The mitochondrial ATPase inhibitor of rat skeletal muscle

by

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ABSTRACT

An inhibitor of mitochondrial ATPase has been partially purified from rat skeletal muscle. Mitochondria were prepared from rat skeletal muscle in solutions containing sucrose, mannitol and EDTA, and purified further by centrifugation in discontinuous sucrose density gradients. Purification of the mitochondria was followed by cytochrome oxidase activity. The inhibitor was released from these mitochondria by alkaline extraction. Ten fold purification was achieved by heat treatment and trichloroacetic acid precipitation, demonstrating that, like other coupling factor ATPase inhibitors, the rat skeletal muscle inhibitor is not destroyed by either of these procedures. Trypsin sensitivity indicated that the inhibitor is a protein. Inhibition of inhibitor-depleted submitochondrial particles from rat skeletal muscle was observed. Also cross-reactivity between the inhibitor and submitochondrial inhibitor-depleted particles of rat skeletal muscle and beef heart was demonstrated.

As a preliminary for the inhibitor study, the K_m and V_{max} of the ATPase activity of inhibitor-depleted submitochondrial particles derived from rat skeletal muscle mitochondria, prepared in Chappell-Perry medium, were estimated. In addition, the oligomycin-sensitive ATPase activity of mitochondria and inhibitor-depleted submitochondrial particles from beef heart and rat skeletal muscle were measured and compared.

ABBREVIATIONS

ADP	- adenosine diphosphate
ATP	- adenosine 5'-triphosphate
GTP	- guanosine 5'-triphosphate
ITP	- inosine 5'-triphosphate
CTP	- cytidine 5'-triphosphate
UTP	- uridine 5'-triphosphate
ATPase	- adenosine 5'-triphosphatase
Pi	- inorganic phosphate
Mg^{2+}	- magnesium ions
Mn^{2+}	- manganese ions
Ca^{2+}	- calcium ions
Na^{+}	- sodium ions
K^{+}	- potassium ions
NH_4^{+}	- ammonium ions
TCA	- trichloroacetic acid
EDTA	- ethylenediamine tetraacetic acid
TES	- N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
DNP	- 2, 4-dinitrophenol
SDS	- sodium dodecyl sulphate
A particles	- submitochondrial particles obtained by sonication at pH 9.2
AS particles	- A particles which have been passed through sephadex G50

I INTRODUCTION

(1) Mitochondrial ATPase

The energy-transducing membranes of mitochondria, chloroplasts and prokaryotes contain ATPase enzymes which are responsible for ATP synthesis in connection with both respiration and photosynthetic electron transport. Mitchell's chemiosmotic theory (1) states that the flow of electrons through a system of carrier molecules drives protons across the coupling membrane creating an electrochemical proton gradient. ATP synthesis is driven by the reverse flow of protons down the gradient. In the mitochondrion the flow of electrons through the respiratory chain results in the outward translocation of protons across the inner mitochondrial membrane. As these protons move inwards through the ATPase complex ATP is generated from ADP and inorganic phosphate.

Many lines of evidence support the postulates of the chemiosmotic theory (2,3,4,5). Mitchell and Moyle (2), for example, suspended mitochondria in a medium lacking oxygen and then monitored the pH as oxygen was injected, the proton concentration then rose rapidly. When oxygen was exhausted, the pH returned slowly to normal. The functioning of uncouplers of oxidative phosphorylation such as dinitrophenol by their action as proton ionophores suggests the importance of the proton gradient in this process. When chloroplasts or mitochondria are subjected

to artificial pH gradients ATP synthesis results. Combination of the ATPase complex in vesicles with the light-driven proton pump, bacteriorhodopsin, has demonstrated that ATP synthesis can be generated by a proton gradient (3). Finally, it has been shown that vesicles reconstituted from the ATPase of a thermophilic bacterium (4) or from a mitochondrial ATPase (5) would hydrolyse ATP forming an electrochemical proton gradient.

Recently, however, the chemiosmotic theory has been challenged by evidence which suggests that the terminal reactions of oxidative phosphorylation involve a series of reactions analogous to substrate-level phosphorylation. Griffiths and coworkers (6) have demonstrated that the isolated ATPase complex from beef heart can synthesise ATP in a reaction which requires dihydrolipoate, a component of the complex, and an unsaturated fatty acid such as oleic acid. In addition Johnston and Criddle (7) have shown that the F_1 ATPase from yeast catalyses the formation of ATP from oleoylphosphate and ADP.

The ATPase enzyme appears as conspicuous globular bodies protruding through the surface of the inner mitochondrial membrane into the matrix (8). The protruding sphere, designated F_1, was first isolated by Racker (9). F_1 is attached to a set of hydrophobic proteins, F_o, embedded in the membrane via a connecting protein, the oligomycin-sensitivity conferring protein (OSCP). When isolated as a whole from submitochondrial particles, the F_1-F_o system catalyses an ATPase activity which is inhibited by oligomycin and N,N'-dicyclohexyl-carbodiimide (DCCD). When the system is incorporated into liposomes it also catalyses an

ATP-dependant proton translocation (5).

The F_1 component which is the catalytically active part of the enzyme, has been purified from several eukaryote sources including beef heart (10), rat liver (11,12), brown adipose tissue (13) and yeast (14) as well as chloroplasts (15) and prokaryotes (16). The F_1 of beef heart is a soluble protein made up of five subunits of molecular weights 54,000 (α) 51,000 (β) 33,000 (γ) 17,000 (δ) and 11,000 (ϵ) (17). In solution purified F_1 catalyses an ATPase activity which is cold labile, the loss of activity being caused by dissociation of F_1 into its subunits (18). Early estimates of subunit composition suggested a complex of $\alpha_3\beta_3\gamma\delta\epsilon$ (19). However, more recent estimates based on titrations of the sulfhydryl groups and cross linking studies are compatible with a subunit structure of $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ (20) or $\alpha_2\beta_2\gamma_2\delta\epsilon$ (21). When comparing the amino acid composition of the F_1 subunits, it was noted (22) that while the γ and δ subunits differ from one another in composition, α, β and $(\gamma+\delta)$ are similar. This prompted the suggestion that the γ and δ subunits are the products of cleavage of a single subunit. An F_1 preparation, lacking the γ and δ subunits did not differ in its catalytic properties from the complete F_1, indicating that the α and β subunits play the main role in the ATPase activity. However, in reconstitution experiments with F_1-depleted submitochondrial particles, this F_1 preparation failed to demonstrate coupling activity. Therefore, Kozlov and coworkers (23) suggest that the γ and δ subunits are necessary for the formation of specific contacts between the F_1 molecule and the components of the mitochondrial membrane.

This is supported by work on the ATPase of E. coli, in which the ∂ subunit has been shown to be the site of attachment of F_1 to the membrane (24).

The catalytic site of F_1 is believed to be on the β subunit. This has been indicated by the work of Nelson et al (25) who showed that the inhibitor, 7-chloro-4-nitrobenzo-2-oxa-1,3 diazol, modifies a tyrosine residue on the β subunit of the ATPase from E. coli. This work has also been repeated with beef heart mitochondrial ATPase (26,27). The alkylating substrate analogue p-N-(2-chloroethylmethyl)-aminobenzylamide γ-^{32}P derivative of ATP, which inhibits the F_1 ATPase, has also been used to study the localisation of the catalytic site of the mitochondrial ATPase complex. Electrophoresis showed that the β subunit had combined with the labelled analogue. ATP protected F_1 from the inhibitory effect of this analogue (28).

The membrane component of mitochondrial ATPase, F_o, contains three or four hydrophobic proteins (29) and the binding sites for the inhibitors oligomycin (30) and DCCD (31). When this component is incorporated into liposomes it induces an increase in permeability which can be prevented by oligomycin, DCCD or the other components of the ATPase complex, F_1 and OSCP (32). These results suggest that the membrane component is a proton channel and that oligomycin and DCCD exert their inhibitory effect by inactivation of this channel. Recently, Criddle et al (33) have shown that one of the membrane proteins, subunit 9 of Tzagoloff's classification (34), of molecular weight 9,500, which may be extracted with chloroform, methanol solution, is the

proton ionophore. Using an artificial planar membrane and light-induced potentials generated by bacteriorhodopsin, they showed that this protein would dissipate the potential. However, when oligomycin was bound to the protein no dissipation of the potential took place.

Tzagoloff and Meagher (34) have shown that the four hydrophobic proteins of the membrane sector, F_o, are synthesised mitochondrially. This was achieved by selective labelling of the mitochondrial or extra-mitochondrial translation products by incorporation of radioactive amino acids in the presence of an inhibitor of cytoplasmic protein synthesis, cycloheximide or an inhibitor of mitochondrial protein synthesis, chloramphenicol. The use of yeast mutants, which were unable to perform mitochondrial protein synthesis, has demonstrated that the F_1 component of the ATPase is synthesised on cytoplasmic ribosomes (35).

The mechanism of ATP synthesis at the active site of the ATPase enzyme complex is not fully understood. Several hypotheses have been formulated but the evidence is not yet sufficient to decide among them. One hypothetical catalytic mechanism was proposed by Mitchell (36). In this scheme a phosphate group and ADP bind to an active site on F_1 near the end of the proton channel through F_o. Two protons are driven down the channel by the electrochemical gradient and attack one of the phosphate oxygens, which is removed as water. An oxygen of ADP makes a nucleophilic attack on the phosphorus centre forming ATP. Finally, the ATP dissociates from the enzyme

complex.

The alternative hypothesis suggests a less direct role for the protons. Boyer (37) and Slater (38) have proposed that the major energy input in ATP synthesis is required, not for the formation of the covalent bond between ADP and inorganic phosphate, but for the dissociation of the bound ATP. This hypothesis has been supported by the demonstration that the exchangeability of the nucleotides ATP and ADP, which are tightly bound to the ATPase is dependant on electron flow (39).

(2) Mitochondrial ATPase inhibitor

The ATPase inhibitor protein was first isolated from beef heart mitochondria by Pullman and Monroy (40). It has further been identified in the mitochondria of rat liver (41) and several species of yeast (42,43,44) and also in the ATPases of bacteria (45) and chloroplasts (46). This inhibitor is a heat stable protein, which exists as an 11,000 molecular weight dimer (17). It is not destroyed by TCA precipitation but is very sensitive to trypsin.

The inhibitor will inhibit both the soluble F_1 ATPase and the ATPase of submitochondrial particles. The conditions necessary for inhibition have been investigated by Horstman and Racker (47) using beef heart. They observed more pronounced inhibition of the ATPase activity of inhibitor-depleted submitochondrial particles when increasing concentrations of Mg^{2+} and ATP were present up to a maximum of 0.1 mM. For the rat liver

inhibitor maximum inhibition was also dependant on the presence of Mg^{2+}-ATP although ATP alone did confer partial inhibitory activity (41). Satre et al (43) showed that the activity of the inhibitor from yeast could be conferred to the yeast ATPase by the addition of Mg^{2+}-ATP or ATP alone. In beef heart (47) several divalent cations including Mn^{2+} and Ca^{2+} could substitute for Mg^{2+}. In the absence of divalent cations the inhibitor had virtually no effect. Monovalent cations such as K^{+}, Na^{+} and NH_4^{+} did not substitute and could prevent inhibition in the presence of Mg^{2+} and ATP. The interaction between the ATPase and its inhibitor exhibits a dependance on nucleoside triphosphates in the order ATP > ITP > UTP > GTP > CTP. These results correlate with the nucleotide and cation specificity of the ATPase. Therefore, the effect of a nucleoside triphosphate is stronger the higher its rate of hydrolysis by the enzyme. ATP analogues which could be bound but not hydrolysed, did not stimulate inhibitor binding (28). The pH optimum for binding of the inhibitor and enzyme is in the range of 5.0 to 6.5 for the inhibitors from beef heart (47), yeast (43) and rat liver (41).

Asmai et al (48) showed that a variety of ATP-driven reactions such as energy-dependant reduction of NAD by succinate and enhancement of 8-anilinonaphthalene-1-sulphonate (ANS) fluorescence were inhibited when the beef heart inhibitor was added to submitochondrial particles. However, oxidative phosphorylation was not inhibited. The results on ANS fluorescence were confirmed by Ferguson (49) who further demonstrated that the ATP-dependant exchange of the nucleotides ADP and ATP, which are

tightly bound to the ATPase enzyme, is suppresed by the inhibitor. Van de Stadt and coworkers (50) showed that the inhibition of the ATPase by the inhibitor is non-competitive and this interaction is diminished when the ATP/ADP ratio is low or when energy is generated by substrate oxidation. These results suggest that the inhibitor exerts a regulatory function in energy conservation. This is achieved by a mechanism in which respiration energises the membrane and decreases the affinity of the enzyme for the inhibitor, thus ATP is generated. On de-energisation the inhibitor binds to the enzyme and ATP degradation is prevented (28).

Kozlov and Skulachev (28) have suggested that there are several conformations of F_1, only a few of which have high affinity for the inhibitor. A high affinity conformation is generated by the degradative reaction. The inhibitor then binds and stabilises F_1 in a conformation in which the ligand binding sites are drawn close together and protected.

The inhibitor may be isolated from beef heart mitochondria in the form of a mixture of the monomeric and dimeric forms (17). Conversion to the monomer depends on the presence of agents such as dithiothreitol, which are capable of reducing disulphide bonds. The inhibitor forms the smallest or ϵ subunit of the F_1 ATPase of beef heart mitochondria (17), chloroplasts (46) and bacteria (45). Its position in F_1 is unknown, however, it is thought to be located near the surface since it is removeable by treatments such as trypsin digestion (42), passage through sephadex (8) or heat treatment (51). Molecular weight determinations have given values of 5,800 (monomer), 11,350 (dimer)

from gel filtration experiments (17), 9,500 (46) and 10,500 (51) from gel electrophoresis and 15,000 from sedimentation experiments (40) for the beef heart inhibitor. SDS gel electrophoresis was used to obtain molecular weights of 9,500 for the rat liver inhibitor (41) and 12,000 for the yeast inhibitor (43) isolated in the absence of sulphydryl compounds. In the case of the yeast inhibitor a monomer of approximately half the molecular weight was observed when dithiothreitol and mercaptoethanol were present during isolation.

Estimates of the amino acid composition of the inhibitor from two beef heart preparations (17,51), Candida utilis (43) and Saccharomyces cerevisiae (44) all vary to some extent, so that further study such as sequence analysis would be of interest to determine the homology between the inhibitors. The isoelectric points of the purified inhibitors are :-

7.6 (51) and 10.4 (17) beef heart,

8.9 (41) rat liver,

9.05 (44) S. cerevisiae and

5.6 (43) C. utilis.

In spite of these apparent differences cross reactivity between both the C. utilis and rat liver inhibitors with the beef heart system has been demonstrated (43,41).

Table 1 shows the relationship between the ATPase inhibitors of mitochondria and chloroplasts as well as the troponin component TN-I which inhibits the actomyosin ATPase. The F_1 inhibitor interacts with the actomyosin ATPase, however, unlike TN-I inhibition, this inhibition is not affected by

tropomyosin or TN-C and a four-fold greater concentration of the F_1 inhibitor is required. The trypsin-digested F_1 inhibitor will also inhibit the actomyosin ATPase activity, as will trypsin-digested TN-I (52). Conversely, TN-I was found to inhibit mitochondrial ATPase in a reaction which was dependant on the presence of Mg^{2+} and ATP and enhanced by tropomyosin. Trypsin-digested TN-I was ineffective as an inhibitor of the mitochondrial ATPase (53). These results indicate that the interaction of the F_1 inhibitor or TN-I with the actomyosin ATPase involves a site which is not sensitive to trypsin and is therefore different from the F_1 interaction site.

The Ca^{2+}-dependant ATPase of chloroplasts (CF_1) is inhibited by the F_1 inhibitor of beef heart and inhibition is retained after digestion of the inhibitor with trypsin (54). In contrast trypsin-digested TN-I is ineffective (55). Nelson et al (46) have stated that the Mg^{2+}-dependant ATPase activity of chloroplasts is not inhibited by F_1 inhibitor and the chloroplast inhibitor is not inhibitory with mitochondrial ATPase. Chan and Barbour (56) have reported that the rat liver mitochondrial ATPase inhibitor can also interact with the outer side of the inner mitochondrial membrane, stimulating the ATP-Pi exchange activity.

Studies on the location of synthesis of the mitochondrial inhibitor of yeast suggest that this takes place on cytoplasmic ribosomes (44). In addition, yeast cells repressed by growth on glucose show a decrease in inhibitor content, together with other components of the ATPase complex, whose synthesis is also

repressed (43). This is relieved by growth of the cells on glycerol. Lloyd and Edwards (57) have shown that when yeast cells are grown on glucose, binding of the mitochondrial ATPase inhibitor takes place after de-repression and is accompanied by increased sensitivity to inhibitors such as oligomycin which bind to the membrane component, F_o, together with a smaller increase of sensitivity to inhibitors binding to F_1.

(3) Skeletal muscle mitochondria

Skeletal muscle fibres have been classified into three groups: red, white, and intermediate (58). Peter et al (59) have redefined these groups as fast-twitch-oxidative-glycolytic, fast-twitch-glycolytic and slow-twitch-oxidative respectively. The fast-twitch-oxidative-glycolytic or red fibres have the highest capacity for aerobic metabolism since succinate dehydrogenase as well as cytochrome and myoglobin concentrations are highest in these fibres. They are also characterised by a moderate to high glycolytic ability since they have the highest glycogen concentration and a moderate lactate dehydrogenase activity. Electronmicrographs (60) have shown that this fibre type is rich in large mitochondria which have complex cristae and are associated with lipid droplets. These mitochondria are located between the myofibrils as longitudinal chains and peripherally as subsarcolemmal and paranuclear aggregates.

Fast-twitch-glycolytic or white fibres exhibit a predominantly anaerobic metabolism. They have a high glycogen

content as well as high phosphorylase, lactate dehydrogenase and α-glycerophosphate dehydrogenase activities. Succinate dehydrogenase activity is low as are cytochrome and myoglobin concentrations, indicating a limited glycolytic capacity (59). Mitochondria are fewer in number and smaller with less complex cristae. They are found in the interfibrillar regions of the fibre as pairs, one either side of the I band (60).

