# Anaerobic Stress in Germinating Castor Bean, Ethanol Metabolism, and Effects on Subcellular Organelles

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## ABSTRACT

Endosperms from castor beans (*Ricinus communis*) germinated for 0 to 6 days were exposed to anoxia for 0 to 15 hours. Ethanol, the only alcohol detected by gas chromatography in the tissue, accumulates to a concentration of 15 millimolar during the first 2 to 4 hours of anoxia and subsequently decreases. The absolute amount of ethanol varies from 10 micromoles per 5-day endosperm after 4 hours anoxia to less than 1 micromole in 2-day endosperm after 4 hours. Lactate content is 2 micromoles or less per endosperm. Alcohol dehydrogenase and pyruvate decarboxylase activities, which are localized in cytosolic fractions, are not greatly affected by anoxia. The recoveries of the marker enzymes and protein in endoplasmic reticulum (ER) and mitochondrial fractions decrease during anoxia. After 15 hours, the recovery of NADPH cytochrome $c$ reductase is 15% of that in controls, fumarase is 50%, and catalase is 75%.

Glyoxysomes and ER are capable of converting ethanol to acetaldehyde which was measured using the fluorogenic reagent, 5,5-dimethyl-1,3-cyclohexanedione. The glyoxysomal activity is dependent on a hydrogen peroxide-generating substrate and the ER is dependent on NADPH. However, these activities are less than 3% of the alcohol dehydrogenase activity.

Hypoxic stress and alcoholic fermentation are common features of plant metabolism. Ethanol is produced by flooded seedlings (1, 18, 19), ice encased leaves (2), root nodules (20), and tissues exposed to $SO_2$ and $O_3$ (10). The endosperm of germinating castor bean endosperm is an appropriate model to study anaerobic stress and alcohol metabolism. This tissue responds to anoxia by generating ethanol and smaller amounts of lactate (11). ER, mitochondria, and glyoxysomes, subcellular components which depend on $O_2$ have been isolated and well characterized (3, 6, 21). The glyoxylate cycle has been shown to participate in ethanol metabolism (17). In the earlier investigation of anoxic castor bean endosperm, Kobr and Beevers (11) followed ethanol accumulation for 2 h in endosperms after 5 d germination. However, plant tissues are often capable of producing ethanol for longer periods of time (1, 4).

One purpose of this study was to determine the extent and regulation of ethanol production in castor bean during prolonged anoxia as a function of seedling age. Also, we examined the possibility that other alcohols might be produced and metabolized during anoxia. Because the endosperm initially contains very little carbohydrate (7), it was expected that little or no ethanol would be generated prior to the establishment of gluconeogenesis. We explored the possibility that the capacity for ethanol generation might be regulated by pyruvate decarboxylase and alcohol dehydrogenase, the enzymes responsible for the final steps of ethanol production.

The second major aim of this study was to examine the subcellular locations of ethanol metabolism and the effects of anoxia on $O_2$ requiring organelles. It has been reported that rat liver microsomal Cyt P-450 and peroxisomal catalase are capable of oxidizing ethanol (14, 16). The significance of these activities relative to the alcohol dehydrogenase is not clearly established. Thus, we examined the capacities of ER and glyoxysomes to metabolize ethanol in castor bean endosperm. We followed the effects of anoxia on the recoveries of marker enzymes and protein in these subcellular fractions as well as mitochondria.

## MATERIALS AND METHODS

Castor beans (*Ricinus communis* var Hale) were grown in water-saturated vermiculite in the dark at 30°C. The seeds were not presoaked because of possible $O_2$ limitations. The endosperms were harvested at the desired stage of germination, 1 to 6 d. Endosperms were placed, without added liquid, in flasks connected in series to a source of nitrogen or air bubbled through distilled $H_2O$. The flasks were placed in a 30°C incubator. At each designated time, the last flask containing the appropriate amount of endosperm was removed such that the flow of gas to subsequent samples was not interrupted during incubation.

