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THE EFFECTS OF ETHANOL ON LABELING OF Y-AMINOBUTYRIC ACID BY PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN OAT LEAVES

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THE EFFECTS OF ETHANOL ON LABELING OF γ-AMINOBUTYRIC ACID BY PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN OAT LEAVES

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CHAPTER I

Introduction

A review of the literature reveals that dilute solutions of ethyl alcohol have commonly been used as solvents for growth regulating substances in studies on growth and metabolism in plants. However within recent years low concentrations of ethanol have been shown to materially affect the growth and metabolic processes in plants. Ber and Moskwa (4) observed substantial increments in both shoot height and shoot weight following treatment of oat seedlings with weak concentrations of ethanol. Later Mer (15, 16) showed that low non-toxic dosages of ethyl alcohol greatly enhanced mesocotyl growth in oats.

Anker (2) reported that upon the addition of Avena coleoptile segments to media containing small quantities of ethanol an increase in oxygen consumption became evident. Similar results with carrot root sections were observed by Lowe and James (14).

Rohrbaugh (19) while studying the effects of plant growth regulators on intermediary metabolism in leaves dissolved certain of these substances in dilute ethanol. Subsequent to pretreatment in water,

dilute ethanol, and solutions of growth regulators in dilute alcohol the leaves were allowed to fix $C^{14}O_2$ photosynthetically. He noted that the dilute alcohol augmented the percentage of label going into some metabolites.

Earlier I (13) studied the effects of ethanol on the labeling of intermediary products in the leaves of oat and pea. The data showed that generally alcohol brought about slight changes in labeling trends in most metabolites but the percentage of C^{14} fixed into γ-aminobutyric acid appeared notably enhanced. This amino acid is known to be widespread among animals, plants, and microorganisms (17, 20, 24, 27).

Generally in animals only tissues of the brain have been shown to contain a significant quantity of γ-aminobutyric acid and it is now believed to play a major role in the neurotransmission mechanism of the central nervous system (1, 9). Among mammals this metabolite seems to be almost completely restricted to the tissues of the brain (18). Hakkinen and Kulonen (11) obtained data which showed that the brain level of γ-aminobutyric acid in Wistar rats was raised by as much as 34% shortly after administering ethanol as a 33% aqueous solution orally by means of a stomach tube. Later Higgins (12) reported a similar rise in brain γ-aminobutyric acid content under the same conditions.

In extracts of various plant tissue, γ-aminobutyric acid has been found to be one of the major constituents of the free amino nitrogen pool (17, 23, 25). Yet, in spite of its abundance and exceedingly broad distribution, the basic role of this compound in the metabolism of plants is not well known.

The present paper deals with a study of the effects of ethanol

on the products formed in short-time photosynthesis in oat leaves with special attention to the metabolism of γ-aminobutyric acid.

CHAPTER II

Materials and Methods

Excised Victory oat (Avena sativa L.) leaves were used in the experiments described in this study. They were taken from seedlings kept under controlled conditions for two-weeks. The seeds were sown in a mixture of loam soil, then watered and placed in an air conditioned room kept at 23 ± 1 C. Two 150-w reflector flood lamps and six "cool white" 40-w fluorescent lamps provided an irradiance of 500 to 800 ft-c at the level of the plants. For each experiment the seedlings were kept on a daily 12 hour photoperiod during the 2-week growing period.

Fifteen days after planting, uniform seedlings were selected and placed in water. From each the first leaf was cut and weighed after blotting away any excess water. The tip of each leaf was also trimmed until the remaining portion of the blade weighed 90 mg. For better contact with the pretreating solutions, about 7 mm of sheath was allowed to remain on each leaf. The pretreating solutions consisted of 1%, 4%, and 16% ethanol with distilled water as the control.

Immediately after weighing, the sheath ends of the leaves were placed into vials containing the appropriate pretreatment solutions. The vials were then arranged under a bank of six "white" and six "daylight" 30-w fluroescent lamps which produced an irradiance of 800 to 1000 ft-c

during the two-hour pretreatment. All pretreating operations were carried out in the control room with the temperature maintained at 23 ± 1 C. To insure adequate transpiration the air in the control room was kept circulating during the pretreatment period. An equal quantity of leaf tissue was used in each pretreatment for individual experiments.

