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Nature of the Fatty Acid Synthetase Systems in Parenchymal and Epidermal Cells of *Allium porrum* L. Leaves[1]

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RENE LESSIRE[2] AND PAUL K. STUMPF
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Fatty acid synthesis was compared in cell-free extracts of epidermis and parenchyma of *Allium porrum* L. leaves. Parenchyma extracts had the major fatty acid synthetase (FAS) activity (70–90%) of the whole leaf; palmitic acid was also the major fatty acid synthesized when acetyl-coenzyme A (CoA) was the primer, but when acetyl-acyl carrier protein (ACP) was employed, $C_{18:0}$ and $C_{16:0}$ were synthesized in equal proportion. With the epidermal FAS system when either acetyl-CoA or acetyl-ACP was tested in the presence of labeled malonyl-CoA, palmitic acid was the only product synthesized. Specific activities of the FAS enzyme activities were determined in both tissue extracts.

The properties of malonyl-CoA:ACP transacylase were examined from the two different tissues. The molecular weights estimated by Sephadex G-200 chromatography were 38,000 for the epidermal enzyme and 45,000 for parenchymal enzyme. The optimal pH was for both enzymes 7.8 to 8.0 and the maximal velocity 0.4 to 0.5 micromoles per milligram protein per minute. These enzymes had different affinities for malonyl-CoA and ACP. For the malonyl-CoA:ACP transacylase of epidermis, the K_m values were 5.6 and 13.7 micromolar for malonyl-CoA and ACP, respectively, and 4.2 and 21.7 micromolar for the parenchymal enzyme. These results suggest that the FAS system in both tissues are nonassociated, that the malonyl-CoA:ACP transacylases are isozymes, and that both in epidermis and in parenchyma tissue two independent FAS systems occur. Evidence would suggest that β-ketoacyl-ACP synthase II is present in the parenchymal cells but missing in the epidermal cell.

Very long chain fatty acids (C_{20}–C_{32}) are synthesized in epidermal cells of *Allium porrum* L. leaves by elongation of a fatty acid precursor (6, 7, 11). Cassagne and Lessire have provided evidence that stearoyl-CoA is the precursor for this elongation (6, 7). However, little data are available concerning the synthesis of palmitic and stearic acids in epidermal cells. Indirect evidence suggested a *de novo* fatty acid synthesis occurring in epidermal cells. For example, isolated epidermal cells were able to utilize [1-^{14}C]acetate for the synthesis of labeled palmitic and stearic acids (4). Although Schmidt's degradation data indicated that palmitic acid was the product of *de novo* synthesis, data from the degradation of labeled stearic acid was equivocal (5). In the presence of acetyl-CoA and [1,3-^{14}C]malonyl-CoA, cell-free extracts of the epidermis synthesized both labeled palmitic and stearic acids (5).

This paper describes a comparison of the FAS[3] system in both parenchymal and epidermal cells and defines the nature of the soluble FAS systems in both cell types. Because of the ultrastructure of leek epidermal cells, intact leucoplasts cannot be prepared by the usual technique. Therefore, the localization of the FAS systems in leucoplasts of epidermal cells could not be pursued.

MATERIALS AND METHODS

Materials. Blue dextran, albumin (BSA), ovalbumin (hen egg), chymotrypsinogen A, Cyt *c*, NADH, NADPH, acetyl-CoA, and DTT were purchased from Sigma Chemical Company. Diketene was the product of Aldrich Chemical Company. Acetic anhydride and glycerol were from Mallinkrodt Inc. Crotonic anhydride was purchased from Eastman Organic Chemicals and trans-2-decenoic acid from ICN K & K. [1,3-^{14}C]Acetyl-CoA (52 Ci/mol), [1,3-^{14}C] (59 Ci/mol), and [2-^{14}C]malonyl-CoA (59 Ci/mol) were purchased from New England Nuclear, Rosechem Products, and the Radiochemical Center Amersham, respectively. Sephadex G-200 was obtained from Pharmacia and *Escherichia coli* from Grain Processing Company.

