been fractionated by differential centrifugation into 2 enzymatic components. Palmitic acid is converted into a coenzyme A derivative by cytoplasmic particles in the presence of coenzyme A, adenosine triphosphate and magnesium ion. Palmityl-coenzyme A is then degraded to acetyl-coenzyme A by soluble proteins in the presence of coenzyme A and diphosphopyridine nucleotide.

Literature Cited

1. BARRON, E. J. AND P. K. STUMPF. 1962. Fat metabolism in higher plants. XIX. The biosynthesis of triglycerides by avocado-mesocarp enzymes. Biochim. Biophys. Acta 60: 329–37.
2. BUCHANAN, M. A. 1959. Paper chromatography of the saturated fatty acids. Anal. Chem. 31: 1616–18.
3. CHERRY, J. H. 1963. Nucleic acid, mitochondria, and enzyme changes in cotyledons of peanut seeds during germination. Plant Physiol. 38: 440–46.
4. DALTON, A. J. 1955. A chrome-osmium fixative for electron microscopy. Anat. Record 121: 281.
5. GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVIS. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751–66.
6. KORNBERG, A. AND W. E. PRICER, JR. 1953. Enzymatic synthesis of the coenzyme A derivatives of long chain fatty acids. J. Biol. Chem. 204: 329–43.
7. MARCUS, A. AND J. VELASCO. 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. J. Biol. Chem. 235: 563–67.
8. MANGOLD, H. K., B. G. LAMP, AND H. SCHLENK. 1955. Indicators for paper chromatography of lipids. J. Am. Chem. Soc. 77: 6070–72.
9. REBEIZ, C. A. AND P. CASTELFRANCO. 1964. An extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of fatty acids. Plant Physiol. 39: 932–38.
10. STUMPF, P. K. Private communication.

Activation and Oxidation of Acetic Acid-1-C^{14} by Cell Free Homogenates of Germinating Peanut Cotyledons [1, 2]

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It is commonly accepted that the generation of acetyl-CoA from fatty acids in animals and higher plants is a mitochondrial function. Stumpf and Barber (15) reported that mitochondrial pellets prepared from germinating peanut cotyledons are capable of degrading long chain and short chain fatty acids to acetyl-CoA, which is subsequently oxidized by the citric acid cycle. Recently an extra-mitochondrial β-oxidation system capable of degrading long chain and short chain fatty acids to acetyl-CoA was prepared from 4.5 day old germinating peanut cotyledons (12). Fractionation of this system indicated that the long chain fatty acid activating enzyme is particulate, while the conversion of the activated fatty acids to acetyl-CoA is catalyzed by soluble enzymes (13). It has been generally accepted that exogenous acetate has the same metabolic fate as the acetyl-CoA generated from long chain fatty acids by β-oxidation. This assumption is made plausible by the ubiquity of acetyl-thiokinase in plant tissues (10), and has warranted the use of labeled acetate as substrate in investigations dealing with the conversion of storage lipids to sugar during seed germination (1, 11). Because of this intimate connection between the β-oxidation and the acetyl-thiokinase pathways—providing alternate routes to the same high-energy intermediate—we felt it was worthwhile to investigate the acetyl-thiokinase activity of germinating peanut cotyledons, particularly with respect to its distribution among the various subcellular constituents.

Materials and Methods

The commercial sources of labeled and unlabeled chemicals have been reported in earlier communications (12, 13). Acetic acid-1-C^{14}, 2.5 μc/μmole and 50.6 μc/μmole were purchased from the New England Nuclear Corporation. The preparation and assay of salt-free NH_2OH, the synthesis of acetyl-1-C^{14}-CoA, acetyl-P, acetyl-GSH, acetyl-hydroxamic acid, the assay for the enzymatic production of activated acetate and of acetyl-hydroxamic acid were described previously (12). Radioactive CO_2 evolved in con-

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ventional Warburg vessels was converted to $BaC^{14}O_3$ at the end of the reaction (14) and counted with a thin window counter.

The particulate fractions (a mitochondrial fraction, P_1; an intermediate fraction, P_2; a microsomal fraction, P_3) and the 2 supernatant fractions (S_1 and S_3) were prepared exactly as described previously (13). Protein contents of the different fractions were determined by the biuret method (13).

