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LIPID AND AMINO ACID CHANGES IN RYE DURING VERNALIZATION

by

LAWRENCE WAYNE THOMSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Lipid and Amino Acid Changes in Rye During Vernalization" submitted by Lawrence Wayne Thomson in partial fulfilment of the requirements for the degree of Master of Science.

Supervisor

Date Sept. 29, 1972

ABSTRACT

The rye varieties used in this study were Sangaste, which exhibited a vernalizing response upon chilling and Prolific which did not. Samples were collected at weekly intervals during chilling. Extracts of the excised embryo tissue were analyzed for lipids and amino acids.

An increased accumulation in the total alcohol soluble amino acids and amides occurred during the initial 3 weeks of the chilling period and was followed by a decline in both varieties. The early accumulation was more pronounced in Sangaste which increased by 142% as compared to 40% for Prolific. Proline, alanine, and glutamine showed a marked accumulation during the initial 3 weeks with the winter variety containing almost double that of the spring.

Total lipids were extracted with chloroform: methanol 2:1 v/v and purified by column chromatography and two-dimensional thin layer chromatography. Quantities of the different lipids were determined by phosphorus and sugar analysis. The total phosphorus content of the total lipid extract expressed on dry tissue weight was found higher for Sangaste suggesting a higher phospholipid content, however, the portions of the individual phospholipid components were similar for both varieties and showed similar responses during the 8 week chilling period.

During the initial 3 weeks an increased accumulation of 18:3 and a corresponding decline in 18:2 was observed for all lipid components analyzed. The glycolipids were found to be more unsaturated than the phospholipids prior to chilling and showed a greater increase in unsaturation during the treatment. In general the fatty acid content of the respective lipid classes was very similar for both varieties.

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INTRODUCTION

Vernalization and photoperiodism are two examples in which external conditions influence plant development. Both regulate the transition from the vegetative to the reproductive growth phase. Vernalization is defined as a chilling effect which either enhances or enables flower differentiation. Photoperiodism is a light effect which also influences flower differentiation and the heading processes which follow. The photo-effect is known in part to be due to a transmittable response from the leaf to the apical regions. Although numerous studies have been undertaken to define the vernalization mechanism, no clear explanation has emerged.

Winter cereals have been favored in studying the vernalizing mechanisms since their seeds can be germinated in the dark and are receptive to the chilling response. This enables the photo-effect to be separated from the cold treatment.

Several recent studies have centered on free amino acid changes accompanying vernalization of cereals (Trione *et al.*, 1967; Jones and Weinberger, 1970; Ste-Marie and Weinberger, 1970; Weinberger and Godin, 1966). Studies on lipid changes during the vernalization of winter cereals were conducted by Redshaw (1968). In the analysis he included both the endosperm and embryo. Thus lipid changes in the embryo might have been masked by the lipids of the endosperm.

In the study reported here rye seedlings were subjected to vernalization temperatures and at weekly intervals the embryos were excised. This material was analyzed for free amino acids and lipid components.

REVIEW OF LITERATURE

A. Definition and Practical Application of Vernalization

Environmental influences on plant development are most noted in the termination of dormant conditions and the transition of plants from vegetative growth to flowering. The induction of flowering in many species is controlled by the daylength. In some species, flower induction could be enhanced by chilling periods followed by the appropriate daylength. Possibly due to the adaptive property of plants, most of those which show an enhancement in flowering by chilling, require the chilling period to be followed by normal growing temperatures and long daylength photoperiods. The chilling requirement and the daylength effect on flowering appear complementary, however they are suggested to be separate processes (Evans 1971). Plants exhibiting this chilling requirement may be broadly classified into winter annuals and biennials. The winter annuals include common varieties of winter cereals, the biennials include vegetable plant species. Others, perennials and woody plant species also show a chilling effect (Chouard, 1960) but will not be included in this review. Winter cereals will flower without chilling when grown under appropriate daylength and an extended growing period. Chilling of the cereals enhances flower formation. For a number of biennial species studied, chilling is a requirement for floral development. A biennial variety of sugar beet will remain in the vegetative stage for an indefinite period of time if it is not subjected to a chilling process (Lang, 1965). This chilling process which either initiates or enhances

flower formation has been termed vernalization. Vernal from the latin vernum meaning spring-like.

The vernalizing effect on winter cereals was reported by Klippart (1857). Gassner (1918) demonstrated that germinating cereal seedlings were receptive to this response. Lysenko (as reviewed by Whyte and Hudson, 1933) reported that imbibed seeds could be vernalized. If the moisture content was restricted so that the seedlings did not break through the seed coat the vernalized seeds could be machine sown. During the 1930's the sowing of vernalized seeds was extensively practiced in Russia (Whyte, 1948). The vernalizing of seeds with a minimum amount of growth does not destroy their frost resistance. Winter varieties are more frost resistant than spring varieties (Gott, 1961, Pugsley 1971). Researchers in India also made practical use of the vernalizing process. The flowering of rice crops was enhanced to enable harvest prior to the annual monsoon season (Whyte, 1948).

In areas which do not experience extreme conditions the natural vernalizing process is put to practical use. Commercial seed production for the sugar beet industry in North America is located in areas where vernalization occurs in the field under the natural environment. Locally winter wheat is grown as an important cash crop in southern areas of Alberta.

Numerous review articles on the vernalization process have been published (Evans, 1971; Redshaw, 1968; Chailakhyan, 1968; Lang, 1965; Purvis, 1961; Chouard, 1960; Whyte, 1948).

B. Requirements for Vernalization

The range of environmental conditions which give an optimum vernalizing response and the growth stage of the plant which is receptive to low temperature treatment varies greatly among plant species. Most biennials exhibit a juvenile period in which they are non responsive. For example *Hyoscyamus niger* has a juvenile period of 10 days (Melchers, 1952; Sarkar, 1958) and Brussels sprouts are non responsive until the plants are 11 weeks old (Stokes and Verker, 1951). However winter cereals respond to the chilling treatment at any stage of growth from fertilization until flower differentiation. In both the biennial and winter annual species the receptor portion of the plant has been shown to be the actively growing meristem (Purvis, 1961; Wellensiek, 1964). Due to the variations in the vernalizing requirements among plant species this review will be restricted to the general requirements of the winter cereals.

The receptiveness of maturing seeds on the mother plant to the vernalizing response was suggested by the work of Kostjucenko and Zarubajlo (1936, 1937) reviewed by Whyte (1948). They found the duration of the cold treatment required for complete vernalization was dependent upon the temperature under which the seed had matured. Seeds harvested from northern areas when compared with those of the same variety harvested from southern areas required a shorter chilling period. By applying artificial chilling to developing ears, either cut or attached to the plant and varying in maturity from 5 to 35 days after anthesis, Gregory and Purvis (1938) reported vernalization was effective from the early stages of seed development but became

progressively less effective as the seed reached maturity. Seeds, in their dormant state cannot be vernalized, however the partially vernalized state attained during the maturation of the seed is able to survive desiccation and storage for several months (Purvis, 1961). Young germinating seedlings were reported to be receptive (Gasser, 1918) and even imbibed seeds showing no visible signs of germination could be vernalized (Lysenko as reviewed by Whyte and Hudson, 1933). Growing plants which have not reached the heading stage can also be vernalized, however a decline in the enhancement of heading was noted in relation to the increase in the age of the plant at the beginning of the cold treatment (Gott, 1957; Purvis, 1961).

The length of the cold treatment required for complete vernalization is also suggested to be correlated to the winter hardiness of the variety (Gott, 1961). Gott reported the more frost resistant the variety, the longer the chilling period required.

The moisture content required to render dormant seeds receptive to the chilling treatment is reported by Purvis (1961) to be 50% of the dry weight of the seed for winter rye. Higher moisture levels were not harmful unless a film of water surrounded the seed and reduced the oxygen supply, or for varieties which required a lengthy chilling period the enhanced growth rate due to high moisture levels depleted the carbohydrate supply of the endosperm (Purvis, 1961).

A series of studies have indicated the endosperm is not required for vernalization. Embryos excised from imbibed seeds were found capable of receiving the chilling response (Gregory and Purvis, 1938). Also apices dissected from the imbibed embryo could be vernalized on

a culture medium (Purvis, 1940). The possibility that some endosperm metabolites, which were translocated to the embryo during the imbibing of the seed, were active in the vernalizing process was ruled out by Purvis (1961) who showed embryos excised from dry dormant seed could be vernalized. This clarified earlier theories concerning the involvement of the endosperm. Cholodny (1935) detected a large supply of auxin-like material in the endosperm of maize and other cereals which upon germination is transferred to the embryo and presumably used for extension growth. The low temperature required for vernalization retards growth, resulting in the build-up of an auxin-like material which according to Choldny (1935) accelerated development.

Purvis (1948) and Hänsel (1953) found the most effective low temperature range to be between 1 to 7°C. Low temperature was not effective below -6°C, slow between -4 to 0°C and above 7°C. Temperatures above 12-14°C were not effective. Trione and Metzger (1970) employing precise temperature control suggested the most rapid vernalization response occurred in the range of 6 to 8°C for the winter wheat and winter barley varieties studied. Seedlings incubated at 4°C or lower or(and) above 8°C gave a slow response. If the chilling period were discontinuous, the enhanced effect was somewhat less than a continuous cold treatment with the same total cold exposure (Purvis and Gregory, 1952). Purvis and Gregory (1952) also showed a devernalizing effect in partially vernalized seedlings if the temperature approached 35°C immediately following the chilling period. Complete devernalization was possible only after short periods of chilling. The stability to heat increased with the duration of the cold

treatment. Friend (1953) showed the devernalizing effect of higher temperature (35°C for 3 days) is halved for each additional week of low temperature. Seedlings which are partially vernalized can be fixed if they are maintained at 12-15°C for a few days (Purvis and Gregory, 1952). Seedlings which are devernalized can be revernalized. Purvis (1961) showed revernalization is a repetition of the same initial process, not a rapid restoration of an inactivated intermediate product.

This devernalizing effect which decreases with increasing duration of cold treatment has been noted in most plants which have been studied (Lang, 1965).

C. Evaluation of the Vernalizing Response

The following review will be confined to features common to winter cereals. Since winter cereals are receptive to the vernalizing stimulus as young germinating seedlings, attempts have been made to evaluate the vernalized condition at this stage rather than measuring the enhancement of the flowering behavior of the plants. Such attempts have not resulted in a reliable parameter for evaluation. Therefore, most researchers still use flower enhancement as the criterion of measurement.

Chemical studies on the germinating seedlings, developed by Ricker (1934) and Basserskaya (1936) as reviewed and applied by Weinberger and Ku (1966) indicate a progressive response as vernalization advanced to completion. Using the ferric chloride-potassium ferrocyanide color test the color of the growing point changed from yellow to green and finally to dark blue as the seedlings reached the vernalized state.

The color gradation was verified by parallel growth experiments. Staining embryo sections with eosine and methylene blue and determining the pH at which the tissue sections stained blue indicated a change in the isoelectric point of the embryo tissues from pH 5.32 to pH 4.88 during the course of vernalization. These techniques are not widely used.

Interest in early morphological characteristics as a reliable parameter was initiated by Sereiskj and Sludskaja (1937) and Cajhlachjan and Zdanova (1938). By comparing vernalized and unvernalized seedlings they suggested a possible correlation between the accelerated first leaf emergence and a reduction ir the first leaf length as the state of vernalization progressed. Other investigations have shown that leaf length is not a reliable guide. Weinberger and Godwin (1963) noted accelerated early root and leaf growth but no reduction in final leaf size. By subjecting a spring variety of rye, with no vernalization requirement, to chilling conditions comparable to those of a winter rye variety the first leaf of both varieties was shortened by the cold treatment (Purvis and Hatcher, 1959). The reduction of the first leaf length of winter rye was shown to depend upon the growth conditions during the vernalizing periods (Hurd, 1964). By adjusting the temperature and moisture content during the chilling process it was possible to obtain a whole range of leaf sizes for any degree of vernalization. The vernalization process itself was much less affected than the leaf length by either temperature or moisture content.

Purvis (1934) indicated, following studies on the vegetative growth after the vernalization period, the progressive acquisition of

the vernalized state was accompanied by the progressive reduction in the leaf number before flower initiation. Further observations on the spike primordia of rye indicated a minimum number of leaves must be formed before differentiation of flower initials can occur. For the spring variety under normal long day conditions the minimum number was 7; for the unvernalized winter variety the minimum number was 25. The progressive effect of vernalization on the enhancement of flowering resulted in a successive decline of the leaf number of the winter variety from 25 to 7. Microscopic analysis of the growing points revealed extensive modifications in the general growth patterns upon the transition to flower production (Purvis, 1961; Friend *et al.*, 1963; Ahrens and Loomis, 1963, Trione and Metzger, 1970). In rye and other cereals with spike inflorescence the growing meristem becomes long and pointed with alternating ridges. In the vegetative state these ridges expand into leaves. When the flower induction processes are complete, swelling appears above each ridge resulting in the appearance of auxilliary buds or double ridges. In each double ridge leaf expansion is suppressed and the auxilliary meristem eventually produces a flower bearing spikelet. Gregory and Purvis devised a 'scoring' mechanism using the condition of the apex as a criterion of the progress to flower initiation (Purvis, 1961). By their method numerical values were assigned to different stages of development. Thus the plants from seeds which were planted at one date were all harvested at the same time and scored. For those which reached heading prior to harvest, the number of days from heading to harvest were accounted for in the scoring system. Various researchers have devised similar

scoring scales to evaluate the vernalizing response (Friend *et al.*, 1963; Halse and Weir, 1970; Trione and Metzger, 1970).

The number of days to flowering following the chilling period has been adapted by many workers as a suitable measurement of detecting general differences in the flowering response. However if more accurate measurements are required the scoring method involving the condition of the apex is suggested to be a more precise parameter (Trione and Metzger, 1970).

D. Mechanisms of Action

The theory of environmental influence on plant development was believed to be first formulated by Klebs (1903, 1904) as reviewed by Lang (1965a). "According to Klebs the characteristic properties of each organism are determined by its 'specific structure'. The interaction of the specific structure with the external (environment) conditions results in continuously changing 'internal conditions' which determine the actual emergence and elaboration of the organisms character." Lang (1965a) suggests if Kleb's 'specific structure' were replaced by DNA and 'internal conditions' with the transmission of DNA information via RNA to protein synthesis, the theory would be similar to the general concept of development understood today. The theory that the cold treatment influences gene expression is possibly justified in that the vernalization requirement is inheritable. Crosses between winter and spring rye suggest a close 3:1 segregation in the F_2 generation with spring dominant (Purvis, 1939). Crossing studies with Australian winter wheats show a more complex type of inheritance sug-

gesting a possible multigene involvement (Pugsely *et al.*, 1971).

Studies concerning the mechanism of the vernalization process have centered on attempts to define the nature of the environmental stimulus, to detect a possible shift in genetic expression induced by cold treatment and to correlate cold induced metabolic changes with the vernalizing process.

1. Nature of the Response to the Environmental Stimulus

Graft unions of a vernalized donor which induced flowering in an unvernalized receptor of the biennial *Hyoscyamus niger* indicated the stimulus is transmittable. Several cold-requiring plant species have since shown successful flower induction by grafting (Lang 1965). However the grafting experiments were restricted to plant species requiring vegetative growth before being receptive to the vernalizing treatment. A transmittable floral stimulus of the photoresponse has been shown by numerous grafting experiments (Chailakhyan, 1968; Lang, 1965). Possibly the stimulus transmitted in the graftings was the stimulus due to the photoresponse. The vernalization response could be a nontransmittable local effect which enables expression of the transmittable photoresponse.

Crude extracts from vernalized wheat and radish plants caused enhanced flowering in unvernalized winter wheat seedlings (Tomita, 1968). The extracts were squeezed from shoot apices of vernalized seedlings grown under photo-inductive conditions. From this method it would be difficult to distinguish whether the extracts contained a vernalizing hormone or a transmittable flowering response whose formation, perhaps was enhanced as a result of the cold treatment. Redshaw (1968)

using aqueous extracts isolated from the vernalized rye seedlings immediately following the chilling period and applied to young unvernalized winter rye seedlings reported no enhancement in flowering.
Tomita (1968) reported nucleotides were effective when injected into the cavity of the empty seed sac of unvernalized winter cereals in the third leaf stage. In contrast to Tomita's findings Trione and Metzger (1971) using 4 wheat cultivars and 3 commercial preparations of nucleotodies similar to those used by Tomita found the nucleotides were ineffective as substitutes for chilling.

Of the hormones and physiologically active compounds known to be present in plants, gibberellin has received the most intensive study concerning hormonal influence on flower formation. As vernalization appears to enable or enhance the plants ability to receive the photoperiodic stimulus this review on gibberellin will also include its influence on the photoperiodic response. In most plants regardless of their daylength requirement for floral induction, the content of gibberellin was always higher under long day conditions when compared to short day (Chailakhyan, 1971). Long day plants grown under short day conditions were induced to flower by an application of gibberellin. However gibberellin did not enhance flowering of short day plants. Chailakhyan (1971) showed that flowering in long day plants was always preceded by stem elongation. He suggested the 'florigen complex' is composed of two growth substances, gibberellins necessary for formation and growth of stems and 'anthesins', substances necessary for flower formation. At present there is no direct evidence for the presence of

anthesins (Chailakhyan, 1971). Studies on the replacement of the required cold treatment with gibberellin preparations showed that under long day conditions gibberellin could induce stem and flower formation in unvernalized one year old plants of the biennial species of henbane, carrot, parsley, turnips, cabbage as well as seedlings of winter forms of rape and lettuce (Lang, 1957). However unlike the photoperiodic effect where gibberellin could replace the long day requirement, gibberellin application failed to induce flowering in the biennial unvernalized species grown under shortday conditions. Gibberellins were also not effective in enhancing flowering in unvernalized rye seedlings, until the seedlings had reached the 9-10 leaf stage (Purvis, 1961). In a comparative study on the content of natural gibberellins Chailakhyan (1968) showed the leaves of the spring forms of wheat, rye and rape were higher than the corresponding winter forms when grown under long days. The content of gibberellin in the leaves of winter forms which had been vernalized approached that of the spring forms. However, the content of spring, winter and vernalized winter varieties were all similar when grown under short days. With this evidence Chailakhyan (1968) suggested there was no formation or accumulation of gibberellins during vernalization, but vernalization is a preparatory process which results in the formation of possible gibberellin precursors. Others (Lang, 1965; Evans, 1971) have suggested that gibberellin may not be involved in the actual thermoinduction process, but may in some way be associated with the state in which the process establishes itself. This is thought to explain the lack of a

promoting effect of gibberellin when it is applied during seed vernalization of winter cereals (Lang, 1965).