**Gas Chromatographic Ethanol Measurement.** Endosperms from three seedlings were placed in an ice-cold mortar containing 6 ml 50 mM Tricine (pH 7.5) and homogenized. The homogenate was quickly transferred to a cold 10-ml screw-capped test tube and centrifuged for 10 min at 1000$g$. The lipid layer was removed and 3.2 ml of the supernatant was transferred to another cold 10-ml tube. Protein precipitation was performed using a modification of the procedure described by Cooper (5). The 3.2 ml of supernatant was vortexed with 0.15 ml of 0.67 N $H_2SO_4$ and 0.15 ml of 10% sodium tungstate and allowed to stand on ice for 5 min. It was then centrifuged for 5 min at 1000$g$. One ml of the supernatant was mixed with 1 ml of 2.65 mM isobutanol, the internal standard, and 2 $\mu$l of this was injected in the gas chromatograph. The sample was analyzed on a Hewlett-Packard 5840A gas chromatograph with a 5840A GC terminal, using a glass column (1.83 m × 2 mm) packed with Supelco Poropak Q, 80/100 mesh. The oven temperature was 170°C, the helium flow was 40 ml/min, and the flame ionization detector temperature was 250°C. The number of moles of ethanol was determined from the peak area relative to the internal standard, isobutanol. All analyses were performed in duplicate.

**Lactate Measurement.** Portions of endosperm from three seedlings were weighed to 0.5 g and homogenized in 4 ml of 8% $HClO_4$ with a mortar and pestle (9). The homogenate was centrifuged at 1000$g$ for 10 min, the lipid layer removed, and the supernatant decanted. The reagents from a Sigma lactic acid kit were used in the analyses. To a 1-ml cuvette were added 50 $\mu$l supernatant, 0.74 ml of the buffer-NAD mixture, 50 $\mu$l of the lactate dehydrogenase suspension. The $A_{340}$ was recorded before

adding the dehydrogenase and after incubating for 40 min at 37°C. The amount of lactate was calculated from the change in absorbance. The recovery of a known amount of lactate added to the homogenate was 86%.

**Cell Fractionation.** The ER, mitochondria, and glyoxysomes were separated from the cytosol by centrifuging the $270g$ supernatant from homogenized endosperm on a sucrose gradient in a vertical rotor as described by Donaldson (8).

**Enzyme Assays.** Alcohol dehydrogenase was measured in a 1-ml reaction mixture containing 60 mM Tris (pH 9.2), 25 mM $MgCl_2$, 0.5 mM NAD, 50 mM alcohol, and 20 $\mu$l of sample. The rate of NADH formation was calculated from the change in $A_{340}$ recorded by a Perkin Elmer 555 spectrophotometer using an extinction coefficient of 6.22 $mM^{-2}$ for the NADH. Routine assays, $K_m$ determinations, and substrate specificity studies were done using samples of $270g$ supernatants of endosperm homogenates.

Pyruvate decarboxylase was measured according to the method of Leblova and Valik (13) using a Perkin-Elmer 240 fluorescence spectrophotometer. Samples were prepared by homogenizing endosperms from three seedlings with 6 ml 0.2 M sodium phosphate (pH 6.0) in a mortar and pestle. After centrifugation at $7000g$ for 10 min, the lipid layer was removed and the supernatant decanted. The enzyme activity in a 0.2-ml sample of the supernatant was determined in a 2-ml reaction mixture containing 60 mM sodium phosphate (pH 6.0), 20 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, and 0.5 mM $MgCl_2$. The mixture was incubated for 10 min at 40°C and then 2 ml of the dimedone reagent was added to the mixture and placed in a boiling water bath for 6 min. (The dimedone reagent consisted of 0.3 g 5,5-dimethyl-1,3-cyclohexanedione, 2.5 g ammonium acetate, 0.4 ml acetic acid made up to 100 ml with water.) The amount of acetaldehyde produced was determined by measuring the fluorescence at 460 nm when excited at 396 nm. The 100% fluorescence was set with a standard consisting of 2.5 mM acetaldehyde mixed with an equal volume of dimedone reagent developed in a boiling water bath for 6 min. The amounts of acetaldehyde produced were calculated from the linear regression equation generated by a series of known acetaldehyde concentrations (up to 2.5 mM) included in the enzyme reaction mixture described above. This compensated for quenching of fluorescence by components of the reaction mixture. The sensitivity and repeatability of the dimedone measurement of acetaldehyde were later improved as described below.