In Experiments 1 and 2, after the two-hour pretreatment, the vials containing the plant material were lowered into separate photosynthesis chambers. Each chamber consisted of an 80 ml glass test tube, a curved glass side arm inserted into a one hole rubber stopper, and a serum vial cap. The chambers containing the leaves were submerged in a water bath to a depth well above the height of the leaves.

In Experiment 3, 16% ethanol and distilled water were the only pretreating solutions used. Some sets of leaves in each of the two solutions were exposed to either a low oxygen or a high oxygen environment. This was accomplished by placing the vials containing the proper solutions and leaves into chambers through which flowed currents of oxygen (high oxygen) or nitrogen (low oxygen). Each chamber consisted of a 110 ml glass test tube plugged with a three hole stopper. Into one hole of each stopper a glass side arm with a receiving well was inserted, and this was later sealed with a serum vial cap. The remaining two holes in each stopper provided ports for the entrance and exit of the proper gas. Air was allowed to flow over the surface of the control leaves. For this experiment prior to lowering the chambers into the bath, it was necessary only to disconnect the tubes from the respective gas streams and seal the ports.

Eight 150-w reflector flood lamps, four on each of two sides of the bath, served as a source of illumination. These lamps produced an irradiance of 2500 to 2800 ft-c at the surface of the leaves. Heat from the lamps was absorbed by running tap water through rectangular glass culture bottles placed between each lamp and the water bath. The temperature of the chambers was kept at 20 ± 1 C by placing ice cubes as needed into the water bath.

One hundred microliters (50 microcuries) of labeled sodium bicarbonate ($NaHC^{14}O_3$) solution were placed in the reservoir of each side arm just before they were sealed with the serum vial caps. Then from 3 to 5 drops of a 3% acid (H_2SO_4) solution were injected into the labeled bicarbonate in the reservoirs by means of a hypodermic needle introduced through the serum vial caps. The acid solution brought about a release of $C^{14}O_2$ from the bicarbonate solution and the gas quickly diffused throughout the chambers.

The leaves were allowed to fix $C^{14}O_2$ photosynthetically for 20 or 30 minutes and then killed immediately by submergence in 5 ml of boiling 85% ethanol for about 3 minutes. After standing in the alcohol solutions overnight the leaves were removed and boiled in 3 ml of distilled water for one hour. The leaf tissue was then removed from each distilled water extract and stored.

The water and alcohol extracts were combined and diluted to 10 ml with distilled water. From the combined extracts 10 microliter aliquots (in triplicate) were taken and plated out on stainless steel planchets. After drying, the radioactivity was counted with a Mylar end window gas flow counter. From the mean counts for the separate planchet

series the total C^{14} fixed into the extractable material was calculated.

Each extract was evaporated to 0.5 ml by slightly heating in a water bath while passing a stream of air over it. Aliquots of the concentrated extracts equivalent to a given weight of tissue were spotted on separate sheets of Whatman No. 1 filter paper (18-1/4 x 22-1/2) inches). These papers were developed two-dimensionally with phenol:water (71: 29 v/v) in the first direction and butanol:propionic acid:water in the second direction. The butanol:propionic acid:water solvent described by Benson et al. (3) was prepared immediately before use by thoroughly mixing equal parts of a solution containing 1246 ml of butanol and 84 ml of water with a solution containing 620 ml of propionic acid and 790 ml of water. The chromatograms were allowed to dry at room temperature and then each was exposed to Kodak no-screen X-ray film (14 x 17 inches) from one to three weeks. The films were developed and all radioactive spots were marked on the papers and counted with a large Fuller (8) type end-window gas flow counter. The percentage of the total C^{14} fixed into the separate compounds on each chromatogram was calculated and recorded.

Some spots on the papers were eluted and the identity determined by co-chromatographing with authentic compounds. Others were identified with spray tests or by a comparison of the relative position on the papers with respect to known compounds. In a few cases a combination of the three methods was used to learn the identity of a spot.

Small segments (2.2 x 4.5 cm) containing the γ-aminobutyric acid were cut from each chromatogram. The segments were eluted separately with deionized water in excess of 2.0 ml and the eluates were concentrated to approximately 0.1 ml . The concentrated eluates were spotted

on separate 1 x 11 inch washed and buffered paper strips (26) and thereafter developed one-dimensionally by ascending chromatography in 77% ethanol. After drying at room temperature the γ-aminobutyric acid area on each strip was located with the use of a chromatogram scanner. These areas were cut from the strips and eluted separately with deionized water in excess of 1.5 ml. The separate eluates were concentrated to a volume of 1.0 ml.

