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Tissue Distribution of Acetyl-Coenzyme A Carboxylase in Leaves[1]

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BASIL J. NIKOLAU[2], EVE SYRKIN WURTELE[3], AND PAUL K. STUMPF*
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Acetyl-CoA carboxylase [acetyl-CoA—carbon dioxide ligase (ADP forming), EC 6.4.1.2] is a biotin-containing enzyme catalyzing the formation of malonyl-CoA. The tissue distribution of this enzyme was determined for leaves of C_3- and C_4-plants. The mesophyll tissues of the C_3-plants *Pisum sativum* and *Allium porrum* contained 90% of the leaf acetyl-CoA carboxylase activity, with the epidermal tissues containing the remainder. Western blotting of proteins fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, using ^{125}I-streptavidin as a probe, revealed biotinyl proteins of molecular weights 62,000, 51,000, and 32,000 in *P. sativum* and 62,000, 34,000, and 32,000 in *A. porrum*.

In the C_4-plant sorghum, epidermal protoplasts, mesophyll protoplasts and strands of bundle sheath cells contained 35, 47, and 17%, respectively, of the total leaf acetyl-CoA carboxylase activity. In *Zea mays* leaves the respective figures were 10% for epidermal protoplasts, 56% for mesophyll protoplasts, and 32% for bundle sheath strands. Biotinyl proteins of molecular weights 62,000 and 51,000 were identified in leaves of sorghum and *Z. mays*.

The results are discussed with respect to each tissue's requirements for malonyl-CoA for various metabolic pathways.

Acetyl-CoA carboxylase is a biotin-containing enzyme, catalyzing the ATP-dependent carboxylation of acetyl-CoA, to form malonyl-CoA (26). Extensive studies over the last 20 years have led to an understanding of the role of this enzyme in the regulation of *de novo* fatty acid biosynthesis in *Escherichia coli*, yeast, and various mammalian sources (26, 43). However, in plants the significance of acetyl-CoA carboxylase in the regulation of fatty acid biosynthesis is unknown. Attempts at correlating acetyl-CoA carboxylase activity with *de novo* fatty acid biosynthesis in plants are complicated because malonyl-CoA is a common intermediate in a number of other metabolic pathways, such as the pathways leading to the biosynthesis of cuticular waxes (23), flavonoids (13), stilbenoids (12), anthroquinones (34), napthoquinones (34), *N*-malonyl-ACC[4] (1), and malonic acid (39).

It has long been known from anatomical studies, that leaves are composed of heterogeneous populations of cells (10). Recent microscopic analysis of leaves of C_3-plants has established that epidermal cells typically comprise 10% of leaf cells, while mesophyll and vascular cells account for 55 and 35%, respectively, of the leaf cell population (19). Obviously, compartmentation at both the cellular and subcellular level plays an important role in the regulation of leaf metabolism.

Previous work in this laboratory has established the chloroplastic localization of fatty acid biosynthesis in mesophyll cells of spinach, a C_3-plant, because both ACP (33) and acetyl-CoA synthetase (24) are present exclusively in this organelle. In C_4-plants, such as *Zea mays*, chloroplasts isolated from bundle sheath and mesophyll cells have been shown to have the capacity for *de novo* fatty acid biosynthesis from acetate (15).

The biosynthesis of the very long chain fatty acids required for cuticular waxes occurs in the epidermal tissue (23). This process is postulated to involve *de novo* biosynthesis of C_{16} and C_{18} fatty acids in the plastids, followed by the cytosolic elongation of these acids to chain lengths of C_{22} to C_{32}. Acetyl-CoA carboxylase is required to supply malonyl-CoA for both the *de novo* synthesis and the elongation reactions.

The compartmentation of flavonoid biosynthesis is not as simple. As with most secondary metabolites, there is variety in the distribution and type of flavonoids found in different plant species (13), which is also reflected in the compartmentation of flavonoid biosynthesis. Some plants such as *Pisum sativum* (18), *Sorghum bicolor* (22), *Sinapsis alba* (38), *Bryophylum crenatum* (44), *Allium porrum* (44), and *Tulipa* (5) sequester flavonoids in epidermal cells. In *Sinapsis*, phenylalanine ammonia lyase and chalcone isomerase, two enzymes required for the biosynthesis of flavonoids, were found mainly in the epidermis (44). More significantly, chalcone synthase, one of the malonyl-CoA requiring enzymes of flavonoid biosynthesis, was present exclusively in the epidermis of *P. sativum* leaves (18). Oat leaves, on the other hand, accumulate flavonoids both in mesophyll and epidermal cells (44), and recent evidence suggests that flavonoid biosynthesis in this plant occurs in both cell types (35).

The biosynthesis of napthoquinones and anthroquinones requires malonyl-CoA (34). Although Kannangara *et al.* (20) demonstrated that chloroplasts are capable of the synthesis of 6-methyl-salicylate from acetate, the role of other compartments in the biosynthesis of these products is unknown.

Stilbene synthase has been demonstrated in extracts of *Rheum rhaponticum* (36). This enzyme catalyzes the biosynthesis of resveratrol, a stilbenoid, from *p*-coumaryl-CoA and malonyl-CoA (36). Recently, the occurrence of the malonated derivative of ACC was reported in plants (17). The requirement for malonyl-CoA in the synthesis of *N*-malonyl-ACC has now been demonstrated (1). Malonic acid, which accumulates in root tissue of *Glycine max*, has been shown to be derived from the carboxylation of acetyl-CoA (39).