Ethanol oxidation activities in ER and glyoxysomal fractions from sucrose gradients were compared to cytosolic alcohol dehydrogenase by measuring the acetaldehyde production using the dimedone reagent described above. The reaction mixture for alcohol dehydrogenase was as described above except that a 5-ml final volume contained 50 $\mu$l of the cytosol fraction. The reaction mixture for ER contained 50 mM K-phosphate (pH 7.4), 0.2 mM NADP, 3 mM glucose-6-P, 0.06 units glucose-6-P dehydrogenase, 50 mM ethanol, and 0.1 to 1.0 ml ER sample in a 5-ml final volume. The glyoxysomal reaction mixture contained 30 mM K-phosphate (pH 7.4), 0.15 mg/ml BSA, 0.01% Triton X-100, 0.1 mM CoA, 0.2 mM NAD, 10 $\mu$M palmitoyl CoA, 50 mM ethanol, and 0.5 ml glyoxysomal fraction in a 5-ml final volume. The three different reaction mixtures were incubated at 30°C. At timed intervals, duplicate 0.5-ml samples were taken from the mixtures, mixed immediately with 1 ml dimedone reagent containing 0.5 ml $H_2O$, and developed in a boiling water bath for 40 min. The time intervals for alcohol dehydrogenase were 1, 3, 4, and 5 min. The intervals for ER and glyoxysomes were 1, 4, 6, and 8 min. The fluorescence was compared to known amounts of acetaldehyde, 0.5 to 100 nmol, developed with dimedone reagent. Fluorescence was read at 345/460 nm. The 40-min development time and the 345-nm excitation wave-

length were found to yield higher and more stable fluorescence readings than the conditions used previously (13).

Protein concentrations were determined by the Lowry method. Catalase (6), fumarase (6), and NADPH Cyt $c$ reductase (21) were assayed as described. All reagents used in the enzyme assays were obtained from Sigma.

## RESULTS

**Gas Chromatography.** This method was used rather than an enzymic technique to allow detection of other substances, for example other alcohols, which might accumulate during anaerobic metabolism. Ethanol was the only product detected. The minor components observed (Fig. 1) were present in the endosperm before anoxia and did not change in amount during anoxia.

The recovery of ethanol was determined by adding a known amount of ethanol to the homogenization of an endosperm sample. The sample was prepared for GC as described. The amount of ethanol recovered was 80% of the amount added indicating that our measurements underestimated the ethanol accumulation by 20%.

**Ethanol and Lactate Production during Anoxia.** When castor bean endosperms are placed in a $N_2$ atmosphere, ethanol and small amounts of lactate accumulate (Table I). Endosperms in later stages of germination produce more ethanol but generate less lactate than endosperms at earlier stages. The amount of accumulated ethanol declines after a few hours of anoxia. The decline begins after 2 h in 2- and 3-d endosperms, after 3 h in 4-d tissue, and after 4 h in 5-d tissue. This indicates that ethanol production is limited in some way related to seedling development.

The concentration of ethanol relative to the weight of water imbibed was estimated to be 15 mM in endosperms regardless of seedling age.

**Pyruvate Decarboxylase and Alcohol Dehydrogenase.** These enzymes are responsible for converting the product of glycolysis, pyruvate, to ethanol. Also, alcohol dehydrogenase initiates the reutilization of alcohol when $O_2$ is restored. Since ethanol production appears to be limited, these enzymes were measured at different stages of germination. Pyruvate decarboxylase activity increases during germination and is enhanced by anoxia (Table II). The alcohol dehydrogenase activity in castor bean is 230 nmol/min per endosperm prior to germination, increases to 650 nmol/min after 1 d and persists at 500 nmol/min from 2 to 5 d. As shown below, anoxia diminishes this activity slightly.

