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STUDIES ON RAT LIVER ATP CITRATE LYASE
AND ACETYLCoA CARBOXYLASE

A Thesis

Submitted to the Faculty of Science
of the University of Glasgow

for the degree of
Doctor of Philosophy
by

Brian Houston, B.Sc.

December 1983

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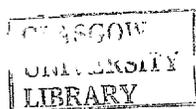
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A drop of water is a little thing

But when will it dry away if united to a lake?

Saskya Pandita

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Summary

ATP citrate lyase and acetylCoA carboxylase catalyse the first two steps in de novo fatty acid synthesis. Both enzymes are substrates for protein kinases in vivo and in vitro. The work described in the first part of this thesis was undertaken in order to study the phosphorylation of purified liver acetylCoA carboxylase. The work reported in the second part was concerned with the effect of phosphorylation on the activity of ATP citrate lyase.

In addition to the work on the liver enzyme the chromatographic behaviour of mammary gland acetylCoA carboxylase was studied. In the course of this work evidence was obtained that the use of polyethylene glycol precipitation during purification results in the irreversible aggregation of the enzyme.

Low amounts of acetylCoA carboxylase were found in pig liver indicating that this tissue would be a poor source of the enzyme. In general, higher levels of acetylCoA carboxylase were found in rat liver, although there was considerable variation in the amounts of activity measured. A number of techniques were investigated in attempts to purify the rat liver enzyme. Some success was achieved using polyethylene glycol precipitation or using chromatography on Phenyl-Sepharose, but these procedures were not reproducible.

Under certain conditions, chromatography on DEAE cellulose resulted in the separation of two peaks of 'acetylCoA carboxylase activity' which corresponded to polypeptides with subunit

M_r s = 125000 and 250000. This was initially interpreted as the separation of proteolytically degraded acetylCoA carboxylase from the undegraded form. Subsequent study indicated that the 125000 M_r polypeptide was ATP citrate lyase and that the acetylCoA carboxylase activity associated with this enzyme was spurious and was the result of the contamination of acetylCoA by CoA. Evidence was then obtained that the purification of acetylCoA carboxylase would be difficult to achieve due to the presence in rat liver of an inhibitor of the enzyme.

Rat liver ATP citrate lyase was purified using ammonium sulphate fractionation and chromatography on DEAE cellulose, Affigel Blue and Ultrogel A2 to a final specific activity of 13.4 U/mg. The enzyme was pure and free from proteolytic degradation as judged by non-denaturing and SDS-polyacrylamide gel electrophoresis. Various physicochemical properties of the enzyme were studied.

The kinetic mechanism of ATP citrate lyase was investigated. Provided that the assays were performed in the absence of ADP, parallel double reciprocal plots were obtained when MgATP and CoA or MgATP and Mg-citrate were varied at non-saturating concentrations of the third substrate. These studies demonstrated that the kinetic mechanism is ping-pong and thus resolved the discrepancy between the ping-pong mechanism implied by enzyme labelling experiments and the sequential mechanism implied by previous kinetic studies.

Purified ATP citrate lyase was phosphorylated by cAMP dependent protein kinase to the extent of 2.08 ± 0.15 moles of phosphate/mole

of ATPCL tetramer. No change in activity upon phosphorylation was detected using either the standard assay or an assay in which the substrate concentrations were approximately physiological. A kinetic study indicated that phosphorylation did not significantly alter the V_{max} nor the K_m s for CoA or Mg-citrate. Phosphorylation resulted in a significant increase in the K_m for ATP (from $90.1 \pm 1.7 \mu\text{M}$ to $193.0 \pm 3.6 \mu\text{M}$; $P < 0.01$). Assay conditions were developed whereby the decrease in activity which accompanies phosphorylation could be monitored directly. The physiological significance of these results is discussed.

Abbreviations

ACC, acetylCoA carboxylase; ATPCL, ATP citrate lyase; BSA, bovine serum albumin; cAMPdPrK, cyclicAMP dependent protein kinase; cAMPiPrK, cyclicAMP independent protein kinase; DTNB, 5, 5' - dithiobis (nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGCoA, hydroxymethylglutarylCoA; LDH: lactate dehydrogenase; lrmgACC, lactating rabbit mammary gland acetylCoA carboxylase; lrmgFAS, lactating rabbit mammary gland fatty acid synthase; MOPS, morpholinosulphonic acid; OAA, oxaloacetate; PEP, phosphoenoylpyruvate; PK, pyruvate kinase; plACC, pig liver acetylCoA carboxylase; rlACC, rat liver acetylCoA carboxylase; rlATPCL, rat liver ATP citrate lyase; SDS, sodium dodecyl sulphate; TEMED, N,N,N',N' - tetramethylethylenediamine.

Chapter 1

Introduction

In most higher organisms the chemical energy contained in food-stuffs which is in excess of the animal's immediate energy requirement is stored as either triglyceride or carbohydrate. Of these two substances triglyceride is the more concentrated energy store: each gram of triglyceride will yield twice as much energy as the corresponding amount of carbohydrate. The energy stored in triglyceride is released by the breakdown of the triglyceride to fatty acids and their subsequent oxidation to CO_2 and H_2O . The synthesis and degradation of triglyceride can therefore be viewed as two opposing processes, one storing energy, the other releasing it. There is now evidence for the existence in liver and adipose tissue of a futile cycle involving the synthesis and degradation of triglyceride. This results in an effective amplification of the reciprocal regulation of the two enzymes involved, triglyceride lipase and glycerolphosphate acyltransferase. However, it would be inappropriate were both fatty acid synthesis and oxidation to occur simultaneously and so it is not surprising to find that both processes are rigorously controlled. In this way the synthesis and degradation of triglyceride are tailored to meet the animal's energy requirements. In animals this homeostasis is maintained by the interaction of the endocrine and nervous systems, which coordinate synthesis and degradation both within a single organ and between several organs.

The physiological processes which maintain this homeostasis are relatively well understood, as are the metabolic pathways by which triglyceride is synthesised and degraded in the cell. At the cellular level our knowledge of the control of these pathways is less complete. For example, it is known that the total amount of lipogenic enzymes in the cell varies in response to the hormonal and dietary status of the animal. The mechanisms by which

variations in the hormonal status result in altered rates of protein synthesis and degradation are, however, not known. Similarly, there is evidence that some lipogenic enzymes respond, in the short term, to the hormonal status of the animal. The work described in this thesis was carried out with the intention of elucidating the nature of the short term responses of two lipogenic enzymes, ATP citrate lyase (ATP: citrate oxaloacetate-lyase (CoA-acetylating and ATP dephosphorylating) (EC 4. 1. 3. 8, hereafter abbreviated to ATPCL) and acetylCoA carboxylase (acetylCoA : carbon dioxide lyase (ADP) (EC 6. 4. 1. 2, hereafter abbreviated to ACC).

In Section 1 of this chapter I describe the metabolic pathways leading to the de novo synthesis of triglycerides and also of cholesterol, whose de novo synthesis involves several of the enzymes involved in fatty acid synthesis. In Section 2 I discuss the physicochemical and enzymatic properties of ATPCL and ACC. In the third section I review what is known regarding the long and short term regulation of lipogenesis and finally, in Section 4, I outline the objectives of the work carried out.

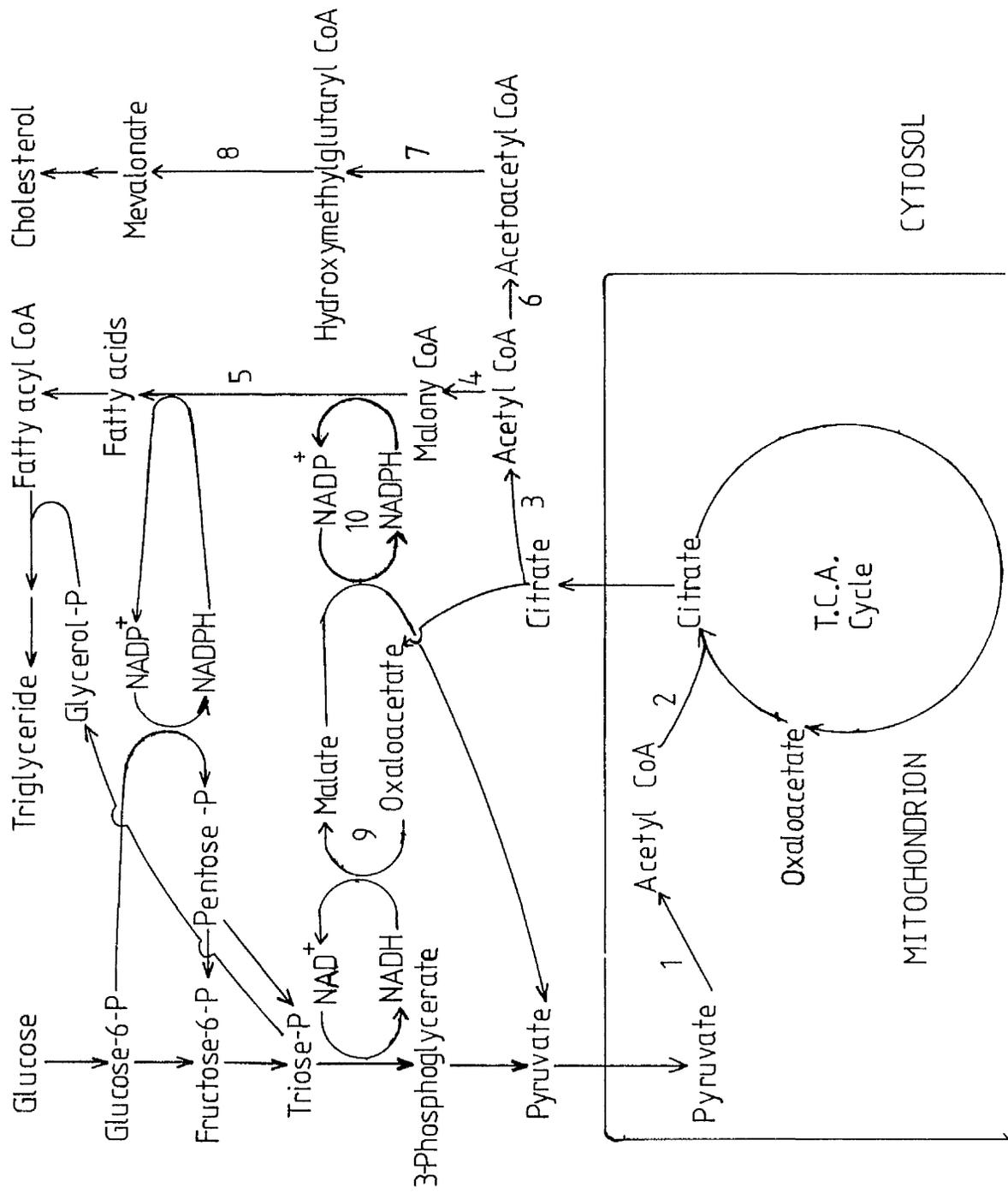
Section 1 Pathways of de novo lipid synthesis in liver

In the liver, dietary carbohydrate which is in excess of the animal's immediate energy requirement has two main fates. It is either converted into glycogen and stored in the liver or it is converted into triglyceride, the bulk of which is then transported out of the liver for storage in adipose tissue. While hexose phosphates can be polymerised directly to form glycogen, synthesis of lipid from carbohydrates involves their degradation to acetyl units followed by the assembly of the acetyl units into fatty acids and cholesterol. The pathway of lipogenesis from glucose is shown in Figure 1.1. It can be seen that de novo lipid synthesis involves most of the major pathways in intermediary metabolism.

Dietary carbohydrate is first converted to pyruvate in the cytoplasm by the enzymes of the glycolytic pathway. Pyruvate is then transported into the mitochondria and in the reaction catalysed by pyruvate dehydrogenase is oxidised to acetylCoA, which is the precursor of lipids in non-ruminants. Note, however, that the enzymes which catalyse the synthesis of lipids from acetylCoA are located in the cytoplasm and on the endoplasmic reticulum. The acetylCoA required for lipid synthesis must, therefore, be transported across the mitochondrial membrane since diffusion of acetylCoA out of the mitochondria is too slow to account for the rates of lipid synthesis observed in vivo. The generally accepted pathway for the transport of acetylCoA into the cytoplasm is shown in Figure 1.1. Mitochondrial acetylCoA first condenses with oxaloacetate (OAA) to form citrate (catalysed by citrate synthase) which is transported out of the mitochondria by the tricarboxylate carrier. Cytoplasmic acetylCoA is generated by the enzyme ATPCL which catalyses the ATP dependent cleavage of citrate to OAA and acetylCoA (Srere, 1962; Daikuhara et al, 1968).

Figure 1.1 . Pathways of de novo lipid synthesis in eukaryotes.

Enzymes mentioned in the text are: (1), pyruvate dehydrogenase; (2), citrate synthase; (3), ATP citrate lyase; (4), acetylCoA carboxylase; (5), fatty acid synthase; (6), β -ketothiolase; (7) hydroxymethylglutarylCoA synthase; (8), hydroxymethylglutarylCoA reductase; (9), malate dehydrogenase; (10), malic enzyme. In the interests of clarity several enzymic steps have been omitted.



There is considerable experimental evidence in support of this mechanism for the translocation of acetylCoA into the cytoplasm. Srere and Bhaduri (1962) found that citrate is a precursor of fatty acids in pigeon liver and experiments with asymmetrically labelled citrate have indicated that it is the fragment derived from acetylCoA which is incorporated into fatty acids (Spencer and Lowenstein, 1962). Further evidence for the involvement of ATPCL in the supply of acetylCoA for fatty acid synthesis comes from the experiments of Watson and Lowenstein (1970) who found that fatty acid synthesis in vivo is inhibited by (pn)-(4S)-4-hydroxycitrate, a potent inhibitor of ATPCL. In addition, tissue levels of ATPCL and the rate of fatty acid synthesis change in parallel in response to changes in the hormonal or dietary status of the animal.

The oxaloacetate formed in the reaction catalysed by ATPCL can be recycled back into the mitochondria after conversion to malate (catalysed by malate dehydrogenase) followed by conversion into pyruvate (catalysed by malic enzyme). (There are many other possible fates of oxaloacetate).

The synthesis of fatty acids from acetylCoA is carried out by two enzymes, ACC and fatty acid synthase (FAS). ACC catalyses the ATP dependent carboxylation of acetylCoA to malonylCoA. This is the first committed step in de novo fatty acid synthesis as malonylCoA has no metabolic fate other than conversion to fatty acids, which is catalysed by FAS. The product of this reaction is generally palmitate although recent evidence (Linn and Srere, 1980) suggests that palmitate is not released as the free acid but rather as the CoA thioester. The long chain fatty acylCoA thioesters are then available for synthesis into triglycerides and phospholipids.

0

It is now clear that ATPCL also provides the acetylCoA which is the precursor of cholesterol. De novo cholesterol synthesis proceeds through the intermediate mevalonate. It was initially believed that the formation of mevalonate from hydroxymethylglutarylCoA (HMGCoA) was the first committed step in cholesterol synthesis since HMGCoA was a precursor for both cholesterol and ketone body formation. There are, however, both cytoplasmic and mitochondrial forms of the enzymes which catalyse the formation of HMGCoA from acetylCoA, β -ketothiolase and HMGCoA synthase (Williamson et al., 1972; Sugiyama et al., 1972). There is now evidence that only cytoplasmic acetylCoA is utilised in cholesterol synthesis, the mitochondrial β -ketothiolase and HMGCoA synthase being concerned exclusively with ketone body production (Dietschy and McGarry, 1974). Thus, it is apparent that the committed step in cholesterol synthesis is the formation of acetoacetylCoA in the cytoplasm. The involvement of ATPCL in the generation of acetylCoA for cholesterol synthesis is indicated by experiments where cholesterol synthesis was inhibited by (m)-(4S)-4-hydroxycitrate a potent inhibitor of ATPCL (Watson and Lowenstein, 1970).

The above discussion has been concerned with the supply of acetylCoA which is the precursor of the carbon skeleton of both fatty acids and cholesterol. Lipid synthesis also requires NADPH as a reducing agent (Numa and Yamashita, 1974). About 50% of the NADPH required for fatty acid synthesis is generated in the pentose phosphate cycle (Flatt and Ball, 1962; Katz et al., 1964). The remainder of the NADPH required is generated from cytoplasmic NADH by a transhydrogenation system involving coupled reactions between malate dehydrogenase and malic enzyme (Pande et al., 1964). As can be seen from Figure 1.1 this series of reactions is also involved in the citrate/pyruvate cycle mentioned above whereby OAA produced in

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the reaction catalysed by ATPCL is returned to the mitochondria as pyruvate. The overall effect is an ATP dependent transhydrogenation. Thus, ATPCL is involved in the generation of both the acetyl units and the reducing equivalents required for lipid synthesis.

In mammals, the pathway of de novo lipid synthesis is apparently identical in all of the major lipogenic tissues; liver, adipose tissue and mammary gland. Variations do occur, however, in the chain length of the fatty acids produced. Most of the fatty acids are then esterified to triglyceride. In mammary and adipose tissue the triglyceride is stored in the fat droplet of the cell. In the liver, however, most of the triglyceride is transported out of the cell and is eventually stored at other sites in the body. The amount of cholesterol synthesised in adipose and mammary tissue is much lower than that synthesised in the liver.

Section 2. Properties of the major lipogenic enzymes

Introduction

Section 1 of this chapter outlined the biochemical pathways leading to the de novo synthesis of fatty acids. The work described in this thesis is largely concerned with only one part of that pathway, the synthesis of fatty acids from cytoplasmic citrate. In particular, because of their apparent involvement in the hormonal regulation of fatty acid synthesis, the work focused on the enzymes ATPCL and ACC. In this section I review the physical and enzymatic properties of these enzymes. A less detailed description of the properties of FAS is also given.

ATP citrate lyase

ATPCL was first discovered in pigeon liver by Srere and Lipmann(1953). Srere subsequently purified the enzyme from chicken liver and determined the stoichiometry of the reaction (Srere 1959, 1961). ATPCL has not been detected in prokaryotic organisms. The enzyme has been found in certain moulds and plants which synthesise unusually high amounts of fatty acids. ATPCL is widely distributed in animal tissues and high levels are found in tissues which have a particularly large capacity for lipogenesis such as liver, adipose and mammary tissue. High levels are also found in brain. This may be because ATPCL is part of the pathway which provides acetyl units in acetylcholine synthesis. However, there is some dispute as to the proportion of the acetyl units that is generated by ATPCL (Tucek and Cheng, 1970).

ATPCL has been purified from a number of sources including rat and chicken liver, rat mammary gland and rat brain. The purified rat liver enzyme has been crystallised (Inoue et al, 1966). ATPCL

is generally considered to be located in the cytoplasmic compartment of the cell (Srere, 1972). Recently, however, some workers have suggested that ATPCL can associate with mitochondria (Janski and Cornell, 1980) or with the endoplasmic reticulum (Avruch et al, 1981).

The M_r of ATPCL has been estimated by a variety of techniques. Inoue et al (1966), using density gradient centrifugation, obtained a value of 500000 for the M_r of the rat liver enzyme. Somewhat later, Srere's group reported an M_r of 440000 for rat liver ATPCL (rlATPCL), a value obtained using sedimentation equilibrium centrifugation (Singh et al, 1976). Similar values have been determined by other workers for lactating rat mammary gland ATPCL (Guy et al, 1981).

There is, therefore, general agreement that the M_r of ATPCL is of the order of 440000 - 500000. Indeed, it is possible that ATPCLs from all sources have a similar M_r since ATPCLs isolated from such diverse sources as mango and the mould Penicillium spiculosporum have M_r s in this range (Srere, 1975). The $S_{20,w}$ as estimated by a number of workers is in the range 13.0 - 15.0 S (Inoue et al, 1966; Singh et al, 1981). This sedimentation coefficient is consistent with the M_r of 500000. There is also some evidence from sedimentation equilibrium and gel filtration experiments that ATPCL can exist in a more aggregated form (Singh et al, 1976; Guy et al, 1981; Corrigan and Rider, 1981). These observations are discussed more fully in Chapter 5.1.

Early studies on ATPCL, where the M_r was estimated by gel filtration in 7 M guanidine-HCl, suggested that the rat liver enzyme was composed of 8 identical subunits with M_r s of approximately 50000 (Srere, 1972). In later studies, analysis of pure rlATPCL on SDS-polyacrylamide gels revealed 3 protein staining bands with M_r s of 110000, 67,000 and 57,000 (Singh et al, 1976). Since there was

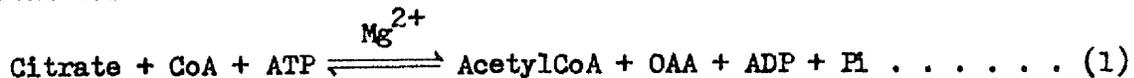
considerable variation in the relative intensities of the 3 bands the authors suggested that the two lower M_r polypeptides were derived from the 110,000 M_r polypeptide by limited proteolysis which had occurred during purification. Later studies, where ATPCL has been purified in the presence of protease inhibitors have shown that this interpretation is correct. In such cases the purified ATPCL shows only a single band on SDS-polyacrylamide gel electrophoresis (Linn and Srere, 1979; Guy et al, 1981; Alexander et al, 1979). Thus, ATPCL is composed of 4 apparently identical subunits. Estimates of the subunit M_r are in the range 110,000 - 125,000. A detailed discussion of the subunit M_r is given in chapter 5.1.

The amino acid composition of ATPCL purified from various sources have been published (see Guy et al, 1981). ATPCL is sensitive to chemical modification by thiol specific reagents such as DTNB. At least one of the thiol groups modified by DTNB would appear to be at the active site since citrate protects against inactivation (Cottam and Srere, 1969). Purified ATPCL tends to lose activity during storage, due apparently to the oxidation of sulphhydryl groups, since activity can be partially recovered by incubation in the presence of reducing agents.

Srere (1959) determined that avian liver ATPCL has a pH optimum of 8.7. This has been confirmed for r1ATPCL by later workers. The pH profile of the enzyme is, to a certain extent, dependent on the buffer used. Ranganathan et al (1980) obtained maximum ATPCL activity at pH 8.7 in Tris-HCl buffer. In HEPES (4 - (2 - hydroxymethyl) - 1 - piperazineethanesulphonic acid) buffer, however, they observed a double peak with optima at pH 7.6 and pH 8.6.

The stoichiometry of the reaction was first determined by Srere (1961) using purified chicken liver ATPCL. The reaction

catalysed by ATPCL is one of the most complex enzyme catalysed reactions known, involving three substrates, four products and a metal ion :



The metal ion requirement is due to the fact that MgATP and Mg-citrate are the active forms of these substrates, rather than non-complexed ATP and citrate (Plowman and Cleland, 1967). The metal ion requirement was initially studied by Srere (1961) who found that Mn^{2+} or Co^{2+} could partially replace Mg^{2+} .

The reaction is reversible although the equilibrium favours cleavage of citrate. The equilibrium constant was initially measured by Plowman and Cleland (1967) as 1.0 - 1.5 M. Later, Guynn and Veech (1973) estimated the equilibrium constant which would be observed under approximately physiological conditions (pH 7.0, I = 0.25). The observed equilibrium constants were 0.98 M at $[\text{Mg}^{2+}] = 0$ and 0.098 M at $[\text{Mg}^{2+}] = 1$ mM. This dependence on Mg^{2+} ions arises because the substrates of the reaction are MgATP and Mg-citrate rather than the non-complexed forms.

The order of binding of the substrates of ATPCL was studied by Plowman and Cleland (1967). The kinetic data obtained by these workers indicated that the mechanism was sequential with MgATP binding first, followed by the random rapid equilibrium binding of Mg-citrate and CoA. However, isotope exchange data obtained by these workers suggested the existence of a ping-pong mechanism with MgADP released from the enzyme before the addition of CoA and Mg-citrate. Later kinetic studies by Farrar and Plowman (1971a) also indicated that the kinetic mechanism was sequential. Thus, there is a discrepancy between the mechanism implied by kinetic data and that implied by

isotope exchange data. Farrar and Flowman (1971b), taking into account all of the available kinetic and non-kinetic data proposed a hybrid mechanism whereby MgADP could be released at several points in the reaction. A more detailed discussion of the discrepancy between the kinetic mechanisms implied by different types of experiments is given in Chapter 5.2.

Evidence in support of the ping-pong mechanism has come from the discovery that ATPCL is phosphorylated extremely rapidly in the presence of MgATP alone (Flowman and Cleland, 1967; Inoue et al, 1968). There is a continuing controversy regarding the nature of the amino acid at the active site which becomes phosphorylated. The phosphoamino acid is acid-labile and Srere's group identified it as phosphohistidine, following alkaline hydrolysis of the phosphoenzyme (Cottam and Srere, 1969 b). More recently Ramakrishna and Benjamin (1979) have confirmed this result. Takeda's group, however, identified the phosphoamino acid as glutamyl phosphate on the basis of the pH stability of the phosphoenzyme and suggested that the result obtained by Cottam and Srere (1969 b) was the result of a rearrangement occurring during alkaline hydrolysis (Inoue et al, 1968). Irrespective of the nature of the phosphoamino acid formed, these results support the ping-pong mechanism, and are in disagreement with the kinetic results of Flowman and Cleland (1967).

Incubation of either phosphoATPCL with Mg-citrate alone, or of ATPCL with MgATP and Mg-citrate results in the formation of citrylATPCL and Pi (Inoue et al 1967, 1968). Furthermore incubation of phosphoATPCL with [^{18}O] - citrate resulted in the incorporation of 1 mole of ^{18}O into each mole of Pi released. Citryl phosphate can replace MgATP and Mg-citrate in reaction (1) and incubation of ATPCL with citryl phosphate alone yields citrylATPCL + Pi (Walsh and Spector

1968, 1969). Taken together these results indicate that phosphoATPCL reacts with Mg-citrate to form phosphocitrylATPCL which then forms citrylATPCL + Pi. (Note that the phosphocitryl moiety is not necessarily covalently bound to the enzyme). Inoue et al (1968) and Walsh and Spector (1969) incubated citrylATPCL with CoA and obtained stoichiometric amounts of acetylCoA and OAA. This reaction most likely proceeds through a citrylCoA intermediate since Eggerer and Remberger (1963) and Srere and Bhaduri (1964) had earlier shown that ATPCL could catalyse the cleavage of citrylCoA to acetylCoA and OAA. A mechanism consistent with all of the results described above is presented in Figure 1.2 (after Srere, 1972).

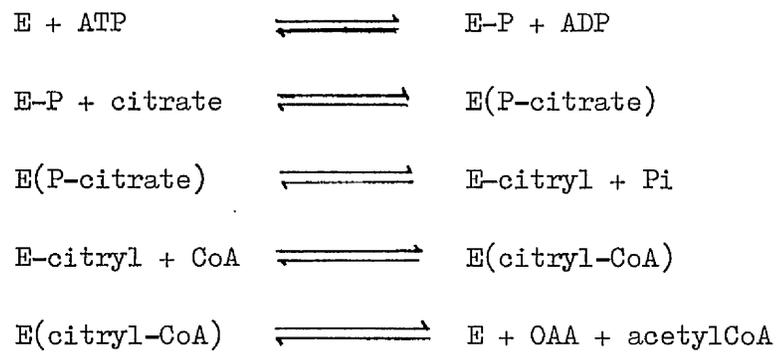
There is some evidence that citrate is an activator of ATPCL as well as a substrate. In their study of the kinetic properties of ATPCL, Plowman and Cleland (1967) obtained non-linear double reciprocal plots when Mg-citrate was the varied substrate and the $[Cl^-]$ was low. At high $[Cl^-]$ the non-linearity was abolished. One interpretation of this non-linearity which was proposed by Plowman and Cleland (1967) was that free citrate was activating the enzyme by binding to a site distinct from the active site. Support for this concept comes from the observation of Eggerer and Remberger (1963) that citrate increased the rate of cleavage of citrylCoA to acetylCoA and OAA. The possible activation of ATPCL by citrate has received scant attention by subsequent workers. Consequently there is no information regarding the nature of this activation,

There is considerable evidence that ATPCL is inhibited by one of the products of the reaction, ADP, which inhibits the enzyme competitively with respect to MgATP (Inoue et al, 1966; Atkinson and Walton, 1967).

Szutowitz et al (1974) reported that ATPCL purified from adult

Figure 1.2. Reaction mechanism of ATP citrate lyase.

A dash (-) indicates a covalent bond. Parentheses around a compound indicate that it is not necessarily covalently bound to ATPCL.



rat brain was inhibited by D- and L-glutamate. The degree of inhibition was time dependent and required the presence of ATP and an excess of Mg^{2+} ions in addition to glutamate. The inhibition was unusual in that while ATP was required for inhibition, the inhibition was said to be competitive with respect to ATP. The mechanism of this inhibition is unclear. The time dependence indicates that it is not the simple case of competitive inhibition which Szutowicz et al (1974) imply. Indeed, the time course and ATP and Mg^{2+} requirement of the inhibition are reminiscent of phosphorylation by a protein kinase. Szutowicz et al (1974) did not explore this possibility. The work described above by Szutowicz et al (1974) was carried out on ATPGL which had been purified from adult rat brain. These authors also studied the enzymes isolated from immature rat brain and the livers of rats which had been starved for 48 h and then refed a fat free, high carbohydrate diet for 72 h and found that they were not inhibited to a great extent. In contrast r1ATPGL from rats which had been fasted for 48 h were inhibited by 40% after 30 min incubation. These results suggest that some other factor (which varies with the nutritional or developmental state of the animal) is involved in this effect of glutamate.

ATPGL is also inhibited by (pn) - (4 S) - 4 - hydroxycitrate which can be isolated from Garcinia. This substance is a potent competitive inhibitor of ATPGL and Watson et al (1969) reported a K_i of $0.6\mu M$. While citrate, ADP and glutamate are potential physiological effectors of ATPGL, (pn) - (4 S) - 4 - hydroxycitrate quite clearly is not. This substance has, however, proved useful in in vivo studies of fatty acid metabolism.

The possible role of these effectors in the in vivo regulation of ATPGL is discussed in Section 3 of this chapter.

AcetylCoA carboxylase

Early studies on fatty acid metabolism in avian liver revealed that two distinct enzymes (termed R_1 and R_2) were required for the synthesis of fatty acids from acetylCoA (Fritz, 1961). Wakil's group showed that fatty acid synthesis was bicarbonate dependent (Gibson *et al.*, 1958) and Wakil (1958) subsequently showed that the R_1 component catalysed the ATP dependent carboxylation of acetylCoA. Following this identification of R_1 as ACC the R_2 component was identified as FAS, which catalyses the synthesis of long chain fatty acids from malonylCoA.

ACC has been detected in plant, animal, and bacterial cells. In birds and mammals it is particularly active in tissues which have a high capacity for lipogenesis. ACC has been purified from a number of mammalian and avian tissues including chicken and rat liver, bovine and rat adipose tissue and rabbit and rat mammary gland. It should be understood that the discussion on the properties of ACC which is given below applies only to the avian and mammalian enzymes unless otherwise stated.

ACC is a soluble enzyme and is generally considered to be located in the cytosol compartment of the cell since it is not pelleted during centrifugation of tissue homogenates at 105000 xg. Furthermore, Meredith and Lane (1978) have shown that ACC is released from digitonin treated cells at the same rate as known cytoplasmic enzymes such as lactate dehydrogenase. However, there are a number of reports which indicate that ACC is associated to a certain extent with the endoplasmic reticulum. This was first reported by Margolis and Baum (1966) who found ACC activity in the microsomal fraction of homogenates of avian livers, provided that Mg^{2+} ions were present in the homogenisation buffer. Little ACC activity was found in the microsomes in

the absence of Mg^{2+} . Using a combination of electron microscopy and histochemistry Yates et al (1969) visualised rat liver ACC (rIACC) in situ and found that it was located primarily on the endoplasmic reticulum. Most workers, however, homogenise the tissue in the presence of EDTA and consequently ACC is found in the 105000 xg supernatant.

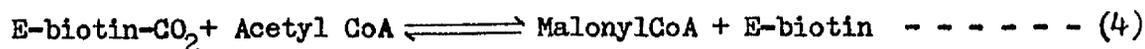
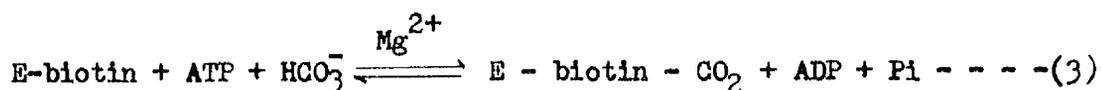
Extensive studies have shown that avian and mammalian ACCs have similar properties with respect to M_r and quaternary structure. ACC can exist in vitro in either an active high M_r form or in an inactive low M_r form. Examination of the high M_r form by electron microscopy indicates that it has a filamentous structure and that each filament is a polymer of some 10 - 20 protomeric units (Gregolin et al, 1966). The variation in the number of protomers in each filament explains the weight heterogeneity of the high M_r form, $4 \times 10^6 - 10^7$. Each filament is approximately 100 nm wide and lengths of up to 5000 nm have been reported. The rod-like nature of the polymeric form is reflected in its hydrodynamic properties: the active form of ACC has a high intrinsic viscosity ($[\eta] = 83 \text{ cm}^3 \text{g}^{-1}$) and exhibits a hypersharp boundary during sedimentation in the analytical ultracentrifuge (Moss and Lane, 1972; Gregolin et al, 1966). As might be expected, the polymeric form of the enzyme has a large sedimentation coefficient: values of around 70 S have been obtained from sedimentation velocity experiments while somewhat lower values are obtained using sucrose density gradient centrifugation (Lane et al, 1974).

Dissociation of the polymer into the inactive protomer can be accomplished by treatment with 0.5 M NaCl at pH 9.0, by exposure to cold, by incubation with malonylCoA or bicarbonate, or by removal of the tricarboxylic acid activators of the enzyme. Early reports of the M_r of the protomer were in the range of 400000 - 560000 (Gregolin

et al, 1966; Moss et al, 1972). More recent work suggests that the M_r is closer to 500000 (Hardie and Cohen, 1978b; Ahmad et al, 1978). The transition from the polymeric form to the protomer is accompanied by a decrease in both the intrinsic viscosity (to $11.5 \text{ cm}^3 \text{ g}^{-1}$) and the sedimentation coefficient (to 13 - 15 S) (Lane et al, 1974).

Early studies on the avian and rat liver enzymes indicated that the protomers were themselves composed of subunits. SDS - polyacrylamide gel electrophoresis of purified rLACC revealed the presence of 3 protein staining bands with M_r s of 215000, 125000 and 118000 (Inoue and Lowenstein, 1972). Purified avian liver ACC could also be resolved into 3 polypeptides under similar conditions (Guchhait et al, 1974). These results led both groups to propose that the protomeric form of ACC was composed of non-identical subunits. It is now clear, however, that the lower M_r polypeptides observed by these workers were caused by partial proteolysis of ACC, which had occurred during purification. This was first demonstrated by Tanabe et al (1975) who showed that the two lower M_r bands were absent from preparations of rLACC which had been purified in the presence of the protease inhibitor PMSF. These workers also demonstrated that treatment of the purified ACC with either trypsin or a lysosomal extract from rat liver could generate the lower M_r fragments. These results have since been confirmed by other workers who have shown that SDS - polyacrylamide gel electrophoresis of ACC purified from a number of different sources reveals only one protein staining band.

The subunit M_r estimated by SDS - polyacrylamide gel electrophoresis is somewhat variable : values from 230000 - 252000 have been reported (Tanabe et al, 1975; Hardie and Cohen, 1978b; Witters and Vogt, 1981). This variation in the estimates of the subunit M_r may be due to some of the preparations being partially proteolysed



Reaction (3), the ATP dependent carboxylation of biotin, occurs in the absence of acetylCoA. The site of this carboxylation is the 1'-N of the uriedo ring (Numa et al 1964, 1965). When the carboxylation of biotin was carried out using [¹⁸O] - HCO₃⁻ one atom of oxygen from the HCO₃⁻ was incorporated into Pi (Kaziro et al, 1962). This result suggests that the reaction involves the nucleophilic attack on the γ-phosphoryl phosphorus by the bicarbonate, coupled with the attack on the bicarbonate carbon by the 1'-N of the uriedo group. Kaziro et al (1962) proposed that the mechanism described above is concerted. More recently Polakis et al (1973) suggested that the mechanism is sequential with carbonic-phosphoric-anhydride as an intermediate.

The second partial reaction (4) can take place in the absence of both ATP and HCO₃⁻. The carboxyl group of ¹⁴C-labelled carboxybiotin-ACC can be transferred to acetylCoA in the absence of ATP and HCO₃⁻. This transfer would seem to be facilitated by electrophilic activation of the carboxylate group which is probably caused by deformation of the uriedo ring brought about by a conformational change at the active site (Lane et al, 1974). The conformational strain induced by the carboxylation of ACC is enough to cause the dissociation of the polymeric form of the enzyme into protomers.

It is appropriate to discuss the ACC from *E. coli* at this point. Pure *E. coli* ACC can be readily resolved into 3 types of subunit, each of which has a distinct function: 1) the carboxyl carrier protein, which contains covalently bound biotin; 2) biotin carboxylase, which catalyses the ATP dependent carboxylation of the carboxyl carrier protein (or of free biotin); 3) carboxyl transferase which catalyses the transfer of CO_2 from the carboxyl carrier protein (or free carboxy biotin) to acetyl CoA. Clearly, in *E. coli* ACC each of the partial reactions occurs at a separate active site. In the currently accepted model of the mechanism of *E. coli* ACC the uriedo ring of biotin oscillates between the two active sites. This is possible since the uriedo ring is located at the end of a flexible side chain on the biotin carboxylase subunit.

In mammalian and avian ACCs both partial reactions would appear to be catalysed by only one polypeptide. It is widely believed, however, that reactions 3 and 4 do occur at distinct active sites on this polypeptide and that the biotin oscillates between them as it does in the *E. coli* enzyme. It is possible that in mammalian and avian ACC these active sites are located on distinct domains.

As mentioned above, ACC from all animal tissues can exist in either a polymeric form or a protomeric form. Addition of citrate or isocitrate to solutions of the protomeric form results in its conversion into the polymer as judged by the changes in the physical parameters of the enzyme. Thus, addition of citrate results in an increase in the sedimentation coefficient from 13 - 15 S to 50 S and an increase in the intrinsic viscosity from $11.3 \text{ cm}^3\text{g}^{-1}$ to $87 \text{ cm}^3\text{g}^{-1}$ (Gregolin et al, 1966; Moss and Lane, 1972). Furthermore, the transition from protomer to polymer has been visualised in the electron microscope (Gregolin et al, 1968). The transition from

the protomeric to the polymeric form is accompanied by an increase in the activity of the enzyme (Numa et al, 1965; Gregolin et al, 1966).

Lane's group have made an extensive study of the mechanism by which citrate and isocitrate activate ACC. These workers observed that citrate increased the V_{\max} of ACC rather than decreasing the K_m for any of the substrates (Moss et al, 1972; Gregolin et al, 1968 a,b). Citrate increases the rate of both of the partial reactions (reactions 3 and 4) (Gregolin et al, 1968) and this suggests that the binding of citrate induces a conformational change in the enzyme. Lane et al, (1974) have proposed that such a conformational change brings the biotinyl group into closer proximity to the substrate binding sites. It has been shown that in the presence of citrate, the biotin group is inaccessible to avidin whereas in the absence of citrate avidin can bind to the biotin and inactivate ACC irreversibly. However, in view of the effects of citrate on the aggregation state of the enzyme it is difficult to interpret the significance of these results.

The activity of ACC is determined by the position of the equilibrium between protomer and polymer. When citrate is removed from solutions of polymeric ACC there is a slow decrease in the proportion of polymer present ($t_{\frac{1}{2}} = 10$ min) as judged by the decrease in the viscosity of the solution. The decrease in viscosity is paralleled by a decrease in activity. In the presence of either bicarbonate or malonylCoA the decreases in the proportion of polymer and the activity are almost instantaneous ($t_{\frac{1}{2}} = 5$ sec) (Moss and Lane, 1972). Both bicarbonate and malonylCoA carboxylate ACC and in the absence of citrate this results in the dissociation of the polymer into protomers. Citrate clearly prevents this dissociation, presumably because the conformation induced by citrate favours polymerization. This explains why citrate increases the rate of decarboxylation of

carboxy-biotin-ACC. The rate of decarboxylation is increased still further if an acceptor for the CO_2 is present. Thus, citrate and acetylCoA have been shown to have a synergistic effect in increasing the rate of decarboxylation (Lane et al, 1970).

ACCs from different sources differ in the rate of transition from protomer to polymer. In the case of chicken liver ACC the transition is virtually instantaneous. The enzymes from rat tissues are polymerised more slowly and these enzymes require preincubation at 37°C in the presence of citrate before maximum activity is expressed (Inoue and Lowenstein, 1972). There is also an absolute requirement for citrate in the ACC assay, whether the enzyme has been preincubated with citrate or not (Inoue and Lowenstein, 1972; Nakanishi and Numa, 1970). As discussed above, in the absence of citrate carboxylation of ACC by bicarbonate would result in the dissociation (and inactivation) of the polymeric form of the enzyme. Citrate also causes a conformational change in the protomer. As described above this change is believed to result in the biotin group becoming more closely associated with the active sites. In this way citrate activates the protomeric form of the enzyme by a classical allosteric mechanism in addition to its effect on the aggregation state.

Yeh and Kim (1980) reported that partially purified rlACC is activated by CoA and that this activation caused a decrease in the K_m of ACC for acetylCoA. Purified rlACC, however, could not be activated. In a subsequent publication they reported that purified rlACC could indeed be activated, provided that the enzyme was free from tightly bound citrate (Yeh et al, 1981). The binding of CoA (1 molecule/subunit) was not affected by the presence of tightly bound citrate, but the presence of citrate prevented activation. PalmitoylCoA, a potent inhibitor of ACC (see below), prevented the

binding of CoA. The effect of the activation on the kinetic parameters of the enzyme is somewhat unclear. CoA activated partially purified ACC by decreasing the K_m for acetylCoA from 200 μ M to 4 μ M. The purified, citrate-free preparations had lower K_m s for acetylCoA and activation appeared to be the result of an increase in the V_{max} since the K_m s for acetylCoA were unaltered. The nature of the CoA activation is therefore unclear at the present time.

Witters et al (1981) have reported that partially purified rlACC is activated by guanine nucleotides, especially 5' - GMP, and have detected an endogenous ACC activator in rat liver with similar properties to 5' - GMP. The effect of both the endogenous activator and 5' - GMP is to alter the ACC citrate dose-response curve. In the absence of the activator or 5' - GMP the citrate dose response-curve is linear up to 5 mM citrate. In contrast, in the presence of the activator or 5' - GMP the dose-response curve is hyperbolic, with ACC being maximally active at citrate concentrations greater than 0.2 mM. Furthermore, the V_{max} in the presence of the activator or 5' - GMP is approximately 2-fold greater than that in their absence. The mechanism of this activation remains to be elucidated, particularly since purified rlACC is not activated by either guanine nucleotides or endogenous activator.

Gregolin et al (1966) reported that malonylCoA is an inhibitor of ACC. Two kinds of inhibition were observed. MalonylCoA was competitive with respect to acetylCoA. This was not unexpected since both substances have similar structures and it would appear to be a classic case of competitive product inhibition. MalonylCoA was also found to be competitive with respect to citrate. This arises because malonylCoA can carboxylate ACC and this (as discussed above) results in depolymerization and inactivation. In this respect malonylCoA

clearly acts in opposition to citrate, which promotes the formation of the polymer.

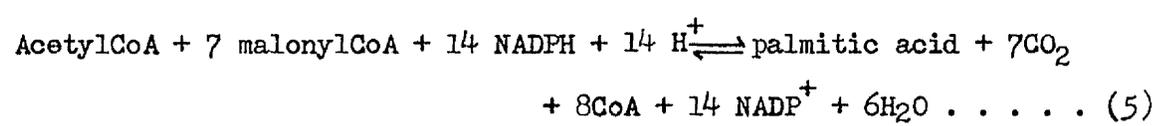
ACC is also inhibited by long chain fatty acyl thioesters (Numa, 1974). Initially some workers interpreted this inhibition as being due to detergent action (Srere, 1965). However, current evidence suggests that long chain fatty acyl thioesters exert their effects by an allosteric mechanism. The inhibition is competitive with respect to citrate, but not with respect to any of the substrates of ACC. Long chain fatty acyl thioesters bind extremely tightly to ACC. In studies using a fluorescent analogue of stearoylCoA Ogiwara et al (1978) and Nikawa et al (1979) found that the K_i was in the region of 5 nM. This was very similar to the dissociation constant measured by Sreekrishna et al (1980) who also reported that 1.2 molecules of thioester was bound per subunit. Like malonylCoA, long chain fatty acyl thioesters are competitive with respect to citrate and promote the depolymerization of ACC. Citrate can in fact displace long chain fatty acyl thioesters from ACC, provided that an acceptor for the thioester is present. Sreekrishna et al (1980) used 6 - O - methyl-glucose polysaccharide as an acceptor: in vivo this function might well be fulfilled by the fatty acid binding protein described by Halestrap and Denton (1974) and Lunzer et al (1977).

In summary, most effectors of ACC alter the activity of the enzyme by increasing or decreasing the amount of the active form. These effectors alter the position of the equilibrium between protomer and polymer by inducing conformational changes which maintain one form or the other. The possible role of these effectors in the regulation of ACC is discussed in section 3 of this chapter.

Fatty acid synthase

Lynen and coworkers identified the R₂ component of the avian liver fatty acid synthesising system as FAS. As with ATPCL and ACC large amounts of FAS are found in mammalian tissues which have a high capacity for lipogenesis. The physical and chemical properties of FAS have been extensively studied. The M_r of the mammalian enzyme is 500,000 and the native enzyme can be resolved into two apparently identical subunits (M_r 250,000) by SDS-polyacrylamide gel electrophoresis.

FAS catalyses the synthesis of long chain fatty acids from malonylCoA:



A great deal of evidence indicates that the overall reaction is composed of 7 partial reactions. These involve the addition of an acetyl group from malonylCoA onto the growing fatty acyl chain and its subsequent reduction by NADPH. Mammalian FAS is extremely interesting since it appears that all 7 partial reactions are catalysed by the one polypeptide.

While FAS is obviously an important and interesting enzyme, this thesis is mainly concerned with the two other major lipogenic enzymes, ATPCL and ACC. For this reason, only the salient properties of FAS have been mentioned above. The reader is referred to several excellent reviews on FAS (Bloch and Vance, 1977; Stoops et al, 1977).

Section 3. In vivo regulation of fatty acid synthesis

The two major functions of fatty acid synthesis are the synthesis of membrane phospholipid and the storage as triglyceride of foodstuffs which are in excess of the animal's needs. Although the regulation of these two pathways is of great interest, a discussion of the regulatory processes involved in phospholipid synthesis is beyond the scope of this chapter. I will therefore confine myself to a discussion of the control of synthesis of fatty acids required for esterification into triglyceride.

It is not surprising to find that the rate of fatty acid synthesis varies with the metabolic state of the animal. Profound changes in the rate of fatty acid synthesis occur when the diet of the animal is manipulated. Fasting results in a decrease in the rate of fatty acid synthesis while a diet which is low in fat or high in carbohydrate causes increased fatty acid synthesis, particularly after starvation (Craig et al, 1972). These changes occur over a period of many hours and are maintained until the dietary status of the animal is altered. For example, Bortz (1967) reported that administration of a high fat diet caused a 6-fold decrease in hepatic fatty acid synthesis after 24 h.

Chronic administration of glucagon or hydrocortisone also causes a decrease in the rate of fatty acid synthesis and such treatment also blunts the increase in the rate of fatty acid synthesis which occurs on feeding the animal a fat-free diet. Fatty acid synthesis is also altered in various pathological conditions. It is decreased in rats which have been made diabetic by administration of alloxan (Brady and Gurin, 1950). This reduction in fatty acid synthesis can be reversed by treatment with insulin.

Hepatic fatty acid synthesis also varies in accordance with

hormonal and developmental changes. Ballard and Hanson (1967) showed that the rate of fatty acid synthesis is high in foetal and adult liver, but low in suckling animals. In rabbit and rat mammary gland fatty acid synthesis increases dramatically around the time of parturition and remains high during lactation (Howanitz and Levy, 1965). These changes in the mammary gland would appear to be due to changes in the level of prolactin (Volpe and Vagelos, 1976).

The changes in the rate of fatty acid synthesis described above can be considered long term in that they occur over 24 - 48 h and are maintained for prolonged periods. Fatty acid synthesis is also subject to short term variation where changes in the rate of synthesis take place over periods of time measured in minutes rather than hours. In the liver these short term changes are controlled primarily by glucagon and insulin, while in adipose tissue adrenaline is much more important than glucagon. Klain and Weiser (1973) reported that injection of glucagon in to rats decreased the rate of fatty acid synthesis by 70% within 30 min. Similar results were obtained when liver slices (Allred and Roehrig, 1973) or hepatocytes (Goodridge, 1973; Cook et al, 1977) were incubated with glucagon or dibutyrylcAMP. The effect of adrenaline on fatty acid synthesis is less clear. Denton and Halperin (1968) found that incubation of fat-pads with adrenaline for 60 min decreased fatty acid synthesis only after prior stimulation by insulin. Rapid insulin-stimulated changes in the rate of hepatic fatty acid synthesis were noted by Brady and Gurin (1950) who found that treatment of liver slices with insulin increased the rate of fatty acid synthesis. More recently Goodridge (1973) showed that insulin increased fatty acid synthesis by 43% within 1 h. Similar results have been obtained by other workers (Geelen et al, 1978; Witters et al, 1979b). Insulin also increases

fatty acid synthesis in adipose tissue. Using epididymal fat-pads, Denton and Halperin (1968) demonstrated a 5-fold increase in fatty acid synthesis within 60 min of incubation with insulin.

From the results described above, it would appear that fatty acid synthesis is subject to two modes of regulation. In long term regulation, changes in the rate of fatty acid synthesis occur over periods of hours or days in response to prolonged metabolic states such as fasting, diabetes or lactation. The rate of synthesis is also modulated in the short term, in response to changes in the levels of insulin, glucagon and adrenaline. The mechanisms involved in both modes of regulation have been extensively studied. These studies have indicated that the mechanisms involved in the two cases are quite different. Long term regulation involves changes in the total amount of lipogenic enzymes while short term regulation involves changes in the catalytic efficiency of key enzymes.

Long term regulation of fatty acid synthesis

In most cases the long term variation is accompanied by parallel changes the activity of the lipogenic enzymes. Kornacker and Lowenstein (1965) and Gibson et al (1972) showed that ATPCL activity parallels the rate of fatty acid synthesis in the liver in that it decreases during starvation and increases upon refeeding. In these experiments the ATPCL returned to pre-starvation levels following refeeding with a normal diet. Refeeding a high carbohydrate/low fat diet greatly elevated the ATPCL activity above pre-starvation levels. Gibson et al (1966) showed that puromycin prevented the increase in ATPCL activity which occurs upon refeeding, suggesting that protein synthesis was involved. This was confirmed by Suzuki et al (1967) who found that the increase in ATPCL activity was accompanied by

an increase in the amount of immunoprecipitable ATPCL in tissue homogenates. This variation in the amount of enzyme protein present is the result of increased or decreased rates of ATPCL synthesis (Gibson et al, 1972). The level of ATPCL activity also varies in parallel with the rate of fatty acid synthesis in other conditions such as diabetes and lactation (Howanitz and Levy, 1965; Kornacker and Lowenstein, 1965; Ballard and Hanson, 1967). Current evidence indicates that these changes are also the result of increased or decreased amounts of ATPCL protein (Numa, 1974).

The activity of ACC in adipose tissue and liver also fluctuates in parallel with the rate of fatty acid synthesis. Starvation, alloxan diabetes or a diet high in polyunsaturated fats lower ACC activity 2-fold. Refeeding a high carbohydrate diet increases ACC activity 6-fold above control levels. Both the increase and the decrease in ACC activity occur over 2 - 3 days (Saggerson and Greenbaum, 1970; Craig et al, 1972). Nakanishi and Numa (1970) and Majerus and Kilburn (1969) showed that these fluctuations in ACC activity were the result of raised or lowered amounts of ACC protein. Using a combination of immunochemical and isotopic techniques they showed that, except in starvation, the changes in the amounts of ACC were the result of increases or decreases in the rate of enzyme synthesis. In normally fed, refed or alloxan-diabetic rats the rate of degradation of ACC was essentially the same ($t_{\frac{1}{2}} = 50$ h). In starvation the rate of degradation was increased ($t_{\frac{1}{2}} = 18 - 30$ h) while the rate of synthesis was decreased. The long term changes in fatty acid synthesis in other metabolic states are also paralleled by changes in the total amount of ACC protein and activity. For example, the increase in ACC activity observed in mammary gland at the onset of lactation is accompanied by an increase in the amount of

immunoprecipitable enzyme (Mackall and Lane, 1977).

Tissue levels of FAS activity also vary in parallel with fatty acid synthesis and as with ACC and ATPCL this variation is due to increases or decreases in the amount of enzyme protein present in the tissue (Gibson et al., 1972). In addition, the levels of the hexose monophosphate shunt enzymes, malate dehydrogenase and malic enzyme all vary in parallel with the rate of fatty acid synthesis. It will be recalled (see Fig. 1.1) that these enzymes are involved in the generation of the NADPH required for fatty acid synthesis (Pande et al. 1964; Lockwood et al., 1970).

These results indicate that the levels of all of the enzymes involved in fatty acid synthesis undergo coordinate changes in response to the nutritional or developmental status of the animal (see Gibson et al., 1972). Furthermore, the changes in the tissue content of these enzymes appear to be the result of increased or decreased synthesis. The factors which govern this coordinate synthesis are not known. Discoordinate changes in the activities of ACC and ATPCL and malate dehydrogenase led to the proposal that a fatty acid synthesis operon does not operate in mammalian tissues (Volpe and Vagelos, 1976). The changes in ACC activity which these authors noted occurred over a period of 1 - 2 h and so may not have been caused by protein synthesis. Thus, the existence of such an operon cannot be ruled out. No inducers or repressors of these enzymes have been identified although several substances have been implicated (Volpe and Vagelos, 1974). In diabetic rats, fructose feeding restores the amount of hepatic FAS to normal levels. This finding indicates that the synthesis of FAS is not completely dependent on the presence of insulin and suggests that the decrease in lipogenic enzymes observed during diabetes is the result of impaired glucose

transport. These authors have interpreted this result as implying that some metabolites of fructose, possibly triose phosphates, are involved in the regulation of FAS synthesis. Other workers have found that in liver there is an inverse relationship between the amount of dietary polyunsaturated fatty acids and the tissue content of FAS and have proposed that these fatty acids (or their metabolites) may be involved in controlling the coordinate expression of the lipogenic enzymes (Muto and Gibson, 1970).

Short term regulation of fatty acid synthesis

I described above how fatty acid synthesis was acutely modulated in response to the level of hormones such as insulin, glucagon and adrenaline. These fluctuations in activity occur within minutes, in contrast to the longer term regulation where the changes occur over a period of days. Studies on the mechanism of the acute regulation of fatty acid synthesis have largely been focussed on ACC. There are two main reasons for this. Firstly, ACC is generally considered to catalyse the rate limiting step in de novo fatty acid synthesis. Secondly, the activity of ACC has been shown to change in parallel with rapid changes in the rate of fatty acid synthesis, in contrast to the two other enzymes directly involved, ATPCL and FAS. ACC catalyses the first committed step in de novo fatty acid synthesis since malonylCoA has no other metabolic fate. AcetylCoA is also the precursor of cholesterol and so ACC affords the first opportunity to regulate fatty acid synthesis alone. A number of workers have reported that in liver and adipose tissue the levels of ACC are considerably lower than those of FAS and ATPCL. There are several studies, however, which indicate that the activities of all three enzymes are comparable. This is true for both liver and mammary gland (Guy et al, 1981; Hardie and Guy, 1980; Chang et al, 1967;

Kornacker and Lowenstein, 1965a) One possible explanation for these conflicting results is that in early studies, optimal conditions for the assay of ACC had not been determined. Another explanation could be the presence of an inhibitor of ACC in unfractionated tissue homogenates (Abdel-Halim and Porter, 1980).

In spite of the evidence that ACC, FAS and ATPCL are found in comparable amounts, ACC is still considered to be the rate limiting enzyme in fatty acid synthesis. In section 2 of this chapter the effects and modes of action of a number of activators and inhibitors of ACC were discussed. Several workers have pointed out that at the concentrations of these effectors found in whole tissues, ACC would be considerably inhibited (Numa, 1974; Carlson and Kim, 1974). In view of this, these authors maintain that the maximum activity of ACC is not expressed in vivo and that consequently the enzyme is rate limiting.

Changes in the activity of ACC distinct from the changes in the total enzyme levels were first observed by Korchack and Masoro (1962) after altering the diet of the animals. It now seems likely that the changes in activity observed by these workers were mediated by changes in the plasma levels of glucagon and insulin.

Glucagon has been shown to decrease the activity of ACC in rat liver. Klain and Weiser (1973) found that ACC activity in rat liver decreased by 70% in 30 min following injection of glucagon. The decrease paralleled the decrease in fatty acid synthesis noted above. More recently a number of workers have used isolated hepatocytes to study the effects of hormones on ACC activity. Using this system Cook et al (1977) and Watkins et al (1977) found that glucagon did not have an effect on ACC activity after 1 h, even though fatty acid

synthesis decreased by 60% during that time. Subsequent studies, however, showed that glucagon did indeed decrease ACC activity in hepatocytes provided that the cells were homogenised at 25 - 37°C and that ACC was assayed in the absence of citrate (Geelen et al, 1978; Witters et al, 1979b).

Insulin causes an increase in ACC activity. Geelen et al (1978) demonstrated that insulin increases ACC activity in hepatocytes in parallel with the increase in fatty acid synthesis. Witters et al (1979b) showed that insulin increased ACC activity by 50% within 15 min.

ACC activity in adipose tissue appears to be regulated by the levels of insulin and adrenaline. Halestrap and Denton (1974) showed that insulin increased ACC activity in rat epididymal fat pads by 100% after 30 min incubation. These workers also showed that adrenaline in the presence of glucose and insulin caused a 66% decrease in ACC activity. Lee and Kim (1978, 1979) subsequently demonstrated that adrenaline alone can inactivate ACC in adipose tissue.

Evidence for the short term regulation of ACC in mammary gland is scarce. For example, there is no direct evidence that the activity of ACC in mammary tissue is regulated by anything other than changes in the total amount of ACC protein in the tissue. Recently Agius and Williamson (1980) noted a rapid inhibition of fatty acid synthesis in lactating rat mammary gland when the animals had a single intra-gastric dose of fat. Since this treatment lowers ACC activity in the liver (Bortz, 1967) it is possible that mammary gland ACC is affected in the same way. Unfortunately, the authors did not measure ACC activity in their experiments.

The results described above show that ACC in liver and adipose tissue is acutely regulated by the hormones insulin, glucagon and adrenaline. The rapidity with which these changes in activity occur indicate that they are not caused by changes in the number of ACC molecules present in the tissue since the half-life of degradation is in the range 2 - 5 days and the time required for de novo synthesis is similar. To explain the hormone dependent changes in ACC activity two different mechanisms have been proposed. In the first of these, changes in ACC activity are secondary to hormone induced changes in the levels of various activators and inhibitors of ACC. The second explanation is that the changes in ACC activity are the result of covalent modification (phosphorylation) catalysed by hormone dependent protein kinases.

In vivo regulation of acetylCoA carboxylase by changes in effector concentration

The in vitro effects of allosteric activators and inhibitors of ACC were described in section 2 of this chapter. These in vitro effects have led to the hypothesis that the in vivo concentrations of these effectors determine the activity of ACC and hence the rate of fatty acid synthesis. Thus, the increase in ACC activity which is caused by insulin would be the result of an increased cytosolic citrate concentration and also the result of a decrease in the concentration of fatty acylCoA. An increased level of fatty acylCoA thioesters would result from the stimulation of lipolysis by adrenaline and glucagon. In order to gain evidence for this hypothesis it is necessary to show that the effectors are present in vivo at the concentrations which affect ACC activity in vitro. In addition, the effector concentrations must be shown to change in the correct direction under a variety of physiological and hormonal conditions.

Early attempts to correlate citrate levels with ACC activity or the rate of fatty acid synthesis in a variety of metabolic states gave conflicting results. Hepatic citrate concentrations were found to be lowered in starvation and diabetes, conditions in which fatty acid synthesis is also decreased (Start and Newsholme, 1968). Administration of insulin, which increases ACC activity, also increased the citrate concentration (Herrera and Freinkel, 1968). Other workers, however, have found that under conditions where ACC activity is modulated, there is no change in citrate levels, or that citrate levels change in the opposite direction to ACC activity (Halestrap and Denton, 1974).

One restraint upon the interpretation of the above results was that for technical reasons, whole cell citrate concentrations were determined rather than cytoplasmic citrate concentrations. It was possible, therefore, that the cytoplasmic citrate concentrations did not change in parallel with the total cellular citrate content. More recently a technique has been developed which allows the determination of metabolites in both the mitochondrial and cytoplasmic compartments of the cell (Zuurendonk and Tager, 1974). Using this technique Watkins et al (1977) found that glucagon drastically lowered the cytoplasmic citrate concentration. Meredith and Lane (1978) showed that this decrease in citrate concentration was accompanied by the depolymerization of ACC in vivo. These results must be treated with caution, however, as other workers, using the same method as Watkins et al (1977) found that glucagon caused only an insignificant decrease in cytoplasmic citrate (Siess et al, 1977).

The in vitro activation of ACC requires citrate concentrations of 5 - 20 mM. These concentrations are considerably higher than the cytoplasmic citrate concentrations measured in the reports cited

above. Both groups found that in the absence of glucagon the citrate concentration was approximately 0.5 mM. It is clear that at this citrate concentration, maximum ACC activity would not be expressed. In this respect the recent report by Witters et al (1981) is of interest. These authors found that ACC is activated by guanine nucleotides and that their effect is to alter the citrate dose-response curve of ACC so that the enzyme is maximally active at known cytosolic citrate concentrations. Furthermore, the activity of ACC is extremely sensitive to citrate in the range of 0.05 - 0.2 mM; for example there was a 14-fold difference in ACC activity between these two values. It is interesting to note that Watkins et al (1977) found that glucagon modulated the cytoplasmic citrate concentrations within this range. These results suggest that cytoplasmic citrate might well have a role in the in vivo regulation of ACC.

The high affinity of ACC for fatty acylCoA thioesters by itself argues that they have some physiological role. The effect of insulin and adrenaline on the concentration of fatty acylCoA thioesters in rat fat pads was studied by Halestrap and Denton (1974). These workers found that hormone dependent changes in ACC activity were accompanied by changes in the protomer/polymer ratio, consistent with a regulatory role for citrate or fatty acylCoA thioesters. Insulin decreased the thioester level while adrenaline caused an increase. Under most conditions an inverse correlation between ACC activity and thioester concentration was observed. In liver fatty acylCoA thioester levels are reduced by insulin and increased by glucagon (Yeh and Leveille, 1971; Goodridge, 1973).

The major problem in interpreting these results is whether the bulk of the thioester is accessible to ACC. In the work mentioned above total fatty acylCoA thioester levels were measured rather than

the free cytosolic concentration. The values obtained were in the range 50 - 150 μM . Clearly, if this were the free cytosolic concentration ACC would be completely inhibited since the K_i for fatty acylCoA thioesters is in the range 1 - 7 nM (Nikawa et al, 1979). It is almost certain, however, that in vivo most fatty acylCoA thioesters are bound to membranes or to protein and that the concentration of free thioesters is very low. While this means that experiments in which the total cellular thioester concentration is measured do not provide much useful information it does make regulation of ACC by fatty acylCoA thioesters more plausible. Halestrap and Denton (1974) proposed that the concentration of free fatty acylCoA thioesters might be regulated by the 12000 M_r fatty acid binding protein (FABP). In their scheme increased concentrations of free fatty acids (resulting from increased lipolysis) would displace fatty acylCoA thioesters from FABP which would in turn inhibit ACC. Lunzer et al (1977) subsequently showed that partially purified FABP protects partially purified ACC from inhibition by palmitoylCoA.

It is not clear that the control of ACC by its allosteric effectors is physiologically relevant. It seems likely, however, that the relative concentrations of fatty acylCoA thioesters and citrate do have a role in regulating ACC activity and through this fatty acid synthesis. It would appear, however, that factors in addition to changes in the concentrations of allosteric effectors are involved in the hormone dependent regulation of ACC. There is now considerable evidence that the activity of ACC is subject to regulation by covalent modification in the form of phosphorylation and dephosphorylation.

Regulation of acetylCoA carboxylase by phosphorylation
and dephosphorylation

In section 3 I described how ACC activity could be decreased by exposure of the tissue to glucagon or β -adrenergic agents. These hormones are believed to act by activating adenylyl cyclase which results in an increase in the intracellular concentration of adenosine - 3':5'-monophosphate (cAMP). Several reports suggested that the glucagon dependent inactivation of ACC was mediated by cAMP. For example, Allred and Roehrig (1973) and Goodridge (1973) showed that treatment of liver slices or hepatocytes with dibutyrylcAMP resulted in a 3-fold decrease in ACC activity. Increased levels of cAMP activate cAMP dependent protein kinase (cAMPdPrK) and hence lead to the phosphorylation of a number of enzymes (see e.g. Nimmo and Cohen, 1977). One possible explanation of the glucagon and β -adrenergic dependent inactivation of ACC was that it was due to cAMP dependent phosphorylation.

The phosphorylation of ACC was first reported by Carlson and Kim (1973) who observed that partially purified rlACC was inactivated by incubation in the presence of ATP, Mg^{2+} and a protein fraction from rat liver. Using immunoprecipitation of ACC the inactivation was shown to be accompanied by the incorporation of ^{32}P into ACC. The kinase responsible for this phosphorylation was not cAMPdPrK since incubation in the presence of cAMP did not increase the rate or extent of phosphorylation. The effects of phosphorylation by this cAMP independent protein kinase on the kinetic properties of rlACC were also described (Carlson and Kim, 1974). The phosphorylated form was less sensitive to activation by citrate and more sensitive to inhibition by palmitoylCoA. In addition the V_{max} of the enzyme was reduced by phosphorylation.

Other workers were unable to find any evidence for the inactivation of ACC by phosphorylation and argued against it on a number of grounds. Lane et al (1974) maintained that rIACC could not be inactivated by phosphorylation since purified rIACC contained 1 mole of phosphate per mole of ACC subunit yet was maximally active. Pekala et al (1978) found that ACC from chicken hepatocytes contained 18 - 20 moles of phosphate per ACC protomer and that treatment of the hepatocytes with dibutyrylcAMP did not alter the extent of phosphorylation. Lent et al (1978) met these criticisms by pointing out that structural phosphate unrelated to changes in enzyme activity was not unusual (Nimmo and Cohen, 1977). The results of Pekala et al (1970) could also be explained if dibutyrylcAMP simultaneously activated cAMPdPrK and a protein phosphatase. It is worth noting that the phosphate content reported by Pekala et al (1978) is somewhat higher than that reported by other workers.

Halestrap and Denton (1974) found that while incubation of adipose tissue extracts with ATP and Mg^{2+} did decrease ACC activity, this was much less pronounced in the presence of citrate. These authors suggested that the results of Carlson and Kim (1973) could be explained by the ATP dependent carboxylation of ACC by endogenous bicarbonate which leads to depolymerization in the absence of citrate. Yeh et al (1980) subsequently showed that AMP stimulated the phosphorylation and inactivation of ACC by the cAMP independent protein kinase (cAMPiPrK) described by Carlson and Kim (1973) and that phosphorylation was inhibited by a high adenylate energy charge in the phosphorylation system. The authors assume that under conditions where fatty acid synthesis is high, the ATP concentration in vivo would also be high. Consequently, ACC would not be phosphorylated. One drawback over this argument is that ATP concentrations vary very

little.

Kim and co-workers subsequently reported that the rate and extent of phosphorylation of partially purified ACC was dependent upon the concentration of citrate in the incubation medium (Lent et al, 1978), high concentrations of citrate decreasing phosphorylation. It is interesting to note that at low citrate concentrations the rate and extent of phosphorylation was increased by cAMP. As with the endogenous cAMPiPrK phosphorylation by the endogenous cAMPdPrK was accompanied by inactivation of ACC. Inactivation was the result of a decrease in the amount of the polymeric form as judged by the decrease in the sedimentation coefficient from 48 S to 17 S. Lent et al (1978) met the criticism of Halestrap and Denton (1974) by demonstrating that phosphorylation and depolymerization occurred in a bicarbonate free medium.

Hardie and Cohen (1978a) studied the phosphorylation of pure lactating rabbit mammary gland (lrmgACC). In the presence of ATP, Mg^{2+} and cAMP the ACC was phosphorylated to the extent of 1 mole of phosphate/mole of ACC subunit. In the absence of cAMP or in the presence of the inhibitor protein of cAMPdPrK, 0.3 moles of phosphate/mole of ACC subunit was incorporated. These results indicated that the ACC used by Hardie and Cohen (1978a) was contaminated by trace amounts of a cAMPdPrK as well as by trace amounts of cAMPiPrK (termed ACC kinase-2). In terms of rate of phosphorylation ACC was comparable to other known substrates of cAMPdPrK, suggesting that this phosphorylation might be important in vivo.

