

Acetyl Coenzyme A Carboxylase Activity in Developing Seedlings and Chloroplasts of Barley and Its Virescens Mutant¹

Received for publication May 16, 1980 and in revised form September 30, 1980

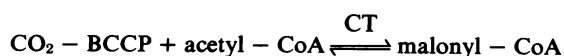
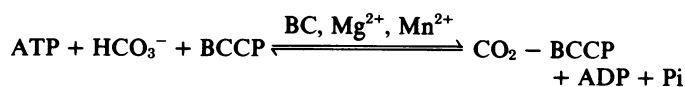
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ABSTRACT

Acetyl coenzyme A (CoA) carboxylase activity of whole tissue homogenates and chloroplast preparations was analyzed as the acetyl-CoA-dependent incorporation of [¹⁴C]bicarbonate into an acid-stable product. The absolute requirement for ATP and MgCl₂, the complete inhibition with avidin, and end-product analysis were consistent with the presence of acetyl-CoA carboxylase activity. Little difference was found between the mutant and normal tissue homogenates from the 1- to 3-day growth stages, during which period both showed a 3-fold increase. However, by 4 days, the activity of the mutant exceeded that of the normal. Fractionation studies showed that the enzyme was a soluble protein present in the stromal fraction of chloroplasts. The biotin content was also highest in the stroma, although it was found in the lamellar fraction as well. For both the mutant and the normal, the highest acetyl-CoA carboxylase activities were obtained in the stromal preparations from 4-day seedlings (54 and 31 nmoles per milligram protein per minute for the mutant and the normal, respectively) with a progressive decline by 6 and 8 days. The difference between the mutant and the normal was not due to the accumulation of an inhibitor in the normal.

Acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA which, in turn, is utilized by the fatty-acid synthetase complex for the *de novo* synthesis of fatty acids (23, 33). The enzyme has been purified from wheat germ (13), barley embryos (6), a variety of animal tissues, yeast, and microorganisms (23). All the carboxylases studied were shown to contain definable subunits, and the animal and yeast complexes required SDS and urea treatment for dissociation, which resulted in inactivation; however, the *Escherichia coli* enzyme complex was readily dissociable into active subunits and the partial reactions have been primarily defined by the study of the *E. coli* system (35). The *E. coli* complex is composed of three proteins, BC,² BCCP, and CT, which catalyze the two half-reactions (35):



The wheat germ (13) and barley embryo (6) enzymes are soluble

¹ This work was supported by a grant to S. Z. from the Natural Sciences and Engineering Research Council of Canada.

² Abbreviations: BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; HABA, 2-(4'-hydroxyazobenzene) benzoic acid; PE, phosphatidylethanolamine.

proteins and have been purified 1,000-fold to a specific activity of 6.3 and 7.4 $\mu\text{mol HCO}_3^-$ incorporated/mg protein·min, respectively. The purified wheat germ protein was partially dissociated into two protein constituents and the partial activities suggested BC and BCCP separated as one complex and CT as the other (13).

Even though chloroplasts are the major or sole site of cellular fatty acid synthesis in green tissue (29), the presence of acetyl-CoA carboxylase in the chloroplast fraction has only been ascribed through indirect methods. Reports describing acetyl-CoA carboxylase activity in green tissue and in isolated chloroplasts are limited, but intact chloroplasts readily incorporate acetate into palmitic and oleic acid by *de novo* synthesis (12, 33), suggesting that the enzyme is functioning effectively. Acetate and acetyl-CoA served as poor substrates for broken chloroplasts, whereas malonyl-CoA was readily incorporated into fatty acids (33). This discrepancy was partially explained by the occurrence of an unidentified inhibitor in disrupted lettuce and spinach chloroplast fractions (33). The buildup of an inhibitor was also suggested from studies of plastids isolated from greening barley seedlings (18). Good acetyl-CoA carboxylase activity was detected if the disrupted spinach chloroplast system was supplemented with *E. coli* carboxyltransferase, suggesting the inhibitor was specific for the CT reaction (21). The procaryotic nature of the spinach acetyl-CoA carboxylase has also been inferred from studies involving CT supplementation in combination with chloroplast fractionation (21). Complete activity required the presence of both stromal and lamellar preparations and the BCCP protein was described as a membrane-bound constituent (21).

In the preceding study (34) of developing leaves of Gateway barley and its virescens mutant, a general lag in the accumulation of the major chloroplast acyl lipids was evident for the mutant. Here, the study has been extended to examine acetyl-CoA carboxylase activity in developing leaves and chloroplasts of the two lines. The enzyme activity which was maximal during the interval of rapid lipid accumulation, declined sharply at leaf maturity but was higher for the mutant than the wild type. Contrary to the findings of Stumpf and co-workers (18, 21, 33), appreciable activity was detectable in disrupted chloroplasts and the complete activity was found in stromal preparations.

MATERIALS AND METHODS

Chemicals. S-Acetyl-CoA (lithium salt), alumina C_γ, ATP-disodium (grade I), avidin (type III, 10 units/mg protein), D-biotin, BSA, (fraction V), DL-dithiothreitol (DL-DTT), 2-HABA and protease (type VI) were obtained from Sigma. Ammonium sulfate (special enzyme grade) was obtained from Schwarz/Mann, hydroxylapatite powder (Bio-Gel HTP) was from Bio-Rad Laboratories, and NaH¹⁴CO₃ was from New England Nuclear. The silica gel impregnated glass fiber sheets (ITLC-SG) were obtained from Gelman Instrument Co.