Results

Radioactive Products of Acetic Acid-1-C^{14} Activation. The $S_{10,000\times g}$ fraction was treated with 4 volumes of saturated neutralized ammonium sulfate as described in detail in an earlier report (12). The $S_{10,000\times g}$ 0 to 80 % ammonium sulfate fraction, thus prepared, was incubated with acetic acid-1-C^{14} in the presence of CoA, ATP and Mg. The reaction mixture was processed and chromatographed descendingly in *n*-butyl alcohol-acetic acid-H_2O (8:3:3) for 22 hours at 4° (12). Three radioactive areas could be detected, with the following R_F values: 0.11, 0.26, and 0.38. These areas were identical with those described in an earlier communication, dealing with the production of acetyl-CoA from long chain fatty acids (12), and identified as acetyl-CoA, acetyl-P, and acetyl-GSH (table I). Upon addition of NH_2OH at the end of the incubation period, the 3 radioactive areas were converted into a single new area (table I). This area moved with acetyl-hydroxamic acid in phenol-H_2O (8:2), *n*-butyl alcohol-acetic acid-H_2O (8:3:3) and isopropyl alcohol-NH_4OH-H_2O (80:15:15). The synthesis of acetyl-1-C^{14}-hydroxamic acid from acetic acid-1-C^{14} showed an absolute requirement for CoA, ATP and Mg (table II). Omission of the potassium phosphate buffer from the reaction mixture yielded a consistently lower percent conversion. This effect might be attributed to the partial omission of the K ion which has been reported to be required for acetyl-thiokinase (7).

Table I. *Distribution of Radioactivity from Acetic Acid-1-C^{14} into Various Radioactive Products*

The reaction mixture contained 100 μmoles of potassium phosphate buffer pH 7.5, 200 μmoles of Tris-HCl buffer, pH 7.5, 0.1 μmole of acetic acid-1-C^{14} (50.6 μc/μmole), 1 μmole ATP, 0.6 μmole CoA, 10 μmoles GSH, 5 μmoles $MgCl_2$, 0.5 ml of the $S_{10,000\times g}$-0-80 % ammonium sulfate preparation in 1.7 ml volume. Time of incubation, 40 minutes, temperature, 37°.

Radioactive products	Without added NH_2OH	With added NH_2OH
	% of total C^{14} in substrate	% of total C^{14} in substrate
Acetyl-C^{14}-CoA	9.6	0
Acetyl-C^{14}-P	0.2	0
Acetyl-C^{14}-GSH	1.1	0
Acetyl-C^{14}-hydroxamic acid	0.0	11.6
Total	10.9	11.6

Table II. *Cofactor Requirements for the Activation of Acetic Acid-1-C^{14}*

The complete reaction mixture and other conditions were the same as in table I, except that acetic acid-1-C^{14} of lower specific activity (2.5 μc/μmole) was used: when the potassium phosphate buffer was omitted it was replaced by an equal amount of the Tris-HCl buffer, and when GSH was omitted, CoA was reduced with H_2S.

Components	% Conversion of C^{14} from acetic acid-1-C^{14} into acetyl-hydroxamic acid
Complete	11.70
Without potassium phosphate buffer	6.20
Without CoA	0.50
Without GSH	11.00
Without ATP	0.80
Without Mg	1.30

Acetyl-Thiokinase Activity of the Subcellular Components. The acetyl-thiokinase activity in the $S_{10,000\times g}$ 0 to 80 % ammonium sulfate fraction is proportional to protein concentration up to 0.6 to 0.7 mg of protein (fig 1).

In order to investigate the distribution of acetyl-thiokinase activity, equivalent volumes of P_1, P_2, P_3 and S_1, were assayed; in this way the results obtained with different particulate and soluble fractions are comparable on a fresh weight basis. For example the amount of each fraction used in table III corresponds to 25 mg of cotyledonary tissue. As shown in table III, all the acetyl-thiokinase activity of the $S_{10,000\times g}$ was recovered in the high speed soluble fraction, S_1. The activity of the particulate fractions was prac-

