ACETYL-CoA CARBOXYLASE IN PHOTOSYNTHETIC TISSUE

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ABSTRACT

Acetyl-CoA carboxylase catalyses the rate-limiting reaction in de novo fatty acid biosynthesis in a wide variety of organisms. In plants however, the significance of this enzyme in regulating de novo fatty acid biosynthesis is unknown.

In this investigation acetyl-CoA carboxylase of maize, barley and spinach leaves has been studied in order to compare some features of this enzyme in the three plants. In maize, acetyl-CoA carboxylase is located in chloroplasts, where it occurs as a soluble enzyme in the stromal fraction. Both mesophyll and bundle sheath cells also exhibit acetyl-CoA carboxylase activity. Activities of acetyl-CoA carboxylase in isolated chloroplasts have been compared to rates of lipid synthesis from acetate in order to investigate the role of acetyl-CoA carboxylase in regulating de novo fatty acid biosynthesis from acetate. Although acetyl-CoA carboxylase activity was higher than that expected from the rate of acetate incorporation into lipids of isolated chloroplasts of maize and barley, the opposite was found for chloroplasts isolated from spinach. In chloroplasts from the emerging leaves of maize seedlings, lipid synthesis was maximal with chloroplasts isolated from the leaf segment corresponding to leaf greening. However, a relationship between leaf development and acetyl-CoA carboxylase activity was less apparent. Consequently a regulatory role for acetyl-CoA carboxylase in de novo fatty acid biosynthesis could not be directly established from a comparison of the rates of lipid synthesis and acetyl-CoA carboxylase activities.

An alternative approach to the establishment of a regulatory role for acetyl-CoA carboxylase was to purify the enzyme from maize and barley leaves, and study its response to variable concentrations of substrates, products and certain cellular metabolites. Purification of both enzymes was per-

formed by identical procedures, including polyethylene glycol fractionation, hydrophobic chromatography and gel filtration. Acetyl-CoA carboxylase from both maize and barley leaves appeared to be an integral enzyme, as no evidence for its dissociation was found, contrary to the findings of the bacterial enzyme. The kinetic properties of the partially purified enzyme from maize and barley were very similar. The apparent Michaelis constants for the substrates, acetyl-CoA and HCO_3^-, were about 0.1mM and 2mM, respectively for both enzymes. The enzymically active form of the substrate, ATP, was found to be Mg.ATP. Furthermore, free ATP inhibited enzymic activity, while free Mg^{2+} activated the enzyme from both plant sources. Monovalent cations, particularly K^+, were positive effectors of acetyl-CoA carboxylase, on the other hand, the products of the acetyl-CoA carboxylase reaction, malonyl-CoA and ADP were inhibitors. ADP inhibition was competitive with respect to ATP, but uncompetitive with respect to acetyl-CoA. Inhibition of acetyl-CoA carboxylase activity by CoA was noncompetitive with respect to acetyl-CoA, while palmitoyl-CoA inhibition was uncompetitive with respect to acetyl-CoA.

From the view point of regulation of acetyl-CoA carboxylase activity, the observed response of activity to changes in pH, and the concentrations of Mg^{2+}, K^+, ATP and ADP *in vitro*, may be of significance. Changes in the chloroplastic levels of these effectors of acetyl-CoA carboxylase activity have been reported to occur during light-dark transition of chloroplasts, and would be consistent in regulating acetyl-CoA carboxylase activity in the light and dark.

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LIST OF ABBREVIATIONS

ACP	acyl-carrier protein
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCCP	biotin carboxyl-carrier protein
BSA	bovine serum albumin
chl	chlorophyll
ρ-CMB	ρ-chloromercuribenzoate
CoA	coenzyme A
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Mes	2[N-Morpholino] ethane sulphonic acid
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NEM	N-ethylmaleimide
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid

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
RuDP	ribulose 1,5-diphosphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	thin-layer chromatography
Tricine	N-tris[Hydroxymethyl]-methyl glycine
Tris	tris (hydroxymethyl) aminomethane

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INTRODUCTION

1.1 General Background

Acetyl-CoA carboxylase [acetyl-CoA-carbon dioxide ligase (ADP forming), EC 6.4.1.2] catalyses the first committed step in the synthesis of fatty acids from acetyl-CoA. The enzyme was found almost simultaneously in avian livers by several workers, and recognised as an essential enzymatic component required for fatty acid synthesis (Porter and Tietz, 1957; Porter *et al*, 1957; Gibson *et al*, 1958a; 1958b; Formica and Brady, 1959). The stoichiometry of the reaction catalysed by the enzyme was described by Wakil (1958) (Reaction 1).

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \xrightarrow{Me^{2+}} \text{malonyl-CoA} + \text{ADP} + \text{Pi} \quad (1).$$

Biotin has long been known as a prosthetic group in certain HCO_3^--fixing reactions; the "Wood-Werkman" reaction of $H^{13}CO_3^-$ incorporation into oxaloacetate by a soluble system from *Micrococcus lysodeikticus* (Lardy *et al*, 1947; Potter and Elvehjem, 1948; Wessman and Werkman, 1950) and the ATP-dependent carboxylation of propionate to form succinate (Lardy, 1952; Lardy and Peanasky, 1953), have implicated biotin as a cofactor. These reactions were inhibited by avidin, a biotin-binding glycoprotein from egg white (Allison *et al*, 1933). Analogous with these reactions, acetyl-CoA carboxylase activity was found to be susceptible to avidin-inhibition (Titchener and Gibson, 1957; Wakil *et al*, 1958; Wakil and Gibson, 1960) and was classified as a biotin-dependent carboxylase.

1.2 Acetyl-CoA Carboxylase from Animal Sources

1.2.1 Molecular properties

Acetyl-CoA carboxylase has been purified to homogeneity from a number of animal sources. The enzyme is active as polymeric filaments of molecular weight $4 - 8 \times 10^6$ (Lane et al, 1974) which are in equilibrium with the inactive protomer. The protomer of the avian liver enzyme has a molecular weight of 410,000 (Gregolin et al, 1968b), whereas the corresponding value for the bovine adipose tissue enzyme is 560,000 (Moss, et al, 1972). Early work on the structural organisation of the protomer indicated that it was a tetramer of four non-identical polypeptides of molecular weights $110 - 125 \times 10^3$ (Gregolin et al, 1968b; Kleinschmidt et al, 1969; Guchhait et al, 1974c). However, later workers found that in fact the protomer was composed of two identical polypeptides of approximately $220 - 240 \times 10^3$ daltons (Inoue and Lowenstein, 1972; Tanabe et al, 1975; Mackall and Lane, 1977: Ahman et al, 1978; Witters et al, 1979a; Hardie and Guy, 1980). The discrepancy with early work has been shown to be due to a limited proteolysis of acetyl-CoA carboxylase during isolation (Tanabe et al, 1975; 1977; Guy and Hardie, 1980).

1.2.2 Regulation of catalytic activity

Acetyl-CoA carboxylase catalyses the first committed step in the formation of fatty acids from acetyl-CoA and has been found to be a key regulatory point in the control of fatty acid biosynthesis. These regulatory mechanisms are discussed below under the headings of long-term and short-term controls of activity.

1.2.2.1 Long-term control of acetyl-CoA carboxylase

The rate of fatty acid synthesis in a number of tissues fluctuates under a variety of metabolic conditions,

including dietary, hormonal, developmental and genetic alterations. These long-term fluctuations appear to be mediated by alterations in the concentrations of the enzymes involved in the biosynthesis of fatty acids, in particular acetyl-CoA carboxylase and fatty acid synthetase. Acetyl-CoA carboxylase activity was lowered in the livers of rats, either when the animals were starved, or fed on a high-fat diet, or made alloxan-diabetic, and was markedly elevated in livers of rats fed a fat-free diet (Numa et al, 1961; Wieland et al, 1963; Allmann et al, 1965). Quantitative immunoprecipitin analysis of crude extracts of livers indicated that these changes in acetyl-CoA carboxylase activity were due to differences in the amount of the enzyme (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970). These increases in enzyme content are due solely to an increase in the rate of synthesis of enzyme, whereas the decline in the enzyme content as a consequence of starvation etc, was due to both diminished rates of synthesis and accelerated degradation of the enzyme (Majerus and Kilburn, 1969; Nakaniski and Numa, 1970). Measurement of the hepatic content of specific polysomes synthesizing acetyl-CoA carboxylase, reflect the changes in the rate of synthesis of this enzyme in vivo, brought about by dietary manipulations and the alloxan-diabetic state (Nakanishi et al, 1976; Horikawa et al, 1977).

Livers of genetically obese hyperglycemic mice (C57BL/6J-ob) (Ingalls et al, 1950) exhibit an increased level of lipogenesis (Jansen et al, 1967) that results from elevated levels of several enzymes involved in long-chain fatty acid biosynthesis, including acetyl-CoA carboxylase, fatty acid synthetase (Chang et al, 1967) and citrate-cleavage enzyme (Kornacker and Lowenstein, 1964). The increased level of acetyl-CoA carboxylase appears to be due to an increased quantity of the enzyme (Nakanishi and Numa, 1971) caused by an elevated number of polysomes containing messenger RNA coding for acetyl-CoA carboxylase, and the consequent increased rate of synthesis of the enzyme (Tanabe et al, 1976).

In rat mammary glands, the onset of lactation is marked by an increase in the activity of enzymes involved in the biosynthesis of milk, including the lactogenic enzyme acetyl-CoA carboxylase (Howanitz and Levy, 1965; Baldwin and Milligan, 1966; Kuhn and Lowenstein, 1967; Gul and Dils, 1969; Gumaa et al, 1973; Baldwin and Young, 1974). Studies into the amount of immunotitratable acetyl-CoA carboxylase, showed that this rise in activity during lactation was due to an increase in the concentration of acetyl-CoA carboxylase enzyme (Mackall and Lane, 1977).

1.2.2.2 Short-term control of acetyl-CoA carboxylase

In contrast to long-term controls of fatty acid biosynthesis which manifest their effects on acetyl-CoA carboxylase over a period of days, variations in the rate of fatty acid biosynthesis have been observed over a period of hours (for reviews see Volpe and Vagelos, 1973; Numa and Yamashita, 1974). These more rapid changes in the rate of fatty acid synthesis appear to be achieved by alterations of the catalytic efficiency of acetyl-CoA carboxylase, which is the rate-limiting enzyme. Changes in the relative proportions of the inactive protomer and active polymer of acetyl-CoA carboxylase, that have been observed in vitro (Gregolin et al, 1966b; Kleinschmidt et al, 1969; Moss et al, 1972) have been implicated as a means of modulating the enzyme's activity in vivo.

The equilibrium between the polymer and protomer in vitro, is affected by a number of metabolites and conditions of incubation (Table 1) (Lane et al, 1974; Lent et al, 1978; Yeh and Kim, 1980). The physiological significance of these effects, in the control of acetyl-CoA carboxylase activity have been studied by a number of groups. The occurrence of the polymer-protomer transition has been demonstrated in vivo by three different methods (Halestrap and Denton, 1974; Meredith and Lane, 1978; Ashcaft et al, 1980). Lane and

TABLE I

PROTOMER-POLYMER TRANSITION OF ANIMAL ACETYL-CoA CARBOXYLASE

PROTOMER	POLYMER
inactive	active
Molecular weight 420,000	Molecular weight $4\text{-}6 \times 10^6$
Equilibrium towards protomer favoured by:	Equilibrium towards polymer favoured by:
$ATP.Mg^{2+} + HCO_3^-$	citrate, isocitrate
malonyl-CoA	phosphate
fatty acyl-CoA	albumin
alkali pH	pH 6.5 - 7.0
NaCl (>0.2M)	high enzyme concentration
low enzyme concentration	dephosphorylation
phosphorylation	CoA

From Lane et al., 1974.

co-workers have suggested that acetyl-CoA carboxylase activity *in vivo* is controlled by the concentration of citrate (Moss and Lane, 1972 ; Lane *et al*, 1974). A positive correlation has been reported between *de novo* fatty acid biosynthesis, acetyl-CoA carboxylase activity and the citrate content of cells (Goodridge, 1973c; Nishikori *et al*, 1973; Geelan and Gibson, 1975; Müller *et al*, 1976; Watkins *et al*, 1977; Geelan *et al*, 1978). However this view has been challenged by a number of workers who found negligible changes in cellular levels of citrate under conditions that appreciably changed acetyl-CoA carboxylase activity and the rate of fatty acid biosynthesis (Spencer and Lowenstein, 1967; Greenbaum *et al*, 1971; Guynn *et al*, 1972; Brunengraber *et al*, 1973; Halestrap and Denton, 1973; Denton, 1975; Harris, 1975; Cook *et al*, 1977). Furthermore, the measured cellular concentration of citrate is about 0.1 - 0.3mM, while 5mM citrate is required for the *in vitro* activation of acetyl-CoA carboxylase.

The cellular levels of fatty acyl-CoA thiosters have been implicated in the regulation of acetyl-CoA carboxylase in mammals. An inverse relationship has been found between the concentration of fatty acyl-CoA and the rate of fatty acid synthesis or acetyl-CoA carboxylase activity in livers (Guynn *et al*, 1972; Goodridge, 1973a; 1973b; 1973c; Nishikori *et al*, 1973; Goodridge *et al*, 1974; Cook *et al*, 1977) and adipose tissues (Halestrap and Denton, 1973; 1974; Denton, 1975). However, some workers have also reported no change in fatty acyl-CoA concentration both in perfused livers (Brunengraber *et al*, 1973) and isolated mouse hepatocytes (Müller *et al*, 1976), in conditions which altered the rate of fatty acid synthesis and acetyl-CoA carboxylase activity. It has been suggested that the activity of acetyl-CoA carboxylase may not be regulated by the total fatty acyl-CoA concentration in the cell, but by the concentration of free fatty acyl-CoA, which may be dependent on specific cellular proteins, similar to albumin, which

bind this class of metabolite (Goodridge, 1972; Lunzer *et al*, 1977).

Kim and co-workers first suggested that acetyl-CoA carboxylase activity was regulated in rat livers by a phosphorylation-dephosphorylation cycle (Carlson and Kim, 1973; 1974a, 1974b), which accompanied enzyme activation-inactivation *in vitro* (Lee and Kim, 1977); the phosphorylated enzyme being inactive. These observations have also been extended to rat epididymal fat pads (Lee and Kim, 1978; Brownsey *et al*, 1979) and mammary glands from rabbit (Hardie and Cohen, 1979) and rat (Hardie and Guy, 1980). The phosphorylation-dephosphorylation cycle appears to be controlled by factors which regulate the *in vivo* rate of fatty acid synthesis. The phosphorylation of the rat liver enzyme is stimulated by cAMP (Lent *et al*, 1978) and glucagon (Witters *et al*, 1979a; 1979b), both of which inhibit fatty acid synthesis in liver (Allred and Roehrig, 1972; Goodridge, 1973a; Geelan and Gibson, 1975; Geelan *et al*, 1978). Similarly in epididymal adipose tissue, phosphorylation has been demonstrated to be stimulated by epinephrine (Lee and Kim, 1978; Brownsey *et al*, 1979; Lee and Kim, 1979) which also inhibits fatty acid synthesis in this tissue (Denton and Martin, 1970).

Some workers however, dispute the physiological significance of the phosphorylation-dephosphorylation cycle in the regulation of acetyl-CoA carboxylase. Pekala *et al* (1978) reported phosphorylation of chick liver acetyl-CoA carboxylase, without a significant change in activity. However Lee and Kim (1979) point out that not all phosphorylation sites are important for regulation of enzyme activity. Indeed recent studies of the tryptic digestion of the phosphorylated acetyl-CoA carboxylase indicates multiple sites of phosphorylation (Brownsey and Hardie, 1980; Brownsey *et al*, 1981). Studies of the regulatory significance of the recently purified acetyl-CoA carboxylase kinase (Shiao *et al*, 1981) and acetyl-CoA carboxylase phosphatase (Krakower and Kim, 1981) should clarify the mechanism involved.

Allosteric activation of acetyl-CoA carboxylase by coenzyme A, at physiological concentrations, is accompanied by polymerisation of the enzyme (Yeh and Kim, 1980). The CoA binding site is not affected by citrate, but may be the same as the palmitoyl-CoA binding site (Yeh et al, 1981), thus CoA-binding modifies the inhibition of acetyl-CoA carboxylase by palmitoyl-CoA. Similar modifications of both the effects of palmitoyl-CoA and citrate on acetyl-CoA carboxylase have been observed with phosphorylation of the enzyme (Carlson and Kim, 1974b). As has been suggested by other workers, acetyl-CoA carboxylase activity may be regulated by an interaction of the different effects discussed in this section (Nishikori et al, 1973; Hardie, 1981).

1.3 Acetyl-CoA Carboxylase from Escherichia coli

1.3.1 Molecular properties

Acetyl-CoA carboxylase from E. coli has been studied extensively in the laboratories of Lane and Vagelos. Initially the enzyme was separated into two protein fractions known as Ea and Eb (Alberts and Vagelos, 1968). Ea was shown to contain biotin, which was carboxylated in a ATP-requiring reaction (Reaction 3) (Alberts et al, 1969). The protein fraction Eb transferred the carboxyl group from biotin to acetyl-CoA forming malonyl-CoA (Reaction 4) (Alberts and Vagelos, 1968).

$$\text{E-biotin} + \text{ATP} + HCO_3^- \xrightarrow[Me^{2+}]{\text{Biotin carboxylase}} \text{E-biotin-}CO_2^- + \text{ADP} + \text{Pi} \qquad (3)$$

$$\text{E-biotin-}CO_2^- + \text{acetyl-CoA} \xrightarrow{E_b} \text{malonyl-CoA} + \text{E-biotin} \qquad (4)$$

Later work demonstrated that acetyl-CoA carboxylase in E. coli was composed of three distinct proteins: biotin carboxylase, biotin carboxyl-carrier protein (BCCP) which together constituted Ea, and carboxyltransferase (E_b), all three of which have been purified separately (Dimroth et al, 1970; Alberts, et al, 1971; Nervi et al, 1971; Guchhait et al,

1974 a).

The BCCP component of the enzyme contains covalently bound biotin which plays a key role in the mechanism of the carboxylation of acetyl-CoA (Alberts et al, 1969). The protein was initially purified as a peptide of molecular weight 9,100, containing one covalently bound biotin prosthetic group (Nervi et al, 1971). Later work, however, showed that proteolysis had probably occurred during the purification. The native form of BCCP is now thought to have a molecular weight of 45,000 and to consist of two subunits of 22,500 daltons, each of which contain a biotin prosthetic group (Fall et al, 1971; Fall and Vagelos, 1972; 1973). The biotin prosthetic group can be carboxylated by biotin carboxylase (Reaction 3) and is thought to act as a carboxyl-carrier (Alberts et al, 1969; Nervi et al, 1971).

Biotin carboxylase has a native molecular weight of 100,000 and is composed of two subunits of 50,000 daltons (Dimroth et al, 1970). The enzyme catalyses the carboxylation of the biotin group of the BCCP component, free d-biotin and a number of biotin derivatives (Dimroth et al, 1970; Polakis et al, 1974). This probably has enabled extensive study of the mechanism of acetyl-CoA carboxylase to be performed (Guchhait et al, 1974b; Polakis et al, 1974).

The carboxyl group from the carboxyl-BCCP is transferred to acetyl-CoA by the enzyme, carboxyltransferase (Alberts et al, 1971; Guchhait et al, 1971), which has been found to have a native molecular weight of 130,000 and is composed of four subunits, two of 30,000 daltons and two of 35,000 daltons (Guchhait et al, 1974a).

1.3.2 Regulation of catalytic activity

Endogenously synthesized fatty acids in E. coli appear to be exclusively utilized for the synthesis of membrane lipids (Cronan and Vagelos, 1972), suggesting that control

of lipogenesis may be coupled to such growth-related processes as protein and RNA synthesis. Protein and RNA synthesis appears to be under genetic control of the *rel* gene (Neidhardt, 1966; Edlin and Broda, 1968), which is inoperative in 'relaxed' (*rel*$^-$) mutants. Fatty acid synthesis has been observed to be under the control of the *rel* gene (Sokawa *et al*, 1973; Golden and Powell, 1972; Polakis *et al*, 1973); amino acid starvation decreased the rate of fatty acid synthesis in *rel*$^+$ cells but not in *rel*$^-$ cells. During amino acid starvation of *rel*$^+$ cells, two unusual nucleotides, ppGpp and pppGpp, accumulate in the cells, which have been postulated to be the mediators of the *rel* gene (Cashel, 1969; Cashel and Gallant, 1969; Lazzarini *et al*, 1971). Polakis *et al* (1973) have shown that (p)ppGpp inhibits the carboxyltransferase component of acetyl-CoA carboxylase, demonstrating the locus of the control of lipogenesis in *E. coli*.

1.4 Acetyl-CoA Carboxylase from Yeast

Acetyl-CoA carboxylase has been purified to homogeneity from both *Saccharomyces cerevisiae* (Sumper and Riepertinger, 1972) and *Candida lypolytica* (Mishina *et al*, 1976a), and has been found to be composed of one type of subunit of molecular weight 230,000, containing one covalently-bound biotin prosthetic group.

The regulatory processes in the control of fatty acid biosynthesis have been studied extensively in both *S. cerevisiae* and *C. lypolytica*, especially as they relate to acetyl-CoA carboxylase. These yeasts are able to grow on glucose or *n*-alkane (or fatty acid) as the sole source of carbon. The activity of acetyl-CoA carboxylase, in cells grown in *n*-alkane was found to be lower than in glucose grown cells. The decrease in acetyl-CoA carboxylase activity was due to a decrease in the cellular content of the enzyme, rather than to a reduced catalytic efficiency of the enzyme (Kamiryo and Numa, 1973; Mishina *et al*, 1976b). Regulation of the rate

of synthesis, rather than the rate of turnover of acetyl-CoA carboxylase has been found to be important in determining the cellular content of the enzyme (Mishina et al, 1976b), and this was reflected in the amount of messenger RNA coding for acetyl-CoA carboxylase (Horikawa et al, 1980). The repression of the synthesis of acetyl-CoA carboxylase in n-alkane grown cells was found to be mediated by fatty acyl-CoAs derived from exogenously supplied n-alkane (Kamiryo et al, 1976; 1977; 1979).

1.5 Acetyl-CoA Carboxylase from Plants

Acetyl-CoA carboxylase was first studied in plant embryonic tissue of wheat (Hatch and Stumpf, 1961) and was later purified to homogeneity (Heinstein and Stumpf, 1969). The enzyme was found to have a molecular weight of 630,000 and to be separable into 7.3S and 9.4S components by ultracentrifugation. The 9.4S component contained biotin which underwent carboxylation in the absence of the 7.3S component (Heinstein and Stumpf, 1969). Comparison with the E. coli enzyme, suggests that the 9.4S component contains the biotin carboxylase and BCCP components, and the 7.3S component catalyses the carboxyltransferase reaction. The subunit composition of the enzyme initially appeared complex (Nielsen et al, 1979). However, this may have been due to proteolytic digestion during isolation, since Egin-Bühler et al (1980) have isolated the enzyme with a molecular weight of 700,000, composed of two types of subunits of molecular weights 240,000 and 98,000; the larger containing biotin. The presence of a similarly sized acetyl-CoA carboxylase in barley embryos has been demonstrated by Brock and Kannangara (1976), with a molecular weight of 610,000.

In green leaves, de novo fatty acid synthesis has been demonstrated to occur exclusively in the chloroplasts (Ohlrogge et al, 1979). However, early attempts to demonstrate the presence of acetyl-CoA carboxylase in isolated chloroplasts were either unsuccessful (Burton and Stumpf, 1966) or the

activity of the enzyme was found to be less than the maximum observed rates of fatty acid synthesis of 2000 nmol/h/mg chlorophyll (Kannangara et al, 1971; 1973; Kannangara and Stumpf, 1973; Roughan et al, 1979a; Browse et al, 1981).

Kannangara and Stumpf (1972) working with spinach suggested that the molecular organisation of the chloroplastic acetyl-CoA carboxylase was similar to that of the E. coli enzyme. They described the presence of three components, biotin carboxylase, carboxyltransferase and BCCP, of which the former two were stromal and the latter bound to the lamellae. The low activity of the enzyme was thought to be due to the instability of the carboxyltransferase component, or to the presence of an inhibitor of this component. A similar distribution of protein-bound biotin was described in plastids from a number of species (Kannangara and Stumpf, 1973), and a biotin-containing protein of molecular weight 21,000 was isolated from barley chloroplast membranes (Kannangara and Jensen, 1975).

However, later workers have reported soluble acetyl-CoA carboxylase activity in cell-free extracts of barley (Reitzel and Nielsen, 1976), which appears to be chloroplastic (Thomson and Zalik, 1981). A soluble acetyl-CoA carboxylase has also been partially purified from spinach chloroplasts and avocado plastids, after stabilization of the enzymes (Mohan and Kekwick, 1980). The enzyme from avocado plastids was found to have a molecular weight of 650,000 with one biotin per mole of enzyme. A similarly large acetyl-CoA carboxylase has been purified from parsley cell cultures, with a molecular weight of 840,000, composed of subunits of 210,000 daltons and 105,000 daltons, with the former subunit containing biotin (Egin-Bühler et al, 1980).

1.6 Acetyl-CoA Carboxylase from Other Sources

Acetyl-CoA carboxylase has also been studied in a number of additional sources. In Pseudomonas citronellolis acetyl-CoA

carboxylase resembles that from E. coli in its molecular organisation, with three separable components, namely biotin carboxylase, carboxyltransferase and BCCP. SDS-polyacrylamide gel electropheresis studies, showed that the biotin carboxylase component was associated with a polypeptide of 53,000 daltons, while the carboxyltransferase component was associated with two polypeptides of 36,000 and 33,000 daltons. The biotin prosthetic group was found to be covalently bound to a 25,000 dalton polypeptide, which was assumed to be the BCCP component (Fall et al, 1975; Fall, 1976). In contrast to the E. coli enzyme, however, acetyl-CoA carboxylase from P. citronellolis could be stabilized and isolated as a complex of all three components (Fall, 1976).

Acetyl-CoA carboxylase from Mycobacterium phlei has been partially purified and appears to be an aggregated complex which does not dissociate into active constituent enzymes. Both acetyl-CoA and propionyl-CoA were carboxylated by the enzyme at approximately the same rate (Erfle, 1973).

A unique structural organisation for acetyl-CoA carboxylase has been found in light-grown Euglena gracilis, in which the enzyme is found as a complex together with phosphenolpyruvate carboxylase and malate dehydrogenase, with a total molecular weight of 360,000 (Wolpert and Ernst-Fonberg, 1975a). When dissociated, the enzymes retained the same activity as when complexed, and had molecular weights of 183,000, 67,000 and 127,000 for phosphoenolpyruvate carboxylase, malate dehydrogenase and acetyl-CoA carboxylase, respectively (Wolpert and Ernst-Fonberg, 1975b). Wolpert and Ernst-Fonberg suggest that this multienzyme complex, in conjunction with malic enzyme, facilitates the formation of substrates, malonyl-CoA and NADPH for fatty acid biosynthesis.

Acetyl-CoA carboxylase purified from the free-living nematode Turbatrix aceti, has molecular weight of 667,000. Analysis

by SDS-gel electropheresis indicates that it consists of two polypeptides having molecular weights 82,000 and 58,000, with the former containing biotin (Meyer et al, 1978). The enzyme carboxylates propionyl-CoA at a faster rate than acetyl-CoA and monovalent cations, especially K^+, activate the enzyme (Meyer and Meyer, 1978).

1.7 Acetyl-CoA Carboxylase as a Biotin Enzyme

Comparison of the molecular organisation and mechanism of carboxylation of a number of biotin-dependent enzymes, such as propionyl-CoA carboxylase, β-methylcrotonyl-CoA carboxylase, geranyl-CoA carboxylase, pyruvate carboxylase, ATP: urea amidolyase, methylmalonyl-CoA-pyruvate transcarboxylase, methylmalonyl decarboxylase, oxaloacetate decarboxylase and acetyl-CoA carboxylase has enabled a better understanding of the properties of these enzymes (for reviews see Moss and Lane, 1971; Wood and Barden 1977; Lynen, 1979). The common feature of all these enzymes is the biotin prosthetic group, which functions as 'HCO_3^--carrier'.

The biotin prosthetic group appears to be covalently-bound to all of the above enzymes through an amide linkage to a lysyl ε-amino group. The carboxylated intermediate has been shown to be 1'-N-carboxy-(+)-biotin for propionyl-CoA carboxylase, β-methylcrotonyl-CoA carboxylase, methylmalonyl-CoA-pyruvate transcarboxylase and acetyl-CoA carboxylase, and is probably identical in all biotin-containing enzymes.

The carboxylated intermediate is formed in an ATP-requiring reaction (Reaction 5) in the carboxylases, however, in the transcarboxylases and decarboxylases (Reaction 6), this reaction is independent of ATP.

$$\text{Enz-biotin} + \text{HO-CO}_2^- + \text{ATP} \rightarrow \text{Enz-biotin-CO}_2^- + \text{ADP} + \text{Pi} \qquad (5)$$

$$\text{Enz-biotin} + \text{R}_1\text{—CO}_2^- \rightarrow \text{Enz-biotin-CO}_2^- + \text{R}_1 \qquad (6)$$

The second half-reaction, in the case of the carboxylases and transcarboxylases involves the transfer of the carboxyl group from the intermediate to an acceptor (Reaction 7). However, with the decarboxylases, the carboxyl group is lost as CO_2 (Reaction 8).

$$\text{Enz-biotin-}CO_2^- + R_2 \rightarrow \text{Enz-biotin} + R_2\text{-}CO_2^- \quad (7)$$

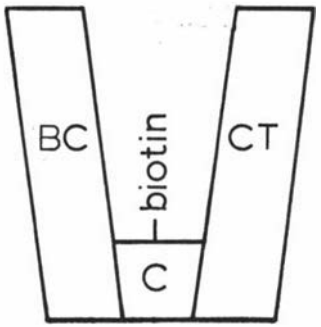
$$\text{Enz-biotin-}CO_2^- \longrightarrow \text{Enz-biotin} + CO_2 \quad (8)$$

Lynen (1979) has identified three classes of biotin-containing enzymes (Fig. 1). Enzymes of the first class readily separate into three active components: biotin-containing component (BCCP), a biotin carboxylase component which catalyses either reaction 5 or 6, and the carboxyltransferase component catalysing either reaction 7 or 8. The second group of enzymes separate into two active components, one of which carboxylates the bound biotin (Reaction 5 or 6), presumably a complex of tightly coupled BCCP and biotin carboxylase. The other component contains no biotin, but catalyses the carboxyltransferase reaction (Reaction 7 or 8). The third class of enzymes do not separate into active components and presumably contain the BCCP, biotin carboxylase and carboxyltransferase combined.

1.8 Fatty Acid Biosynthesis in Plants

All plant extracts that carry out *de novo* fatty acid biosynthesis have been shown to be stimulated by the addition of acyl-carrier protein (ACP), indicating that *de novo* fatty acid biosynthesis in plants is an ACP-dependent process (Stumpf, 1977; 1980). The demonstration that the sole detectable site of ACP in spinach protoplasts is the chloroplast, suggests that this organelle carries out all *de novo* fatty acid biosynthesis in photosynthetic tissues (Ohlrogge *et al*, 1979).

Although the ultimate carbon precursor of acetyl-CoA in plants is CO_2, the process by which acetyl-CoA becomes avail-

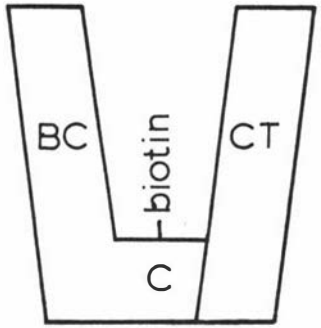


Acetyl-CoA carboxylase:

E. coli
Achromobacter
P. citronellolis

Methylmalonyl-CoA-pyruvate transcarboxylase:

P. shermanii



Acetyl-CoA carboxylase:

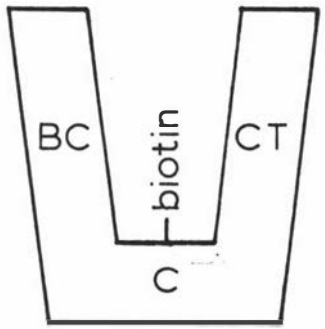
wheat germ
T. aceti

Pyruvate carboxylase:

P. citronellolis

β-methylcrotonyl-CoA carboxylase:

Achromobacter



Acetyl-CoA carboxylase:

C. lypolytica
S. cerevisiae
mammalian liver,
adipose tissue,
mammary gland.

Pyruvate carboxylase:

yeast

FIGURE 1: Structural classification of biotin containing enzymes.
BC = biotin carboxylase; CT = carboxyltransferase; C = BCCP (from Lynen, 1979).

able for de novo fatty acid biosynthesis in chloroplasts is not known. Recently evidence has accumulated to support the formation of acetyl-CoA within chloroplasts, via the pathway: CO_2 → 3-PGA → 2-PGA → PEP → Pyruvate → Acetyl-CoA (Yamada and Nakamura, 1975; Murphy and Leech, 1977; 1978). Phosphoglyceromutase for the conversion of 3-PGA, formed by the carboxylation of ribulose 1,5-diphosphate, to 2-PGA although not found in chloroplasts, has been demonstrated in proplastids of castor bean (Simcox et al, 1977). Enolase, catalysing the reaction 2-PGA → PEP has been found in castor bean proplastids (Simcox et al, 1977) and pea chloroplasts (Stitt and Rees, 1979). Pyruvate kinase, catalysing the conversion of PEP to pyruvate, has been demonstrated in castor bean proplastids (Simcox et al, 1977; De Luca and Dennis, 1978) and chloroplasts isolated from green leaves of castor bean (Ireland et al, 1979). The presence of this enzyme has also been shown in etioplasts (Ireland et al, 1979) and chloroplasts (Stitt and Rees, 1979) isolated from pea leaves. Pyruvate dehydrogenase, the final enzyme in the proposed pathway for the formation of acetyl-CoA, has been found in the proplastids of castor bean (Reid et al, 1977; Thomson et al, 1977a; 1977b), and in pea chloroplasts (Elias and Givan, 1979; Williams and Randall, 1979).

However, the suggestion that chloroplasts are able to synthesize acetyl-CoA and thus fatty acids from CO_2 has not gone unchallenged (Sherratt and Givan, 1973; Roughan et al, 1979b). Roughan et al (1979a; 1979b), favour the view that acetyl-CoA is derived from an extrachloroplastic source. Acetate derived from mitochondrial acetyl-CoA, could become available to the chloroplast after transportation as either free acetate or citrate. As both the mitochondrial membrane and chloroplast envelope are permeable to acetate, this molecule would be able to freely diffuse from the site of synthesis, in the mitochondria, to the chloroplast, for activation to acetyl-CoA by the stromal enzyme acetyl-CoA synthetase (Jacobson and Stumpf, 1972; Roughan and Slack, 1977). This suggestion also implies a function for acetyl-

CoA synthetase, which would not be required if acetyl-CoA was derived via a chloroplastic pyruvate dehydrogenase.

The possibility that chloroplastic acetyl-CoA is derived from mitochondrial citrate has been suggested by the work of Nelson and Rinne (1975; 1977a; 1977b), who found the enzyme citrate lyase in the cytosol of developing soybean seeds. They were also able to demonstrate fatty acid synthesis from [1,5-^{14}C] citrate in this tissue. In contrast, however, avocado mesocarp extracts (Weaire and Kekwick, 1975), and isolated spinach chloroplasts (Yamada and Nakamura, 1975) were not able to synthesize fatty acids from citrate.

Following the carboxylation of acetyl-CoA by acetyl-CoA carboxylase in the chloroplast (see Section 1.5), de novo fatty acid synthesis is thought to take place in the chloroplast by a series of reactions catalysed by soluble enzymes, which convert acetyl-CoA and malonyl-CoA to palmitic acid (Reaction 9)

$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{palmitic acid} + 8\text{CoA} + 7\text{CO}_2 + 14\text{NADP}^+ + 6\text{H}_2\text{O} \qquad (9)$$

Studies to elucidate the nature of this pathway have been mainly carried out by following incorporation of radioactive label, from [^{14}C] acetate into fatty acids by isolated chloroplasts. Rates of up to 2000 nmol acetate incorporated/ h / mg chl have been reported (Roughan et al, 1979a; Browse et al, 1981) with isolated spinach chloroplasts, which is approaching the rates found in the intact spinach leaves. However, label from [^{14}C] acetyl-CoA or [^{14}C] malonyl-CoA was poorly incorporated by intact chloroplasts into fatty acids (Stumpf et al, 1967; Weaire and Kekwick, 1975). This is thought to be due to the impermeability of the chloroplast envelope to CoA esters. However, the removal of the chloroplast envelope results in preparations which show poor rates of fatty acid synthesis from acetate and acetyl-CoA, but higher rates from malonyl-CoA (Brooks and Stumpf, 1965;

1966; Stumpf, 1977). This discrepancy was explained by implicating an inhibitor to acetyl-CoA carboxylase released upon disruption of chloroplasts (Burton and Stumpf, 1966).

Fatty acid biosynthesis by isolated chloroplasts has been shown to be a light-dependent process, by a number of workers (Smirnov, 1960; Mudd and McManus, 1962; Stumpf and James, 1962; 1963; Stumpf et al, 1967). The supply of ATP and NADPH was suggested to cause the light-dependency of this process (Stumpf and James, 1962; 1963). Recently, Nakamura and Yamada (1979) observed that [^{14}C] acetate supplied to isolated chloroplasts in the light was incorporated into malonyl-CoA and acetyl-CoA as well as fatty acids, but only acetyl-CoA was labelled in darkness. They suggested therefore that the carboxylation of acetyl-CoA could be the light-dependent reaction in fatty acid synthesis. However, Roughan et al (1980) suggest that the accumulation of labelled acetyl-CoA from [^{14}C] acetate both in the light and dark is due to the presence of a thiokinase in an outer chloroplast compartment and not due to a dark-block of acetyl-CoA carboxylase.

Although the enzymes involved in the synthesis of palmitic acid, in isolated chloroplasts, have not been studied individually, the ACP requirement for this process suggests that de novo fatty acid biosynthesis is carried out by a system of individual soluble enzymes, similar to that found in E. coli (Stumpf, 1977; 1980; Majerus and Vagelos, 1967).

CHAPTER 2

MATERIALS

2.1 Plant Materials

Maize (*Zea mays* var. XL45) and barley (*Hordeum vulgare*) seeds were obtained from Arthur Yates and Co., Ltd, NZ. The seeds were soaked overnight in water and sown in trays of peat/pumice (1:2, v/v) potting mixture supplied with Hoagland's solution A (Hoagland and Arnon, 1938). Plants were grown in a controlled environment with day/night temperatures, vapor pressure deficits, and equivalent relative humidities at 25/20^{o}C, 10/5 mbar and 68/78%, respectively. Day-length was 12h and photosynthetically active radiation of 400-700nm range was 170 W.m^{-2}. After 8 days of growth plants were harvested by cutting the shoots at the base. Maize acetyl-CoA carboxylase was purified from whole seedling tissue, but in experiments using maize seedlings, the coleoptile and first leaf was removed. In experiments on barley acetyl-CoA carboxylase the whole seedling was used.

Spinach (*Spinacia oleracea*), field-grown, was obtained from the local market and was used on the day of purchase.

2.2 Reagents

The following chemicals were obtained from Sigma Chemical Co., St Louis: ADP, AMP, ATP, avidin, acrylamide, biotin, bis-acrylamide, BSA Fraction V, CoA, Coomassie Brilliant Blue G250 and R250, cyanogen bromide, dithiothreitol, glutathione, Hepes, malate, Mes, NADH, NADPH, palmitic acid, PEP, POPOP, PPO, Tricine, Tris.

Cellulase (Cellulysin^{TM}) and Miracloth were from Calbiochem,

La Jolla.

Cellulose powder MN300 was from Macherey, Nagel and Co., Duren, Germany.

Separose 2B and Dextran T40 were obtained from Pharmacia Fine Chemicals, Uppsala.

Bio-Gel P6 was from Bio-Rad Laboratories, Richmond.

Ultrogel AcA22 was from LKB-Produkter, Sweden.

The radiochemicals, [1-^{14}C]acetate and $NaH^{14}CO_3$, were obtained from The Radiochemical Centre, Amersham.