The cytosolic alcohol dehydrogenase of castor bean endosperm has the capacity to oxidize a variety of alcohols as do other plant
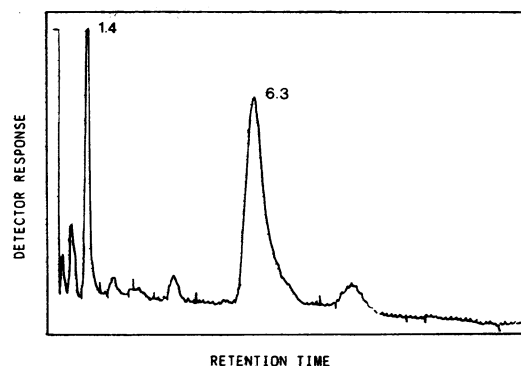


RETENTION TIME

FIG. 1. GC of deproteinized homogenate of endosperm after exposure to 2 h anoxia. The peak at 1.4 min is ethanol. The peak at 6.3 min is isobutanol, the internal standard which was added in known amounts to the homogenate. The chromatography was performed on Poropak Q, 170°C, 40 ml He/min.

Table I. *Accumulation of Ethanol and Lactate in Excised Endosperm during Exposure to Anoxia*

The sample for each time point consisted of three endosperms in a single flask. Each value represents an average of duplicate samples. The range did not exceed 25% of the mean.

| Time of Germination | Accumulation of Ethanol and Lactate at Following Times in Anoxia | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 20 min | 40 min | 1 h | 2 h | 3 h | 4 h | 5 h |
| *d* | *nmol ethanol/endosperm* | | | | | | | |
| 2 | 0 | 0.1 | 0.3 | 0.6 | 1.8 | 0.9 | 0.7 | 0.8 |
| 3 | 0 | 0.3 | 1.1 | 1.8 | 2.5 | 2.3 | 1.7 | 1.5 |
| 4 | 0 | 0.4 | 1.2 | 1.9 | 3.2 | 5.2 | 4.8 | 3.6 |
| 5 | 0 | 0.4 | 2.1 | 2.8 | 6.5 | 8.9 | 10.0 | 6.1 |
| | *nmol lactate/endosperm* | | | | | | | |
| 2 | 0.0 | ND[a] | ND | 0.3 | 0.3 | 1.9 | ND | 1.1 |
| 3 | 0.5 | ND | ND | 0.7 | 1.9 | 2.1 | ND | 2.3 |
| 4 | 1.5 | ND | ND | 0.9 | 1.1 | 1.6 | ND | 1.9 |
| 5 | 0.2 | ND | ND | 0.5 | 0.3 | 0.3 | ND | 0.2 |

[a] Not determined.

Table II. *Pyruvate Decarboxylase in Germinating Castor Bean Endosperm*

Castor beans were germinated from 2 to 5 d and the excised endosperms were exposed to anoxia for the time indicated. Pyruvate decarboxylase activity was measured in endosperm homogenates using the dimedone reagent described in "Materials and Methods."

| Time of Germination | Activity of Pyruvate Decarboxylase at Following Hours in Anoxia | | |
|---|---|---|---|
| | 0 | 3 | 5 |
| *d* | $\mu mol \cdot endosperm^{-1} \cdot h^{-1}$ | | |
| 2 | 7.8 | 7.4 | 8.3 |
| 3 | 12.4 | 13.2 | 14.2 |
| 4 | 16.6 | 17.2 | 17.9 |
| 5 | 15.7 | 15.1 | 17.7 |

tissues (12). However, ethanol is the only alcohol detected in castor bean and is the preferred substrate. The dehydrogenase activity decreases with increasing chain length of the alcohol. Relative to ethanol the activities are as follows, propanol and butanol 50%, pentanol 30%, hexanol 10%, and heptanol 3%. Also, the $K_m$ is proportional to carbon number, ranging from 10 mM for ethanol to 80 mM for pentanol. The greatest activity is obtained with 50 mM ethanol; higher concentrations are inhibitory.

