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Citrate Synthetase in Mitochondria and Glyoxysomes of Maize Scutellum

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ABSTRACT

Mitochondria and glyoxysomes were isolated from scutella of maize (*Zea mays* L.) by density gradient centrifugation. Citrate synthetase was partly solubilized from the organelles by sonication. The sonicated organelle suspensions were centrifuged at high speed, and the supernatants were used as enzyme preparations without further purification. The enzymes of the two organelles differ in all properties examined (pH activity curve, Km for substrates, elution volume on Sephadex G-100, mobility on starch gel at pH 7). Both enzymes are inhibited by ATP, but the inhibition is stronger for the mitochondrial enzyme. The inhibition is competitive for the mitochondrial enzyme and noncompetitive for the glyoxysomal enzyme. The glyoxysomal, but not the mitochondrial enzyme, is inhibited 40% with 1 mM ADP and cytidine triphosphate.

Citrate synthetase of germinating fatty seeds is present both in mitochondria and glyoxysomes (2). The reaction catalyzed by this enzyme belongs to the Krebs cycle in mitochondria and to the glyoxylate cycle in glyoxysomes. The function of citrate synthetase is similar in both cases: the enzyme introduces into the cycles acetyl units derived from breakdown of foodstuffs (in the glyoxylate cycle this function is performed also by malate synthetase). The mitochondrial enzyme has been crystallized from several sources and its regulation by ATP or NADH has been demonstrated in several organisms (7, 8, 20). The properties of the glyoxysomal citrate synthetase are comparatively little known. Data by Axelrod and Beevers (1) show that in castor bean endosperm the response of the glyoxysomal citrate synthetase to ATP is quite different from that of the mitochondrial enzyme. In this paper, we show that the enzymes of the two organelles are widely different in many basic properties.

MATERIALS AND METHODS

The strain of maize (*Zea mays* L.) used was De Kalb XL-640 kindly supplied by De Kalb Italiana, Mestre. Glyoxysomes and mitochondria were isolated essentially as described in a previous paper (9). A Spinco SW-27 rotor was substituted for the Spinco SW-25.1 rotor and a 10-ml cushion of 52% (w/w) sucrose was layered at the bottom of the gradient instead of a 3-ml one. The rotor was run for 2.5 hr at 27,000 rpm at 2 C. Under these conditions, the glyoxysomes are pelleted while the mitochondria form a band in the gradient. The glyoxysomal pellet was resuspended in 0.5 M sucrose; the mitochondrial band was collected by means of a Pasteur pipette, diluted with 0.5 M sucrose, and pelleted. The mitochondrial pellet was resuspended in 0.5 M sucrose. The final protein concentration of the mitochondrial and glyoxysomal suspensions was 3 to 5 mg/ml. Cross-contamination of glyoxysomes and mitochondria was monitored by assaying the activity of cytochrome oxidase in the glyoxysomal fraction and of malate synthetase in the mitochondrial fraction (Table I). Some malate synthetase (probably derived from broken glyoxysomes) is present in the mitochondria, while cytochrome oxidase is practically absent in the glyoxysomes. The presence of some glyoxysomal contamination in the mitochondrial fraction should have no significant influence on the results, since the specific activity of citrate synthetase is 3 to 5 times higher in the mitochondria. This means that even if glyoxysomes were mixed with mitochondria in a 1:1 ratio at least three-fourths of the total activity would still be of mitochondrial origin.

The suspensions of both organelles (minimal volume: 3 ml) were sonicated at 20 kcycles/sec for 20 times of 10 sec each with an interval of 5 sec between each sonication. The sonicated suspensions were centrifuged for 30 min at 50,000 rpm in a Spinco 50 Ti rotor. The supernatants were used as a source of the enzyme without further purification. Their specific activity ranged between 1 and 2 units/mg protein for mitochondria and 0.2 to 0.5 units/mg protein for glyoxysomes. A unit is the amount of enzyme that catalyzes the breakdown of 1 μmole acetyl-CoA/min. The sonication treatment releases 70 to 80% of the enzyme activity from the mitochondria and 50 to 60% from the glyoxysomes.