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

C H A P T E R 3

METHODS

3.1 Preparation of Acyl-CoA Esters

Acetyl-CoA and propionyl-CoA were synthesized by reacting CoA with acetic anhydride and propionic anhydride, respectively (Stadtman, 1957), in 0.1M $NaHCO_3$ at 4°C for 15 min. The solutions were then adjusted to pH 4.0 with 2M HCl and dried by lyophilization.

Butyryl-CoA and palmitoyl-CoA were synthesized under nitrogen by the procedure of Young and Lynen (1969). Approximately 20 µmoles of acid was dissolved in tetrahydrofuran and reacted with 15% excess of triethylamine, after which the mixed anhydridewas formed by reacting with ethylchloroformate. The CoA ester was finally synthesized by reacting the mixed anhydride of the acid, with free CoA. Palmitoyl-CoA was precipitated by addition of perchloric acid to 1% and the precipitate recovered by centrifugation and dried under a vacuum. Unreacted palmitic acid was extracted three times with diethyl ether and the residue dried under a stream of nitrogen. The solution of butyryl-CoA was adjusted to pH 4.0 with 2M HCl and dried under vacuum.

The synthesis of malonyl-CoA was carried out by the method of Rutkoski and Jaworski (1978). Five hundred µl of 0.1M thiophenol dissolved in tetrahydrofuran was added to 10 µmoles of malonic acid, followed by the addition of 0.5 ml 0.1M dicyclohexylcarbodiimide in tetrahydrofuran over a period of 1h, to form monothiophenylmalonate. After 3h, 16 µmoles of CoA dissolved in 1M Na_2CO_3 / $KHCO_3$ buffer, pH 9.2, was added over a period of 4h. Finally, 200 µl of glacial acetic acid was added to stop the reaction and the mixture dried under vacuum.

The acyl-CoA esters were dissolved in about 0.2 ml of water and purified by cellulose TLC in butanol:acetic acid:water (5:2:3) (Pullman, 1973). After drying the TLC plates, the CoA esters were located under a UV-lamp and the appropriate areas of cellulose were transferred to centrifuge tubes. The CoA esters were extracted three times with 2 ml of water, and the combined extracts were dried by lyophilization. Finally the products were dissolved in water to the appropriate concentrations, as determined by the absorbance at 260 nm, assuming ε_{260} was 16400.

All CoA esters were more than 95% pure as determined from absorbances at 232 nm and 260 nm. The yield of acetyl-CoA and propionyl-CoA was in the range of 80-95%. Palmitoyl-CoA and butyryl-CoA were obtained in approximately 55% yield, and malonyl-CoA in a yield of 40%.

3.2 Enzyme Assays

Acetyl-CoA carboxylase activity was assayed as the acetyl-CoA-dependent, acid-stable radioactivity derived from $H^{14}CO_3^-$. The assay was carried out in a volume of 200 ml containing: 0.1M Tricine-KOH (pH 8.0), 1mM ATP, 2.5 mM $MgCl_2$, 50mM KCl, 1mM dithiothreitol, 0.3mM acetyl-CoA and either 10mM or 30mM $NaH^{14}CO_3$ (1 Ci/mol). The reaction was started by the addition of acetyl-CoA and incubated at 30°C with shaking for 5 min, and then stopped by the addition of 50 µl 6M HCl. A 50 µl aliquot was dried on a 1 cm x 1 cm square of Whatman 3MM paper, and the residual radioactivity determined. Assays without added acetyl-CoA were used as blanks.

NADPH-malate dehydrogenase was assayed spectrophotometrically by the change in absorbance at 340 nm due to the oxidation of NADPH when oxaloacetate was converted to malate (Hatch and Slack, 1969). The assay was carried out in a 1 ml cuvette containing: 50mM Tricine-KOH (pH 8.0), 3mM oxaloacetate and 0.2mM NADPH. The enzyme was first activated at 20°C

with 25mM dithiothreitol, and then added to the remaining reactants in the cuvette to start the assay.

RuDP carboxylase and PEP carboxylase, respectively, were assayed as the RuDP- and PEP-dependent, acid-stable radioactivity derived from $NaH^{14}CO_3$ (Wishnick and Lane, 1971). The assays were carried out in a volume of 200 µl containing: 0.2M Tris-HCl (pH 7.8), 0.06mM EDTA, 5mM DTT, 10mM $MgCl_2$, 50mM $NaH^{14}CO_3$ (0.5 Ci/mol) and either 0.5mM RuDP or 0.5mM PEP. The assay mixtures with enzyme, but without the substrate, were incubated at 30°C for 2 min, before adding either RuDP or PEP. The assay was allowed to continue for a further 5 min, before the addition of 50 µl 6M HCl, to stop the reaction. The acid-stable radioactivity was determined as described above for the assay for acetyl-CoA carboxylase.

Catalase activity was followed by the change in absorbance at 240 nm, due to the disappearance of H_2O_2 (Lück, 1963). The assay was carried out in a 1 ml cuvette, containing 50mM phosphate buffer (pH 7.0) and 0.05% H_2O_2. The assay was started by the addition of the solution containing the enzyme.

Fumarase activity was assayed by following the absorbance change at 250 nm due to the formation of fumarate from malate (Hill and Bradshaw, 1969). The assay was carried out in a volume of 1 ml containing 50mM phosphate buffer and 50mM malate (pH 7.8). The assay was started by the addition of the enzyme.

Mg^{2+}-ATPase was assayed as the Mg^{2+}-dependent release of inorganic phosphate from ATP (Douce et al., 1973). Enzyme preparations were incubated in 1 ml solutions containing 10mM ATP and 10mM $MgCl_2$ at 37°C for 20 min. At the end of the incubation, 1 ml of 20% trichloroacetic acid was added and the mixture centrifuged at 2000g for 5 min. An aliquot of the supernatant was assayed for inorganic phosphate by the procedure of Taussky and Shorr (1953).

Biotin carboxylase activity was assayed as the biotin- and ATP-dependent, radioactivity derived from $NaH^{14}CO_3$ which was stable to "CO_2 bubbling" (Guchhait *et al*, 1974a). The assay was carried out in a volume of 0.5 ml, containing: 0.1M triethanolamine-HCl (pH 8.0), 1mM ATP, 8mM $MgCl_2$, 10mM biotin, 3mM glutathione, 0.3 mg BSA, 50 µl ethanol, 8mM $NaH^{14}CO_3$ (1.25 Ci/mol). The assay was started by the addition of enzyme and was incubated at 30°C for 10 min, after which the reaction was quickly cooled by the addition of 1.0 ml of ice-cold water, and following the addition of a drop of *n*-heptanol, CO_2 was bubbled through the solution for 30 min at 2°C to remove excess $H^{14}CO_3^-$ and the residual radioactivity determined. Assays without added ATP were used as blanks.

The biotin prosthetic group of acetyl-CoA carboxylase was labelled by carboxylation with $NaH^{14}CO_3$, using excess partially purified biotin carboxylase from *E. coli* (Guchhait *et al*., 1974a). The assay was carried out in a volume of 0.5 ml, containing: 0.1M triethanolamine-HCl (pH 8.0), 1mM ATP, 8mM $MgCl_2$, 0.18mM $NaH^{14}CO_3$ (60 Ci/mol), 0.3 mg BSA, 2 milliunits of biotin carboxylase (see Section 3.6). The assay was started by the addition of extract to be tested for the biotin prosthetic group and incubated at 37°C for 10 min. The assay was stopped and radioactivity in the biotin prosthetic group determined as described for the assay of biotin carboxylase.

3.3 Preparation of Cell-Free Extracts

Cell-free extracts from plant tissues were prepared at 4°C by homogenising about 5g of leaf material, cut into about 5 mm segments, in a chilled mortar and pestle with 1g of acid-washed sand and two volumes of cold 0.1M Tris-HCl buffer at pH 8.0 containing 20mM β-mercaptoethanol and 1mM EDTA (0.1M Tris buffer). The brei was filtered immediately through a layer of Miracloth and 1 ml of filtrate was de-salted on a Biogel P6 column, which had been previously

equilibrated with 0.1M Tris buffer. The green eluate of approximately 2 ml was collected and stored on ice.

3.4 Preparation of Chloroplasts

Chloroplasts were isolated from leaves, by homogenisation at $4^{o}C$, for 3 sec and 5 sec, with mixing if necessary in between, in a Waring Blendor at full speed with four volumes of 0.5M sucrose buffer (0.5M sucrose, 50mM Tricine, 20mM β-mercaptoethanol, 1mM $MgCl_2$, adjusted to pH 8.0 with NaOH). The brei was filtered through two layers of Miracloth, and the chloroplasts pelleted at 2000g for 1 min (Hawke et al., 1974a). The chloroplasts were resuspended in a minimal volume of 0.5M sucrose buffer, filtered through a layer of Miracloth and centrifuged through 0.6M sucrose at 800g for 15 min (Leese et al., 1971). The final chloroplast pellet was resuspended in 0.5M sucrose buffer.

Disruption of isolated chloroplasts was carried out by passage of a 1 ml aliquot of isolated chloroplasts through a column (bed volume 10 ml) of Biogel P6, previously equilibrated with 0.1M Tris-HCl (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA. The green eluate was collected and homogenised in a glass Ten-Broek homogeniser to ensure complete disruption. This procedure not only removed the low molecular weight osmoticum, sucrose, causing disruption of the chloroplasts, but also the low molecular weight substrate, RuDP, whose carboxylation by RuDP carboxylase interfered with the assay for acetyl-CoA carboxylase activity.

3.5 Preparation of Mesophyll Protoplasts and Bundle Sheath Strands

Maize leaves were cut into 0.5 mm transverse segments and digested with a 2% solution of Cellulysin in 0.6M sorbitol, 20mM MES buffer and 5mM $MgCl_2$ adjusted to pH 5.5 with NaOH,

for 3h at 30°C with gentle shaking at 30 rev/min (Kanai and Edwards, 1973a). At the end of the incubation the leaf segments were filtered through an 80 µm nylon net and washed with a solution of 0.6M sorbitol, 50mM Tricine, 5mM $MgCl_2$ adjusted to pH 8.0 with NaOH (0.6M sorbitol buffer). The combined filtrate and washings were centrifuged at 200g for 3 min and the supernatant discarded. The pellet of crude mesophyll protoplasts was resuspended in 0.6 ml of 0.6M sorbitol buffer and dispersed in a mixture of 1.65 ml 30% (w/w) polyethylene glycol 6000, 4.5 ml 20% (w/w) Dextran T40, 1.5 ml 2.4M sorbitol and 0.45 ml 0.2M sodium phosphate buffer (pH 7.8). The dispersed protoplasts were centrifuged in a swinging bucket rotor at 300g for 6 min at 4°C (Kanai and Edwards, 1973b). The purified mesophyll protoplasts collected at the interface of the two-phase system, were recovered by means of a Pasteur pipette. The two-phase purification procedure was repeated and the protoplasts finally suspended in 0.6M sorbitol buffer and pelleted by centrifugation (300g for 3 min) and suspended in a small volume of 0.6M sorbitol buffer.

The bundle sheath strands were obtained from the material on the 80 µm nylon net and were purified by the filtration and sedimentation procedures of Kojima et al (1979). The material on the 80 µm nylon net was suspended in 12 ml of 0.6M sorbitol buffer and was allowed to settle for 5 min in a 15 ml centrifuge tube. The cuticular fragments floated to the top of the solution and were removed with a Pasteur pipette. The volume was made to 12 ml with 0.6M sorbitol buffer, mixed gently and again allowed to settle, and the cuticular material removed. The settling procedure was repeated once more, and the strands were collected by centrifugation at 400g for 1 min. The supernatant fluid was removed and the whole procedure repeated. Finally, the strands were collected by centrifugation and resuspended in 0.6M sorbitol buffer.

Extracts of mesophyll protoplasts and bundle sheath strands

were prepared by homogenisation in a glass Ten-Broek homogeniser, with a small volume of 50mM Tricine-NaOH (pH 8.0), containing 20mM β-mercaptoethanol and 1mM EDTA (50mM Tricine buffer) and filtering through a layer of Miracloth.

3.6 Preparation of E. coli Acetyl-CoA Carboxylase Components

The biotin carboxylase and biotin carboxyl-carrier protein (BCCP) of acetyl-CoA carboxylase of E. coli was partially purified by the procedure of Guchhait et al (1974a). E. coli B cells were grown in 30ℓ batches in a Fermacell Fermenter (New Brunswick Scientific Co., Inc.) in a medium containing: 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NaCl, 0.4% $(NH_4)_2SO_4$, 0.07% $MgSO_4.7H_2O$, 0.05% sodium citrate, 0.5% glucose and 0.05% peptone. At about full log-phase of growth, cells were harvested by centrifugation at 7,000g for 10 min, and washed twice with 0.1M potassium phosphate buffer (pH 7.0) containing 5mM β-mercaptoethanol and 1mM EDTA. Cells were broken by two passages through a French Pressure Cell at 20,000 psi and centrifuged at 20,000g for 30 min. The supernatant was fractionated with ammonium sulphate, and the protein fraction precipitated between 25% and 42% saturation of ammonium sulphate was dissolved in 5mM potassium phosphate buffer (pH 7.0) containing 20% glycerol, 5mM β-mercaptoethanol and 1mM EDTA, and dialysed overnight against the same buffer. The biotin carboxylase and BCCP components were separated by calcium phosphate gel fractionation. Sufficient calcium phosphate gel was added to give a gel: protein of 1:1 and stirred for 30 min. The gel was collected by centrifugation (10,000g, 15 min) and washed three times with 5mM potassium phosphate buffer (pH 7.0). The washings and supernatant containing BCCP were combined, and the protein precipitated at 80% saturation of ammonium sulphate was pelleted by centrifugation and stored at $-80^{o}C$.

Biotin carboxylase was eluted, by washing the gel three times with 0.12M potassium phosphate buffer (pH 7.0) con-

taining 5mM β-mercaptoethanol and 1mM EDTA. The washings were pooled, and the protein precipitated at 50% ammonium sulphate was collected by centrifugation and stored at -80°C.

3.7 Incorporation of [1-^{14}C] Acetate into Lipids

3.7.1 [1-^{14}C] acetate incorporation into lipids by isolated chloroplasts

The incorporation of [1-^{14}C] acetate into lipids by isolated chloroplasts was carried out in 15 ml stoppered tubes in a volume of 250 µl, containing: 0.3M sorbitol, 50mM Tricine-KOH, pH 7.8, 30mM $NaHCO_3$, 2.0mM ATP, 0.5mM CoA, 1mM $MgCl_2$, 2.5mM DTT, 0.2mM NADPH, 0.2mM NADH, 0.25mM [1-^{14}C] acetate (16 Ci/mol) and chloroplasts equivalent to 30 - 70 µg of chlorophyll. Incubations were carried out at 20°C under an illumination of 20,000 lux from tungsten lamps, with agitation at 78 cycles/min for 20 - 30 min. Reactions were stopped by the addition of sufficient chloroform:methanol (2:1) to form a single phase. Water was added, and the chloroform layer washed separately with 1% acetic acid, 0.1M NaCl and three times with water. The final chloroform solution was dried under a stream of N_2 and the residue dissolved in 1 ml of chloroform. A suitable aliquot was dried in a scintillation vial and radioactivity determined.

3.7.2 [1-^{14}C] acetate incorporation into lipids by leaf slices

One gram of fresh leaf tissue was sliced transversely into 1 mm strips and incubated in 5 ml of 0.1M phosphate buffer pH 7.4, 50mM $NaHCO_3$ and 5 µCi of [1-^{14}C] acetate (0.08 µmoles), for 1h at 30°C under 20,000 lux illumination. Lipid was extracted with chloroform:methanol (2:1) by the method of Bligh and Dyer (1959), and washed successively with 1% acetic acid, 0.1M NaCl and three times with water. The final chloroform solution was dried under a stream of

nitrogen and the residue dissolved in 1 ml of chloroform. An appropriate aliquot was dried in a scintillation vial and radioactivity determined.

3.8 Preparation of Maize Leaf Sections

Lipid synthesis and acetyl-CoA carboxylase activity was measured in defined segments of the emerging second leaf from 8 day old, light-grown maize plants. These leaves show successive stages of cell and plastid differentiation, with immature tissue near the base and mature tissue in the distal sections of the leaves (Leech et al, 1973). Each leaf from about 500 - 700 plants was cut transversely into four sections of 2 cm length (leaf sections A - D) from the base and the remaining distal section (E) was 2 - 5 cm in length.

3.9 Preparation of Sepharose-N-Propane

Hydrophobic column chromatography as described by Shaltiel (1974), with Sepharose-N-propane was used to purify acetyl-CoA carboxylase. 100g of Sepharose 2B was activated with 10g of cyanogen bromide at pH 11. The activated Sepharose was suspended in 100 ml of 0.1M $NaHCO_3$ (pH 9.5) and mixed with 25 ml of propylamine, 50 ml $NaHCO_3$ (pH 9.5) and 50 ml N,N-dimethylformamide. The coupling was allowed to proceed for 10h at room temperature, then the resin was washed successively with 500 ml of water, 0.2M acetic acid, water, 50mM NaOH, water, dioxane-water (1:1), 0.2M acetic acid and finally with 2ℓ of water. When not in use the resin was stored in 0.02% NaN_3 at $4^{o}C$.

3.10 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in 0.7 cm

x 10 cm glass tubes, according to the system of Davis (1964). The separating gel was 7% acrylamide, 0.23% bis-acrylamide dissolved in buffer (0.06M HCl, Tris to pH 8.9) and N,N,N', N'-tetramethylethylenediamine (TEMED) (50 µl per 100 ml). The stacking gel was 2.5% acrylamide, 0.625% bis-acrylamide, 1% sucrose dissolved in buffer (0.062 Tris-HCl, pH 6.7) and TEMED (50 µl per 100 ml). Gels were polymerised with ammonium persulphate (100 mg per 100 ml), which was dissolved in the gel just before pouring.

Protein samples were dialysed for 2h against electrode reservoir buffer (38.5 mM glycine, Tris to pH 8.3), before electrophoresis, and were applied to the gels in a solution containing 20% glycerol and 0.005% bromophenol blue. Electrophoresis was carried out with a current of 2mA per gel, until the tracking dye entered the separating gel, then at 4mA per gel. At the end of electrophoresis, the gels were removed from the tubes with a fine needle, the tracking dye marked with a piece of fine wire, and the gels stained for 2h at 50°C in a solution of 0.25% Coomassie Brilliant Blue R250 in methanol/acetic acid/water (9:1:10). Excess stain was removed by diffusion in methanol/acetic acid/water (9:1:10) and the gels were stored in 7% acetic acid, for up to 2 weeks, before being photographed.

3.11 Centrifugation

Ultracentrifugations and sucrose density gradient centrifugations were carried out on a Beckman Model L2-65B Ultracentrifuge. Centrifugations at low speeds were performed in Sorvall RC-2B or RC-3 centrifuges. All centrifugations were carried out at 4°C, and all centrifugal forces are quoted as g max values.

Isolated chloroplasts (Section 3.4) were centrifuged on a discontinuous sucrose density gradient (Miflin and Beevers, 1974), composed of a 4 ml cushion of 60% (w/w) sucrose, overlayed with 6 ml of a linear gradient from 60% (w/w) to 42% (w/w) sucrose, 5 ml of 42% (w/w) sucrose, 10 ml of a linear gradient from 42% (w/w) to 30% (w/w) sucrose and a final

3 ml of 30% (w/w) sucrose. Isolated chloroplasts were overlayed on the gradient and centrifuged in a SW27 rotor at 4,000 rpm for 5 min and then at 10,000 rpm for a further 10 min. Gradients were fractionated on an ISCO Density Gradient Fractionator, into 1.2 ml fractions.

Isolated disrupted chloroplasts (Section 3.4) were separated into stromal and membrane fractions by centrifugation on a discontinuous sucrose density gradient composed of 2 ml of 60% (w/w) sucrose and 9 ml of 15% (w/w) sucrose. Disrupted chloroplasts were overlayed on the gradient and centrifuged in an SW41Ti rotor at 286,000g for 1h. Fractionation of the gradient into 1.2 ml fractions was carried out on an ISCO Density Gradient Fractionator.

All sucrose solutions were prepared in 50mM Tricine-NaOH pH 8.0 buffer containing 20mM β-mercaptoethanol and 1mM EDTA.

3.12 Analytical Methods

3.12.1 Determination of protein

Protein was determined by the Coomassie dye binding method of Bradford (1976), and by absorbance measurements at 280nm. Bovine serum albumin was used as the standard protein in the protein dye binding procedure. Solutions of bovine serum albumin were prepared in 0.01M phosphate buffer (pH 7.2) and standardized assuming $A^{1\%}_{1cm}$ at 279nm of 6.67 (Foster and Sterman, 1956).

3.12.2 Determination of chlorophyll

Chlorophyll was determined in 80% (v/v) acetone as described by Arnon (1949).

3.13 Determination of Radioactivity

^{14}C radioactivity was determined with a Beckman Model LS8000 Scintillation Counter. Aqueous samples were counted in Triton X-100/toluene (1:2, v/v) containing 0.4% PPO and 0.01% POPOP. Non-aqueous samples were counted in toluene containing 0.4% PPO and 0.01% POPOP. Carboxylase activities were determined by drying reaction samples on 1 cm x 1 cm squares of 3MM paper and counting as a non-aqueous sample.

Radioactivity on chromatograms was detected by scanning on a Packard Model 7200 Radiochromatogram Scanner.

3.14 Chromatographic Procedures

3.14.1 Gel filtration chromatography

Gel filtration chromatography was carried out in a Pharmacia K15/90 column, packed with Ultrogel AcA22 as instructed by the manufacturer. Buffer was pumped with an LKB Model 4912A Peristaltic Pump, at a rate of 4 ml/h and eluents were collected on an ISCO Model 328 Fraction Collector. Ultraviolet absorbance of the eluate was monitored with an ISCO Model UA-5 Absorbance Monitor.

3.14.2 Thin-layer chromatography

MN300 cellulose plates were prepared as instructed by the manufacturer, to a thickness of 1 mm, and allowed to air-dry at room temperature for 2 days.

All thin-layer chromatography was carried out at room temperature.

3.14.3 Paper chromatography

Paper chromatography was carried out in the ascending

direction in glass jars using Whatman No. 1 and 3MM paper.

3.15 Purification of Acetyl-CoA Carboxylase from Leaves

Acetyl-CoA carboxylase from leaves was purified by two procedures, Procedure I and II, to approximately the same degree of purity. The initial purification steps were identical in both procedures and are described below. All purification steps were carried out at $4^{o}C$.

450g of plant leaves were harvested and homogenised in a stainless steel Waring Blendor at full-speed for 30 sec, with two volumes of 0.1M Tris-HCl buffer (pH 8.0), containing 20mM β-mercaptoethanol and 1mM EDTA. The brei was filtered through a layer of Miracloth and one of nylon bolting cloth, and the filtrate centrifuged at 30,000g, in an SS34 rotor for 30 min. The pellet was discarded, and to the supernatant was added finely ground polyethylene glycol 6000 (PEG) to a level of 6g/100 ml, with constant stirring. After stirring for a further 15 min the precipitated protein was pelleted by centrifugation at 12,000g for 20 min in a GS3 rotor and discarded. PEG was added to the supernatant to a level of 8g/100 ml with constant stirring and the precipitated protein was pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA (10mM Tris buffer).

Procedure I:

The PEG fraction was loaded on to a column of Sepharose-N-propane (100 ml bed volume) which was previously equilibrated with 10mM Tris buffer. Unbound protein was washed off the column with 10mM Tris buffer and elution was carried out overnight with a linear gradient from 10mM Tris buffer (300 ml) to 0.2M KCl in 10mM Tris buffer (300 ml), at a rate of 30 ml/h.

The fractions with acetyl-CoA carboxylase at a specific activity greater than 80 nmol/min/mg were pooled and concentrated by ultrafiltration through a Diaflo PM30 ultrafiltration membrane, to a final volume of about 7 ml. Of this fraction, 5 ml was purified further by gel filtration through a column of Ultrogel AcA22, equilibrated with 10mM Tris buffer (Section 3.14.1). Fractions containing acetyl-CoA carboxylase of high specific activity were pooled and used to characterize the enzyme.

Procedure II:

In this procedure the purification steps up to the PEG fractionation were as described above, except only 100g of leaves were usually used. To the PEG fraction solid ammonium sulphate was added at pH 8.0 to a level of 24.4 g/100 ml. The precipitated protein was collected by centrifugation and resuspended in 10mM Tris buffer, to give a final volume of about 5 ml. This fraction was purified further by gel filtration on a column of Ultrogel AcA22 as in Procedure I. Fractions which contained acetyl-CoA carboxylase were pooled and used to characterize the enzyme.

CHAPTER 4

RESULTS

GENERAL FEATURES AND STABILITY OF LEAF ACETYL-CoA CARBOXYLASE

4.1 General Features of Acetyl-CoA Carboxylase Activity

Since acetyl-CoA carboxylase is a poorly characterized enzyme in all except a few plant species, namely spinach (Mohan and Kekwick, 1980) and barley (Thomson and Zalik, 1981), initial work in this study on maize tissue was concerned with the demonstration of acetyl-CoA carboxylase activity in cell-free extracts. An acetyl-CoA-dependent, acid-stable radioactive product derived from $NaH^{14}CO_3$ was found to accumulate in maize leaf cell-free extracts. The product was identified as malonic acid after alkali hydrolysis, by thin-layer chromatography (Huang, 1970) (Fig. 2) and paper chromatography (Denison and Phares, 1952) (Fig. 3). The $H^{14}CO_3^-$-fixing activity showed an absolute requirement for $MgCl_2$ and ATP, and was stimulated to some extent by DTT and KCl. Avidin, a biotin-binding protein, was found to be a potent inhibitor, but pretreatment of avidin with biotin prevented the inhibition (Table II). These features are characteristic of previously-characterized acetyl-CoA carboxylases from other sources (Lane *et al.*, 1974), thus confirming that this $H^{14}CO_3^-$-fixing activity in maize leaf cell-free extracts was due to acetyl-CoA carboxylase.

Acetyl-CoA carboxylase activity in cell-free extracts was found to be proportional to the amount of protein in the assay, up to 100 µg (Fig. 4) and was also linear with time of incubation, up to about 15 min (Fig. 5). Therefore subsequent assays contained less than 100 µg of protein and were incubated for 5 min.

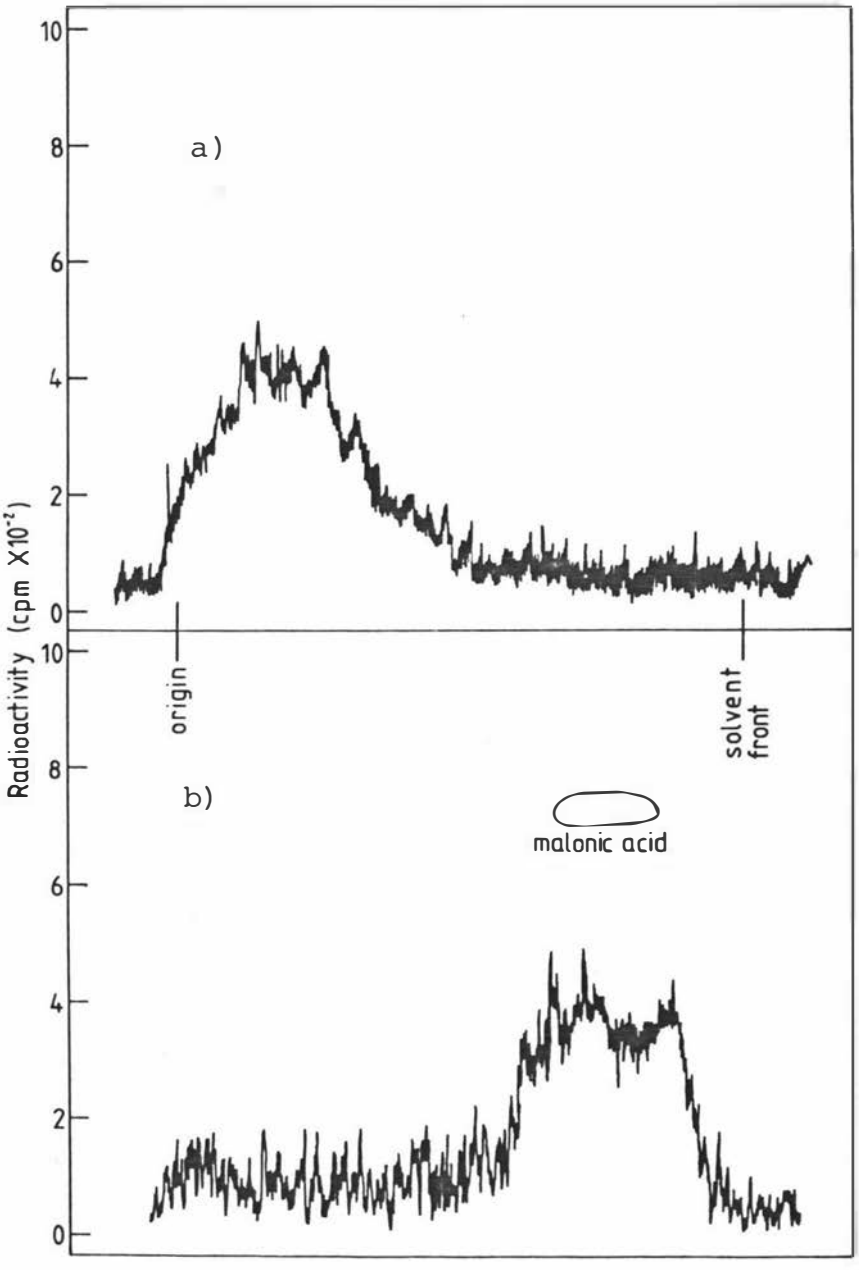


FIGURE 2: Identification of the product of the acetyl-CoA carboxylase assay by TLC.

50 µl of the products of a standard acetyl-CoA carboxylase assay was chromatographed on a Gelman TLC plate (ITLC-SG, Type 20), (a) before and (b) after hydrolysis with 2M KOH. The chromatogram was developed in water-saturated ether/formic acid (7:1). After drying, the plates were scanned for radioactivity and malonic acid detected by spraying with with a neutral to slightly alkaline solution of bromocresol green (400 mg/l) in 95% ethanol. Authentic malonic acid (2 µmoles) was used as a standard.

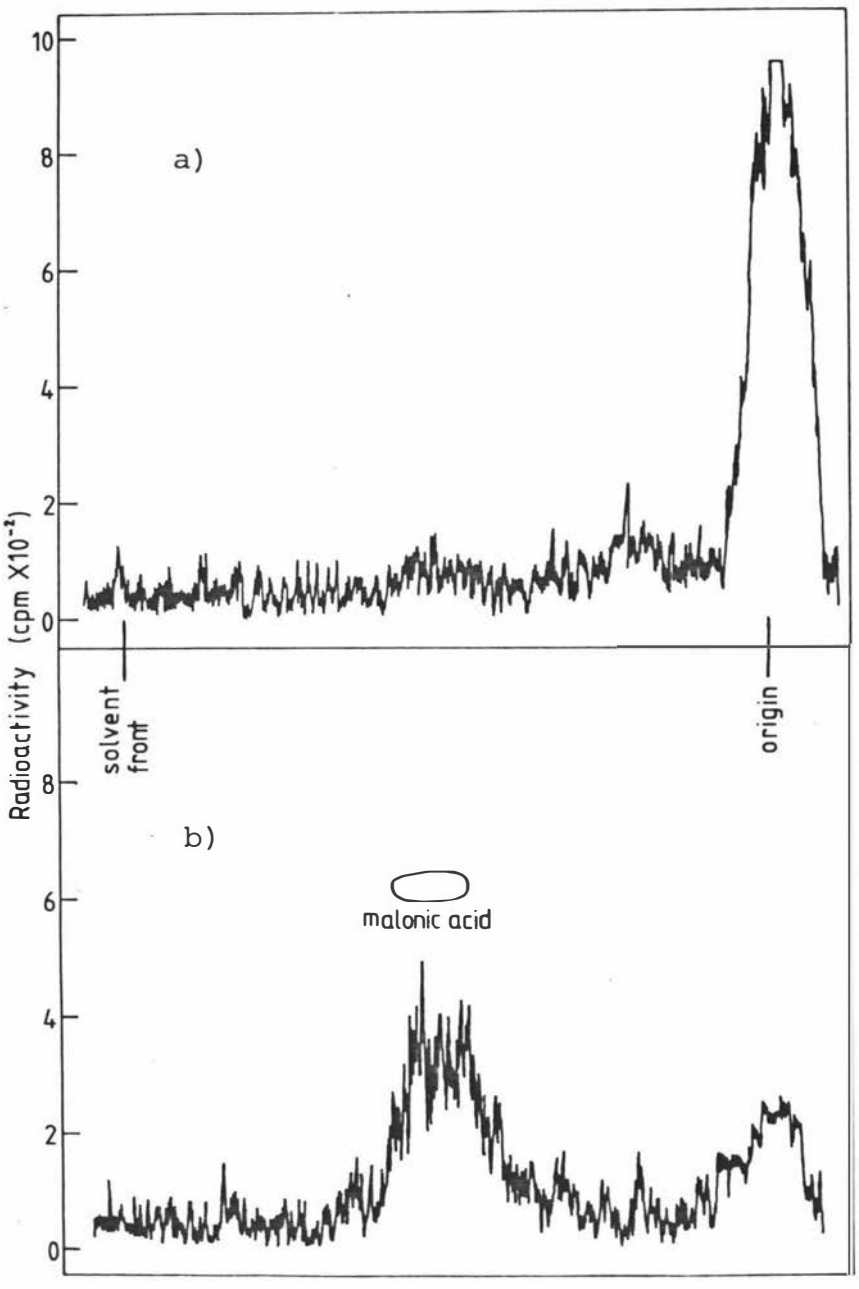


FIGURE 3: Identification of the product of the acetyl-CoA carboxylase assay by paper chromatography.

50 µl of the products of a standard acetyl-CoA carboxylase assay was chromatographed on Whatman No. 1 paper (20 cm x 5 cm), (a) before and (b) after hydrolysis with 2M KOH. The chromatogram was developed in diethyl ether/glacial acetic acid/water (13:3:1), and after drying was scanned for radioactivity. Malonic acid was detected with a neutral to slightly alkaline solution of bromocresol green (400 mg/l) in 95% ethanol. Authentic malonic acid (2 µmoles) was used as a standard.

TABLE II

COFACTOR REQUIREMENTS OF ACETYL-CoA CARBOXYLASE ACTIVITY IN CELL-FREE EXTRACTS OF MAIZE

Omission	$H^{14}CO_3^-$ fixed/assay (dpm)
None	16,025
Acetyl-CoA	885
ATP	120
$MgCl_2$	190
$H^{14}CO_3^-$	0
DTT	14,475
KCl	14,775
Maize leaf extract (70µg protein)	80
None + Avidin (0.2 units)	65
None + Avidin (0.2 units) pretreated with biotin	15,086

Assay conditions were as described in Section 3.2, with 30mM $NaHCO_3$.

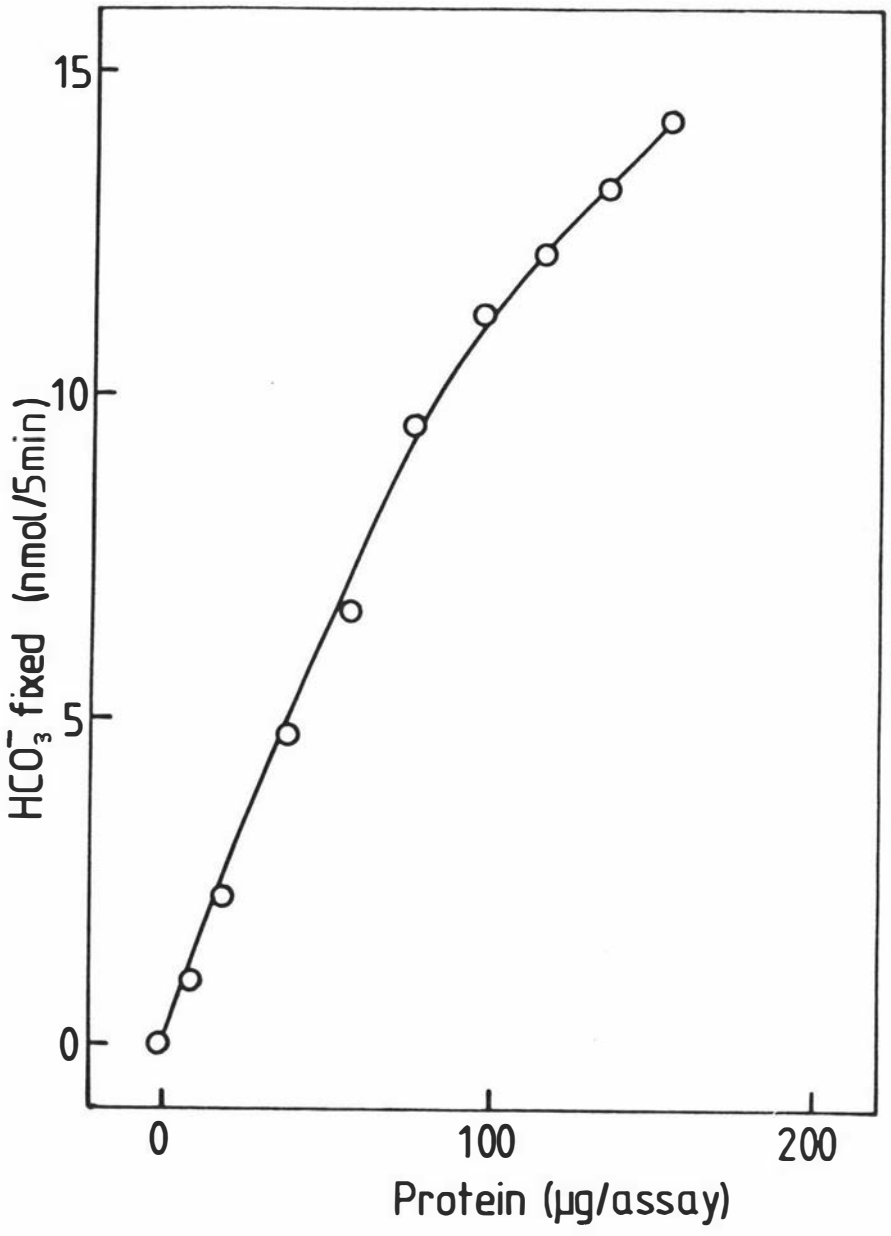


FIGURE 4: Dependence of acetyl-CoA carboxylase activity on protein levels of cell-free extracts of maize leaves.

Cell-free extracts of maize leaves were prepared as in Section 3.3. Assay conditions: as described in Section 3.2 using 10mM HCO_3.

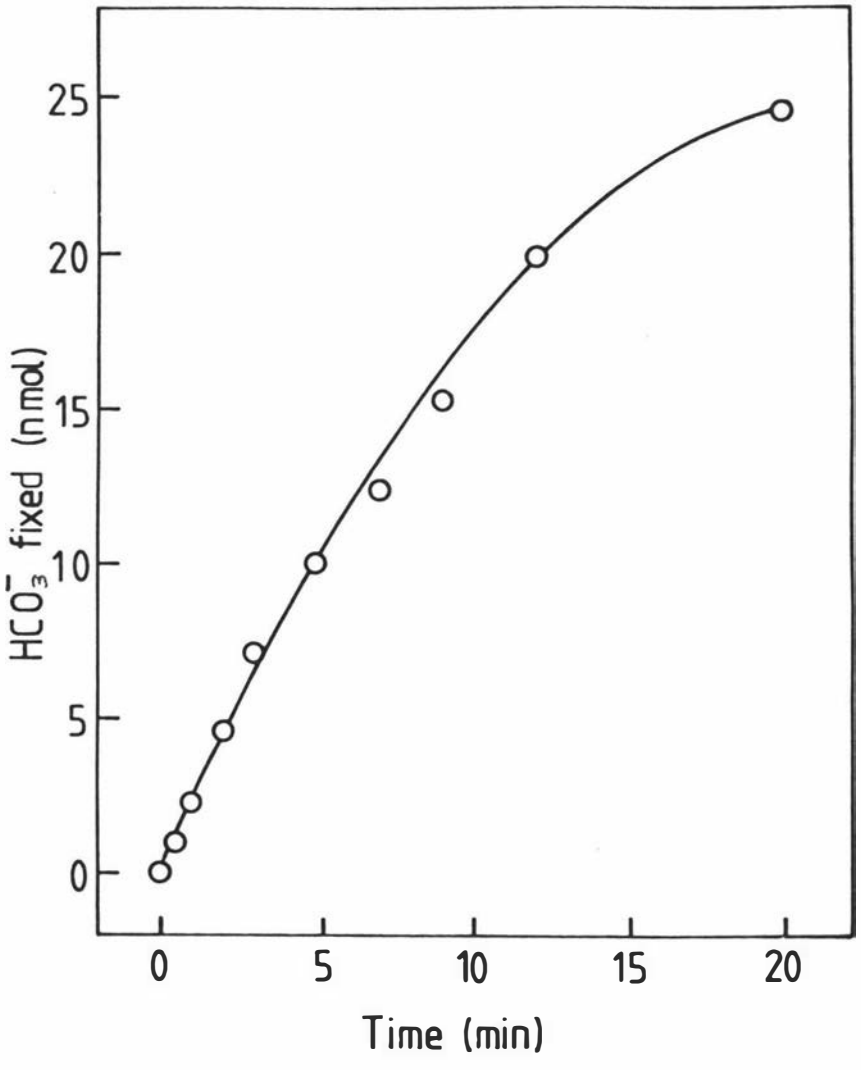


FIGURE 5: Effect of time on acetyl-CoA carboxylase activity in cell-free extracts of maize leaves.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, and cell-free extract of maize leaf containing 98 µg of protein, prepared as in Section 3.3.

