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FREE AMINO ACID CHANGES ASSOCIATED WITH VERNALIZATION OF WHEAT*

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Abstract—Changes in the free amino acids in the leaf tissues of a spring and an obligate winter wheat were determined. The plants were grown for 0–5 weeks at 2° and at 25°. Seventeen known and at least seven unidentified ninhydrin positive components were detected. Only glutamic acid and lysine were more abundant at 25° than at 2°. At 2° the spring variety contained higher quantities of 14 of the amino acids, and the winter wheat contained higher quantities of alanine, glutamic acid, proline and arginine; proline being outstanding. At 25° the differences were not so marked. A general increase in the neutral and acidic amino acids in both varieties grown at 2° during the first 2 weeks was followed by a rapid decline during the subsequent week. Usually, a gentle to rapid increase followed during the final 2 weeks of growth. The basic amino acids did not follow this general pattern. The effect of growing temperature on the amino acid levels was usually greater than the varietal effect of spring vs. winter wheat. But large differences between these varieties were detected in the levels of phenylalanine, proline, tryptophan and unknown No. 1. Glutamine and asparagine + serine, which were all eluted together, usually accounted for 30–80 per cent of the total free amino acids. Alanine and proline were both very high in the cold-grown winter wheat; alanine contributed 30–35 per cent and proline 16–18 per cent of the total free amino acids during the latter part of the growth period. Similar and parallel changes were observed in some of the amino acids during the 5 weeks of growth, but there was no apparent correlation between these patterns of similar change and the common biosynthetic families of amino acids. The patterns and rather dramatic changes in levels of several of the soluble nitrogen compounds may well be significant clues to biochemical mechanisms involved in vernalization of wheat. Components which this study suggests may be of particular interest are proline, glutamine and asparagine, ammonia plus overlapping unknowns, other basic amino acids and an acidic substance tentatively suggested to be glutathione.

INTRODUCTION

SOME unique biochemical changes take place during the low temperature induction of winter wheat. Clues to these important biochemical events can probably be found in the soluble substances of low molecular weight which exhibit rapid turnover. Thus, information on vernalization can be sought by observing changing patterns in low molecular weight substances.

The total free amino acid level in wheat plants grown at 2° changes dramatically during the middle portion of the vernalization period, just before the physiological expression of these biochemical changes.[1] Since amino acids serve as important biosynthetic precursors of proteins, coenzymes and many other substances, qualitative and quantitative changes of the free amino acids in spring and winter wheat were investigated.

Pauli and Mitchell[2] found that total free amino acids in winter wheat plants increased

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[1] E. J. TRIONE, *Plant Physiol.* **41**, 277 (1966).

[2] A. W. PAULI and H. L. MITCHELL, *Plant Physiol.* **35**, 539 (1960).

during the first 2 weeks of cold treatment. Qualitative studies of free amino acids of cold-grown winter wheat plants by Kirillova[3] showed that some acids increased while others decreased. Moskov and Bozova[4] found that glutamic acid increased in a winter variety of barley during cold treatment but decreased in a spring variety; proline increased only in the winter variety. Grzesiuk and Kulka[5] found the free amino acids glutamic acid and lysine in highest concentrations in young vernalized rye seedlings, whereas aspartic acid was highest in non-vernalized rye seedlings.

