Massey University Library

Thesis Copyright Form

Title of thesis: Acetyl-CoA Carboxylase in the photosynthetic tissue of maize.

(1) (a) I give permission for my thesis to be made available to readers in the Massey University Library under conditions determined by the Librarian.

(b) I do not wish my thesis to be made available to readers without my written consent for _____ months.

((a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

(b) I do not wish my thesis, or a copy, to be sent to another institution without my written consent for _____ months.

(3) (a) I agree that my thesis may be copied for Library use.

(b) I do not wish my thesis to be copied for Library use for _____ months.

Signed Shane Rutherford

Date 17/10/88

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS	DATE

ACETYL-CoA CARBOXYLASE IN THE PHOTOSYNTHETIC TISSUE OF MAIZE

A thesis presented in partial fulfilment of the requirement for the degree of Master of Science in Biochemistry at MASSEY UNIVERSITY

SHANE McARTNEY RUTHERFURD

1988

ABSTRACT

The aim of this study was, a). to examine further, aspects of the role of acetyl-CoA carboxylase in the regulation of fatty acid synthesis in the provision of acyl lipid for plastid development, and b). to purify acetyl-CoA carboxylase from maize leaves using the affinity methods which have been used successfully to purify the enzyme from animal tissues.

In a constant weight of tissue, carboxylase activity decreased 7.6-fold over the period of 4 to 12 days after sowing, while total acetyl-CoA carboxylase activity increased 9-fold in maize seedlings over the period of 4 to 8 days with no further increase up to day 12. Protein levels decreased 3-fold over the growth period examined, while specific activity was constant at 27.2 to 28.3nmol/min/mg of protein between 4 and 6 days, before increasing to a maximum of 33.2nmol/min/mg of protein at day 7, then decreasing to one third of the maximum value on day 12. Chlorophyll levels in a constant weight of tissue increased 260-fold over the period of 4 to 11 days.

The changes in the level of acetyl-CoA carboxylase activity paralleled changes in fatty acid levels in tissue along the length of the 9-day-old maize leaf. The levels of both biochemical parameters increased in the region from the leaf

base to 15mm along the leaf. After which they both decreased to a minimum at 25-30mm along the leaf before increasing to a maximum at 60mm along the leaf, and finally decreasing towards the leaf tip.

A 5-fold increase in acetyl-CoA carboxylase activity was observed from the least favourable chloroplast stromal concentrations of ATP, ADP, Mg^{2+} and H^{+} in the dark, to the most favourable concentrations of these metabolites present in the chloroplast stroma during light periods.

These findings are consistent with, 1). a role for acetyl-CoA carboxylase in the regulation of fatty acid synthesis in maize photosynthetic tissue and, 2). control of acetyl-CoA carboxylase activity via light-dependent changes in the pH and concentrations of ATP, ADP and Mg^{2+} found in the stroma of chloroplasts.

Several attempts were made to purify acetyl-CoA carboxylase using avidin-affinity chromatography. However, after the initial, apparently successful attempt, active enzyme could not be recovered from the avidin-affinity column upon elution with biotin. Changes were made to several chromatographic conditions, and although ionic strength in the range of 0.1 to 1.0M KCl, did not affect the elution of active acetyl-CoA carboxylase from the column; lowering the column flow rates from 1.5ml/hr/ml of gel to 0.15-0.3ml/hr/ml of gel did appear to enhance the binding of the enzyme to the column. Using this flow rate, a 62 000 dalton protein and a 54 500 dalton protein were eluted in a fraction found to contain biotin-containing

proteins. Since it is feasible that the 62 000 dalton is biotin-containing and since this protein has a similar molecular weight to 60 000-62 000 dalton biotin-containing subunit of maize leaf acetyl-CoA carboxylase, the potential for purifying acetyl-CoA carboxylase from maize leaves using avidin-affinity chromatography seems to exist. However, further investigation is necessary in order to facilitate the recovery of active carboxylase from the avidin-affinity column.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr J.C.Hawke, for his advice, encouragement and assistance throughout the course of this study.

I would also like to thank the staff of the Plant Physiology Division, DSIR, for their assistance in using the climate control rooms for growing the maize plants, and Mr J.E.Ormsby for his care of the rats.

Thanks also go to Miss C.C.Mackle for carrying out fatty acid analyses.

Finally, thanks go to my wife, Kay, and my mother, Barbara, for assistance in the preparation of this manuscript.

LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCCP	biotin carboxyl-carrier protein
BSA	bovine serum albumin
CoA	coenzyme A
DGDG	digalactosyl diglyceride
DMCS	dimethyl dichlorosilane
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Mes	2[N-morpholino] ethane sulphonic acid
MGDG	monogalactosyl diglyceride
PBS	phosphate buffer-saline
PEG	polyethylene glycol
POPOP	1,4-bis[2(5-phenyloxazolyl)]benzene
ppGpp	guanosine 5'-diphosphate-3'-diphosphate
PPO	2,5-diphenyloxazole
pppGpp	guanosine 5'-triphosphate-3'-diphosphate
RNA	ribonucleic acid
Rubisco	ribulose 1,5-bisphosphate carboxylase oxygenase
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tricine	N-tris[hydroxymethyl]-methyl glycine
Tris	tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylene sorbitan monolaurate

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

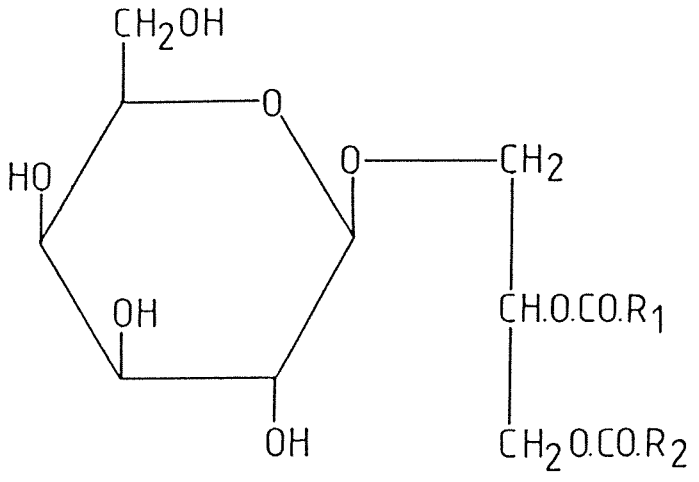
INTRODUCTION

1.1 Nature and Role of Lipids in Leaf Tissue

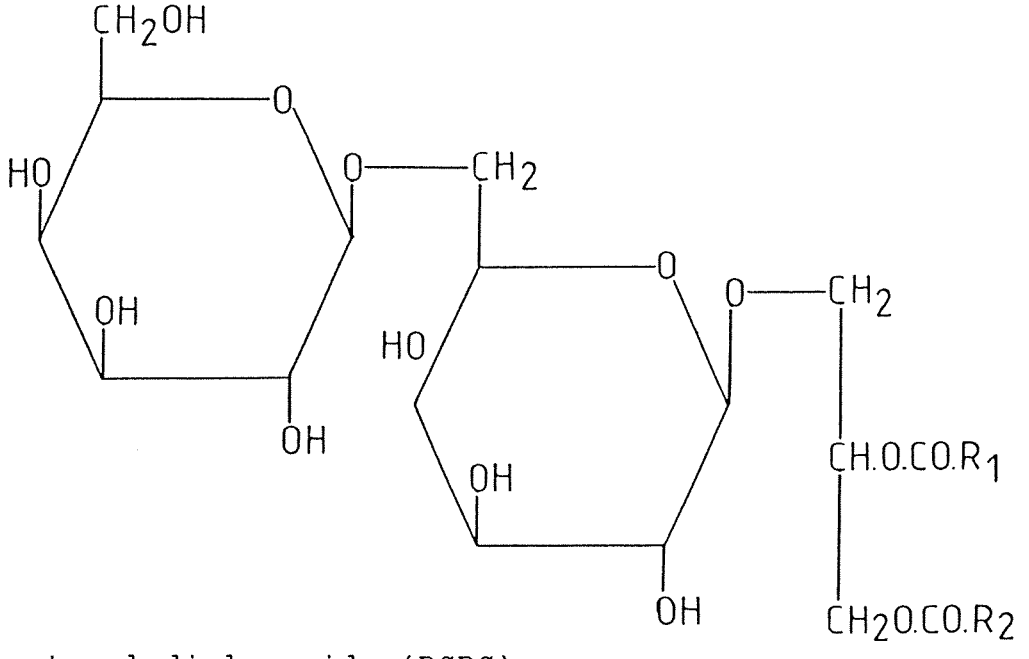
Lipid constitutes 10% of the dry weight of photosynthetic tissue, the main components being phospholipids, which constitute 15% to 35% (w/w) of total lipid, and galactolipids, which constitute 61% to 76% (Douce and Joyard, 1980). Chloroplasts contain a large proportion of the lipids, particularly the galactolipids and some types of phospholipids, for example phosphatidyl glycerol. In some species, such as beet and tobacco, chloroplasts contain nearly all the galactolipid present in the leaf (Wintermanns, 1960; Ongun et al., 1968).

The precise functions of galactolipids are uncertain. However, it is thought that galactolipids form the main components of the lipid bilayer of the thylakoid membrane, as galactolipids comprise 80% of the polar lipids in chloroplasts. The lipid bilayer provides a permeability barrier to polar molecules, and a flexible framework capable of housing a variety of proteins (Stumpf, 1980).

The two galactolipids, monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) (Fig.1) are the major galactolipids present in plant tissue, having been detected in



Monogalactosyl diglyceride (MGDG)



Digalactosyl diglyceride (DGDG)

FIGURE 1: Structure of Monogalactosyl Diglycerides (MGDG) and Digalactosyl Diglicerides (DGDG)

many plants including runner beans (Sastry and Kates, 1964), pumpkin (Roughan, 1970), barley (Gardiner, 1968), squash, white clover, lettuce, lucerne and maize (Roughan and Batt, 1969).

MGDG's contain a higher proportion of trienoic fatty acids than DGDG's (Leech et al., 1973), and plants may be grouped into two categories according to whether the galactolipids in the photosynthetic tissue contains 16:3 and 18:3, or, 18:3 as the sole trienoic fatty acid. Plants belonging to the former category are referred to as "16:3 plants" and include rape (Brassica rapus) and spinach (Spinacea oleracea), plants in the latter category, "18:3 plants" include barley (Hordeum vulgare), runner beans (Phaseolus multiflorus), and pumpkin (Curcurbita pepo). The "16:3 plants" usually have 16:3 fatty acid at the R_1 position, and 18:3 fatty acid at the R_2 position of the galactolipid, while the "18:3" plants have 18:3 fatty acids at both the R_1 and R_2 positions. Most of the fatty acids synthesized in photosynthetic tissue are destined to become the fatty acid moieties of the lipid constituents of the thylakoid membranes.

1.2 Sources of Acetyl-CoA for Fatty Acid Synthesis

The pyruvate dehydrogenase complex catalyses the conversion of pyruvate to acetyl-CoA, and until recently this enzyme complex was thought to be absent from the plastids of plants. This implied that the sole source of acetyl-CoA for fatty acid synthesis was exogenous acetyl-CoA formed in the mitochondria. Acetyl-CoA can be hydrolysed to form acetate in a reaction catalysed by acetyl-CoA hydrolase. Acetate then passes into the

cytosol, and through the chloroplast membrane where it may be re-esterified to form acetyl-CoA in a reaction catalysed by acetyl-CoA synthetase (Murphy and Stumpf, 1981). Sizeable incorporation of acetate into long chain fatty acids in the intact chloroplast has been observed in many plants, including barley (Appelqvist et al., 1968), lettuce (Brooks and Stumpf, 1965; Brooks and Stumpf, 1966), and maize (Hawke et al., 1974a). The presence of an active hydrolase and synthetase in spinach (Murphy and Stumpf, 1981), suggested that the above pathway was used for producing acetyl-CoA for fatty acid synthesis in the chloroplast.

The recent isolation of a chloroplastic form of the pyruvate dehydrogenase complex (Camp and Randall, 1985), indicates another possible source of acetyl-CoA; that is, formation directly from pyruvate in the chloroplast (Fig.2). Chloroplastic pyruvate dehydrogenase complex has a sharp alkaline pH optimum, as well as a requirement for Mg^{2+} (Camp and Randall, 1985). These requirements for activity suggest that the pyruvate dehydrogenase complex may be regulated by light, since illumination of chloroplasts causes an increase in both Mg^{2+} concentration and pH. De novo fatty acid synthesis occurs in the light, at which time the pyruvate dehydrogenase complex would be activated to supply the acetyl-CoA needed. Whether both pathways operate in vivo to supply acetyl-CoA for fatty acid synthesis is still unclear.

This proposal is similar to the earlier postulate that acetyl-CoA carboxylase is regulated by light, thereby, allowing an increased carbon flow along the fatty acid synthesis pathway

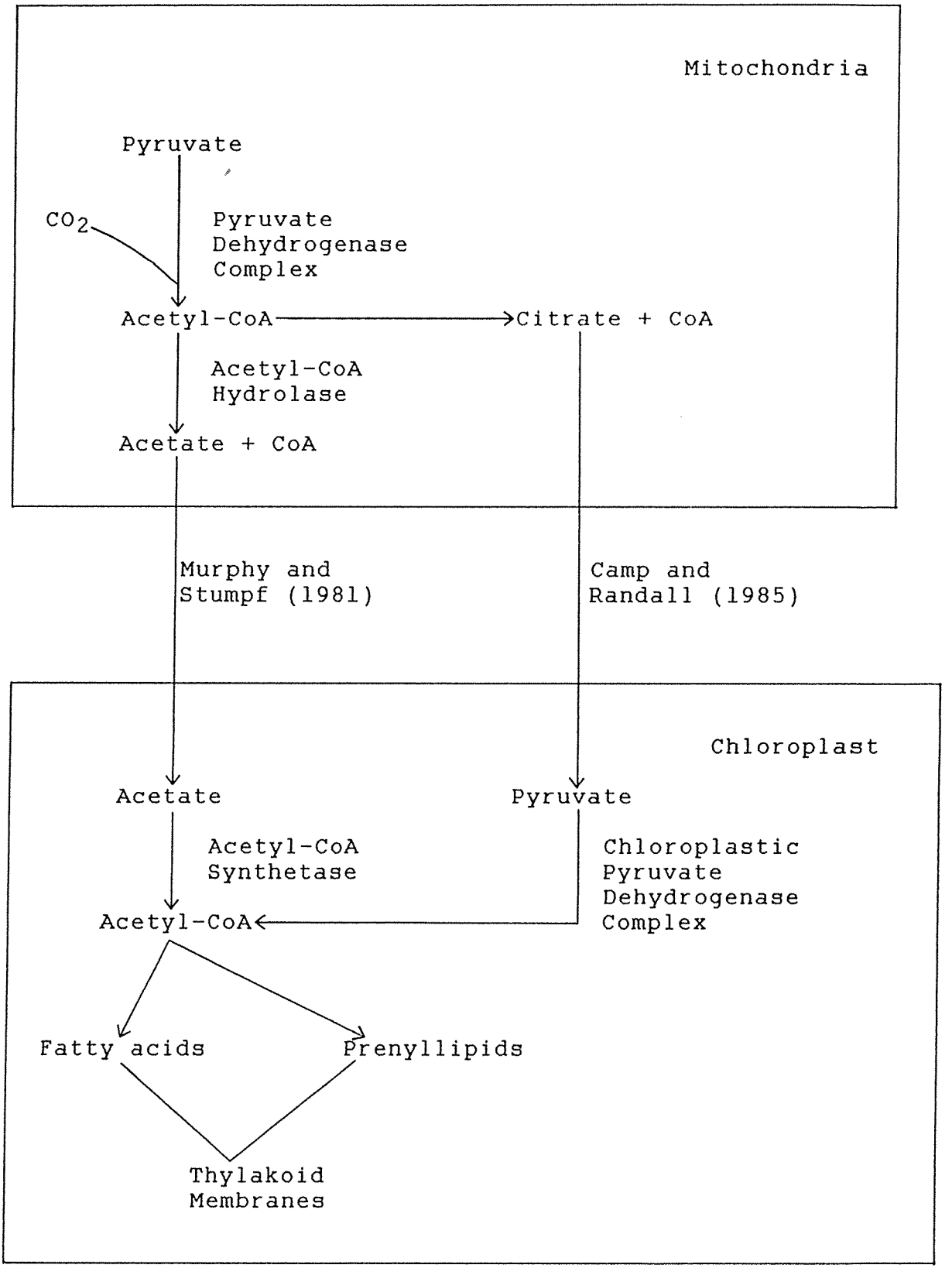


FIGURE 2: Alternative Proposals for the Provision of Acetyl-CoA in Mature Spinach Chloroplasts (Murphy and Stumpf, 1981; Camp and Randall, 1985)

in the presence of light (Nikolau and Hawke, 1984).

1.3 Nature of Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase [acetyl-CoA-carbon dioxide ligase (ADP forming) EC 6.4.1.2] is the enzyme responsible for the conversion of acetyl-CoA to malonyl-CoA, by the addition of carbon dioxide derived from bicarbonate. The reaction catalysed by acetyl-CoA carboxylase is the first committed step of fatty acid synthesis and consists of two half reactions (Reactions 1 and 2).

$$HCO_3^- + ATP + \text{E-biotin} \xrightarrow{Mg^{2+}} \text{E-biotin-}CO_2^- + ADP + Pi \quad (1)$$

$$\text{acetyl-CoA} + \text{E-biotin-}CO_2^- \longrightarrow \text{malonyl-CoA} + \text{E-biotin} \quad (2)$$

$$\text{acetyl-CoA} + HCO_3^- + ATP \xrightarrow{Mg^{2+}} \text{malonyl-CoA} + ADP + Pi$$

1.3.1 Acetyl-CoA Carboxylase in E. coli

The carboxylation of acetyl-CoA in E. coli involves three proteins; biotin carboxylase, carboxyltransferase and a biotin carboxyl carrier protein. The first half reaction (Reaction 3), involves the ATP-dependent carboxylation of the biotin prosthetic group of the carboxyl carrier protein (BCCP), which is catalysed by biotin carboxylase. The second half reaction

$$\text{CCP-biotin} + HCO_3^- + \text{ATP} \underset{\text{carboxylase}}{\overset{Me^{2+}\ \text{biotin}}{\rightleftharpoons}} \text{CCP-biotin-}CO_2^- + \text{ADP} + \text{Pi} \quad (3)$$

$$\text{CCP-biotin-}CO_2^- + \text{acetyl-CoA} \underset{\text{transferase}}{\overset{\text{carboxyl-}}{\rightleftharpoons}} \text{malonyl-CoA} + \text{CCP-biotin} \quad (4)$$

$$\text{acetyl-CoA} + HCO_3^- + \text{ATP} \overset{Me^{2+}}{\rightleftharpoons} \text{malonyl-CoA} + \text{ADP} + \text{Pi}$$

(Reaction 4), involves the transfer of carbon dioxide from the biotin carboxyl carrier protein to acetyl-CoA, producing malonyl-CoA. This reaction is catalysed by carboxyl-transferase. Biotin carboxylase is composed of two 50 000 dalton subunits, while carboxyltransferase has a molecular weight of 130 000 daltons, and is composed of four subunits, two of 30 000 daltons, and two of 35 000 daltons. The biotin carboxyl carrier protein is made up of two 22 000 dalton subunits, and contains one biotin molecule per 22 000 dalton subunit (Lane et al., 1974). It is thought that the biotin carboxyl carrier protein, biotin carboxylase and the carboxyltransferase exist as a ternary complex, since biotin carboxylase, although not required for Reaction 4, activates the reaction (Guchhait et al., 1974).

1.3.2 Acetyl-CoA Carboxylase in Animals

Acetyl-CoA carboxylase has been isolated from several different animal sources, including rat liver (Nakanishi and Numa, 1970; Tipper and Witters, 1982; Holland et al., 1984), rat mammary gland (Ahmad et al., 1978), rabbit mammary gland (Hardie and Cohen, 1979), bovine adipose tissue (Moss et al., 1972) and the free-living nematode Turbatrix aceti (Meyer et al., 1978).

In animals, the molecular weight for the acetyl-CoA carboxylase polymer ranges from 4 to 8 x 10^6 daltons. The protomer consists of two subunits of molecular weight 230 000 to 260 000 daltons. Each subunit contains an active site for biotin carboxylase and carboxyltransferase activity, as well as the biotin carboxyl carrier protein, and allosteric effector sites (Wakil et al., 1983).

1.3.3 Acetyl-CoA Carboxylase in Plants

Acetyl-CoA carboxylase has been isolated from several plant sources, including barley embryos (Brock and Kannangara, 1976), wheat germ (Heinstein and Stumpf, 1969; Nielsen et al., 1979; Egin-Bühler et al., 1980), avocado plastids, spinach chloroplasts (Mohan and Kekwick, 1980), parsley cell cultures (Egin-Bühler et al., 1980) and maize (Nikolau and Hawke, 1984). The molecular weight of acetyl-CoA carboxylase from these sources vary from 500 000 to 840 0000 daltons, the number and size of the constituent subunits also varies.

Using polyacrylamide gel electrophoresis under dissociating conditions, it has been shown that acetyl-CoA carboxylase in barley embryos (Brock and Kannangara, 1976) and avocado plastids (Mohan and Kekwick, 1980) contains three subunits, with molecular weights ranging from 21 000 to 41 000 daltons. Wheat germ acetyl-CoA carboxylase was thought to contain six subunits ranging from 21 000 to 135 000 daltons (Nielsen et al., 1979), but it has since been found that there are only two subunits, of molecular weight 240 000 and 98 000 daltons (Egin-Bühler et al., 1980). The identification of six subunits of wheat germ

acetyl-CoA carboxylase was probably due to limited proteolysis of the two large subunits. Maize acetyl-CoA carboxylase was estimated to have a molecular weight of 500 000 daltons, made up of a number of unique subunits of molecular weight 61 000 to 62 000 daltons (Nikolau and Hawke, 1984). Acetyl-CoA carboxylase from parsley cell cultures has two subunits, of molecular weight 210 000 and 105 000 daltons (Egin-Bühler et al., 1980).

Until recently the nature of the biotin-containing subunits of acetyl-CoA carboxylase in higher plants had been unclear. Nikolau et al. (1985), examined 14 different plant sources for biotin-containing proteins including such diverse sources as leaf tissue from spinach, maize, leeks, peas, sorghum, beet and lima beans, callus tissue cultured from sorghum and lima beans, cotyledons from cucumbers, rose petals and potato tubers. They used western blotting analysis to detect biotin-containing proteins, and found four major biotin-containing proteins from the different sources. These four proteins have molecular weights of 30 000 to 32 000, 33 000 to 35 000, 50 000 to 51 000, and 60 000 to 62 000 daltons. There seems to be little conformity as to the subunit composition of acetyl-CoA carboxylase in the different plant tissues. It is not known whether the presence of multiple biotin-containing proteins is due to the existence of other biotin-containing enzymes, or to isoenzymes of acetyl-CoA carboxylase. Purified acetyl-CoA carboxylase from maize tissue has a biotinyl subunit of 60 000 daltons, yet a 50 000 to 51 000 dalton biotin-containing protein was also found in maize leaf extracts. In maize epidermal protoplasts, which contain acetyl-CoA carboxylase

activity, the 50 000 to 51 000 dalton protein is the only biotin-containing protein present (Nikolau *et al.*, 1984), suggesting that, in maize at least, isoenzymes of acetyl-CoA carboxylase do exist. Although it has been found that *in vitro* cleavage of one large biotin-containing protein to give a variety of smaller biotin-containing proteins does not occur, the multiple biotin-containing subunits may result from the degradation of acetyl-CoA carboxylase *in vivo* during protein turnover (Nikolau *et al.*, 1985).

1.3.3.1 Tissue Distribution of Acetyl-CoA Carboxylase

Malonyl-CoA is not only the precursor of fatty acid synthesis, but also the synthesis of other compounds including flavonoids, cuticular waxes, stilbenoids and malonic acid. Some of these biosynthetic systems are compartmentalized in different cells in the leaves, so it is not unexpected to find acetyl-CoA carboxylase distributed in different amounts amongst the different types of cells. In C_3-plants such as *Pisum sativum* and *Allium porrum*, the mesophyll cells contained 90% of the leaf acetyl-CoA carboxylase activity, with the epidermal cells containing the remainder (Nikolau *et al.*, 1984). However, in C_4-plants such as sorghum and maize, acetyl-CoA carboxylase activity is distributed more equally throughout the mesophyll, epidermal and bundle sheath tissues. In maize the distribution of acetyl-CoA carboxylase activity between these three tissues is 56%, 10% and 32% respectively (Nikolau *et al.*, 1984).

1.3.3.2 Stromal Nature of Acetyl-CoA Carboxylase

It was originally thought that acetyl-CoA carboxylase in plant tissue existed as three separate components, with the biotin carboxylase and carboxyltransferase moieties located in the stroma, and the biotin carboxyl carrier protein bound to the lamallae (Kannangara and Stumpf, 1972). However, Reitzel and Nielsen (1976), found 88% of the total acetyl-CoA carboxylase activity of barley leaf homogenates was present in supernatants, following centrifugation to remove all membranes. Nikolau et al. (1981), found acetyl-CoA carboxylase activity, and 96% of carboxylatable biotin in stromal fractions of isolated maize chloroplasts, while in isolated spinach chloroplasts 94% of the total acetyl-CoA carboxylase activity was found in the stromal fraction (Nikolau et al., 1985), with the remainder of the activity loosely associated with the thylakoid membranes. Therefore, it is evident that plant acetyl-CoA carboxylase is a soluble enzyme located in the stroma of the chloroplast.

1.4 Proposed Mechanisms for Acetyl-CoA Carboxylase Activity

Acetyl-CoA carboxylase from avian liver (Wakil and Gibson, 1960), and rat liver (Kosow et al., 1962) contains a biotin prosthetic group covalently attached to the ϵ-amino group of a lysine residue, via the carboxyl group of the biotin side chain (Kosow et al., 1962). Protein-bound biotin binds to carbon dioxide at the 1'-N position, in the first step of the two step reaction catalysed by acetyl-CoA carboxylase (Waite and Wakil, 1966; Numa et al., 1964). Kinetic mechanisms for this reaction proposed by Lynen et al. (1961) and Knappe et al. (1961a,b)

suggest an ordered kinetic mechanism for the reaction catalysed by acetyl-CoA carboxylase as cited by Beaty and Lane (1982), (Fig.3a) with ATP and HCO_3^- binding to acetyl-CoA carboxylase and reacting to form the carboxylbiotinyl intermediate. Acetyl-CoA then binds to produce a quarternary complex between the carboxylbiotinyl intermediate, ADP, Pi and acetyl-CoA. Transfer of carbon dioxide to acetyl-CoA occurs, then malonyl-CoA, the product of carboxylation of acetyl-CoA, is released. This is followed by the release of Pi and finally ADP. However, Hashimoto and Numa (1971), have proposed a ping-pong (double displacement) mechanism for the reaction catalysed by acetyl-CoA carboxylase in rat liver (Fig.3b), with the existence of two independent catalytic sites, one at which the biotin prosthetic group is carboxylated, and the other where carboxyl transfer from carboxyl-biotinyl-enzyme to acetyl-CoA occurs. While this mechanism may apply to rat liver acetyl-CoA carboxylase, kinetic studies have indicated that it does not hold for chicken liver acetyl-CoA carboxylase (Beaty and Lane, 1982), and although this difference could result from species-dependence, it is still uncertain.

1.5 Regulation of Acetyl-CoA Carboxylase

1.5.1 Regulation in E. coli

Fatty acids synthesized in E. coli are used almost exclusively for membrane synthesis and maintenance. Therefore, fatty acid synthesis is likely to be coupled to protein and RNA synthesis, as these, as well as membrane synthesis, are growth-related processes. The linking factor between these

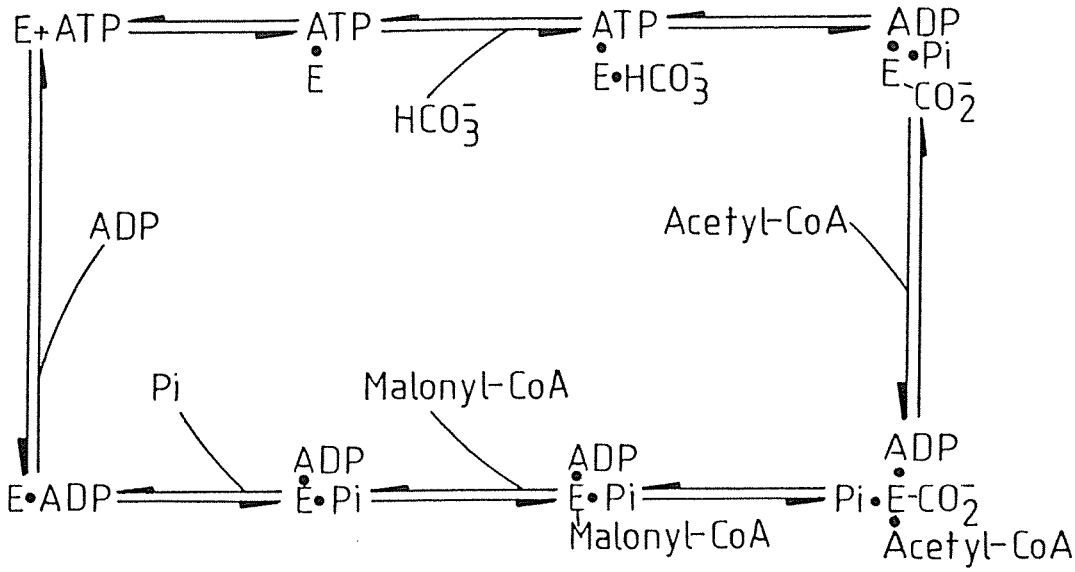


FIGURE 3a: Ordered Kinetic Mechanism of the Reaction Catalysed by Acetyl-CoA Carboxylase in Chicken Liver. Proposed by Lynen et al., (1961); Knappe et al., (1981a,b).

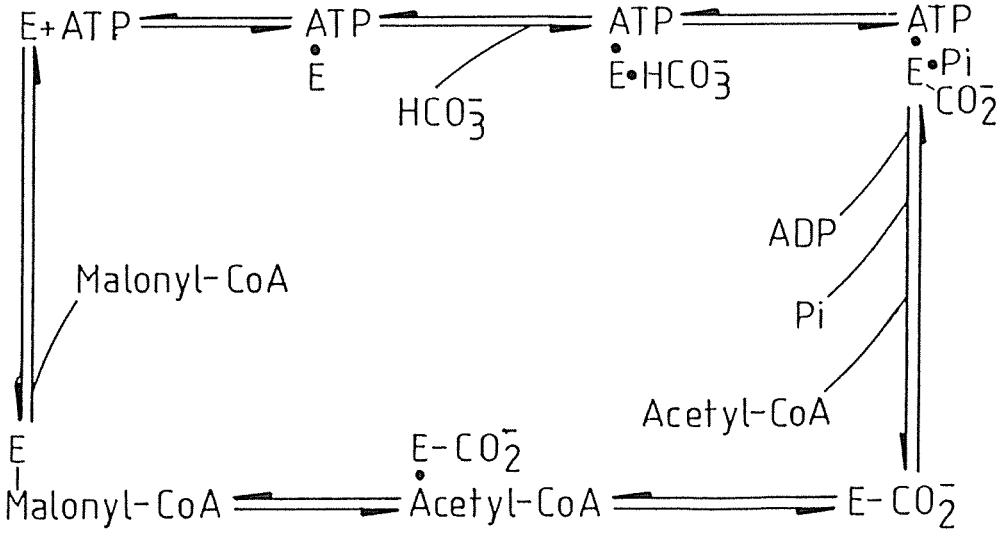


FIGURE 3b: Ping-Pong Kinetic Mechanism of the Reaction Catalysed by Acetyl-CoA Carboxylase in Rat Liver. Proposed by Hashimoto and Numa, (1971). From Beaty and Lane, (1982).

