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SOME ASPECTS OF THE UPTAKE AND UTILIZATION OF GLUTAMIC ACID BY TOBACCO

by
Howard Jay Stein

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of the requirements for the degree of
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INTRODUCTION

From the eighteenth century until well into the nineteenth century botanists generally accepted the Humus Theory as the explanation of the source from which plants derive their nutrients. According to this theory, the organic components of the soil provide all of the necessary substances for the growth of plants. Although plant physiologists of that time gradually came to recognize that the atmosphere serves as the primary source of carbon for green plants, the idea that nitrogen may be derived solely from humus persisted.

In 1840 Justus von Liebig attacked the Humus Theory, declaring that ammonia in the atmosphere and soil serves as the source of nitrogen for plants. Theodore de Saussure, who had earlier established the failure of plants to assimilate atmospheric nitrogen, and others vigorously opposed Liebig's interpretation. J. B. Boussingault resolved the controversy by publishing the results of experiments in which nitrates and the ash of plants satisfactorily supported the growth of plants in soil from which the organic compounds had been removed by heating. Subsequent experimentation has firmly established the suitability of nitrates, and ammonia in some cases, as sources of nitrogen. Indeed, these inorganic compounds provide nearly all of the nitrogen derived from soil by the higher plants.

Upon the verification of Boussingault's results, investigation into the ability of plants to derive nitrogen from organic compounds was extended. Coming before the development of modern bacteriology, these studies were conducted without regard to the influence of soil microbes. Because organisms which grow in soil are capable of converting organic nitrogen to inorganic forms, experiments in which organic nitrogen compounds were presented to plants in non-sterile root environments were meaningless.

More recent investigations using modern techniques have provided support for both sides of the early controversy regarding the suitability of organic and inorganic nitrogen sources. In particular, some plants were found to utilize the nitrogen of amino acids fully as well as inorganic forms, whereas other plants fail to grow on amino acids as sole nitrogen sources.

The ability of the higher plants to utilize the nitrogen of amino acids has particularly been investigated by A. I. Virtanen and his associates. Their results prompted him to suggest in 1937 that among the higher plants there is a fundamental difference between legumes and non-legumes, namely, that the legumes are capable of deriving nitrogen from amino acids supplied to their roots and that the others are not. Subsequent work in Virtanen's laboratory seemed to support this hypothesis. Data from other sources have sometimes been at variance with it. The tissue culture technique, for example, has provided abundant evidence that

excised tissues from many plant families could be grown with amino acids as the sole source of nitrogen.

The question arose, then, as to whether the behavior of plants not apparently utilizing amino acids could be due to the inability of the amino acids to enter their roots or instead to some physiological property of these roots which prevents the nitrogenous moiety after entry from being transferred to the rest of the plant.

The leakage of organic compounds from intact roots, now considered to be a normal phenomenon, has been recognized for many years. Prominent among the compounds released are amino acids. If amino acids can diffuse from the free space of a root into the external medium, one might expect these compounds to enter the root by the same path. In this case the ability to utilize the amino acids could be dependent upon the properties of the cell membranes.

The occurrence of amino acids is universal in living systems. Amino acids are utilized not only in the synthesis of proteins but also of hormones, pigments, the nitrogenous bases of nucleic acids, and some coenzymes. Glutamic acid, aspartic acid, and alanine are associated intimately with the respiratory pathways of the cell. Thus it would seem unlikely that once one of these compounds enters the cell it could not be assimilated. Moreover, inorganic nitrogen is converted quickly into amino acids in many plants. Analyses of the xylem sap of some trees show little inorganic

nitrogen to be present, the bulk of the total soluble nitrogen being represented by amino acids and amides. Consequently it seems likely, a priori, that permeability barriers preclude entry of externally presented amino acids into those plants unable to utilize such compounds.

However, other facts in the literature contradict such a simple explanation. The toxicity to some tissues of even those amino acids which participate in the primary metabolic pathways is not necessarily ascribable to permeability or related phenomena. Also, mixtures of amino acids frequently are stimulatory in cases in which the compounds presented singly are of little value.

The knowledge that plants derive their nitrogen from soil primarily in an inorganic form has removed, in a sense, some of the incentive to examine the abilities of higher plants to absorb organic nitrogenous compounds. The purpose of the present investigation is to evaluate these capabilities in several selected plants and to attempt to correlate those results with their capacity to derive nitrogen from the absorbed compounds.

The compound chosen was glutamic acid because of its ubiquity, its close association with the Krebs cycle, its participation in protein synthesis, and its function as a nitrogen donor in the formation of other amino acids. The tobacco plant was selected on the basis of experiments by Ghosh and Burris published in 1950 in which aseptically

grown tobacco plants were shown to be unable to derive nitrogen solely from glutamate. For the sake of comparison a plant which is known to have this ability and which is a legume seemed desirable. An easily grown and much studied plant, the pea, was used. In addition, some of the experiments were performed with barley which, like tobacco, fails to grow on some common amino acids.

Three approaches to the problem were employed: (1) The effect of glutamic acid on the respiratory rates of excised roots was used as an index of its penetration and subsequent utilization. (2) The uptake of the amino acid was investigated by direct measurement. (3) The transfer of absorbed glutamate to the shoot was examined, and the chemical form in which it was transported was determined.

LITERATURE REVIEW

In beginning the study of the uptake of chemical substances by living cells, the neophyte is inevitably impressed by the great volume of the literature on the subject. Later, after gaining some insight into the problem, he is more likely to be impressed by the lack of definitive knowledge of the mechanisms by which this fundamental process is accomplished in different organisms and systems.

According to the currently accepted theories, there are three basic means by which the entry of exogenous substances into cells is effected: (1) diffusion of small particles through pores in the bounding membrane, (2) diffusion of fat-soluble molecules through lipid components of the membrane (for both possibilities cf. Bonner and Galston, 1952), and (3) mediated transport (as defined by Christensen, 1959). Mediated transport can be further subdivided into passive and active types, the former providing for a flux without establishing a concentration gradient, the latter requiring the expenditure of energy by an exergonic reaction and leading to the accumulation of the substance against a concentration gradient. Active transport, which is usually necessary for the maintenance of the cell <u>in vivo</u>, is often considered to involve carriers

in the membrane (Danielli, 1954) but has also been explained by the presence in the membrane of pores within which are adsorption sites, the placement of which results in a one-way transport of solutes (Burgen, 1957).

The Uptake of Amino Acids by Animal Cells and Tissues

Because a large amount of work has been done with animal tissues in attempting to elucidate the actual mechanism of amino acid uptake, a short review of what has been accomplished is appropriate here.

These investigations have been primarily carried out by Christensen and his co-workers and are adequately summarized by Noall *et al*. (1957) and Christensen (1959).

The uptake of glycine by Ehrlich Ascites tumor cells can be accelerated by the addition of pyridoxal, a cofactor in the transamination reaction. Surprisingly, pyridoxal also stimulates the influx of divalent manganese ions, with the addition of one of several amino acids producing an even greater response. These data point to a combination of the amino acid with pyridoxal, of manganese with pyridoxal, or of all three in one complex. Neither zinc, iron, nor copper ions are similarly involved. Metallic ions are further implicated in that there is an outward flux of potassium ions with the uptake of glycine. By loading the cells with potassium beforehand, one can bring about an increase in the uptake of the amino acid. The possibilities

are that the outward flux of potassium constitutes the exergonic reaction that drives the influx of glycine or that the carrier moves outward in preferential combination with potassium.

The structural requirements for amino acid uptake have been determined. The amino group in the alpha or beta position is preferable to one farther removed from the alpha carboxyl; the mechanism involves the un-ionized amino group; the carboxyl group can be replaced by the sulfonic group; and alpha-methyl groups intensify transport, the last indicating that there is no requirement for the loss of an alpha-hydrogen atom as in transamination.

Through the use of an unmetabolizable amino acid, alpha-aminoisobutyric acid, stimulation of amino acid uptake has been shown to occur in a variety of tissues in response to the addition of divers animal hormones. Recently, Christensen (in a verbal, unpublished presentation) speculated on the relationship between the action of these hormones and their properties as surface active agents.

Burgen's (1957) theory of transport relies on the spacing of binding sites within pores in the membrane. The ability of the hormones to deform the membrane by surface action may, according to Christensen, explain their effect on amino acid transport in terms of altering the spacings between binding sites. Weiss (1960) has presented a theory based on the effects on transport of the orientation of

macromolecules at the cell surface. Modification of the surface controlled by the movement of charged particles within the cell would be responsible for changes in uptake rates, moieties of the surface macromolecules being the regulators of specificity. This mechanism does not exclude Christensen's interpretation.

The Uptake of Amino Acids by Bacteria

A priori, the most likely explanation of the failure of some plants to utilize amino acids as nitrogen sources seemed to be one derived by analogy from the phenomenon of crypticity in bacteria. A thoroughly documented and more comprehensive discussion of the succeeding remarks can be found in the review by Cohen and Monod (1957).

Crypticity can be generally defined as the situation in which an exogenous substrate is not metabolized by a cell which contains the relevant enzyme systems. The classic example of crypticity is the behavior of Escherichia coli and two of its mutants toward beta-galactosides. The normal cell is capable of growing on a variety of these compounds as carbon sources; the two mutants are not. One of the mutants, when homogenized, contains no demonstrable beta-galactosidase. On the other hand, the second mutant, the cryptic type, contains the normal amount of beta-galactosidase after being grown on a suitable enzyme inducer, but the intact cells respond to exogenous galactosides at an extremely slow rate compared to the normal type.

Because E. coli is incapable of deriving energy, carbon, or sulfur from thiogalactosides, the discovery that these compounds are accumulated by this organism provided an excellent opportunity to study the uptake mechanisms without the interference of subsequent metabolism of the substrate. Through the use of radiosulfur-labeled thiogalactosides, cells of the normal type and of the first mutant mentioned above are found to accumulate galactosides in the same manner, the kinetics of which can be described by the Michaelis-Menten analysis of enzyme reactions. The uptake by cryptic cells, however, follows the course of a diffusion process, i.e., it is linear with respect to galactoside concentration. Results of measurements of the hydrolysis of galactosides by normal and cryptic types also support the hypothesis that uptake in normal cells is enzyme-mediated, if one accepts the assumption that the rate of hydrolysis in the intact cell is controlled by the rate of entry of the substrate.

Some of these data can be explained by either the presence of specific receptor sites within the cell or the occurrence in the membrane of "permease" sites which catalyze the accumulation of galactosides. Since the accumulated galactosides may exceed five per cent of the dry weight of the cells, the former interpretation requires an inordinately large number of specific sites within the cell. Because of the stereospecificity of uptake, Cohen and Monod

(1957) assumed that the sites are proteinaceous and calculated that one site for each unit of molecular weight 2,000 of cellular protein would be required. This led to the acceptance of the permease system as an explanation.

Permeases are considered to exist both as passive mediators of uptake and as active mechanisms deriving their energy from coupled exergonic reactions. The expenditure of energy would be required by a system which accumulates a substrate within the cell to a chemical activity above that in the medium. The action of uncouplers of phosphorylation supports this interpretation in that they inhibit accumulation without preventing the passive entry of the substrate.

Amino acid permeases have been investigated, and workers at the Pasteur Institute in Paris have found specific, independent uptake systems for valine, methionine, tyrosine, and phenylalanine. Unlike the galactoside permease, these systems are constitutive. The permease concept has been invoked to explain the alleviation of valine toxicity by leucine and isoleucine in sensitive strains of E. coli, the leucines being considered to compete for the permease carrier.

From a study of aspartate uptake by Bacterium cadaveris, Williams (1960) has concluded that the aspartase activity exhibited by intact cells is limited by the rate at which the amino acid enters and that the system resembles

the permease in all respects studied. Holden and Holman (1959) found accumulation of freely extractable glutamic acid by species of Lactobacillus, Streptococcus, and Leuconostoc, the amount taken up exceeding ten per cent of the total dry weight of the Lactobacillus. Each of these organisms required glucose, i.e., respiratory energy, for operation of the accumulation mechanism. An extension of these data by Holden (1959) revealed that the uptake of glutamic acid by vitamin B_6-deficient cells was considerably less than that by normal cells. Unlike mouse tumor cells (Christensen, 1959), the bacterium failed to respond to the addition of the vitamin (pyridoxal). Full activity could be restored by the addition of 0.5 M sucrose or a mixture of acetate, NH_4^+, and the vitamin. Holden hypothesized that vitamin B_6, and, by implication, transamination, is only indirectly involved in the uptake mechanism.

According to Mandelstam (1956a, b) diamines inhibit the growth of certain coliform bacteria by blocking the uptake of basic amino acids. These findings, he suggests, implicate the amine group in the uptake mechanism, probably through the formation of hydrogen bonds between the amine group and cell wall sites.

In summary, the currently dominant theories of the means by which bacteria remove amino acids from the milieu are in general accord with the classical carrier mechanism.

Amino Acids as Nitrogen Sources for Higher Plants

Inorganic compounds of nitrogen are ordinarily considered to supply that element to plants grown in soil. The nitrogen that plants remove from the soil is replenished primarily through the decomposition of organic materials made available by the death of living organisms. The major portion of the nitrogen thus made available to soil microbes is derived from proteins, the first step in the degradation of which is considered to release amino acids. This digestive process has been referred to as "aminization" (Tisdale and Nelson, 1956). Further transformations produce ammonia and, eventually, nitrate. The formation of humus, a nitrogenous substance more resistant to microbial attack, is not considered here.

When proteolysis proceeds at a more rapid rate than ammonification, as occurs sometimes in acid soils, amino acids may accumulate (Thimann, 1955). Studies on the amount of free amino acids in soil indicate that ordinarily their concentration is low, but Paul and Schmidt (1960) stated their belief that many reports showing little or no amino acids to be present in soil were due to the use of inefficient methods of extraction. Putnam and Schmidt (1959) found that amino acids added to soils rapidly disappeared.

The main consideration here is that amino acids do occur in soil and that, although their concentration at a given moment is low, the total amount of nitrogen which

passes through the amino acid stage is large. Under such conditions the roots of plants capable of accumulating amino acids will probably be able to do so, even though most of these compounds may be taken up by micro-organisms.

In his classic review of nitrogen fixation P. W. Wilson (1940) described the practice of mixed cropping and modern explanations of its effectiveness. The planting of legumes in association with non-legumes results in increased growth of the non-legume. Investigation of the phenomenon has shown that some of the nitrogen fixed in the legume is "excreted" and becomes available to the associated plant. When the two are grown together in the absence of rhizobia, no enhancement of growth of the non-legume is evident. The question then arose as to the chemical form of the "excreted" nitrogen. Virtanen (1938) was able to detect amino acids, particularly L-aspartic acid, in the medium of rhizobium-inoculated, but otherwise sterile, pea plants. He had previously established the ability of peas to grow in sterile culture with aspartate as the sole nitrogen source. He concluded that aspartate is the main product of nitrogen fixation and that the legume derives its nitrogen by the utilization of that amino acid.

It is now known that the release of amino acids from intact roots is not confined to nodulated legumes. Indirect evidence for the presence of metabolites and both stimulators and inhibitors in the vicinity of roots has been

derived from the behavior of soil microbes (Clark, 1949) and root-infecting nematodes (Ellenby, 1958; Wieser, 1955) as well as from the effects of associated growth mentioned above. The presence of metabolites around roots results in greatly enhanced numbers of micro-organisms when compared with the population of unplanted soil. The region of high microbial activity is known as the rhizosphere (Clark, 1949). The chemical nature of the free metabolites has been deduced from the activity and requirements of bacteria within the rhizosphere (e.g., Rovira, 1956a and b; Katznelson and Bose, 1959). Among the compounds which have been demonstrated within the rhizosphere by more direct means are cardiac glycosides (Ellenby, 1958), nucleotides and flavanone (Lundegårdh and Stenlid, 1944), thiamin and biotin (West, 1939), inhibitors of root growth (Eliasson, 1959), and amino acids. Katznelson et al. (1954) and Linder et al. (1957) have shown that compounds applied to leaves can be transported to and lost by roots. Slankis (1957) reported that radioactive sugars, amino acids, and organic acids have been detected in the root medium after feeding $C^{14}O_2$ to pine seedlings. Andal and co-workers (1956) found as many as eight amino acids lost by roots of rice, and Börner (1955) recovered six amino acids from water in which seeds had been allowed to swell. Frenzel (1957) concluded that, because amino acids were released only from plants supplied with inorganic nitrogen, there is

a spatial separation of newly synthesized and stored amino acids. An investigation of the physiology of the loss of metabolites from roots of several species conducted by Rovira (1959) revealed that the rate of loss decreased during the first two weeks of growth, increased light intensity caused increases in the recovery of some amino acids and decreases in others, heat generally stimulated the loss, and changes in calcium concentration and aeration had little effect. Most of these compounds are excreted in quantities too smallto have a noticeable effect on the nutrition of neighboring macroscopic plants.

Returning to Virtanen's investigation of associated growth, he failed to obtain evidence that aspartic acid can act as the sole nitrogen source for barley or wheat. This led him to the conclusion (Virtanen, 1938, p. 34) that "the difference between the legumes and non-legumes regarding N-nutrition is thus of fundamental nature."

As will be shown below, Virtanen's generalization is no longer considered correct since some non-legumes utilize amino nitrogen, but it is applicable in other cases.

Investigations on the assimilation of amino acids as nitrogen sources go back as far as 1853, according to Hutchinson and Miller (1911). They summarized the early work using organic compounds, showing that the majority failed as nitrogen sources or gave uncertain results. Frequently the results of different investigators conflicted. In the

description of their own experiments, Hutchinson and Miller stressed the necessity for sterility. Vickery, thirty years later (1941), seemed to imply by a comment about internal infection of seeds that he distrusted all of the successful results with organic nitrogen sources.

Table 1 contains a compilation of data obtained under sterile conditions with various amino acids, the effectiveness of which as nitrogen sources was measured by several methods. Interpretation of these results is hampered by failure of many of the investigators to provide adequate controls or to use equimolar amounts of nitrogen in the divers treatments. Moreover, one can question the suitability of some of the methods used to estimate the efficiency of the compounds. Molliard (1910), for example, showed that the fresh weight increase of radish plants when supplied with aspartate was 26-fold that in leucine, yet the respective dry weights differed by less than a factor of five. Similarly, Newton's data (1957) contain several instances in which the quantitative evaluations on a fresh weight basis do not lead to the same conclusions as those derived from height measurements. A perusal of Table 1 reveals decreases in the dry weight of several plants in the presence of amino acids which increased the nitrogen content. It is significant, however, that increases in dry weight did not occur without increases in nitrogen content.

TABLE 1

A COMPILATION OF THE SIGNIFICANT LITERATURE ON THE UTILIZATION OF AMINO ACIDS BY INTACT ANGIOSPERMS

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
alpha-alanine				
Barley	...	I	...	10
Clover	I	I	I (NH_4)	2
Maize	I	...	D (NO_3)	1
Maize	...	I	D (?)	7
Pea	I	I	D (NH_4)	3
Pea	...	I	...	10
Radish	D	...	...	5
Tomato	I	I	I (NH_4)	2
Wheat	...	I	D (NO_3)	10
Wheat	...	...	D (NO_3)	6
Wheat	I	I	D (NO_3)	11
beta-alanine				
Barley	I	I	...	9
Pea		"poor"	...	9
Tomato	S	I	D (NH_4)	2

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
alpha-amino-n-butyric acid				
Tomato	D	I	D (NH_4)	2
arginine				
Clover	I	I	I (NH_4)	2
Maize	...	I	D (?)	7
Sunflower	...	I	D (?)	7
Tomato	I	I	I (NH_4)	2
asparagine				
Barley	I	I	I (NO_3)	11
Clover	I	I	I (NO_3)	11
Clover	I	I	I (NH_4)	2
Maize	I	...	I (NO_3)	1
Pea	I	I	D (NO_3)	11
Radish	I	...	...	5
Sisyrinchium	...	I	...	8
Tomato	I	I	I (NH_4)	2
Wheat	I	I	D (NO_3)	11

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		aspartic acid		
Barley	D	I	D (NO_3)	11
Clover	I	I	I (NO_3)	9
Clover	I	I	I (NH_4)	2
Maize	...	I	D (?)	7
Pea	I	I	D (NH_4)	3
Pea	I	I	...	9
Pea	I	I	...	10
Pea	I	I	I (NO_3)	11
Radish	I	...	...	5
Sunflower	...	I	D (?)	7
Tomato	I	I	I (NH_4)	2
Wheat	D	I	D (NO_3)	11
Wheat	D	S	D (NO_3)	10
		cysteine		
Tomato	I	I	D (NH_4)	2

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		cystine		
Clover	D	I	D (NH_4)	2
Tomato	I	I	D (NH_4)	2
Wheat	...	I	D (NO_3)	10
		glutamic acid		
Clover	I	I	I (NH_4)	2
Maize	...	I	D (?)	7
Sunflower	...	I	D (?)	7
Tomato	I	I	I (NH_4)	2
Wheat	D	S	D (NO_3)	10
		glutamine		
Tomato	I	I	S (NH_4)	2
		glycine		
Barley	...	I	...	10
Clover	I	I	I (NH_4)	2
Cnicus	...	S	...	4

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		glycine		
Cucumis	...	S	...	4
Maize	I	...	D (NO_3)	1
Maize	...	I	D (?)	7
Pea	...	I	...	10
Radish	I	...	...	
Radish	I	...	...	5
Sisyrinchium	...	I	...	8
Sunflower	...	I	D (?)	7
Tomato	I	I	S (NH_4)	2
Wheat	...	I	D (NO_3)	10
Wheat	...	...	D (NO_3)	6
		histidine		
Clover	I	I	S (NH_4)	2
Tomato	I	I	S (NH_4)	2
		isoleucine		
Clover	I	I	D (NH_4)	2
Tomato	D	I	D (NH_4)	2

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		leucine		
Clover	I	I	D (NH_4)	2
Ipomaea	...	S	...	4
Radish	I	...	...	5
Tomato	I	I	D (NH_4)	2
Wheat	...	...	D (NO_3)	6
		lysine		
Clover	I	I	S (NH_4)	2
Maize	...	I	D (?)	7
Tomato	I	I	D (NH_4)	2
		methionine		
Tomato	I	I	D (NH_4)	2
		phenylalanine		
Clover	I	I	S (NH_4)	2
Maize	D	...	...	7
Tomato	D	I	D (NH_4)	2

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		proline		
Tomato	I	I	S (NH_4)	2
		serine		
Tomato	D	I	D (NH_4)	2
		threonine		
Clover	I	I	I (NH_4)	2
Tomato	I	I	I (NH_4)	2
		tryptophane		
Tomato	D	I	D (NH_4)	2
		tyrosine		
Cucumis	...	S	...	4
Maize	...	I	D (?)	7
Radish	D	...	...	5
Tomato	D	S	D (NH_4)	2

TABLE 1 - Continued

Plant	Response			Reference[b]
	Dry Weight[a]	Nitrogen Content[a]	Compared with Inorganic Form[a]	
		valine		
Clover	I	I	S (NH_4)	2
Tomato	D	I	D (NH_4)	2

[a]Key: I increased
D decreased
S remained the same
The dry weight and nitrogen content data were compared to corresponding figures obtained in the absence of added nitrogen.

