AN ABSTRACT OF THE THESIS OF

Richard P. Sandstrom for the degree of Master of Science

in Forest Science presented on April 29, 1977

Title: INCORPORATION OF $^{15}NO_3$ INTO AMINO ACIDS OF DOUGLAS-FIR XYLEM SAP

Abstract approved: ______________________

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Roots are considered to be the major production site of amino acids that appear in the xylem sap of conifers. The carbon skeleton of amino acids is derived from photosynthetically reduced carbon dioxide while the nitrogen is derived from the inorganic forms taken up by the roots. An experiment was conducted to determine the extent of amino acid synthesis from the products of photosynthesis and root assimilated nitrogen.

Douglas-fir seedlings were exposed to $^{14}CO_2$ in the atmosphere and $^{15}NO_3$ in both a solution and a soil rooting medium during a 24-hour period; 12 hours of light followed by 12 hours of dark. Amino acids were extracted from the xylem sap of both roots and stems. The N-TFAA n-butyl esters of the amino acids were formed to allow the separation and quantification of individual amino acids by gas-liquid chromatography. A gas-liquid chromatograph-ionization chamber system was designed to

determine the ^{14}C specific activity of individual amino acids eluting from a GLC column. The percent enrichment of ^{15}N was determined with a mass spectrometer.

Of the xylem sap amino acids analyzed, ^{14}C was found to be present in only one amino acid, believed to be proline. The only significant ^{15}N enrichment occurred in alanine. The majority of amino acids showed a diurnal fluctuation in their concentration with a maximum that occurred at the end of the 12-hour light period and a low that occurred at the end of the following 12-hour dark period.

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Incorporation of $^{15}NO_3$ into Amino Acids of Douglas-fir Xylem Sap

by

Richard P. Sandstrom

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 1977

Commencement June 1977

APPROVED:

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in charge of major

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Date thesis is presented ______April 29, 1977______

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ACKNOWLEDGEMENT

I first want to thank Dr. Warren Webb for making this project possible. His support and patience were greatly appreciated. A special note of gratitude goes to Don Griffin who collected the mass spectra data and whose help and advice allowed me to overcome many technique problems. I am also grateful for the fruitful discussions between myself and Dr. Te May Ching, Dr. W.D. Loomis, and Dr. Robert Becker. A very special thank you to Kathy, for putting up with the rather frantic life of a graduate student.

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INCORPORATION OF $^{15}NO_3$ INTO AMINO ACIDS OF DOUGLAS-FIR XYLEM SAP

I. INTRODUCTION

Nitrogen is often a limiting factor in tree growth and metabolism. Both inorganic and organic nitrogenous compounds are involved either directly or indirectly in every biochemical reaction in the living tree. Enzymes, for example, are composed of amino acids in genetically specified sequences and they are essential in catalyzing biochemical reactions. Non-enzyme proteins are either membrane components responsible for selective permeability or factors for synthesis. Nucleotides, which contain nitrogen, provide substrates, energy, and coenzymes for biosynthesis. Alkaloids and many other nitrogen containing compounds are ubiquitous in the plant world. Consequently, nitrogen has been the subject of intensive investigation in plants.

Nitrogen uptake and metabolism are inseparable from carbon metabolism; an exception may be passive uptake of nitrogen by root tissue. Photoassimilated carbon dioxide is the ultimate source of carbon utilized in the assimilation of nitrogen by the plant root. Therefore, any discussion of nitrogen's role in plant metabolism must eventually include the role of carbon.

Much information is available on the separate metabolism of carbon and nitrogen in plants; however, very little is known about how tree metabolic systems utilize carbon in the assimilation of nitrogen into biochemical constituents. Amino acids have been shown to be the principle form of nitrogen found in the xylem sap of trees, suggesting that these compounds are the major transporting agents for nitrogen assimilated by the root tissues. If the amino acids found in the xylem sap are produced in the root tissues, then the major constituents of these compounds, carbon and nitrogen, must be transported either directly or indirectly to the production site from their respective assimilation points. It is obvious that the production of amino acids in the roots and their subsequent transport in the xylem sap is dependent upon the interaction of photoassimilated carbon with nitrogen assimilated by the root.

Most of the current knowledge about carbon and nitrogen utilization and assimilation in plants comes from the use of tracers, e.g. ^{14}C and ^{15}N. These two tracer elements have the advantages of being directly utilizable by plant systems and easily detectable with modern equipment. A combination of gas-liquid chromatography (GLC) with gas ionization and mass spectrometry provides techniques with which ^{14}C and ^{15}N can be simultaneously analyzed in the individual compound using a GLC-ionization chamber system to analyze the

β (beta) emitter (^{14}C) and GLC-mass spectrometer system for analyzing the heavy isotope (^{15}N).

A study was initiated to define some of the characteristics of the link between photoassimilated carbon and root assimilated nitrogen in Douglas-fir seedlings and to test the applicability of GLC for the quantitative and qualitative analysis of amino acids. The tree seedlings were exposed to $^{14}C\text{-}CO_2$ in the atmosphere and $^{15}N\text{-}NO_3$ in a rooting medium (both hydroponic and soil). An association between the fixed $^{14}CO_2$ and $^{15}NO_3$ was sought by quantifying these tracers in amino acids extracted from the xylem sap using gas-ionization and mass spectrometry respectively.

II. LITERATURE REVIEW

Nitrogen Forms Available to Plant Roots

The nitrogen nutrition of plants has been studied extensively over the years. The ionic forms of nitrogen, most commonly nitrate (NO_3^-) and ammonium (NH_4^+), are the major forms of nitrogen available to the plant root tissue. Nitrate is a common constituent of nitrogen fertilizers but is also produced naturally through the process of nitrification of organic material by soil microorganisms. Ammonium is perhaps the most readily utilizable form of nitrogen by the plant because it is already in reduced form. It is a common constituent in fertilizers and is also produced in significant amounts by soil microorganisms through the process of mineralization. Organic nitrogen, more commonly amino acids, is the prevailing form of nitrogen in the world in both the soil and in living organisms (10). As plant and animal remains decompose, organic nitrogen is added to the soil as well as being produced from inorganic nitrogen already present. This organically bound nitrogen is essentially immobile with a relatively small but highly significant percentage being put back into usable, inorganic form through mineralization (10).

Nitrate nitrogen is generally considered the prevailing form of inorganic nitrogen in soils (73). This statement should be qualified,

however. The factors determining nitrate availability in soils vary markedly. Soil aeration, temperature, and moisture content are the major factors controlling the form of nitrogen in soils (10, 64). These conditions are controlled naturally by climate; however, they can also be manipulated by man.

Being an anion, nitrate is present in the soil solution because of its poor ion exchange capacity with the net negatively charged soil particles. The availability of this ion is, therefore, dependent upon conditions favorable to its production and subsequent appearance in the soil solution.

Agricultural crops utilize nitrate to a great extent in their growth and development, thus requiring the soil conditions to be favorable for nitrate stability. Agricultural practices readily manipulate soil conditions for favorable nitrate production and quiescence. This is not the case for forest sites.

Forested areas are generally relegated to areas where the soils are characteristically acidic. Nitrate availability in these soils is generally dependent upon temperature and soil moisture content which partly determines soil aeration. Miller (64) has shown that nitrate availability in mid-Pacific Coast Range soils is controlled, to a large extent, by the dominant tree cover and soil drainage. Red alder sites transform more ammonium to nitrate through nitrification than do comparable Douglas-fir sites. He also shows that

increased denitrification occurs in soils where the water content is near saturation levels. Drainage of nitrate from watersheds does not result in a significant loss of nitrate.

In discussing the form of nitrogen present in Douglas-fir forest sites, reduced nitrogen can be considered the prevailing form of nitrogen available to them. Miller (64) shows that ammonium is the prevalent form of soluble nitrogen in the soils of the Coast Range exceeding nitrate by a factor of 10 or more. Ammonium nitrogen is easily utilized by microorganisms and is often the most readily utilized form of nitrogen by plants (44, 61, 93). Mycorrhizae must be mentioned at this point since it is of widespread opinion that the affiliation of the fungi with roots is of considerable importance to tree nutrition (6, 7). Mycorrhizae show more of an affinity to assimilate ammonium nitrogen than nitrate nitrogen and can also assimilate nitrogen in the form of amino acids (53).

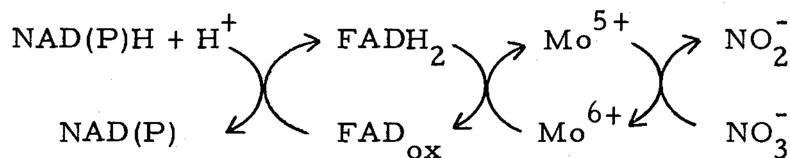
Assimilation of Nitrate and Ammonium Ions by Plants

Under controlled conditions, nitrate nitrogen is readily absorbed by plant root tissue. The actual uptake mechanism of nitrate or any other ion is still unclear; however, general opinion holds that there is both an active and a passive mode of uptake of ions by plant roots with the expenditure of energy occurring in active uptake. In general, ion absorption occurs most rapidly in actively

growing and expanding cells above the root tip (34). Mycorrhizae are also important in this respect because they can absorb nitrate (less so than ammonium) and transfer it to the root tissue (53, 60).

Once nitrate is in the root, its reduction to ammonium by nitrate and nitrite reductases is a prerequisite to its incorporation into organic compounds, primarily amino acids. This reduction of nitrate can occur in both roots and leaves of many plants (12, 34, 35, 45). In woody plants, however, nitrate is believed to be reduced primarily in the roots (5, 73).

The nitrate reductase enzyme, believed to be located in the cytosol, is generally thought of as a complex containing dehydrogenase and nitrate reducing activities (34, 35). The reduction process is dependent on a supply of electrons from a readily oxidizable source or a photosynthetically reduced electron carrier. The scheme for nitrate reduction in plants is shown below.



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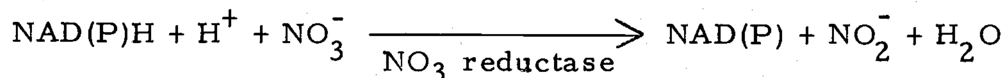
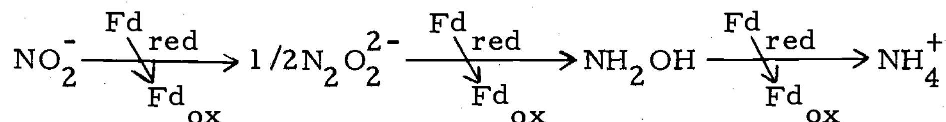


In the leaves, nitrate reductase is dependent on photosynthesis through the shuttle of carbon compounds from the chloroplast to the

cytoplasm where NAD(P) H (reduced nicotinamide dinucleotide [phosphate]) is produced (12). In the roots, NADH (reduced nicotinamide dinucleotide) can be supplied from varied sources such as glycolysis, and the tricarboxylic acid cycle in mitochondria. NADPH may also be produced in the root by the pentose phosphate pathway because evidence exists that nitrate stimulates this path (81). Flavin may be needed in the light dependent reduction; however, questions still remain about its actual function (35). Molybdenum functions in the enzyme complex as an electron carrying intermediate, perhaps the immediate electron donor to nitrate (34). The enzyme complex is regulated by many factors not pertinent to this discussion. In general, however, nitrate ion can induce the enzyme in both leaf and root tissue. Ammonium ion can cause inhibition in the root but does not seem to affect the leaf enzyme (35).

Nitrite reductase reduces nitrite to ammonium and differs from nitrate reductase in that the electron donors are different. Reduced nicotinamide adenine dinucleotide (phosphate) does not support the reduction of nitrite in higher plants. Reduced ferredoxin is generally considered the electron supplier of the system in a series of steps shown below.



Only NO_2^- and NH_4^+ of the above scheme are observed; the hyponitrite and hydroxyl-amine are theoretical intermediates which have never been found in living systems (35).

The nitrite reductase system is closely associated with the chloroplast lamellae through monocyclic electron transport in leaf tissue (35, 54, 55, 62), and can be induced by nitrite and in some cases nitrate (34, 35). In root tissue, leucoplasts are found to support a degree of nitrite reduction agreeing with the observation that plastid membranes are associated with the reduction processes (15, 62). There is disagreement, however, as to the electron donor of the root nitrite system. Ferredoxin has not been conclusively found in root tissue suggesting that nitrite reduction may not involve the presently described systems (15, 35). Miflin (62) shows that in vitro, glucose-6-phosphate is able to support some nitrite reduction in the root plastid fraction, lending support to the theory that the pentose phosphate pathway may be involved. Srakissian and Fowler (81) show a shift from glycolysis to the pentose phosphate pathway during nitrate assimilation in roots. The shift may facilitate the production of NADPH for nitrite production; however, the evidence is not conclusive. NADPH works as an electron donor only when ferredoxin is added to the reaction mixture (35). The final product of nitrate-nitrite reduction is ammonium which must be

assimilated into carbon compounds or it would build up to toxic levels in the plant.

Ammonium ion is probably absorbed into the root in a manner similar to the nitrate ion although the ionic charge of the two is reversed. The fact that ammonium is already in reduced form when it enters the root tissue has a profound influence on the assimilation rate of this ion. Ammonium can be incorporated directly into organic compounds, specifically amino acids and amides.

Amino Acid Synthesis in Plants

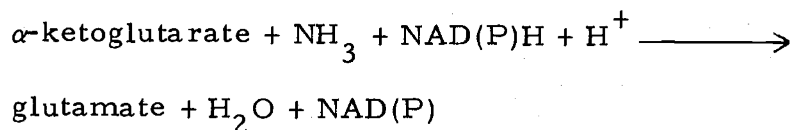
Amino acids are the primary products of reduced nitrogen (NH_4^+) and α-keto acids; therefore, any discussion of amino acid biosynthesis must include nitrogen metabolism as well as carbon metabolism. In green tissues and in roots, the biosynthesis of amino acids is closely associated with nitrate-nitrite reduction to ammonium. Klepper et al. (45) show conclusively that reduced NAD is needed in the reduction of nitrate to nitrite in the cytoplasm of green tissue. Magalhaes et al. (54) and Miflin (62) show that nitrite is reduced in the chloroplast lamellae. Canvin and Atkins (12) show that the assimilation of $^{15}NO_3$ into amino nitrogen in leaf tissue is strictly dependent on light and, as mentioned earlier, this dependence is based on a carbon shuttle across the chloroplast membrane. In finalizing the evidence, Givan et al. (31) and Kirk and Leech (43)

show conclusively that amino acids are synthesized in chloroplasts and the process is to some extent light dependent.

The biosynthesis of amino acids in root tissue has also been shown to be closely associated with nitrate-nitrite reduction, which is regulated by NADH, thus being dependent on carbon metabolism (40, 41, 46, 66, 67, 81). A special emphasis must be made, however, to point out that ammonium incorporation into amino nitrogen is significant in roots (44, 61, 62, 93). Root tissue not only encounters ammonium from the nitrate-nitrite reducing process but also must absorb reduced nitrogen from the rooting medium. As mentioned earlier, ammonium is toxic to plants, so a means to detoxify is imperative. Kirkby (44) states that detoxification of ammonium by binding with carbon compounds should take precedence over all other metabolic processes in roots.

Reductive Amination

Glutamate dehydrogenase, catalyzing the reductive amination of α-ketoglutaric acid, is considered the primary enzyme responsible for incorporating nitrogen into amino acids:



$$\alpha\text{-ketoglutarate} + NH_3 + NAD(P)H + H^+ \longrightarrow$$
$$\text{glutamate} + H_2O + NAD(P)$$

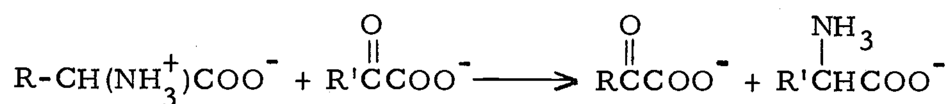
The enzyme is found in both photosynthetic and non-photosynthetic cells and has a specificity for reduced NAD (35, 49, 55, 91). Givan

et al (31) and Kirk and Leech (43) show photoreductin of α-ketoglutarate to glutamate in isolated chloroplasts. Miflin (63) shows that isolated leucoplasts from root cells also support the glutamate dehydrogenase reaction. An ambiguity exists, however, as to the actual role of glutamate dehydrogenase. In many plant cells, the enzyme has a low activity coupled with a generally high K_m value for ammonium (48, 62, 72). The significance of this will be discussed later.

In the primary assimilation of nitrogen, glutamic acid plays a major role in nitrogen dissemination through transamination because other amino acid dehydrogenase enzymes, which function in a similar manner to glutamate dehydrogenase (supporting the direct amination of other keto acids with ammonium), are rare. Once formed, glutamic acid can support the synthesis of a variety of other amino acids through a variety of transaminase reactions.

Transamination

Transaminases or aminotransferases catalyze the transfer of the α-amino group of one amino acid to the α-carbon of an α-keto acid in the reaction shown below:



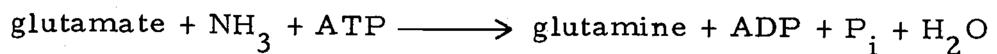
$$R\text{-}CH(NH_3^+)COO^- + R'\overset{O}{\overset{\|}{C}}COO^- \longrightarrow R\overset{O}{\overset{\|}{C}}COO^- + R'\overset{NH_3}{\overset{|}{C}}HCOO^-$$

The enzyme requires the prosthetic group, pyridoxal phosphate,

which forms a Schiffs base (-N=C-) between itself and the amino donor. Further reaction forms the new amino acid plus regenerates pyridoxal phosphate (35, 50). Both particulate and soluble aminotransferases have been isolated from plant tissue.

Amide Formation

Glutamine synthetase is another important enzyme involved in the assimilation of ammonium. The synthetase enzyme catalyzes the amidation of glutamic acid:



$$\text{glutamate} + NH_3 + ATP \longrightarrow \text{glutamine} + ADP + P_i + H_2O$$

Glutamine synthetase is located in both photosynthetic and non-photosynthetic tissue. In green tissue, much of the enzyme is associated with the chloroplasts (35). Fowler et al. (25) find that approximately one-third of the glutamine synthetase activity in sycamore root culture is associated with the particulate fraction.

Glutamine formation functions to incorporate ammonium directly into organic compounds, as does glutamate dehydrogenase, thus providing a means of storage and transport as well as detoxification of the ion. Cocking and Yemm (14) indicate that glutamine synthetase along with glutamate dehydrogenase are the primary enzymatic mechanisms for the assimilation of ammonium in roots. Barnes (1, 2) shows that glutamine is the major nitrogenous compound in the xylem sap of seven species of pine, accounting for 80% of the

total amino acids. Lewis (51) shows glutamine supplies the seed of Datura stramonium with most of the reduced nitrogen required for amino acid synthesis. Durzan (17-21, 23) shows major changes in levels of glutamine and other basic amino acids over a variety of conditions, including mineral nutrition, seasonal variations, diurnal variation, and light intensity.

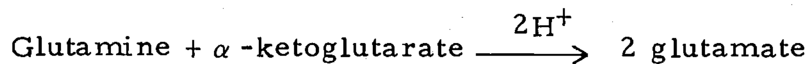
Glutamine synthetase (GMS) is also present in the chloroplast. O'Neal and Joy (71) show that the K_m value for ammonium of GMS in pea leaves is much lower than pea leaf glutamate dehydrogenase (GDH) (48), thus providing a more effective sink for ammonium assimilation. Since labeled ammonium (^{15}N) is rapidly incorporated into glutamic acid in chloroplasts (43, 54, 55), a means must be present that transfers the amide group of glutamine to the α-amino group of glutamate. Lea and Miflin (49), Fowler et al. (25) and Miflin and Lea (63) describe an enzyme system in higher plants which accounts very well for the preceding information.

Glutamate Synthetase

Miflin (62) shows that the level of glutamate dehydrogenase in intact chloroplasts does not account for the rapid formation of amino acids in this organelle. He and other researchers, Pahlick and Joy (72) and Lea and Thurman (48), show that the K_m value for chloroplastic glutamate dehydrogenase is high; a concentration high enough

at times to uncouple photosynthetic phosphorylation. The low activity and high K_m of GDH would then necessitate another means by which ammonium could be detoxified.

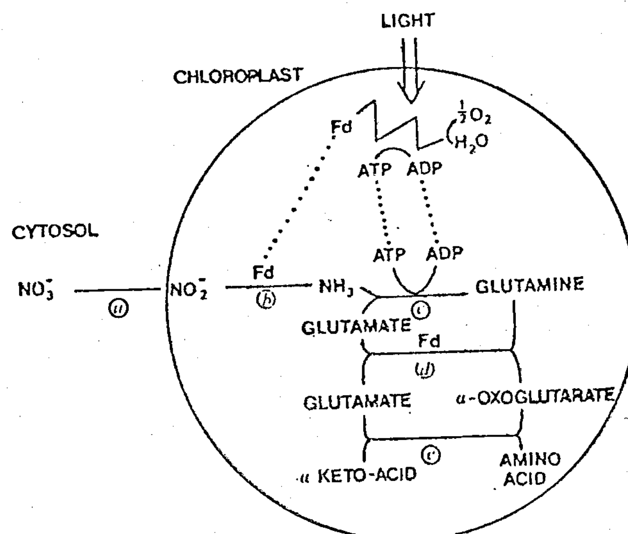
The enzyme, glutamate synthetase (GOGAT), catalyzes the amination of α-ketoglutarate by transfering the amide group of glutamine to the α-carbon of the keto acid:



$$\text{Glutamine} + \alpha\text{-ketoglutarate} \xrightarrow{2H^+} 2\ \text{glutamate}$$

Since glutamine is involved in the reaction, glutamine synthetase activity would be imperative for its continued function. Lea and Miflin (49) show that chloroplasts can convert α-ketoglutarate to glutamate in the light and that added ammonium stimulates the reaction. However, when glutamine is supplied, they find a 17-fold increase in the rate of glutamate formation. This reaction is still light dependent but is not dependent on NADPH. Reduced ferredoxin is found to be an excellent source of electrons, confirming the dependence of the reaction in green tissues upon light. The two authors suggest that this is the major route of nitrogen into amino acids in leaves because it ties in well with the reduction of nitrate and nitrite. Their scheme is shown in Figure 1.

Fowler and Sarkissian (25) and Miflin and Lea (63) show the existence of a glutamate synthetase system in root cells. The enzyme is similar in most respects to the leaf enzyme but can utilize both NADH and NADPH as electron donors. The glutamate



The proposed route of entry of nitrate into amino acids in leaves. The enzymes are, *a*, nitrate reductase; *b*, nitrite reductase; *c*, glutamine synthetase; *d*, glutamate synthase and *e*, transaminase.

Figure 1. From Lea and Miflin (40).

synthetase system, however, may not be as important in the roots as in the leaves. Concentrations of ammonium ion exposed to the root would make the glutamate dehydrogenase reaction feasible there.

The finding that a glutamate synthetase system exists in plants is important in two respects. It provides an alternative method of ammonium incorporation into amino acids, especially when concentrations of the ion are low, and it may be a major route for the assimilation of nitrate nitrogen into amino acids.

Asparagine and arginine, although not definitely linked to primary nitrogen incorporation into α-ketoacids, function in a major role of nitrogen nutrition. Both have a high nitrogen to carbon (N/C) ratio relative to most of the other amino acids, implying they have a capacity for storage and particularly transport of reduced

nitrogen. Durzan (17-19) shows arginine increases at the onset of dormancy in Picea glauca and Pinus banksiana. During quiescence, it is the predominant amino acid present. The onset of bud break in Picea glauca shows a diurnal fluctuation and net depletion of arginine with an increase in other amino acids, most predominantly glutamine and asparagine (20). Durzan (21) also shows low light intensities divert soluble nitrogen into arginine. Muhammad and Kumazawa (65), Kretovich (46), Ivanko (40), and many others (1, 24, 39, 41, 43, 52) show that the amides and arginine are important in transporting and disseminating nitrogen throughout the plant.