From the individual concentrated eluates 100 microliter aliquots were taken (in triplicate) and plated out on stainless steel planchets. After drying the planchets were counted with a Mylar end-window counter and the amount of γ-aminobutyric acid-C^{14} (based on counts per second) was calculated for each eluate. Later the amount of γ-aminobutyric acid (based on weight per ml) was determined colorimetrically with ninhydrin (6) on duplicate 250 microliter aliquots from the separate eluates. The optical density of each sample was measured with a Spectronic 20 colorimeter set at 570 mμ. The specific activity (counts per second per microgram) of γ-aminobutyric acid was calculated for the separate eluates.

CHAPTER III

Results

In Experiments 1 and 2, three 90 mg leaves were placed in vials and each set pretreated for two hours in either distilled water, 1%, 4%, or 16% ethanol. They were then permitted to fix $C^{14}O_2$ photosynthetically for thirty minutes. The total C^{14} incorporated into alcohol-water extractable material was determined. No attempt was made to measure the non-extractable C^{14} in the tissue. The data are shown in tables I and IV.

Slight variations in amount of C^{14} fixed were apparent even among the controls. But in general the total label incorporated by the plant tissue appeared not to be materially affected by alcohol in this range of concentrations. However, one set of leaves treated in 4% ethanol (table IV) incorporated notably less C^{14} than generally found among other pretreatments.

Tables II and V show the percentages of C^{14} fixed into different metabolites during the periods of photosynthesis. Treatments "a" and "b" represent different sets of leaves which were similarly pretreated for each ethanol concentration. The data for replicates 1 and 2 were obtained from separate chromatograms made from equal volumes of the same extract. Aliquots equivalent to approximately 46 mg of fresh tissue

were placed on the origin of separate chromatograms. Larger aliquots were found generally not to be satisfactorily resolved on the paper.

Generally definite labeling trends can be seen in the individual metabolites, but the trend for γ-aminobutyric acid seemed to be more consistent than that found for other compounds. For both experiments this amino acid accumulated the least C^{14} in the control leaves. Increases in ethanol concentration consistently enhanced the percentage of C^{14} going into this metabolite (tables III and VI).

The amount (weight) of γ-aminobutyric acid present in the leaves appears to have been little changed by the different alcohol pretreatments. Some variations were found but the differences for similarly treated leaves are as great as for those treated differently.

The amount of C^{14} fixed into γ-aminobutyric acid was greatly increased by the pretreatment with ethanol. Leaves pretreated with 16% ethanol incorporated over 5 times as much C^{14} into γ-aminobutyric acid as did the controls. Since the total amount of γ-aminobutyric acid was not changed, the specific activity of the γ-aminobutyric acid in the leaves closely followed the trend for C^{14} incorporation.

For Experiment 3 the pretreating solutions consisted of only 16% ethanol and distilled water. During pretreatment with these solutions, sets of leaves in each were exposed to an atmosphere of oxygen, nitrogen, or air during pretreatment. After the two-hour pretreatment the leaves were allowed to fix $C^{14}O_2$ photosynthetically for twenty minutes. In each case those leaves pretreated in 16% ethanol while being exposed to oxygen, nitrogen, or air fixed less C^{14} than their counterparts in distilled water (table VII). The data indicate that pretreatment in an atmosphere

with high oxygen concentration greatly retards the photosynthetic fixation of $C^{14}O_2$ by oat leaves, but that pretreating in an atmosphere of nitrogen with very little oxygen had little effect.

Shown in table VIII are the percentages of the total fixed C^{14} incorporated into the separate metabolic products in Experiment 3. The effects of pretreatment with alcohol were generally the same regardless of whether the leaves were kept in an atmosphere of high oxygen concentration, high nitrogen concentration, or air.

The specific activity of γ-aminobutyric acid as determined for this exper'ment is shown in table IX. In each case more C^{14} was fixed into this amino acid by the control leaves than by those exposed to oxygen or nitrogen. The amount of γ-aminobutyric acid appeared not to be materially altered by either treatment. The results indicate that nitrogen may have caused a reduction in the synthesis of this amino acid. In most cases the specific activity of γ-aminobutyric acid showed a decrease when leaves were placed in an atmosphere of oxygen or nitrogen during pretreatment with or without ethanol. The data indicate that the specific activity of this compound is enhanced by the treatment of oat leaves with low concentrations of ethyl alcohol. These findings are in agreement with those observed for Experiments 1 and 2.

