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MALATE SYNTHETASE IN HIGHER PLANTS.[1]

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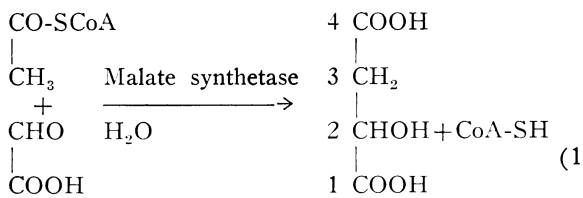
Malate synthetase brings about the condensation of glyoxylate and acetyl CoA to form malate, according to reaction 1.



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The enzyme was first discovered in microorganisms by Wong and Ajl (14) and is one of the key enzymes of the glyoxylate cycle described by Kornberg and Krebs (6). In earlier work we have shown that the enzymes of the glyoxylate cycle are present in the endosperm of the germinating castor bean (Kornberg and Beevers 8) and one of these, isocitritase, has been examined in some detail (Carpenter and Beevers 5). In this report a comparable investigation has been carried out on malate synthetase. Its distribution in a variety of plant tissues has been determined and some of the properties of the castor bean enzyme have been investigated. The results reinforce the earlier suggestions that malate synthetase and isocitritase, as components of the glyoxylate cycle are an essential part of the machinery by which fats are converted to carbohydrate.

[1] Received May 11, 1959.

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MATERIALS AND METHODS

PLANT MATERIALS Germination of the castor beans and the preparation of crude extracts (castor bean preparation) were carried out by methods described previously (8). Other seeds were germinated in a similar fashion on vermiculite and grown in the dark at 30° C for the stated periods. Mature parts were cut from plants growing in the greenhouse.

Lactobacillus arabinosus, adapted to malate, was grown by the method of Nossal (9) as modified by Stiller (11). Lyophilized cells were stored at −15° C.

Special chemicals Sodium glyoxylate was obtained from Mann Biochemicals. Acetate-1-C^{14} and acetic-1-C^{14} anhydride at a rated specific activity of 1 millicurie per millimole were supplied by Nuclear of Chicago.

Acetyl-1-C^{14}-CoA was prepared from the anhydride by the method of Simon and Shemin (10). All measurements of radioactivity were made on $BaCO_3$ in a windowless gas flow counter and the results are corrected for background and self absorption. Combustions to determine the C^{14} content of substrates were carried out according to Stutz and Burris (13).

Aceto-CoA-kinase was prepared from yeast using Berg's (3) method to the stage of the first ammonium sulfate precipitation. It was stored in the frozen condition and maintained its activity over several months. The kinase was shown to have a low but detectable malate synthetase activity. In the results of those experiments in which it was used, these blank values (150–250 cpm) have been subtracted.

All enzyme incubations were carried out at 30° C.

Results

Assay of Malate Synthetase: In earlier work malate synthetase activity was detected by allowing the reaction to proceed in the presence of acetate-2-C^{14}, separating the products on a two-dimensional chromatogram and counting the C^{14} in malate. Isocitritase was present in the extracts, and isocitrate usually was used as the source of glyoxylate for the malate synthetase reaction (8).

This method suffers as an assay system for malate synthetase itself because, in addition to its slowness, the synthesis of malate depends on the ability of the extracts to convert acetate into acetyl-CoA, and on the activity of isocitritase. In the earlier work (8) it was shown that isocitritase activity was higher than that of the acetate activating system and of the malate synthetase in the crude extracts. In addition, it seemed that glyoxylate in substrate amounts was inferior to isocitritase + isocitrate as a source of glyoxylate.

In the present work, with different reagents, it was shown that glyoxylate was just as effective as isocitrate + isocitritase. The progress of acetate-1-C^{14} incorporation into malate is shown in figure 1. In all subsequent work, therefore, glyoxylate was added as the acceptor for acetyl CoA, and the synthesis of malate was thus independent of isocitritase activity.

