

Mobilization and Metabolism of Protein and Soluble Nitrogen during Spring Growth of Apple Trees

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Abstract. The extractable protein in bark tissue of 1-year-old shoots of apple trees (*Malus domestica* Borkh. cv. Golden Delicious on Malling (M) 26 rootstocks) declined dramatically during spring growth, while amino acids increased. Extensive degradation of protein was first visible at the silver-tip stage of growth. Prior to this period, some redistribution of nitrogenous compounds was indicated by the increase in amino acids while protein remained unchanged. Total extractable nitrogen declined during spring growth, indicating that it was mobilized to developing tissues. *In vitro* activity of an acid endoprotease increased rapidly upon regrowth, but its increase preceded the *in vitro* decline in proteins. The autolytic activity, however, did not change in the early growth stage, and protein had already declined 50% when this activity significantly increased. Electrophoretic separation of intact proteins indicated a substantial shift in relative mobility from higher to lower molecular weights as the season advanced. However, the majority of proteins showed little evidence of net breakdown during early spring growth. Two polypeptides of 38,000 and 56,000 daltons which were present up until mid-April were not detected in extracts of shoots collected in May. The 65% decline in total protein observed by May 10, however, could not be accounted for by the loss of these 2 peptides. It is thus concluded that the immediate requirement of nitrogen at the early stage of growth (up to silver-tip) is met by the transport of soluble nitrogen present in adjacent bark and that redistributed from the wood. The large requirement of nitrogen after silver-tip stage of growth is then met by massive breakdown of storage proteins.

The utilization of storage proteins is one of the early processes of germinating seeds in supplying N for developing tissues. The mechanisms involved in the degradation of these storage proteins have recently received considerable attention (1, 3). Various proteolytic enzymes have been characterized in some detail, though the activity profiles of these enzymes are not always correlated with the depletion of proteins during growth (1, 3, 5). While most of the research on the use of protein N has been focused on annual seed plants, comparable information in woody deciduous fruit trees is extremely limited.

For the past several years, we have studied the annual cycling of N transformations in the apple: mobilization, conservation and reutilization of nitrogenous compounds. It has been established that mobilization of leaf N during autumnal senescence involves a series of biochemical and enzymatic changes in senescing leaves, and those changes are closely related to environmental conditions such as daylength and temperatures (16). The majority of the imported N, if not all, is incorporated into proteins in woody structural parts of the tree, especially in the bark (7, 12, 16). We have demonstrated specific enzymatic steps which lead to a substantial accumulation of bark proteins in the autumn (8). The reutilization of these proteins the following season is catalyzed by proteolytic enzymes. An acid endoprotease present in dormant apple shoot bark has been characterized in some detail (6). A summary of our current knowledge on N recycling has been presented elsewhere (17).

However, the mechanisms involved in N recycling of apple trees are not well established, especially those involved in the

reutilization of stored N in bark in the spring. In this communication, we report some steps in the sequence of events occurring in the spring in relation to the use of N derived from proteins and amino acids in shoot bark tissue of the apple. The isolation of those proteins which may function primarily as storage forms of N is also examined. A clear understanding of the use of protein and amino acid N in woody tissues of fruit trees at the early stage of spring growth is of particular importance since this process takes place when environmental conditions for absorption of nutrients and their translocation to tree-tops are not always optimal (2, 4, 9).

Materials and Methods

Plant materials. One-year-old shoots were collected randomly from 160 of 7-year-old 'Golden Delicious' apple trees on M 26 rootstocks, about 100 shoots on each sampling date. Bark was peeled off from mid-shoot sections, pooled, and extracted for proteins, enzymes, and amino acids. The sampling period extended from January to May 10 of 1980, but data presented are those from March 10 to May 10 at 10-day intervals, since the data obtained in January and February were almost the same as those of March 10.