TABLE I

EFFECTS OF ETHANOL ON PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN THE LEAVES OF VICTORY OATS DURING THIRTY MINUTES (Experiment 1)

Two hour pretreatment	Sets of 3 leaves each	Fresh weight (mg)	c/s/mg ft wt tissue
Water	a	270.0	452
	b	270.0	342
1% Ethanol	a	270.0	437
	b	270.0	420
4% Ethanol	a	270.0	400
	b	270.0	309
16% Ethanol	a	270.0	332
	b	270.0	312

TABLE II

EFFECTS OF ETHANOL ON THE LABELING OF INTERMEDIARY METABOLITES DURING THIRTY MINUTES OF PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN VICTORY OAT LEAVES (Experiment 1)

Metabolites	Replicates	Percentages of the total C^{14} fixed into separate metabolites during 30 minutes photosynthesis after a 2-hour pretreatment							
		Pretreatment solution							
		HOH		1% ETOH		4% ETOH		16% ETOH	
		a	b	a	b	a	b	a	b
		%	%	%	%	%	%	%	%
Glycerate	1	1.3	1.1	1.0	1.2	1.1	1.2	0.5	1.6
	2	1.1	1.1	1.1	1.2	1.0	1.1	0.6	0.6
Malate	1	4.8	4.0	5.2	4.3	4.3	4.2	2.2	2.6
	2	4.0	4.0	4.6	4.5	4.7	4.1	2.5	2.8
Citrate	1	0.3	0.3	0.5	0.2	0.6	0.3	0.3	0.3
	2	0.3	0.3	0.4	0.3	0.7	0.3	0.3	0.3
Glutamate	1	0.8	0.6	0.7	0.7	1.1	0.6	1.0	0.9
	2	0.7	0.6	0.7	0.7	0.9	0.7	0.9	1.0
Aspartate	1	0.6	0.5	0.5	0.5	0.3	0.4	0.1	0.1
	2	0.6	0.6	0.5	0.5	0.3	0.4	0.1	0.1
Alanine	1	1.3	1.0	1.2	0.7	0.8	0.6	0.9	0.5
	2	1.0	1.0	1.1	0.6	0.8	0.6	0.7	0.4
Glycine, Serine, Glucose *	1	13.8	10.9	14.0	13.0	18.3	16.9	23.6	25.1
	2	11.0	10.9	13.3	11.8	18.2	16.7	24.0	26.2
Sucrose	1	66.0	72.2	67.8	70.5	64.6	65.7	61.7	59.0
	2	71.8	72.0	69.3	71.3	63.6	65.7	60.8	57.4
γ-Amino-butyrate	1	0.3	0.2	0.4	0.5	0.8	1.0	2.0	2.3
	2	0.2	0.2	0.4	0.5	0.8	0.8	2.1	2.5
Fructose	1	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2
	2	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1
Phosphate esters	1	8.1	6.9	6.4	6.5	5.8	6.7	5.3	5.2
	2	7.0	7.4	6.3	6.4	6.5	7.2	5.4	5.6

*These compunds were not well enough resolved on chromatograms to permit individual counting.

TABLE III

EFFECTS OF ETHANOL ON THE SPECIFIC ACTIVITY OF γ-AMINOBUTYRIC ACID IN VICTORY OAT LEAVES AFTER THIRTY MINUTES OF PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ (Experiment 1)

Two hour pretreatment		Replicates	C^{14} fixed per 46 mg fr wt	Weight of amino acid	Specific activity
			c/s	µg	c/s/ µg
Water	a	1	4.0	1.4	2.8
		2	5.0	1.9	2.6
	b	1	10.0	1.3	7.7
		2	8.0	1.1	7.3
1% Ethanol	a	1	18.0	2.7	6.7
		2	28.0	1.5	18.7
	b	1	23.0	2.1	10.9
		2	21.0	1.3	16.2
4% Ethanol	a	1	24.0	2.4	10.0
		2	39.0	0.3	130.0
	b	1	31.0	2.0	15.5
		2	36.0	1.7	21.1
16% Ethanol	a	1	98.0	0.5	196.0
		2	117.0	*	-
	b	1	121.0	0.3	403.3
		2	137.0	1.0	137.0

*The weight of γ-aminobutyric acid was not determined for this fraction.