In an attempt to make the assay independent of the acetate activating activity of the extracts, acetyl-1-C^{14}CoA was employed. As shown in table I, malate synthesis was achieved with this substrate, in the absence of ATP and CoA. However, the instability of the acetyl-CoA and the necessity for chromatographic separation immediately before use make it an undesirable substrate for assay of malate synthetase. The use of aceto-CoA-kinase from yeast to augment the native acetate activating enzyme is described in a later section.

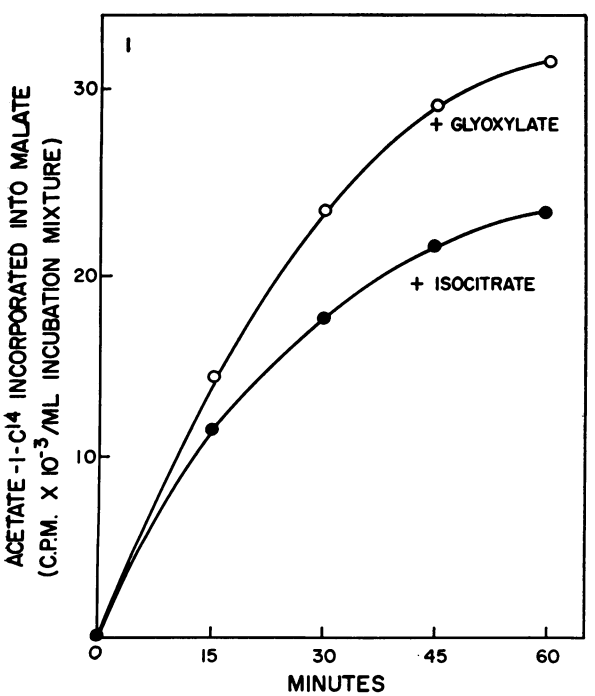


Fig. 1. Progress of acetate incorporation into malate. Standard assay conditions (table II) were used, except that an equimolar amount of D-isocitrate was substituted for glyoxylate in one of the digests.

Table I

Degradation of Malate Samples Produced from Acetyl-1-C^{14} CoA and Acetate-1-C^{14} by Castor Bean Preparations

Substrate		C^{14} (cpm/mg $BaCO_3$) in individual carbons			% of total in C-4
		COOH-CHOH-CH_2-COOH			
		1	2 and 3	4	
Acetyl-CoA	1)	2.1	1.0	38.1	93 %
	2)	1.3	0	12.0	90 %
Acetate	1)	0.9	1.5	35.8	94 %
	2)	5.6	0.4	151.0	96 %

The malate was isolated chromatographically from digests in which glyoxylate and cofactors were supplied to those containing acetate-1-C^{14}. No ATP or CoA was included in those digests in which acetyl-CoA was the substrate. One and two are separate experiments.

An important fact which was established by the acetyl-CoA experiment and the parallel experiments with acetate-1-C^{14} (table I) is that the C^{14} is almost exclusively confined to the C-4 COOH group of malate. This establishes that the condensation occurs as indicated in reaction 1 and further, that, once formed, the malate is stable; there is a negligibly small amount of randomization between the COOH groups of malate as might be expected if fumarase were active in the extracts. In addition, of course, the maintenance of this inequality of labelling in the COOH groups of malate precludes any possibility of its having arisen secondarily from succinate in a hypothetical direct back to back condensation of acetate.