Synthesis of Acyl-ACP Derivatives. ACP was purified from *E. coli* by the method of Rock and Cronan (15). Acetyl-ACP (2), aceto-acetyl-ACP (24), crotonyl-ACP (24), and 2-decanoyl-ACP (25) were synthesized by reacting reduced ACP with acetic anhydride, diketene, crotonic anhydride, and mixed anhydride of 2-decenoic acid, respectively. The ACP products were precipitated with 10% of TCA, washed, and purified according to the method of Shimakata and Stumpf (16). Concentrations of acyl-ACPs were determined by measuring the level of thiol groups liberated after interaction with neutral hydroxylamine (8, 20).

Crude Extracts Preparation. About 6 g of epidermis of *Allium porrum* L. leaves were carefully separated from parenchyma and homogenized at 4°C in 50 ml of a 0.1 M Tris-HCl buffer (pH 7.5) containing 3 mM DTT and 20% glycerol using a Braun razor blade homogenizer for 3 × 10 s at low speed. The extract was then filtered through five layers of Miracloth and used as enzyme source. The corresponding parenchyma tissue free of epidermis (about 60 g) was homogenized and filtered under the same conditions.

For each enzyme activity measurement, the proportionality to the protein content and incubation time were verified. The activities were calculated as the initial velocity of the reaction.

Acetyl-CoA:ACP Transacylase and Malonyl-CoA:ACP Transacylase Assays. Ten mM DTT, 100 μM ACP, and a suitable amount of enzyme were incubated 10 min at 25°C in a 0.1 M Tris HCl buffer (pH 8.1). The reaction was started by adding 8.2 μM [1,3-^{14}C] or [2-^{14}C]malonyl-CoA. The final volume was 60 μl. Acid precipitation, filtration with Millipore filters, and other procedures were done as described by Alberts *et al.* (1).

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[2] Permanent address: Centre National de la Recherche Scientifique, Institut de Biochimie Cellulaire et Neurochimie, 1 rue Camille-Saint-Saens, 3307 Bordeaux Cedex, France.

[3] Abbreviations: FAS, fatty acid synthetase; ACP, acyl-carrier protein.

The acetyl-CoA:ACP transacylase activity was measured under the same conditions except that the [1,3-^{14}C]acetyl-CoA concentration was 22.4 μM and the final volume was 90 μl.

Other Enzyme Assays. The β-ketoacyl-ACP synthetase activity was assayed by the method of Garwin *et al.* (9). *E. coli* ACP (20 μM) was incubated 10 min at 25°C in presence of 1 mM DTT, 20 munits of malonyl-CoA:ACP transacylase from avocado, and 20 μM of [2-^{14}C]malonyl-CoA in a 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Ten μl of enzyme were added and the reaction was started by the addition of decanoyl-ACP at a final concentration of 30 μM. The final volume was 50 μl. After 30 min of incubation at 37°C, the reaction was stopped by adding 0.8 ml of the reductant.

β-Ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase I activities were measured with a Cary 14 spectrometer under the same conditions used by Shimakata and Stumpf (16).

Protein Determination. Protein concentrations were determined by the Bradford method (3).

Sephadex G-200 Chromatography. Before chromatography, the crude extracts of parenchyma and epidermis tissues were centrifuged at 10,000*g* for 20 min. The pellets were discarded and the supernatants were recentrifuged at 68,000*g* for 2 h. $(NH_4)_2SO_4$ was added to the resulting supernatant to a final concentration of 80%. After 15 min of gentle shaking, the extracts were centrifuged at 10,000*g* for 20 min. The pellets were resuspended in a 0.1 M Tris-HCl (pH 7.5) buffer containing 3 mM DTT and 20% glycerol and applied on a Sephadex G-200 column (1.5 × 87 cm) equilibrated and eluted with the same buffer. Fractions of 2.1 ml were collected. Before each chromatography, the column was calibrated using marker proteins; no change in the elution volume were detected. Under these conditions, the void volume of the column was 53.7 ml and the elution volumes for BSA (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and Cyt *c* (12,500) were 76.5, 91.2, 110.21, and 126.3 ml, respectively.

