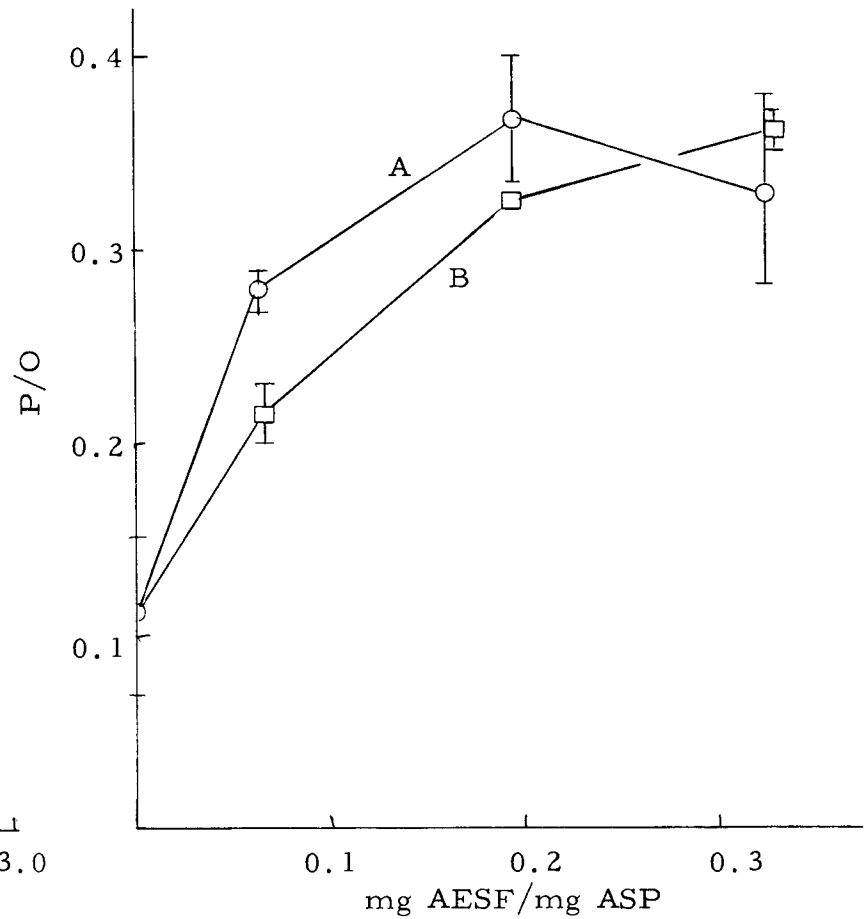
Figure 65. Response of ASP to crude AESF.

ASP and AESF prepared fresh according to Materials and Methods.

Figure 66. Repeated incubation of ASP with a sample of purified AESF.

Purified AESF obtained as described in Figure 64. ASP was freshly prepared. Appropriate amounts of AESF were added to centrifugates with 7.1 mg ASP. The incubation samples were centrifuged. The supernatant fluids were retained and stored on ice until the pellets had been resuspended and assayed as usual. Then 7.1 mg ASP was added again to the same incubation samples and again sedimented and pellets assayed to give the second incubation data. Line A, first incubation, line B, second incubation.


0.4
0.3
0.2
0.1
P/O
1.0
2.0
3.0
mg AESF/mg ASP


0.4
0.3
0.2
0.1
P/O
A
B
0.1
0.2
0.3
mg AESF/mg ASP

It was desirable to see if the AESF purified with ASP as the test particle retains its broad specificity, expecially towards cHMP. Figure 67 indicates the activity of a purified AESF preparation towards ASP. Figure 68 indicates the activity of the same AESF with cHMP. There is little stimulation of oxidative phosphorylation. Again the scapegoat of coupling factor multiplicity may be cited, or, cHMP may simply be inactive.

B. The Energy-linked Reactions of the Keilin-Hartree Heart Muscle Preparation

Recently Lee, Azzone and Ernster (45) showed that non-phosphorylating preparations still retain the energy conservation apparatus without catalyzing the synthesis of ATP or allowing the reversal of oxidative phosphorylation. The work reported here was at exploratory stages at that time. After the report by Lee *et al.*, Slater (74), and Kettman (33) reported the retention of the energy-linked reactions in the heart muscle preparation. Shortly thereafter, Haas (20) published preliminary reports of his experiments. More extensive data has been reported by van Dam and ter Welle (85). About the same time, Gawron's group also reported similar findings (17), in addition, they reported the reversal of oxidative phosphorylation in HMP. This last finding was contrary to the Haas report and the following data. The detailed results from Gawron's group remain unpublished and

cannot be analyzed here. Part of the data examined below was the subject of a preliminary note as well as previously examined information concerning AESF.

