SOME STUDIES ON THE BIOGENESIS OF THE PYRIDINE RING OF ANABASINE IN EXCISED *NICOTIANA GLAUCA* LEAVES

by

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Some Studies on the Biogenesis of the Pyridine Ring of Anabasine in Excised Nicotiana glauca Leaves.

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(date)

ABSTRACT

The origin of the pyridine ring in higher plants is not definitely known. In most animals and microorganisms, the pyridine ring arises as nicotinic acid and in some higher plants, this has also been shown to be the case. Nicotinic acid itself is thought to arise by at least two major pathways: (a) by degradation of tryptophan, (b) by condensation of glyceraldehyde and aspartic acid.

Nicotiana alkaloids (nicotine, nornicotine and anabasine primarily) are synthesized in both the roots and leaves of different species of Nicotiana. Root cultures of some species use nicotinic acid as the precursor for the pyridine moeity of the alkaloids. Aspartic acid and glyceraldehyde have been proposed as near precursors of nicotinic acid although some experimental observations cannot be explained by this hypothesis. The present study was undertaken to investigate whether the leaves of N. glauca could use nicotinic acid for anabasine biosynthesis and if so, what precursors are likely in its biosynthesis. γ-Methylene glutamic acid was proposed to provide all the carbons for nicotinic acid. Several γ-substituted derivatives of glutamic acid, including the one considered here, are found in relatively large con-

centrations in plants and their biosynthesis is thought to involve the condensation of two pyruvic acid molecules.

The excised leaves were first checked for anabasine synthesis by $^{14}CO_2$ feeding and then the following compounds were introduced into separate leaves via the transpirational stream: (a) nicotinic acid-U-^{14}C; (b) aspartic acid-U-^{14}C; (c) γ-methylene glutamic acid-2-^{14}C; (d) sodium pyruvate-1-^{14}C; (e) sodium pyruvate-2-^{14}C; (f) sodium pyruvate-3-^{14}C. After a fixed time of metabolism, anabasine was isolated from each leaf, oxidized to nicotinic acid and the specific activity determined.

The criterion used here to determine the immediacy of a precursor was the "dilution factor", which takes into account the specific activity of the substrate administered, its pool size within the leaf, as well as the specific activity of the nicotinic acid isolated. The smaller the dilution factor, the more immediate the precursor. The following dilution factor values were obtained: nicotinic acid-U-^{14}C = 11.5; aspartic acid-U-^{14}C = 1.0×10^{4}; γ-methylene glutamic acid-2^{14}C = 1.3×10^{4}; sodium pyruvate-1-^{14}C = 1.1×10^{5}; sodium pyruvate-2-^{14}C = 3.1×10^{4}; sodium pyruvate-3-^{14}C = 7.1×10^{3}. The largest pool was detected for aspartic acid -- almost 400 μg, whereas

nicotinic acid and pyruvate showed pools of 184 µg and 67 µg respectively. γ-methylene glutamic acid could not be detected within the leaf.

The low dilution factor of nicotinic acid proves not only that the leaf of Nicotiana glauca could use this for anabasine biosynthesis, but also that it is by far the most efficient precursor. The fact that the label is introduced from $^{14}CO_2$ to the pyridine ring indicates that nicotinic acid need not be transported from another part of the plant to the leaf. γ-methylene glutamic acid, though not detected within the leaf, is almost as efficient as aspartate in serving as a precursor for nicotinic acid, suggesting that there could be another pathway involving other intermediates. Pyruvate could be a major intermediate in a second pathway since we observe that carbon-3 labelled pyruvate is incorporated even more efficiently than aspartate. The fact that the label is introduced from carboxyl labeled pyruvate (though to a lesser extent lends further support to such a hypothesis, since if pyruvate were to be incorporated by conversion to aspartate, it would have lost the label through decarboxylation. It is of course possible that pyruvate is converted to 3-phosphoglyceraldehyde by the reversal of glycolysis and the label is incorporated via the 3-carbon unit.

