Ethyl Alcohol Metabolism in Leguminous Seedlings[1, 2]

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Introduction

The problem of the alcoholic fermentation in meristematic tissues has been reviewed by Betz (3). This investigator has also shown that the alcohol content in pea root cells drops markedly with increasing distance from the tip, and that pea root segments are able to metabolize exogenous ethyl alcohol quite rapidly particularly in the presence of high O_2 concentrations (2).

Cossins (6, 7, 8, 9) has made an extensive study of the metabolism of ethyl alcohol in a variety of higher plant tissues. In general the metabolites he describes in feeding studies with labeled substrate are those expected if the exogenous ethanol is converted into acetyl-CoA, which then gives rise to other substances by the known metabolic reactions. Alcohol dehydrogenase is ubiquitous in seedlings (2, 3, 7). The enzymatic oxidation of acetaldehyde to acetate (or acetyl-CoA) has been described in a variety of tissues of animal and microbial origin (11); but, except for a report by Davies on pea seedling mitochondria (10), it has hardly been studied in higher plants. Castelfranco (4, 5) has stressed the difference in the fates of labeled ethyl alcohol and acetate when these 2 substrates are fed to plant tissue segments.

In this study we have observed that while the metabolic fates of exogenous ethanol and acetate fed to pea roots are in general quite similar, there is a major metabolite of ethyl alcohol and acetaldehyde, which is hardly formed from acetate. This metabolite has been isolated and has been identified as ethyl β-glucoside.

A DPN-dependent dehydrogenase which is able to oxidize acetaldehyde to acetate has been found to be present in peanuts and peas, both in the cotyledons and in the embryo axis. This enzyme has been partially purified and its properties are under study.

These 2 processes, the conjugation of ethyl alcohol to yield glycosidic derivatives, and its oxidation by alcohol dehydrogenase and aldehyde dehydrogenase to the level of acetate, help to explain the metabolism of exogenous labeled alcohol and the drop in endogenous alcohol content as tissues mature.

Materials and Methods

Ethyl alcohol-1-C^{14}, 2.5 and 5.4 $\mu c/\mu mole$; ethyl alcohol-2-C^{14}, 2.5 $\mu c/\mu mole$; Na acetate-1-C^{14}, 2.0 $\mu c/\mu mole$; Na acetate-2-C^{14}, 2.5 $\mu c/\mu mole$, were purchased from New England Nuclear Corporation. Acetaldehyde-1,2-C^{14}, 1 $\mu c/\mu mole$, was obtained from Calbiochem. Yeast α-glucosidase, and rabbit muscle lactic dehydrogenase, a 2 % slurry in sat. $(NH_4)_2SO_4$, were obtained from Sigma. Almond β-glucosidase, yeast alcohol dehydrogenase (A grade), a 3 % slurry in sat. $(NH_4)_2SO_4$ and protamine sulfate were obtained from Calbiochem. DPN and crystalline bovine serum albumen were obtained from Nutritional Biochemicals Corporation.

Glucose was determined quantitatively with Worthington glucostat following the procedure recommended by the manufacturer. This involved the oxidation of glucose by glucose oxidase with the formation of an equivalent amount of H_2O_2 which was used to oxidize a reduced chromogen. The absorbancy of the oxidized chromogen was read in a colorimeter at 400 mμ.

Norit A was washed several times with water by decantation to remove fine particles, it was then washed with 2 M HCl, 10 % NH_4OH in 95 % ethyl alcohol, 5 % HCl in 95 % ethyl alcohol, ethyl alcohol, and water; it was finally dried.

The radioactivity on chromatograms was detected by means of a Vanguard strip counter Model 800 Autoscanner, or by radioautography on Kodak Royal Blue X-ray film. Quantitative determinations of radioactivity were done mostly in a Tri-carb scintillation counter using a scintillation solution of the following composition: 0.3 g dimethyl POPOP, 15 g PPO and 150 g naphthalene in 1140 ml toluene, 1140 ml 1,4-dioxane and 720 ml abs. ethyl alcohol. The balance study shown in tables I and II was done using a thixotropic suspension in which water-soluble, lipid-soluble and insoluble radioactivities are counted with approximately the same efficiency (Xylene: 1,4 dioxane: abs. ethyl alcohol, 3:3:2, containing 8 % naphthalene, 0.5 % PPO, 0.005 % dimethyl POPOP, and 4 % Cab-O-sil). The samples were diluted in 0.1 N NaOH and 1.0 ml of the alkaline sample was added to 15 ml of the scintillation fluid (14). Because of the intense color, the radioactivity in acetaldehyde 2,4-dinitrophenyl hydrazone could not be determined in the scintillation counter. Instead, the radioactive precipitate was collected on a glass filter and counted in a thin-window gas flow counter,

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Plant Material. Pea seeds (*Pisum sativum L.* var. Alaska) were soaked for 6 hours and germinated for 36 hours between moist filter paper in the dark at 23°. The roots were then cut off with a razor blade. In some studies only the apical 7 mm were used, otherwise the roots were cut 15 mm long. Peanuts (*Arachis hypogea* var. Virginia Jumbo) were germinated and harvested as described elsewhere (16).