Of the many energy-linked reactions (7), only two were examined in HMP, however, they were examined in several ways. The "energy linked transhydrogenase" was assayed using both energy derived from the oxidation of succinate and energy from the hydrolysis of ATP. Additional experiments were attempted using energy derived from oxidation through the terminal region of the respiratory chain. This was attempted with the TMPD-ascorbate system.

The reversal of oxidative phosphorylation was studied by the ATP driven reduction of NAD by succinate. Besides the use of ATP as the energy source, attempts were made to utilize the TMPD-ascorbate system to generate energy from the terminal region of the respiratory chain to drive the succinate reduction of NAD.

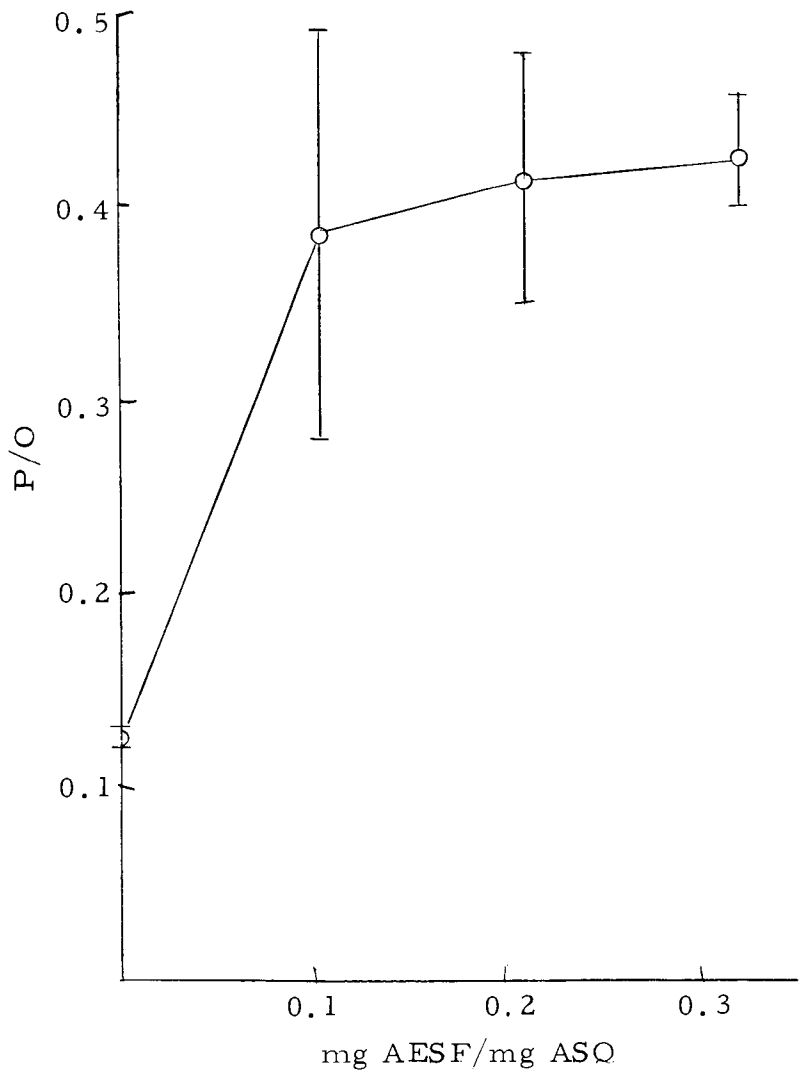
For brevity, only the experiments involving the succinate reduction of NAD driven by ATP and the ATP and succinate oxidation driven transhydrogenase are reported. Assays for these reactions are described in the Materials and Methods section. The results generally varied with the kind of HMP examined. The cHMP usually had higher activities than the HMP as normally prepared.