To

my parents

and

my wife

for

their

understanding and encouragement

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1. INTRODUCTION

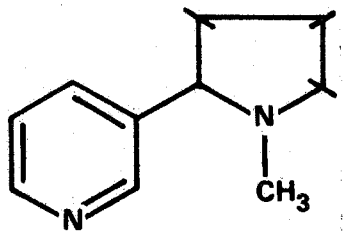
1.1 General Survey

The origin of the pyridine ring in nature is a problem that has concerned biochemists for a long time. Several biologically important compounds contain a pyridine moiety, those of special importance being: (a) Nicotiana alkaloids, (b) nicotinic acid (and its amide) and (c) the vitamin B_6 group.

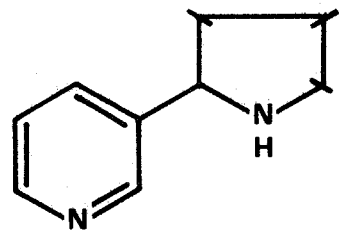
The alkaloids present in certain Nicotiana species, commonly referred to as "tobacco alkaloids", are of relatively simple structure, consisting of a pyridine ring substituted in the β position by another nitrogen-containing heterocyclic ring. The three most abundant alkaloids of this group are: nicotine, nornicotine and anabasine. Another common pyridine-containing compound related to the tobacco alkaloids is ricinine, found mostly in Ricinus communis. The structures of these alkaloids are given in Figure 1. Different species of Nicotiana contain the three above-mentioned alkaloids to different extents. Nicotine is the major constituent of the alkaloid fraction of many strains of N. tabacum, and a minor constituent of certain strains of this species and of most other species of Nicotiana known. Nornicotine is the major alkaloid in most species that contain nicotine in small quantities, whereas anabasine is the major constituent of the alkaloid fraction in N. glauca. These alkaloids are present in different amounts in the roots, the stem and the leaves of the same

FIGURE 1

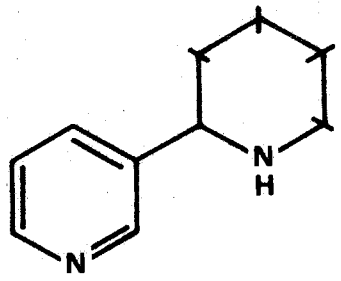
The structures of nicotine, nornicotine, anabasine and ricinine.



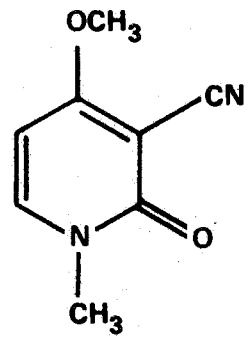
(a) Nicotine



(b) Nornicotine



(c) Anabasine



(d) Ricinine

plant and it has also been shown that they can be synthesized in the roots as well as in the leaves.[1]

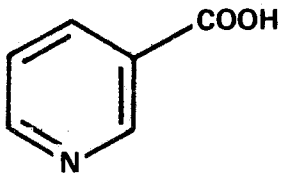
Nicotinic acid (niacin), which is biologically equivalent to its amide, is widely distributed in nature but not usually in the free state: it is a constituent of two important hydrogen transfer co-enzymes, NAD^+ and $NADP^+$ (Figure 2). The acid was first isolated from yeast and from rice bran whereas the amide was obtained from liver concentrates. Deficiency of nicotinic acid (or nicotinamide) in man leads to a condition termed pellagra which is characterized by patches of dermatitis, thickening and abnormal pigmentation of the skin, disfunction of the digestive and nervous systems, loss of memory, insanity and eventually death. Therefore, nicotinic acid is termed the pellagra-preventive (PP) factor.

Vitamin B_6 refers to a group of very closely related compounds, pyridoxine, pyridoxal and pyridoxamine (Figure 3), which, in the form of their phosphates, are interconvertible in vivo. These are involved in a number of extremely important metabolic reactions of the α-amino acids including transaminations, racemizations, decarboxylations and eliminations.

