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PHYSIOLOGICAL CHANGES IN THE WHEAT ROOT TIP FOLLOWING GROWTH AND VERNALIZATION

BY

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A thesis
submitted to the
University of Ottawa
in partial fulfillment of the requirements for the degree
Doctor of Philosophy in Biology.

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ABSTRACT

Sequential changes in the alcohol soluble amino acid and carbohydrate fractions of vernalized and control root tips of *Triticum aestivum* var. Marquis (spring) and Rideau (winter) wheats have been followed. Substantial changes in these fractions could be related to developmental changes in the root tip. Vernalization superimposed additional, consistent changes mainly in the content of the amino acids asparagine, glutamine, glycine, and glutamic acid, and of the sugars fructose and sucrose. Incorporation studies using ^{14}C glutamic acid and aspartic acid served to emphasize the distinct metabolic differences between the mitotic and elongating regions of the root tips. In the mitotic region, the highest incorporation of label was in the protein fraction, whereas in the elongating region, the highest activity was recovered in the free amino acid pool. Two enzyme systems, namely amylase and glutamic acid-alanine transaminase were also studied.

The possibility of using the uptake of ^{51}Cr as an indicator of metabolic change in wheat root tips, as it is in animal tissues, was also examined. A consistent pattern of ^{51}Cr incorporation was observed in the serial sections of both wheat varieties. The changed metabolic state attendant on vernalization was reflected by a higher level of ^{51}Cr incorporation. Histochemical studies of sulphydryl group distribution along the wheat root tip

were also carried out in order to determine whether there exists a relationship between sulphydryl groups and the pattern of ^{51}Cr uptake. No such correlation could be established.

The effect of the water content of the grain during vernalization which is known to affect the subsequent root growth, was also examined. Incomplete imbibition led to a lessened metabolic response to the cold treatment.

RESUME

Nous avons étudié les effets superposés de la croissance et de la vernalisation sur la teneur en acides aminés et en hydrates de carbone de la racine de deux variétés de blé, Triticum aestivum var. Marquis (blé de printemps) et Rideau (blé d'hiver). Les stades progressifs de la différentiation de la racine s'accompagnent de changements marqués de la concentration de ces substances. La vernalisation produit un changement additionnel, surtout dans la concentration de l'acide aspartique, l'asparagine, la glutamine, la glycine, le glucose et le fructose.

L'incorporation des acides aspartique et glutamique marqués au ^{14}C diffère dans les différentes régions de la racine. Dans la région méristématique, c'est dans les protéines que l'incorporation est le plus élevé, tandis que dans la région de l'élongation, c'est dans la fraction des acides aminés libres que se retrouve la plus grande partie du ^{14}C.

Nous avons aussi étudié la possibilité d'utiliser le ^{51}Cr comme indicateur de changements métaboliques dans la racine du blé, comme il est employé dans les tissus d'animaux. Il existe une relation entre le taux d'incorporation du ^{51}Cr et le stade métabolique des cellules de la

racine du blé. Les changements métaboliques liés à la vernalisation se manifestent par une augmentation du niveau d'incorporation du ^{51}Cr . Aucune corrélation n'a pu être établie entre la distribution du ^{51}Cr et la répartition des groupes sulphydryles dans la racine.

Une imbibition incomplète du grain durant la vernalisation aboutit à une diminution de la réaction métabolique au traitement par le froid.

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Section 1 - General Introduction and Procedures

INTRODUCTION

Vernalization, a term coined by Lysenko, in 1928, may be defined as the " acquisition or acceleration of the ability to flower by a chilling treatment " (Chouard,1961). The capability to respond to a cold treatment is not universal. It is mainly found in long day plants with a protracted vegetative period (Purvis, 1961). Some species, especially cereals, exist in two forms: a spring form, which if sown in the spring will bloom during mid-summer of the same year, and a winter form, which in temperate countries needs to be sown in the fall in order to flower during the following summer. Without the over-wintering, the winter form would come to ear only in late November, too late for harvesting.

The age at which a plant can be successfully vernalized varies. In cereals, the mature grain as well as the grain ripening in the ear is sensitive to the cold treatment (Gregory and Purvis, 1938 a ; Kostjucenko and Zarubailo, 1936). In the case of most biennials and perennials the seed is not vernalizable (Melchers, 1952).

The physiology of vernalization is known mainly through two large bodies of work: that of Melchers and Lang on _Hyoscyamus niger_ and that of Gregory and Purvis on the rye Petkus (Ruhland, 1961). The fundamental rules of vernalization derived for rye are also applicable to the

other cereals, especially wheat (Chouard, 1961).

The effective temperature of chilling is in the range 1°C to 6°C with a widespread optimum (Hansel, 1953; Purvis, 1948). It decreases from 0°C to -5°C and also from 7°C to 14°C. At -6°C and 15°C no vernalization occurs. However, low temperature is without effect on dry seed. The ideal water content, according to Lysenko (1932) and Tolmacev (1927), would be an amount sufficient to insure the beginning of germination while low enough to prevent seedling growth. For cereals 50-60% of the air dry weight of the seed has proved adequate. Higher levels of imbibition do not prevent vernalization. They promote seedling growth and are thus impractical with respect to a later sowing of the seed.

The presence of oxygen is also absolutely necessary for vernalization (Gregory and Purvis, 1938 b).

Although the characteristics and the requirements of vernalization for different species are known, the mechanism of vernalization is still not fully understood. In order to help elucidate the process, many studies have been concerned with the consequences of the cold treatment on the morphology and biochemistry of different plant organs.

Morphological changes induced by vernalization in cereals concern mainly the behaviour of the first leaves. These emerge earlier and mature more rapidly (Konovalov, 1944; Sereiski and Sludskaja, 1937) and there is also a shortening of the blade (Purvis and Hatcher, 1959). A reduction in the number of leaf primordia formed before the

inception of flower primordia has also been reported (Purvis, 1934). Chakravarti (1950) has found also a more extensive development of vascular tissue during seed germination.

Physiological changes in the seed during the low temperature treatment are numerous. Physico-chemical differences in the protoplasm such as a shift in the iso-electric point of proteins (Richter, 1934) increased permeability (Chestakov and Sergeev, 1937; Filipenko, 1936), alteration of staining characteristics (Bassarskaya, 1936) and a modified plasmolytic response (Bassarskaya, 1936) have been reported. Differences in metabolism include modified enzyme activity (Chouard, 1960), change in vitamin C and B (Noggle, 1946; Séchet, 1953), altered lipid content (Dupéron, 1952, 1953), reduction in auxin level (Chailakhyan and Zdanova, 1938). Important changes in carbohydrate and nitrogen patterns have also been reported (Chouard, 1960). This is of interest since vernalization accelerates the subsequent flowering and it has been suggested that nitrogen metabolism is related to the attainment of " ripeness to flower " (Klebs, 1918; Runger, 1961).

There is a possibility that the metabolic changes observed during the chilling treatment are simply the result of cold exposure and are not directly related to vernalization itself. Less numerous have been studies dealing with biochemical differences in plants grown from vernalized seed i.e. with only the non germinated seed exposed to the low temperature treatment. A study of this type was made by

Weinberger and Godin (1966) who have found qualitative and quantitative changes in the free amino acid content of the leaves of wheat following vernalization.

The apical meristem ordinarily perceives the vernalization stimulus (Purvis, 1961). Purvis (1940) has shown that the excised embryo and even apices dissected from the embryo could be successfully vernalized. Wellensiek's discovery that the leaves of Lunaria biennis could perceive the cold treatment (Wellensiek, 1961) and that the site of perception was the meristematic tissue at the base of the petiole (Wellensiek, 1962) led to the generalisation that dividing cells are the site of vernalization. Once a meristem has been vernalized, the after effect is transmitted by subsequent cell division.

During the germination of wheat, meristematic activity results first in the emergence of the primary root. The coleoptile appears later. Little information is available, however, on the effect of vernalization on the root. Wort (1939) noted that vernalization was followed by more vigorous root growth and by an increase in the dry weight of whole root systems. Weinberger and Godin (1963) reported an increase in the mitotic activity of vernalized roots. More detailed research of Weinberger and Ku (1966) revealed a prolongation and stimulation of the cell division phase of growth, resulting from the chilling treatment of wheat seeds. Differences in dry weight and fresh weight were also observed.

Amongst the different organs of the plant, the root

provides excellent material for the study of growth and development. It is an axial structure, uncomplicated by the formation of lateral appendages. Furthermore, division does not occur throughout the organ, but is limited to a certain zone, the apical meristem, at the tip of the root. This is followed by a short transition region after which no further division occurs. Subsequent changes in the cell may be attributed to the process of expansion and later differentiation. Thus, in the same root at any one time, cells may be found in a variety of stages of development, with the different steps spatially separated.

Advantage has been taken of this serial succession of stages in cell development in order to study some biochemical aspects of cell growth. Various experimental methods have been used. These include direct examination of the intact root, cutting the root into segments of various sizes, and dealing with individual cells isolated from a given region (Heyes and Brown, 1965; Torrey, 1965). Histochemical studies on longitudinal or cross sections have also been made (Avers, 1961; Jensen, 1955a).

The serial section technique, which involves a manual cutting of the root into segments has been used extensively. It permits a comparison between regions of the root of varying ages. Furthermore, the size of the sections can be adjusted to suit the needs of a particular experiment. The parameters studied with this method are numerous: nitrogen

content (Baldovinos, 1953; Brown and Broadbent, 1950), oxygen uptake (Baldovinos, 1953; Brown and Broadbent, 1950; Karlsson and Eliasson, 1955), carbohydrates and cell wall constituents (Baldovinos, 1953; Brown and Sutcliffe, 1950), nucleic acids (Holmes, Mee and Hornsey, 1955; Jensen, 1958), enzyme activities such as peroxidases (Pilet and Galston, 1955), dipeptidases (Robinson and Brown, 1952), phosphatases (Robinson and Brown, 1952) and respiratory enzymes (James and Boulter, 1955).

No studies have yet been made of changing metabolic patterns along the root following vernalization. The main purpose of this work is to investigate the superimposed effects of growth and vernalization on two main biochemical parameters, namely free amino acid content and carbohydrate composition. Some related enzyme systems were also studied. In animal tissues, the uptake of ^{51}Cr was found to be a useful index of metabolic modifications. The possibility of using this compound as an indicator of metabolic change in wheat root tips was also examined.

Carbohydrates and nitrogen compounds were chosen because of their essential role in cellular metabolism, their importance in cold hardiness (Pauli and Mitchell, 1959; Zech and Pauli, 1960, 1962), their known involvement during seed vernalization (David, 1945; David and Séchet, 1947; Dupéron, 1952, 1953) and subsequent flowering (Chailakhyan, 1968). Features of nitrogen and carbohydrate metabolism in the root will be discussed later in the

introduction to their respective sections.

In order to determine whether there is a difference in response between a spring wheat and a winter wheat, two varieties were selected: Triticum aestivum variety Rideau (winter wheat), and Marquis (spring wheat).

The effect of the water content of the seed during the vernalization treatment, which is known to affect the subsequent root growth (Weinberger and Ku, 1966) was also investigated.

MATERIALS AND METHODS

Treatment of grain and seedlings

Two varieties of wheat, Marquis, a spring wheat, and Rideau, a winter wheat, were used in parallel experiments. Grain was obtained from the Canadian Department of Agriculture through the courtesy of Mr. Gefeller.

Imbibition: The grains were allowed to imbibe glass distilled water to 60% of their air dry weight. Imbibition took place at 25°C in black painted 9cm Petri dishes lined with Whatman no. 1 filter paper. In the case of Rideau wheat, two periods of imbibition were used, namely 5 and 14 hours. Marquis wheat was given only one 5 hour imbibition period. A longer imbibition resulted in the emergence of the primary root (Ku, 1965).

Vernalization: The imbibed grains were stored in a refrigerator maintained at a temperature of 2 ± 1°C. The grains were aerated daily by lifting the lids of the Petri plates. Whereas vernalization of Rideau wheat required 5 weeks' chilling to complete the thermophase requirement, that of Marquis required one week (Ku,1965).

Germination: Following the above treatments, the grains were allowed to germinate on cheese-cloth covered screens fitted on top of 250 ml beakers. Germination took

place at 25°C in the dark in a controlled environment chamber. Drying of the grains was prevented by covering them with a layer of cotton wool wetted with distilled water.

The seedlings selected for further study were those with a straight primary root measuring 1.5 ± 0.2 cm. Selection was made on the basis of root length to insure that the material chosen was of the same morphological age.

Cutting of the roots: The roots were cut into seven 0.5 mm serial segments using a specially designed root cutter (Ku, 1969). The cutter consists basically of a series of razor blades inserted on two metal rods. Sections of a required length are obtained by placing metal blocks of a given thickness between the blades.

Treatment of root segments

Subsequent treatment of the root sections depended on the biochemical parameter which it was desired to assay.

Unless otherwise stated, all determinations were made on duplicate groups of 100 segments. When replication was not within 12%,the determination was repeated two more times.

EXPRESSION OF THE DATA

Four different parameters were used to express the data, namely, per segment, per mg protein, per cell and per dry weight.

Segment basis

The 0.5 mm segments were numbered 1 to 7 from the tip to the base of the root. Histological studies have shown that segment 1 consists mainly of root cap, segments 2 and 3 contain the meristematic region, segments 4 to 7 cells in the process of elongation and differentiation.

Protein basis

The protein content of each segment was obtained from the data of Ku (1969). Protein was extracted using sodium borate, and the quantity determined by means of the Folin reagent (Lowry et al, 1951).

Cell basis

The average cell number in each section was obtained by incubating groups of each of the 7 segments in 10% chromic acid for 48 hours at room temperature (0.5 cc/10 segments). The tissue was then further macerated by repeatedly sucking the suspension into a pipette and blowing out rapidly. The cell counts were made on a haemocytometer slide (Brown and Rickless, 1949).

Dry weight basis

Dry weights were obtained by heating the segments in an oven for 24 hours at 110°C. After equilibration with atmospheric humidity, the weights were taken to an accuracy of ± 0.01 mg on an analytical balance.

Code used

$R_{14}K$ - Rideau wheat imbibed 14 hours, control

$R_{14}V$ - Rideau wheat imbibed 14 hours, vernalized

R_5K - Rideau wheat imbibed 5 hours, control

R_5V - Rideau wheat imbibed 5 hours, vernalized

M_5K - Marquis wheat imbibed 5 hours, control

M_5V - Marquis wheat imbibed 5 hours, vernalized

Section 11 - Studies on Nitrogen Metabolism

INTRODUCTION

The discovery of asparagine in 1806 (Vauquelin and Robiquet, 1806) and of glutamine in 1883 (Schulze and Bosshard, 1883) focused attention on the importance of free amino acids as plant cell constituents. However the lack of suitable techniques for the isolation and identification of amino acids hampered early workers in the field. Schulze in 1906 listed only 10 amino acids known to occur free in plant tissues. With the subsequent development of paper chromatography, knowledge of the range of free amino acids present in plants has increased considerably. New plant amino acids are being characterized every year and there seems to be no foreseeable limit to their number.

The free soluble amino acid fraction contains all the protein amino acids together with a number of other amino acids which have not been characterized to date as occurring in plant proteins. The role of many of these free amino acids is still obscure. Some are thought to be end products of metabolism. Since several occur in storage organs or organs of perennation, they may provide inert storage forms of nitrogen (Steward and Durzan, 1965). The distribution of the non-protein amino acids varies with the species. Among the more important non-protein amino acids is γ amino butyric acid which was first isolated from potato tuber (Thompson, Pollard and Steward, 1953) and is now known to be ubiquitous in plants (Steward and Durzan, 1965).

The amino acid metabolism in plants is intricate and

involves far more than the simple combination of the amino acids to form proteins. Plants can utilize inorganic forms of nitrogen such as nitrate or ammonia for their protein synthesis, with the necessary carbon atoms ultimately derived from photosynthesis. However, individual growing cells may receive their nitrogen for synthesis in already elaborated forms. The most important of these nitrogen rich forms are glutamine and asparagine (Steward and Bidwell, 1962). Furthermore, the elimination of wastes does not occur in plants as it does in animals and the end products are stored, either in the cell vacuole or in specialized storage organs, to be translocated as the need arises. Thus there is a continual recycling with translocation, storage, and re-entry of the soluble amino acids in metabolism.

The fate of the free amino acids is varied. The pathways of amino acid metabolism have been explored using isotopically labelled nutrients. Incorporation of ^{14}C labelled amino acids has been followed in a variety of plant species and organs. These tracer methods have demonstrated that the free amino acids incorporated into protein arise not from the total pool of amino acids but from small isolated active metabolic pools spatially different from the larger " inactive " pools (Steward and Bidwell, 1962). Investigations of Vittorio et al (1954) and of Vickery and Zelitch (1960) have led to the concept that " there exists different compartments in the cells in which the fate of a given metabolite may be different ". Furthermore, different

pathways may exist for the synthesis of any particular amino acid depending on the metabolic situation. A good example is the case of glutamine which originates differently in growing cells (Steward, Bidwell and Yemm, 1958) and in a storage organ (Hood, Lyman and Tatum, 1951).

Incorporation studies of labelled precursors into growing root tips have been made by Jensen (1957) and by Clowes (1958). In this way, some ideas of differences in the rate of protein synthesis can be gained with respect to different tissue types or individual cell types. This may contribute to the elucidation of problems of cell differentiation.

The metabolism of amino acids also involves extensive interconversions. Such transformations are of two main types: group transfer reactions such as transaminations, transamidations, and transpeptidations, and interconversions where the whole amino acid undergoes reaction with a modification of the carbon skeleton. Transaminases, which transfer an amino group are common in plants. Most of them involve glutamic acid since the incorporation of inorganic nitrogen occurs vis this compound (Loomis, 1958). The more documented transaminases are the glutamic acid - aspartic acid, the aspartic acid-alanine and the glutamic acid - alanine transaminases (Loomis and Stumpf, 1958). Most if not all the known amino acids can participate in transaminations.

Glutamic acid - alanine transaminase has been demonstrated in crushed pea plants (Virtanen and Laine, 1941)

and in pea seedlings (Rautanen, 1946). Giri et al (1952) found that the activity of the glutamic acid - alanine transaminase increased during the germination of the green gram seed. Leonard and Burris (1947), who made a survey of transaminase activity (glutamic acid - aspartic acid and glutamic acid - alanine) in plants found the greatest activity in the roots.

Several factors affect the composition of the soluble nitrogen fraction in plants. The first variation is from species to species and is most probably due to genetic factors (Steward and Durzan, 1965). It has been suggested that certain plant families may be characterized by the occurence of particular amino acids i.e. liliaceous family by the presence of γ methylene compounds (Fowden and Steward , 1957), legumes by piperidine compounds (Steward and Durzan, 1965).

Variation is also encountered from organ to organ of a single plant and within an organ, differences occur during its growth and development. The accumulation of material as in seeds and storage organs, its mobilisation during germination and growth is reflected in the free amino acid composition. The study of free amino acid changes can thus contribute to a more detailed knowledge of growth and development.

The amino acid composition in relation to growth has been studied in leaves (McKee, 1958), during floral initiation of a variety of species such as tulip (Zacharius,

Cathey and Steward, 1957), and during the development of several fruits such as apple (Hulme, 1936), banana (Ram, Ram and Steward, 1962), pine cones (Durzan, 1964). In all these cases, recognizable changes such as senescence, climacteric rise, floral initiation were reflected by definite changes in the amino acid content. These studies point to a correlation between nitrogen metabolism and development. Changes in a particular amino acid or differences in accumulation parallel sequential stages of development.

Growing regions have also been investigated and certain features have been found to characterize dividing versus non dividing cells. Steward et al (1954) who analysed the shoot apex of lupine found in this small growing point, essentially the same range of amino acids as is found in mature tissues. The basic amino acids were prominent in the region of cell division where they constituted 75% of the soluble amino acid fraction. The tendency of basic amino acids to dominate in meristems was also noted in a fern, _Adiantum_ (Steward, Wetmore and Pollard, 1955). The data for both lupine and _Adiantum_ indicate that the glutamyl family (glutamic acid, glutamine and γ amino-butyric acid) prevails over the aspartyl family (aspartic acid, asparagine) in the regions where growth by division is most active.

The analysis of growing and non-growing cells of tissue cultures has also shown that the nitrogen composition of proliferating cells differs from that of mature non-

dividing cells. Glutamine was found to be associated with conditions favorable to growth and protein synthesis, alanine with conditions adverse to growth (Steward and Durzan, 1965).

Some amino acids have profound morphogenetic effects. Alanine given to pea plants in the nutrient solution makes the plant bushy and the root system short and tuftlike (Virtanen and Linkola, 1946). Isoleucine and leucine inhibit stem and branch elongation in tobacco seedlings (Steinberg, 1947). Waris (1957) has reported that, under the influence of glycine, new plantlets are formed from detached cells of the root tips of Oenanthe aquatica.

The nutrition and the environment of a plant determine to a large extent the composition of the free amino acid fraction. Crane (1951), studying the effect of mineral deficiency in the mint plant has reported that lack of sulfur results in the accumulation of glutamine and asparagine. The daily photoperiod and diurnal fluctuation of temperature affects the free nitrogen content of certain species, such as the mint plant. The night temperature specifically determines the ratio of glutamine and asparagine (Rabson, 1956).