Hardie and Cohen (1979) subsequently purified lrmgACC in the presence of 50 mM NaF in order to prevent dephosphorylation by protein phosphatases during purification. This preparation differed

from ACC prepared in the absence of NaF in two respects. Firstly, the specific activity was lower (1.2 U/mg as against 3.0 U/mg). Secondly the phosphate content was higher (6.2 molecules of phosphate/subunit as against 4.8 molecules of phosphate/subunit). Treatment of the ACC prepared in the presence of NaF with protein phosphatase-1, a protein phosphatase now known to have a broad substrate specificity (Cohen, 1982), removed 1 molecule of phosphate/subunit. This was accompanied by an increase in the specific activity to a level similar to that of ACC prepared in the absence of NaF. These results indicated that purification of lrmgACC in the absence of NaF results in the loss of a phosphate group the presence of which inactivates the enzyme. In an attempt to identify the kinase responsible for the phosphate removed by protein phosphatase - 1, Hardie and Guy (1980) incubated the dephosphorylated form of ACC in the presence of ATP and Mg^{2+} and in the presence or absence of cAMP. Neither cAMPdPrK nor ACC kinase-2 could reverse the activation caused by protein phosphatase-1. This result suggests that a third protein kinase is involved in the phosphorylation of lrmgACC.

Hardie and Guy (1980) also provided evidence that the phosphorylation system of rat mammary gland ACC may be different from that of lrmgACC. Rat mammary gland ACC was phosphorylated to the extent of 2 moles of phosphate/mole of ACC subunit by exogenous cAMPdPrK with concomitant inactivation. Addition of protein phosphatase-1 reactivated the ACC in a reaction in which approximately 1 molecule of phosphate/subunit was removed. Thus, rat mammary gland ACC differs from lrmgACC both in the stoichiometry of phosphorylation by cAMPdPrK and in the identity of the phosphate group removed by protein phosphatase-1. Given that rat and rabbit mammary gland mammary ACCs have different subunit M_r s (Hardie and Guy, 1980), the differences in the phosphorylation of the two enzymes is not surprising. It is

interesting to note that a similar situation occurs with liver fructose 1,6 bisphosphatase where the rat, but not the rabbit enzyme is a substrate for cAMPdPrK (Hosey and Marcas, 1981).

The results described above indicate that ACC can be phosphorylated and inactivated in vitro by protein kinases present in mammary gland and liver. There is now evidence that such a mechanism operates in vivo. Avruch et al (1978) observed that treatment of adipocytes with adrenaline stimulated the incorporation of ^{32}P into a polypeptide with an M_r of 220,000. Witters et al (1979a) observed a similar phenomenon in isolated hepatocytes and using immunoprecipitation identified the 220,000 M_r polypeptide as ACC. The glucagon stimulated phosphorylation of rLACC was paralleled by a decrease in the activity of the enzyme. Brownsey et al (1979) subsequently reported that ACC is phosphorylated in adipocytes in response to adrenaline.

By analogy with other enzymes which are phosphorylated in response to glucagon and adrenaline it seems likely that the phosphorylation of ACC in response to these hormones is catalysed by cAMPdPrK. The factors involved in the regulation of the various cAMP independent kinases described by Hardie and Cohen (1978a), Hardie and Guy (1980) and Carlson and Kim (1973) are not known. Some more recent work on the phosphorylation of ACC is discussed in Chapter 7.

Hormone dependent phosphorylation of ATP citrate lyase

Although the activity of ATPCL in liver, adipose and mammary tissue varies in the long term in response to changes in the nutritional or developmental status of the animal, there is no evidence to suggest that ATPCL activity is subject to short term regulation.

The amount of ATPCL activity found in lipogenic tissues is comparable to those of ACC and FAS (see section 3, this chapter). It is not inconceivable, therefore, that under some conditions the reaction catalysed by ATPCL is rate limiting for fatty acid synthesis.

Recently there has been a renewal of interest in the short term regulation of ATPCL since it has been found that ATPCL becomes phosphorylated in vivo in response to insulin, glucagon and adrenaline.

The first indication that ATPCL contains covalently bound phosphate came from Linn and Srere (1979). These workers found covalently bound ^{32}P in rATPCL which had been purified from animals which had been injected with ^{32}Pi prior to being killed. This phosphate was acid stable and base labile, and so was distinct from the acid labile phosphate which becomes bound to ATPCL during catalysis. Linn and Srere (1979) also showed that rat liver contained a phosphatase which was capable of removing the structural (acid stable) phosphate. No difference in activity was found between the phosphorylated and dephosphorylated forms of ATPCL. This is not altogether surprising since protein phosphorylation unconnected with regulation of activity is not unknown. There was no evidence to suggest that the phosphorylation observed by Linn and Srere (1979) was hormone dependent.

The first demonstration of hormone dependent phosphorylation came from studies of hormone action on intact cells. In studies of the mechanism of action of insulin on isolated adipocytes, Benjamin and Singer (1974, 1975) noted that this hormone stimulated the incorporation of ^{32}P into a polypeptide with an M_r of 140000 (termed IPP - 140). Subsequent work revealed that insulin stimulated the phosphorylation of a number of adipocyte polypeptides, one of which, phosphopeptide - 2 (M_r 128000), corresponded to

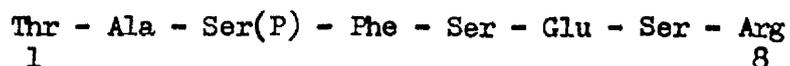
IPP - 140. This effect of insulin was confirmed by Avruch and co-workers (Avruch et al, 1976) who also reported that β -adrenergic agonists also stimulated the incorporation of ^{32}P into a protein corresponding to phosphopeptide - 2. In similar studies on hepatocytes, Alexander et al (1979) observed that both insulin and glucagon stimulated the incorporation of ^{32}P into a 123000 M_r polypeptide (corresponding to phosphopeptide - 2). These workers purified this phosphoprotein to homogeneity and demonstrated a concomitant purification of ATPCL activity. In addition, the specific ATPCL activity of the pure phosphopeptide was similar to that reported for pure ATPCL. Shortly after this, Ramakrishna and Benjamin (1979) identified adipocyte phosphopeptide - 2 as ATPCL, using a similar approach to that of Alexander et al (1979). Using immunoprecipitation, Janski et al, (1979) confirmed that glucagon stimulated the incorporation of ^{32}P into ATPCL, but found no change in the activity of the enzyme.

The results of these in vivo studies provided no insight into the nature of the kinases catalysing the phosphorylation of ATPCL. Furthermore, in view of the report (Alexander et al, 1979) that the insulin and glucagon dependent phosphorylations were additive, it was of interest to study the phosphorylation of ATPCL in vitro.

Guy et al (1980) purified ATPCL from rat mammary gland and demonstrated that it could be phosphorylated in vitro by the catalytic subunit of cAMPdPrK. A maximum of 2.8 moles of phosphate/mole of ATPCL tetramer was incorporated. In a subsequent report (Guy et al, 1981) these workers showed that rat mammary gland ATPCL is also a substrate for cGMP dependent protein kinase although the rate of phosphorylation was only 1/40 th of that with cAMPdPrK. In addition, the presence of a cAMPiPrK which phosphorylated ATPCL was also reported.

Alexander *et al* (1981) studied the in vitro phosphorylation of r1ATPCL. cAMPdPrK was found to phosphorylate ATPCL to the extent of 2 - 3 moles of phosphate/mole of ATPCL tetramer. Extracts of hepatocytes were also capable of catalysing the phosphorylation of ATPCL and this was stimulated by prior treatment of the hepatocytes with glucagon. No phosphorylation was observed in the presence of the inhibitor protein of cAMPdPrK.

The results of Alexander *et al* (1981) suggested that the glucagon stimulated phosphorylation of ATPCL was mediated by cAMP via cAMPdPrK. In order to confirm that an enzyme is the substrate in vivo of a hormone dependent protein kinase it is necessary to show that the site phosphorylated by the kinase in vitro is identical to the site phosphorylated in vivo in response to the hormone. Alexander *et al* (1981) initially reported that identical patterns of phosphopeptides were obtained when samples of ATPCL which had been phosphorylated either in vitro, or in vivo in response to glucagon were subjected to limited proteolysis followed by SDS-polyacrylamide gel electrophoresis. Pierce *et al* (1981) subsequently purified a phosphorylated octapeptide following tryptic digestion of ATPCL which had been phosphorylated in vitro by cAMPdPrK and reported the sequence:



These workers also isolated a phosphopeptide from ATPCL which had been phosphorylated in vivo in response to glucagon and showed that the amino acid composition of the peptide was identical to that of the octapeptide isolated from ATPCL which had been phosphorylated in vitro. These results clearly show that the sites phosphorylated in vitro by cAMPdPrK and in vivo in response to glucagon both reside on the same

octapeptide. These results do not, however, show that both sites are identical. Pierce et al (1981) did not sequence the phosphopeptide isolated from the ATPCL which had been phosphorylated in response to glucagon. This must be done before the identity of both sites is confirmed since it is possible that either Ser 5 or Ser 7 in the octapeptide are the residues phosphorylated in vivo.

ATPCL is also a substrate for a cAMPiPrK. This was observed by Guy et al (1981) and Ramakrishna and Benjamin (1981). The latter workers subsequently purified this kinase (termed ATPCL kinase) and showed that it was distinct from the cAMPiPrK present in rat liver. The maximum amount of phosphate incorporated into ATPCL by ATPCL kinase was 1.6 moles of phosphate/mole of ATPCL tetramer. In a later study Ramakrishna et al (1981) compared the phosphorylation of ATPCL catalysed by cAMPiPrK and ATPCL kinase. Each kinase phosphorylates ATPCL to a similar extent (approximately 2 moles of phosphate/mole of ATPCL tetramer), while a combination of both kinases results in an incorporation of 4 moles of phosphate/mole of ATPCL tetramer. This result is interesting since it is known that the effects of insulin and glucagon on the phosphorylation state of ATPCL are additive in vivo (Alexander et al, 1979), and suggests that each kinase has a distinct phosphorylation site specificity. Limited proteolysis of the phosphorylated ATPCL revealed that the site phosphorylated by cAMPiPrK was located on a different peptide from the site phosphorylated by ATPCL kinase. These authors also reported that acid hydrolysis of ATPCL which had been phosphorylated by cAMPiPrK yielded only one phosphoamino acid, phosphoserine. In contrast acid hydrolysis of ATPCL which had been phosphorylated by ATPCL kinase yielded both phosphoserine and phosphothreonine.

The results described above show that it is almost certain

that cAMPdPrK is responsible for the glucagon stimulated phosphorylation of ATPCL. The identity of the kinase responsible for the insulin stimulated phosphorylation is not known. The obvious candidate is ATPCL kinase but this is by no means certain. Pierce et al (1981) mention that the site of insulin stimulated phosphorylation is located on the same tryptic peptide as the cAMPdPrK site. Ramakrishna et al (1981) found that the cAMPdPrK and the ATPCL kinase sites were located on different tryptic peptides, which suggests that the insulin stimulated kinase and ATPCL kinase are not identical.

The evidence discussed above shows that ATPCL is phosphorylated in vivo, and in vitro studies have gone some way in identifying the protein kinases involved. It is not known, however, whether this phosphorylation has any physiological role. The activity of ATPCL does not change after treatment of hepatocytes with glucagon (Janski et al, 1979) or after in vitro phosphorylation by cAMPdPrK (Guy et al, 1981). The effects on ATPCL activity of either insulin treatment of hepatocytes or phosphorylation by ATPCL kinase have not been reported. A more detailed discussion on this point is given in Chapter 6.2.

Section 4.

Objectives

Mammals have three major lipogenic organs: liver, adipose tissue and mammary gland. The major function of both adipose and mammary tissue is the synthesis, storage and release of triglyceride. In liver, however, there are a number of important metabolic pathways reflecting the importance of this organ in maintaining homeostasis. It is obvious that in order for liver metabolism to proceed efficiently there must be integrated control of the various metabolic processes. The opposing effects of glucagon on hepatic glycogen synthesis and glycogenolysis is well known. More recently it has been observed that glucagon stimulates both lipolysis and ketogenesis in liver: the glucagon dependent inhibition of fatty acid synthesis was discussed in section 3 of this chapter. This raises the possibility that in liver there is reciprocal control of a number of anabolic and catabolic processes. The study of the regulation of fatty acid synthesis in liver appeared more interesting and important than its study in other tissues.

A considerable body of evidence indicates that in vivo hepatic ACC is a substrate of cAMPdPrK. However, the in vitro phosphorylation of pure liver ACC has not been reported. My initial objectives were therefore to study the phosphorylation of pure liver ACC using cAMP dependent and independent protein kinases. During the course of the work the objectives were expanded to include a study of rLATPCL.

Chapter 2

Materials and Methods

Section 1MaterialsChemicals

Ammonium sulphate (specially purified for enzyme work), dioxan, ethoxyethylene, ethylenediaminetetraacetic acid (EDTA), glycinamide, hydrogen peroxide, β -mercaptoethanol, naphthalene, *o*-nitrophenyl- β -D-galactoside, potassium citrate, potassium dihydrogen orthophosphate and triethanolamine were obtained from BDH Chemicals, Poole, Dorset, U.K. 5,5' - dithiobis (nitrobenzoic acid) (DTNB), dithiothreitol (DTT), morpholinosulphonic acid (MOPS) and tris (hydroxymethyl) aminomethane (Tris) were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Dimethyl suberimidate dihydrochloride was a kind gift of Dr. J.R. Coggins of this laboratory. All other chemicals were obtained from BDH Chemicals or Fisons Scientific Apparatus, Loughborough, Lancs., U.K. and were of the highest quality available.

Electrophoresis components

Ammonium persulphate, acrylamide (electrophoresis grade), N,N' - methylenebisacrylamide (electrophoresis grade), sodium dodecyl sulphate (SDS) and N,N,N',N' - tetramethylethylenediamine (TEMED) were obtained from BDH Chemicals.

Radiochemicals

Adenosine 5' - [γ - 32 P]-triphosphate ([γ - 32 P]-ATP), specific activity > 5000 Ci/mole, and sodium [14 C]-bicarbonate, specific activity 0.1 mCi/mole, were obtained from Amersham International, Amersham, Bucks, U.K.

Chromatographic materials

Diethylaminoethyl cellulose (DE 52), (DEAE cellulose), carboxymethylcellulose (CM 52), and phosphocellulose (P11) were products of Whatman Biochemicals, Maidstone, U.K. Affigel-Blue and Bio-gel HPT were obtained from Bio-Rad Laboratories Ltd., Watford, Herts, U.K. Agarose-hexane-CoA was obtained from P-L Biochemicals Inc., Milwaukee, U.S.A. Ultrogel A2 and Ultrogel ACA44 were purchased from LKB Instruments Ltd., South Croydon, Surrey, U.K. Sephadex G-25, Sephadex G-50, Sephadex G-200, Sepharose CL-6B, Octyl Sepharose CL-4B and Phenyl Sepharose CL-4B were obtained from Pharmacia (G.B.) Ltd., London, U.K. Procion Red-Sepharose 4B, prepared by direct coupling of Procion Red HE-3B to Sepharose 4B, was a gift from Mr. D.W. Meek of this laboratory.

Protease inhibitors

Antipain, Aprotinin, benzamidine-HCl, Leupeptin, Lima Bean Trypsin Inhibitor type II and phenylmethylsulphonyl fluoride (PMSF) were obtained from Sigma (London) Chemical Co.

Biochemicals

Acetyl-coenzyme A (trilithium salt) (acetylCoA), acetyl phosphate (potassium-lithium salt), adenosine-5'-diphosphate (monopotassium salt), adenosine-5'-triphosphate (disodium salt), Coenzyme A (trilithium salt), β -nicotinamide-adenine dinucleotide, reduced (disodium salt), β -nicotinamide-adenine dinucleotide phosphate (disodium salt), β -nicotinamide-adenine dinucleotide phosphate, reduced (tetrasodium salt), phosphoenolpyruvate (monosodium salt) (PEP) and 3-phosphoglycerate (monosodium salt) were purchased from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Haemocyanin and ferritin were obtained from G.P. Laboratories, Bishop's Stortford, Herts., U.K. Bovine serum

albumin (fraction V) (BSA), histone (type IIa) and malonyl-coenzymeA (malonylCoA) were obtained from Sigma (London) Chemical Co.

Enzymes

β -galactosidase (E.C. 3.2.1.23), Hexokinase/Glucose-6-phosphate dehydrogenase (E.C. 2.7.1.1./E.C.1.1.1.49), lactate dehydrogenase (E.C. 1.1.1.27), malate dehydrogenase (E.C. 1.1.1.37), pyruvate kinase (E.C. 2.7.1.40), and phosphoglycerate kinase (E.C. 2.7.2.3) were purchased from Boehringer Corp. (London). Phosphotransacetylase (E.C. 2.1.3.8) was obtained from Sigma (London) Chemical Co.

Pyruvate dehydrogenase (E.C. 1.2.4.1) and the 'arom' complex of *N. crassa* were kind gifts from Dr. J.R. Coggins of this laboratory. Phosphorylase kinase (E.C. 2.7.1.38) was a gift from Prof. P. Cohen, University of Dundee. AcetylCoA carboxylase, cyclicAMP dependent protein kinase, fatty acid synthase and glyceraldehyde-3-phosphate dehydrogenase were purified as described in section 2 of this chapter.

Miscellaneous

Polyethylene glycol 6000 and Triton X-100 were obtained from B.D.H. Chemicals, 2,-5-diphenyloxazole was purchased from Koch-Light Laboratories Ltd., Colnbrook, U.K. Agarose and Coomassie Brilliant Blue G-250 were obtained from Sigma (London) Chemical Co. Dialysis tubing was obtained from the Scientific Instrument Centre Ltd., London, U.K. Millipore filters were obtained from Millipore S.A., 67120 Molsheim, France.

Animals

Male and female Sprague-Dawley rats (180-200 g), obtained from the departmental animal house, were used throughout this work. The

rats were normally starved for 24h then refed a fat-free diet for 48 h prior to being killed by cervical dislocation. In the initial stages of this work the fat-free food was prepared in the laboratory using components described in Halliburton (1966). In the latter stages of the work commercial fat-free food was used and was obtained from B.P. Nutrition (U.K.) Ltd., Stepfield, Essex, U.K.

Lactating New Zealand White rabbits were obtained from the departmental animal house and were killed by an intravenous injection of Nembutal at 14 days post partum.

Sheep used to raise antibodies against acetylCoA carboxylase were kept at Glasgow University Veterinary Field Station, Cochno Farm, Dumbartonshire. Injection of antigen and the bleeding of the sheep were carried out by members of the Scottish Antibody Production Unit.

Livers from newly slaughtered pigs and cattle were obtained from a local abattoir and transported to the laboratory on ice.

Section 2

Methods

Routine methods

Glassware

All glassware was washed in a solution of Haemosol (Alfred Cox (Surgical) Ltd., Coulsden, Surrey, U.K.), rinsed thoroughly with tap water and finally with distilled water. The glassware was then dried in a 60°C oven. Pipettes were soaked for at least 24 h in a solution of Decon 75 (Decon Laboratories Ltd., Hove, Sussex, U.K.) then rinsed extensively with tap water in an automatic pipette washer. The pipettes were rinsed several times with distilled water and then dried in a heated pipette drier.

pH and conductivity measurements

All pH measurements were made on a Radiometer pH meter (type PHM 26c) using a GK 2301 glass electrode. The meter was standardised regularly using standard buffer solutions prepared with standard buffer tablets. All conductivity measurements were made at 4 °C on a Radiometer CDM 2e conductivity meter.

Radioactivity measurement

Radioactivity was measured using an Intertechnique SL 4000 Liquid Scintillation Spectrometer using external standardisation to check that quenching did not vary between samples.

Spectrophotometry

All spectrophotometric measurements were made using a Unicam SP 500 monochromator fitted with a Gilford 252 photometer, a Gilford manual cuvette positioner type 2450 and a Gilford chart recorder. Polyacrylamide gels were scanned using the above spectrophotometer fitted with a Gilford 2520 gel scanner.

Centrifugation

All centrifugation at forces less than 40000 x g was carried out on an MSE High Speed 18 centrifuge with either a 6 x 250ml rotor or an 8 x 50ml rotor. Centrifugation at forces greater than 40000 x g was carried out on an MSE PrepSpin 50 ultracentrifuge using a 6 x 100ml or a 10 x 10ml aluminium angle rotor. Centrifugal forces are quoted in g(ave) ignoring acceleration and deceleration times.

Micropipetting

Volumes less than 10 μ l were dispensed with an S.G.E. glass syringe (Scientific Glass Engineering PTY Ltd, North Melbourne, Australia). Volumes from 10 to 500 μ l were routinely dispensed with either fixed volume Oxford pipettes (BCL Ltd., Lewes, Sussex, U.K.) or variable volume Finnpiettes (Jencons Ltd., Hemel Hempstead, Herts, U.K.). During kinetic studies on ATP citrate lyase volumes from 10 to 50 μ l were dispensed using S.G.E. glass syringes.

Preparation and storage of buffers

Stock solutions of 1M potassium phosphate, pH 7.0 were prepared by dissolving 272.2g of KH_2PO_4 in distilled water, adjusting to pH 7.0 with 5N KOH and making up to a final volume of 2l. Stock solutions of 1M Tris-HCl were prepared by dissolving Tris base (121 g/l) in distilled water, adjusting to the required pH with 6N HCl and making up to the required volume. A stock solution of 1M triethanolamine base (TEA) was prepared by diluting 149 g of triethanolamine to 1.0 l with distilled H_2O . Stock solutions were stored at room temperature. Solutions buffered with TrisHCl and potassium phosphate were prepared as required by diluting the stock solutions with distilled H_2O , adding any other components (eg. EDTA, DTT) and adjusting the pH if necessary. The dilute buffer solutions

were stored at 4°C. Solutions buffered with MOPS were made up as required by dissolving MOPS in distilled water, adding any other components, and adjusting the pH with 5N KOH. MOPS buffers were stored at 4°C. Solutions buffered with triethanolamine-HCl (TEA-HCl) were prepared as required by diluting 1M TEA with distilled H₂O, adding any other components and adjusting the pH with 6N HCl.

Preparation of chromatographic materials

DEAE cellulose: 200 g of DE 52 was suspended in 2 l of 0.2M potassium phosphate buffer, pH 7.0. Fines were removed by the method described by the manufacturer and the suspension was brought to a volume of 1.0 l with 0.2 M potassium phosphate, 0.05% (w/v) NaN₃, pH 7.0, and stored at 4°C.

Phosphocellulose: 100 g of P11 was suspended in 1500 ml of distilled water. The resin was allowed to settle and the supernatant was decanted off. This was repeated several times to remove fines and the resin was finally collected on a Buchner funnel. The resin was resuspended in 750 ml of 0.5 N NaOH, stirred for exactly 5 min then collected on a Buchner funnel and rinsed with 5 l of distilled H₂O. This procedure was repeated using 1500 ml of 0.25 N HCl. The P11 was finally resuspended in 1500 ml of 0.2 M potassium phosphate, 0.05% (w/v) NaN₃, pH 7.0 and stored at 4°C.

Gel filtration media: Sephadex G-25 and Sephadex G-50 were suspended in 0.05% (w/v) NaN₃ and allowed to swell for at least 24 h at 4°C. Sephadex G-200 was treated similarly but allowed to swell for at least 3 days. Sepharose CL-4B, Ultrogel A2 and Ultrogel AcA44 are supplied preswollen: these were diluted with distilled H₂O before use. All gel filtration media were degassed on a water pump before use.

All other chromatographic media were treated according to the manufacturers' recommendations before use. All materials were equilibrated before use by pumping the required buffer through the poured column. Equilibration was judged to be complete when both the pH and conductivity of the effluent were identical to those of the buffer.

Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was carried out essentially by the method of Davis (1964). Stock solutions were prepared as follows:

Tris-HCl/TEMED :	36.3 g Tris
	0.23 ml TEMED
	48 ml 1N HCl
	H ₂ O to 100 ml
Acrylamide/Bis :	30.0 g acrylamide
	0.8 g N,N'-methylenebisacrylamide (Bis)
	H ₂ O to 100 ml
Ammonium persulphate :	1.6 mg of ammonium persulphate/ml H ₂ O
Tris/Glycine Stock Solution :	60 g Tris
	288 g glycine
	H ₂ O to 1 l

The Tris-HCl/TEMED and acrylamide/Bis solutions were stored at 4°C. Tris/glycine stock solution was stored at room temperature. Ammonium persulphate solution was prepared immediately before use.

The gel mixture was prepared by mixing the components in the proportions given below. The ammonium persulphate solution was added last.

Final percentage of acrylamide	Tris-HCl/TEMED (ml)	Ammonium persulphate (μ l)	Acrylamide/bis (ml)	H ₂ O (ml)
3	5	20	4.0	11.0
4	5	20	5.6	9.7
5	5	20	6.7	8.3
6	5	20	8.0	7.0
7	5	20	9.3	5.7

The gel mixture was poured into 0.5 cm x 10 cm glass tubes to a height of 7 cm using a Pasteur pipette. The mixture was overlaid with distilled water and allowed to set.

The electrophoresis buffer was 25-fold diluted Tris/glycine stock solution made 0.1% (v/v) with β -mercaptoethanol. The gels were pre-electrophoresed at 3mA/tube for 30 min at 4°C. 5 μ l of tracking dye (0.1%(w/v) bromophenol blue in 20% (v/v) glycerol) was layered onto the surface of each gel and the current was switched on until the dye had just penetrated the surface of the gel. Samples were applied in 20% (v/v) glycerol in a volume of 10 - 40 μ l. Electrophoresis was carried out at 4°C until the tracking dye was less than 1 cm from the bottom of the gel.

Gels were removed from the tubes by 'rimming' with a syringe needle and the position of the tracking dye was marked with a piece of wire. The gels were then stained for either protein or enzyme activity.

Electrophoresis under denaturing conditions in the presence of SDS was carried out either by the method of Weber and Osborn (1969) (Method A) or with the discontinuous system of Laemmli (1970) (Method B).

Method A : the following stock solutions were prepared.

1M sodium phosphate buffer, pH 6.5 : 67.0 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
 219.6 g $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$
 H_2O to 1 l

10% SDS : 10 g SDS
 H_2O to 100 ml

Acrylamide/Bis : 30 g acrylamide
 0.8 g N,N'-methylenebisacrylamide
 H_2O to 100 ml.

The 1M sodium phosphate and 10% SDS were stored at room temperature. Acrylamide/Bis was stored at 4°C. The gel mixture was prepared by mixing the components in the proportions given below.

Final percentage of acrylamide	1M sodium phosphate (ml)	Acrylamide/bis (ml)	H_2O (ml)	10% SDS (ml)	TEMED (μl)	Ammonium persulphate (mg)
3	5	5	39.5	0.75	25	50
4	3.75	5	28.4	0.375	20	40
5	3.0	5	21.7	0.3	15	30

Gels were poured as described above and allowed to set.

Prior to electrophoresis samples were dissolved in a buffer (denoted NaPi sample buffer) consisting of :

0.5 ml 10% SDS
 1.0 ml glycerol
 0.05 ml 1 M sodium phosphate
 0.1 ml 0.5% (w/v) bromophenol blue
 H₂O to 5 ml

β -mercaptoethanol was added to this buffer immediately before use (10 μ l β -mercaptoethanol/250 μ l buffer). Samples were diluted at least 2-fold in this buffer and boiled for 2 - 5 min.

The electrophoresis buffer was :

200 ml 1 M sodium phosphate, pH 6.5
 20 ml 10% SDS
 H₂O to 2 l

Samples, 20 - 100 μ l, were layered on top of each gel and electrophoresis was carried out at room temperature at a current of 7 mA/tube until the tracking dye was less than 1 cm from the bottom of the gel. Gels were removed as described above and stained for protein.

Method B : the following stock solutions were prepared.

1.5 Tris-HCl, pH 8.8 : 181.8 g Tris
 pH to 8.8 with 6N HCl
 H₂O to 1 l

Once the separating gel had set the stacking gel was poured to a height of 2 cm, overlaid with water and allowed to set.

Prior to electrophoresis samples were dissolved in a buffer (denoted Laemmli sample buffer) consisting of:

10 ml 0.5 M Tris-HCl, pH 6.8
10 ml 10% SDS
5.0ml 0.5% (w/v) Bromophenol Blue
10 ml glycerol
65 ml H₂O

Prior to use, β -mercaptoethanol was added to the sample buffer (10 μ l/250 μ l of buffer) and samples (diluted at least 2-fold) were boiled for 2-5 min. Samples (20-200 μ l) were layered on top of the gel.

The electrophoresis buffer was:

200 ml Tris/glycine stock solution
20 ml 10% SDS
H₂O to 2 l

Electrophoresis was carried out at room temperature. Samples were run into the stacking gel at a current of 2 mA/tube until they had 'stacked', and then at 4 mA/tube until the tracking dye was less than 1 cm from the bottom of the gels. Gels were removed and stained for protein.

Fixing and staining gels for protein

Protein bands on polyacrylamide gels were located using Coomassie Brilliant Blue G-250.

Staining solution: Methanol 5 l
 Acetic acid 1 l
 H₂O 4 l
 10 g Coomassie Brilliant Blue G-250

Gels were immersed in 6 ml of this solution for 45 min at 40°C then destained in 12 ml of a solution consisting of:

Methanol 1 l
 Acetic acid 1 l
 H₂O 8 l

The destaining solution was changed at least twice during the first 12 h. Destaining was complete after 2 days. The gels could then be photographed or scanned at 600 nm.

SDS-polyacrylamide gels were calibrated using proteins of known subunit M_r . These included : lrngFAS , M_r 250000 (Hardie and Cohen, 1978b); myosin (heavy chain), M_r 212000 Harrington and Kerr (1965); *N. crassa* 'arom complex', M_r 165000 (Lumsden and Coggins, 1977); phosphorylase kinase α subunit, M_r 145000; phosphorylase kinase β subunit, M_r 140000; phosphorylase kinase γ subunit, M_r 128000 (Cohen, 1973); *E. coli* β -galactosidase, M_r 118000 (Fowler and Zabin, 1977); pyruvate decarboxylase subunit of *E. coli* pyruvate dehydrogenase, M_r 100000; dihydrolipoyl transacetylase subunit of *E. coli* pyruvate dehydrogenase, M_r 80000 (Brown and Perham, 1976); bovine serum albumin, M_r 68000 (Tanford et al, 1967); rabbit muscle GAPDH, M_r 36000 (Harris and Waters, 1976). The positions of the marker

proteins were located by scanning the gels at 600 nm after the gels had been stained for protein and the R_f of each protein was calculated (R_f = distance migrated by protein/distance migrated by bromophenol blue). A calibration curve of $\log_{10} M_r$ against R_f was constructed.

Activity stain for ATP citrate lyase

ATP citrate lyase was located after electrophoresis on non-denaturing polyacrylamide gels by the general method of Nimmo and Nimmo (1982). In this method inorganic phosphate produced during the hydrolysis of ATP is precipitated as calcium phosphate.

Immediately after electrophoresis the gels were soaked for 20 min at 37°C in 100mM TEA-HCl, 30 mM β -mercaptoethanol, pH 8.7. ATPCL activity was located by soaking the gels in a mixture containing 100 mM TEA-HCl, 200 μ M CoA, 20 mM potassium citrate, 5 mM ATP, 40 mM $MgCl_2$, 15 mM $CaCl_2$, 30 mM β -mercaptoethanol, pH 8.7.

The precipitate generally appeared after 30 min and reached a maximum intensity after 3 - 4 h. Activity stained gels were stored at 4°C in 200 mM triethanolamine (pH 10.5). Gels which had been stained for activity could be photographed against a black background or scanned at 600 nm.

Location of radioactivity on polyacrylamide gels

Gels which had been previously stained for protein or ATPCL activity after electrophoresis of samples containing ^{32}P were used. Each gel was quickly frozen with powdered dry ice and cut into 1 mm slices with a gel slicer. Slices were placed in scintillation vials (2 slices/vial) and covered with 0.3 ml H_2O_2 . The vials were capped and incubated at 40°C overnight. The vials were allowed to cool and 3 ml of scintillation fluid (4g 2,5-diphenyloxazole/l of

toluene: Triton x-100 2:1 v/v) was added. The vials were then counted for ^{32}P .

Protein estimation

Protein was estimated by the method of Bradford (1976) using bovine serum albumin ($A_{280}^{1\%} = 6.5$) as standard. Protein reagent was prepared:

100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% ethanol

100 ml 85% (w/v) phosphoric acid

H₂O to 1 l

The reagent was filtered and stored at room temperature.

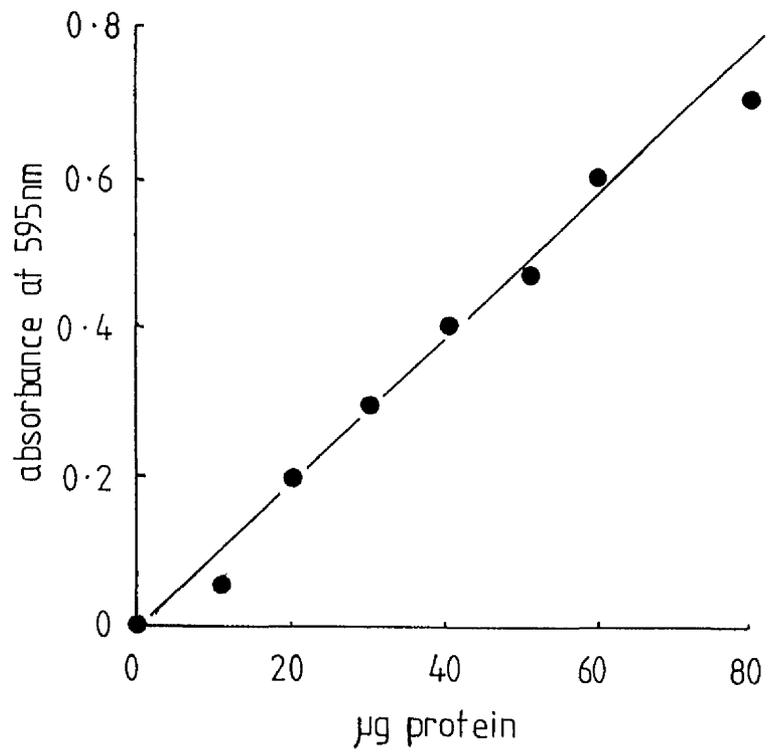
10 - 100 μg of protein in a volume of 100 μl was added to 2.5 ml of the protein reagent and mixed. The E_{595} was read in disposable plastic cuvettes between 2 and 60 min later. The amount of protein/sample was determined by reference to a standard curve of E_{595} against amount of bovine serum albumin (Figure 2.1). A new standard curve was constructed for each set of assays.

The protein concentrations of purified lrmgFAS and r1ATPCL solutions were determined using absorption coefficients for 1% solutions of 10.0 (Hardie and Cohen, 1978b) and 11.4 (Singh *et al.*, 1976) respectively.

Standardisation of solutions

ATP was assayed by the method of Lamprecht and Trautschold (1963). Each cuvette contained, in a final volume of 1.0 ml, 100 mM TrisHCl, pH 7.5, 20 mM glucose, 40 mM MgCl₂, 0.3 mM NADP⁺, 0.28 U hexokinase, 0.14 U glucose-6-phosphate dehydrogenase and 0-50 μM ATP. The reaction was started by the addition of ATP and the reduction of NADP⁺ was monitored at 340 nm. The amount of ATP in the sample

Figure 2.1. Standard curve for Bradford (1976) protein assay



(equal to the amount of NADPH produced) was calculated from the increase in absorbance after the reaction had gone to completion.

CoA was assayed by the phosphotransacetylase end point method (Biochemica Information volume 1, p 63, Boehringer). Each cuvette contained, in final volume 3.0 ml, 93 mM Tris-HCl, pH 7.6, 3.3 mM acetyl phosphate, 10U phosphotransacetylase and 0-50 μ M CoA. The reaction was started by the addition of phosphotransacetylase and allowed to go to completion. The formation of acetylCoA was monitored at 233 nm and the amount of CoA in the sample calculated (ϵ_{233} for acetylCoA = 4440 mol litre⁻¹ cm⁻¹).

Citrate was assayed using the standard ATPCL assay (Linn and Srere, 1979) with a limiting amount of citrate present (0 - 50 n moles).

Enzyme assays

cAMP dependent protein kinase was assayed by the method of Witt and Roskoski (1975) using histone type IIa as a substrate. Each incubation contained, in a final volume of 80 μ l, 31 mM Tris-HCl pH 7.5, 10 μ M cAMP, 2 mg/ml histone type IIa, 2 mM MgCl₂ and 0.2 mM [γ -³²P]-ATP (specific activity 5 Ci/mole). All components except ATP and MgCl₂ were incubated at 30°C for 5 min and the reactions were started by the addition of ATP and MgCl₂. After 15 min at 30°C 50 μ l samples were removed and placed on 2 cm x 2 cm pieces of phosphocellulose paper and the papers were dropped into a beaker containing 1l of ice-cold water. When all of the assays had been completed the papers were rinsed together 5 times with 1l tap water, twice with 200 ml acetone and then dried with a hair-drier. Each phosphocellulose square was placed in a scintillation vial containing 10 ml of scintillation fluid and counted for ³²P. The

scintillation fluid was composed of 30 g naphthalene, 4 g 2,5-diphenyloxazole, 300 ml ethoxyethylene, 300 ml dioxan, toluene to 1 l.

1 unit of cAMP dependent protein kinase is defined as the amount of enzyme that catalyses the transfer of 1 picomole of phosphate from ATP to histone type IIa/min at 30°C.

Fatty acid synthase was assayed by the method of Carey and Dils (1970). Each cuvette contained in a final volume of 1.0 ml, 200 mM potassium phosphate pH 7.0, 1 mM β -mercaptoethanol, 1 mM EDTA, 0.1 mM NADPH, 0.1 mM acetylCoA and 0.2 mM malonylCoA. All components except acetylCoA were preincubated at 37°C prior to assay. The background rate of NADPH oxidation was measured and the reaction was started by the addition of acetylCoA. The oxidation of NADPH was monitored at 340 nm.

1 unit of FAS is the amount of enzyme that utilises 1 μ mole of malonylCoA/min at 37°C. This is equivalent to 2 μ moles of NADPH oxidised/min.

β -Galactosidase was assayed by the method of Wallenfals et al., (1959). Each cuvette contained, in a final volume of 1.0 ml, 10 mM Tris-acetate pH 7.5, 10 mM MgCl₂, 10 mM β -mercaptoethanol, 100 mM NaCl and 1.5 mM *o*-nitrophenol- β -D-galactoside. These components were preincubated at 37°C for 5 min and the reaction was then started by the addition of β -galactosidase. The production of *o*-nitrophenol was monitored at 412 nm. β -galactosidase activity is quoted in arbitrary units.

Pyruvate kinase was assayed by the method of Gutmann and Bernt (1974). Each cuvette contained, in a final volume of 1.0 ml, 50 mM TEA-HCl pH 7.2, 100 mM KCl, 20 mM MgSO₄, 1 mM PEP, 2 mM ADP,

0.2 mM NADH and 1.0 U lactate dehydrogenase. All components except PEP were preincubated at 37°C for 5 min prior to assay. The reaction was started by the addition of PEP. The oxidation of NADH was monitored at 340 nm.

1 unit of pyruvate kinase is defined as the amount of enzyme that catalyses the oxidation of 1 μ mole of NADH/min at 37°C.

ATP citrate lyase was assayed routinely by the method of Linn and Srere (1979). Each cuvette contained, in a final volume of 1.0 ml, 100 mM Tris HCl pH 8.7, 20 mM potassium citrate, 10 mM MgCl₂, 0.2 mM NADH, 5 mM ATP, 0.2 mM CoA and 2 U malate dehydrogenase. All components except the ATPCL sample were preincubated at 37°C for 5 min. The reaction was started by the addition of the sample. The oxidation of NADH was followed at 340 nm. One unit of ATPCL is the amount of enzyme that catalyses the formation of 1 μ mole of acetylCoA/min at 37°C.

Three different methods were used to assay for ACC.

Method 1 : (Majerus et al, 1968). In this method the rate of formation of [¹⁴C]-malonylCoA from acetylCoA and [¹⁴C] -HCO₃⁻ is measured. Each incubation contained, in a final volume of 400 μ l, 100 mM Tris HCl pH 7.5, 0.1 mM DTT, 20 mM MgCl₂, 20 mM sodium citrate, 0.75 mg/ml B S A, 20 mM [¹⁴C]-Na HCO₃ (specific activity 0.05 μ Ci/ μ mole, 2.5 mM ATP and 0.15 mM acetylCoA. All components except acetylCoA were preincubated at 37°C for at least 5 min prior to assay. The reaction was started by the addition of acetylCoA. After 2 min the reaction was stopped by the addition of 100 μ l of 5 N HCl. The reaction mixture was evaporated to dryness under a stream of nitrogen and the residue was then redissolved in 500 μ l of distilled water. This was placed in a scintillation vial containing 3 ml scintillation fluid (4 g 2,5-diphenyloxazole/l of toluene : triton X-100 2:1) and

counted for ^{14}C .

Method 2 : (Manning et al, 1976). The production of malonylCoA is coupled to the oxidation of NADPH by FAS. Each assay contained, in a final volume of 1.0 ml, 100 mM Tris-HCl pH 7.5, 20 mM MgCl_2 , 25 mM potassium citrate, 20 mM Na HCO_3^- , 0.2 mM NADPH, 5 mM ATP, 0.75 mg/ml B S A , 0.5 U FAS and 0.2 mM acetylCoA. All components except acetylCoA were preincubated at 37°C for 5 min prior to assay. The reaction was started by the addition of acetylCoA.

Method 3 : (modified from Nakanishi and Numa, 1974). The production of ADP is coupled to the oxidation of NADH with pyruvate kinase and lactate dehydrogenase. Each assay contained, in a final volume of 1.0 ml, 100 mM Tris-HCl pH 7.5, 20 mM MgCl_2 , 25 mM sodium citrate, 10 mM NaHCO_3^- , 0.2 mM NADH, 5 mM ATP, 0.75 mg/ml B S A , 1.0 mM PEP, 7 U pyruvate kinase, 7 U lactate dehydrogenase and 0.2 mM acetylCoA. All components except acetylCoA were preincubated at 37°C for 5 min prior to assay, the reaction being started by the addition of acetylCoA.

One unit of ACC is the amount of enzyme that catalyses the production of $1\ \mu\text{mole}$ of malonyl CoA/min at 37°C .

Enzyme purification

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12) was purified by the method of Sheek and Slater (1978). The purification involved the preparation of a 10000 x g supernatant from the homogenised tissue, ammonium sulphate fractionation and chromatography on carboxymethyl cellulose. The purified enzyme gave a single band on non-denaturing and SDS-polyacrylamide gels.

The catalytic subunit of cAMP dependent protein kinase (E.C. 2.7.1.37) was purified by the procedure of Sugden et al (1976) as far as the second hydroxylapatite column. The catalytic subunit prepared in this way was at least 50% pure as judged by SDS-polyacrylamide gel electrophoresis. The enzyme was stored at 4°C in 350 mM potassium phosphate, 0.1 mM DTT, pH 6.8. The preparation was stable for at least two months.

Lactating rabbit mammary gland acetylCoA carboxylase (E.C.6.4.1.2) was purified by the method of Hardie and Cohen (1978a) except that the second polyethylene glycol precipitation was omitted. The final preparation was stored at 4°C in a buffer containing 100 mM potassium phosphate, 25 mM potassium citrate, 1 mM EDTA, 0.4 mM DTT, 1mM benzamidine-HCl, pH 7.0. The results of a typical purification are given in Table 2.1. The specific activity of the final material was lower than that reported by Hardie and Cohen (1978a). Analysis of the 3% polyethylene glycol precipitate on SDS-polyacrylamide gels revealed only one protein staining band. No FAS activity could be detected in the final preparation. A second polyethylene glycol precipitation did not affect either the specific activity or the purity as judged by SDS-polyacrylamide gel electrophoresis.

Lactating rabbit mammary gland fatty acid synthase was purified essentially by the method of Hardie and Cohen (1978b) from the 3% polyethylene glycol supernatant obtained during the purification of ACC. The gel filtration step and second ammonium sulphate precipitation were omitted. The purified enzyme was stored in 0.5 ml aliquots at -20°C in a buffer containing 250 mM potassium phosphate, 1mM EDTA, 0.1 mM DTT, 1 mM benzamidine-HCl, pH 7.0. The results of a typical purification are given in Table 2.2. The enzyme was pure as judged by the criteria of a single band on both non-denaturing (Fig.5.5) and

Table 2.1. Purification of lactating rabbit mammary gland acetylCoA carboxylase

Step	Volume (ml)	Protein (mg)	Activity U	Specific Activity U/mg	Purification - fold	Yield %
105000 x g supernatant	770	11080	192	0.016	1	100
35% (NH ₄) ₂ SO ₄ precipitate	135	1485	112	0.075	4.6	58
3% polyethylene glycol precipitate	10	230	77	0.336	21	40

The data refer to a preparation using 2 rabbits (336 g of tissue)

Table 2.2. Purification of lactating rabbit mammary gland fatty acid synthase.

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Purification -fold	Yield % %
105000xg supernatant	770	11080	1309	0.12	1	100
35% $(\text{NH}_4)_2\text{SO}_4$ precipitate	135	1485	827	0.38	3.1	63
3% polyethylene glycol supernatant	420	1568	682	0.43	3.6	52
DEAE cellulose pool	208	865	509	0.58	4.8	39
30% $(\text{NH}_4)_2\text{SO}_4$ precipitate	6.4	159	352	2.2	18.3	27

The data were obtained with a preparation from 2 rabbits (336 g of tissue).

SDS-polyacrylamide gel electrophoresis. The specific activity of the purified FAS was slightly higher than that obtained by Hardie and Cohen (1978b), although the overall yield was somewhat less.

Immunological techniques

Antibodies to purified lrmgACC were raised in sheep. Purified lrmgACC (0.04 mg/ml) was dialysed extensively against 20 mM potassium phosphate, 0.15 M NaCl, pH 7.0. 2.5 ml of this solution was diluted 2-fold with Freund's complete adjuvant and an emulsion was prepared by sonication. Two sheep were used. 2.5 ml of the lrmgACC/adjuvant emulsion was injected intramuscularly into each animal. This procedure was repeated 4 weeks later using Freund's incomplete adjuvant. Two weeks later, i.e. 6 weeks after the initial injection, both sheep were bled. The plasma was stored frozen.

Analysis of the plasma obtained indicated that only one sheep had produced antibodies to lrmgACC. The antibody fraction from this plasma was partially purified by the method of Cohen et al (1976). 20 ml of the plasma was brought to 45% saturation with ammonium sulphate and stirred for 15 min at 4°C and the precipitate was collected by centrifugation at 20000 x g for 15 min. The pellet was dissolved in 7 ml of buffer A (see above) and dialysed against 2 l of this buffer overnight at 4°C. The dialysate was then brought to 35% saturation with ammonium sulphate and stirred at 4°C for 15 min and the precipitate was harvested as before. The pellet was dissolved in 5 ml of buffer A. Following clarification by brief centrifugation the 35% ammonium sulphate pellet was applied to a column of Sephadex G-200 equilibrated in buffer A. The column was developed with Buffer A at a flow rate of 5 ml/h. Fractions from the large peak of material which absorbed at 280 nm were pooled and concentrated by

ammonium sulphate precipitation. The antibody solution was stored in buffer A at 4°C.

Immunodiffusion was carried out by the method of Ouchterlony (1953). Glass microscope slides were covered with 1% (w/v) agarose containing 20 mM Tris-HCl, 0.15 M NaCl, 0.1% (w/v) NaN₃, pH 7.5. Once the agarose had set, rosettes (one centre well surrounded by six peripheral wells) were cut using a punch. Samples were loaded using an S.G.E. glass syringe and the slides were incubated overnight at room temperature in a plastic box lined with a moist paper towel. Precipitin bands were visible after 16 h.

Antibody-antigen complexes were analysed on Weber and Osborn (1969) type gels as described by Cohen *et al* (1976). The protein solution under analysis was diluted to 10 - 12 mg/ml with 20 mM potassium phosphate, pH 7.0, then diluted 2-fold with 3 M NaCl 2% (v/v) Triton x-100. Any turbidity was removed by centrifugation at 12000 xg in an Eppendorf bench centrifuge. 200 µl of the clarified protein solution was added to 200 µl of antibody solution and mixed carefully. After standing for 1 h on ice the precipitate was collected by centrifugation for 2 min at 12000 x g in a bench centrifuge. The pellet was redissolved in 200 µl NaPi sample buffer, boiled for 5 min and analysed by SDS-polyacrylamide gel electrophoresis.

Density gradient centrifugation

Centrifugation through sucrose gradients was performed by the method of Martin and Ames (1961). Gradients from 5% to 20% sucrose, in the required buffer, were poured in 14 ml tubes (total volume of gradient 13.5 ml). 0.5 ml of enzyme solution, which had been dialysed into the required buffer, was carefully layered on top of

the sucrose. Samples were centrifuged for 16 h at 25000 rpm in an M.S.E. 6 x 14ml titanium swing-out rotor. After centrifugation the contents of the tubes were harvested by displacement with 40% sucrose containing 0.5% (w/v) bromophenol blue. Fractions (10 drops/fraction) were collected and assayed for enzyme activity.

Cross-linking of proteins

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was cross-linked with dimethyl suberimidate for use as a molecular weight marker on SDS-polyacrylamide gels. Prior to cross-linking, the enzyme was dialysed into 100 mM TEA-HCl, 0.1 mM DTT, pH 8.0. A solution of 50 mM dimethyl suberimidate was prepared by first mixing equal volumes of 0.200 N NaOH and 500 mM TEA-HCl, pH 8.0. The dimethyl suberimidate was dissolved in this and used immediately. The final conditions were 10 mM dimethyl suberimidate, 50 mM TEA-HCl, 1 mg/ml GAPDH, 20 mM NaCl, pH 8.0. The reaction was allowed to proceed for 2 h at room temperature whereupon the incubation mixture was diluted 2-fold with either NaPi or Laemmli sample buffer and boiled for 5 min. The denatured, cross-linked GAPDH was stored at 4°C.

ATP citrate lyase was cross-linked with dimethyl suberimidate. Prior to cross-linking, 150 μ l of ATPCL was dialysed into 100 mM TEA-HCl, 15 mM β -mercaptoethanol, pH 8.0. The protein concentration after dialysis was 1.6 mg/ml. A 10 mM solution of dimethyl suberimidate was prepared by mixing equal volumes of 500 mM TEA-HCl pH 8.0 and 0.040 N NaOH. The dimethyl suberimidate was dissolved in this and used immediately. Each incubation contained, in a volume of 50 μ l, 50 mM TEA-HCl, 2 mM dimethyl suberimidate, 4 mM NaCl, 1 mg/ml ATPCL, pH 8.0. The reactions were carried out at room temperature and stopped at various times by the addition of 10 μ l of 1.0 M

glycinamide, pH 8.0.

Incorporation of ^{32}P into protein

The incorporation of ^{32}P into protein was measured by the method of Nimmo et al (1976). The exact conditions of the reaction and times of removal of samples are given in Chapter 4.4 and Chapter 6.1.

Reactions were stopped by removing samples to 1 ml Eppendorf centrifuge tubes containing 10 μl of 10 mg/ml B S A and adding 1 ml portions of 5% (w/v) trichloroacetic acid. The tubes were kept on ice for 10 min, then centrifuged for 2 min in an Eppendorf 5412 bench centrifuge and the supernatants were discarded. The pellets were dissolved in 0.5 ml portions of 0.1 N NaOH and then reprecipitated with 0.5 ml 10% (w/v) trichloroacetic acid. After standing on ice for a further 10 min the tubes were centrifuged for 2 min and the supernatants were discarded. Each pellet was rinsed with 1 ml of (w/v) trichloroacetic acid and then dissolved in 100 μl of 90% (v/v) formic acid. The dissolved pellets were placed in scintillation vials containing 2 ml of scintillation fluid (4 g 2,5 - diphenyl-oxazole/litre of toluene : Triton x-100 2:1 (v/v)) and counted for ^{32}P .

Kinetic studies on ATP citrate lyase

In the kinetic studies on ATPCL described in Chapter 5.2, initial velocity measurements were made using either a malate dehydrogenase coupled assay (Method a) or a pyruvate kinase/lactate dehydrogenase coupled assay (Method b).

Method a : each cuvette contained, in a final volume of 1.0 ml, 50 mM morpholinolinosulphonic acid (MOPS-NaOH), 0.25 M KCl, 15 mM β -mercaptoethanol, 0.05 mM NADH, 2.5 U malate dehydrogenase

0.424 mM free Mg^{2+} , pH 7.0. The concentrations of MgATP, CoA and Mg-citrate used are given in figure legends.

Method b : each cuvette contained, in a final volume of 1.0 ml, 50 mM MOPS-NaOH, 0.25 M KCl, 15 mM β -mercaptoethanol, 0.05 mM NADH, 1.0 mM phosphoenolpyruvate, 7 U pyruvate kinase, 7 U lactate dehydrogenase, 0.424 mM free Mg^{2+} pH 7.0. The concentrations of MgATP, Mg-citrate and CoA used are given in the appropriate figure legends.

The total amounts of ATP, potassium citrate and magnesium acetate required to give a free Mg^{2+} concentration of 0.424 mM and the required concentrations of MgATP and Mg-citrate were determined from the relationship:

$$\text{Total } Mg^{2+} = 0.424 \text{ mM} + 0.867 [\text{Total ATP}] + 0.5 [\text{Total potassium citrate}]$$

$$\text{where } 0.867 [\text{Total ATP}] = [\text{MgATP}]$$

$$\text{and } 0.5 [\text{Total potassium citrate}] = [\text{Mg-citrate}]$$

The above relationship was derived using the dissociation constants of 0.065 mM for MgATP and 0.424 mM for Mg-citrate quoted in Plowman and Cleland (1967). In this way it was possible to vary both [Mg-citrate] and [MgATP] from assay to assay and to keep the free Mg^{2+} concentration constant at 0.424 mM.

During the kinetic studies on ATPCL assays were performed at 37°C with the chart recorder set to give a full scale deflection of 0.025 absorbance units. This full scale deflection corresponds to an NADH concentration of approximately 4 μ M. The lowest substrate concentration used in the experiments was 2 μ M.

All solutions used in the kinetic studies were filtered through Millipore filters (0.22 μm pore size) prior to being used. This filtration was necessary to remove dust particles which contribute significantly to the background 'noise' when the spectrophotometer is operated at such high sensitivity.

The results of the kinetic studies are reported as double reciprocal plots. In these studies assays were normally performed in duplicate although in some cases, especially at low substrate concentrations, assays were performed in triplicate. In some instances only one datum point is shown indicating that the measured initial velocities were identical in each of the duplicate assays.

Chapter 3

Studies on acetylCoA carboxylase from pig liver
and lactating rabbit mammary gland.

Section 1. Studies on pig liver acetylCoA carboxylase

The strategy adopted in attempting to purify pig liver ACC (pLACC) was to follow the initial stages of well established procedures for the purification of rLACC. The two most frequently cited procedures were those of Inoue and Lowenstein (1972) and Nakanishi and Numa (1970).

I initially prepared fractions from pig liver and attempted to detect ACC activity in these. A 90000 x g supernatant was prepared by the method of Inoue and Lowenstein (1972). 200 g of fresh pig liver was homogenised in 400 ml of a buffer containing 5 mM Tris-HCl, 20 mM sodium citrate, 0.5 mM EDTA, 5 mM β -mercaptoethanol, pH 7.5. The homogenate was centrifuged for 15 min at 20000 x g. The resulting supernatant was then centrifuged for 45 min at 90000 x g and the supernatant was retained. ACC from rat liver requires activation in the presence of citrate and reducing agents before maximum activity is expressed (Inoue and Lowenstein, 1972). I therefore attempted to activate the ACC by incubation in the presence of citrate. Prior to incubation a 5 ml sample of the 90000 x g supernatant was gel filtered into 20 mM Tris-HCl, 1 mM DTT, pH 7.5 on Sephadex G-25 (2.2 cm x 30 cm) to remove any low M_r substances which might inhibit the enzyme.

'Citrate activated' 90000 x g supernatant was prepared by incubating 0.5 ml of the gel filtered material in 50 mM Tris-HCl, 20 mM sodium citrate, 20 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, pH 7.5 (final volume 1 ml) at 37°C for 30 min. ACC activity was assayed by the bicarbonate fixation assay of Majerus et al (1968). No activity could be detected in the 90000 x g supernatant before or after gel filtration or after 'citrate activation'. Although no

activity could be found in the 90000 x g supernatant, or fractions derived from it, a 0 - 30% ammonium sulphate precipitation was carried out on it (Nakanishi and Numa, 1970). Inoue and Lowenstein (1972) state that such fractionation reduces the requirement of rACC for citrate activation. The 0 - 30% ammonium sulphate precipitate was prepared by adding solid $(\text{NH}_4)_2\text{SO}_4$ to the 90000 x g supernatant (175 g/l), stirring for 15 min and collecting the precipitate by centrifugation at 20000 x g for 15 min. The pellet was redissolved in 20 mM potassium phosphate, 1 mM EDTA, 5 mM β -mercaptoethanol, pH 7.0. The ammonium sulphate precipitate was gel filtered on Sephadex G-25 M and activated by citrate as described above for the 90000 x g supernatant, but again no ACC activity could be detected. No ACC activity was detected at any of the above stages on the first three occasions on which this experiment was carried out. On a fourth occasion (see below) a small amount of activity (0.004 U/mg) was detected in the ammonium sulphate precipitate.

In order to show that the bicarbonate fixation assay was capable of detecting ACC, the linearity of the assay with respect to time and enzyme concentration was checked using extracts from rat liver. The level of ACC activity detected in samples of 'citrate activated' 90000 x g supernatants prepared from fed rat liver was 0.15 U/g liver (wet weight). This is rather low compared with the amount of activity quoted by Inoue and Lowenstein (1972) : 2.5 U/g liver (wet weight). It was possible, therefore, that the level of ACC activity was being underestimated. In my hands the assay method of Majerus et al (1968) was not straightforward, the problem being the evaporation to dryness of the samples once the reaction had been stopped. Drying the samples under a stream of air or nitrogen was very slow unless the samples were heated. If

the samples were heated, the residue tended to turn a yellow/brown colour indicating, perhaps, that the samples were charring. This could have resulted in colour quenching.

By this time a method for the purification of large amounts of lrmgACC and lrmgFAS had been reported (Hardie and Cohen, 1978a,b). It was possible, therefore, to measure the activity of pure lrmgACC using the method of Majerus et al (1968) and compare it with the activity measured by the method of Manning et al (1976) in which the production of malonylCoA is coupled to the oxidation of NADPH using FAS. Figure 3.1 indicates that the bicarbonate fixation assay is linear for up to 4 min. Figure 3.2 shows that the rate of incorporation of bicarbonate into malonylCoA is proportional to the amount of enzyme added. The activity of one sample of lrmgACC measured by the method of Majerus et al (1968) was 10.5 U/ml while the method of Manning et al (1976) gave a value of 9.7 U/ml. The two assay methods clearly gave comparable results.

The procedure described above i.e. the preparation of a 90000 x g supernatant and 0 - 30% ammonium sulphate precipitate from pig liver was repeated using the assay of Manning et al (1976). Again, no activity could be detected in any fraction with or without 'citrate activation'. Continuation of the purification through the DEAE cellulose step described by Nakanishi and Numa (1970) which is reported to obviate 'citrate activation', revealed no ACC activity.

Two attempts were made to purify plACC using the procedure for the purification of lrmgACC (see Chapter 2.2.). The procedure was followed up to and including the polyethylene glycol step. No activity was detected using the FAS coupled assay.

These results raised the question of the limits of sensitivity

Figure 3.1. Time course of [^{14}C] - bicarbonate
fixation assay

Lactating rabbit mammary gland acetylCoA carboxylase (30 mg/ml)
was diluted 4-fold. 10 μl samples were assayed using Method 1
described in Chapter 2.2 except that the reactions were stopped at
the times indicated. (●), ACC activity.

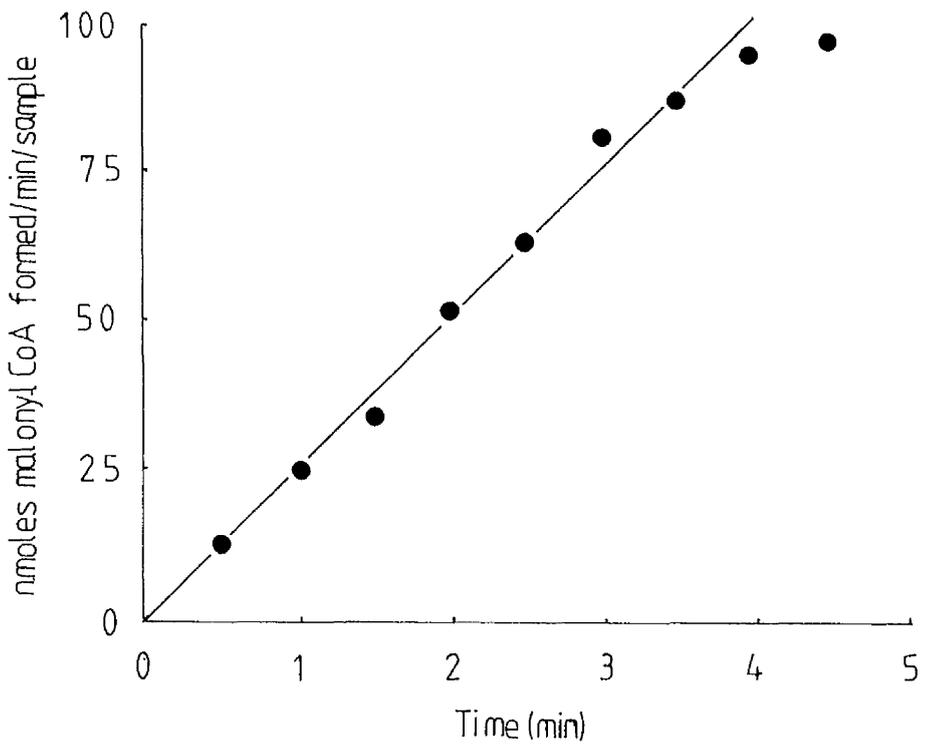
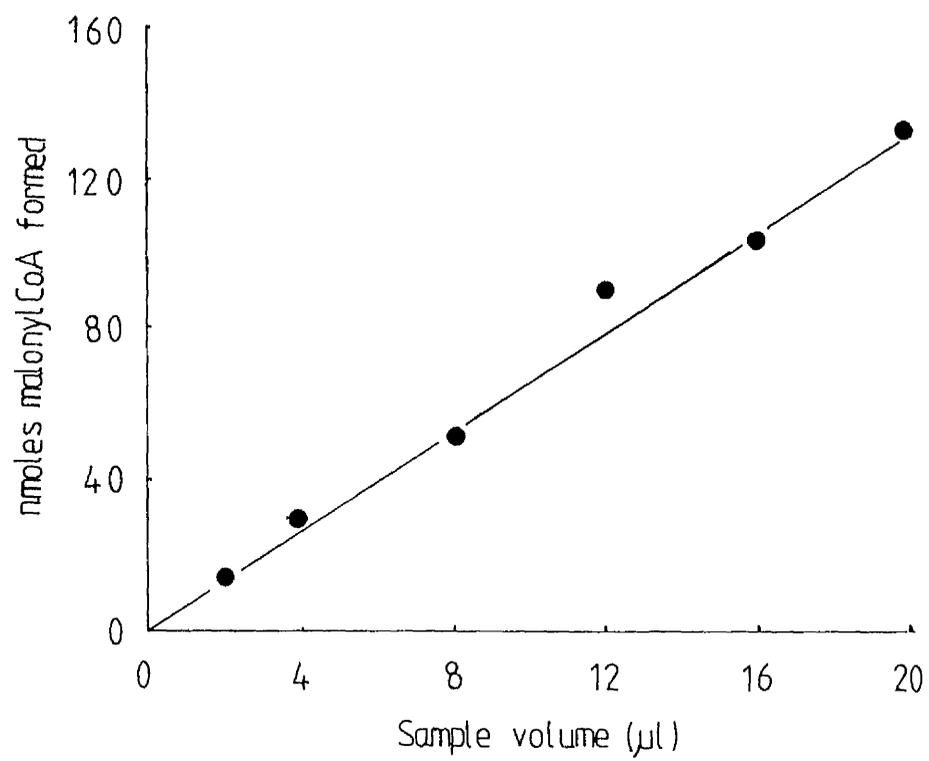


Figure 3.2. Dependence of rate of production of malonylCoA upon the amount of acetylCoA carboxylase added.

Lactating rabbit mammary gland acetylCoA carboxylase (30 mg/ml) was diluted 4-fold. Samples were assayed by Method 1 described in Chapter 2.2 for 3 min.



of the assays used. For most assays coupled to the oxidation of NADPH one would expect to be able to detect activities of 0.01 U/ml or lower. The limit of sensitivity of the bicarbonate fixation assay is 0.05 U/ml or lower. I therefore concluded that essentially no ACC activity could be detected in extracts of pig liver.

These results suggested that either there was no ACC protein in pig liver or the enzyme was present in an inactive form. One could envisage that there might be allosteric inhibitors, such as fatty acylCoA thioesters, very tightly bound to the enzyme, or that an active ACC kinase was present in the fractions to be assayed. Since the assay necessarily includes ATP and Mg^{2+} ions such as kinase could inactivate the ACC very rapidly in the assay. I sought to resolve this problem by using antibodies raised against purified lrmgACC to assay for ACC protein in pig liver extracts.

Immunological studies

Antibodies against pure lrmgACC were raised in sheep as described in Chapter 2.2. When analysed by Ouchterlony double diffusion the antibody preparation gave rise to a single precipitin line against lrmgACC, indicating that the antibody was specific for only one antigen. No precipitin line was produced when control serum was used. It was possible that the lrmgACC was contaminated with trace amounts of FAS and that the antibody had been raised against this. Ouchterlony double diffusion of the antibody against lrmgFAS did not result in the formation of a precipitin line. The antibody preparation was therefore specific for ACC. The nature of the antigen-antibody complex was also analysed on SDS-polyacrylamide gels by the method of Cohen et al (1976). This method has an advantage over Ouchterlony double diffusion. If the subunit M_r of the protein of interest is known, analysis of the antigen-antibody precipitate on SDS-polyacrylamide

gels can confirm that the antibodies have not been raised against some impurity in the antigen preparation.

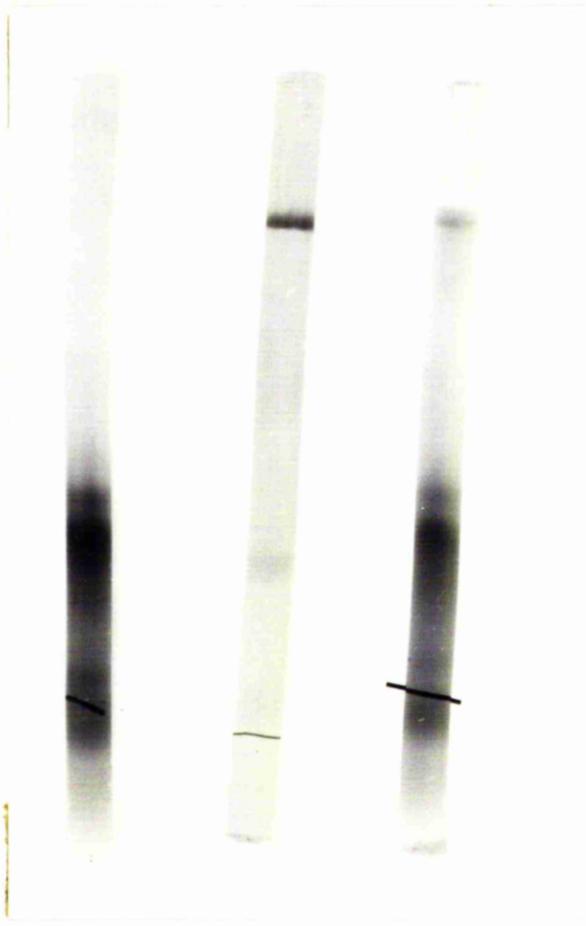
Figure 3.3 shows the results of an experiment where a sample of the anti-ACC fraction was incubated with pure lrmgACC as described in Chapter 2.2. No precipitate formed when ACC was replaced with lrmgFAS or when the anti-ACC was incubated with buffer. In both of these cases no protein was visible on the polyacrylamide gels when the contents of the incubation vials were analysed as if a precipitate had been formed. This indicated that there was no carry over of unprecipitated protein. The results displayed in Figure 3.3 show that the antibody precipitates a protein with a subunit M_r of 250000. Assuming that ACC and FAS are the only two proteins in mammalian tissues which have subunit M_r s of 250000, this result indicates that the antibody is specific for ACC since FAS is not precipitated by the antibody. Gel 3 (Fig. 3.3) shows a doublet perhaps indicating that partial proteolysis of the ACC occurred during the precipitation with antibody.