Fatty Acid Synthesis Assay. Fatty acid synthesis was measured according to the procedure of Jaworski *et al.* (10). The concentrations used were acetyl-CoA, 12 μM; NADH, 500 μM NADPH, 500 μM, ACP, 50 μg; [2-^{14}C]malonyl-CoA (59 Ci/mol), 2.5 μM; and enzyme, 0.5 mg in a 0.1 M Tris-HCl (pH 8.1) buffer. The volume of the reaction mixture was 1 ml. The incubation was carried for 1 h at 37°C and stopped by adding 0.5 ml of KOH, 8 M. After 30 min of saponification at 80°C, 0.2 ml of concentrated H_2SO_4 were added slowly and the fatty acids were extracted with 4 ml of chloroform. After evaporation of the solvent, the methyl esters were prepared in presence of 2 ml of 2.5% of H_2SO_4 in anhydrous methanol containing 5% benzene at 70°C for 20 min. After cooling and adding 2.5 ml of 5% NaCl in water, the methyl esters were extracted with 5 ml of petroleum ether and analyzed by radio-GLC using a 10% DEGS column at 170°C on a Varian 920 chromatograph equipped with a proportional Nuclear Chicago radioactivity counter.

RESULTS

FAS Synthesis in Epidermis and Parenchyma Crude Extracts. All enzyme activities required for FAS synthesis were present in crude extracts prepared from parenchyma and epidermis tissue of leek leaves (Table I). The specific activities for the acetyl-CoA:ACP transacylase, malonyl-CoA:ACP transacylase, β-ketoacyl-ACP synthetase, and β-ketoacyl-ACP dehydrase were of the same order in both tissue extracts. The β-ketoacyl-ACP reductase was 4.8 times more active in epidermis extract than in parenchymal extracts. The same observation was observed from the enoyl-ACP reductase I which had a higher specific activity in the epidermis than in the parenchymal cells. The data for the specific activities for the different enzyme activities are in good agreement with those reported for safflower seeds and spinach leaves (16, 17). Acetyl-CoA:ACP transacylase and β-ketoacyl-ACP synthetase I had the lowest activity suggesting that also in leek leaves these enzymes may have a regulatory function as well (16, 17).

Table I. *Specific Activities of FAS Enzyme in Parenchymal and Epidermal Cells*

Enzymes	Epidermis	Parenchyma
	nmol/mg protein·min	
Acetyl-CoA:ACP transacylase	0.087	0.133
Malonyl-CoA:ACP transacylase	57.3	44.5
β-Ketoacyl-ACP synthetase I	0.076	0.105
β-Ketoacyl-ACP reductase	19.7	4.1
β-Ketoacyl-ACP dehydrase	6.9	9.7
Enoyl-ACP reductase I	8.0	3.6

Table II. *Enzyme Distribution in Parenchyma and Epidermis Expressed as Percentage in Total Leaf Tissue*

A unit is defined as the amount of enzyme to form 1 nmol/min.

Enzymes	Epidermis		Parenchyma		Epidermis/Parenchyma
	units/g fresh tissue	%[a]	*units/g fresh tissue*	%	*ratio*
Acetyl-CoA:ACP transacylase	0.64	6.5	0.71	93.5	0.9
Malonyl-CoA:ACP transacylase	132.5	15.7	97.9	84.3	1.4
β-Ketoacyl-ACP synthetase I	0.48	9.0	0.54	91.0	0.9
β-Ketoacyl-ACP reductase	104.7	31.8	22.4	68.2	4.7
β-Ketoacyl-ACP dehydrase	34.6	8.0	44.4	92.0	0.8
Enoyl-ACP reductase I	38.8	20.8	16.6	79.2	2.3
Primers for Fatty Acid Synthesis	Activity[b]		Activity		
		%[a]		%	
Acetyl-CoA	0.60	7.3	7.66	92.7	0.08
Acetyl-ACP	1.96	31.3	4.30	68.7	0.46

[a] Relates to distribution of FAS system in epidermis and parenchyma.
[b] Activity is defined as nmol malonyl-CoA incorporated into fatty acids/h·g fresh weight tissue.