[b]References:
1 Brigham, 1917
2 Ghosh and Burris, 1950
3 Hutchinson and Miller, 1911
4 Lutz, 1898
5 Molliard, 1910
6 Newton, 1957
7 Ratner et al., 1956
8 Tanaka, 1931
9 Virtanen, 1938
10 Virtanen and Linkola, 1946
11 Virtanen et al., 1933

The ability of peas to assimilate glutamic acid and to use its nitrogen more advantageously than that of ammonia or nitrate was published in a report by Virtanen which was not available to the present author (cf. Virtanen and Linkola, 1946; Miettinen, 1959).

Further inspection of Table 1 discloses many examples in which the response to amino acids equals or excels that to inorganic nitrogen, but the plants were clover and tomato in all of these cases except two (barley and maize grown on asparagine). Of 76 entries under "nitrogen content" only seven show no increase. This should be contrasted with the comparable data for tobacco shown in Table 2.

For the sake of simplicity no mention was made in Table 1 of the stereoisomers used. In most of the older work the isomers were not specified, but the assumption that the racemic mixtures was used is probably justified. Virtanen and Linkola (1946) stated that peas and clover were unable to distinguish between the optical forms of aspartic acid, and Ghosh and Burris (1950) failed to find significant differences in dry weight or nitrogen content of tomato plants grown on L-glutamic acid and on the racemate. On the other hand, Newton (1957) found large differences in the response of wheat seedlings to the enantiomorphs of alanine and of leucine, the D-form of the former exhibiting strong toxicity and the L-enantiomorph of the latter showing a less obvious, but still significant, inhibition.

Toxicity of D-amino acids is not uncommon, the antibiotic properties of some peptides being attributed to their "unnatural" structural configuration (Fruton and Simmonds, 1953).

That some plants are retarded or deformed by L-amino acids is more surprising. Reports by Miettinen and Waris (1958), Waris (1959), and Audus and Quastel (1947) show these effects must be interpreted carefully since very high concentrations of normal metabolites, far in excess of the usual physiological concentrations, were required in most cases to bring about these abnormalities. Miettinen and Waris found that glycine treatment produced profound morphological changes accompanied by a tenfold increase in the concentrations of amino acids and a lowering of the glucose and fructose concentrations of the entire plant. Later Waris concluded that the effect was closely concerned with differentiation and that there was no irreversible genetic change involved. Audus and Quastel's experiments showed inhibition of the growth of roots by L-amino acids as measured by dry weight.

These effects produced by normal metabolites could be the result of disturbances of the equilibria of the reactions involving the metabolites. An interpretation which should not be overlooked is that deduced by Cohen (1958) from the inhibition of growth of certain susceptible strains of Escherichia coli by L-valine. In the presence

of this amino acid and an inducer of galactosidase the bacterium synthesizes protein which has an abnormally high valine content and none of which is an active form of the galactosidase.

Data on the effects of amino acids on the growth of excised tissues and embryos are less consistent than those on intact plants. Robbins (1951) summarized the situation thus: "For almost any amino acid for which utilization is claimed, other evidence showing that it is not utilized or is toxic can be cited." He suggested that some of the conflicting evidence arose from such diverse causes as failure to account for sterility, lack of purity of the amino acids, secondary effects of the acids (such as buffering), stereospecificity, imbalance of mixtures of amino acids, differences in the stage of development of the plants, effects of the carbon content of the amino acid, light intensity, pH, temperature, and concentration. One might add to this list that the literature contains many examples in which the effects of contiguous tissues are ignored. The inability of an excised tissue or organ to respond to an added compound may often be due to the lack of cofactors which are supplied in vivo by adjoining tissues or organs. Lack of a response, then does not unequivocally indicate failure of the tested compound to enter the cell or absence of the enzymes necessary to utilize it. For example, Nitsch and Nitsch (1957) published evidence that the increment of growth of Jerusalem

artichoke tissue due to the addition of casein hydrolysate is enhanced 40-fold by auxin. An even more striking example is provided by the experiments of Fries (1954) on the growth of decotylized pea seedlings. Both the dry weight and length of excised roots far exceeded those of roots still attached to shoots.

Bonner (1943) and Harris (1959) each found clonal variations in the responses of excised roots. The variation between the two clones of clover used by Harris was so great that he concluded that several amino acids which are effective as sole nitrogen sources for one clone are not adequate for the other.

In general, the response of excised tissues to mixtures is greater than to individual amino acids (Gautheret, 1955). Most of the investigations in this field have been with the intention of seeking amino acids with growth stimulating properties rather than the testing of them as nitrogen sources.

A series of publications on the uptake of amino acids by radish root slices (Said and Younis, 1952 and 1953; Younis, 1960) reveals that alanine, leucine, phenylalanine, and aspartic acid contribute to substantial increases in the total nitrogen content of the tissues, but glutamic acid is less efficient in this respect. Because these data were obtained by incubating the slices for 48 hours, the question of sterility can be raised. Although all of the solutions

and apparatus were sterile, the root slices had merely been rinsed several times in sterile water.

Fries (1954) decided from his work with decotylized peas that only glycine, arginine, and "probably" glutamic acid of all the amino acids in casein hydrolyzate support the growth of the roots. With the exception of glutamic acid all of the amino acids tested were inhibitory at high concentrations. On the basis of the interchangeability of these compounds, he further suggested that the ornithine-citrulline-arginine cycle was present in pea roots.

A study of the efficiency of various nitrogen sources in the growth of crown gall tissue cultures from sunflower revealed a peculiar response to some amino acids (Riker and Gutsche, 1948), the plot of growth vs. amino acid concentration having two maxima of stimulation flanking a trough showing strong inhibition. The inhibitory concentration in most cases was approximately 10^{-3}M. These results prompted Eberts et al. (1954) to investigate the pathways by which these compounds are assimilated. Although the investigation failed to explain all of the results of the earlier report, the ability of the tissue to incorporate nitrogen simultaneously from nitrate and one of the several amino acids used was established. This was true even at concentrations of amino acid which are inhibitory to growth. No correlation between the activities of transaminases and growth could be found at several levels of glutamate, aspartate, or alanine

in the medium. At 10^{-3}M glycine was utilized more readily than glutamate, aspartate, and alanine.

Working with cultures of carrot root tissue, Steward and his collaborators (1956) interpreted their results to indicate that amino acids are not directly incorporated into protein to any great extent, but that protein synthesis is independent of the free amino acid pool. They suggested that the free amino acids are in some manner separated from the site of protein synthesis, their nitrogen being transferred to those sites primarily in the form of glutamate. The direct oxidation of amino acids was thought to involve the amino acids released during protein degradation. This hypothesis was based on the failure of absorbed, C^{14}-labeled amino acids to give rise to labeled carbon dioxide quickly. Subsequently, Birt and Hird (1958) showed that of eleven amino acids tested only alanine, aspartic acid, glutamic acid, and phenylalanine underwent substantial metabolic destruction upon being absorbed by carrot root disks. In reiterating their earlier interpretation, Steward and Bidwell (1958) showed that the amino acid content of protein in their tissue cultures was remarkably constant regardless of the amino acid supplied or the rate of growth. They also found that the carbon of protein was derived principally from carbohydrates.

The ability of mitochondria isolated from bean hypocotyl homogenates to incorporate glycine, glutamic acid, and

aspartic acid into protein was established by Webster (1954a). Protein synthesis was enhanced by Krebs cycle intermediates, cyanide inhibiting that enhancement. Stimulation of the incorporation of these amino acids into protein was produced by the addition of adenosine triphosphate. This stimulation was unaffected by cyanide. Webster concluded that the Krebs cycle substrates yielded energy which was necessary for the synthetic reaction, that the inhibitor prevented energy transfer from the organic acids to the synthetic mechanism, and that adenosine triphosphate was a suitable energy source for the reaction. He later observed (1954b) that glutamic acid is accumulated by sections of the bean hypocotyl, carrot root, and potato tuber. In bean hypocotyls 15.5% of the absorbed glutamate was found in protein, 43% as the free acid, and 18.1% as glutamine after incubation for one hour. The incorporation of glutamate into protein was also observed in slices of carrot root and potato tuber, leaves of spinach, and seedlings of oat and pea. Fractionation of homogenates of bean hypocotyl by centrifugation revealed varying capacities for incorporation of glutamate, the microsomal fraction having the greatest and the nuclear fraction the least. These data are not in accord with the interpretations of Steward et al. (1956) and Steward and Bidwell (1958) which are noted above.

The Uptake of Amino Acids by the Tissues of Higher Plants

Investigations of the uptake of amino acids by intact higher plants are few. Experiments designed to test the ability of plants to utilize amino acids as nitrogen sources were seldom intended to cast any light on the means by which the nitrogen entered.

Virtanen and Linkola (1946), in addition to measuring the effectiveness of aspartic acid in supplying nitrogen to pea and clover plants, investigated the relative rates of disappearance of carbon and nitrogen from the medium while aspartate was being absorbed. They found no evidence for the breakdown of aspartate before uptake. Ratner and co-workers (1956) also decided that amino acids are taken up intact, basing their conclusion on the failure of roots to alter the carbon chains of radioactive amino acids during uptake.

In a broad study of the ability of wheat roots to remove glycine and tyrosine from the medium, Ratner and Besermen'i (1959) found that the former amino acid is taken up at roughly twice the rate of the latter during the first five hours after presentation. The curve plotting the time-course of uptake of glycine showed a tendency to level off at about four hours, assuming a somewhat sigmoid shape; the curve for tyrosine uptake, however, indicated a slowly rising rate throughout the whole experimental period. Both

alanine and phenylalanine proved to be competitive inhibitors of the absorption of glycine, alanine being the more effective. These two competitors were poor inhibitors of tyrosine uptake. These workers also examined the fate of glycine taken up by wheat roots, and found that 38% of the activity in roots that had been washed after an hour in C^{14}-labeled glycine was in protein; after five hours in the amino acid the root protein contained only 20% of the radioactivity.

A series of papers on the metabolism of nitrogen in storage roots has originated in the university at Cairo. Four of these have dealt specifically with the uptake and utilization of amino acids. Although sterile solutions and apparatus were used, the introduction of contaminated tissue leaves the results of experiments which ran as long as 48 hours open to question on the grounds of possible involvement of bacteria. Because some precautions were taken, however, these data should not be completely ignored. Said and Younis (1953) found that slices of radish root remove much more alanine, leucine, and phenylalanine from 0.014% solutions of each in the first 24 hours of incubation than in the succeeding 24 hours. Tissue analyses suggested that nearly half of the absorbed alanine and over two-thirds of the leucine and phenylalanine were accumulated as the free amino acids. The appearance of large amounts of ammonia in both the tissue and the medium when alanine was presented

was considered to be a result of oxidative deamination. The carbon dioxide output of the tissues was measured daily, revealing an enhancement of respiration which was strikingly greater during the second 24 hour period than the first in leucine and alanine.

Radish roots fed aspartic acid at various concentrations showed sharp decreases in ammonia production with the raising of the amino acid concentration (Said and Younis, 1952). Conversely, protein, peptide, and amino nitrogen increased under the same conditions. The enhancement of carbon dioxide production was greater at low concentrations of the amino acid (0.008% aspartic acid nitrogen or less), higher concentrations being inhibitory during the first day and stimulatory thereafter. The authors suggested that deamination of amino acids may occur at cytoplasmic surfaces. Because aspartate was presented without buffering, the medium was initially acidic. They suggested that the observed increases in deamination with time result from increases in pH concomitant with uptake of the amino acid.

Younis (1960) reported that the uptake and utilization of L-glutamic acid by radish root slices is in many respects comparable to that of aspartic acid. The greatest enhancement of carbon dioxide evolution was at the concentration showing maximum uptake and ammonia production, namely 8.58 mM. A more concentrated solution, 11.44 mM, was less effective.

Using carrot root disks washed four days in distilled water, El-Shishiny and Nosseir (1957) noted that the uptake of D-glutamate in 24 hours was one-third that of the L-enantiomorph. About 70% of the absorbed L-glutamate was assimilated, but the D-form was accumulated without further transformation. The simultaneous presentation of one mole each of D- and L-glutamic acid led to greater total uptake of amino nitrogen than from one mole of either form, but the uptake from the combination was not equivalent to the sum of that from the separate solutions. Enhancement of the respiratory rate by both stereoisomers was mentioned, but no quantitative data were included.

Birt and Hird (1956, 1958a and b) collaborated on a study of the uptake of amino acids by carrot root slices. They found that washing enhanced both the uptake of amino acids and the respiratory response of the tissue to these compounds. After 20 hours of washing the enhancement of oxygen consumption during 6 hours incubation was only 1% of the control value in either 5 or 50 mM L-phenylalanine even though uptake of the amino acid in the higher concentration was 2.5 times that from the lower. After 168 hours of washing, however, the observed respiratory stimulation was as high as 19%, the value rising with concentration up to 25 mM and falling off somewhat at 50mM. Stereospecificity of the respiratory response varied with the compound used, D- and L-leucine being comparable and D-phenylalanine being

less effective than its natural isomer. A comparison of the respiratory response and the amount of a given amino acid taken up showed that those subject to greater metabolic destruction, namely glutamate and aspartate, produced a larger respiratory response per mole than those which are less readily degraded, such as alanine, phenylalanine, and histidine. Using C^{14}-labeled leucine and phenylalanine, they found that both of these compounds contribute to the carbon dioxide production of the tissue. Of eleven amino acids tested, including the D-isomers of six, all but L-alanine were concentrated against a gradient. It is interesting that D-alanine was accumulated to 2.5 times its external concentration. Since glucose and L-phenylalanine inhibit the production of carbon dioxide from each other after prolonged washing, the respiratory enhancement was attributed to depletion of endogenous reserves of metabolizable substrate. That a part of the respiratory stimulation was due to the uptake process itself was not unequivocally demonstrated. Respiratory stimulation by 20 mM potassium chloride did occur. The uptake of histidine was inhibited by potassium chloride, potassium phosphate at pH 6-8, 10^{-4} M 2,4-dinitrophenol, 10^{-3} M sodium cyanide, phenylalanine, and arginine. From these data the authors concluded that the uptake mechanism requires the expenditure of respiratory energy, is not highly specific, and may be similar or the same for both inorganic ions and amino acids. They also

noted that those amino acids with charges on the side chain were less readily absorbed than those with lipophilic side chains.

Oudman (1936) found that organic nitrogen is absorbed by leaves of Drosera only when there is a sufficient supply of other nutrients. The rate of uptake was decreased by high concentrations of minerals and increased by raising the temperature. Asparagine was taken up by Drosera leaves at a rate above that which could be attributed to diffusion alone (Arisz and Oudman, 1937), whereas caffein entered by diffusion. A lag period in the uptake of asparagine was seen. The removal of oxygen resulted in slowing the absorption of asparagine without affecting that of caffein (Arisz and Oudman, 1938).

Amino Acids as Nitrogen Sources for Tobacco

Some of the literature on the ability of amino acids to provide nitrogen for tobacco plants is summarized in Table 2. All of the compounds studied by Ghosh and Burris (1950) produced increases in the nitrogen content by the plants, but the amino acids which caused decreased dry weights yielded only very small increases in the nitrogen content. None of the amino acids were as effective as ammonium or nitrate in stimulating the growth of tobacco, the greatest dry weight attained with an amino acid being 31% of that with ammonium sulfate.

TABLE 2

A COMPILATION OF THE SIGNIFICANT LITERATURE ON THE UTILIZATION OF AMINO ACIDS BY INTACT TOBACCO PLANTS

No Growth	Decrease Dry Weight	Increase Dry Weight
Ghosh and Burris, 1950: Variety Spanish Comstock 7.35×10^{-4} M amino acid in nitrogen		
DL-aspartate	beta-alanine	DL-alpha-alanine
L-hydroxyproline	L-glutamate	L-arginine
	glycine	L-asparagine
	DL-isoleucine	casein hydrolyzate
	DL-leucine	cysteine
	L-leucine	L-cystine
	DL-methionine	DL-glutamate
	DL-norleucine	glutamine
	DL-serine	L-histidine
	DL-threonine	L-lysine
	DL-tryptophane	DL-phenylalanine
	DL-valine	L-proline
		L-tyrosine
Beaumont et al., 1931[a]: Variety Havana Seed 2.78×10^{-2} M amino acid nitrogen		
alanine	arginine	asparagine
aspartate	histidine	cystine
glutamate	lysine•HCl	
glycine		
leucine		
lysine•2HCl		
phenylalanine		
proline		
tyrosine		

TABLE 2 - Continued

No Growth	Decrease Dry Weight	Increase Dry Weight
Steinberg, 1947: Variety Maryland Medium Broadleaf 200 mg per l amino acids		
DL-alpha-amino-n-butyrate L-hydroxyproline DL-serine	DL-alanine L-arginine·HCl L-asparagine D-aspartate L-glutamate glycine L-leucine DL-isoleucine L-proline L-tryptophane L-tyrosine others	none

[a]Some liberties have been taken in reporting this paper. Because no control figures were included, those acids rated as causing slight or poor growth were assigned to the decrease column and those which permitted growth as high as 10 cm to the increase column.

The data of Beaumont *et al.* (1931) in Table 2 show toxicity which probably resulted from the relatively high concentrations used. In this respect their experiments resemble those of Audus and Quastel (1947).

Steinberg (1947) also found tobacco to be unable to grow well on amino acids (Table 2). Because he added equal weights of the various compounds, his results are not comparable on an equal nitrogen basis, but all the concentrations were 2.67×10^{-3} M or less. He interpreted the toxicity of aspartate, glutamate, histidine·2HCl, and lysine·2HCl to be partially due to excessive acidity. Failure of growth of the plants in the presence of such common metabolites as alanine, asparagine, glutamate, and glycine to reach even 5% of the control value is striking. DL-isoleucine produced morphological abnormalities which were remarkably similar to those of the physiological disorder commonly known as frenching. Several other morphological responses produced by other amino acids seem to be comparable to those reported by Waris (1959) for another species. Steinberg *et al.* (1950) showed later that frenching symptoms produced by mineral deficiency are accompanied by increases in the amino acid content of the deformed leaves.

Pratesi and Ciferri (1946) found the effectiveness of amino acids as nitrogen sources to decrease in the following series: beta-alanine, leucine, aspartate, and gluta-

mate.

Tobacco callus tissue cultures were found by Frank et al. (1951) to grow on glutamate as a sole nitrogen source, the optimum concentration being near 4×10^{-3} M. The fresh weight produced on that concentration of glutamate was less than half the maximum produced on a mixture of alanine and nitrate. Alanine alone produced slight growth at concentrations below 5×10^{-4} M, was toxic at 10^{-3} M, and stimulated growth strongly at higher concentrations. More recently, Sandstedt and Skoog (1960) discovered that minute amounts of aspartate and glutamate, equivalent to those in 0.1% yeast extract solutions, promote growth of tobacco callus to a greater extent than a synthetic mixture containing all of the amino acids of yeast extract. In this case, however, the amino acids are not serving as nitrogen sources.