Slow-twitch-oxidative fibres rely mainly on aerobic metabolism. They have a low glycogen concentration, low phosphorylase, lactate dehydrogenase and α-glycerophosphate dehydrogenase. Their cytochrome concentration and succinate dehydrogenase activity are intermediate between those of the two other fibre types (59). The mitochondria are variable in number (60).

Therefore, the different skeletal muscle fibre types vary in the extent to which they utilise aerobic or anaerobic metabolism and this can be correlated with the appearance, number and location of their mitochondria (61). By comparison cardiac muscle exhibits a rate of aerobic metabolism higher than that of skeletal muscle (62) and is correspondingly richer in large mitochondria, which have the highest density of cristae found in vertebrate tissues (63). Skeletal muscle fibres also differ from one another in their genotypes, as shown by the different isoenzyme patterns of lactate dehydrogenase, AMP deaminase, light chains of myosin, phosphorylase kinase and cyclic AMP-dependant protein kinase found in the different muscle fibres (64).

Some evidence exists that mitochondria from different types of muscle fibres have different biochemical properties. Kark et al (65) found that equal amounts of mitochondria from slow-twitch-oxidative (red) fibres oxidised β-hydroxybutyrate eight times as rapidly as mitochondrial fractions from fast-twitch-glycolytic (white) fibres. Pande and Blanchaer (66) have shown that slow-twitch-oxidative muscle mitochondria from the rabbit have a greater capacity for fatty acid oxidation than mitochondria from fast-twitch-glycolytic muscles. In the case of cardiac muscle (67) two populations of mitochondria exist, one beneath the sarcolemma and the other between the myofibrils. These can be isolated separately and have been shown to differ metabolically. The specific activities of succinate dehydrogenase and citrate synthetase were higher in the interfibrillar mitochondria and these mitochondria oxidised substrates at a faster rate than the subsarcolemmal mitochondria.

The composition of skeletal muscle mitochondria is not static and changes can be induced. Oscai and Holloszy (68) have demonstrated that the mitochondrial enzymes of fatty acid oxidation, the citric acid cycle, components of the respiratory chain and the mitochondrial ATPase increase in response to exercise. Therefore, the capacity to oxidise pyruvate and long chain fatty acids to obtain energy is a feature of the adaptation of skeletal muscle to exercise. However, the glycolytic enzymes, creatine phosphokinase and adenylate kinase were unchanged, indicating that anaerobic ATP generation is unaffected by exercise.

Changes in skeletal muscle mitochondria also take place during cold-acclimatisation (69). Skeletal muscle of cold-acclimated rats contained an increased number of smaller mitochondria, however, cytochrome oxidase and mitochondrial protein concentration per gram of muscle was unchanged.

In man several abnormalities in skeletal muscle mitochondrial metabolism have been reported (70,71,72). In non-thyroidal hypermetabolism ,Luft's disease, (70,71) skeletal muscle mitochondria were increased in number and larger with tightly packed cristae. Loose coupling of oxidative phosphorylation, a high mitochondrial ATPase activity and decreased calcium retention suggested that cycling of divalent cations between the mitochondria and cytoplasm might be the cause of the hypermetabolism. Schotland et al (72) have reported a neuromuscular disorder associated with a defect in mitochondrial energy supply. Skeletal muscle mitochondrial ATPase activity was reduced in comparison to normal, however, this might have been secondary to replacement of functional inner mitochondrial membrane by crystalline inclusions.

(4) Aims of research

It is apparent from the evidence sited that the mitochondrial ATPase is a key enzyme in cellular energy transduction and conservation, the activity of which is regulated by its inhibitor protein subunit. The enzyme and its inhibitor of

beef heart, rat liver and yeast mitochondria have been isolated and studied. However, this is not the case for skeletal muscle, where the enzyme has only been measured, for example with respect to changes in response to exercise. Skeletal muscle differs from cardiac muscle in both structure and metabolism. In addition the mitochondria from the skeletal muscle fibres show differences from those of cardiac muscle.

The aim of this research was to devise a method of preparing skeletal muscle mitochondria in which the ATPase and its inhibitor could be studied and then to isolate the inhibitor following the methods used for the beef heart and rat liver inhibitors. It was found that the skeletal muscle inhibitor could not be prepared by the steps used for the beef heart and rat liver inhibitors but a combination of steps unique to this preparation was required.

II METHODS AND MATERIALS

(1) Materials

Chemical	Type	Supplier
ATP	sodium salt grade 1	Sigma
Phospho(enol) Pyruvate	trisodium salt	Sigma
Pyruvate kinase	from rabbit muscle type 11	Sigma
Sucrose	special enzyme grade	Schwarz/Mann
Ammonium sulphate	special enzyme grade	Schwarz/Mann
Oligomycin	contained oligomycin A, B and C	Sigma
Rutamycin	oligomycin analogue	Dr. R.L.Mann, Eli Lilly Co.
EDTA	disodium salt	Sigma
Cytochrome c	from horse heart type 111	Sigma

(2) Preparation of mitochondria from rat skeletal muscle

Male rats weighing between 250 and 500 g were killed by cervical section. All subsequent operations were carried out in a cold room at 4°C unless otherwise specified. A refrigerated Sorvall RC-2B set at 0°C was generally used for centrifugation.

(a) Chappell-Perry medium (73): The excised skeletal

muscle from the hind limbs was placed in cold 0.15 M potassium chloride solution. It was then freed of fat and connective tissue and minced finely. The mince was blotted, weighed and then suspended in 10 volumes of Chappell-Perry medium (0.1 M potassium chloride, 0.05 M Tris-hydrochloride buffer, pH 7.4, 0.001 M Na-ATP, 0.005 M magnesium sulphate and 0.001 M EDTA). It was then homogenised in 50 ml aliquots using a PT 10 OD polytron at half maximum speed for 30 seconds in the cold. The homogenate was centrifuged at 650 x g. for 5 minutes. The supernatant was saved and the pellet was resuspended, homogenised and centrifuged as before. The supernatants were pooled and centrifuged at 14,000 x g. for 10 minutes. The brown mitochondrial pellet obtained was suspended in 0.25 M sucrose solution.

(b) Sucrose/mannitol solutions: The solutions were prepared as described by Chance and Hagihara (74). However, the use of the enzyme nagarse was omitted since the mitochondrial ATPase inhibitor is easily destroyed by trypsin (40). The pieces of skeletal muscle were placed in cold stock medium (0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, pH 7.4). The fat and connective tissue was removed and the muscle was rinsed with cold stock solution. It was then minced very finely with scissors and weighed. About 27 ml of homogenising medium (stock medium containing 0.01 M Tris-phosphate, pH 7.6) was added to 3 g of minced tissue. This mixture was then homogenised using the PT 10 OD polytron for 30 seconds at maximum speed. The homo-

genate was centrifuged at 450 x g. for 5 minutes. The supernatant was carefully poured off and centifuged at 12,000 x g. for 10 minutes. The walls of the centrifuge tubes were wiped to remove fat and the mitochondrial pellets were combined by resuspending them in about 1 ml of suspending medium (stock medium containing 0.01 M Tris-chloride, pH 7.4) for each initial gram of muscle. The suspension was then centrifuged at 8,000 x g. for 5 minutes and the brown pellet was suspended in 0.25 M sucrose solution.

(c) Discontinuous density gradient centrifugation: This procedure was carried out on rat skeletal muscle mitochondria prepared using sucrose/mannitol solutions. The final 8,000 x g. centrifugation step was omitted and the mitochondrial pellet was suspended in solution A(0.1 M Tris-chloride, pH 7.4, 0.21 M mannitol, 0.07 M sucrose, 0.01 M EDTA, pH 7.4) using about 0.5 ml per initial gram of muscle. Aliquots of 6.5 ml of this suspension were layered onto 45.5 ml discontinuous density gradients containing Tris, mannitol and EDTA at the same concentration as solution A and ranging in 6 stages, each of 6.5 ml, from 1.07 M sucrose to 1.32 M sucrose over a cushion of 1.97 M sucrose, as described by Bullock et al (75). Centrifugation was carried out at 105,000 x g. for 45 minutes by the use of a SW 25.2 Spinco rotor. After centrifugation the gradient could be divided into 3 sections:- an upper clear zone, an intermediate zone of 3 brown mitochondrial bands and a lower zone containing a white band, F3. These 3 zones were removed separately using a hypodermic syringe with a U-shaped needle. Each was then diluted

with solution A and centrifuged at 20,000 x g. for 15 minutes. The pellets obtained were suspended in 0.25 M sucrose solution.

(3) Preparation of beef heart mitochondria

Beef heart mitochondria were prepared as stated by Quilliam (76). This was a modification of the method described by Smith (77) in which triethylamine chloride was substituted for Tris in the sucrose isolation medium.

(4) Preparation of A particles (78)

Mitochondria were suspended at approximately 20 mg/ml in a solution containing 0.18 ml 0.25 M sucrose/ml and 0.00625 ml 0.1 M EDTA, pH 7.4/ml. The pH was adjusted to 9.2 by the use of pH paper with freshly diluted 1.0 N ammonium hydroxide and the mixture was allowed to stand in ice for 15 minutes. It was then sonicated in an ice bath at half maximum speed on a Bronwill sonicator for 2 minutes in 15 second intervals, with cooling of the mixture and probe between each burst of sonication. The suspension was then allowed to stand in ice for a further 15 minutes. Centrifugation was carried out at 26,000 x g. for 10 minutes in a Spinco Type 65 rotor. The pellet obtained was discarded and the supernatant was recentrifuged at 100,000 x g. in a Spinco Type 65 rotor for 60 minutes. The resulting supernatant was discarded and the pellet was homogenised in 2/3 the original

volume of 0.25 M sucrose-1 mM EDTA, pH 7.4, solution. The suspension was centrifuged at 100,000 x g. for 30 minutes. The pellet was then homogenised in the same volume of 0.25 M sucrose solution and centrifuged as before. Finally, the pellet was suspended in 0.25 M sucrose solution at a concentration of about 50 mg/ml.

(5) Preparation of AS particles (8)

A particle suspensions were homogenised with an equal volume of equilibrating buffer (30 mM Tris-sulphate, pH 8.0, 75 mM sucrose, 250 mM potassium chloride and 2 mM EDTA, pH 8.0). The sample was then applied to a column of coarse G50 Sephadex which had been equilibrated at room temperature. The column was of radius 1.3 cm and length 4 cm/ml of sample. The flow rate was adjusted so that the passage time was 40 to 60 minutes. The particles were centrifuged at 100,000 x g. in a Spinco Ti 65 rotor for 15 minutes and then suspended in 0.25 M sucrose solution.

(6) ATPase assay (47)

The assay mixture contained 50 mM Tris-sulphate, pH 7.7, 5 mM magnesium sulphate, 5 mM ATP, 5 mM phospho(enol) pyruvate and 32 µg pyruvate kinase, 5 to 10 µg of enzyme preparation and water to a final volume of 1 ml. Oligomycin or rutamycin, dissolved in absolute ethanol, was added at a concentration of 10 to

100 µg/mg enzyme protein, other assay mixtures contained equivalent amounts of ethanol. The enzyme-buffer mixtures were equilibrated on a shaking waterbath at 30°C and the reaction was started by the addition of substrate. After 10 minutes incubation the reaction was stopped by the addition of 0.25 ml 50% TCA. The tubes were allowed to stand in ice for 10 minutes and were then centrifuged at 6,000 x g. for 10 minutes. Aliquots of 0.5 ml were taken for phosphate determination. A unit of enzyme activity was defined as the quantity that catalyses the formation of 1 µmole of phosphate per minute. Specific activity was defined as the units of enzyme activity per mg of enzyme protein.

(7) Assay of ATPase inhibitor (47)

In a final volume of 0.5 ml AS particles, containing approximately 0.06 units of ATPase activity, were incubated for 20 minutes at room temperature with various amounts of inhibitor in the presence of 0.5 mM magnesium sulphate, 0.5 mM ATP, pH 7.0, 15 mM Tris-TES, pH 6.7. The volume was adjusted with 0.25 M sucrose solution. The order of addition was sucrose, buffer, AS particles, inhibitor, magnesium sulphate and ATP. In each assay a blank without AS particles or inhibitor was measured so that non-enzymic production of phosphate could be taken into account. Also a control containing inhibitor but without AS particles was set up for each aliquot of inhibitor tested. After incubation 2 samples of 0.2 ml were taken from each tube, each

sample was added to a 0.803 ml ATPase assay mixture. To obtain a zero time control 0.25 ml 50% TCA was added to one of these and it was immediately placed in an ice bath. The second tube was incubated at 30°C for 10 minutes and the ATPase assay was carried out as described before. One unit of activity was defined as the amount of inhibitor which gave 50% inhibition of 0.2 units of ATPase.

(8) Protein assay

Protein was estimated by the method of Lowry et al (79).

(9) Phosphate assay

Phosphate was assayed by a modification of the method of Baginski and Zak (80). Eikonogen (0.25 g 1-amino-2-naphthol sulphonic acid, 0.25 g sodium sulphite, 14.65 g sodium hydrogen sulphate) was prepared weekly by dissolving the mixture in approximately 100 ml of hot distilled water. This was then cooled to room temperature, filtered and made up to 100 ml. 250 µl of 2.5% ammonium molybdate and 100 µl of Eikonogen were added to each 500 µl sample with mixing. After 2 minutes 500 µl of arsenite-citrate reagent (2% sodium arsenite, 2% sodium citrate, 2% glacial acetic acid) was added with mixing. The optical density was read at 700 nm on a Beckman DU spectrophotometer after 45 minutes. Standards containing 0 to 161 nmoles

of potassium dihydrogen phosphate in 10% TCA were determined in each assay.

(10) Cytochrome oxidase assay

A solution of 1.0 mM reduced cytochrome c was prepared as described by Wharton and Tzagoloff (81) by dissolving cytochrome c in 2/3 the final volume of 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and making up to volume by the addition of 1 M ascorbic acid containing 3 mM EDTA. This solution was then dialysed overnight at 4°C against the phosphate buffer to remove excess ascorbate. The change in absorbance of the appropriate concentration of the ferrocytochrome c solution, after oxidation by potassium ferricyanide was measured and the percentage reduction, which was always greater than 90%, was taken into account when calculating cytochrome oxidase activity.

In the cytochrome oxidase assay carried out by the method of Yonetani (82), reaction mixtures were made up in cuvettes of 1 cm light path. These contained 0.5 ml 0.2 M potassium phosphate buffer, pH 6.0, containing 1 mM EDTA, 0.92 mM ferrocytochrome c, which was sufficient to give an optical density of 1.0 and distilled water to a final volume of 1 ml. The measurement was carried out at 25°C in a recording Beckman DB-G spectrophotometer at 550 nm. The reaction was initiated by mixing in 0.01 ml containing an appropriate amount of the enzyme, which had been diluted in 0.01 M potassium phosphate

buffer, pH 7.0, containing 0.1% Tween-80. In the blank cuvette the substrate, ferrocytochrome c, was replaced by an equivalent amount of 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The initial rate of absorbance decrease at 550 nm, which was linear for up to 5 minutes, was recorded and from this the initial turnover rate of the enzyme was calculated using the extinction coefficient:

$$\Delta_\epsilon \text{ at } 550 \text{ nm} = 0.0196 \text{ M}^{-1} \text{ cm}^{-1}$$

One unit of enzyme activity was defined as the quantity which catalyses the reduction of one μmole of ferrocytochrome c per minute. Specific activity was defined as the units of enzyme activity per mg of enzyme protein.

(11) Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out on 13% slab gels with β-mercaptoethanol according to O'Farrell (83) using α-chymotrypsin, ovalbumin, cytochrome c, bovine serum albumin and alcohol dehydrogenase as standards. Staining with Coomassie Brilliant Blue was carried out as described by Maizel (84).

III RESULTS

(1) ATPase activity of skeletal muscle mitochondria, A and AS particles prepared using Chappell-Perry medium

(a) Inhibition by rutamycin:

(i) Mitochondrial ATPase: Mitochondria, prepared using Chappell-Perry medium were assayed for ATPase activity in the presence of varying amounts of rutamycin. This reagent was used to determine mitochondrial ATPase activity specifically since this activity, but not extra-mitochondrial ATPases, are inhibited by oligomycin and its analogue rutamycin. The titration curve (Figure 1) shows that the maximum inhibition obtained was 64%. Since maximum inhibition was reached by 40 µg rutamycin/mg mitochondrial protein, this concentration was used in subsequent determinations of the mitochondrial ATPase activity. Rutamycin-sensitive ATPase activity was calculated by subtraction of the activity remaining after rutamycin treatment.

(ii) A particle ATPase: The ATPase activity of A particles was titrated with rutamycin. As shown in the graph (Figure 2) a plateau has been reached by 60 µg rutamycin/mg A particle protein with a maximum inhibition of 77% for this preparation.

(iii) AS particle ATPase: When the ATPase activity of AS particles was titrated with rutamycin a maximum inhibition of 71% was achieved (Figure 3). This was reached by a concentration

of 100 μg rutamycin/mg AS particle protein. Therefore, this rutamycin concentration was used in subsequent assays of the AS particle ATPase.

(b) AS particle ATPase: AS particles were isolated from rat skeletal muscle mitochondria, prepared in Chappell-Perry medium.

(i) Time study: AS particles were incubated for 0, 1, 2, 5 or 10 minutes in the regenerating system assay mixture. Tubes containing AS particles and rutamycin were also incubated for the same time intervals. Figure 4 shows that the ATPase activity of the AS particles was linear up to 10 minutes in the presence or absence of rutamycin.

(ii) Enzyme concentration study: When increasing amounts of AS particles were incubated in the regenerating system assay mixture, the amount of inorganic phosphate released was linear in both the presence and absence of rutamycin over the range measured (Figure 5).

(iii) Determination of kinetic parameters: The ATPase activity of the rat skeletal muscle AS particles was determined over the range 0 to 7 mM ATP in the presence and absence of rutamycin using the standard ATPase assay. The kinetic parameters were estimated using two graphical representations (Figures 6, 7, 8). The values for the K_m and V_{max} obtained are given in Table 2.

