THE ACCUMULATION AND ELIMINATION OF ETHANOL IN ROOTS OF SUGAR BEET PLANTS

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This is to certify that the

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DONALD G. KENEFICK ABSTRACT

A study was made on the effect of an anaerobic environment upon the metabolism of intact sugar beet plants. The volatile substance which accumulated in the root tissue was oxidizable with dichromate and was shown, by enzymatic characterization, to be primarily ethanol.

Considerable quantities of ethanol accumulated when the complete plant was placed under oxygen stress. Only slight increases were observed when an attempt was made to limit the oxygen supply to the roots by flooding the soil with water. In the latter case, it seemed probable that the foliage was supplying oxygen to the root system, particularly that portion of the root which was analyzed.

The ethanol which accumulated in root tissue was eliminated exponentially when the plants were returned to aerobic conditions. A study of this elimination mechanism showed that ethanol which was supplied to the root system was transpired from foliar tissue in considerable quantity. It was further demonstrated that ethanol was transpired from the top portions of the plant when the taproot was placed under anaerobic stress.

THE ACCUMULATION AND ELIMINATION OF ETHANOL IN ROOTS OF SUGAR BEET PLANTS

By

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TABLE OF CONTENTS

INTRODUCTION

Soil aeration has been a topic of considerable interest since plant roots are dependent on the soil for a source of oxygen as well as water and nutrients. The complex nature of soil-plant relationships has not permitted positive research data on oxygen requirements of plants. However, before the dependence of plants upon soil oxygen can be established, the direct effect of oxygen stress in plants must be determined. This requires a study of plant respiration.

The common-path theory of respiration has been well established in recent years. This is based on the idea that respiration has an anaerobic phase (glycolysis) and an aerobic phase (citric acid cycle). Under normal aerobic conditions both glycolysis and the citric acid cycle are operative and glucose is converted to carbon dioxide and water. However, under anaerobic conditions glycolysis terminates in the accumulation of anaerobic end-products.

The purpose of this study was: 1) to determine the pattern of accumulation of an anaerobic end-product in sugar beet root tissue and its elimination during an aerobic recovery period, 2) to observe possible injurious effects of anaerobiosis on sugar beet plants, 3) to identify the principal anaerobic end-product in sugar beets,

and 4) to determine the principal means of eliminating this material during an aerobic recovery period.

LITERATURE REVIEW

Russell (14) has cited several references where various anatomical and morphological responses as well as physiological processes were altered in plants receiving inadequate amounts of oxygen to the root system. He concludes that even with the lack of quantitative verification, the large volume of qualitative information seems to justify the conclusion that soil aeration, in many instances, is a major factor in plant growth.

In contrast to this view, James (6) suggests that lack of oxygen is something that happens to plants in laboratories and its occurrence in the field is rather rare. He further suggests that under conditions of high water tables, plants may not endure anaerobiosis, but rather evade it. Aquatic plants have natural stem adaptations for gaseous transport to the root system and represent plants which normally grow under flooded conditions.

Sifton (15,16) has cited various references establishing the role of highly developed intercellular spaces from leaf to root that permit the flow of photosynthetic oxygen to areas of low concentration. Atmospheric oxygen may also be transported to anaerobic sites. The development of lysigenous ducts in roots has

been associated with poor soil aeration in various upland crops grown in Japan (20).

It is feasible that most plants will survive for considerable periods of time with their root systems under anaerobic conditions as suggested by James. The problem, however, takes on different proportions with economic crops. Where optimum growing conditions are desirable, slight anaerobiosis may affect the efficiency of a growing plant, not in terms of an energy differential between anaerobic and aerobic respiration (19) but rather in terms of essential processes characteristic of aerobic metabolism and the effect of toxic substances formed during anaerobiosis.

There are several references available (2,4,6) which develop the history of alcoholic fermentation from carbohydrates. In 1861 Pasteur demonstrated that yeast cells derived energy from glucose in the absence of oxygen. Following this classical discovery in 1897 Hans and Edward Buchner accidently discovered fermentation when they attempted to preserve yeast extracts by the addition of large amounts of sucrose. This discovery was the basis of modern enzyme chemistry for it was the first demonstration of fermentation in the absence of living cells.

Harden and Young in 1905 demonstrated that a rapid increase of fermentation occurred from yeast juice when inorganic phosphate was added to the fermentation solution. This suggested that organic phosphate esters were probably formed and they succeeded in isolating fructofuranose-1, 6-diphosphate (Harden-Young ester). Following this

discovery glucopyranose-6-phosphate (Robison ester), fructofuranose-6-phosphate (Neuberg ester) and glucopyranose-1-phosphate (Cori ester) organic phosphates were isolated.

In a second major contribution Harden and Young discovered that yeast extract lost its activity if dialysed and that activity could be restored either by adding the dialysate or small portions of boiled yeast extract. Thus they were able to show that the extract contained a non-dialysable thermolabile enzyme and a thermostable co-enzyme later known as DPN.

Subsequent establishment of the glycolytic pathway was dependent upon the research of many workers. Among the researchers associated with the present day concept of glycolysis are Embden, Neuberg, Meyerhof, Parnas, D. M. Needham and Warburg. A summary of the glycolytic scheme may be found in most biochemistry textbooks.

The reactions involved in the formation of anaerobic end-products of carbohydrate metabolism are of specific importance to this study. Ethanol, lactic acid and glycerol are the most common products of anaerobiosis. Small quantities of glycerol are known to be formed during fermentation by yeast. This is attributed to the small amount of DPNH which reacts with dihydroxyacetone phosphate to form phosphoglycerol. Phosphoglycerol is in turn hydrolyzed forming free glycerol and phosphate. Neuberg increased the yield of glycerol from fermenting liquors by two methods termed his "second" and "third" fermentations. In both cases the amount of acetaldehyde reduced to ethanol was restricted. This increased the amount of

DPNH available for the reduction of dihydroxyacetone phosphate and glycerol was formed as described above.

Ethanol and lactic acid are the most common anaerobic end-products in higher plants (18). Lactic acid is formed in those systems where pyruvic acid is not decarboxylated. In 1907 Fletcher and Hopkins demonstrated lactic acid formation during muscle contraction in frogs. Meyerhof in 1927 showed that the amount of lactic acid formed was chemically equivalent to glycogen break down. It was shown later that the enzyme lactic acid dehydrogenase was involved where DPNH was oxidized and pyruvic acid reduced to lactic acid.

Neuberg demonstrated that yeast was capable of decarboxylating pyruvic acid with the formation of acetaldehyde. His "first" fermentation showed the reduction of acetaldehyde to ethanol. Again, an oxidation-reduction system is involved where DPNH is oxidized and the aldehyde reduced.

Anaerobic respiration theories proposed by physiologists for higher plants and animals paralleled the discovery of these basic fermentation mechanisms. Shortly after Pasteur's discovery, workers became interested in fermentation studies on sugar beets and other plants. During the early 1900's much confusion developed (10) from the lack of appreciation of the complex enzyme systems involved in fermentation. Pfeffer, Kostychev and Blackman were finally credited with showing the relationship between anaerobic and aerobic respiration or the common-path theory.