On the basis of these literature reports it would appear that amino acids are not effective nutrients for tobacco.

METHODS AND MATERIALS

Respirometry

Measurement of Oxygen Uptake

Standard techniques for the determination of oxygen consumption with the Warburg respirometer were employed (Umbreit et al., 1957).

In all of these experiments the total liquid volume of the flasks was 3.2 ml, of which 0.2 ml was 10% KOH, and the remainder consisted of roots and the medium. A filter paper wick was placed in the alkali to facilitate the removal of carbon dioxide. Root volumes were measured by one of three methods: (a) For peas the average volume occupied by ten 2 cm. root tips (0.2 ml) was found by displacement of water, and the volume of medium was reduced by that amount in each flask. (b) When barley roots were used, their volume was not measured directly, but the portion of roots to be added to a Warburg flask was placed in a graduated test tube, and medium was added to the desired total volume. (c) With tobacco, portions of roots were weighed on tared waxed paper strips, and, assuming a density of 1 g/ml for both roots and medium, the volume of medium was adjusted accordingly.

Addition of reagents was accomplished by placing in the sidearm 0.5 ml of a solution which when added to the con-

tents of the main chamber produced the desired final concentration.

All respirometry experiments were run at 25° C. After filling, the flasks were placed in the water bath for at least 15 minutes with shaking before the stopcocks were closed. The Warburg apparatus used, a rotary model manufactured by American Instrument Co., was adjusted to produce a shaking rate of 112 cycles per minute with an amplitude of 4 cm. The pivotal shaking mechanism allowed readings to be taken without stopping agitation. Respiration was calculated on the basis of oxygen uptake per unit oven dry weight, the roots being dried on aluminum moisture dishes at least overnight at 100 - 105° C.

Measurement of Carbon Dioxide Production

Evolution of carbon dioxide was measured by the direct method described by Umbreit *et al.* (1957). Corrections were made in the calculations for unequal amounts of tissue, and the data were adjusted to account for the initial bound carbon dioxide in the medium.

The method relies on the measurement of total gas exchange in flasks containing roots, substrate, and no alkali. Other flasks containing 0.2 ml of 10% KOH in the center well were used to measure oxygen consumption. The difference between these two values is the volume of carbon dioxide evolved. Carbon dioxide bound by the medium was released by the addition of 0.5 ml of 2 N HCl from a sidearm immediately

before taking a reading. Double sidearm flasks were used, the substrate being tipped in all flasks at the beginning of the experiment; the second sidearm contained the acid solution. All other conditions were as described above for measurement of oxygen consumption.

The calculation of carbon dioxide production was as follows:

CO_2 evolution = (total gas exchange) - (O_2 uptake) - (initial bound CO_2).

Radioisotope Experiments

Counting

In all of the experiments in which the uptake of glutamate was followed by changes in radioactivity counting was done on a Nuclear-Chicago gas flow counter with automatic sample changer and printer-timer. Although a windowless detector was used for most of the counting, several experiments contain data derived from thin-window counting. Each experiment was carried out completely with either one or the other instrument. All samples were plated on stainless steel planchets and placed within a ring drawn with a wax pencil on the bottom of the planchet.

As recommended by Umbreit et al. (1957) a minimum of 5000 counts above background were recorded. Under such conditions the probable statistical counting error is below the sampling error. The additional statistical errors involved

in low counting rates were avoided by adjusting the quantities so that all samples had disintegration rates above approximately ten times the background rate (15-20 cpm).

Uptake by Excised Roots

Two methods, similar in principle, were used to measure the absorption of C^{14}-labeled glutamic acid. In the first, the roots were placed in polystyrene containers 86 mm in diameter and 36 mm high containing the experimental solution. A narrow bore polyethylene tube inserted through a hole in the lid provided an inlet for water-saturated compressed air which served both to aerate and stir the solution. At the end of the experiment the containers and aeration tubes were rinsed, the washings being added to the bulk of the solution for analysis. The second method involved placing the roots and solution on a reciprocal shaker in 125 ml Erlenmeyer flasks. The shaker speed was adjusted as high as possible without running the risk of losing solution by splashing from the top of the flasks.

Carefully measured aliquots of a common solution containing the labeled amino acid were pipetted into each container, thus assuring that both the concentration of glutamate and the amount of radioactivity were uniform within all of the flasks used in a given experiment. Excess water was removed from the roots in a porcelain basket centrifuge. Then equal portions of roots were weighed out and placed in the experimental vessels for specified periods. At the end

of the interval both roots and solution were filtered in a funnel, the filtrate being caught in a volumetric flask. Distilled water and washings from the experimental containers were poured over the roots. No appreciable activity was detected in subsequent washings of the roots. Thus, the roots were thoroughly washed. They were then placed in tared moisture dishes and dried at 100 - 105° C.

After the volumetric flasks had been filled, 0.5 ml aliquots were removed and placed on one x 5/16 inch stainless steel planchets and dried in a stream of air. Rings made with a wax pencil were placed on the bottom of the planchets to prevent creeping of the sample up the sides. This procedure assured uniform distribution of the material.

Portions of the original solutions were diluted to the same extent as the final solutions and sampled in similar fashion. In several of the early experiments the original solutions were sampled directly without dilution. The resulting apparent increases in activity during the uptake period suggested that self absorption phenomena were distorting the results. Comparison of the relative activities of aliquots of a solution and those of a diluted portion of the same solution confirmed the suspicion. Thereafter all of the samples within an experiment were diluted to the same degree.

This development raised the question of the possibility of large errors arising from the apparent dilution or depletion that results from uptake. The effects of self absorption were estimated by the method suggested by Hendler

(1939). This entails the sampling and counting of a series of dilutions of the same solution, providing differing weights of material on a given area. The specific activity (as cpm per unit weight) of each sample is divided into that of an arbitrary reference, producing a correction factor. The correction factor multiplied by the counting rate at any weight per unit area yields the counting rate that sample would produce if it were of the same density as the reference sample. The data for two such comparisons are presented in Figure 1. Because very small weights are involved, the densities are calculated from the concentration of the original solution instead of from direct weighings. The points designated as the original solutions represent the concentrations actually used for the uptake experiments; those used as the reference points are of the concentrations resulting from the dilution required by the procedure for measuring uptake described above. Although much lower densities were used here than those of Hendler (1959), trends are apparent.

In the experiments dealing with uptake of glutamic acid by excised roots the concentrations and volumes of solutions and the amount of roots were so adjusted as to avoid the uptake of more than half of the radioactive glutamate in a given container; that limit was exceeded only once (Figure 17, uptake by washed roots at 6 hours). In the majority of cases less than one-third was depleted. The curves in Figure 1 each indicate corrections of 5% or less when a sample contains half as much material as its reference sample. Thus there is

Figure 1. Self-absorption correction factors for the counting of solutions of C^{14}-glutamic acid, uniformly labeled.

The highest weight value on each curve represents 5×10^{-4} M glutamate and 10^{-3} M NaN_3 (•) or 5×10^{-5} M 2,4-dinitrophenol (x). The values for lighter weights were derived from dilutions of these original solutions. Enlarged symbols indicate the references points for which the average of three determinations was used.

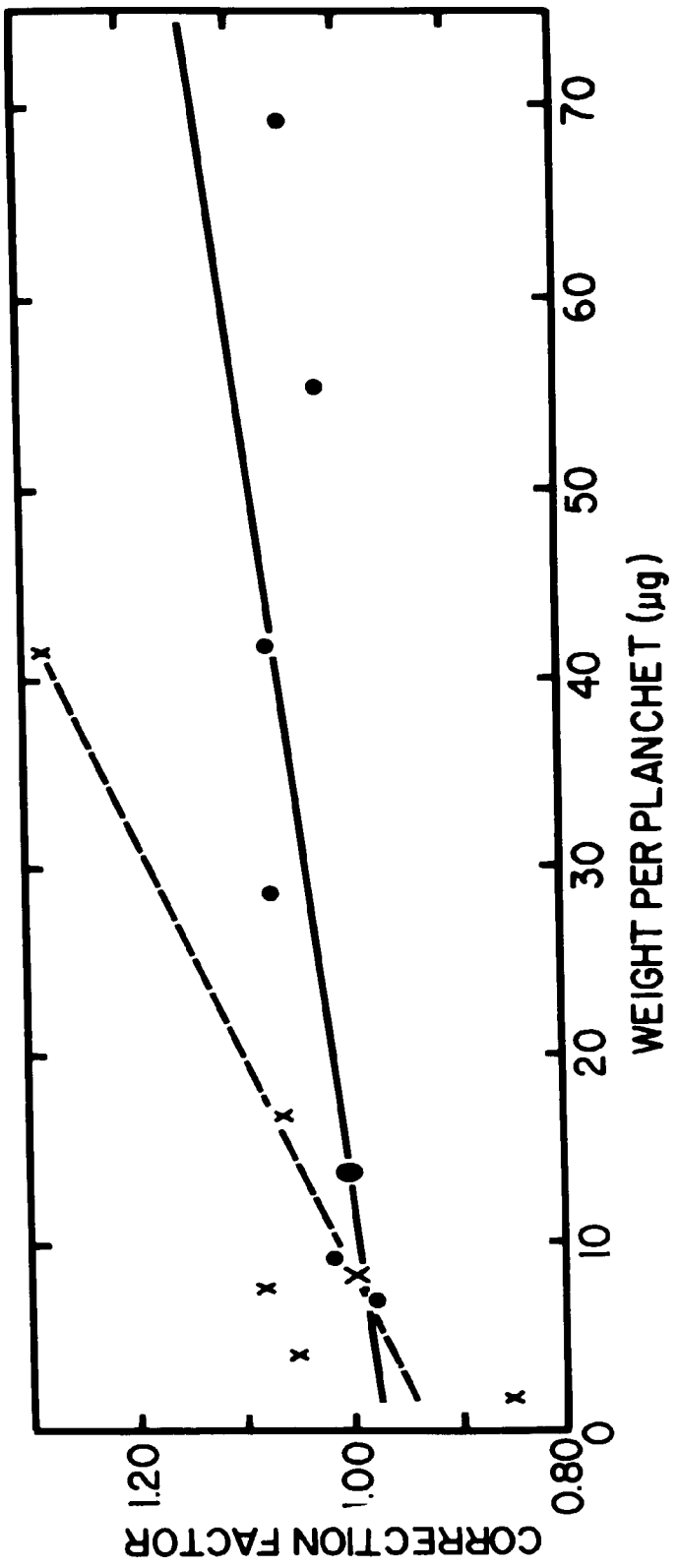

CORRECTION FACTOR
0.80
1.00
1.20
0
10
20
30
40
50
60
70
WEIGHT PER PLANCHET (µg)

Figure 1

reasonable assurance that the self-absorption errors are not excessive.

However, one must note that these correction curves can be applied properly only when all of the solutes are absorbed at the same rate. In the experiments on the uptake of glutamate in the presence of inhibitors there is reason to believe that some of the inhibitors penetrate very slowly in the comparison to the amino acid. Because each planchet contains too little material to permit accurate weighing, there is no effective way accurately to determine the self-absorption correction factor.

Uptake by Intact Plants

In an attempt to relate the uptake of glutamic acid to transpiration simultaneous measurements of both processes were made. With minor variations the method consisted of placing the root system of a plant or group of plants in a solution of known volume and composition and measuring the changes in volume and composition after a given period of time. With peas and barley the roots were allowed to drain for several minutes, large drops of water being removed gently with a glass rod immediately before the roots were placed in the experimental solution. The same procedure was followed at the end of the experiment with care that all of the excess solution was returned to its container. In this way the volume of water adhering to the roots at the onset

of the experiment and added to the system was roughly compensated for by the amount that was removed at the end.

The volume transpired was measured indirectly by weighing the container and its solution before and after the uptake period. A density of 1.0 g/ml was assumed for all solutions. Aeration and stirring of the solution was accomplished by a water-saturated air stream flowing through a capillary tube. Before removing samples for analysis, the solutions were returned to their initial volumes.

The transpiration stress was varied in some cases by enclosing the shoots in polyethylene bags which were closed tightly to restrict the transpiration rate or through which a dried air stream was passed when increased rates were desired. These experiments were all carried out in the light, the light sources being either sun or a combination of fluorescent and incandescent light.

Scanning of Chromatograms

Chromatograms containing radioactive compounds were run through a Vanguard model 800 strip-scanner which produces a record of the relative radioactivity along a linear scale identical to distances along the chromatogram. A pair of opposed detectors above and below the chromatogram make this a particularly sensitive instrument. The scanning record was later compared with spots visually located after the strips were sprayed with chromogenic reagents.

Chromatography

Both one- and two-dimensional chromatograms were used. All chromatograms were run at approximately 25° in cylindrical glass containers 100 mm in diameter and 300 or 500 mm high. Whatman No. 1 filter paper was used. After spotting, the papers were allowed to equilibrate with the solvent vapors for at least three hours before the ascent or descent of solvent was begun.

The two-dimensional chromatograms were run on 21 cm squares which had been held in cylindrical shape with staples. The first solvent was water-saturated phenol; the second consisted of a fresh mixture of equal volumes of solutions containing 84 ml of water in 1246 ml n-butanol and 620 ml propionic acid in 790 ml of water. According to Rabson and Tolbert (1958) this combination of solvents in conjunction with 0.1% ninhydrin in 95% ethanol containing 2 drops of 2,6-lutidine per 100 ml for spraying enables the simultaneous detection of keto- and amino acids. The amino acids yield a color with ninhydrin, and the keto-acids in the presence of lutidine fluoresce under 366 mu ultraviolet light.

The unidimensional chromatograms were developed in the butanol-propionic acid-water solvent mentioned above.

Suction Technique

This is a modification of the technique used by Kramer (1932) to measure the permeability of roots to water. The

top of a tobacco plant was removed leaving a stump approximately 4 cm long. Leaf bases and minor surface irregularities were removed with a razor blade, and the lateral surfaces of the stump were coated thoroughly with Dow-Corning high vacuum grease, care being taken to keep the grease off the transverse surface of the stump. A short piece of gum rubber tubing of slightly smaller inside diameter than the width of the stump was worked down to the point of insertion of the uppermost roots, and a glass tube of suitable size was inserted in the opposite end of the rubber tube, the end of the glass tube touching the stump. Hose clamps were firmly attached near the ends of the rubber tube. A vacuum of about one-half atmosphere was applied at the open end of the glass tube. In this manner it was found possible to draw a solution through the intact root system and to recover the so-called xylem sap exudate by breaking the vacuum and removing the accumulated solution with a pipet.

Plant Materials

Seed

All of the plants used were of varieties commonly used in physiological studies:

Pisum sativum L. var. Alaska

Hordeum vulgare L. var. Atlas 46

Nicotiana tabaccum L. var. Maryland Mammoth

Growth of Plants

Pea roots for respirometric studies

The pea seeds were surface sterilized by placing them in 10% Chlorox solution containing a drop of Ivory liquid detergent. After 40 minutes the seeds were rinsed in distilled water. For germination the seeds were placed between two wet paper towels which rested on a stainless steel grid which just broke the surface of distilled water through which compressed air was bubbled.

Within 24 to 48 hours after the beginning of sterilization the seeds were suspended on grids above distilled water held on the base of a polystyrene Petri dish one inch high and six inches in diameter. The grids were made from one-quarter inch galvanized hardware cloth which was painted with Glyptal enamel (manufactured by General Electric Co.) and baked at 125° C. for two hours. The grids were periodically sprayed with Krylon acrylic spray (Krylon Co., Norristown, Pa.) as an additional precaution to eliminate heavy metal ions. The seeds were covered with paper towels which were kept moist until the elongating shoots lifted them free of the seeds. Both germination and growth were carried out at 25° C. in the dark.

Six days after originally wetting the seeds, unless otherwise specified, 2-cm root tips were excised. Washing, when performed, was accomplished as described below.

Intact pea plants for uptake studies

The seeds were sterilized and germinated as described in the preceding section and were then transferred to the two-zone root culture system described by Norman et al. (1959). This consisted of a waxed paper cup, the bottom of which was replaced by 1/8 inch hardware screen cloth which had been treated as noted in the preceding section. The bases of the cups rested on cylindrical polystyrene containers in which approximately 400 ml 10^{-4} M calcium sulfate solution was placed and periodically renewed.

About 50 pregerminated seeds were placed on top of the following nutrient mixture in each cup and were covered with a 1/4 inch layer of vermiculite. For each container nutrients were supplied by these amounts of ion exchange resins and clays thoroughly mixed with 10 g. of vermiculite:

0.86 g. Ca bentonite

0.43 g. Mg bentonite

0.17 g. K bentonite

0.86 g. Duolite exchange resin A-7 (NO_3)

0.86 g. Duolite exchange resin A-2 (SO_4)

0.21 g. Amberlite exchange resin IRA-400 (PO_4)

10 g. vermiculite

By watering carefully the vermiculite was kept moist without leaching any nutrients into the lower container. Only the roots which extended into the lower container were used for uptake studies.

Pea roots for uptake studies

These roots were produced in the same manner as those for respirometry with the difference that the roots were excised after 14 days of growth and that the plants were supported on a stainless steel grid above a stainless steel tank of 26 liter capacity. All metal parts were sprayed with two thin coats of Krylon acrylic spray. The tank contained 7.5×10^{-5} M calcium sulfate and 2.5×10^{-5} M magnesium sulfate. This solution was replaced on alternate days.

Barley roots for respirometric studies

These were grown as described above for peas with three exceptions: (a) the seeds were not surface sterilized, (b) the pregermination period was not allowed to exceed 24 hours in order to avoid damaging tender roots during handling, and (c) the grids used were of 12 mesh Saran screen cloth.

Intact barley plants for uptake studies

These were grown in a manner analogous to that for intact pea plants.

Tobacco

Tobacco seeds were planted in sand and grown in a greenhouse by the staff of The University of Michigan Botanical Gardens. Soluble nutrients were added periodically. Plants one to four inches high were removed from their pots, and the sand was washed free in a gentle stream of lukewarm water. The plants were then placed in liquid culture. Each

plant was placed in a one inch hole in the lid of 1.5 liter cylindrical polystyrene container. These containers were painted black to prevent the growth of algae in the nutrient solution. The plants were supported by their own lower leaves. Aeration was supplied through narrow bore polyethylene tubing.

The culture solution used was that recommended by Hoagland and Arnon (1938) containing all of its nitrogen as nitrate. The solution was used at half strength, except in some of the earlier work, and ferric tartrate was added at full strength. Periodically their micronutrient mixture was included. The solution lost through transpiration was renewed once or twice daily, depending on the size of the plants and the weather. The entire solution was replaced in clean containers every three to six days.

Plants grown in this manner served as a source of material for all of the experiments utilizing tobacco.

Washing of Excised Roots

As is shown in the Results and Discussion, it was necessary to wash the roots for a day after excision before a respiratory response to glutamic acid could be elicited consistently. This was accomplished by placing the roots in a beaker, the mouth of which was covered by a piece of Saran screen cloth secured by a rubber band. Distilled water or a solution was fed slowly by gravity into the beaker which was clamped at a 45^{o} angle over a sink. In this manner the liquid volume in the beaker was kept relatively small. Compressed

air introduced through a capillary tube provided both aeration and stirring. No effort was made to control the rate of flow of water within narrow limits, but the total volume used always exceeded 18 liters.

Reagents

Materials and sources were as listed below:

L-glutamic acid-U-C^{14}	Volk Radiochemical Co. Chicago, Illinois
Telomycin	Bristol Laboratories Syracuse, New York
Strophanthin-k	Nutritional Biochemicals Corp. Cleveland, Ohio
alpha-aminoisobutyric acid	Sigma Chemical Co. St. Louis, Missouri
All other amino acids	Nutritional Biochemicals Corp. Cleveland, Ohio

RESULTS AND DISCUSSION

Respiratory Response of Excised Roots to Glutamic Acid

Measurement of the metabolism of an organic compound is commonly approached by investigating the changes which the compound produces in the respiration of the organism under study. One might expect, therefore, to find an enhancement of the uptake of oxygen and evolution of carbon dioxide with the presentation of glutamic acid to roots if it enters and is metabolized.

Preliminary Experiments

A series of experiments in which the utilization of oxygen by pea root tips in water was compared with that in 10^{-3} M L-glutamic acid gave conflicting results. Although the endogenous respiratory rates of root tips of the same age agreed well between experiments, the response to the amino acid ranged from stimulation to inhibition, with some experiments showing no effect. The results of such an experiment are shown in Figure 2. This illustration cannot be considered as typical; the wide range in the observed responses both qualitatively and quantitatively prevents the selection of a typical reaction.

Because the pea seedlings had been grown without added nutrients, the possibility of a mineral deficiency was

Figure 2. Effect of 10^{-3} M L-glutamic acid on the oxygen uptake of freshly excised pea root tips.