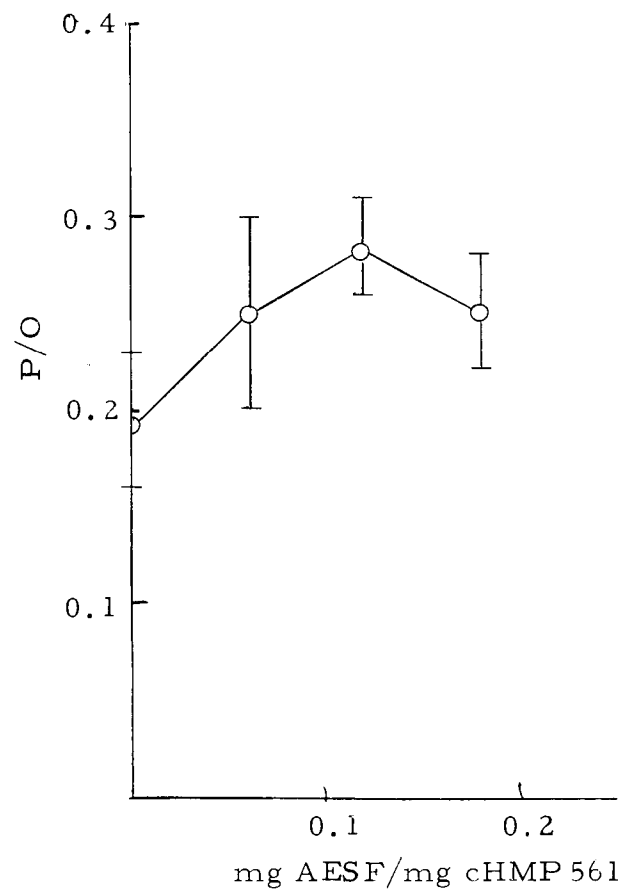
Figure 67. Response of ASP with purified AESF.

The same preparation of purified AESF as used in Figure 68 was first assayed with a recent preparation of ASP to ascertain activity.

Figure 68. Response of cHMP 561 with purified AESF.

Purified AESF which was known to be active (Figure 67) was reconstituted with freshly thawed cHMP 561.


0.5
0.4
0.3
0.2
0.1
P/O
0.1
0.2
0.3
mg AESF/mg ASO


0.4
0.3
0.2
0.1
P/O
0.1
0.2
mg AESF/mg cHMP 561

C. ATP Driven Transhydrogenase

The results of the investigations with ATP driven transhydrogenase can be easily summarized. The three sub-mitochondrial particles studied and their characteristic activities are listed in Table 39. The reaction was dependent on NAD and a NADH regenerating system. Oligomycin was found to inhibit the reaction more than 90%. The reaction was additionally sensitive to triiodothyronine. As others have found (7), the reaction was best demonstrated by preincubation with ATP. Magnesium ions were required for the reaction. The reaction was insensitive to anaerobsis.

Table 39. The ATP driven transhydrogenase activity of sub-mitochondrial particles.*

Particle	$A_{340\ m\mu}$ min./g
HMP 447	32
HMP 443	40
ASP	47
ETPH	60

*Assayed as outlined in Materials and Methods. The rates are those which are oligomycin sensitive. ETPH was prepared by the method of Smith and Hansen (66).

When AESF was added as a purified preparation, the reaction was lightly affected, usually inhibited.

D. ATP Supported Reduction of NAD by Succinate

Three sub-mitochondrial particles were examined for this reaction. As reported in the literature, neither ASP nor cHMP (or HMP) exhibited any activity. A phosphorylating particle, ETPH, was examined to establish the validity of the assay technique. With ETPH, the activity behaved as has been described in the literature. The reaction was dependent on Mg^{++}, ATP, succinate and NAD. Rates of 220 delta $A_{340\ m\mu}$ per min. at 1 g ETPH/ ml in the assay media were obtained. At 177 mμg oligomycin per mg of ETPH, the reaction was inhibited 88%. In the course of experiments with HMP, a stimulation of the succinate-NAD reaction by AESF was observed when energy was derived or supported by aerobic TMPD-ascorbate. However, the reaction was not repeatedly observed and some interaction between AESF and the TMPD-ascorbate system has been observed. It was not possible to cleanly separate the effects. Slater (74) claims the demonstration of the reaction in this manner, this is possible, but AESF stimulation of the reaction is not clearly demonstrable.