To satisfy the requirements of alcohol fermentation the carbon

dioxide-ethanol ratio must equal unity with glucose as a substrate. Several workers have studied alcoholic fermentation in higher plants. Stiles and Dent (17) and Bennet- Clark (3) attempted to show true fermentation in beet root slices. They obtained ratios near unity. Phillips (12) also demonstrated approximately equivalent amounts of carbon dioxide and ethanol from carbohydrate fermentation in rice and barley seedlings under anaerobic conditions. Nance (11) obtained ethanol values close to theoretical from known concentrations of glucose in barley roots, however, the carbon dioxide concentrations were much higher than expected. The discrepancy was attributed to a non-carbohydrate source of carbon dioxide. Recently (1) high ADH activity has been correlated with ethanol concentrations in rice shoots. These workers suggested the role of ADH in rice was one of an adaptive enzyme since increased enzymatic activity was associated with low oxygen concentrations.

These references provide a basis for suspecting ethanol as a primary end-product in anaerobic metabolism. The continued accumulation of any anaerobic product with physiological activity eventually will be injurious. It therefore seems logical that plants would have a protective mechanism whereby these products are inactivated and/or eliminated, yet there is little of this type of information available. The elimination of ethanol was not detected in rice and barley seedling studies described above (11,12).

TERMS AND ABBREVIATIONS USED

"Sugar beet" or "beet" will be used for Beta Vulgaris. "Intact plant" as used in this study will mean tap root, crown and the youngest two to three leaves. Tap root will be designated as "root" and will be separated from the hypocotyl by a transverse cut at the origin of the first lateral roots. "Anaerobiosis" will be used, however only partial anaerobic conditions will be obtained due to residual oxygen in the plant tissue and a slight contamination of oxygen in the nitrogen gas and water. The term "distillation" will be used to mean steam distillation, the process used in this study to recover ethanol from tissue.

The following abbreviations were used; ADH- lyophilized yeast alcohol dehydrogenase (Worthington Biochemical), DPN- diphosphopyridine nucleotide (Sigma). APDPN-3-acetylpyridine diphosphopyridine nucleotide (Pabst Laboratories).

MATERIALS AND METHODS

Preparation of Beet Root Tissue for Anaerobic Treatment. The sugar beets were harvested and the roots washed and scrubbed with a brush under a stream of tap water. All leaves were then removed except the youngest 2 or 3. The intact plant, including the leaves was

then placed in a dark chamber for anaerobic treatment.

Preparation of Beet Root Tissue for Distillation. After completing the anaerobic treatment the beet roots were washed and blotted dry and the root removed for use in distillation.

Uniformity of samples was maintained by halving, quartering and trimming the root longitudinally until desired sample weight was obtained. The same size sample was used for all quantitative ethanol determinations within each experiment. The root was immediately placed in a Waring Blender with sufficient distilled water to give good agitation during grinding for 30 seconds. The pH of the sample was lowered to 2.5 - 3.0 by the addition of tartaric acid crystals.

The total volume of distilled water added to the sample was determined by the amount required to quantitatively wash the tissue into the distillation flask, but again was constant with each experiment.

Distillation of Beet Root Tissue. The rate of distillation was maintained constant by employing a manometer on the gas line to the steam generator. The duration of distillation was determined by the quantity of tissue and the water added. It was found that 95-97% of the known ethanol could be recovered from beet root tissue by collecting 5-10 ml. of distillate in excess of the water volume initially added. Foaming was prevented by applying a trace of Dow Antifoam A on the inside of the distillation flask.

Storage of Distillate. The method for storing the beet distillate

was dependent on the procedure used for quantitative determination of ethanol. For the dichromate procedure, 1 drop of concentrated sulphuric acid lowered the pH of 50-60 ml. of distillate to 2.5-3.0. For the ADH determination of ethanol, the distillate was buffered with 0.1 M pyrophosphate at pH 9.5. In both procedures the distillate was stoppered and stored at 40° F.

Dichromate Determination of Ethanol. Harger's method (5) for ethanol determination was used. In those instances where further dilution of the distillate was required it was determined by using a series of three dilutions of the original distillate and reacting 5 ml. from each dilution with 1 ml. of standard dichromate and 5 ml. of concentrated sulphuric acid. A visual check of the intensity of yellow color indicated the proper dilution. A known concentration of ethanol was used to standardize the dichromate for each series of determinations.

Procedure for Enzymatic Determination of Ethanol. Actual identification of ethanol in sugar beet distillate was accomplished enzymatically. The high degree of specificity of yeast ethanol dehydrogenase for ethanol provides a quantitative method of characterizing this alcohol in tissue distillate. The procedure involves oxidation of the ethanol to acetaldehyde. The oxidation is accompanied by an equimolar reduction of DPN. Since the DPN is all in the oxidized form initially, the amount of ethanol oxidized is equal to the amount of DPN reduced. The quantity of reduced DPN can be determined

spectrophotometrically at a wave length of 340 mu. The reduced form has a fairly high plateau of absorbency at this band while the oxidized form does not absorb in this region of the spectrum. The reaction is reversible and is presented in the following equation where k_1 is the velocity constant of the forward and k_2 is the velocity constant of the reverse reaction at any given temperature.

$$CH_3CH_2OH + DPN \underset{k_2}{\overset{k_1}{}} CH_3CHO + DPNH + H^+$$

According to the law of mass action, the velocity of each reaction is equal to the product of the activities of the reactants (approximately the concentrations for dilute solutions) raised to the power of their coefficients (one in this case) times the velocity constant. Equilibrium is attained when the velocity of the forward and reverse reactions are equal. The proportion of the components of the reaction at equilibrium is given by the following equation where K is the equilibrium constant and is equal to the ratio of the velocity constants.

$$\frac{(CH_3CHO)(DPNH)(H^+)}{(CH_3CH_2OH)(DPN)} = K = 1.1 \times 10^{-11} \quad (13)$$

The greater the displacement from equilibrium, the more energy is available to drive the reaction to equilibrium. The velocity with which equilibrium is approached is dependent upon the catalytic activity of the enzyme, alcohol dehydrogenase. The reaction was buffered at pH 9 since the low hydrogen ion concentration favors

oxidation of the ethanol and a higher pH seriously interferes with enzymatic activity. Therefore, the hydrogen ion concentration can be included with the equilibrium constant and the equation simplified to the following.

$$\frac{(CH_3CHO)(DPNH)}{(CH_3CH_2OH)(DPN)} = K_2$$

Racker's assay (13) was used to evaluate the catalytic activity of commercial enzyme preparations. A zero order reaction was attained by using excess ethanol and the initial reaction velocity determined. An enzyme preparation precipitated with ammonium sulfate was found to be inactive however sufficient activity was obtained from a lyophilized preparation.

A quantitative determination of ethanol required modification of this assay so that more than 95% of the ethanol present in dilute solution would be oxidized to acetaldehyde. The final proportion of aldehyde to ethanol was evaluated from the equilibrium equation. Semicarbazide was introduced since lowering the acetaldehyde concentration by formation of the semicarbazone would tend to drive the oxidation of ethanol to completion. It was not successful since less than 10 per cent of the ethanol was oxidized at equilibrium. The next obvious step was to increase the concentration of DPN. At the same time the pH was increased one half unit to make the equilibrium more favorable. The loss of catalytic activity due to the change in pH was overcome by increasing the enzyme concentration. An increase of DPN concentration to 10 times the original assay

concentration resulted in an increase in ethanol oxidation from less than 10 per cent to about 40 per cent.