Carbohydrate metabolism in plants has a profound role in nitrogen metabolism and amino acid formation. Carbohydrates, through oxidation, provide the electrons necessary for the reduction of oxidized nitrogen and for the incorporation of reduced nitrogen into amino acids. They also provide energy in the form of ATP (adenosine triphosphate) for amide synthesis and lastly, the carbon backbone into which nitrogen is incorporated for the production of amino acids.

In the majority of reactions described above, NADH and NADPH provide the electrons necessary to complete the reactions. These reduced coenzymes are produced in the glycolytic pathway, the tricarboxylic acid cycle and the pentose phosphate pathway in plants where carbohydrates are oxidized (25, 35). NADPH can also

be produced in photosynthetic electron transport. As mentioned earlier, the pentose phosphate pathway may be of particular importance due to its putative role in nitrate assimilation.

Keto acids, generated from glycolysis and the tricarboxylic acid cycle, are utilized as carbon backbone structures for amino acids. As mentioned earlier, nitrogen incorporation into these acids can occur by reductive amination or by transamination. The importance of keto acids is particularly acute when high concentrations of ammonium are encountered. To detoxify a large amount of ammonium, a concomitant amount of keto acids must be available. This could tax reserve carbohydrates causing considerable depletion, which in seedlings could be disastrous since carbohydrate reserves may not be developed. Plants grown with ammonium nitrogen show greater amounts of amino acids in their tissues but lower accumulations of organic acids (44). Michael et al. (61) show that plants denied carbohydrates from shoots absorb less nitrogen than intact plants and that ammonium uptake is reduced more than nitrate uptake. These plants are found to contain lower amounts of soluble carbohydrates and starch. Continuation of this condition would lead to an unproductive use of carbohydrates where under optimal conditions these same carbohydrates could be used for the synthesis of other cellular components. Acute exposure to ammonium in high concentrations could slow down or stop respiration altogether because of a depletion of respiratory organic acids.

The ultimate source of carbon for energy of synthesis and the skeletons of amino acids is photosynthesis. Rangnekar and Forward (76) and Schier (82) show that photoassimilated $^{14}CO_2$ is assimilated into organic acids and amino acids. This situation is especially pronounced (in general) in C-4 plants where $^{14}CO_2$ is initially incorporated into malate and aspartate (9).

Translocation of Amino Acids in Plants

The assumption can be made that roots are the major producers of amino acids in tree seedlings, and that, at least in trees, is the major form of nitrogen after its assimilation. There must then be transport of amino acids from the roots to different regions of the plant. Intercellular transport can occur over short distances by plasmodesmata through the cellular cytoplasm and perhaps over long distances through the phloem. The other major transport system for amino acids occurs outside of the protoplasm through the water carrying vessels of the xylem (73).

A number of authors show that xylem transport of amino acids is the predominant mode of movement of this compound (1, 2, 5, 39-41, 51, 66, 67, 70, 73, 80, 88). However, this should be qualified. Xylem sap is relatively easy to extract whereas determination of intra- and intercellular and mass phloem transport is extremely difficult due to the delicate nature of these tissue types.

Nitrate is found in varying amounts in the xylem sap of herbaceous species along with amino acids; however, in woody species, even though nitrate is present in the xylem sap, amino acids predominate (2, 5). Ammonium is found only in very small amounts in the xylem sap of tree species, for reasons previously mentioned (5).

Transport of amino acids from root to shoot does not necessarily have to end in the shoot. Oghoghorie and Pate (70) show that considerable amounts of nitrogen received by the shoot from the roots is cycled back to the root by the downward translocation stream. However, very little nitrogen returned to the root, from the shoot, is incorporated back into the stream of nitrogen leaving the roots.

Amino acids in the xylem sap show seasonal as well as diurnal fluctuations in quantity and quality. Barnes (1) shows that seasonal peaks occur in the amino acid concentrations of trees in the spring and fall. Diurnal variation of tree xylem sap amino acids has not been found in the literature.

Isolation and Separation of Free Amino Acids in Plants

In the physiological fluids of plants, amino acids exist in the dipolar or zwitterion form (50), thus many of the more popular methods of isolation of amino acids use the acid-base properties of

these compounds to facilitate their isolation. In determining free amino acids in plant fluids, the ion character of the amino acids is used to separate these compounds from other physiological substances. Ion exchange chromatography is used to separate both acidic and basic amino acids from plant solutions (26, 50, 74). Cation exchange chromatography separates out the more basic amino acids when the separation is carried out in near neutral pH conditions; the opposite is true of anion exchange chromatography (94) Both acidic and basic amino acids can be separated simultaneously on a cation exchange column when the pH of their solution is low enough that all the amino acids are present in protonated form (42, 87). Under acidic conditions, all the amino acids are absorbed onto the cation exchange resin of the column and then eluted with a base and collected. The amino acids at this point are still in a mixture so further work must be done to separate individual compounds.

Only a brief summary of separation techniques used in amino acid analysis will be presented here. The techniques are much more involved, so the reader is encouraged to go to the references cited for a more complete discussion of instrumentation and procedures.

High pressure liquid chromatography (HPLC), also called high performance liquid chromatography, has rapidly become an important method for amino acid analysis (83). The system is

characterized by a pump which can feed, under high pressure (1,000-10,000 psi), eluent into a closed tubular column packed with a solid sorption medium; ion exchange resins are used to facilitate high resolution separations of amino acids. When the eluent flows out it enters a detection system and is then disposed of or collected for further identification. The technique involves injecting the amino acid solution into a sample valve where it is mixed with a buffer. Under high pressure, the buffered solution is forced through the chromatographic column. When separation of amino acid constituents is complete, the individual compounds elute from the column into a reaction-detection system where each amino acid is mixed with ninhydrin and the absorbance of the characteristically colored solution is read with a spectrophotometer (83, 84).

Paper electrophoresis, paper and thin layer chromatography are three widely used techniques for separating and identifying amino acids. Electrophoresis utilizes the ionic properties of amino acids to facilitate their separation. A paper strip, soaked with buffer, has both ends placed into electrode vessels containing buffer. The amino acid sample to be separated is placed in the middle of the strip. A direct current flows across the strip for a predetermined amount of time after which the strip is removed and dried. A chromogenic agent is then sprayed on the strip to locate the individual amino acids. Movement of the amino acids from the origin

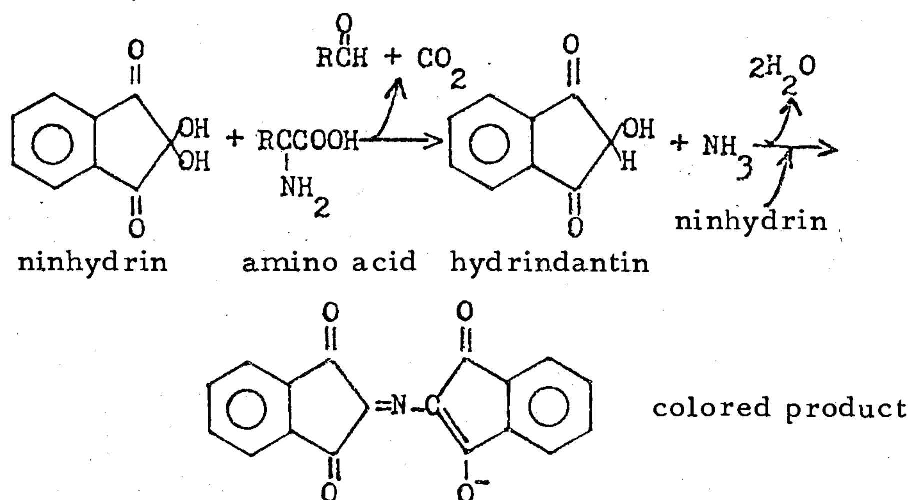
depends on their ionic charge (dependent on the pH of the buffer) and their mobility in the electric field (dependent on current and voltage) (84).

Paper chromatography uses a paper strip as an inert support for an eluting solvent (26, 84). A sample mixture of amino acids is spotted onto the paper near one edge. The edge is placed in a vessel filled with an eluting solvent which begins to ascend or descend the paper depending on the system used. The amino acids are separated by their solubility in the eluting solvent or by direct absorption by the paper or both. The elution phase of the analysis takes place in a closed container.

Thin layer chromatography (TLC) involves spotting a sample mixture of amino acids onto a thin layer of dry adsorbant powder spread over a glass plate. The edge of the plate is placed into a closed vessel containing an eluting solvent. The amino acids are separated by their solubility in the eluting solvent and their adsorption onto the adsorbent (26, 84). TLC is generally quicker and gives better resolution than paper chromatography. Once separated, amino acids can be detected by spraying a chromogenic agent directly onto the chromatograms. All three techniques allow elution of the amino acids from the chromatogram for further quantification.

The most common chemical means of detection and quantifying amino acids isolated by the preceding techniques is to generate a

colored compound with the reaction between amino acids and ninhydrin (50, 64).



The first molecule of ninhydrin oxidatively decarboxylates the amino acid. The generated ammonium and a molecule of hydrindantin react with another molecule of ninhydrin to form a purple colored compound; proline and hydroxyproline form colored compounds which are yellow.

Column chromatographic systems usually employ ninhydrin detection systems for the eluted amino acids. The light absorbance of the resulting colored complex is read at a wavelength of 570 nm (OD_{570}) (4, 65). This reading is then compared with a concentration vs. absorbance curve generated using known amounts of amino acids.

Gas-liquid Chromatography of Amino Acids

Of pertinent interest to this study is the separation and characterization of free plant amino acids by gas-liquid chromatography

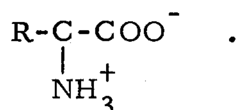
and mass spectrometry (26, 59). Volatile derivatives of amino acids are carried through a packed column by an inert carrier gas. The sample components separate by their differential partition coefficient in the thin layer of a liquid-stationary phase coated over an inert solid support by the carrier gas. The liquid phase retards each component at different rates at each theoretical plate. With their passage through a number of theoretical plates, the components are completely separated and eluted by the carrier gas. The separated components are further carried through a detector which sends an amplified electrical signal to a recording device.

The selection of a detector is a prime consideration in the analysis of any compound by GLC. The detector should have a high sensitivity, low noise level and a wide linearity of response (59). For the analysis of amino acids, three detector types are available; thermal conductivity (TC), flame ionization (FID), and electron capture (EC). Each has its distinct advantages and disadvantages (26, 59).

Flame ionization is the most widely used detection system in use today for the detection of amino acids (36). Effluent gas coming off the column is mixed with hydrogen and burned in air. The ions and electrons produced are collected on an electrode creating a current which is amplified and recorded. The FID possesses a high sensitivity, being able to detect 10^{-10} moles and lower (36, 59). It

has the widest linear range (detector response vs. sample size) of the three detectors. However, the response of the FID must be calibrated by known quantities of samples since the response varies with the operating conditions of the instrument.

To separate and quantify amino acids by gas-liquid chromatography, one major obstacle must be overcome; the insufficient volatility of free amino acids. Amino acids have a general formula



The carboxyl and amino group are characteristic of amino acids with some exceptions (i.e. the amino nitrogen of proline is substituted into the R group to form an imino acid--a cyclic compound). These two constituent groups make it possible to derivatize the amino acid into a volatile ester capable of GLC analysis.

Easily formed derivatives with suitable volatility are most desirable for GLC separation. Gehrke and Stalling (27) go further and define the criteria for a suitable derivative to be used in the qualitative and quantitative determination of amino acids:

1. The derivative should be simple in its formation, no rearrangements or structure alteration.
2. The derivatization reaction should go to 95 to 100% completion.
3. There should be no sample loss on vacuum concentration of the sample solution.
4. The derivative should be stable with respect to time and temperature.

5. The derivative must have increased volatility, thus lower retention time.

6. The derivative must be in a form with little or no reactivity with the solid-inert support, and resolvable.

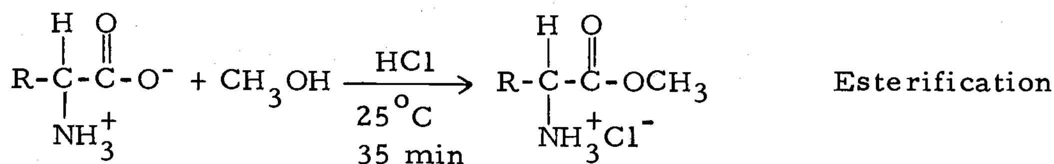
There is no general agreement on the type of derivatization procedure to be used since so many are available (36). The most popular methods are those that require the least amount of time and still provide a suitable derivative. A general method involves the two-step conversion of an amino acid to an N-substituted ester, e.g. the esterification of the carboxyl group and the acylation of the amino group.

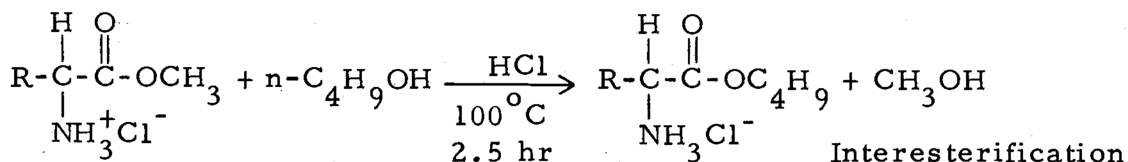
The preferred method of reducing the polarity of an amino group of an amino acid is to acylate it. Acid anhydrides, most commonly trifluoroacetic anhydride (TFAA), are the primary agents in use today for the acylation of the amino group. The acylating medium containing TFAA has the advantage of being able to be injected into the gas chromatograph without prior evaporation. This reduces the possibility of hydrolysis and volatility losses of the derivatives from concentration caused by evaporation of the medium's solvent.

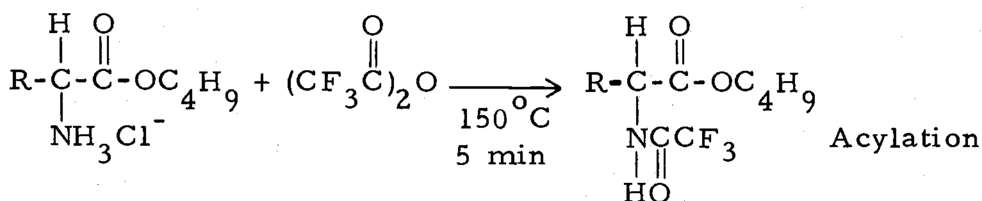
The polarity of the carboxyl group is dealt with by esterifying the group with an alcohol in acid medium. A wide variety of amino acid esters has been used in the GLC analysis of amino acids (36).

The most popular esterifying agents range from the one carbon methyl alcohol to the five carbon amyl (pentyl) alcohol.

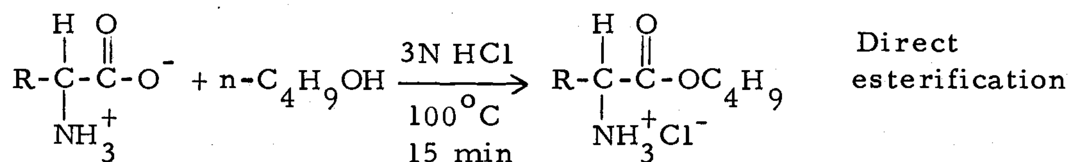
A comprehensive study on the GLC of amino acids has been completed by Gehrke and associates (27-29, 42, 47, 77, 79, 94, 95). They have developed a technique utilizing the N-trifluoroacetyl n-butyl esters of protein amino acids. Their first method of esterification involves forming the methyl ester of the amino acid followed by interesterification in n-butanol (27, 47). The butyl ester is then acylated in a closed tube with TFAA.

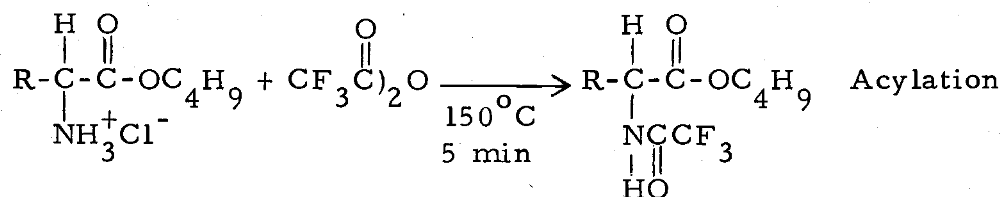






Roach and Gehrke (79) refined the procedure by eliminating the interesterification step which was needed to solve the solubility problem of cystine and other basic amino acids in n-butanol. Direct esterification of an amino acid to its n-butyl ester is now possible:





The solubility problems mentioned above are eliminated by dissolving the amino acid sample in a solution of 3N HCl in n-butanol and immersing the reaction flask into a sonic water bath to facilitate solubilization. Recoveries of amino acids using this technique averaged 99.7%. The refined procedure also cuts three hours off the reaction time making it possible to complete esterification and acylation in approximately 30 minutes instead of the previous three and one-half hours for the interesterification procedure.

The column packing support can vary in an analysis; however, it should meet the criteria of having a large surface area with a strong affinity for the liquid phase while still remaining inert to the sample. Acid washed (AW) Chromasorb W (Johns Manville Co.), a calcined diatomite support, is the most widely used solid support for the separation of the polar amino acids because its surface is relatively non-absorptive. The acid washing rids the support of trace metals. Chromasorb G was used in earlier work by Gehrke (28) but was replaced with acid washed Chromasorb W because it

produced a better separation (79). High performance (HP) Chromasorb G (long heat treatment of Chromasorb G) is still used as the support to separate the basic amino acids. Silane treated supports such as chrom-Q (Cabot Co.) may also be used in GLC analysis. They offer even greater resolution because any reactive surface is blocked with silane (42).

The stationary phase is of foremost importance to the separation of the amino acid derivative. The nature of the amino acid derivative coupled with the column temperature determines which stationary phase to use. Therefore, the liquid phase should completely separate the amino acid and possess a high temperature stability (36). Analyses using the N-TFAA n-butyl esters require a temperature program range from 90°C to about 230°C. The stationary phase should not be volatile at the upper temperature limit.

The polarity of the liquid phase generally controls the resolution; that is, the more polar the liquid phase, the greater the resolution (36). Silicones and polyesters are the two classes of compounds used as liquid phases for amino acid separation. Histidine, arginine, cystine and tryptophan must be separated on a silicone phase because they are not eluted from polyester phases. The remaining amino acids are eluted on a polyester phase (78).

In their earlier work, Gehrke and Stalling (27) used a polyester coated packing of diethylene glycol sebacate/ethylene glycol succinate methyl silicone polymer (DEGS/EGSS-X) for the separation of the majority of amino acids and DC-550, a methyl silicone polymer, for the separation of histidine, arginine and cystine. Later work by Gehrke et al. (28) shows ethylene glycol adipate (EGA)--0.65% w/w)--to be a more suitable polyester phase. OV-17 was shown to be superior over DC-550 as the silicone phase; OV-17 is a methyl phenyl silicone. EGA has remained, up to the present time, the prime choice of a polyester phase. OV-17, however, has been replaced with a mixed silicone phase consisting of 2.0% (w/w) OV-17 and 1.0% (w/w) OV-210 (a methyl trifluoropropyl silicone) showing even greater resolving ability over OV-17 alone.

The percentage of liquid phase on the inert support makes a difference in the resolving power of the system. Borstlap (8) uses three layer percentages of EGA on Chromasorb W (AW)-DCMS (silane) treated. Each layering gives different separation patterns. Thom and Parsons (87) find similar results.

The nature of the two types of column packing allows them to be used simultaneously in a gas chromatograph. Two columns, one containing the polyester phase and the other the silicone phase, can be placed in the same oven allowing the simultaneous elution and

characterization of all the amino acids without having to change columns and GLC operating conditions.

The column material itself cannot be overlooked. Because of their relative inertness over other materials, Pyrex glass columns are preferred over metal columns. Metal, including stainless steel, can catalyze the destruction of some N-TFAA n-butyl amino acid esters; most pronounced is the destruction of tryptophan (47). Teflon lined stainless steel is not satisfactory because absorbance into the plastic coating itself can cause tailing of the esters plus change elution patterns.

There are inherent problems with the analysis of N-TFAA n-butyl esters of amino acids. Many of the N-TFAA n-butyl esters were found to decompose in metal injection ports (47), necessitating the use of on-column injection (preferred) or glass lined injection ports. Inorganic salts present during the esterification procedure may affect quantification of the amino acids (36). Exogenous sources of amino acids, such as dust, fingerprints and dandruff, can significantly affect quantitative as well as qualitative descriptions of amino acids when the analysis is carried out at the nanogram level (77). Solubility of amino acids in n-butanol must be overcome to assure complete formation of derivatives. Cancalon and Klingman (11) find oxidation of tryptophan, methionine, phenylalanine and tyrosine to

occur during the ion exchange cleanup procedure prior to derivative formation.

The amide nitrogen of glutamine and asparagine is very subject to hydrolysis in the acidified esterification mixture making it impossible to detect these compounds when normal esterification procedures are used in the analysis. Therefore, the peaks appearing for glutamic and aspartic acids are actually mixtures of glutamate and aspartate coupled with the hydrolysis products of glutamine and asparagine. Hediger et al. (33), to a large extent, overcome this problem by modifying the esterification procedure. They perform a direct esterification to the n-butyl ester in the n-butanol containing 1.25M HCl at 100°C for seven minutes. Hydrolysis of the amides still occurs, but they are present in the chromatograms.

The N-TFAA n-butyl esters of amino acids are shown to be the most reliable derivatives in GLC analysis of these compounds; however, they were developed primarily for the analysis of protein amino acid. Raulin (78) expands the procedure to include non-protein amino acids. He separates all of the protein amino acids as well as 18 non-protein amino acids using the N-TFAA n-butyl esters on an EGA column. This is particularly encouraging because the procedure can now be applied extensively to the study of plant extracts which contain many non-protein amino acids. Nijholt (69) uses the N-TFAA n-butyl amino acid esters to determine the free

amino acid composition of Douglas-fir needles and bark. Borstlap (8) uses the same procedure and extends it for use with mass spectrometry.

The GLC analysis of amino acids opens up new avenues of research capabilities not achieved by conventional analysis. GLC coupled with other analytical tools makes it possible to observe and quantify constituent elements present in amino acids. Isotope labeling (^{14}C and ^{15}N) is extremely important for investigating the metabolism of amino acids in plants. The analysis of these labeled compounds by GLC enables determinations to be made of content, specific activity, isotope enrichment, radiochemical and chemical purity (13, 30, 57).

The most common method of analysis of ^{14}C labeled compounds by GLC is collecting the separated compound in the effluent followed by counting (57). Generally a splitting device is attached to the end of a column so a portion of the sample can go to the detector and a larger volume to the collector. A similar method absorbs the split sample in a flowing liquid scintillator which can be collected and counted separately or proceed to a flow-through scintillation counting device.

The preceding method may be extrapolated to gas ionization counting where the split sample effluent flows through an ionization chamber. The ^{14}C-β radiation ionizes gas atoms inside the chamber.

The charged particles are collected on an electrode and the chamber wall due to a potential put across them. The current that develops from the build up of the charged particles is amplified and monitored describing the specific activity of the radioactive material in the sample.

Gas-liquid chromatography coupled with mass spectrometry offers perhaps the most useful application of GLC to the analysis of amino acids. The volatility of the amino acid esters provides the means with which an amino acid can enter a mass spectrometer. The volatile esters are ionized by an electron source, accelerated by an electrostatic field, deflected by a magnetic field, collected, amplified and recorded (13).

Mass spectrometry offers ready identification of amino acids because fragmentation patterns of the compounds are characteristic for each compound under the same operating conditions. In other words, a reproducible fingerprint can be compiled for each amino acid under predetermined operating conditions. Summons et al. (86) and Gelpi et al. (30) have published mass spectra data for the N-TFAA n-butyl esters of many amino acids. Borstlap (8) uses GLC-mass spectrometry to identify 4-amino butyric acid in Spirodola polyrhiza (L.) Schleiden.

III. MATERIALS AND METHODS

Plant Materials

Three-year-old Douglas-fir seedlings (Pseudotsuga menziesii [Mirb.] Franco) were used in the experiment. The seedlings were large, two-year-old nursery grown stock when they were obtained in the spring of 1975. They were transplanted into a sandy loam soil in two pound coffee cans and grown outside in partial shade for one year.