Exposure of plants to cold temperature also results in marked alteration of their nitrogen composition. Studies on cold hardiness first drew attention to this relationship. In the wheat plant, a general increase in soluble non-protein nitrogen was found to accompany the cold treatment (Pauli and Mitchell, 1959; Pauli, Kolp and Stickler, 1961; Zech and Pauli, 1960, 1962).

Vernalization was later discovered to affect nitrogen metabolism. The free amino acid composition of vernalized grain (Grzesiuk and Kulka, 1963; Jones, 1969; Sparmann, 1961), seedlings (Trione, 1966) and leaves (Trione, Young and Yamamoto, 1967; Weinberger and Godin, 1966) of full grown winter wheat plants differs both qualitatively and quantitatively from that of the unvernalized material. Glutamic acid, glutamine and proline seem to be the amino acids most affected.

The present work is concerned with a study of the free amino acid composition of the wheat root tips. Roots from both vernalized and non-vernalized grain are compared in an attempt to determine whether the acceleration of root growth following vernalization is correlated with alterations in nitrogen patterns. The incorporation of two selected amino acids, namely glutamic acid and aspartic acid was also examined.

MATERIALS AND METHODS

A - Free Amino Acid Composition of the Root Tips

Amino acid analysis

The seven serial root tip segments were placed in 0.3 ml 70% ethanol. The sections were homogenized and centrifuged for 15 minutes at 6700 rpm. The debris was further extracted twice, the last extraction lasting overnight. The combined supernatants were evaporated to dryness by means of a warm air draft, picked up in 0.5 ml of 12.5% sucrose solution and analysed by means of a Technicon amino acid analyser. Readings for asparagine and glutamine were obtained after hydrolysis in IN HCL for 3 hours in sealed evacuated tubes (Borsook and Dubnoff, 1939).

B - ^{14}C Studies Involving Glutamic Acid and Aspartic Acid

Incubation and protein extraction

One hundred root tips were cut into seven 0.5 mm serial sections. Segments 2 and 3, and 6 and 7 (counting from the tip) were combined and placed in 1 ml of an isotonic sucrose solution. The above regions were chosen in order to allow a comparison between meristematic cells (segments 2 and 3) and more mature cells (segments 6 and 7). The segments

were then incubated 6 hours at room temperature with either 0.1 ml ^{14}C glutamic acid (specific activity 7.5 mc/mM) or ^{14}C aspartic acid (specific activity 6.1 mc/mM) both in sucrose solution yielding 800,000 counts/ml. All solutions were kept frozen until use.

A long incubation period was necessary because incorporation of label was extremely low. Furthermore, it assured availability of ^{14}C to the more deeply lying layers of the root, those not in immediate superficial contact with the labelled amino acid. Roberts and Butts (1967) have shown that in _Zea Mays_ root tips, 4 hours are needed for ^{14}C glucose to penetrate to the pith region. Although bacterial contamination may be a serious problem in plant incorporation studies (Wilson, 1966), sterile conditions were not used since uptake by the root tip and not disappearance of label in the solution was measured. Decomposition of original label by bacteria was slight, as shown by the autoradiogram (Fig.5).

After the incubation period, the segments were washed 3 times with 5 ml of a saturated solution of unlabelled amino acid and homogenised in sodium borate (1.1g per litre) (Fowden, 1952). Following centrifugation for 15 minutes at 6700 rpm extraction of protein by sodium borate was repeated twice and the supernatants combined. The protein was then precipitated by adding TCA to 10 percent.

The protein precipitate was washed with acetone and ether, dried, and the radioactivity of the different fractions

i.e. debris, protein, organic washings and protein auperna-tant was assessed by means of a Nuclear Chicago deep well liquid scintillation counter. Quench corrections were made where necessary.

Autoradiography

The root segments of M_5K alone were incubated with ^{14}C glutamic or ^{14}C aspartic acid as described above. They were then homogenised in 70% ethanol and the free amino acids extracted as previously described.

A chromatogram was made of the alcohol extract on Whatman No. 1 filter paper using butanol: acetic acid: water (4:1:5) as solvent. ^{14}C glutamic acid and ^{14}C aspartic acid were also spotted. A radioautograph of the chromatogram was obtained using Kodak X-ray film. Exposure lasted 8 weeks.

C - Glutamic Acid - Alanine Transaminase

Determination of activity

The cut root segments were placed individually in ice cold 0.1M phosphate buffer, pH 7.5, and homogenized. The homogenate was allowed to stand 1 hour at 2°C in a refrigerator and centrifuged at 6700 rpm on a clinical centrifuge for 12 minutes. The transaminase activity of the supernatant was determined by estimating the pyruvic acid formed using the method of Friedemann and Haugen (1943), as described by Smith and Williams (1951).

The reaction mixture consisted of 0.2 ml of 0.2 M α ketoglutaric acid with 0.5 ml of 0.2 M phosphate buffer pH 7.5 and 0.2 ml of the homogenate supernatant. These reagents were placed in a small test-tube in a constant temperature water bath at 38°C. Then 0.2 ml of 1 M alanine, also at 38°C was added and the reaction was allowed to proceed for 20 minutes. The enzyme reaction was stopped by the addition of 1 ml of 2,4 dinitrophenylhydrazine reagent (0.1% in 2M HCL). The concentration of pyruvic acid was then determined colorimetrically by the method of Lichstein and Umbritt (1947). Three milliliters of toluene were added to the incubation medium, the resulting mixture well shaken, and the layers allowed to separate. The top layer was removed. To 2 ml of top layer was added 3 ml of 1.5% KOH in 95% alcohol. The resulting colored solution was read at 570 mμ on a Beckman DU Spectrophotometer. A calibration curve was established using pyruvic acid in the concentration range of 10 to 140 μg

RESULTS

A - Free Amino Acid Composition of the Wheat Root Tip

The content of free amino acids in the root tip is expressed in five different ways, namely: quantity per 3.5 mm root tip, per segment, per dry weight, per protein and per cell. This is to ensure a more complete picture of the physiological changes occuring along the root, since the method of expressing the data will clearly influence the interpretation of the results.

Total free amino acid content of the 3.5 mm wheat root tip

Table 1 gives the total content of amino acids found in the 3.5 mm root tip. The spring and winter varieties differ in that Marquis, the spring wheat, contains more of each free amino acid except for ornithine, histidine and asparagine. Approximatly 65% of the total amino acids found in both Rideau and Marquis wheats, control and vernalized, can be accounted for by glutamine, glutamic acid, aspartic acid and alanine. These amino acids, especially the dicarboxylic acids are the main starting points for nitrogen metabolism (Meister, 1965). Methionine and tyrosine are present in very low concentrations and neither of the sulfur amino acids, i.e., cystine and cysteine were detected. According to Fowden (1959), these rarely occur in plants in the free state except under conditions of rapid protein breakdown. The amount

Table 1. Total free amino acid content of the 3.5 mm wheat root tip (10^{-5} micromoles)

	$R_{14}K$	$R_{14}V$	R_5K	R_5V	M_5K	M_5V
Alanine	127	136	126	102	122	188
Arginine	1	4	1	8	6	3
Asparagine	42	70	8	42	12	16
Aspartic acid	201	238	264	133	289	293
Glutamic acid	287	425	328	271	542	632
Glutamine	488	418	496	410	407	649
Glycine	62	49	97	43	112	69
Histidine	65	62	66	48	51	32
Isoleucine	34	30	36	28	55	44
Leucine	49	37	56	36	78	66
Lysine	17	17	15	15	21	15
Methionine	4	2	4	3	8	8
Ornithine	45	38	32	30	34	29
Phenylalanine	21	21	20	20	26	23
Proline	98	110	187	100	241	214
Serine	96	108	110	99	119	144
Threonine	41	40	51	37	49	54
Tyrosine	13	6	7	14	24	15
Valine	47	55	57	39	81	59
γ Amino butyric acid	38	43	25	30	67	36
Total	1776	1909	1986	1508	2344	2589

of asparagine, which is generally considered to be a storage product (Steward and Bidwell, 1952) is also very low.

Although the total amount of free amino acids in both vernalized and unvernalized roots is similar, the concentration of the individual acids is different. Vernalized roots of R_{14} wheat show a 33% increase in glutamic acid over the unvernalized control. Bilinski and McConnell (1957 a,b), while studying the utilisation of ^{14}C labelled acetate by intact plants, have shown that the TCA cycle plays a major role in the formation of the dicarboxylic acids. Enhanced assimilation of gaseous nitrogen (Allison and Burris, 1957)or of carbon dioxide (Poel, 1953) would also result in the accumulation of glutamic acid. Vernalization increases the asparagine content by 64% in R_{14} wheat and by 400% in R_5 wheat, whereas the content of this amino acid was unaffected in Marquis wheat. However, a comparable increase (60%) is found in the other major amide i.e. glutamine, the level of which is less affected in Rideau wheat. Glycine, valine and γ amino-butyric acid are significantly decreased by vernalization in Marquis wheat but are not consistently affected in R_{14} wheat.

Proline, which was found by Markowski et al (1962), Trione et al (1967), and Jones (1969) to increase markedly with vernalization in the grain, and was therefore considered to be a good indicator of the vernalization process, is not affected in the R_{14} root tip, while it decreases in both the R_5 and M_5 root tips.

The length of the imbibition period has no great effect on the free amino acid content of unvernalized Rideau wheat. Asparagine, glycine and proline are the only amino acids which differ significantly. The asparagine content is increased 5 fold with an increase in the imbibition period. Comparing the vernalized R_5 and R_{14} wheats large differences are noted in aspartic acid, glutamic acid and asparagine. The R_{14} Rideau wheat always has the lower concentration. There is a 2 fold increase in the content of aspartic acid in R_5, but not in R_{14}, where it is unaffected. The imbibition period thus has an effect on the free amino acid content of vernalized wheat.

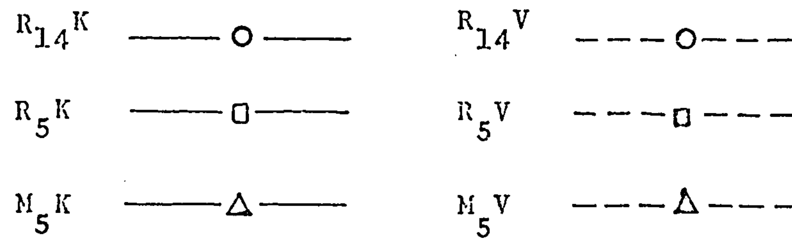
Total free amino acid content per segment

There is a continual increase in the total amino acid content from segment 1 to segment 4 with slight increments thereafter in both Rideau and Marquis wheats (Fig. 1). This increase with growth probably reflects the accumulation of free amino acids in the vacuole. Vernalized R_5 wheat has a lower concentration of free amino acids than R_{14} in segments 3 to 7. This could result from a lower metabolic rate due to the insufficient imbibition period.

Individual free amino acids per segment

Although there is a continuous increase in the total content of free amino acids from segment 1 to segment 7, there

Fig. 1. Change in the total free amino acid content of the wheat root tip with growth, imbibition, vernalization and wheat variety.



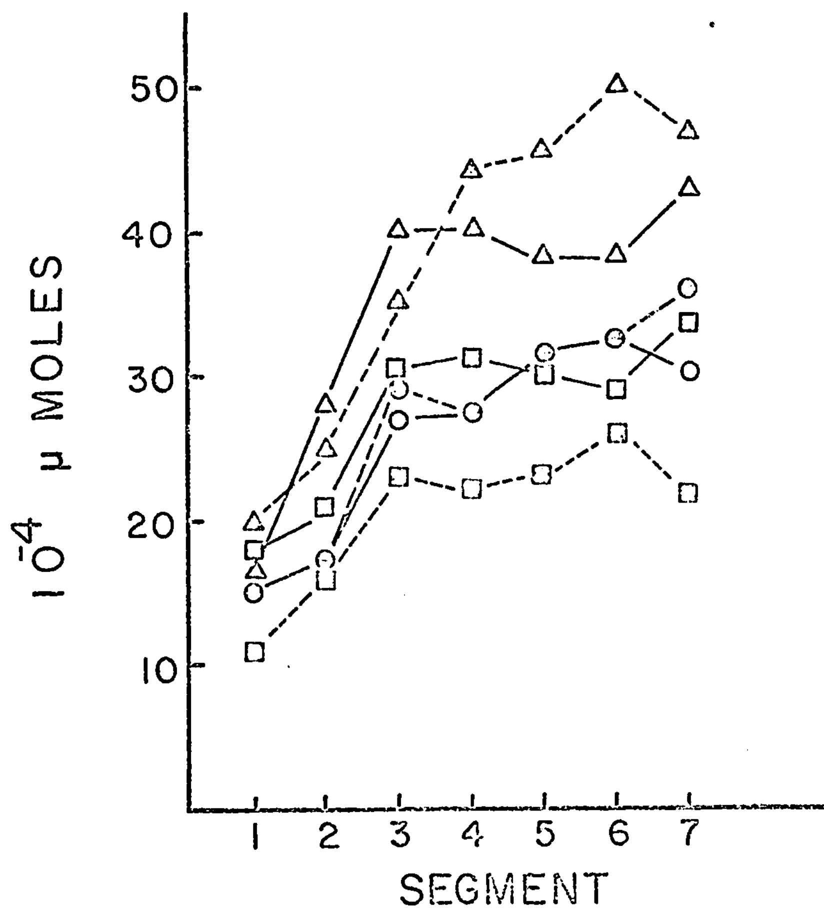
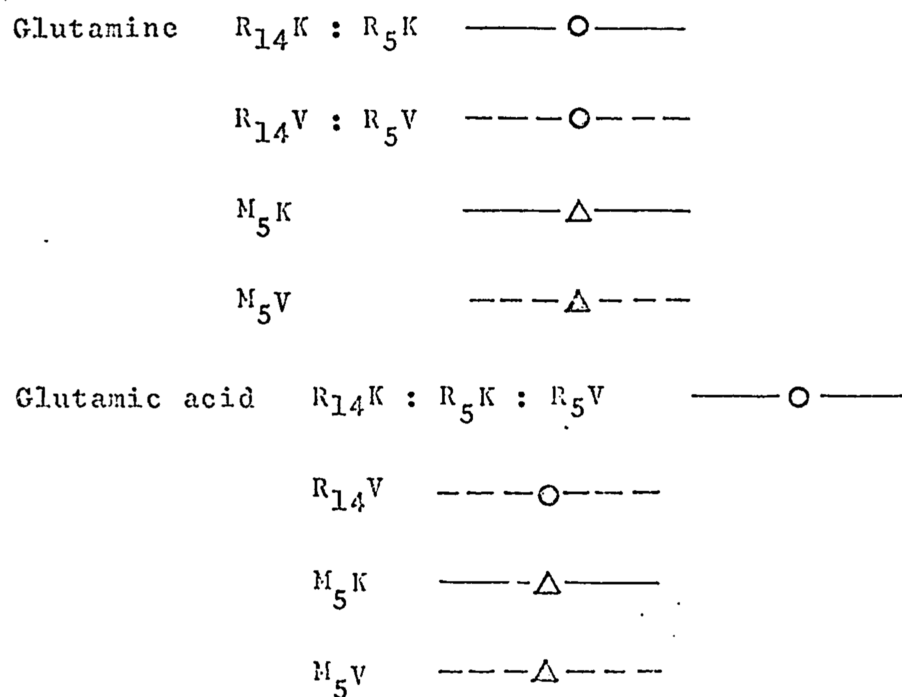

50
40
30
20
10
10^{-4} μ MOLES
1
2
3
4
5
6
7
SEGMENT

Fig. 2a. Change in the concentration of the individual free amino acids of the wheat root tip with growth, imbibition, vernalization and wheat variety.



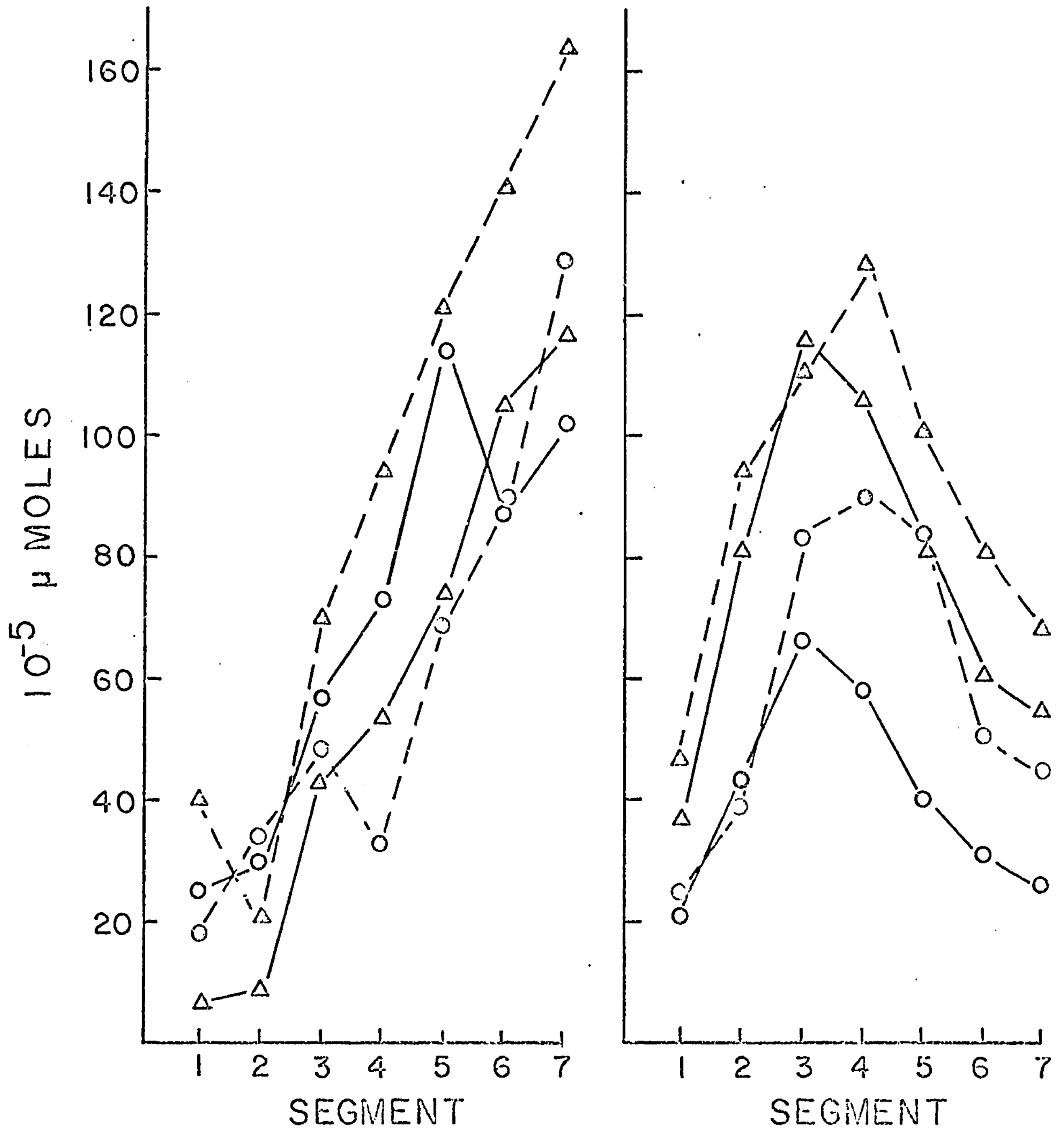
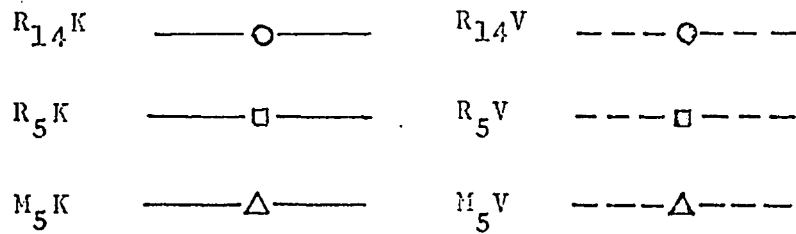
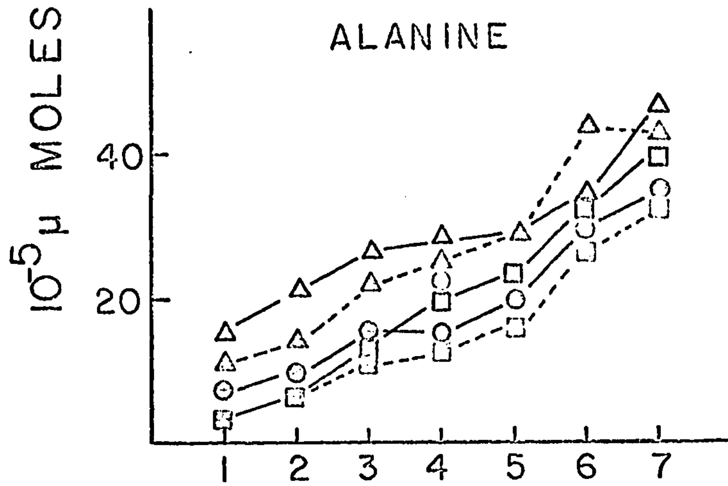
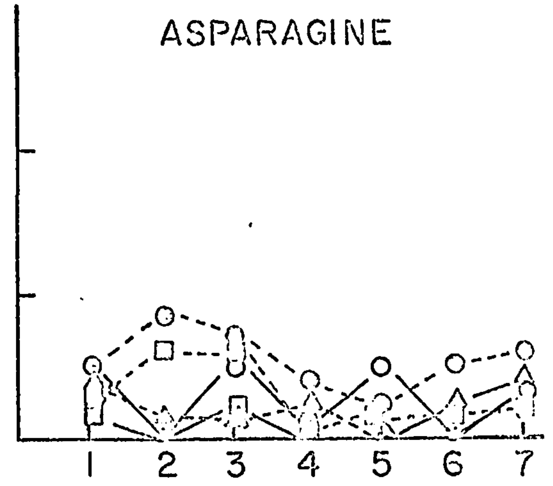
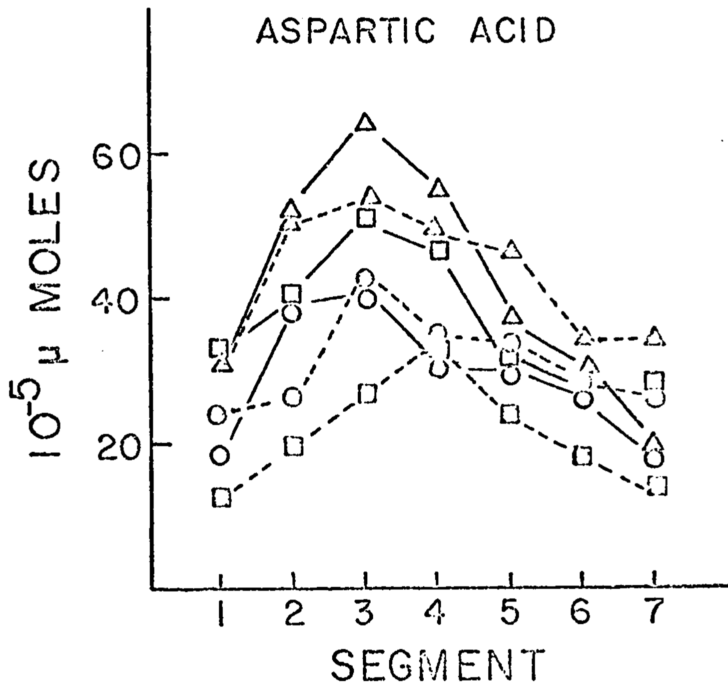

GLUTAMINE
GLUTAMIC ACID
160
140
120
100
80
60
40
20
10^{-5} μ MOLES
1 2 3 4 5 6 7
SEGMENT
1 2 3 4 5 6 7
SEGMENT

Fig. 2b. Change in the concentration of the individual free amino acids of the wheat root tip with growth, imbibition, vernalization and wheat variety.