In the meantime, the acetylpyridine analog of DPN was obtained since it has a more favorable equilibrium constant (8). Using a high concentration of the analog in place of the DPN proved successful for the quantitative determination of ethanol.

The assay procedure used was essentially the same as that reported by Kaplan (9). 1.2 mgs. of APDPN were weighed into 3" assay tubes followed by the addition of 0.6 ml. of 0.1 M pyrophosphate buffer (pH 9.5) containing the ethanol sample. After complete mixing with the analog 0.5 ml. was withdrawn and pipetted into a 1 ml. cuvette. To this 0.1 ml. (100 ugm) of ADH was added and the reaction incubated at 37° C for 30 minutes. At the end of the incubation period the sample was diluted to 1 ml. with glass distilled water and the optical density determined at 365 mu. in a Beckman D.U. spectrophotometer. The reference cell contained all reactants except the APDPN. A blank containing no added ethanol was included with each group of determinations since ethanol was present as a contaminant in the reagents. The blank value was substracted from all final optical density readings on known and unknown samples. A standard curve with known ethanol dilutions was obtained with each group of unknowns.

EXPERIMENT 1. ANAEROBIC ACCUMULATION OF ETHANOL AND ITS AEROBIC ELIMINATION

The purpose of this experiment was to study the accumulation of ethanol after anaerobiosis and its rate of elimination during an aerobic recovery period. Selection of anaerobic time intervals was influenced by concern over possible contamination from anaerobic microorganisms. This was particularly true in the case of the three hour treatment. It was assumed there would be only slight increases in the anaerobic microorganism populations during this time interval. In addition the thorough washings in running water which each root received both before and after treatment would minimize this type of contamination. No visual decomposition occurred in any of the samples either before or after treatment.

Experimental Procedure. Sugar beets (variety 401) were pulled on August 18, 1958 from a field planted May 15th on the University Farm. The plant roots selected varied from 80-100 grams. The roots were washed with a brush under running tap water and all leaves were removed except the youngest two.

The treatments were applied by the use of the apparatus shown in Figure 1. Aluminum screening was wrapped around each beet root for support and held in place by a rubber band. The beet roots and

Figure 1. Apparatus used for Anaerobic Treatment in Experiment 1.

supports were then placed on the metal container and the container placed, with stopcock up, into the earthenware crock. With the stopcock open, tap water was forced into the system at the bottom replacing the air inside. When the water level reached the top of the earthenware crock it was turned off and the stopcock opening was attached to a tank of dry nitrogen. The stopper was removed from the bottom opening and nitrogen gas replaced the water. When the water level was approximately two inches from the bottom, the stopcock was again closed and the stopper replaced in the crock. The procedure was repeated once more to minimize the oxygen present in the metal container. By maintaining water in the bottom of the container it acted as a seal from the atmosphere.

Nine plants were placed in each of four containers. Two separate anaerobic treatments were used, three and six hours, with eighteen plants per treatment.

Two beets were distilled immediately after harvesting from the field and represented the non-treated beets. (The distillation schedule did not permit distilling non-treated beets at the end of each aerobic time interval, however previous analysis of beets wrapped in damp paper toweling and placed on the laboratory bench for 12 and 24 hours showed no increase in ethanol.)

At the end of the three and six hour anaerobic treatment one beet from each container and therefore two from each treatment were harvested and prepared for distillation. (Throughout the remaining distillations and ensuing titrations data on beets from each container were kept separate.) The remaining beets were washed and returned to

aerobic conditions by transplanting in poly-ethylene sacks containing sand as shown in Figure 2. Subsequent distillations of beets were made at various aerobic time periods.

Preparation of Beet Roots for Distillation. At scheduled time intervals the beets were harvested and washed with tap water, excess water being removed by blotting the roots. The tap root was removed and approximately 20 gram samples were obtained for distillation. The sample was weighed to the closest 1/10 gram. Approximately 10 grams of remaining tissue was placed in tared weighing bottles and placed in an oven at 85° C for 12 hours for moisture determination.

The 20 gram sample for distillation was cut into small pieces and placed in the Waring Blender for grinding. Approximately 35 ml. of distilled water was added to the sample with enough tartaric acid to lower the pH to 2.5 - 3.0. The sample was ground for 30 seconds and then transferred to a 500 ml. ground glass distillation flask. The blender container was washed with distilled water to bring the total volume of added water to 75 ml.

The samples were steam-distilled for 13 minutes at which time 85-90 ml. of distillate was obtained. The distillate was condensed by a Graham intercoiled vertical condenser with ground glass joints.

Experimental Results. The data from this experiment are plotted in Figure 3. Each point in the graph represents a different sugar beet and an average of three titrations per distillate sample. Even with the nitrogen flushing precautions used, there apparently was a considerable difference in oxygen present within each of the

Figure 2. Aerobic Recovery of Sugar Beets Transplanted in Sand and Poly-ethylene Sacks, Treated with 3, 6, 9, 12 Hrs. of N Gas. Top row - 30 Hrs., Middle row - 7 Days, Bottom row 12 Days after Treatment.

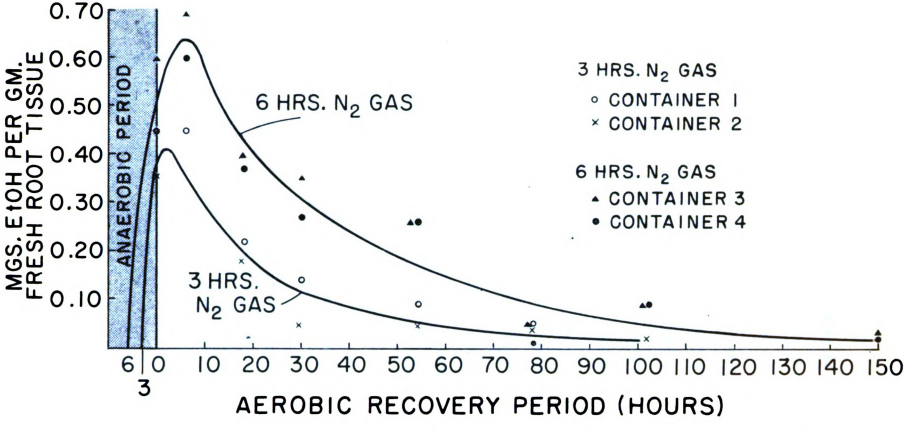

Figure 3. Accumulation of Ethanol in Sugar Beets Treated to 3 and 6 hours of Nitrogen Gas and its Elimination during an Aerobic Recovery Period.

apparatus. This would tend to explain differences in points within treatments. Part of this may also be explained on the basis of variation in level of the water seal at the bottom of each container.

There was a maximum of 5% variation in moisture content between beets which was considered to be within the range of experimental error. An estimated 10% inherent biological variation of this heterogeneous material was considered the greatest source of error.

This experiment was repeated again with the addition of two extra anaerobic treatments of 9 and 12 hours. The purpose of this experiment was to observe any visual damage which might result from longer exposure to anaerobiosis. The only dichromate determinations made were at the initial and 7th day intervals. This data is shown in Table 1. Five beets were used per treatment in which one from each treatment was selected for initial ethanol levels at zero aerobic time. The remaining four per treatment were used for observations.