E. Succinate Oxidation Supported Transhydrogenase

This has been the most extensively studied reaction. Table 40 lists several preparations, their activities, and oligomycin stimulation. The reaction is dependent on NAD, NADP, succinate (with

reservation), and aerobis. Some preparations exhibit significant activity in absence of succinate (50% of activity in presence of succinate). This activity is completely dependent on aerobsis and stimulated to the same extent as the activity in the presence of succinate. The easiest interpretation is that some NADH is slipping pass the rotenone block. This oxidation is sufficient to support the transhydrogenase. Figure 69 lists the stimulation of the reaction by oligomycin. In contrast to the report of van Dam and ter Welle (85), the reaction is inhibited by higher levels of oligomycin.

Table 40. Succinate oxidation supported transhydrogenase activity of sub-mitochondrial particles.*

Particle	$A_{340\ m\mu}\ min^{-1}\ g^{-1}$	Oligomycin stimulation
HMP	20-70	30-100%
ASP	42	240%
ETPH	200	100%

*Assayed as indicated in Materials and Methods. The HMP value is representative of many preparations.

AESF stimulates the succinate oxidation supported transhydrogenase of almost all of the HMP preparations investigated. Figure 70 shows the titration of the activity with a HMP preparation. This titration was carried out in the presence of magnesium ion. If magnesium ion is left out of the assay, the results obtained are indicated in Figure 71. When magnesium ion is added to a system containing both AESF and HMP, the results obtained are recorded in Figure 72. This

clearly demonstrates the necessity for magnesium ion for the interaction of AESF and HMP. Note that the reaction stimulated does not require magnesium. For this result a K_m (Mg^{++}) of about one mM can be estimated. As is indicated in Table 41, the oligomycin and AESF stimulations are not mutually exclusive. Bovine serum albumin does not replace AESF. If the preparation is fully stimulated by oligomycin, AESF may inhibit the rate. If the particle is partly stimulated by AESF, then oligomycin will continue to stimulate the reaction, but less strongly, This partial antagonism of oligomycin and AESF as well as partial replacement bears further study.

Table 41. Stimulation of succinate supported transhydrogenase activity: oligomycin and AESF.

Preparation	Stimulation by AESF	Stimulation by oligomycin	Stimulation by oligomycin of the AESF stimulated particle
ASP (activity, 40)	55%	300%	140%
HMP	34%	73%	9%
HMP	203%	29%	5% inhibition

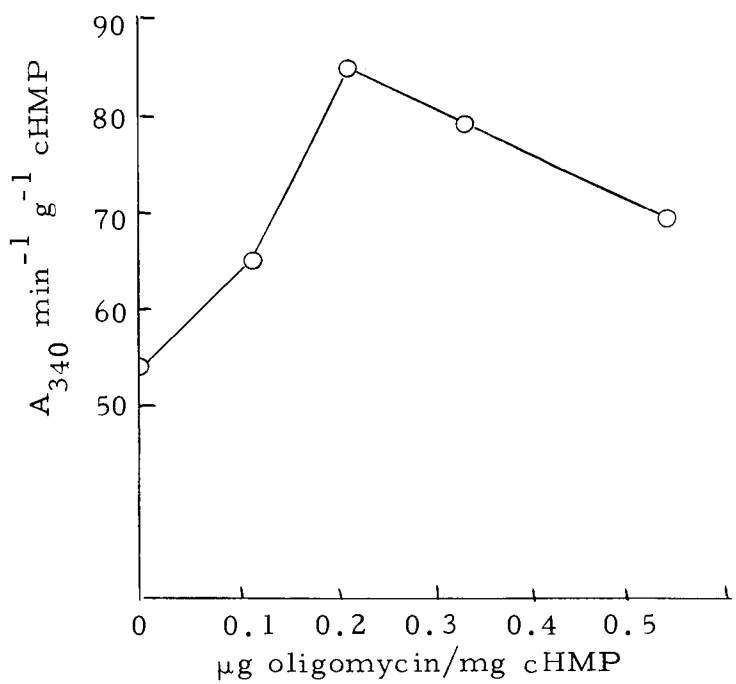
Stimulation of the transhydrogenase by AESF when supported by TMPD-ascorbate oxidation has been observed. There is again an interesting reaction between AESF and TMPD-ascorbate under the condition of the assay. This reaction prohibits conclusive statements about the stimulation.

