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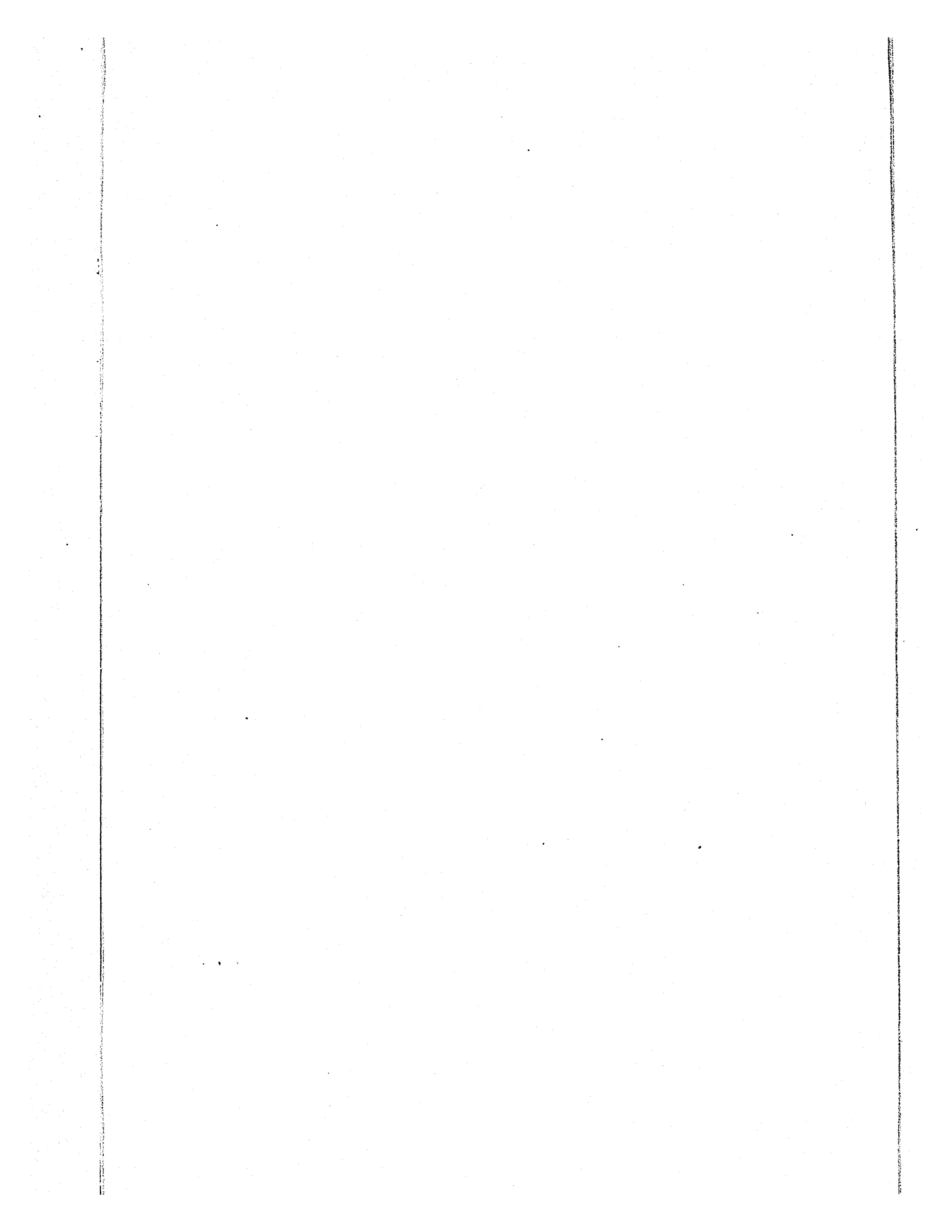

VERNALIZATION OF TRITICUM VULGARE (var. RIDEAU):

MORPHOLOGICAL AND BIOCHEMICAL STUDIES.

by

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A Thesis

submitted to the

University of Ottawa

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

April 1962

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ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. L.P. Dugal and Dr. C. Godin for their invaluable advice and encouragement.

I am also indebted to my colleagues, especially Dr. A. DesMarais for many discussions.

The help of Mr. A. Lutz and Mr. C. Pouyez is gratefully acknowledged.

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STATEMENT OF THE PROBLEM

Studies in plant growth have hitherto been pursued for the most part in separate fields of botany. On the one hand there has been the physiological-biochemical approach as exemplified by the recent work on the variation during growth of the total protein nitrogen and alcohol soluble amino acid pool in the leaves of the banana, (Friedberg and Steward, 1960), daffodil, (Bryant and Fowden, 1959) and barley (Pleshkov and Fowden, 1959). On the other hand morphological aspects have been used to establish responses to vernalization. Such work has shown that besides affecting the time of earing, vernalization induces shorter lengths in the initial one or two emergent leaves of many varieties of spring cereals.

In the present work the physiological and morphological methods for studying leaf ontogeny and vernalization have been employed concurrently on spring wheat, Triticum vulgare var. Rideau.

It was attempted to seek information on the change in some growth indices with time under greenhouse conditions following vernalization of the imbibed seed. The growth indices selected were leaf length and weight, root weight and cell count of a 10 mm segment of the root tip. The biochemical factors followed in parallel experiments were changes in total (bulk) protein, alcohol soluble amino acids and the enzyme glutamic decarboxylase.

ABSTRACT

The effect of vernalization on seedling growth of spring wheat, *Triticum vulgare* (var. Rideau) has been studied by observing morphological and biochemical changes in the first three leaves. Leaf length, dry weight and total protein measurements indicated that vernalization accelerated all stages of leaf development although the maximum leaf size was unaffected.

The composition of the alcohol amino acid pool varied with leaf ontogeny in both untreated and vernalized plants. The most pronounced effect of vernalization was to depress the relative content of arginine which was one of the major pool constituents in untreated leaves. The concentrations of other important amino acids, γ-aminobutyric acid and glutamic acid varied with the physiological age of the leaf, and vernalization did not alter this relationship. The activity of the enzyme glutamic decarboxylase was also mainly dependent on the physiological leaf age.

Vernalization caused a great increase in meristematic activity of the root tip.

RESUMÉ

On a étudié les effets de la vernalisation sur la pousse des plants de blé hâtif (Triticum vulgare var. Rideau) en observant les changements morphologiques et biochimiques des trois premières feuilles. Des mesures de la longueur de la feuille, de son poids sec et de son contenu en protéines ont démontré que la vernalisation accélère toutes les étapes du developpement de la feuille bien que sa taille maximum reste inchangée.

La composition du mélange d'acides aminés solubles dans l'alcohol varie avec l'ontogénie de la feuille des plants normaux et vernalisés. L'effet le plus marqué de la vernalisation est de diminuer la quantité d'arginine, un des principaux constituants du mélange chez les feuilles non-traitées. La concentration des autres acides aminés importants, soient les acides γ-aminobutyrique et glutamique, varie selon l'âge physiologique de la feuille et la vernalisation ne change pas cette relation. L'activité de la décarboxylase de l'acide glutamique varie aussi de la même façon.

La vernalisation cause une grande augmentation de l'activité méristématique du bout de la racine.

List Of Code Used In The Text:

G: - Control plants - exposed to greenhouse temperatures only

C_1: - Seeds vernalized one week at 2 ± 1°C

C_2: - Seeds vernalized two weeks at 2 ± 1°C

C_3: - Seeds vernalized three weeks at 2 ± 1°C

(C1–C3: Subsequently brought into the greenhouse)

L1: - First emergent leaf

L2: - Second emergent leaf

L3: - Third emergent leaf

L1-G, L2-G, L3-G: - 1st, 2nd and 3rd leaf respectively of the G Series

L1-C_1, L2-C_1, L3-C_1: - 1st, 2nd and 3rd leaf respectively of the C_1 Series

L1-C_2, L2-C_2, L3-C_2: - 1st, 2nd and 3rd leaf respectively of the C_2 Series

L1-C_3, L2-C_3, L3-C_3: - 1st, 2nd and 3rd leaf respectively of the C_3 Series

L1-9, L1-16, L1-30 - 1st leaf: - 9, 16 and 30 days old respectively

L2-9, L2-16, L3-30 - 2nd leaf: - 9, 16 and 30 days old respectively

L3-9, L3-16, L3-30 - 3rd leaf: - 9, 16 and 30 days old respectively

PART ONE

INTRODUCTION

In temperate countries the seeds of winter cereals must be planted before the end of winter for the plants to flower during the following summer. Spring cereal seeds which generally cannot survive the prolonged cold of winter are sown in the spring, and the cereals flower in the summer of the same year. These empirical agricultural methods led to the study of vernalization.

The term, originally coined by Lysenko (1928) describes the acceleration of the reproductive stage of growth by chilling the seed or seedling. The chilling treatment affects the meristematic cells of the embryo (Gregory and Purvis, 1938[a]; Purvis, 1947), but the latent changes only become morphologically evident during subsequent growth and development. Cereal plants can be vernalized in the seed stage. The effectiveness of chilling both for winter and spring cereals is dependent on water supply as well as temperature. The level of imbibition should be sufficient to allow the vernalization process to operate but must be low enough to hinder seedling growth (Lysenko, 1932; Gregory and de Ropp, 1938). To initiate respiration imbibition is first carried out at 15-20°C in the presence of oxygen and the seeds subsequently chilled to -3 to +10°C (Gregory and Purvis, 1938[ab]; Hänsel, 1953[a]).

Cold nights occurring during late summer maturation can induce vernalization of the immature embryo within the milky caryopse whilst the seed is still in the ear (Gregory and Purvis, 1938[ab]). Under natural conditions, the various cereal species have different chilling time requirements which depend on their native climate (Purvis and Gregory, 1952; Wort, 1940). It follows then that cereal seeds may be partially

vernalized before reaping. Because of this fact the same variety of wheat, grown in different regions, may have different requirements for the duration of the cold treatment. Vernalization characteristics are borne by a few Mendelian factors (Purvis, 1939). Although vernalization in the cereal is completed within the grain, its evaluation is necessarily delayed until flowers are initiated. Biochemical tests have been devised to detect the vernalized condition in the seed (Richter, 1934; Bassarskaya, 1936), but at present there is no satisfactory criterion for predicting the growth pattern of the germinating grain or seedlings from such data.

In winter cereals vernalization invariably accelerates flowering but in spring cereals this is not always the case. Wort (1940) showed that in spring wheats flowering was accelerated in some instances but unaffected or even retarded in others. Studies on morphological modifications in spring cereals following vernalization have also yielded diverse results. Sereiskij and Sludskaya (1937) found accelerated emergence and reduction in length of the first leaf only, whilst other workers (Purvis and Hatcher, 1959; Hänsel, 1953[b]) observed length reductions in the first and second leaves or no change at all (Hänsel, 1953[a]).

Little information is available on the effect of vernalization on root growth. Wort (1939) noted that more vigorous root growth followed vernalization.

The methods used in most of the studies in this field have been essentially physical measurements. In other fields of plant physiology plant development has been followed by observing changes in the number of cells of the tissues (Brown and Rickless, 1949; Sunderland

and Brown, 1956; Sunderland, 1960) and the enzyme content (Weinberger and Clendenning, 1952; Robinson and Brown, 1954; Smillie and Fuller, 1960). Changes in the bulk nitrogen (Bryant and Fowden, 1959; Pirie, 1959; Smillie and Krotkov, 1961) and alcohol soluble amino acid pool have also been observed (Bryant and Fowden, 1959; Pleshkov and Fowden, 1959; Friedberg and Stewart, 1960).

The main object of the present work was to establish whether recognizable biochemical changes within the tissues accompanied morphological changes attendant on vernalization.