Studies of acetyl-CoA carboxylase from plant sources were initially hampered by low activities of this enzyme (6, 21). Later

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[2] Present address: Department of Cellular, Viral and Molecular Biology, University of Utah, Salt Lake City, UT 84132.

[3] Present address: Native Plants, Inc., 417 Wakara Way, Salt Lake City, UT 84108.

[4] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACP, acyl carrier protein; PEP, phosphoenolpyruvate; RuBP, ribulose 1,5-bisphosphate.

workers achieved stabilization and purification of this enzyme from embryonic tissue of wheat (9, 16) and barley (4), from avocado fruit mesocarp (30), and from parsley cell culture (9). The only reported purification of this enzyme from leaves is that from maize leaves (31). Native mol wt within the range from 5×10^5 to 8.4×10^5 were reported for all plant acetyl-CoA carboxylases. The data regarding the subunit organization of these enzymes for different plant species is confusing and no trend is evident. Biotin-containing subunits of mol wt 240,000, 21,000, 240,000, and 60,000 have been reported for acetyl-CoA carboxylase from wheat germ (9), barley embryos (4), parsley cell culture (9), and maize leaves (31), respectively.

Because acetyl-CoA carboxylase in leaves is required in a number of different cell types to supply malonyl-CoA for at least six different biosynthetic pathways, and because purified acetyl-CoA carboxylase activities from different tissues and species differ in their molecular organizations, there is a possibility that these activities represent isozymes of acetyl-CoA carboxylase.

In this publication, we present data on the distribution of acetyl-CoA carboxylase among the leaf tissues of C_3- and C_4-plants.

MATERIALS AND METHODS

Chemicals. Acetyl-CoA was synthesized by reacting CoA with acetic anhydride as described by Stadtman (37). Excess acetic anhydride was removed by extraction with diethyl ether and the purity and concentration of the product was judged by its absorbance at 232 and 260 nm. Streptavidin was a kind gift from Dr. E. O. Stapley, Merck Institute. Iodination of streptavidin with $Na^{125}I$ was carried out by the method of Fracker and Speck (11). Excess [^{125}I]iodide was removed by gel filtration (42). Biochemicals were purchased from Sigma, except Cellulysin and Macerase which were obtained from CalBiochem. The radiochemicals $NaH^{14}CO_3$ (54 Ci/mol) and $Na^{125}I$ (carrier-free) were purchased from Amersham.

Plant Material. *Pisum sativum Argentum* mutant seeds (29) were the generous gift of Dr. G. A. Marx. Seeds of *P. sativum*, sorghum (*Sorghum* × *Sudangrass* Hybrid, var WAC Forage 99), and *Zea mays* (var Golden Hybrid Blend) were soaked in aerated water overnight at room temperature. They were rinsed with water and germinated in water-saturated vermiculite. Approximately 10 d after planting, *P. sativum* seedlings were transplanted into individual pots of sterilized soil mixture, and watered daily.

Plants were grown in a controlled environment with day/night temperatures of 26.5/22°C with a 16-h light period provided by fluorescent light (1700 ft-c). Fully expanded *P. sativum* leaves were harvested from 20- to 30-day old plants. Sorghum leaves were used 5 d after planting. The second true leaf of *Z. mays*, 7 d after planting, was used.

Allium porrum plants were obtained from a local market. The section 7 to 10 cm above the meristem was used in all experiments.

Separation of Leaf Tissues. *A. porrum* leaf segments were snapped in half longitudinally and the intact epidermis was peeled from the mesophyll layer of cells. The epidermal layers are referred to as inner (adaxial) and outer (abaxial) epidermis, depending upon which side of the leaf they were peeled from.

P. sativum leaves were peeled immediately after harvesting to yield intact upper (adaxial) and lower (abaxial) epidermal tissues and mesophyll tissue with a low degree of cross-contamination (18).

Immediately upon peeling all material was frozen in liquid nitrogen.

Preparation of Tissue and Leaf Extracts. Epidermal, mesophyll, bundle sheath strands, and whole leaf tissues were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle while being kept at the temperature of evaporating liquid nitrogen. Upon the evaporation of the nitrogen, 2 to 8 ml of 50 mM Tris-HCl pH 8.0, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1% PVP-40 (buffer A) was added, which immediately froze. As the mixture thawed, it was further homogenized. The brei was filtered through a 20-μm mesh nylon net. An aliquot of the filtrate was immediately passed through a short column of Sephadex-G25 using a rapid centrifugation procedure (42). Columns were equilibrated with buffer A containing 0.01% Triton-X100. Eluates were either assayed for enzyme activity immediately or frozen dropwise in liquid nitrogen until required.

Preparation and Purification of Mesophyll and Epidermal Protoplasts. Leaves of *Z. mays* were digested for 2.5 h with a Cellulysin and Macerase solution and the released mesophyll protoplasts were purified by flotation on a cushion of sucrose solution as described by Day *et al.* (8).