Biochemical changes induced by exposure of seedlings to chilling temperature could also be included as an environmental stimulus, these will be discussed later.

2. Nucleic Acids and Vernalization

Studies by Bonner (1965) and Varner *et al.* (1965) suggest differentiation in higher plants results from shifting in patterns of gene repression and derepression. Bonner demonstrated globulin synthesis in pea seedlings is localized in cells differentiated as cotyledon tissue. The repression of DNA template activity specific for globulin synthesis in root and stem tissues was suggested to be due to the binding of specific proteins, histones, to DNA rendering the genes inactive for transcription. The induction of α-amylase synthesis by the application of gibberellic acid results in an enhancement of RNA synthesis (Varner *et al.*, 1965). Orthophosphate-^{32}P incorporation studies showed an enhanced synthesis of specific RNA fractions suggested to be mRNAs. Both mRNA and α-amylase synthesis was inhibited by the application of actinomycin D and nucleic acid base analogs. Loening (1962) demonstrated the presence of messenger-like RNA in higher plant systems and with the observation that genetic information for protein synthesis is carried by mRNA Loening (1968) expressed hope that the identification of specific messengers in higher organisms would be a means of understanding control mechanisms in cell differentiation. Review articles by Lang (1965a), Heslop-Harrison (1967) and Tsanev and Sendov (1971)

suggested that cells in multicellular organisms retain their full genetic potential during growth and differentiation. Thus, development would result from a shift in template activity.

The transition from vegetative to reproductive growth must at some stage involve a shift of genetic expression (Evans, 1971). Environmental influences on the transition are most noted for the vernalization and photoperiodic responses. The effect of both responses appears to be in the conditioning of the apex which enables a change in the developmental pattern of future growth. The leaves are the main receptors of the photoresponse and the stimulus in turn is transmitted to the apex. The actively dividing meristematic cells have been shown to be a requirement for the chilling response (Wellensiek, 1964).

Studies on nucleic acid metabolism relative to flower induction have shown an enhanced RNA synthesis and accumulation at the apex when activated by either stimulus. Studies on the 'conditioning' effect of the photostimulus were aided with the discovery of plants requiring single inductive daylength cycles (Bernier, 1963; Bernier *et al.*, 1970; Bronchart *et al.*, 1970). Labelled precursors for RNA synthesis applied to the apex during the approximated time of arrival of the photostimulus showed a general enhanced synthesis of all RNA fractions. Application of inhibitors to RNA synthesis (actinomycin D) and base analogs which result in fraudulent RNA (2-thiouracil) inhibited flowering when applied during this activation period (Evans, 1971; Bernier *et al.*, 1970; Bronchart *et al.*, 1970). Autoradiography studies showed the stimulated RNA synthesis was general throughout the apex area, occurring in leaf primordia as well as flower primordia

(Bronchart *et al.*, 1970). This general stimulation of RNA synthesis led Evans (1971) to suggest the arrival of the photostimulus at the apex results in an overall stimulation of the metabolic activity in the area.

The vernalizing response was suggested to be a direct effect on the meristematic regions (Heslop-Harrison, 1967). The vernalized state is progressively acquired through prolonged chilling. By measuring orthophosphate-^{32}P uptake into nucleic acid fraction, Jones *et al.* (1971) reported an increased synthesis of nucleic acid in germinating embryos of wheat seedlings compared to spring seedlings when both were grown at low temperatures. Previously Konarvo (1954) reported an increased synthesis and accumulation of RNA during vernalization. Dévay (1965) reported ribonuclease (RNase) activity with a pH optimum of 5.6 localized in the apical region which reached an optimum level during the first 2 weeks of cold treatment. Babenko *et al.* (1971) also report the accumulation of acidic RNase activity during the initial stages of vernalization. Philips and Fletcher (1969) suggest RNase activity is correlated with the rate of turnover of RNA rather than the absolute levels present in the tissue. Whether this acidic RNase indicates a 'turnover' of RNA specific to the vernalizing process, or if during vernalization RNA synthesis characteristic for vegetative state of the plant is inactivated is hypothetical.

Teraoka (1967), using polyacrylamide disc electrophoresis for protein separation, demonstrated that as the winter wheat embryo acquired the vernalized state the soluble protein fractions increased and approached the composition of the spring variety. Initial studies

on the histone content of winter wheat embryos showed a change in their chromatography pattern in the process of vernalization. Teraoka (1967) suggested the possibility of histone suppression of gene action. However a further purification of the histone preparations isolated from spring, vernalized and unvernalized wheat embryos showed the changes in histones during vernalization were mainly quantitative (Teraoka 1968). The theory that histones act as gene repressors poses the question as to the nature of their specificity. It was shown that histones complex with RNA as well as DNA (Huang *et al.*, 1965). Some suggest (Clever, 1961; Allfrey *et al.*, 1963) histone removal is effective in derepression by exposing large segments of DNA for transcription and the transcription would be under the control of more refined devices. Others (Bonner and Huang, 1964) suggest histones play a more specific role in repression of template activity and suggest specific binding to base sequences in DNA. Repression and derepression would require a qualitative changes in histone content. Teraoka (1968) does not exclude the possibility that minor qualitative changes noted in the histone components are not associated with the vernalization process.

3. Metabolic Changes During Vernalization

Changes in the chemical composition of plants during growth at low temperatures have been reported by many. Possibly due to the adaptive ability of winter cereals, those which exhibit hardiness to cold temperatures also display a vernalizing response. The active meristematic regions of the plant are shown to be receptive to the chilling stimulus (Purvis, 1940; Wellensiek, 1964). In a review on cell

culture studies Heslop-Harrison (1967) suggested that cells lose their differentiated state during mitotic division. The fate of the newly divided cells was suggested to be determined by extra-nuclear factors present at the time, or in multicellular systems by neighboring cells through intercellular connections. This theory was based on culturing studies. If single differentiated cells were cultured they returned to the ground state upon a few cell divisions, however, if clones of differentiated cells were cultured the differentiated state persisted through many although limited cell generations. Heslop-Harrison (1967) suggested this persistance was maintained by growth factors and nutrients originally present within the clone environment. Differentiation at the shoot apex was suggested to be dependent on nutrient substances moved from the more mature areas (Wardlaw, 1965). Ste-Marie and Weinberger (1970, 1970a) reported no accumulation of carbohydrates and amino acids in the root apex area, the main accumulation occurred in the more mature region of the root.

The vernalization process has similarities to the adaptive ability of cultured bacterial cells when transfered to a new medium. Following the transfer of bacteria to a new medium several subcultures on the new medium are required before growth reaches an optimum level (Dean and Hinshelwood, 1964). Once the strain has adapted to the new medium transfer back to the original medium would again require an adaptive time interval. However, after a relatively short period on the new medium, the condition was easily lost when the bacteria were transferred back to the original conditions. Dean and Hinshelwood (1964) suggested a direct correlation between the stability of the adaptive

condition and the time interval of growth on the new medium. Similarly, the vernalized state is gradually acquired through prolonged chilling. Partially vernalized plants become devernalized when subjected to higher temperatures (Purvis, 1961; Lang, 1965). Also the stability of the vernalized state increases with the duration of the chilling treatment (Friend, 1953).

The initial research to determine the requirements for vernalization of cereals by Gregory and Purvis as reviewed by Purvis (1961) indicated that the plant must retain a metabolically active state during the chilling requirement. Conditions which suppressed growth, such as, short day (Purvis, 1961) and high pressure (Tomita, 1964) were reported to partially replace the cold treatment requirement. This led Evans (1971) to suggest a possible conditioning effect on the apex. Conditions which make assimilates readily available will enhance flowering. However, Trione and Metzger (1971) did not show an enhancement due to high pressure treatment.

Several attempts have been made to relate specific biochemical changes to the vernalization mechanism. However, the plant material analyzed has not been consistent among the different researchers. Dévay (1962) vernalized imbibed seeds and analyzed the embryo and endosperm at various stages of vernalization for carbohydrate fractions. Trione (1966, 1967) analyzed green leaf material of 6 day old seedlings which were vernalized in the light. The lower leaves were collected at intervals during the chilling period and analyzed for carbohydrates, soluble protein and free amino acids. In Weinbergers' studies plants were subjected to various periods of chilling and then grown at normal

temperatures prior to sample collection. Comparisons in the growth patterns and chemical composition were made between those receiving and those not receiving previous chilling. The first three leaves were harvested for analysis of soluble nitrogen compounds (Weinberger and Godin, 1966). Ste-Marie and Weinberger (1970, 1970a) analyzed the carbohydrate and free amino acid content of the first primary root. Jones and Weinberger (1970) analyzed the imbibed unvernalized and fully vernalized embryo and endosperm for protein and free amino acid content.

a. Carbohydrates

Analysis on samples collected during the chilling period suggest a relationship between carbohydrate metabolism and vernalization (Dévay, 1962; Trione, 1966). Both reported that the winter varieties accumulated more soluble carbohydrates than spring varieties when grown at low temperatures. Dévay (1962) using several varieties with varying chilling requirements reported the extent of the accumulation was dependent on the vernalizing requirement. The longer the chilling period required the larger the accumulation. Dévay (1962) reported amylase and maltase activity in the winter varieties at low temperatures between 0 and 5°C but little activity in the spring variety at the low temperature. She suggested the increase in soluble sugars during the cold treatment was due to depolymerization and transport of storage polysaccharides.

Culturing studies on excised embryos by Purvis showed that sugars could replace the endosperm. Of the carbohydrates tested fructose,

sucrose and ribose gave the best response. A culture medium containing 2% sugar gave the optimum response. Trione (1966) reported a slightly higher accumulation of fructose, a large accumulation in sucrose and a very marked increase in oligosaccharides in the winter variety as compared to the spring when grown at chilling temperatures. In cold-hardiness studies Babenko and Gevorkyan (1967) reported a direct relationship in oligosaccharide content and frost resistance. Studies by Green (1972) on sugar induced frost-tolerance suggested frost-resistance is more than a simple osmotic effect. Incubation with mannitol increased the osmotic pressure but had little effect on frost-hardiness. Purvis (1944) reported little enhancement of flowering when embryos were cultured with mannitol or mannose medium. However, in contrast between frost-tolerance and vernalization Green (1972) reported an increase in frost-hardiness when plants are grown on a sugar enriched medium at normal temperatures. Purvis (1944) indicated embryos cultured on sugar enriched medium at normal temperatures showed no enhancement in flowering.

b. Proteins and Amino Acids

Soluble nitrogen components were found to increase during growth at low temperatures. Discrepancies as to the nature and extent of accumulation appear in the literature. Two theories appear to be evident, one would be the winter or hardy variety response to low temperature with a rapid accumulation of low molecular weight metabolites. These are suggested to increase the viscosity of the cell sap and prevent the extreme desiccation common to frost damage. Another theory

would be the winter or hardy varieties exhibit higher respiratory activity than the cold-sensitive and spring varieties. An accumulation does occur in the latter instance however the nature of the compounds accumulated differ between the hardy and sensitive varieties.

The discrepancies may be partially accounted for if consideration is given to the portion of the plant analyzed, and the method of presenting results. Zech and Pauli (1962) were able to show a high association between the amount of soluble protein accumulated and the cold-tolerance of winter wheat plants if crown tissue was analyzed but variability occurred if leaf material was sampled. Pauli and Mitchell (1960) suggested that comparisons based on fresh weight of sample may be in error because of the differing moisture contents which occur between varieties at normal and lower temperatures.

Trione (1966), sampling lower leaves and basing analysis on sample fresh weight reported an increase in soluble protein content in the winter wheat varieties when compared with spring wheat varieties grown under the same low temperature conditions. Others (Pauli and Mitchell, 1960; Gerloff *et al.*, 1967; Jones and Weinburger, 1970) did not show a varietal difference, although the soluble protein fraction did increase during growth at low temperatures. Pauli and Mitchell (1960) based their studies on total nitrogen, the others were on a dry weight basis.

Pauli and Mitchell (1960) reported an increase in total free amino acids in winter wheat varieties during the first two weeks of cold treatment. This accumulation and the degree of frost-tolerance declined upon the return to normal growth temperatures. Specific

increases in some amino acids occurred as winter hardiness developed in alfalfa roots Wielding *et al.*, 1960). Arginine for example increased 246% in the non-hardy variety and 363% in the hardy variety. Similar changes were noted for alanine. Trione *et al.* (1967) comparing spring and winter wheat varieties and sampling at intervals during the vernalizing process reported an increase in alanine, glutamine, proline and arginine, with the proline increase being outstanding. Jones and Weinburger (1970) also reported an accumulation of proline as well as the amides asparagine and glutamine in vernalized winter wheat embryos. Choline and betaine derivatives used to increase frost resistance of potato varieties, were noted to intensify the accumulation of free amino acids especially proline, serine and glycine in the potato leaves (Bokarev and Ivanova, 1971). Leaf samples from the more resistant potato variety had a concentration of proline 1.5 times that of the less resistant variety studied (Bokarev and Ivanova, 1971).

Analysis of protein and free amino acid content of both endosperm and embryo suggest hydrolysis of the storage protein in the endosperm and some transport of free amino acids to the embryo (Sane and Zalik, 1968; Jones and Weinburger, 1970; Beletskya, 1971). In a comparison of the temperature effect on amino acid accumulation during germination of maize hybrids Beletskaya (1971) showed a higher accumulation of free amino acids in both the endosperm and embryo during germination at 10°C than at 20°C. The extent of the accumulation was less pronounced in the hardy variety. She suggested these results indicated the lower accumulation was not due to reduced hydrolysis and transport of storage protein, but the free amino acids were utilized

more readily in the cold-resistant hybrid. Proline, although not the major amino acid present, occurred in a considerably greater amount in the hardier maize variety.

The high accumulation of proline in winter and hardy varieties has resulted in some speculation as to its functional role. It has been hypothesized that due to its high hydrophilic nature its accumulation during periods of drought or low temperatures serves a protective role preventing extreme desiccation. Savitskaya (1967) was unable to relate moisture content and proline accumulation in studies with barley grown under drought conditions. Singh *et al.* (1972) reported the leaf water potential was similar for all barley varieties studied when the plants were subjected to a water deficit, however proline accumulated at much higher levels in the drought resistant varieites. Proline has been reported to considerably stimulate respiration in leaf tissue (Britikov and Linskens, 1970). The accumulation of proline in plant tissue is not unique to growth under low temperature or drought conditions but is also present in tissues prior to the beginning of intensive growth and differentiation processes (Britikov and Linskens, 1970).

The effect of protein and amino acid accumulation on plant development is not known. Early vernalization studies on cultured rye embryos by Purvis (1944) showed an enhanced response with the addition of nitrate to a concentration of 2mM in the culture medium. The replacement of nitrate with other sources of nitrogen; ammonium sulfate, urea, asparagine and glycine gave similar responses.

c. Lipids

Few studies have been concerned with lipid changes during vernalization. Redshaw and Zalik (1968) reported notable changes in lipids during the chilling period, the proportion of the polar lipids present in the total lipid fraction increased, with a corresponding decrease in the non-polar lipid fraction. An overall increase in the percent of linolenic (18:3) present in the total fatty acid fraction occurred in both lipid fractions. Comparisons were made on spring and winter varieties of wheat and rye. In their study germinating seedlings containing both endosperm and embryo were collected at weekly intervals during the vernalization process and analyzed. However, the noted lipid changes occurred in both varieties and the response was attributed to the low temperature conditions and was suggested not to bear any direct relationship with the vernalization process.

Several studies on the changes in lipid content of plants associated with a variation in temperature have attempted to relate the findings to cold sensitivity. However non-temperature induced lipid changes normally occur during development. A brief discussion of these would be of value in interpreting temperature induced changes.

During the greening of etiolated plants a large increase in the portion of galactolipids occurs (Trémolières and Lepage, 1971; Roughan and Bordman, 1972). Fatty acid analysis revealed a high accumulation of linolenic acid (18:3). Galactolipids originally present in minor quantities in etiolated plants were high in 18:3. The noted increase in 18:3 in greening tissue was suggested as being due to the overall increase in the galactolipid portion (Trémolières and Lepage, 1971).

During the ripening of grains of corn (Weber, 1969; 1970), and oats (Beringer, 1971), and during the maturation of alfalfa leaves (Klopfenstein and Shigley, 1967) an increase in the saturation level of fatty acids has been noted. Specific changes in the lipid components during the maturation of corn found by Weber (1969) were an increase in the non-polar lipid fraction and a corresponding decrease in the polar lipid fraction. Triglycerides constituted 10-17% of the total lipids 10 days after pollination and increased to 75-92% by 75 days. The polar lipid fraction represented 70-72% at 10 days and 4-12% at 75 days.

Harwood and Stumpf (1970) showed that during the early stages of germination the new fatty acids synthesized do not show any relation to the fatty acid composition of the mature seeds. Studies on germinating pea seeds showed palmitic (16:0) and stearic (18:0) were the first fatty acids synthesized during the first 20 hours of germination. The majority of these fatty acids were found in the phospholipid fractions, which in turn were localized in the particulate fraction (Harwood and Stumpf, 1970).

Low temperatures were noted to increase the unsaturation of fatty acids. This has been shown by Harris and James (1969, 1969a) to be due to the increase in the available oxygen, which is the rate limiting factor for desaturation. The absorption coefficient of oxygen in water (volume of oxygen at standard conditions, 760mm and 0°C, dissolved in one volume of water) is 0.0440 at 4°C and 0.0261 at 30°C (Lange, 1967). Flax seeds which contain chloroplasts in their seed tissue, intact green algal cells and green leaf material did not show an increase in the synthesis of unsaturated fatty acids with a decrease

in temperature. However bulb tissue, safflower and castor bean seeds responded to low temperature with an increased synthesis of unsaturated fatty acids. Incubation of these seeds and plants in an oxygen enriched environment at normal temperatures resulted in an increase in unsaturated fatty acid synthesis in the non photosynthetic tissue. Tissues containing chloroplasts did not respond. James and Harris (1969a) suggested that the photosynthetic tissue produced an excess oxygen which was required for desaturase activity.

