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Partial Purification and Characterization of DCCD-Sensitive ATPase from Pea Cotyledon Mitochondria

by

© Mark Burnett WHISSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Partial Purification and Characterization of DCCD-Sensitive ATPase from Pea Cotyledon Mitochondria submitted by Mark Burnett WHISSON in partial fulfilment of the requirements for the degree of Master of Science in Plant Biochemistry.

Mary Spencer
Supervisor

Date April 27, 1981

ABSTRACT

The DCCD-sensitive ATPase of pea (*Pisum sativum* L.) cotyledon mitochondria was solubilized from submitochondrial particle (SMP) membranes. The solubilized ATPase (FoF1) was partially purified and characterized.

To release FoF1 from SMP membranes, the detergents sodium cholate, Triton X-100, and sodium deoxycholate were investigated. The latter two detergents were found to either interfere with the ATPase assay, or deactivate the pea FoF1. Sodium cholate was thus chosen for the solubilization of the enzyme. The parameters of FoF1 solubilization were varied, and it was concluded that 0.6 mg cholate/mg protein in 10% saturated ammonium sulphate resulted in solubilization of 85% of the total ATPase activity. Sensitivity of the enzyme to DCCD was maintained. Incubation of SMP in the detergent medium decreased the sensitivity of the solubilized enzyme to DCCD, and its specific activity.

Purification of the crude enzyme preparation (CE) by affinity chromatography with Affigel Blue was attempted, but this procedure caused a 50% inactivation of the FoF1, and no active FoF1 could be released from the Affigel Blue column. Ammonium sulphate precipitation did result in an increase in specific activity. At between 38% and 45% saturated ammonium sulphate, 20% of the ATPase activity was precipitated, with a specific activity 4 to 5 times higher than that of the CE. The precipitate, designated NSE ATPase, was highly sensitive to DCCD, and had a specific activity 12 to 13 times higher than the specific activity of pea SMP.

Methods for further purification of NSE were investigated with CE. Ammonium sulphate precipitation chromatography of CE was attempted, and although a separation of FoF1 from other proteins was observed, over 90% of the ATPase activity was lost. A separation of CE from other proteins was also obtained by sucrose density gradient centrifugation, but over 80% of the ATPase activity was lost. It was thought that the deactivation may have been a result of the long centrifugation times that are required for sucrose density gradients because of their high viscosity. Accordingly, CE was separated by centrifugation

through Percoll density gradients, which have low viscosities even at high densities. It was found that the Percoll density gradients that resulted in poorer separations maintained much more ATPase activity than Percoll gradients that gave better separations.

When the Percoll density gradient technique was used on NSE, or when the ATPase peak from a Percoll density gradient was fractionated with ammonium sulphate, all of the ATPase activity was destroyed. It was suggested that the deactivation may be caused by removal of one or more factors that are necessary to either the function or the stability of the pea FoF1, such as phospholipids. Crude soybean phospholipids stimulated NSE only slightly however.

The properties of the NSE preparation were investigated. Preliminary experiments revealed that NSE contained levels of cytochrome and NADH dehydrogenase contamination comparable to those of the highly purified FoF1 preparations in the literature. The pea FoF1 was found to break down during polyacrylamide gel isoelectric focusing. Preliminary examination by SDS electrophoresis also suggested that the NSE may contain as few impurities as the highly purified mitochondrial FoF1 preparations in the literature.

It was found that in most respects, the pea FoF1 had properties similar to pea SMP, rather than pea F1. These included specificity for nucleotide triphosphates, anion effects, stimulation of the ATPase activity by aging at 25 C, inhibition of ATPase activity by DCCD, and biphasic kinetic properies. It was suggested that the biphasic kinetics displayed by pea FoF1 may have physiological significance. It seemed that Fo modified many properties of F1 by binding to it.

In other respects (cold lability and cation specificity), the FoF1 was intermediate between pea SMP and purified pea F1. It was suggested that the pea FoF1 preparation may be in equilibrium with free F1 and Fo.

INTRODUCTION

Thirty years ago Lehninger and coworkers proved that the chemical energy of Krebs cycle intermediates is conserved by the coupling of electron flow along the respiratory chain to oxygen, with oxidative phosphorylation. The subsequent development of three competing hypotheses (Racker, 1977) in the following 15 years spurred an ever increasing volume of research into the mechanism by which the energy of electron flow through the electron transport chain is coupled to the endothermic phosphorylation of ADP. In the early 1960s Racker and his collegues discovered a coupling factor (F1), that when released from mitochondrial membranes displayed ATPase activity. Coupling factor preparations were able to restore oxidative phosphorylation capabilities to coupling factor-depleted membranes and were able to hydrolyse ATP, but could not catalyse ATP synthesis or any of the exchange reactions that are associated with ATP synthesis in mitochondria (Pedersen, 1975). During the late 1960s other workers isolated coupling factors remarkably similar to F1 from a tremendous variety of sources, including rat liver mitochondria, yeast mitochondria, bacteria, and even chloroplasts (Kagawa *et al*, 1979).

According to Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1966) the membrane protein (Fo) that anchors F1 to the membrane also functions as a proton pore and perhaps gate. The membrane itself is a barrier that allows the buildup of a proton gradient that the hypothesis states drives ATP synthesis on F1 during proton leakage back through the ATPase (FoF1). It is now generally accepted (Boyer *et al*, 1977) that FoF1 embedded in a closed membrane that enables the formation of a proton gradient (F1 side alkaline) is the minimal requirement to observe ATP synthesis and many of the associated exchange reactions. Consequently submitochondrial particles (SMP), subbacterial particles (SBP) and subchloroplast particles (SCP) have become the standard tools for investigating ATP synthesis and exchange reactions, while F1, which has been highly purified from a variety of sources in the last 10 years, has become the most common tool for investigating the ATP hydrolysis function (Pedersen, 1975).

However, many properties of F1 (kinetics, specificity, nucleotide binding) are dissimilar to the membrane bound enzyme (Nelson, 1976, Pedersen, 1975), and it has even been suggested that the ATPase reaction may not in fact be the reverse of the ATP synthetase reaction (Penefsky, 1974b). On the other hand, data obtained from SMP, SBP, or SCP are rarely conclusive because of the presence of unknown lipids, proteins, and carbohydrates. Consequently, many investigators have attempted to purify the FoF1 complex in a soluble form (see Chapter 1). However, since "mitochondrial ATPase is perhaps the most complex enzyme system known to man" (Pedersen, 1975), this approach has met with limited success until recently, because it is a very labile system.

Although chloroplasts are a popular source of F1 and more recently of FoF1, plant mitochondria have received very little attention in this regard, perhaps because of the low yields of F1 obtained. To the author's knowledge, only two groups have purified F1 from plant mitochondria (Yoshida and Takeuchi, 1970 and Malhotra and Spencer, 1974, Grubmeyer and Spencer, 1979), and no one has yet investigated a solubilization of FoF1 from this ubiquitous and important source.

Since F1 from peas has been shown to have properties that differ in important points to those of animal mitochondrial F1 (Grubmeyer and Spencer, 1978), it was concluded that a FoF1 preparation from plant mitochondria may eventually yield valuable insights into the mechanism of FoF1 function. Thus the aim of this research project was to solubilize the FoF1 of pea mitochondria. The FoF1 extract was then to be purified if possible, and at least partially characterized.

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List of Abbreviations

ADP	:	adenosine-5'-diphosphate
ATP	:	adenosine-5'-triphosphate
ATPase	:	adenosine triphosphatase
CCCP	:	carbonyl cyanide m-chlorophenyl hydrazine
CE	:	cholate extract of SMP
DCCD	:	N,N'-dicyclohexyl carbodiimide
Fo	:	membrane portion of proton-ATPase of SBP,SCP or SMP
FoF1	:	proton-ATPase of SBP,SCP or SMP
F1	:	soluble portion of proton-ATPase of SBP,SCP or SMP
GTP	:	guanosine-5'-triphosphate
Km	:	Michaelis constant
MES	:	2(N-morpholino)ethane sulphonic acid
NADH	:	reduced nicotinamide adenine dinucleotide
NSE	:	ammonium sulphate enzyme
NTP	:	nucleoside-5'-triphosphate
OD	:	optical density
PEP	:	phosphoenolpyruvate
PMF	:	proton motive force
Pi	:	inorganic phosphate
SBP	:	subbacterial particles
SCP	:	subchloroplast particles
SDS	:	sodium dodecyl sulphate
SMP	:	submitochondrial particles
TEMED	:	N,N,N',N'-tetramethylethylene diamine
TES	:	N-tris (hydroxymethyl)-methyl-2-amino ethane sulphonic acid
ATPase unit	:	1 umole of Pi released/min

1. LITERATURE SURVEY

1.1 Reviews

There is today a very large body of literature on the coupling ATPase, derived in the main from work with SMP, SBP, SCP, and F1 preparations from mitochondria, bacteria, and chloroplasts. Instead of repeating material in the many excellent reviews that are available on F1 and membrane-ATPase preparations, it is the intention of the author to refer the reader to a few of the better ones and then summarize the literature, first, on the solubilization of FoF1, and second, on the enzymatic properties of FoF1.

For F1 from chloroplasts, the reader is referred to Baird and Hammes (1979) and Nelson (1976, 1977). For F1 from mitochondria, or all sources, one should read Senior (1973) and Pedersen (1975), both of which are very comprehensive. Kozlov and Skulachev (1977) have also published an excellent review, in which they attempt to reconstruct the mechanism of ATPase action. The review of Harris (1978) summarizes the hundreds of nucleotide binding experiments that have been reported, and their possible significance to ATPase mechanism models. Many of these reviews contain sections on the membrane bound ATPase, i.e. of SMP, SBP, and SCP, since their properties are often quite different from those of F1. Membrane bound ATPases are reviewed by Baird and Hammes (1979), Harris (1978), Kozlov and Skulachev (1977), and Nelson (1976).

There are available two comprehensive reviews (Kagawa, 1978 and Kagawa *et al*, 1979) on the structural and functional aspects of the FoF1 complex. Fillingame's (1979) review contains a large section on the subunits of FoF1 and the function of Fo. Pedersen (1975) includes small sections on the properties of the isolated FoF1.

1.2 FoF1 Solubilization

In 1966, a preparation designated Fo was isolated by sonication and enzyme digestion from beef heart SMP (Kagawa and Racker). Fo could be combined with F1 preparations to confer sensitivity to oligomycin on them, as well as other properties previously only found in SMP, SBP, or SCP. However, the specific activity of the combined preparations was low and the purity unknown.

The first recorded detergent extraction of an FoF1 preparation is that of Tzagoloff *et al* (1968). The procedure was fairly lengthy but produced a preparation free of all respiratory components aside from some cytochromes and flavins. The preparation had a high specific activity of 5-8 umoles Pi released/min/mg protein and was 90% inhibited by oligomycin.

It was not until 1971, however, that two groups (Kagawa and Racker, and Tzagoloff and Meagher) isolated FoF1 preparations (from SMP), that could be reconstituted with phospholipids into vesicles. These vesicles could then catalyze ATP-Pi exchange, an important reaction that SMP, SBP, and SCP but no previous enzyme preparations were capable of catalyzing. Both methods required only two or three steps although the Tzagoloff and Meagher preparation was not highly sensitive to energy transfer inhibitors and the Kagawa and Racker preparation was greatly stimulated by the addition of other factors, such as F1. The preparation from Tzagoloff and Meagher (1971) was subsequently modified to purify a very active FoF1 from yeast SMP (Ryrie, 1975a). The principle modifications consisted of the addition of ATP during isolation to stabilize the complex, and the addition of a molecular sieving step that removed high molecular weight contaminants.

Swanljung and Frigeri (1972) published an isolation technique that used as its major purification step affinity chromatography. SMP were incubated in 0.3% Triton X-100 and centrifuged at high speed to remove remaining vesicles. The supernatant layer was passed through a column to which purified ATPase inhibitor had been covalently bound. The subsequently eluted enzyme complex had a very high specific activity, but because of problems in binding the enzyme to the column and later releasing it, the yield was only 20% or so. In

view of suggestions that the binding of the ATPase inhibitor to the enzyme may be controlled by membrane energization (Van de Stadt *et al*, 1973) this result is not surprising. Furthermore, the purified enzyme complex was not inhibited by oligomycin, except at very high concentrations.

Later workers have also used affinity chromatography, but with ADP rather than the ATPase inhibitor bound to the support (Brodie *et al*, 1979 and Higashi *et al*, 1975). Although the binding and release of the enzyme to the ADP-column can be easily manipulated, resulting in higher yield, there are other membrane proteins that also bind ADP and may therefore bind to the column, eg. the adenine nucleotide antiporter. A further purification step in the form of a sucrose density gradient, perhaps modified from the glycerol gradient of Tzagoloff and Meagher (1971), was thus added.

Carmeli and Racker (1973) reported the first solubilization of FoF1 from chloroplasts. This was acheived by incubating chloroplasts with 2% cholate and 0.4 M ammonium sulphate and spinning down the remaining vesicles and SCP at high speed. When dialysed, this crude extract formed vesicles that catalysed an uncoupler- and DCCD- sensitive ATP-Pi exchange . When the crude extract was fractionated by ammonium sulphate precipitation, it gave an ATPase with an apparent molecular weight lower than that of the ATPase of the crude extract. However, the ATP-Pi exchange catalysed by the vesicles after removal of ammonium sulphate by dialysis was not sensitive to uncouplers or DCCD. They also experimented with molecular sieving but although an ATPase peak was found, no purification resulted. Later authors (Clarke and Morris, 1976) also tried to purify detergent FoF1-extracts by molecular sieving with only slightly more success (4 x purification).

Bragg and [redacted] (1976) were the first workers to report a large purification by the use of molecular sieves. After chromatography of a cholate extract of *E. coli* membranes on Sepharose 6B followed by chromatography on Sepharose 4B, a 16 fold purification was obtained. However, Hare (1975) (see below) obtained a higher specific activity of the FoF1 preparation from the same source and a higher purification (20-25 fold) with one spin through a sucrose density gradient. The former preparation contained 4 more polypeptide

bands (as shown by SDS electrophoresis) than the latter, but was stimulated more by added phospolipids. It was stated that the former FoF1 preparation contained no respiratory chain components although this was not shown.

An "ATP-Pi Exchangease" was prepared (Sadler *et al*, 1974) from SMP by disintegration of the particles with lysolecithin and removal of large membrane fragments by a short high speed centrifugation. Because the exchange reaction is believed to require closed vesicles (Boyer *et al*, 1977) and because the "exchangease" itself was spun down at 100,000 g (the speed usually used to rid preparations of vesicles and large membrane fragments), the lysolecithin treatment probably did not even solubilize the enzyme although it did reduce contamination by many electron transport components. The procedure resulted in a four fold increase in ATPase activity.

Hatefi *et al* (1974) reported a preliminary preparation, derived from that of Tzagoloff *et al* (1968), of complex V from beef heart SMP. Complex V has very similar properties to FoF1 and is most probably the same enzyme (see Stiggall *et al*, 1978). Hatefi's preparation had a lower specific activity, but was able to catalyse an ATP specific ATP-Pi exchange without addition of or reconstitution with any other factors. This method was later made very complex by the addition of even more detergent extraction and salting in and out steps (Stiggall *et al*, 1978). The resulting complex V had a high specific activity (8-10 umoles Pi released/min/mg protein), low contamination by respiratory components and a variable high ATP-Pi exchange activity that was inversely related to the concentration of cholate used in the second detergent extraction. The cholate concentration also caused the amount of phospholipid to vary from 5-13 ugm phosphorous/ mg protein. If it was less than 7 ugm/mg, the ATP-Pi exchange had an absolute requirement for added phospholipid. If it was greater than 7 ugm/mg, the preparation catalysed ATP-Pi exchange, although the exchange was always stimulated by phospholipid added up to concentrations of 100 ugm/mg protein.

Jackl and Sebald (1975) attempted to use immunoprecipitation as the principle purification step after solubilization of the membrane with Triton X-100. Although the yield and the specific activity were not reported, they did

find 9 proteins other than the 5 normally attributed to F1. This is 5-7 more than are found by SDS electrophoresis of the highly purified preparations.

Probably the most important contribution to the field of FoF1 research to date was made by Sone *et al*, (1975). After much searching, they chose as their source material a thermophilic bacterium, PS3. The F1 and FoF1 ATPases from PS3 are highly stable, even under conditions that cause rapid dissociation or denaturation of ATPases from other sources, e.g. high temperature or 4 M urea. Consequently, Kagawa and his group were able to develop for the PS3 FoF1 a fairly long procedure that utilized principally ion exchange chromatography, to which many other FoF1s appear to be sensitive. This gave a 15 fold purification (18 umoles Pi released/min/mg protein) with a 40% yield and 75% inhibition by DCCD. SDS electrophoresis revealed only 8 bands, i.e. 5 from F1 and 3 from Fo. The fact that the source was available in large quantities, coupled with the high yield procedure and the stability of the enzyme, has allowed the completion of many elegant experiments by Kagawa's team since 1975, including reconstitution of active FoF1 entirely from individual subunits, electron density mapping of crystallized F1 and so on. Their discoveries will be discussed later in this chapter.

Soper and Pedersen (1976) investigated solubilization of FoF1 from rat liver SMP. They concluded that deoxycholate was superior to Triton X-100 and other detergents in terms of specific activity and sensitivity to DCCD. They did not attempt to purify the deoxycholate extract any further.

The procedure of Carmeli and Racker (1973) was further refined by Serrano *et al* (1976) principally by careful optimization of the cholate extraction conditions and by addition of a sucrose density gradient centrifugation. The resulting mitochondrial FoF1 preparation had a specific activity of 15 umoles Pi released/min/mg protein (which was dependent on added phospholipids), 0-3% respiratory components, and some adenine nucleotide transporter contamination. It could be reconstituted with phospholipid into vesicles with a very high ATP-Pi exchange and the ability to function as reversible ATP driven proton pumps. This was the first purified FoF1 from mitochondria that was shown to have this capability. It showed 11 bands on SDS gel electrophoresis, 3 of which

were very faint and which were concluded to be trace contaminants. This procedure still produces perhaps the best FoF1 available from mitochondria.

Recently Berden and Voorn-Brouwer (1978) have reported the isolation of an FoF1 from beef heart SMP with properties very similar to those of the FoF1 of Serrano *et al* (1976). It had a similar specific activity, even though it showed 4 more bands on SDS gels than the FoF1 of Serrano *et al* (1976). When reconstituted into vesicles, the preparation also catalyzed ATP driven proton translocation. The notable point about the method of Berden and Voorn-Brouwer is that it is appreciably shorter and gives a very high yield (88% compared to 30% for that of Serrano *et al* (1976), which itself is high in comparison to that obtained by many other methods). After removal of Triton X-100 by dialysis the procedure consisted merely of fractionation with ammonium sulphate in cholate.

With the exception of the two reports mentioned above, since 1976 investigators appear to have been concentrating on purifying FoF1 preparations from different sources by slightly modifying fractionation and purification techniques that had been previously published.

The technique of Carmeli and Racker (1973) was barely altered by Winget *et al* (1977) to extract and partially purify an FoF1 from chloroplasts that could be put into synthetic phospholipid vesicles with bacteriorhodopsin. These vesicles were able to catalyse ATP-Pi exchange and light driven ATP synthesis. Pick and Racker (1979) modified the procedure of Serrano *et al* (1976) to obtain a highly purified FoF1 from chloroplasts.

The Tzagoloff *et al* (1968) procedure was somewhat streamlined and adapted to extraction and partial purification of FoF1 from *E. coli* membranes by Freidl *et al* (1977). In 1979, Freidl *et al* used a novel detergent (Aminoxid WS35) and a procedure based on that of Sone *et al* (1975) to highly purify the FoF1 of *E. coli*. The ATPase activity was increased 21 fold and the preparation showed only 8 bands after SDS gel electrophoresis.

Another FoF1 was purified from *E. coli* membranes by Foster and Fillingame (1979), who used a partially modified method of Hare (1975) with only the addition of an ammonium sulphate precipitation step. This precipitate

also showed 8 subunits after SDS gel electrophoresis and had a specific activity of 16-18 umoles Pi released/min/mg protein.

Oren and Gromet-Elhanan (1977) used a simple Triton X-100 extraction coupled with a glycerol gradient to solubilize a very low activity FoF1 from the nonsulphur purple bacterium *Rhodospirillum rubrum*. Schneider *et al* (1980) purified a considerably higher specific activity FoF1 from the same bacterium by adapting the method of Freidl *et al* (1979). Even so, it still showed 12 or so bands after SDS electrophoresis and quite low activity (2 umoles Pi released/-min/mg protein) when compared with other purified preparations.

The mitochondria of the mould, *Aspergillus nidulans*, were used as a source by Marahiel *et al*, (1977). The purification procedure was based on that of Tzagoloff and Meagher (1971) and resulted in a 6 fold purification.

1.3 Properties of the Isolated FoF1

1.3.1 Purity

The first and most important question that should be asked of a new enzyme purification is, of course, how pure is the enzyme preparation that it produces? This question has normally been addressed with direct assays of FoF1 preparations for specific impurities (eg. spectophotometric assays for cytochromes or enzymatic assays for NADH dehydrogenase) and/or SDS polyacrylamide gel electrophoresis of FoF1 preparations.

Kagawa and Racker (1971) found in their FoF1 preparation no cytochrome a + a3, 79% of the mitochondrial cytochrome b in SMP, 37% of the cytochrome c + c1 in SMP, and 20% of the mitochondrial phospholipid in SMP (i.e. 0.64 umol Pi/mg protein). The preparation of Tzagoloff *et al* (1968) had a considerably higher specific activity than that of Kagawa and Racker (1971) (10 fold higher) and not surprisingly, lower impurity levels. It also had no cytochrome a, 8% of the mitochondrial amount of cytochrome b, and only 4% of the cytochrome c + c1.

The more recent highly purified preparations have specific activities over 2 times higher than that of Tzagoloff and Meagher (1971). Analyses commonly

reveal 0 or less than 0.5% cytochrome a (3% in the case of Sone *et al* (1975)), 2% NADH dehydrogenase, 2% cytochrome b, 1-3% cytochrome c + c1, and 5% or less phospholipid, relative to the source membranes (Sone *et al* 1975, Serrano *et al* 1976, Stiggall *et al* 1978, Brodie *et al* 1979).

Most workers, however, have judged the purity of their preparation by the specific activity and by the number of bands it shows after SDS-electrophoresis. Early preparations often showed 12 or more bands, e.g. Sadler *et al* (1974). In fact, some later preparations have shown even more bands, e.g. 16 (Marahiel *et al* 1977, Bragg and Hou 1976), even some of the highly purified FoF1s (Stiggall *et al*, 1978).