Buffers and Reagents. The pH of all buffers was adjusted at 20

C. Buffers containing DTT were prepared in bulk without DTT. The DTT was added fresh daily to new portions of the buffers. HABA was recrystallized from aqueous methanol.

Plant Material. *Hordeum vulgare* (cv. Gateway) and its virescens mutant which were described in the preceding paper (34) were used in this study. For analysis of seeds germinated for 1 and 2 days, the embryos were excised from the endosperms, whereas whole shoots from 3- and 4-day-old seedlings were excised at the point of seed attachment. To facilitate the isolation of embryos, the seeds were dehusked essentially as described by Brock and Kannangara (6) and then were grown on filter paper in a clear Plexiglas germination box. The box was placed in a growth chamber under a 12-cm water shield, with the growth chamber kept under continuous light at $90 \mu\text{E}/\text{m}^2 \cdot \text{s}$ (fluorescent, cool-white) and 20 C. The light intensity in the growth box was about $80 \mu\text{E}/\text{m}^2 \cdot \text{s}$. Whole seeds (not dehusked) were surface-sterilized with NaOCl and grown in vermiculite under continuous light at $90 \mu\text{E}/\text{m}^2 \cdot \text{s}$. The apical 3-cm leaf segments of these were used for the analysis of 4-, 5-, and 6'-day-old seedlings (Table I) and for plastid isolation of 4-, 6-, and 8-day-old seedlings (Table V).

Whole Tissue Analysis. Embryos or leaf segments were washed with distilled H_2O and homogenized in 0.1 M K-phosphate (pH 8.3) containing 1 mM EDTA and 1 mM DTT, using a TenBroeck tissue grinder. The homogenate was filtered through one layer of nylon cloth (Nitex; pore size, $25 \mu\text{m}$) and centrifuged at 1,000g for 2 min. The supernatant was made to volume in the grinding buffer and aliquots were taken for enzyme activity estimations and protein determinations.

Plastid Isolation. Leaf segments were washed with distilled H_2O and chilled at 4 C for 15 min. All subsequent operations were carried out at 4 C. The leaf segments were homogenized in a 50-ml volume Waring Blendor with two successive 1-s bursts, followed by one 3-s burst using a 4:1 (v/w) grinding medium to sample ratio. The grinding medium was 67 mM K-phosphate buffer (pH 8.0) containing 0.5 M sucrose, 1 mM MgCl_2 , and 0.1% w/v BSA. The homogenate was gently squeezed through two layers of Miracloth (Calbiochem), and gravity was filtered through eight layers of $25\text{-}\mu\text{m}$ pore size nylon cloth. The filtrate was centrifuged for 2 to 5 min at 3,000g. The pellet was resuspended in a small volume of grinding medium and centrifuged through a 10-ml layer of 67 mM phosphate buffer (pH 8.0) containing 1 mM MgCl_2 and 0.6 M sucrose at 440g for 15 min in an IEC model BD-2 centrifuge using the SW 969 rotor. The chloroplast pellet was resuspended in 0.6 M buffered sucrose for plastid counts or disrupting buffer for the isolation of chloroplast fractions. Plastids were counted with a haemocytometer and plastid isolates were monitored for bacterial contamination by plating serial dilutions on several types of agar media.

Chloroplast Fractionation. The chloroplast pellet was rinsed gently with the disrupting buffer and then suspended in the disrupting buffer, 20 mM K-phosphate (pH 7.0) containing 1 mM EDTA and 1 mM DTT, to give a Chl concentration of 0.5 to 1.5 mg/ml. The suspension was disrupted in a TenBroeck tissue grinder and the stroma and lamellae fractions were separated by centrifugation for 1 h at 122,000g at 4 C in a Beckman model E ultracentrifuge using the SW 50E rotor. The pigment-free stroma fraction was concentrated to approximately 1.3 mg protein/ml for enzyme activity studies, using an Amicon B15 miniconcentrator. The unwashed pellet was taken as the lamellar fraction and resuspended in the disrupting buffer to give a protein concentration of approximately 1.3 mg/ml.

Partial Purification of Acetyl-CoA Carboxylase Subunits from *E. coli*. *E. coli* B cells were cultured from single colonies on Bacto-Penassay Broth (Difco) and harvested at one-half to three-quarters log phase (by pelleting at 9,000g), washed once with 0.1 M K-phosphate (pH 7.0) containing 250 mM NaCl, and collected again

by centrifugation. They then were resuspended in a minimum volume of the phosphate-buffered saline solution and the slurry was transferred to screw-capped vials and stored at -20 C .

When required, the thawed cells were washed again with phosphate-buffered saline solution and then suspended in 20 mM K-phosphate (pH 7.0) containing 1 mM EDTA and 1 mM DTT (0.5 g wet weight cells/ml buffer). Approximately 1 to 2 g packed cells were suspended per test tube. The tubes were packed in an ice slurry and sonicated for 90 s at 30% maximum with a Sonic 300 Dismembrator, (Artek Systems Corp.) using a precooled microtip. Cellular debris was removed by centrifugation at 20,000g for 30 min. Acetyl-CoA carboxylase subunits were partially purified by scaling down the method of Alberts *et al.* (1) All steps were performed at 4 C.