Subjecting oat plants to low temperature during seed maturation was shown to influence the unsaturation level of fatty acids in seeds both directly and indirectly (Beringer, 1971). Low temperatures slowed the maturation process, plants kept at 12°C were less mature and synthesized more unsaturated fatty acids than plants kept at 28°C. A direct effect was noted if seeds at the same stage of maturity and grown under the same temperature conditions were incubated with $^{14}CO_2$ and then subjected to 12°C or 28°C for 2 hours. Seeds kept at the higher temperature had high labeling in the 16:0 and 18:0 fractions, while in those at 12°C the activity was associated mainly with the 18:1 fraction (Beringer, 1971). Stearic acid (18:0) was suggested to be a precursor for oleic acid (18:1) in higher plants (Gurr 1971). Also labeling studies by Huber and Zalik (1963) suggested the more unsaturated fatty acids were formed from less unsaturated ones during lipid synthesis in developing flax seeds. In contrast to Beringer's study Kuiper (1970) reported no increase in the level of fatty acid unsaturation with a decrease in temperature when studying alfalfa leaves grown at 15 and 30°C. However the samples were collected from green photosynthetic

tissue which according to Harris and James (1969, 1969a) would produce oxygen in excess of that required for desaturase activity. Kuiper (1970) did report a change in polar lipid components. When comparing a hardy alfalfa variety with a cold sensitive variety, the hardier variety showed a higher content of MGDG, DGDG, Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). These lipid components increased in both varieties as the temperature decreased. Phosphatidyl glycerol, phosphatidyl inositol and sulfolipid were higher in the cold sensitive varieties. These lipid components increased in both varieties as the temperature increased.

Some interesting studies by Lyons and coworkers suggested that the composition of the lipid moieties present in the mitochondrial membrane depicted the chilling resistant nature of the plant. Fatty acid analysis on mitochondria isolated from cold-resistant and cold-sensitive plants showed the fatty acids from the cold resistant to be more unsaturated (Lyons *et al.*, 1964). Also the mitochondria from the resistant forms showed a greater flexibility, they were able to readily swell or contract when placed in hypotonic, hypertonic or salt solutions (Lyons *et al.*, 1964). Arrhenius plots of the respiratory rates showed a linear decrease with a decrease in temperature from 25 to 1.5°C for the cold resistant mitochondria. The cold-sensitive showed a marked deviation in the slope at approximately 9-12°C with an increase in the slope as the temperature approached 1.5°C (Lyons and Raison, 1970). Arrhenius plots for the activity of mitochondrial respiratory enzymes also showed a discontinuity for the cold-sensitive varieties (Raison, Lyons and Thomson, 1971). The authors suggested the discontinuity

was due to a phase change and a possible involvement of membrane lipids. Previous work by Lyons and Asmundson (1965) showed that an increase in the mole % of unsaturated fatty acids beyond 60% markedly decreased the freezing point of the mixture when comparing fatty acid mixtures of 16:0 and 18:2, and 16:0 and 18:3. Disruption and fragmentation of the mitochondrial membrane did not affect the discontinuity of the Arrhenius plots for enzyme activity. A change in membrane permeability to enzyme substrates was thought unlikely to explain the phenomenon (Raison, Lyons and Thomson, 1971). However treatment with detergent (Triton X-100, 0.1% v/v; sodium deoxycholate 0.05%) resulted in a continuous linear plot for all respiratory enzyme systems studied in the cold-sensitive varieties. References cited by the authors suggested that the detergents acted by splitting the membrane bilayer longitudinally. The action of the detergent is suggested to disperse the enzymes embedded within the membrane. Raison, Lyons and Thomson (1971) suggested that the temperature induced change in enzyme activity was likely associated with a temperature induced change in the physical state of the membrane lipids.

SECTION I

EVALUATION OF THE VERNALIZATION PROCESS

Materials and Methods

A. Plant Material

Two rye varieties were used in this study, Sangaste winter rye which exhibited a vernalizing response and Prolific spring rye which did not show an enhancement in flowering when subjected to the corresponding chilling period.

The Sangaste seed was received from Mr. R. Berg, Department of Plant Science, University of Alberta. The Prolific seed was Grade No. 1 Canada Seed purchased from Canwest Seed Company, Edmonton.

B. Vernalizing Conditions

The seeds of both varieties were surface sterilized with 2.6% sodium hypochlorite (Perfex) for 10 minutes, rinsed in glass distilled water, then soaked for 12 hours in the dark at 24 ± 3°C. Following this the seeds were transferred to a plexiglass germination box layered with moist filter paper and germinated in the dark for a further 36 hours. Any seeds which had not germinated after this period were discarded. The box with the germinated seeds was then placed in a dark cold room at 4 ± 2°C. At weekly intervals samples were collected and planted in 17.5 cm pots in a mixture of loam: peat: sand of 3:2:1. The pots were placed in a growth chamber at 20°C, 55% relative humidity and 1100 foot-candles of continuous light. The vernalizing response was evaluated by recording the time required for

heading. The time of heading was taken as the first visible extrusion of ears on the main shoot of each plant.

Results and Discussion

The effect of the length of the cold treatment on the time required for heading is shown in Figure 1. The unvernalized Sangaste variety did flower when grown under continuous light, however it required an extended growing period of 200 days. An increase in the chilling time for the Sangaste variety resulted in a corresponding decrease in the time required for heading. This enhancement in heading was most notable between 2 and 7 weeks of cold treatment. The spring variety showed no enhancement in flowering as a result of the chilling treatment, it headed consistently between 40-45 days following transfer to the growth chamber.

If total days from germination to heading were considered, rather than days from planting, the chilling treatment showed a definite decrease in the flowering time of Sangaste (Fig. 2). This enhancement was noted for the first 7 weeks of the cold treatment. Early development in the spring variety appeared to be completely halted during the chilling period. An increase of approximately 1 week in the time required for flowering was noted for each additional week of cold treatment.

The growth stage of the seedlings which were collected for the planting-out experiments and for the analysis conducted in Sections II and III of this study are shown in Figure 3. The seedlings exceed the growth stage which would enable them to be machine sown using

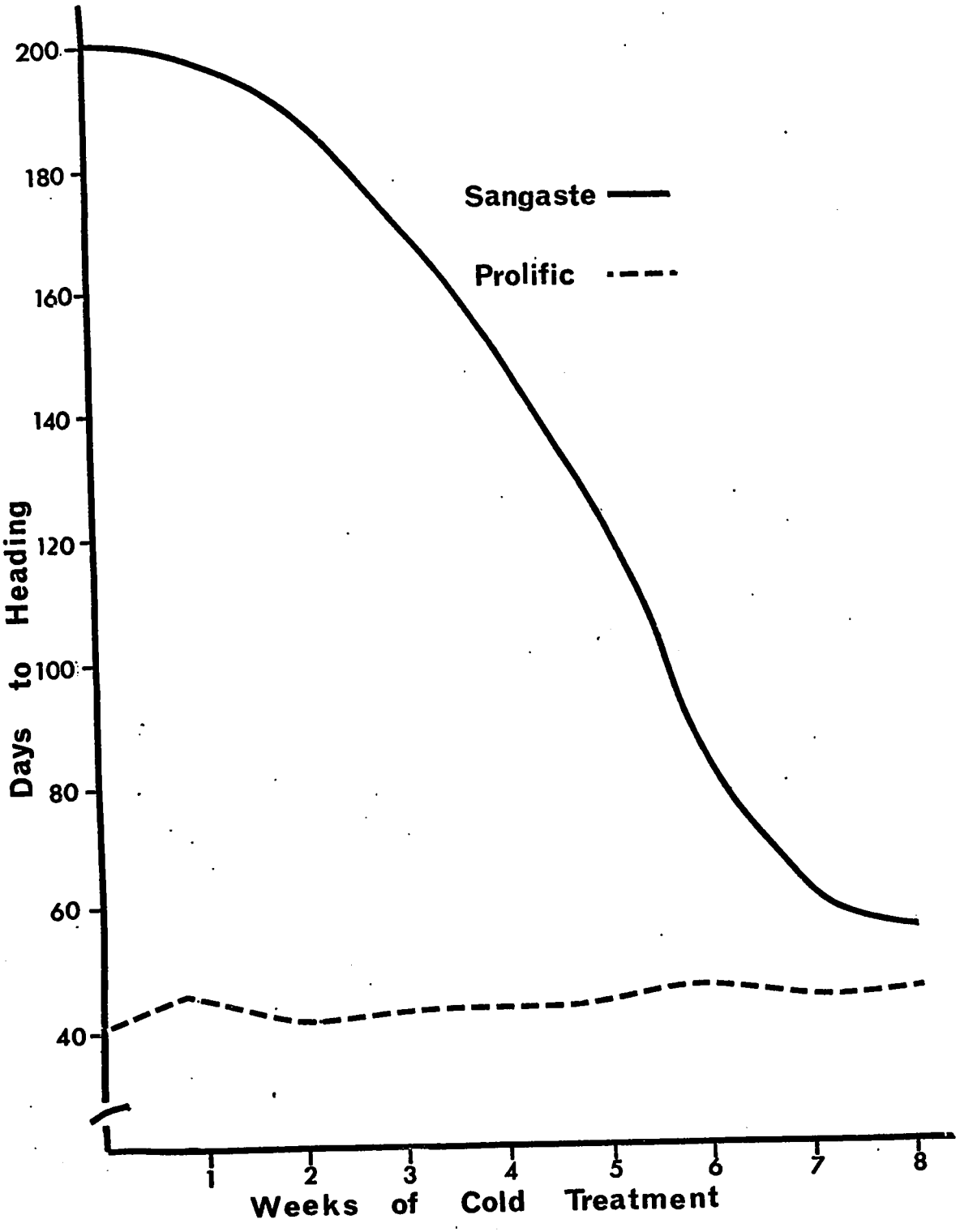


FIG. 1. The effect of the length of the chilling treatment on the time required for heading of two rye varieties, recorded are the number of days from the end of the chilling period to heading.

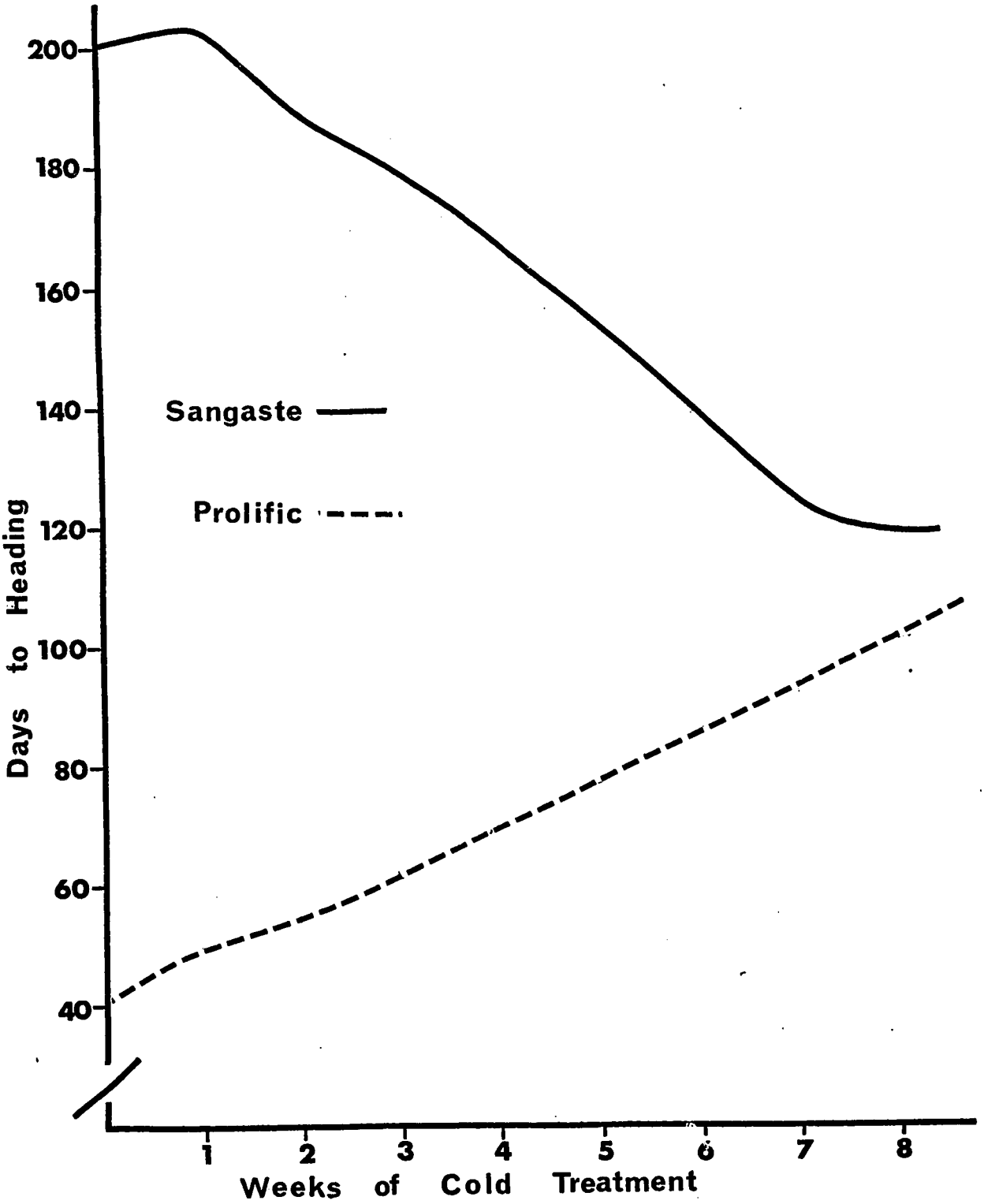
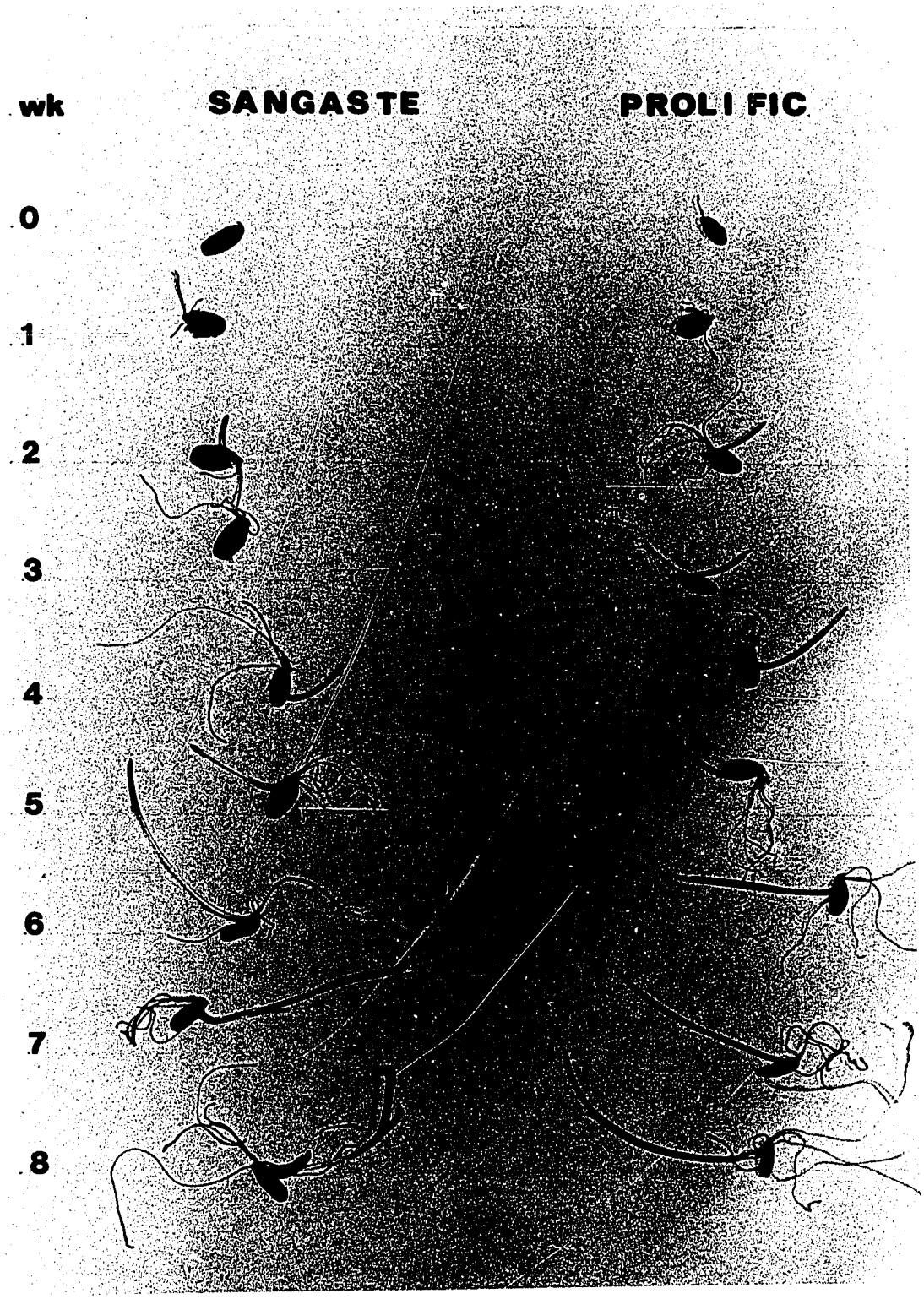


FIG. 2. The effect of the length of the chilling treatment on the time required for heading of two rye varieties, recorded are the total number of days from germination to heading.