Two-cm root tips from 6-day old dark-grown seedlings; respiration at 25° C.; glutamate added at time zero; (1) in water; (2) in glutamate, pH 6.0; (3) in glutamate and Hoagland's solution, pH 6.0.

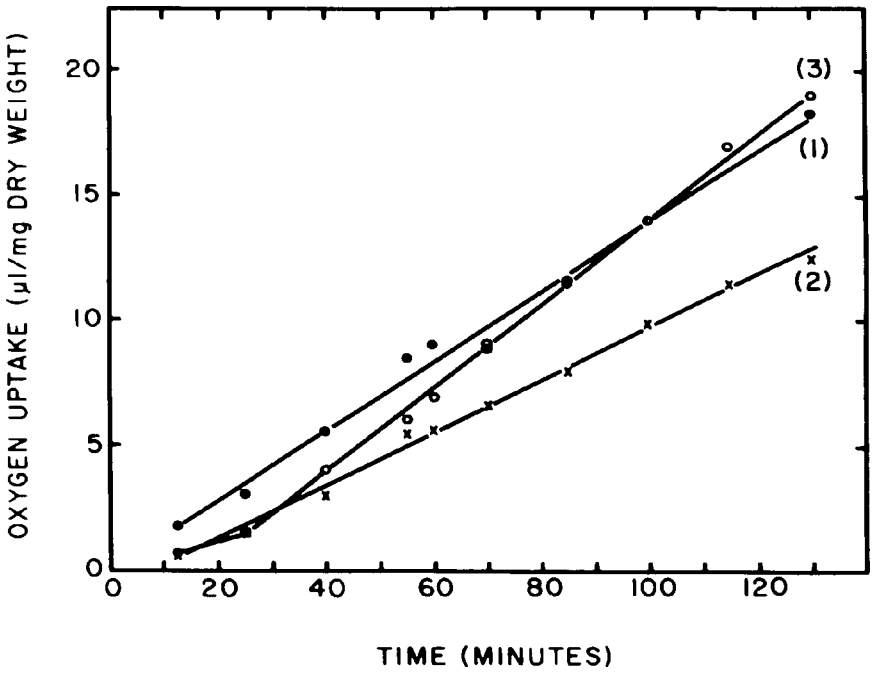

OXYGEN UPTAKE (µl/mg DRY WEIGHT)
0
5
10
15
20
0
20
40
60
80
100
120
TIME (MINUTES)
(1)
(2)
(3)

Figure 2

tested by supplying Hoagland's solution (the same as described for growing tobacco plants) including the micronutrient mixture during the incubation period. The results, plotted in Figure 2, provided no support for the deficiency hypothesis. That there was a long-term deficiency that could not be relieved immediately was ruled out by the response of washed roots (see below).

In order to eliminate pH effects, buffers were used to keep the pH within the "physiological" range. Solutions of unbuffered glutamic acid had a pH of 3.4, but buffering the solution at 5.0 to 6.6 did not improve the consistency of the results.

Lest a reaction was being missed because of a long time lag, the incubation periods were increased to over six hours, but to no avail.

A single experiment using glutamine and aspartic acid showed that oxygen uptake of pea roots in 10^{-3} M solution of each of these at pH 6.5 was not significantly different from the control after 6.5 hours of incubation. Asparagine, however, produced a small stimulation (Table 3).

The Response of Washed Roots

In examining the uptake of amino acids by carrot root disks Birt and Hird (1956) showed that washing progressively increased the ability of the tissue to accumulate these compounds. They later found (1958a) enhancement of the respiratory response by washing. It seemed likely, therefore, that

TABLE 3

EFFECTS OF SEVERAL AMINO ACIDS ON OXYGEN UPTAKE BY SEVEN DAY OLD PEA ROOT TIPS

(Two cm. root tips from dark-grown seedlings; 25° C.; glutamate added at time zero)

Compound (10^{-3} M)	Oxygen uptake in 390 min. ($\mu l.O_2$/mg. dry weight)	Standard error
None	58.5	±0.7
L-Glutamine	61.1	±5.0
L-Asparagine	66.8	±4.8
DL-Aspartic acid	53.1	±8.6

pea roots might behave in a similar fashion.

Washing at low temperature

Pea roots washed for one day in distilled water at 3-5° C. gave results similar to those of unwashed roots. For example, in one experiment the oxygen consumption in 300 minutes was 20.8 ± 0.5, 17.9 ± 0.7, and 19.9 ± 1.1 µl O_2 per mg dry weight in water, pH 3.4 glutamate, and pH 6.0 glutamate respectively. Washing in dilute $CaSO_4$ produced comparable results.

Washing at room temperature

In contrast, glutamic acid produced a strong and consistent stimulation of the oxygen uptake of pea roots washed approximately 24 hours in distilled water at room temperature (Figure 3). If the response is plotted on a semi-log scale, its logarithmic nature becomes apparent.

Figure 4 contains a comparison of the oxygen consumption of washed and of freshly excised barley roots in the presence and absence of L-glutamate. The endogenous respiration of the freshly excised roots exceeded that of both groups in washed roots, but notice that those washed at low temperature retained a higher proportion of the original metabolic activity than did those washed at room temperature. The decrease as a result of the washing is probably due to the decreased availability of metabolic substrates rather than to removal of materials from the roots. The cold

Figure 3. Stimulation of oxygen uptake of washed pea roots by 10^{-3} M L-glutamic acid.

Respiration at 25° C.; pH 6.0; glutamate added at time zero; two-cm root tips from 6-day old dark-grown seedlings; open circles, semi-logarithmic; closed circles, linear scale; corrected by subtraction of values in the absence of glutamate (26.8 µl/mg dry weight at 8 hours 5 minutes).

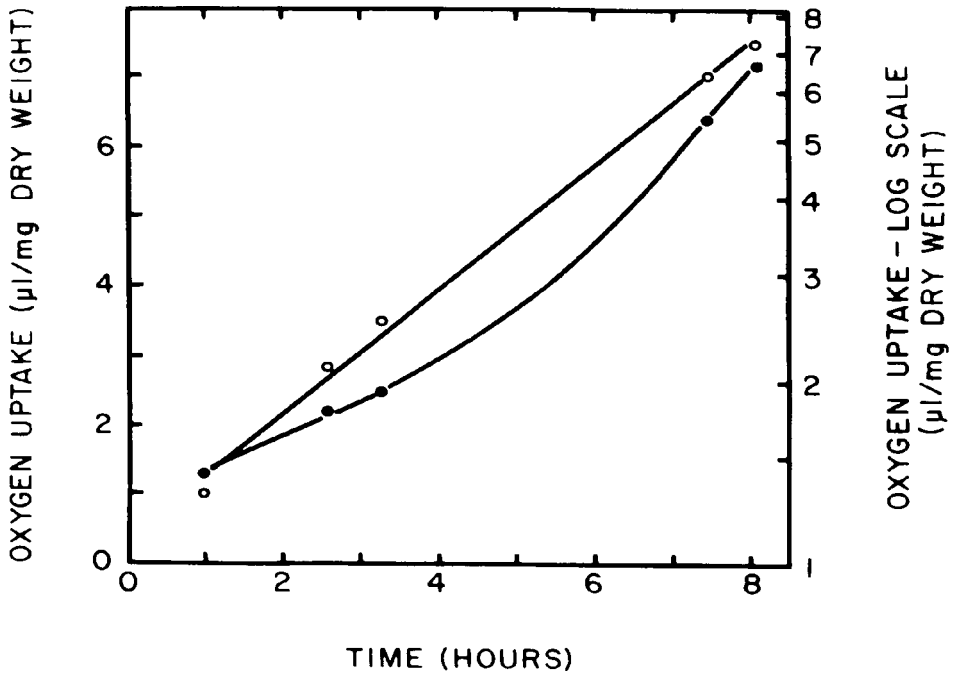

OXYGEN UPTAKE (µl/mg DRY WEIGHT)
0
2
4
6
0
2
4
6
8
TIME (HOURS)
OXYGEN UPTAKE - LOG SCALE
(µl/mg DRY WEIGHT)
1
2
3
4
5
6
7
8

Figure 3

Figure 4. Stimulation of oxygen uptake of excised barley roots by 10^{-3} M L-glutamic acid.

Roots from 6-day old dark-grown seedlings; respiration at 25° C.; glutamate added at time zero; values after 145 minutes in water (clear bar) and in glutamate at pH 6.0 (stippled bar).

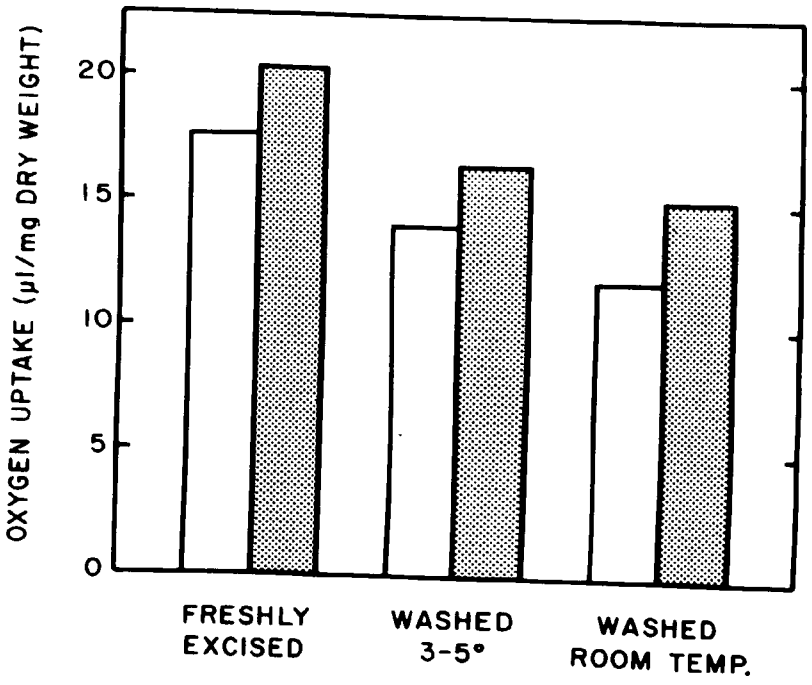

OXYGEN UPTAKE (µl/mg DRY WEIGHT)
20
15
10
5
0
FRESHLY EXCISED
WASHED 3-5°
WASHED ROOM TEMP.

Figure 4

treatment retarded substrate depletion by slowing down respiratory activity. Unlike peas, barley gave no indication of an enhancement of the response to glutamate by washing.

As is shown in Figure 5, tobacco roots metabolize L-glutamic acid when it is presented shortly after excision, the activity increasing markedly with washing for one day at room temperature. On the same graph are the values for the response when the initial pH of the solution is altered one full pH unit above or below the standard value used in these experiments. The titration curve of glutamic acid (cf. Fruton and Simmonds, 1953, p. 93) reveals that there is little change in ionic species over the pH range used. Thus it is more likely that the diminished response at pH 7.0 is due to effects on the activity of an enzyme related to a carrier, or on the internal metabolism, than to changes in the availability of a preferred ionic species.

The respiration of washed tobacco roots in 5×10^{-4} M glutamate is introduced in Figure 6. The Q_{O2} value at this concentration is 2.5, whereas the value at 10^{-3} M is 3.3.

The respiratory quotient for the response to glutamate can be derived from Figure 6. The R.Q. for tobacco roots in water is unity, and the addition of the amino acid produces a net R.Q. of 1.4. Although the respiratory quotient cannot be considered to indicate the nature of the metabolic pathway without equivocation (Umbreit et al., 1957), a value of unity is suggestive that carbohydrate is the principal

Figure 5. Stimulation of the oxygen consumption of tobacco roots by 10^{-3} M L-glutamic acid.

Respiration at 25° C.; freshly excised roots at pH 6.0 (1); roots washed for a day at room temperature, respiration at pH 5.0 (2), 6.0 (3), and 7.0 (4); corrected for endogenous respiration by subtraction of values in the absence of glutamate; correction at 5 hours for freshly excised roots 11.2 µl/mg dry weight, for washed roots 15.2; glutamate added at time zero.

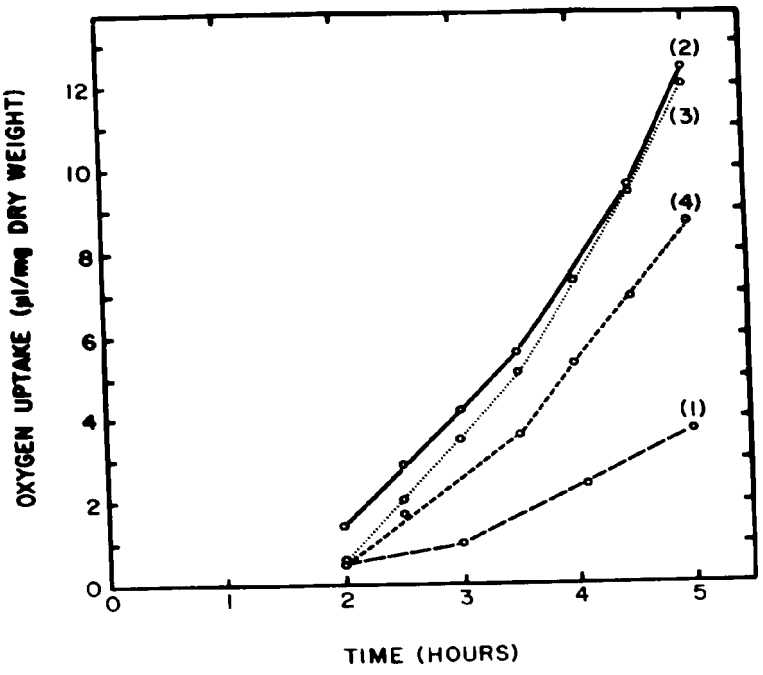

OXYGEN UPTAKE (µl/mg DRY WEIGHT)
0
2
4
6
8
10
12
0
1
2
3
4
5
TIME (HOURS)
(1)
(2)
(3)
(4)

Figure 5

Figure 6. Respiration of washed tobacco roots in 5×10^{-4} M L-glutamic acid.

Respiration at 25° C.; glutamate added at time zero; pH 6.0; corrected for endogenous respiration by subtraction of values in the absence of glutamate; correction at 6 hours for O_2 uptake 18.1 µl/[illegible] weight, for CO_2 evolution 17.7; CO_2 line contains no points because it was derived from the mean difference of slope of two pairs of lines.

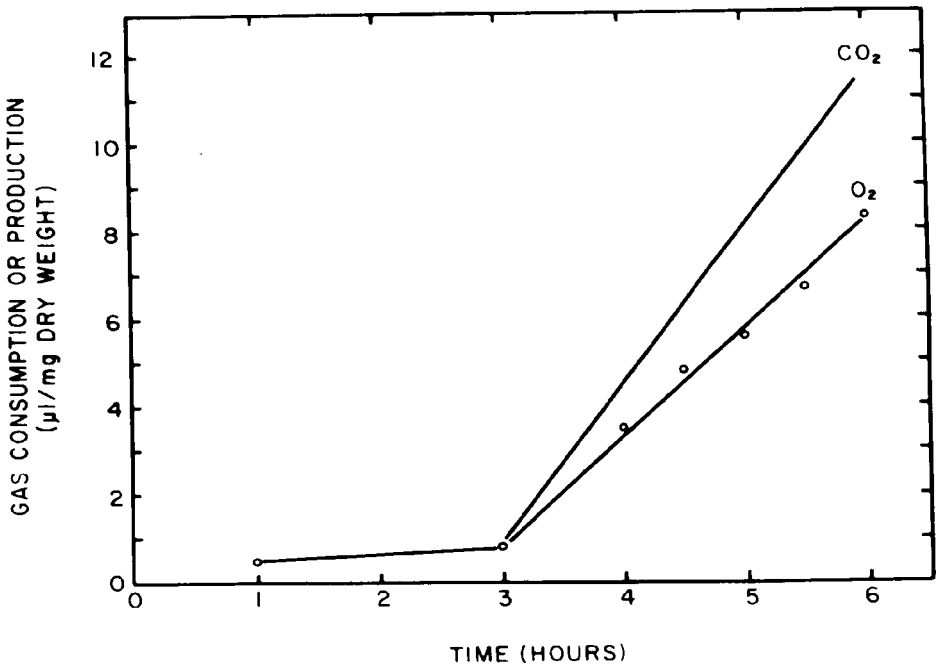

GAS CONSUMPTION OR PRODUCTION
(µl/mg DRY WEIGHT)
0
2
4
6
8
10
12
0
1
2
3
4
5
6
TIME (HOURS)
CO_2
O_2

Figure 6

substrate. The complete oxidation of glutamic acid, calculated as alpha-ketoglutarate, yields a R.Q. of 1.25. If the amino acid is oxidatively deaminated in its conversion to the keto acid instead of being transaminated, the value would be 1.11. The observed value for the net response, then, may indicate that oxidation of the amino acid is not the sole source of the stimulation. Since some plants are known to possess the enzymes necessary for the anaerobic decarboxylation of amino acids (Rogers, 1955), the participation of such a system in producing the high R.Q. should be considered.

Washing with calcium sulfate

Divalent cations are considered to have the capacity of making biological membranes less permeable, presumably by acting as binding agents. In several experiments designed to test the effect of washing in $CaSO_4$ or of presenting this compound to water-washed pea roots no decrease in the reaction to glutamate was observed. Solutions of 10^{-4} M $CaSO_4$ were used in each case.

Adding mineral ions to washed roots

Since washing in distilled water obviously removes ions from the free space of roots, and since ions are known to leak from roots (Lundegårdh, 1959), the possibility that the effects of washing were related to the removal of mineral salts was tested by adding salt solutions with the amino acid to pea roots. The response to glutamate remained

unaffected by the following solutions: Hoagland's solution + micronutrient mixture at concentrations of 0.16 and double that described by Hoagland and Arnon (1938), or 10^{-3} and 5×10^{-3} M KCl. Moreover, none of these solutions changed the endogenous respiration.

Aging the Roots Without an Extensive Wash

In both tobacco and pea there appear from the experiments above that there are changes brought about by subjecting the roots to a day of intensive washing, but the changes do not seem to be related to washing per se. The hypothesis that aging is the significant factor was checked by excising roots and keeping them for a day in a relatively small volume of water before measuring their response to glutamic acid.

Pea roots treated in this manner showed no signs of glutamate utilization. However, the liquid in which the roots were kept became extremely turbid, presumably from the presence of large numbers of bacteria. Bacteria did not become troublesomein the experiments with washed roots because the constant flow of water served to remove both substrates and suspended bacteria.

To circumvent contamination problems an antibiotic, telomycin, was introduced into the container in which the roots were aged. The concentration used, 100 ppm, has been found to have no effect on the growth of cucumber and barley seedling roots (Norman, 1959). The roots were rinsed briefly

to remove excess telomycin before measuring their respiration. Although roots that were exhaustively washed show a shorter lag than those which were not, the respiratory rates of both sets of roots after about five hours of incubation were nearly the same (Figure 7). Regardless of the differences in the two responses, these data support the idea that aging participates in the induction of the increased response of roots to glutamic acid.

In a discussion of the effects of aging on the metabolism of sections of fleshy roots and tubers, Laties (1957) suggested that the differences in respiration of fresh and aged sections could be due to starvation and its effects on substrate availability or to the demise of one respiratory system leaving a second system in operation. Although equating the physiological responses of fibrous roots and storage organs may not always be proper, Laties' conclusion seems particularly apt in regard to the present discussion because it applies to Avena coleoptiles as well and since in both types of tissue it was the failure of Krebs cycle acids to elicit respiratory responses from freshly excised sections that led to the explanation. One would expect the stimulation of respiration by glutamic acid to result, at least in part, from its conversion to alpha-ketoglutarate, the carbon skeleton of which would be degraded through action of the Krebs cycle.

Figure 7. Stimulation of the oxygen consumption by L-glutamic acid of pea roots aged without a thorough wash.

Respiration at 25° C.; two-cm root tips from 6-day old dark-grown seedlings; Roots (1) aged for a day after excision in 100 ppm telomycin or (2) washed for a day after excision in running distilled water (from Figure 3); (1) corrected by subtraction of respiration in the absence of glutamate (23.6 µl/mg dry weight at 6 hours).