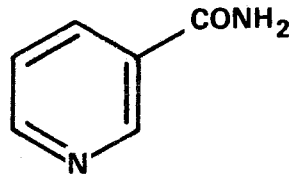
The Nicotiana alkaloids have been particularly useful in the studies of pyridine ring biogenesis, due to their relatively simple structure and also the availability of

FIGURE 2

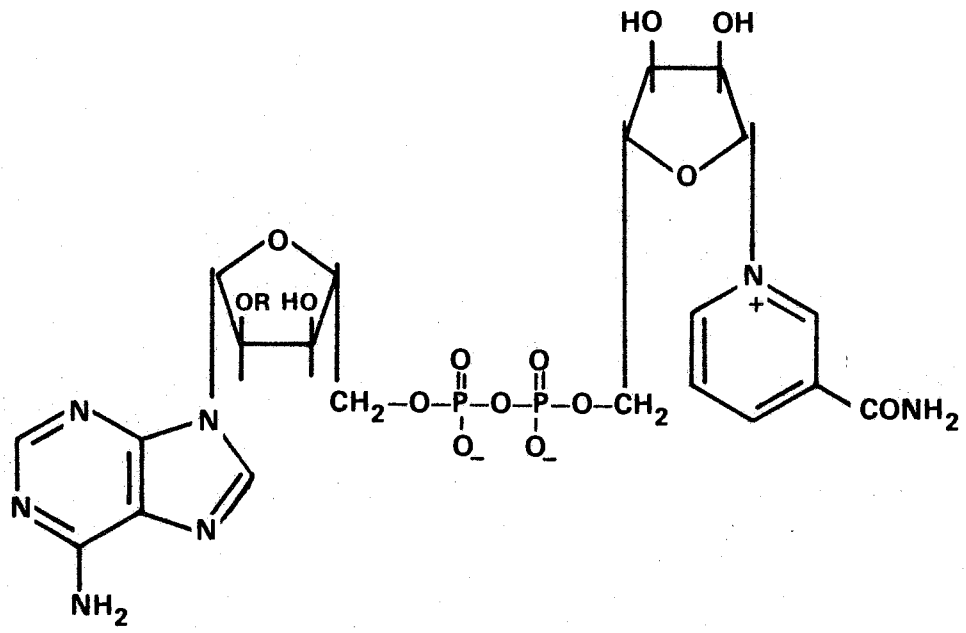
Structures of nicotinic acid and
its biologically important derivatives.



(a) Nicotinic acid



(b) Nicotinamide

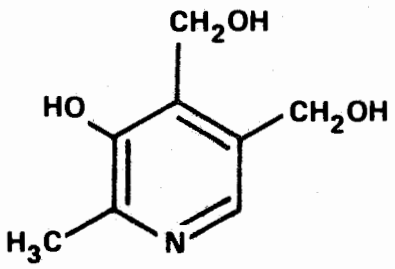


(c) R=H : Nicotinamide - adenine dinucleotide $\left[NAD^{+}\right]$

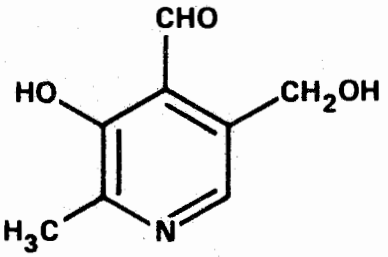
(d) R= $\left[-O-\underset{\underset{O}{\|}}{P}(OH)_2\right]$: Nicotinamide - adenine dinuceleotide phosphate $\left[NADP^{+}\right]$

FIGURE 3

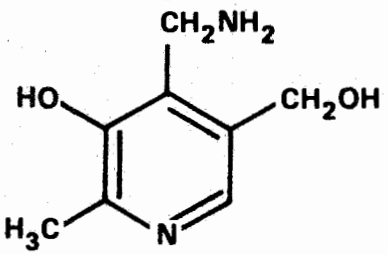
Vitamin B_6 group.