However, Capaldi (1973) compared FoF1 made by the methods of Tzagoloff *et al* (1968) and Kagawa and Racker (1966) with SDS-gel elecrophoresis. He also fractionated the preparations by repeated NaBr precipitation and ran the fractions and the FoF1 reconstituted from these fractions on SDS gels. He concluded that as well as the 5 F1 subunits, there were 4 subunits in mitochondrial FoF1. This conclusion has been strengthened by the results from recent highly purified FoF1s (Serrano *et al*, 1976, Pick and Racker, 1979), although other workers (Sone *et al*, 1975, Freidl *et al*, 1979, Schneider and Altendorf, 1980) have purified fully functional FoF1 from bacteria with only 8 subunits. There is even at present some controversy over whether or not the third Fo subunit is necessary for bacterial Fo function (Sone *et al*, 1978, Fillingame *et al*, 1980).

1.3.2 Structure-Function Relationships of FoF1

The FoF1 complex catalyses the last step in the processes of oxidative and photosynthetic phosphorylation. Therefore its function is to provide a channel that protons are able to travel through as they move down the protonmotive gradient, and to use the energy released by these protons to drive the endergonic synthesis of ATP. In prokaryotes it probably also uses the energy of ATP hydrolysis to form a protonmotive gradient, that is then used used for active uptake of substrates (Kaback, 1976, Boyer *et al*, 1977).

Reconstitution has been a powerful tool in the investigation of structure-function relationships. Some of the reviews mentioned at the beginning of this chapter detail the use of reconstitution to elucidate the function of each subunit of F1. The reviews of Kagawa (1978), Kagawa *et al* (1979), and Fillingame (1980), detail the same for the FoF1, principally of the thermophilic bacterium PS3. They concluded that the D and E subunits bind the rest of F1 to Fo, which itself is composed probably of 3 different subunits, although only 2 appear to be necessary, to reconstitute ATP-Pi exchange and proton translocation (Sone *et al*, 1978). The number (e.g. Foster and Fillingame, 1979, Freidl *et al*, 1979) and even the molecular weight of the subunits of Fo (Schneider and Altendorf, 1980) have been confirmed from eukaryotic sources, but it should be noted that other workers have shown that Fo from higher sources, beef heart SMP (Alfonzo and Racker, 1979) and chloroplasts (Pick and Racker, 1979), may well contain 4 different types of subunits. Although it seems unlikely from an evolutionary point of view (Hasan and Rosen, 1979), the Fo from higher eukaryotic sources may differ from that of PS3, from which the vast majority of our knowledge to date has come.

1.3.3 Catalytic Properties of FoF1: ATPase Activity

ATPase activity is the most convenient way to monitor purification of the enzyme, and when used in conjunction with inhibition by DCCD or similar inhibitor, is very specific for FoF1. Aside from the maximum rate of ATP hydrolysis measurable, however, few workers have investigated this activity in FoF1 preparations, which is rather surprising in view of the amount of literature on the same activity in SMP and purified F1.

Soper and Pedersen (1976) found that deoxycholate-solubilized FoF1 from beef heart showed biphasic ATP kinetics, with Kms of 29 and 313 uM. Other workers have found only linear Michaelis-Menten kinetic plots with Kms from 13 uM (Schneider *et al*, 1980) to 300 uM (similar to the membrane bound enzyme)(Oren and Gromet-Elhanan, 1977) for FoF1 from a nonsulphur purple bacterium, and from 140 uM (Swanljung *et al*, 1973) to 160 uM (Stigall *et al*, 1978) for beef heart FoF1. At present, therefore, the general consensus would

appear to indicate a Km of 10-300 uM, although one should keep in mind that many variables can alter the Km drastically. For example, the use of Ca++ in place of Mg++ as the cation raised the Km by 400% (Schneider *et al*, 1980) and activation with cardiolipin instead of lysolecithin increased the Km by 500% (Swanljung *et al*, 1973). It may also vary with the type of detergent used, the amount of membrane still present, and so on. However, the Km of FoF1 is roughly one order of magnitude lower than that of purified F1, and almost the same as that for membrane bound ATPase (i.e. SMP, SBP, etc.) (Pedersen, 1975).

Carmeli and Racker (1973) and Swanljung *et al* (1973) have both done pH profiles of ATPase activity of chloroplast and mitochondrial FoF1 respectively , and found the optimum pH to be 8.0, with or without added phospholipids, the same as purified F1 and membrane bound ATPase.

Only three groups have so far investigated the nucleotide specificity for hydrolysis by FoF1. Ryrie (1975) reported that yeast FoF1 hydrolysed ITP, ATP, and GTP at almost the same rates. UTP was hydrolysed at 20% of the previous rates, and other nucleotides (TTP, CTP, and ADP) were hydrolysed at less than 5% of this rate. Stiggall *et al* (1978) found that GTP and ITP were hydrolysed at 66% and 44% respectively, of the rate of ATP hydrolysis. UTP was not hydrolysed. Tzagoloff *et al* (1968) found that with beef heart FoF1 the rate of hydrolysis of GTP and ITP was only 30% of the rate of ATP hydrolysis. Thus SMP from yeast and beef heart show specificities similar to the FoF1 preparation from beef heart by Tzagoloff *et al* (1968) while purified F1 characteristically shows specificities similar to those found by Ryrie (1975). The most highly purified FoF1 preparation (Stiggall *et al*, 1978) falls in between the two.

With both beef heart FoF1 (Tzagoloff *et al*, 1968) and yeast FoF1 (Ryrie, 1975), Ca++ has been found to be relatively ineffective in combining with ATP to form a substrate complex, compared to Mg++ (and Mn++). It is similar in this respect to the membrane bound and F1 forms of the enzyme (Pedersen, 1975). Pick and Racker (1979) found that Ca++ was almost as effective as Mg++ in forming a substrate complex for chloroplast FoF1. Chloroplast FoF1 would thus appear to be intermediate between membrane bound and soluble F1

(Nelson, 1976).

1.3.4 Catalytic Properties of FoF1: Energy Linked Reactions

The presence of, or the potential for reconstitution of ATP–Pi exchange has been used as a marker to indicate the capability for energy–linked reactions, which of course, are essential features of the *in vivo* enzyme that the well studied F1 apparently lacks. Early workers (eg. Sadler *et al*, 1974) purified "exchangease particles" that were able to catalyse ATP–Pi exchange without the addition of extra factors or phospholipids. It was soon realized, however, that if extracted and purified gently enough, purified FoF1 could catalyse ATP–Pi exchange merely by reconstitution with phospholipids into vesicles in accordance with the predictions of Mitchell (1961, 1966). Since then, exchange rates from 2.3 nmol Pi/min/mg protein (Schneider *et al*, 1980) to 410 nmol Pi/min/mg protein (Stiggall *et al*, 1978) for FoF1 reconstituted into vesicles have been recorded. Most exchange rates fell between 25 nmol Pi/min/mg protein and 200 nmol Pi/min/mg protein, with the higher rates catalysed by the more highly purified preparations (e.g. Ryrie, 1975), irrespective of the source or detergents used. Carmeli and Racker (1973) found that the optimum pH of the exchange was 8.0 or 8.5, much the same as the optimum for ATPase activity. Stiggall *et al* (1978), Pick and Racker (1979), Hatefi *et al* (1974), and Carmeli and Racker (1973) have investigated the nucleotide specificity of the exchange and found it to be highly specific for ATP. Other nucleotide exchanges were less than 10% of the rate of ATP exchange, with the exception of the chloroplast FoF1 of Pick and Racker (1979) that could catalyse a GTP–Pi exchange about 30% as fast as the ATP–Pi exchange. This is much the same as the exchange and hydrolytic specificity of the membrane bound enzyme. Pick and Racker (1979) also observed that the exchange was dependent on Mg++ and that Ca++ was ineffective. Stiggall *et al* (1978) found that the Km (ATP) of the exchange was approximately 2.5 mM, 250 times higher than the Km (ATP) for the hydrolysis reaction.

In all cases tested, the exchange was sensitive to uncouplers and DCCD. Workers have also demonstrated ATP driven proton translocation into vesicles

reconstituted with purified FoF1 from mitochondria, *E. coli*, and PS3 (Schneider and Altendorf, 1980, Ryrie and Blackmore, 1976, Sone *et al*, 1975), either by direct pH measurement (e.g. Kagawa *et al*, 1973), fluorescent dye quenching (e.g. Freidl *et al*, 1979), or fluorescent dye enhancement (Sone *et al*, 1975). Kagawa *et al* (1973) found that the phospholipids used in the reconstitution with mitochondrial FoF1 must contain unsaturated fatty acid side chains. Kagawa and coworkers have used the more stable FoF1 to investigate in detail the electrochemical gradient that is formed during ATP hydrolysis (Kagawa *et al*, 1979), and have concluded that the gradient is larger than predicted by Mitchell's (1966a) postulate that 2 protons are pumped/ATP. As was the case with ATP-Pi exchange, ATP driven proton exchange was found to be inhibited by low concentrations of uncouplers, DCCD or oligomycin in all instances.

ATP synthesis by FoF1 preparations (eg. Racker and Stoeckenius, 1974) and by purified FoF1 (see below) in vesicles has been demonstrated and shown to be dependent upon a correctly oriented electrochemical gradient. A sufficient gradient was formed by: acid to base transition (Pick and Racker, 1979), illumination of purified bacteriorhodopsin that was reconstituted into the membrane with FoF1 (Winget *et al*, 1977), or by a chemical redox potential across the membrane (external ascorbate and internal ferricyanide) coupled to a permeable proton carrier (PMS) (Ryrie and Blackmore, 1976). Synthesis of ATP was insensitive to all specific electron transport inhibitors, but sensitive to uncouplers or the absence of any part of the gradient production system. Although none of these experiments utilized purified lipids for reconstitution, they did provide strong support for Mitchell's (1966) chemiosomotic hypothesis. Sone *et al* (1977) did substantiate the primary role of proton translocation in phosphorylation by using the highly purified FoF1 of Sone *et al* (1975) and reconstituting it with a defined mixture of phospholipids from PS3. The phosphorylation was driven by acid-base transition and was concluded to be 40 times faster/mg protein than phosphorylation by mitochondria, SMP, or SBP (Kagawa *et al*, 1979).

2. MATERIALS AND METHODS

2.1 General

ATP was obtained from Terochem Inc., Edmonton. Sephadex G-25 and Percoll were from Pharmacia, Uppsala, Affigel Blue from Bio-Rad, ammonium sulphate (Enzyme Grade) from Serva, Heidlberg, and Ampholines from LKB, Bromma, Sweden. All other chemicals and biochemicals were obtained from Sigma or Fisher and were of the highest purity available. pH was measured at 25 C. All other manipulations were done at 0-2 C except where stated otherwise.

2.2 Tissue

Pea seeds (*Pisum sativum* L. cv Homesteader) were soaked in tap water for 6 hours and then planted in trays of vermiculite (horticultural grade). The trays were incubated at 27 C (high humidity) in the dark for 4 days. The plants were harvested, rinsed in tap water and the cotyledons removed by hand and kept on ice until use (within 2 hours).

In early experiments various growing methods that could have decreased the time required for the removal of the cotyledons were tried. For example, peas were grown for one or two days on trays between sheets of moistened blotting paper at 27 C (high humidity), or suspended in constantly agitated and aerated vats of water at 25 C. However, all of these methods resulted in lower yields and/or specific activities, than did the vermiculite procedure (see Appendix 2).

2.3 Preparation of Mitochondria

Mitochondria were prepared essentially by the method of Solomos *et al.* (1972) as follows. One and a half litres of freshly prepared pea cotyledons (see Appendix 2) was divided into 3 lots of 500 ml, and ground with 3 volumes of 0.5 M mannitol, 5 mM EDTA, 0.5% BSA, 0.05% cyseine, and 50 mM TES (pH 7.4) in a large mortar and pestle. The homogenate was strained

through 2 layers of Miracloth (Calbiochem) and centrifuged at 900 g for 8 min. The supernatant layer was removed and centrifuged at 19,000 g for 20 min. The pellet was suspended in 50 ml of 0.3 M mannitol, 0.3% BSA, and 25 mM TES (pH 7.2), and centrifuged at 23,000 g for 15 min. The pellet thus obtained was resuspended in 250 mM sucrose and 50 mM TES (pH 7.0)

2.4 Preparation of SMP

One batch of mitochondria, preferably fresh (see Appendix 2), was divided into two lots and each was diluted to 60 ml (120 ml total) with 250 mM sucrose and 50 mM TES (pH 7.0). The diluted mitochondria were sonicated on ice at 90% full power with an Artek Sonic Dismembrator (model 300 large tip) for 2 one min bursts separated by a one minute cooling period. The temperature of the sonicate was thus kept below 5 C. The same beaker was always used to contain preparations during sonication. The sonicate was centrifuged at 23,000 g for 15 min. to remove unbroken mitochondria. The supernatant layer was centrifuged for one hour at 100,000 g and the pellet was resuspended in 2-4 ml of 250 mM sucrose and 50 mM TES (pH 7.0) and frozen at -20 C in 1 ml containers sealed with rubber bungs. For storage for longer than two weeks, the frozen SMP was stored in liquid nitrogen in unsealed tubes.

Some batches of mitochondria were resuspended in 10 mM magnesium chloride, 30 mM mercaptoethanol, and 20 mM TES (pH 6.9). These mitochondria were sonicated for four one minute bursts with a one minute cooling period between each sonication. The SMP were resuspended in 0.5 mM EDTA, 1 mM magnesium sulphate, 0.5 mM dithiothreitol, and 10 mM TES (pH 7.5) and stored as above. The resulting SMP (SMP2) had a specific activity 5 to 10 times higher (0.3-1.0 umoles Pi released/min/mg protein) than ordinary SMP (0.05-0.1 umoles Pi released/min/mg protein) but the total protein content was lower. This will be discussed further in a later chapter.

2.5 Preparation of Cholate Extract (CE)

ATPase was solubilized from SMP by the method of Serrano *et al* (1976), slightly modified as follows. Five ml of SMP or SMP2 (protein content 15-25 mg/ml) were thawed and brought to 10% saturation of ammonium sulphate by the addition of solid ammonium sulphate. Sodium cholate (25% w/v) was added to give a concentration of 0.6 mg cholate /mg protein and gently stirred until the ammonium sulphate had dissolved and the cholate dispersed. The resulting dark orange brown mixture was immediately centrifuged at either 100,000 g for one hr or 235,000 g for 35 min. The supernatant layer (CE) was divided into 1 ml aquilots and frozen at -20 C. If it was to be stored for more one week the frozen aquilots were stored in liquid nitrogen as described above.

Initial experiments to determine the extraction conditions were performed under the conditions described in Chapter 3 in a Beckman Airfuge with an A100 rotor spun at 145,000 g at 25 C.

2.6 Column Chromatography with Affigel Blue

Affigel Blue was rinsed and degassed at room temperature in double distilled water. The slurry was packed into a 1 cm by 10 cm column and washed with five volumes of Eluting Buffer (250 mM sucrose, 1 mM magnesium sulphate, 1.0% sodium cholate, and 50 mM TES (pH 7.0 or pH 8.0)), at 0.5 ml/min. At either 2 C or 25 C, 0.35-0.70 ml of CE were loaded onto the column. One volume of Eluting Buffer was then pumped through, followed by three volumes of Eluting Buffer whose pH increased linearly from 7.0 (or 8.0) to 11.0 (or 11.3) and one volume of pH 11 (or 11.3) buffer (the high pH wash). The pH 11.0 (or 11.3) buffer was identical to the Eluting Buffer except that it was buffered with 50 mM glycine instead of TES. The OD of the eluant was monitored at 280 nm with a Pharmacia Duo Optical Monitor and collected in one ml fractions by an LKB Ultrorac Fraction Collecter. Fractions were assayed for ATPase activity and protein content by the methods described below.

2.7 Ammonium Sulphate Precipitation

This procedure was modified slightly from that of Serrano *et al* (1976). Cholate extract (0.5 or 1.0 ml) was brought to 38% saturation by the addition of 0.45 ml of saturated ammonium sulphate/ ml CE while stirring constantly. The solution was immediately spun at 23,000 g for 20 min. The supernatant layer was carefully decanted and brought to 45% saturation by the slow addition of 0.127 ml of saturated ammonium sulphate/ ml of beginning CE. The solution was stored on ice for 20-30 min with brief mixing every five min and subsequently spun at 23,000 g for 20 min. The supernatant layer was discarded and the small pellet resuspended in 50 ul of 50 mM sucrose, 0.5 mM EDTA, 1.0 mM magnesium sulphate,0.5 mM dithiothreitol and 10 mM TES (pH 7.5).

2.8 Ammonium Sulphate Precipitation Chromatography

Expanded and degassed Sephadex G-25 (48 ml) was packed into a 1.5 cm by 30 cm column and equilibrated with two volumes of 50 mM sucrose, 0.5 mM EDTA, 1.0 mM magnesium sulphate, 0.5 mM dithiothreitol, 1.0% sodium cholate, 10 mM TES (pH 7.5), and 50% saturated ammonium sulphate. One and a half ml of CE that had been centrifuged in 38% ammonium sulphate was loaded onto the column, and was subsequently eluted with 50 ml of a 50% to 38% saturation linear ammonium sulphate gradient in equilibration buffer at a flow rate of 0.5 ml/min. Fractions were collected and analysed as described above.

2.9 Sucrose Density Gradient Centrifugation

The procedure of Serrano *et al* (1976) was modified as follows. CE (0.8 ml) was layered onto 4.7 ml linear sucrose gradient, from 0.35 M to 0.70 M, containing 0.5 mM EDTA, 1 mM magnesium sulphate, 0.5 mM dithiothreitol, 1.0% sodium cholate, and 10 mM TES (pH 7.5). The gradient was centrifuged for 6 hr at 235000 g and collected by siphoning off measured fractions. The fractions were assayed as described below.

2.10 Percoll Density Gradient Centrifugation

Two different types of Percoll density gradients were used: preformed linear gradients and self-generating gradients.

Ten ml preformed gradients from 0-95% Percoll, 25-75% Percoll, and 0-50% Percoll were made with a Buchler gradient maker and contained 0.5 mM EDTA, 1 mM magnesium sulphate, 0.5 mM dithiothreitol, from 0 to 3% sodium cholate, and 10 mM TES (pH 7.5). Half a ml of CE was layered onto the gradient and centrifuged at various speeds for various lengths of time (see Chapter 3).

For experiments using self-generating gradients 7.5 ml of medium A (0.5 mM EDTA, 1 mM magnesium sulphate, 0.5 mM dithiothreitol, and 10 mM TES (pH 7.5)) in 95% Percoll, 2.1 ml of medium A, 0.4 ml of 25% sodium cholate, and 0.5 ml of CE were mixed together in a tube and centrifuged at various speeds for various times (see Chapter 3).

The gradients were removed by siphoning and fractioned into one ml volumes. ATPase activity and protein were assayed as described below.

2.11 Polyacrylamide Gel Isoelectric Focusing

The method of Eastwell (1980) was used as follows. Gels (7.5% acrylamide/ 2.5% BIS- acrylamide) containing 0.19% N,N'-methylene bis-acrylamide (Bio-Rad, Richmond, Calif.), 7.31% acrylamide, 5% glycerol (v/v), 1.0% (v/v) Ampholine pH 5/7, 1.0% (v/v) Ampholine pH 7/9, 0.1% (v/v) TEMED, and 0.5 mg/ml ammonium persulphate were mixed and cast in 3.5 mm ID glass tubes at 5 C after 20 min degassing. The gels were 10 cm long.

After curing the gels for 16 hr the sample was applied as a 20% (v/v) glycerol solution, and overlaid with 25 ul of 1.0% (v/v) Ampholine pH 7/9, and 5% (v/v) glycerol. The anode electrolyte was 40 mM glutamate, and the cathode electrolyte was 100 mM ethanolamine. The gels were run at 18.8 V/cm for 17 hrs, then 75 V/cm for 30 min, and finally at 100 V/cm for one hr with a Buchler model 3-1014 power supply. Immediately after focusing, the gels were placed on ice to reduce diffusion.

The gels were stained overnight for protein with Reisner's stain (Reisner *et al*, 1975), which contains 0.04% (w/v) Coomassie Brilliant Blue G-250 in 3.5% perchloric acid. The gels were then soaked in 7% (v/v) acetic acid for 2 hr and then transferred to 7% (v/v) acetic acid and 5% (v/v) methanol at approximately 45 C. The solution was stirred continuously for two days in a diffusive destainer (model 172A, Bio-Rad Laboratories, Richmond, California. The gels were scanned at 490 nm in a 10 cm gel boat with a Cary 219 Spectrophotometer.

Gels were stained for ATPase activity for two days at room temerature with Horak's stain (Horak, 1972) (300 mM sucrose, 50 mM calcium chloride,5 mM ATP (added separately), and 25 mM TES (pH 8.0)). The gels were scanned at 578 nm. ATPase activity was revealed by a white band of calcium phosphate precipitate.

2.12 SDS Polyacryamide Gel Electrophoresis

Slab gels were made by the method of Laemmli (1970), as follows. A 10 cm long separation gel was cast in a Bio-Rad model 220 Dual Vertical Slab Gel Electrophoresis Cell. The gel consisted of 12.5% acrylamide, 0.33% N,N'-methylene bis-acrylamide, 0.1% SDS, 0.025% (v/v) TEMED, 0.025% ammonium persulphate, and 0.375 M Tris-HCl (pH 8.8). A stacking gel 1 cm long was cast on top of the separation gel and consisted of 3% acrylamide, 0.08% N,N'-methylene bis-acrylamide, 0.1% SDS, 0.025% (v/v) TEMED, 0.025% ammonium persulphate, and 0.125 M Tris-HCl (pH 6.8). The electrolyte contained 0.1% SDS, 0.192 M glycine, and 0.025 M Tris-HCl (pH 8.3). The sample, in 2% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.0001% Bromophenol blue, and 0.0625 M Tris-HCl (pH 6.8), was incubated at 100 C for 90 sec before application onto the gel.

The gels were run at room temperature with a Bio-Rad model 500 power supply at 20 mV for 1 hr and at 70 mV for 1.5 hr. Upon completion of the run, the slabs were immediately stained with Reisner's stain as described above.

2.13 ATPase Assays

The ATPase assay that was used in the initial stages (up to the studies on the effects of detergents on ATPase) of this research was that of Malhotra and Spencer (1974). The assay medium contained 300 mM sucrose, 3 mM magnesium sulphate, and 25 mM TES (pH 8.0). The reaction was started by the addition of SMP or enzyme, or ATP to a concentration of 3 mM and a final volume of 2 ml. The reaction ran for 10 min at 30 C and was quenched by 2 ml of ice cold 120 mM glycine, 1.8 M sodium perchlorate and 0.3 N HCl. After incubation on ice for 6 min the tubes were centrifuged to remove protein, and 2 ml were removed to 2 ml of Mozersky's (1966) molybdate reagent (2.1 N sulphuric acid, 600 mM sodium perchlorate, and 12.5 mM ammonium molybdate). Four ml of isobutanol:benzene (1:1) were added to the mixture, which was then stirred vigorously for 10 sec. The two phases were separated by centrifugation, and the presence of oxidized phosphomolybdate complex in the hydrocarbon phase was quantified by measurement of the OD at 313 nm in a quartz cuvette.