ALANINE
10^{-5} μ MOLES
40
20
1
2
3
4
5
6
7


ASPARAGINE
1
2
3
4
5
6
7


ASPARTIC ACID
10^{-5} μ MOLES
60
40
20
1
2
3
4
5
6
7
SEGMENT

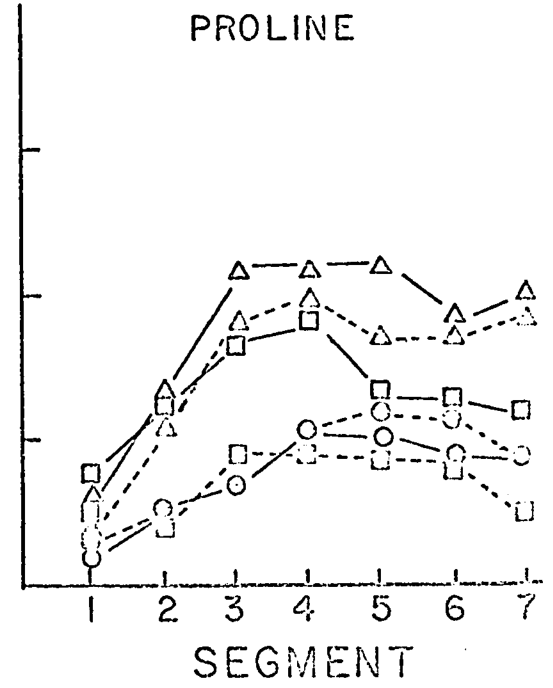
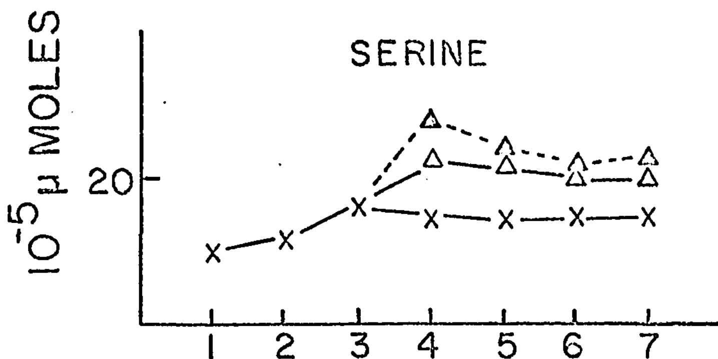
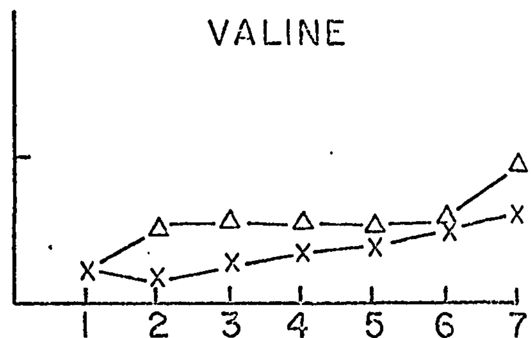

PROLINE
1
2
3
4
5
6
7
SEGMENT

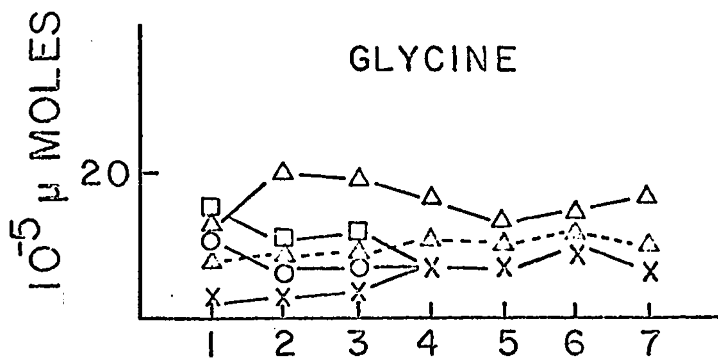
Fig. 2c. Change in the concentration of the individual free amino acids of the wheat root tip with growth, imbibition, vernalization and wheat variety.

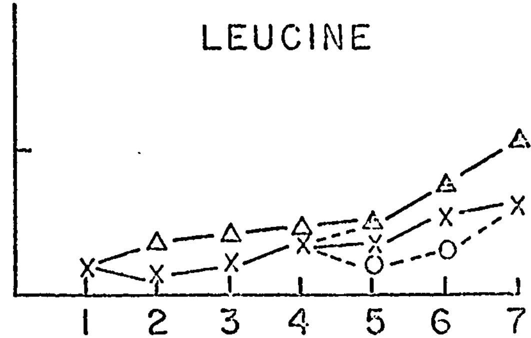
$R_{14}K$	——O——	$R_{14}V$	– – –O– – –
R_5K	——□——	R_5V	– – –□– – –
M_5K	——△——	M_5V	– – –△– – –

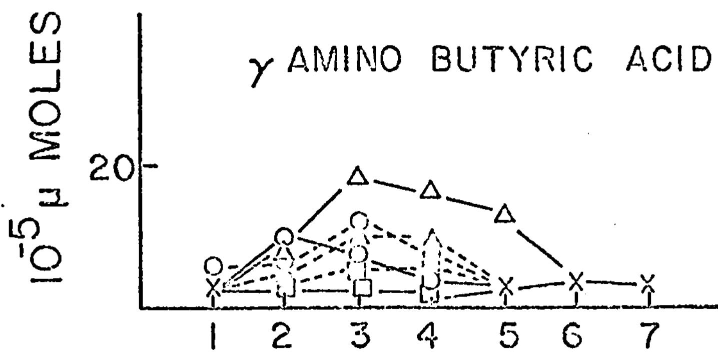
all treatments except those specifically shown ——X——


SERINE
10^{-5} μ MOLES
20
1 2 3 4 5 6 7


VALINE
1 2 3 4 5 6 7


GLYCINE
10^{-5} μ MOLES
20
1 2 3 4 5 6 7


LEUCINE
1 2 3 4 5 6 7


γ AMINO BUTYRIC ACID
10^{-5} μ MOLES
20
1 2 3 4 5 6 7
SEGMENT

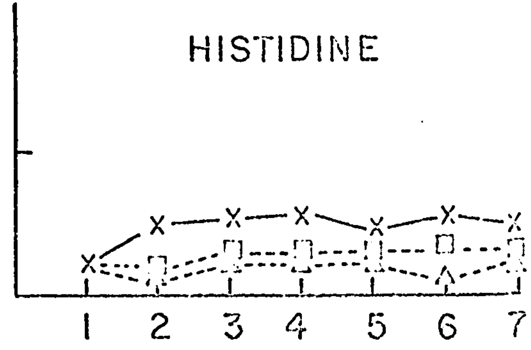
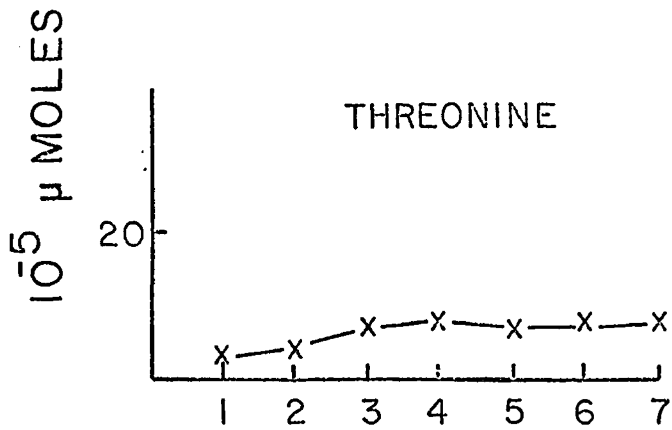
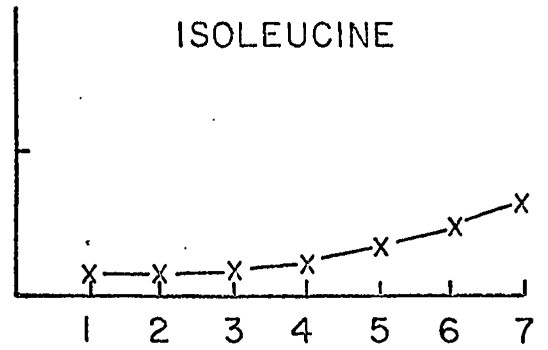
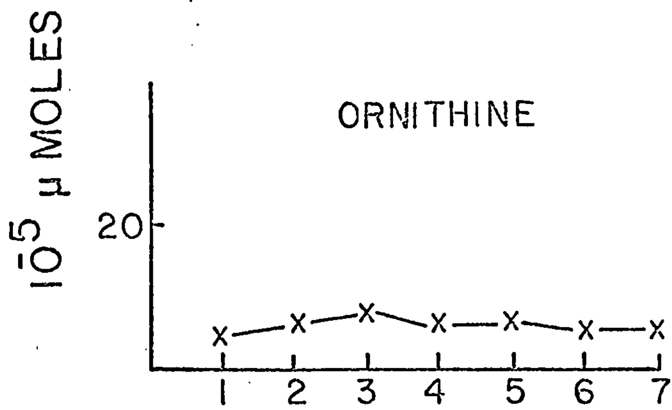

HISTIDINE
1 2 3 4 5 6 7
SEGMENT

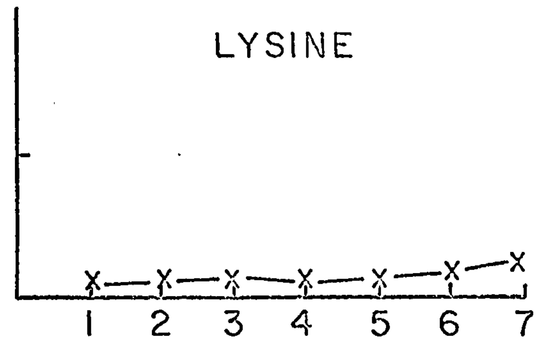
Fig. 2d. Change in the concentration of the individual free amino acids of the wheat root tip with growth, imbibition, vernalization and wheat variety.

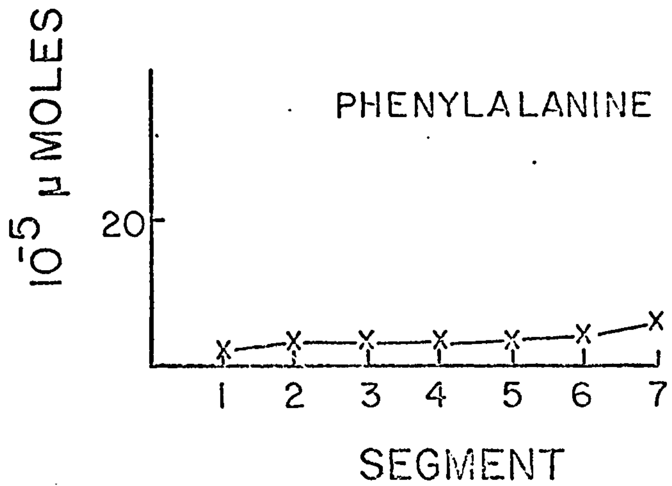
all treatments ——X——

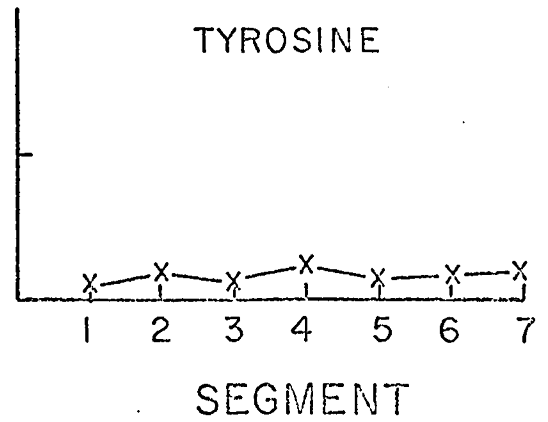

THREONINE
10^{-5} μ MOLES
20
1 2 3 4 5 6 7


ISOLEUCINE
1 2 3 4 5 6 7


ORNITHINE
10^{-5} μ MOLES
20
1 2 3 4 5 6 7


LYSINE
1 2 3 4 5 6 7


PHENYLALANINE
10^{-5} μ MOLES
20
1 2 3 4 5 6 7
SEGMENT


TYROSINE
1 2 3 4 5 6 7
SEGMENT

are three main patterns of change in the concentration of the individual free amino acids during the earlier stages of root growth (Fig. 2 a, b, c, d).

1- Some amino acids (aspartic and glutamic acid) show an increase from segment 1 to a maximum in segments 3 and 4 followed thereafter by a decrease to segment 7.

2- Others (glutamine and alanine) increase continually from segment 1 to segment 7.

3- The remainder, i.e. all the neutral and some of the basic amino acids show only a slight fluctuation along the root.

Vernalization does not alter the pattern of change of the individual amino acids with growth. Rather, it affects the quantity of a particular amino acid present in a particular segment. In R_{14} wheat, segment 5 is the most markedly affected with respect to glutamine, glutamic acid and proline. Vernalized and control roots of R_5 wheat differ markedly in their content of aspartic acid in all segments. In Marquis wheat, except for glutamine and to a far lesser extent glycine, there are no pronounced differences between vernalized and control roots.

Total free amino acids per dry weight

Computing the data on a dry weight dry basis (Table 2) yields the same relationship between the different amino acids as that previously detailed in Table 1. The pattern of change

Table 2. Free soluble amino acid composition of the 3.5 mm wheat root tip (10^{-3} micromoles per gram dry weight)

	$R_{14}K$	$R_{14}V$	R_5K	R_5V	M_5K	M_5V
Alanine	168	189	154	125	138	178
Arginine	1	5	1	7	8	3
Asparagine	56	97	10	51	13	15
Aspartic acid	265	331	321	163	327	277
Glutamic acid	379	592	400	332	613	598
Glutamine	645	582	604	502	461	614
Glycine	82	68	118	52	126	65
Histidine	86	86	80	59	58	30
Isoleucine	45	42	44	34	62	42
Leucine	63	51	68	44	88	62
Lysine	21	23	18	18	24	14
Methionine	5	3	5	4	9	7
Ornithine	59	53	39	37	38	28
Phenylalanine	28	29	24	24	29	22
Proline	130	153	208	122	273	202
Serine	128	150	134	121	135	136
Threonine	54	56	62	45	55	51
Tyrosine	17	8	8	17	27	14
Valine	62	77	70	42	96	56
γ Amino butyric acid	50	60	30	36	76	34

of the individual amino acids is also very similar to that observed on a segment basis (Fig. 2). The main reason for expressing the data per dry weight is to permit a comparison with the results of Steward et al (1954) who determined the free amino acid content of the shoot apex of lupine and expressed his results per dry weight. This comparison will be discussed later.

Total free amino acids per μg protein

The change in total content of amino acid per μg protein with growth is shown in Fig. 3

The ratio of free amino acid to protein is low in the meristematic region and increases with increasing age of the root sections. In Marquis wheat, there is a decrease in the ratio with vernalization. In R_{14} wheat, there is no difference between vernalized and control roots in segments 1 to 4. However, there is an increase in the ratio for vernalized roots above the control in segments 5 to 7. Vernalized roots of R_5 and R_{14} wheats differ mainly in segments 6 and 7, where the total free amino acids content of R_5 per protein is lower than that of R_{14}.

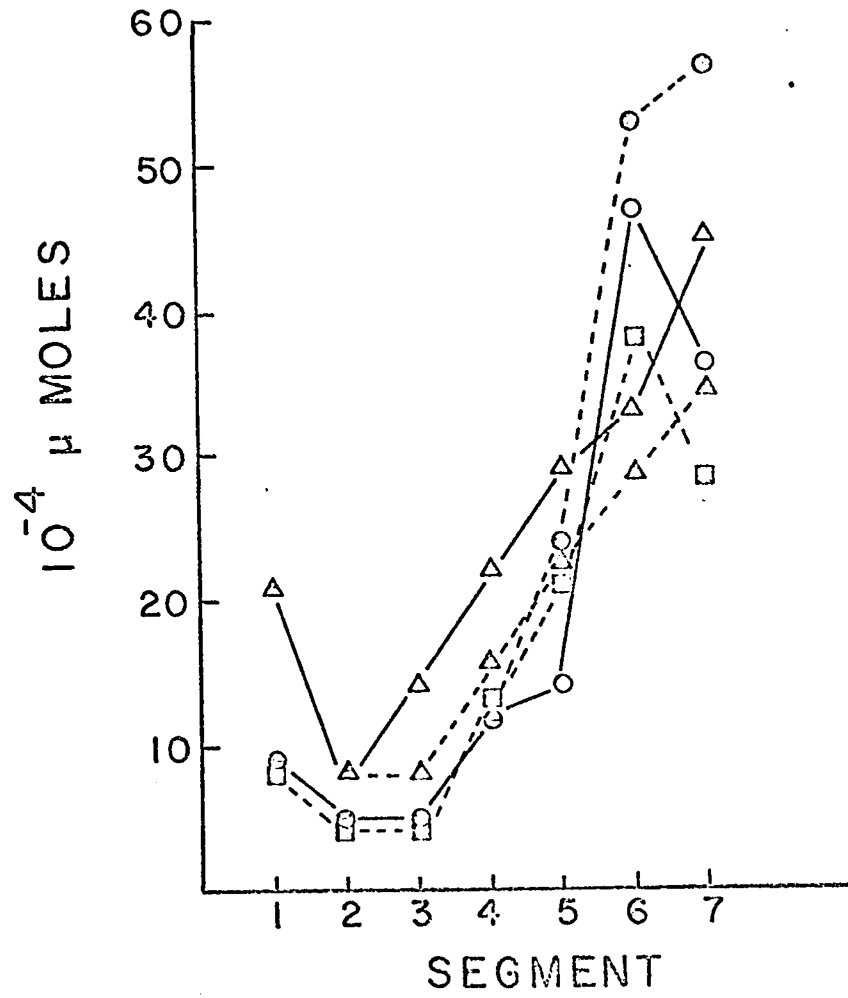
The decreased ratio of free amino acids to protein in vernalized Marquis roots suggests an increase in protein synthesis. However, comparing the two curves more closely, it can be seen that the individual segments of the vernalized roots have the same protein ratio as the lower numbered segment of the control root, i.e., segment 3 vernalized corresponds to

Fig. 3. Change in the total free amino acid content per μg protein of the wheat root tip with growth, imbibition, vernalization and wheat variety.

