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THE PURIFICATION AND IMMUNOLOGICAL ISOLATION OF ATP CITRATE LYASE FROM RAT LIVER

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ABSTRACT

ATP CITRATE LYASE (E.C 4.1.3.8) has been purified from rat hepatocyte cytoplasm by a combination of existing published procedures. The final purification method produced homogeneous ATPCL with specific activity of 10-16 units/mg.

Antibodies were raised in rabbits against purified ATPCL eluted from reactive Blue Sepharose CL-6B or DEAE anion exchange column.

The purified antibodies were tested for their specificity for ATPCL. This was accomplished by Ouchterlony double diffusion analysis and also by disruption of antibody-antigen complexes and visualizing the generated protein bands on detergent gels.

The equivalence point of the purified antibody was determined by immunotitration with both purified enzyme and crude extract. The equivalence point was later confirmed by immunotitration of radiolabelled proteins.

Antibodies were then used to immunochemically isolate and quantitate the amount of (35-S) methionine or (14-C) radiolabelled ATPCL in the cytosolic fraction of rat liver.

Pulse labelling of rat liver proteins in vivo and then precipitation of radiolabelled proteins demonstrated that the purified antibodies precipitated proteins other than just the ATPCL subunit. The amount of ATPCL present in the cytosolic fraction could be calculated after immunoprecipitation and excision of radiolabelled ATPCL subunition SDS-PAGE. The proportion of ATPCL protein to the total TCA precipitable protein could then be calculated since the immunoprecipitation was carried out under conditions of antibody excess.

Radiolabelled ATPCL was then immunoprecipitated from the cytosolic fractions of rats that had been subjected to different nutritional regimes.

The results of this set of experiments showed that induction of ATPCL activity resulted from an increase in immunologically reactive

protein. Increasing amounts of radiolabelled immunoreactive ATPCL protein could be precipitated by antibodies as the enzyme was induced. Induction of ATPCL activity resulted from increased rate of synthesis or decreased rate of degradation of immunoreactive protein and not from the activation of pre-existing enzyme protein.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'- triphosphate
BPB	Bromophenol blue
cDNA	Circular DNA
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
HIgG	Immunoglobulin heavy chain
LIgG	Immunoglobulin light chain
	mRNA Messenger RNA
PBS	Phosphate buffered saline (10 mM Na_2HPO_4, 150 mM NaCl pH 7.4)
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N",N"Tetramethylethylenediamine
Tris	Tris(Hydroxymethyl)aminomethane

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CHAPTER 1

1.1 INTRODUCTION AND HISTORICAL REVIEW

ATP citrate lyase (E.C 4.1.3.8) catalyses the following reaction:

citrate + CoASH + ATP ----------> Oxaloacetate + AcetylCoA + ADP + Pi

(12,89,113,117,150,164,168,176,199,211)

This enzyme is of physiological importance because of its role in providing acetyl units for lipogenesis from glucose.

In animals acetylCoA is formed by the oxidative decarboxylation of pyruvate within the mitochondria, whereas lipogenesis occurs in the cytosol (19). Since acetylCoA cannot penetrate the inner mitochondrial membrane its export to the cytoplasm is accomplished by condensation with oxaloacetate to form citrate which is then transported across the mitochondrial membrane by a specific carrier (19). In the cytosol, citrate is reconverted to oxaloacetate and acetylCoA by ATP citrate lyase (ATPCL) (12,50,71,73,124,156,169,174,200). The acetylCoA formed in the cytosol can then be utilized for fatty acid biosynthesis.

Thus ATPCL can be regarded as the first committed enzyme of lipogenesis from carbohydrate in the cytosol in non-ruminant mammals (40, 43,89,94,98,99,104).

ATPCL has been shown to be widely distributed in nature. The enzyme was initially found in lipogenic tissues such as liver (2,5,12,14,15,19, 40,63,87,89,92,98,99,111,140,147,164,168,172,173,174,178, 189, 194, 205, 209,211,213) adipose tissue (2,13,14,15,18,19,29,95, 135, 136, 140, 143, 164,166,180,184,185) and lactating mammary gland (70,71,172).

ATPCL has also been demonstrated in non lipogenic tissues such as brain (12,74,157,172,186,187,188,189,190,191), adrenal (29,172), and testicular tissue (10,11,29,57). The enzyme was also shown to be present in microbiological sources. ATPCL activity is present when these bacteria (9,91,165,179) and fungal (24,25,26,28,69,119,165,179) sources are grown under optimal conditions when lipogenesis is occurring.

Two plant sources that contain ATPCL are mango fruit (121, 172) and

germinating castor bean endosperm (58).

ATPCL has been purified to homogeneity from several tissues including rat liver (89,113,150,189), adipose tissue (140, 148), mammary gland (71) and brain (189).

Mammalian ATPCL is a 440-480,000 molecular weight phosphoprotein (2,5,14,18,113,140,148,151). The enzyme is a tetramer of four identical subunits (12,71,111,113,140,148). It has been suggested that the enzyme has multiple chromatographic forms (40). The tetrameric form mentioned above is stable at basic pH and is the predominant form of the enzyme. About 10% of the enzyme in rat liver exists as a polymer with an apparent molecular weight of ten million which is stable at acid pH (40). ATPCL has also been reported to exist in a high molecular weight complex with fatty acid synthetase and acetylCoA carboxylase (61,175,177).

The activity and concentration of ATPCL has been shown to change with variation in diet (1,8,12,56,59,71,88,98,104,105,124,151,156,167, 168,170,193,211,212,213). Induction of the enzyme is also sensitive to the administration of hormones (8,11,28,29,59,64,88,90,95,103,105, 124,167,168,170,) and the expression of cellular programmes for differentiation (16,68,98,105,106,164,174,207,208).

The change in the activity of ATPCL has been shown to parallel the change in the rate of lipid synthesis in live (59,92,104,151,167,212), adipocytes (208), and lactating mammary gland (71) when previously starved rats are refed a high carbohydrate diet.

The amount of ATPCL found in a liver homogenate declines when rats are starved for two or more days (2,8,56,59,98,102,104,124,167,170,211, 212) as shown by enzyme assay, rocket immunoelectrophoresis or radial immunodiffusion. Induction of alloxan diabetes in rats also results in a pronounced decrease in the ATPCl activity when compared to rats fed a standard chow diet. When alloxan induced diabetic or starved rats are refed a high carbohydrate diet (2,8,59,102,104,105,113, 140,167,170,193, 204,211,212) or administered insulin (40,59,78,174, 185,211), ATPCL is restored to the same level as that found in chow fed rats.

The levels of ATP citrate lyase, Fatty acid synthetase, Malate

dehydrogenase, AcetylCoA carboxylase, and Glucose-6-phosphate dehydrogenase have been shown in rat liver to rise and fall co-ordinately in response to dietary variation (59,171,201,204). Thus ATPCL and the other lipogenic enzymes are coordinately induced.

In a number of cases it has been shown that the induction of ATP Citrate lyase is paralleled by proportional increases in translatable messenger RNA's (35,59,71,138,155,181,208).

Recent dot translation hybridization analysis show that the relative content of rat liver ATPCL mRNA (4.9kb) increased when previously starved mice are refed a high carbohydrate diet. There was a 25 fold increase in ATPCL mRNA after a 15 hour refeeding in rat liver and a 30 fold increase in murine ATPCL. The increased abundance of ATPCL immunoreactive protein paralleled this induction of the mRNA (53,155,206,208).

The blockage of the induction of immunoreactive protein by puromycin (59,71), actinomycin D (90), cordycepin (60) and @-amanitin (an inhibitor of RNA polymerase II (52)), is indirect evidence that de novo synthesis of ATPCL messenger and protein are necessary for induction (60,99,211).

Previous work, (Gibson 1973 (71)) has shown that the change in the total ATPCL activity in rat liver is a result of a change in the rate of synthesis of the enzyme with very little change in the rate of degradation of the protein.

These reports indicate that the synthesis of ATP citrate lyase is controlled at a pretranslational level (155).

The plasma signal which mediates the observed effect of diet on the level of the "lipogenic" enzymes is generally considered to be insulin, which is elevated in the blood of well fed animals. However glucagon, which is elevated in the blood of starved animals inhibits the synthesis of the lipogenic enzymes (reviewed 124). In cultured hepatocytes, insulin and thyroxine stimulate the accumulation of ATPCL and this stimulation is counteracted by glucagon (99,185).

The mechanism by which these individual hormones regulate the synthesis and/or degradation of ATPCL mRNA in vivo is the subject of conti-

nuing investigation using hormone sensitive cultures of rat hepatocytes (206,208).

These data provide evidence that the regulation of ATPCL depends on the long term adaptive changes based on hormone dependent (123,168) and diet dependent (59,156,193,212) induction and degradation (59,104,169,212).

The overall rate of fatty acid biosynthesis is subject to short term regulatory control by regulation of acetylCoA carboxylase. The activity of this enzyme is controlled by allosteric effectors and by reversible phosphorylation (30,35,72,73,79,100). ATPCL has also been shown to be subject to reversible phosphorylation in response to the actions of hormones (2,5,8,12,15,43,70,95,140,143,145).

Several workers have suggested that phosphorylation of ATPCL may well be significant in the regulation of the enzyme, which represents a crucial link between carbohydrate and lipid metabolism. Inactivation and activation of ATPCL at the crossroads of the fate of dietary carbon is therefore vitally important in considering the control of lipogenesis. Phosphorylation requires an expenditure of energy in the cellular economy and are usually strictly controlled and have a defined function.

However, to date, no regulatory significance has been conclusively assigned to the phosphorylations within ATPCL.

The phosphorylation sites within ATPCL are separate and can be distinguished by their chemical properties, and by their different susceptibilities to phosphoprotein phosphatases (4,71,137).

The phosphorylation sites of ATP citrate lyase are defined as:

1. Catalytic phosphate.

A phosphorylated histidine (0.5molP/mol of subunit)(42,140,151,164, 165) is found at the catalytic site. The phosphate is derived directly from ATP as a result of the reaction mechanism (42,89,90,134, 147,205). This phosphate is acid labile (5) as well as being sensitive to hydrolysis by hydroxylamine and substrates (4,42,46,89,90,113,140,143,151,165).

The catalytic phosphate is located on a 68,000 molecular weight tryptic peptide from the enzyme that catalyses acylCoA lyase activity.

3S-citrylCoA ----------> AcetylCoA + Oxaloacetate

2. Structural phosphate.

The reversible phosphorylation of ATPCL in the structural site is the subject of active investigation, and as a consequence, the published literature is often confusing and controversial. There is also a great deal of confusion and uncertainty as to the specific role and mechanism of the structural site phosphorylations. Different preparative methods and analytical techniques for the preparation of ATPCL from different tissues may also contribute to the inconsistency of the published data.

Structural phosphates are at sites on the enzyme distant from the catalytic site. Structural site phosphorylations are catalysed by kinases and fall into two classes:

(a) Those catalysed by glucagon-cAMP dependent protein kinase(s).
(b) Those catalysed by insulin sensitive-cAMP independent protein kinase, ATPCL kinase, calcium - independent and calmodulin - insensitive kinase(s).

In rat liver both structural phosphates reside on an acid stable phospho-serine residue (113,143,164). In rat adipose tissue, structural phosphates reside in both serine and threonine residues (148).

In rat liver and fat pad there is 0.5 mol P/mol of subunit (113,149)

but only 0.2 mol P/ mol of subunit in rat mammary gland ATPCL (71).

Labelling of structural serine phosphate can be detected after 32P injection of orthophosphate in vivo (2,111,113).

In rat liver the structural phosphate, resulting from labelling with 32P orthophosphate in vivo, resides in a 57,000 molecular weight peptide. This peptide is formed by the action of trypsin on native ATPCL (2,113) and contains citrate thiokinase activity (111).

citrate + ATP + CoASH ----------> (3S)-citrylCoA

ATPCL is phosphorylated at sites distinct from the catalytic site in response to the actions of glucagon, insulin, and B-agonists but by apparently different mechanisms in different tissues.

Glucagon and B-adrenergic agonists (2,3,4,5,13,14,15,18,21,31, 54,71,85,86,95,113,140,168) mediate phosphorylation through a cAMP dependent proteinkinase (4,5,12,70) which increases the amount of serine structural phosphate in the enzyme from hepatocytes (148), but not in the enzyme from mammary gland (71). This difference may be a tissue specific phenomenon and not a difference in enzyme structure, since the physiochemical properties (subunit molecular weight, subunit structure, absorbance index and specific activity) and the amino-acid composition of rat liver is very similiar to rat mammary ATPCL (71).

cAMP-dependent protein kinase phosphorylates ATPCL to the extent of 0.5 mol P/ mol of ATPCL subunit from hepatocytes (12). The rate of phosphorylation of ATPCL by the cAMP-dependent protein kinase is similiar to the observed rate of phosphorylation of the enzyme caused by glucagon in hepatocytes (5).

The site of phosphorylation by purified cAMP-dependent protein kinase has been shown to be located in a unique tryptic peptide of 1,000 molecular weight (132,133,146,185) with the following sequence

-thr-ala-ser[p]-phe-ser-glu-ser-arg- (4,12,132)

In rat hepatocytes (2,95,113) and adipocytes (14,21,54,85,140) this phosphate can be removed by a specific phosphatase which has been par-

tially purified from rat liver (113,164).

Experimental evidence suggests that insulin stimulates the phosphorylation of a specific serine residue of ATPCL (2,4,14,18,19,20,21, 49,54,95,133,140,142,185) via an insulin-sensitive protein kinase (cAMP-independent protein kinase, ATPCL kinase) (4,12,15,19,20,21,71,143,144).

Ramakrishna and Benjamin 1981 (143,144) have reported the partial purification of a cAMP-independent protein kinase from rat adipose tissue. This kinase phosphorylates rat adipose tissue ATPCL at a site distinct from that phosphorylated by the cAMP-dependent protein kinase.

The site phosphorylated by the insulin-sensitive cAMP-independent protein kinase occurs on a tryptic peptide of 8,500 molecular weight. This has been determined by phospho-aminoacid analysis of radio-labelled tryptic peptides separated by HPLC (135,145,154). Both a serine and a threonine residue in this peptide are phosphorylated in the enzyme from rat adipo-cytes, (135,136,145,146,148).

The cAMP-dependent protein kinase phosphorylates only a single serine residue in a peptide of 1,000 molecular weight (135,136).

Phosphorlyation of the larger peptide was shown to be absolutely dependent upon the prior phosphorylation of a residue within the smaller peptide by the cAMP-dependent protein kinase. The degree of phosphorylation of ATPCL by both kinases together is more than their sum when incubated separately (146). Whether the smaller peptide is contained within the larger peptide is still the subject of investigation.

A number of reports suggest that within rat liver ATP citrate lyase, the same site is phosphorylated in response to either insulin or hormones that increase cAMP (12,133). These workers have used a number of different techniques including analysis of phosphopeptides released from ATPCL isolated from liver cells or 3T3-Li preadipocytes by partial proteolysis,analyses of complete tryptic digests of [32P]-ATPCL by reverse phase HPLC and a combination of electrophoresis and chromatography.

These observations are consistent with the possibility that phosphorylation of one serine (or threonine) within ATPCL phospho-peptide

excludes the subsequent phosphorylation of a second residue (185). These data are inconsistent if the phosphorylations are of regulatory significance because these hormones have entirely different overall effects on lipogensis.

Avruch (2,14) and Benjamin (19) raise the possibility that one pathway of response of ATPCL to insulin might involve one or more of the following :

(a) inhibition of a phosphatase,
(b) the activation of a cAMP - independent protein kinase,
(c) an alteration in the ability of ATPCL to serve as a substrate for the protein phosphatase or
(d) an indirect effect on cAMP - dependent protein kinase.

The dephosphorylation of the insulin sensitive phosphorylation site in rat liver ATP citrate lyase can be catalysed by protein phosphatases 1, 2a and 2c in vivo (214). Isolated rabbit skeletal muscle phosphorylases can dephosphorylate this site in lactating mammary gland ATPCL (71). In both cases the specific dephosphorylations of ATPCL has no observable effect on the activity of the enzyme (214).

The following roles have been proposed for the phosphorylated and dephosphorylated forms of ATP citrate lyase :

(a) Dephosphorylation could be a signal for the association of ATPCL with the outer mitochondrial membrane.

Only 4-10% of the total ATPCL has been shown to bind to mitochondria (39,92,94,147).

Janski and Cornell (92,94) reported that citrate would release 80% of the ATPCL bound to the mitochondria in the absence of magnesium in vitro.

When added simultaneously, ATP, citrate and CoA released 98% of the ATPCL bound to the mitochondria.

In the presence of magnesium ions, CoA released 100% whereas ATP, CoA, and citrate released 91% of ATPCL bound to the mitochondria.

They also showed (94) that in starved rats, 52% (0.6 of 1.22 units) of ATPCL was bound to the mitochondria and this is a higher proportion than rats refed a fat free diet (7.8 of 32 units). The increase was attributed to elevation of CoA levels in the fed state.

This result was confirmed by the use of the fatty acid analogue 5-(tetradecyloxy) 2 - furoic acid, which lowers CoA levels in liver cells and results in an increase in binding of ATPCL to mitochondria.

Inactivation of the enzyme by binding to the membrane may represent a mechanism for controlling the rate of citrate cleavage either in the cytosol or as it leaves the mitochondria (39,92,93,113,143,147).

(b) An association with microsomes (112,114) or endoplasmic reticulum (114).

In vitro, ATPCL binds to isolated rat liver microsomes (114). This binding phenomenon appears to be both temperature and time and dependent and is a saturable process that requires the presence of a specific membrane protein. However individual preparations of microsomes bind ATPCL to variable extents. When purified rough and smooth endoplasmic reticulum were prepared both fractions bound ATPCL to the same extent. There is sufficient microsomal protein in the hepatocyte to allow most, if not all, the ATPCL to be bound to the endoplasmic reticulum even when the enzyme has been induced over ten fold by dietary manipulations (114).

In vitro relatively high salt concentrations inhibit binding of ATPCL to microsomes. At 50 mM potassium phosphate and 60 mM KCl, 40% of the enzyme is bound, whereas 10uM CoASH completely eliminates binding (114).

There is no compelling evidence for the association of ATPCL with microsomes in vivo.

(c) A signal for protein degradation or that phosphorylation affects turnover or subcellular localization (2,71,113,147,185,208).

Dunaway and Segal (51) have isolated a 3500 Mr stabilizing factor that protects phosphofructokinase (PFK) from lysosomal and thermal inactivation.

Phosphorylation of PFK does not alter the allosteric properties of this enzyme but decreases the affinity for the stabilization factor which in turn controls the activity of PFK.

Osterland and Bridger (126,127) isolated the same stabilizing factor and showed that this peptide also stabilizes ATPCL against thermal and lysosomal degradation. No evidence was presented to suggest that phosphorylation of ATPCL affects either the affinity of ATPCL for the stabilizing factor, or any turnover phenomenon.

To date, no alteration in ATPCL function has been demonstrated to result from hormone stimulated phosphorylation of the enzyme (12,95). In vitro, there has been no observed alteration in the kinetic properties of the various phosphorylated and dephosphorylated forms of ATPCL (12,70,71,95,98,113,117,142,144,146,147,185,208).

Ranganathan et al (1980) (147) could demonstrate no difference in the manner in which they are degraded by lysosomal proteases (147). Even complete removal of the phosphopeptide from ATPCL by proteolytic nicking does not alter any kinetic property of the enzyme (164).

The phosphorylation of ATPCL is slow and hence any effect on regulation would have to be long term (149).

The reversible phosphorylations of ATPCL in both sites is of relevance in this thesis if these phosphorylations affect the affinity of the enzyme for the DEAE anion exchange chromatography column or if it alters its behaviour in the various preparative procedures used.

Hoffman et al (76) located only one peak of activity after anion exchange chromatography. Corrigan et al (40) located 3 individually resolvable peaks. Two of these peaks were retained by anion exchange

columns while the first was eluted in the void volume peak. There was no data presented comparing the degree of phosphorylation between these protein peaks.

1.2 OBJECTIVES OF THIS INVESTIGATION

The purpose of this thesis is to investigate the molecular basis of the regulation of ATP citrate lyase by dietary manipulation. To achieve this aim, it was found necessary to isolate milligram quantities of homogeneous ATPCL and prepare high-titre ATP citrate lyase antiserum.

The antibodies can then be used to confirm that the increase in inducible enzyme activity paralleled the increase in ATPCL immunoreactive protein.

CHAPTER 2.

GENERAL METHODS

INTRODUCTION.

This chapter represents the general methods used in the purification of ATPCL.

2.1 BUFFERS

Aqueous solutions were made up in deioinized glass distilled water. Buffers were cooled to the 4 C before being adjusted to the required pH.

2.2 EXPERIMENTAL ANIMALS

Sprague-Dawley strain rats were obtained from the Small Animal Production Unit at Massey university. Animals were maintained at 23 C in a room illuminated from 6.00 a.m. to 6.00 p.m. daily. Food and water were normally available ad libitum.

Rabbits (New Zealand White) were also bred by the Small Animal Production Unit at Massey University.

2.3 CENTRIFUGATION

Ultracentrifugation was carried out on a Beckman model L2-65B ultracentrifuge. Low speed centrifugation was performed in a Sorvall RC-2B centrifuge.

All centrifuge forces are quoted as g max values.

2.4 SPECTROPHOTOMETRY

The spectrophotometric determinations were made on a Cecil 292 or 392 recording spectrophotometers.

2.5 PROTEIN DETERMINATIONS.

Protein concentrations were determined by the Biuret procedure (Gornall et al; 1949). The protein was precipitated by the addition of four volumes of cold 10% tricholoroacetic acid and placed on ice for at least one hour. The precipitated protein was collected by centrifugation, the supernatant removed to waste and the pellet solubilised in 0.1 M NaOH. Three milliliters of Biuret reagent was added and the absorbance was determined at 540 nm after allowing 20 minutes reaction time .

Bovine serum albumin solution was used as the standard. Solutions of bovine serum albumin were made up in 0.01M phosphate buffer, pH 7.2 and standardized assuming an E(1%, 1 cm) at 279 nm of 6.67 (Foster and Sherman, 1956).

Microgram quantities of protein were determined by the protein dye binding method using Coomassie Blue G 250 (Bradford,1976). Bovine serum albumin was used as a standard for this method.

Immunoglobulin fractions were quantitated by assuming an E(1%,1 cm) at 280 nm of 13.4 for rabbit immunoglobulin (197).

2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was carried out in 13 x 15 x 0.15cm linear slab gels. Electrophoresis was carried out in a vertical electrophoresis unit (similiar to LKB 2001,Bromma Sweden).

2.6.1 Discontinuous non denaturing gels

These were prepared essentially as described by Davis (1964), Reisfield (1962), and Hedrick & Smith (1968).

2.6.2 Resolving gel

Sufficient acrylamide and N,N'-methylene-bis-acrylamide to give a 7 percent gel concentration and an acrylamide to bis acrylamide ratio of 30:1 were dissolved in small pore buffer [1.5 M Tris, pH to 8.8 with HCl, 12.5 ml per 100ml]; N,N,N,N'tetramethylethylenediamine [TEMED],50 ul per 100 ml. Water was added to a final volume of 100 ml give. The solution was degassed for 20 min prior to polymerization.

2.6.3 Stacking gel

Sufficient acrylamide and bis-acrylamide to give a gel concentration of 10 percent and an acrylamide to bis-acrylamide ratio of 10:1 were dissolved in a large pore buffer [0.495 M Tris, pH to 6.7 with HCl], 12.5 ml per 100 ml; TEMED 50 ul per 100 ml. Water was added to give the required volume and then the solution degassed for 20 minutes prior to polymerization.

2.6.4 Polymerization

A 7.5% solution of ammonium presulphate was freshly prepared. Ammonium persulphate (300 ul per 50 ml of acrylamide solution) was added to initiate polymerization. The solutions were mixed by swirling for 10-15 seconds. Formation of polymerized gel occurs within 20-25 minutes.

2.6.5 Electrode reservoir buffer

Electrode reservoir buffer (38.5 mM glycine, Tris to pH 8.3) was used in both the upper and lower reservoirs.

2.6.6 Sample preparation

Protein samples (5-150 ug in up to 100 ul) were applied to each gel track in a solution containing 20% glycerol, bromophenol blue (5 ul of 0.001% per 100 ul) and the same ionic components as the stacking gel. Samples were applied by layering between the upper reservoir

buffer and the stacking gel with a glass microsyringe.

2.6.7 Sodium dodecyl sulphate continuous denaturing gels

Discontinuous polyacrylamide gels containing SDS were prepared essentially as described by Laemlli (1970), Cleveland (1976), and Anderson B. L. (1983). The solutions used for the denaturing gels were the same as those for the non denaturing gels except that 0.1% SDS was added to the electrode buffer, resolving and stacking gels.

2.6.8 Sample preparation

Protein samples (5-150 ug in up to 100 ul) were applied to each gel track in a solution containing 6 M urea, 1% SDS, 0.001% bromophenol blue, 2 - mercaptoethanol (1 ul per 20 ul of sample). Before application to the gel, the samples were boiled for 15 minutes to denature the proteins.

Samples were allowed to cool and then were applied by layering between the upper reservoir buffer and the stacking gel with a glass microsyringe.

2.6.9 Electrophoresis

Electrophoresis was performed at room temperature. Slab gels were electrophoresised at 50 volts (5-10 mA) until the bromophenol blue had entered the resolving gel after which the voltage was increased to 150 volts (30 mA) for 4-5 hours.

2.6.10 Gel staining

Gels were stained for 1 hour at 60 C or overnight at room temperature in freshly prepared 0.125% Coomassie Brilliant blue R 250 in methanol water, glacial acetic acid (10:10:0.2 v/v/v).

Gels were destained for several hours at 60 degrees or overnight at

room temperature. Destaining was aided by frequent changes of destaining solution (acetic acid, methanol, water, and glycerol 1:9:10:0.2 v/v/v/v).

2.6.11 Gel scanning

Destained gels were scanned at 580 nm on a double beam gel scanner (ISCO Model 1310).

2.7 ESTIMATION OF PROTEIN SUBUNIT MOLECULAR WEIGHTS.

The molecular weights of protein subunits were estimated from SDS gels calibrated with proteins of known subunit molecular weight (6,32,45, 47,75,202).

Mixtures of standard proteins (each 10 - 60 ug) were run on each gel. The migration of each standard protein relative to the bromophenol blue dye front was plotted against the log molecular weight. The subunit molecular weight of the unknown proteins were estimated by interpolation from the standard regression line.

The standards used were :
bovine serum albumin (66,000), transferrin (72,000), phosphorylase b (97,400), B-galactosidase (116,000), fatty acid synthatase (250,000).

In some gels cross linked bovine serum albumin was used (bands in multiples of 66,000) as the standard.

2.8 DETERMINATION OF RADIOACTIVITY

Beta emittors were determined by liquid scintillation spectrophotometry in a Beckman Model LS-850 scintillation spectrophometer.

Aqueous samples were counted in a scintillation fluid of Triton X-100, toluene (1:2,v/v), containing 0.4% 2, 5 - diphenyloxazole (PPO) and 0.01% 1,4-di-(2-(5-phenyloxazoyl)) benzene (POPOP).

Counting efficiency was determined by external standardization.

2.9 DETERMINATION OF THE RADIOACTIVITY WITHIN GEL SLICES

Each track was sliced into 2 mm horizontal sections with a multiple razor blade cutter. Slab gels were sliced into 10 vertical strips with a razor blade.

Each slice was then transferred to a scintillation vial and 1 ml of a mixture of concentrated ammonia, and fresh 30% hydrogen peroxide (1:99, v/v) was added.

Vials were loosely capped and incubated at 60 C overnight, after which time the gels had completely dissolved.

Radioactivity was determined by liquid scintillation spectrophotometry after addition of 5 ml of Triton - toluene scintillation fluid. Sufficient water was added to give a stable monophasic emulsion.

2.10 DETERMINATION OF RADIOLABELLED TRICHLOROACETIC ACID PRECIPITABLE PROTEIN

Radiolabelled TCA insoluble protein was precipitated from solution by the addition of 5 volumes of 10% TCA containing 10 mM methionine and allowing the mixture to stand at 0 C overnight.

TCA precipitated protein was recovered by centrifugation for 15 minutes in a bench microfuge.

The supernatant was removed by pasteur pipette, and the pellet resuspended in homogenisation buffer. The protein pellet was then reprecipitated and washed twice with cold TCA/methionine. The sample was solubilised with 3 M NaOH, and transferred to a scintillation vial.

Radioactivity present in these samples was determined after the addition of 5 ml triton-toluene liquid scintillation fluid (section 2.8) and sufficient water to give a single phase emulsion.

Protein concentration was routinely determined as previously indicated (section 2.5).

2.11 CALIBRATION AND MAINTENANCE OF CHROMATOGRAPHY COLUMNS

2.11.1 Ion exchange chromatography columns

Precycling of anion exchange chromatography columns (Whatman DEAE 52 and DEAE 32) was performed as described by the Whatman Information Handbook.

The anion exchange resin was stirred into excess 0.1 M HCl (20 ml/ 5 ml preswollen gel) and allowed to swell for 30 minutes. The supernatant was filtered off, washed with 1 liter of distilled water, and then 0.1M NaOH (20 ml/ 5 ml of preswollen DEAE). After 30 minutes, the supernatant was filtered off, and the alkali treatment repeated. The DEAE was then washed well with water, and degassed for 30 minutes. The column was then packed and equilibrated with buffer.

2.11.2 Calibration of the gel filtration column

The molecular weight of protein peaks, obtained after elution from Sephacryl-S200, were determined by the method of Andrews et al (1965), and Whitaker (1963).

The void volume of these columns was determined in a separate experiment by the elution of Blue Dextran (Mr 2,000,000) (146,155,156).

The column was calibrated using standard proteins of known molecular weight, Blue Dextran, and 300 mM sodium and potassium chloride.

Included volume was determined after addition of 12 ml of Sephacryl S-200 buffer containing 300 mM potassium and sodium chloride. Flame spectroscopy was used to determine the fraction with the largest level of sodium and potassium ions.

2.11.3 Regeneration of Sephacryl S-200 column

Sephacryl-S200 was precycled after 5 uses. This was accomplished by washing the Sephacryl S200 with 5 volumes of 0.2 M NaOH on a sintered glass filter. The gel media was resuspended in 300 ml of water and neutralized with 0.1 M HCL. After degassing for 30 min the column was repacked and equilibrated with Sephacryl S-200 buffer.

2.11.4 Regeneration of Blue Sepharose CL-6B affinity chromatography column.

This column was precycled as described by Redshaw and Loten (1979) and Pharmacia Information Handbook. The Blue sepharose can be reused after 4-5 washing cycles of 10 volumes of 0.1 M Tris-HCl, 0.5 M sodium chloride 6 M urea and 1% Triton X-100 (pH 8.5) then 10 volumes of 0.1 M sodium acetate, 0.5 M chloride, 6 M urea, 1% Triton X-100 (pH 4.5) followed by equilibration with buffer.

This can be conveniently performed in a funnel with a fine sinter.