4.2 Stabilization of Acetyl-CoA Carboxylase in Cell-Free Extracts by Sulfhydryl Protecting Agents

It was clearly advantageous to stabilize acetyl-CoA carboxylase activity in cell-free extracts, in order to proceed with the purification of this enzyme. As in almost all previous studies of acetyl-CoA carboxylase, workers had included a sulfhydryl protecting agent in the isolation buffer, a study was carried out to investigate the effect of these agents on the activity of the maize leaf enzyme.

In the absence of a sulfhydryl reagent, acetyl-CoA carboxylase in cell-free extracts was relatively unstable. Acetyl-CoA carboxylase activity was lost within 200 min of the preparation of the maize cell-free extract (Fig. 6), and in barley cell-free extracts activity was reduced by 70% in 6h (Fig. 7). The addition of β-mercaptoethanol to the cell-free extracts of both maize (Fig. 6) and barley (Fig.7) leaves stabilized acetyl-CoA carboxylase activity. Storage of the cell-free extracts under an atmosphere of nitrogen appeared to improve the stability of the enzyme, although not to the extent of β-mercaptoethanol.

Acetyl-CoA carboxylase activity in cell-free extracts of spinach leaves was found difficult to determine due to the very low levels present. This problem was overcome by precipitating the enzyme with polyethlene glycol and resuspending the precipitate in a small volume of buffer. Spinach leaf acetyl-CoA carboxylase activity was unstable, and was lost during the time of the experiment (about 1h), when 0.1M Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA was used (Table III). Following the publication of Mohan and Kekwick's (1980) work on acetyl-CoA carboxylases from avacado plastids and spinach chloroplasts, it was confirmed that inclusion of 15% glycerol and 0.1% BSA in the isolation buffer stabilized acetyl-CoA carboxylase in cell-free extracts of spinach leaves (Table III). The inclusion of 15% glycerol and 0.1% BSA in buffers did

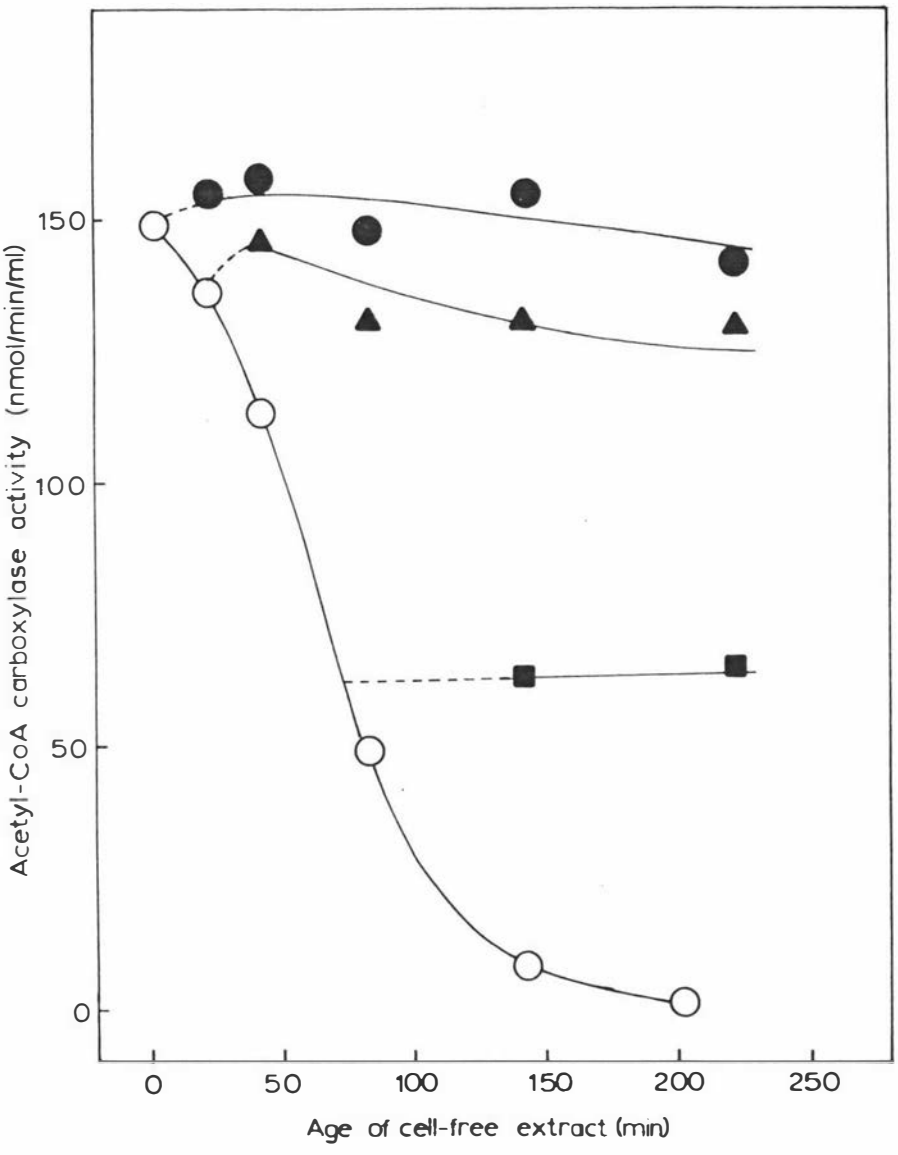


FIGURE 6: Stabilization of acetyl-CoA carboxylase activity in cell-free extracts of maize by β-mercaptoethanol.

Activity of acetyl-CoA carboxylase in the cell-free extracts of maize without β-mercaptoethanol (○). 2 min (●), 30 min (▲), and 70 min (■) after preparation the cell-free extracts contained 20mM β-mercaptoethanol. Assay conditions: as described in Section 3.2 using 30mM HCO_3^-.

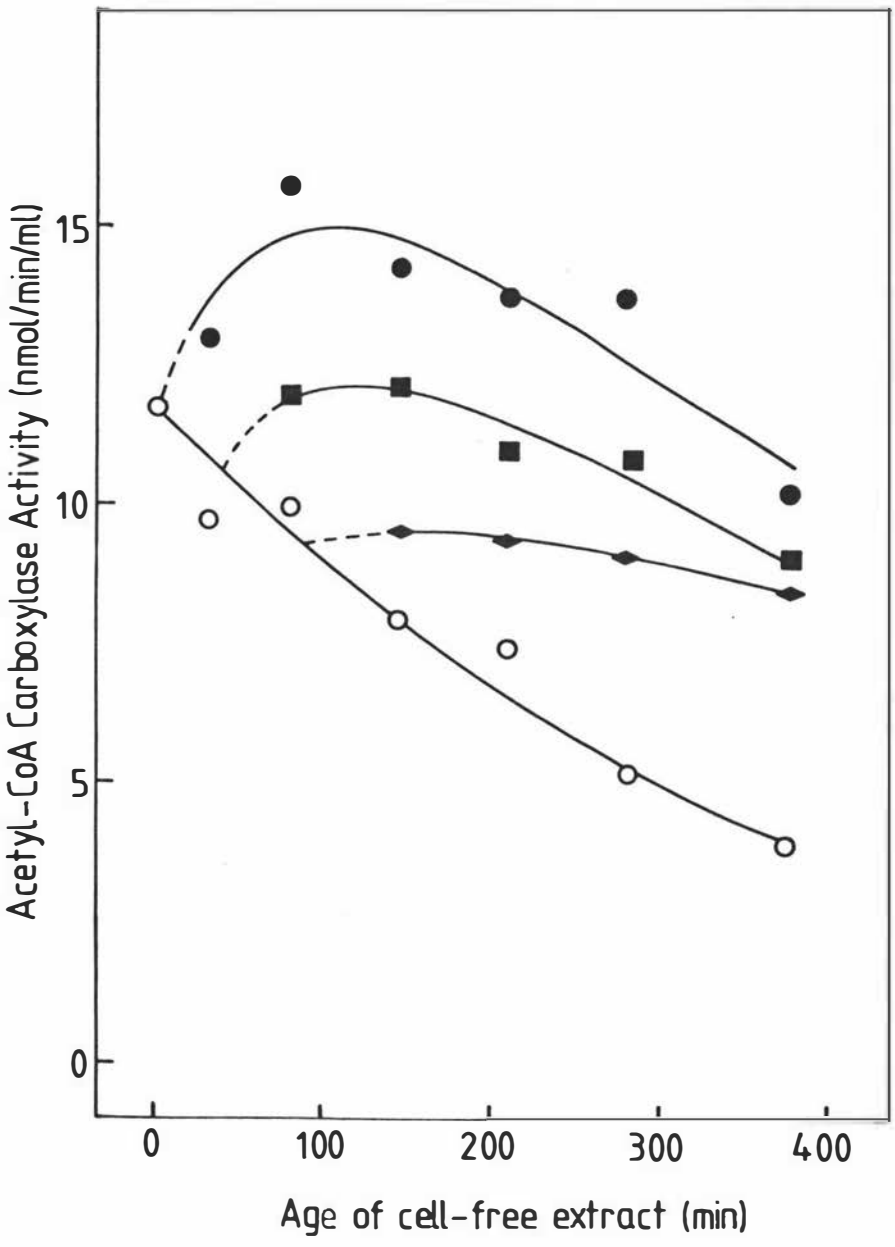


FIGURE 7: Stabilization of acetyl-CoA carboxylase activity in cell-free extracts of barley by β-mercaptoethanol.

Activity of acetyl-CoA carboxylase in the cell-free extracts of barley without β-mercaptoethanol (○). 1 min (●), 40 min (■), and 90 min (◆) after preparation the cell-free extracts contained 20mM β-mercapto-ethanol. Assay conditions: as described in Section 3.2 using 10mM $NaHCO_3$.

TABLE III

ACETYL-CoA CARBOXYLASE ACTIVITY IN CELL-FREE EXTRACTS OF SPINACH LEAVES

Fraction	Acetyl-CoA Carboxylase Activity (nmol/min/40g tissue)	
	A	B
Cell-free extract	460	623
30,000g supernatant	zero	412
6-14% PEG	zero	122

Two batches, 40g fresh weight of spinach leaves (A and B), were homogenised, centrifuged at 30,000g and fractionated with polyethylene glycol (PEG), as described in Section 3.15. The buffer used with batch A was 0.1M Tris-HCl (pH 8.0), containing 20mM β-mercaptoethanol and 1mM EDTA, while with batch B the buffer was 0.1M Tris-HCl (pH 8.0), containing 20mM β-mercaptoethanol, 1mM EDTA, 15% glycerol and 0.1% BSA. Acetyl-CoA carboxylase activities were determined in standard assays (Section 3.2), using 10mM $NaHCO_3$.

not enhance the stability of acetyl-CoA carboxylase in cell-free extracts of either maize or barley leaves.

LOCATION OF ACETYL-CoA CARBOXYLASE IN MAIZE LEAVES

4.3 Location of Acetyl-CoA Carboxylase in Chloroplasts of Maize Leaves

Chloroplasts isolated from the second leaf of maize seedlings were centrifuged on the discontinuous sucrose density gradient described in Section 3.11. Fractionation of the gradient showed two clearly defined regions containing chlorophyll (Fig. 8). The chlorophyll peak of higher density contained RuDP carboxylase (83% of the activity applied to the gradient) and NADPH-malate dehydrogenase (95% of the activity applied to the gradient), which are commonly used enzymes for the characterization of intact chloroplasts from bundle sheath and mesophyll cells, respectively, of C_4 plants. Observation by phase-contrast microscopy confirmed the intact nature of the chloroplasts in this region of the gradient. The absence of these enzymes from the chlorophyll band of lighter density indicated that this band contained broken chloroplasts, which was consistent with their appearance in phase-contrast microscopy. The single zone of acetyl-CoA carboxylase activity (107% of the activity applied to the gradient) corresponded to the intact chloroplast band as indicated by the marker enzymes. In contrast, no detectable acetyl-CoA carboxylase activity was found in the fractions containing broken chloroplasts.

A high purity of the chloroplast preparation was indicated by the absence of detectable PEP carboxylase, fumarase and catalase, which are cytoplasmic, mitochondrial and peroxisomal enzymes, respectively. Centrifugation of the chloroplast preparation through a 0.6M sucrose layer, prior to density gradient centrifugation, removed non-chloroplastic

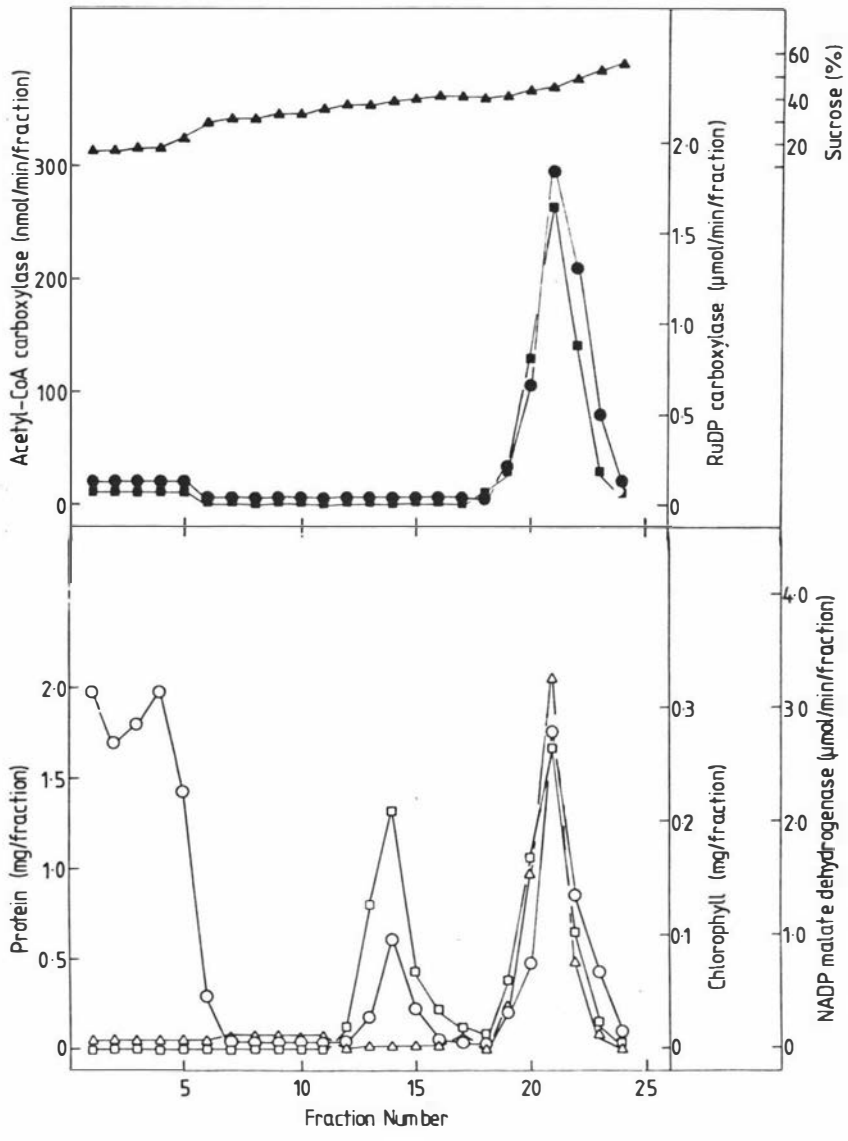


FIGURE 8: Distribution of enzymes, chlorophyll and protein following the centrifugation of purified maize chloroplasts in a sucrose density gradient.

Chloroplasts isolated from maize leaves (Section 3.4) were centrifuged in a sucrose density gradient described in Section 3.11. Fractionation of the gradient was carried out on an ISCO Gradient Fractionator. Sucrose (▲), acetyl-CoA carboxylase (●), RuDP carboxylase (■), protein (o), chlorophyll (□), and NADPH-malate dehydrogenase (△).

contaminants and enzymes released from chloroplasts broken during the isolation procedure. The lack of chloroplastic enzymes at the top of the sucrose density gradient was indicative of the minimal breakage of chloroplasts during density gradient centrifugation. The large protein band at the top of the gradient was due to the BSA which was included in the chloroplast isolation buffer. From the proportion of chlorophyll, in the two chlorophyllous bands, it was estimated that about 60% of the chloroplasts were intact, which agrees with phase-contrast microscopy observation of the chloroplast preparation prior to density gradient centrifugation (Fig. 9).

4.4 Solubility of Maize Chloroplastic Acetyl-CoA Carboxylase

Although there is now increasing evidence that chloroplastic acetyl-CoA carboxylase is a stromal enzyme (Mohan and Kekwick, 1980; Thomson and Zalik, 1981), at the time this investigation was begun the soluble nature of this enzyme was not established. In isolated maize chloroplasts, which were disrupted by passage through a desalting column (Section 3.4), the solubility characteristics of acetyl-CoA carboxylase were investigated by centrifugation on a discontinuous density gradient (Section 3.11). The stromal content of the chloroplasts was efficiently separated from the membrane fractions by this procedure, as judged by the distribution of the marker enzymes in the gradient (Fig. 10). The coincidence of RuDP carboxylase (107% of the activity applied to the gradient) and acetyl-CoA carboxylase (111% of the activity applied to the gradient) activities in the gradient confirmed the recent findings of the stromal location of acetyl-CoA carboxylase.

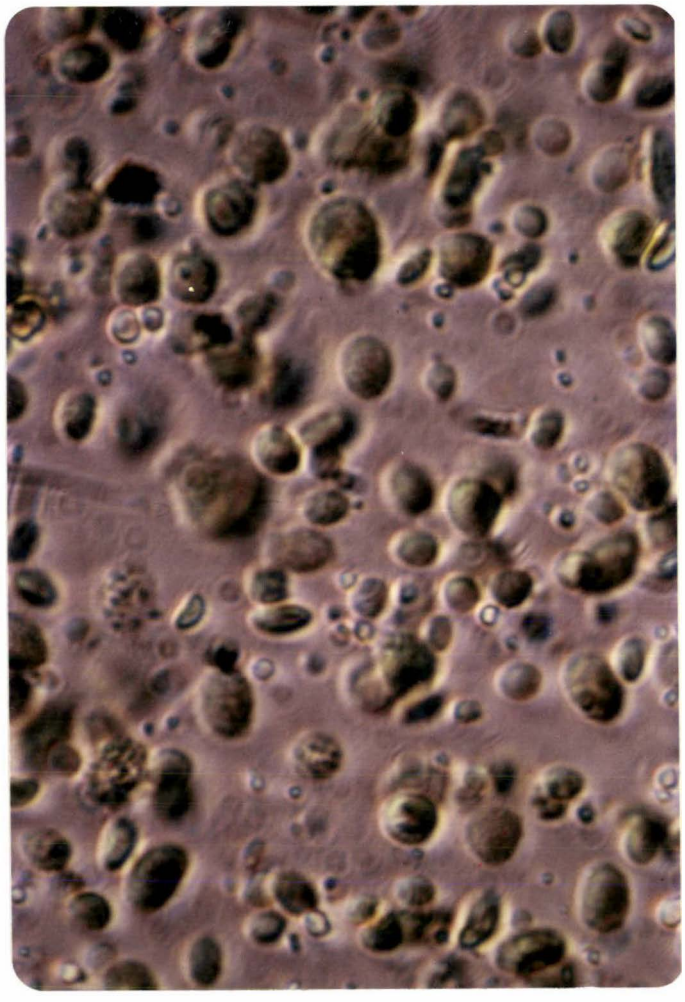


FIGURE 9: Representative field of a preparation of isolated maize chloroplasts.

Chloroplasts were isolated from maize leaves as described in Section 3.4, and photographed under phase-contrast. Magnification 1600x.

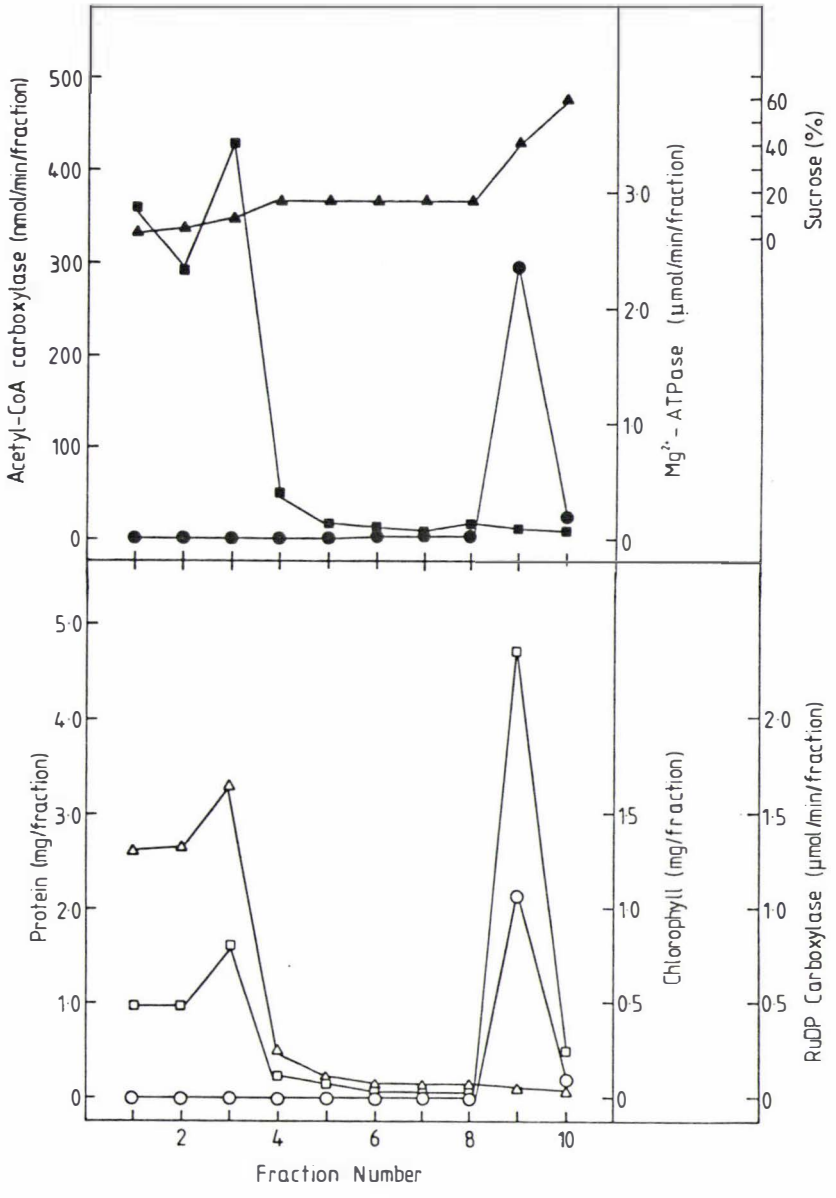


FIGURE 10: Distribution of enzymes, chlorophyll, and protein following the centrifugation of disrupted maize chloroplasts in a discontinuous sucrose density gradient.

Chloroplasts isolated from maize leaves were disrupted by a desalting procedure (Section 3.4) and centrifuged in a discontinuous sucrose density gradient as described in Section 3.11. Fractionation of the gradient was carried out on an ISCO Gradient Fractionator. Sucrose (▲), acetyl-CoA carboxylase (■), Mg^{2+}-ATPase (●), RuDP carboxylase (△), protein (□) and chloroplyll (○).

4.5 Location of the Biotin Prosthetic Group of Chloroplastic Acetyl-CoA Carboxylase

Although a stromal acetyl-CoA carboxylase was demonstrated in chloroplasts of maize (Section 4.4), the possibility of a second enzyme requiring membrane bound biotin still existed (Kannangara and Stumpf, 1972). The location of the biotin prosthetic group of acetyl-CoA carboxylase within maize chloroplasts was therefore investigated, by carboxylation of the biotin moiety with $H^{14}CO_3^-$ using a biotin carboxylase preparation from E. coli (Section 3.6). Biotin carboxylase was prepared free of all other components of the E. coli acetyl-CoA carboxylase, BCCP and carboxyltransferase, by adsorption on to a calcium phosphate gel (Section 3.6). Prior to separation of the components of the enzyme, total acetyl-CoA carboxylase activity increased during purification (Table IV), perhaps due to the removal of the inhibitor reported previously (Guchhait et al., 1974a).

As well as carboxylating free biotin, biotin carboxylase appeared to carboxylate the biotin prosthetic group of acetyl-CoA carboxylase of disrupted maize chloroplasts. This reaction required ATP and $MgCl_2$ and was inhibited by avidin (Table V), properties which were analogous to the carboxylation of BCCP in E. coli (Guchhait et al., 1974a). In order to demonstrate that the above reaction carboxylated the biotin prosthetic group of maize acetyl-CoA carboxylase, the transference of the radioactive label from carboxyl-biotin to acetyl-CoA was examined. Following the carboxylation of the biotin prosthetic group in disrupted maize chloroplasts with biotin carboxylase of E. coli and $H^{14}CO_3^-$, in the absence of acetyl-CoA, by the procedure described in Section 3.2, 5250 dpm were stable to gassing with CO_2 (Table VI). Most of this radioactive label was unstable in acid conditions, consistent with previous findings of the stability of carboxyl-biotin (Guchhait et al., 1974b). However, upon further incubation with added acetyl-CoA and fresh maize chloroplast extract, 85% of the radioactivity fixed to

TABLE IV

PREPARATION OF BIOTIN CARBOXYLASE FROM *E. coli*

Fraction	Total Protein (mg)	Acetyl-CoA Carboxylase		Biotin Carboxylase	
		Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)
Cell-free extract	1135	36.3	0.032	n.d.	-
20,000g supernatant	928	180.4	0.194	n.d.	-
25 - 42% ammonium sulphate	240	489.3	2.040	n.d.	-
5mM phosphate wash of gel	102	none	-	none	-
0.12M phosphate wash of gel	85	none	-	861	10.1

Details of the purification procedures used are given in Section 3.6. Acetyl-CoA carboxylase and biotin carboxylase activities were assayed as the acetyl-CoA- and biotin-dependent fixation of $H^{14}CO_3^-$, respectively, as described in Section 3.2. n.d. = not determined.

TABLE V

COFACTOR REQUIREMENTS FOR THE CARBOXYLATION OF THE BIOTIN PROSTHETIC GROUP IN MAIZE CHLOROPLASTS BY BIOTIN CARBOXYLASE OF *E. coli*

Omission	$H^{14}CO_3^-$ fixed/assay (dpm)
None	15,113
ATP	579
$MgCl_2$	300
Biotin carboxylase	387
Chloroplast extract (29µg chlorophyll)	285
None + Avidin (0.2 units)	788

Details of the conditions for the carboxylation of the biotin prosthetic group of acetyl-CoA carboxylase are given in Section 3.2. Chloroplasts were isolated from maize leaves and disrupted by a desalting procedure (Section 3.4). Biotin carboxylase of *E. coli* was prepared as described in Section 3.6.

TABLE VI

MALONYL-CoA FORMATION FROM CARBOXYL-BIOTIN BY MAIZE CHLOROPLASTS

Treatment of assay	$H^{14}CO_3^-$ fixed/assay (dpm)
Gassing with CO_2	5250
Acidified with 100µl 6M HCl	320
Gassing with CO_2 + incubation with acetyl-CoA and maize chloroplast extract and then acidified with 100µl 6M HCl	4450

The biotin prosthetic group in three separate aliquots of disrupted maize chloroplasts, equivalent to 10µg chlorophyll, was carboxylated with biotin carboxylase (Section 3.2). Following which, the assays were either gassed with CO_2 or acidified with 100µl 6M HCl or incubated further at 30°C for 15 min after the addition of acetyl-CoA (0.3mM) and disrupted maize chloroplasts equivalent to 20µg of chlorophyll, and then acidified with 100µl 6M HCl.

carboxyl-biotin became acid-stable, consistent with the formation of ^{14}C-malonyl-CoA (Table VI).

Chloroplasts isolated from maize, barley and spinach leaves were disrupted by passage through a desalting column (Section 3.4), and the stromal and membrane fractions separated by centrifugation on discontinuous sucrose density gradients described in Section 3.11. 91 - 106% of the biotin carboxylated by biotin carboxylase of E. coli in disrupted chloroplasts, was recovered in the stromal fractions, while the membrane fractions contained only 3 - 7% of the carboxylated biotin (Table VII).

4.6 Distribution of Acetyl-CoA Carboxylase Between Mesophyll and Bundle Sheath Cells of Maize Leaves

Plants which fix CO_2 by the so-called 'C_4-pathway', such as maize, distribute certain enzymes differentially between the two types of chlorophyllous cells, mesophyll and bundle sheath (Slack et al., 1969). Following the digestion of maize leaves with cellulase, mesophyll protoplasts and strands of bundle sheath cells were obtained (Section 3.5) virtually free of cross-contamination, as judged by the marker enzymes PEP carboxylase and RuDP carboxylase (Slack et al., 1969) (Table VIII) and observation by phase-contrast microscopy (Fig. 11 and 12). Acetyl-CoA carboxylase activity was found in both cell types, with the specific activity somewhat greater in the mesophyll protoplasts.

LEVELS OF ACETYL-CoA CARBOXYLASE ACTIVITY IN LEAVES

4.7 Investigation of the Relationship Between Leaf Development, Acetyl-CoA Carboxylase Activity and [1-^{14}C]acetate Incorporation into Lipids

The partially emerged second leaves of maize seedlings have

TABLE VII

DISTRIBUTION OF THE BIOTIN PROSTHETIC GROUP IN CHLOROPLASTS ISOLATED FROM THE LEAVES OF THREE PLANTS

Chloroplast Source	dpm $H^{14}CO_3^-$ fixed		
	Broken Chloroplasts	Stroma	Membranes
Maize	1,005,007	1,069,133	40,238
Spinach	491,096	480,203	14,966
Barley	88,173	84,491	6,760

Chloroplasts were isolated from maize, spinach and barley leaves and disrupted by a desalting procedure (Section 3.4). The biotin prosthetic group of acetyl-CoA carboxylase was carboxylated with biotin carboxylase (Section 3.2) in the chloroplast extract, and the stromal and membrane fractions prepared by centrifugation on discontinuous sucrose density gradient (Section 3.11). Disrupted chloroplasts of maize, spinach and barley equivalent to 590µg, 420µg and 175µg of chlorophyll, respectively, were used in these experiments.

TABLE VIII

DISTRIBUTION OF ENZYMES BETWEEN MESOPHYLL PROTOPLASTS AND BUNDLE SHEATH STRANDS

Enzyme	Activity (nmol/min/mg protein)	
	MP	BSS
PEP carboxylase	1105	28
RuDP carboxylase	15	1670
Acetyl-CoA carboxylase	121	85

Extracts of mesophyll protoplasts and bundle sheath strands were prepared by enzymic digestion of maize leaves (Section 3.5). Assay conditions are described in Section 3.2. 10mM $NaHCO_3$ was used in the assays for acetyl-CoA carboxylase activity. MP = mesophyll protoplasts; BSS = bundle sheath strands.

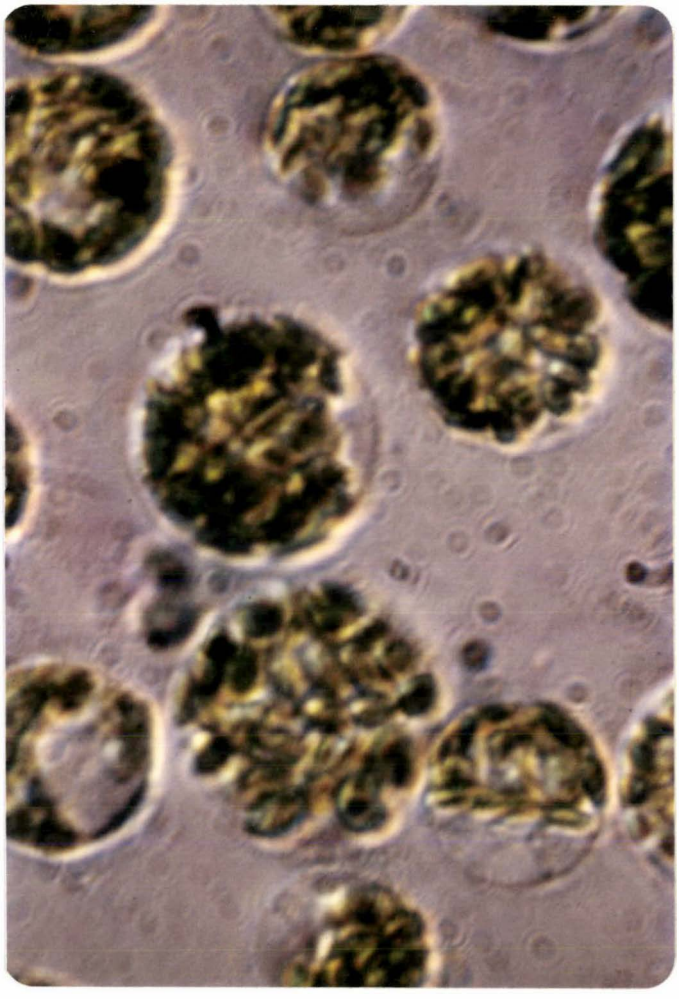


FIGURE 11: Representative field of a preparation of isolated maize mesophyll protoplasts.

Mesophyll protoplasts were isolated from maize leaves as described in Section 3.5, and photographed under phase-contrast. Magnification 800x.

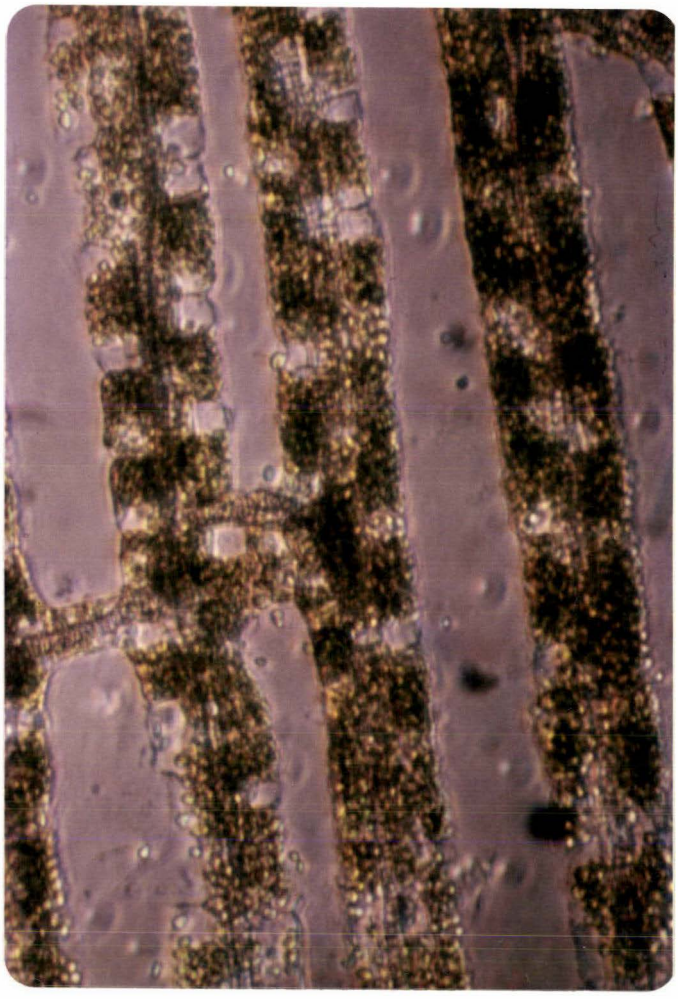


FIGURE 12: Representative field of a preparation of maize bundle sheath strands.

Strands of bundle sheath cells were prepared from maize leaves as described in Section 3.5, and photographed under phase-contrast. Magnification 400x.

been used by a number of workers to investigate the process of leaf and plastid development (Leech et al., 1973; Hawke et al., 1974b; Baker and Leech, 1977). Such leaves show successive levels of cell and plastid development, with the least differentiated cells and plastids nearest the base of the leaf, the fully differentiated cells in the distal part of the leaf, and the intermediate sections of the leaf show a linear progressive sequence of cell and plastid differentiation. Serial segments from base to tip of such leaves therefore, offered a natural system in which the relationship between leaf development, acetyl-CoA carboxylase activity and the rate of [^{14}C] acetate incorporation into lipids could be studied.

4.7.1 Incorporation of [1-^{14}C] acetate by maize leaf slices

The second emerging leaves of 8 day-old maize seedlings were sectioned as described in Section 3.8. Tissue from each section of the leaves was sliced and incubated with [1-^{14}C] acetate in the light, to determine lipid synthesis (Section 3.7.2). The rate of [^{14}C] acetate incorporation into lipids of the leaf slices, expressed on a fresh weight basis, was at a minimum in the basal sections (sections A and B) which contain the least differentiated cells (Table IX). Section C, which corresponds to the region of the leaf in transition between undeveloped and developed tissue, showed the maximum rate of acetate incorporation. In the two distal sections (D and E), which contain more developed tissue, the rate of [^{14}C] acetate incorporation was found to decrease from that found in section C.

4.7.2 Incorporation of [1-^{14}C] acetate into lipids by isolated chloroplasts from developing maize leaves

Chloroplasts at successive levels of development were isolated by the procedure described in Section 3.4, from sectioned leaves of maize (Section 3.8). Each of the chloro-

TABLE IX

EFFECT OF LEAF DEVELOPMENT ON [1-^{14}C] ACETATE INCORPORATION INTO LIPIDS BY LEAF SLICES OF MAIZE

Leaf Section	[1-^{14}C] acetate incorporation into lipids (nmol/h/g fresh weight)
A	13.1
B	13.0
C	22.5
D	15.8
E	14.7

Maize seedlings grown for 8 days as described in Section 2.1, were cut into 2 cm sections (Section 3.8). Tissue from each leaf section was sliced into 1mm strips and assayed for the capacity to incorporate [^{14}C] acetate into lipids (Section 3.7.2).

plast preparations were assayed for their capacity to incorporate [1-^{14}C] acetate into lipids. The rates of lipid synthesis from acetate with reference to chlorophyll levels, the usual basis for expression of activity, showed a maximum with chloroplasts isolated from the least developed tissue (section A), while chloroplasts isolated from tissue of increasing maturity and differentiation (sections B - E), showed progressively lower rates. However, the changing levels of chlorophyll in isolated chloroplasts at different developmental stages (Leech et al, 1973; Baker and Leech, 1977) confuses the above comparison. Therefore, use has been made of data on plastid numbers obtained in maize seedlings grown under almost identical conditions by Leech and co-workers (Leese et al., 1971; Baker and Leech, 1977), which enables the comparison of the rates of acetate incorporation to be made in relation to plastid development. The chlorophyll content of 150×10^6 plastids, isolated from sections A, B, C, D and E of maize leaves has been defined as being equivalent to 45, 50, 75, 125 and 200 µg of chlorophyll, respectively. On the basis of plastid numbers (Table X), the rate of de novo lipid synthesis was highest in chloroplasts isolated from sections C and D, which are the sections corresponding to the transition region of the leaf between undeveloped and developed tissue.