$R_{14}K$: R_5K ———O——— $R_{14}V$ – – –O– – –

M_5K ———△——— M_5V – – –△– – –

R_5V – – –□– – –



60
50
40
30
20
10
10^{-4} μ MOLES
1
2
3
4
5
6
7
SEGMENT

segment 2 control, and so on. Thus it seems that in the spring wheat, vernalization retards the pattern of metabolic differentiation by prolonging the mitotic activity of cells which would otherwise have passed over to the phase of elongation.

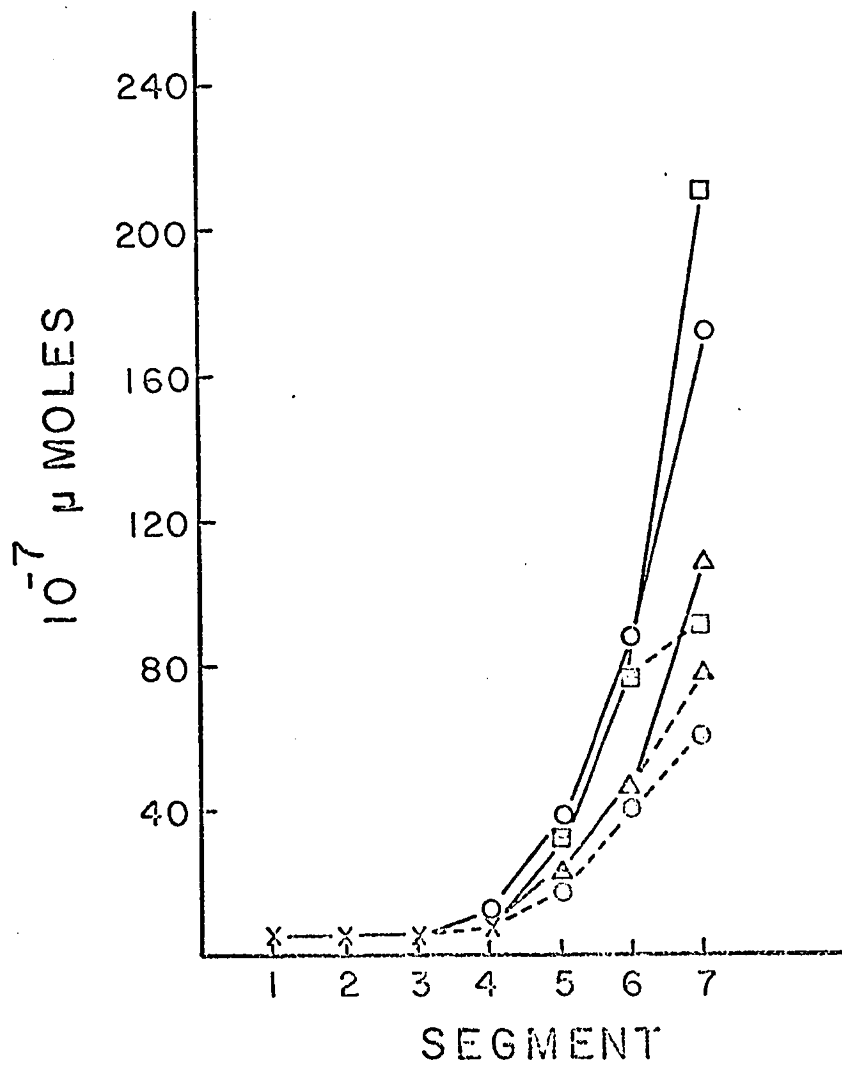
Total free amino acids per cell

From Fig. 4, we see that the total content of free amino acids per cell is very low in the meristematic region, segments 1 to 4, and then increases to segment 7. This is true of both wheat varieties, vernalized and control. The meristematic region is generally characterized by its relative lack of a wide range of metabolic activity (Torrey, 1965). Furthermore, in actively growing tissues, much of the protein balance is directed towards synthesis (Folkes, 1959) and any free amino acid would probably be immediately incorporated into protein.

Only the roots of R_{14} wheat show a major change with vernalization, namely a decrease in the free amino acid content of the cells of segments 5, 6 and 7, relative to the control. Both R_5V and M_5V differ from the controls only in segment 7. Previous work of Ku (1965) has indicated that, following vernalization, these older portions of the root still contain a large number of actively dividing cells. It is therefore difficult to compare activity on a cell basis, for the corresponding segments of vernalized and control roots may not contain cells in the same physiological state. The

Fig. 4. Change in the total free amino acid content per cell of the wheat root tip with growth, imbibition, vernalization and wheat variety.

$R_{14}K$	——○——	$R_{14}V$	– – –○– – –
R_5K	——□——	R_5V	– – –□– – –
M_5K	——△——	M_5V	– – –△– – –



240
200
160
120
80
40
10^{-7} μ MOLES
1
2
3
4
5
6
7
SEGMENT

cells of the root differentiate continually and the population of any one segment is not homogeneous. Thus, in this type of study it is more informative to compare the physiology of corresponding regions of the root rather than compare two different cell types.

B - ^{14}C Studies Involving Glutamic Acid and Aspartic Acid

These two amino acids were chosen for study because of their relatively high concentration in the root tip and because they were the only two amino acids which decreased with growth, suggesting a high rate of utilisation.

Incorporation of ^{14}C label into the protein fraction

The incorporation of ^{14}C into protein is always higher in the region of mature cells (segments 6-7) than in the region of meristematic cells (segments 2-3)(Table 3). This supports the contention that in roots, the main region of protein synthesis is not in the meristematic zone but rather in the zone of elongation and maturation.

Vernalization does not affect the incorporation of ^{14}C aspartic and glutamic acids in a similar fashion. With respect to glutamic acid, there is practically no change between control and vernalized roots in the amount of label recovered in protein for R_{14} and M_5 wheats. However, the incorporation into R_5V is decreased relative to control in both segments 2-3 and 6-7. The incomplete imbibition

Table 3. Incorporation of ^{14}C label into the protein fraction

	Segment	Glutamic acid	Aspartic acid
		(counts /10 min.	per mg protein
$R_{14}K$	2-3 6-7	3728 27984	507 8580
$R_{14}V$	2-3 6-7	3285 29240	1363 16800
R_5K	2-3 6-7	3761 31492	1234 16940
R_5V	2-3 6-7	1172 17800	606 16800
M_5K	2-3 6-7	1319 10984	1376 9391
M_5V	2-3 6-7	1178 8300	669 4375

of the grain during the vernalization process seems to have a carry over effect on enzymes controlling the incorporation of this amino acid.

Vernalization has a greater influence on the incorporation of aspartic acid than on that of glutamic acid. Here, all the treated series (R_5V, $R_{14}V$, M_5V) differ from the controls, except for segments 6 - 7 of R_5V. However, the spring and the fully imbibed winter wheat react in opposite ways. Following vernalization, incorporation in Marquis spring wheat is reduced by half, while it is doubled in the Rideau (R_{14}) winter wheat.

<u>Distribution of radioactivity in the different fractions</u>

<u>Glutamic acid</u>: There is a different pattern of distribution of radioactivity in the supernatant and protein fractions of segments 2 - 3, and 6 - 7 (Table 4). In the meristematic zone (segments 2 - 3) the activity in the protein is always higher than that in the supernatant. In the region of mature cells, the opposite is true i.e. the higher activity lies in the supernatant. Although there is a higher specific activity in the protein of the older parts of the root (Table 2), the above results seem to indicate that in the meristematic region, the greater proportion of the glutamic acid supplied goes into protein, while in the more mature regions it remains in the pools in the vacuole. There it is probably

Table 4. Percentage distribution of ^{14}C glutamic acid in the different fractions

	Segment	Debris	Organic	Protein	Supernatant
$R_{14}K$	2-3 6-7	25 23	3 3	51 20	22 54
$R_{14}V$	2-3 6-7	21 21	4 1	46 14	28 63
R_5K	2-3 6-7	23 19	2 2	52 21	22 58
R_5V	2-3 6-7	39 24	3 2	36 14	22 57
M_5K	2-3 6-7	36 27	4 2	31 12	29 59
M_5V	2-3 6-7	34 32	8 4	33 11	25 52

metabolized to a variety of amino acids because of its central role in transamination reactions (Loomis and Stumpf, 1958). This interpretation is also supported by the autoradiograph (Fig. 5) of an alcohol extract of root homogenate, where in segments 6 - 7 no trace of the original glutamic acid was found, while some traces of other unidentified compounds are visible. The elongation zone thus seems to participate in more active protein and amino acid cycling than the meristematic zone.

The distribution of the radioactivity does not differ significantly between vernalized and control roots.

Aspartic acid: The pattern of distribution of radioactivity in segments 2 - 3 also differs from that in segments 6 - 7 (Table 5). The percentage radioactivity in the protein of segments 2 - 3 is always equal to or greater than that found in the supernatant apart from $R_{14}V$. In segments 6 - 7 there is a 3 to 8 fold higher radioactivity in the supernatant fraction than in the protein fraction.

Following vernalization, there is an increase in incorporation in the supernatant fraction of R_{14} wheat, namely from 20 to 34% in segments 2 - 3 and from 64 to 80% in segments 6 - 7. This gain is made at the expense of the debris fraction. Marquis wheat behaves differently from Rideau wheat in that there is a decrease, not an increase, in the supernatant fraction of segments 6 - 7

Table 5. Percentage distribution of ^{14}C aspartic acid in the different fractions

	Segment	Debris	Organic	Protein	Supernatant
$R_{14}K$	2-3	53	6	21	20
	6-7	25	1	10	64
$R_{14}V$	2-3	36	6	24	34
	6-7	8	2	10	80
R_5K	2-3	42	8	28	21
	6-7	22	2	10	66
R_5V	2-3	46	7	31	16
	6-7	13	1	9	75
M_5K	2-3	31	9	42	18
	6-7	20	1	13	65
M_5V	2-3	41	5	36	17
	6-7	40	2	9	49

of vernalized roots, again at the expense of the debris fraction. These opposite reactions cannot presently be explained.

Comparing the pattern of distribution of radioactivity of glutamic acid (Table 4) with that of aspartic acid (Table 5), it can be seen that the two patterns are dissimilar. On the whole, vernalization affects the metabolism of aspartic acid more than that of glutamic acid. These two compounds are the heads of two different families of amino acids (Yemm and Folkes, 1958). It would thus be of interest to follow the effect of vernalization on the other members of their respective families.

Autoradiography

Very little activity is found in the alcohol soluble fraction (Fig. 5). The only activity detected is in segments 6 - 7 following incubation with ^{14}C glutamic acid. The original ^{14}C amino acids were not recovered. Because of this low rate of recovery and incorporation, further expriments along this line were not pursued.

C - Glutamic Acid - Alanine Transaminase

The activity of this particular transaminase was assessed in order to test the hypothesis that it may be responsible for the observed decrease in the free glutamic acid concentration along the root associated with a concomitant increase in alanine concentration (Fig. 2 a, b).

Fig. 5. Autoradiograph of an alcohol extract of Marquis wheat root homogenate.

A. segments 2-3 incubated with ^{14}C glutamic acid

B. ^{14}C glutamic acid, authentic sample

C. segments 6-7 incubated with ^{14}C glutamic acid

D. segments 2-3 incubated with ^{14}C aspartic acid

E. ^{14}C aspartic acid, authentic sample

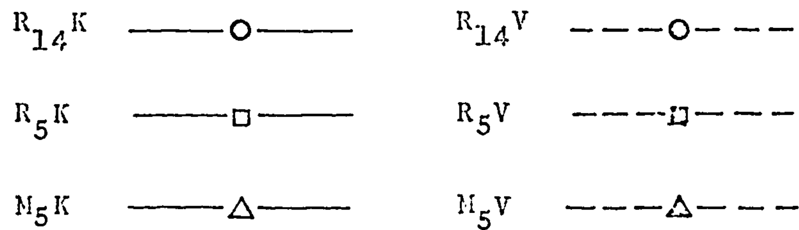
F. segments 6-7 incubated with ^{14}C aspartic acid

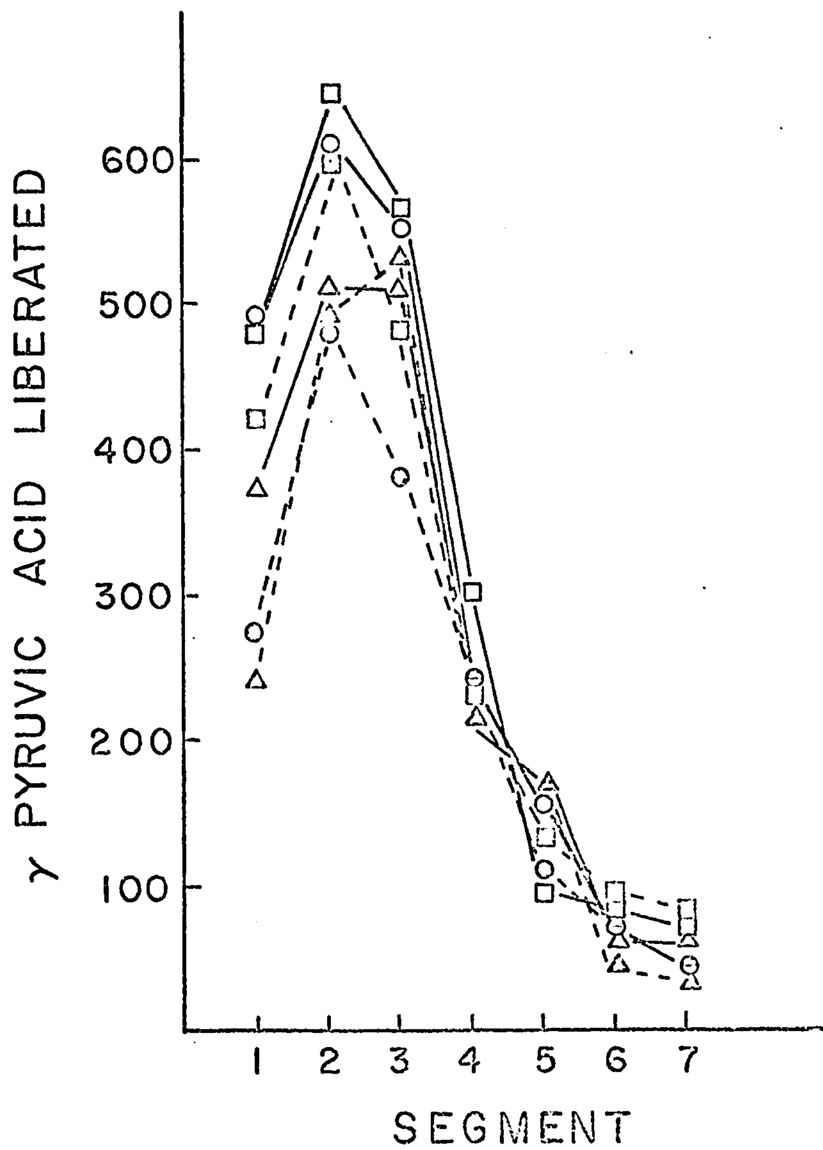
A B C D E F

Transaminase activity was detected in all of the seven sections (Fig. 6). The peak of activity, in both Marquis and Rideau wheats is found in segments 2 and 3 and then decreases to segment 7. The meristematic zone is thus the most active site with respect to glutamic acid - alanine transaminase action. This region also contains the highest concentration of free glutamic acid and the pattern of transaminase activity correlates with that of the occurence of glutamic acid, not with that of alanine.

Vernalized roots of both R_5 and R_{14} wheat show a decrease in the transaminase activity of segments 1 to 3 relative to unvernalized roots. This decrease is more pronounced in R_{14} than in R_5 and is not observed in Marquis wheat. Vernalized roots of R_{14} also contain a greater quantity of free glutamic acid in these segments (Fig. 2a), while the amount incorporated into protein (Table 3) remains the same. It would thus seem that vernalization affects the metabolism of glutamic acid by favoring a greater accumulation of this compound as a result of a reduced transaminase activity.

Fig. 6. Change in the glutamic acid -alanine transaminase activity of the wheat root tip with growth, imbibition, vernalization and wheat variety.




γ PYRUVIC ACID LIBERATED
600
500
400
300
200
100
1
2
3
4
5
6
7
SEGMENT

DISCUSSION

The array of free amino acids in wheat root tips is similar to that found by Oaks (1965) for maize root tips and by Lawrence and Grant (1963) for pea root tips. However, although the relative proportion of the amino acids in the maize root tip corresponds to that found in the wheat root tips (i.e. glutamic acid, aspartic acid and their amides predominate while arginine, lysine, tyrosine, γ amino butyric acid and phenylalanine occur in low concentration), it differs considerably from that in pea roots. Here the dominant amino acid is homoserine (not detected in this analysis) with high levels of alanine, threonine, glutamic acid, arginine and serine. Aspartic acid was found to occur only in relatively low quantities. The pattern of free amino acids in root tips thus seems to be governed by genetic factors acting at the species or family level, resulting in different patterns of nitrogen metabolism.

The distribution of free amino acids in the segments is altered by growth of the root tip. The extent of this alteration varies for each individual amino acid and also depends upon the wheat variety. Glutamic acid and aspartic acid are the only amino acids which progressively increase along the root tip (to a maximum obtained at a region 2 mm form the apex) and thereafter decrease in content, while the other amino acids increase or remain more or less constant. This could indicate that glutamic acid and aspartic

acid are being rapidly metabolised and possibly transformed into a variety of other amino acids, particularly alanine and glutamine. Boulter and Barber (1963) who found a decrease in the dicarboxylic acids during the germination of pea seedlings, have suggested that a decrease in the above compounds could also be linked with their role in purine and pyrimidine synthesis. Furthermore, the dicarboxylic acids have the greatest possibility for transamination and deamination and can be metabolised to a whole series of amino acids (McKee, 1962). The glutamine content exhibits the most pronounced increase with growth, especially in the spring variety. An 8 fold increase is observed in M_5V between segments 2 and 7. Since glutamine characteristically accumulates in conditions favorable for protein synthesis, this suggests that the main site of protein synthesis is not in the strictly meristematic region but rather in the elongation zone. This is consistent with Kopp's (1948) data which indicated that in wheat roots as much as half of the protein synthesis of the cell occurred during cellular elongation.

The different imbibition periods used for Rideau wheat, namely 5 and 14 hours result in parallel changes in the metabolism of vernalized roots. The observed difference is one of degree, with the reaction of R_{14} always more pronounced than that of R_5. Since hydration of the grain initiates the metabolic processes subsequently leading to germination, insufficient water levels during the cold treatment may retard or prevent completion of the reaction leading to full vernalization.

Steward (1954) has determined the free amino acid content of serial sections of the shoot apex of lupine. His data is included here (Table 6), for the behavior of the free amino acids in the shoot apex contrasts sharply with that of the amino acids found in the root apex. The major points of comparison are (Table 6 and Table 7):

1) The array of amino acids in both the root and shoot apices is the same.
2) In the shoot, the basic amino acids are prominent in the meristem and their concentration decreases sharply in more mature regions. In the wheat root, the basic amino acids are not prominent, either in the meristem or in the more mature regions.
3) The total content of glutamic acid and glutamine collectively and separately is high in the shoot meristem and is reduced when passing into the area of elongation and differentiation (unexpanded leaves). The opposite trend is observed in the root for glutamine while glutamic acid increases from the meristem to the zone of elongation and decreases slightly in the zone of maturation.
4) Tryptophan, the precursor of indole acetic acid is prominent in the shoot meristem. It was not detected in the root tip.
5) The content of most of the amino acids present in the shoot apex (except asparagine and aspartic acid) decreases from the meritem to the older

Table 6. Amino acid composition of non-protein nitrogen fraction of Lupinus albus (micrograms of amino acid per mg dry weight of sample) ✧

	Shoot meristem	Leaf primordia	Unexpanded leaves
Aspartic acid	0.94	0.77	1.35
Glutamic acid	4.9	2.1	1.8
Serine	2.8	1.6	0.75
Glycine	-	-	-
Asparagine	65.5	56.2	96.8
Threonine	2.2	3.0	0.75
Alanine	2.8	1.6	0.87
Glutamine	3.1	2.2	2.1
Lysine	3.8	0.54	0.18
Arginine	5.2	4.3	0.39
Proline	4.1	3.5	1.1
Valine	10.3	7.1	1.7
Leucines	5.5	2.4	1.2
Phenylalanine	8.7	8.9	1.1
Tryptophan	1.4	0.6	0.39
Tyrosine	1.4	-	0.24
γ Amino butyric acid	1.9	1.7	0.49

✧ reproduced from Steward (1954)

Table 7. Free soluble amino acid composition of Rideau wheat root tip (10^{-2} micromoles per gram dry weight)

	Meristem	Zone of elongation	Zone of maturation
Aspartic acid	48	61	69
Glutamic acid	67	95	90
Serine	16	32	27
Glycine	9	18	29
Asparagine	6	15	12
Threonine	6	16	18
Alanine	16	33	92
Glutamine	53	96	300
Lysine	4	4	5
Arginine	-	-	2
Proline	17	31	53
Valine	5	14	33
Leucines	5	23	70
Phenylalanine	1	6	15
Trytophan	-	-	-
Tyrosine	1	2	12
γ amino butyric acid	10	14	6

leaves. In the root (except for glutamic acid) the reverse was found i.e. the trend is towards an increase from the meristematic region to the zones of elongation and maturity.