FIG. 3. Photograph showing the growth stage of the rye seedlings collected at weekly intervals during the chilling period.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PROLI FIC
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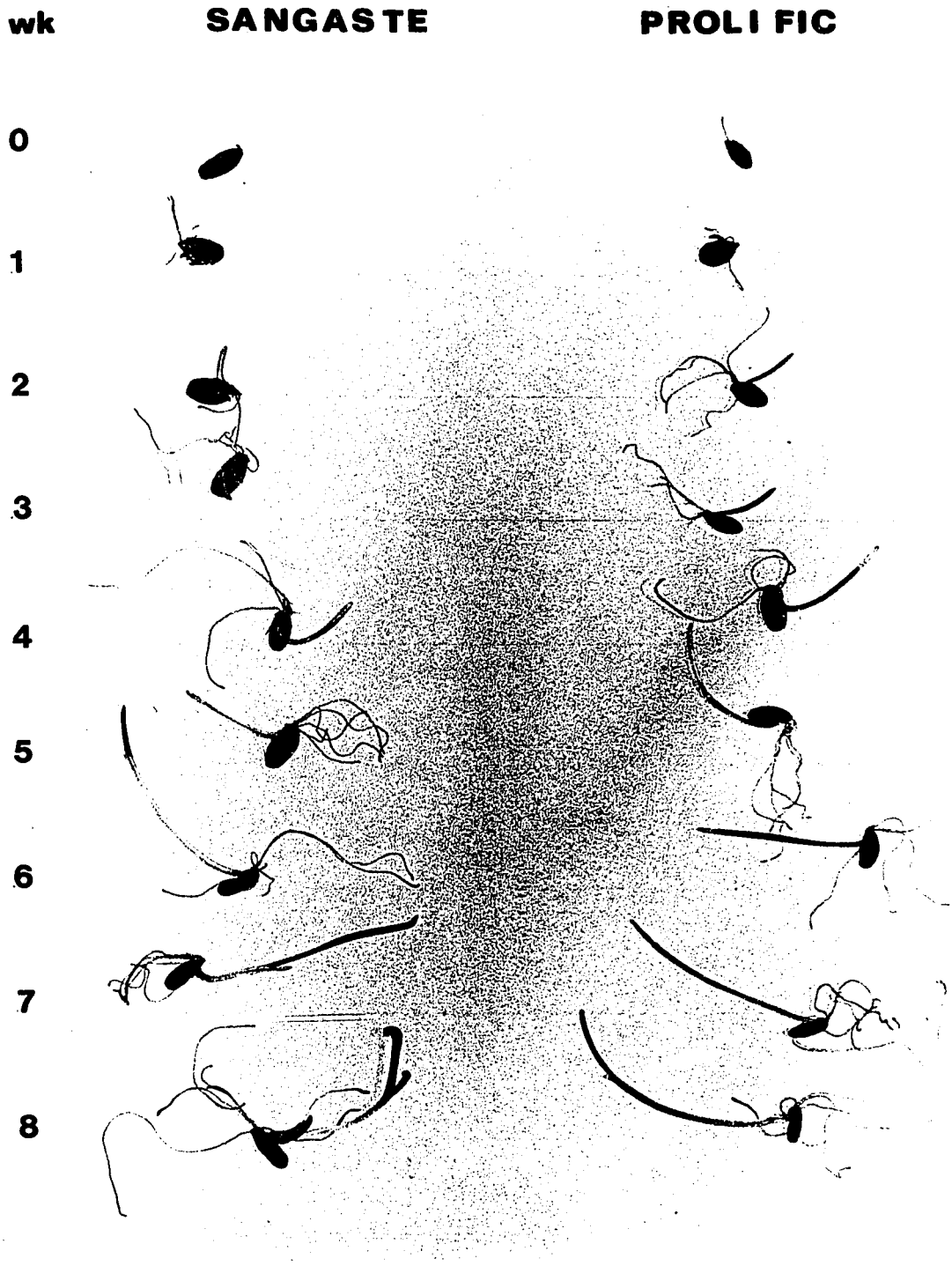
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the method devised by Lysenko. However Purvis (1961) indicated Lysenko's method of restricting growth by reducing the moisture content was not a requisite condition for the vernalizing process. She reported vernalization was equally effective at higher moisture levels providing the growth rate did not deplete the carbohydrate supply of the endosperm. Early growth of the seedling was desirable for this study to facilitate separation of the embryo from the endosperm as the embryo was used for the analysis conducted in Sections II and III. A comparison of the growth rate of spring and winter variety during the chilling period revealed an enhanced development of the winter variety (Fig. 3). By 8 weeks of cold treatment the Sangaste variety had reached the two-leaf-stage while the first leaf appeared to be just emerging from the coleoptile sheath in the spring variety.

One of the disadvantages in using the number of days required until heading to evaluate the vernalizing effect is that the time required for heading is influenced by other factors, the most noted being the daylength. The plants in this study were grown under continuous light because Gott *et al.* (1955) had reported that flower initiation was enhanced under continuous light when comparisons were made with similar plants grown under long day and short day photoperiods. In their study initiation occurred for both the spring rye and the vernalized winter rye following 2, 3 and 7 weeks of growth under continuous light, long day and short day respectively. For the unvernalized winter rye initiation occurred at 8, 15 and 12 weeks

respective to the above conditions. A photoperiodic effect on flower initiation was also noted by Purvis (1961) who revealed that if spring, unvernalized and vernalized winter rye plants were subjected to short days for different periods followed by long days only the heading of the unvernalized winter rye variety was enhanced by the short day treatment. Purvis (1961) also showed the spring and winter rye did not head when grown under short day (10 hour). Upon dissection of the apical region she noted the ears had differentiated but died before emerging from their protective sheath. It appears from the studies of Gott *et al.* and Purvis that prior to flower differentiation unvernalized winter rye responds as a short day plant but following differentiation long days are required for stem elongation and the following heading processes; whereas the spring and vernalized winter varieties respond as long day plants throughout their developmental sequence. This optimum enhancing effect by continuous light on flower induction of unvernalized rye noted by Gott *et al.* is surprising since short days showed a greater enhancement than long days. However Evans (1971) in a review on the role of phytochrome on flower induction suggests that flower formation in both short day and long day plants may be enhanced under continuous light depending on the red/far red ratio of the light source. In this study growth under continuous light was thought to minimize any photoeffect which may influence the heading time. No attempt was made to determine the red/far red ratio of the light source or the phytochrome content of the seedlings.

SECTION II

AMINO ACID CHANGES DURING VERNALIZATION

Materials and Methods

A. Plant Material

The seed material was vernalized as described in Section I. At weekly intervals samples of both varieties were collected and the embyros separated from the endosperm. The separation was carried out in a cold room at 4°C using a green safelight. The embryos, including scutellum, roots and shoots were frozen in liquid nitrogen, lyophilized and stored at -20°C until required.

To compare the changes noted during the cold treatment with changes which might normally occur during early seedling growth, seeds from both varieties were surface sterilized, soaked for 12 hours and grown in the dark at 24 ± 3°C for 4 days. The growth of the plants after 4 days at this temperature approximated the 3 to 4 week growth stage for the cold treated seedlings as shown in Figure 3.

B. Free Amino Acid Estimation

Amino acids were extracted from the embryo tissue with 80% ethanol (v/v). Approximately 0.3 grams of sample were homogenized using a Polytron (Brinkman instrument) with a PT-20ST (saw-tooth) generator. The homogenate was made to a volume of 25 ml and 3 ml aliquots were withdrawn in triplicate for nitrogen determinations by the micro-Kjeldahl method (official methods; AOAC, 1955). The remaining suspension was centrifuged at 10,000 rpm for 10 minutes. The super-

natant was decanted and the pellet resuspended in 15 ml of 80% ethanol (v/v) and recentrifuged. This procedure was repeated for a total of 3 extractions. The 4th extraction showed no color development when treated with ninhydrin. The combined supernatants from the three extractions for each sample were concentrated almost to dryness using a rotary vacuum evaporator maintained at room temperature. The sample was diluted with a sample diluting buffer pH 2.2 (see appendix for composition). The pH of the solution was adjusted to 2.0 and the final solution made to a volume of 5 ml. An aliquot of 300 µl was analyzed with a Beckman/Spinco amino acid analyzer. Neutral and acidic amino acids were separated on a 50 cm column packed with Beckman type custom research resin AA-15. The amino acids were eluted from the resin with a sodium citrate buffer, pH 3.25 followed by a pH 4.25 buffer. The basic amino acids were separated on a 10 cm column of Beckman type TA-35 resin using a citrate buffer at pH 5.28 (see appendix for composition of buffers). The columns were operated at 55°C and samples forced into the resin with nitrogen pressure. Sodium cyanide was added at a concentration of 0.00004 M to the eluting buffer just prior to use. This was used as a catalyst for color development. The flow rate of the eluting buffer was 68 ml/hr, and that of the ninhydrin reagent 34 ml/hr. The overall flow for the mixture through the reaction coil maintained at 100°C for color development was 102 ml/hr. The length of the reaction coil was such to allow a reaction time of approximately 7 minutes per ml. The optical density of the solution was measured at 440 and 570 nm and recorded on a Honeywell 3 channel, dot-type recorder. The area under the curves were measured by the

height x width method and calibrated using a standard mixture containing 0.5 μ moles/300 μl of each amino acid. For the unknown peaks an average calibration factor calculated from the acidic and neutral amino acid standards excluding proline and cystine was used.

With this procedure the amides glutamine and asparagine were eluted together with serine and threonine. To obtain resolution of these compounds a 1 ml portion of the sample in the diluting buffer was hydrolyzed at 110°C for 3 hours with 1 ml of 2N HCl. The acid was removed on a rotary vacuum evaporator, the sample collected with the sample diluting buffer, pH adjusted and made to a volume of 5 ml. This hydrolysis results in the conversion of glutamine and asparagine into glutamic acid and aspartic acid respectively. The amide content was then taken as the difference between the values for glutamic and aspartic acid obtained before and after hydrolysis (Miller, 1965).

C. Nitrogen Determinations

The total nitrogen content of the aliquots taken from the sample homogenate and the nitrogen content of the whole embryos based on the dry weight of the embryo were determined using the micro-kjeldahl method.

Results

The changes in the free amino acid content in the embryo during germination at vernalizing temperatures are shown for both varieties in Figures 4-26. The data are presented as micromoles of amino acid per millimole of nitrogen. Proline, alanine, glutamic acid, glutamine and asparagine were the major amino acids and amides present in the

embryos of both varieties following 36 hours of germination at room temperature (0 weeks). During the chilling period the content of proline, alanine and glutamine increased, reaching maximum levels at 3 to 4 weeks and then declined during the remainder of the cold treatment. They showed much higher accumulation in the Sangaste variety. Asparagine (Fig. 7) accumulated gradually in both varieties but no varietal difference was apparent. Glutamic acid (Fig. 8), although a major amino acid prior to treatment, gradually declined in content during the chilling period showing a similar trend for both varieties. Arginine (Fig. 9) showed a similar response to the change in glutamic acid content, however it was present at approximately half the concentration. Lysine, histidine and valine (Figs. 10-12) increased in concentration in both varieties during the treatment period with only histidine showing a slightly higher accumulation in the Sangaste variety. The other amino acids analyzed (Figs. 13-24) were present in low amounts and showed little variation during the vernalizing period. Two fractions were not identified with the standards used and are reported as unknowns 1 and 2. Unknown 1 (Fig. 25) was eluted immediately after ammonia, however it was present at a low concentration, approximately 5 μm/mmole N and showed little variation during the chilling period. Unknown 2 (Fig. 26) was eluted before aspartic acid; it showed a general decline in both varieties.

The content of the ethanol soluble amino acids and amides for both varieties at 3 weeks of cold treatment and at 4 days without chilling are shown in Table 1. At this stage of vernalization

FIGS. 4-26. Changes in the content of alcohol soluble amino acids isolated from embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period.
Sangaste ——— Prolific - - - - - - - - -

FIG. 27. Total nitrogen content determined by the micro-Kjeldahl method, of embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period.
Sangaste ——— Prolific - - - - - - - - -

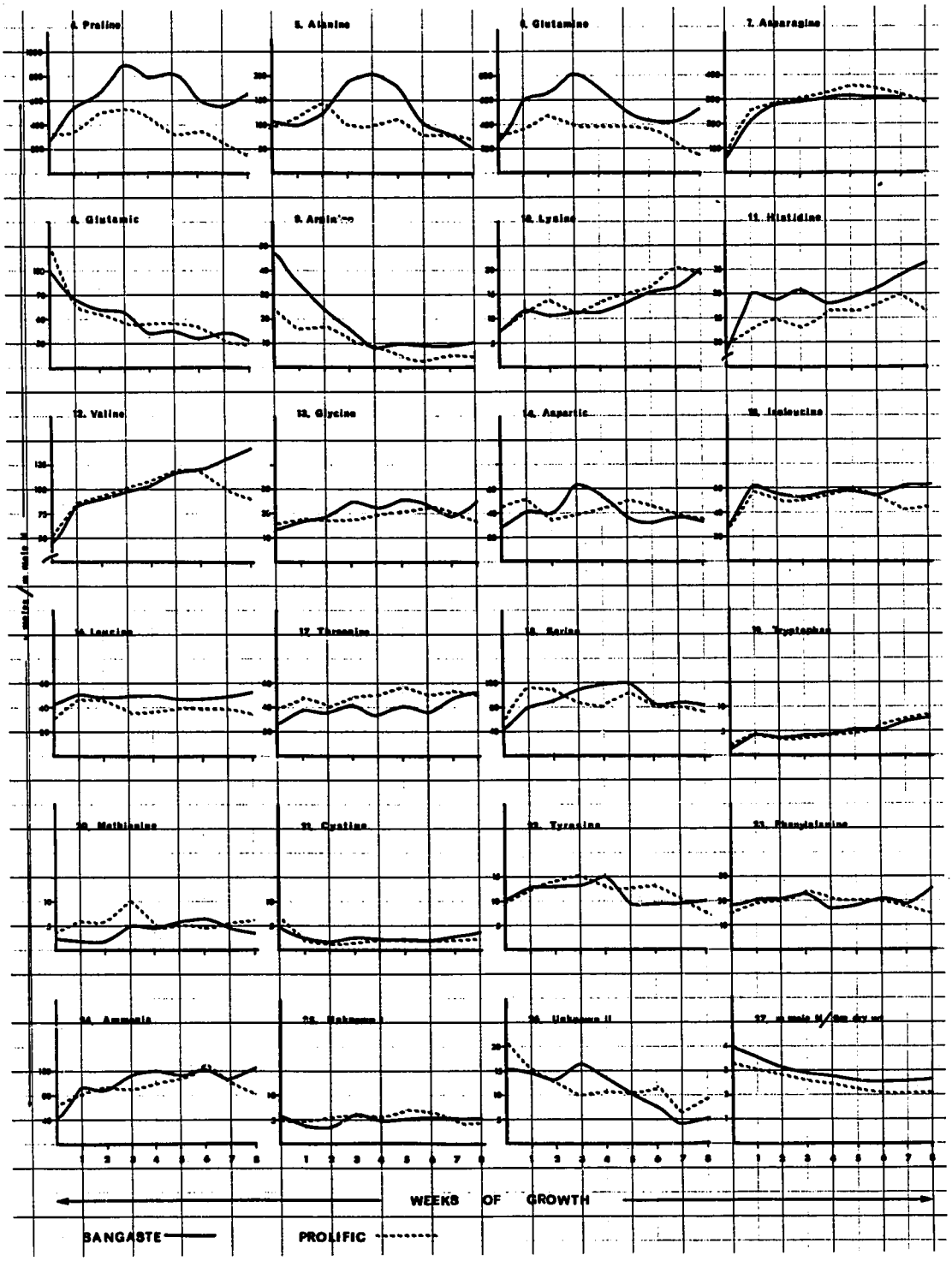

4. Proline
5. Alanine
6. Glutamine
7. Asparagine
8. Glutamic
9. Argin'
10. Lysine
11. Histidine
12. Valine
13. Glycine
14. Aspartic
15. Isoleucine
16 Leucine
17 Threonine
18. Serine
19. Tryptophan
20. Methionine
21. Cystine
22. Tyrosine
23. Phenylalanine
24. Ammonia
25. Unknown I
26. Unknown II
27. m mole N/gm dry wt
m mole N
1 2 3 4 5 6 7 8
WEEKS OF GROWTH
SANGASTE
PROLIFIC

Table I. Free amino acids of embryo tissue of rye seedlings grown at vernalizing temperatures for 3 weeks or at 24°C for 4 days.

		μ moles/m mole N					
Amino acid		4°C	24°C	Amino acid		4°C	24°C
Lysine	S	11.3	14.0	1/2 Cystine	S	2.6	2.3
	P	11.2	12.8		P	1.5	2.3
Histidine	S	31.8	27.6	Valine	S	98.8	86.5
	P	23.4	28.2		P	100.8	66.2
Ammonia	S	114.2	163.8	Methionine	S	5.9	3.4
	P	89.6	96.1		P	10.5	3.8
Arginine	S	16.0	6.6	Isoleucine	S	52.5	55.6
	P	11.0	2.3		P	50.2	48.3
Tryptophan	S	3.8	T	Leucine	S	49.0	49.9
	P	4.1	T		P	36.7	39.0
Aspartic	S	65.3	38.9	Tyrosine	S	12.8	7.7
	P	39.9	34.4		P	15.3	6.8
Threonine	S	43.7	45.0	Phenylalanine	S	24.7	11.9
	P	48.1	50.2		P	25.1	12.2
Serine	S	111.0	61.7	Unknown I	S	6.6	-
	P	87.1	62.4		P	6.0	-
Glutamic	S	72.5	148.8	Unknown II	S	17.2	T
	P	53.2	141.2		P	9.5	T
Proline	S	911.6	367.8	Asparagine	S	282.3	307.2
	P	566.6	284.3		P	303.6	304.2
Glycine	S	25.0	18.4	Glutamine	S	846.3	587.3
	P	17.4	15.5		P	397.8	343.3
Alanine	S	190.1	128.2				
	P	95.7	100.0				

S = Sangaste, P = Prolific, T = Trace

proline and glutamine show the highest accumulation in both varieties with Sangaste accumulating approximately double the level reached in Prolific. Although the growth state of the 4-day unvernalized seedlings approximated that reached by the 3 to 4 week chilled seedlings, differences in the content of some amino acids occurred between the two growing temperatures and there were varietal differences. Most noted differences are evident in the content of proline, glutamine, serine and alanine. These accumulated at much higher levels in the Sangaste variety grown at 4°C and in general the amino acid content of Prolific was less affected by the growing temperatures. Glutamic acid showed a marked higher level in both varieties grown at room temperature.

The total nitrogen content based on the dry weight of the embryo was noted to be higher for the Sangaste variety, however the level in both varieties showed parallel decline during growth at the cold temperature (Fig. 27). This decline is perhaps due to the increased accumulation of non nitrogen components which may include soluble carbohydrates and cell wall constituents.

Discussion

A. Methods

Initial results basing the amino acid content on the dry weight of the sample were erratic. An unknown amount of ethanol insoluble material was noted to precipitate on the polytron generator and glassware during the homogenizing procedure. It was found the results were reproducible if the calculations were based on the nitrogen

content and the nitrogen determinations were made on aliquots taken after the sample was homogenized.

Two replicates were used for each analysis, the difference between the replicates was within 10% in most cases. Differences between replicates were usually higher for the amino acids present in small amounts. Following the hydrolysis to obtain resolution of threonine and the amides, the proline content was determined and compared to the amount obtained before hydrolysis. The differences between the two determinations were within 10% and this was taken as evidence that either proteins and peptides were not solublized by the 80% ethanol or they were not hydrolyzed by the method used.