Pea Root Incubations; Small Scale. Thirty root apices 7 mm long (170 mg) were incubated in each of 2 Warburg vessels for 1 hour at 25°. The center wells contained 0.25 ml of 14 % KOH and the main compartments contained the plant tissue and the radioactive substrates (2 μmoles of ethyl alcohol-1-C^{14} or Na acetate-1-C^{14}, 2.5 μc/μmole) in 2 ml of 0.05 M potassium phosphate buffer pH 5.0. After the incubation in a metabolic shaker the KOH was drawn into a conical centrifuge tube, Ba acetate was added, and the precipitated $BaCO_3$ was washed 3 times with 50 % ethyl alcohol. The root apices were transferred to a tea strainer and washed well with tap and distilled water. Each tissue sample was placed in a glass centrifuge tube covered with 2 ml of boiling methyl alcohol and thoroughly disrupted with a stirring rod. Two ml of chloroform were added and the brei was centrifuged. The residue was extracted again with 2 ml of methyl alcohol: chloroform, 1:1. To the combined extracts 3 ml of chloroform and 1.8 ml of water were added, the resulting 2-phase mixture was allowed to settle and the upper aqueous phase was drawn off. The lower phase was washed 3 times with 0.9 ml of water. The chloroform-rich phase was stored under N_2 until it could be counted. The 4 aqueous washes were recombined with the methyl alcohol-chloroform insoluble residue and 2 ml of methanol were added. The mixture was centrifuged and the supernatant was drawn off. The residue was then extracted twice with 5 ml of 80 % ethyl alcohol; the methanolic and ethanolic extracts were combined and evaporated to dryness under vacuum. The residues from evaporation were dissolved in 2 ml of water. Thus each incubated pea root tissue sample gave rise to 4 fractions: 1, the $BaCO_3$ precipitate containing the respiratory CO_2; 2, the chloroform-soluble fraction containing the lipids; 3, the water-soluble fraction containing the amino acids, organic acids, sugars, etc.; 4, the insoluble residue consisting of denatured proteins, cell wall polysaccharides, etc.

The radioactivity in each fraction was determined by scintillation counting in the thixotropic gel. Aliquots of fractions 2, 3, and 4 were dried under acid conditions to insure the removal of labeled, acid-volatile, substrates and metabolites. Fraction 3 was separated further into basic, acidic and neutral components by passing through ion exchangers. Columns 3 cm long and 6 mm internal diameter of Dowex-50-H^+ and Dowex-1-formate were used; the former was eluted with 30 % NH_4OH, and the latter with 40 % formic acid. The radioactivity in each fraction was determined. Two-dimensional descending paper chromatography on Whatman 1 was employed for the separation and identification of the labeled organic constituents. The constituents of the basic fraction were separated using phenol: water, 5:1, in the short direction, followed by *n*-butyl alcohol: 2-butanone: water, 2:2:1 (a petri dish containing a 5 % solution of cyclohexylamine was placed in the bottom of the cabinet). The amino acid spots were detected by spraying with 0.2 % ninhydrin and 7 % acetic acid in acetone. Organic acids were separated using phenol: water, 4:1, in the long direction followed by *n*-amyl alcohol saturated with an equal volume of 5 M formic acid. The acid spots were detected by spraying with 0.04 % bromophenol blue in ethyl alcohol adjusted to approximately pH 6.7. The neutral compounds were separated using phenol: water, 5:1, in the short direction followed by *n*-butyl alcohol: acetic acid: water, 12:3:5. The reducing sugars were detected by reaction with alkaline $AgNO_3$. The chromatograms were dipped in a $AgNO_3$ bath made by adding 1 ml of sat. aq. $AgNO_3$ to 200 ml acetone, and adding H_2O dropwise to redissolve the white precipitate. The chromatogram was air dried, sprayed lightly with 0.5 M NaOH in 95 % ethyl alcohol and allowed to react for 3 minutes at room temperature. The chromatogram was finally dipped in $Na_2S_2O_3$ (photographic hypo fixer) to clear the background. The spots appeared dark brown or black on a light beige background. An alternative procedure was to dip the chromatogram in a mixture of 2 % aniline in acetone, 2 % diphenylamine in acetone and 85 % phosphoric acid (5:5:1). The paper was air-dried and heated at 100° for about 2 minutes. Reducing sugars gave characteristically colored spots, blue-gray for glucose and reddish-brown for fructose.