This method was subsequently modified. The reaction volume was reduced to 1 ml, as was the volume of quench medium. The protein precipitation step was omitted, and the entire 2 ml reaction/quench mixture treated as before.

The removal of the protein precipitation step had two advantages. Firstly, because the volume was halved, the amounts of enzyme and substrate required for the same reading were halved. Secondly, the time taken to perform an assay was reduced as a result of removal of a pipetting step and a centrifugation step, and because the capacity of the centrifuge had previously limited the assay to only twelve tubes at a time.

The protein (if any) that was previously precipitated, banded at the hydrocarbon/water interface in the last step of the assay. When using SMP or higher specific activity preparations, the assay was found to give identical results to the unmodified method of Malhotra and Spencer (1974).

Later in the project a new assay was developed to even further reduce the length of the time required (see Appendix 1). The reaction conditions were left unchanged and the Pi detection method of Serrano *et al* (1976) was used,

modified as follows. One ml of reaction medium was quenched with 2 ml of 0.72 N sulphuric acid and 0.7% ammonium molybdate. Any resulting turbidity was cleared by addition of 100 ul of 10% (w/v) SDS. To develop colour, 50 ul of freshly prepared 1%(w/v) sodium ascorbate was added. After 10 min, the reduced phosphomolybdate complex was measured by recording the OD at 750 nm.

This assay was considerably faster than either of the previous assays. Turbidity from protein was rarely a problem, so that after quenching the reaction, only one step i.e. adding and mixing in ascorbate, was required before reading the amount of Pi released. The assay was thus found to be more reproducible, and was linear to over 300 nmoles of Pi (see Appendix 1). The sensitivity of this assay (100 nmoles Pi=0.14 OD at 750 nm) was approximately one fifth of that of the previous assay (100 nmoles Pi=0.70 OD at 313 nm) and one third of the first assay (100 nmoles Pi=0.40 OD at 313 nm). However, because of its better reproducibility, and speed, it was considered much more useful. It was used in all of the experiments done after the cholate extraction procedure was optimized.

One unit of ATPase was defined as 1 umole of Pi released/min under the assay conditions described in this chapter.

2.14 ATPase Assay with Regeneration of ATP

During kinetic experiments an enzyme trap was employed to remove ADP and maintain the initial ATP concentration. One ml of the assay medium (2 mM magnesium sulphate, 50 mM KCl, 2 mM PEP, and 25 mM TES (pH 8.0)) contained 50 ugm of pyruvate kinase. The enzyme was added and the mixture incubated at 30 C for 10 min. Then the reaction was started by the addition of equal volumes of 100 mM sodium ATP and 100 mM magnesium sulphate. After 10 or occasionally 15 min the reaction was quenched and assayed for released Pi with the reduced molybdate assay.

2.15 Protein Assay

Protein was assayed by the method of Sedmak and Grossberg (1977) as follows. A sample containing from 10 to 50 ugm of protein was diluted to 2 ml with double distilled water. To this, 2 mls of protein dye containing 0.06% Coomassie Brilliant Blue G-250 and 3.0% (w/v) perchloric acid were added and after a timed wait of 5 to 10 min, the OD at 625 nm was read.

2.16 Cytochrome Determination

Cytochromes a, b, c1, and c were determined by the method of Williams (1964). To each of two 1 cm light path cuvettes were added 100 to 200 ul of sample, 200 ul of 120 mM potassium phosphate (pH 7.4 and 200 ul 10% sodium deoxycholate. To one cuvette 100 ul of 50 mM potassium ferricyanide was also added, and to the other was added 100 ul of 50 mM sodium ascorbate and a few grains of sodium hydrosulphite. The contents of both cells were mixed by inversion and a difference spectrum recorded from 500 to 630 nm. From the curve, the optical density differences of the following wavelength pairs were calculated: Aa=550-535 nm, Ab=554-540 nm, Ac=563-577 nm, and Ad=605-630 nm. From these values the following mathematical expressions were calculated;

$$B=Aa/21.0$$

$$C=(Ab-6.51B)/15.6$$

$$D=(Ac+1.16B-1.48C)/13.8$$

$$E=(Ad+0.22B+0.482C-0.076D)/12.0$$

From these expressions the concentration of each of the cytochromes was calculated as follows;

cyt a conc.(umol/ml)=Xd=E

cyt b conc.(umol/ml)=Xc=D+0.0263Xd

cyt c1 conc.(umol/ml)=Xb=C-0.0484Xd-0.225Xc

cyt c conc.(umol/ml)=Xa=B-0.03Xd+0.149Xc-0.491Xb

2.17 Assay for NADH dehydrogenase

The NADH dehydrogenase assay that was used was modified slightly from that of King and Howard (1967) by reducing the reaction volume to 2.88 ml and increasing the concentration of the reactants accordingly. The OD of the reaction media (1.74 or 0.87 mM potassium ferricyanide, 41.7 mM potassium phosphate (pH 7.4), and 0.16 mM NADH at 30 C) was recorded at 420 nm against a water blank. After the non-enzymatic decrease in OD became linear with time, 10 to 50 ugm of enzyme was added and the absorbance decrease recorded for 1 min. To convert the decrease in OD 420 nm to mM NADH a factor of 0.5 was used.

3. RESULTS AND DISCUSSION: SOLUBILIZATION AND PURIFICATION OF FoF1

3.1 Preliminary Testing of Detergents

In late 1978 when this project was begun, the three best FoF1 solubilizations and purifications that were available were those of Sone *et al* (1975), Serrano *et al* (1976), and Stiggall *et al* (1978). To solubilize FoF1, the procedures used pre-extraction of the membranes with cholate and then solubilization with 2% Triton X-100, solubilization with 1.5% cholate, and solubilization with 0.3 mg deoxycholate/g protein respectively. Therefore, cholate, deoxycholate, and Triton X-100 were investigated as suitable agents for the solubilization of FoF1.

3.1.1 Effects of Detergents on the ATPase Assay

Sodium cholate was found to have little effect on the ATPase assay when used at concentrations of 1.0% or less (Fig. 1). The detergent Triton X-100 caused cloudiness of the isobutanol-benzene mixture at concentrations greater than 0.1%, and thus increased the OD at 313 nm. Deoxycholate also increased the OD at 313 nm, but at all concentrations. The increases in OD may have been caused by a reaction of the detergent with the molybdate or phosphomolybdate complex. Triton X-100 may even react with isobutanolbenzene, or perhaps the Kraft point (Helenius and Simons, 1975) of Triton X-100 in isobutanol-benzene is above 25 C and thus the cloudiness was a crystalline suspension of Triton X-100.

3.1.2 Effects of Detergents on the ATPase

Increasing the concentration of cholate in the presence of a fixed amount of SMP resulted in a 200% increase in the OD at 313 nm (Fig. 2). Since, as shown in Figure 1, cholate itself does not affect the assay except at the higher concentrations, cholate must have stimulated the ATPase. This could have been achieved through breakdown of the membrane structure and thus the PMF, or by release of the ATPase inhibitor protein (Serrano *et al*, 1976). Previous experiments in this laboratory showed that the uncoupler CCCP, did not

Figure 1. Effects of Detergents on the ATPase Assay

The standard assay mixture (in 1 ml final volume) was 300 mM sucrose, 3 mM magnesium sulphate, and 25 mM TES (pH 8.0). One hundred nmoles of Pi and appropriate amounts of detergent (25% w/v) were added to the final concentrations shown. The assay was performed as described in Chapter 2. (□): sodium cholate; (○): Triton X-100; (△): sodium deoxycholate.

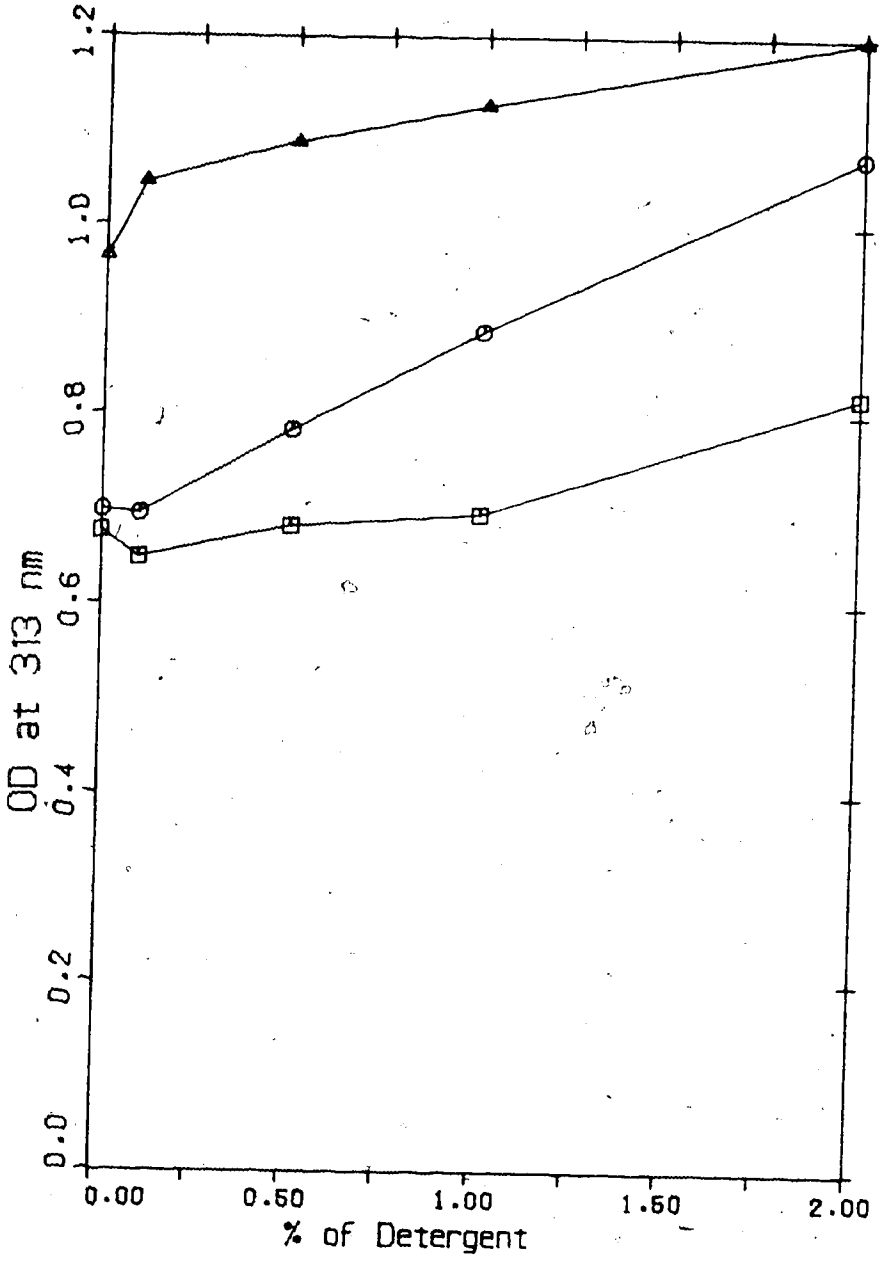
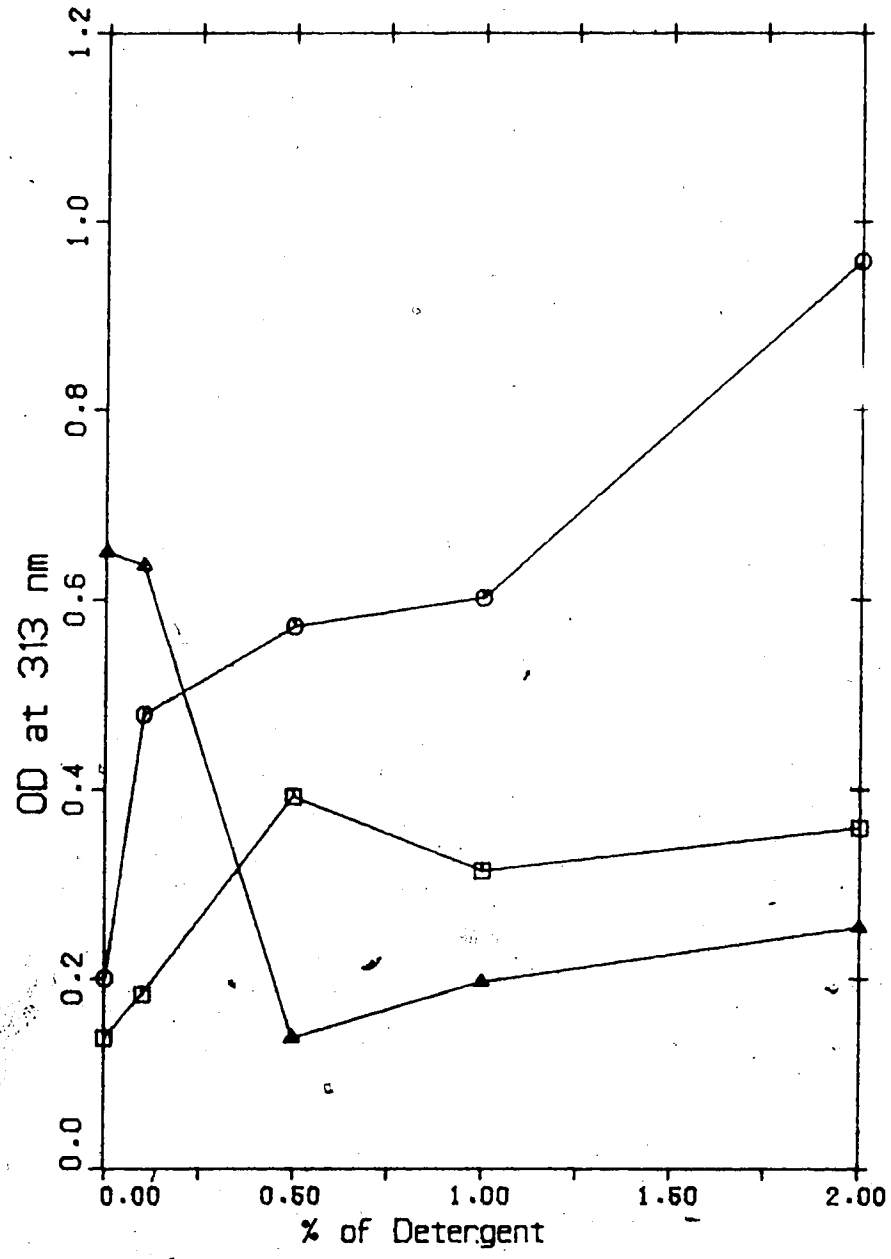

OD at 313 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.50
1.00
1.50
2.00
% of Detergent

Figure 2. Effects of Detergents on the Apparent Activty of the ATPase

Assay conditions as in Figure 3 except that 100 nmoles of Pi was replaced by 19 ugm of protein. After incubation of the assay mixture at 30 C for 10 min each reaction was started by addition of ATP to a final concentration of 3 mM. The Pi detection assay was performed as described in Chapter 2. (□): sodium cholate; (○): Triton X-100; (△): sodium deoxycholate.


OD at 313 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.50
1.00
1.50
2.00
% of Detergent

stimulate the ATPase of SMP in this system (Grubmeyer, 1978). Subsequent experiments (see Chapter 4) demonstrated that aging caused only a 40% stimulation of activity in CE, whereas aging of SMP stimulated the ATPase activity 10 fold. Aging is believed to cause release of the ATPase inhibitor (Grubmeyer, 1978). This suggests that the activation of ATPase by cholate was a result of dissociation of the inhibitor polypeptide. As would be expected if this was true, the stimulation did not continue indefinitely as the concentration increased.

Between 0 and 0.1%, Triton X-100 caused a rapid increase in the apparent activity of the ATPase and from 0.1% onwards, a steady increase in apparent activity at about the same rate as Triton X-100 affected the assay itself (Fig. 2). Therefore, low concentrations of Triton X-100 also caused a large increase in the apparent activity of the ATPase, perhaps by the same mechanism as did cholate.

In order to reconstitute energy-transducing activities, it is necessary to remove most of the detergent used for solubilization. It has been found that non-ionic detergents such as Triton X-100 are more difficult to remove by mild methods, eg. dialysis, than cholate and deoxycholate (Helenius and Simons, 1975). Although reconstitution of these activities was not attempted in this study, it was concluded that Triton X-100 would be unsuitable for FoF1 solubilization since it also affected the ATPase assay itself. (See below also).

Increasing concentrations of deoxycholate progressively deactivated the enzyme (Fig. 2). Deactivation by deoxycholate has been reported by other authors (eg. Soper and Pedersen, 1976) and although the mechanism is unknown, it may possibly be caused by an unfavourable interaction between Fo and the hydrophobic environment created by deoxycholate. However, this inhibition may be released by removal of the deoxycholate (Tzagoloff *et al*, 1968).

3.2 Preliminary Detergent Extractions

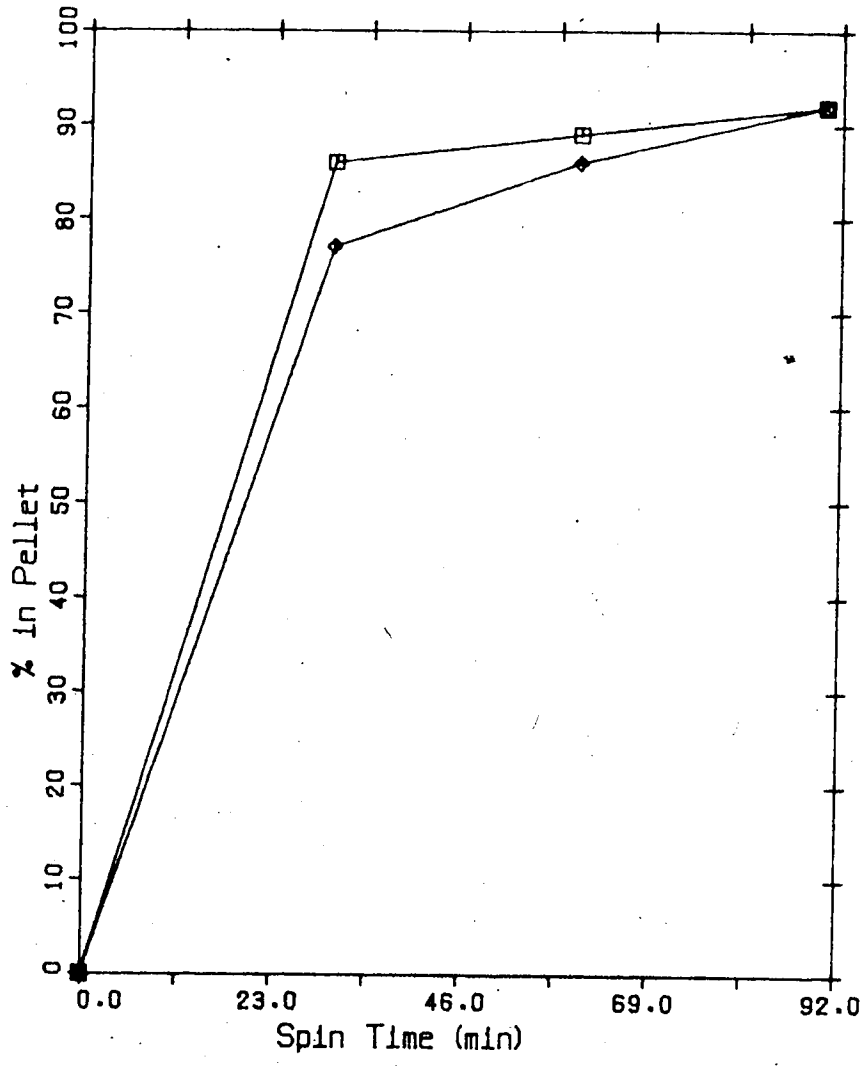
Detergent extraction of FoF1 from membranes usually requires high protein concentrations (from 10-30 mg/ml). Therefore, in order to reduce the amount of SMP used in the experiments to determine the optimum detergent extraction conditions, these experiments were performed in a Beckman Airfuge as described in Materials and Methods. Each tube in the Airfuge rotor has a capacity of 150 ul and can be spun at speeds of up to 145,000 g. In control spins it was found that almost 90% of activity sedimented in less than 30 min and that a further 5% could be sedimented by 90 min of centrifugation (Fig. 3). It was suggested (personal communication with Beckman, Inc.) that this latter finding may be caused by "wall effects" resulting from the small size of the tubes.

A scaled down cholate extraction by the method of Serrano *et al* (1976) resulted in 82% of the total protein, and 93% of the total ATPase activity remaining in the supernatant layer after 30 min at 145,000 g. Since the activity was still in the supernatant layer, it was judged to be soluble (Serrano *et al*, 1976). Upon addition of cholate, the ATPase activity became insensitive to oligomycin although sensitivity to DCCD was not affected (data not shown). There was no activation of the enzyme by cholate. Deoxycholate extraction of SMP under the conditions described by Stiggall *et al* (1978) resulted in deactivation of 80% of the ATPase (data not shown). Furthermore, although 89% of the protein was solubilized, there was no detectable ATPase activity in the supernatant layer. It would seem, therefore, that the latter method was too harsh for this enzyme, while the former gave good results.

The procedure of Sone *et al* (1975), which produced the most highly purified FoF1 preparation, involved pre-extraction of the membranes with 1% cholate and 4.5% saturated ammonium sulphate and subsequent solubilization of the ATPase with Triton X-100. Since the preextraction conditions are almost the same as those used successfully in the paragraph above, and because Triton X-100 affected the assay itself (Fig. 1) and is difficult to remove (Helenius and Simons, 1975), solubilization with FoF1 by Triton X-100 was not pursued further.

Figure 3. Rate of Precipitation of SMP during Centrifugation in Airfuge A100 Rotor

One hundred and fifty ul of SMP (23 mg/ml) were centifuged at 145,000 g for the time indicated at 25 C. Protein and ATPase activity were determined as described in Chapter 2. (□): % of total ATPase activity present after centrifugation that was in the pellet; (◇): % of total protein in the pellet.


100
90
80
70
60
50
40
30
20
10
0
% In Pellet
0.0
23.0
46.0
69.0
92.0
Spin Time (min)

The cholate extraction procedure was thus the most promising technique.

3.3 Optimization of the Cholate Extraction Procedure

Since the FoF1 of peas may have properties different to those of the FoF1 of beef heart, the parameters of cholate extraction were varied to determine the optimum extraction conditions for pea SMP. Serrano *et al* (1976) incubated beef heart SMP in cholate and 10% saturated ammonium sulphate for 7 min before centrifugation down of insoluble protein. It was found that incubation of pea SMP in the same solution reduced the specific activity of the ATPase, the yield of ATPase in the supernatant layer, and especially the sensitivity of the enzyme to DCCD (Fig. 4). Incubation thus appeared to allow the detergent to damage the ATPase.