I then analysed a fresh pig liver extract for ACC activity and for ACC protein immunologically. A 105000 x g supernatant and a 0 - 35% ammonium sulphate precipitate were prepared using the procedure described for the purification of lrmgACC. ACC activity was detected in the pig liver extracts used in this experiment. Table 3.1 compares the ACC activity measured in the 105000 x g supernatant with that reported by Inoue and Lowenstein (1972). There is at least 10 - fold less activity in pig liver than in rat liver.

A sample of the 0 - 35% ammonium sulphate precipitate was incubated with anti-ACC antibody as described in Chapter 2.2 and the

Figure 3.3 Analysis of precipitate from anti-acetylCoA
carboxylase/lactating rabbit mammary gland
acetylCoA carboxylase incubation.

SDS-polyacrylamide gel electrophoresis on 4% polyacrylamide
gels was performed by the method of Weber and Osborn (1969). The
samples analysed were: (1), 30 μg anti-acetylCoA carboxylase;
(2), 4 μg pure lactating rabbit mammary gland acetylCoA carboxylase;
(3), precipitate formed using 600 μg lrmgACC and 60 μg anti-acetylCoA
carboxylase.



1

2

3

Table 3.1 Comparison of acetylCoA carboxylase activity
in pig and rat liver.

	Pig liver	Rat liver
Specific activity (U/mg)	0.004	0.03
Enzyme activity (U per gram of liver wet weight)	0.14	2.5

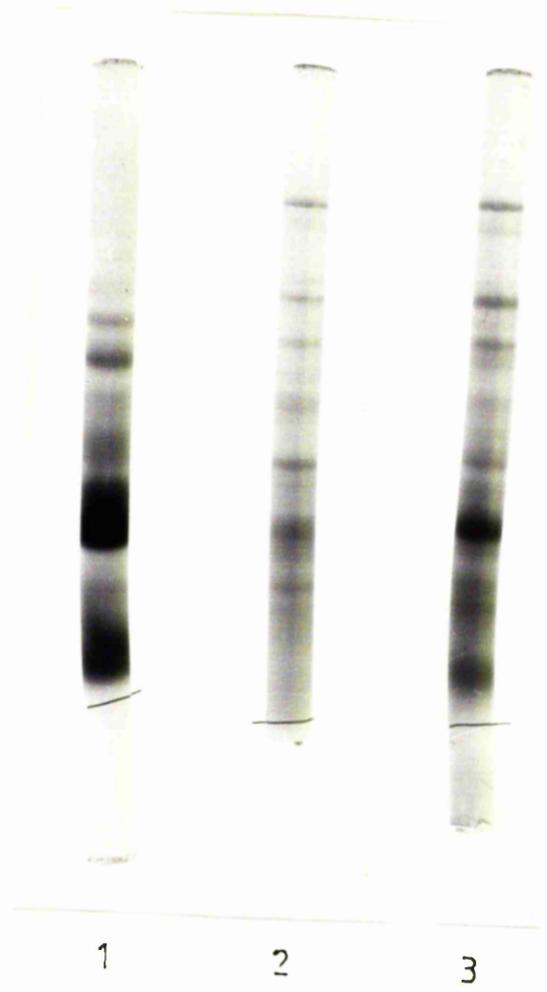
Data refer to a 105000 x g supernatant prepared from pig liver (this work) and a 90000 x g supernatant prepared from rat liver (Inoue and Lowenstein, 1972).

resulting precipitate was analysed by SDS-polyacrylamide gel electrophoresis (Fig. 3.4). On this occasion there was considerable carry-over of the liver proteins which were not precipitated : all of the proteins visible on gel 2 are also visible on gel 3. Thus, it is impossible to say whether any ACC was precipitated by the antibody. This problem could have been overcome either by using Ouchterlony double diffusion analysis or by using Staphylococcus aureus membranes to precipitate the antigen-antibody complex (Kessler, 1975) and hence isolate pure antigen. However, because only low levels of ACC activity were present in pig liver I did not consider it worthwhile to perform these experiments.

The work described above indicates that pig liver contains very little ACC activity. It is tempting to speculate that this was due to the dietary status of the animals. One disadvantage in using pig liver as a source of ACC was that the animals' diet could not be manipulated to induce a high enzyme level. Another disadvantage was that pigs were not suitable animals in which to manipulate hormone levels when studying the hormone dependent regulation of enzyme activity. In view of this it was decided to study ACC from rat liver rather than pig liver.

Figure 3.4. Analysis of precipitate from anti-acetylCoA carboxylase pig liver extract incubation.

SDS-polyacrylamide gel electrophoresis on 4% polyacrylamide gel was performed by the method of Weber and Osborn (1969). The samples analysed were: (1) 30 μg anti-acetylCoA carboxylase; (2) 17 μg 0 - 35% ammonium sulphate precipitate (see text); (3) precipitate from anti-acetylCoA carboxylase / 0 - 35% ammonium sulphate precipitate incubation, (formed using 600 μg anti-acetylCoA-carboxylase and 850 μg of 0 -35% ammonium sulphate precipitate).



Section 2. Experiments on lactating rabbit mammary gland acetylCoA
carboxylase and fatty acid synthase

I studied the behaviour of lrmgACC on a variety of chromatographic media in the hope that the information gained would enable a purification scheme for rlACC to be devised. In the course of the work on rlACC it became desirable to find a method which would separate ACC from FAS. The behaviour of FAS on various chromatographic media was therefore studied.

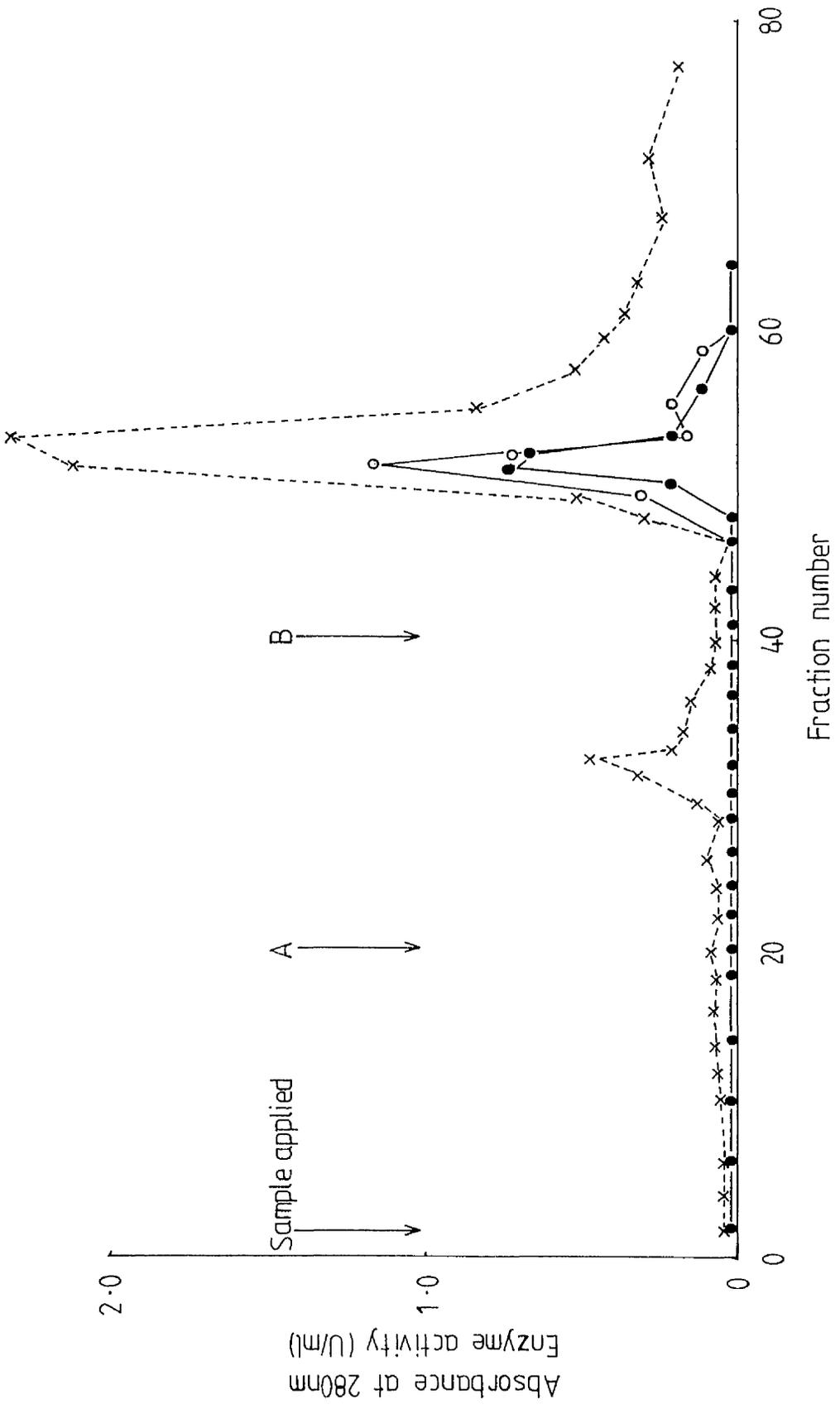
Ion-exchange chromatography

The procedure used to purify lrmgFAS indicates that FAS will bind to DEAE cellulose at pH 7.0 and can be eluted with 125 mM potassium phosphate. The binding of lrmgACC to DEAE cellulose was therefore studied to determine whether this might provide a separation of ACC and FAS.

All buffers used contained 1 mM EDTA and 15 mM β -mercaptoethanol and were adjusted to pH 7.0 before use. lrmgACC and FAS were partially purified from one rabbit as described in Chapter 2.2. as far as the 0 - 35% ammonium sulphate precipitation. The precipitate was then gel filtered into 10 mM potassium phosphate on Sephadex G-25 M (2.2 cm x 30 cm). The gel filtered material which contained ACC and FAS activity was then applied to a column of DEAE cellulose (4.4 cm x 9.0 cm) equilibrated in the above buffer. The column was washed with the equilibration buffer until the E_{280} of emerging fractions was less than 0.05. The column was then washed with 50 mM potassium phosphate followed by 125 mM potassium phosphate. The elution of enzyme activity is shown in Figure 3.5. Both ACC and FAS activity bound to the DEAE cellulose and both were eluted by the 125 mM potassium phosphate wash.

Figure 3.5. Chromatography of lactating rabbit mammary gland acetylCoA carboxylase and fatty acid synthase on DEAE cellulose.

Lactating rabbit mammary gland ACC and FAS were partially purified as described in the text. 93 U of ACC and 150 U of FAS were applied to the column at a flow rate of 11.7 ml/h. 11.8 ml fractions were collected and monitored for absorbance at 280 nm (x), ACC activity (●) and FAS activity (o). (A) indicates start of 50 mM potassium phosphate wash, (B) indicates start of 125 mM potassium phosphate wash.



This experiment indicated conditions under which ACC would bind to DEAE cellulose. In a similar experiment (not shown) the 0 - 35% precipitate was applied to the DEAE cellulose in 20 mM potassium phosphate. The separation of ACC from FAS was attempted by developing the column with a linear gradient from 20 mM - 200 mM potassium phosphate. No separation of the two activities was achieved, the elution profile being similar to that of Fig. 3.5.

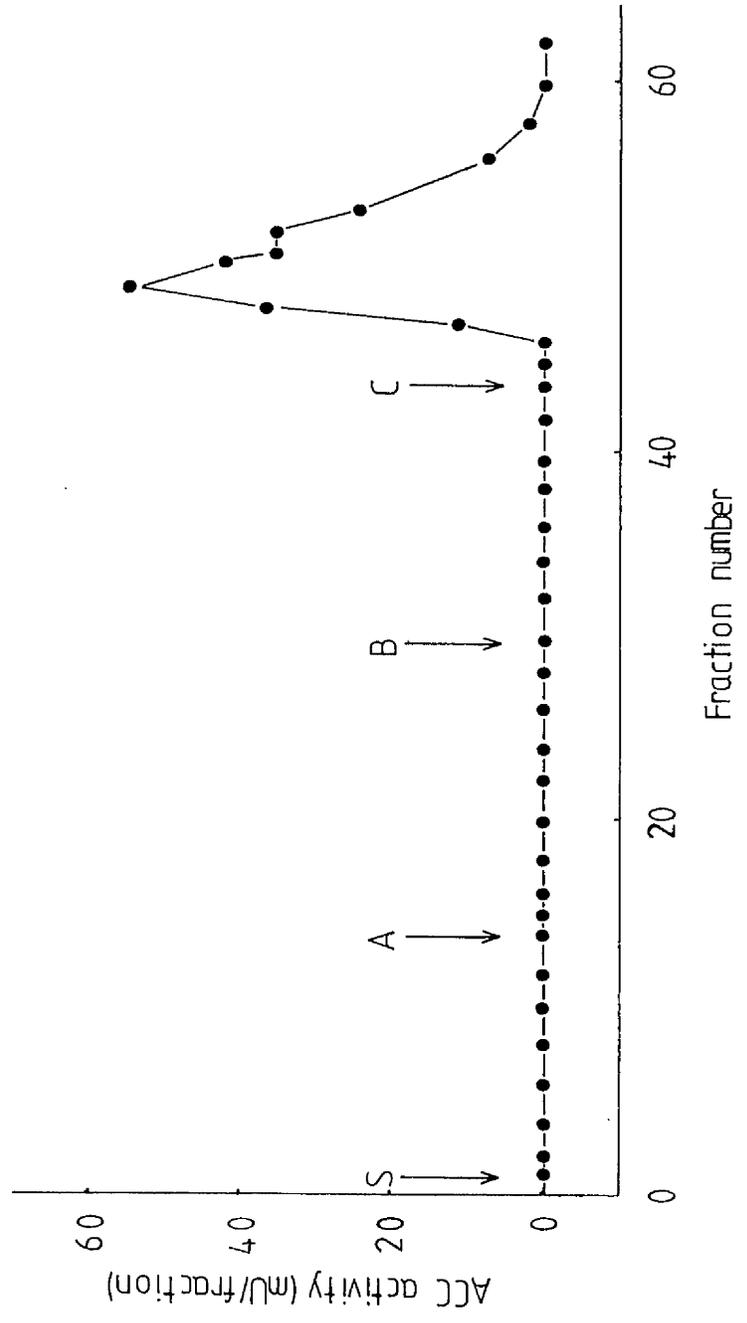
The procedures for the purification of rIACC described by Nakanishi and Numa (1970) and Inoue and Lowenstein (1972) used fractionation on calcium phosphate. I wanted to determine whether phosphocellulose could replace calcium phosphate as it was felt that chromatography on the latter medium could lead to proteolysis of ACC through the activation of Ca^{2+} ion dependent proteases.

Two experiments where lrmgACC was chromatographed on phosphocellulose are reported here. In each experiment a 0.9 cm x 2.5 cm column of phosphocellulose was equilibrated with 20 mM potassium phosphate and lrmgACC which had been previously dialysed into this buffer was applied to the phosphocellulose. The column was then washed with 10 column volumes of the equilibration buffer followed by 10 column volumes each of the buffers indicated in Fig. 3.6. Phosphocellulose did not resemble calcium phosphate in that ACC activity could not be eluted with 200 mM potassium phosphate. The ACC activity was eluted from the column with 100 mM potassium phosphate, 0.5 M NaCl.

A second experiment was performed to determine whether there was a more specific way of eluting ACC from phosphocellulose. The column was equilibrated and lrmgACC was applied as described above. Elution of ACC activity was then attempted by washing the column with

Figure 3.6. Chromatography of lactating rabbit mammary gland acetylCoA carboxylase on phosphocellulose (1).

0.9 U of acetylCoA carboxylase was applied to the column at the point indicated by S. The arrows indicate the points at which the column was washed with the following: A, 100 mM potassium phosphate; B, 200 mM potassium phosphate; C, 100 mM potassium phosphate, 0.5 NaCl. (●), ACC activity.



the buffers described in Fig. 3.7. Two of the substrates of ACC, ATP and acetylCoA, failed to elute the enzyme. Similarly, citrate, which is known to induce a conformational change in ACC failed to elute ACC activity. ACC was eluted from the column using a gradient from 0.1 - 0.5 M NaCl. The recovery of ACC was 45%. The enzyme did not elute from the column in a sharp peak and I concluded that there would be no advantage in using gradient elution in the rat liver purification.

Dye-ligand chromatography

Since two of the substrates of ACC contain adenyl groups I investigated the use of dye-ligand chromatography (Thompson et al., 1975). A 0.9 cm x 3.5 cm column of Cibacron Blue-Sepharose equilibrated in 50 mM potassium phosphate was used. 0.3 U (1 mg) of pure lrmgACC which had been dialysed overnight into the equilibration buffer and then diluted 100-fold with it to a volume of 10.0 ml was applied to the column. No ACC activity bound to the column (Figure 3.8(a)); the recovery of ACC activity was 108%. The experiment was repeated using 10 mM potassium phosphate. Once again ACC did not bind. On the basis of experience with other proteins in this laboratory it is unlikely that this lack of binding was due to the column being overloaded.

I studied the behaviour of lrmgFAS on Cibacron Blue-Sepharose to investigate whether this could be used as a negative step in the purification of rlACC. A 1.4 cm x 4.0 cm column of Cibacron Blue-Sepharose equilibrated in 10 mM potassium phosphate was used. 55 U (25 mg) of pure lrmgFAS was diluted 10-fold with this buffer to a volume of 10.0 ml and applied to the column. As with ACC, FAS activity did not bind (Figure 3.8 (b)). The recovery of FAS activity in fractions 5 - 12 was 89%.

Figure 3.7. Chromatography of lactating rabbit mammary gland acetylCoA carboxylase on phosphocellulose (2)

0.45 U of lrmgACC was applied to the column as described in the text. The arrows indicate the points at which the column was washed with the following solutions: S, sample application and wash with equilibration buffer; A, 2 mM ATP; B, 0.1 mM acetyl CoA; C, 25 mM potassium citrate; D, linear gradient from 0.1 - 1.0 M NaCl. All solutions except the equilibration buffer contained 100 mM potassium phosphate. The NaCl concentration of the fractions was determined from their conductivities. (o), ACC activity; (x), absorbance at 280 nm; (•), NaCl concentration.

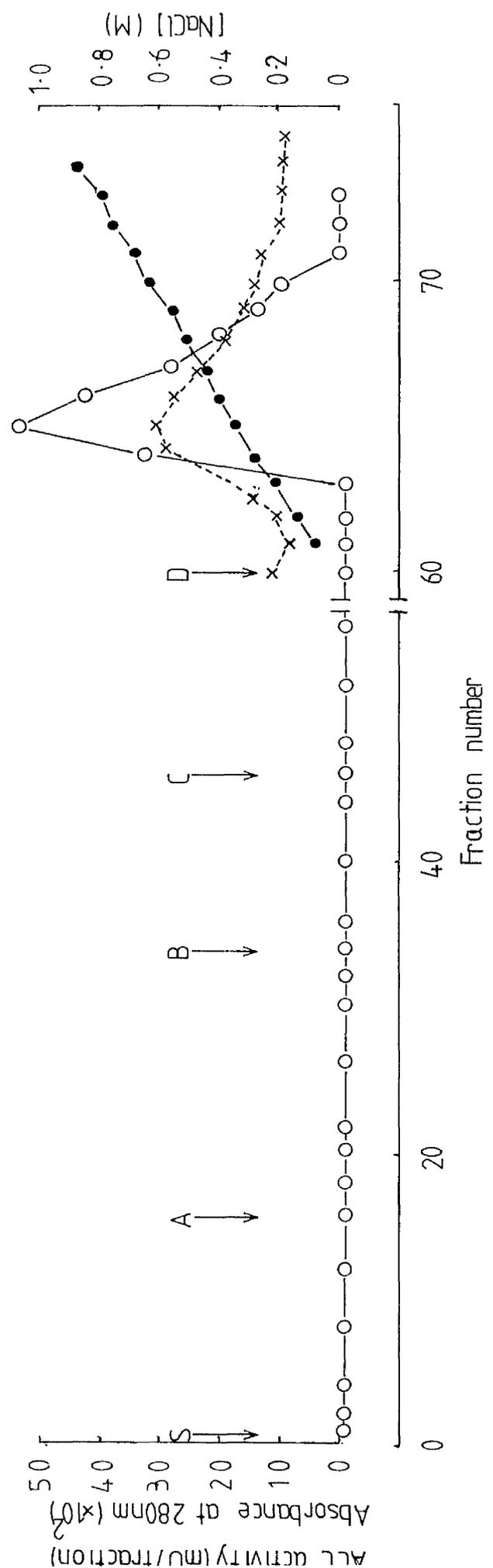
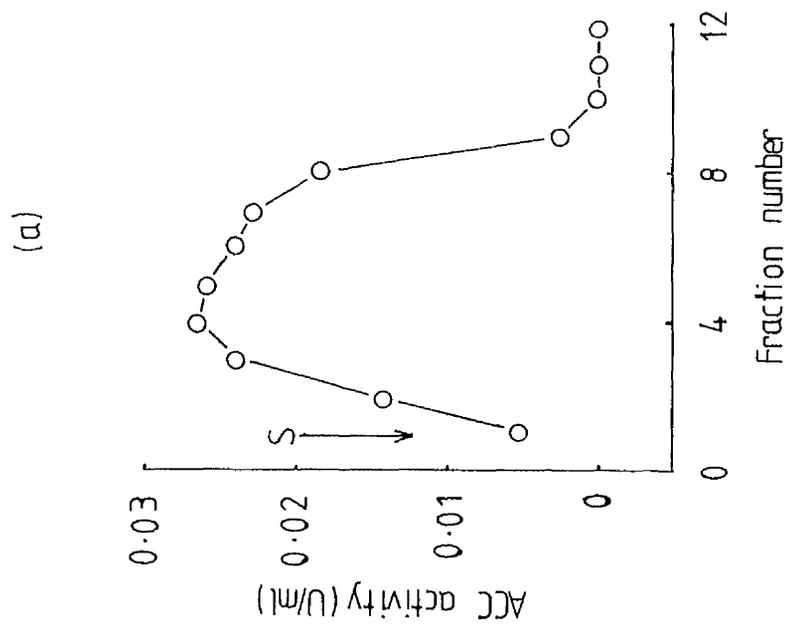
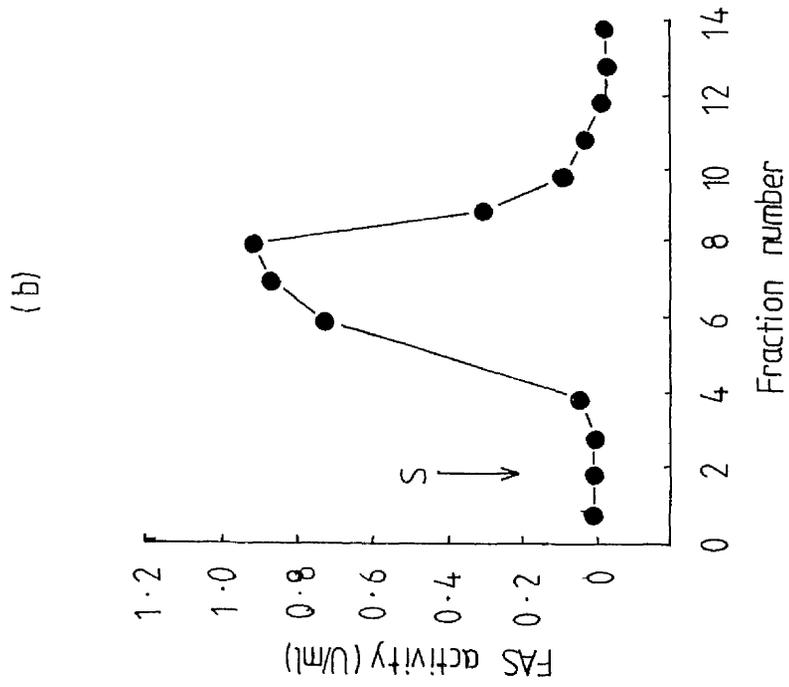


Figure 3.8. Chromatography of lactating rabbit mammary gland acetylCoA carboxylase and fatty acid synthase on Cibacron Blue F3G-A-Sepharose.

(a) AcetylCoA carboxylase was applied to the column at the point indicated by S and the column was then washed with the equilibration buffer. Flow rate: 20 ml/h, 2 ml fractions were collected. (o), ACC activity.

(b) Fatty acid synthase was applied to the column at the point indicated by S and washed through with equilibration buffer. Flow rate: 20 ml/h, 1.8 ml fractions were collected. (●) FAS activity.



This experiment indicated that FAS did not bind to Cibacron Blue Sepharose. It has been reported that NADP^+ dependent enzymes bind more strongly to Procion Red HE - 3B than they do to Cibacron Blue F3G-A (Watson et al 1978). I therefore studied the behaviour of 1rmgFAS on Procion Red-Sepharose. A column 0.9 cm x 7.0 cm equilibrated in 100 mM potassium sulphate was used. 5.8 U of pure 1rmgFAS was diluted to 20 ml with the equilibration buffer and applied to the column at a flow rate of 22 ml/h. No FAS activity emerged unretarded. The column was then washed with 5 column volumes of equilibration buffer containing (a) 100 μM NADPH, (b) 100 μM NADP^+ and (c) 2 M NaCl. No FAS activity was detected in any of the fractions and no protein was eluted by the 2 M NaCl wash. FAS therefore binds tightly to Procion Red-Sepharose.

I next studied whether ACC would bind to (and could be eluted from) this medium. A 0.9 cm x 7.0 cm column equilibrated in 100 mM potassium phosphate was used. 0.3 U (1 mg) of pure 1rmgACC which had been dialysed overnight into the equilibration buffer and diluted with this buffer 100-fold to a volume of 10 ml was applied to the column at a flow rate of 25 ml/h. No ACC activity emerged unretarded. The column was then washed with (a) 100 mM potassium phosphate, 5 mM ATP; (b) 500 mM potassium phosphate and (c) 100 mM potassium phosphate, 3 M NaCl. No ACC activity or absorbance at 280 nm was detected in any of the fractions.

These results show that ACC and FAS bind strongly to Procion Red Sepharose and suggest that the enzymes cannot be eluted using reasonably mild conditions.

Hydrophobic interaction chromatography

Both ACC and FAS bind molecules which have hydrophobic moieties. I therefore studied the behaviour of ACC and FAS on Phenyl-Sepharose and Octyl-Sepharose.

A column of Octyl-Sepharose (0.9 cm x 7.0 cm) equilibrated in 100 mM potassium phosphate was used. 55 U (25 mg) of pure 1rmgFAS was diluted with the equilibration buffer and applied to the column. The column was then washed with (a) 2 column volumes of equilibration buffer followed by 5 column volumes each of : (b) 5 mM potassium phosphate, 20 μ M palmitoylCoA; (d) 5 mM potassium phosphate, 25% (v/v) ethanediol; (e) 5 mM potassium phosphate, 50% (v/v) ethanediol. All of the FAS bound to the column. Lowering the ionic strength of the buffer, inclusion of palmitoylCoA or reducing the polarity of the buffer with 25 or 50% (v/v) ethanediol failed to elute FAS, as judged by absorbance at 280 nm and FAS activity.

This experiment was repeated using 1.0 U of 1rmgACC. The ACC was applied to the column and the column was then washed with 5 column-volumes of (a) equilibration buffer; (b) 5 mM potassium phosphate; (c) 5 mM potassium phosphate, 20 μ M palmitoylCoA; (d) 5 mM potassium phosphate, 25% (v/v) ethanediol; (e) 5 mM potassium phosphate, 50% (v/v) ethanediol. All the ACC bound to the column. Washing the column with the above solutions failed to elute the ACC from the column as judged by absorbance at 280 nm and ACC activity.

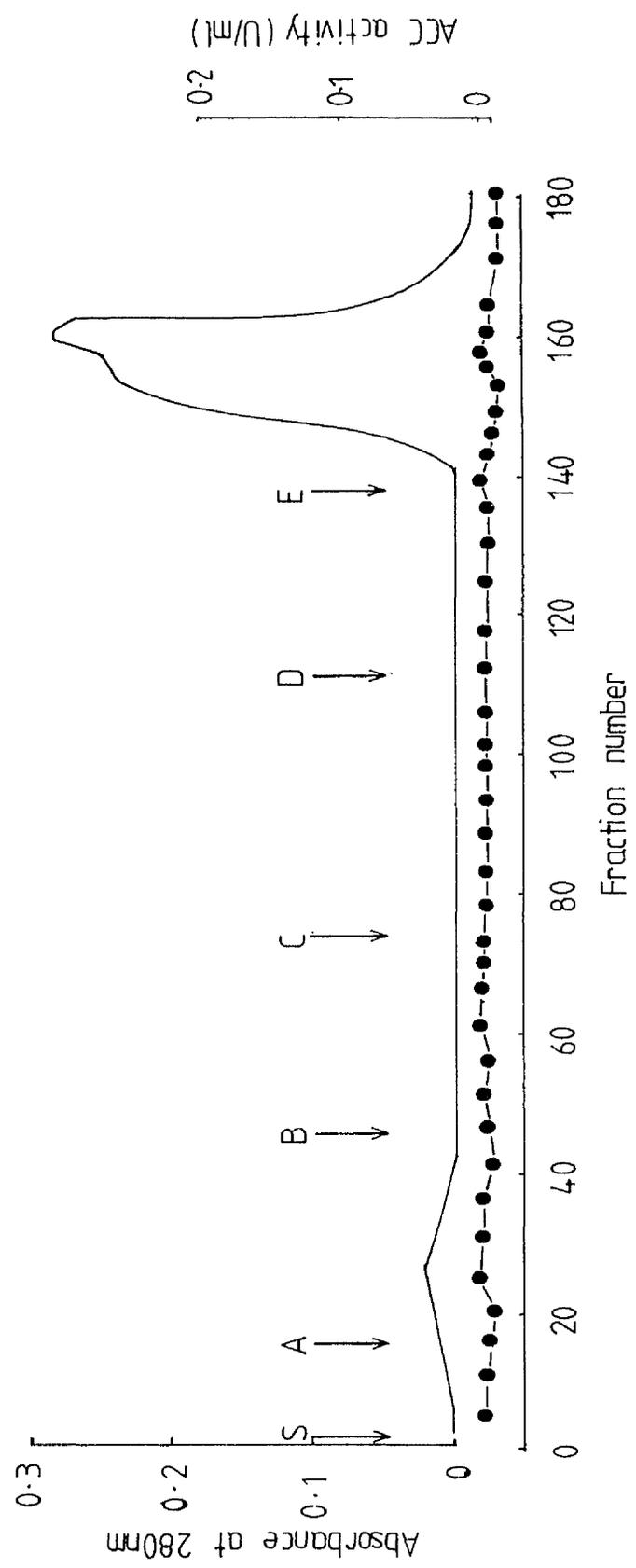
These results indicated that both FAS and ACC bound very tightly to Octyl-Sepharose, in keeping with the observation that octyl groups are strongly hydrophobic. I therefore studied the behaviour of ACC on a less hydrophobic medium, Phenyl-Sepharose. A column of Phenyl-Sepharose (0.9 cm x 4.0 cm) equilibrated in 100 mM potassium phosphate

was used. 3 U (10 mg) of ACC was diluted 10-fold with this buffer to a volume of 3.9 ml and applied to the Phenyl-Sepharose. The column was then washed with buffers containing the components indicated in Fig. 3.9. No ACC activity was detected in any fraction. Fractions 140 - 165, which contained protein but not ACC activity, were pooled and dialysed overnight into 100 mM potassium phosphate, 20 mM potassium citrate. Even after this treatment no ACC activity was found. The protein eluted from the Phenyl-Sepharose would, however, appear to be ACC since analysis on SDS-polyacrylamide gels revealed a single band which migrated to the same position as lrmgACC (not shown). I concluded therefore that elution of ACC with 25% (v/v) ethanediol somehow inactivates the enzyme.

The information gained in the experiments on the chromatographic properties of lrmgACC and lrmgFAS enabled me to devise a scheme for the partial purification of rLACC which involved chromatography on DEAE cellulose and phosphocellulose. The experiments suggested that dye-ligand chromatography and chromatography on octyl-Sepharose would be of little value in the purification of rLACC.

Figure 3.9. Chromatography of lactating rabbit mammary gland
acetylCoA carboxylase on Phenyl-Sepharose CL - 4B.

ACC was applied to the column (arrow S) as described in the text. The arrows indicate the points at which the column was washed with buffers containing: A, 100 mM potassium phosphate; B, 5 mM potassium phosphate; C, 5 mM potassium phosphate, 10 μ M palmitoylCoA; D, 5 mM potassium phosphate, 0.1% (w/v) Triton X-100; E, 5 mM potassium phosphate, 25% (v/v) ethanediol. Flow rate, 6 ml/h; 0.33 ml fractions were collected. (—), absorbance at 280 nm; (—●—), ACC activity.



Section 3. Physical properties of lrmgACC purified using polyethylene glycol precipitation

In my hands solutions of lrmgACC which had been purified using the procedure of Hardie and Cohen (1978a)(hereafter referred to as 'PEG purified' ACC) always had a slightly turbid appearance. This turbidity was noticeable even in freshly prepared solutions and increased during storage at 4°C. This precipitation did not itself result in a loss of activity of the solution (expressed on a U/ml basis). After brief centrifugation to pellet the insoluble material, assay of the supernatant always revealed that it had lost activity compared to the turbid solution. The pellet, which was active, could be resuspended, but not redissolved. Extensive dialysis of the freshly prepared 3% polyethylene glycol precipitate into either 100 mM potassium phosphate, 25 mM potassium citrate (buffer A) or citrate-free buffer A did not prevent the appearance of the turbidity.

Scant attention was paid to this phenomenon at first : Hardie and Cohen (1978b) refer to the slightly turbid appearance of the lrmgACC solutions which they prepared so it was not unexpected. One problem was that solutions of ACC required clarification immediately before use which led to a loss of activity as the still-active precipitate was removed. Over a period of time, however, several observations were made which suggested that 'PEG purified' ACC had different properties to ACC purified from other sources using conventional methods. These observations are described below.

Several attempts were made to electrophorese 'PEG purified' ACC on 3% polyacrylamide gels. This percentage of polyacrylamide has been found suitable for the electrophoresis of FAS and ATPCL which have M_r s similar to that of the protomeric form of ACC. On no occasion did lrmgACC penetrate into the gel and a very dense layer of protein

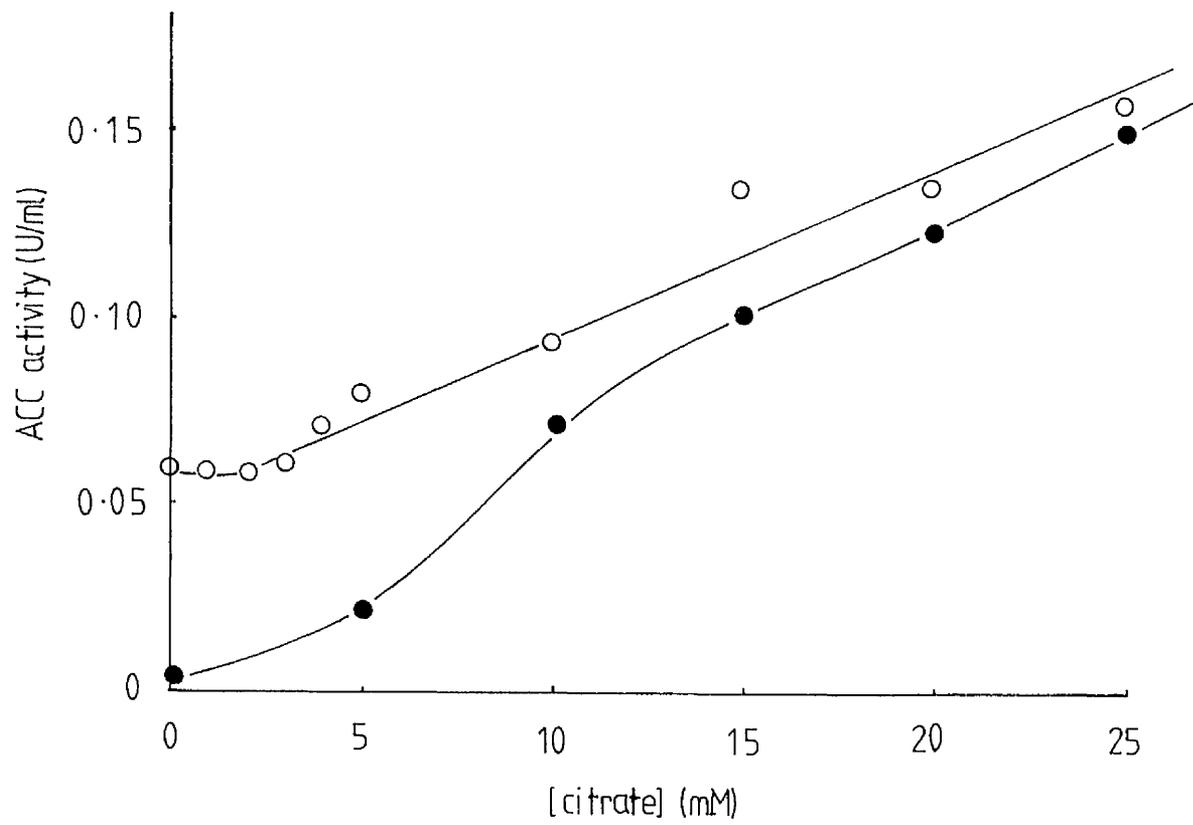
was observed on the top surface of the gel after staining. The explanation of this could be that the purified lrmgACC was stored in the presence of 25 mM potassium citrate and hence would have been in the polymeric form and therefore too large to penetrate into the gel. However, the 'PEG purified' ACC did not penetrate into the gel after dialysis into either 100 mM potassium phosphate or 100 mM potassium phosphate, 20 μ M palmitoylCoA. These procedures are known to result in the depolymerization of ACC (see Chapter 1.2). These results suggested that 'PEG purified' ACC could not be depolymerised by established procedures.

ACC purified from all sources is reported to have an absolute requirement for citrate in the assay (Lane et al., 1974). This is distinct from the activation in the presence of citrate and reducing agents which is required during the early stages of the purification procedures. 'PEG purified' ACC does not have this absolute requirement for citrate in the assay. This was first noted in assays of pure ACC where the enzyme had been stored in the presence of 25 mM potassium citrate, and it was possible that the small amount of citrate carried into the assay (final concentration 0.25 mM) would meet the requirements of the enzyme. The citrate requirement of 'PEG purified' ACC was therefore studied using enzyme which had been dialysed into a citrate-free buffer, namely 100 mM potassium phosphate.

ACC activity was then assayed over a range of citrate concentrations (given in Fig. 3.10) by the method of Manning et al. (1976). The ionic strength of the assay medium was kept constant by the addition of KCl. Fig. 3.10 shows that 25 mM citrate causes a 2.5 - fold increase in the activity of 'PEG purified' ACC. On the other hand this concentration of citrate causes a 30 - fold increase in the activity of ACC partially purified without the use of poly-

Figure 3.10. Citrate dependence of lactating rabbit mammary gland acetylCoA carboxylase.

AcetylCoA carboxylase purified using polyethylene glycol precipitation (o) and acetylCoA carboxylase partially purified as far as the DEAE cellulose stage described in Table 3.2 (●) were assayed at the citrate concentrations indicated. Both samples were diluted to the same activity (as measured by the standard assay prior to the experiment.



ethylene glycol. Clearly, the degree of lrmgACC activity afforded by citrate is much lower for enzyme prepared using polyethylene glycol than that which has not been exposed to polyethylene glycol.

The observations described above, i.e. the tendency of lrmgACC to come out of solution and its inability to penetrate into non-denaturing polyacrylamide gels under depolymerizing conditions, suggested that 'PEG purified' ACC was maintained in a highly aggregated state. This would explain the third observation, the small effect of citrate: the enzyme is present in the aggregated form whether citrate is present or not. It was of interest to examine the aggregation state of 'PEG purified' ACC more quantitatively under a variety of conditions. The two most convenient methods for the determination of the aggregation state were measurement of the sedimentation coefficient or measurement of the M_r by gel filtration. As is described more fully in Chapter 4.1., in my hands gel filtration of ACC led to loss of activity so that this method was ruled out. I therefore determined the sedimentation coefficient for the enzyme under various different conditions.

Density gradient centrifugation of 'PEG purified' ACC was performed on 5 - 20% sucrose gradients as described in Chapter 2.2. Three centrifugation runs were carried out. The buffer composition used in each run is given below:

First Run : 100 mM potassium phosphate, 25 mM potassium citrate

Second Run: 100 mM potassium phosphate

Third Run : 100 mM potassium phosphate, 20 μ M palmitoylCoA.

Samples were dialysed for 16 h at 4^oC into the appropriate buffer before each run. In the first and second runs 2 U (7.1 mg) of ACC was centrifuged. In the third run 0.5 U (7.1 mg) of ACC was centrifuged. The difference between the specific activities of the

ACC preparations used is due to the fact that the ACC used in the third run lost activity during dialysis. In each run 3.75 U of β -galactosidase and 4.0 U of pyruvate kinase were centrifuged together but separately from the ACC. In the third run no ACC activity could be detected. The protein content of each fraction was therefore assayed by a modification of the method given in Chapter 2.2 in which 200 μ l of each fraction was added to 1 ml of the protein reagent.

No suitable markers with sedimentation coefficients greater than 16 S were available and so the value for ACC could not be accurately determined. Approximate values were obtained using β -galactosidase and pyruvate kinase as markers as illustrated in Fig. 3.11.

The sedimentation coefficients reported for the protomeric form of ACC are approximately 13 S. The sedimentation coefficients for the polymeric form are 45 S. Hardie and Cohen (1978b) report a value of 51 S for 1rmgACC. No value was quoted for the sedimentation coefficient of their enzyme measured in the absence of citrate. The sedimentation coefficient for the main peak obtained in the first run (approximately 42 S) is lower than that reported by Hardie and Cohen (1978b) but in good agreement with that reported for ACC from other sources (Fig. 3.11). Sedimentation in the absence of citrate (Fig. 3.12) resulted in only a small change in the sedimentation coefficient of the main peak from 42 S to 32 S. In both cases there was an obvious shoulder to the ACC peak indicating the presence of ACC with slightly lower sedimentation coefficients. However, it is clear that even in the absence of citrate the ACC was largely in the polymeric form. The fact that no activity was detected in the third run (Fig. 3.13) was probably owing to the dialysis in the presence of palmitoylCoA which resulted in a decrease in the specific activity of

Figure 3.11. Density gradient centrifugation of lactating rabbit mammary gland acetylCoA carboxylase (first run).

Centrifugation conditions are given in the text. After the run was completed the contents of each tube were harvested and fractions were monitored for pyruvate kinase activity (\square), β -galactosidase activity (\circ) and ACC activity (\bullet). The inset shows a replot of the data in the main diagram where the peak fractions of pyruvate kinase and β -galactosidase activities are plotted against their sedimentation coefficients. The position of the peak ACC fraction is indicated.

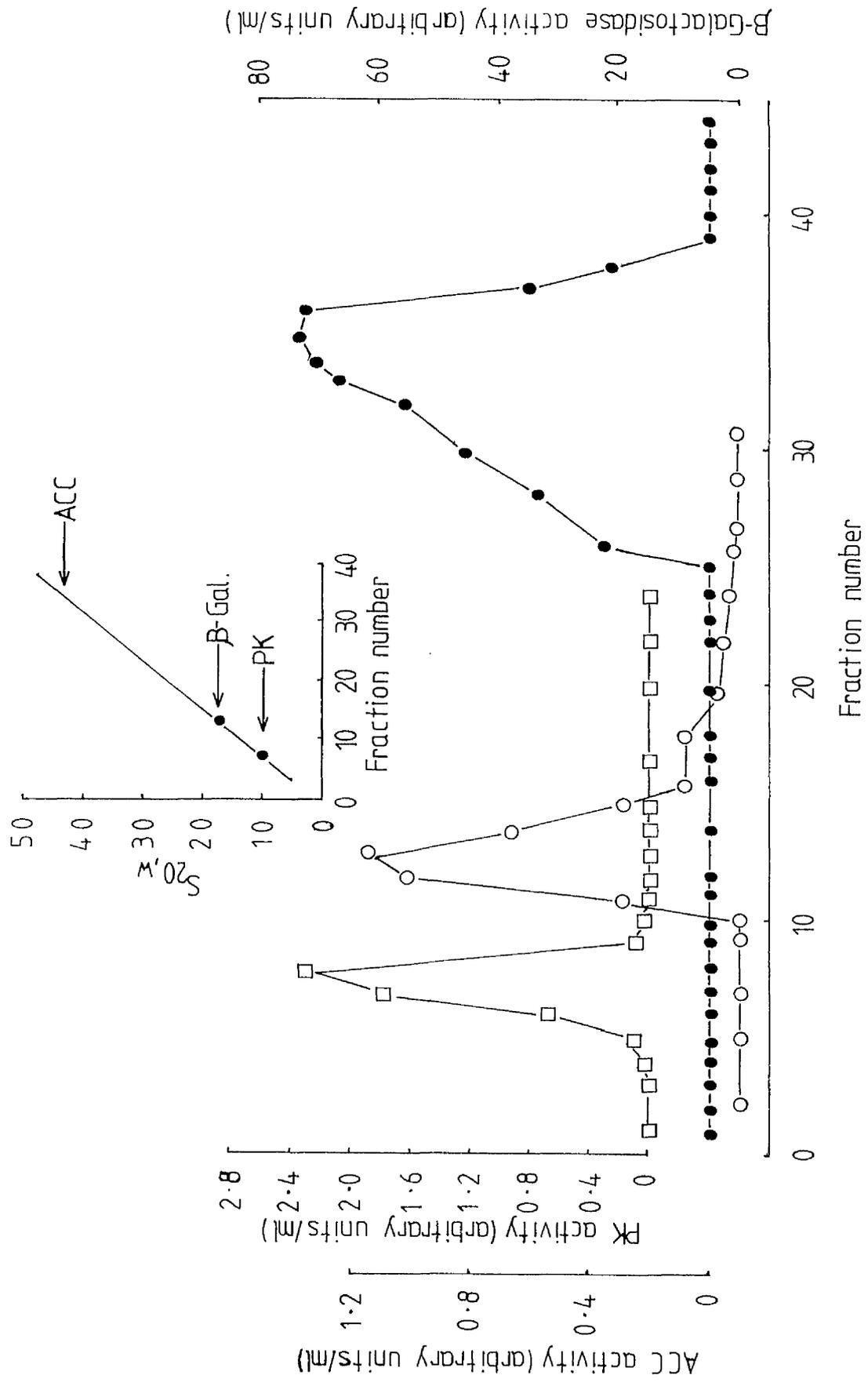


Figure 3.12. Density gradient centrifugation of lactating rabbit mammary gland acetylCoA carboxylase (second run).

Centrifugation conditions are given in the text. After centrifugation the contents of each tube were harvested and assayed for pyruvate kinase activity (x) β -galactosidase activity (o) and ACC activity (•).

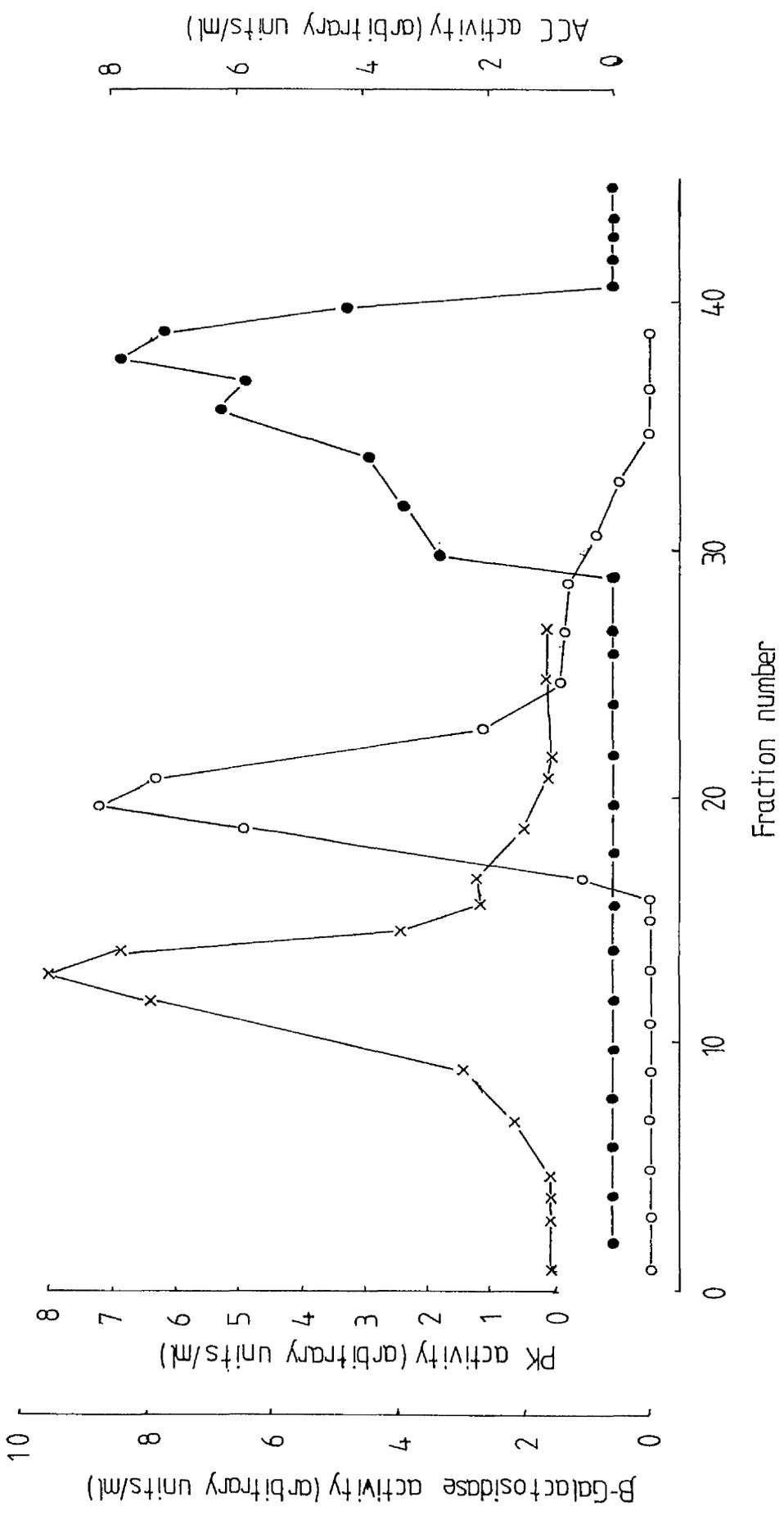
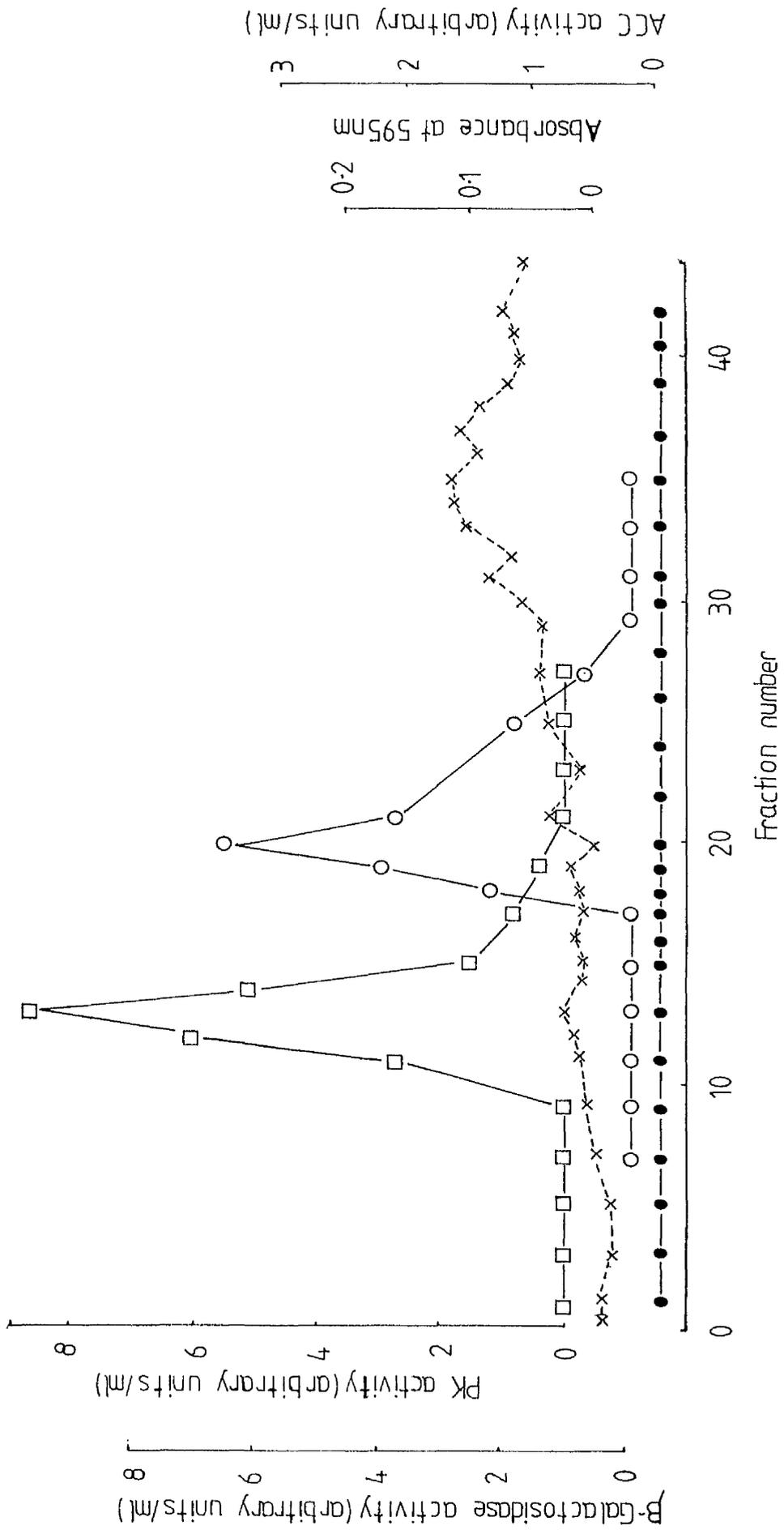


Figure 3.13. Density gradient centrifugation of lactating
rabbit mammary gland acetylCoA carboxylase (third run

The centrifugation conditions are described in the text. After centrifugation the contents of each tube were harvested and fractions were assayed for pyruvate kinase activity (\square), β -galactosidase activity (\circ), ACC activity (\bullet) and protein (described in text) (\times).



the ACC. The inevitable dilution of the sample during the centrifugation would mean that this low level of activity was difficult to detect. Incubation of the fractions at 37°C for 30 min in the presence of 25 mM potassium citrate, 20 mM MgCl₂, 0.1 mM DTT did not result in an increase in activity. Estimation of the protein distribution in the fractions suggested a peak around those fractions corresponding to a sedimentation coefficient of 29 - 32 S.

The density gradient centrifugation results indicate that 'PEG purified' ACC exists in the polymeric form both in the absence of citrate and in the presence of palmitoylCoA. This explains one of the original observations made, that the lrmgACC would not penetrate into 3% polyacrylamide gels. There are no reports of the sedimentation properties of lrmgACC other than that of Hardie and Cohen (1978b) so one cannot say with certainty that this continued aggregation is not a unique property of lrmgACC. Ahmad et al (1978), however, have purified rat mammary gland ACC by a method which does not involve the use of polyethylene glycol. Their procedure results in an enzyme which has a sedimentation coefficient of 40 S in the presence of citrate and 12 - 13 S in its absence. It seems unlikely that there should be such a fundamental difference between rabbit and rat mammary gland ACC and so it would appear that it is the use of polyethylene glycol in the purification which results in the continued aggregation of pure lrmgACC. Support for this view comes from the experiment on the citrate requirement of lrmgACC: only the 'PEG purified' ACC did not require citrate in the assay.

It may be that trace amounts of polyethylene glycol remain in lrmgACC preparations even after extensive dialysis since one would expect the removal of polyethylene glycol by dialysis to be slow. Thus it is possible that trace amounts of polyethylene glycol could

be responsible for maintaining ACC in the polymeric form. If this is so it could explain the results regarding the aggregation state of 'PEG purified' ACC described above. Alternatively the effect of polyethylene glycol on the aggregation state could be irreversible even if all of the polyethylene glycol was removed.

The results of the experiments described in this section indicated that purification of ACC using polyethylene glycol results in an enzyme preparation which is maintained in a highly aggregated state even under conditions which promote the depolymerization of ACC purified using conventional methods. This meant that it was desirable to find a method for the purification of rLACC which did not involve the use of polyethylene glycol, since one objective of this work was to study the effect of phosphorylation on the polymeric state of the liver enzyme. Clearly, such experiments would not be possible with an enzyme preparation which was artefactually maintained in an aggregated form. The experiments leading to a purification scheme for rLACC are described in the following chapter.

Chapter 4

Attempted purification of rat liver

acetylCoA carboxylase

Section 1. Initial attempts to purify rat liver acetylCoA
carboxylase

Partial purification of rat liver acetylCoA carboxylase

Rat liver was chosen as a source of ACC since the diet of the animals could easily be manipulated to induce high levels of enzyme and because the hormonal state of the animals could readily be manipulated. In this section I describe a partial purification procedure based on the experiments described in Chapter 3.2 and my attempts to purify rlACC beyond this stage. The main objective was to isolate rlACC free from proteolytic digestion.

Unless otherwise stated all buffers used included 1.0 mM EDTA, 1 mM benzamidine-HCl, 15 mM β -mercaptoethanol and were adjusted to pH 7.0 prior to use. ACC was assayed by the method of Manning et al (1976), 6 - 10 rats, which had been starved for 24 h and then refed a fat-free diet for 48 h were used. The animals were killed by cervical dislocation and the livers were removed. All subsequent procedures were performed at 4°C.

The livers were rinsed in ice-cold 0.25 M sucrose, weighed, and then homogenised (3 x 15 sec in an Atomix blender) in 3 volumes of 0.25 M sucrose, 1.2 mM PMSF. The homogenate was centrifuged at 20000 x g for 15 min whereupon the supernatant was filtered through muslin and then centrifuged at 90000 x g for 60 min.

The 90000 x g supernatant was applied to a column of DEAE cellulose (4.4 cm x 11.0 cm) equilibrated in 20 mM potassium phosphate. Unbound protein was washed through the column using the equilibration buffer until the E_{280} of the emerging solution was less than 0.05. The column was then developed with a linear gradient from 20 - 500 mM potassium phosphate (500 ml/reservoir). Fractions containing ACC

activity were pooled.

The pooled fractions from the DEAE cellulose step were applied to a phosphocellulose column (4.4 cm x 9.0 cm) equilibrated in 100 mM potassium phosphate. Unbound protein was washed through the column with the equilibration buffer until the E_{280} of emerging fractions was less than 0.05. ACC activity was eluted by washing the column with 100 mM potassium phosphate, 0.5 M NaCl. Fractions containing ACC activity were pooled.

I took great care to minimise the activity of proteases during this procedure. This involved homogenization in an isotonic medium which contained PMSF and the inclusion of EDTA and benzamidine-HCl in all buffers. ACC activity was often undetectable in the 90000 x g supernatant but was usually detectable after the DEAE cellulose step. However, the total amount of activity at this stage was somewhat variable (see Table 4.1). The elution of ACC from DEAE and phosphocellulose is shown in Figures 4.1 and 4.2. In both cases the elution conditions were very similar to those which eluted lrmgACC (Chapter 3.2). The specific activity of the ACC after DEAE cellulose chromatography was generally in the range of 0.01 - 0.03 U/mg. Chromatography on phosphocellulose gave an approximately 10 - fold purification: the specific activity after this step was 0.2 - 0.3 U/mg. Although this specific activity is close to that obtained for pure lrmgACC (Table 2.1) analysis by SDS-polyacrylamide gel electrophoresis indicated that the preparation was not pure (not shown). I therefore investigated the further purification of rlACC.

Attempted purification of rat liver acetylCoA carboxylase beyond the phosphocellulose stage

The most obvious method to try when attempting to further purify ACC from the phosphocellulose fraction was precipitation with

Table 4.1. The amounts of acetylCoA carboxylase measured in the initial stages of 11 consecutive preparations.

Preparation	AcetylCoA carboxylase activity (U) measured in	
	90000xg supernatant	DEAE cellulose pool
1	0	12.5
2	0	12
3	16	12
4	30	22
5	0	0
6	0	0
7	7.3	15
8	9.5	8
9	0	42
10	23	N.D.
11	0	5.4

The preparation of the 90000xg supernatants and DEAE cellulose pools is described in Chapter 4.1. AcetylCoA carboxylase activity was measured by the method of Majerus et al (1976). Values given refer to the amount of acetylCoA carboxylase activity measured in preparations using 10 rats. N.D. indicates that the acetylCoA carboxylase activity was not measured.

Figure 4.1. Chromatography of 90000 x g supernatant
on DEAE cellulose.

A 90000 x g supernatant (200 ml) was prepared as described in the text and applied to a column of DEAE cellulose (4.4 cm x 11 cm) equilibrated in 20 mM potassium phosphate. The column was washed with this buffer as far as fraction 12 whereupon a linear gradient from 20 - 500 mM potassium phosphate (500 ml/reservoir) was applied (A). 100 ml fractions were collected in fractions 1 - 12: 16 ml fractions were collected from fraction 13 onwards, (x), absorbance at 280 nm; (●) ACC activity; (o) conductivity.

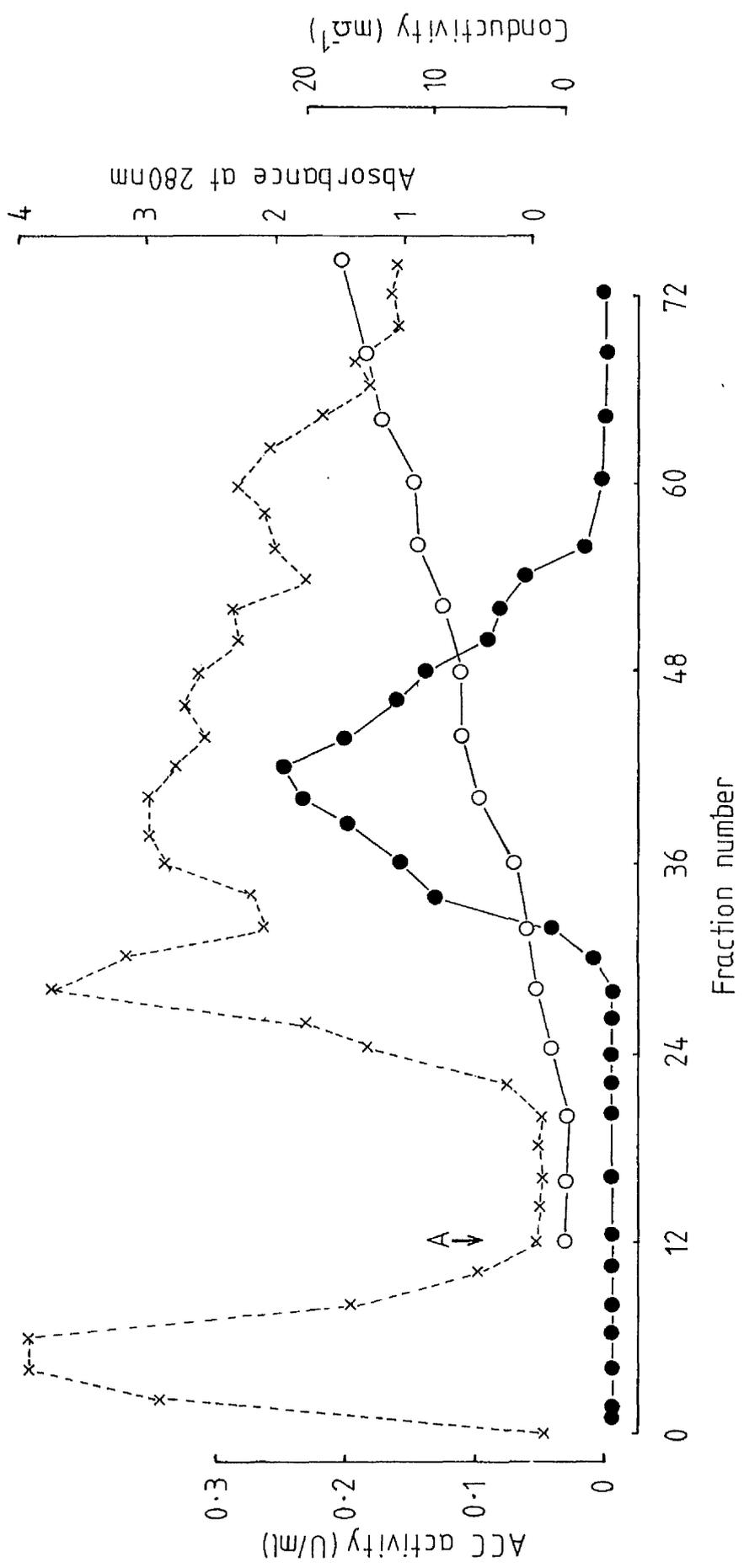
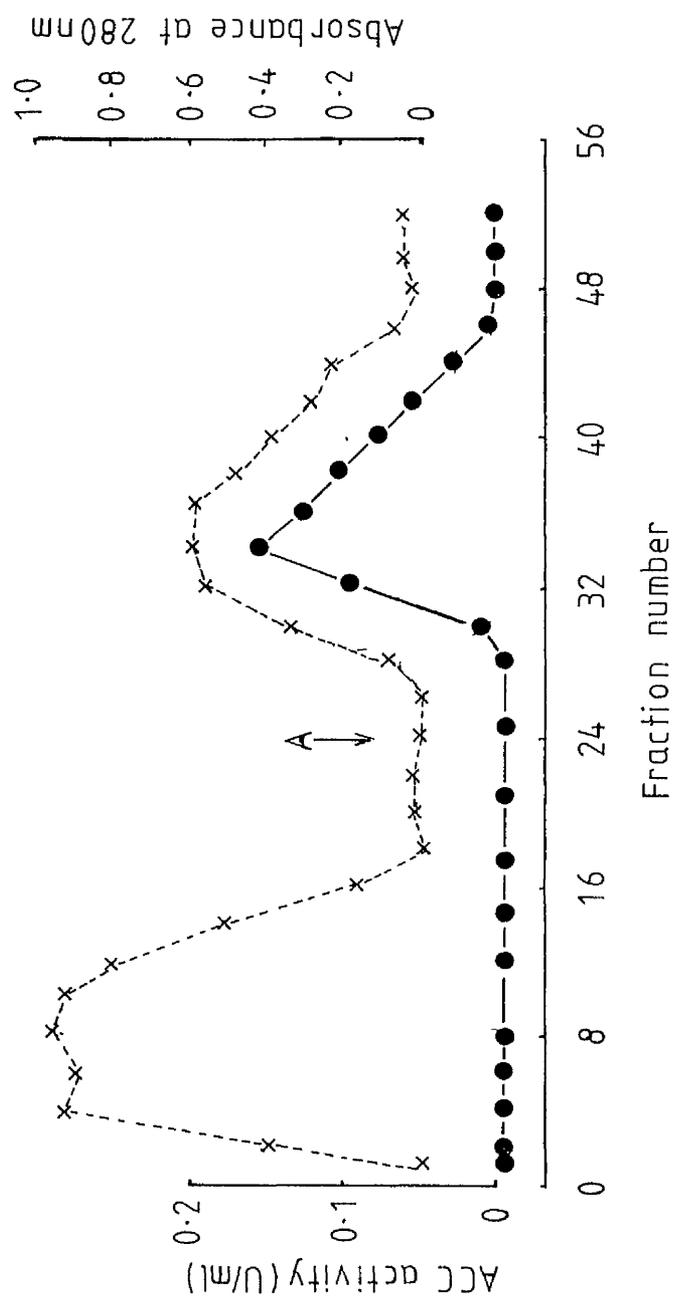


Figure 4.2. Chromatography of DEAE cellulose pool on phosphocellulose column.

A DEAE cellulose pool was prepared as described in the text using 10 rat livers. The pool (40 U) was applied to a phosphocellulose column (4.4 cm x 8 cm) equilibrated in 100 mM potassium phosphate and washed with this buffer. At (A) the column was washed with 100 mM potassium phosphate, 0.5 N NaCl. 50 ml fractions were collected between 1 and 21; 10 ml fractions were collected from 22 onwards. (c), absorbance at 280 nm; (●), ACC activity.



3% (w/v) polyethylene glycol, a procedure which specifically precipitates ACC in mammary gland extracts, Hardie and Cohen (1978a). These experiments were performed before it was realised that polyethylene glycol altered the physicochemical properties of lrmgACC (see Chapter 3.3).

The procedure was carried out at room temperature. The pooled phosphocellulose fractions were brought to 3% (w/v) in polyethylene glycol by the dropwise addition of 0.064 volumes of 50% (w/v) polyethylene glycol 6000. The solution was stirred slowly for 3 h and the precipitate was collected by centrifugation at 40000 x g for 15 min. The pellet was resuspended in 100 mM potassium phosphate, 25 mM potassium citrate. The purification factor and yield at each stage of the procedure are given in Table 4.2. Samples from each stage of the purification were analysed on 4% SDS-polyacrylamide gels.