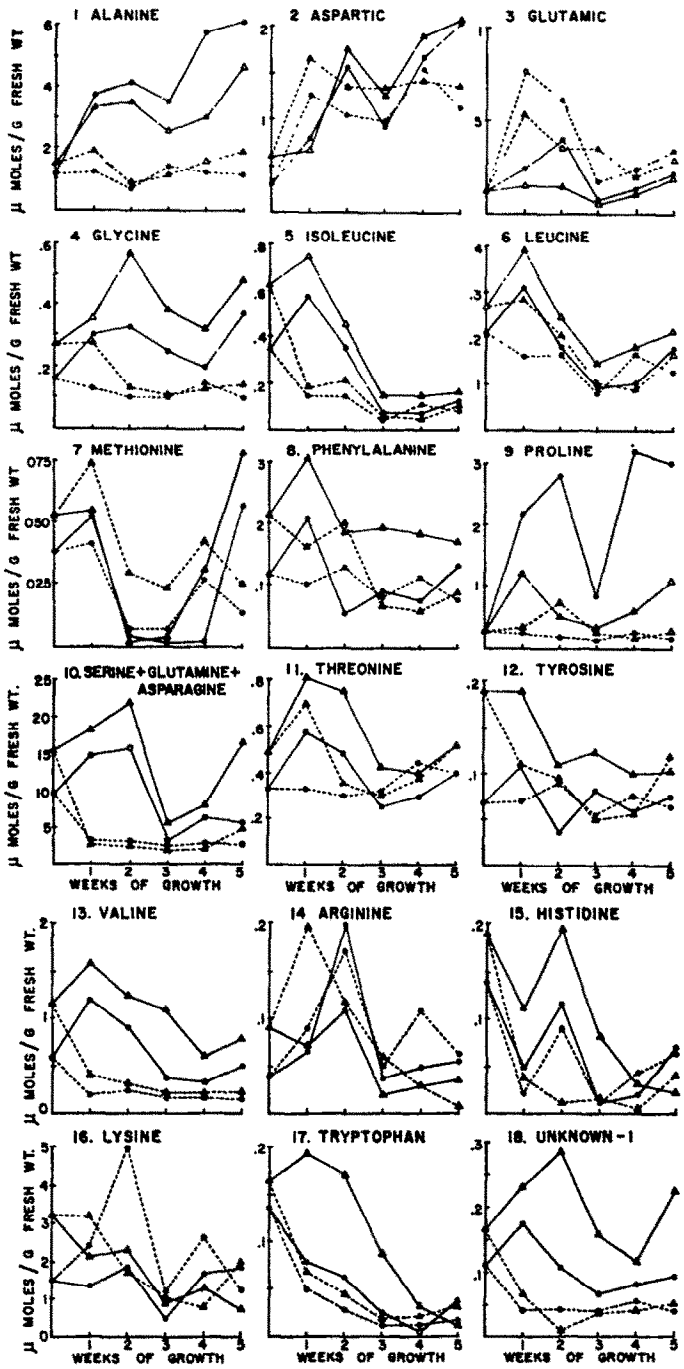
RESULTS AND DISCUSSION

The results obtained are shown in Figs. 1–18. Data are expressed as micromoles of each amino acid per gram fresh weight of leaf tissue. In addition to the seventeen known amino acids which were identified, at least seven unidentified ninhydrin positive compounds were detected. Most of these unknowns were present in very small quantities, their appearance was not consistent, and hence data on them are not presented herein. An unknown which was present in significant quantity in all tissues, however, is illustrated in Fig. 18. This unknown component came off the column about 35–45 min before aspartic acid, and calculations of its quantity were based on the aspartic acid standard. Other prominent unknowns overlapping the ammonia peak are discussed below.

The free amino acid content differed in the two wheat varieties and changed with temperature. The general effect of temperature on amino acid levels indicates that in both varieties alanine, glycine, isoleucine, proline, serine + the amides, valine, and the major unknown were present in greater quantity at 2° than at 25°, whereas only glutamic acid and possibly lysine were more abundant at 25° that at 2°. Alanine, glutamic acid, arginine and particularly proline were in greater amounts in the winter wheat than in the spring wheat at 2°, but aspartic acid, glycine, isoleucine, leucine, methionine, phenylalanine, serine + amides, threonine, tyrosine, valine, histidine, lysine, tryptophan and unknown No. 1 were all more abundant in the spring wheat than in the winter wheat. At 25°, the differences in amino acid patterns between these two varieties were not so marked; histidine and lysine were slightly higher in the winter wheat, but aspartic acid, methionine, phenylalanine, and tyrosine were more abundant in the spring wheat.

The amino acid patterns changed with time as well as with variety and temperature. At the beginning of the temperature treatment the young etiolated seedlings of the spring wheat contained more of every free amino acid except glutamic acid and proline than the winter wheat seedlings. In both varieties grown at 2°, during the first 2 weeks there was a general increase in all except the basic amino acids histidine, lysine and tryptophan. Nearly all of the amino acids declined rapidly during the middle portion of the growth period at 2°. This was usually followed by a gentle to rapid increase in amino acid level during the final 2 weeks of growth. In the initial 2 weeks of growth at 25°, the patterns of change were more variable: in both varieties: four amino acids increased during this period, six maintained the same level, and eight tended to decrease. During the final 2–3 weeks of growth at 25°, most of the amino acid levels did not change appreciably in either the spring or winter wheat. However, arginine and lysine did change significantly during this period and it is noteworthy that the arginine and lysine curves followed similar patterns.

