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Oxidation of NADH in Glyoxysomes by a Malate-Aspartate Shuttle[1]

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ABSTRACT

Glyoxysomes isolated from germinating castor bean endosperm accumulate NADH by β-oxidation of fatty acids. By utilizing the glutamate: oxaloacetate aminotransferase and malate dehydrogenase present in glyoxysomes and mitochondria, reducing equivalents could be transferred between the organelles by a malate-aspartate shuttle. The addition of aspartate plus α-ketoglutarate to purified glyoxysomes brought about a rapid oxidation of accumulated NADH, and the oxidation was prevented by aminooxyacetate, an inhibitor of aminotransferase activity. Citrate synthetase activity in purified glyoxysomes could be coupled readily to glutamate: oxaloacetate aminotransferase activity as a source of oxaloacetate, but coupling to malate dehydrogenase and malate resulted in low rates of citrate formation. Glyoxysomes purified in sucrose or Percoll gradients were permeable to low molecular weight compounds. No evidence was obtained for specific transport mechanisms for the proposed shuttle intermediates. The results support a revised model of gluconeogenic metabolism incorporating a malate-aspartate shuttle in the glyoxysomal pathway.

Two reactions generate NADH in the glyoxysomes of germinating oil seeds. Isolated glyoxysomes accumulate 1 mol of NADH for each mol of acetyl CoA generated during β-oxidation of fatty acids (5). Additionally, the conversion of 2 mol of acetate to 1 mol of succinate in the glyoxylate cycle requires the oxidation of malate to OAA[2] and, thus, the production of 1 mol of NADH (2). Inasmuch as isolated intact mitochondria, but not glyoxysomes, are capable of reoxidizing NADH, it was concluded that the NADH produced in the glyoxysomes is oxidized by the mitochondria (1, 5, 13).

However, the presence of highly active GOT in glyoxysomes and mitochondria suggests that aspartate and malate may be utilized in a shuttle system transferring reducing equivalents generated from NADH between the organelles (1, 4). In this paper, we have investigated this possibility further. Shuttling systems of this type have been described in other organelles (3, 6, 8, 18).

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[2] Abbreviations: ADH: alcohol dehydrogenase; AOA: aminooxyacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GOT: glutamate oxaloacetate aminotransferase; αKG: α-ketoglutarate; MDH: malate dehydrogenase; MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; OAA: oxaloacetate; PMS: phenazine methosulfate.

MATERIALS AND METHODS

Isolation of Organelles. Seeds of castor bean (*Ricinus communis* L. cv. Hale) were soaked overnight and germinated in Vermiculite for 4 days at 30 C. Endosperm tissue was chopped with razor blades in homogenizing buffer (0.45 M sucrose, 150 mM Tricine-KOH [pH 7.5], 0.1 mM $MgSO_4$, 0.1 mM KCl, 0.05 mM $CaSO_4$, and 0.1% BSA); 1 ml was used per g fresh weight of tissue. The homogenate was filtered through nylon mesh and centrifuged at 150*g* for 10 min to remove plastids and cell debris.

For sucrose gradient isolation of organelles, the homogenate (15–20 ml) was layered on top of a linear sucrose gradient (15–60% sucrose [w/w] in 2.5 mM Tris-Mes [pH 7.2]) and centrifuged for 1 h at 80,000*g* in a Beckman SW 25.2 rotor. Purified organelles were collected by fractionation of the gradient. Sucrose concentrations were determined refractometrically. For transport studies, glyoxysomes were diluted with 10 volumes of dilution buffer (13.5% [w/w] sucrose, 0.1 mM $MgSO_4$, 0.1 mM KCl, 0.05 mM $CaSO_4$, 10 mM Tris-Mes [pH 6.8], and 0.05% BSA) while gently stirring on ice. The dilution buffer was metered by an Isco model 380 programmed gradient pump over a 40-min period so that the sucrose concentration was reduced by less than 1%/min.