Starch gel electrophoresis was run according to Scandalios (16) modified as described by Longo and Scandalios (11). Horizontal gel slices were stained for citrate synthetase using the histochemical reaction described by Trelease and Becker (19) for malate synthetase. Two hundred mM OAA[1] was used, however, as a substrate in place of glyoxylate, Mg^{2+} was omitted, and the final concentration of acetyl-CoA was 630 μM instead of 50 μM. In these conditions, enzyme activity appeared as purple bands after about 1 hr incubation at 30 C. The color faded away after about 3 hr. No bands appeared if either acetyl-CoA or OAA was omitted. The high concentration of acetyl-CoA was absolutely required for obtaining a visible reaction. Higher concentrations would probably have produced a stronger stain, but their use was prevented by the high cost of this reagent.

Citrate synthetase was assayed by the method of Srere (18) with some modifications. The buffer used was 20 mM glycyl-

[1] Abbreviations: OAA: oxaloacetate; DTNB: 5-5'-dithiobis(2-nitrobenzoic acid).

Table I. *Specific Activities of Some Enzymes from Mitochondria and Glyoxysome Fractions of Maize Scutellum*

Mitochondria and glyoxysomes were purified from the crude particulate fraction by density gradient centrifugation.

	Citrate Synthetase	Malate Synthetase	Cytochrome Oxidase
	nmoles p-nitrothiophenol ion formed/mg protein·min		*nmoles Cytc oxidized/mg protein·min*
Glyoxysomes	250	584	2
Mitochondria	1480	17	485

glycine (final concentration), pH 8.5, instead of 100 mM tris, pH 8.1; the final concentration of acetyl-CoA was 120 μM. We found it important to prepare fresh DTNB and OAA solutions each day, in order to have high and reproducible reaction rates.

Malate dehydrogenase was assayed by the method of Ochoa (14), catalase by the method of Chance and Maehly (3), and cytochrome oxidase by the method of Smith (17). Protein was determined by the method of Lowry *et al.* (12) or by measuring the absorbance at 260 and 280 nm.

RESULTS

Changes in Activity and Compartmentation. The variations in the level of citrate synthetase in maize scutellum during germination are quite similar to those of other enzymes related to the glyoxylate cycle (9). The activity per scutellum is low in the dry seed; it rises quickly after imbibition, reaches its peak at the 5th day of germination, and declines during the following 3 days. The variations of specific activity in the isolated mitochondrial and glyoxysomal fractions follow a similar pattern (Fig. 1).

The compartmentation of citrate synthetase in the scutellum was followed during the first 9 days of germination. The activity was assayed in the following fractions: crude homogenate, crude particulate fraction (mitochondria + glyoxysomes), microsomes and postmicrosomal supernatant. (We call microsomes the material sedimenting between 10,800*g* × 10 min and 105,000*g* × 2 hr). The results are shown in Figure 2. At the 1st day of germination, 65% of the total activity is in the soluble fraction (postmicrosomal supernatant), about 30% in the crude particulate fraction, and 5% in the microsomes. The fraction of citrate synthetase activity that is compartmented in the crude particulate fraction rises quickly from the 3rd to the 5th day of germination and declines steadily thereafter. The activity present in the supernatant behaves in the opposite way: it decreases in the first 5 days until reaching a minimum in coincidence with the peak of activity in the crude particulate fraction and rises again in the following days. The activity in the microsomes remains very low during the first 4 days: it increases after this time and reaches a peak between the 7th and the 8th day. This pattern of compartmentation is similar to that of malate synthetase (10) and may be explained in a similar way. The increase of activity in the supernatant and microsome fractions after the 5th day is probably due to breakdown of mitochondria and glyoxysomes. It is possible that the supernatant activity derives mostly from mitochondria, while the microsomal activity is of glyoxysomal origin because citrate synthetase seems to be retained more firmly by the latter type of organelles.

In the light of these results, all other experiments were done using scutella at the 5th day of germination. At this time the compartmentation of the enzyme in the mitochondria and glyoxysomes is maximal and also the specific activity in the two types of organelles has reached its peak. The total citrate synthetase activity present at this stage of development in the glyoxysomes is about 20% of that in mitochondria.