TABLE IV

EFFECTS OF ETHANOL ON PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN THE LEAVES OF VICTORY OATS DURING THIRTY MINUTES (Experiment 2)

Two hour pretreatment	Sets of 3 leaves each	Fresh weight (mg)	c/s/mg ft wt tissue
Water	a	270.0	369
	b	270.0	416
1% Ethanol	a	270.0	309
	b	270.0	390
4% Ethanol	a	270.0	261
	b	270.0	385
16% Ethanol	a	270.0	379
	b	270.0	372

TABLE V

EFFECTS OF ETHANOL ON THE LABELING OF INTERMEDIARY METABOLITES DURING THIRTY MINUTES OF PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN VICTORY OAT LEAVES (Experiment 2)

Metabolites	Replicates	Percentages of the total C^{14} fixed into separate metabolites during 30 minutes photosynthesis after a 2-hour pretreatment							
		Pretreatment solution							
		HOH		1% ETOH		4% ETOH		16% ETOH	
		a	b	a	b	a	b	a	b
		%	%	%	%	%	%	%	%
Glycerate	1	1.5	1.3	1.8	1.2	1.6	1.0	0.8	0.9
	2	1.6	1.2	1.9	1.1	1.7	1.0	0.[illegible]	0.9
Malate	1	6.4	5.3	5.5	5.1	5.9	4.8	3.7	4.3
	2	5.3	4.5	5.1	5.2	6.1	5.0	3.1	4.0
Citrate	1	0.6	0.5	0.5	0.5	0.7	0.6	0.5	0.5
	2	0.5	0.4	0.5	0.6	0.7	0.6	0.5	0.5
Glutamate	1	1.4	1.3	1.3	1.4	1.6	1.4	1.8	1.7
	2	1.7	1.1	1.2	1.3	1.7	1.4	1.7	1.9
Aspartate	1	0.6	0.4	0.3	0.4	0.4	0.3	0.1	0.2
	2	0.6	0.4	0.3	0.4	0.4	0.3	0.1	0.2
Alanine	1	1.4	0.9	0.9	0.9	0.8	0.6	0.5	0.7
	2	1.3	0.8	0.8	0.9	0.8	0.6	0.5	0.7
Glycine, Serine, Glucose *	1	19.0	12.5	24.6	17.0	32.1	19.5	23.1	26.9
	2	15.8	13.0	24.4	17.0	33.4	18.7	20.1	31.3
Sucrose	1	53.7	67.3	49.6	62.3	41.1	59.9	57.4	49.7
	2	60.6	68.4	46.1	61.8	40.2	61.0	62.3	45.7
γ-Amino-butyrate	1	0.5	0.4	1.0	0.6	1.5	1.0	2.4	3.3
	2	0.5	0.3	1.1	0.6	1.3	1.0	2.1	3.1
Fructose	1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1
	2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phosphate esters	1	11.0	6.9	10.8	7.6	10.3	8.2	6.0	8.0
	2	9.1	7.5	10.2	8.1	10.1	7.7	5.5	7.8

*These compounds were not well enough resolved on chromatograms to permit individual counting.

TABLE VI

EFFECTS OF ETHANOL ON THE SPECIFIC ACTIVITY OF γ-AMINOBUTYRIC ACID IN VICTORY OAT LEAVES AFTER THIRTY MINUTES OF PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ (Experiment 2)

Two hour pretreatment	Replicates		C^{14} fixed per 46 mg fr wt	Weight of amino acid	Specific activity
			c/s	μg	c/s μ g
Water	a	1	19.0	1.5	12.7
		2	20.0	2.0	10.0
	b	1	17.0	1.6	10.6
		2	17.0	2.0	8.5
1% Ethanol	a	1	22.0	1.5	14.7
		2	29.0	1.4	20.7
	b	1	24.0	1.5	16.0
		2	25.0	1.9	13.2
4% Ethanol	a	1	18.0	2.0	9.0
		2	24.0	1.5	16.0
	b	1	50.0	1.3	38.5
		2	42.0	1.0	42.0
16% Ethanol	a	1	101.0	2.3	43.9
		2	114.0	1.8	63.3
	b	1	129.0	1.1	126.4
		2	120.0	0.4	300.0