For Percoll gradient separations, 5 to 10 ml of homogenate were layered on top of a Percoll gradient (0–45% [v/v] Percoll [Pharmacia] in 0.5 M sucrose, 2.5 mM Tris-Mes [pH 7.2]) and centrifuged for 30 min at 40,000*g* in a swinging bucket rotor. The glyoxysomal fraction was collected and diluted with three volumes of dilution buffer. Diluted glyoxysomes from sucrose or Percoll gradients were pelleted by centrifugation at 10,000*g* for 10 min and gently resuspended in 2 to 4 ml dilution buffer prior to transport measurements.

Enzyme Assays. GOT was measured spectrophotometrically by coupling the production of OAA to the oxidation of NADH by endogenous MDH (4). The reaction was initiated by the addition of aspartate or αKG.

Citrate synthetase was measured spectrophotometrically by the reaction of DTNB with released CoA (4). The reaction mixture contained 50 mM K-phosphate (pH 7.4), 1.5 mM DTNB, 5 mM $MgCl_2$, 0.05 mM acetyl CoA, and 5 to 10 μg protein. The reaction was initiated by the addition of 0.1 mM OAA or OAA generated by coupled reactions.

β-oxidation was followed spectrophotometrically at 340 nm by the production of NADH upon the addition of 5 nmol palmityl-CoA to a reaction mixture containing 50 mM K-phosphate (pH 7.4), 0.05 mM CoA, 2.5 mM DTT, 0.1 mM NAD, and 10 to 20 μg glyoxysomal proteins. For β-oxidation experiments, 2 mM DTT was added to the homogenization buffer and sucrose gradient.

MDH, catalase, and fumarase were determined spectrophotometrically (14). ATP hydrolyzing activity was determined by release of Pi (15). Proteins were determined with the Folin phenol

reagent. O_2 consumption of purified mitochondria or tissue segments was measured with a Clark O_2 electrode at 25 C.

Determination of NAD, NADH. Intact endosperm tissue was frozen and ground in a mortar containing liquid N_2. NAD or NADH from isolated organelles or frozen tissue was extracted in acid or alkali essentially as described (12). The NAD and NADH in the extracts were measured by a modification of the method of Nisselbaum and Green (16). This method utilizes a cycling mixture composed of MTT, PMS, ethanol, and ADH. The rate of MTT reduction to the purple-colored formazan was measured at 556 nm at 35 C. The assay mix contained in a volume of 1 ml, 100 μg MTT, 250 μg PMS, 50 mM glycylglycine (pH 7.4), 50 mM nicotinamide, 250 mM ethanol, 10- to 200-μl sample, and 250 μg ADH. Since the nonspecific background rate of MTT reduction varied significantly between samples, the change in rate of color formation upon addition of ADH was used to determine nucleotide content. Between 1 and 200 pmol NAD or NADH could be determined by comparison to a linear standard curve produced by known quantities of NADH.

NAD and NADH were also determined by native fluorescence with an Eppendorf photofluorimeter essentially as described (12).

Uptake Measurements. Purified glyoxysomes in dilution buffer (0.1 ml containing 80–100 μg protein) were incubated at room temperature for 10 min. Uptake was initiated with 20 μl of the desired ^{14}C-labeled compound (0.1 μCi) and ^{3}H-labeled H_2O (0.25 μCi). Samples were removed and layered on top of 0.1 ml silicone oil (Dow Corning 704) in 0.4 ml microcentrifuge tubes and centrifuged for 30 s in a Beckman Microfuge B. The bottom of the tube containing the pelleted glyoxysomes was cut off and the ^{14}C and ^{3}H assayed in 5 ml of 0.5% PPO-toluene-Triton X-100-H_2O (20:10:3).

RESULTS

Glyoxysomal NADH Oxidation. As shown in Figure 1, isolated glyoxysomes accumulate NADH as a product of β-oxidation of fatty acid. Addition of aspartate or αKG alone did not affect NADH production. When added together, they caused the rapid oxidation of NADH accumulated by isolated glyoxysomes. The requirement for both aspartate and αKG strongly suggested that GOT was producing OAA which could then be used as a substrate for MDH, thus oxidizing NADH. This was confirmed by the addition of the potent inhibitor of aminotransferase, AOA, which completely prevented the oxidation of NADH induced by aspartate and αKG (Fig. 1). Addition of a small amount of OAA stoichiometrically oxidized NADH with or without AOA. Excess OAA completed the oxidation of NADH (Fig. 1).