PART TWO

EXPERIMENTAL

A. METHODS

1. Treatment of Seeds and Seedlings

Method of Seed Planting - Fifty medium sized seeds were selected per flat (7" x 9") and planted equally spaced in loam soil half an inch beneath the soil surface. Sowings were arranged so that the control and treated plants were taken into the greenhouse at the same time.

Watering Procedures - During low temperature treatment growth was purposely limited by restricting moisture to approximately fifty percent of the dry weight of the seeds (Wort, 1939). The seeds were allowed an eight hour imbibition period at room temperature before being transferred to the cold. The flats were covered with polythene to prevent evaporation. After transfer to the greenhouse at the end of the cold treatment the polythene was removed, and the flats were then watered and maintained at "field capacity". Water stress was kept the same for all series (Mothes, 1931). A similar eight hour imbibition period for the control series of plants was allowed at room temperature before transfer to the greenhouse.

Conditions of Vernalization and Greenhouse Temperatures - Vernalized seeds were exposed at 2 ± 1°C for one, two or three weeks. Following this treatment they were taken together with the control series and placed in the greenhouse under natural short day conditions (September - March). Temperatures fluctuated somewhat, maximum temperatures of 90°F and

minimal temperatures of 45°F were sometimes recorded. The average day time temperature was 75 ± 3°F and night temperature 60 ± 5°F.

Selection of Plants - Only those seedlings showing emergent coleoptiles after three days greenhouse growth were used in the study. These seedlings were identified by tagging. Slower growing seedlings were discarded. This gave a reasonably homogeneous plant population. As 5 different determinations were to be made at each of the four periods (5, 7, 14, 21 days) six flats were used per period (this allowed one spare). The whole population of a flat was used for a determination. Flats were randomly selected for the measurements. Experiments were carried out in triplicate unless otherwise stated.

Time of Leaf Detachment - Leaves were always detached from the plants between 9.30 and 10.30 a.m. Diurnal variation, if a factor (Krotkov and Bennett, 1952), would therefore affect all series equally.

Method of Leaf Disintegration - Leaves were homogenized in all glass hand homogenizers with minimal additions of solvent and using vertical movements parallel to the axis (Anderson, 1956).

2. Leaf and Root Measurements

Leaf Length - The first three leaves alone were examined. Of these, only those which were macroscopically evident were measured. When very small, leaf length was taken as length from leaf tip to point of insertion on the stem. Where a ligule was discernible with a hand lens, leaf length was taken as the length from leaf tip to the ligule. Measurements were made with a ruler and a dissecting microscope with a moving stage

and micrometer eye piece to give lengths to ± 0.1 mm. Leaves defined in this way were also used to obtain leaf weights and in the study on the nitrogen compounds.

Leaf Weight - Leaves were quickly rinsed clean of adhering soil particles, blotted dry and their fresh weight obtained. Dry weights were taken after heating the leaves at 80°C for 24 hours. Weights were taken to ± 0.1 mg after equilibrating with atmospheric humidity.

Cell Counting Technique (Roots) - Adventitious roots of comparable length (± 5%) were selected from each plant of the experimental unit. They were handled very carefully and most of the soil particles were easily removed by immersing the flat in water and floating off the adhering soil. Final rinsing was done in large trays using a continuous flow of tepid water and carefully brushing the root tips with a fine camel hair brush. In this way the root tip area was cleaned of visible adhering soil particles. (Whole roots were similarly cleaned for dry weight determinations but the method was extremely laborious and was not judged too satisfactory). Kept moist and placed on the stage of a dissecting microscope the root tip was lightly brushed until completely free of all soil. The technique used for the estimation of cell numbers was that previously established by the author (Brown and Rickless, 1949). Using a sharp edged razor a 10 mm root tip segment was cut from each physiologically similar root of the experimental series. These were placed in 5% chromic acid (2 cc per four roots) and left to stand at room temperature for 12 - 18 hrs. After treatment with the acid solution the tissue was broken up into a fine slurry by manipulating it with a

thin slightly roughened glass rod against the sides of the vessel. The slurry of cells was mixed with the fluid and the final suspension, on which the count was to be made, was obtained by squirting the fluid several times rapidly through the narrow orifice of a pipette. Counts were made on a haemocytometer slide.

Statistical Data - Most of the data presented in Part 2, B1 were subjected to an analysis of variance. Wherever the F value (Dixon and Massey, 1951) was found to be significant at the 5% level the Duncan multiple range test (Duncan, 1955) was used to locate the significant difference at the 5% level.

3. Extraction and Estimation of Total Leaf Protein

The method of extraction was based on that used for Chlorella proteins (Fowden, 1951). Leaves were quickly detached from the plants, rinsed in distilled water to remove adhering soil particles, blotted dry on filter paper and weighed. The leaf tissue was macerated and exhaustively extracted with 0.2M sodium borate buffer at pH 9.0. The pH of the macerate was kept at 9.0 by addition of N KOH. After grinding, the cell debris was removed by centrifugation. The supernatant was decanted and the debris re-extracted three times with borate buffer. The extracted proteins were flocculated by adjusting the pH to 4.5. After the third extraction no protein precipitation was obtained, indicating that a good extraction of protein had been achieved. The precipitated protein was warmed to 70°C for 3-5 minutes and removed by centrifuging. The coagulated protein was then twice washed with dilute acetic acid, pH 4.5, followed by six washings with boiling ethanol. Little pigment

adhered to the protein after this treatment. After drying at room temperature the protein was finely powdered in a glass mortar. The powder was later redissolved in a known volume of water. Nitrogen determinations on this solution using Folin's reaction (Lowry et al, 1951), when compared with Kjeldahl nitrogen determinations on the whole leaf indicated that the proteins extracted represented 85-90 percent of the total nitrogen originally present in the leaf. The Folin method was used throughout as it is very rapid, and provided sufficiently exact data for the type of comparison used in this work.

4. Extraction and Estimation of Alcohol Soluble Leaf Amino Acids

Extraction - Free amino acids were extracted from the leaves with 70 percent ethanol w/v in an ice cooled glass homogenizer. The tissue was homogenized with minimal additions of ethanol. The debris, after centrifuging was further extracted. The combined supernatants were kept in the cold overnight. The debris was covered with ethanol (the amount used varied with the fibrous character of the leaves) and left in the cold overnight. The final volume of the combined extracts, obtained after centrifuging at 20,000 g for twenty minutes was recorded. Using a warm air draught, extracts were reduced to a known volume. When not used immediately, extracts were stored in polythene screw cap bottles in a freezer at -25°C.

Chromatographic Procedures - Prior to use the leaf extracts were thawed and mixed thoroughly. Aliquots of 25 - 100 μl were used for one dimensional paper chromatography. In this way a variety of loadings were applied simultaneously.

The chromatograph tank used in this study was of standard design and could accomodate six sheets of filter paper. The paper used in all cases was Whatman grade No. 1, 18-1/4" x 22-1/2" and the solvent, butanol : acetic acid : water 4 : 1 : 5. The amino acids used in the co-chromatographed spots were chromatographically pure stock supplies of amino acids.

Chromatograms were pre-conditioned for 1 - 2 hours in the chromatography chamber before developing. Initially they were allowed to develop for 54 hours continuously. Later in the study the method successfully used by Pasieka (1960) was followed using two thirty six hour development periods separated by drying and sometimes cold storage.

Analysis of Chromatograms - The method of estimation of amino acids commonly used for paper chromatograms is spraying with an alcoholic solution of ninhydrin. In calibrating runs this method gave variable values, sometimes as much as 30% lower than the employed concentration of amino acids. Moore and Stein (1948, 1954) have shown that use of ninhydrin-hydrindantin reagent gave very close estimates of the concentration of amino acids in solution, but they had not used the reagent with paper chromatograms.

A successful adaptation of the Moore and Stein technique to paper chromatograms was developed in the present work*.

One, or both of the following two reagent mixtures were finally used to aid spot identification and the quantitative and qualitative estimation of amino acid content:

---

* After completion of the present research an adaptation was published which is applicable to paper chromatography with reproducible results. (Matheson et al, 1961; Tigane et al, 1961).

Reagent A: 2.0 gm ninhydrin
0.3 gm hydrindantin
75 cc methyl cellosolve
25 cc 0.025M sodium acetate buffer pH 5.5

Immediately after mixing, nitrogen gas was bubbled into the reagent mixture, and the air above the mixture was replaced as far as possible with nitrogen. The reagent bottle was then tightly stoppered and stored in the cold.

Reagent B: 50 ml absolute ethanol
0.05 gm ninhydrin
2 ml collidine
0.5 ml glacial acetic acid

Spot Identification - Spot identification was facilitated by spraying one chromatogram with Reagent A and a duplicate with Reagent B. When required, rapid visualization of the amino acids in the latter was achieved by heating the chromatogram at 60°C for 5 min; in other cases the collidine-ninhydrin paper was left in the dark for twenty-four hours. Identification was further aided by co-chromatographing standard amino acids. Identified spots were ringed with a pencil mark, a margin of approximately 0.5 mm being left where possible around the colored area.

Estimation of Amino Acids - The concentration of the following seventeen commonly occuring amino acids were evaluated: leucine, iso-leucine, phenylalanine, valine, tyrosine, methionine, γ-aminobutyric acid, proline, alanine, glutamic acid, threonine, glycine, serine,

aspartic acid, arginine, histidine and lysine. Although cystine was always recognized, its concentration was not evaluated. Under the conditions employed here it did not move far from the starting point, and its brownish colored area was sometimes overlaid with unknown amino acids giving a blue color. Even the utilisation of the Pasieka modification did not allow clear spot delineation in this case.

The developed amino acids were visually compared with a concentration series of leucine (0.01 - 4μ moles/l) to give semi-quantitative estimates.

In quantitative work two methods for blank estimation were used, the choice depending upon the number of amino acid spots to be identified at any one time. For a small number of amino acids each colored pencil ringed area and an identical blank were cut out and treated as below. For larger numbers of amino acids each colored pencil ringed area was weighed alone. At the same time a single blank area was cut out and weighed, one blank being weighed per chromatogram. The blank correction was adjusted according to the weight of the colored paper area containing an individual amino acid.

In all cases blank areas were treated similarly to colored areas. The spots were cut into small pieces, put into boiling tubes and 2.00 cc Reagent A, and 1.00 cc of a 1 : 1 methyl cellosolve water mixture was added. If the colored areas were large, twice the volume of both reagents was used in order to cover the pieces of paper. The boiling tubes were topped with 5 cc beakers or aluminum foil caps and heated on a rack in boiling water for twenty minutes. After heating they were shaken in a rack at 26° C for 5 minutes in order to oxidize the excess

hydrindantin red. The resulting colored solution was found to be stable for at least 48 hours.

Readings were taken on a Beckman D.U. spectrophotometer at 570 mμ for all amino acids except histidine. The latter gave a brown color and was read at 330 mμ. Corrections were made for the background reading from determinations on the blank areas. Where the weighing method for blank determinations was used weights were measured to 0.1 mg.

Calculation of Amino Acid Values

As results were to be expressed on a leucine equivalent basis, conversion factors were obtained by comparing the optical densities of the developed solutions from standard concentrations of the amino acids with that from leucine. Using these factors the optical density values from the leaf extracts were converted to corresponding leucine values. From a calibration graph of optical density vs. concentration of leucine in μmoles/l the leucine equivalent concentrations were then obtained.