CHAPTER 3

PURIFICATION OF ATP-CITRATE LYASE FROM RAT LIVER

3.0 INTRODUCTION

Some indication of the difficulties which have been encountered in the purification of ATP-citrate lyase may be gained from the number of different purification methods cited in the literature. Virtually every group which has worked on this enzyme has devised their own purification method, either by considerable modification of existing methods or by developing a totally new series of steps. These difficulties have also been experienced in the course of this work and consequently an extremely high proportion of the experimental work reported in this thesis has involved repeated investigations into methods for obtaining pure enzyme in high yield.

Several procedures for the purification of ATP citratelyase have been published (2,89,113,139,141,142,143,146,147,150,151,168,185,211). The specific activity of ATPCL reported in this literature has a range of values from 0.8 units/mg (Plowman 1967) to 13.59 units/mg (Szutowicz 1981) at 25 C. These discrepancies appear to arise from a number of sources.

Different techniques have been used to purify the enzyme and to determine enzyme activity. The criterion used for purity of the enzyme varies between groups and the degree of protein degradation occurring during purification can also contribute to the variation in published specific activity. The instability of this enzyme, which has previously been described by Linn and Srere (1979), may also be the cause of erroneous data.

The most common method for ascertaining them homogeneity of the purified enzyme is by visualization of the 110,000 molecular weight subunit on detergent polyacrylamide gels. The amount of protein applied to the gel has varied considerably and obviously affects the visualization of any contaminating protein.

Purification of this enzyme by published methods has proved to be very difficult. The diversity of the methods utilized in purification schemes for this enzyme gives an indication that no single scheme has been proved to give consistent results. In this work the enzyme was purified by a modification of the procedure of Linn and Srere (1979). However a large number of purification techniques were tried in attempts to improve both the yield and the purity of the final product

3.1 ELUTION OF ATPCL FROM REACTIVE BLUE SEPHAROSE CL-6B.

Affinity chromatography on Reactive 2-Blue Sepharose CL-6B, using the method of Redshaw and Loten (1981), was used as the final step of the purification protocol.

The binding of ATPCL to the Blue sepharose is due to both hydrophobic and electrostatic interactions with the immobilized triazine dye, Cibacron F3-GA (23,76). These interactions occur between the multiple aromatic and polar sulphonate groups on the dye with amino acid residues in the protein that may be involved in the active site cleft (48). However immobilized Cibacron F3-GA dye is not a uniquely specific analogue of nucleotide coenzyme A and there is some degree of latitude in the binding of other enzymes to this dye (116).

The binding of enzymes to Blue Sepharose was concluded by Thompson et al (195,196) and Bohme (1972) (23) to be dependent upon the presence of the dinucleotide fold. ATP citrate lyase does not possess binding sites for NAD or NADPH. Dehydrogenases that are present which bind NAD or NADPH will also have a high affinity for Blue Sepharose.

Unlike most enzymes that hydrolyses ATP, ATP citrate lyase also has a binding site for CoA. The interaction of ATPCL with Blue Sepharose is strong and ATPCL has an affinity for this material even in 0.2 M phosphate buffer. AcetylCoA, ADP, and Pi elute ATPCL from Blue Sepharose more effectively than when the enzyme's substrates are used (211). However Coenzyme A has a greater eluting capacity than ATP. This is in agreement with the higher affinity of ATPCL for CoA than for ATP (77,89, 134,164). The cost of Coenzyme A, compared to ATP, severely restricts the use of this as an eluent.

ATPCL can be eluted from Blue Sepharose with either CoA, ATP, ADP, 3'- AMP or NADP but not with NAD, 5'-AMP or phosphate (76). Elution occurs because the negative charge in the 2' or 3' position of the adenosine moiety (CoA, NADP, 3'-AMP) is responsible for breaking the electrostatic and steric interactions between the immobilized triazine dye and ATPCL. A chain of 2 or 3 phosphate groups in the 5' position of adenosine (ADP, ATP) are sterically similar to CoA, NADP, and 3'-AMP and will elute ATPCL. A single 5' phosphate (5'-AMP) or a phosphate chain coupled to another ligand (NAD) is sterically dissimilar to the 2' or 3' phosphate groups and is insufficient to elute ATPCL from the dye matrix (76).

For this reason, ATPCL was routinely eluted from Blue Sepharose Cl-6B using 5 mM ATP.

3.2 MATERIALS

Adenosine 5'triphosphate, dithiothreitol, benzamidine hydrogen chloride, malate dehydrogenase, Blue-2 Sepharose CL-6B, bovine serum albumin, Coenzyme A, Sodium dodecyl sulphate, and molecular weight standards were obtained from the Sigma Chemical company, St Louis, Missouri.

Sephacryl-S200 was obtained from Pharmacia AB Fine Chemicals, Uppsala, Sweden.

DEAE 32 and 52 were obtained from Whatman, England. Polyethylene glycol 6000 was obtained from Merck-Schuchardt, Munich, Germany.

3.3 METHODS

3.3.1 Assay for ATP-Citrate lyase

The ATP citrate lyase activity was determined using an assay in which the oxaloacetate produced by the reaction was converted to malate by malate dehydrogenase. The coupled reaction was followed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH [41,172,194].

ATP citrate lyase

citrate + CoASH + ATP ----------> acetylCoA + oxaloacetate + ADP + Pi.

malate dehydrogenase

oxaloacetate + NADH ----------> malate + NAD

The assay conditions were essentially as described by Linn and Srere (1979) and Szutowicz (1983) except that 10 mM dithiothreitol was used as well as 2-mercapto ethanol in the assay.

Highly purified or highly reactive preparations of ATP citrate lyase were diluted to the appropriate concentration with 200 mM KCL containing 5 mM dithiothreitol prior to assay.

Assays were preincubated for 5 minutes at 25 C, and the reaction initiated by the addition of ATP. When assaying fractions from the Reactive Blue-Sepharose CL-6B column CoA was used to initiate the reaction.

The oxidation of NADH was monitored continuously at 340nm in a Cecil 292 or 392 recording spectrophotometer. For all of the enzymes assayed, a unit of activity is defined as the amount of enzyme required to transform 1 umol of substrate per min at 25 C.

The conversion ratios for determining the ATP citrate lyase activity at different temperatures (113,189) are

20/25	C	0.43
25/25	C	1.00
30/25	C	1.86

The assay mixture contained -

	Stock Soln	Volume added	Final Conc
	mM	ul	mM
Tris/HCl pH=8.7	200	500	100
Sodium Citrate.	500	40	20
* DTT	0.5	*20	10
* CoA	5	*20	0.1
MgCl2	500	20	10
NADH	70	20	0.14
ATP	0.25	20	5
KCl	4.0 M	20	80
2-mercaptoethanol.	500	20	10
Malic Dehydrogenase	100 Units/ml	20	2

	Volume of ASSAY	= 0.68 ml	
	Volume of Sample + water	= 0.32 ml	

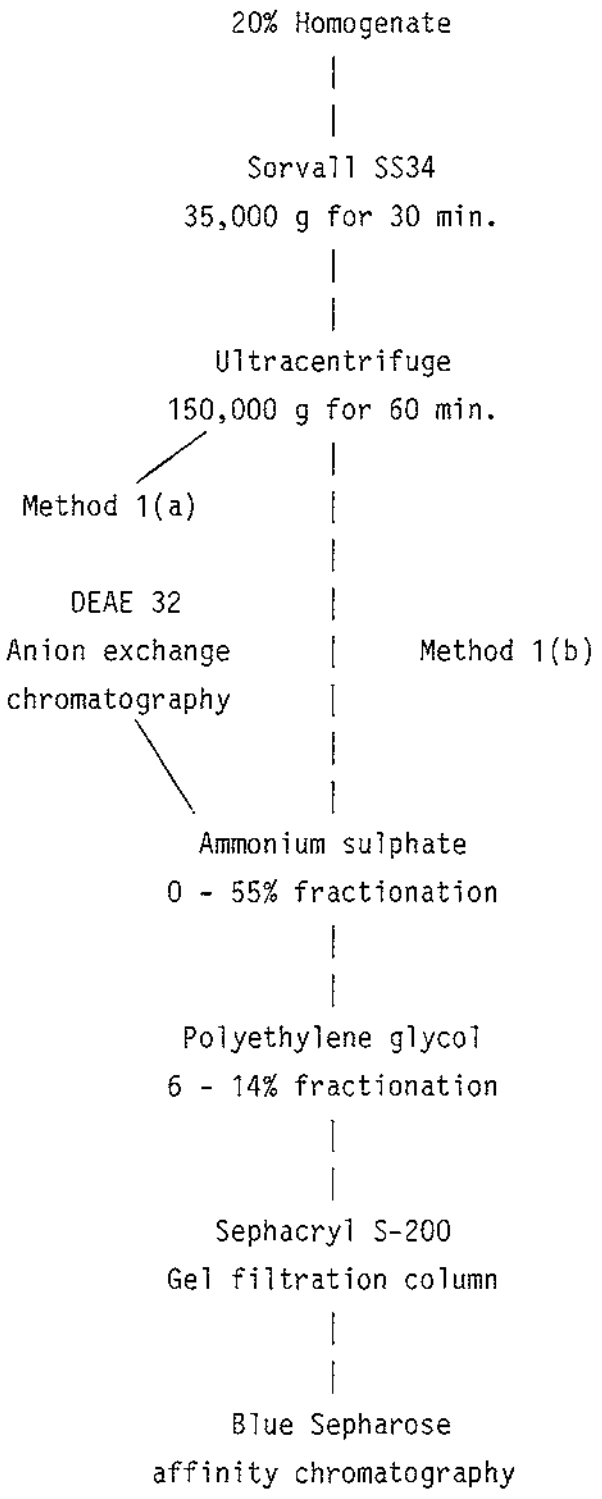
	Total Volume	= 1.00 ml	

3.3.2 Purification methods of ATP-Citrate lyase.

METHOD 1.

This was a modification of the methods of Redshaw and Loten [1981] and Linn and Srere [1979].

Flow diagram of Methods 1(a) and 1(b)



Male Sprague-Dawley rats of approximately 250g size were caged singly, maintained on a twelve hour light/dark rhythm, fasted for two nights, and then refed a fat free, high carbohydrate diet [66% sucrose, 33% casein hydrolysate, 0.15 vitamin/mineral mix, 0.85% water] for three nights (113,164,193).

This procedure has been shown to increase the activity of ATP citrate lyase in rat liver (Suzuki et al;1967 (183)).

Drinking water was available ad libitum throughout this time. During the feeding periods the diet was also provided ad libitum.

The rats were killed at the start of the light phase of day 6 by cervical dislocation and the livers excised.

The individual livers were chilled on ice until all rats were sacrificed. Subsequent operations were carried out at 0 C.

The liver tissue was passed through a chilled stainless steel tissue press into a tared beaker. The weight of the pulped livers was determined and five volumes of homogenisation buffer (see BUFFER TABLE appendix 1) were added to give a 20% homogenate. The volume was recorded and a one ml aliquot removed for determination of enzyme activity and protein concentration. This aliquot was labelled as 20% homogenate.

The 20% homogenate was then subjected to a further homogenisation by 4 passes in a Citenco motor driven teflon/glass Potter/Elvehjem homogeniser. (0.23 mm clearance, rotating at 6000 rpm, total usable volume of 50 ml).

This fraction was then loaded into 45 ml polycarbonate centrifuge tubes and centrifuged at 34,800g for 15 mins in a Sorvall RC-5B centrifuge, SS-34 rotor 17,000 rpm).

The supernatant was decanted through a layer of Miracloth to trap the floating coagulated fat particles. The volume was recorded and a 1ml sample taken for protein determination and enzyme activity. This sample was labelled as SS-34 supernatant.

The supernatant was then centrifuged using Sorvall 30ml polycarbo-

nate ultracentrifuge tubes in a 50.2Ti or 60Ti rotor in a Beckman L2-65B ultracentrifuge for 60 minutes at 35,000k (50Ti = 158,000g 50.2Ti =212,000g). The supernatant was removed with a pasteur pipette and filtered through 'Miracloth' or glass wool. The volume was recorded and a 0.5ml sample taken for protein analysis and determination of enzyme activity. This sample corresponds to ultracentrifuge supernatant (50.2Ti) or "cytosol".

An equal volume of supernatant dilution buffer was added to the supernatant before anion exchange chromatography.

DEAE - anion exchange chromatography

The diluted cytosol fraction was applied to a DEAE-32 column (DEAE cellulose, 35mm radius x 150 mm). This column was equilibrated with 500 ml of DEAE (ammonium chloride) buffer (see appendix 1), and then 150 ml DEAE benzamidine buffer (see appendix 1).

The column was eluted with 150 ml DEAE benzamidine buffer, and then 500ml of DEAE (ammonium chloride) buffer. ATP citrate lyase activity coeluted with the red coloured material from the column.

The volume of this fraction was determined and a 1.0 ml aliquot was taken for enzyme activity and protein analysis. This fraction is referred to as DEAE-32.

Ammonium sulphate fractionation.

Solid ammonium sulphate was added to the DEAE -32 to give 55% saturation (351.1 g/l). The solid was added over a time period of 30 minutes with gentle stirring. The mixture was then stirred for an additional 60 mins, and the precipitate was recovered by centrifugation at 9750 g for 25 minutes (Sorvall RC-5B centrifuge, SS-43 rotor, 9000 rpm). The 55% ammonium sulphate supernatant was retained and the enzyme activity and protein concentration were determined before discarding this fraction.

The 55% ammonium sulphate precipitate was resuspended in homogeni-

sation buffer(containing 1 mM dithiothreitol) to a volume of half that of the cytosol fraction and a 0.6 ml sample taken and used for enzyme analysis and protein determination.

This fraction was labelled as 0- 55% $(NH_4)_2\ SO_4$.

Polyethylene glycol (PEG) 6000 fractionation.

It is essential to maintain the solution at 0 C while adding PEG. This avoids possible denaturation during this step. Finely powered PEG was added to the DEAE 32 fraction to give a final concentration of 6% (6g/100ml).

During the addition of PEG the solution was placed in a -20 degree methanol bath and maintained at 0 C. Care was taken not to freeze the sample. The PEG was added slowly over a period of 15 minutes, and stirred for an additional 15 minutes at 0 C. The solution was then left for a further 15 minutes. Unwanted protein was collected by centrifugation at 34,800g for 15 mins (Sorvall RC5-B centrifuge, SS-34 rotor, 17000 rpm). The supernatant was decanted, and the volume recorded. A 0.2 ml sample was taken and labelled as PEG >6%.

The PEG >6% supernatant was then subjected to an additional PEG precipitation. PEG powder was added to give a final concentration of 14% (8g/100ml) over a 15 minute period while the solution remained in the -20 C methanol bath. Care was taken to keep the temperature at 0 C. The solution was stirred for an additional 15 minutes at 0 C. The precipitate was obtained by centrifugation as above. The supernatant was poured off and retained for protein and enzyme activity analysis before discarding. This fraction is referred to as PEG >14%.

The protein precipitated between 6 and 14% PEG concentrations was resuspended in a volume of 10 ml of Sephacryl buffer. The final volume determined and a 0.2 ml sample taken and used for protein and activity analysis. This fraction is referred to as PEG 6-14%.

Gel filtration using Sephacryl S-200

The PEG 6-14% fraction was then applied to a column of Sephacryl - S200 column (800 mm x 32 mm) which had been equilibrated with 1 litre of Sephacryl buffer (see appendix 1). The column was eluted with 500 ml Sephacryl buffer at rate of 20 ml/hrs. Thirteen ml fractions were collected. ATP citrate lyase activity eluted from the column slightly before a yellow brown material.

The protein eluted from Sephacryl-S200 column was determined by measurement of the absorbance at 280 nm using Sephacryl buffer as a blank at 280nm, or by the Biuret method of Gornall (60) after precipitation of the protein with cold 10% trichloroacetic acid (as in section 2.11). The protein concentration and the ATP citrate lyase activity of each fraction was determined and those fractions with a specific activity of higher than 0.90 units/mg of protein were combined and used for the next stage of the purification.

Affinity chromatography using Blue - 2 Sepharose Cl-6B

The fractions with the highest ATP citrate lyase specific activity from the Sephacryl - S200 column were purified further by chromatography on a Reactive Blue-2 Sepharose CL-6B column (1.5 x 9 cm).

The procedures followed were that of Redshaw and Loten (1981). The fraction obtained from the Sephacryl column was applied to a Reactive Blue 2 Sepharose CL-6B column equilibrated with 500 ml Blue Sepharose buffer (see appendix 1).

Under these conditions most of the ATPCL was retained by the column. After sample loading the column was washed with 50 ml of Blue-Sepharose buffer, at a flow rate of 25 ml/hr. Fractions of six ml were collected. The column was then eluted with 50 ml of Blue Sepharose buffer which contained 5mM NAD, followed by 20 ml of Blue Sepharose buffer which contained 1mM DTT.

At this stage the absorbance of the eluent at 280 nm was less than 0.05, indicating that all the unbound protein had been removed.

The ATP citrate lyase activity was then eluted with 50 ml of Blue sepharose buffer containing 5mM ATP, 1mM DTT (pH 7.5).

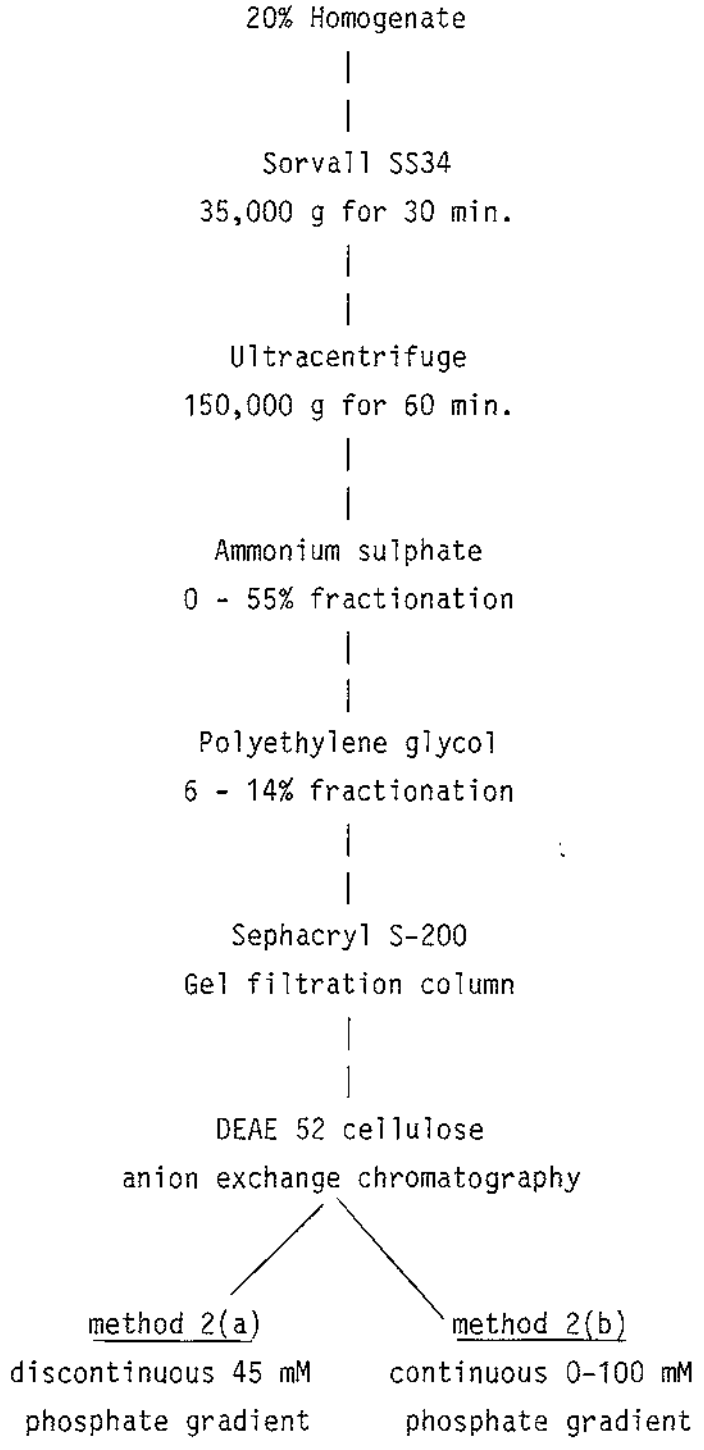
The enzyme requires thiol protection in the form of dithiothreitol, both in the elution buffer and in the buffer into which the chromatographic fractions are collected (Cottam & Srere, 1969). Therefore 6.0 ml fractions were collected into test tubes containing 2 ml of 10 mM DTT. All fractions eluted from the Blue Sepharose column were assayed for enzyme activity and protein determination on the same day.

The fractions containing ATPCL with high specific activity were dialyzed overnight against Blue Sepharose buffer that contained 50 mM benzamidine, 10 mM DTT, and stored at -20 degrees in 2 ml aliquots. A sample of the eluted sample was reduced to approximately 0.1 ml by the use of Aquacide 1-A, and an aliquot used for 7.5% S.D.S polyacrylamide gel electrophoresis (see section 2.6). If 20 ug of protein showed a single band with an apparent Mw of 123,000 then the remaining samples were used for the preparation of antibodies to ATP citrate lyase (chapter 6).

3.3.3. Preparative method 2.

This was a modification of the methods of Linn and Srere [1979], Guy & Cohen (1981), and Ramakrishna and Benjamin (1983).

Flow diagram of Methods 2(a) and 2(b)



Affinity chromatography using Reactive Blue-2 sepharose CL 6B as described in method 1 resulted in a product with variable purity and stability. In order to overcome this problem the final stage of purification was replaced by elution from a DEAE 52 anion exchange chromatography column.

ATP citrate lyase was purified as in method 1(b) up to elution from the Sephacyl S-200 gel filtration column. Fractions of high specific activity from this step (0.9-1.5 units/mg of protein) were diluted with 5 volumes of 20 mM sodium phosphate buffer which contained 1.0 mM magnesium acetate, 0.1 mM disodium EDTA, 1.0 mM benzamidine, 1.0 mM dithiothreitol, 10% glycerol (v/v) at a final pH of 7.50. The resulting solution was then dialyzed against the same buffer overnight.

The dialyzed solution was centrifuged 34,800g for 30 minutes at 4 C (Sorvall SS-34 rotor, 17,000 rpm), and the supernatant applied to a column of DEAE 52 anion exchange column (55 mm by 12.5 mm) that had previously been precycled and then pre-equilibrated with 20 mM sodium phosphate buffer. The conductivity of a 1 ml sample of the last washing after equilibration was checked to ensure that it was the same as that of the equilibration buffer. The best results were obtained when less than 1 unit of enzyme activity was loaded per 10 ml of swollen exchanger. The column was washed with 20 mM sodium phosphate buffer until the absorbance at 280 nm of the effluent equalled that of the 20 mM phosphate buffer.

ATP citrate lyase was eluted from the column using either 45 mM sodium phosphate buffer (discontinuous phosphate gradient Method 2(a) or a 20-100 mM phosphate gradient (Method 2(b)). The gradient volume was 6 times that of the column.

The elution buffer contained 1.0 mM magnesium acetate, 0.1 mM disodium EDTA, 1.0 mM Benzamidine, 1.0 mM DTT, 10% glycerol (v/v) at final pH of 7.50.

The following protease inhibitors were added to this buffer at the concentrations indicated leupeptin 4ug/ml, antipain 4 ug/ml, L-1-tosyl amido-2-phenyl-ethylchloromethyl ketone 0.1 mM, and N -p-tosyl-L-lysine chloromethyl ketone 0.1mM.

3.3.4 Preparative method 3.

This method involved the addition of a Reactive Blue Sepharose column step after the final DEAE 52 anion exchange step in method 2(a).

Flow diagram of Methods 3

20% Homogenate
|
Sorvall SS34
35,000 g for 30 min.
|
Ultracentrifuge
150,000 g for 60 min.
|
Ammonium sulphate
0 - 55% fractionation
|
Polyethylene glycol
6 - 14% fractionation
|
Sephacryl S-200
Gel filtration column
|
DEAE 52 cellulose
anion exchange chromatography
|
method 2(a)
discontinuous 45 mM
phosphate gradient
|
|
Blue Sepharose
affinity chromatography

ATP citrate lyase fractions which eluted from DEAE 52 which had a specific activity greater than 9.0 were pooled and dialyzed overnight against Blue Sepharose buffer overnight.

ATP citrate lyase was applied to a column of Reactive Blue-2 Sepharose CL-6B as described in method 1. The washing procedures for the column were the same as in method 1.

ATP citrate lyase was eluted from Blue Sepharose with a buffer containing 5 mM ATP, 1 mM DTT, 5 mM NAD. The eluted enzyme normally has a specific activity of 14-16 units per mg of protein.

RESULTS AND DISCUSSION

3.4 DISCUSSION OF THE ASSAY

The assay overestimates the level of ATP citrate lyase. The correction of ATPCL activity is more significant in chow and starved rats because of the lower levels of ATP citrate lyase in these animals.

When fractions prior to the ammonium sulphate step were assayed without CoA being added, significant NADH oxidation occurred. This rate needed to be subtracted from the rate obtained after CoA is added. This eliminates an overestimate of the total amount of ATPCL being present.

When no ATP is present in the assay then background NADH oxidation is negligible. The activity of the NADH oxidases increased with increasing ATP concentration. The concentration range of ATP from 5 to 200 mM was tested. These ATP dependent NADH oxidases were separated from the ATPCL preparation during the ammonium sulphate fractionation step.

3.5 RESULTS AND DISCUSSION OF THE PURIFICATION PROCEDURES.

3.5.1 Addition of Magnesium ions to the homogenisation buffer.

It has been reported that the addition of 10 mM magnesium chloride disrupts the high molecular weight complex liberating tetrameric ATP citrate lyase (40).

When 10 mM magnesium chloride was added to the homogenisation buffer no significant elevation of ATP citrate lyase activity in the ultracentrifuged supernatant could be detected. At this concentration it has also been reported as stabilizing ATPCL binding to the mitochondrial membrane (40).

3.5.2 DEAE 32 cellulose chromatography.

Recovery of enzymatic activity from the 50.Ti supernatant using various techniques can be seen in Table 1.

In early purification attempts only 67% (n=4 expts) of total enzyme activity applied to the DEAE-cellulose column was recovered in the eluent.

If an ammonium sulphate precipitation step (25-50 % saturation) was used in place of the DEAE-cellulose step 81% could be recovered (N=8 expt). Subsequent work revealed that the addition of 0.1 M sodium fluoride to the buffers used in the DEAE-cellulose step increased the recovery to 94%.

TABLE 1

Alternative strategies for recovery of ATPCL activity from the 50.Ti supernatant

Fraction	a Total Protein mg	b Total Activity units	Specific Activity units/mg	c Purification factor	Recovery 1%
Starting material 50.Ti supernatant	2280	221	0.097	4.22	100
d Initial system DEAE 32 50 mm NaF in elution buffer	907	146	0.161	7.00	67
e Method 1(a) DEAE 32 100 mM NaF in elution buffer	1851	209	0.113	4.91	94
f Method 1(b) Ammonium sulphate 0-55% saturation	1227	173	0.141	6.13	81

a Protein determined with Biuret reagent.

b Activity determined by malate dehydrogenase coupled assay. A unit is defined as the umol of acetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm at 25 C.

c Purification factor is relative to 20% homogenate.

d For every gram of protein applied to the column there was at least 150 ml of DEAE cellulose w/v.

e Preparative method 1(a) involves elution with 100 mM sodium fluoride in the elution buffer.

f In preparative method 1(b) the DEAE 32 column is replaced by an ammonium sulphate fractionation.

Fluoride (0.1 M) has the added advantage of being a phosphatase inhibitor. ATP citrate lyase is a phosphoenzyme and structural site dephosphorylation occurs at low fluoride concentrations within 15 minutes (151).

Structural site dephosphorylation may be responsible for retention of ATPCL to the DEAE-cellulose column under these eluting conditions. It is advantageous to maintain the fluoride concentration at a final concentration of 0.1 M throughout the early stages of the preparation as the enzyme is stable in this buffer and it affects good recovery on DEAE-cellulose or with ammonium sulphate fractionation. Therefore the enzyme was diluted with a stock solution of homogenisation buffer containing 0.2 M fluoride before DEAE 32 fractionation or the ammonium sulphate fractionation.

Purification method 1(a) involves chromatography of the 50.Ti supernatant on DEAE-cellulose. In method 1(b) the DEAE cellulose step is replaced with ammonium sulphate fractionation. This substitution caused no significant change in the specific activity but reduced the time of the preparation.

3.5.3 Ammonium sulphate and polyethylene glycol fractionation.

Losses of activity from both the ammonium sulphate and PEG steps can be minimized by maintaining the solution as cold as possible without freezing and by adding the ground solid PEG over an extended period of time (at least 30 minutes) and then resuspending the precipitate in buffers containing 1mM DL-dithiothreitol.

Several different ammonium sulphate concentrations were tested for effectiveness in precipitating enzyme activity. The best results were obtained with a 30-50% saturation fraction since 30-40% saturation resulted in large amounts of enzyme activity remaining in the supernatant. In the work of Houston (1984) which used a different buffer system, a 30-40% ammonium sulphate fractionation gave equivalent results to the use of a 30-50% saturation described above.

Large losses of enzyme activity can occur when using solid PEG 6000.

The results of 30 experiments show that it is advisable to collect a 6-14% precipitate, resuspend it and then reprecipitate with a 0-10% fractionation. The resulting pellet was resuspended and then centrifuged at 35,000g (Sorvall SS34 rotor 17,000 rpm for 15 minutes at 4C). The clear supernatant contained the enzyme activity which could then be loaded on to the DEAE 52 or the reactive Blue-2 Sepharose CL-6B column.

3.5.4 Gel filtration

The use of the Sephacryl S-200 column was found to be unsatisfactory.

The exclusion limit of this gel filtration medium is 200,000 Mr, which is far too small for this protein (480,000). Sephacryl 300 or 400 would have been a better choice since the exclusion limits are one and ten million respectively.

When the gel filtration column was calibrated with standards of known molecular weight, ATPCL eluted with a molecular weight of 500,000 +/- 20,000. This is in agreement with previous workers (2,84,164).

3.6 ELUTION OF ATPCL FROM BLUE SEPHAROSE USING PREPARATIVE METHODS 1(a) and 1(b)

The final purification step first used was affinity chromatography on Blue Sepharose using the method of Redshaw and Loten (1981).

The results obtained using different methods for the elution of ATP citrate lyase from Blue Sepharose can be seen in Table 2. The best method of elution of ATPCL, based on specific activity, was using 5 mM ATP, 1 mM DTT.

In all subsequent experiments 5 mM ATP, 1 mM DTT was used to elute ATPCL from Blue Sepharose.

Increasing the ATP concentration in the elution buffer to 10 mM decreases the purity of the eluted enzyme. No individual fraction eluted with 10 mM ATP, 1 mM DTT had a specific activity greater than 7.5 units/mg.