E = enzyme, . = non covalent band, - = covalent band

processes is the rel gene (RC locus) in E. coli. This gene controls ribonucleic acid synthesis and has been implicated in the control of lipid synthesis.

During amino acid starvation protein synthesis ceases, as does RNA synthesis (Neilhardt, 1966), however, this dependence of RNA synthesis on protein synthesis, or "stringency", is lost in the rel^- (relaxed) mutant.

In protein-dependent RNA synthesis two nucleotides have been detected during amino acid starvation (Cashel and Gallant, 1969). These two nucleotides ppGpp and pppGpp, have not been detected in the rel^- mutant, and it has been determined that they mediate stringent control of lipid synthesis in E. coli, by acting as negative effectors on acetyl-CoA carboxylase (Polakis et al., 1973). Furthermore, it has been determined that ppGpp and pppGpp bind to a common site on the carboxyltransferase causing 50 to 60% inhibition of the carboxyltransferase activity, thereby inhibiting lipid synthesis.

1.5.2 Regulation in Animals

Animal acetyl-CoA carboxylase, in its protomeric form, is a dimer made up of identical monomers. The inactive protomer can undergo reversible association to become the polymeric, active form of the enzyme.

The main allosteric effectors of acetyl-CoA carboxylase are citrate, isocitrate, malonyl-CoA, fatty acyl-CoA's and free

CoA. Citrate and isocitrate activate chicken liver acetyl-CoA carboxylase by increasing the V_{max}, without altering the Km of the enzyme for any of its subtrates (Gregolin et al., 1968a), while citrate activation is accompanied by polymerisation of the enzyme in chicken liver (Gregolin et al., 1966a, Gregolin et al., 1966b; Gregolin et al., 1968b). Acetyl-CoA carboxylase has similar binding constants for citrate and isocitrate, and similar V_{max} values when citrate or isocitrate are bound (Lane et al., 1974). Therefore, citrate and isocitrate are almost equally effective in activating acetyl-CoA carboxylase. Free CoA also activates acetyl-CoA carboxylase. There is one CoA binding site per subunit of acetyl-CoA carboxylase, and CoA binding, while unaffected by citrate, is inhibited by palmitoyl-CoA (Yeh et al., 1981). Unlike citrate and isocitrate, CoA activation of acetyl-CoA carboxylase is not accompanied by enzyme polymerisation (Yeh et al., 1981). CoA reduces the Km of acetyl-CoA carboxylase for acetyl-CoA from 400μM to as low as 4μM (Yeh and Kim, 1980). The Km value of the activated enzyme for acetyl-CoA is close to the acetyl-CoA concentration in rat liver which is reported to be 5μM (Yeh and Kim, 1980).

Malonyl-CoA is a potent inhibitor of acetyl-CoA carboxylase from avian liver (Lane et al., 1974), and rat liver (Ogiwara et al., 1978). It inhibits competitively with respect to citrate and acetyl-CoA. The competitive inhibition between malonyl-CoA and acetyl-CoA is of the classical product-substrate competitive inhibition type. The competitive relationship between malonyl-CoA and citrate is thought to result from the opposing effect of citrate and malonyl-CoA on the polymer-protomer

equilibrium (Gregolin et al., 1966b).

Long chain fatty acyl-CoA's are potent inhibitors of hepatic acetyl-CoA carboxylase (Lane et al., 1974; Kim, 1983). Inhibition by palmitoyl-CoA is competitive with respect to citrate, but non-competitive with respect to substrates (Goodridge, 1972).

1.5.2.1 Regulation by Covalent Modification

In 1973, it was reported that acetyl-CoA carboxylase activity from rat liver was regulated by covalent modification involving the interconversion between a phosphorylated (inactive), and dephosphorylated (active) form of the enzyme (Carlson and Kim, 1973). Later, it was reported that the phosphorylation and inactivation of acetyl-CoA carboxylase was accompanied by the conversion of the polymeric (active) form of the enzyme to the protomeric (inactive) form (Lent et al., 1978; Shiao et al., 1981). Other studies indicated that the polymeric form is the favoured species for phosphorylation (Kim, 1983), and that the depolymerised enzymes are the phosphorylated forms rather than protomers of unphosphorylated species (Lee and Kim, 1979). The phosphorylated acetyl-CoA carboxylase is more susceptible to inhibition or inactivation by a variety of negative effectors such as palmitoyl-CoA, avidin and ATP (Carlson and Kim, 1974; Brownsey et al., 1979), while the dephosphorylated enzyme is more sensitive to citrate activation (Brownsey et al., 1979; Hardie and Guy, 1980). Therefore covalent modification makes the allosteric control mechanisms functional at the physiological concentrations of cellular metabolites.

Cyclic AMP and its derivative dibutyryl cyclic AMP inactivate acetyl-CoA carboxylase. However the mechanism by which cyclic AMP inactivates the enzyme is unclear. In epididymal fat pads of rats (Carlson and Kim, 1974), and rat liver (Lent and Kim, 1982), phosphorylation of acetyl-CoA carboxylase has been reported to be unaffected by cyclic AMP-dependent protein kinase, indicating that the endogenous protein kinase present in acetyl-CoA carboxylase preparations is cyclic AMP-independent. It has been found that in chicken liver, cyclic AMP inhibits the formation of cellular citrate. It is now thought that it is this reduction in citrate concentration that causes inactivation of chicken liver acetyl-CoA carboxylase. In rat liver, the phosphorylating and inactivating effect of cyclic AMP can be duplicated by AMP (Yeh et al., 1980). It has been suggested that AMP is the major inactivating compound, and that acetyl-CoA carboxylase therefore, is inactivated by low energy status in the cell. This suggestion is supported by the knowledge that acetyl-CoA carboxylase is concerned with the deposition of excess cellular energy equivalents as lipid. However, if this were the case, one would expect acetyl-CoA carboxylase to be active under conditions of high ATP, yet the enzyme is inactivated by an ATP-dependent phosphorylating mechanism (Carlson and Kim, 1974). This paradox can be rationalised by assuming that at high concentrations of ATP, the enzyme is maintained in the dephosphorylated, active form by means of allosteric regulation. Chen and Kim (1982), found three adenylate nucleotide binding sites on acetyl-CoA carboxylase, one catalytic ATP site, one regulatory ATP site and one regulatory site for cyclic AMP and AMP. The discovery of three adenylate nucleotide binding sites supports the above

hypothesis. However, other workers have reported phosphorylation, and inactivation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase, and cyclic AMP-independent protein kinases in rabbit mammary gland (Hardie and Cohen, 1978). Phosphorylation and inactivation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase has also been reported in adipocytes and hepatocytes of rats (Holland et al., 1984; Holland et al., 1985). Therefore the mechanism by which cyclic AMP inactivates acetyl-CoA carboxylase is still uncertain, and may indeed involve phosphorylation by cyclic AMP-dependent and independent protein kinases.

1.5.3 Regulation in Plants

While the regulation of acetyl-CoA carboxylase by covalent modification has been well documented in animals, there is no evidence of this form of regulation of acetyl-CoA carboxylase in plant systems. Acetyl-CoA carboxylase requires both ATP and Mg^{2+} for maximal activity (Nikolau and Hawke, 1984). Wheat germ (Nielsen et al., 1979), avocado plastid (Mohan and Kekwick, 1980) and maize (Nikolau and Hawke, 1984) acetyl-CoA carboxylase require the Mg^{2+}-ATP complex for activity, while free Mg^{2+} activates the enzyme, and free ATP has an inhibitory effect on acetyl-CoA carboxylase (Nielsen et al., 1979).

The dependence of acetyl-CoA carboxylase from different plant sources on acetyl-CoA and HCO_3^- for activity is quite uniform, as the Km values obtained for acetyl-CoA and HCO_3^- are of the same order of magnitude respectively, independent of the plant source (Table 1).

TABLE I

Km VALUES OF ACETYL-CoA AND HCO_3^- FOR ACETYL-CoA CARBOXYLASE OBTAINED FROM DIFFERENT PLANT SOURCES

Plant Source	Km Values	
	Acetyl-CoA (mM)	HCO_3^- (mM)
Maize leaves	0.11[2]	1.4[2]
Wheat Germ	0.15[3]	1.05[3]
Barley leaves	0.11[4]	2.1[4]
Spinach leaves	0.15[1]	3.0[1]
Avocado plastid	0.26[1]	8.0[1]

1. Mohan and Kekwick, 1980

2 Nikolau and Hawke, 1984

3 Hatch and Stumpf, 1961

4 Nikolau, 1981

1.5.3.1 Effects of Monovalent Cations on the Activity of Acetyl-CoA Carboxylase

HCO_3^--dependent activation of acetyl-CoA carboxylase by certain monovalent cations has been found in wheat germ (Nielsen et al., 1979) and maize (Nikolau and Hawke, 1984). Maximal activation occurs with K^+ and Rb^+, while smaller cations such as Na^+ and Li^+ are either inhibitory, or neutral in their effect on acetyl-CoA carboxylase activity. The mechanism is uncertain, but it has been suggested that activation is due to the formation of a ternary complex between the substrate, enzyme and cation (Seulter, 1970). Not only is monovalent cation activation of acetyl-CoA carboxylase HCO_3^--dependent but it is also dependent on the atomic radii of the cation. However, the activation curve for NH_4^+ is a different shape to that of K^+ or Rb^+ even though NH_4^+ has a radius between that of K^+ and Rb^+. It has been shown that monovalent anions have no effect on the activity of acetyl-CoA carboxylase in plants (Nielsen and Stumpf, 1976).

1.5.3.2 Effects of Light on the Activity of Acetyl-CoA Carboxylase

Nakamura and Yamada (1979), found that incorporation of radioactively labelled acetate into acetyl-CoA and fatty acids occurred in the light, while in the dark, labelled acetate was incorporated only into acetyl-CoA. This suggests that light stimulates fatty acid synthesis at the site of acetyl-CoA carboxylase.

Reitzel and Nielsen (1976), found that while total amounts of acetyl-CoA carboxylase activity increased in both light-grown and etiolated barley seedlings over the first week after germination, the rate of increase in light-grown seedlings was approximately five times greater than the increase in etiolated tissue. It was also found that in etiolated barley tissue, while the total activity increased during the first week after germination, the specific activity remained the same. In light-grown seedlings, the specific activity increased over the first week concomitantly with the increase in total activity. It was also found that approximately twelve hours after etiolated seedlings had been placed in the light, acetyl-CoA carboxylase activity was detected, and this increased (over twelve hours) to the level of activity in green seedlings. It has been suggested that the increase in activity of plant acetyl-CoA carboxylase in response to light can occur by short-term and long-term mechanisms, with the latter involving increased synthesis of acetyl-CoA carboxylase in response to light (Reitzel and Nielsen, 1976). In short-term regulation by light it is proposed that regulation of acetyl-CoA carboxylase activity is mediated by the availability of photochemically generated ATP (Eastwell and Stumpf, 1983) and by light-dependent changes in stromal pH and Mg^{2+} concentrations (Nikolau and Hawke, 1984; Hawke and Leech, 1987).

In the dark, ATP and ADP concentrations in the chloroplast stroma are approximately 0.3 to 0.9mM and 0.7 to 1.2mM respectively (Stitt et al., 1982; Hampp et al., 1982; Kobayashi et al., 1979). On illumination of chloroplasts there is photophosphorylation of ADP to form ATP. This process is driven

by a proton gradient across the thylakoid membrane (Heldt et al., 1973; Werdan et al., 1975), and Mg^{2+} ions enter the stroma to provide a constant ionic environment. As a result of these processes, the concentration of ATP rises to 0.8-1.4mM while the ADP concentration falls to 0.3-0.4mM (Stitt et al., 1982; Hampp et al., 1982; Kobayashi et al., 1979). The pH changes from approximately 7.1 to 8.0, and the concentration of Mg^{2+} ions rises from 2mM in the dark (Portis, 1981) to approximately 5mM in the light (Portis et al., 1976; Hind et al., 1974).

Nikolau and Hawke (1984), found a two-fold increase in maize leaf acetyl-CoA carboxylase activity resulted from the dark-light changes in concentration of each metabolite: ATP, ADP and Mg^{2+}, while a three-fold increase in activity resulted from the dark-light change in pH. If all changes in concentration of metabolites and appropriate changes in pH are co-ordinated and the effects are cumulative then a possible 24-fold increase in activity of acetyl-CoA carboxylase would result. Subsequently Hawke and Leech (1987), have reported a 10-fold increase in acetyl-CoA carboxylase activity in wheat leaves from the least favourable to the most favourable conditions that result from dark-light transitions.

1.6 Purification of Acetyl-CoA Carboxylase

1.6.1 Non-Affinity Methods

Acetyl-CoA carboxylase has been purified to varying levels of purity from many sources, using a variety of methods.

Guchhait et al. (1974), purified the components of E. coli acetyl-CoA carboxylase using non-affinity methods. They subjected the 25-42% ammonium sulphate precipitate of the cell-free extract to two successive calcium phosphate gel fractionations. Biotin carboxylase was eluted from the calcium phosphate gel with 0.12M potassium phosphate at pH 7.0, and carboxyltransferase was eluted using 0.5M potassium phosphate at pH 7.0. Biotin carboxylase was then subjected to DEAE-cellulose chromatography and cellulose phosphate chromatography before crystals were collected following dialysis. Carboxyltransferase was further purified by successive column chromatographic fractionations on DEAE-cellulose, phosphocellulose and DEAE-cellulose (type 20).

A purification of 900-fold was reported for the biotin carboxylase, and 480-fold for the carboxyltransferase component. Recovery of biotin carboxylase activity was 12%, with most activity being lost at the ammonium sulphate fractionation and cellulose phosphate chromatography steps. While recovery of carboxyltransferase activity was 3.2% with most activity being lost at the phosphocellulose chromatography step.

Modifications to this method of purification have been carried out on tissues from different sources with varying degrees of success. For example, Nakanishi and Numa (1970), achieved a 1700-fold increase in specific activity of rat liver acetyl-CoA carboxylase by precipitation in 30% ammonium sulphate, followed by elution from a column of calcium phosphate gel with 0.2M potassium phosphate pH 7.3, then 25% ammonium sulphate

fractionation. The precipitate was dialysed in the presence of citrate before the supernatant was subjected to DEAE-cellulose and Sepharose 2B chromatography. A recovery of 17% was achieved, with most loss in activity occurring at the DEAE-cellulose chromatography step.

In the purification of acetyl-CoA carboxylase from maize leaf tissue, the 6-14% polyethylene glycol precipitate of the crude extract was subjected to 40% ammonium sulphate precipitation. The redissolved precipitate was subjected to filtration on an Ultragel AcA 22 column. Fractions containing acetyl-CoA carboxylase activity were concentrated using a Diaflo PM 30 filtration membrane and then subjected to a second fractionation on the Ultragel AcA 22 column. A 90-fold purification of acetyl-CoA carboxylase and a recovery of 3% was obtained. Ion-exchange chromatography, and, CoA and avidin-affinity chromatography gave inferior purifications of maize acetyl-CoA carboxylase compared to the procedure above. (Nikolau and Hawke, 1984).

1.6.2 Avidin-Affinity Chromatography

Avidin is a glycoprotein found in egg-white, and is well known for its inhibitory effect on biotin-containing enzymes. The biotin-binding ability of avidin makes it a potential affinity column ligand, for purifying many biotin-containing enzymes. Avidin in its native tetrameric form has a Kd for biotin of 10^{-15}M (Moss and Lane, 1971), while monomeric avidin has a much weaker affinity for biotin. Monomeric avidin is therefore routinely used for avidin-affinity chromatography, as

tetrameric avidin will only release biotin-containing proteins under denaturing conditions. Avidin-affinity chromatography has been successfully used to purify acetyl-CoA carboxylase from rat adipocytes (Holland et al., 1985), rat hepatocytes (Holland et al., 1984; Tipper and Witters, 1982; Thampy and Wakil, 1985), chicken liver (Beaty and Lane, 1982), rape seed (Slabas and Hellyer, 1985), and parsley cell cultures (Egin-Bühler and Ebel, 1983). However, this method has proved relatively unsuccessful in the purification of acetyl-CoA carboxylase from maize leaf tissue (Nikolau and Hawke, 1984).

1.6.2.1 Preparation of the Avidin-Affinity Chromatography Column

Green and Toms (1973), coupled the avidin tetramer to Sepharose 4B following activation by cyanogen bromide. The column was then washed with guanidium chloride to dissociate the avidin-tetramer and elute non-covalently bound subunits. On dissociation of the tetramer, it was found that three classes of binding sites existed; 25% had a low affinity for biotin ($K=5 \times 10^{-8}M$), about 33% had an intermediate affinity ($K=10^{-10}M$), while the rest had the affinity of the native tetramer ($K=10^{-15}M$) (Green and Toms, 1973). Although not proven, the existence of three classes of binding sites would seem to be due to the presence of monomer, dimer and tetramer forms of avidin bound to the Sepharose. This could be the result of: 1). the ligand concentration being too high, resulting in association of covalently bound subunits, 2). incomplete dissociation of the native tetramer by guanidium chloride, 3). a temperature-dependent association of the

covalently bound avidin monomers, resulting from a temperature-dependent mobility of the agarose matrix (Green and Toms, 1973).

To block any high or intermediate affinity sites, the avidin-affinity column can be washed with biotin prior to use. This process saturates all binding sites with biotin. By washing the column with 0.1M glycine-HCl at pH 2.0 the biotin bound to the lower affinity sites can be removed, making them available for the binding of biotin-containing proteins. This method protects the proteins from binding to high or intermediate affinity sites, since if this was to occur, elution would only be possible under denaturing conditions. Blocking the high and intermediate affinity sites results in increased recoveries of biotin-containing proteins purified by avidin-affinity chromatography (Henrikson et al., 1979).

Since little is known about the regulation of fatty acid synthesis in plants, this study aims to investigate the relationship between acetyl-CoA carboxylase activity and fatty acid synthesis in developing maize leaves. Also, it has been suggested that this regulation is via light-dependent changes in stromal pH and concentrations of ATP, ADP and Mg^{2+}. The effect of these changes on acetyl-CoA carboxylase activity from maize leaves will also be investigated. Finally, maize leaf acetyl-CoA carboxylase has yet to be purified to homogeneity, and an attempt will be made, using avidin-affinity chromatography, to achieve this.

CHAPTER 2

MATERIALS

2.1 Plant Materials

Maize (Zea mays var. XL45) seeds were obtained from Arthur Yates and Co., Ltd, N.Z., while maize (Zea mays var. Pioneer 3709) seeds were obtained from Elders Pastoral, N.Z. Ltd. The seeds were soaked for 24 hr and sown to a depth of 1.5cm in a tray of 80% pumice, 20% peat mixture. Seedlings were grown in either climate control cabinets with day/night temperatures of 22/20°C and day/night humidity of 68/78%, or climate control rooms with day/night temperatures of 22/18°C and similar humidity. Day length was 12 hr. The seeds were watered every morning for the first four days after sowing, and then daily with Hoagland's solution A (Hoagland and Arnon, 1938) until harvested.

Seedlings were harvested after nine days by cutting off the seedlings at the basal node. The coleoptiles and first leaves were then removed and the remaining leaves used as the source of acetyl-CoA carboxylase. In experiments investigating levels of acetyl-CoA carboxylase activity in developing nine-day-old leaves and leaves of different ages, only the second leaves were used.

2.2 Reagents

The following chemicals were obtained from Sigma Chemical Co., St. Louis: acrylamide, ADP, ATP, avidin, biotin, bis-acrylamide, BSA fraction V, CoA, Coomassie Brilliant Blue G-250 and R-250, cyanogen bromide, dithiothreitol, glutaraldehyde, Hepes, Mes, POPOP, PPO, Tricine and Tris.

Biogel P6 was from Bio-Rad Laboratories, Richmond, U.S.A.,

Sepharose 4B-CL was from Pharmacia Fine Chemicals, Uppsala.

SDS (Specially pure) was from BDH Chemicals Ltd., Poole, England,

Hyperfilm βmax was from Amersham International PLC., Amersham

Nitrocellulose paper was from Schleicher and Schuell, Dassel, F.R.G.

The radiochemicals, [^{14}C]-biotin, NaH$^{14}CO_3$ and ^{35}SLR-streptavidin, were obtained from Amersham International PLC., Amersham.

All other reagents were obtained as Analar grade from either BDH Chemicals Ltd., Poole, England, or May and Baker Ltd., Dagenham, England.

METHOD

3.1 Analytical Method

3.1.1 Determination of Protein Levels

Protein levels were determined by using the Coomassie Blue dye binding method. Coomassie Blue was prepared by dissolving 100mg of Coomassie Blue G-250 in 50ml of 95% (v/v) ethanol, then adding 100ml of 85% (w/v) phosphoric acid and diluting with water to 1000ml (Read and Northcote, 1981). Aliquots of between 25-400µl were added to 5ml of Coomassie Blue and the absorbance read at 595nm. Bovine serum albumin (BSA) was used to standardise the Coomassie Blue.

3.1.2 Determination of the Levels and Composition of Fatty Acids in Maize Leaves.

The levels and composition of fatty acids in nine-day-old maize leaves were determined by gas-liquid chromatography. Ten maize leaves were sectioned (Section 3.5) and fatty acids from each section were trans-esterfied by refluxing in 1ml of methanol containing 2% (v/v) H_2SO_4. 25µl to 100µl of 26.3µg/25µl methyl heptadecanoate was added as an internal standard before refluxing, and 5ml of 5% NaCl was then added before methyl esters were extracted twice in 5ml of hexane. The hexane extract was then washed with 4ml of 2% (w/v) K_2CO_3 before adding anhydrous Na_2SO_4 to dry the hexane.

Hexane was then evaporated under N_2 and methyl esters dissolved in 50µl to 100µl of hexane prior to separation on a Shimadzu GC-8A chromatograph using a 2m by 4mm glass column containing 15% DEGS on Chromosorb W, AW-DMCS (mesh 60/80) with "on-column injection". Column temperature was 175°C and the N_2 flowrate approximately 24ml/min, while the injection temperature was 200°C.

3.1.3 Determination of Chlorophyll Levels

Chlorophyll was extracted from leaf tissue in 80% (v/v) acetone and the extract filtered through Whatman No.1 filter paper. Chlorophyll levels were then determined as described by Arnon (1949).

3.1.4 Determination of Radioactivity

^{35}S- and ^{14}C-radioactivity was determined using a Beckman LS8000 Scintillation Counter. Water-soluble samples were counted either in 5ml of Triton X-100/toluene (1:2, v:v) containing 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.02% (w/v) 1,4-bis[2(5-phenyloxazolyl)] benzene (POPOP) or 5ml of dioxane containing, 6% (w/v) naphthalene, 0.4% (w/v) PPO, 0.02% (w/v) POPOP, 10% (v/v) methanol and 2% (v/v) ethylene glycol.

In the assay of acetyl-CoA carboxylase, [^{14}C]-malonyl-CoA was determined as the acid-stable radioactivity on 1.5cm squares of Whatman 3MM filter paper and was counted in 5ml of toluene containing 0.4% (w/v) PPO and 0.02% (w/v) POPOP.

^{35}SLR-streptavidin-acetyl-CoA carboxylase complex bound to nitrocellulose paper was counted in 5ml of the toluene-based scintillation solvent.

3.2 Preparation of Acetyl-CoA

Acetyl-CoA was synthesized by reacting CoA and acetic anhydride as described by Stadtman (1957). 8mg of CoA was dissolved in 1ml of ice-cold water, and 0.2ml of ice-cold 1M $NaHCO_3$ was added before adjusting the pH to 7.5 with 1M KOH if necessary. 0.13-0.15ml of freshly prepared ice-cold 0.1M acetic anhydride was then added and the mixture shaken on ice for 30 min. The solution was then adjusted to pH 6.0 by the gradual addition of 1M HCl, before freeze-drying. The yield and purity of redissolved acetyl-CoA was determined spectrophotometrically at 232nm and 260nm, as the thioester and free CoA absorb at 260nm (molar absorption coefficient 16 400), while the thioester alone absorbs at 232nm (molar absorption coefficient 8 700).

3.3 Preparation of Avidin-Sepharose

Two different avidin-Sepharose preparations were used for the purification of acetyl-CoA carboxylase. The first was purchased from Sigma and poured into a 3.7cm x 1.6cm plastic syringe barrel fitted with a layer of Miracloth (Column A), and washed with 0.1M Tris buffer at pH 8.0 containing 0.5M KCl, 1mM ethylenediamine tetraacetic acid (EDTA), 15mM β-mercaptoethanol and 0.02% (w/v) sodium azide (Column Buffer). Column A had an exchangeable biotin-binding capacity of 25nmol/ml of gel. The

second avidin-Sepharose was prepared according to the procedure described by Holland (personal communications). 30ml of Sepharose-4B-CL was washed under suction in a scintered glass funnel with approximately 900ml ice-cold distilled deionised water. The gel was then resuspended in 275ml of ice-cold water plus 275ml of ice-cold 10mM sodium phosphate buffer at pH 7.0. The pH of the Sepharose gel suspension was adjusted to pH 11.0 with 2M NaOH just prior to activation with approximately 15g of ground CNBr (28mg/ml of gel suspension). The pH was maintained at pH 11.0 with 2M NaOH, and the solution was kept at 2^{o}C, and stirred constantly. After the pH stabilized at 11.0, the gel suspension was then poured over ice into a scintered glass funnel, and the liquid removed by suction. Avidin coupling was carried out by very quickly washing the gel with 1300ml of ice-cold 10mM sodium phosphate buffer at pH 7.0 under suction, and resuspending it in 275ml of 10mM sodium phosphate buffer at pH 7.0 containing 100mg of avidin, before tumbling the suspension for 20 hr at 0-4^{o}C. The sodium phosphate buffer was then suctioned off, and the coupled gel washed with 1M ethanolamine-HCl at pH 7.0 which was resuspended in 1M ethanolamine-HCl at pH 7.0 for 2-3 hr at room temperature, before removing the ethanolamine-HCl by suction. In order to dissociate the avidin tetramer, the gel was washed with 3M guanidine-HCl at pH 1.5 containing 0.2M KCl before resuspending in the same buffer for 16-20 hr at room temperature. The gel was then poured into a 20cm by 1.8cm glass column fitted with a glass frit and washed with Column Buffer at a rate of 10ml/hr, until the absorbance at 280nm was less than 0.01. The high affinity binding sites were then blocked with 0.8mM biotin in the above buffer, and the biotin from the lower

affinity sites was removed by three alternate washings with 5 vol of glycine-HCl at pH 2.0 and 5 vol of Column Buffer. The column was equilibrated with Column Buffer prior to use (Column B)(Holland, personal communication; Gravel et al., 1980). Column B had an exchangeable biotin-binding capacity of 4-6nmol/ml of gel.

3.3.1 Determination of the Exchangeable Biotin-Binding Capacity of Avidin-Sepharose

Prior to column use, 10µl of 10mM [^{14}C]-biotin, containing approximately 50 000 cpm was applied, per 10ml of avidin-Sepharose gel, to a column previously equilibrated with 5 vol of Column Buffer. The column was left to equilibrate for 10-15 min before washing with Column Buffer at a flow rate of 10ml/hr to elute unbound [^{14}C]-biotin. The bound [^{14}C]-biotin was eluted with 5 vol of Column Buffer containing 2mM biotin, and the radioactivity determined in Triton X-100/toluene scintillation solvent.

3.4 Acetyl-CoA Carboxylase Assay

Acetyl-CoA carboxylase activity was assayed as the incorporation of $H^{14}CO_3^-$ into acid-stable malonyl-CoA.

3.4.1 Assay of Rat Liver Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase from rat liver was assayed in an assay mixture of 150µl, containing 50mM Tris buffer at pH 7.5,

1mM dithiothreitol (DDT), 5mM $MgCl_2$, 2mM sodium citrate, 1.0mg/ml BSA, 0.2mM acetyl-CoA, 12mM $NaHCO_3$, approximately 2µCi $NaH^{14}CO_3$, 4mM ATP and 40µl of enzyme solution (Lent and Kim, 1982). Acetyl-CoA carboxylase was preincubated for 30 min at 37°C in the above mixture with the omission of the ATP, $NaH^{14}CO_3$, $NaHCO_3$ and acetyl-CoA. These four components were added to start the reaction, which was carried out at 37°C for 5 min. Non-specific carboxylations were measured by omitting acetyl-CoA from the reaction mixtures. The reaction was stopped by the addition of 50µl of 5M HCl, which inactivated the enzyme and converted any unfixed HCO_3^- to CO_2. 50µl of the reaction mixture was spotted onto 1.5cm square of Whatman 3MM filter paper and the radioactivity incorporated into acid-stable products was determined using the toluene scintillation solvent.

3.4.2 Assay of Maize Leaf Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase from maize leaf tissue was assayed in an assay mixture of 200µl containing 0.1M Tricine buffer at pH 8.0, 1mM ATP, 2.5mM $MgCl_2$, 50mM KCl, 1mM DTT, 0.3mM acetyl-CoA, 10mM $NaHCO_3$, approximately 2µCi $NaH^{14}CO_3$ and 50µl of enzyme solution containing no more than 100µg of protein (Nikolau *et al.*, 1981). Acetyl-CoA was added to start the reaction, which was carried out at 30°C for 5 min. Non-specific carboxylations were measured by omitting acetyl-CoA from the reactions. 50µl of 5M HCl was used to stop the reaction and 50µl of the reaction mixture was spotted onto Whatman 3MM filter paper and radioactivity determined using the toluene scintillation solvent.

3.5 Preparation of Maize Leaf Sections

90-110mm long second leaves of nine-day-old maize seedlings were cut into 12 transverse sections as detailed in Fig.4. In the determination of acetyl-CoA carboxylase activity and protein levels, 20 leaves were used for sections A to G, ten leaves for sections H to I and five leaves for sections K and L. Chlorophyll was determined using ten leaves for sections A to I and five leaves for sections K and L, while fatty acids were determined using ten leaves for all leaf sections.

3.6 Preparation of Cell-Free Extracts

Cell-free extracts were prepared by homogenisation of maize leaf tissue in 0.4ml of 0.1M Tris buffer at pH 8.0 containing 1mM EDTA and 20mM β-mercaptoethanol. Homogenisation was carried out at 4°C in a chilled Eppendorf centrifuge tube containing 0.1g of acid-washed sand, using a ground glass rod with the same taper as the tube. Cellular debris was removed by centrifugation for 3 min in an Eppendorf centrifuge. In order to remove endogenous substrates, 0.2ml of the supernatant was passed through a 0.9cm x 3.3cm Biogel P6 column (exclusion limit 6000) previously equilibrated with the same 0.1M Tris buffer at pH 8.0. The green eluant was collected and stored on ice.