Assay System for Acetyl-CoA Carboxylase Activity. Enzyme activity was assayed by the acetyl-CoA-dependent incorporation of [^{14}C]bicarbonate into an acid-stable product. All constituents, excluding the enzyme preparations, were made up as individual solutions in deionized H_2O and stored at -20 C until required. Solutions of ATP and MgCl_2 were brought to pH 8.2 with NaOH. Samples of $\text{NaH}^{14}\text{CO}_3$, NaHCO_3 , and acetyl-CoA sufficient for a single experiment were stored in glass-sealed vials and screw-capped vials, respectively. They were thawed just before use. Solutions of ATP, DTT, and BSA were refrozen a maximum of three to four times. The reaction mixture contained, in a final volume of 200 μl : 5 μmol Tris-HCl (pH 8.3), 1 μmol ATP, 2 μmol MgCl_2 , 8 μmol KCl, 0.50 μmol DTT, 0.2 mg BSA, 0.74 μmol $\text{NaH}^{14}\text{CO}_3$ (5 μCi), 0.5 μmol acetyl-CoA, and plant extract containing 10 to 40 μg protein. In all cases, two to three replications at differing protein concentrations were employed and the reaction rates were linear to the protein concentrations over the range used. Acetyl-CoA was added to initiate the reaction after the other constituents had equilibrated for 3 min at 30 C. The reactions were terminated by the addition of 40 μl concentrated HCl. The incorporation of $\text{NaH}^{14}\text{CO}_3$ was linear for an incubation period of approximately 20 min for chloroplast stromal preparations and for approximately 10 min for whole tissue homogenates. Thus, a reaction time of 10 min was used for the enzyme assays. Fifty- μl aliquots of the assay mixture were spotted on Whatman No. 1 filter paper discs, 21 mm diameter, and dried in a fume hood with the aid of a heat lamp at 60 C for 30 min. The acid-stable radioactivity was counted using a scintillation counter. Samples lacking acetyl-CoA served as controls.

The specific radioactivity, in dpm/ μmol , of the $\text{NaH}^{14}\text{CO}_3$ solution was determined as outlined by Miller and Levy (26). Protein estimations were determined by the method of Bradford (5) with BSA fraction V as the standard, and Chl was determined according to Arnon (2).

Identification of Reaction Product. The reaction product was analyzed by Gelman TLC as described by Huang (14). The reaction was terminated with concentrated HCl and then brought to 3 N KOH to hydrolyze the thioester. Following hydrolysis, the samples were neutralized with HCl. Aliquots of unhydrolyzed, hydrolyzed, and malonic acid standards were spotted on Gelman ITLC-SG sheets, dried in a fume hood at 60 C for 15 min under a heat lamp, and developed with the solvent system of water-saturated ether: formic acid (7:1, v/v). After chromatography, the sheets were dried in the fume hood at 60 C for 30 min. Unlabeled malonic acid was detected with slightly alkaline bromocresol green solution. For radioactivity determinations, 1-cm sections were cut from the sheet and the activity was determined as previously described.

Biotin Determination. The biotin content of the chloroplast stroma and lamellar fractions was determined by a spectrophotometric assay based on the binding of a dye by avidin, essentially as outlined by Green (11). Stromal fractions were defatted with two washings of diethyl ether; lamellar fractions were resuspended

in 1 to 2 ml 50 mM K-phosphate (pH 6.8) using the TenBroeck tissue grinder and defatted with diethyl ether:methanol (4:1, v/v). The stroma and lamellar fractions then were made to a volume of 5 or 10 ml with 50 mM K-phosphate (pH 6.8) and heat-denatured at 70 C for 15 min. After cooling to room temperature, the samples were incubated with 10 mg protease for 48 h at 30 C. The samples were again heat-denatured and then centrifuged at 12,000g for 30 min. The supernatant was freeze-dried and the biotin content was determined. The freeze-dried samples were dissolved in 450 μ l 100 mM phosphate buffer (pH 6.8) and 150 μ l containing 242 μ g avidin and 22 μ g HABA in the same buffer was added. The *A* was read at 500 nm and from this the *A* at 600 nm was subtracted. Biotin was quantified by comparison with standards which were also subjected to the protease digestion.

RESULTS

Acetyl-CoA Carboxylase Activity of Whole Tissue Homogenates. The acetyl-CoA carboxylase activity of whole cell homogenates is shown in Table I. The mutant and normal showed similar activities during the initial 3 days growth, when compared on the basis of protein content, fresh weight, and seedling count. A marked increase in specific activity from approximately 6 to 20 nmol/mg protein·min and on a per 100 seedling basis from approximately 12 to 43 μ mol/100 seedlings·h occurred during this interval. Increases were, however, less evident when expressed on a fresh weight basis. By 4 days, the activity of the mutant exceeded that of the normal on all comparisons. These differences were more evident on samples taken from the apical 3-cm leaf segments designated by 4', 5', and 6' days. The difference between the 4 and 4' days, which is most evident for activity expressed as seedling counts, is likely due mainly to the sampling method as outlined above. On samples taken from leaf segments (4', 5', and 6' days), the activity of the normal was maximal at 4' days and showed a rapid decline at 5' and 6' days. The activity of the mutant peaked at 5' days and showed a decline by 6' days.