Let this concentration be c μmoles/l

the volume of eluate and diluent be Vcc

the volume of original extract from n leaves weighing W gms be $V_o$cc

the aliquot (50-100 μl) taken from $V_o$cc for chromatography be δ μl

then amount of amino acid eluted from δ μl = $\frac{Vc}{1000}$ μmoles

∴ amount of amino acid in $V_o$cc i.e. n leaves = $\frac{1000\, VcV_o}{\delta}$ μmoles

$$\frac{\text{Amount of amino acid}}{\text{weight of leaves in gm}} = \frac{1000\ V\ c\ V_o}{W\ \delta}$$

5. Extraction of Glutamic Decarboxylase

The leaves were freshly collected and chilled prior to maceration in a cold room at 2 ± 1°C. The undiluted cell fluid was filtered through nylon and the chlorophyll content and volume recorded. It had previously been established that glutamic decarboxylase of wheat is located in the supernatant fraction (Weinberger and Clendenning, 1952) obtained by centrifugation at 20,000 g. Therefore this fraction was used in routine measurements of enzymatic activity. Although impure, these extracts were easily prepared and did not require the addition of pyridoxal phosphate as co-enzyme.

Activity measurements were made at the pH optimum (5.5) in the presence of excess substrate. All values were corrected for thermo-stable activity, if any, and for enzyme activity without substrate or with the decarboxylation product. Enzymatic capacities were calculated as mm^3 CO_2/hr/ml of original cell fluid and mm^3 CO_2/hr/twenty leaves. Activity measurements were made manometrically in a nitrogen atmosphere at 30°C and corrected for endogenous carbon dioxide production (enzyme in 2 ml phosphate buffer, pH 5.5; 10µmoles/l glutamate, 30°C).

B. RESULTS

1. Physical Measurements on Leaves and Roots

After exposure of imbibed Rideau wheat seeds to temperatures of 2° ± 1°C for periods of one to three weeks, growth under greenhouse conditions was investigated. Measurements were made on the following parameters: - leaf length and weight, root weight and meristematic root tip activity. Observations were made after 5, 7, 14 and 21 days of growth.

a. Leaf Length

The lengths of the first emergent leaf (L1) are given in Table 1. In all cases 60 leaves were measured for each observation (triplicate experiments of 20 leaves each).

Table 1

First Leaf (L1): - Variation of leaf length (mm) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G (Control)	C_1	C_2	C_3	
5	22.0 ± 3.8^xx	24.4 ± 4.8	31.5 ± 4.2	39.8 ± 2.9	12.20
7	51.0 ± 1.8	53.0 ± 1.9	63.6 ± 1.5	63.8 ± 2.1	15.70
14	55.8 ± 4.8	54.7 ± 3.3	60.5 ± 4.4	60.8 ± 3.1	4.04
21	58.9 ± 4.1	56.0 ± 2.2	59.6 ± 5.0	62.8 ± 2.8	4.06

*F 5% = 4.07 F 0.5% = 9.6 xx Standard Deviation

Duncan's Test (5% level)

Days	G	C_1	C_2	C_3
5	22.0	24.4	31.5	39.8
7	51	53	63.6	63.8

Significant differences in length were found only between the

G and C_3 series after five days growth and between the G,C_1 and C_2,C_3 series after seven days. No significant differences were found between treatments in the length of the first leaf after 14 and 21 days. It would appear that the longer periods of vernalization affect only the growth to early maturity of the first leaf and not the ultimate length.

The lengths of the second leaf (L2) for all treatments are shown in Table 2.

Table 2

Second Leaf (L2): - Variation of leaf length (mm) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G (Control)	C_1	C_2	C_3	
5	3.7 ± 1.5 xx	28.0 ± 7.1	25.2 ± 3.9	30.5 ± 4.3	20.97
7	20.5 ± 6.8	91.9 ± 17.5	84.5 ± 13.5	96.5 ± 3.2	26.82
14	106.6 ± 6.2	131.5 ± 6.8	127.3 ± 8.6	124.7 ± 7.4	11.49
21	127.9 ± 15.7	137.5 ± 3.3	134.3 ± 6.1	130.1 ± 9.1	4.01

*F 5% = 4.07 F 0.5% = 9.6 xx Standard Deviation

Duncan's Test (5% Level)

Days	Treatment			
5	G 3.7	C_2 25.2	C_1 28.0	C_3 30.5
7	G 20.5	C_2 84.5	C_1 91.9	C_3 96.5
14	G 106.6	C_3 124.7	C_2 127.3	C_1 131.5

Vernalization caused a significant increase in the length of the five, seven and fourteen day old leaves over those of the control.

Unlike the case of L1, the duration of vernalization appeared to be immaterial.

No significant difference was found between the lengths of

any of the leaves after twenty-one days growth. As for the first leaf (L1) vernalization only affected growth to early maturity and not the final leaf lengths.

The lengths of the third leaf (L3) under similar conditions are shown in Table 3.

Table 3

Third Leaf (L3): - Variation of leaf length (mm) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G (Control)	C_1	C_2	C_3	
5	0.4 ± 0.3[xx]	5.5 ± 1.8	5.1 ± 3.0	9.2 ± 2.6	8.29
7	2.4 ± 1.8	21.9 ± 10.4	18.4 ± 4.1	26.3 ± 10.3	5.48
14	122.9 ± 5.2	171.9 ± 15.0	163.0 ± 6.5	166.8 ± 7.4	17.28
21	162.3 ± 13.5	173.4 ± 7.3	173.7 ± 11.1	174.8 ± 9.1	0.96

*F 5% = 4.07 F 1% = 7.59 F 0.5% = 9.6 xx Standard Deviation

Duncan's Test (5% Level)

Days	Treatment			
5	G 0.4	C_2 5.1	C_1 5.5	C_3 9.2
7	G 2.4	C_2 18.4	C_1 21.9	C_3 26.3
14	G 122.9	C_2 163.0	C_3 166.8	C_1 171.9

Significant increases in the leaf length were found for all the treated plants up to two weeks growth. The duration of vernalization had no apparent effect on leaf length. After twenty-one days growth there was no significant difference between the lengths of any of the leaves as was found for L1 and L2.

The duration of vernalization was thus of significance only in the first leaf (L1) up to seven days of growth. The second and

third leaves of all the vernalized plants showed no inter-treatment variation. Vernalization accelerated early and intermediate growth phases but never affected the final leaf length. The effect of treatment on leaf length is shown in Fig. 1.

b. Leaf Dry Weight

The variations of dry weight of the first three leaves were investigated for the same conditions as the lengths. Triplicate weighings were made on sets of 10 leaves. The data for the first leaf (L1) are shown in Table 4.

Table 4

First Leaf (L1): - Variation in dry weight (mg per 10 leaves) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G(Control)	C_1	C_2	C_3	
5	11.1 ± 3.6[xx]	21.4 ± 3.2	19.0 ± 3.5	21.4 ± 2.5	6.60
7	33.9 ± 8.3	45.8 ± 5.0	48.4 ± 7.7	43.3 ± 4.8	4.06
14	68.7 ± 5.0	70.6 ± 2.7	78.7 ± 4.7	81.8 ± 6.1	4.03
21	77.7 ± 8.8	51.8 ± 9.0	39.0 ± 7.9	18.0 ± 4.1	30.67

*F 5% = 4.07 F 1% = 7.59 F 0.5% = 9.6 xx Standard Deviation

Duncan's Test (5% Level)

Days	Treatment			
	G	C_2	C_1	C_3
5	11.1	19.0	21.4	21.4
	C_3	C_2	C_1	G
21	18.0	39.0	51.8	77.7

During the experimental period the control leaves continuously increased in dry weight. In the vernalized series, however, the weights reached a maximum after fourteen days and then decreased. During the first week of greenhouse growth, when vernalization has been

shown to influence leaf length a significant difference was observed in the dry weight between treated and control leaves. The effect of vernalization became evident again after twenty-one days. At this period, the duration of vernalization affected a progressive decrease in dry weight i.e. $G > C_1 > C_2 > C_3$. This suggests that in L1 the onset of senescence with accompanying translocation of material from the leaf was accelerated by longer periods of vernalization.

Dry weights for the second leaf are presented in Table 5.

Table 5

Second Leaf (L2):- Variation in dry weight (mg per 10 leaves) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G(Control)	C_1	C_2	C_3	
5	5.4 ± 0.7[xx]	15.8 ± 3.9	12.0 ± 0.5	13.2 ± 3.7	8.01
7	16.4 ± 5.2	56.0 ± 8.1	55.8 ± 8.9	51.9 ± 10.3	50.13
14	84.8 ± 8.5	86.3 ± 5.7	86.6 ± 9.9	84.9 ± 4.7	0.49
21	99.6 ± 9.0	99.3 ± 7.2	108.4 ± 3.4	102.0 ± 12.1	0.39

*F 5% = 4.07 F 1% = 7.59 F 0.5% = 9.6 xx Standard Deviation

Duncan's Test (5% Level)

Days	Treatment			
5	G 5.4	C_2 12.0	C_3 13.2	C_1 15.8
7	G 16.4	C_3 51.9	C_2 55.8	C_1 56.0

The second leaves progressively increased in weight during the entire experimental period, irrespective of treatment. Dry weights in the vernalized series up to seven days of growth were much higher than those of the control. The duration of vernalization appeared to be immaterial. During the latter part of the experimental period (fourteen and twenty-one days) the dry weights of all leaves were similar.

The lack of a significant variation in dry weight of young second leaves from seeds vernalized for different periods indicates that only a portion of the translocates from senescent L1 reached L2.

Dry weights for the third leaf for all treatments are found in Table 6.

Table 6

Third Leaf (L3): - Variation in dry weight (mg per 10 leaves) with age and vernalization

Days from transfer to greenhouse	Treatments				F* value
	G (Control)	C_1	C_2	C_3	
5	** xx	1.7 ± 0.4	1.3 ± 0.14	1.8 ± 0.6	11.13
7	0.08 ± 0.03	13.6 ± 2.5	11.6 ± 2.6	13.8 ± 4.2	16.77
14	61.1 ± 5.7	120.9 ± 7.5	118.8 ± 6.4	128.2 ± 9.8	49.91
21	168.1 ± 7.8	162.4 ± 14.7	183.5 ± 7.3	191.2 ± 13.8	3.12

*F 5% = 4.07   F 0.5% = 9.6
** Not macroscopically evident
xx Standard Deviation

Duncan's Test (5% Level)

Days	Treatment			
	G	C_2	C_1	C_3
5	**	1.3	1.7	1.8
	G	C_2	C_1	C_3
7	0.08	11.6	13.6	13.8
	G	C_2	C_1	C_3
14	61.1	118.8	120.9	128.2

Dry weight increased progressively with time for all treatments. The duration of vernalization was not significant but vernalization did substantially increase the dry weights to fourteen days of growth.

Again after twenty-one days there was no material difference between the weights of any of the leaves.

c. Leaf Fresh Weight

The fresh weights of the leaves of all series were observed and are shown in Table 7, where each value represents the mean of thirty determinations. The large standard deviations obtained in many cases vitiated the use of leaf fresh weight as a stable parameter in the present study.

Table 7

First, second and third leaves (L1, L2 and L3): - Variation of fresh weight (mg per 10 leaves) with age and vernalization

Days from transfer to greenhouse	Treatments					
	G			C_1		
	L1	L2	L3	L1	L2	L3
7	362 ± 71xx	55 ± 28	6 ± 2	470 ± 60	564 ± 57	81 ± 14
14	370 ± 57	538 ± 43	198 ± 20	665 ± 34	699 ± 64	1109 ± 114
21	421 ± 59	675 ± 40	1063 ± 174	457 ± 81	757 ± 40	1036 ± 227

Days from transfer to greenhouse	Treatments					
	C_2			C_3		
	L1	L2	L3	L1	L2	L3
7	445 ± 94	521 ± 68	85 ± 7	401 ± 42	598 ± 74	100 ± 38
14	567 ± 70	651 ± 95	725 ± 63	626 ± 47	878 ± 33	538 ± 43
21	300 ± 74	751 ± 74	1379 ± 113	374 ± 76	934 ± 69	1133 ± 160

xx Standard Deviation

d. Root Measurements

Visual comparison with the controls indicated that root development was more pronounced in the case of vernalized seeds during early growth. The increase in root length was, however, impossible to follow meaningfully owing to the formation of adventitious roots, laterals, and the death of the apical meristem of the primary root during the experimental period. Growth was also hard to assess by root weight

measurements because it was difficult to free the laterals and root hairs completely of soil particles. Large standard deviations were obtained for fresh and dry weights of the roots. Some of these results are shown in Table 8.