The seedlings were germinated and grown on filter paper in the dark in an attempt to minimize any variability not associated with the vernalizing response. Photosynthetic intermediates serve as a ready carbon source for the amino acids which may influence the free amino acid composition. Another reason for using etiolated seedlings was to keep the conditions comparable to those required for the lipid analysis (Section III).

Studies by Purvis (1944) indicated that the endosperm was not required for vernalization. She was able to vernalize embryos, which were excised from ungerminated grain on a culture medium containing a carbon and nitrogen source. For the study reported here the seedlings were vernalized intact so the endosperm would furnish the required nutrients. Although the chilling temperatures might affect the rate of hydrolysis and transport of the nitrogen metabolites from the endosperm (Beletskaya, 1971), any variation in the amino acids or

amides which may be active in the vernalizing response would likely occur in the actively growing embryo. For these reasons and also to avoid the masking effect of storage material from the endosperm, only the embryos were analyzed in this study.

B. Results

The unknown which was eluted immediately after ammonia (Fig. 25) was present in both varieties grown at 4°C, however it was absent in both varieties if they were grown at room temperature (Table 1). This is in agreement with the findings of Trione *et al.* (1967), for wheat, however unlike their findings the amount remained low in both varieties of rye and did not vary during the chilling period. Trione *et al.* noted the unknown reached maximum levels in the winter variety during the first 2 weeks then declined sharply and was absent by the 5th week. They suggested this disappearance may reflect the removal of an inhibitory substance but they also noted a similar response for the spring variety during the initial 2 weeks and an increase rather than decline at the 5th week. In this study it appears the formation of the unknown is a direct result of the low temperature treatment.

When the total amino acid and amide levels for the weekly intervals are tabulated (Table II) an increased accumulation is noted for both varieties grown at 4°C. For the Sangaste variety maximum levels are reached by 3 weeks, showing an increase of 108% in the free amino acid level and 228% in the amide content. These decline slowly during the remainder of the treatment period. The initial increase was less pronounced for the spring variety, at 3 weeks the amino acids and

Table II. Total free amino acids and amides of embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period and a comparison with seedlings grown for 4 days at 24°C.

	μ moles/m mole N									
Weeks	0	1	2	3	4 day	4	5	6	7	8
Sangaste										
Amino acid	898.8	1352.7	1397.8	1876.7	1238.4	1544.4	1618.8	1316.7	1354.4	1470.3
Amide	344.1	837.9	917.6	1128.6	898.4	618.7	828.2	747.0	741.3	
Prolific										
Amino acid	1035.1	1178.5	1387.5	1304.5	1006.2	1221.0	1173.1	1222.4	908.3	766.4
Amide	401.4	614.8	756.3	701.4	647.5	713.0	798.2	759.1	545.2	434.6

amides were noted to increase by 26% and 75% respectively. This increase in soluble amino acids and amides is not unique to early growth at low temperature. In a comparable study at room temperature (insert in Table II) an increase of 37% and161% in the free amino acid and amide content was noted for the Sangaste variety. The corresponding changes for Prolific were nil and a 61% increase. These findings are in general agreement with other studies on cold grown plants and germinating seedlings. Wilding *et al.* (1960) reported specific increases in some amino acids during the chilling of winter-hardy alfalfa roots when comparisons were made with a non-hardy variety. Trione *et al.* (1967) also report specific increases in alanine, proline and the amides in the leaves of a winter wheat variety when comparisons were made to a spring wheat variety when both were grown at 2°C. Beletskaya (1971) and Sane and Zalik (1968) report a several fold increase in the level of most free amino acids in the embryonic tissue during germination at normal growing temperatures with maximum levels occurring on the 8th to 10th day for barley (Sane and Zalik, 1968) and on the 6th day for maize seedlings (Beletskaya, 1971).

In this study it is seen that the increase in total soluble amino acids and amides in the cold treated Sangaste seedlings greatly exceeded the increase which occurred during the early growth at normal temperatures when seedlings at comparable growth stages were analyzed and also greatly exceeded the increase noted in the spring variety when it was grown at the vernalizing temperatures.

The general fluctuation in total amino acids and amides during the chilling period was that of a rapid increase in content during the initial stages followed by a gradual decline during the remainder of the treatment period. If the individual amino acids were examined (Figs. 4-26) a general response is not evident. Some amino acids increased throughout the treatment period, some declined, others changed little and a few showed marked increases followed by a gradual decline. This variation in response is not in agreement with the findings of Trione *et al.* (1967) who reported an increase in most of the free amino acids during the initial 2 weeks of cold treatment followed by a rapid decrease during the 3rd week and a general increase through the remainder of the treatment period. The stage of growth of the plant material sampled could possibly account for the differences between these studies. They (Trione *et al.*, 1967) suggested a possible relationship between the decline in most free amino acids at the 3rd week and the vernalizing mechanism and indicated this decline occurred just prior to flower differentiation. However, flower initiation does not necessarily occur during the chilling period. Gott *et al.* (1955) noted during studies on the vernalization of imbibed rye seeds that flower initiation did not occur in fully vernalized seedlings until 2 weeks after the plants were grown under normal temperatures.

The possible involvement of the noted free amino acid changes in the vernalizing mechanism is speculative. With the belief that morphological changes result from previous biochemical changes, the enhancement in flower initiation due to the cold treatment perhaps

results from biochemical changes which occurred during the chilling period. Little is understood about the intracellular distribution of free amino acids in plant tissues. The review by Oaks and Bidwell (1970) suggested that metabolites accumulate in pools and a metabolite may undergo different fates depending on its locale. Bidwell *et al.* (1964) gave evidence which suggested that proteins are in a continuous state of turnover and the amino acids which are readily incorporated into protein are not in association with those resulting from protein breakdown. Sane and Zalik (1968) found the amino acid composition of the endosperm protein and the embryonic protein in germinating barley seeds to be very different and suggested some interconversion of amino acids must have taken place. Thus the main source of the free amino acids analyzed in this study likely resulted from the hydrolysis and transport of storage protein from the endosperm, from protein turnover in the embryo tissue and possible interconversions within the soluble metabolic pools.

The marked accumulation of proline, alanine and glutamine observed in this study is not unique to the vernalizing mechanism. Under climatic stress increases in specific free amino acids have been noted for several species, however it is the more hardy species which usually show the more significant increases (Taylor *et al.*, 1972; Singh *et al.*, 1972). Thus it appears the accumulations noted under the stress conditions are directly related to the plant's adaptive ability. The estimate of the increases in free proline in barley seedlings grown under water stress conditions is suggested by Singh

et al., (1972) to be a useful criterion in the selection of drought-tolerant varieties. As the cold injury of plants is suggested to result from extreme tissue water loss (Levitt, 1972) the increased proline content in the Sangaste variety could be a feature of the cold-tolerance of the winter variety.

SECTION III

LIPID CHANGES DURING VERNALIZATION

Materials and Methods

A. Plant Material

The source of cold treated and untreated plant material was the same as that detailed under methods in Section II.

B. Solvents

All solvents used were of analytical reagent grade. Methanol and acetic acid were redistilled. The ethanol used as preservant in the chloroform was removed by refluxing chloroform with calcium chloride for 45 minutes and distilling the mixture. Phenol used for the sugar determinations was redistilled and allowed to crystallize at room temperature.

C. Lipid extractions

Lipids were extracted from the freeze-dried embryos by homogenizing the tissue in chloroform: methanol (2:1, v/v) using a polytron (Brinkmann instrument) with a 20ST (saw tooth) generator. Approximately 90 ml of solvent per 0.3 gm of sample material was used. This allowed adequate volume for the reextraction procedures and washings required. Para-chloromercuribenzoate(PCMB) was added as an enzyme inhibitor (Yang, *et al.*, 1967) to the extraction solvent at a concentration of 10^{-4}M. Solubilization of the PCMB required low heating in methanol prior to the addition of chloroform. The initial homogenate was

centrifuged at 10,000 rpm for 10 min., the supernatant decanted and the pellet resuspended using the polytron. This procedure was repeated 3 times. The combined supernatants were filtered, followed by washing of the pellet over the filter paper.

The non-lipid contaminants were removed from the chloroform: methanol extract using the method devised by Williams and Merriless (1970). This method utilizes the ability of Sephadex G-25 to swell in water and at the same time absorb water soluble contaminants. As the sample material was lyophilized the addition of water was required to cause the Sephadex to swell. To each sample extract 1 gram of Sephadex G-25 was added followed by 1 ml of water. The solution was dried on a rotary vacuum evaporator at room temperature until no free liquid remained in the flask. To ensure the complete absorption of the water and contaminants, the Sephadex was resuspended in chloroform and concentrated once more, after which it was again resuspended in chloroform and the suspension poured into a narrow chromatographic column (1 cm ID) with the end drawn to a capilliary and layered with glass wool. The lipids were washed from the Sephadex with 100 ml of chloroform. By monitoring further washings containing methanol (C:M, 2:1 v/v) with thin layer chromatography (TLC) traces of polar lipid components were noted to have remained on the Sephadex following the washings with 100 ml of chloroform. An additional 10 ml of chloroform methanol (2:1 v/v) was found to adequately remove the polar lipids. This resulted in the elution of non-lipid contaminants, however when the lipid components were separated on TLC plates

the contaminants remained close to the origin and did not interfere with the polar lipid separations.

The washings from the Sephadex column were concentrated to dryness under vacuum, resuspended and made up to a volume of 5 ml in chloroform.

D. Separation of Polar Lipids

The polar lipids were separated by two-dimensional TLC using a method similar to that devised by Nichols (1964). The neutral lipid components were not separated, but were taken as the band which moved with the solvent front when the total lipid extract was chromatogrammed on the TLC plates.

Thin layer plates (20 cm x 20 cm) were coated with approximately 250 u layer of silica gel HR (w/o binder N, Merck). They were dried for at least 3 hours before activation and were activated just prior to use at 110°C for 1 hour, and cooled in a dessicating chamber.

The solvent systems were chloroform: methanol: 7N ammonium hydroxide (65:25:4 v/v) for the first dimension followed by chloroform: methanol: acetic acid: water (85:15:10:3 v/v) in the second direction. Following development in the 1st direction the solvent was removed from the plate by placing the plate under vacuum for approximately 20 minutes. The vacuum was released under nitrogen, and the chamber flushed with nitrogen for several seconds. A faint smell of ammonia was noted after this drying period indicating all of the first solvent was not removed, but if the drying time and vacuum pressure were consistent clear reproducible separations were obtained.

The chromatogram tanks were lined with filterpaper and solvents were freshly prepared daily.

A 0.5 ml aliquot of the total lipid extract was concentrated under a stream of nitrogen and applied with a fine capilliary as a spot to the lower left-hand corner of each plate approximately 2 cm from either edge. Based upon gravimetric determinations which will be explained in the discussion of the extraction procedures, the amount of total lipid applied to each plate was approximated at 2 mg.

During the application of the sample to the TLC plate, the sample and the plate were maintained under a nitrogen atmosphere. A special applicator box was fabricated to enable a continous stream of nitrogen to be passed over the plate.

E. Identification of Phospholipids and Glycolipids

Spraying the plate lightly with 50% sulfuric acid and charring in an oven at 180°C for 15 min. was used to visualize all lipid spots on the TLC plate. Authentic samples, specific color tests and comparisons to the migration patterns shown in published chromatograms were used to identify lipid classes and individual spots.

F. Phospholipid and Galactolipid Determinations

Spots on the chromatograms which were to be used for quantitative determinations of phospholipids and galactolipids were detected with iodine vapor. The spots were circled in pencil and the iodine allowed to vaporize. For the phosphorus determinations one plate was adequate to supply a sufficient amount of the individual phospholipid components

for analysis, however for the galactolipid analysis the pooled contents of corresponding spots from 2 plates were required.

The amount of phosphorus was determined by analyzing for the phosphorus content using the method devised by Bartlett (1959). As the highly purified silica gel HR which does not interfere with the phosphorus assay was used, elution of the lipid from the adsorbent was not required. The spots were scraped into pyrex test tubes and blanks were taken from comparable zones. To the phospholipid plus silica gel HR, 0.5 ml of 10N sulfuric acid were added. The tubes were then placed in a metal heating block which had holes bored to tightly fit the test tubes. The metal block was welded to a hot plate and heated at 150-160°C for 3 hours. Following the 3 hours digestion a few drops of 30% H_2O_2 were added and the solution was further heated for 1.5 hours to complete combustion and decompose all the peroxide. To the hydrolysate 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of the Fiske SubbaRow reagent were added and the contents mixed thoroughly and heated for 7 minutes in a boiling water bath. The samples were centrifuged at 10,000 rpm for 5 minutes to pellet the absorbent and the optical density of the clear solution was determined at 830 nm and recorded with a Beckmann DK-1 spectrophotometer. The region of maximum absorbance of the phosphomolybdate complex was noted to occur at 830 nm as shown in Figure 28. The concentration of the phosphorus in the samples was determined from a standard curve (Fig. 29). A linear equation from the curve ordinates was calculated by the least squares method and programmed on an Olivetti 101 Programma. Using

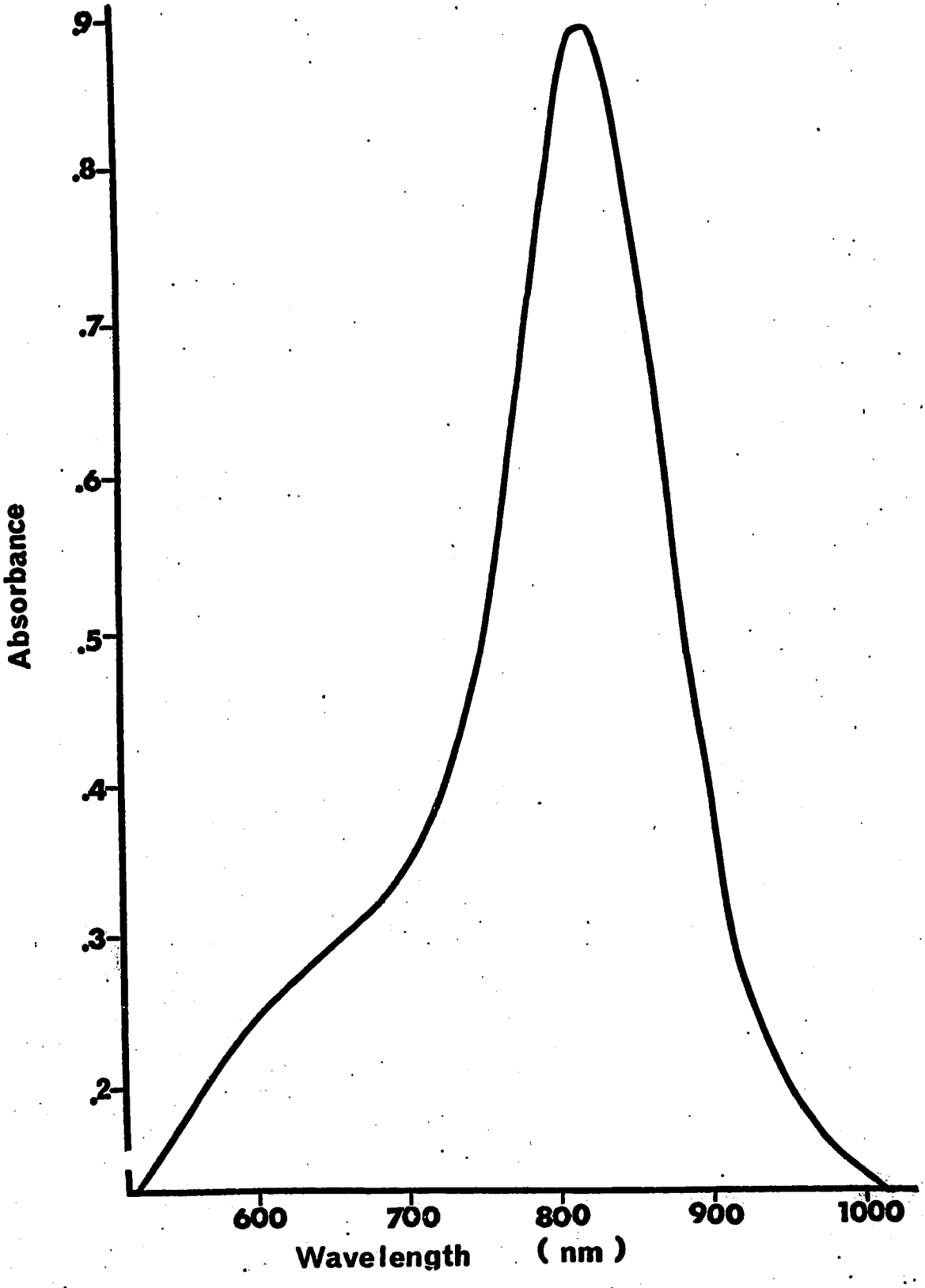


FIG. 28. Absorption spectrum of the phosphomolybdate complex as recorded with a Beckman DK-1 spectrophotometer.