Pea Root Incubation; Preparative Scale. In order to collect a quantity of ethyl β-glucoside sufficient to permit its chemical characterization, pea roots were incubated under the following conditions: 14 g of roots were shaken at 25° in 30 ml of 0.05 M potassium phosphate buffer pH 5 containing 50 μc of ethyl alcohol-1-C^{14} or other radioactive substrate. After 60 minutes the pea roots were drained, washed with water, blotted and killed in 100 ml of boiling 80 % ethanol. After cooling to room temperature the suspension was blended in a Virtis homogenizer and filtered with suction. The extract was evaporated to dryness under reduced pressure and the residue was dissolved in 25 ml of H_2O and extracted 3 times with an equal volume of ethyl ether. The ether phase was discarded and the aqueous phase was applied to a Dowex-50-H^+ column, approximately 25 ml of resin bed volume. The column was washed with 100 ml of H_2O and the effluent was concentrated to 25 ml and applied to a Dowex-1-formate column of equal size. This column was also washed with 100 ml of H_2O and the effluent was concentrated under reduced pressure to 15 ml and treated 3 times with 400 mg of washed charcoal. Each char-

coal treatment involved shaking the solution and the charcoal for 20 minutes at room temperature and centrifuging. The charcoal was pooled and washed twice with 15 ml of H_2O; the washes were discarded. The charcoal was then eluted 4 times with 15 ml of 10 % ethyl alcohol. The eluate was evaporated to dryness under reduced pressure and the residue was spotted on chromatography paper.

In the purification of the ethyl β-glucoside from the pea root extract the following unidimensional chromatographic techniques were used: descending chromatography on methanol-washed Whatman 3 MM using *n*-butyl alcohol:acetic acid:water, 12:3:5, or 4:1:1 and ascending chromatography on methanol-washed Whatman No. 1, using ethyl acetate:pyridine:water, 3.17:1.0:1.15 (upper phase).

Purification of Acetaldehyde Dehydrogenase. Peanut acetone powder was prepared by homogenizing the precooled cotyledons in 20 volumes of reagent grade acetone at −15° in a Waring blendor for 1 minute. The acetone was filtered and the powder was washed with cold acetone, cold peroxide-free ethyl ether and ethyl ether at room temperature. The powder was then dried in a vacuum desiccator and stored at −10°. The acetone powder was extracted in a mortar and pestle with 9 times its weight of cold buffer of the following composition: 0.05 M KCl, 0.001 M Tris pH 7.3, 0.001 M Na_2 EDTA, 0.0015 $MgCl_2$; the pH of this buffer was 5.4. The extraction was repeated and the 2 extracts combined; further extractions failed to dissolve any additional enzymatic activity. The enzyme solution was treated with cold acetone, maintaining the temperature between −2 and 0°. The precipitate between 10 % and 50 % acetone contained all the activity. It was redissolved in one-fifth the volume of the acetone powder extract, in a buffer of the following composition: 0.04 M KCl, 0.01 M Tris pH 7.3, 0.001 M Na_2 EDTA, 0.0015 $MgCl_2$. Two-tenths ml of 2 % protamine sulfate solution was then added per ml of enzyme. The inactive precipitate was discarded and the supernatant was acidified with 0.5 M acetic acid to pH 4.7. The inactive supernatant was discarded, and the precipitate was resuspended in 0.05 M phosphate buffer pH 7, using a volume one-fifth as large as that of the protamine sulfate supernatant.

Assay of Acetaldehyde Dehydrogenase. The enzyme was incubated at 37° for 30 minutes in a metabolic shaker with 0.04 μmole acetaldehyde-1,2-C^{14}, 1.5 μmole DPN, and 100 μmoles phosphate buffer pH 8.5 in 1.0 ml total volume. The reaction was stopped by the addition of 25 ml of 95 % ethyl alcohol. The denatured proteins were centrifuged down and washed with 2 ml 80 % ethyl alcohol. The combined ethyl alcohol fractions were dried, first on a steam bath and then in a vacuum desiccator. The residue was suspended in 1.0 ml of water. The suspension was saturated with $(NH_4)_2SO_4$, acidified to pH 2 with 6 M H_2SO_4, and extracted 3 times with 1.0 ml of *n*-butyl alcohol. The combined butyl alcohol fractions were mixed with 1.1 ml of 10 % aq. NH_4OH and 3 ml petroleum ether. Under these conditions radioactive Na acetate was recovered quantitatively in the NH_4OH phase. Two 0.1 ml aliquots were taken from the aqueous ammonia layer and placed into 2 liquid scintillation vials. One vial was filled with scintillation solution and counted. The other vial was dried, the residue resuspended in 5 drops of glacial acetic acid and dried again, before it was filled with scintillation solution and counted. The difference between these 2 counts measured the radioactivity in acetate. From this figure the percent of acetaldehyde-1,2-C^{14} oxidized to acetate-1,2-C^{14} was calculated. To check the identity of the acid volatile product, the NH_4OH phase was chromatographed according to the method of Kennedy and Barker (12). All the radioactivity moved as a single spot corresponding to authentic ammonium acetate.