Exposure of Seedlings to $^{15}NO_3^-$ and $^{14}CO_2$ in a Controlled Environment Chamber

Four nutrient solutions were prepared with the following minerals and their concentrations (37):

Solution 1

Mineral	Grams/30 L H_2O
$FeCl_3$	0.225
$MnCl_2$	0.027
H_3BO_3	0.045
$ZnCl_2$	0.0018
$CuCl_2$	0.00225

Solution 2

Mineral	Grams/30 L H_2O
$NaH_2PO_4 \cdot 2H_2O$	4.545
KCl	6.345
KH_2PO_4	3.960
$NaSO_4 \cdot 10H_2O$	9.360
HNO_3 [1/]	6.435

[1/] 6.435 grams of HNO_3 with a 30 atom percent excess of ^{15}N (40, 41, 70) were used as the nitrogen source in the nutrient solution.

Solution 3

Mineral	Grams/30L H_2O
$MgSO_4$	6.930
$MgCl_2$	6.525

Solution 4

Mineral	Grams/30L H_2O
$CaCl_2$	9.855

Ten round, plastic buckets, approximately four liters each, were used to hold the nutrient solution and seedlings in the controlled environment chamber. Styrafoam sheets, cut to fit the bucket and slotted to accommodate the seedlings, were used to hold the seedlings upright in the nutrient solution.

On the night prior to the experiment, 20 seedlings were removed from their pots, washed free of soil, and placed two each in the buckets filled with aerated distilled water. The following morning, the distilled water of eight buckets was replaced with three liters of nutrient solution aerated through a plastic tube linking each bucket with a central air tube from an air pump. The seedlings, along with four still in pots and watered with 300 ml of the nutrient solution, were placed in the 1,780 liter controlled environment chamber and exposed to $^{14}CO_2$. The four seedlings in the two buckets not placed in the growth chamber served as controls.

Conditions in the growth chamber were as follows: light from a single 6,000 watt xenon arc lamp reduced to 0.441 ly/min (from 0.790 ly/min) by a polyester fiberglass screen between the light source and the seedlings (90); temperature constant at 25°C; $^{12}CO_2$

held between 320 and 340 ppm; and specific activity of $^{14}CO_2$ held between 0.029 and 0.039 μCi/L.

The seedlings were treated and labeled as follows:

Four plants used as controls: A-1, 2, 3, 4

Four plants removed after 4 hours of light: B-1, 2, 3, 4

Four plants removed after 8 hours of light: C-1, 2, 3, 4

Four plants removed after 12 hours of light: D-1, 2, 3, 4

Four plants removed after 12 hours of light and 12 hours of dark: E-1, 2, 3, 4

Four potted plants removed after 12 hours of light and 12 hours of dark: F-1, 2, 3, 4

Extraction of Xylem Sap

The controls, then the treated seedlings, were harvested after each treatment. Each seedling was cut in two just above the root collar. The two halves labeled "S" for stem and "R" for root, were immediately placed in plastic bags and brought to a cold room. While in the cold room, the stems and roots of two seedlings were stripped of all phloem for two inches beyond the cut stems. The seedling parts were then placed in a pressure bomb chamber with the stripped buds protruding from a rubber gasket seal. Tygon tubing was placed over the cut ends to form a small reservoir to aid in collecting the xylem sap. Chamber pressure was slowly increased to 300 psi and held until no more sap could be extracted. The sap was collected into labeled, 30 ml storage vials and immediately frozen in liquid

nitrogen. The sap from stems of each seedling pair was combined, as was that from the roots, and labeled 1, 2-SX and 1, 2-RX. After sap extraction, the seedlings were placed back in their plastic bags and stored in a freezer. The remaining two plants, three and four, and the remaining plants in each exposure, were treated in the same manner.

Purification of Xylem Sap

Each sample of xylem sap was thawed, the volume measured, then poured into 30 ml of chloroform in a 60 ml separatory funnel for an organic-aqueous phase separation. A measured volume of the aqueous fraction and a known amount of n-butyl stearate in n-butyl alcohol (an internal standard for quantification of the derivatized amino acids) was placed in a 100 ml round bottom flask and evaporated to dryness in a 60°C water bath using a Rinco Buchi rotary evaporator. The residue was redissolved in 5 ml of 0.1N HCl to acidify the amino acids.

Amino Acid Extraction: Cation Exchange

The amino acids were collected as follows: approximately one ml of Dowex 50-X8-strong acidic cation exchange resin, 50-100 mesh, was placed in a 10.0 ml graduated cylinder and "washed" with 10.0 ml of 4N HCl. The now acidified resin was poured into a

10.5 x 150 mm ion exchange column with a glass wool plug placed in the bottom. The resin was then washed with 20 ml of distilled water after the acid had been drained. The liquid level in the column was never allowed to drop below the surface of the resin.

The acidified sap residue was placed on the resin and allowed to flow through at a rate of 5-10 drops a minute. The column was then rinsed with 20 ml of distilled water; the eluate was discarded. The amino acids on the resins were eluted with 10 ml of 2N NH_4OH and collected in a 100 ml round bottom boiling flask. Twenty milliliters of distilled water used to rinse the resin was also collected in the flask. The eluate was evaporated to dryness in a 60°C water bath.

Formation of Amino Acid Derivatives

The dried amino acids from the eluate were derivatized to their N-TFAA n-butyl esters for separation by gas-liquid chromatography (69, 78, 79, 94; see also Literature Review). One hundred and fifty microliters of acidified (3N HCl) n-butyl alcohol was added to the dried eluate for each 100 µg of total amino acids (prior to this experiment, ninhydrin quantification tests for the total xylem sap amino acids [65] were conducted utilizing seedlings from the above stock). The flask was then immersed in an ultrasonic water bath for 15 seconds to dissolve the amino acids in the solution. After

mixing, the flask was placed into a 100°C oil bath for 15 minutes to esterify the amino acids to their n-butyl esters. After esterification, the mixture was transferred to a 3/8 inch x 3 inch acylation tube where the excess butanol was evaporated by passing dry nitrogen gas over the sample. Next, 100 µl of TFAA in methylene chloride was added to the residue for each 100 µg of amino acid present. The tube was tightly sealed, placed in the ultrasonic water bath for thorough mixing of the solution, then immersed in a 150°C oil bath for five minutes to acylate the amino nitrogen of the amino acid esters. After the mixture was cooled, the solvent was evaporated with a stream of dry nitrogen gas and replaced with 20-50 µl of purified chloroform. The chloroform mixture was transferred to a 0.3 ml tapered reaction vial for handling and storage.

Gas-Liquid Chromatography (GLC) of Amino Acid Esters

A Hewlett-Packard 5750B Research Chromatograph with flame ionization detector was used to detect the derivatized amino acids. A ready-made column packing, Tabsorb (Regis Chemical Co.), ethylene glycol adipate (EGA) on acid washed Chromasorb W, 0.65% (w/w), was used to pack a six foot Pyrex glass column (69).

Prepurified helium carrier gas had a flow rate of 40 ml/min through the column. The injection port temperature, column temperature range and detector temperature were 230°C, 90°-230°C,

and 250°C respectively. The column temperature was increased 6°C/min after a five minute post injection interval. The chart speed was 0.5 in/min. Injection volumes of 0.3 μl to 1.0 μl were made with a 10 μl syringe (Hamilton Co.).

Quantifying the amino acids extracted from the seedlings involved an initial preparation of standards. A standard solution consisting of 1.25 μM of L-cystine and 2.5 μM of the following amino acids in 0.1N HCl was used: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine. One set of the standards was derivatized directly without going through the cation exchange purification step. Another set of the standards was processed through the cation exchanger and then derivatized. Both the standard amino acid derivatives and the xylem sap amino acid derivatives were injected into the gas chromatograph. After GLC separation, the peak area of each amino acid derivative was determined using a Hewlett-Packard model 9821A calculator combined with a Hewlett-Packard model 9864 digitizer.

Two factors must be determined from the gas chromatography for each of the standard amino acid derivatives in order to quantify the amino acids in the xylem sap. They are the relative molar

response (RMR) and the percent recovery of each amino acid from the purification step.

The relative molar response for each amino acid standard was determined using the following equation:

$$RMR_{(aa\ std)} = \frac{\text{Area of the Amino Acid in an equal molar mixture of standards}}{\text{Area of the Internal Standard (butyl stearate) in an equal molar mixture of standards}}$$

The relative molar response of each amino acid is the value obtained from the area of the amino acid peak relative to the internal standard peak. When this value is determined for standards, both the amino acid and the internal standard have the same molarity. The resulting ratio or RMR is constant for each amino acid under the same operating conditions of the gas chromatograph. The peak areas for the xylem sap amino acids were next determined and their RMR values determined relative to a known amount of internal standard added to the sample:

$$RMR_{(sample\ aa)} = \frac{\text{Area of Amino Acid}_{(sample)}}{\text{Area of Internal standard}_{(sample)}}$$

The RMR of the sample amino acid was then compared with the RMR of the standard amino acid to determine the amount of the amino acid in the sample by the following equation:

$$\frac{\text{Moles of Internal Standard}_{(sample)}}{RMR_{(standard)}} \times RMR_{(sample)}$$

$= \mu$moles amino acids

The percent recovery of amino acids from the ion exchange cleanup was determined by comparing the molar concentrations of the amino acid standards taken through the purification process with the original concentration of the standard. The molar concentrations of the sample amino acids were then determined by the following equation:

$$\mu\text{moles/ml}_{(\text{sample})} = \frac{\dfrac{\text{Moles of Internal Standard}_{(\text{sample})}}{\text{RMR}_{(\text{standard})}} \times \text{RMR}_{(\text{sample})}}{\%\ \text{Recovery} \times \text{ml of xylem sap}}$$

Determination of ^{14}C in Amino Acids and Plant Tissues

Total ^{14}C in each amino acid mixture was determined by using liquid scintillation counting (89). Labeled carbon in individual amino acids was determined using a GLC ionization chamber system.

The scintillation counting involved adding a measured volume of the derivatized amino acids into a glass scintillation vial containing 15 ml of scintillation fluid and counting the mixture in a Packard model 3380 Scintillation Counter. Disintegrations per minute per microliter (dpm/μl) were determined by dividing counts per minute (cpm) by the percent counting efficiency and then dividing by the injected volume. Background dpm/μl, determined by combusting

and counting a known amount of the derivatized control amino acids, were subtracted from the radioactive samples.

The percent counting efficiency must be determined for each sample to eliminate any quenching effects occurring in the scintillation fluid. The determination of counting efficiency is thus an estimation of the degree of quenching (89). Two methods, internal standard and external standard ratio, were used in the present study to determine the percent counting efficiency. The first method alone was used to correct quenching in the derivatized samples, whereas a combination of the two methods was used to correct for quenching in the whole tissue samples.

Ten scintillation vials were filled with 15.0 ml of scintillation fluid. A measured amount of chemical quenching agent (chloroform) was added to each vial; volumes of added chloroform ranged from 0.0 ml to 0.5 ml. Two replications were made for each quench treatment. An internal "spike" of ^{14}C-toluene of known specific activity was added to each of the quench standard vials. The solutions were counted and the percent counting efficiency for each sample was determined as follows:

$$\frac{\text{CPM of quenched sample - CPM of background radiation} \times 100}{\text{DPM of toluene spike}} = \text{\% counting efficiency}$$

An external standard ratio was determined for each quench standard, to be used for the whole tissue samples, whose counting procedures

are described later. The number obtained is the ratio of the shift in pulse height of electrons in the scintillation fluid from an external gamma-ray source when measured in two different regions of the pulse height spectrum (89). This external standard ratio (AES) was automatically determined by the scintillation counter prior to the counting.

The AES and the percent counting efficiency of each quench standard were then plotted on a graph and a regression analysis for AES vs. percent counting efficiency was conducted. The equation for the regression line of the standards was used to determine the percent counting efficiency for each sample that had an AES ratio recorded prior to counting.

The radioactive carbon in individual amino acids was determined by connecting the eluate from the gas chromatograph to an ionization chamber system (Figure 2). The ^{14}C detection system consisted of a 250 ml Carey-Tolbert ionization chamber with an output connection to a Carey 401 Vibrating Reed Electrometer; a 90 V potential was maintained across the chamber's electrodes.

A measured volume of the derivatized amino acid mixture was injected into the column and separated. As each derivative came off the column, it was mixed with preheated argon before its entry into the ionization chamber; argon was used as the purge gas as well as the ionizing gas. The electrical signal collected on the chamber

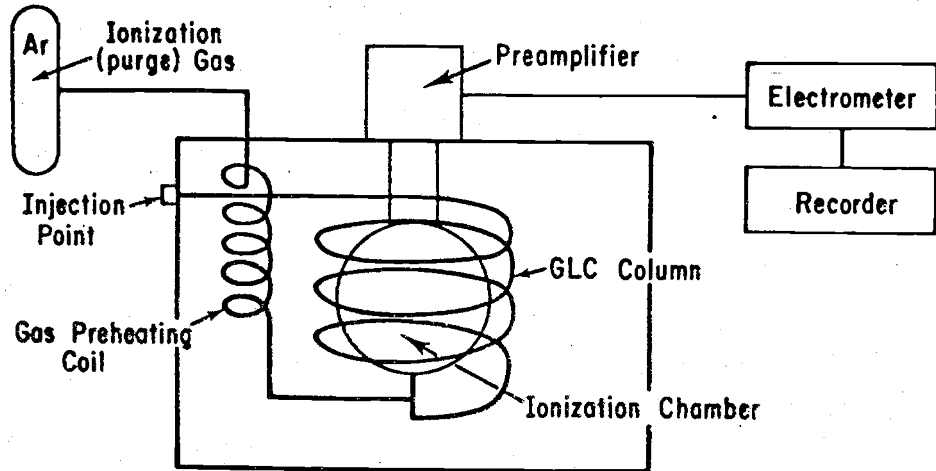


Figure 2. Hewlett-Packard gas chromatograph with ionization chamber system.

electrodes was amplified by a preamplifier and electrometer and recorded on an Easterline-Angus model T171B Port-a-Graph Recorder. The number of disintegrations per second (dps) was calculated by using the following formula:

$$\frac{\text{No. of Radioactive Events x Ave. Energy Present}}{\text{Energy Required to Make an Ion Pair}} = \frac{\text{DPS x eV}}{\text{eV}} = \text{DPS}$$

$$\frac{\text{Total No. of electrons } (e^-) \text{ produced}}{6.28 \times 10^{18} \text{ e/sec}} = \text{Amps (current)}$$

$I = E/R$

I = Ionization Current (Amps)
E = Electrometer Reading (Volts)
R = Resistance (Ohms)

$$I = \frac{\text{Area under Curve } (\text{cm}^2) \text{ x Operating Range of Electrometer (V)}}{\text{Resistance}} = \text{Amps}$$

$$\text{No. of Radioactive Events} = \frac{\text{Energy req'd to Make an Electron Pair} \times 6.28 \times 10^{18}\ \text{e/sec} \times \text{Amps}}{\text{Average Energy Present}}$$

$$\text{DPS} = \frac{\text{eV} \times 6.28 \times 10^{18}\ \text{e/sec}}{\text{eV}}$$

DPS x 60 DPM/chamber efficiency = actual DPM

Chamber efficiency was determined by estimating the actual efficiency of the 250 ml chamber, as it was operating in the described system, from the manufacturer's specifications. The efficiency of a 1,000 ml chamber, according to the manufacturer's specifications, is 85%. When linked to the Carey 401 Vibrating Reed Electrometer used in this study, the chamber had a recalculated efficiency of 64%; 76% of the manufacturer's value (Warren Webb, personal communication). The drop in efficiency is attributed to the resistor used in the electrometer. A 250 ml chamber has a counting efficiency of 45% according to the manufacturer. When linked to the Carey 401 Vibrating Reed Electrometer, the efficiency was taken to be 76% of the manufacturer's value or 34%.

Total ^{14}C in the seedling tissue was also determined using liquid scintillation counting. The plant tissue was separated into roots, old needles, new needles and stems. Each tissue was dried at 73°C for 24 hours, weighed to the nearest 0.1 mg and ground in a Wiley mill through a 20 mesh screen. A 10-30 mg subsample of each of the ground tissues was placed in a small paper cone and

combusted in a Packard Tricarb model 306 Sample Oxidizer. DPM/ mg for each sample was determined as described earlier.

Determination of ^{15}N in Amino Acids with GLC-Mass Spectrometry

^{15}N enrichment in individual amino acids appearing in Douglas-fir xylem sap was determined by GLC-mass spectrometry of the N-TFAA n-butyl esters of each amino acid. A Varian MAT Ch-7 mass spectrometer, differentially pumped and tied into a System Industries System 150 data system, was used in the present study. A Varian 1200 Gas Chromatograph with a glass jet separator (Finigan Corp.) was used to channel the N-TFAA n-butyl amino acid esters to the flame ionization detector and the mass spectrometer.

A 4 ft x 1 mm ID Pyrex glass column was packed with Tabsorb. The carrier gas (He) flow rate was between 15 and 24 cc/min. The initial column temperature was 100°C and was programmed to increase to 220°C at 6°/min. The injector temperature was 250°C and the separator temperature was 260°C. The mass spectrometer source temperature was approximately 175°C with a 300 μamp trap current at 70 eV. Two runs were made. The first scan was 50 to 400 mass/charge (M/e) units for all the amino acids present in the sample. The second scan was slowed down to a scan of approximately 15 M/e units to look at alanine, aspartic acid,

4-amino butyric acid and glutamic acid. Data were collected for selected masses on the first run. The ratio of $M+1/M^+$ was determined for all scans of good intensity for each peak. M^+ was chosen as the largest nitrogen containing ion fragment present not a doublet (two different fragments with the same nominal mass). Data collected for the second run were collected for the selected masses. Selected ion chromatograms were integrated for the masses of interest. The $M+1/M^+$ ratio of run two is the ratio of the integrated area counts.

The percent increase in ^{15}N enrichment for each amino acid was determined by calculating the increase in the $M+1/M^+$ ratio, for the selected fragment of the amino acid from the $^{15}NO_3$ treated seedlings, over the $M+1/M^+$ ratio for the same fragment of the amino acid from the control.

IV. RESULTS

Extraction of Xylem Sap

Table 1 shows the average volume of xylem sap collected from the two roots and two stems at each collection period. On the average, there is little difference in the total amount of sap collected from each seedling grown in either the nutrient solution or in soil. In both treatments more sap was collected from roots than stems. The volume of sap collected after the 8 hour and 12 hour harvest of the hydroponic plants varies markedly from the mean; the 8 hour volume is only half the 12 hour volume.

GLC Separation of Amino Acids

Figure 3 shows the GLC chromatogram of 15 amino acids in the standard amino acid solution. The chromatogram shows that the N-TFAA n-butyl amino acid esters separate very well on the EGA column. Table 2 shows the relative molar response (RMR) values of the quantifiable amino acids found in the xylem sap of the Douglas-fir seedlings as determined from the reference standards. In general, the RMR values are smaller than the published values. The RMR values for phenylalanine and lysine correspond well with published values. The experimental RMR value for 4-aminoburytic acid is only estimated. No standard was present for this amino

Table 1. Average milliliter of sap extracted from either two roots or two stems from each treatment.

Treatment	Average ml/2 roots or 2 stems	
	Root	Stem
A	9.2	2.5
B	9.3	3.9
C	6.8	2.0
D	13.3	3.3
E	8.0	2.4
F	9.4	5.8
Ave. A-F	9.3	3.3
Ave. A-E	9.3	2.8

Table 2. Relative molar response values (RMR) of amino acids[1] with respect to glutamic acid.

Amino Acid	Experimental Column	Roach and Gehrke (78)	Borstlap (8)
ala	0.41	0.52	
val	0.42	0.73	
gly	0.36	0.43	
ile	0.41	0.83	
leu	0.77	0.83	
thr	0.45	0.64	
4-abu	0.50	--	0.64
met	0.72	0.56	
phe	1.16	1.14	
asp	0.83	0.91	
glu	1.00	1.00	
lys	0.84	0.86	

[1]Amino acids listed are those found in Douglas-fir xylem sap.

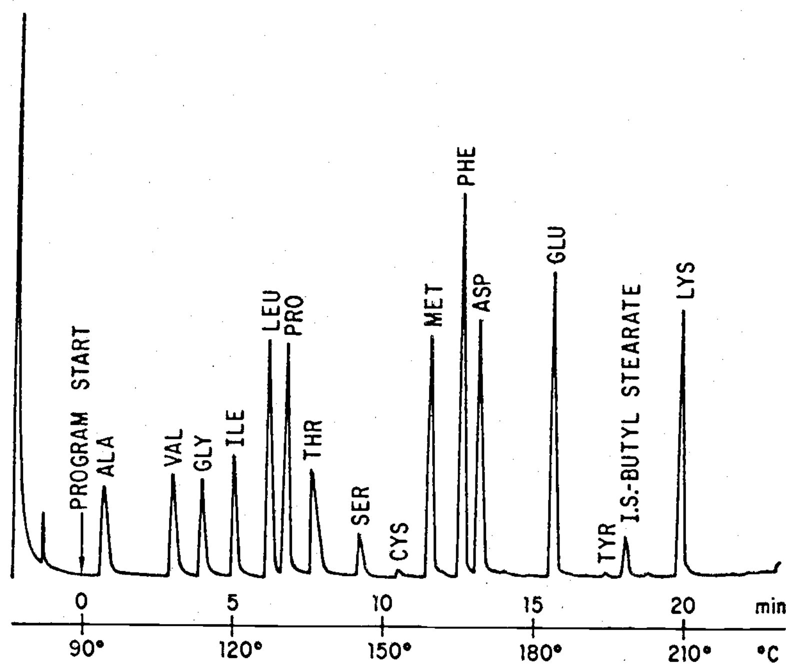


Figure 3. GLC chromatogram of 15 amino acids in the standard amino acid solution.

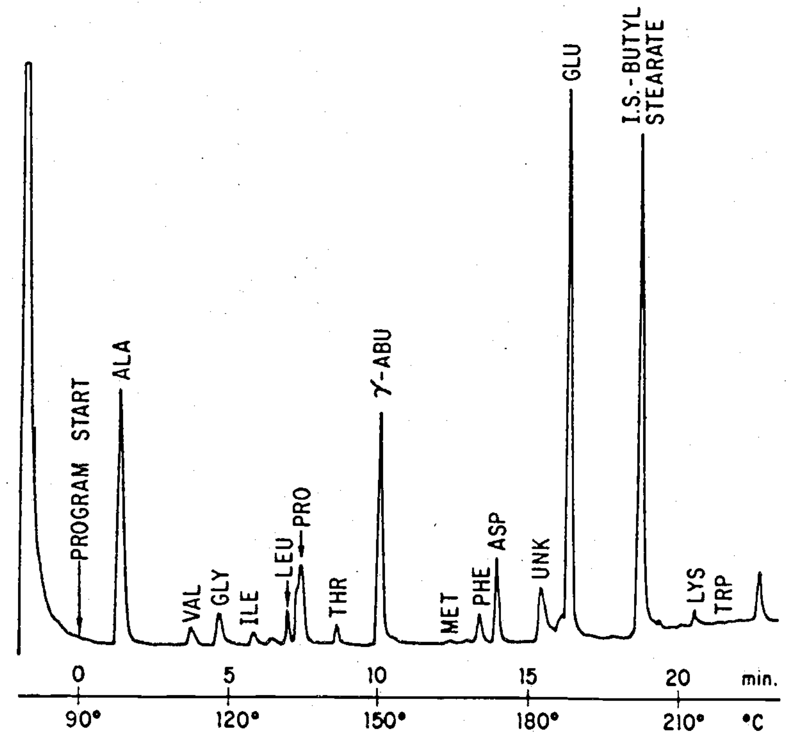


Figure 4. GLC chromatogram of amino acids present in the control seedling xylem sap (A-3, 4RX).

acid. The basic amino acids, arginine, tryptophan and histidine, are not represented in Figure 3 because they do not elute from the polyester EGA column.