Fig. 4.3 shows that the material precipitated by polyethylene glycol has only one protein staining band on SDS-polyacrylamide gels although it is possible that additional bands would be visible if a greater amount of protein had been applied to the gel. Polyethylene glycol precipitation does not work as well for rlACC as it does for lrmgACC principally because the recovery at this step is very low, only 7%. In contrast, the recovery during the same step in the lrmgACC procedure is 70%. It is interesting to note that the polyethylene glycol step in the preparation of rlACC causes a loss of some 70% of the ACC activity; it would seem that some sort of inactivation of ACC takes place.

In spite of the low recoveries obtained with this procedure the results were encouraging. The specific activity (0.44 U/mg) was similar to that obtained for pure lrmgACC (see Chapter 2.2 and Hardie and Cohen, 1979). It was, however, considerably lower than

Table 4.2 Purification of rat liver acetylCoA carboxylase using polyethylene glycol precipitation

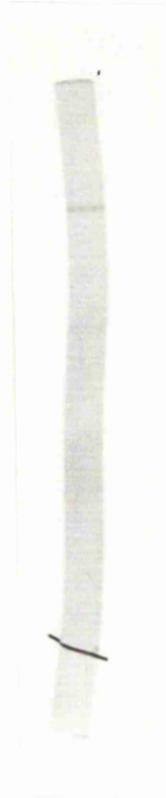
Step	Volume (ml)	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Purification (-fold)	Yield (%)
1	90000xg supernatant	5320	16.0	0.003	1	100
2	DEAE-cellulose pool	700	12.0	0.017	5.6	75
3	phosphocellulose pool	45	9.5	0.21	70	59
4	polyethylene glycol precipitate	1.5	0.66	0.44	146	4.1
5	polyethylene glycol super- natant	N.D.	2.7	N.D.	-	-

The livers from 10 rats were used. N.D. indicates that this parameter was not determined.



Fig 4.3. SDS-polyacrylamide gel electrophoresis of rat liver acetylCoA carboxylase purified using polyethylene glycol precipitation.

rlACC was purified as described in the text. 2 μ g was analysed on an SDS-polyacrylamide gel by the method of Weber and Osborn (1969)



specific activities reported for rlACC by other workers (Inoue and Lowenstein, 1972; Nakanishi and Numa, 1970; Tanabe et al, 1975). The pure rlACC prepared by these workers had specific activities of 7 - 15 U/mg. At the time that the work reported here was carried out the low specific activity of the rlACC purified using polyethylene glycol was interpreted as being due to the presence of substantial amounts of inactive ACC, since the preparation was apparently pure as judged by SDS-polyacrylamide gel electrophoresis. An alternative explanation was that the preparation was contaminated by FAS. No FAS activity could of course be detected, as PMSF, which irreversibly inactivates FAS, was included in the homogenisation buffer. Given that 1mgFAS does not precipitate with 3% (w/v) polyethylene glycol it is perhaps unlikely that this preparation of rlACC should be contaminated with substantial amounts of FAS. This could have been checked if an antibody to FAS had been available. Subsequent to this work being carried out, Witters and Vogt (1981) reported a specific activity of 1.2 U/mg for rlACC purified using a method which also involved polyethylene glycol precipitation. Thus, the specific activity of rlACC purified by Witters and Vogt (1981) is of the same order of magnitude as that of rlACC purified using the method described above.

Attempts to improve upon the recovery obtained during the polyethylene glycol precipitation were unsuccessful. Indeed the procedure proved to be difficult to reproduce: on occasions ACC was not precipitated at 3% (w/v) polyethylene glycol but could be precipitated at 7% (w/v) polyethylene glycol. The recovery of ACC after 7% (w/v) polyethylene glycol precipitation was poor and analysis of the precipitate on SDS-polyacrylamide gels indicated that the preparation was impure.

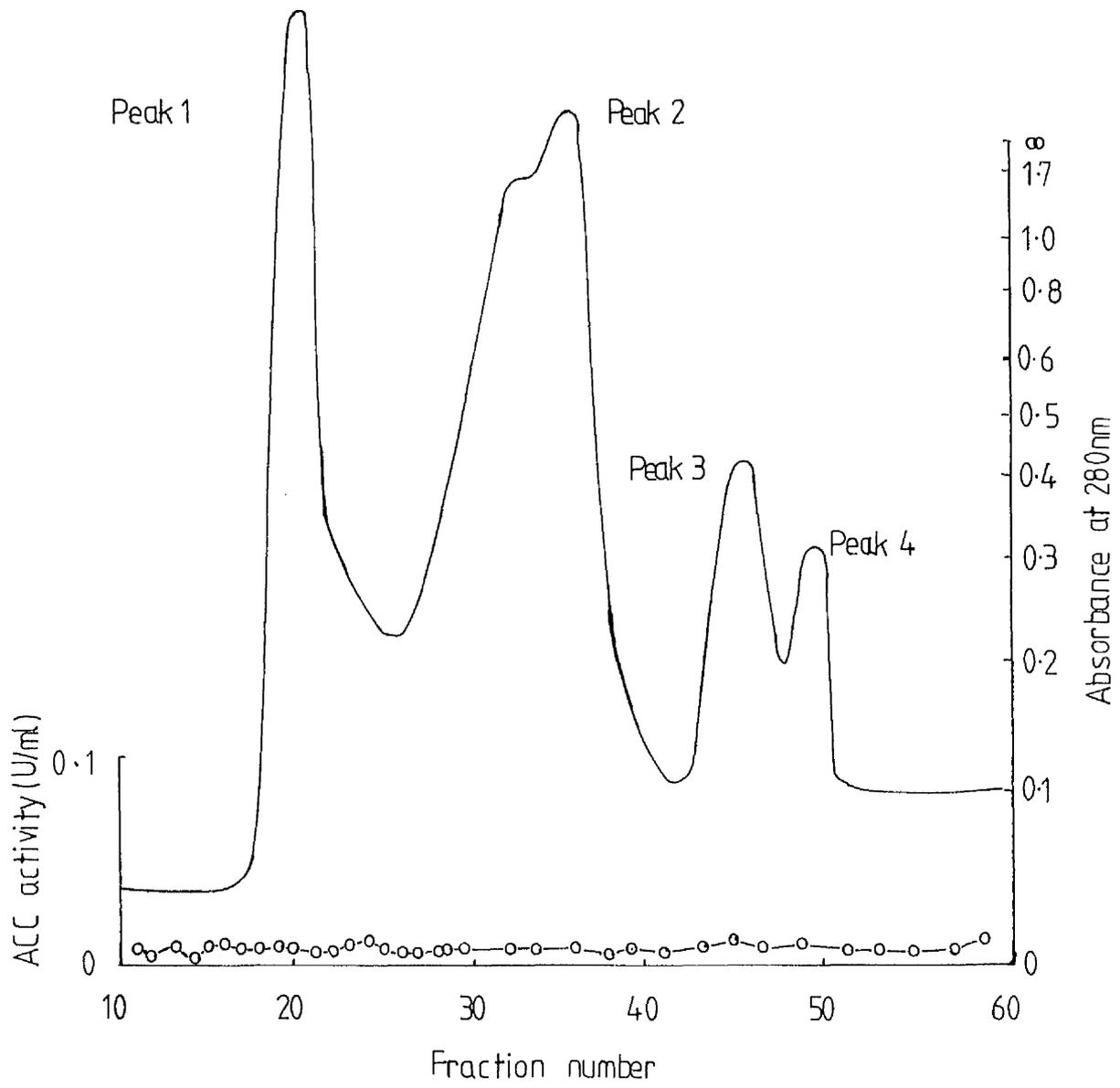
The reason for the variable behaviour of rlACC during the polyethylene glycol step is not known. It is possible that it was the result of variations in either the total protein concentration of the pooled phosphocellulose fractions or the aggregation state of the ACC. The procedure of Witters and Vogt (1981) is different from that reported here in that polyethylene glycol precipitation is used at an early stage in order to remove proteases. This perhaps explains why their procedure is more reproducible. In any case the use of polyethylene glycol in the purification of rlACC was not pursued further for two reasons: the irreproducibility of the precipitation and the results of the experiments reported in Chapter 3.3 which indicated that polyethylene glycol should not be used in the purification of rlACC.

Gel filtration on a variety of media has been used as a final step in a number of ACC purification procedures (Nakanishi and Numa, 1970; Inoue and Lowenstein, 1972; Ahmad et al., 1978). In the presence of citrate ACC has an M_r of approximately 10^7 and should be readily separated from proteins with lower M_r s.

The phosphocellulose pool from a rat liver preparation (using 6 rats) carried out up to step 3 of Table 4.2 was concentrated by vacuum dialysis to a volume of 8.0 ml. The concentrated phosphocellulose pool (6.5U) was applied to a column of Sepharose CL-6B (2.2 cm x 78 cm) equilibrated in 100 mM potassium phosphate, 25 mM potassium citrate. The column was developed with the equilibration buffer at a flow rate of 12 ml/h, at room temperature. Fractions (6 ml) were collected and assayed for ACC activity. The absorbance at 280 nm of the emerging solution was monitored continuously. The results of this experiment are illustrated in Fig. 4.4. No ACC activity could be detected in any of the fractions. Samples were removed from the fractions corresponding to each of the 280 nm

Figure 4.4. Chromatography of partially pure rat liver
acetyl CoA carboxylase on Sepharose CL - 6B.

The experimental procedure is given in the text. Fractions
were monitored for absorbance at 280 nm (continuous line) and assayed
for ACC activity (o).



absorbance peaks and analysed on SDS-polyacrylamide gels. Only the sample from peak 1 had a protein band corresponding to ACC (not shown). It appears that the ACC was inactivated during gel filtration. 'Citrate activation' or assaying in the presence of biotin (0.1 mg/ml) failed to reveal any ACC activity in any of the peak fractions.

As mentioned above, other workers have successfully gel filtered ACC in conditions similar to those described above. It was difficult to imagine what caused this inactivation: the gel filtration was carried out in the presence of a reducing agent and the allosteric activator, citrate. The ACC activity was stable in the buffer used. Although it seemed unlikely that the ACC was losing its biotin moiety, the gel filtration was repeated in the presence of 0.1 mg/ml biotin. As before no ACC activity could be detected in any of the fractions. As discussed in Chapter 3 .1 the sensitivity of the assay used (Manning et al, 1976) was quite adequate to detect levels of ACC as low as 0.01U/ml, so that it is unlikely that the absence of activity was due simply to the dilution of the sample during gel filtration. No explanation for the inactivation has yet been found.

Since ACC has a binding site for CoA I attempted affinity chromatography. Agarose-hexane-CoA equilibrated with 100 mM potassium phosphate, 2.5 mM potassium citrate was used. The phosphocellulose pool from a rat liver preparation carried out as far as step 3 in Table 4.2 was used. The phosphocellulose pool (6.5 U) was dialysed into the equilibration buffer being applied to the column. Under these conditions no ACC activity bound to the column.

The experiment was repeated using 0.5 ml of Agarose-hexane-CoA equilibrated in 100 mM potassium phosphate, 0.15 M NaCl. The phosphocellulose pool (4.5 U) from a rat liver preparation, carried

out as far as step 3 of Table 4.2 using 6 rats, was applied directly to the column. The column was then washed with 20 column volumes of the equilibration buffer. Under these conditions no ACC activity emerged in the flow through fractions. Elution was attempted using 20 volumes of 100 mM potassium phosphate, 25 mM potassium citrate and then this buffer containing firstly 0.2 mM CoA, then 0.2 mM acetylCoA and finally 5 mM ATP. ACC activity was not eluted by washing the column with 100 mM potassium phosphate, 25 mM potassium citrate even though the enzyme would not bind under these conditions. Similarly, washing the column with buffer containing CoA, acetylCoA or ATP was unsuccessful. No further attempts to use affinity chromatography were made.

The binding of ACC to CoA is reversible and ACC ought to be readily eluted from Agarose-hexane-CoA. One possible explanation for the failure to achieve elution is that the ACC may have bound to the Agarose-hexane-CoA by a hydrophobic interaction with the hexanyl spacer arm since lrmgACC interacts strongly with hydrophobic groups: the binding in the presence of NaCl would support this. Alternatively the ACC might have bound to the Agarose-hexane-CoA by the palmitoylCoA binding site since hexane CoA is a reasonably good analogue of a fatty acylCoA thioester.

The experiments reported in Chapter 3.2 indicated that lrmgACC would bind to Phenyl-Sepharose and could be eluted with 25% (v/v) ethanediol, albeit with total loss of activity. The use of Phenyl-Sepharose chromatography as a step in the purification of rlACC was therefore investigated.

A column of Phenyl-Sepharose CL - 4B (0.9 cm x 6.7 cm) equilibrated in 100 mM potassium phosphate, 0.5 NaCl was used. The phospho-

cellulose pool (19 U) from a rat liver preparation carried out as far as step 3 of Table 4.2 was applied directly to the column. The column was then washed with buffer solutions containing the components indicated in Fig. 4.5. Fractions 42 - 45 were pooled and analysed on 4% SDS-polyacrylamide gels. As was the case with lrmgACC, rlACC could only be eluted from Phenyl-Sepharose with 25% (v/v) ethanediol. With rlACC, however, virtually all of the activity applied to the column was recovered (Table 4.3). One difference between the two experiments was that citrate was present in the buffer used to elute the rlACC. Note that in Fig. 4.5 the ACC is eluted in a sharp peak which corresponds to the initial part of a much broader E_{280} peak. The reason for this is not clear. It may be that the absorbance at 280 nm which continues after the ACC peak is due to the elution of proteins which interact more strongly with the Phenyl-Sepharose. Even though the final specific activity shown in Table 4.3 is similar to that found for rlACC purified using polyethylene glycol, the preparation was not pure, as judged by SDS-polyacrylamide gel electrophoresis. Using this method the purity of the ACC was estimated to be 10 - 15%.

Although the recovery over the Phenyl-Sepharose step was excellent, the degree of purification of the phosphocellulose pool afforded by this step was poor (2 - fold). It is possible that further experiments might have identified conditions under which a greater degree of purification could have been achieved. It was clear, however, that further purification beyond the Phenyl-Sepharose stage was required. Studies along these lines were not pursued since several problems concerning the reproducibility of the early stages of the partial purification procedure had arisen.

Figure 4.5. Chromatography of partially purified rat liver acetylCo
carboxylase on Phenyl-Sepharose CL - 4B.

The experiment was performed as described in the text. Elution
was attempted using the buffers indicated: A, 20 mM potassium phosph
B, 20 mM potassium phosphate, 10 μ M palmitoylCoA; C, 20 mM potassium
phosphate, 0.1% Triton X - 100; D, 100 mM potassium phosphate, 25 mM
potassium citrate, 25% (v/v) ethanediol. Continuous line, absorbanc
at 280 nm; (o), ACC activity. 6 ml fractions were collected.

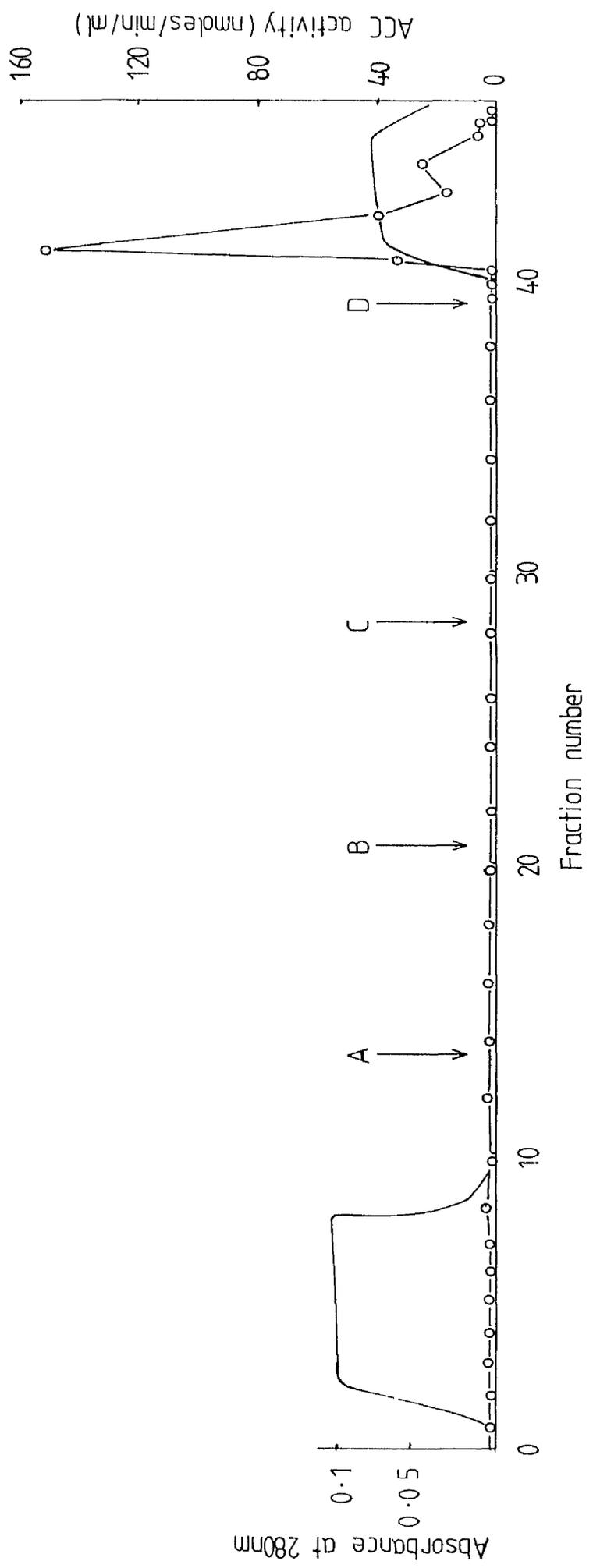


Table 4.3. Partial purification of rat liver acetylCoA carboxylase using Phenyl-Sepharose CL - 4B.

Step	Volume (ml)	Activity (U)	Protein (mg)	Specific Activity (U/mg)
1 90000 x g supernatant	200	0	3600	
2 DEAE cellulose pool	560	56	869	0.064
3 Phosphocellulose pool	220	19.1	70.7	0.27
4 Phenyl-Sepharose pool	50	18.5	29	0.64

The livers from 10 rats were used. No activity was detected in the 90000 x g supernatant.

Section 2. Problems associated with the partial purification procedure described in Chapter 4.1.

Table 4.1 shows the amounts of ACC activity in 90000 x g supernatants and DEAE cellulose pools (prepared from rat livers as described in Chapter 4.1) from a number of different preparations. The following points should be noted:

- 1) On a few occasions no activity was detected in either the 90000 x g supernatant or the DEAE cellulose pool.
- 2) In the majority of cases activity was detectable only after chromatography on DEAE cellulose: the amount of activity present was highly variable.
- 3) On those occasions where activity was detected in the 90000 x g supernatant comparable amounts were found in the DEAE cellulose pool. Once again there was great variation in ACC levels.

Clearly, ACC activities varied from one preparation to another. There was no obvious pattern in this variation: the fractions were all prepared in an identical manner. The large variations in ACC activity were reminiscent of those caused by changes in the animals' dietary status. As has been discussed previously the rats used in this work had been starved for 24 h then fed a fat-free diet for 48 h prior to being killed. The fat-free food was prepared by mixing powdered components to a recipe given in Halliburton (1966). The food was then mixed with water and presented to the animals as a slurry. The inclusion of biotin (0.02 mg/ml) in both the rats' drinking water and the water used to prepare the slurry made no difference to the variability of the ACC activity.

The amount of food consumed varied from one group of rats to the next. Since it was likely that the amount of food consumed after

fasting would influence the amount of ACC present in the livers, commercially prepared fat-free rat food was obtained in the hope that more reproducible results would be obtained.

A second major problem was the variable behaviour of ACC on DEAE cellulose and phosphocellulose. ACC activity, on occasion, did not bind to DEAE cellulose under the conditions described in Chapter 4.1. When this occurred the run-through fractions, which contained the ACC activity, were noticeably turbid even though the 90000 x g supernatant applied to the column had been clear. Re-application of the pooled run-through fractions to an identical DEAE cellulose column produced the same result: both ACC activity and the material causing the turbidity did not bind.

The turbid run-through fractions could be clarified by centrifugation at 105000 x g for 1 h. All of the ACC activity remained in the supernatant indicating that the precipitated material was not ACC. When the 105000 x g supernatant was then applied to DEAE cellulose all of the ACC activity bound and could be eluted from the column as described in the previous section. This suggested that it was the presence of the insoluble material which prevented the binding of the ACC to the ion exchange resin.

One possible explanation for the appearance of turbidity is that a change in pH may have occurred when the 90000 x g supernatant was applied to the ion-exchange resin. The DEAE cellulose was always equilibrated at pH 7.0 but the 90000 x g supernatant was not buffered at all. When measured, the pH of the 90000 x g supernatant was generally found to be about pH 6.0. It appeared likely that in order to prevent the insoluble material from interfering with the binding of ACC it would be necessary to homogenise the tissue in a

solution buffered at pH 7.0 and to apply the ACC to the column in such a buffer.

The homogenisation buffer described in the previous section was therefore modified: it was made 20 mM in potassium phosphate and adjusted to pH 7.0. The other components were unchanged. In order to ensure that the ACC was applied to the DEAE cellulose in the equilibration buffer, the ACC was precipitated by 30% saturation with ammonium sulphate and the resuspended precipitate was then gel filtered on Sephadex G 25 into 10 mM potassium phosphate, pH 7.0 (buffer B). After gel filtration into the equilibration buffer, the ammonium sulphate precipitate was occasionally turbid. The turbidity was removed by centrifugation at 105000 x g for 1 h before application of the supernatant to DEAE cellulose.

The third problem encountered concerned the lrmgFAS used in the ACC assay. Several preparations of this were contaminated with ACC activity, which resulted in a fairly high background ACC activity in each assay. The levels of ACC (in terms of U/ml) were always rather low in the fractions prepared from rat liver and it was undesirable to have a background rate similar to or even greater than that being measured.

Nakanishi and Numa (1970) reported a novel method of assaying ACC by coupling the production of ADP to the oxidation of NADH with the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH). PK and LDH contain no ACC activity. Moreover, the background rate due to 'NADH oxidase' was never excessive and no background rate was detected after ion exchange chromatography. This assay was used instead of the FAS coupled assay. Table 4.4 shows the activities in various preparations which contained ACC measured either by the FAS coupled assay or by the modification of the method of Nakanishi and

Table 4.4 Comparison of assays of Manning et al (1976) and Nakanishi and Numa (1974).

Tissue	Preparation	ACC activity (U/ml) measured by:	
		method (2)	method (3)
rabbit mammary gland	pure ACC	8.1	8.0
rabbit mammary gland	pure ACC	7.7	7.4
rat liver	DEAE cellulose pool	0.054	0.06
rat liver	0-30% ammonium sulphate pellet	0.39	0.44

Samples were prepared as described in Chapter 2.2 (1mgACC), Chapter 4.1 (DEAE cellulose pool) and Chapter 4.3 (0-30% ammonium sulphate pellet) and assayed by method (2) (Manning et al, 1976) and method (3) (Nakanishi and Numa, 1974). Each value is the mean of two separate measurements.

Numa (1970) described in Chapter 2.2. It can be seen that the two methods give virtually identical results.

Overcoming the problems described in the previous section resulted in what was effectively a complete revision of the partial purification procedure described above. It should be stressed that the changes were not made simultaneously but were introduced over a period of several months. Once all of the modifications had been made the initial stages of the procedure were quite reproducible. The revised procedure is described in Chapter 4.3.

Section 3. Revised partial purification procedure for rat
liver acetylCoA carboxylase

Six rats were subjected to the fasting/refeeding regime described in Chapter 4.1 except that the fat-free rat food used was obtained from a commercial source. The rats were killed and the livers were removed and rinsed in ice cold homogenisation buffer. All subsequent procedures were carried out at 4°C. All buffers used contained 1 mM EDTA, 15 mM β -mercaptoethanol and were adjusted to pH 7.0 prior to use. Unless otherwise stated ACC activity was assayed by the modification of the PK/LDH coupled assay of Nakanishi and Numa (1970) described in Chapter 2.2.

The livers were homogenised by 3 x 15 sec bursts in an Atomix blender in 3 volumes of a buffer containing 20 mM potassium phosphate, 10 mM potassium citrate, 0.25 M sucrose, 1 mM benzamidine-HCl, and 1.2 mM PMSF. A 90000 x g supernatant was prepared and brought to 30% saturation with ammonium sulphate, maintaining the pH at 7.3 - 7.4 by the addition of 5N NH₃. After stirring for 30 min, the precipitate was collected by centrifugation at 20000 x g for 15 min and the pellet was then resuspended in buffer B to a final volume of 8 - 10 ml. The resuspended pellet was then applied to a column of Sephadex G-25M (2.2 cm x 30 cm) equilibrated in buffer B and the column was developed with this buffer. Fractions containing material absorbing at 280 nm were pooled. Occasionally the pooled fractions were turbid, resembling the DEAE cellulose run-through fractions described in Section 2 of this chapter in that the ACC activity in these fractions would not bind to DEAE cellulose. On such occasions the turbidity was removed by centrifugation at 150000 x g for 1 h. In the experiments reported below the ammonium sulphate precipitate treated in this way is referred to as 'the gel filtered 0 - 30% ammonium sulphate precipitate'.

Table 4.5 shows the amounts of ACC activity recovered in the 90000 x g supernatants and gel filtered 0 - 30% ammonium sulphate precipitate in 4 different preparations. Activity was always detectable in the 90000 x g supernatants and the amounts of activity recovered in the 0 - 30% ammonium sulphate precipitates were much less variable than previously. Thus, the modifications seemed to be effective. Recovery of ACC activity in the gel filtered 0 - 30% ammonium sulphate precipitate was always greater than 100%. This phenomenon was more marked when the 90000 x g supernatant was brought to 35% saturation with ammonium sulphate and the precipitate harvested and gel filtered as described above. (This fraction is referred to as the 'gel filtered 0 - 35% ammonium sulphate precipitate' in the following text). The recovery of ACC activity in the gel filtered 0 - 35% ammonium sulphate precipitate was in the range 200 - 300%. This high recovery was attributed to difficulties in assaying the activity in crude extracts.

Chromatography on DEAE cellulose

Preliminary experiments indicated that the ACC activity in the gel filtered ammonium sulphate precipitates would bind to a smaller amount of DEAE cellulose than had been required when the 90000 x g supernatant was applied directly to the resin. These experiments also indicated that the ACC could be eluted by washing the column with 50 mM potassium phosphate. The results described below are from the first preparation where all of the modifications to the procedure were used.

A gel filtered 0 - 30% ammonium sulphate precipitate containing 19.3 U of ACC activity was applied to a column of DEAE cellulose (1.4 cm x 2.9 cm) equilibrated in buffer B. The column was washed

Table 4.5. Amounts of acetylCoA carboxylase activity measured in 4 different preparations from rat liver.

Preparation	90000 x g supernatant (U)	0 - 30% ammonium sulphate precipitate (U)
a	2.0	4.4
b	17	19.3
c	15	15
d	9.5	14.1

90000 x g supernatants and 0 - 30% ammonium sulphate precipitates were prepared as described in the text. Six rats were used in each preparation. AcetylCoA carboxylase was assayed using the pyruvate kinase/lactate dehydrogenase coupled assay.

with this buffer until the E_{280} of the eluate was 0.05 whereupon the column was washed with 50 mM potassium phosphate, 1 mM benzamidine-HCl. Fractions were monitored for absorbance at 280 nm and ACC activity (Fig. 4.6). Fractions 18 - 21 were pooled.

Analysis of the pooled fractions on SDS-polyacrylamide gel electrophoresis (Fig. 4.7) revealed that the major protein staining band had a subunit M_r of 250000. A number of minor bands were also visible on the gel indicating that further purification was required. In a number of experiments chromatography on phosphocellulose proved to be unsatisfactory. Either all of the activity was lost during the step or no purification was achieved as judged by SDS-polyacrylamide gel electrophoresis. This was surprising since phosphocellulose chromatography had been performed successfully in earlier work. The failure of phosphocellulose chromatography was, at the time, attributed to variations in either the batch of phosphocellulose or in the pre-cycling procedure.

As indicated in Chapter 4.1 attempts to find a reproducible method for the further purification of rLACC had been unsuccessful although chromatography on Phenyl-Sepharose appeared to be potentially useful. Further studies in this area were not pursued as evidence was obtained that ACC activity could, under certain conditions, be separated from the bulk of the contaminating protein in the gel filtered 0 - 30% ammonium sulphate precipitate by chromatography on DEAE cellulose.

A gel filtered 0 - 30% ammonium sulphate precipitate (containing 14.1 U of ACC activity) was applied to a DEAE cellulose column (0.9 cm x 9.5 cm) equilibrated in buffer B. The column was then washed with Buffer B and then developed with 50 mM potassium phosphate,

Figure 4.6. Chromatography of 0 - 30% ammonium sulphate precipitate on DEAE cellulose.

The experiment was carried out as described in the test. 2 ml fractions were collected and assayed for protein (●) or ACC activity (○). The arrow indicates the fraction at which the 50 mM potassium phosphate wash was started.

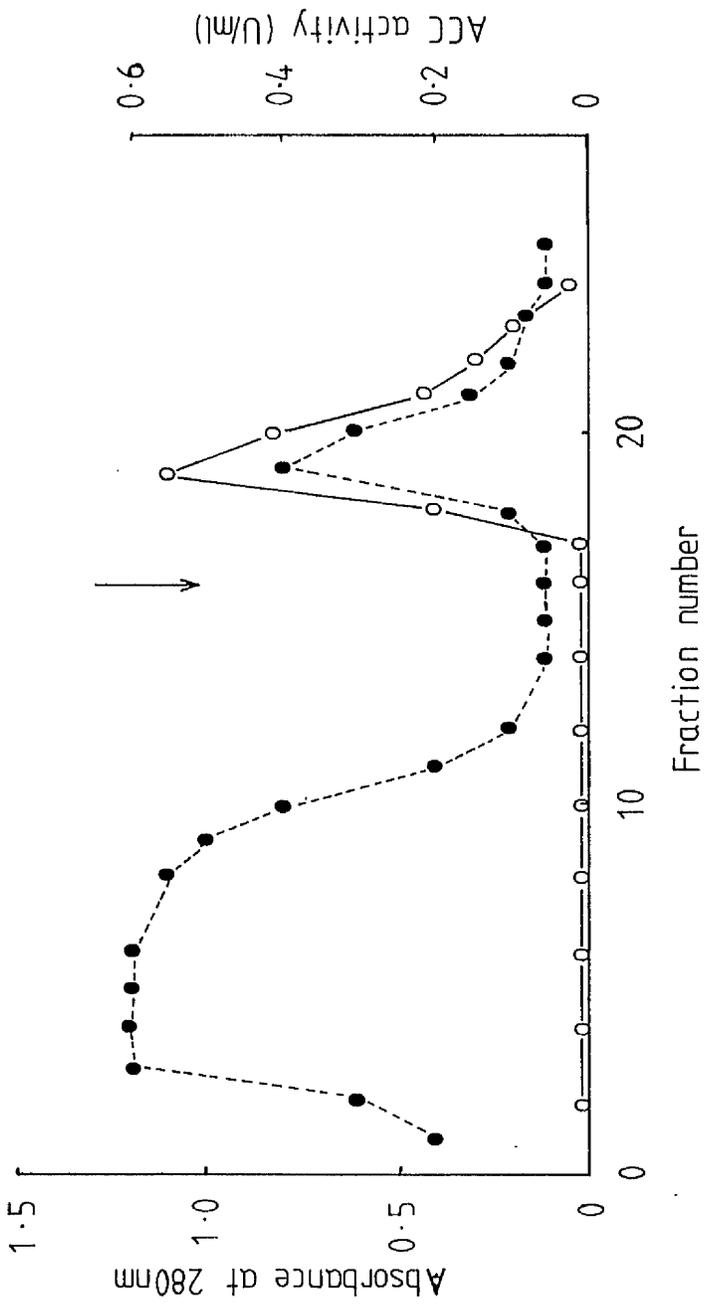


Figure 4.7. SDS-polyacrylamide gel electrophoresis of fractions derived from rat liver.

The preparation of the rat liver fractions is described in the text. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969). The samples electrophoresed were 90000 x g supernatant (40 μg) (1); 0 - 30% ammonium sulphate precipitate (70 μg) (2), gel filtered 0 - 30% ammonium sulphate precipitate (10 μg) (3); DEAE cellulose pool (5 μg , 2 μg) (4,5).



1 mM benzamidine-HCl as described above. The elution of ACC activity and material absorbing at 280 nm was monitored. The elution profile is shown in Fig. 4.8. In contrast to the result illustrated in Fig. 4.6 two protein peaks were detected and ACC activity was associated with only one of these. The specific activity of the ACC in fraction 3 (approximately 0.5 U/mg) was similar to that obtained for apparently pure rlACC prepared using either precipitation with polyethylene glycol or chromatography on Phenyl-Sepharose.

Samples from each fraction were analysed on SDS-polyacrylamide gel electrophoresis. This revealed an interesting pattern in the distribution of protein staining bands amongst the fractions which contained ACC activity (Fig. 4.9). The peak of ACC activity correlated with a polypeptide whose subunit M_r (by comparison with cross-linked GAPDH) was approximately 120000. The second protein peak correlated with a polypeptide with a subunit M_r of approximately 250000. Although the specific activity of fractions 5 and 6 was low it was assumed that this was ACC. The proportion of the 120000 M_r protein decreased from fraction 3 onwards and this band was absent from fraction 6. The decrease in the proportion of the 120000 M_r polypeptide was accompanied by an increase in the proportion of the 250000 M_r polypeptide present.

This observation, that the peak of ACC activity corresponded to a polypeptide with a subunit M_r of 120000 was interpreted as being due to the proteolysis of native ACC which is believed to have a subunit M_r of 250000. Since precautions had been taken against proteolysis during the homogenisation of the livers and in subsequent procedures, I checked that the pattern seen on the gels was not the result of proteolysis during the denaturation of the samples. In the experiment shown in Fig. 4.9 the samples had been added to the

Figure 4.8. Elution of acetylCoA carboxylase activity from
DEAE cellulose by 50 mM potassium phosphate pH 7.0.

The experiment was performed as described in the text. The
50 mM wash was applied at fraction 0. 3 ml fractions were collected
and monitored for absorbance at 280 nm (●) and ACC activity (○)

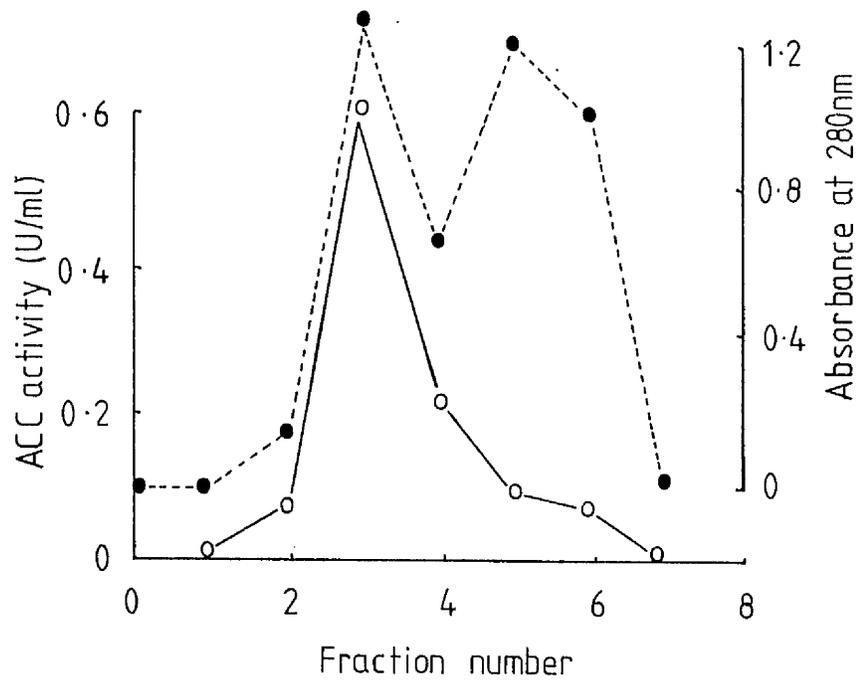
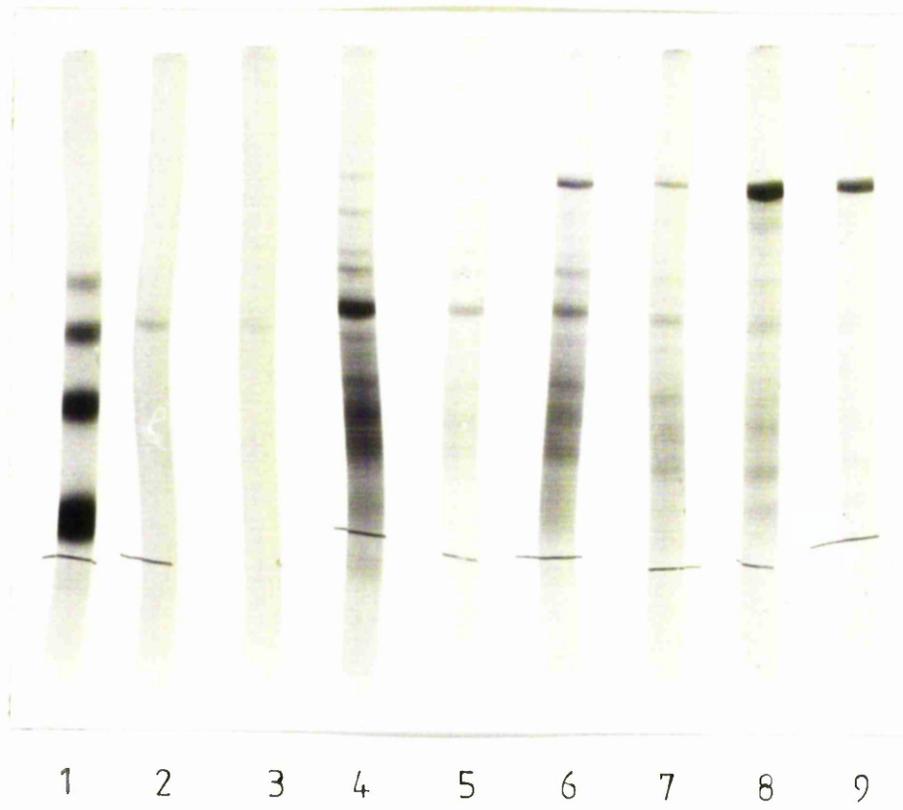


Figure 4.9. SDS-polyacrylamide gel electrophoresis of fractions containing acetylCoA carboxylase from DEAE cellulose column.

Electrophoresis was performed by the method of Weber and Osborn (1969). The samples electrophoresed were: GAPDH (cross-linked with dimethyl suberimidate, gel 1) and fractions from the DEAE cellulose column described in the legend to Fig. 4.8. A sample of each fraction was diluted 2-fold with NaPi sample buffer and boiled for 5 min. The following amounts after dilution were electrophoresed, fraction 2 (40 μ l, 15 μ l) (2,3); fraction 3 (40 μ l, 15 μ l) (4,5); fraction 4 (40 μ l, 15 μ l) (6,7); fraction 5 (30 μ l) (8); fraction 6 (30 μ l) (



sample buffer in the order 2 to 6 and only then were the samples boiled.

Adding the samples to the sample buffer in the reverse order (i.e. 6 to 2) or adding the samples to boiling sample buffer did not change the pattern observed (result not shown). Since the pattern observed did not appear to be an artefact of the method used to prepare the samples for electrophoresis it was concluded that in Fig. 4.8 proteolysed ACC was separated from native ACC on the DEAE cellulose. It was believed that the geometry of the column was important in this respect. The dimensions of the column were 0.9 x 9.5 cm, resulting in a column which was relatively long, but had a narrow diameter. It seemed possible that the observed separation was achieved by 'true chromatography'.

These results suggested that it might be possible to purify rIACC by chromatography on a long, narrow column of DEAE cellulose. For example, in the experiment described above, fraction 6 (gel number 9 in Fig. 4.9) is apparently pure. It was possible that the 250000 M_r band seen on SDS-polyacrylamide gels might have contained FAS in addition to ACC. However, since it is known that proteolysis activates ACC the low specific activity of the material was attributed to the success of the precautions taken to prevent proteolysis.

In the next section of this chapter, I report the results of experiments which were designed to achieve a greater purification of native ACC and to further characterise the 120000 M_r and 250000 M_r polypeptides.

Section 4. Characterisation of 120000 M_r and 250000 M_r polypeptides

Gradient elution of acetylCoA carboxylase from DEAE cellulose

The elution of ACC shown in Fig. 4.8 was achieved by developing the DEAE cellulose column with 50 mM potassium phosphate. The separation of the two protein peaks (which I assumed to contain proteolysed and native ACC) was not complete. Since greater resolution can generally be achieved with gradient elution, the use of this method was studied. During this work I noticed that more ACC activity was recovered in 0 - 35% saturation ammonium sulphate precipitates than in 0 - 30% precipitates and so in subsequent experiments I used 0 - 35% saturation ammonium sulphate precipitates.

A gel filtered 0 - 35% ammonium sulphate precipitate (41 U) was applied to a DEAE cellulose column (2.1 cm x 12 cm) equilibrated in buffer B. The column was washed with buffer B until the E_{280} of the eluate 0.05 and it was then developed with a linear gradient from 10 mM to 200 mM potassium phosphate (150 ml/reservoir). Fractions were monitored for ACC activity and absorbance at 280 nm (Fig. 4.10). The results show that two protein peaks were eluted from the column. Clearly, gradient elution gave much better resolution than the stepwise elution shown in Fig. 4.8. The fractions with the highest activity (13 - 17) and those with the greatest absorbance at 280 nm (20 - 27) were pooled. These are defined as DEAE cellulose peaks 1 and 2 respectively. Analysis on SDS-polyacrylamide gels showed that DEAE cellulose peak 1 contained essentially pure 120000 M_r material and that DEAE-cellulose peak 2 contained essentially pure 250000 M_r material (Fig. 4.11). The specific activity of the ACC in the DEAE cellulose peak 1 pool was 1.2U/mg, comparable with the specific activities of pure r1ACC and lrmgACC reported in the previous

Figure 4.10. Gradient elution of acetylCoA carboxylase activity from DEAE cellulose.

The experiment was performed as described in the text. 6 ml fractions were collected and monitored for absorbance at 280 nm (●) and ACC activity (○). 1 indicates DEAE cellulose peak 1, 2 indicates DEAE cellulose peak 2.

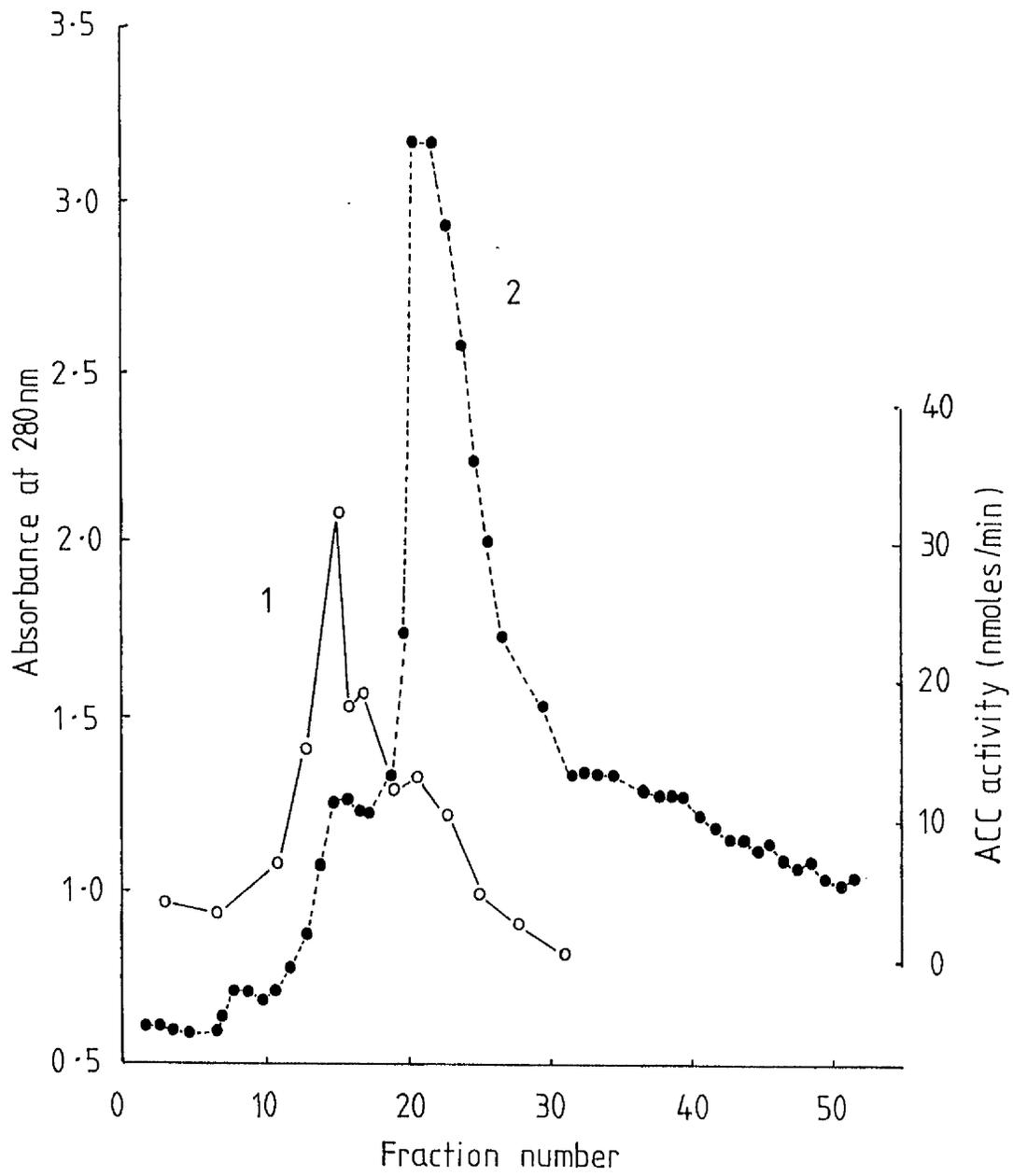
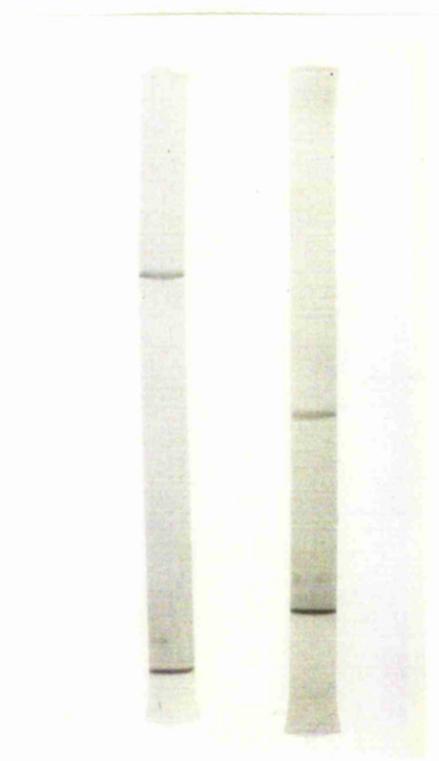


Figure 4.11. SDS-polyacrylamide gel electrophoresis of
DEAE cellulose peak 1 and 2.

Electrophoresis on 6% polyacrylamide gels in the presence of SDS was performed by the method of Laemmli (1970). DEAE cellulose peak 1 and 2 were prepared by the method described in the text. The samples analysed were : (1), DEAE cellulose peak 2 (3 μ g) and (2) DEAE cellulose peak 1 (3 μ g).



1

2

chapter. The specific activity of the ACC in the DEAE cellulose peak 2 was much lower (0.13 U/mg). Since the DEAE cellulose peak 2 pool showed only one polypeptide on SDS-polyacrylamide gel electrophoresis whose mobility corresponded to that expected for undegraded ACC (Fig. 4.11), the low specific activity implied that either the ACC was inactive or it was contaminated with FAS.

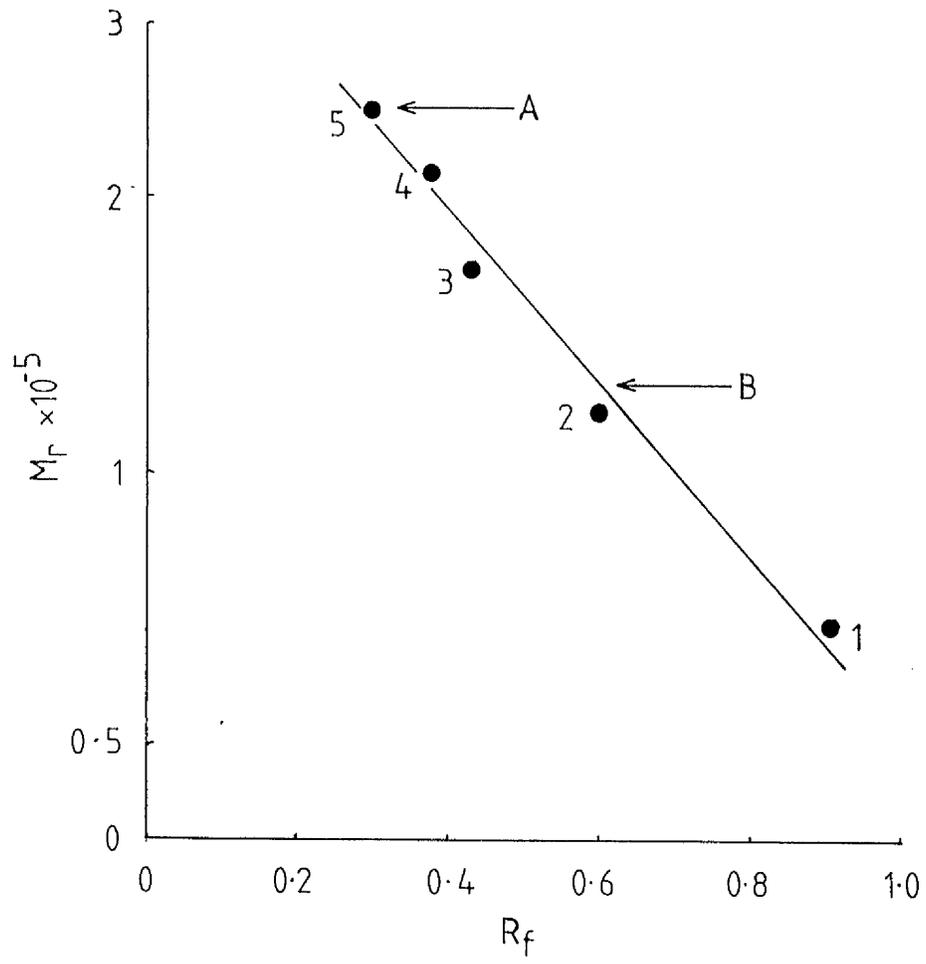
Relative molecular masses of DEAE cellulose peak 1 and 2 polypeptides

The subunit M_r of the DEAE cellulose peak 1 polypeptide has been reported as approximately 120000. This value was estimated by visual inspection of its migration on SDS-polyacrylamide gels relative to cross-linked GAPDH (subunit M_r 36000). In order to determine its M_r more accurately, proteins of known M_r were used as markers and the position of each protein band was located exactly by densitometry.

Samples were analysed on 6% polyacrylamide gels in the presence of SDS by the method of Laemmli (1970). After electrophoresis the gels were stained for protein and then scanned at 600 nm. The R_f of each protein staining band was calculated as described in Chapter 2.2 and a standard curve of $\log_{10} M_r$ versus R_f was constructed (Fig. 4.12). An M_r of 250000 was found for the DEAE cellulose peak 2 polypeptide while a value of 125000 was obtained for the DEAE cellulose peak 1 polypeptide. These results suggested that the protease believed to be responsible for the proteolysis of ACC must cleave it into two polypeptides of equal size. This pattern of proteolysis was in marked contrast to that observed by earlier workers on rLACC. These workers found that native rLACC was cleaved into polypeptides with M_r s of 124000 and 118000 which were derived from native ACC with a subunit M_r of 230000 (see Tanabe *et al.*, 1975). In this present work only one polypeptide with an M_r of 125000 was

Figure 4.12. Estimation of the relative molecular masses of DEAE cellulose peak 1 and 2 polypeptides.

SDS-polyacrylamide gel electrophoresis was carried out as described in the text. The M_r markers used were: (1), bovine serum albumin (68000); (2), β -galactosidase (118000); (3) *N. crassa* "arom complex" (165000); (4), myosin (212000); (5), lactating rabbit mammary gland FAS (250000). References to these values are given in Chapter 2.2. The arrows indicate the R_f s of the DEAE cellulose peak 1 (B) and peak 2 (A) polypeptides.



observed. The absence of the 118000 M_r polypeptide was interpreted as being due to its rapid degradation to much smaller polypeptides.

Phosphorylation of DEAE cellulose peak 1 and 2 polypeptides
by cAMP dependent protein kinase

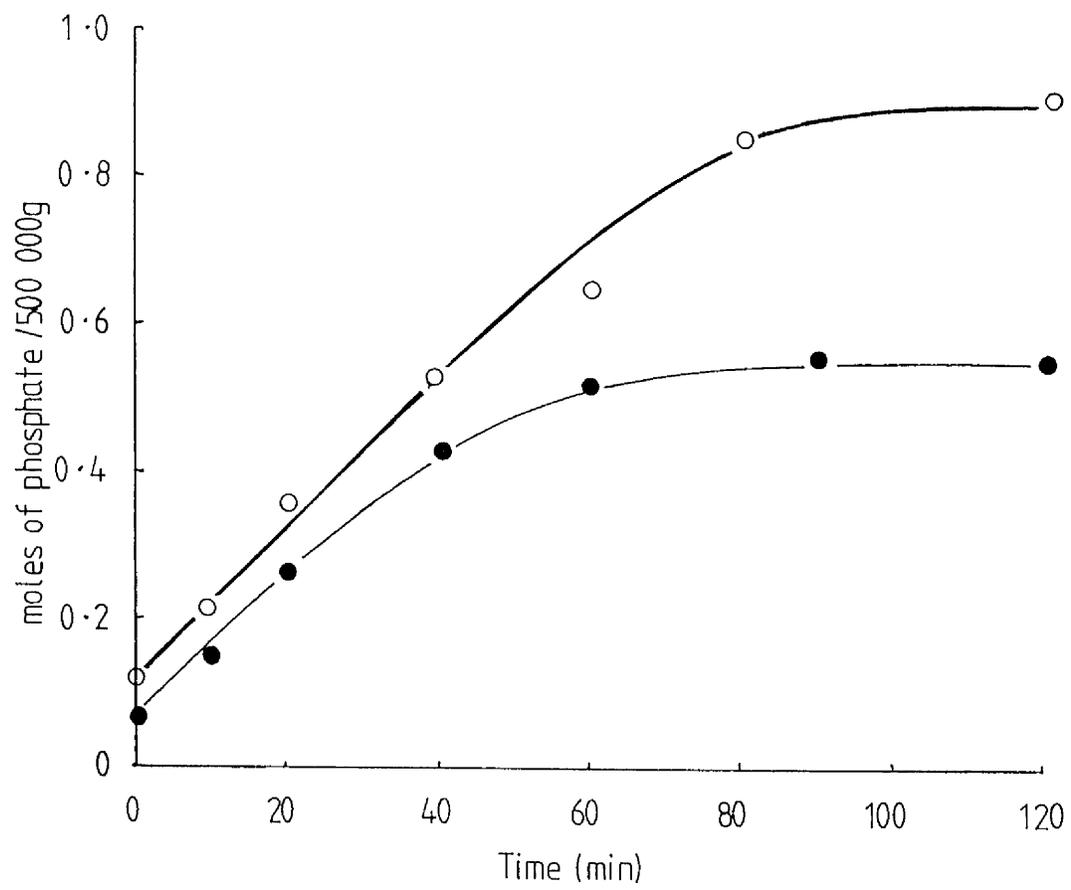
Guy and Hardie (1981) have reported that lrmgACC is sensitive to limited proteolysis involving the removal of a 20000 M_r peptide containing the cAMPdPrK phosphorylation sites. The ACC in the DEAE cellulose peak 2 had a subunit M_r of 250000 suggesting that such proteolysis had not taken place. It was of interest to determine whether the DEAE cellulose peak 2 polypeptide could be phosphorylated by cAMPdPrK : the phosphorylation of the DEAE cellulose peak 1 polypeptide was also studied.

The incorporation of phosphate into each of the polypeptides is illustrated in Fig. 4.13. Each incubation contained, in a final volume of 0.5 ml, 0.2 mM [γ - ^{32}P]-ATP (10 μ Ci/ μ mole), 20 mM NaF, 10000 U of cAMPdPrK catalytic subunits (hereafter referred to simply as catalytic subunits) and 0.25 mg peak 1 or peak 2 protein. Control incubations, without the catalytic subunits or without the protein substrate were also carried out. The amount of phosphate incorporated into protein was calculated assuming an M_r of 500000 for ACC. The maximum amounts of phosphate incorporated into the DEAE cellulose peak 1 and 2 polypeptides were 0.9 moles/500000 g and 0.54 moles/500000 g respectively. No incorporation was found in the absence of substrate indicating that the catalytic subunits did not autophosphorylate. No incorporation of phosphate into ACC was found in the absence of catalytic subunits indicating that there was no endogenous kinase in either of the two samples. Similar results were obtained in other experiments where the concentrations of catalytic subunits and ATP were varied.



Figure 4.13. Phosphorylation of DEAE cellulose peak 1 and 2 polypeptide by cAMP dependent protein kinase.

Samples from DEAE cellulose peak 1 (o) and peak 2 (•) were incubated with the components described in the text. Samples (50 μ l) were removed at the times indicated and the amount of phosphate incorporated into protein was determined as described in Chapter 2.2.



Both of these values are much lower than those reported for mammary gland ACC. 2 moles of phosphate/500000 g of ACC were incorporated into the rabbit enzyme (Hardie and Cohen, 1978a) while 4 moles of phosphate/500000 were incorporated into the rat enzyme (Hardie and Guy, 1980). It was considered unlikely that the cAMPdPrK phosphorylation sites were partially occupied, as no precautions had been taken against phosphatase activity during the purification procedure. The alkali labile phosphate contents of the DEAE cellulose peak 1 and 2 polypeptides used in this experiment were not determined but this parameter would not have provided any information about the occupancy of the cAMPdPrK sites since structured phosphate unrelated to hormone dependent protein kinases is not unknown (Nimmo and Cohen, 1978).

Samples from each of the incubations described were analysed on 6% polyacrylamide gels in the presence of SDS. After electrophoresis the gels were stained for protein and scanned at 600 nm, and radioactivity was located as described in Chapter 2.2. Fig. 4.14 indicates that all of the radioactivity was incorporated into the 125000 M_r polypeptide (DEAE cellulose peak 1 incubation) or the 250000 M_r polypeptide (DEAE cellulose peak 2 incubation). This result showed that in both cases, the phosphate was all incorporated into the expected band and not into some highly phosphorylatable protein present in only trace amounts.

Samples from each incubation were also analysed on non-denaturing 3% polyacrylamide gels. After electrophoresis the gels were stained for protein and then scanned at 600 nm. Radioactivity was located as described in Chapter 2.2. Only one protein-staining band was visible after electrophoresis of the DEAE cellulose peak 1 polypeptide, confirming that this preparation was pure. In contrast two bands of approximately equal intensities were visible after

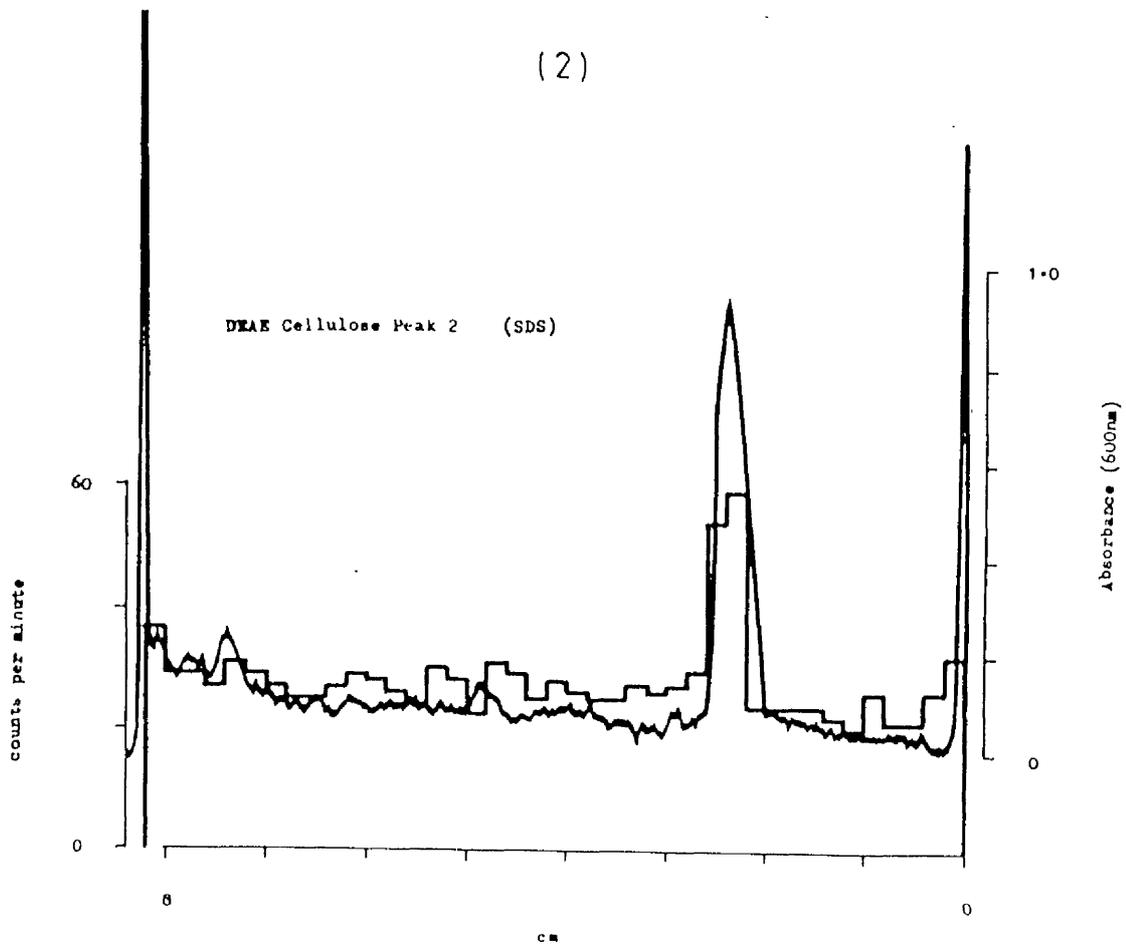
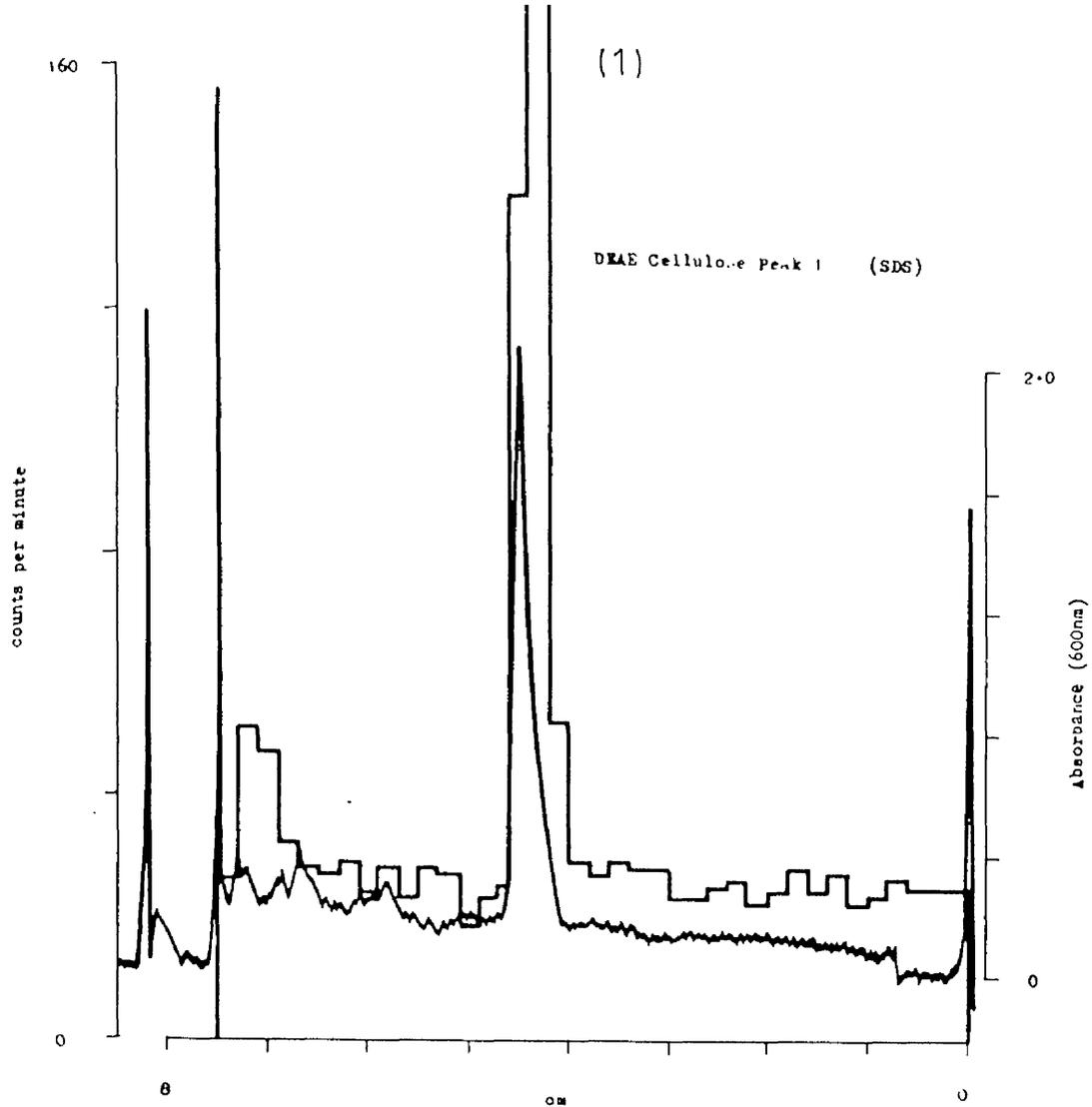
Figure 4.14 Location of ^{32}P after SDS-polyacrylamide gel electrophoresis of DEAE cellulose peak 1 and 2 polypeptides phosphorylated with cAMP dependent protein kinase.

The experiment was performed as described in the text.

(1) Electrophoresis of DEAE cellulose peak 1 polypeptide.

(2) Electrophoresis of DEAE cellulose peak 2 polypeptide.

Each gel was scanned at 600 nm (continuous line) and then sliced and the radioactivity in each slice measured (crenellated line).



electrophoresis of the DEAE cellulose peak 2 sample (Fig. 4.15). Comparison of gels 1 and 2 (Fig. 4.15) shows that one of the two bands in the DEAE cellulose peak 2 sample migrated to the same position as lrmgFAS.

Fig. 4.16 shows the location of the ^{32}P after electrophoresis of the phosphorylated DEAE cellulose peaks 1 and 2 polypeptides on the non-denaturing gels shown in Fig. 4.15. As was to be expected for the peak 1 sample all of the radioactivity was located in the single protein-staining band. In contrast, neither of the two protein staining bands visible after electrophoresis of the DEAE cellulose peak 2 sample were associated with significant amounts of radioactivity. Surprisingly, most of the radioactivity on that gel was located in the first gel slice suggesting that the protein to which the radioactivity was bound had not penetrated into the gel. This was reminiscent of the behaviour of lrmgACC purified using polyethylene glycol on non-denaturing polyacrylamide gel electrophoresis. It was, therefore, possible that the ^{32}P that did not penetrate the gel was associated with ACC. Either (or both) of the bands that did penetrate into the gel could be some sort of 'non-phosphorylatable' ACC. In an attempt to determine whether either of these two proteins was ACC I attempted to stain replicate gels for ACC activity but was unsuccessful. It seemed likely that the upper of the two bands was FAS. The DEAE cellulose peak 2 contained no FAS activity but since the material had been exposed at an early stage of the purification procedure to PMSF, an irreversible inhibitor of the thioesterase component of FAS, the result was not conclusive. It was, therefore, necessary to carry out the purification in the absence of PMSF so that the hypothesis could be tested.

A gel filtered 0-35% ammonium sulphate precipitate was prepared

Figure 4.15. Polyacrylamide gel electrophoresis of DEAE
cellulose peaks 1 and 2.

Electrophoresis on 3% non-denaturing polyacrylamide gels was performed by the method of Davis (1964). DEAE cellulose peaks 1 and 2 were prepared as described in the text. The samples electrophoresed were: (1), lrmgFAS (5 μ g); (2), DEAE cellulose peak 2 (7 μ g); DEAE cellulose peak 1 (5 μ g).