The unit of enzyme activity was defined as the amount of enzyme which oxidizes 1 % of the radioactive acetaldehyde to acetate under the conditions of the assay. Usually 20 to 50 enzyme units were present in each assay. Proteins were determined by the biuret method (13), using bovine serum albumen as the standard.

Results

Radioactivity Distribution from Ethyl Alcohol-1-C^{14} and Na Acetate-1-C^{14}. Tables I and II indicate the distribution of radioactivity which were obtained when pea roots were incubated with ethyl alcohol-1-C^{14} and Na acetate-1-C^{14}. A few differences are worth noting: A) almost 4 times as much acetate was converted into nonvolatile metabolites as ethanol (table I); B) the insoluble residue consisting mainly of cell wall materials and denatured proteins was labeled to a greater relative extent from acetate than from ethanol (table I); no explanation for this difference can be given at present; C) the neutral fraction was labeled to a greater extent from ethanol than from acetate (table II). Paper chromatography and autoradiography of the basic fraction showed no striking qualitative differences in the patterns of metabolites obtained from ethyl alcohol and acetate.

Table I. *General Pattern of Metabolites Obtained from Pea Roots Incubated with Ethyl Alcohol-1-C^{14} or Na Acetate-1-C^{14}*

Tissue 170 mg was incubated 1 hour, at 25° with 2 μmoles of labeled substrate (2.5 μc/μmole).

	Ethyl alcohol-1-C^{14}	Na Acetate-1-C^{14}
% of added C^{14} recovered in all fractions	2.9	11.1
% of recovered C^{14} found in each fraction		
Respiratory CO_2	15.5	18.0
Chloroform soluble	22.0	17.8
Water soluble	57.8	52.2
Insoluble residue	4.7	12.0

Table II. *Separation of Water-Soluble Metabolites Upon Ion-Exchange Resins*

The materials applied to the ion exchange columns were the water soluble fractions shown in table I.

	Ethyl alcohol-1-C^{14}	Na acetate-1-C^{14}
% of total water soluble C^{14} recovered in each fraction		
Basic fraction	58.5	56.5
Acid fraction	33.7	41.7
Neutral fraction	7.8	1.8

In both cases the principal radioactive spot corresponded with glutamic acid followed by aspartic, glutamine, alanine, valine, serine and glycine in order of decreasing intensity on radioautographs. Paper chromatography and autoradiography of the acid fraction show no qualitative differences between the 2 substrates; in both cases the radioautographs showed labeled citric, malic, succinic, α-ketoglutaric and a trace of fumaric acids.

Preliminary analysis of the lipids by thin layer chromatography and reversed phase paper chromatography revealed no qualitative differences in the composition of the radioactive components[3].

Paper chromatography and autoradiography of the neutral fractions brought out some striking qualitative differences in the fate of the 2 substrates. Ethyl alcohol-1-C^{14} gave rise to 1 single major neutral metabolite (fig 1) while Na acetate-1-C^{14} was converted into at least 4 neutral radioactive substances (fig 2). However the label in the alcohol metabolite was greater than the label in all the acetate metabolites judging by intensity of the dark

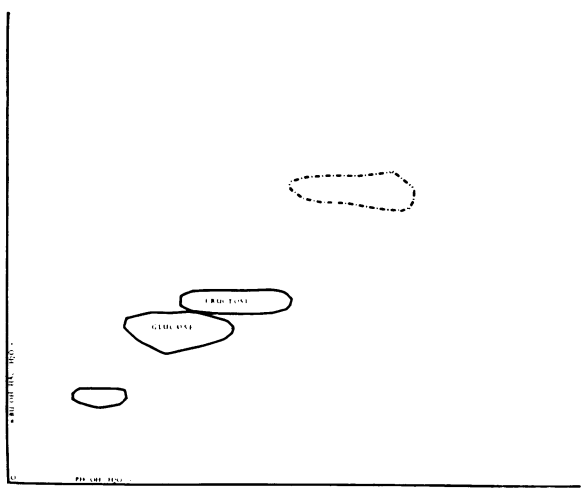

FIG. 1. Radiochromatogram of the neutral fraction obtained from pea roots incubated with ethyl alcohol-1-C^{14}. The solid outlines mark the spots detected by alkaline $AgNO_3$; the shaded outlines mark radioactivity.

[3] We are indebted to Mr. R. W. Breidenbach for this analysis.