Soluble Leaf Acetyl-CoA Carboxylase. An increase in the specific activity of acetyl-CoA carboxylase in the high-speed supernatant fraction was found following centrifugation of 5-day normal barley (apical, 5-cm) leaf homogenates at 110,000g for 60 min. The results, in nmol NaHCO₃ incorporated/mg protein, were: whole leaf homogenate, 9.6; 110,000g supernatant, 17.1; 110,000g pellet, 1.4. This is contrary to what would be expected if one of the constituents of the enzyme complex were membrane-bound, as was previously reported (20, 21), and is in agreement with the results of Reitzel and Nielsen (31) who reported an increase in the specific activity when whole barley-leaf homoge-

nates were centrifuged at 150,000g for 45 min.

Chloroplast Acetyl-CoA Carboxylase. Since several studies have indicated that the plastid fraction is the major site of fatty-acid synthesis (29, 36), chloroplasts were isolated and the enzyme activity was assayed in both membrane and stromal fractions. These results (Table II) suggest that the complete enzyme complex occurs in the soluble fraction of the chloroplast. Further comparisons between the mutant and normal were therefore made using isolated plastids.

Both the Chl and protein content were shown to change considerably during the growth interval. The Chl content on a leaf fresh-weight basis showed a 9-fold increase in the mutant and a 2-fold increase in the normal (Table IV). The protein content also increased (34) and ribulose - 1,5-bisP carboxylase, the major chloroplast stromal protein, increased 1.5- and 5-fold, respectively, for the normal and the mutant between 4 and 8 days (3). In an attempt to obtain a suitable parameter for comparisons, plastid numbers were determined for both the normal and mutant plant types. Because Chl is confined to the chloroplast membranes, calibration curves were established expressing Chl concentration against plastid number. A typical curve is shown in Figure 1. Such curves were established for both mutant and normal seedlings at 4, 6, and 8 days. From the slope of each line, the number of plastids corresponding to the Chl concentration could be determined. The Chl content per plastid for both plant types at 4, 6, and 8 days is shown in Table III.

The Chl content per plastid of the normal reached a maximum at 6 days; however, the plastid Chl content of the mutant was still increasing at 8 days, where it represented 54% of the level reached by the normal at its maximum. Evidence that selection of plastids occurred during the isolation of the mutant plastids was indicated by a comparison of a ratio of the Chl content of the mutant to the normal on a whole-leaf and a per plastid basis (Table III). These differences, however, were less evident by 8 days, presumably due to the general increase in the mutant plastid size and the over-all mutant chloroplast population becoming more homogeneous.

Bacterial contamination in the plastid isolates was minimal. Using the highest counts obtained for the isolates monitored, it was estimated at 0.007 and 0.04% for the 6-day normal and mutant, respectively. Those of the 8-day normal and mutant were slightly lower, 0.008 and 0.002%, respectively.

When activity was expressed on a plastid number basis (Table IV), both plant types showed maximum activity at 4 days; however, the normal contained only 18% of the level reached by the mutant. The activity in both decreased to very low levels by 8 days. This decrease could likely be accounted for in part by the loss of chloroplast-soluble proteins during plastid isolation and

Table I. *Acetyl-CoA Carboxylase Activity of Whole Cell Homogenates.*

Homogenates were prepared from 1 to 6 day-old mutant and normal barley seedlings and the activities were expressed on protein, fresh weight, and per seedling basis. Days 1 to 4 are for seedlings germinated from dehusked barley seeds and harvested as outlined. Days 4' to 6' are for the apical 3-cm leaf segments of seedlings grown on vermiculite. Values for 1- to 4-day plant material are the mean \pm SE of two experiments with three replicates per experiment. Values for 4' to 6' days are the mean \pm SE of one experiment with two to three replications.

Days	HCO ₃ ⁻ Incorporated in					
	Mutant			Normal		
	nmol/mg protein·min	μ mol/g fresh wt·h	μ mol/100 seedlings·h	nmol/mg protein·min	μ mol/g fresh wt·h	μ mol/100 seedlings·h
1	6.50 \pm 0.21	23.48 \pm 1.97	12.50 \pm 0.94	5.82 \pm 0.15	24.16 \pm 2.16	15.52 \pm 1.05
2	7.46 \pm 0.72	16.05 \pm 0.68	25.92 \pm 3.56	8.03 \pm 0.46	16.32 \pm 1.31	33.72 \pm 3.09
3	20.38 \pm 1.54	28.28 \pm 3.14	40.72 \pm 2.92	19.28 \pm 0.80	25.67 \pm 1.16	43.06 \pm 4.34
4	25.53 \pm 1.21	24.58 \pm 0.78	84.90 \pm 7.68	20.84 \pm 1.18	20.30 \pm 0.81	75.85 \pm 6.60
4'	19.63 \pm 0.23	23.17 \pm 1.37	42.70 \pm 2.55	15.25 \pm 1.65	21.33 \pm 1.98	37.80 \pm 3.52
5'	19.93 \pm 1.48	21.37 \pm 0.96	44.33 \pm 2.33	10.87 \pm 1.22	15.90 \pm 1.57	29.13 \pm 2.90
6'	12.53 \pm 0.79	16.57 \pm 0.92	33.97 \pm 2.38	6.40 \pm 0.49	10.03 \pm 0.92	16.97 \pm 1.58

Table II. Acetyl-CoA Carboxylase Activity of Chloroplast Stroma and Membrane Fractions

The chloroplasts were isolated from the apical 3-cm leaf segments of 4- and 5-day-old normal barley seedlings. Values for 4 days are the mean \pm SE of three determinations. Values for 5 days are single estimates.