TABLE 2

Alternative strategies for the
recovery of ATPCL from the Blue Sepharose

Fraction	a Total Protein mg	b Total Activity units	Specific Activity units/mg	c Purification factor	Recovery (%)
d Starting material Sephacryl S-200 fraction applied	14.2	14	0.983	43	100
Elution system (1) Blue sepharose 5 mM ATP, 1 mM DTT					
Best result	0.168	2.56	15.24	662	18
Typical result	0.180	1.35	7.50	330	9.6
Elution system (2) Blue sepharose 5 mM ATP, 1 mM DTT 5 mM NAD					
Best result	0.366	2.46	6.72	292	18
Typical result	0.158	1.03	6.52	283	7.4
Elution system (3) 10mM ATP, 1mM DTT					
Pooled fraction	1.82	12.34	6.93	300	88

a Protein determined by Gornval or Bradford method.

b Activity determined by malate dehydrogenase coupled assay. A unit of enzyme activity is defined as umol of AcetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm.

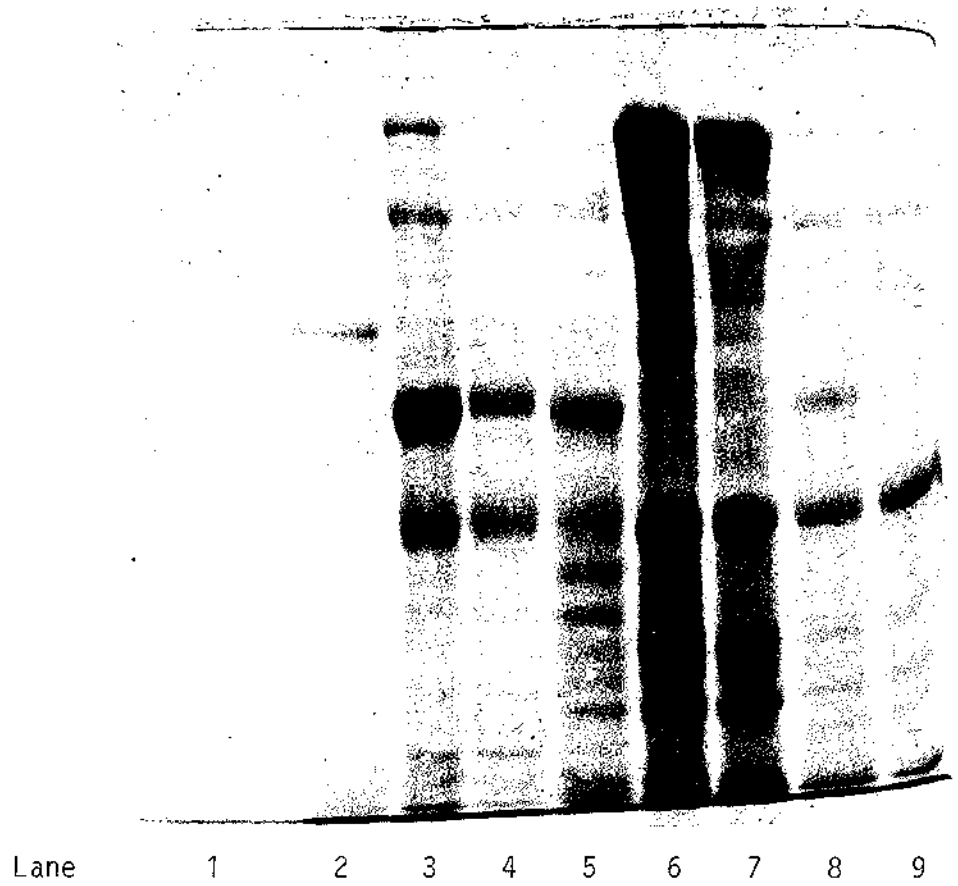
c Purification factor is compared relative to 20% homogenate.

d Sephacryl S200 fraction was obtained by purification method 1(b). Sample was dialyzed against Blue Sepharose buffer and then centrifuged before application to the Blue Sepharose column. 14 units of enzyme activity were applied. Total recovery of the applied units was greater than 95%.

A photograph of the SDS gel electrophoretic separation of proteins from the individual purification steps can be seen in Fig 1.

Figure 1

SDS Polyacrylamide gel of individual purification fractions obtained using Method 1(b).



Lane 1 ATP citrate lyase eluted from Blue Sepharose. Subunit represents a loading of 2 ug Coomassie staining protein. Specific activity of this fraction was 6.56 units/mg.

2 Supernatant of 14% PEG fractionation.

3 Sephacryl - S200 fraction.

4 Resuspended Polyethylene glycol 4-14% fractionation.

6 & 7 50Ti ulracentrifuge supernatant.

8 & 9 SS34 35,000 g supernatant.

A typical elution profile and purification table summarizing the purification methods 1(a) and 1(b) can be seen in Fig 2 and Table 3 and 4.

The yield and purity obtained by these single methods was variable. The relatively large losses of total enzyme activity observed on the Blue Sepharose column was due to pooling the fractions of high specific activity (Tables 2, 3, and 4). Later fractions which contained enzymes of lower specific activity were discarded. This resulted in the overall yield being lower than the published literature value of 21% (150).

ATP citrate lyase was eluted from reactive Blue -2 Sepharose CL-6B column in a major single symmetrical peak with 6.0 ml fractions. By decreasing the volume to 1.0 ml fractions 2 major peaks occurred (Fig 2). The pooled fractions from the major peak from the Blue Sepharose column had an average specific activity of 6.56 units/mg of protein (Method 1(a)), and 7.50 (Method (1(b)).

TABLE 3.

PURIFICATION OF RAT LIVER ATP CITRATE LYASE
METHOD 1(a).

Fraction	a Total Protein mg	b Total Activity units	Specific Activity units/mg	Purification factor	Yield (%)
20% homogenate	12950	298	0.023	1	100
SS34 supernatant	3408	237	0.070	3.04	80
50.Ti supernatant	2280	221	0.097	4.22	74
DEAE 32 50 mm NaF in elution buffer	907	146	0.161	7.00	49
Ammonium sulphate 0-55%	432	98	0.227	9.86	33
Polyethylene glycol 4-14%	252	69	0.274	11.91	23
c Sephacryl S-200 peak fraction	19	16	0.842	36.61	5.4
d Blue sepharose best result	0.53	4.72	8.91	387	1.6
typical	0.34	2.23	6.56	285	0.75

a Protein was determined with Biuret reagent after TCA precipitation, or Coomassie dye binding method of Bradford.

b Activity determined by malate dehydrogenase coupled assay. A unit is defined as the umol of acetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm at 25 C.

c Recovery from a Sephacryl S-200 column was 85% of the activity applied.

d Blue Sepharose data represent rechromatography of the peak Sephacryl S-200 fraction only. Elution of ATPCL using 5 mM ATP, 1 mM DTT. Best result represents 1 fraction of 5 ml from 1 experiment. Typical result represents mean of 4 expts.

TABLE 4.

PURIFICATION OF RAT LIVER ATP CITRATE LYASE
a METHOD 1(b).

Fraction	Total Protein mg/ml	b Total Activity units	c Specific Activity units/mg	Purification factor	Yield (%)
20% homogenate	1295	298	0.023	1	100
SS34 supernatant	3408	237	0.070	3.04	80
50.Ti supernatant	2280	221	0.097	4.22	74
Ammonium sulphate 0-55% saturation	1227	173	0.141	6.13	60
Polyethylene glycol 4-14%	427	152	0.356	15.48	51
d Sephacryl S-200 peak fraction	60	59	0.983	42.74	20
Blue sepharose					
e best result	0.168	2.56	15.24	662	0.86
f typical	0.180	1.35	7.50	330	0.45

a Preparative method 1(b) is method 1(a) without the DEAE 32 column.

b Protein was determined with Biuret reagent after TCA precipitation, or Coomassie dye binding method of Bradford.

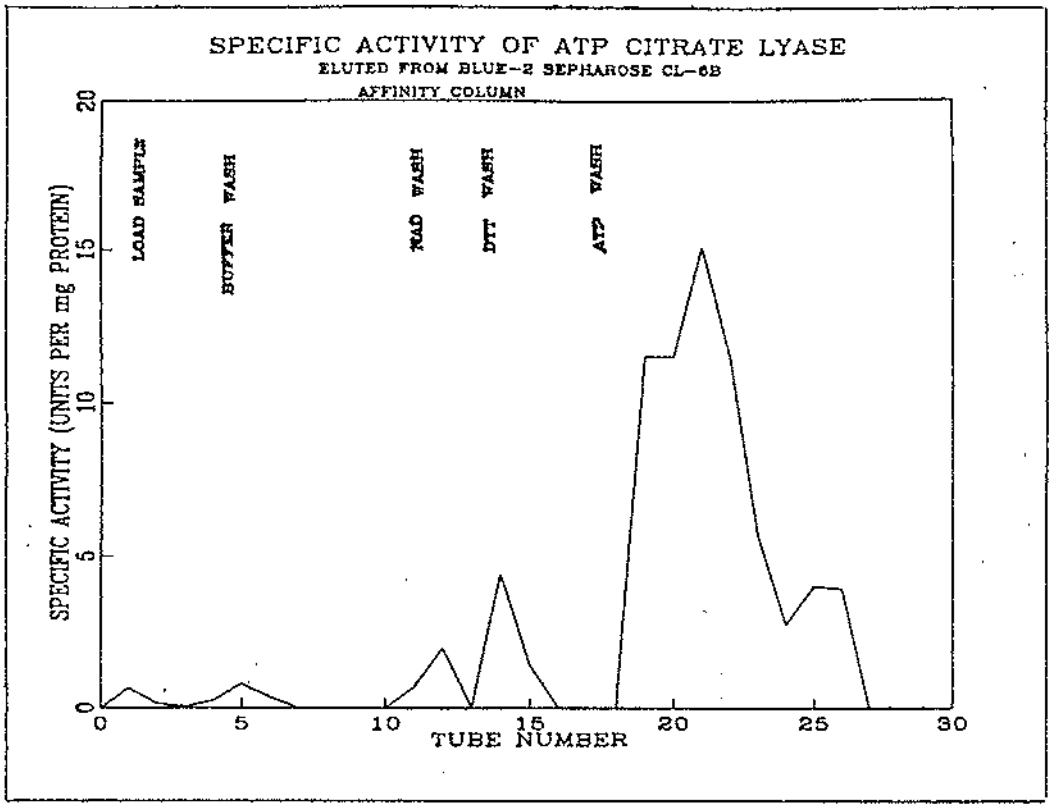
c Activity determined by malate dehydrogenase coupled assay. A unit is defined as umol of acetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm at 25 degrees.

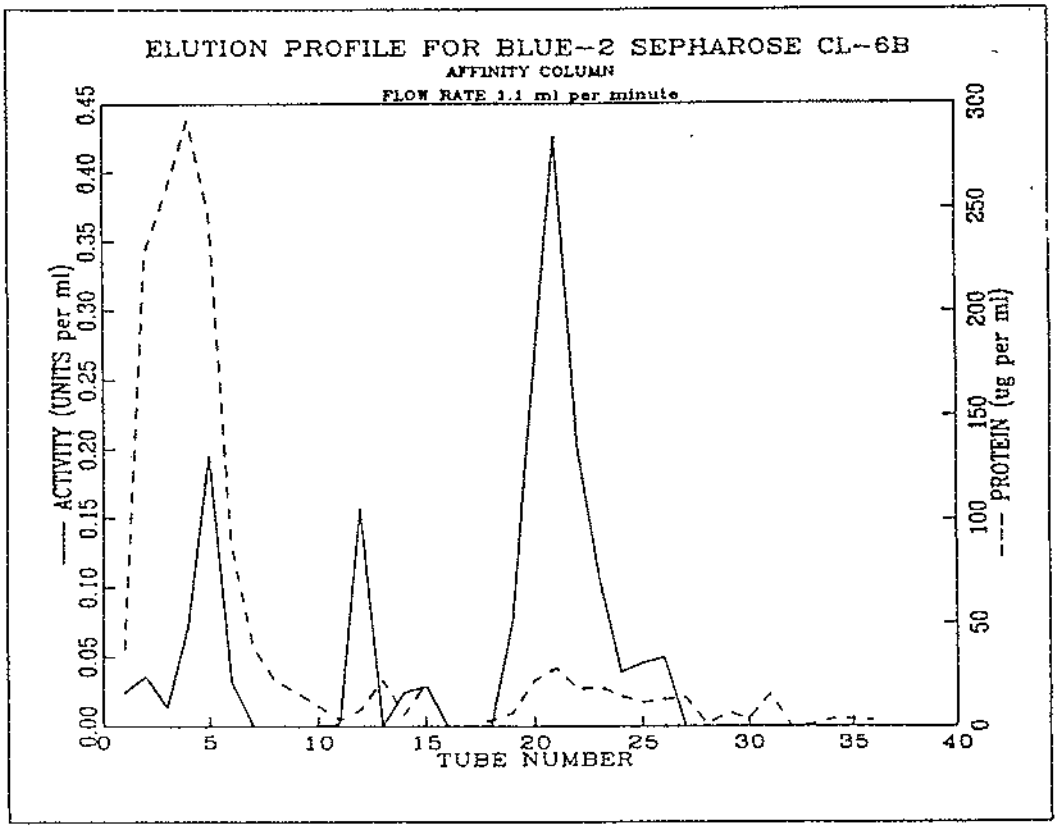
d Sephacryl S200 column recovered 95% of the total enzymatic activity from the PEG step.

e Blue Sepharose data represent rechromatography of only 14 units of enzyme activity using the peak Sephacryl S-200 fraction. ATP citrate lyase eluted from Blue Sepharose using 5 mM ATP, 1 mM DTT. 12.3 units were recovered overall.

f Best result represents 1 fraction in 1 experiment. Typical result represents mean of 12 experiments.

Figure 2. Elution pattern of purified ATP citrate lyase from from Blue Sepharose CL-6B column. Fourteen milligrams of protein were applied in a volume of 5.2ml. The flow rate was 66ml/hr and 1.0 ml fractions were collected. Enzyme activity was determined by the malate dehydrogenase coupled assay (as described in section 3.3.1).


SPECIFIC ACTIVITY OF ATP CITRATE LYASE
ELUTED FROM BLUE-2 SEPHAROSE CL-6B
AFFINITY COLUMN
LOAD SAMPLE
BUFFER WASH
NAD WASH
DTT WASH
ATP WASH
SPECIFIC ACTIVITY (UNITS PER mg PROTEIN)
TUBE NUMBER


ELUTION PROFILE FOR BLUE-2 SEPHAROSE CL-6B
AFFINITY COLUMN
FLOW RATE 1.1 ml per minute
ACTIVITY (UNITS per ml)
PROTEIN (ug per ml)
TUBE NUMBER

The results presented are directly comparable to those obtained by Redshaw and Loten (1981) who obtained similar recoveries and specific activities. Individual fractions obtained from the Blue Sepharose column varied in specific activity (2.36-15.69 units/mg of protein). In order to get consistency in the purity of the eluted enzyme the fraction volume had to be reduced from 6.0 ml to 1.0 ml. Only fractions with a specific activity of greater than 10 units/mg were pooled for immunization into rabbits.

The eluted ATP-Citrate lyase lost 54% of activity over a 24 hour dialysis period. The resulting protein was not suitable for immunization into rabbits since the loss of activity was accompanied by the generation of degradation products. Losses of activity could be partially remedied by the addition of 10 mM DTT, 50 mM benzamidine pH 7.5 into fractions prior to collection and in the dialysis buffer.

It must be emphasized that ATPCL is an unstable enzyme that readily undergoes changes (-SH group oxidation, proteolytic cleavage, loss of phosphate groups) even under optimal storage conditions (114).

The degree of homogeneity of the enzyme was estimated by electrophoresis on both detergent (SDS) and native gels. A photograph of the separation of the ATP citrate lyase subunit on SDS PAGE comprises Fig. 3. The quantitative scanning of this track is shown in Fig. 3. This fraction was loaded so that the ATP citrate lyase 120,000 Mr subunit represented 4 ug of the protein. The specific activity of this fraction was 6.9 units/mg of protein.

Redshaw and Loten (1981) obtained a single band on SDS gel electrophoresis of a 7 ug loading of enzyme with the same specific activity, as shown in Fig 2.

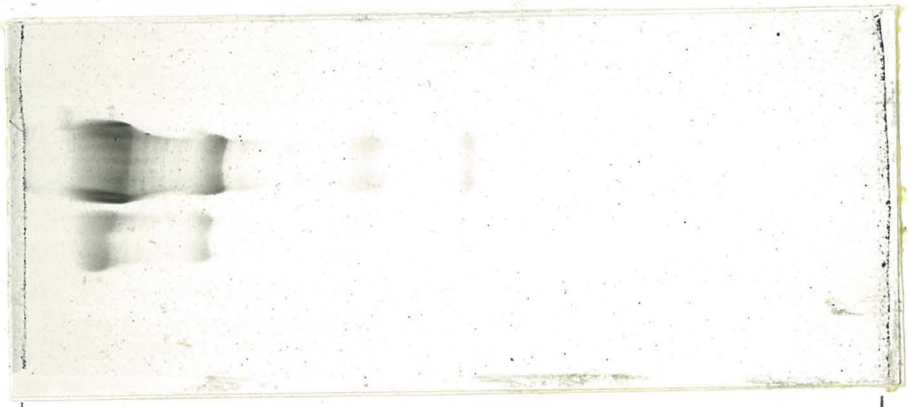
The minimum ATP citrate lyase concentration loaded onto these gels was 7 ug of protein. When pooled samples with a specific activity of 6.9 units/mg were electrophoresed on 7.5% SDS gels at greater than 7 ug of ATP citrate lyase protein loading other major protein bands of both high and low molecular weights were seen.

The major protein contaminants were high molecular weight proteins with minor contamination by the low Mr degradation products of 52,000 and 57,000 from the ATP citrase lyase subunit.

Figure 3. Microdensitometer tracing of Coomassie stained protein at 580 nm of a SDS polyacrylamide slab gel track. Electrophoretic separation of ATP citrate lyase eluted from Blue Sepharose purified by Method 1(a). Electrophoresis was performed as described in section 2.6 using a resolving gel concentration of 7.5% acrylamide. Four ug of protein was applied. The gel was stained with Coomassie Blue R250 as described in section 2.6.2. Relative migration (Rf) was determined in comparison to the Bromophenol blue dye front.

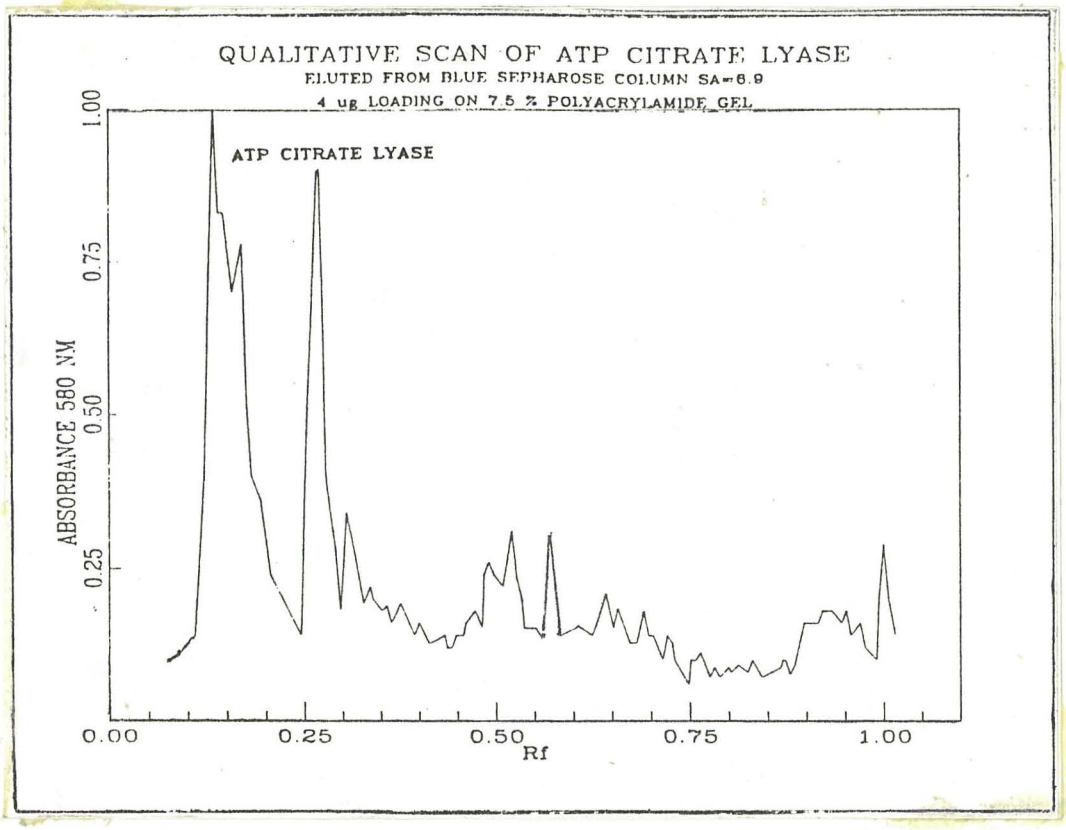
Figure 3

SDS polyacrylamide gel electrophoresis (7.5%) of ATP citrate lyase prepared by purification method 1(a).



origin

Bromophenol blue dye front



At a protein loading of greater than 7 ug of ATP citrate lyase subunit on SDS gels two high molecular weight contaminants of 240,000 and 250,000 were clearly visible. These protein contaminants were not Fatty acid synthetase or acetylCoA carboxylase as judged by their specific assay. The contaminants were not present in fractions which had a specific activity of greater than 9.

Recently Swutowicz (1981) obtained enzymes which showed multiple bands when using a single Blue Sepharose column as the final stage in purification. Subsequent removal of these protein contaminants by chromatogrpahy on DEAE 52, followed by rechromatography on Blue Sepharose increased the specific activity from 6.8 to 8.6 - 11.3 units/mg protein. It is interesting to note that even with a specific activity of 11.3, multiple bands were demonstrated on electrophoresis of the purified enzyme on detergent gels.

The major disadvantages with purification methods 1(a) and 1(b) are that only small amounts of pure enzyme are produced.

The best experiments using these methods (n=3/30) produced 170 ug of enzyme protein having a specific activity of 15.24 +/- 2.1 units/mg. The instability of the enzyme and appearance of the proteolytic fragments that resulted made these fractions unsuitable for immunisation into rabbits.

A purification was attempted using a modification of a method reported by Houston et al (1984). This method avoids the use of polyethylene glycol fractionation. PEG treatment has been shown to irreversibly alter the kinetic properties of Phosphofructokinase (152) and mammary gland AcetylCoA carboxylase (84). ATPCL could not be purified from rat liver using this method because an unsatisfactory large loss of activity occured when the 25-50% ammonium sulphate fraction was eluted from the Sephacryl-S200 column. This occured even when 0.1 M sodium fluoride was substituted for the protease inhibitors. Sodium fluoride should not be used and the specific proteolytic inhibitors should be used instead.

A single Blue Sepharose column of the size used is insufficient for generation of a reasonable amount of antigen (1 mg) of high specific activity. The ATP citrate lyase that was eluted was of variable purity and yield. For these reasons the alternative methods 2(a) and 2(b) were

investigated for the final step of purification.

3.7 RESULTS AND DISCUSSION OF THE ELUTION OF ATPCL FROM DEAE 52 ANION EXCHANGE CELLULOSE. PURIFICATION METHOD 2

Due to problems encountered with using Blue Sepharose as the final step in the purification procedure it was decided to use a final DEAE 52 column using a modified procedure according to Guy et al (1981), and Linn and Srere (1979).

In separate experiments, ATP citrate lyase was eluted from DEAE 52 using either a 45 mM discontinuous phosphate gradient or a continuous 0 - 100 mM phosphate gradient. A comparison of the recovery of activity applied to this column and degree of purification using these two methods can be seen in Table 5. The 45 mM discontinuous phosphate gradient elutes ATP citrate lyase with a specific activity of 10.8 units/mg with a yield of 1 mg.

Using the continuous gradient ATP citrate lyase is eluted with a lower specific activity of 7.6 units/mg.

Best results were obtained using a 45 mM discontinuous phosphate gradient containing proteolytic inhibitors - leupeptin(4ug/ml), antipain (4 ug/ml), benzamidine (1.0 mM), L-1-Tosylamino-2-phenyl-ethyl chloromethyl ketone (0.1 mM), N-p-Tosyl-l-lysine chloromethylketone (0.1 mM).

TABLE 5

Recovery of activity from the Sephacryl S200 column using a DEAE 52 column.

Fraction	a Total Protein mg	b Total Activity units	Specific Activity units/mg	c Purification factor	Recovery (%)
d Sephacryl S00 peak fraction	15.2	14	0.921	40.0	100
DEAE 52 45 mM discontinuous phosphate gradient					
e best result	0.258	4.05	15.69	680	28
f typical result	0.363	3.98	10.98	480	28
pooled fractions	1.03	10.84	10.78	470	77
DEAE 52 0-100 mM continuous phosphate gradient best result	0.60	4.57	7.60	330	32
g pooled fraction	2.22	10.80	4.86	211	75

a Protein determined with Biuret reagent or Coomassie dye binding.

b Activity determined by malate dehydrogenase coupled assay. A unit is defined as the umol of acetylCoA prod uced/min/ml as monitored by NADH oxidation at 340 nm at 25 C.

c Purification factor is compared relative to 20% homogenate.

d Sephacryl S200 fraction was applied to the DEAE 52 column after dialysis against DEAE 52 buffer overnight and centrification. 14 units of activity were applied to the column.

e Best result represents a single fraction in 1 experiment.

f Typical result represents the mean of at peak fractions at least 4 experiments.

g Pooled fraction represents pooling of fractions to recover 75% of the activity.

Attempts to replace these inhibitors with 20 - 100 mM sodium fluoride was unsuccessful. The enzyme preparation had an absolute requirement for the above proteolytic inhibitors during purification. ATP citrate lyase was not retained on the DEAE 52 column in the presence of 30-100 mM fluoride, and a concentration of 20 mM is insufficient to stop losses of activity.

A purification table and elution profile for preparations using method 2 is seen in Table 6 and Fig 4.

These results show that ATPCL was successfully eluted from the DEAE anion exchange column. The enzyme was obtained with increased yield and at a greater specific activity than when it was eluted from Blue Sepharose (Prep methods 1(a and b)).

ATP citrate lyase eluted from DEAE 52 with a specific activity of 10.88 +/- 0.10 units/mg with a yield of 0.3 to 1.1 mg. Linn and Srere report a specific activity of 10 units/mg for ATP citrate lyase purified by this procedure.

TABLE 6.

PURIFICATION OF RAT LIVER ATP CITRATE LYASE
METHOD 2.

Fraction	b Total Protein mg/ml	c Total Activity units	Specific Activity units/mg	Purification factor	Yield (%)
20% homogenate	1295	298	0.023	1	100
SS34 supernatant	3408	237	0.070	3.04	80
50.Ti supernatant	2280	221	0.097	4.22	74
Ammonium sulphate 0-55% saturation	1227	173	0.141	6.13	60
Polyethylene glycol 4-14%	427	152	0.356	15.48	51
d Sephacryl S00 peak fraction	15.6	14.37	0.921	40.1	21
e DEAE 52					
f best result	0.26	4.08	15.69	680	1.4
typical result	1.03	11.10	10.78	470	3.70

a Preparative method 2 represents method 1(b) with the DEAE 52 column substituted for the Blue Sepharose column.

b Protein was determined with Biuret reagent after TCA precipitation, or Coomassie dye binding method of Bradford.

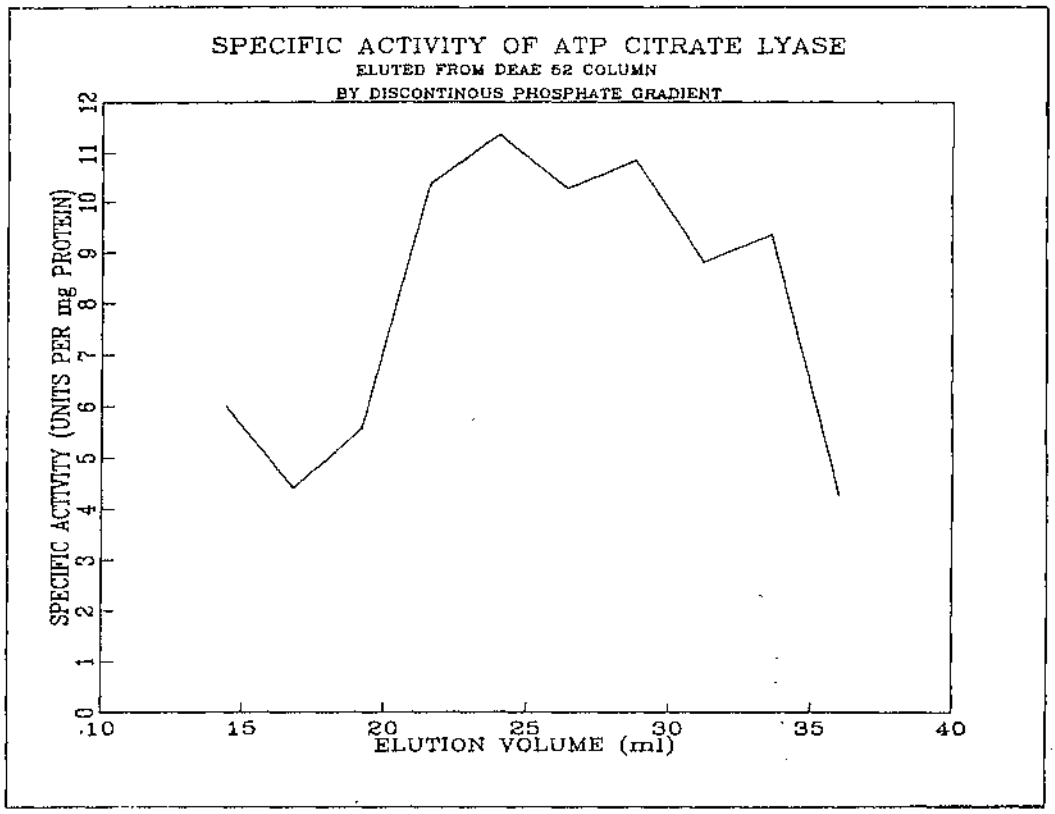
c Activity determined by malate dehydrogenase coupled assay. A unit is defined as umol of acetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm at 25 degrees.