[3] G. A. KIRILLOVA, *Fiziol. Rast.* **5**, 175 (1958).
[4] I. MOSKOV and L. BOZOVA, *Comp. Rend. Acad. Bulgare Sci.* **15**, 559 (1962).
[5] S. GRZESIUK and K. KULKA, *Acta Soc. Botan. Polon.* **32**, 313 (1963).



FIGS. 1–18. THE EFFECTS OF TIME AND TEMPERATURE ON THE CHANGES IN THE FREE AMINO ACIDS IN SPRING AND WINTER WHEAT VARIETIES. (Weeks of growth at indicated temperature following 6-day germination in the dark.)

○ ———— ○ Winter wheat grown at 2°;
△ ———— △ Spring wheat grown at 2°;
○ - - - - - ○ Winter wheat grown at 25°;
△ - - - - - △ Spring wheat grown at 25°.

Methionine was the only sulfur-containing amino acid identified in significant but very low amounts. However, cysteine, the other common sulfur amino acid, was occasionally present in trace amounts. Cysteine is also present in the tripeptide glutathione, the only soluble small molecular weight sulfhydryl compound found in detectable quantities in wheat leaves.[6] Unknown-1 eluted where reduced glutathione emerges. *S*-methylcysteine sulfoxides and sulfones also overlap the position, however, so that identification is not clear.

The amounts of the individual amino acids at the different growth stages varied considerably (Figs. 1–18), but when calculated as a percentage of the total free amino acids the variation was much less. The amides + serine usually accounted for 30–80 per cent of the total free amino acids; alanine 10–20 per cent; aspartic acid 5–20 per cent; valine 3–7 per cent; proline 2–8 per cent; glutamic acid 1–5 per cent; glycine, isoleucine, leucine, threonine and lysine individually accounted for less than 5 per cent; cysteine, methionine, phenylalanine, tyrosine, arginine, histidine, tryptophan and unknown-1 individually accounted for less than 1 per cent. Alanine and proline were both very high in the cold-grown winter wheat; alanine contributed 30–35 per cent and proline 16–18 per cent of the total free amino acids during the latter part of the growth period. This level of individual free amino acids is in agreement with other analyses of plant tissues,[7] except that proline and valine were much higher, and arginine much lower than expected.

The chromatographic method normally used with protein hydrolysates does not resolve gamma-aminobutyric acid. This is unfortunate since it is now considered unusual not to find it among the free amino acids of plants. Often it appears in appreciable quantities and may be an important indicator since it derives from the decarboxylation of glutamic acid. This takes on added interest in view of the present results on glutamic acid, glutamine, proline, ammonia and the known interrelationships between these components.

Similar and parallel changes were observed in some of the amino acids during the 5 weeks of growth, but there appears to be little or no relation between these patterns of similar change and the common biosynthetic relationships of the amino acids. For example, alanine and valine are closely related biosynthetically but do not follow parallel changes in the wheat plants. Since these data represent gross changes in free amino acids in green leaves, it is unlikely that any direct relation between biosynthesis of amino acids and total free amino acid levels could be detected.

Most of the changes in amino acid levels were in the same direction and of the same order of magnitude in both the spring and winter wheat leaves. In general, the effect of the growing temperature on the amino acid levels was greater than the varietal effect of spring vs. winter wheat. Substantial differences in the levels of phenylalanine, proline, tryptophan and unknown No. 1 between spring and winter wheat leaves were detected however.

The significance of the dramatic proline accumulation is unknown, but it may be a useful indicator to the vernalization process. The rapid decrease in many of the amino acids during the 2nd and 3rd week of growth at 2° suggests an enhanced synthesis of proteins or peptides in which unusually large amounts of proline may be involved. During this period of growth the total soluble proteins of both the spring and winter wheat leaves were increasingly at a gentle uniform rate. At week 3, there was a distinct increase in soluble protein of the 2° grown winter wheat concurrently with a rapid decrease in total free amino acids.[1] This is consistent with the rapid decrease in individual amino acids during this same period as

[6] Y. WAISEL, H. KOHN and J. LEVITT, *Plant Physiol.* 37, 272 (1962).
[7] F. C. STEWARD, F. CRANE, K. MILLAR, R. M. ZACHARIUS, R. RABSON and D. MARGOLIS, *Symp. Soc. Expl. Biol.* 13, 148 (1959).

noted above. Even if there had not been an evident increase in soluble protein nitrogen, this would not preclude the possible synthesis of specific protein or peptide which could easily have gone undetected in the overall protein data.