(2) Comparison of ATPase and cytochrome oxidase activities of mitochondria and submitochondrial particles from rat skeletal muscle prepared by different techniques

(a) ATPase activities and yields of rat skeletal muscle mitochondria prepared by different techniques: A comparison of the properties of skeletal muscle mitochondria, prepared using Chappell-Perry medium and sucrose/mannitol solutions, is given in Table 3. Although the yield of mitochondria prepared using Chappell-Perry medium is high, only 48% of the ATPase activity of this preparation is sensitive to oligomycin. The yield of mitochondria prepared using sucrose/mannitol solutions is smaller and again the sensitivity to oligomycin is less than 100%. However, it is evident that purification of the sucrose/mannitol mitochondria is achieved by sucrose density gradient centrifugation since the gradient mitochondria have the highest ATPase activity and the highest sensitivity to oligomycin. This sensitivity had a mean value of 66% and varied up to 80% in some preparations. However, the yield is low, 0.49 mg/g of muscle, and the use of sucrose density gradients restricts the amount of mitochondria that can be prepared at one time. The average yield of units of the oligomycin-sensitive ATPase was 61% of the total number of units applied to the gradient.

(b) Cytochrome oxidase activities of rat skeletal muscle mitochondria prepared using sucrose/mannitol solutions and gradient purification: The mitochondrial enzyme, cytochrome oxidase, was assayed to estimate the recovery and purification of the skeletal muscle mitochondria. Table 4 shows the specific

activities and yields of the homogenate, the mitochondria without gradient purification and after gradient purification. There has been an 18 fold purification of the mitochondria from the homogenate and a further 2 fold purification by the sucrose density gradient step. Therefore, overall the gradient mitochondria have been purified 36 fold relative to the homogenate. The F3 fraction, found as a white band near the bottom of the sucrose density gradient, probably contained myofibrils since it exhibited an ATPase activity with very little sensitivity to oligomycin and a low cytochrome oxidase activity. For the beef heart mitochondria used in this study the cytochrome oxidase activity was 4.1 μmoles/min/mg. It was apparent that purification of the skeletal muscle mitochondria by gradient centrifugation was a necessary step in order to remove actomyosin ATPase and the troponin component TN-I both of which would have interfered with assays of the mitochondrial ATPase and its interaction with its inhibitor.

(c) Comparison of mitochondrial and AS particle ATPase activities from skeletal muscle and beef heart: Table 5 gives a comparison between the ATPase activities of the mitochondrial and AS particle fractions. With Chappell-Perry rat skeletal muscle mitochondria there was little increase in the specific activity of the oligomycin-sensitive ATPase of the AS particles compared to that of the mitochondria. Also these two fractions showed the lowest sensitivity to oligomycin. In the case of rat skeletal muscle mitochondria, prepared using sucrose/mannitol

solutions and purified on the gradient, a 2 fold stimulation of activity was seen during the preparation of the AS particles. These particles showed a high sensitivity to oligomycin. With beef heart mitochondria, as expected from the work of Racker and Horstman (8), a large increase in specific activity was obtained during the preparation of the AS particles, giving an 8 fold stimulation of the oligomycin-sensitive ATPase activity relative to the mitochondria. The ATPase activity of both the beef heart mitochondria and AS particles was highly sensitive to oligomycin.

(3) The ATPase inhibitor from rat skeletal muscle

(a) Alkaline extraction:

(i) Method: This was carried out as described by Horstman and Racker (47). To 10 mg mitochondrial protein in 0.25 M sucrose solution was added 2.5 µl 1.0 M Tris-sulphate, pH 7.4, and 10 µl 0.1 M EDTA, pH 7.4. The volume was adjusted to 0.5 ml with 0.25 M sucrose solution. The mixture was placed in an ice bath and 25 µl cold 1.0 N potassium hydroxide solution was added quickly with mixing. After exactly 1 minute 2.5 µl 10 N acetic acid was added with vigourous mixing. The mixture was allowed to stand in ice for 2 minutes before the pH was brought to 5.4 with 1.0 N acetic acid by the use of pH paper. Within 1 minute the pH was adjusted to 7.4 with 1.0 N potassium hydroxide solution. The mixture was centrifuged at 14,000 x g. for 10 minutes and the supernatant containing the

ATPase inhibitor protein was retained.

(ii) Results: AS particles and an alkaline extract were prepared from mitochondria isolated using Chappell-Perry medium. However, when these were titrated in an inhibitor assay no significant levels of inhibition could be obtained. Therefore, to test the inhibitor preparation technique and assay method, AS particles and an alkaline extract were prepared from beef heart mitochondria, the system originally used by Horstman and Racker (47). Titration of beef heart alkaline extract with beef heart AS particles gave an inhibition curve (Figure 9). The specific activity of this alkaline extract was calculated to be 314 units/mg.

Since other AS particle and inhibitor preparations such as those from rat liver (41) and yeast (43) had been shown to cross-react with the beef heart system, a series of cross-reactivity experiments between the rat skeletal muscle and beef heart systems was carried out. Again no significant levels of inhibition could be obtained when a rat skeletal muscle mitochondrial alkaline extract or AS particle preparation was used with the corresponding beef heart fraction.

Therefore, it was decided to prepare skeletal muscle mitochondria by a method more similar to that used for beef heart mitochondria. This involved the use of sucrose/mannitol solutions. These mitochondria were subjected to alkaline extraction and beef heart AS particles were used in the assay of this extract. Figure 10 shows that this alkaline extract

contained measureable levels of an inhibitor capable of reacting with beef heart mitochondrial ATPase. To insure that this inhibition was mitochondrial in origin, gradient purified mitochondria were prepared and used for alkaline extraction. In some cases double extraction of these mitochondria was required to release the inhibitor. Titration of the gradient mitochondria alkaline extract with beef heart AS particles gave higher levels of inhibition than the alkaline extract from non-gradient mitochondria at the 3 points tested (Figures 10 and 11). Therefore, gradient centrifugation achieved some purification of the inhibitor protein and facilitated its release by alkaline extraction.

AS particles and alkaline extracts were prepared from skeletal muscle gradient mitochondria and beef heart mitochondria concurrently. The 4 titration curves obtained are shown in Figures 11 and 12. These graphs indicate that there is cross-reactivity between rat skeletal muscle and beef heart mitochondrial ATPases and their inhibitor proteins. The specific activity of the rat skeletal muscle inhibitor was 2 units/mg with the skeletal muscle ATPase and 3.6 units/mg with the beef heart ATPase. To obtain this skeletal muscle alkaline extract mitochondria were repeatedly extracted with alkali and the active fractions (numbers 1 and 2) were pooled. Table 6 lists the properties of the alkaline extract.

Chan and Barbour (41) observed that the use of 0.15 M hypotonic sucrose, rather than 0.25 M, during alkaline extraction

of rat liver mitochondria gave rise to a 50% increase in inhibitor yield. Therefore, an alkaline extract was prepared from rat skeletal muscle gradient mitochondria suspended in 0.15 M sucrose. This led to an increase in yield of 20%. The specific activity of this alkaline extract was similar to that of the alkaline extract prepared from mitochondria suspended in isotonic sucrose, when assayed with approximately the same number of units of ATPase (Table 6). Increasing the units of ATPase per tube in the inhibitor assay gave an increased value for the specific activity of the inhibitor. As shown in Table 6 the specific activity increased from 2.2 to 4.8 units/mg when the units of enzyme per tube were increased from 0.02 to 0.109. Therefore, at the lower concentration of enzyme dilution is such that either enzyme-inhibitor binding is reduced or the activity itself is lost. Titration of the rat skeletal muscle AS particle ATPase used in these experiments with oligomycin gave a maximum inhibition of 69% and allowed recalculation of inhibitor specific activities on this basis (Table 11).

(b) Heat treatment:

(i) Method: The protein concentration of the alkaline extract, prepared with hypotonic sucrose, was adjusted to 0.9 to 1.0 mg/ml. 1 to 2 ml aliquots of the solution were placed in 75 x 12 mm pyrex test tubes and incubated in a water bath at 80°C for 3 minutes with constant agitation. After cooling on ice for 15 minutes the precipitated protein was removed by centrifugation at 24,000 x g. for 10 minutes. The supernatant

was retained for protein and inhibitor assays.

(ii) Results: Heat treatment of alkaline extract resulted in a white precipitate of denatured protein. When this was removed by centrifugation the yield of protein in the supernatant was 0.19 mg/mg alkaline extract protein. The specific activity of the inhibitor was increased 3.6 fold relative to the alkaline extract. The yields of inhibitor units and specific activities for 2 preparations are given in Tables 9 and 10. Heat treatment of the TCA precipitate at a concentration of 1 mg/ml caused only a small increase in specific activity (Table 8).

(c) TCA precipitation:

(i) Method: 50% cold TCA was added dropwise to the inhibitor solution to a final concentration of 10%. The mixture was shaken and immediately centrifuged at 20,000 x g. for 5 minutes. The supernatant was removed and the precipitate was homogenised in 1 to 2 ml of cold distilled water using an all-glass Potter-Elvehjem homogeniser. The pH was adjusted to 5.0 with 1.0 N potassium hydroxide by the use of pH paper and the suspension was centrifuged at 24,000 x g. for 5 minutes. The supernatant was saved, neutralised to pH 7.4 with 1.0 N potassium hydroxide and 2.0 M sucrose was added to make a final solution 0.25 M in sucrose.

(ii) Results: When the heat treated 0.15 M sucrose alkaline extract was subjected to TCA precipitation the resulting fraction showed a 2.2 fold increase in the specific

activity of the inhibitor. The yield of protein in this procedure was 0.1 mg/mg alkaline extract protein. Specific activities and yields of inhibitor units for 2 preparations are given in Tables 9, 10, and 11 and titration curves in Figures 13, 14, and 15. TCA precipitation was also successfully applied to an alkaline extract prepared from gradient mitochondria in 0.25 M sucrose (Table 8). In this case a 3 fold purification was achieved with a yield of 0.49 mg/mg alkaline extract protein. The apparent increase in the total units may have been due to the greater number of units of enzyme per tube in the TCA precipitate assay.

(d) Ammonium sulphate fractionation:

(i) Method: Solid ammonium sulphate was added to the inhibitor fraction in small amounts while stirring on ice until the required level of saturation was reached. Stirring was continued for 20 minutes and the resulting precipitate was obtained by centrifugation at 14,000 x g. for 10 minutes. The precipitate was then dissolved in 1 to 2 ml 0.25 M sucrose and dialysed against 500 volumes of this solution.

(ii) Results: Following the procedure of Horstman and Racker (47) the protein precipitating between 40% and 70% saturation was obtained using a 0.25 M sucrose alkaline extract derived from non-gradient rat skeletal muscle mitochondria. Initially this fraction failed to show inhibition. However, on dialysis of the fractions inhibition was obtained as shown in Table 7. Titration of the 40% to 70% fraction with skeletal muscle AS particles gave a specific activity of 1.5 units of

inhibitor/mg. When this procedure was repeated with ammonium sulphate fractions derived from gradient mitochondria the expected increase in specific activity relative to the alkaline extract was not obtained. The 0% to 40% and 40% to 70% fractions showed comparable levels of inhibition with a decrease in the slope of the curve when larger aliquots of the fractions were used in the assay (Figure 16). A shorter dialysis time of 1 hour rather than overnight dialysis gave a slight improvement in inhibition levels, suggesting some loss of inhibitor during dialysis.

Chan and Barbour (41) using rat liver mitochondria found it necessary to use the 0% to 50% ammonium sulphate fraction at this stage of the purification. Therefore, a 0% to 50% ammonium sulphate fraction was made from a 0.25 M sucrose alkaline extract of rat skeletal muscle gradient mitochondria. This fraction was immediately precipitated with TCA to remove ammonium sulphate and therefore avoid the need for dialysis. However, ammonium sulphate fractionation and TCA precipitation gave a titration curve showing less inhibition than when TCA precipitation alone was used. Lack of success in achieving purification by ammonium sulphate fractionation is probably due to the small aliquots and protein content of the inhibitor fractions as well as interference by ammonium sulphate in the assay of inhibitor activity.

(e) Fractionation with ethanol:

(i) Method: The TCA fraction, derived from the heat

treated 0.15 M sucrose alkaline extract was brought to a concentration of 0.8 M ammonium sulphate by the addition of the solid salt. To each ml of this solution 1.7 ml of ice-cold 95% ethanol was added dropwise in the cold and the mixture was centrifuged at 9,000 x g. for 10 minutes. The precipitate was homogenised in 1 to 2 ml of 0.25 M sucrose buffered with 10.0 mM Tris-sulphate, pH 7.4, and insoluble matter was removed by centrifugation as before. Solid ammonium sulphate was added to give a concentration of 0.4 M and 1.7 ml of ethanol were added per ml of solution. The precipitate was collected by centrifugation and dissolved in a minimum volume of buffered sucrose as before.

(ii) Results: The yield of protein in this procedure was 0.009 mg/mg alkaline extract protein. When titrated with AS particle ATPase the fraction showed less inhibition than the TCA precipitate. However, low ATPase units were used in these assays and contaminating ammonium sulphate was also probably present.

(f) Gel electrophoresis:

(i) Results: Gel electrophoresis of the 0% to 40% and 40% to 70% ammonium sulphate fractions showed several high molecular weight proteins in the range 25,000 to 70,000 and two protein bands which migrated approximately the same distance as the marker protein, cytochrome c, molecular weight 12,400. When isotonic sucrose alkaline extracts from beef heart and rat skeletal muscle mitochondria were subjected to electrophoresis the banding patterns obtained were similar to each other and to

those of the ammonium sulphate fractions. Each showed two low molecular weight protein bands. These two bands were also seen in the electrophoretic patterns of the TCA precipitate and heat treated TCA precipitate derived from the rat skeletal muscle alkaline extract. This TCA precipitate, in which the specific activity of the inhibitor had increased 3 fold relative to the alkaline extract (Table 8) also showed high molecular weight protein bands. However, some of these were reduced in intensity.

These results were confirmed by electrophoresis of a hypotonic sucrose alkaline extract, heat treated alkaline extract and TCA precipitate (Figure 17). The specific activities of these fractions are given in Tables 10 and 11. The use of marker proteins (Figure 18) allowed the calculation of molecular weights (Figure 19). This gave values of 13,700, 12,500 and 10,500 for the protein bands which migrated furthest in these 3 fractions (Figure 17).

The electrophoretic pattern of a TCA precipitate derived from a 0% to 50% ammonium sulphate fraction of an isotonic sucrose alkaline extract (Figure 18) also showed low molecular weight protein bands. However, it is apparent that heat treatment of the alkaline extract (Figure 17) is more efficient in removing high molecular weight proteins than the ammonium sulphate fractionation.

Figure 17 also shows the electrophoretic pattern obtained from the ethanol precipitate. Only 9 μg of protein were available for electrophoresis. However, a low molecular weight protein band was observed.

(g) Trypsin digestion:

(i) Method: Trypsin digestion was carried out at 30°C for 15 minutes. The assay mixture contained 0.25 M sucrose, 15 mM Tris-TES, pH 6.7, an aliquot of the TCA precipitate and trypsin at 50 µg/µg of TCA precipitate, dissolved in 150 mM Tris-TES, pH 6.7. Digestion was terminated by the addition of trypsin inhibitor at 2 µg/µg trypsin, dissolved in 150 mM Tris-TES, pH 6.7. After 15 minutes at room temperature the ATPase inhibitor assay was initiated by the addition of AS particles, magnesium sulphate and ATP.

(ii) Results: As shown in Table 12 digestion of the TCA precipitate with trypsin significantly reduced the level of inhibition. This indicated that inhibition of the AS particle ATPase was due to a protein component of the TCA precipitate. The specific activity of this TCA precipitate before trypsin digestion is given in Tables 10 and 11.

IV DISCUSSION

(1) ATPase activity of skeletal muscle mitochondria, A and AS particles prepared using Chappell-Perry medium

(a) Inhibition by oligomycin: The inhibition levels obtainable with oligomycin and the ATPase of rat skeletal muscle mitochondria have been demonstrated in the present work. For submitochondrial particles maximum levels of inhibition greater than 70% were achieved. These results can be compared with those of Tzagoloff and Meagher (34) and Johnston et al (85). Both of these groups, working with yeast submitochondrial particles, obtained inhibition curves similar to those of skeletal muscle submitochondrial particles over the same range of oligomycin concentration. Maximum levels of inhibition were 90% (34) and 80% (85). The availability of mutants resistant to oligomycin has prompted the study of the oligomycin sensitivity of the yeast mitochondrial ATPase. In the case of mitochondria from non-resistant organisms a high sensitivity to oligomycin may be an indication of a mitochondrial preparation which is not contaminated by other ATPase enzymes such as Ca^{2+}-dependant ATPase, Na^{+}-K^{+} ATPase or actomyosin. Therefore, it appears that the mitochondrial ATPase prepared from rat skeletal muscle is acceptable in this respect. However, the level of inhibition in different preparations is somewhat variable and can be altered by the addition of various reagents. Potassium ions, dihydrolipoic

acid and lipoic acid enhance oligomycin inhibition of yeast mitochondrial ATPase, whereas H^+ ions decrease inhibition. Addition of low levels of mercuric ions and iodoacetic acid block oligomycin inhibition but not enzyme activity (85). These results were interpreted in terms of a model in which the oligomycin binding site exists in a high or a low affinity form, the relative amounts of these two forms being governed by the added reagents. The effect of these reagents on oligomycin binding suggests that certain aspects of proton translocation, conformational change and chemical coupling may be amalgamated by further study of oligomycin inhibition.

(b) AS particle ATPase: The K_m and V_{max} values obtained for the oligomycin-sensitive ATPase of rat skeletal muscle may be compared with those obtained by Van de Stadt *et al* (50), who measured the ATPase activity of beef heart AS particles by determining change of pH in the reaction medium. For the skeletal muscle AS preparation the reaction was measured under the standard conditions at 30°C and pH 7.7, while Van de Stadt *et al* (50) used 25°C and pH 7.4. Nevertheless, the value of 0.7 mM for the K_m of the beef heart enzyme is in good agreement with the result of 0.8 mM obtained in this study. The V_{max} of 3.7 μ equivalents of H^+/min/mg for the beef heart enzyme is intermediate between the values of 5.2 and 1.0 μmoles phosphate/min/mg obtained for the uninhibited and oligomycin-sensitive ATPases respectively from skeletal muscle. The V_{max} of the oligomycin-sensitive ATPase might be expected to be closer to that of the

uninhibited ATPase activity. However, as shown in Figures 6, 7, and 8 some variability in data was observed. This was probably due to the use of the ATP regenerating system in the assay.