Chloroplast Fraction	HCO_3^- Incorporated in Following Growth Stages	
	4 days	5 days
	<i>nmol/mg protein · min</i>	
Whole (disrupted)	4.33 \pm 0.98	6.45
Stroma	24.30 \pm 4.22	19.02
Lamellar	1.28 \pm 0.27	0.62
Stroma + lamellar	6.00 \pm 1.37	7.84

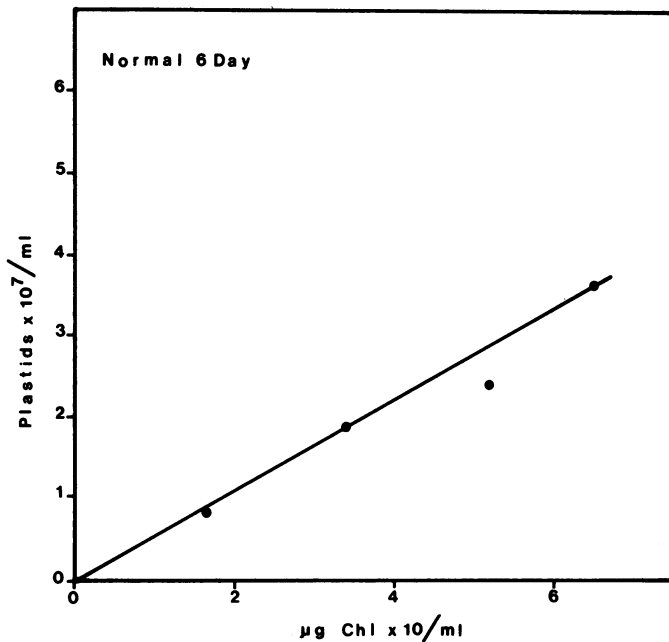


FIG. 1. Regression line used to determine the number of plastids from the Chl concentration of an isolated plastid fraction of 6-day normal barley. The correlation coefficient was $r_{xy} = +0.97$ and the regression coefficient was $b_{yx} = 0.55$ plastids/ μg Chl. At each growth stage, three separate analyses were conducted for the normal and the mutant. The average of the three was used to obtain the individual regression lines.

Table III. Chl Content per Plastid and Percentage Chl in Mutant Relative to Normal.

The Chl content was determined from the apical 3-cm leaf segments of normal and mutant barley seedlings at 4, 6, and 8 days. Results are the mean \pm SE for three experiments with three to four concentrations used per experiment as shown in Figure 1. Whole leaf estimates are averages of two experiments.

Days	Chl		Mutant Chl/Normal Chl	
	Normal	Mutant	Whole leaf	Plastids
	<i>pg/plastid</i>			<i>%</i>
4	1.35 \pm 0.18	0.51 \pm 0.05	8.16	37.8 \pm 1.9
6	1.74 \pm 0.04	0.70 \pm 0.11	26.2	40.2 \pm 7.3
8	1.56 \pm 0.05	0.94 \pm 0.10	48.6	60.2 \pm 6.8

also perhaps due to an inhibitor build-up during greening (21). Estimates of soluble protein:Chl ratios of the plastid isolates (Table IV) were considerably lower for the mutant at 8 days and the normal at 6 and 8 days than the protein:Chl ratios reported for intact isolated plastids: 8.9 for spinach (16) and 5.2 to 6.4 for tobacco (30). Also, a cursory comparison of the ability to evolve O_2 in the presence of ferricyanide before and after osmotic shock (24) of a 7-day normal plastid preparation suggested that only 25% of the plastids were intact.

Tests to Determine Presence of an Inhibitor. The addition of the partially purified *E. coli* carboxyltransferase subunit (CT subunit) to a 5-day normal stromal preparation did not enhance $\text{NaH}^{14}\text{CO}_3$ incorporation (Table V). This is contrary to the findings of Kannangara and Stumpf (21) who reported a substantial formation of malonyl-CoA upon the addition of the *E. coli* carboxyltransferase subunit to disrupted spinach chloroplast preparations. In a further test for inhibitor activity, aliquots from normal and mutant stromal preparations were pooled. Inasmuch as the pooled activities (shown in Table VI) were additive, the lower activity from the normal stromal preparations was not due to the action of an inhibitor.

Assay Constituents. The effect of the various assay constituents on the enzyme activity is shown in Table VII. The absolute requirement for ATP, MgCl_2 , and acetyl-CoA is in agreement with the known cofactor requirements for acetyl-CoA carboxylase activity (23). The mild stimulation by KCl, DTT, and BSA was not always experienced and, in some instances (as shown in Table VII), BSA at 0.2 mg was slightly inhibitory.