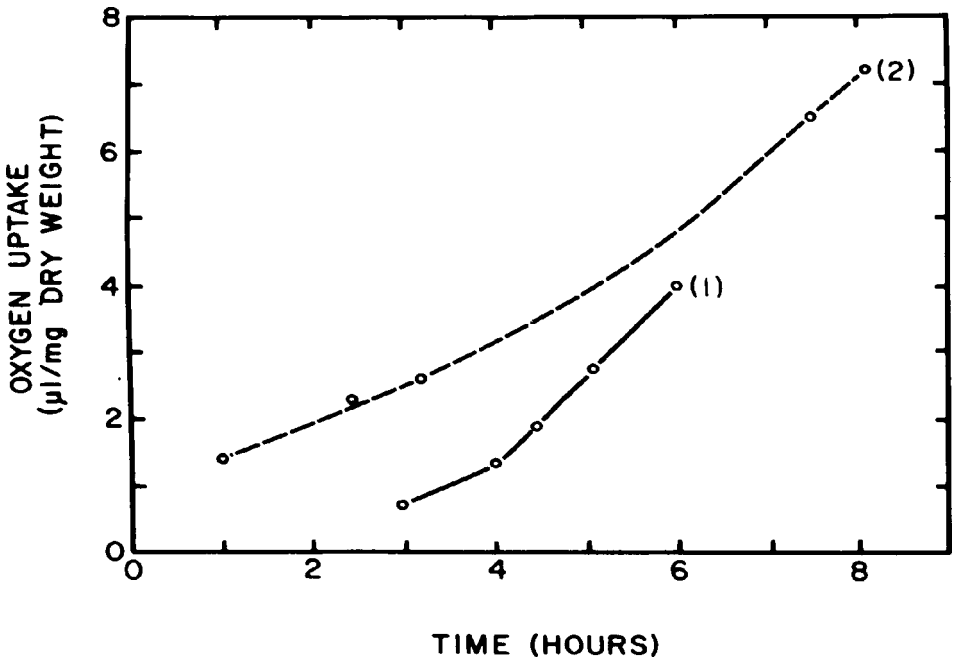


Figure 7

Respiratory Stimulation by D-Glutamic Acid

It is of interest to determine the response of roots to the "unnatural" optical isomer of glutamic acid. Presumably this isomer cannot participate directly in protein synthesis since no proteins have been found containing demonstrable amounts of amino acids with the dextro configuration. As described in the review of the literature above, the roots of some higher plants exhibit no strong preference between the stereoisomers of amino acids while others either fail to utilize the D-enantiomorph or are adversely affected by it.

The washed roots of pea, barley and tobacco each respond to the unnatural isomer of glutamic acid, but the responses of pea and tobacco are not as vigorous as those to the L-enantiomorph (Figures 8 and 9). When compared with the data of Figure 2, the curve for pea with the D isomer shows a longer lag period, but the Q_{O2} from the seventh to eighth hours is about 1.6 µl/mg/hr for D-glutamic acid and about 1.2 for its enantiomorph. Tobacco roots, on the other hand, exhibit a much stronger preference for the laevo form, the Q_{O2} for the L and D-isomers respectively being 3.4 and 0.4.

The reduced responses in both cases may be explained by a slower rate of entry of the D-enantiomorph. In peas, however, the eventual approximation of the respiratory rate in response to the D and L forms suggests that either the rate of entry is limiting only during the early stages or

Figure 8. Stimulation of oxygen uptake of washed pea and tobacco roots by 10^{-3} M D-glutamic acid.

Respiration at 25° C.; pH 6.0; glutamate added at time zero; corrected by subtraction of values in the absence of glutamate : 20.3 µl/mg dry weight for pea at 7 hours, 30.2 for tobacco at 9 hours.

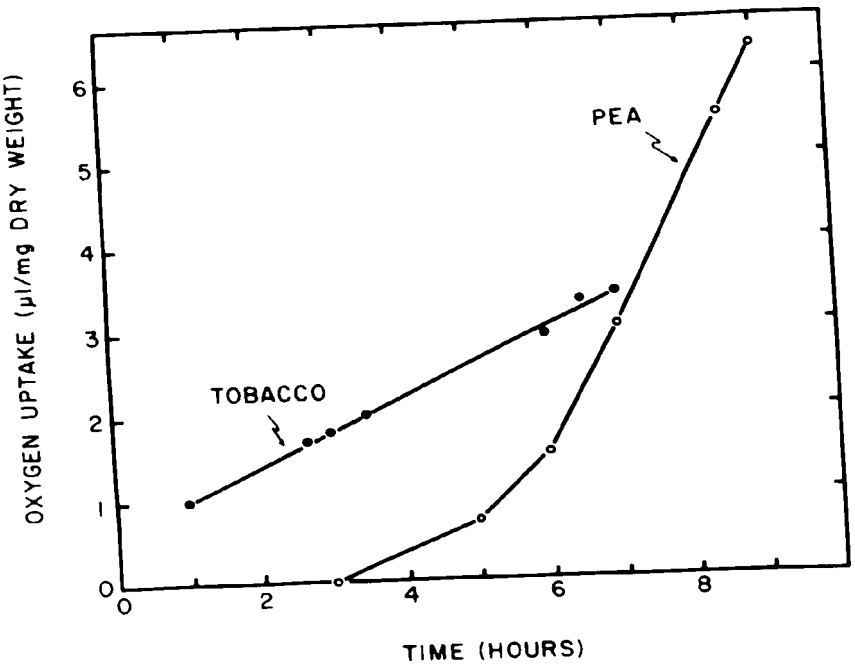

OXYGEN UPTAKE (µl/mg DRY WEIGHT)
0
1
2
3
4
5
6
0
2
4
6
8
TIME (HOURS)
PEA
TOBACCO

Figure 8

Figure 9. Respiratory stimulation of washed barley roots by 10^{-3} M D-glutamic acid.

Respiration at 25° C.; pH 6.0; roots excised from 6-day old dark-grown seedlings; corrected for endogenous respiration by subtraction of values in the absence of glutamate; correction 7.0 µl/mg dry weight at 1.6 hours; glutamate added at time zero.

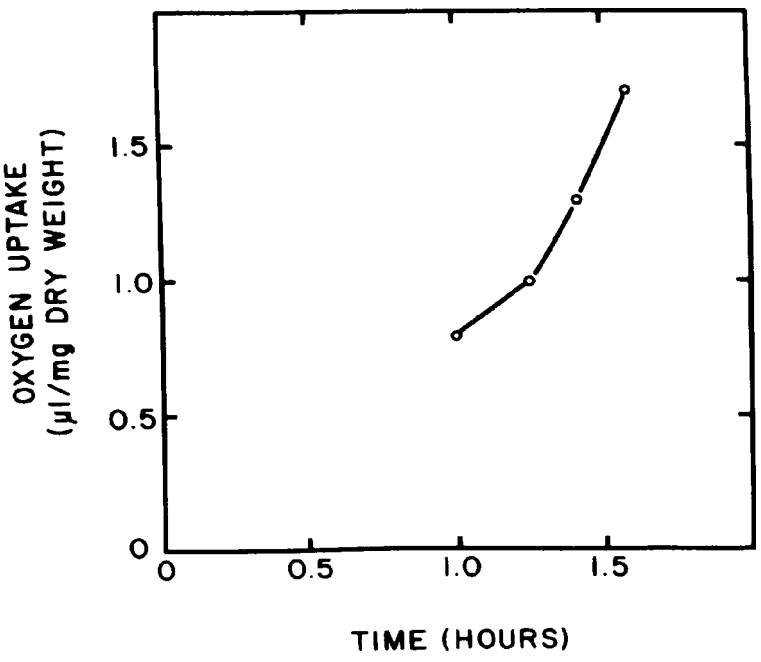


Figure 9

that the rate of utilization of the D-enantiomorph increases with time, reaching a maximum near that for the laevo form. Dependence of the rate of respiratory response on the maximum rate of utilization attainable in the tissue is equally plausible.

Respiratory Stimulation by L-Glutamine

Because of their structural similarity, comparison of the responses to glutamine and glutamic acid was essayed. At equal concentrations the glutamine brings about a greater rate of oxygen uptake (Figure 10). Since the energy content of the amide is the greater, its enhanced ability to stimulate respiration is not surprising. However, the possibility that it is nitrogen that is being supplied instead of carbon must not be overlooked. In that event, the greater nitrogen content of the amide would be the reason for its enhanced ability to evoke the response.

Pyridoxal and the Response to L-Glutamic Acid

Christensen (1959) discussed the stimulation of amino acid uptake by animal cells with pyridoxal. He postulated that amino acids may enter the cells in combination with the vitamin. Figure 11 shows a stimulation of the respiration of pea roots by pyridoxal alone. The addition of glutamate does not result in additional stimulation. Directly or otherwise, then, pyridoxal at 6×10^{-3} M is inhibitory to the respiratory response to glutamate. Christensen _et al._ (1954)

Figure 10. Stimulation of oxygen consumption of washed pea roots by 10^{-3} M L-glutamine.

Respiration at 25° C.; two-cm root tips from 6-day old dark-grown seedlings; washed for a day after excision in distilled water; (1) L-glutamine, (2) L-glutamate (from Figure 3); (1) corrected for respiration in the absence of glutamine (13.3 µl/mg dry weight at 4.5 hours).

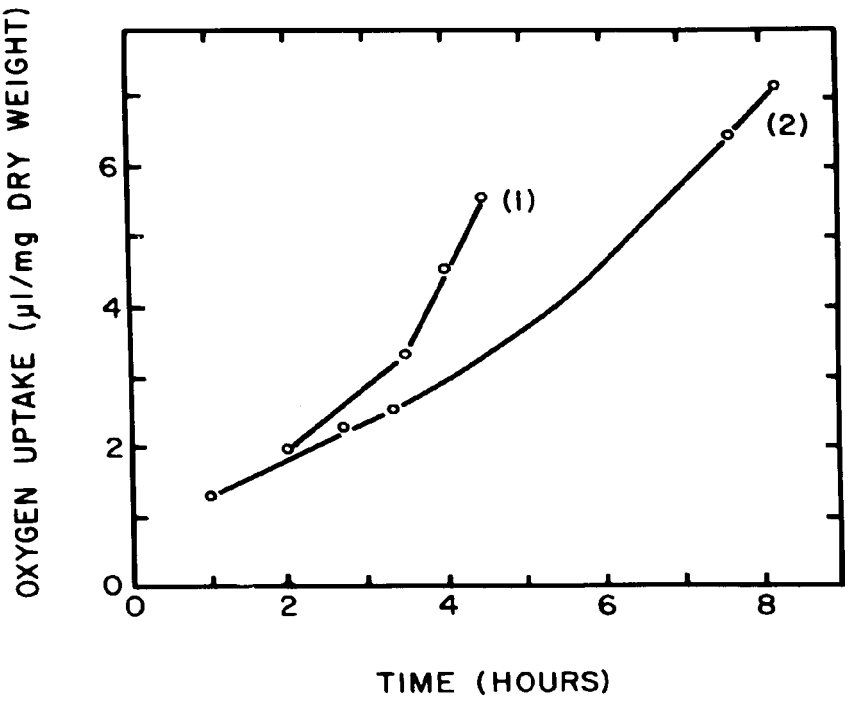


Figure 10

Figure 11. Effect of pyridoxal on the respiration of washed pea roots in the presence and absence of glutamic acid.

Respiration at 25° C.; pH 6.0; glutamate added at time zero; in water (●), in 6 x 10^{-4} M pyridoxal (X), in 6 x 10^{-4} M pyridoxal plus 10^{-3} M L-glutamic acid (O); correlation coefficient of the net stimulation by pyridoxal 0.988 $\pm$ 0.847 at the 95% level.

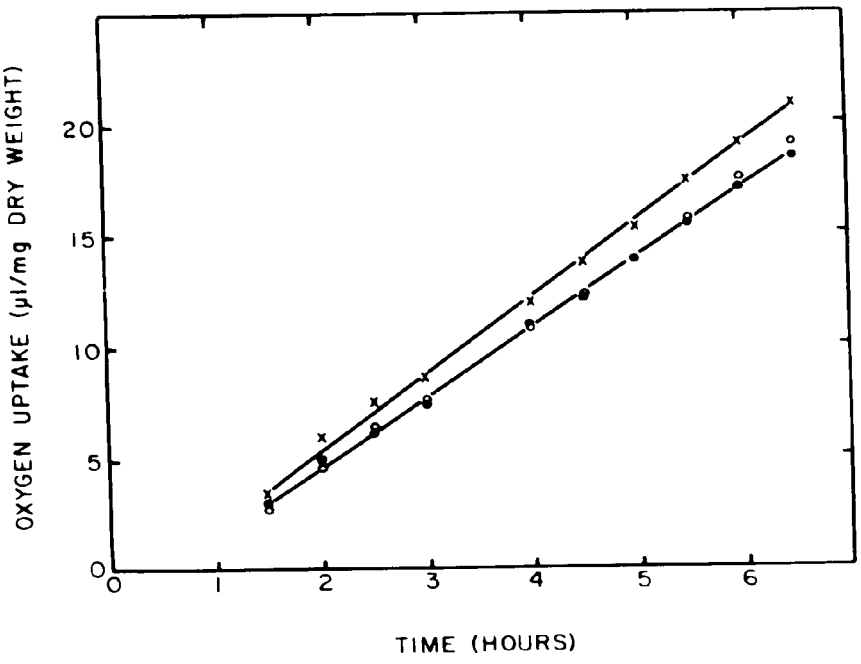


Figure 11

found the opposite effect: the same concentration of pyridoxal used here stimulated glycine uptake by tumor cells but repressed respiration somewhat.

This surprising result is not inconsistent with the possibility that by stimulating the activity of a pyridoxal-requiring enzyme system the vitamin prevents the glutamate from being involved in the reactions that produce the respiratory response. Such an enzyme system could conceivably occur either on the cell surface or inside.

If one approaches these results from the opposite direction, the conclusion would be that the amino acid inhibits the reaction of the cells to pyridoxal. The entry of both compounds in combination, i.e., as a Schiff's base, would be expected to lead to the amination of endogenous keto-acids, thus returning the vitamin to the aldehydic form, which presumably is the molecular species responsible for the increase in oxygen uptake when pyridoxal is presented alone.

If the amino acid and the vitamin combined at the cell surface and for some reason could not penetrate as the combined form, neither moiety of the complex could reach the sites at which each produces respiratory stimulation alone. The results also indicate by the lack of a response that the combination in some manner blocks the entry of the free molecules of each compound. Therefore, a second, and perhaps more likely, explanation of these data would require the combination of both glutamate and pyridoxal at the site at

which each would be able to combine individually with a carrier. The amino acid-vitamin-carrier complex must either be too stable to allow release of the metabolites into the interior of the cell or be unable to traverse the membrane.

Since the experiment was conducted with solutions containing only the two reagents plus sodium ions added in titration, a vital mineral ion may be lacking at the cell surface. The participation of pyridoxal phosphate in transamination (Fruton and Simmonds, 1953) may indicate the need for phosphate or for the presentation of pyridoxal phosphate for this reaction to proceed.

On the other hand, Christensen and coworkers (1954) found stimulation of amino acid uptake by pyridoxal itself in the absence of phosphate. Pal and Christensen (1959) found evidence for the entry of manganese ions along with lysine and pyridoxal, with either the metal or the amino acid apparently entering in combination with the vitamin. These data were derived from studies with animal cells, however, and attempts by the present author to find other bases for comparison between the root and erythrocyte or tumor cells with respect to amino acid uptake failed. Specifically, the efflux of potassium concurrent with the influx of amino acid into the animal cells mentioned (Christensen, 1959) did not occur when the roots of intact pea plants were presented with glutamate or aspartate, and the total uptake of potassium in the presence of glutamate exceeded that in the absence of glutamate.

Alpha-Aminoisobutyric Acid and Its Effect on the Response of Roots to Glutamic Acid

In his investigation of the uptake of amino acids by animal tissues, Christensen (1959) used a non-metabolizable compound, alpha-aminoisobutyric acid, to dissociate the results of transport from those involving further utilization of the transported molecules. Using Jerusalem artichoke explants, Nitsch and Nitsch (1957) found that this compound not only failed as a nitrogen source but exhibited toxicity in the presence of nitrate.

Aminoisobutyric acid proved to be strongly inhibitory when presented to washed pea roots (Figure 12). Glutamate in equal concentration nearly completely relieved the inhibition, producing a stimulation which, in the later stages of the experiment, approximated that produced by glutamate alone. With tobacco roots, however, the inhibition was less striking (Figure 13), and the inhibition of the basal respiration was of only slightly less magnitude than that of the response to glutamate. The effects of this compound on both species was one of lengthening the lag without decreasing the ultimate rate of the reaction.

The original hope in employing this compound was to find respiratory stimulation which could be attributed to active uptake without the interference of further degradation of the amino acid. No such evidence was found. The data do support the view that the aminoisobutyrate enters, in

Figure 12. Effect of alpha-aminoisobutyric acid on the oxygen consumption of washed pea roots in the presence and absence of L-glutamic acid.

Respiration at 25° C.; both amino acids at 10^{-3} M, pH 6.0; amino acids added at time zero; curves corrected for endogenous respiration by subtraction of values in the absence of the amino acids (32.5 µl/mg dry weight at 8.5 hours).

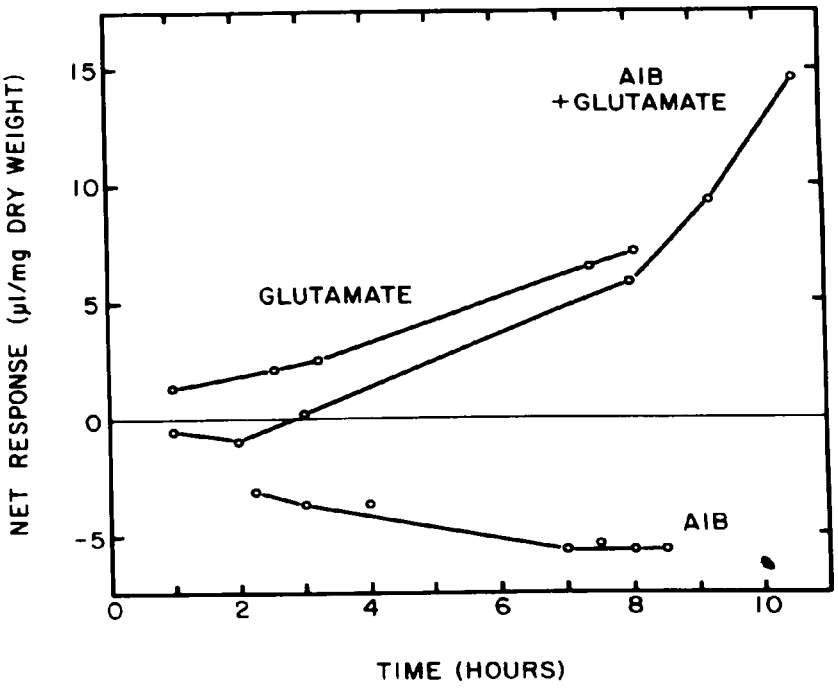

NET RESPONSE (µl/mg DRY WEIGHT)
15
10
5
0
-5
0
2
4
6
8
10
TIME (HOURS)
AIB
+GLUTAMATE
GLUTAMATE
AIB

Figure 12

Figure 13. Effect of alpha-aminoisobutyric acid on the oxygen consumption of washed tobacco roots in the presence and absence of L-glutamic acid.

Respiration at 25° C.; both amino acids at 10^{-3} M, pH 6.0; amino acids added at time zero; curves corrected for endogenous respiration by subtraction of values in the absence of the amino acids (17.2 µl/mg dry weight at 5 hours).

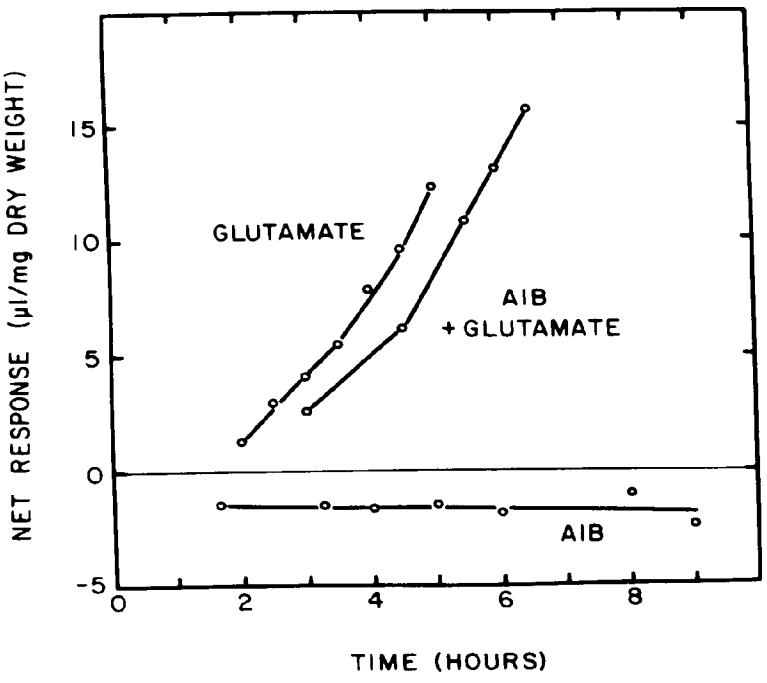

NET RESPONSE (µl/mg DRY WEIGHT)
15
10
5
0
-5
0
2
4
6
8
TIME (HOURS)
GLUTAMATE
AIB
+ GLUTAMATE
AIB

Figure 13

that there is a strong inhibition of the basal respiration. The reversal of the inhibition by glutamate suggests that the two compounds may compete for an enzyme or carrier, the glutamate being the stronger competitor.