Table 1. Ethanol Levels in Anaerobically Treated Sugar Beets at Initial and Seventh Day Aerobic Intervals.

Anaerobic Treatment	Mgs. Etoh/gm. fresh tissue	
Hours	Initial Aerobic	7th day Aerobic
3	0.27	0.01
6	0.61	0.09
9	0.65	0.64
12	1.76	0.14

Pictures taken at various stages of regrowth are shown in Figure 2. Only slight necrosis of the foliage occurred in the three and six hour treatments but damage was very evident in the nine and twelve hour treatments. It was also observed that injury occurred between the six and nine hour intervals with little or no apparent difference between the three and six or between the nine and twelve hour nitrogen treatments.

The high ethanol level observed for the nine hour treatment after seven days was caused by spoilage. All other values approached check levels at this time.

EXPERIMENT 2. ETHANOL ACCUMULATION IN FLOODED SUGAR BEETS

A flooding experiment was devised to simulate anaerobic conditions brought about by high water tables under field conditions.

Experimental Procedure. Sugar beets (Variety 401) were planted in coarse sand in 9 x 25 inch metal cylinders in the greenhouse on February 6th and as the second pair of primary leaves emerged the plants were thinned to 3 per cylinder. On April 13th two cylinders containing six plants were placed in a metal can which was equal in height to the plant containers. Water at room temperature was then slowly added to the can until the water level in the sand reached the base of the leaves. This indicated a complete flooding of the plants and the starting time for the treatment. The greenhouse temperature ranged from 68° F at night to 90° F during the day.

This experiment differed from the previous experiment and also the following ones in that no leaves were removed and the plants were not pulled from the soil until after the anaerobic treatment. Also the leaves were exposed to normal atmospheric oxygen concentration and were actively photosynthesizing. There was no aerobic recovery period in this experiment. Check plants were harvested with each treated beet.

The method of washing, sampling, and preparing the root tissue

for distillation was the same as in Experiment 1. The distillation and dichromate procedures for ethanol determination were also described.

Experimental Results. The results obtained from this experiment are shown in Table 2. While the difference in accumulation between treated and untreated beets were not as pronounced as in Experiment 1 the ethanol concentrations were consistently higher for all flooded beets. Perhaps the significance of the values of 0.03 obtained for the 1 and 2.25 hour interval could be questioned. At the 3.75 hour interval however, 0.06 mg. ethanol was obtained for the flooded compared to 0.01 mg. for the check plant. This difference was magnified as the end of the light period was approached as shown by the 9 hour flooding interval. After 21 hours of flooding (end of a dark period) a value of 0.14 mg. ethanol was obtained. The ethanol concentration was back to 0.07 mg. at the end of the second light period and 35 hours of flooding. This decrease was not readily explainable.

Table 2. The Effect of Root Flooding upon Ethanol Accumulation in Sugar Beet Root Tissue.

Sample	Flooding Treatment (Hours)	mgs. Etoh/gm. Fresh Root Weight
Check	----	0.01
Treated	1	0.03
Check	----	0.01
Treated	2.25	0.03
Check	----	0.01
Treated	3.75	0.06
Check	----	0.02
Treated	9.0	0.12
Check	----	0.01
Treated	21.0	0.14
Check	----	0.01
Treated	35.0	0.07

EXPERIMENT 3. ENZYMATIC CHARACTERIZATION OF ETHANOL IN BEET DISTILLATE

The purpose of this study was to determine what portion of previous dichromate values was attributable to ethanol.

Experimental Procedure. The sugar beets used for this study were planted in the greenhouse October 4, 1958 in 10 inch clay pots. The plants were grown in sand media, watered with nutrient solution and supplied with four hours of additional light per day. When the second pair of primary leaves emerged the plants were thinned to four per pot.

Two harvest dates were used in this experiment, January 29th and February 4th at which time the tap roots weighed 15-20 grams. The plants were pulled, roots washed and leaves removed as previously described.

The anaerobic treatments were applied by placing one intact plant into each of four 12 inch test tubes wrapped in a manner to eliminate light. With the exception of the check beet each of the other tubes were closed with rubber stoppers containing two ground glass stopcocks. One stopcock was connected to a vacuum pump and the other to a cylinder containing dry nitrogen. With both stopcocks open and the valve on the nitrogen tank closed, the system was

evacuated to 15 inches of mercury for the first five minutes and then increased to 25 inches for the final five minutes. At the end of the ten minute evacuation period the valve on the nitrogen cylinder was opened and the vacuum pump turned off. When the system reached atmospheric pressure, determined by the vacuum gauge, the stopcocks were closed.

Differences between each of the three treated beets were obtained by using one, two and three hour intervals in the nitrogen atmosphere. The check beets were distilled in one instance one hour after harvest and on the other two hours after harvest. Immediately after termination of each treatment the root tissue was prepared for steam distillation. To 12 grams of root tissue a total of 45 ml. of distilled water was added, approximately 60 ml. of distillate being collected from each sample.

A 25 ml. portion of the distillate was buffered with 0.1 M pyrophosphate at pH 9.5, stoppered and stored at 40° F for enzymatic ethanol determination. The remaining portion was acidified to pH 2.5 - 3.0 with 1 drop of concentrated sulphuric acid, stoppered and stored at 40° F for dichromate analysis.

Experimental Results. Three known ethanol concentrations were used to obtain a standard curve. A standard curve determination was made with each group of unknowns. Optical density values for blank determinations (no added ethanol) were substracted from known and unknown samples. There was no noticeable loss of enzyme activity during the three hours required to complete nine determinations.

A theoretical standard curve was obtained by multiplying the

extinction coefficient of APDPN (7.8×10^3) (7) by the molar concentration of each known ethanol dilution. As is shown in Figure 4 there was good agreement between the observed data and the theoretical curve.

The results of this experiment are shown in Table 3. According to enzymatic assay there was a range of 46.7 to 88.4 per cent of the dichromate values for the six treated sugar beets. Only one of the beets gave a value of 46.7 per cent which was considerably lower than the other five. This same sugar beet also gave a dichromate value which was considerably higher than the beet harvested January 29th which also received two hours of nitrogen gas. It suggested an abnormal beet. Another explanation for the variation in this data may be the presence of an inhibitor thus influencing enzyme activity. Disregarding the low value the data indicated 60-90% of the dichromate values were due to ethanol. The dichromate values again were averages of three titrations.