Table 8

Fresh Weight: - Variation of root weight (mg per 10 roots) with age and vernalization

Days from transfer to greenhouse	Treatments			
	G(Control)	C_1	C_2	C_3
7	456 ± 90.5xx	503 ± 94	563 ± 154	587 ± 71
14	1395 ± 113	1764 ± 284	1065 ± 265	1604 ± 196
21	3046 ± 197	2876 ± 269	2814 ± 228	4106 ± 202

xx Standard Deviation

Root vigor was therefore estimated by counting the cells of the meristematic root tip segment of comparable roots of all series at weekly intervals. The results shown in Table 9 are expressed as total cell count (T.C.), meristematic cell count (M.C.) and vacuolated cell count (V.C.). The triplicate determinations for each result varied by ± 4 - 6%.

Table 9

Variation of cell count (x 10^{-5}) with age and vernalization in meristematic root tips (10 mm segment)

Days from transfer to greenhouse	Treatments											
	G			C_1			C_2			C_3		
	T.C.	M.C.	V.C.	T.C.	M.C.	V.C.	T.C.	M.C.	V.C.	T.C.	M.C.	V.C.
7	5.5	4.8	0.7	16.7	16.0	0.7	33	32.1	0.9	30	29.1	0.9
14	21.1	18.0	3.1	23.2	22.1	1.1	27	24.1	2.9	24	20.2	3.8
21	17.0	12.0	5.0	18.0	13.2	4.8	18	12.8	5.2	17	12.5	4.5

These data were not subjected to an analysis of variance as part of the root caps sometimes sloughed away during handling. Even without statistical analysis, however, it is evident that the total cell count in the root tips was greatly affected by vernalization during early growth. After seven days little difference was observed. The vacuolated cell counts were unaffected by vernalization, but increased in all cases over the experimental period. The major portion of the root tip cells were meristematic and these are taken as a measure of root vigor. The duration of vernalization influenced the number of these cells in the order $G < C_1 < C_2 = C_3$ up to seven days. At fourteen to twenty-one days the effect of vernalization was no longer observable although in the case of the control and C_1 series meristematic cell counts increased up to two weeks and then decreased. A progressive decrease in meristematic cell count was observed for the C_2 and C_3 series.

2. Chemical Determinations on Leaf Extracts

a. The Variation in Total Protein with Age and Vernalization

Triplicate values for total nitrogen per 100 mg dry weight of tissue were found for all the leaf series at the stated, experimental periods and are shown as arithmetic means in Table 10.

Table 10

Total leaf protein nitrogen for control and vernalized leaf series (data expressed as mg nitrogen per 100 mg dry weight of tissue)

Series **	Age of leaves in days			
	5	7	14	21
L1-G (Control)	3.87	3.94	3.70	3.58
L1-C_1	4.06	4.00	3.96	3.04
L1-C_2	3.93	3.98	3.90	3.26
L1-C_3	3.74	3.82	3.89	2.89
L2-G (Control)	3.90	3.92	3.88	3.79
L2-C_1	4.00	4.01	3.98	3.87
L2-C_2	3.91	4.00	4.00	3.95
L2-C_3	3.95	4.01	4.00	3.89
L3-G (Control)	*	*	4.00	3.97
L3-C_1	4.97	4.06	4.02	4.00
L3-C_2	6.01	4.06	4.01	4.00
L3-C_3	5.04	4.05	4.00	3.98

* Leaf too small to give adequate sample
** Code as previously detailed (p.vi)

The variation in the total nitrogen content of the leaves over the growth period falls within the limits of experimental error. In the exceptional cases of L3-C_1, 2, 3, levels of total nitrogen at age 5 days were higher than for any other leaves but by seven days had decreased to the general values. These results are consistent with those found by other authors (Bryant and Fowden, 1959).

The change in total nitrogen with time thus appears to be quite small when the data are expressed as above on a common weight basis. The emphasis in this work has been on the effects of vernalization on the growth of specific leaves, rather than on an estimate of changes with respect to lumped dry weight of many leaves. In order to maintain this focus on the leaves estimates for the bulk nitrogen per ten leaves were computed. These values which are arithmetic means of triplicate

determinations are found in Table 11. The data expressed in this way give a different picture of the distribution of total nitrogen in all the leaf series.

Table 11

Total nitrogen (mg per 10 leaves) for all ontogenetic and vernalized leaf series

Series	Age of leaves in days			
	5	7	14	21
L1-G (Control)	0.43	1.33	2.46	2.78
L1-C_1	0.83	2.15	2.78	1.58
L1-C_2	0.75	1.90	3.07	1.27
L1-C_3	0.80	1.80	3.19	0.52
L2-G (Control)	0.21	0.64	3.29	3.78
L2-C_1	0.63	2.85	3.43	3.76
L2-C_2	0.47	2.28	3.46	4.28
L2-C_3	0.52	2.08	3.38	4.05
L3-G (Control)	*	*	2.44	6.46
L3-C_1	0.83	0.55	4.86	6.50
L3-C_2	0.62	0.47	4.56	7.29
L3-C_3	0.67	0.45	5.12	7.60

* Leaf too small to give adequate sample

The data are also shown in Fig. 2. The experimental period encompasses within one or more of the leaves various portions of the sigmoid growth curve (leaf length vs time). These growth phases are mirrored in the substantial changes in the total nitrogen of the leaves as they progress through periods of exponential growth to final maturity and senescence (Fig. 1). In the first leaf the more rapid onset of senescence in the vernalized series is paralleled by a more rapid loss in total leaf nitrogen. In all the vernalized second and third leaf series (L2 and L3) the earlier onset of growth (as compared with control) can be correlated with correspondingly large increases in total nitrogen.

b. Calibrations for Amino Acid Analyses

In order to estimate the margin of error in the visual method of rating amino acids (p. 11) duplicate concentration series of leucine were co-chromatographed and quasi-quantitatively assayed. In the concentration range of 0.5 - 2.5 µmoles/l the divergence from the known concentrations was ± 5%. Concentrations above and below these limits gave results within only ± 10 - 15% of the known values.

To evaluate the accuracy of the quantitative method (p. 11) estimations were again made on known concentrations of leucine. The optical density measurements, together with the comparable data of Moore and Stein (1948, 1954) are shown in Fig. 3. There are many references in the literature on the variable oxidation of Ruhemann's purple when ninhydrin alone is used as the identifying amino acid spray (Kay et al, 1956). The results with ninhydrin indicate that within any one series of chromatograms processed at the same time, there is a good reproducibility of results. However, between chromatograms processed at different times the variability was sometimes such as to require a new calibration line for each separate development (Fig. 3). This has also been found by other workers (Thompson et al, 1951) and has led to highly refined techniques to duplicate similar conditions.

The hydrindantin method on the other hand gave some variation of results both within and between series, but the variations were never such as to necessitate a different calibration. The requirement of only a single calibration line, together with increased sensitivity due to higher optical density at a given concentration led

us to adopt the hydrindantin method for use on the leaf extracts.

Leucine Equivalents - In order to express the amino acids as leucine equivalents, solutions containing seventeen amino acids in equal concentrations were chromatogrammed and the optical density measurements compared with that of leucine. In this way the leucine calibration line served to convert optical density measurements of all amino acids to concentrations.

These results are given in Table 12.

Table 12

Color Densities at 570 mμ of Amino Acids relative to Leucine

Amino Acid	Relative Color Density	Amino Acid	Relative Color Density
Leucine	1.00	Glutamic acid	1.02 ± 0.03
Isoleucine	1.00 ± 0.02	Threonine	0.90 ± 0.02
Phenylalanine	1.04 ± 0.04	Glycine	0.94 ± 0.03
Valine	1.04 ± 0.02	Serine	0.96 ± 0.02
Tyrosine	0.98 ± 0.03	Aspartic Acid	0.84 ± 0.02
Methionine	0.92 ± 0.03	Arginine	0.92 ± 0.03
γ-Aminobutyric acid	0.94 ± 0.03	Histidine (330 mμ)	0.88 ± 0.04
Proline	0.95 ± 0.04	Lysine	0.99 ± 0.02
Alanine	1.02 ± 0.02		

c. The Variation in Some Alcohol Soluble Amino Acids with Age and Vernalization

The first three leaves of wheat under study here contain a number of soluble amino acids of which seventeen (see Table 12) were evaluated at various time intervals. This afforded information on changes in the soluble amino acid pool during the development of vernalized and untreated leaves.

Total Soluble Amino Acids (Control Series only) - The total concentration of the amino acids was calculated by adding their individual

values and its variation with time is shown in Fig. 4 (cf also Table 13). During the rapid exponential growth of the second leaf, its initially high soluble nitrogen content was depleted. The lowest level was reached at the phase of most active growth. Thereafter, the amino acid content was regained continuously. The onset of senescence in the first leaf (L1) is characterized by a steady decline in soluble amino acids.

Individual Amino Acids - The amino acid concentrations (μmoles/gm dry weight) during exponential growth and maturation are given in Table 13. The results have also been evaluated as amino acid content in μmoles per 20 leaves in order to demonstrate the behaviour of a leaf as a unit. These are shown in Table 14.

The latter way of expressing concentrations gives a truer picture of the amino acid level than the dry weight basis. During growth photosynthetic products (mainly carbohydrates) accumulate and thus "dilute" the amino acids. This is evident on comparing Tables 13 and 14. As an example we could cite arginine where the concentration in μmoles/20 leaves was approximately constant during the growth of L2 whilst the concentration in μmoles/gm dry weight apparently decreased by a factor of 2.7.

Both methods of expressing the amino acid concentrations lead of course to similar values for the relative composition in terms of individual amino acids. The changes in the relative amounts of amino acids are shown in Fig. 5.