Other amino acids are found to be present in the xylem sap; however, they were not present in large enough quantities to be quantified by the GLC method. These amino acids, which include cysteine, proline, serine, methionine, and tyrosine, were identified by mass spectrometry; 4-aminobutyric acid was also identified by mass spectrometry. Cysteine is found to elute after isoleucine followed by proline. Serine elutes just before 4-aminobutyric acid which elutes just before methionine. Tyrosine elutes between glutamic acid and the internal standard. Mass spectra for selected amino acids found in the xylem sap of the control seedlings are shown in the Appendix. The spectrum for the internal standard, n-butyl stearate, is also shown.

Figures 4 through 6 show representative chromatograms of the xylem sap amino acids present in the control (A) seedlings, the 24-hour solution-treated seedlings (E) and the 24-hour potted seedlings (F) respectively. The controls and the solution treated seedlings are very similar but differ from the potted seedlings which show a very large amount of glutamic acid present. The double peak present in Figure 6 may be due to glutamic acid plus glutamine;

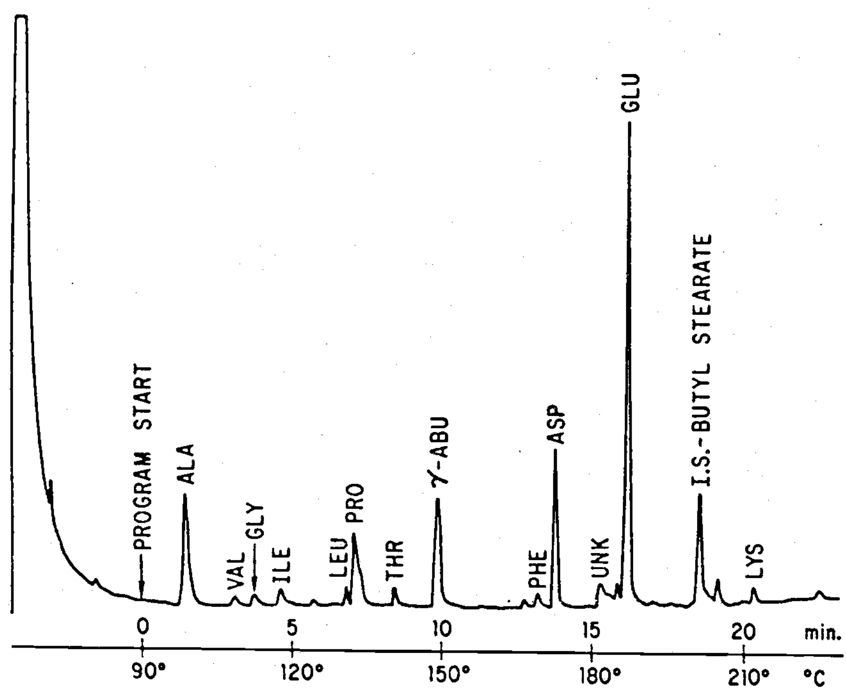


Figure 5. GLC chromatogram of amino acids present in the xylem sap of the seedlings exposed to the nutrient solution (E).

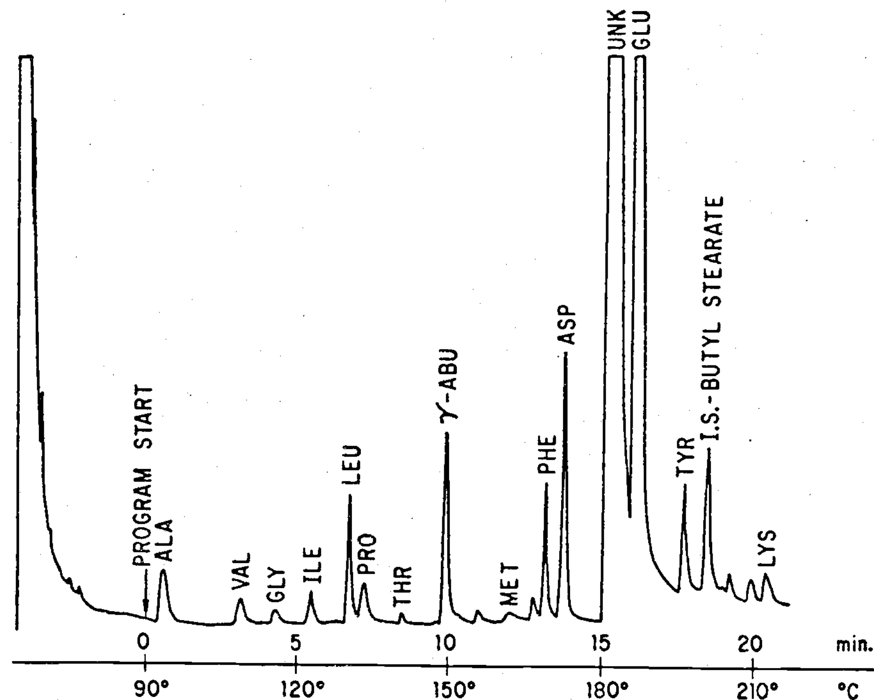


Figure 6. GLC chromatogram of amino acids present in the xylem sap of the potted seedlings (F).

an earlier attempt by this author to derivatize pure glutamine resulted in a chromatogram showing two peaks very similar to the double peak associated with glutamic acid in Figure 6.

Cation Exchange Recovery of Amino Acids

Table 3 shows the percent recovery of the amino acids from the cation exchange procedure. The recoveries are low and on the whole averaged 39%. The recoveries of alanine and cysteine are especially low with recoveries of 19 and 22% respectively. In contrast, tyrosine shows an 88% recovery from the ion exchanger.

Diurnal Concentrations of Amino Acids

Figures 7 through 10 show the diurnal concentrations of glutamic acid (glu), aspartic acid (asp), 4-aminobutyric acid (4-abu), alanine (ala), isoleucine (ile), threonine (thr), glycine (gly), valine (val), lysine (lys), leucine (leu), and phenylalanine (phe) in the root and stem xylem sap of the solution treated seedlings. In general, there is a peak concentration when the lights are turned off and a low, four hours after the lights are turned on. The concentrations of the amino acids represented in the chromatograms range from a high of approximately 3 μmoles/ml of xylem sap to a low of 0.001 μmoles/ml. The concentrations of each amino acid at time zero and 24 hours are generally the same.

Table 3. Concentration of standard amino acids before and after cation exchange cleanup for percent recovery determination.

Amino Acid	Molarity of Recovered Standard AA Mix.	Molarity after Cation Exchange Cleanup	% Recovery
ala	0.75	0.1439	19
val	0.75	0.3034	40
ile	0.75	0.2915	39
leu	0.75	0.3015	40
pro	0.75	0.2610	35
thr	0.75	0.2651	35
ser	0.75	0.3336	44
cys	0.75	0.1674	22
met	0.75	0.3015	40
phe	0.75	0.2856	38
asp	0.75	0.3093	41
glu	0.75	0.2859	38
tyr	0.75	0.6623	88
lys	0.75	0.2557	34
gly	0.75	0.2804	37

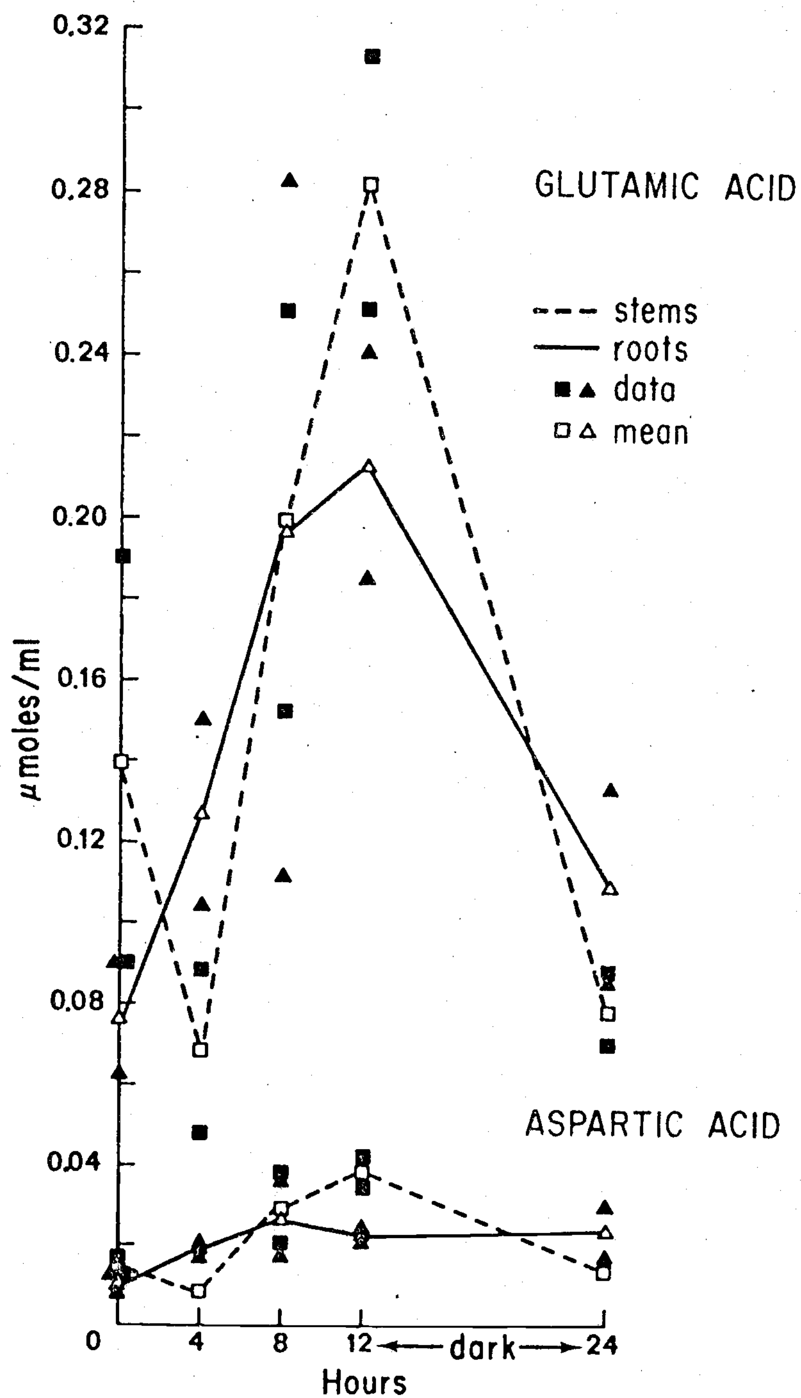


Figure 7. Diurnal concentrations of glutamic acid and aspartic acid.

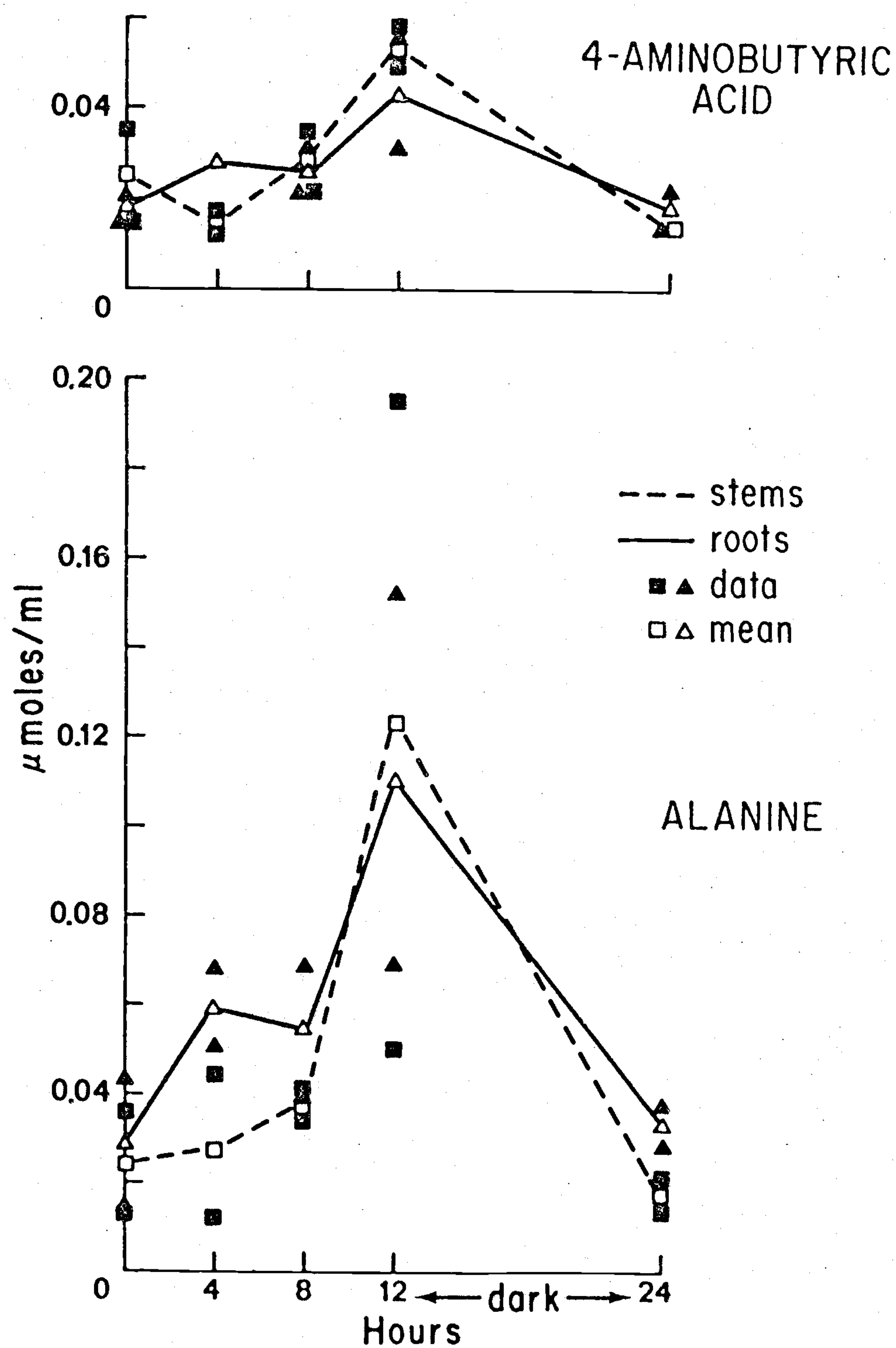


Figure 8. Diurnal concentrations of 4-aminobutyric acid and alanine.

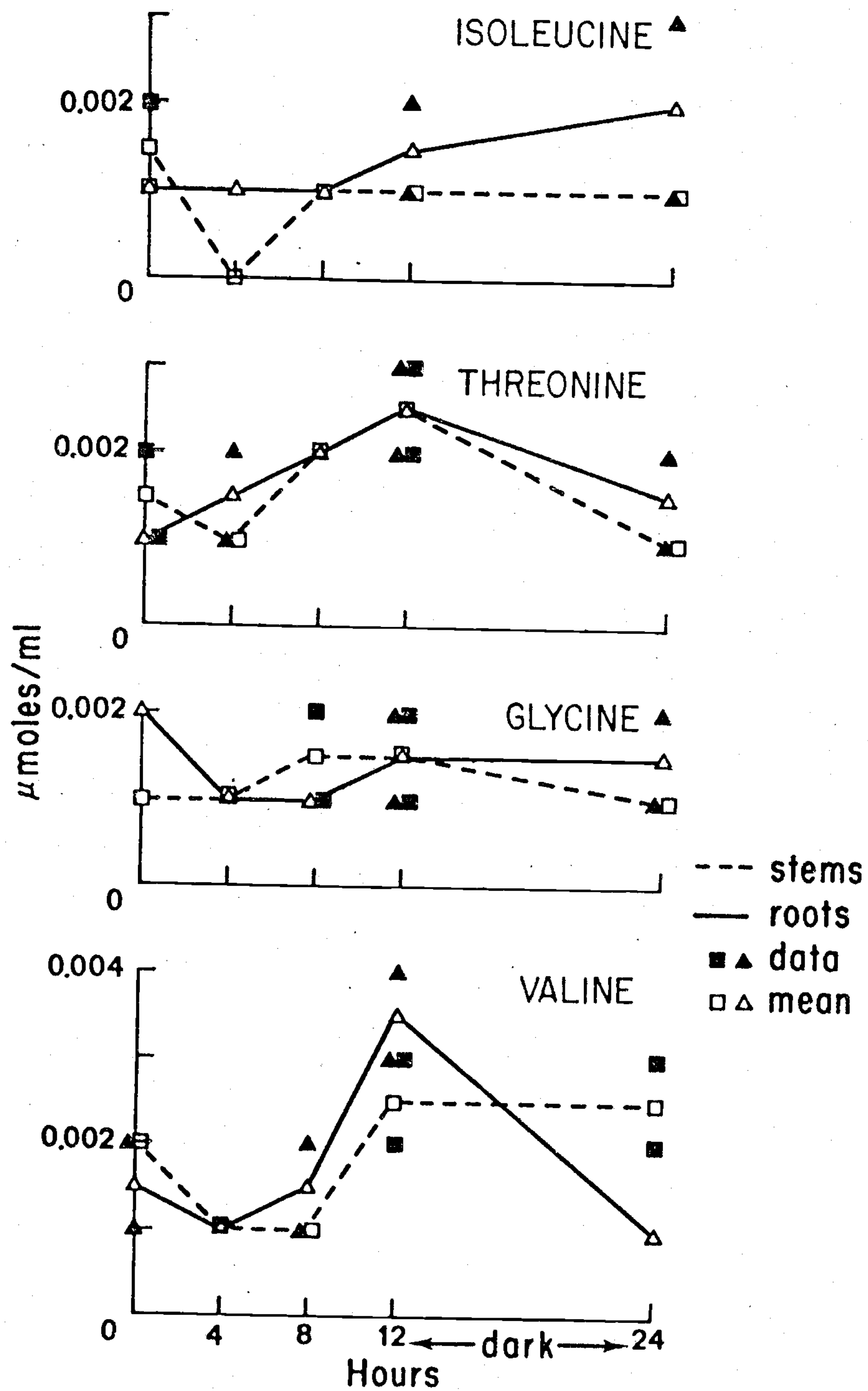


Figure 9. Diurnal concentrations of isoleucine, threonine, glycine and valine.

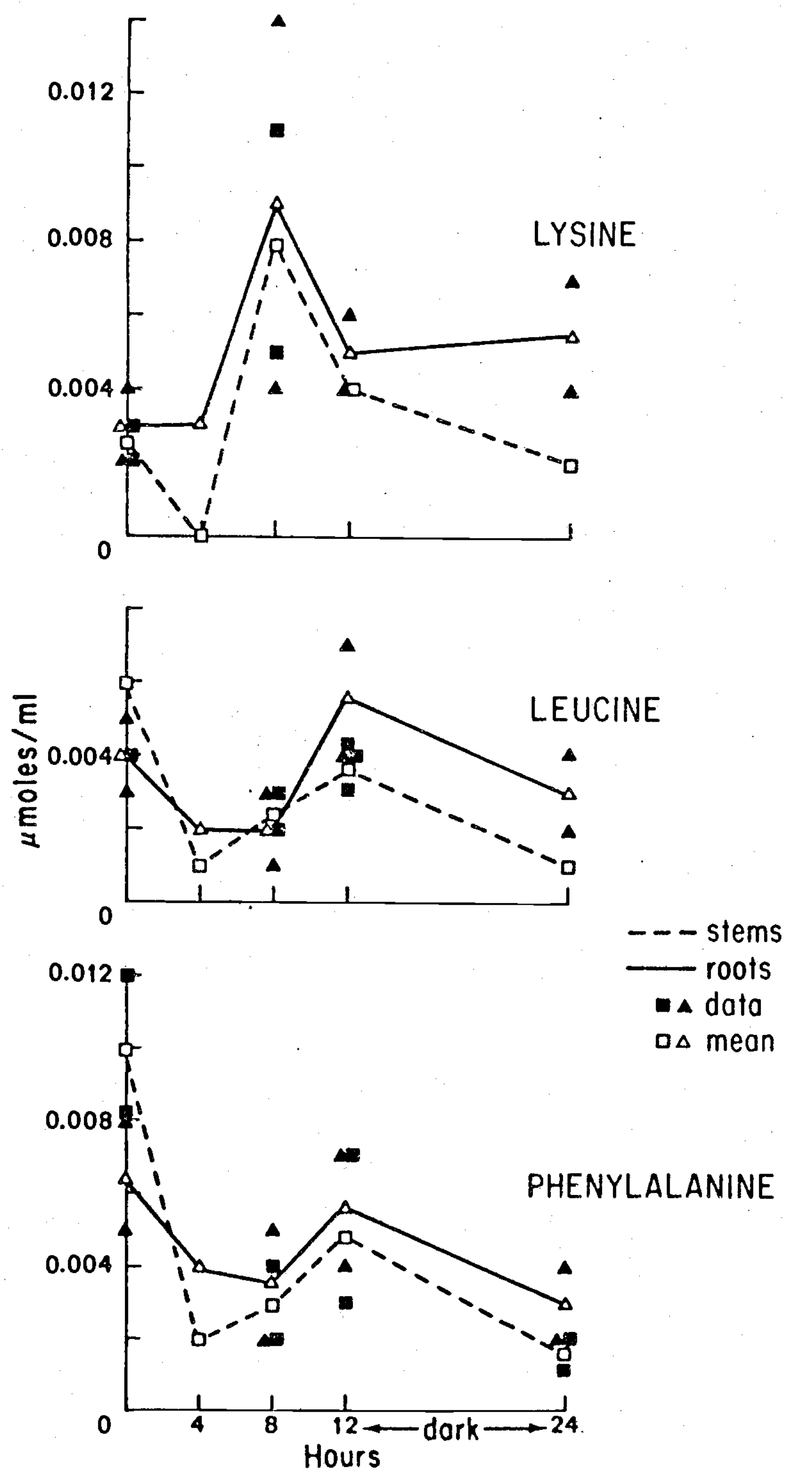


Figure 10. Diurnal concentrations of lysine, leucine and phenylalanine.

The data for the potted plants are not included in these graphs because data were taken for this treatment only at the 24 hour exposure. Of the amino acids analyzed in the potted seedlings, aspartic acid, 4-amino butyric acid, alanine, threonine, and glycine were found to have xylem sap concentrations commensurate with the solution treated seedlings. However, isoleucine, valine, lysine, leucine, and phenylalanine extracted from the potted seedlings had concentrations roughly 10 times greater than found in the solution treated seedlings; glutamic acid concentration could not be measured because its corresponding peak on the chromatogram could not be kept on scale.

Table 4 shows the average μmoles of amino acids present in the xylem sap of two roots or two stems of each treatment. The data for the solution grown plants show the same diurnal trends as shown in Figures 7 through 10.

Glutamic acid: Glutamic acid has the highest concentration in the xylem sap of any amino acid measured. The average concentration, for root plus stem sap, ranges from 0.1 μmole/ml at time zero to about 0.24 μmole/ml at 12 hours when the lights are turned off. The concentration drops to about 0.1 μmole/ml at the end of 24 hours (12 hours light and 12 hours dark). The concentration of glutamic acid is greater in the stem sap than the root sap at time

Table 4. Total μmoles/2 roots (or 2 stems) of each amino acid in each treatment.