Protein extraction and enzyme purification. The extraction of protein from the bark and the purification of a hemoglobin-hydrolyzing enzyme (Hgb-ase) were as described previously (6). The extraction buffer (0.1 M phosphate-sodium citrate, pH 6.0) contained 0.4 M NaCl, 6 mM cysteine, and insoluble polyvinylpyrrolidone (PVP). The protease isolation involved (NH₄)₂SO₄ fractionation followed by gel filtration through a Sephacryl S-200 Superfine column, then hemoglobin-coupled Sepharose 4B affinity column chromatography.

Enzyme assay. Hgb-ase activity was measured at pH 4.6 as described previously (6), by using approximately 40-fold purified enzyme preparations. The autolytic activity (which may be defined as the activity of enzyme which degrades its native proteins) was measured by monitoring the changes (increases) in ninhyd-

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rin-positive compounds before and after the incubation of a portion of crude extracts at 40°C for 2 hr. The 0.9-ml reaction mixtures were adjusted to contain about 0.2 mg protein in the assay buffer, 0.1 M sodium citrate, pH 4.6. The reaction was terminated by adding 0.1 ml of 50% (w/v) trichloroacetic acid. The protein precipitate was removed immediately by centrifugation for 3 min (Beckman Microfuge B), and a portion of supernatant was used to determine the ninhydrin-positive compounds. To insure measurement of autolytic activity, assays were also conducted over different time intervals and with varying amount of extracts added to the assay buffer.

The proteolytic activities are expressed as μ moles of L-leucine equivalent/g fresh tissue/hr under the standard assay conditions described above.

Polyacrylamide gel electrophoresis. The undenatured proteins were electrophoresed using 7.5% polyacrylamide gels with Tris buffer at pH 8.9, according to Davis (5). Approximately 0.1 mg of protein was applied to the gels, and the stock buffer was diluted to 1/5 rather than 1/10. The gels were run at 2 mA/gel for 2.5 hr.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted according to Weber and Osborn (20). The running buffer contained 0.1% SDS in a Tris-glycine system at pH 8.3. Separation was not improved by increasing the concentration of SDS in the running buffer. The proteins were stacked at 1 mA/gel and separated at 2 mA/gel. Bromophenol blue was used as a tracking dye. Gels were stained with Coomassie blue R (0.5% in 7% acetic acid), destained in acetic acid-methanol-water (1:3:9, v/v/v), and then scanned at 600 nm with a gel scanning attachment on a spectrophotometer (Beckman Model 25). The absorbance scale shown in the scanning profiles (Fig. 3 and 4) is relative. For molecular weight (MW) estimations, phosphorylase b, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, soybean trypsin inhibitor, and lysozyme were used as marker proteins.

Fractionation of total bark proteins. For these experiments, the total bark protein was extracted with 0.1 M Tris-HCl, pH 8.0, containing 0.4 M NaCl, 6 mM cysteine, and insoluble PVP. The proteins extracted with this medium were then fractionated by diethylaminoethyl (DEAE)-cellulose column chromatography (7, 13). The column was eluted using a stepwise gradient of 0.1, 0.2, and 0.3 M NaCl in sodium borate buffer (pH 9.5), eluting peaks I, II, and III proteins in that order.

Protein, protein-N, and amino acid determinations. Protein was determined as described previously (6), using BSA as a standard. Protein-N was estimated by dividing the amount of protein by 6.25. The amino acids in the bark prepared as above were extracted with 80% boiling ethanol, and the ninhydrin-positive compounds were determined by the method of Yemm and Cocking (21). Standards were prepared from L-leucine.

Replication of the experiments. Except for the protein fractionation experiments which are the averages of 2 determinations on each sampling date, all analytical data presented are averages of at least 9 readings; at least 3 determinations from 3 independently prepared samples on each date. Wherever appropriate, standard deviations are presented by vertical bars.

Results

Protein decline started in early April, followed by an extensive decline up to early May (Fig. 1). Protein declined 65% during this 2-month period, from 14.2 to 5 mg/g, at a rate of about 2 mg/g fresh tissue/10 days. This decline began to level off in early May. An increase in amino acids was observed 10 days before the protein decline, although the amount of amino acids increased in this

period is not considered significant (Fig. 1). Amino acids were the highest in the sample collected on April 20, after which they decreased rapidly. The total extractable N also started to decline in early April (Fig. 1), suggesting that N was mobilized to developing tissues.