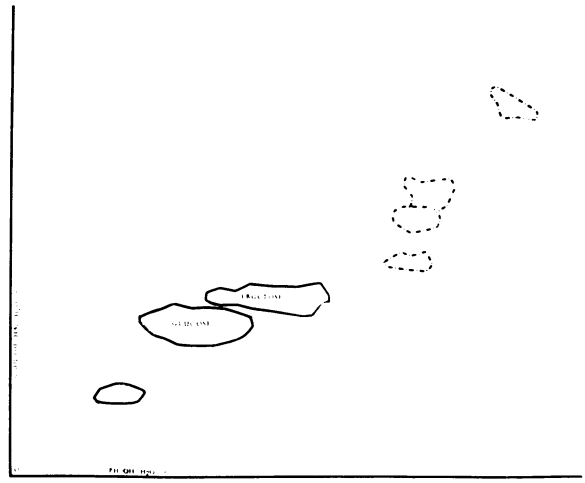

FIG. 2. Radiochromatogram of the neutral fraction obtained from pea roots incubated with Na acetate-1-C^{14}. The solid outlines mark the spots detected by alkaline $AgNO_3$; the shaded outlines mark radioactivity.

spots on the radioautographs. All the labeled compounds moved with high R_F values in both solvents indicating that they were considerably less polar than the common sugars, sucrose, glucose, and fructose, which were not radioactive.

Isolation of Ethyl β-Glucoside. Since the main neutral metabolite from ethyl alcohol-1-C^{14} was found to be adsorbed on charcoal and eluted with 10 % aqueous ethanol, this procedure was used to accumulate quantities of the unknown material by incubating 14 g lots of pea root tissue with trace amounts of radioactive substrates. Ethyl alcohol-1-C^{14}, ethyl alcohol-2-C^{14} and acetaldehyde-1,2-C^{14} behaved very similarly. In all 3 cases roughly 80 % of the neutral radioactivity was adsorbed on charcoal and eluted with 10 % ethanol. On paper chromatography the charcoal eluate was shown to contain a single radioactive spot moving with an R_F value of 0.6 in *n*-butyl alcohol: acetic acid: water, 12: 3: 5, and with an R_F value of 0.25 in ethyl acetate: pyridine: water, 3.17: 1.0: 1.15 (upper phase).

These observations suggested that the bond between carbons 1 and 2 of ethyl alcohol was not broken (or if it was broken, that the 2 fragments had equal chance of being incorporated into the metabolite in question). The similarity in the behaviors of ethyl alcohol and acetaldehyde could be explained by the action of alcohol dehydrogenase.

The charcoal eluates obtained from the incubations with Na acetate-1 or 2-C^{14} gave on chromatography with *n*-butyl alcohol: acetic acid: H_2O, 2 radioactive bands, a fast moving band, R_F = 0.8, and a slow radioactive band, R_F = 0.5. The slow-moving band was eluted with methanol and rechromatographed with ethyl acetate: pyridine: water. In this system it was resolved into 2 components, a major spot, R_F = 0.30, and a minor spot, R_F = 0.25, which agreed with the ethyl β-glucoside spot obtained from labeled ethyl alcohol and labeled acetaldehyde.

When the chromatograms were sprayed with the aniline-diphenylamine reagent, which detects sugars having free anomeric carbons and glycosides undergoing acid hydrolysis during the test, several colored spots were obtained. Two of these spots were chromatographically similar to the ethyl β-glucoside, their R_F values being 0.55 and 0.66 in *n*-butyl alcohol: acetic acid: water (vs. 0.60 for ethyl β-glucoside) and 0.30 and 0.16 in ethyl acetate: pyridine: water (vs. 0.25 for ethyl β-glucoside). These impurities were removed by controlled acid hydrolysis. The charcoal eluate was evaporated to dryness, redissolved in 0.5 % oxalic acid and heated at 100° for 20 minutes. The solution was cooled, saturated with K_2CO_3 and extracted 4 times with 1 volume of 95 % ethyl alcohol. Under these conditions the ethyl β-glucoside was extracted quantitatively into the alcohol phase while the free sugars formed by the acid hydrolysis remained in the saturated salt solution. Chromatography of the ethyl alcohol extract in both solvent systems and treatment of the chromatograms with the aniline-diphenylamine reagent failed to reveal either of the 2 contaminants. The monosaccharides liberated during this controlled acid hydrolysis were identified chromatographically and by means of color reactions as glucose and fructose.

Identification of Ethyl β-Glucoside. Upon prolonged heating in 1 M HCl or $HClO_4$, the radioactive metabolite was destroyed; the decomposition was made evident by the progressive decrease of total radioactivity during the heating process (fig 3). The lost radioactivity could be recovered by sweeping the vapors with N_2 during the hydrolysis into a trap of scintillation fluid surrounded by an ice bath. When this volatile radioactive unknown was trapped in ice water it could not be precipitated by addition of $Ba(OH)_2$, nor was it rendered less volatile by treatment with base or with semicarbazide and 2,4-dinitrophenylhydrazine. Thus CO_2, volatile acids, acetaldehyde, and other volatile carbonyl compounds were excluded as possible identities for this volatile unknown. The nonradioactive residue formed during acid hydrolysis was identified as glucose on the basis of the yellow color obtained with the cysteine-H_2SO_4 reagent which changes to green on standing (1), reaction with glucose oxidase, cochromatography with authentic glucose in 5 different solvents.