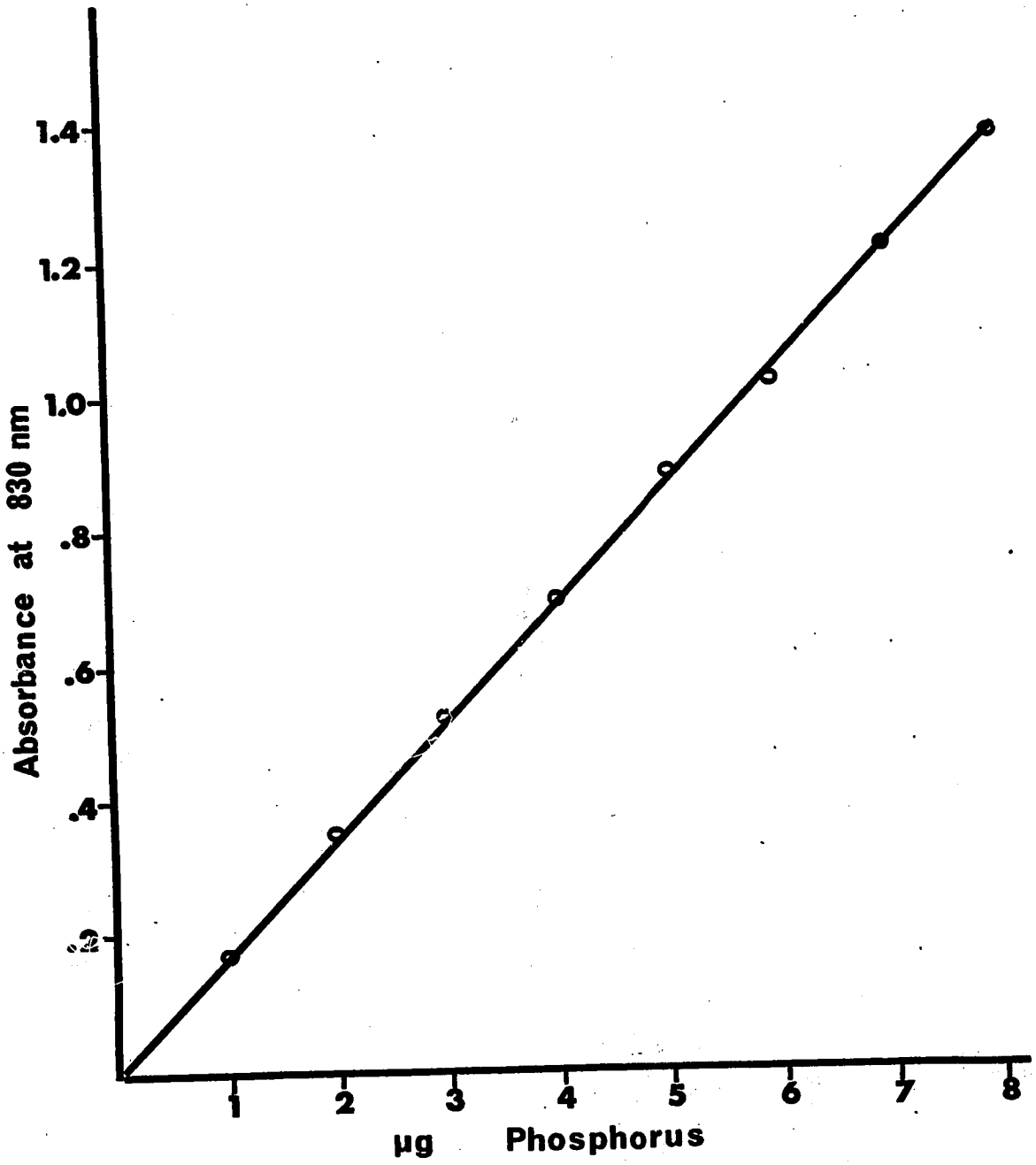


FIG. 29. Standard curve for phosphorus.

the linear equation the phosphorus content was determined directly from the O.D. reading.

Galactolipid content was determined using the method devised by Roughan and Batt (1968). The galactolipid containing spots and corresponding blanks were scraped from the plates into centrifuge tubes. To each tube 2 ml of 2N sulfuric acid was added and the tubes were placed in a boiling water bath for 60 minutes, during which time they were shaken 4 to 5 times. Following centrifugation at 10,000 rpm for 5 minutes to pellet the adsorbent, 1 ml aliquots were withdrawn from each tube. To each aliquot 1 ml of 2% phenol and 4 ml of concentrated sulfuric acid were added followed by immediate mixing on a vortex mixer. This concentration of phenol and sulfuric acid was reported by Pullishy (1970) in this lab, to give the maximum absorbance for galactose standards and galactose moieties of glycolipids. The maximum absorbance of the yellow-orange complex was at 490 nm as shown in Figure 30. This has been shown by Dubois *et al.* (1956) to be the characteristic absorption maximum for hexoses. They reported pentoses showed an absorption maximum at 480 nm. The absorption spectrum of spots 3, 11 and 12 plus 13 is also indicated in Figure 30. The content of the galactolipid present was determined using a standard curve (Fig. 31) prepared by adding known amounts of galactose to tubes containing silica gel HR and treating them similarly to the samples. The galactolipid content was determined directly from the O.D. reading using a linear equation calculated from the standard curve.

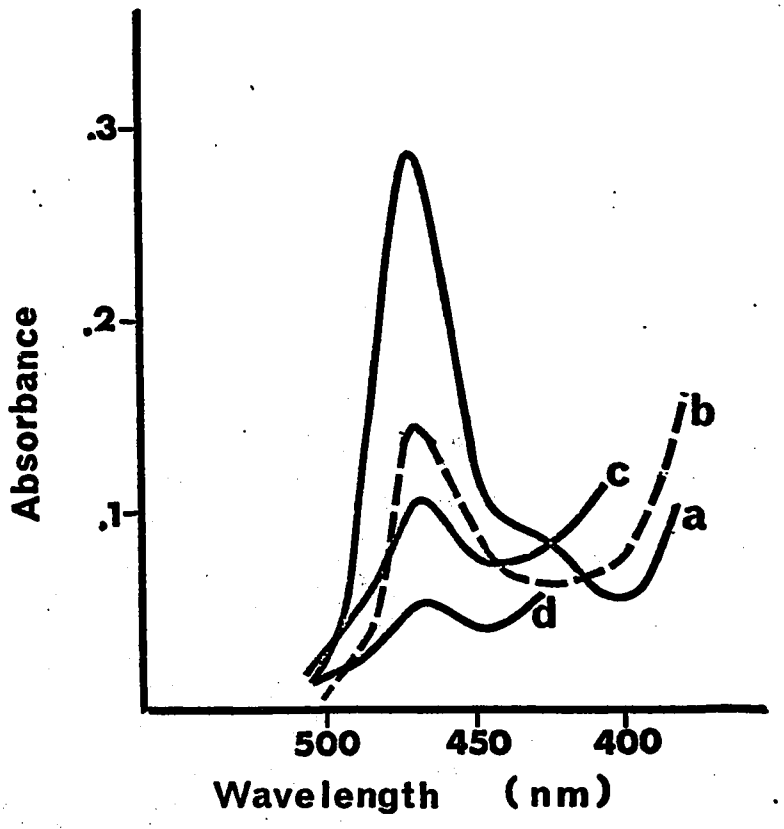


FIG. 30. Absorption spectra of galactose standard and the galactose moieties of glycolipids isolated from 4 day Sangaste rye embryo tissue. (a) galactose; (b) digalactosyl diglyceride; (c) monogalactosyl diglyceride; (d) unidentified glycolipid. The galactose was prepared by adding 1 ml of the standard solution (60 µg/ml) to a centrifuge tube containing a small amount of silica gel HR. To this 1 ml of 4 N sulfuric acid was added and the solution was heated at 100°C for 60 minutes. Following cooling the tubes plus contents were centrifuged to pellet the silica gel. A 1 ml aliquot was withdrawn and to this 1 ml 2% phenol and 4 ml cone sulfuric acid were added. The solution was shaken immediately and allowed to cool before scanning. The glycolipids were scraped off the silica gel HR plates into centrifuge tubes and 2 ml of 2 N sulfuric acid added. They were then treated the same as the standard.

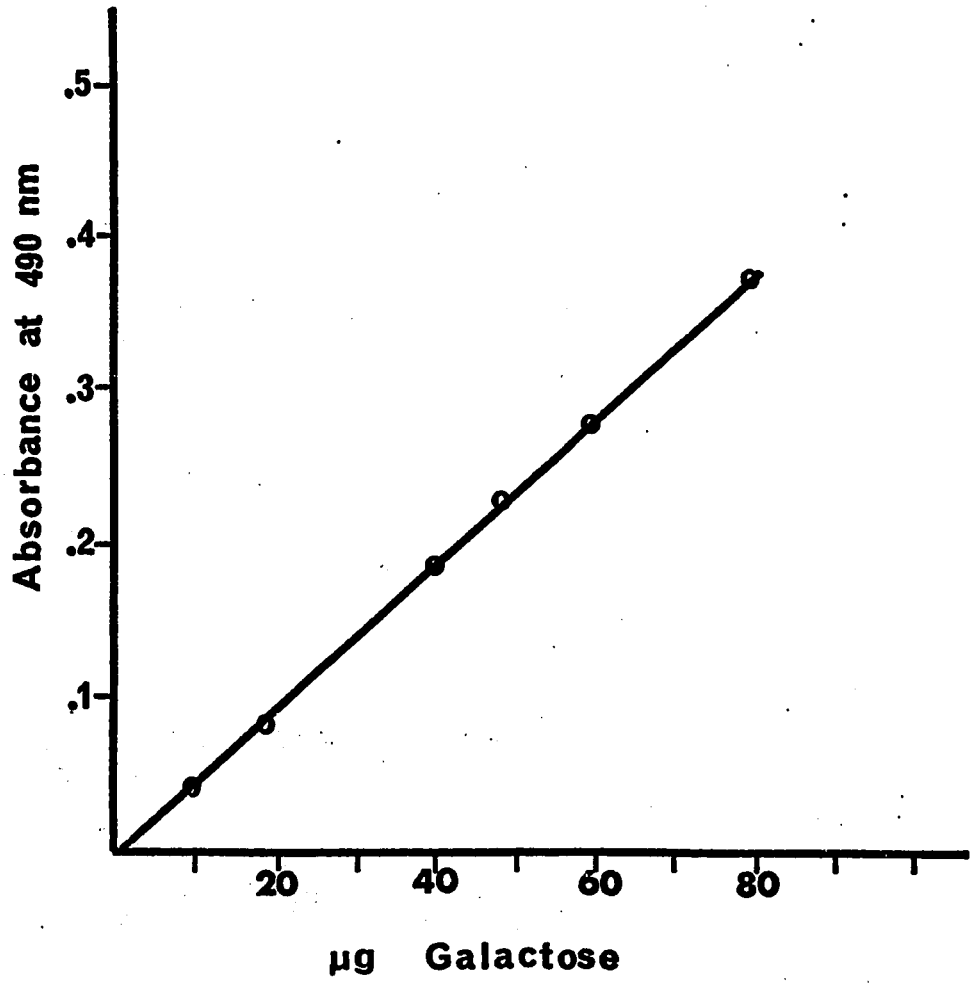


FIG. 31. Standard curve for galactose.

G. Fatty Acid Determinations

Spots on the developed chromatograms which were to be used to determine the fatty acid content of the separated phospholipids and galactolipids were detected by spraying the plates with 2% 2',7'-dichlorofluorescein (Eastman) in 95% ethanol (Skipski and Barclay, 1969; Renkonen and Varo, 1967). The plates were allowed to dry for approximately 15 minutes following spraying and viewed under an ultra violet light. The spots were scraped into glass test tubes; the pooled contents of the corresponding spots from 2 plates were required for each lipid component to attain adequate sample size for the detection of the fatty acids. However for the spots identified as phosphatidyl choline and neutral lipid one plate was adequate. To each tube containing the lipid component plus the adsorbent 2 ml of methanol and 0.1 ml of sulfuric acid were added. The tubes were refluxed, using a cold finger condenser, at 70°C for 2 hours. The contents were then diluted with 3 ml of water and the methyl esters extracted with 3 washings of petroleum ether. The petroleum ether was evaporated off under nitrogen and the methyl esters taken up in methanol. The fatty acid methyl esters were analyzed on an Aerograph model 200 gas chromatograph equipped with a hydrogen flame ionizing detector. A coiled stainless steel 9' by $\frac{1}{8}$" column packed with 20% ethylene glycol adipate on Anakrom SD 60/70 mesh, P. (Analabs) was used. The operating conditions of the gas chromatograph were: column temperature 195°C, injector temperature 240°C and the flow rate of nitrogen maintained at 25 ml per minute.

The peaks were identified by matching the retention times with known methyl ester fatty acid standards (Analabs). The peak areas were measured by the triangulation method. When a known mixture of standards were chromatographed the peak areas were found to be proportional to the amount added.

Results

A. Lipids Identified

The lipid spots visualized following charring of the developed TLC plates with sulfuric acid are shown in Figure 32. This photograph is representative of the lipid separations obtained for both varieties at all growing stages analyzed.

Spots 1, 2, 4, 5, 6 and 9 were identified as phosphorus containing lipids by spraying the plates with the molybdenum blue reagent which is noted to be specific for the detection of phospholipids (Dittmer and Lester, 1964). A trace of phosphorus also appeared around spot 10. The Dragendorff test (Wagner *et al.*, 1961) identified #2 as a choline containing lipid. Ninhydrin spray (Skipski *et al.*, 1962) indicated #5 contained a free amino group. Authentic samples (Analabs) confirmed #2 and #5 to be phosphatidyl choline and phosphatidyl ethanolamine respectively and also indicated #1 to be phosphatidyl inositol. Comparisons to the lipid migration patterns shown in the published chromatograms of Nichols (1964) and Galliard (1968) suggested #4 and #9 to be phosphatidyl glycerol and phosphatidic acid respectively and provided further support to the identity of spots 1, 2 and 5. Spot #6 was not identified.

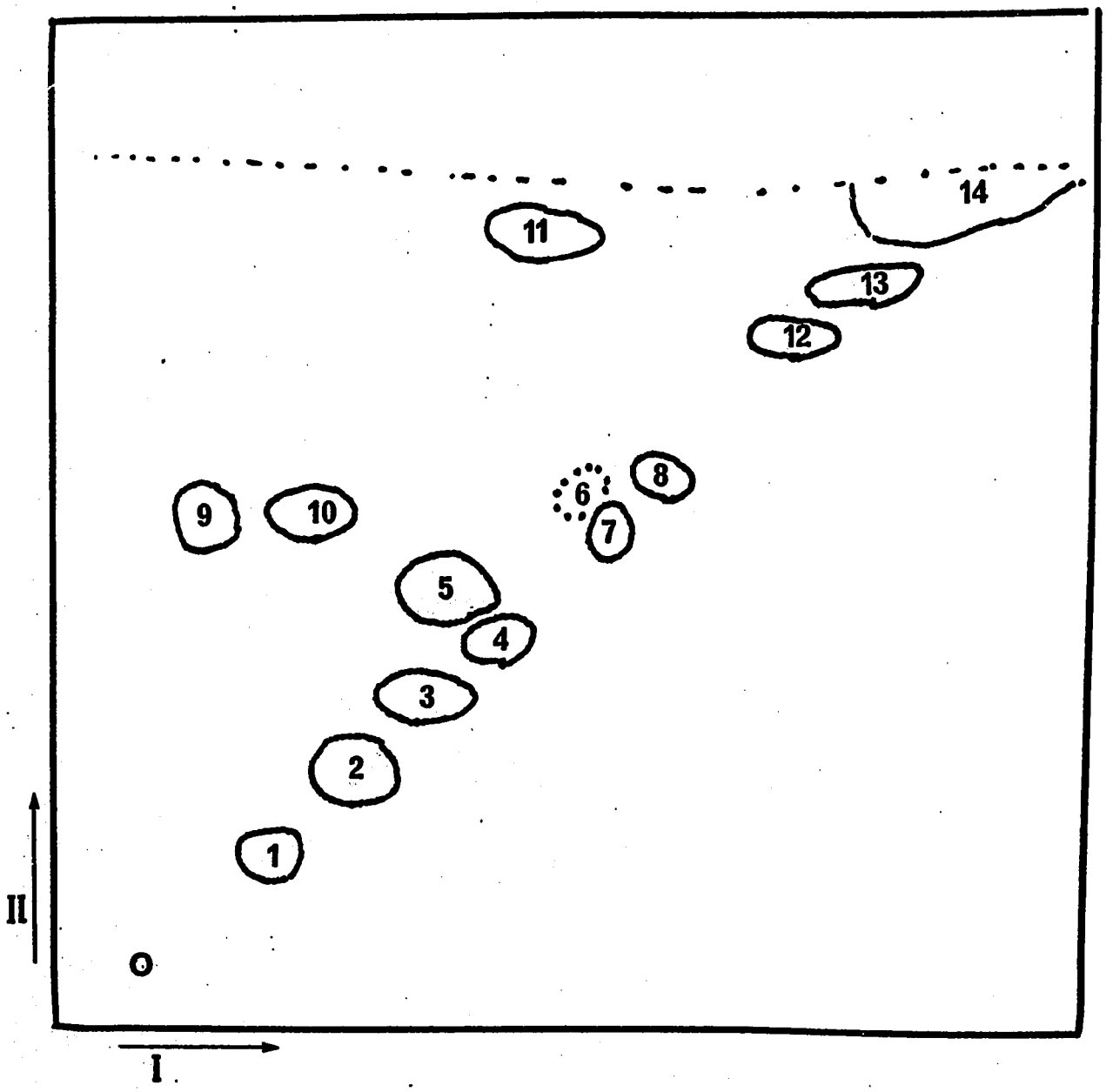
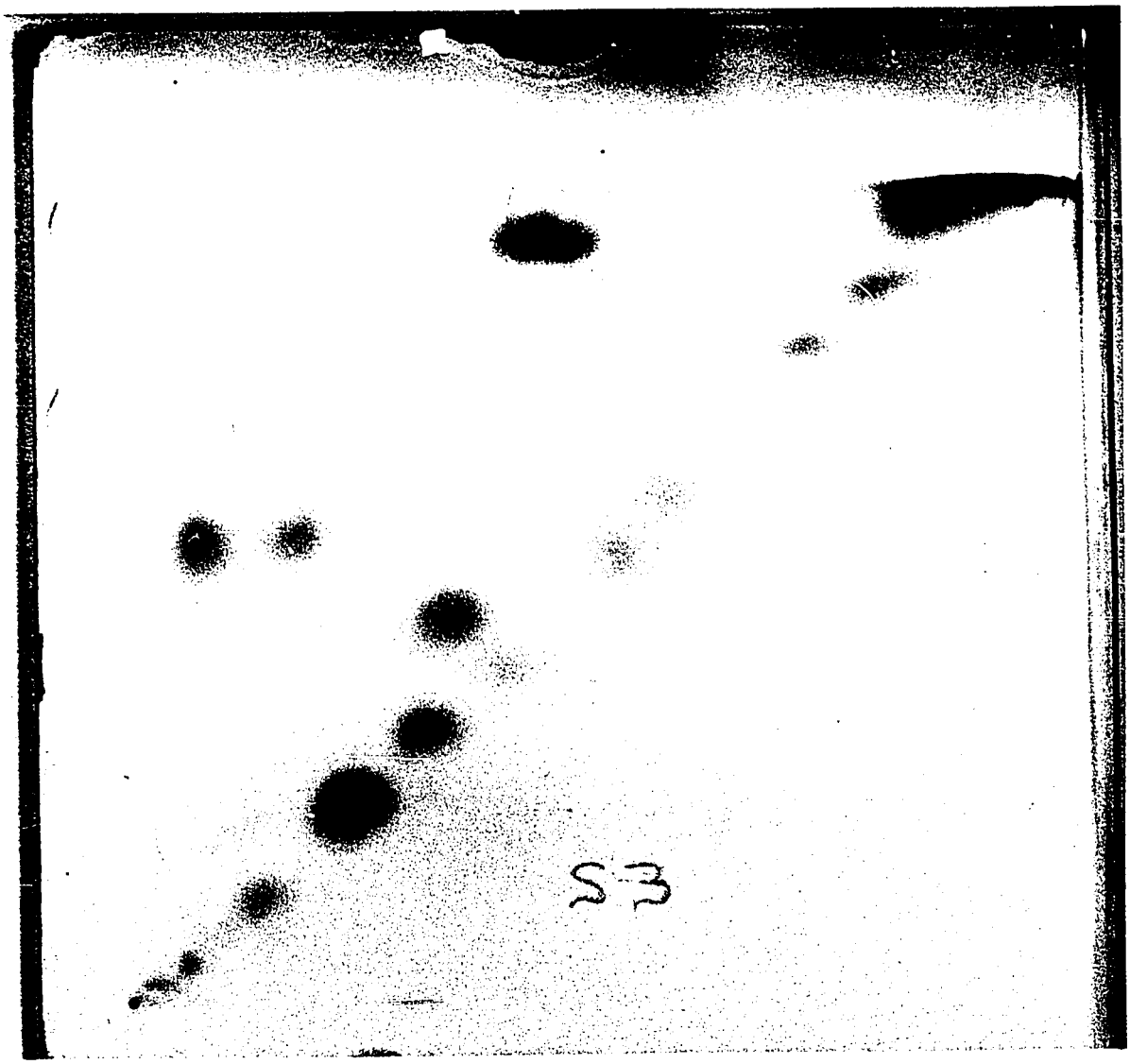


FIG. 32. Photograph showing a two-dimensional thin layer chromatogram of polar lipids isolated from Sangaste embryo tissue at 3 weeks of cold treatment. This chromatogram is typical of the separations obtained for both varieties at the growing stages analyzed. Spots were visualized by charring after spraying with 50% sulfuric acid. The silica gel HR plate was developed in direction I with CMAm (65:25:4) followed by CMAcW (85:15:10:3) in direction II. The numbered spots were identified as follows: O = origin, 1 = PI, 2 = PC, 3 = DGDG, 4 = PG, 5 = PE, 6, 7 and 8 unidentified, 9 = PI, 10 unidentified, 11 = UGL, 12 and 13 taken as MGDG, 14 = Neutral lipid.