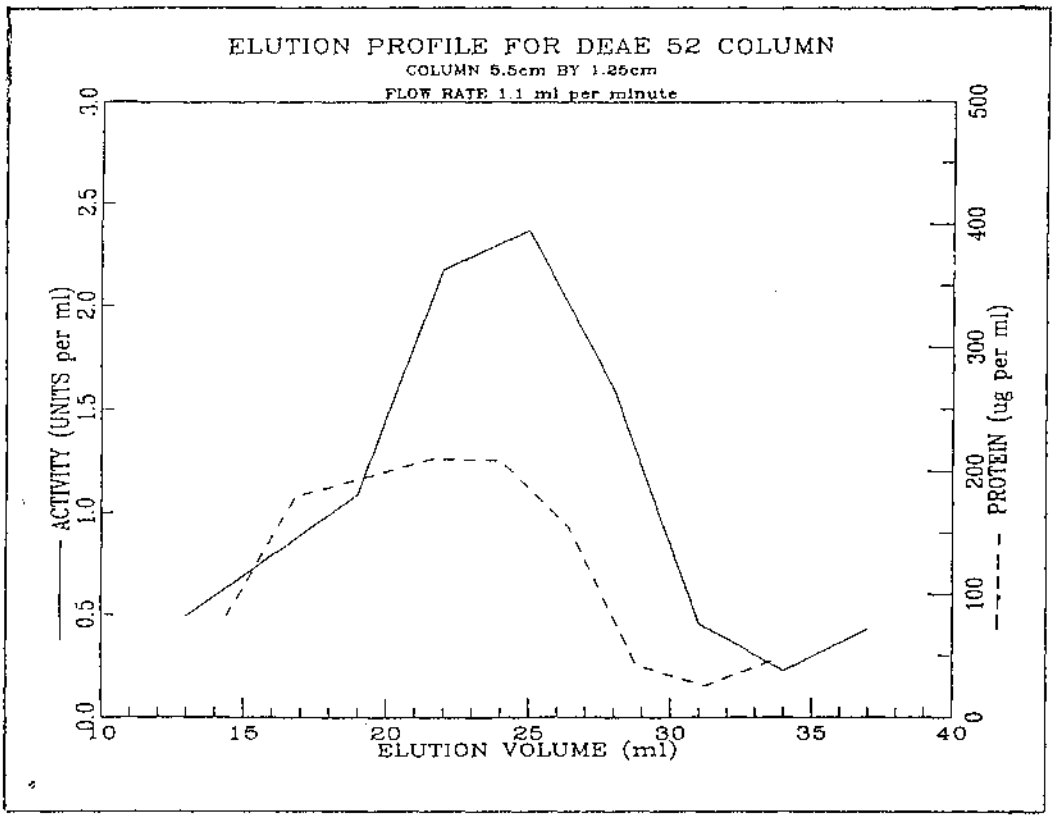
d Sephacryl S200 column recovered 95% of the total enzymatic activity from the PEG step.

e Sephacryl S200 fraction was applied to the DEAE 52 column after dialysis against DEAE 52 buffer overnight and centrification. DEAE 52 data represent rechromatography of the peak Sephacryl S-200 fraction only. Elution of ATPCL using 45 mM discontinuous phosphate gradient.

f Best result represents a single fraction in 1 experiment. Typical result represents mean of 6 expts.

Figure 4. Elution pattern of purified ATP citrase lyase from the DEAE 52 column. Fifteen milligrams protein were applied in a volume of 12.8 ml. Elution of ATP citrate lyase was by use of a 45 mM discontinuous phosphate gradient. The flow rate was 66 ml/hr and 3.3 ml fractions were collected. Enzyme activity was determined by the malate dehydrogenase coupled assy as described in section 3.3.1.


SPECIFIC ACTIVITY OF ATP CITRATE LYASE
ELUTED FROM DEAE 52 COLUMN
BY DISCONTINOUS PHOSPHATE GRADIENT
SPECIFIC ACTIVITY (UNITS PER mg PROTEIN)
0 1 2 3 4 5 6 7 8 9 10 11 12
10 15 20 25 30 35 40
ELUTION VOLUME (ml)


ELUTION PROFILE FOR DEAE 52 COLUMN
COLUMN 5.5cm BY 1.25cm
FLOW RATE 1.1 ml per minute
ACTIVITY (UNITS per ml)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
PROTEIN (ug per ml)
0 100 200 300 400 500
0 15 20 25 30 35 40
ELUTION VOLUME (ml)

The elution profile (Fig 4) shows that the ATPCL activity elutes from DEAE 52 in a single peak with an additional peak eluted at the void volume. This non-absorbed form accounts for 8-14% of the total ATPCL activity. Corrigan (40) report a similiar proportion of ATPCL which is not retained during chromatography on DEAE-Sephadex A-25, and 2 peaks eluted with a 0-0.4 M KCl gradient.

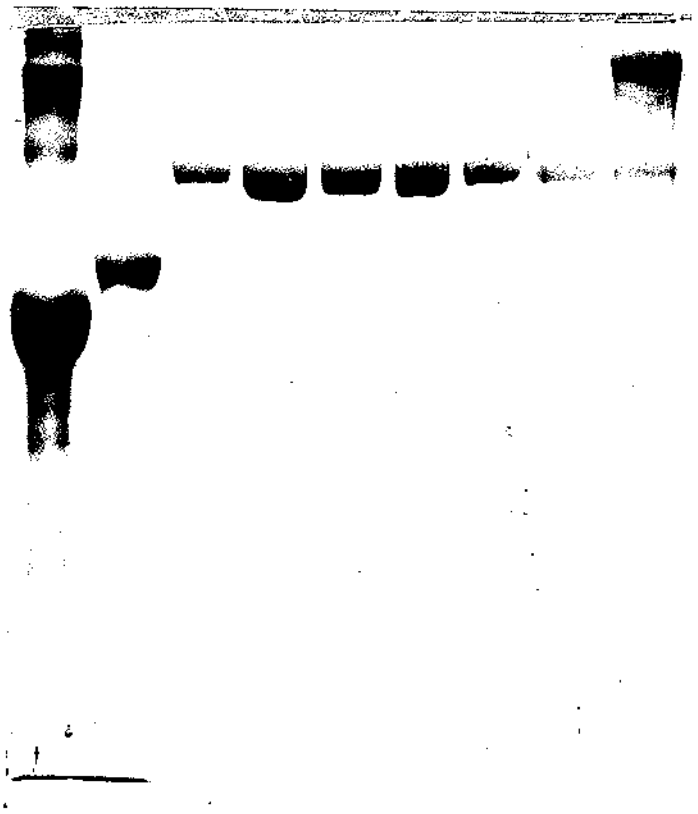
The SDS gel electrophoretic separation of protein eluted from this column can be seen in Figure 5. A qualitative scan of the gel separation of ATP citrate lyase with a specific activity of 11.0 units/mg can be seen in Figure 6. This gel shows that only minimal amounts of proteolytic fragments are present when the enzyme is prepared in this way.

When eluted samples were electrophoresed on detergent gels, no secondary protein bands were observed at 25 ug loading. The molecular weight of the ATP citrate lyase subunit, as estimated from standard protein markers, correlates well with the reported literature value of 123,000 +/- 5,000 (see Fig 5.)

The enzyme is more stable in the 45 mM sodium phosphate elution buffer than the Blue sepharose elution buffer, and hence can be subjected to an additional gel filtration step, or can be pooled for rechromatography on Blue Sepharose if necessary.

Figure 5. SDS gel electrophoresis of fractions eluted from the DEAE - 52 column using a 45 mM discontinuous phosphate gradient. Electrophoresis was performed as described in section 2.6 using a resolving gel concentration of 7.5% acrylamide. The protein concentration was calculated assuming a specific activity of 10 unit/mg. Gel was stained with Coomassie R250 as described in section 2.6.2. Rf was determined relative to the Bromophenol blue dye front.

SDS gel electrophoresis of ATP citrate lyase eluted from DEAE 52. (Preparative Method 2).

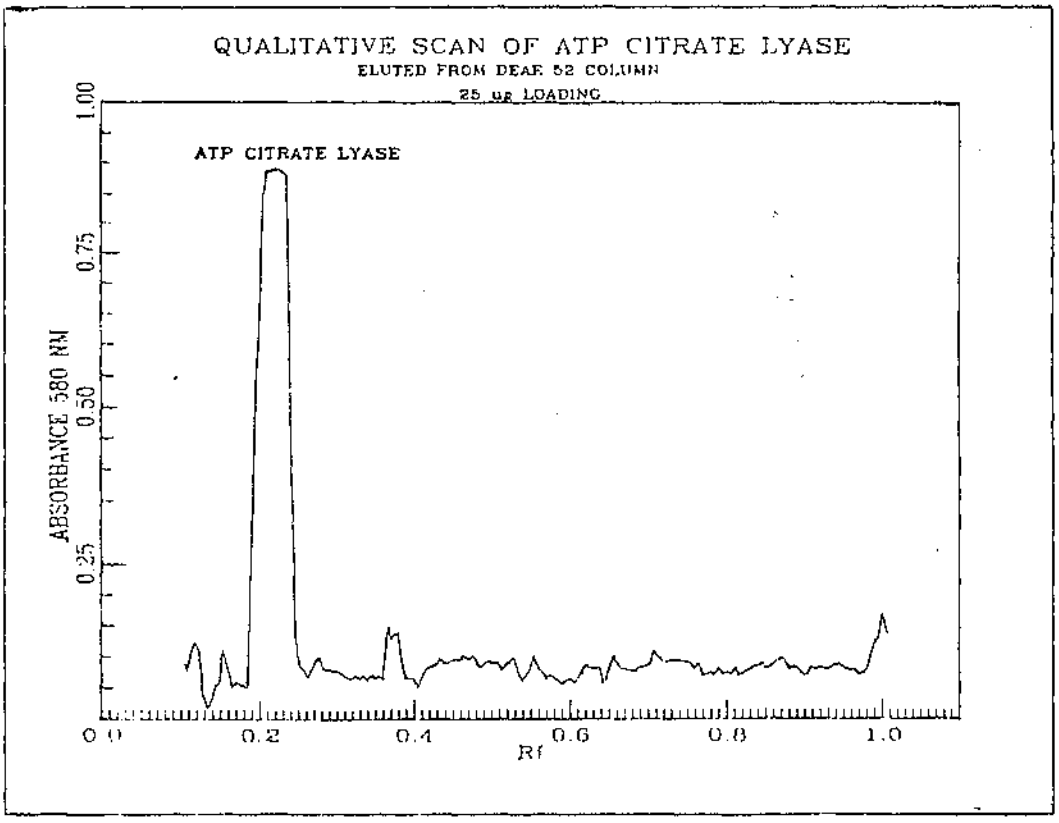


Lane 1 2 3 4 5 6 7 8 9

		Mw
Lane 1	BSA	66,000
2	Human Transferrin	72,000
3-8	ATP citrate lyase fractions	123,000
9	Fatty acid synthetase	250,000
	Subunit	125,000

Lane	Specific Activity Units/mg	Protein loaded ug
3	10.2	16
4	11.0	25
5	10.0	19
6	10.6	18
7	8.5	8
8	8.8	6

Figure 6. Microdensitomer tracing at 580 um of a SDS slab gel track. Electrophoretic separation of ATP-citrate lyase purified on DEAE - 52. This fraction contained ATP citrate lyase with a specific activity of 11.0 units/mg. Twenty five ug of ATP citrate lyase protein was applied to the gel. The gel was stained with Coomassie R250 as described in section 2.6.2. The Rf was calculated relative to the Bromophenol blue dye front.


QUALITATIVE SCAN OF ATP CITRATE LYASE
ELUTED FROM DEAE 52 COLUMN
25 ug LOADING
ATP CITRATE LYASE
ABSORBANCE 580 NM
0.25
0.50
0.75
1.00
0.0
0.2
0.4
0.6
0.8
1.0
Rf

3.8 RESULTS AND DISCUSSION OF PREPARATIVE METHOD 3.

When peak fractions obtained from the DEAE 52 column (9.8 - 12.2 units/mg) were rechromatographed on Blue Sepharose, the final specific activity of the eluted product was 13.72 + 2.1 units/mg.

To affect a reasonable recovery of the enzyme from this column ATP citrate lyase was eluted using 5 mM ATP, 1 mM DTT and 5 mM NAD. Increasing the ATP concentration to 10 mM increased the recovery to 63% but lowered the specific activity to 8.68 units/mg.

A typical purification using this method is shown in Table 7. Total recovery was low, and the enzyme was unstable if left undialyzed. The major degradation product is a 22,000 molecular weight fragment (Figure 7).

The best result obtained had a specific activity of 13.71 +/- 2.1 units/mg. This result is comparable to the specific activity obtained by Houston et al (1984). A SDS electrophoretic separation of protein eluted from Blue Sepharose can be seen Fig 8.

TABLE 7.

PURIFICATION OF RAT LIVER ATP CITRATE LYASE
METHOD 3.

Fraction	a Total Protein mg	b Total Activity units	Specific Activity units/mg	c Purification factor	Recovery (%)
d DEAE 52 pooled fraction Method 2	1.04	11	10.78	470	100
e Blue Sepharose 5 mM ATP, 1mM DTT, 5 mM NAD elution buffer					
best result	0.14	1.92	13.71	596	17
typical result	0.06	0.81	13.50	590	7
pooled result	0.21	2.49	11.85	516	23

a Protein determined with Coomassie R250 dye binding.

b Activity determined by malate dehydrogenase coupled assay. A unit is defined as the umol of acetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm at 25 C.

c Purification factor is relative to 20% homogenate.

d DEAE 52 fraction was dialyzed against Blue Sepharose buffer and centrifuged 17,000 g before application on to Blue Sepharose column.

e Samples eluted from Blue Sepharose were assayed immediately. The eluted enzyme was very unstable. Best result represents 1 fraction in 1 experiment. Typical result represents mean of 3 experiments. Pooled result represents combining fractions to give a specific activity of greater than 11 units/mg.

Figure 7. Electrophoretic separation of ATP citrate lyase prepared by Preparative Method 3. Sample was obtained by elution from Blue Sepharose column but was not dialysed against Blue Sepharose buffer. Resolving gel concentration of 7.5% acrylamide. ATP citrate lyase subunit loading represents 6 ug of Coomassie staining protein. Major proteolytic fragment present is of 22,000 Mw.

Figure 8. Electrophoretic separation of ATP citrate lyase prepared by Preparative Method 3. Sample was dialysed against Blue Sepharose buffer containing 5 mM DTT, and 50 mM Benzamidine. The specific activity of this sample was 13.7 units/mg. The ATP citrate lyase subunit represents 36 ug (track 1) and 72 ug (track 2) Coomassie staining protein.

Figure 7 Electrophoretic separation of undialysed ATP citrate lyase prepared as in Preparative Method 3.

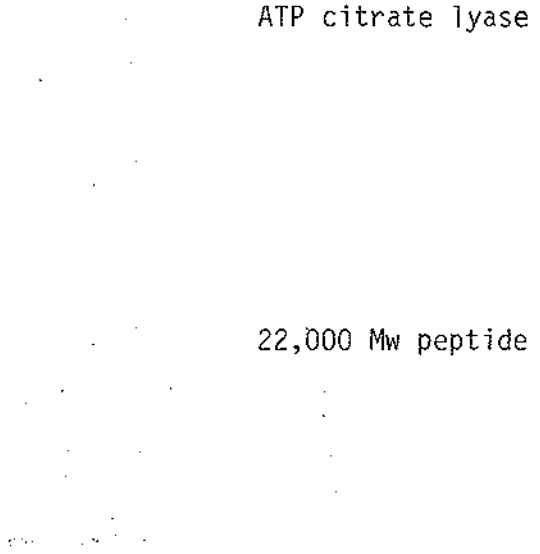
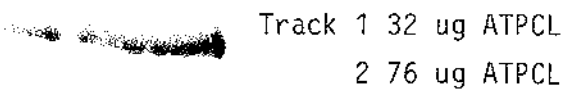


Figure 8 Electrophoretic separation of ATP citrate lyase prepared as in Preparative Method 3. Sample was dialysed to remove ATP. Specific activity of sample was 13.7 units/mg.



ATPCL elutes from the DEAE-52 anion exchange column with a specific activity lower than 10 units/mg protein then it can be further purified by rechromatography on a Blue Sepharose column to greater than 10 units/mg protein. However these fractions require additional gel filtration steps after the second Blue Sepharose column to remove proteolytic fragments.

3.9 CONCLUSION OF PURIFICATION PROCEDURES.

A comparison of the results obtained by the individual experimental methods of purification is shown in Table 8.

It can be seen that the removal of the DEAE 32 step (method 1(b)) from preparative method 1(a) increased the specific activity from 6.56 to 7.50 units/mg but this was at the expense of a lower yield than in method 1(b). The reason for the difference in specific activity may only be a factor of time saving in method 1(b), and hence less proteolytic cleavage by proteases took place.

Method 2(a) produced ATP citrate lyase with a significantly higher specific activity than method 1 or 2(b).

The use of a discontinuous phosphate gradient in method 2(a) produced an ATP citrate lyase of higher specific activity than the use of a discontinuous phosphate gradient (method 2(b)). A similar result may have been obtained if the continuous gradient had consisted of a larger volume, and smaller samples had been collected. Alternatively the gradient could have been restricted to 20 - 50 mM instead of the range of 0 - 100 mM used.

TABLE 8

COMPARISON OF EXPERIMENTAL METHODS.

a Purification Method c Typical Result	b Specific Activity units/mg	Yield ug
d METHOD 1(a)	6.56	300
METHOD 1(b)	7.50	180
METHOD 2(a)	10.78	1030
METHOD 2(b)	4.86	2200
METHOD 3	13.50	80
METHOD 3 pooled	11.85	270

a All experiments have been scaled so that 14 units were applied to the column used in the last step of protocol.

b Protein determined by Coomassie G250 dye binding method. Activity determined by malate dehydrogenase coupled assay as described section 3.3.1.

c Typical result represents the mean of at least 4 experiments.

d Method 1(b) Represents removal of DEAE 32 column from Method 1(a).

2(a) Represents substituting a DEAE 52 column for the Blue Sepharose as the final step. Elution of ATPCL from DEAE 32 using a 45 mM discontinuous phosphate gradient.

2(b) Represents Method 2(a) but elution of ATPCL from DEAE 52 using a 0 - 100 mM continuous phosphate gradient.

3 Represents Method 2(a) but an additional rechromotography of ATPCL on another Blue Sepharose column.

3 pooled Represents the pooling of fractions that showed only 1 band on SDS PAGE at 20 ug loading. This ATPCL fraction was used for immunization into rabbits.

The results of the different methods of purification show that method 2(a), which used a discontinuous gradient, produces 3 times the quantity of ATP citrate lyase as methods 1(a) and 6 times that of method 1(b).

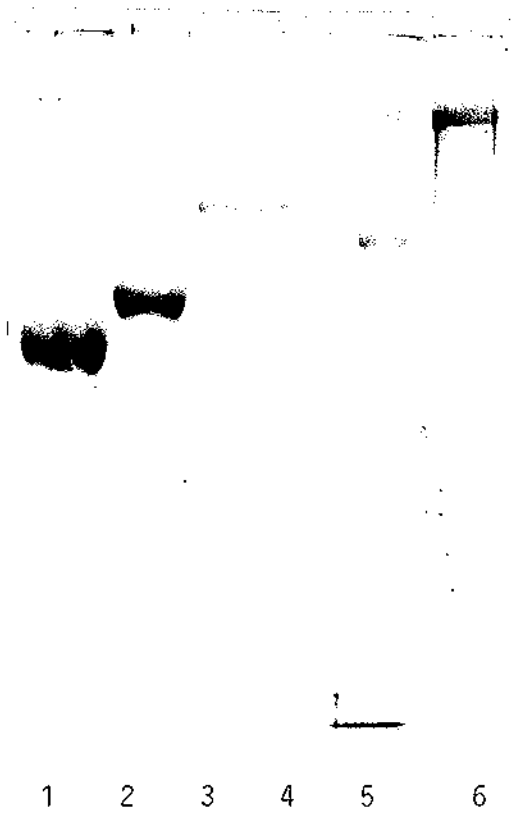
Method 2(a) was the preferred method of obtaining enough ATP citrate lyase for subsequent rechromatography on Blue Sepharose.

When samples eluted from either Blue sepharose (Method 1(b) or DEAE 52 (method 2a)) were electrophoresed on a SDS slab gel, the ATP citrate lyase subunit migrated the same Rf (Figure 9). The molecular weight of the ATPCL subunit, as estimated from standard protein markers, correlates well with the reported literature value of 125,000 +/- 5,000 (Figure (10).

Method 3 produces ATP citrate lyase with a specific activity of 13.5 units/mg. The enzyme is eluted with a low yield, and is very unstable if undialyzed. A thirty percent increase in specific activity resulted in a 5 fold decrease in yield.

Figure 9. Comparison of the electrophoretic mobility of the ATPCL subunit on SDS PAGE prepared by 2 individual methods. Purified ATPCL was obtained after elution from Blue Sepharose, as in Preparative Method 1(b) or after elution from DEAE 52 cellulose, as in Preparative Method 2(a). Four ug of ATPCL subunit (method 2(a) and two ug (method 1(b) were applied. Electrophoresis was performed as in section 2.6 using a resolving gel concentration of 7.5% acrylamide. The gel was stained with Coomassie R250 as described in section 2.6.2. Relative migration of samples and standards was determined in comparison to the bromophenol dye front.

Figure 9. Comparison of the relative migraton of the ATPCL subunit when the enzyme is prepared by Preparative Method 1(b) or 2(a).



Lane 1 BSA.

2 Human Transferrin.

3 ATPCL eluted from DEAE specific activity 11.0 4 ug loading.

4 ATPCL eluted from Blue Sepharose specific activity 7.50 2ug loading.

5 Phosphorylase b.

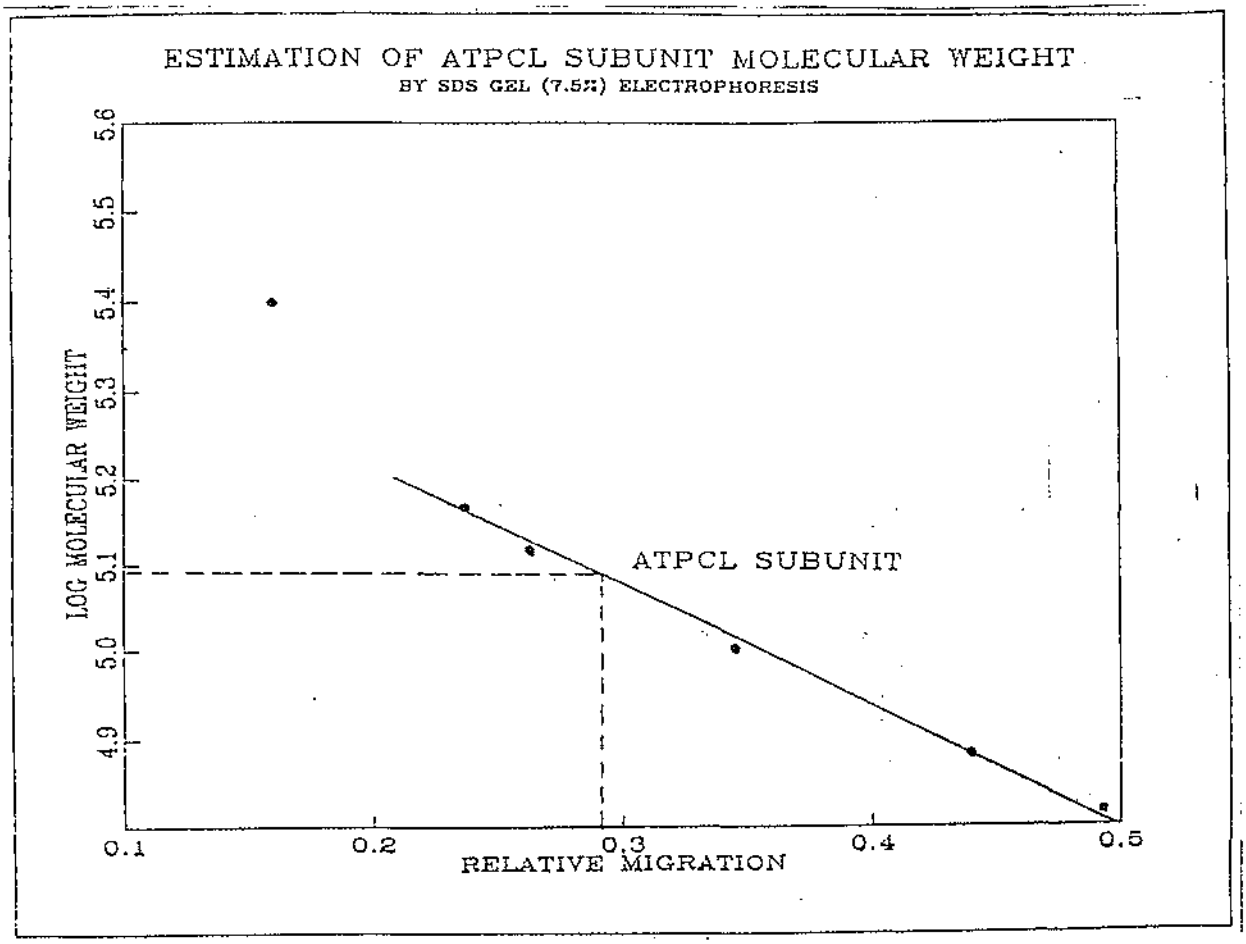
6 Fatty acid synthetase.

Figure 10. Estimation of the ATP citrate lyase subunit molecular weight.

The relative migration (Rf) of the molecular weight markers in Figure 9 were calculated relative to the Bromophenol blue dye fornt.

The logarithmetic molecular weight of the subunit of the standard was then plotted against its Rf, and a least linear squares regression line generated.

ATPCL subunit molecular weight was then determined by interpolation of this line.



Markers of known molecular weight in descending order are ....

Rat liver Fatty acid synthetase	250,000 Mr
Human Transferrin dimer	144,000 Mr
Bovine Serum Albumin dimer	132,000 Mr
Rabbit Muscle Phosphorylase b	94,000 Mr
Human Transferrin monomer	72,000 Mr
Bovine Serum Albumin monomer	66,000 Mr

Stabilizing the enzyme after it has been eluted from Blue sepharose with 5 mM ATP and 1 mM DTT should be the subject of further investigation. Stabilizing the final product may perhaps be accomplished by

(1) Immediate freeze drying.

(2) Rapid dilution of the pooled enzyme with Blue sepharose buffer, followed by vacuum dialysis.

(3) Addition of higher concentrations of B-mercaptoethanol or of DTT into those test tubes into which ATP citrate lyase is to be collected.

Secondly, the overall concentration of ATP citrate lyase carried through to the final step of purification may be increased by the addition of a carrier protein such as BSA. The BSA would then be a substrate for the proteases that are present in the early stages.

Coenzyme A would be alternative to ATP as a means of eluting ATP citrate lyase from Blue Sepharose, but it is more expensive as an eluting agent. Houston et al (1984) successfully eluted ATP citrate lyase from an Affi - gel Blue column using 5 mM ATP and 0.25 mM CoA. Gel filtration increased the specific activity to 13.6 units/mg. Coenzyme A has been previously reported to have a greater eluting capacity than ATP when eluting ATP citrate lyase from Blue Sepharose (211). Increased yield may have resulted if 0.25 mM Coenzyme A and 5 mM ATP had been used to elute ATP citrate lyase from Blue Sepharose.

Since ATP citrate lyase has a binding site for Coenzyme A perhaps Coenzyme A-agarose should be investigated as an alternative to Blue Sepharose.

During the preparation of this thesis a new method for the purification of ATPCL from the liver of rats was published (Wraight et al 1985). This method describes a rapid 2 step purification procedure based on dye-ligand binding chromatography. ATPCL was purified using a Yellow MX-6G Sepharose CL-6B column, followed by ion-exchange chromatography using a DEAE-Triacryl column. The final product has a specific activity of 10 units/mg with a yield in excess of 50%. ATPCL isolated by this procedure has little proteolytic nicking and is stable.

Production of only small amounts of pure antigen by the procedures described in this section caused severe limitations to the production of high titre antiserum.

CHAPTER 4

EFFECT OF DIET ON THE LEVEL OF INDUCTION OF ATP CITRATE LYASE

4.1 INTRODUCTION

The activity of ATP-Citrate lyase in rat liver has been shown to be dependent on both the hormonal and nutritional state of the animal (102-105). Starvation for 2 days followed by refeeding a high carbohydrate diet has been reported to result in greatly increased levels of ATP citrate lyase (2,60,99,171).

4.2 ANIMALS FEEDING REGIMES

Young male Sprague Dawley rats weighing about 200 g were used in all these studies. Animals were maintained on N.M.R rabbit pellets and are referred to as the 'normal' group. The group which was starved for 48 hours are called the 'fasted' group; and the group fasted for 2 and refed a high carbohydrate (66% sucrose, 33% casein hydrolysate, 0.15% vitamin mix, 0.85% water) diet are referred to as the 'induced' rats.

4.3 METHODS

Sprague-Dawley rats were obtained from the Small Animal Production unit at Massey University at the beginning of day 1. The animals were single caged and maintained at 23 C in a room illuminated from 6.00 am to 6.00 pm daily.

These animals had been maintained on a chow diet (ad libitum) since weaning. Water and diet were available ad libitum.

The chow diet was removed at 6.00 pm on day 1, and the animals were starved for 48 hours. On the evening of day 3 the rats were refed either a chow diet or the high carbohydrate diet for 2 or 3 days. The level of enzyme in the fasted rats was determined in animals sacrificed on the evening of day 3.

The livers were then processed as in section 3.3.2 steps 1-5. After ultracentrifugation at 45,000 RPM in a Beckman 60Ti rotor the clear central section of the supernatant was carefully removed and recentrifuged. The central section of this fraction was then carefully aspirated with a pasteur pipette. Care was taken not to transfer the fatty floating material or particulate precipitate.

The recentrifuged 'cystolic' fraction was assayed for enzyme activity, and protein content. The results were expressed as units of ATP citrate lyase activity per gram of liver (wet weight).

In order to decide how much antisera to add in immunoprecipitation experiments the specific activity of pure ATP citrate lyase is assumed to be 15 units/mg.

4.4 DISCUSSION OF THE EFFECT OF DIET ON ATP citrate lyase INDUCTION.

The effect of different dietary regimes on enzyme induction can be viewed in Table 9.

There is an observed reduction in ATP citrate lyase activity during starvation and a reappearance of activity when the rats are refed the chow diet.

The reduction in activity per gram of liver in starved rats compared to the rats refed a diet chow seems to be modest (1.00 vs 0.86 fold induction). This is because the livers total mass declines significantly during starvation.

There is a significant induction of enzyme activity per total liver during starvation and refeeding a high carbohydrate diet. When the feeding regime is extended to 3 days of starvation, 2 days refeeding, there is a significant elevation in the level of the total enzyme activity at the end of the refeeding period (36 fold). This was probaly caused by hunger forcing the rats to eat more of the high carbohydrate diet. The diet is rather unappealing to the rats.

TABLE 9

THE EFFECT OF DIETARY REGIME ON THE LEVEL OF ATP CITRATE LYASE IN RAT LIVER

Dietary regime	Units of Enzyme Activity /gram of liver wet weight	Induction Fold
Normal	0.359 +/- 0.019	1.00
Fasted	0.309 +/- 0.016	0.86 +/- 0.09
Chow	0.365 +/- 0.026	1.02 +/- 0.12
Induced	5.29 +/- 2.12	14.7 +/- 6.9
Induced 2	12.85 +/- 0.41	35.8 +/- 3.0

The "cytosolic" fraction was recentrifuged 150,000g for 1 hour and the supernatant assayed for activity. The units used are units of enzyme activity per gram of liver wet weight. The data represents at least 3 expts per regime. Activity determined by malate dehydrogenase coupled assay. A unit of activity is the amount of enzyme required to transform 1 umol of NADH/min.

Normal : represents rats maintained on chow diet.
Fasted : starved for 2 days.
Chow : fasted 2 days then refed a chow diet for 2 days.
Induced : fasted for 2 days then refed a carbohydrate diet for 2 days.
Induced 2 : starved for 3 days then refed a high carbohydrate diet for 2 days.