Mesophyll protoplasts of *P. sativum* were obtained from leaves whose epidermis had been removed. The mesophyll tissue was floated on a solution of 1% (w/v) Cellulysin, 0.6 M mannitol, 20 mM Mes-KOH pH 5.6. Following a 2-h digestion period at 30°C, leaf material was agitated and the released protoplasts were collected by centrifugation at 300*g* for 3 min and were washed twice with 0.6 M mannitol, 20 mM Mes-KOH pH 5.6.

Details of the procedure used to isolate epidermal protoplasts from *Z. mays* and sorghum is being published separately; a brief description is included here. Leaves were digested in a solution of Cellulysin and Macerase for 2 h (46). Protoplasts were separated from undigested leaf fragments by filtration through a 44-μm mesh nylon net. Protoplasts were pelleted (300*g*, 3 min) and epidermal protoplasts were separated from mesophyll protoplasts by centrifugation on a Ficoll (dialyzed and lyophilized, obtained from Sigma) density step gradient. Epidermal protoplasts which were visualized with Neutral Red dye (22) were collected from the top of the 7% (w/w) Ficoll step. Sorghum mesophyll protoplasts were recovered from the 10% (w/w) and 20% (w/w) Ficoll interface. Isolated protoplasts were washed with isotonic buffer and pelleted by centrifugation at 300*g* for 3 min.

Epidermal protoplasts of *P. sativum* were obtained from the peeled epidermal tissue. Digestion was for 3 h as described for mesophyll protoplasts. The released protoplasts were collected and washed as described above.

Preparation of Bundle Sheath Strands. Leaf blades of *Z. mays* and sorghum were abraded with carborundum, washed three times with distilled H_2O, and cut transversely in 1- to 2-mm strips. Digestion of *Z. mays* (8) and sorghum (46) leaf blades and isolation of bundle sheath strands (22) were carried out by described procedures. Strands of bundle sheath cells of sorghum were lightly homogenized with a Teflon pestle to remove remaining mesophyll contamination. Strands were collected on a 144-μm mesh nylon net.

Preparation of Protoplast Extracts. Isolated mesophyll and epidermal protoplasts were lysed (4°C) in 0.01% Triton-X100 and immediately passed through a Sephadex-G25 column as described above. Eluates were either assayed for enzyme activity or frozen dropwise in liquid nitrogen until required.

Assays. Protoplast fractions and tissue extracts were analyzed for acetyl-CoA carboxylase (32) and NADP-malate dehydrogenase (14) activities. RuBP carboxylase activity was assayed according to Wishnick and Lane (45) following 10-min activation at 30°C in the assay buffer without RuBP. PEP carboxylase was assayed as for RuBP carboxylase, without prior activation and replacing RuBP with PEP. Chl was assayed by the method of Arnon (2) and protein by a dye-binding method (3).

Preparation of Samples for SDS-PAGE. Epidermal, mesophyll, bundle sheath strands, and whole leaf tissues were frozen in liquid nitrogen immediately after isolation. The material was ground to a fine powder in a mortar and pestle while being kept at the temperature of evaporating liquid nitrogen. Upon the

evaporation of the nitrogen, 1 ml of 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1 mM EDTA, 10 mM Tris-HCl pH 8.0 was added. As the mixture thawed, it was homogenized thoroughly. The brei was heated to 100°C for 5 min following which solid material was removed by centrifugation at 10,000*g* for 3 min in a Microfuge. The supernatant was either used for electrophoresis immediately or stored at −20°C. Isolated mesophyll and epidermal protoplasts were pelleted by centrifugation at 300*g* for 3 min. The supernatant was removed, and the pellet resuspended in a small volume of 1% SDS, 5% 2-mercaptoethanol, 1 mM EDTA, 10% glycerol, 10 mM Tris-HCl pH 8.0. After heating at 100°C for 5 min, the samples were centrifuged in a Microfuge (10,000*g* for 3 min) and the supernatant was used for electrophoresis.

Electrophoresis was carried out by the method of Laemmli (25) in 10% (w/v) acrylamide/0.27% (w/v) bisacrylamide slab gels.

Western Blotting. Following SDS-PAGE, proteins were transferred from the gel onto nitrocellulose paper electrophoretically essentially as described by Towbin *et al.* (41). The transfer was carried out in 20 mM Tris-HCl (pH 8.3), 150 mM glycine, 20% (v/v) methanol at 60 v (300 mamp) for 4 h. The nitrocellulose paper was then soaked overnight in a solution of 3% (w/v) BSA in 10 mM Tris-HCl (pH 7.4), 0.9% (w/v) NaCl, and washed with 8.0×10^5 cpm of [^{125}I]streptavidin (about 8.0×10^7 cpm/mg protein) for 2 h in a buffer containing 3% (w/v) BSA. Unbound [^{125}I]streptavidin was removed by washing the paper three times with 10 mM Tris-HCl (pH 7.4), 0.9% NaCl. After air-drying, the nitrocellulose paper was exposed to Kodak X-Omat G film at −70°C, using a DuPont Cronex Lightning Plus Intensifying Screen. Exposed film was developed as recommended by the manufacturer with Kodak GBX Developer.

RESULTS

Isolation of Protoplasts. Pea leaf protoplasts, obtained after enzymic digestion, were examined microscopically as described above. Epidermal preparations were greater than 98% pure with 2×10^5 protoplasts obtained from 10 leaves. Mesophyll protoplasts were greater than 99% pure, and 5×10^5 protoplasts were obtained from 3 leaves.