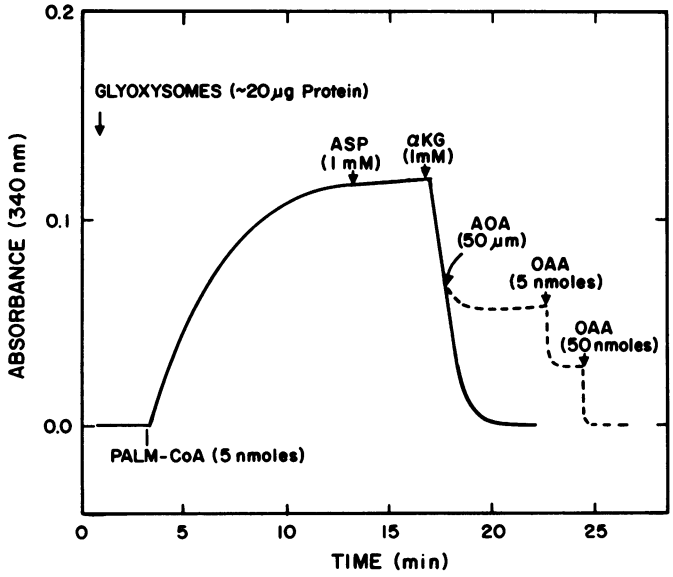

FIG. 1. Accumulation and oxidation of NADH by isolated glyoxysomes from castor bean endosperm. The initial reaction mixture (1 ml) contained 50 mM K-phosphate (pH 7.2), 0.05 mM CoA, 0.1 mM NAD, and 2.5 mM DTT. Experimental compounds (in 10 μl) were added at the indicated times and final concentrations. PALM-CoA, palmityl-CoA; ASP, aspartic acid.

As has been noted in other systems (9), the degree of inhibition by AOA varies as a function of assay pH. There was a noticeable difference between the glyoxysomal and mitochondrial forms of GOT in pH optimum and sensitivity to AOA inhibition (Table I). The K_i of AOA inhibition of glyoxysomal and mitochondrial enzymes was 2.8 and 1.4 μM, respectively, at pH 7.2 and 5 mM aspartate.

As an *in vivo* indication of the operation of a shuttle system involving the transaminases in the glyoxysomes and mitochondria, the rate of O_2 uptake by 4-day endosperm tissue was measured in the presence of AOA. Since the metabolism of germinating castor bean endosperm is almost totally directed toward the process of gluconeogenesis (1), inhibition of the gluconeogenic pathway should also lower the observed respiration rate. The addition of 5 mM AOA caused a rapid drop in the respiration rate of endosperm tissue (Fig. 2). The O_2 uptake of castor bean primary root tissue, in which gluconeogenesis does not occur, was unaffected by 5 mM AOA (Fig. 2).

Citrate Synthetase Activities. Citrate synthetase, one of the key enzymes in the glyoxysomal pathway, produces citrate from acetyl

Table I. *GOT Activity and Its Inhibition by Aminooxyacetate in Isolated Glyoxysomes and Mitochondria as a Function of pH*

Assay mixture contained 30 mM Tris-Mes, 2.5 mM αKG, 5 mM aspartic acid, 0.1 mM NADH, and 3–5 mg organelle protein. AOA concentration was 10 μM where applied.