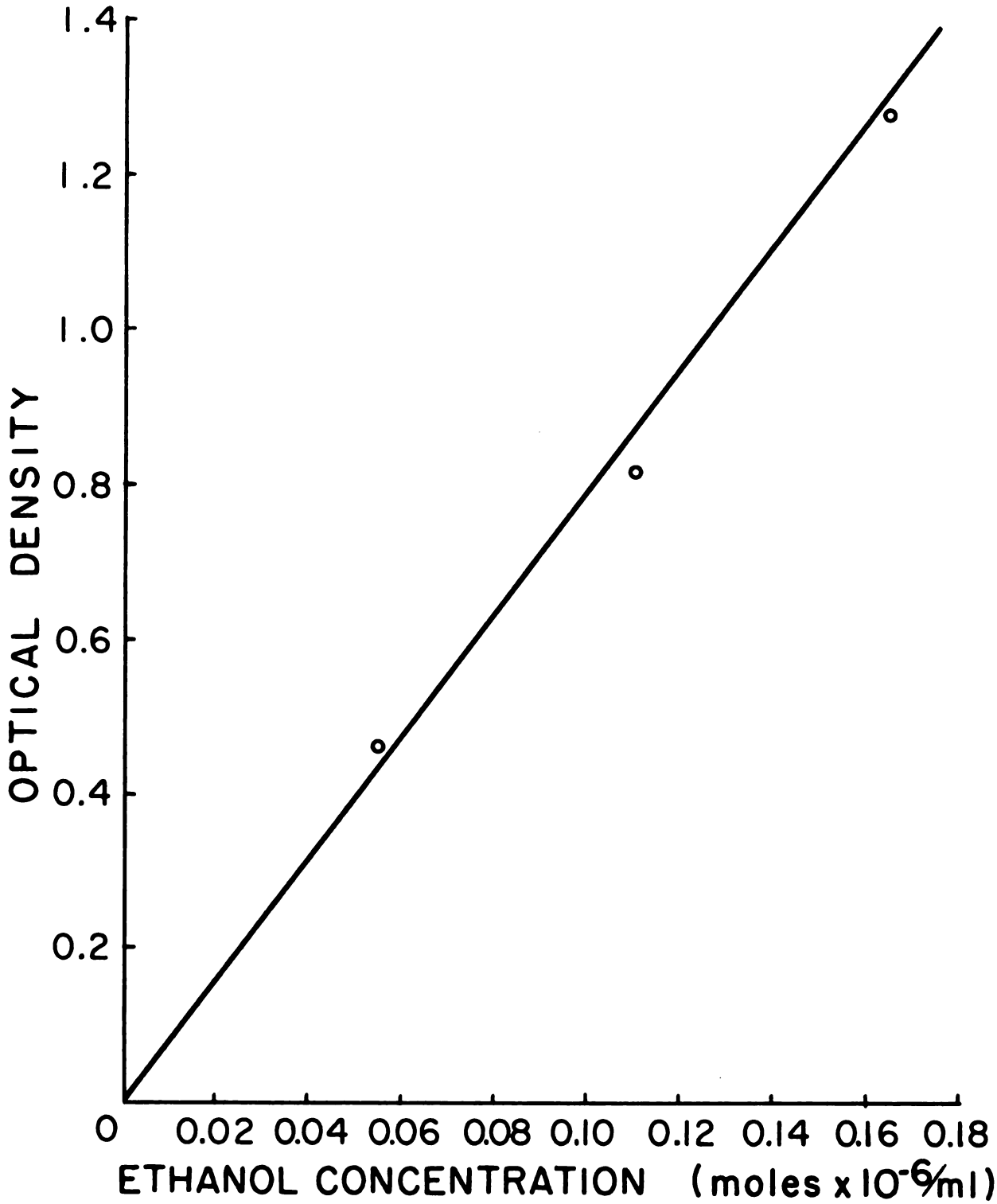

Figure 4. A Comparison of a Theoretical Standard Curve to Optical Density Values obtained from Three Known Ethanol Concentrations.

Table 3. Comparisons of Dichromate and Enzymatic Determined Ethanol Levels from Sugar Beet Distillate

Beet Treatment	Harvest Dates					
	January 29			February 4		
	Dichromate	ADH Assay	% of Dichromate	Dichromate	ADH Assay	% of Dichromate
Check	0.010*	0.003*	---	0.020*	----	
1 hr N_2gas	0.189	0.167	88.4	0.158	0.098*	62.0
2 hr N_2gas	0.290	0.235	81.0	0.368	0.172	46.7
3 hr N_2gas	0.534	0.422	79.0	0.472	0.359	76.1

* Expressed as mgs. Ethanol per gm. Fresh Weight of Root Tissue

EXPERIMENT 4. EVOLUTION OF ETHANOL FROM ANAEROBIC SUGAR BEETS

In general there are two possible mechanisms where plants could eliminate products of anaerobic respiration. One possibility might be actual metabolism of these materials and a second, the secretion of ethanol or closely allied substances from roots, leaves or both. This experiment was conducted to see if ethanol could actually be recovered from treated beets after their return to an aerobic environment.

Experimental Procedure. Sugar beets which were grown in the greenhouse were exposed to three hours of nitrogen gas in a dark chamber as described in the previous experiment. At the end of the treatment interval the roots were washed, wrapped in damp paper toweling and each plant placed in an aerobic chamber as shown in Figure 5. Air was passed over the surface of the plant by attaching the outlet to an aspirator. At the inlet the air was dehydrated and decontaminated by bubbling it through a tube of concentrated sulphuric acid. Condensation on the inside of the plant chamber was minimized by placing this tube in an ice bath. Air from the plant chamber, containing evaporated and transpired materials, was bubbled through 5 ml. of 70% sulphuric acid at the outlet. Water, ethanol and

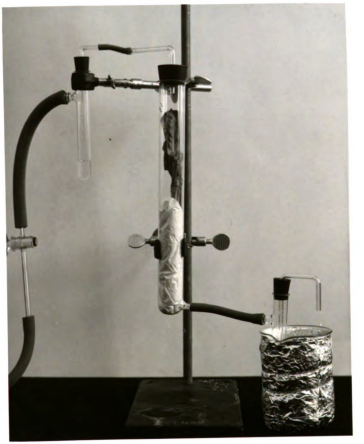

Figure 5. Apparatus Used for Collecting Ethanol from Sugar Beets which had Received an Anaerobic Treatment.

possibly other volitile materials were condensed in this trap.

At appropriate intervals the ethanol trap was removed and replaced with a fresh trap. Five milliliters of the removed sample were pipetted into a cuvette and 1 ml. of standard dichromate added (equivalent to 0.5 mg. of ethanol), mixed and allowed to react for 30 minutes at room temperature. At the end of the reaction time 2 ml. of 70% sulphuric acid were added and mixed in to bring the total volume to 8 ml. Optical density of the sample was determined at 440 mu. on a Coleman Jr. spectrophotometer. The procedure for recovery of ethanol was not standardized therefore only semi-quantitative results were obtained.

In the dichromate determination of ethanol, the reaction is one of oxidizing ethanol to acetic acid and in turn the dichromate is reduced. During this process the intensity of the characteristic yellow color of dichromate decreases as the ethanol in the sample increases. Thus the intensity of yellow color was inversely proportional to ethanol concentration. Optical density groupings of 0.00-0.10; 0.10-0.20; 0.20-0.30; 0.30-0,40; 0.40-0.50 were rated as 0, 1, 2, 3, 4, respectively.

Experimental Results. Two specific gravity determinations were made on condensate from each of the four ethanol traps. These values are compared to the specific gravity of the original 70% sulphuric acid dilution in Table 4. Differences in rate of air movement as well as total evaporating surface within the chamber could have influenced the dilution of the original 70% sulphuric acid. However,

differences in specific gravities of samples within each treatment were small ($\pm$ 0.001) when compared to differences in samples between treatments (more than $\pm$ 0.028). This suggests a much lower transpiration rate for the beets anaerobically treated during the time interval of this experiment.