The induction of ATP citrate lyase was variable. When rats were starved for 2 days and then refed a high carbohydrate diet the level of total induction was 100-260 units per liver. This corresponds to 2.91 to 10.02 units of enzyme activity per gram of liver. The maximal induction of total enzyme activity never exceeded 260 units per liver.

In the chow fed rats or rats starved 2 days the ATP citrate lyase activity is overestimated in the assay. This can be corrected by subtracting the blank rate if CoA is omitted from the assay.

In previous studies Finklestein (53) obtained statistically higher values for fasted rats (0.93 +/- 0.099 units of enzyme activity per gram of liver) and chow refed (1.36 +/- 0.37U/g). The values obtained for the carbohydrate refed rats are similiar 15.9 +/- 1.73 vs 12.85 +/- 0.41 U/g. The difference may lie in the use of different commercially prepared chow diets in the experiments.

CHAPTER 5

PURIFICATION OF RAT ALBUMIN AND PRODUCTION OF RABBIT ANTI-RAT ALBUMIN ANTIBODY PRODUCTION.

INTRODUCTION

Albumin synthesis in serum has shown to vary in rats refed a wide range of protein-deficient and energy-deficient diets (101,118). The level of hepatic albumin mRNA and albumin has recently been reported to vary with diet.

In liver, both albumin mRNA level and rate of albumin synthesis are diminished in response to low levels of dietary protein. The level of albumin mRNA in the livers of rats refed a fat-free diet (carbohydrate-rich) diet was 30 to 45% of the level in rats refed the basal 4% fat diet (37).

In this chapter, the time course of the experiment was 1/2 to 1/12 that of the published reports. For this reason, the variation in serum albumin levels was only 3%. The relative constancy of serum albumin synthesis means that it can be utilized as an internal control when studying the induced synthesis of proteins in liver.

Since serum albumin represents a major circulating protein, the incorporation of the isotope into albumin can be used as a convenient radiolabelled standard. This assumes that all of the proteins are synthesized from the same amino acid pool and that the albumin is reasonably representative of serum proteins and total subcellular proteins in its uptake of labelled amino acid.

Rat albumin was purified to homogeneity to be used in the production of rabbit anti-rat albumin antibodies which were then used to immunoprecipitate radiolabelled albumin in vivo.

In individual experiments albumin was immunoprecipitated from the serum, and ATPCL was immunoprecipitated from the liver cytosolic fraction.

Albumin's rate of synthesis was normalized to 65% of the total TCA-precipitable protein in sera.

5.2 MATERIALS

Commercial rat albumin fraction V powder (A 6272) was obtained from Sigma Chemical Co, St Louis, Missouri.

5.3 METHODS

Commercial rat albumin was purified to homogeneity by native gel electrophoresis as described in section 2.6.1

Samples of 200 ug of commercial albumin were dissolved in 50 ul of sample buffer (section 2.6.1) and laid into 5 wells. Electrophoresis was performed on a 7.5% acrylamide resolving gel with a 10% acrylamide stacking gel.

After 5 hours the gel was removed from the plates and 2 tracks from either side of the gel were excised and stained with Coomassie Blue R250 stain and then destained as in section 2.6.1.

Remaining gel, containing electrophoresised rat albumin 66,000 Mr subunit and contaminating proteins, was stored at 4 C in electrode buffer.

Molecular weights of the visualized protein bands were determined from standards of known relative migration.

Albumin's 66,000 Mr subunit was then excised from the stored gel. Remaining gel, containing contaminating proteins, was stained and destained to show that only the albumin subunit band had been removed.

A 1cm sample of the native gel section containing the albumin subunit was then homogenized in 0.1 ml of stacking gel buffer. Liquid was drawn off and the protein denatured as in section 2.6.1. A sample was electrophoresised on 7.5% SDS PAGE slab gels as in section 2.6.1. Only a single band at 66,000 was present after staining and destaining of this gel.

The remaining protein band was then injected into rabbits after homogenisation in 1 ml of PBS. This was accomplished with the use of two glass 2 ml syringes connected by a microtip. The same immunization protocol was used as in section 6.3.1, and 6.3.2.

Rabbit gamma globulin was prepared as in section 6.3.

Equivalence point of the anti-albumin antibody was determined by immunoprecipitation of radiolabelled rat albumin from rat sera as in section 6.4.2.

Ouchterlony plates were prepared, loaded, and stained as described in section 6.4.3

5.4 RESULTS AND DISCUSSION OF ANTI-ALBUMIN IgG PRODUCTION AND IMMUNOPRECIPITATION OF ALBUMIN FROM RAT SERA.

Antisera raised against purified rat albumin showed a single precipitation line by Ouchterlony double diffusion when challenged against commercially prepared albumin or rat serum. A typical Ouchterlony plate can be seen in Figure 11.

Data for the immunoprecipitation of radiolabelled (35-S) rat albumin with purified antibody can be viewed in Table 10. The immunotitration showed that 1 ml of antiserum is saturated at greater than 190 +/- 5 ul of serum. The radioactivity that is present in the supernatant is linear up to 150 ul of serum. After 200ul of sera the radioactivity that can be located in the precipitate is 5800 +/- 150 cpm. Radioactivity found in the precipitate is constant for tubes 5-7 despite a 1.5 fold increase in added serum. Complete immunoprecipitation of rat albumin when antibody was in excess was checked by SDS PAGE of the supernatant. No albumin subunit was visualized on SDS PAGE for the supernatant of tubes 1-4.

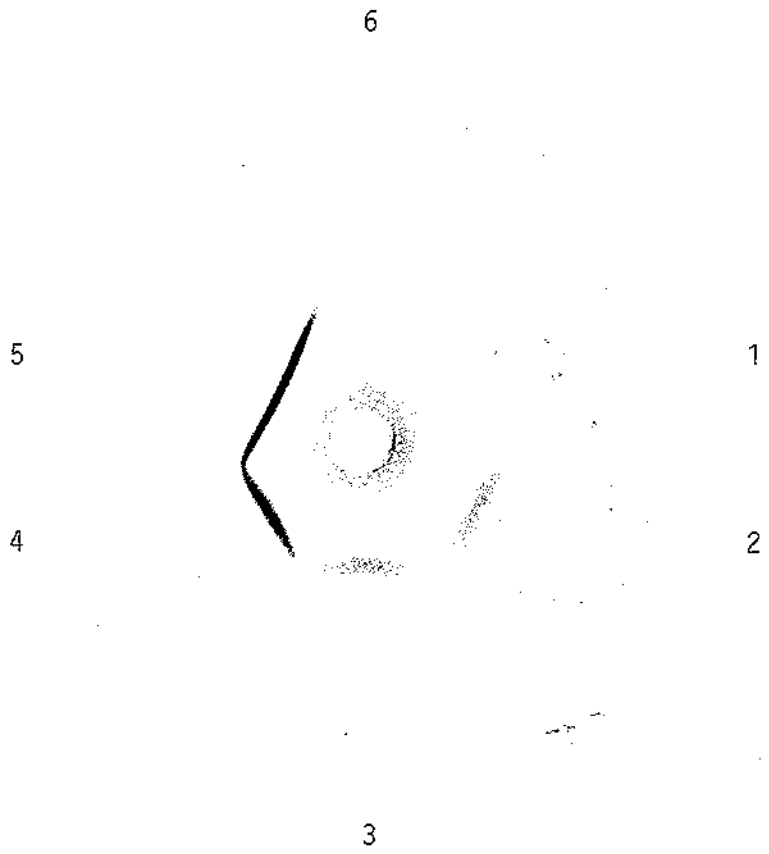
Immunoprecipitation of radiolabelled albumin from rat serum derived from rats with different nutritional status can be seen in Table 11.

Figure 11

Ouchterlony plate of purified IgG
challenging rat serum.

60 ug of rat serum was loaded into the central well of an Ouchterlony plate. 50-200 ul of antibody was added to the outer wells. Immunodiffusion was allowed to proceed for 24 hours in a water saturated atmosphere at room temperature for 2 hours and then overnight at 4 C. Plates were washed in PBS, 2% salt overnight, then photographed.

Figure 11



1 : 20 ul PBS vs 20 ug purified IgG.
2 : 5 ul rat serum vs 20 ug purified IgG.
3 : 7 ul rat serum vs 20 ug purified IgG.
4-5 : 15 ul rat serum vs 20 ug purified IgG.
6 : 2 ul rat serum vs 20 ug purified IgG.

TABLE 10

Immunotitration of radiolabelled rat serum
with purified rabbit anti-rat albumin.

Tube number	1	2	3	4	5	6	7
Volume of serum added (ul)	0	50	100	150	200	250	300
Volume of antiserum added (ul)	10	1000	1000	1000	1000	1000	1000
PBS added	0	950	900	850	800	750	700
Radioactivity present in immunoprecipitate cpm	0	1500	3300	4800	5800	5900	5600
Radioactivity present in supernatant cpm	0	900	1800	2400	3800	6100	8800

a Radioactivity in fractions determined by liquid scintillation spectroscopy after addition of 10 ml triton/toluene scintillant.

b Radioactivity in antibody-antigen complex was determined as follows. After resuspending antigen-antibody complex in PBS and centrification through a discontinuous sucrose gradient 3 times the immunoprecipitates were disrupted and electrophoresised on 7.5% SDS polyacrylamide gels. The 66,000 Mr albumin subunit was excised from the gel and dissolved in hydrogen peroxide/ammonia. Radioactivity was determined as in (a).

c Radioactivity in TCA precipitable protein of serum was determined by precipitating protein and washing with 10% TCA 3 times. Neutralization with 1 M NaOH and counting as in (a). Serum contained 48,000 cpm/ml. Protein concentration of serum is 63 mg/ml as determined by Biuret method.

d Proteins labelled with (35-S) Methionine administered interperitoneally, with a labelling time of 24 hours.

TABLE 11

Immunoprecipitation of (35-S) rat albumin from rat serum using rabbit anti-rat albumin IgG.

Nutritional status albumin immuno-precipitated from	Radioactivity present in Albumin subunit cpm/ml	total protein cpm/ml	Percentage of total protein
Normal	38200	56600	67.5
	35000		61.8
Induced 1	28200	40600	69.5
	24500		60.3
Induced 2	32600	48000	67.9
	31100		65.8
	32900		68.5
Fasted	50000	80000	62.5

a Albumin was immunoprecipitated from rat serum. Enough Anti-rat IgG was added to precipitate a 10 fold excess of albumin in Induced 2 rats as determined by the previous immunotitration. 500-1000 cpm of albumin was typically immunoprecipitated.

b Albumin radioactivity determined per ml of serum after excision of the 66,000 Mr subunit from 7.5 % polyacrylamide slab gels, dissolving gel slices in hydrogen peroxide/ammonia and addition of 10 ml of scintillant.

c Radioactivity present in total protein was determined after precipitation with cold 10% TCA, centrification and washing twice with cold 10% TCA. Neutralization with 1 M NaOH and addition of 10 ml of scintillant.

d Nutritional status is represented by the following:

Normal : represents rats refed a chow diet for 2 days.
Induced 1 : represents starved 2 days refed carbohydrate diet for 2 days.
Induced 2 : represents starved 2 days refed a carbohydrate diet for 6 weeks.
Fasted : represents starved for 3 days.

The immunoprecipitation of serum albumin from rats with different nutritional status show that rat albumin synthesis varies slightly with respect to its uptake of methionine. In all experiments, albumin represented 65 +/-3% of the total TCA precipitatable protein. This value was constant despite a 2 fold increase in the label incorporated and a wide range of dietary extremes.

Rat albumin has been reported to be present at a concentration of 36.3 +/- 3.4 mg/ml in serum (83). In this set of experiments the total TCA precipitable protein content of serum was found to be 63 +/-2 mg/ml. Scaling the albumin concentration to 65% of the total protein is slightly higher than the 57 +/- 7% predicted from the physiological data. Therefore albumin is not a perfect example of a labelled control. However it does offer the advantage of being consistent, in this set of experimental conditions, despite a change in dietary protocol.

In all additional immunological experiments, albumin was immunoprecipitated from sera and ATPCL from recentrifuged rat liver cytosolic fraction. In individual experiments the percentage of albumin in the serum was then scaled to 65% of the TCA precipitable protein. This is a convenient method for reducing the variability between immunological isolations of rat liver ATPCL.

CHAPTER 6

PREPARATION OF ANTI-ATP CITRATE LYASE ANTIBODIES.

6.1 INTRODUCTION

Antibodies were raised in rabbits by procedures modified from those of Schimke (1975, 1976), Crowle (1973), Campbell (1975) and reviews (108,109,197).

In separate challenges antibodies were raised against rat liver ATP citrate lyase and against rat albumin.

Gamma globulin (IgG) was prepared from immune sera by ammonium sulphate fractionation. Purified IgG was then used to immunoprecipitate ATPCL from the recentrifuged cytosolic fraction or purified by preparative method 2(a). The specificity of the antibody for the antigen was then examined using 3 methods.

6.2 MATERIALS

Freund's complete adjuvant was from Sigma No F4258. Electrophoresis grade acrylamide was from BDH Laboratory Chemicals England.

6.3 IMMUNOLOGICAL PROTOCOLS

6.3.1 Preparation of anti-ATP citrate lyase sera

ATP citrate lyase obtained from rat liver by a modified method of Redshaw and Loten (1981) or Linn and Srere (1979), and which showed only one band on 7% SDS-PAGE gels, was used for immunization into rabbits.

Two white, female New Zealand rabbits were injected intradermally and intramuscularly with 20-400 ug of purified ATPCL (1 ml in phosphate buffered saline (PBS); 10 mM sodium hydrogen phosphate, 150 mM sodium chloride pH 7.4) after emulsifying with an equal volume of Freund's

adjuvant. Emulsification was accomplished by the use of two 5 ml glass syringes into which a microtip had been introduced.

On days 1, 7, and 21 half of the emulsion was injected subcutaneously into the back skin flap, and half intradermally into the back leg of the rabbits.

On days 28, 29, 30 increasing amounts of ATPCL (20-200 ug) were administered in a volume of 1 ml PBS into the marginal ear vein, or emulsified with 1 ml of Freund's adjuvant and injected intradermally and intramuscularly (109).

6.3.2 Preparation of serum

Serum collection began 49 days after the initial injection, then at one week intervals. The rabbits were bled from the marginal ear vein, or by non-lethal cardiac puncture into Sorvall 50 ml centrifuge tubes (22,96,97).

Marginal ear vein bleeding was aided by wiping the ear with xylene to produce vasodilation (22,67). Samples of 40-50 ml of blood were routinely obtained per bleed. The rabbits were bled over a 5 week period.

The blood was allowed to clot for 2-3 hours at room temperature. The clot was detached from the side of the centrifuge tube and then allowed to stand overnight at 4 C. The retracted clot was separated from the serum by decanting.

Collected serum was then centrifuged at 35,000 g in a Sorvall SS-34 rotor for 30 minutes.

The straw coloured supernatant was then heated to 55-57 C for 10 minutes to inactivate the complement. The heated supernatant was then recentrifuged as above and stored in 1-5 ml aliquots at -20 C. A sample of non-inoculated rabbit blood was also obtained and used to check for non-specific precipitation of albumin and ATPCL.

6.3.3 Preparation of the Gamma globulin fraction.

After thawing the serum was filtered through glass wool to remove any precipitated material and then centrifuged at 10,000g for 20 minutes at 4 C. This step immediately preceded ammonium sulphate precipitation as further material precipitated at 4 C or after thawing.

The gamma globulin fraction was obtained from the serum by the purification procedure according to Finkelstein et al (53) except that a 40% ammonium sulphate fractionation was used instead of sodium sulphate for the precipitation of the gamma globulin.

The solution was left undisturbed at 4 C overnight to allow the immunoglobulin precipitate to flocculate. The precipitate was centrifuged 35,000 g (Sorvall SS34 17,000 rpm for 30 minutes) at 4 C, and then dissolved in 2.5 volumes (as compared to the original sera) using PBS.

The resulting solution was then reprecipitated and recentrifuged. The precipitate was redissolved in 5 mM disodium phosphate pH 7.4 to a volume equal to half that of the original serum.

The immunoglobulin fraction was dialyzed extensively against several changes of PBS to remove ammonium sulphate. Residual sulphate ions were tested for by adding a drop of immunoglobulin into a test tube containing a solution of barium chloride. Any cloudiness indicated the need for further dialysis.

The volume was measured and the protein concentration calculated by measuring the absorbance at 279 nm of a 1:25 dilution using E1%, 1cm = 14.9 (115,197).

Protein concentration (mg/ml) = (O.D x 25)/1.49

Better results were obtained with samples which had not been frozen, and when there was no delay between steps.

The immunoglobulin solution was either frozen in 1-2 ml aliquots at -20 C, or dialyzed against ammonium bicarbonate (0.01 M containing 0.001% sodium azide) in the ratio of 10ml of IgG solution to 4 litres of

ammonium bicarbonate.

After 3 changes of buffer the purified immunoglobulin was placed in small, acid-washed test tubes, freeze dried, and sealed under vacuum and then stored at -20 C. All antibody samples were reconstituted to 3 mg/ml with PBS before use.

6.4 CHARACTERISATION OF THE ANTI-ATP CITRATE LYASE ANTIBODY INTERACTION.

6.4.1 Introduction

The characterization of the antibody-antigen interaction and specificity of the purified immunoglobulin fraction was determined using the following methods:

(1) Immunotitration of a constant amount of antibody with varying amounts of antigen.

(2) Qualitative scanning of electrophoretically disrupted antibody-antigen complexes.

(3) Immunodiffusion in Ouchterlony plates.

6.4.2 Determination of the equivalence point : antibody titer.

The determination of the equivalence point for the antibody was performed using a constant amount of antibody and varying the amount of antigen in a constant volume of 0.55 ml (81,86,159,213).

Antigen (100 ug of purified ATPCL prepared as in method 1(b) steps 1-5, or rat albumin fraction V) and antiserum (1-100 ul of anti-ATPCL, anti-albumin, or non-inoculated sera) were mixed and incubated for 60 min at room temperature, and then left to stand overnight at 4 C.

The antigen-antibody complexes were collected by centrifugation for 10 minutes in an Eppendorf bench centrifuge, after addition of 100 ul of 1M sucrose containing 1% Triton X-100, 1% deoxycholate in PBS (sucrose gradient mix). This creates a discontinuous sucrose gradient that helps to minimize non-specific of protein trapping by the complex (Schmike et al 1974).

The supernatant was then removed and an aliquot assayed for ATPCL activity (see section 3.2). Results are expressed as ug of pure ATPCL removed from solution per ml of antiserum. It was assumed that the specific activity of pure ATPCL is 16 units/mg of protein.

The precipitated antigen-antibody complex was resuspended in 0.5 ml of PBS and then recentrifuged through 100 ul of sucrose gradient mix. After 2 more successive resuspensions and centrifugations the immunoprecipitate was then disrupted for analysis on 7% polyacrylamide gels. The pellet was resuspended and denatured by the procedure described in section 2.7-2.10.

Purified native antigen was also subjected to the same denaturing procedure so that the banding pattern obtained from the disrupted subunits could be used for comparison.

Electrophoresis was carried out according to Laemmli (1970). Polyacrylamide slab gels were stained and destained as previously described in section 2.6. The gel was then sectioned into 10 vertical tracks and scanned at 580 nm.

6.4.3 Immunodiffusion

Ouchterlony double diffusion was also used as a means of testing for the specificity of the reaction of antigen with antibody (128,129,130).

Microscope slides were coated with 1 ml of hot 0.1% electrophoresis grade agarose in sodium barbital buffer and heated to dryness. This procedure aids binding of the agarose to the slide. Slices were then covered with 2.5 ml of hot 1.0% agarose in sodium barbital, ionic strength 0.025, pH 8.2, and allowed to set on a level surface.

Coated slides were stored at 4 C in a water saturated atmosphere. Wells were punched with a Gelman well punch after at least 4 hours at 4 C. Plugs were removed by gentle suction from a pasteur pipette attached to a water vacuum pump.

Suitable volumes of antigen and antibody were then placed in the wells. The wells could be loaded with several separate applications.

Immunodiffusion proceeded at 4 C in a water saturated atmosphere overnight. After the precipitin lines had formed the slides were washed overnight in PBS, and then photographed.

RESULTS AND DISCUSSION

6.5 RESULTS AND DISCUSSION ON THE IMMUNISATION PROTOCOL

The practice of intravenous boosters via the marginal ear vein is not recommended. One rabbit that was immunised with antigen died after intravenous marginal ear vein boosting. The booster injection should have been administered in the back skin flap, and there should have been more boosters administered.

There may be a problem with enzyme stability while it is within the rabbit. The enzyme loses a large proportion of its activity over a 24 hour period and this is concomitant with the generation of proteolytic fragments. It is not known whether there is a loss of antigenicity concomitantly with the loss of enzymatic activity. Antibodies derived from the proteolytic fragments were unable to immunoprecipitate native ATPCL, nor did these fragments boost the titre of antibodies in rabbits. It can be concluded that the proteolytic fragments did not have the same antigenic sites as tetrameric ATPCL. The problem of generation of IgG's that are specific to proteolytic fragments will need to be eliminated for the production of a monospecific antisera to ATPCL. Perhaps a cell myeloma system could be used instead of rabbits as a source of IgG.

Immunological experiments were limited by the availability of high titre antisera. Further radiolabelling work was hindered because of this.

Rabbit anti-rat ATPCL serum or purified IgG fraction quantitatively immunoprecipitated rat liver ATPCL whereas the pre-immune serum did not. The antiserum effectively immunoprecipitated ATPCL purified from either Blue Sepharose CI-6B, DEAE 52, and the rat liver cytosol (ultracentrifuge fraction).

Rabbit anti-rat albumin quantitatively immunoprecipitated rat

albumin from rat sera.

6.6 IMMUNOTITRATION

The equivalence point of the antibody for the antigen was determined by immunotitration. Partially purified ATPCL eluted from the gel filtration column and the respun 60Ti ultracentrifuge supernatant (cytosolic fraction) were both used.

A typical immunotitration result assaying the supernatant activity after immunoprecipitation can be seen in Figure 12.

The graph shows that nine percent of the total activity remained in solution in the ultracentrifuge supernatant.

All of the activity could be titrated when fractions from the gel filtration column were used. Not all the ATPCL could be titrated from the supernatant ultracentrifuge fraction even when a four fold excess of antibody was used.

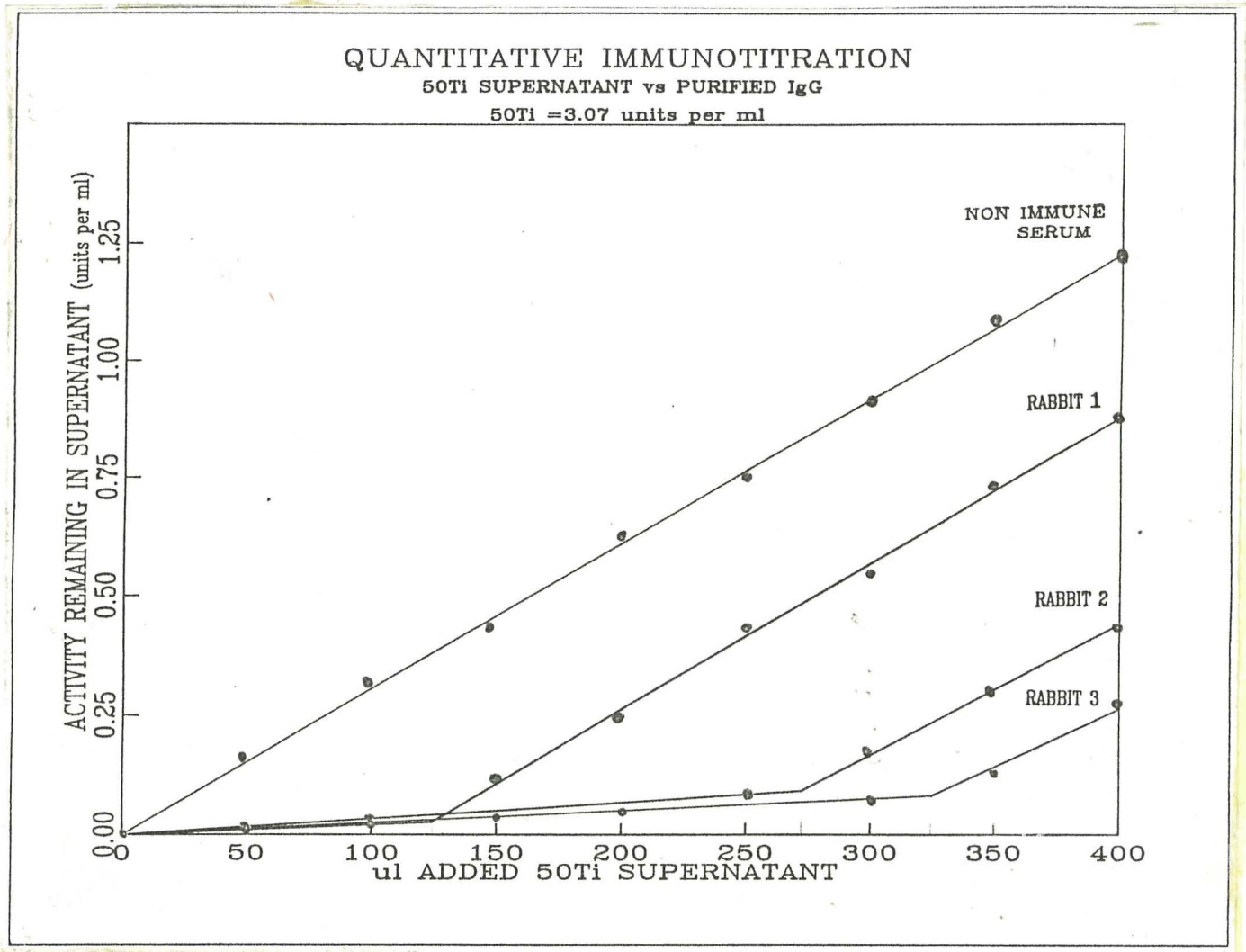
The reason for this finding could be due to different immunological forms of ATPCL, or due to the presence of NADH oxidases in the cytosol. If this form of ATPCL is immunologically different then it is not precipitated in the 0-55% ammonium sulphate fractionation step, but is separated by gel filtration since all of the ATPCL activity could be immunoprecipitated in all fractions following the gel filtration step. It should be noted that no antibodies were generated from fractions not retained on either Blue Sepharose or DEAE 52 columns. Nine to fourteen percent of the ATPCL activity in the cytosol does not bind to DEAE 52 despite increasing the gel to protein ratio 6 fold (see section 3.7).

Alternatively the presence of ATP-dependent NADH oxidases in the supernatant may be interfering with the assay (see section 3.4.1). The equivalence point was determined at the break point of the graph.

The equivalence point is expressed as milligrams of ATPCL immunoprecipitated per ml of antiserum. The total amount of antiserum and the equivalence point is given in Table 12.

Figure 12

Immunotitration of ATP citrate lyase activity from a recentrifuged ultracentrifuge supernatant. Antibody and antigen were added togther in a fixed volume of 0.55 ml. The immunoprecipitate was collected by centrifugation through a discontinuous sucrose gradient. Supernatant was removed and assayed for enzyme activity.



Non immune sera (0.1 ml) prepared from non innoculated rabbit

Rabbit 1 represents using 50 ul of Rabbit 1 antibody
Rabbit 2 represents using 100 ul of Rabbit 2 antibody
Rabbit 3 represents using 50 ul of Rabbit 3 antibody

Rabbit 1 and 2 antibodies were generated from Blue Sepharose eluted ATPCL using preparative method 1 (b).
Rabbit 3 antibodies were generated from DEAE 52 eluted ATPCL using preparative method 2 (a).

TABLE 12

Equivalence point and total amount of purified IgG prepared using different sources of ATPCL.

ANTIBODIES GENERATED FROM ATPCL ELUTED FROM mg ATPCL/ml antiserum	EQUIVALENCE POINT ml	TOTAL VOLUME OF PURIFIED IgG
BLUE SEPHAROSE CL-6B		
RABBIT 1	0.48	20
RABBIT 2	0.53	22
DEAE 52		
RABBIT 3	1.25	25

a Equivalence point determined from 5 immunotitrations.

b Equivalence point units are milligrams of ATPCL immunoprecipitated per ml of antiserum. It is assumed pure ATPCL specific activity is 16 units/mg protein.

The values for the equivalence points listed in Table 12 were later confirmed to be the same order of magnitude by immunoprecipitating radiolabelled ATPCL.

6.7 DISRUPTED ANTIBODY-ANTIGEN IMMUNOPRECIPITATES

The supernatant of the immunotitration was used to determine the equivalence point. Precipitates were collected by centrifugation through a discontinuous sucrose gradient, washed and resuspended 3 times. The antigen - antibody complexes were then disrupted by the use of 6M urea and 1% SDS. Disruption was followed by boiling for 15 min and then electrophoresis in 7% polyacrylamide slab gels.

A typical gel of the disrupted immunoprecipitated complexes can be seen in Figures 13 and 15. Gels stained with Coomassie blue were then scanned as in section 2.6.10. Typical results are shown in Figures 14(a, b) and 16. These antibodies from different rabbits appear to precipitate different protein from the cytosolic fraction (Figure 13, 14(a) and 14(b).

6.7.1 Discussion of proteins immunoprecipitated IgG purified from rabbit 1.

Antibodies from rabbit 1 were induced from ATPCL eluted from Blue sepharose CL-6B using preparative method 1(b). The ATPCL eluted from this column had a specific activity of 6.5 -8.9 units/mg and was used for immunization of this rabbit only if a single band was seen on SDS PAGE at 123,000 Mr at 7 ug loading.

The results shown in Figure 13 and 14(a) show that the immunoprecipitate prepared from rat liver cytosol using the IgG fraction from rabbit 1 contains a number of proteins in addition to the IgG heavy and light chains. The major additional bands in the immunoprecipitate has a Mr of 123,000 which agrees well with the published value for the ATPCL subunit (2,4,5,31,84,164). There is a smaller amount of a protein of Mr 67,000 and trace amounts of protein of 250,000 and 260,000. Later experiments in which radiolabelled proteins from rat liver cytosol were immunoprecipitated using this antibody preparation showed that the Mr

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Figure 13

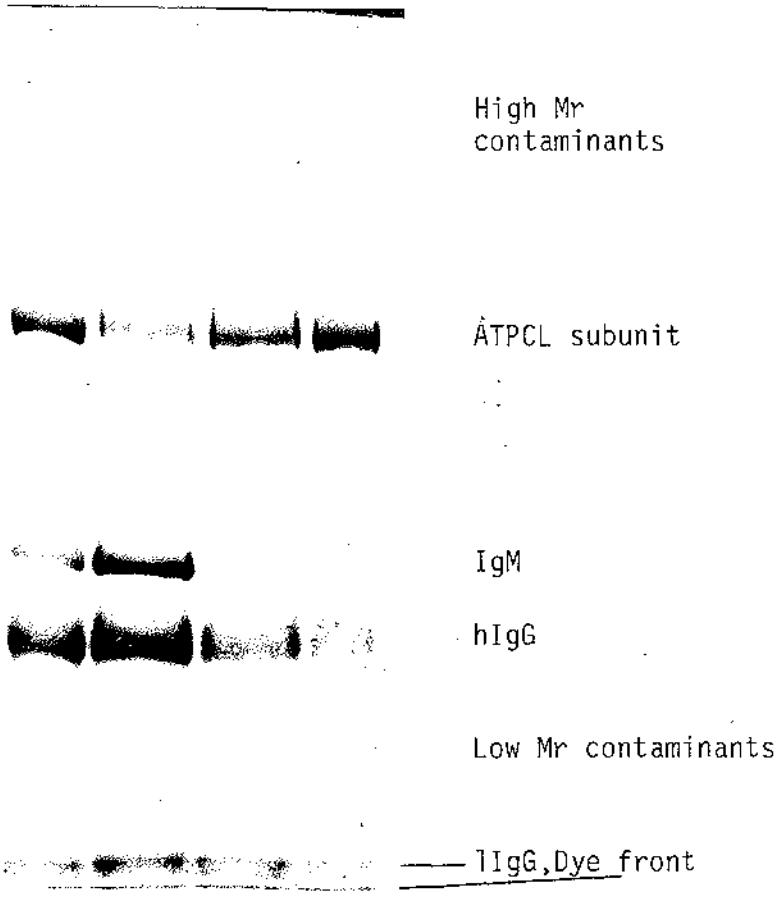
Electrophoretic separation of disrupted antibody - antigen complexes.