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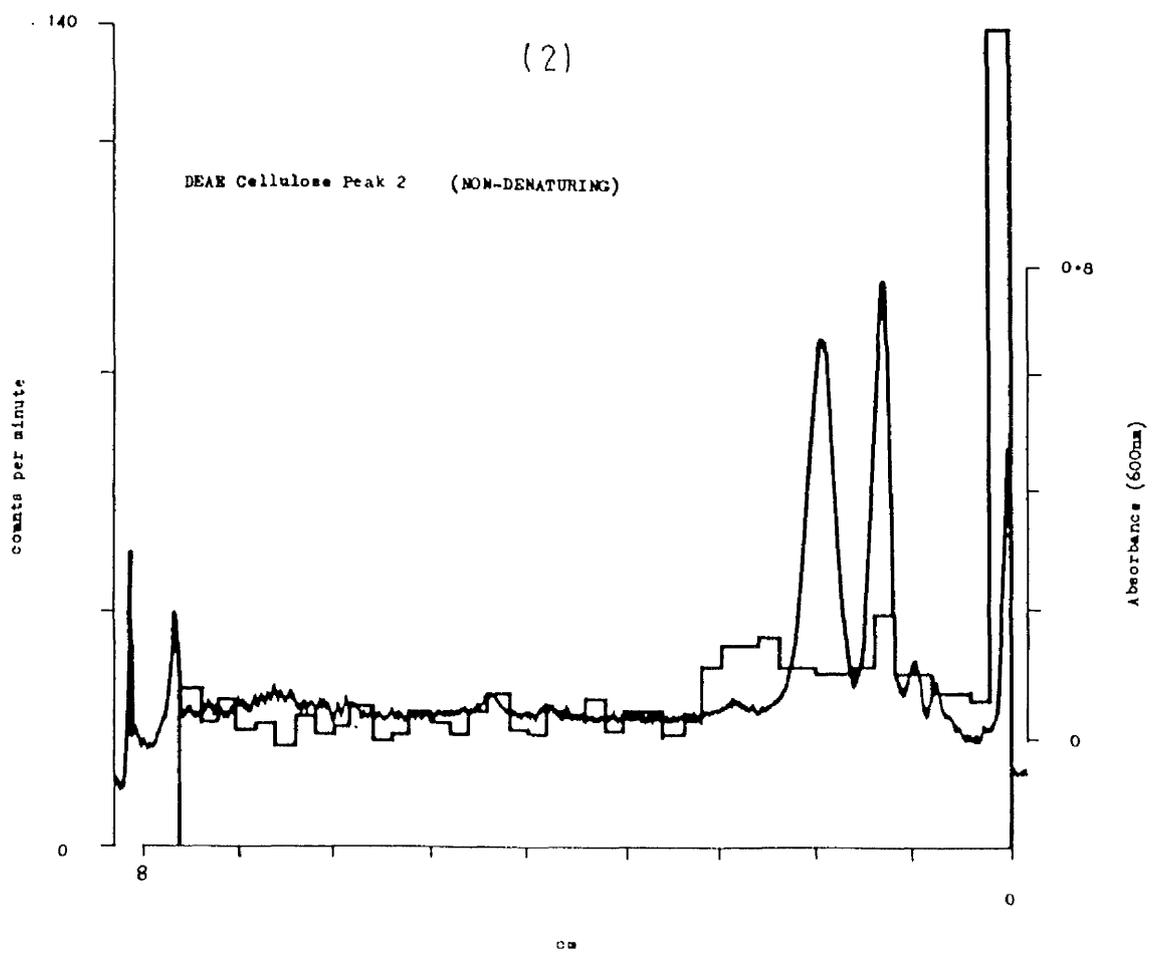
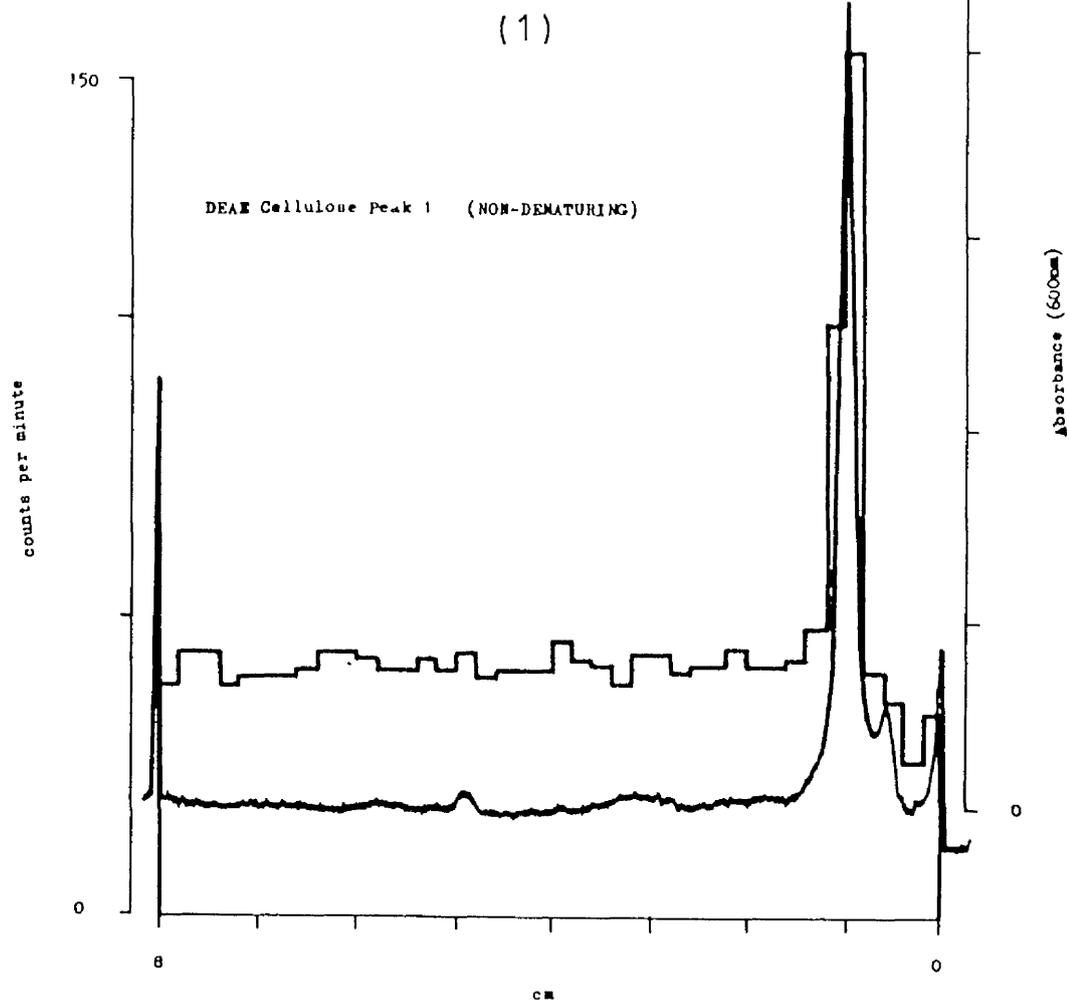
Figure 4.16. Location of ^{32}P after non-denaturing polyacrylamide gel electrophoresis of DEAE cellulose peak 1 and 2 polypeptides phosphorylated with cAMP dependent protein kinase.

The experiment was performed as described in the text.

(1), Electrophoresis of DEAE cellulose peak 1 polypeptide.

(2), Electrophoresis of DEAE cellulose peak 2 polypeptide.

Each gel was scanned at 600 nm (continuous line) and then sliced and the radioactivity in each slice measured (crenellated line).



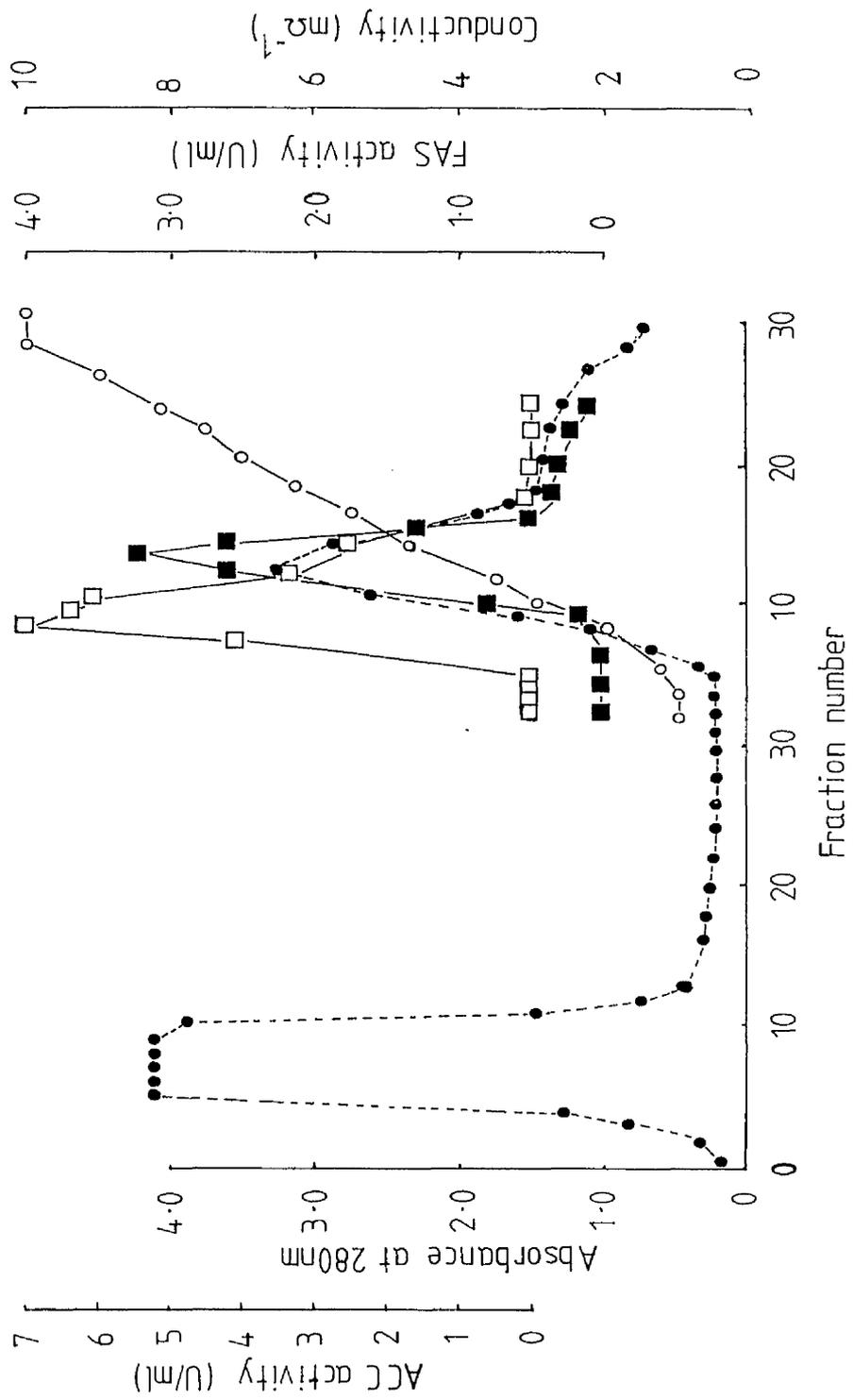
as described in section 3 except that PMSF was omitted from the homogenisation buffer. Chromatography of the gel filtered 0-35% ammonium sulphate precipitate on a column of DEAE cellulose (1.5 cm x 12.6 cm) was carried out as described in section 4. Fractions were assayed for FAS and ACC. The elution of protein and enzyme activity is illustrated in Fig. 4.17. On this occasion only one protein peak was observed. A peak of FAS activity coincided with this and a peak of ACC activity emerged before this. A 'shoulder' of ACC activity seemed to coincide with the main protein peak which probably corresponded to the DEAE cellulose peak 2 defined above. The specific activity of the FAS (approximately 1 U/mg) suggested that it was fairly pure (Stoops et al, 1979).

This result supported the hypothesis made above, that the upper band in gel 2 in Fig. 4.15 was FAS. The identity of the lower band in gel 2 is not known. In view of the subunit M_r of this protein it is possible that it was ACC. Alternatively it is possible that the ACC in peak 2 did not penetrate into the gel. This would agree with the low specific activity of the ACC in the DEAE cellulose peak 2 (0.13 U/mg) which would suggest that the ACC was approximately 10% pure. If this is so it would imply that the ACC was extensively phosphorylated, of the order of 5 moles/500000 g of ACC. I therefore attempted to reduce the proteolysis of ACC and to find a method which would separate the ACC in DEAE cellulose peak 2 from FAS.

In the purification procedure described above all of the buffers used contained benzamidine-HCl. In an attempt to reduce the proteolysis of ACC I increased the number of protease inhibitors in all buffers. The protease inhibitors used were: benzamidine-HCl (1 mM), Lima Bean Trypsin Inhibitor (2 μ g/ml), leupeptin, antipain and aprotinin (all at 0.2 μ g/ml). PMSF was not included in the homogen-

Figure 4.17. DEAE cellulose chromatography of 0 - 35% ammonium sulphate precipitate prepared in the absence of phenylmethanesulphonyl fluoride.

The experiment was performed as described in the text. Fractions (7 ml) were monitored for: ACC activity (□), absorbance at 280 nm (●), conductivity (○) and FAS activity (■). Note that only one peak of absorbance at 280 nm was observed on this occasion.



isation buffer as it inactivates FAS: in any case a comparison of Figs. 4.10 and 4.17 revealed that PMSF did not prevent the proteolysis. In addition a more gentle method of homogenising the livers (compared with the use of an Atomix blender) was used in the hope that this would minimise the rupture of lysosomes. Homogenisation of the livers was therefore carried out using a glass homogeniser with a Teflon pestle.

Neither of these two modifications to the procedure, either singly or together, increased the amount of undergraded ACC in DEAE cellulose peak 2 as judged by the relative activities in peaks 1 and 2.

Attempts to separate fatty acid synthase and acetylCoA carboxylase on Utrogel A2.

The most obvious way to separate ACC from FAS was by gel filtration in the presence of citrate where the polymeric form of ACC ($M_r = 10^7$) ought to be well separated from FAS ($M_r = 0.5 \times 10^6$). As described in Chapter 4.1, in my hands gel filtration of ACC in the presence of citrate led to irreversible inactivation. The reason for this inactivation was never determined. It was noted, however, that both the medium, Sepharose CL - 6B, and the dimensions of the column used (2.4 cm x 90 cm) necessitated the use of rather slow flow rates. The chromatography took at least 16 h. I felt that if this time could be reduced ACC activity might be preserved. I therefore used a gel filtration medium which could be used at a higher pressure than Sepharose CL - 6B.

0.5 ml of a 0 - 35% ammonium sulphate precipitate which had been redissolved to a final volume of 5.2 ml in buffer B was applied to a column of Ultrogel A2 (1.2 cm x 30 cm) equilibrated in buffer B. The column was developed with buffer B at a flow rate of 8.4 ml/h. The column was calibrated with proteins of known M_r . Due to the

fractionation range of Ultrogel A2 it was not possible to determine the void volume of the column. This would have required a substance with an $M_r = 2.5 \times 10^8$; such a substance was not available. It was, therefore, not possible to construct the usual calibration curve for a gel filtration column where V_e/V_o is plotted against $\log_{10} M_r$ of the standard proteins.

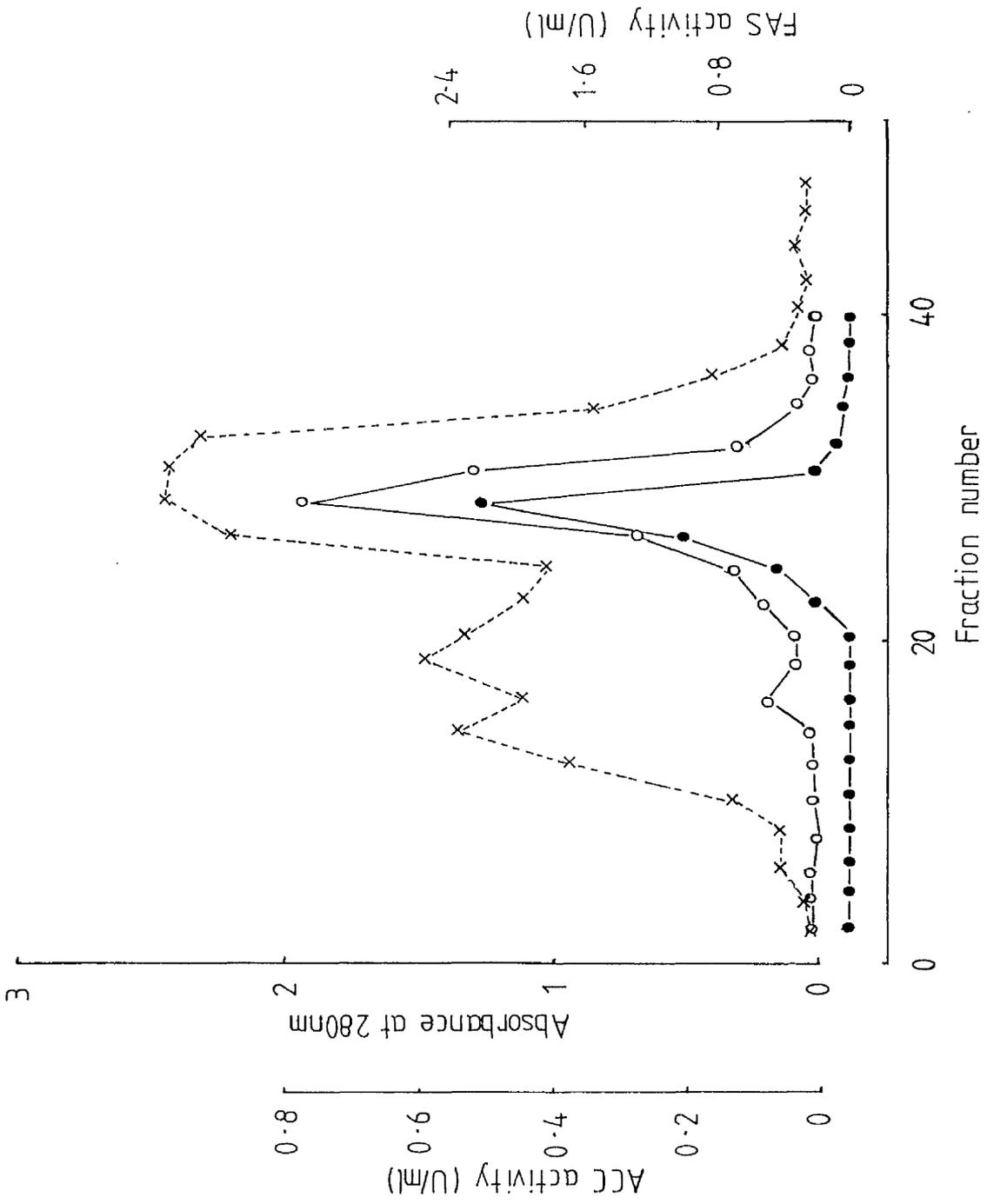
At least 90% of the ACC activity co-eluted with FAS (Fig. 4.18) indicating an M_r of 500000. Only a small amount of the ACC activity (< 5%) eluted at a volume corresponding to an M_r of several million. Analysis of the peak ACC and FAS-containing fractions (27 - 30) on SDS - polyacrylamide gels indicated that the major polypeptides present had M_r s of 125000 and 250000. After chromatography of the fractions containing ACC and FAS (24 - 32) on DEAE - cellulose (0.9 cm x 10 cm) the ACC activity was detected in peak 1. Gel filtration of a sample of the 90000 x g supernatant gave a similar elution profile to that shown in Fig. 4.18 in that ACC and FAS activity co-eluted (not shown).

The results of the gel filtration experiments were unexpected. It seemed unlikely that proteolysis would prevent the polymerisation of ACC in the presence of citrate. Limited proteolysis of large proteins such as ACC is thought to occur at exposed regions of the polypeptide chain which link the larger structural domains. Such cleavage does not usually result in drastic changes in the tertiary structure of the protein. Indeed, in the case of ACC, the early work on the physicochemical properties of the enzyme which demonstrated the polymerisation of citrate, was carried out using preparations of ACC which were later shown to be proteolysed.

I then reconsidered my initial interpretation that the DEAE cellulose peak 1 polypeptide was proteolysed ACC. The gel filtration

Figure 4.18. Gel filtration of 0 - 35% ammonium sulphate precipitate on Ultrogel A2.

The experiment was performed as described in the text. 1 ml fractions were collected and monitored for absorbance at 280 nm (x), ACC activity (●), and FAS activity (○).



results suggested that the peak 1 polypeptide was not, in fact, ACC.

The correct identification of this protein is described in the following section.

Section 5. Identification of the DEAE cellulose peak 1 protein

The results in the previous section implied that the ACC activity detected in DEAE cellulose peak 1 was an artefact. In considering the identity of the DEAE cellulose peak 1 polypeptide the following points were considered:

- 1) The protein produced oxidation of NADH in the presence of the components required for the PK/LDH coupled assay of ACC.
- 2) The protein had a subunit M_r of 125000 and a native M_r of 500000.
- 3) The enzyme was a substrate for cAMPdPrK.
- 4) The enzyme was induced in parallel to FAS in rat liver by starvation followed by refeeding with a fat-free diet, implying that the enzyme was lipogenic.

All of these properties are possessed by the enzyme ATPCL. Assay of a sample of the DEAE cellulose peak 1 (containing 0.28 U/ml of ACC activity) by the method of Linn and Srere (1979) revealed 0.54 U/ml of ATPCL activity. The specific activity of the ATPCL was 1.6 U/mg (at 37°C). Given that some authors have reported specific activities of 2 U/mg or less for homogeneous ATPCL and that DEAE cellulose peak 1 contained only one band which was of the correct M_r it seemed likely that DEAE cellulose peak 1 was substantially pure ATPCL.

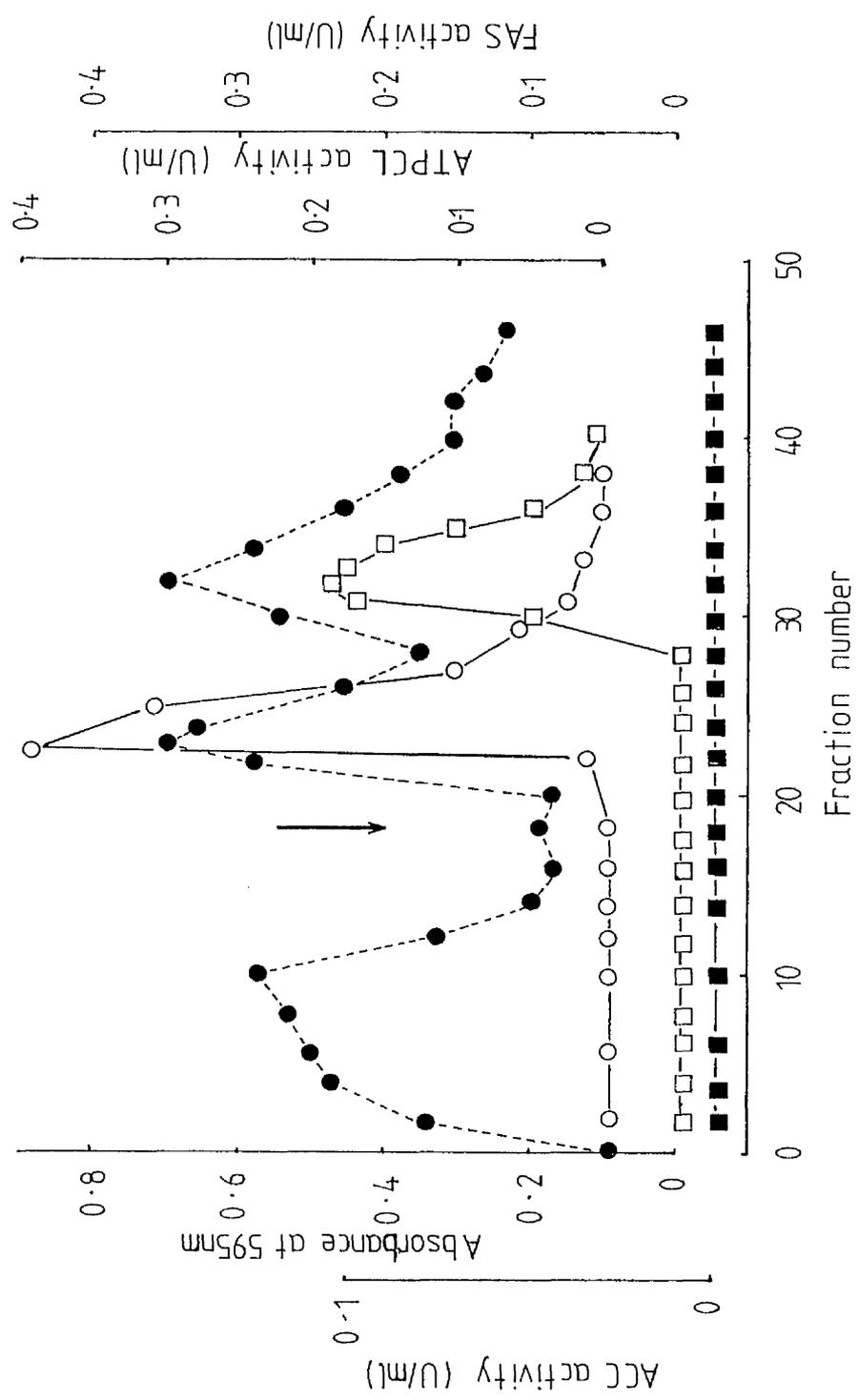
This result implied that in the work reported in the preceding sections of this chapter I had been selecting for ATPCL rather than ACC. It was not difficult to discover the reason for this. ADP is a product of the reactions catalysed by both ATPCL and ACC. Both enzymes can, therefore, be assayed using a PK/LDH coupled system provided that in each case all of the substrates are present. Two

of the substrates of ATPCL, ATP and citrate were components of the ACC assay. It only required one of the components to be contaminated with CoA to convert an assay specific for ACC into one which would detect both ACC and ATPCL. Assay of the acetylCoA used to assay ACC revealed that it was contaminated with 40% CoA.

In theory, the 'ACC' activities previously quoted in this chapter are the sum of genuine ACC activity present plus the ATPCL activity measured under the (non-optimal) assay conditions. One would expect, however, to have seen a second pronounced peak of (genuine) ACC activity eluted from the DEAE-cellulose in peak 2. On no occasion was such a second activity peak obvious. This suggests that some factor prevented the detection of genuine ACC activity in the fractions eluted from DEAE cellulose. Confirmation of this was obtained from an experiment where a gel filtered 0 - 35% ammonium sulphate precipitate was chromatographed on DEAE cellulose as described above and fractions were assayed for ACC (by the method of Manning et al, 1976), FAS and ATPCL. The elution of these enzymes from the column is illustrated in Fig. 4.19. This shows clearly that no ACC activity was detected, that ATPCL correlated with the first peak of protein and that FAS activity correlated with the second peak of protein. It seems likely that the 'ACC' activity in the DEAE cellulose peak 1, as studied in this chapter, was always due to ATPCL. Assay of the 90000 x g supernatant and 0 - 35% ammonium sulphate precipitate in this experiment indicated that no ACC activity was present in these fractions. The absence of ACC activity was observed on other subsequent occasions and strongly suggests that all of the ACC activity measured with the PK/LDH coupled assay was attributable to ATPCL.

Figure 4.19. Elution of ATP citrate lyase, fatty acid synthase and acetylCoA carboxylase from DEAE cellulose.

The experiment was performed as described in the text using a DEAE cellulose column (1.5 cm x 16 cm). The column was developed with a gradient from 10 to 200 mM potassium phosphate, pH 7.0 (150 ml/reservoir). 7 ml fractions were collected and assayed for protein (●), ATPCL activity (○), FAS activity (□) and ACC activity (■). The arrow indicates the point at which the gradient was started.



Inhibition of acetylCoA carboxylase by extracts from rat liver

The reason why no ACC activity was detected in rat liver fractions was discovered fortuitously when rlACC was assayed with FAS contaminated with lrmgACC. This meant that each assay contained some endogenous ACC activity. In the experiment shown in Figure 4.20, a gel filtered 0 - 35% ammonium sulphate precipitate was chromatographed on DEAE cellulose and assayed for ATPCL and ACC. The endogenous ACC activity corresponded to 7 nmoles NADPH oxidised/min/assay. The data in Figure 4.20 show the total ACC activity detected for each fraction. The values have not been corrected for the endogenous ACC activity in the assay. It is evident that there was no positive peak of ACC activity but rather that some component that inhibits ACC activity was eluted from the column in peak 2. It seems likely that this inhibitor was responsible for the absence of activity, or low activity, noted in the 90000 x g supernatant throughout this work. As reported in Section 3 ACC activity had been detected in 0 - 30% ammonium sulphate precipitates though not, in the work reported in this section, in 0 - 35% ammonium sulphate precipitates. This observation suggested that it might be possible to separate ACC from the inhibitor using ammonium sulphate fractionation.

This was attempted using the liver from one rat. A 90000 x g supernatant was prepared and brought to 30% saturation with ammonium sulphate. After stirring for 30 min the precipitate was removed by centrifugation. The resultant supernatant was brought to 40% saturation with ammonium sulphate, stirred for 30 min and the precipitate was collected by centrifugation. Both precipitates were resuspended in buffer B to final volumes of 1.25 ml. Samples of the 0 - 30% precipitate were then assayed in the presence of various amounts of the 30 - 40% precipitate (Table 4.6). This shows clearly

Figure 4.20. Inhibition of acetylCoA carboxylase activity by fractions from DEAE cellulose peak 2.

Chromatography on DEAE cellulose was carried out as described in the text using a column 1.5 cm x 16 cm. The column was developed using a linear gradient from 10 - 200 mM potassium phosphate (150 ml/reservoir). 10 ml fractions were collected and monitored for protein (●), ATPCL activity (○) and ACC activity (×). The endogenous ACC activity in each assay was 7 pmoles NADPH/min as indicated. 10 μl samples were assayed for ACC.

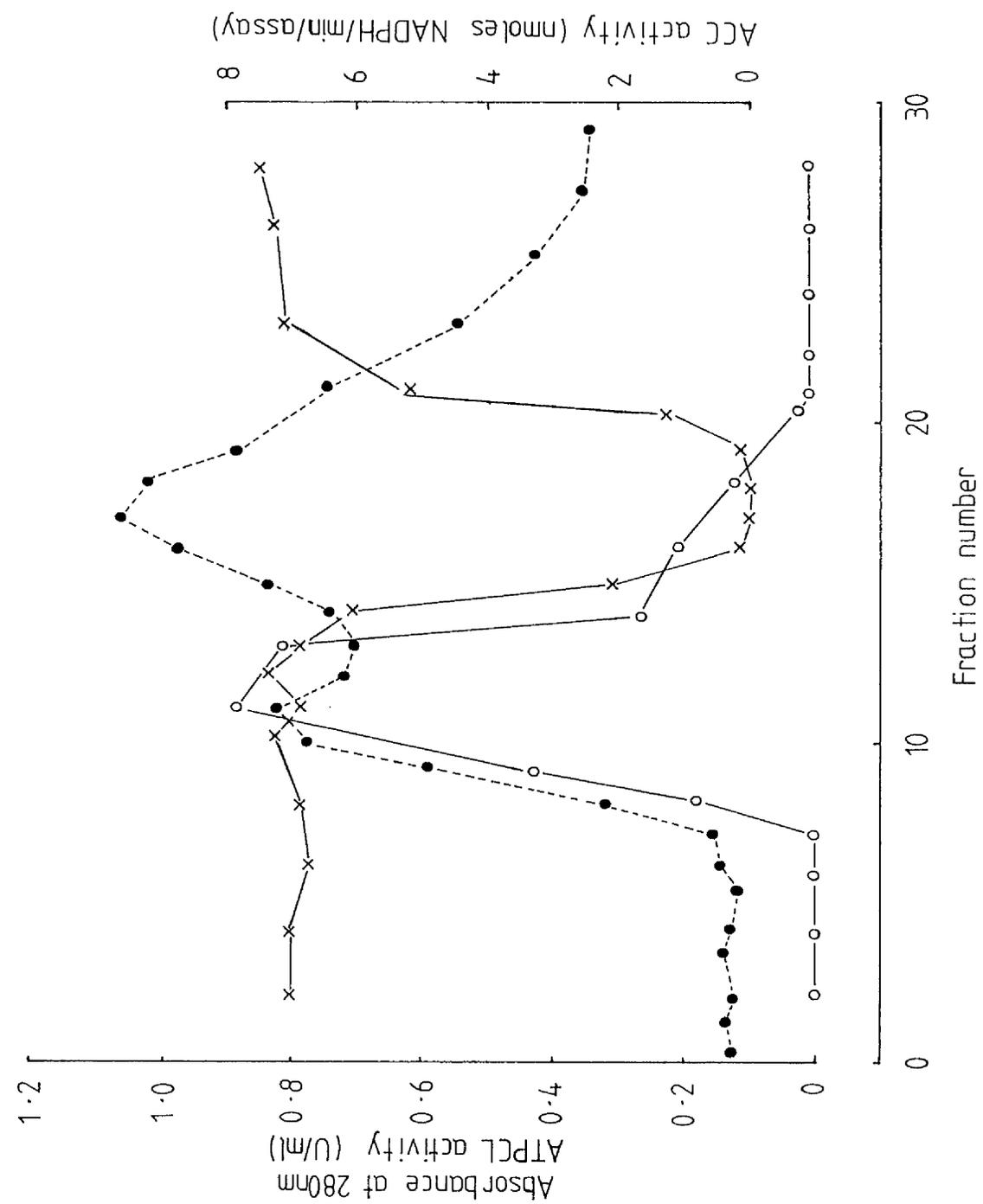


Table 4.6. Inhibition of rat liver acetylCoA carboxylase by
30 - 40% ammonium sulphate precipitate.

Components in assay	ACC activity (nmoles/min)
5 μ l lrmgFAS	7.5
5 μ l lrmgFAS + 10 μ l 0 - 30% ASP	82
5 μ l lrmgFAS + 10 μ l 0-30 ASP + 10 μ l 30 - 40 % ASP	27
5 μ l lrmgFAS + 10 μ l 0-30 ASP + 20 μ l 30 - 40 % ASP	5.6
5 μ l lrmgFAS + 10 μ l 0-30 ASP + 30 μ l 30 - 40 % ASP	0

Acetyl CoA carboxylase was assayed by the method of Manning et al (1976) using 5 μ l of lrmgFAS as coupling enzyme. The lrmgFAS was contaminated with ACC (activity 7.5 n moles/min/assay). ACC activity was assayed in the presence of the coupling enzyme only or the coupling enzyme plus the amounts of the other components indicated. 0 - 30% ASP and 30 - 40% ASP refer to the 0 - 30% and 30 - 40% ammonium sulphate precipitates respectively.

that the 30 - 40% precipitate contains some factor that inhibits or inactivates the ACC present in the 0 - 30% precipitate. The conclusion drawn from this result was that the inhibitor could be separated from ACC by ammonium sulphate fractionation. It was not possible to determine whether all of the ACC was precipitated at 30% saturated ammonium sulphate.

Discussion

In the work described in this chapter little progress was made in the purification of rlACC. This lack of progress was due to two factors; the lack of specificity of the assay method used and the presence of an inhibitor of ACC in the rat liver. The initial checks on the validity of the PK/LDH coupled assay were made to compare it with the FAS coupled assay. They were not designed to test its specificity. The preparations used in the initial checks did not contain any ATPCL activity. Had this not been so the discrepancy between the activities detected by the two methods would have been noted and studied further. The PK/LDH coupled method clearly detects both enzyme activities. Since ACC co-elutes from DEAE cellulose with FAS there should have been two 'ACC' activity peaks on the elution profile of the DEAE cellulose column (eg. Fig. 4.17) corresponding to ATPCL (DEAE cellulose peak 1) and genuine ACC (corresponding to DEAE cellulose peak 2). Due to the presence of the inhibitor and the non-specificity of the assay, the only 'ACC activity' detected was that generated spuriously by ATPCL. The difference between the M_r of the polypeptide associated with this activity and that expected for ACC was explained as being due to the proteolysis of ACC. At the time, this seemed a reasonable interpretation. One of the flaws in this part of the work was not that the interpretation of proteolysis was made but that no other possible interpretation was considered. This meant that time and effort were wasted in characterisation of the DEAE cellulose peak 1 polypeptide and in the rather futile task of trying to reduce the amount of it present by taking further precautions against proteolysis.

No attempts were made to characterise the ACC inhibitor. It seems likely, however, that it is identical to the ACC inhibitor

reported by Porter and co-workers (Abdel-Halim and Porter, 1980; Shiao et al, 1981). These workers initially reported an inhibitor of ACC with similar properties to that reported in the present work in that it could be separated from ACC by ammonium sulphate fractionation. In their hands, ACC was precipitated at 30% saturation with ammonium sulphate while the inhibitor was precipitated between 30 and 40% saturation with ammonium sulphate (Abdel-Halim and Porter, 1980). In a subsequent report (Shiao et al, 1981) the inhibitor was identified as a cAMPiPrK and its further purification was reported. Since the kinase only appears to require MgATP in order to inactivate ACC and MgATP is a substrate for ACC, it is clearly difficult or impossible to detect ACC activity until the ACC has been separated from the kinase. This almost certainly is an explanation for the low amounts of (genuine) ACC activity detected in 90000 x g supernatants throughout this work. It might also explain the variability in the amounts of ACC detected. The measurable ACC activity would depend not only on the amount of ACC present but also on the degree of inhibition provided by the inhibitor.

At this stage I decided to stop the work on rIACC for 2 reasons. Firstly, I had made little progress towards the purification of undegraded ACC; in particular I had not managed to separate ACC from FAS or from the inhibitory kinase. Secondly, I had, albeit fortuitously, devised a purification procedure for ATPCL. Since this enzyme can also be phosphorylated I decided to concentrate on a study of the effects of phosphorylation by cAMPiPrK on the kinetic parameters of ATPCL.

Chapter 5

Physical and kinetic characterization
of purified rat liver ATP citrate lyase.

Introduction

In Chapter 4 I described a procedure whereby apparently homogeneous ATPCL could be isolated from rat liver using ammonium sulphate fractionation and chromatography on DEAE cellulose. This procedure had not been designed specifically for the isolation of ATPCL and so the first two stages were modified to maximise the yield of ATPCL. As a result the ATPCL which was eluted from the DEAE cellulose was not pure by the criterion of SDS-polyacrylamide gel electrophoresis. This necessitated the use of further purification steps. In Section 1 I report the purification procedure together with the results of experiments on the physical properties of the purified enzyme. The second section is devoted to a study of the kinetic properties of ATPCL.

Section 1 Purification and physical characterisation of
rat liver ATP citrate lyase

Purification

Six rats were starved for 24 h and then fed a fat-free diet for 48 h. They were then killed by cervical dislocation. The livers were removed and rinsed in ice-cold homogenisation buffer and they were then blotted dry and weighed. All subsequent procedures were carried out at 4°C. Unless otherwise stated all buffers contained 1 mM EDTA, 15 mM β -mercaptoethanol, 2 mM benzamidine-HCl, 200 μ g/ml Lima Bean Trypsin Inhibitor, 20 μ g/ml Antipain, Leupeptin and Aprotinin.

The livers were homogenised for 3 x 15 sec in an Atomix blender in 3 volumes of 20 mM potassium phosphate, 0.25 M sucrose, 10 mM potassium citrate, pH 7.0. The homogenate was centrifuged for 10 min at 20000 x g. The supernatant was filtered through glass wool to remove fat particles and then centrifuged for 2 h at 105000 x g. The supernatant was retained and filtered through glass wool.

The 105000 x g supernatant was brought to 30% saturation with ammonium sulphate (175 g/l) and stirred for 30 min. The pH was maintained at 7.3 - 7.4 by the cautious addition of 5 N NH₃. The precipitate was removed by centrifugation at 20000 x g for 15 min. The supernatant was brought to 40% saturation with ammonium sulphate (61 g/l) and stirred for 30 min. The precipitate was collected by centrifugation at 20000 x g for 15 min. The supernatant was discarded and the precipitate was redissolved in a minimal volume of 10 mM potassium phosphate, pH 7.0 (buffer B). The redissolved 40% ammonium sulphate precipitate was applied to a column of Sephadex G-25

(2.2 cm x 30 cm) equilibrated in buffer B. The column was developed with buffer B and fractions which contained protein and had a conductivity of less than 0.9 mmho were pooled.

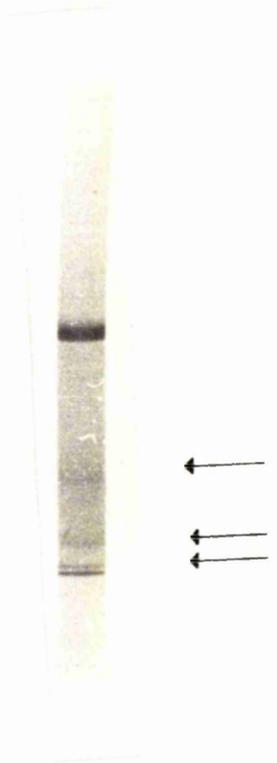
The pooled fractions from the Sephadex G-25 column were applied to a column of DEAE cellulose (2.2 cm x 16 cm) equilibrated in buffer B. The column was washed with buffer B until the E_{280} of the eluate was less than 0.05. The column was then developed with a linear gradient from 10 - 150 mM potassium phosphate, pH 7.0 (150 ml/reservoir). Fractions were assayed for ATPCL and FAS activities and monitored for absorbance at 280 nm. Those fractions which contained ATPCL activity but not FAS activity were pooled.

The pooled fractions from the DEAE cellulose column were applied to a column of Affigel Blue (1.7 cm x 10.0 cm) equilibrated in 150 mM potassium phosphate, pH 7.0 (buffer C). The column was washed with buffer C until the E_{280} of the eluate was less than 0.05. ATPCL activity was eluted by washing the column with 250 ml of buffer C containing 0.25 mM CoA and 5 mM ATP. Preliminary experiments had indicated that both substances together gave better elution than either alone. The ATPCL was then concentrated by vacuum dialysis to between 2 and 5 ml.

Analysis of pooled fractions from the Affigel Blue column by SDS-polyacrylamide gel electrophoresis generally revealed the presence of low M_r contaminants. (Fig. 1.)⁵ These minor contaminants were removed by gel permeation chromatography. The pooled fractions were then applied to a column of Ultrogel Aca 44 (1.2 cm x 50 cm) equilibrated in 100 mM potassium phosphate, 10 mM potassium citrate, pH 7.0 and the column was developed with this buffer. Fractions were analysed on SDS-polyacrylamide gels and those which contained pure ATPCL were pooled and

Figure 5.1. Analysis of Affigel Blue by SDS-polyacrylamide gel electrophoresis.

The Affigel Blue pool was prepared as described in the text and 6 μ g was analysed on a 6% polyacrylamide gel by the method of Laemmli (1970). The low M_r contaminants are indicated by arrows.



dialysed against 2 x 21 of 100 mM TEA-HCl, 0.4 mM DTT, 1 mM benzamidine-HCl, pH 7.8 (buffer D) (β -mercaptoethanol and the other protease inhibitors used throughout the procedure were omitted from buffer D). The ATPCL was finally dialysed into 500 ml of buffer D containing 40% (v/v) glycerol. The purified ATPCL was stored at -20°C in 0.5 ml aliquots.

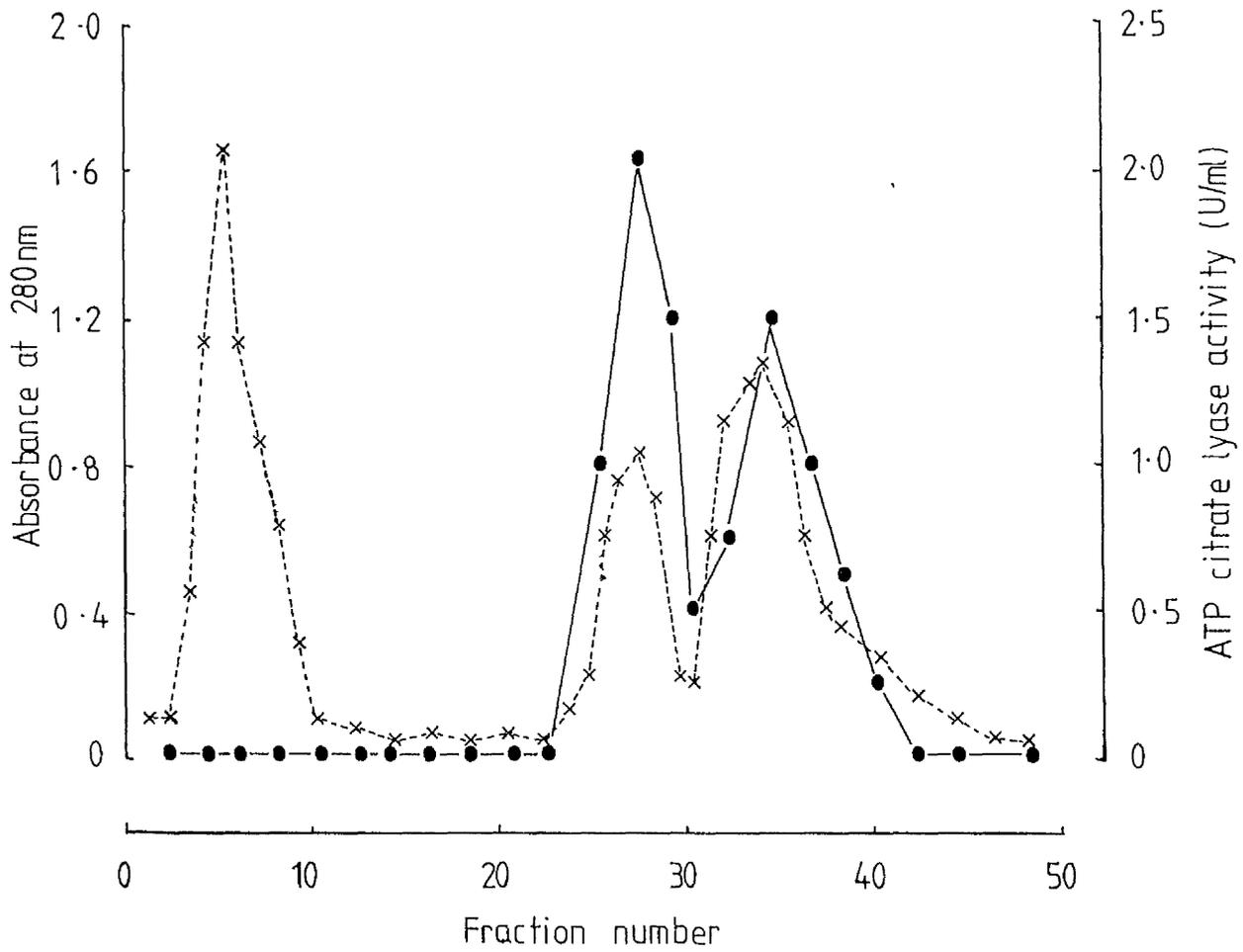
ATPCL is susceptible to limited proteolysis by endogenous proteases (Singh *et al.*, 1976). In view of this extensive measures were taken to minimise proteolytic activity. These measures included carrying out the purification as rapidly as possible at 4°C and homogenising the livers in an isotonic medium. Moreover, EDTA and a number of protease inhibitors were present in all of the buffers used during the purification.

As discussed in Chapter 4.2, rLACC would not always bind to DEAE cellulose apparently as a result of the presence of turbid material in the redissolved 0-30% ammonium sulphate precipitate. Preliminary experiments indicated that this material also prevented the binding of ATPCL to DEAE cellulose. Centrifugation of the homogenate at $105000 \times g$ for 2 h seemed to remove this material.

The elution profile of ATPCL from the DEAE cellulose column was generally similar to that shown in Fig. 4.19. By using a gradient from 0 - 150 mM potassium phosphate rather than 0 - 200 mM potassium phosphate it was possible to separate ATPCL from FAS completely. On two occasions, however, ATPCL was eluted from DEAE cellulose in two peaks; one of these is illustrated in Fig. 5.2. On these occasions only the first peak was further purified since the second peak contained FAS. The elution profile shown in Fig. 5.2 suggests that two forms of ATPCL are present. A similar result was obtained by

Figure 5.2. Elution of ATP citrate lyase from DEAE cellulose
in two peaks.

24.5 ml of desalted 30 - 40% ammonium sulphate precipitate
(260 U) was applied to a column of DEAE cellulose (1.7 cm x 21 cm)
and then eluted using a 10 - 150 mM potassium phosphate gradient
as described in the text. 11.5 ml fractions were collected and
monitored for absorbance at 280 nm (x) and ATPCL activity (●)

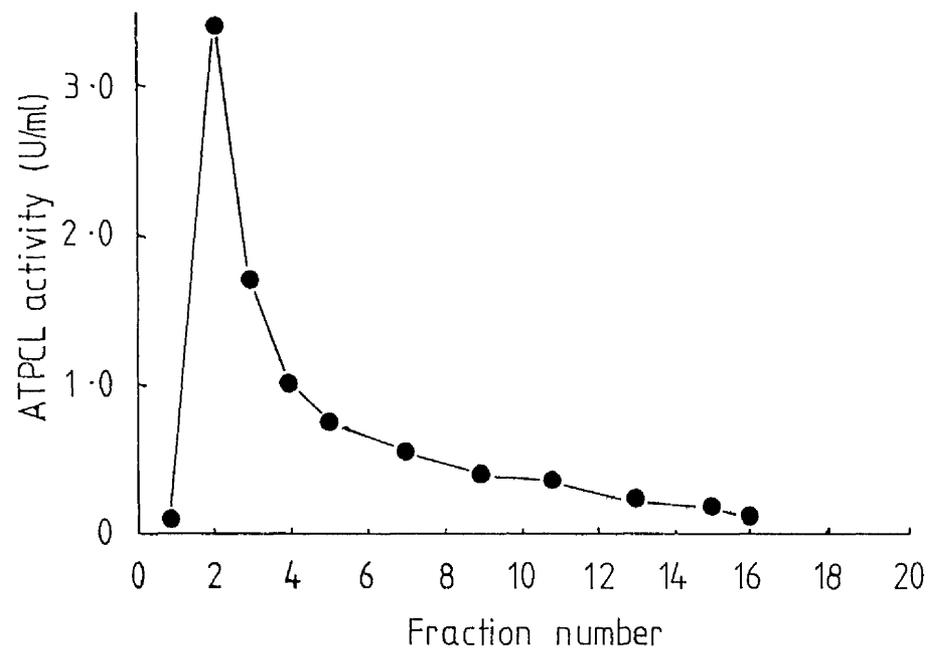


Corrigan and Rider (1981). These authors observed that during chromatography of a 45000 x g supernatant prepared from rat liver on DEAE Sephadex A-25, 10 - 15% of the ATPCL activity did not bind to the column. They also observed two peaks of ATPCL on gel filtration with apparent M_r s of 10^7 and 4.5×10^5 . On the basis of the relative amounts of ATPCL in each peak and the degree of inhibition of the activity in each peak by L- glutamate, they concluded that the aggregated form of ATPCL corresponded to the form which did not bind to DEAE Sephadex. The relationship between the two forms of enzyme observed in this work and those observed by Corrigan and Rider (1981) is not known. The limited occurrence of the phenomenon in this work perhaps confirms the suggestion made by Corrigan and Rider (1981) that one form is unstable and is converted into the other. While no evidence was found in this present work for an aggregated form of ATPCL using gel filtration or cross-linking with dimethyl suberimidate, traces of an aggregated form were observed during density gradient centrifugation (see below). It is tempting to speculate about the nature of the two forms observed on anion-exchange chromatography. Linn and Srere (1979) state that they have observed two forms of rLATPCL on isoelectric focusing and suggest that the two forms may differ in their content of structural phosphate. It is possible that this could also explain the two forms of ATPCL noted above although a simple charge difference alone would be unlikely to cause such good separation of the two forms. A comparison of the M_r s of the two peaks of ATPCL activity in Fig. 5.2 could have given some useful information.

The elution of ATPCL from Affigel Blue is illustrated in Fig.5.3. ATPCL did not elute from the column in a sharp peak. The peak of ATPCL activity is eluted after 1.5 column volumes

Figure 5.3. Chromatography of ATP citrate lyase on Affigel Blue.

90 ml of a DEAE cellulose pool (200 U) was applied to an Affigel Blue column (1.7 cm x 9 cm) and ATPCL was eluted from the column as described in the text. 15 ml fractions were collected and assayed for ATPCL activity (•).



but prolonged washing (12 column volumes) with the elution buffer is required to elute the ATPCL fully. This phenomenon, that immobilised dyes require prolonged washing in order to elute the bound enzyme completely has been noted by other workers in this laboratory. The protein concentration of the eluate from Affigel Blue columns was too low to allow concentration by ammonium sulphate precipitation. The concentration was therefore carried out using vacuum dialysis.

The chromatography of ATPCL on Ultrogel ACA 44 is illustrated in Fig. 5.4. ATPCL is eluted from the column in the void volume. I found that, provided care was taken during the DEAE cellulose step to ensure that the preparation was not contaminated with FAS, no other protein was eluted with ATPCL. Fractions in the trailing edge of the ATPCL activity peak were analysed on SDS-polyacrylamide gels to ensure that no low M_r contaminants were included when the fractions containing ATPCL were pooled.

The data shown in Table 5.1 are from a typical ATPCL preparation. The overall yield of ATPCL using this procedure (10.5%) was rather low compared with those of other workers. Yields of 73% (Hoffman *et al.*, 1979), 52% (Linn and Srere, 1979) and 21% (Redshaw and Loten, 1981) have been reported. Most of the activity was lost during steps 4 and 5. The recovery over the Affigel Blue step was generally about 50% while two thirds of the activity present in the Affigel Blue pool was regularly lost between that stage and the final preparation. Activity was always lost during vacuum dialysis, presumably due to the adherence of protein to the dialysis membrane. Activity was lost at the gel filtration step since only the most pure fractions were retained. A small amount of activity (< 5.0%) was lost during the subsequent dialysis.

Figure 5.4. Chromatography of ATP citrate lyase on Ultrogel AcA44.

1 ml of a concentrated Affigel Blue pool, prepared as described in the text was applied to a column of Ultrogel AcA44 (1.2 cm x 50 cm) and eluted using the buffer described in the text at a flow rate of 1.2 ml/min. 1.2 ml fractions were collected and monitored for absorbance at 280 nm (x) and ATPCL activity (o).

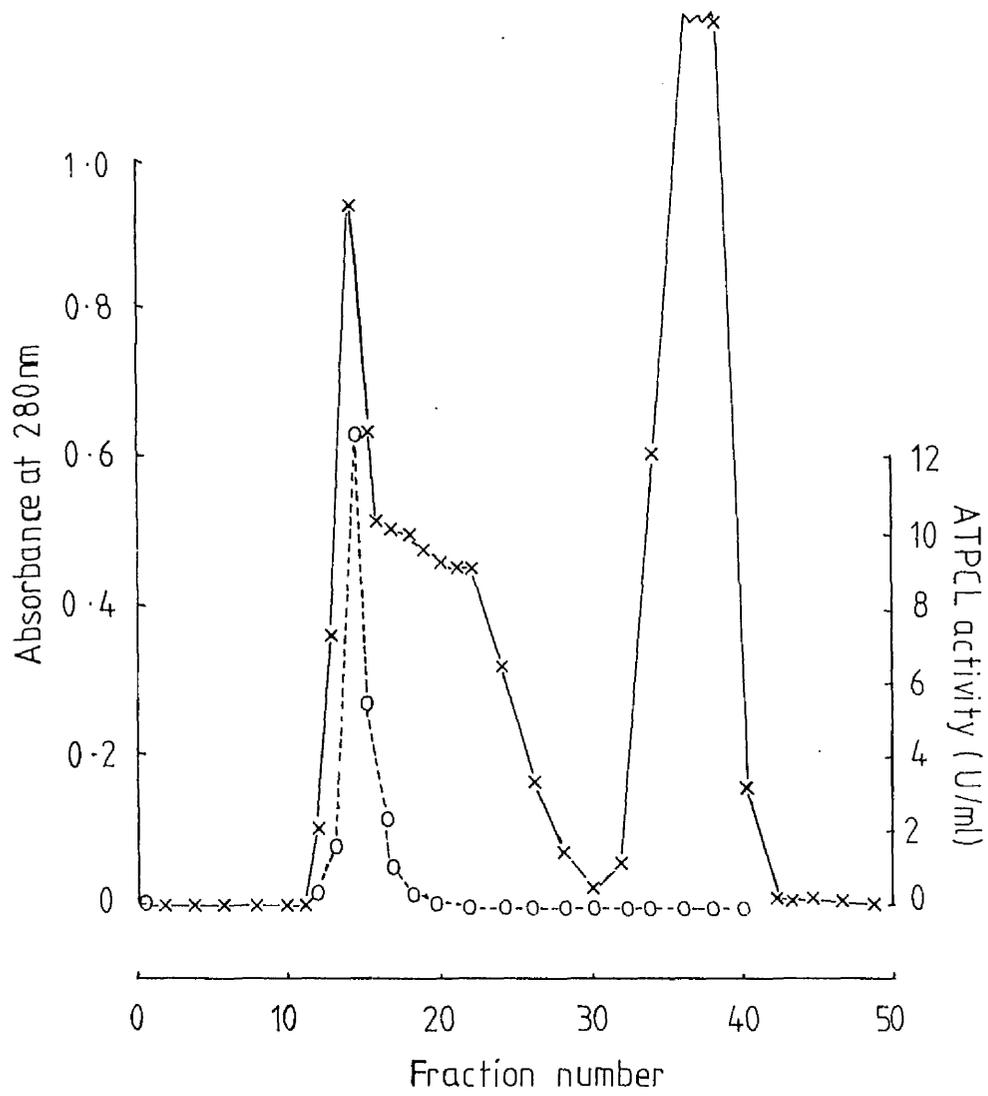


Table 5.1 Purification of ATP citrate lyase from rat liver

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Purification (-fold)	Yield (%)	
1	105000xg supernatant	129	240	380	0.14	1	100
2	30-40% ammonium sulphate precipitate	8	380	344	0.9	6.4	90
3	DEAE cellulose	125	54	241	4.4	32	63
4	Affigel Blue	230	9.4	103	10.9	78	27
5	Ultrogel AcA44 and dialysis	4.6	2.8	40	13.6	97	10.5

The data above was obtained with a preparation from 6 rats (46 g of tissue). In steps 1 - 3 the protein concentration was determined by the method of Bradford (1976). In steps 4 and 5 the absorption coefficient of 11.4 (Singh *et al* 1976) was used.

The specific activity of the purified ATPCL ranged from 12.4 to 15.6 U/mg (mean 13.4 U/mg). This specific activity is similar to, or higher than those reported by other workers. The specific activities of pure r1ATPCL reported by other workers recently are shown in Table 5.2. There is considerable variation in the values reported. It is interesting to note that there is a two-fold difference between specific activities for which the protein concentration was determined using either absorbance at 280 nm or the method of Lowry et al (1951), and those for which the method of Bradford (1976) was used. Alexander et al (1979) state that estimates of the protein concentration of solutions of pure ATPCL by the method of Bradford (1976) were twice those made by the method of Lowry et al (1951). In this present study the protein concentration of pure ATPCL determined by the method of Bradford (1976) was twice that determined by absorbance at 280 nm using an $A_{280}^{1\%}$ of 11.4. This absorption coefficient was reported by Singh et al (1976) and was determined by measuring the absorption at 280 nm of a solution which contained a known dry weight of pure ATPCL. While this is not a good method for the determination of absorption coefficients, the value obtained by Singh et al (1976) is similar to the value of 11.9 reported for lactating rat mammary gland ATPCL which was determined refractometrically in the analytical ultracentrifuge (Guy et al, 1980). It would appear, therefore, that the method of Bradford (1976) overestimates the protein concentration of ATPCL solutions by a factor of two. This suggests that the difference in specific activity between the preparation of Hoffman et al (1979) and those of other workers is due to the different methods used to estimate the protein concentration. There is, however, a two-fold variation in the specific activities where the protein concentration was determined from the absorbance at 280 nm. This may be due to partial proteolysis of ATPCL which while having no immediate effect

Table 5.2. Specific activities reported for pure ATP citrate lyase.

Reference	Specific Activity (U/mg)	Protein Estimation Method
Singh et al (1976)	11.0 - 12.6	absorbance at 280 nm
Linn and Srere (1979)	13.8	absorbance at 280 nm
Hoffman <u>et al</u> (1979)	6.4	Bradford (1976)
Alexander <u>et al</u> (1979)	12-14	Lowry <u>et al</u> (1951)
"	6-7	Bradford (1976)
Redshaw and Loten (1981)	10	Lowry <u>et al</u> (1951)
Ramakrishna and Benjamin (1981)	9.5	absorbance at 280 nm
Present study	13.4	absorbance at 280 nm

Specific activities were corrected to U/mg at 37°C using a factor of 2 for assays performed at 25°C (see text). Where the absorbance at 280 nm was used to estimate the protein concentration an absorption coefficient of 11.4 was used (Singh et al, 1976).

on the activity of ATPCL, does decrease the stability of the enzyme (Linn and Srere, 1979). It is interesting to note that the preparation with the highest specific activity (Linn and Srere, 1979) was prepared using polyethylene glycol fractionation in the early stages of the procedure. As discussed in Section 1 of this chapter such treatment might help to minimise limited proteolysis.

The entire purification procedure takes 3 - 4 days. Steps 1 - 4 can be completed in 14 - 15 h. The vacuum dialysis, which is commenced immediately after the enzyme has been eluted from Affigel Blue takes between 36 - 48 h. The gel filtration and analysis of the fractions on SDS-polyacrylamide gels can be completed within 12 h. The remainder of the time is taken up by the dialysis. The ATPCL was stable for 14 - 21 days when stored at -20°C in the buffer described above. After this time the preparation lost activity and it could not be reactivated by prolonged dialysis into buffer containing fresh DTT. Enzyme preparations which had lost activity were not used in studies on the phosphorylation and kinetic properties of ATPCL described in Section 3 of this chapter and in Chapter 6.

Physical characterisation

The material after step 5 showed a single protein staining band after non-denaturing polyacrylamide electrophoresis (Fig. 5.5). No other protein staining bands were visible when up to $10\ \mu\text{g}$ of ATPCL was electrophoresed. The R_f of ATPCL is slightly less than that of FAS under the electrophoresis conditions used. A single band was visible after staining for ATPCL activity (Fig. 5.6). The activity stained band had the same R_f as the band visible after staining for protein. The material after step 5 showed a single protein staining band after SDS-polyacrylamide gel electrophoresis either by the method

Figure 5.5. Non-denaturing polyacrylamide gel electrophoresis of pure ATPCL.

Polyacrylamide gel electrophoresis on 3% gels was performed by the method of Davis et al (1964). The samples electrophoresed were: (1), 5 μ g ATPCL; (2), 10 μ g ATPCL; (3) 5 μ g 1rmgFAS. The specific activity of the ATPCL was 14.2 U/mg.



Figure 5.6. Activity staining of ATP citrate lyase

Non-denaturing polyacrylamide gel electrophoresis on 3% gels was performed by the method of Davis (1964). (1), 10 μ g ATPCL stained for protein; (2), 20 μ g ATPCL stained for ATPCL activity as described in Chapter 2.2.

The specific activity of the ATPCL used was 13.6 U/mg.



1



2

of Weber and Osborn (1969) (Fig. 5.7) or the method of Laemmli (1970) (Fig. 5.8). The purified ATPCL was apparently free from proteolytic degradation as judged by the absence of protein staining bands with M_r s of approximately 57000 and 70000. This indicates that the precautions taken to minimise proteolysis were successful. The preparation after step 5 was free of FAS as judged by the absence of FAS activity in the preparation and the absence of protein staining bands corresponding to FAS on polyacrylamide gels.

Density gradient centrifugation on 5 - 20% sucrose gradients was performed by the method of Martin and Ames (1962) as described in Chapter 2.2. All buffers contained 1 mM EDTA and 15 mM β -mercaptoethanol. Each tube contained in addition to the sucrose, either 100 mM potassium phosphate, pH 7.0 or 100 mM potassium phosphate, 25 mM potassium citrate, pH 7.0. Samples (0.5 ml, 4.0U) of ATPCL were centrifuged in the presence or absence of 25 mM potassium citrate. Samples (0.5 ml) of β -galactosidase (4.0U) or pyruvate kinase (10U) were centrifuged in the presence of 25 mM potassium citrate. After centrifugation, the contents of each tube were harvested and fractions were assayed for the appropriate enzyme (Figure 5.9). The positions of the peaks of activity of β -galactosidase and pyruvate kinase were plotted against their sedimentation coefficients. Using this calibration curve the sedimentation coefficient for ATPCL in the presence and absence of citrate was found to be 13.5 S. This value is identical to that reported by Inoue et al (1966) for rLATPCL and similar to that reported for rat mammary gland ATPCL by Guy et al (1980). Slightly higher values for rLATPCL have been reported by Singh et al (1976) and Ramakrishna and Benjamin (1979). Small amounts of a more highly aggregated form of ATPCL may occur, especially in the presence of citrate.



Figure 5.7. SDS-polyacrylamide gel electrophoresis of ATP citrate lyase by the method of Weber and Osborn (1969).

ATPCL was analysed on 4% polyacrylamide gels in the presence of SDS by the method of Weber and Osborn (1969). (1), 3 μg ATPCL; (2) 6 μg ATPCL.





Figure 5.8. SDS-polyacrylamide gel electrophoresis of ATP citrate lyase by the method of Laemmli (1970).

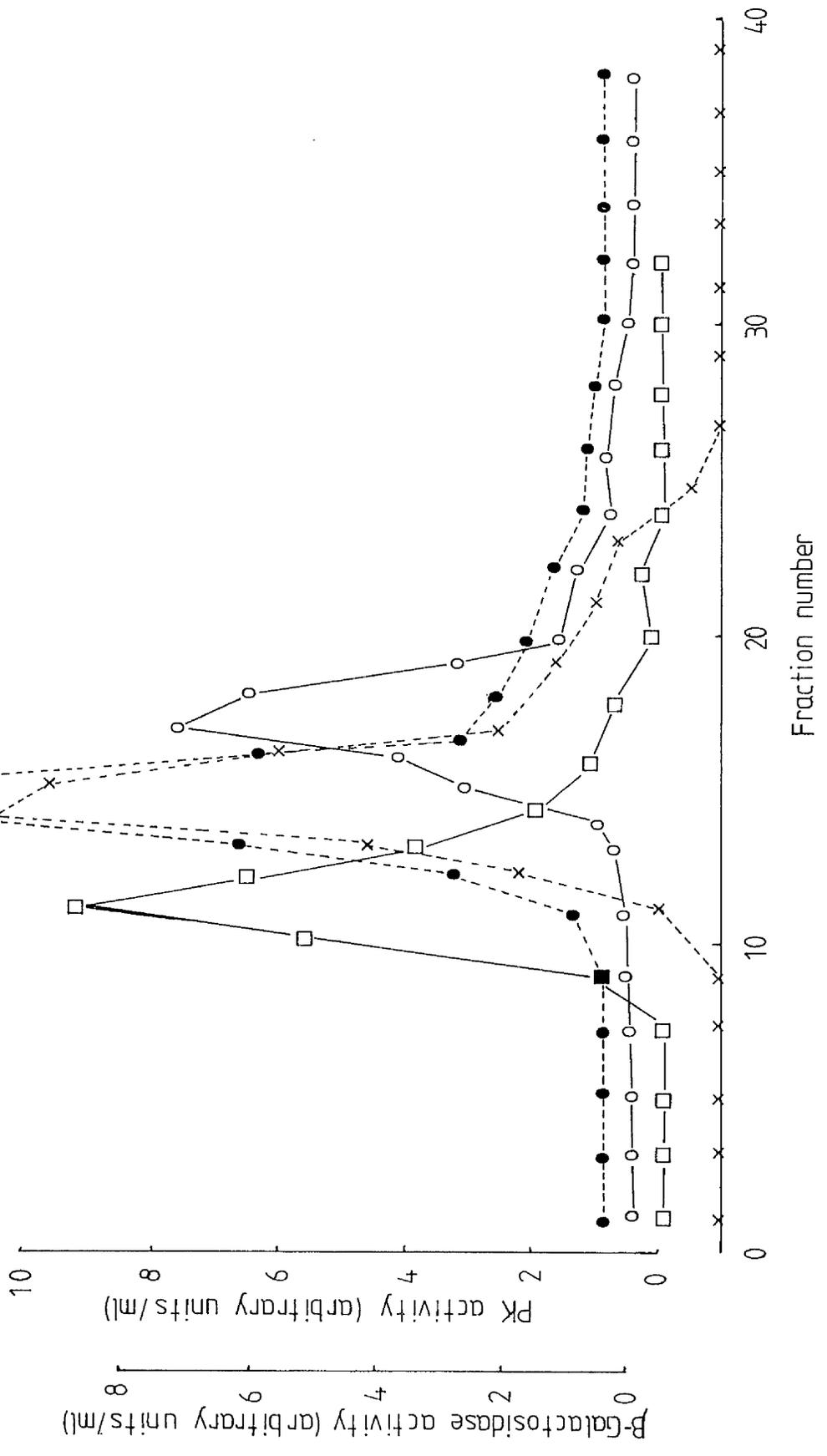
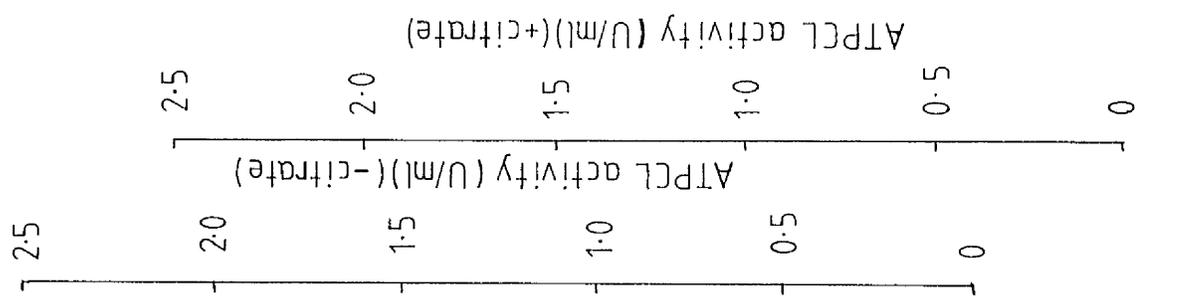
Pure ATPCL was analysed on 6% polyacrylamide gels in the presence of SDS by the method of Laemmli (1970). (1), 5 μ g ATPCL



1

Figure 5.9. Density gradient centrifugation of ATP citrate lyase.