**Subcellular Distribution of Alcohol Metabolism.** Figure 2 shows that alcohol dehydrogenase and pyruvate decarboxylase are located in the cytosolic fractions of germinating castor bean endosperm. These fractions contain other cytosolic components such as most of the enzymes of reversed glycolysis and the pentose cycle as previously reported (8, 15). Other subcellular components may contribute to ethanol oxidation. Both the ER and glyoxysomal fractions will convert ethanol to acetaldehyde which can be detected fluorometrically using the dimedone reagent (Fig. 3). In each case, the spectral characteristics of the product are the same as acetaldehyde, maximum excitation at 395 nm and maximum emission at 460 nm. The ER activity is dependent upon an NADPH-generating system and is inhibited by CO (Table III), indicating that a Cyt P-450 monooxygenase is responsible (21). The glyoxysomal activity requires a $H_2O_2$ source, such as fatty acid oxidation. The acetaldehyde produced in the absence of ethanol may be derived from acetate released
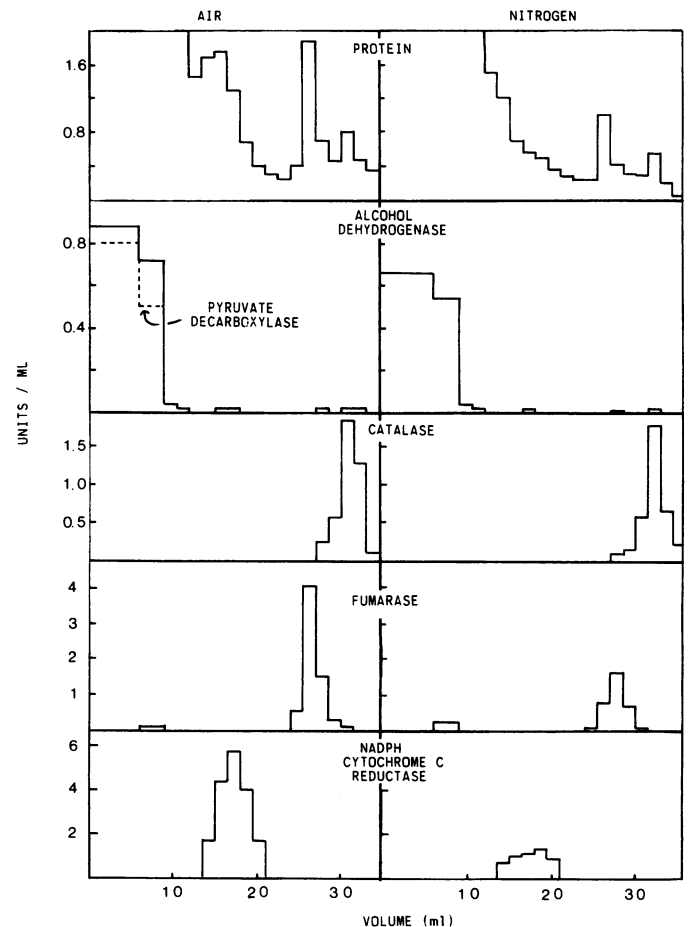


FIG. 2. Subcellular fractionation of excised 4-d endosperm exposed to air or $N_2$ for 15 h. After exposure, 15 g of endosperm from each treatment was homogenized and centrifuged at 270g, 10 min, to remove the fat, plastids, and nuclei. Then 13.5 ml of the supernatant was layered on a 20 ml, 18% to 59% (w/w) linear sucrose gradient and centrifuged in a vertical rotor for 25 min at 25,000 rpm as described previously (8). The units are: protein, mg; alcohol dehydrogenase, $\mu mol/min$; pyruvate decarboxylase, $\mu mol/min \cdot 10$; catalase, mmol/min; fumarase, $\mu mol/min$; NADPH Cyt c reductase, nmol/min.

from the fatty acid. Under the conditions used here, the glyoxysomal oxidation of ethanol exhibits saturation kinetics and an apparent $K_m$ value of 20 mM for ethanol (Fig. 4) which is in the same range as the cytosolic alcohol dehydrogenase. The kinetics of the glyoxysomal activity are probably a function of the rate of $H_2O_2$ production.

Total ethanol oxidation capacity was compared in subcellular fractions obtained from normal, aerobically germinated castor bean endosperms (Fig. 5). The cytosolic alcohol dehydrogenase has the greatest capacity to convert ethanol to acetaldehyde. The glyoxysomal system has a lower capacity and the ER has the least.