TABLE VII

EFFECTS OF HIGH OR LOW OXYGEN LEVEL ON PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN VICTORY OAT LEAVES DURING TWENTY MINUTES AFTER PRETREATMENTS WITH AND WITHOUT ETHANOL (Experiment 3)

Two hour pretreatment	Sets of 3 leaves each	Fresh weight (mg)	c/s/mg fr wt tissue
Air & HOH	a	270.0	256
	b	270.0	271
Air & 16% ETOH	a	270.0	255
	b	270.0	234
Oxygen & HOH	a	270.0	101
	b	270.0	79
Oxygen & 16% ETOH	a	270.0	66
	b	270.0	37
Nitrogen & HOH	a	270.0	315
	b	270.0	304
Nitrogen & 16% ETOH	a	270.0	296
	b	270.0	278

TABLE VIII

EFFECTS OF HIGH OR LOW OXYGEN LEVEL ON LABELING OF INTERMEDIARY METABOLITES DURING TWENTY MINUTES OF PHOTOSYNTHETIC FIXATION OF $C^{14}O_2$ IN VICTORY OAT LEAVES PRETREATED WITH AND WITHOUT ETHANOL (Experiment 3)

Metabolites	Percentage of total C^{14} fixed into separate metabolites during 20 minute photosynthesis after a 2-hour pretreatment											
	Pretreatment											
	Air & HOH		Air & 16%ETOH		Oxygen & HOH		Oxygen & 16%ETOH		Nitrogen & HOH		Nitrogen & 16% ETOH	
	a	b	a	b	a	b	a	b	a	b	a	b
	%	%	%	%	%	%	%	%	%	%	%	%
Glycerate	1.5	1.7	1.7	0.6	4.7	1.4	0.9	0.7	0.2	0.2	0.3	0.7
Malate	0.4	1.0	2.2	1.9	3.8	2.3	5.0	4.6	2.2	1.2	3.2	2.0
Citrate	0.1	0.1	0.3	0.2	0.3	0.3	0.5	0.7	0.1	0.1	3.2	2.0
Glutamate	0.3	0.5	1.1	1.2	0.9	0.7	1.6	1.9	0.2	0.2	0.4	0.3
Aspartate	0.1	0.3	0.2	0.3	3.2	4.9	7.7	8.1	1.0	0.9	1.5	1.3
Alanine	0.6	1.1	1.6	2.0	1.5	2.0	3.1	3.7	1.7	2.0	3.4	3.6
Glycine, Serine, Glucose *	11.9	12.9	27.4	21.9	15.6	19.7	17.4	23.9	2.7	2.7	3.8	9.7
Sucrose	75.1	71.7	51.6	59.0	43.2	47.6	40.2	32.2	81.1	80.1	71.1	69.2
γ-Aminobutyrate	0.2	0.5	1.6	1.5	0.3	0.5	2.1	3.5	0.2	0.1	0.7	0.8
Fructose	0.1	0.1	0.1	0.1	0.3	0.3	0.5	0.3	0.1	0.1	0.1	0.2
Phosphate esters	8.1	8.7	9.8	8.4	23.1	17.3	17.4	16.5	9.4	10.6	13.1	9.9

*These compounds were not well enough resolved on chromatograms to permit individual counting.

TABLE IX

EFFECTS OF HIGH OR LOW OXYGEN LEVEL ON THE SPECIFIC ACTIVITY OF Y-AMINO-BUTYRIC ACID IN VICTORY OAT LEAVES PRETREATED WITH OR WITHOUT ETHANOL DURING TWENTY MINUTES PHOTOSYNTHESIS (Experiment 3)

Two hour pretreatment	Sets of 3 leaves each	C^{14} fixed per 46 mg fr wt	Weight of amino acid	Specific activity
		c/s	µg	c/s µg
Air & HOH	a	17.1	1.5	11.4
	b	10.3	1.0	10.3
Air & 16% ETOH	a	54.8	1.0	54.8
	b	47.7	1.5	31.8
Oxygen & HOH	a	4.5	1.1	4.1
	b	6.7	0.8	8.4
Oxygen & 16% ETOH	a	17.6	1.3	13.5
	b	21.2	1.5	14.1
Nitrogen & HOH	a	7.5	0.3	24.3
	b	5.9	0.6	9.8
Nitrogen & 16% ETOH	a	33.5	1.0	33.5
	b	26.8	0.9	29.8