Figure 69. Stimulation of the succinate oxidation supported transhydrogenase activity of cHMP by oligomycin.

cHMP assayed as indicated in Materials and Methods. Oligomycin added sequentially as concentrated ethanol solution. Activity expressed in accordance with common practice in the literature.

Figure 70. Stimulation of the succinate oxidation supported transhydrogenase of HMP by AESF.

Ammonium sulfate fraction of AESF used as prepared in Materials and Methods. AESF added sequentially as a 27 mg/ml solution to 0.4 mg HMP 447. $MgCl_2$ concentration was 10 mM.


90
80
70
60
50
A_{340} min^{-1} g^{-1} cHMP
0
0.1
0.2
0.3
0.4
0.5
μg oligomycin/mg cHMP

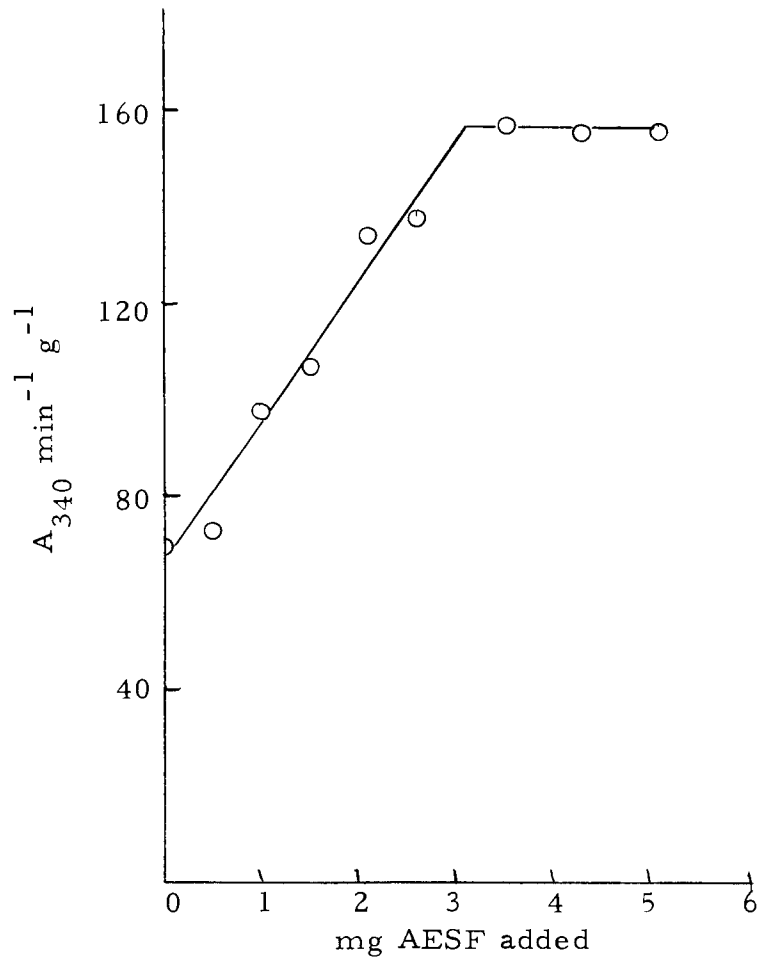
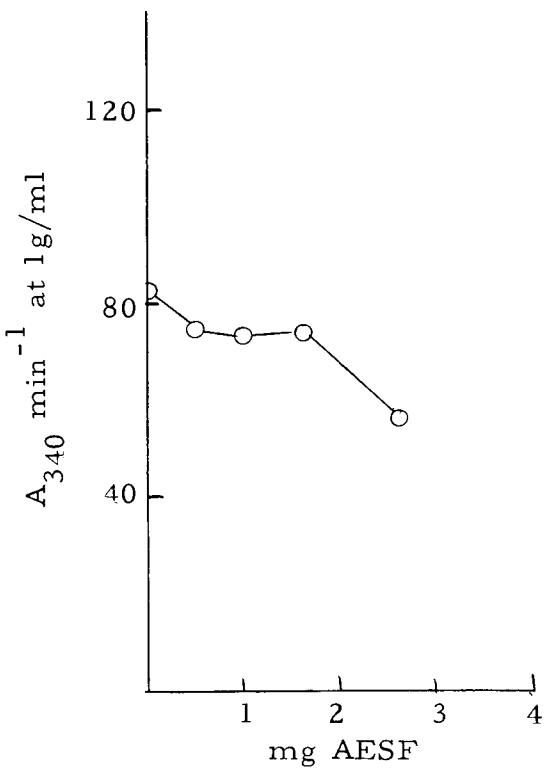