**Effect of Anoxia on Subcellular Fractions.** The ability of the various subcellular components to oxidize ethanol following anoxia and return to $O_2$ requires that they survive anoxia. Subcellular fractions were isolated from endosperms which had been germinating 4 d and then exposed to anoxia for up to 15 h. These were compared to fractions obtained from endosperms which were excised but maintained in air (Fig. 2). The protein associated with the ER fractions is diminished during anoxia to 75% at 6 h and 60% at 12 h. Some of the protein in these fractions is cytosolic and the per cent recovery of ER protein is overestimated. The ER band is no longer visible in the centrifuge
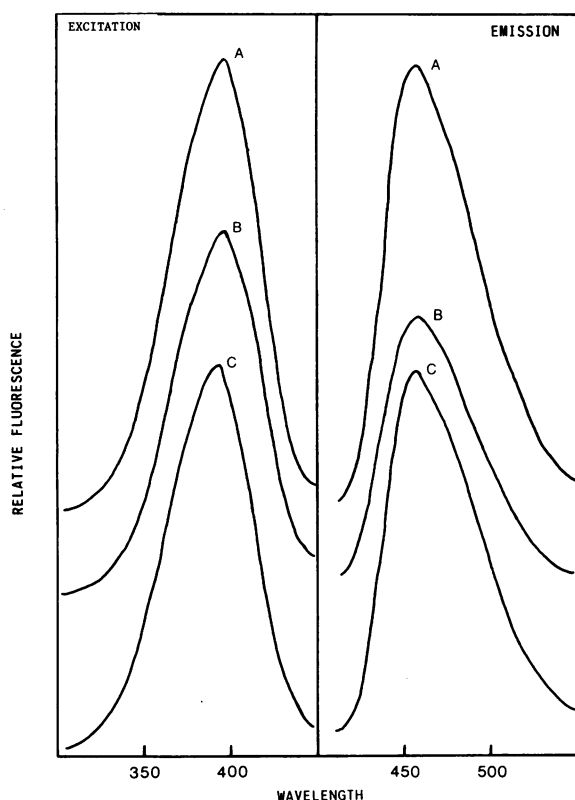
FIG. 3. Fluorescence spectra of ethanol oxidation products after reaction with the dimedone reagent. Excitation: the emission or analyzer wavelength was held constant at 460 nm while the excitation wavelength was varied from 300 to 450 nm. Emission: the excitation wavelength was held constant at 394 nm while the emission wavelength was varied from 400 to 550 nm. The three pairs of peaks shown represent (A) the glyoxysomal palmitoyl CoA-dependent ethanol oxidation product, (B) the ER NADPH-dependent ethanol oxidation product, and (C) acetaldehyde.

Table III. *Conversion of Ethanol to Acetaldehyde by ER and Glyoxysomes*

The complete reaction mixture was as described in "Materials and Methods." The ER fractions from a sucrose gradient were diluted with 2 volumes of 50 mM Tricine buffer and centrifuged at 40,000 rpm for 30 min in a Dupont A840 rotor. The pellet was resuspended in a volume of 50 mM Tricine equal to the original volume of the combined fractions. The centrifugation and resuspension were repeated.

| Assay Condition | Rate of Acetaldehyde Formation in | |
|---|---|---|
| | ER | Glyoxysomes |
| | $nmol \cdot ml^{-1} \cdot min^{-1}$ | |
| Complete | 13.5 | 560 |
| Omit subcellular fraction | 0.0 | 113 |
| Omit ethanol | 0.0 | 213 |
| Omit substrate[a] | 0.0 | 0 |
| Omit cofactors[b] | 0.0 | 133 |
| Complete, CO treated[c] | 4.5 | ND[d] |

[a] Substrates: ER, glucose-6-P and glucose-6-P dehydrogenase. [b] Cofactors: ER, NADP; glyoxysomes, NAD and CoA. [c] CO treatment: CO gas was bubbled through the complete reaction mixture in the cuvette for 30 s. [d] Not determined.
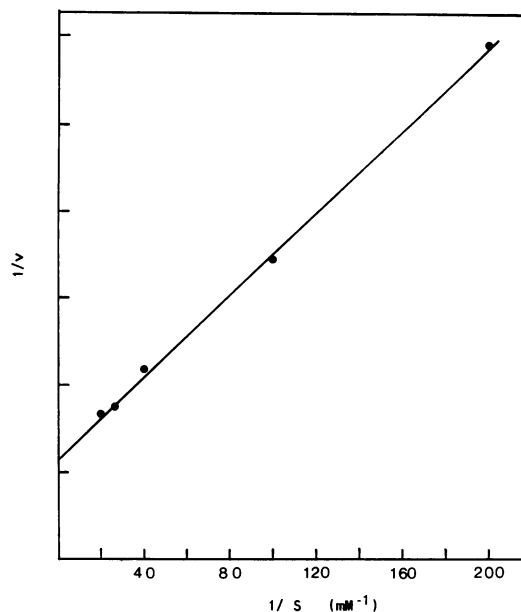