(2) ATPase and cytochrome oxidase activities of mitochondria and submitochondrial particles from rat skeletal muscle prepared by different techniques

(a) Yields and ATPase activities of rat skeletal muscle mitochondria prepared by different techniques: Several methods are available for the preparation of skeletal muscle mitochondria (73, 74, 86-92). Skeletal muscle homogenised in non-electrolyte medium often assumes a gelatinous consistency which makes it difficult to obtain sufficient disintegration of the myofibrils and release the mitochondria, therefore, mitochondrial protein yields are low. To eliminate this problem Chappell and Perry (86) introduced the use of isotonic salt solutions and showed that good yields of mitochondria could be obtained by this method. However, mitochondria prepared in this way appear to have lost some cytochrome c, since added cytochrome c will stimulate respiration with all substrates. Also this preparation contains not only intact mitochondria but also submitochondrial particles (93).

More recently a method devised by Chance and Hagihara (74) for preparation of cardiac muscle mitochondria has been successfully applied to skeletal muscle tissue (89, 90, 95).

Here sucrose/mannitol/EDTA solutions are used in conjunction with the proteolytic enzyme nagarse. In some studies (87, 88, 90, 92) heparin and/or bovine serum albumin were reported to preserve skeletal muscle mitochondria during isolation. A direct comparison of the oxidative activity and respiratory control ratios of mitochondria produced using different solutions cannot be made since these parameters were measured at 25°C in early studies using Chappell-Perry medium (73, 91, 93, 94) and at 37°C when sucrose/mannitol/EDTA solutions were used (89, 90).

Previously several methods of homogenisation have been used to disrupt skeletal muscle, for example, by the all-glass Potter-Elvehjem homogeniser (73), the Bronwill mechanical cell homogeniser (91), a ground glass and Teflon homogeniser (90) and an Ultra-Turrax hand disintegrator (92). In present work preliminary studies indicated that good yields of mitochondria could be obtained by the use of the polytron as specified by Kidwai (96). This instrument disrupts the tissue by mechanical shearing and localised sonication. With rat cardiac tissue it has been shown that very gentle polytron treatment releases subsarcolemmal mitochondria and further treatment with nagarse will release the interfibrillar mitochondria (67).

Methods used in the current study to prepare skeletal muscle mitochondria followed those of Chappell and Perry (86) and Chance and Hagihara (74). In the latter case sucrose density gradient centrifugation was used to further purify the preparation. The yield of mitochondria prepared using Chappell-Perry medium

of 3.1 mg mitochondrial protein per gram of muscle compares favourably with the yield stated by Ernster and Nordenbrand (73) of 2 to 3 mg/g for rat skeletal muscle mitochondria prepared in this medium. The yield of 2.27 mg/g for mitochondria prepared using sucrose/mannitol/EDTA solutions is higher than that of Bullock et al (89) who obtained 0.84 to 1.27 mg/g when using this isolation medium together with proteolytic enzymes instead of mechanical homogenisation. After purification on a sucrose gradient the yield in the present study decreased to 0.49 mg/g for the pooled mitochondrial fractions. Three bands of mitochondria were obtained in the sucrose gradient, in positions similar to those reported by Bullock et al (75), for the different subpopulations of skeletal muscle mitochondria. The uppermost band represents small interfibrillar mitochondria from the white fibres and the lower two bands represent larger subsarcolemmal mitochondria from the red and white fibres. If polytron homogenisation is not releasing all the mitochondria from this mixture of muscle fibre types from the rat hind limb, then an increased yield of mitochondria could be obtained by treatment of the low speed centrifugation pellet with proteolytic enzymes. However, in the present study this would have been unsuitable since the ATPase inhibitor is very suspectible to proteolytic digestion.

The oligomycin-sensitive ATPase activities of the rat skeletal muscle mitochondrial preparations listed in Table 3 may be compared with results from other studies. Oscai and

Holloszy (68) prepared rat skeletal muscle mitochondria in a medium of 175 mM potassium chloride containing 0.1 mM EDTA. When assayed for ATPase activity using an ATP regenerating system a value of 0.4 μmoles/min/mg was obtained for the oligomycin-sensitive activity of an aged, sonicated mitochondrial preparation. This is lower than the value of 1.28 μmoles/min/mg obtained in the present study using mitochondria prepared in Chappell-Perry medium. Rat skeletal muscle mitochondria have also been isolated by Dow (90) using the sucrose/mannitol medium of Chance and Hagihara (74) plus heparin. In this case the oligomycin-sensitive ATPase activity was 0.8 μmoles/min/mg. Mitochondria isolated in the absence of heparin had an oligomycin-sensitive ATPase activity of 0.3 μmoles/min/mg with an oligomycin sensitivity of 42%. These results were achieved using a 15 minute incubation without an ATP regenerating system. Therefore, some inhibition of the coupling ATPase by ADP would be expected. In addition, the assay medium contained albumin, which was shown to lower the ATPase activity. Therefore, the oligomycin-sensitive activity of 0.8 μmoles/min/mg for mitochondria prepared in the present study using the same sucrose/mannitol medium and without gradient purification, is comparable. Also the oligomycin sensitivity of 43% for these mitochondria is in agreement with the value reported by Dow (90). At present no comparison is available in the literature for the ATPase activity of gradient purified rat skeletal muscle mitochondria which showed a 2.7 fold increase from that of the non-gradient mitochondria.

(b) Cytochrome oxidase activities of rat skeletal muscle mitochondria prepared using sucrose/mannitol solutions and sucrose gradient purification: Cytochrome oxidase is an enzyme found in the inner membrane of the mitochondrion as a terminal member of the electron transport chain. This enzyme was used as an index of purification and recovery for the rat skeletal muscle mitochondria. The increase in specific activity between the homogenate and gradient mitochondria indicates that a 36 fold purification of the mitochondria$_{\text{A}}^{\text{cytochrome oxidase}}$ has been achieved (Table 4). This may be compared with the increases in specific activity of 23 fold for succinate dehydrogenase and 21 fold for α-glycerophosphate dehydrogenase obtained when a pure preparation of rat cardiac subsarcolemmal mitochondria was isolated using a polytron tissue processor (67). The 50% recovery of cytochrome oxidase units in the non-gradient mitochondria suggest that either some mitochondria, present in the homogenate, are lost during differential centrifugation, possibly in the low speed pellet, or alternatively cytochrome oxidase may be lost into the 14,000 x g. supernatant if the mitochondria have been damaged. Nevertheless, the 50% and 28% recovery in the non-gradient and gradient mitochondria respectively compares favourably with the results of Behrens and Himms-Hagen (69) who achieved 28% to 40% recoveries of this enzyme when isolating rat skeletal muscle mitochondria by a similar method using protease and a Duall homogeniser. After sucrose density gradient purification a 57% recovery and doubling in specific activity of cytochrome oxidase compared to the non-gradient mitochondria

was achieved (Table 4). This correlates with the 61% yield and 2.7 fold increase in specific activity of the oligomycin-sensitive ATPase obtained during this procedure (Table 3).

The specific activity of the beef heart mitochondrial cytochrome oxidase of 4.1 μmoles/min/mg is intermediate between that of the gradient and non-gradient rat skeletal muscle mitochondria. Beef heart mitochondria might be expected to show a higher cytochrome oxidase activity since heart mitochondria have more dense cristae and higher repiratory activity than skeletal muscle mitochondria. However, these beef heart mitochondria have not been purified on a sucrose gradient.

(c) Comparison of mitochondrial and AS particle ATPase activities from rat skeletal muscle preparations and beef heart: Rat skeletal muscle mitochondria prepared using sucrose/mannitol solutions were superior to those prepared using Chappell-Perry medium since the former exhibited an ATPase activity which was more sensitive to oligomycin. In addition this ATPase activity could be activated by sonication at alkaline pH and passage through sephadex to give a specific activity of 3.3 μmoles/min/mg (Table 5). Oscai and Holloszy (68) have obtained the same result by preparing rat skeletal muscle mitochondria in which the ATPase activity was activated by incubation in either 0.5 M Tris, pH 7.4, or 0.05 M Tris, pH 9.0. This gave oligomycin-sensitive ATPase activities of 3.2 and 3.3 μmoles/min/mg respectively.

A comparison can be made between the uninhibited ATPase activity of the AS particles obtained in this study and values

found in the literature. For the beef heart enzyme Racker and Horstman (8) obtained 10 to 15 μmoles/min/mg, while other workers (41, 43) have stated values of 3.3 and 3.7 μmoles/min/mg. For rat liver Chan and Barbour (41) give a value of 0.8 umoles/min/mg and for yeast Satre et al (43) obtained 2.7 μmoles/min/mg. Therefore, the specific activity of 8.4 μmoles/min/mg obtained for beef heart AS particles in the present work compares favourably with the literature values. The values of 5.2 μmoles/min/mg for the gradient purified rat skeletal muscle mitochondria and 4.6 μmoles/min/mg for the rat skeletal muscle mitochondria prepared using Chappell-Perry medium also lie within the expected range.

When the ATPase activities of sucrose/mannitol rat skeletal muscle mitochondria are compared with those from beef heart (Table 5) it is apparent that, although the sensitivities to oligomycin are similar, the specific activity of the rat skeletal muscle mitochondria is twice that of the beef heart mitochondria. The ATPase activity of a mitochondrial preparation will depend on the density of the inner membrane cristae, in which the enzyme is located, the purity of the preparation and the extent to which the ATPase inhibitor has been removed from the enzyme complex. Therefore, rat skeletal muscle mitochondria and beef heart mitochondria prepared in this study would be expected to differ since beef heart mitochondria have a higher density of cristae and the two types of mitochondria were isolated by different methods.

Beef heart AS particles show an increase in specific activity and oligomycin sensitivity of the ATPase compared to that of the mitochondria (Table 5). In the case of rat skeletal muscle both of these parameters show a smaller increase. The magnitude of activation of the ATPase attained during preparation of AS particles depends in part on the initial activity of the mitochondria and their purity, assuming that this activation reflects the removal of the inhibitor subunit from the ATPase molecule. Therefore, differences in inhibitor content of the mitochondrial fractions as well as ease of removal of the inhibitor will influence the extent of activation. Oscai and Holloszy (68) report an 8 fold stimulation of the ATPase activity of rat skeletal muscle mitochondria as compared to the 2 fold stimulation seen in the present work. The difference lies in the lower initial mitochondrial ATPase activity attained in the former study. This might be due to greater retention of the inhibitor in the mitochondria. However, in present studies mitochondria prepared using Chappell-Perry medium, similar to the potassium chloride and EDTA medium used by Oscai and Holloszy (68) could not be activated by AS particle preparation.

(3) The ATPase inhibitor from rat skeletal muscle mitochondria

(a) Alkaline extraction: Results from this study indicate that the ATPase inhibitor cannot be measured when rat skeletal muscle mitochondria isolated in Chappell-Perry medium

are used. Initial work showed that when AS particles and alkaline extracts, prepared from these mitochondria, were titrated together little inhibition of the ATPase activity was observed. The use of beef heart mitochondrial alkaline extracts and AS particles demonstrated that the preparative technique and assay method were not responsible for this lack of inhibition.

The lack of cross-reactivity between the beef heart and rat skeletal muscle systems suggests that both the AS particles and alkaline extract of the skeletal muscle mitochondria were deficient. Since the beef heart inhibitor has been shown to bind to both rat liver (41) and yeast (43) AS particles, it appeared probable that it would also bind to rat skeletal muscle AS particles. This was found to be the case when AS particles were prepared from rat skeletal muscle mitochondria isolated in sucrose/mannitol medium. Therefore, the failure of AS particles derived from Chappell-Perry mitochondria to bind the inhibitor indicates that the use of this isotonic salt solution alters the mitochondria so that inhibitor binding is prevented. Alkaline extracts from Chappell-Perry mitochondria did not cause inhibition of the beef heart AS particles. This could have been due to either loss of the inhibitor during preparation of the mitochondria or tight binding of the inhibitor to the enzyme. The former alternative appears more likely since Horstman and Racker (47) have shown that inhibitor-enzyme binding is prevented by 40 mM potassium chloride, a concentration lower than that found in Chappell-Perry medium. In addition, the

preparation of AS particles from Chappell-Perry mitochondria by sonication at alkaline pH followed by passage through sephadex, a process designed to remove the inhibitor, had little effect on the ATPase activity of these mitochondria. Whereas the ATPase activity of skeletal muscle mitochondria prepared in sucrose/mannitol solutions could be stimulated by this procedure (Table 5).

Cross-reactivity experiments (Figures 11, 12) between the beef heart ATPase-inhibitor system and that of rat skeletal muscle mitochondria isolated in sucrose/mannitol solutions, indicate that ; (a) the beef heart alkaline extract is more active in inhibiting both the beef heart and skeletal muscle ATPases, and (b) the rat skeletal muscle alkaline extract is more active towards the beef heart enzyme than the homologous enzyme, even when differences in the oligomycin sensitivity of the two enzyme preparations are taken into account. Other cross-reactivity studies of the beef heart system with rat liver (41) and with yeast (43) have used the purified inhibitors. In these cases the beef heart inhibitor was more potent in inhibiting the membrane-bound ATPases from both beef heart and the heterologous mitochondria, as found in this study. Therefore, it may be concluded that the beef heart inhibitor has a high affinity for the mitochondria ATPases from different sources. These cross-reactivity experiments suggest that the ATPase-inhibitor system has been conserved during evolution, probably due to its key role in cellular energy metabolism.

The specific activity of the inhibitor fraction appears to be dependant on the units of enzyme per tube used in the assay (Table 6). Therefore, this must be taken into account when comparing results from different inhibitor purifications (Table 13). The specific activity of 4.8 units/mg for the skeletal muscle alkaline extract may be compared with the value of 9.0 units/mg for the rat liver inhibitor (41), both obtained using approximately 0.1 units of the homologous AS particle enzyme per tube. The specific activity of a yeast alkaline extract of 4.3 units/mg was obtained with 0.2 units of enzyme per tube (43). If the maximum oligomycin sensitivity of 70% for the rat skeletal muscle AS particles is taken into account then the specific activity of the alkaline extract increases from 4.8 to 7.9 units/mg (Table 11). Therefore, the activity of the rat skeletal muscle alkaline extract is in the same range as both the rat liver and yeast alkaline extracts. Horstman and Racker (47), using 2 to 3 units of enzyme per tube obtained a specific activity of 855 units/mg for the beef heart alkaline extract. A value of 314 units/mg was obtained in the present work using 0.075 units of enzyme per tube. Therefore, the beef heart alkaline extract has a much higher specific activity than the yeast, rat liver or rat skeletal muscle alkaline extract.

(b) Heat treatment: Like the ATPase inhibitors from beef heart (47), rat liver (41), chloroplasts (46) and E. coli (44) the rat skeletal muscle inhibitor has been shown to be stable to heat. As a purification technique, heat treatment

was most successfully applied to a solution of approximately 1 mg/ml concentration. Heat treatment has been used as a final step in the purification of the beef heart (47) and rat liver (41) inhibitors at this protein concentration but was most useful at an earlier stage in the present purification scheme.

(c) TCA precipitation: It can be seen from Tables 9, 10 and 11 that the specific activity of the TCA precipitate is dependant on the number of units of enzyme per tube in the assay, the highest activity of 51 units/mg being achieved with 0.1 units of enzyme. This constitutes a 10.6 fold purification of the inhibitor relative to the alkaline extract. If the oligomycin sensitivity of the AS particles is taken into account then the specific activity has a value of 88 units/mg (Table 11).

(d) Ammonium sulphate fractionation: The use of ammonium sulphate fractionation in the purification of the ATPase inhibitors from beef heart (47), rat liver (41) and yeast (43) mitochondria indicated that this technique might be useful in the isolation of the rat skeletal muscle inhibitor. However, when various ammonium sulphate fractions were prepared, no increase in specific activity was achieved. Initially, no inhibition was achieved with the ammonium sulphate fractions unless dialysed. This may be explained by the observation of Horstman and Racker (47) that 15 mM ammonium ions cause release of the ATPase from inhibition. This effect may have been accentuated at the low levels of enzyme used in the present inhibitor assay. However, even after dialysis or removal of the

ammonium sulphate by TCA precipitation no purification of the inhibitor was detected.

V CONCLUSIONS

In this study mitochondria were prepared from rat skeletal muscle using Chappell-Perry medium and from these sub-mitochondrial particles depleted of ATPase inhibitor were derived. The kinetic parameters of this membrane-bound ATPase were estimated and found to be comparable with those of the beef heart enzyme. However, this preparation was not suitable for inhibitor studies.

In order to measure the rat skeletal muscle mitochondrial ATPase inhibitor it was necessary to prepare mitochondria in sucrose/mannitol solutions. These mitochondria were further purified by sucrose density gradient centrifugation and a purification of 36 fold relative to the muscle homogenate was measured using the marker enzyme cytochrome oxidase. Inhibitor-depleted submitochondrial particles were prepared from these mitochondria and their activities were compared with those of the beef heart enzyme.

Alkaline extracts of these mitochondria were capable of causing inhibition of the inhibitor-depleted ATPase enzyme preparations. Cross-reactivity between the ATPase-inhibitor systems of rat skeletal muscle and beef heart was demonstrated. A 10 fold purification of the ATPase inhibitor from rat skeletal muscle mitochondria was achieved by alkaline extraction in

hypotonic sucrose, heat treatment and TCA precipitation. Therefore, the rat skeletal muscle mitochondrial ATPase inhibitor is similar to the inhibitors from other mitochondria in that it is heat and TCA stable. In addition, it has been shown that this inhibitor is protein in nature since like other ATPase inhibitors it is destroyed by a brief exposure to trypsin.