Treatment		Total	ala		val		gly		ile	
			R	S	R	S	R	S	R	S
C	A	2.009	0.262	0.061	0.014	0.005	0.018	0.002	0.009	0.004
4L	B	2.782	0.553	0.109	0.009	0.004	0.009	0.004	0.009	0
8L	C	2.835	0.367	0.075	0.010	0.002	0.007	0.003	0.007	0.002
12L	D	7.208	1.470	0.406	0.046	0.008	0.020	0.005	0.020	0.003
12L + 12D	E	1.931	0.264	0.041	0.008	0.006	0.012	0.002	0.016	0.002
12L + 12D (potted)	F	2.975 (- glu)	0.099	0.034	0.056	0.093	0.009	0.014	0.042	0.090

Treatment		Total	leu		thr		4-abu		phe	
			R	S	R	S	R	S	R	S
C	A	2.009	0.037	0.015	0.009	0.004	0.166	0.062	0.060	0.025
4L	B	2.782	0.019	0.004	0.014	0.004	0.260	0.056	0.037	0.008
8L	C	2.835	0.014	0.005	0.014	0.004	0.180	0.057	0.024	0.006
12L	D	7.208	0.073	0.012	0.033	0.008	0.572	0.176	0.073	0.016
12L + 12D	E	1.931	0.024	0.002	0.012	0.002	0.148	0.035	0.024	0.004
12L + 12D (potted)	F	2.975 (- glu)	0.263	0.426	0.014	0.032	0.179	0.139	0.367	0.357

Treatment		Total	asp		glu		lys	
			R	S	R	S	R	S
C	A	2.009	0.092	0.036	0.699	0.395	0.028	0.006
4L	B	2.782	0.177	0.031	1.180	0.267	0.028	0
8L	C	2.835	0.180	0.058	1.340	0.403	0.061	0.016
12L	D	7.208	0.299	0.125	2.830	0.932	0.066	0.013
12L + 12D	E	1.931	0.188	0.032	0.872	0.188	0.044	0.005
12L + 12D (potted)	F	2.975 (- glu)	0.357	0.238	--	--	0.056	0.110

zero and at 12 hours, but is lower in the stem than the root at 4 hours and 24 hours. At 8 hours, the concentration is equal in both fractions.

Aspartic acid: Aspartic acid shows a steady increase from zero to 12 hours of light with concentrations ranging from 0.012 μmole/ml to 0.030 μmole/ml respectively, for root plus stem sap. At the end of the 12 hours of dark the concentration is about 0.018 μmole/ml. The concentration of sap in the separate root and stem sap fractions shows the same trend as found in glutamic acid.

4-Aminobutyric acid: 4-Aminobutyric acid is another amino acid present in large amounts relative to the others. Its initial concentration, for root sap plus stem sap, is about 0.021 μmole/ml. The concentration gradually increases to about 0.05 μmole/ml at 12 hours and then drops to about 0.016 μmole/ml at the end of 24 hours. The concentration of this amino acid, in the separate root and stem sap fractions, shows a trend similar to glutamic acid throughout the exposure period, except for the 24 hour period, where both fractions appear to have equal concentrations of the amino acid.

Alanine: The concentrations represented for alanine, throughout the day, are only exceeded by glutamate. In general, for root plus stem sap, there is a steady increase in concentration from about 0.025 μmole/ml at time zero to about 0.1 μmole/ml at 12 hours. At the end of the 12 hour dark period, the concentration drops to its

zero hour value. The alanine concentration in the root sap is greater than in the stem sap at 4, 8 and 24 hours of the exposure period. At zero and 12 hours, the alanine concentration in both fractions appears to be the same.

Isoleucine: Isoleucine is present in very low amounts throughout the exposure period. The concentration of this amino acid drops from zero hours to 4 hours and increases steadily to a high at the end of the 24 hour light and dark exposure. The average concentration only reaches a maximum of 0.0015 µmole/ml at 24 hours. This occurs because the concentration of this amino acid in the root sap increases. Isoleucine concentration in the stem shows a high at zero hours and a low at 4 hours. It increases again at 8 hours and then levels off for the remainder of the exposure period. The concentration in the root sap is constant for the first 8 hours of exposure and then steadily increases to a high at the end of 24 hours.

Threonine: Threonine shows a steady increase in the average concentration from 0.0012 µmole/ml at time zero to 0.0025 µmole/ml at 12 hours of light. The concentration then drops to the zero value at the end of the dark period. The data suggest that the concentration of threonine in the separate root and stem fractions is equal throughout the experiment.

Glycine: Glycine, like isoleucine, is also present in low amounts. The initial concentration at time zero is about 0.0015

μmole/ml. A drop occurs at 4 hours to a low of about 0.001 μmole/ml. The concentration then steadily increases to 0.0015 μmole/ml at 12 hours. After 24 hours the concentration drops to 0.00125 μmole/ml. It would be difficult to contend that there is a difference in the concentration of glycine in the root and stem fraction for the 4 hour period and beyond. However, the initial glycine concentration in the root sap is twice as high as in the stem sap.

<u>Valine</u>: The average valine concentration shows a drop from about 0.002 μmole/ml to 0.001 μmole/ml at 4 hours. The concentration then steadily increases to a high of 0.003 μmole/ml at 12 hours. At the end of 24 hours, the concentration drops to about its zero time value. The data show that the valine concentration in the stem sap appears to be the same in the root sap for the first 8 hours. The 12 hour period shows the valine concentration to be higher in the root than the stem sap fraction. This is reversed at the 24 hour period.

<u>Lysine</u>: Lysine shows an average concentration drop from about 0.003 μmole/ml at time zero to about 0.001 μmole/ml at 4 hours of light. Between 4 hours and 8 hours of light there is an increase to about 0.0085 μmole/ml. The concentration drops to about 0.0045 μmole/ml at 12 hours of light. This contrasts with the other amino acids in that the peak concentration is at 8 hours of light instead of 12 hours. After 12 hours of light and 12 hours of

dark, the concentration levels off to about 0.004 μmole/ml; slightly higher than the zero hour value. The lysine concentrations in the separate sap fractions appear to be nearly equal throughout the exposure period with the 4 and 24 hour periods the exception. There is no detectable lysine in the stem fraction at 4 hours and the 24 hour concentration in the stem is at its initial value, but lower than the concentration found in the roots.

Leucine: Leucine shows an average concentration low of about 0.0015 μmole/ml, which occurs 4 hours after the light is turned on. The concentration then increases to a high of about 0.0045 μmole/ml at 12 hours of light (approximately the same level found at time zero). After the 12 hour light and 12 hour dark periods, the concentration drops to less than half the starting value at time zero. The leucine concentration, in the separate root and stem sap fractions, shows the same trend throughout the exposure period as with glutamic acid.

Phenylalanine: Phenylalanine shows a drop in the average concentration at the end of 4 hours of light; 0.008 μmole/ml to 0.003 μmole/ml. The concentration levels off until 8 hours when it increases to about 0.005 μmole/ml at 12 hours. At the end of 24 hours the concentration drops down to about 0.002 μmole/ml. The phenylalanine concentration of the separate root and stem sap fractions, throughout the exposure period, shows a trend similar

to glutamic acid. The exception is the 12 hour period which shows the two fractions to have about equal concentrations of phenylalanine.

In general, the diurnal concentrations of the amino acids in Douglas-fir xylem sap decrease in the following order: glu > ala > 4-abu and asp > lys > phe > leu > val and thr > gly > ile. Five amino acids show similar concentration trends in the separate root and stem sap fraction throughout the exposure period; these are glu, asp, 4-abu, leu, and phe.

^{14}C Incorporation into Amino Acids

Liquid scintillation counting: Table 5 shows the average dpm/ml of ^{14}C incorporated in amino acids present in Douglas-fir xylem sap. Seedlings treated with the nutrient solution show no ^{14}C incorporation into amino acids of the xylem sap throughout the entire exposure period. The data for the potted seedlings are included to show that these plants did have ^{14}C incorporated into their xylem sap amino acids.

GLC-ionization chamber counting: Figure 11 shows the strip chart recording the ionization chamber output for the N-TFAA n-butyl ^{14}C-glutamic acid ester used as a standard. The graph shows a definite peak with the output tracing returning to the original baseline.

Table 5. Average dpm/ml of ^{14}C in extracted amino acids from Douglas-fir xylem sap derivatized to their N-TFA n-butyl esters.

Treatment	Sap Source	Ave. dpm/ml
A	Root	0
A	Stem	0
B	Root	6.6
B	Stem	0
C	Root	0
C	Stem	6.5
D	Root	0
D	Stem	0
E	Root	0
E	Stem	0
F	Root	1499.6
F	Stem	1964.2

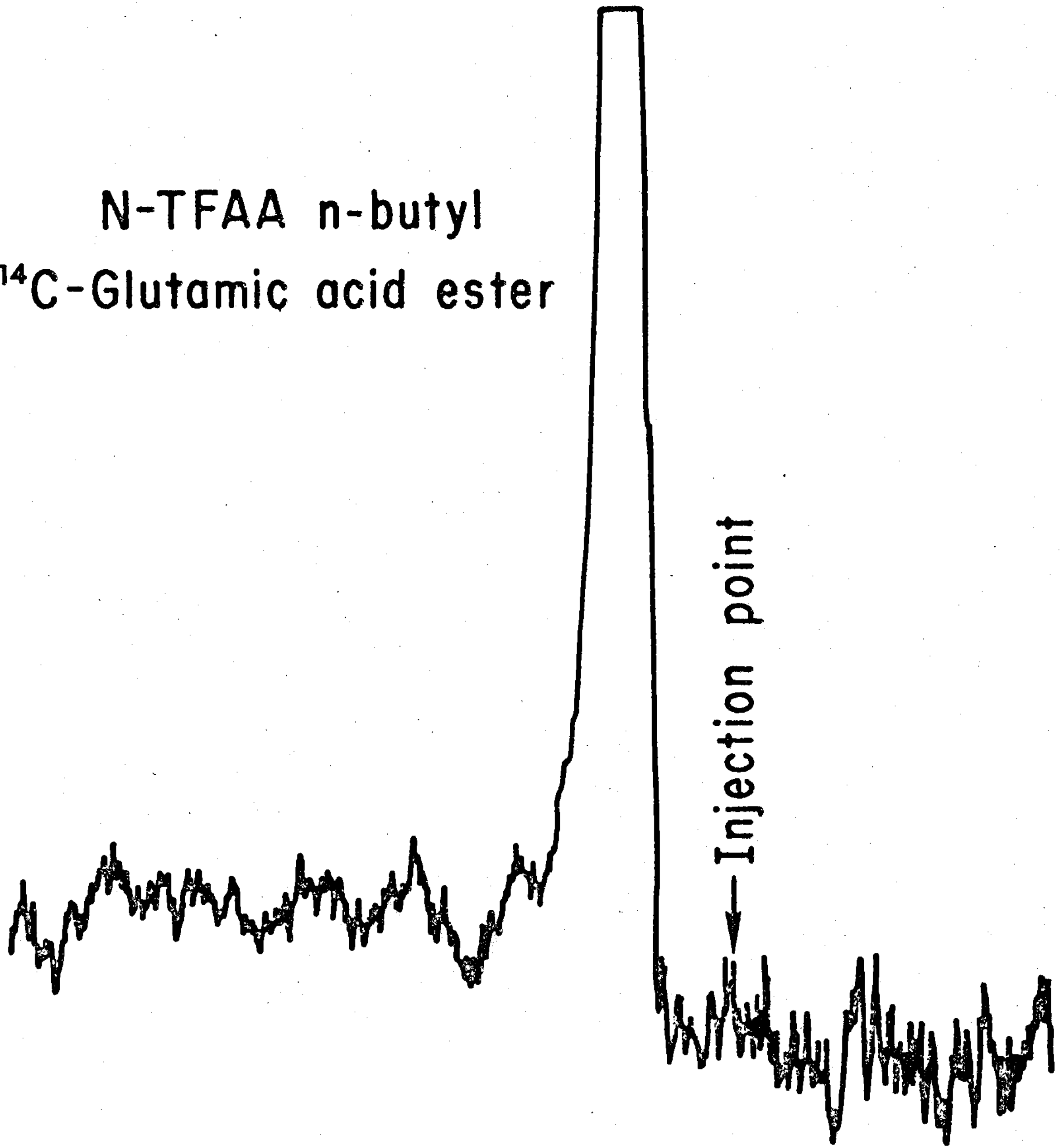


Figure 11. Strip chart recording of ionization chamber output for N-TFAA n-butyl ^{14}C-glutamic acid ester.

Figure 12 shows the strip chart recordings of the output from the ionization chamber for the amino acid samples from the 24 hour solution treated and potted seedlings. The amino acids from the solution treated seedlings have no ^{14}C (confirming the liquid scintillation method) as evidenced by the graph. What appear to be peaks on these graphs can be accounted for by noise in the system as seen in the irregularities of the peaks. The sample from the potted seedlings, on the other hand, shows ^{14}C present in the derivatized amino acids, as the peaks are very distinct and rise well above the noise level.

Table 6 shows the estimated dpm/ml in each peak for the F sap in Figure 12. These values are about 50% greater than the values obtained from the liquid scintillation counting method (if the F-12RX value is not considered). The root samples give a lower estimate than the stem samples because it is difficult to ascertain the true area of the curve for the F-1,2RX sample shown in Figure 12.

Figure 13 shows the ionization chamber output for a temperature programmed run of one of the F samples. The graph shows a main ^{14}C peak eluting at about 133°C. The 133°C elution peak corresponds to the peak identified as proline in Figure 6, as verified by the retention temperatures of the standard amino acid mixture and by mass spectrometry.

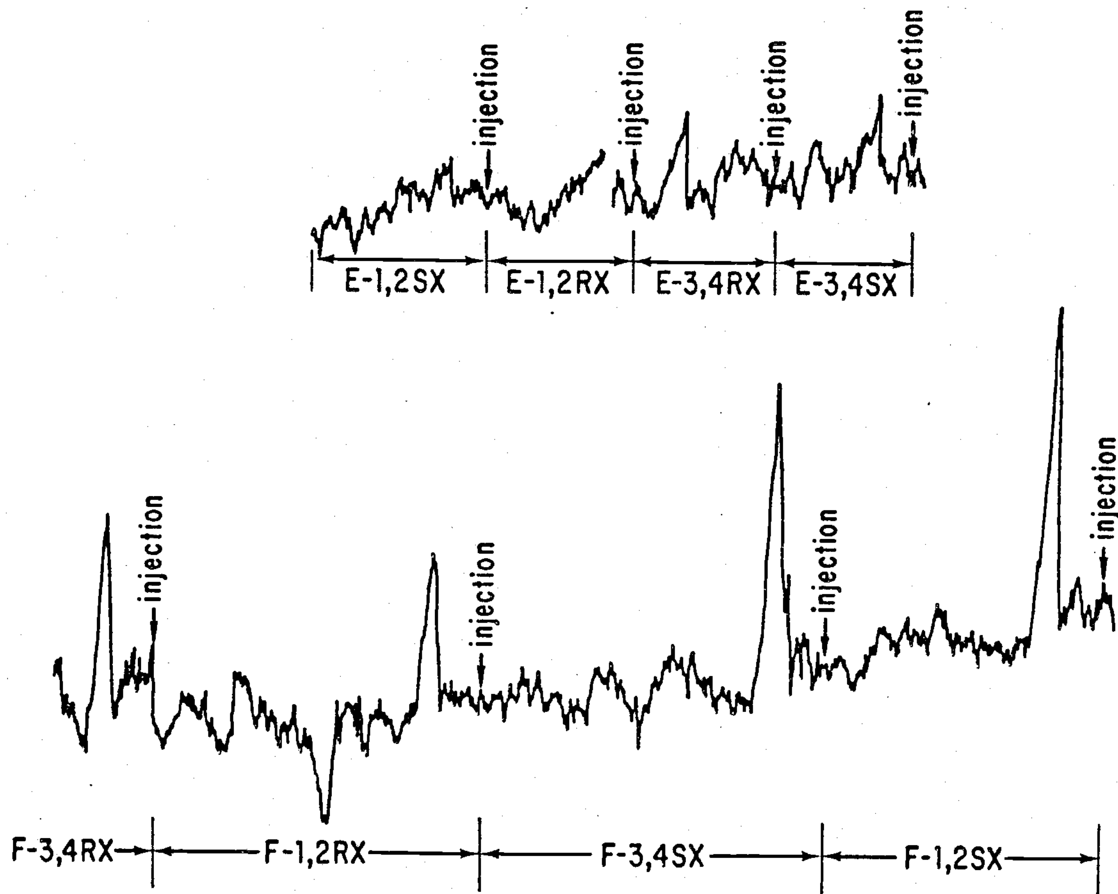


Figure 12. Strip chart recording of ionization chamber output for all the N-TFAA n-butyl esters of the xylem sap amino acids from the E treatment (top) and the F treatment (bottom) seedlings.

Table 6. Estimated dpm/ml for ^{14}C in peaks shown in Figure 12.

Xylem Sap Source	Estimated dpm/ml	Mean	% of Liquid Scintillation
F-1, 2SX	2624.8	2827.3	1.43
F-3, 4SX	3029.8		
F-1, 2RX	805.0	1579.3	1.05
F-3, 4RX	2353.6		

Table 7. Average specific activity (dpm/mg) of roots, old needles, new needles, stems and total plant tissue for the control (A), solution treated (E) and potted (F) seedlings.

Tissue Type	A	E	F	Significance between Means of E and F
Roots	5.4	5.4	143.6	Significant at $t_{.01}$
Old needles	4.4	2235.4	1370.3	Significant at $t_{.02}$
New needles	3.5	4127.6	5248.1	Significant at $t_{.3}$
Stems	3.9	195.6	404.4	Significant at $t_{.001}$
Total plant	17.2	809.6	679.6	Significant at $t_{.4}$

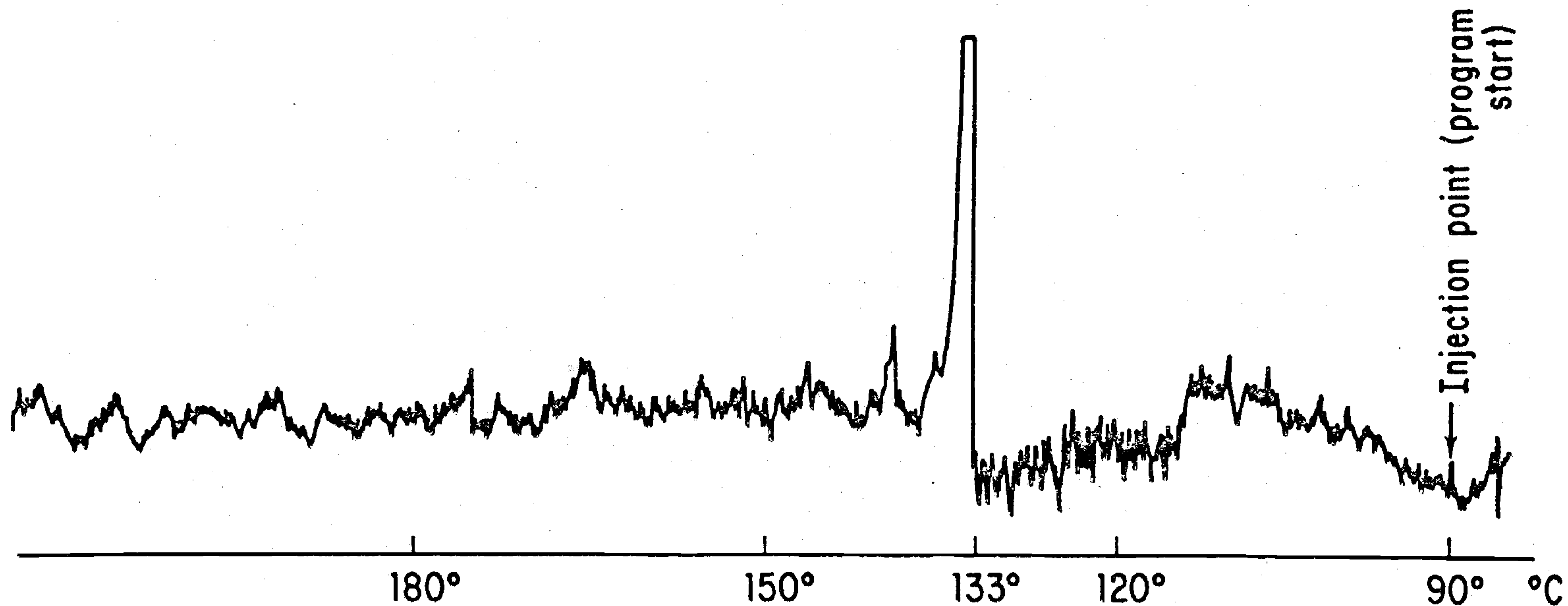


Figure 13. Strip chart recording of ionization chamber output for N-TFAA n-butyl esters of xylem sap amino acids from treatment F (F-1,2SX): GLC-ionization chamber system, temperature program.

^{14}C Incorporation into Plant Tissue

Table 7 shows the average ^{14}C specific activity (dpm/mg) for the roots, old needles, new needles and stems for the control and the two different 24 hour treatments, E and F. The ^{14}C specific activity in the control tissues is background only, as would be expected. The tissues from the solution treated and potted seedlings, E and F respectively, show distinct differences in the specific activities of each tissue type. The roots of the solution treated seedlings show essentially no incorporation of ^{14}C, whereas the roots of the potted seedlings did. There is no significant difference in the specific activity of the new needles between the solution treated and potted seedlings. The stems of the potted seedlings have over two times more ^{14}C present than do the stems of the solution treated seedlings.

Table 7 also shows the average total seedling specific radioactivity for the control, solution treated and potted seedlings. The potted seedlings incorporate the same amount of ^{14}C as the E seedlings do.

^{15}N Incorporation in Amino Acids

Figure 14 shows the percent increase over the control plants of ^{15}N in alanine, 4-aminobutyric acid, aspartic acid and glutamic acid present in the root and stem extracted xylem sap of the E and F

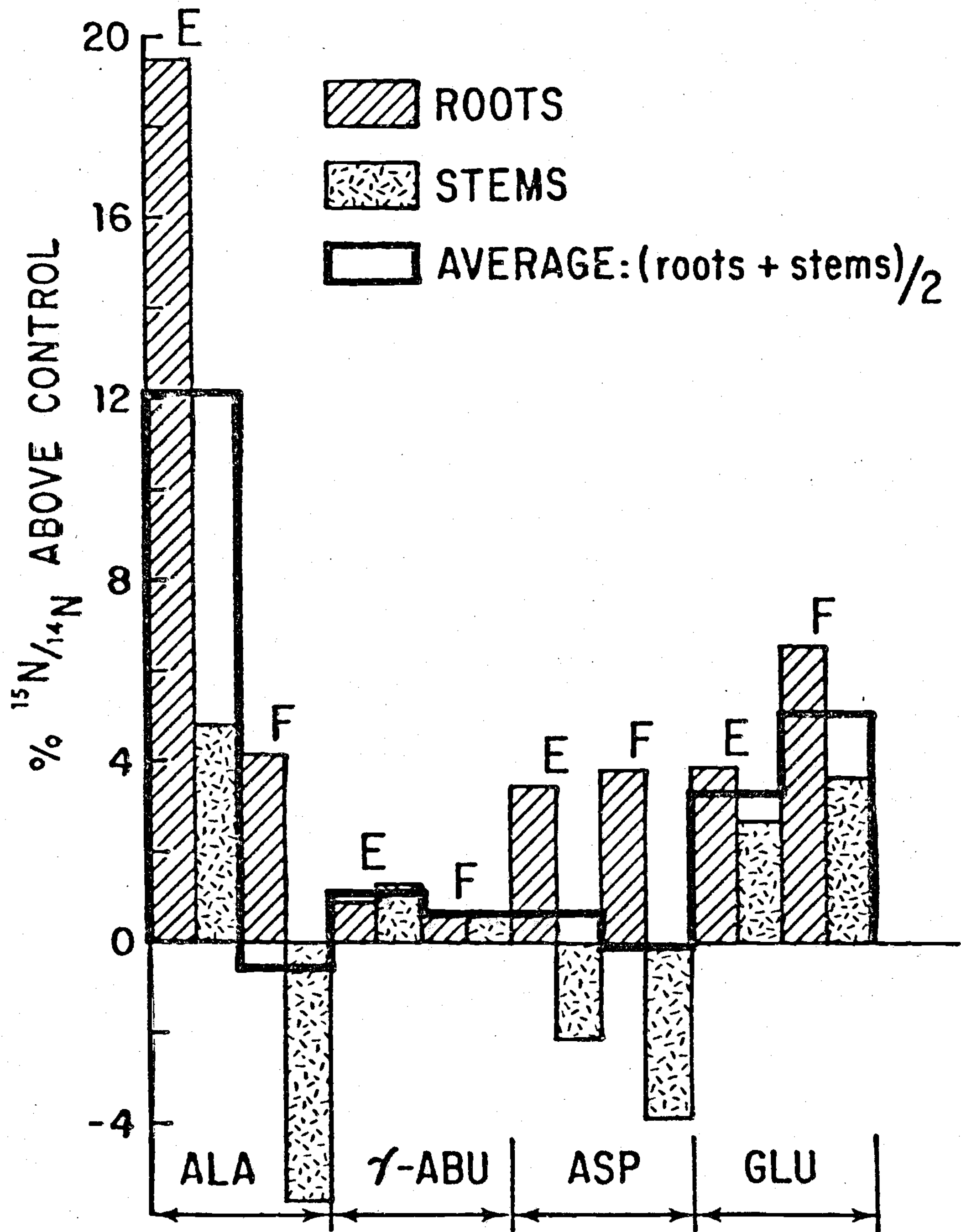


Figure 14. The percent increase, over the control, of ^{15}N in alanine, 4-aminobutyric acid, aspartic acid and glutamic acid present in xylem sap extracted from roots and stems of the E and F treatment seedlings.

seedlings. The average ^{15}N increase in each amino acid of both root and stem is also shown. These four amino acids were chosen for the ^{15}N study because they were the most important major amino acids found in the extracts and were the most possible compounds to show any increase in ^{15}N. They were the only amino acids to show a significant increase in ^{15}N enrichment. The mass spectrometer was operating at its limit in detecting any ^{15}N increases in the experimental treatments; therefore, it is difficult to discern whether ^{15}N enrichment occurred in these amino acids. There must be at least a 5% increase of ^{15}N above the control to be certain enrichment has occurred in an amino acid.