160
120
80
40
A_{340} min^{-1} g^{-1}
0
1
2
3
4
5
6
mg AESF added

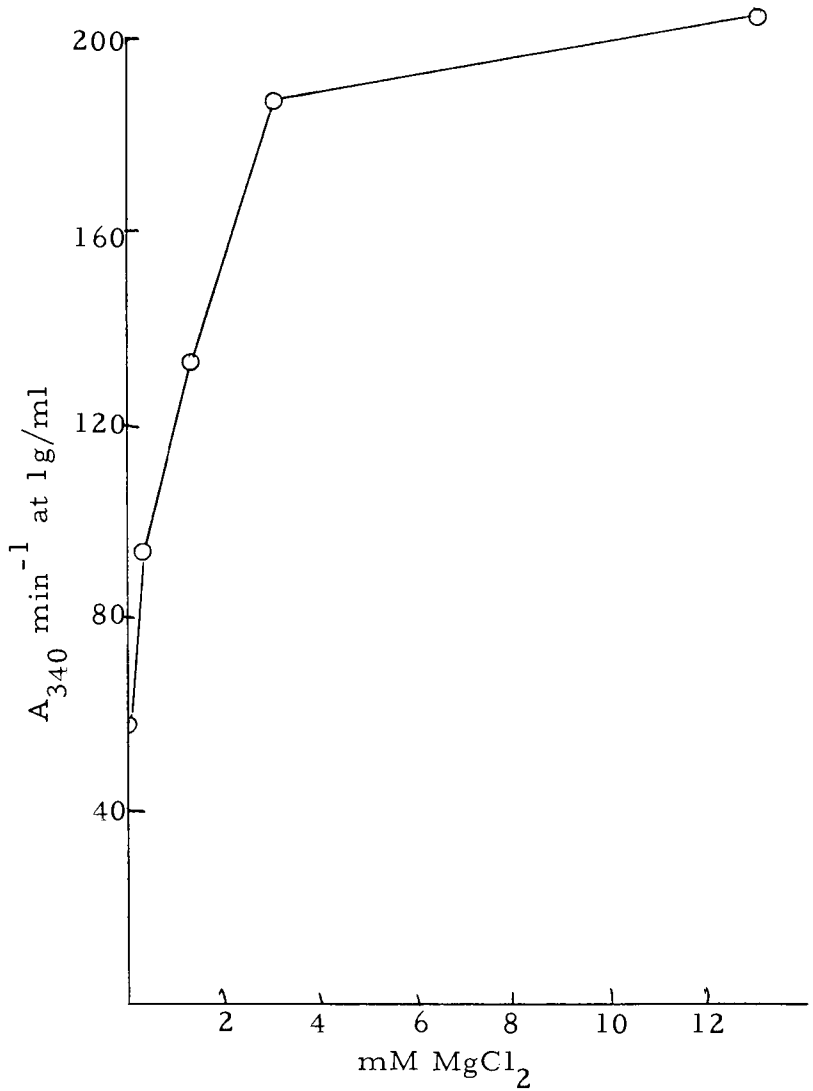
Figure 71. Stimulation of the succinate oxidation supported transhydrogenase of HMP by AESF in the absence of magnesium ions.

Conditions as in Figure 70 except no $MgCl_2$ added.

Figure 72. Stimulation of the succinate oxidation supported transhydrogenase of HMP by magnesium ions in the presence of AESF.

The system contained 0.4 mg HMP 447 and 5.4 mg AESF. $MgCl_2$ was added sequentially as aliquots of a 1 M solution.


120
80
40
A_{340} min^{-1} at 1g/ml
1
2
3
4
mg AESF


200
160
120
80
40
A_{340} min^{-1} at 1g/ml
2
4
6
8
10
12
mM $MgCl_2$

A few attempts were made to physically demonstrate the stimulation of transhydrogenase. Since the transhydrogenase reaction is not easily uncoupled like oxidative phosphorylation, it was hoped that clear demonstration could be affected. Using a system that was marginally stimulated, (delta P/O of 0.1), the resultant particles were examined for transhydrogenase in the presence and absence of oligomycin. The results are indicated in Figure 73. Stimulation of the transhydrogenase is observed as well as continual stimulation by oligomycin.