Since 50 fold and 80 fold purifications from the alkaline extract have been reported for the beef heart (47) and rat liver (41) inhibitors respectively, it is apparent that a further 5 to 8 fold increase in the specific activity of the rat skeletal muscle inhibitor may be expected. This might be achieved by techniques such as gel filtration on sephadex in which the low molecular weight inhibitor could be separated from higher molecular weight contamination. This method has proved successful in the preparation of inhibitor-depleted submitochondrial particles.

In further work an investigation of the effect of the troponin component TN-I on the skeletal muscle mitochondrial ATPase would be of interest to confirm reports (53) that this component can inhibit the enzyme and to establish the comparative levels of inhibition by TN-I and the mitochondrial ATPase inhibitor since both are found in skeletal muscle. The work of Dow (90) on the effect of calcium on the skeletal muscle mitochondrial ATPase might be extended to clarify the relative effects of magnesium and calcium ions on the oligomycin-sensitive ATPase and on inhibitor binding. The effect of pH, nucleotide and ion concentration should also be investigated for the skeletal muscle ATPase system.

REFERENCES

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

2. Hinkle,P.C. and McCarty,R.E. (1978) Sci. Amr. 238, 104-124.

3. Racker,E. (1976) A New Look at Mechanisms in Bioenergetics, pp. 124-126.

4. Sone,N.,Yoshida,M.,Hirata,H.,Okamoto,H. and Kawaga,Y. (1976) J. Memb. Biol. 30, 121-134.

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

6. Griffiths,D.E. and Hyams,R.L. (1977) Biochem. Soc. Trans. 5, 207-208.

7. Johnston,R. and Criddle,R.S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4919-4923.

8. Racker,E. and Horstman,L.L. (1967) J. Biol. Chem. 242, 2547-2551.

9. Pullman,M.E.,Penefsky,H.S.,Datta,A. and Racker,E. (1960) J. Biol. Chem. 235, 3322-3329.

10. Penefsky,H.S.,Pullman,M.E.,Datta,A. and Racker,E. (1960) J. Biol. Chem. 235, 3330-3336.

11. Lambeth,D.O. and Lardy,H.A. (1971) Eur. J. Biochem. 22, 355-363.

12. Catterall,W.A. and Pedersen,P.L. (1971) J. Biol. Chem. 246, 4587-4594.

13. Cannon,B. and Vogel,G. (1977) FEBS Letters 76, 284-289.

14. Schatz,G.,Penefsky,H.S. and Racker,E. (1967) J. Biol. Chem. 242, 2552-2560.

15. Lien,S. and Racker,E. (1971) in San Pietro,A. ed. Methods in Enzymology XXIII, pp. 547-555.

16. Abrams,A. and Smith,J.B. (1974) in Boyer,P. ed. The Enzymes 10, pp. 395-429.

17. Knowles,A.F. and Penefsky,H.S. (1972) J. Biol. Chem. 247, 6624-6630.

18. Racker,E. (1976) A New Look at Mechanisms in Bioenergetics, p. 10.

19. Senior,A.E. (1973) Biochim. Biophys. Acta 301, 249-277.

20. Senior,A.E. (1975) Biochem. 14, 660-664.

21. Baird,B.A. and Hammes,G.G. (1977) J. Biol. Chem. 252, 4743-4748.

22. Kozlov,I.A. and Mikelsaar,H.N. (1974) FEBS Letters 43, 212-214.

23. Kozlov,I.A.,Kondrshin,A.A.,Konoenko,V.A. and Metalsky,S.T. (1976) J. Bioenerg. 8, 1-7.

24. Sternweis,P.C. and Smith,J.B. (1977) Biochem. 16, 4020-4025.

25. Nelson,N.,Kanner,B.I. and Gutnick,D.L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2720-2724.

26. Ferguson,S.J.,Lloyd,W.J.,Lyons,N.H. and Radda,G.K. (1975) Eur. J. Biochem. 54, 117-126.

27. Ferguson,S.J.,Lloyd,W.J. and Radda,G.K. (1975) Eur. J. Biochem. 54, 127-133.

28. Kozlov,I.A. and Skulachev,V.P. (1977) Biochim. Biophys. Acta 463, 29-89.

29. Tzagoloff,A.,Rubin,M.S. and Sierra,M.F. (1973) Biochim. Biophys. Acta 301, 71-104.

30. Tzagoloff,A. (1970) J. Biol. Chem. 245, 1545-1551.

31. Cattell,J.K.,Lindop,C.R.,Knight,I.G. and Beechey,R.B. (1971) Biochem. J. 125, 169-177.

32. De Pierre,J.W. and Ernster,L. (1977) Ann. Rev. Biochem. 46, 201-262.

33. Criddle,R.S.,Packer,L. and Sheih,P. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4306-4310.

34. Tzagoloff,A. and Meagher,P. (1972) J. Biol. Chem. 247, 594-603.

35. Sebald,W. (1977) Biochim. Biophys. Acta 463, 1-27.

36. Mitchell,P. (1977) Ann. Rev. Biochem. 46, 996-1005.

37. Boyer,P.D. (1974) in Ernster,L.,Estabrook,R.W. and Slater,E.C. eds., Dynamics of Energy-Transducing Membranes, pp. 289-301.

38. Slater,E.C. (1974) in Ernster,L.,Estabrook,R.W. and Slater,E.C. eds., Dynamics of Energy-Transducing Membranes, pp. 1-20.

39. Harris,D.A.,Radda,G.K. and Slater,E.C. (1977) Biochim. Biophys. Acta 459, 560-572.

40. Pullman,M.E. and Monroy,G.C. (1963) J. Biol. Chem. 238, 3762-3769.

41. Chan,S.H.P. and Barbour,R.L. (1976) Biochim. Biophys. Acta 430, 426-433.

42. Landry,Y. and Goffeau,A. (1975) Biochim. Biophys. Acta 376, 470-484.

43. Satre,M.,De Jerphanion,M.-B.,Huet,J. and Vignais,P.V. (1975) Biochim. Biophys. Acta 387, 241-255.

44. Ebner,E. and Maier,K.L. (1977) J. Biol. Chem. 252, 671-676.

45. Nieuwenhuis,F.J.R.M. and Bakkenist,A.R.J. (1977) Biochim. Biophys. Acta 459, 596-604.

46. Nelson,N.,Nelson,H. and Racker,E. (1972) J. Biol. Chem. 247, 7657-7662.

47. Horstman,L.L. and Racker,E. (1970) J. Biol. Chem. 245, 1336-1344.

48. Asmai,K.,Juntti,K. and Ernster,L. (1970) Biochim. Biophys. Acta 205, 307-311.

49. Ferguson,S.J.,Harris,D.A. and Radda,G,K, (1977) Biochem. J. 162, 351-357.

50. Van de Stadt,R.J.,De Boer,B.L. and Van Dam,K. (1973) Biochim. Biophys. Acta 292, 338-349.

51. Brooks,J.C. and Senior,A.E. (1971) Arch. Biochem. Biophys. 147, 467-470.

52. Yamazaki,S.,Hasebe,H.,Takisawa,H.,Tamaura,Y. and Inada,Y. (1977) Biochem. Biophys. Res. Commun. 75, 1104-1110.

53. Tamaura,Y.,Yamazaki,S.,Hirose,S. and Inada,Y. (1973) Biochem. Biophys. Res. Commun. 53, 673-679.

54. Hasebe,H.,Yamazaki,S.,Almazan,A.M.,Tamaura,Y. and Inada,Y. (1977) Biochem. Biophys. Res. Commun. 77, 932-938.

55. Yamazaki,S.,Takisawa,H.,Tamaura,Y.,Hirose,S. and Inada,Y. (1975) FEBS Letters 56, 248-251.

56. Chan,S.H.P. and Barbour,R.L. (1976) Biochem. Biophys. Res. Commun. 72, 499-506.

57. Lloyd,D. and Edwards,S.W. (1976) Biochem. J. 160, 335-342.

58. Padykula,H.A. and Gauthier,C.F. (1967) in Milhorat,A.T. ed., Exploratory Concepts in Muscular Dystrophy, pp. 117-128.

59. Peter,J.B.,Barnard,R.J.,Edgerton,V.R.,Gillespie,C.A. and Stempel,K.E. (1972) Biochem. 11, 2627-2633.

60. Shafiq,S.A.,Gorycki,M.,Goldstone,L. and Milhorat,A.T. (1966) Anat. Rec. 156, 283-302.

61. Price,H.M. (1974) in Walton,J.N. ed., Disorders of Voluntary Muscle, p. 51.

62. De Haan,E.J.,Groot,G.S.P.,Scholt,H.R.,Tager,J.M. and Wit-Peeters,E.M. (1973) in Bourne,G.H. ed., The Structure and Function of Muscle, Vol.3, pp. 418-471.

63. Munn,E.A. (1974) The Structure of Mitochondria, p. 29.

64. Burchell,A.,Cohen,P.T.W. and Cohen,P. (1976) FEBS Letters 67, 17-22.

65. Kark,R.A.P.,Blass,J.P.,Avigan,J. and Engel,W.K. (1971) J. Biol. Chem. 246, 4560-4566.

66. Pande,S.V. and Blanchaer,M.C. (1971) Am. J. Physiol. 220, 549-553.

67. Palmer,J.W.,Tandler,B. and Hoppell,C.L. (1977) J. Biol. Chem. 252, 8731-8739.

68. Oscai,L.B. and Holloszy,J.O. (1971) J. Biol. Chem. 246, 6968-6972.

69. Behrens,W.A. and Himms-Hagen,J. (1977) J. Bioenerg. Biomemb. 9, 41-63.

70. Luft,R.,Ikkos,D.,Palmieri,G.,Ernster,L. and Afzelius,B. (1962) J. Clin. Invest. 41, 1776-1804.

71. DiMauro,S.,Bonilla,E.,Lee,C.P.,Schotland,D.L.,Scarpa,A.,Conn Jr.,H. and Chance,B. (1976) J. Neurol. Sci. 27, 217-232.

72. Schotland,D.L.,DiMauro,S.,Bonilla,E.,Scarpa,A. and Lee,C.P. (1976) Arch. Neurol. 33, 475-479.

73. Ernster,L. and Nordenbrand,K. (1967) in Estabrook,R.W. and Pullman,M.E. eds., Methods in Enzymology X, pp. 86-88.

74. Chance,B. and Hagihara,B. (1963) Proc. 5th. Inter. Congr. Biochem., Moscow, 1961, Vol.5, pp. 3-26.

75. Bullock,G.R.,Carter,E.E. and White,A.M. (1973) Biochim. Biophys. Acta 292, 350-359.

76. Quilliam,N. (1974) M.Sc. Thesis, Univ. of Manitoba, (Unpub.)

77. Smith,A.L. (1967) in Estabrook,R.W. and Pullman,M.E. eds., Methods in Enzymology X, pp. 81-84.

78. Fessenden,J.M. and Racker,E. (1966) J. Biol. Chem. 241, 2483-2489.

79. Lowry,O.H.,Rosebrough,N.J.,Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275.

80. Baginski,E. and Zak,B. (1960) Clin. Chim. Acta 5, 834-838.

81. Wharton,D.C. and Tzagoloff,A. (1967) in Estabrook,R.W. and Pullman,M.E. eds., Methods in Enzymology X, pp. 245-246.

82. Yonetani,T. (1967) in Estabrook,R.W. and Pullman,M.E. eds., Methods in Enzymology X, pp. 332-333.

83. O'Farrell,P.H. (1975) J. Biol. Chem. 250, 4007-4021.

84. Maizel,Jr.,J.V. (1972) Methods in Virology 5, 179-246.

85. Johnston,R.,Scharf,S. and Criddle,R.S. (1977) Biochem. Biophys. Res. Commun. 77, 1361-1368.

86. Chappell,J.B. and Perry,S.V. (1954) Nature 173, 1094-1095.

87. Max,S.R.,Garbus,J. and Wehman,H.R. (1972) Anal. Biochem. 46, 576-584.

88. Thakar,J.H. and Ashmore,C.R. (1975) Anal. Biochem. 69, 545-551.

89. Bullock,G.,Carter,E.E. and White,A. M. (1970) FEBS Letters 8, 109-111.

90. Dow,D.S. (1967) Biochem. 6, 2915-2922.

91. Peter,J.B. and Lee,L.D. (1967) Biochem. Biophys. Res. Commun. 29, 430-436.

92. Makinen,M.W. and Lee,C.P. (1968) Arch. Biochem. Biophys. 126, 75-82.

93. Hedman,R. (1965) Exptl. Cell Res. 38, 1-12.

94. Azzone,G.F. and Carafoli,E. (1960) Exptl. Cell Res. 21, 447-467.

95. Rigault,M.Y.A. and Blanchaer,M.C. (1970) Can. J. Biochem. 48, 27-32.

96. Kidwai,A.M. (1974) in Fleischer,S. and Packer,L. eds., Methods in Enzymology XXXI, pp. 134-144.

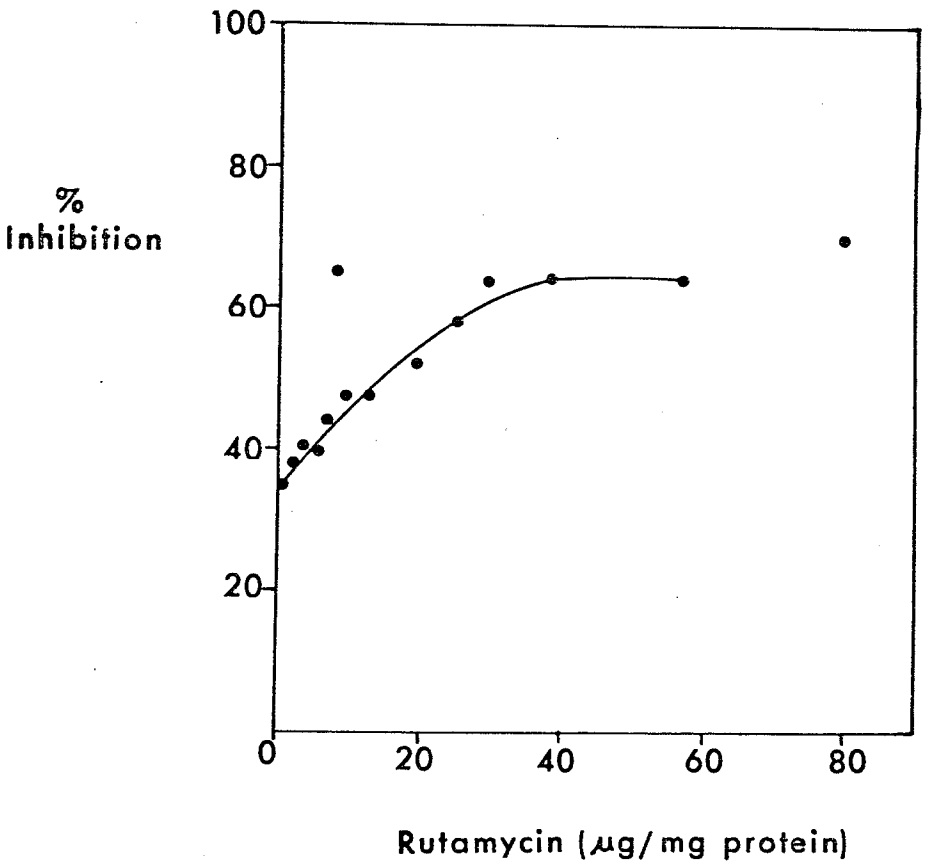

Figure 1: Inhibition of mitochondrial ATPase of rat skeletal muscle prepared using Chappell-Perry medium by rutamycin.

Mitochondria (33.5 µg protein/tube) were assayed for ATPase activity in the presence of 1.0 mM DNP and 0-80 µg rutamycin/mg mitochondrial protein. The graph shows results from 5 experiments in which the average specific activity of the enzyme was 1.87 umoles/min/mg. In each assay controls (1) without enzyme (2) with enzyme but without rutamycin or ethanol were set up.

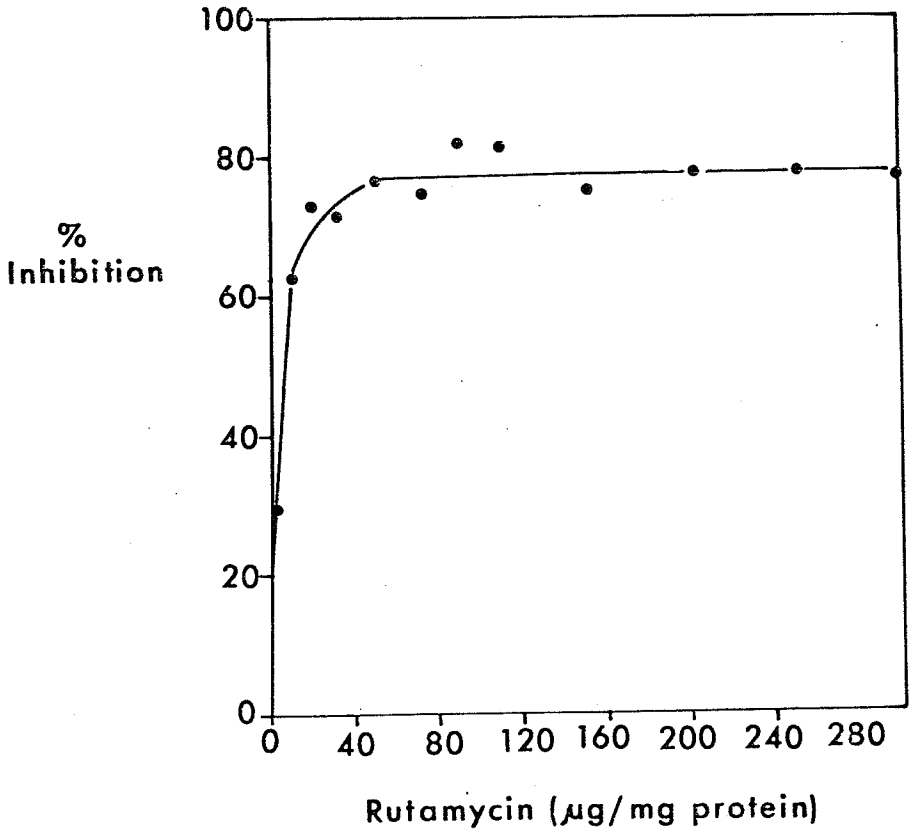

Figure 2: Inhibition of A particle ATPase from rat skeletal muscle by rutamycin.