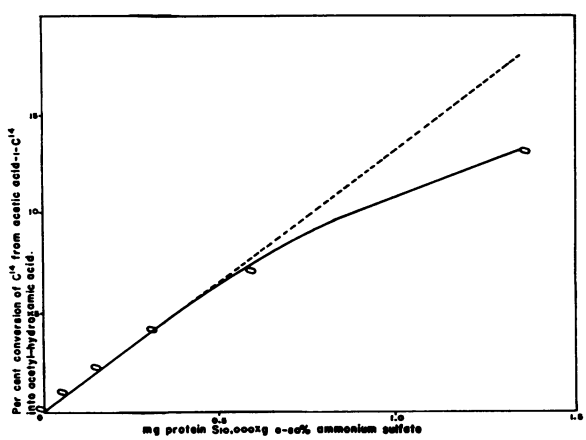

FIG. 1. Acetyl-thiokinase activity in the presence of different concentrations of the $S_{10,000\times g}$ 0 to 80 % ammonium sulfate fraction. The concentrations and other incubation conditions were as in table I.

Table III. *Acetyl-Thiokinase Activity of the Different Subcellular Fractions*

The complete reaction mixture and other conditions were the same as in table I. Equivalent volumes of the particulate and S_1 fractions were assayed corresponding to 25 mg of fresh cotyledonary tissue.

Fraction	mg Protein	% Conversion of C^{14} from acetic acid-1-C^{14} to acetyl-hydroxamic acid
$S_{10,000\times g}$	0.7	5.9
S_1	0.4	7.5
P_1	0.04	0.2
P_2	0.03	0.1
P_3	0.02	0.1
$P_2 + P_3$		0.2
$S_1 + P_1$		6.1
$S_1 + P_2$		6.3
$S_1 + P_3$		6.0
$S_1 + P_2 + P_3$		7.6

tically nil. Furthermore no synergistic effect could be detected upon recombing S_1 with any of the particulate fractions (table III).

Oxidation of Palmitic Acid-11-C^{14}, Acetic Acid-1-C^{14} and Acetyl-1-C^{14}-CoA. The mitochondrial pellet, P_1, obtained from 43 g of cotyledon tissue was suspended in a small volume of Tris-sucrose buffer and adjusted to contain 100 mg protein per ml (3.2 ml total volume). Half an ml of the P_1 suspension, containing 50 mg of protein, and 0.5 ml of the $S_{10,000\times g}$ fraction, corresponding to 250 mg of cotyledonary tissue, were assayed individually and in combination for $C^{14}O_2$ production. Release of $C^{14}O_2$ from both acetic acid-1-C^{14} and palmitic acid-11-C^{14} required the presence of both the mitochondrial pellet, P_1 and the extra-mitochondrial fraction $S_{10,000\times g}$ (table IV).

Table IV. *Oxidation of Acetic Acid-1-C^{14} and Palmitic Acid-11-C^{14} by the Mitochondrial Pellet, P_1, in the Absence and Presence of the $S_{10,000\times g}$ Fraction*

The reaction mixture contained in a total volume of 2 ml: 100 μmoles potassium phosphate buffer pH 7.5, 200 μmoles Tris-HCl buffer pH 7.5, 0.1 μmole palmitic acid-11-C^{14} (15,000 cpm) or 0.1 μmole acetic acid-1-C^{14} (27,500 cpm), 1 μmole α-ketoglutaric acid, 0.01 μmole cytochrome c, 1 μmole $MnSO_4$, 5 μmoles $MgCl_2$, 10 μmoles GSH, 0.15 μmole DPN, 0.13 μmole TPN, 0.2 μmole TPP, 0.6 μmole CoA, 1 μmole ATP, 400 μmoles sucrose, 13 μmoles KCl, 0.5 ml of the P_1 suspension and/or 0.5 ml of $S_{10,000\times g}$. Time of incubation 1 hour. Temperature 30°.

Fraction	% C^{14} from palmitic acid-11-C^{14} in $BaC^{14}O_3$	% C^{14} from acetic acid-1-C^{14} in $BaC^{14}O_3$
P_1	2.2	2.0
$S_{10,000\times g}$	1.0	0.7
$P_1 + S_{10,000\times g}$	9.6	8.5

Table V. *Oxidation of Acetyl-1-C^{14}-CoA by the Mitochondrial Pellet, P_1, in the Presence and Absence of the Soluble Protein*

The reaction mixture and other incubation conditions were the same as in table IV, except that 50,000 cpm of acetyl-1-C^{14}-CoA were used.