4.7.3 Acetyl-CoA carboxylase activity in cell-free extracts of developing maize leaves

Tissue from each section of maize leaves (Section 3.8) were used to prepare cell-free extracts (Section 3.3). Acetyl-CoA carboxylase activity was determined in these extracts, to investigate the effect of leaf development on activity. On the basis of leaf fresh weight, acetyl-CoA carboxylase activity showed no clear trend in the different sections (Table XI). However, when based on protein content activity increased from the least differentiated tissue in section A to reach a maximum in the intermediate section C, and then decline in the more fully differentiated tissue in

TABLE X

EFFECT OF LEAF DEVELOPMENT ON [1-^{14}C] ACETATE INCORPORATION INTO LIPIDS BY ISOLATED CHLOROPLASTS OF MAIZE

Leaf Section	[1-^{14}C] acetate incorporation into lipids	
	nmol/h/mg chl	nmol/h/150x10^{6} plastids
A	52	2.3
B	36	1.8
C	38	2.9
D	26	3.3
E	13	2.6

Eight day old maize seedlings grown as described in Section 2.1, were cut into 2 cm sections (Section 3.8). Chloroplasts were isolated from tissue of each section (Section 3.4), and assayed for their capacity to incorporate [^{14}C] acetate into lipids (Section 3.7.1).

TABLE XI

ACETYL-CoA CARBOXYLASE ACTIVITY IN CELL-FREE EXTRACTS OBTAINED FROM SECTIONS OF MAIZE LEAVES

Leaf Section	Acetyl-CoA carboxylase activity	
	nmol/min/mg protein	nmol/min/g fresh weight
A	11.6	87
B	18.6	130
C	31.0	104
D	21.6	98
E	19.0	132

Maize plants were grown and sectioned as described in Table X. Acetyl-CoA carboxylase activity was determined in cell-free extracts (Section 3.3) of tissue from each section, by the standard assay (Section 3.2), using 10mM $NaHCO_3$.

sections D and E (Table XI).

4.7.4 Acetyl-CoA carboxylase activity in isolated chloroplasts from developing maize leaves

Isolated chloroplast preparations from successive sections of maize seedlings, which were used to determine the rate of [^{14}C] acetate incorporation into lipids (Section 4.7.2), were disrupted by a desalting procedure (Section 3.4), and the activity of acetyl-CoA carboxylase determined in the extracts. Activities were related to chlorophyll and plastid numbers (Leese et al., 1971), and in both comparisons (Table XII) acetyl-CoA carboxylase activity was at a maximum in chloroplasts from the least developed tissue (section A) and decreased in chloroplasts from the successively more developed tissue (sections B to E). However, when activity was expressed on the basis of plastid numbers this trend was not as clear as when expressed on the basis of chlorophyll.

4.8 Acetyl-CoA Carboxylase Activity and Lipid Synthesis in Leaves of Maize, Barley and Spinach

Acetyl-CoA carboxylase activities in cell-free extracts of maize, barley and spinach leaves showed wide differences. Activity was highest in extracts prepared from whole leaves of 8 day-old maize, which was about twice that found in barley extracts prepared by the identical procedure (Section 3.3) (Table XIII). In order to determine the acetyl-CoA carboxylase activity in cell-free extracts of spinach leaves, 15% glycerol and 0.1% BSA was included in the buffer (Mohan and Kekwick, 1980; Section 4.2), thus precluding the expression of the activity on a protein basis. However, on the basis of fresh weight, acetyl-CoA carboxylase activity in spinach leaves was only 15% of that found in maize. Preparation of maize and barley cell-free extracts with buffers containing 15% glycerol and 0.1% BSA did not affect acetyl-CoA carboxylase activity.

TABLE XII

ACETYL-CoA CARBOXYLASE ACTIVITY IN CHLOROPLASTS ISOLATED FROM SECTIONS OF MAIZE LEAVES

Leaf Section	Acetyl-CoA carboxylase activity	
	nmol/min/mg chl	nmol/min/150x10^6 plastids
A	550	24.7
B	208	10.4
C	167	12.6
D	69	8.6
E	18	3.6

Acetyl-CoA carboxylase activity was determined by the assay described in Section 3.2, using 30mM $NaHCO_3$, in the same isolated chloroplasts used in the experiment described in Table X, after disruption by the desalting procedure described in Section 3.4.

TABLE XIII

[1-^{14}C] ACETATE INCORPORATION INTO LIPIDS OF CHLOROPLASTS AND ACETYL-CoA CARBOXYLASE ACTIVITIES IN CELL-FREE EXTRACTS AND CHLOROPLASTS FROM MAIZE, BARLEY AND SPINACH

Plant	[^{14}C] acetate incorporation into lipids of chloroplasts nmol/min/mg chl	Acetyl-CoA carboxylase activity		
		Isolated Chloroplasts	Cell-free extracts	
		nmol/min/mg chl	nmol/min/mg protein	nmol/min/g fresh weight
Maize	0.33	34.5	27.2	172
Barley	0.12	8.5	11.2	95
Spinach	6.00	2.8	n.d.	24

Chloroplasts were isolated and cell-free extracts prepared from leaves of maize, barley and spinach by the procedures described in Sections 3.4 and 3.3, respectively. Acetyl-CoA carboxylase activity was assayed using 10mM HCO_3^-, as described in Section 3.2, following disruption of chloroplasts by desalting (Section 3.4). [^{14}C] acetate incorporation into lipids by intact chloroplasts was performed as in Section 3.7.1. n.d. = not determined.

The presence of an inhibitor to acetyl-CoA carboxylase has been reported in a number of previous studies (Burton and Stumpf, 1966; Kannangara and Stumpf, 1972). However, the nearly complete recovery of acetyl-CoA carboxylase activity upon mixing of cell-free extracts of maize and spinach leaves (Table XIV), would appear to rule out the presence of an inhibitor to acetyl-CoA carboxylase in spinach leaves.

Acetyl-CoA carboxylase activity in isolated chloroplasts was determined, and compared to the rate of [^{14}C] acetate incorporation into lipids (Section 3.7.2) by the same chloroplast preparations from maize, barley and spinach leaves. Chloroplasts isolated as in Section 3.4 from maize, barley and spinach leaves, contained 50 - 60%, 30 - 50% and 80 - 90% intact chloroplast, respectively, as judged by examination under phase-contrast microscopy. Acetyl-CoA carboxylase activity was determined following disruption of the chloroplasts by the desalting procedure described in Section 3.4, except that spinach chloroplasts were desalted in an elution buffer containing 15% glycerol and 0.1% BSA. Maize chloroplast extracts showed the highest acetyl-CoA carboxylase activity, followed by extracts of barley and spinach, which showed 25% and 8% of the activity found in maize chloroplast extracts, respectively (Table XIII). However, [^{14}C] acetate incorporation into lipids was highest with chloroplasts of spinach, which was 17-fold and 50-fold higher than the rates shown by chloroplasts of maize and barley, respectively. Comparison of acetyl-CoA carboxylase activity with the rate of [^{14}C] acetate incorporation into lipids by the different chloroplasts, indicated that maize and barley chloroplastic acetyl-CoA carboxylase activity was 60-fold and 100-fold in excess of the rate of [^{14}C] acetate incorporation, respectively. Spinach chloroplasts, on the other hand, appeared to contain acetyl-CoA carboxylase activity which was only half that expected from the rate of [^{14}C] acetate incorporation.

TABLE XIV

ACETYL-CoA CARBOXYLASE ACTIVITIES IN MAIZE AND SPINACH LEAVES

Source of cell-free extract	Volume (ml)	Acetyl-CoA carboxylase activity (nmol/min)
Maize leaves	1.0	23.85
Spinach leaves	1.0	2.05
Maize + spinach leaves	2.0	24.83

Cell-free extracts were prepared from maize and spinach leaves as described in Section 3.3, except the spinach cell-free extract was prepared in a buffer containing 15% glycerol and 0.1% BSA. 1ml aliquots of each extract were mixed and acetyl-CoA carboxylase activity was assayed by the standard procedure (Section 3.2), using 10mM $NaHCO_3$.

4.9 Effect of Light on Acetyl-CoA Carboxylase Activity in Maize Leaves

Recently Nakamura and Yamada (1979) have suggested, on the basis of the concentration of intermediates, that acetyl-CoA carboxylase is the light-dependent enzyme in de novo biosynthesis of fatty acids in chloroplasts. It might be expected therefore to find different acetyl-CoA carboxylase activities in leaves subjected to different light-dark treatments. Maize plants, grown for 8 days under a standard light-dark regime (Section 2.1), were placed in the dark for 18h. While half the batch of plants (D) were kept in the dark during the sampling period, to serve as a control, the remaining plants (L) were placed in sunlight for 26 min, and then returned to the dark. At each sampling time cell-free extracts were prepared from about 3g of leaves (Section 3.3) and acetyl-CoA carboxylase activity determined. There was no obvious difference between the enzyme's activity in the extracts from dark and illuminated leaves (Fig. 13).

PURIFICATION OF LEAF ACETYL-CoA CARBOXYLASE

4.10 Purification of Acetyl-CoA Carboxylase from Maize Leaves

The purification of acetyl-CoA carboxylase from maize leaves was carried out to enable the kinetic characteristics of the enzyme to be investigated. The instability of the enzyme hampered the isolation of the enzyme to homogeneity, and consequently the subsequent investigation of its molecular structure was precluded. Partial purification was obtained by two procedures (Procedure I and II, see Section 3.15), each of which resulted in preparations of the enzyme of similar specific activity.

In both procedures, purification commenced by the fraction-

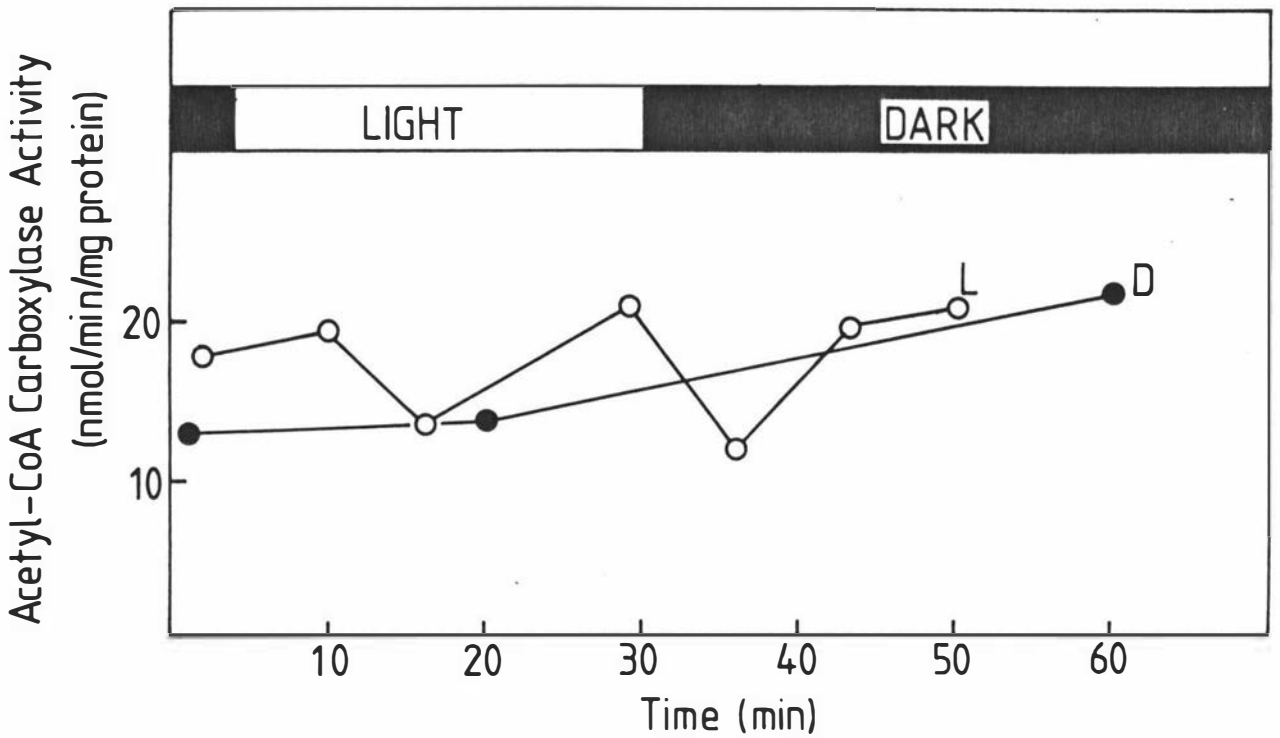


FIGURE 13: Effect of light on the activity of acetyl-CoA carboxylase in maize leaves.

Maize plants were kept in the dark continuously (●) or transferred in to sunlight for 26 min and then back to darkness (○). Acetyl-CoA carboxylase activity was assayed as in Section 3.2 using 10mM HCO_3^-, in the cell-free extracts of leaves prepared as in Section 3.3.

ation of the 30,000g supernatant of the homogenate with polyethylene glycol 6000 (PEG). Protein precipitated between 6% and 14% saturation of PEG contained all of the enzyme activity found in the 30,000g supernatant, with a four-fold increase in specific activity (Table XV and XVI).

In Procedure I, the next purification step was hydrophobic column chromatography on Sepharose-N-propane. This method of fractionation is dependent on the hydrophobic interaction between the ligand (propane) and the protein being chromatographed. Shaltiel and co-workers (Er-el et al., 1972) first described this procedure in the purification of glycogen phosphorylase b, and since then it has become a widely used method in protein purification.

Sepharose-N-propane was chosen after investigation of the binding of acetyl-CoA carboxylase to a series of columns containing a homologous series of hydrocarbon-coated agarose (Shaltiel Hydrophobic Chromatography Kit I, from Miles-Yeda Ltd, Rehovot, Israel). Each column (bed volume 1 ml) was equilibrated with 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA, and loaded with a 0.2 ml solution containing acetyl-CoA carboxylase in the above buffer. Unbound protein was eluted from each column with 2 ml of the start buffer and the bound proteins were eluted with 2 ml of 1M KCl in start buffer. None of the added enzyme bound to agarose without any ligands, however 85% and nearly 100% of the added enzyme was bound to agarose-N-ethane and agarose-N-butane, respectively (Table XVII). Agarose with hexane, octane and decane as ligands also bound all of the added enzyme, and in all cases 1M KCl eluted approximately 90% of the bound enzyme. Sepharose-N-propane was chosen as it would appear from these results to just bind all of the added carboxylase, and would thus result in the greatest purification. During purification, this procedure gave a recovery of 40% accompanied with a 10-fold increase in specific activity (Table XV). A typical elution profile of protein and acetyl-CoA carboxylase acti-

TABLE XV

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

Fraction	Volume (ml)	Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification (fold)
Homogenate	1350	16,200	5063	3.2	100	1
30,000g supernatant	1290	9,040	3225	2.8	56	0.9
6 - 14% PEG	91	10,062	1031	9.8	62	3
Sepharose-N-Propane Affinity chromatography	68	4,156	41	101.9	26	32
Ultrogel AcA 22 Gel filtration	7	1,323	3.0	473.2	8	136

The purification of acetyl-CoA carboxylase was carried out from 500g of maize leaves as described in Section 3.15.

TABLE XVI

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

Fraction	Volume (ml)	Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification (fold)
Homogenate	284	5220	710	7.4	100	1
30,000 g supernatant	263	5274	447	11.8	101	1.6
6 - 14% PEG	25	5921	145	40.8	113	5.5
40% ammonium sulphate precipitate	5.2	1999	50	40.0	38	5.4
Ultrogel AcA 22 Gel filtration	5	1308	8.5	153.9	25	20.8

The purification of acetyl-CoA carboxylase was carried out from 100g of maize leaves as described in Section 3.15.

TABLE XVII

INTERACTION OF ACETYL-CoA CARBOXYLASE WITH SEPHAROSE-N-C_x

Ligand chain length (x)	Acetyl-CoA carboxylase activity (%)	
	10mM Tris wash	1M KCl wash
0	100	0
2	15	80
4	2	90
6	0	92
8	0	91
10	0	90

Shaltiel Hydrophobic Chromatography Kit I columns (from Miles-Yeda Ltd, Rehovot, Israel), were equilibrated with 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol, 1mM EDTA and loaded with acetyl-CoA carboxylase (total activity of 30 nmol/min). The columns were successively eluted with 2ml each of equilibration buffer and 1M KCl in equilibration buffer. Total activity in each eluate, as a percentage of the applied activity, is given above.

vity from Sepharose-N-propane, with a linear salt gradient from zero to 0.2M KCl, is shown in Fig. 14.

The fractions containing acetyl-CoA carboxylase activity from the above procedure were pooled and concentrated to about 7 ml, by ultrafiltration through a Diaflo PM30 Ultrafilter (Section 3.15), without affecting acetyl-CoA carboxylase activity. The final purification step was obtained by gel filtration on a column of Ultrogel AcA22. Ultrogel beads are composed of three-dimensional polyacrylamide lattice with an interstitial agarose gel, resulting in beads of high rigidity. This enables a higher flow rate than more conventional media for gel filtration, such as Sepharose. The medium used in this study, Ultrogel AcA22, has a fractionation range of 100,000 to 1,200,000 daltons.

A typical elution from a column of Ultrogel AcA22 is shown in Fig. 15, and typically gel filtration on this medium resulted in a four-fold increase in specific activity, however recovery was only 30% of the applied activity. Overall, Procedure I resulted in a 130-fold purification of acetyl-CoA carboxylase, with an 8% recovery of the initial activity (Table XV).

In Procedure II, the polyethylene glycol fractionation described above, was followed by a further fractionation with ammonium sulphate. The protein precipitated at 40% saturation of ammonium sulphate (Section 3.15) was subsequently fractionated by gel filtration on Ultrogel AcA22. Although the ammonium sulphate fractionation did not result in a further increase in specific activity, it enabled the PEG fraction to be concentrated to a small volume necessary for the subsequent gel filtration step. Furthermore, if the ammonium sulphate precipitation was omitted, chromatography on the Ultrogel AcA22 column resulted in a poor recovery of the enzyme.

A typical elution profile from Ultrogel AcA22 obtained with

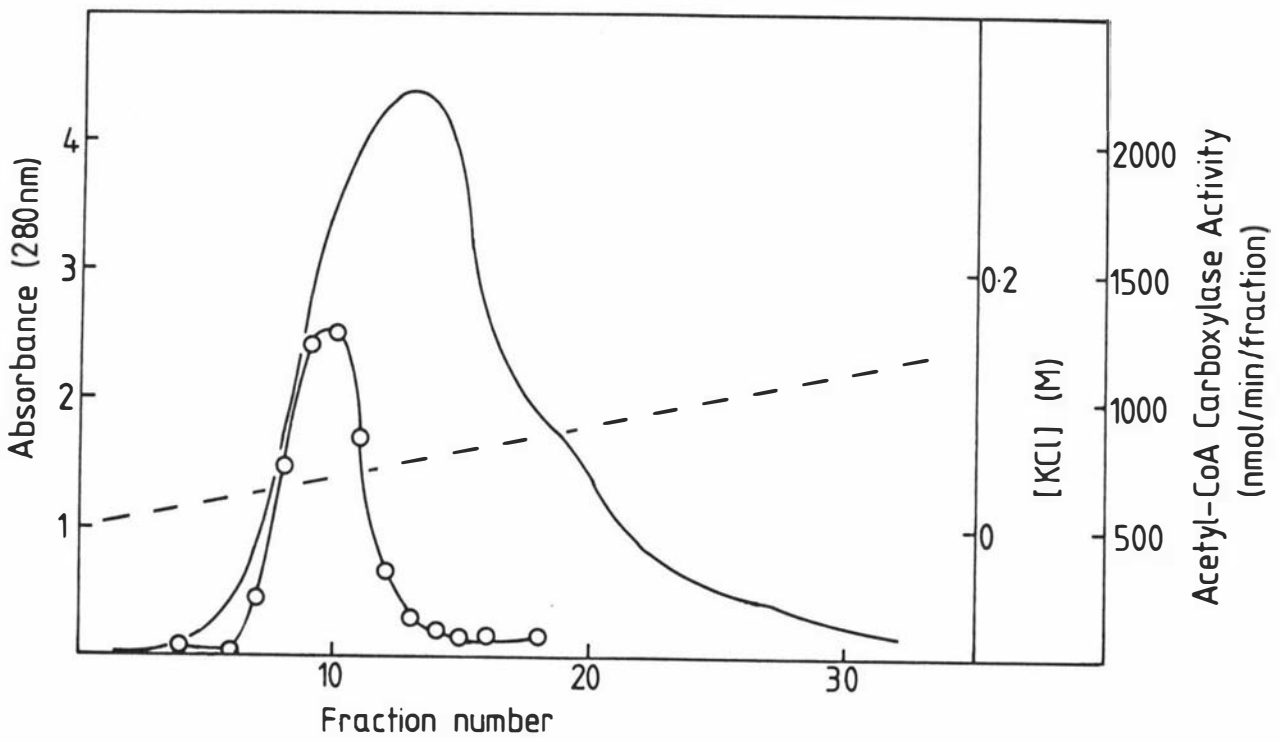


FIGURE 14: Elution of acetyl-CoA carboxylase of maize from Sepharose-N-Propane.

Acetyl-CoA carboxylase precipitated between 6% and 14% PEG was resuspended in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA (10mM Tris buffer) and was loaded on to a column of Sepharose-N-Propane, equilibrated with 10mM Tris buffer. Unbound protein was washed off with 10mM Tris buffer and elution was carried out with a linear gradient from zero to 0.2M KCl (- -) in 10mM Tris buffer, at a rate of 30 ml/h. Fractions of approximately 10 ml were collected and acetyl-CoA carboxylase activity (o), and A_{280} (—) determined.

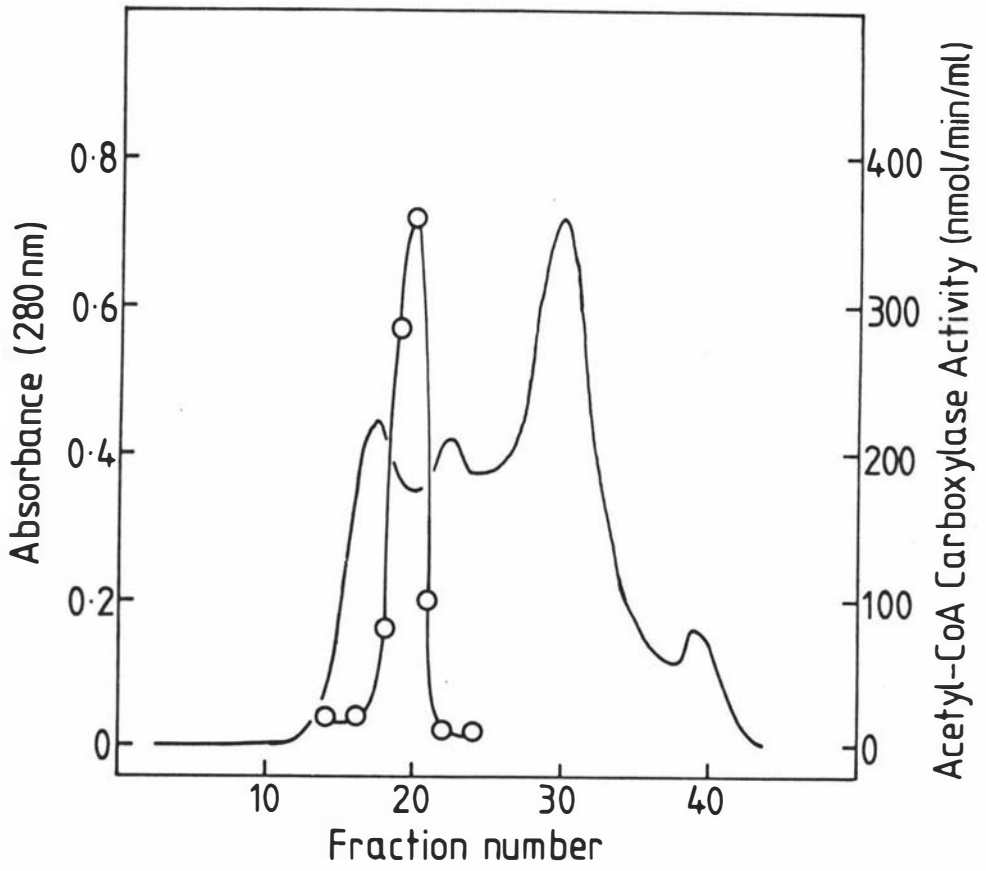


FIGURE 15: Gel filtration of acetyl-CoA carboxylase of maize on Ultrogel AcA 22.

Acetyl-CoA carboxylase (5 ml), recovered from hydrophobic chromatography, was subjected to gel filtration on a column of Ultrogel AcA 22, with 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA (Section 3.14.1). Acetyl-CoA carboxylase activity (o) and A_{280} (—) was determined in each fraction (4 ml).

Procedure II is shown in Fig. 16. The recovery and purification obtained was similar to that obtained during this step in Procedure I. Procedure II resulted in a 20- to 40-fold increase in specific activity with a 25% recovery of the initial activity (Table XVI).

The specific activity of acetyl-CoA carboxylase obtained by both purification procedures was in the range of 200 - 500 nmol/min/mg of protein. Analysis of the final enzyme preparation was carried out by polyacrylamide gel electrophoresis. Following electrophoresis, an unstained gel was sliced transversely into 2 mm slices and the enzyme located by assaying each slice for acetyl-CoA carboxylase activity in 0.5 ml solution containing: 0.1M Tris-HCl buffer (pH 8.0), 5mM β-mercaptoethanol, 1mM ATP, 5mM $MgCl_2$, 50mM KCl, 2mM $NaH^{14}CO_3$ (5Ci/mol) and 0.1mM acetyl-CoA. Following incubation at 30°C for 30 min, 0.1 ml of 6M HCl was added to stop the reaction and 0.3 ml from each assay was dried on a 2 cm x 2 cm square of Whatman 3MM paper and the radioactivity fixed into malonyl-CoA determined. Acetyl-CoA carboxylase activity coincided with the position of the heavily stained protein band on an identical gel (Fig. 17). The area under the absorbance peak corresponding to acetyl-CoA carboxylase activity compared with the total area of the absorbance profile suggested that the enzyme was 40 - 60% pure. The partially purified acetyl-CoA carboxylase contained no detectable PEP carboxylase or RuDP carboxylase.

Acetyl-CoA carboxylase prepared by both procedures was relatively unstable, losing 50% of the initial activity in 5 days when stored at 4°C in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA in an atmosphere of nitrogen gas (Table XVIII). Inclusion of glycerol in the buffer at 20% concentration had little effect on the stability, and freezing at -20°C caused complete inactivation of the enzyme. These instability problems made further purification difficult and consequently the molecular weight and subunit organisation of the enzyme was not investigated.

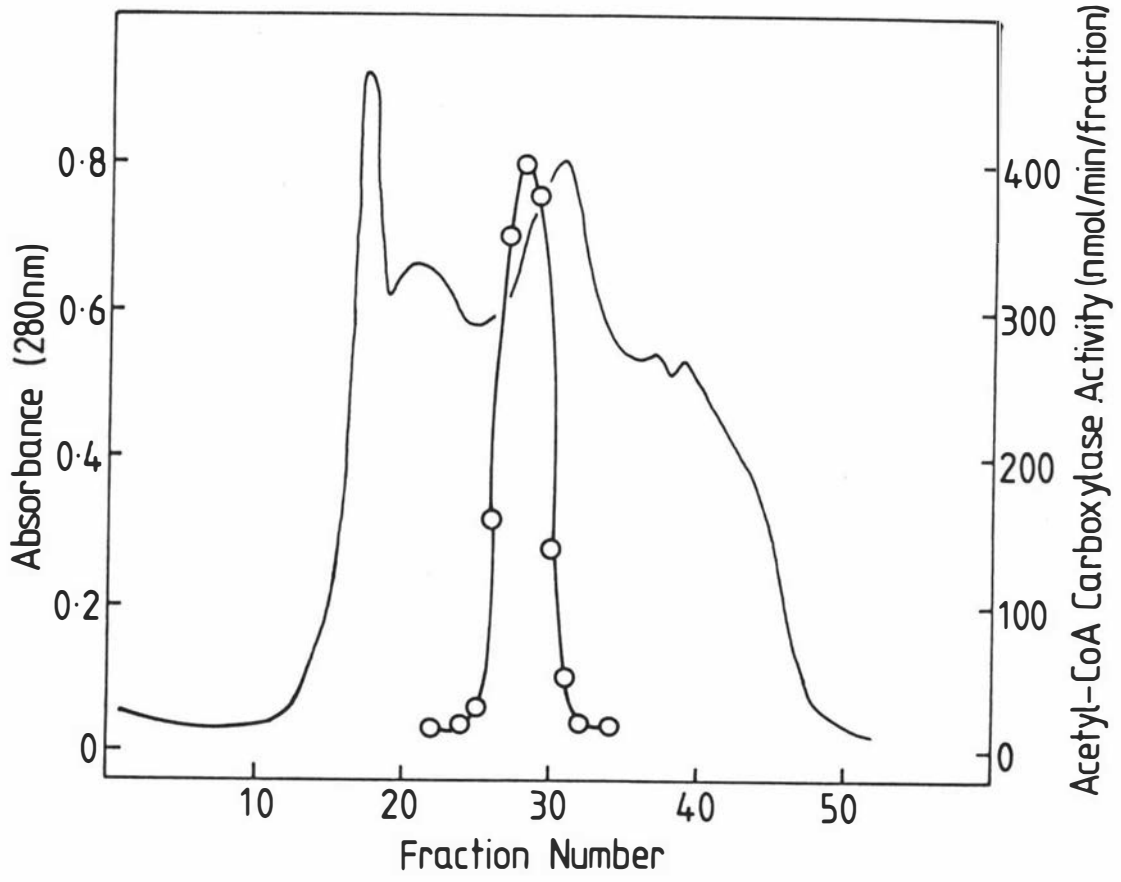


FIGURE 16: Gel filtration of acetyl-CoA carboxylase of maize on Ultrogel AcA 22.

Acetyl-CoA carboxylase precipitated at 40% ammonium sulphate, in purification Procedure II (Section 3.15), was re-suspended in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA, was subjected to gel filtration on Ultrogel AcA 22 under the conditions described in Fig. 15. Acetyl-CoA carboxylase activity (o) and A_{280} (—) was determined in each fraction (4 ml).

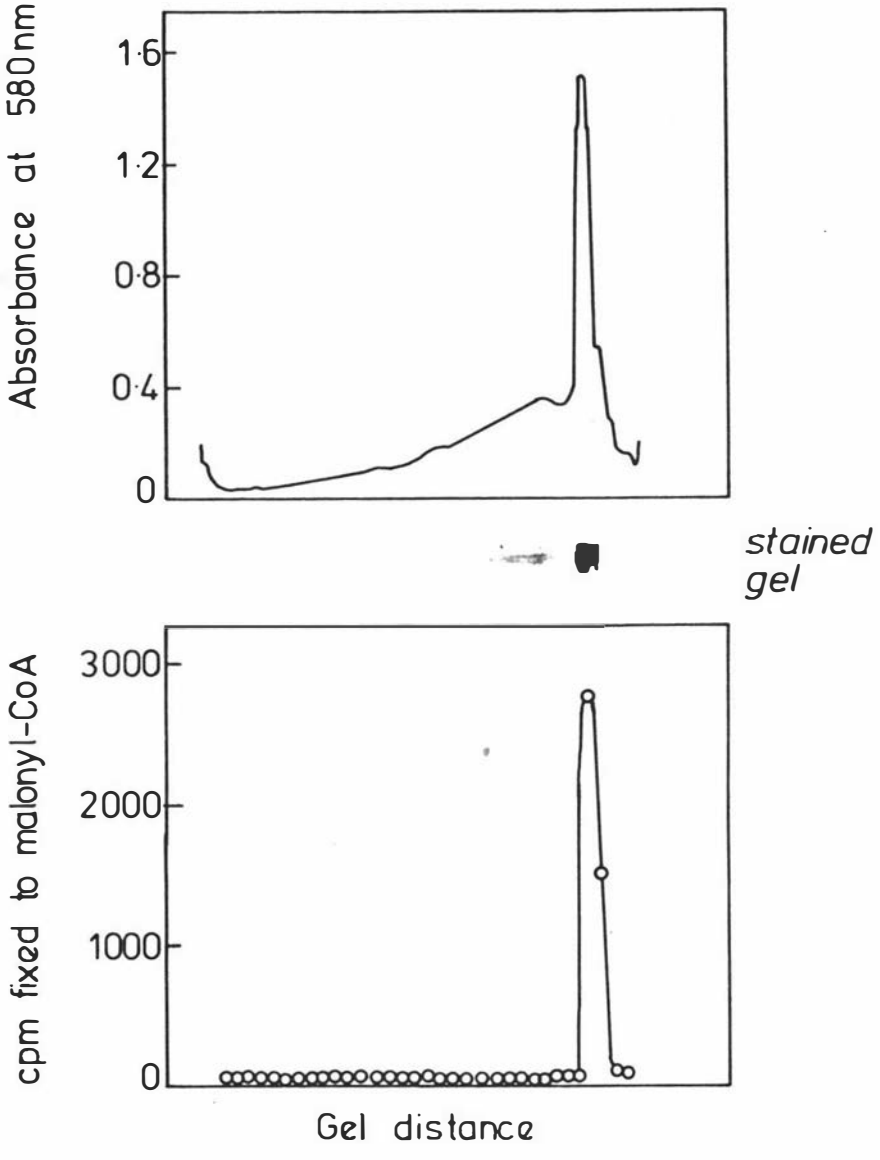


FIGURE 17: Polyacrylamide gel electropheresis of acetyl-CoA carboxylase of maize leaves.

Upon polyacrylamide gel electropheresis (Section 3.10) of partially purified acetyl-CoA carboxylase of maize leaves (50 µg protein), the gel was either sliced into 2 mm sections and each slice assayed for acetyl-CoA carboxylase activity (o) as described in Section 4.10, or stained with Coomassie Brilliant Blue (Section 3.10) and the A_{580} (—) scan was obtained on an ISCO Gel Scanner Model 1310.

TABLE XVIII

STABILITY OF PARTIALLY PURIFIED ACETYL-CoA CARBOXYLASE

Age of preparation (days)	Acetyl-CoA carboxylase activity (nmol/min/mg)
0	413
3	289
5	222
7	176
9	122
12	77

Acetyl-CoA carboxylase of maize purified by Procedure I, after gel filtration on Ultrogel AcA 22 (day 0), was stored at 4^{o}C in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA under an atmosphere of nitrogen.

The characterization of the kinetic properties of the enzyme were investigated within 5 days of the end of the purification.

In developing the two procedures that were finally adopted to purify acetyl-CoA carboxylase, a number of alternative procedures were investigated, but none were found satisfactory. When the 30,000g supernatant of the total homogenate was fractionated with ammonium sulphate, only the protein fraction precipitated between 20% and 40% ammonium sulphate saturation contained acetyl-CoA carboxylase. However, this comprised only 20 - 30% of the original activity, and the increase in specific activity was small. Acetyl-CoA carboxylase after PEG fractionation was not absorbed to either DEAE-cellulose, DEAE-Sephadex or CM-Sephadex when applied in 10mM Tris-HCl buffer between pH 7.5 and 8.5. On the other hand, the enzyme appeared to be either irreversibly bound to or completely inactivated by phosphocellulose, since no activity could be eluted even at 5M NaCl. Affinity chromatography using Sepharose-CoA (Chibata et al., 1974) and Sepharose-avidin (Henrikson et al., 1979), respectively, have been used to purify enzymes that require CoA, and are biotin-dependent carboxylases. However, neither of these affinity media proved satisfactory in purifying maize leaf acetyl-CoA carboxylase. Only 50% of the enzyme after hydrophobic chromatography was bound to Sepharose-CoA and elution of the bound enzyme with a salt gradient gave no increase in specific activity. Acetyl-CoA carboxylase did not bind to Sepharose-avidin.

4.11 Purification of Acetyl-CoA Carboxylase from Barley Leaves

In order to ensure that acetyl-CoA carboxylase of maize leaves was not atypical of acetyl-CoA carboxylases from photosynthetic tissue, and also for a comparison of the enzyme from C_4 and C_3 plants, acetyl-CoA carboxylase was also

purified from barley leaves. The barley leaf enzyme exhibited very similar properties to that of the enzyme from maize leaves during each stage of purification with Procedure I (see Sections 3.15, 4.10) (Table XIX). Approximately a 20-fold increase in specific activity was obtained during purification, with the final specific activity of enzyme being about 200 nmol/min/mg of protein. As with the enzyme purified from maize leaves, the major problem encountered during purification and subsequent storage of the barley enzyme was that of stability. Storage at 4°C under a nitrogen atmosphere in 10mM Tris-HCl buffer (pH 8.0) containing 20mM β-mercaptoethanol and 1mM EDTA led to 40% loss of activity in 5 days, while freezing at -20°C resulted in complete inactivation of the enzyme. Due to this problem the kinetic properties of acetyl-CoA carboxylase from barley was studied within 5 days of purification.

KINETIC PROPERTIES OF ACETYL-CoA CARBOXYLASE

4.12 Effect of Acetyl-CoA and HCO_3^- Concentrations on Acetyl-CoA Carboxylase Activity

Increasing the concentration of acetyl-CoA in the assay from 0.015mM to 0.3mM, resulted in a six-fold increase in the activity of acetyl-CoA carboxylase of maize (Fig. 18) and barley (Fig. 19). Maximum activity of the enzymes from both sources occurred above 0.15mM acetyl-CoA, but the maximum rate attained was dependent on the HCO_3^- concentration. Lineweaver-Burk plots (Lineweaver and Burk, 1934) of the data indicated very similar $K_m^{\text{acetyl-CoA}}$ values, namely 0.11 mM (Fig. 20) and 0.10mM (Fig. 21), for acetyl-CoA carboxylase of maize and barley, respectively.

Activity of the maize (Fig. 22) and barley (Fig. 23) acetyl-CoA carboxylase increased five- and seven-fold, respectively, when the HCO_3^- concentration was increased from 0.5mM to 20mM.

TABLE XIX

PURIFICATION OF ACETYL-CoA CARBOXYLASE OF BARLEY LEAVES USING PROCEDURE I

Fraction	Volume (ml)	Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification (fold)
Homogenate	940	20,337	2,350	8.6	100	1
30,000g supernatant	895	22,727	1,924	11.8	112	1.4
6 - 14% PEG	67	16,586	898	18.5	82	2.2
Sepharose-N-Propane Affinity chromatography	196	7,303	66	110.5	36	13
Ultrogel AcA 22 Gel filtration	13.5	2,550	13.5	188.9	13	22

The purification of acetyl-CoA carboxylase was carried out from 300g of barley leaves as described in Section 3.15.

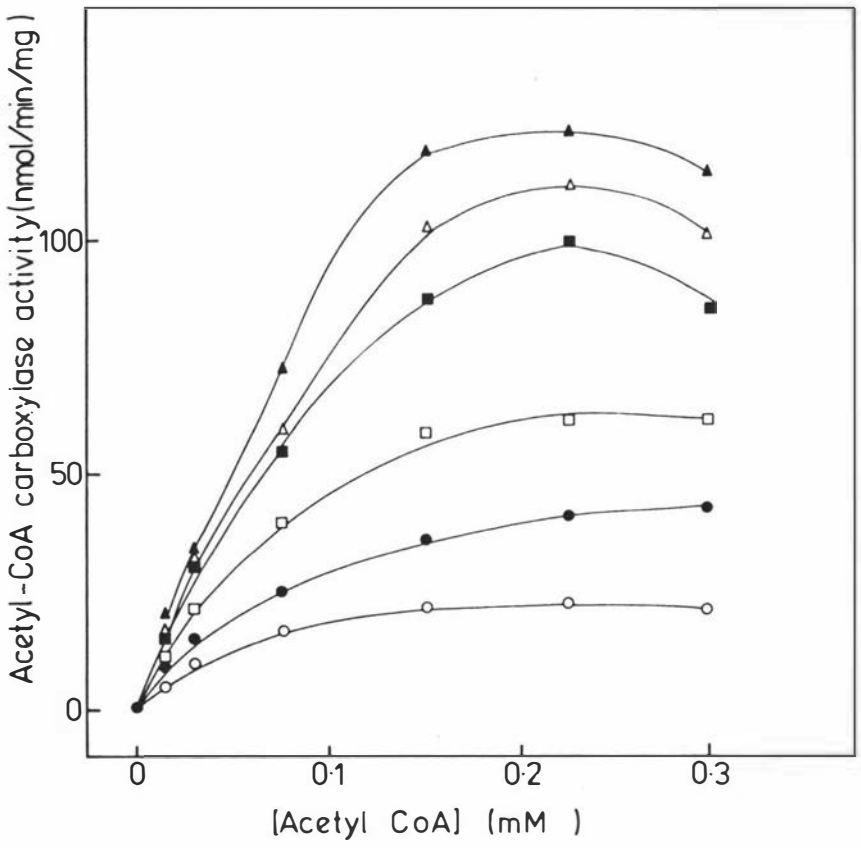


FIGURE 18: Relationship between HCO_3^-, acetyl-CoA concentrations and the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2, except the concentration of acetyl-CoA was varied, with HCO_3^- concentrations at 0.5mM (o), 1.0mM (●), 2.0mM (□), 5.0mM (■), 10mM (△) and 20 mM (▲).