The enzyme assay system which was developed makes use of this localization of C^{14} in the malate and the specific decarboxylation at C-4 which is brought about by malate adapted *Lactobacillus arabinosus*. Acetate-1-C^{14} was used as substrate and at the end of the incubation the malate production was estimated by first stopping the reaction by boiling and then adding the incubation mixture (or an appropriate aliquot) with 50 micromoles of unlabeled malate (pH 5.7) to a Warburg vessel. A suspension of Lactobacillus cells was added after filling with N_2. The CO_2 from C-4 was collected in KOH and the C^{14} content of the carbonate was determined. The total radioactivity in the carbonate was a measure of malate production from acetate; since the specific activity of the acetate-1-C^{14} was known, malate formation in micromoles can also be calculated. It was established that residual acetate was not decarboxylated by the Lactobacillus. Malate synthetase activity of Lacto-

TABLE II

CO-FACTOR REQUIREMENTS FOR MALATE SYNTHESIS

	ACETATE-1-C^{14} INCORPORATED INTO MALATE (CPM)		
	FRESH PREPARATION	AFTER 16 HR DIALYSIS AT 3° C.	
		A (STILL)	B (STIRRED)
Complete system[1]	31,700	8,750	6,140
— CoA	122	115	85
— ATP	371	127	...
— Mg^{++}	10,121	...	...
— Glyoxylate	10,452	1,740	379
— GSH	23,172	...	...

[1] The complete system (standard assay conditions) contained the following constituents in the stated amounts in micromoles. Potassium phosphate, pH 7.6, 60; glutathione, (GSH) 3.3; $MgCl_2$, 10; CoA, 1.3; $CH_3C^{14}OONa$ 2.5 (1.7×10^5 cpm); ATP, 2.7; glyoxylate, 3; castor bean preparation 0.25 ml in a final volume of 1.5 ml. Incubation time 30 min.

bacillus preparations was shown to be virtually zero. The sensitivity of the method depends on the initial specific activity of the acetate. In most of our experiments 2.5 μM acetate containing about 100,000 cpm C^{14} were added. Since malate decarboxylation is quantitative, the formation of more than 0.001 μM of malate can be readily detected.

MALATE SYNTHETASE FROM CASTOR BEANS: a) *Co-factors for incorporation of acetate into malate*: Previously (7, 8) the incubations were carried out in the presence of CoA, ATP, Mg^{++} and glutathione (GSH). The essentiality of these components is established by the results in table II. The effects of omitting CoA or ATP, components of the activating system, are particularly striking; in the absence of either, the incorporation was reduced to less than 1 % of the control rate. The omission of glyoxylate from the digests did not completely abolish malate synthesis by the fresh preparation, which presumably contained some endogenous source of glyoxylate (7). The essentiality of the glyoxylate was clearly established by the results with the dialyzed enzyme, in which malate formation was eventually reduced to about 6 % of the control rate. The decline in the activity of malate synthetase during dialysis was not appreciably greater than that which occurred when the enzyme was stored at the same temperature for a similar period.

The influence of CoA and ATP is shown in figure 2. The curves relating malate synthesis to co-factor concentration show a linear phase at concentrations lower than 1 mg/ml; at levels greater than this, saturation of the enzyme is approached. In the subsequent experiments 1.5 mg. ATP (2.7 micromoles) and 1 mg CoA (1.3 micromoles) were added to each reaction mixture.

b) *Effect of enzyme concentration*: The lower curve in figure 3 shows that malate synthesis is proportional to the amount of enzyme added over a wide range of concentrations. The amount of enzyme usually added in the experiments was 0.25 ml (about 2.5 mg protein).

c) *Effect of exogenous aceto-CoA-kinase*: The capacity of the system to produce malate from acetate can be strikingly increased by addition of the acetate activating enzyme from yeast (figure 3). In the presence of a fixed amount (0.2 ml) of aceto-CoA-kinase (determined to be an adequate amount in preliminary experiments) higher rates of malate formation were obtained; the rate of the reaction was again proportional to the amount of castor bean preparation which was added. These results confirm that the native acetate activating system is less vigorous than the malate synthetase; the maximum ability to produce malate was not measured in the previous assay system, as suspected earlier (8).