Fraction	% C^{14} from acetyl-1-C^{14}-CoA in $BaC^{14}O_3$
P_1	2.1
$S_{10,000\times g}$	0.1
S_1	0.2
Boiled $S_{10,000\times g}$*	0.0
$P_1 + S_{10,000\times g}$	6.5
$P_1 + S_1$	8.6
P_1 + boiled $S_{10,000\times g}$	0.5

* Five minutes at 100°.

This observation could be easily accounted for if the $S_{10,000\times g}$ were required to generate acetyl-1-C^{14}-CoA from both substrates and if the oxidation of this activated intermediate took place in the mitochondria. However the oxidation of synthetic acetyl-1-C^{14}-CoA by P_1 is also stimulated by the $S_{10,000\times g}$ fraction (table V). This stimulation is lost upon boiling (5 min, 100°; the boiled enzyme is actually inhibitory) but is retained when the dialyzed high speed supernatant S_1 is substituted for the $S_{10,000\times g}$ (table V). This stimulation is therefore probably due not to an additional cofactor but to a soluble protein. The addition of glyoxylic acid doubles the oxidation of acetyl-CoA by this recombined particulate-soluble system (table VI). The cofactors used for tables IV, V and VII are the cofactors described by Stumpf and Barber (15) for coupled β-oxidation and tricarboxylic acid cycle activities. Research is underway to determine the minimum cofactor requirement for the oxidation of acetyl-CoA to CO_2 in the presence and absence of glyoxylate.

Discussion

Millerd and Bonner (10) made a survey of acetyl-

Table VI. *Oxidation of Acetyl-1-C^{14}-CoA in the Absence and Presence of Added Glyoxylic Acid*

The reaction mixture and other incubation conditions were the same as in table IV except that 50,000 cpm of acetyl-1-C^{14}-CoA were added. Five μmoles of glyoxylic acid were added where indicated.

Fraction	Without added glyoxylic acid	With added glyoxylic acid
	% C^{14} from acetyl-1-C^{14}-CoA in $BaC^{14}O_3$	% C^{14} from acetyl-1-C^{14}-CoA in $BaC^{14}O_3$
P_1	1.7	6.1
S_1	0.9	1.9
$P_1 + S_1$	8.6	17.5

thiokinase activity in acetone powders of various plant tissues, and concluded that it is widely distributed among higher plants. Hiatt and Evans (6) and Hiatt (7) studied in greater detail the metal cofactor requirement for the spinach-leaf enzyme. Hatch and Stumpf (5) reported a malonyl-thiokinase in germinating peanut cotyledons and implied the presence of an acetyl-thiokinase. To our knowledge, our report is the first detailed study on acetyl-thiokinase from germinating cotyledons of a fat-storing seed. The method of preparation used by us is unlikely to solubilize a particulate enzyme, hence we have presumptive evidence that this enzyme is soluble also in vivo. This is in contrast with the palmityl-thiokinase which occurs in various particulate fractions and especially in the microsomes (13). It appears therefore that in peanut cotyledons readily soluble acetic acid is activated by a soluble enzyme, and sparingly soluble palmitic acid is activated by a particulate enzyme.

The nature of the stimulation of $C^{14}O_2$ evolution by the soluble proteins is not fully understood at this time. Obviously this $C^{14}O_2$ evolution is of the same order of magnitude whether acetyl-1-C^{14}-CoA is generated from long chain fatty acids by β-oxidation or generated from acetic acid by acetyl-thiokinase or simply added to this system. The particulate component of this system has the same sedimentation rate as plant mitochondria. However electron microscopy performed on the tissue from which it was prepared failed to show the presence of organelles having a typical mitochondrial structure (13). The $C^{14}O_2$ evolution by this system doubles upon addition of glyoxylic acid (table VI). Since we know that the glyoxylate cycle is operating in peanut cotyledons (2, 4, 9) we might consider the possibility that this pathway is functioning in the production of $C^{14}O_2$ (possibly at the oxalacetate to phosphoenolpyruvate step). Indeed Canvin and Beevers (3) have reported that while labeled acetate is converted to sugar and CO_2 in castor bean endosperm, only a very small amount of the label is found in glutamate. These workers therefore suggested that the enzymes isocitric dehydrogenase and α-ketoglutaric dehydrogenase, although present, may contribute relatively little to the dissimilation of isocitrate in castor bean (3). Oaks and Beevers (11) stress that, contrary to the condition in fatty seeds (castor bean endosperm) both the tricarboxylic acid and the glyoxylic acid cycles contribute to acetate utilization in cereals (corn scutella), as they do in many microorganisms.