	Assay pH				
	5	6	7	8	9
	μmol/min·mg protein				
Glyoxysomes					
Control	0.08	1.8	6.6	8.9	8.2
% inhibition by AOA	100	99	99	35	12
Mitochondria					
Control	0.97	5.3	11.4	15.7	16.8
% inhibition by AOA	100	100	100	94	42

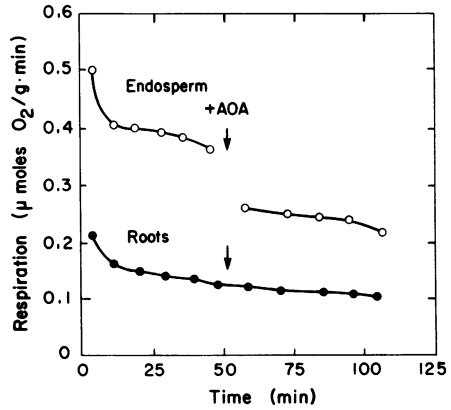

FIG. 2. Effect of AOA on the respiration of castor bean endosperm and primary root tissue. Endosperm tissue slices (40 mg) or primary root segments (70 mg) were incubated in 1 ml 50 mM K-phosphate (pH 5) at 25 C and O_2 consumption was monitored with an O_2 electrode. 5 mM AOA was added where indicated (arrows).

CoA and OAA. Acetyl CoA can readily be formed in the glyoxysomes by β-oxidation of fatty acids. It has been assumed that the OAA required for citrate synthesis is produced by the oxidation of malate by the highly active MDH present in glyoxysomes. However, the equilibrium constant for the conversion of malate to OAA is highly unfavorable, and the presence of NADH results in the near complete conversion of OAA to malate. The action of GOT on aspartate and αKG could provide an alternate means of producing OAA in the glyoxysome for citrate synthesis. The ability of these two pathways to support citrate synthetase activity is demonstrated in Figure 3. The addition of 0.1 mM OAA or 1 mM aspartate and 1 mM αKG resulted in rapid citrate synthesis by purified glyoxysomes, whereas the rate of product formation in the presence of up to 10 mM malate and 0.1 mM NAD was much slower and, after 10 min, citrate synthesis virtually ceased.

We suspected that the drop-off in citrate synthetase activity supported by malate was caused by a reduction in the equilibrium concentration of OAA as the concentration of NADH (originally zero) increased. Citrate synthetase activity in isolated mitochondria did not decrease with time when coupled to malate, presumably because mitochondria are able to oxidize the NADH formed, preventing inhibition. Calculations using the equilibrium constant for MDH of 1.2×10^{-12} (11) demonstrate that small changes in NADH concentration could have a large effect on the equilibrium concentration of OAA. The effect that this would have on the initial rate of citrate synthesis in glyoxysomes was tested experimentally by the addition of NADH. The total concentration of pyridine nucleotides was kept constant at 100 μM. When OAA was present in excess, NADH did not inhibit citrate synthesis. However, the addition of NADH strongly inhibited the initial rate of citrate synthesis when this was coupled to malate oxidation (via MDH). Citrate synthesis coupled to aspartate and αKG transamination (via GOT) was much less affected by NADH (Fig. 4). The rate of citrate synthesis coupled to aspartate and αKG rapidly returned to the rate in the absence of NADH but, as previously seen (Fig. 3), citrate production coupled to malate oxidation slowly declined. These results graphically demonstrated that malate oxidation by MDH can provide only very low equilibrium levels of OAA for citrate synthesis and that, even in the presence of low concentrations of NADH, MDH competes with citrate synthesis for available OAA.

NAD and NADH Concentrations. Since the proportion of NAD and NADH may strongly influence the rate and direction of metabolic reactions, we measured the amounts of NAD and

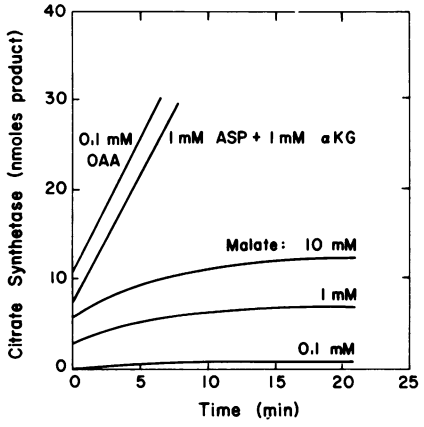

FIG. 3. Coupling of GOT or MDH to citrate synthetase activity in glyoxysomes. Reaction mixture contained 50 mM K-phosphate (pH 7.4), 1.5 mM DTNB, 5 mM $MgCl_2$, 0.05 mM acetyl CoA, and 8 μg glyoxysomal protein. Activity was initiated with OAA, OAA produced by coupling to GOT activity with ASP and αKG, or by coupling OAA production to MDH activity with malate and 0.1 mM NAD.