S3

Spots 3, 7, 8, 11, 12 and 13 gave a purple color characteristic of glycolipids and sterols during early color development when the plates were sprayed with 50% sulfuric acid and placed in a 110°C oven. The orcinol-sulfuric acid spray (Svennerholm, 1956) gave the characteristic blue color of glycolipids and sterols to the above mentioned spots. Comparisons to the lipid migration patterns shown in the published chromatograms of Nichols (1964) and Galliard (1968) suggested #3 to be digalactosyl diglyceride (DGDG) and either #12 or #13 to be monogalactosyl diglyceride (MGDG). The pooled contents of #12 and #13 were taken for the analysis of MGDG content. Galliard was unable to identify a component with a similar migration pattern as spot 11 and referred to it as an unidentified galactolipid (UGL). Spots 7 and 8 were not further identified.

Spot 10 did not appear when the chromatogram was exposed to iodine vapor or to 2',7'-dichlorofluorescein and was not further identified, however a trace of phosphorus was evident at the edge of the spot nearest the PA spot. Spot 10 was included in the phosphorus assay.

B. Phospholipid and Glycolipid Content

The total phosphorus and sugar content of the lipid extracts for both varieties sampled during the treatment period and expressed on the dry weight of the embryo tissue are shown in Tables III and IV respectively. Sangaste is noted to contain more total phosphorus in the lipid extract than the Prolific however, the trend is similar for both varieties showing a gradual decline throughout the treatment period. Initially Sangaste had a higher sugar content in the lipid

Table III. Phosphorus content of total lipid extracts from embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period.

Values are expressed as µg P/g tissue dry weight

Weeks	Sangaste	Prolific
0	1081.0	899.7
1	966.1	681.5
2	586.4	526.3
3	484.4	424.6
4	376.1	319.5
5	375.7	342.4
6	415.5	305.3
7	355.1	292.4
8	304.5	286.6

Figures are averages of 4 aliquots from the same sample. The standard error of the estimate did not exceed 2% for any of these determinations.

Table IV. Sugar content of total lipid extracts from embryo tissue of rye seedlings sampled at intervals during the vernalizing period.

Values are expressed as µg galactose/g tissue dry weight

Weeks	Sangaste	Prolific
0	3836	2860
1	2984	3101
2	2917	2159
4	2379	2567
6	2349	2459
8	2517	2489

Figures are averages of 4 aliquots from the same sample. The standard error of the estimate ranged as high as 12% for some of these determinations.

extract but there was not a consistent difference throughout the treatment. This variability may have been due to the high error of estimation.

The components contributing to the total phospholipid and glycolipid content were separated by chromatographing aliquots of the total lipid extract by TLC and the possibility of expressing the contents of the individual lipid components within the subclasses as a percentage of the total lipid content of the subclass was explored. However the recovery rates from the TLC plates were found to be inconsistent (Table V and VI) as considerable variability occurred among sampling dates and between chromatograms spotted with aliquots from the same original sample. If the phosphorus content of the separated phospholipids was expressed as a percentage of the total phosphorus recovered from the chromatograms (Table VII), the standard error of the estimate among chromatograms from the same original sample was within 2% for most determinations. To determine the total phosphorus recovered the phosphorus content of the separated phospholipids were summed, the entries used in the summation were the same for all samples analyzed. Since no trend in the recovery rates over sampling intervals was evident (Table V) the presence of undetected components markedly influencing the ratio between the amount applied to the chromatogram and the amount recovered was thought unlikely. Additionally because the percentages of phosphorus recovered were relatively high they were taken to be representative of the total phosphorus content of the sample. With this method of presentation the Sangaste variety con-

Table V. Recovery of phosphorus from two-dimensional thin layer chromatograms. Chromatograms were spotted with the total lipid extract from rye seedlings sampled at weekly intervals during the vernalizing period.

$$\% \text{ Recovery} = \frac{\text{Sum of phosphorus recovered from all phosphorus containing lipids separated by TLC}}{\text{Total phosphorus applied}} \times 100$$

Weeks	Sangaste	Prolific
0	66.7	77.3
1	74.1	81.5
2	89.1	88.5
3	95.6	85.3
4	75.7	85.4
5	81.5	85.0
6	88.7	79.4
7	80.0	84.0
8	83.2	78.7

Figures are averages of 3 or 4 plates spotted with aliquots from the same sample. The standard error of the estimate did not exceed 6% for any of these determinations. The same 7 phospholipid components were summed for each determination.

Table VI. Recovery of sugar from two-dimensional thin layer chromatograms. Chromatograms were spotted with the total lipid extract from rye seedlings sampled at intervals during the vernalizing period.

$$\% \text{ Recovery} = \frac{\text{Sum of galactose content of DGDG, MGDG and UGL separated by TLC}}{\text{Total galactose applied}} \times 100$$

Weeks	Sangaste	Prolific
0	25.1	22.3
1	27.3	33.0
2	28.1	41.9
4	41.1	36.7
6	36.9	42.7
8	46.4	44.8

Figures are averages of 2 determinations using 4 plates, the pooled contents of 2 plates were required for each determination. All the plates at the weekly intervals were spotted with aliquots from the same sample. The standard error of the estimate ranged as high as 15% for some of these determinations.

tained higher total phospholipid on the dry weight basis of embryo tissue (Table III), however the percentages of the lipid components within the phospholipid classes were similar for both varieities (Table VII). The percentages of PI, PE, PG and unknowns 6 and 10 were very similar for both varieties and showed little variation throughout the treatment period. The PC and PA content also showed a similar response for both varieties except at 1 week. The content of PC showed a gradual almost linear decline during the first 7 weeks from 46 to 31% for the Prolific variety and from 44 to 30% for the Sangaste variety. The PA content showed a corresponding increase from 13 to 21% for the Prolific variety and 14 to 24% for the Sangaste variety.

The phospholipid composition of the embryo tissue following growth at room temperature for 4 days is shown as an insert in Table VII. There was little difference between the varieties grown at room temperature, however noted differences were evident between the two growing temperatures when seedlings of comparable growth stages were analyzed. The PI content was higher in both varieties grown at room temperature in comparison to 4°C (16% as compared to 6-9%) while the PA content was very low (3-4% as compared to 12-18%).

Changes in the glycolipid content during the treatment period were determined similar to the procedure outlined for the phospholipids. However the recovery rates were low (Table VI) and a trend in the percentages recovered was evident increasing from 22 to 46% as the duration of the treatment period increased. Therefore the percentages of the components analyzed were considered not to be representative

Table VII. Phospholipid content of embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period and at 4 days following growth at 24°C.

Values are expressed as a percent of total phospholipid

		Lipid Class						
Weeks		PI	PC	PG	PE	#6	PA	#10
0	S	10.8	44.5	5.9	16.1	4.5	14.7	3.4
	P	9.6	46.6	5.7	17.9	4.0	13.5	2.7
1	S	7.7	44.8	7.9	23.1	5.1	6.9	4.6
	P	7.3	33.8	5.7	17.9	4.0	13.5	2.7
2	S	7.4	40.0	9.0	21.1	5.6	14.1	2.7
	P	7.9	40.5	8.2	19.0	5.6	16.2	2.6
3	S	9.1	38.3	8.2	19.7	4.6	18.6	1.7
	P	6.7	40.3	7.8	19.8	5.6	12.5	7.3
4 day	S	16.1	47.4	9.3	21.5	1.2	4.4	T
	P	16.1	47.0	12.9	19.9	1.1	2.9	T
4	S	9.2	37.0	8.9	19.2	T	23.2	2.4
	P	7.5	35.0	7.7	16.6	5.8	21.7	5.5
5	S	10.2	35.7	8.6	22.0	4.7	18.6	T
	P	8.3	33.2	7.6	16.1	8.9	22.5	6.8
6	S	8.8	30.3	6.7	18.6	8.8	21.5	2.5
	P	8.9	34.5	6.9	17.8	6.0	21.7	5.8
7	S	8.9	30.5	7.3	14.0	11.7	24.8	2.7
	P	9.1	31.2	6.9	13.1	12.9	20.8	5.9
8	S	8.9	34.8	6.0	18.0	6.2	22.8	3.3
	P	8.3	34.4	7.3	14.9	8.3	20.8	5.9

T = Trace S = Sangaste P = Prolific

Figures are the averages of 3 or 4 determinations. All plates at the weekly intervals were spotted with aliquots from the same sample. The standard error of the estimate was within 4% for these determinations.

Table VIII. Galactolipid content of the embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period and at 4 days following growth at 24°C.

Values are expressed as a percent of the three galactolipids

	Sangaste			Prolific		
Weeks	MGDG	DGDG	UGL	MGDG	DGDG	UGL
0	33.0	48.1	18.9	39.7	39.7	20.7
1	36.9	41.7	21.4	39.3	39.3	21.6
2	30.6	44.3	25.1	34.2	37.3	28.6
3	23.3	49.1	27.6	31.2	33.7	34.6
4 day	38.2	52.0	9.7	34.7	54.3	10.9
4	34.2	34.9	31.1	36.6	33.3	31.0
5	26.5	36.6	36.9	31.7	34.5	33.7
6	25.3	37.9	36.8			
7	25.6	43.2	31.1	35.4	38.6	25.9
8	27.5	38.3	32.2			

Figures are averages of two determinations using 4 plates. The pooled contents of 2 plates were required for each determination. All plates at the weekly intervals were spotted with aliquots from the same sample. The standard error of the estimate ranged as high as 15% for some of these determinations.

of the total glycolipid content.

The phenol-sulfuric acid method used in this study for sugar determinations has a sensitivity range from 10 to 100 µg of sugar (Dubois *et al.* 1956). The variability noted in the recovery rates, even between the duplicates from the same original extract (Table VI), is believed largely attributable to the low galactolipid content. Most samples had readings near the lower limit of the sensitivity scale, and the pooled contents of the corresponding spots of two chromatograms were required. Whereas for the phosphorus determinations one plate was adequate. The low recovery can be attributed in part to the presence of sugar containing lipids which were detected with the specific spray reagents, but not further identified and not included in the analysis. Early color development during charring of the chromatogram following spraying with 50% sulfuric acid indicated spots 7 and 8 contain a glycolipid moiety. Galliard (1968) and Nichols (1964) using similar solvent systems to separate plant polar lipids reported spots of similar migration patterns and suggested these components to be cerebrosides. Plant cerebrosides are reported by Galliard (1968) to contain a glucose moiety. Color development following the orcinol-sulfuric acid spray also indicated the presence of sugar containing contaminants just off the origin. In retrospect this method of quantitating microamounts of glycolipids by their sugar content appears less sensitive than the method described by Kuksis (1966) and Allen and Good (1971) in which quantitation was based on the fatty acid content using an internal fatty acid standard.

However from the method used in this study trends appear evident. The glycolipid classes recovered from the plates presented as percentages of the three glycolipids recovered are shown in Table VIII. The results are presented on the basis of sugar content and conversion into their respective molar ratios were not applied because of the unidentified glycolipid (UGL).

The amounts of MGDG and DGDG differed between the two varieties. DGDG was present in higher levels than MGDG in the Sangaste variety throughout the treatment period whereas the fractions were present in similar proportions in the Prolific variety. The amounts of the respective glycolipids in 4 day-old seedlings grown at 24°C were similar for both varieties. In comparison with cold treated seedlings the level of DGDG was rather high and was low for UGL.

C. Fatty Acid Content

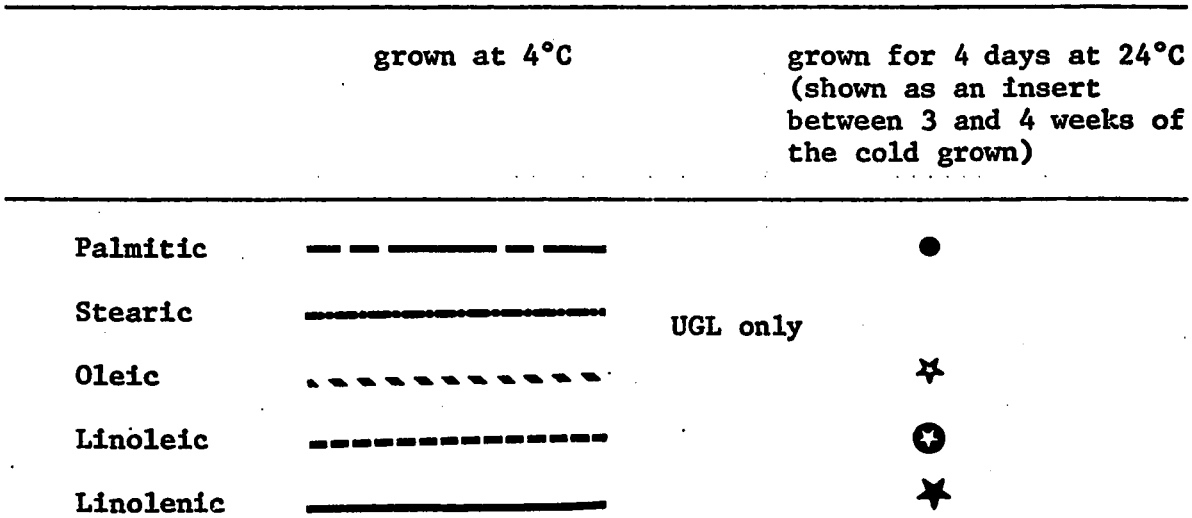
The changes in the fatty acid content within the lipid classes during the treatment period are shown in Figures 33 to 42. The amount of the individual fatty acids are expressed as a percentage of the total fatty acid content of the sample. The major fatty acids detected were palmitic (16:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). Stearic (18:0) was present in trace amounts in some samples. Short chain fatty acids containing less than 16 carbon atoms did appear in trace amounts in the neutral lipid fraction and to a lesser extent in the glycolipid fractions, however they were not present in sufficient amount to enable quantitation. A trace of palmitoleic (16:1) appeared in the PG and PE fractions in both varieties at the

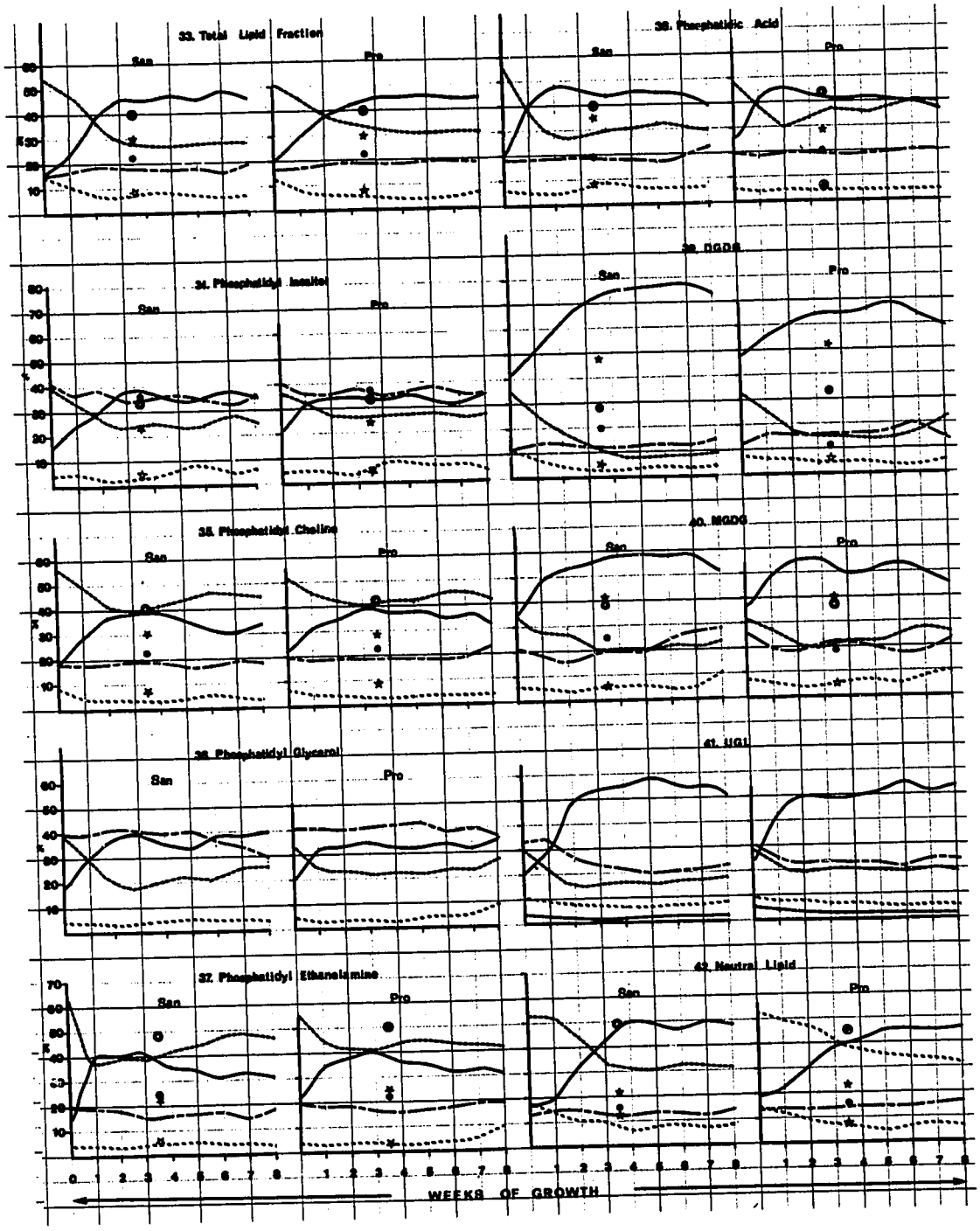
third week of cold treatment and was evident throughout the remainder of the treatment period.