Inactivation by Heat. The citrate synthetase of the glyoxysomes is more sensitive to heat than that of the mitochondria: after 2 min at 50 C, its activity is reduced by 40% while the

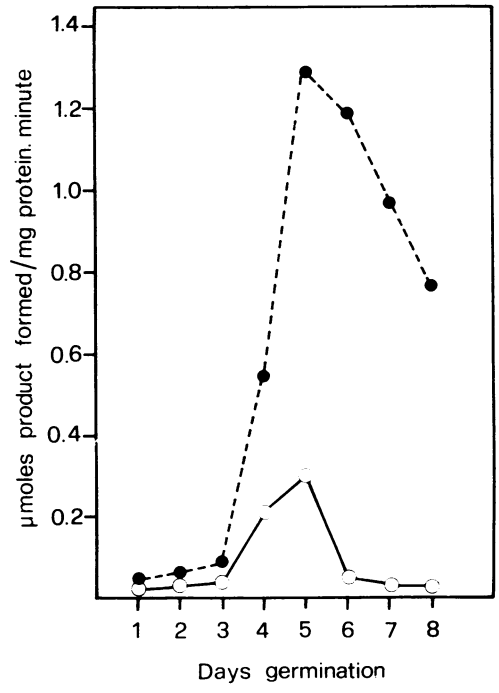

FIG. 1. Variations of the specific activity of citrate synthetase in mitochondria (--●--) and glyoxysomes (—○—) during germination.

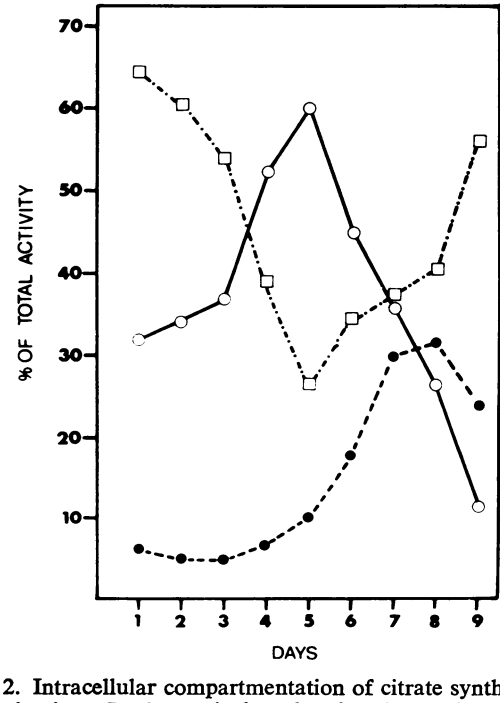

FIG. 2. Intracellular compartmentation of citrate synthetase during germination. Crude particulate fraction (—○—); microsomes (- -●- -); postmicrosomal supernatant (- · □- ·).

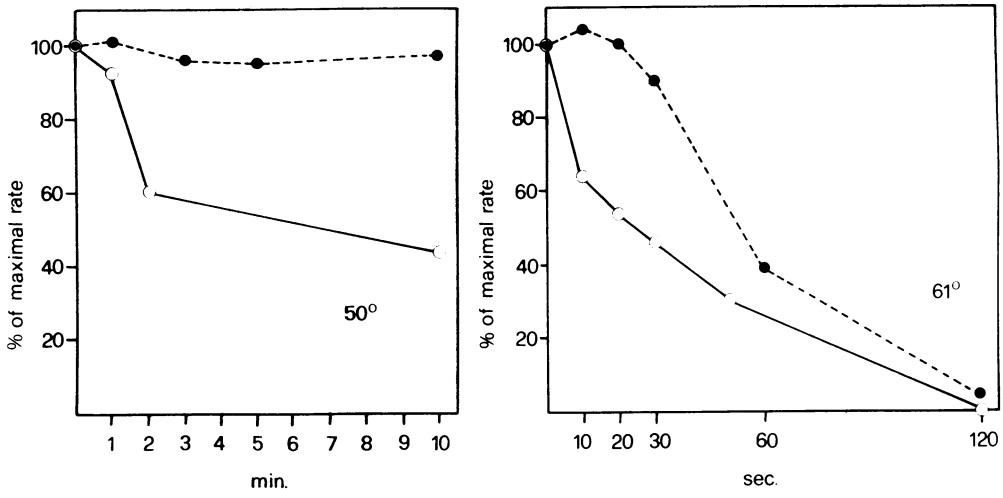

FIG. 3. Heat inactivation of citrate synthetase from mitochondria (--●--) and glyoxysomes (—○—). The samples were heated in a constant temperature bath; aliquots were removed at the indicated times and immediately chilled in an ice-water bath before assaying.