Since the cholate molecules must bind to the protein and lipid molecules to "cover" the hydrophobic regions, it is not the concentration of cholate that is important, but the ratio of cholate to protein. When less than 0.6mg of cholate/mg of protein was used, the percentage and the specific activity of FoF1 solubilized were significantly reduced (Fig. 4). Higher ratios of cholate:protein lower the specific activity and DCCD sensitivity of FoF1 (Fig. 4), both of which are obviously undesirable. At these higher levels of cholate, the detergent molecules may displace too many phospholipids from around Fo and thereby interfere with the functioning of FoF1, or the detergent molecules may even be so numerous as to bind in positions that effectively block proton movement through the pore. In any case, more cholate did not solubilize more ATPase, and 0.6g cholate/g protein was thus concluded to be the optimal ratio. This was the same ratio as used by Serrano *et al* (1976).

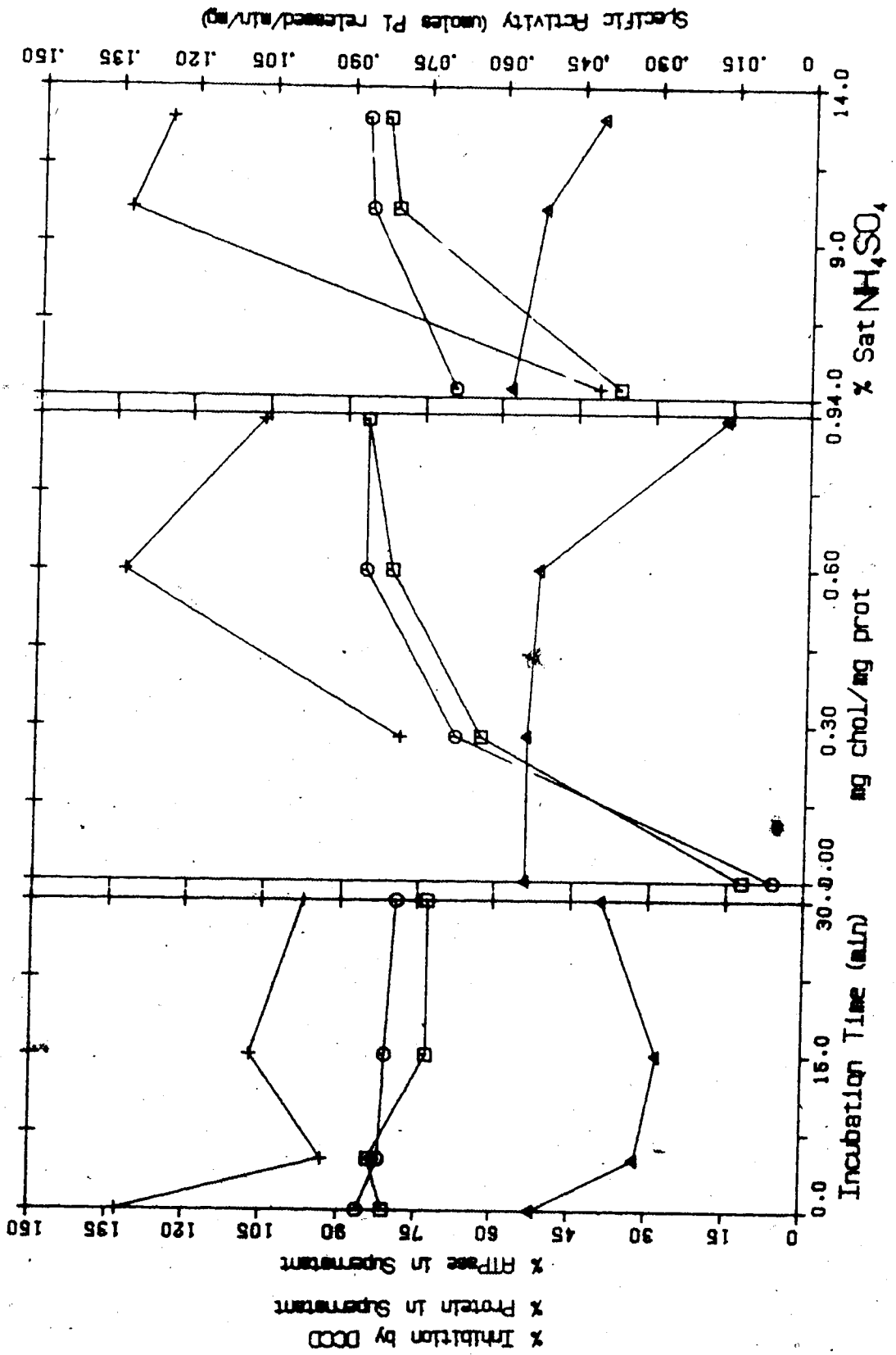
Ionic strength is known to greatly affect the effectiveness of the bile salts (Tzagoloff and Penefsky, 1971) probably by changing the CMC [1] and micellar properties (Helenius and Simons, 1975). Serrano *et al* (1976) noted that the use of 2% saturated ammonium sulphate resulted in the solubilization of only 20% of the ATPase. In our system, it was found that a lower concentration of

---

[1] critical micellar concentration

Figure 4. Optimization of the Cholate Extraction Procedure

Solid ammonium sulphate and sodium cholate (25% w/v) were added to final concentrations of 10% saturated and 0.6 mg/mg protein (or as otherwise specified), to 150 ul of SMP (23 mg/ml) in 250 mM sucrose and 50 mM TES (pH 7.0). The ammonium sulphate and cholate were quickly dissolved by pipetting, and the resulting translucent mixture was either immediately centrifuged, or incubated at 0 C for the indicated time interval. The mixture was centrifuged in a Beckman Airfuge at 145,000 g for 30 min at 25 C. Protein and ATPase activity were determined as described in Chapter 2. In appropriate assays, DCCD was added to a concentration of 0.1 mM ???? (○): % of total ATPase activity after centrifugation, that was in the supernatant layer; (□): % of total protein that was in the supernatant layer; (△): % inhibition by DCCD of the ATPase activity of the supernatant layer; (+): specific activity of the supernatant layer (umoles Pi released/min/mg protein).


% Inhibition by DCCD
% Protein in Supernatant
% ATPase in Supernatant
Incubation Time (min)
mg chol/mg prot
% Sat NH_4SO_4
Specific Activity (moles Pi released/min/mg)

ammonium sulphate also solubilized much less ATPase, although the solubilized FoF1 did have a higher sensitivity to DCCD than FoF1 released with 10% ammonium sulphate (Fig. 4). When the concentration of ammonium sulphate was raised above 10% saturation, no release of extra FoF1 was observed, and the sensitivity of the enzyme to DCCD was reduced. Thus the optimal ammonium sulphate concentration was 10% saturated, the same as used by Serrano *et al* (1976).

Interestingly, when scaled up to a 5 ml volume (see Chapter 2), the optimized procedure increased the total ATPase activity 2-3 fold. A similar effect was noted earlier (see Testing of Detergents) and by Serrano *et al* (1976) , who suggested that it may be caused by dislocation of the inhibitor protein. Three results supported this sugestion. First, as discussed earlier (see Testing of Detergents), the cholate stimulation was probably not caused by release of inhibition by proton motive back pressure. Secondly, the ATPase activity of CE was increased by only 40% (see Chapter 4) by agin[illegible]ch dissociates the inhibitor protein. Thirdly, SMP2, which were made [illegible] the presence of dithiothreitol, did not show an increase in total ATPase activity after extraction with cholate. Dithiothreitol is commonly used to release the inhibitor protein of chloroplast ATPase (Nelson, 1976) and is probably responsible for the 5-10 fold higher specific activity of SMP2 than is found in SMP.

3.4 Chromatography of CE on Affigel-Blue

Probably no other technique can purify a protein as mu[illegible]h in a single step, as affinity chromatography is potentially able to do. Failure of the technique is most often caused by poor binding of the protein to the immobilized ligand, or by poor release of the protein from the immobilized ligand. Often the ligand is inactivated during or by its immobilization.

In order to avoid at least this last pitfall, affinity chromatography with Affigel-Blue was attempted. Affigel-Blue consists of agarose beads to which Cibacron Blue F3GA is covalently bound. Cibacron Blue is a dye whose structure

is similar to that of adenosine, so Affigel-Blue will bind to all enzymes that have a dinucleotide fold, ie. most dehydrogenases and kinases.

High substrate/product concentrations, high pH, or high ionic strengths could be used to elute FoF1 from the dye, once bound. ADP is a potent inhibitor of the ATPase (Lowe *et al*, 1979), and ATP and Pi would require removal before fractions could be assayed. At high ionic strengths, the enzyme appeared to be unstable (see below). Therefore, a pH gradient was used to release the FoF1 from the dye.

When the column was not pre-equilibrated with cholate, the enzyme was inactivated (Table 1) probably because the agarose support acted as a molecular sieve that separated the enzyme from the cholate, thus causing aggregation and deactivation. Previous workers have noted an increase in apparent molecular weight, and partial deactivation upon detergent removal without its replacement by phospholipids or other detergents (eg. Carmeli and Racker, 1973; Pick and Racker, 1979). The total deactivation that ocurred suggests that pea FoF1 may be more fragile than FoF1 from other sources.

Pre-equilibration of the column with 1% cholate resulted in elution of 3 major protein peaks, one of which, the peak in the void volume, was considerably larger than either of the remaining peaks (Table 1). All of the ATPase activity (50% of the amount applied) coincided with the large protein peak in the void volume. Since the pH optimum of FoF1 is 8.0 and the activity drops sharply as the pH rises (see Chapter 4), it was concluded that FoF1 should be eluted between pH 8.0 and 11.3. The pH at which CE was loaded onto the column was raised to 8 to promote better binding of the enzyme. The protein peak eluted between pH 8.0 and 11.3 was considerably larger than that eluted with a pH 7-11 gradient, but the ATPase activity eluted was still found only in the void volume. To improve binding to the column even further, after the CE was loaded the column was incubated at 25 C for 30 min. Although the protein peak was even larger and squewed, no activity at all was eluted from the column (Table 1). The void volume was always cloudy, as if the previously solubilized intrinsic membrane proteins had for some reason come out of solution (Swanljung *et al*, 1973). It seemed unlikely that much FoF1 had

Table 1. *Affigel Blue Chromatography of CE*

The 1 cm by 10 cm column was prepared and run as described in Chapter 2. All manipulations were performed at 0 to 2 C except that in run 4 0.35 ml of CE was loaded onto the column and incubated at 25 C for 30 min. The eluant was fractionated and assayed as described in Chapter 2. In all runs, 0.7 ml of CE containing 1.550 units of ATPase activity, was loaded onto the column, except in run 4, which had only 0.35 ml of CE loaded.

	Conditions	ATPase activity			OD_{280} peak		
		void vol.	grad. vol.	high pH wash	void vol.	grad. vol.	high pH wash
1.	no cholate	none	none	none	-	-	-
2.	1% cholate, pH7-11	0.75	none	none	3.4	0.05	1.1
3.	1% cholate, pH8-11.3	0.08	none	none	3.6	0.1	0.3
4.	1% cholate, pH8-11.3, incubate at 25 C	none	none	none	3.4	0.2	0.1

bound to the dye but if it did it was either denatured by the high pH before it was released, or some factor that is necessary to maintain the active state of FoF1 may have been removed by the column.

3.5 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation is a gentle separation technique that has been used for many FoF1 purifications. Most FoF1s are precipitated between 25 and 50% saturation ammonium sulphate. Serrano *et al* (1976) found that precipitation of CE between 38 and 45% saturation ammonium sulphate gave a 200% increase in specific activity over CE, with 43% yield.

When this ammonium sulphate precipitation protocol was followed with pea CE, only 8% of the activity was recovered in the 38–45% precipitate fraction, but the specific activity was increased almost 5 fold (Table 2). Two different approaches were tried to increase the yield of FoF1 without significantly reducing the specific activity: incubation of the solution at 45% saturation ammonium sulphate before precipitation, and an increase in ammonium sulphate concentration for the second precipitation. A wait of 45 min increased the yield to 20% (Table 2), which is lower than that of Serrano *et al* (1976), but the specific activity was increased over CE by 350%, twice the increase obtained by Serrano *et al* (1976). Further work with ammonium sulphate precipitations showed that after approximately 20 min the yield did not continue to rise, while the specific activity declined.

These experiments suggested that the pea FoF1 was not highly stable at 0 C and high ionic strengths. This conclusion was supported by precipitation of the enzyme from higher ammonium sulphate concentrations. Both the 38–50% precipitate and the 38–55% precipitate fractions had reduced specific activities and reduced sensitivities to DCCD, although the yields were not increased (Table 2). Furthermore, the 70% or so of FoF1 activity that previously remained in solution after the 45% ammonium sulphate precipitation, was apparently destroyed by the higher ionic strengths. The beef heart FoF1 is more stable at ammonium sulphate concentrations of over 55% saturation (Serrano *et al*, 1976). Pick and

Table 2. *Ammonium Sulphate Precipitation of CE*

Precipitations were performed as described in Chapter 2, except for the following details. In experiment 1, the mixture was centrifuged immediately after bringing to 45% saturation of ammonium sulphate. In experiments 3 and 4, the concentration of ammonium sulphate was brought to 50% and 55% saturation, respectively, instead of 45% saturation, and the mixtures were centrifuged immediately. The specific activity of the CE was 0.105 units/mg.

Conditions	Specific activity (units/mg)			% of total Original ATPase			% ATPase recovered
	% saturation			% saturation			
	10-38	38-45	>45%	10-38	38-45	>45	
1. Serrano *et al*	0.053	0.580	0.089	12	8	80	100
% sens. to DCCD	100	100	63				
2. wait 45 min (control)	0.065	0.450	0.049	10	20	34	64
% sens. to DCCD	100	100	26				
3. to 50% sat.	0.044	0.174	0	10	22	0	32
% sens. to DCCD	100	40	-				
4. to 55% sat.	0.033	0.190	0	8	22	0	30
% sens. to DCCD	100	40	-				

Racker (1979) have, however, noted that although fractions from over 45% saturated ammonium sulphate had ATPase activity, they had low energy transducing capabilities.

Therefore, ammonium sulphate precipitations were done by the method of Serrano *et al* (1976) except for an incubation of 20 min in 45% saturated ammonium sulphate before the second spin. The 38-45% precipitate fraction was designated NSE (ammonium sulphate enzyme).

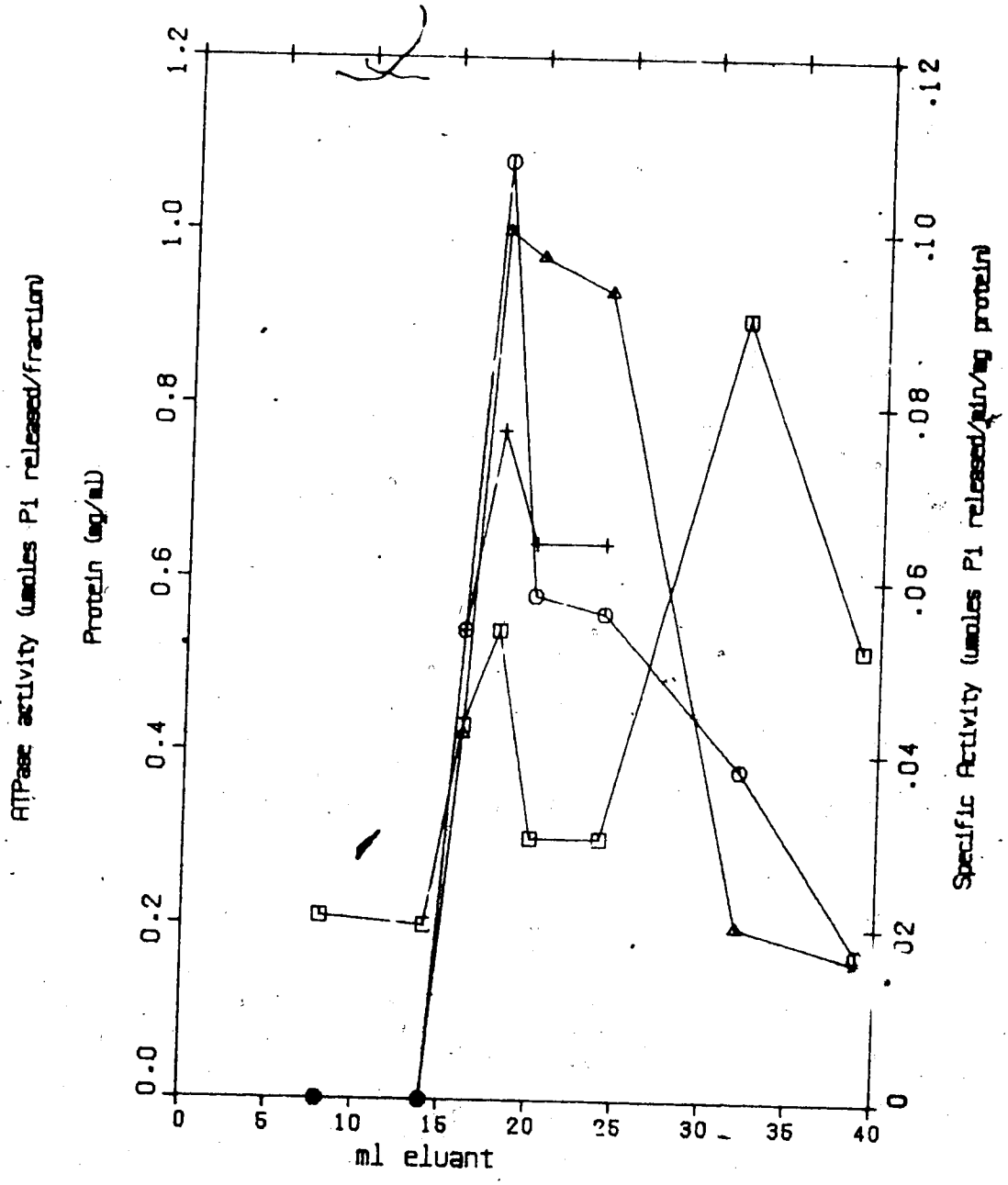
3.6 Ammonium Sulphate Precipitation Gel Chromatography

Since the ammonium sulphate precipitation step was successful, a modification of it was attempted to increase the purification even further. CE in 50% ammonium sulphate was washed through a molecular sieve column that excluded the FoF1, but not the ammonium sulphate, with a decreasing ammonium sulphate gradient. At 50% ammonium sulphate, FoF1 precipitates (Table 2) and thus should deposit on the column. As the ammonium sulphate gradient passes by the FoF1, a lower ammonium sulphate concentration at which FoF1 is soluble should be reached. When it redissolves, FoF1 would move into the void volume and should repass the critical ammonium sulphate concentration at which it becomes soluble (since ammonium sulphate would not also move in the void volume) and thus reprecipitate. Other proteins in CE should have different critical ammonium sulphate concentrations and should therefore be separated from FoF1 (see also Chapter 2).

Upon elution of the ammonium sulphate gradient, two protein peaks were observed, one of which overlapped a peak of ATPase activity (Fig. 5). However, the specific activity of the peak was 0.1 umoles Pi released/min/mg protein, which was 10 times lower than that of the CE loaded onto the column. This inactivation may have been caused by many factors, such as removal of a factor required for stability of the enzyme, or by the high ionic strength. The latter, however, should not by itself cause a 10 fold inactivation (see Table 2), so this technique was discontinued in favour of previously published procedures, such as sucrose density gradient centrifugation.

Figure 5.Ammonium Sulphate Precipitation Chromatography

The Sephadex G-25 column was prepared, equlibrated, loaded, and run as described in Chapter 2. The specific activity of the CE used was 0.68 umoles Pi released/min/mg protein. The volume of each fraction was 2 ml. (□): mg protein/fraction; (○): ATPase activity per fraction (umoles of Pi released); (△): specific activity (umoles Pi released/min/mg protein).


ATPase activity (umoles Pi released/fraction)
Protein (mg/ml)
Specific Activity (umoles Pi released/min/mg protein)
ml eluant
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
.02
.04
.06
.08
.10
.12
0
5
10
15
20
25
30
35
40

3.7 Sucrose Density Gradient Centrifugation

The method of Serrano *et al* (1976) was modified as described in Materials and Methods. The modification consisted of omitting lysolecithin, methanol and deoxycholate from the gradient. Serrano *et al* (1976) included these compounds in the gradient to prevent inactivation of the enzyme, but the compounds also destroyed the sensitivity of the enzyme to DCCD (Serrano *et al*, 1976). However, sensitivity to DCCD was necessary because it provided the only specific assay for FoF1, since no energy transformation reactions were measured during this research project.

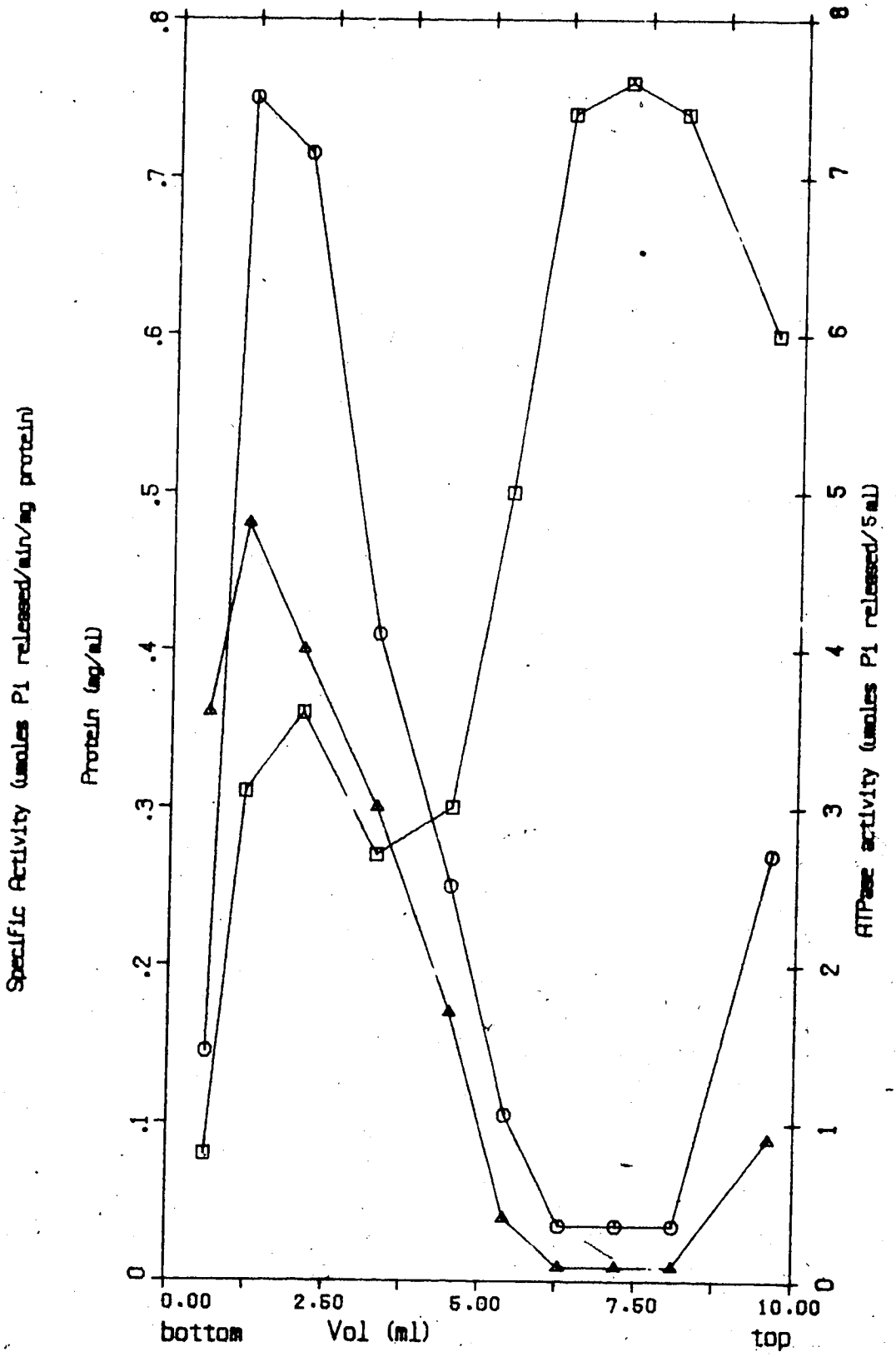
Two well separated protein peaks were obtained, the smaller of which had high ATPase activity (Fig. 6). The second larger protein peak probably contained adenine nucleotide transporter (Serrano *et al*, 1976). Floating on the meniscus of the tube was a smaller peak of ATPase activity, which possibly contained membrane fragments.