The purity of the protoplast and bundle sheath strand preparations from *Z. mays* and sorghum were monitored by both microscopic examination and assaying for marker enzyme activities. Bundle sheath contamination of mesophyll protoplast preparations was determined by assaying for RuBP carboxylase, an enzyme exclusive to bundle sheath cells of C_4-plants. Contamination was less than 2%. PEP carboxylase and NADP-malate dehydrogenase activities were utilized to determine the contamination of the bundle sheath strands by mesophyll cells. Contamination was routinely less than 10%.

Preparations enriched with epidermal protoplasts were usually 60 to 80% pure as determined by counting of protoplasts with a hemacytometer. The only contamination was mesophyll protoplasts. Mesophyll preparations were usually less than 5% contaminated by epidermal protoplasts.

Acetyl-CoA carboxylase activity in epidermal and mesophyll protoplasts was corrected for cross-contamination by solving the following simultaneous equations:

$$n_E^M \cdot X + n_M^M \cdot Y = a_M$$
$$n_E^E \cdot X + n_M^E \cdot Y = a_E$$

where

n_E^E = number of epidermal protoplasts in the epidermal preparation/ml;
n_M^E = number of mesophyll protoplasts in the epidermal preparation/ml;
n_M^M = number of mesophyll protoplasts in the mesophyll preparation/ml;
n_E^M = number of epidermal protoplasts in the mesophyll preparation/ml;
X = acetyl-CoA carboxylase activity/epidermal cell;
Y = acetyl-CoA carboxylase activity/mesophyll cell;
a_M = acetyl-CoA carboxylase activity of mesophyll preparation/ml;
a_E = acetyl-CoA carboxylase activity of epidermal preparation/ml.

Table I. *Distribution of Acetyl-CoA Carboxylase Activity in Leaf Tissue of Pisum sativum*

Tissue	Activity		Distribution
	nmol/min·mg protein	*nmol/min·leaf*	%
Upper epidermis	14.7 ± 2.5	0.26 ± 0.04	8.6
Lower epidermis	9.4 ± 1.5	0.18 ± 0.02	6.0
Mesophyll	1.8 ± 0.3	2.6 ± 0.4	85.4
Whole leaf	0.65 ± 0.1	2.8 ± 0.3	

Table II. *Acetyl-CoA Carboxylase Activity in Mesophyll and Epidermal Protoplasts Isolated From Pisum sativum Leaves*

Assay	Protoplast Type	
	Epidermal	Mesophyll
Protein (μg/10^6 cells)	493 ± 60	357 ± 40
Chl (μg/10^6 cells)	0	84 ± 10
Acetyl-CoA Carboxylase		
nmol/min·10^6 cells	2.5 ± 0.5	0.060 ± 0.01
nmol/min·mg protein	5.1 ± 1.0	0.165 ± 0.03
nmol/min·mg Chl		0.71 ± 0.10

Table III. *Distribution of Acetyl-CoA Carboxylase Activity in Leaf Tissues of Allium porrum*

Tissue	Activity			
	nmol/min·mg protein	*nmol/min·g fresh wt*	*nmol/min·g leaf*	%
Outer epidermis	0.58 ± 0.09	7.3 ± 1.4	0.22 ± 0.04	5.4
Inner epidermis	0.52 ± 0.09	5.3 ± 1.0	0.15 ± 0.03	3.6
Mesophyll	1.00 ± 0.07	3.1 ± 0.5	3.73 ± 0.46	91.0
Whole leaf	0.84 ± 0.11	3.3 ± 0.5	3.33 ± 0.46	

Distribution of Acetyl-CoA Carboxylase Activity in Leaf Tissue and Protoplasts of *P. sativum*. The *Argentum* mutant of *P. sativum* which was used in these experiments has an enlarged airspace between the epidermal and mesophyll layer of cells (29). This morphology simplifies the separation of the epidermis since it can be peeled intact from the mesophyll layer without cross-contamination (18). The specific activities of acetyl-CoA carboxylase were 5- and 8-fold higher in extracts of the lower and upper epidermal tissues, respectively, than in extracts of mesophyll tissue (Table I). In each leaf, 86% of the total acetyl-CoA carboxylase activity was located in the mesophyll cells, with the remainder approximately equally divided between the upper and lower epidermal tissue. The sum of the activities in the epidermal and mesophyll tissues was equal to the acetyl-CoA carboxylase activity found in whole leaf extracts.

The specific activity of acetyl-CoA carboxylase was 4-fold higher in epidermal protoplasts than in mesophyll protoplasts (Table II). The distribution and specific activity of acetyl-CoA carboxylase in the epidermal protoplast preparations were similar to those found in the epidermal tissue extracts. The specific activity of acetyl-CoA carboxylase in isolated mesophyll protoplasts was about one-sixteenth of that found in mesophyll tissue extracts (compare Tables I and II). Vascular cells account for approximately 40% of the cells in the mesophyll tissue (19),

Table IV. *Distribution of Acetyl-CoA Carboxylase Activity in Mesophyll, Epidermal Protoplasts, Strands of Bundle Sheath Cell Isolated From Leaves of Sorghum*

The results are the average of seven determinations.