Inhibitory Effect of Avidin. Avidin completely inhibited enzyme activity (Table VIII). This inhibition was prevented if the reaction medium containing avidin was preincubated in the presence of biotin before the addition of $\text{NaH}^{14}\text{CO}_3$. This observation indicated that biotin was participating in the reaction. Biotin is known to serve as a prosthetic group covalently bound to the enzyme (23, 35).

End-product Analysis. Further evidence that the incorporation of $\text{NaH}^{14}\text{CO}_3$ was a measure of acetyl-CoA carboxylase activity is provided by end-product analysis. TLC on ITLC-SG plates suggested the presence of a CoA ester because the activity remained at the origin in the solvent system used (14) (Fig. 2). Upon hydrolysis, the radioactive compound showed the same migration pattern as malonic acid, which is the known acetyl-CoA-dependent product of acetyl-CoA carboxylase activity.

Biotin Content of Chloroplast Fractions. The biotin content in the stroma and lamellar preparations was also determined as a further test for the location of the enzyme within the chloroplast. The biotin content was highest in the stromal fraction, although the unwashed membrane fraction also contained a considerable quantity (Table IX). In contrast to the activity estimates, the biotin content of the stromal preparations of the normal exceeded the concentration detected in the mutant.

DISCUSSION

In the present study, the cofactor requirements (Table VII), the complete inhibition by avidin (Table VIII), and the end product obtained (Fig. 2) corroborate the known reaction mechanism for acetyl-CoA carboxylase.

The enzyme activities of the whole tissue homogenate of embryo isolates from 1-day seedlings (Table I) are comparable to those reported for wheat germ (13) and barley embryo extracts (6). The increased activity during development (Table I) was expected because acyl lipids are important membrane constituents. The nearly identical values for the mutant and normal at 2 and 3 days and the higher activity at 4 days for the mutant was not anticipated. Electron microscopic observations (data not presented) of sections of 2-day normal shoots revealed developing chloroplasts with lamellae in groups of four traversing the plastid, whereas the

Table IV. Acetyl-CoA Carboxylase Activity of Chloroplast Stroma

The chloroplasts were prepared from apical 3-cm leaf segments of 4-, 6-, and 8-day-old mutant and normal barley seedlings. Enzyme activity values are the mean \pm SE of two experiments with two to three replicates per experiment.

Day	HCO ₃ ⁻ Incorporated in						Protein/Chl		Chl	
	Mutant			Normal			Mutant	Normal	Mutant	Normal
	nmol/mg protein·min	nmol/mg Chl·min	nmol/plastid × 10 ⁸ ·min	nmol/mg protein·min	nmol/mg Chl·min	nmol/plastid × 10 ⁸ ·min	ratio		mg/g fresh wt	
4	54.2 \pm 1.8	1,730 \pm 178	88.3 \pm 9.1	31.4 \pm 3.1	120.7 \pm 17	16.3 \pm 2.3	32.6 \pm 5.2	4.0 \pm 0.7	0.12 \pm 0.02	0.99 \pm 0.14
6	34.3 \pm 0.8	163 \pm 38	28.4 \pm 6.7	7.7 \pm 0.5	4.1 \pm 0.5	0.5 \pm 0.2	4.8 \pm 1.9	0.9 \pm 0.3	0.62 \pm 0.07	1.98 \pm 0.27
8	16.6 \pm 1.3	19 \pm 3	1.8 \pm 0.3	4.8 \pm 0.3	1.1 \pm 0.1	0.2 \pm 0.0	1.2 \pm 0.2	0.3 \pm 0.1	1.08 \pm 0.03	2.10 \pm 0.03

Table V. Effect of Addition of *E. coli* Carboxyltransferase Subunit to Stromal Preparation from 5-Day Normal Barley on Acetyl-CoA Carboxylase Activity

Enzyme System ^a	Activity Incorporated
	dpm
Ea (<i>E. coli</i>)	3,011
Eb (<i>E. coli</i>) ^b	45
Ea + Eb	77,109
Stroma ^c	30,669
Stroma + Eb	29,700

^a Ea, BC + BCCP subunits; Eb, CT subunit.

^b Eb, 23 μ g protein.

^c Stroma preparation, 75 μ g protein.

Table VI. Effect of Pooling Stromal Preparations on Acetyl-CoA Carboxylase Activity

Stromal preparations from 4-, 6-, and 8-day-old mutant and normal barley seedlings were pooled. The numbers in parentheses indicate the number of determinations and the expected values are the mean of the specific activities of the unpooled enzyme preparations.