Although the density gradient provided very good separation, it also inactivated over 80% of the ATPase that was added (data not shown). The specific activity of the ATPase peak was 0.48 umoles Pi released/min/mg protein (compared to the CE, which was 0.68 umoles Pi released/min/mg protein), and contained 3–4% of the activity that was loaded onto the gradient. The deactivation may have been caused by removal of some protective factor(s), as suggested before, or the enzyme may be unstable and thus be dissociated during the long spin. The long spin time is required because dense sucrose solutions are also quite viscous, and molecular movement is thus slowed by high sucrose concentrations.

In a recent paper by Pick and Racker (1979), deactivation of chloroplast FoF1 during sucrose density gradient centrifugation was eliminated by addition of phospholipids to the gradient. The phospholipids apparently did not alter the position of FoF1 on the gradient. For future work with this enzyme, it might be fruitful to try this approach with sucrose density gradients, and perhaps with Percoll density gradients also (see below).

Figure 6.Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was performed and the fractions assayed as described in Chapter 2. The specific activity of the CE used was 0.68 umoles Pi released/min/mg protein. (□): mg protein/ml; (○): ATPase activity (umoles Pi released/ml); (△): specific activity (umoles Pi released/min/mg protein).


Specific Activity (umoles Pi released/min/mg protein)
Protein (mg/ml)
ATPase activity (umoles Pi released/5 ml)
0
.1
.2
.3
.4
.5
.6
.7
.8
0
1
2
3
4
5
6
7
8
0.00
2.50
5.00
7.50
10.00
bottom
Vol (ml)
top

3.8 Percoll Density Gradient Centrifugation

Percoll is a colloidal silica sol coated with polyvinylpyrrolidone to reduce its toxicity to biological systems. At 100% concentration (as it is supplied from Pharmacia), it has a density of 1.13 gm/ml, a viscosity of only 10 cP, and a low osmolality (less than 20 mOs/kg water). It therefore overcomes many of the problems normally associated with sucrose gradients by allowing separation of organelles by density gradients in as little as 20 min at as low as 10,000 g. It will even spontaneously form gradients at moderate g forces and spin times. Although successful separation by Percoll of particles smaller than large viruses has not been reported, purification of FoF1 on Percoll gradients was attempted because it was hoped that the short centrifugation times that would be necessary would minimize FoF1 inactivation.

It was expected that self generating Percoll gradients would give the best separation of FoF1 from contaminants , since such a gradient should range from 0–100% Percoll. Table 3 shows that no activity was recovered from self generated Percoll gradients. FoF1 may have been inactivated by the dilution, or as suggested above, by removal of some factor(s) that are neccessary for maintenance of an active conformation. The former is unlikely in view of the observation that when CE was applied into preformed Percoll gradients by pipetting through a bent pasteur pipette at the density at which the CE banded, the specific activity of the peak was no higher than when CE was layered on top of the gradient (data not shown).

It was found that if cholate was absent from preformed gradients, no separation was observed (Table 3). This is in agreement with previous authors (Carmeli and Racker, 1973, and Helenius and Simons, 1975) who have shown that removal of detergent from solutions of detergent solubilized proteins, eg. by dialysis, results in aggregation. Cholate concentrations of 0.5 and 1.0% resulted in good separations with the former giving a peak of slightly higher specific activity. At higher concentrations, cholate deactivated the enzyme. In most of the successful published procedures the detergent concentration is reduced, typically to 0.5%, after extraction of FoF1 from the membranes (eg. Sone *et al*, 1975, Foster and Fillingame, 1979). Higher detergent concentrations

Table 3. *Percoll Density Gradient Centrifugation*

Percoll gradients were made and assayed as described in Chapter 2. The gradients were centrifuged at the g force indicated for the time indicated. Conditions are expressed as the concentration range of the gradient in % undiluted Percoll.

Conditions	Centrifugation time (min)	Centrifugation force (g x 1000)	% Cholate	Separation	Specific Activity (% of CE)
self formed	30	60	1	none	0
self formed	55	80	1	none	0
self formed	30	100	1	none	0
25 to 75	45	60	0.0	none	0
25 to 75	40	30	0.5	good	180
25 to 75	40	30	1.0	good	120
25 to 75	40	30	2.0	—	0
25 to 75	80	30	3.0	—	0
25 to 75	20	30	1.0	medium	250
25 to 75	40	30	1.0	good	120
0 to 95	60	30	1.0	medium	60
25 to 75	40	30	1.0	good	120
0 to 50	40	30	1.0	good	68

may alter the hydrophobic environment of Fo, or interact more directly with the enzyme (eg. by blockage of the proton pore, or by disruption of hydrophobic bonds within FoF1.

A centrifugation step 40 min long at 30,000 g was observed to be sufficient to cause the FoF1 to move down into the gradient (see Table 3 and Fig. 7). Longer and/or faster centrifugations did not improve separation and usually precipitated a portion of the silica sol. A 20 min centrifugation gave a higher specific activity peak but did not result in a separation equivalent to a 40 min centrifugation. The 20 min centrifugation moved The ATPase only one cm or so into the gradient while other proteins moved no more than 4 cm into the gradient (Table 3). Once again it appeared that the pea FoF1 was inactivated by either dilution, or by removal of necessary stabilization factors.

This problem was encountered again while experimenting with different Percoll density gradient ranges. FoF1 banded at approximately 30% Percoll on 25–75% gradients (Fig. 7). This meant that on 0–95% and 0–50% gradients, the enzyme moved all the way down through a large portion of the gradient (Fig. 7), and apparently this caused deactivation. The ATPase peaks of both of these gradients had a lower specific activity than either the peak on 25–75% gradients or the CE.

3.9 Further Purification

Deactivation was observed again when the 38–45% saturated ammonium sulphate fraction (NSE) was centrifuged through a 25–75% Percoll density gradient, or on fractionation with 38–45% saturated ammonium sulphate of the ATPase peak of a 25–75% Percoll gradient (Table 4).

Therefore, before further work on the purification of this FoF1 is attempted, the nature of this deactivation must be elucidated. Is it caused simply by dilution or by removal of stabilization factor(s), as suggested above? Recent authors have shown that the presence of phospholipid is essential to ATPase activity (Berden and Voorn-Brouwer, 1978). Asolectin (an undescribed partially purified phospholipid preparation from soybean) has been shown to be effective

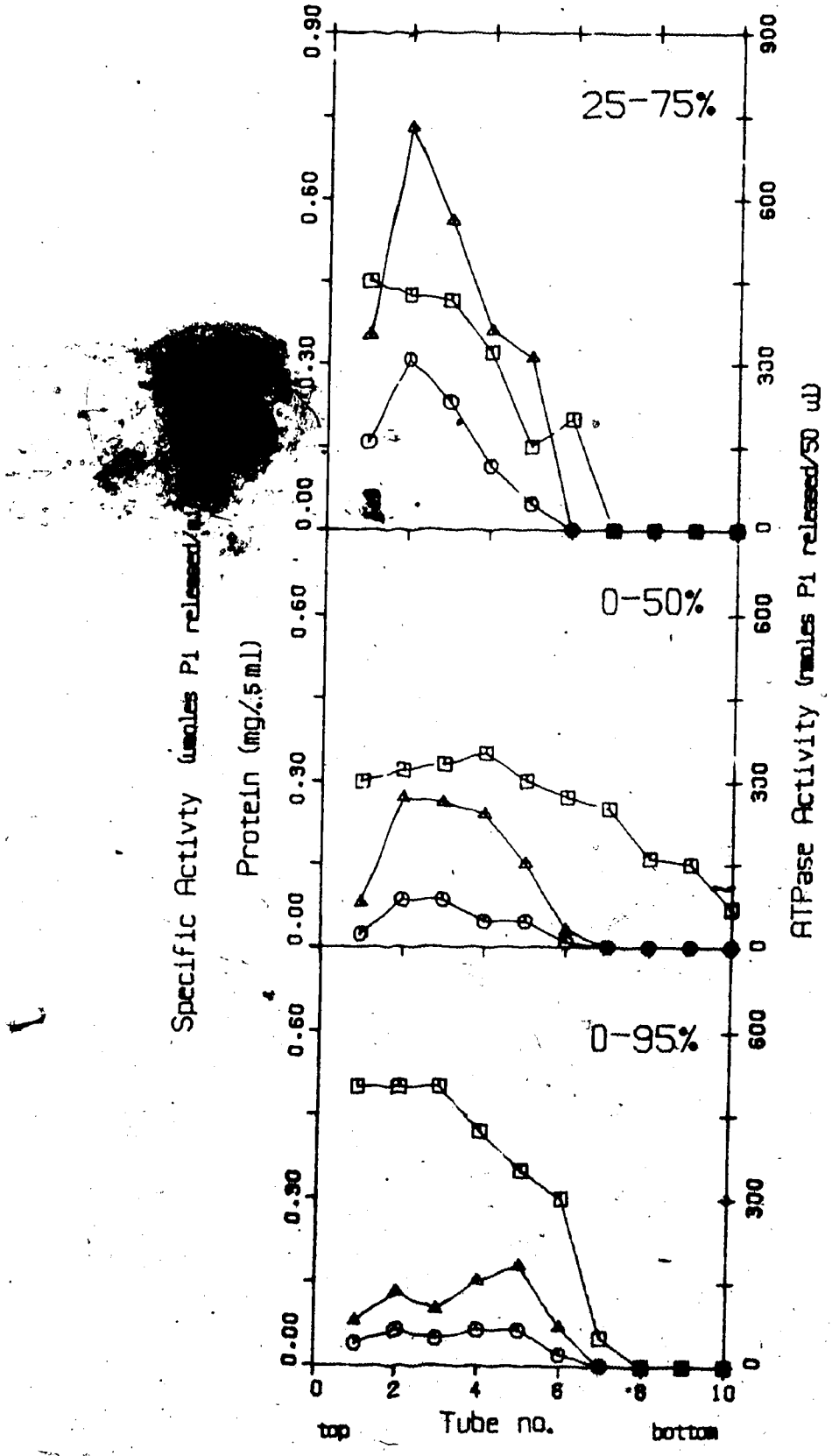
Table 4. *Further Purification of NSE*

The ATPase activity peak (1 ml) from a 25 to 75% Percoll density gradient was brought to 10% saturated ammonium sulphate and centrifuged at 23,000 g for 40 min. The supernatant layer was fractionated by ammonium sulphate precipitation as described in Chapter 2. In the second treatment, 100 µl of NSE was layered on top of a 25 to 75% Percoll density gradient (Chapter 2) and centrifuged at 30,000 g for 40 min. Assays were performed as described in Chapter 2.

Conditions	Specific activity before (units/mg)	Specific activity of target fraction after second step	% inactivation of total activity in second step
Percoll gradient then ammonium sulphate precipitation	0.70	0	63
Ammonium sulphate precipitation then Percoll gradient	1.2	0	100

Figure 7. Representative Examples of Percoll Density Gradients

Percoll density gradients of the ranges specified were made and centrifuged as described in Chapter 2, at 30,000 g for 40 min. Assays were performed as described in Chapter 2. (□): mg protein/ml; (○): ATPase activity (nmoles Pi released/50 ul); (△): specific activity (umoles Pi released/min/mg protein).


25-75%
0-50%
0-95%
Specific Activty (nmoles Pi released/
Protein (mg/.5 ml)
ATPase Activity (nmoles Pi released/50 ul)
0.90
0.60
0.30
0.00
900
600
300
0
0
2
4
6
8
10
top
Tube no.
bottom

in restoration of ATPase activity of phospholipid depleted preparations (Serrano *et al*, 1976). Since the pea FoF1 may be quite similar in such properties as phospholipid content to the FoF1 prepared by Serrano *et al* (1976), it is possible that the deactivation is caused by phospholipid depletion. (The FoF1 preparation of Serrano *et al* (1976) contains only 0.08 umole phospholipid/mg protein and is stimulated 10 fold by asolectin). However, the NSE was found to be stimulated by only 20% by addition of 100 ugm of soybean phospholipids (Sigma, 40% lecithin)/mg protein.

In any case, an increase in specific activity from 0.07 umoles Pi released/min/mg protein (SMP) to 0.93 umoles Pi released/min/mg protein (NSE) represents a 13 fold purification, barring stimulation and deactivation, which is a high purification compared to many other preparations. Highly purified FoF1 from mitochondria usually is purified 5-10 fold (eg. Serrano *et al*, 1976, Stiggall *et al*, 1978). Preparations of Fof1 from bacteria, gg. *E. coli*, when highly purified show a 15-20 fold purification (eg. Foster and Fillingame, 1979, Schneider *et al*, 1980). These figures presumably reflect the greater number of transport and other proteins in the latter source materials.

These results led us to forgoe further purification attempts, and to instead characterize the NSE preparation.

4. RESULTS AND DISCUSSION: FoF1 PROPERTIES

4.1 Purity of NSE

4.1.1 Enzymatic Assays for Impurities

In an effort to determine the purity of the NSE, the level of NADH dehydrogenase activity and the amount of cytochromes present were quantified. However, in all but a few experiments, the assays were not sufficiently sensitive to give accurate data with the amount of sample that was available. This was particularly true of the cytochrome assay. The phospholipid assay of Dittmer and Wells (1969) was also experimented with, but was found to require too much sample to obtain readings.

NADH dehydrogenase activity, although lower in activity in pea SMP than in other SMP (Serrano *et al*, 1976), was reduced 80% by extraction with cholate and fractionation with ammonium sulphate (Table 5). This compares favourably with the results of Serrano *et al* (1976) who reported an 87% reduction of NADH dehydrogenase activity after detergent extraction and ammonium sulphate fractionation. Sone *et al* (1975) showed only 0.05 umol of NADH were reduced per minute by one mg of their highly purified PS3 FoF1.

Negligible amounts of cytochrome a were detected in NSE (Table 5), comparable to the FoF1 preparations of Serrano *et al* (1976) and Stiggall *et al* (1978). Sone *et al* (1975) reported 0.02 nmoles cyt a/mg FoF1 preparation. The content of cytochrome b of NSE was reduced approximately 90% from that of SMP (Table 5). Serrano *et al* (1976) obtained a similar purification. However, the highly purified preparations of Sone *et al* (1975) and Stiggall *et al* (1978) both had significantly higher cytochrome b contents (0.06 nmol/mg and 0.15 nmol/mg respectively). The measured levels of cytochromes may be lower than the levels actually present because of the difficulties that were mentioned above of obtaining data. Cytochromes c1+c were reduced approximately 80% by the solubilization and ammonium sulphate fractionation procedure detailed in this thesis. Serrano *et al* (1976) obtained identical results with their procedure. The FoF1 purified by the procedure of Stiggall *et al* (1978) contained approximately twice as much cytochromes c1+c as the pea NSE, while FoF1 from PS3 (Sone

Table 5. *Recovery of Respiratory Components during Isolation of NSE*

The assays were performed as described in Chapter 2 on between 14 to 80 µgm of protein. (-) indicates that data were not measurable.

Sample	NADH dehydrogenase (µmoles/min/mg)	Cyt *a* (n mol/mg)	Cyt *b* (n mol/mg)	Cyt *c* (n mol/mg)	Cyt *a1* (n mol/mg)
Mitochondria	0.66	-	-	-	-
SMP	1.71	0.13	0.46	0.11	0.09
CE	0.79	-	-	-	-
10 to 38% sat. ammonium sulphate		0.14	0.09	0.20	0.08
NSE	0.38	0.00	0.03	0.02	0.02

et al, 1975) contained less than half of the contamination shown by NSE.

In summary, the reduction of cytochrome content of NSE, by our procedure appeared to be very similar to that obtained by some of the best currently available FoF1 purification procedures (Serrano *et al*, 1976, Sone *et al*, 1975, and Stiggall *et al*, 1978), although the absolute cytochrome contents were often lower than expected. It would seem that a large proportion of the cytochromes were precipitated between 10 and 38% saturated ammonium sulphate (Table 5), with the exception of cytochrome b. This is in contrast the results of Kagawa and Racker (1971), which indicated that beef heart cytochromes c, b, and c1 precipitated above 33% saturated ammonium sulphate. Serrano *et al* (1976) found that most cytochromes were not precipitated by 45% saturated ammonium sulphate. The contrast in results may reflect intrinsic differences in the properties of the cytochromes from pea and from beef heart, or the previously mentioned difficulties with the cytochrome assay.

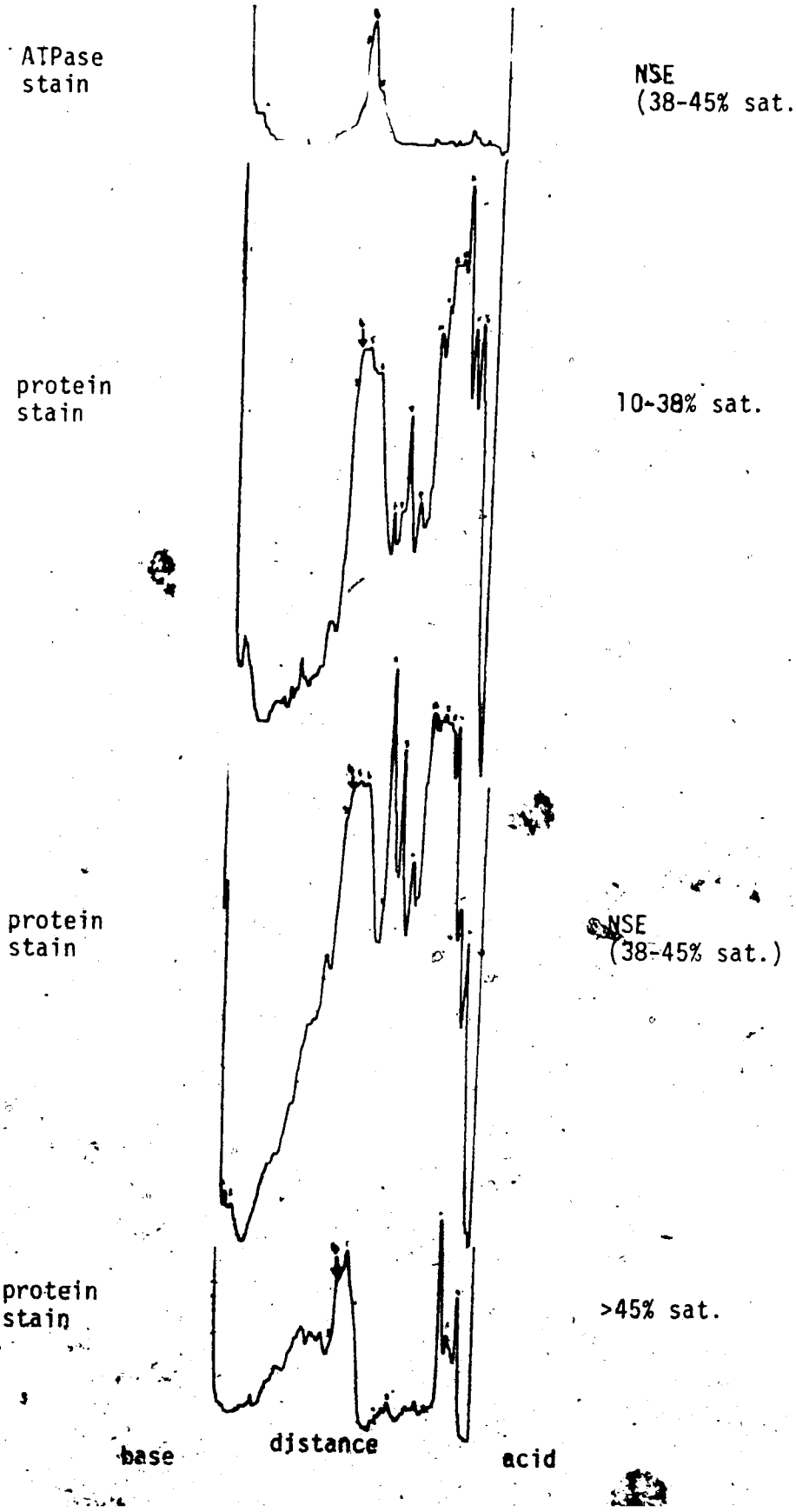
4.1.2 Gel Separations

It was originally thought that gel isoelectric focusing would be a good indicator of how pure NSE was, since each contaminating protein should have a discrete different isoelectric point. Furthermore, the FoF1 should not be entirely inactivated during the focusing thereby allowing staining for ATPase activity to be done to pinpoint FoF1.

Approximately 16 protein peaks were observed on the gels (Fig. 8), 14 of which appeared in the 38–45 precipitate fraction. However, when the gel was stained for ATPase activity, one sharp peak with two prominent shoulders was observed (Fig. 8). Hence, the FoF1 was present on the gels in three slightly different active forms, and after consideration of its evident fragility (see Chapter 3), most probably it was also present in one or many more inactive forms, eg. dissociated subunits. In fact, gel isoelectric focusing over a different pH range produced 6 peaks in 3 major groups, that reacted with the ATPase stain (data not shown), one of which was insensitive to DCCD. We therefore concluded that gel isoelectric focusing was unsuitable for our purposes because we could draw no conclusions from the data that it yielded.

Figure 8. Polyacrylamide Gel Isoelectric Focusing of Ammonium Sulphate Fractions of CE

Three hundred and fifty ul of 10 to 38% saturated ammonium sulphate fraction, 100 ugm of NSE (38 to 45% saturated ammonium sulphate), or 300 ugm of protein not precipitable by 45% saturated ammonium sulphate were loaded onto polyacrylamide gels prepared as described in Chapter 2. The latter fraction was centrifuged through a 1 ml Biogel-P column preequilibrated with 0.25 M sucrose, and 50 mM TES (pH 7.0), to remove ammonium sulphate and cholate. The gels were stained for either protein or ATPase activity as described in Chapter 2.