The nonvolatile radioactive unknown was also hydrolyzed by β-glucosidase, while α-glucosidase failed to release any radioactivity (fig 4).

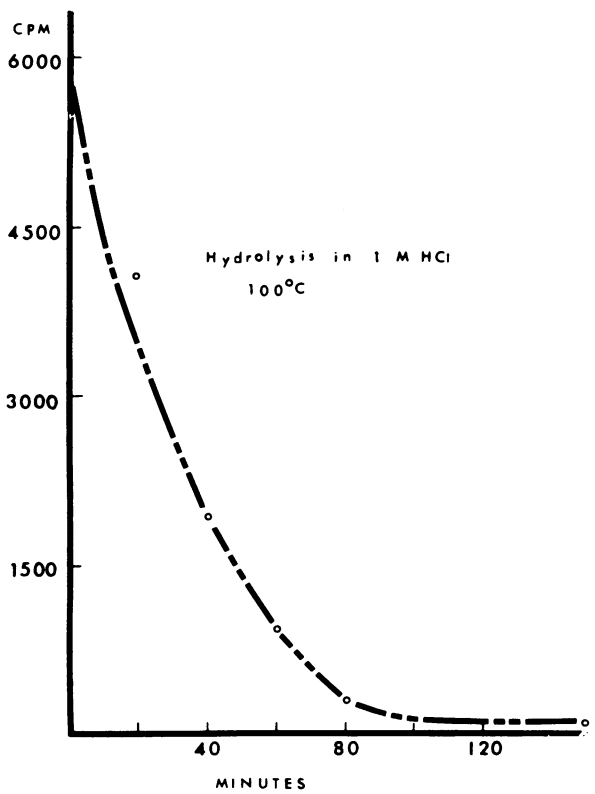

FIG. 3. Acid hydrolysis of the labeled ethyl glucoside isolated from pea roots incubated with C^{14}-ethyl alcohol.

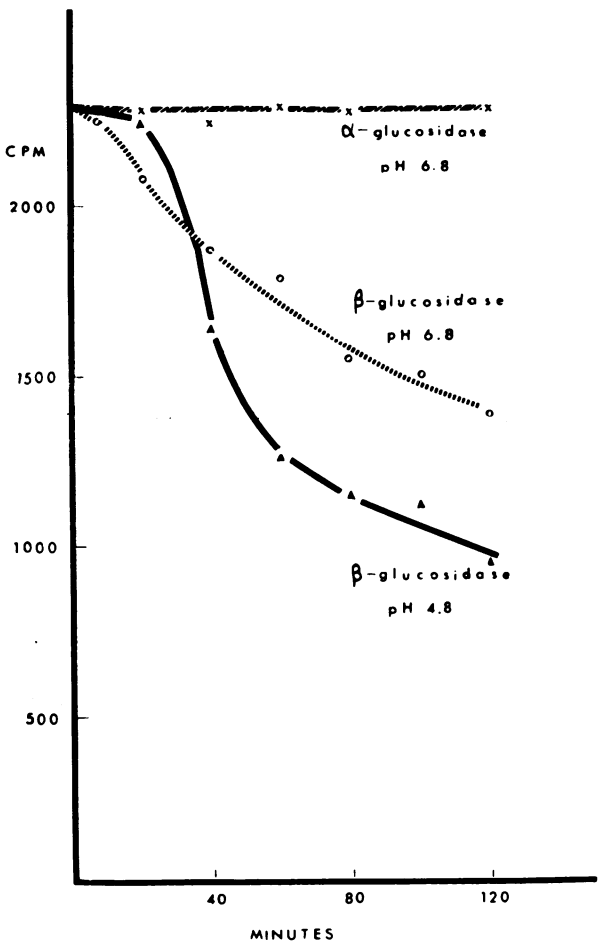

FIG. 4. Enzymatic hydrolysis of the labeled ethyl glucoside isolated from pea roots incubated with C^{14}-ethyl alcohol.

The volatile radioactive hydrolysis product was identified as ethyl alcohol by means of the following observations. A sample of the nonvolatile neutral product formed from ethyl alcohol-2-C^{14}, purified by charcoal adsorption, paper chromatography, and mild acid hydrolysis was hydrolyzed with 1 M $HClO_4$ in an evacuated Van Slyke-Folch 2-legged tube. The reaction mixture was allowed to stand overnight in 1 side of the tube after which the volatile constituents were distilled over to the other side. An aliquot of the distilled hydrolysis product containing 12,000 cpm, was added to 5 μmoles DPN, 500 μmoles Na pyruvate, 50 μmoles of potassium phosphate buffer pH 7.5 and 0.2 ml of 0.1 M Tris [this last addition was required to neutralize the $(NH_4)_2SO_4$ added with the enzyme mixture] in a total volume of 3.0 ml. The reaction vessel was a 75 ml distilling flask fitted with an inlet of N_2 gas and a microcondenser. The receiver contained 4 ml of saturated 2,4-dinitrophenylhydrazine in 1 M H_2SO_4 surrounded by an ice bath. The reaction was begun by adding 0.1 ml of alcohol dehydrogenase and 0.1 ml of lactic dehydrogenase diluted to 0.5 ml with water.