Pyridoxine



Pyridoxal



Pyridoxamine

suitable plants. It is known that the biosynthesis of these alkaloids involves a separate construction of the pyridine ring and the other heterocyclic ring followed by a condensation of the two. Results of previous biosynthetic studies (which will be given later) suggest that the pyridine ring arises as nicotinic acid in most animals, microorganisms and some higher plants.

Nicotinic acid biosynthesis is therefore of major importance and a considerable amount of work has been done to ascertain its possible immediate precursors and the biosynthetic pathway(s). Two major advances in methodology account for the present knowledge of the pathways of nicotinic acid biosynthesis. The older of these, "the induced-mutant method", was employed to demonstrate the possible sequence of precursors in Neurospora. The other, the use of isotopic tracers, has flourished in more recent years and has proved to be by far the more powerful of the two. By the use of isotopes, it has been shown, for example, that the Neurospora pathway is not the sole route to nicotinic acid in nature and that it may be replaced or at least quantitatively overshadowed by other pathways in certain organisms.

Special interest attaches to the study of biosynthetic routes to nicotinic acid in higher plants such as Nicotiana species, for the following reasons. First, the experimental

plants are sufficiently complex that the array of mutants needed for detection and identification of intermediates by the older methods could not have been obtained without enormous effort. Second, the nicotinic acid produced in these cases is converted to terminal metabolites, the pyridine alkaloids, which subsequently accumulate in very large quantities in the plant organs. Third, these plants do not respond to the introduction of intermediates with accelerated rates of terminal metabolite production. Fourth, classical methods of enzymology have not been applied successfully to a single step of the suggested biosynthetic sequences. This being the case, the employment of isotopic tracer methods is both expedient and mandatory.

At the same time, it is important to note that the actual pathway normally used by a given biological system to synthesize a given compound may not be uniquely clarified by means of studies with isotopically labelled compounds for a number of reasons. First, it is possible that enzymatic alteration of the compound may occur by metabolism prior to its reaching the site of biosynthesis. Second, it would be possible that the compound may not reach the site of biosynthesis at all, depending on the feeding process or for permeability reasons. Third, the feeding of the compound may alter the pool size (if any)

of this and closely related compounds and thereby alter the normal metabolism of the system. This becomes very important if there are several pools ("compartmentations") of the same compound for different metabolic processes of the system under study. As such, the fact that the label is transferred from, for example, compound "A" but not from compound "B" does not necessarily confirm that "A" is a precursor/intermediate whereas "B" is not. "A" might be an actual precursor or it may be an alternative to it. Compound "B", although not incorporated, could well be a precursor which is unable to reach the site of biosynthesis intact, due to one or more reasons given above. Nevertheless, when comparable and fairly consistent results are obtained in several different systems and when they are based on common ubiquitous compounds, it is probable that the resulting picture is at least close to the way in which these systems normally do synthesize the compound under study.

Another aspect that should be emphasized is that there could be more than one pathway for the biosynthesis of a compound in the same plant. Not all parts of a plant may utilize the same biosynthetic pathway for the synthesis of the same compound. There may be different enzyme systems involved and hence different precursors and different routes for the synthesis of a compound in

different parts. Some parts may even show partial synthesis. This could be quite possible in the biogenesis of Nicotiana alkaloids, where one of the two heterocyclic ring systems could be synthesized elsewhere and transported to the site of synthesis of the other, for condensation to take place.