In the light of these facts we would like to suggest that in young germinating peanut cotyledons (4–5 days, 23°) where no mitochondria are as yet noticeable, acetyl-CoA, produced by extra-mitochondrial β-oxidation (12, 13) and extra-mitochondrial acetyl-thiokinase, is oxidized by a sequence of reactions which is either the by now classical glyoxlate cycle (3, 8) or some reasonable modification thereof. Furthermore we suggest that the enzymes necessary to catalyze this whole sequence are distributed between the particulate and the soluble cell components.

Summary

A soluble enzyme preparation having acetyl-thiokinase activity is described in homogenates of germinating peanut cotyledons. This enzyme is capable of activating acetic acid to acetyl coenzyme A in the presence of coenzyme A, adenosine triphosphate and magnesium ion.

The oxidation of palmitic acid-11-C^{14} and acetic acid-1-C^{14} to $C^{14}O_2$ requires the presence of both a particulate fraction sedimenting like mitochondria, and a soluble protein fraction. Likewise synthetic acetyl coenzyme A requires both soluble and particulate components for oxidation of CO_2. The extent of this oxidation is increased markedly by addition of glyoxylate.

Literature Cited

1. Beevers, H. 1961. Metabolic production of sucrose from fat. Nature 191: 433–36.
2. Bradbeer, C. and P. K. Stumpf. 1959. Fat metabolism in higher plants. XI. The conversion of fat into carbohydrate in peanut and sunflower seedlings. J. Biol. Chem. 234: 498–501.
3. Canvin, D. T. and H. Beevers. 1961. Sucrose synthesis from acetate in the germinating castor bean: kinetics and pathway. J. Biol. Chem. 236: 988–94.
4. Cherry, J. H. 1963. Nucleic acid, mitochondria, and enzyme changes in cotyledons of peanut seeds during germination. Plant Physiol. 38: 440–46.
5. Hatch, M. D. and P. K. Stumpf. 1962. Fat metabolism in higher plants. XVII. Metabolism of malonic acid and its α-substituted derivatives in plants. Plant Physiol. 37: 121–26.
6. Hiatt, A. J. and H. J. Evans. 1960. Influence of certain cations on activity of acetic thiokinase from spinach leaves. Plant Physiol. 35: 673–77.
7. Hiatt, A. J. 1964. Further studies on the activation of acetic thiokinase by magnesium and univalent cations. Plant Physiol. 39: 475–79.
8. Kornberg, H. L. and H. A. Krebs. 1957. Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. Nature 179: 988–91.
9. Marcus, A. and J. Velasco. 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. J. Biol. Chem. 235: 563–67.
10. Millerd, A. and J. Bonner. 1954. Acetate activation and acetoacetate formation in plant systems. Arch. Biochem. Biophys. 49: 343–55.
11. Oaks, A. and H. Beevers. 1964. The glyoxylate cycle in maize scutellum. Plant Physiol. 39: 431–34.
12. Rebeiz, C. A. and P. Castelfranco. 1964. An extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of fatty acids. Plant Physiol. 39: 932–38.
13. Rebeiz, C. A., P. Castelfranco, and A. H. Engelbrecht. 1965. Fractionation and properties of an extra-mitochondrial enzyme system from peanuts catalyzing the β-oxidation of palmitic acid. Plant Physiol. 40: 281–86.
14. Stumpf, P. K. 1955. Fat metabolism in higher plants. III. Enzymic oxidation of glycerol. Plant Physiol. 30: 55–58.
15. Stumpf, P. K. and G. A. Barber. 1956. Fat metabolism in higher plants. VII. β-oxidation of fatty acids by peanut mitochondria. Plant Physiol. 31: 304–08.