Sucrose density gradient centrifugation of ATPCL was carried out as described in the text. (—□—) pyruvate kinase activity; (—○—) β -galactosidase activity; (----●----) ATPCL activity (sample run in absence of citrate); (----x----) ATPCL activity (sample run in presence of citrate).

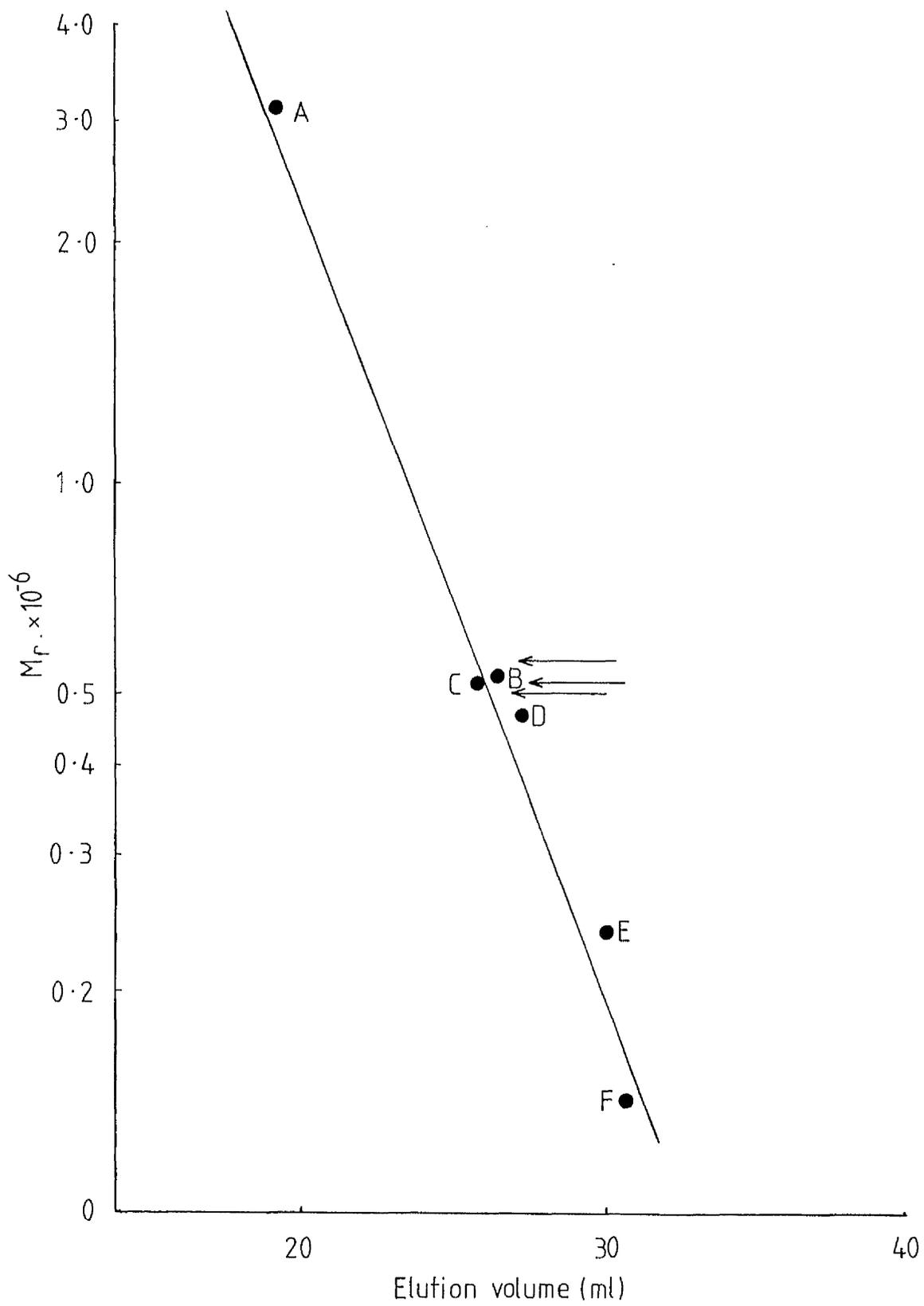


The M_r of ATPCL was estimated by gel filtration on a column of Ultrogel A2 (1.2 cm x 30 cm) equilibrated with 50 mM potassium phosphate, 20 mM potassium citrate, pH 7.0. It was necessary to include citrate in this buffer to maintain ATPCL activity during the gel filtration. An 0.2 ml aliquot of ATPCL (6.0U) was applied to the column and the column was then developed with equilibration buffer at a flow rate of 15 ml/h. 0.5 ml fractions were collected and assayed for ATPCL activity. The column was calibrated by measuring the V_e of proteins of known M_r . As discussed in Chapter 4.4 it was not possible to determine the void volume of this column. The results of this experiment are shown in Fig. 5.10. The V_e of ATPCL was 25.9 ± 0.35 (mean and SD of 3 separate experiments). The V_e of LmgFAS was 25.8, almost exactly equal to that of ATPCL. During gel filtration of partially purified ATPCL, which contained rLFAS, ATPCL and FAS activity were eluted together (result not shown). Thus the results in Fig. 5.10 indicate that ATPCL has an M_r of 500000 ± 20000 in agreement with the results of Inoue et al (1966). Singh et al (1976) using sedimentation equilibrium centrifugation obtained an M_r of 440000 for ATPCL. As Fig. 5.10 indicates, ATPCL is eluted from Ultrogel A₂ before the M_r marker apoferritin (M_r 440000), and so the M_r is clearly greater than 440000.

The subunit M_r of ATPCL was estimated on 6% polyacrylamide gels run in the presence of SDS by the method of Laemmli (1970) using the M_r standards indicated in the legend to Fig. 4.12. The subunit M_r of ATPCL was estimated in this experiment to be 125000 (result not shown). An identical result was obtained when the M_r of the DEAE cellulose peak 1 polypeptide described in Chapter 4.4 (later identified as ATPCL) was estimated.

Figure 5.10. Calibration of Ultrogel A2.

The experiment was performed as described in the text. The M_r markers used were: (A), haemocyanin, M_r 3×10^6 (Marrink and Gruber, 1969); (B), β -galactosidase, M_r 520000 (Weber *et al.*, 1964); (C), lrmgFAS, M_r 510000 (Hardie and Cohen, 1978b); (D) apoferritin, M_r 440000 (Bjork and Fish, 1971); (E), pyruvate kinase, M_r 237000 (Biochemica Information, vol 2, Boehringer); (F), rabbit muscle GAP, M_r 144000 (Harris and Waters, 1976). The arrows indicate the volume at which ATPCL eluted in 3 separate experiments.

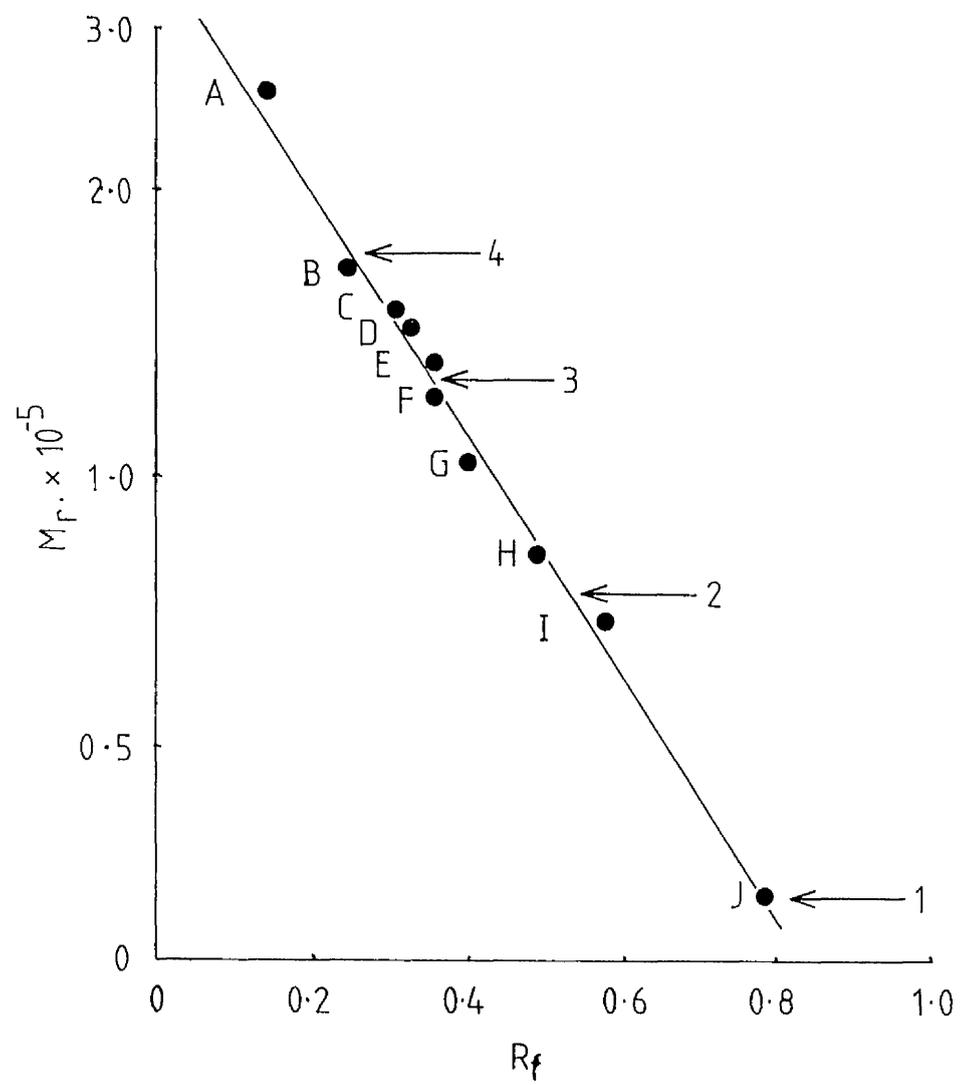


The subunit M_r of ATPCL was estimated on 4% polyacrylamide gels run in the presence of SDS by the method of Weber and Osborn (1969) using cross-linked GAPDH as an M_r marker. A calibration curve of $\log_{10} M_r$ against R_f was constructed and the subunit M_r of ATPCL was estimated to be 108000. This value differs considerably from that estimated in the experiment described above. The difference could have been due to either the different buffer systems used or the different M_r standards employed. To resolve this question I estimated the subunit M_r by both methods using the same M_r markers for each.

Various uncrosslinked M_r markers and ATPCL were electrophoresed in the presence of SDS by the method of Laemmli (1970) or the method of Weber and Osborn (1969). The calibration curve for the M_r markers electrophoresed by the method of Weber and Osborn (1969) is shown in Fig. 5.11. The subunit M_r of ATPCL was estimated from this plot to be 123000. An identical M_r was obtained when the M_r markers were electrophoresed by the method of Laemmli (1970) (result not shown). Cross-linked GAPDH was electrophoresed by the method of Weber and Osborn (1969). The apparent M_r s of the cross-linked GAPDH polypeptide were estimated from Fig. 5.11 to be monomer, 36000; dimer, 73000; trimer, 123000; tetramer, 170000. This result explains the discrepancy between the subunit M_r of ATPCL estimated using cross-linked GAPDH and that estimated using uncrosslinked M_r standards. The cross-linked polypeptides migrate more slowly than their M_r s predict. One explanation for this is that the cross-linked polypeptides bind less SDS per unit length than uncrosslinked polypeptides. This would happen if the cross-linking occurred in such a way that it prevents complete denaturation of the polypeptides. This result indicates that caution should be exercised when using

Figure 5.11 Calibration of polyacrylamide gels run in the presence of SDS by the method of Weber and Osborn (196)

The M_r standards used were: (A), lrmgFAS (250000); (B), N.cras: "arom complex" (165000); (C), phosphorylase kinase α subunit (145000); (D), phosphorylase kinase α' subunit (140000); (E), phosphorylase kinase β subunit (128000); (F), β -galactosidase (118000); (G), E.coli pyruvate dehydrogenase (pyruvate decarboxylase subunit) (100000); (H), E. coli pyruvate dehydrogenase (dihydrolipoyl transacetylase subunit)(80000); (I), bovine serum albumin (68000); (J), rabbit muscle GAPDH (36000). The arrows 1 - 4 indicate the positions to which the monomer (1), dimer (2), trimer (3), and tetramer (4) of cross-linked GAPDH migrated. Arrow 3 also indicate the R_f of ATPCL.



cross-linked proteins as M_r standards and that if possible, the M_r s of the cross-linked species should be checked against proteins of known M_r .

Table 5.3 shows the subunit M_r s of ATPCL reported by a number of recent workers. The value of 123000 obtained in this work is identical to that obtained by Alexander et al (1979) and Singh et al (1976) using SDS-polyacrylamide gel electrophoresis and that obtained by Singh et al (1976) using gel filtration in 6 M guanidine-HCl. The value of 128000 reported by Ramakrishna and Benjamin (1979) is probably an overestimate since in their M_r standards these authors used a value of 130000 for the subunit of β -galactosidase. The value of 105000 reported by Redshaw and Loten (1981) is much lower than estimated by other workers. It is difficult to comment on this value since the authors used only 2 markers, phosphorylase b and egg ovalbumin, and they gave no indication of the M_r s they assigned to these proteins. These workers should obviously have used more markers. It is apparent, therefore, that most workers who estimate the subunit M_r of ATPCL using SDS-polyacrylamide gel electrophoresis obtain a value in the region of 123000. Singh et al (1976) also estimated the subunit M_r by sedimentation equilibrium in 6 M guanidine-HCl and obtained a value of 110000. They believed that this value was more accurate than those obtained by other methods on the grounds that reliable M_r markers in excess of 100000 were not available.

The subunit M_r of 123000 measured in this study is consistent with the native M_r of 500000 determined by gel filtration and the fact that ATPCL seems to be a tetramer (see below). This value (123000) was used in calculating the stoichiometry of phosphate incorporation into ATPCL in the experiments reported in Chapter 6.

Table 5.3. Recent estimates of the subunit relative molecular mass of ATP citrate lyase.

Reference	Method	Tissue	M_r
Singh <u>et al</u> (1976)	A	rat liver	110000
	B	rat liver	123000
	E	rat liver	123000
Alexander <u>et al</u> (1979)	C	rat liver	123000
	C	rat A.T.	123000
Ramakrishna and Benjamin (1979)	C	rat A.T.	128000
	C	rat liver	128000
Guy <u>et al</u> (1980)	F	rat M.G.	116000
Redshaw and Loten (1981)	D	rat liver	105000
Present work	C, E	rat liver	123000

The methods referred to are: A, sedimentation equilibrium centrifugation in 6 M guanidine-HCl; B, gel filtration in 6 M guanidine-HCl; C, SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970); D, SDS-polyacrylamide gel electrophoresis by the method of Neville (1971); E, SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (1969); F, SDS-polyacrylamide gel electrophoresis by the method of Shapiro and Maizel (1969). Rat A.T., rat adipose tissue; rat M.G., rat mammary gland.

ATPCL was cross-linked with dimethyl suberimidate as described in Chapter 2.2. The reactions were stopped at the times indicated in the legend to Figure 5.12 and the contents of each reaction vessel were diluted 2-fold with NaPi sample buffer and boiled for 5 min. Samples from each incubation were analysed by SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (1969) using 3% polyacrylamide gels. The result of this experiment is illustrated in Figure 5.12. At 0 min only one band, corresponding to the monomer, is visible. After 5 min, bands corresponding to the dimer and trimer are visible while after 15 min the tetramer is also visible. The intensity of the tetramer band increases with time in the 30 min and 45 min samples while the intensities of the lower M_r bands decrease. After 60 min only one band, corresponding to the tetramer is visible. This result confirms that ATPCL is a tetramer. On the basis of a subunit M_r of 123000 the tetramer has an M_r of 492000 in close agreement with the M_r estimated by gel filtration.

It is interesting to note the ease with which ATPCL can be cross-linked. The concentration of dimethyl suberimidate used in this experiment was one fifth of that used to cross-link the GAPDH used as an M_r marker. Even with this low dimethyl suberimidate concentration, ATPCL is cross-linked approximately four times more quickly than GAPDH.

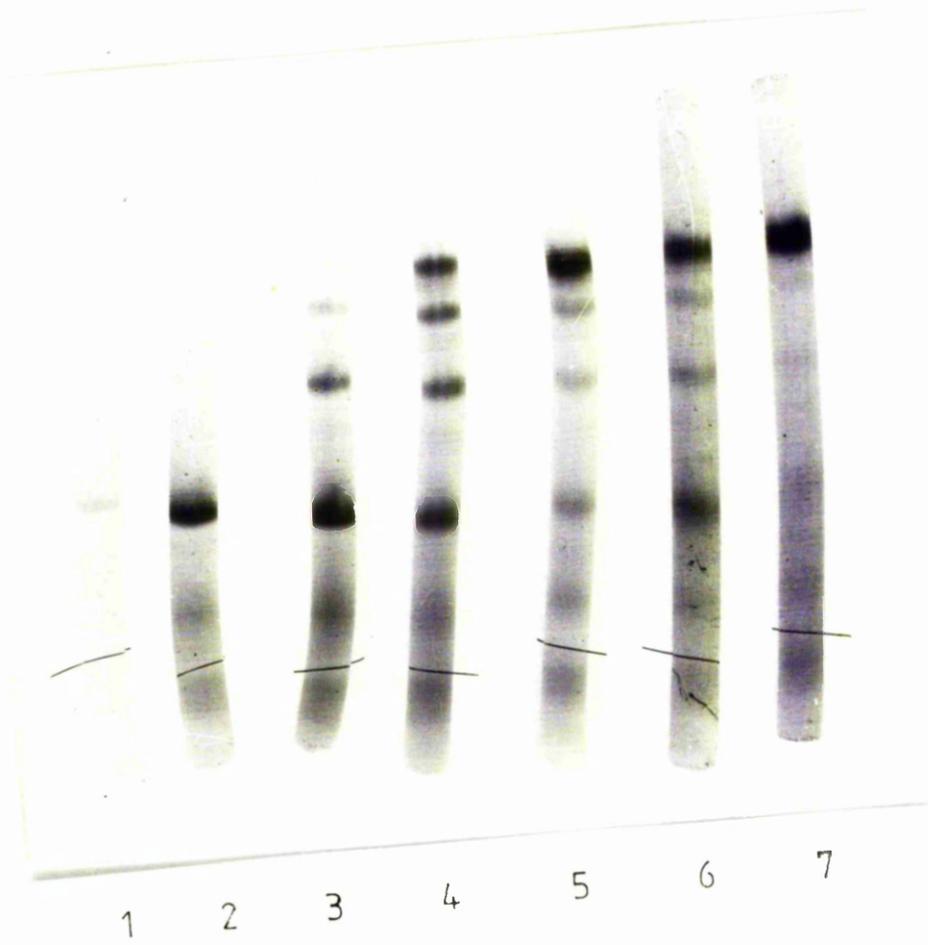
Stability of ATP citrate lyase in Tris-HCl

In the work described in this chapter ATPCL was assayed by the spectrophotometric method of Linn and Srere (1979). This assay could be performed in two ways:

Method (a) : All assay components except the enzyme sample were preincubated at 37°C for 5 min. The reaction was then started by

Figure 5.12. Cross-linking of ATP citrate lyase with dimethyl suberimidate.

ATPCL was cross-linked as described in Chapter 2, section 2 (b) The reactions were stopped at the following times: (1), 0 min; (2), 5 min; (3), 15 min; (4), 30 min; (5), 45 min; (6), 60 min. 50 μg of ATPCL was applied to each gel.



the addition of the enzyme sample. This method was used routinely for assay of fractions from chromatography columns and of purified enzyme.

Method (b) : The enzyme sample and all of the assay components except CoA were preincubated at 37^oC for 5 min. The reaction was then started by the addition of CoA. This method was used to assay samples taken during the early stages of ATPCL purifications since the background rate of NADH oxidation could be determined during the last 2 min of the preincubation period.

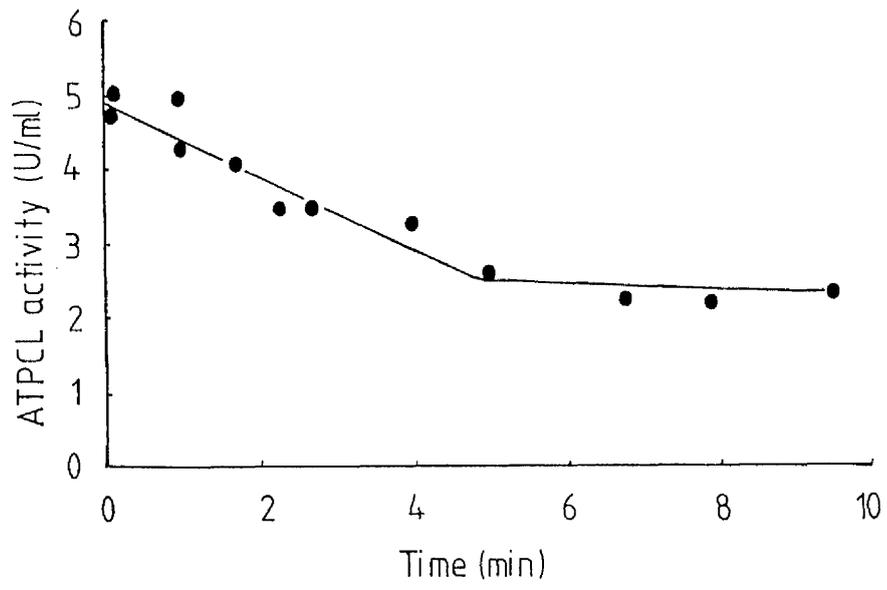
During the course of the work it was noted that estimates of ATPCL activity made by Method (b) were lower than those made by Method (a). The activities detected after preincubation for 5 min of some samples (105000 x g supernatant, 30 - 40% ammonium sulphate precipitate and the DEAE cellulose pool) were generally between 50 and 60% of those measured without preincubation.

When the samples were assayed by Method (a) the progress curve was linear for at least 10 min, indicating that inactivation did not occur when all three of the substrates were present. The data presented in Figure 5.13 indicate that ATPCL lost activity during the preincubation. The inactivation was not complete but levelled off after approximately 50% of the activity had been lost. Since the assay components include ATP and MgCl₂ it was possible that ATPCL was inactivated by a protein kinase during the preincubation. When ATPCL was preincubated for 5 min in the absence of ATP and the reaction started by the addition of ATP, the ATPCL lost only 20% of its activity during the preincubation, supporting the idea that a kinase might be responsible for the inactivation.

I next attempted to inactivate ATPCL by incubation with ATP and

Figure 5.13. Inactivation of ATP citrate lyase during preincubation prior to assay.

Cuvettes containing all of the assay components except ATPCL and CoA were incubated at 37°C for 5 min. ATPCL samples (from a DEAE cellulose pool prepared by the method described in Chapter 5.1.) were then added and preincubated for the times indicated. Each assay was started by the addition of CoA.



Mg²⁺ alone. A 30 - 40% ammonium sulphate precipitate was prepared as described in Section 1 of this chapter and diluted to a protein concentration of 1.7 mg/ml with 10 mM potassium phosphate, pH 7.0. This was then incubated at 37°C in the presence of 10 mM ATP and 100 mM MgCl₂ in a final volume of 140 μl. 10 μl samples were removed at various times and assayed by Method (a). There was no change in activity over a 60 min period. This experiment was repeated on one further occasion with similar results. This demonstrated that the inactivation was not caused by ATP.

During the initial stages of the work on ATPCL, when the purification procedure was being developed, ATPCL was dialysed into 20 mM Tris-HCl, 100 mM KCl, pH 8.0 prior to being applied to Cibacron Blue Sepharose. Table 5.4 shows the results of an experiment in which a DEAE cellulose pool, prepared as described in Section 1 of this chapter, was dialysed overnight at 4°C into the above buffer. As a control, a sample was stored overnight at 4°C without dialysis. ATPCL activity was measured before and after the dialysis by both Method (a) and Method (b). These results show that ATPCL lost activity during dialysis into Tris-HCl when assayed using Method (a). No activity was lost during storage of the control sample at 4°C. The ATPCL in the newly prepared DEAE cellulose pool and in the DEAE cellulose pool after storage at 4°C could be inactivated by approximately 33% during preincubation in the assay mixture. It was noted that a similar degree of inactivation had occurred during dialysis into Tris-HCl and that the dialysed ATPCL could not be further inactivated. This suggested that in both cases the inactivation was caused by the same factor.

The only components common to both the assay mixture and the dialysis buffer were Tris-HCl and β-mercaptoethanol. I therefore

Table 5.4. Stability of ATP citrate lyase during dialysis into Tris-HCl buffer.

Treatment	activity method (a) (U)	activity method (b) (U)
newly prepared	225	140
overnight storage	220	140
overnight dialysis at 4°C	126	116

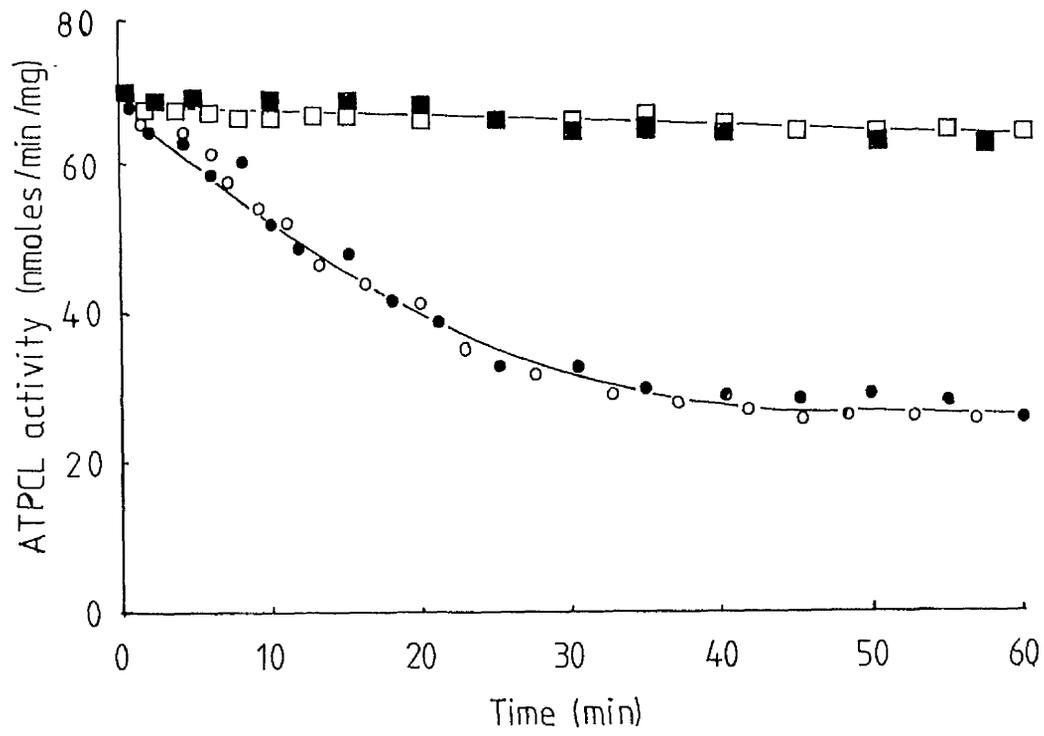
The experiment was carried out as described in the text.

studied the stability of ATPCL in 100 mM Tris-HCl in the presence and absence of β -mercaptoethanol. Samples of ATPCL (140 μ g) purified as far as the Affigel Blue step (specific activity 11.3 U/mg) were incubated at 25°C under the conditions described in the legend to Figure 5.14. Samples were removed and assayed by Method (a). ATPCL was inactivated in a time dependent manner after addition to 100 mM Tris-HCl, pH 8.0. No inactivation occurred when ATPCL was incubated in 100 mM potassium phosphate pH 7.0 with or without 15 mM β -mercaptoethanol.

These results indicated that ATPCL was inactivated in Tris-HCl buffers. This inactivation occurs fairly slowly but can be prevented by the presence of all three of the enzyme's substrates. The amount of inactivation caused by the presence of Tris-HCl was never more than 50% (Figs. 5.13 and 5.14). One interpretation of these results is the existence of a low activity conformer of ATPCL favoured by the presence of Tris-HCl and that the presence of all three substrates prevents the conformational change to the low activity form. This stabilisation by the substrates could be either kinetic or thermodynamic. It is possible that these two mechanisms could be distinguished by studying the effect of dialysis in the presence of substrate on ATPCL activity. The specificity of this inactivation by Tris-HCl was not investigated. It would be of interest to study the effect of other amines on ATPCL activity.

Figure 5.14. Inactivation of ATP citrate lyase by Tris-HCl.

ATPCL was purified as far as the Affigel Blue stage and then dialysed overnight into 100 mM potassium phosphate pH 7.0 at 4°C. Aliquots of ATPCL were incubated at 25°C in: 200 mM potassium phosphate pH 7.0 (□); 200 mM potassium phosphate, 15 mM β-mercaptoethanol pH 7.0 (■); 200 mM Tris-HCl, pH 8.7 (○); 200 mM Tris-HCl, 15 mM β-mercaptoethanol, pH 8.7 (●). Samples were removed at the times indicated and assayed for ATPCL activity by the method of Linn and Srere (1979).



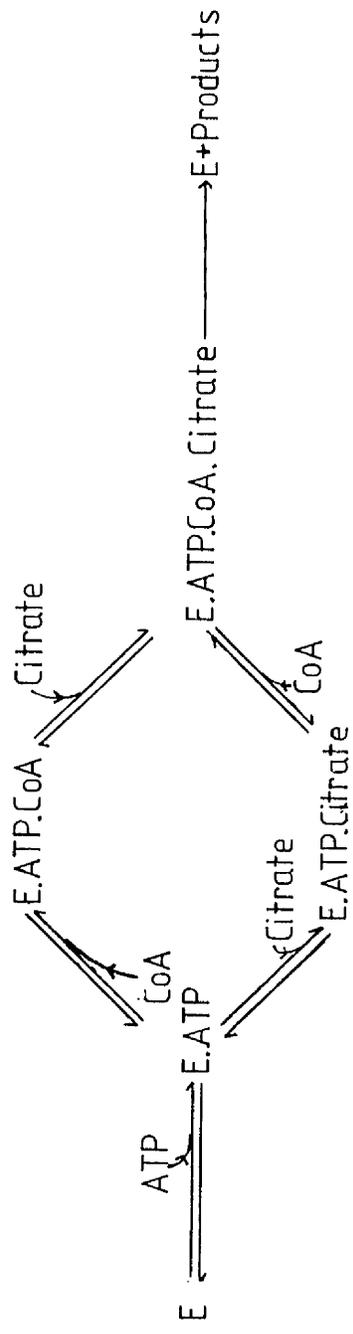
Section 2 Kinetic studies on ATP citrate lyase

The experiments described in this section were carried out in order to elucidate the kinetic mechanism of the reaction catalysed by ATPCL and to determine the values of the kinetic constants involved. Other workers had been unable to detect any changes in ATPCL activity associated with the phosphorylation of the enzyme. However, no careful analysis of the effects of phosphorylation on the kinetic parameters had been carried out. Moreover, the kinetic mechanism of the enzyme (Plowman and Cleland, 1967) is such that the assay conditions, unless chosen carefully, could actually conceal a change in activity (see below).

Previous studies on the kinetic properties of ATP citrate lyase

Studies to determine the mechanism of ATPCL were originally performed by Plowman and Cleland (1967). These authors measured the initial velocity of the forward reaction at several different concentrations of MgATP, CoA and Mg-citrate and analysed their data in subsets consisting of initial velocities measured when the concentrations of two of the substrates were varied at either a high or a low constant level of the third. Parallel double reciprocal plots were obtained when MgATP and CoA or MgATP and Mg-citrate were varied at saturating constant concentrations of Mg-citrate and CoA respectively. However, the double reciprocal plots were intersecting at low levels of the third substrate. Intersecting double reciprocal plots were obtained when CoA and Mg-citrate were varied at all levels of MgATP. These results suggested that the kinetic mechanism was sequential with MgATP binding first followed by the random rapid equilibrium binding of CoA and Mg-citrate (Figure 5.15). Plowman and Cleland (1967) also found that ATPCL could catalyse the exchange of

Figure 5.15. Sequential kinetic mechanism of ATP citrate lyase proposed by Plowman and Cleland (1967).



^{14}C -ADP into ATP in the absence of Mg-citrate and CoA. These workers were also able to isolate a phosphoenzyme resulting from the incubation of ATPCL with ^{32}P -ATP. These results implied a ping-pong mechanism which proceeded through a phosphoenzyme intermediate. Farrar and Plowman (1971, a,b) studied the kinetic mechanism of the reverse reaction. Their initial velocity studies indicated that the mechanism was sequential, but their isotope exchange experiments were unable to distinguish between a sequential or a ping-pong mechanism. In order to account for all of the experimental evidence, these authors proposed a mechanism whereby MgADP could be released from ATPCL at several different stages in the reaction. Thus, there is a discrepancy between the results of initial velocity studies, which indicate a sequential mechanism and enzyme labelling experiments which indicate that the mechanism is ping-pong.

I wished to repeat the initial velocity experiments of Plowman and Cleland (1967) for three reasons.

1) To resolve the discrepancy concerning the kinetic mechanism of the reaction. The kinetic study of Plowman and Cleland (1967), which suggested a sequential mechanism, can be criticised on the grounds that the so-called 'saturating substrate' was not truly saturating. For example, when MgATP and CoA were varied the Mg-citrate concentration was only 28 times the Michaelis constant; when Mg-citrate and MgATP were varied CoA was only 12.5 times the Michaelis constant. Cleland (1970) himself has emphasised that concentrations of at least 100 times the Michaelis constant are required for the true saturation.

2) The enzyme preparations used by Plowman and Cleland (1967) and Farrar and Plowman (1971 a,b) were almost certainly proteolytically degraded since these workers had taken no precautions against

proteolysis during the purification and they present no physical characterization of their preparation. I wished to study the kinetic properties of ATPCL using enzyme which had not been proteolytically degraded (see Section 2 of this chapter).

3) I wished to be able to compare the kinetic properties of phosphorylated and non-phosphorylated forms of the enzyme.

Initial velocity studies

Plowman and Cleland (1967) performed their assays in 100 mM Tris-HCl, pH 8.1. In view of the results presented in Section 1 of this chapter, which show that ATPCL is inactivated by Tris-HCl, I wished to avoid the use of this buffer. I also wished to perform the assays at a more physiological pH (pH 7.0). Preliminary results indicated that 50 mM MOPS (pKa = 7.1) did not inactivate ATPCL.

Plowman and Cleland (1967) noted that in the absence of KCl, double reciprocal plots of initial velocity against Mg-citrate or non-complexed citrate were convex-up curves. In the presence of chloride ions, however, the plots were linear. The explanation of these results favoured by Plowman and Cleland (1967) was that the non-complexed citrate binds at a regulatory site and increases both the K_{m1} for Mg-citrate and the maximum velocity. Presumably chloride ions can also bind to this site. 0.25 M KCl was therefore included in all assays so that double reciprocal plots of initial velocity against Mg-citrate would be linear.

The concentration of free Mg^{2+} ions was 0.424 mM in all assays. This value was chosen to facilitate the calculation of the total amount of Mg^{2+} (added as magnesium acetate) required in each assay. This was calculated using the relationship given in Chapter 2.2.

The initial velocity measurements were made under the conditions described in Chapter 2.2 (Method a). The concentrations of MgATP, Mg-citrate and CoA used are given in Figures 5.16 - 5.21. Three pairs of experiments were performed:

- 1) MgATP and Mg-citrate were varied at high and low concentrations of CoA (Figures 5.16 and 5.17 respectively).
- 2) MgATP and CoA were varied at high and low concentrations of Mg-citrate (Figures 5.18 and 5.19 respectively).
- 3) Mg-citrate and CoA were varied at high and low concentrations of MgATP (Figures 5.20 and 5.21 respectively).

In the first pair of experiments the initial velocity pattern was parallel at the high concentration of CoA (indicating an irreversible step between the addition of MgATP and Mg-citrate) and intersecting at non-saturating levels of CoA (indicating no irreversible steps between the addition of MgATP and Mg-citrate). In the second pair of experiments the initial velocity pattern was parallel at the high concentration of Mg-citrate (indicating an irreversible step between the addition of MgATP and CoA) and intersecting at non-saturating levels of Mg-citrate (indicating no irreversible steps between the addition of MgATP and CoA). In the third pair of experiments an intersecting pattern was observed at both saturating and non-saturating levels of MgATP, indicating that the steps between the binding of CoA and Mg-citrate are reversible under all conditions. These results show that the binding of substrates is sequential. Taken together, the data presented in Figures 5.16 - 5.21 suggest that MgATP binds first followed by the random order binding of Mg-citrate and CoA. One can reach this conclusion without any additional studies such as product inhibition because of the odd observation that at high fixed levels of CoA, varying MgATP and

Figure 5.16. Double reciprocal plots of initial velocity against Mg-citrate concentration at a high constant CoA concentration.

Assays were performed using Method (a) described in Chapter 2.2 and at a CoA concentration of $200 \mu\text{M}$. MgATP concentrations used were: $400 \mu\text{M}$, (\bullet); $76 \mu\text{M}$, (\circ); $40 \mu\text{M}$ (\blacksquare); $26 \mu\text{M}$ (\square); $20 \mu\text{M}$ (\times).

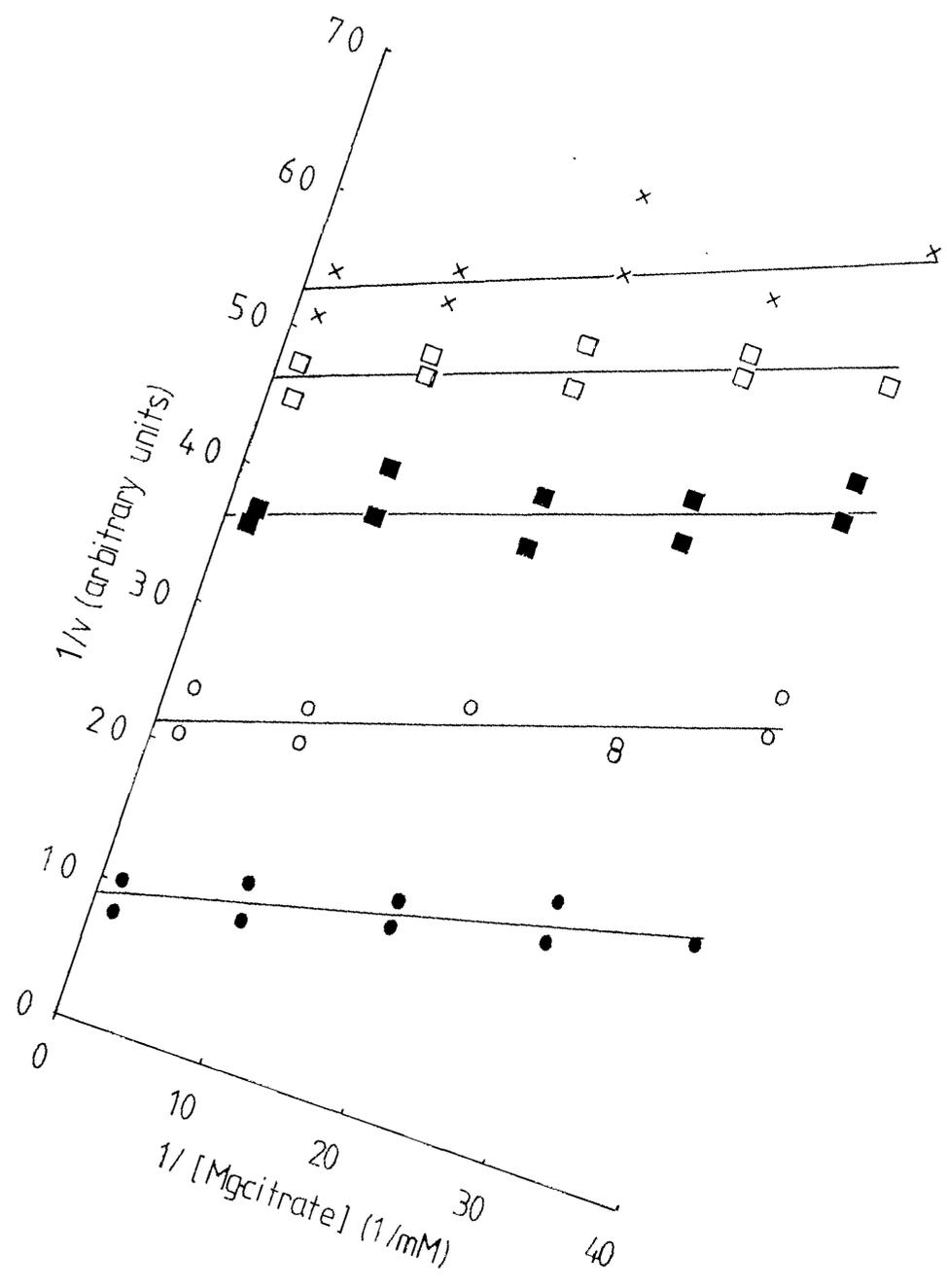


Figure 5.17. Double reciprocal plot of initial velocity against Mg-citrate concentration at a constant low CoA concentration.

Assays were performed using Method (a) described in Chapter 2.2 and at a CoA concentration of $10 \mu\text{M}$. The MgATP concentrations used were: $400 \mu\text{M}$, (●); $76 \mu\text{M}$, (○); $40 \mu\text{M}$, (■); $20 \mu\text{M}$, (□).

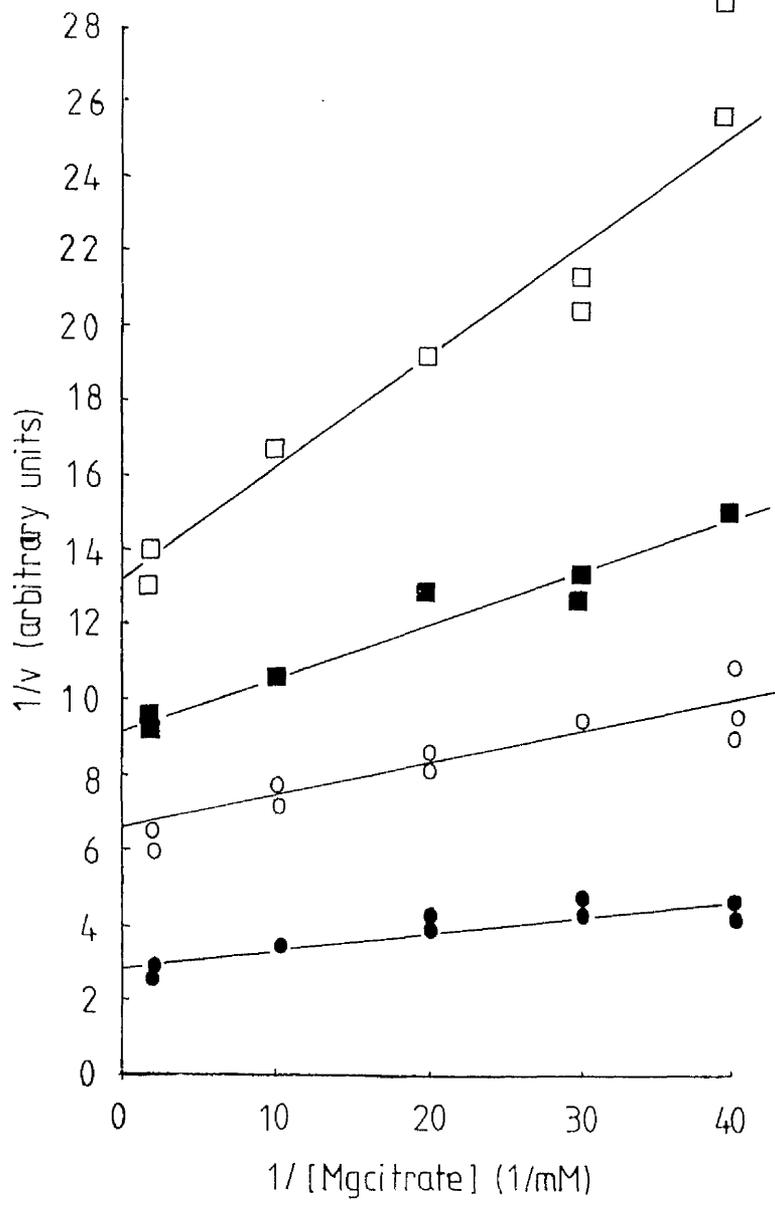


Figure 5.18. Double reciprocal plots of initial velocity against CoA concentration at a constant high Mg-citrate concentration.

Assays were performed using Method (a) described in Chapter 2. and at a Mg-citrate concentration of 9 mM. MgATP concentrations used were: $400\ \mu\text{M}$ (\bullet); $76\ \mu\text{M}$ (\circ); $40\ \mu\text{M}$, (\blacksquare), $20\ \mu\text{M}$ (\square).

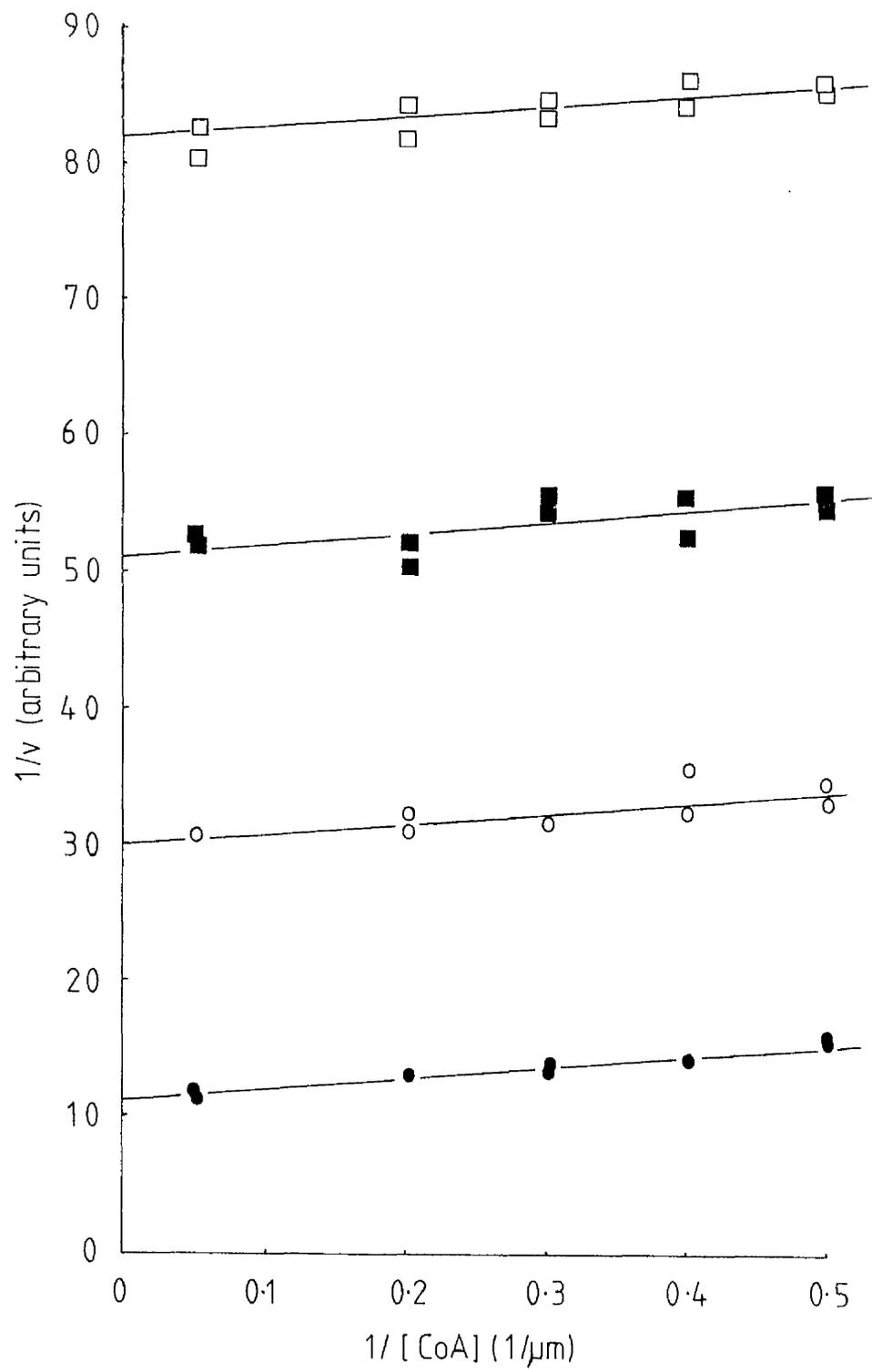


Figure 5.19. Double reciprocal plots of initial velocity against C concentration at a constant low Mg-citrate concentration

Assays were performed using Method (a) described in Chapter 2.2 and at a Mg-citrate concentration of 0.15 mM. MgATP concentrations used were: 400 μM , (\bullet); 76 μM , (\circ); 40 μM , (\blacksquare); 20 μM , (\square).

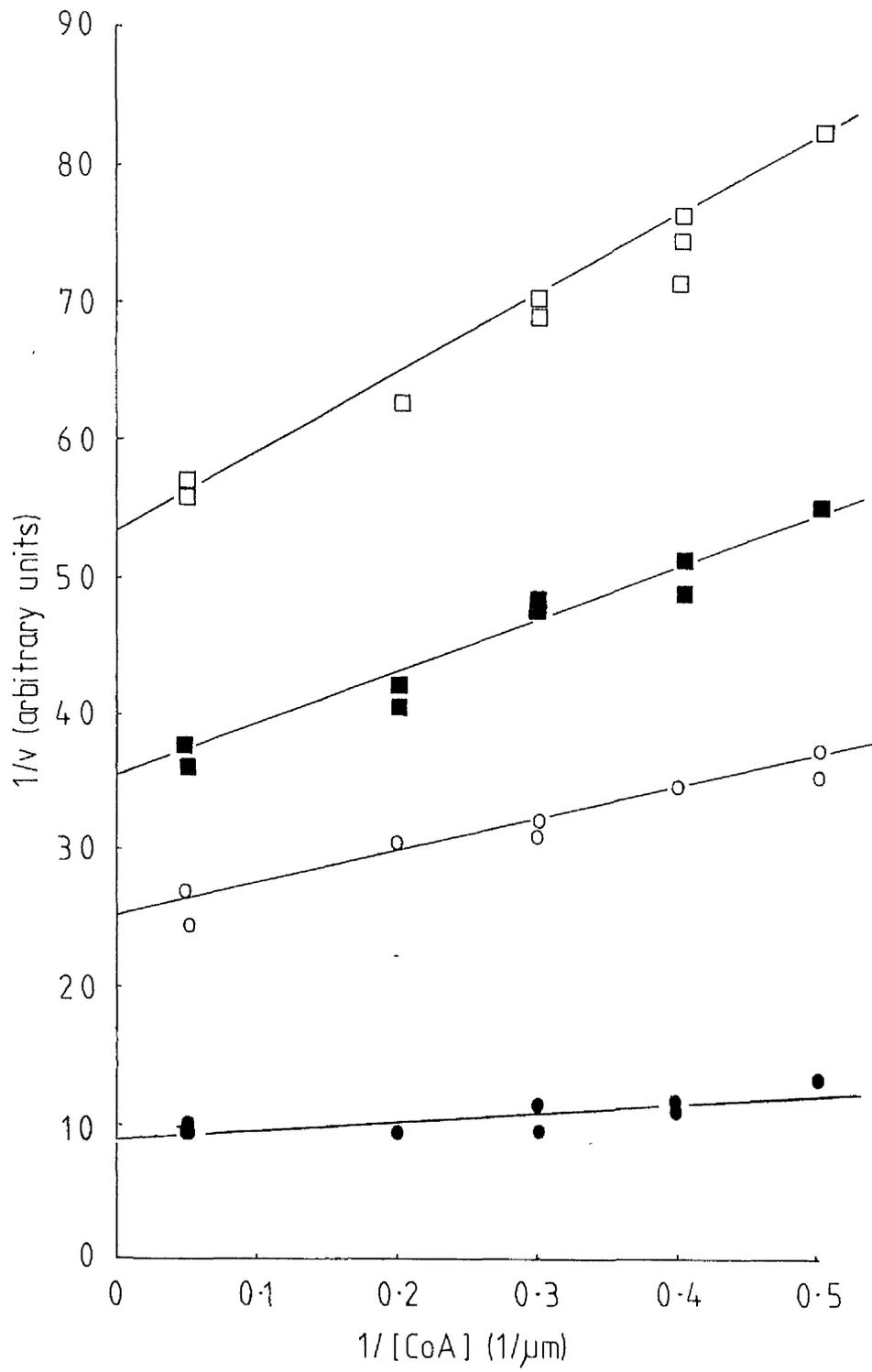




Figure 5.20. Double reciprocal plots of initial velocity against Mg-citrate concentration at a constant high MgATP concentration.

Assays were performed using Method (a) described in Chapter 2. and at a MgATP concentration of 17.3 mM. CoA concentrations used were: 20 μ M, (\bullet); 5 μ M, (\circ); 3 μ M, (\blacksquare); 2 μ M, (\square)

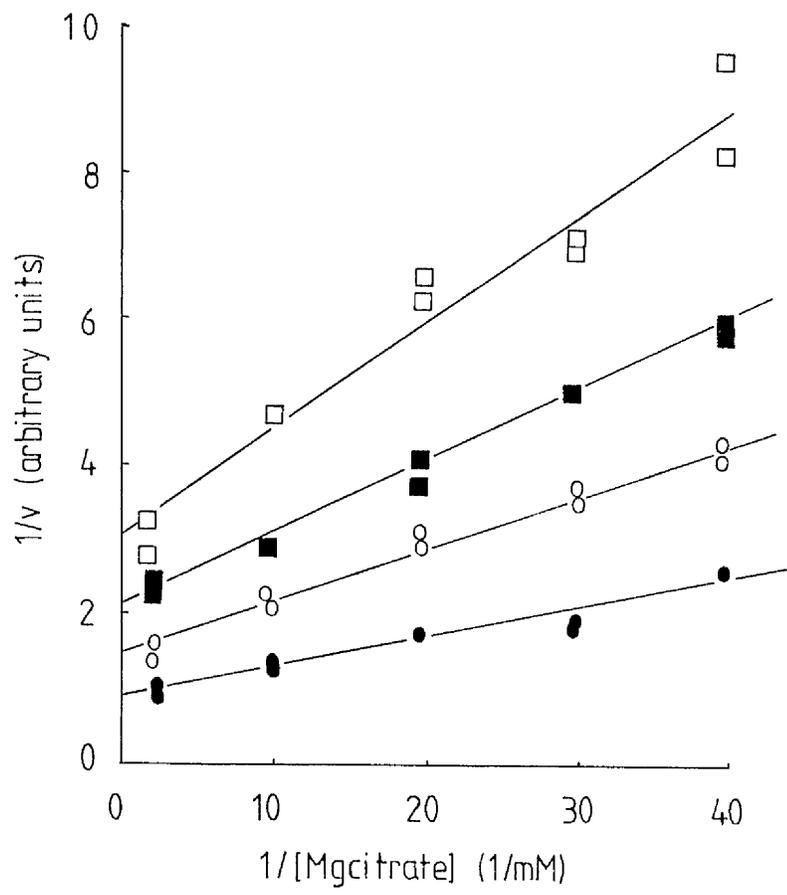
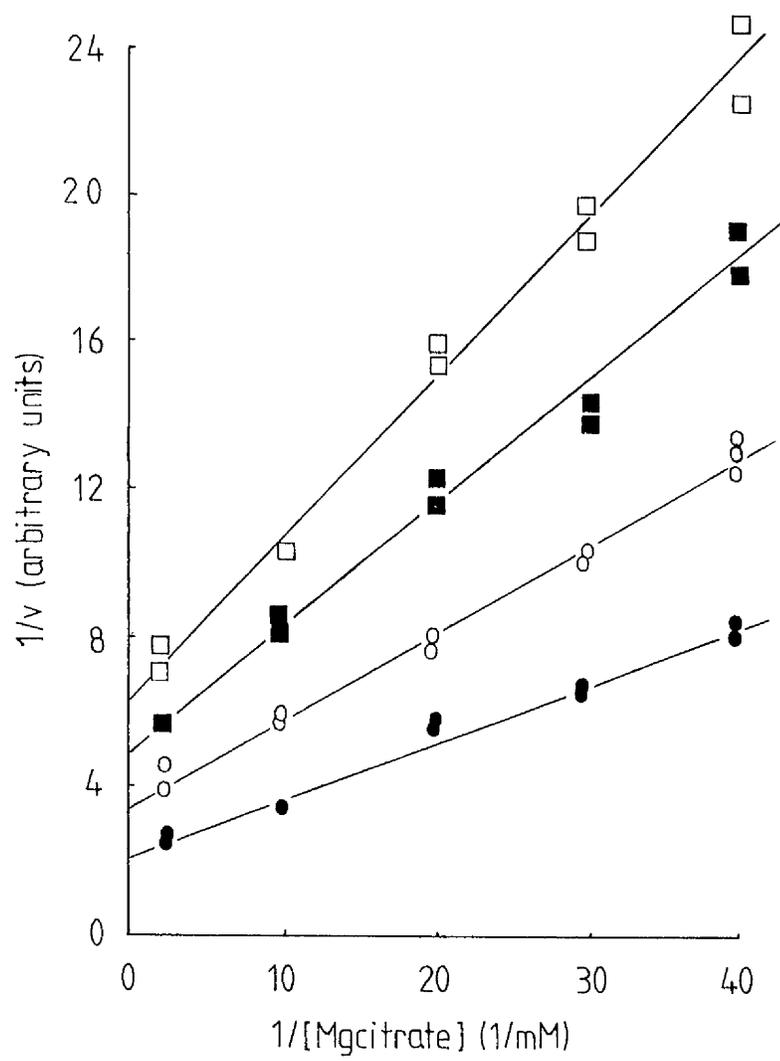


Figure 5.21. Double reciprocal plots of initial velocity against Mg-citrate concentration at a constant low MgATP concentration.

Assays were performed using Method (a) described in Chapter 2, and at a MgATP concentration of $200 \mu\text{M}$. CoA concentrations used were: $20 \mu\text{M}$, (\bullet); $5 \mu\text{M}$, (\circ); $3 \mu\text{M}$, (\blacksquare); $2 \mu\text{M}$, (\square)



Mg-citrate gave parallel plots while at a high fixed level of Mg-citrate varying MgATP and CoA also gave parallel plots. These apparently contradictory findings result from the fact that when one substrate in a random order mechanism is saturating essentially all of the flux is through one arm of the pathway and the reaction effectively becomes compulsory order. The saturation with Mg-citrate or CoA creates an effectively irreversible step between the binding of MgATP and CoA or MgATP and Mg-citrate respectively.

These results for unproteolysed ATPCL agree with those of Plowman and Cleland (1967). Thus, the discrepancy between the sequential mechanism indicated by initial velocity studies, and the ping-pong mechanism indicated by enzyme labelling and isotope exchange data was not resolved by these experiments.

By replotting the slopes and intercepts of the lines in Figures 5.16 - 5.21 against the reciprocal of the concentration of the changing fixed substrate it would be possible to determine numerical values for the kinetic parameters such as the Michaelis constants for the substrates. It is more appropriate that these parameters of ATPCL be dealt with in Chapter 6 in which the kinetic properties of phosphorylated and non-phosphorylated ATPCL are compared.

Kinetic evidence in favour of a ping-pong mechanism

In the kinetic mechanism implied by the results of enzyme labelling and isotope exchange experiments there is an irreversible step, the formation of a phosphoenzyme intermediate prior to the binding of CoA and Mg-citrate. The pattern of double reciprocal plots predicted for this mechanism is that plots of MgATP against CoA or MgATP against Mg-citrate will be parallel at all concentrations of Mg-citrate and CoA respectively. The results illustrated in

Figures 5.17 and 5.19 show, however, that at low concentrations of the fixed substrate, the double reciprocal plots are intersecting, indicating that there are no irreversible steps between the binding of MgATP and Mg-citrate or CoA. I therefore concluded that the mechanism was sequential.

Subsequent to the initial velocity studies described above being performed it was realised that a substantial amount (approximately 20% by weight) of ADP was present in the solutions of ATP used. This renders the patterns observed in Figs. 5.17 and 5.19 ambiguous. This is because in the presence of ADP the formation of the phosphoenzyme is reversible and consequently an intersecting pattern of double reciprocal plots would be expected. This is analogous to a case mentioned by Cleland (1970): in a ping-pong mechanism the presence of the product released between addition of two substrates that normally give parallel lines, converts the pattern to an intersecting one. Thus the results shown in Figs. 5.17 and 5.19 are compatible with the ping-pong mechanism. Since Plowman and Cleland (1967) did not specially purify the ATP used in their work, it seems likely that their initial velocity studies were performed in the presence of ADP and their results are also compatible with a ping-pong mechanism. I therefore repeated certain initial velocity studies in the absence of ADP in an attempt to distinguish between the sequential and ping-pong mechanisms.

ATPCL was partially purified as far as the DEAE cellulose step described in Section 1 of this chapter. The peak activity fraction from the DEAE cellulose column (10 ml, 55U) was then dialysed overnight into 2l of 100 mM TEA-HCl, 0.4 mM DTT, 1 mM EDTA, 1 mM Benzamidine-HCl, pH 8.0. The dialysed enzyme was then diluted to a suitable concentration with the dialysis buffer and used for the initial

velocity studies.

The initial velocity studies were carried out as described in Chapter 2.2 using Method b, and at the concentrations of MgATP, Mg-citrate and CoA given in the legends to Figures 5.22 and 5.23. Two experiments were carried out. In the first experiment MgATP and CoA were varied at 0.15 mM Mg-citrate. This value is approximately 3 times the K_m of ATPCL for Mg-citrate (see Chapter 6.2). The results of this experiment are shown in Figure 5.22. In the second experiment MgATP and Mg-citrate were varied at 10 μ M CoA. This value is twice the K_m of ATPCL for CoA. The results of this experiment are shown in Figure 5.23.

In the assay method used in these experiments, the reaction catalysed by ATPCL is followed by coupling the production of ADP to the oxidation of NADH using PK and LDH. Using this method the initial velocity can be measured in the absence of ADP since any contaminating ADP is removed by the coupling enzymes during the preincubation period (approximately 15 min).

In these studies, performed in the absence of ADP, parallel double reciprocal plots were obtained when MgATP and CoA or MgATP and Mg-citrate were varied at non-saturating concentrations of Mg-citrate or CoA respectively. Thus, the pattern obtained in the absence of ADP is the opposite to that obtained in the presence of ADP. The results given in Figures 5.22 and 5.23 show that there is an irreversible step between the binding of MgATP and CoA and between the binding of MgATP and Mg-citrate, even at low concentrations of Mg-citrate and CoA respectively. The kinetic mechanism would, therefore, appear to be ping-pong. The fact that in the presence of ADP there are no irreversible steps between the binding of all three substrates is evidence that the irreversible step observed in the absence of ADP is the formation of the phosphoenzyme intermediate.

Figure 5.22. Double reciprocal plots of initial velocity against CoA concentration at a constant low Mg-citrate concentration.

Assays were performed using Method (b) described in Chapter 2.2 and at a Mg-citrate concentration of 0.15 mM. MgATP concentrations used were: 400 μ M, (x); 76 μ M, (■); 40 μ M, (●); 20 μ M, (o).

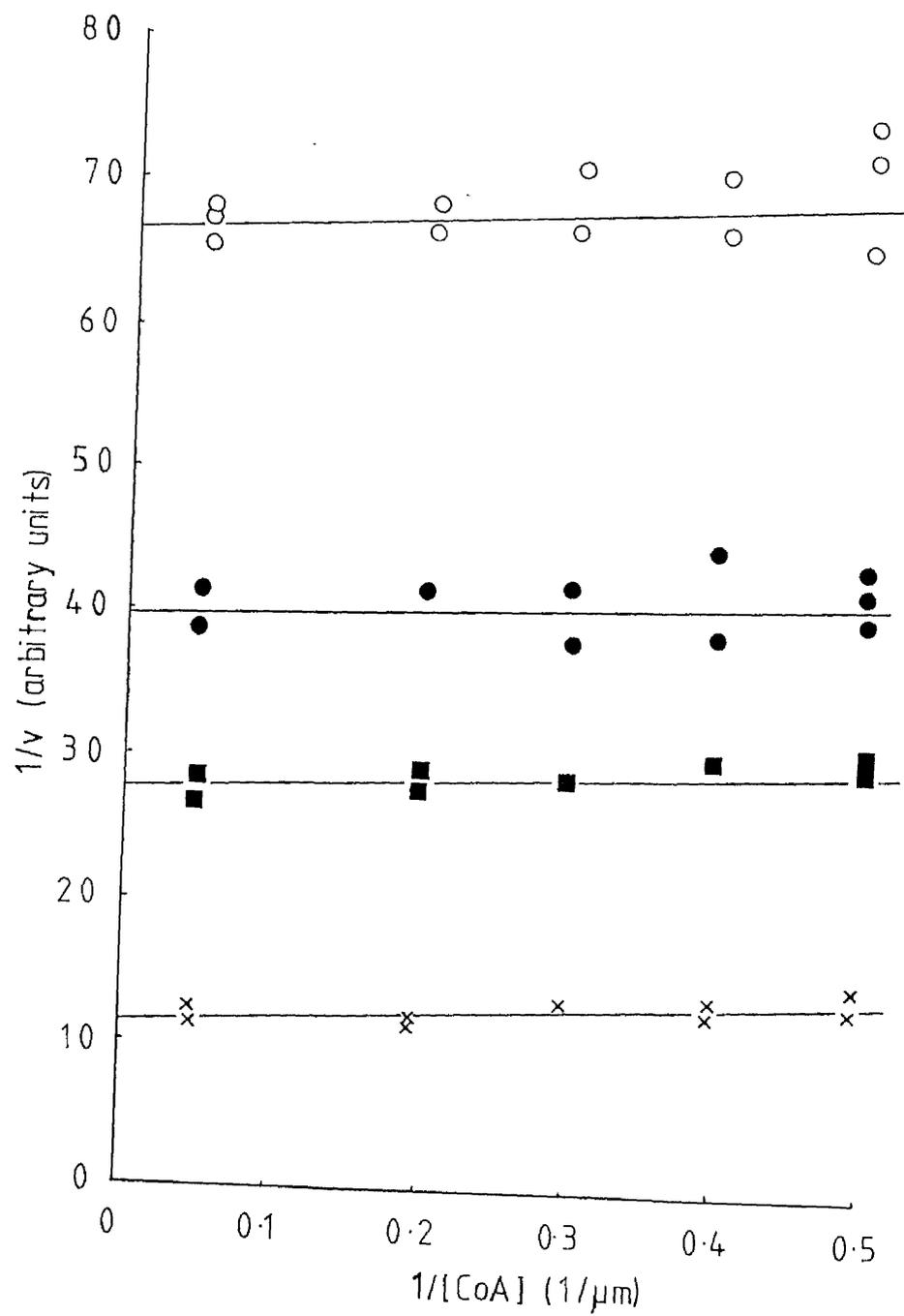
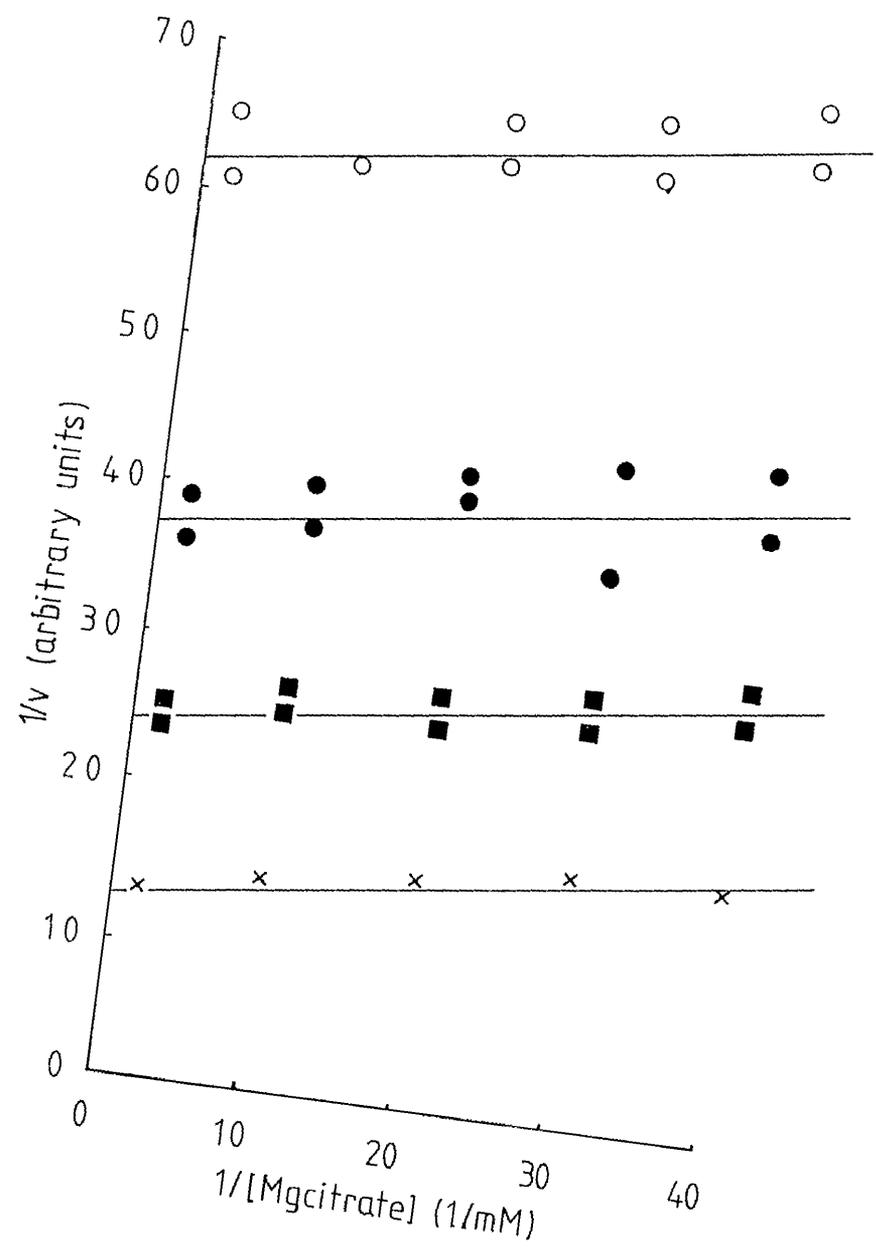


Figure 5.23. Double reciprocal plots of initial velocity against Mg-citrate concentration at a constant low CoA concentration.