3.7 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of leaf proteins and enzyme preparations was carried out using slab gels 140mm wide, 180mm

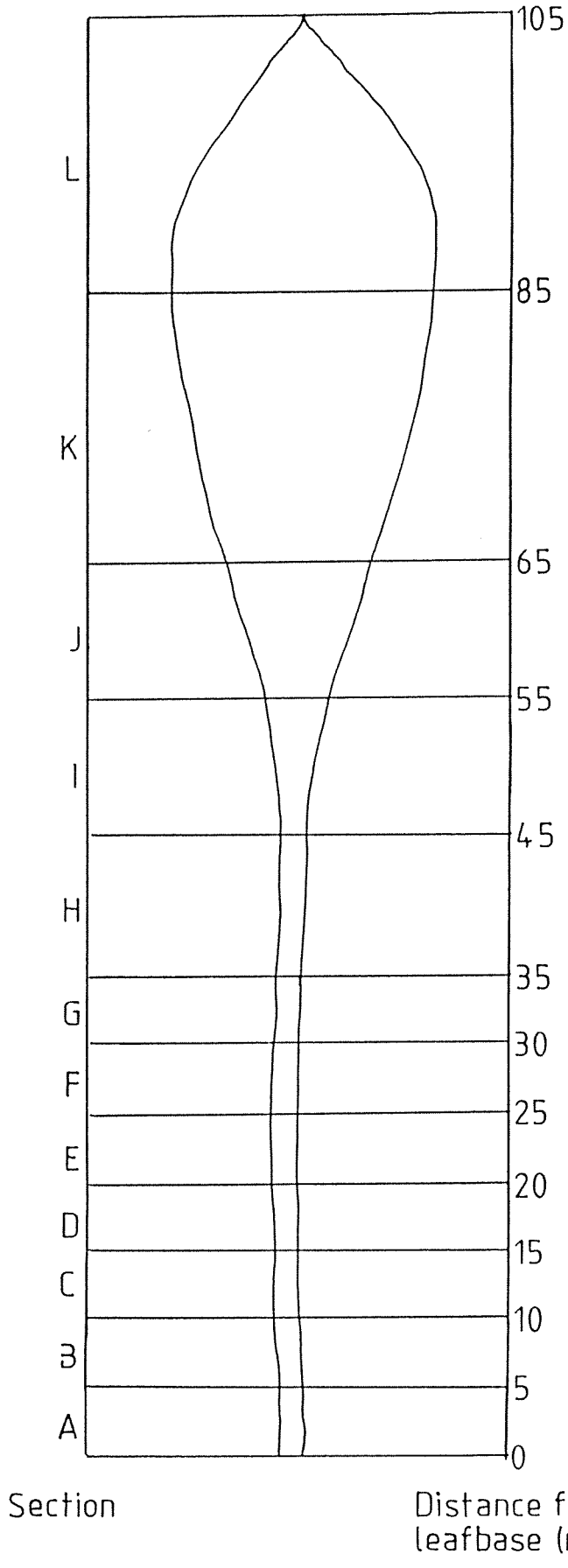


FIGURE 4: Sectioning of a Second Leaf of a Maize Seedling

long and 1.5mm thick. The separating gel was 15% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide dissolved in 0.375M Tris buffer at pH 8.8, while the stacking gel was 5% (w/v) acrylamide and 0.03% (w/v) bis-acrylamide dissolved in 0.06M Tris buffer at pH 6.8. Dissociating gels included 0.1% (w/v) sodium dodecyl sulphate (SDS) in both stacking and separating gels. Both gels were degassed before polymerisation with 0.05% (v/v) N.N.N.'N'-tetramethylethylenediamine (TEMED) and 0.05% (w/v) ammonium persulphate for the separating gel and 0.1% (v/v) TEMED and 0.05% (w/v) ammonium persulphate for the stacking gel, just prior to pouring. Electrode buffer contained 0.025M Tris buffer and 0.192M glycine, with the addition of 0.1% (w/v) SDS for dissociating gels.

Protein samples with a concentration greater than 4mg/ml were passed through a Biogel P6 (ex 6000) column, and the filtrate then added to an equal volume of 60mM Tris buffer at pH 6.8, 5% (w/v) β-mercaptoethanol, 10% (w/v) sucrose, and 0.001% bromophenol blue with the addition of 2% (w/v) SDS when using dissociating gels. Samples with protein concentrations less than 4mg/ml were dialysed overnight to remove any low molecular weight compounds, and concentrated by freeze-drying and redissolving the protein in 60mM Tris buffer at pH 6.8. Samples prepared for dissociating gels were then heated to 100°C for 3 min before being stored at -20°C. For convenience, gels were stored at 4°C for up to one week. Electrophoresis was at 15mA until the tracking dye had entered the separating gel, and then at 20mA for 5 hr. However, if transfer of proteins onto nitrocellulose paper (Section 3.8) was to follow gel electrophoresis it was convenient for the gels to be made fresh and

electrophoresed at 6-8mA overnight.

Gels were stained overnight with 0.1% (w/v) Coomassie Blue R-250 in a solution of 50% (v/v) methanol, 5% (v/v) acetic acid, and destained by diffusion in 40% (v/v) methanol and 7% (v/v) acetic acid. During staining and destaining, gels were agitated constantly. Destaining solution was recycled by passing through a column of decolourising charcoal which removed the stain. If the amount of protein in the gels was insufficient for visualisation using Coomassie Blue staining, then gels were subsequently silver stained according to the method of Oakley et al. (1980). This procedure involved soaking the gel in 10% glutaraldehyde for 30 min before rinsing the gel with two changes of 500-1000ml of glass-distilled water over 10 min and soaking overnight. After draining off the water, the gel was soaked for 15 min in freshly made ammonical silver solution. Ammonical silver solution was prepared by mixing 1.4ml of ammonium hydroxide and 21ml of 0.36% (w/v) sodium hydroxide, and slowly adding 4ml of 19.4% (w/v) silver nitrate with constant stirring, before making up to 100ml with distilled water. Subsequently the gel was placed in an aqueous solution of 0.005% citric acid and 0.019% formaldehyde until bands appeared, and then washed with glass-distilled water.Gels were then photographed.

3.8 Transfer of Dissociated Proteins from Gels to Nitrocellulose paper

Transfer of dissociated protein from gels to nitrocellulose paper was carried out on the unstained polyacrymide slab gels. Immediately after electrophoresis, the stacking gel was removed

and proteins were transferred using a Transphor Electroblotting Unit LKB2005 as instructed by the manufacturer. Electroblotting was carried out in a 4°C room at 0.6A for 1 hr in 25mM Tris buffer at pH 8.3, 150mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol.

3.9 Biotin-Specific Probing

Protein samples were either spotted directly onto nitrocellulose paper or transferred by transblotting (Section 3.8). Non-specific protein binding sites were blocked by washing the nitrocellulose paper with 3% (w/v) BSA in 10mM phosphate buffer-saline (PBS) for 1 hr. Unbound BSA was removed by washing the nitrocellulose paper with PBS containing 0.1% (v/v) Tween 20 several times over 30 min and finally with PBS not containing Tween 20. Biotin-containing proteins were then probed overnight with 1-2µCi of ^{35}SLR-streptavidin in 1% (w/v) BSA in PBS containing 2.5mM $MgCl_2$. Unbound ^{35}SLR-streptavidin was removed by several washes, over a 30 min period, in PBS containing 0.1% (v/v) Tween 20. Finally the nitrocellulose paper was washed with PBS and distilled water, then air-dried and autoradiographed using Hyperfilm βmax for no less than 10 days. The blocking, probing and washing procedures are those recommended by Hawke and Leech (1987), except that probing was carried out overnight rather than for 1 hr, and that probing buffer contained 2.5mM $MgCl_2$ (see Section 4.7.1).

3.9.1 Detection of Protein on Nitrocellulose Paper

After the nitrocellulose paper had been autoradiographed,

proteins on the nitrocellulose paper were stained with 0.1% (w/v) Amido Black 10B in a solution of 25% (v/v) propan-2-ol, 10% (v/v) acetic acid for 15-30 sec (Gershoni and Palade, 1982). The paper was destained by diffusion over 20 min in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid with five changes of the destaining solution. Staining for longer than 30 sec or failing to change the destain often enough resulted in a stained background.

3.10 Purification of Rat Liver Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was purified from the livers of five male Wistar rats, fed a high carbohydrate low fat diet, and which had been killed by cervical dislocation. The livers were removed and rinsed in 50mM potassium phosphate buffer at pH 7.5 containing 2mM EDTA, 10mM β-mercaptoethanol and 0.25M sucrose before homogenisation in the same buffer in a Waring blender at full speed for 1 min at 4°C. The homogenate was then made up to 3% (w/v) PEG by the slow addition of 50% (w/v) PEG with stirring over 20 min. Stirring was continued for a further 20 min, and the homogenate allowed to stand for 30 min before being centrifuged at 16 000xg for 30 min. The supernatant was decanted through a layer of glass wool and cheesecloth and adjusted to 5% (w/v) PEG by the slow addition of 50% (w/v) PEG before stirring for 20 min, standing for 30 min and centrifuging at 16 000xg for 30 min. The precipitate was resuspended in a minimum volume of 10mM potassium phosphate buffer at pH 7.5 containing 2mM EDTA and 10mM β-mercaptoethanol before centrifuging at 20 000xg for 10 min to remove any insoluble material (Lent and Kim, 1982). The precipitate was further

purified using avidin-affinity chromatography as described in Section 3.12.

3.11 Purification of Maize Leaf Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was purified from nine-day-old maize seedlings with the coleoptiles and first leaves removed. The harvested leaves were immersed in 2 vol of buffer containing 0.1M Tris buffer at pH 8.0, 1mM EDTA and 20mM β-mercaptoethanol and homogenised for 30 sec in a Waring blender at full speed. The homogenate was filtered through a layer of nylon muslin cloth (mesh 125μ) and nylon bolting cloth (mesh 25μ) to remove fibrous material. The filtrate was then centrifuged at 30 000xg for 30 min in a Sorval RC2-B centrifuge using an SS34 rotor, to precipitate cellular debris and most organelles, leaving microsomes and soluble enzymes.

Sufficient $(NH_4)_2SO_4$ was added to the supernatant to give 35% saturated $(NH_4)_2SO_4$ (19.4g $(NH_4)_2SO_4$ per 100ml of supernatant). After equilibration for 30 min the solution was centrifuged at 12 000xg for 20 min. The supernatant was adjusted to 50% saturated $(NH_4)_2SO_4$ (8.7g per 100ml of supernatant) and allowed to equilibrate for 30 min before centrifuging as before.

The 35%-50% $(NH_4)_2SO_4$ precipitate was resuspended in a minimum volume of 10mM Tris buffer at pH 8.0 containing 1mM EDTA and 20mM β-mercaptoethanol. The resuspended precipitate was passed through a 40cm x 3cm Biogel P6 (ex6000) column at a flow rate of 70ml/hr, previously equilibrated with 10mM Tris buffer.

Fractions containing protein were pooled and concentrated to approximately 6ml using an Amicon ultrafiltration unit equipped with a Diaflo PM 30 filtration membrane. Ultrafiltration was carried out under a pressure of 25-30 p.s.i. producing a flow rate of about 10-15ml/hr. After concentrating the enzyme solution, 0.5ml aliquots were frozen in liquid air and stored at -80°C (Fig.5).

3.12 Purification of Acetyl-CoA Carboxylase Using Avidin-Affinity Chromatography

Either freshly prepared or frozen maize leaf acetyl-CoA carboxylase concentrate was loaded onto an avidin-affinity column at a flow rate of 0.15-0.3ml/hr/ml of gel. Unbound protein was subsequently washed through the column with approximately 4 vol of Column Buffer containing 0.1M Tris pH 8, 0.5M KCl, 1mM EDTA, 15mM β-mercaptoethanol and 0.02% (w/v) sodium azide until the absorbance of the eluant at 280nm was less than 0.01. The column was then washed with Column Buffer containing 2mM biotin and 1ml fractions from affinity Column A and 3ml fractions from affinity Column B were collected. The columns were regenerated by three alternate washings with 5 vol of 0.1M glycine buffer at pH 2.0 and 5 vol of Column Buffer. Fractions were assayed for the presence of acetyl-CoA carboxylase activity and protein levels determined, all steps were carried out at 4°C.

Rat liver acetyl-CoA carboxylase was purified by avidin-affinity chromatography as described above, except that the column buffer contained 0.1M Tris buffer at pH 7.5, 0.5M NaCl, 1mM EDTA, 0.1mM DTT and 5% (v/v) glycerol (Song and Kim, 1981).

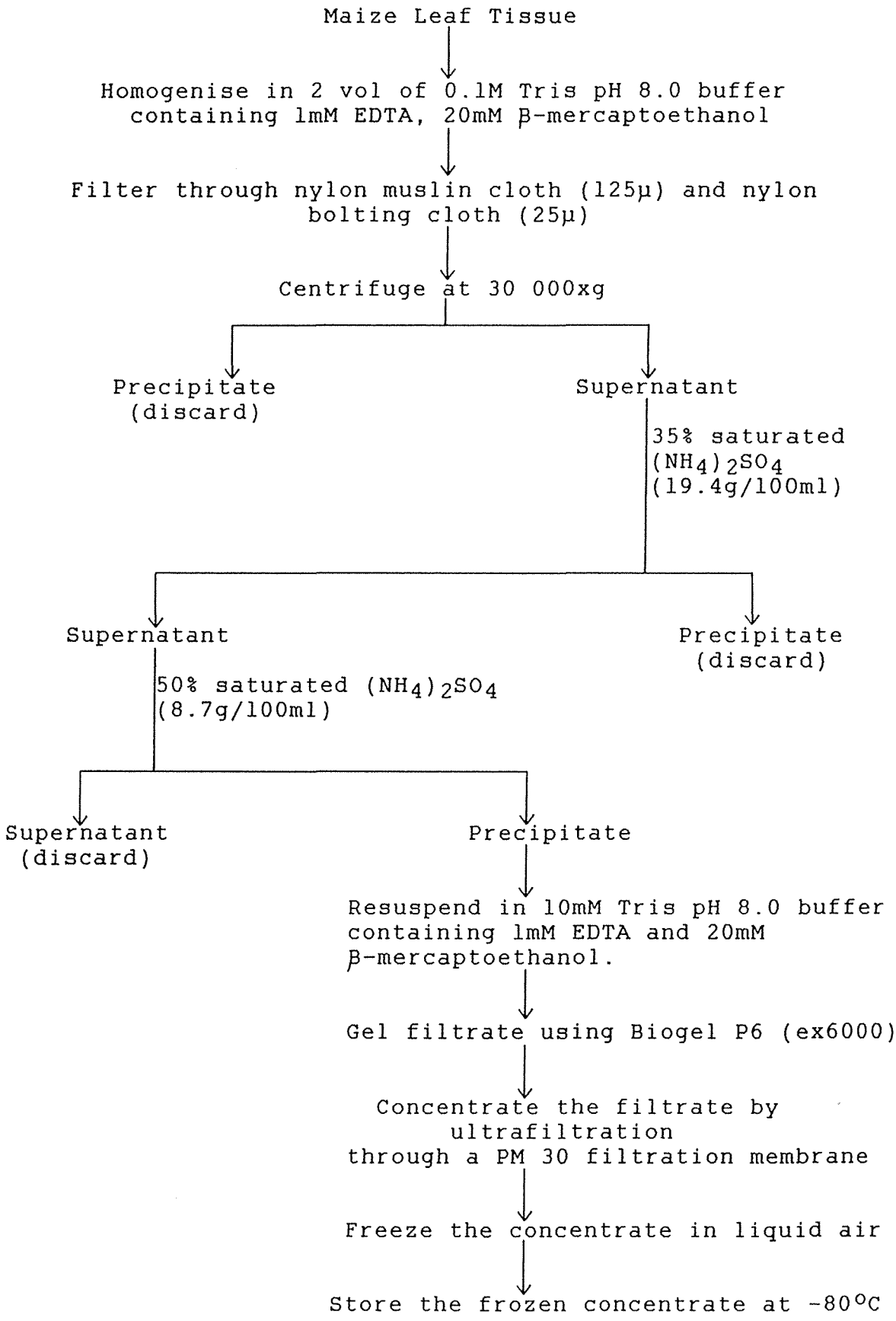


FIGURE 5: Purification of Maize Leaf Acetyl-CoA Carboxylase (Procedure II)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 General Features of the Maize Leaf Acetyl-CoA Carboxylase Assay

Acetyl-CoA carboxylase activity was assayed by measuring the incorporation of $H^{14}CO_3^-$ into the acid-stable product malonyl-CoA. The pH and concentration of assay components were those which gave maximum activity of acetyl-CoA carboxylase (Nikolau and Hawke, 1984). When the effect of time and protein concentration on the assay of acetyl-CoA carboxylase from maize leaf cell-free extracts was examined, the amount of HCO_3^- fixed, increased linearly up to 8 min (Fig.6). For convenience, all subsequent assays involved 5 min incubations.

Nikolau (1981), reported that in maize leaf cell free extracts, there was a linear relationship between the amount of HCO_3^- fixed and the amount of protein present per assay up to 100µg. When assaying partially purified acetyl-CoA carboxylase a linear relationship existed with protein levels up to 150µg (Fig.7). In order to utilize conditions which complied with both the above situations, protein levels were kept below 100µg per assay when assaying acetyl-CoA carboxylase activity.

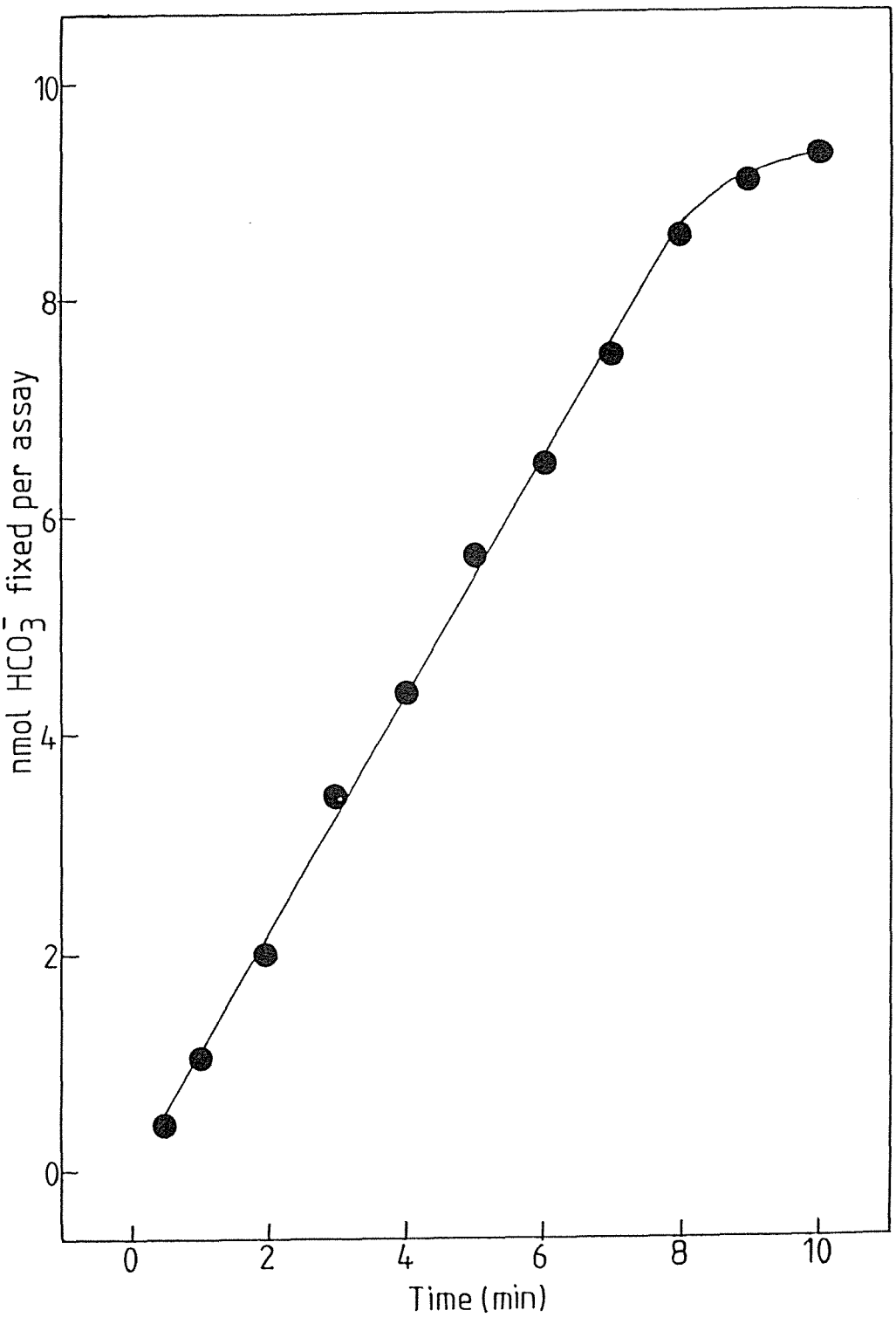


FIGURE 6: Effect of Time on Acetyl-CoA Carboxylase Activity

Acetyl-CoA carboxylase activity was determined from cell-free extracts (Section 3.6) of the 2nd leaves of 9-day-old maize seedlings.
Assay conditions were as described in Section 3.4.2. Reaction mixtures contained 65µg of protein.
Values shown are the average of duplicate assays.

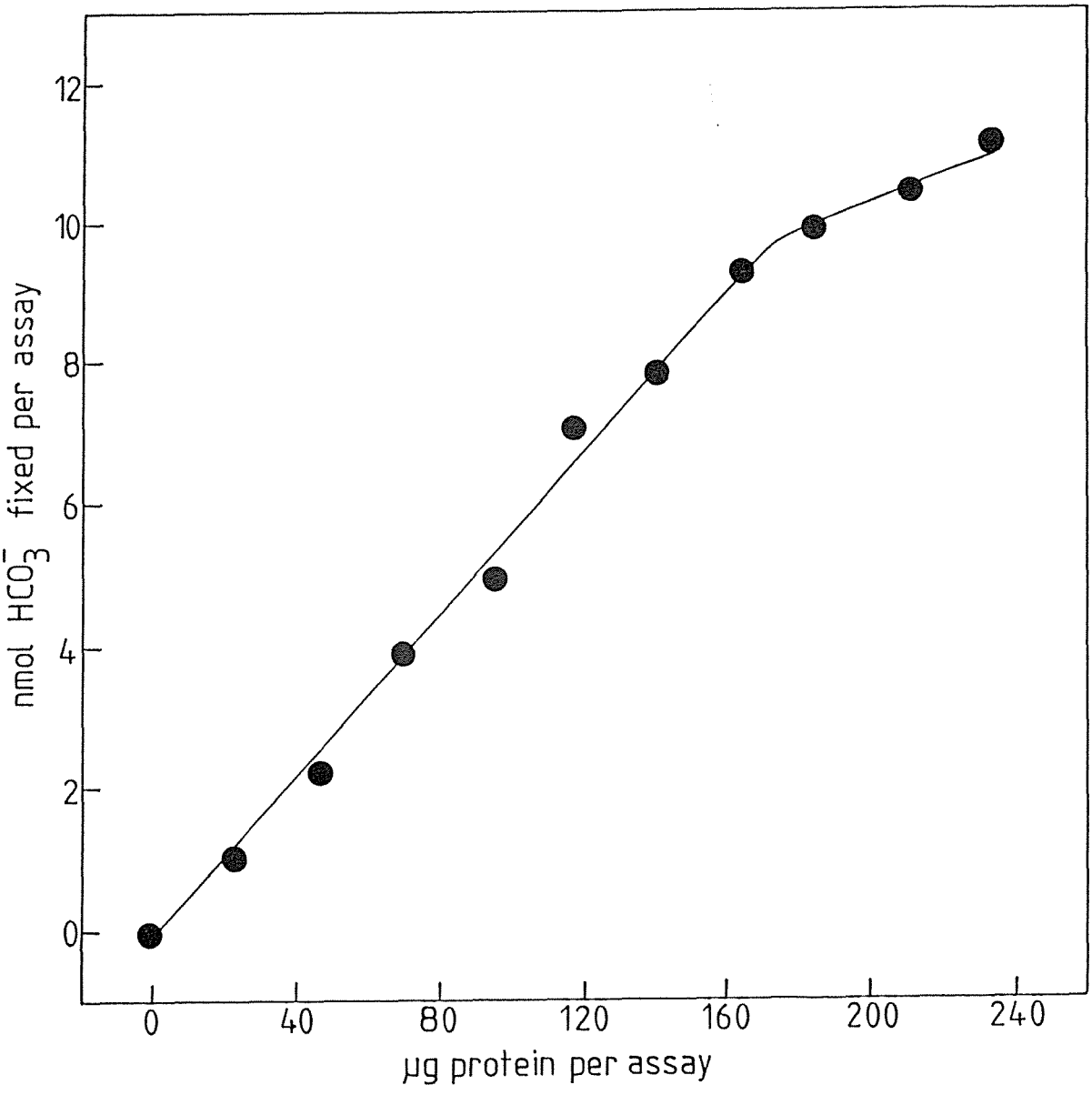


FIGURE 7: Effect of Protein Levels on Acetyl-CoA Carboxylase Activity

Acetyl-CoA carboxylase activity was determined from cell-free extracts of the 2nd leaves of 9-day-old maize seedlings (Section 3.6).
Assay conditions were as described in Section 3.4.2.
Values shown are average of duplicate assays from 2 experiments.

4.2 Stability of Acetyl-CoA Carboxylase extracted from Maize Leaves

Acetyl-CoA carboxylase activity from cell-free extracts stored on ice was assayed intermittantly over a period of 32 hr. Half the carboxylase activity was lost after 6 hr, while 98% of activity was lost after 32 hr (Fig.8). Cell-free extracts containing 0.6, 1.2 and 4.8mg/ml of protein each lost acetyl-CoA carboxylase activity at the same rate, therefore, acetyl-CoA carboxylase stability appeared to be unaffected by the protein concentration over this range.

The partially purified Acetyl-CoA carboxylase concentrate (Section 4.8) was frozen in liquid air and subsequently stored at -80^{o}C. Under these conditions acetyl-CoA carboxylase could be stored up to 5 weeks with only 50% loss in activity.

4.3 Levels of Acetyl-CoA Carboxylase in Maize Seedlings of Different Ages

The level of acetyl-CoA carboxylase activity was investigated in the second leaf of maize seedlings grown for 4 to 12 days. Four days was the lowest practical limit under the growth conditions used, since the shoots were only 4mm long at this stage.

Total acetyl-CoA carboxylase activity in the 4-day-old leaf was about 7nmol/min (Fig.9, line A), this increased to 13nmol/min after 6 days and over the next 2 days increased more than 4-fold to reach 59nmol/min at day 8. During the following 3-day period

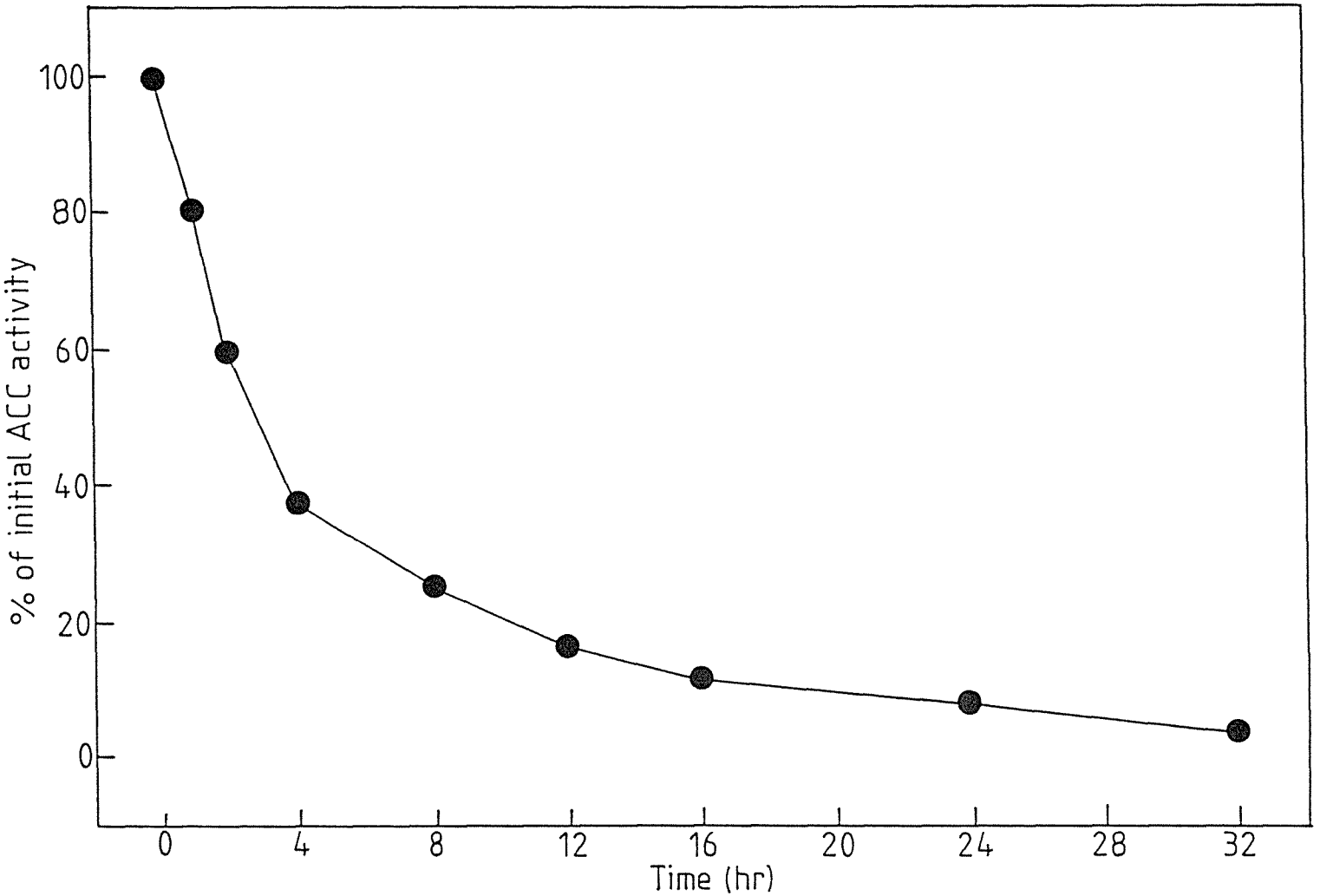


FIGURE 8: Stability of Acetyl-CoA Carboxylase Activity in Cell-Free Extracts of Maize Leaves

Acetyl-CoA carboxylase activity was assayed from cell-free extracts (Section 3.6) over a 32 hr period. The assay involved a 5 min incubation at 30°C in 0.1M Tris buffer at pH 8.0 containing 1mM ATP, 2.5mM $MgCl_2$, 50mM KCl, 1mM DTT, 0.3mM acetyl-CoA and 10mM $NaHCO_3$ containing approximately 2µCi $NaH^{14}CO_3$. The protein concentration of the cell-free extract was 4.8mg/ml. Values shown are the average of duplicate assays from 2 experiments.

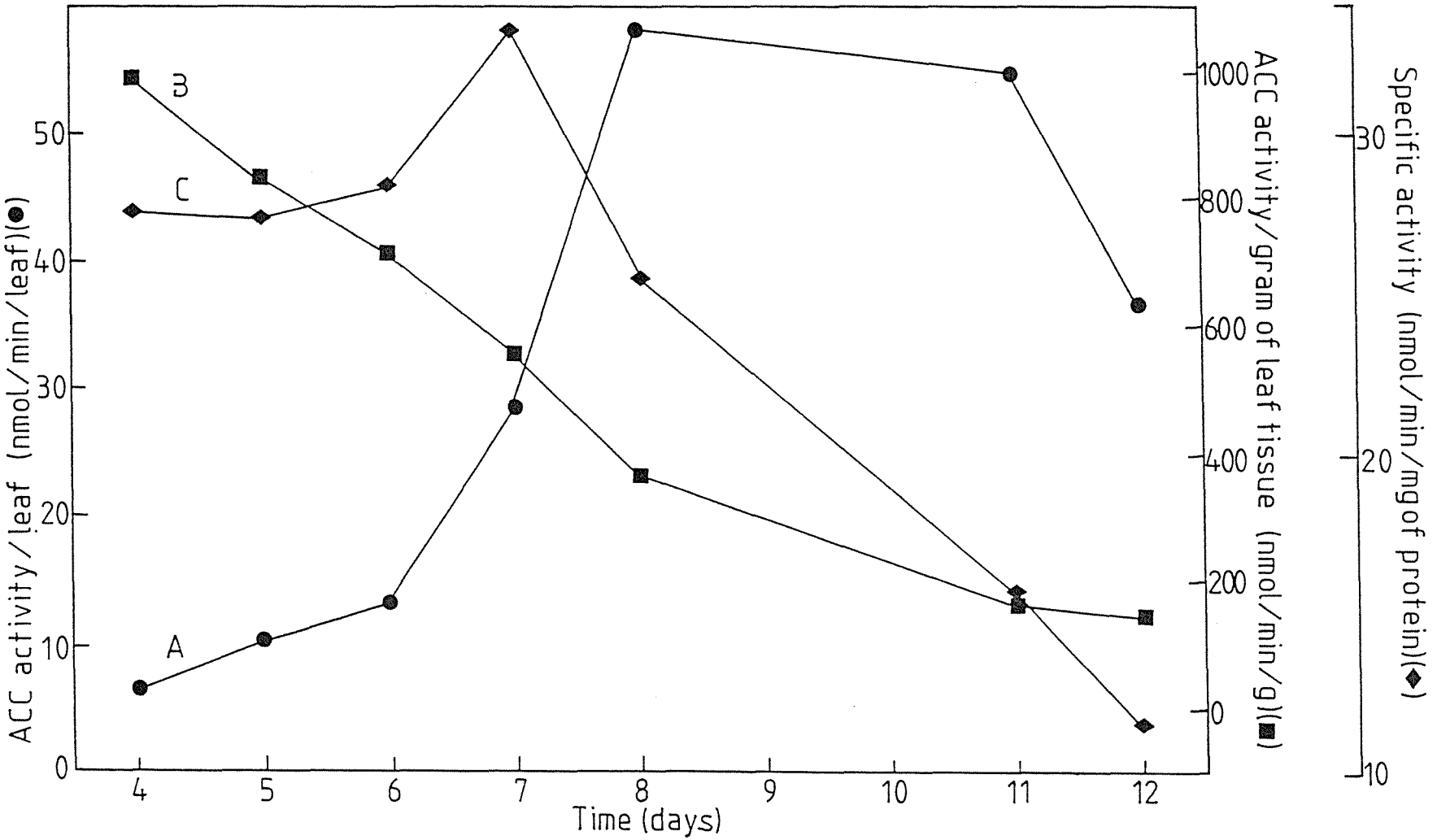


FIGURE 9: Levels of Acetyl-CoA Carboxylase Activity in Developing Maize Leaves.