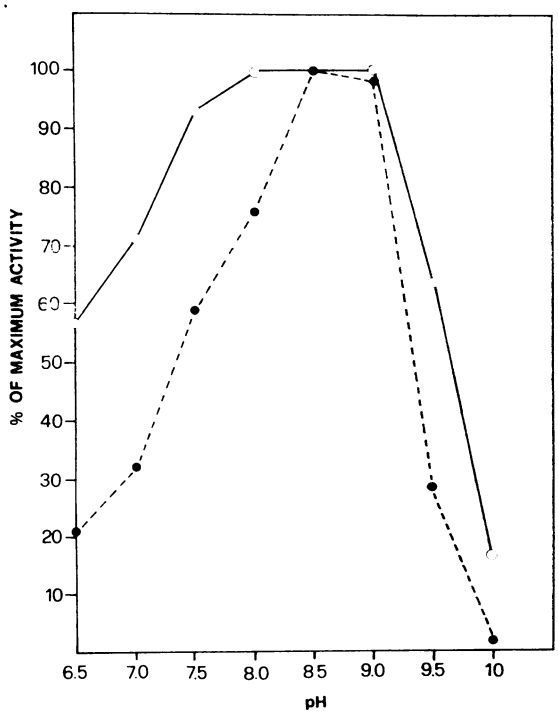

FIG. 4. pH dependence of the activity of mitochondrial (--●--) and glyoxysomal (—○—) citrate synthetase. Glycylglycine buffer (20 mM) was used in the pH range from 6.5 to 8.5, 20 mM NaOH-glycine from pH 8.5 to 10. The activity values did not differ significantly at pH 8.5 with either of the two buffers.

activity of the mitochondrial enzyme remains constant for 10 min at this temperature. At 61 C both enzymes are completely inactivated after 2 min, but the inactivation proceeds at a higher rate in the glyoxysomal enzyme (Fig. 3).

pH Sensitivity. The pH optimum is the same for both enzymes (8.5), but the glyoxysomal enzyme maintains a high activity in a broader range of pH. This difference between the two enzymes is more clear-cut at the acid side of the curve; at pH 7.5 the glyoxysomal enzyme retains 93% of the activity at pH 8.5 while the activity of the mitochondrial enzyme has dropped to 59% (Fig. 4). The measurements of enzyme activity at the extreme pHs of the curve may be misleading because the reaction with DTNB can be confidently used only between pH 7.4 and 9 (18), but the difference in the behavior of the two enzymes remains significant even in this more restricted range.

Gel Filtration. The elution volume of the glyoxysomal enzyme on Sephadex G-100 is significantly larger than that of the mitochondrial enzyme (Fig. 5). The elution volume of the mitochondrial enzyme is very close to that of the commercial pig heart enzyme (mol wt 96,000), while the elution volume of the glyoxysomal citrate synthetase is somewhat larger than that of bovine serum albumin (mol wt 67,000). Both enzymic activities are eluted as a single symmetrical peak.

Affinity for Substrates. As illustrated in Figure 6, the mitochondrial and glyoxysomal citrate synthetases have different affinities for acetyl-CoA. The apparent *Km* values for acetyl-CoA are 4 μM for the mitochondrial enzyme and 29 μM for the glyoxysomal enzyme. The *Km* values for OAA are respectively 34 and 100 μM.

Effects of Nucleotides and NADH. ATP inhibits the mitochondrial enzyme. The inhibition is competitive with respect to acetyl-CoA, as stated by other research workers. Also the glyoxysomal enzyme is inhibited by ATP, but the inhibition is smaller and noncompetitive (Figs. 6 and 7).

Both enzymes are inhibited by AMP, ADP, and CTP, but, contrary to what happens in the case of ATP, the inhibition is stronger for the glyoxysomal enzyme. Significant inhibitions of the latter can already be observed at concentrations of ADP and CTP as low as 1 mM (Fig. 7).