Data in Table 4 shows the effect of anaerobic treatment upon ethanol recovery from intact sugar beets. There is strong evidence here to indicate that ethanol formed during anaerobiosis may be evolved from intact sugar beets under aerobic conditions. All non-treated beets rated either a 3 or 4 optical density whereas those treated were rated as 0. It was also noted that partial removal of oxygen resulted in an intermediate rating of 2.

Table 4. Evolution of Ethanol from Anaerobic Sugar Beets after Exposure to 32-36 hours of Normal Air.

Treatments	Oxygen Displacement	Specific Gravity 1	Specific Gravity 2	Optical Density Ratings
1. 70% H_2SO_4 Blank	----	1.559	1.554	4
2. Check - Feb. 16	None	---	---	4
3. 3 hrs N_2 Feb. 16	Complete	---	---	0
4. Check Feb. 18	None	1.478	1.477	3
5. Check Feb. 18	None	1.456	1.457	3
6. 3 hrs N_2 Feb. 18	Approx. 50%	1.507	1.506	2
7. 3 hrs N_2 Feb. 18	Complete	1.507	1.506	0

EXPERIMENT 5. TRANSPIRATION OF ETHANOL FROM SUGAR BEETS WHEN THE ROOTS WERE PLACED IN ETHANOL CONTAINING MEDIA.

The previous experiment established that at least a portion of the alcohol formed during anaerobic respiration could be secreted by the sugar beet plant. Since both the root and leaves were within the anaerobic chamber it was impossible to determine the exact mechanism of elimination. The assumption was made however, that a considerable amount would be transpired from the leaves. Also since data from Experiment 1 was concerned with ethanol elimination from root tissue it was necessary to demonstrate transport of ethanol from the root to leaf tissue. In the previous experiment the ethanol trapped could have been formed in the anaerobic leaf tissue and thus would not represent ethanol produced in anaerobic root tissue. The purpose of the following experiments was to study movement of ethanol from the root to the leaves.

Experimental Procedure. Three experiments were conducted (Experiments 5A, 5B, and 5C) in which the roots were immersed in media containing dilute ethanol solutions. The leaves were placed inside a glass chamber similar to the system shown in Figure 6.

A seal was made between the hypocotyl and the leaf chamber

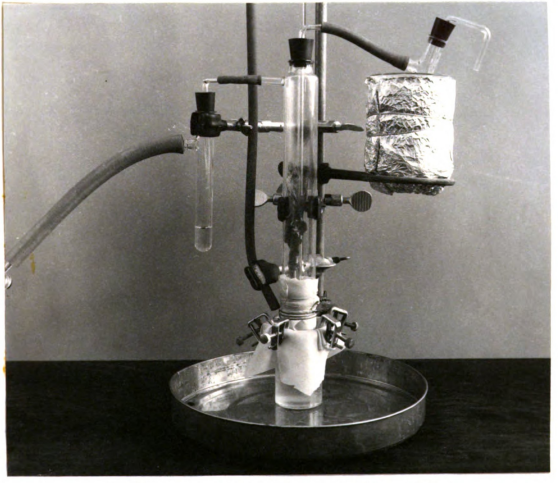

Figure 6. Apparatus used for Collecting Ethanol Transpired from Sugar Beet Leaves.

in the following manner. For Experiment 5A and 5B beets were selected in which the diameter of the hypocotyl was large enough to fit tightly into the base of the leaf chamber. To eliminate ethanol contamination from the media the glass-hypocotyl juncture was smeared with silicone stopcock grease. This joint was then wrapped firmly several times with Dow Saran Wrap. The seal was finally secured by wrapping rubber bands tightly on both sides of the joint and tying. This procedure provided a reasonably air-tight seal since the rate of bubbling, on both the inlet and outlet traps of sulphuric acid was constant indicating little if any leakage at the hypocotyl-glass seal. The method of cleaning, drying and moving the air through the leaf chamber was described in the previous experiment.

Two intact sugar beets were used in Experiment 5A. The ends of the tap root were cut off diagonally below a water surface and discarded. These cuts were made approximately 3 inches below the crown. The remaining root portion of one plant was then quickly transferred to a 1.5% ethanol solution and the other to a 0.5% solution.

In Experiment 5B two beets were transplanted into a quartz sand medium. In this experiment the roots were not cut off. The sand of the treated plant was moistened with a 1.5% solution of ethanol while distilled water was used for the check plant.

The hypocotyl-glass seal in Experiment 5C was improved by the use of rubber sheeting in addition to a water seal on the inside of the leaf chamber of the treated beet. A glass capillary was extended into the leaf chamber which permitted periodic replacement of

this water. The water seal was not used for the check beet since it would have created a small anaerobic area on the plant.

The hypocotyl-glass seal consisted of two separate seals similar to the upper seals shown in Figure 7. A small hole was punched in each of two 4 x 4 inch rubber sheets. Each sheet was stretched, expanding the hole, and pulled on over the tap root. One was placed on the upper portion of the hypocotyl and the other on the lower portion. A quarter inch rubber sheeting flange was formed at the juncture with the root. These flanges were wrapped tightly and tied with rubber bands. After placing the leaves inside the chamber the two rubber sheets were folded over the base of the leaf chamber and tightly secured with fine wire. A third rubber sheeting seal was made below the hypocotyl area in a similar manner and fastened around the sand container. Similar seals were made on both the treated and check beets, but again the additional water seal was not used for the check beet. Here as in Experiment 5B whole roots were used and quartz sand moistened with a 1.5% solution of ethanol for the treated beet and distilled water for the check beet.

The same procedure was used for ethanol analysis as described in Experiment 4. Optical density ratings were also used in summarizing this data.

Experimental Results. Data obtained from these experiments are combined in Table 5. In Experiment 5A, 13 hours after starting the treatment, a considerable amount of ethanol was trapped from the treatment with 1.5% ethanol (optical density rating of 0) while only

a slight amount (optical density rating of 3) was collected from the beet treated with 0.5% ethanol. However after 17 hours and for the remainder of the experiment a rating of 0 was obtained for both treatments. The results of this experiment were somewhat questionable due to this first attempt at making a seal.

The result from Experiment 5B showed a rating of 0 after 12 hours for the treated beet and this high ethanol value continued for the remainder of the experiment. The untreated beet however, showed no transpiration of ethanol.

Data from Experiment 5C was considered more reliable than the data from 5A and 5B due to the improvement made in the seals. In this experiment the treated beet gave the same optical density rating as the untreated after 9.5 hours indicating no transpired ethanol. At the end of 22 hours a rating of 1 was obtained for the treated compared to 4 for the untreated plant. For the 30 and 51.4 hour intervals no difference was obtained between the treated and untreated beets, however after 55.5 hours 0 and 4 ratings were observed for the treated and untreated beets respectively. The lack of differences at the two previous intervals could not be explained, but it did suggest ethanol contamination through the seal was no longer a problem.

Wet spots occurred on the leaves of the plant in 1.5% ethanol solution in Experiment 5A. These spots developed into necrotic areas similar to those on the leaves of plants at the 6, 9, and 12 hour anaerobic treatments in Experiments 1 and 2. In Experiments 5B and 5C the foliage of treated plants showed pronounced stimulation in growth when compared to the untreated plants. This was also observed

for the intermediate anaerobic treatment of 6 hours in Experiment 1. No visual damage was apparent on the roots of any of these plants, however in 5C the check plant had started regrowth of hair roots compared with no regrowth for the treated plant.