The Effect of Inhibitors on the Respiratory Response of Roots to L-Glutamic Acid

In an effort to relate the respiratory response of roots in the presence of amino acids to the uptake of these compounds, the effects of respiratory inhibitors on both the respiratory response itself and the uptake of glutamic acid was compared. Data on the uptake will be presented in a later section.

2,4-Dinitrophenol

Dinitrophenol exerts its action upon the respiration of certain cells by uncoupling respiration from phosphorylation (Laties, 1957). In so doing, the compound deprives the cell of its principal agent of energy transfer, adenosine triphosphate. Laties further describes a possible involvement of adenosine triphosphate in transport through the formation of a phosphorylated metabolic intermediate which combines with the transported ion at the outer surface of the bounding membrane. The phosphorylated compound would be the carrier.

The concentration of 2,4-dinitrophenol used in the present experiments, 5×10^{-5} M, was chosen primarily on the

basis of data presented by Russell (1954) which show this concentration to be strongly inhibitory to the uptake of phosphate by barley plants.

Inhibition of the response of washed pea roots to glutamate is shown in Figure 14. Note that there was an increased lag with little effect, if any, on the ultimate rate of the reaction. The corresponding data for tobacco are illustrated in Figure 15, the effect being comparable to that on pea.

Sodium Azide

James (1953) discussed the action of sodium azide on respiration under two categories, namely, the complexing of transition metals and the uncoupling of phosphorylation. The metal complexes it forms are similar to those with hydrogen cyanide but less stable. In its effects on phosphorylation azide resembles dinitrophenol. Prevention of the formation of adenosine triphosphate during triosephosphate oxidation is presumed. The action of azide is considered to depend on its ability to form hydrazoic acid and is therefore not independent of pH, acidity favoring increased inhibition. In the present investigation it was considered advisable to use only one pH, 6.0, in all of the experiments in order to avoid direct effects of hydrogen ion concentration on the uptake of glutamate.

The concentration of azide used here was 10^{-3} M, the level at which Russell (1954) found roughly 60% inhibition

Figure 14. Effects of inhibitors on the respiratory response of washed pea roots to 10^{-3} M L-glutamic acid at an initial pH of 6.0.

Respiration at 25° C.; roots pretreated approximately 90 minutes in inhibitors before addition of glutamate; glutamate added at time zero; (1) in the absence of inhibitors, (2) in 10^{-3} M NaN_3, (3) in 5×10^{-5} M 2,4-dinitrophenol; corrected for values in the absence of glutamate by subtraction: (1) 26.8 µl/mg dry weight at 8 hours 5 minutes, (2) 19.3 at 8 hours, (3) 24.8 at 7.5 hours.

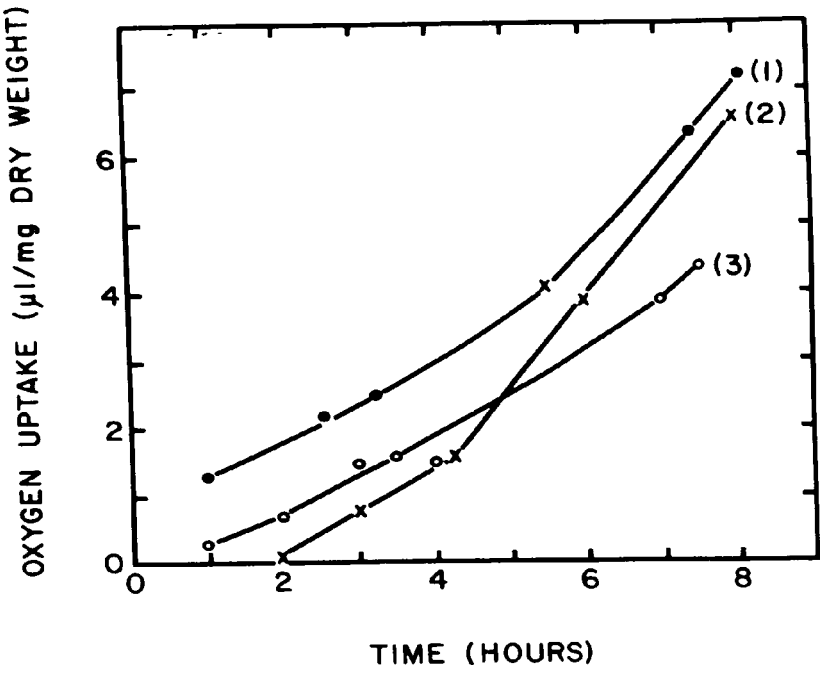


Figure 14

Figure 15. Effects of inhibitors on the respiratory response of washed tobacco roots at 10^{-3} M L-glutamic acid at an initial pH of 6.0.

Respiration at 25° C.; roots pretreated approximately 90 minutes in inhibitors before addition of glutamate; glutamate added at time zero; (1) in the absence of inhibitors, (2) in 10^{-3} M NaN_3, (3) in 5×10^{-5} M, 2,4-dinitrophenol, (4) in 10^{-3} M benzimidazole; corrected for values in the absence of glutamate by subtraction: (1) 15.2 µl/mg dry weight at 5 hours, (2) 19.2 at 6 hours, (3) 36.0 at 6.5 hours, (4) 18.8 at 7 hours.

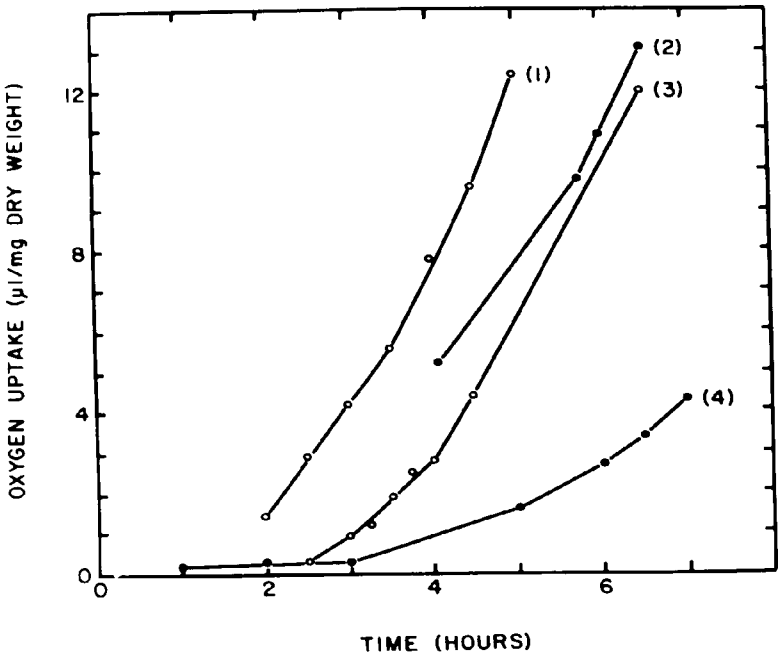


Figure 15

of the uptake of phosphate by roots and about 90% inhibition of the transfer of phosphate to the shoot in barley.

The initial action of azide on the response of pea roots to glutamate (Figure 14) seems to be overcome after several hours, the stimulation increasing to as great a rate as that by glutamate alone between four and eight hours after the addition of the amino acid.

Tobacco roots show a close parallel in the effects of azide and dinitrophenol (Figure 15).

Benzimidazole

Following the discovery in this laboratory that benzimidazole stimulates the uptake of potassium by excised barley roots (Klingensmith, 1959; Klingensmith and Norman, 1960) this compound was tried at the concentration at which the maximum stimulation of uptake was found, 10^{-3} M. Instead of stimulation, strong inhibition of the respiratory response of tobacco roots to glutamate was found (Figure 15).

That benzimidazole can affect both respiration and amino acid-related metabolism is known from the studies of Person et al. (1957) which revealed the ability of benzimidazole to prevent the rise in respiratory rate normally associated with the detachment of wheat leaves and to produce decreases in the rate of protein degradation.

Strophanthin-k

Cardiac glycosides are potent inhibitors which are used in small doses as stimulators of the mammalian heart muscle. Investigations of their mode of action on several types of animal cells show that they inhibit ion transport without inhibiting respiration (Schatzmann, 1953; Post and Jolly, 1957). The advantages of using so specific an inhibitor are obvious.

The addition of strophanthin-k to washed pea roots has no inhibitory effect on their response to L-glutamate, as is shown in Figure 16. The inhibitor, which is actually a mixture of glycosides (Stecher, 1960), cannot be considered to prevent the membrane transport of glutamate under these conditions. The apparent trend toward stimulation after the first four hours of incubation requires further investigation.

A recent report by Paine and Heinz (1960) has shown that ouabain, another cardiac glycoside, fails to inhibit the transport of glycine into tumor cells at the concentration used here but is effective at concentrations of 0.01 mM (ca. 7.3 mg/l) or higher.

The Effects of Inhibitors on the Endogenous Respiration of Excised Pea and Tobacco Roots

With the establishment of the ability of some inhibitors to delay the respiratory response of roots to glutamic acid the question arose as to whether the site of their action is the uptake mechanism *per se*, the systems which degrade the

Figure 16. Effect of strophanthin-k, 5 mg/l, on the stimulation of respiration of washed pea roots by 10^{-3} M L-glutamic acid at pH 6.0.

Respiration at 25° C.; two-cm root tips from 6-day old dark-grown seedlings; glutamate curve from Figure 3; glutamate + strophanthin curve corrected for values in the absence of glutamate (30.8 µl/mg dry weight at 8 hours).

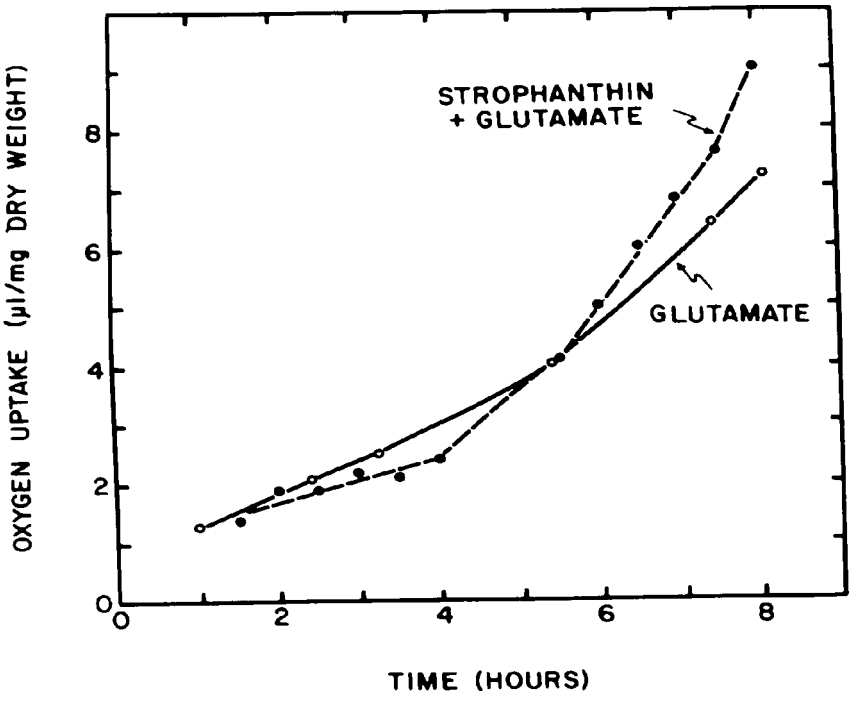

STROPHANTHIN
+ GLUTAMATE
GLUTAMATE
OXYGEN UPTAKE (µl/mg DRY WEIGHT)
TIME (HOURS)
0
2
4
6
8

Figure 16

already accumulated amino acid, or the general respiratory system. A useful approach to the answer is to compare the effects of the inhibitors in the absence of glutamate with those in its presence.

The respiration rates for washed roots of tobacco under various treatments are given in Table 4. Comparable data for pea roots were too variable from one experiment to the next to be analyzed in the same fashion. Dinitrophenol stimulated the consumption of oxygen by tobacco roots, but sodium azide and benzimidazole had no effect.

Laties (1957) discussed the mode of action of dinitrophenol in detail, attributing its effects to its ability to prevent the regeneration of high energy phosphate bonds during electron transport. The respiration rate, he stated, is dependent on both the supply of phosphate acceptors and the presence of high energy phosphate donors. An uncoupler of phosphorylation, such as dinitrophenol, will exert an influence over the rate of respiration only when the turnover of phosphate is limiting. When such is the case, the addition of the inhibitor will increase the concentration of phosphate acceptors, thus increasing the rate of oxygen uptake. The action under these conditions is one of inhibition of energy transfer and <u>not</u> of the consumption of oxygen.

Shacter (1955) investigated the changes in oxygen consumption by mouse tumor cells elicited by various concentrations of dinitrophenol. He found stimulation at levels ranging from 10^{-5} to slightly less than 2×10^{-4} M, with higher

TABLE 4

EFFECTS OF INHIBITORS ON THE ENDOGENOUS RESPIRATION OF WASHED TOBACCO ROOTS

Excised roots washed for a day in running distilled water; 25° C.; inhibitors titrated to pH 6.0 with NaOH, no buffer.

Treatment	Q_{O2}	Standard Error
NONE	3.0	±0.24
5×10^{-5} M DNP[a]	5.1	±0.44
10^{-3} M NaN_3	3.2	±0.34
10^{-3} M benzimidazole	2.8	±0.39

[a] 2,4-dinitrophenol

concentrations producing strong inhibition. The stimulation of tobacco root respiration reported here may be attributed to a lower sensitivity or to a relatively low internal concentration of the inhibitor.

Let us focus our attention on the matter of the internal concentration of dinitrophenol. James (1953a) and Stenlid (1949) point out that the penetration of weakly acidic inhibitors is highly dependent on pH. Dinitrophenol was shown by Stenlid to inhibit oxygen consumption by carrot leaves at the pH of 5 or lower and to stimulate it in the range of 6.7 to 7.0. He attributed this effect to the greater ability of the undissociated molecule of dinitrophenol to penetrate, the cell membranes being almost impermeable to polar molecules.

Although the present experiments were run at pH 6.0, there was no buffer included in the milieu. Consequently, changes in pH would be expected. The changes in pH observed during incubation of tobacco and pea roots are given in Table 5. There was little change in the hydrogen ion concentration in the presence of pea roots, but tobacco roots in each case caused the pH of the medium to shift to 8.0. The explanation of this phenomenon may lie in the report by Dawson (1942) that cultured tobacco roots released nicotine into the surrounding medium. Perhaps the loss of this alkaline compound causes the observed rise in pH. Regardless of the source of the increased alkalinity, the change most certainly

TABLE 5

CHANGES IN pH DURING THE INCUBATION OF WASHED ROOTS

Species	Hours of Incubation	Glutamate Content	Original pH	Final pH
Pea	6	10^{-3} M	6.00	6.06[a]
Tobacco	5	none	5.11	8.06[b]
	5	10^{-3} M	5.00	8.09[b]
	5	10^{-3} M	6.00	7.80[b]
	5	10^{-3} M	7.00	8.01[b]

[a]Mean of 5 samples

[b]Mean of 3 samples

must have had an effect on the ability of the inhibitor to penetrate.

Thus we have reason to believe that the penetration of dinitrophenol into the pea roots used was slow and that its entry into the tobacco roots was even more limited. One would expect, however, that entry of the inhibitor occurred during the lapse of time between putting the roots into the Warburg flasks and the onset of the experiment proper. The results bear out this thought since there is no sign of increasing inhibition of the response to glutamate as would be expected if the internal concentration of the inhibitor were being built up (Figures 14 and 15)

Many of the same remarks concerning the effects of dinitrophenol could be repeated for azide, the penetration of which is also highly dependent on pH (Stenlid, 1949; James, 1953a). Azide produced no inhibition of the endogenous respiration of tobacco roots (Table 4). This does not parallel the effects of azide on the respiratory enhancement produced by glutamic acid (Figure 15). The failure of azide to change the endogenous respiration while decreasing the rate at which the roots respond to an exogenous substrate may possibly be an indication of action of the inhibitor at the cell surface.

Benzimidazole proved to be the most potent inhibitor of the response of tobacco roots to glutamic acid (Figure 15) while exhibiting no effect on the endogenous respiration

(Table 4). This will be discussed later. Its failure to change the respiratory rate when presented alone is in accord with the results of Klingensmith (1959) and Hillman (1955) who showed the same to be true for corn root tips and Lemna plants, respectively.

Uptake of L-Glutamic Acid by Excised Roots

A compound cannot act as a nitrogen source for a plant unless it or its nitrogenous moiety can enter the cells of the plant. Entry of the compound, however, is not the only condition to be satisfied. Rather, one must also consider the rate at which the nitrogen source enters in relation to the needs of the whole plant, the presence of the appropriate metabolic systems to convert the nitrogen to the necessary chemical forms, and the existence of mechanisms to translocate these compounds from the roots to other parts of the plant. Possible toxicity of the nitrogen source in high concentrations must not be overlooked. Ammonia is inhibitory in high concentrations, yet in lower concentrations is the preferred nitrogen source for many plants.

The experiments described in this section concern the removal of glutamic acid from the medium by roots. The methods employed yield no information about the metabolic fate of the entering molecules.

Comparison of Glutamate Uptake by Washed and Freshly Excised Roots

The stimulation of respiration elicited by washing excised roots in distilled water for a day was shown (Figure 7) to be produced to nearly the same degree by merely allowing the excised roots to age. The question arises as to whether the effect is one on the respiratory mechanism, on uptake, or on both.

Freshly excised pea roots and those which had been washed for about 24 hours in distilled water were put into aliquots of the same solution containing 5×10^{-4} M L-glutamic acid and uniformly labeled C^{14}-L-glutamate. As can be seen in Figure 17, uptake of the amino acid by the washed roots greatly exceeded that by the freshly excised group. Similarly obtained data for tobacco roots, in Figure 18, show the same kind of response.

Comparison of these graphs with the corresponding ones for the respiratory response (Figures 1 and 2 for pea; 5 and 6 for tobacco) reveals two interesting parallels. For both plants the enhanced respiratory rate is coupled with increased uptake of the amino acid, and there is a suggestion of similarity in the shapes of the uptake curves and their respiratory counterparts. It should be pointed out that the concentration of glutamate in the uptake experiments was half that in these respiratory assays with the exception of the experiment plotted in figure 6, in which it was the same.

Figure 17. Effect of washing on the uptake of L-glutamic acid by excised pea roots.

Original concentration 5 x 10^{-4} M; labeled with L-glutamate-U-C^{14}; pH 6.0; roots of 14-day old dark-grown seedlings; freshly excised roots (O), roots washed for a day in distilled water (●); 95% confidence limits: freshly excised at 5 hours 47 min. 9.00 $\pm$ 0.04, washed at 6 hours 22.17 $\pm$ 7.31.

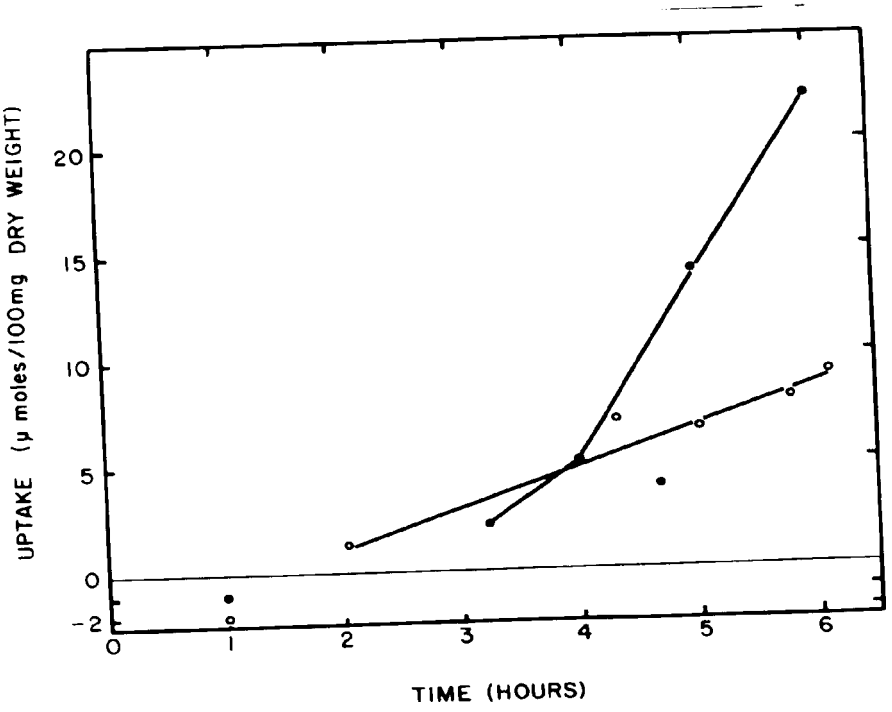

UPTAKE (μ moles/100mg DRY WEIGHT)
20
15
10
5
0
-2
0
1
2
3
4
5
6
TIME (HOURS)

Figure 17

Figure 18. Effect of washing on the uptake of L-glutamic acid by excised tobacco roots.