d) *Effect of pH*: When the malate synthesis reaction was carried out at different pH levels the data in table III were obtained. A broad optimum in the region of pH 6.8 to 8.0 is apparent. Effects on the acetate activating enzyme and the malate synthetase are compounded in this curve for the over all reaction. The standard enzyme assay was carried out at pH 7.6.

e) *Effect of age of seedlings*: Rapid fat breakdown begins only after 3 to 4 days of germination; several of the enzymes of the endosperm (1) as well as isocitritase (5) show very striking increases in activity at this time. A similar, though smaller rise is evident in the malate synthetase activity, measured in the absence of exogenous aceto-CoA-kinase (lower curve of figure 4). In the presence of the acetate activating enzyme a more striking rise is evident between days 1 and 3; the activity per seedling remains at this high level, in contrast to the system in which

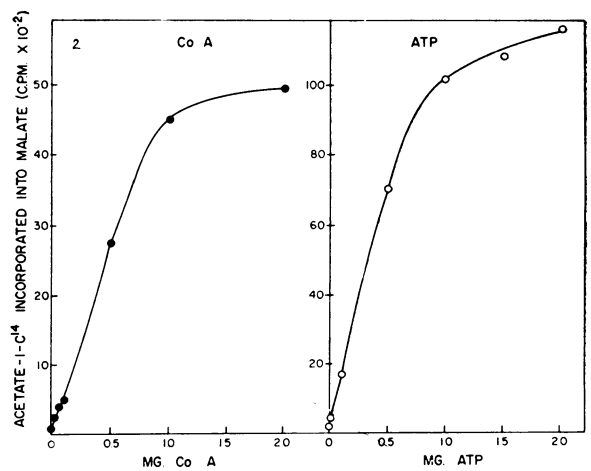
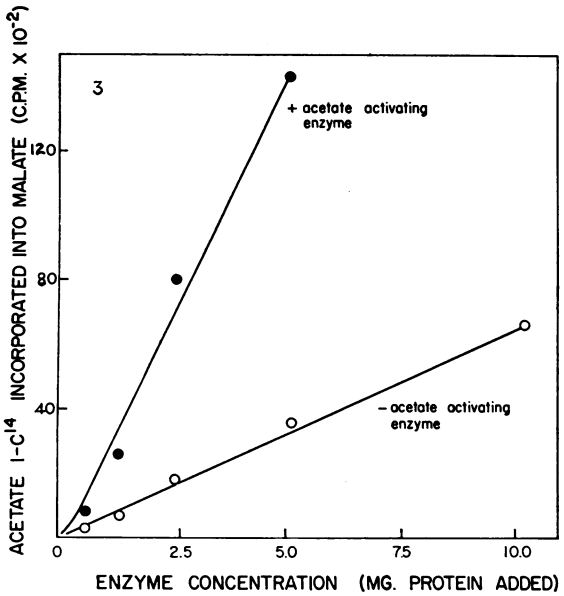


FIG. 2. CoA and ATP requirements for the production of malate from acetate and glyoxylate. In the experiment in which CoA concentration was varied, 1.5 mg ATP was added to all digests. In the (separate) ATP experiment, 1 mg CoA was added to all digests. Other conditions as in table II. Acetate: Sp.Act.: 5×10^4 cpm/μM.