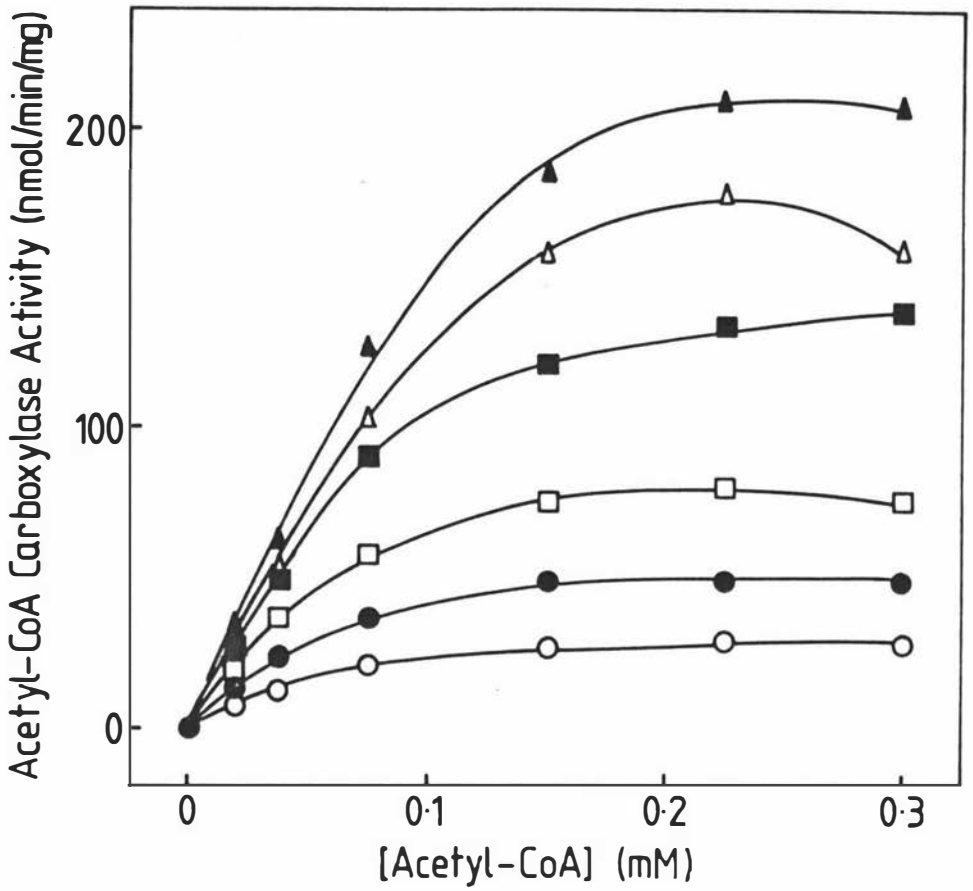


FIGURE 19: Relationship between HCO_3^-, acetyl-CoA concentrations and the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions : as in Fig. 18.

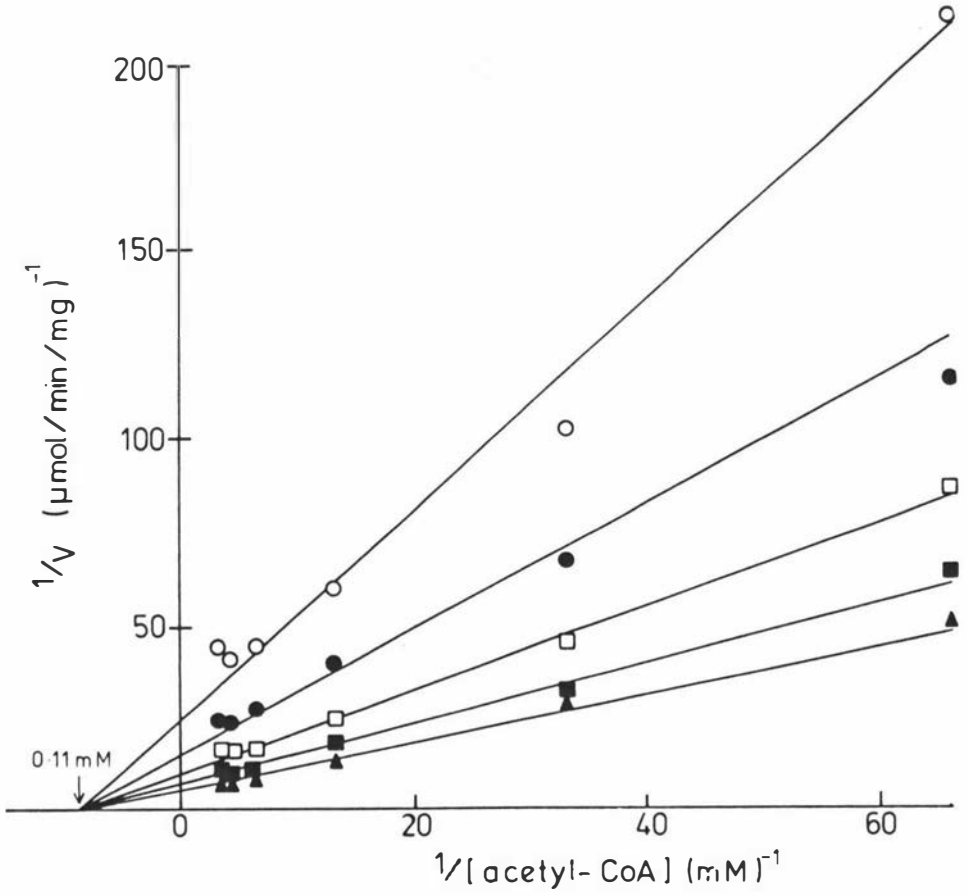


FIGURE 20: Lineweaver-Burk analysis of the effect of acetyl-CoA on the activity of maize leaf acetyl-CoA carboxylase. Analysis of the data presented in Fig. 18.

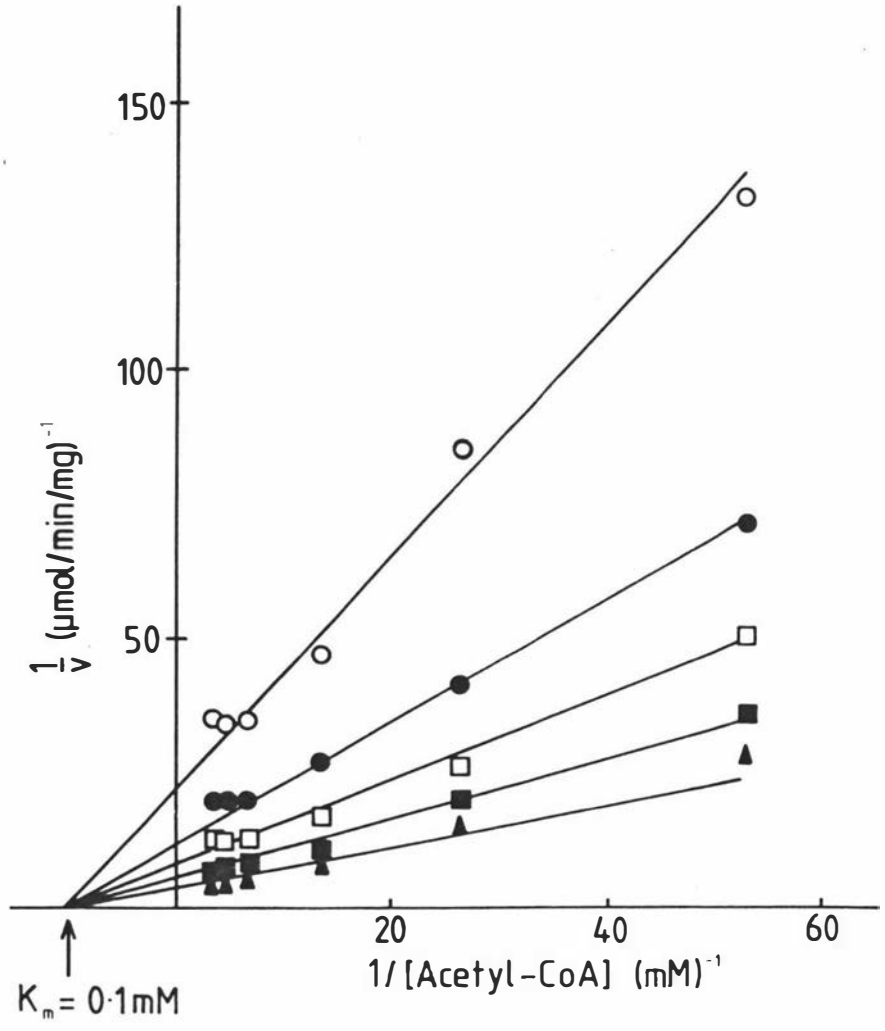


FIGURE 21: Lineweaver-Burk analysis of the effect of acetyl-CoA on the activity of barley leaf acetyl-CoA carboxylase.

Analysis of the data presented in Figure 19.

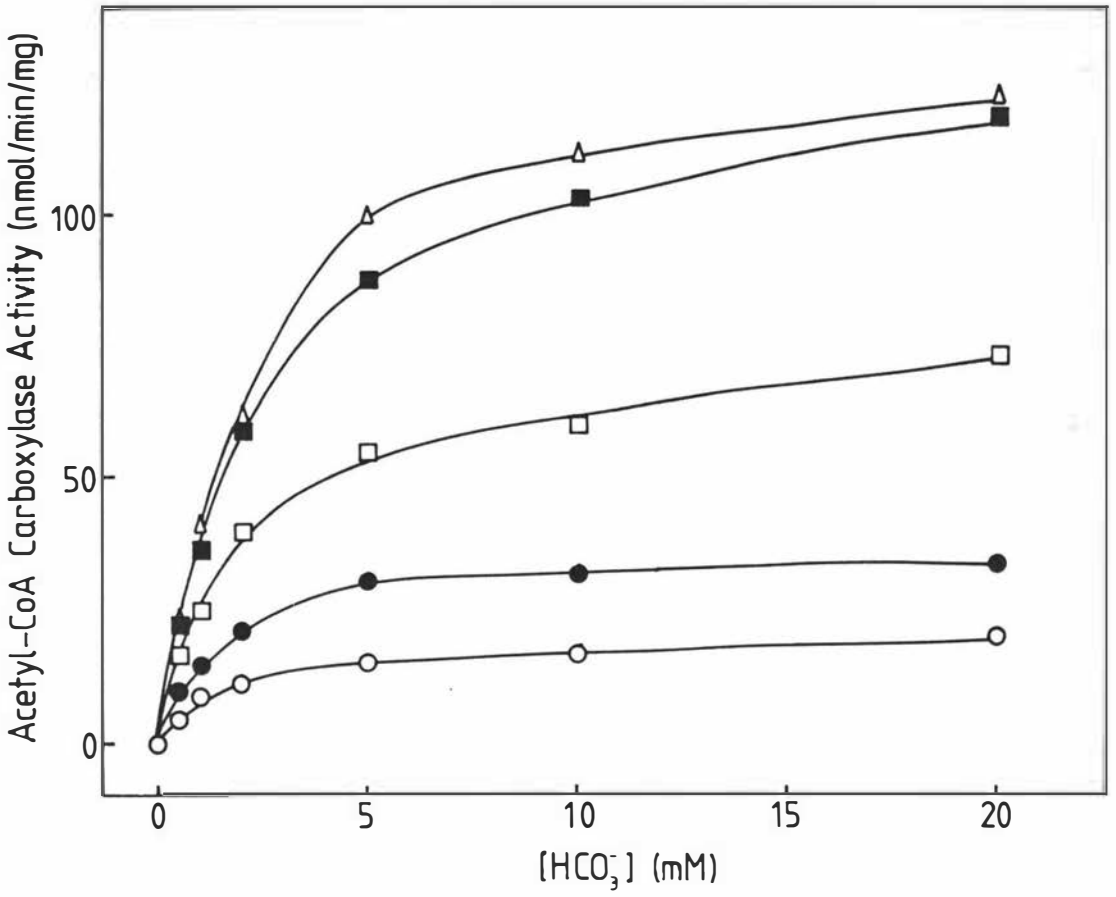


FIGURE 22: Relationship between HCO_3^-, acetyl-CoA concentrations and the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2, except the concentration of HCO_3^- was varied, with acetyl-CoA concentrations at 0.015mM (o), 0.030mM (●), 0.075mM (□), 0.150mM (■) and 0.225mM (△).

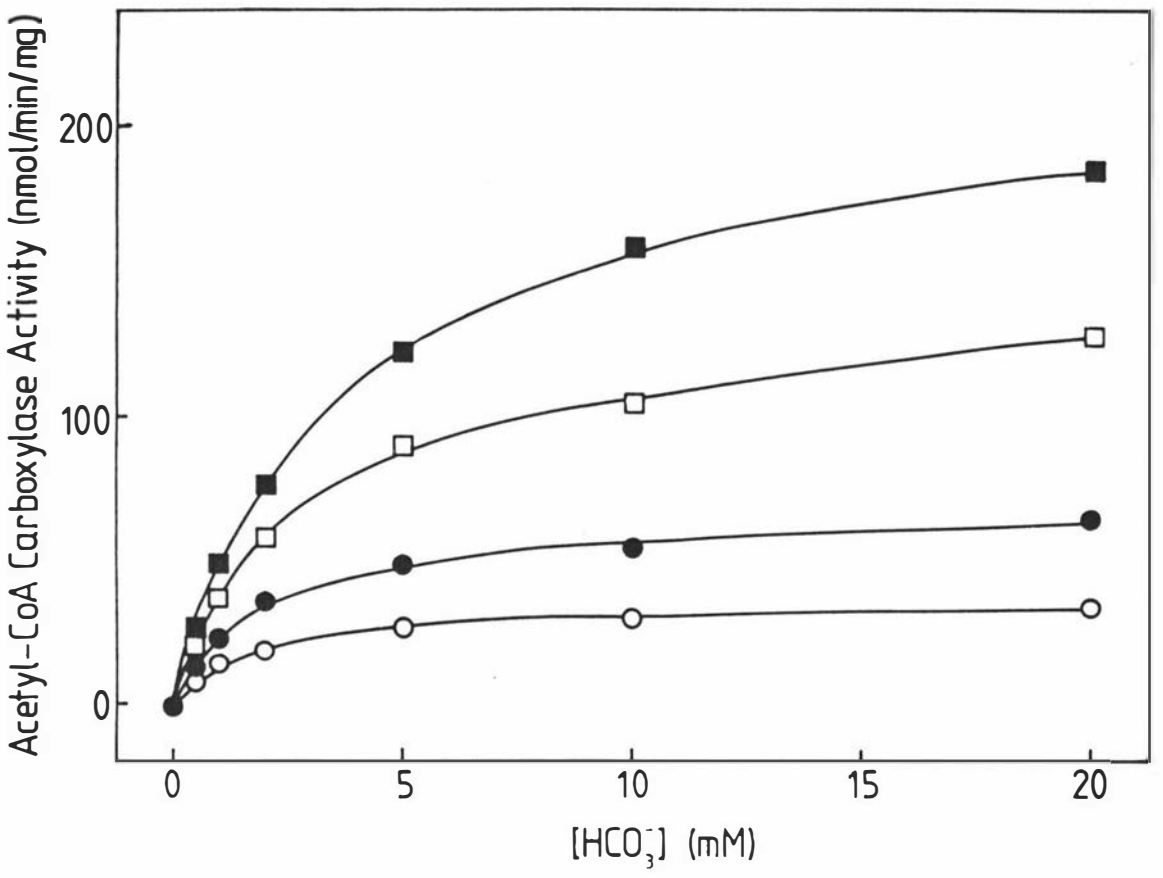


FIGURE 23: Relationship between HCO_3^-, acetyl-CoA concentrations and the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions: as in Fig. 22, except acetyl-CoA concentrations were 0.019mM (o), 0.038mM (●), 0.075mM (□) and 0.150mM (■).

Lineweaver-Burk plots gave values of $K_m^{HCO_3^-}$ of 1.4mM and 2.1mM for the maize (Fig. 24) and barley (Fig. 25) enzymes, respectively.

4.13 Effect of ATP and $MgCl_2$ Concentrations on Acetyl-CoA Carboxylase Activity

Initial characterization of the cofactor requirement of acetyl-CoA carboxylase activity in cell-free extracts of maize, indicated an absolute requirement for ATP and $MgCl_2$ (Section 4.1). As the biologically active form of ATP in a number of enzymes is Mg.ATP, the relationship between Mg^{2+}, ATP and the activity of acetyl-CoA carboxylase was investigated.

At constant ATP concentrations, increased $MgCl_2$ concentrations resulted in a sigmoidal increase in the activity of acetyl-CoA carboxylase of both maize (Fig. 26) and barley (Fig. 27). Maize acetyl-CoA carboxylase activity was maximal at 3mM and 4mM $MgCl_2$ when the ATP concentrations were maintained at 1mM and 3mM, respectively. Maximum activity of barley acetyl-CoA carboxylase at 1mM ATP was attained when the $MgCl_2$ concentration was increased to 4mM.

In the presence of 2.5mM and 5.0mM $MgCl_2$, increasing the ATP concentration up to about 2.0mM resulted in hyperbolic increases in the activities of both maize (Fig. 28) and barley (Fig. 29) acetyl-CoA carboxylases. Activities of both enzymes were maximal in the range of about 0.1 - 1.5mM ATP, when the $MgCl_2$ concentration was 2.5mM. While at 5.0mM $MgCl_2$, maximum activity was over a larger range of ATP concentrations, between 0.1mM and 3.5mM. Higher concentrations of ATP resulted in inhibition of the activity of the acetyl-CoA carboxylases.

The interdependence of acetyl-CoA carboxylase activity with Mg^{2+} and ATP concentrations was also investigated by varying

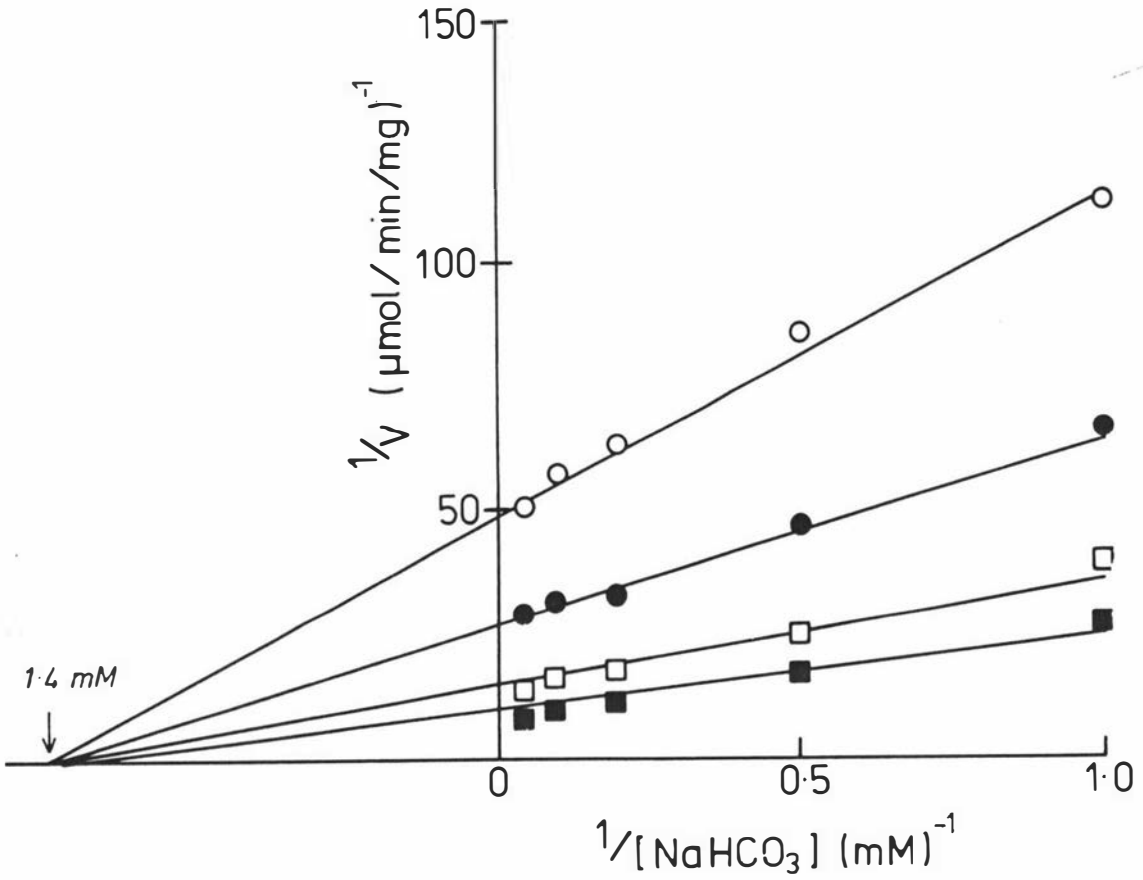


FIGURE 24: Lineweaver-Burk analysis of the effect of HCO_3^- on the activity of maize leaf acetyl-CoA carboxylase. Analysis of the data presented in Fig. 22.

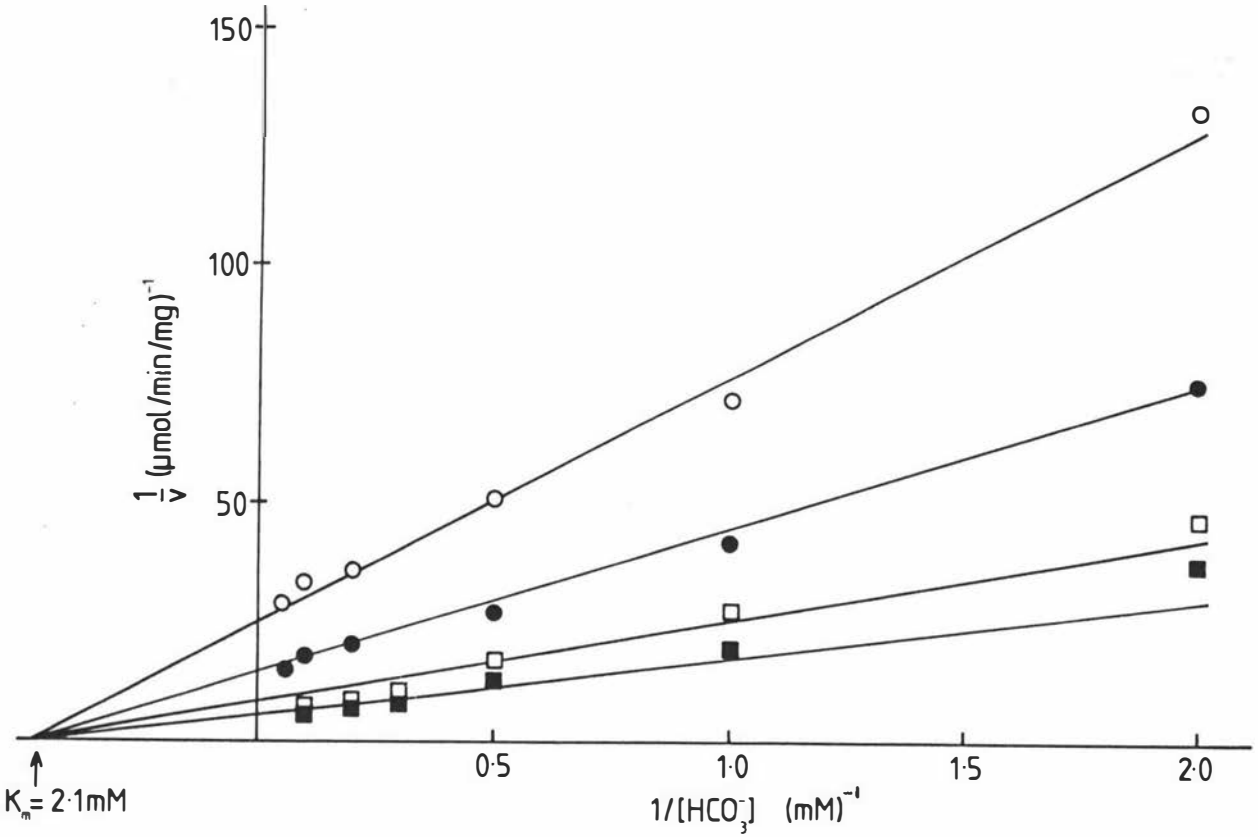


FIGURE 25: Lineweaver-Burk analysis of the effect of HCO_3^- on the activity of barley leaf acetyl-CoA carboxylase. Analysis of the date presented in Fig. 23.

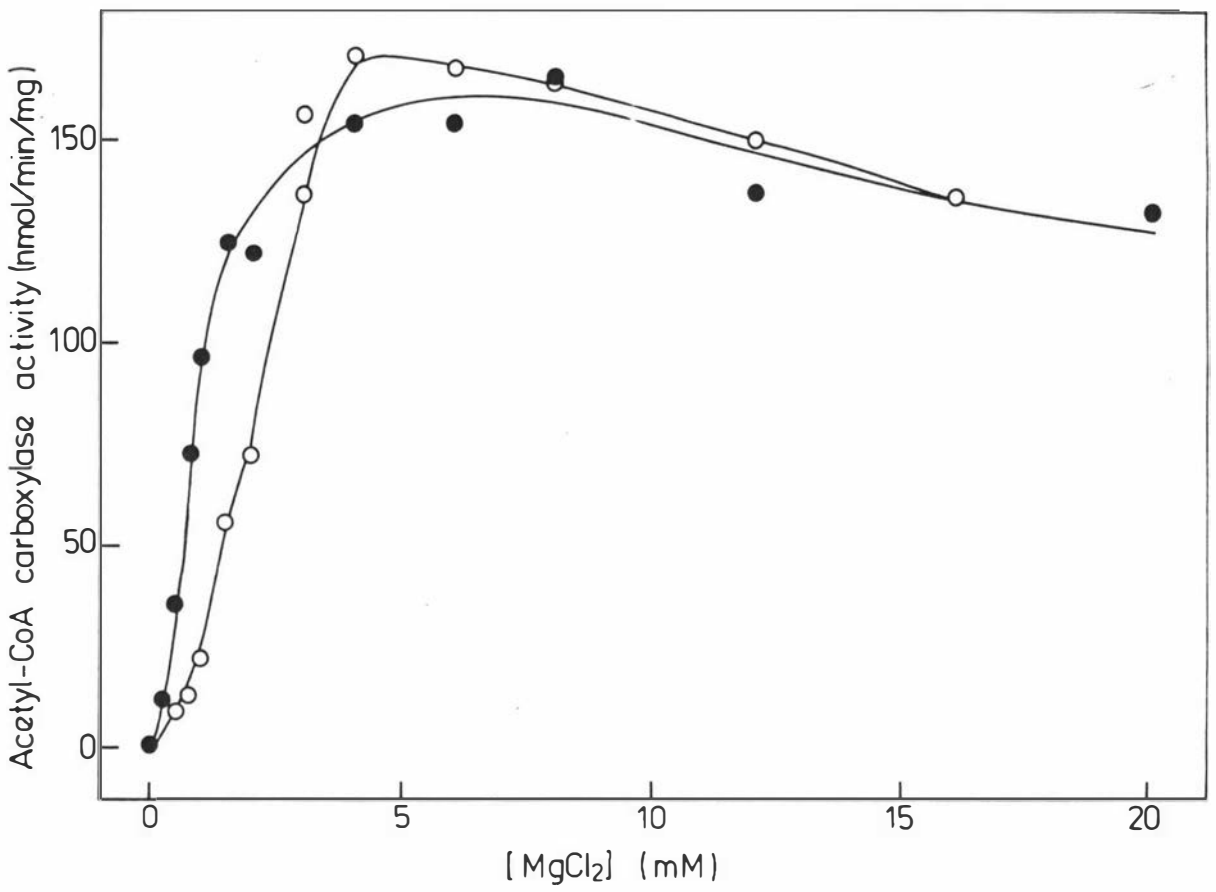


FIGURE 26: Effect of $MgCl_2$ on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, 1mM (●) or 3.0mM (o) ATP and varying concentrations of $MgCl_2$.

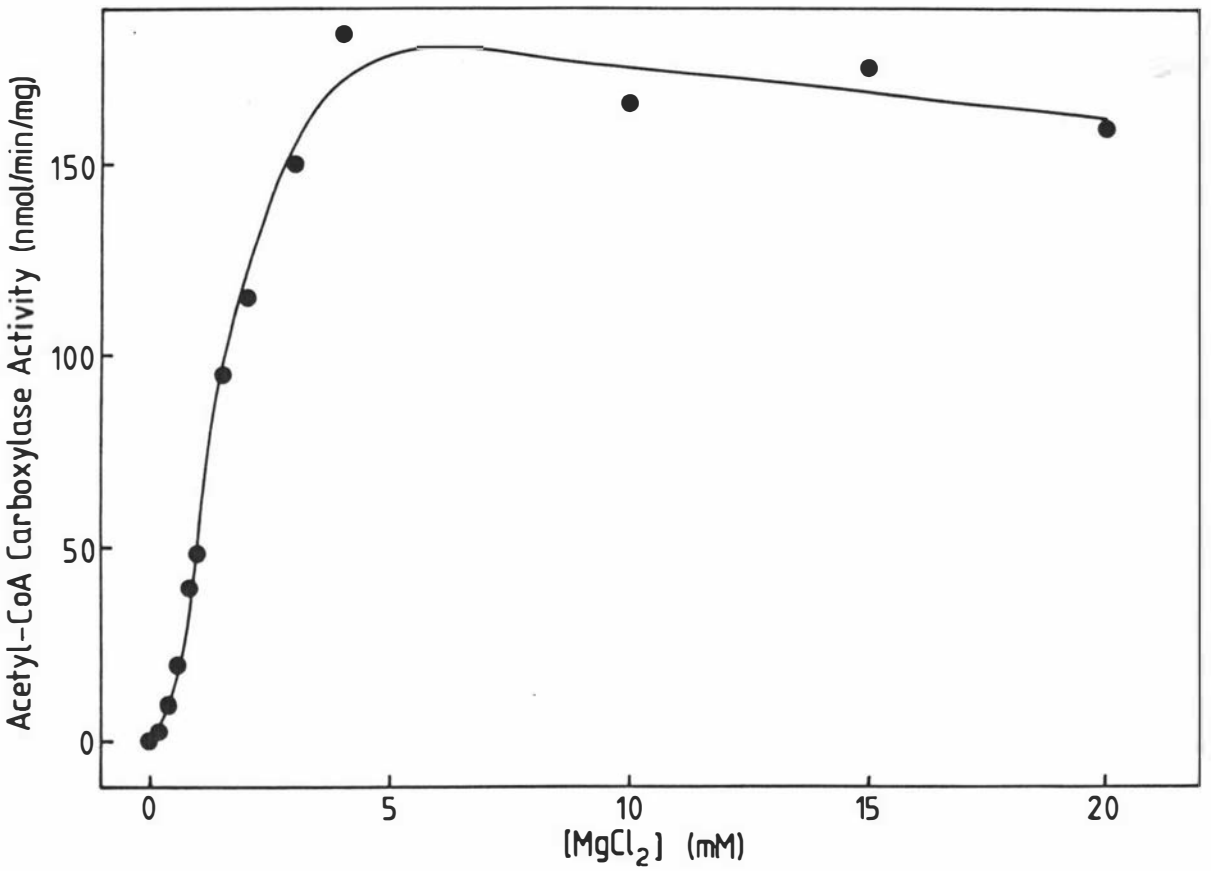


FIGURE 27: Effect of $MgCl_2$ on the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, 1mM ATP and varying concentrations of $MgCl_2$.

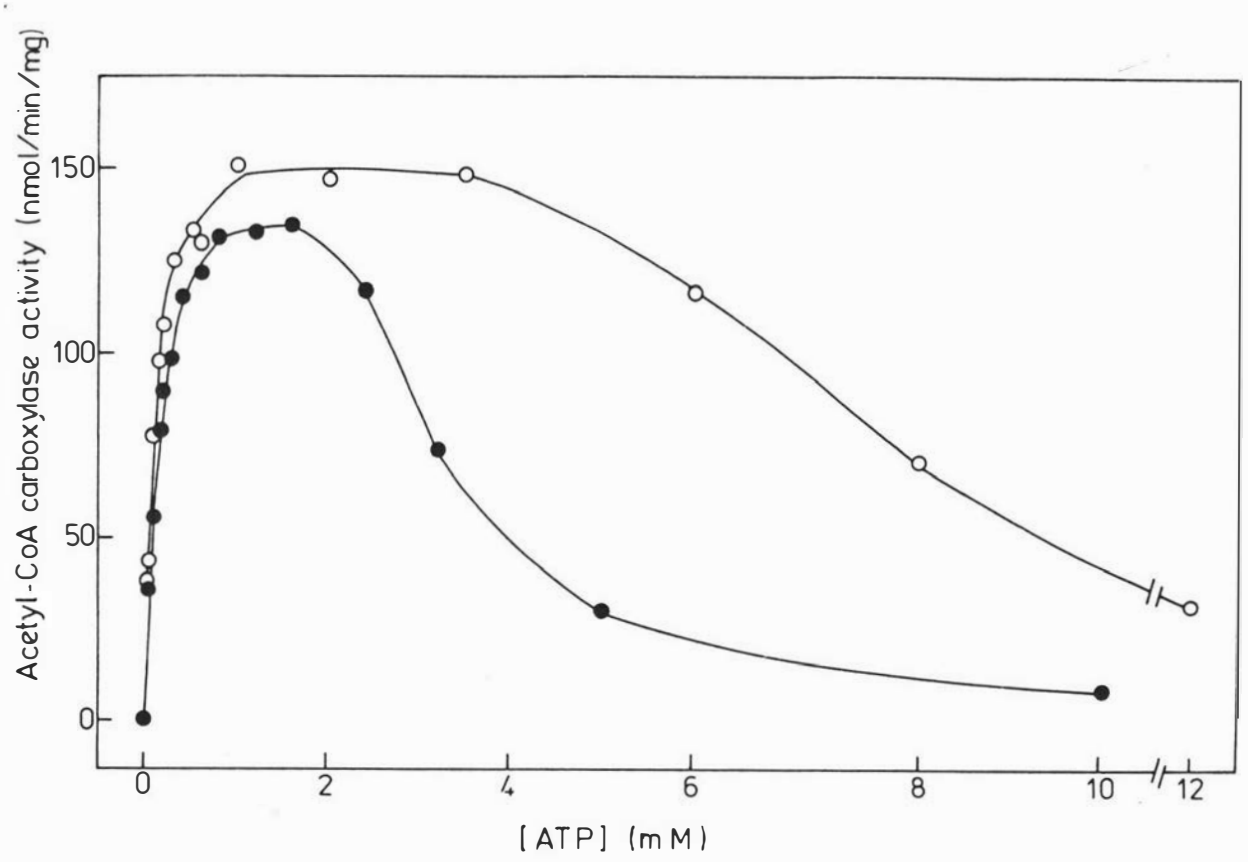


FIGURE 28: Effect of ATP on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, 2.5mM (●) or 5.0mM (o) $MgCl_2$ and varying concentrations of ATP.

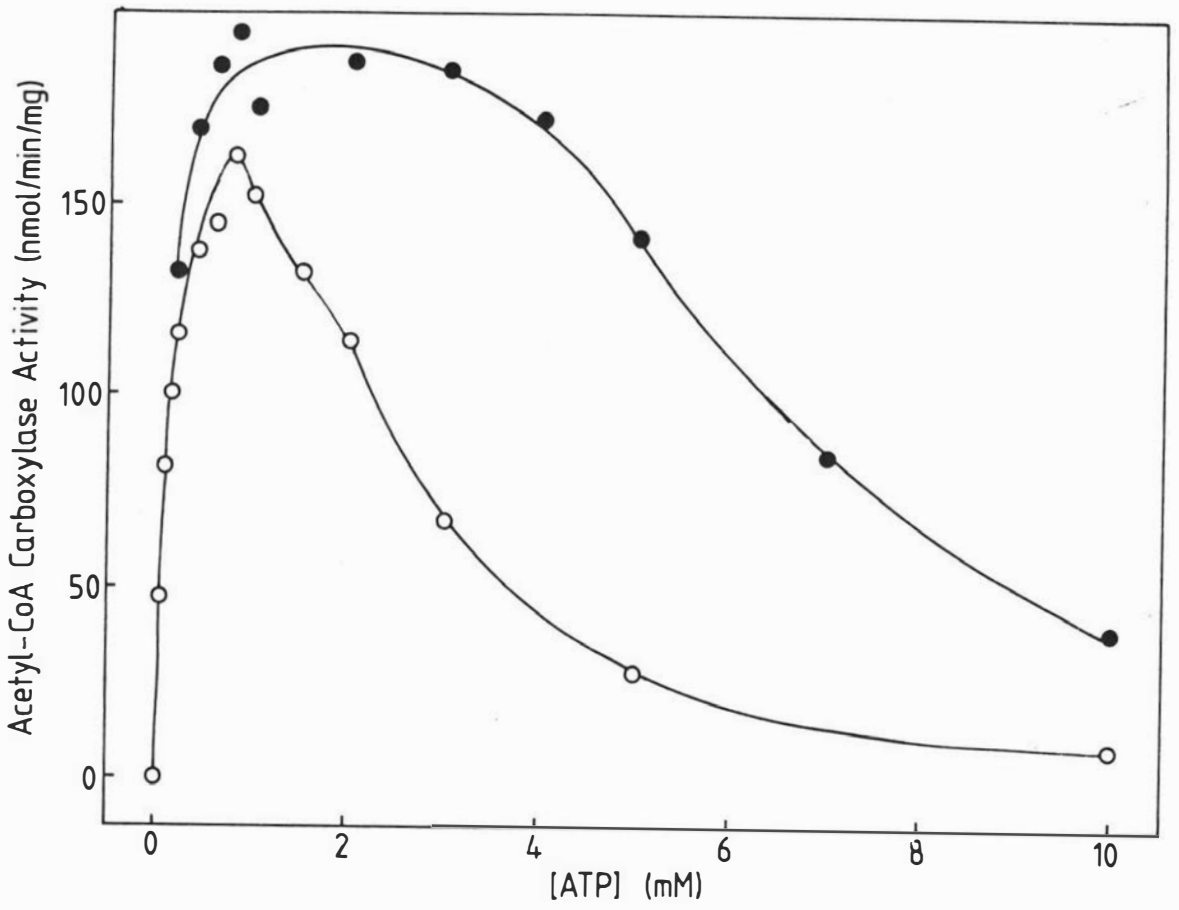


FIGURE 29: Effect of ATP on the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, 2.5mM (o) or 5.0mM (●) $MgCl_2$ and varying concentrations of ATP.

both Mg^{2+} and ATP concentrations, and maintaining a constant relative difference between the two cofactors. When the $MgCl_2$ concentration was always 1mM less than that of ATP, activity of both maize (Fig. 30) and barley (Fig. 31) leaf acetyl-CoA carboxylases showed a sigmoidal response to increasing ATP concentration. However, upon increasing the relative concentration of $MgCl_2$, so that it is either equal to, or in 2.5mM excess of the ATP concentration, activities of acetyl-CoA carboxylases of both maize and barley showed increasingly hyperbolic responses to increasing ATP concentration. The Hill-coefficient (n), which can be taken as a measure of 'sigmoidicity', decreased from 2.7 to 1.2 to 1.1 when the $MgCl_2$ concentration was 1mM less than, equal to and in 2.5mM excess of the ATP concentration, respectively, for the maize enzyme (Fig. 32). Concurrently the value of $ATP_{0.5v}$ decreased from 3.4mM to 1.9mM to 0.2mM when the relationship between the ATP and $MgCl_2$ concentrations were altered as described above (Fig. 32). The barley leaf acetyl-CoA carboxylase showed almost identical behaviour, with the Hill-coefficients decreasing from 2.3 to 1.5 to 1.2, while the value of $ATP_{0.5v}$ changed from 3.2mM to 1.5mM to 0.15mM, as the relative $MgCl_2$ concentration was altered from 1mM less than, to be equal to, and 2.5mM in excess of ATP, respectively (Fig. 33).