For all lipid classes analyzed from both varieties during the chilling period the most marked changes in content were for 18:3, and 18:2. The percentage of 18:3 increased during the initial 3 to 4 weeks of the chilling period and a corresponding decrease occurred in the percentage of 18:2. The percentage of 16:0 present in the individual lipid classes showed little variation throughout the treatment period. The increases noted in the percentage of 18:3 in the total lipid fraction (Fig. 33), showing an increase of approximately 25%, were not common for all the lipid classes. In general a less marked increase occurred in the phospholipid fractions (Figs. 34 to 38) and a more marked increase in the glycolipid fractions (Figs. 39 to 41). Within the phospholipid fractions variations in the fatty acid content were noted to be specific to the individual lipid classes analyzed. In the PI and PG fractions (Figs. 34 and 36) 16:0 was the major fatty acid in the Prolific variety representing approximately 40% of the total fatty acid content. Palmitic was also a major fatty acid in the Sangaste variety, however due to the larger increase in the 18:3 content it shared the predominant role with 18:3. In the PC and PE fractions (Figs. 35 and 37) 18:2 was the predominant fatty acid, and increases in 18:3 content during the initial 3 weeks approximated 15% in the PC fraction and 20% in the PE fraction. In the PA fraction (Fig. 38) 18:3 became the major fatty acid at the 2 week period, showing an increase of approximately 15

FIGS. 33-42. Changes in fatty acid composition of lipid fractions isolated from rye embryo tissue sampled at weekly intervals during the chilling period and at 4 days following growth at 24°C.

SAN = Sangaste, PRO = Prolific.




33. Total Lipid Fraction
San
Pro
38. Phosphatidic Acid
San
Pro
34. Phosphatidyl Inositol
San
Pro
39. DGDG
San
Pro
35. Phosphatidyl Choline
San
Pro
40. MGDG
San
Pro
36. Phosphatidyl Glycerol
San
Pro
41. UGL
San
Pro
37. Phosphatidyl Ethanolamine
San
Pro
42. Neutral Lipid
San
Pro
WEEKS OF GROWTH

to 20%. In the glycolipid fractions analyzed 18:3 was the most predominant fatty acid in all fractions except in the UGL fraction prior to chilling (Figs. 39 to 41). The percentage of 18:3 was noted to increase from approximately 40% to 75% in the DGDG fraction during the initial 4 weeks for the Sangaste variety and from approximately 50 to 70% for the Prolific variety (Fig. 39). Large increases in the 18:3 content were also noted for the MGDG and UGL fractions (Figs. 40 and 41). The variations in the fatty acid content of the neutral lipid fraction (Fig. 42) showed a similar response to that shown for the total lipid fraction (Fig. 33) except that the initial increase in unsaturation showed a delay of 1 week in the neutral lipid fraction.

The fatty acid content of the lipid classes separated from 4 day old seedlings, shown as an insert in the figures indicate a slight increase in unsaturation during the normal growth processes at room temperature. This is evident by comparing the fatty acid content recorded following germination for 36 hours at room temperature (0 weeks in above figures) to the fatty acid content recorded following 4 days of growth. However these increases are only a fraction of the increases recorded when compared to plants grown to a comparable stage at 4°C. The DBI was calculated according to the method outlined by Lyons *et al.* (1964) in which the summation of the weight percent of each acid is multiplied by the number of double bonds it contains per molecule and divided by 100. The larger the numerical values attained the more unsaturated the sample. The

values presented in Table IX indicate increased unsaturation levels for both varieties during the cold treatment.

In summary this study indicated lipid changes occurred during early growth at low temperatures and marked differences were evident between the two growing temperatures when seedlings at comparable growth stages were analyzed. The total phosphorus content of the lipid extract on a dry weight basis was higher in Sangaste suggesting a higher phospholipid content for the winter variety, however the percentages of phospholipid components contributing to the total phospholipid content showed similar variations between the two varieties. An increase in the percentage of PA present in the total phospholipid fraction occurred in both varieties during the chilling period with a corresponding decline in the percentage of PC. In contrast the percentage of PC was high and that of PA was very low in the seedlings grown at room temperature. The changes noted in the respective lipid classes isolated from the two varieties were very similar.

Discussion

A. Plant Material

The embryo tissue including the scutellum, roots and shoots were used in this analysis. Others, as discussed in Section II, have shown the excised embryo can be vernalized in the absence of the endosperm if they were cultured on a medium containing a carbon and nitrogen source. Earlier studies on lipid changes in cereal seedlings during the vernalizing period have been reported (Redshaw, 1968), however whole plants including the endosperm were used in the analysis.

Table IX. Double bond index of lipid extracts from the embryo tissue of rye seedlings sampled at weekly intervals during the vernalizing period and at 4 days following growth at 24°C.

Values are expressed as the summation of the percent of fatty acid multiplied by the number of double bonds it contains per molecule and divided by 100.

	Lipid Class							
	Total Lipid		Phospholipid		Galactolipid			
Weeks	SAN	PRO	SAN	PRO	SAN		PRO	
0	1.71	1.75	1.55	1.62	1.79		1.84	
1	1.78	1.83	1.74	1.79	1.90		2.04	
2	1.95	1.96	1.81	1.82	2.16		2.12	
3	2.02	1.99	2.03	1.83	2.26*	2.18	2.16*	2.10
4-day	1.79	1.77	1.75	1.66	2.05*		2.09*	
4	1.99	2.03	1.84	1.81	2.24*	2.18	2.14*	2.08
5	2.07	2.05	1.77	1.81	2.28		2.11	
6	2.00	2.06	1.82	1.77	2.16		2.15	
7	2.12	2.01	1.87	1.72	2.26		2.01	
8	1.96	1.97	1.80	1.77	2.01		2.04	

Phospholipid values represent fatty acids from PI, PC, PE, PG and PA fractions. Galactolipid values represent fatty acids from MGDG, DGDG and UGL fractions except values marked with * are from MGDG and DGDG classes only.

SAN = Sangaste PRO = Prolific

The lipid changes studied included gravimetric determination of the total polar and non polar lipid and the fatty acid composition of the respective subclasses. In this study interest was concentrated on the polar lipid components which are known to be important membrane constituents. The endosperm was discarded in an attempt to remove any masking effect of the endosperm lipids. The plants were vernalized in the dark in order to limit pigment formation which is also soluble in the organic solvents used. Also during greening a marked increase in the chloroplast lipids, in particular the glycolipids has been noted (Trémolières and Lepage, 1971: Roughan and Boardman, 1972). This would introduce a further variable which was reduced by growing the seedlings in complete darkness during the cold treatment.

B. Lipid Extractions

Various solvents for the extraction of complex lipids have been cited in the literature. The extraction solvent chloroform: methanol 2:1 (v/v) proposed by Folch *et al.* (1957) was used in this study since it has given satisfactory extraction of chloroplast (Pullishy, 1970), mitochondrial (Rouser and Fleicher, 1967) and seedling lipids (Redshaw, 1968). Following the repeated extraction procedure described in the methods, hydrolysis of the cell residues with 5% sulfuric acid in methanol liberated only trace quantities of fatty acids suggesting very little lipid remained in the tissue residue.

Para-chloromercuribenzoate (PCMB) was added to the extraction solvent in an attempt to minimize lipid alterations during extraction. Various plant tissues are noted to contain phospholipase D (Chapman,

1969; Yang *et al.*, 1967) which splits the terminal phosphate ester bond of the phospholipids and hydrolyses phospholipids to phosphatidic acid or if other phosphatidyl derivatives are present it catalyzes transphosphatidylations. Also if any alcohol is present in the extraction solvent the methyl ester of the alcohol may be formed (Yang *et al.*, 1967). Yang *et al.* (1967) report steaming the tissue prior to extraction or the use of PCMB at 10^{-4}M will stop the enzyme activity. Both methods were tried in this study and the TLC plates compared with extracts in which no precautions were taken, however no difference was noted in the pattern of phosphorus containing lipids on the chromatograms. If the enzyme were active presumably it would form phosphatidylmethanol (PM). The chromatographic properties of PM were reported by Roughan and Batt (1969), to be very similar to those of diphosphatidyl glycerol (DPG). The only phosphorus containing unknown made visible which could be either DPG or PM would be component #6 shown in Figure 32 and it remained very low in amount as indicated in Table VII.

Three of the common methods for the removal of contaminants from the lipid extracts were compared to determine their suitability for the embryo tissue used. These included the method devised by Bligh and Dyer (1959) and the method of Maxwell and Williams (1967) using Sephadex LH-20 as well as a method using Sephadex G-25 (Williams and Merriless, 1970).

Freeze-dried Sangaste embryo tissue following 36 hours of germination at room temperature was extracted by each of the three methods.

The tabulated results are the means ± the half range for 2 replicates for the chloroform soluble lipids expressed as percent of tissue dry weight.

Bligh and Dyer	4.97 ± 0.93
LH-20	6.72 ± 0.26
G-25 (course)	6.59 ± 0.21

The low recovery and the poor reproducibility for the Bligh and Dyer method were likely due to incomplete recovery of the lipids from the polar lipid phase because of the heavy emulsion formation. The yield and reproducibility by the LH-20 and G-25 methods were similar and there was no apparent difference between the TLC chromatograms obtained from these two methods. The G-25 method was selected because it required less handling time.

C. Fatty Acid Analysis

The iodine vapor used to locate spots for phosphorus and sugar analysis was previously reported to give erroneous fatty acid results (Nichaman *et al.*, 1963). Initial analysis in this study also indicated a loss of polyunsaturated fatty acids when the chromatograms were exposed to iodine vapor. The commonly used detector Rodamine 6G, which is reported not to interfere with fatty acid content was found insensitive. This was possibly due as suggested by Allen and Good (1971) to the presence of acetic acid from the second solvent system remaining on the chromatogram. Lipids which were to be used for the analysis of their fatty acid content were therefore detected on the

TLC plates by spraying the plates with 2,7-dichlorofluorescein (Renkonen and Varo, 1967).

D. Assay Methods

The phenol-sulfuric acid method used to determine glycolipid content was reported to be more sensitive than the anthrone method for micro-determinations of sugars from glycolipids separated by TLC (Roughan and Batt, 1968). This reagent reacts quantitatively with most carbohydrates, however it does not react with all sugars to the same extent (Dubois *et al.*, 1956). Spectrophotometric readings were referred to a galactose standard curve since the major glycolipids present were DGDG and MGDG.

The galactose content could be determined to a sensitivity of 10 µg which would correspond to 43 µg MGDG. The phosphorus assay used was sensitive to 0.5 µg of phosphorus which would correspond to 12 µg of PG. To compensate for the differing sensitivity of the two assay methods the corresponding glycolipids of two chromatograms were pooled whereas one was adequate for the phosphorus determinations. Higher lipid applications on the plate caused overloading. The range in the content of the lipid classes produced some difficulty. The high content of PC resulted in readings close to the upper limit for phosphorus from single plate determinations while the low content of MGDG caused readings close to the lower limit for galactose determinations using the pooled contents of two plates.

E. Lipid Content

The major lipid components detected by the two-dimensional TLC separation of the total lipid extract from etiolated rye embryos (Fig. 32) are typical of plant tissues; lettuce leaves (Nichols, 1964), potato tubers (Galliard, 1968) and apple pulp (Galliard, 1968a). The high levels of PC and PE in the phospholipid fractions were also reported for potato tuber (Galliard, 1968) and for etiolated pea seedlings (Trémolières and Lepage, 1971; Roughan and Bordman, 1972). The higher unsaturation levels of the galactolipid fractions in comparison with the phospholipid fractions is also typical of other plant tissues (Galliard, 1968; Trémolières and Lepage, 1971; Roughan and Bordman, 1972). The similarity in the fatty acid content of PC and PE and the high level of 16:0 in the PI and PG fractions is in agreement with Galliards' (1968) findings for potato. The increase in 18:3 and the corresponding decline in 18:2 and 18:1 during the initial stages of the chilling period of cereals was also reported by Redshaw and Zalik (1968). Labeling studies on developing flax seed by Huber and Zalik (1963) suggested 18:1 may be a precursor of 18:2 and 18:3. Gurr (1971) gives evidence which indicates the polyunsaturated C_{18} fatty acids arise by the sequential desaturation of 18:1. The data here might be interpreted in terms of such conversions.

The increased level of unsaturation of the polar lipid components during the initial 3 to 4 weeks of chilling and the general increase in the percentage of PA throughout the treatment period could perhaps

reflect a change in membrane properties. Studies by Shah and Schulman (1968) on phospholipid monolayer systems indicated both the nature of the phospholipid derivative and the degree of unsaturation influenced the physical properties measured. The level of unsaturation and the polar derivatives are suggested to control the cavity size formed between the planar arranged phospholipid molecules. Stowe and Dotts (1971) postulated the enhancement of pea stem elongation following the addition of alkane derivatives of a molecular length greater than 20 angstroms as due to the added alkanes migrating into the cavities and thereby influencing the membrane characteristics. Based on these postulates an increased unsaturation level would increase the membrane flexibility. Lyons *et al.*, (1964) in a comparison of mitochondria isolated from cold-resistant and cold-sensitive plants relates the greater swelling and contracting ability of the cold-resistant mitochondria to the higher unsaturation level of their lipid components.

The lipids analyzed in this study reflect the total lipid content of the tissue. This may have masked lipid changes specific to plasma membranes or intracellular organelles. Since the lipid changes were similar for both varieties these changes appear to reflect a general response to low temperature.

CONCLUSION

Two rye varieties were used in this study, Sangaste which showed a vernalizing response and Prolific which did not. The spring variety was used as a control to differentiate between changes which normally occur during growth at low temperatures and those which might be attributed to the vernalization response.

Seeds showing visible signs of germination were grown on moist filter paper in the dark at 4°C. To check the effectiveness of the artificial chilling method and to follow the vernalizing response, seedlings were collected at weekly intervals during the chilling period and grown to heading. When the number of days from the end of the chilling period to heading were recorded a definite enhancement in the heading time of Sangaste with an increase in chilling time up to 8 weeks was evident. There is a possibility that the chilling time merely served as an extension of the growing period. However, when the total days from imbibing the seed to heading were recorded a definite vernalizing response was evident for the Sangaste seedlings.

In an attempt to gain insight into biochemical changes occurring during vernalization, the free amino acid content was determined for embryo tissue collected at weekly intervals during the cold treatment. A marked increase in the total free amino acids and amides occurred during the initial 3 weeks in the cold treated Sangaste seedlings. This increase greatly exceeded the increase noted during early growth at room temperature when seedlings at comparable growth stages were analyzed and also greatly exceeded the increase in the cold-grown spring

variety. In general the amino acid content of Prolific was less affected by the growing temperature. The higher accumulation in the cold-grown Sangaste was largely attributed to the marked accumulation of proline, alanine and glutamine. These accumulated in Sangaste to approximately double the level reached in the cold-grown Prolific.

Lipids were extracted from embryo tissue collected at weekly intervals during the chilling period. The total phosphorus and sugar content of the lipid extract was determined, the polar lipid components were separated by 2D-TLC and the individual components quantitated on the basis of their sugar and phosphorus content. The fatty acid composition of the separated lipid components was also determined. Since polar lipids are important membrane constituents any alterations in the percentage composition of the individual components or their degree of unsaturation may reflect changes in membrane properties. This study indicated lipid changes occurred during early growth at low temperatures and marked differences were evident between the growing temperatures when seedlings at comparable growth stages were analyzed. The total phosphorus content of the lipid extract on the dry weight basis was higher in Sangaste suggesting a higher phospholipid content for the winter variety, however the percentage of the individual phospholipid components contributing to the total phospholipid content showed similar variations in the two varieties.

The phenol-sulfuric method used to quantitate glycolipids is less sensitive than the phosphorus assay used in the quantitation of phospholipids. The low amount of glycolipid in the etiolated seedlings

resulted in several readings close to the lower limit of the sensitivity scale. This would likely account for the high error of estimate associated with these determinations. In retrospect this method of quantitating micro amounts of glycolipids by their sugar content is not recommended.

The unsaturation level of the fatty acid moieties of all the lipid components analyzed increased during the initial 3 weeks of chilling treatment. This corresponded with the increase in proline, alanine and glutamine. The unsaturation level was much higher for the glycolipid than the phospholipid fractions. In general changes noted in the respective lipid classes isolated from the two varieties were very similar.

Both varieties responded to low temperature with marked biochemical changes which may be a reflection of the hardening process, on the other hand these may be part of a sequence of events of the vernalizing mechanism.

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APPENDIX

Table i. Buffers for amino acid analysis

pH	2.2	3.25	4.25	5.28
Use	Sample dilution buffer	Long column eluent	Long column eluent	Short column eluent
Na Conc.	0.2N	0.2N	0.2N	0.35N
Citric H_2O	21 g	840 g	840 g	491 g
NaOH (97%)	8.4 g	330 g	330 g	288 g
Conc. HCl	16 ml	426 ml	188 ml	136 ml
Caprylic	0.1 ml	4.0 ml	4.0 ml	2.0 ml
Thiodiglycol	20 ml	200 ml	200 ml	--
BRIJ-35 sol.	2.0 ml	80 ml	80 ml	40 ml
Final Volume	1 litre	40 litres	40 litres	20 litres