Acetyl-CoA carboxylase activity and protein levels were determined from cell-free extracts (Section 3.6) of the 2nd leaves of 4 to 12-day-old maize seedlings. Assay conditions were as described in Fig.8. Values shown are the average of triplicate assays from 2 experiments.

the carboxylase activity appeared to plateau. However, when the increase in seedling weight was taken into account, the carboxylase activity per gram of leaf tissue was greatest in the 4-day-old seedling and thereafter it declined with increasing seedling age (Fig.9, line B). There was an approximately linear relationship between acetyl-CoA carboxylase activity per gram of leaf tissue and seedling growth between 4 and 8 days. At 4 days the carboxylase activity was 1000nmol/min/g, but thereafter decreased to 380nmol/min/g at 8 days. From days 8 to 12, the rate of decline in activity decreased slightly with the activity being 130nmol/min/g on day 12. This represents an overall 7.6-fold decrease in acetyl-CoA carboxylase activity per gram in maize seedlings from 4 to 12 days. The increase in acetyl-CoA carboxylase levels as the leaf develops is most likely in response to the demand for fatty acids required for thylakoid membrane formation which is occurring at increasing levels as the leaf increases in size. The decrease in activity per gram of leaf tissue is most likely due, mainly, to the accummulation of cellulose, and low lipid turnover in the developing leaf.

The specific activity of acetyl-CoA carboxylase in cell-free extracts was constant between 4 and 6 days, at 27.2 to 28.3nmol/min/mg of protein (Fig.9, line C). By day 7 the specific activity had increased to 33.2nmol/min/mg protein, and thereafter decreased linearly to 11.2nmol/min/mg of protein at day 12.

The similar specific activities from days 4 to 6 and the slight increase in total activity (Fig.9, lines A,C), and total protein levels (Fig.10a), suggests that acetyl-CoA carboxylase was being

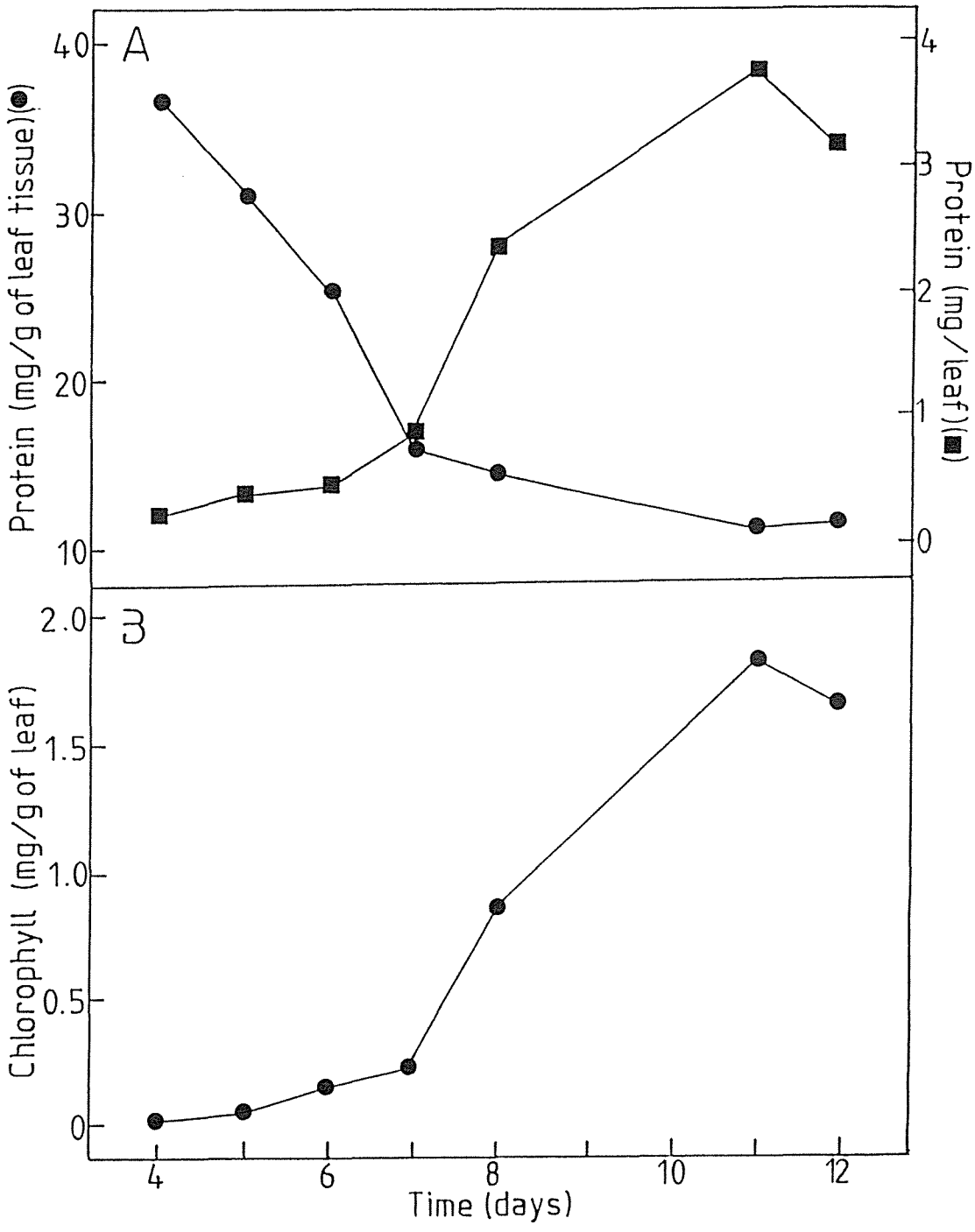


FIGURE 10: Protein and Chlorophyll Levels in Developing Maize Leaves

Protein levels were determined from cell-free extracts of the 2nd leaves of 4 to 12-day-old maize seedlings. Chlorophyll was extracted from leaf tissue and levels determined as described in Section 3.1.3.
Values shown are the average of triplicate assays of 2 experiments.

synthesized concomitantly with total protein. The decrease in specific activity from days 7 to 12 (Fig.9 line C) can most likely be attributed to an increase in the level of proteins associated with photosynthesis in the chloroplasts of maturing leaf tissue.

Chlorophyll levels in a constant weight of tissue, increased 30-fold over days 4 to 7 from 0.007mg/g to 0.224mg/g (Fig.10b), followed by an 8-fold increase to reach a maximum level of 1.8mg/g at day 11. This increase in chlorophyll levels is indicative of the chloroplast development which occurred over the 8-day growth period examined.

Although the amount of total protein in the leaf increases as the leaf increases in size (Fig.10a), the amount of protein relative to the amount of leaf tissue decreases over 3-fold from day 4 to 12 from 36.5mg/g to 11.5mg/g. This reduction in the amount of protein per gram of leaf tissue as the seedling ages, implies an accummulation of other cellular compounds, such as cellulose, which makes up the cell walls, and lipids which contribute to the thylakoid membranes.

4.4 Biochemical Changes During Development of the Maize Leaf

In maize, cells are produced from the basal intercalary meristem, and are therefore arranged in a gradient of cellular and plastid development along the leaf; with the youngest tissue nearest the base of the leaf and the mature tissue near the tip (Leech and Baker, 1983; Leech et al., 1973). In order to investigate the relationship between acetyl-CoA carboxylase

activity, fatty acid synthesis and plastid development, it is useful to examine the levels of acetyl-CoA carboxylase activity and fatty acid levels along a developing maize leaf.

Acetyl-CoA carboxylase activity was examined along the second leaves of 9-day-old maize (Zea mays var. XL45 and Zea mays var. Pioneer 3709) seedlings, while fatty acid levels were examined in similar seedlings from the later hybrid only. It was necessary to use both hybrids since the var. XL45 hybrid became unavailable during the course of this investigation.

4.4.1 Fatty Acid Content

The total amount of fatty acids per 5mm section increased from 21.7µg at the leaf base to 26.5µg between 10 to 15mm from the leaf base before decreasing to a minimum of 21.0µg between 20-25mm (Fig.11). The fatty acid levels increased from the minimum value to a maximum of 30.5µg at approximately 60mm from the leaf base and falling away to 28.6µg at the leaf tip. Fatty acids per gram of leaf tissue decreased 2.2-fold from 5.9mg/g at the leaf base to 2.9 to 3.2mg/g at 20 to 40mm along the leaf (Fig.11). This was followed by a sharp rise to 5.6mg/g at the leaf tip. The high fatty acid levels per gram of leaf tissue at the leaf base reflects the high proportion of membranes in this region, due to the small cell and plastid size. The levels of fatty acids were generally 2 to 5.5 times greater along maize leaves when compared to wheat leaves of a similar size (Hawke and Leech, 1987), apart from near the leaf tips where the levels in wheat leaves are similar to that of maize leaves.

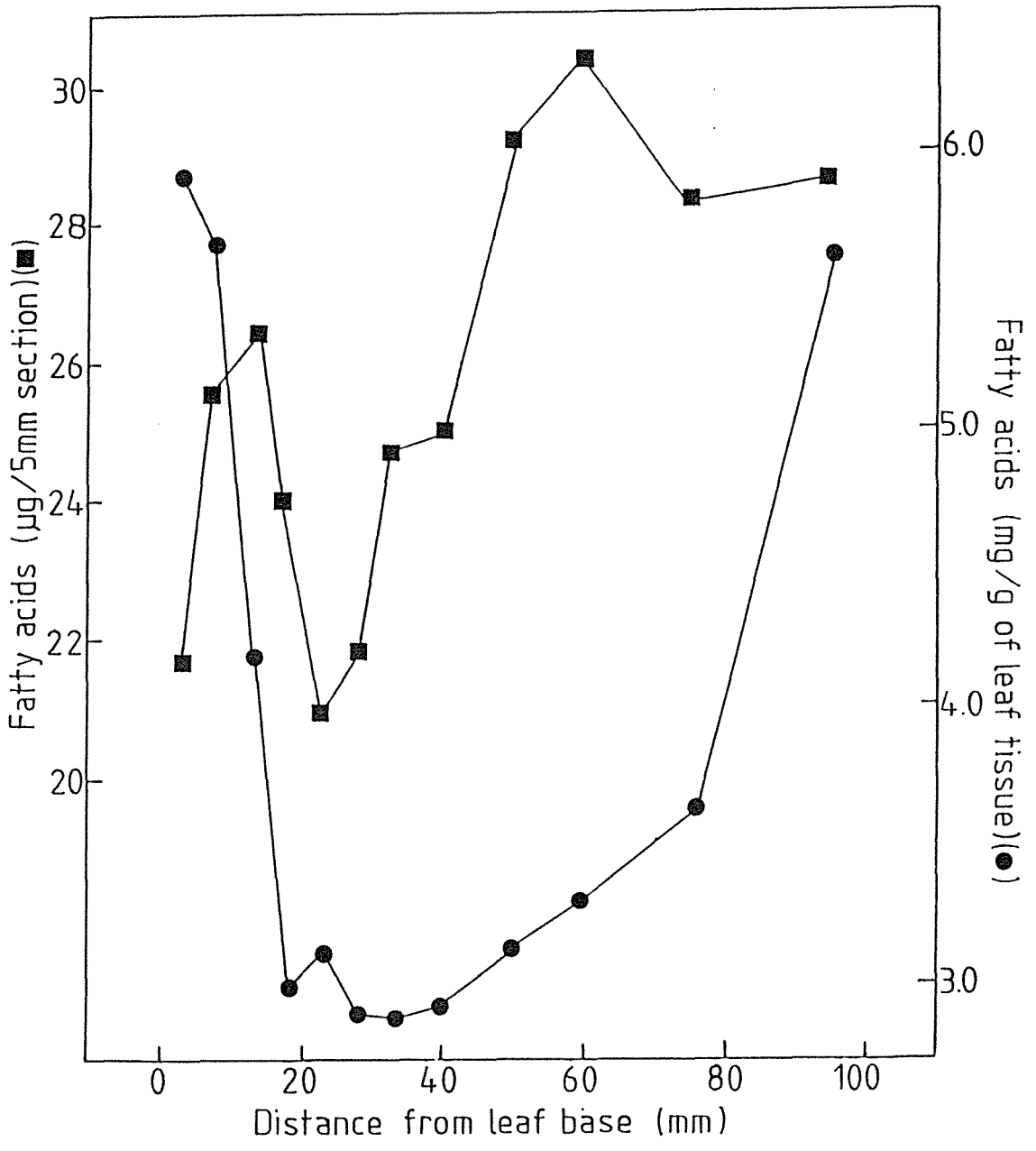


FIGURE 11: Changes in Fatty Acid Levels During Cell Development in the Maize Leaf

The 2nd leaves of maize (Zea mays var. Pioneer 3709) seedlings were sectioned as described in Section 3.5. Fatty acids were extracted, and their levels determined as described in Section 3.1.2.
Fatty acid levels per 5mm section were calculated for the average weight of the 5mm leaf sections used.
Values are the average of 3 analyses of different tissue batches.

Fatty acid levels per 5mm section reached a minimum between 20 to 25mm from the leaf base, while fatty acid levels per gram of leaf tissue were minimal between 20 to 40mm. On the basis of other work with maize tissue (Leech et al., 1973; Hawke et al., 1974a; Leese and Leech, 1976; Leech and Baker, 1983), these low fatty acid levels reflect the cell and plastid elongation that occurs in this region, since cell elongation effectively lowers the membrane density and dilutes cellular components. In contrast, in wheat leaves of similar size, fatty acid levels per 5mm section were found to be minimal at about 10 to 15mm from the leaf base (Hawke and Leech, 1987). Thus suggesting that cell elongation in wheat leaves occurs nearer the leaf base than in maize leaves. The increase in fatty acid levels in maize from 40mm along the leaf to the leaf tip is likely to be in response to the increased demand for fatty acids necessary for thylakoid formation. In wheat leaves fatty acid levels per 5mm section also increase over this region and coincide with the major phase of chloroplast enlargement (Hawke and Leech, 1987).

Linoleic acid (18:2) was the most abundant fatty acid constituent of lipids at the leaf base; making up 56% of the fatty acids present, while linolenic acid (18:3) was the most abundant fatty acid at the leaf tip making up 59% of the fatty acids in this region (Table II). The level of 18:3 was relatively constant from the leaf base to 25 to 30mm along the leaf, thereafter increasing about 5.5-fold, from approximately 3µg to 16.8µg at the leaf tip (Fig.12). The level of 18:2 decreased 3.2-fold from 14µg at 5-10mm from the leaf base to 4.4µg at the leaf tip. Apart from a slight increase over the first 15mm of the leaf, the level of 16:0 in constituent lipids

TABLE II

FATTY ACID COMPOSITION OF LIPIDS IN THE SECOND LEAF OF THE MAIZE SEEDLING

Distance from leaf base (mm)	Acyl Content (µg/5mm portion)	Fatty Acid Composition *(weight %)			
		16:0	18:1	18:2	18:3
0-5	21.7	25.6	4.2	55.8	11.5
5-10	25.6	25.2	4.7	54.3	14.3
10-15	26.5	28.3	5.7	50.3	13.9
15-20	24.0	34.7	9.9	37.8	10.7
20-25	21.0	28.0	9.2	41.7	16.3
25-30	21.9	30.7	14.9	33.1	14.7
30-35	24.7	27.5	8.1	38.2	20.4
35-45	25.0	24.7	6.5	35.9	28.0
45-55	29.2	23.2	3.1	34.0	36.9
55-65	30.5	21.8	2.8	26.1	45.9
65-85	28.3	21.1	1.9	18.6	55.3
85-105	28.6	21.6	1.5	15.4	58.6

Fatty acid levels were determined as described in Section 3.1.2 from leaves sectioned as described in Section 3.5.
Values are the average of 3 analyses of different tissue batches.
*Minor components e.g. 14:0, 18:0 are omitted.

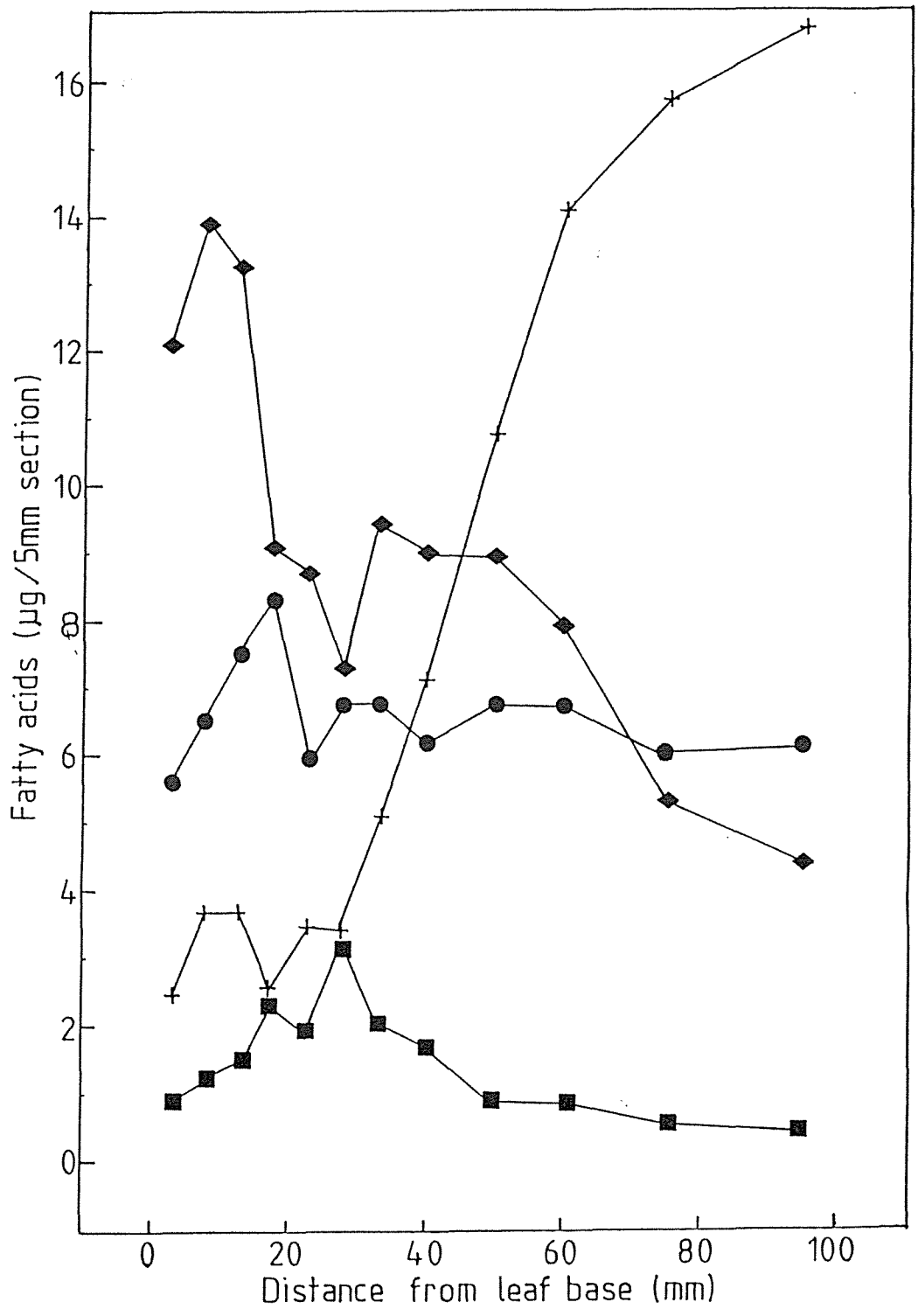


FIGURE 12: Changes in the Levels of Individual Fatty Acids During Cell Development in the Maize Leaf

The levels of the four major fatty acids, 16:0 (●), 18:1 (■), 18:2 (◆) and 18:3 (+) along 9-day-old second leaves of maize (Zea mays var. Pioneer 3709) seedlings.
Values are the average of 3 analysis of different tissue batches.

was relatively constant over the entire length of the leaf (Fig.12). A similar trend occurred in the levels of 18:1, with a maximum level occurring in the 15-20mm region of the leaf. 14:0 and 18:0 were present in minor amounts in maize leaves with 14:0 constituting less than 1.5% of total fatty acids and 18:0 constituting less than 6.5%.

The leaf base contains cells that lack chloroplasts, and this is reflected in the high proportion of the more saturated fatty acids present in this region (Table II). These fatty acids are mainly constituents of the phospholipids, which are major components of structural membranes (Leech et al., 1973), and are therefore quantitatively more important in the immature tissue. The increasing levels of 18:3 from 20mm along the leaf to the leaf tip reflects the plastid development occurring in this region since plastid development is closely paralleled by an increase in galactolipids (Leech et al., 1973), and galactolipids, particularly MGDG's, contain very high proportions of 18:3. The increasing level of 18:3 in particular, indicates the increased formation of galactolipid-rich thylakoid membranes.

4.4.2 Protein Levels

Protein levels at the leaf base were 25mg/g of leaf tissue and 21mg/g in the XL45 and Pioneer 3709 hybrids respectively (Fig.13). These levels decreased 2.5 to 3-fold to 8.5 to 10mg/g between 20 to 50mm along the leaf, and then increased to 21 to 22mg/g at the leaf tip in both hybrids. The high protein levels at the leaf base, reflect the large number of small cells

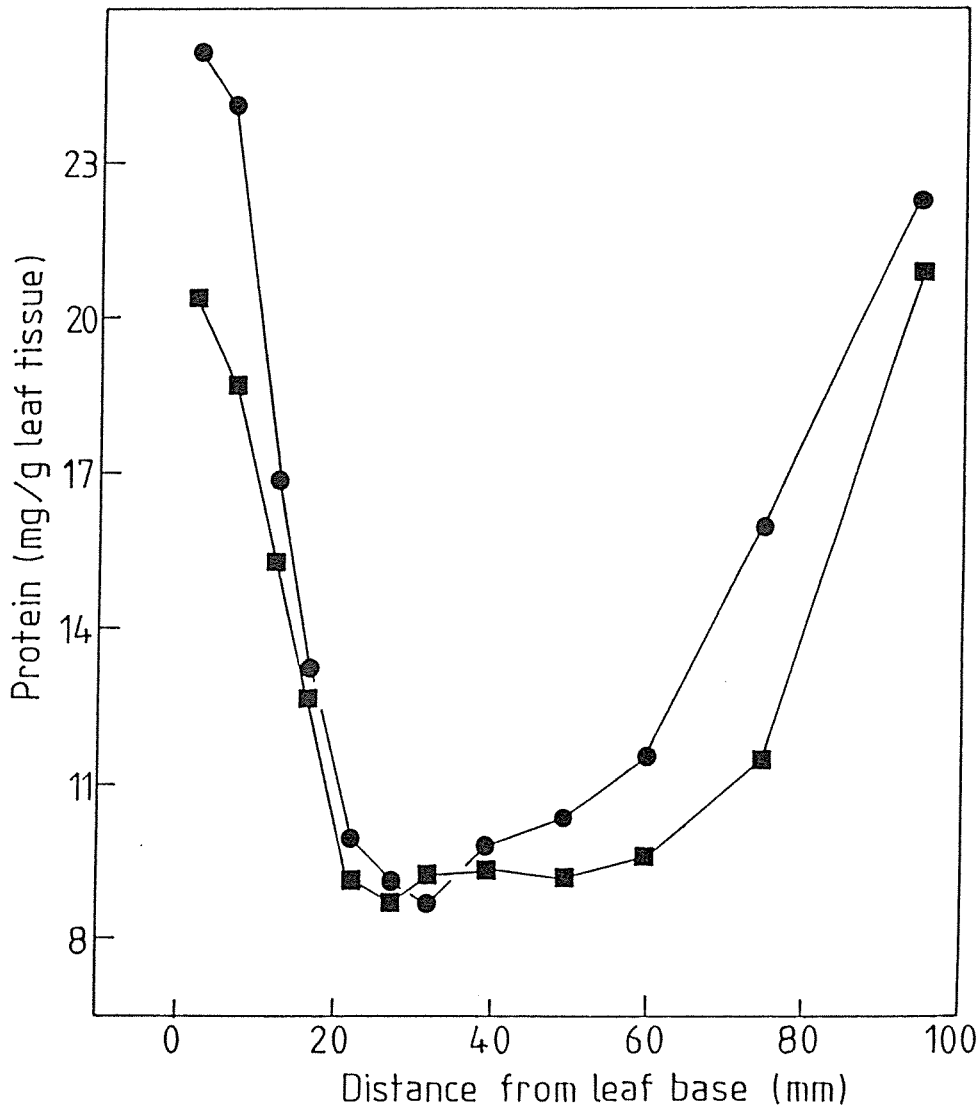


FIGURE 13: Changes in Protein Levels During Cell Development in the Maize Leaf

Protein levels were determined from cell-free extracts (Section 3.6) of leaf sections (Section 3.5) of 9-day-old Zea mays var. XL45 (●), and Zea mays var. Pioneer 3709 (■) seedlings. Protein levels were then calculated for a constant weight of leaf tissue.
Values are averages of duplicate determinations from 5 experiments for Zea mays var. XL45, and 3 experiments for Zea mays var. Pioneer 3709.

present in this region, whereas in the region of low protein levels, it is likely that there are a smaller number of larger cells that have undergone cell elongation. The region exhibiting low protein levels is also the region of low levels of fatty acids/g (Fig.11). The rise in protein levels that occurred in the more distal regions of the leaf reflects the increased levels of proteins associated with photosynthesis and in particular ribulose bisphosphate carboxylase oxygenase (Rubisco), which makes up 70% to 90% of the soluble leaf protein (Dean and Leech, 1982; Leech and Baker, 1983).

4.4.3 Acetyl-CoA Carboxylase Activity

Total acetyl-CoA carboxylase activity increased 3-fold and 2.6-fold from the base of the leaf to 60-80mm along the leaf, in the XL45 and Pioneer 3709 maize hybrids respectively (Fig.14). In the XL45 hybrid, peak activity occurred between 65 to 85mm from the leaf base, while in the Pioneer 3709 hybrid peak activity occurred in the region 55 to 65mm from the base. The level of acetyl-CoA carboxylase activity throughout the leaf in the Pioneer 3709 hybrid was about 60% of the activity present in the XL45 hybrid. Carboxylase activity measured on a per gram of leaf tissue basis showed a 2 and 3-fold drop in activity between 5-30mm along the leaf in the XL45 and Pioneer 3709 hybrids respectively (Fig.15), before increasing approximately 2.5-fold towards the leaf tip in both hybrids. The high acetyl-CoA carboxylase activity per gram of leaf tissue at the leaf base, reflects the high fatty acid requirement per gram of leaf tissue in this region. The decrease in carboxylase activity per gram of leaf tissue that occurs between 5mm and

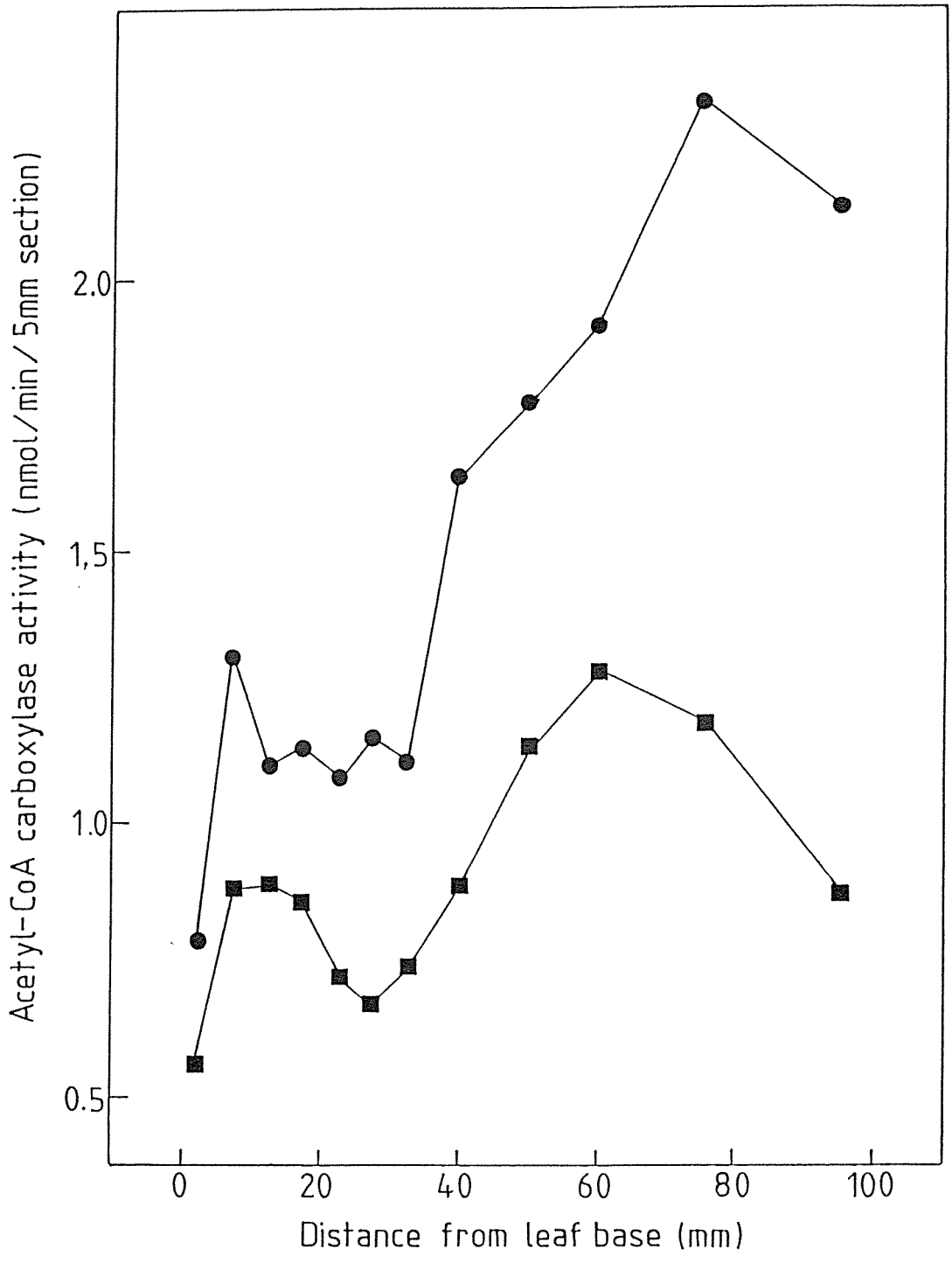


FIGURE 14: Changes in Acetyl-CoA Carboxylase Activity During Cell Development in the Maize Leaf

Acetyl-CoA carboxylase activity was determined from cell-free extracts (Section 3.6) of sections of the 2nd leaf of 9-day-old Zea mays var. XL45 (●), and Zea mays var. Pioneer 3709 (■) seedlings. Acetyl-CoA carboxylase activity was calculated per 5mm leaf section. Values are averages of duplicate assays from 5 experiments for Zea mays var. XL45 and 3 experiments for Zea mays var. Pioneer 3709.