All of the above amino acids from the root and stem xylem sap, with the exception of aspartate, extracted from the solution treated seedlings, show an increase in ^{15}N abundance over the controls. The data for the potted seedlings are also shown in Figure 14 for comparison. These amino acids extracted from the root xylem sap of the potted seedlings show a greater percent in ^{15}N than those from the stem xylem sap. Alanine shows the greatest percent increase of ^{15}N in all the amino acids from the solution treated seedlings. The root fraction is especially high showing about a 19.5% increase over the control. The ^{15}N enrichment of alanine in the stem fraction is only 25% of the root fraction. Four-amino butyric acid shows a very small increase in ^{15}N. Both the root and stem

fraction appear to be about equal, however, due to the limits of the mass spectrometer no definite conclusions can be made. Aspartic acid shows about a 3.5% increase of ^{15}N in the root fraction of the solution treated seedlings. Conversely, there was a 2% loss in abundance of ^{15}N in the fraction thus indicating possible error present in the assay. Glutamic acid shows a 4% and a 3% increase in ^{15}N abundance in the root and stem fractions of the solution treated seedlings.

The data for the potted seedlings show more variation than the data from the solution treated seedlings. Perhaps this should be expected because the solution treated control seedlings were used as the controls for the potted seedlings as no potted controls were provided. Also, the roots of the potted seedlings were in soil and not directly in contact with the nutrient solution.

Alanine, from the root fraction of the potted seedlings, shows only a 4% increase in ^{15}N abundance. However, this contrasts with the near 6% loss in the stem fraction, again indicating possible error in the assay. Four-aminobutyric acid shows less than a 1% increase in both the root and stem fraction. Aspartic acid shows the same pattern as alanine with a near 4% increase of ^{15}N in the root sap compared to a 4% loss in the stem sap. Glutamic acid shows the largest increase in ^{15}N of all the amino acids present in the potted seedlings. The root fraction shows a 6.5% increase and the

stem fraction shows a 4.5% increase. Also, the glutamic acid in the potted seedlings shows a higher ^{15}N enrichment than the solution treated seedlings.

V. DISCUSSION

This study demonstrates that gas-liquid chromatography can be applied in studying amino acid biosynthesis and metabolism in tree seedlings, because GLC can be linked to mass spectrometry and gas ionization systems for the quantification of ^{15}N and ^{14}C in amino acids. With this system of instruments it is now possible to discern the metabolic link between photoassimilated carbon and root assimilated nitrogen.

The experimental data show that the N-trifluoroacetyl n-butyl esters of the amino acids in Douglas-fir xylem sap can be resolved by the specified GLC column under the conditions described. This makes it possible to study not only the diurnal concentration changes in each amino acid in the xylem sap, but also the radioactive carbon (^{14}C) and heavy nitrogen (^{15}N) in each.

Amino Acid Composition in Douglas-fir Xylem Sap

The amino acid composition in the Douglas-fir xylem sap of this study corresponds very well with a study conducted by Webber and DeBell (unpublished data). Their data also includes histidine and arginine which was not analyzed in this study because they do not elute from the EGA column. The average concentrations of the xylem sap amino acids in this study agree, in magnitude, to the

concentrations of the Webber and DeBell study even though their work did not analyze diurnal fluctuations.

It is not surprising that glutamic acid (glutamate + glutamine) appears in the greatest concentration of all the amino acids. As reported in the literature review, conclusive evidence shows that glutamic acid and glutamine are the principle products of nitrogen assimilation in tree roots and that both of these compounds are present in large amounts in the xylem sap of conifers. This would especially hold true in the present study since it was performed in the spring during budbreak; a time when the actively growing needles and shoots require a readily available source of nitrogen. Glutamine and glutamate have the capability of responding to this need very well. Their production and transport are essential because they are easily transportable and are involved in a multitude of secondary reactions requiring either nitrogen or carbon or both.

Earlier work by this author (unpublished) shows that xylem sap collected from stems of older Douglas-fir trees (approximately 30 years in age) during budbreak contained glutamic acid in amounts exceeding the other amino acids present. The collection, extraction and derivatization procedures of the amino acids in these samples were identical to the procedures outlined in Materials and Methods. The amino acid derivatives were eluted from an EGA column;

however, the column material was teflon lined stainless steel tubing instead of glass.

In this study, glutamic acid is also a primary indicator that there is a distinct difference between the results of the two treatments E and F. The plants exposed to the nutrient solution have only a fraction of the glutamic acid that is present in the seedlings remaining in the soil. The F seedlings can be considered to be normal in this study because their root systems were never disturbed. The evidence suggests, therefore, that glutamate metabolism, formation and transport is adversely affected by the handling and conditions present in the E treatment.

It can be expected that the roots of the solution treated seedlings were improperly aerated thus adversely affecting respiration. Alpha-ketoglutaric acid is the immediate precursor to glutamate and is also a respiratory intermediate. Therefore, if the experimental conditions were inhibiting respiration they would also be negatively influencing glutamic acid production and the production of any other compound requiring keto acids for its formation.

Canvin and Atkins (12) show in leaf tissue that the absence of oxygen has no effect on nitrate reduction. They contend that glytolytic respiration can fulfill the electron needs of the reduction process. Also, NADH levels may increase in the tissues subject to low oxygen levels, because the electron transport chain can no longer

accept electrons for the reduction of oxygen to water. Therefore, enough NADH is present to reduce nitrate (32). Taking this into account, the anaerobic conditions present in this experiment would do more to divert the formation and flow of carbon metabolites rather than directly diminish nitrate reduction.

Any reduction in glutamate formation would necessarily have to result in the reduction of glutamine since this compound is synthesized from glutamic acid and ammonia. This is perhaps the greatest, single factor for the drop in glutamic acid (glutamine) concentration as shown in the GLC chromatograms of the control and solution treated seedlings as compared with the chromatogram of the potted seedlings (Figures 4, 5 and 6).

A reduction in adenosine triphosphate (ATP) production may also help explain the loss of glutamine. ATP production is to a large extent dependent upon aerobic respiration. Without oxygen, the flow of electrons through the respiratory electron transport chain is virtually stopped, resulting in a loss of ATP production. Without ATP, glutamine formation is also stopped.

To speculate, there may be two pools of glutamic acid in the roots; one whose function is to produce compounds which are transportable in the xylem sap for use in other plant tissues, and a second pool which produces compounds that are metabolized in the root tissue for growth and maintenance that can also appear in the xylem

sap. The production of the first pool is sensitive to oxygen levels around the root. When oxygen decreases below optimum levels, the TCA cycle slows, restricting the production of α-ketoglutarate and ATP. A reduction in the keto acid would slow the production of new glutamic acid and a reduction in ATP would slow glutamine production as mentioned earlier. The second pool may be produced independent of root conditions. Alpha-ketoglutarate could be formed outside the root tissue, from the glutamate dehydrogenase reaction or by transamination of the amino group of glutamate to another keto acid, and transported into the roots for glutamate production. Under anaerobic conditions, glutamic acid and glutamine may still be produced (on a reduced scale) in the roots but their intended function may be for tissue maintenance rather than large scale export to other regions of the seedling.

Neither glutamic acid or aspartic acid showed any trace of ^{14}C incorporation in either the E or the F treatment seedlings. This is difficult to explain because it would be expected that ^{14}C incorporation would occur in these two compounds because of their importance in metabolic reactions throughout the plant. Both glutamate and aspartate show ^{15}N incorporation as would be expected. However, it must be kept in mind that the $^{15}NO_3$ fed to the potted seedlings had to pass through the soil; every little ^{15}N would be expected to occur in any amino acids extracted from these seedlings

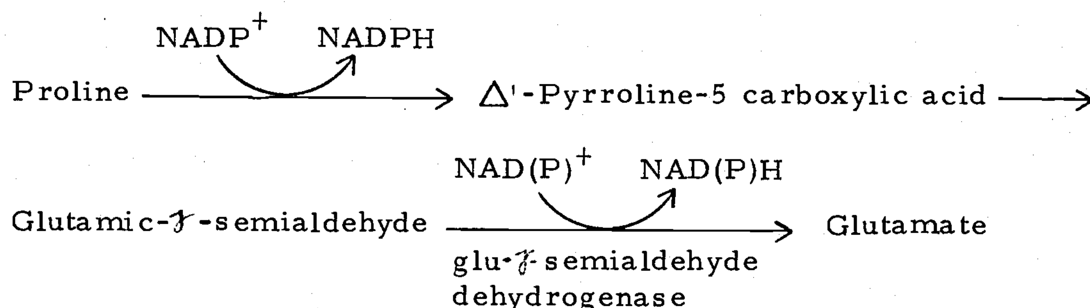
as compared to amino acids extracted from the solution treated seedlings. This problem is even more pronounced since no potted controls were provided for. Also, since the amide groups of glutamine and asparagine are hydrolyzed, an important source of detectable ^{15}N is eliminated.

Aspartic acid (asp + asn) was not present in very large amounts when compared with glutamic acid in both the solution treated and potted seedlings. This is surprising in light of its potential capacity to carry and transport extra nitrogen, as does glutamate and glutamine. This suggests that glutamic acid (more likely glutamine) is the major nitrogen transporting compound in Douglas-fir in the spring. This is not, however, a conclusive statement. Arginine, which was not analyzed for, but is found in Douglas-fir xylem sap (Webber and DeBell, unpublished), has an even greater potential function as a nitrogen transporter. Of all the amino acids, arginine has the highest N/C ratio.

Proline is included in this discussion because it is thought to be the only amino acid to have ^{14}C incorporated into it. This occurred only in the F seedlings since no ^{14}C was incorporated into any amino acid in the E seedlings. These results were unexpected and are difficult to explain.

Durzan and Ramaiah (22) and Durzan (23) show that proline is an active metabolite during budbreak in conifers. They explain that

in expanding buds, proline is metabolized to glutamine via glutamic acid by the following reaction:



The precise intermediates have not been found. Durzan further suggests that during spring budbreak, proline could be a readily available energy source because its conversion to glutamate would yield NADPH. Therefore, proline may be a transportable form of readily utilizable energy for early tissue growth providing reducing power for the reduction of CO_2 during photosynthesis, while at the same time, providing glutamic acid and glutamine which can be utilized in a multitude of other reactions.

Proline is a product of glutamic acid metabolism(35, 58). This fact alone can account for the ^{14}C showing up in proline, but it does not explain why proline is the exclusive recipient of the label. Because glutamic acid is normally found to be the precursor to proline, and glutamic acid is formed from the respiratory intermediate α-ketoglutaric acid, one would expect that the ^{14}C label would show up in the other amino acids formed directly from respiratory intermediates such as alanine and aspartic acid. The problem is even more perplexing because glutamic acid (glu + gln)

is present in the xylem sap in the highest concentration. Obviously a more in-depth study, exploring the many facets of proline formation and metabolism, must be completed before any conclusions are drawn about this particular amino acid's function.

Improper aeration of the nutrient solution for the E seedlings is best established by examining alanine. The concentration of alanine in the xylem sap of these seedlings is exceeded only by glutamic acid. Also, the ^{15}N enrichment into alanine present in the root fraction of the solution treated seedlings far surpasses any other amino acid. Both of the preceding factors suggest that there is a block to aerobic respiration. This is substantiated by the fact that the glutamic acid concentration in the xylem sap from the solution treated seedlings is much less (approximately 10 times less) than that present in the sap from the potted seedlings. As explained earlier, without proper amounts of oxygen present for aerobic respiration, the respiratory intermediates of the tricarboxylic acid cycle would be depleted. With a block to the TCA cycle in effect, glycolysis would be expected to be the major mode of respiration occurring in the roots. Normally, pyruvate from glycolysis would be drawn into the TCA cycle to be further metabolized. However, with the TCA cycle inoperative, pyruvate must be metabolized to other products; ethanol, lactate or alanine may be produced (50).

Alanine is obviously a product of importance since ^{15}N enrichment into this compound is very pronounced. The question remains, however, as to how the alanine is formed and where the ^{15}N comes from. Direct incorporation of ^{15}N from reduced $^{15}NO_3$ is a possibility. As mentioned above, however, an alanine dehydrogenase, although reported to occur in some plant cells, has never been purified (35). The mode of alanine production is considered to be a transaminase reaction catalyzed by an alanine aminotransferase which has been isolated and purified. Alanine may also be formed by the decarboxylation of aspartic acid.

Guinn and Brinkerhoff (32) have shown that the amino acid composition in roots and xylem sap of cotton plants change drastically when the roots are subject to anaerobic conditions. Alanine accumulated more than any other amino acid in their study. Streeter and Thompson (85) assert that alanine accumulates under anaerobic conditions by the decarboxylation of aspartic acid. Their results, after feeding ^{14}C-aspartate to radish leaves, showed that the label appeared in alanine. However, the ^{15}N labeling of alanine in this study disputes the contention that alanine is formed from aspartate. There is no significant ^{15}N enrichment in aspartic acid, which would be expected if aspartate was the actual precursor to alanine.

The most logical explanation (based on the literature) for the increased ^{15}N enrichment in alanine would be the flow of reduced nitrogen to α-ketoglutaric acid to form glutamic acid, via reductive amination, which then forms alanine from pyruvate by a transaminase reaction. The data would support this hypothesis only by assuming that pyruvate accumulation, from anaerobic conditions, was creating a strong sink for the transaminase reaction to occur, thus causing the flow of newly assimilated ^{15}N to go toward alanine production. Pyruvate is most likely the only nitrogen acceptor under anaerobic conditions to accumulate, thus giving it the capability of being a very strong sink for newly assimilated nitrogen.

Four-aminobutyric acid is another puzzling aspect of this experiment. Streeter and Thompson (85) and Dubinina (16) show that this compound accumulates under anaerobic conditions in leaf tissue. The data indicate that, at least in the xylem sap, this does not necessarily happen. The reverse may hold true in this experiment; the 24 hour molar concentration of this compound in the solution treated seedlings is less than the level in the controls at the start of the experiment. This experiment, however, dealt with xylem sap amino acids that may function differently than those found in intact plant tissues.

The diurnal concentration of the xylem sap amino acids is interesting. The data suggest that the amino acids are formed in

response to photoperiod and appear in the xylem sap accordingly; their concentration increases as long as the light is on and then decreases when it is turned off.

All the amino acids, except alanine, quantified in the stem xylem sap show a decrease in concentration during the first four hours. This may occur if the processes of photosynthesis and growth in the above-ground sections of the seedlings are drawing amino acids from the xylem sap to be used in these processes. After four hours, enough photosynthate may reach the roots for a resumption of amino acid production and transport. This could explain the increase in amino acid concentration after four hours.

Amino acid decrease and increase in the xylem sap in response to photoperiod seems logical. However, this study shows that, in E, no new photosynthate reaches the roots to be used in amino acid synthesis. Also, no appreciable amounts of ^{15}N enrichment occurred in any amino acid in the xylem sap except alanine. If newly assimilated carbon and nitrogen are not involved in the production of the xylem sap amino acids, then their increase would have to be derived from reserve materials in the root tissues or amino acids already present. Protein breakdown fits this explanation since amino acids derived from protein, produced prior to the experiment, would show no ^{14}C or ^{15}N incorporation. This would also explain why only major protein and not non-protein amino acids are found in the xylem sap. The exceptions to this are glutamic acid, aspartic acid and alanine, because their concentrations in the xylem sap are too large to account for by protein breakdown alone. These particular amino acids may

be produced from reserve carbohydrates present in the roots by the processes described earlier. The nitrogen for these amino acids would be obtained by transamination at the expense of other amino acids.

The concentration of amino acids found in the root sap follows a diurnal pattern similar to the stems, except there is a steady increase in the concentration of amino acids from the time the lights are turned on until they are turned off. The initial four hour drop that occurs in the stem sap and not the root sap may be because a strong sink, such as photosynthesis, is not present to deplete the sap amino acids. From zero to four hours the stem metabolic processes may be pulling more amino acids out of the sap present in the stem than can be replaced by the roots.

The above discussion leads to the speculation that there may be an amino acid concentration gradient in the xylem sap along the length of the xylem vessels, especially pronounced during the first few hours of light. Loading of amino acids into the root sap may be in response to this gradient; as the amino acid concentration in the stem sap decreases, their concentration in the root sap increases until the deficit in the stem sap is made up. At this point the rate of concentration increase of amino acids in the root sap begins to slow. The exceptions to this are glycine, valine, leucine and phenylalanine which show a concentration drop in both the root and stem sap during the first four hours.

The literature shows that whole tissue amino acid concentration follows a different diurnal pattern. Durzan (20) shows there is a diurnal variation in the concentration of amino acids in expanding buds of 20-year-old white spruce (Picea glauca). He shows that amino nitrogen increases (in general) in leaf tissue from sunrise to sunset but that there is more than one peak in concentration throughout the day. Amino acid peaks occur at sunset and sunrise with lows occurring about four hours after sunset and four hours after sunrise. The latter characteristic appears in some of the amino acids present in the xylem sap as shown. More sampling times would be needed in this study to determine whether there is more than one concentration high in amino acids of the xylem sap during a 24 hour period.

Extraction, Derivative Formation and Separation of Amino Acids

The data show that the extraction and derivatization of the amino acids was not without problem. The low recovery of the standard amino acids can most likely be attributed to improper preparation of the cation exchange resin. Kaiser et al. (42) first mixed the resin in deionized distilled water to form a slurry; this was stirred for about one hour. The water was removed from the resin which was then covered with 7N NH_4OH and stirred for one hour; this step was repeated two more times. The resin was next washed with deionized water until neutral and then regenerated to the H^+ form by adding 3N HCl to form a slurry which was stirred for about one hour. The resin used in this study was new, but was

cleaned by stirring it in 4N HCl for approximately five minutes; this is obviously an insufficient cleaning procedure.

The low RMR values shown in this study as compared to other published values may be due to a combination of things. The most prominent cause of the low RMR values may be insufficient dissolution of the amino acids in the n-butanol during the direct derivatization procedures. Roach and Gehrke (79) said they overcame the solubility problem by a 15 second sonification of the amino acid n-butanol mixture. Cancalon and Klingman (11) found however, that even after 30 seconds of sonification the molar response values for each amino acid were not even 50% of the RMR values from the interesterification method. They showed that only after 30 minutes of sonification did the RMR values equal the interesterification values. Also, the N-TFAA n-butyl amino acid derivatives are subject to hydrolysis, especially the serine, threonine and cysteine derivatives. Therefore, all water must be excluded during derivatization and chromatography (74).

Not all of the amino acids present in the xylem sap were sufficiently resolved enough to be quantified. Proline could be identified but not quantified in the solution grown plants because it eluted in a multiple peak. Cancalon and Klingman (11) show proline coelutes with an unidentified compound. They attribute this to a

contaminant in the ion exchange resin when it is not thoroughly cleaned. This may be the cause of the multiple proline peak in Figures 4 and 5. Serine, cysteine, methionine and tryptophan were not present in large enough quantities to be properly analyzed; threonine, tyrosine and phenylalanine may also show lower than normal molar concentrations. As mentioned earlier, serine, threonine and cysteine have very unstable N-TFAA n-butyl derivatives in the presence of moisture. Methionine, phenylalanine, tyrosine and tryptophan may be lost due to oxidation during ion exchange chromatography (11).

The GLC ionization chamber and the GLC mass spectrometry results show that these two joint procedures can be very helpful in elucidating the quantity of carbon and nitrogen incorporated into each individual amino acid. It was very difficult to obtain proper functioning conditions with the ionization chamber technique. As can be seen in Figures 11, 12 and 13, the electrical noise is significant to the extent that low levels of ^{14}C present in the amino acid would essentially be non-detectable. More experimentation is needed to solve this problem. This procedure is also extremely sensitive to breakdown of the amino acid derivatives while they are going through the chromatographic separation. If the derivatives have their ionic characteristics changed by even a partial breakdown, they will foul up the ionization chamber output. The chamber has a potential

across the chamber wall and an electrode. If the derivatives are charged they will collect on the chamber wall or electrode and remain there, thus destroying any symmetry of the output peak, and result in a baseline that is at a new level equal to the peak height; in other words, the peak will not drop down to the original baseline. This problem is overcome by silanizing all stainless steel fittings and tubing in the chromatograph so no reactive surfaces are present to break down any derivatives.

The results presented in Table 6 are only an estimation of the ^{14}C present in the amino acids because the counting efficiency of the 250 ml ionization chamber was not properly determined. The chamber must be calibrated, while it is in the system, with known amounts of a derivatized ^{14}C amino acid. The reason this was not done at the time of the data collection is because the system was working only for a very short time and the decision was made to collect data on the derivatized xylem sap amino acids.

The results of the mass spectrometry analysis are disappointing in this study because it is not sensitive enough to determine to any great reliability the ^{15}N enrichment of the individual amino acids studied in this experiment. This is due to not enough $^{15}NO_3$ being available to the plant roots to be assimilated in sufficient enough quantities to be detected. A minimum of 5% enrichment of ^{15}N into an amino acid is needed to reliably demonstrate that

enrichment has occurred. The mass spectrometry of these amino acid derivatives was, however, very pleasing in other ways. This technique offers an incontestable and convenient method of identifying amino acids. No two amino acids have the same fragmentation pattern under a predetermined set of operating conditions. Published data is all that is needed to identify the compound. Standards do not necessarily have to be used. The mass spectrometry of amino acid derivatives is convenient in yet another way; the determination of ^{15}N (when enough is present) in amino acids can be done without having to do a Kjeldahl digestion to generate and collect ammonia gas (to be introduced into the mass spectrometer), which is a very drawn out procedure (13).

Both of the above techniques work and should be utilized in further physiological work with amino acids to elucidate their production and function. However, quantifying amino acids should be accomplished by the use of high pressure liquid chromatography. The technique is as sensitive as GLC and has the added attraction of being almost trouble-free in comparison to derivatizing the amino acids and chromatographing them (Dr. Robert Becker, OSU Biochemistry Dept., Personal communication).

VI. SUMMARY AND CONCLUSIONS

The techniques described above are workable and offer a powerful means by which amino acid production and metabolism can be observed. More work must be done to get the GLC ionization chamber system stabilized. The major problems, derivative stability and adverse electrical noise levels, are correctable. The entire system can be made compatible to derivative stability by eliminating all water and reactive surfaces anywhere the derivative may come in contact with them. The noise level can be reduced by stabilizing the ionization chamber with respect to the column oven. The problems represented here were a result of vibration from the oven fan and any movement of the ion-chamber itself.