FIG. 4. Double reciprocal plot of palmitoyl CoA-dependent ethanol oxidation in glyoxysomes.

| | PERCENT OF TOTAL ETHANOL OXIDATION |
|---|---|
| CYTOSOLIC ALCOHOL DEHYDROGENASE | |
| ETHANOL + NAD$^+$ $\longrightarrow$ ACETALDEHYDE + NADH + H$^+$ | 96.9 |
| ER CYTOCHROME P450 | |
| ETHANOL + NADPH + H$^+$ + O$_2$ $\longrightarrow$ ACETALDEHYDE + NADP$^+$ + 2H$_2$O | 0.5 |
| GLYOXYSOMAL FLAVIN OXIDASE AND CATALASE | |
| ACYL CoA + O$_2$ $\longrightarrow$ ENOYL CoA + H$_2$O | |
| ETHANOL + H$_2$O$_2$ $\longrightarrow$ ACETALDEHYDE + 2H$_2$O | 2.6 |

FIG. 5. Ethanol oxidation in cytosol, ER, and glyoxysomes. Fractions were isolated from castor bean endosperm after 4 d germination. The cytosolic fractions were combined, the ER fractions were combined, and the glyoxysomal fractions were combined. The acetaldehyde production rate was determined in each and reported as a percentage relative to the total activity in all three. The rate of acetaldehyde production from 50 mM ethanol was measured in the appropriate reaction mixtures for alcohol dehydrogenase, NADPH mixed function oxidase, and palmitoyl CoA oxidation, respectively (see "Materials and Methods"). Acetaldehyde was measured using the dimedone reagent. No correction was made for acetaldehyde produced without ethanol.

tube after 12 h. NADPH Cyt $c$ reductase activity in the 25 to 35% sucrose region of the gradient, where ER is usually found, is much lower after 9 h and is almost immeasurable after 12 h (Fig. 6). The recovery of protein in mitochondrial fractions is 50% at 6 h and 18% at 12 h. The marker enzyme, fumarase, is also diminished by anoxia. Alcohol dehydrogenase activity is decreased slightly as are glyoxysomal protein and catalase.

## DISCUSSION

This investigation shows that excised castor bean endosperm accumulates ethanol and smaller amounts of lactate during anoxia. No other alcohols are detected by GC, although the alcohol dehydrogenase is capable of oxidizing C3 through C7 alcohols. Endosperms in earlier stages of germination accumulate less total ethanol than the 5-d tissue. However, the ultimate concentration achieved is about the same in all stages, 15 mM. The production of ethanol is sustained for 2 to 4 h and then declines.
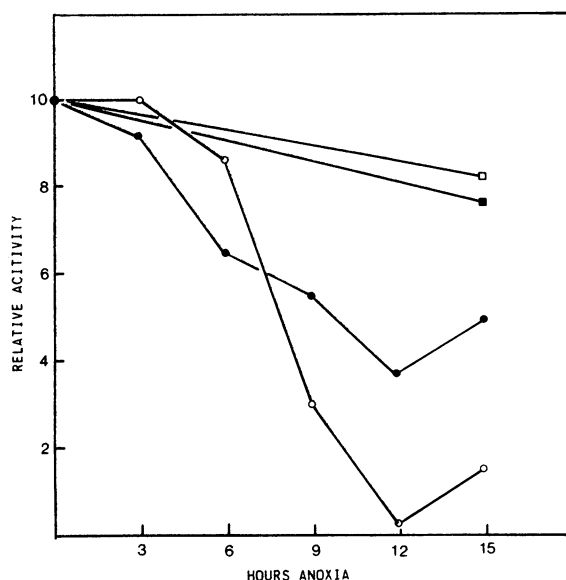
FIG. 6. Effects of anoxia on the recovery of marker enzymes in subcellular fractions from castor bean endosperm. The tissue was excised after 4 d germination, exposed to anoxia for the period of time indicated, and fractionated as shown in Figure 2. The marker enzyme activities in the appropriate fractions were totaled and compared to the total activity in the corresponding fractions obtained from the unexposed endosperm. Cytosol fractions: alcohol dehydrogenase (□); glyoxysomal fractions: catalase (■); mitochondrial fractions: fumarase (●); ER fractions: NADPH Cyt c reductase (O).