Some bacterial citrate synthetases which are not regulated by ATP are inhibited by NADH (20). We tested the effect of NADH at 76 and 210 μM concentration on both scutellum enzymes. No effect was observed in either case if the enzyme preparations were partially freed from malate dehydrogenase by gel filtration on Sephadex G-100. If the sonicates were assayed without this purification step a consistent inhibition of both mitochondrial and glyoxysomal citrate synthetase was observed. This effect is probably due to removal of OAA from the reaction medium by malate dehydrogenase. The comparatively low affinity of both enzymes for OAA supports this hypothesis.

Starch Gel Electrophoresis. The mitochondrial and the glyoxysomal citrate synthetase both show three anodic bands on the zymogram. Two of these bands do not seem to differ sig-

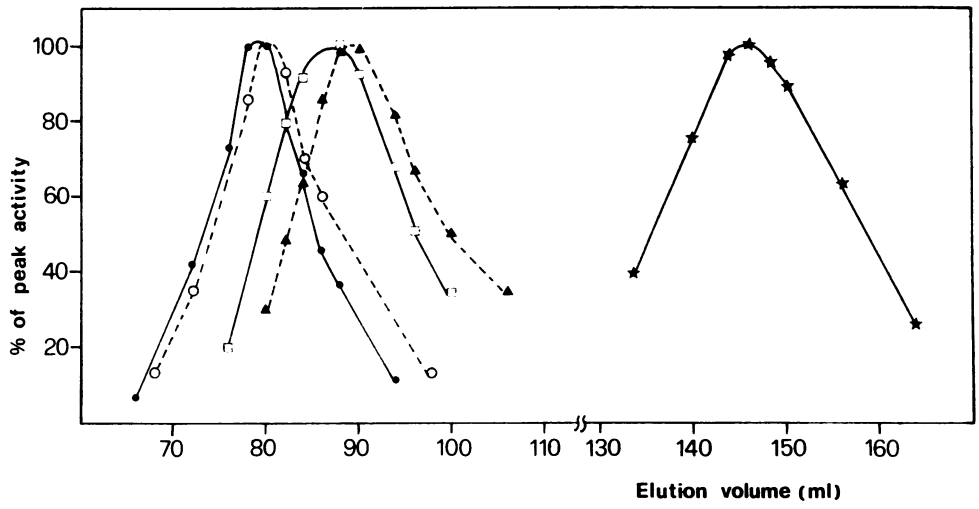

FIG. 5. Gel filtration of citrate synthetase from mitochondria (--○--) and glyoxysomes (--▲--). The column (Sephadex G-100; 2.5 × 50 cm) was calibrated with pig heart citrate synthetase (—●—; mol wt 96,000), bovine serum albumin (—□—; mol wt 67,000) and horse heart cytochrome *c* (—★—; mol wt 12,384). The column was eluted with 20 mM phosphate buffer pH 7.6.