Distribution of FAS Enzyme Activities in Leek Leaves. Table II shows that the enzyme level in each tissue expressed as units per g of fresh tissue is about the same with the exception of the β-ketoacyl-ACP reductase and the enoyl-ACP reductase which are elevated as indicated in Table I. The highest levels of the FAS enzymes were localized preferentially in parenchymal tissue. In each case, the parenchyma contained at least 68% but more generally 90% of the total activity for each enzyme. These results can not be interpreted as a contamination of epidermal extract by parenchymal tissue because no Chl was detected in epidermal crude extracts. The Chl content of parenchymal extracts was 2.4 μg/mg of protein. Furthermore, in the two tissue extracts the FAS enzymes had about the same specific activity, whereas β-ketoacyl-ACP reductase and enoyl-ACP reductase specific activities were higher in epidermal extracts than in parenchymal extracts.

Characteristics of the FAS System in Epidermal and Paren-

chymal Extracts. The cofactor requirements for the FAS synthesis using epidermal and parenchymal extracts with [2-^{14}C]malonyl-CoA as the labeled substrate were determined (Table III). The omission of NADPH, ACP, or NADPH and NADH resulted in markedly decreased activity for synthesis. Of interest, in the absence of NADH, the remaining activity with the epidermal crude extract was 48% whereas with the parenchymal enzyme, omission of NADH led to an 88% decrease in synthesis. These results suggest a fairly broad specificity for NADPH by the two reductases in epidermal extracts but a greater NADH specificity with the parenchymal tissue for one of the reductases. The addition of cerulenin at a concentration of 50 μM completely inhibited the FAS synthesis in both extracts. With acetyl-CoA as the primer, [^{14}C]palmitic acid was the major product, *i.e.* 82% was found with epidermal FAS system and 59% with the parenchymal extract (Table IV). In both cases, radioactive $C_{12:0}$ and $C_{14:0}$ were synthesized, but with the parenchymal extract, $C_{18:0}$ made up 14%. When acetyl-ACP was employed as the primer, fatty acid synthesis increased about 3-fold with epidermal extract but decreased about 44% with the parenchyma (Table III). Analysis of fatty acid methyl esters (Table IV) showed that the products of fatty acid synthesis using the epidermal extract was practically unchanged whereas with the parenchyma $C_{18:0}$ was now formed equal to that of palmitic acid. These results indicated that an increase of the acetyl-ACP concentration could change the distribution of synthesized fatty acids only with parenchymal systems. This observation is consistent with the possible regulatory role of the acetyl-CoA:ACP transacylase (19). Moreover, the data suggest that β-ketoacyl-ACP synthetase II, responsible for the conversion of C_{16} fatty acids to C_{18}, is not functioning (or present) in the epidermal extracts but is in the parenchymal system.

Table III. *Cofactor Requirements for FAS Synthesis in Epidermal and Parenchymal Cells*

Conditions	Epidermis		Parenchyma	
	Amount	Activity	Amount	Activity
	nmol/mg·h	%	*nmol/mg·h*	%
Complete	0.087	100	1.87	100
−NADH	0.042	48.3	0.21	11.2
−NADPH	0.006	6.9	0.03	1.6
−ACP	0.006	6.9	0.02	1.1
−NADH −NADPH	0.007	8.0	0.01	0.5
−NADH + Cerulenin (50 μM)	0.001	1.1	0.03	1.6
−Acetyl-CoA + Acetyl-ACP (5.6 μM)	0.298	342.5	1.04	55.6

Table IV. *Fatty Acid Analysis of [^{14}C]Fatty Acids Formed from [^{14}C] Malonyl-CoA (+ACP) and the Indicated Primers*

The fatty acids were analyzed by radio GLC.