7.8 µg protein/tube A particles derived from mitochondria prepared in Chappell-Perry medium were assayed for ATPase activity in the presence of 0-300 µg rutamycin/mg A particle protein as described for the mitochondrial ATPase (Figure 1).

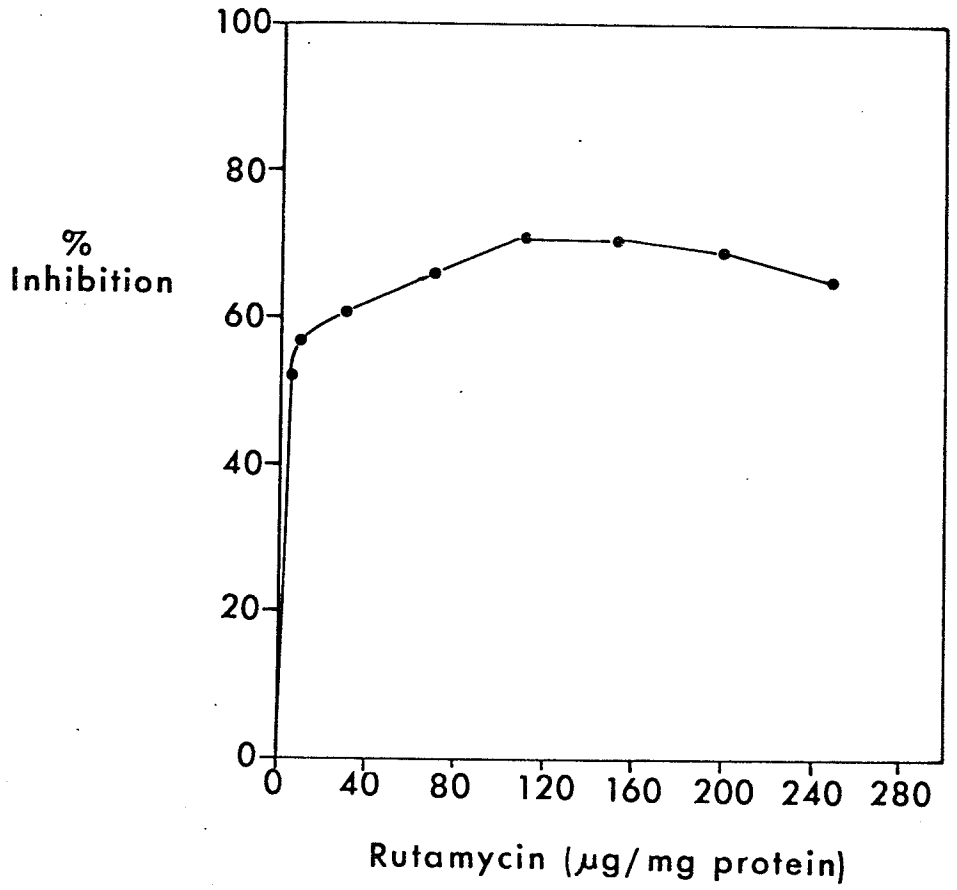

Figure 3: Inhibition of AS particle ATPase from rat skeletal muscle by rutamycin.

The ATPase activity of 6.9 µg protein aliquots of AS particles, derived from mitochondria prepared in Chappell-Perry medium, was determined in the presence of 0-250 µg rutamycin/mg AS particle protein as described in Figure 1. Averages of 2 determinations at each rutamycin concentration were plotted.

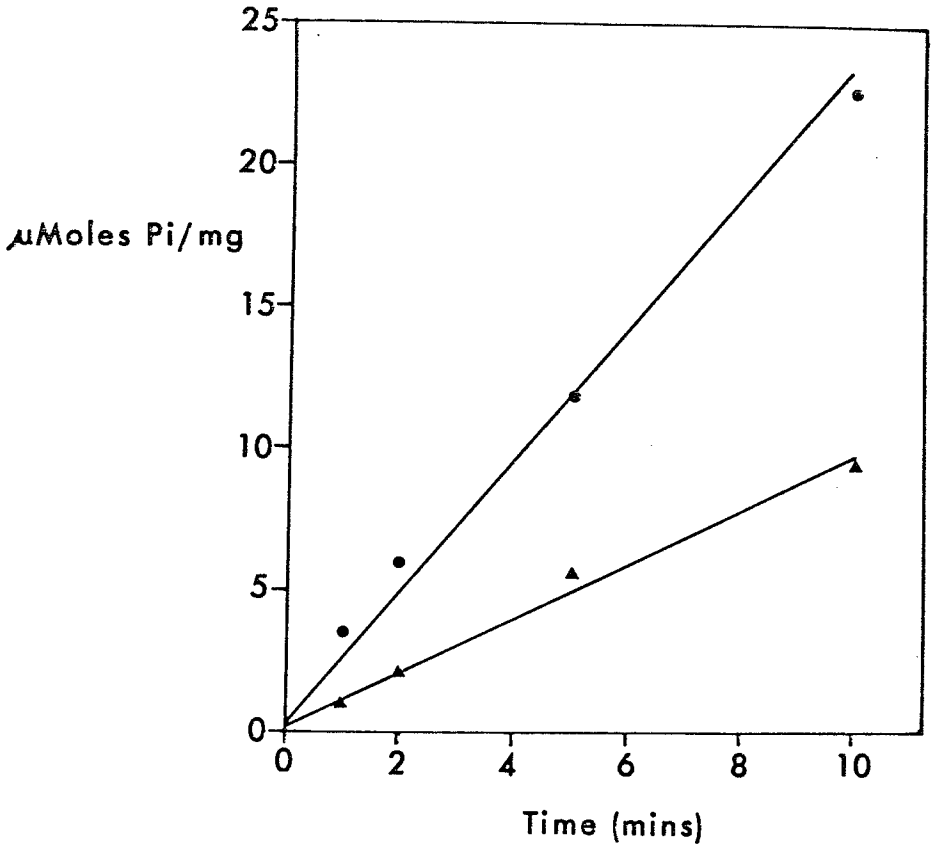

Figure 4: Time study of AS particle ATPase activity.

6.9 µg protein aliquots of AS particles derived from rat skeletal muscle mitochondria prepared in Chappell-Perry medium were incubated for 0-10 minutes and assayed as described for Figure 1. Results graphed were averages of 2 duplicate experiments.

Circles - without rutamycin

Triangles - with rutamycin (10 µg/mg)

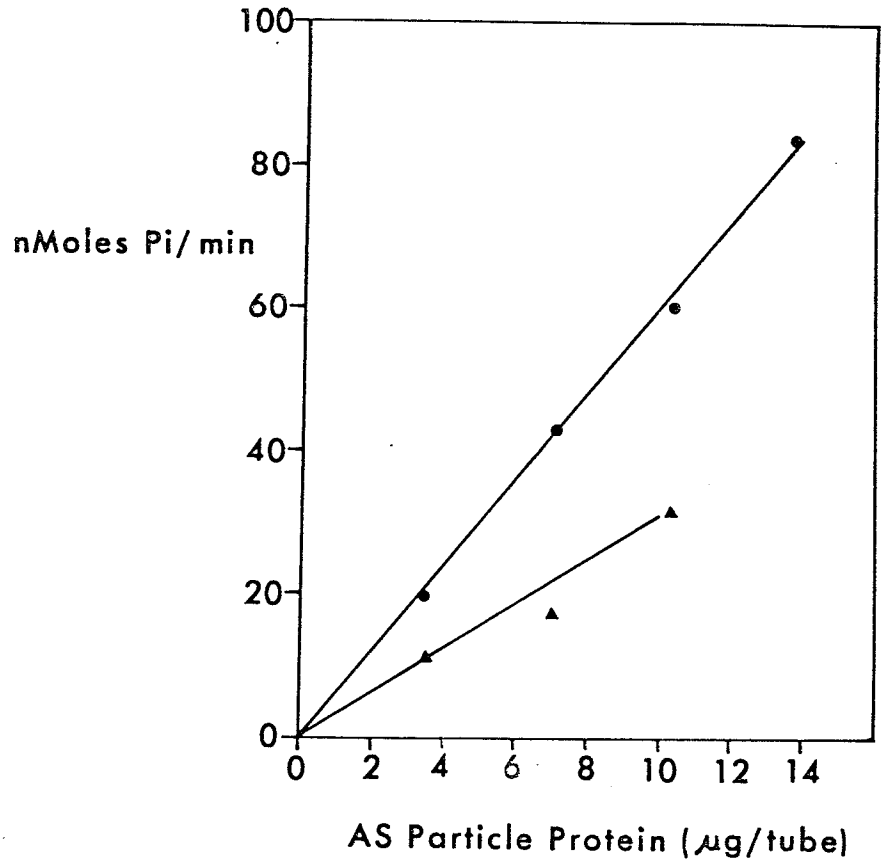

Figure 5: AS particle enzyme concentration study.

0-13.8 µg AS particles derived from rat skeletal muscle mitochondria prepared in Chappell-Perry medium, were incubated for 10 minutes in the assay as described in Figure 1.

Circles - without rutamycin

Triangles - with rutamycin (10 µg/mg)

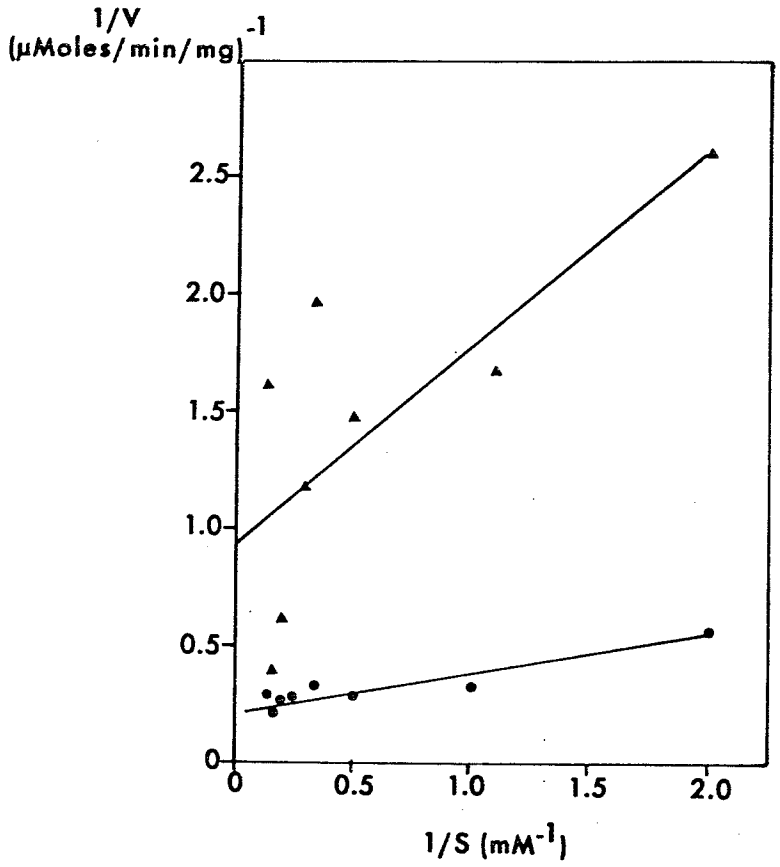

Figure 6: Determination of ATPase K_m and V_{max} for AS particles derived from rat skeletal muscle mitochondria prepared in Chappell-Perry medium (A).

The ATPase activity of AS particles (4.9 μg/tube) was determined over the range 0-7 mM ATP in duplicate experiments as described in Chapter II. When present 100 μg rutamycin/mg AS particle protein was used.

Circles - uninhibited ATPase

Triangles - rutamycin-sensitive ATPase

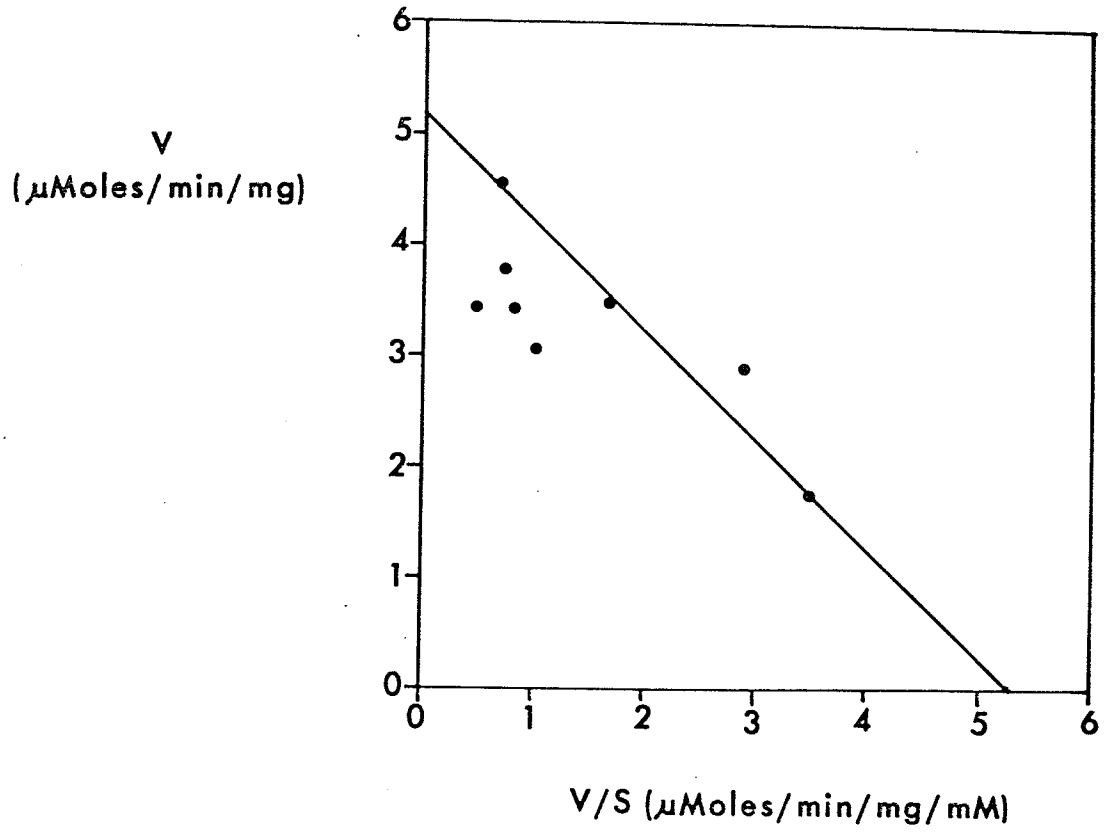

Figure 7: Determination of ATPase K_m and V_{max} for AS particles derived from rat skeletal muscle mitochondria prepared in Chappell-Perry medium (B).

(See Figure 6)

Circles - uninhibited ATPase

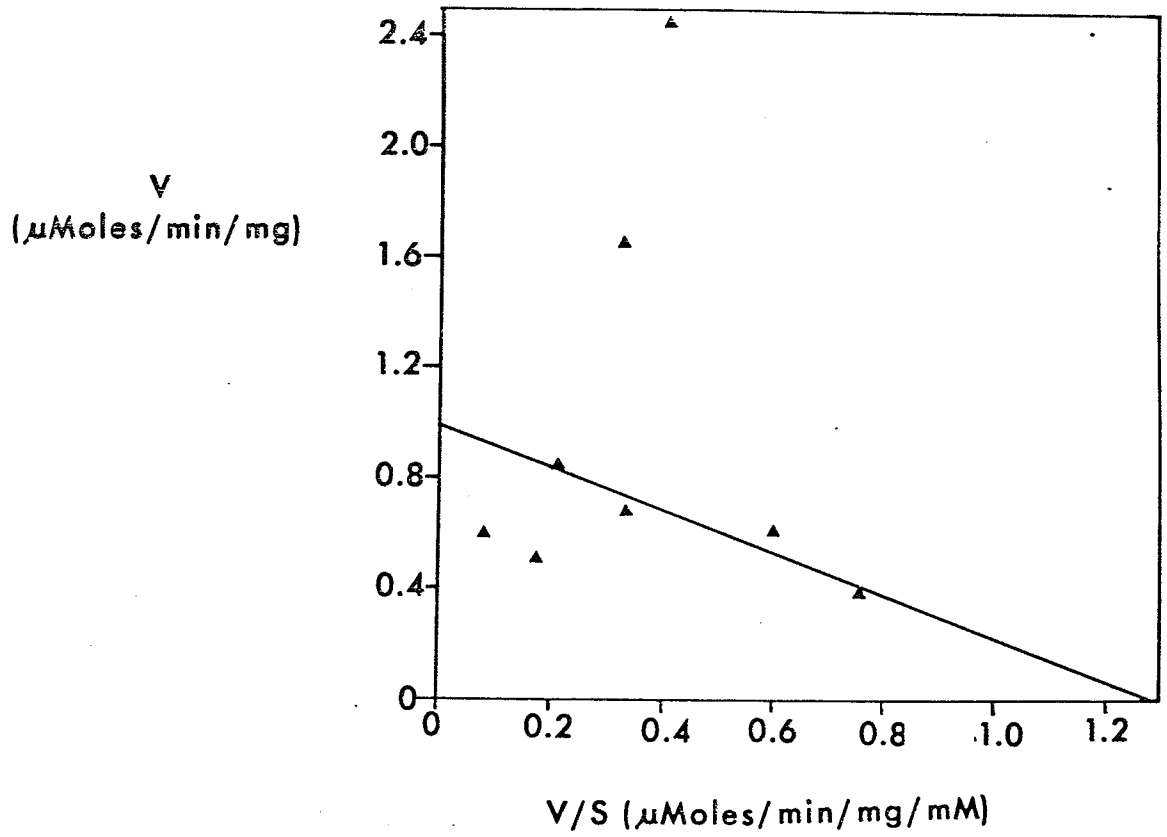

Figure 8: Determination of ATPase K_m and V_{max} for AS particles derived from rat skeletal muscle mitochondria prepared in Chappell-Perry medium (C).