Whether these synthetic events at week 3, including an equally dramatic accumulation of oligosaccharides,[1] are directly or only coincidentally related to mechanism(s) of vernalization is a sizeable question yet to be answered. That these events happen most strikingly in the winter wheat at 2° as contrasted to activity at 25°, and that they occur just prior to the physiological expression of flower induction suggests something more than mere coincidence. Certainly the changes offer more solid leads for deeper probing than have been available heretofore.

Other possibly important clues reside with ninhydrinpositive constituents overlapping the ammonia peak. Both varieties grown at 2° show large quantities of at least one unknown on the trailing edge of the ammonia elution peak. This unknown(s) was very high in the winter variety during the first 2 weeks of growth at 2° but decreased sharply by week 3 and was virtually absent after 5 weeks. In the spring variety at 2° this unknown receded to a less pronounced minimum at 3 weeks but then increased substantially by week 5, giving a very long, broad-based tail on the trailing edge of the strong ammonia peak. The amount of the unknown was somewhat greater in the spring than the winter variety. Initial quantities of ammonia and unknown(s) appear roughly equivalent at several micromoles each per gram fresh weight of tissue. Precise values could not be calculated because of the overlapping and non-symmetry of the peaks. The patterns of ammonia and unknown(s) would roughly parallel those of the amides + serine, except that maximums would appear at week 1 rather than week 2.

At 25° the unknown(s) on the trailing edge of the ammonia peak was not evident in either variety, but in both varieties at 25° a roughly constant ammonia peak was partially overlapped on the leading edge by a variable intensity peak. (This elution position on the leading edge of the ammonia peak is normally occupied by ethanolamine.) The quantities of ammonia and of unknown were much less at 25° than at 2°. The behavior of the unknowns on the leading and trailing edges of the ammonia peak indicate that they are different substances.

The possible presence of an inhibitory substance as a factor contributing to the vernalization requirement should be considered. It is tempting to postulate that the disappearance of the prominent unknown(s) trailing ammonia in the cold grown winter wheat may reflect removal of inhibitory substance. The relatively high initial levels of ammonia (not shown), amides + serine, methionine, tyrosine, histidine, and tryptophan, indicate protein degradation prior to the synthetic or utilization events evident at week 3. This is in line with Fowden's observation[8] that certain amino acids such as methionine, cysteine, tyrosine, and histidine are rarely found in the free state except under conditions of rapid protein breakdown.

It is interesting to compare the amino acid patterns in the spring wheat grown at 25° and the winter wheat grown at 25°. The spring wheat was induced to flower and was in the boot stage of heading after 5 weeks of growth, whereas the winter wheat remained vegetative. This physiological difference as not reflected in the levels of the neutral and acidic amino acids, but marked differences were observed in the levels of the basic amino acids, arginine, histidine and lysine. It has been suggested that these basic amino acids play lead roles in dormancy and senescence, or at least are sensitive indicators.[9]

[8] L. Fowden, *Symp. Soc. Expl. Biol.* **13**, 283 (1959).
[9] A. W. Naylor, *Symp. Soc. Expl. Biol.* **13**, 193 (1959).

These variations in basic amino acids may reflect differences in histone metabolism between the variety which remained vegetative and the variety undergoing reproductive development. It is a basic tenet of modern biology that physiological changes are the expression of prior biochemical changes at the genetic level. The correlation of histone metabolism with genetic function, and with observable differentiation processes, is noteworthy.[10]

Little is known about the pools of amino acids in plant tissues; namely, whether large quantities of amino acids can be stored in more than one way or whether significant quantities of peptides are stored. MacLennan *et al.*[11] indicated the existence of pools of amino acids in wheat leaves which were not in equilibrium with the turnover pools. The intracellular location of the various functioning pools in plant tissues remains conjectural although there are several recent reports on the subject.[12,13]

We hope that the interesting leads suggested by these data will encourage further exploration with other plants having obligate cold requirements for flower induction. Because they appear most dominantly involved and because of the analytical difficulties, predominant attention is indicated for the resolution of serine, asparagine, glutamine, proline, the basic amino acids including ornithine, gamma-aminobutyric acid, ethanolamine and the prominent unknowns leading and trailing the ammonia peak. More rigorous amino acid separation procedures than are satisfactory for hydrolysates of purified protein must be used to resolve these components.