Acid (Products)	Distribution of Radiolabel in Presence of Different Primers			
	Epidermis		Parenchyma	
	Acetyl-CoA	Acetyl-ACP	Acetyl-CoA	Acetyl-ACP
	%			
$C_{12:0}$	4.9	ND[a]	7.8	1.9
$C_{14:0}$	13.1	14.3	17.8	3.8
$C_{16:0}$	82.0	85.7	59.7	46.8
$C_{18:0}$	ND	ND	14.7	47.4

[a] Not detected.

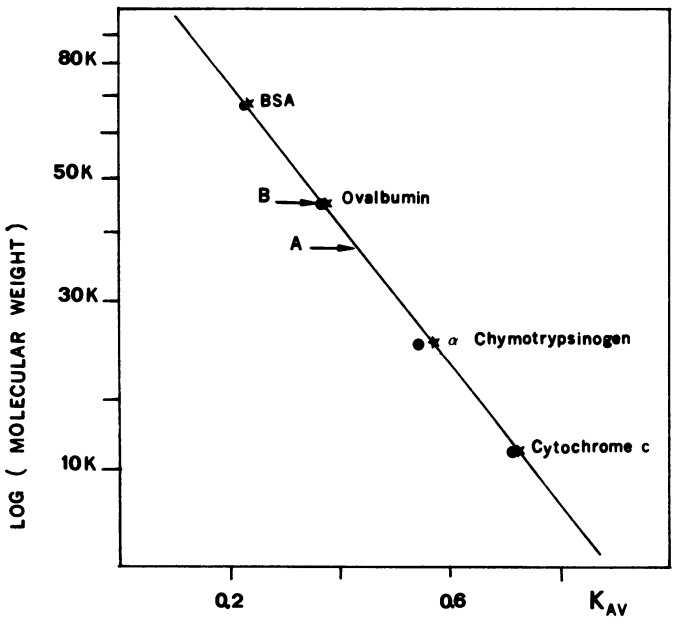

FIG. 1. Mol wt determination of malonyl-CoA:ACP transacylase. The elution volumes from Sephadex G-200 column were estimated as described in "Materials and Methods." (A) and (B) Elution volumes of epidermal and parenchymal enzymes, respectively. (●) and (★), Elution volumes of marker proteins before epidermal and parenchymal protein chromatography, respectively.

Comparison of Properties of Malonyl-CoA:ACP Transacylases from Parenchymal and Epidermal Tissues. The above results indicated several differences between epidermal and parenchymal FAS systems. Using malonyl-CoA:ACP transacylase as an enzyme marker for the FAS, several properties of this enzyme were studied to determine more precisely what, if any, differences occurred between epidermal and parenchymal FAS systems.

By Sephadex G-200 chromatography, the mol wt of the malonyl-CoA:ACP transacylase extracted from both tissues has been estimated. The elution volumes with the same column were 95 and 90 ml for the epidermal and parenchymal enzymes, respectively, which corresponded (Fig. 1) to mol wt of 38,000 and 45,000. These mol wt were of the same order as reported earlier for the spinach leaf (30,000) (17), barley leaves (41,000) (13), and somewhat higher than the safflower enzyme seed (22,000) (16). These results also indicated that, as with other plants (13, 16, 17), the FAS system in the epidermis and in the parenchyma was of a nonassociated procaryotic nature.