(See Figure 6)

Triangles - rutamycin-sensitive ATPase

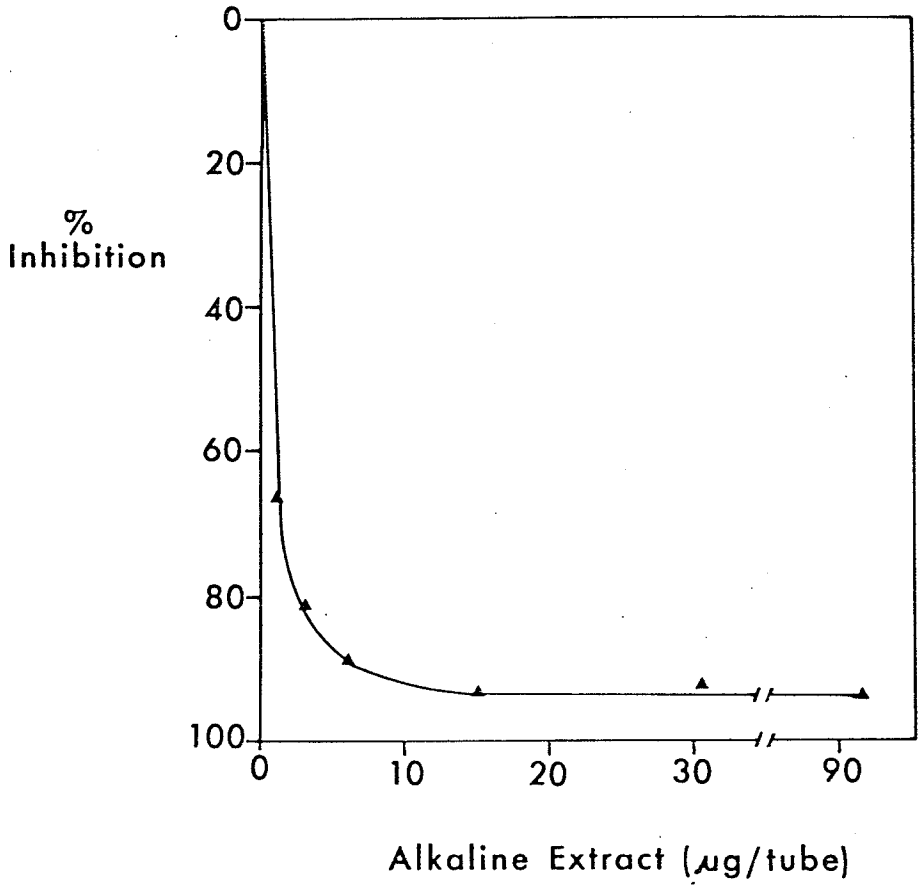

Figure 9: Titration of beef heart alkaline extract with beef heart AS particle ATPase.

Beef heart alkaline extract was titrated with beef heart AS particles in the standard inhibitor assay. The average initial ATPase activity was 0.075 units/tube.

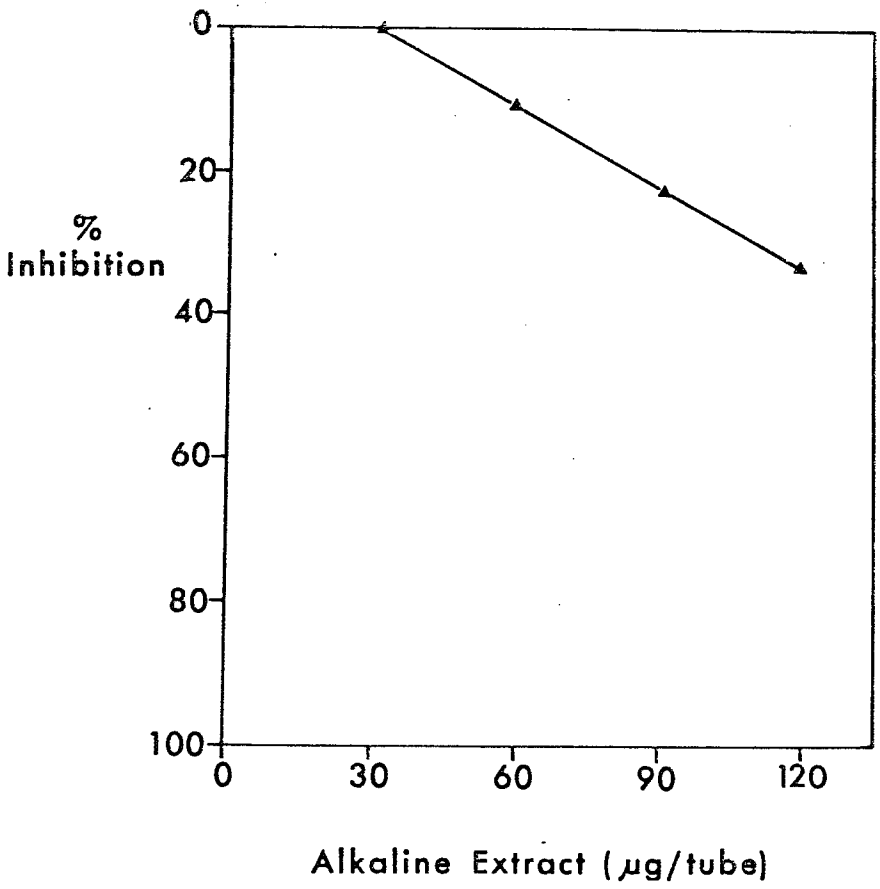

Figure 10: Titration of alkaline extract from non-gradient rat skeletal muscle mitochondria with beef heart AS particle ATPase.

The graph shows the means of 4 determinations. ATPase activity was 0.056 units/tube.

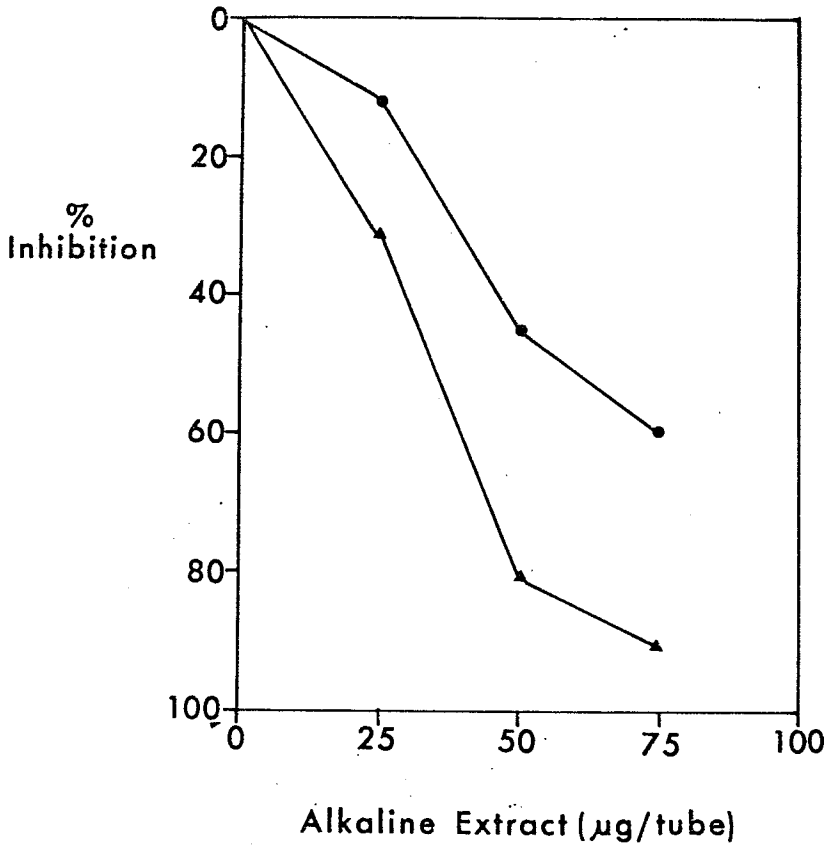

Figure 11: Titration of rat skeletal muscle gradient mitochondria alkaline extract with beef heart and rat skeletal muscle AS particle ATPases.

Specific activity of inhibitor was 2.0 units/mg with rat skeletal muscle ATPase and 3.6 units/mg with beef heart ATPase.

Circles - with rat skeletal muscle ATPase (0.022 units/tube)

Triangles - with beef heart ATPase (0.025 units/tube)

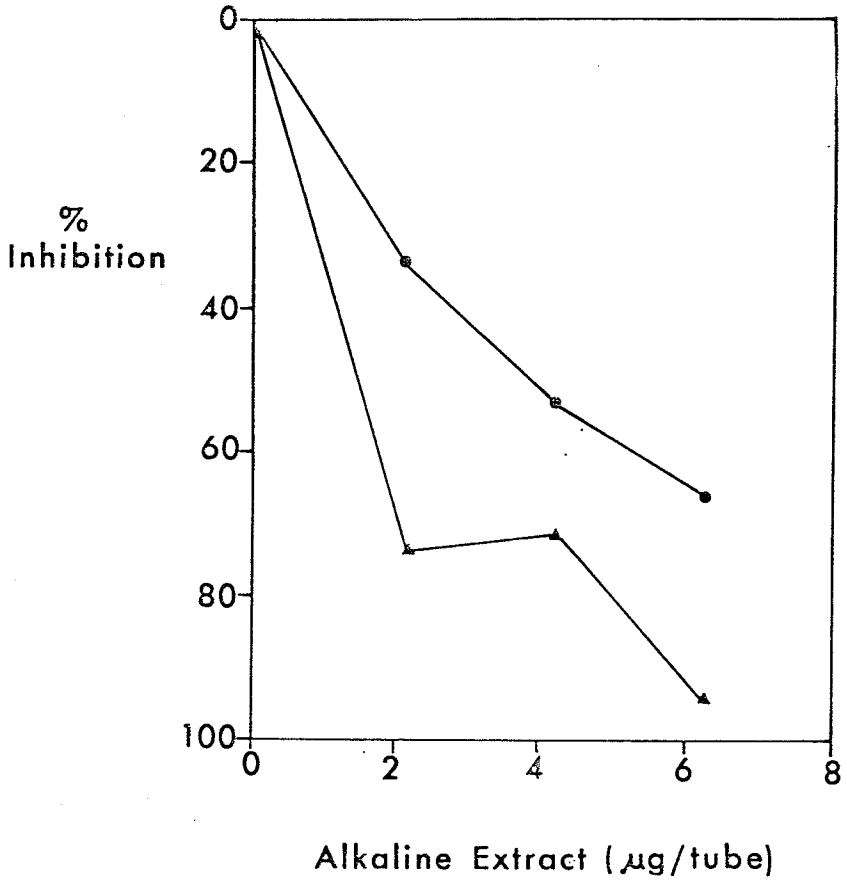

Figure 12: Titration of beef heart mitochondria alkaline extract with beef heart and rat skeletal muscle AS particle ATPases.

Specific activity of inhibitor was 23.3 units/mg with rat skeletal muscle ATPase and 72.6 units/mg with beef heart ATPase.

Circles - with rat skeletal muscle ATPase (0.018 units/tube)

Triangles - with beef heart ATPase (0.021 units/tube)

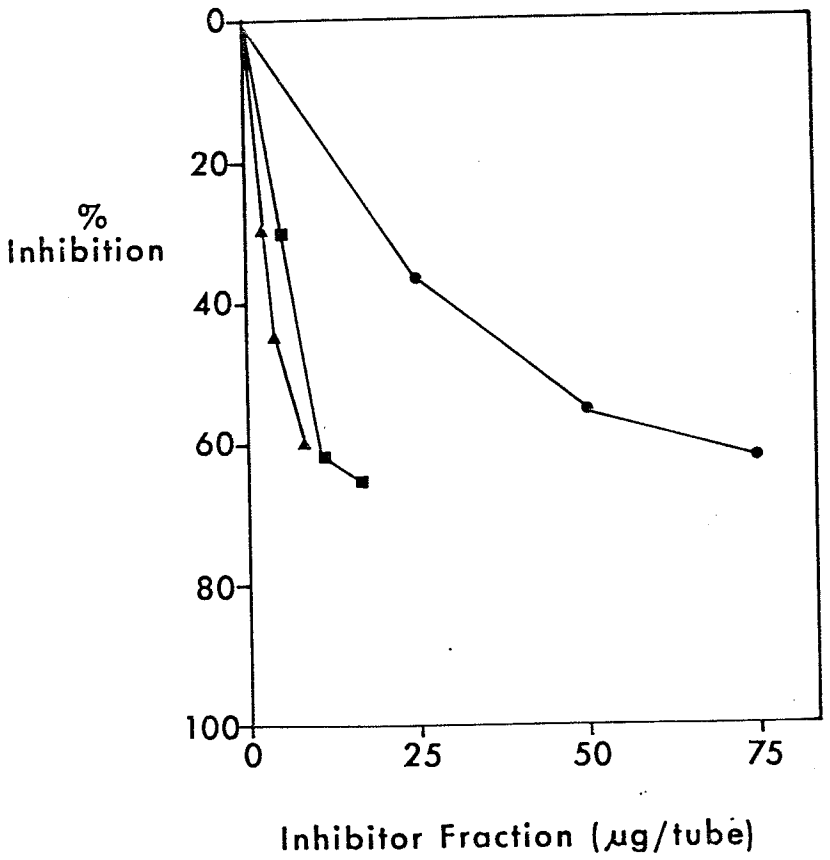

Figure 13: Titration curves for rat skeletal muscle mitochondrial inhibitor fractions with rat skeletal muscle AS particle ATPase (A).

(See Table 9)

Circles - alkaline extract (hypotonic)

Squares - heat treated

Triangles - TCA precipitate

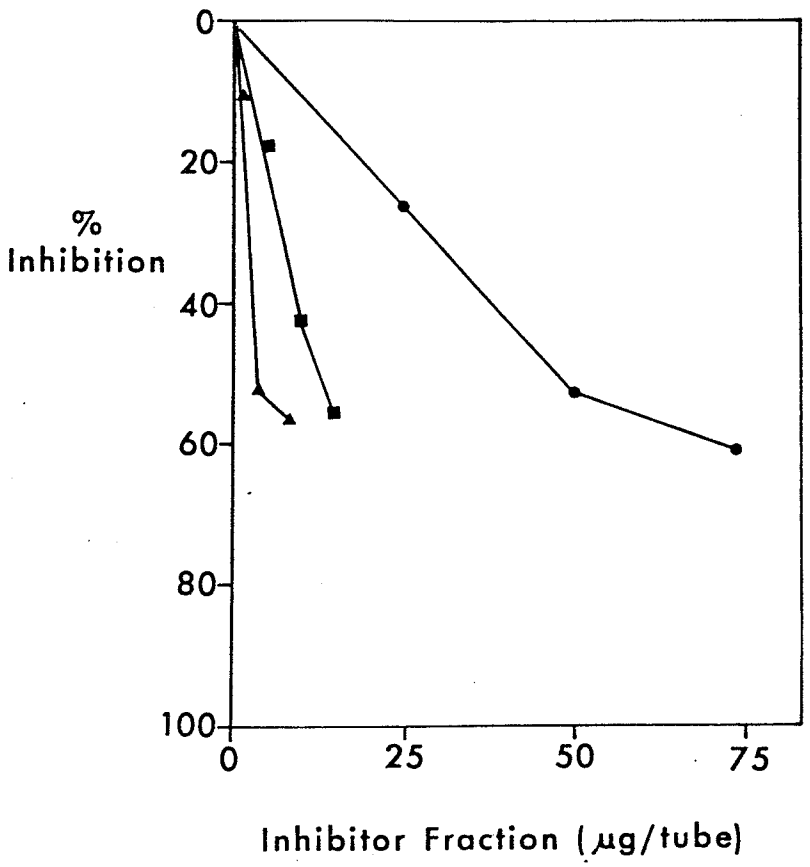

Figure 14: Titration curves for rat skeletal muscle mitochondrial inhibitor fractions with rat skeletal muscle AS particle ATPase (B).

(See Table 10)

Circles - alkaline extract (hypotonic)

Squares - heat treated

Triangles - TCA precipitate

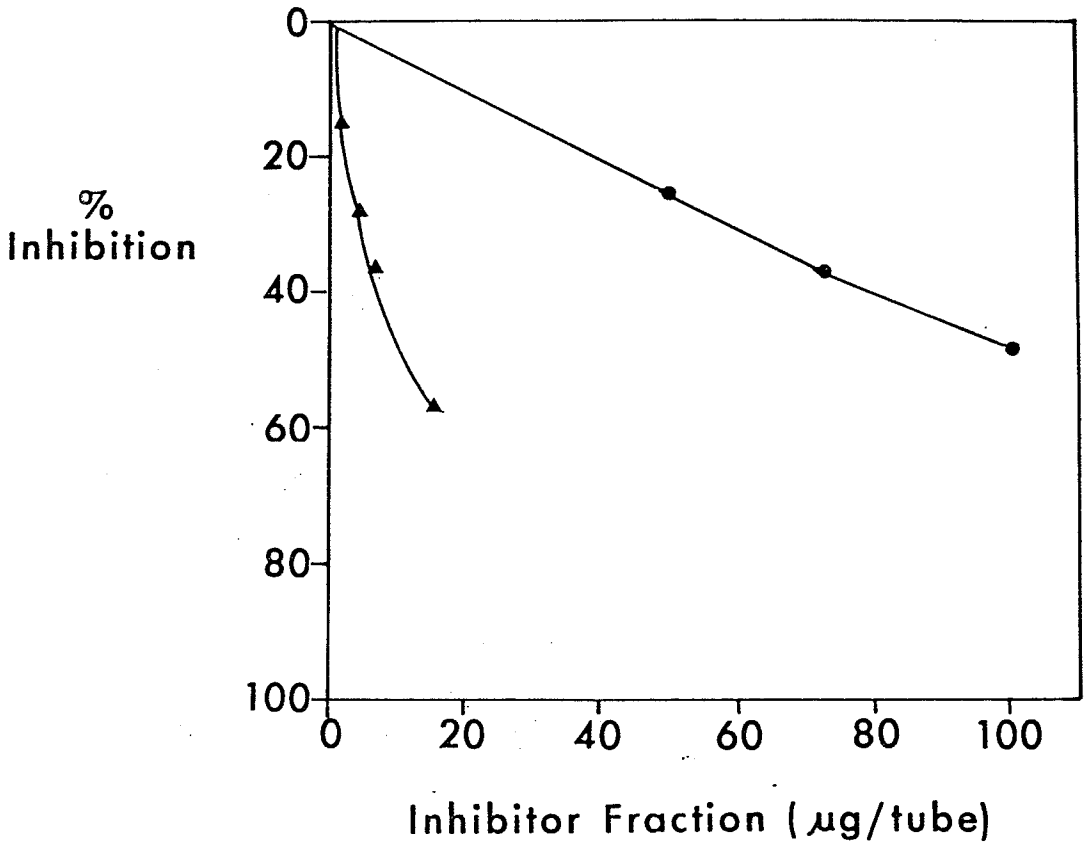

Figure 15: Titration curves for rat skeletal muscle mitochondrial inhibitor fractions with rat skeletal muscle AS particle ATPase (C).