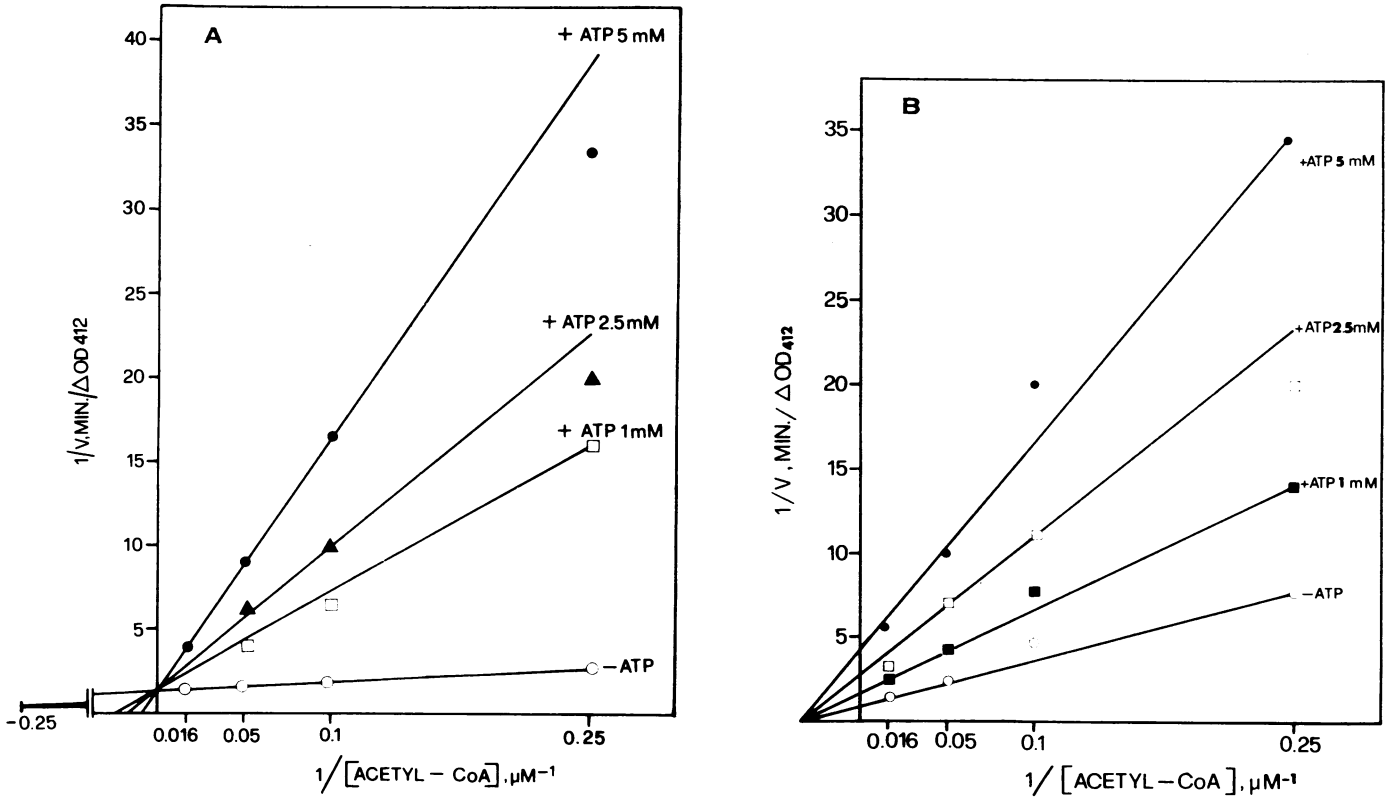

FIG. 6. Double reciprocal plot of reaction rate *versus* acetyl-CoA concentration in presence and absence of ATP. A: mitochondrial citrate synthetase; B: glyoxysomal citrate synthetase.

nificantly in their velocities of migration, while the third is faster than the other two in the mitochondria and much slower in the glyoxysomes (Fig. 8).

DISCUSSION

The pattern of compartmentation of citrate synthetase in the crude particulate and microsomal fractions suggests that the enzyme must be bound rather tightly to at least one of the two types of organelles. Experiments of lysis of glyoxysomes by sonication or detergents suggest that citrate synthetase is only partially released from these organelles (G. P. Longo, E. Bernasconi, and C. P. Longo unpublished data). The sonic treatment we used for solubilizing the enzyme releases citrate synthetase more easily from mitochondria than from glyoxysomes. This can be expected because citrate synthetase is assumed to be present in the mitochondrial matrix rather than being bound to mitochondrial membranes (5).

The citrate synthetase of the glyoxysomes differs from that of mitochondria in all properties that have been examined by

us so far (pH optimum, molecular size, thermal stability, affinity for substrates). There should be no doubt that there are two completely different enzymes. The properties of the mitochondrial enzyme (K*m* for acetyl-CoA, inhibiting concentrations of ATP, type of inhibition by ATP and mol wt) are reasonably similar to those of the enzyme from animal mitochondria (8). The only exception seems to be the high K*m* for OAA. The glyoxysomal enzyme, on the contrary, seems to be quite different from all types of citrate synthetase that are known until now. There are, however, some analogies between the glyoxysomal citrate synthetase and malate dehydrogenase from microbodies of fatty seeds or green leaves. Both enzymes are much more thermolabile than their mitochondrial counterparts and both show at least one band that migrates very slowly on starch gel at pH 7 (11, 15). It remains to be established whether this is only a coincidence or whether it will also hold true for other enzymes that are present both in microbodies and in other cell compartments.