There is a general correspondence of ethanol production capacity with increased activities of pyruvate decarboxylase and alcohol dehydrogenase during germination. However, neither of these enzymes is rate limiting in ethanol generation. Our measurements and those previously reported (11) indicate that ethanol is generated at a rate of about 4 $\mu$mol/h per endosperm in a 5-d castor bean. Pyruvate decarboxylase activity at 5 d is 15 $\mu$mol acetaldehyde/h per endosperm. Alcohol dehydrogenase has an activity of 30 $\mu$mol/h per endosperm, at least in the reverse direction. The decline in ethanol generation is not associated with a decrease in these activities during anoxia. Therefore, the process must be regulated elsewhere.

The potential to accumulate larger amounts of ethanol is correlated with the development of gluconeogenic activity and increased carbohydrate content in castor bean. Seedlings which contain mostly stored carbohydrate such as rice and oats are capable of sustaining ethanol production for longer periods of time, 24 to 92 h, and achieve concentrations in the range of 40 mM (1, 4). In castor bean, the amount of ethanol produced is much less than the amount of available carbohydrate. Desveaux and Kogane-Charles (7) observed that the greatest accumulation of sugar is detected in the endosperm at the point in time when about half of the fat has been degraded. After 6 d germination at 25°C, they measured 260 $\mu$mol sucrose per endosperm and 96 $\mu$mol glucose. We detected only 10 $\mu$mol ethanol per endosperm at about the same stage of germination, 5 d at 30°C.

The rate and extent of ethanol production are not directly determined by pyruvate decarboxylase and alcohol dehydrogenase activities or by carbohydrate availability as discussed above. Glycolysis may be regulated by enzymes such as phosphofructokinase and pyruvate kinase. The measurements made by Kobr and Beevers (11) of glycolytic intermediates in castor bean endosperm implicate these two enzymes in establishing the rate of glycolysis at the onset of anoxia. Such regulation would not,

however, explain the eventual decline in ethanol during anoxia. Compartmentation of sugar, in the vacuole, could limit the extent of ethanol production.

The second purpose of our study was to investigate the subcellular location(s) of ethanol oxidation and the effects of anoxia on subcellular organelles. Our studies show that ethanol oxidation may take place in several subcellular locations involving three different mechanisms. In the cytosol, the NAD-dependent alcohol dehydrogenase is responsible. The ER is capable of NADPH-dependent oxidation of ethanol. Glyoxysomes can conduct the peroxidation of ethanol when provided with a $H_2O_2$ generating substrate such as fatty acid. The greatest capacity, however, seems to reside with the cytosolic dehydrogenase.

We observed a diminished recovery of marker enzymes and protein in ER and mitochondrial fractions after 6 to 15 h anoxia. This suggests that mitochondria and ER are degraded during anoxia while the glyoxysomes survive to a greater extent. This increases the possibility that the glyoxysomes could make a significant contribution to ethanol metabolism during recovery from anoxia.

The rate of NADH generation by alcohol dehydrogenase may exceed the capacity of mitochondrial oxidation especially during the initial stages of recovery when the ethanol concentration is relatively high, 15 mM. Thus, the amount of free NAD available could limit cytosolic flux and increase the proportions of ethanol metabolized in the glyoxysomes. Also, there are a variety of $H_2O_2$-generating oxidases in glyoxysomes in addition to the acyl CoA oxidase which was supplying $H_2O_2$ in our measurements. Thus, the total rate of peroxide production, and ethanol peroxidation, is potentially greater than we measured. In vivo measurements of catalase status in liver indicate that peroxisomes may be responsible for up to 30% of the ethanol metabolized (16).

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