Storer and Cornish-Bowden (1976) have calculated the concentrations of the Mg.ATP complex, with different proportions of $MgCl_2$ and ATP. Maximum concentration of Mg.ATP occurs when $MgCl_2$ is in excess of ATP by about 1 - 3mM, and as maximum acetyl-CoA carboxylase activity occurs under such conditions (Fig. 26 - 29), Mg.ATP appears to be the substrate of this enzyme. However, both free Mg^{2+} and free ATP can affect the activity of the enzyme. London and Steck (1969) have described a number kinetic models of enzyme reactions, in which $MgCl_2$ and ATP combine with the enzyme and each other. The possibilities investigated were of an enzyme with Mg.ATP as the substrate, which was either inhibited by free ATP and free Mg^{2+}, or inhibited by free ATP and acti-

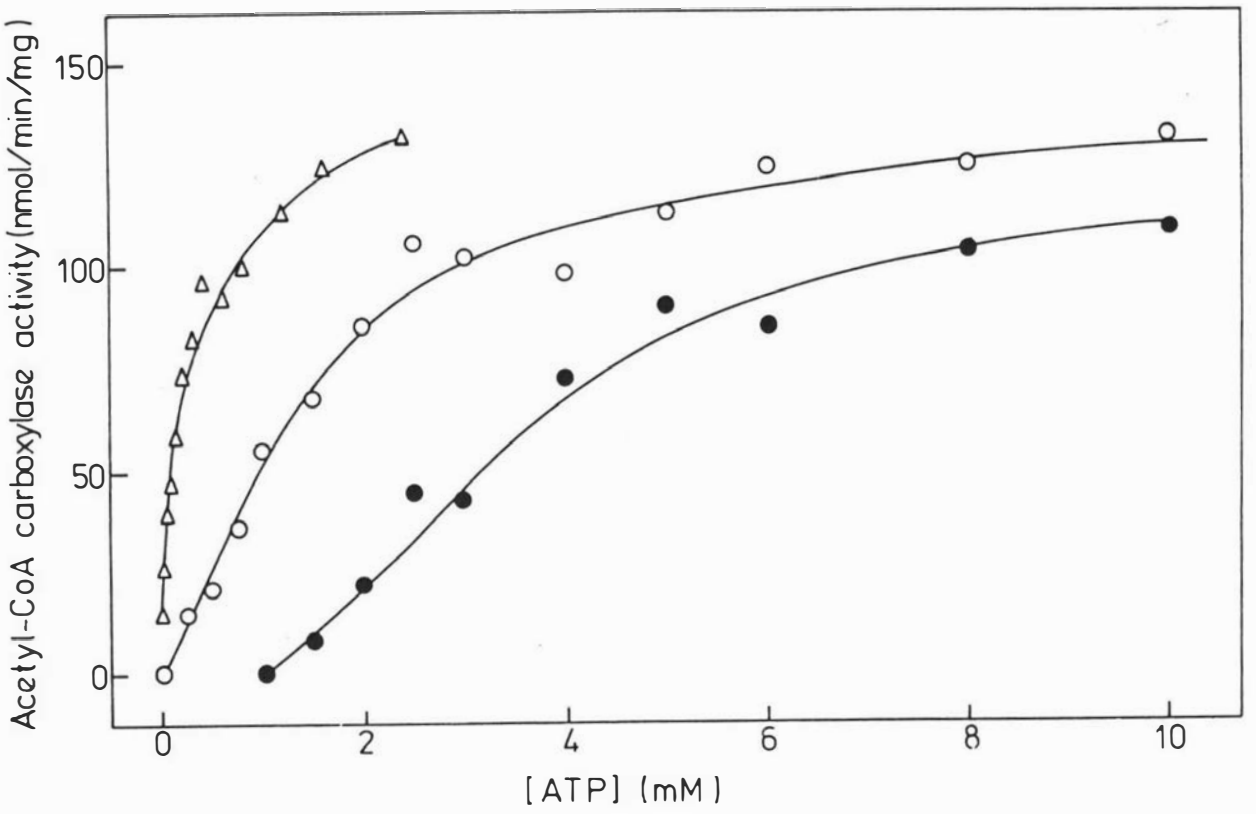


FIGURE 30: Relationship between ATP, $MgCl_2$ concentrations and the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^- and varying ATP and $MgCl_2$ concentrations. The relationships between $MgCl_2$ and ATP were: $[MgCl_2] = [ATP] - 1mM$, (●); $[MgCl_2] = [ATP]$, (o); $[MgCl_2] = [ATP] + 2.5mM$, (Δ).

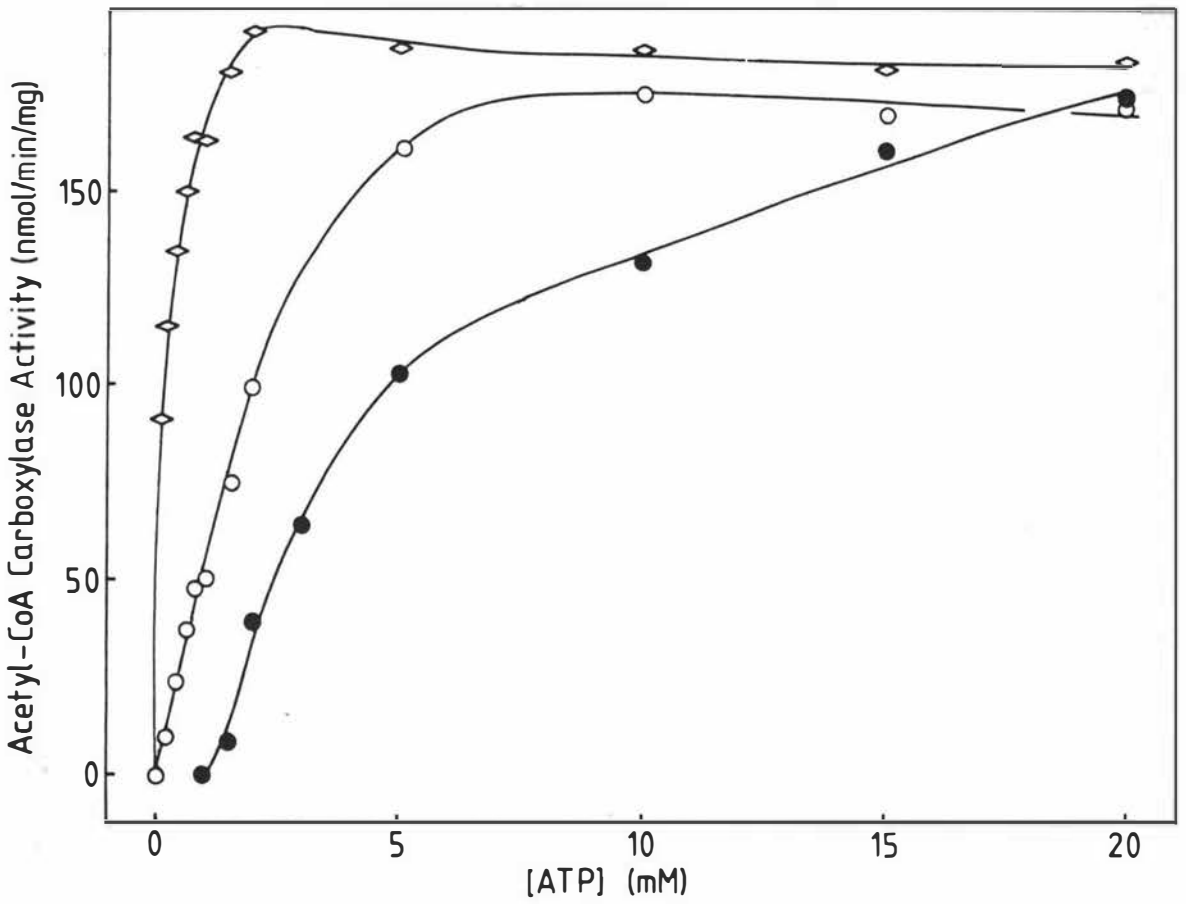


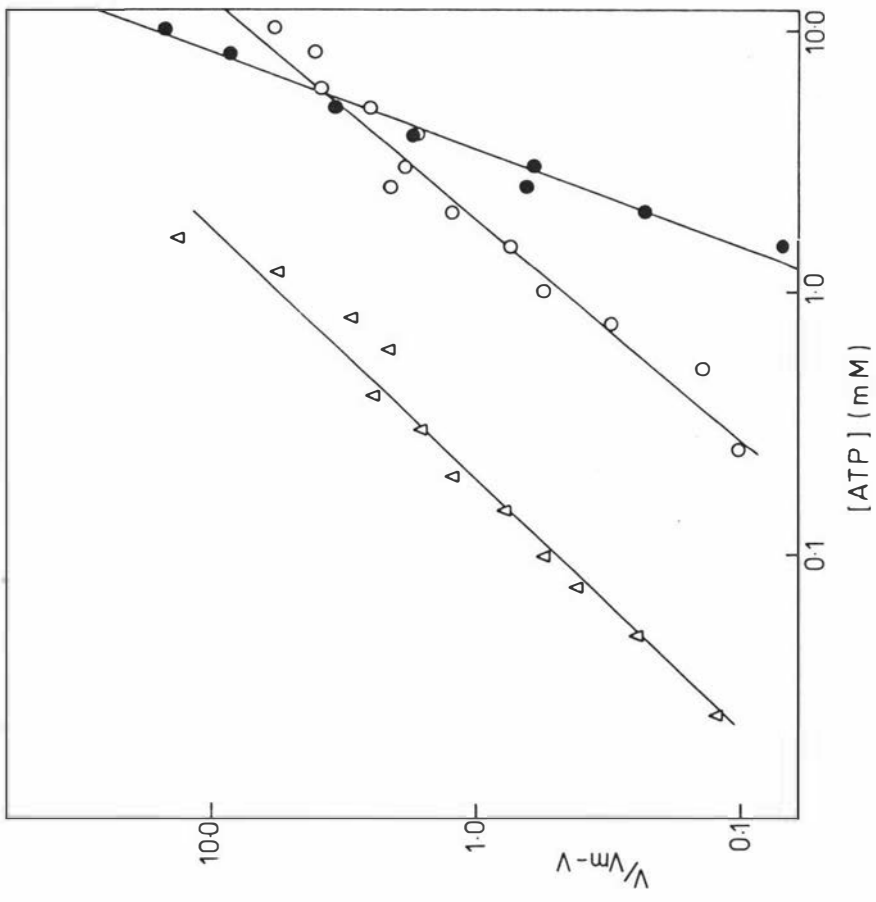
FIGURE 31: Relationship between ATP, $MgCl_2$ concentrations and the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^- and varying ATP and $MgCl_2$ concentrations. The relationships between $MgCl_2$ and ATP were: $[MgCl_2] = [ATP] - 1mM$, (●); $[MgCl_2] = [ATP]$, (o); $[MgCl_2] = [ATP] + 2.5mM$, (◇).

FIGURE 32: Analysis by Hill-plot of the relationship between ATP, $MgCl_2$ concentrations and the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 30. Vm as obtained by the method of Endrenyi et al (1975). The Hill-plot constants are shown below.

Relationship between $MgCl_2$ and [ATP]	Hill Coefficient	$ATP_{v0.5}$ (mM)
[$MgCl_2$] = [ATP] - 1mM (●)	2.7	3.4
[$MgCl_2$] = [ATP] (o)	1.2	1.9
[$MgCl_2$] = [ATP]+ 2.5mM (Δ)	1.1	0.20


10·0
1·0
0·1
V/Vm-V
0·1
1·0
10·0
[ATP] (mM)

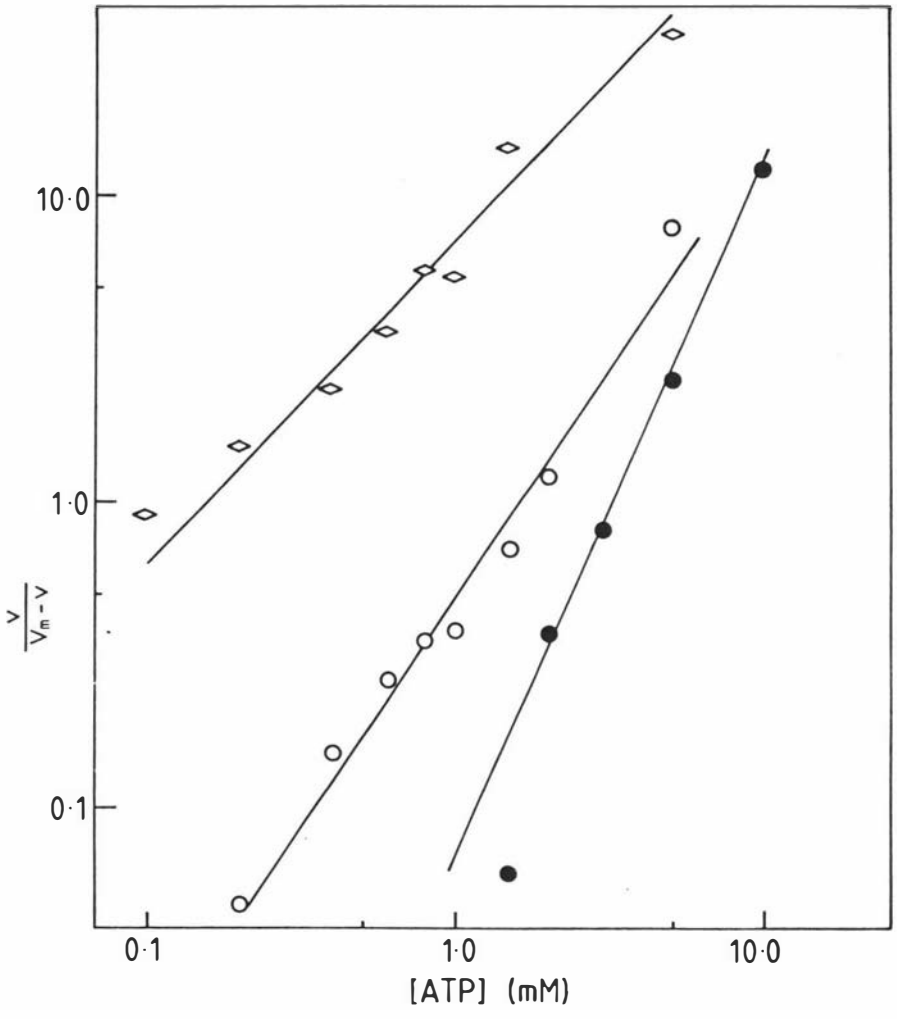


FIGURE 33: Analysis by Hill-plot of the relationship between ATP, $MgCl_2$ concentrations and the activity of barley leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 31. Vm was obtained by the method of Endrenyi et al (1975). The Hill-plot constants are shown below.

Relationship between $MgCl_2$ and ATP	Hill Coefficient	$ATP_{v0.5}$ (mM)
$[MgCl_2] = [ATP] - 1mM$ (●)	2.3	3.2
$[MgCl_2] = [ATP]$ (o)	1.5	1.5
$[MgCl_2] = [ATP] + 2.5mM$ (◇)	1.2	0.15

vated by free Mg^{2+}, or activated by free Mg^{2+} only. The properties of acetyl-CoA carboxylase of maize and barley appear to fit the second model, with free Mg^{2+} acting as an activator, and free ATP behaving as an inhibitor. Thus, inhibition of acetyl-CoA carboxylase by ATP was responsible for the decrease in the activity when the ATP concentration was increased above that of Mg^{2+} (Figs 28 and 29), and also the sigmoidal increase in activity with increasing Mg^{2+} concentrations (Figs 26 and 27). Mg^{2+} activation of acetyl-CoA carboxylase on the other hand was responsible for the decrease in the Hill-coefficients and in the values of $ATP_{v0.5}$, when the relative Mg^{2+} concentration was increased, from 1mM less than the ATP concentration, to be equal to, and 2.5mM in excess (Figs 30 - 33).

These interesting effects of Mg^{2+} and ATP concentrations on acetyl-CoA carboxylase, may have physiological significance with regard to the effect of light and dark on the activity of this enzyme, and with its possible effect on the rate of fatty acid biosynthesis in chloroplasts.

4.14 Specificity of the Divalent Metal Ion Required for Acetyl-CoA Carboxylase Activity

Of the divalent cations tested as alternatives to Mg^{2+}, only Mn^{2+} supported acetyl-CoA carboxylase activity, but the maximum activity attained was only 25% of that observed with Mg^{2+} (Fig. 34). Maximum activity when the ATP concentration was 1mM, occurred at 1mM Mn^{2+}, compared with 3mM Mg^{2+}. Ca^{2+}, Ni^{2+} and Co^{2+} were ineffective in replacing Mg^{2+}.

4.15 Effect of pH on Acetyl-CoA Carboxylase Activity

Initial attempts to investigate the effect of pH on acetyl-CoA carboxylase were carried out using the buffers Mes, Hepes, Tricine and phosphate individually. As acetyl-CoA

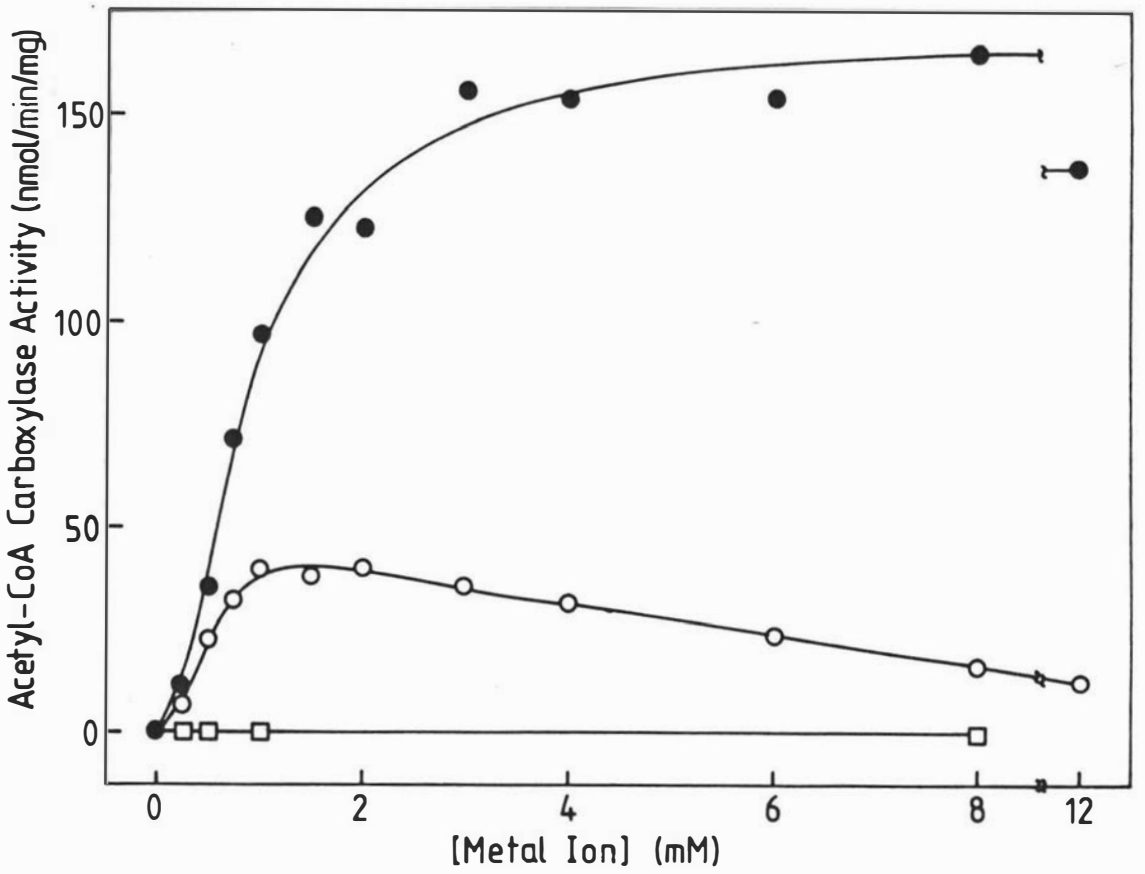


FIGURE 34: Effect of divalent metal ions on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^- and varying concentrations of $MgCl_2$, (●); $MnCl_2$, (o); $CoCl_2$, $NiCl_2$, $CaCl_2$, (□).

carboxylase activity was affected differently by each buffer, difficulty was encountered in determining the effect of pH on activity. This problem was overcome by using a mixture of the buffers Mes, Hepes and Tricine adjusted to the appropriate pH with NaOH.

Acetyl-CoA carboxylase from both maize (Fig. 35) and barley (Fig. 36) showed very similar dependence on pH, with the pH optimum at 8.4. The activities decreased sharply on either side of the optimum and showed minimal activities below 6.5 and above pH 9.0.

4.16 Effect of Temperature on Acetyl-CoA Carboxylase Activity

Precipitation of proteins by heat denaturation is a widely used procedure in the purification of enzymes. However, the instability of maize leaf acetyl-CoA carboxylase at high temperatures precluded the use of this procedure in the purification of the enzyme. In 5 min assays, the activity of maize leaf acetyl-CoA carboxylase increased rapidly as the temperature was raised from 20^{o}C to 40^{o}C. However, above 40^{o}C activity decreased rapidly as the enzyme was unstable at these higher temperatures, so that 54^{o}C only 15% of the activity at the optimum temperature remained. Routine assays in this study were carried out at the more physiological temperature of 30^{o}C, where activity was 70% of maximum.

4.17 Activation of Acetyl-CoA Carboxylase Activity by Monovalent Cations

Acetyl-CoA carboxylase from wheat germ showed a HCO_3^--dependent activation by monovalent cations, especially K^+ (Nielsen et al., 1979). Similarly, the inclusion of K^+ in an otherwise standard assay solution, enhanced the activity of the

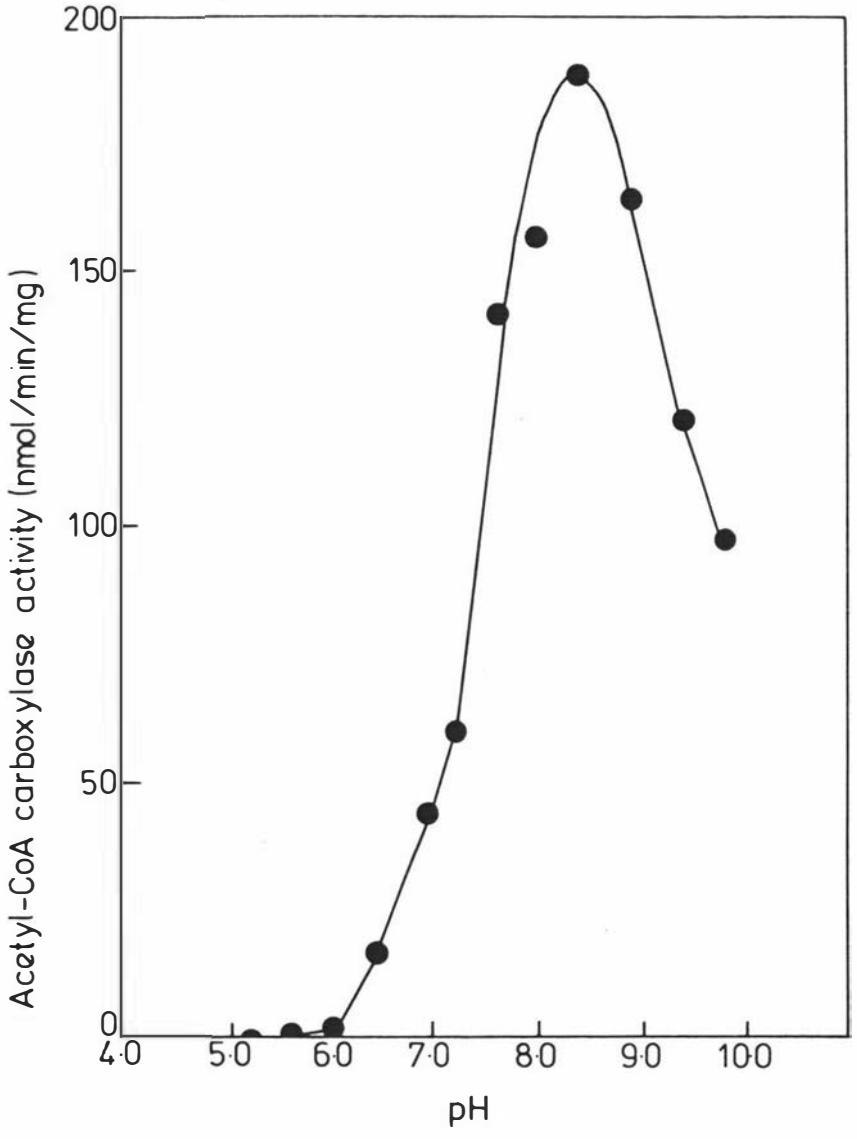


FIGURE 35: Effect of pH on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2, using 10mM HCO_3^-, except the buffer was a mixture of 0.1M Mes, 0.1M Hepes, 0.1 Tricine adjusted to the final pH with NaOH.

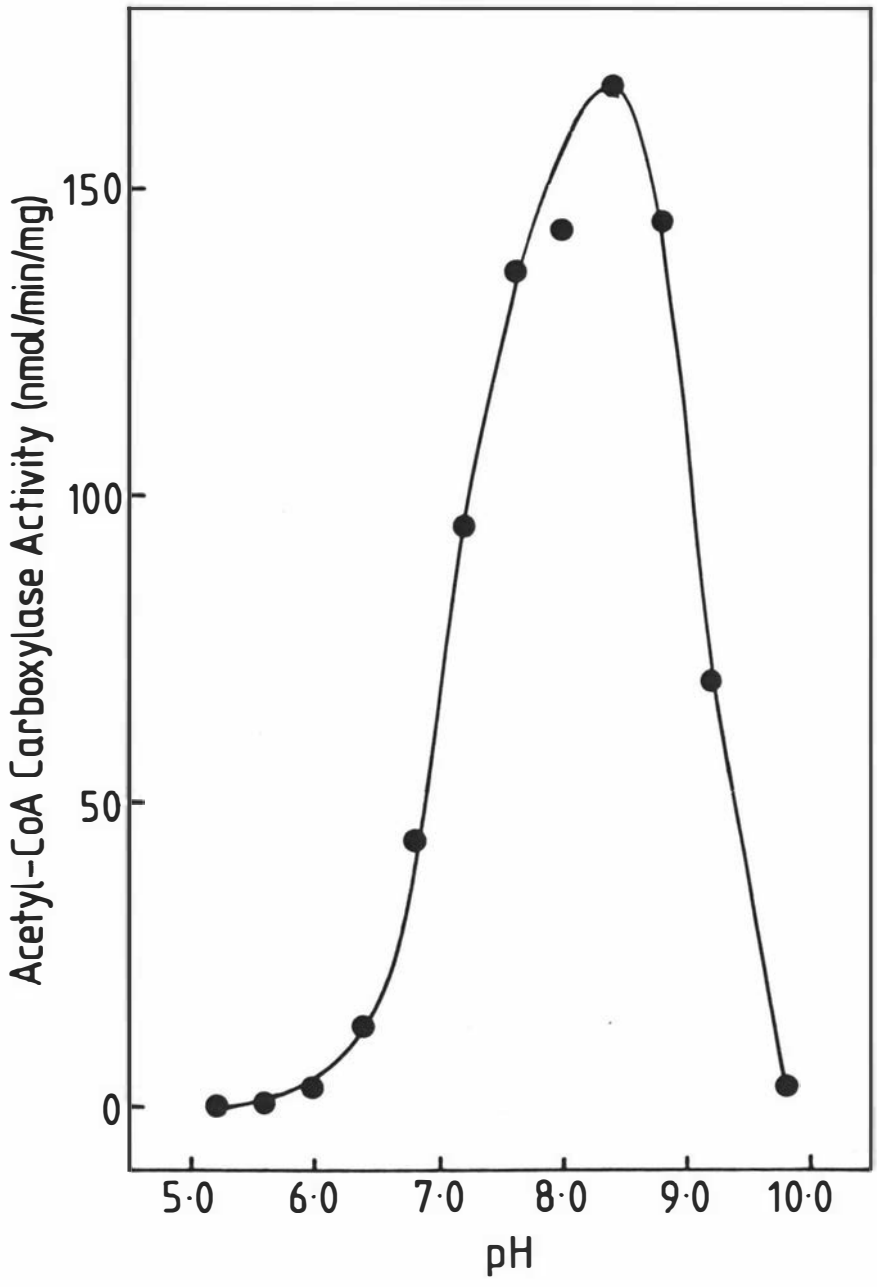


FIGURE 36: Effect of pH on the activity of barley leaf acetyl-CoA carboxylase.

Assay conditions: as in Fig. 35.

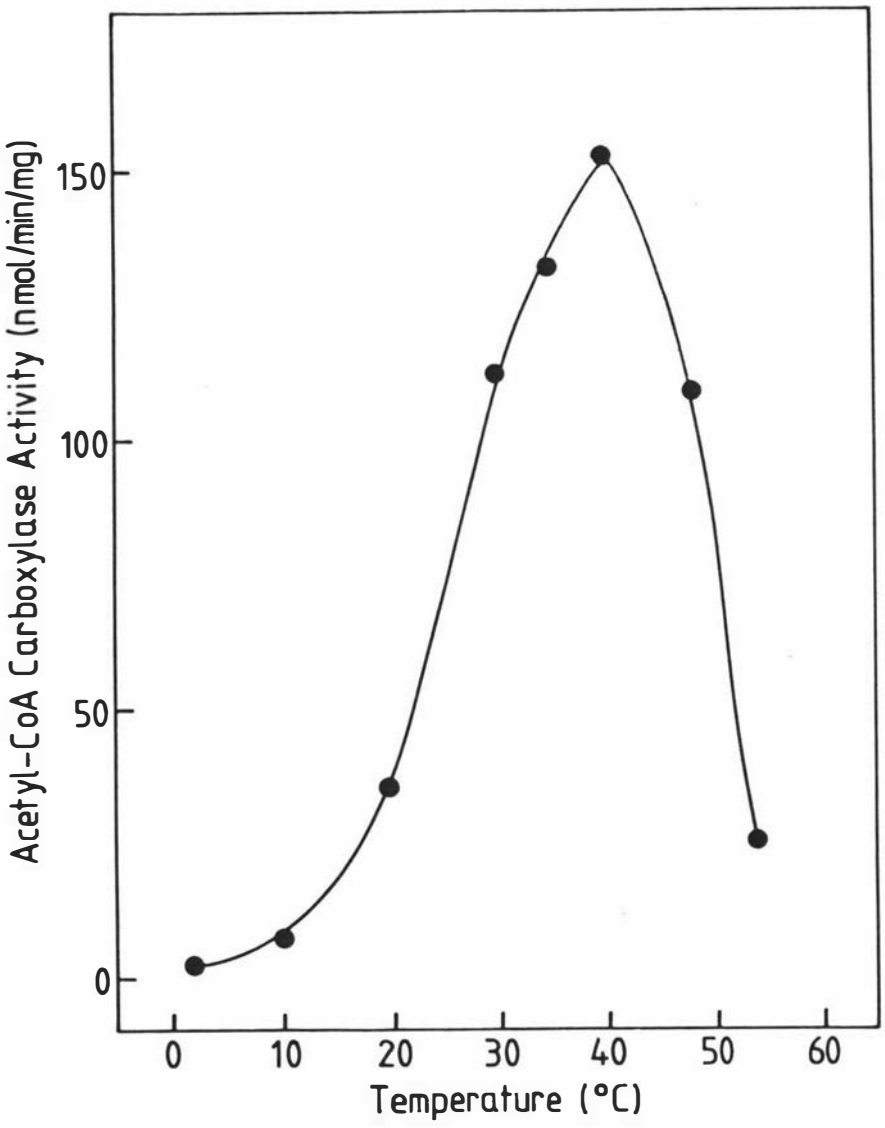


FIGURE 37: Effect of temperature on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2, using 10mM HCO_3^-, except the assays were performed at the indicated temperatures.

maize leaf enzyme. Greatest activation by K^+ was observed at 0.18mM HCO_3^- (Fig. 38), which was the lowest HCO_3^- concentration examined. Compared with a 3-fold stimulation obtained at this HCO_3^- concentration, a 2- and 1.4-fold activation by K^+ was observed at 1mM and 10mM. Maximum activation of acetyl-CoA carboxylase at each HCO_3^- concentration examined occurred above 12 - 15mM K^+.

The specificity of the monovalent cation required for activation of acetyl-CoA carboxylase was tested by substitution of K^+ with Li^+, Cs^+ and NH_4^+, using chloride salts with each cation. At 1mM and 10mM HCO_3^-, K^+ was the most effective activator followed in order by NH_4^+ and Cs^+ (Table XX). Monovalent cation activation of acetyl-CoA carboxylase was greater at 1mM than at 10mM HCO_3^-. Li^+ inhibited acetyl-CoA carboxylase at both of these HCO_3^- concentrations.

4.18 Substrate Specificity of Acetyl-CoA Carboxylase

Partially purified acetyl-CoA carboxylase of maize leaves, carboxylated propionyl-CoA as well as acetyl-CoA. Below 0.1mM, propionyl-CoA was almost as effective as acetyl-CoA as a substrate, but was inhibitory at higher concentrations (Fig. 39). The rate of propionyl-CoA carboxylation was about 45% that of acetyl-CoA, at the optimum concentration for each substrate. Butyryl-CoA was not carboxylated by acetyl-CoA carboxylase under these standard conditions.

4.19 Inhibition of Maize Leaf Acetyl-CoA Carboxylase by Malonyl-CoA

Malonyl-CoA, the carboxylated product of the acetyl-CoA carboxylase catalysed reaction, strongly inhibited the activity of the enzyme from maize (Fig. 40). Malonyl-CoA, at a concentration of 1.5mM, inhibited acetyl-CoA carboxylase activity by 95%, while 50% inhibition occurred at about 0.35mM.

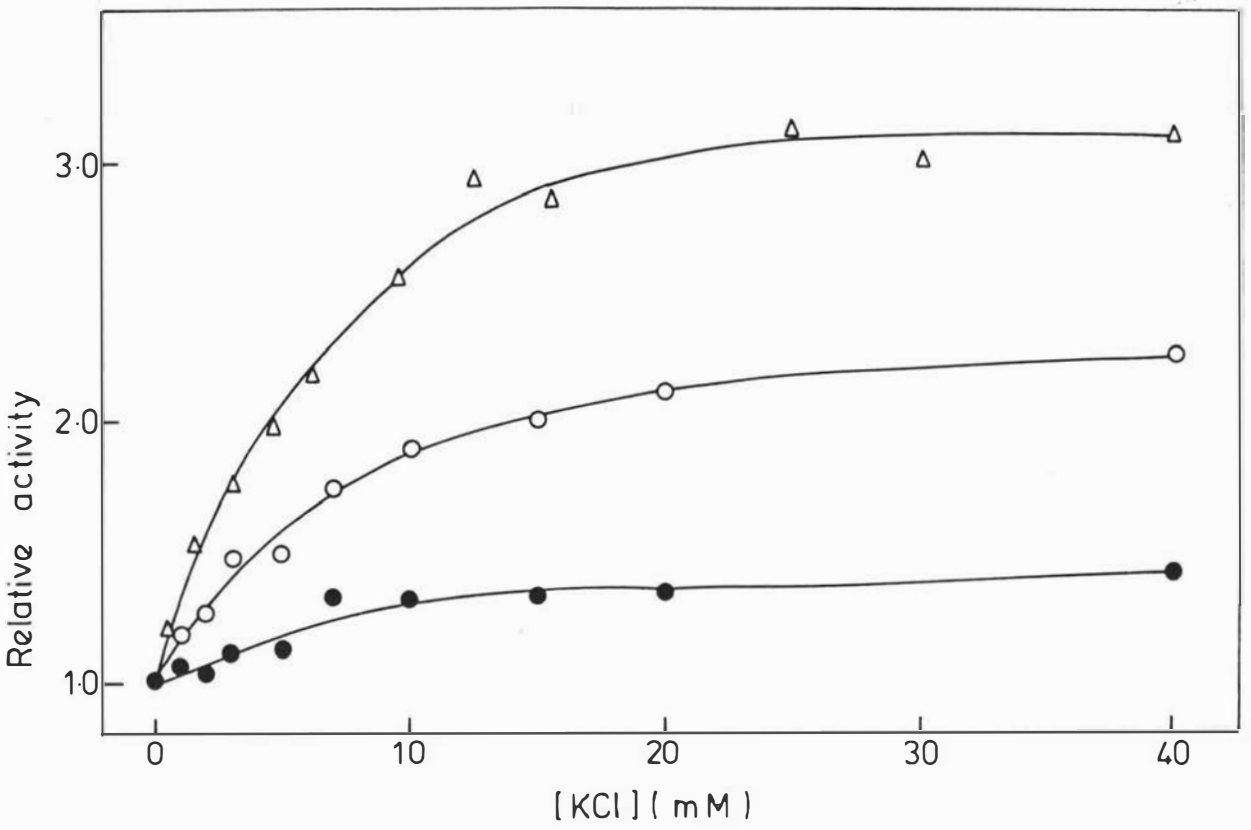


FIGURE 38: Effect of KCl on the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2, except NaOH was used for pH adjustments and the KCl concentration varied as shown. Acetyl-CoA carboxylase activities were: 12.1 nmol/min/mg at 0.18mM HCO_3^- (Δ), 23.1 nmol/min/mg at 1mM HCO_3^- (o), and 123 nmol/min/mg at 10mM HCO_3^- (●), without added KCl.

TABLE XX

ACTIVATION OF MAIZE LEAF ACETYL-CoA CARBOXYLASE BY MONOVALENT CATIONS

Cation	Relative acetyl-CoA carboxylase activity	
	1mM HCO_3^-	10mM HCO_3^-
None	1.00	1.00
K^+	2.11	1.34
Li^+	0.90	0.64
Cs^+	1.16	1.09
NH_4^+	1.35	1.24

Acetyl-CoA carboxylase assays were performed with 1mM or 10mM HCO_3^- (Section 3.2), using NaOH for adjustment of pH. The selected cation was added to a final concentration of 20mM. Without the added cation, acetyl-CoA carboxylase activity was 23.1 and 123 nmol/min/mg at 1mM and 10mM HCO_3^-, respectively.

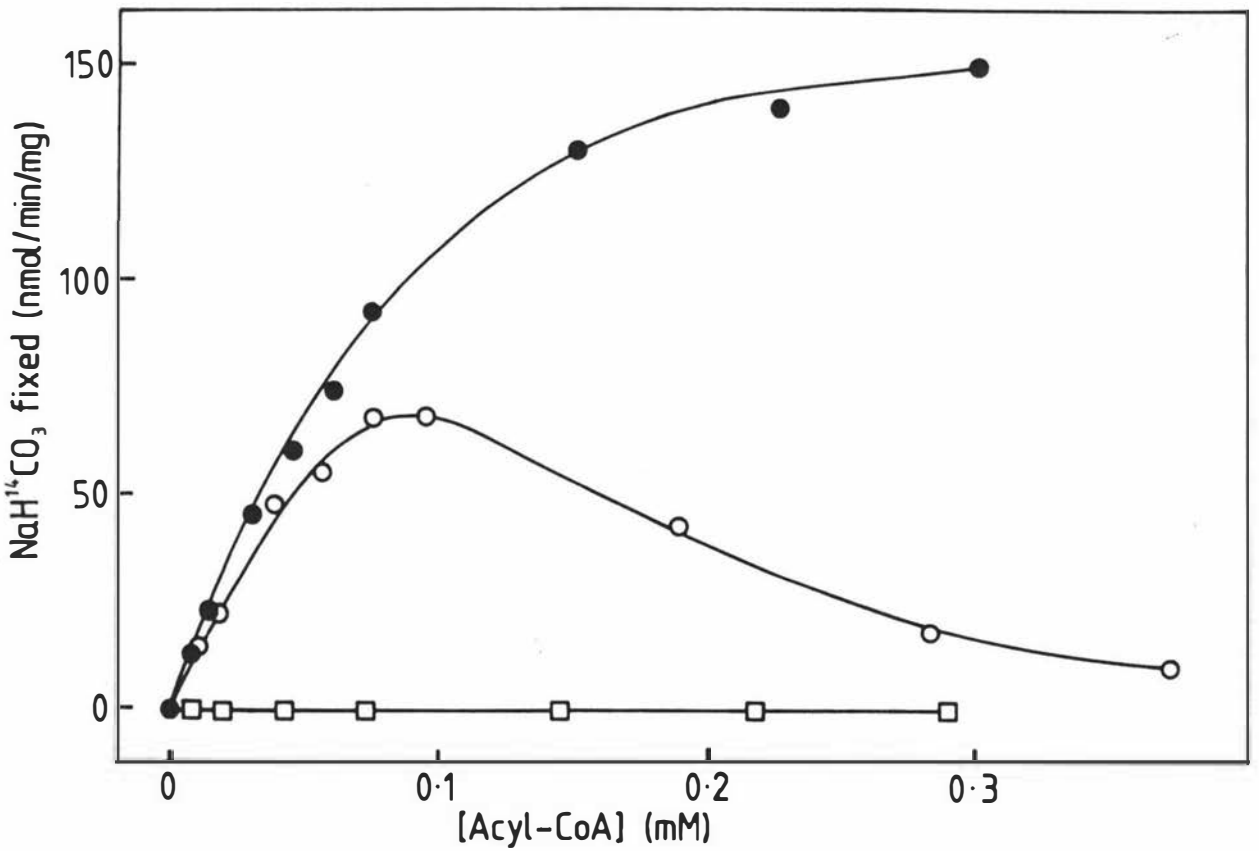


FIGURE 39: Specificity of carboxylation by maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^- and either acetyl-CoA (●) or propionyl-CoA (o) or Butyryl-CoA (□) as substrates.