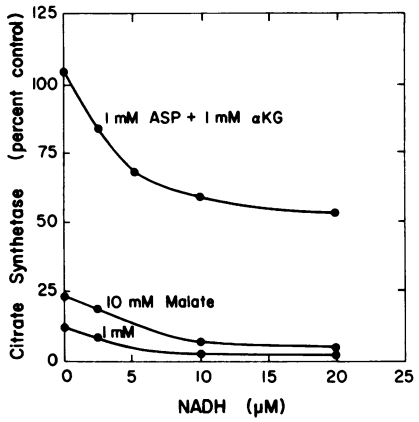

FIG. 4. Inhibition by NADH of glyoxysomal citrate synthetase activity coupled to GOT or MDH. Initial rates of citrate synthetase activity determined as described (Fig. 3). Total concentration of NAD and NADH maintained at 100 μM. Control rate determined in presence of 0.1 mM OAA (496 nmol product/min·mg protein).

Table II. *NAD and NADH Content of Castor Bean Endosperm and Purified Mitochondria and Glyoxysomes Treated with AOA*

	Control			+ 100 μM AOA		
	NAD	NADH	NADH/NAD	NAD	NADH	NADH/NAD
	nmol/mg protein		%	*nmol/mg protein*		%
Endosperm	0.70	0.18	26	0.70	0.16	23
Supernatant	0.55	0.10	18	0.52	0.10	19
Mitochondria	4.0	0.16	4	3.2	0.19	6
Glyoxysome	0.22	<0.004	<2	0.40	0.15	38

NADH in isolated organelles and intact endosperm and investigated their response to the transaminase inhibitor, AOA. The inclusion of 100 μM AOA during organelle purification greatly increased the proportion of reduced pyridine nucleotide in isolated glyoxysomes but did not significantly alter the NAD and NADH content of the mitochondria, supernatant, or intact endosperm (Table II). Based on estimates of compartment size, the concentration of pyridine nucleotides are calculated to be 0.2 to 0.4 mM in cell cytoplasm (10% tissue weight), 1.5 to 2.0 mM in mitochondria (2 μl matrix volume/mg protein), and 0.15 to 0.4 mM in glyoxysomes (1.5 μl inulin inaccessible space/mg protein).

Uptake Studies with Isolated Glyoxysomes. The proposed shuttle system would require a large flux of organic and amino acids across the organelle membranes and raises the possibility of special transport systems in the glyoxysomal membrane. For transport studies, glyoxysomes purified on sucrose gradients at their buoyant density of 1.25 g/cm^3 (52% w/w) must first be diluted. Unfortunately, glyoxysomes are very sensitive to osmotic shock and, even with gradual dilution over a 40-min period, they lost 20 to 40% of their catalase content. Electron microscopy (not shown) also indicated considerable loss of microbody contents during dilution.

Glyoxysomes in 14% sucrose were incubated with labeled metabolites and separated from nonabsorbed tracer by centrifugation through a layer of silicone oil in microcentrifuge tubes. [^{3}H]H_2O was included routinely as an estimate of total H_2O space in the sedimented glyoxysomes. Intact glyoxysomal volume could then be calculated by the difference in uptake of [^{14}C]methylinulin (external solution and broken organelles). The incorporation of labeled compounds was very high at the shortest time interval of 15 s and is ascribed to occluded water and nonspecific binding. Subsequently, uptake occurs more slowly until it equilibrates after 5 to 10 min. This slower phase was used for rate determinations.

The percentage of H_2O space occupied by the inulin remains essentially constant over the experimental time period. Aspartate equilibrated at the same concentration as the external solution but αKG appeared to be accumulated to a higher concentration by the glyoxysome. A comparison of the uptake ratios for a variety of compounds (Table III) suggests that the observed accumulation may be related to the charge of the molecule and therefore simply represents binding to nonspecific sites. The glyoxysomal membrane appeared equally permeable to the low mol wt compounds tested, giving similar rates of uptake (above initial binding) for the carboxylic acids, amino acids, as well as sorbitol and NAD.