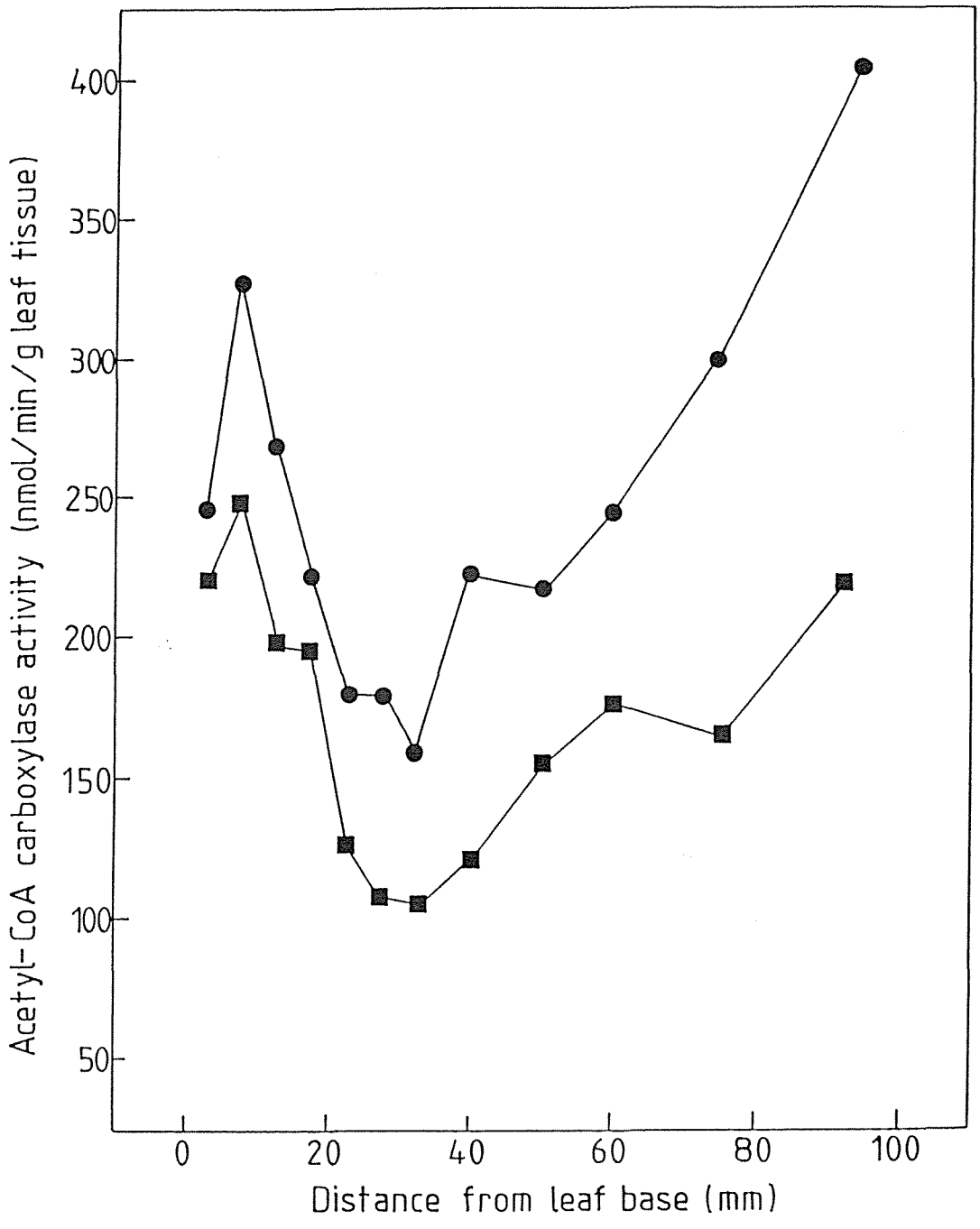


FIGURE 15: Changes in Acetyl-CoA Carboxylase Activity During Cell Development in the Maize Leaf

Acetyl-CoA carboxylase activity was determined from cell-free extracts (Section 3.6) of leaf sections of the 2nd leaf of 9-day-old Zea mays var. XL45 (●) and Zea mays var. Pioneer 3709 (■) seedlings. Acetyl-CoA carboxylase activity was then calculated for a constant weight of leaf tissue.
Values are the averages of duplicate assays from 5 experiments for Zea mays var. XL45, and 3 experiments for Zea mays var. Pioneer 3709.

30mm from the leaf base is due to cell elongation since elongation effectively dilutes cell components. The increase in activity beyond 30mm reflects the increased demand for fatty acids, necessary for thylakoid membrane formation. When acetyl-CoA carboxylase activity was expressed for a constant weight of protein the activity increased 2.5-fold and 2-fold in the XL45 and Pioneer 3709 hybrids respectively, from the leaf base to 40 to 50mm along the leaf (Fig.16). Since the leaf base is the site of maximum development, it is likely that the low specific activity in this region results from the presence of other enzymes necessary for cell and plastid development. The increase in specific activity occurring between the leaf base and 30mm along the leaf results from the protein levels decreasing at a greater rate than the acetyl-CoA carboxylase activity. From 30mm to 50mm from the leaf base, the carboxylase activity is increasing while the protein levels remain relatively constant (Fig.13,15). In the region 40-50mm from the leaf base to the leaf tip, acetyl-CoA carboxylase activity per mg of protein decreased 1.3 to 2-fold in the XL45 and Pioneer 3709 hybrids respectively. The decrease in specific activity along this section of the leaf is most likely due to the increased levels of enzymes involved with photosynthetic processes, in particular Rubisco (Leech and Baker, 1983).

There is a significant correlation between the fatty acid levels and acetyl-CoA carboxylase activity in the developing maize leaf. Both biochemical parameters increase from the leaf base to a peak between 5-15mm along the leaf, thereafter decreasing up to 20-30mm from the leaf base, before increasing to their maximum values in the leaf at 55-65mm from the leaf base, and

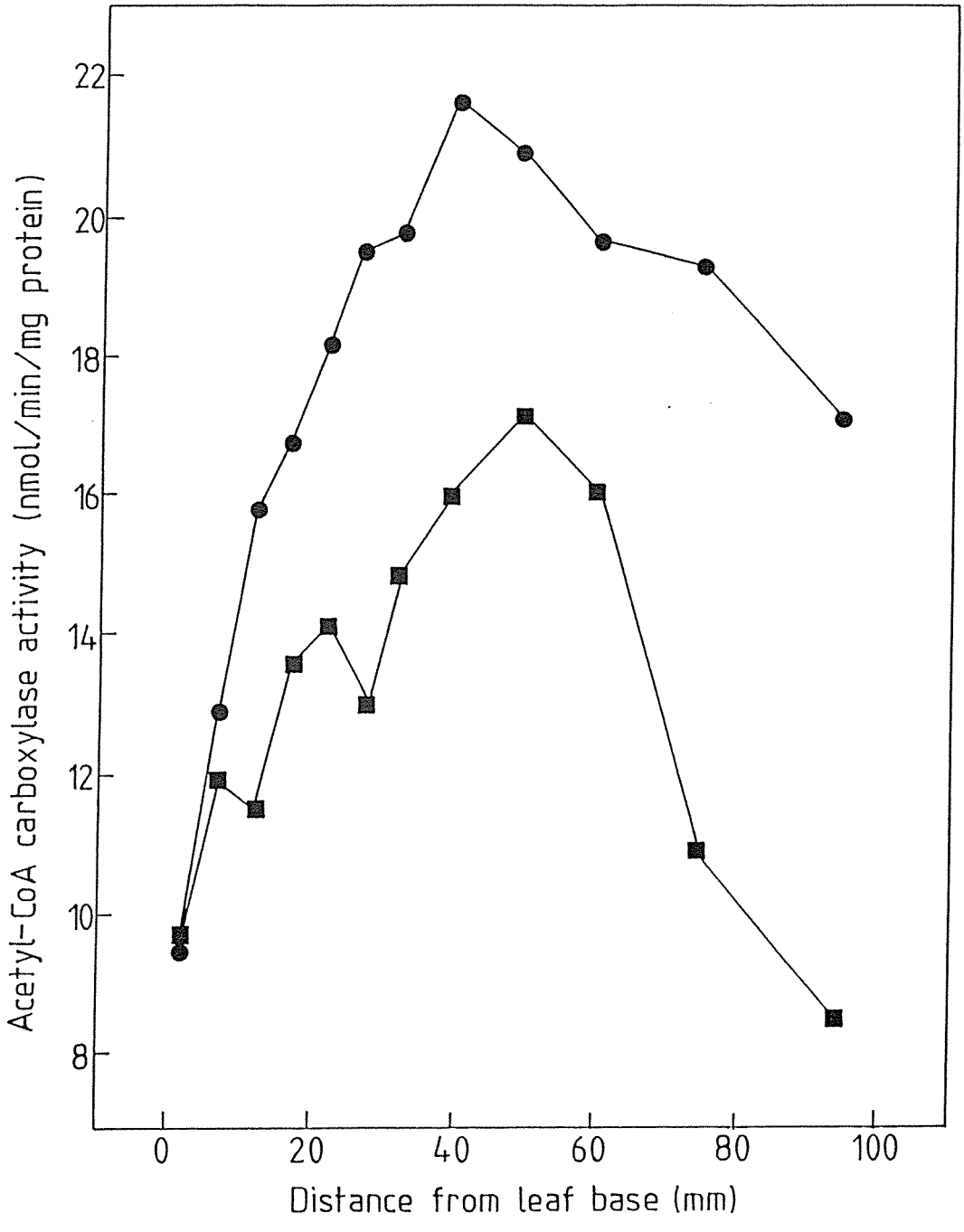


FIGURE 16: Acetyl-CoA Carboxylase Activity in the Developing Leaf of Maize Seedlings

Acetyl-CoA carboxylase activity and protein levels were determined from cell-free extracts (Section 3.6) of sections of the 2nd leaf of 9-day-old Zea mays var. XL45 (●) and Zea mays var. pioneer 3709 (■) maize seedlings. Acetyl-CoA carboxylase activity was calculated for a constant weight of protein.

then decreasing towards the leaf tip (Fig.11,14). Confirmation of the correlation between fatty acid levels and acetyl-CoA carboxylase activity is obtained by comparing both biochemical parameters on a per weight basis (Fig.11,15). The correlation between acetyl-CoA carboxylase activity and fatty acid levels reflects the possible role of acetyl-CoA carboxylase in the regulation of fatty acid synthesis in maize leaf tissue.

4.4.4 Chlorophyll Levels

Chlorophyll levels increased 32-fold along the leaf, with the greatest increase occurring between 20mm from the leaf base, to the leaf tip (Fig.17). The low level of chlorophyll in the basal region is indicative of tissue containing immature plastids, while increasing chlorophyll levels reflect the maturation of chloroplasts associated with increasing tissue differentiation. In a fixed weight of leaf tissue acetyl-CoA carboxylase activity, fatty acid levels and protein levels all increase from 20-30mm along the leaf to the leaf tip, reflecting a correlation between these biochemical parameters with chloroplast maturation. It is likely that the increase in acetyl-CoA carboxylase activity and fatty acid levels are in response to thylakoid membrane formation, while increasing protein levels reflect the increase in levels of photosynthetic enzymes in the chloroplasts.

4.5 Role of Light in the Regulation of Acetyl-CoA Carboxylase

Although fatty acid biosynthesis in photosynthetic tissue is a light-dependent process (Nakamura and Yamada, 1979), the

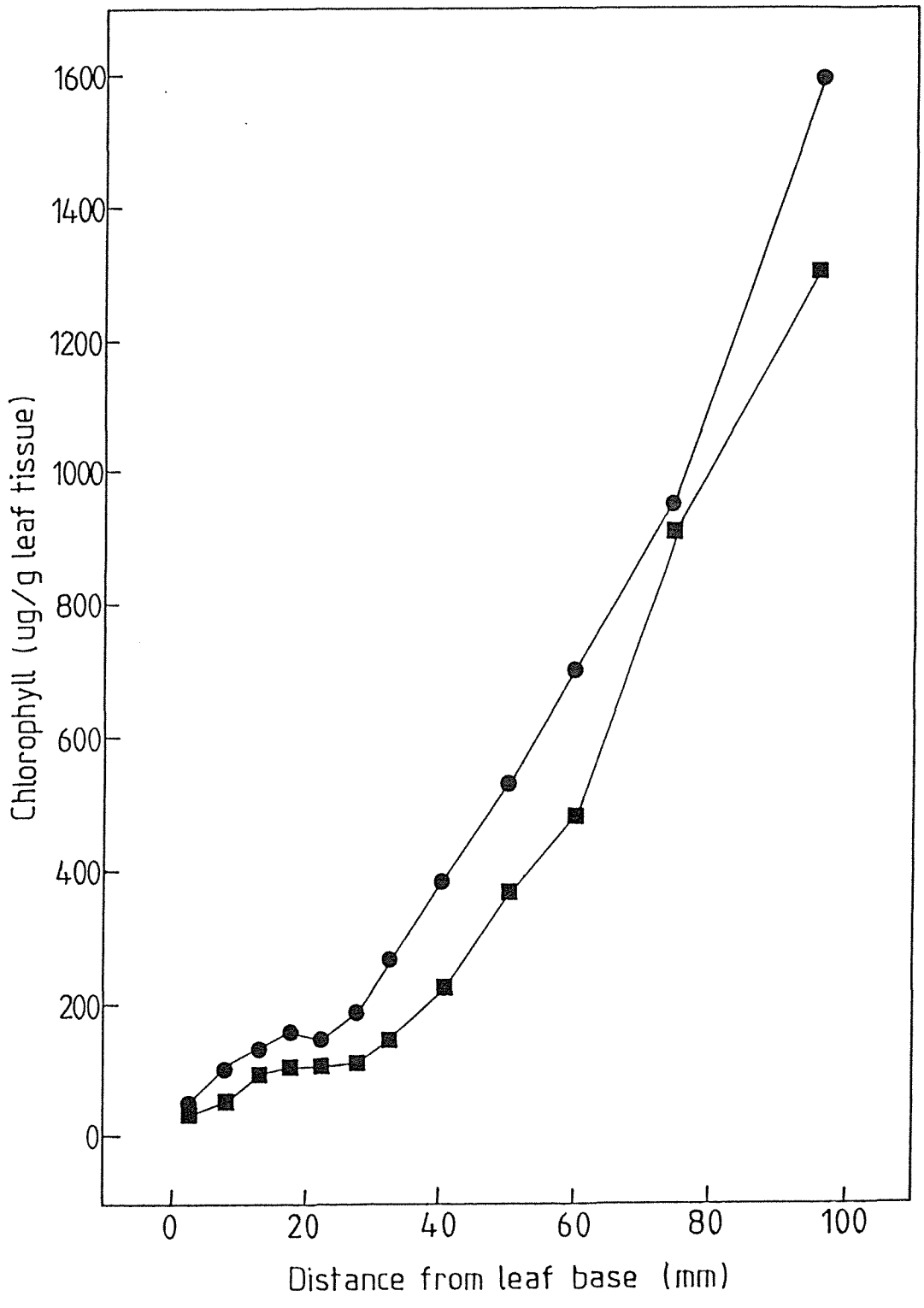


FIGURE 17: Changes in Chlorophyll Levels During Cell Development in the Maize Leaf

Chlorophyll levels were determined as described in Section 3.1.3 from Zea mays var. XL45 (●), and Zea mays var. Pioneer 3709 (■). Leaves sectioned as described in Section 3.5.
Chlorophyll levels were then calculated for a constant weight of leaf tissue.
Values are the averages of 5 experiments for Zea mays var. XL45 and 3 experiments for Zea mays var. Pioneer 3709.

mechanism by which light regulates fatty acid synthesis and the enzymes involved in regulation is uncertain. Nikolau and Hawke (1984), on the basis of kinetic studies, have suggested that acetyl-CoA carboxylase in maize leaves is regulated by the observed changes in stromal pH and concentrations of Mg^{2+},ATP and ADP that occur during dark-light transitions. Subsequently, Hawke and Leech (1987), have reported evidence to suggest that these changes in pH and metabolite concentrations regulate acetyl-CoA carboxylase in wheat leaf tissue. In order to confirm the role of light in the regulation of maize leaf acetyl-CoA carboxylase, the enzyme was extracted from the second leaves of 8-day-old maize seedlings (Section 3.6), and carboxylase activity assayed at the pH's and concentrations of ATP, ADP and Mg^{2+} that occur in the stroma during dark-light transitions (Table III).

An overall 5-fold difference in acetyl-CoA carboxylase activity was observed between the change from the least favourable (Table III, Column 1), to the most favourable (Table III, Column 9) conditions within the range of physiological concentrations of ATP, ADP, Mg^{2+} and H^{+} that occur in response to dark-light transitions. This change in activity is of the same order of magnitude as the 8-fold decrease in [1-^{14}C]-acetate incorporation into lipid that occurs upon removal of illumination from 7-day-old maize chloroplasts, reported by Hawke et al. (1974b).

When all the metabolite concentrations were similar (Table III, Columns 3,5,7), acetyl-CoA carboxylase activity was similar at pH's 7.1 and 7.5, but at pH 8.0 carboxylase activity was almost double. The greater acetyl-CoA carboxylase activity at pH 8.0

TABLE III

ACETYL-CoA CARBOXYLASE ACTIVITY OBSERVED IN THE PHYSIOLOGICAL RANGE OF ATP,ADP, Mg^{2+} CONCENTRATIONS, AND pH PRESENT IN THE STROMA OF CHLOROPLASTS IN THE LIGHT AND DARK

	"DARK"			"INTERMEDIATE"			"LIGHT"		
pH	7.1	7.1	7.1	7.5	7.5	7.5	8.0	8.0	8.0
ATP (mM)	0.3	0.5	0.8	0.3	0.8	1.4	0.8	1.1	1.4
ADP (mM)	1.0	0.8	0.6	1.0	0.6	0.3	0.6	0.45	0.3
Mg^{2+} (mM)	1.8	2.0	2.3	1.8	2.0	5.0	2.3	2.5	5.0
Acetyl-CoA Carboxylase Activity (nmol/min/mg Protein)	3.0	4.8	6.1	5.0	6.4	14.2	10.0	11.2	15.4

Acetyl-CoA carboxylase activity was assayed in cell-free extracts of 8-day-old maize (Zea mays var. Pioneer 3709) seedlings. Assay conditions were as described in Section 3.4.2 except that the pH concentrations of ATP, ADP and Mg^{2+} were those used by Hawke and Leech (1987) which are shown above.
Values are averages of triplicate assays from 3 experiments.

is consistent with a pH optimum at 8.0 for acetyl-CoA carboxylase activity in maize (Nikolau and Hawke, 1984). The results from Table III, columns 6 and 9 do not reflect the sharp pH optimum for acetyl-CoA carboxylase activity, as only a slight increase in enzyme activity occurs between pH 7.5 and 8.0. Although this may be due to the presence of ADP, it may also be because the enzyme is more sensitive to pH changes at the more favourable metabolite concentrations.

The standard assay conditions described in Section 3.4.2 most closely resemble those in Column 8 (Table III), without the inclusion of ADP. Acetyl-CoA carboxylase activity assayed in this experiment was only 45% of the activity from similar tissue assayed under standard conditions (Fig.9). The inclusion of ADP may have reduced the activity since ADP is an inhibitor of maize acetyl-CoA carboxylase (Nikolau and Hawke, 1984).

Incorporation of [1-^{14}C]-acetate into lipids by maize chloroplasts is also pH-dependent (Hawke et al., 1974b) with a 2.3-fold greater [1-^{14}C]-acetate incorporation at pH 8.0 compared to pH 7.1. It is possible that the pH-dependence resulted from a greater acetyl-CoA carboxylase activity under these conditions.

Hawke and Leech (1987), reported a 10-fold difference in acetyl-CoA carboxylase activity in 7-day-old wheat leaves using similar conditions to those presented in Table III. While the specific activity of the maize enzymes was 5 to 10-fold higher than wheat acetyl-CoA carboxylase, the latter was 2 times more sensitive to the metabolite and pH changes investigated.

The evidence presented suggests that acetyl-CoA carboxylase from maize leaf tissue is regulated by light-dependent changes in pH and concentrations of ATP, ADP and Mg^{2+}. Therefore it is possible that the regulation of fatty acid synthesis by light is at the site of acetyl-CoA carboxylase.

4.6 Distribution of Acetyl-CoA Carboxylase in Maize Seedlings

In order to determine which tissue section of the maize seedling provided the best source of acetyl-CoA carboxylase for purification of this enzyme, levels of carboxylase activity were determined in cell-free extracts of epicotyl, coleoptile and leaf tissue of 9-day-old maize seedlings (Fig.18) (Table IV).

The epicotyl, coleoptile and leaf tissues contributed 10%, 16% and 74% respectively of the total acetyl-CoA carboxylase activity, while the leaves contained 5 times more carboxylase activity per gram of tissue than the epicotyl or coleoptile. The specific activity of acetyl-CoA carboxylase in the second and subsequent leaves was 2.7 times greater than in the first leaf. In addition, the first leaf contained a large proportion of fibrous tissue near the leaf base which was difficult to homogenise (Fig.18, Section A). Consequently, the first leaf and coleoptile were discarded and the second and subsequent leaves of 9-day-old maize seedlings were used as the source of maize acetyl-CoA carboxylase for preparative and purification procedures.

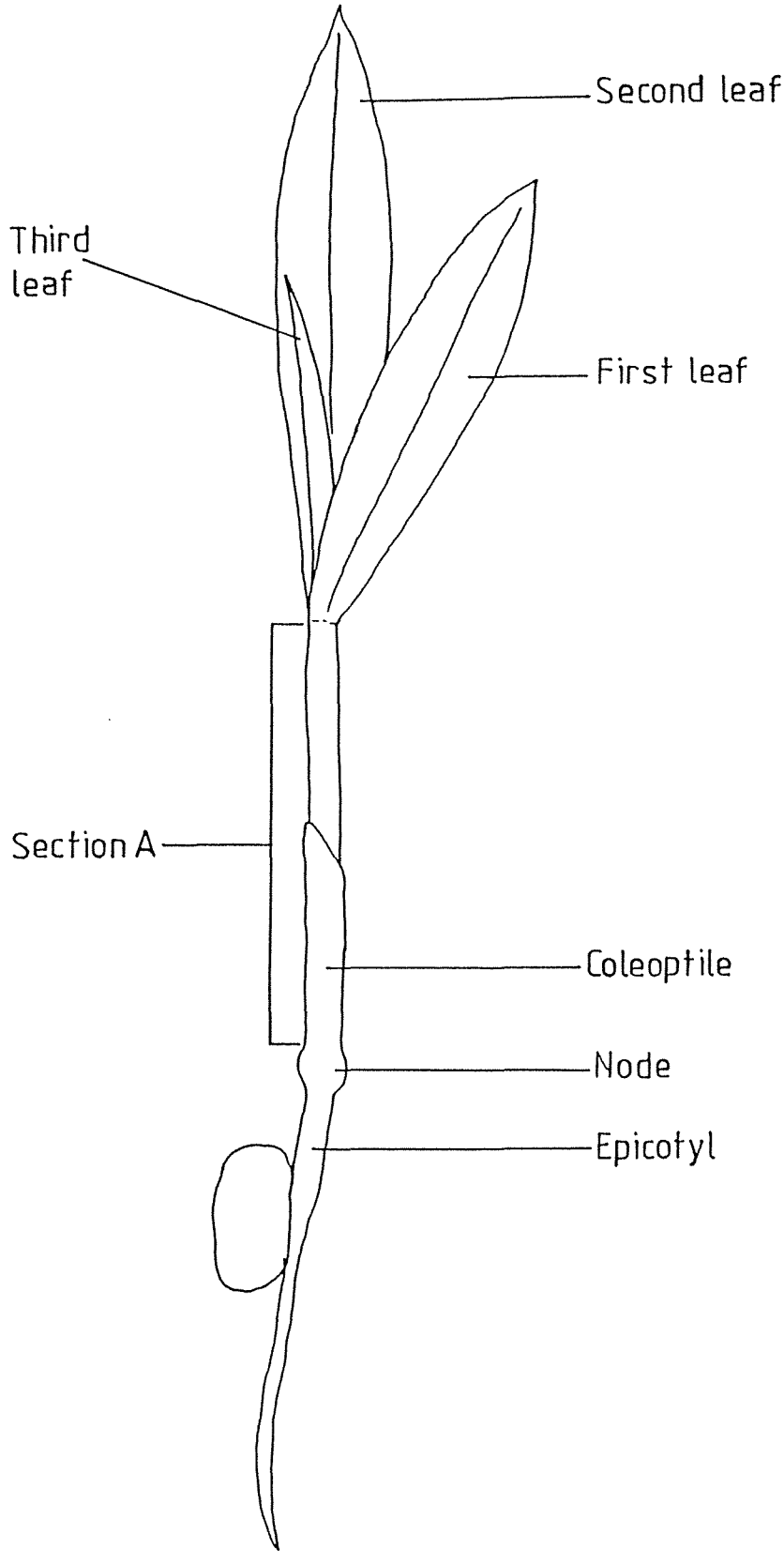


FIGURE 18: Schematic Diagram Showing Various Sections of a Nine-Day-Old Maize Seedling

TABLE IV

DISTRIBUTION OF ACETYL-CoA CARBOXYLASE ACTIVITY BETWEEN TISSUES OF NINE-DAY-OLD MAIZE SEEDLINGS

Tissue	Weight per seedling (g)	Acetyl-CoA Carboxylase		
		(nmol/min)	(nmol/min/g wet of tissue)	(nmol/min/mg of protein)
Stalk	0.221	5.29	23.94	2.37
Coleoptile	0.228	8.21	36.01	10.53
1st Leaf	4.144	16.47	114.38	8.90
2nd and subsequent leaves	0.116	20.96	180.69	23.82

Acetyl-CoA carboxylase activity and protein levels were determined from cell-free extracts of each tissue. Tissue was obtained from 9-day-old seedlings as shown in Figure 18.
Values are the average of duplicate assays of 1 experiment using 4 seedlings.

4.7 Western Blotting Analysis of Biotin-Containing Proteins

Acetyl-CoA carboxylase is the only biotin-containing protein reported in maize, and therefore western blotting analysis using a ^{35}SLR-streptavidin probe for biotin appeared an ideal method for detecting the presence of acetyl-CoA carboxylase. The detection of biotin-containing proteins has been achieved in previous studies of plant acetyl-CoA carboxylase using [^{135}I]-streptavidin (Nikolau et al., 1984; Nikolau et al., 1985) and [^{35}S]-streptavidin (Hawke and Leech, 1987). However, in this study, when western blotting analysis was carried out on polyacrylamide gels containing acetyl-CoA carboxylase from maize leaves, no radioactive bands were detected on the developed film. Failure of this technique could be due to either acetyl-CoA carboxylase subunits not being transferred from the polyacrylamide gel to the nitrocellulose, or the subunits passing through, or not adhering to the nitrocellulose, or, the ^{35}SLR-streptavidin not binding to the biotin-containing proteins. These possibilities were subsequently investigated.

4.7.1 Effect of ATP and $MgCl_2$ on the Acetyl-CoA Carboxylase-^{35}SLR-Streptavidin Interaction

Direct application of ^{35}SLR-streptavidin onto nitrocellulose, in an 8mm diameter spot (5µl), established that 60cpm (70pmoles) of ^{35}SLR-streptavidin was easily detectable on Hyperfilm βmax following autoradiography for 17 days (Fig.19). 4 to 12nmol/min of partially purified acetyl-CoA carboxylase (Procedure II) was then spotted directly onto nitrocellulose,

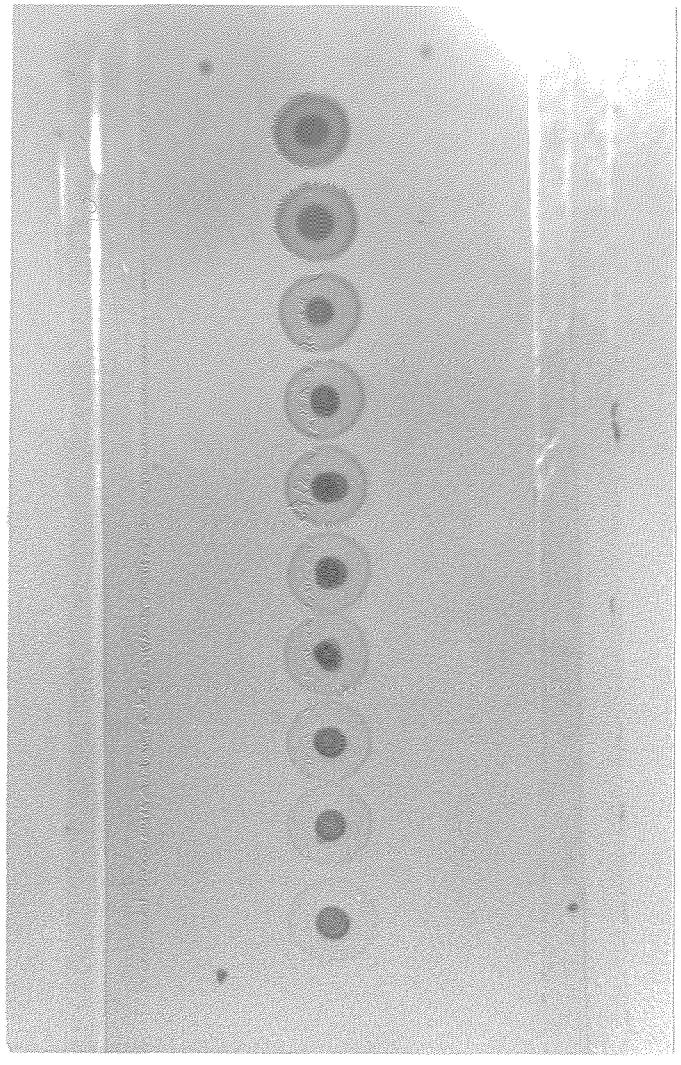


FIGURE 19: Autoradiograph of ^{35}SLR-Streptavidin Spotted Directly onto Nitrocellulose

^{35}SLR-streptavidin-nitrocellulose was autoradiographed against Hyperfilm βmax for 17 days.

which was then blocked, and probed with ^{35}SLR-streptavidin as described in Section 3.9, except that probing was carried out in the absence of 2.5mM $MgCl_2$ for a period of 1 hr (Hawke and Leech, 1987). After autoradiography for 10 days, very faint darkened spots were detected on the film (Fig.20, film 1).

Although it was apparent that biotin-containing proteins could be detected using ^{35}SLR-streptavidin probing, the method was not sensitive enough for practical use. Therefore, in order to improve the streptavidin-acetyl-CoA carboxylase interaction, the more potent avidin-acetyl-CoA carboxylase interaction was investigated. Initial studies showed that if avidin was added to maize acetyl-CoA carboxylase assay mixtures just prior to assaying, then there was only a 5% loss in activity. Consequently, acetyl-CoA carboxylase was incubated with avidin for 30 min at 30^{o}C before assaying. Since ATP and $MgCl_2$ are required for carboxylase activity, the effect of the presence of these compounds on inhibition of acetyl-CoA carboxylase by avidin was investigated. Concentrations of ATP and $MgCl_2$ were those which gave maximum activity, while concentrations of avidin were in the order of those used by Nikolau and Hawke (1984), (Table V). Acetyl-CoA carboxylase activity in the presence of 2.5mM $MgCl_2$ was 110% of the activity in the absence of $MgCl_2$. However, enhancement of carboxylase activity in the presence of both 4mM ATP and 2.5mM $MgCl_2$ was 106.6%, which was not as great as with $MgCl_2$ alone. 4mM ATP inhibited acetyl-CoA carboxylase activity to 98.6% of the activity in the absence of ATP.