ATPase stain
NSE (38-45% sat.)
protein stain
10-38% sat.
protein stain
NSE (38-45% sat.)
protein stain
>45% sat.
base
distance
acid

There are no reports in the literature of FoF1 being subjected to this technique.

Many authors have, however, used SDS polyacrylamide gel electrophoresis to determine FoF1 purity (eg. Alfonzo and Racker, 1980, Clarke and Morris, 1976, Kagawa *et al*, 1978). It is now well known that F1 has 5 types of subunits (Pedersen, 1975 and Nelson, 1976) that will be referred to here as A, B, C, D, and E. Subunits A and B have molecular weights in the range of 50,000 to 60,000, depending on the source, while C, D, and E are smaller and constitute a small proportion of F1. In reconstitution experiments, Sone *et al* (1978) showed that a fully functional FoF1 from PS3 could be made from only 7 types of subunits, although the conclusion that FoF1 consists of only 7 types of subunits *in vivo*, has been challenged on genetic grounds by Fillingame *et al* (1980), who worked with *E. coli*. The most highly purified bacterial preparations of FoF1 all contain 8 types of subunits (eg. Sone *et al*, 1975). FoF1 from higher sources, that is, chloroplasts (Pick and Racker, 1979) and mitochondria (Serrano *et al*, 1976, and Capaldi, 1973) appears to consist of 9 different subunits (therefore, 4 in Fo), although the best preparations still contain more than this (Serrano *et al*, 1976; 12 subunits, Stiggall *et al*, 1978; 13 subunits).

Preliminary SDS gel electrophoresis of NSE and the ATPase peak from Percoll gradients suggested that there are 9 different subunits in NSE, and 8 in the gradient purified enzyme (data not shown). Their approximate molecular weights were as follows; 1 (72,000), 2 (58,000), 4 (44,000), 5 (36,000), 6 (28,000), 7 (19,000), 8 (12,000), and 9 (5,000). Grubmeyer (1978) showed that the A and B subunits of pea F1 ran together in a very prominent band at 58,000 molecular weight on SDS electrophoresis. Band 2 thus probably contains the A and B subunits of F1. Grubmeyer (1978) also showed that subunits C and D of pea F1 had apparent molecular weights of 36,000 and 22,000. Thus bands 5 and 7 probably correspond to subunits C and D respectively. Band 9 was observed to be diffuse and probably contains both subunit E of the F1, and the smallest of the Fo subunits (the DCCD binding protein), which in bacteria has a molecular weight of 5,000 to 6,000 (Sone *et al*, 1975). Alternatively, the DCCD binding protein (DBP) may be in band 8, which was also

observed to be diffuse. Workers have found that mammalian mitochondrial DBP has a molecular weight of 11,000 to 12,500 (Serrano *et al*, 1976 and Stiggall *et al*, 1978). Serrano *et al* (1976) suggested that bands of higher molecular weight than A and B were respiratory components, as most probably is band 1 in our gels and the unexplained 65,000 band that is observed in SDS electrophoresis of pea F1 (Grubmeyer, 1978). Band 4 was absent from density gradient purified ATPase and is, therefore, probably a contaminant. Serrano *et al* (1976) found that purification of FoF1 by density gradient centrifugation reduced contamination by the adenine nucleotide transporter. Thus band 4 may be the nucleotide transporter, although its molecular weight in animal mitochondria is believed to be approximately 30,000 to 32,000 (Kiehl, 1980 and Serrano *et al* (1976)). Kiehl (1980) also showed that incubation of FoF1 preparations with carboxyatractylate removed nucleotide transporter contamination. It could be fruitful to try this technique in future work.

Capaldi (1973), after comparison of two beef heart FoF1 preparations, concluded that Fo contained a 28,000 subunit, which indicates that band 6 (28,000) may be part of FoF1. Alfonzo and Racker ,(1979) have since shown that the 28,000 subunit of beef heart Fo is necessary for Fo functioning. Capaldi (1973) also concluded that the oligomycin sensitivity conferring protein (OSCP) had a molecular weight of 19,000. If the pea mitochondrial OSCP has the same molecular weight, it may be in band 7 along with D. Pick and Racker (1979) found that a subunit that was attributed to Fo had the same apparent molecular weight as subunit D of chloroplast F1 (ie. 17,500).

In summary, preliminary SDS gel electrophoresis suggested that there are at least 9 different proteins in NSE. Two of these appeared to be contaminents (bands 1 and 4) and five of the rest were attributed to F1 (bands 2, 3, 5, 7, and 9). The remaining two proteins (band 6, molecular weight=28,000 and band 8, molecular weight= 12,000) were thought to be components of Fo. It was also thought that band 7 (molecular weight=19,000) and band 9 (molecular weight= 5,000) may contain more than one protein each. Since bands 1 and 4 were small (data not shown) the NSE preparation may be as pure or purer than the FoF1 preparations of Serrano *et al* (1976) and Stiggall *et al* (1978).

4.2 Specificity

4.2.1 Cations

Pea FoF1 was able to catalyse ATP hydrolysis in the presence of either Mg++ or Ca++, the latter suporting slightly higher rates (Table 6). This is in contrast to the work of some previous authors, who have found that Ca++ is relatively ineffective as a cosubstrate for ATP for animal and yeast mitochondrial FoF1 (Tzagoloff *et al*, 1968, Ryrie, 1975), as it is for membrane bound mitochondrial ATPase and purified mitochondrial F1 (Pedersen, 1975). Pea SMP also have low Ca++-ATPase activity, but pea F1 shows rates of Ca++ATP activity 3 fold higher than Mg++ATP activity. FoF1 was thus intermediate in cation specificity between SMP and F1. Pick and Racker (1979) found that chloroplast FoF1 was able to catalyse Ca++-ATP hydrolysis almost as well as Mg++-ATP hydrolysis, and was therefore also intermediate between thylakoid bound ATPase and chloroplast F1 (chloroplast F1 is specific for Ca++) (Nelson, 1976). Schneider *et al* (1980) concluded that the cation requirements of purified FoF1 were similar to those of membrane bound ATPase in photosynthetic bacteria.

Therefore, although Fo from animal and yeast mitochondria determines the cation specificity of these F1s, it would seem that the cation specificity of the pea FoF1 is only partly determined by the Fo portion. The remainder is presumably determined by the membrane and/or a membrane protein(s).

4.2.2 Nucleotide Triphosphates

NSE was slighty more specific in its nucleotide requirements than CE (Table 6). Both however, had similar specificities to pea SMP, rather than pea F1 (Grubmeyer, 1978). Other workers have found that the specificity of mitochondrial FoF1 is either the same as that of F1 (Ryrie, 1975a), intermediate between F1 and SMP (Stiggall *et al*, 1978), or the same as SMP (Tzagoloff *et al*, 1968). Pedersen (1975) has advised cautious interpretation of specificity data reported at one NTP concentration and at one point in time. Of the above studies including this one, only Stiggall *et al*, (1978) have reported Vmax, Km and so forth. It is probable therefore, that the specificity of FoF1 is

Table 6. *Specificity of NSE and CE*

The standard assay mixture (in 1 ml final volume) was 300 mM sucrose, 3 mM magnesium sulphate or calcium sulphate, 25 mM TES (pH 8.0) and 3 mM NTP. From 5 to 18 µgm of protein were added to start the reaction. After 10 min the reaction was stopped and free Pi measured as described in Chapter 2.

Conditions	NTPase activity			
	CE	NSE		
	% of control	Specific activity (units/mg)	% of control	
Mg^{++}, ATP		0.35	100	
Ca^{++}, ATP		0.40	114	
Mg^{++}, ATP	100		100	
Mg^{++}, GTP	183		161	
Mg^{++}, ITP	275		188	
Mg^{++}, UTP	85		33	
Mg^{++}, CTP	10		2	
Mg^{++}, ADP	10		3	
Mg^{++}, IDP	13		4	

intermediate between that of SMP and F1. The pea enzyme may be different to the beef heart FoF1, ie. be similar to the pea SMP enzyme, or it may only appear to be so, since Grubmeyer (1978) found that activation of pea SMP (by removal of the inhibitor protein) reduced the GTPase activity of the SMP. If the stimulation of the ATPase by cholate (Chapter 3) is caused by dissociation of the inhibitor protein, then one might expect a higher specificity for ATP from an FoF1 that otherwise might be more like F1 (ie. have relatively low specificity for ATP). It should be noted though, that dissociation of sufficient inhibitor to decrease GTPase by 40% resulted in a 10 fold increase in ATPase activity, and, that stimulation by cholate typically resulted in a twofold increase in specific activity.

4.3 pH Optimum

In common with pea SMP and pea F1 (Grubmeyer, 1978) pea NSE displayed a sharp ATPase activity peak at pH 8.0 (Fig. 9). Most other ATPases from mitochondria, bacteria, and chloroplasts are also assayed at pH 8.0 (eg. Stiggall, *et al* 1978, Sone *et al*, 1975, Carmeli and Racker, 1973), although Ryrie (1975a) has reported that the partially purified FoF1 of yeast mitochondria had a pH optimum of 9.5. Previous workers with FoF1 had shown pH optima of 8.0 (Carmeli and Racker, 1973) and 8.0–9.0 (Tzagoloff *et al*, 1968). It is therefore probable that the pH optimum of purified FoF1 is the same as that of the other forms of the enzyme, ie. 8.0, although it may be more pronounced in some cases, eg. this one.

4.4 Kinetic Properties of FoF1

Pea CE and pea NSE were found to have biphasic Michaelis–Menten kinetics (Fig. 10 and 11). CE had a Vmax of 0.58 umoles Pi released/min/mg protein, and Kms of 41 uM and 430 uM (Fig. 10). NSE had a Vmax of 1.33 umoles Pi released/min/mg protein and Kms of 76 uM and 480 uM (Fig. 11).

On closer examination one may observe that each biphasic line could be more closely approximated by a curve. Christensen (1975) has pointed out that

Figure 9. pH Profile of NSE ATPase

The reactions were performed as described in Chapter 2 with either 3.5 ugm or 7.0 ugm protein, except that the pH was adjusted as shown. At pH 6.5, 50 mM TES was replaced with 50 mM MES. At pH 8.5 and 9.0, 50 mM TES was replaced with 50 mM glycine. Separate blanks were provided at each pH.

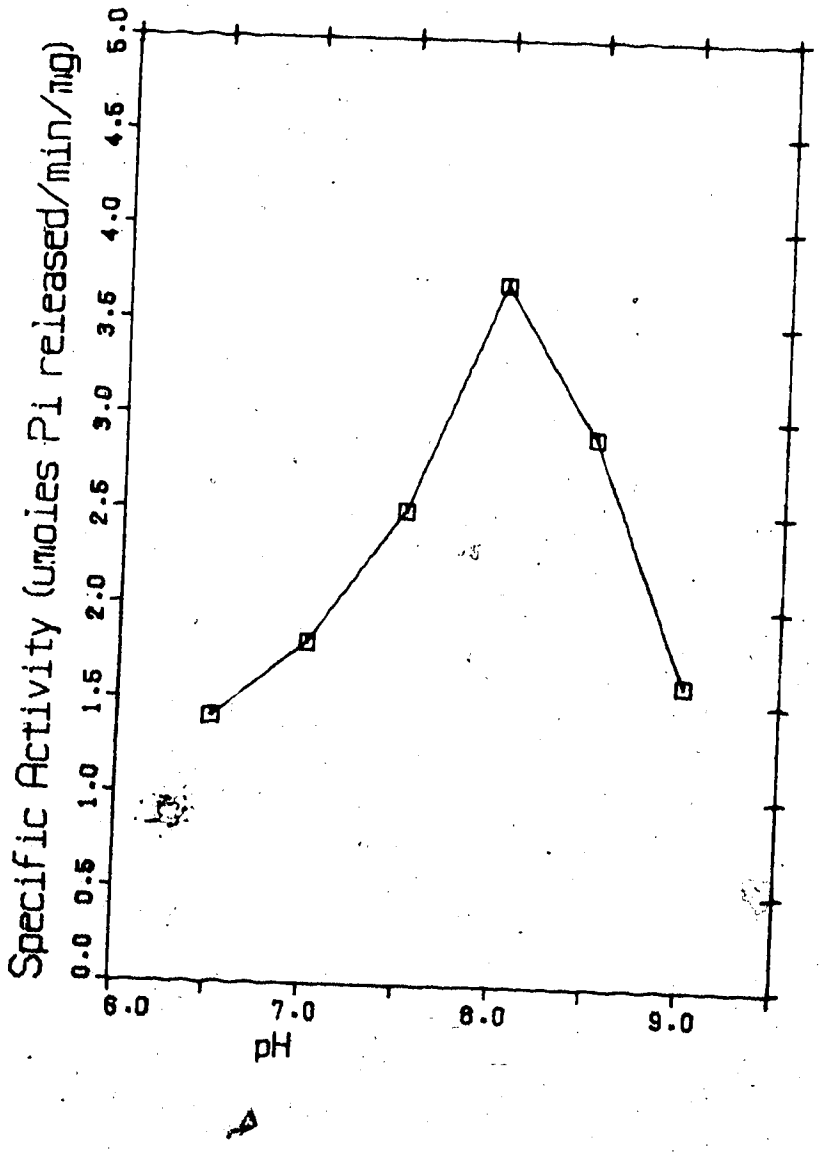

Specific Activity (umoles Pi released/min/mg)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
6.0
7.0
8.0
9.0
pH

Figure 10. ATP Kinetics of CE

The standard ATPase assay with regeneration, was used as described in Chapter 2. Seventy five ugm of CE was incubated in the medium at 30 C for 10 min. The reactions were begun by addition of MgATP to the concentrations indicated. After 10 min the reactions were terminated and free Pi measured as described in Chapter 2. Blanks were provided for each MgATP concentration. Because of the large range of MgATP concentrations required, duplicates of each concentration could not be performed. Instead, duplicates at 0.01 mM MgATP were done to ensure that reproducibility was within 10%. CE was stored at −20 C between experiments. (□): CE at day 1 plus values from day 4 and day 5 CE corrected to day 1 by proportionally increasing the specific activity at each MgATP concentration so that the activity at 1 mM MgATP was always the same; (○): CE at day 4, uncorrected; (△): CE at day 5, uncorrected.

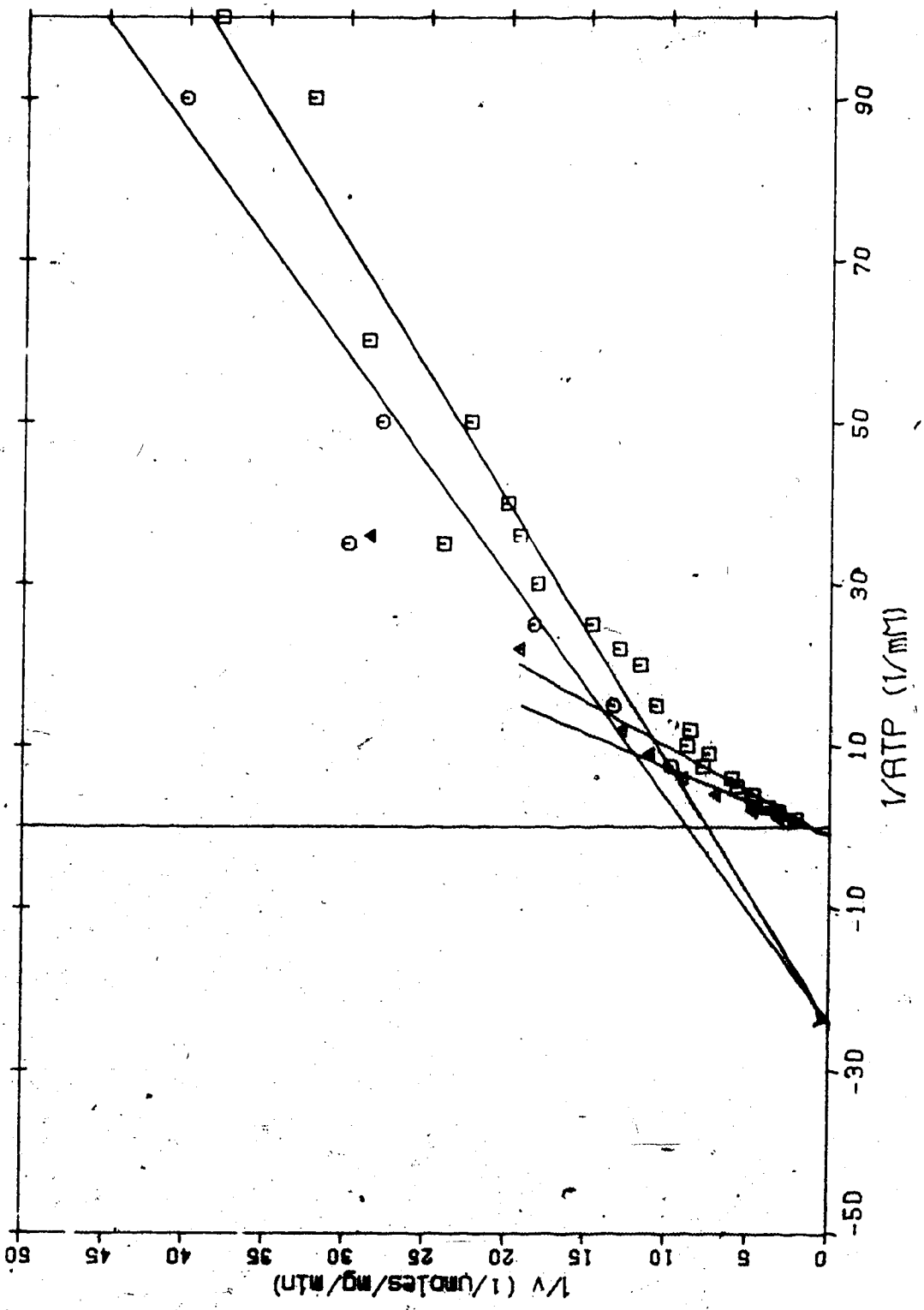
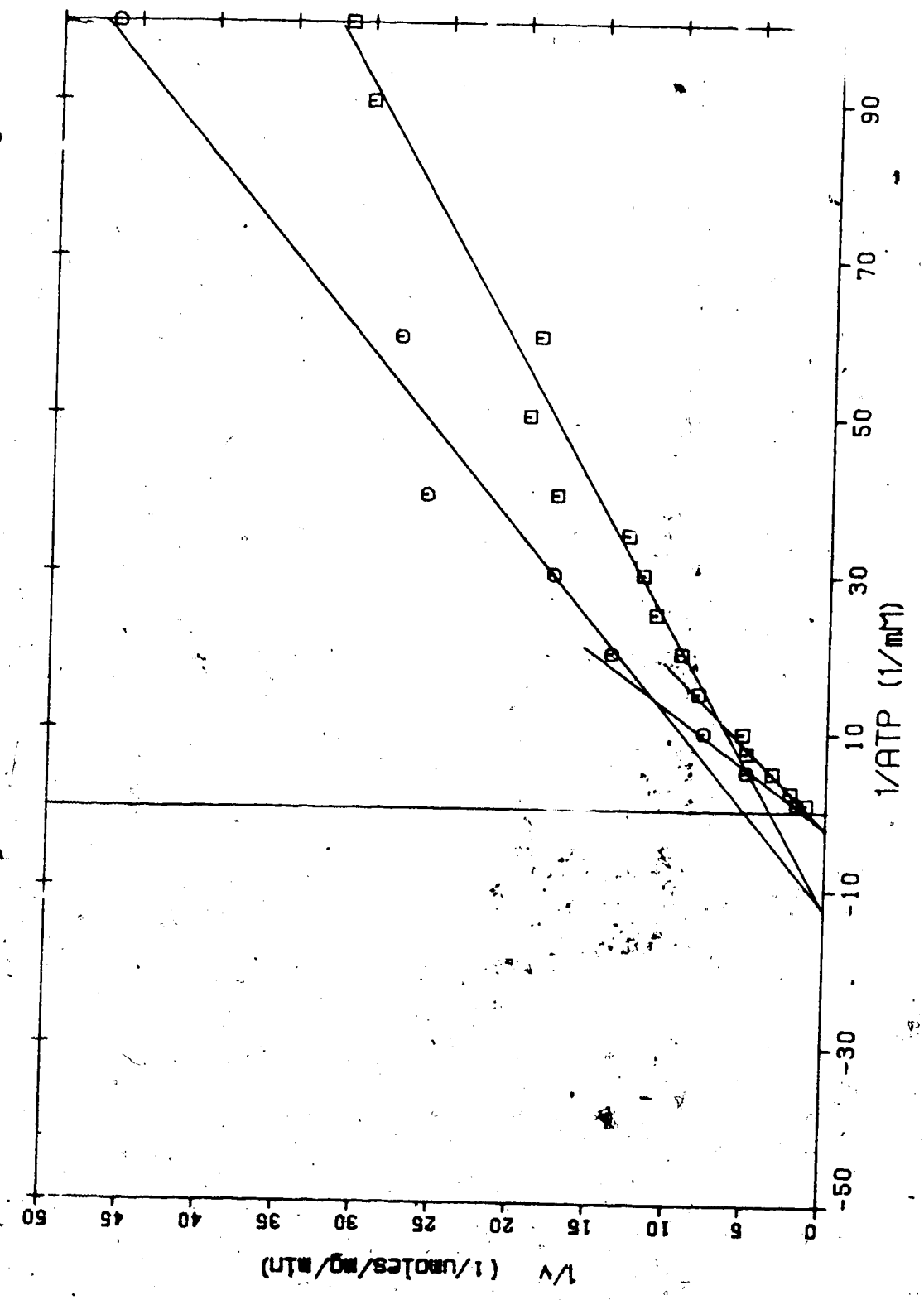

1/V (1/umoles/mg/min)
0
5
10
15
20
25
30
35
40
45
50
1/ATP (1/mM)
90
70
50
30
10
-10
-30
-50

Figure 11. ATP Kinetics of NSE

Assay as Figure 10 except that the reactions were run for 20 min and each contained 20 ugm of protein. (□): NSE aged 1.5 hr at 0 C plus data from NSE aged 3.5 hr at 0 C, corrected as in Figure 10; (○): NSE aged 3.5 hr at 0 C, uncorrected.


1/V (1/uMoLes/mg/mLn)
0
5
10
15
20
25
30
35
40
45
50
1/ATP (1/mM)
-50
-30
-10
10
30
50
70
90

if there are two catalytically active sites on an enzyme, Lineweaver-Burke plots should show curves such as those in Figures b and c, and that each point on the curve would be a product, to varying degrees, of both sites. Therefore, in order to determine the Km of each site, the velocity at each substrate concentration must be split into its two components. This technique (computerized) was used by Melanson and Spencer (1981) and Grubmeyer *et al* (1979) for analysis of kinetic data of ATP synthesis by pea SMP. The calculations raised the higher Km considerably, from 30 uM to 160uM, but did not greatly affect the lower Km, which was lowered from 9 uM to 4.8 uM. This treatment was not applied to the data shown in Fig. 10 and 11, partly because no one else has applied it to ATPase data and it thus could not be compared to previous work. That the Kms shown in this thesis may not be real should, therefore, be kept in mind by the reader. The data was also plotted in the form of Eadie-Hofstee plots (v/s versus v) (plots not shown) but graphical analysis was even more difficult. NSE from SMP2 also displayed curvilinear kinetics (data not shown).