TABLE 13

Control Series: - Variation of Alcohol Soluble Amino Acids (μmoles amino N/gm dry weight) with Age and Ontogeny

Amino Acid	Leaf Series and Age in Days							
	L1			L2			L3	
	Mature	Mature	Late mature	Exponential	Late exponential	Mature	Exponential	Near mature
	9 days	16 days	30 days	9 days	16 days	30 days	16 days	30 days
Leucine	1.53	2.41	-	-	-	-	-	-
Isoleucine	-	-	-	-	-	-	-	-
Phenylalanine	0.60	1.00	-	-	-	-	-	-
Valine	-	-	-	-	-	-	-	-
Tyrosine	0.20	0.32	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-
γ-Aminobutyric acid	15.13	10.94	11.88	4.24	1.49	7.68	1.94	5.45
Proline	0.65	-	-	-	0.32	0.02	0.20	-
Alanine	0.06	4.43	4.53	6.87	-	3.20	-	2.33
Glutamic acid	11.42	6.90	6.47	2.06	0.74	2.37	0.86	3.72
Threonine	0.68	1.64	-	2.63	-	1.36	-	0.01
Glycine	0.68	1.67	3.56	2.52	0.01	2.06	0.01	1.15
Serine	2.62	0.19	1.29	-	-	0.92	-	0.11
Aspartic acid	7.54	5.52	2.52	1.49	1.38	1.93	1.76	1.41
Arginine	15.08	10.77	10.59	16.04	6.88	5.85	6.52	3.60
Histidine	10.97	4.14	4.17	2.86	1.60	3.91	1.76	1.30
Lysine	1.20	0.46	1.49	1.49	1.60	1.01	1.06	0.50
μmoles amino N/gm dry weight	68.36	50.39	46.50	40.20	14.02	30.31	14.11	19.58

- ... Detected in trace amounts

TABLE 3.4

Control Series: - Variation of Alcohol Soluble Amino Acids (µmoles in leucine equivalents per 20 leaves) with Age and Ontogeny

Amino Acid	Leaf Series and Age in Days							
	L1			L2			L3	
	Mature	Mature	Late mature	Exponential	Late exponential	Mature	Exponential	Near mature
	9 days	16 days	30 days	9 days	16 days	30 days	16 days	30 days
Leucine	0.28	0.72	-	-	-	-	-	-
Isoleucine	-	-	-	-	-	-	-	-
Phenylalanine	0.12	0.30	-	-	-	-	-	-
Valine	-	-	-	-	-	-	-	0.01
Tyrosine	-	0.80	-	-	-	-	-	-
Methionine	-	0.80	-	-	-	-	-	-
γ-Aminobutyric acid	3.80	3.26	3.67	0.74	0.56	3.64	0.87	4.20
Proline	0.12	-	-	-	0.12	0.08	0.09	0.01
Alanine	0.01	1.32	1.40	1.20	-	1.52	-	1.80
Glutamic acid	2.00	2.10	2.00	0.36	0.28	1.08	0.39	2.90
Threonine	0.12	0.50	-	0.16	-	0.62	0.01	0.01
Glycine	0.12	0.51	1.10	0.44	0.06	0.94	0.05	0.90
Serine	0.46	0.06	0.40	-	-	0.42	-	0.09
Aspartic acid	1.32	1.68	0.78	0.26	0.52	0.98	0.80	1.12
Arginine	2.64	3.28	3.27	2.80	2.58	2.66	2.95	2.81
Histidine	1.92	1.26	1.29	0.50	0.16	1.78	0.80	1.02
Lysine	0.21	0.14	0.46	0.26	0.60	0.46	0.48	0.39
Total per 20 leaves	13.12	15.93	14.37	6.72	4.88	14.18	6.44	15.25

- ... Detected in trace amounts

The percentage contribution to the total of the various amino acids were also derived from the results of the visual rating method (p. 11) and substantially similar values obtained. In addition this method was used to investigate the variation in relative amino acid content of the first three leaves of wheat vernalized for one, two and three weeks respectively. The results are shown in Tables 15, 16 and 17 for the three leaves.

Both the quantitative results in Tables 13 and 14 and the quasi quantitative values in Tables 15, 16 and 17 indicate that the first three leaves whether from vernalized or untreated seeds contain very little leucine, isoleucine, phenylalanine, valine, tyrosine, methionine and proline at any growth stage. Other workers have considered alanine, arginine, glutamic acid, aspartic acid and γ-amino-butyric acid to be the most prevalent amino acids in plants (Stewart and Pollard, 1957). In the Rideau wheat leaves these amino acids also make up the major portion of the amino acid pool.

The earliest growth phases were found in the third leaf (L3). Here at 1 week only alanine, glutamic acid and γ-amino-butyric acid contributed more than 2% to the pool. At the two week stage which corresponded approximately to the end of the exponential growth phase the pool contained also threonine, glycine, serine, aspartic acid, arginine, histidine and lysine in amounts higher than 2%. This growth stage was also found in the second leaf (L2 - 2 weeks) when the amino acid distribution was similar except for serine.
The effect of vernalization on amino acid distribution during the exponential growth phase is difficult to deduce from the results.

The most pronounced effect of vernalization was on the arginine content of the leaves. Whilst in the control series this was 30 - 50% of the total pool, vernalization caused a drastic reduction to 5% or less. This great difference vitiates comparison of the contributions of the other amino acids and makes the effects of vernalization on them difficult to assess. It does not appear, however, that vernalization simply advances in time the growth phases of the untreated plants.

The high level (~30%) at leaf maturity of γ-amino-butyric acid and lower level of glutamic acid generally found in both the control and vernalized leaf series may arise as follows. At late maturity the total protein level of the leaf declines (L1, Table 10) whilst the total amino acid concentration increased (Fig. 4). Thus hydrolysis of proteins into their constituent amino acids probably took place at this stage leading to increased levels of glutamic acid. This acid on decarboxylation would yield γ-aminobutyric acid.

The author and Clendenning (1952) have shown that a glutamic decarboxylase is present in wheat (var. Coronation) leaves and that its activity was highest during leaf maturity and early senescence. The existence of such an enzyme in the present wheat variety (Rideau) would lend support to the ideas outlined above. The observations described in the next chapter were therefore made on the effect of ontogeny and vernalization on the glutamic decarboxylase activity of Rideau wheat.

TABLE 15

First Leaf (L1): - Variation in Relative Soluble Amino Acid Content* (per cent) with Age and Vernalization

Weeks from transfer to greenhouse	1				2				3			
Amino Acid	G	C_1	C_2	C_3	G	C_1	C_2	C_3	G	C_1	C_2	C_3
Leucine) Isoleucine)	-	2-5	-	-	-	2-5	2-5	-	-	2-5	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Valine) Tyrosine)	-	-	-	-	-	-	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-	-	-	-	-
γ-Aminobutyric acid	25-30	25-30	30-35	35-40	15-20	20-25	25-30	40-45	25-30	10-15	20-25	20-25
Proline	-	-	-	-	-	-	-	-	-	-	-	-
Alanine	-	-	5-10	5-10	15-20	5-10	15-20	5-10	15-20	10-15	15-20	15-20
Glutamic acid	15-20	15-20	20-25	20-25	10-15	10-15	15-20	20-25	10-15	5-10	5-10	5-10
Threonine	-	-	-	-	2-5	-	-	-	-	-	-	-
Glycine	-	-	-	-	5-10	-	-	-	5-10	-	-	-
Serine	2-5	2-5	2-5	-	-	2-5	2-5	-	2-5	-	-	-
Aspartic acid	5-10	15-20	5-10	2-5	2-5	15-20	5-10	2-5	5-10	20-25	10-15	10-15
Arginine	15-20	15-20	5-10	5-10	45-50	15-20	5-10	5-10	20-25	20-25	10-15	10-15
Histidine	10-15	10-15	2-5	5-10	5-10	5-10	-	-	5-10	-	-	-
Lysine	-	2-5	-	2-5	2-5	-	-	-	2-5	-	-	-

* Amino acids as a percentage of the total alcohol soluble amino acids

The dash represents 0 - 2%

TABLE 16

Second Leaf (L2): - Variation in Relative Soluble Amino Acid Content* (per cent) with Age and Vernalization

Weeks from transfer to greenhouse	1				2				3			
Amino Acid	G	C_1	C_2	C_3	G	C_1	C_2	C_3	G	C_1	C_2	C_3
Leucine) Isoleucine)	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Valine) Tyrosine)	-	-	-	-	-	-	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-	-	-	-	-
γ-Aminobutyric acid	10-15	10-15	10-15	10-15	10-15	20-25	25-30	25-30	25-30	30-35	30-35	30-35
Proline	-	-	-	-	2-5	-	-	-	-	-	-	-
Alanine	15-20	10-15	10-15	10-15	-	10-15	2-5	5-10	10-15	10-15	5-10	5-10
Glutamic acid	5-10	10-15	10-15	10-15	5-10	10-15	10-15	20-25	5-10	30-35	25-30	30-35
Threonine	2-5	10-15	5-10	10-15	-	10-15	10-15	5-10	2-5	2-5	5-10	2-5
Glycine	5-10	-	-	-	-	5-10	5-10	2-5	5-10	5-10	2-5	5-10
Serine	-	15-20	5-10	35-40	-	20-25	25-30	20-25	2-5	5-10	5-10	-
Aspartic acid	2-5	-	-	-	5-10	-	-	-	5-10	-	-	-
Arginine	40-45	-	-	-	45-50	2-5	2-5	-	15-20	€	-	-
Histidine	5-10	-	-	-	2-5	-	-	-	10-15	-	2-5	-
Lysine	2-5	-	-	-	10-15	-	-	-	2-5	-	-	-

* Amino acids as a percentage of the total alcohol soluble amino acids

The dash represents 0 - 2%

TABLE 17

Third Leaf (L3): - Variation in Relative Soluble Amino Acid Content* (per cent) with Age and Vernalization

Weeks from transfer to greenhouse	1				2				3			
Amino Acid	G**	C_1	C_2	C_3	G	C_1	C_2	C_3	G	C_1	C_2	C_3
Leucine) Isoleucine)	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-
Valine) Tyrosine)	-	-	-	-	-	2-5	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-	-	-	-	-
γ-Aminobutyric acid	-	35-40	35-40	35-40	10-15	30-35	25-30	25-30	25-30	35-40	35-40	35-40
Proline	-	-	-	-	-	-	-	-	-	-	-	5-10
Alanine	-	25-30	25-30	20-25	-	2-5	5-10	2-5	10-15	2-5	2-5	2-5
Glutamic acid	-	35-40	35-40	40-45	20-25	15-20	10-15	15-20	15-20	20-25	15-20	25-30
Threonine	-	-	-	-	-	10-15	5-10	5-10	-	10-15	10-15	5-10
Glycine	-	-	-	-	-	2-5	5-10	2-5	5-10	5-10	-	2-5
Serine	-	-	-	-	-	5-10	5-10	5-10	-	10-15	10-15	5-10
Aspartic acid	-	-	-	-	10-15	-	-	2-5	5-10	-	-	-
Arginine	-	-	-	-	30-35	2-5	2-5	-	15-20	-	-	2-5
Histidine	-	-	-	-	10-15	2-5	2-5	-	5-10	-	-	-
Lystine	-	-	-	-	5-10	2-5	-	2-5	2-5	-	-	-

* Amino acids as a percentage of the total alcohol soluble amino acids

** Not macroscopically evident

The dash represents 0 - 2%

3. Enzyme Study: - Glutamic Decarboxylase

The glutamic decarboxylase activity of the first three leaves, vernalized and untreated, was estimated after 5, 7, 14 and 21 days of greenhouse growth. The activity of the enzyme increased with increasing age of the leaves in all cases. The maximum activity which was reached at maturity was dependent on leaf ontogeny, i.e. it increased in the order L1 < L2 < L3. These results are similar to those found for Coronation wheat (Weinberger and Clendenning, 1952).

In Table 18 are listed the glutamic decarboxylase activity measurements expressed as mm^3 CO_2/ml supernatant of the leaf tissue extract.

Table 18

Glutamic decarboxylase activity in control and vernalized wheat leaves (mm^3 CO_2/ml supernatant)

Age in days after transfer	G			C_1			C_2			C_3		
	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
5	nil	nil	*	nil	20	20	nil	10	20	nil	10	22
7	52	50	10	48	55	92	63	60	105	55	58	95
14	52	140	326	55	162	520	68	175	450	70	155	480
21	78	160	470	75	180	565	75	200	540	82	183	525

* Leaf not macroscopically evident

The enzyme activity measurements are also expressed on a twenty leaf basis, and these are shown in Table 19. The data indicate that the maximum enzyme activity attained increased roughly three times from leaf to leaf in the ontogenetic series for all treatments.