Hgb-ase activity had increased with the March 20 sample (Fig. 2A). This activity further increased about 2-fold within the following 10 days and then remained at a relatively constant level of activity at about 35 units throughout April. Autolytic activity did not increase significantly until late April, exhibiting its highest activity with the May 1 sample (Fig. 2B). Both Hgb-ase and autolytic activity dropped rapidly in May to the level obtained with the samples from dormant bark.

Specific activities of both enzymes did not change much until early April (A and B of Fig. 2). They increased rapidly in late April apparently due to both increases in enzyme activities and a decline in proteins. Specific activities levelled off in early May in both cases.

Fractionation of proteins into 3 groups by DEAE-cellulose column chromatography and the changes in their proportion to total proteins revealed a partial loss of each elution peak protein as compared to its level in dormant tissue (Table 1). Although total protein declined as growth resumed, not all 3 groups of proteins exhibited the same rate of breakdown. While the peak I proteins had declined 15% between March 20 and April 1, the proteins in peaks II and III remained unchanged. Both peaks I and II lost approximately 30% of their proteins in early April, the peak III proteins were at the early March level. The peak I proteins did not decrease after April 20, whereas the other 2 fractions, especially the peak III proteins, had undergone extensive hydrolysis after that date. Such a differential rate of hydrolysis affected the relative proportion of each group to total proteins. At the dormant stage, total protein was composed of 45, 43, and 12% of the elution

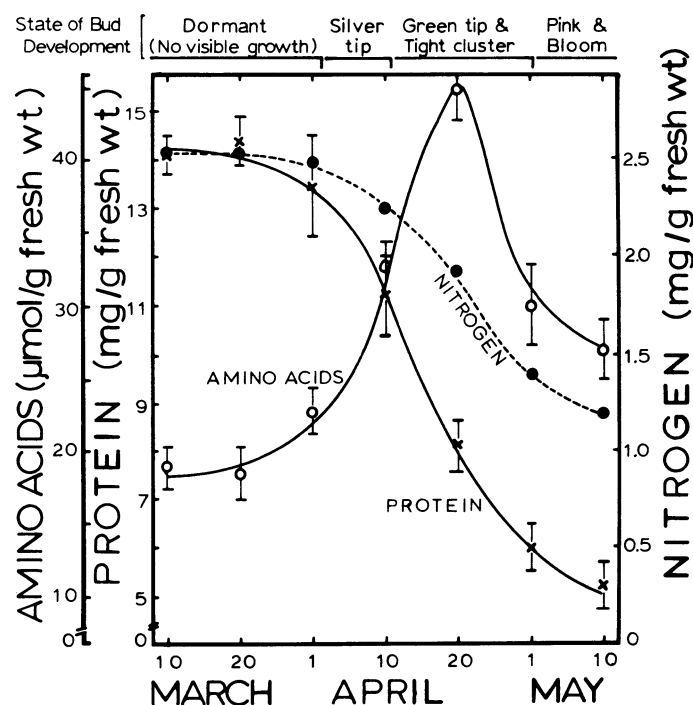


Fig. 1. Changes in proteins and amino acids extracted from shoot bark of the apple during the spring growth period. Also shown are the changes in the extractable nitrogen found in protein and amino acids.