The activity of these partially purified enzymes was studied as a function of pH. Figure 2 shows that the optimum pH for the two different enzymes was about the same, 7.8 and 8.0. Nevertheless, when the pH was increased from 6.9 to 7.8, only a 1.7-fold stimulation occurred with the malonyl-CoA:ACP transacylase from parenchyma, whereas the activity of the epidermal source enzyme was 3-fold higher.

The influence of malonyl-CoA and ACP concentrations were also studied. Lineweaver-Burk plots gave K_m values of 4.2 and 5.6 μM for malonyl-CoA for parenchymal and epidermal enzymes, respectively (Fig. 3), the the maximal velocities were 0.48 and 0.40 μmol/min·mg, respectively.

Figure 4 shows the double reciprocal plots for varying concentrations of ACP. The K_m for ACP calculated was 13.7 and 21.7 μM for epidermal and parenchymal enzymes, respectively. The maximal velocities were in this case 0.53 and 0.47 μmol/min·mg. Table V summarized the properties of the partially purified malonyl-CoA:ACP transacylase from epidermis and paren-

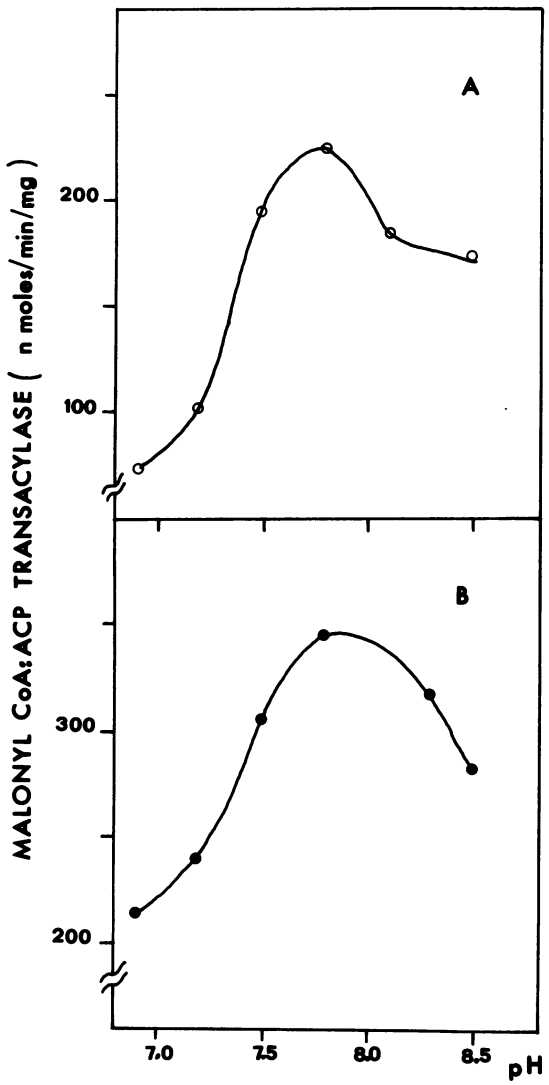

FIG. 2. pH Dependence of the purified malonyl-CoA:ACP transacylase. The activity of each enzyme was measured in presence of Tris-buffers 0.1 M at the indicated pH, in the conditions described in "Materials and Methods." A and B, Results obtained using enzyme from epidermis and parenchyma, respectively.