The kinetics of aspartate influx was determined to indicate the mechanism of uptake. The influx of aspartate was proportional to the concentration and strongly suggested diffusive entry (Fig. 5). Further measurements of influx from concentrations of 5 μM to 1 mM were consistent with diffusive influx. No evidence for saturable uptake kinetics was obtained. Similar results were obtained for the influx of citrate, although the high level of binding made influx computations more hazardous.

ATP was investigated as a possible source of energetic transport across the glyoxysomal membrane. Low levels of ATP hydrolytic activity were detected in glyoxysomal fractions and in association with purified glyoxysomal ghost membranes, but since low levels of Cyt *c* oxidase, a mitochondrial marker, were also present, the ATPase activity was probably due to contamination. The addition of 0.5 mM ATP or 10 μM dinitrophenol did not affect the influx of ^{14}C-labeled compounds.

Table III. *Uptake of Various Compounds by Purified Glyoxysomes from Castor Bean Endosperm*

Purified glyoxysomes (80 mg protein) were incubated in 13.5% sucrose (w/w), 5 mM Tris-Mes (pH 6.5), 0.1 mM $MgSO_4$, 0.1 mM $CaSO_4$, 0.1 mM KCl, 0.05% BSA, [^{3}H]H_2O, and 0.1 mM ^{14}C-labeled compound.

^{14}C-label	Uptake Time (min)		
	0.25	1	5
	% ^{14}C absorbed/% ^{3}H absorbed		
Citrate	5.1	6.4	5.6
α-KG	3.9	3.7	4.9
Succinate	2.1	2.1	2.3
Malate	1.6	1.6	1.7
Glutamate	0.96	1.0	1.05
NAD	0.89	0.98	1.04
Sorbitol	0.87	0.90	1.01
Aspartate	0.76	0.86	0.94
[^{14}C]Methylinulin, 60 μg/ml	0.65	0.67	0.66

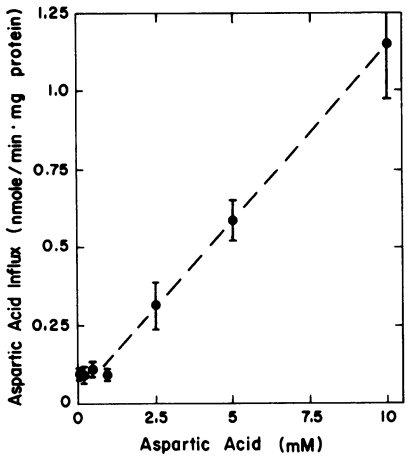

FIG. 5. Asp influx into isolated glyoxysomes as a function of aspartate concentration. Influx was determined by increase in label incorporation after initial binding. Bars represent ±SE.

Percoll Gradient Separations. To obtain purified glyoxysomes that had not been osmotically stressed by sucrose gradient centrifugation, we separated the organelles on a Percoll gradient. Discontinuous Percoll gradients have recently been used successfully for the purification of mitochondria (10). Figure 6 presents a comparison between organelles purified in a 0 to 45% (v/v) Percoll gradient and a standard sucrose gradient. The homogenate was divided, and equal volumes were layered on top of similar gradients, centrifuged as described, fractionated, and assayed for mitochondria (fumarase) and glyoxysomes (catalase). The Percoll gradient did not separate the organelles as well as the sucrose gradient. The organelle bands were noticeably broader and tended to overlap in Percoll. It was also noted in other experiments that Percoll gradients were more sensitive to overloading, resulting in poor separation of the organelle fractions.

The glyoxysomes from the Percoll gradients showed the same uptake characteristics as those from the sucrose gradients. They remained quite permeable to low mol wt compounds.