ATPCL was immunoprecipitated from the cytosolic fraction using antibodies generated from ATPCL eluted from Blue Sepharose.

Electrophoretic separation was performed as described in section 2.6. The gel was loaded so that the ATPCL subunit represents 20 ug of Coomassie stained protein.

Figure 13

Electrophoretic separation of antibody-antigen complexes immunoprecipitated from the cytosolic on a 7% SDS PAGE slab gel.



Track 1 & 2	Rabbit 1 vs 'cytosolic' fraction.
Track 3	Rabbit 2 vs 'cytosolic' fraction.
Track 4	Rabbit 1 & 2 vs purified ATPCL eluted from DEAE 52 column.

Table

hIgG heavy chain immunoglobulin.

lIgG light chain immunoglobulin.

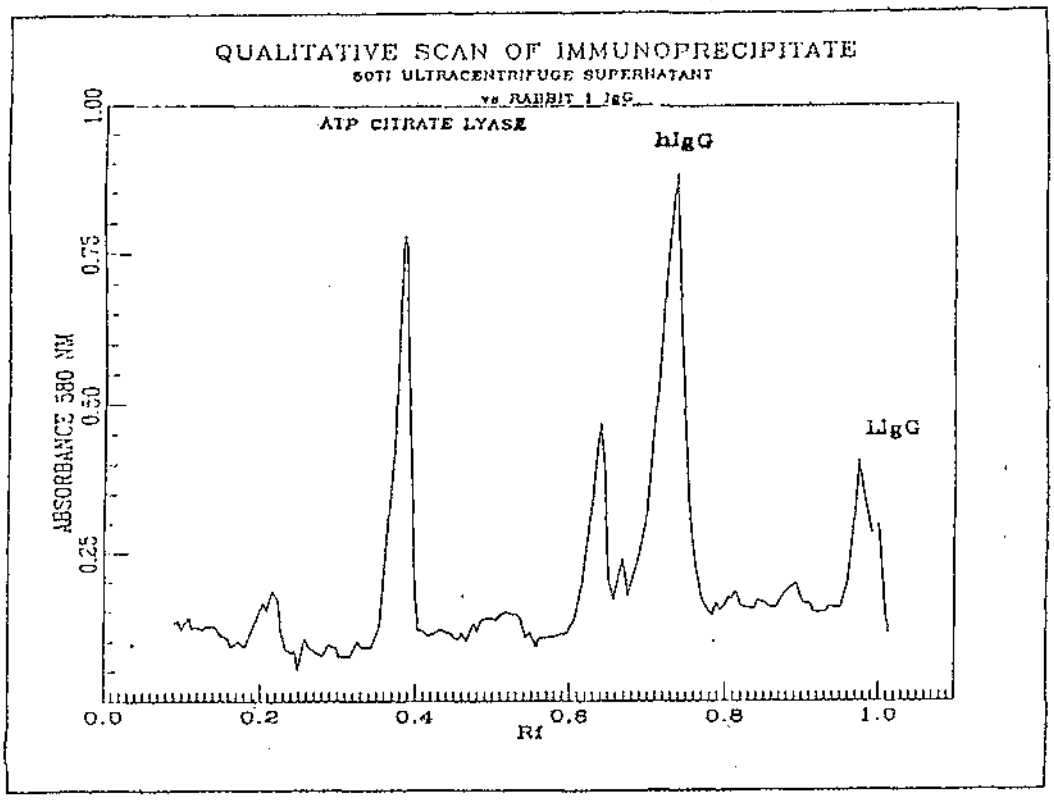
IgM immunoglobulin M.

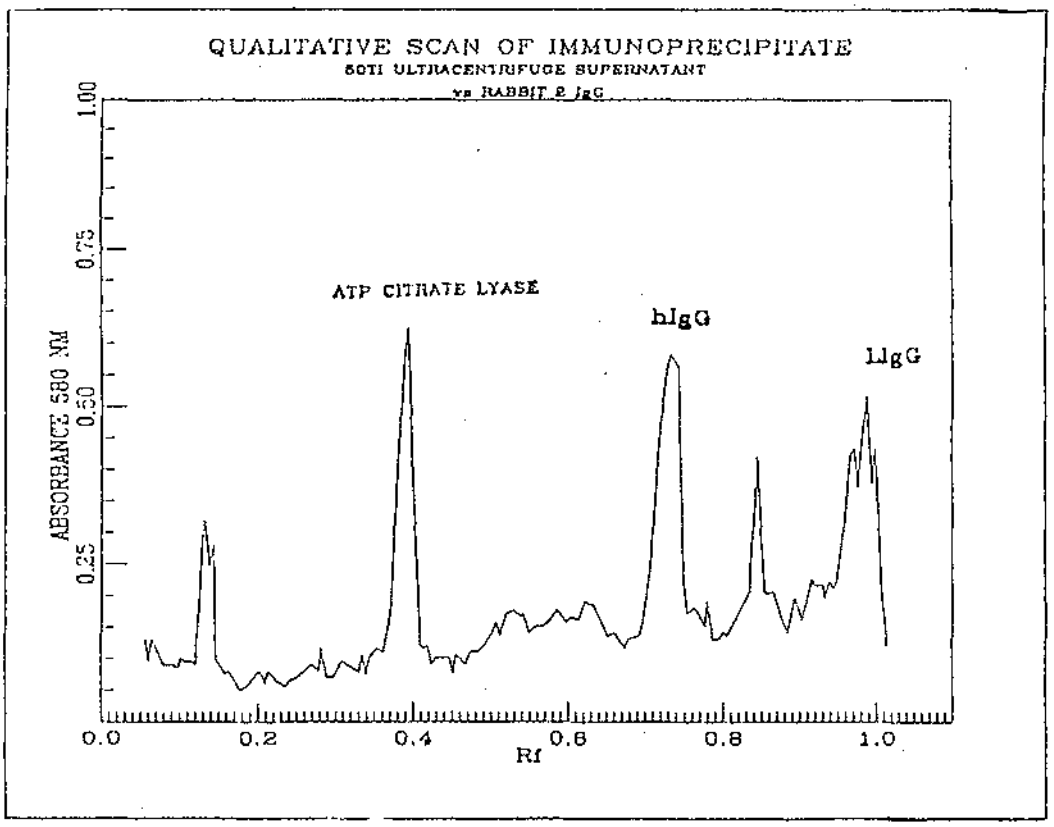
Figure 14 (a) and 14 (b).

Microdensitometer tracings of gel tracks from Figure 12 scanned at 580 nm. 13 (a) = track 1 and 13 (b) = track 3 Figure 12. Proteins were immunoprecipitated from cytosolic fraction as described in section 6.4.2. Twenty ug of ATPCL subunit was immunoprecipitated. The Rf was calculated relative to the Bromophenol blue dye front.

Figure 14 (a)
Purified IgG from rabbit 1 vs recentrifuged cytosol.

Figure 14 (b)
Purified IgG from rabbit 2 vs recentrifuged cytosol.


QUALITATIVE SCAN OF IMMUNOPRECIPITATE
50Ti ULTRACENTRIFUGE SUPERNATANT
vs RABBIT 1 IgG
ATP CITRATE LYASE
hIgG
LIgG
ABSORBANCE 580 NM
0.25
0.50
0.75
1.00
0.0
0.2
0.4
0.6
0.8
1.0
Rf


QUALITATIVE SCAN OF IMMUNOPRECIPITATE
50Ti ULTRACENTRIFUGE SUPERNATANT
vs RABBIT 2 IgG
ATP CITRATE LYASE
hIgG
LIgG
ABSORBANCE 580 NM
0.25
0.50
0.75
1.00
0.0
0.2
0.4
0.6
0.8
1.0
Rf

67,000 band was not labelled. It is probable that this band is rabbit IgM heavy chain.

It is not unusual for IgM to be present with IgG in early bleeds after a primary antigenic challenge since IgM is the first antibody produced.

The major protein band immunoprecipitated is the 123,000 Mr subunit of ATPCL (Rf 0.38). This band is clearly separate from any other contaminating protein band. This was of importance when considering the sectioning of radioactive ATPCL subunit from other labelled protein bands. It was not known if the antibodies were specific for the tetramer and/or for the subunit.

The 123,000 Mr band of the ATPCL subunit was well separated from any other protein bands. This facilitated the subsequent sectioning of this band from the gel and enabled the radioactivity of this band to be determined.

The major proteolytic fragments of 53,000 and 57,000 Mr, which are generated by the action of trypsin on native ATPCL, could not be seen in these gels because they comigrate with the heavy chain of IgG (55,000 Mr). The sectioning of SDS PAGE gels containing immunoprecipitated radiolabelled proteins was subsequently used to test whether these major ATPCL fragments were present.

The gel scan of the disrupted antigen-antibody complexes shown in Figure 13 and Figure 14 shows that the antibody from rabbit 1 was more selective in immunoprecipitating ATPCL than that from Rabbit 2.

6.7.2 Discussion of proteins immunoprecipitated by Rabbit 2 purified IgG.

The scan of Coomassie stained protein derived from disrupted antibody-antigen complexes from track 3 Figure 13 can be seen in Figure 14(b). This scan shows that antibodies from rabbit 2 immunoprecipitate 2 proteins of high Mr. These proteins are the same Mr (266,000 and 250,000) as the contaminants precipitated by Rabbit 1 IgG. These proteins could be fatty acid synthase or Acetyl CoA carboxylase despite

there being no activity for these enzymes in the fractions used for inoculation.

ATPCL has been reported to exist as a complex with these enzymes (61,175,177) and it is possible that they may be precipitating as a complex.

Purified IgG from Rabbit 2 also precipitates small amounts of two proteins of low molecular weight. One of these has a Mr of 37,000. The other protein band occurs just ahead of the light chain immunoglobulin band (Rf 0.97) with an Mr of 26,000.

The major degradation products of proteolysis of ATPCL (53,000 & 57,000 Mr) could not be seen as these are hidden in heavy chain IgG (55,000 Mr).

ATPCL subunit (Mr 123,000) constituted the major protein precipitated by this antibody. The subunit is distinct (Rf 0.38) and not surrounded by contaminating protein peaks.

When 60 ug of ATPCL subunit was immunoprecipitated with purified Rabbit 2 IgG additional protein bands were seen on SDS PAGE slab gels. These results can be seen in Figure 15.

Three additional minor bands are seen at 85-95,000 and 2 at 68,000. Multiple bands are seen at below 55,000 Mr.

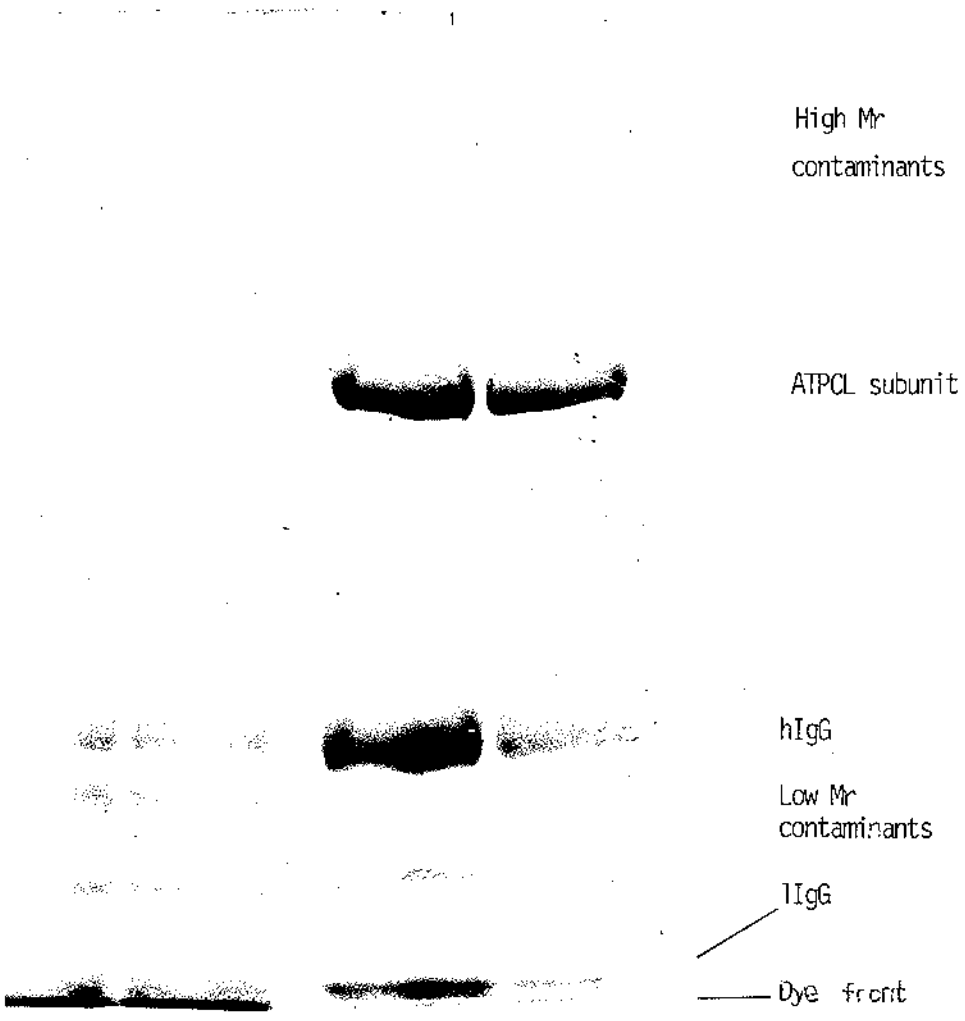
It can be concluded from both the gel scan of disrupted antibody-antigen complexes (Figures 14(b) and Figure 15 that the antibody was not monospecific towards ATPCL. This occurred despite a strict selection process in choosing fractions that were to be used for immunization.

6.7.3 Discussion of proteins immunoprecipitated by purified IgG from rabbit 3.

ATPCL active fractions obtained after elution from the DEAE-52 cellulose column, which had a specific activity of greater than 10 units/mg and which showed only the ATPCL subunit at 20 ug loading on SDS PAGE slab gels (as seen in Figure 5), were used for inoculation into

Figure 15

Detail of Rabbit 2 disrupted antibody-antigen complexes separated on SDS PAGE slab gels.



Tracks 1 & 2 20 ul TCA precipitated recentrifuged cytosol.
Tracks 3 & 4 Rabbit 2 vs cytosolic fraction.

Table

hIgG heavy chain immunoglobulin.
lIgG light chain immunoglobulin.
IgM immunoglobulin M.

this rabbit.

The antibodies that were generated were purified and the specificity towards ATPCL was tested by precipitating proteins from the recentrifuged cytosolic fraction. A typical scan of the Coomassie blue stained proteins from the disrupted antibody-antigen complexes after separation on SDS-PAGE can be seen in Figure 16.

The gel scan of proteins precipitated by purified IgG from rabbit 3 shows that the ATPCL is the major protein band precipitated (Rf 0.385). In addition to the ATPCL subunit there are 3 proteins of high Mr and 7 other minor protein bands precipitated by purified Rabbit 3 antibodies.

These protein contaminating proteins have a subunit molecular weight of

260,000, 250,000, 245,000, 95,000, 75,000, 53,000
51,500, 48,000, 42,000, 39,000.

There is also a contaminating protein that has the same Mr as light chain of IgG (23,000). The LIgG band is significantly elevated in the scan when compared to all of the other scans (Figures 14(a) & (b)). This protein band is probably the final degradation product of ATPCL (22,000 Mr) as seen in Figure 7(a).

The gels scan shows that this antibody is not monospecific towards ATPCL. The contaminating proteins that are immunoprecipitated are distinctly separate in molecular weight from the ATPCL subunit.

6.7.4 Immunoprecipitation of purified ATPCL

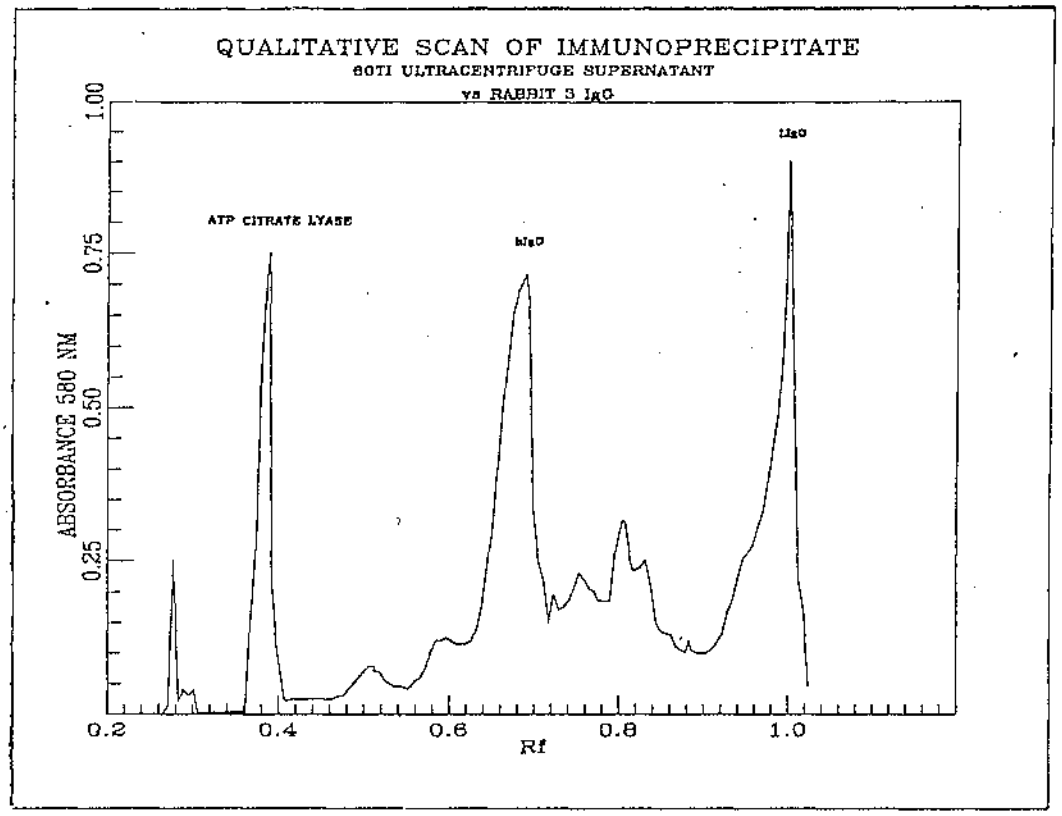
One hundred ul of the IgG fraction from Rabbit 1 and Rabbit 2 were mixed and used to immunoprecipitate purified ATPCL (specific activity 10 units/mg). The enzyme had previously been purified by elution from the DEAE 52 column (preparative method 2(a)).

The antibody-antigen complex was precipitated, disrupted and the

Figure 16

Microdensitometer tracing of the SDS-PAGE separation of the proteins from an immunoprecipitate from rat liver cytosol with the IgG fraction from rabbit 3.

Proteins were immunoprecipitated from the cytosolic fraction as described in 6.4.2 using purified Rabbit 3 IgG. Antibody-antigen complexes were collected as described in section 6.4.2. An amount of cytosol which contained 20 ug of ATPCL was immunoprecipitated.


QUALITATIVE SCAN OF IMMUNOPRECIPITATE
60TI ULTRACENTRIFUGE SUPERNATANT
vs RABBIT 3 IgG
ATP CITRATE LYASE
ABSORBANCE 580 NM
0.25
0.50
0.75
1.00
0.2
0.4
0.6
0.8
1.0
Rf

proteins electrophoresised on SDS-PAGE gels as previously described. A photograph of the electrophoresed protein bands can be seen in Figure 13 (track 4).

The scan of the Coomassie staining protein from the disrupted antibody-antigen complex (as seen in Figure 13) can be viewed in Figure 17.

This scan shows that only the 123,000 Mr ATPCL subunit is immunoprecipitated.

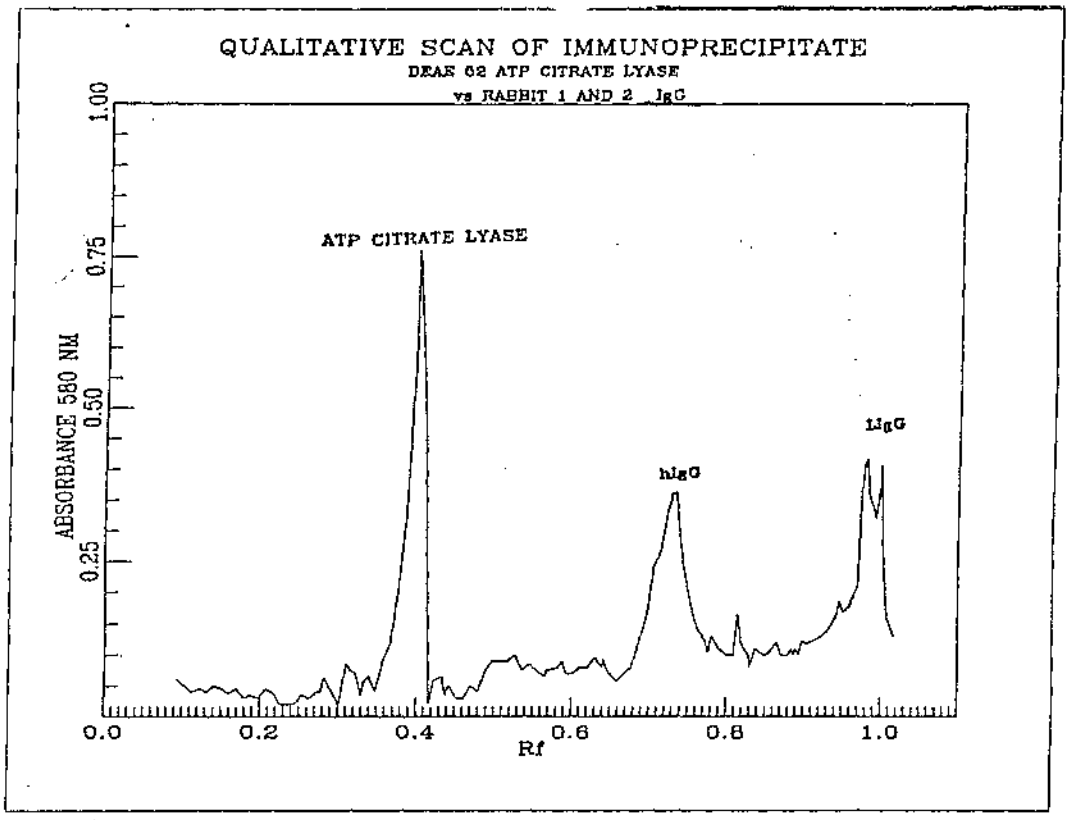
The scan shows that there is a possible mechanism for the production of a monospecific antibody to rat liver ATPCL by first producing antibodies from ATPCL via preparative method 1(b). Secondly, purifying ATPCL by preparative method 2(a). The antibody and the antigen could then be mixed and the immunoprecipitate collected as described in section 6.4.2. After collecting and washing the immunoprecipitate could be resuspended in PBS, emulsified with adjuvant and then immunized into rabbits.

It is interesting to note that no high molecular weight proteins or IgM heavy chain is immunoprecipitated when purified ATPCL was immunoprecipitated. Two high molecular weight proteins are precipitated when Rabbit 1 and 2 IgG are added to the cytosolic fraction (Figure 14(a) and 14(b)).

Rabbit 3 antibodies precipitate 3 proteins of high molecular weight when challenging the cytosolic fraction (Figure 16). High Mr proteins must be present with the ATPCL eluted from DEAE 52 eluted to generate an immunological response in Rabbit 3. The contaminants precipitated by Rabbit 1 and 2 are either not present (immunologically different) or in low concentration in the purified ATPCL fractions from DEAE 52. The IgM heavy chain, that is present when antibodies from rabbit 1 are used to immunoprecipitate ATPCL from the cytosol (Figure 13), may be precipitating only the contaminant proteins or degradation products. These proteins are not present with the purified ATPCL by Method 3.

Additional experiments using purified radiolabelled ATPCL will be required to confirm that no radioactivity is present in the proteolytic fragments (57,000, 53,000, and 22,000 Mr). This information cannot be obtained directly from the gels because the major fragments are obscured

Figure 17


QUALITATIVE SCAN OF IMMUNOPRECIPITATE
DEAE 62 ATP CITRATE LYASE
vs RABBIT 1 AND 2 IgG
ATP CITRATE LYASE
hIgG
lIgG
ABSORBANCE 580 NM
0.25
0.50
0.75
1.00
0.0
0.2
0.4
0.6
0.8
1.0
Rf

by the light and heavy chain of IgG (23,000 and 55,000).

6.8 Results and discussion of Ouchterlony Double immunodiffusion

Antisera raised against purified hepatocyte ATPCL showed a single precipitin line by Ouchterlony double diffusion (Ouchterlony 1948) when challenged against partially purified ATPCL. A typical Ouchterlony plate can be seen Figure 18, 19 and 20.

When the purified antibodies were challenged against the recentrifuged cytosolic fraction, multiple bands were observed. This observation alone, does not provides evidence that the antibodies are not monospecific since multiple bands may be present in Ouchterlony plates if the enzyme exists in multiple polymeric states.

Ouchterlony plates of purified IgG challenging partially purified ATPCL and recentrifuged cytosolic fraction.

Partially purified ATPCL (eluted from the Sephacryl S-200 column as described in Prep. method 1(b)) or recentrifuged cytosolic fraction was used as a source of ATPCL protein. 5-60 ug of ATPCL protein was loaded into the central well of an Ouchterlony plate. 50-200 ul of antibody was added to the outer wells. Immunodiffusion was allowed to proceed for 24 hours in a water saturated atmosphere at room temperature for 2 hours & then overnight at 4 C. Plates were washed in PBS, 2% salt overnight, then photographed.

6.9 CONCLUSION OF THE IMMUNOLOGICAL PROTOCOLS AND QUANTIFICATION OF THE ANTIBODY-ANTIGEN INTERACTION.

Antibodies to rat liver ATPCL were raised in Rabbits 1, 2 and 3. These antibodies were initiated from immunological responses to ATPCL prepared by preparative method 1(b) (Rabbit 1 and 2) or method 2(a) (Rabbit 3).

Figures 18, 19 and 20.

Ouchterlony plates of purified IgG challenging partially purified ATPCL and recentrifuged cytosolic fraction.

Partially purified ATPCL (eluted from the Sephacryl S-200 column as described in preparative method 1 (b) or recentrifuged cytosolic fraction was used as a source of ATPCL protein. 5-60 ug of ATPCL protein was loaded into the central well of an Ouchterlony plate. 50-200 ul of antibody was added to the outer wells. Immunodiffusion was allowed to proceed for 24 hours in a water saturated atmosphere at room temperature for 2 hours and then overnight at 4° C. Plates were washed in PBS, 2% salt overnight, then photographed.

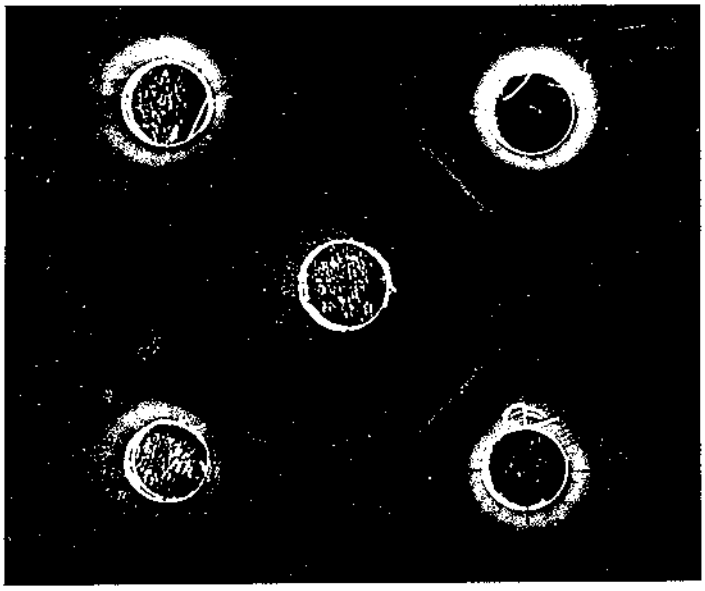


Figure 18

Rabbit 1 IgG vs ATPCL partially purified.

LHS		RHS
5 ug ATPCL		30 ug ATPCL
	70 ul IgG	
5 ug ATPCL		30 ug ATPCL

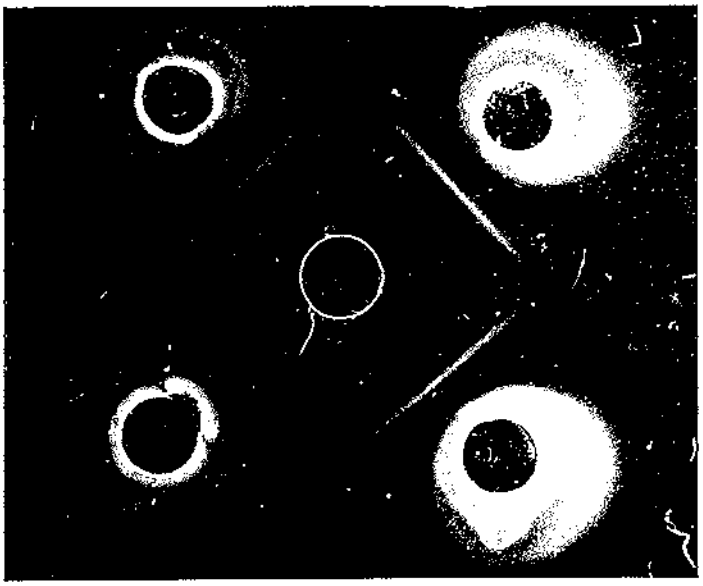


Figure 19

Rabbit 2 IgG vs ATPCL partially purified

LHS		RHS
60 ug ATPCL		60 ug ATPCL
	70 ul IgG	
60 ug ATPCL		60 ug ATPCL

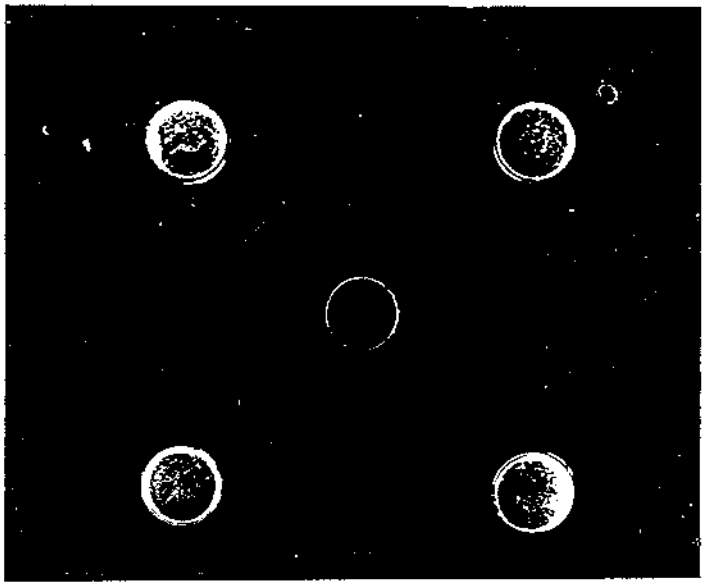


Figure 20

Rabbit 1 IgG vs Cytosolic fraction

LHS		RHS
30 ug ATPCL		30 ug ATPCL
	70 ug IgG	
30 ug ATPCL		30 ug ATPCL

No rabbits were immunized with ATPCL prepared by method 3 due to lack of time.