Table 19

Glutamic decarboxylase activity in control and vernalized wheat leaves (mm^3 CO_2/20 leaves)

Age in days after transfer	G			C_1			C_2			C_3		
	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
5	nil	nil	*	nil	2.6	2.0	nil	1.2	2.0	nil	1.1	3.3
7	5.7	5.5	0.3	8.6	8.2	18.4	9.3	9.0	15.7	8.2	8.6	17.9
14	5.2	25.0	65.0	8.2	28.9	104.0	10.0	33.0	90.0	10.4	27.7	105.0
21	7.8	22.9	94.0	13.4	32.1	113.0	11.0	40.0	108.0	12.2	32.7	120.0

* Leaf not macroscopically evident

The maximum enzyme activity in comparable leaves of all vernalized plants was approximately the same and was independent of the period of vernalization. The control leaves all had somewhat lower values than the vernalized series. The development of enzyme activity with time was similar for all ontogenetically equivalent leaves of the series. This is illustrated in Fig. 6. In L2 and L3 an "induction" period during the time corresponding to early exponential growth was followed by a sudden rise of the activity which continued to maturity before levelling off. In L1 the activity developed was always relatively small and remained essentially constant during late maturity and early senescence.

In Fig. 6 are also shown the concentrations of glutamic acid and γ-aminobutyric acid which were obtained by the visual rating method. With the exception of L3-C_2 these increased during the period of exponential growth and early maturity as found in L2 and L3. At late maturity (L1-G, C_1) the concentration of the acids remained approximately constant and then decreased during early senescence (L1-C_1, 2, 3).

The two amino acids also did not show the large ontogenetic effect which was so marked in the case of the enzyme activity.

FIGURE I.