(See Table 11)

Circles - alkaline extract (hypotonic)

Triangles - TCA precipitate

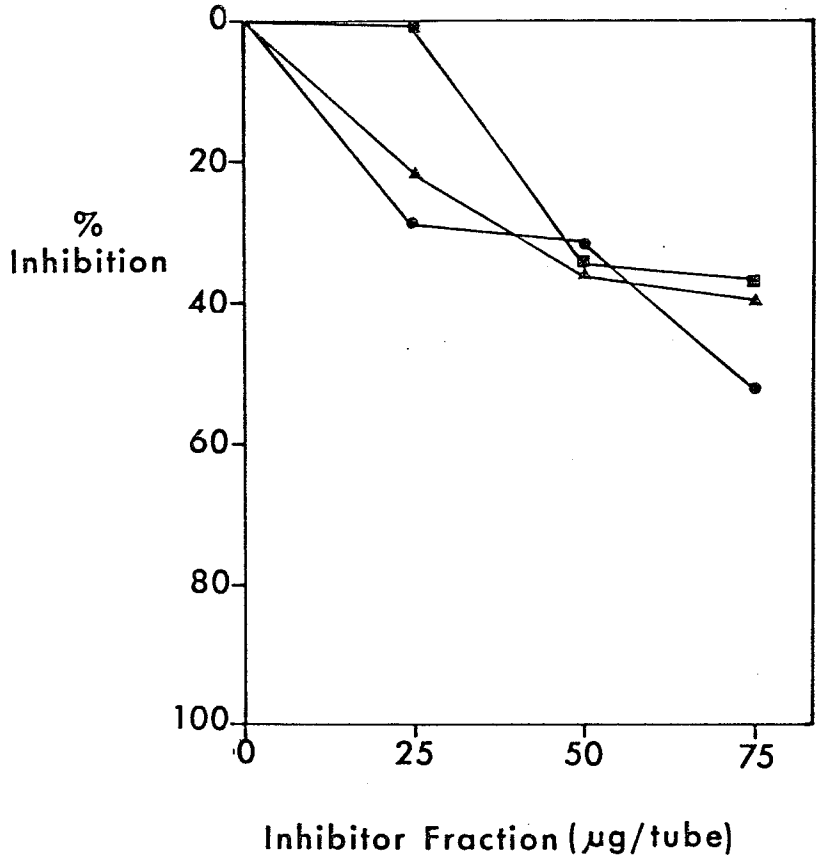

Figure 16: Titration curves for rat skeletal muscle mitochondrial inhibitor fractions with rat skeletal muscle AS particle ATPase (D).

Average values for 2 determinations.

Circles - alkaline extract (isotonic)

Triangles - 0% to 40% ammonium sulphate fraction

Squares - 40% to 70% ammonium sulphate fraction

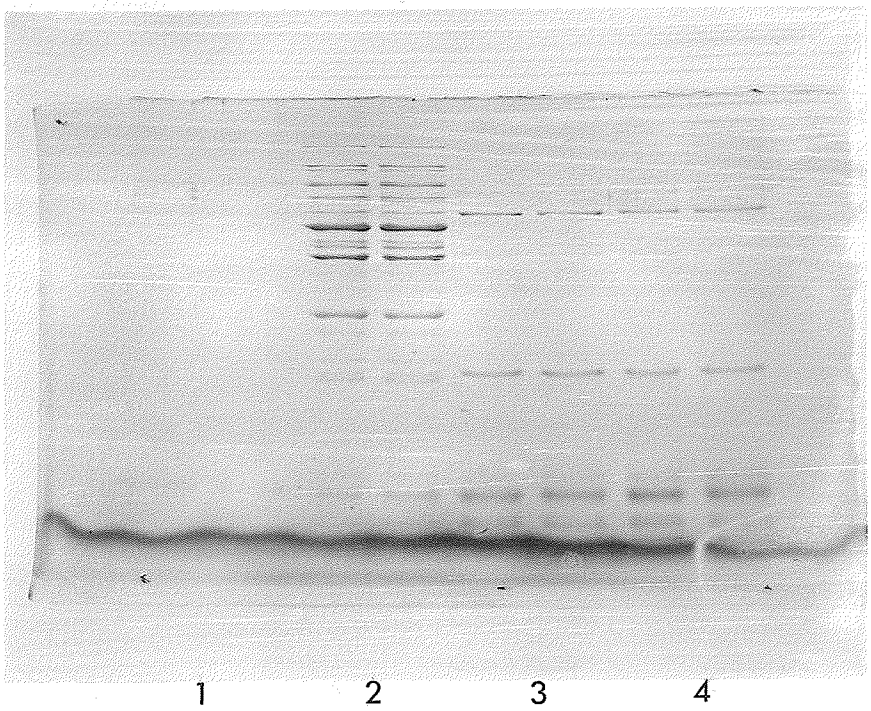

Figure 17: Gel electrophoresis of rat skeletal muscle mitochondrial inhibitor fractions.

1 - ethanol fraction (3µg)

2 - hypotonic sucrose alkaline extract (30µg)

3 - heat treated alkaline extract (31µg)

4 - TCA precipitate (29µg)

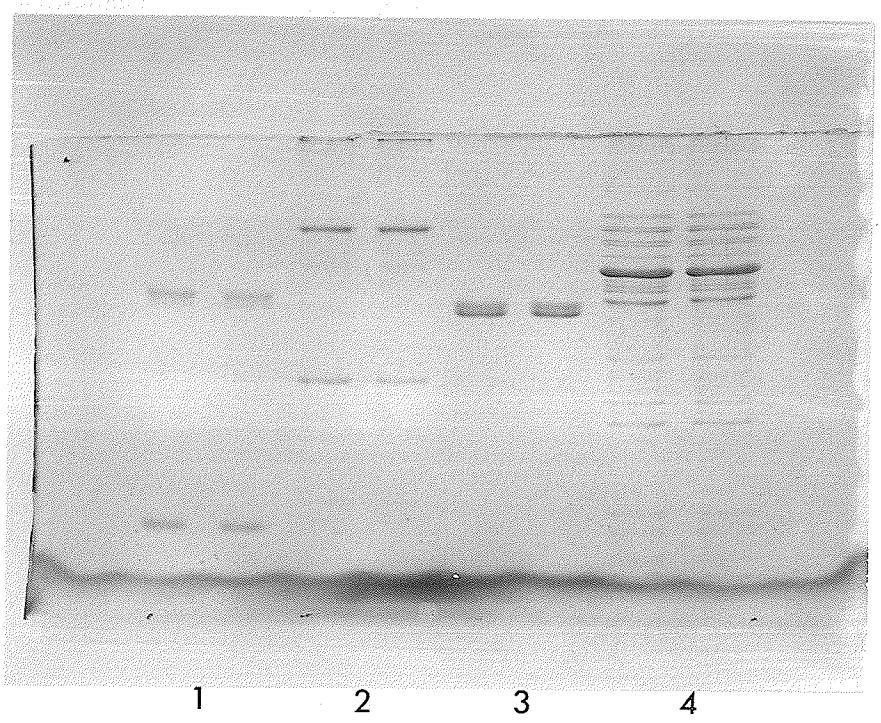

Figure 18: Gel electrophoresis of rat skeletal muscle mitochondrial inhibitor fraction and marker proteins.

1 - ovalbumin (45,000), cytochrome c (12,400)

2 - bovine serum albumin (68,000)
-chymotrypsin (25,700)

3 - alcohol dehydrogenase (37,000)

4 - TCA precipitate (30 µg) derived from 0% to 50% ammonium sulphate fraction of an isotonic sucrose alkaline extract

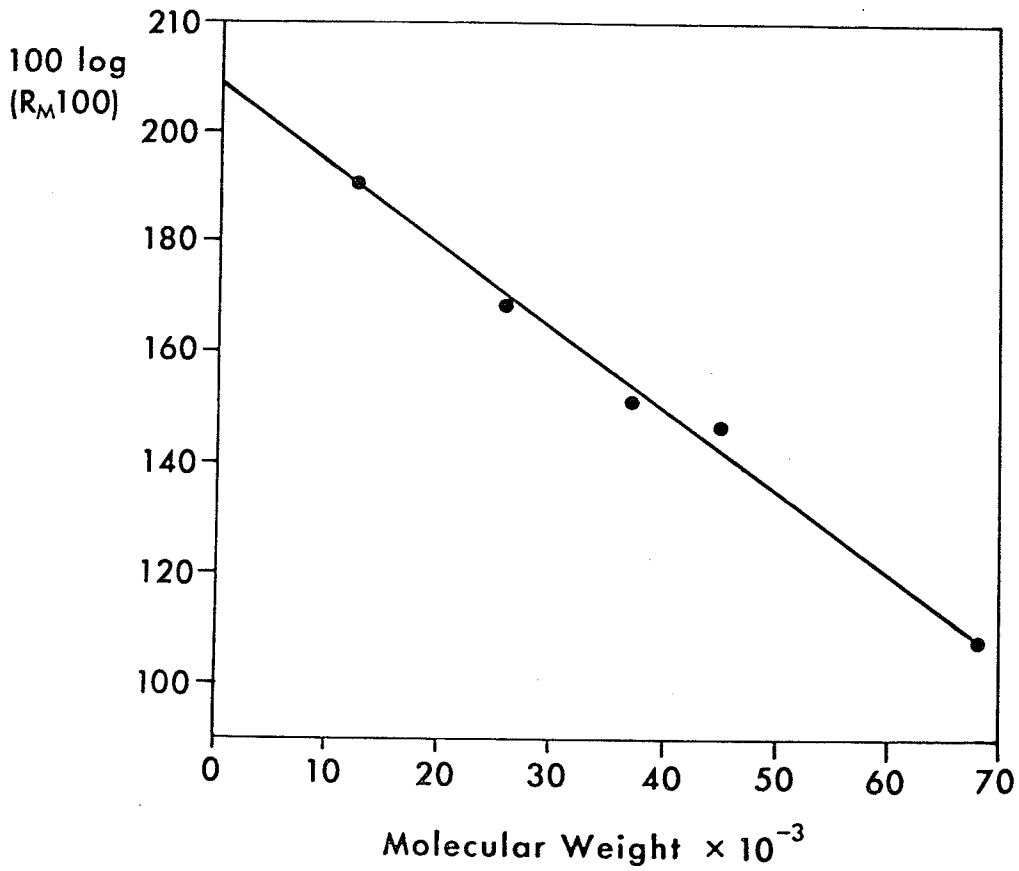

Figure 19: Graph of molecular weight marker proteins from gel electrophoresis.

TABLE 1: (52). INHIBITORY EFFECTS OF MITOCHONDRIAL ATPase INHIBITOR (F_1 INHIBITOR), CHLOROPLAST ATPase INHIBITOR (CF_1 INHIBITOR), AND TROPONIN COMPONENT TN-I ON THE ATPase ACTIVITIES OF VARIOUS BIOLOGICAL ENERGY TRANSDUCING SYSTEMS.

	Beef heart F_1 inhibitor	Spinach CF_1 inhibitor	Rabbit skeletal muscle TN-I
Rabbit skeletal muscle Actomyosin ATPase	+		+
Beef heart Mitochondrial ATPase	+	-	+
Spinach Chloroplast ATPase	+	+	+

+ ; inhibition - ; non-inhibition

TABLE 2: KINETIC PARAMETERS OF AS PARTICLE ATPase

Graph axes	Rutamycin-sensitive ATPase		Uninhibited ATPase	
	V_{max} (μmoles/min/mg)	K_m (mM)	V_{max} (μmoles/min/mg)	K_m (mM)
1/V, 1/S	1.1	0.9	5.3	1.0
V, V/S	1.0	0.8	5.2	1.0

TABLE 3: ATPase ACTIVITY AND YIELD OF RAT SKELETAL MUSCLE MITOCHONDRIA

Method of preparation ( ) number of preparations	Specific activity (oligomycin-sensitive)** (μmoles/min/mg)	% inhibition by oligomycin	Yield of mitochondrial protein (mg/g muscle)
A (2)	1.28	48*	3.10
B (6)	0.80	43	2.27
C (5)	2.22	66	0.49

A - Chappell-Perry medium

B - sucrose/mannitol solutions

C - sucrose/mannitol solutions and sucrose density gradient

* - rutamycin used

** - total minus non-inhibited

TABLE 4: CYTOCHROME OXIDASE ACTIVITIES OF RAT SKELETAL MUSCLE FRACTIONS PREPARED IN SUCROSE/MANNITOL SOLUTIONS

Fractions ( ) number of determinations	Specific activity (μmoles ferrocytochrome c oxidised/min/mg)	Yield %
Homogenate (3)	0.18	100
Non-gradient mitochondria (4)	3.20	50
Gradient mitochondria (4)	6.50	28
F3* (4)	0.42	3

* - the bottom band found after sucrose density gradient centrifugation

TABLE 5: ATPase ACTIVITY OF MITOCHONDRIA AND AS PARTICLES

Fraction ( ) number of preparations	Specific activity (μmoles/min/mg)	Specific activity (oligomycin sensitive) (μmoles/min/mg)	% inhibition by oligomycin
A* (2)			
Mitochondria	2.6	1.3	50
AS	4.6	1.4	30
B (2)			
Mitochondria	2.9	1.8	62
AS	5.2	3.3	64
C** (2)			
Mitochondria	1.5	0.9	60
AS	8.4	7.6	90

A - rat skeletal muscle (Chappell-Perry medium)

B - rat skeletal muscle (sucrose/mannitol solutions)

C - beef heart

* - with rutamycin

** - one preparation with rutamycin

TABLE 6: RAT SKELETAL MUSCLE MITOCHONDRIAL ALKALINE EXTRACTS

Sucrose (M) in medium ( ) number of preparations	Protein (mg/ml)	Yield of protein (mg/mg mitochondrial protein)	Inhibitor Specific activity (units/mg)	Initial units of ATPase/tube
0.25 (2)	1.5	0.13	2.1	0.019
0.15 (1)	1.4	0.13	2.2	0.020
0.15 (1)	1.4	0.13	4.8	0.109
0.15 (1)	0.9	0.09	3.8	0.030

TABLE 7: AMMONIUM SULPHATE FRACTIONATION OF RAT SKELETAL MUSCLE ALKALINE EXTRACT

Fraction	Undialysed		Dialysed	
	% inhibition	µg/tube in assay	% inhibition	µg/tube in assay
0 - 40%	-15	60	10	60
40 - 70%	-17	60	63	60
70 -100% *	72	4	38	8

* - smaller amount of protein used in assay because of low protein yield of this fraction

TABLE 8: PURIFICATION OF RAT SKELETAL MUSCLE MITOCHONDRIAL ATPase INHIBITOR (A)

Fraction	Volume ml	Protein mg	Total units	Specific activity units/mg	Yield %	Units enzyme per tube
Alkaline extract (isotonic)	16.0	13.4	26.9	2.0	100	0.016
TCA precipitate	1.4	6.6	38.9	5.9	100	0.025
Heat treated	6.8	5.1	35.2	6.8	100	0.031

TABLE 9: PURIFICATION OF RAT SKELETAL MUSCLE MITOCHONDRIAL ATPase INHIBITOR (B)

Fraction*	Volume ml	Protein mg	Total units	Specific activity units/mg	Yield %
Alkaline extract (hypotonic)	8.6	7.8	29.8	3.8	100
Heat treated	8.2	1.5	20.6	13.9	69
TCA precipitate	0.8	0.7	21.2	32.2	71

* - assayed with an average of 0.028 units of enzyme per tube

TABLE 10: PURIFICATION OF RAT SKELETAL MUSCLE MITOCHONDRIAL ATPase INHIBITOR (C)

Fraction*	Volume ml	Protein mg	Total units	Specific activity units/mg	Yield %
Alkaline extract (hypotonic)	7.2	9.9	21.5	2.2	100
Heat treated	10.0	1.9	14.2	7.6	66
TCA precipitate	1.6	1.0	15.9	16.0	74

* - assayed with an average of 0.021 units of enzyme per tube

TABLE 11: PURIFICATION OF RAT SKELETAL MUSCLE MITOCHONDRIAL ATPase INHIBITOR (D)

Fraction*	Volume ml	Protein mg	Total units	Specific activity units/mg	Yield %	Specific** activity units/mg
Alkaline extract (hypotonic)	7.2	9.9	47.5	4.8	100	7.9
TCA precipitate	1.6	1.0	49.9	51.1	100	88.0

* - assayed with an average of 0.111 units of enzyme per tube

** - maximum inhibition of ATPase enzyme by oligomycin of 69% taken into account

TABLE 12: TRYPSIN DIGESTION OF TCA PRECIPITATE FROM RAT SKELETAL MUSCLE MITOCHONDRIA

Fraction*	% inhibition
TCA precipitate (15µg)	75.7
TCA precipitate (15µg) and trypsin	36.5
TCA precipitate (15µg) trypsin inhibitor and trypsin	63.8

* - assayed with 0.03 units of AS particle ATPase per tube

TABLE 13: COMPARISON OF SPECIFIC ACTIVITIES OF ALKALINE EXTRACTS

Alkaline extract	AS particles	Units of enzyme/tube	Specific activity (units/mg)
Beef heart (47)	Beef heart	2 - 3	855
Rat liver (41)	Rat liver	0.1	9.0
Yeast (43)	Beef heart	0.2	4.3
Beef heart	Beef heart	0.07	314
Rat skeletal muscle	Rat skeletal muscle	0.1	4.8