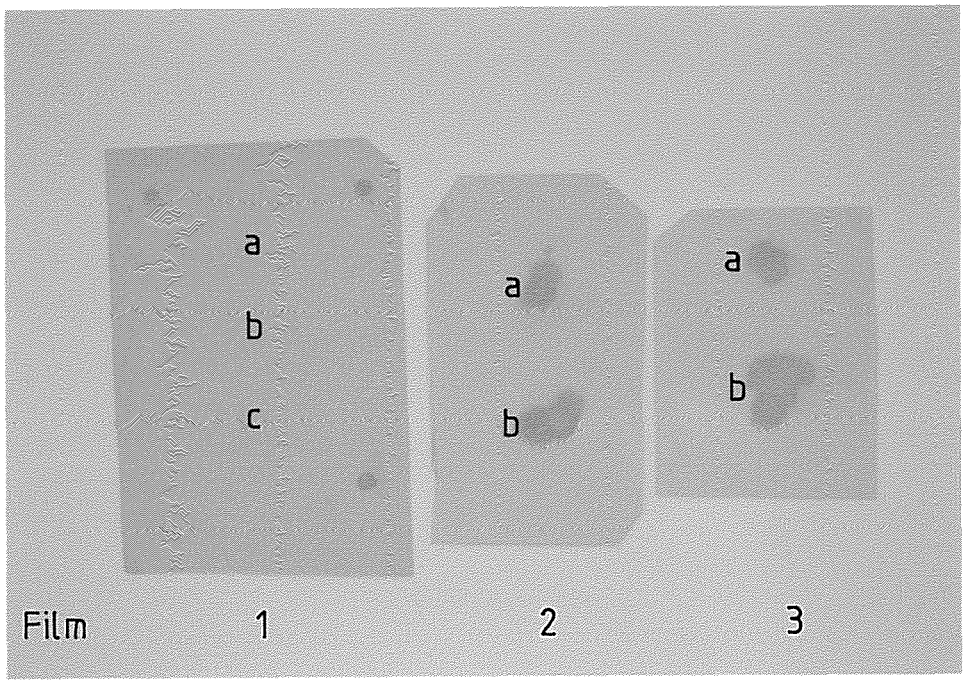


FIGURE 20: Autoradiographs of Biotin-Specific Probes of Partially Purified Acetyl-CoA Carboxylase

Film 1: 4nmol/min (a), 8nmol/min (b) and 12nmol/min (c) of partially purified acetyl-CoA carboxylase activity was spotted onto nitrocellulose, and subsequently washed and probed as described in Section 3.9, except that 2.5mM $MgCl_2$ was omitted from the probing buffer and probing was carried out for 1 hr. The film was autoradiographed against Hyperfilm βmax for 10 days.

Film 2 & 3: 10nmol/min (a) and 20nmol/min (b), acetyl-CoA carboxylase activity, from the same preparation as used above, was spotted onto nitrocellulose, and subsequently washed, and probed overnight, as described in Section 3.9, in the absence (Film 2) and presence (Film 3) of 2.5mM $MgCl_2$ in the probing buffer. The films were both autoradiographed against Hyperfilm βmax for 13 days.

TABLE V

EFFECT OF ATP AND $MgCl_2$ ON AVIDIN INHIBITION OF ACETYL-CoA CARBOXYLASE FROM MAIZE

	ACC Activity (nmol/min/mg protein)			
		4mM ATP	2.5mM $MgCl_2$	4mM ATP + 2.5mM $MgCl_2$
Without Avidin	22.5	22.2	24.8	24.0
With Avidin				
0.2 units	20.8	18.1	15.4	20.2
0.4 units	18.0	14.9	12.4	20.2
(per incubation tube)				

Partially purified acetyl-CoA carboxylase (Procedure II) was incubated with avidin in 0.1M Tricine buffer at pH 8.0 containing 50mM KCl and 1mM DTT for 30 min at 30°C before assaying, as described in Section 3.4.2.

In the presence of either $MgCl_2$ or ATP, 0.4 units of avidin inhibited acetyl-CoA carboxylase up to 11% more than 0.2 units of avidin, however, when $MgCl_2$ and ATP were added together this concentration dependence was lost. The maximum of 48% inhibition of acetyl-CoA carboxylase occurred in the presence of 2.5mM $MgCl_2$ and 0.4 units of avidin. On the basis of the avidin-acetyl-CoA carboxylase interaction the inclusion of 2.5mM $MgCl_2$ into the ^{35}SLR-streptavidin probing buffer (PBS) would only marginally improve the ^{35}SLR-streptavidin-acetyl-CoA carboxylase binding. This was confirmed when acetyl-CoA carboxylase, spotted onto nitrocellulose was probed overnight in the presence and absence of 2.5mM $MgCl_2$ (Fig.20, Films 2,3).

4.7.2 Effect of Probing Time on the Acetyl-CoA Carboxylase-^{35}SLR-Streptavidin Interaction

Since the avidin-acetyl-CoA carboxylase interaction was found to be time-dependent (Section 4.7.1), it is likely that this finding can be extended to the streptavidin-acetyl-CoA carboxylase interaction. Therefore, the effect of increasing the period of ^{35}SLR-streptavidin probing on radioactive spot intensities was investigated. Partially purified acetyl-CoA carboxylase (Procedure II) was spotted onto nitrocellulose, which was blocked, and then probed overnight with ^{35}SLR-streptavidin. After autoradiography for 13 days, dark spots were detected on the film (Fig.20, Film 2). By comparing Fig. 20, Films 1,2 and 3 it is apparent that the increased probing time had a much greater effect on the ^{35}SLR-streptavidin-acetyl-CoA carboxylase interaction than the inclusion of 2.5mM $MgCl_2$, however, since $MgCl_2$ was found to

enhance avidin inhibition of acetyl-CoA carboxylase, all subsequent ^{35}SLR-streptavidin probes were carried out in the presence of 2.5mM $MgCl_2$ overnight.

4.7.3 Effect of Blotting Time on Protein Transfer from Polyacrylamide Gels to Nitrocellulose

Partially purified acetyl-CoA carboxylase (Procedure II) was electrophoresed on an SDS-15% polyacrylamide gel, after which the gel was cut into 3 sections. The 3 sections were then transblotted against 2 layers of nitrocellulose for 1,2 and 2.5 hr respectively (Fig.21). The nitrocellulose papers were subsequently stained for protein using Amido Black 10B. The side of the first sheet of nitrocellulose closest to the gel during protein transfer (Fig.22,1A) shows a variety of protein bands with varying molecular weights. The side of the second sheet of nitrocellulose furthest from the gel (Fig.22,1D) shows primarily proteins of lower molecular weight. This is consistent with protein transfer being inversely proportional to the molecular weights of the proteins undergoing transblotting, (Gershoni and Palade, 1983). The protein-nitrocellulose interaction is not clearly understood as at pH 8.3, the pH at which protein blotting is usually performed, nitrocellulose is negatively charged, as are most of the proteins being transblotted (Gershoni and Palade, 1983). Hydrophobic interaction probably plays a role in the protein-nitrocellulose interaction as non-ionic detergents such as Triton X-100 are known to facilitate removal of proteins from nitrocellulose (Schneider, 1980; Farrah et al., 1981).

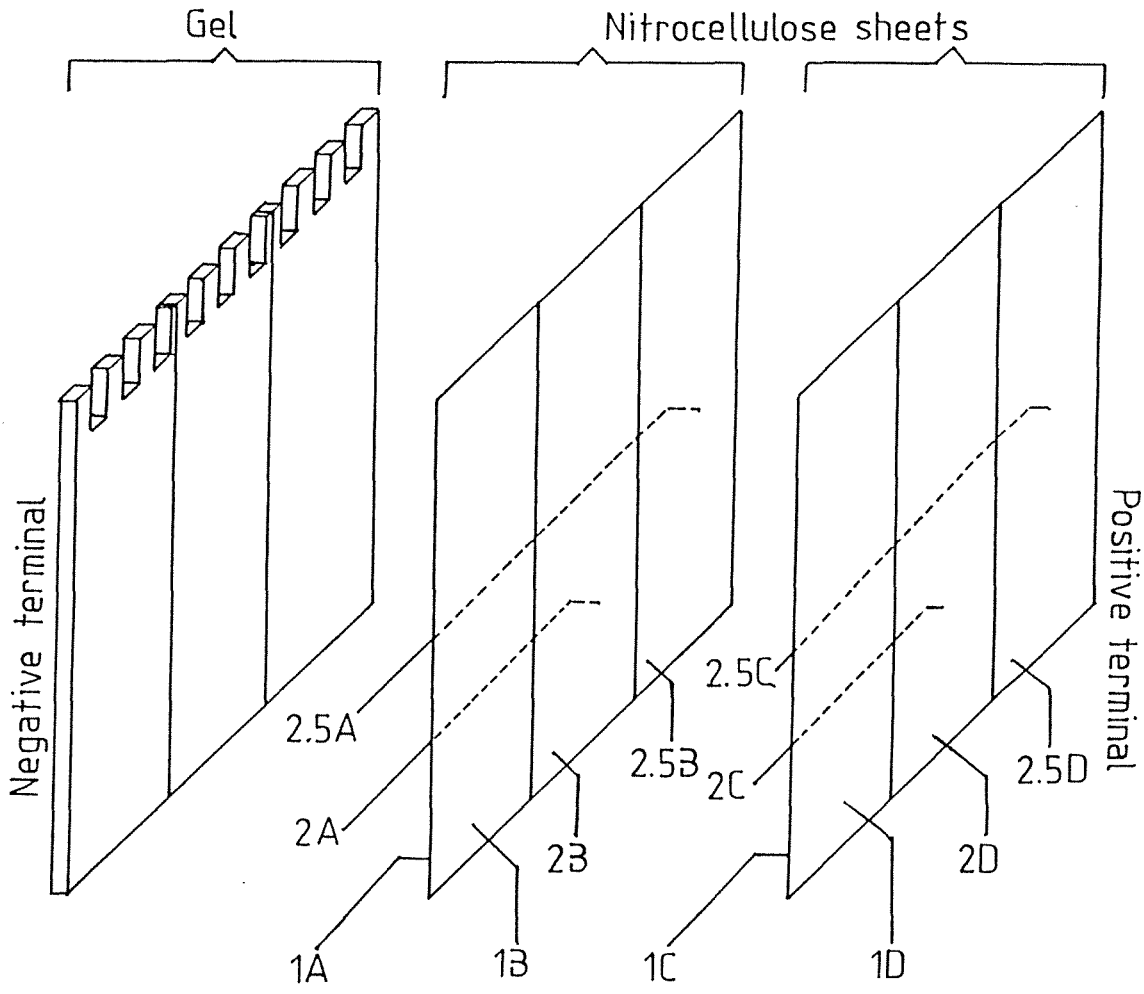


FIGURE 21: Arrangement of Gel and Nitrocellulose Sheets in the Transblotting Apparatus During Transblotting, for the Investigation of Transblotting Time on Protein Transfer on to Nitrocellulose

Labelling Code: 1, 2, 2.5 = transblotting time (hr)
A, B, C, D = side of nitrocellulose sheet

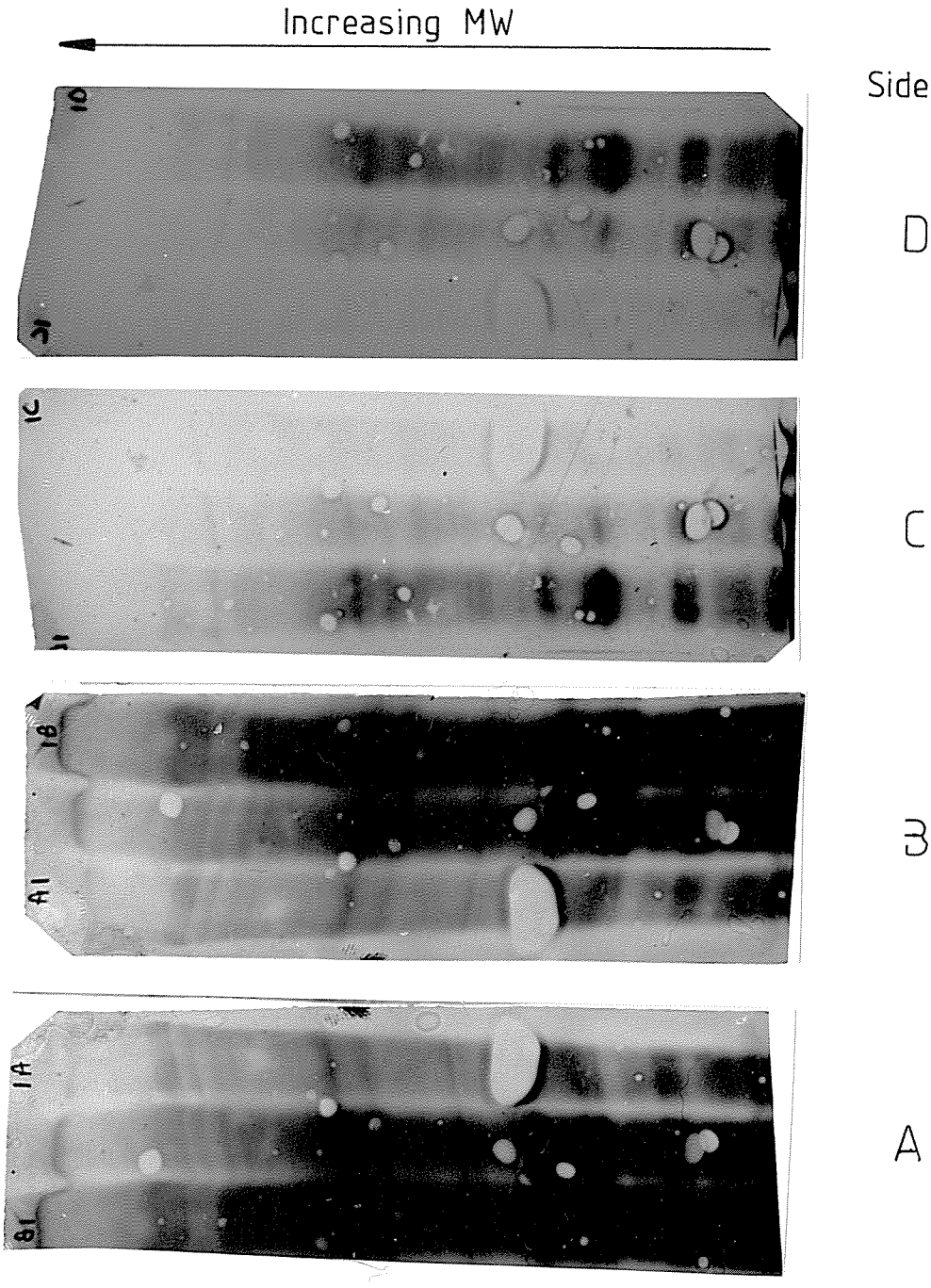


FIGURE 22: Proteins Transferred from a Polyacrylamide Gel to 2 Sheets of Nitrocellulose Paper

Nitrocellulose paper arranged as in Fig.21 contains proteins transferred from a polyacrylamide gel by transblotting for 1 hr (Refer to Section 4.7.3).
Proteins on the nitrocellulose were visualised using Amido Black 10B stain.

Under the electrophoretic conditions used, acetyl-CoA carboxylase migrates to a position approximately 20% of the distance migrated by the tracking dye. The most intense protein bands around this region of the gel were detected on the nitrocellulose sheet nearest the gel after transblotting for 1 hr (Fig.22, 1A and 1B). Only proteins of very high molecular weights (R_f<0.1), remained on the gel after transblotting for 1 hr.

Transblotting for 1 hr against 1 sheet of nitrocellulose appeared to provide the best conditions for transferring acetyl-CoA carboxylase from the polyacrylamide gels. However, when gels were transblotted for 1 hr against 2 sheets of nitrocellulose, followed by overnight probing with ^{35}SLR-streptavidin in probing buffer containing 2.5mM $MgCl_2$, only very faint bands could be detected on the film after autoradiography for 18 days.

4.8 Purification of Acetyl-CoA Carboxylase from Maize Leaf Tissue

During initial experiments, the acetyl-CoA carboxylase in maize leaves was partially purified using the first 4 steps of the purification procedure described by Nikolau and Hawke (1984) (Procedure I). A stepwise description of the procedure (Fig.23), is as follows: 1). homogenisation of the second and subsequently developed leaves of 9-day-old maize seedlings in 2 vol of homogenising buffer containing 0.1M Tris buffer at pH 8.0, 1mM EDTA and 20mM β-mercaptoethanol, followed by filtration through Miracloth (or nylon muslin cloth (mesh 125μ) when

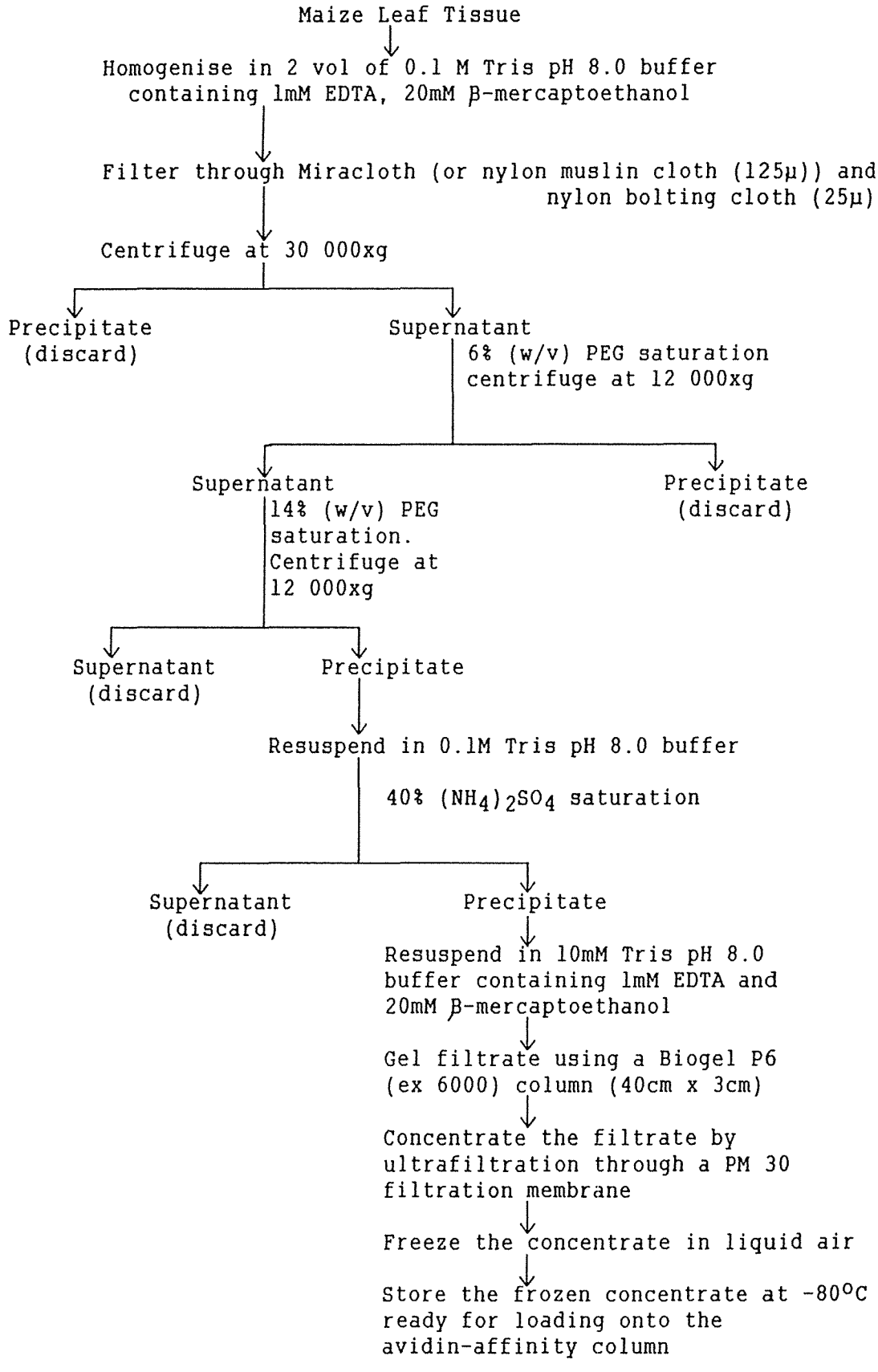


FIGURE 23: Purification of Maize Leaf Acetyl-CoA Carboxylase (Procedure I)

Procedure I as described in Section 4.8

Miracloth became unavailable) and nylon bolting cloth (mesh 25μ)

2). Removal of precipitated material by centrifugation at 30000xg for 30 min in a Sorvall RC2-B centrifuge.

3). Precipitation and fractionation of proteins in the supernatant by 6 to 14% polyethylene glycol (PEG) and solubilization of this precipitate, in 10mM Tris buffer at pH 8.0 containing 1mM EDTA and 20mM β-mercaptoethanol.

4). Precipitation of proteins with 40% ammonium sulphate, and solubilization of the precipitate in 10mM Tris buffer (Nikolau and Hawke, 1984). The ammonium sulphate was then removed from the redissolved precipitate by passage through a Biogel P6 (ex 6000) gel filtration column (3cm x 40cm), equilibrated with 10mM Tris buffer at pH 8.0, containing 1mM EDTA and 20mM β-mercaptoethanol. The volume of the sample for desalting was no more than 5% of the column volume, and activity was eluted in a single peak which coincided with a single protein peak. When required, the volume of the gel filtrate was reduced by ultrafiltration in an Amicon Ultrafiltration unit equipped with a PM 30 filtration membrane, under a pressure of 25-30 p.s.i. (Table VI).

After five attempts using this procedure, only 17 to 20% of the original acetyl-CoA carboxylase activity was retained in the 6-14% PEG precipitate at step 3, while on average, 60% of the original carboxylase activity remained in the 14% supernatant. Since Nikolau and Hawke (1984), had reported precipitation of 73% of the original carboxylase activity when using this precipitant during the isolation of maize leaf acetyl-CoA carboxylase, the precipitation of acetyl-CoA carboxylase was further investigated.

TABLE VI

PARTIAL PURIFICATION OF ACETYL-CoA CARBOXYLASE FROM MAIZE LEAVES USING PROCEDURE I

Fraction	Volume (ml)	Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification (fold)
Homogenate	210	4146.9	620.0	6.7	100	1
30000xg supernatant	200	4932.7	291.0	17.0	118.9	2.5
6-14% PEG	20	708.1	28.2	25.1	17.1	3.2
40% ammonium sulphate precipitate	5.8	383.0	17.4	22.0	9.2	3.3
Gel filtrate (a)	56	525.6	15.7	33.5	12.7	5.0
Ultrafiltration (b) (concentrate)	6	328.2	12.9	25.4	7.9	3.8

45g of leaf tissue used.

(a) Eluant from the Biogel P6 column.

(b) Concentration through the PM 30 filtration membrane.

4.8.1 Effect of Polyethylene Glycol on Precipitation of Maize Leaf Acetyl-CoA Carboxylase

The precipitation of acetyl-CoA carboxylase from maize by PEG was measured over the range of 0-50% PEG, in steps of 5% PEG. Figure 24 shows that the amount of acetyl-CoA carboxylase activity precipitated at each PEG concentration closely followed the amount of total protein precipitated, without any substantial enhancement of specific activity at any point. 35% of the original acetyl-CoA carboxylase activity remained in solution in the 50% PEG supernatant, which was the highest practical limit due to the high viscosity of 50% PEG. The fractions containing the highest specific activity were between 10 to 20% PEG, this is in contrast to the 6 to 14% PEG fractionation used by Nikolau and Hawke (1984). The recovery of carboxylase activity in the fractions between 5-15% PEG, which are those most similar to the fractionation used by Nikolau and Hawke (1984), was only 13%, which differs markedly from the 73% recovery reported by these workers. Although differing from the results of Nikolau and Hawke (1984), the results presented in this study suggest that PEG is not an ideal means of fractionating acetyl-CoA carboxylase.

The precipitating effects of PEG are affected by the ionic strength of the solution undergoing precipitation (Polson et al.,1964), therefore, it is possible that the broad nature of the acetyl-CoA carboxylase activity peak is due to the presence of 0.1M Tris in the homogenising buffer.

Not all of the acetyl-CoA carboxylase activity was precipitated

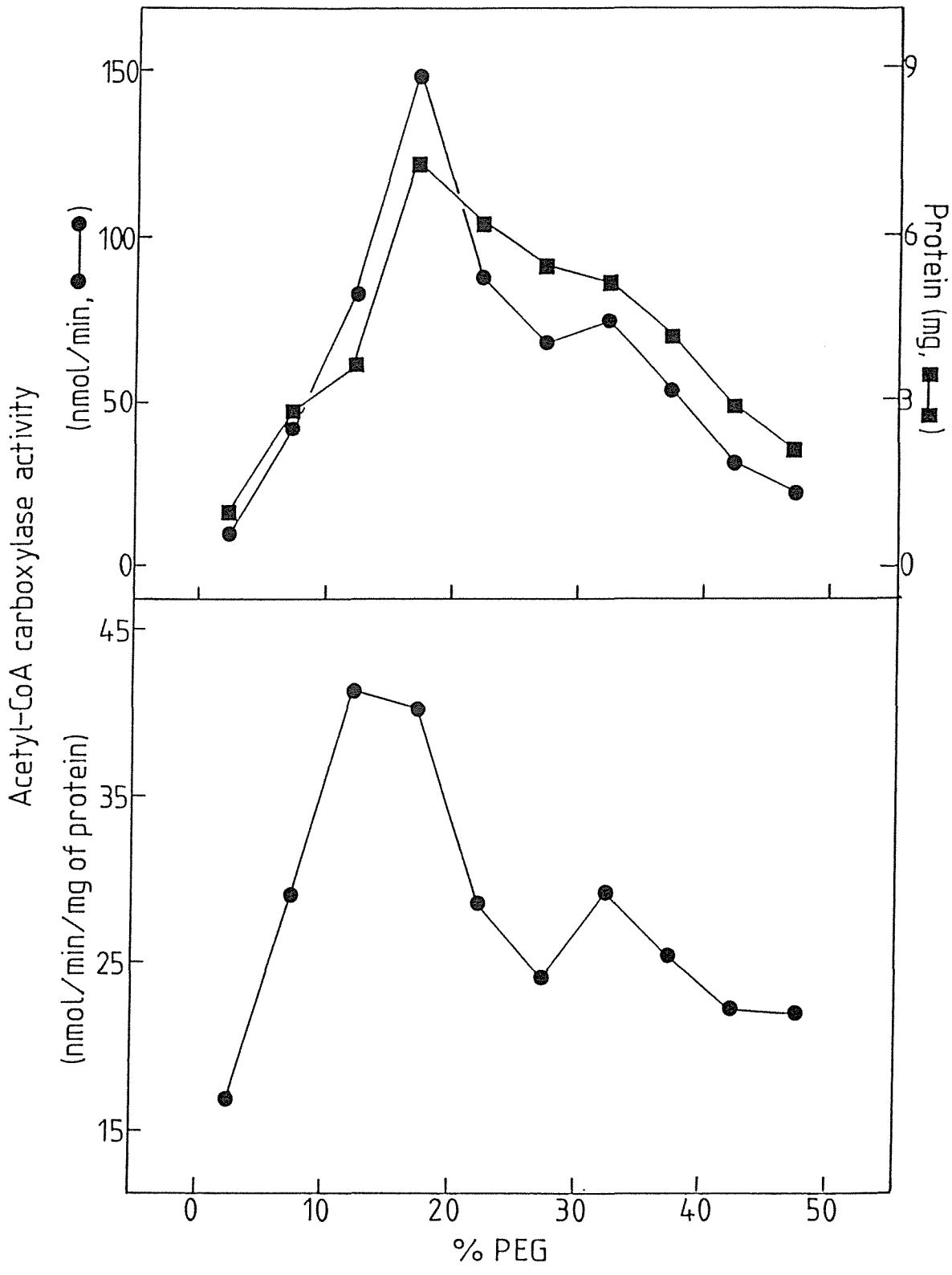


FIGURE 24: Precipitation of Acetyl-CoA Carboxylase and Protein by Polyethylene Glycol (PEG)

0-50% PEG was added in steps of 5% PEG to the 30000xg supernatants of maize leaf homogenates (Section 3.11) over a 20 min period, and allowed to equilibrate for 30 min, before centrifuging at 12000xg. Acetyl-CoA carboxylase activity and protein levels were determined in the precipitates after they had been resuspended in 10mM Tris buffer at pH 8.0.
74g of leaf tissue was used.

by 50% PEG, even though the peak of activity precipitated by 50% PEG appears to tail off towards 50% PEG. An explanation for this finding is that isoenzymes of acetyl-CoA carboxylase exist in maize leaf tissue, one isoenzyme being precipitated by 50% PEG and the other one remaining in solution. This evidence supports other suggestions of acetyl-CoA carboxylase isoenzymes existing in maize tissue (Nikolau et al., 1984).

Since PEG did not appear to be an ideal means of fractionating maize leaf acetyl-CoA carboxylase, alternative procedures were investigated.

4.8.2 Effect of Ammonium Sulphate on the Precipitation of Maize Leaf Acetyl-CoA Carboxylase

The precipitation of acetyl-CoA carboxylase activity and protein was determined in the range 0-60% ammonium sulphate, in stepwise increases of 5%, in a similar manner to that described for PEG above (Fig.25). All the carboxylase activity was precipitated by 60% ammonium sulphate, with 80% of the activity precipitated in 2 sharp peaks. The first peak occurred between 35 to 40% ammonium sulphate, and the second between 45 to 50% ammonium sulphate. A similar distribution of acetyl-CoA carboxylase activity was obtained in a second fractionation with ammonium sulphate. Confirmation that most of the acetyl-CoA carboxylase was precipitated at two distinct ammonium sulphate concentrations was obtained during a subsequent acetyl-CoA carboxylase purification, where relatively equal amounts of carboxylase activity were recovered from a 35 to 40% ammonium sulphate precipitate and supernatant. Precipitation of

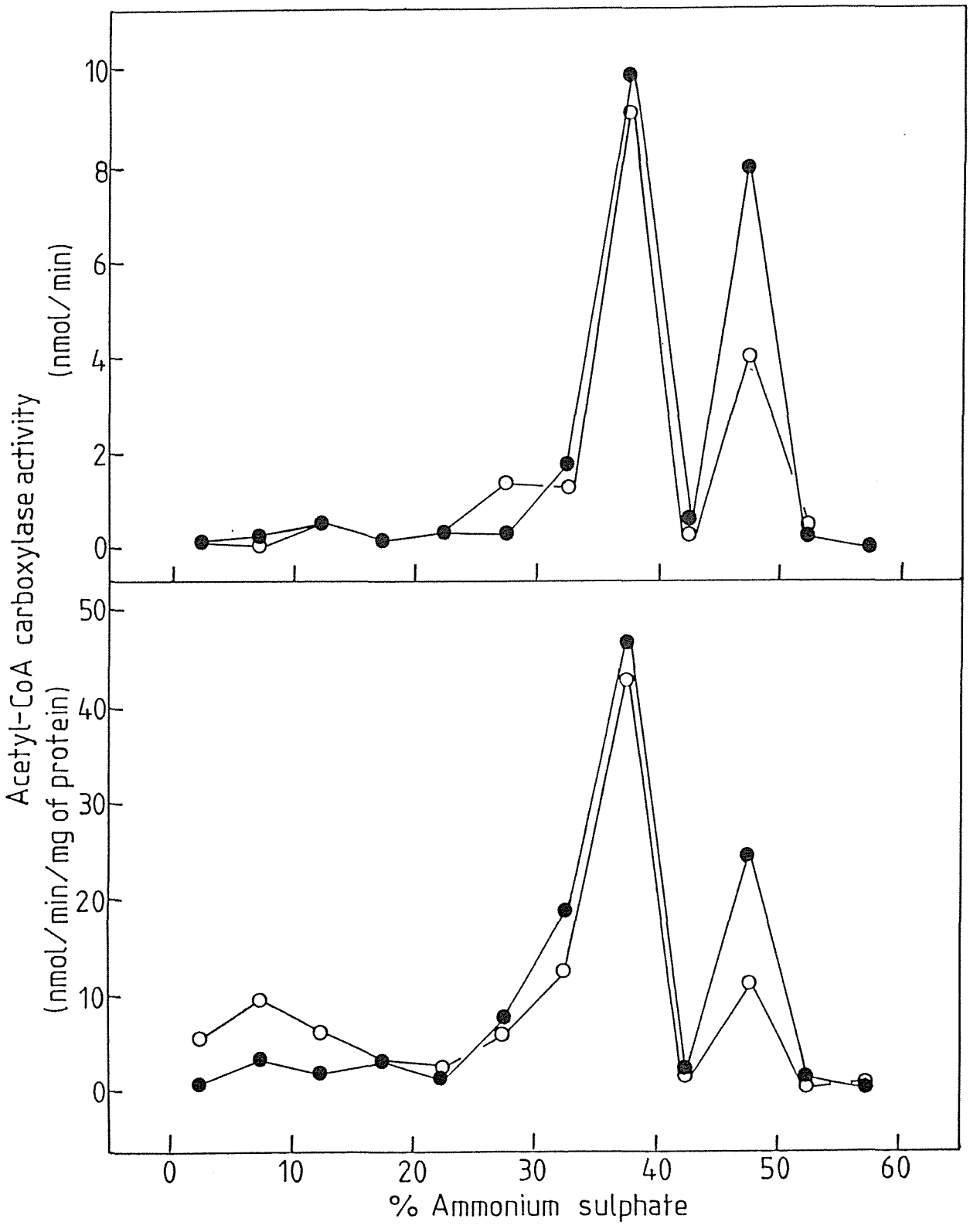


FIGURE 25: Precipitation of Acetyl-CoA Carboxylase and Protein by Ammonium Sulphate

0-60% Ammonium sulphate was added in steps of 5%, to the 30000xg supernatants of maize leaf homogenates (Section 3.11) over 20 min, and allowed to equilibrate for 30 min before centrifugenation at 12000xg. Precipitates were resuspended in 10mM Tris buffer at pH 8.0, and passed through a Biogel P6 column equilibrated with the same buffer. Acetyl-CoA carboxylase activity and protein levels were then determined.
20g of leaf tissue was used in experiment 1 (O), while 28g of leaf tissue was used in experiment 2 (●).

acetyl-CoA carboxylase at two distinct ammonium sulphate concentrations is consistent with the results obtained from PEG precipitation studies (Section 4.8.1), suggesting the existence of acetyl-CoA carboxylase isoenzymes in maize.