EXPERIMENTAL

Plant Material

The wheat varieties used in this study were "Elgin", a winter wheat (*Triticum compactum* Host), and "Red Bobs", a spring wheat (*T. aestivum* L.). This winter wheat has an obligate cold requirement of about 4–5 weeks for flower induction.[1] Seeds of both varieties were obtained from R. J. Metzger, plant geneticist, U.S. Department of Agriculture, Corvallis, Oregon. The plants were cultured as previously described.[1] Germination and initial seedling growth occurred in the dark at 20°. Six days after seeding, half of the boxes containing the young etiolated seedlings were moved into a 2°, 10,700 lux controlled environment; the other half were moved into a 25°, 100 lux, controlled environment. This procedure was repeated once each week until five such sets of seedlings were available in each environment. Thus, the seedlings ranged from 0 to 5 weeks in length of time grown at the 2° and 25° temperatures. The seedlings grew uniformly and vigorously. Healthy, turgid, uniform, lower leaves from plants grown in the cold or warm environments for 0–5 weeks were harvested 5 hr after the beginning of the daily photoperiod and used for analyses.

*Analytical Methods**

Immediately after harvest uniform wheat leaves (4·00 g fresh weight) were selected and were frozen in liquid nitrogen and ground in a mortar to powder form; 20 ml of 80% ethanol was added, and the mixture was homogenized in a Duall, conical, glass-to-glass homogenizer

* Mention of a commercial product is for identification of material used, and does not imply endorsement by the U.S. Department of Agriculture.

[10] J. BONNER and P. Ts'o, *The Nucleohistones*, Holden-Day, San Francisco (1964).
[11] D. H. MACLENNAN, H. BEEVERS and J. L. HARLEY, *Biochem. J.* **89**, 316 (1963).
[12] A. OAKS, *Plant Physiol.* **40**, 142 (1965).
[13] A. OAKS, *Plant Physiol.* **41**, 173 (1966).

at 1000 rev/min for 2 min. The homogenate was centrifuged at 17,000 g and the residue discarded. The supernatant was lyophilized and then stored for several days at −15°.

The free amino acids in the lyophilized samples were partially purified and separated into basic, and neutral and acidic fractions according to the procedures of Thompson *et al.*[14] The basic amino acids were retained on a Dowex 50W-X4 ion exchange resin in the NH_4^+ form and were eluted with 2 N NH_4OH. Neutral and acidic amino acids were retained on a Dowex 50W-X4 resin in the H^+ form, and were also eluted with 2 N NH_4OH, followed by deionized water. All procedures were carried out in a cold room at 2–4°. The effluents were frozen immediately and lyophilized within a few hours.

The lyophilized samples were each taken up in a small amount of deionized water, and the total amino acids in an aliquot of each sample were determined by Rosen's ninhydrin method.[15] From these data the appropriate volumes required in the amino acid analyzer were calculated. The individual amino acids in each sample were then separated and quantitatively determined in a Phoenix Amino Acid Analyzer, Model K-8000, which operated according to the procedures for protein hydrolysates described by Spackman, Stein and Moore.[16]

The amides, glutamine and asparagine, nearly always emerge together and are particularly difficult to resolve from other components with present chromatographic systems. In the standardized procedure used here, the amides eluted with serine. Serine value of plants commonly are the same order of magnitude as threonine and glycine, and there is no evidence or reason to suspect sharp divergence from that pattern here. Such information coupled with recorded values for soluble serine and amide content of plant leaves under a range of environmental conditions[7] leaves little doubt that glutamine is the predominant constituent in our serine and amides peak.

[14] J. F. Thompson, C. J. Morris and R. K. Gering, *Anal. Chem.* **31**, 1028 (1959).
[15] H. Rosen, *Arch. Biochem. Biophys.* **67**, 10 (1957).
[16] D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.* **30**, 1190 (1958).