CHAPTER IV

Discussion

The results presented in this paper show that ethanol affects the distribution of C^{14} among many intermediary metabolites of Victory oat leaves. Alcohol seems to have increased the percentage of label incorporated into some compounds but decreased it for others. Its most apparent effect was the decided enhancement of C^{14} going into γ-amino-butyric acid by increasing concentrations of the ethanol. Similar results were observed in a previous study (13). Other investigators have demonstrated that in plant tissue, growth and metabolic processes may be materially affected by low concentrations of ethyl alcohol.

Gudjonsdotter and Burstrom (10) stated that weak solutions of ethanol and other aliphatic alcohols counteracted the retarding effect of light on wheat roots. They pointed out that the alcohols primarily stimulated cell elongation rather than multiplication, an effect which was observed only in concentrations approaching toxic levels. They suggested that probably these alcohols enhanced elongation by stimulating the production of active growth substances.

Mer (15, 16) found that when the carbon dioxide concentration in air flowing over etiolated oat seedlings was raised to 5%, growth of the mesocotyl was substantially increased while that of the coleoptile was depressed. He reported further that when dilute ethanol was supplied

to the roots, strikingly similar responses were produced. In both cases the enhancement effect was attributed to the extension of the meristematic activity of the modal meristems.

Working with Avena coleptile sections Anker (2) showed that oxygen uptake induced by indoleacetic acid could be further enhanced by low amounts of ethyl alcohol. He also reported that the presence of ethanol (40 mg/liter) in media without an added source of carbon increased oxygen consumption 8 to 23%. This response would seem to indicate the use of ethanol as a substrate, but Anker suggested that perhaps it stimulated endogenous sucrose metabolism which subsequently may have been indirectly responsible for the increased uptake of oxygen.

Lowe and James (14) reported results from work with carrot root tissue which appeared to be in harmony with Anker's suggestion. They found that upon addition of ethyl alcohol, in small amounts, to media containing carrot discs a sudden rise in oxygen consumption followed. Yet when ethanol-1-C^{14} was used only negligible quantities of C^{14} were incorporated into organic acids and carbon dioxide. They suggested that probably the intracellular spatial separation of the appropriate enzymes (known to be present in carrot roots) may have prevented the aerobic oxidation of the alcohol.

In general the data appearing in tables III, VI, and IX show that the specific activity of Y-aminobutyric acid was enhanced by the ethanol solutions. But at the same time the pool size of this amino acid appears not to have been materially changed. These findings suggest that ethanol stimulated the synthesis of Y-aminobutyric acid in addition to its utilization within the leaf tissue. That this compound actively participates

in amino acid and protein metabolism of plants has become evident within recent years (9, 20, 22).

Shortly after the discovery of γ-aminobutyric acid in higher plants by Dent et al. (7) and subsequent confirmation by Stewart et al. (24), this compound was thought to be merely an end product of glutamic acid metabolism formed principally under the influence of the enzyme, glutamic acid decarboxylase (27, 29). Although this method of synthesis is feasible and has been thoroughly substantiated (22, 24), yet frequently in plants there is found no correlation between the amount of γ-aminobutyric acid present and the activity of this enzyme (27).

Steward (20) questions whether γ-aminobutyric acid is formed by glutamic acid decarboxylase in growing tissue cultures. From an intensive study of nitrogen metabolism, respiration, and growth of carrot root tissue Steward et al. (21) cited data which they considered evidence that γ-aminobutyric acid served as a source for glutamic acid and glutamine. Furthermore they considered γ-aminobutyric acid to be an exceedingly active metabolite and therefore was intimately associated (as a precursor of glutamic acid and glutamine) with the general nitrogen metabolism of plants. Succinic semialdehyde, probably arising from succinic acid of the citric acid cycle, was presumed by them to be the apparent precursor of this amino acid.