Tissue	Activity			
	nmol/min·10⁶ cells	*nmol/min· mg protein*	*nmol/min· mg Chl*	*nmol/min·g fresh wt*
Epidermal protoplasts	2.9 ± 0.7	12.3 ± 3.0		
Mesophyll protoplasts	0.48 ± 0.1	1.5 ± 0.3	8.6 ± 1.8	
Bundle sheath strands		1.2 ± 0.3	13.2 ± 3	
Whole leaves		3.2 ± 1.0	37.4 ± 10	40.5 ± 12

Table V. *Distribution of Acetyl-CoA Carboxylase Activity in Leaf Tissue of Sorghum*

See text for the description of the method used to calculate these figures.

Tissue	Distribution	
	nmol/min· g of leaf	%
Epidermal	10.5 ± 2.5	35
Mesophyll	13.9 ± 3.0	47
Bundle sheath	5.2 ± 1.1	17

suggesting that these cells may contain substantial acetyl-CoA carboxylase activity.

Distribution of Acetyl-CoA Carboxylase Activity in Leaf Tissue of *A. porrum*. The specific activity of acetyl-CoA carboxylase was about twice as high in extracts of mesophyll tissue relative to the epidermal tissue extracts from *A. porrum* (Table III). Approximately 90% of the leaf acetyl-CoA carboxylase activity was located in the mesophyll tissue of the leaf. The outer and inner epidermal tissues accounted for 5.4 and 3.6% of the leaf acetyl-CoA carboxylase activity. The activity of the whole leaf extract was similar to the sum of the activities of the different tissues of the *A. porrum* leaf.

Distribution of Acetyl-CoA Carboxylase in Leaf Tissue of Sorghum. Isolated epidermal protoplasts of sorghum showed the highest specific activity of acetyl-CoA carboxylase compared to the isolated mesophyll protoplasts and strands of bundle sheath cells (Table IV). The specific activity of the enzyme in isolated mesophyll protoplasts and bundle sheath strands was about 10% of that found in isolated epidermal protoplasts.

The sorghum leaves used in this study contained 3.9×10^6 epidermal cells and 29.0×10^6 mesophyll cells, respectively, per gram of fresh weight; 25% of the total leaf Chl (1.73 mg/g fresh weight) was found in bundle sheath cells (E. S. Wurtele and B. J. Nikolau, unpublished work). Using these data, the distribution of acetyl-CoA carboxylase activity was calculated in the different tissues of sorghum leaves (Table V). The epidermal tissue contained 35% of the leaf acetyl-CoA carboxylase activity, while the mesophyll and bundle sheath cells contained 47 and 17%, respectively. The sum of the acetyl-CoA carboxylase activity in the different cell types was calculated to be 29.6 ± 6.6 nmol/min·g fresh weight, which agrees quite well with the experimentally obtained figure from whole-leaf extracts of 40.5 ± 12 nmol/min·g fresh weight (Table IV).

Acetyl-CoA Carboxylase Activity in Leaf Tissue of *Z. mays*. Activity of acetyl-CoA carboxylase in the various isolated leaf cells from *Z. mays* is shown in Table VI. Activities are expressed on a basis of cell numbers, protein, and Chl to allow comparison among the different tissues. Highest activity was found in isolated strands of bundle sheath cells. Mesophyll protoplasts contained approximately half the activity of bundle sheath strands both on the basis of protein and Chl. Isolated epidermal protoplasts had the lowest activity of acetyl-CoA carboxylase. The activity of acetyl-CoA carboxylase shown by whole leaf extract was within the range of activities shown by the various fractions.

The relative distribution of acetyl-CoA carboxylase activity for different cell types of *Z. mays* leaves could not be determined, as information on the ratio of epidermal:mesophyll:bundle sheath cells for this plant is not available. However, an estimate made by using the data obtained for sorghum leaves suggested epidermal cells contained about 11% of the total leaf acetyl-CoA carboxylase activity in *Z. mays*; 56 and 32% of the total activity may be in mesophyll and bundle sheath tissue, respectively.

Identification of Biotin-Containing Proteins. As an alternative to examining the distribution of acetyl-CoA carboxylase by assaying for enzyme activity, a system was developed to detect this enzyme (and other biotin-containing proteins) by assaying for the presence of biotinyl proteins. The principle of the method utilized a combination of two procedures, Western blotting (41) and streptavidin-biotin conjugation (7). Following SDS-PAGE, proteins were transferred to nitrocellulose paper electrophoretically. [^{125}I]Streptavidin was used to probe for biotin-containing proteins; the location of [^{125}I]streptavidin-biotin complex on the nitrocellulose paper was determined by autoradiography.

Biotinyl Proteins in *P. sativum* and *A. porrum*. Three biotin-containing proteins were detected in leaf extracts of *P. sativum* (Fig. 1) and *A. porrum* (Fig. 2). Epidermal and mesophyll tissues of both species contained a biotinyl protein with a mol wt of approximately 62,000 ± 5,000. *P. sativum* leaves also contained biotinyl proteins of 51,000 ± 5,000 and 32,000 ± 3,000 D, the latter of which was only faintly seen (Fig. 1); these biotinyl proteins were present in both epidermal and mesophyll tissues.

Table VI. *Acetyl-CoA Carboxylase Activity in Epidermal, Mesophyll Protoplasts, and Bundle Sheath Strands Isolated From Leaves of Zea mays*

The results are the average of seven determinations.