VARIATION IN LEAF LENGTH WITH TIME

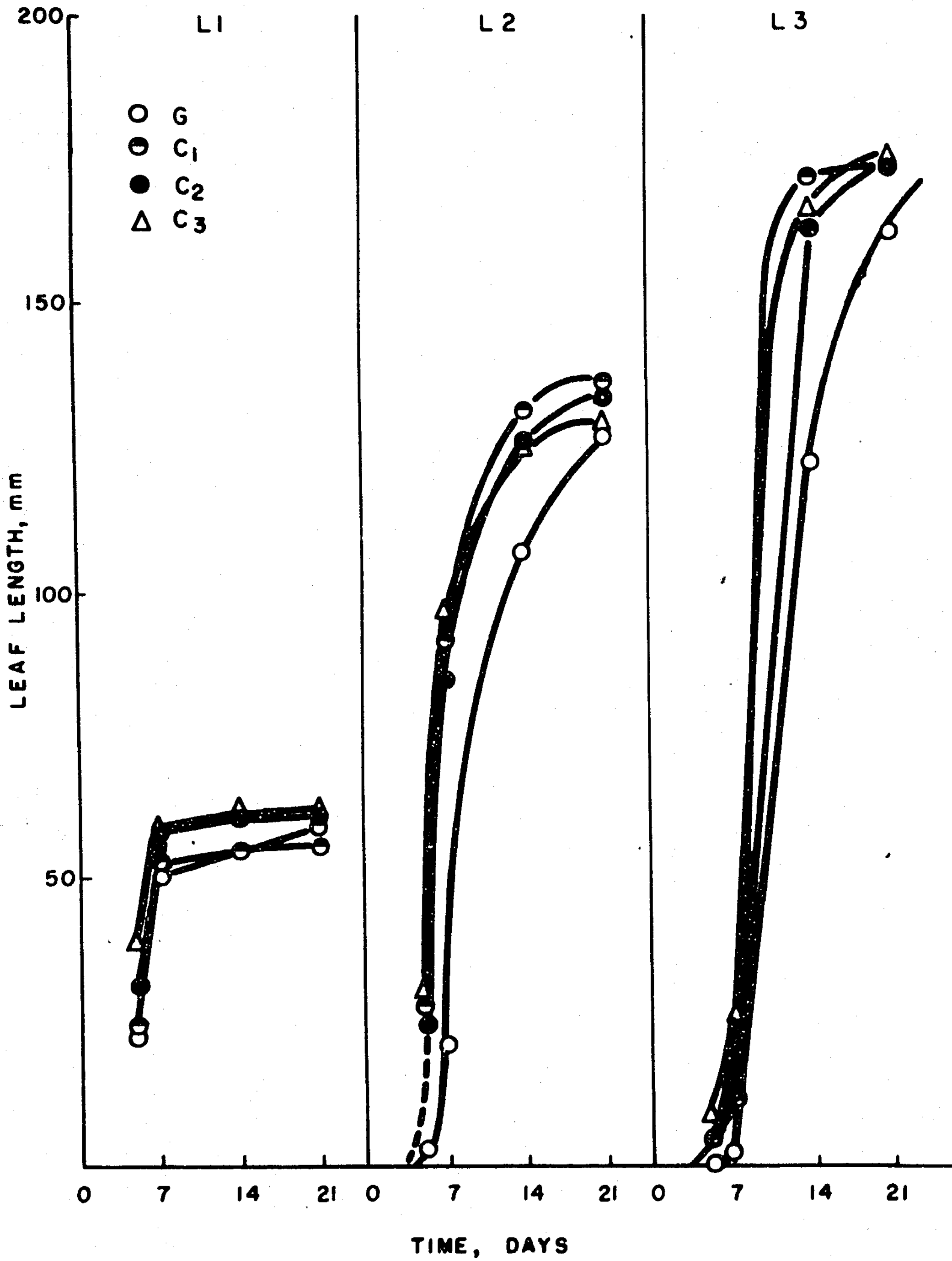


FIGURE 2

VARIATION OF TOTAL LEAF NITROGEN WITH TIME

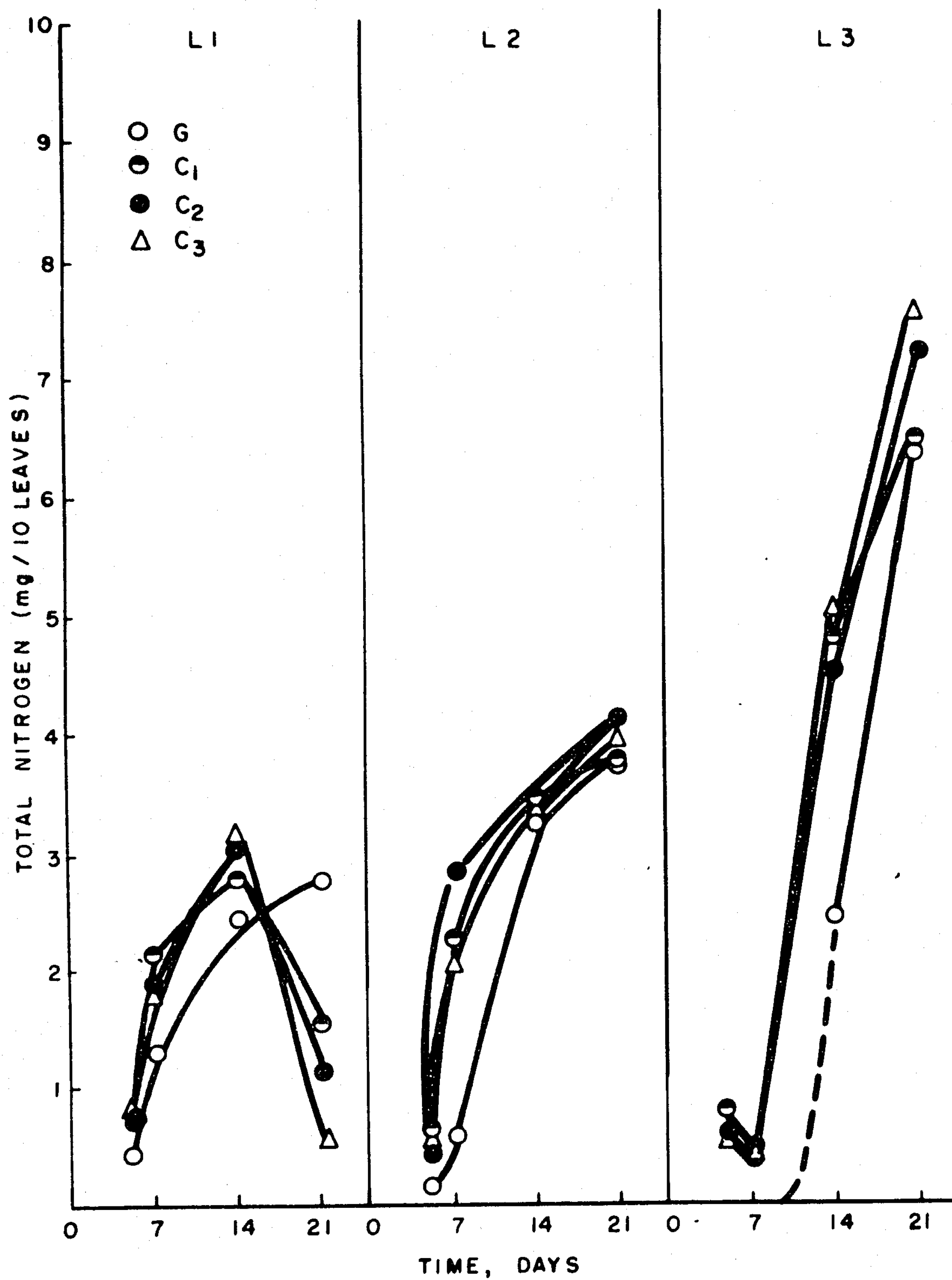


FIGURE 3

OPTICAL DENSITY (570 mμ) VS. LEUCINE CONCENTRATIONS

A COMPARISON OF METHODS

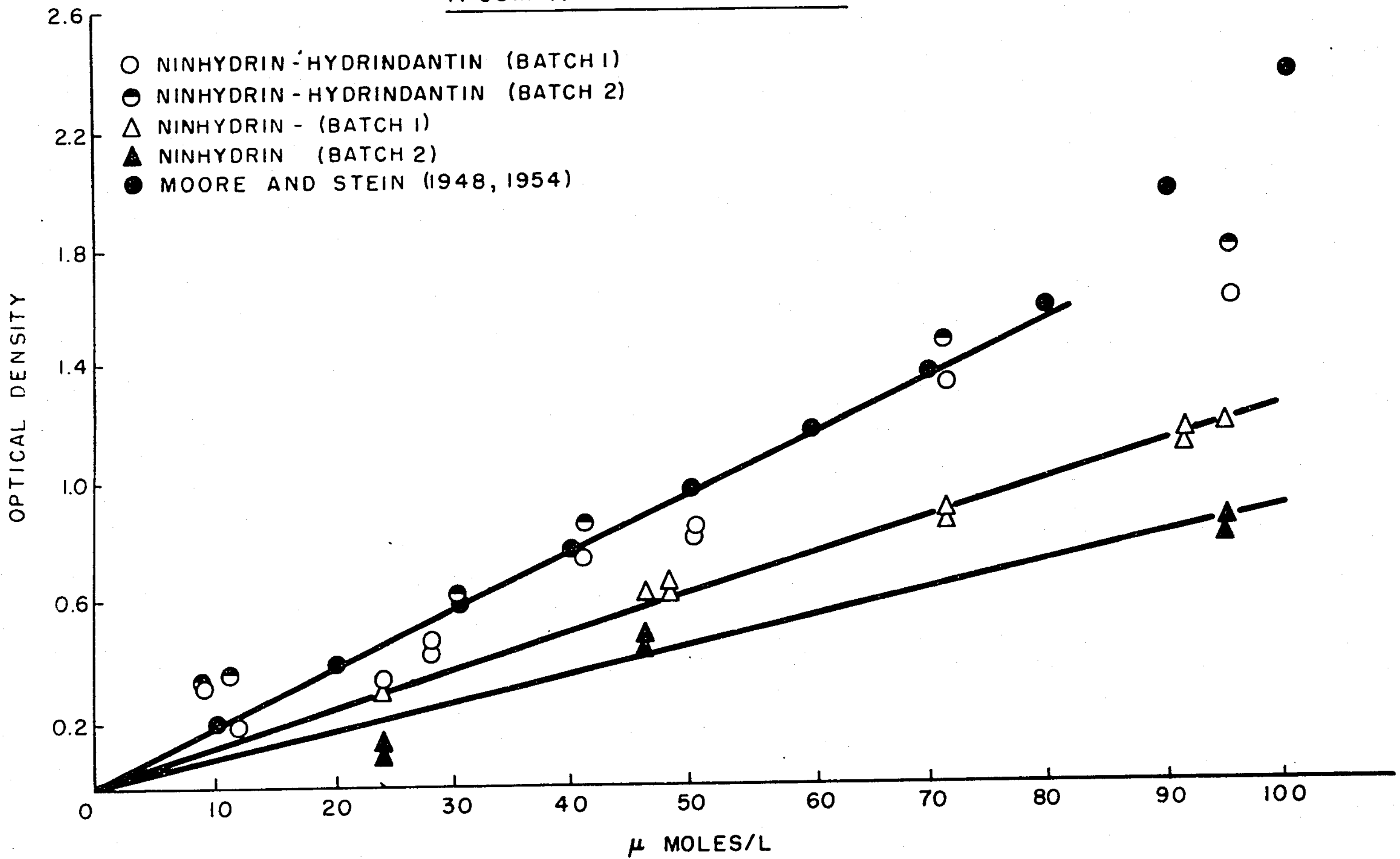


FIGURE 4

CHANGE IN TOTAL CONCENTRATION OF SOLUBLE AMINO ACIDS OF LEAVES (L1 AND L2) DURING DEVELOPMENT OF CONTROL SERIES (G)

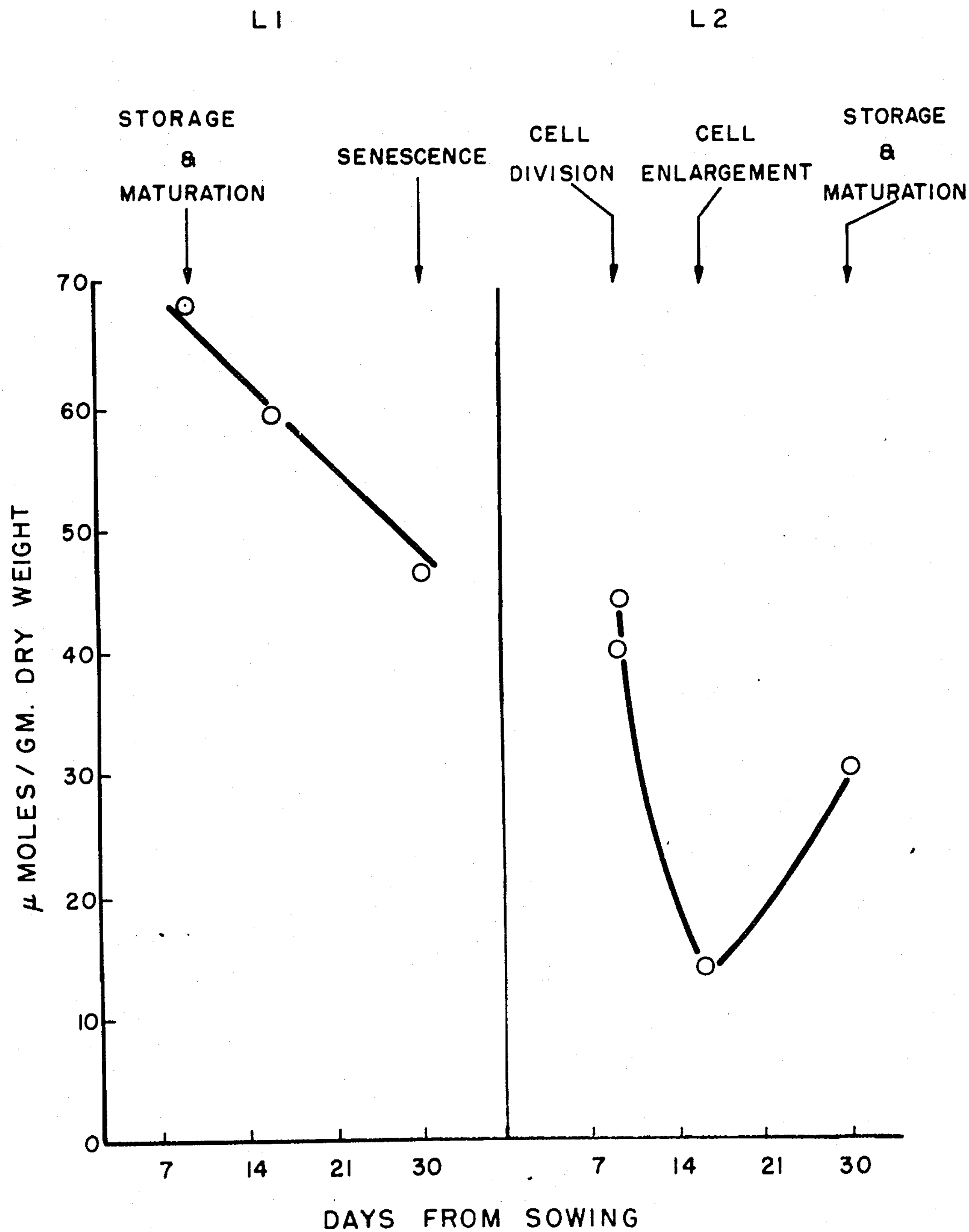
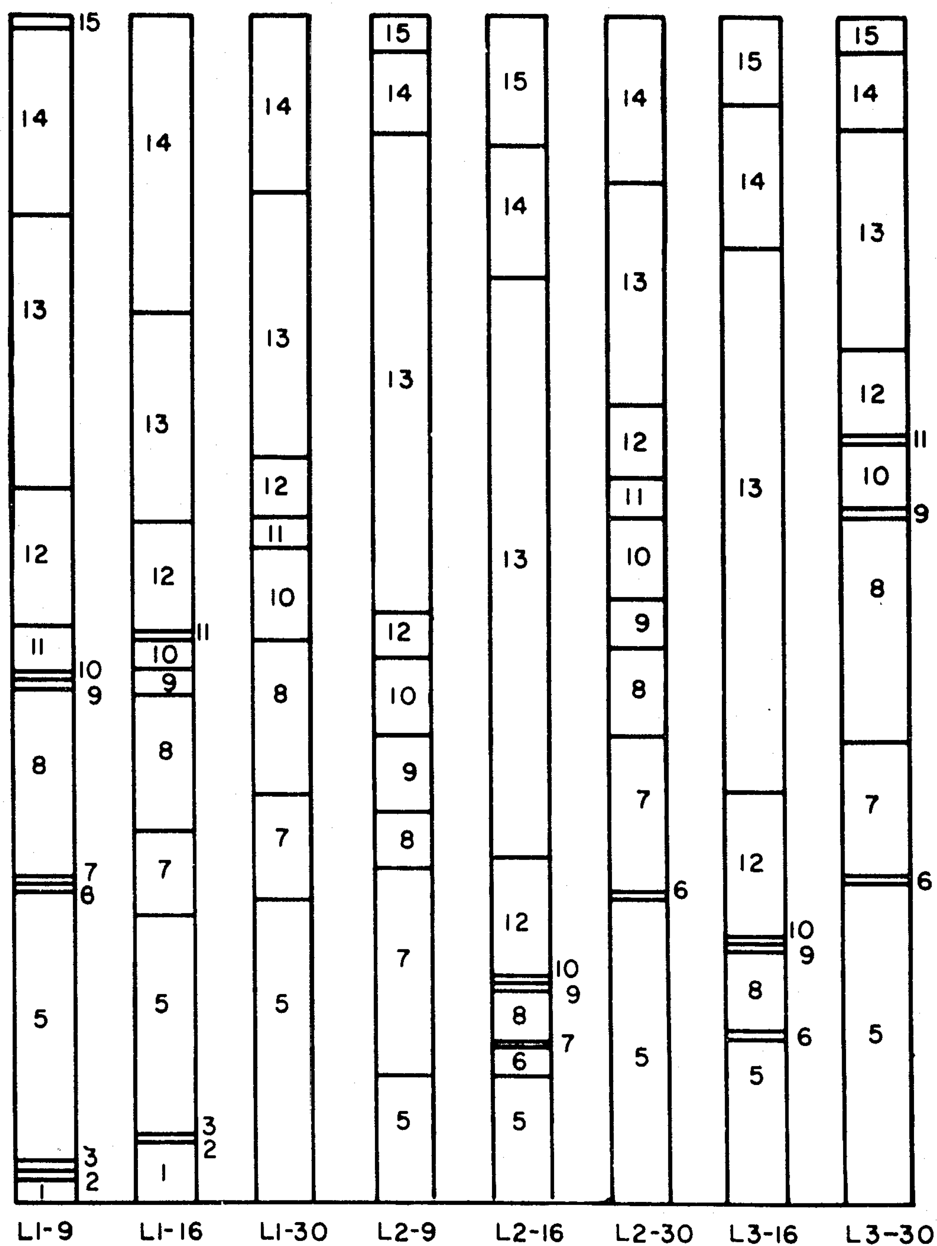


FIGURE 5

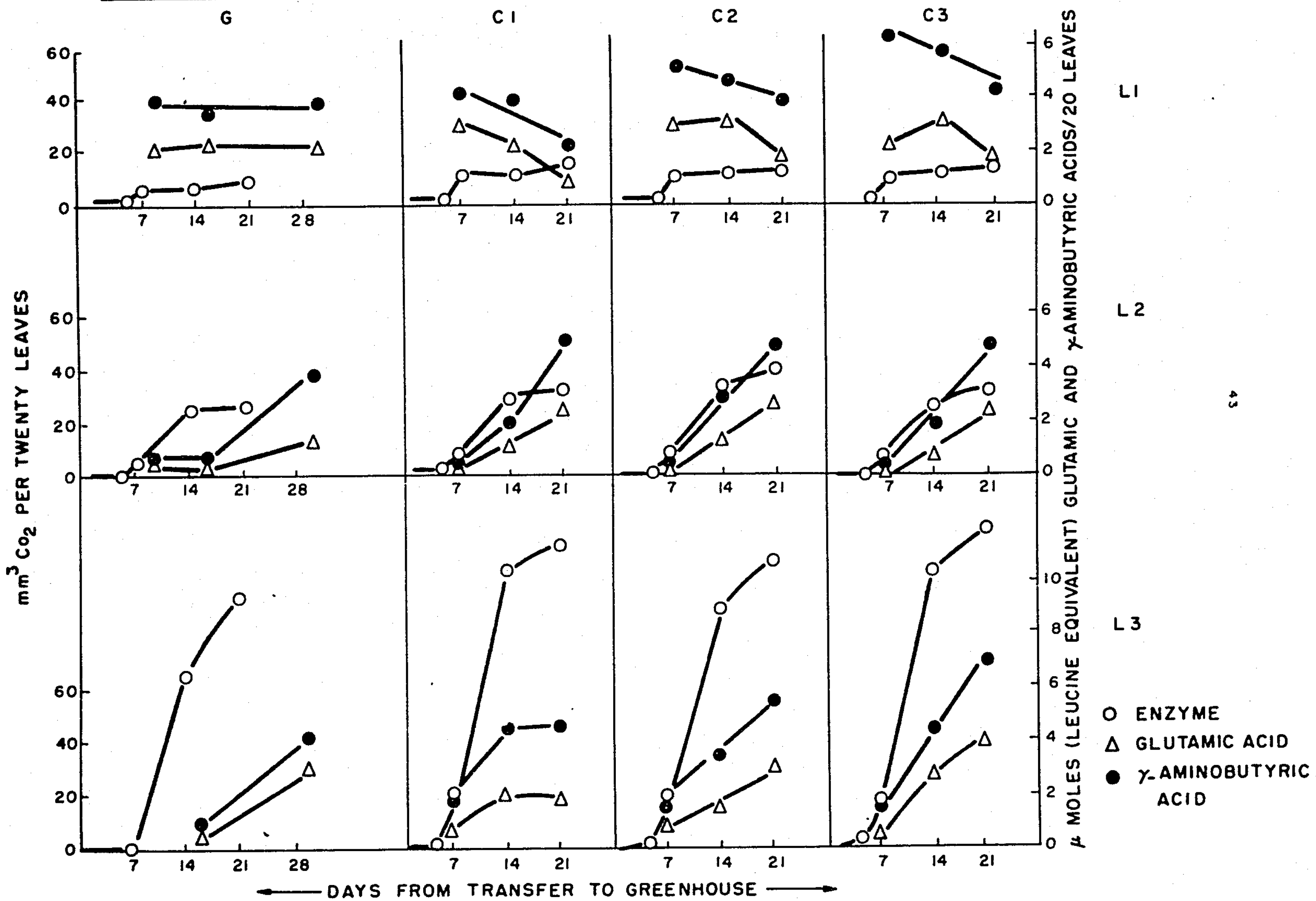
RELATIVE AMOUNTS OF AMINO ACIDS AT VARIOUS LEAF AGES
(% OF TOTAL AMINO ACIDS)