DISCUSSION

Glyoxysomes contain the five sequential enzymes of the glyoxylate cycle, citrate synthetase, aconitase, isocitrate lyase, malate synthetase, and malate dehydrogenase, and do not contain succinate dehydrogenase, fumarase, or an electron transport system linking NADH to O_2 (4, 13). Since the mitochondria do have these latter capabilities, it seemed most likely that these organelles accounted for not only the oxidation of succinate generated in the glyoxylate cycle in the glyoxysomes, but for the direct oxidation of NADH generated there in the glyoxylate cycle and in the β-oxidation of fatty acids.

The experiments in the present paper show how it is possible to account for a sustained regeneration of NAD in the glyoxysomes without a direct transport of NADH to the mitochondria. All of the glyoxysomal enzymes are involved, as before (2), but the reducing equivalents are transferred to mitochondria for oxidation in the form of malate, and a compensating shuttle of glutamate, αKG, and aspartate, utilizing the aminotransferase, known to be present in mitochondria and glyoxysomes, maintains the required carbon balance. In this representation of the reactions in the glyoxysome (Fig. 7), malate is not converted to OAA, as previously assumed (2). Instead, we suggest that the MDH in the glyoxysome

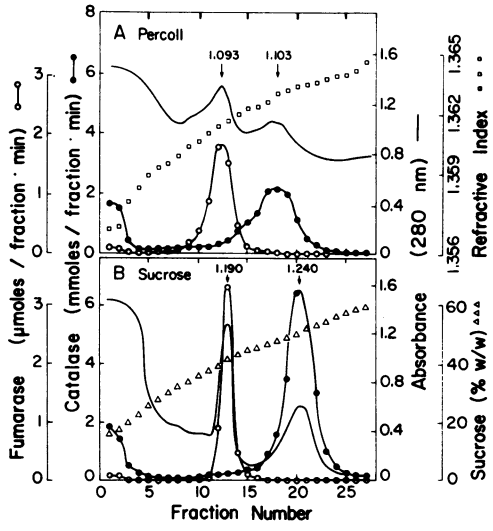

FIG. 6. Distribution of fumarase and catalase obtained when a castor bean endosperm homogenate was centrifuged in a 0–45% Percoll or 15–60% sucrose gradient. The peak density (g/cm^3) and location of the organelle bands are indicated by arrows.

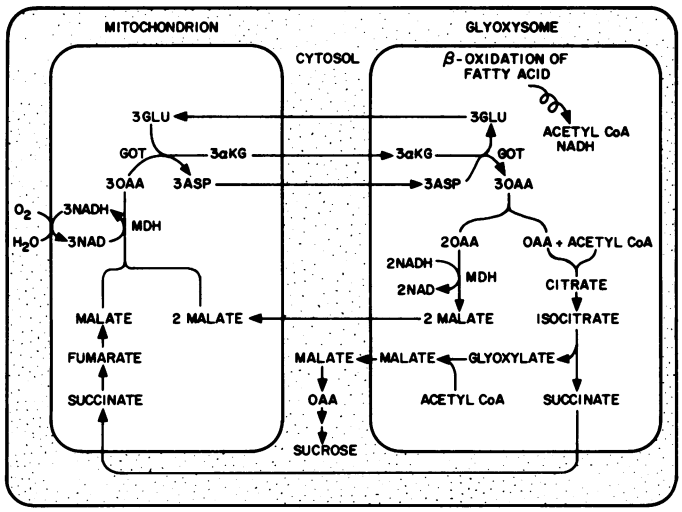

FIG. 7. Proposed malate-aspartate shuttle for the transfer of reducing equivalents between glyoxysomes and mitochondria providing for *in situ* oxidation of NADH produced by glyoxysomal β-oxidation.

functions in the reductive direction to consume NADH and generate malate. This malate, along with that generated by the malate synthetase reaction, is transported out of the glyoxysome.

Figure 7 presents the overall stoichiometry of the hypothesized shuttle between glyoxysomes and mitochondria during gluconeogenic metabolism in germinating castor beans. Three equivalents of aspartate and αKG are transaminated in the glyoxysomes to form glutamic acid and OAA. The glutamate is returned to the mitochondria, but the OAA is used for two reactions within the glyoxysome. Two units of OAA are consumed by MDH to produce malate and oxidize NADH to NAD. The remaining OAA is used as a substrate for the rest of the glyoxylate pathway in which two units of acetyl CoA are introduced, producing malate and succinate. The mitochondria complete the shuttle by oxidation and transamination reactions to reform three units of aspartate and αKG. The overall process results in the formation of one unit of malate (to be used for sucrose synthesis in the cytosol) and the transfer of three equivalents of NADH to the mitochondria for oxidation.