Tissue	Activity			
	nmol/min·10⁶ cells	*nmol/min· mg protein*	*nmol/min· mg Chl*	*nmol/min·g fresh wt*
Epidermal protoplasts	0.8 ± 0.5	3.3 ± 2.0		
Mesophyll protoplasts	2.8 ± 1.0	5.6 ± 2.0	30.0 ± 10	
Bundle sheath strands		8.0 ± 1.0	60.0 ± 7.0	
Whole leaf		3.5 ± 0.8	36.0 ± 5.0	41.0 ± 6.0

The other biotinyl proteins in *A. porrum* leaves had mol wt of 34,000 ± 3,000 and 30,000 ± 3,000 (Fig. 2). Mesophyll tissue of *A. porrum* contained low amounts of the 34K D biotin-containing protein. Both the 30K and 34K D biotinyl proteins were detected in epidermal tissue of *A. porrum*.

Biotinyl Proteins of *Z. mays* and Sorghum. In whole leaf extracts of sorghum (Fig. 3) and *Z. mays* (Fig. 4), two major biotinyl proteins were detected. The mol wt of these proteins are 62,000 ± 5,000 and 51,000 ± 5,000.

In *Z. mays* leaves, both biotinyl proteins were found in extracts of bundle sheath strands and mesophyll protoplasts. Interestingly, epidermal protoplast preparations only contained the 51K D protein.

The distribution of the biotinyl protein in sorghum leaves differed from that in *Z. mays*. Mesophyll protoplast preparations contained only the 62K D protein, while both the 62K and 51K D biotinyl proteins were detected in the epidermal protoplast and bundle sheath strand preparations.

DISCUSSION

Most previous studies of acetyl-CoA carboxylase have been concerned with the relationship of this enzyme to fatty acid biosynthesis (30, 32, 40). However, in plants the product of the reaction catalyzed by this enzyme, malonyl-CoA, is also an intermediate common to the biosynthesis of cuticular waxes (23), flavonoids (13), stilbenoids (12), anthroquinones and napthoquinones (34), *N*-malonyl-ACC (1), and malonic acid (39). As some of these biosynthetic systems are known to be compartmented in different cells of leaves, an investigation of the distribution of acetyl-CoA carboxylase among the different cell types of leaves was of interest.

Acetyl-CoA carboxylase activity in the plants which were examined ranged between 0.8 and 3.5 nmol/min·mg of protein, and was present in all leaf tissues. However, the distribution of this enzyme among the different leaf tissues showed some variation between plant species. In the C_3-plants *P. sativum* and *A. porrum*, the majority of the leaf activity was in the mesophyll tissue. The epidermal tissue of these leaves contained 15 and 9% of the leaf activity, respectively. In C_4-plants the distribution of acetyl-CoA carboxylase activity was determined among epidermal protoplasts, mesophyll protoplasts, and bundle sheath strands. Epidermal protoplasts of *Z. mays* contained 10% of the leaf acetyl-CoA carboxylase activity, similar to that found in the epidermis of C_3-plants. However, sorghum epidermal protoplasts contained over one-third of the leaf acetyl-CoA carboxylase activity (despite the fact that these cells comprise only a fraction of the total leaf cells). Recently, *in vivo* studies with *Clivia maniata* leaves demonstrated maximum synthesis of cutin to occur in the expanding leaf regions (27). The sorghum leaves used in this study were still rapidly elongating and, thus, presumably rapidly synthesizing cutin. The high acetyl-CoA carboxylase activity in the epidermal cells of sorghum could be required to supply malonyl-CoA for cutin biosynthesis in these leaves. The distribution of acetyl-CoA carboxylase activity between mesophyll and bundle sheath cells was similar in both sorghum and *Z. mays* leaves, with approximately half of the total leaf activity

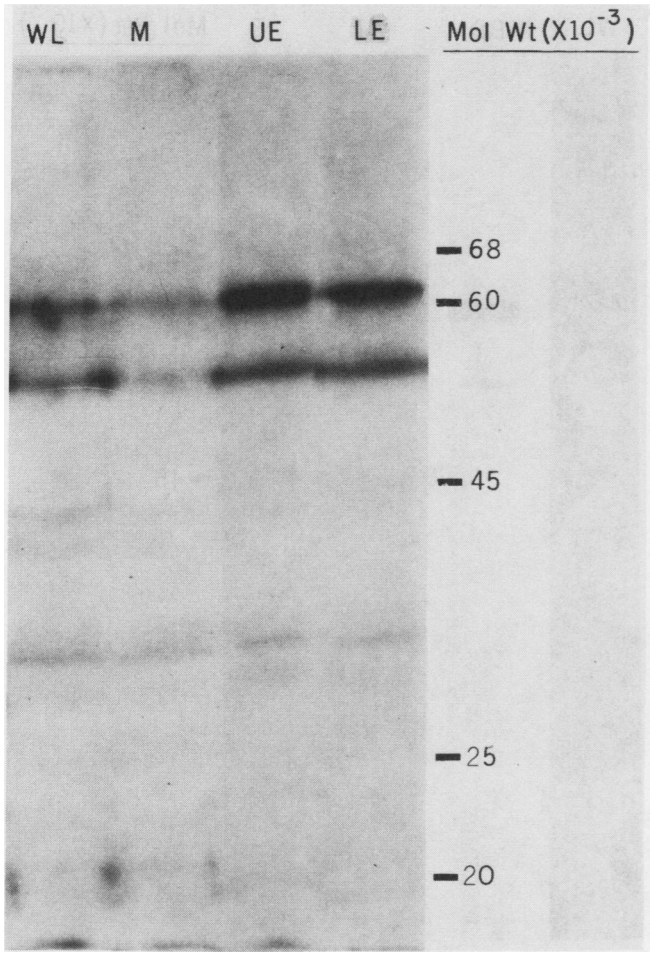

FIG. 1. Biotinyl proteins in whole leaf (WL), mesophyll (M), upper epidermal (UE), and lower epidermal (LE) tissues of *P. sativum* leaves.