The work of Gonzalez et al. (9) generally supported the suggestion of Steward et al. (21) relative to the active role of γ-aminobutyric acid in plants. They found it to be readily metabolized by segments of Helianthus tuberosus tubers suspended in liquid media. On chromatograms made with extracts from the tuber tissue the highest label noted among the

amino acids, excluding γ-aminobutyric acid, was alanine, proline, aspartic acid, and glutamic acid. Only malic acid in the organic acid fraction showed any appreciable accumulation of C^{14}. From these results they arrived at the same conclusion as Steward et al, that γ-aminobutyric acid is an active metabolite and is closely associated with the intermediates of the citric acid cycle.

The experimental data presented in table VII show that the photosynthetic fixation of $C^{14}O_2$ in leaves of oat is greatly retarded in an atmosphere with high oxygen concentration. At the same time $C^{14}O_2$ fixation in the leaves exposed to an atmosphere with very little oxygen appeared not to differ materially from that in the controls. These results closely resemble the findings of Turner et al. (28) which showed that photosynthesis was greatly inhibited by high oxygen concentrations. These workers stated, "If glyceraldehyde phosphate dehydrogenase acted as a pacemaker in photosynthesis (e.g. at high light intensity and high CO_2 concentration) then inhibition of the enzyme by oxygen could inhibit photosynthesis (i) by depressing the rate of re-oxidation of reduced pyridine nucleotide derived from the photolysis of water, so that this process and oxygen evolution would slow down, (ii) by causing a block at the 3-phosphoglyceric acid level which could interfere with the operation of the carbon cycle." According to Carr (5), Tamiya and Huzizige in 1949, and Miyachi et al. in 1955 were in agreement with this point of view. I believe that photosynthesis was probably inhibited by the pretreatment of leaves in an atmosphere of high oxygen concentration in my study.

Turner et al. (28), furthermore, noted no significant difference between the rates of photosynthesis in air and nitrogen. These results

are likewise supported by my data.

The most apparent effect of oxygen on the metabolism of γ-aminobutyric acid was the decided reduction in amount of C^{14} fixed. Oat leaves exposed to nitrogen accumulated less label into this metabolite than the controls but the difference is not great and may not be significant. The pool size of γ-aminobutyric acid appeared not to have been affected by either oxygen or nitrogen. Therefore it appears that the lowering of the specific activity of this metabolite in each case resulted from an inhibition of the photosynthetic fixation of $C^{14}O_2$. In spite of these conditions the specific activity of γ-aminobutyric acid appeared definitely to be increased by ethanol under each atmospheric condition.

CHAPTER V

Summary

Victory oat seeds, Avena sativa L., were germinated and grown under controlled conditions. Fifteen days after planting, selected leaves from uniform seedlings were cut and pretreated for two hours in distilled water, 1%, 4%, and 16% ethanol. Immediately after each pretreating period the leaves were permitted to fix $C^{14}O_2$ photosynthetically for twenty or thirty minutes. Then they were extracted with boiling 85% ethanol and further extracted with boiling distilled water for one hour. The respective ethanol and water extracts were combined, concentrated, and aliquots equivalent to a given amount of tissue were placed on the origin of separate chromatograms. After developing two-dimensionally, the percentages of C^{14} fixed into the separate intermediary metabolites were determined.

For one experiment distilled water and 16% ethanol were the only pretreating solutions used. But, in this case, some leaves in each kind of solution were exposed to a high oxygen or low oxygen (nitrogen) environment during the pretreating period. They were extracted and treated as above.

The γ-aminobutyric acid from each chromatogram was purified by rechromatography on washed and buffered paper. Then the radioactivity

(based on counts per second per ml) and the amount (based on weight per ml) of γ-aminobutyric acid in each sample was determined. The specific activity (counts per second per microgram) was calculated for each eluate.

The data showed that ethanol generally affects the distribution of C^{14} among the intermediary metabolites. Its most apparent effect was the decided enhancement of C^{14} going into γ-aminobutyric acid, with increasing concentrations.

The pool size of γ-aminobutyric acid appeared not to be materially affected by the alcohol solutions. But the data indicate that the amount of C^{14} fixed into this compound and therefore its specific activity were augmented by raising the concentration of the ethanol solution.

The photosynthetic fixation of $C^{14}O_2$ in oat leaves was definitely retarded by a high oxygen environment. In leaves exposed to nitrogen (low oxygen) this process appeared not to differ significantly from the controls. Except for a reduction in the total amount of C^{14} fixed in an atmosphere with a high level of oxygen, the different atmospheric conditions appeared to have little effect on the metabolism of γ-aminobutyric acid.

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