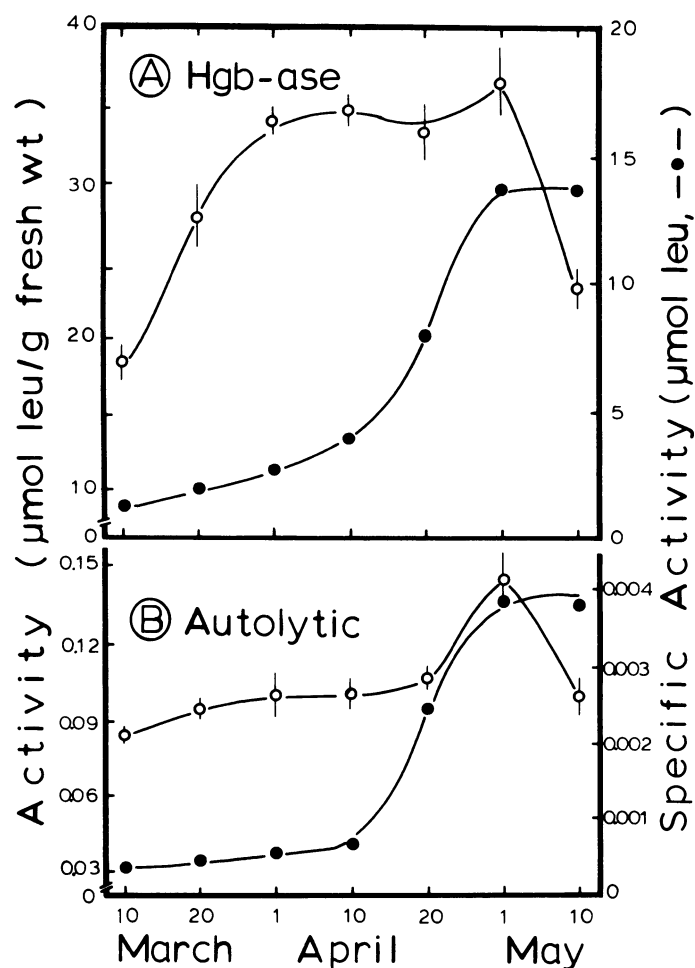


Fig. 2. Changes in the activities of the Hgb-ase and autolysis during spring growth. The enzymes were extracted from the shoot bark and, in the case of the Hgb-ase, purified approximately 40-fold as described in the text.

peaks I, II, and III proteins, respectively. These proportions had changed to 52, 34, and 13% in early May.

Fig. 3 shows the scanning profiles of total extractable proteins separated by electrophoresis. Two typical electrophoregrams are shown: the March 10 and May 1 samples. Prominent protein species were found concentrated at a relative mobility (R_m) of 0.39 on March 10. However, the R_m of the highest protein peak shifted 0.15 toward the anode on May 1. The difference in MW calculated from the peak shift between these 2 samples was about 140,000 daltons. Although not shown, the shift in this prominent protein peak was first visible on April 10. It was difficult to sepa-

rate single protein species in the undenatured condition due to highly diffused nature of the protein bands.

Changes in protein profiles are much more evident when the proteins were electrophoresed in the presence of SDS (Fig. 4). The majority of the peptides were separated within MW ranges of 20,000 and 100,000 daltons, i.e., the R_m values between 0.2 and 0.6, with the March 10 samples, and they exhibited little modification throughout the season. On May 1, however, 2 peptides at R_m values of 0.225 and 0.34 had nearly disappeared. The MWs of these peptides were estimated at 56,000 and 38,000 daltons, respectively. Peptides of less than 20,000 daltons first disappeared and later accumulated. The relative proportion of those peptides with R_m values between 0.6 and 0.9 to total proteins generally and gradually increased as the season advanced.

Discussion

The amount of N in proteins and amino acids and their relative importance in supplying N in the spring have been of great concern (7, 10, 16, 19). Of the total extractable N in dormant shoot bark, about 90% of the N was found in proteins with about 10% in the ethanol soluble fractions (Fig. 1), in agreement with our previous report (7). This proportion of protein-N to total extractable N decreased to approximately 70% at mid- to late April. The ratio remained essentially unchanged during the rest of the experimental period. Thus, the decline in the ratio of protein-N to total N was not as dramatic as the decline in the total protein content (65%) during the whole period. It is likely that during the later stages of the spring growth, new protein synthesis occurred in the bark, and this might have contributed in maintaining the ratio of protein-N to total N at a relatively constant level. However, it is more likely, in addition to the amino acids released by protein hydrolysis, the active absorption of soil N and its translocation to tree-tops also as amino acids are responsible for the constant ratio of protein-N in the bark. When there exists an excess amount of N, the rate of protein synthesis may not keep up with the N supply, resulting in an increase in the soluble N (18) which can be mobilized to developing tissues.