Table 5. Transpiration of Ethanol from Sugar Beets with Roots in an Ethanol-Containing Media.

Experiment	Hrs. from Start of Experiment	Hrs. Between Samples	Optical Density Ratings		
			1.5% Etoh	0.5% Etoh	Check
5A	3	3	4	4	-
	13	10	0	3	-
	17	4	0	0	-
	34	17	0	0	-
5B	12	12	0	-	4
	17	5	0	-	4
	19	2	0	-	4
5C	9.5	9.5	4	-	4
	22.0	12.5	1	-	4
	30.0	8.0	4	-	4
	41.5	11.5	4	-	4
	55.5	14.0	0	-	4

EXPERIMENT 6. FLOWING WATER SEAL TO ELIMINATE ETHANOL CONTAMINATION IN THE LEAF CHAMBER.

With the various types of seals used in Experiment 5 there was still a question of seepage or diffusion of ethanol from the media through the seals into the plant chamber. The purpose of the following experiment was to develop a seal which would reduce possible ethanol contamination in the system.

Experimental Procedure. The system was essentially the same as described in Experiment 5A where the lower portion of the root was cut off diagonally below a water surface and discarded and the remaining root portion was placed in a 1.5% ethanol solution. However a better seal was developed to demonstrate that all the ethanol which was trapped was actually transpired by the enclosed leaves.

The seal is shown in Figures 6 and 7. Two 4 x 4 inch rubber sheets were placed on the upper portion of the hypocotyl as described in Experiment 5C. The leaves and crown were then placed in the chamber as previously described.

Two more rubber sheets were cut as before and placed on the lower portion of the beet root. The first sheet, which would eventually be fastened around the jar containing the ethanol solution, was placed on the root with the flange upward (about ½" of root tissue

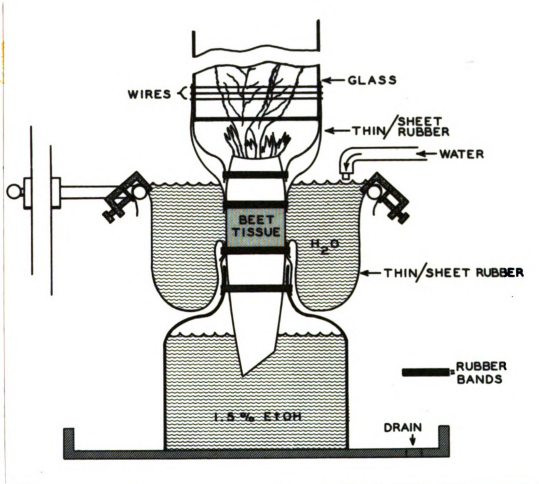

Figure 7. Diagram of the "Flowing-Water Seal" used to Eliminate Ethanol Contamination in the Leaf Chamber. (See Figure 6.)

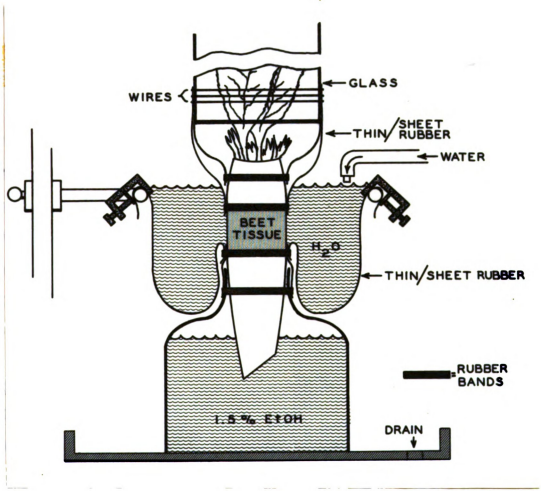

Figure 7. Diagram of the "Flowing-Water Seal" used to Eliminate Ethanol Contamination in the Leaf Chamber. (See Figure 6.)

was exposed between the upper seal and this seal). The final rubber sheet, the one which formed a cup for the water bath, was superimposed over the flange on the root created by the jar cover,however, this flange was turned in the opposite direction. Both seals were then secured by wrapping and tying with rubber bands.

The lower portion of the tap root was then removed as previously described. The root was quickly transferred into 1.5% ethanol solution. The jar-cover-sheet was fastened tightly around the lip of the jar containing the ethanol solution. The next sheet was formed into a cup supported by a $2\frac{1}{2}$ inch ring. Distilled water at room temperature supplied at constant pressure was then allowed to pass over the exposed root tissue with the excess flowing to the drain. By continuous washing of an exposed $\frac{1}{2}$ inch section of the root located between a double latex seal on the ethanol solution bottle and another double seal fastened to the plant chamber, contamination was minimized.

Experimental Results. Data obtained from this study are shown in Table 6. No check plant was included since it was well established that plants under normal aerobic conditions did not transpire ethanol. During the first 16 hours no ethanol was recovered. At the end of 23 hours however, the plant had started to transpire ethanol as indicated by an optical density of 0.32. The following 7.5 hour interval a considerably larger quantity was trapped yielding an optical density of 0.10 suggesting ethanol was being transpired at a much faster rate.

At the end of 30.5 hours a water soluble dye was injected into the ethanol solution. The experiment was terminated at the end of 45

hours at which time an optical density of 0.06 was obtained from condensate in the ethanol trap. The root and foliage were dissected and the dye was traced in the vascular system to the extremities of the leaves. Thus it was established that the four water tight seals on the root had not disrupted the functioning of the vascular system. In addition no visible breakdown of tissue could be detected. As a final check 1 ml. of standard dichromate was added to the scrub and no change in color intensity occurred.

Table 6. Transpiration of Ethanol from Beet Leaves with the Root placed in an Ethanol Solution. (Flowing H_2O Seal)

Hours from Start of Experiment	Hours Collection Per Sample	Optical Density at 440 (mu)
4.5	4.5	0.46
16.0	11.5	0.46
23.0	7.0	0.32
30.5	7.5	0.10
45.0	14.5	0.06

EXPERIMENT 7. TRANSPIRATION OF ETHANOL FROM A SUGAR BEET WITH ITS ROOT IMMERSED IN WATER.

This experiment was a final attempt to demonstrate a transfer of ethanol from the root to the leaves. The source of plant material and method of harvesting has been described in Experiment 6.

Experimental Procedure. Two sugar beet plants were used in this experiment, one treated, the other untreated. The treatment was imposed in the following manner. After washing the root, the leaves were removed as previously described. Two 4 x 4 inch rubber sheets were fastened to the hypocotyl as in Experiment 5C. The tap root was placed in a 4 inch test tube having a diameter of 1 inch. This test tube was then filled with water and the rubber sheeting folded over the outside of the tube and sealed by wrapping with fine wire. The sugar beet plant with the sealed test tube was then placed inside a plant chamber as shown in Figure 5. Dry decontaminated air was then passed over the leaf surfaces and the transpired material condensed in 70 per cent sulphuric acid as before. The same system was used for the untreated plant except in this case the root was wrapped with damp paper toweling and placed in the 4 inch test tube without sealing or adding water.