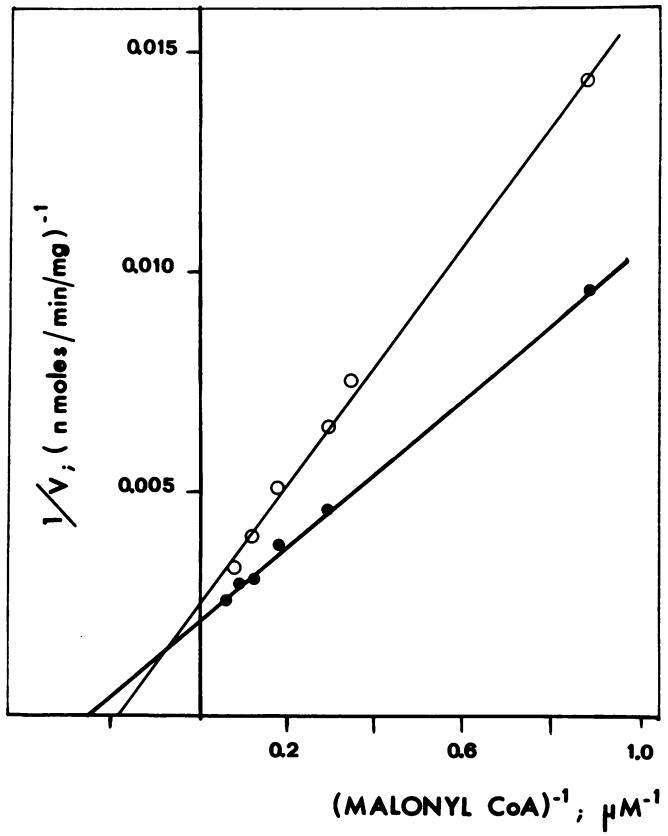

FIG. 3. Influence of malonyl-CoA concentration on malonyl-CoA:ACP transacylase activities. Malonyl-CoA:ACP transacylase activity was measured at different concentrations of malonyl-CoA. The results were expressed by Lineweaver-Burk plots. (O) and (●), Results obtained using enzyme from epidermis and parenchyma, respectively.

chyma.

These results suggested that the malonyl-CoA:ACP transacylase from either the epidermal and the parenchymal cells were quite similar in several characteristics and that the activity was associated with proteins ranging from 38,000 to 45,000 which is very similar to proteins found in a number of other plant systems containing nonassociated FAS systems (13, 16, 17).

DISCUSSION

Both in parenchymal cells and in epidermal cells of *Allium porrum*, a *de novo* FAS system occurs requiring ACP, NADPH, and also NADH (Table III). Both FAS systems are inhibited by a 50 μM cerulenin concentration. As shown in Table I, all enzymes (acetyl-CoA:ACP transacylase, malonyl-CoA:ACP transacylase, β-ketoacyl-ACP synthetase I, β-ketoacyl-ACP reductase, β-ketoacyl-ACP dehydrase, and enoyl-ACP reductase I) are present in the two FAS systems. The estimation of mol wt of malonyl-CoA:ACP transacylase is in good agreement with the procaryotic nature of FAS found previously for different systems (13, 16, 17). These proteins have a mol wt of about 40,000 which is inconsistent with the polyfunctional polypeptide dimeric organization found for the yeast FAS having mol wt for each polypeptide of around 200,000 (22) or for the homodimeric organization reported for mammalian systems (21). Each of the FAS component enzymes is equally distributed in both cell types expressed as units per g of fresh tissue. Table II shows a great similarity between the two tissues. Nevertheless, in the total leaf, the major activity for fatty acid synthesis occurs in the parenchymal tissue, *i.e.* about 90% of the fatty acids of the total leaf are synthesized by the green parenchymal tissue.

The principal fatty acid formed by the epidermal FAS system (either with acetyl-CoA or acetyl-ACP as the primer) is palmitic acid (Table IV). Under the same conditions, the parenchyma extract formed both palmitic and stearic acid which would suggest the presence of an active β-ketoacyl-ACP synthetase II in the parenchymal cells. The data were supported by the fact that [1-^{14}C]palmitoyl-ACP could be elongated in presence of NADPH and malonyl-CoA using parenchymal cell-free extract; even under these conditions, no stearic acid formation was observed with epidermis extract.