A 35 to 50% ammonium sulphate fractionation step was introduced into the purification procedure in order to precipitate as much of the acetyl-CoA carboxylase activity as possible. The specific activity of the 35 to 50% ammonium sulphate precipitate was 2.2-fold greater than that of the 30000xg supernatant, and contained on average 58% of the carboxylase activity of the 30000xg supernatants. In comparison, the specific activity of the 6-14% PEG precipitate was 1.5-fold greater than the 30000xg supernatants, and this fraction contained only 18% of the carboxylase activity.

A further problem with Procedure I which was encountered following PEG fractionation was the presence of green solids on the surface of the supernatant of the 40% ammonium sulphate precipitate. The supernatant, precipitate and the floating solid contained 7%, 48% and 45% respectively of the acetyl-CoA carboxylase activity, while the distribution of protein was 45%, 38% and 17% in the supernatant, precipitate and floating solid respectively. As the floating solid contained almost half the total carboxylase activity and only 17% of total protein, it was kept and pooled with the precipitate. However, it was very difficult to remove the supernatant without breaking up the floating solid. Even when using a Pasteur pipette to remove the supernatant it was difficult to keep the floating solid intact. No such green floating solid was encountered when ammonium

sulphate fractionation was carried out directly on the 30000xg supernatant (Procedure II).

Following these investigations, PEG fractionation was omitted and 40% ammonium sulphate precipitation replaced by a 35 to 50% ammonium sulphate fractionation (Procedure II) (Section 3.11) (Table VII).

Ammonium sulphate was then removed from the precipitate using a Biogel P6 gel filtration column (ex 6000)(3cm by 40cm), before the eluant was concentrated to approximately 10% of its volume using an Amicon Ultrafiltration unit equipped with a Diaflo PM 30 filtration membrane, and stored at -80^{o}C.

4.9 Purification of Maize Leaf Acetyl-CoA Carboxylase Using Avidin-Affinity Chromatography

Further purification of a partially purified acetyl-CoA carboxylase concentrate was attempted using avidin-affinity chromatography. 850nmol/min of acetyl-CoA carboxylase activity and 16mg of protein was loaded onto Column B (8cm by 1.8cm, with an exchangeable biotin binding capacity of 4 to 6nmol/ml of gel), at a flow rate of 6ml/hr. 140nmol/min of acetyl-CoA carboxylase activity, and 10mg of protein passed unbound through the column during loading. 27nmol/min of acetyl-CoA carboxylase activity, 3% of the activity loaded, was eluted after approximately 3 vol of Column Buffer (Section 3.3) containing 2mM biotin had passed through the column (Fig.26). No protein could be detected in eluted fractions containing carboxylase activity using Coomassie Blue. However, after this initially successful

TABLE VII

PARTIAL PURIFICATION OF ACETYL-CoA CARBOXYLASE FROM MAIZE LEAVES USING PROCEDURE II

Fraction	Volume (ml)	Activity (nmol/ min)	Protein (mg)	Specific Activity (nmol/min mg)	Recovery (%)	Purification (fold)
Homogenate	246	6532.2	720.8	9.1	100	1.0
30000xg supernatant	230	4685.0	450.8	10.4	71.7	1.1
35-50% Ammonium sulphate	13.8	1735.3	120.1	14.5	26.6	1.6
Gel filtrate (a)	77	2740.0	121.0	22.6	42.0	2.5
Ultrafil-tration (b) (concentrate)	6.6	2711.3	121.3	22.4	41.5	2.5

(a) Eluant from the Biogel P6 column.

(b) Filtrate concentrated through the PM 30 filtration membrane.

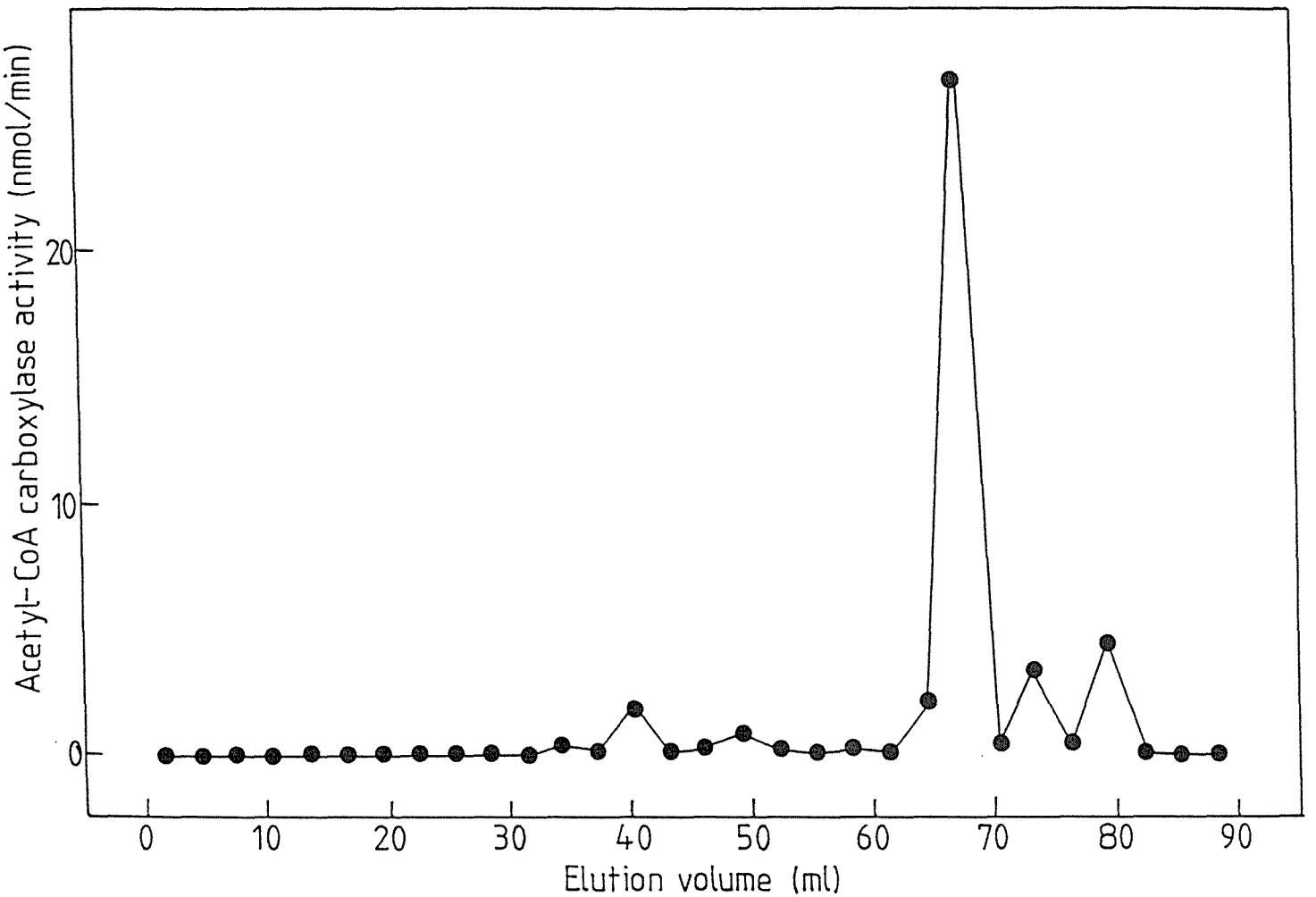


FIGURE 26: Elution Profile From Avidin-Affinity Column

Partially purified acetyl-CoA carboxylase (Procedure I) was loaded onto Column B at a flow rate of 6ml/hr. The graph shows the elution of acetyl-CoA carboxylase activity from the Column after washing with Column Buffer containing 2mM biotin. Protein levels were too low for detection using Coomassie Blue. Fraction size was 3ml.

attempt, acetyl-CoAcarboxylase could not be eluted from the column in an active state.

Failure to elute active acetyl-CoA carboxylase using avidin-affinity chromatography could have been due to: 1). failure of acetyl-CoA carboxylase to bind to the column, 2). acetyl-CoA carboxylase was binding irreversibly to column unless denaturing conditions were used, or, 3). the instability of the enzyme was such that all activity was lost before it could be eluted from the column. The interaction between acetyl-CoA carboxylase and the avidin-affinity chromatography was investigated in order to obtain information to improve the purification of acetyl-CoA carboxylase using avidin-affinity chromatography.

The performance of monomeric-avidin-sepharose with regard to the binding and exchange of biotin is affected very little by pH changes over the range of 5.5 to 9.0 (Henrikson et al., 1979), therefore pH effects were not investigated. However, the effects of ionic strength and column flow rates on the avidin-acetyl-CoA carboxylase interaction were investigated.

4.9.1 Effect of Ionic Strength and Flow Rate on the Binding of Acetyl-CoA Carboxylase to the Avidin-Affinity Column

Since KCl is included in the Column Buffer (usually 0.5M (Henrikson et al., 1979; Gravel et al., 1980; Tipper and Witters, 1982)) to prevent non-specific interactions between protein and the avidin-affinity column, the effect of varying KCl concentrations within the range of 0.2M to 1.0M on

acetyl-CoA carboxylase binding to the column was examined. Acetyl-CoA carboxylase activity could not be eluted from the column using any concentration of KCl. However, since workers routinely use 0.5M KCl in column buffers used for the purification of acetyl-CoA carboxylase from rat liver (Tipper and Witters, 1982) and parsley cell cultures (Egin-Buhler and Ebel, 1983) by avidin-affinity chromatography (Holland, personal communications), 0.5M KCl was included in the Column Buffer.

When Column B (8cm by 1.8cm) was run at 10ml/hr for the first 20ml and 30ml/hr thereafter, all the acetyl-CoA carboxylase activity and protein loaded onto the column was eluted in the break through volume (Table VIII). However, when the flow rate was reduced to 3ml/hr for the first 20ml, then 6ml/hr, only half the carboxylase activity, and 74% of the protein loaded, was eluted in the breakthrough volume, suggesting that at the slower flow rate acetyl-CoA carboxylase bound to the column. Even when acetyl-CoA carboxylase appeared to have bound to the avidin-affinity column, no activity could be eluted with Column Buffer containing 2mM biotin, and therefore the dependence on activity for detecting the presence of acetyl-CoA carboxylase was unsatisfactory. Since acetyl-CoA carboxylase is the only known biotin-containing enzyme in plant tissue, biotin-specific probing was used to detect the presence of acetyl-CoA carboxylase in column eluants. Since Column A was smaller than Column B and had a superior biotin-binding capacity, (25nmol of biotin bound per ml of gel compared to 4-6nmol of biotin bound per ml of gel), it was used for the following experiments. Column A was loaded with partially purified acetyl-CoA carboxylase (Procedure II) and washed with 20ml of Column Buffer

TABLE VIII

EFFECT OF FLOW RATE ON THE BINDING OF ACETYL-CoA CARBOXYLASE TO THE AVIDIN-AFFINITY COLUMN

	Flow rate	
	3ml/hr for the 1st 20ml then 6ml/hr	10ml/hr for the 1st 20ml then 30ml/hr
ACC activity loaded (nmol/min)	30.1	126.1
ACC activity in breakthrough volume (nmol/min)	15.1	127.1
Protein loaded (mg)	5.28	9.01
Protein in breakthrough volume (mg)	3.92	8.91

Partially purified acetyl-CoA carboxylase was loaded onto Column B (8cm by 1.8cm, with an exchangeable biotin binding capacity of 4 to 6nmol/ml of gel) which was run as described in Section 3.12 except that the column flow rates were those shown above. Carboxylase activity and protein were determined from all fractions and summated to give total carboxylase activity and protein in the breakthrough volume.
The same enzyme preparation was used to test both flow rates.

at 2 to 4ml/hr. Then the column was eluted with 30ml of Column Buffer containing 2mM biotin followed by 30ml of 0.1M glycine buffer at pH 2.0. 1ml fractions were collected throughout. The elution profile of biotin-containing proteins revealed 4 major peaks (Fig.27,28), however, since the levels of radioactivity present on the nitrocellulose were so low (maximum of 37cpm) the results are not quantitative. Protein was detected in peaks 1, 2 and 4 by Amido Black 10B protein stain (Fig.27).

Polyacrylamide gel electrophoresis of each peak showed that Peak 1 contained many proteins (results not shown), while Peak 2 contained only 2 major proteins (Fig.29), which had estimated molecular weights of 62 000 (Band 1) and 54 500 (Band 2) (Fig.30). Since a biotin-positive reaction was observed in these fractions and since the Band 1 and Band 2 proteins were eluted with 2mM biotin it is likely that at least one, if not both of these proteins are biotin-containing. The biotin-containing subunit of maize leaf acetyl-CoA carboxylase has a molecular weight of 60 000 to 62 000 (Nikolau and Hawke, 1984) so it is quite possible that the 62 000 dalton (Band 1) protein purified by avidin-affinity chromatography is the biotin-containing subunit of acetyl-CoA carboxylase. However the identity of the 54 500 dalton (Band 2) protein is less certain. No protein could be detected in Peak 3 by either Amido Black 10B protein staining or silver staining of SDS-15% polyacrylamide gels containing Peak 3 fractions, suggesting that this peak was probably an artifact of the ^{35}SLR-streptavidin probing procedure. An SDS-15% polyacrylamide gel of Peak 4 fractions showed that it contained 5 proteins (results not shown),

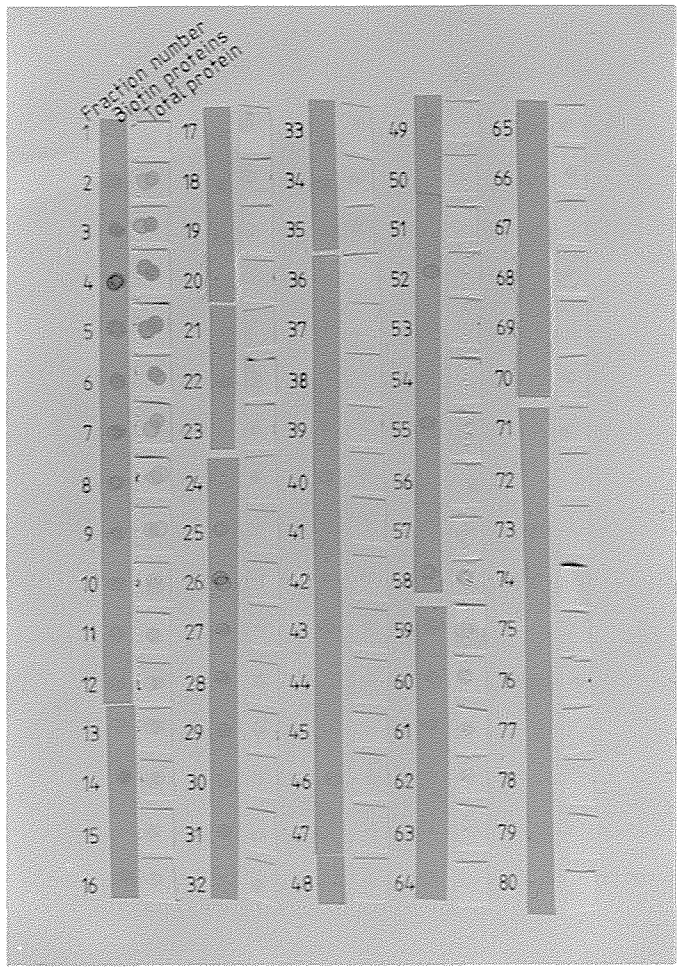


FIGURE 27: Autoradiograph of Biotin-Containing Protein and Total Protein Eluted from the Avidin-Affinity Column

140nmol/min of partially purified maize leaf acetyl-CoA carboxylase (Procedure II) was loaded onto Column A in 20ml of Column Buffer at 2-3ml/hr. The column was eluted with 30ml of Column Buffer containing 2mM biotin followed by 30ml of 0.1M glycine buffer at pH 2.0. 1ml fractions were collected throughout. Biotin-containing proteins were detected by spotting 10µl of each fraction onto nitrocellulose. The nitrocellulose was then blocked and probed as described in Section 3.9 and autoradiographed against hyperfilm βmax for 2 months. Total protein was detected by spotting 10µl of each fraction onto nitrocellulose and staining with Amido Black 10B as described in Section 3.9.1

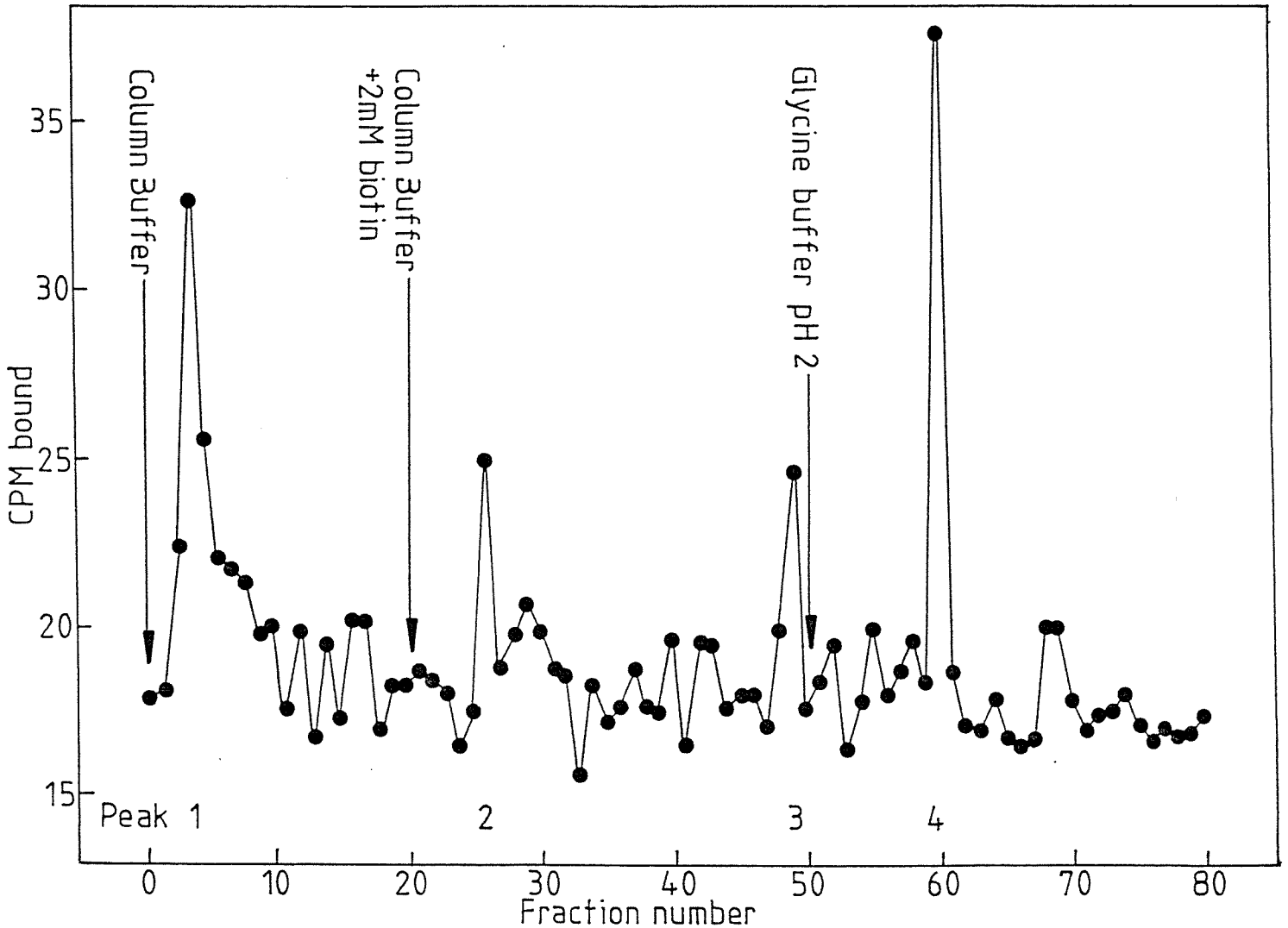


FIGURE 28: Elution of Biotin-Containing Protein from the Avidin-Affinity Column

After the nitrocellulose paper had been autoradiographed (Fig. 27), each area of sample application on nitrocellulose was cut out and the radioactivity determined in 5ml of toluene scintillation solvent (Section 3.1.4) by counting for 50 min. Values are accurate to ±6CPM.

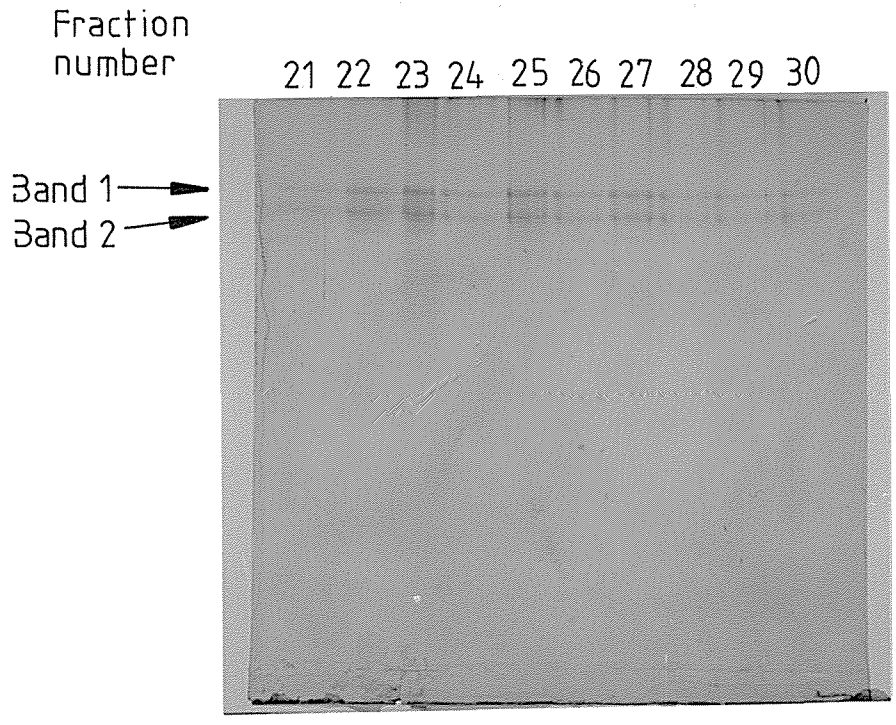


FIGURE 29: SDS-15% Polyacrylamide Gel of The Fractions Containing Peak 2

25µl of the first 10 fractions collected after eluting with Column Buffer containing 2mM biotin were loaded onto an SDS-15% poyacrylamide gel prior to electrophoresis as described in Section 3.7. The gel was then silver stained as described in Section 3.7.
Peak 2 corresponds to Fraction No. 26.

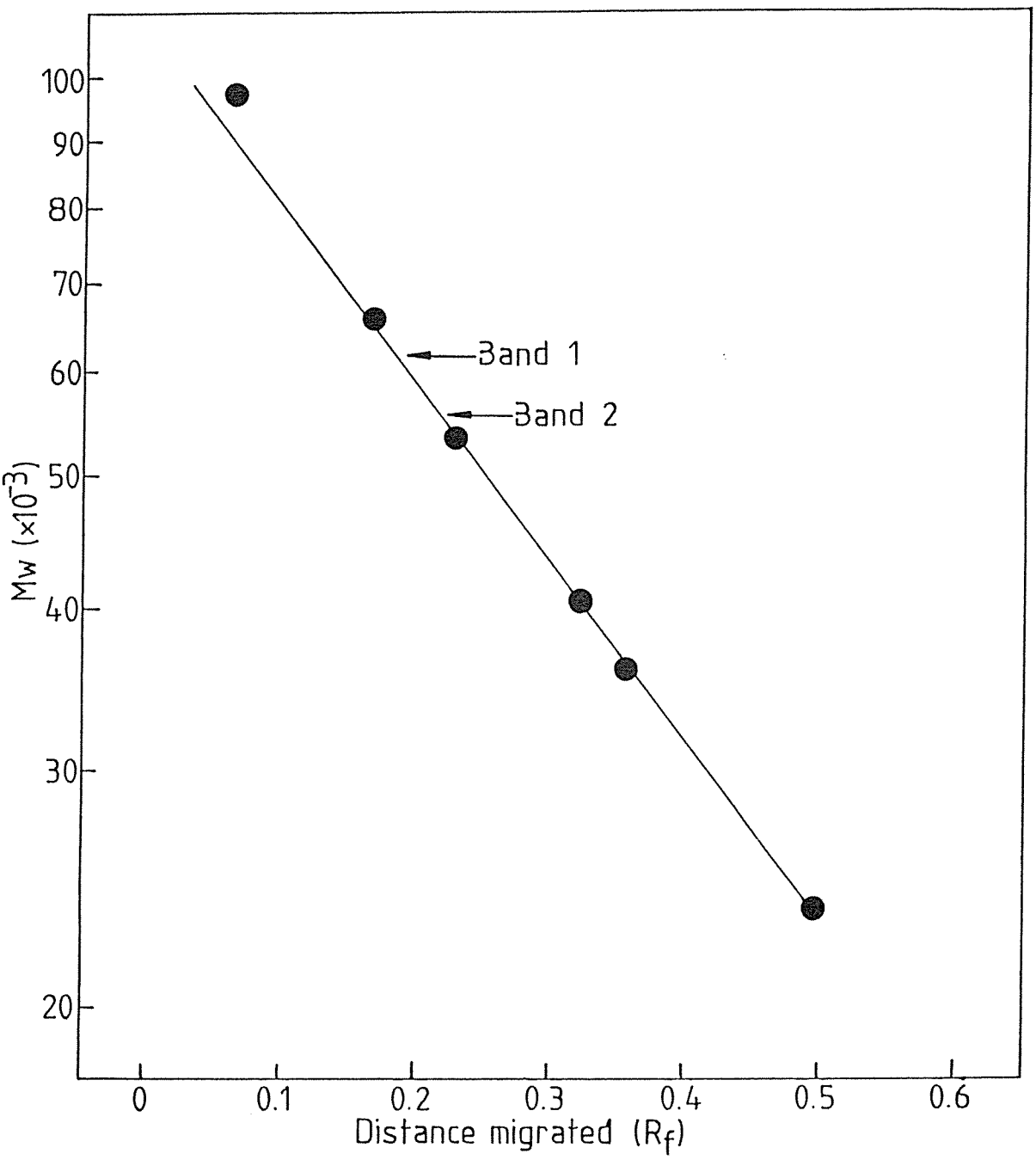


FIGURE 30: Estimation of the Molecular Weights of Protein Bands from 15% Polyacrylamide Gels

5µg of each protein mw marker:- phosphorylase B (97 400), Bovine serum albumin (66 000), glutamate dehydrogenase (53 000), Aldolase (40 000), glyceraldehyde 3-phosphate dehydrogenase (36 000) and trypsinogen (24 000), was prepared for electrophoreses as described in Section 3.7 before being loaded onto as SDS-15% polyacrylamide gel. Electrophoresis and staining with Coomassie Blue was as described in Section 3.7.

including the same Band 1 and Band 2 proteins eluted in Peak 2, and 3 other proteins in lesser amounts with approximate molecular weights of 19 000, 21 000, 28 000.

A similar multi-peaked elution profile was obtained by Guchhait et al. (1974), who used avidin-affinity chromatography to purify the biotin carboxyl carrier protein from E. coli. They found that biotin-containing proteins and carboxylase activity were eluted after each successive wash with column buffer containing 10mM biotin; 6M guanidine containing 50mM phosphate buffer at pH 7.0 and 1mM EDTA, and finally the same buffer at pH 2.0. They suggested that the multi-peaked elution profile was due to the presence of multiple forms of biotin-binding sites on the column. Although the avidin-sepharose prepared for this investigation had been exposed to biotin in order to irreversibly block the high affinity sites, it does not exclude the possibility of biotin-binding sites of differing affinities being present on the avidin-affinity column. Therefore, since the two eluants containing Band 1 and Band 2 proteins gave a biotin-positive reaction, it is feasible that the Band 1 and Band 2 proteins are biotin-containing, and that they are bound to biotin-binding sites with different biotin affinities. Consequently the protein bound to the lower affinity sites being eluted with Column Buffer containing 2mM biotin, and the protein bound to the higher affinity sites being eluted with 0.1M glycine buffer at pH 2.0.

4.10 Purification of Rat Liver Acetyl-CoA Carboxylase Using Avidin-Affinity Chromatography

Avidin-affinity chromatography has been successfully used to purify acetyl-CoA carboxylase from a number of animal tissues including rat adipocytes (Holland et al., 1985), rat liver (Holland et al., 1984; Tipper and Witters, 1982; Thampy and Wakil, 1985; Song and Kim, 1981) and chicken liver (Beaty and Lane, 1982). In view of the failure to purify maize leaf acetyl-CoA carboxylase using this method it was desirable to attempt to reproduce the procedure used with the rat liver enzyme. Rat liver acetyl-CoA carboxylase was partially purified 2.5-fold by conventional methods and then further purified using avidin-affinity Column A (Table IX). Major losses of acetyl-CoA carboxylase activity occurred at the PEG fractionation step, where 99% of acetyl-CoA carboxylase activity and 95% of the protein present in the homogenate was lost, and at the avidin-affinity chromatography step, where only 3% of the carboxylase activity and 0.1% of the protein loaded onto the column was recovered following elution with Column Buffer containing 2mM biotin.