Such curvilinear plots may be caused by any one of a number of factors, such as negative cooperativity (Cleland, 1967), two kinetically different ATPase sites (Pedersen, 1976, Soper and Pedersen, 1976), or a subpopulation of damaged enzyme (Melanson and Spencer, 1981) caused by detergent or some other treatment. Since the pea F1 showed curvilinear ATP kinetics when purified (Grubmeyer, 1978) and curvilinear ADP and ATP kinetics when membrane bound (Melanson and Spencer, 1981, Grubmeyer, 1978), it is unlikely that the curvilinear kinetics are caused by damage to some ATPases by detergent although Kms, etc. were different (see below). Pedersen (1976) found biphasic kinetics in SMP isolated by detergent fractionation, in which case damage could not have been caused by sonication (since there was none). Pedersen (1976) thus concluded "that at least two kinetically distinct classes of ATP binding sites must be present on (rat liver) mitochondrial ATPase...". It seems most probable that the same is true of pea FoF1.

Although many workers report linear ATP kinetics for other proton-ATPases, curvilinear kinetics are not unknown. As mentioned above,

Melanson and coworkers (1981 and 1979) have shown that pea membrane bound ATPase shows curvilinear kinetics with respect to ADP esterification, although the two Kms were approximately one order of magnitude lower than the Kms reported here. It should be noted that Swanljung *et al*, (1973) concluded that stimulatory phospholipids lowered the Km (ATP) of FoF1 by over 80%. The Kms reported here thus may be variable. Grubmeyer (1978) has shown that pea F1 displays "negative cooperativity", ie., curvilinear kinetics. The high Km was calculated graphically to be 170 uM (40% of that of CE and NSE), but the low Km was not calculated. Pea SMP also displayed curvilinear kinetics (Grubmeyer, 1978), although the range of ATP concentrations was insufficient to allow calculation of the higher Km. The lower Km was approximately 70 uM, which is quite similar to that of CE and NSE (Fig. 10 and 11). This is interesting in view of the data of Kayalar *et al*, (1976) who also showed that addition of an uncoupler increased the Km of SMP with respect to ADP and Pi. Since the FoF1 should be entirely uncoupled, it is evident that uncoupling does not also increase the Km (ATP).

Soper and Pedersen (1976) also found little difference between the Kms (ATP) of SMP and solubilized (but not purified) FoF1. Furthermore, the Kms (29 uM and 310 uM for the FoF1) were quite close to those of pea NSE and CE (Fig. 10 and 11). The remaining publications on FoF1 kinetics all show linear ATP kinetics and considerably lower ATP affinities (22 mM, Stiggall, *et al* 1978; 300 uM, Oren and Gromet-Elhanan, 1977; and 140 - 9,000 uM, Swanljung *et al*, 1973). The absence of two ATP sites in the latter two preparations may be traced to the TX-100 that was used for solubilization of these complexes, since Soper and Pedersen (1976) found that TX-100 apparently destroyed one site and thus gave linear kinetics for rat liver FoF1. The FoF1 of Stiggall *et al* (1978) was only assayed at very high ATP concentrations (the lowest was 2 mM), which accounts for the lack of biphasic kinetics (see Fig. 10 and c).

Aging of CE at subzero temperatures for up to 5 days and aging of NSE on ice for up to 4 hr slowly deactivated the enzyme (Fig. 10 and 11), although in each case the Kms were unchanged. Lower enzyme activity indicates that either all enzyme molecules have been partially deactivated, or that some

molecules have been entirely deactivated while the rest are unchanged. A decrease in Vmax with no concommitant decrease of the Kms would tend to support the latter conclusion. Some molecules may be more sensitive to such deactivation than others within the same population, because they may be closely associated with different amounts and compositions of phospholipids and other impurities.

4.5 Anion Effects

Previous work from this laboratory (Grubmeyer, 1978) has shown that the ATPase of pea mitochondria possess a unique intrinsic stimulation by chloride ions, in addition to the bicarbonate stimulation that both membrane bound ATPases and purified F1 from other sources normally possess.

Preliminary results (Table 7) indicated that NSE was stimulated by both 40 mM NaCl (17%) and 40 mM sodium bicarbonate (43%), but much less than F1 was (125% and 220% respectively), and slightly less than were SMP (73% by 100 mM NaCl and 64% by 20 mM sodium bicarbonate). This provides further evidence that the chloride ion stimulation of pea F1 may be of physiological significance, and in addition suggests that the Fo portion somehow exerts a modifying effect on these anion stimulations, possibly by restricting conformational changes.

Although pea F1 is stimulated 210% by 250 mM NaCl (Grubmeyer, 1978), pea FoF1 was not (Table 7). This lack of stimulation may reflect the instability of the pea FoF1 at higher ionic strengths (see Chapter 3). Instability at higher ionic strengths may also have been the reason for the lower stimulations under the other treatments, as compared to SMP, although one would not expect 10 mM NaCl to accelerate enzyme dissociation.

Calcium ATPase activity was inhibited by chloride ions as was pea F1 (Grubmeyer, 1978).

Table 7. *Anion Effects on ATPase Activity of NSE*

Assay conditions as in chapter 2. Each tube contained 10 µgm of protein, which was incubated in the assay medium for 10 min before initiation of the reaction by addition of ATP to a concentration of 3 mM. Sodium bicarbonate or sodium chloride was added as shown. In experiments 6 and 7, 3 mM magnesium sulphate was replaced with 3 mM calcium sulphate.

Conditions	Specific activity (units/mg)	% of control
1. Mg^{++}	0.35	100
2. Mg^{++}, 40 mM $NaHCO_3$	0.50	143
3. Mg^{++}, 10 mM NaCl	0.38	108
4. Mg^{++}, 40 mM NaCl	0.41	117
5. Mg^{++}, 250 mM NaCl	0.36	101
6. Ca^{++}	0.40	114
7. Ca^{++}, 40 mM NaCl	0.36	100 (91% of Ca^{++})

4.6 Effects of Temperature on the Stability of CE and NSE

4.6.1 CE

When stored in liquid nitrogen for less than a week, CE lost approximately 10% of its original activity (Fig. 12). This 10% loss of activity appeared to be intrinsic to the liquid nitrogen since repeated freezing and thawing at each assay did not decrease the activity any more than one freeze and thaw (Fig. 12). If stored for longer than 7 days in liquid nitrogen, the CE loses further activity.

CE was also stored at −20 C. There was an initial 15−25% loss of activity (Fig. 12), but further storage of up to a week at −20 C did not increase the loss. As with storage in liquid nitrogen, there was no apparent difference in stability between samples that were thawed and refrozen between assays and those that were not.

At 0 C it was observed that CE lost 86% of its original activity within 4 days (Fig. 12). This loss was considerably more rapid than in either of the two treatments mentioned above, but much slower than normally observed with the cold labile F1. It is, therefore, probably reasonable to conclude that the CE is not cold labile.

CE is more unstable than pea SMP, which can be stored for up to 2 weeks at −20 C without loss of its most labile property− the capacity for oxidative phosphorylation (Grubmeyer, 1978). Other workers routinely store membrane extracts at −70 C (Stiggall *et al*, 1978, Serrano *et al*, 1976).

The sensitivity of pea FoF1 to DCCD was found to be sustained for more than 4 days only if stored in liquid nitrogen (data not shown). This was taken as an indication that the native conformation of FoF1 was maintained for longer periods when kept at liquid nitrogen temperatures than at −20 C.

Therefore, in this study CE was divided into 1 ml volumes and frozen in stoppered cellulose nitrate test tubes at −20 C. When the CE was solid, the stoppers were removed and the tubes and the frozen CE were stored in liquid nitrogen.

During incubation at 30 C, CE showed a stimulation of ATPase activity (Fig. 13) similar to that of SMP during aging (Grubmeyer, 1978), although the

Figure 12. Storage Stability of CE

CE was frozen at −20 C and stored for the indicated length of time in unstoppered testtubes in liquid nitrogen. Tubes containing 'bulk' CE were thawed, assayed, and refrozen (at −20 C and then placed in liquid nitrogen), whereas 'individual' CE tubes contained sufficient CE for one ATPase assay only. CE was stored at −20 C in stoppered tubes for the indicated lengths of time. 'Bulk' CE was thawed and refrozen for each assay, while 'individual' CE was only frozen and thawed once. CE was also stored in stoppered tube on ice for the indicated time periods.

Assays were as described in Chapter 2. (□): 'bulk' CE in liquid nitrogen; (○): 'individual' CE in liquid nitrogen; (△): 'bulk' CE at −20 C; (+): 'individual' CE at −20 C; (x): CE at 0 C.

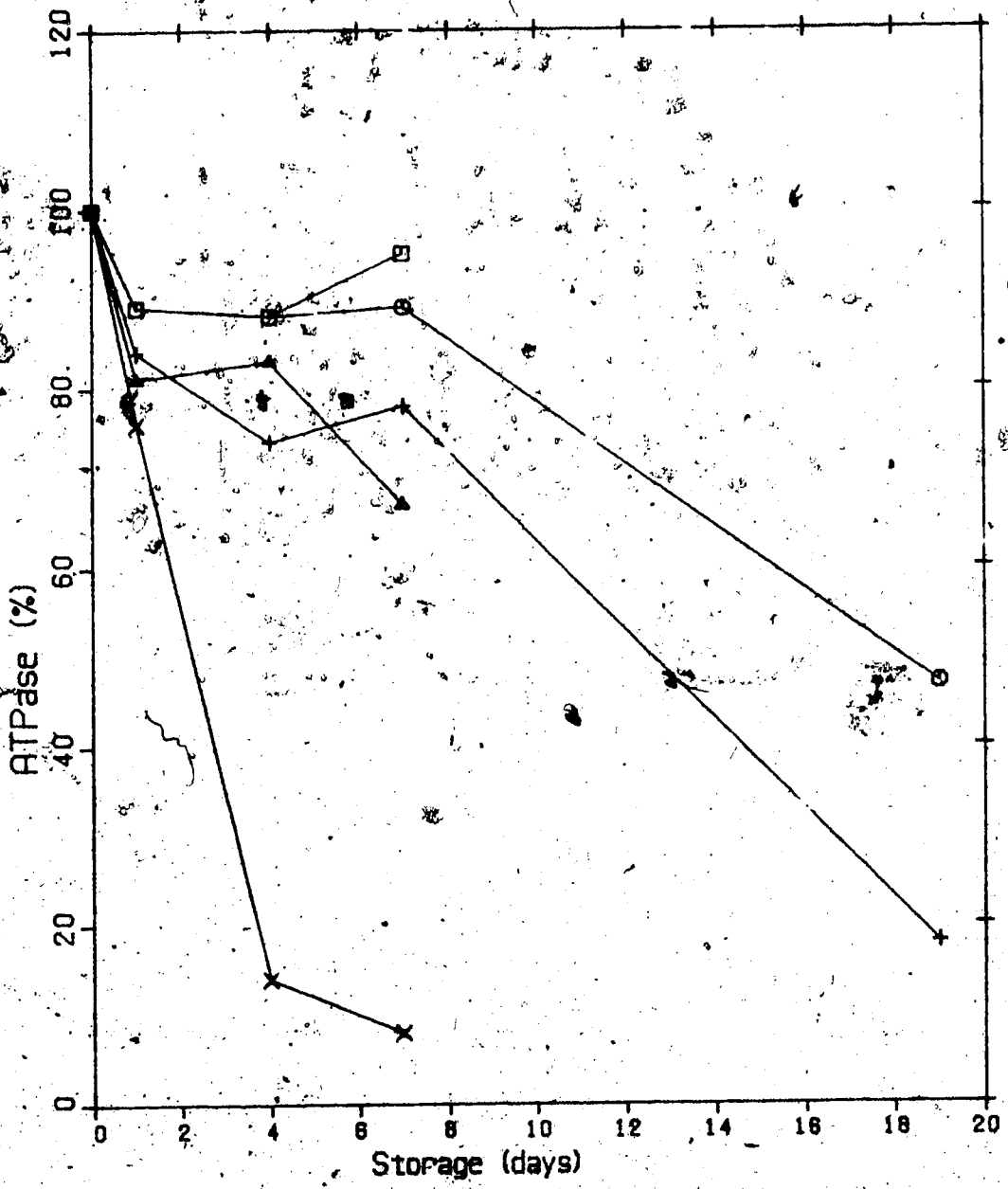
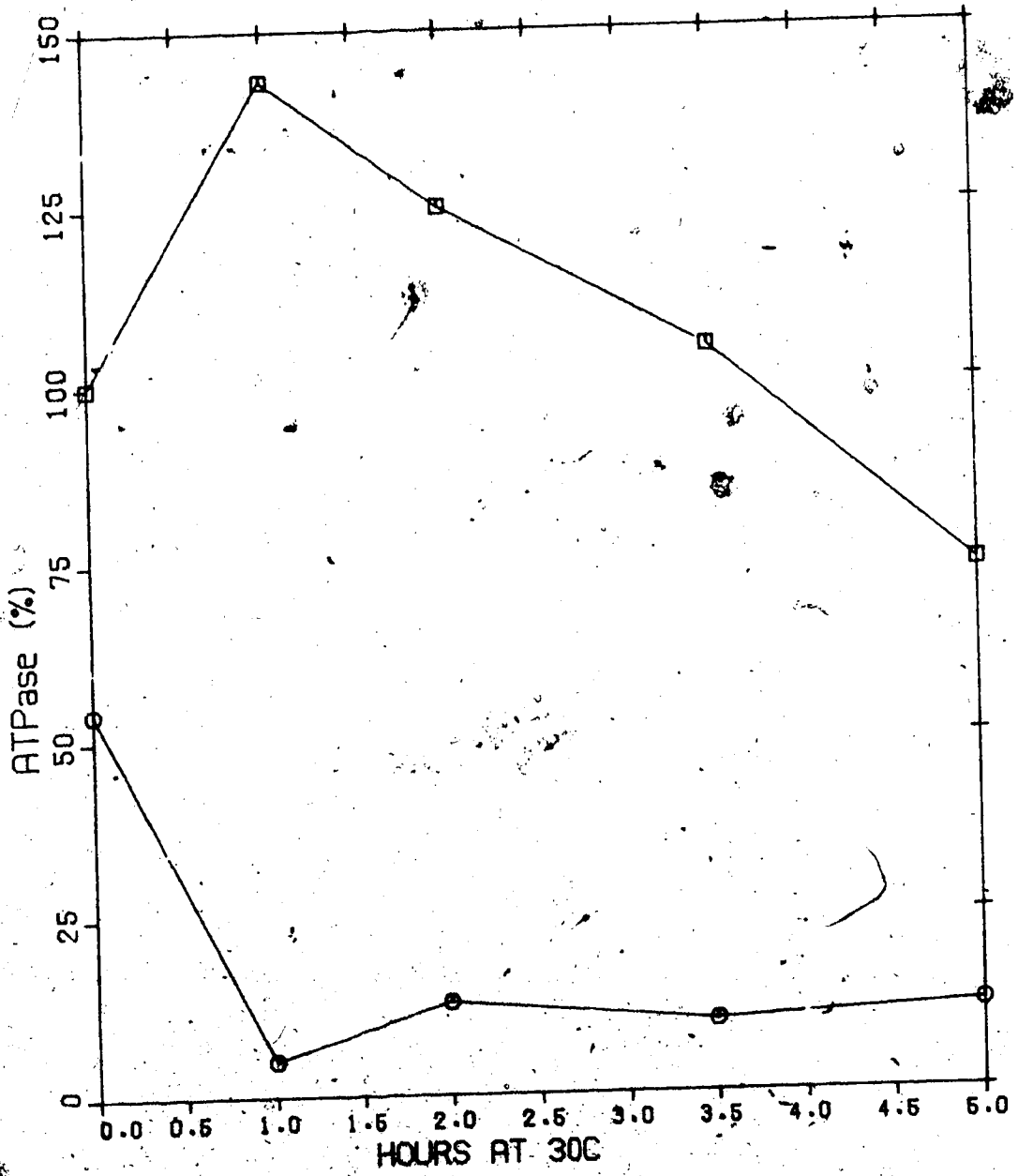

ATPase (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Storage (days)

Figure 13. Aging of CE at 30 C

Assays as in Chapter 2. Undiluted CE was incubated at 30 C for the time indicated. In appropriate reactions, DCCD was added to a concentration of 0.1 mM. (□): ATPase activity of CE; (○): sensitivity to DCCD of the ATPase activity of CE.


ATPase (%)
150
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
HOURS AT 30C

latter is stimulated considerably more [illegible]fold compared to 43% for CE). The stimulation of pea ATPase has been suggested to be caused by dissociation of an inhibitor protein (Grubmeyer, 1978). Thus, the solubilized FoF1 may be depleted in inhibitor protein. This could be caused by the high ionic strength that was used during detergent solubilization, as suggested in Chapter 3. Alternatively, the Fo portion may be quickly dissociated at 30 C (the sensitivity to DCCD decreased rapidly - Fig. 13) leaving a population that was predominately F1. Grubmeyer (1978) showed that pea F1 is not stimulated by aging.

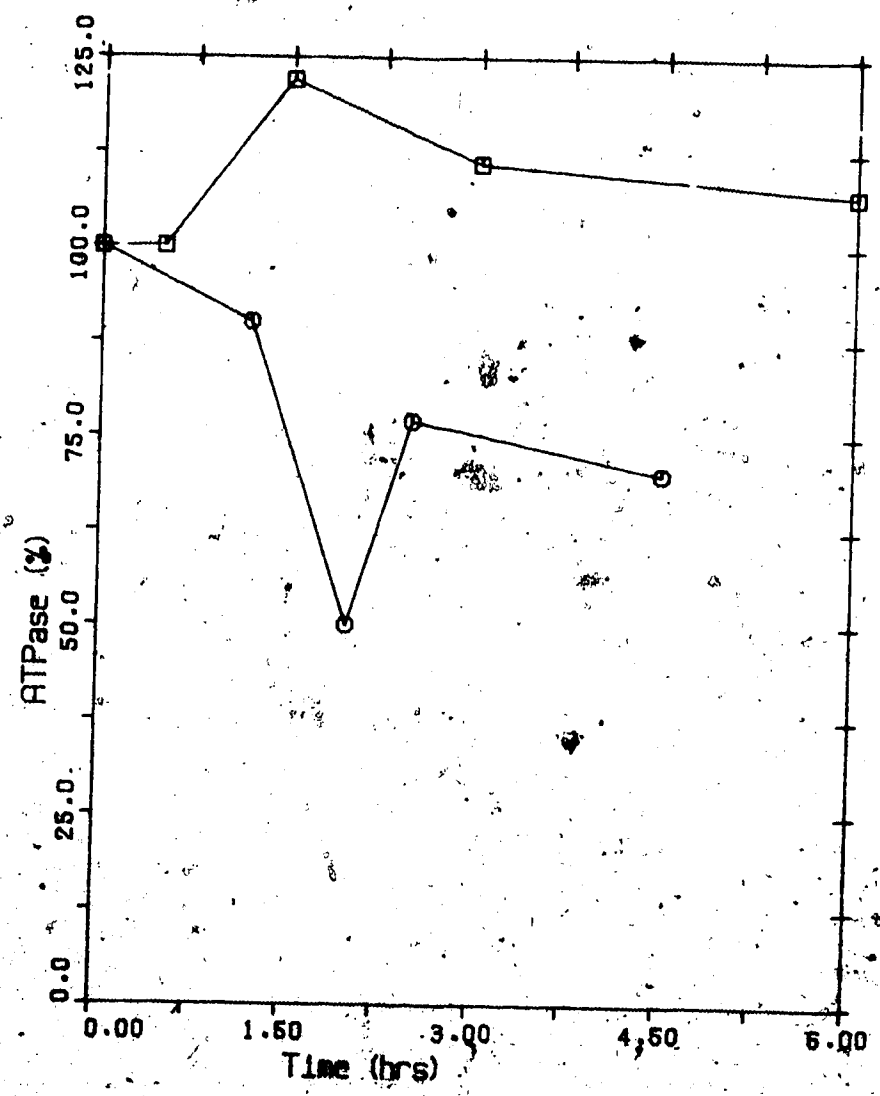
4.6.2 NSE

Some previous workers (eg. Stiggall *et al*, 1978) have routinely stored purified FoF1 at −70 C for short periods before use. One must assume that many other workers sto[illegible] only membranes or membrane extracts, and purify fresh FoF1 for every experiment, since storage conditions for membrane extracts but not purified [illegible] are specified (eg. Serrano *et al* (1976), Foster and Filligame, 1979). Occasionally, left over NSE was frozen at −20 C at the end of an experiment. Usually 3–5 days storage resulted in a 40% reduction in ATPase activity (data not shown), and this, coupled with the fact that most experiments used almost all the NSE precipitated for that experiment, led us to precipitate NSE fresh from stored CE for every experiment.

The partially purified FoF1 preparation was found to be slightly stimulated by more than 30 min incubation at 25 C (Fig. 14). This stimulation was most probably also caused by protein inhibitor dissociation. In any case, numerous NSE preparations were assayed for detectable stimulation by incubation at 25 C, over the time span of the ATPase assay but stimulation was never found. Ryrie (1975a) also noted a stimulation of FoF1 ATPase by aging, and likewise attributed it to release of the inhibitor protein. In contrast to its evident stability at room temperature, NSE was steadily deactivated at 0 C (Fig. 14). Although more rapid than the deactivation of CE at 0 C, it was still much slower than that of pea and other purified F1s, which may lose up to 90% activity in 20 min at 0 C (Grubmeyer, 1978, Penefsky, 1974). It is possible that this gradual

Figure 14. Aging of NSE

Assays as in Chapter 2. NSE was aged undiluted. (□): ATPase activity during incubation at 25 C; (○): ATPase activity during incubation at 0 C.


125.0
100.0
75.0
50.0
25.0
0.0
ATPase (%)
0.00
1.50
3.00
4.50
6.00
Time (hrs)

decrease in activity of NSE is caused by a slow interchange between the FoF1 form and the F1 and Fo forms. The decrease may therefore be prevented by addition of p-amino benzamidine since Freidl *et. al* (1979) have suggested that this compound inhibits release of F1 from *E. coli* membranes. However, Ryrie (1975a) found that yeast FoF1 showed only one band during analytical ultracentrifugation after cold inactivation. Approximately 50% of FoF1 ATPase activity was lost within 5-10 hrs but none was lost thereafter.