Effect	Activity in Stroma Preparations of Following Ages		
	4 days	6 days	8 days
	nmol/mg protein·min		
Obtained	47.4(2)	18.1(3)	13.2(2)
Expected	41.6(2)	20.8(3)	11.3(2)

2-day mutant shoots contained only undifferentiated proplastids. Because the mutant displays a lag in chloroplast development relative to the normal (15) and lipid synthesis in leaf segments is most active during the stage of chloroplast development (4), an increased acetyl-CoA carboxylase activity in the normal would be expected. The findings of Reitzel and Nielsen (31) on studies with developing leaves of Chl-deficient barley mutants were in agreement with this in that the enzyme activities of the mutants they studied remained below those of the wild type. A possible explanation for the higher activity in the virescens mutant is an increased synthesis in the mutant of other cellular membranes relative to those of the chloroplast. Another possibility is that the enzyme product, malonyl-CoA, was directed into other synthetic pathways, biotin (8), flavonoids (38), and 6-methyl salicylic acid (19). However, the higher biotin content in the normal relative to the mutant (Table IX) rules out the channelling of increased enzyme product into biotin in the mutant. The higher PE content as a percentage of total acyl lipids for the mutant in comparison to the normal at 4 days (34) would suggest proportionally more mitochondrial to chloroplast membranes for the mutant inasmuch as chloroplasts lack PE and the mitochondria are enriched in PE

Table VII. Cofactor Requirements and Effect of Other Additives on Acetyl-CoA Carboxylase Activity

The enzyme preparations added were 28 μ g protein from 6-day normal chloroplast stroma and 34 μ g protein from 2-day normal whole tissue homogenate prepared as outlined. The values in parentheses for KCl, DTT, and BSA represent one-half the concentrations of those used in the complete assay mixture.

Assay Mixture	Activity Incorporated in	
	6-day normal	2-day normal
	dpm	
Complete	6,093	4360
-ATP	57	185
-MgCl ₂	20	49
-KCl (or 4 μ mol KCl)	5,280(5,747)	5037
-DTT (or 0.25 μ mol DTT)	5,708(6,054)	4414
-BSA (or 0.1 mg BSA)	5,597(6,204)	5902
-KCl, -DTT, -BSA	5,031	
-Enzyme	31	49
-NaH ¹⁴ CO ₃	26	12
-Acetyl-CoA	41	66

(25). However, the PE content of both plant types was similar at 4 days when comparisons were made on a fresh-weight basis (34), suggesting a similar mitochondrial content.

The enzyme activity of the chloroplast preparations from 4-day seedlings of 31 nmol/mg protein·min for the normal and 54 nmol/mg protein·min for the mutant (Table IV) represents, to our knowledge, the first data showing appreciable acetyl-CoA carboxylase activity from chloroplast isolates. Activities of 1.7 nmol/mg protein·min (17) and 0.25 μ mol/mg Chl·h (32) have been reported for chloroplasts isolated from immature spinach leaves. Acetyl-CoA carboxylase activity has also been reported in the proplastids of developing castor bean endosperm at an activity of 10.7 nmol/mg protein·min (7).

The very high activity of the 4-day mutant stromal preparation relative to the normal, based both on Chl and protein content, is likely mainly due to the much higher ribulose-1,5-bisP carboxylase protein present in the normal relative to the mutant at 4 days (3) and the very low Chl content per plastid of the 4-day mutant (Table III). Accurate comparisons on a per plastid basis are not possible due to the large differences in the protein:Chl ratios of the isolated plastids (Table IV). The very high protein:Chl ratio of the 4-day mutant reflects its low Chl content per plastid. The decline in the protein:Chl ratio evident in the 6- and 8-day normal and 8-day mutant is probably due to excessive loss of soluble protein from the more mature plastids during isolation. On the other hand, the decrease in specific activity on a protein basis for the normal between days 4 to 8 and for the mutant between days 6 and 8 probably represents a true decline inasmuch as the major

Table VIII. Effect of Addition of Avidin to Stromal Preparations on Acetyl-CoA Carboxylase Activity

The stroma were prepared from 5-day normal and 6-day mutant and from 2-day normal and 2-day mutant whole-tissue homogenates.

Assay Medium	Activity Incorporated dpm
5-day normal (18 μ g protein)	
Complete	8,725
+Avidin (1.4 units) ^a	55
+ Biotin (1.5 μ g) + avidin (1.2 units) ^b	7,589
6-Day mutant (38 μ g protein)	
Complete	19,989
+Avidin (1.1 units)	43
+Biotin (1.6 μ g) + avidin (1.1 units) ^b	19,972
+Biotin (1.6 μ g)	19,088
2-Day normal (34 μ g protein)	
Complete	4,598
+Avidin (1.1 units)	59
+Biotin (1.6 μ g) + avidin (1.1 units) ^b	3,433
2-Day mutant (29 μ g protein)	
Complete	4,199
+ Avidin (1.1 units)	71
+ Biotin (1.6 μ g) + avidin (1.1 units) ^b	3,495

^a One unit will bind 1 μ g D-biotin.

^b The avidin and avidin-biotin mixtures were preincubated 3 min with the complete medium minus sodium bicarbonate. $\text{NaH}^{14}\text{CO}_3$ then was added to initiate the reaction.