The root and shoot apices thus behave in opposite ways with respect to free amino acid content. Unfortunately we are dealing with two different species and although the spectrum of amino acids found in the apex of both species is similar, one cannot be sure that the differences in concentration are metabolic rather than varietal. It would therefore be worthwhile to compare the shoot and root apices of the same species. Such a comparison is not yet available.

The free amino acids found in wheat root tips are not necessarily the result of synthesis _in situ_. The work of Oaks (1965) has shown that in maize root tips, asparagine and the amino acids of the neutral and basic fractions were preferentially transported to the root tip. These amino acids occurred in low concentration in the wheat root tips and changed relatively little with growth. Oaks also found that the synthesis _in situ_ of glutamic acid, aspartic acid and glutamine was relatively easy. These acids, which are closely associated with the TCA cycle (McLennan, Beevers and Harley, 1963) were found in large quantities in the wheat root tips. They are probably extensively metabolised as evidenced from their pattern of distribution with growth. It has been suggested that the synthesis of amino acids in the root tip region is controlled by the supply of amino acids from the

transport system. This could be achieved by a repression of the involved biosynthetic enzymes as a result of end product imhibition. During vernalization, the embryo and endosperm were subjected to a cold treatment. Since these regions are known to supply the root tip with some free amino acids, vernalization could act by regulating the endogenous synthesis of these amino acids in the growing region. Limiting the synthesis of certain amino acids could be an important factor in sparing glucose carbon for cell-wall synthesis and energy production which would result in the higher growth rate observed in the vernalized root.

There is a greater incorporation of both glutamic acid and aspartic acid into the proteins of segments 6 and 7 than in those of segments 2 and 3. However, there is also a corresponding decrease in the amount of both free aspartic and glutamic acids in segments 6 and 7 relative to segments 2 and 3. It would therefore point to a higher synthesis of protein in the maturing regions of the root rather than in the strictly meristematic zones. Jensen (1957) who followed the incorporation of ^{14}C phenylalanine and ^{14}C adenine in root tip cells of Allium cepa also found a marked increase in incorporation during cellular elongation and has interpreted his results as indicative of steady protein synthesis.

There does not seem to be a definite pattern of change in the amino acids following vernalization. Some increase in concentration, others decrease, while still others do not vary at all. Furthermore, the magnitude of the change varies with particular segments i.e. with the stage of development. This could suggest differences in the composition of the proteins being synthesised. Steward et al (1965) using serial 1 mm sections of pea roots have shown that different proteins are characteristic of different stages of development. Furthermore, growth and development entail changing activity of different enzymes, as demonstrated by the work of Robinson and Brown, 1952). The changing activity of at least one enzyme with growth namely glutamic acid-alanine transaminase has been demonstrated during the present study.

Section 111 - Studies on Carbohydrate Metabolism

INTRODUCTION

Carbohydrates are among the most important naturally occurring organic compounds. They make up more than half the dry weight of plants, and, as respiratory substrates, play a central role in metabolism. Depending on their function, the carbohydrates can be divided into two groups, structural and nutritional. The main structural carbohydrates of higher plants are cellulose, hemicelluloses and pectins. The nutritional carbohydrates include starch, inulin and small chain sugars especially sucrose and glucose.

The sugars are usually defined as a group of polyhydroxy aldehydes or ketones normally possessing an unbranched carbon chain. They are divided into three main classes according to the degree of polymerisation: monosaccharides or simple sugars which cannot be hydrolysed to smaller units, oligosaccharides which contain fewer than ten monosaccharide units, and the polysaccharides which contain more than ten of these units.

A wide variety of monosaccharides are found in plants. However, not all occur in the free state; the majority are present in a combined form.

Trioses and tetroses owe their importance to their significant role as intermediate products in the glycolytic pathway. Tetroses also form the transitory fission products of higher sugars (Shafizadeh and Wolfrom, 1958). Three pentoses, D-xylose, D-ribose and L-arabinose occur normally

in higher plants, usually in the combined state (Gibbs,1966). The hexoses, D-glucose, D-galactose, D-fructose, D-mannose are the most frequently encountered sugars,and are usually present in all higher plants. Glucose is by far the most widely distributed sugar. It occurs free and also as a constituent of many important compounds such as starch, cellulose, sucrose and coniferin. Fructose also occurs both in the free state, and in the bound state, as in inulin. Both galactose and mannose principally exist in the form of polymers, mannan and galactan. Two seven carbon sugars, D-mannoheptulose and D-sedoheptulose, have been detected in plants. The latter is a significant intermediate in the fixation of CO_2 in photosynthesis. (Benson, Bassham and Calvin, 1951).

The more important oligosaccharides are sucrose, raffinose, maltose and cellobiose. The monosaccharide residues of these sugars can either be alike (as in maltose) or different (as in raffinose). Maltose and cellobiose are the repeating units of two important glycosides, starch and cellulose. Maltose has also been detected free in germinating wheat (Axelrod, 1965). Raffinose is, next to sucrose, the most common sugar found in higher plants, and is a constituent of many seeds (Axelrod, 1965). Sucrose is universal in distribution. It is a major product of photosynthesis and a usual prominent form of storage and accumulation of carbohydrates. There is also evidence that sugars are translocated largely in the form of sucrose (Gibbs, 1966).

Polysaccharides constitute the bulk of the structural and storage material of higher plants. Most of them are found in the cell wall with cellulose as the major component. Starch is the most important reserve carbohydrate and is widely distributed throughout the body of the plant. It is stored primarily in roots, tubers and seeds. The latter may contain as much as 80% of their dry weight as starch (Whelan, 1958) which will serve for the nutrition of the developing seedling before the onset of photosynthesis.

The major pathway of carbohydrate formation is photosynthesis. Part of this process involves the light catalysed conversion of carbon dioxide into fructose-6-P and glucose-6-P with the subsequent transformation of these compounds to glucose, sucrose, fructose and starch. These four sugars are then metabolised into other carbohydrates and finally into carbon dioxide and water. However, in a germinating seed in the dark, photosynthesis does not occur. Carbohydrate metabolism is then mostly concerned with the breakdown of the reserve polysaccharides of the grain, interconversions among the sugars, and their utilisation through glycolysis or the hexose monophosphate shunt. Furthermore, in growing plant tissues, many of the intermediates of carbohydrate catabolism may be diverted to the synthesis of other important organic substances, including amino acids (Yemm,1965). Glutamate, aspartate, alanine and glycine may arise directly from α ketoglutarate, oxalacetate, pyruvate and glyoxalate by reductive amination or transamination (Bonner and Varner, 1965).

Plant cells possess enzymatic systems capable of interconverting monosaccharides. Sucrose can be synthesized in vivo at the expense of glucose and fructose. This has been demonstrated in potato (Nelson and Auchincloss, 1933), red clover and wheat plants (Virtanen and Nordlund, 1934). Nurmia (1935) has shown that in living plants, sucrose could also be synthesized from D-glucose, D-fructose, D-galactose and maltose. Glucose and fructose are readily interconvertible (Nurmia, 1935; Putman and Hassid, 1954), and so are galactose and glucose (McCready and Hassid, 1941; Putman and Hassid, 1954). Pentose sugars arise from the oxidation of hexose phosphates through the hexose monophosphate shunt. This pathway is known to function in roots (Axelrod and Bandurski, 1952; Gibbs, 1952).

Sucrose is degraded in vivo by the enzyme invertase which produces one molecule each of glucose and fructose. In the corn radicle, Hellebust and Forward (1962) have found that the distribution curve for invertase follows that of the growth rate for the corn root tip.

Starch is hydrolysed by the amylases. In the grain the end product of B-amylase action is maltose. α-amylase yields a mixture of sugars, including maltose and glucose. The maltose in both cases is usually further broken down (Mayer and Poljakoff-Mayber, 1963).

Analyses have been made of the carbohydrate content of growing root tips. Baldovinos (1953) has found that total sugars and cellulose show a progressive increase per cell

along the axis of the corn root. In the root of Vicia faba, Jensen (1955b), who measured total carbohydrate, glucose and fructose, on a cellular basis found that the root cap cells were high in starch and cellulose while the cells of the apical meristem were low in total carbohydrates and glucose. Total carbohydrate was also found to increase two fold between the meristem and the region of cell elongation.

Cold temperatures are known to affect carbohydrate metabolism in cereal plants. In wheat, exposure to cold temperature, often results in an increase in reducing sugars (Kneen and Blisk, 1941; Kruzhilin, 1963; Zech and Pauli, 1960). There also exists differences in sugar content between winter and spring cereal varieties, when grown in cold environments. Trione (1966) found highly significant differences in the level of sucrose, oligosaccharides and starch between winter and spring wheat varieties grown at 12°C. The winter wheat seedlings accumulated more of these carbohydrates.

Refrigeration treatment also activates the amylase of wheat embryos in germination (Fleishman, 1959). Ikeda (1961) has found that, in wheat, the amylase activity in seedlings was closely related to cold resistance, increasing with storage of the seedlings at -5°C. Amylase activity in winter wheat varieties is initiated at 0°C, while in spring wheat it can be shown only above 10°C (Devey, 1963).

During the vernalization process, sugar changes also occur. One of the major effects is an increase in soluble carbohydrates in vernalized seeds (Dupéron, 1949, 1950). David (1949) has also found an increase in reducing and non-reducing soluble sugars in vernalized cereal grains with a concomittant decrease in insoluble polysaccharides. This suggests a depolymerization of carbohydrates during vernalization. Enzyme acitivities are also modified with relative increases in the activity of sugar hydrolases in particular amylase and invertase (Chouard, 1960).

The importance of soluble carbohydrates in vernalization has also been demonstrated by Purvis (1944). Excised embryos deprived of their residual sugar and grown on a sugar free medium showed no response to vernalization. If the vernalization treatment preceeded the sugar depletion, then the response was positive. Morever, she found that the activities of different carbohydrates differed with regards to the vernalization response. Sucrose and fructose were the most potent.

In wheat the primary root is the first structure to emerge following the vernalization treatment. The purpose of the present investigation is to determine whether the sugar changes elicited in the grain by vernalization are carried over to the root and to determine the pattern of variation of the individual sugars during the growth of the root. Both a spring and a winter wheat variety were studied.

MATERIALS AND METHODS

Determination of the individual free sugars

Paper chromatography: An alcohol extract of root homogenate was chromatographed on Whatman No. 1 filter paper using butanol: acetic acid: water (4:1:5) as solvent (Hough, 1962).

Column chromatography: The seven root segments (150 roots per determination) were homogenized and extracted 3 times with 80% ethanol. The combined extracts were evaporated to dryness and redissolved in 0.5 ml of 0.1 M boric acid. The free soluble sugar content was determined by means of a Technicon carbohydrate analyser.

Determination of total free sugar content

Total sugar was determined using the method of Dubois et al (1956). To 2 ml of an alcohol extract of root homogenate was added 1 ml phenol and 5 ml sulfuric acid. The resulting colored solution was left to stand one and a half hours at room teperature and read at 490 mμ on a Beckman D.U. spectrophotometer. A calibration curve was established using maltose as standard.

Determination of starch content

Starch determinations followed the total sugar analysis. The debris remaining after sugar extraction was covered with 1.5 ml perchloric acid and allowed to stand 30 minutes in an ice water bath. After centrifugation at 6700 rpm, the supernatant was removed and the debris further extracted. The combined supernatants were made up to 5 ml with distilled water and the sugars resulting from the hydrolysis of starch were determined according to the method of Dubois et al as described above.

Determination of amylase activity

The method of Bernfeld (1955) was used to determine amylase activity. The root sections were homogenized in acetate buffer (pH 5.2) and extracted three times. Two ml of the enzyme extract were incubated 3 hours with 1 ml of 75% starch solution. The reaction was stopped by the addition of 2 ml dinitrosalicylic acid reagent. (1 gram dinitrosalicylic acid and 30 gr. of Rochelle salt in 100 ml 0.5 N NaOH). A blank was prepared in which the dinitrosalycilic acid was added to the enzyme extract before the addition of starch. Color was developed by heating the mixture 5 minutes in boiling water, followed by cooling in running tap water. The optical density was read at 540 mμ on a Beckman D.U.spectrophotometer. A calibration curve was established with maltose in the concentration range 0.2 - 2 mg.

Separation of amylase activity into α and B components

Inactivation of B amylase (Clum, 1967): 0.05 ml of 8% Ca acetate were added to 2 ml of the enzyme extract and the solution heated 15 minutes at 70%.

Inactivation of α amylase (Clum, 1967): The enzyme extract was brought to pH 3.3 with I N HCL and placed overnight in a refrigerator at 2°C. The pH was then adjusted to 4.6 with I N NaOH.

Following both of the above treatments the amylase activity was determined as previously described.

RESULTS

Paper chromatography

Paper chromatography did not yield meaningful results. Because of the low free sugar content of the root sections, a great number of roots (i.e. 400) were needed in order to assess the chromatogram quantitatively. The number required proved to be too large to handle successfully. Furthermore, the large number of determinations needed (due to the number of root segments, the different treatments, and varieties of wheat) made this method of analysis impractical.

Column chromatography (Technicon carbohydrate analyser)

The Technicon automatic carbohydrate analyser is a fairly recent development and some difficulties were encountered. Two columns were used throughout the following expriments and discrepancies were noted in their respective analyses. Furthermore, the apparatus has not been used extensively on plant material and this made identification of the peaks difficult. This method of analysis was nevertheless preferred to paper chromatography because despite the above mentioned drawbacks, it still proved to be more sensitive, accurate and easier to apply to root sections.

Total free sugar content of the 3.5 mm wheat root tips

The sugar content of the 3.5 mm root tips, obtained by totalling the individual sugars of the seven 0.5 mm segments, is shown in Table 8.

Four sugars, fructose, glucose, raffinose and sucrose, were detected in relatively high concentration in the root tips of both Rideau and Marquis wheats. The Rideau variety has more of both glucose and fructose than the Marquis variety. The opposite is found for raffinose while the concentration of sucrose is similar in both varieties.

The sugar identified as xylose appeared in the analysis of only one of the two columns used. Its concentration was quite constant (~4 μg) and it was present in every determination made with that particular column. It cannot be ascertained whether it is an artifact or a sugar actually present in the extract. Both columns gave comparable readings for the xylose standard.

One sugar could not be identified. It occurred next to ribose in the analysis chart with a peak height similar to that of ribose (0 - 1.8 μg in terms of ribose). This unknown, and also galactose, were detected in small quantities and did not appear in every segment. No concentration is shown for these two sugars in Table 7. Galactose occurred only in trace amounts which could not be calculated. However, their presence in specific segments is noted (Table 9).

Table 8. Total free sugar content of the 3.5 mm wheat root tip (10^{-2} micrograms)

	$R_{14}K$	$R_{14}V$	M_5K	M_5V
Fructose	368	519	263	238
Galactose	+	+	+	+
Glucose	324	377	297	274
Raffinose	427	408	584	588
Ribose	9	5	5	6
Sucrose	234	213	207	262
Xylose	+ +	+ +	+ +	+ +
Unknown	+	+	+	+
Total	1362	1522	1357	1368

\+ occured only in trace amounts and was not detected in every segment.

\+ + was detected only in the analysis made with one of the two columns.

Table 9. Occurence of galactose and of the unknown sugar

	Segments in which detected			
	$R_{14}K$	$R_{14}V$	M_5K	M_5V
Galactose	3,4,5,6	6,7	6,7	6,7
Unknown	2,3,4,5,6,7	3,4,5,6,7	4,5,6	5,6,7

The roots of Marquis wheat, control and vernalized, and those of control Rideau wheat have the same total sugar content. However, the roots of vernalized Rideau wheat show an increase in total sugar over the control. This increase is mainly due to fructose.

Individual free sugars per segment.

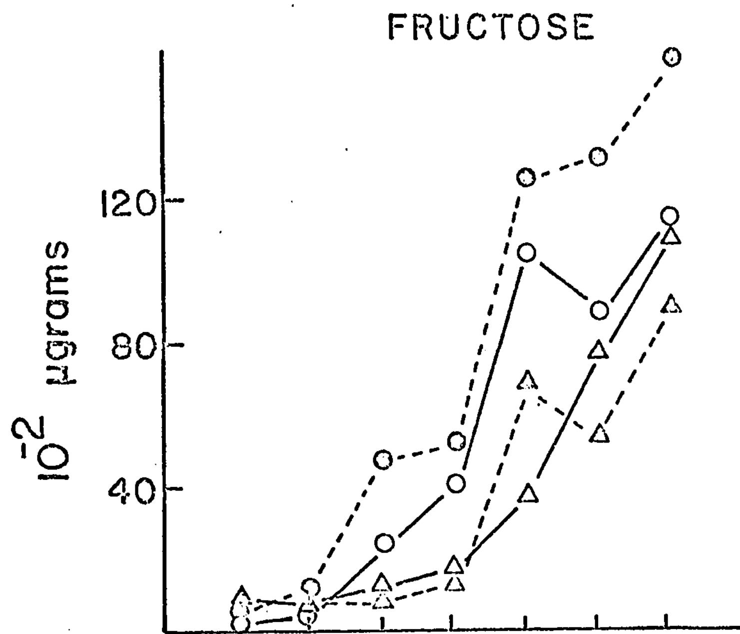
Occurence of all sugars except the unknown and galactose: The variation in individual sugars with growth is shown in Fig. 7. Fructose and glucose follow the same pattern of change with growth, namely an increase from the apex (segment 1) to the zone of mature cells (segment 7). In Marquis wheat, the concentration of both fructose and glucose is similar in every segment. In Rideau wheat, the concentration of fructose is markedly higher than that of glucose in segments 5, 6, and 7.

Apart from segments 1 and 2, the vernalized roots of Rideau wheat have a higher concentration of fructose in every segment. The difference in fructose concentration observed between vernalized and control roots is especially pronounced in the region of more mature cells, segments 6 and 7. Data for Marquis wheat is however more erratic. While the glucose content is always lower in cold treated Marquis wheat roots, the fructose content fluctuates. It is markedly higher in segments 3 and 5 of vernalized roots, but lower in segments 6 and 7.

Fig. 7. Change in the concentration of the individual free sugars of the wheat root tip with growth, vernalization and wheat variety.