The IgG was purified from the sera and then tested against either purified ATPCL or recentrifuged cytosolic fraction.

The equivalence point of the antibody for ATPCL was determined by immunotitration. Rabbits 1 and 2 had a lower titer than Rabbit 3. The 2.5 fold increase in titer of Rabbit 3 can be directly attributed to the greater amount of antigen injected into this rabbit. Ten percent of the total activity in the cytosol fraction could not be titrated from solution. This may be due to either an interference from ATP-dependent NADH oxidases in the assay or an immunologically different form of ATPCL. No antibodies were made to ATPCL active fractions that are not retained on Blue Sepharose or DEAE 52.

When purified IgG was used to immunoprecipitate proteins from the recentrifuged cytosolic fraction all antibodies were shown not to be monospecific towards ATPCL. This was determined by scanning of the SDS PAGE gel tracks of the disrupted antibody-antigen complexes (Figures 14a & b, and 16).

The immunological response in Rabbit 1 was different from Rabbit 2 even though both rabbits received antigen derived from preparative method 1(b). Rabbit 1 has a large amount of circulating IgM whereas Rabbit 2 antibodies are predominantly IgG (Figure 13).

When the amount of ATPCL subunit that was precipitated by Rabbit 2 antibodies was increased from 20 to 60 ug multiple bands are seen on SDS PAGE gels (Figure 15). The purified IgG precipitated at least two high and several low Mr protein subunit with a 20 ug loading of the ATPCL subunit on SDS PAGE.

Rabbit 1 and 2 purified IgG were mixed and then used to immunoprecipitate purified ATPCL, eluted from the DEAE 52 column (preparative method 2(a). Only three Coomassie staining protein bands were seen on SDS PAGE of the disrupted antibody-antigen complex. These were the ATPCL subunit, heavy and light chain IgG (Figures 13 and 17).

This method should be used for the production of monospecific antiserum towards ATPCL since preparative method 2(a) is insufficient to supply homogenous ATPCL.

The heavy chain IgM is absent from this gel scan even though it is present with the IgG fraction of rabbit 1. The circulating IgM found in antibodies from rabbit 1 may be immunoprecipitating proteins other than ATPCL.

Immunodiffusion experiments showed that when using partially purified ATPCL no secondary precipitin lines were seen on Ouchterlony plates with a 60 ug loading of ATPCL (Figures 18 and 19). When antibody was used to challenge the recentrifuged cytosolic fraction 2 precipitin lines were seen (Figure 20).

Because of the incomplete specificity of antibodies prepared against ATPCL it was necessary, when examining the synthesis of labelled protein, to separate each immunoprecipitate by SDS-PAGE and examine the amounts of radioactivity in the ATPCL subunit band.

CHAPTER SEVEN.

LABELLING OF LIVER PROTEINS IN VIVO.

7.1 INTRODUCTION

The capacity of rat liver to synthesize albumin and ATP citrate lyase *in vivo* can be determined by immunochemical isolation of these proteins after administration of radioactive isotope.

The accumulation of label in ATPCL in rat liver cytosol and serum albumin in serum was examined after 24 hour intraperitoneal labelling with 14-C protein labelling or 35-S methionine. The antibody preparations described, in the previous chapter were used for immunochemical isolation of labelled protein.

In separate experiments, purified antibodies from Rabbit 1 and 2 were used to immunoprecipitate (14-C) or (35-S)-radiolabelled ATPCL. Antibodies from Rabbit 3 were used to immunoprecipitate only (35-S) methionine radiolabelled ATPCL. Rabbit anti-rat albumin IgG was used to precipitate albumin from rat sera.

To reduce experimental error the amount of albumin was normalized to 65% of the total TCA precipitable protein in rat sera as described in chapter 5.

The antibody/radiolabelled antigen complexes were disrupted and examined on SDS PAGE. These gels were sectioned into small slices and the radiolabel within each section determined as in section 2.9.

This procedure is the most sensitive technique for determining the specificity of antibody for the antigen (Cashman and Pitot 1971).

7.2 MATERIALS

L-[35 S]Methionine (250 uCi per mmol) and [14 C]protein hydrolysate (50 mCi per mol) were obtained from the Radiochemical Center, Amersham, England and were stored at -70 C until just prior to use.

The antibody preparations used have been described in section 6.3

7.3 METHODS

7.3.1 Interperitoneal (24 hour) labelling.

A male rat (150 gram) was starved for 2 days, then refed a high carbohydrate diet for either 2 or 3 days. A day prior to the completion of the dietary regime the rat was injected intraperitoneally at 9.00 am with 1 ml of PBS containing 50 uCi of [14-C] protein hydrolysate or 250 uCi [35-S] methionine. Food was returned to the animal at 6.00 pm. At 9.00 am the following day the rat was killed by cervical dislocation.

Cardiac blood was collected at this time and the serum obtained (section 6.3.2) was used for determination of the radioactivity in the serum protein after immunoprecipitation. In all experiments the albumin content of the serum was normalized to 65 % as previously described.

The liver was perfused via the portal vein with 10.0 ml of 250 mM sucrose, 1mM magnesium chloride to remove the blood. It was then excised, blotted dry, weighed and passed through a stainless steel tissue press to disrupt the tissue.

The minced tissue was then processed as in section 3.2 except that the homogenisation buffer contained 0.1% Triton X-100 and 0.1% sodium deoxycholate and the middle portion of the ultracentrifuge supernatant was recentrifuged 250,000g for 2 hours (Beckman 60.Ti rotor 40,000 rpm). The mid-portion of the respun fraction was removed by pasteur pipette and labelled as "recentrifuged cytosol".

The recentrifuged cytosolic fraction was used for:

(1) the determination of the total incorporation of radioactivity into the tricholoroacetic acid precipitable protein. (section 2.10),
(2) assayed for ATP citrate lyase activity,
(3) used for the immunoprecipitation of radiolabelled ATP citrate lyase using purified IgG antibody.

7.3.2 Immunoprecipitation of ATP citrate lyase

The procedure for the immunoprecipitation of ATPCL labelled with [14-C] protein hydrolysate or [35-S] methionine from rat liver cytosol was modified as described by Schmike (1975), Gifforn (1984), Benjamin 1975 & 1978, and Hopkirk (1979).

The immunoprecipitation was performed by incubation of the recentrifuged cytosol with constant amount of antibody at 2.8 times the concentration required at the equivalence point (as determined in preliminary immunotitration experiments, section 6.4.2 and 6.6).

Antigen was precipitated from the recentrifuged ultracentrifuge supernatant solution containing Triton and deoxycholate by adding 0.33 ml of antisera to 0.18 ml of extract. This was allowed to stand for 1 hour at 37 C, and then overnight at 4 C. Immunoprecipitated antibody-radiolabelled antigen complexes were collected by centrifugation in an Eppendorf Microcentrifuge for 15 minutes through a discontinuous 1 M sucrose gradient and washed 5 times with 0.5 ml potassium phosphate containing 100 mM sodium chloride, 2 mM DTT, 0.1 mM EDTA, 1 mM Magnesium sulphate (pH 7.0) and again respun through a discontinuous sucrose gradient.

The precipitate was then disrupted and electrophoresed on SDS-PAGE as described in section 2.6.8. The radioactivity within any band was determined as described in section 2.9.

A radiolabelled protein sample from the second ultracentrifuge supernatant was obtained routinely by TCA precipitation and then subjected to SDS-PAGE (section 2.6) after sample preparation.

This sample was used to compare :

(1) the proportion of the radioactivity present in any protein band compared to the total in the recentrifuged cytosolic fractions supernatant.
(2) the proportion of the total radioactivity that is immunoprecipitated by the antibody.
(3) the amount of radioactivity present in the immunoprecipitated protein band compared to the amount found in that band in the ultracentrifuge fraction.

7.4 IMMUNOPRECIPITATION OF ATPCL FROM RAT LIVER CYTOSOL.

7.4.1 Results and discussion of 24 hour intraperitoneal labelling of a rat refed a high carbohydrate diet for 2 days.

The recentrifuged cytosolic fraction derived from a rat refed a high carbohydrate diet for 2 days had the following experimental characteristics:

TABLE 13

Fraction	Volume ml	a ATPCL Activity units/ml	b Protein mg/ml	c Radioactivity DPM/ml
recentrifuged cytosol	11	1.93 +/-0.10	25.84 +/- 0.25	53850 +/-1570

a Activity determined by malate dehydrogenase coupled assay. A unit of activity is defined as the number of umol of AcetylCoA produced/min/ml as monitored by NADH oxidation at 340 nm.

b Protein determined with Biuret reagent after TCA precipitation as described in section 2.5.

c Radioactivity in TCA precipitable protein was determined as described in section 2.10. Result represents 24 determinations.

The disrupted antigen/radiolabelled immunoprecipitates were subjected to SDS-PAGE. Gels were then cut into 2 mm wide tracks and the radiolabel within each track determined as in section 2.9.

The Rf was determined relative to the bromophenol blue which migrated 110.5 mm from origin. The regression line for mobility (Rf) versus molecular weight (Mw) derived from standards of known molecular weight was:

$$Rf = -0.6618 \log Mw + 3.767$$

This gives reasonable accuracy for the determination of molecular weights between 19,000 to 260,000. The Molecular weights of unkown protein bands were calculated from the center of the sliced section. The first 10 mm of a gel represents the stacking gel and no molecular weights can be determined in this region.

The results represent the mean of at least two individual experiments. The gel loading was such that the ATP citrate lyase subunit represented 20 ug of protein.

The (14-C) radioactive protein bands immunoprecipitated by Rabbit 1 and 2 antibodies can be seen in table 14.

TABLE 14

Gel slice Rf	Molecular Weight	Radioactive proteins precipitated from recentrifuged cytosol by a TCA	b RABBIT 1 IgG	RABBIT 2 IgG	
		DPM/ml of recentrifuged cytosol			
0.030	stacking gel	1850	0	0	
0.060	stacking gel	1650	1977	0	
0.090	stacking gel	1740	198	12	
0.121	320,000	2090	38	12	
0.152	290,000	1160	182	32	
0.182	266,000	1500	321	26	
0.212	235,000	2120	19	0	
0.242	212,000	2690	45	12	
0.273	190,000	1610	27	39	
0.303	170,000	2920	193	11	
0.364	139,000	1710	13	9	
0.394	125,000	1320	284	300	ATPCL
0.424	112,000	2430	61	76	
0.455	101,000	1450	56	72	
0.485	91,000	2860	34	89	
0.515	82,000	1590	48	0	
0.545	74,000	1630	57	0	
0.576	66,000	3320	0	0	
0.606	60,000	3380	109	0	
0.636	54,000	1830	133	20	
0.666	48,500	1720	61	40	
0.727	39,000	2120	7	140	
0.818	28,600	1590	0	15	
0.849	26,000	1350	0	168	
0.879	23,000	1030	6	60	
0.909	21,000	1740	203	377	
0.939	19,000	1310	59	60	
0.970	17,000	970	0	140	
0.999	15,000	1150	0	0	
1.020		120	0	0	
Total radioactivity in whole track (Dpm)		53830			

a TCA track scan represents total radioactivity present in Trichloroacetic acid ppt protein.

b Rabbit 1 and 2 represent the proteins immunoprecipitated from the recentrifuged cytosolic fraction using purified IgG. Data has been scaled so that radioactive albumin control represents 65% of the total TCA precipitable protein.
Background has been removed for all slices (29 +/-2 DPM).

7.4.2 Discussion of the (14-C) radio-labelled proteins that were precipitated by TCA from the recentrifuged cytosolic fraction.

The total amount of radioactivity present in protein bands in the TCA precipitated sample from a rat refed a high carbohydrate diet for 2 days was 53830 DPM/ml of solution. This total is in good agreement with experiments in which rat cytosol was precipitated by TCA and then counted (53850 +/- 1570 DPM/ml (N=19 determinations).

If we assume the specific activity of pure ATPCL is 16 units per mg of protein, then the ATPCL determined by the assay represents 0.467 +/- 0.073% of the total TCA precipitable protein.

If the 110-125,000 band (Rf 0.394 to 0.424) represents ATPCL then it represents 6.96% of the total amount of TCA precipitable protein. This proportion is very high when compared to that obtained from the assay of ATPCL, and is an overestimate due to the presence of other proteins other than ATPCL at this position in the gel.

7.4.3 Discussion of the immunoprecipitated (14-C) radiolabelled proteins.

Anti-ATP citrate lyase antibodies induced in Rabbit 1 by ATPCL purified through Blue Sepharose (Method 1(b)) precipitated 4130 +/- 250 DPM per ml of ultracentrifuge supernatant, which represents 7.7% of the total radioactivity.

By comparison antibodies from Rabbit 2 immunoprecipitated 1710 DPM/ml of ultracentrifuge supernatant. This represents 3.2% of the total.

However 48% of all of the radioactive proteins immunoprecipitated with antibodies from Rabbit 1 remained in the second 2 mm slice. This high molecular weight contaminant was not due to insufficient urea, B-mercaptoethanol, or boiling as this band was present at this position even after increasing the urea concentration to 6M, a 5 fold increase in B-mercaptoethanol concentration, and increasing the boiling time to 15 minutes. Antibodies from rabbit 2 did not immunoprecipitate these high molecular weight contaminants to the same extent as indicated in Figure

14(b).

The 240-260,000 band present in the scan is actually a doublet, and these two bands could be separated by finer sectioning of the gel. When the two bands were separated the radioactivity was distributed evenly between the two slices. IgG from Rabbit 2 did not immunoprecipitate any significant amount of radiolabelled proteins in this area. Increasing the amount of cytosol so that 60 ug of ATPCL was immunoprecipitated would have shown additional protein bands similiar to the result obtained in section 6.6.2.

Purified IgG from rabbit 1 also immunnoprecipitates radioactive proteins that are equivalent in molecular weight to immunoglobulin heavy chain IgG (Mr 55,000). These are presumably the two proteolytic fragments of ATPCL with molecular weights of 53,000 & 57,000 formed by trypsin like proteolysis of ATPCL. These two fragments represent 2.6% of the total radioactivity precipitated by these antibodies.

Purified IgG from rabbit 2 did not immunoprecipitate these proteins.

The reason for this difference may lie in the location of the immuno-determinants, as well as stability of the sample used for immunisation.

Antibodies produced in rabbit 2 immunoprecipitate proteins of 39,000, 26,000, 17,000 and a 21,000 Mr. These non-ATPCL protein contaminants represented 48% of the radioactivity precipitated by the antibodies.

The 21,000 molecular weight protein is precipitated by all the antibodies raised against ATPCL and may be the final degradation product or a binding protein (210). A protein of molecular weight was observed when ATPCL was purified by preparative method 3 (Figure 7(a)).

It is interesting to note that when ATP citrate lyase was immunoprecipitated from solution it did not contain the 3,500 molecular weight stabilising factor (127,130). No significant amounts of radioactivity were located in gel segments near the bromophenol blue dye front. It is not known whether the binding of the stabilising factor affects the immunological recognition of the antibody for ATPCL. If it does, then

it could explain why all the ATPCL activity could not be titrated from solution despite a ten fold excess of antibody.

Because the purified IgG produced by both rabbits were not monospecific towards ATPCL the proportion of radioactivity in ATPCL compared to the total amount of radioactivity should be made after excising the ATPCL subunit from the SDS PAGE gel.

7.5 CALCULATION OF THE PROPORTION OF IMMUNOREACTIVE ATP CITRATE LYASE IN THE ULTRACENTRIFUGE SUPERNATANT.

In the experiment described in the previous section antibody was present to excess. Consequently the proportion of ATPCL to the total TCA precipitable protein can be determined. The proportion of ATPCL in a rat that has been starved 2 days then refed a high carbohydrate diet for 2 days is 292/53830 DPM = 0.542 +/- 0.048%.

The above experiment was totally repeated 16 times using Rabbit 1 antibodies and 12 times using Rabbit 2 antibodies using the same radiolabelled rat. In these experiments only the 125,000 +/- 7,000 molecular weight band was excised from the gel. This was confirmed by staining the remaining gel. The radioactivity present within this slice was then determined as before.

Antibodies from Rabbit 1 immunoprecipitated 279 +/- 24 DPM/ml of recentrifuged cytosol supernatant when present in 3 fold excess. ATP citrate lyase represents 0.518 +/- 0.060 % of the total TCA precipitatable protein (N=16 determinations). Antibodies from Rabbit 2 immunoprecipitated 259 +/- 13 DPM/ml from the ultracentrifuge supernatant when present in a threefold excess. ATP citrate lyase represents 0.481 +/- 0.048 % of the total TCA precipitable protein (N= 12 determinations).

7.6 RADIOIMMUNOTITRATION

In these experiments 0.6 mg of Rabbit 1 purified antibody was used to precipitate radiolabelled ATPCL from 0.18 ml of recentrifuged cytosolic supernatant. The immunoprecipitated radiolabelled ATPCL subunit

was collected, excised and counted from SDS PAGE gel slices as previously described.

TABLE 15

Tube number	1	2	3	4	5	6
cytosol supernatant (ul)	0	100	200	400	800	1600
homogenisation buffer (ul)	1800	1700	1600	1400	1000	200
Rabbit 1 purified IgG (ul)	200	200	200	200	200	200
a Radioactivity in (14-C) ATPCL subunit (DPM/ml of supernatant)						
b	0	29	50	121	189	209

a Radioactivity immunoprecipitated in ATPCL subunit has been scaled to 1 ml of recentrifuged cytosol.

b Samples were in triplicate. Albumin immunoprecipitated from sera has been scaled to 65% of the total TCA precipitable protein.

The results of Table 15 show that 200 ul of antibody is neutralised by 800 and 1600 ul of supernatant. The equivalence point for 0.2 ml of antisera is 209 +/- 25 DPM/ml of supernatant. Therefore a ml of antisera will neutralise 1045 +/- 125 DPM in the ATPCL subunit.

In the previous section, using the similar cytosolic fractions, ATPCL represented 0.518 +/- 0.060% of the total TCA precipitable protein. Therefore a ml of antisera will neutralise 3.75 +/- 0.10 ml of supernatant or 450 +/- 140ug of ATPCL. The assumptions that were made were that ATPCL represents a typical (14-C) radiolabelled protein and that the specific activity of pure ATPCL is 16 units/mg. The supernatant was shown to have similiar characteristics as those in Table 13. The results obtained here are in agreement with the value of 0.48 mg/ml of antisera obtained in section 6.6.

The antibody was in excess for tubes 1-5, therefore the proportion of immunoreactive ATPCL to the total protein could be determined. ATPCL represents 0.501 +/- 0.077% of the total TCA precipitable protein.

7.7 RADIOACTIVE IMMUNOPRECIPITATION OF (35-S) METHIONINE LABELLED ATPCL FROM A RAT REFED A HIGH CARBOHYDRATE DIET FOR 3 DAYS BY RABBIT 3 DEAE PURIFED IgG.

The recentrifuged cytosolic supernatant containing (35-S) methionine radiolabelled proteins obtained from a rat that had been starved 2 days and refed a high carbohydrate diet for 3 days, was prepared as described in section 7.3.1.

The recentrifuged cytosolic supernatant 60Ti(2) fraction characteristics were as follows :

TABLE 16

Fraction	Volume ml	a Activity units/ml	b Protein mg/ml	c Radioactivity cpm/ml
recentrifuged cytosol	8.5	5.09 +/-0.10	35.28 +/- 0.25	12200 +/-1120

a Activity determined by malate dehydrogenase coupled assay. A unit of activity is defined as the umol of AcetylCo produced/min/ml as monitored by NADH oxidation at 340 nm.

b Protein was determined with Biuret reagent after TCA precipitation as described in section 2.5.

c Radioactivity in TCA precipitable protein was determined as described section 2.10. Result represents 12 determinations.

In this experiment 100 ul of antibody was mixed with 100ul of supernatant and 50 ul of homogenisation buffer in a constant volume of 0.25 ml.

Antigen-antibody complexes were collected and treated as in section 7.3.2. Gel slices were processed as in sections 2.8, 2.9, 2.10 except that samples were counted in 10 ml scintillation fluid. Radioactive proteins precipitated by Rabbit 3 purified IgG are listed in Table 17.

TABLE 17

a (35-S) methionine radiolabelled proteins immunoprecipitated by RABBIT 3 antibodies.

Molecular weight of the excised gel slice	Radioactivty present in gel slice cpm precipitated/ml of cytosol
260,000	113 +/- 7
250,000	110 +/- 4
160,000	116 +/- 19
125,000 (ATPCL)	155 +/- 5 (N=3)
110,000	40 +/- 5
96,000	94 +/- 9
88,000	86 +/- 8
78,000	91 +/- 14
57,000	77 +/- 6
55,000 (HIgG)	89 +/- 8
45,000	94 +/- 5
30,000	97 +/- 9
25,000 (LIgG)	77 +/- 4
20,400	101 +/- 6
19,000	98 +/- 4
DYE front	77 +/- 4

b Radioactivity precipitated above = 1495 cpm/ml
Total cpm/ml precipitated = 1832 cpm/ml

a Albumin was immunoprecipitated as a control and scaled to 65% of the TCA precipitable protein in sera.

b Background counts have been removed (35+/-5 cpm).

Radioactive (35-S) methionine was used because only 300 cpm could be precipitated in the ATPCL subunit per ml of recentrifuged cytosolic fraction when carbon labelling was used.

When (35-S) methionine replaced (14-C) protein hydrolysate in the labelling experiments the same magnitude of counts were precipitated in the ATPCL subunit. This is because methionine is rare in rat liver ATPCL (89,164) as in most other proteins.

Methionine represents 2% of the residues in the ATPCL subunit in rat liver whereas cysteine represents 10% (89,164). Therefore 5 times the radioactivity per mg of protein could have been precipitated if (35-S) cysteine had been used as the radiolabel.

ATPCL subunit represents only 8.5% of the radioactivity precipitated by these antibodies. This is despite injecting ATPCL eluted from DEAE with a specific activity of greater than 10 units/mg which showed only the ATPCL subunit on SDS PAGE at 25 ug loading.

The contaminating proteins have subunits of variable molecular weight (260-12,000 Mr). This is in contrast with the data in Figure 16 which shows that predominantly subunits of low molecular weight were preferentially precipitated by these antibodies. The reason for the difference may be due to the different methionine contents of different proteins.

A significant amount of radioactivity is present in both the heavy and light chains of immunoglobulin and the 21,000 Mr protein. The appearance of these bands can be directly contributed to the instability of the ATPCL in the sample injected into the rabbits. These bands were not present in the samples used for inoculation.

7.8 CALCULATION OF THE PROPORTION OF ATPCL TO THE TOTAL TCA PRECIPITABLE PROTEIN.

The results show that Rabbit 3 (antibodies initiated from ATP citrate lyase eluted from the DEAE 52 column) immunoprecipitated 155 +/- 5 cpm per ml of recentrifuged cytosol for a rat refed a high carbohydrate diet for 3 days (Table 17). These experiments were conducted with

a four fold excess of antibody to antigen as previously determined by immunotitration (section 6.6). Therefore the proportion of immunoreactive ATPCL to the total TCA precipitable protein can be determined.

ATPCL represents 1.270 +/- 0.158% of the total TCA precipitable protein. This is somewhat higher than 0.902 +/- 0.024% derived from the assay (if the specific activity of pure ATPCL is taken as 16 units/mg).

7.9 CONCLUSION OF THE IMMUNOPRECIPITATION OF (14-C) LABELLED ATPCL FROM A REFED RAT.

The proportion of (14-C)-ATP citrate lyase present in a rat starved 2 days then refed a high carbohydrate diet for 2 days to the total TCA precipitable protein was determined by the following methods tabulated in Table 18.

TABLE 18

	Method of estimation	Source of antibody	Proportion of ATPCL to total
a	ASSAY	---	0.467 +/- 0.073 %
	RADIOIMMUNOTITRATION	RABBIT 1	0.501 +/- 0.077 %
	RADIOIMMUNOTITRATION	RABBIT 2	0.446 +/- 0.087 %
b	IMMUNOPRECIPITATION	RABBIT 1	0.518 +/- 0.060 %
	IMMUNOPRECIPITATION	RABBIT 2	0.481 +/- 0.048 %
c	RADIOACTIVE GEL SLICES	RABBIT 1	0.555 +/- 0.027 %
	RADIOACTIVE GEL SLICES	RABBIT 2	0.548 +/- 0.056 %

a ASSAY assumes specific activity of 16 units/mg protein.

b Immunoprecipitation represents 1 individual data obtained in section 7.5 from the radioactive gel slices obtained in 7.4.1

c Radioactive (14-C) gel slices represent excision of the ATPCL sub-unit from SDS PAGE gels after disruption of the antigen-antibody complex. 16 determinations were made using Rabbit 1 IgG and 12 determinations using Rabbit 2 IgG.

ATP citrate lyase represents 0.508 +/- 0.059 % of the total TCA precipitable protein for a rat that has been starved 2 days, then refed a high carbohydrate diet for 2 days.

It can be concluded from the radioactive gel slices (Table 14) that the antigen used to immunise the rabbits was impure (section 7.4.3). In particular, the Commassie staining proteins seen on 7% SDS PAGE gels (as described in sections 6.7) directly correlate with radioactivity immunoprecipitated by the antibodies. The exception is the Rabbit 2 purified IgG which does not precipitate radioactivity in the two high molecular weight proteins (250-260,000 Mr) to the same extent as predicted from previous gels. These proteins may have been counted if the amount of ATPCL subunit had been increased from 20 to 60 ug. Rabbit 1 IgG or IgM precipitates a very high Mr contaminant that does not enter the resolving gel.

The radioactivity scans of the gels also show that Rabbit 2 IgG precipitates less of the major proteolytic fragments than Rabbit 1. These bands are obscured in non-labelled experiments by HIgG.

Both sets of purified antibodies precipitate a 21,000 Mr protein that is either a binding protein or the final degradation product.

Overall, ATPCL eluted from the Blue Sepharose column was not homogeneous and contained traces of contaminating proteins of which some are not visualised by Coomassie staining of the polyacrylamide gels (Figure 3). These proteins may have been visualised with the more sensitive silver staining method. Contaminating proteins, which were eluted with the Blue sepharose column fractions containing ATPCL, generated separate immunological responses and hence were precipitated with the purified IgG obtained from Rabbit 1 or 2 (Figure 14).

7.10 CONCLUSION OF THE IMMUNOPRECIPITATION OF (35-S) METHIONINE LABELLED ATPCL FROM A RAT ON A REFED REGIME.

The proportion of ATP citrate lyase present in the liver of a rat that has been starved for 2 days then refed a high carbohydrate diet for 3 days to the total TCA precipitable protein was determined by the following methods:

TABLE 19

Method of estimation	Source of antibody	Proportion of ATPCL to total
a ASSAY	---	0.902 +/- 0.024 %
b RADIOACTIVE GEL SLICES	RABBIT 3	1.270 +/- 0.158 %

a ASSAY assumes specific activity of 16 units/mg protein. Recentrifuged cytosol had a protein concentration of 32.5 mg/ml and activity of 5.09 units/ml.

b RADIOACTIVE (35-S) GEL SLICES represent excision of the ATPCL subunit from SDS PAGE gels after disruption of the antigen - antibody complex. 3 determinations were made using Rabbit 3 IgG.

It can be concluded from the radioactive gel slices (Table 17) that Rabbit 3 purified IgG is not monospecific towards ATPCL and additional proteins are precipitated by these antibodies. The subunit molecular weights of the contaminating proteins are of both high and low Mr. Major contaminating proteins that were precipitated in non-labelling experiments (section 6.6) were less than 55,000 Mr (Figure 15). Proteins, other than the ATPCL subunit, represent 90% of the (35-S) radioactivity precipitated by these antibodies. A significant amount of radioactivity was present in the proteolytic fragments of ATPCL which were precipitated by Rabbit 3 purified IgG.

When (35-S) methionine replaced (14-C) protein hydrolysate there was no significant elevation of the radioactivity immunoprecipitated in the ATPCL subunit. A better choice of radiolabel to use would have been (35-S) cysteine because there are 4-5 times more cysteine residues than methionine in the ATPCL subunit (71,89,164).

When Table 19 is compared to Table 18 it can be seen that the increase in induction of ATPCL is concomitant with an increase in immunological protein. The comparison of these results is complicated because the two radiolabels were different and the antibodies that were used to precipitate the ATPCL subunit were also different. In the following chapters, (35-S) ATPCL is immmunoprecipitated from rats with a different nutritional status or when a different labelling time is used.

Because the antibodies to ATPCL are not monospecific the analysis of the radioactivity present in ATPCL needs to be determined after excising the 110-125,000 Mr subunit from the SDS PAGE gel. There will be an overestimate of the ATPCL protein if there are contaminating proteins present within the gel slice.

CHAPTER 8

INTRAPORTAL LABELLING OF A RAT ON THE STARVATION/REFED REGIME.

8.1 INTRODUCTION

The rate of synthesis of albumin and ATPCL was measured *in vivo* after a 30 minute pulse of (35-S) methionine. Incorporation of label into specific proteins was expressed as a percentage of the label in the total TCA precipitable protein. The incorporation of label into albumin was used as a control in the ATPCL immunoprecipitation experiments.

This is because albumin synthesis is constitutive under these experimental conditions (chapter 5).

Radioisotope was administered by intraportal injection as this has been shown to be an excellent route (62,163). The high specific activity of the isotope used gives a true tracer dose after intravenous injection (215), and produces only negligible changes in plasma methionine levels. Free isotope has been shown to be cleared from the blood within 5 minutes of injection (131) and maximal specific activity of nascent protein occurs within 1 to 1.5 minutes after injection (192).

METHODS.

A male rat (150 g) was starved over two nights and refed a high carbohydrate diet for 3 days. At the end of this dietary regime the rat was anaesthetized by the intraperitoneal injection of 10.0 mg of Nembutal in 1.00 ml of PBS. The peritoneal cavity was exposed and 250 uCi of L-[35S] methionine in 0.1 ml of PBS was introduced into the portal vein. Ten minutes later the syringe needle was removed.