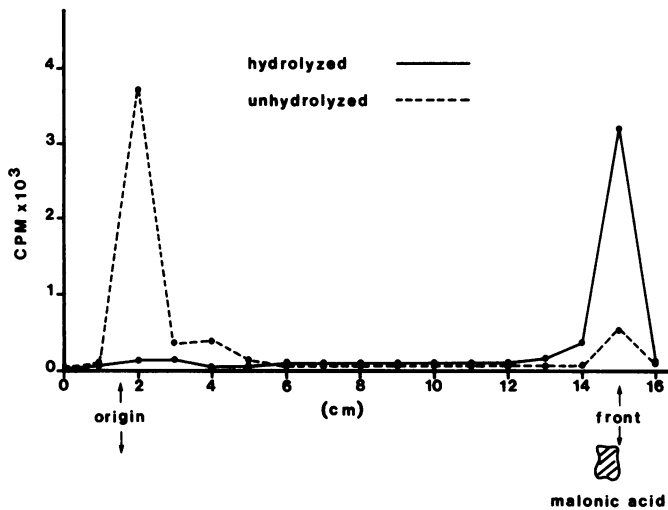


FIG. 2. Fractionation of the product of the acetyl-CoA carboxylase enzyme assay from a 6-day mutant stromal preparation. The acid-stable product was chromatogrammed on ITLC-SG plates as outlined by Haug (14). One-cm segments of the developed chromatogram were counted in a scintillation counter. Unhydrolyzed represents an aliquot taken after the reaction was terminated with concentrated HCl. Hydrolyzed represents an aliquot from the acid-terminated mixture made to 3 N with KOH as outlined.

chloroplast-soluble protein ribulose-1,5-bisP carboxylase showed minor increases during these stages (3) and a selective loss of chloroplast-soluble protein is not likely. Acyl lipid synthesis is probably coordinated with the synthesis of various macromolecules, organelle development, and cell growth. This is supported by the results of acetate labeling studies where an increase in the rate of acyl-lipid synthesis corresponded to the period most active in chloroplast development (4, 12).

The lower specific activity in the normal relative to the mutant

Table IX. Biotin Content of Chloroplast Stromal and Lamellar Fractions

The fractions were obtained from the apical 3-cm leaf segments of 5-day mutant and normal barley seedlings. Biotin was assayed by the avidin-dye binding method (11). Values represent the mean \pm SE of three experiments.

Chloroplast Fraction	Biotin Content	
	Mutant	Normal
	$\mu\text{g}/\text{mg protein}$	
Stroma	1.01 \pm 0.17	3.12 \pm 0.72
Lamellar	0.47 \pm 0.14	0.77 \pm 0.06

was shown not to be due to the presence of an inhibitor in the normal by experiments in which normal and mutant stromal isolates from seedlings of the same age but different physiological stages were pooled (Table VI). Also, an increase in activity was not evident upon the addition of an active *E. coli* carboxyltransferase subunit (Table V), suggesting a fully functional stromal enzyme complex.

The higher biotin content in the stromal fraction relative to the lamellar fraction (Table IX) is consistent with the localization of the biotin containing enzyme to the stromal fraction (Table II). However, the much higher levels in the normal relative to the mutant is not consistent with the activities found (Table IV). Biotin estimations as performed were presumed adequate for the localization of the acetyl-CoA carboxylase because it is the only biotin enzyme described for plants. The biotin levels of 1.0 to 3.1 $\mu\text{g}/\text{mg protein}$ (Table IX) exceed those reported for purified barley-embryo acetyl-CoA carboxylase (6); however, they are somewhat less than the biotin content of purified BCCP (6, 10). The high readings obtained probably reflect a high free-biotin content in the chloroplast stromal preparations. High levels of soluble biotin were also reported in lettuce, maize, pea, and tobacco chloroplasts (22). The results, however, do not rule out the possibility that the functional BCCP component of the acetyl-CoA carboxylase complex is also associated with the lamellar membrane, as was found by others (20, 21).

Although the origin of acetyl-CoA, a required substrate for the carboxylase enzyme is still uncertain (33), recent evidence has shown that bicarbonate, pyruvate and acetate each serve as effective precursors for fatty acid biosynthesis with isolated intact chloroplasts (28, 39). The more recent finding that a pyruvate dehydrogenase complex is localized in the chloroplast fraction (9, 37) suggests the chloroplasts can provide their own acetyl-CoA. The data presented here for the localization of the acetyl-CoA carboxylase enzyme to the chloroplast stromal fraction at activity levels comparable to the incorporation rates achieved for pyruvate, HCO_3^- , and acetate is consistent with the tenet that the complete fatty acid biosynthetic pathway resides within the chloroplast. In a preliminary study, using the same procedure for plastid isolation and acetyl-CoA carboxylase assay, very low specific activities were detected for expanding lettuce, spinach, and tobacco leaves and for greening cucumber cotyledons (data not presented). The lowest activity obtained for barley was at least 4 times higher than the levels obtained for these dicotyledonous plants. Our values obtained for spinach approximate those recently reported by Mohan and Kekwick (27) for spinach chloroplast homogenates. Apart from possible enzyme inhibitors in the other plants (33), barley, with its intercalary meristem, has an advantage over dicotyledons in providing tissue of uniform physiological age for developmental studies. This is of value in assaying for acetyl-CoA carboxylase activity inasmuch as it is highest during the period of rapid lipid accumulation and declines sharply as the tissue matures. The virescens mutant, with its high specific activity of acetyl-CoA carboxylase in chloroplasts isolated from young leaves, is a useful system for the further characterization of the chloroplast acetyl-

CoA carboxylase.

Acknowledgments—We thank Dr. Jill Williams for kindly monitoring the plastid isolates for bacterial contamination and Dr. Fred Cook for the generous supply of *E. coli* cells.

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