The mass spectrometer offers certain identification to any amino acid that can be derivatized. It is also the most indisputable tool available for directly analyzing the components of individual amino acids. One must remember, however, that the mass spectrometer has limitations. The instrument is only as good as the samples presented to it. If ^{15}N is being analyzed for, then enough has to be present in the amino acids to be detected with confidence and analyzed by the machine.

The experimental procedure has many gaps that, at the time, were difficult to comprehend. Better precautions must be made to

insure that root shock or damage does not occur when examining the metabolism of the roots. This then poses the question--just how is root shock defined?

It must be kept in mind that only the amino acids in the xylem sap were examined. What appears in the sap may have little relationship to what occurs in compartmentalized tissue fractions. This is an extremely important concept because this study employs an extremely sensitive method for amino acid analysis, the mass spectrometer. Only the protein amino acids plus 4-aminobutyric acid were present in large enough quantities to be detected. This is a small fraction of the possible amino acids, both protein and non-protein, that may be present in Douglas-fir, that can be derivatized and eluted on an EGA column.

Amino acid metabolism in plants is a very complex subject. The formation, transport and metabolism can be categorized separately but are far from being independent entities. To understand the function of amino acids in plants, none of the preceding three categories can be discounted. This study has shown that the amino acids present in Douglas-fir xylem sap are far from being understood. One fact will remain indisputable; the appearance of amino acids in plants is based on carbon and nitrogen metabolism and their response to the demands of the seedlings' immediate

environment. Hopefully, the techniques described here will help to further the understanding of these diverse compounds.

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APPENDIX

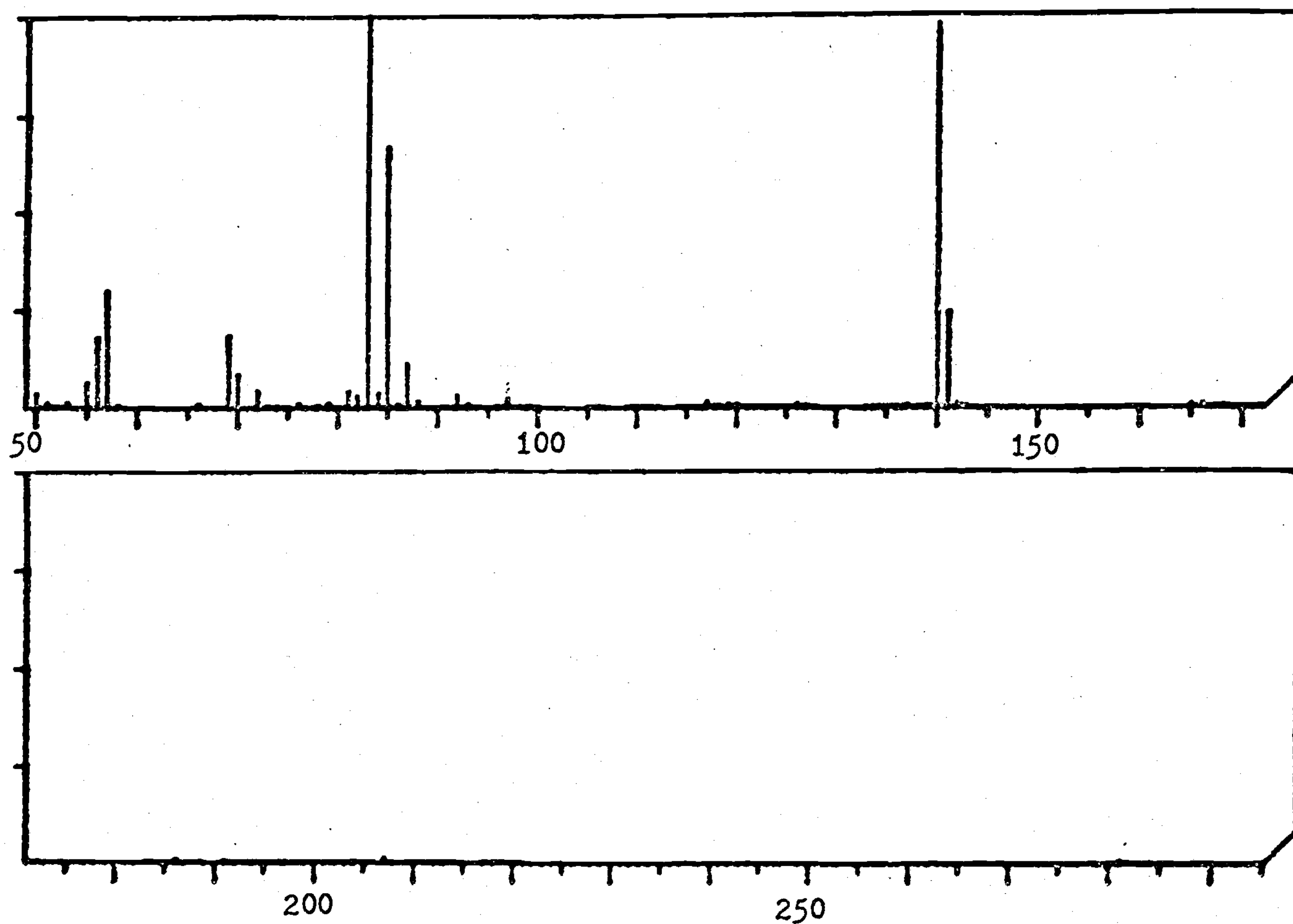

Figure 15. Mass spectrum for N-TFAA n-butyl alanine ester.

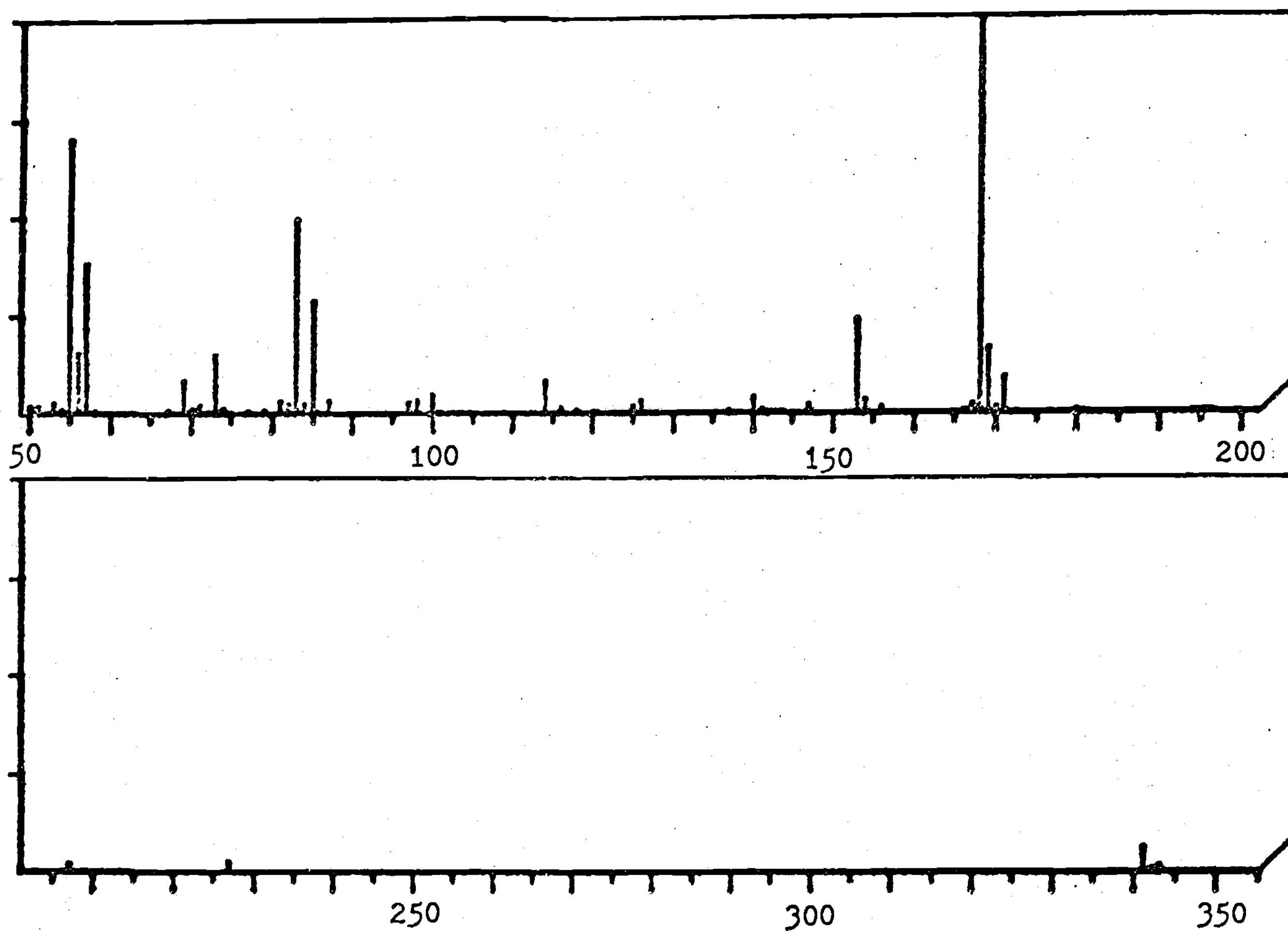

Figure 16. Mass spectrum for N-TFAA n-butyl valine ester.

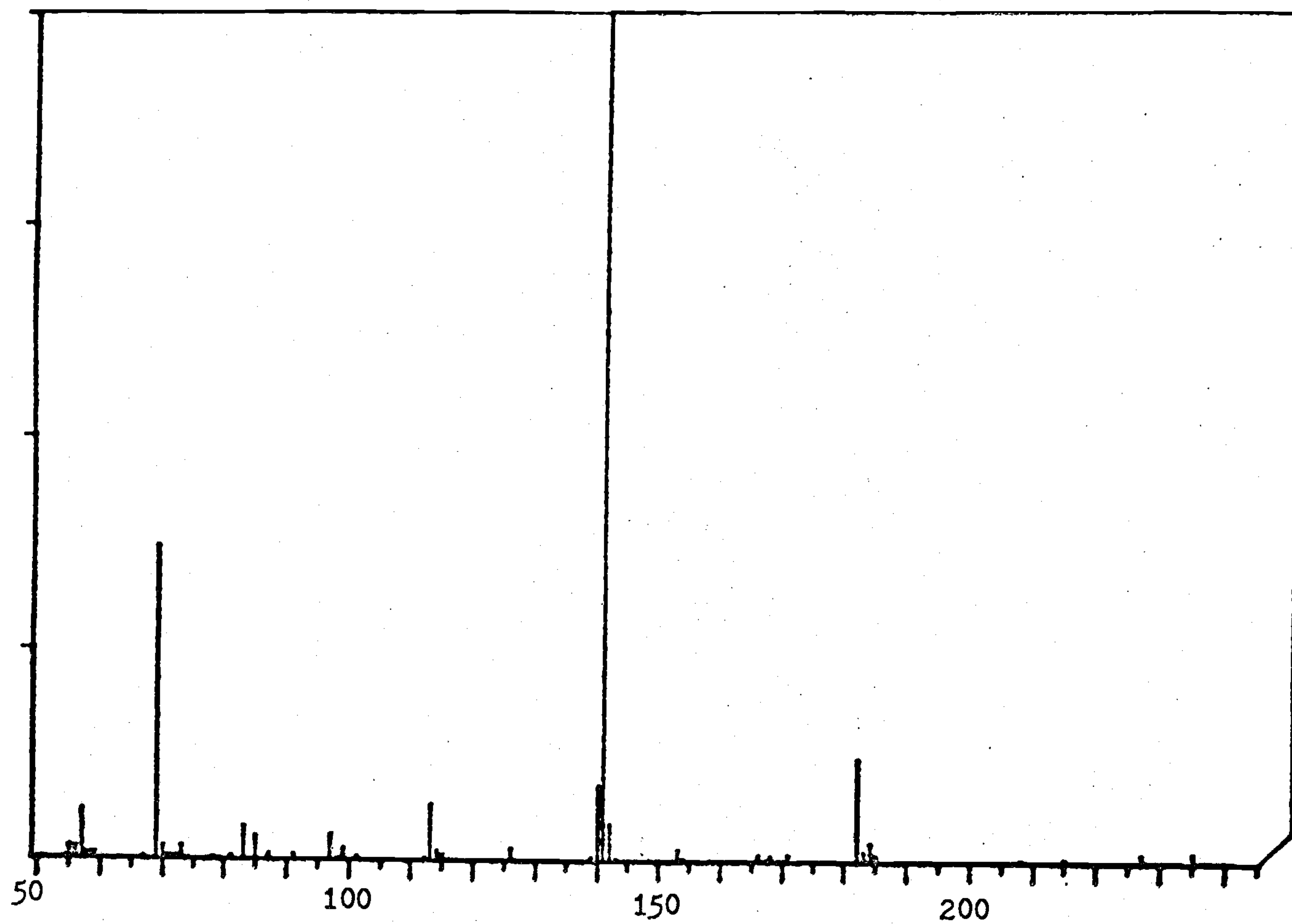

Figure 17. Mass spectrum for N-TFAA n-butyl leucine ester.

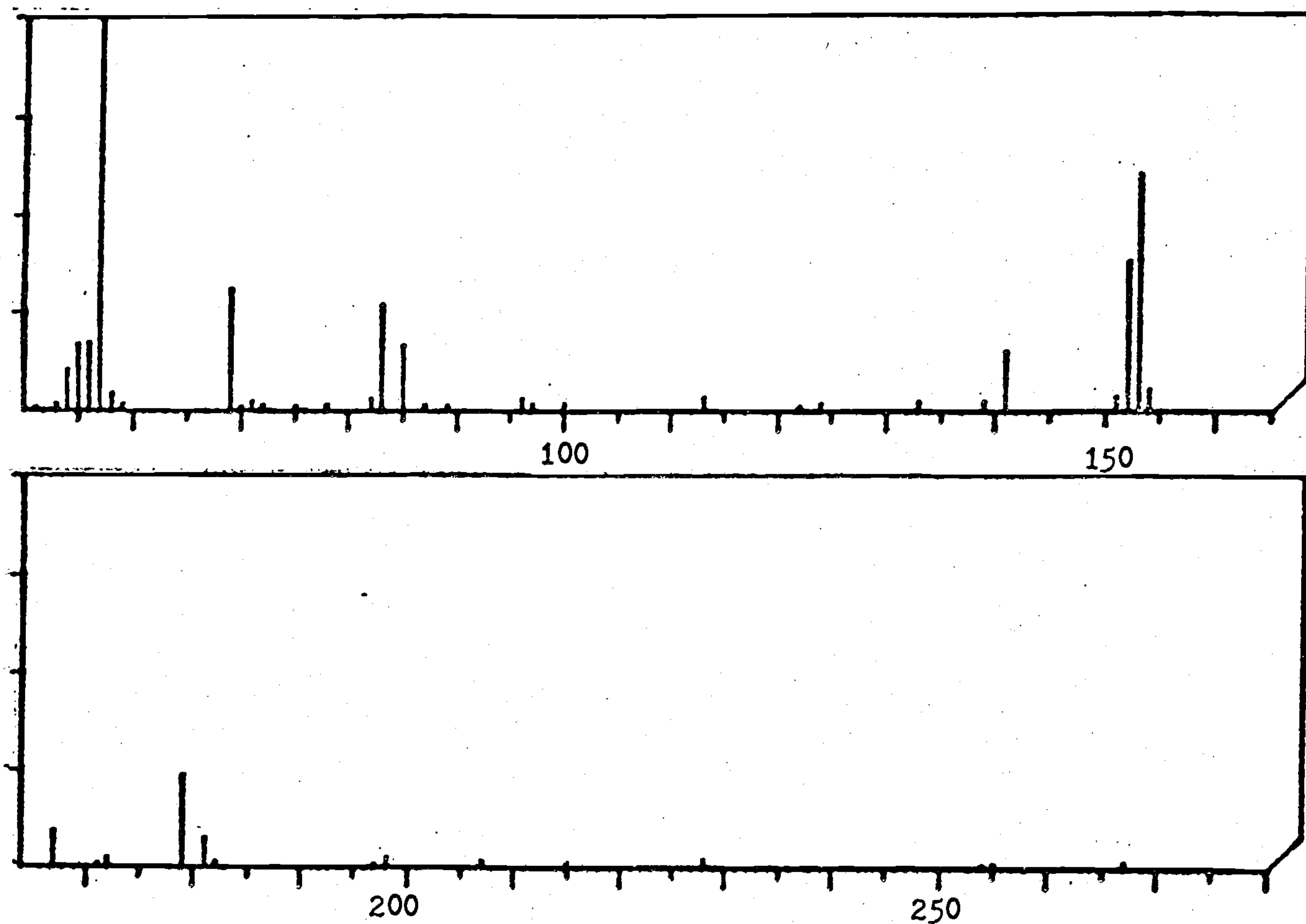

Figure 18. Mass spectrum for N-TFAA n-butyl threonine ester.

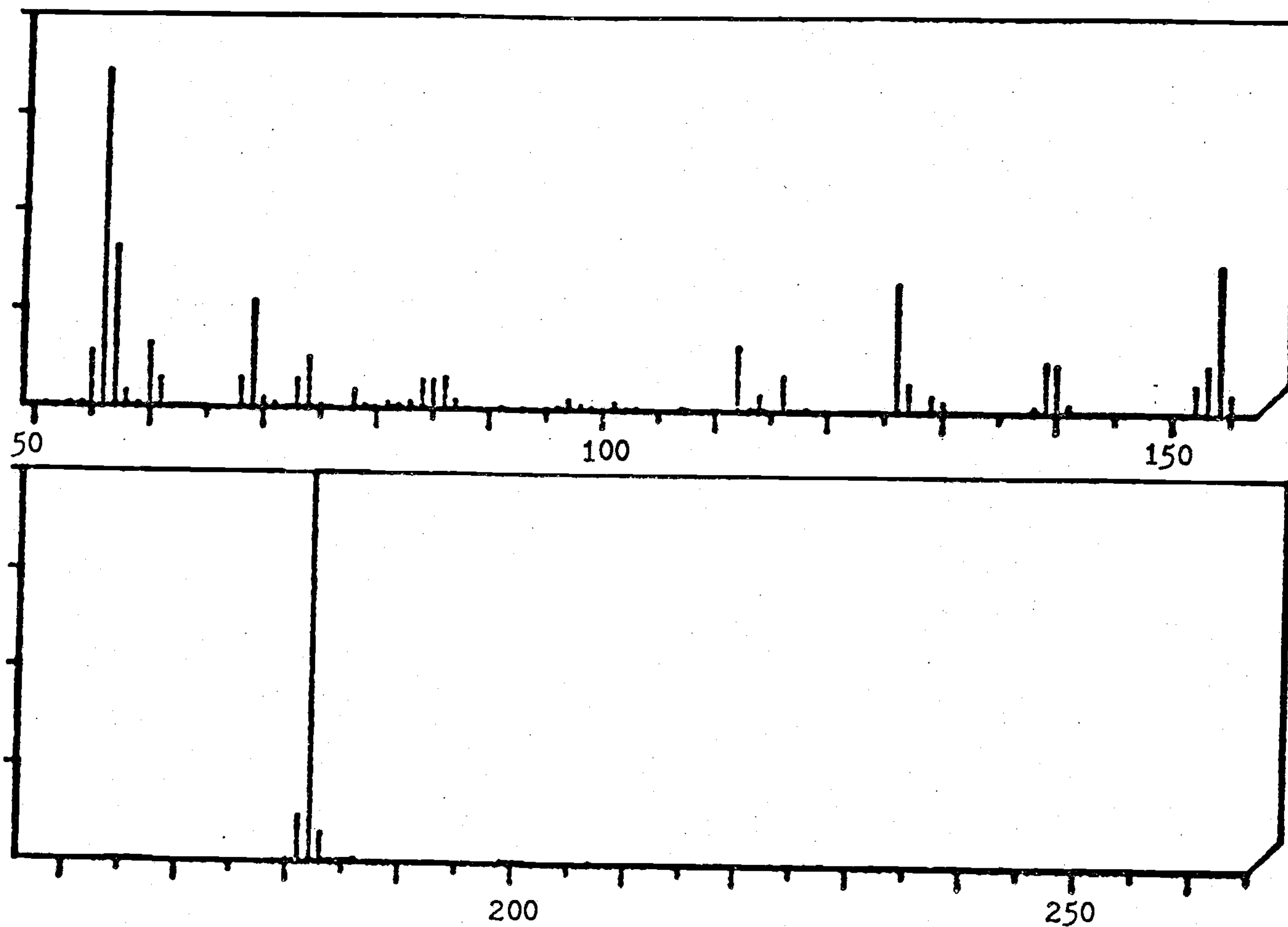

Figure 19. Mass spectrum for N-TFAA n-butyl 4-aminobutyric acid ester.

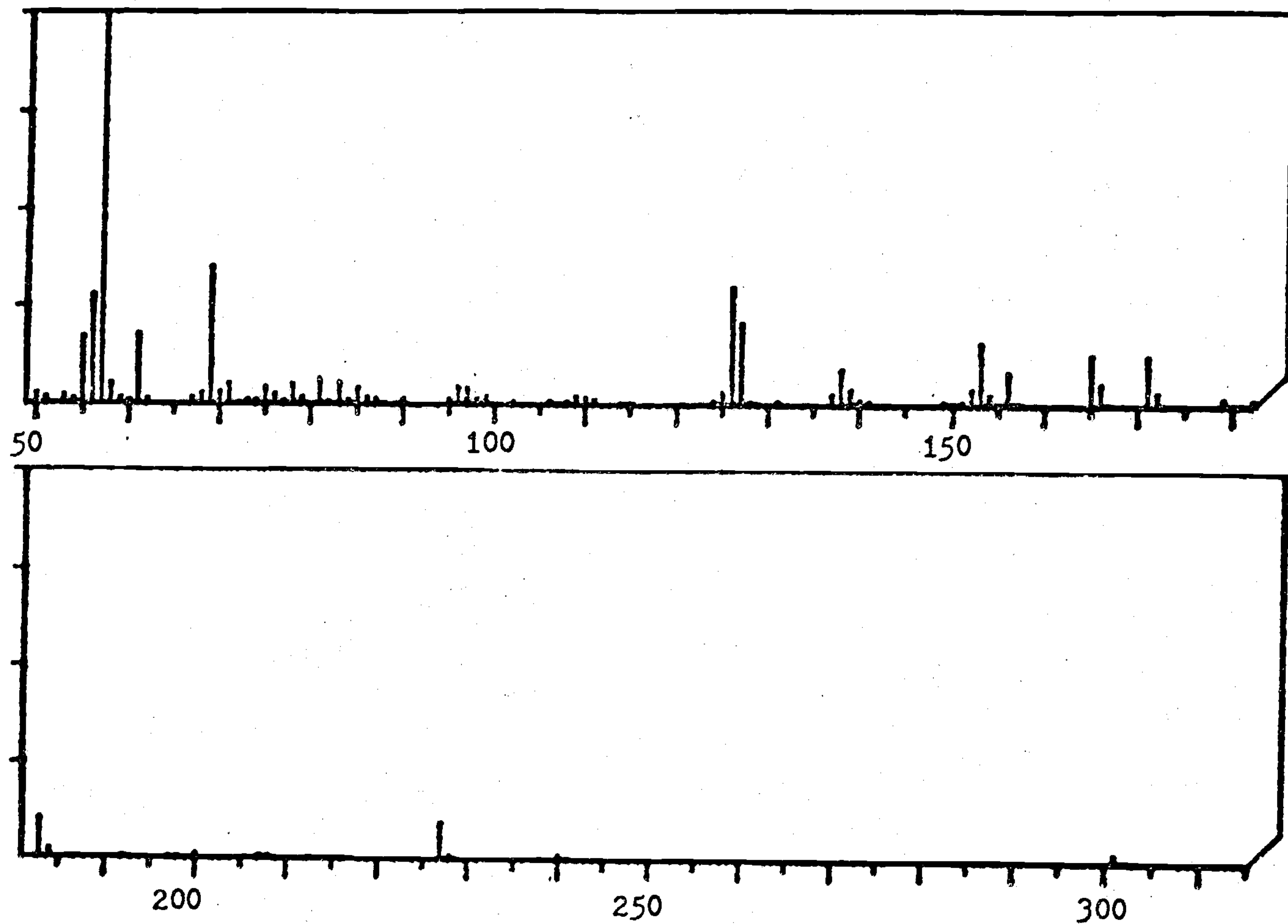

Figure 20. Mass spectrum for N-TFAA n-butyl methionine ester.