$R_{14}K$	——o——	$R_{14}V$	- - -o- - -
M_5K	——△——	M_5V	- - -△- - -

* curve similar for all treatments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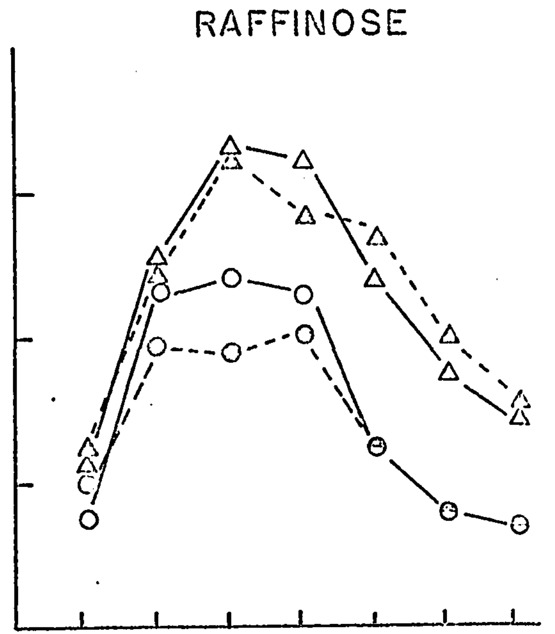
80
40
10^{-2} µgrams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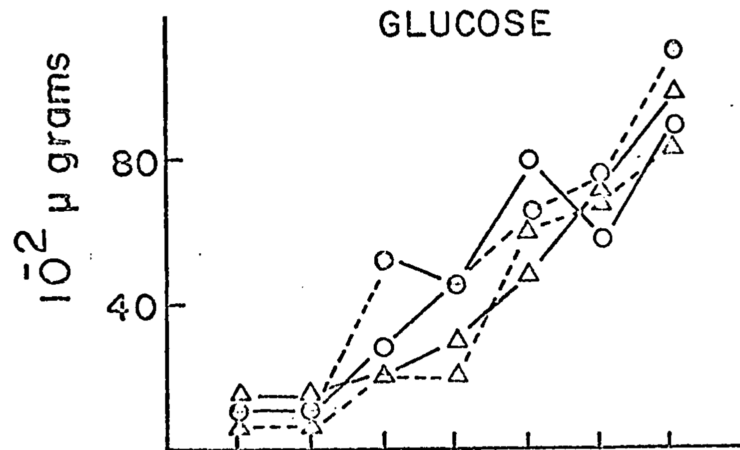
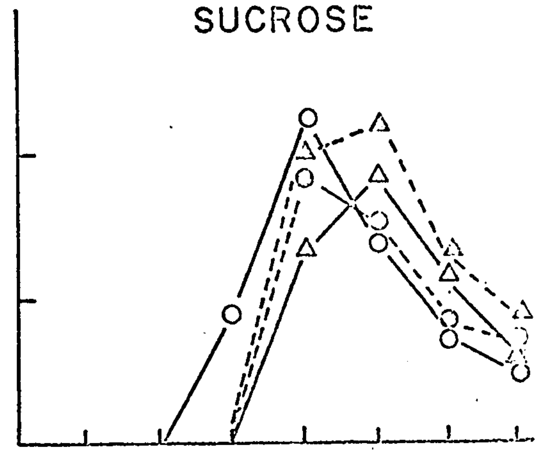
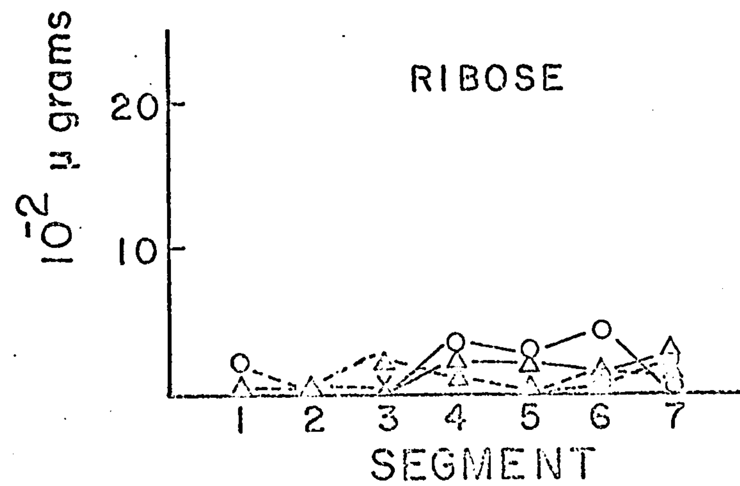
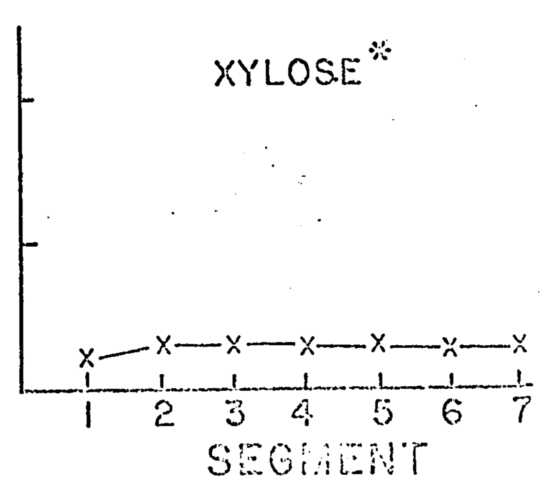

GLUCOSE
80
40
10^{-2} µ grams


SUCROSE


RIBOSE
20
10
10^{-2} µ grams
1 2 3 4 5 6 7
SEGMENT


XYLOSE*
1 2 3 4 5 6 7
SEGMENT

Raffinose and sucrose yield slightly different patterns according to the species. In Marquis wheat, raffinose concentration increases from segment 1 to segment 3, than decreases to segment 7. In Rideau wheat, the concentration increases from segment 1 to segment 2, thereafter remains constant to segment 4, and decreases to segment 7. The level of raffinose is higher in Marquis wheat roots than in Rideau wheat roots in all segments except 1 and 2.

Vernalization does not affect the raffinose content of Marquis roots. In Rideau wheat, there is a 20% decrease in this sugar in the meristematic zone (segments 2 and 3) following vernalization.

Sucrose attains a peak concentration in segment 5 of Marquis wheat. In Rideau wheat the maximum is in segment 4. Sucrose was not detected in sections 1, 2 and 3 of the roots of M_5K, M_5V and $R_{14}V$, and in sections 1 and 2 of $R_{14}K$.

The level of sucrose is lower in vernalized Rideau roots than in control roots in both segments 3 and 4. The decrease is less pronounced in segment 4. A similar pattern is observed in Marquis wheat roots for segments 4 and 5.

The fact that sucrose concentration decreases from segment 4 to segment 7 while that of glucose and fructose increases suggests a conversion of sucrose to these two sugars. The enzyme necessary for this hydrolysis is known to occur in root tips (Hellebust and Forward, 1962). The continuous increase in free hexoses with growth indicates

storage in the cell vacuole for subsequent utilisation.

Ribose concentration does not vary, remaining low in every segment. The graph for xylose is fragmentary since this sugar was detected with only one column. However, it would seem to follow the same pattern as that of ribose. Both these sugars do not change detectably with vernalization.

Occurence of the unknown and galactose: As previously mentioned, these two sugars are not present in every segment. Their occurence is shown in Table 9. Rideau wheat roots contain more of the unknown than Marquis roots. There is no apparent change in this carbohydrate following vernalization, except that there seems to be a segment shift: i.e. in $R_{14}K$ it is first detected in segment 2, while in $R_{14}V$, in segment 3. The same is found in Marquis wheat with segments 4 and 5.

Galactose is present only in the older segments of both Marquis wheats. In untreated Rideau wheat it occurs in segments 3 to 6, whereas it is detected solely in segments 6 and 7 of the vernalized roots.

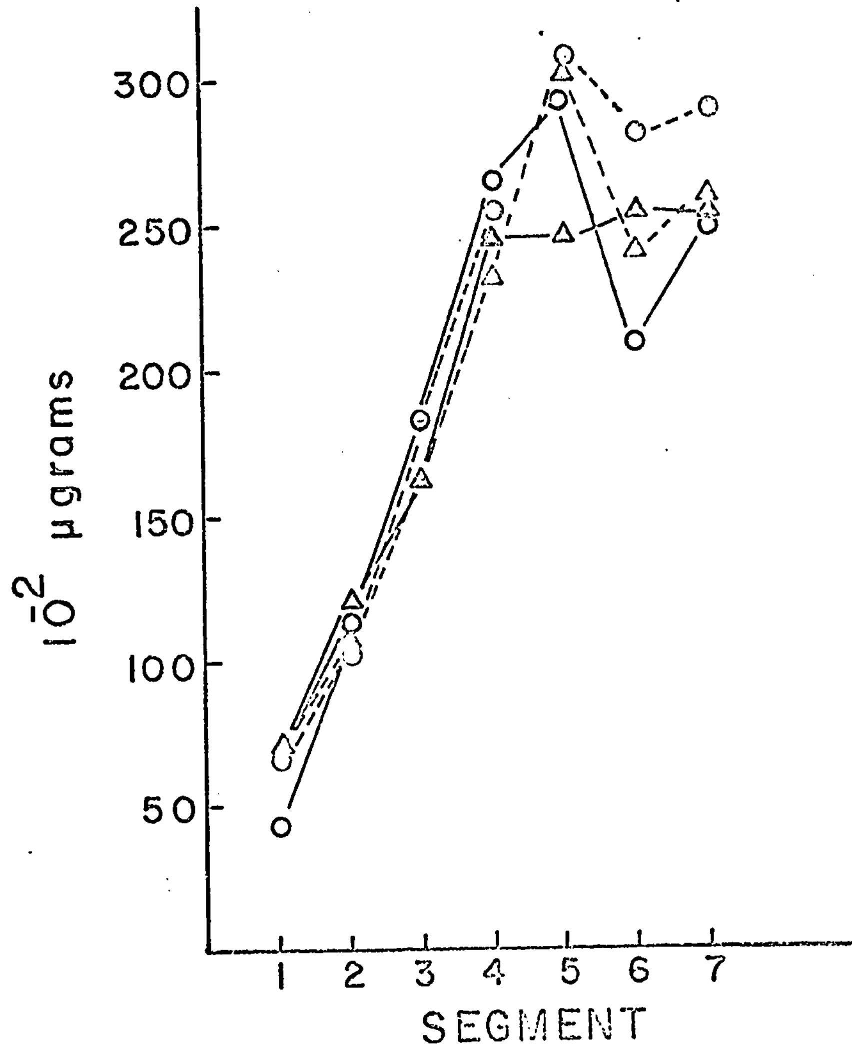
Total free sugar content per segment

The total sugar content, obtained by adding the individual sugar concentrations in each segment (Fig. 8) increases 5 fold from segment 1 to segment 5 and then remains constant (M_5K), or decreases (M_5V, $R_{14}K$, $R_{14}V$). This change in pattern coincides with the zone of maximum elongation and could result from cell wall deposition. Vernalized roots of Rideau wheat differ markedly from the

Fig. 8. Change in the total free sugar content of the wheat root tip with growth, vernalization and wheat variety.

$R_{14}K$ ——o—— $R_{14}V$ - - - o - - -

M_5K ——△—— M_5V - - - △ - - -


300
250
200
150
100
50
10^{-2} µgrams
1
2
3
4
5
6
7
SEGMENT

control roots in segments 6 and 7. There is no difference between control and vernalized Marquis roots, except in segment 5.

Total free sugar content as determined by the Dubois method

The phenol sulfuric acid method detects all sugars: monosaccharides, polysaccharides, oligosaccharides and sugar alcohols (Fig. 9). It thus differs from the column analysis which only reveals monosaccharides and small chain sugars.

The two methods give a similar pattern of change of total sugar with growth. However, they give different relationships between the wheat varieties. When the Dubois method is used, control Marquis wheat roots have a higher sugar concentration than those of Rideau wheat. The results obtained following vernalization are also different. Vernalized Rideau roots consistently have more total sugar than control roots, not only in segments 6 and 7 but also in segments 4 and 5. Marquis roots show a decrease in soluble sugar following vernalization.

These differences would seem to be due to oligosaccharides and polysaccharides. Furthermore, they are probably connected with cell elongation since they occur in the segments further removed from the apex.

Starch content

The curve for total starch content per segment is the reverse of that for total sugar i.e., there is a decrease from

Fig. 9. Change in the total free sugar content (Dubois method) of the wheat root tip with growth, vernalization and wheat variety.

Fig. 10. Change in the total starch content of the wheat root tip with growth, vernalization and wheat variety.

$R_{14}K$	——o——	$R_{14}V$	– – –o– – –
M_5K	——△——	M_5V	– – –△– – –

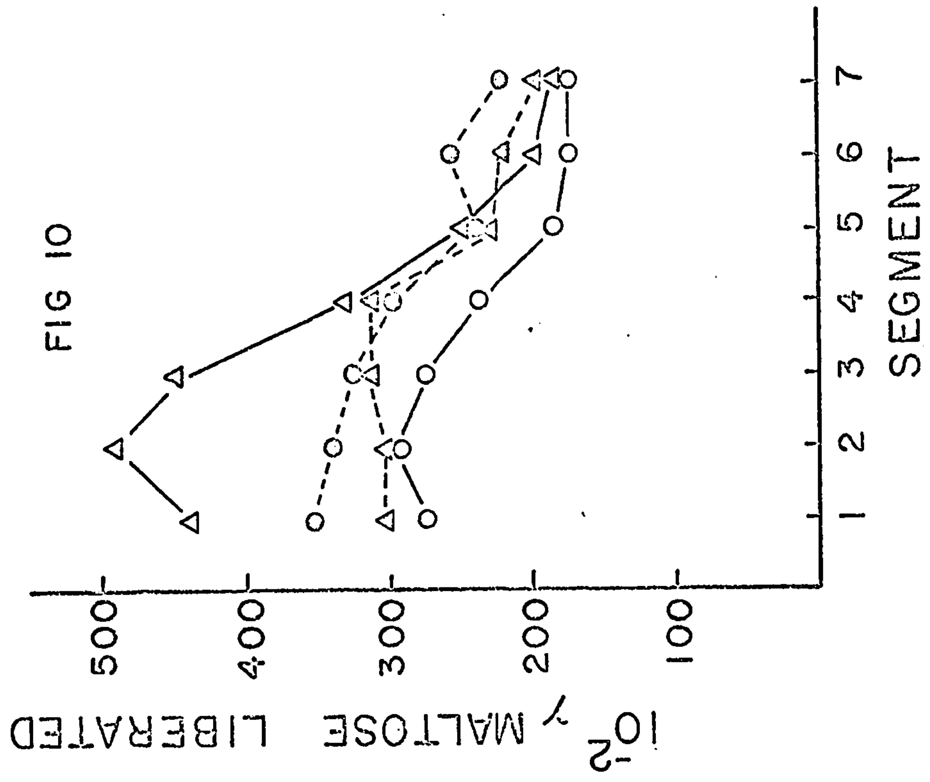


FIG 10
SEGMENT
1 2 3 4 5 6 7
500 400 300 200 100
10^{-2} γ MALTOSE LIBERATED

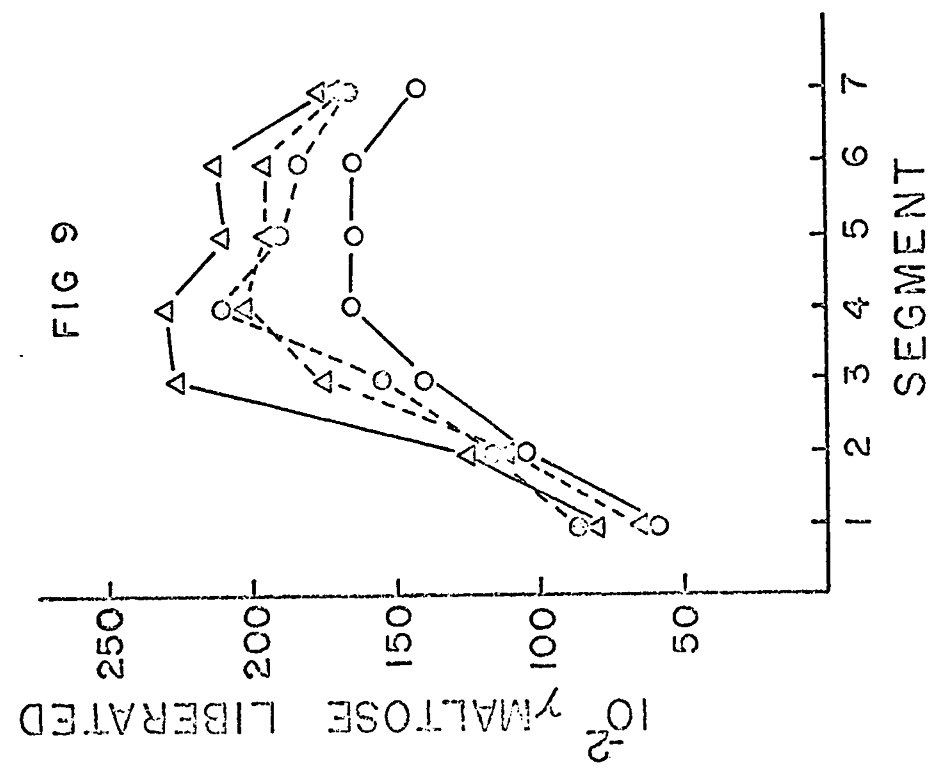


FIG 9
SEGMENT
1 2 3 4 5 6 7
250 200 150 100 50
10^{-2} γ MALTOSE LIBERATED

segment 1 to segment 7 (Fig. 10). The higher starch concentration is found in the meristematic zone. Since the segments with the least sugar also contain the most starch, this seems to indicate a starch to sugar conversion, less pronounced in the apical segments and increasing with cell elongation. This could provide material for cell wall formation or as respiratory substrates. The relationship between the curves is similar to that for total sugar: the highest starch content is in the Marquis variety and the roots of vernalized Marquis wheat have less starch than those of the control, while the roots of vernalized Rideau wheat have more.

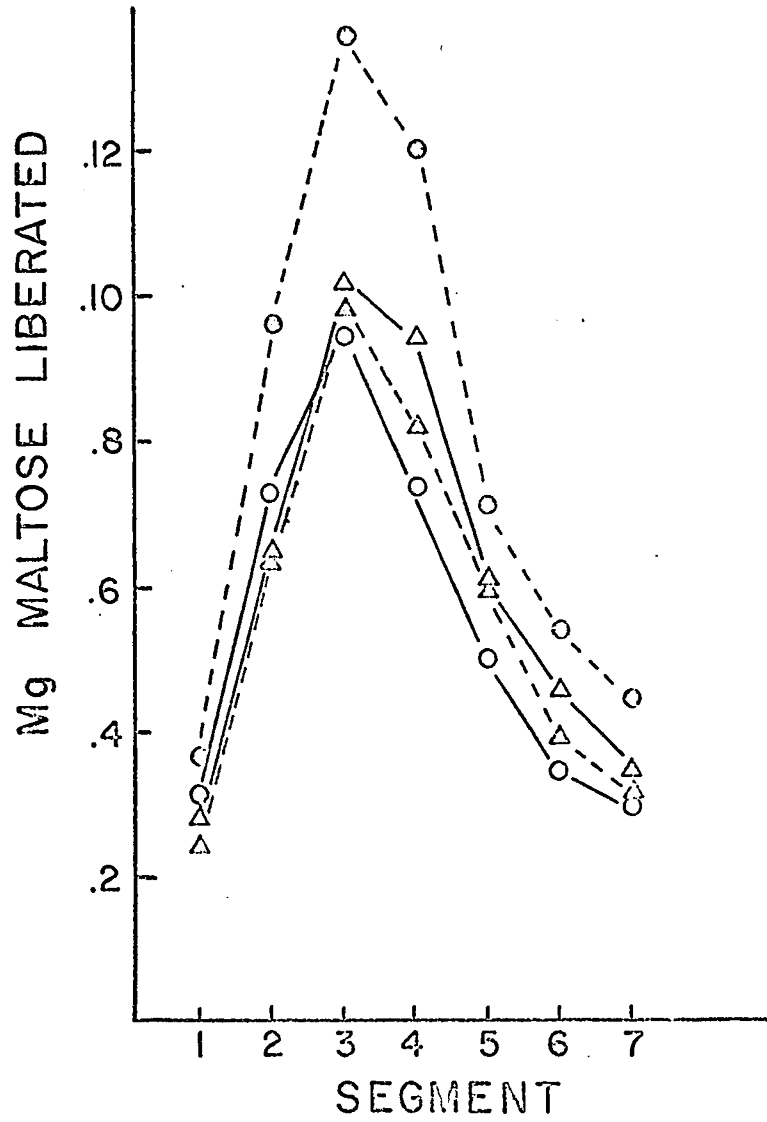
Amylase activity

Amylase activity is at a peak in segment 3 (Fig. 11), There is a 5 fold difference in activity from either segment 1 or segment 7 to the maximum in segment 3. Vernalization does not change the amylase activity in Marquis wheat. In Rideau wheat it is increased in every segment. The increased amylase activity with vernalization in Rideau wheat parallels the higher starch content of the vernalized roots. The fact that amylase activity is higher in the meristematic zone of vernalized Rideau roots while there is no corresponding increase in sugar suggests that the sugars liberated are utilised as they are being formed.

Fig. 11. Change in the amylase activity of the wheat root tip with growth, vernalization and wheat variety.

$R_{14}K$ ——o—— $R_{14}V$ - - -o- - -

M_5K ——△—— M_5V - - -△- - -



Mg MALTOSE LIBERATED
.12
.10
.8
.6
.4
.2
1
2
3
4
5
6
7
SEGMENT

Following the inactivation of α amylase, no amylase activity could be detected. Following inactivation of B- amylase, traces of activity remained. It would therefore seem that the inactivation treatments were not specific. Since the amylase content of the sections is extremely low, even a small inactivation of one amylase by the treatment inhibiting the other would result in the apparent lack of activity obtained here. To test this hypothesis, pure α-amylase solution was treated so as to inactivate B-amylase. This resulted in a 19% decrease in activity. A concentrated enzyme extract was then prepared using 100 whole root tips, 3.5 mm in length. The enzyme activity of this solution was assayed before and after the inactivation treatment for B-amylase. The decrease in acivity was 21% comparable to that obtained for the pure α-amylase solution. Amylase activity in the wheat root tips therefore seems to be due entirely to the action of α-amylase.

DISCUSSION

In young seedlings, such as those used in the above expriments, where photosynthesis is not yet operative, sugar metabolism depends entirely on the carbohydrate reserves of the grain.

The carbohydrates found in the wheat grain include starch, cellulose and soluble sugars (Peterson, 1965). Starch is the most abundant constituent and is confined almost entirely to the endosperm. Cellulose occurs in the fibrous material of the pericarp, seed coat and the aleurone layer. The embryo, according to Horder et al (1954), contains 20% soluble carbohydrate, chiefly sucrose and raffinose. Other sugars found in wheat grains are glucose, fructose, maltose and melibiose (Peterson, 1965).

Of the above mentioned sugars, four i.e., glucose, fructose, sucrose and raffinose, were found in the root tip. The others were either not present or occurred in amounts too low to be detected. In addition, three " new " sugars, i.e., galactose, xylose and ribose appeared. This indicates extensive interconversion and metabolism of sugars during the growth of the primary root. The two pentoses, xylose and ribose, most probably arise from the oxidation of hexose phophates by means of the hexose monophosphate shunt.

The accumulation in plants of galactose is unusual (Axelrod, 1965). It could arise from the hydrolysis of the trisaccharide raffinose (Axelrod, 1965).This is supported by the fact that raffinose concentration decreases from

segment 4 to segment 7, that of both glucose and fructose,(which together with galactose forms the raffinose molecule) increases while galactose, which was not present in the younger segments, is detected.