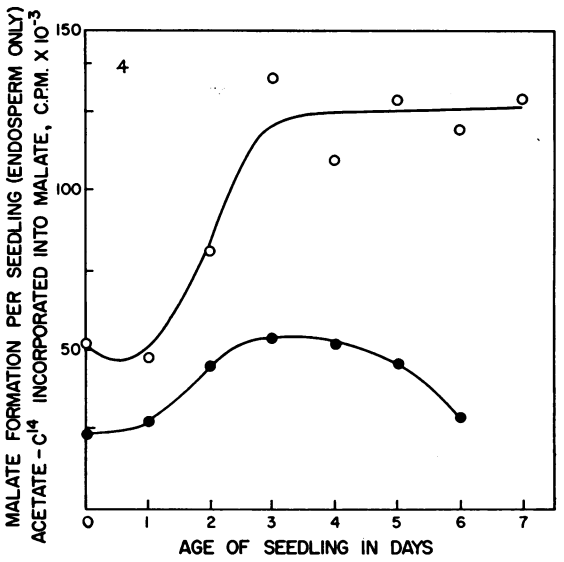


FIG. 3 (*left*). Effect of enzyme concentration on malate production. The lower curve was obtained under the conditions shown in table II, except that the amount of enzyme was varied as indicated. The upper curve was obtained in a parallel series of digests to which 0.2 ml aceto-CoA-kinase were also added. Acetate: Sp.Act.: 2.7×10^4 cpm/μM.

FIG. 4 (*right*). Variation in activity of malate synthetase during germination of castor beans. Extracts were made at the times indicated; activity was measured under the standard assay conditions (lower curve). The upper curve was obtained when 0.2 ml aceto-CoA-kinase were also present. Acetate: Sp.Act.: 5×10^4 cpm/μM.

only native aceto-CoA-kinase is present. This latter fall can thus be ascribed to a progressive failure in the native acetate activating system, rather than to a decline in malate synthetase. It is important to note that malate synthetase can be extracted from ungerminated seeds, and that this can be done in the early stages of growth. Isocitritase was not detectable at these times (5).

f) *Intracellular localization of malate synthetase.* Enzyme extracts were usually prepared in dilute phosphate and centrifuged at 10,000 × G for 15 minutes. However, if the blending was carried out in the medium employed earlier for preparing mitochondria and microsomes (1) the final supernatant had very feeble activity. (table IV). On the other hand, the particulate fractions were highly active in malate synthesis when aceto-CoA-kinase was provided. The striking effect of the exogenous acetate activating enzyme indicates that there had been a separation of endogenous activating enzyme and malate synthetase during the centrifuging (table IV). Although the microsomes have a high specific activity of malate synthetase, the total amount of protein in these fractions is very small, (1) and a much higher percentage (about 60 %) of the total activity in the original homogenate was recovered with the mitochondrial fraction (which had been washed by resuspension in sucrose phosphate). The enzyme so recovered can be readily displaced or eluted from the particles by simply transferring them to 0.01 M potassium phosphate, pH 7.6.

MALATE SYNTHETASE IN OTHER PLANT TISSUES: A variety of plant materials, listed in table V was tested for malate synthetase activity in the standard assay system (table II). The materials are listed in decreasing order of effectiveness in inducing malate synthesis in the presence of exogenous aceto-CoA-kinase. The ability to produce malate without benefit of this addition is shown in the first column of figures. Clearly, the enzyme is widespread, but there are very large differences in the malate synthetase activity in the tissues examined. It is noteworthy that those which gave extracts with high specific activity are

TABLE III

EFFECT OF pH ON MALATE SYNTHESIS

pH	5.6	6.0	6.4	6.8	7.2	7.6	8.0
Malate formation (cpm acetate-C^{14} incorporated)	111	964	2,330	3,740	2,990	3,290	3,280

Standard assay conditions (table II) were used, except that the potassium phosphate component was adjusted to the pH levels indicated. Acetate: Sp.Act.: 5×10^4 cpm/micromole.

TABLE IV

MALATE SYNTHETASE ACTIVITY OF CELL FRACTIONS OF CASTOR BEAN ENDOSPERM

FRACTION	MALATE SYNTHETASE ACTIVITY (CPM ACETATE-C^{14} INCORPORATED /MG PROTEIN)	
	−ACETO-CoA-KINASE	+ACETO-CoA-KINASE (0.2 ML)
Washed mitochondria	393	3,450
Microsomes	169	7,175
Supernatant	172	290

Standard assay conditions were used (table II).