Experimental Results. The data from this experiment are shown in Table 7. No differences were obtained between the treated and untreated plants 26.5 hours after the start of the experiment. An optical density of 0.35 was obtained for the treated plant after 37.5 hours compared to 0.44 for the untreated plant indicating transpired ethanol from the treated beet. At the end of the experiment (50.5 hours) 5 ml. of the condensate from the treated beet reduced nearly all the dichromate while the optical density for the untreated plant remained unchanged. Thus this experiment showed conclusively that ethanol formed anaerobically in a sugar beet root could be transpired.

Table 7. Transpiration of Ethanol Formed in an Anaerobic Sugar Beet Root.

Hours from Start of Experiment	Collection Time (Hours)	Optical Density	
		Treated	Untreated
14.0	14.0	0.46	0.46
26.5	12.5	0.44	0.46
37.5	11.0	0.35	0.44
50.5	13.0	0.06	0.44

DISCUSSION

The elimination of toxic materials accumulated during periods of stress is obviously an important process in all organisms. Not only is it important from the standpoint of survival but also with respect to recovery after any particular stress has been removed. Like most major biological processes this perhaps is not a single step but rather a series of reactions controlled by a specific system characteristic of the organism. Although ethanol is toxic to living systems, its physiological activity is reduced considerably compared to its precursor acetaldehyde. The function of alcohol dehydrogenase in sugar beets then may be considered as a first step in the detoxification of a more physiologically active substance. Alcohol dehydrogenase apparently functions as an adaptive enzyme as was suggested in the study on rice (1).

The amount of ethanol which accumulated in sugar beet root tissue was dependent upon the length and type of anaerobic treatment. In Experiment 1 the rate of increase was much greater for the 3 hour than for the 6 hour treatment with nitrogen gas. There are three apparent explanations for this. First, the amount which accumulates in 3 hours may represent stress in the bulk of the root tissue which is actively metabolizing while the additional 3 hours in the 6 hour

treatment would involve some of the less active tissue. This argument does not appear valid because of the alternation of meristematic and vascular tissue throughout the radius of the root tissue. It may be possible that some of the inner meristematic tissue, because of age, was not respiring as rapidly. The proportion of meristematic to vascular tissue within each ring decreases from the periphery to the inside of the root. This also represented the direction of oxygen stress with time, for a root immersed in an anaerobic atmosphere. Second, the reduced rate of ethanol accumulation for the additional 3 hour period may have been influenced by some elimination mechanism which was initiated during this interval. Third, the metabolism of the root tissue may have been reduced due to the accumulation of toxic levels of anaerobic end-products resulting in injury to the plants. The decreased rate of ethanol accumulation during the second 3 hour period could have resulted from any one or a combination of these factors.

The amount of ethanol which accumulated in root tissue when only the root system was flooded (Experiment 2) was strikingly reduced. In this experiment the foliage extended into normal air and carried on normal photosynthetic activity whereas in Experiment 1 the leaves,as well as the root system, were in an anaerobic environment and did not receive light. Another major difference between Experiments 1 and 2 was that additional leaves were left on in Experiment 2 thus providing more surface for oxygen supply and ethanol elimination.

The most important factor responsible for the difference in

ethanol accumulation in these two experiments was attributed to the influence of the foliage being in normal air. The leaves are capable of supplying oxygen to root from two sources; (1) by diffusion from the normal atmospheric concentrations and (2) by diffusion as a by-product of photosynthesis. Both of these sources are important for plants under flooded conditions, however, the data in Experiment 2 particularly suggests the role of photosynthetic oxygen under these conditions. Ethanol accumulated slowly during high light and temperature conditions of the day, however, the amount doubled as the end of the light period was approached. The ethanol concentrations did not increase much during the following dark period and actually decreased at the end of the second light period. This does not seem consistent but by this time elimination mechanisms could have been functioning and perhaps there was a redistribution of the ethanol from the root to other plant parts.

A study of the formation of anaerobic end-products in plants growing under high water table conditions has considerable potential as a means of detecting oxygen stress. If the root system of a plant is flooded and then the whole plant pictured as an oxygen gradient system, the leaves would represent tissue with normal oxygen concentrations while the extremities of the roots would perhaps represent tissue subjected to complete anaerobiosis. If such a system actually exists in plants then perhaps the taproot does not represent a tissue site of severe anaerobiosis and only a small difference in ethanol levels would be expected.

A study of a possible oxygen gradient system in plants under

flooded conditions would require micro-quantitative ethanol determinations in root hairs. Microdiffusion techniques could be used to great advantage in this type of study. Once the site of severe oxygen stress is established greater differences would likely be obtained than were found in this particular flooding experiment. The system of root hairs represents an essential part of a growing plant and an interference in their normal metabolism would seriously curtail plant efficiency.

Data from this study (Figure 3) clearly indicated sugar beets were capable of eliminating ethanol after being returned to an anaerobic environment. The slight increase in ethanol which occurred after returning the plant to normal air may have been due to three factors. First, and most obvious, when comparing the 3 and 6 hour treatment, the lag represented the time required for oxygen to diffuse back into the tissue. With the 6 hour treatment anaerobiosis penetrated deeper and therefore it took longer for oxygen to re-enter these areas. Second, it may have been due to a slow conversion back to normal aerobic metabolism suggesting possible injury at these ethanol levels. Finally the lag could have been attributed to an increase in anaerobic microorganism population, however, a much greater increase would be expected if organisms were involved. The 1.76 mgs. ethanol per gram of fresh root tissue which occurred in the treatment with 12 hours of nitrogen (Table 1) may indicate that such was the case.

The pattern of elimination was, in general, an exponential function. This was particularly true for the 3 hour treatment. For the 6 hour treatment, however, a plateau occurred between the 18 and

54 hour aerobic interval which suggests a second limiting factor in ethanol elimination. Further study is required to verify this point and establish its significance.

The detection of injury was dependent upon growth comparisons during recovery, macroscopic and microscopic examination of tissue sections. It was not possible from the data to determine a threshold concentration of ethanol where injury occurred. It was however, possible to observe injury due to the direct effect of high ethanol concentration in sugar beet leaf tissue.

Dark necrotic spots developed in leaves of plants receiving 6 or more hours of nitrogen gas. These spots were also apparent when plants roots were placed in 1.5 per cent ethanol solution. It was assumed that the ethanol increased the permeability of cell membranes since these spots first appeared as wet areas and then developed into necrotic spots.

A stimulation of foliage elongation was suggested at the intermediate treatment of 6 hours and actually occurred in plants whose roots were in an ethanol containing medium. This could be interpreted as injury in the sense that abnormal growth occurred. Foliage retardation occurred beyond the 6 hour anaerobic period however the direct cause was not apparent.

This study has shown the important role of leaves in the elimination process. Perhaps a closer study of this particular function of foliage would provide valuable information on the metabolism of plants.

CONCLUSION

A volatile substance was obtained from sugar beets which were treated anaerobically. Enzymatic characterization of this substance established the primary anaerobic end-product as ethanol. Ethanol accumulated rapidly under high oxygen stress and the pattern of elimination approximated an exponential relationship. Transpiration from foliage was the only detected means of elimination.

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