After 20 minutes incubation at 25°, the flask was cooled to 0° and N_2 was bubbled slowly to sweep the vapors into the 2,4-dinitrophenylhydrazine trap. Another aliquot of the enzyme mixture was added and was allowed to react for another 20 minutes at 25°. This cycle was repeated 4 times.

Ten μmoles of carrier acetaldehyde was added to the receiver and the precipitated 2,4-dinitrophenylhydrazone was collected on a fiber glass filter, washed, dried and counted in a thin window gas flow counter. From the initial and final counts and the efficiencies of both counters the percent conversion of labeled volatile hydrolysis product into a 2,4-dinitrophenylhydrazine precipitable derivative was calculated. Table III shows that this conversion is dependent on the presence of alcohol dehydrogenase. This finding indicates that the volatile radioactive hydrolysis product is a primary alcohol. On paper chromatography with 3 solvents (dibutyl ether: dimethyl formamide: tetrahydrofuran, 85:15:4; abs. ethanol: petroleum ether, 4:1; 2-octanol: formic acid: H_2O, 3:1:3) the radioactivity was found to coincide with the colored spot of carrier acetaldehyde 2,4-dinitro-phenylhydrazone.

Table III. *Identification of the Volatile Radioactive Compound Released by Acid Hydrolysis*

	% C^{14} converted to a 2,4-dinitrophenyl hydrazine precipitable form
Incubated with ADH, LDH, DPN, pyruvate	66
Same without ADH	1.3

Quantitative Estimation of the Endogenous Ethyl β-Glucoside. Fourteen g of pea root tissue were incubated with ethyl alcohol-1-C^{14}, 5.4 μc/μmole, and the labeled ethyl β-glucoside was extracted and purified by the procedure which has been described. The glucose content of this extract was determined quantitatively before and after hydrolysis with 1 M $HClO_4$. The difference between these 2 values indicates the bound glucose that was released by acid hydrolysis; glucose after hydrolysis, 1.36 mg-(glucose before hydrolysis, 0.10 mg) = glucose released, 1.26 mg.

From the total radioactivity found in this fraction (20,250 cpm), the counting efficiency (34 %) and the specific activity of the labeled substrate (5.4 μc/μmole) we calculated that 5×10^{-3} μmoles of ethyl-β-glucoside were formed from the exogenous ethyl alcohol during the incubation, accounting for approximately 1 μg of bound glucose released during the acid hydrolysis. This amount is negligible with respect to the total bound glucose which was 1.26 mg. Obviously the purification procedure is rather laborious and the yield of isolated glucoside is anything but quantitative. However, it is safe to conclude that the endogenous content of ethyl β-glucoside in pea roots is of the order of 100 μg/g of fresh tissue.

Purification of Acetaldehyde Dehydrogenase. Peanut cotyledon homogenates in a hypertonic Tris-sucrose were prepared as described previously and fractionated into a mitochondrial pellet (30 min, 10,000 × *g*), a microsomal pellet (1 hr, 144,000 × *g*), and a high speed supernatant (16, 17). All the acetaldehyde-oxidizing activity was found in the supernatant and the only metabolite which was not volatile

Table IV. *Purification of Acetaldehyde Dehydrogenase from Germinating Peanut Cotyledon Acetone Powder*

Purification step	Total volume ml	Units of enzyme activity	Total protein mg	Units / mg protein
Acetone powder suspension	220	...	8350	...
pH 5.4 extract	153	4900	1150	4
10–50 % acet ne precipitate	31.5	12,900	850	13
Protamine sulfate supernatant	33.6	13,400	300	45
pH 4.7 precipitate	6.7	10,700	100	107

at the pH of the incubation mixture was identified as acetate by paper chromatography. The dry acetone powder proved to be a more convenient source of the enzyme for further purification. The results of the 4-step purification procedure are summarized in table IV. The overall purification is about 180-fold on a protein basis starting from the crude acetone powder. Most of the nonprotein contaminants were also removed. The enzyme has an absolute requirement for DPN which cannot be replaced by TPN, but thus far, side reactions have prevented the utilization of a spectrophotometric assay. The enzyme is strongly inhibited by heavy metals and iodoacetamide indicating the presence of essential sulfhydryls. CoA is not required and is even slightly inhibitory. All attempts to demonstrate an active acetate intermediate have been unsuccessful. This acetaldehyde dehydrogenase was found not only in cotyledon but also in embryo axis tissue (table V). Germinating peas were actually a richer source than peanuts, although in our hands the peanut enzyme proved easier to purify.