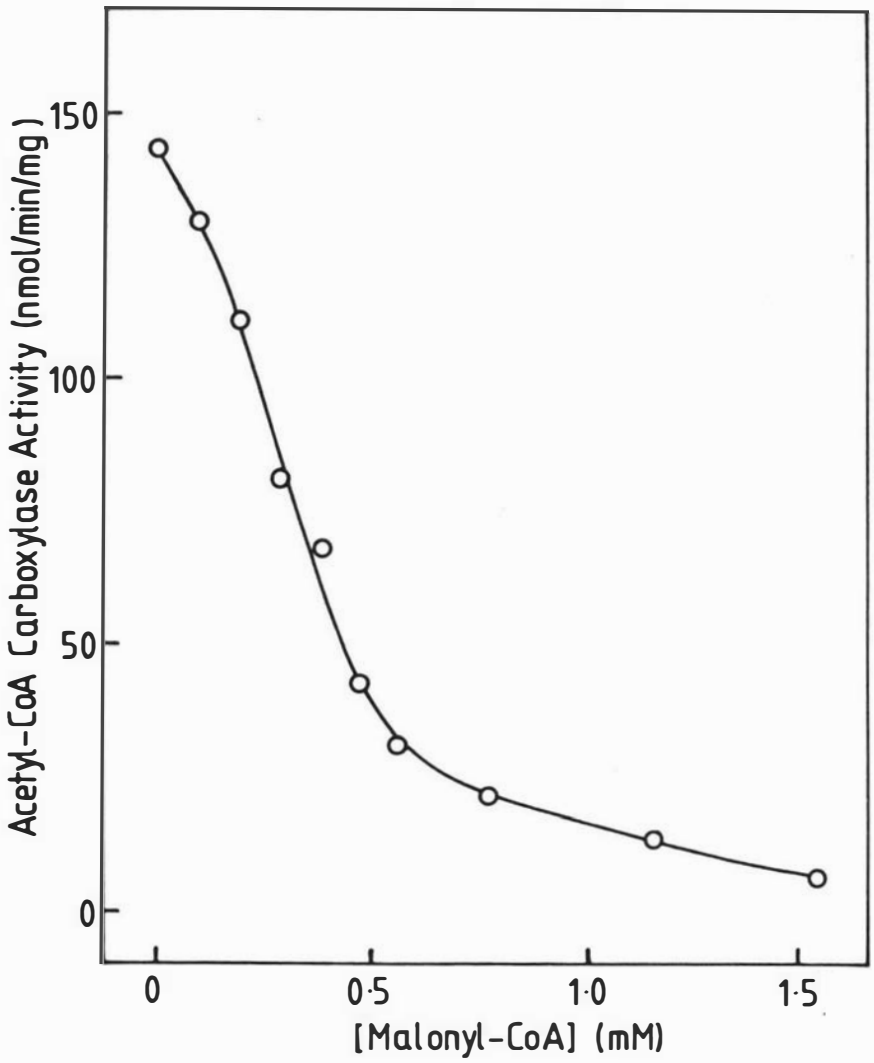


FIGURE 40: Effect of malonyl-CoA on the activity of maize leaf acetyl-CoA carboxylase

Assay conditions: as described in Section 3.2, using 10mM HCO_3^- and varying concentration of added malonyl-CoA.

4.20 Effect of ADP on Acetyl-CoA Carboxylase Activity

Preliminary studies showed that acetyl-CoA carboxylase from maize was inhibited by ADP, a product of the reaction. This inhibition by ADP was investigated in detail (Fig. 41). A Lineweaver-Burk plot of the data showed that the inhibition was competitive with respect to ATP (Fig. 42). The values of K_m^{ATP} increased from 0.15mM in the absence of ADP, to 3.0 mM in the presence of 5.0mM ADP. Analysis of the same data as described by Dixon (1953), confirmed the competitive nature of the inhibition with respect to ATP and provided the inhibition constant, K_i^{ADP} of 0.4mM (Fig. 43).

Inhibition of the carboxylation of acetyl-CoA by ADP, was also investigated at different concentrations of acetyl-CoA (Fig. 44). Analysis of the data by the Lineweaver-Burk method (Fig. 45), indicated that inhibition was uncompetitive with respect to acetyl-CoA. Increasing concentrations of ADP decreased the values of V_{max} and $K_m^{acetyl-CoA}$.

4.21 Effect of CoA on Acetyl-CoA Carboxylase Activity

Recent investigations in the laboratory of Kim have shown CoA activation of rat liver acetyl-CoA carboxylase (Yeh and Kim, 1980; Yeh *et al.*, 1981). As maize acetyl-CoA carboxylase was found to interact with CoA, immobilized on Sepharose (Section 4.10), an investigation of the effect of CoA on acetyl-CoA carboxylase activity was carried out. In contrast to the rat liver enzyme, acetyl-CoA carboxylase from maize leaves was inhibited by free CoA (Fig. 46). A Lineweaver-Burk plot of the data shown in Fig. 46, indicated that increasing concentration of CoA did not affect the value of $K_m^{acetyl-CoA}$, but decreased the V_{max} of the reaction (Fig. 47). Treatment of the data according to Dixon (1953) indicated that the inhibition of acetyl-CoA carboxylase activity by CoA was noncompetitive with respect to acetyl-CoA, and the inhibition constant, K_i^{CoA} was 1.58mM (Fig. 48).

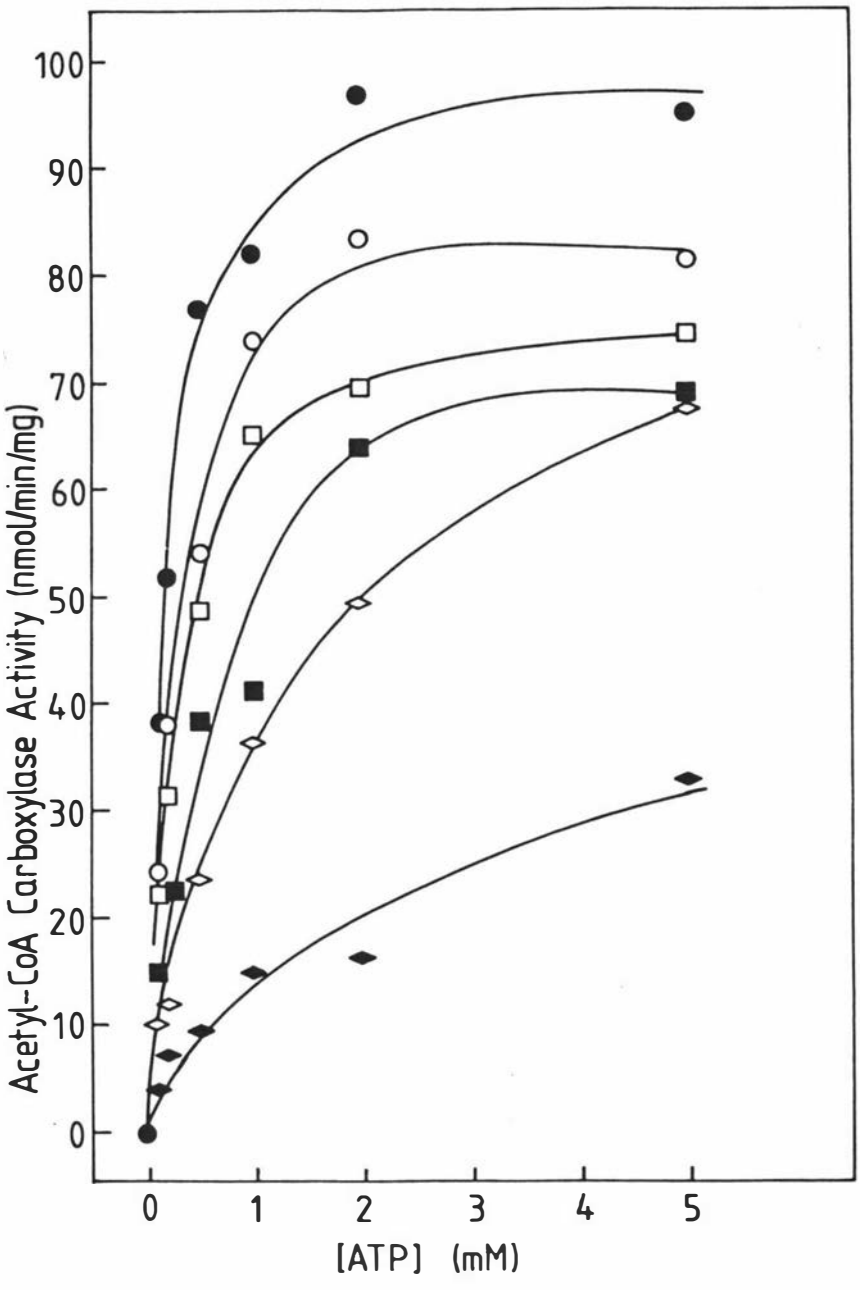


FIGURE 41: ADP inhibition of the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, except the $MgCl_2$ concentration was always 2.5mM in excess of ATP, and ADP was added to final concentrations of 0mM (●), 0.2mM (○), 0.4mM (□), 1.0mM (■), 2.0mM (◇) and 5.0mM (◆).

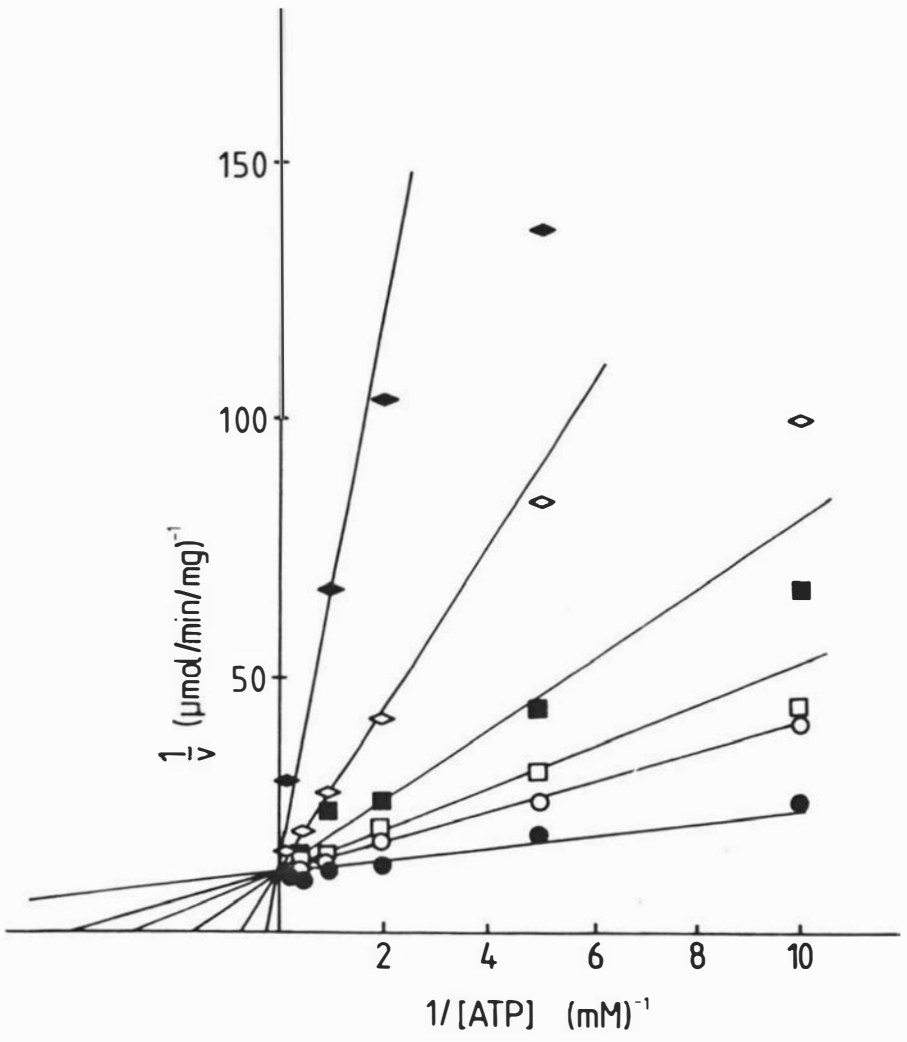


FIGURE 42: Lineweaver-Burk analysis of the effect of ADP on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 41.

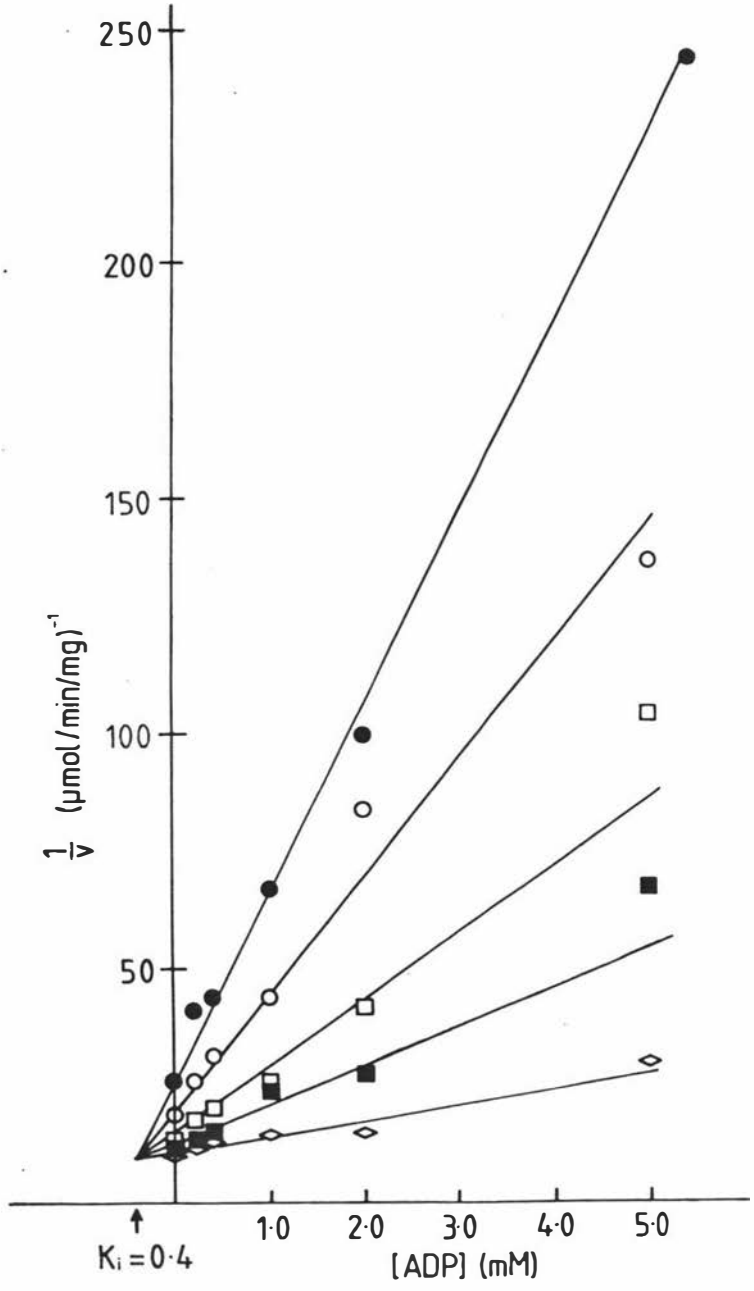


FIGURE 43: Analysis by Dixon-plot of the effect of ADP on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 41 by the method of Dixon (1953). ATP concentrations were: 0.1mM (●), 0.2mM (o), 0.5mM (□), 1.0mM (■) and 5.0mM (◇).

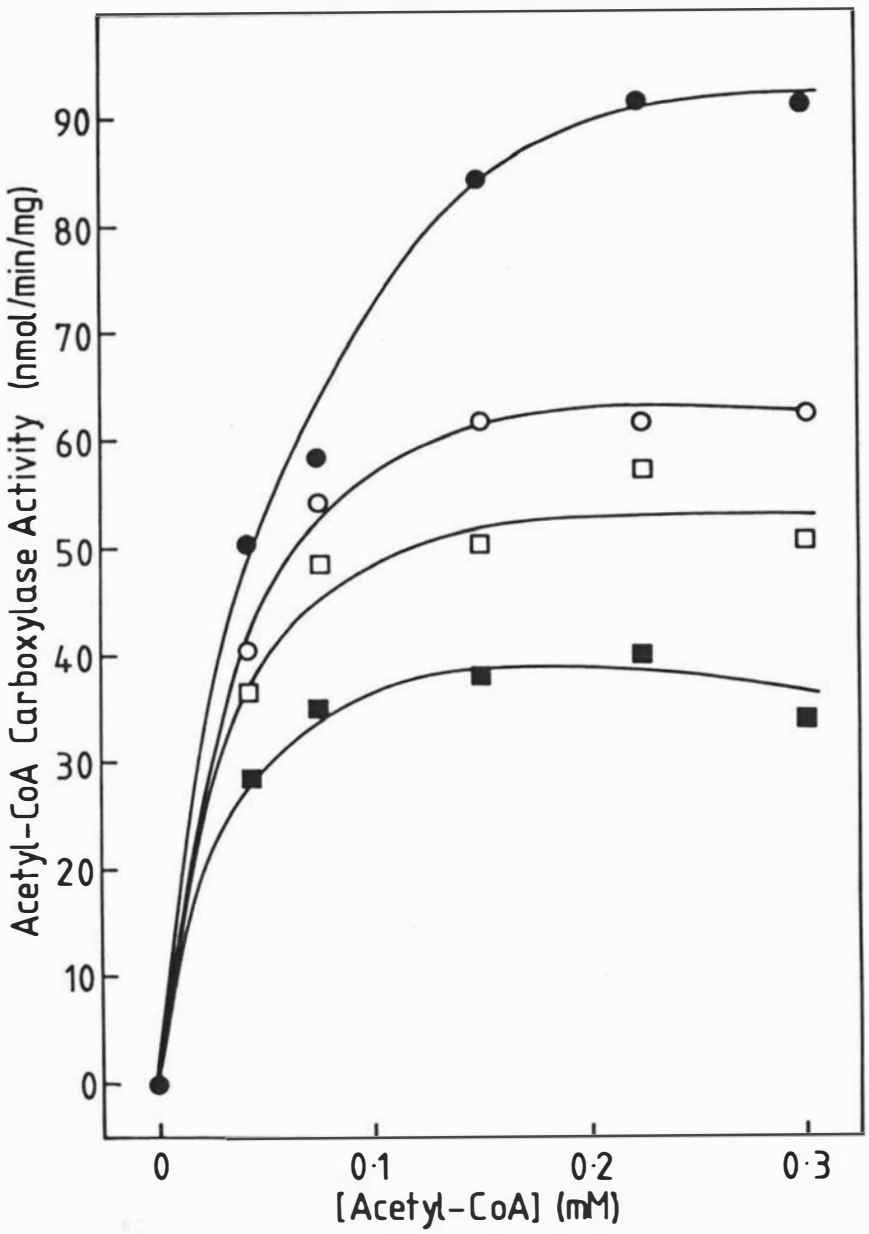


FIGURE 44: ADP inhibition of the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, except the acetyl-CoA concentration was varied and ADP was added to a final concentration of 0mM (●), 0.2mM (o), 0.4mM (□) and 2.0mM (■).

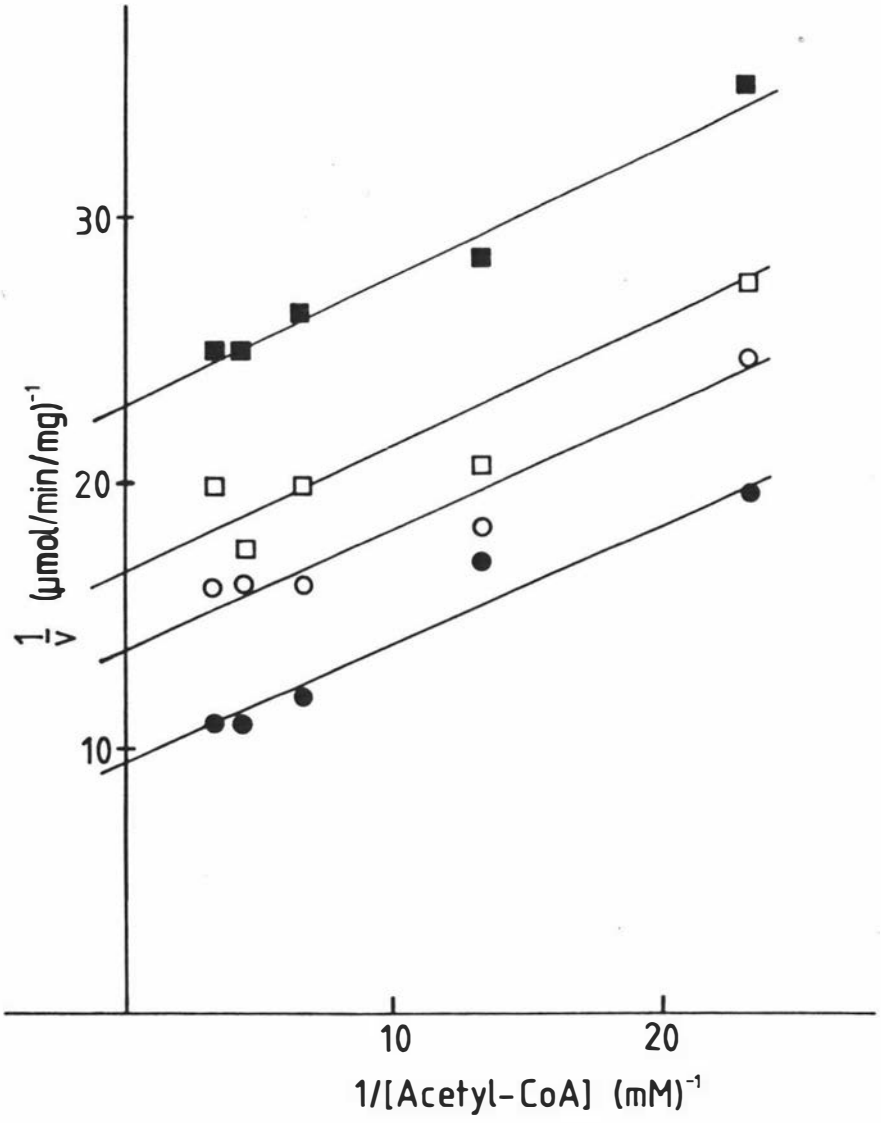


FIGURE 45: Lineweaver-Burk analysis of the effect of ADP on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 44.

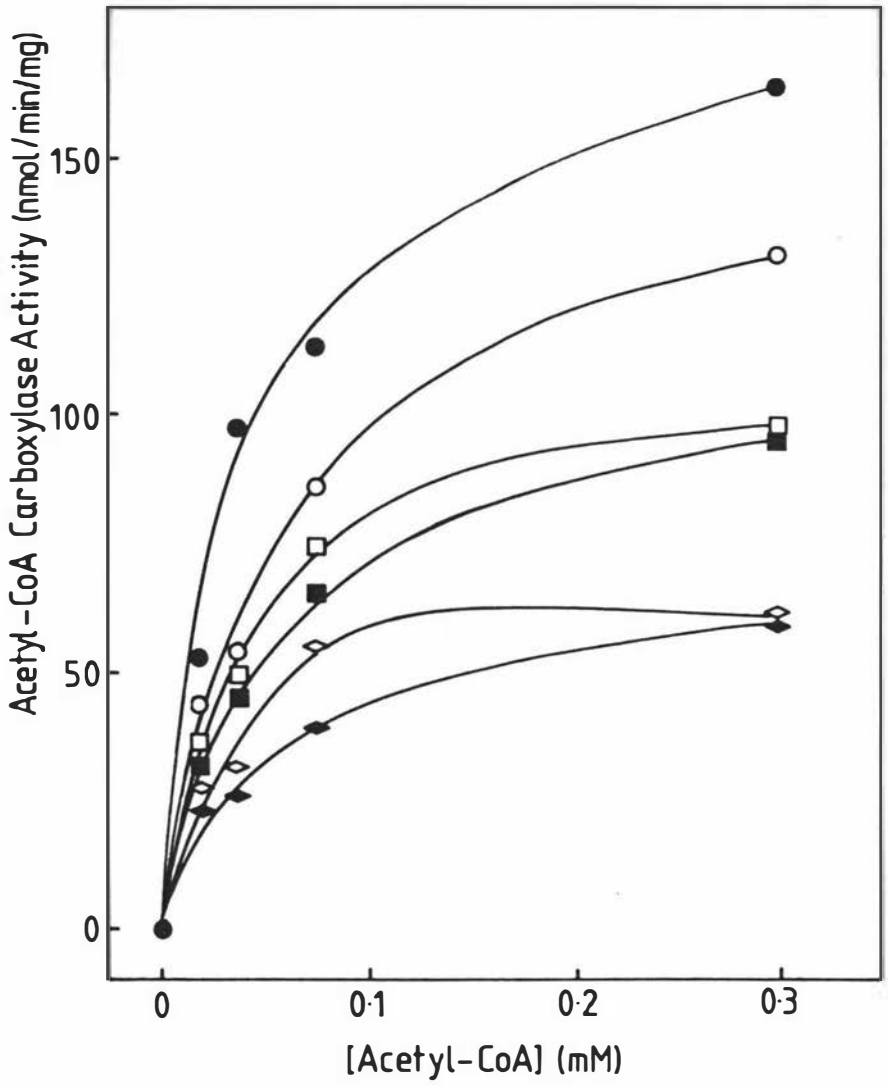


FIGURE 46: CoA inhibition of the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, except the acetyl-CoA concentration was varied and CoA was added to a final concentration of 0mM (●), 0.2mM (○), 0.5mM (□), 1.0mM (■), 1.5mM (◇) and 2.0mM (◆).

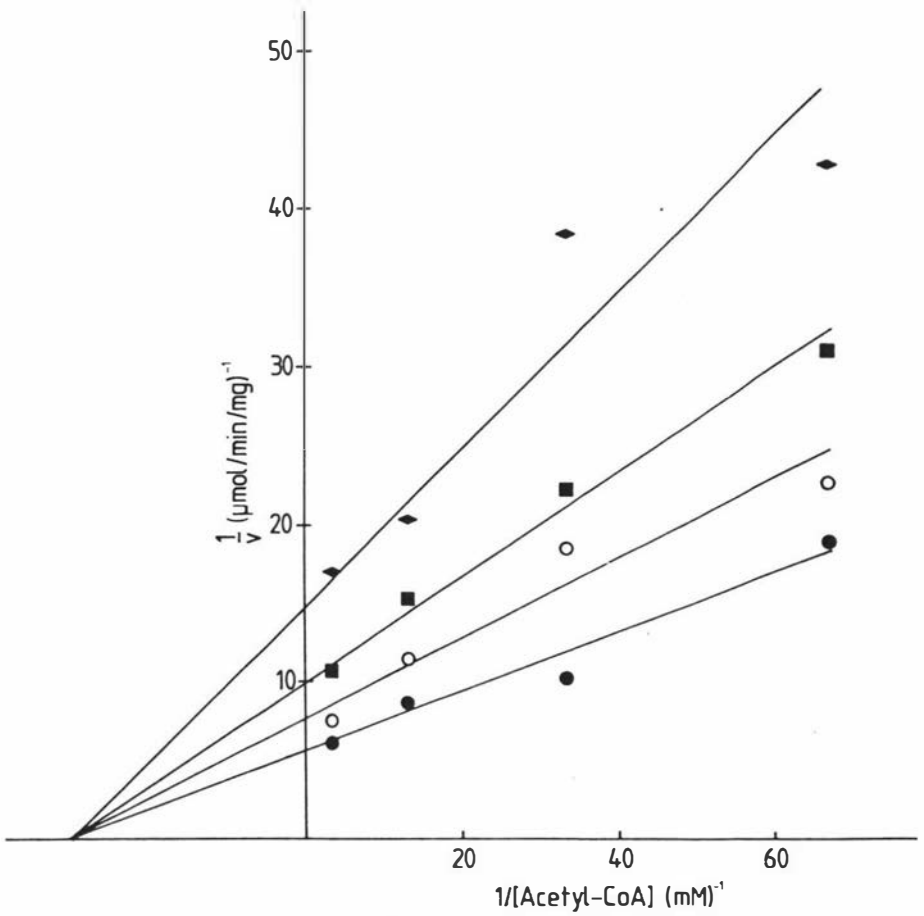


FIGURE 47: Lineweaver-Burk analysis of the effect of CoA on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 46.

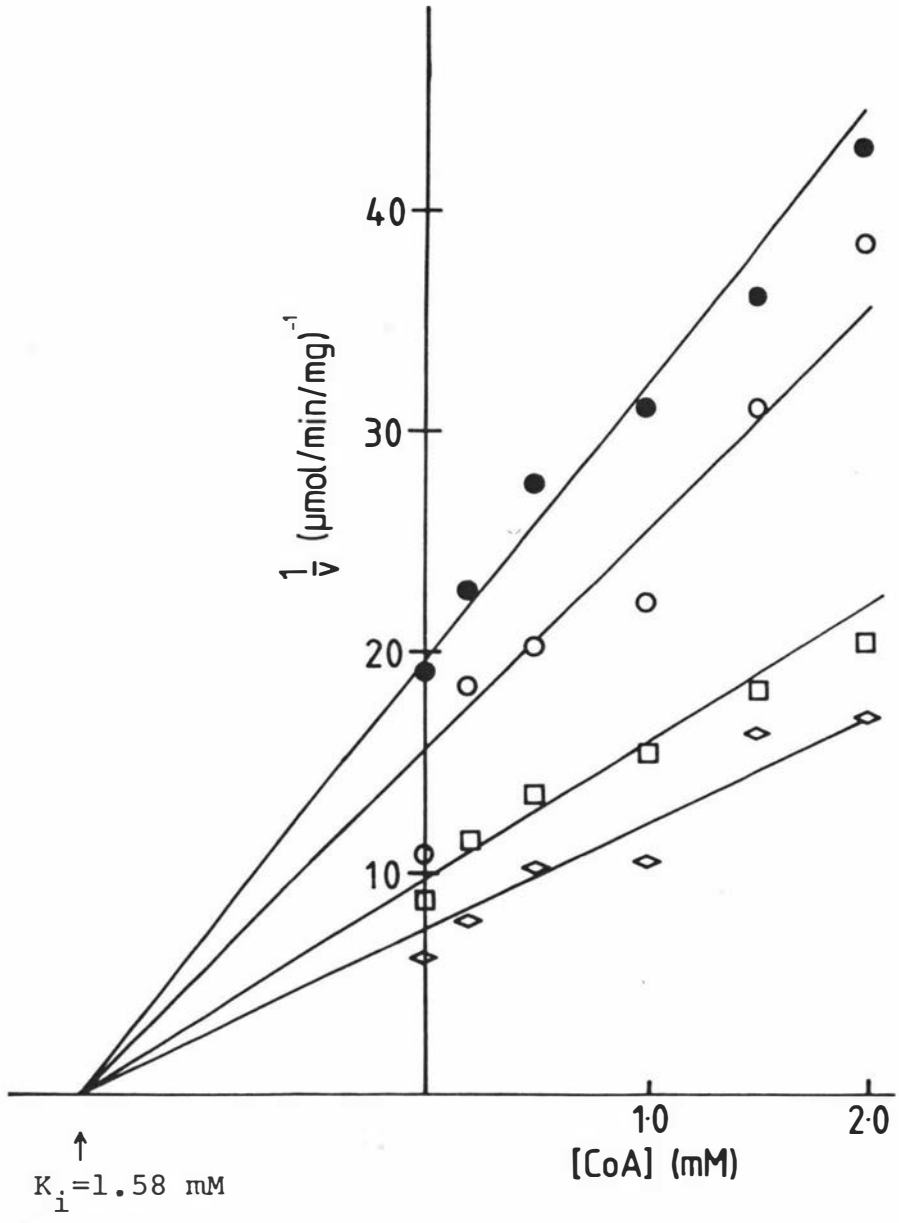


FIGURE 48: Analysis by Dixon-plot of the effect of CoA on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 46 by the method of Dixon (1953). Acetyl-CoA concentrations were: 0.075mM (●), 0.030mM (o), 0.075mm (□) and 0.300mM (◇).

4.22 Effect of Palmitoyl-CoA on Acetyl-CoA Carboxylase Activity

Acetyl-CoA carboxylases from mammalian sources have been shown by a number of workers to be susceptible to inhibition by fatty acyl-CoA esters (Lane et al., 1974). These metabolites being the end product of de novo fatty acid biosynthesis, may play an important role in the regulation of this process. Palmitoyl-CoA was found to inhibit acetyl-CoA carboxylase from maize, with almost complete inhibition at 37.5μM (Fig. 49). Analysis of the inhibition with respect to acetyl-CoA, by the Lineweaver-Burk method indicated that the inhibition was uncompetitive (Fig. 50), decreasing both the V_{max} of the reaction and $K_m^{acetyl\text{-}CoA}$.

4.23 Effect of AMP, Phosphoenolpyruvate, Oxaloacetate and Citrate on Acetyl-CoA Carboxylase Activity

In C_4 plants, such as maize, phosphoenolpyruvate and oxaloacetate play an important role in the fixation of CO_2. It was of interest therefore to determine if these compounds have any regulatory significance in acetyl-CoA carboxylation. Only slight inhibition was observed with both compounds: 2mM PEP and 5mM oxaloacetate resulted in 15% and 20% inhibition of activity, respectively. Maize leaf acetyl-CoA carboxylase was also inhibited by AMP, with 10mM AMP causing 38% inhibition. As these effects on activity were only slight, further characterization was not carried out.

Acetyl-CoA carboxylases from mammalian sources are allosterically activated by citrate (Lane et al., 1974). However, 5mM citrate inhibited maize acetyl-CoA carboxylase by about 65%. Addition of equimolar amounts of $MgCl_2$ with citrate restored full activity of the enzyme, indicating that the cause of inhibition by citrate was likely to be due to the complexing of free Mg^{2+} by citrate.

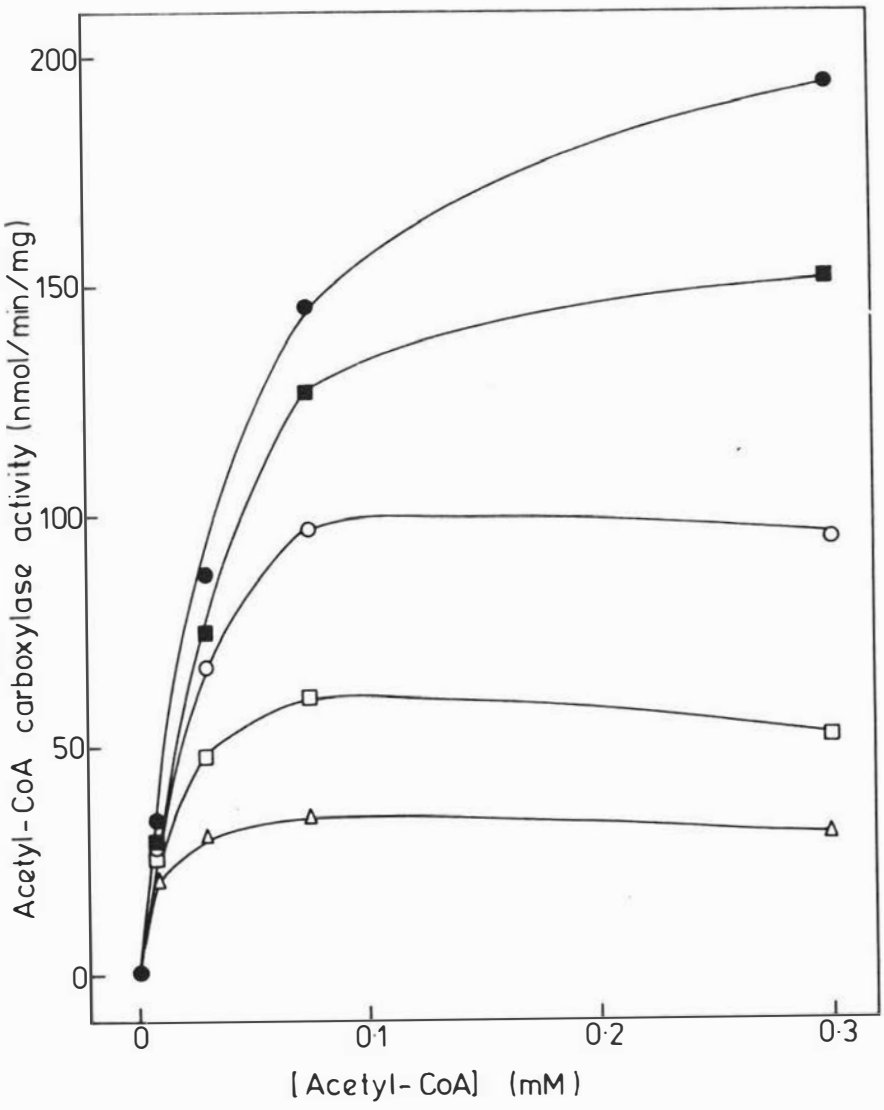


FIGURE 49: Palmitoyl-CoA inhibition of the activity of maize leaf acetyl-CoA carboxylase.

Assay conditions: as described in Section 3.2 using 10mM HCO_3^-, except the acetyl-CoA concentration was varied and palmitoyl-CoA was added to a final concentration of 0µM (●), 4.2µM (■), 12.6µM (○), 25.0µM (□) and 37.5µM (△).

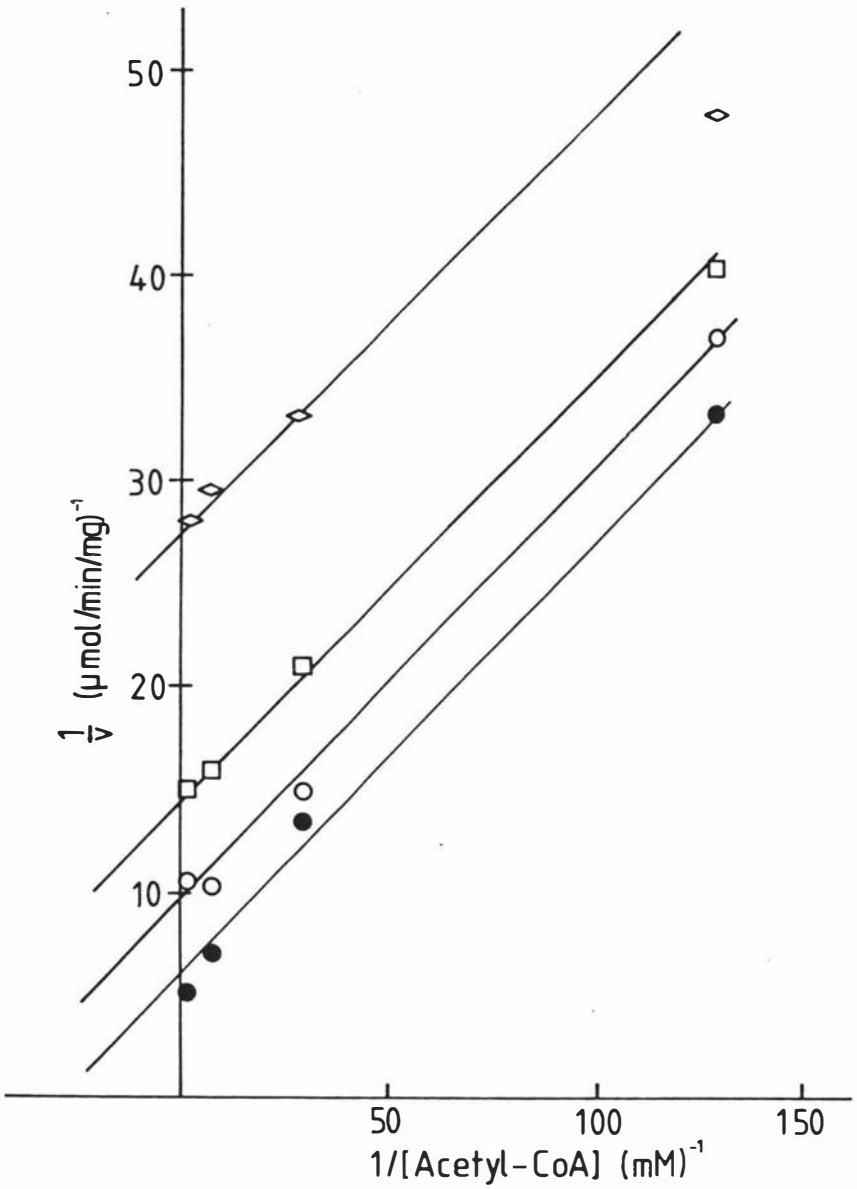


FIGURE 50: Lineweaver-Burk analysis of the effect of palmitoyl-CoA on the activity of maize leaf acetyl-CoA carboxylase.