Assays were performed using Method (b) described in Chapter 2.2 and at a CoA concentration of $10\ \mu\text{M}$. MgATP concentrations used were: $400\ \mu\text{M}$, (\times); $76\ \mu\text{M}$, (\blacksquare); $40\ \mu\text{M}$, (\bullet); $20\ \mu\text{M}$ (\circ).



The results presented in this section resolve the discrepancy concerning the mechanism of ATPCL that has been mentioned above. It is now clear that the initial velocity studies that suggested a sequential mechanism were performed in the presence of ADP. This had the effect of converting parallel plots to intersecting ones. In the absence of ADP parallel plots are obtained. The kinetic mechanism is clearly ping-pong, in agreement with enzyme labelling and isotope exchange data.

Chapter 6

Studies on phosphorylated and non-phosphorylated ATP citrate lyase

Introduction

The phosphorylation of ATPCL in vivo in response to glucagon and insulin is well documented (Alexander et al, 1979; Ramakrishna and Benjamin, 1979). As discussed in Chapter 1 the enzyme which catalyses the glucagon mediated phosphorylation of ATPCL is apparently cAMPdPrK (Alexander et al, 1981; Pierce et al, 1981). This chapter describes the phosphorylation of purified r1ATPCL by cAMPdPrK in vitro, together with the results of a detailed study of the effects of phosphorylation upon the kinetic parameters of ATPCL.

Section 1 Phosphorylation of ATP citrate lyase by cAMP-
dependent protein kinase

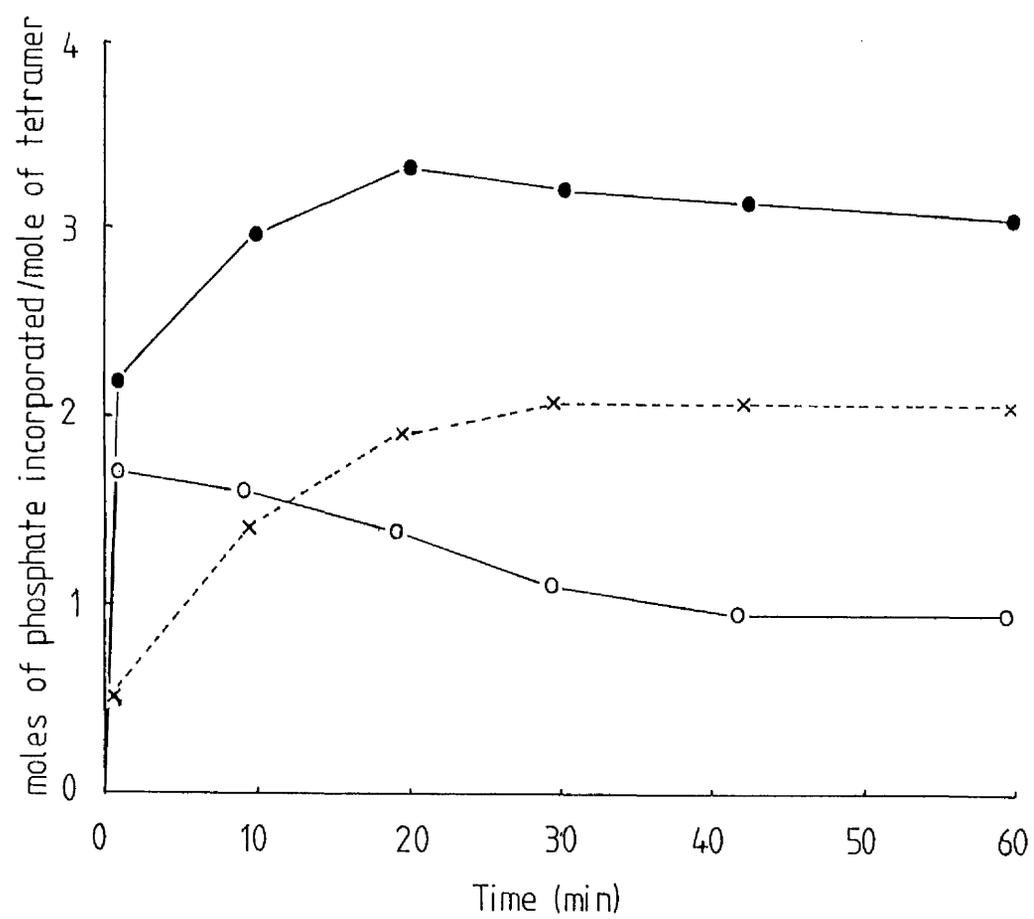
Phosphorylation of ATP citrate lyase

The phosphorylation of ATPCL was carried out at 30°C. Each incubation contained 0.5 mg/ml ATPCL, 5 mM MgCl₂, 0.5 mM [γ -³²P]-ATP (100000 cpm/n mole) and the components described in the legend to Figure 6.1. Reactions were started by the addition of 5 mM ATP/50 mM MgCl₂ and samples (usually 25 μ l) were removed after various times. The incorporation of ³²P into protein was measured after precipitation with 5% TCA by the method given in Chapter 2.2. The results from a typical experiment are illustrated in Figure 6.1. These results show that in the presence of cAMPdPrK the phosphate incorporated into ATPCL reaches a level of 3.0 moles of phosphate / mole of tetramer after 20 min and remains at this level for at least 40 min. A significant amount of phosphate is incorporated into ATPCL in the absence of cAMPdPrK. After 1 min 1.6 moles of phosphate are incorporated: this level gradually falls off so that only 1 mole of phosphate/mole of tetramer remains after 60 min. By subtracting the incorporation in the absence of cAMPdPrK from the incorporation in its presence it is possible to determine the net incorporation due only to cAMPdPrK. This reached a level of 2.1 moles of phosphate/mole of tetramer after 30 min.

One interpretation of the incorporation of phosphate into ATPCL in the absence of cAMPdPrK would be that the preparation was contaminated with a highly active protein kinase. As discussed in Chapter 1.2, however, the active site of ATPCL can be phosphorylated when the enzyme is incubated in the presence of ATP and Mg²⁺ ions. This catalytic phosphate is acid-labile and I had assumed that in the

Figure 6.1. Phosphorylation of ATP citrate lyase in the presence and absence of cAMP dependent protein kinase.

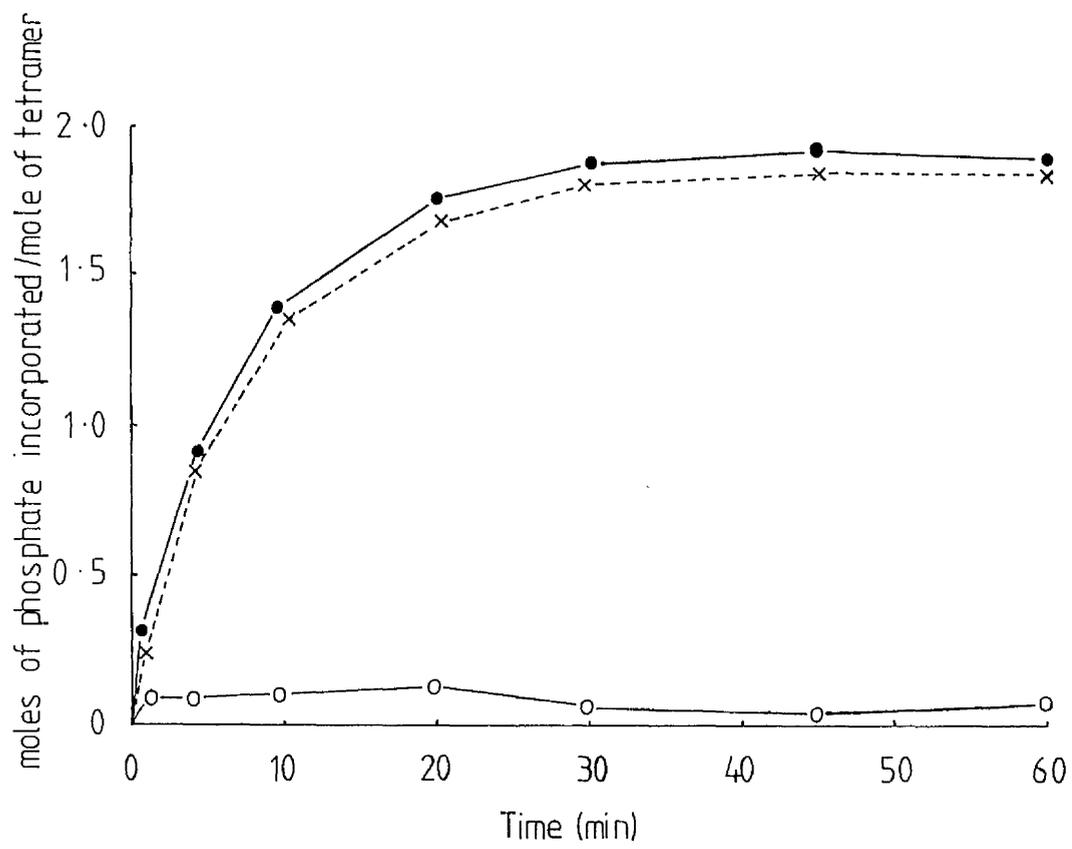
ATPCL was incubated with ATP and $MgCl_2$ in the presence (•) or absence (o) of the catalytic subunit of cAMPdPrK (30000 U/ml) as described in the text. The net phosphorylation due to cAMPdPrK determined by subtracting the incorporation in the absence of cAMPdPrK from that in its presence is indicated by (x).



experiment reported in Fig. 6.1, the precipitation with TCA would have been sufficient to remove any catalytic phosphate. It was possible, however, that incubation in 5% TCA for 20 min at 4°C was not sufficiently harsh to remove catalytic phosphate. In order to determine whether the phosphate incorporated into ATPCL in the absence of cAMPdPrK was all acid-labile (and hence presumably catalytic phosphate) the above experiment was repeated except that two samples were taken at each time point. In one set of samples the initial 5% TCA precipitates were redissolved in 0.1 N NaOH and then immediately reprecipitated with 10% TCA as described in Chapter 2.2. In the second set of samples the initial 5% TCA precipitates were redissolved in 100 μ l of 15% (v/v) acetic acid, 0.2 N HCl and then incubated at 40°C for 16 h (modified from Ramakrishna and Benjamin, 1981). After this incubation the protein was precipitated with 10% TCA and the remainder of the procedure given in Chapter 2.2 was followed. The results of this experiment are illustrated in Figure 6.2. This shows that in samples treated with HCl and acetic acid no phosphate is found in the ATPCL incubated in the absence of cAMPdPrK. The time course of and maximum level of phosphorylation of ATPCL incubated in the presence of cAMPdPrK as judged by the samples treated with HCl, is identical to the net incorporation (defined as incorporation $+cAMPdPrK$ less incorporation $-cAMPdPrK$) in the samples which had been treated with NaOH. These results confirm that the phosphate incorporated into ATPCL in the absence of cAMPdPrK is acid-labile and hence has presumably been incorporated into the active site of the enzyme. Fig. 6.1 shows that this 'catalytic' phosphorylation is extremely rapid. The reason for the subsequent slow decrease in the amount of catalytic phosphate incorporated is not known. A similar pattern of catalytic phosphorylation was noted by Ramakrishna and Benjamin (1981).

Figure 6.2. Phosphorylation of ATP citrate lyase by cAMP dependent protein kinase.

ATPCL was incubated in the presence or absence of the catalytic subunit of cAMPdPrK as described in the legend of Fig.6.1. Samples were removed at the times indicated and the amount of phosphate incorporated into ATPCL was determined after treatment with acetic acid/HCl or with NaOH as described in the text. (●), ATPCL incubated in the presence of cAMPdPrK and treated with acetic acid/HCl; (○), ATPCL incubated in the absence of cAMPdPrK and treated with acetic acid/HCl; (x) net phosphorylation due to cAMPdPrK in the NaOH treated samples.



Somewhat surprisingly, Guy *et al* (1980) working with rat mammary gland ATPCL did not observe any incorporation of catalytic phosphate into their preparation. This cannot be due to differences in the methods used to measure incorporation of phosphate into protein since a similar TCA precipitation procedure was used in both this present study and that of Guy *et al* (1980).

The results shown in Fig. 6.2 indicate that it is, therefore, not necessary to involve a cAMP independent protein kinase to explain the high incorporation of phosphate in the absence of cAMPdPrK. However, it is not possible to rule out completely the possibility that such a kinase is present and can phosphorylate ATPCL on, for example, a glutamyl residue thus giving an acid-labile phosphate.

Location of ^{32}P on polyacrylamide gels

Samples of ATPCL were incubated with ATP and MgCl_2 in the presence or absence of cAMPdPrK as described in Figs. 6.1 and 6.2. Samples were removed after the incorporation of acid-stable phosphate had reached a plateau of 2 moles of phosphate/mole of tetramer and then electrophoresed on 6% polyacrylamide gels in the presence of SDS. The gels were then stained for protein. After destaining the gels were scanned at 600 nm. The gels were then frozen with dry ice and cut into 2 mm slices and the ^{32}P content of each slice was measured as described in Chapter 2.2. (Figs. 6.3 and 6.4). In both cases only one protein staining band, with an M_r equal to that of the subunit of ATPCL (123000), was apparent. In the sample which had been incubated with cAMPdPrK all of the ^{32}P was located in the protein staining band. No radioactivity was located in the gel corresponding to the sample incubated without cAMPdPrK. These results confirm that the ^{32}P incorporated into protein in the



Figure 6.3. SDS-polyacrylamide gel electrophoresis of ATP citrate lyase incubated with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and MgCl_2 in the absence of cAMP dependent protein kinase.

The experiment was performed as described in the text. SDS-polyacrylamide gel electrophoresis was performed on 6% gels by the method of Laemmli (1970). The gel was scanned at 600 nm (continuous line) then sliced and the ^{32}P content of each slice measured (crenellated line). $5\ \mu\text{g}$ of protein was applied to the gel.

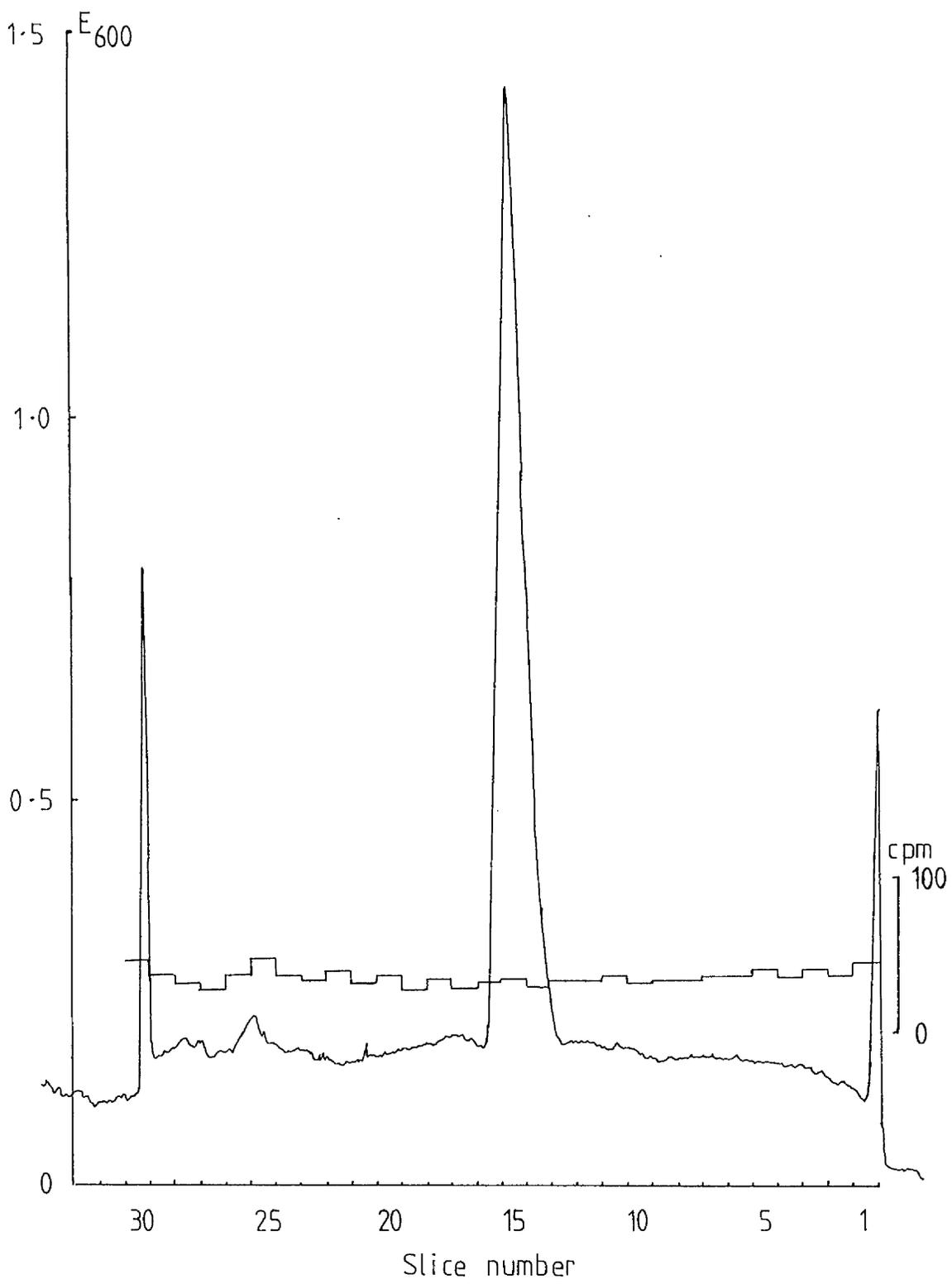
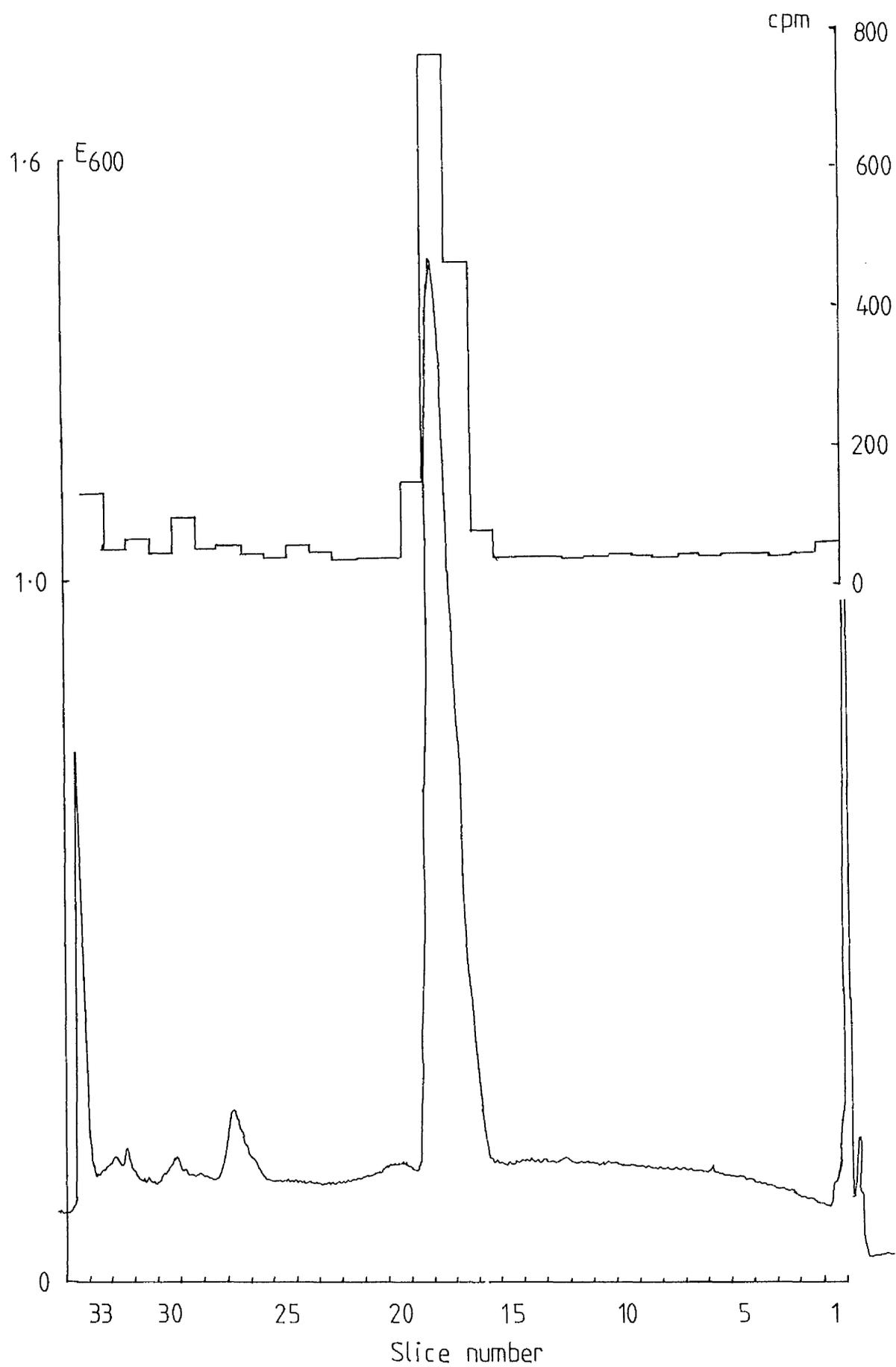


Figure 6.4. SDS-polyacrylamide gel electrophoresis of ATP citrate lyase incubated with [γ - 32 P]-ATP and $MgCl_2$ in the presence of cAMP dependent protein kinase.

The experiment was performed as described in the text. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Gels were scanned at 600 nm (continuous line) and the 32 P content of each slice measured (crenellated line). 5 μ g of protein was applied to the gel.



experiments shown in Figs. 6.1 and 6.2 is indeed incorporated into ATPCL. They also show that the ^{32}P is incorporated into undegraded ATPCL rather than into a proteolytic fragment. No trace of catalytic phosphate was found in the sample which had been incubated without cAMPdPrK. This implies that the conditions used to destain the gels (10% acetic acid at 40°C for at least 36 h) were sufficient to remove the acid-labile catalytic phosphate. Indeed, this method of removal of catalytic phosphate has been used for quantitation of the amount of phosphate incorporated into ATPCL by autoradiography of electrophoresed samples (Alexander *et al.*, 1981; Ramakrishna and Benjamin, 1981).

In another experiment samples of ATPCL were incubated with ATP and MgCl_2 in the presence or absence of cAMPdPrK as described in Figs 6.1 and 6.2 and then analysed by non-denaturing polyacrylamide gel electrophoresis. Samples from each incubation were removed after the incorporation due to cAMPdPrK had reached a plateau of 2 moles of phosphate/mole of tetramer and electrophoresed on 3% polyacrylamide gels. After electrophoresis the gels were stained for ATPCL activity and then scanned at 600 nm. The gels were quickly frozen with dry ice and cut into 2 mm slices and the ^{32}P content of each slice was then determined by the method given in Chapter 2.2. The results of this experiment are shown in Figures 6.5 and 6.6. Both samples were successfully stained for ATPCL activity. In the sample which had been incubated with cAMPdPrK all of the radioactivity was located in the ATPCL activity band. A much smaller amount of radioactivity co-migrated with the ATPCL activity in the sample incubated in the absence of cAMPdPrK. This is almost certainly catalytic phosphate since the samples which had been subjected to non-denaturing polyacrylamide gel electrophoresis followed by staining for ATPCL activity had not been treated in any way that

Figure 6.5. Non-denaturing polyacrylamide gel electrophoresis of ATP citrate lyase incubated with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ in the absence of cAMP dependent protein kinase.

The experiment was performed as described in the text.

Polyacrylamide gel electrophoresis was performed by the method of Davis (1964). After electrophoresis the gel was stained for ATPCL activity and then scanned at 600 nm (continuous line). The gel was then sliced and the ^{32}P content of each slice measured (crenella line). $10\ \mu\text{g}$ of protein was applied to the gel.

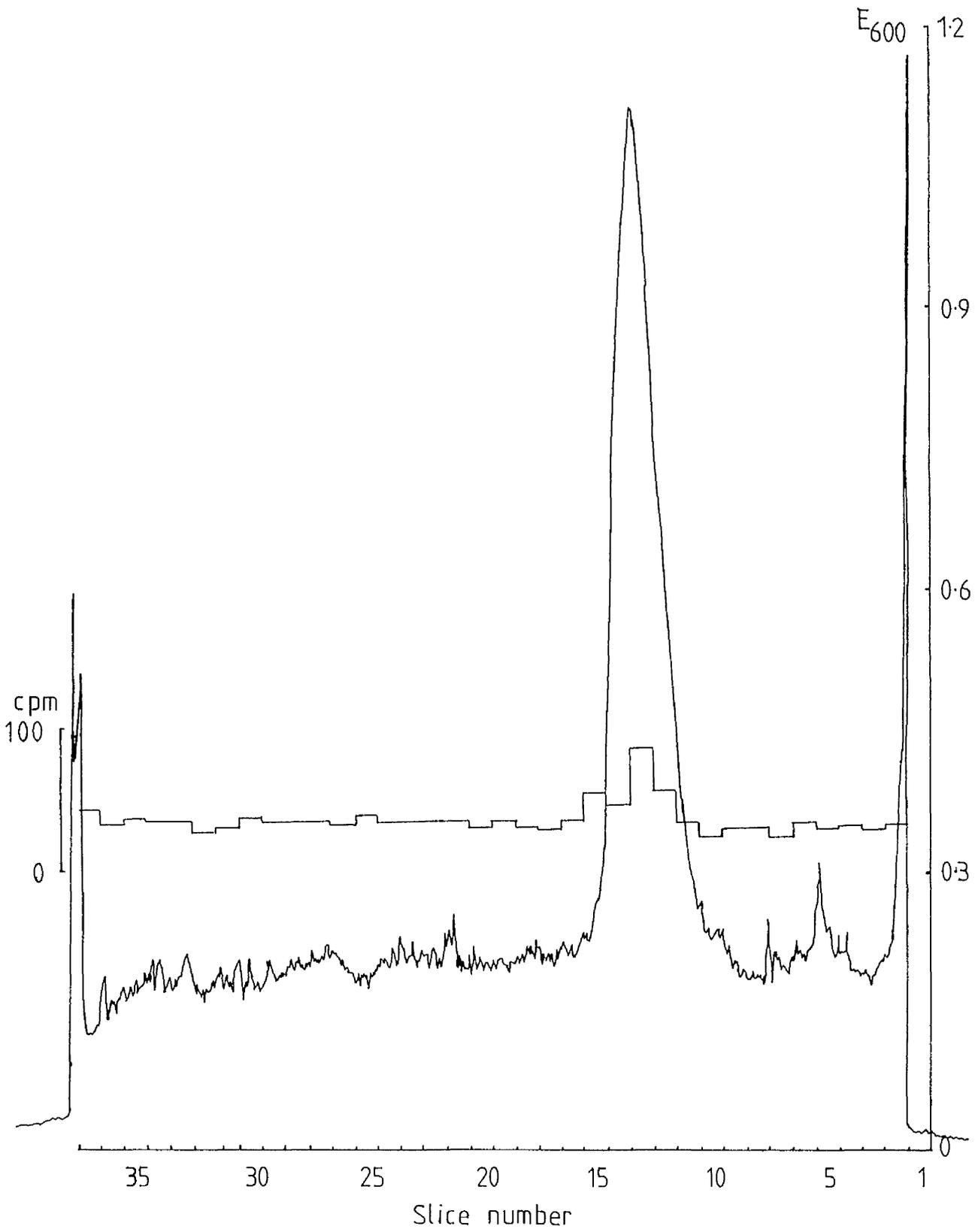
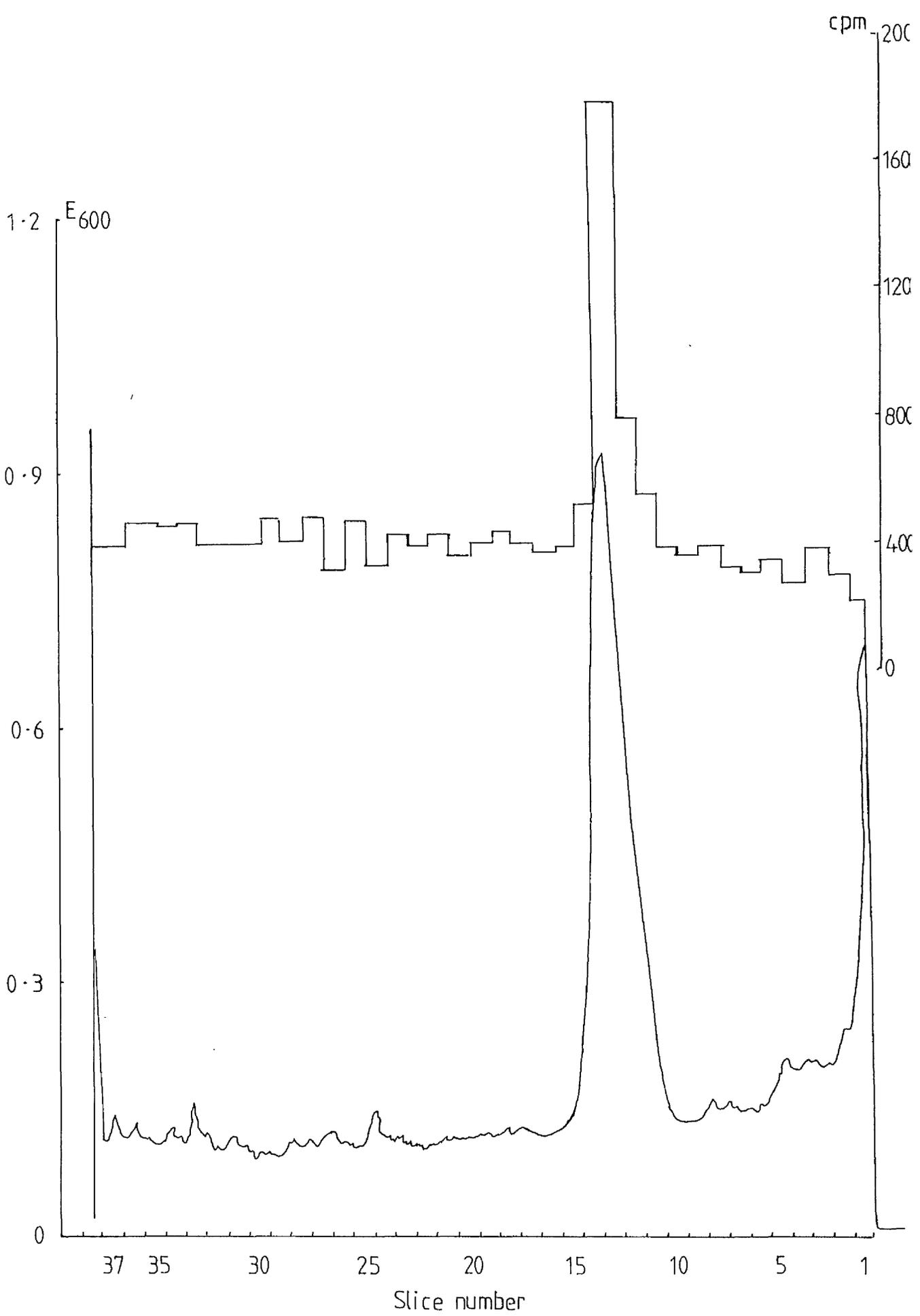


Figure 6.6. Non-denaturing polyacrylamide gel electrophoresis of ATP citrate lyase incubated with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ in the presence of cAMP dependent protein kinase.

The experiment was performed as described in the text. Polyacrylamide gel electrophoresis was performed by the method of Davis (1964). After electrophoresis the gel was stained for ATPCL activity and then scanned at 600 nm (continuous line). The gel was then sliced and the ^{32}P content of each slice measured. $10\ \mu\text{g}$ of protein was applied to the gel.



would remove catalytic phosphate. When samples of ATPCL which had been incubated in the presence of cAMPdPrK were subjected to non-denaturing polyacrylamide gel electrophoresis and then stained for protein only one band was visible (cf. Fig. 5.5). This band had an identical R_f to the ATPCL activity stain band shown in Fig. 5.6. After the gels had been sliced, the radioactivity in each slice was determined and it was found that all of the ^{32}P was located in the protein stained band. No radioactivity was located in samples which had been incubated in the absence of cAMPdPrK (results not shown).

These results show quite conclusively that in the experiments described in Figs. 6.1 and 6.2 the phosphate is incorporated into ATPCL rather than into some minor contaminant.

Stoichiometry of phosphate incorporation into ATP citrate lyase

In order to obtain a reliable value for the stoichiometry of phosphorylation of a protein it is necessary to have an accurate knowledge of both the protein concentration and the specific radioactivity of the ATP used. In this work the protein concentration of pure ATPCL was determined spectrophotometrically using the absorption coefficient of 11.4 given in Singh *et al.*, 1976. The concentration of ATP in stock solutions of [γ - ^{32}P]-ATP was assayed as described in Chapter 2.2. The cpm of 50 μl aliquots of the stock [γ - ^{32}P]-ATP solution was measured during each experiment. Thus the specific radioactivity of the ATP used could be calculated.

Figs 6.1 and 6.2 indicate that rATPCL is phosphorylated by cAMPdPrK to the extent of approximately 2 moles of phosphate/mole of tetramer. In a series of experiments the amount of phosphate incorporated into ATPCL was 2.08 ± 0.15 moles of phosphate/mole of

tetramer (mean and standard deviation of data from 9 experiments). The stoichiometry of phosphate incorporation into ATPCL was calculated using a subunit M_r of 123000. The degree of incorporation could not be increased by raising the concentration of ATP/MgCl₂ or by increasing the amount of cAMPdPrK relative to ATPCL.

The level of phosphorylation found in this work is in keeping with the results of other workers. Similar levels of incorporation have been reported by Guy et al (1980) (2.8 moles of phosphate/mole of tetramer) and by Alexander et al (1981) (2 - 3 moles of phosphate/mole of tetramer). These results seem to indicate that only half of the supposedly identical subunits of ATPCL are phosphorylated by cAMPdPrK. One possible reason for this, proposed by Pierce et al (1981) is that half of the phosphorylation sites are sequestered from the kinase by virtue of the tertiary or quaternary structure of ATPCL. In support of this proposal they state that under 'mildly denaturing' conditions ATPCL can be phosphorylated by cAMPdPrK to the extent of 4 moles of phosphate/mole of tetramer and that the additional phosphate is located on the same tryptic peptide as that which accepts the first 2 moles of phosphate. No indication was given of the conditions used to denature ATPCL. In my hands, phosphorylating ATPCL in the presence of 1 M urea did not increase the extent of phosphorylation beyond 2.2 moles of phosphate/mole of tetramer. The sequestration hypothesis might also explain the finding that ATPCL is phosphorylated to the same extent (2 moles of phosphate/mole of tetramer) by the cAMP independent protein kinase described by Ramakrishna and Benjamin (1981). Preliminary work by Avruch's group (Pierce et al, 1981) suggests that the amino acid phosphorylated in response to insulin is located on the same tryptic peptide as the serine phosphorylated by cAMPdPrK. It must be stated, however, that it is

far from certain that the kinase described by Ramakrishna and Benjamin (1981) is in fact responsible for the increased phosphorylation in response to insulin.

One other possible explanation for the substoichiometric incorporation is that the cAMPdPrK sites are partially occupied in purified ATPCL. Analysis of the alkali-labile phosphate content of one batch of ATPCL used in this work revealed a phosphate content of 2.5 moles of phosphate/mole of tetramer even though no precautions had been taken against phosphatase activity during the purification. A similar level of structural phosphate was observed by Linn and Srere (1979). Clarification of the reasons for the incorporation of only 2 moles of phosphate/mole of tetramer must await the determination of the sites phosphorylated by kinases in vitro and the site(s) occupied by the basal alkali-labile phosphate present in purified ATPCL.

Effect of cAMP dependent protein kinase dependent phosphorylation on ATP citrate lyase activity

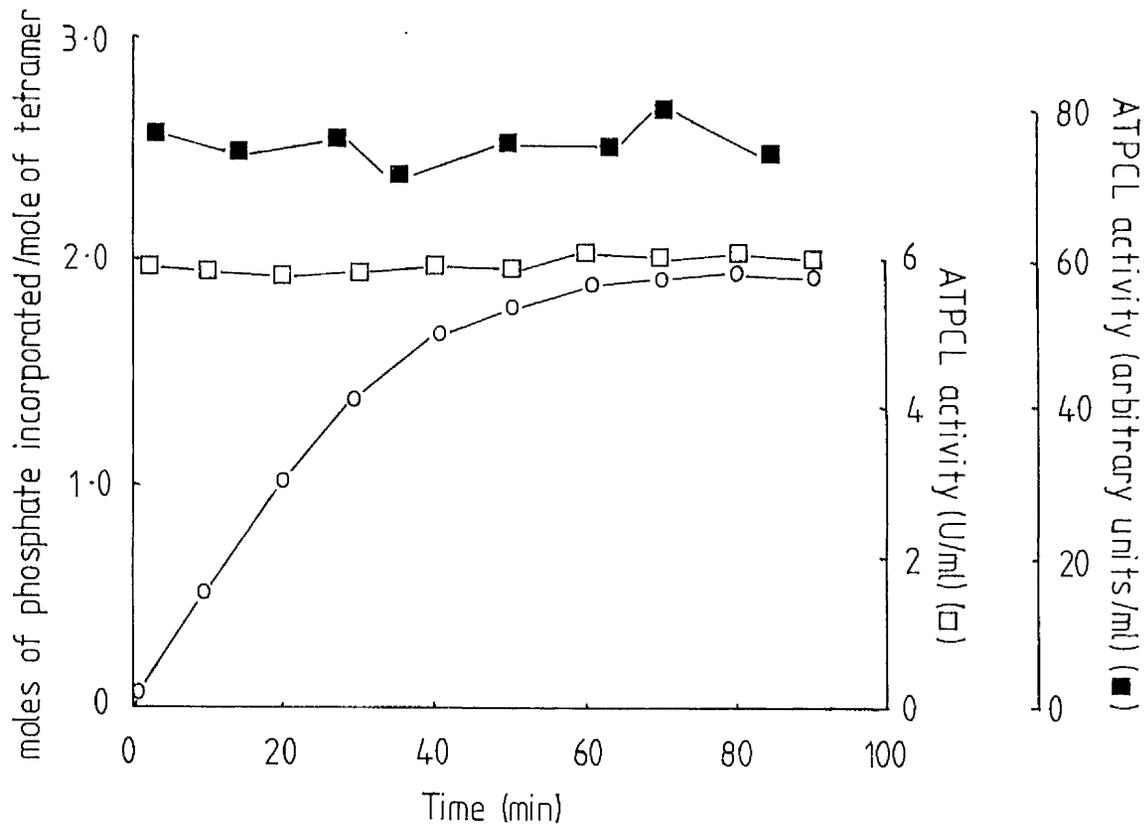
As discussed in Chapter 1.3, Guy et al (1981) were unable to detect changes in the activity of ATPCL following phosphorylation with cAMPdPrK using the assay method of Linn and Srere (1979). These are the only workers who have attempted to detect changes in ATPCL activity concomitant with cAMPdPrK dependent phosphorylation in vitro. Linn and Srere (1979) purified rATPCL which had an endogenous structural phosphate content of 2 moles of phosphate/mole of tetramer. No change in ATPCL activity was observed following the removal of the structural phosphate using a partially purified phosphatase from rat liver. It must be borne in mind, however, that the phosphorylated ATPCL purified by Linn and Srere (1979) was not necessarily phosphorylated in vivo by cAMPdPrK. The assay method used in the two studies discussed above (Linn and Srere, 1979)

measures the V_{\max} of ATPCL. The concentrations of substrates employed in this assay are: ATP, 5 mM; potassium citrate, 20 mM; CoA, 200 μ M. These concentrations are respectively 17.6, 285 and 123 times the Michaelis constants quoted by Plowman and Cleland (1967). This degree of saturation with substrates makes it unlikely that any change in the activity of ATPCL would be observed, other than one due to a change in the V_{\max} . For example, if the K_m of ATPCL for CoA was increased 10-fold upon phosphorylation, the enzyme would still be saturated with CoA and the observed reaction velocity would not change. In most cases phosphorylation by cAMPdPrK does result in a change in K_m rather than V_{\max} .

It seemed desirable therefore that when attempting to detect changes in ATPCL activity the assays be performed under conditions which would more accurately reflect the concentrations of substrates found in the cell. Each cuvette contained, in a final volume of 1.0 ml, 50 mM MOPS, 1.5 mM β -mercaptoethanol, 4.25 mM MgATP, 0.35 mM Mg-citrate, 1 mM free Mg^{2+} , 7 μ M CoA, 0.05 mM NADH, 2 units of MDH, pH 7.0. Assays were performed at 37°C with the chart recorder set to give a full scale deflection of 0.025. Figure 6.7 shows the results of an experiment in which the activity of ATPCL was monitored during the phosphorylation reaction by either the method of Linn and Srere (1979), or the assay method described above. The incorporation of phosphate reached a level of 1.9 moles of phosphate/mole of tetramer after 60 min and remained at this level for the next 30 min. No change in the activity of ATPCL was observed using either assay method. These results confirm those of Guy et al (1981) that changes in ATPCL activity cannot be detected using the assay of Linn and Srere (1979). The results also appear to contradict those of Roehrig (1980) who reported in an abstract that phosphorylation

Figure 6.7. Activity of ATP citrate lyase during phosphorylation by cAMP dependent protein kinase.

The experiment was carried out as described in the text. Samples were removed at the times indicated and the incorporation of phosphate into ATPCL was determined (○). Samples were also removed and assayed for ATPCL activity using either the method of Linn and Srere (1979) (□) or the 'physiological' assay described in the text (■).



dependent changes in ATPGL activity could be detected by assaying at low CoA concentrations; further details of the experiment were not given. No other workers have reported monitoring the activity of ATPGL during phosphorylation by cAMP_iPrK using an assay which employs low or physiological concentrations of substrates.

From the results described above it is apparent that changes in ATPGL activity concomitant with phosphorylation by cAMP_iPrK cannot be detected using either the standard V_{\max} assay or an assay in which the substrates are at physiological concentrations. In order to determine whether phosphorylation by cAMP_iPrK does alter any of the kinetic parameters of ATPGL, a detailed comparison of the kinetic parameters of the phosphorylated and non-phosphorylated forms of ATPGL was undertaken. This work is described in the following section.

Section 2 A study of the kinetic properties of ATP citrate lyase after phosphorylation by cAMP dependent protein kinase

As discussed in Section 1 of this chapter, I was unable to detect changes in the activity of ATPCL concomitant with phosphorylation by cAMPdPrK using either of the two sets of assay conditions described, a result in keeping with that of Guy et al (1981). It was apparent, therefore, that a detailed study of the kinetic properties of ATPCL before and after phosphorylation by cAMPdPrK would be required to determine the effect (if any) of phosphorylation upon the activity of the enzyme.

Studies by other workers

Only two groups have attempted to make a detailed comparison of the kinetic parameters of phosphorylated and non-phosphorylated ATPCL. Guy et al (1981) phosphorylated rat mammary gland ATPCL to the extent of 2.8 moles of phosphate/mole of tetramer in vitro using cAMPdPrK. They then determined the Michaelis constants of the phosphorylated and non-phosphorylated ATPCL for ATP, citrate and CoA. This was done using a modification of the assay of Linn and Srere (1979) where one of the substrates was varied while the concentrations of the other two were held constant. These workers found no difference between the phosphorylated and non-phosphorylated forms with respect to the V_{\max} or the K_m s for ATP, citrate and CoA. One can criticise this work, however, on the grounds that the substrates of ATPCL are MgATP and Mg-citrate rather than the non-complexed forms (Flowman and Cleland, 1967). Furthermore the complexed and non-complexed forms, and free Mg^{2+} , are in equilibrium. This means that when Guy et al (1981) varied the amount of ATP added to each assay the concentrations of ATP, MgATP, citrate, Mg-citrate and free Mg^{2+} would change. It is not possible, since insufficient experimental details are

given, to determine the levels of MgATP, Mg-citrate and free Mg^{2+} actually used. It is possible that, since the concentrations of the substrates were not rigorously controlled, a change in the K_m for MgATP or Mg-citrate would have gone undetected.

A more extensive comparison of the kinetic properties of phosphorylated and non-phosphorylated ATPCL was made by Ranganathan et al (1980). These workers purified r1ATPCL containing 2 moles of phosphate/mole of tetramer and using a partially purified phosphatase, prepared dephosphorylated ATPCL. They then compared the kinetic parameters of the phosphorylated and dephosphorylated forms using two different buffer systems (100 mM Tris-HCl, pH 8.7 and 10 mM HEPES, pH 7.6). These authors found no difference between the dephosphorylated and phosphorylated forms with respect to the K_m s for ATP, citrate and CoA using the assay buffered with Tris-HCl. Using the HEPES assay they found that the K_m s for citrate and CoA did not change but they observed a change in the K_m for ATP from 54 μ M (phosphorylated) to 292 μ M (dephosphorylated). Ranganathan et al (1980) state that it is not clear why the change in the K_m was apparent in only the HEPES buffered assay. The two buffer systems were not comparable, however. In order to obtain approximately physiological conditions, the substrate concentrations in the HEPES system were much lower than those in the Tris-HCl system. Furthermore, ADP (1 mM) and Pi (4 mM) were present in the HEPES system. As Ranganathan et al (1980) point out, the change in the K_m for ATP is the opposite to what one would expect, based on the known effects of phosphorylation by cAMPdPrK on lipogenic enzymes such as ACC (see Chapter 1.3 for a discussion of this).

At the time that the work by Ranganathan et al (1980) was published there was no information (either in that report or in the

earlier one by Linn and Srere, 1979) concerning the identity of the kinase which introduced the endogenous phosphate onto the ATPGL. There was also no information as to whether the amount of the endogenous phosphate altered in response to changes in the hormonal status of the animal. Hence, the physiological significance of the endogenous phosphate was unclear. In a subsequent report, however, Ranganathan et al (1982) provided evidence that the phosphate group removed by the partially purified phosphatase was located on the same tryptic peptide as the cAMPdPrK phosphorylation site, a finding which the authors interpreted as indicating that the incorporation of the endogenous phosphate was catalysed by cAMPdPrK.

Irrespective of the nature of the phosphorylated and dephosphorylated ATPGL in the work reported by Ranganathan et al (1980), the Michaelis constants were determined for non-complexed ATP and citrate. In addition, no attempt was made to keep the free Mg^{2+} concentration constant. The same criticisms which were made about the work by Guy et al (1981) can therefore be made about the work of Ranganathan et al (1980).

The common criticism of the work discussed above is that the kinetic studies were not carried out sufficiently well to enable one to say conclusively that there was no change in the Michaelis constants for MgATP and Mg-citrate. In the kinetic experiments reported in Chapter 5.2, two of the substrates were varied at either a high or a low concentration of the third. These experiments were performed using an assay system in which the concentrations of MgATP, Mg-citrate and free Mg^{2+} ions could be calculated from the amounts of ATP, citrate and magnesium acetate added. The concentrations of MgATP and Mg-citrate could therefore be systematically varied and the concentration of free Mg^{2+} kept constant. Thus, this assay system

is suitable for studying the kinetic properties of phosphorylated and non-phosphorylated ATPCL.

Preparation of phosphorylated and non-phosphorylated
ATP citrate lyase

ATPCL was phosphorylated by cAMPdPrK under the conditions described in Section 1 of this chapter in a final volume of 1.0 ml. As a control a second sample of ATPCL was incubated under identical conditions except that the cAMPdPrK was omitted. At intervals throughout the incubations 25 μ l samples were removed and the radioactivity incorporated into ATPCL was measured as described in Chapter 2.2. The remainder of the procedure was performed at 4^oC. After the net incorporation of phosphate catalysed by cAMPdPrK had reached a plateau of 2 moles of phosphate/mole of tetramer, the incubation mixtures (800 μ l) were applied to columns of Sephadex G-50 (Fine) (1.1 cm x 25 cm) equilibrated in 100 mM TEA-HCl, 20 mM NaF, 2 mM benzamidine-HCl, 1 mM DTT, pH 7.5 (Buffer E). After application of each sample, the column was developed with Buffer E (flow rate 1 ml/min) and 1 ml fractions were collected. The radioactivity in each fraction was measured and the fractions from the first peak of radioactivity to emerge from the column (corresponding to phosphorylated ATPCL) were pooled. They were dialysed against 2l of buffer E overnight and then against 250 ml of buffer E containing 40% (v/v) glycerol for a further 6 h. Control and phosphorylated ATPCL were stored at - 20^oC in buffer E containing 40% (v/v) glycerol prior to use. As expected, the control ATPCL contained covalently bound phosphate. ATPCL which had been incubated in the presence of cAMPdPrK had approximately 4 times as much phosphate as the control ATPCL.

There was no difference between the specific activities of the

control and phosphorylated ATPCL when they were assayed using the method of Linn and Srere (1979). There was no loss of phosphate from the phosphorylated ATPCL, nor a change in the specific activities of the control and phosphorylated enzymes during the period in which the kinetic measurements were performed (6 days).

Initial velocity measurements

All initial velocity measurements were made under the conditions given in Chapter 2.2 using the MDH coupled assay and at the substrate concentrations given in Figures 6.8 - 6.13. The results are presented as double reciprocal plots of initial velocity against variable substrate concentration. The results shown in Fig. 6.8 (control) and Fig. 6.9 (phosphorylated) are from an experiment where Mg-citrate was varied at different fixed levels of MgATP and at a constant high CoA concentration. The results shown in Fig. 6.10 (control) and Fig. 6.11 (phosphorylated) are from an experiment where Mg-citrate was varied at different fixed levels of MgATP and at a constant high CoA concentration. The results shown in Fig 6.12 (control) and Fig. 6.13 (phosphorylated) are from an experiment where CoA was varied at different fixed levels of MgATP and at a constant high Mg-citrate concentration.

The patterns of lines obtained in these experiments are similar to those in Figs. 5.15 - 5.21 in Chapter 5.2. The lines are parallel at constant high concentrations of Mg-citrate and CoA and intersecting at a constant high concentration of MgATP. This is true for both the phosphorylated and non-phosphorylated ATPCL. Note, however, that while there is no noticeable difference between Figs. 6.8 and 6.9 or between Figs 6.10 and 6.11, there is an obvious difference between Figs. 6.12 and 6.13. The results presented in

Figure 6.8. Initial velocity measurements of unphosphorylated
ATP citrate lyase at a constant high CoA concentration.

ATPCL was incubated in the absence of cAMPdPrK then gel filtered and dialysed as described in the text. The initial velocity measurements were made using Method (a) described in Chapter 2.2 and using the Mg-citrate concentrations indicated. The MgATP concentrations used were: 20 μM (\square); 40 μM (\blacksquare); 76 μM (\circ); 400 μM (\bullet). The CoA concentration was 200 μM .

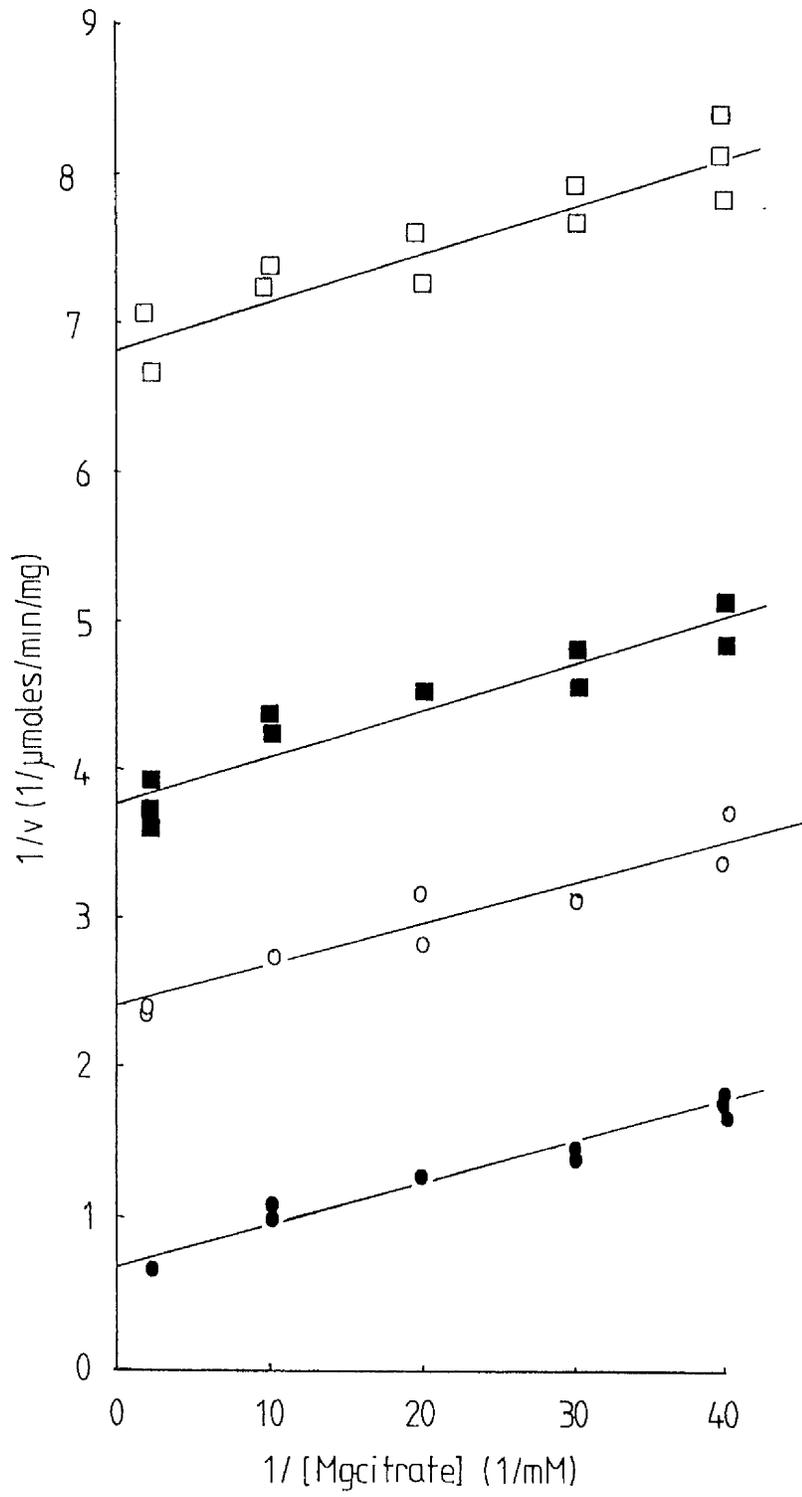


Figure 6.9. Initial velocity measurements of phosphorylated ATP citrate lyase at a constant high CoA concentration.

ATPCL was incubated in the presence of cAMPdPrK then gel filter and dialysed as described in the text. The initial velocity measurements were made using Method(a)described in Chapter 2.2 and the Mg-citrate concentrations indicated. The MgATP concentrations used were: 20 μ M, (\square); 40 μ M (\blacksquare); 76 μ M (\circ); 400 μ M (\bullet). The CoA concentration was 200 μ M.

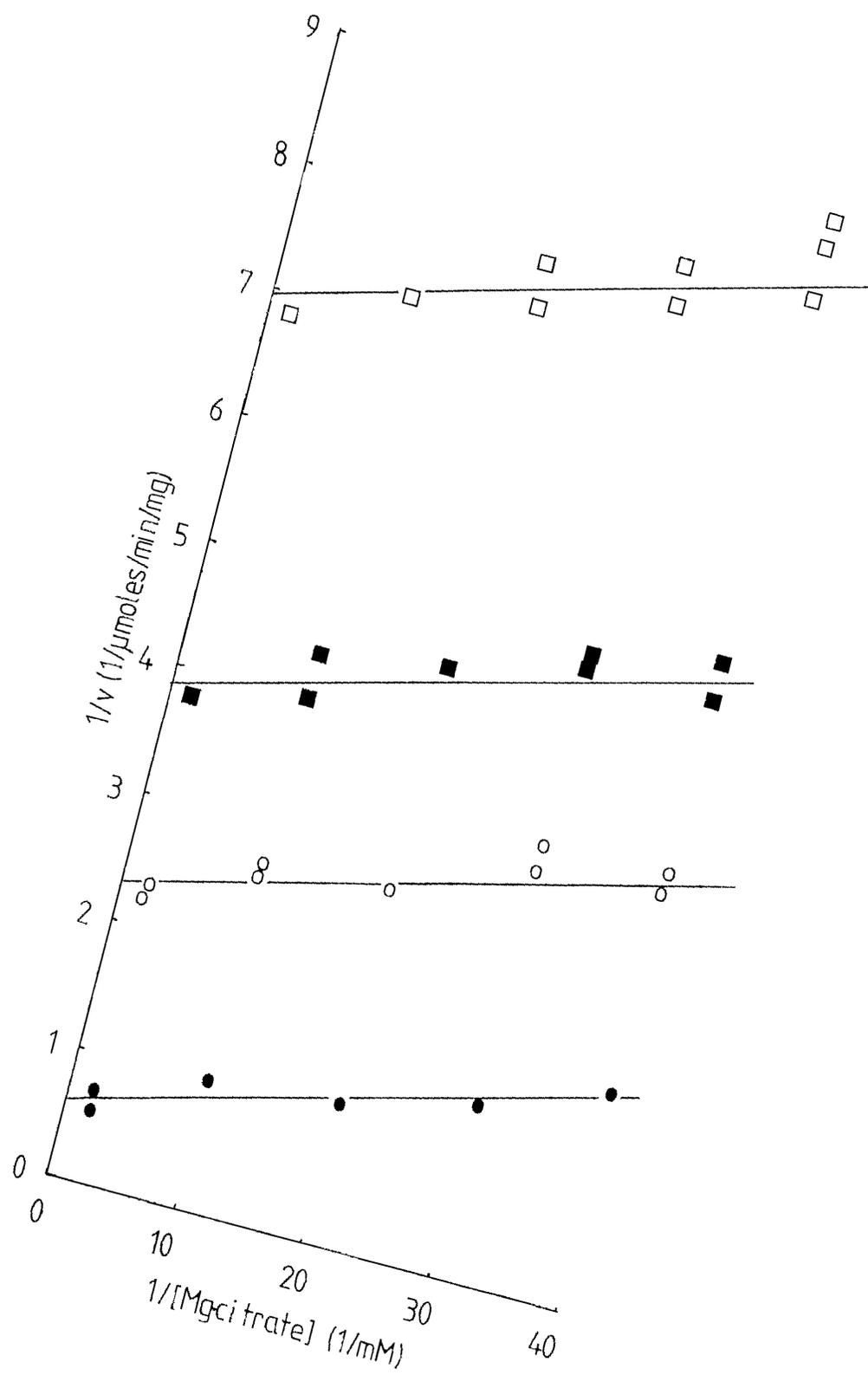


Figure 6.10. Initial velocity measurements of unphosphorylated ATP citrate lyase at a constant MgATP concentration.

ATPCL was incubated in the absence of cAMPdPrK then gel filtered and dialysed as described in the text. The initial velocity measurements were made using Method (a) described in Chapter 2.2 and using the Mg-citrate concentrations indicated. The CoA concentrations used were: $2 \mu\text{M}$ (\square); $3 \mu\text{M}$ (\blacksquare); $5 \mu\text{M}$ (\circ); $20 \mu\text{M}$ (\bullet). The MgATP concentration was 17.6 mM .

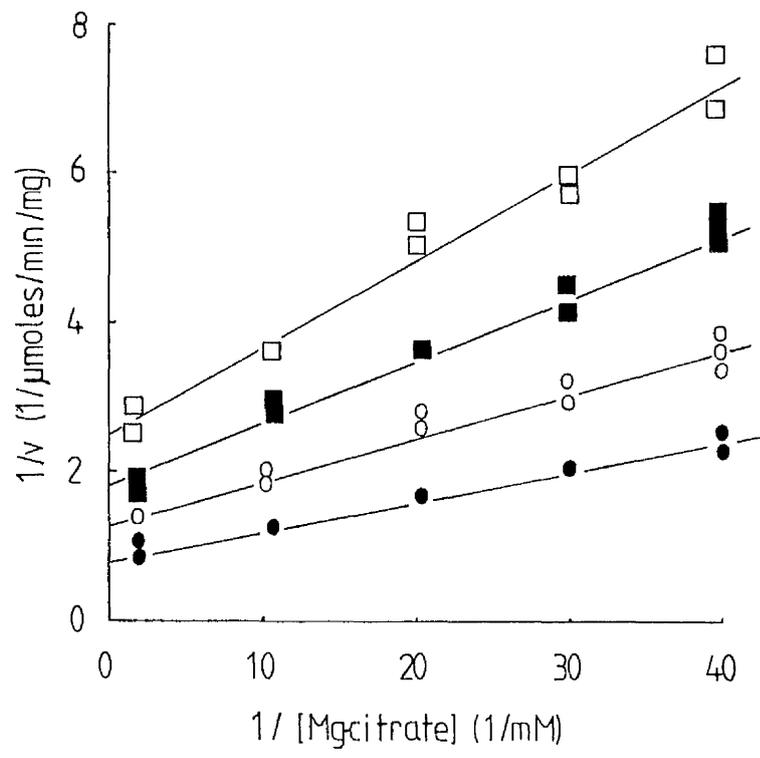


Figure 6.11. Initial velocity measurement of phosphorylated ATP
citrate lyase at a constant high MgATP concentration.

ATPCL was incubated in the presence of cAMPdPrK then gel filtered
and dialysed as described in the text. The initial velocity measure-
ments were made using Method (a) described in Chapter 2.2. The CoA
concentrations used were: $2\ \mu\text{M}$ (\square); $3\ \mu\text{M}$ (\blacksquare); $5\ \mu\text{M}$ (\circ);
 $20\ \mu\text{M}$ (\bullet). The MgATP concentration was 17.6 mM.

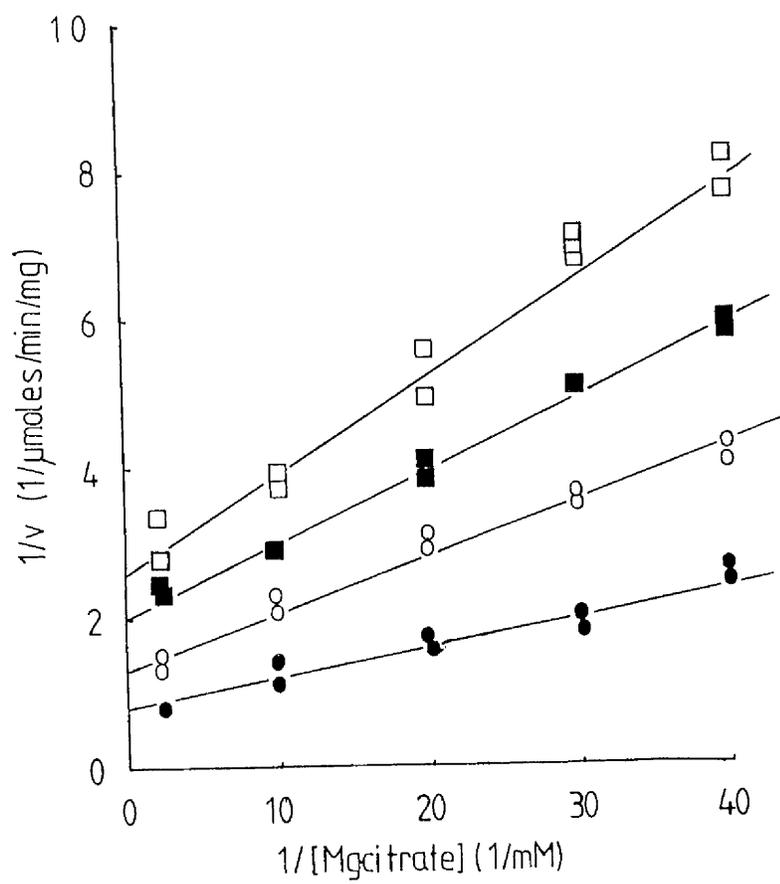


Figure 6.12. Initial velocity measurements of unphosphorylated ATP citrate lyase at a constant high magnesium citrate concentration.

ATPCL was incubated in the absence of cAMPdPrK then gel filtered and dialysed as described in the text. The initial velocity measurements were made using Method (a) described in Chapter 2.2 and at the CoA concentrations indicated. The MgATP concentrations used were: 20 μM (\square); 40 μM (\blacksquare); 76 μM (\circ); 400 μM (\bullet). The Mg-citrate concentration was 9 mM.