Starch is the only carbohydrate which has its maximum concentration in the younger zones of the root. The root cap region with its high starch concentration could thus be serving as a storage area. Starch is not transported as such in the plant; its presence in the root tip presupposes synthesis from simpler units, probably sucrose. Vernalized roots of Rideau wheat contain both more starch and more soluble sugars in every segment. This suggests a greater mobilisation of reserves during germination and a faster rate of transport to the growing radicle. This agrees indirectly with David's (1944) data which show that in wheat grains during vernalization, part of the starch in the endosperm disappears while starch appears in the plumule and radicle of the embryo. The higher starch concentration in vernalized roots could thus also be a carry-over from this original re-distribution.

There are conflicting reports, regarding the effect of vernalization on carbohydrate content. Séchet (1949) and Dupéron (1949,1950) have found that cold treatment causes an accumulation of sugars in the grain of a variety of cereals. However, Devey (1963) reports that the carbohydrate content of plants derived from partially to fully vernalized seeds decreases with the process of vernalization. Our data for roots parallel those obtained by Séchet and Dupéron for the grain.

The curve for amylase distribution along the root does not follow that of starch nor that of total sugar. Rather, it is more closely related to that of sucrose. Porter (1953) who studied starch synthesis and degradation in vivo has observed that in situations where starch is degraded, sugars appear, especially sucrose. Moreover the fluctuations in sucrose content are always more pronounced than those of hexoses. He concludes that sucrose should be regarded as a product of starch degradation. This would seem to be the case in the growing wheat root tips.

The increase in total free soluble sugars parallels cell elongation. However, this probably should not be considered as an increase per se, but rather as an indication of lessened utilisation. The reserve polysaccharides are hydrolysed in the grain and are transported along the root. They would be maximally utilised in the meristematic region in initial cell wall formation. Elongation would seem to involve no further withdrawal of carbohydrates, as evidenced by the flat part of the curve in segments 5 to 7.

The decrease in both raffinose and sucrose content in the meristematic zone of vernalized Rideau roots may result from the higher mitotic rate observed in this region following the cold treatment (Weinberger and Ku, 1966), through a withdrawal of sugar for cell wall formation and increased metabolic utilisation. Sucrose is also significantly absent from the younger segments of the roots of both Rideau and Marquis wheat,

Sucrose has been shown to be closely associated with respiration in germinating barley (McLeod, Travis and Wreay, 1953). Furthermore, Goddard and Bonner (1960) who have determined the respiration rates of 1 mm slices of Zea mays root tips have shown that respiration is higher in elongating cells than in meristematic cells. This could account in part for the decrease in sucrose concentration observed in the older segments of the root.

The two wheat varieties, Marquis and Rideau, do not contain the same total amount of soluble sugars (as determined by Dubois method) in their roots. However, the pattern of carbohydrate distribution along the root is the same. On the whole, vernalization affects the carbohydrate content of Rideau roots more than that of Marquis. There is a decrease in both the starch and sugar content of Marquis wheat following the cold treatment. Since Marquis wheat does not require the vernalization treatment, the cold temperature might simply be slowing down the enzymatic reactions leading to hydrolysis of polysaccharides.

Section lV - Uptake of ^{51}Cr as an indicator of metabolic change in wheat root tips.

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INTRODUCTION

A - Uptake of ^{51}Cr By The Wheat Root Tip

The occurence of chromium in plant and animal tissues has long been established (Baetjer, 1956; Davis, 1956). To date, its essentiality is still unproven but there is increasing evidence that it may be an indispensable micronutrient for the mammalian organism (Schwarz and Mertz, 1959; Glinsmann, Feldman and Mertz, 1966).

In the plant field, most of the tissues which have been analyzed have contained at least trace amounts of chromium (Davis, 1956). The concentration of this element in normal vegetable tissue has been reported to be within the range of 0.01-1 p.p.m. (Saint-Rat, 1948). Gericke (1944) has shown that the addition of trace amounts of chromium to the soil increased the yield of nearly all plants.

Although the exact metabolic role of chromium is still unknown, interest in this element has been spurred by its discovery in several compounds of physiological importance. Wacker and Vallee (1959) found a significant concentration of chromium in RNA and suggested that it plays a role in maintaining the configuration of the RNA molecule, perhaps by linking purine or pyrimidine bases through covalent bonds. Schwarz and Mertz (1959) have shown that chromium is important in maintaining normal glucose tolerance in the rat. Chromium has also been found to catalyse the phosphoglucomutase

reaction (Strickland, 1949) and to activate the succinic dehydrogenase - cytochrome system (Horecker, Stotz and Hogness,(1939). In animal tissues, work with nucleoproteins (Hermann and Speck, 1954) and with red blood cells (Gray and Sterling, 1950) has revealed that chromium passes through the cell membrane as the hexavalent form but is bound as the trivalent form.

Except for the labelling of blood components to determine their life-span or survival rate, chromium has not been used extensively as a tracer isotope. Recently, however, Vittorio, Wight and Sinnott (1962), working with mice injected with sodium radiochromate, have shown that the incorporation of ^{51}Cr followed a consistent pattern in all the organs studied. This, together with the greater incorporation of chromium by young or rapidly proliferating tissues than by older ones, led them to the successful utilization of ^{51}Cr as an index of repair in tissues that had suffered X-radiation damage (Vittorio, Wight and Sinnott, 1963; Vittorio and Wight, 1963; Vittorio and Dziubalo, 1964). Metabolic imbalances were thus detectable even before gross morphological changes were evident.

No specific work had previously been done relating aspects of plant metabolism and cellular development to radioactive chromium uptake. The present work was undertaken to determine whether a relationship exists between the stages of cellular development in plant tissues and ^{51}Cr incorporation. Previous studies of Weinberger and Godin (1963,1966)

and Weinberger and Ku (1966) had indicated that vernalization accelerated all stages of wheat development. Thus information was also sought as to whether a changed pattern of incorporation would parallel metabolic changes coincident with vernalization.

Since prior work has shown that the growth rate of the root tip was substantially increased following vernalization (Weinberger and Ku, 1966), this region was chosen for the present study.

B - Sulphydryl - Disulfide Patterns Along the Wheat Root Tip

Following completion of the experiments on the uptake of ^{51}Cr by the wheat root tip, it was found that this process involved a reduction of chromium from the hexavalent to the trivalent state. The possibility therefore exists that patterns of distribution of reducing agents in the root before and after vernalization could be correlated with patterns of chromium uptake.

Sulphydryl groups are potent reducing agents known to be functionally active in plant cells. Moreover, it has been found that cold treatment can elicit changes in their distribution. In wheat, Levitt and co-workers (Levitt et al, 1961; Schmutz, Sullivan and Levitt, 1961) have shown that -SH content increases with cold hardening and that -SH changes parallel frost resistance. Both the -SH and -SS content of winter and spring varieties are altered during the vernalization process (Kohn, Waisel and Levitt, 1963)

and these changes can be ascribed in part to SH⇄SS oxidation reduction.

Many reports have appeared on the relationship between sulphydryl and growth. It has been postulated that they are linked to both cell division and cell elongation (Barron,1951; Brunel-Capelle, 1956). Thiol compounds accumulate in meristematic tissues (Hammett, 1929) and in regions of rapid growth (Binet and Magrou, 1931). Mazia (1954) has presented definite evidence for a determining role of protein -SH groups in growth by cell division in animal tissues. Sulphydryl groups are also thought to be the primary point of attachment of growth hormones (Muir and Hansch, 1952; Siegel and Galston, 1953) and it has often been suggested that the auxin regulation of growth may involve specific sulphydryl requirements (Pilet, 1957; Thimann, 1951; Marré and Arrigani, 1957). The early stages of seed germination are accompanied by rapid increases in glutathione, a major -SH containing peptide (Hopkins and Morgan, 1943).

Histochemical localisation of sulphydryl groups was undertaken to determine whether a correlation exists between the pattern of distribution of this reducing agent and the uptake of chromium. The effect of growth and vernalization on the distribution of both -SS and -SH was also investigated.

MATERIALS AND METHODS

A - Uptake of ^{51}Cr by the wheat root tip

The roots were cut in seven 0.5-mm serial sections and placed in an isotonic 0.21 M sucrose solution. They were then incubated with 0.02 Ci of sodium radiochromate (specific activity, 210 mCi/mg Cr) for 60 minutes at room temperature. The samples were centrifuged at 1800 rpm for 5 minutes, the supernatants pipetted off and the section washed three times with an excess of isotonic sucrose solution. Radioactivity was assessed by means of a Nuclear Chicago deep-well crystal scintillation counter.

To determine whether trivalent chromium is also able to penetrate intact cells, an excess of sodium ascorbate was added to duplicate sections before incubation with $Na^{51}CrO$. This procedure reduced the chromate from the hexavalent to the trivalent form. The sections were washed with nonradioactive NaCrO after the incubation period to obtain data solely relating to the binding of ^{51}Cr at the cut surfaces of the segments, thus reducing the possibility of counts due to adsorption.

Autoradiographs of the root tips were also obtained by using the liquid emulsion technique (Kopriwa and Leblond, 1962), with Kodak NTB-3 emulsion.

B - Sulphydryl - Disulfide Patterns Along the Wheat Root Tip

The histochemical tests described below were made on formalin fixed paraffin embeddded longitudinal root sections. Control sections for -SH were treated with a blocking agent, iodoacetate.

The detailed procedures followed were those described by Pearse (1960).

Histochemical localisation of -SH groups

Dihydroxy - Dinaphtyl - Disulfide (DDD) method: This procedure is based on the reaction of sulphydryl groups with naphthols present in the DDD reagent (Barrnett and Seligman, 1952). The product of this reaction is colorless but coupling with a diazo dye (diazo blue B) gives a visible blue color.

Mercury Orange method: The mercaptide formation test is based on the ability of sulphydryl groups to react with mercuric compounds to form colored mercaptides. The mercury compound used is 1-(4-chloromercuriphenylazo) naphthol -2 (R S R) (Bennett, 1951).

Ferric Ferricyanide method: This method depends on the reduction of a fresh solution of ferricyanide in acid solution at pH 2.4 by sulphydryl groups in the tissues. (Chèvremont and Frédéric, 1943). The resulting ferrocyanide combines with ferric iron (provided as ferric sulphate) to give a precipitate of insoluble Prussian blue.

Histochemical localisation of -SS groups

Dihydroxy - Dinaphtyl - Disulfide (DDD) method: This is the same procedure as described above, except that the -SH groups in the tissue were blocked with iodoacetate and the -SS groups were subsequently reduced to -SH with a 10% solution of KCN. This reagent will not unblock the original -SH groups.

Performic acid - Alcian blue method: The -SS groups are first oxidized using performic acid. Combination with an acid solution of the phthalocyanin dye Alcian blue yields a blue color (Adams and Sloper, 1956).

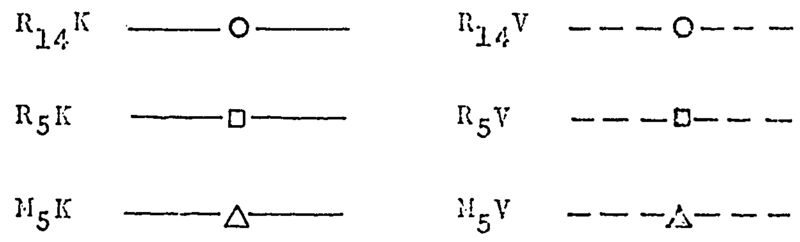
RESULTS AND DISCUSSION

A - Uptake of ^{51}Cr by the Wheat Root Tip

The uptake of chromium in each of the seven root tips segments was assessed on a per segment and on a per cell basis. The values were expressed as percentage of segment 1 which was assigned a value of 100%. Segment 1 was specifically chosen because chromium incorporation in this section, on a per cell basis, was found to be most constant throughout the series. Furthermore, Weinberger and Ku (1966) have shown that it is the segment least affected by vernalization. This stability may be related to the anatomy of the region, the greater part of which consists of root cap.

As can be observed in Fig. 13, there is a consistent pattern of ^{51}Cr incorporation in all series, in spite of the different imbibition periods and variety of grain used. In every case, the uptake was at a minimum in segments 2 and 3 and increased thereafter to segment 7. The metabolic change which follows vernalization was paralleled by a changed pattern of uptake. In every case, the incorporation of ^{51}Cr obtained in those root tips derived from vernalized grain was higher than that in the control series. This is even more strikingly apparent when the data are plotted on a segment basis Fig. 14. Since it has been shown that vernalization increases the mitotic activity and growth rate of the

Fig. 13. Incorporation of ^{51}Cr (counts /min. per cell) in the wheat root tip.



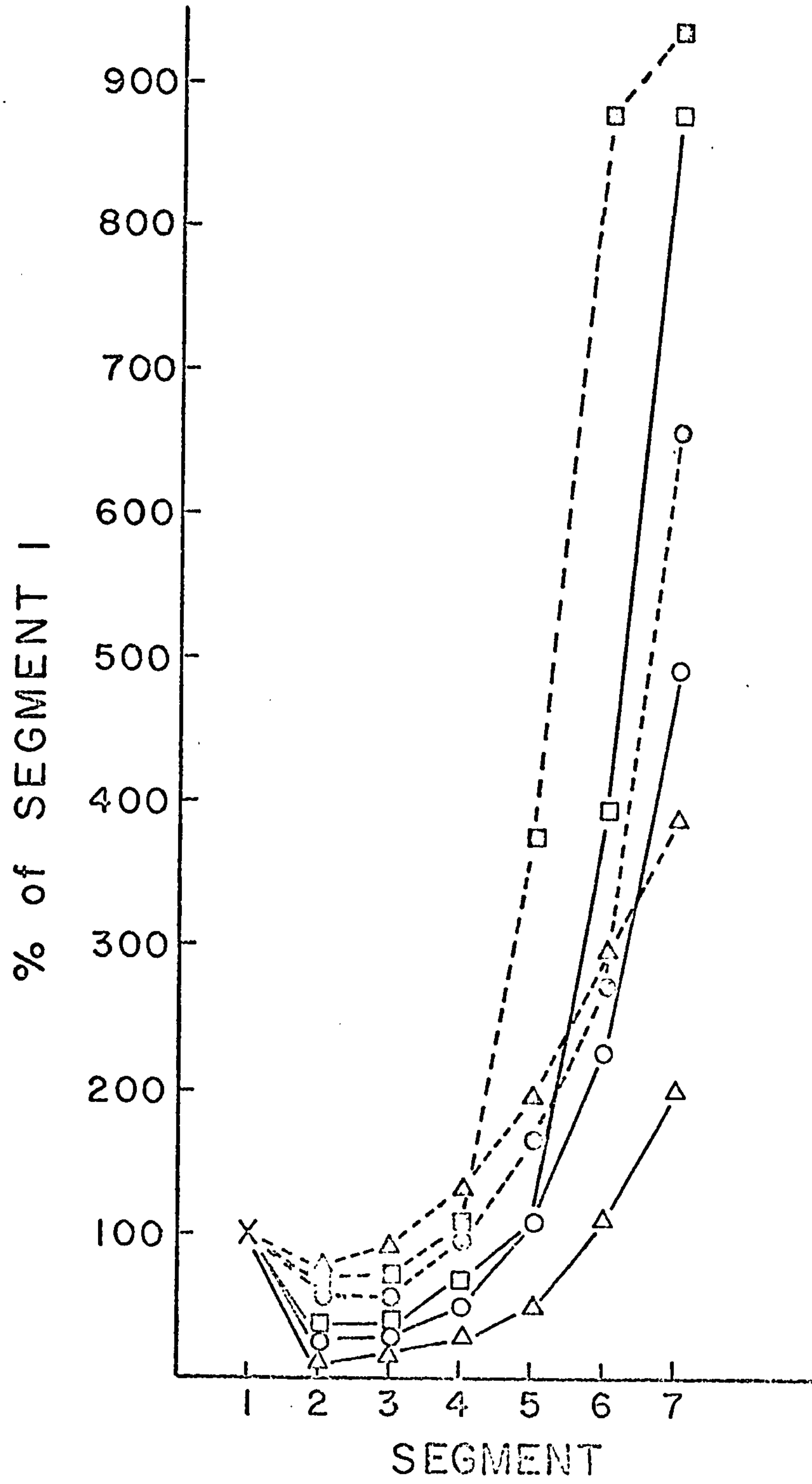
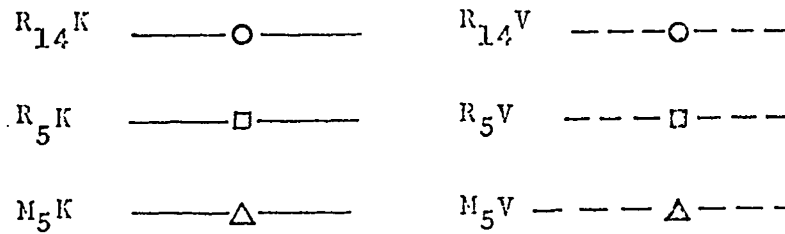
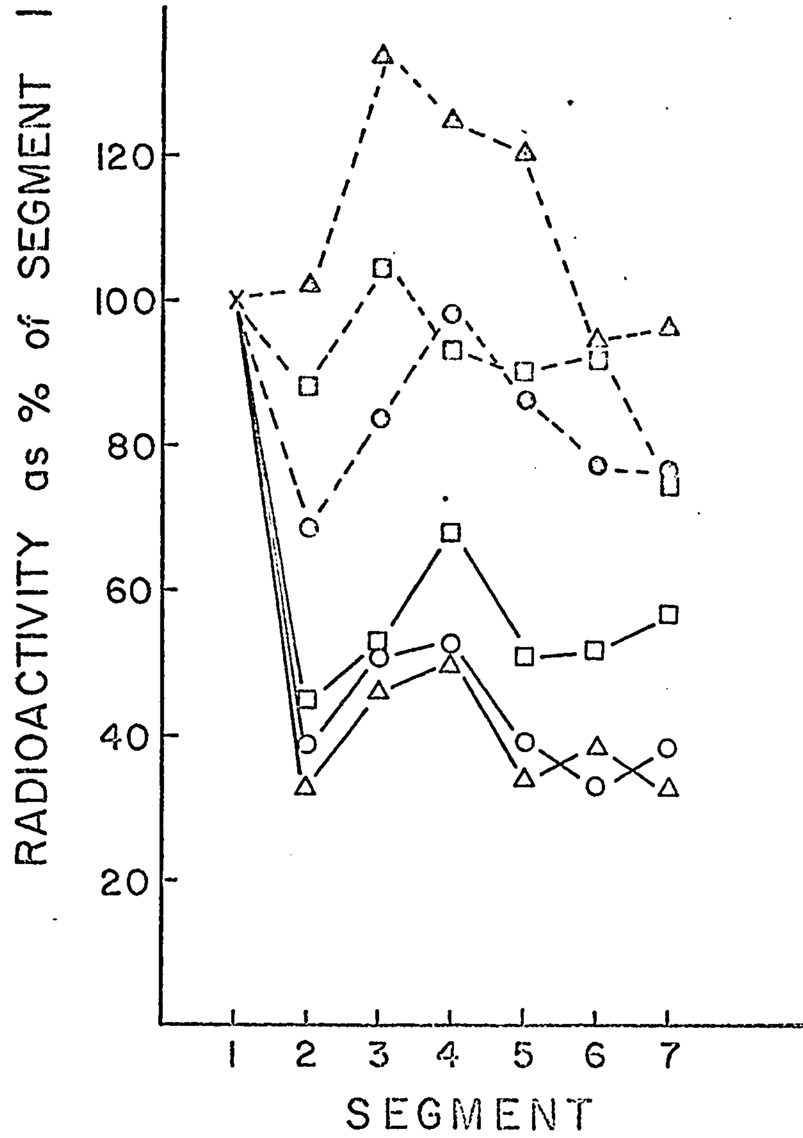

900
800
700
600
500
400
300
200
100
% of SEGMENT I
1
2
3
4
5
6
7
SEGMENT

Fig. 14. Uptake of ^{51}Cr (counts /min.) by the wheat root tip.