Original concentration 5×10^{-4} M; labeled with L-glutamate-U-C^{14}; pH 6.0; freshly excised roots (●), roots washed for a day in distilled water at room temperature (X), roots aged for a day in a small volume of 100 ppm telomycin (O); 95% confidence limits at 5 hours: freshly excised 3.67 $\pm$ 1.81, washed 8.69 $\pm$ 2.01, telomycin 9.32 $\pm$ 0.82; at 4.5 hours: freshly excised 5.66 $\pm$ 2.63.

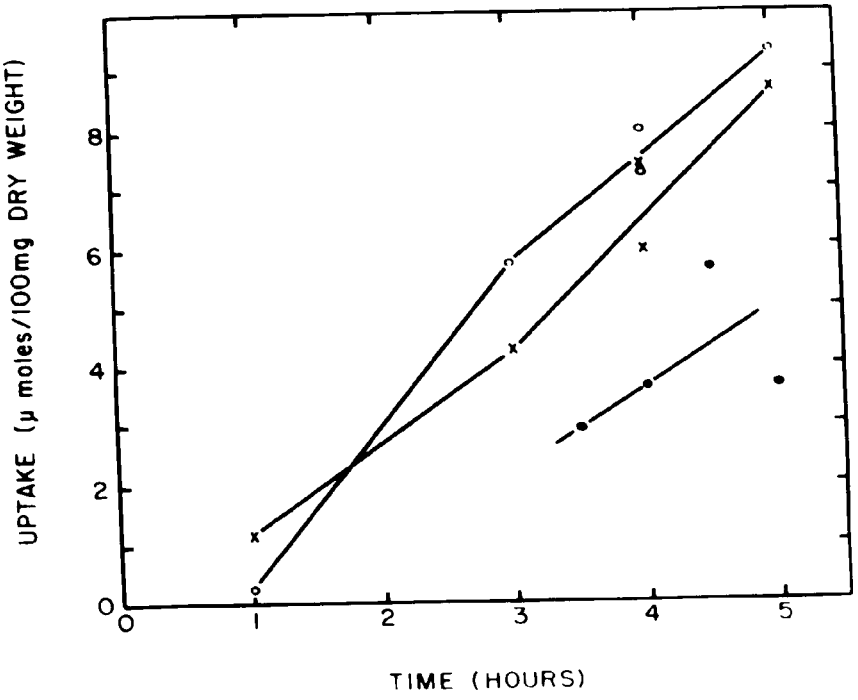


Figure 18

Comparison of Glutamate Uptake by Washed and Unwashed, Aged Roots

Recorded in an earlier section (Figure 7) is an attempt to distinguish between washing and aging in their effects on the respiratory response of roots. With the same endin mind the uptake of glutamate by tobacco roots, one set washed in running, aerated distilled water at room temperature, another allowed to incubate in a small volume of 100 ppm telomycin, was measured. The results, illustrated in Figure 18, lead to the conclusion that there is no difference in the abilities of the two sets of roots to absorb glutamic acid. This is in accord with the earlier conclusion based on respirometric data.

Effects of Inhibitors on the Uptake of Glutamic Acid by Excised Roots

The inhibition of the respiratory response of roots to glutamic acid by a variety of inhibitors suggests that they may act by blocking the uptake of the amino acid, so their effect on uptake was studied.

In Figure 19 are presented the data for the uptake of glutamate by pea roots which have been washed in distilled water for a day. Those to which dinitrophenol or azide were added seem to absorb the amino acid at about the same rate as the untreated roots during the first 4 hours. Thereafter, however, the strong increase characterizing the untreated roots is not evident in the behavior of the treated samples.

Figure 19. Effect of respiratory inhibitors on the uptake of L-glutamic acid by excised pea roots.

Original concentration of glutamate 5×10^{-4} M; labeled with L-glutamate-U-C^{14}; pH 6.0; roots of 14-day old dark-grown seedlings; no inhibitor (●), 5×10^{-5} M 2,4-dinitrophenol (X), 10^{-3} N NaN_3 (O); 95% confidence limits: no inhibitor at 6 hours 22.17 ± 7.31, dinitrophenol at 5 hours 5.58 ± 1.09, azide at 5 hours 14 min. 0.43 ± 8.5.

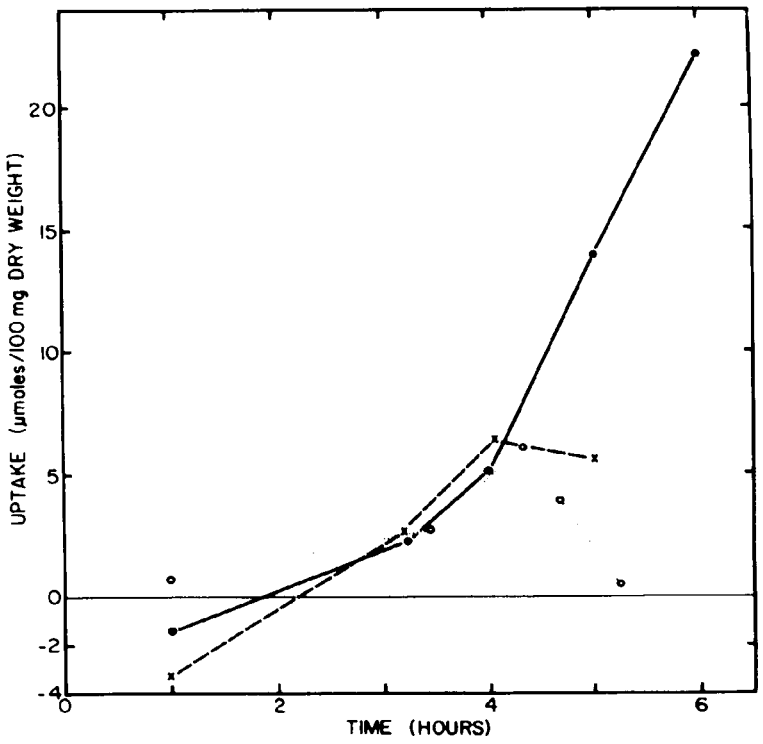


Figure 19

This effect is unlike that on the respiratory response which shows inhibition at the early stages followed by apparent recovery (Figure 14).

Tobacco roots behave differently under the same conditions. Figure 20 provides little or no indication of inhibition of uptake by the concentration of dinitrophenol, 5×10^{-5} M, which increased the lag period of the respiratory response (Figure 15). At 10^{-3} M there is no sign of an increase in uptake between two and five hours. The results of azide treatment provide difficulties in interpretation. The one point showing strong uptake seems to be aberrant. If that point is ignored, the data can be construed as indicating complete cessation of absorption of glutamate after the second hour.

The literature contains an abundance of examples in which respiratory inhibitors block the uptake of both organic and inorganic compounds. Cohen and Monod (1957) mention the ability of dinitrophenol and of azide to inhibit the uptake of galactosides, glucuronides, and amino acids by Escherichia coli. Cyanide, azide, and dinitrophenol at 10^{-3} M each inhibited the uptake of glutamate into bean hypocotyl sections and slices of carrot root and potato tuber to a greater extent than anaerobiosis (Webster, 1954b). Birt and Hird (1958b) found found that the uptake of L-leucine by carrot root disks was repressed within the first half hour by 1.0 mM cyanide, 20 mM fluoride, or 5 mM iodoacetate. The absorption was unaffected

Figure 20. Effect of respiratory inhibitors on the uptake of L-glutamic acid by tobacco roots.

Original concentration of glutamate 5×10^{-4} M; labeled with L-glutamate-U-C^{14}; pH 6.0; roots washed for a day in distilled water; no inhibitor (●), 5×10^{-5} M 2,4-dinitrophenol (●), 10^{-3} M dinitrophenol, (○), 10^{-3} M NaN_3 (X); 95% confidence limits: without inhibitor at 5 hours 8.69 ± 2.01, 5×10^{-4} M dinitrophenol at 5 hours 9.80 ± 0.62, 10^{-3} M dinitrophenol at 5 hours 1.35 ± 0.17, NaN_3 at 5 hours 15 min. 7.56 ± 6.16 and at 5 hours 25 min. 2.28 ± 2.33.

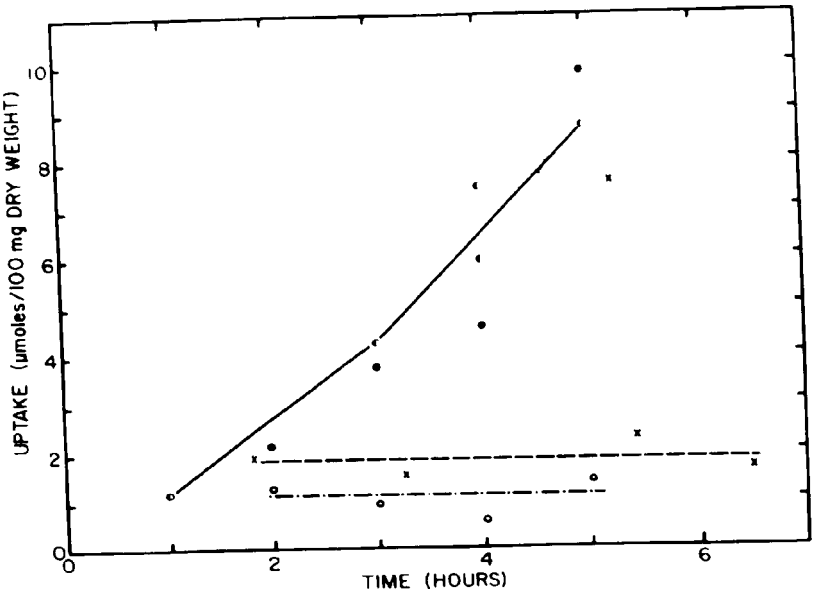


Figure 20

by 0.1 mM dinitrophenol for the first 30 minutes, after which inhibition resulted but not as pronounced as that caused by the other inhibitors. At higher concentrations of dinitrophenol, however, the uptake was more affected. Rowlands et al. (1957) observed a 50% decrease in the rate of accumulation of glutamic acid by Staphylococcus aureus in 0.45 mM dinitrophenol.

Carrot root tissue in approximately 0.2 mM dinitrophenol completely loses its ability to accumulate potassium chloride. One quarter of that concentration of inhibitor is almost as effective (Robertson et al., 1951). When untreated tissue has been allowed to accumulate salts and is then placed in distilled water, leakage of salts occurs. This leakage was observed by Robertson and his colleagues to be increased by 140% when 0.2 mM dinitrophenol was added. The rate of leakage is too low to account for the decreased rate of accumulation in the presence of this inhibitor. Birt and Hird (1956) observed the same phenomenon in connection with the absorption of L-histidine by carrot root disks. These data may provide the explanation for an experiment performed early in the present investigation in which the flux of glutamic acid was measured colorimetrically with ninhydrin. During the uptake of this amino acid by intact pea plants there was an increase in the amount of ninhydrin positive material in the medium in the case of several of the samples which had been treated with 2,4-dinitrophenol or 2,4-dichloro-

phenoxyacetic acid at 10^{-3} M each. These results were too erratic to warrant inclusion here, but they may be an indication that pea roots behave the same in this respect as carrot disks.

The Uptake of L-Glutamic Acid by Intact Root Systems

The lack of a good method of correlating the behavior of excised roots with that of those still attached makes it difficult to estimate the capabilities of roots in the nutrition of intact plants. In addition to the effects of substrates, hormones, and cofactors supplied by the shoot, excised roots are not subject to the influence of the transpiration stream. The nature of the increase in ion uptake attendant with increases in the rate of transpiration is the subject of considerable controversy. Russell and Shorrocks (1959) listed some of the literature supporting the view that the two processes are independent and that showing inter-relationship. Perhaps the most widely accepted explanation is that proposed by Broyer and Hoagland (1943) which relates the interaction of these processes to the nutrient status of the root. When the roots are low in salts, much of the incoming supply is retained within the root cells themselves, with relatively small amounts being transferred to the shoot. Under such conditions, changes in the rate of flow of water through the roots have little effect upon total salt uptake. On the other hand, roots of high salt status accumu-

late relatively little, most of the absorbed ions being transported to the shoot. In this situation the enhancement of salt uptake by increments in the rate of transpiration is obvious. If the ions are being tranported through living cells between the external medium, or the free space of the cortex, and the xylem elements, the increased flow of water can be envisioned as a diluant which tends to lower the concentration gradient between the medium and the stele. The effect on the gradient would thus be the stimulus supplied by the transpiration stream.

Other investigators, notably Hylmö (1955 and 1958), have argued in favor of transfer of at least part of the nutrient supply of the shoot through the cortex by mass flow. Acceptance of this interpretation became more general with the advent of the free space concept, some of the proponents of which believe in the continuity of free space throughout the plant (Epstein, 1955; Kramer, 1957). This view received further experimental support from the report of Kuzin et al. (1956) who, by use of heavy water and C^{14}-labeled acetate, found the content of acetate in bean leaves to be proportional to water absorption at all rates of transpiration tested. It should be noted, however, that by measuring the radiocarbon activity in the leaves one follows not the uptake of acetate but the resultant of its translocation from root to shoot. Contrast these data with the report by Martos (1959) showing the nitrogen and phosphorus

content of shoots of wheat to be greater in plants with roots attached than in rootless ones. This affords good evidence that the roots contribute more than a conducting system in their participation in ion uptake.

Intact Plants

By comparing the uptake of water and glutamate simultaneously by the roots of intact plants an attempt was made to determine the effect of transpiration on the absorption of the amino acid. That the two processes did not proceed at proportional rates can be seen in Table 6.

Under the assumption that the total uptake is the result of both transpirational and accumulative processes, the former being proportional to the volume transpired and the latter being proportional to the dry weight of the roots, some of the data were analyzed by the two-term equation total uptake=k_1 (dry weight of roots)+k_2(volume transpired). Since three of the components of this equation are known, the other two can be calculated by solving two simultaneous equations. This treatment yielded a large number of negative values for the constants, and, therefore, the approach was abandoned. Hylmö (1953, 1955, and 1958) and Kylin and Hylmö (1957) have had success in applying a similar equation to data derived from both their own experiments and from published figures from other investigators. Their method, which was recently clarified by Pettersson (1960), divides the total ion transport to the shoot into two components, one

TABLE 6

THE UPTAKE OF GLUTAMIC ACID BY INTACT PLANTS

COMPARED WITH THE VOLUME TRANSPIRED

Uptake measured by change in radioactivity of solutions containing both nonradioactive and C^{14}-L-glutamate presented to the roots of intact plants.

Plant	Glutamate Uptake (arbitrary units)[a]	Root Dry Weight (mg)	Volume Transp. (ml)	Units per 100 mg Dry Weight
Barley (Expt. 35)	11.1	90.7	11.5	12.2
	4.4	79.2	10.1	5.5
	9.7	64.8	9.2	15.0
Pea (Expt.28)	3.7	140	13.5	2.7
	1.8	120	9.1	1.5
Pea Expt. 41)	21.2	102.4	2.2	20.7
	20.0	96.1	1.8	20.8
	8.0[b]	97.0	7.3	8.3

[a]These units are comparable within experiments only.

[b]Treated with 10^{-3} M 2,4-dinitrophenol.

representing that portion of the transported ions which is independent of transpiration, the other being an index of the transpiration-linked component. The plot of the transport of ions to the shoot versus the volume of water transpired yields a straight line, the y-intercept of which is interpreted as the transpiration-independent transfer of ions to the shoot. Russell and Shorrocks (1959) criticized this interpretation on the grounds that it "depended on extrapolation from data, the quantitative validity of which is doubtful because of inadequate statistical control."

Russell and Shorrocks (1959) analyzed their results differently. They calculated the transpiration stream concentration factor (TSCF), which is the quotient of the concentration of an ion in the transpiration stream divided by its concentration in the external medium. They did not measure the concentration in the transpiration stream directly but calculated it from the amount of the ion that was transported to the shoot divided by the volume that was concurrently transpired. Pettersson (1960) made the cogent observation that this method fails to account for any increase in the ion content of the shoot which is ascribable to a cell to cell transfer of salt from root to shoot.

Referring again to Table 6, one group of plants in experiment 41 was treated with dinitrophenol. This inhibitor at 10^{-3} M has already been shown to be a potent deterrent of the uptake of glutamic acid by excised tobacco roots (Figure

20). Assuming that its effect on attached pea roots is similar, one can roughly estimate the participation of cellular metabolism in the uptake of glutamate by intact pea plants by comparing the values of the treated group with those which were not inhibited. Note that transpiration was encouraged in the treated group and slowed down in the others. Despite the relatively high rate of transpiration in dinitrophenol, the treated plants absorbed no more than 40% as much amino acid as the controls. This provides evidence in favor of the strong participation of energy-requiring metabolic processes in the total uptake by the plant, transpiration alone being relatively ineffectual.

The same interpretation was advanced by Laties (1959) who went on to suggest that the release of ions into the stele after their active uptake and transport through the cortex is effected by an endogenous volatile inhibitor which prevents the development in cells near the stele of the type of metabolism required for salt accumulation. He did not explain how changes in the rate of transpiration might interact with such a system.

Detopped Roots

Using the suction technique described under Methods and Materials, one can not only recover materials from the transpiration stream but measure uptake by the intact root system. The results of two such determinations are compared

with the performances of excised roots in Table 7. It is obvious that the uptake per unit of excised root is far below that of the intact root system.

During the uptake of substances which are transported to the shoot in an intact plant, at least part of the absorbed material is delivered to the lumen of xylem elements and is passively carried up by the transpiration stream. In the case of excised roots, however, that portion of the absorbed material is returned to the medium. This interpretation is supported indirectly by the experiments of Zhurbitskii and Wiei-Nan (1959) who found more P^{32} in the shoots of intact plants than could be recovered from the bleeding sap of those the shoots of which had been removed.

Transfer of Amino Acid from the Medium to the Shoot by Intact Root Systems

The transfer of nutrients is a topic closely related to the preceding discussion of the interaction between transpiration and ion uptake. If there is a direct path by which the components of the external solution can be pulled passively by transpirational forces, one would expect a large part of the nutrient supply of the shoot of a rapidly transpiring plant to be of the chemical forms supplied externally. If, on the other hand, the nutrients must interact with the metabolic machinery of root cells before reaching the shoot, chemical transformations would not be unexpected.

TABLE 7

UPTAKE OF GLUTAMIC ACID BY EXCISED ROOTS AND INTACT ROOT SYSTEMS OF TOBACCO IN FOUR HOURS

Uptake measured by the change in radioactivity of solutions containing both nonradioactive and C^{14}-L-glutamate; solutions aerated continuously during uptake period; original concentration 5 x 10^{-4} M glutamate.

Treatment	Uptake (μmoles per 100 mg Dry Weight)	Volume removed by suction (ml)
Freshly excised	3.6	. . .
Excised and aged in telomycin	7.6	. . .
Excised and washed	6.7	. . .
Intact; suction method	24.6	5.13
	13.1	?[a]

[a]The exact value here is not known, but it is in excess of 4 ml.

In this respect the nitrogen nutrition of higher plants is particularly interesting. Examination of the xylem sap of many species led to the surprising discovery that nitrates constitute only a minor fraction of the total nitrogen, amino acids and amides accounting frequently for over 90% (Bollard, 1958 and 1960). Wieringa (1958) on the other hand found the bleeding sap of uninoculated, sterile-grown pea plants to be free of amino acids.

Russian investigators have shown much interest in the occurrence of amino acids in the transpiration stream. Mokronosov et al. (1959) found a diurnal rhythm in the amino acid content of the sap of potato, the daytime amount being high. Short photoperiods favored higher concentrations of these compounds but had no qualitative effect. Stoev and coworkers (1959) reported ten amino acids in the sap of grape. Using an interesting approach, Khotyanovich (1958) showed that the six amino acids in the sap of pine seedlings were reduced to three when the oxygen supply of the roots was severely restricted. Complete anaerobiosis decreased the number to one, glycine.

Not all of the transported organic nitrogen has been found in the form of amino acids. A broad spectrum of nitrogenous compounds has been detected (Bollard, 1960), among which are the tobacco alkaloids. Wada et al. (1959) reported finding eight alkaloids in the roots and xylem sap of two varieties of Nicotiana tabacum.

In the present series of experiments interest was centered on the nitrogenous compounds the appearance or increase of which could be directly attributed to the presentation of glutamic acid to the roots. Samples of xylem sap were withdrawn from tobacco roots by the suction method described under Methods and Materials. The aliquots were chromatographed, and the chromatograms were cut longitudinally into three pieces, each of which was treated with a different chromogenic reagent. When this was done with the roots in glutamate solution instead of water, no qualitative change was detected. By repeating this technique using uniformly labeled glutamate, the carbon skeleton was found to be transferred as three compounds, all of which were ninhydrin positive. For this reason only the radioactive compounds were positively identified.

The roots in distilled water produced sap containing six (or possibly seven) ninhydrin positive spots when developed on paper with butanol-propionic acid-water as the solvent. Treatment of the paper with bromcresol green (Block et al., 1952) revealed one acidic compound which did not react with ninhydrin. Formation of 2,4-dinitrophenylhydrazones on the developed chromatograms by Dobinson's (1959) method showed one faint spot which was apparently too dilute to be detected by ultra-violet fluorescence of the reaction product with ninhydrin (cf. Methods and Materials).