The drastic protein decline was first visible on April 10, when the shoot samples were at the silver-tip stage of growth. However, amino acids increased prior to the decline in proteins, suggesting that there was a redistribution of amino acids from wood to bark tissue. O'Kennedy et al. (12) reported that amino acids recovered from dormant wood tissue were redistributed in the spring, resulting in an increase in soluble N in the bark while protein remained unchanged. Even if the quantitative contribution of the redistributed N to total N level is small, its physiological significance should be emphasized, especially at the early stage of growth. It is also noteworthy that, when interpreting the seasonal changes in the level of amino acids, the ninhydrin-positive compounds with leucine as a standard do not necessarily reflect total soluble N

Table 1. Total extractable protein and elution peaks I, II, and III proteins recovered from a DEAE-cellulose column. The amount of protein is expressed as mg/g fresh weight of bark tissue.

Season	State of bud development	Total		Peak I			Peak II			Peak III		
		mg	% loss	mg	% total	% loss	mg	% total	% loss	mg	% total	% loss
March 10	Dormant	14.2	0	6.4	45	0	6.1	43	0	1.7	12	0
March 20	(No visible growth)	14.5	0	6.6	45	0	6.2	43	0	1.7	12	0
April 1		13.5	7	5.6	41	15	6.2	46	0	1.7	13	0
April 10	Silver-tip	10.9	25	4.8	44	27	4.4	40	29	1.7	16	0
April 20	Green-tip	8.1	44	3.0	37	55	3.6	44	42	1.5	19	12
May 1	Tight cluster	6.1	58	3.2	52	52	2.1	34	66	0.8	13	53
May 10	Pink & bloom	5.1	65	2.8	55	58	1.8	35	71	0.5	10	71

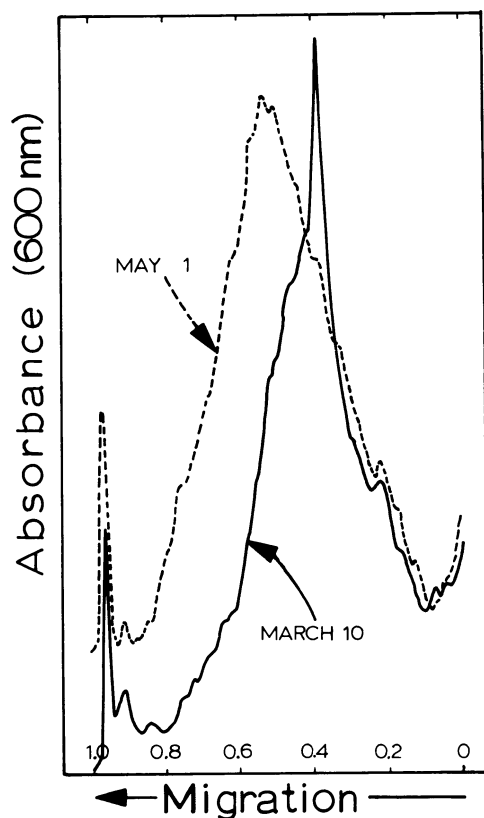


Fig. 3. Scanning profiles of total bark protein extracts (undenatured) prepared from the samples of March 10 and May 1.

levels. Interconversions between high (such as arginine and the amides) and low N (such as alanine) amino acids can always occur.