In contrast, the epidermal cell appears to form only palmitic acid from acetyl-CoA (ACP) and malonyl-CoA (ACP). The data support the presence of two independent FAS systems, one localized in parenchymal tissue and another in epidermal cell layers. The physical and enzymic characteristics of malonyl-CoA:ACP transacylase for the two cells (Table V) indicate that a slight difference in mol wt occurs. While maximal velocities are about the same, the K_m values for the substrates, particularly for ACP, are quite different. These results seem to indicate that these two enzymes are isozymic and that the FAS system in parenchy-

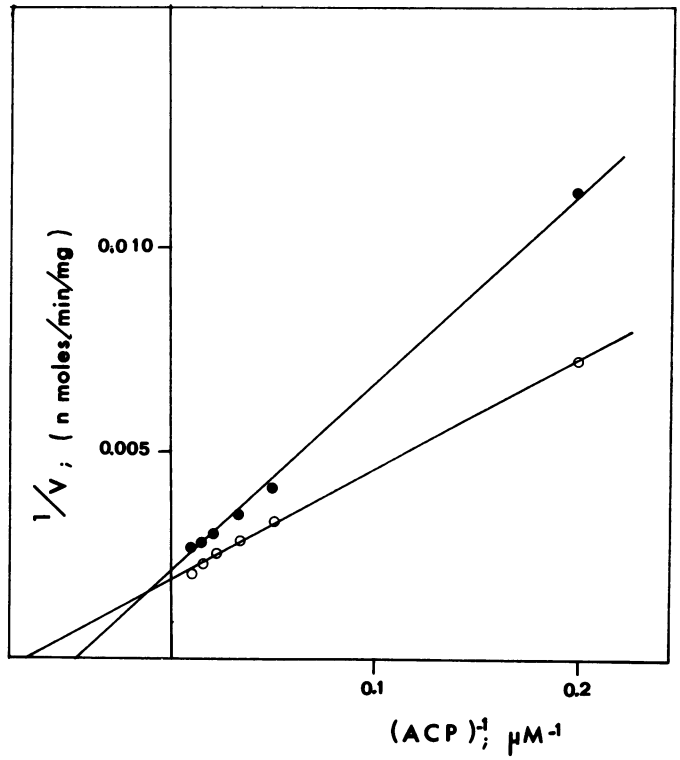

FIG. 4. Influence of ACP concentration on malonyl-CoA:ACP transacylase activities. Malonyl-CoA:ACP transacylase activity was measured at different concentration of ACP. The results were expressed by Lineweaver-Burk plots. (○) and (●), Results obtained using enzyme from epidermis and parenchyma, respectively.

Table V. *Properties of Malonyl-CoA:ACP Transacylase*

	Epidermis	Parenchyma
Mol wt	38,000	45,000
Optimal pH	7.8	7.8–8.0
K_m Malonyl-CoA	5.6 μM	4.2 μM
K_m ACP	13.7 μM	21.7 μM
V_m	0.45 μmol/min·mg	0.47 μmol/min·mg

mal and epidermal cells are nonassociated. Nevertheless, the localization of the FAS system in epidermal cells is still unknown. It has been established that, in the leaf cell, ACP was localized only in chloroplasts (14) which were therefore the site of FAS (23). In leaves of leek, epidermal tissue is devoid of chloroplasts and contains only one or two leucoplastids per cell (11). Because of the ultrastructure of the epidermal cell which has a very thick cell wall, intact leucoplastids can not be prepared to determine the localization of the FAS. However, the microsomes of epidermal cells are the site for the formation of very long chain fatty acids and are able to form stearic acid from malonyl-CoA.

Although previous results (6, 7, 11) indicate that stearoyl-CoA is the primer for elongation by the microsomal system in leek epidermal cells, the observations cited in this paper indicate that the ACP-dependent system is limited to the synthesis of palmitic acid. Recent evidence in this laboratory (Agrawal, Lessire, and Stumpf, unpublished data) indicates that the microsomal elongation system may have a rather broad primer acyl-CoA specificity. Thus, the *de novo* ACP-dependent system may supply a range of acyl moieties which channel into the microsomal system for rapid conversion to the very long chain fatty acids, the precursors for the hydrocarbons.

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