Mitochondria were obtained by centrifuging for 30 min. at 10,000 × G after an initial 10 min. spin at 800 × G. The microsomal fraction was that sedimenting in 60 min. at 54,000 × G after removal of the mitochondria. Acetate: Sp. Act.: 2.7×10^4 cpm/micromole.

largely ones which have a high fat content and in which, furthermore, conversion to carbohydrate occurs. In some of the closely neighboring tissues, such as the castor bean embryo, malate synthetase activity is very low, and it is uniformly low in mature leaves.

However, several other non-fatty tissues show clearly detectable activity, e.g., corn embryo and the pea cotyledon. It is of interest too that activity was still quite strong in the watermelon cotyledon even after eight days of growth in the light, but after a longer period it had virtually disappeared from the sunflower cotyledon. The enzyme is present in the castor bean endosperm when it is maturing on the parent plant.

It can be concluded from the different degrees of stimulation induced by the exogenous aceto-CoA-kinase that the relative amounts of endogenous activating enzyme and malate synthetase were not the same in each tissue. The most striking stimulation (70-fold) was that in sunflower seed which had just begun to germinate, but sizeable increases were observed in most of the other tissues.

It should be emphasized that tissues other than the castor bean endosperm were not investigated intensively to find the age for best yields of malate synthetase. It is quite possible then that at some stage in its development the sunflower or even the peanut might be a superior source.

DISCUSSION

In the present work, by the use of acetyl-1-C^{14}-CoA it has been established that the malate synthetase reaction occurs as written in equation 1, and that the C^{14} in the malate which is produced remains almost exclusively in C-4. Parallel results of a similar kind have recently been described by Bradbeer and Stumpf (4) for peanut and sunflower preparations. Glyoxylate and (isocitritase + isocitrate) are now shown to be equally effective as acetyl acceptors in the malate synthetase reaction. Absolute requirements for CoA and ATP were established when acetate, rather than acetyl-CoA was included.

It was shown that the endogenous acetate activating system in the castor bean extracts was incapable of producing acetyl-CoA at a sufficiently rapid rate to saturate the malate synthetase reaction; the addition of yeast aceto-CoA-kinase greatly improved the rates of malate synthesis and allowed a more realistic picture of the capacity and distribution of malate synthetase itself.

The possible limitation of the endogenous activating system was recognized in an earlier report (8) in which the rates of malate synthesis were considerably lower than those which would be required in vivo if the malate synthetase and glyoxylate cycle were major steps in the conversion of fat to carbohydrate. The present results show that this is in fact so, and when this limitation was removed, initial rates

TABLE V

MALATE SYNTHETASE ACTIVITY[1] OF PLANT TISSUES

PLANT MATERIAL	ACETATE-C^{14} INCORPORATED INTO MALATE (CPM/MG PROTEIN)	
	WITHOUT EXOGENOUS ACETO-CoA-KINASE	ADDITIONAL INCORPORATION ON ADDING YEAST ACETO-CoA-KINASE
Castor bean endosperm, 5-day-old seedling	2,500	2,700
Soybean cotyledon, 5-day-old seedling	598	4,072
Sunflower seed, 24 hr germination	33	2,217
Watermelon cotyledon, 5-day-old seedling	173	1,917
Pea cotyledon, 5-day-old seedling	497	1,383
Peanut cotyledon, 5-day-old seedling	128	1,694
Watermelon cotyledon, 8 days in light	213	1,149
Vigna sesquipedalis cotyledon, 2-day-old seedling	445	840
Corn embryo, 5-day-old seedling	82	488
Watermelon hypocotyl, 5-day-old seedling	16	325
Castor Bean, maturing seed	84	148
Vigna sesquipedalis, hypocotyl 5-day-old seedling	193	0
Wheat coleoptile, 5-day-old seedling	56	62
Castor bean embryo, 5-day-old seedling	50	0
Sunflower cotyledon, 3 weeks in light	96	0
Castor bean, mature leaf	2	29
Tomato, mature leaf	60	0
Tobacco, mature leaf	38	0
Barley, mature leaf	18	0
Soybean, mature leaf	7	0
Sunflower, mature leaf	1	0

[1] The various plant extracts were prepared by grinding a measured weight with an appropriate volume of 0.05 M potassium phosphate pH 7.6.