The AESF is not acting in the same manner as oligomycin is now indicated both from the necessity of Mg^{++} for the AESF stimulation and antagonism of oligomycin in the AESF stimulated system.

F. Purification of AESF

The purification of AESF has proceeded on the basis that a modified preparation of F_2 (14) contains stimulating activity, e.g. stimulates the energy-linked transhydrogenase of HMP. From this point, ammonium sulfate fractionation has purified the active fraction. This information is included in an abbreviated fashion in Figure 4 of Materials and Methods. This fraction is called AESF. During the fraction of the crude AESF reported in discussion of stimulation of oxidative phosphorylation, a similar purification was indicated although not as extensive as in Figure 4. The procedure repeated there did not

proceed to the ammonium sulfate fractionation but did go through a differential extraction. Other methods for the purification of the transhydrogenase stimulating factor were investigated and are presented here.

It was found that 0.3 M potassium phosphate buffer was not necessary for extraction of the active fraction, as little as 0.1 M buffer was sufficient. This modified extraction proceeded with slight purification.

If the purified extract were dialyzed against 10 mM Tris-acetate, pH 7.4 containing 0.1 mM ATP and 0.1 mM EDTA, and passed through a carboxymethylcellulose column equilibrated against the same buffer, the activity would elute with the "breakthrough" peak. Considerable amounts of inactive protein were retained on the column giving a net 3-fold purification. The carboxymethyl-cellulose column was 1.8 by 20 cm. The chromatography was carried out in the cold.

As might be expected from the above, the active fraction was absorbed onto DEAE cellulose under the same conditions. The breakthrough peaks contained no stimulatory activity. When the column was eluted with 0.1 or 0.3 M tris-acetate buffers, no activity was eluted, but the activity was eluted by 1.0 M tris-acetate buffer with the breakthrough peak. There was at this point a 6-fold increase in specific activity but with a large net loss in activity compared to the dialyzed ammonium sulfate fraction applied to the column.