Thirty minutes after injection of the isotope the rat was killed by cervical dislocation and the liver was excised, perfused and processed as described in section 6.2.

A sample of blood from the heart was also obtained at this time for immunoprecipitation of serum albumin.

The central section of the recentrifuged 60Ti supernatant was used for determination of radioactive incorporation into the TCA precipitatable protein, immunoprecipitation of ATPCL, and assayed for ATPCL activity.

8.3 ANALYSIS OF THE LABELLED PRODUCTS.

Labelled ATP citrate lyase and albumin were immunoprecipitated by purified antibodies as described in section 6.2. Sufficient antibody was added to precipitate 60 ug of antigen. Radioactive albumin and ATPCL protein bands were identified by comparison with standards of known molecular weight after SDS PAGE (section 2.7.). The incorporation of radioactivity within individual gel slices was determined after they had been dissolved in ammonia/hydrogen peroxide (1:99 v/v) as described in section 2.9.

Protein was determined with Biuret reagent after precipitation with 10% cold TCA as described in section 2.10. Protein determinations were in triplicate using 20 ul and 100 ul fractions. Radioactivity incorporated into total TCA precipitable protein was determined as in section 2.10.

8.4 RESULTS AND DISCUSSION OF 30 min PULSE CHASE EXPERIMENT

The recentrifuged cytosolic fraction obtained from a rat labelled for 30 minutes with (35-S) methionine after being starved 2 days then refed a high carbohydrate diet for 3 days had the following characteristics :

TABLE 20

Fraction	Volume ml	a Total Activity units	b Total Protein mg	Specific Activity units/mg	c Specific Radioactivity cpm/mg protein
d Homogenate	28.0	41.16	1180	0.035	242
SS34	21.0	32.97	507	0.065	278
60Ti(1)	12.0	28.68	274	0.104	343
60Ti (2)	8.0	21.12	176	0.120	316

a The unit of ATPCL activity is the umol acetylCoA produced per minute as determined in the coupled malate dehydrogenase assay at 340 nm by the oxidation of NADH.

b Protein determined with Biuret reagent after TCA precipitation.

c Radioactivity in TCA precipitable protein determined as described in section 2.10.

d Liver wet weight was 6.88 grams, after tissue press 5.60 g.

e 60Ti (1) represents the ultracentrifuge supernatant cytosolic fraction. 60Ti (2) represents recentrifuged cytosolic fraction.

f Concentration of ATPCL per liver 6.01 units/gram wet weight liver. After connective tissue removed 7.39 units/gram wet weight liver.

8.4.2 Immunoprecipitation of Albumin from rat serum.

Albumin was immunoprecipitated from 50 ul of serum using rabbit anti-rat albumin IgG. Complete immunoprecipitation of the albumin was confirmed by SDS PAGE of the supernatant serum.

Incorporation of radioactivity into the TCA precipitable protein from rat serum was 36500 +/- 1900 cpm/ml. Of this total, the radioactivity incorporated into albumin was 22800 +/- 500 cpm/ml of blood.

Radioactivity incorporated into albumin represents 62.47 +/- 4.62% of the total TCA precipitable serum protein. For the immunoprecipitation experiments albumin was arbitrarily scaled to 65% of the total TCA precipitable protein.

8.4.3 Proportion of ATPCL in TCA precipitable protein.

The radioactivity immunoprecipitated from rat liver cytosol and present in individual gel slices can be seen in Table 21.

TABLE 21

Molecular weight range of protein in gel slice	Purified antibody used	a Radioactivity precipitated per ml 60Ti(2)	Percent of total TCA precipitable protein
ATPCL subunit			
110-125,000	R1	68	0.976
	R1	50	0.717
	R1	48	0.689
	R1	39	0.560
	R2	46	0.660
	R2	48	0.689
	R2	62	0.890
	R2	68	0.976
	R3	40	0.574
	R3	52	0.746
	R3	64	0.918
	OTHER PROTEIN BANDS THAT ARE IMMUNOPRECIPITATED		
260,000	R2	17	0.244
	R3	22	0.316
250,000	R1	44	0.631
	R3	23	0.330
240,000	R1	54	0.775
160,000	R2	6	0.086
94,000	R1	41	0.588
	R2	31	0.445
83,000	R2	20	0.287
78,000	R3	77	1.105
53-57,000	R1	64	0.918
	R2	40	0.574
	R3	41	0.588
	R3	40	0.574
45,000	R1	23	0.330
45,000	R3	19	0.273
DYE FRONT	R1	11	0.158
	R2	261	3.75
	R3	79	1.13

a Radioactivity determined in the excised gel slice after dissolving in hydrogen peroxide/ammonia, and addition of 10 ml scintillation fluid as described section 2.10

The radioactive counts immunoprecipitated from the 60Ti(2) supernatant was 53.2 +/- 10.1 cpm/ml (N=11 determinations). The concordant data shows that 49 +/- 2.0 cpm are precipitated per ml of recentrifuged cytosolic supernatant (N = 5). The total amount of radioactivity incorporated in the TCA precipitable protein was 6970 +/- 196 cpm/ml.

Since the antibody was in a ten fold excess over the antigen, the proportion of ATPCL to the TCA precipitable protein can be calculated. ATPCL represents 49/6970 = 0.703 +/- 0.049 % of the total TCA precipitable protein.

The proportion of ATPCL to the total TCA precipitable protein was significantly lower than the result obtained in section 7.8 and 7.10, (0.703 vs 1.27 +/- 0.158%).

The difference lies in the variability of induction of ATPCL in individual experiments. In this chapter ATPCL was induced to 2.64 +/- 0.06 units/ml, whereas in chapter 7, the level of ATPCL induction was 5.09 +/- 0.10 units/ml recentrifuged cytosolic fraction. If the induction of ATPCL is scaled to 5.09 units/ml then the two results become comparable (1.355 +/-0.125% vs 1.270 +/-0.158%).

This result indicates that the induction of ATPCL activity is concomitant with an increase in the amount of immunoreactive protein.

The data shows that the amount of incorporation of (35-S) methionine into albumin occurs at a rate 90 fold faster than into the ATPCL subunit under this set of experimental conditions. This gives some indication of the relative rate of synthesis of ATPCL and of albumin. The proportion will vary widely as ATPCL is induced or degraded under different dietary conditions.

CHAPTER 9

IMMUNOPRECIPITATION OF ATP CITRATE LYASE FROM RATS WITH DIFFERENT NUTRITIONAL STATUS

9.1 INTRODUCTION

In the experiments described in Chapter 8 it was shown that an increase in the activity of ATPCL corresponded with an increase in immunoreactive radiolabelled protein. This was established by immunoprecipitation of an increasing amount of radiolabelled ATPCL subunit, as the activity of the enzyme in liver is increased. This result was observed in experiments involving rats that had been starved for 2 days and refed a high carbohydrate, low fat diet for 3 days.

In this set of experiments the relative rate of synthesis of ATPCL was measured for five different dietary treatments by following the incorporation of (35-S) methionine into immunoreactive ATPCL protein and into trichloroacetic acid precipitated material. Incorporation of radioactivity into ATPCL was determined after precipitation with purified IgG and excision of the labelled ATPCL from SDS PAGE gels.

Incorporation of radioactivity into TCA insoluble protein was taken as a measure of total protein synthesis i.e the rate of incorporation of isotope into synthesized enzyme relative to that incorporated into the total soluble protein. This calculation corrects for the size of the amino-acid pool and the amount of radioactivity administered (Cashman and Pitot, 1971).

9.2 METHODS.

In separate experiments, (35-S)-methionine radiolabelled ATPCL was immuno precipitated from the recentrifuged cytosolic fraction using purified IgG. These fractions were obtained in individual experiments from rats that had been starved for 2 days then refed a

(1) chow diet for 2 days,
(2) starved an additional day,
(3) high carbohydrate diet for 2 days,

(4) high carbohydrate diet for 3 days,
(5) high carbohydrate diet for 6 weeks.

Labelling and analysis of immunoprecipitated proteins was accomplished as in chapter 6. ATPCL was precipitated from the cytosolic fraction using a ten fold excess of antibody.

To standardize the radioactivity recovered in the ATPCL subunit, the albumin concentration was scaled to 65.0% of the total TCA precipitable protein in sera. The radioactivity in the recentrifuged cytosolic fraction was scaled to 120,000 cpm per milliliter of solution.

9.3 RESULTS AND DISCUSSION

The amount of immuno-reactive ATPCL compared to the level of induction can be seen in Table 22 and 23. Actual experimental data is presented in Appendix 2.

The results show that an increase in ATPCL activity correlates with an increase in the amount of immunoreactive protein. When ATPCL is induced there is an increased amount of radiolabelled ATPCL subunit precipitated (Table 23).

These results strongly suggest that an adaptive increase in the level of ATPCL was observed during starvation and refeeding. This finding was observed in the livers of animals maintained on both the high carbohydrate and chow refeeding.

Previous radiochemical studies (53,59,99,208) have shown that the long term carbohydrate dependent regulation of ATPCL is via enzyme induction, and not by the activation of pre-existing enzyme. The increase in ATPCL activity by de novo synthesis of new protein is directly proportional to an increased amount of immuno-reactive protein rather than by an allosteric activation or interconversion of pre-existing enzyme (60,99,208).

TABLE 22

IMMUNOREACTIVE RADIOLABELLED (35-S) METHIONINE ATPCL
IN RELATION TO ENZYME ACTIVITY.

Dietary Status	Liver wet weight g	Enzyme activity units/ml	Protein mg/ml	a Radioactivity precipitated in the immunoreactive ATPCL subunit/ ml cytosolic fraction scaled to (b) cpm/ml	
				Total data Mean +/- SD	Concordant data Mean +/- SD
c Normal	5.62	3.22	25.9	851 +/- 80	d 843 +/- 48 (N=7)
e Fasted	1.51	2.00	28.5	391 +/- 36	391 +/- 36 (N=3)
Induced 1	4.78	2.45	26.9	611 +/- 33	611 +/- 33 (N=3)
Induced 3	5.60	2.64	22.2	916 +/- 174	840 +/- 35 (N=5)
Induced 2	8.60	5.09	35.3	1522 +/- 84	1530 +/- 36 (N=4)
Induced 4	10.88	5.36	27.8	1368 +/- 199	1245 +/- 14 (N=3)

a Actual experimental data appears in Appendix 2.

b Data scaled to 120,000 cpm/ml of supernatant so that albumin represents 65% TCA precipitable protein.

c Nutritional status key:

Fasted represents starved 3 days (24 hr radiolabel pulse).
Normal represents fasted 2 days then chow refeeding for 3 days.
Induced represents fasted 2 days then refeeding carbohydrate diet.
Induced 1 refeeding CHO diet for 2 days (24 hr radiolabel pulse).
Induced 2 refeeding CHO diet for 3 days (24 hour pulse).
Induced 3 refeeding CHO diet for 3 days (30 minute pulse chase expt).
Induced 4 refeeding CHO diet for 6 weeks (24 hr radiolabel pulse).

d Concordant data represents subset of the total data.
Errors represent one standard deviations.

e Starved 3 days and Induced 1 N=3 experiments only.

TABLE 23

IMMUNOREACTIVE (35-S) METHIONINE LABELLED ATPCL SUBUNIT IN RELATION TO ENZYME SPECIFIC ACTIVITY.

Dietary Status	Liver wet weight g	Specific Activity units/mg	a Specific Radioactivity b cpm in ATPCL subunit/mg Protein cpm/mg	
			Total data Mean +/- SD	Concordant data Mean +/- SD
c Normal	5.62	0.124	32.86 +/- 3.09	d 32.55 +/- 1.85 (N=7)
e Fasted	1.51	0.070	13.72 +/- 1.26	13.72 +/- 1.26 (N=3)
f Induced 1	4.78	0.090	22.71 +/- 1.22	22.71 +/- 1.22 (N=3)
Induced 3	5.60	0.119	41.26 +/- 7.84	37.84 +/- 1.23 (N=5)
Induced 2	8.60	0.144	43.11 +/- 2.38	43.34 +/- 1.02 (N=4)
Induced 4	10.88	0.193	49.21 +/- 7.16	44.78 +/- 0.51 (N=3)

a Actual data derived from Table 22.

b Data scaled to 120,000 cpm/ml of supernatant and so that albumin represents 65% TCA precipitable protein.

c Nutritional status key:

Fasted represents starved 3 days (24 hr radiolabel pulse).
Normal represents fasted 2 days then chow refeeding for 3 days.
Induced represents fasted 2 days then refeeding carbohydrate diet.
Induced 1 refeeding CHO diet for 2 days (24 hr radiolabel pulse).
Induced 2 refeeding CHO diet for 3 days (24 hour pulse).
Induced 3 refeeding CHO diet for 3 days (30 minute pulse chase expt).
Induced 4 refeeding CHO diet for 6 weeks (24 hr radiolabel pulse).

d Concordant data represents subset of the total data.
Errors represent one standard deviation.

e Starved 3 days and Induced 1 N=3 experiments only.

The overall data shows a correlation between the activity present in the supernatant with the amount of radioactivity precipitated with immunoreactive ATPCL.

The increase in activity per ml of cytosolic fraction is consistent with an increase in the radioactivity precipitated in immunoreactive protein (Table 22). This result confirmed that the adaptive increase of ATPCL is not a result of increased liver growth and general protein synthesis.

The relative rate of synthesis of ATPCL varied greatly depending on the length of time of refeeding the high carbohydrate diet (Table 23). It was found that the enzyme level significantly decreased during starvation and increased over a three day refeeding.

Refeeding a chow diet returned the enzyme activity to a level equivalent to refeeding a high carbohydrate diet for 2.5 days. The overall induction of enzyme activity was lower than obtained in Chapter 4. Lower levels of induction may be due to stress caused from the intraperitoneal injection.

Statistical analysis of Table 23 showed that the rat refed a carbohydrate diet for 6 weeks had significantly lower levels of incorporation of radioactivity into the ATPCL subunit than predicted from a regression line. This result can be seen in Table 24.

TABLE 24

Statistical analysis of data in Table 23
Correlation of radioactivity present with immunoreactive ATPCL compared with the induction of enzyme activity.

Nutritional Status	Specific Activity units/mg	Specific Radioactivity Observed Data cpm/mg	(a) Fitted Data cpm/mg
(b) Normal	0.124	(c) 32.86 +/- 3.09	34.50 +/- 3.24
Fasted	0.070	13.72 +/- 1.26	13.86 +/- 1.27
Induced 1	0.090	22.71 +/- 1.22	21.51 +/- 1.15
Induced 2	0.144	43.11 +/- 2.38	42.14 +/- 2.33
Induced 3	0.119	41.26 +/- 7.84	32.59 +/- 6.19
Induced 4	0.193	49.21 +/- 7.16	60.87 +/- 8.85

Slope of best fit = 38.22 Correlation coefficient = 0.9955
Intercept on the Y-axis (Specific radioactivity) = - 12.89
Intercept on the X-axis (Specific activity) = 0.03372

a Best correlation obtained without Induced 3 or Induced 4.
Correlation coefficient for all points = 0.9223.

b Nutritional status key:

Fasted represents starved 3 days (24 hr radiolabel pulse).
Normal represents fasted 2 days then chow refeeding for 3 days.
Induced represents fasted 2 days then refeeding carbohydrate diet.
Induced 1 refeeding CHO diet for 2 days (24 hr radiolabel pulse).
Induced 2 refeeding CHO diet for 3 days (24 hour pulse).
Induced 3 refeeding CHO diet for 3 days (30 minute pulse chase expt).
Induced 4 refeeding CHO diet for 6 weeks (24 hr radiolabel pulse).

c Errors represent one standard deviation.

The lack of radioactivity in immunoreactive ATPCL subunit in rat liver which has been refed a high carbohydrate diet for 6 weeks (Induced 4 in Table 23) is not due to experimental error but is due to incorporation of the labelled methionine into other proteins. Changes in the level of an enzyme takes place against a background of continuous synthesis and degradation (157,198).

After 6 weeks of refeeding a carbohydrate diet, ATPCL is no longer in the "burst" phase of induction and a lower steady state rate of synthesis and degradation has been attained. Because ATPCL is no longer being actively synthesized the rate of incorporation of (35-S) methionine into the ATPCL subunit will be lower than in those experiments in which ATPCL is still being induced. Lower levels of methionine incorporation may also be due to the mobilization of non-labelled methionine derived from degradation of other proteins.

A significantly greater incorporation of radiolabelled methionine into the ATPCL subunit was observed in the 30 minute pulse chase experiment. The 20% increase in the level of immunoreactive protein, as determined by radioactivity precipitated in the ATPCL subunit, is due to active synthesis of ATPCL within the short pulse time. Because the pulse time is short and ATPCL is being very actively synthesized, a greater proportion of label is incorporated with the immunoreactive subunit than with constitutive proteins. Therefore the overall specific radioactivity (cpm/mg protein) is higher than predicted. When the time scale of the pulse is increased to 24 hours, the rate of incorporation into other proteins becomes significant, thereby lowering the specific radioactivity.

Additional experiments are required to determine the effect of degradation of the enzyme and the amount of reutilisation of label.

9.4 THE PROPORTION OF ATPCL TO TCA PRECIPITABLE PROTEIN.

The proportion of radioactivity immunoprecipitated as ATPCL subunit to the total radioactivity was compared to the proportion of ATPCL as judged by the enzyme assay to the total protein. This assumes a specific activity of pure ATPCL is 16 units/mg. The results can be seen in Table 25.

TABLE 25

Comparison of the proportion of ATPCL to the total protein for different nutritional states

Nutritional status	a Proportion of ATPCL to total protein as determined from (1) Enzyme assay	(2) b cpm in ATPCL/total cpm Total data Mean +/- SD	(2) b cpm in ATPCL/total cpm c concordant data Mean +/- SD
d Normal	0.777	0.709 +/- 0.067	0.703 +/- 0.001
Fasted	0.439	0.326 +/- 0.030	0.326 +/- 0.030
Induced 1	0.569	0.509 +/- 0.027	0.509 +/- 0.027
Induced 3	0.746	0.763 +/- 0.145	0.700 +/- 0.029
Induced 2	0.901	1.268 +/- 0.070	1.275 +/- 0.030
Induced 4	1.205	1.114 +/- 0.162	1.038 +/- 0.012

a Calculation of proportion of ATPCL to total protein from assay (specific activity/16 units per mg) x 100.

b Total cpm is scaled to 120,000 cpm/ml as in table 22.

c Concordant data represents subset of total data with lowest statistical error.

d Nutritional status same as in Table 22 or Table 23.

The results in Table 25 show that the proportion of ATPCL to the total protein predicted from the assay directly correlates with a specific induction of immunoreactive ATPCL, as determined from radio-labelling experiments. The amount of immunoreactive ATPCL is slightly lower than predicted by the specific activity. This is probably due to the choice of radio-amino acid, or else the specific activity of pure ATPCL must be lower than 16 units/mg. Methionine may be in a lower proportion in ATPCL than the average of all the proteins that are being synthesized.

The data presented in Table 25 agrees with previously published data. These reports used the identical techniques for the quantitation of the (35-S) methionine, and 4, 5 (3-H) leucine (12) incorporated into ATPCL subunits.

It has also previously been shown that the accumulation of ATPCL, AcetylCoA carboxylase and the other lipogenic enzymes, is caused by a real increase in the rate of synthesis (59,60,120,122,208).

The antibodies produced in this study can now be used to confirm whether the enhancement of the enzyme protein was affected by a specific increase of the rate of synthesis rather than a decrease in the rate of degradation.

Additional studies will also be required to determine whether the induction of immunologically reactive protein is controlled at the translational or transcriptional level.

CHAPTER 10

CONCLUSION.

This investigation has aimed at elucidating several aspects of the purification and biochemistry of rat liver ATP citrate lyase. The development of a reliable and reproducible purification method for the enzyme and the use of this enzyme in a series of studies to ascertain the effect on ATPCL of different dietary regimes, were specific aims of this work.

Rat liver cytosolic enzyme ATPCL has been purified from rats refed a high carbohydrate diet ad libitum. Chapter 3 details the investigation of the existing purification methods available. In the three methods investigated, a 200 - 600 fold purification was obtained. The reason for the variation in purity is probably due to proteolytic cleavage, loss of phosphyl groups, and -SH oxidation of ATPCL during the long purification time course, as well as the effect of pooling a large number of fractions to obtain a reasonable yield.

Purification method 3 involves anion exchange chromatography on DEAE 52, followed by affinity chromatography on Reactive Blue Sepharose CL-6B. The method gave highly purified ATPCL. Specific activity of the final purified product was 10-16 activity units/mg of protein at 25° C (21-28 units/mg at 30°C). Problems were encountered with stability and yield of the final product after elution of ATPCL from the Blue Sepharose column. A 500-600 fold purification gave rise to homogeneous preparations when examined on denaturing gel electrophoresis at 72 microgram loading. Serious problems with proteolytic cleavage and inactivation of the enzyme were circumvented by using specific protease inhibitors and increasing the dithiothreitol concentration throughout the preparative scheme. The very rapid inactivation of enzymic activity was paralleled by the generation of proteolytic fragments visible on SDS gel electrophoresis.

The elution of ATPCL from DEAE 52 anion exchange column (Method 2(b)) had the advantage of obtaining enzyme of high specific activity (9.8-12 units/mg) that was more stable, and in greater yield than that obtained from a single elution from Blue Sepharose. This activity is

directly comparable with the work of Linn and Srere (1979), and Ramakrishna & Benjamin (139). This purification step should be used to pool the fractions of ATPCL with high specific activity before the additional step of affinity chromatography on Blue Sepharose.

Redshaw and Loten (1979) obtained a single band on SDS PAGE at 4 ug loading. This single band was obtained using pooled fractions with a specific activity of 6.9 units/mg. In the present work, multiple bands were obtained on SDS PAGE with ATPCL fractions with the same specific activity with only a two fold increase in the protein loaded.

Later purification methods showed that these additional proteins could be eliminated if, first, the enzyme was eluted from the DEAE 52 anion exchange column.

Szutowicz et al also confirmed multiple bands on SDS PAGE of rat liver ATPCL after the use of a single Blue Sepharose column. In their work, purification of enzyme to homogeneity showed only one band on SDS PAGE at 20 ug loading with a specific activity of 14 whereas Szutowicz shows multiple banding at 11-15 units/mg protein. Multiple bands may have been the result of using the more sensitive silver staining method.

ATPCL eluted from Blue Sepharose (Preparative method 1(b)) or DEAE 52 (method 2(B) migrated with the same Rf on reducing SDS PAGE gels.

The appearance of multiple peaks in the elution profile on elution of Blue Sepharose CL-6B and DEAE 52 provided evidence for different chromatographic forms, separable by adsorption or non-adsorption on affinity chromatography or anion exchange. Multiple peaks were apparent on reduction of column fractions from 6 to 1 ml.

ATPCL has been previously reported to exist in multiple chromatographic forms separable by DEAE anion exchange chromatography (40). This finding may explain the fact that not all the ATPCL activity could be immunoprecipitated from the respun ultracentrifuge supernatant, since no antibodies were raised against ATPCL that did not bind to either the Blue Sepharose or DEAE anion exchange column. The proportion of ATPCL not immunoprecipitated by purified antibodies in the liver of a starved refed rat is 10% of the total. This proportion is identical to that

reported by Corrigan et al (40) to be the proportion of highly polymerized ATPCL. Alternatively, the inability to titrate all the enzymic activity may be due to the presence of NADH oxidases interfering with the assay.

Previous reports have indicated that ATPCL shows an adaptive response to refeeding a high carbohydrate diet. The reported induction of the enzyme in rats by fasting and then refeeding a high carbohydrate diet, was found to be repeatable. The level of ATPCL (as determined by the assay) decreases in liver during fasting and is increased 35 fold by refeeding a high carbohydrate diet for 2 days. Individual experiments show that induction is variable but consistent with the reported literature values.

The lack of production of a large amount of homogeneous antigen, due to its rapid inactivation in solution, provided a severe handicap to the production of high titre antibodies. Antibodies raised against ATPCL were not monospecific, despite a high selection of pure fractions for the immunization of the rabbits.

Antibodies raised against Blue Sepharose eluted ATPCL, elicited immunological responses against both high and low molecular weight contaminating proteins.

Antibodies generated against ATPCL fractions eluted from the DEAE anion exchange column were more selective toward ATPCL than antibodies initiated from fractions eluted from Blue Sepharose. This increase response can be attributed to both increased stability of ATPCL after elution from DEAE 52 and the amount of antigen used for inoculation. Rabbit 1 antibodies contained both IgM and IgG that immunoprecipitated ATPCL from rat liver.

When radiolabelled proteins were immunoprecipitated from recentrifuged cytosolic fractions a large amount of radioactivity was found in major proteolytic fragments (54, 57, and 22,000). The banding pattern obtained on SDS PAGE is such that these breakdown proteins were masked in the heavy chain IgG band (55,000) as well as the light chain IgG (23,000).

It was found that antibodies prepared against ATPCL purified by Blue

Sepharose chromatography immunoprecipitated purified ATP citrate lyase from the DEAE 52 column, only 3 bands could be visualized on SDS-PAGE. These bands corresponded to the light and heavy immunoglobulin chains and the ATPCL subunit. Whether the antibody - ATP citrate lyase immunoprecipitate complex was capable of eliciting an immunological response in vivo was not investigated. The method looked promising as a mechanism for the production of monospecific antibodies against ATPCL.

Because the antibodies were not monospecific the determination of the amount of radiolabelled ATPCL present in the recentrifuged cytosolic supernatants had to be determined after immunoprecipitation and excision of the ATPCL subunit on SDS PAGE.

The antibodies were used to determine the proportion of immunoreactive ATPCL in rats that had been subjected to different dietary regimes. The proportion of immunoreactive radiolabelled (35-S) methionine ATPCL directly correlates with the proportion of ATPCL determined from the assay.

The overall conclusion of this series of experiments clearly demonstrates that ATPCL induction is proportional to a specific increase in the immunoreactive protein and not an activation of pre-existing enzyme.

10.2 EXTENSIONS OF THIS WORK.

A logical extension of the present work on production of monospecific antibodies against ATP citrate lyase would include:

(1) The demonstration that the observed increase in enzyme protein did result from an increased rate of ATPCL synthesis rather than a decreased rate of degradation. The level at which this regulation occurs could then be investigated to see whether it occurs at the level of transcription (messenger RNA synthesis) or translation (protein synthesis).

(2) The examination of the rate of synthesis and degradation of ATPCL in rats under different dietary conditions.

(3) The isolation of small quantities of ATPCL by means of immobilized

antibody columns in order to determine the phosphorylation state of this enzyme under the different dietary states.

(4) The determination of the synthesis of ATPCL in an *in vitro* cell free system which has been programmed with isolated rat liver mRNA. The experiment should indicate whether changes in the rate of protein synthesis correlate with changes in the amount of mRNA for ATPCL, providing evidence for regulation at the transcriptional level.

A long term objective is to prepare a cDNA clone for rat liver ATPCL. This would involve the immunological detection of ATPCL protein synthesis, in an expression vector for E. Coli. Once isolated these clones could be used as a direct probe to investigate mRNA levels and eventually to isolate the gene coding for ATPCL.

APPENDICIES

1.0 TABLE OF BUFFERS

- 1.1 Homogenisation buffer 400 ml -

10 mM Sodium Phosphate
2500 mM Sucrose
100 mM Sodium Fluoride
5 mM Sodium EDTA
50 mM Benzamidine
1 mM Magnesium Chloride
10% Glycerol
0.1% B-mercaptoethanol

- 1.2 Supernantant Dilution buffer 400 ml-

10 mM Sodium Phosphate
250 mM Sucrose
5 mM EDTA (diSodium)
50 mM Benzamidine

- 1.3 DEAE 32 (Ammonium Chloride) buffer -

10 mM Sodium Phosphate
5 mM EDTA (diSodium)
50 mM Ammonium Chloride
50 mM Sodium Fluoride

- 1.4 DEAE 32 (Benzamidine) buffer 300ml -

10 mM Sodium Phosphate
50 mM Sodium Fluoride
50 mM Benzamidine
5 mM EDTA (diSodium)

- 1.5 Sepharcyl S-200 buffer 2 litre -

10 mM Sodium Phosphate
100 mM Sodium Fluoride.
5 mM EDTA (diSodium)
50 mM Sodium Citrate
1 mM Dithoithreitol

- 1.6 Blue Sepharose buffer 1 litre -

10 mM Sodium Phosphate
100 mM Sodium Fluoride
5 mM EDTA (diSodium)
50 mM Sodium Citrate

- 1.7 DEAE 52 Equilibration buffer

20 mM Sodium Phosphate
1.0 mM Magnesium Acetate
0.1 mM EDTA (disodium)
1.0 mM Benzamidine HCL
1.0 mM Dithiothreitol

10% (v/v) Glycerol

- 1.8 DEAE 52 Elution buffer

(a) 20 - 100 mM Phosphate Gradient
All contents in buffer 1.8 except
Reservior 1 20 mM Sodium phosphate 700 ml
Reservior 2 100 mM Sodium Phosphate 700 ml
Protease inhibitors

(b) 45 mM Discontinous gradient
All contents in buffer 1.8 except
45 mM Sodium Phosphate
Protease inhibitors

- 1.9 Protease inhibitors used in Buffer 1.8 -

4 ug/ml Leupeptin
4 ug/ml Antipapain
0.1 mM L-1-tosyl amido-2-phenyl-ethylchloro-methyl ketone
0.1 mM N -p-tosyl-L-Lysine chloromethyl ketone

** All Buffers were cooled and then adjusted to pH=7.5 **

APPENDIX 2

(35-S) Methionine labelled ATPCL immunoprecipitated from recentrifuged cytosolic fractions of rats with different nutritional status.

Dietary Status	Liver Wet weight	Enz. Act. units/ml	Prot. mg/ml	Radioactivity present in TCA precipitable protein cpm/ml (1)	ATPCL Subunit cpm/ml (2)	Albumin % of total serum proteins
Normal	5.62	3.22	25.9	19,500	136 130 132 112 121 148 129 140 136 130 150	67.5 61.8
Fasted	1.51	2.00	28.48	113,000	399 321 342	62.5
Induced 1	4.78	2.45	26.9	26,000	139 152 116	69.5 60.3
Induced 2	8.60	5.09	35.3	12,200	155 160 150 145 145 151 171	65.0
Induced 3	5.60	2.64	22.12	6,970	68 50 48 39 46 48 62 68 40 52 64	65.0
Induced 4	10.88	5.36	27.78	10,700	111 108 110 104 157 156	65.8 67.8

a Dietary status:

Normal : represents fasted 2 days then chow refeeding for 3 days.
Fasted : represents starved 3 days.
Induced : represents fasted 2 days then feeding carbohydrate diet.

Induced 1 : refeeding CHO diet for 2 days (24 hr intraportal labelling).
Induced 2 : refeeding CHO diet for 3 days (24 hr intraportal labelling).
Induced 3 : refeeding CHO diet for 3 days (30 minute pulse chase expt).
Induced 4 : refeeding CHO diet for 6 weeks (24hr intraportal labelling).

b The values such as 156 represents data that is statistically different at one standard deviation. This data is eliminated in concordant data.

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