0.25 ml. of the supernatant solution obtained after centrifuging at 10,000 × G for 15 min was used in the standard assay conditions (table II). 0.2 ml of aceto-CoA-kinase was added as required.

Acetate: Sp.Act.: 2.7×10^4 cpm/μM.

of malate synthesis more than ten times those reported earlier, i.e., (about 10 micromoles/hr individual bean) were achieved. While this rate is still not in excess of that required to be completely convincing, it is sufficiently high to warrant the view that it is of importance and we regard it as subject to improvement by different extraction procedures. Using a different procedure, high rates of malate synthesis have been achieved in extracts of peanut cotyledons, (Marcus, personal communication). The limitation imposed by the endogenous activating system when the malate synthetase reaction is measured in crude extracts would not be a consideration in vivo, when acetate units, already in the activated (CoA) form, are presumably provided during β-oxidation of the long chain fatty acids (12).

Malate synthetase is present at the earliest stages of germination but increases strikingly during days 1 to 3. This increase is similar to that observed in mitochondrial enzymes (1) and in isocitritase (5). In contrast to isocitritase, however, the level of malate synthetase per seedling does not decline in the later stages of germination, and furthermore, malate synthetase is present in the ripening and ungerminated seed.

A substantial portion (60 %) of the enzyme present in a sucrose phosphate homogenate can be recovered with the separated mitochondria. Although this association with the particles persists through washing in sucrose phosphate and sedimenting, it is readily broken on transfer to dilute phosphate (0.01 M) and the enzyme is found in the clear supernate. When homogenates are prepared in dilute phosphate, the enzyme is recovered in the soluble phase. At the present time, the association of the enzyme with the mitochondria is regarded merely as a fortunate circumstance in purification attempts (Yamamoto and Beevers, unpublished); other criteria for regarding the enzyme as a genuine mitochondrial component have not yet been met.

From the experiments on distribution of the enzyme, it is clear that germinating fatty seeds are the best sources of the enzyme (table V). However, other tissues such as pea cotyledons and corn seedlings have definite enzyme activity, and in general the distinction between fatty and non-fatty tissues, though quite marked, is not as complete as it was for isocitritase (5). The enzyme appears to persist longer in germinating seeds of watermelon than does isocitritase, and it is present in the ripening and ungerminated castor bean. Nevertheless, it is clear that both isocitritase and malate synthetase are present together in the most active conditions in all of those tissues which are converting fat into carbohydrate and thus the present results are in accord with the proposed importance of the glyoxylate cycle in this conversion. In those non-fatty tissues in which it occurs, malate synthetase may play a role by replenishing C-4 dicarboxylic acids at the expense of glyoxylate generated in some reaction other than that catalyzed by isocitritase.

Summary

The malate produced from acetate-1-C^{14} (or acetyl-1-C^{14} CoA) and glyoxylate by malate synthetase from castor bean endosperm contains C^{14} almost exclusively in C-4. An assay system for malate synthetase was developed which makes use of this fact and of the ability of Lactobacillus cells to release C-4 of the malate so produced as $C^{14}O_2$. Absolute requirements for CoA and ATP were demonstrated for malate synthesis when acetate was used as substrate.

The capacity of crude extracts of castor bean endosperm and other plant tissues to produce malate from acetate and glyoxylate is limited by their ability to convert acetate to acetyl-CoA. When aceto-CoA-kinase prepared from yeast was added, the rates of malate synthesis were considerably enhanced. Malate synthetase was shown to be present in widely different amounts in a variety of plant materials. Although the enzyme is not confined to those tissues converting fats in sugars, the best sources are fatty seeds in which this change is occurring, and which are known to contain isocitritase.