Several lines of evidence support the operation of such a shuttle mechanism. The required enzymes are present in both organelles and are highly active. *In vitro* experiments showed that glyoxysomal GOT can be coupled with MDH to oxidize NADH in purified glyoxysomes (Fig. 1). Isolated mitochondria from castor bean endosperm readily oxidize succinate. Oxidation of malate alone is much slower but is stimulated more than 2-fold by the addition of glutamate. Malate oxidation and particularly the glutamate stimulation is strongly inhibited by the transaminase inhibitor, AOA. The more rapid O_2 uptake in the presence of succinate (largely due to oxidation to malate) is not affected (Table IV). Using radioactive tracers to study the gluconeogenic potential of various amino acids in endosperm tissue, Stewart and Beevers (17) demonstrated that added aspartate was rapidly converted to organic acids (principally malate) and then to sugars in a classic pattern for precursor, intermediate, and final product.

The operation of GOT in glyoxysomes as we hypothesize would also solve a metabolic problem that previously has received little attention: how to maintain an adequate level of OAA for citrate synthetase activity in an organelle which produces NADH but does not have a mechanism to oxidize it to NAD. The formation of OAA from malate by MDH is a highly unfavorable reaction especially in the presence of NADH. As shown by actual extraction and quantification of the pyridine nucleotides, castor bean endosperm contains a high percentage of NADH (Table II).

Table IV. *Effect of Aminooxyacetate on Oxidation of Succinate, Glutamate, and Malate by Mitochondria from Castor Bean Endosperm*

Mitochondria purified in a sucrose gradient were diluted and O_2 uptake measured in a 1-ml solution containing 0.4 M sucrose, 5 mM $MgSO_4$, 10 mM K-phosphate (pH 7.2), 0.1% BSA (w/v), 0.1 mM ATP, and 50–100 μg mitochondrial protein.

Substrate	Rate of O_2 uptake		RC
	State 3	State 4	
	μmol O_2/min·mg protein		*ratio*
Succinate, 5 mM	146	73	2.0
+ AOA, 100 μM	135	65	2.1
Malate, 5 mM	35	25	1.4
+ glutamate, 2.5 mM	85	54	1.6
+ glutamate + AOA, 100 μM	24	10	2.4
+ succinate, 5 mM	196	100	2.0

Although our results indicate that the pyridine nucleotides in isolated mitochondria and glyoxysomes are highly oxidized, a recent report has indicated a more reduced condition (7). The presence of the transaminase inhibitor, AOA, greatly increased the proportion of reduced nucleotides in glyoxysomes. The addition of NADH reduced even further the low rate of citrate synthesis which could be coupled to malate and MDH but only partially and transiently inhibited activity coupled to aspartate, αKG, and GOT (Figs. 3 and 4). This evidence strongly suggests that GOT serves as a source of OAA for citrate synthetase activity in glyoxysomes.

Since MDH would compete with citrate synthetase for OAA in the presence of NADH, the question of regulating or directing the use of OAA produced in the glyoxysome could be raised. By following the stoichiometry of the glyoxysomal reactions depicted in Figure 7, it becomes evident that the proposed pathway would automatically regulate the division of OAA usage. Citrate synthetase is quite capable of competing with MDH for OAA (Fig. 4) and has a lower K_m for OAA as substrate (20 μM for citrate synthetase and 45 μM for MDH).

Considered in relation to the proposed shuttle, our failure to observe special transport systems in the glyoxysomes is disappointing. It is possible that our experimental manipulations may have damaged the fragile glyoxysomal membrane and so prevented detection of a transport system which operates *in vivo*. Nevertheless, it is clear that the various components of the shuttle can traverse the membranes of isolated glyoxysomes by diffusion, and this may be adequate to sustain its operation *in vivo*.

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