Analysis of the data presented in Fig. 49.

4.24 Effect of Sulfhydryl Reacting Reagents on Acetyl-CoA Carboxylase

The stabilization of acetyl-CoA carboxylase activity in cell-free extracts of maize by sulfhydryl protecting agents, such as β-mercaptoethanol (Section 4.2), suggests that this enzyme may require sulfhydryl group(s) for activity. Examination of the susceptibility of the partially purified acetyl-CoA carboxylase of maize to inhibition by sulfhydryl reacting reagents appeared to support this possibility. Following exhaustive dialysis of the partially purified enzyme against 10mM Tris-HCl buffer (pH 8.0) containing 1mM EDTA, to remove β-mercaptoethanol, aliquots of the enzyme were incubated with different concentrations of either ρ-chloromercuribenzoate or N-ethylmaleimide. The remaining acetyl-CoA carboxylase activity was then assayed by the standard method (Section 3.2). Complete inhibition of activity was observed with 60μM ρ-chloromercuribenzoate, while N-ethylmaleimide required a concentration of 0.5mM for complete inhibition (Table XXI).

TABLE XXI

INHIBITION OF MAIZE LEAF ACETYL-CoA CARBOXYLASE ACTIVITY BY SULFHYDRYL REACTING REAGENTS

Treatment before assaying	Acetyl-CoA carboxylase activity (nmol/min/mg)
None	101.8
0.5mM NEM	4.0
1.0mM NEM	3.4
30μM ρCMB	33.4
60μM ρCMB	5.4
120μM ρCMB	2.2

Maize leaf acetyl-CoA carboxylase preparation was dialysed for 16h against two changes of 50 volumes of 10mM Tris-HCl buffer (pH 8.0) containing 1mM EDTA, and treated for 20 min at 20°C with the appropriate reagent. Acetyl-CoA carboxylase activity was determined with 10mM HCO_3^-.
NEM = N-ethylmaleimide; ρCMB = p-chloromercuribenzoate.

CHAPTER 5

DISCUSSION

5.1 Acetyl-CoA Carboxylase - its General Features and Stability

The short-term regulation of de novo biosynthesis of fatty acids in animal tissues and E. coli is greatly influenced by the activity of acetyl-CoA carboxylase (reviewed in Volpe and Vagelos, 1973; Lane et al., 1974; Numa and Yamashita, 1974). Fundamental to this regulatory role is the influence of metabolites such as pyrophosphate derivatives of guanosine in E. coli, citrate and fatty acyl-CoA esters in animals, and phosphorylation-dephosphorylation of the enzyme in animal tissues.

Acetyl-CoA carboxylase from plant sources is less-well characterized: indeed its low activity, in the few photosynthetic tissues examined, has greatly hindered a detailed study of the properties of this enzyme. Considerable difficulty was experienced in early attempts to demonstrate acetyl-CoA carboxylase activity in chloroplast preparations from spinach (Kannangara and Stumpf, 1972), and barley (Kannangara and Stumpf, 1973) unless supplemented with acetyl-CoA carboxylase components of E. coli. Due to this difficulty the regulatory role of acetyl-CoA carboxylase in fatty acid biosynthesis by photosynthetic tissues has not been established.

Despite the early reports of low activity of acetyl-CoA carboxylase in chloroplast preparations from barley (Kannangara and Stumpf, 1973), considerably higher activities of this enzyme were subsequently found in cell-free extracts prepared from barley leaves (Reitzel and Nielsen, 1976). The application of similar isolation methods in this

study to maize and barley leaves gave equally promising results, although initial difficulties were experienced with the maintenance of activity. Previous investigations of acetyl-CoA carboxylase of avian liver (Gregolin et al., 1968b) wheat germ (Hatch and Stumpf, 1961), Turbatrix aceti (Meyer and Meyer, 1978), and yeast (Matsuhashi et al., 1964) reported the requirement of free sulfhydryl group(s) for activity of the enzyme. In the present study with maize and barley tissues, the inclusion of a sulfhydryl protecting agent such as β-mercaptoethanol in the homogenisation buffer was effective in increasing the stability of acetyl-CoA carboxylase. This stabilization of acetyl-CoA carboxylase was probably partly due to the protection of readily oxidisable sulfhydryl group(s) on the enzyme, which are reduced in the active enzyme, since sulfhydryl attacking reagents such as N-ethylmaleimide and ρ-chloromercuribenzoate inhibited the partially purified enzyme from maize.

Although the presence of sulfhydryl protecting agents during the isolation of maize and barley leaf acetyl-CoA carboxylase resulted in the isolation of active enzyme, the application of the same isolation procedure to spinach leaf failed to isolate the active enzyme. Mohan and Kekwick (1980) recently reported the stabilization of spinach chloroplast acetyl-CoA carboxylase by the inclusion of glycerol, BSA, citrate and $NaHCO_3$ in the isolation buffer. A reinvestigation of the spinach enzyme confirmed that it was very labile in buffers not containing glycerol and BSA. Whilst the lability of the spinach enzyme explains the earlier difficulties in the detection of this enzyme in spinach chloroplast preparations (Kannangara and Stumpf, 1972), the measured levels of acetyl-CoA carboxylase activity are still relatively low, even when isolated in the presence of glycerol and BSA, compared to the activity of the enzyme in extracts of maize and barley. The activities of acetyl-CoA carboxylase were also undetectable in extracts of broad bean and pea leaves, when prepared with buffers lacking glycerol and BSA (J E Tularam, personal communication). The effect of using the

Mohan and Kekwick (1980) buffer in isolating broad bean and pea acetyl-CoA carboxylase has not been studied. More extensive investigations of acetyl-CoA carboxylase from broad bean and pea may indicate interesting trends between species.

5.2 Cellular Location of Acetyl-CoA Carboxylase in Maize Leaves

The restriction of ACP to chloroplasts isolated from spinach protoplasts, suggests that this organelle may be the sole site of de novo fatty acid biosynthesis in leaves (Ohlrogge et al., 1979). It would be expected therefore that acetyl-CoA carboxylase is similarly distributed. Indeed, centrifugation of purified maize chloroplasts on a sucrose density gradient demonstrated the location of acetyl-CoA carboxylase in intact chloroplasts. The high purity of the isolated chloroplasts was indicated by the absence of detectable cytoplasmic, mitochondrial and peroxisomal enzymes in the density gradient. These chloroplast preparations contained a mixture of mesophyll and bundle sheath chloroplasts as both NADPH-malate dehydrogenase and RuDP carboxylase, enzymes restricted to the mesophyll and bundle sheath chloroplasts, respectively (Slack et al., 1969), occurred in the preparations. However, comparison of the activities of these two enzymes in the density gradient relative to those reported in preparations of isolated mesophyll protoplasts and strands of bundle sheath cells (Kanai and Edwards, 1973a; Gutierrez et al., 1974) indicates the predominance of mesophyll-derived chloroplasts, probably resulting from the easier release and disruption of mesophyll cells compared with bundle sheath cells during homogenisation of the leaves (Björkman and Gauhl, 1969; Berry et al., 1970; Bucke and Long, 1971).

Certain enzymes involved in the fixation of CO_2, by C_4 plants such as maize, are restricted to one of the two chlorophyllous

cell types, mesophyll and bundle sheath cells (Slack et al., 1969). The specific activities of acetyl-CoA carboxylase in extracts of mesophyll protoplasts and strands of bundle sheath cells were found to be similar, indicating the location of this enzyme in both cell types. Although mesophyll chloroplasts can now be purified from isolated mesophyll protoplasts (Day et al., 1981), the purification of bundle sheath chloroplasts has not been achieved. Consequently it was not possible to demonstrate unequivocally the location of acetyl-CoA carboxylase in both chloroplast types. Since Hawke et al (1975) demonstrated that both mesophyll and bundle sheath chloroplasts were able to incorporate [^{14}C] acetate in to fatty acids, it seems reasonable to assume that acetyl-CoA carboxylase occurs in chloroplasts of both cell types.

Although the present studies have demonstrated that acetyl-CoA carboxylase occurs in chloroplasts, they have not shown that the enzyme is restricted exclusively to these organelles. An extra-chloroplastic acetyl-CoA carboxylase may have a function in the supply of malonyl-CoA required for the elongation of fatty acids in the biosynthesis of cuticular waxes (for review see Kolattukudy et al., 1976). In the cell fractionation studies employed many chloroplasts were broken, resulting in contamination of non-chloroplastic fractions by chloroplastic enzymes. Perhaps a more detailed study with gently lysed protoplasts may in the future characterize the location of acetyl-CoA carboxylase.

Earlier investigations of the nature of spinach chloroplastic acetyl-CoA carboxylase indicated that this enzyme was readily dissociable into three protein components, similar to the E. coli enzyme. The biotin-containing protein was membrane-bound whereas the other two proteins, one catalysing a biotin carboxylase reaction and the other a carboxyltransferase reaction, were located in the stroma (Kannangara and Stumpf, 1972). These findings were later extended to a number of other plant tissues such as tobacco, pea, butter

lettuce, maize and barley (Kannangara and Stumpf, 1973). Furthermore, a biotin-containing protein was identified on the chloroplast lamellae of barley seedlings which had been grown in a medium containing [^{14}C]-biotin (Kannangara and Jensen, 1975). However, later workers have reported the presence of soluble acetyl-CoA carboxylases in photosynthetic tissues. Reitzel and Nielsen (1976) first isolated a soluble enzyme in cell-free extracts of barley and subsequently the chloroplastic location of this enzyme was established (Thomson and Zalik, 1981). The stromal location of acetyl-CoA carboxylase has also been demonstrated in chloroplasts of spinach and plastids of avocado (Mohan and Kekwick, 1980). Consistent with these recent reports, acetyl-CoA carboxylase of maize chloroplasts was located in the stroma, without any membrane requirement for full activity. However, the possibility of two isoenzymes of acetyl-CoA carboxylase, one completely soluble and the other with a membrane component as described by Kannangara and Stumpf (1972), is not totally excluded, although it appears unlikely as all but 4% of the biotin carboxylated by biotin carboxylase of E. coli, was present in the stroma of maize chloroplasts. Parallel experiments, in this study, with disrupted chloroplasts isolated from barley and spinach also confirmed the stromal location of the biotin prosthetic group of acetyl-CoA carboxylase in these tissues. Recent measurements of the biotin content of barley chloroplasts (Thomson and Zalik, 1981), showed that 80% of chloroplast biotin was located in the stroma, agreeing with the present findings.

A definitive explanation for the identification of a membranous biotin-protein on the one hand (Kannangara and Stumpf, 1972), and a stromal holoprotein on the other, is not possible at present. However, it may be relevant that Mohan and Kekwick (1980) reported a loss of activity and also of a protein fragment containing biotin, when the avocado plastid acetyl-CoA carboxylase was transferred to buffers not containing BSA. It is conceivable that such a biotin-protein released upon disruption of spinach chloro-

plasts in the absence of BSA, may bind to lamellae membranes by non-specific hydrophobic or ionic interactions. However, the amino acid analysis of the BCCP component of E. coli acetyl-CoA carboxylase (Fall and Vagelos, 1972), which may be similar to the biotin-protein of spinach chloroplasts, does not indicate any unusual enrichment of hydrophobic amino acid residues to account for excessive hydrophobic interactions. Rather BCCP of E. coli has an acidic isoelectric point and consequently an overall negative charge at physiological conditions (Fall and Vagelos, 1972). If the biotin-protein fragment of spinach chloroplastic acetyl-CoA carboxylase reported by Kannangara and Stumpf (1972) is similarly charged, repulsive forces would be anticipated between this protein fragment and thylakoid membranes, which are also negatively charged at pH 7.8 (Westrin et al., 1976). It is still possible however, that isolated areas on the surface of the biotin-protein fragment or thylakoid membranes may be oppositely charged and thus lead to non-specific binding. Examples of non-specific binding of soluble enzymes to membranes have been reported previously. Dalling et al (1972) found that non-specific binding of nitrate reductase to isolated chloroplasts was prevented by the inclusion of BSA in buffers, and recently McNeil and Walker (1981) reported the binding of RuDP carboxylase to thylakoid membranes when spinach chloroplasts were disrupted in the presence of Mg^{2+}. Although the above possibilities may explain the presence of a biotin-containing protein in thylakoid preparations from spinach chloroplasts (Kannangara and Stumpf, 1972), the difference in the solubility of acetyl-CoA carboxylase of maize and barley chloroplasts reported in recent studies, and those of Kannangara and Stumpf (1973) and Kannangara and Jensen (1975) cannot as yet be explained.

5.3 Relationship Between Acetyl-CoA Carboxylase Activity and Lipid Synthesis in Isolated Chloroplasts

One of the long-standing problems in the investigation of

lipid synthesis by isolated chloroplasts has been the low levels of acetyl-CoA carboxylase activity compared to the rates of de novo fatty acid synthesis. For example, isolated spinach chloroplasts have been observed to incorporate [^{14}C] acetate into lipids at rates of up to 20 - 30 nmol/min/mg chl (Roughan et al., 1979a; Browse et al., 1981). However, reported levels of acetyl-CoA carboxylase activity in spinach chloroplasts are relatively low: Roughan et al. (1979a) reported the activity at 4.1 nmol/min/mg chl, while Mohan and Kekwick (1980) found the activity at 0.13 nmol/min/mg protein, which assuming a chlorophyll : protein ratio of 15 for isolated spinach chloroplasts (Kirk and Tilney-Bassett, 1978a), converts to 2.0 nmol/min/mg chl. In this study, isolated chloroplasts of spinach leaves showed similar activities, with [^{14}C] acetate incorporation into lipids at 6.0 nmol/min/mg chl, while acetyl-CoA carboxylase activity was only 2.8 nmol/min/mg chl. This relatively low activity of acetyl-CoA carboxylase may have been due to the presence of an inhibitor to the enzyme, which was first suggested to explain the absence of acetyl-CoA carboxylase activity in lettuce chloroplasts (Burton and Stumpf, 1966). However, the lack of inhibition of acetyl-CoA carboxylase activity in maize leaf cell-free extracts following mixing with cell-free extract of spinach leaves appears to rule out this possibility.

Isolated maize chloroplasts in this study had an acetyl-CoA carboxylase activity of 34.5 nmol/min/mg chl, while [^{14}C] acetate incorporation into lipids was only 1% of this rate. A similar excess of acetyl-CoA carboxylase activity above that of lipid synthesis was also obtained with barley chloroplasts: 8.5 nmol/min/mg chl for acetyl-CoA carboxylase, compared with 0.12 nmol/min/mg chl of [^{14}C] acetate incorporation into lipids.

Although isolated maize and barley chloroplasts appear to show excess acetyl-CoA carboxylase activity above that of [^{14}C] acetate utilization, the measured rates of [^{14}C] acetate

incorporation into lipids are probably reduced from those found *in vivo*. In contrast however, improved isolation techniques have resulted in increased measured rates of [^{14}C] acetate incorporation into lipids by isolated chloroplasts of spinach *in vitro* (Roughan *et al.*, 1979a). The relatively low activity of acetyl-CoA carboxylase in isolated chloroplasts of spinach therefore is puzzling. It may be significant however, that acetyl-CoA carboxylase activity in isolated chloroplasts of maize and barley is of the same order as the observed rate of lipid synthesis by isolated spinach chloroplasts.

5.4 Relationship Between Acetyl-CoA Carboxylase Activity, Lipid Synthesis and Leaf Development

The morphological changes during development of the monocotyledonous leaf includes differentiation of small agranal proplastids into chloroplasts by enlargement of the organelle and accumulation of lipid-rich lamellae (Leech *et al.*, 1973). A pronounced and rapid increase in plastid lipids occurs immediately following emergence of the expanding leaf, from the surrounding sheathing leaves, into the light. In this segment of the leaf there is presumably an elevated rate of lipid synthesis, compared with that in the less mature segments below and the more mature segments above. These variations in the cellular requirements for lipid should also be reflected by altered capacities for malonyl-CoA synthesis.

In the present study, the rate of [^{14}C] acetate incorporation into lipids was maximal with leaf slices prepared from the region of the leaf corresponding to lamellae synthesis in chloroplasts. Consistent with this finding were the measured rates of [^{14}C] acetate incorporation into lipids by isolated chloroplasts, which were maximal with chloroplasts isolated from the same region of the leaf. These studies confirm earlier work on the effect of leaf development on lipid

synthesis by leaf slices of maize, barley and wheat (Hawke et al, 1974b; Bolton and Harwood, 1978) and isolated chloroplasts of maize leaves (Hawke et al., 1974a; McKee, 1979).

However the effect of leaf development on acetyl-CoA carboxylase activity was not as clear. Chloroplasts isolated from the youngest tissue of maize leaves showed maximum acetyl-CoA carboxylase activity. But the variable degree of intactness of chloroplasts isolated from tissues of varying age possibly confuses this comparison. The greater activity in chloroplasts isolated from the younger tissue may be due to the higher degree of intactness of these preparations compared to chloroplasts isolated from the older tissue of the leaves. In cell-free extracts, where this problem does not occur, no clear relationship was seen between leaf development and acetyl-CoA carboxylase activity when related to fresh weight of tissue. However, on a protein basis, acetyl-CoA carboxylase activity was maximal in cell-free extracts prepared from tissue in which lamellae synthesis is taking place. Recently, Thomson and Zalik (1981) reported a similar relationship between leaf development and acetyl-CoA carboxylase activity in barley. As in this study, this relationship was best shown in cell-free extracts only when the activity was expressed on a protein basis, whereas on the basis of fresh weight the relationship is not so clear. Similarly, with isolated chloroplasts, the relationship between leaf development and acetyl-CoA carboxylase activity was unclear. The uncertainty which appears to exist in the relationship between lipid synthesis and acetyl-CoA carboxylase activity in isolated chloroplasts of variable development, may possibly be due to the lack of techniques to isolate chloroplasts, from maize and barley leaves, which are able to synthesize lipids at rates comparable to those in vivo, and are thus minimally altered from their in vivo situations.

Alternatively, greening of dark-grown plant tissues have

been utilized to study the effect of leaf development on acetyl-CoA carboxylase activity. The morphological and biochemical changes which take place during the greening of etiolated tissues are very different from those occurring in plants grown under a normal light-dark regime (Weier and Brown, 1970; Leech et al., 1973; Leech, 1977; Kirk and Tilney-Bassett, 1978b; Boffey et al., 1980). During growth of plants in the dark, proplastids differentiate into etioplasts containing the prolamellar body, which upon illumination is dispersed and replaced by thylakoid membranes as the etioplasts develop into chloroplasts. Using this system, rather contradictory results have been reported. Despite the increased capacity of isolated barley chloroplasts to incorporate [^{14}C] acetate into lipids, the activity of acetyl-CoA carboxylase decreased with the age of the etiolated tissue and with increasing time of illumination of etiolated seedling, from which chloroplasts were isolated (Kannangara et al., 1971). However, later work indicated increasing acetyl-CoA carboxylase activity in cell-free extracts, with increasing age of etiolated and light-grown barley seedlings, and time of greening of etiolated barley seedlings, from which the extracts were prepared (Reitzel and Nielsen, 1976). This later finding of increasing acetyl-CoA carboxylase activity in greening tissue, is consistent with the increased capacity of chloroplasts, isolated from this tissue, to synthesize lipids from acetate.

5.5 Purification of Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase has been purified to homogeneity from a wide number of sources. From animal tissues the enzyme has been purified from liver (Gregolin et al., 1968a; Nakanishi and Numa, 1970; Inoue and Lowenstein, 1972), mammary gland (Miller and Levy, 1969; Manning et al., 1976; Ahmad et al., 1978; Hardie and Cohen, 1978) and adipose tissue (Moss et al., 1972) to specific activities in the range of 5 - 15 μmol/min/mg of protein, which were about 300-

to 2000-fold greater than the specific activity in the initial extracts. From the yeasts C. lipolytica (Mishina et al., 1976b) and S. cerevisiae (Sumper and Riepertinger, 1972), acetyl-CoA carboxylase was purified to a final specific activity of 8 µmol/min/mg of protein.

The only preparations of acetyl-CoA carboxylase from plant sources which attained specific activities as high as those from animals and yeasts have been prepared from embryonic tissues. Heinstein and Stumpf (1969) reported a 1000-fold purification of acetyl-CoA carboxylase of wheat germ, by column chromatography on ion-exchange resins followed by sucrose density gradient centrifugation, to give a final specific activity of about 6.3 µmol/min/mg of protein. Application of this procedure to barley embryos resulted in a preparation of acetyl-CoA carboxylase with a specific activity of 7.1 µmol/min/mg of protein (Brock and Kannangara, 1976). More recently, acetyl-CoA carboxylase of wheat germ was purified 2,200-fold with a modified procedure, which included affinity chromatography on a column of Blue-Sepharose (Egin-Bühler et al., 1980), to a final specific activity of 1.4 µmol/min/mg of protein. The low specific activity of acetyl-CoA carboxylase in this preparation, relative to that of Heinstein and Stumpf (1969), was due to the low specific activity of the enzyme in the initial extract. Application of the same purification procedure to acetyl-CoA carboxylase of parsley cell culture, resulted in a 300-fold purification of the enzyme, to a specific activity of 0.4 µmol/min/mg of protein.

The only reported purification of acetyl-CoA carboxylase from leaves, is that of spinach chloroplasts. Using ammonium sulphate fractionation, followed by gel filtration, Mohan and Kekwick (1980) obtained a 200-fold increase in specific activity, to 0.025 µmol/min/mg of protein. The identical purification procedure applied to plastids of avocado, resulted in nearly a 2000-fold purification of acetyl-CoA carboxylase, to a specific activity of 0.084 µmol/min/mg of

protein.

In this study, the final purification procedures adopted to purify acetyl-CoA carboxylase of maize leaves, followed extensive attempts at purifying this enzyme by some of the procedures used previously in purifying acetyl-CoA carboxylase from other sources. Ammonium sulphate fractionation, ion-exchange chromatography, CoA and avidin affinity chromatography were either unsuccessful or gave inferior purifications compared with the procedures finally adopted. Acetyl-CoA carboxylase of maize leaves was purified by two procedures which utilized combinations of polyethylene glycol fractionation, ammonium sulphate precipitation, hydrophobic chromatography and gel filtration. The two procedures that were finally used to purify acetyl-CoA carboxylase, resulted in preparations of similar specific activities, in the range of 0.2 - 0.5 μmol/min/mg of protein. Analysis by polyacrylamide gel electropheresis indicated the purity of acetyl-CoA carboxylase to be in range of 40 - 60%. Acetyl-CoA carboxylase of barley leaves was also purified by one of the two alternative procedures used to purify the enzyme from maize. This enzyme behaved identically to that from maize during purification, reaching a final specific activity of 0.2 μmol/min/mg of protein, which may be indicative of the similarity of acetyl-CoA carboxylases from maize and barley leaves.

Acetyl-CoA carboxylase from both maize and barley leaves was relatively unstable during purification, which limited the degree of purification obtained. The specific activities of the preparations from maize and barley leaves were, as expected, less than the specific activities of pure acetyl-CoA carboxylase from animals, yeasts and embryonic tissue of wheat and barley. However, compared to the recently reported preparations of this enzyme from spinach chloroplasts and avocado plastids (Mohan and Kekwick, 1980), acetyl-CoA carboxylase purified from maize and barley leaves, in this study, showed specific activities 10-fold

higher.

Following polacrylamide gel electropheresis of the purified maize leaf acetyl-CoA carboxylase, the coincidence of enzymic activity with a single stained protein band discounted the possibility that this enzyme dissociates into separate active protein components, as occurs in E. coli (Guchhait et al., 1974a) and was suggested for the spinach chloroplast enzyme (Kannangara and Stumpf, 1972).

5.6 Kinetic Properties of Acetyl-CoA Carboxylase

5.6.1 Effect of substrates on acetyl-CoA carboxylase

Acetyl-CoA carboxylase of maize and barley leaves showed very similar dependence of activity on the concentration of the substrates, acetyl-CoA and HCO_3^-. Both enzymes had very similar affinities for these substrates, as judged by the apparent Michaelis constants, 0.10mM and 0.11mM for acetyl-CoA and 1.4mM and 2.1mM for HCO_3^-, for the maize and barley enzymes, respectively. Comparison of these constants with those of acetyl-CoA carboxylases from other plant sources indicates the similarity of these enzymes. The Michaelis constant for acetyl-CoA were determined as 0.15mM, 0.10mM and 0.26mM for the enzyme from wheat germ (Hatch and Stumpf, 1961), spinach chloroplasts and avocado plastids (Mohan and Kekwick, 1980), respectively, while the K_ms for HCO_3^- were 1.05mM, 3.0mM and 8.0mM for the same enzymes, respectively.

In contrast, acetyl-CoA carboxylases from animal sources have K_ms for acetyl-CoA in the range of 16 - 30 µM (Miller and Levy, 1969; Moss et al., 1972), about an order of magnitude below that found for the plant enzymes, and for HCO_3^- the K_ms are in the range of 10 - 15mM (Miller and Levy, 1969; Maragoudakis, 1970), about an order of magnitude greater than the enzyme from plants. Acetyl-CoA carboxylase

from yeasts, which are evolutionally closer to plants than animals, show apparent K_ms for acetyl-CoA and HCO_3^- of 0.26mM and 3.7mM, respectively (Mishina et al., 1976a), which are similar to those of acetyl-CoA carboxylases from plants.

All acetyl-CoA carboxylases, that have been studied, require both ATP and $MgCl_2$ for maximum activity (Lane et al., 1974). In line with a number of other enzymes which utilize ATP, the biologically active form of this substrate in the acetyl-CoA carboxylase reaction appears to be Mg.ATP complex. Both acetyl-CoA carboxylases from maize and barley show similar properties, with Mg.ATP as active substrate for the reaction. Furthermore, comparison of the kinetic properties of the maize and barley enzyme, with the kinetic models of London and Steck (1969), indicate that these two enzymes are activated by free Mg^{2+} and inhibited by free ATP. Detailed studies of the Mg^{2+} and ATP requirements of acetyl-CoA carboxylases of wheat germ (Nielsen et al., 1979) and avocado plastids (Mohan and Kekwick, 1980), have shown very similar results. Both these enzymes require Mg.ATP as the substrate and whereas free ATP inhibits activity, free Mg^{2+} was found to be an activator.

5.6.2 Substrate specificity of acetyl-CoA carboxylase

A number of acetyl-CoA carboxylases have been shown to carboxylate other acyl-CoA esters, as well as acetyl-CoA. Propionyl-CoA carboxylation has been demonstrated to occur with acetyl-CoA carboxylase of rat mammary gland (Miller and Levy, 1969), bovine adipose tissue (Moss et al., 1972), chicken liver (Gregolin et al., 1966a), yeast (Matsuhashi et al., 1964), T. aceti (Meyer and Meyer, 1978) and wheat germ (Hatch and Stumpf, 1961). All these enzymes, except the enzyme from T. aceti, carboxylated propionyl-CoA less efficiently than acetyl-CoA, and the enzyme from wheat germ was also able to carboxylate butyryl-CoA. Although acetyl-CoA carboxylase of maize did not carboxylate butytyl-CoA, propionyl-CoA carboxylation occurred at up to 60% the rate

of acetyl-CoA carboxylation.

Recently, Mohan and Kekwick (1980) reported an apparent lack of discrimination between Mg^{2+} and Mn^{2+} by acetyl-CoA carboxylase of avocado plastids. Similarly, the biotin carboxylase component of E. coli acetyl-CoA carboxylase is able to carry out its enzymic function at the same efficiency with either Mg^{2+} or Mn^{2+} (Guchhait et al., 1974a). However, acetyl-CoA carboxylases of chicken liver (Gregolin et al., 1968a), rat mammary glands (Miller and Levy, 1969) and wheat germ (Hatch and Stumpf, 1961) show reduced activity when Mg^{2+} is replaced by Mn^{2+}. Acetyl-CoA carboxylase of maize behaved similarly to these later enzymes, as Mn^{2+} replacement of Mg^{2+} caused a 75% reduction in activity at optimum concentrations of metal ions.

5.6.3 Effectors of acetyl-CoA carboxylase activity

Monovalent cations, in particular K^+, were found to activate acetyl-CoA carboxylase of maize. Almost identical activation of acetyl-CoA carboxylase of wheat germ has been reported, with activation being inversely proportional to the concentration of HCO_3^- (Nielsen et al., 1979). Activation by K^+ could be mimmicked by NH_4^+ and to a lesser extent by Cs^+ with both acetyl-CoA carboxylases of maize and wheat germ. However, Li^+ was found to inhibit the enzyme from maize. A number of other enzymes are also activated by monovalent cations, in particular kinases and ligases (Suelter, 1970). The cation ionic radius appears to be important in this activation, with ions larger than K^+, such as Rb^+, Cs^+ and NH_4^+ activating, whereas cations with a smaller ionic radius, such as Li^+ and Na^+, are either inhibitory or neutral in their effect on enzymic activity (Mildvan, 1970). Seulter (1970) in his review of monovalent activation of enzymes, suggests that activation may be due to the formation of a functional ternary complex between substrate, enzyme and the cation, which facilitates the enzymic reaction.

A number of important metabolites involved in de novo fatty acid biosynthesis were found to inhibit the activity of acetyl-CoA carboxylase of maize, in particular the products of acetyl-CoA carboxylation, malonyl-CoA and ADP. Malonyl-CoA inhibition of the avian liver acetyl-CoA carboxylase has been reported, and has been suggested to have significance in the regulation of the activity of this enzyme (Gregolin et al., 1966b; Chang et al., 1967). However, inhibition of acetyl-CoA carboxylase by ADP has not been reported previously, and detailed investigation of this inhibition showed that it was competitive with respect to ATP, and uncompetitive with respect to acetyl-CoA. Alteration of the K_ms of both these substrates in the presence of ADP, suggests that ADP binds either to the binding sites of ATP and acetyl-CoA or that ADP binding alters these binding sites so that binding of ATP and acetyl-CoA becomes more difficult.

Fatty acyl-CoA esters, which are the final products of de novo fatty acid biosynthesis, are thought to play an important role in the control of acetyl-CoA carboxylase activity in animal tissues (Lane et al., 1974). Inhibition of acetyl-CoA carboxylase activity by palmitoyl-CoA, and a number of other fatty acyl-CoAs, has been investigated by a number of workers and appears not to be due to a general detergent effect of this class of metabolites (Bortz and Lynen, 1963; Numa et al., 1965; Greenspan and Lowenstein, 1968). In the case of acetyl-CoA carboxylase of maize, palmitoyl-CoA was a potent inhibitor of enzymic activity. The uncompetitive nature of this inhibition with respect to acetyl-CoA indicated that palmitoyl-CoA binding to the enzyme altered the binding site for acetyl-CoA. The inhibition of acetyl-CoA carboxylase activity in chloroplasts by acyl-CoA esters may be significant in avoiding the accumulation of this metabolite, as its strong detergent properties would cause major disruption in chloroplasts (Bertrams et al., 1981), where membrane structure appears to be very important in the maintenance of functional integrity. However, de novo fatty acid biosynthesis in chloroplasts is an ACP-

dependent process (Stumpf, 1977; 1980), and the level of acyl-CoA esters in the stroma is still to be determined. Thus the physiological significance of this effect is uncertain.

Recently acetyl-CoA carboxylase of rat liver has been shown to be activated by free CoA (Yeh and Kim, 1980; Yeh *et al.*, 1981). These workers have speculated that this activation may be important in the control of the activity of this enzyme and thus fatty acid synthesis *in vivo*. However, acetyl-CoA carboxylase of maize showed the opposite behaviour, with free CoA causing inhibition of the activity of this enzyme. Further investigations of the CoA concentration in the stroma of chloroplasts is required to ascertain if this effect has any significance *in vivo*.

Acetyl-CoA carboxylases of animal tissues are activated by citrate *in vitro* (Lane *et al.*, 1974). As this metabolite is the direct precursor of cytoplasmic acetyl-CoA, citrate activation of this enzyme is thought to be significant in the regulation of acetyl-CoA carboxylase activity *in vivo*. Although the *in vivo* precursor of chloroplastic acetyl-CoA has not as yet been identified, Mohan and Kekwick (1980) have reported the activation of acetyl-CoA carboxylases of spinach chloroplasts and plastids of avocado by citrate. Previous reports had suggested the lack of such activation of acetyl-CoA carboxylase from plant tissues (Burton and Stumpf, 1966). In the present study, citrate did not activate acetyl-CoA carboxylase of maize, but inhibition arose, probably due to complexing of free Mg^{2+}. Support for an inhibitory effect due to complexing of free Mg^{2+} comes from a similar response to other Mg^{2+} complexing compounds, such as phosphate.

5.7 Light-Dark Modulation of Acetyl-CoA Carboxylase Activity

Although fatty acid biosynthesis from acetate by isolated

chloroplasts is a light-dependent process (Smirnov, 1960; Mudd and McManus, 1962; Stumpf and James, 1963), the exact nature of this light-dependency is unknown. The limitation of ATP and NADPH in the dark was first suggested to cause the light-dependency of fatty acid biosynthesis (Stumpf and James, 1962; 1963), however, later work demonstrated that reduction of ATP and NADPH levels in chloroplasts did not greatly affect fatty acid synthesis (Givan and Stumpf, 1971). Recently, acetyl-CoA carboxylase has been suggested to be the light-dependent reaction in the biosynthesis of fatty acids (Nakamura and Yamada, 1979). Although Roughan et al (1980) challenged the arguments of Nakamura and Yamada (1979), the importance of acetyl-CoA carboxylase in regulating fatty acid biosynthesis in almost all other tissues (Lane et al., 1974; Volpe and Vagelos, 1973; Numa and Yamashita, 1974), makes this enzyme a logical candidate to regulate fatty acid biosynthesis in chloroplasts as well.

The kinetic properties of acetyl-CoA carboxylase, of maize and barley, suggest that the activity of this enzyme may be modulated by light-dependent changes of metabolite levels in chloroplasts. Although the determination of metabolite levels in chloroplasts has been a difficult task, there is now increasing amount of published data available on this subject, particularly in spinach chloroplasts. Isolated spinach chloroplasts in the dark appear to show levels of ATP and ADP in the range of 0.1 - 1.5mM and 0.3 - 0.8mM, respectively (Keys, 1968; Heber, 1973; Reeves and Hall, 1973; Miginiac-Maslow and Champigny, 1974; Krause and Heber, 1976; Lilley et al., 1977; Kaiser and Urbach, 1977; Inoue et al., 1978; Kobayashi et al., 1979). Despite the considerable variation in the levels found by different workers, there is general agreement that upon illumination of chloroplasts the concentrations of ATP increases and that of ADP decreases. Concurrently with the changes in the levels of ATP and ADP in chloroplasts during the dark-light transition, the stromal pH changes from about 7.1 to 8.0 as protons are

taken up into the thylakoid space (Heldt et al., 1973; Werdan et al., 1975). An ionic balance across the thylakoid membrane is maintained by a redistribution of ions, particularly of Mg^{2+}. Isolated chloroplasts in the dark show a stromal Mg^{2+} concentration in the range of 1 - 3mM (Portis Jr. and Heldt, 1976; Portis Jr., 1981) which increases by 1 - 5mM on exposure of chloroplast to light (Hind et al., 1974; Bulychev and Vredenberg, 1976; Chow et al., 1976; Portis Jr. and Heldt, 1976; Krause, 1977). Under certain conditions an increase in the stromal concentration of K^{+} has also been reported during dark-light transition of chloroplasts (Hind et al., 1974; Chow et al., 1976).

Assuming that these changes in the metabolite levels during light-dark transitions also occur in the chloroplasts of maize and barley, they would have the effect of increasing the activity of acetyl-CoA carboxylase in the light. A number of chloroplastic enzymes involved in the Calvin cycle, including RuDP carboxylase, fructose 1,6-diphosphatase and sepoheptulose 1,7-diphosphatase, appear to be regulated by such changes in chloroplastic metabolite levels during the light-dark transition (Kelley et al., 1976; Walker, 1976; Jensen and Bassham, 1968). It is possible that regulation of CO_2 fixation and de novo fatty acid biosynthesis may be by such similar means. Although the kinetic properties of acetyl-CoA carboxylase suggest a light-dependent modulation of activity, this could not be demonstrated in cell-free extracts of maize leaves. However, the light-dependent changes in the levels of metabolites, which affect acetyl-CoA carboxylase activity, could have been destroyed during the preparation of the cell-free extracts.

5.8 Summary and Suggestions for Further Study

At the time this study was begun, chloroplastic acetyl-CoA carboxylase was thought to be similar to that of E. coli, dissociable into three components, one of which, the biotin-

containing protein was membrane bound. Although a soluble acetyl-CoA carboxylase was reported in cell-free extracts of barley leaves, it was considered to be a possible isoenzyme. However, soluble acetyl-CoA carboxylases have since been demonstrated in the chloroplasts of barley, spinach and plastids of avodaco. This study has confirmed these findings in spinach and barley leaves, and has established a soluble chloroplastic acetyl-CoA carboxylase in maize leaves, which is present in both mesophyll and bundle sheath cells. The demonstration of the soluble nature of the biotin-containing protein in chloroplasts of maize, barley and spinach, rules out the possible presence of isoenzymes, one soluble and the other requiring membrane-bound biotin.

Acetyl-CoA carboxylase has now been demonstrated in a number of plants, and in particular in this study, the activity of this enzyme has been found to be in excess of the rate of fatty acid synthesis from acetate in chloroplasts isolated from maize and barley. However, isolated chloroplasts of spinach which show the highest rates of *in vitro* fatty acid synthesis, and are thus preferred for the study of fatty acid biosynthesis, appear to show lower acetyl-CoA carboxylase activity than that expected from the rate of fatty acid biosynthesis from acetate. Further research of the nature of acetyl-CoA carboxylase in spinach chloroplasts is required to explain this discrepancy.

Acetyl-CoA carboxylase has been purified to some extent from six plant sources, namely wheat germ, barley embryos, spinach chloroplasts, avocado plastids, maize and barley leaves. Comparison of the reported properties of these enzymes, in particular their kinetic properties, indicate certain similarities, such as the requirement for Mg.ATP as the substrate, activation by free Mg^{2+} and by K^{+}, and the almost identical apparent Michaelis constants for the substrates acetyl-CoA and HCO_3^-. Despite these similarities between the enzyme from the different sources, available data on the structural nature of these enzymes is limited and confusing. Further investigations of the molecular structure of acetyl-CoA

carboxylases from plant sources, could confirm the similarities between these enzymes which are hinted at by their kinetic properties.

The light-dependency of de novo fatty acid biosynthesis in chloroplasts has been known for a number of years, but the exact nature of this dependency is as yet unknown. The kinetic properties of acetyl-CoA carboxylase of maize, investigated in this study, suggest that this enzyme may be light-activated by changes in the levels of chloroplastic ATP, Mg^{2+}, ADP, K^{+}, along with changes in the stromal pH, during light-dark transition of chloroplasts. However, further investigations are required to establish the occurrence of light-dependent changes in the activity of acetyl-CoA carboxylase in vivo and to establish if such changes in activity regulate de novo fatty acid biosynthesis in chloroplasts.

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