Table V. *Distribution of Acetaldehyde Dehydrogenase*

This comparison was made on buffer extracts of acetone powders. The buffer contained 0.04 M KCl, 0.01 M Tris pH 7.3, 0.001 M Na_2 EDTA, 0.0015 M $MgCl_2$.

	mg protein	Enzyme units	Enzyme units
	g fr wt	g fr wt	mg protein
Peanut cotyledon	40	189	4.7
Peanut embryo	5	3.4	0.7
Pea cotyledon	45	540	12.0
Pea embryo	15.5	86.5	5.6

Discussion

The pea root experiments described in this report involved fairly long incubation times and subsequent fractionation procedures during which all volatile metabolites (except CO_2) were lost. It was not the object of these studies to confirm an earlier preliminary report (5) that exogenous ethyl alcohol is converted during short-time incubations into free butyric and caproic acids without dilution by acetate. That phase of ethyl alcohol metabolism is still under investigation.

The physiological role of ethyl β-glucoside is not obvious at this point. It is conceivable that the synthesis of this compound is related to the variations in alcohol content which take place in young tissues. Meristematic cells tend to ferment actively with production of alcohol which is rapidly dissimilated as the cells mature. In pea roots, Betz (2, 3) found that the endogenous ethanol content dropped in the following way: meristematic zone (0–2 mm), 254 μg/g fresh weight; zone of elongation (2–5 mm), 85 μg/g fresh weight; zone of differentiation (5–10 mm), 45 μg/g fresh weight. In our own work, the ability of pea root tissue to convert ethyl alcohol-1-C^{14} to neutral nonvolatile metabolites (ethyl glucoside) was slightly higher for the second 7-mm segment than for the first (apical) 7-mm segment (9.8 cpm/mg tissue vs. 7.4 cpm/mg tissue under identical incubation conditions). It appears therefore possible that the conjugation of ethyl alcohol is one of the reactions responsible for the drop of alcohol content. Precise information on the concentration of ethyl glucoside as a function of cell age should prove interesting.

Possibly the ethyl β-glucoside could play a role in maintaining the delicate balance between oxidized and reduced pyridine nucleotides. If the apical meristem is dependent for its energy requirements on the fermentation of translocated sugars, it might be advantageous to the meristematic cells to maintain a high DPN/DPNH ratio without increasing the acetaldehyde concentration to toxic levels. According to the equation $(DPN)/(DPNH) \times (CH_3\text{-}CH_2OH)/(CH_3\text{-}CHO) = K$, this aim could be accomplished by combining the alcohol in some metabolically inert form. Later in the course of the development, as the energy metabolism changes from fermentative to respiratory, the ethyl glucoside could be hydrolyzed and the ethyl alcohol oxidized via acetaldehyde and acetate with production of DPNH. Mitochondria from various plant tissues are able to promote the oxidation of extramitochondrial DNPH coupled to phosphorylation. This has been shown by Wiskich and Bonner (18) for sweet potato and white potato mitochondria, and has been confirmed recently in our own laboratory for peanut cotyledon mitochondria.

Incubation of pea roots with either ethyl alcohol or acetate failed to label the main soluble hexose pool of glucose, fructose and sucrose. The ethyl alcohol metabolite (fig 1) has been identified as ethyl β-glucoside; the neutral metabolites formed from acetate (fig 2) have not yet been identified. They are only weakly labeled, run with high R_F values in both solvents, and behave as relatively nonpolar sugar derivatives. While we were unable to find any reference to the presence of ethyl β-glucoside in biological systems, a recent report by Moreno and Cardini (15) has described the occurrence of ethyl β-D-fructofuranoside in wheat germ in fairly large amounts. The chromatographic properties of this compound, which we synthesized by the action of invertase on sucrose in aqueous ethyl alcohol, are similar to those of the β-glucoside. The fructoside moves slightly ahead of the glucoside in all the solvent systems which we tried. It is also much more sensitive to acid hydrolysis and shows some of the reactions of free ketoses. It is possible that the stable glycosides of simple aliphatic alcohols are fairly widely distributed in nature, but that they have so far escaped detection because of their lack of striking chemical properties.

Summary

An investigation in which excised pea roots were incubated with ethyl alcohol-1 or -2-C^{14}, and with correspondingly labeled sodium acetate, has indicated certain qualitative differences in the metabolites formed from these 2 substrates. One notable difference is the formation of appreciable quantities of a neutral nonvolatile metabolite from ethyl alcohol labeled in either carbon. This metabolite has been purified by ion exchange, charcoal adsorption, paper chromatography and mild acid hydrolysis and has been identified as ethyl β-glucoside.

An enzyme which is able to catalyze the oxidation of acetaldehyde to acetate by diphosphopyridine nucleotide has been demonstrated in pea and peanut seedlings. Triphosphopyridine nucleotide cannot substitute for diphosphopyridine nucleotide. This enzyme has been purified over 100-fold from acetone powder of germinating peanut cotyledons by acetone precipitation, protamine sulfate treatment and isoelectric precipitation.

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