Chromatograms of the radioactive compounds were scanned at a strip speed of 3/4 inch per minute. When the machine was adjusted to its highest sensitivity (full deflection equivalent to about 100 cpm), a broad peak, the top of which was off scale, and a smaller, sharper peak of radioactivity were found. The broad peak was later identified as glutamic acid or glutamine, one or both of which could have been active. The second peak proved to be alanine.

Redevelopment of the chromatogram in water-saturated phenol separated the spots produced by glutamate and its amide, but additional scanning for radioactivity failed to show the source of activity without equivocation. The strip was then cut transversely into half-centimeter pieces, each of which was placed in the center of a planchet and counted on a thin window gas-flow counter. The results are shown in Figure 21. The major portion of the activity was in glutamine. This is in accord with the finding of others cited above.

This result can be construed as evidence, but not proof, for the passage of the bulk of the transferred glutamate through cells where amination occurred.

Miettinen (1959) obtained similar information on the assimilation of alanine by pea plants. The presentation of radioactive alanine to intact pea plants resulted in rapid removal of the amino acid from the medium, the major portion of the activity in both root and shoot being

Figure 21. Radioactivity in chromatographically separated glutamine and glutamic acid derived from the xylem sap of an intact tobacco root system placed in C^{14}-glutamic acid.

Shaded areas represent spots detected by ninhydrin on the original chromatogram; bars show activities of 0.5 cm strips cut from the chromatogram after detection of the spots by ninhydrin.

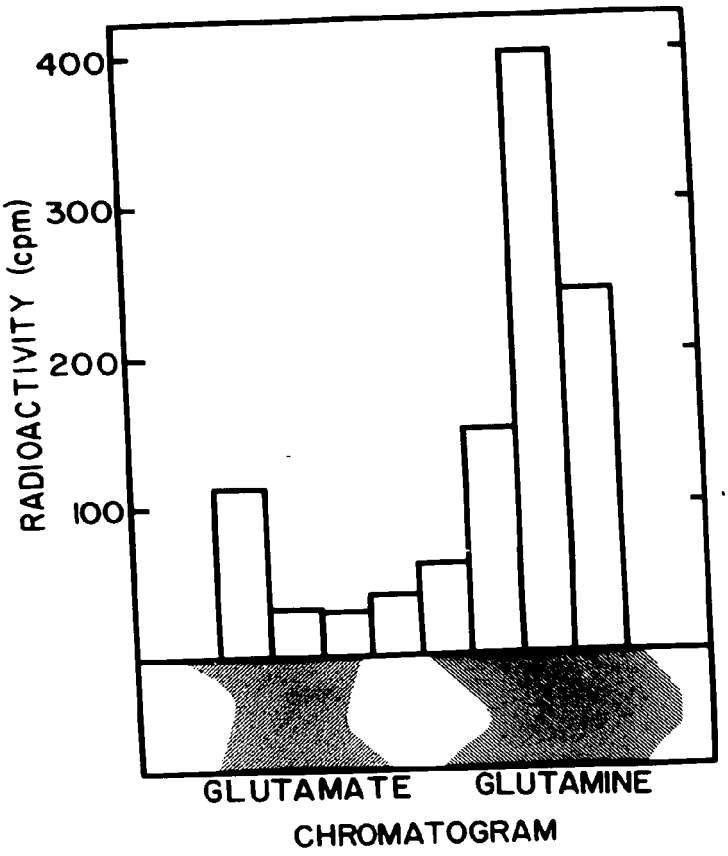

400
300
200
100
RADIOACTIVITY (cpm)
GLUTAMATE
GLUTAMINE
CHROMATOGRAM

Figure 21

found as free alanine. The second most active compound in the root was a peptide which contained radioalanine and unlabeled glycine, serine, and glutamate. This peptide did not appear in the shoot. Radioactivity in both shoot and root also appeared in several amino acids and sugars. If nodulated, but otherwise sterile, plants were used, much of the radioactivity recovered from the root was in two peptides, one being the same as that described above. Uptake of alanine by rootless shoots, however, results in a very different pattern of labeling than was found in the shoots of intact plants. This led to the conclusion that, although the amino acid is absorbed without degradation, it is transferred to the shoot either as a peptide or the free amino acid, the free acid being transported along a route which prevents the compound from coming in contact with decarboxylating and oxidizing enzymes. Thus, as was shown in the present work with tobacco, a considerable portion of an amino acid taken up by the root system of pea undergoes modification before being transported to the shoot.

GENERAL DISCUSSION AND CONCLUSIONS

The Respiratory Response to Glutamic Acid

Glutamic acid has been shown to elicit enhancement of the respiratory rates of excised roots of tobacco, pea, and barley. With the exception of barley the magnitude of the response was augmented by lapse of time after excision. The response itself was characterized by a time lag after which the rate increased logarithmically in pea and barley and linearly in tobacco. The D-enantiomorph of this amino acid produced a similar, though smaller, increase. Two structural analogs of glutamate, glutamine and alpha-amino-isobutyrate, were also employed, the former being more stimulatory than glutamate and the latter being inhibitory both in the presence and in the absence of glutamate. Repression of the respiratory response to glutamate by 2,4-dinitrophenol, sodium azide, and benzimidazole was not complete at the concentrations used. In each case the salient feature appeared to be a lengthening of the lag phase without much change in the ultimate rate.

Nature of the Time Lag

One can infer from the existence of a time lag followed by increased activity of the observed process that the lag represents the time necessary to build up some

limitating component in the reaction.[1] Thus in the case of enzyme adaptation, for instance, the limiting factor is the amount of the active enzyme that is available. The shapes of the respiratory curves plotting the response of roots to glutamate (Figure 3, 5, and 6, for example) show strong resemblance to those characteristic of enzyme adaptation (Oginsky and Umbreit, 1959, chapter 17). This similarity is certainly not conclusive evidence that enzyme activity is limiting in this instance, but the possibility that this is so has not been eliminated.

If the internal concentration of glutamic acid is the limiting factor, one could rationalize that there is no change in the respiratory rate until sufficient amino acid has been absorbed to raise its concentration above the minimum level necessary for its further metabolism. The data of Figure 6 and 18 seem to lend credence to this interpretation in that there was significant uptake of glutamate long before a change in respiration was observed. Note that this holds true for both oxygen consumption and carbon dioxide production.

However, this explanation does not agree with the accepted interpretations of permeability. By and large the passive permeation of substances is dependent on their lipid

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[1]This is not necessarily true of a reaction in which an inhibitor governs the rate.

solubility (Collander, 1959), the ability to permeate increasing with the lipophilic properties of the compound. Since amino acids are "among the most lipid-insoluble substances known" (Collander, 1959, p. 52), the membranes of cells are essentially impermeable to them. Thus, mediated transport (in the sense employed by Christensen, 1959) is necessary for entry of these compounds. Transport of the amino acid into a region of the cell having a higher concentration of the compound than is found in the external solution would require the expenditure of energy. Many investigators in this field would expect such transport against a gradient to result in effects on the rate of respiration (e.g., Laties, 1957). Steward and Sutcliffe (1959) contend that cells that have attained a minimal level of metabolic activity would be able to absorb ions without a significant increase in the respiratory rate. They attributed this ability to the "overall properties of a dynamic system." Only Steward and Sutcliffe's theory is consistent with the possibility of active absorption of the glutamic acid prior to the respiratory response.

James (1953b) considered the enhancement of the respiratory rate of excised roots through aging to be a result of starvation, i.e., depletion of utilizable substrates. If this view is correct, the factor limiting that rate is substrate concentration. The lag, under such conditions, could be attributed to the building up of the

internal glutamate concentration.

Significance of Optical Isomerism

The poor ability of the D-enantiomorph of glutamic acid to elicit a respiratory response in contrast to the L-isomer may be a consequence of steric specificity of either the uptake mechanism, the respiratory enzymes, or both. Birt and Hird (1958a) found that excised, aerobically incubated carrot root tissue absorbed both optical isomers of several amino acids, but that only the L-isomers were metabolically degraded. If the same situation obtains for the relationship between tobacco roots and the enantiomorphs of glutamic acid, the difference in response elicited by these isomers may be largely dependent upon differences in the metabolism of the molecules subsequent to absorption.

Respiratory Inhibitors and the Response

All of the inhibitors used had essentially the same effect, that of delaying the ultimate response. Because this effect on the respiratory response to glutamate occurred even under conditions in which the endogenous respiratory rate remained unchanged, the assumption that the inhibitors acted more or less directly upon the uptake mechanism is reasonable. The eventual recovery to a rate approximating that of the uninhibited roots can then be attributed to that fraction of the uptake which was not inhibited. The lengthened lag is thus interpreted as an

increase in the time necessary for the entry of enough glutamate to produce a measurable increment in the rate of respiration.

The uninhibited fraction of respiration may actually be insensitive to the inhibitors used (Laties, 1957) or may represent a portion of an inhibitor-sensitive mechanism which would be affected only by higher concentrations of the inhibiting compound or at a different pH.

The observed inhibition of the endogenous respiratory rate without an effect on the response to glutamate and vice versa indicates a separation, spatial or functional, of the mechanism responding to glutamate and at least a part of the endogenous respiratory system. This concept of separation resembles the proposal made by Steward and coworkers (1956) that the synthesis and degradation of proteins and the oxidation of protein-derived amino acids occur in the cell of cultured carrot root tissue at a locus to which the free amino acids of the cell cannot penetrate. On the assumption that a similar mechanism prevails in pea and tobacco roots, one might hypothesize that entering glutamate molecules do not act directly in producing a respiratory response but are transformed into another molecular species which is capable of penetrating to an "inner" locus at which there is stimulation of some respiratory mechanism. This "inner" locus could conceivably be inaccessible to some inhibitors which enter

other parts of the cell and affect the endogenous respiration. The lag in the response of such a system could be due in part to the time required to synthesize the second molecular species from glutamate.

The objection that a comparison such as that made above between storage roots and ordinary roots may not be valid can be countered by the data of Frenzel (1957). He found that roots of sunflower plants lose amino acids to the medium only when the medium contains inorganic nitrogen even though there are free amino acids in the root tissues. This suggests a spatial separation of the stored and the newly synthesized amino acids. We can infer from this that the respiratory enzymes in the aged roots used in the present investigation may be located in an area of the cell inaccessible to the bulk of the free amino acids of that cell. In other words, the presence of free amino acids is not synonymous with their availability as respiratory substrates. Presumably, newly absorbed amino acids can be metabolized in the cytoplasm, the most likely locus of the stored compounds being the vacuole.

It should be noted that the two cellular spaces mentioned in regard to Frenzel's (1957) data are not necessarily identical with those proposed by Steward *et al*. (1956) since the latter suggest that absorbed amino acids enter the free amino acid pool whereas Frenzel does not.

Aging

The data presented in this dissertation, which indicate an increase in both the uptake of and respiratory response to glutamate as a result of lapse of time after excision of pea or tobacco roots, parallel the results of others, particularly those working with storage tissues (Sutcliffe, 1954; Birt and Hird, 1956, 1958a, 1958b; Laties, 1957; Dalgarno and Hird, 1960). Although it has been subjected to a great deal of investigation, this increase is still only poorly understood.

In storage tissues the changes in response to salts with aging is accompanied by an increase in the endogenous respiratory rate. Laties (1957) pointed out that this change is not merely quantitative but that the incuced respiratory increment is malonate-sensitive, whereas the original respiration is completely malonate-insensitive. His suggestion that the metabolic changes induced by aging of tissue slices do not occur in the intact storage organs because of the presence of a volatile inhibitor certainly is not applicable to the present data. Furthermore, the results of Dalgarno and Hird (1960) showing that such changes are not induced by the aging of carrot root slices anaerobically rule out the volatile inhibitor mechanism for that tissue.

The observation of Ap Rees and Beevers (1960) that in both carrot root and potato tuber tissue slices there is

a change in the respiratory pathways concurrent with the development of induced respiration provides a significant clue to the fundamental changes which occur. They found during aging an increase in the relative proportion of glucose oxidized through the pentose phosphate pathway as opposed to the Embden-Meyerhof-Parnas glycolytic pathway. Other changes, namely increases in activity of some enzymes associated with electron transport and in the ability to accumulate and esterify phosphate, have been reported by Hackett et al. (1960) and Loughman (1960), respectively. The dependence of these changes on respiratory energy is shown by the rapid esterification of phosphate (Loughman, 1960), by the inhibition of induction of 2,4-dinitrophenol (Ap Rees and Beevers, 1960), and by the inhibition of induction by anaerobiosis (Dalgarno and Hird, 1960). Similarly, dependence on respiration was shown in the present work by failure of the response to glutamate to develop in roots washed at low temperature. Thus, although excised tobacco and pea roots do not exhibit an induced increase in endogenous respiration during aging, there is good evidence that the changes which occur in these roots are not unrelated to those observed in storage organs. Further investigation of this apparent similarity is necessary.

The failure of pea and tobacco roots to develop an increased respiratory rate after excision may be a result of their relatively low reserve of respiratory substrates as compared with storage organs.

The Uptake of Glutamic Acid

Excised Roots

The absorption of glutamate by excised roots of both pea and tobacco was, like their respiratory response, enhanced by aging. Inhibition of that uptake by respiratory inhibitors indicates that an energy-dependent process is involved. As mentioned in the preceding discussion of the changes in respiration produced by aging, there are indications that more energy is made available by the type of respiration induced by aging than by that in freshly excised roots. It is not surprising, therefore, that an energy-dependent process such as absorption of ions would be stimulated by the induction of such a respiratory mechanism.

Dalgarno and Hird (1960) suggested that the stimulation of the uptake of amino acids by carrot root disks results from the removal during incubation of an endogenous substrate which may compete with exogenous amino acid for a carrier. They documented this interpretation by citing the discovery by Dale and Sutcliffe (1959) that extracts of red beet root inhibit the uptake of inorganic ions by that organ. However, there is no evidence that the inhibitor in the extract is effective *in vivo*.

It would be interesting to know whether there is a connection between the rate of utilization of glutamate and the rate of its absorption. In the preceding paragraphs the energy supply has been assumed to be the factor limit-

ing the rate of uptake. Any process which speeds up the removal of glutamate from the site of its release by the transport mechanism would tend to increase the rate of uptake of that compound. Such removal could involve the movement of the unchanged molecule to the vacuole or some other space bounded by a suitable membrane, the degradation of the molecule, or its incorporation into another compound.

Intact Root Systems

The uptake of labeled glutamate by intact root systems has been shown substantially to exceed that by excised roots. Why should this be so?

Absorbed glutamate is transported from intact root systems to their shoots. That much of the transported amino acid can be conducted extracellularly is evident from the results of the suction experiments. If similar translocation occurs in excised roots, some of the glutamate absorbed would be returned to the medium. Thus the uptake measured by using excised roots is probably indicative of their ability to accumulate rather than their total absorptive capacity.

It was pointed out in Results and Discussion that roots do more than provide an inert tube through which the plant can remove nutrients from soil and that it is likely that most of the absorbed ions pass through living cells before reaching the shoot. Under these circumstances one

would expect a part of the observed respiratory response to be associated with that fraction of the absorbed glutamate which was returned to the medium. This fraction could not be detected by the methods used.

Because there is conversion of some of the translocated glutamate to glutamine, the loss of some of the absorbed amino acid could possibly be followed by analyzing for glutamine in the medium after allowing absorption of glutamic acid to occur for several hours.

The Toxicity of Glutamic Acid to Tobacco

The ability of tobacco plants to absorb glutamic acid was established by Ghosh and Burris (1950) and confirmed in the present work. In view of the stimulation of respiration of tobacco roots elicited by this amino acid, its toxicity to tobacco, as reported by Beaumont *et al.* (1931), Steinberg (1947), and Ghosh and Burris (1950), does not seem to result from a specific inhibition of respiration.

Several alternative explanations can be proposed. Each of the investigators listed above grew tobacco from seed in the presence of glutamic acid. The observed inhibition of growth by the amino acid could be due wholly or in part to effects on germination rather than on the subsequent growth of the seedlings.

The occurrence of relatively large amounts of glutamine in the xylem sap is indicative that some of the absorbed glutamic acid is degraded, the nitrogenous moiety

being transferred to other glutamate molecules to form the amide. The transferred nitrogen is likely to pass through ammonia. Since ammonia is toxic to plants in high concentration (Meyer et al., 1960), the inhibition of growth of tobacco by glutamate may reflect the production of free ammonia in the tissues.

A third alternative is that there may be an inhibitory effect of amino acids on tobacco seedlings, but not on mature plants. All of the tobacco tissues used in the present investigation were taken from large plants which had begun rapid growth; in previous work in which toxicity of glutamate was observed small seedlings were used. This suggestion could be checked by growing the plants initially without glutamate and adding the amino acid after the plants had become well established.

One might hypothesize further on the possible lack of toxicity of amino acids to the larger tobacco plants by drawing an analogy with the work of Andreae and Van Ysselstein (1960) on the inhibition of elongation of organs by indoleacetic acid. They consider the accumulation of free auxin in the tissues to be responsible for the typical inhibition of elongation produced by high concentrations of the hormone. They suggest that the conjugation of auxin with aspartic acid acts as a detoxification mechanism in that it serves to remove the free hormone molecules from the tissue. Degradation of these molecules has the same

effect. Thus, it is a balance between the rate of import of indoleacetic acid and the rate of its conjugation and degradation which determines its effect on growth. When an approximate balance has been achieved, growth stimulation is at its maximum; when the rate of import exceeds that of detoxification, the free hormone accumulates, and inhibition of growth occurs.

By analogy, one might postulate that the presence of high concentrations of glutamic acid in the tissues of tobacco may cause inhibition of growth because of toxicity of either the amino acid itself or some product of its metabolism. Detoxification could depend on either the incorporation of the glutamate into other compounds or its degradation by some pathway which does not produce toxic end products. Degradation of a possible inhibitory product would also constitute detoxification. If, then, the balance of the rates of uptake and utilization of glutamate in small tobacco seedlings is different than that in larger plants, differences in the responses of the two sizes of tobacco plants would be observed. Further investigation along this line is necessary.

SUMMARY

Because investigators have found that glutamic acid is not an effective nitrogen source for tobacco, the possibility that this key amino acid either fails to enter the plant or is not available for metabolism subsequent to entry was investigated. The effect of glutamate on the respiratory rates of excised roots was used as an index of its entry and utilization, and uptake of glutamate was measured directly. The transfer of absorbed glutamate and the chemical form in which it was transported were also examined. The behavior of tobacco roots in some of these respects was compared with that of pea and barley.

The respiratory rate of excised roots of tobacco, pea, and barley was enhanced by L-glutamate. Except in barley the responses increased with time after excision if held at room temperature. Aging at low temperature was not similarly effective. This effect resembles that exhibited by storage root slices. The response was characterized by an initial lag followed by a logarithmic increase in pea and barley or by a linear increase in tobacco.

Glutamine produced a greater stimulation than L-glutamate, but the D-amino acid was less effective than its L-form. Respiratory inhibition by alpha-aminoisobutyric acid was reversed by L-glutamate. Pyridoxal and glutamate anta-

gonized the respiratory response that each separately elicits in pea roots.

Repression of the respiratory response to glutamate by 2,4-dinitrophenol, sodium azide, and benzimidazole was not complete at the concentrations used. Each tended to lengthen the lag phase without much change in the final rate, but neither benzimidazole nor azide changed the endogenous respiratory rate of tobacco roots, and dinitrophenol caused in sharp stimulation. This differential response to the inhibitors indicated a spatial or functional separation between the endogenous respiratory mechanism and the system which is affected by glutamate. The relative ineffectiveness of the classical respiratory inhibitors was attributed to the increase of pH of the medium during incubation of tobacco roots, a high pH slowing down the rate of penetration of the weakly acidic inhibitors.

In both pea and tobacco roots the enhanced respiratory response to glutamate induced by aging was coupled with increased uptake of that compound; there was a possibility of correlation between the uptake and respiratory curves.

The uptake by intact tobacco root systems greatly exceeded that by excised roots on a dry weight basis. This difference probably depended at least partly on the effects of transpiration in intact plants and on the return to the medium through the stele of glutamate which had been absorbed through the outer tissues of excised roots. Severe inhibition

of uptake by intact pea plants in the presence of dinitrophenol indicated that the passive entry of glutamate with the transpiration stream was a relatively small component of the total uptake. The total glutamate uptake could not be related quantitatively to the volume of water transpired or to the dry weight of the roots.

When labeled glutamate was drawn by suction through an intact, shootless tobacco root system, all of the radioactivity recovered in the xylem sap was in glutamine, glutamate, and alanine, most of the activity being in the amide and very little in alanine. This was construed as evidence for the passage of the bulk of the transferred glutamate through cells, where amination occurred.

The reported toxicity of glutamate to tobacco was explained on the basis of the probable accumulation of inhibitory concentrations of either glutamate or a toxic metabolic product thereof, possibly ammonia.

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