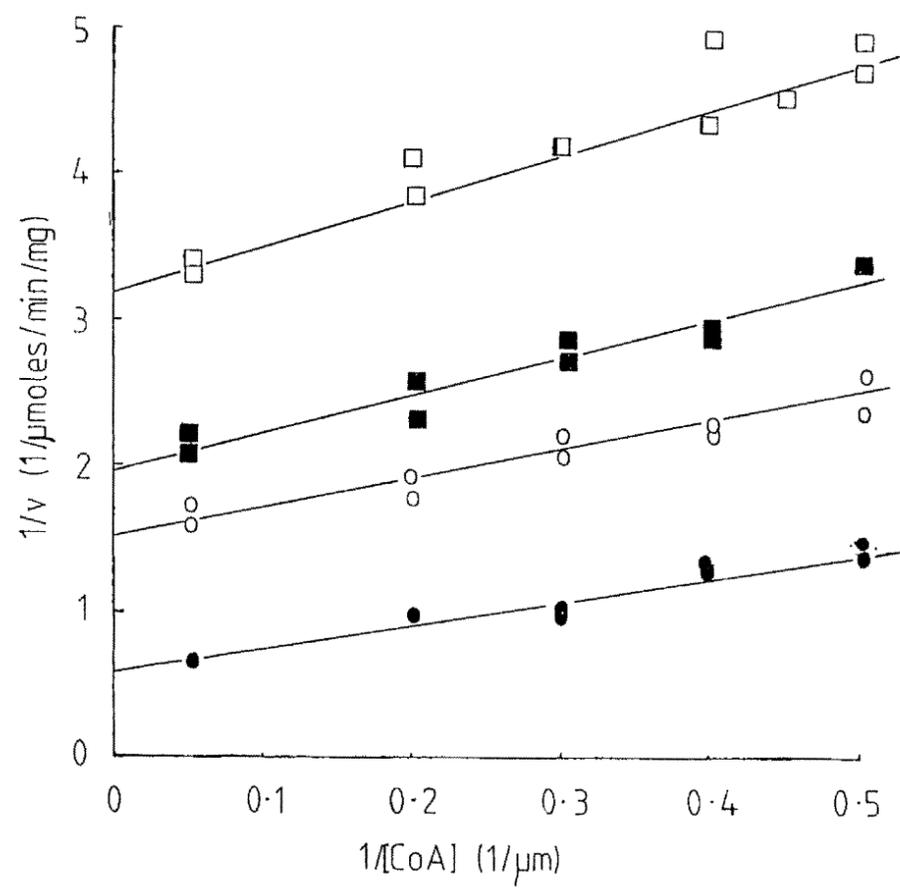
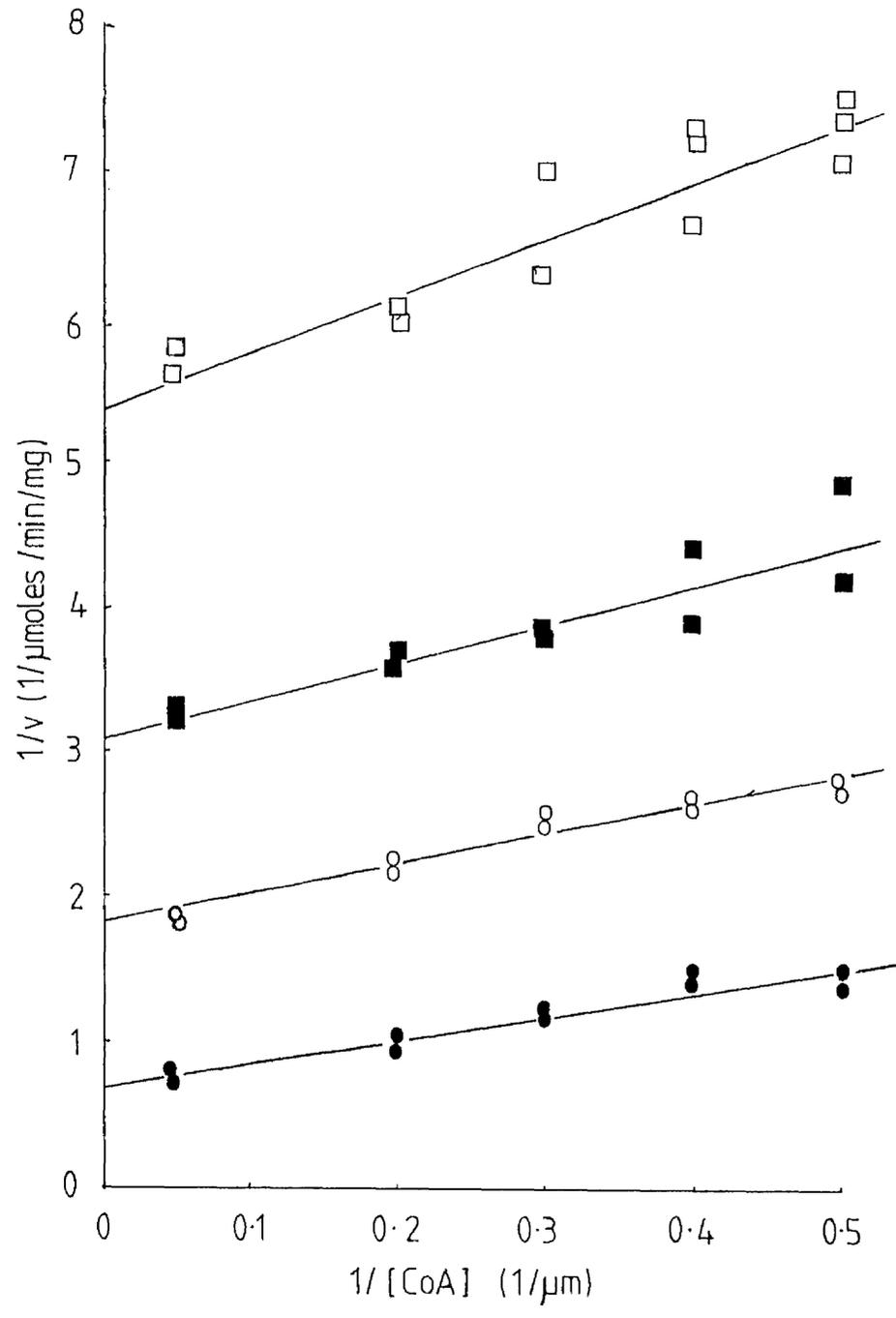


Figure 6.13. Initial velocity measurements of phosphorylated ATP citrate lyase at a constant high Mg-citrate concentra

ATPCL was incubated in the presence of cAMPdPrK then gel filter and dialysed as described in the text. The initial velocity measurements were made using Method (a) described in Chapter 2.2 and at the CoA concentrations indicated. The MgATP concentrations used were: $20\ \mu\text{M}$ (\square); $40\ \mu\text{M}$ (\blacksquare); $76\ \mu\text{M}$ (\circ); $400\ \mu\text{M}$ (\bullet). The Mg-citrate concentration was 9 mM.



Figs. 6.12 and 6.13 indicate that the phosphorylated ATPCL is less active than the control ATPCL at low MgATP concentrations. Note also that if the lines in Figs. 6.10 and 6.11 are extrapolated beyond the $1/v$ axis, the lines in both figures intersect on the $1/[Mg-citrate]$ axis at a point corresponding to a K_s for Mg-citrate of $48 \mu M$. This would suggest that the K_s for Mg-citrate does not change upon phosphorylation by cAMPdPrK.

The results presented in Chapter 5.2 indicate that the binding of CoA and Mg-citrate to ATPCL is random equilibrium. Under the experimental conditions described in the legends to Figs. 6.10 and 6.11, ie. saturating MgATP, the appropriate rate equation is:

$$V = \frac{V_{max}}{1 + \frac{K_m^{Mg-citrate}}{[Mg-citrate]} + \frac{K_m^{CoA}}{[CoA]} + \frac{K_s^{Mg-citrate} K_m^{CoA}}{[Mg-citrate][CoA]}}$$

where V_{max} is the maximum velocity, $K_m^{Mg-citrate}$ and K_m^{CoA} are the Michaelis constants for Mg-citrate and CoA respectively and $K_s^{Mg-citrate}$ is the apparent dissociation constant for Mg-citrate. Numerical values for these parameters were obtained by plotting the slopes and $1/v$ intercepts of the lines in Figs. 6.10 and 6.11 against $1/[CoA]$ (Figure 6.14). Since the binding of Mg-citrate and CoA to ATPCL is random equilibrium it was possible to calculate a numerical value for K_s^{CoA} , the apparent dissociation constant for CoA, as in this case:

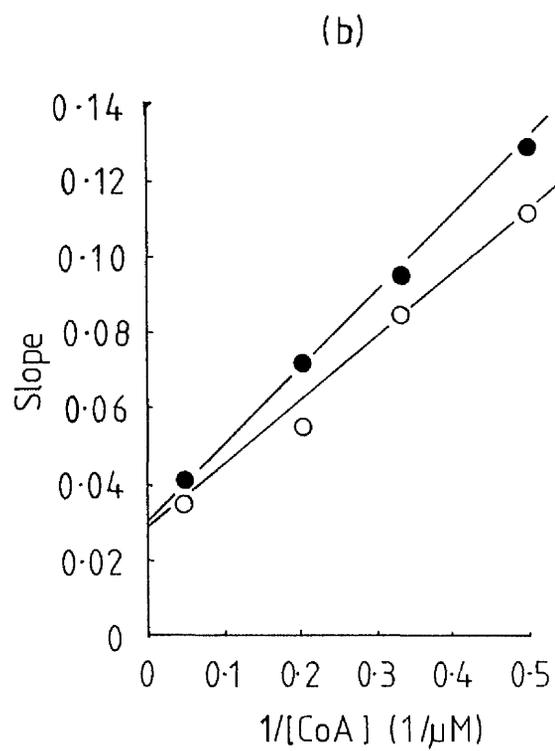
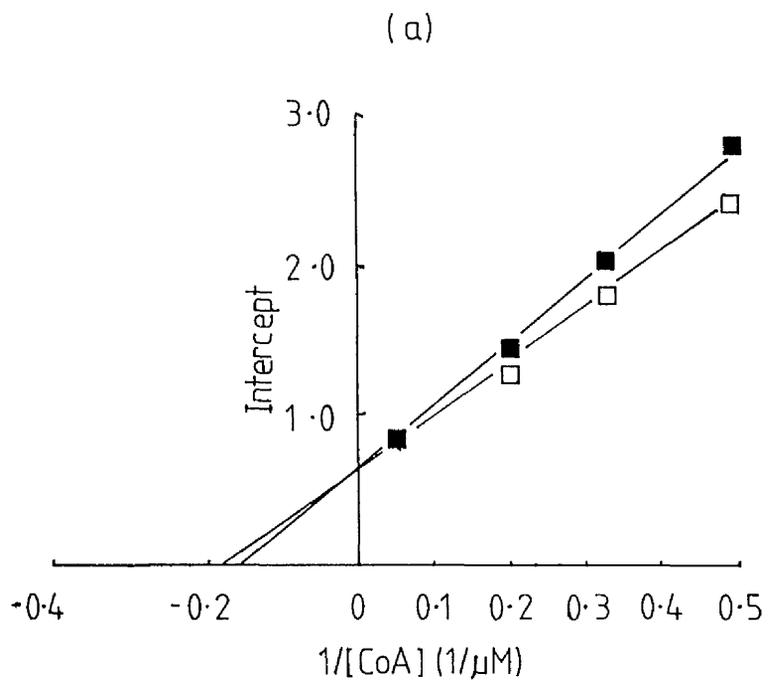
$$K_s^{Mg-citrate} \cdot K_s^{CoA} = K_s^{Mg-citrate} \cdot K_m^{CoA}$$

Chapter 5.2 also indicated that the binding of MgATP and CoA, and MgATP and Mg-citrate is ordered with, in each case, MgATP binding first. Since there is an irreversible step between the binding of

Figure 6.14. Replot of data in Figures 6.10 and 6.11.

(a) Replot of the $1/v$ intercepts of the lines in Fig. 6.10 (non-phosphorylated ATPCL) (\square) and Fig. 6.11 (phosphorylated ARPC (\blacksquare).

(b) Replot of the slopes of the lines in Fig. 6.10 (non-phosphorylated ATPCL) (\circ) and Fig. 6.11 (phosphorylated ATPCL) (\bullet).



MgATP and CoA, and between the binding of MgATP and Mg-citrate, the apparent dissociation constant for MgATP is 0. The appropriate rate equations are therefore:

$$V = \frac{V_{\max}}{1 + \frac{K_m^{\text{MgATP}}}{[\text{MgATP}]} + \frac{K_m^{\text{Mg-citrate}}}{[\text{Mg-citrate}]}}$$

$$V = \frac{V_{\max}}{1 + \frac{K_m^{\text{MgATP}}}{[\text{MgATP}]} + \frac{K_m^{\text{CoA}}}{[\text{CoA}]}}$$

K_m^{MgATP} is the Michaelis constant for MgATP and the other parameters are defined above. Numerical values for these parameters were obtained from the slopes of the lines in Figs. 6.8, 6.9, 6.12 and 6.13 and by plotting the $1/v$ intercepts of these lines against $1/[\text{MgATP}]$ (Figure 6.15). The numerical values of the kinetic parameters of phosphorylated and non-phosphorylated ATPCL are displayed in Table 6.1.

When estimated from Figs. 6.14 and 6.15 the V_{\max} of the phosphorylated ATPCL was found to be different from that of the control ATPCL. The mean V_{\max} of the phosphorylated ATPCL was 1.86 ± 0.08 $\mu\text{moles}/\text{min}/\text{mg}$ (mean \pm SEM of the values shown in Table 6.1) and that of the control ATPCL was 1.66 ± 0.03 $\mu\text{moles}/\text{min}/\text{mg}$ (mean \pm SEM of the values shown in Table 6.1). This difference is not significant ($P < 0.7$). This confirms the results in Section 1 of this chapter and also the results of other workers (Guy *et al.*, 1981; Ranganathan

Figure 6.15. Replot of data from Figures 6.8, 6.9, 6.12 and 6.13.

(a) Replot of the $1/v$ intercepts of the lines in Fig. 6.8 (non-phosphorylated ATPCL) (o) and Fig. 6.9 (phosphorylated ATPCL) (•).

(b) Replot of the $1/v$ intercepts of the lines in Fig. 6.12 (non-phosphorylated ATPCL) (o) and Fig. 6.13 (phosphorylated ATPCL) (•).

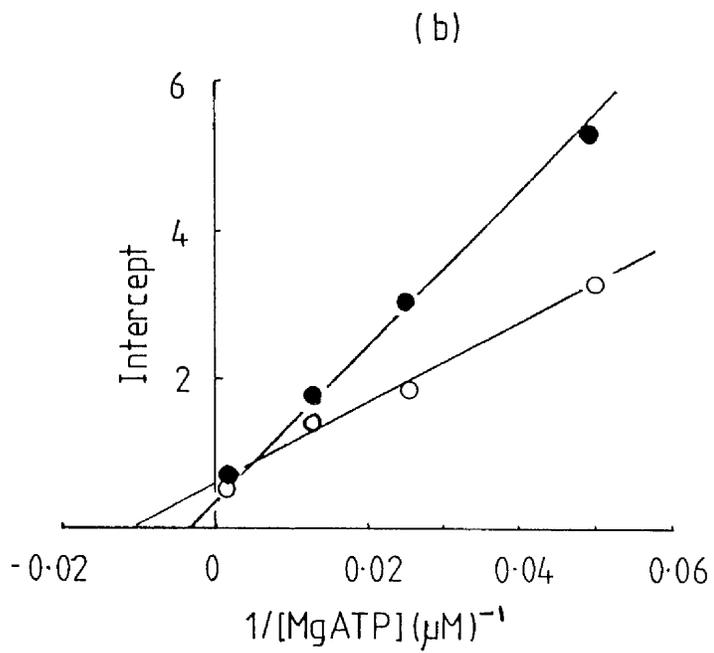
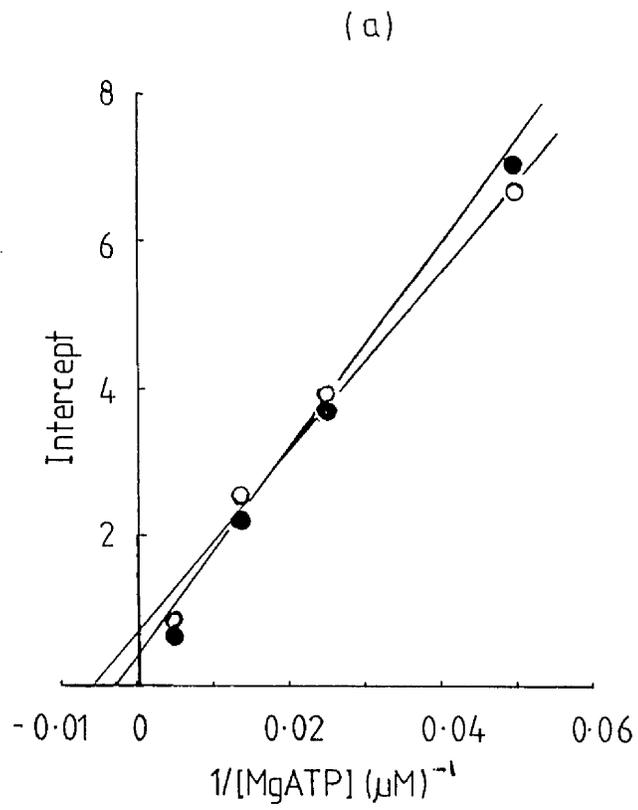


Table 6.1. Numerical values of the kinetic parameters of phosphorylated and non-phosphorylated ATP citrate lyase.

Figure from which the kinetic parameters were determined	Phosphorylation state of ATPCL	Kinetic parameters						
		Mg-citrate K_m (μM)	Mg-citrate K_s (μM)	Mg-citrate K_m (μM)	CoA K_s (μM)	CoA K_m (μM)	MgATP K_m (μM)	V_{max} (moles/min/mg)
6.14	non-phosphorylated	51.0	47.5	6.5	6.1	-	-	1.73
6.14	phosphorylated	46.7	45.4	7.1	6.9	-	-	1.66
6.8, 6.15(a)	non-phosphorylated	49.3	-	-	-	161	-	1.61
6.9, 6.15(a)	phosphorylated	49.9	-	-	-	250	-	1.97
6.12, 6.15(b)	non-phosphorylated	-	-	3.8	-	88.5	-	1.64
6.13, 6.15(b)	phosphorylated	-	-	5.0	-	191	-	1.96

et al, 1981), that the V_{\max} of ATPCL is not altered by phosphorylation of the enzyme by cAMPdPrK.

The values of the K_m for Mg-citrate determined from Figs. 6.8, 6.9 and 6.14 in good agreement (range 46 - 51 μM). These values are somewhat lower than those reported by Plowman and Cleland (1967) who found the K_m for Mg-citrate to be 71 μM . This difference might well be due to the different assay conditions used. The work reported by Ranganathan et al (1981) suggests that some of the kinetic parameters of ATPCL vary according to the buffer and pH of the assay used. No significant difference was found between the control and phosphorylated ATPCL with respect to the K_m for Mg-citrate. The mean K_m of the control enzyme was $50.1 \pm 0.85 \mu\text{M}$ while the K_m of the phosphorylated ATPCL was $48.3 \pm 1.5 \mu\text{M}$ (mean \pm SEM of the values in Table 6.1; $P < 0.5$). This demonstrates that phosphorylation by cAMPdPrK does not result in a change in the K_m of ATPCL for Mg-citrate.

The values of the K_m for CoA determined from Figs. 6.12, 6.13 and 6.14 (range 3.8 - 7.1 μM) are larger than those obtained by other workers (Plowman and Cleland, 1967: 1.6 μM ; Hoffman et al, 1979: 3 μM ; Ranganathan et al, 1981: 1.1 - 2.6 μM). The reason for this is not known. It is unlikely to be due to errors in the concentrations of CoA used in the initial velocity studies since the concentrations of all of the substrates used were checked prior to use (described in Chapter 2.2). The mean K_m for CoA of control ATPCL was $5.2 \pm 0.4 \mu\text{M}$ while that of the phosphorylated enzyme was $6.0 \pm 1.0 \mu\text{M}$ (mean \pm SEM of the values displayed in Table 6.1). This difference is not significant ($P < 0.3$), indicating that phosphorylation by cAMPdPrK has no effect on the K_m of ATPCL for CoA.

The values of the apparent dissociation constants of phosphorylated

and non-phosphorylated ATPCL for Mg-citrate and CoA are presented in Table 6.1. These values are very close to those measured for the Michaelis constants for Mg-citrate and CoA respectively. This finding indicates that each of the substrates binds to a distinct site on the enzyme and that the binding of one substrate does not alter the affinity of ATPCL for the other.

The K_m of the control ATPCL for MgATP determined from Fig. 16(b) was $88.5 \mu\text{M}$ while that of the phosphorylated form was $191 \mu\text{M}$. Similar values were obtained with two other preparations of pure ATPCL. The K_m for MgATP of non-phosphorylated ATPCL was $90.1 \pm 1.7 \mu\text{M}$ (mean \pm SEM of 3 separate experiments) while the K_m for MgATP of the phosphorylated form was $193 \pm 3.6 \mu\text{M}$ (mean \pm SEM of 3 separate experiments). Note that these values were obtained at a constant high Mg-citrate concentration. When CoA was kept at a constant high concentration (Fig. 16(a)) somewhat higher values for the K_m for MgATP were obtained: $161 \mu\text{M}$ and $250 \mu\text{M}$ for the non-phosphorylated and phosphorylated forms respectively. The reason for these differences is not clear, since both methods should have given the same values. An increase in the K_m for MgATP upon phosphorylation was noted in both cases. The difference between the K_m for MgATP (determined at a constant Mg-citrate concentration) of the phosphorylated form and that of the non-phosphorylated form is highly significant ($P < 0.01$). These results show that phosphorylation of ATPCL by cAMPdPrK is accompanied by a 2-fold increase in the K_m of ATPCL for MgATP. This change is consistent with the facts that glucagon inhibits lipogenesis and that phosphorylation by cAMPdPrK reduces the activity of ACC.

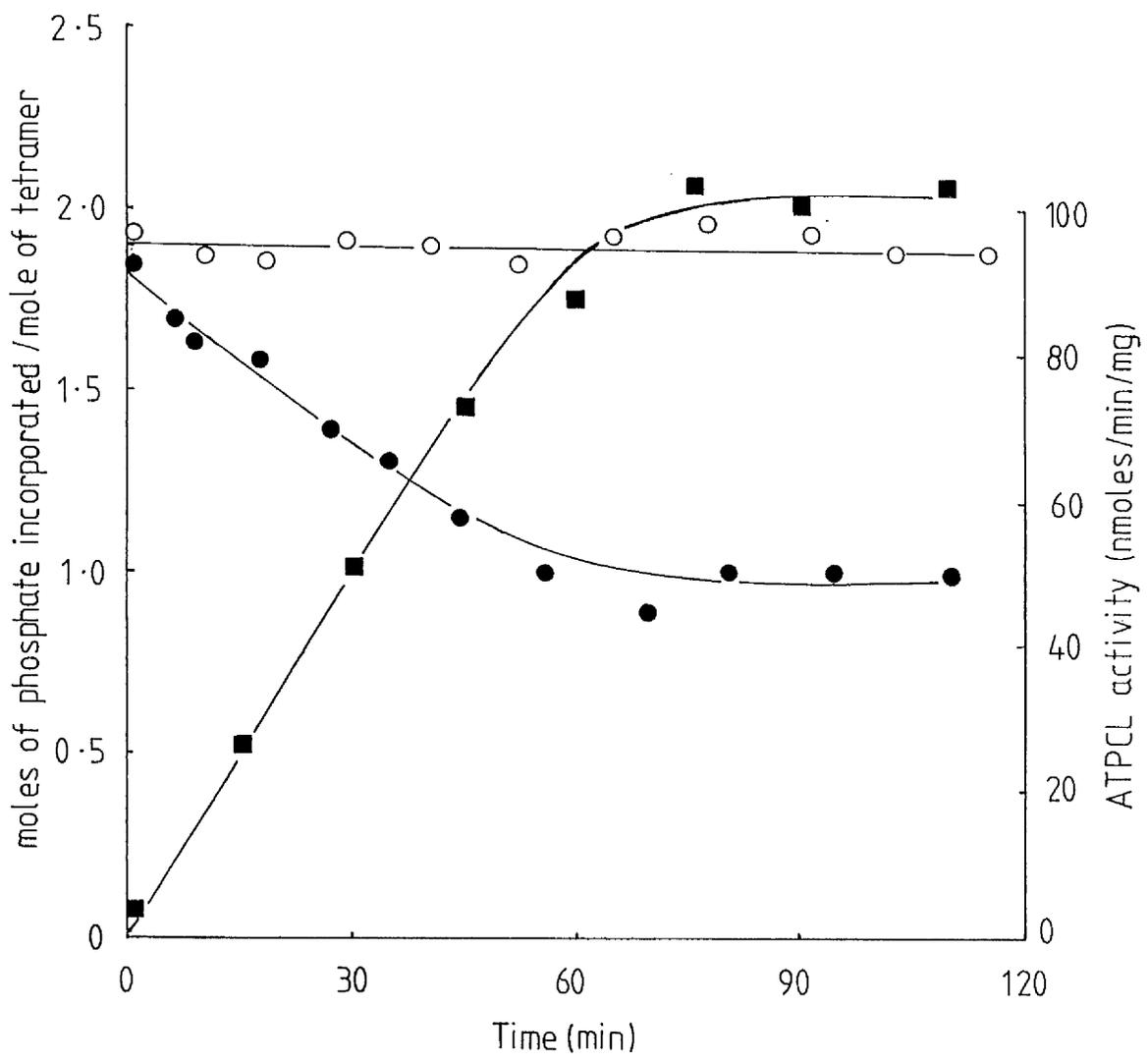
Changes in ATP citrate lyase activity concomitant with phosphorylation by cAMP dependent protein kinase

The results described above, especially those illustrated in Fig. 6.15(b) indicate that at low concentrations of MgATP, ATPCL which has been phosphorylated by cAMPdPrK is less active than non-phosphorylated ATPCL. This suggested that under suitable assay conditions it should be possible to follow the decrease in ATPCL activity during the phosphorylation reaction. Consideration of the data in Fig. 6.15(b) indicated that it would be desirable to use as low a concentration of MgATP as possible.

The phosphorylation reaction was carried out at 20°C. The incubation contained, in a final volume of 300 μ l, 0.4 mg/ml ATPCL, 5000 U catalytic subunit of cAMPdPrK, 5 mM magnesium acetate and 0.5 mM [γ - 32 P]-ATP (110000 cpm/ nmole). A control incubation where cAMPdPrK was omitted was carried out. The reaction was started by the addition of ATP and magnesium acetate. Samples (12.5 μ l) were removed at various times and the incorporation of 32 P into ATPCL measured as described in Chapter 2.2. Samples (10 μ l) were also removed at various times and assayed by a modification of the assay procedure used in the kinetic studies on ATPCL. Each cuvette contained, in a final volume of 1.0 ml, 50 mM MOPS, 0.25 M KCl, 1.5 mM β -mercaptoethanol, 0.05 mM NADH, 9 mM Mg-citrate, 0.424 mM Mg^{2+} , 5.0 μ M CoA, 4.8 U malate dehydrogenase, 4.4 μ M MgATP, pH 7.0. Other conditions and procedures were identical to those used during the kinetic studies. The concentration of MgATP was the lowest that could be used: all of the ATP in the assay had been carried over from the phosphorylation incubation in the 10 μ l sample. The results of this experiment are shown in Figure 6.16. ATPCL was phosphorylated to the extent of 2.1 moles of phosphate/mole of

Figure 6.16. Phosphorylation and inactivation of ATP citrate lyase by cAMP dependent protein kinase.

ATPCL was incubated as described in the text in the presence or absence of cAMPdPrK. Samples were removed at the times indicated and the incorporation of phosphate into ATPCL measured. For clarity on the incorporation of phosphate due to cAMPdPrK is shown (■). It was obtained by subtracting the phosphate incorporated in the absence of cAMPdPrK from the phosphate incorporated in its presence. Other samples were removed at the times indicated and assayed for ATPCL activity as described in the text: (o) incubation in the absence of cAMPdPrK; (●) incubation in the presence of cAMPdPrK



tetramer over a period of 90 min. During this time the activity of the enzyme decreased from 92 nmoles/min/mg to 50 nmoles/min/mg. The concentration of ATP in the incubation remained constant throughout the incubation period. There was no decrease in the activity of the control ATPCL which had been incubated in the absence of cAMPdPrK. Similar results were obtained on two other occasions. These results demonstrate that it is possible to detect changes in ATPCL activity concomitant with phosphorylation by cAMPdPrK. In order to do this, however, it is necessary to perform the assays at a low MgATP concentration. This explains why previous workers, who used much higher MgATP concentrations, were unable to detect any change in activity.

The results reported in this section indicate that phosphorylation of ATPCL by cAMPdPrK results in a change in only one of the kinetic parameters of the enzyme, the K_m for MgATP. The V_{max} and the Michaelis constants for Mg-citrate and CoA are unchanged. The results described above also demonstrate that under appropriate assay conditions the change in activity during phosphorylation can be followed. The magnitude of this decrease in activity is not great, however, (46% at a MgATP concentration of $4.4 \mu M$). Furthermore, the concentration of MgATP in the cytoplasm is much higher than the concentration of MgATP which is required to observe a decrease in activity. These points cast considerable doubt as to the physiological relevance of the change in the K_m for MgATP caused by phosphorylation by cAMPdPrK. This aspect of the work will be discussed more fully in Chapter 7.

Chapter 7

Discussion

Section 1. Experiments on lactating rabbit mammary gland
acetylCoA carboxylase

Purification experiments

The amounts of activity measured in extracts prepared from rat liver were always low and varied considerably (Chapter 4.2). As a result I often had difficulty in detecting ACC activity in dilute solutions, particularly during elution of the enzyme from chromatography columns. In contrast, large amounts of ACC could readily be purified from lactating rabbit mammary gland. I therefore studied the behaviour of lrmgACC on a variety of chromatographic media (Chapter 3.2). It was also of interest to study the behaviour of lrmgFAS on these media in order to determine a method that would separate ACC from FAS. These studies were performed on the assumption that the rabbit mammary gland and rat liver enzymes had similar properties.

The experiments indicated conditions under which ACC would bind to and could be eluted from DEAE cellulose and phosphocellulose. I therefore included chromatography on these media in the partial purification scheme for rlACC. The experiments also suggested that chromatography on Phenyl-Sepharose might be used in the purification of rlACC, provided that activity could be preserved. However, in these studies I was unable to find a method that would separate ACC from FAS.

Studies of aggregation state

Polyethylene glycol is believed to act by steric exclusion, confining the protein to small pockets of solvent and increasing the activity coefficient of the solution. This may result in aggregation

particularly if the protein is large or part of a naturally aggregating system. Thus, polyethylene glycol is known to promote the polymerisation of tubulin and fibrinogen (Lee and Lee, 1979; Fenton and Fasco, 1974) and has been used in the purification of mammalian 2-oxo acid dehydrogenases (Linn et al., 1972).

The experiments performed in Chapter 3.3 indicated that lrmgACC which had been purified using precipitation with polyethylene glycol has properties which were different to those reported for ACC which had been purified using other methods. Thus, in Fig. 3.10, it can be seen that 'PEG purified' lrmgACC was active in the absence of citrate, in contrast to lrmgACC which had been partially purified without the use of polyethylene glycol. One possible criticism of this experiment is that the partially purified ACC might have contained some factor which can inhibit ACC at low citrate concentrations.

In addition to its activity in the absence of citrate, 'PEG purified' lrmgACC tended to come out of solution during storage at 4°C and was unable to penetrate 3% polyacrylamide gels even after dialysis into buffer containing palmitoylCoA, a treatment which promotes depolymerization of ACC which has been purified without the use of polyethylene glycol.

These observations suggested that 'PEG purified' ACC was in an aggregated state and unable to depolymerize into the protomeric form. Support for this hypothesis comes from the experiments reported in Chapter 3.3 which indicate that the sedimentation coefficient of ACC does not decrease to that of the protomeric form after dialysis into citrate-free buffer, or citrate-free buffer containing palmitoylCoA. One could confirm the above hypothesis by repeating the experiment using lrmgACC which had been purified without the

use of polyethylene glycol precipitation. This would rule out the possibility that the irreversible aggregation is a property of lrmgACC rather than ACC purified using polyethylene glycol precipitation. However, it seems unlikely that this is so since Ahmad et al (1978) have reported that mammary gland ACC, purified without the use of polyethylene glycol, can exist in both protomeric and polymeric forms.

The observation that the use of polyethylene glycol alters the properties of a naturally aggregating enzyme by preventing dis-aggregation is not without precedent. Reinhardt (1980) has reported that the presence of polyethylene glycol prevents the spontaneous dissociation of tetrameric rat liver phosphofructokinase into monomers and dimers which normally occurs at low protein concentrations. This result, together with those presented in this work, imply that the use of polyethylene glycol should be avoided when purifying an enzyme which forms aggregates, particularly if aggregation affects the kinetic properties of the enzyme.

Work on the purification of rlACC using polyethylene glycol precipitation was carried out before the studies described in Chapter 3.3 had been completed. As it happened, the polyethylene glycol precipitation step in the rlACC purification proved difficult to reproduce. In view of the anomalous behaviour of 'PEG purified' lrmgACC, I did not attempt to make the procedure more reproducible, and I discontinued the use of precipitation with polyethylene glycol in the rlACC purification procedure. In addition, I avoided the use of polyethylene glycol when devising a purification procedure for rlATPGL since polyethylene glycol might have altered the physico-chemical properties of this enzyme. There was a small possibility

of this happening in view of the reports that ATPCL can exist in an aggregation state greater than that of tetramer (Corrigan and Rider, 1981; Singh et al., 1976).

Section 2. Experiments on liver acetylCoA carboxylase

Initial attempts to detect activity in pig liver extracts were unsuccessful (Chapter 3.1). This was not due to the insensitivity of either of the two assay methods used. On the sole occasion that I was able to detect ACC activity the amount was approximately one tenth of that measured in rat liver by other workers. This low level of ACC activity in pig liver might be due to the diet of the animals prior to slaughter or to the presence of an inhibitor of ACC similar to that found in rat liver (Chapter 4.5). The experiment in which I attempted to detect pig liver ACC immunologically was performed badly and no conclusion could be drawn from it. However, the low levels of activity detected indicated that pig liver was a poor source of ACC and so I decided not to repeat the experiment.

In my early attempts to purify rat liver ACC the enzyme was partially purified using chromatography on DEAE cellulose and phosphocellulose and purification beyond this step was attempted using a variety of methods (Chapter 4.1). The purification involving polyethylene glycol precipitation, although initially promising, was not reproducible. It is possible that the aggregation state is important in determining whether ACC is precipitated by polyethylene glycol and that this varied between experiments. In my hands gel filtration of ACC, even in the presence of citrate and a reducing agent, led to loss of activity. The reason for this is not known.

The ion-exchange chromatography of the procedure described in Chapter 4.1 also proved to be not reproducible. It is interesting to note that the binding of ACC to DEAE cellulose was prevented by material which precipitated during the chromatography of the 90000 x g supernatant. This precipitate also prevented the binding of rLATPCL

to DEAE cellulose. It is possible that the insoluble material was glycogen, large amounts of which are presumably synthesised in rat liver upon refeeding a low fat/high carbohydrate diet, though it is not clear why glycogen should prevent the binding of ACC and ATPCL to DEAE cellulose in this way. One interesting possibility is that ACC and ATPCL are associated with glycogen particles and that in vivo the substrates of the enzymes which synthesise fatty acids are derived from glycogen.

The variable levels of ACC activity detected during this part of the work were attributed to variations in the dietary status of the animals. Another possibility is that the activity of the inhibitory kinase also varied. The presence of this kinase might well explain why ACC activity was low or absent in the 90000 x g supernatants used in these experiments.

In order to increase the amount of ACC activity and to improve the reproducibility of the ion-exchange chromatography I revised the original partial purification procedure (Chapter 4.2). In the revised scheme the ACC was applied to DEAE cellulose after ammonium sulphate fractionation. This itself caused further problems as it later became clear that the inhibitory kinase co-purifies with ACC in this procedure. Unfortunately, at the same time I started to use the PK/LDH assay. Due to contamination of the acetylCoA by CoA this assay was not specific for ACC. Therefore, the overall result of the modifications which I made at this time was that I monitored mainly ATPCL activity.

A considerable amount of time was spent in analysing the properties of the components of the two protein peaks which were resolved on DEAE cellulose (Chapter 4.3 and 4.4).

My initial interpretation was that the first peak contained proteolysed ACC while the second peak contained undegraded ACC. However, it is now clear that the first peak contained ATPCL and that the second peak contained FAS. Given that both of the proteins present in peak 2 had a subunit M_r of 250000 it is likely that this peak also contained ACC.

The major flaw in this part of the work was not the interpretation of the DEAE cellulose peak 1 polypeptide as proteolysed ACC but the interpretation of the trailing edge of 'ACC' activity as intact ACC with low specific activity. This misinterpretation was compounded by the presence of the inhibitory kinase in the second peak (Fig.4.20). This meant that no genuine ACC activity was detected. It is not clear why the effects of the inhibitory kinase should have become so apparent when they did. In the work described in Chapter 4.1 and 4.2 ACC activity, albeit at a low level, was always detected at some stage of the procedure. In the later work it would appear that no genuine ACC activity was detected. If the absence of ACC activity is due to the presence of the inhibitory kinase it would appear that it is more active in the later work. It is difficult to imagine what would have caused this increase in activity.

Section 3. Recent work on the phosphorylation of acetylCoA carboxylase

It is appropriate at this point to review some recent studies on the phosphorylation and inactivation of ACC as one might envisage that some of the objectives outlined in Chapter 1.4 have been met in recent studies. Guy and Hardie (1981) reported that limited proteolysis of lrmgACC results in a decrease in its subunit M_r from 250000 to 225000. This treatment resulted in the loss of phosphate from the enzyme and mimicked the effects of dephosphorylation by protein phosphatase-1. This result implied that the phosphate removed by protein phosphatase-1 is located on the 25000 M_r peptide which is removed during partial proteolysis. This suggests that the subunit M_r of rlACC reported by other workers (230000) might be the result of partial proteolysis which had occurred during purification and that such proteolysis might explain the inability of other workers to phosphorylate pure rlACC.

There are two more recent reports on the purification of rlACC in which the authors report a subunit M_r greater than 230000. Witters and Vogt (1981) purified rlACC using polyethylene glycol fractionation, ion exchange chromatography and gel filtration. Their preparation had a subunit M_r of 240000 and appeared to be free from proteolysis. The authors point out the possibility of a genuine difference between the rabbit and rat enzymes since Hardie and Guy (1980) have reported that rat mammary gland ACC also had a subunit M_r of 240000. The authors do not comment on whether this pure rlACC can be phosphorylated in vitro. Song and Kim (1981) have reported the purification of rlACC with a subunit M_r of 260000 using polyethylene glycol and ammonium sulphate fractionation and chromatography on immobilised monomeric avidin. Their preparation contained 6 moles of alkali labile phosphate/subunit compared with the 2 moles of phosphate/subunit which previous

preparations (subunit $M_r = 230000$) contained. There is no indication in this report that their purified enzyme can be phosphorylated in vitro. The reason for the discrepancy between the subunit M_r reported by Song and Kim (1981) and that reported by Witters and Vogt (1981) is not clear. It may be that the preparation of the latter workers is itself partially proteolysed. A more likely explanation is that the discrepancy is due to the use of different SDS-polyacrylamide gel systems and marker proteins.

It is clear, therefore, that rlACC does have a subunit M_r greater than 230000 and that the peptide which was absent from earlier preparations contains at least one phosphorylation site. In spite of this there have been no reports on the phosphorylation of pure rlACC by cAMPdPrK. Consequently, there has been no detailed study of the effects of such phosphorylation on the kinetic properties and aggregation state of the enzyme. Thus, the primary objective of the work on rlACC (Chapter 1.4) has still to be achieved. In future work I would, therefore, continue with the project outlined in Chapter 1.4 provided that I was able to prepare pure rlACC with a subunit M_r greater than 230000. Given that the two proteins in DEAE cellulose peak 2 are FAS and ACC and that both migrate to the same position on SDS-polyacrylamide gels it would appear that the subunit M_r of rlACC is 250000. This would imply that the ACC in DEAE cellulose peak 2 has not lost the peptide which contains the phosphorylation site(s). The phosphorylation by cAMPdPrK of rlACC which has been purified beyond the DEAE cellulose stage is, therefore, quite feasible.

Future work on rat liver acetylCoA carboxylase

My first task upon resuming work on rlACC would be to devise a procedure for the purification of the inhibitor described in

Chapter 4.5 using the inhibition of purified lrmgACC as the basis of an assay for the inhibitor. By assaying for rlACC at each stage of the purification it should be possible to determine a step in which the ACC is separated from the inhibitor. This step could then be incorporated into a purification procedure for rlACC.

The next task would be to obtain pure rlACC using a procedure based on that described in Chapter 4.3, i.e. some combination of ammonium sulphate fractionation, chromatography on DEAE cellulose and a step to separate ACC from its inhibitor. As discussed in Section 2 of this chapter I believe that ACC co-purifies with FAS up to and including elution from DEAE cellulose. Figure 4.11 (gel 1) and Figure 4.15 (gel 2) indicate that only two proteins, ACC and FAS, are eluted in DEAE cellulose peak 2 and this implies that at this stage a step which separates FAS from ACC will yield pure ACC. In the experiments described in Chapter 3.2 I had no success in devising a method which would separate FAS from ACC using the rabbit mammary gland enzymes as models for the rat liver enzymes. Recently, other workers have introduced methods which ought to separate ACC from FAS. Song and Kim (1981) have purified rlACC using chromatography on immobilised monomeric avidin, which binds ACC by virtue of its affinity for the enzymes biotinyl prosthetic group. Elution is achieved by washing the column with free biotin. Since FAS does not contain a biotinyl group it is probable that ACC could be separated from FAS using this method. Another potentially useful method is that of Brownsey and Denton (1982) who reported that following incubation in the presence of citrate, ACC could be separated from FAS by centrifugation at 160000 x g for 20 min.

Another area for future work concerns the inhibitor of ACC

described in Chapter 4.5. I have tentatively identified this inhibitor as a cAMPiPrk and it is clearly important to confirm this experimentally. As discussed above I believe that the purification of the presumed kinase will be a prerequisite to the purification of rIACC. Thus, it should be possible to compare the effects of phosphorylation by cAMPiPrK with phosphorylation catalysed by a cAMPiPrK. It would also be of interest to study the factors which regulate the activity of this presumed kinase. One possible regulatory factor is Ca^{2+} as a Ca^{2+} -stimulated kinase has been implicated in the phosphorylation and inactivation of rIACC which occurs when isolated hepatocytes are treated with α -adrenergic agents such as noradrenaline and phenylephrine (Ly and Kim, 1981).

Two groups have, in fact, reported the purification from rat liver of protein kinases which phosphorylate and inactivate rIACC (Shiao et al, 1981; Lent and Kim, 1982). The enzyme isolated by Lent and Kim (1982) binds to DEAE cellulose and is a tetramer with an M_r of 700000. It is therefore distinct from the kinase of Shiao et al (1981) which does not bind to DEAE cellulose and has an M_r of 160000. Neither of these kinases is stimulated by cAMP; their dependence on Ca^{2+} does not appear to have been studied. The cAMPiPrK described by Lent and Kim (1982) is interesting in that it can only phosphorylate and inactivate rIACC which has been preincubated in the presence of CoA. Yeh et al (1981) have reported that this treatment also activates rIACC by lowering the K_m for acetylCoA. This area clearly requires more study as we have the interesting situation that the presence of a substance which activates ACC is necessary for the phosphorylation and inactivation of the enzyme.

It is apparent that in addition to the phosphorylation by

cAMPdPrK which is implied by in vivo studies, the activity of rLACC is also regulated by a number of cAMPiPrKs. The kinases purified by Shiao et al (1981) and Lent and Kim (1982) appear to be distinct and the results of Ly and Kim (1981) imply the existence of a Ca^{2+} dependent kinase. Thus, there are possibly 3 cAMP independent ACC kinases. This situation is not unusual: lrmgACC appears to be a substrate for 2 cAMPiPrKs in addition to cAMPdPrK.

In addition to these kinases which inactivate it, it is possible that rLACC can be phosphorylated and activated by a distinct insulin-stimulated protein kinase similar to that found in adipose tissue. The in vivo stimulation of rLACC activity by insulin was reviewed in Chapter 1.3. A similar activation occurs when isolated adipocytes are exposed to insulin. The work of Denton and co-workers has indicated that this activation is accompanied by phosphorylation. Brownsey and Denton (1982) initially observed that the increase in ACC activity which results from the exposure of adipocytes to insulin persists through purification of the enzyme. This result indicated that the activation was due to covalent modification. The insulin-stimulated activation was accompanied by a small, but significant increase in the phosphate content of the ACC. Peptide mapping of purified ACC which had been phosphorylated in vivo in response to either insulin or adrenaline or both hormones together, indicated that the site phosphorylated in response to insulin was distinct from that phosphorylated in response to adrenaline. Furthermore, the effects of the two hormones on the stoichiometry of phosphorylation were additive. The kinase responsible for this activation might well be that described in a previous report by these workers (Brownsey et al, 1981) who found that incubation of partially purified rat adipose tissue ACC in the presence of plasma membranes and ATP resulted in

the phosphorylation and activation of ACC. The activation was similar to that which occurs in vivo in response to insulin in that it was only demonstrable if the ACC had not been incubated with citrate prior to assay.

The studies by Brownsey and co-workers on the insulin-stimulated phosphorylation of ACC are the most interesting aspect of the recent work on this enzyme. Their importance lies in the fact the study of the regulation of this phosphorylation might well lead to the elucidation of the mechanism whereby the effects of insulin are mediated in the cell. This topic is discussed further in Section 5 of this chapter in which the insulin-stimulated phosphorylation of ATPCL is considered.

In summary, while there is now incontrovertible evidence that the activity of rlACC is regulated by a variety of protein kinases, the factors which regulate these enzymes and their precise effects on the kinetic properties of ACC have not yet been elucidated. Future work, using rlACC which is free from proteolytic degradation, would study these points.

Section 4. Purification and properties of rat liver
ATP citrate lyase

Purification

In Chapter 5.1 I describe a novel procedure for the purification of rlATPCL which uses ammonium sulphate fractionation, chromatography on DEAE cellulose and immobilised Cibacron Blue F3G-4 and finally gel filtration on Ultrogel A2. This method allowed the purification of ATPCL which was pure by the criterion of giving a single band on both SDS and non-denaturing polyacrylamide gel electrophoresis. During the purification care was taken to ensure that the final preparation would be free from proteolytic degradation. The absence of any other protein staining bands in gel 2 Fig.5.7 or gel 2 Fig.5.8 demonstrates that proteolysis of ATPCL has not occurred.

The mean specific activity of the pure ATPCL was 13.4 U/mg. As discussed in Chapter 5.1, this value is similar to those reported for rlATPCL by other recent workers. One difficulty in comparing the specific activities reported by different workers is that in some studies ATPCL was assayed at 25°C while in others the assays were performed at 37°C. In the course of this work I observed that the activity of ATPCL measured at 37°C is approximately twice that measured at 25°C. In Table 5.2 specific activities measured at 25°C have been converted to specific activities at 37°C using a factor of 2.

Table 5.2 shows that there is considerable variation in the specific activities reported for pure rlATPCL. This is most likely the result of the different methods used to estimate the protein concentration of the pure ATPCL. However, Linn and Srere (1979) reported a specific activity of 18.8 U/mg yet used the same method to

estimate the protein concentration of pure ATPCL as was used in this present study. This difference implies that my preparation contained some inactive ATPGL since both preparations were of the same degree of purity as judged by polyacrylamide gel electrophoresis.

Using the purification procedure described in Chapter 5.1, workable amounts of ATPCL could be obtained from the livers of 6 rats. This does not alter the fact that the overall yield for the procedure was low (10%), especially when compared with yields reported by other workers. Paradoxically, it would appear that this low yield of pure ATPCL is a result of the steps taken to increase recovery during the early stages of the procedure. In Chapter 4.3 and 4.4 I was able to prepare apparently pure DEAE cellulose peak 1 polypeptide (later identified as ATPCL) from 0-30% or 0-35% ammonium sulphate precipitates. In order to improve the yield of ATPCL at the ammonium sulphate stage I modified the procedure so that a 30%-40% ammonium sulphate precipitate was used. It appears that as a result of this, the ATPCL was not pure when eluted from DEAE cellulose. This is presumably because a greater number of contaminating proteins are precipitated between 30 and 40% saturation with ammonium sulphate than are precipitated between 0-30% and 0-35% saturation. The impurity of the ATPCL at the DEAE cellulose stage necessitated further purification on Affigel Blue and Ultrogel A₂, together with a time-consuming vacuum dialysis to concentrate the Affigel Blue pool. The Affigel Blue pool could not be concentrated by ammonium sulphate precipitation because of its low protein concentration.

It is possible that a better purification procedure would be to reduce the recovery in the ammonium sulphate precipitate in order to obtain pure ATPCL from the DEAE cellulose column. This would not

require further purification (see for example gel 2, Fig.4.11) and could then be concentrated by ammonium sulphate precipitation. This procedure would be much more rapid than that described in Chapter 5.1 and the entire procedure could be carried out in one day. More importantly the losses which occur during chromatography on Affigel Blue and Ultrogel A2 and during vacuum dialysis would be avoided.

Physical characterization of rat liver ATP citrate lyase

The purified rLATPGL had an M_r of 500000 when estimated by gel filtration on Ultrogel A2. The sedimentation coefficient determined by sucrose density gradient centrifugation was 13.5 S. The subunit M_r was estimated using two different SDS-polyacrylamide gel electrophoresis methods (Weber and Osborn, 1969; Laemmli, 1970). A subunit M_r of 123000 was estimated by both methods. These results indicated that purified rLATPGL is a tetramer. Direct evidence of this was obtained by cross-linking purified ATPGL with dimethyl suberimidate (Fig.5.12).

These results are in good agreement with those obtained by most other workers. Singh et al (1976), however, using sedimentation equilibrium centrifugation in the presence and absence of guanidine-HCl, obtained M_r s of 440000 and 110000 for the tetramer and subunit respectively. As shown in Fig. 5.10, when chromatographed on Ultrogel A2 the ATPGL used in this present study had the same elution volume as lrmgFAS and rat liver FAS ($M_r = 500000$). Moreover, the ATPGL had a smaller elution volume than the M_r marker apoferritin ($M_r = 440000$). The M_r of the tetramer when calculated using the subunit M_r of 123000 is 490000 and is clearly in very good agreement with the native M_r estimated by gel filtration. These results indicate that Singh et al (1976) underestimated the native and

subunit M_r s. It may be that the partial specific volume which these authors used was incorrect.

Other than the very small shoulder of higher M_r ATPCL activity noted on sucrose density gradient centrifugation I found no evidence that ATPCL can exist in an aggregation state greater than that of tetramer, as reported by Corrigan and Ryder (1981). Evidence for such an aggregation state would, perhaps, have been most noticeable in the cross-linking experiment in view of the ease with which r1ATPCL can be cross-linked with dimethyl suberimidate. In Fig.5.12 there is, however, no trace of bands corresponding to cross-linked species greater than that of tetramer.

Studies on the kinetic mechanism of rat liver ATP citrate lyase

As discussed in Chapter 1.3 and in Chapter 5.3, there was some uncertainty regarding the kinetic mechanism of ATPCL. Briefly, the reason for this uncertainty was that there was a discrepancy between data obtained from initial velocity experiments and data from isotope exchange and enzyme labelling experiments. The former indicated a sequential mechanism while the latter indicated a ping-pong mechanism with the formation of a phosphoenzyme intermediate prior to the binding of CoA and Mg-citrate (Plowman and Cleland, 1967). It is probable that the ATPCL used by Plowman and Cleland (1967) was not homogeneous since the specific activity of their preparation was only 1.6 U/mg. Furthermore, more recent studies (Singh *et al.*, 1976) have made it clear that the enzyme used by Plowman and Cleland (1967) was almost certainly proteolytically degraded. I therefore repeated the initial velocity studies of Plowman and Cleland (1967) using r1ATPCL which was homogeneous (specific activity 13.4 U/mg) and free from proteolytic degradation and was eventually able to resolve the

above discrepancy.

The initial velocity studies were first performed using assay method (a) (Chapter 2.2). Parallel lines were obtained in double reciprocal plots when Mg-citrate and MgATP were varied at a high fixed concentration of CoA and when CoA and MgATP were varied at a high fixed concentration of Mg-citrate. In contrast, sets of intersecting lines were varied at a low fixed concentration of the third substrate. Intersecting lines were obtained when Mg-citrate and CoA were varied at both high and low concentrations of MgATP. These results indicated that the kinetic mechanism was sequential with MgADP binding first, followed by the random equilibrium binding of CoA and Mg-citrate.

These results and hence the kinetic mechanism inferred from them were similar to those obtained by Plowman and Cleland (1967). Thus, it appeared that the discrepancy between the initial velocity and isotope exchange data was not due to the fact that earlier workers had used impure or proteolytically degraded ATPCL in their studies.

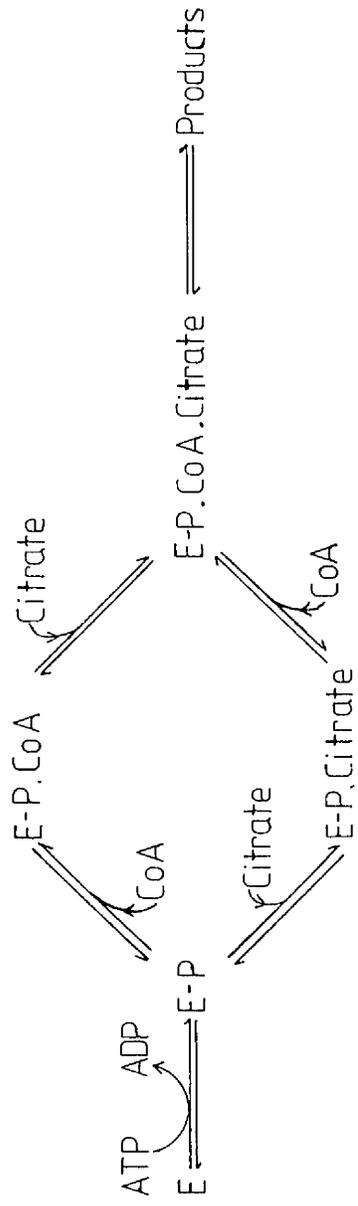
I realised, however, that the ATP which I had used in these experiments was contaminated with ADP, a product of the reaction catalysed by ATPCL. In the experiments where Mg-citrate and MgATP or CoA and MgATP were varied at a low fixed concentration of the third substrate, the presence of ADP in the assay rendered the results ambiguous. An intersecting pattern of double reciprocal plots indicates that all steps between the points of addition of the varied substrates are reversible. The presence of ADP would render formation of phosphoenzyme and ADP release reversible. Thus, the intersecting pattern is compatible both with a sequential mechanism and a ping-pong mechanism where ADP was released from the enzyme

before the binding of CoA and Mg-citrate.

I therefore repeated the experiments described in Figs. 5.17 and 5.19 in the absence of ADP. One way of doing this would have been to purify the ATP using chromatography prior to use. A more simple method was to repeat the experiments using assay method (b) (Chapter 2.2). This is a novel method for assaying ATPCL, in which the formation of ADP is coupled to the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. This method has the advantage that any contaminating ADP can be removed before the reaction is started, i.e. during the preincubation period, and so it was very suitable for my purposes.

Using assay method(b) parallel double reciprocal plots were obtained when MgATP and Mg-citrate or MgATP and CoA were varied at non-saturating concentrations of the third substrate. Thus it appears that in the absence of ADP there is an irreversible step between the binding of MgATP and CoA or Mg-citrate even at non-saturating concentrations of the third substrate. Moreover, it is clear that this irreversible step is the hydrolysis of ATP to free ADP. From this results and those reported in Chapter 5.3, which indicate the order of binding of the substrates, it would appear that the kinetic mechanism of ATPCL is that shown in Figure 7.1. This mechanism is consistent with the results of the isotope exchange and enzyme labelling experiments, which indicate the rapid formation of a phosphoenzyme intermediate.

These studies effectively resolve the discrepancy between the mechanism implied by enzyme labelling studies and that implied by the initial velocity studies of earlier workers, particularly Plowman and Cleland (1967). These workers obtained initial velocity



data similar to those obtained in this present study using assay method (a). However, they did not, apparently, purify their ATP before use and performed the initial velocity studies using an assay which measured the production of acetylCoA and oxaloacetate, rather than the production of ADP. Thus it would appear that the results of their initial velocity studies can be explained by the presence of ADP in the assay.

Phosphorylation of rat liver ATP citrate lyase

The process by which an enzyme is discovered to be regulated by phosphorylation in response to hormones usually follows a predictable pattern. In the first instance the activity of the enzyme is shown to change in the short term in response to one or several hormones. Later the enzyme is shown to be phosphorylated in vitro with a concomitant change in activity and the in vivo changes are shown to be accompanied by changes in the phosphorylation state of the enzyme. ATPCL is unusual in that its in vivo phosphorylation in response to insulin, glucagon and adrenaline has been demonstrated in the absence of evidence of short term changes in activity.

Following the demonstration that the phosphorylation state of ATPCL is hormone dependent several workers attempted to detect a change in the activity of ATPCL following in vitro or in vivo phosphorylation. Other workers attempted to compare the kinetic parameters of the phosphorylated and non-phosphorylated forms of ATPCL. These studies failed to reveal a difference between the phosphorylated and non-phosphorylated forms of the enzyme. The methods used in these studies were, however, open to criticism (discussed fully in Chapter 6.1).

The major objective in this work was to purify rlATPCL and then to perform a very careful study of the effect of phosphorylation by

cAMPdPrK on the kinetic parameters of ATPCL. Such a study could best be carried out using the procedures used in the study of the kinetic mechanism of rLATPCL. Moreover, a knowledge of the kinetic mechanism of ATPCL (indicated by the kinetic studies) was necessary in order to use the relevant rate equation when determining numerical values for the kinetic parameters.

The purified ATPCL was phosphorylated in a time-dependent manner by the catalytic subunit of cAMPdPrK. In this reaction 2.08 ± 0.15 moles of phosphate was incorporated into each mole of ATPCL tetramer (mean and standard deviation of 9 experiments). This stoichiometry implies that only half of the subunits of ATPCL are phosphorylated in the reaction. Pierce et al (1981) have proposed that two of the possible cAMPdPrK phosphorylation sites are sequestered in the native enzyme and can be exposed by 'mild denaturation'. However, cAMPdPrK is known to be capable of phosphorylating denatured proteins at non-regulatory sites, which might possibly explain the results of Pierce et al (1981). More recently Ranganathan et al (1982) have obtained evidence which casts doubt on the sequestration hypothesis of Pierce et al (1981). These workers purified rLATPCL which contained 2 molecules of endogenous phosphate per tetramer, and found that, as in this present study, cAMPdPrK catalysed the incorporation of only two molecules of phosphate per tetramer. However, if the endogenous phosphate was first removed by a protein phosphatase which these workers have purified, cAMPdPrK could catalyse the incorporation of 4 molecules of phosphate per ATPCL tetramer. Peptide mapping of the ^{32}P -labelled ATPCL indicated that the cAMPdPrK phosphorylated both the phosphatase-treated and untreated ATPCLs at the same site.

The results of Ranganathan et al (1982) suggest that the

sub-stoichiometric incorporation noted in both their own study and this present study is the result of the partial occupancy of the cAMPdPrK phosphorylation sites. It is not clear, however, why this presumably metabolically labile phosphate was not lost during purification, as no precautions were taken to minimise protein phosphatase action. The stoichiometry of the in vitro phosphorylation of ATPCL by cAMPdPrK would, therefore, appear to depend on the activity of protein phosphatases during the isolation of the ATPCL. This might explain the recent result of Swergold et al (1982) who found that purified mouse liver ATPCL could be phosphorylated to the extent of 4 molecules of phosphate per tetramer.

No change in the activity of ATPCL was observed following phosphorylation by cAMPdPrK (Fig. 6.7) using either the assay of Linn and Srere (1979) or a modification of that assay in which the substrates of ATPCL are at their presumed physiological concentrations. These results are in keeping with those of other workers who used either rat liver or rat mammary gland ATPCL (Chapter 6.1). The absence of a change in activity does not appear to be the result of substoichiometric phosphorylation as Ranganathan et al (1982) and Swergold et al (1982) who phosphorylated ATPCL to the extent of 4 molecules of phosphate per tetramer were also unable to detect a change in activity.

I next examined the effect of phosphorylation by cAMPdPrK upon the various kinetic parameters of ATPCL. Table 6.1 indicates that the V_{\max} , the Michaelis constants for Mg-citrate and CoA and the dissociation constants for Mg-citrate and CoA are unchanged by phosphorylation. The fact that phosphorylation did not affect the V_{\max} of ATPCL was not unexpected given that no change in activity

had been observed using the assay of Linn and Srere, in which ATPCL is operating under V_{\max} conditions. The fact that no change was observed in the K_m for CoA casts doubt on the report by Roehrig (1980) that a change in ATPCL activity resulting from phosphorylation by cAMPdPrK can only be detected at low CoA concentrations. As shown in Figure 6.15 (b) and Table 6.1 the phosphorylation catalysed by cAMPdPrK is accompanied by a change in the K_m for MgATP. This changed from $90.1 \pm 1.7 \mu\text{M}$ to $193 \pm 3.6 \mu\text{M}$ (mean and S.E.M. of three separate experiments). This change was highly significant and indicated that at low MgATP concentrations, the phosphorylation catalysed by cAMPdPrK caused a decrease in the activity of ATPCL. This was verified in the experiment described in Fig. 6.16 where ATPCL was assayed at a MgATP concentration of $4.4 \mu\text{M}$. Phosphorylation resulted in a 50% decrease in ATPCL activity which correlated with the incorporation of 2 molecules of phosphate/tetramer.

These results are the first clear demonstration that the phosphorylation catalysed by cAMPdPrK does cause a change in one of the kinetic parameters of ATPCL. It is difficult to see how this change by itself can be physiologically relevant. The cytosolic MgATP concentration in rat liver is approximately 4 mM (Siess *et al.*, 1977) and so both forms of ATPCL will be saturated by MgATP in vivo. Hence, phosphorylation by cAMPdPrK would not be expected to alter the in vivo activity of rATPCL since both forms of the enzyme would be operating at V_{\max} . This assumes of course that the in vivo activity of ATPCL is regulated only by the concentrations of its substrates. I believe that this is unlikely. The regulation of ATPCL activity by other metabolites has not received a great deal of attention but it is clear that ATPCL does interact with factors other than its substrates. It is quite possible that phosphorylation by cAMPdPrK

alters the response of ATPCL to one or more of these factors. In Section 5 I discuss future work on ATPCL in the light of evidence for the existence of these factors.

Section 5 Future work on the phosphorylation of ATP citrate lyase by cAMP dependent protein kinase

The results discussed above suggest that although phosphorylation by cAMPPrK does have an effect on the kinetic properties of ATPCL, this change is unlikely to alter the activity of the enzyme in vivo. In this study, and in studies by other workers, ATPCL was assayed only in the presence of its substrates. This is a somewhat artificial situation as in vivo ATPCL is free to interact with other components in the cytosol. It is pertinent to ask therefore, whether no dramatic change in activity was noted because some essential component was not present in the assay. A discussion of such components is of course largely speculative, but given that phosphorylation does not appear to cause a physiologically relevant change in the interaction of ATPCL with its substrates, the effects of such components must now be considered.

The best studied effector of ATPCL is ADP which is a competitive inhibitor of the enzyme (Inoue et al, 1966). The effect of phosphorylation on the kinetic properties of ATPCL in the presence of ADP should be studied as it is possible that phosphorylation might decrease the K_i for ADP. The change in the K_m for MgATP noted in these studies might simply be a result of a change in the K_i for ADP since presumably both bind to the same site. Even if the K_i for ADP does not change, the effect of ADP on the in vivo activity should not be overlooked. In vivo, the presence of ADP will effectively increase the K_m for MgATP. On the basis of the K_i of 171 μ M reported by Inoue et al (1968) and the cytosolic ADP concentration reported by Siess et al (1977), one can estimate that in vivo the apparent K_m of ATPCL for MgATP would be 5.5 times that measured in the absence of ADP. Thus phosphorylation

will change the apparent K_m for MgATP from approximately 0.5 mM to 1.0 mM and so in vivo, the phosphorylated form will not be completely saturated. Thus, phosphorylation would be expected to cause a decrease in the activity of ATPCL in vivo.

In the kinetic study the assays were carried out in the presence of 0.25 M KCl which linearizes the otherwise hyperbolic double reciprocal plots of ATPCL activity against citrate concentration. As discussed in Chapter 1.2 this might indicate that citrate is an allosteric activator. It would therefore be of interest to study the effects of phosphorylation on the response of ATPCL to citrate in the absence of KCl.

Vogel and Bridger (1981) have recently reported that NADPH can protect ATPCL from thermal and proteolytic inactivation. These authors suggest that the decrease in NADPH concentration which would be expected to accompany starvation would result in the rapid degradation of ATPCL in vivo and that this would ensure that the generation of acetylCoA is coupled to the availability of NADPH. However, such a regulatory mechanism would not allow for a rapid increase in ATPCL activity if the concentration of NADPH were to rise. In any case, Guynn et al (1972) have shown that the cytoplasmic NADPH:NADP⁺ ratio does not change upon fasting, refeeding or administration of a high fat diet. A more plausible explanation is that NADPH is an allosteric effector of ATPCL: this would allow rapid modulation of ATPCL activity in response to any change in the level of NADPH. Unfortunately, Vogel and Bridger (1981) did not determine whether NADPH had any effect on the activity of ATPCL. The interaction of ATPCL with NADPH is clearly another area which phosphorylation by cAMPdPrK might affect, and in future work this should be studied.

In this study I have concentrated exclusively on the possible regulation of ATPCL by phosphorylation catalysed by cAMPdPrK. ATPCL is also phosphorylated in vivo in response to insulin, which implies the existence of an insulin-stimulated protein kinase. Ramakrishna and Benjamin (1981) have purified a cAMPiPrK from rat liver which can phosphorylate ATPCL to the extent of 2.4 molecules of phosphate/ATPCL tetramer. In a subsequent study Ramakrishna et al (1981) showed that the site phosphorylated by their kinase is located on a different tryptic peptide from that phosphorylated by cAMPdPrK. The authors suggest the cAMPiPrK which they have isolated might, therefore, be the kinase which mediates the effects of insulin. To prove this it will be necessary to show that the sites phosphorylated in vitro and in vivo are identical. A recent report by Swergold et al (1982) indicates that the sites phosphorylated in vivo in response to insulin or to β -agonists (and presumably glucagon) are located on the same 1000 M_r tryptic peptide. If insulin and glucagon are shown to cause the phosphorylation of the same serine residue then it follows that the phosphorylation of ATPCL is almost certainly without function since it is difficult to rationalize both hormones having the same effect on the enzyme. This result of Swergold et al (1982) suggests that the kinase isolated by Ramakrishna and Benjamin (1981) is distinct from the insulin stimulated kinase. Somewhat surprisingly the effects on ATPCL activity of phosphorylation in vivo in response to insulin, or in vitro by the cAMPiPrK of Ramakrishna and Benjamin (1981) have not been reported. This is clearly of great interest. The insulin-stimulated phosphorylation of ACC is accompanied by an increase in the activity of the enzyme. It is important to determine whether ATPCL is activated in a similar manner.

The insulin-stimulated phosphorylation of cytosolic proteins is

now well established. In addition to ATPCL and ACC there is evidence that ribosomal protein S6 and a 22000 M_r polypeptide (tentatively identified as phosphoprotein phosphatase inhibitor-1) are also phosphorylated in response to insulin (Belsham et al, 1980). However, neither the kinases which catalyse these reactions nor the factors which regulate the activity of the kinases have been identified. Brownsey et al (1981) have reported the presence of a cAMPiPrK in adipocyte plasma membrane preparations which can phosphorylate ACC and increase its activity in a similar fashion to insulin. This kinase may be similar to that present in hepatocyte plasma membranes which, in response to insulin, phosphorylates and activates the low K_m phosphodiesterase associated with these membranes (Marchmont and Housley 1980 a, b). It would be of interest to determine whether this membrane-associated kinase can also phosphorylate ATPCL and whether the site on each enzyme which is phosphorylated in vitro is identical to that phosphorylated in vivo.

Much of the work on the mechanism of action of insulin is aimed at identifying a second messenger for the hormone. Several workers have had some success in this area and have described a peptide-like substance which is released from plasma membranes in response to the binding of insulin and insulin agonists. In cell-free systems this substance mimics the effects of insulin on the activities of cAMPdPrK, pyruvate dehydrogenase and phosphoprotein phosphatase (Iarner et al, 1979; Jarret and Seals, 1979; Kiechle et al, 1981). It is now clear that the binding of insulin also results in the phosphorylation of the insulin receptor (on a Tyrosine residue) and that this phosphorylation is catalysed by the receptor itself (Kasuga et al, 1983). However, it is not known whether this phosphorylation is linked to any post-receptor event. One possibility is the existence of an

insulin-activated kinase cascade.

If it can be shown that the cAMPiPrK described by Brownsey et al (1981) is responsible for the insulin-stimulated phosphorylation of ACC and ATPCL (and that a single enzyme catalyses the phosphorylation of both enzymes), this would represent a considerable advance in the study of the mechanism of action of insulin. The insulin-dependent phosphorylation of ATPCL and ACC is a more convenient system than those used by Larner et al (1979) and Jarret and Seals (1979). The enzymes are soluble and can be fairly easily purified for use in a cell-free system. Moreover the insulin-stimulated phosphorylation of ATPCL and ACC can be more easily monitored than the effects of insulin on other systems. ATPCL is suitable for monitoring the direct incorporation of ^{32}P into protein since insulin-stimulated phosphorylation results in a marked increase in the amount of phosphate incorporated. In contrast, ACC has the advantage that phosphorylation is accompanied by a measurable increase in the activity of the enzyme. By using purified ACC or ATPCL as a substrate it should be possible to assay for, and subsequently purify the insulin-stimulated kinase. The availability of pure kinase will greatly facilitate the study of its regulation. Thus, even though the metabolic effects of the insulin-stimulated phosphorylation of ATPCL and ACC are important, this may be overshadowed by the potential of the system as a probe in the study of insulin action .

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