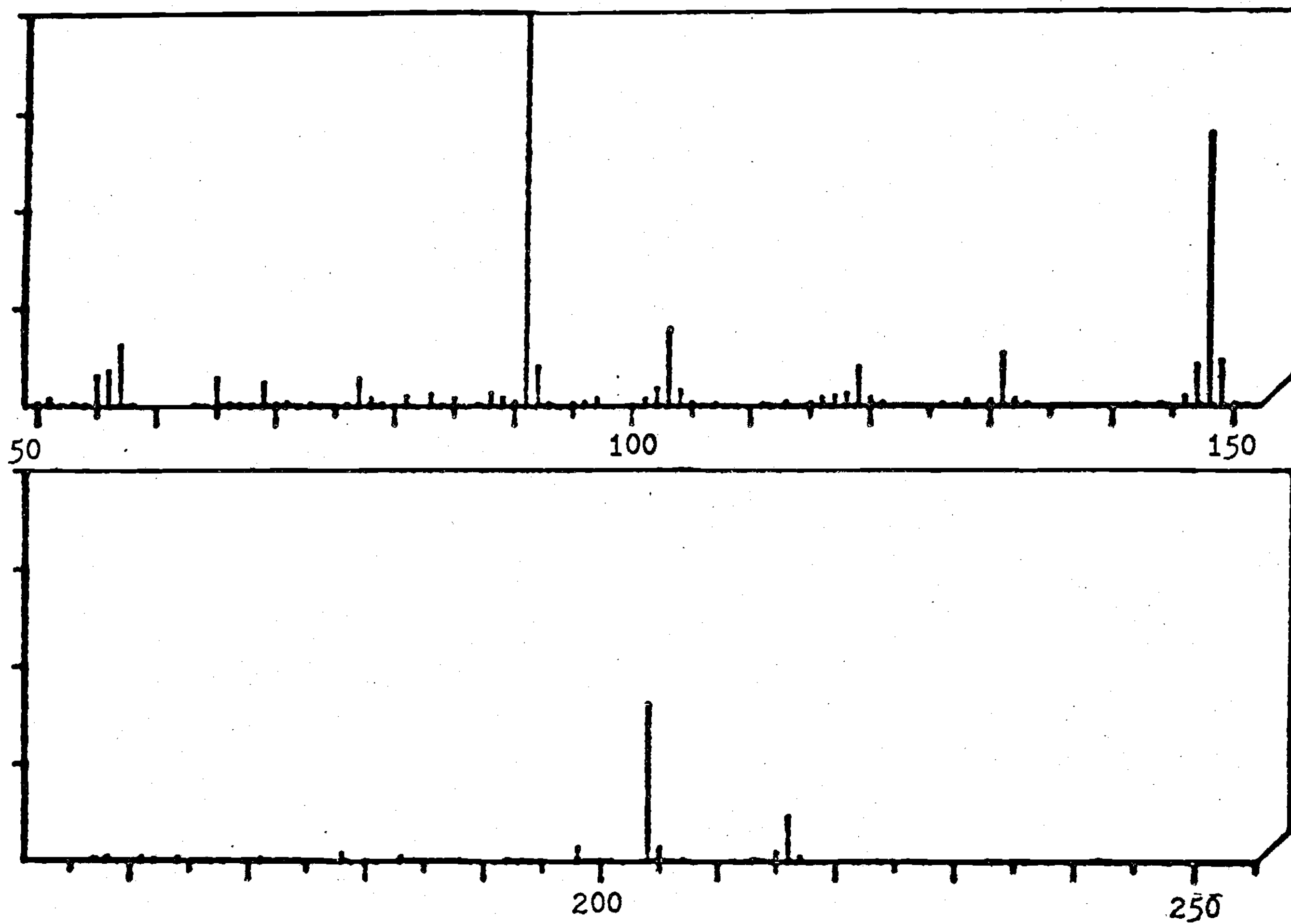

Figure 21. Mass spectrum for N-TFAA n-butyl phenylalanine ester.

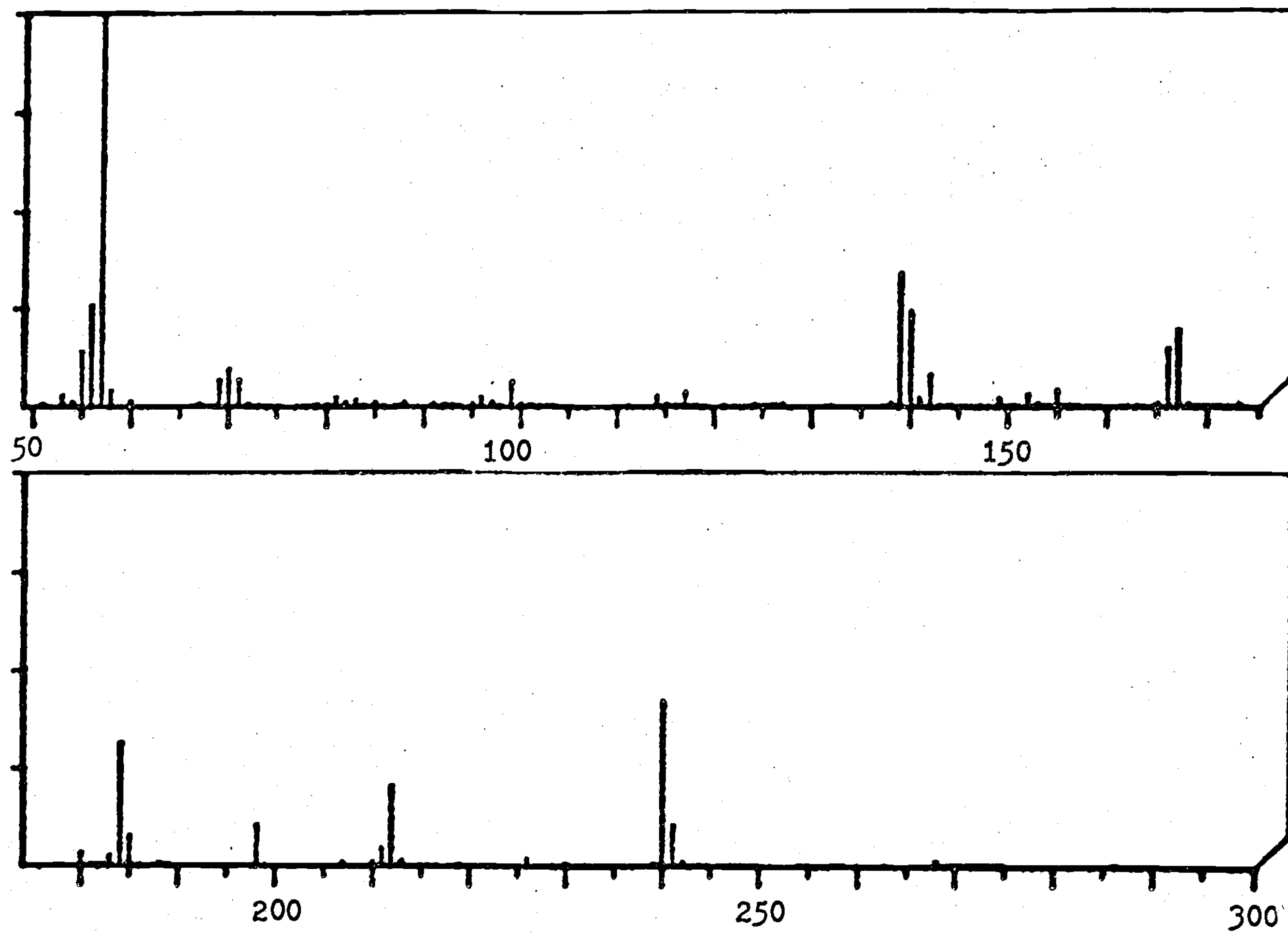

Figure 22. Mass spectrum for N-TFAA n-butyl aspartic acid ester.

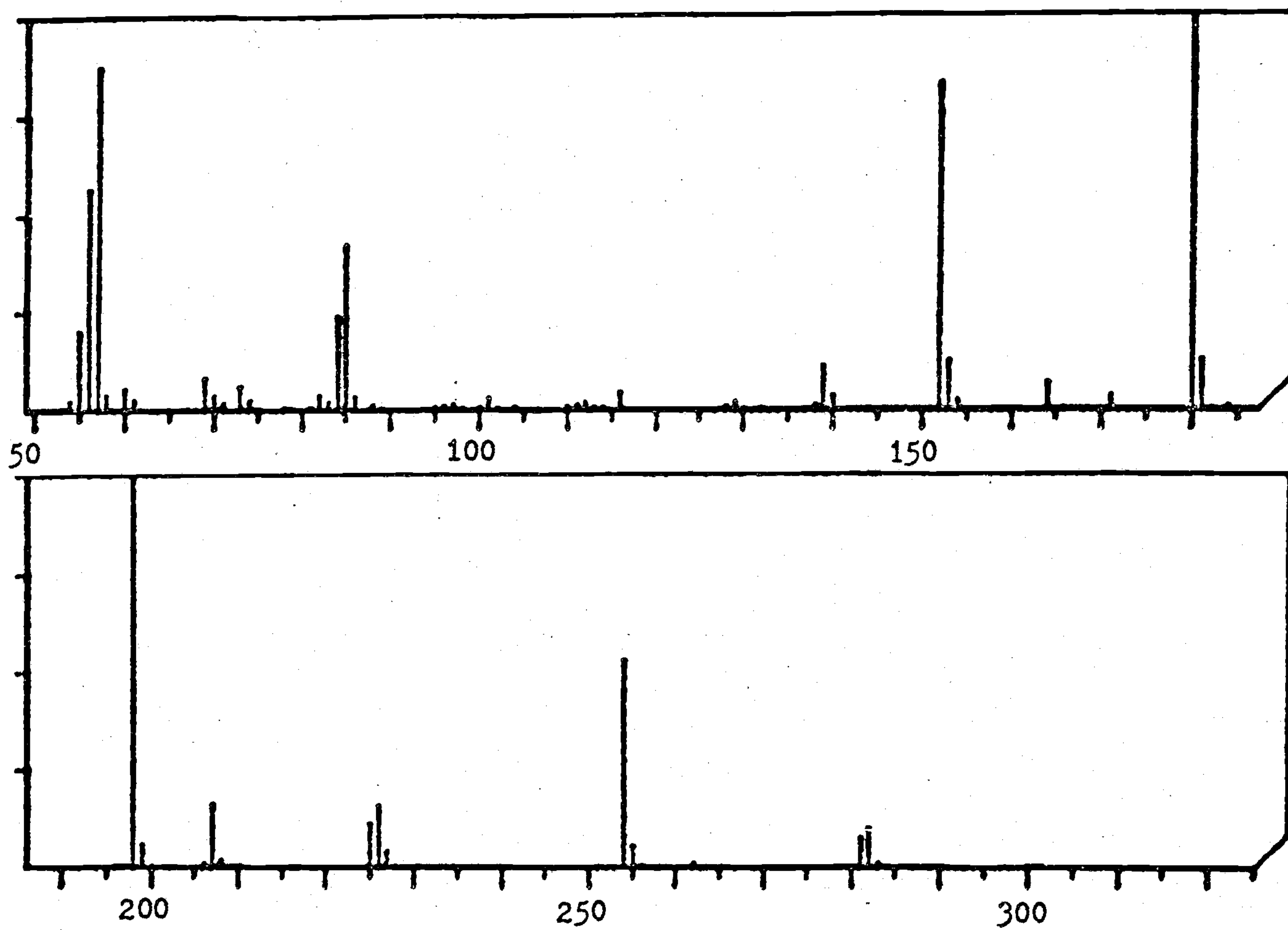

Figure 23. Mass spectrum for N-TFAA n-butyl glutamic acid ester.

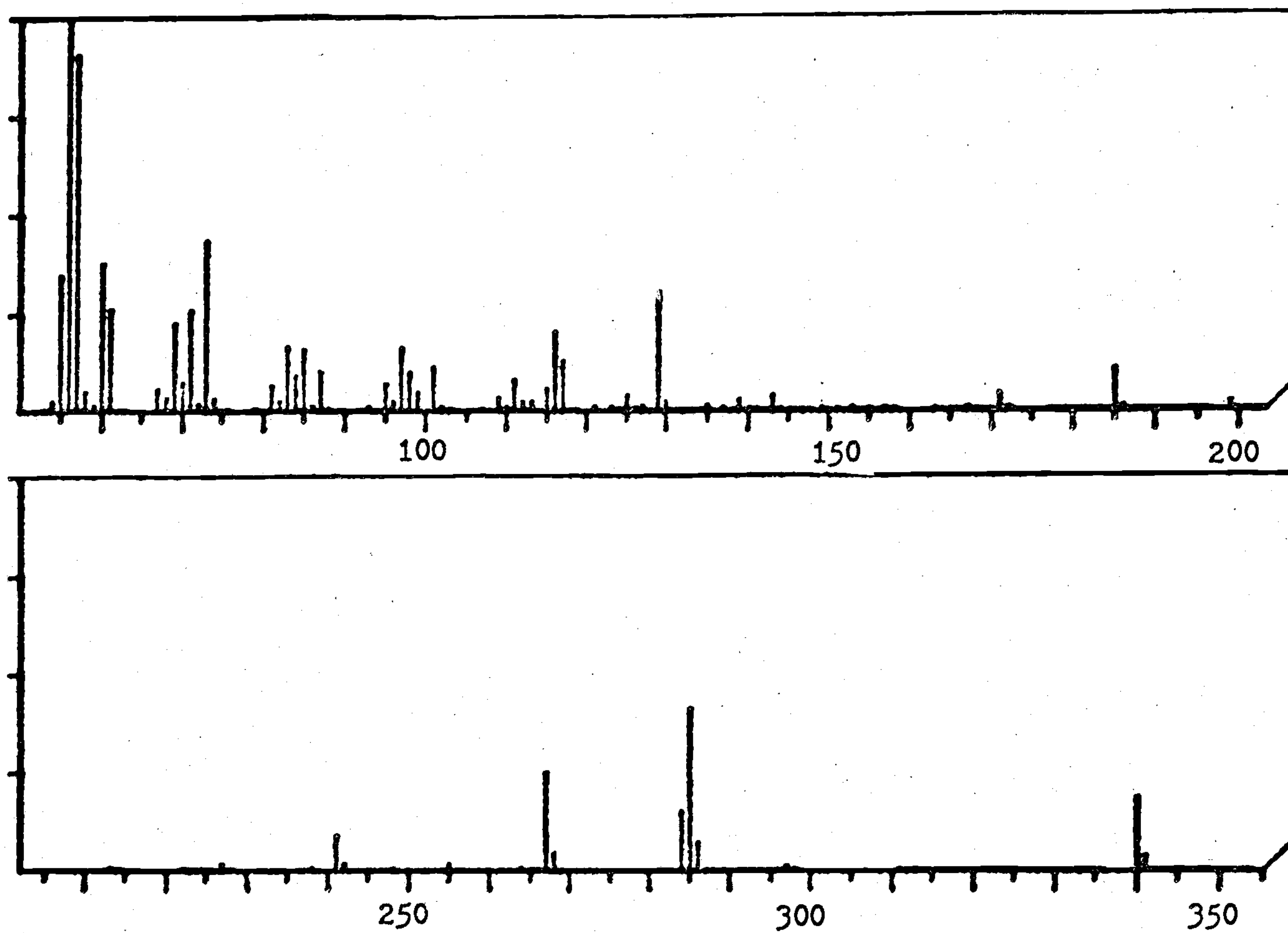

Figure 24. Mass spectrum for N-TFAA n-butyl butylstearate ester.

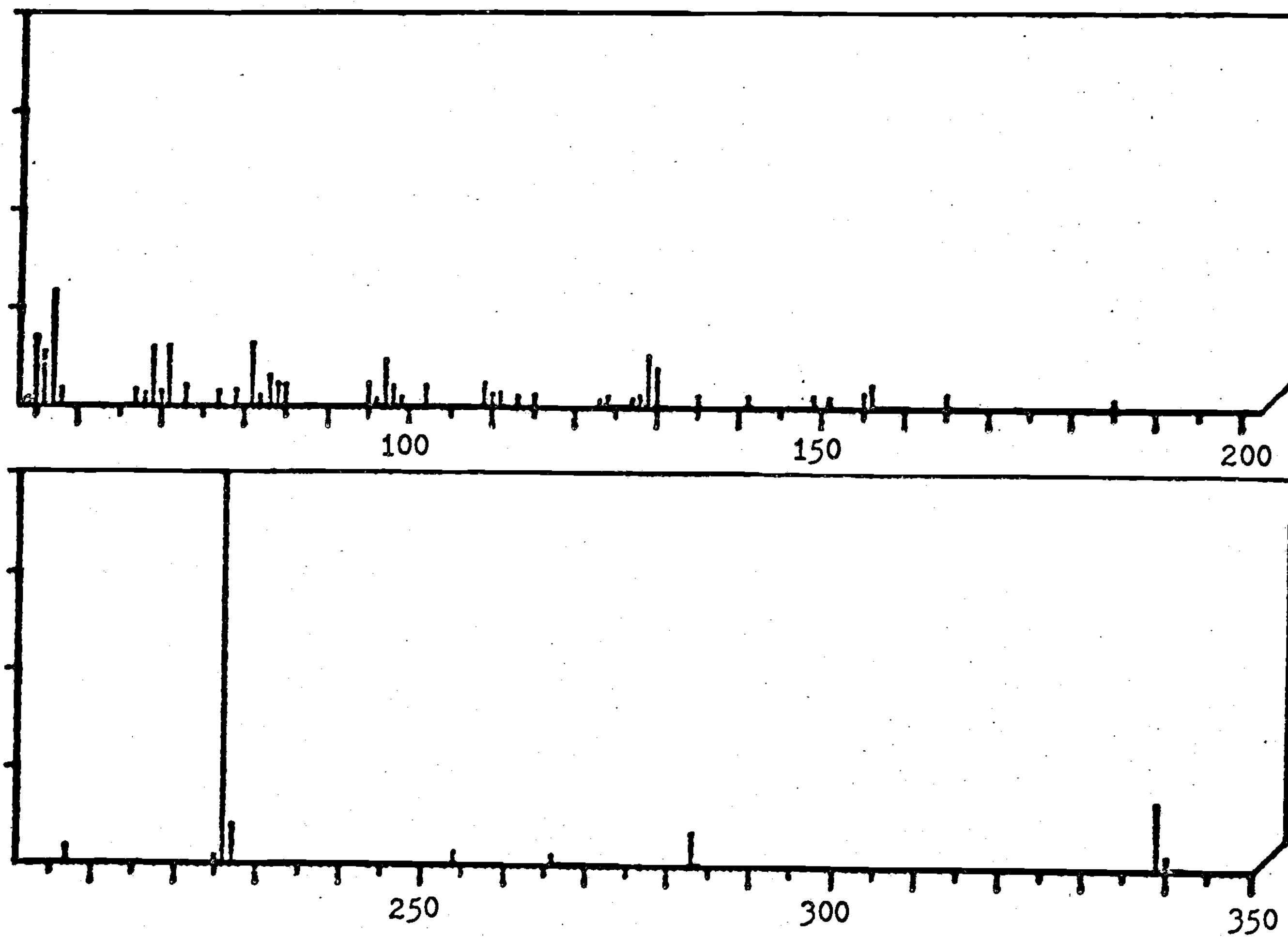

Figure 25. Mass spectrum for N-TFAA n-butyl tryptophan ester.

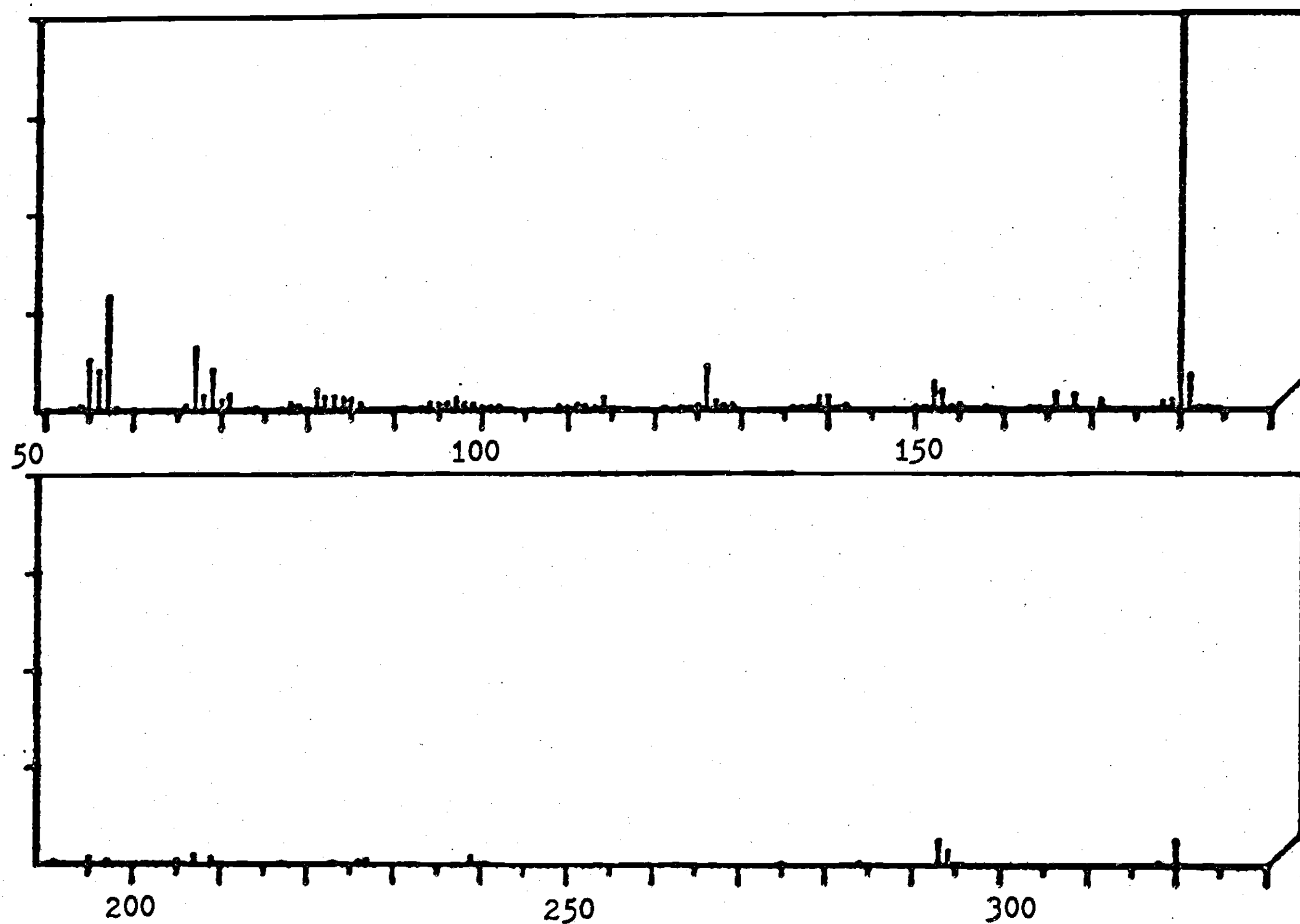

Figure 26. Mass spectrum for N-TFAA n-butyl lysine ester.