During the second and third days of germination of the castor bean, the malate synthetase, which is present in the ripened seed, shows a striking increase in activity; it is maintained at this level for several days. The bulk of the malate synthetase activity of castor bean homogenates can be recovered with the mitochondria.

The results reinforce earlier suggestions about the importance of malate synthetase and the glyoxylate cycle in the conversion of fats to carbohydrates.

Acknowledgements

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DIFFERENCES BETWEEN LIGNIN-LIKE POLYMERS FORMED BY PEROXIDATION OF EUGENOL AND FERULIC ACID IN LEAF SECTIONS OF PHLEUM [1]

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Coniferyl alcohol, eugenol, and ferulic acid can be converted to lignin-like polymers in the presence of H_2O_2 via a peroxidase-catalyzed oxidation. This reaction can be brought about by tissue sections or cell-free extracts of woody and herbaceous plants (6, 10, 14). Furthermore, Neish and his coworkers (5) have shown that ferulic acid-βC^{14} is an efficient precursor of the guaiacyl moiety of lignin of wheat, presumably by conversion to coniferyl alcohol.

Since the position of eugenol in the scheme of lignin biosynthesis has been questioned by Higuchi (10), the lignin-like polymers produced by peroxidation in the presence of eugenol and ferulic acid have been restudied by means of different analytical techniques. The quantitative method for lignin determinations used in the previous studies (10, 14) is based on the weight of the residue insoluble in 72 % H_2SO_4, a technique originally devised for woody tissues. In order to obtain more reliable data for young, herbaceous tissues, determinations used in the present investigation are based on extraction of the lignin in 2 % NaOH (4), followed by an estimation of the free phenolic groups (7), and determination of the ultraviolet difference spectra of the lignin extracts (1, 2, 8). Timothy grass, *Phleum pratense*, was chosen as the test material because it is easy to grow and to study anatomically and biochemically, and mature shoots have been reported to contain as much as 30 % of their dry weight as lignin (13). Results were obtained which show that there are both qualitative and quantitative differences in products from eugenol and ferulic acid.

MATERIALS AND METHODS

Tissue samples were dried first in an oven at 70° C and then in a vacuum desiccator over $CaCl_2$ and H_2SO_4. Dry weight values were either determined directly or were calculated from comparable samples. Lignin was determined as follows: after moistening with distilled water, the samples, containing 30 to 40 mg dry weight, were ground in a mortar with ether until all the chlorophyll was removed, and then were thoroughly extracted with distilled water. The residue was extracted for about 16 hours in 2 to 3 ml of 0.5 N NaOH at about 70° C. This technique is based on the method of Bondi and Meyer (4), who consider the extraction to be quantitative for young annuals. The supernatant and washes of the centrifuged residue were combined, neutralized to about pH 8.5 to 9, and were analyzed within 3 hours for their phenolic and ultraviolet absorbing contents. Some of these components were unstable after about 24 hours under these alkaline conditions. Re-extraction of the residue yielded no significant amount of ultraviolet absorbing compounds. The residue was dried, weighed and tested for materials reacting with phloroglucinol and with Cl_2-Na_2SO_3 (9, 10, 14).

Ultraviolet absorption spectra were determined on aliquots, one diluted with 0.05 N NaOH and the other with 0.05 M phosphate buffer at pH 7, the difference spectrum being obtained by subtraction (1, 2). Optical density readings were made in a Beckman spectrophotometer at intervals of 5 to 10 mμ from 230 to 450 mμ.

Phenol analyses were made by a modification of the method of Gierer for native lignin preparations (7). Suitable aliquots (containing 1 to 3 μg phenol) of the extract in 0.55 ml of distilled water were added to 0.4 ml of 0.5 M tris (hydroxymethyl) aminomethane buffer at pH 9.0 and 0.05 ml of a freshly prepared

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