We have previously reported the presence of an acid endoprotease in dormant apple shoot bark, and that the enzyme is associated with the metabolism of storage proteins which accompanies bud break upon regrowth (6). The first rapid increase in the endoprotease activity was observed prior to the measurable decline in proteins (Fig. 2A). This is not unexpected since a high activity of the endoprotease does not necessarily involve the decline in proteins unless exoproteases act upon the new termini of protein molecules generated by the endoprotease. On the other hand, the measured activity of the endoprotease could be questioned by the fact that such a pre-existing enzyme could be activated *in vitro*. It is possible that the disruption of compartmentation, for instance, liberated the enzyme which exhibited *in vitro* activity without reflecting actual *in vivo* activity.

The autolytic activity would be more meaningful in interpreting the developmental data simply because it involved no foreign substrates. However, its activity was too low compared to the endoprotease, and the significant increase in its activity was observed only after protein had already declined more than 50%. Also, the specific activity of the endoprotease far exceeded that of autolysis, although they both increased 10-fold during the regrowth period. Such a discrepancy between proteolytic activity and protein depletion is not unusual (1, 3, 5). The choice of substrate as well as the assay conditions have been of concern in detecting the real developmental changes in proteolytic activity (1, 15). Changes in pH optimum of autolytic activity at different developmental stages may also be involved (11). Nonetheless, the possible inhibition of autolysis due to the accumulation of end

products *in vitro* is ruled out in this study since the assays were conducted in those ranges where the reaction products are directly proportional to the amount of protein extracts added to the reaction mixtures.

Despite the fact that there is no widely accepted definition of storage proteins in woody plants, their presence has been suggested (10, 18). O'Kennedy and Titus (13) were the first to isolate those proteins which may function primarily as storage forms of N in apple bark tissue. The preparative separation of total extractable protein into 3 groups of proteins by DEAE-cellulose column chromatography has been employed. Here, we have monitored changes in groups of such proteins during the spring growth period (Table 1). The elution peak I proteins appeared to be composed of proteins which were the most sensitive to breakdown, although the peak II proteins exhibited about the same degree of loss as the peak I proteins at the silver-tip stage of growth (April 10). At this time, however, there was little change in the peak III proteins.

The finding that the peak III proteins were the last to be degraded is in contrast to that reported by O'Kennedy and Titus (13). They reported the opposite results with MM 106 rootstocks in a controlled environment. In that study, peak III proteins were the most sensitive, losing more than 60% of their proteins within 7