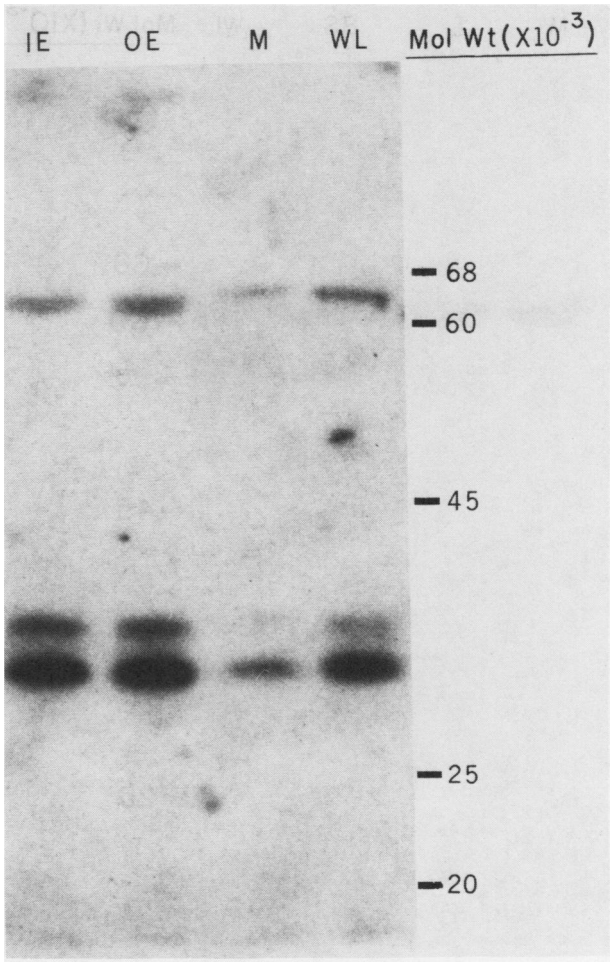

FIG. 2. Biotinyl proteins in inner epidermal (IE), outer epidermal (OE), mesophyll (M), and whole leaf (WL) tissues of *A. porrum* leaves.

being localized in mesophyll cells and the remainder in bundle sheath cells. The differences in the distribution of acetyl-CoA carboxylase activity among the leaf tissues in the plants studied probably reflect the requirements of each cell type for malonyl-CoA in the biosynthetic pathways outlined above.

The SDS-PAGE/Western blotting procedure was utilized to determine the mol wt of the biotin-containing subunits of acetyl-CoA carboxylase in the leaf tissues examined. Since acetyl-CoA carboxylase is the only known biotinyl enzyme in plants, it was a surprise to us to detect multiple biotin-containing proteins in leaves of *P. sativum*, *A. porrum*, sorghum, and *Z. mays*. In total, four biotinyl proteins were detected with mol wt of 62,000, 51,000, 34,000, and 32,000. The 62K D biotinyl protein has been identified as the biotin-containing subunit of acetyl-CoA carboxylase in maize (31) and barley leaves (unpublished data). In barley, this 62K biotinyl protein is the only biotin-containing protein, and it is located in the chloroplasts of mesophyll protoplasts. The other biotin-containing proteins may be subunits of isozymes of acetyl-CoA carboxylase. Interestingly, in preparations of epidermal protoplasts from *Z. mays*, which contain acetyl-CoA carboxylase activity, only the 51K D biotinyl protein was detectable, supporting this hypothesis. The presence of isozymes of acetyl-CoA carboxylase would allow for the differential regulation of the supply of malonyl-CoA required for the six known biosynthetic pathways.

The presence of other biotin-containing enzymes, such as pyruvate carboxylase and propionyl-CoA carboxylase (28) would also lead to the observed multiplicity of biotinyl proteins in leaves. These enzymes have not as yet been detected in plants.

Proteolytic degradation during the preparations of extracts for SDS-PAGE would also result in multiple biotinyl proteins. However, the very rapid procedure used to prepare whole leaf samples for SDS-PAGE gives a similar pattern of biotinyl proteins to preparations which take hours to prepare (mesophyll, epidermal protoplasts, and bundle sheath strands). Furthermore, a leaf extract prepared without SDS and incubated at 30°C did not show any change in the pattern of biotinyl proteins over a period of 6 h. These results suggest that the biotinyl proteins observed in these experiments are present *in vivo* in the leaf. The results do not rule out the possibility that these biotinyl proteins are *in vivo* degradation products occurring during the turnover of acetyl-CoA carboxylase in the leaf.