Although the elution profile of biotin-containing proteins from the avidin-affinity column shows 4 peaks (Fig.32,A) only the first 2 peaks (Peaks $A^X$and B^X) can be seen on the autoradiograph of the elution profile (Fig.31), suggesting that Peaks C^X and D^X are artifacts. Acetyl-CoA carboxylase activity and protein were also eluted in Peaks A^X and B^X (Fig.32 B,C) Peak A^X contained many proteins as determined by gel electrophoreses including acetyl-CoA carboxylase as determined by the acetyl-CoA

TABLE IX

PURIFICATION OF ACETYL-CoA CARBOXYLASE FROM RAT LIVER

Fraction	Volume (ml)	Activity (nmol/ min)	Protein (mg)	Specific Activity (nmol/ min/mg)	Recovery (%)	Purification (fold)
Homogenate	190	5693.6	8431.3	0.68	100	1
3-5% PEG precipitate	4.8	48.53	119.4	0.41	0.85	0.61
20000xg supernatant	4.0	110.39	65.6	1.68	1.94	2.47
biotin eluant from avidin-affinity Column A	3.0	1.63	0.034	47.9	0.03	71

69g rat liver used.

(a) Half the 20000xg supernatant was loaded onto the column.

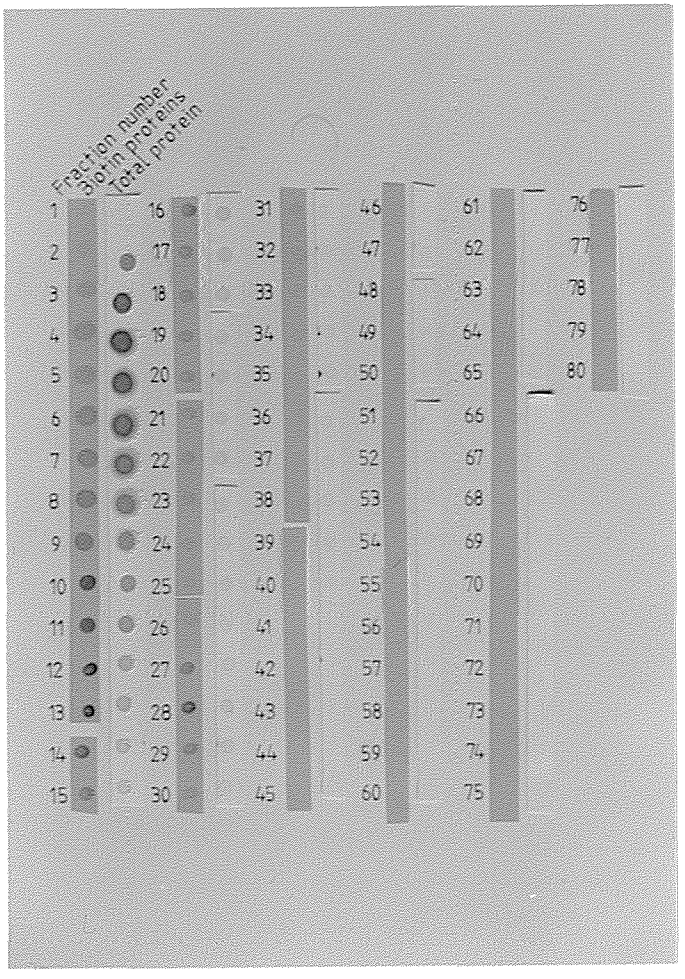


FIGURE 31: Biotin Containing Protein and Total Protein from Rat Liver, Present in the Eluted Fractions From the Avidin-Affinity Column

110nmol/min of partially purified rat liver acetyl-CoA carboxylase was loaded onto Column A in 22ml of Column Buffer at 2-3ml/hr. The column was eluted with 28ml of Column Buffer containing 2mM biotin followed by 30ml of 0.1M glycine buffer at pH 2.0. 1ml fractions were collected throughout. Biotin-containing proteins were detected by spotting 5µl of each fraction onto nitrocellulose, which was subsequently blocked and probed as described in Section 3.9. The nitrocellulose was then autoradiographed against Hyperfilm βmax for 21 days. Total protein was detected by spotting 5µl of each fraction onto nitrocellulose and staining with Amido Black 10B as described in Section 3.9.1

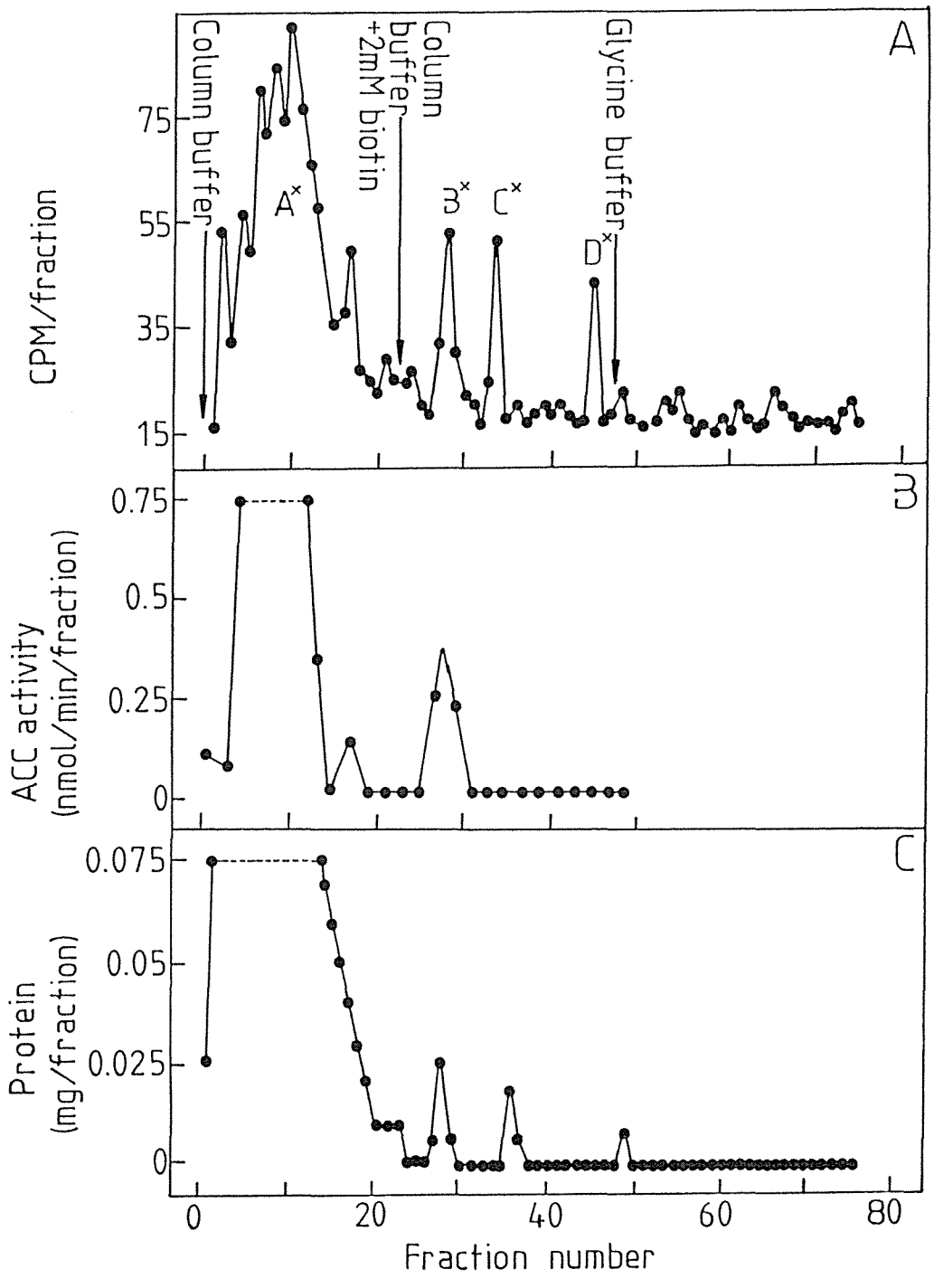


FIGURE 32: Elution of Acetyl-CoA Carboxylase Activity and Biotin-Containing Protein from the Avidin-Affinity Column

A) Biotin-containing proteins from rat liver eluted from the avidin-affinity column. After the nitrocellulose paper had been autoradiographed (Fig.31), each fraction spot on the nitrocellulose was cut out and radioactivity counted in 5ml of toluene scintillation solvent (Section 3.1.4).

B) Acetyl-CoA Carboxylase activity eluted from the avidin-affinity column assay conditions are those described in Section 3.4.1.

C) Protein eluted from the avidin-affinity column. Protein levels determined using Coomassie Blue (Section 3.1.1.).

carboxylase activity assay. Although the levels of carboxylase activity and protein present in Peak B^X fractions were at the limit of detection of the methods used, the evidence suggests that Peak B^X contained purified acetyl-CoA carboxylase, since acetyl-CoA carboxylase activity and proteins including biotin-containing proteins were detected in Peak B^X fractions.

An overall 71-fold increase in specific activity was achieved, with a 29-fold increase in specific activity at the avidin-affinity chromatography step, comparing favourably with the 10-fold increase in specific activity at this step reported by Song and Kim (1981). However, the total recovery of 0.03% of the original carboxylase activity compares very poorly to similar purifications carried out by other workers (Lent and Kim, 1982; Song and Kim, 1981). The reason for the poor recovery at the PEG fractionation step is unknown. However, the poor recovery at the avidin-affinity chromatography step was most likely due to the poor binding of acetyl-CoA carboxylase to the avidin-affinity column, which was in turn probably due to the column flow rate being too high when the 20 000xg supernatant was loaded.

Although the interaction between acetyl-CoA carboxylase, from both rat liver and maize leaves, and the avidin affinity column was poor, the evidence suggests that the rat liver enzyme binds to the column better that the maize enzyme. Support for a weaker interaction between avidin and maize acetyl-CoA carboxylase was obtained by incubating both rat liver and maize enzymes with 0.4 units of avidin for 30 min, prior to assay, resulting in 100% inhibition of rat liver acetyl-CoA carboxylase

activity and only 50% inhibition of maize acetyl-CoA carboxylase. Since the native tetrameric form of avidin binds more firmly to biotin-containing enzymes than the monomeric form, it is probable that the interaction between acetyl-CoA carboxylase and the monomeric-avidin affinity column will be much weaker than the interaction with tetrameric avidin. This is reflected by the poor binding of rat liver, and in particular, maize acetyl-CoA carboxylase to the avidin-affinity column.

CHAPTER 5

GENERAL DISCUSSION

Acetyl-CoA carboxylase is the key regulatory enzyme in the synthesis of fatty acids in animals (Kim, 1983). A similar role for acetyl-CoA carboxylase has been proposed for rape-seeds (Turnham and Northcote, 1983) and some photosynthetic tissues including spinach leaves (Nakamura and Yamada, 1979), maize leaves (Nikolau and Hawke, 1984) and wheat leaves (Hawke and Leech, 1987). Evidence provided in this study on acetyl-CoA carboxylase in developing maize leaves is consistent with this role.

In the present study, total acetyl-CoA carboxylase activity increased 8.5-fold with age in leaf tissue from 4 to 8-day-old maize seedlings, afterwhich, the activity appeared to remain relatively constant up to 11 days. Although the total activity increased between 4 and 8 days, the greater increase in tissue weight resulted in a 7.6-fold decrease in activity in a given weight of tissue. Specific activity was constant between 4 and 6 days, but decreased approximately 3-fold between 7 and 12 days. This decrease was most likely due to an increase in the levels of proteins associated with photosynthesis. Both total protein and chlorophyll levels increased in leaves from 4 to 11 days of age, but decreased slightly on day 12.

Leaves from maize seedlings contain a gradient of plastid and

cellular development with immature tissue at the leaf base and mature tissue at the leaf tip, therefore, sectioned leaves can be better used to investigate plastid development than whole leaves. In order to determine the regions of maximal fatty acid biosynthesis within the leaf, the acyl content of each leaf section was determined. Fatty acid levels increased most rapidly between the leaf base and 15mm along the leaf, and between 25mm and 60mm from the leaf base, with the later increase resulting from an increase in 18:3 levels in response to thylakoid membrane formation. Acetyl-CoA carboxylase activity also increased in these regions and the distribution of activity closely paralleled fatty acid levels throughout the leaf. This is consistent with acetyl-CoA carboxylase having a role in the regulation of the synthesis of fatty acids in maize leaves, and supports a similar finding in wheat leaves (Hawke and Leech, 1987). In a constant weight of tissue, fatty acids, proteins and acetyl-CoA carboxylase activity levels all decreased from the leaf base to 25mm along the leaf, the decrease being most likely due to cell elongation occurring in this region of the leaf. From approximately 30mm from the leaf base to the leaf tip all three biochemical parameters subsequently increased. The increase in protein levels is most likely due to increased levels of photosynthesis associated proteins. While the increase in fatty acid levels, were most likely, due to an increased requirement for fatty acids for thylakoid membrane formation. The increase in acetyl-CoA carboxylase activity was in response to the increased demand for fatty acids.

In 1978, Nielsen reported a 2.4-fold increase in the specific

activity of acetyl-CoA carboxylase upon illumination of etiolated barley seedlings over a 12 hr period, suggesting a light-induced activation of acetyl-CoA carboxylase. Eastwell and Stumpf (1983), suggested acetyl-CoA carboxylase as the site of regulation of fatty acid synthesis in the chloroplasts of spinach and Swiss chard, and that regulation of acetyl-CoA carboxylase was via light-dependent changes in adenylate levels in the chloroplast. Nikolau and Hawke (1984), reported, on the basis of separate catalytic effects of pH and ATP, ADP and Mg^{2+}, a possible 24-fold increase in acetyl-CoA carboxylase activity from the physiological changes in the concentration of these metabolites in the chloroplast stroma in response to dark-light transitions. While Hawke and Leech (1987), reported a 10-fold increase in wheat acetyl-CoA carboxylase activity from the least favourable to most favourable physiological concentrations of these metabolites, indicating that acetyl-CoA carboxylase in wheat leaves is regulated by light-dependent changes in stromal pH and concentrations of ATP, ADP and Mg^{2+}. In this study a 5-fold increase in carboxylase activity occurred between the least favourable concentrations of H^{+}, ATP, ADP and Mg^{2+} to the most favourable, that occur in the chloroplast stroma during dark-light transitions. This increase was less than the possible 24-fold increase suggested by Nikolau and Hawke (1984), indicating that the effect of each light-dependent change on acetyl-CoA carboxylase activity was not cummulative. The 5-fold increase in activity confirms the light-dependence of acetyl-CoA carboxylase in maize, previously found in wheat (Hawke and Leech, 1987).

Therefore it is likely that the light-dependence of fatty acid

synthesis is mediated by acetyl-CoA carboxylase via light-dependent changes in stromal pH and concentrations of ATP, ADP and Mg^{2+}.

It has been suggested that factors affecting thylakoid membrane formation, such as light, may also control acetyl-CoA carboxylase activity (Nielsen, 1978), and evidence presented in this study is consistent with this suggestion. Therefore if the requirement for fatty acids, and in particular 18:3, for thylakoid membrane formation was the limiting factor of plastid development, it could be expected that light, and therefore acetyl-CoA carboxylase, would control plastid development. This is consistent with the presence of etioplasts in etiolated tissue, which lack thylakoid membranes, but develop into mature chloroplasts upon illumination (Leech and Baker, 1983).

Chloroplastic pyruvate dehydrogenase complex, like acetyl-CoA carboxylase, has a sharp pH optimum at 8.0, and a requirement for Mg^{2+}, and is thought to be regulated by light (Camp and Randall, 1985), in a similar manner to that proposed for acetyl-CoA carboxylase (Nikolau and Hawke, 1984; Hawke and Leech, 1987). It would appear that illumination leads to a co-ordinated increase in acetyl-CoA formation from pyruvate and acetyl-CoA carboxylase activity. Both of which are required for the observed increase in fatty acid synthesis when leaf tissue or chloroplasts are transferred from dark to light conditions.

Acetyl-CoA carboxylase has been purified using conventional methods from several different plant sources, including wheat germ (Heinstein and Stumpf, 1969; Nielsen et al., 1979;

Egin-Bühler et al., 1980), avocado and spinach plastids (Mohan and Kekwick, 1980), and parsley cell cultures (Egin-Bühler et al., 1980), while avidin-affinity chromatography has been used to purify acetyl-CoA carboxylase from parsley cell cultures (Egin-Bühler and Ebel, 1983) and rape seed (Slabas and Hellyer, 1985). Acetyl-CoA carboxylase was first purified from maize leaves by Nikolau and Hawke (1984), and Procedure I used here consisted of the first 4 steps of this purification procedure. However, the yield of acetyl-CoA carboxylase activity after the PEG fractionation reported by Nikolau and Hawke (1984), could not be achieved in this study. The inclusion of the PEG fractionation led to the formation of a low density fraction in the subsequent precipitation with ammonium sulphate which floated on the supernatant. This floating solid contained appreciable acetyl-CoA carboxylase activity and may have resulted from the aggregation of lipid. This low density fraction was not formed when PEG fractionation was omitted. The 35 to 50% ammonium sulphate fraction (Procedure II) yielded acetyl-CoA carboxylase with a specific activity 2 times less than that achieved with Procedure I, but the yield was 5 times greater. Since the final step in purification involved avidin-affinity chromatography, it was considered that a greater yield in the preliminary fraction was of more importance than a higher purification, therefore the PEG fractionation was omitted.

In this study, purification of acetyl-CoA carboxylase using avidin-affinity chromatography was attempted, however, after one apparently successful attempt, acetyl-CoA carboxylase could not be eluted from the column in an active state. Failure to elute

active enzyme from the column could be ascribed to either 1). acetyl-CoA carboxylase not binding to the column, 2). the enzyme binding so tightly, it could only be eluted under denaturing conditions, or, 3). the instability of the enzyme was such that all activity was lost before the enzyme could be eluted. The column flow rate was found to be important in facilitating the binding of acetyl-CoA carboxylase to the avidin-affinity column. When flow rates of 2-4ml/hr, depending on column size (Column A (3.7cm by 1.6cm) was run at approximately 2ml/hr while Column B (8cm by 1.8cm) was run at approximately 4ml/hr), were used, biotin-containing proteins were found to bind to the column and were eluted with 2mM biotin or 0.1M glycine buffer at pH 2.0. SDS-gel electrophoresis of eluted fractions showed two proteins of molecular weights 62 000 and 54 500 were present in both eluants. Since both proteins were eluted with biotin, and biotin-containing proteins were present in this eluant, it is likely that the 62 000 dalton protein is the 60 000-62 000 dalton biotin containing subunit of maize acetyl-CoA carboxylase (Nikolau and Hawke, 1984). The identity of the 54 500 dalton protein is less certain. These two proteins were also eluted with 0.1M glycine buffer at pH 2.0, probably from high affinity biotin-binding sites on the column, along with three other proteins which were probably non-specifically bound to the column.

Although these results suggest that acetyl-CoA carboxylase from maize can be purified using avidin-affinity chromatography, no carboxylase activity could be found in the biotin and glycine eluants. This lack of activity is most likely due to the instability of the enzyme and a study of conditions that

increase stability may improve purification of acetyl-CoA carboxylase using avidin-affinity chromatography. Investigation of the monomeric avidin-acetyl-CoA carboxylase interaction may also lead to an improved performance of the column.

It has been suggested that isoenzymes of acetyl-CoA carboxylase exist in maize tissue (Nikolau et al., 1984). This study provides evidence that is consistent with this suggestion, since two peaks containing acetyl-CoA carboxylase activity were obtained during ammonium sulphate fractionation, and only 65% of activity undergoing PEG fractionation was precipitated by 50% PEG, while the remaining activity was present in the supernatant.

Further investigation into the failure of western blotting analysis would be particularly useful. Initial results suggested that acetyl-CoA carboxylase bound poorly to nitrocellulose paper under the conditions used. Therefore investigation of the binding of acetyl-CoA carboxylase to other membranes may lead to improved sensitivity of western blotting analysis. Zeta-bind is a nylon membrane which is positively charged and should therefore bind the negatively charged proteins more tightly, resulting in a proposed 4-fold greater binding capacity than nitrocellulose (Gershoni and Palade, 1982).

Many of the problems encountered in the purification of acetyl-CoA carboxylase from maize would seem to be related to the instability of the enzyme. Therefore conditions that stabilize the enzyme must be found in order to facilitate a successful purification.

BIBLIOGRAPHY

Ahmad, F., Ahmad, P.M., Pieretti, L. and Watters, G.I. (1978). J. Biol. Chem. 253, 1733-1737.

Appelqvist, L.A., Stumpf, P.K. and Von Wettstein, D. (1968). Plant Physiol. 43, 163-187.

Arnon, D.I. (1949). Plant Physiol. 24, 1-15.

Beaty, N.B. and Lane, M.D. (1982). J. Biol. Chem. 257, 924-929.

Brock, K. and Kannangara, C.G. (1976). Carlsberg Res. Commun. 41, 121-129.

Brooks, J.L. and Stumpf, P.K. (1965). Biochim. Biophys. Acta 98, 213-216.

Brooks, J.L. and Stumpf, P.K. (1966). Arch Biochem. Biophys. 116, 108-116.

Brownsey, R.W., Hughes, W.A. and Denton, R.M. (1979). Biochem. J. 184, 23-32.

Camp, P.J. and Randall, D.D. (1985). Plant Physiol. 77, 571-577.

Carlson, C.A. and Kim, K-H. (1973). J. Biol. Chem. 248, 378-380.

Carlson, C.A. and Kim, K-H. (1974). Arch. Biochem. Biophys. 164, 478-489.

Cashel, M. and Gallant, J. (1969). Nature 221, 838-841.

Chen, S-L. and Kim, K-H. (1982). J. Biol. Chem. 257, 9953-9957.

Dean, C. and Leech, R.M. (1982) Plant Physiol. 69, 904-910.

Douce, R. and Joyard, J. (1980). in Biochemistry of Plants, A Comprehensive Treatise, (Stumpf, P.K. ed.), Vol. 4, pp 321-362, Academic Press, New York.

Eastwell, K.C. and Stumpf, P.K. (1983). Plant Physiol. 72, 50-55.

Egin-Bühler, B. and Ebel, J. (1983). Eur. J. Biochem. 133, 335-339.

Egin-Bühler, B., Loyal, R. and Ebel, J. (1980). Arch. Biochem. Biophys. 203, 90-100.

Farrah, S.R., Shah, D.O. and Ingram, L.O. (1981) Poc. Nat. Acad. Sci. U.S.A 78, 1229-1232.

Gardner, H.W. (1968). J. Lipid Res. 9, 139-141.

Gershoni, J.M. and Palade, G.E. (1982). Anal. Biochem. 124, 396-405.

Gershoni, J.M. and Palade, G.E. (1983). Anal. Biochem. 131, 1-15.

Goodridge, A.G. (1972). J. Biol. Chem. 247, 6946-6952.

Gravel, R.A., Lam, K.F., Mahuran, D. and Kronis, A. (1980). Arch. Biochem. Biophys. 201, 669-673.

Green, N.M. and Toms, E.J. (1973). Biochem. J. 133, 687-700.

Gregolin, C., Ryder, E., Kleinschmidt, A.K., Warner, R.C. and Lane, M.D. (1966a). Proc. Natl. Acad. Sci. U.S.A. 56, 148-155.

Gregolin, C., Ryder, E. and Lane, M.D. (1968a) J. Biol. Chem. 243, 4227-4235.

Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K., Chang, H-C. and Lane, M.D. (1968b). J. Biol. Chem. 243, 4236-4245.

Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K. and Lane, M.D. (1966b). Proc. Natl. Acad. Sci. U.S.A. 56, 1751-1758.

Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D. (1974). J. Biol. Chem. 249, 6633-6645.

Hampp, R., Goller, M. and Ziegler, H. (1982). Plant Physiol. 69, 448-455.

Hardie, D.G. and Cohen, P. (1978). FEBS Lett. 91, 1-7.

Hardie, D.G. and Cohen, P. (1979). FEBS Lett. 103, 333-338.

Hardie, D.G. and Guy, P.S. (1980). Eur. J. Biochem. 110, 167-177.

Hashimoto, T.H. and Numa, S. (1971). Eur. J. Biochem. 18, 319-331.

Hatch, M.D. and Stumpf, P.K. (1961). J. Biol. Chem. 236, 2879-2885.

Hawke, J.C. and Leech, R.M. (1987). Planta 171, 489-495.

Hawke, J.C., Rumsby, M.G. and Leech, R.M. (1974a). Plant Physiol. 53, 555-561.

Hawke, J.C., Rumsby, M.G. and Leech, R.M. (1974b). Phytochem. 13, 403-413.

Heinstein, P.F. and Stumpf, P.K. (1969). J. Biol. Chem. 244, 5374-5381.

Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973). Biochim. Biophys. Acta. 314, 244-241.

Henrikson, K.P., Allen, S.H.G. and Maloy, W.L. (1979). Anal. Biochem. 94, 366-370.

Hind, G., Nakatani, N.Y. and Izawa, S. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 1484-1488.

Hoagland, D.R. and Arnon, D.I. (1938). Calif. Agric. Exp. Stn. Circ. 347, 1-39.

Holland, R., Witters, L.A. and Hardie, D.G. (1984). Eur. J. Biochem. 140, 325-333.

Holland, R., Hardie, D.G., Clegg, R.A and Zammit, V.A. (1985). Biochem. J. 226, 139-145.

Kannangara, C.G. and Stumpf, P.K. (1972). Arch. Biochem. Biophys. 152, 83-91.

Kim, K-H. (1983). Curr. Top. Cell. Reg. 22, 143-176.

Knappe, J., Schlegel, H.G. and Lynen, F. (1961a). Biochem. Z. 335, 101-122.

Knappe, J., Ringelmann, E. and Lynen, F. (1961b). Biochem. Z. 335, 168-176.

Kobayashi, Y., Inoue, Y., Furuya, F., Shibata, K. and Heber, U. (1979). Planta 147, 69-75.

Kosow, D.P., Huang, S.C. and Lane, M.D. (1962). J. Biol. Chem. 237. 3633-3639.

Lane, M.D., Moss, J. and Polakis, S.E. (1974). Curr. Top. Cell. Reg. 8, 139-195.

Lee, K-H. and Kim, K-H. (1979). J. Biol. Chem. 254, 1450-1453.

Leech, R.M. and Baker, N.R. (1983). in The Growth and Functioning of Leaves pp 271-307, Dole, J.E., Milthorpe, F.L., eds., Cambridge University Press U.K.

Leech, R.M., Rumsby, M.G. and Thomson, W.W. (1973). Plant Physiol. 52, 240-245.

Leese, B.M. and Leech, R.M. (1976). Plant Physiol. 57, 789-794.

Lent, B.A. and Kim, K-H. (1982). J. Biol. Chem. 257, 1897-1901.

Lent, B.A., Lee, K-H. and Kim, K-H. (1978). J. Biol. Chem. 253, 8149-8156.

Lynen, F., Knappe, J., Larch, E., Jutting, G., Ringelmann, E. and Lachance, J.P. (1961). Biochem. Z. 335, 123-167.

Meyer, H., Nevaldine, B. and Meyer, F. (1978). Biochemistry 17, 1822-1827.

Mohan, S.B. and Kekwick, R.G.O. (1980). Biochem. J. 187, 667-676.

Moss, J. and Lane, M.D. (1971). Adv. in Enzymol. 35, 321-442.

Moss, J. Yamagishi, M., Kleinschmidt, A.K. and Lane, M.D. (1972). Biochemistry 11, 3779-3786.

Murphy, D.J. and Stumpf, P.K. (1981). Arch. Biochem. Biophys. 212, 730-739.

Nakamura, Y. and Yamada, M. (1979). Plant Sci. Lett. 14, 291-295.

Nakanishi, S. and Numa, S. (1970). Eur. J. Biochem. 16, 161-173.

Neidhardt, F.C. (1966). Bacteriol. Rev. 30, 701-719.

Nielsen, N.C. (1978). in Chloroplast Development pp 315-322, Akoyunoglou, G. and Argyroudi-Akoyunoglou, J.H. eds., Elsevier/North-Holland Biomedical Press.

Nielsen, N.C., Adee, A. and Stumpf, P.K. (1979). Arch. Biochem. Biophys. 192, 446-456.

Nielsen, N.C. and Stumpf, P.K. (1976). Biochem. Biophys. Res. Commun. 68, 205-210.

Nikolau, B.J. (1981). Ph.D. Thesis, Massey University.

Nikolau, B.J. and Hawke, J.C. (1984). Arch. Biochem. Biophys. 228, 86-94.

Nikolau, B.J., Hawke, J.C. and Slack, C.R. (1981). Arch. Biochem. Biophys. 211, 605-612.

Nikolau, B.J., Wurtele, E.S. and Stumpf, P.K. (1984). Plant Physiol. 75, 895-901.

Nikolau, B.J., Wurtele, E.S. and Stumpf, P.K. (1985). Anal. Biochem. 149, 448-453.

Numa, S., Bortz, W.M. and Lynen, F. (1964). Adv. Enz. Reg. 3, 407-423.

Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980). Anal. Biochem. 105, 361-363.

Ogiwara, H., Tanabe, T., Nikawa, J. and Numa, S. (1978). Eur. J. Biochem. 89, 33-41.

Ongun, A., Thomson, W.W. and Mudd, J.B. (1968). J. Lipid. Res. 9, 409-415.

Polakis, S.E., Guchhait, R.B. and Lane, M.D. (1973). J. Biol. Chem. 248, 7957-7966.

Polson, A., Potgieter, G.M., Largier, J.F., Mears, G.E.F and Joubert, F.J. (1964). Biochim. Biophys. Acta 82, 463-475.

Portis, Jr. A.R. (1981). Plant Physiol. 67, 985-989.

Portis, Jr. A.R., and Heldt, H.W. (1976). Biochim. Biophys. Acta 449, 434-446.

Read, S.M. and Northcote, D.H. (1981). Anal. Biochem. 116, 53-64.

Reitzel, E. and Nielsen, N.C. (1976). Eur. J. Biochem. 65, 131-138.

Roughan, P.G. (1970). Biochem. J. 117, 1-8.

Roughan, P.G. and Batt, R.D. (1969). Phytochemistry 8, 363-369.

Sastry, P.S. and Kates, M. (1964). Biochemistry 3, 1271-1280.

Schneider, Z. (1980). Anal. Biochem. 108, 96-103.

Seulter, C.H. (1970). Science 168, 789-795.

Shiao, M.S., Drong, R.F. and Porter, J.W. (1981). Biochem. Biophys. Res. Commun. 98, 80-87.

Slabas, A.R. and Hellyer, A. (1985). Plant Science 39, 177-182.

Song, C-S. and Kim, K-H. (1981). J. Biol. Chem. 256, 7786-7788.

Stadtman, E.R. (1957). in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds), vol.3, pp 931-941, Academic Press, New York.

Stitt, M., M^{c}C Lilley, R. and Heldt, H.W. (1982). Plant Physiol. 70, 971-977.

Stumpf, P.K. (1980). in Biochemistry of Plants, A Comprehensive Treatise, (Stumpf, P.K. ed), vol. 4, pp 177-204, Academic Press, New York.

Thampy, K.G. and Wakil, S.J. (1985). J. Biol. Chem. 260, 6318-6323.

Tipper, J.P. and Witters, L.A. (1982). Biochim. Biophys. Acta 715, 162-169.

Turnham, E. and Northcote, D.H. (1983). Biochem. J. 212, 223-229.

Waite, M. and Wakil, S.J. (1966). J. Biol. Chem. 241, 1909-1914.

Wakil, S.J. and Gibson, D.M. (1960). Biochim. Biophys. Acta. 41, 122-129

Wakil, S.J., Stoops, J.K. and Joshi, V.C. (1983). Ann. Rev. Biochem. 52, 537-579.

Werdan, K., Heldt, H.W. and Milovancev, M. (1975). Biochim. Biophys. Acta 396, 276-292.

Wintermanns, J.F.G.M. (1960). Biochim. Biophys. Acta 44, 49-54.

Yeh, L-A., Lee, K-H. and Kim, K-H. (1980). J.Biol. Chem. 255, 2308-2314.

Yeh, L-A. and Kim, K-H. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 3351-3355.

Yeh, L-A., Song, C-S. and Kim, K-H. (1981). J. Biol. Chem. 256, 2289-2296.