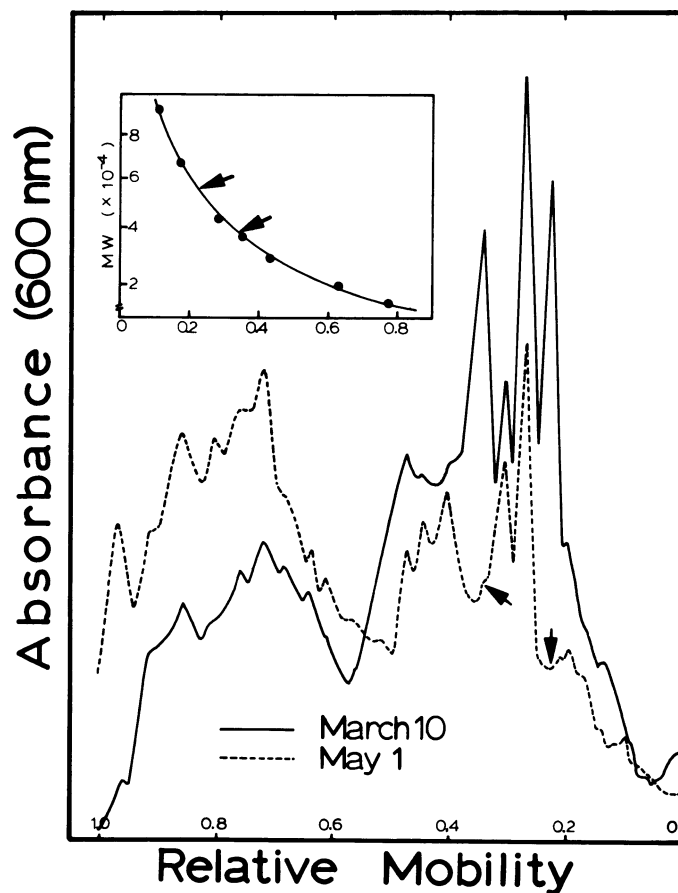


Fig. 4. Scanning profiles of total bark protein extracts (denatured) prepared from the samples of March 10 and May 1. Proteins were electrophoresed in the presence of 0.1% sodium dodecyl sulfate, using 12.5% polyacrylamide gels. Inset shows the standard curve obtained from co-running marker proteins (see Materials and Methods). Arrows indicate the peptides which disappeared during the later stages of spring growth.

days at 25°C, while peak I and II proteins lost about 20%. It has been observed for the past several years that the rate and the degree of accumulation of peak III proteins in the autumn and their disappearance during regrowth appear to be temperature-dependent. The vigor of the shoots is also involved. The proportion of this group of proteins to total proteins observed in this study with the dormant sample (12%) was significantly less than that in the MM 106 rootstocks (30%) (13). It thus appears that the level of peak III proteins in the dormant trees may be the key factor in determining the rate of degradation among those groups of proteins. The rate of disappearance might have been different if the level of peak III proteins was that high with the dormant sample in this study. It is therefore extremely difficult to designate which peak protein represented the majority of storage proteins. All 3 elution peak proteins declined upon regrowth of shoots, even though not at the same rate.

The electrophoretic separation of total extractable protein may more accurately reflect the seasonal changes in protein composition during spring growth. With the intact proteins, it is evident that the major changes in the protein profiles were the shift of protein peaks from higher to lower MW ranges (Fig. 3). Some of the major proteins might have undergone some modification as the season advanced, resulting in the more lower MW species of proteins. It is difficult to clearly identify individual proteins under these conditions. The association of carbohydrate molecules to approximately two-thirds of total proteins has been demonstrated in apple bark (13).

Under dissociated conditions, however, it was possible to identify 2 peptides which were present during dormant state but absent in the samples of May 1 (Fig. 4). Those peptides, 38,000 and 56,000 daltons, are considered as storage proteins which function to supply N for developing tissues. However, their proportions to total protein were not large enough to account for the overall 65% decline in proteins observed during the experimental period. Furthermore, these peptides were still present in the April 20 sample, indicating that they were not the first ones to disappear upon regrowth. These peptides, therefore, may be significant components of storage proteins in apple bark, but others must be involved.

Scanning profiles of low MW peptides of less than 20,000 daltons, exhibited a substantial fluctuation during the regrowth period. A possibility exists that protein molecules are constantly turning over, which gives rise to such fluctuation. In addition, the action of endoprotease(s) would have generated such low MW peptides initially, which then would have been attacked by exoproteases. This may be a logical assumption, although as yet not demonstrated. Also, during the warm spells at the later stages of spring growth, there may be new protein synthesis, generating the lower MW proteins.

It is generally believed that protein hydrolysis is one of the early processes in germinating seeds (1, 3). However, the role of proteins in supplying N for developing tissues of an apple tree at the early stages of regrowth appears to be in need of careful evaluation. The results of this study suggest that soluble N may be more important than protein-N at least up to the silver-tip stage of growth. The rapid increase in amino acids which preceded the decrease in protein (Fig. 1), the small increase in autolytic activity (Fig. 2), and lack of massive breakdown of proteins at the early stage of regrowth (Figs. 3 and 4) support this conclusion. Therefore, a significant finding in this study is that the immediate requirement for N during early regrowth of woody tissues may be met by the transport of soluble N present in adjacent bark tissue and that redistributed from the wood. When the terminal buds go through the silver-tip stage of growth and afterwards, the massive

requirement of N may then be met by the soluble N set free from protein hydrolysis. This conclusion, however, does not necessarily mean that the period of regrowth is separated into 2 distinct phases with regard to the use of N. It does emphasize the relative importance of amino acids in supplying N especially at the early stage of spring growth of an apple tree.

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