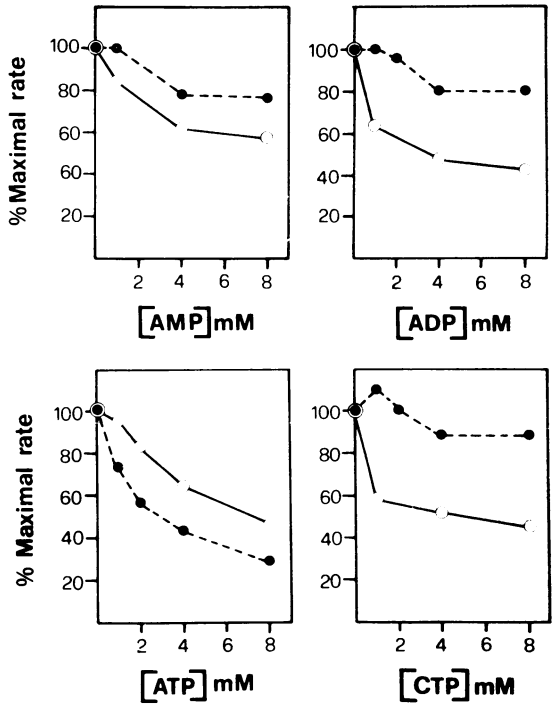

FIG. 7. Inhibition of mitochondrial (--●--) and glyoxysomal (—○—) citrate synthetase by AMP, ADP, ATP, and CTP. Concentration of acetyl-CoA in assay medium: 120 μM.

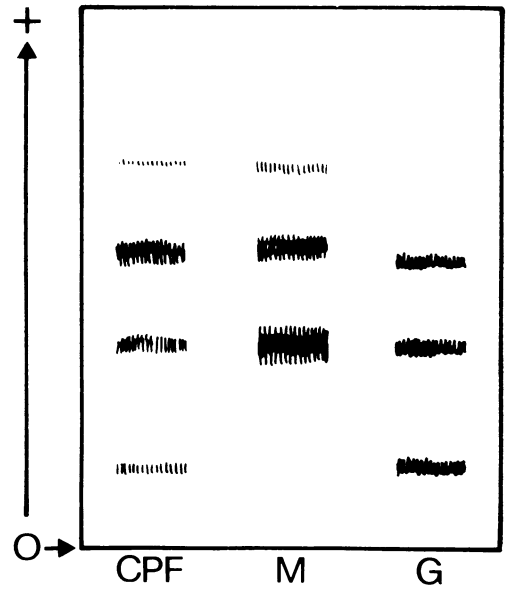

FIG. 8. Electrophoretic pattern of citrate synthetase from maize scutella. CPF: crude particulate fraction; M: purified mitochondria; G: purified glyoxysomes. Arrow indicates the direction of migration at pH 7. ○ indicates the point of insertion of the sample in the starch gel.

Our data on the inhibition of the two citrate synthetases by ATP are somewhat different from those of Axelrod and Beevers (1). They found that only the mitochondrial enzyme from castor bean endosperm is inhibited by ATP. We found that both the mitochondrial and the glyoxysomal enzyme are inhibited, but the degree and type of inhibition (competitive *versus* noncompetitive) are different. The discrepancy between our results and those of Axelrod and Beevers may depend on the plant material used or on some slight difference in the assay method. If we consider, however, not only the effect of ATP, but also that of ADP, our results tend in the same direction. Since the total concentration of ATP + ADP + AMP is fairly constant in a given tissue, we can expect that the ADP level decreases when that of ATP increases and vice versa. The mitochondrial enzyme should be more inhibited when the ATP level is high and the ADP level is low. In opposite conditions, the inhibition should be higher for the glyoxysomal enzyme. A more definite statement on the regulation of both citrate synthetases by adenine nucleotides *in vivo* will be possible only if the concentrations of nucleotides and substrates involved in the two types of organelles are known.

The K*m* of the mitochondrial enzyme for acetyl-CoA is comparable to that of the mitochondrial enzyme from animal sources, *e.g.*, pig heart and rat liver (13). The K*m* of the glyoxysomal enzyme is larger, of the same order of magnitude as that of the mitochondrial enzyme from other plant tissues (4). This comparatively smaller affinity of the glyoxysomal citrate synthetase for its substrate should have no physiological importance, since during the first days of germination, the scutellum should contain high concentrations of acetyl-CoA which derives from hydrolysis and β-oxidation of storage fats. Moreover, electron microscopic examination of other fat-storing tissues suggests that glyoxysomes are often in intimate contact with lipid bodies (6). This should result in high concentrations of fatty acids readily available for β-oxidation inside the glyoxysomes.

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