5. CONCLUSIONS AND FUTURE WORK

5.1 Similarities to Pea SMP and Pea F1

Previous workers have found that purified F1 and membrane bound ATPase differ significantly in many properties, eg. cold lability, Km(ATP), nucleotide specificity (Pedersen, 1975). Consequently, some workers (eg. Soper and Pedersen, 1976) have used only solubilization and purification procedures that resulted in FoF1 preparations with similar properties to the membrane bound ATPase. Although that approach to solubilization and purification was not adopted in this study, most NSE properties were similar to, or the same as those of pea SMP rather than pea F1 (Chapters 3 and 4). These included specificity for nucleotide triphosphates, anion effects, stimulation of ATPase activity by aging at 25 C, sensitivity of ATP hydrolysis to DCCD, and kinetic properties. Some properties however (ie. cation specificity and cold lability), were concluded to be intermediate between pea SMP and pea F1 (Chapter 4). It was suggested that this may have been caused by spontaneous dissociation of some FoF1 into F1 and Fo. If the dissociation was reversible with the equilibrium lying to the FoF1 side, one could expect FoF1 to be intermediate in cold lability, but in all other properties be similar to SMP, since under the cold lability experimental conditions the dissociation to F1 and Fo would be irreversible. (At 0 C, F1 dissociates rapidly and thus would displace the equilibrium to the F1 side.) This hypothesis could be tested in at least two ways. The cold deactivation of FoF1 could be monitored until it ceased. If there normally was an equilibrium between FoF1 and free F1 and Fo, all of the FoF1 ATPase activity would eventually be lost, although FoF1 itself may be slightly unstable at 0 C. Alternatively, FoF1 could be incubated with p-amino benzamidine, which is thought to stop dissociation of FoF1 (Freidl *et al*, 1979). If p-amino benzamidine caused FoF1 to become stable at 0 C, one could conclude that the usual cold deactivation was caused by spontaneous dissociation into F1 and Fo. It is unlikely that NSE is contaminated by significant amounts of free F1, because all steps in the solubilization and purification of NSE were performed at 0-2 C (as described in Chapter 3).

It is unclear however why the cation specificity of FoF1 is intermediate to that of F1 and SMP. Perhaps the specificity in SMP is in part determined by one or more membrane lipids or proteins (by restricting conformational changes, Grubmeyer, 1978) as well as by Fo.

In contrast to both pea F1 and pea SMP, the ATPase activity of FoF1 has been shown to be quite labile (Chapter 3). The lability may be caused by removal during purification of one or more factors required for stability of the enzyme or maintenance of ATPase activity, such as phospholipids, as as was suggested in Chapter 3. Alternatively, it may be caused by dissociation of FoF1 to Fo and F1 as discussed above.

5.2 Similarities to Other FoF1 Preparations

Pea FoF1 appeared to be more easily deactivated than FoF1 from other sources. It was found to be partially cold labile and may have been sensitive to removal of one or more factors during purification that were essential to ATPase activity.

In spite of this, solubilization and fractionation with ammonium sulphate increased the specific activity of FoF1 12 to 13 fold. DCCD was shown to be a potent inhibitor of NSE. Thus under the assay conditions used, free F1 was not a significant impurity of the NSE. Very clean preparations of bacterial FoF1 usually have a specific activity 15 to 20 fold higher than the membranes from which they are purified (eg. Schneider *et al*, 1980, Foster and Fillingame, 1979, Sone *et al*, 1975). The most pure FoF1 preparations from mitochondria (Stiggall *et al*, 1978, and Serrano *et al*, 1976) have specific activities that are 5 to 10 fold higher than the SMP from which the FoF1 was purified. Such differences in degree of increase of specific activity probably reflect differences in the number of different proteins *in vivo* in each source and consequently, the proportion of FoF1 to total protein. Plant mitochondria are very similar in most properties to animal mitochondria (Wiskich, 1977), so the 12 to 13 fold increase in specific activity observed in pea mitochondrial FoF1 preparations probably indicates that NSE is also highly purified.

NSE was also shown to contain low levels of NADH dehydrogenase, and although lack of sensitivity of the cytochrome assays precluded conclusive data, low levels of cytochromes a, b and c1+c were found that were comparable to the highly purified FoF1s of Serrano *et al* (1976), Sone *et al* (1975), and Stiggall *et al* (1978). The % reduction of cytochrome content by purification of pea SMP was also comparable to that of the above purifications.

Polyacrylamide gel electrofocusing was found to give inconclusive data for analysis of NSE, because of enzyme dissociation. However, preliminary results with SDS polyacrylamide disc electrophoresis (by the method of Laemmli, 1970) indicated that there may be as few as 10 or 11 different proteins in 9 bands in NSE. Two minor bands were deduced to be contaminants (bands 1 and 4). Heavier gel loading or two dimensional gel techniques, such as were used by Pick and Racker (1979), may well show more contaminant bands, but should also allow separation of the small molecular weight proteins (discussed in Chapter 4). In any case, these data indicated that our FoF1 preparation could possibly have as little contamination as the highly purified FoF1 preparations in the literature. The preparation of Serrano *et al* (1976) showed 12 bands on SDS electrophoresis, and that of Stiggall *et al* (1978) showed 13 bands. Pea mitochondrial Fo may even be shown to contain less than the four types of subunits found in animal mitochondrial Fo (Capaldi, 1973, Alfonzo and Racker, 1979).

FoF1 preparations from other sources (Stiggall *et al*, 1978, Tzagoloff *et al*, 1968, Ryrie, 1975 and 1975a, etc.) show properties that are much the same as those of membrane bound ATPase, but not F1. This indicates that Fo is able to modify some of the catalytic properties of F1, perhaps by restricting conformational changes.

5.3 Future Work

The first priority of future work on pea FoF1 should be to elucidate the cause of the ATPase inactivation that has hampered further purification of the enzyme in the present study. As suggested earlier in this study, experiments

with the antidissociation compound p-amino benzamidine (Freidl *et al*, 1979) may prove fruitful in this regard. The NSE may also be inhibited by a lack of certain phospholipids. To this end, a study on the stimulatory effects of a variety of types of phospholipids should be done.

If stabilization of the FoF1 could be accomplished, the enzyme could be further purified. The NSE contained measurable amounts of NADH dehydrogenase and cytochromes, and it is unlikely that one ammonium sulphate precipitation step could remove all other contaminations, especially since almost identical procedures have not done so for detergent extracts from chloroplasts (Pick and Racker, 1979) and animal mitochondria (Serrano *et al*, 1976). If stabilizing procedures were developed, eg. addition of phospholipids to density gradients, Percoll density gradient centrifugation could be most useful as a suitable final purification step for the pea FoF1.

A major reason for purifying FoF1 instead of just F1, is to obtain a tool for investigations of energy transducing reactions, ie. phosphorylation of ADP, ATP-Pi exchange and so on. Accordingly, NSE, whether it is purified further or not, should be incorporated into vesicles. There are many new systems available that when incorporated into vesicles, are able to produce a PMF suitable for driving ADP phosphorylation by FoF1, such as the purified Photosystem 1 reaction centre (Bengis and Nelson, 1975).

There is a great deal still to be learned about how the FoF1 catalyses ATP synthesis and hydrolysis, and the FoF1 incorporated into vesicles is an ideal tool for such studies. For example, Choate *et al* (1979) have used two different FoF1 preparations to invesigate isotopic oxygen exchanges in FoF1. Baird *et al* (1979) experimented with singlet-singlet resonances of FoF1 reconstituted into vesicles and concluded that FoF1 protrudes 3 nm from the membrane surface *in vivo*. There are many possibilities for research with such a system.

The pea FoF1 has some properties that are apparently unique, such as the stimulation of ATP hydrolysis by chloride ions. Grubmeyer (1978) has hypothesized that chloride ion stimulation may indicate a type of enzyme control different to that of other FoF1s. Elucidation of chloride ion stimulation of

ATP-Pi exchange or ATP synthesis, if any, may yield information on the mechanism of this stimulation, and thus on the mechanism of action of the enzyme itself.

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APPENDIX 1

5.4 Development of New Pi Assay

The third ATPase assay that was developed in this study consisted of the ATPase reaction conditions used in the second assay, coupled with a modified form of the Pi assay of Serrano *et al* (1976). As explained in Materials and Methods, colour is developed by reduction of the phosphomolybdate complex by ascorbate, and the OD is read at 750 nm.

The OD at 750 nm increases with time (Fig. 15). At low molybdate concentrations (0.1% w/v) the OD increases steadily for over an hour. At higher molybdate concentrations (0.35% and 0.7% w/v) the OD reached a plateau within 5 minutes and only increased slowly from there on. Samples left overnight always turned blue/black by morning. The time interval that elapsed between ascorbate addition and OD reading therefore gave rise to a further source of variability and so was strictly limited to a convenient period, 10 minutes. The highest (0.70% w/v) concentration of molybdate apparently resulted in a more level OD plateau and was therefore used in subsequent assays.

Construction of a standard curve showed the assay to be linear to approximately 400 nmoles (Fig. 16). Figure 16 also shows that the components of the assay are stable and they will give reproducible results over a period of at least 3 months.

In any case, to eliminate any variability caused by the change in OD with time of reduction, or by aging of assay solutions, Pi standards (usually 100 nmoles) accompanied every ATPase assay.

Figure 15. Colour Development by the Reduced Phosphomolybdate Complex

The assay mixture contained 125 nmoles of Pi in 1 ml (final volume) of water. Two ml of quench medium (0.72 N sulphuric acid and appropriate concentrations (w/v) of ammonium molybdate) were added, along with 50 ul of 1% (w/v) sodium ascorbate, and mixed. The optical density at 750 nm was measured immediately and as indicated. The mixtures were stored at 25 C between readings. (□): 0.1% (w/v) ammonium molybdate; (○): 0.35% (w/v) ammonium molybdate; (△): 0.7% ammonium molybdate.

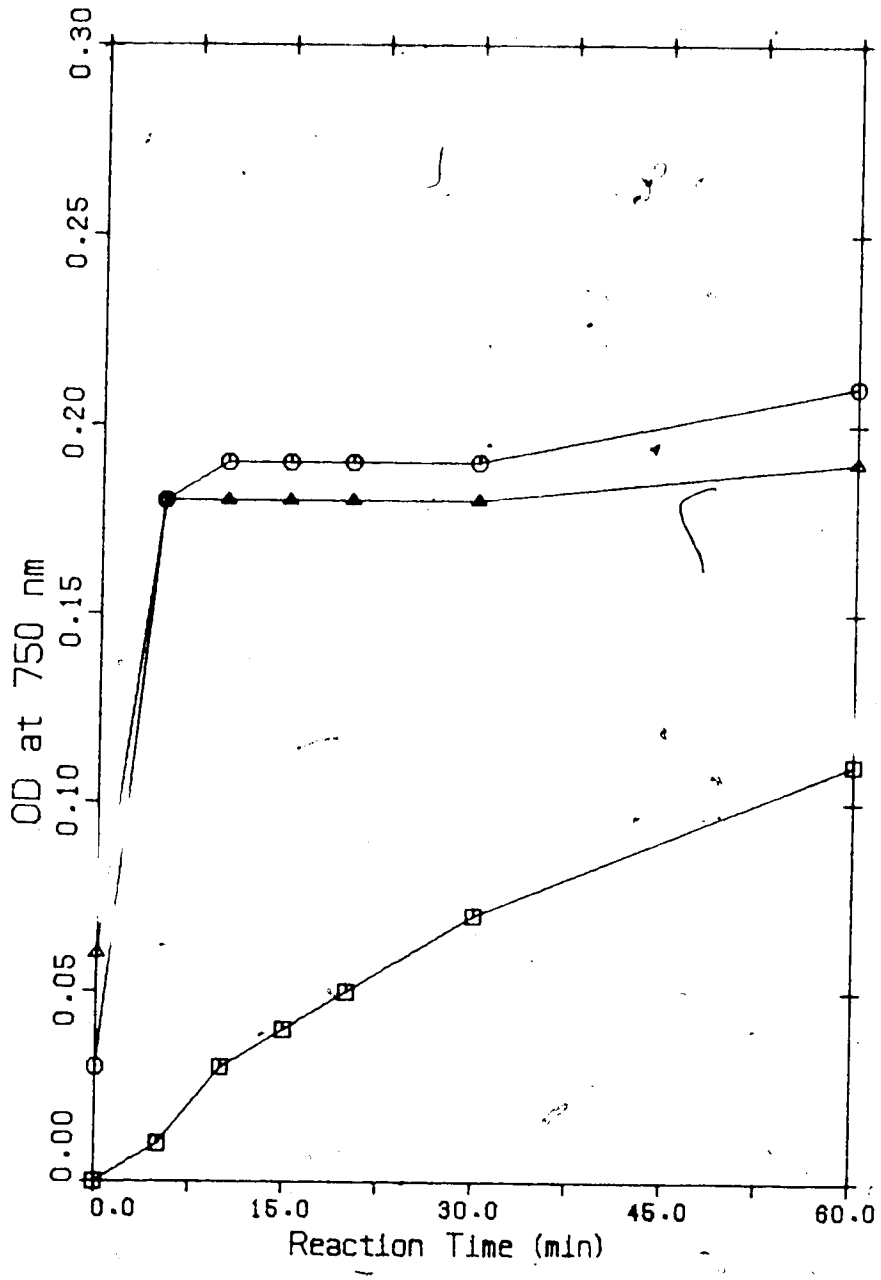
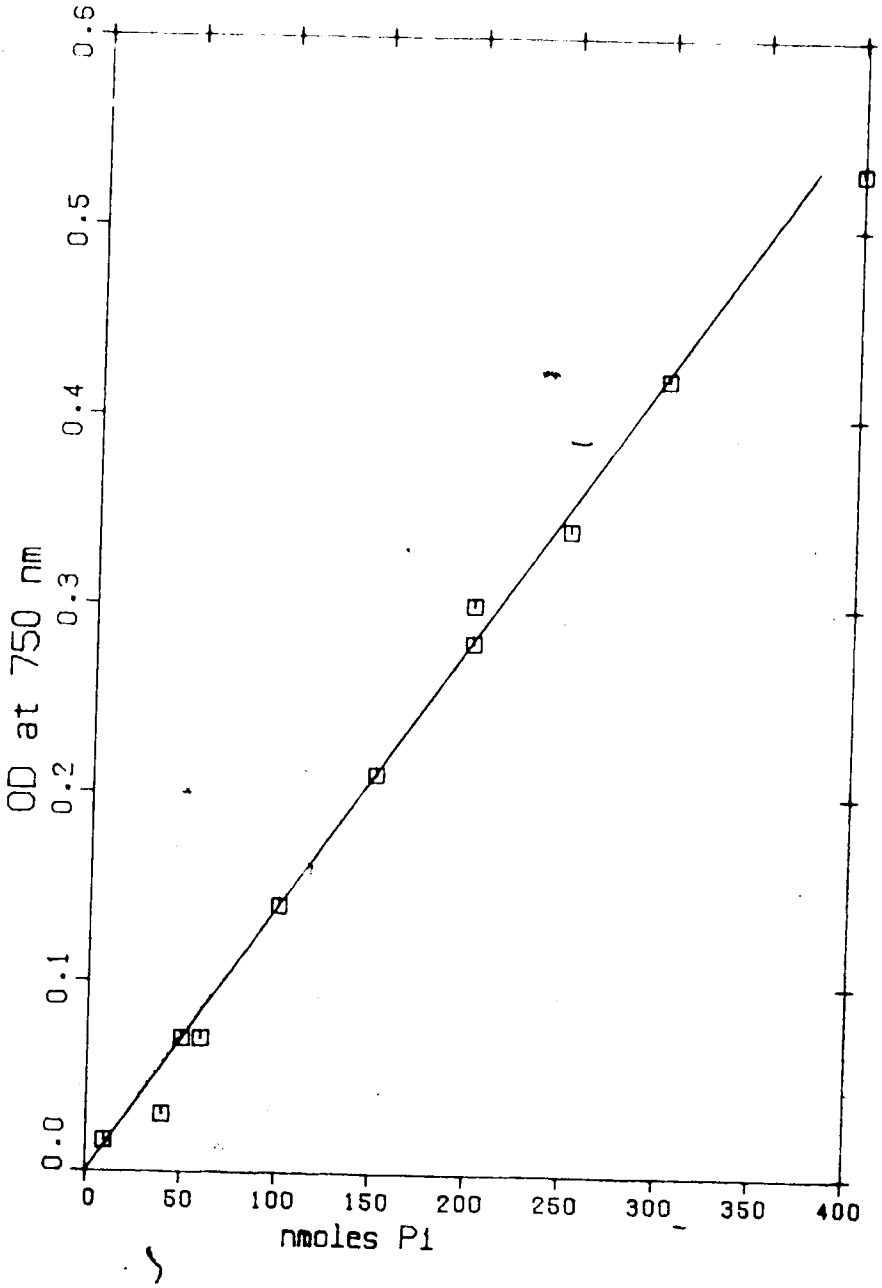

OD at 750 nm
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.0
15.0
30.0
45.0
60.0
Reaction Time (min)

Figure 16. Standard Curve for the New Pi Assay

One ml of assay mixture (300 mM sucrose, 3 mM magnesium sulphate, 25 mM TES (pH 8.0) and an appropriate concentration of Pi) was mixed with 2 ml of quench medium (0.72 N sulphuric acid, and 0.7% (w/v) ammonium molybdate). To develope colour 50 ul of 1% (w/v) sodium ascorbate was added. The solution was incubated at 25 C for 10 min before measurement of the optical density at 750 nm.


OD at 750 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
50
100
150
200
250
300
350
400
nmoles Pi

APPENDIX 2

5.5 Development of Tissue Growth and Organelle Isolation Procedures

The first goal of the project was recognized to be an increase in the scale of production of mitochondria. Most previous projects in this lab have required small amounts of freshly prepared mitochondria. The established procedure was, therefore, most suitable for the growth and grinding of 100 ml of expanded pea cotyledons (or up to 300 ml for SMP production). The most time consuming part of this procedure was the separation of the cotyledons from the shoot, root, and testa. In the present project, to eliminate this procedure mitochondria were isolated from peas that were imbibed for only 24-48 hours (instead of 4 days). After 24-48 hr, the root and shoot were 2-4 mm long and the testa usually unbroken. Whole peas had the disadvantages of not being a homogeneous tissue, and of the ATPase perhaps not being fully functional (Solomos *et al*, 1972). However, the great savings in time in harvesting and peeling peas were considered to outweigh the disadvantages, at least for the preparation of tissue for preliminary experiments. Most of these preliminary experiments, eg. optimization of cholate extraction procedures, and Affigel Blue chromatography, required large amounts of SMP. SMP used in later experiments (from ammonium sulphate precipitation chromatography on) were always 4 day old greenhouse peas (see Chapter 2).

Peas used in technique 1 (Table 8) were imbibed for 24 hr, while suspended in aerated tap water in a large round bottom flask (the bubbler technique). Various methods were tried to keep the peas suspended and therefore sufficiently aerated (magnetic stirrer, mechanical stirrer, air jets). As the amount of peas in the flask was scaled up it was progressively more difficult to keep all of the peas suspended without incurring mechanical damage to the peas. Furthermore, the specific activity of the SMP, although variable, was low (Table 8). After experimentation with different grinding methods, the amount of grinding was concluded to be the major factor in maintenance of the yield of protein (data not shown). Centrifuge tube and bottle shapes were also varied to reduce contamination of the mitochondrial and SMP pellets by the lipid cake

Table 8. *Development of Growth and Organelle Isolation Procedures*

Peas were imbibed and grown and mitochondria were isolated as described in Chapter 2, except for treatment 3. For this procedure, the wash step was omitted. The mitochondria were instead loaded immediately onto a step gradient containing 40 ml of 0.6 M sucrose with 50 mM TES (pH 7.0) and 20 ml of 1.6 M sucrose with 50 mM TES (pH 7.0). The gradient was centrifuged at 30,000 g for 35 min in a swingout head. Approximately 60% of the ATPase activity was recovered at the 0.6 M to 1.6 M interface. SMP2 were made as described in Chapter 2. Protein and ATPase activity were measured as described in Chapter 2. The data in this table were from representative experiments. ---=not determined.

Technique	Dry peas (gm)	SMP obtained (mg)	Specific activity of SMP (units/mg)
1. bubbler	200	---	0.017
2. tray	200	---	0.059
3. tray, step gradient	500	---	0.059
4. tray, roots & testa removed	1,700	150	0.036
5. greenhouse	900	110	0.072
6. greenhouse	1,000	37	1.045

which normally floated on top of the supernatant layer after the second spin. However, this problem eventually appeared (data not shown) to be correlated to over long grinding of tissues, which possibly caused organelle and membrane destruction. The best results were obtained when the tissue was pounded in a mortar and pestle until on 90% of strokes the pestle did not hit any unground cotyledons. Neither of these findings, however, enabled the specific activity of the SMP to be raised any further, and so the bubbler technique was abandoned in favour of the tray method that is described below.

For the tray technique, peas were soaked for 6 hrs in tap water and then sandwiched in a single layer between 2 layers of paper towels soaked in deionized water. The paper towels and peas were laid in fiberglass trays that were covered with plastic mesh and filled to the bottom layer of paper with deionized water. The trays were kept in a high humidity environment at 27 C until the peas were removed for isolation of mitochondria.

This technique was easily scaled up to as much as 1700 ml of peas, and gave SMP with only slightly below average specific activity (see Table 8). A sucrose step gradient (Malhotra and Spencer, 1973) was used to try and further purify the mitochondria before sonication, however, no increase in ATPase activity was apparent (Table 8). This may have been caused by inactivation by high sucrose concentrations, or by the undeveloped mitochondria having a different density to that of the mature mitochondria used by Malhotra and Spencer (1973). Removal of the roots, shoots, and testa of tray grown peas was also experimented with, but this resulted in a slight decrease in specific activity (Table 8).

Gradient "purified", tray SMP (48 hr) and 24 hr bubbled, "unpurified" SMP were both used for preliminary detergent extractions. The former type of SMP was also used for the optimization of cholate extraction, Affigel Blue chromatography, and ammonium sulphate precipitation. The susceptibility of both types of preparations to detergents, etc., was found to be identical.

In a direct comparison, with the same dried peas, media, grinding methods, etc., 4 day old greenhouse SMP (Chapter 2) had a 10% higher specific activity than SMP from 48 hr tray peas (preparations 2 and 5, Table

8), therefore, SMP2 (see Chapter 2) were made from 4 day old greenhouse peas by the method of Coleman and Palmer (1972), with modifications as described in Materials and Methods. SMP2 had a specific activity up to 10 times higher than that of SMP, although the protein yield was less than half of the usual yield (Table 8). It was found that four 1 min bursts of sonication raised the yield 20% (data not shown) and that sonication of fresh mitchondria (rather than previously frozen mitochondria) raised the specific activity even more (60%) (data not shown). Furthermore, the extraction of SMP2 with cholate resulted in CE with a slightly lower specific activity than SMP2, whereas cholate extraction of SMP normally resulted in CE with a 2-3 fold higher specific activity than SMP. (This was discussed in the section on detergent extraction, Chapter 3.) Nevertheless, SMP2 was used for the remaining experiments detailed in chapter 3 while other possible ways of increasing the yield were being explored. Four day old greenhouse SMP were exclusively used for the experiments in Chapter 4.