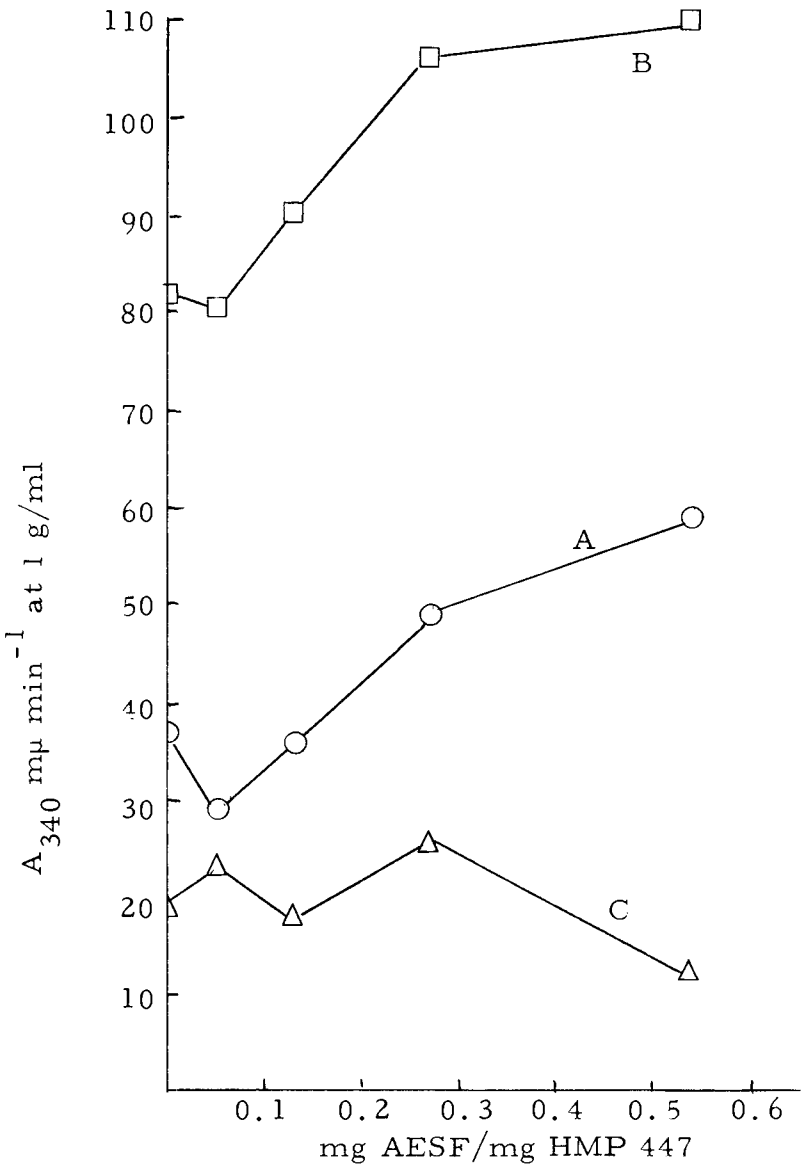


Figure 73. Reconstitution of the succinate oxidation supported transhydrogenase of HMP by AESF.

The necessary amounts of ammonium sulfate fractionated AESF were incubated with 10 mg of HMP 447 with $MgCl_2$ at 10 mM and centrifuged at 50,000 rpm. The pellets were resuspended and assayed. Line A is the usual assay, line B the same assay after addition of 4.3 μg oligomycin per mg HMP 447, line C is the ATP supported transhydrogenase activity.

GENERAL DISCUSSION

A few words of summary may help to put into perspective the main results reported in the thesis. Section I gives the results of investigations with the "shunt" system of Lee, Nordenbrand and Ernster. It was found that the results with succinate oxidase and TMPD were similar to these in the literature and this system was extended to include TCHQ as a shunt compound. The inhibition of succinate oxidase by TCHQ was investigated and the inhibitor effect examined. NADH oxidase yielded more information and it was possible to show differences between TMPD and TCHQ in shunting mechanisms by use of several respiratory inhibitors.

Section II pursued the interactions of TMPD with succinate oxidase. The use of Wurster's Blue was examined here with both soluble and particulate enzymes. Using Wurster's blue, the relationships between soluble succinate dehydrogenases and particulate enzymes was investigated. Along the same lines, the as yet unexplained inhibition of succinate; acceptor oxidoreductase by TTA was investigated with DCIP, WB and ferricyanide as acceptors. The experiments with succinate-ferricyanide were extended to the use of Antimycin A in an attempt to further pinpoint the mode and site of action of TTA. During these experiments, the discovery of a previously unreported activity of SDH was made. This activity was a succinate-ferricyanide

reductase that had somewhat different kinetic constants from the known and well investigated activity. Further, the new activity was extremely labile while the traditional activity has a moderate, well characterized stability. The third section of the thesis extends the observations made in the second section to a wide series of redox enzymes. The prominent result is that only flavoenzymes appear to catalytically reduce WB. In addition, initial observations were made on a system which demonstrates reductase activity with a stable neutral free radical, the well characterized, DPPH.

Where the first three parts of the thesis were concerned mostly with redox properties of HMP and its derivative enzymes, the last two parts were concerned mostly with the "energy coupled reactions" of HMP and other sub-mitochondrial particles, including the restoration of oxidative phosphorylation.

The fourth section first investigated the nature and state of the ATPase in HMP. The solubilization of the enzymes was then described and the characteristics of the soluble enzyme investigated. Restoration of oligomycin sensitivity to the soluble enzyme is then described and finally, a functional role in oxidative phosphorylation is demonstrated through reconstitution of the soluble ATPase with the Nossal particles of Penefsky _et al_.

The final section of the thesis describes the investigation of the energy-linked reactions in HMP. In addition, a soluble protein

fraction was found that stimulated these reactions considerably. The soluble fraction also had a weak stimulatory activity for oxidative phosphorylation with HMP. A series of sub-mitochondrial particles was investigated to find a particle more responsible to the coupling protein. Ammonia sonic particles were found to be most suitable and the coupling protein was purified using this particle as the test particle. The interaction between the soluble protein and the particle was investigated.

Although unsuccessful in the original problem of restoring oxidative phosphorylation in HMP, success was found in restoring energy-linked reactions to the HMP. The weak restoration of oxidative phosphorylation found with the soluble factor was not because the coupling protein was inactive (its activity could be shown with at least three other sub-mitochondrial particles), but because HMP is lacking an additional factor. The basic energy conservation apparatus seemed intact. However, no reversal of oxidative phosphorylation was demonstratable with HMP, perhaps this indicates a lesion in the sequence of reactions leading to oxidative synthesis of ATP.

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