1. LEUCINE & ISOLEUCINE
2. PHENYLALANINE & VALINE
3. TYROSINE
4. METHIONINE
5. γ-AMINOBUTYRIC ACID
6. PROLINE
7. ALANINE
8. GLUTAMIC ACID
9. THREONINE
10. GLYCINE
11. SERINE
12. ASPARTIC ACID
13. ARGININE
14. HISTIDINE
15. LYSINE

FIGURE 6

VARIATION IN ENZYME, GLUTAMIC AND γ-AMINOBUTYRIC ACIDS WITH TIME



PART THREE

GENERAL DISCUSSION

The response to vernalization is undoubtedly connected with the degree to which the thermophase has been completed at seed maturity (Gregory and Purvis, 1938; Purvis and Gregory, 1952), but it also depends on the after-sowing conditions (Wort, 1939, 1940; Gott et al, 1955). The same environment thus may be expected to have a different effect on the development of vernalized and unvernalized plants sown simultaneously. This difference in response is illustrated by the results presented here.

In the case of leaf growth a significant acceleration of the growth of the leaf primordia was observed for all vernalized leaves, even though the maximum leaf lengths (at 21 days) were in all cases similar (Tables 1, 2, 3). This more rapid leaf development in vernalized plants was paralleled by significant larger increases in the dry weight of the leaves after five days growth (Tables 4, 5, 6). By twenty-one days, however, there was no difference between the dry weights of the leaves from untreated and vernalized plants. Besides stimulating leaf growth, vernalization also increased the onset of senescence of the first leaf. The observed drying of the leaf tips was accompanied by significant changes in the leaf dry weight after 21 days of greenhouse growth. The longer the period of vernalization, the more rapid was the onset of senescence (Table 4). Vernalization thus accelerated the whole sequence of developmental changes during the life of the leaf, but had no effect on the length or weight of the mature leaf.

For reasons mentioned in Part 2, B, d (p.21) cell counts were used as an index of root growth. During the first week of greenhouse growth vernalization caused a great increase in meristematic activity. The duration of vernalization was important (Table 9). A close relationship between root vigor and leaf growth has previously been observed (De Ropp, 1946; Chibnall, 1954). Although it is known that the plumule "perceives" the cold treatment, it is at present impossible to say whether the leaf growth expedited by vernalization occurs because of the improved root system or whether the whole plant is directly affected by the cold treatment.

Not all responses to environmental conditions can be assessed by purely morphological criteria, and therefore the effect of vernalization on the total (bulk) protein content of the first three leaves was also followed in the present investigation. It would be natural to assume that growth and protein synthesis proceed concomitantly. Neither our results on protein nitrogen content of 100 mg dry weight of the leaves (Table 10) nor the observations of Bryant and Fowden (1959) on daffodil leaves (also expressed on a dry weight basis) bear out this assumption.

A close correspondence between protein content and growth state appears, however, when our results are expressed on a per leaf basis (Table 11). It is concluded therefore that for comparison with morphological measures of plant development, content of metabolites should be expressed on organ rather than dry weight basis. As already pointed out (p.24) the total (bulk) protein showed an accelerated increase in the three leaves of the vernalized plants parallelling

their accelerated growth due to vernalization. Similarly, the accelerated onset of senescence of the first leaf was accompanied by a progressive decrease in total (bulk) protein after twenty-one days of growth. During the near mature stages of growth in the second and third leaf when the effect of vernalization on leaf length and dry weight was no longer significant, it was found that the total protein content of the leaves from control and treated seeds did not appreciably differ from one another.

Changes following vernalization in the soluble amino acid pool were also followed. The major components of the soluble amino acid pool in Rideau wheat leaves were similar to those observed in other species (Bryant and Fowden, 1959). In the untreated leaves these were arginine, γ-aminobutyric acid, glutamic acid, aspartic acid, histidine and alanine. Development affected the individual as well as the total concentrations of the soluble amino acids.

The onset of senescence in the first leaf was characterized by a decrease in the total soluble amino acids (Fig. 4). A similar trend was also observed in leaves of barley (Pleshkov and Fowden, 1959) and banana (Friedberg and Steward, 1960). This can be explained by assuming translocation of amino acids from the mature leaves into young growing tissues. In the second leaf the total soluble amino acids decreased up to sixteen days of growth and then increased (Fig. 4). The initially higher level of soluble amino acids was presumably derived from translocates. As exponential growth continued rapid utilization of metabolites led to a lower level of total soluble amino acids. As growth in length and frequency of cell division

(Sunderland, 1960) slowed down amino acids accumulated in the leaf cytoplasm. Translocation of amino acids into the leaf at this stage was thus more rapid than their utilization. The increase in total soluble amino acids continued to maturity. Steward et al (1958[b]) found that in actively growing cells of carrot root and potato tuber too, the soluble nitrogen was at a lower level than in mature cells.

These variations with time of the amino acid content of the untreated leaves depict some of the internal changes accompanying the visual stages of development.

Vernalization affected the composition of the soluble amino acid pool. The major change was in the level of arginine which contributed as much as 40 per cent to the pool in the leaves of untreated plants but only represented 10 per cent or less of the total amino acids in the case of vernalized plants (Table 16). A similar effect was noted by Zacharius et al (1957) in the study of the amino acid distribution in tulip bulbs during development and floral initiation. In tulip bulbs (T. gesneriana), arginine may account for 45 per cent of the soluble nitrogen. On floral initiation, however, striking changes ensued: - the arginine contribution dropped and that of compounds typical of the tulip leaf (especially glutamic acid and glutamine) increased. The decrease in relative arginine content may thus be linked to a change from the vegetative to the floral stage of growth.

In fact it has been observed quite generally in higher plants (Naylor, 1959) that it is unusual to find appreciable quantities of arginine in rapidly growing tissues. This can be easily understood

considering that auxin promotes the rapid transformation of the nitrogen of arginine into many of the other amino acids (Duranton and Morel, 1958). Larger amounts of arginine on the other hand are associated with dormancy, senescence and are also found in plants which are growing poorly because of mineral deficiences (Steward et al, 1959).

It is therefore not surprising that during accelerated growth of the leaves of Rideau wheat following vernalization a substantial decrease in arginine content was found. In wheat the metabolic utilization of arginine is believed to occur via conversion to ornithine which then yields glutamic semi-aldehyde and ultimately proline and glutamic acid (Kasting and Delwiche, 1957; McConnell, 1959). These amino acids are incorporated into proteins in the growing leaves. At the same time arginine would serve as a reserve source of nitrogen (Oland and Yemm, 1956; Bollard, 1959).

Fewer amino acids made up the pool in the leaves from vernalized plants (especially L2 and L3, Tables 16 and 17).

In the young third leaf (L3-C_1, C_2, C_3) only alanine, glutamic acid and γ-aminobutyric acid contributed more than 2% of the pool. These findings generally support those of Steward et al (1958[b]) observed on the dividing cells of carrot root tissue culture. It is possible here, as in yeast (Steiner, 1959) that alanine is formed directly from γ-aminobutyric acid so that it too may be traced back to glutamic acid.

The observed low levels of tyrosine, leucine, isoleucine, phenylalanine and methionine in all series was not unexpected. These

amino acids have been shown to occur as only trace amounts in the free state in the leaves of plants given a full complement of nutrients (Steward et al, 1959). Leucine, phenylalanine and glycine have been shown to exert powerful morphogenic effects on plant tissues even in very low concentrations (Steinberg, 1947; Miettenen and Waris, 1958; Miettenen, 1959).

The plant cell vacuole does not contain amino acids in the proportions to form protein. For example it contains γ-aminobutyric acid which is never incorporated as such into protein. The observations of Steward et al (1958[a, b, c]) on potato tuber and carrot explant tissue cultures also indicate that the soluble amino acids cannot be directly correlated with the amino acid content of plant proteins.

From Table 14 and Fig. 1 it is evident that the concentrations of γ-aminobutyric acid and glutamic acid varied with the physiological age of the leaf in all cases. Vernalization did not alter the relationship between the concentration of glutamic acid or γ-aminobutyric acid and the phase of leaf growth. An increase in γ-aminobutyric acid concentration during growth has also been found in the leaves of barley (Pleshkov and Fowden, 1959), daffodil (Bryant and Fowden, 1959) and banana (Friedberg and Steward, 1960). However, large inter-species differences were observed in the behaviour of the other constituents of the amino acid pool with increasing age and no general statements can be made.

Glutamic acid presently regarded as a primary product of amination in plants (Allison and Burris, 1957; Folkes, 1959), and γ-aminobutyric acid, now recognized as one of the "key" amino acids

(Steward et al, 1956; Steiner, 1959), are mainly related by the activity of glutamic decarboxylase which when present always occurs in the supernatant fraction of plant extracts (Dixon and Fowden, 1961). However, no clear relationship between the level of enzyme activity and the concentration of substrate and product was evident in Fig. 6. None should perhaps be expected as other workers have found a similar lack of correlation between glutamic decarboxylase activity and the γ-aminobutyric acid content of a tissue (Thompson et al, 1953; Dixon and Fowden, 1961).

The amount of γ-aminobutyric acid in plant tissue may be controlled in part by the rate of its intracellular movement (Pietruszko and Fowden, 1961; Dixon and Fowden, 1961). Glutamic acid is released from the mitochondria where it is synthesized by the action of glutamic dehydrogenase (Bone, 1959) into the non particulate cytoplasm where glutamic decarboxylase is present. Here decarboxylation to γ-aminobutyric acid takes place. Then, before γ-aminobutyric acid degradation can proceed, it migrates back to the mitochondria where the highest concentration of transaminases is found. Dixon and Fowden suggest that the balance between these two transport mechanisms tends to regulate γ-aminobutyric acid turnover.

In conclusion, the present work indicates that in spring wheat (var. Rideau), vernalization of the seed caused a marked change in the metabolism of the seedling. Internal changes were accompanied by accelerated leaf development and an initial large increase in root growth.

Further research might be directed towards elucidating some of the biochemical changes occuring in the embryo during the course of vernalization.

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