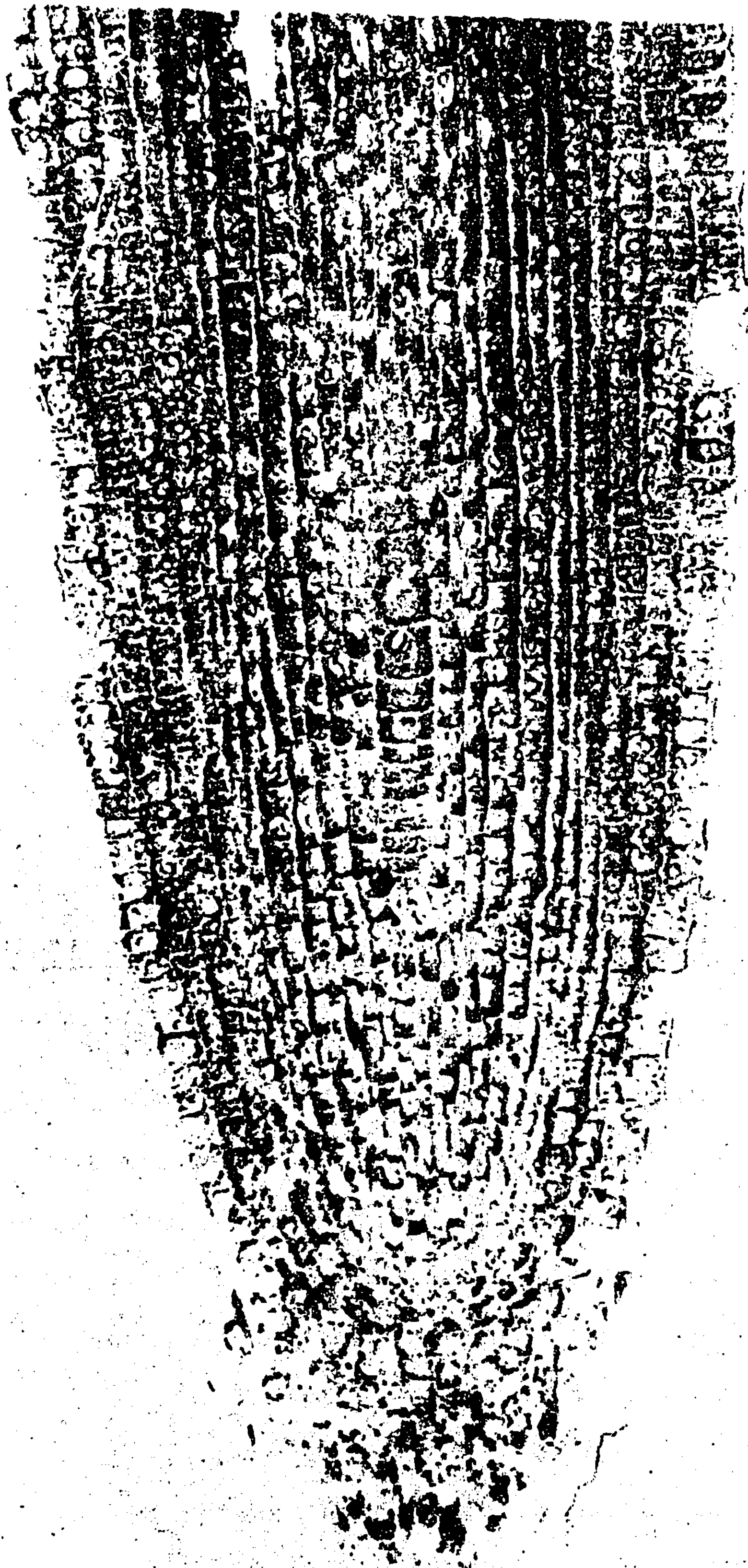

RADIOACTIVITY as % of SEGMENT I
120
100
80
60
40
20
1
2
3
4
5
6
7
SEGMENT

root tips, one can reason that in rapidly proliferating tissue there are more binding sites and hence greater complexing of chromium with these sites. These results agree with those of Vittorio, Wight and Sinnott (1963) who found an increased chromium uptake in tissues recovering from X-radiation damage.

A preliminary analysis of the root tip revealed that the chromium becomes bound predominantly to the protein fraction. This is also the case in animal tissue (Vittorio and Wight, 1963b). More specifically, Pierce (1965), who studied the interaction of trivalent chromium with proteins of human serum, has shown that the carboxylate groupings are responsible for the binding of this element. The data of Ku (1969) and Jones (1969) indicate that vernalization increases the amount of acidic proteins both in the root and the grain of wheat. Hence, the higher uptake of chromium observed in the roots of the vernalized material could well be the result of an augmentation in carboxyl groups.

Concurrent autoradiographic studies indicate that ^{51}Cr is incorporated in all the root tip tissues (Fig. 15). Unfortunately, it is not possible at this time to determine whether the chromium is bound preferentially by any particular cell structure. Chromium is a γ emitter, and shadowing prevents a finer resolution. The uptake in the sub-apical region is interesting particularly since it seems to indicate that ^{51}Cr is incorporated in the quiescent center but to a lesser degree than in the surrounding tissues. Other indicators of change

Fig. 15. Autoradiograph of a control (unvernalized) Marquis wheat root tip following incubation with ^{51}Cr.



have also typified this region as being metabolically sluggish (Clowes, 1954; Jensen, 1958).

In the sections incubated with ascorbate (which reduces chromium from the hexavalent to the trivalent state), there was a 93% decrease in radioactivity. The remaining activity can be attributed to the irreversible absorption of trivalent chromium at the cut surface of the segments. Thus only the hexavalent form seems able to penetrate the intact cell. Gray and Sterling (1950) in their work with red blood cells have reported that although the chromium enters the cell in the hexavalent form, it becomes bound in the trivalent form. Thus the incorporation of chromium may be thought of as occuring in three steps: there is first, the entry of hexavalent chromium into the cell; then its reduction to the trivalent state; and finally its binding to certain cell constituents.

B - Sulphydryl - Disulfide Patterns Along the Wheat Root Tip

The distribution of -SH groups in the wheat root tips as determined by the DDD method is shown in Fig. 16 a,b,c.

The two other methods, which were used to confirm the results obtained with DDD, did not reveal the presence of -SH groups in the root tips. The mercury orange method is very specific but unfortunately the color of the end product is weak (Jensen, 1962). The sensitivity of the ferric cyanide method is also not as high as that of DDD (Pearse, 1960).

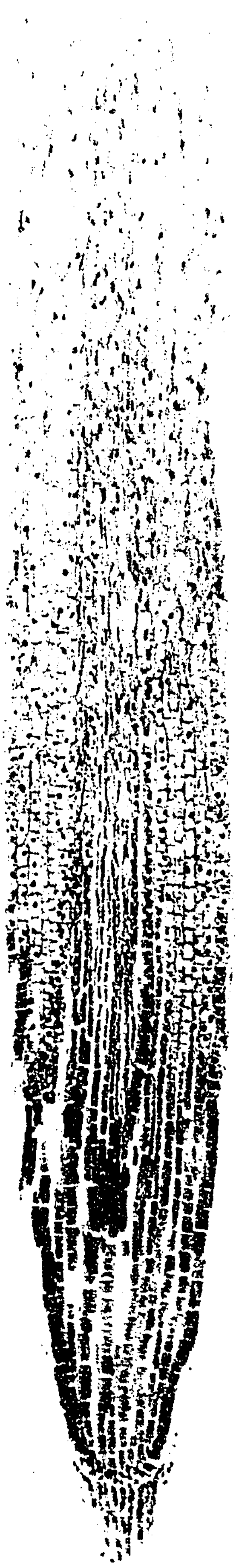
No -SS groups could be detected either by the DDD method

Fig. 16. Histochemical localization of -SH groups.
(DDD method)

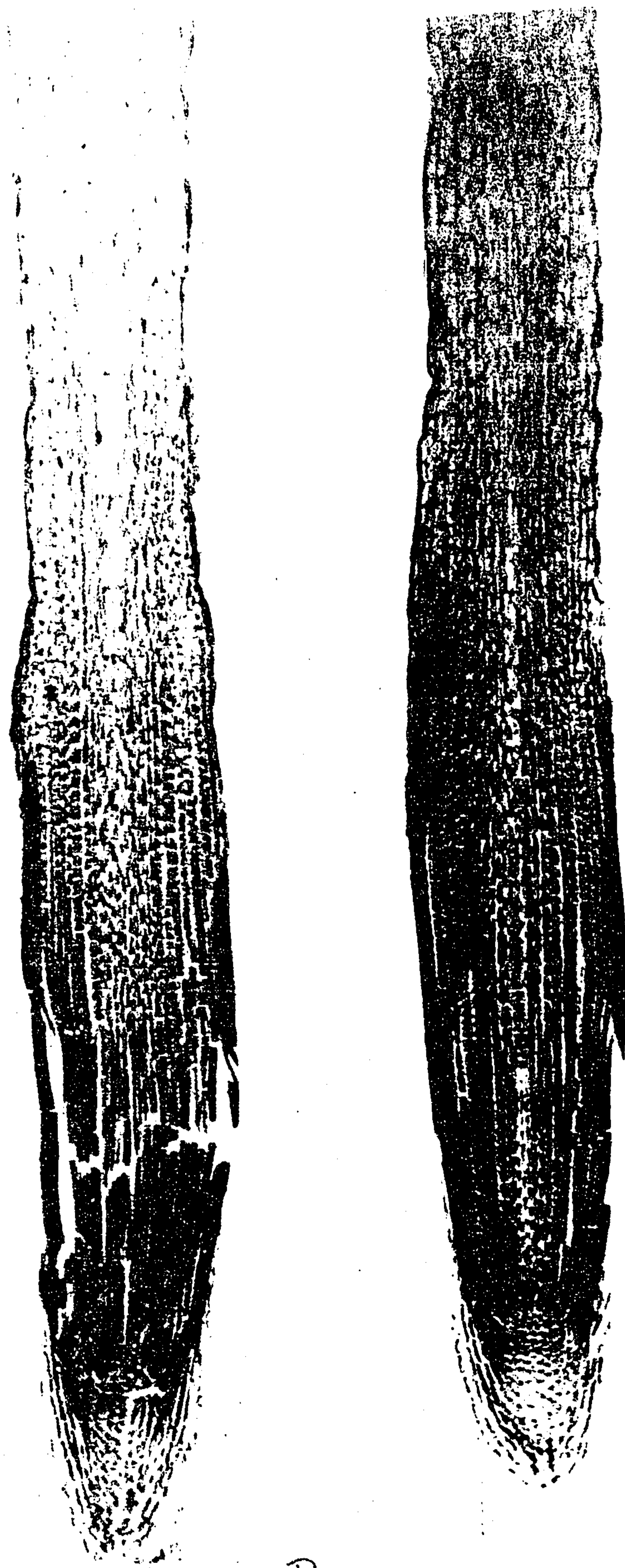
A. $R_{14}K$ and $R_{14}V$

B. R_5K and R_5V

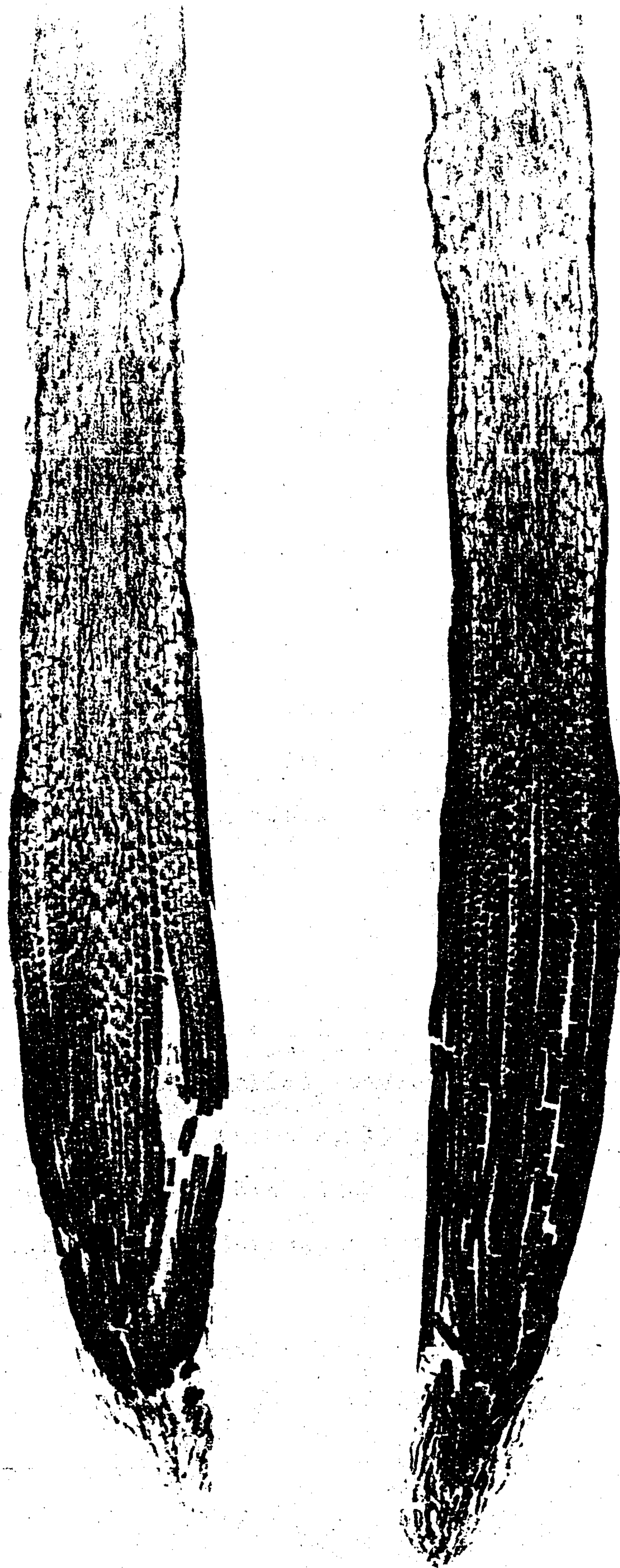
C. M_5K and M_5V



A



B


C

or by the performic-acid method.

The DDD reaction is highly sensitive and can reveal very small concentrations in the tissues. Therefore, disulfide groups, if present in the wheat root tips, occur only in minute quantities.

Comparing the pattern of Cr uptake as given by the autoradiograph (Fig. 15) and that of -SH groups, we find that in both cases, the concentration is greater near the tip of the root and lessens towards the more mature regions. Such a distribution was also found by Roberts (1950) who studied the histochemical localization of reducing activity (dehydrogenase enzymes) in a number of roots. However, these results can be misleading. The meristematic region contains small and numerous cells which will give a greater staining density than cells in the elongation region where the cells are longer and less numerous. These larger cells will then "dilute" the stain.

Other bodies of work on the distribution of -SH in roots, using the serial section technique (Goas, 1955; Pilet, 1957; Hammet and Chapman, 1938) have also shown that the concentration of this reducing agent is high in the meristematic region and decreases progressively along the length of the root.

Vernalization does not seem to affect the -SH distribution in R_5 or M_5 roots. On the other hand, there is a marked decrease in -SH content of R_{14} roots following the cold treatment. These results do not parallel the Cr

data where in all cases, an increase in uptake was observed in the vernalized roots. The different reaction of R_{14} and R_5 roots could result from the fact that a 5 hour imbibition period is not sufficient for the complete process of vernalization to take place.

Since -SH groupings are prominent in regions of active cell division, one would have expected an increase in -SH concentration in the vernalized roots where growth is more active. This was not found to be the case.

Ellis and Trione (1967) who have analyzed the sulphydryl and disulfide patterns associated with the vernalization of wheat have found that the plumules of winter wheat seedlings grown at 3°C contain more -SH groups than those grown at 20°C. My results with the root tip show an opposite trend, namely a decrease in -SH groups following vernalization. However, both sets of data are not strictly comparable, since morphologically different tissues were investigated. Furthermore, Ellis and Trione (1967) actually grew their wheat at the cold temperature while, in this experiment, only the grain was subjected to the cold treatment. Germination took place at 25°C.

Waisel, Kohn and Levitt (1962) have found that glutathione oxidizing activity of the wheat grain increases several fold during exposure to vernalizing conditions. This would then result in a decrease in -SH content. Kohn et al. (1963) have further analyzed the nature of the change in

-SH during vernalization of wheat and have concluded that two mechanisms could be operative: 1) an SH $\rightleftharpoons$ SS oxidation reduction and 2) a splitting off or adding on non-protein SS mainly in the form of oxidized glutathione. No increase in -SS were detected in R_{14} following vernalization. However, there is a possibility that glutathione can be removed from the tissues during fixation, prior to histochemical determinations. (Pearse, 1960).

CONCLUSION

Previous morphological measurements of Weinberger and Ku (1966) have shown that cold treatment of the wheat grain accelerates the subsequent root growth. The present work shows that the physiology of the root is concomitantly altered following vernalization. Growth of the root tips involves both a morphological and a physiological differentiation. The biochemical differentiation is reflected in the changing concentration of free sugars and amino acids along the root axis. Growth of the cells may in turn be thought of as a succession of metabolic states, with each state involving a changing enzyme complement (Brown and Robinson, 1955). This observable differentiation of cells at the biochemical level has its basis in the genetic constitution. Since all cells of an organism possess the same complement of genes, these must then necessarily be activated or repressed during the course of development as the need arises.

My work shows that vernalization alters the metabolic differentiation of the root. The pattern of both free sugars and free amino acids along the root is altered following the cold treatment. When considering free sugars or amino acids as a group, the total amount does not seem to be important: rather it is the composition which is highly relevant. All the parameters studied do not change to the same degree, nor in the same direction. Some increase

some decrease while still others are not affected at all. Variation is also encountered with the age of the cell. The effect is sometimes more pronounced in the young meristematic cells, other times in the more mature cells.

Since vernalization of the wheat grain subsequently accelerates the flowering process, this presupposes a preservation of the after-effect which is perpetuated through several generations of cells. The time between perception of the cold stimulus and the appearance of flowers spans almost the total life cycle of the wheat plant. However, the flowers produce new grain which itself is not vernalized. The transmission of the stimulus seems to stop at the level of meiosis. What is then the halting mechanism?

Both the reaction to vernalization and the biochemical growth patterns vary with the wheat variety. Although the same metabolites were found in both varieties, their concentrations along the root differed. In the majority of cases, Marquis, the spring wheat, had higher amounts of the compounds studied than the Rideau variety. Vernalization of the grain did not affect the roots of Marquis wheat to the same extent as those of Rideau wheat. Although Marquis is a spring wheat and does not absolutely require the cold treatment, chilling can accelerate flowering by as much as 21 days (Wort, 1939). Vernalization would then completely unmask the potentialities which were only partially expressed.

The length of the imbibition period also affected

the reaction to vernalization. The difference is one of degree: incomplete imbibition leads to a lessened response in the root. Water entry in the grain initiates the sequence of reactions leading to germination. De-novo synthesis of enzymes occurs together with an activation of enzymes already present (Mayer and Poljakoff-Mayber, 1963). My results suggest that insufficient water levels prevent the complete biochemical sequence of reactions leading to vernalization.

The changes in the free amino acid composition which were noted following vernalization could be significant in themselves, apart from reflecting alterations in metabolism. Some amino acids have morphogenetic effects (Steinberg, 1947; Waris, 1957) and modifications in the concentration of these compounds in the cells could then induce an altered growth rate or an altered sequence of differentiation.

The effect of vernalization on the physiology of the root is also strikingly illustrated by the modified pattern of ^{51}Cr uptake. Although this and the other differences in metabolism observed between control and vernalized roots can be thought of as metabolic consequences, rather than as the basis of vernalization, they can still provide valuable background information to help elucidate the biochemical and molecular nature of the vernalization process.

The physiological changes observed in the root following the vernalizing treatment of the grain are probably the expression of prior modifications at the genetic level.

The vernalization requirement in wheat is transmitted in a complex manner. It exhibits a quantitative inheritance with a number of intergradations between an absolute requirement, as in all winter wheats, and no requirement at all, as in some spring wheats (Salisbury, 1963).

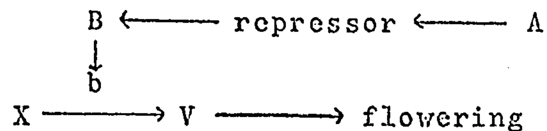
Temperatures below 10°C are effective in vernalization (Purvis, 1948; Hansel, 1953). Above 15°C devernalization occurs (Purvis and Gregory, 1952). However, with increasing duration of the low temperature treatment, there is a point after which reversal of vernalization is no longer possible (Purvis and Gregory, 1952). After devernalization of partially vernalized grain, further exposure to low temperature effects a revernalization (Purvis and Gregory, 1952). My experiments indicate that more than one metabolic pathway seems to be involved. However, no qualitative differences between vernalized and non-vernalized roots were detected.

Ku (1969) reports an increase in RNA in the roots of vernalized Rideau and Marquis wheats following vernalization. Terakoa (1967) and also Ku (1969) have found differences in nuclear histone concentration associated with the cold treatment. This strongly suggests a derepression of genes which would then code for the production of new RNA.

One can postulate a case where the key compound in vernalization is a substance V. This would be the substance initiating the sequence of reactions leading to a subsequent acceleration of flowering. V is produced

from X, the reaction catalysed by enzyme b.

The formation of b is regulated by gene B. In a winter wheat, formation of b is repressed by a repressor substance formed under the control of gene A.



In a spring wheat, B would normally be derepressed. Cold would be the stimulus that derepresses B in the winter wheat allowing V to accumulate. To account for devernalization, we can assume that V, at"normal" temperatures is metabolized to another compound D. Vernalization would be complete only when a certain threshold concentration of V is reached. After this point, devernalization would no longer be possible.

The reaction $V \xrightarrow{c} D$ would not occur at low temperatures. There could be a feedback imhibition of D on c, the enzyme necessary for its formation. This could also be an equilibrium reaction, with cold favoring the production of V.

In some spring wheats such as Marquis vernalization can also slightly accelerate flowering. In this case, there would still be a slight repression of D.

It has also been hypothesized that hormones are involved in vernalization, and in some cases, application of

gibberellic acid can replace the vernalization treatment (Lang, 1957). It is therefore possible that cold could act indirectly, by promoting the formation of an effector substance, a molecular agent of derepression. In higher plants, hormones are known to act as effectors (Bonner, 1965). However no hormone has yet been found to completely replace vernalization in wheat.

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