The mol wt of the biotinyl subunit of purified acetyl-CoA carboxylase has been determined in wheat germ (9), barley embryos (4), parsley cell culture (9), and maize leaves (31). The purified maize leaf acetyl-CoA carboxylase contained a biotinyl subunit of 60K D (31). This subunit was found in all the leaves that were examined. Indeed, a 60K D biotin-containing protein has been found in approximately 10 other leaves which were examined (data not shown). Biotin-containing peptides of 240,000 and 21,000, which have been reported in wheat germ (9), barley embryos (4), and parsley cell culture (9) were not detected in this study. Possibly, acetyl-CoA carboxylase has a different sized biotinyl subunit in embryos and in tissue culture.

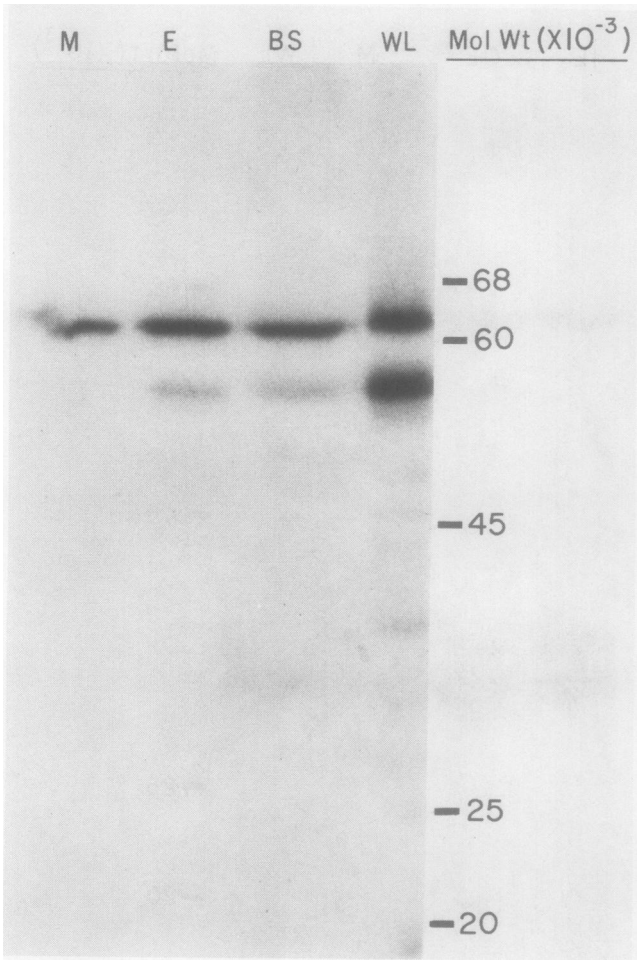

FIG. 3. Biotinyl proteins in mesophyll protoplasts (M), epidermal protoplasts (E), bundle sheath strands (BS), and whole leaf extract (WL) from sorghum leaves.

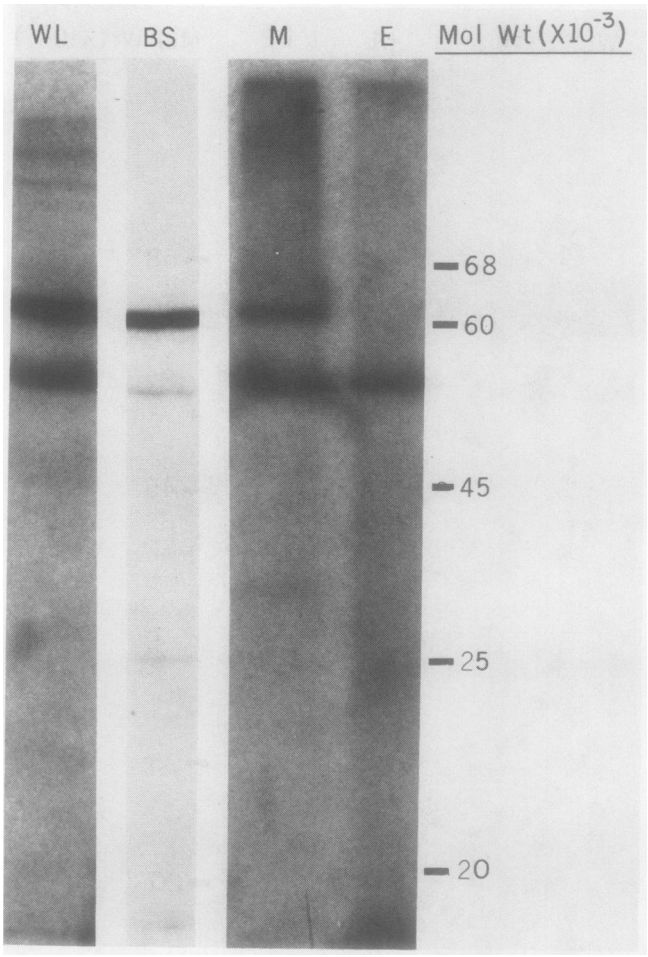

FIG. 4. Biotinyl proteins in the whole leaf extract (WL), bundle sheath strands (BS), mesophyll protoplasts (M), and epidermal protoplasts (E) from *Z. mays* leaves.

Finally, further biochemical characterization of the individual biotinyl proteins is required to identify the function of these proteins. Such data would also enhance our understanding of the metabolism of malonyl-CoA in leaf tissues.

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