1.2 Scope of the investigation

The alkaloid selected for this study was anabasine (Figure 1c), the major alkaloid in N. glauca, primarily for two reasons. First, the biosynthesis of anabasine has not been subjected to as rigorous an investigation as that of nicotine. There have been only a few experiments reported, relating to anabasine biosynthesis and in such cases, suggested intermediates of nicotine biogenesis have been administered to N. glauca plants. Since in most instances, similar incorporation patterns had been observed, it has been concluded that whatever applies to the pyridine ring biosynthesis of nicotine, also applies to that of anabasine. Second, anabasine is synthesized in the roots as well as in the leaves of N. glauca, but most of the work has been done with root cultures, as this has been the commonly used experimental system in the studies with nicotine biogenesis. As such, it was of interest to find out whether the leaves of N. glauca would also use the suggested

pathway for anabasine biosynthesis (refer to Chapter 2) and also to look into the possibility of alternative pathways from different precursors.

It was first necessary to establish whether the pyridine ring of anabasine does arise from nicotinic acid even in this experimental system. We then went on to determine whether γ-methyleneglutamic acid could serve as a direct precursor for nicotinic acid in an alternative or probably a simultaneous route. This is in a sense, the reversal of the known alkaline degradation of nicotinic acid to α-hydroxymethylglutaric acid, which on vacuum distillation is converted to the corresponding methylene derivative.

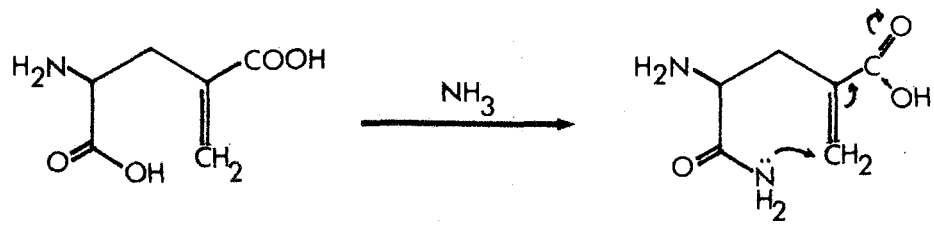
Several γ-substituted derivatives of glutamic acid have been found in large concentrations in plants. These include γ-methyleneglutamic acid and its amide[2,3], γ-methylglutamic acid[4], γ-methyl-γ-hydroxy glutamic acid[4,5] and γ-hydroxyglutamic acid[6]. The structural similarity among these compounds has led to the suggestion that the initial biosynthetic pathway of these involves the condensation of two pyruvate molecules (one, probably in the form of phosphenolpyruvate) to yield γ-methyl-γ-hydroxy-α-ketoglutarate which would be metabolised further either by dehydration or reduction followed by transamination. Shannon and Marcus[7] have reported the isolation of an enzyme system that catalysed the aldol cleavage of γ-methyl-γ-hydroxy-α-keto-

glutarate to two pyruvate molecules.

Considering the above factors, it seemed logical to find out whether pyruvate itself could serve as a direct precursor for nicotinic acid. For this purpose, the incorporation of specifically labeled pyruvate was studied, and the results were compared with those of the incorporation studies using aspartic acid (a suggested precursor) and γ-methyleneglutamic acid. Figure 4 shows a possible scheme for the conversion of γ-methylene glutamic acid to nicotinic acid with the former providing all the carbons while the nitrogen atom is picked up from the ammonia pool.

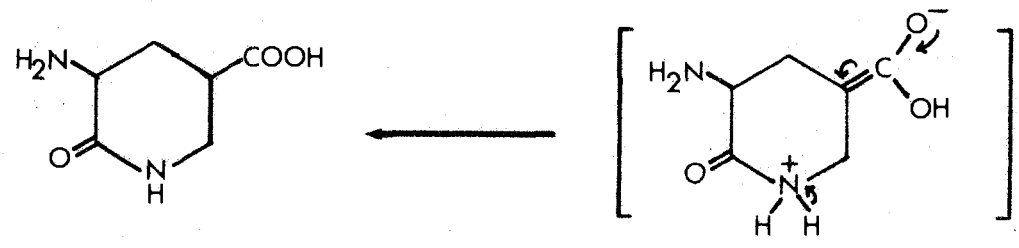
FIGURE 4

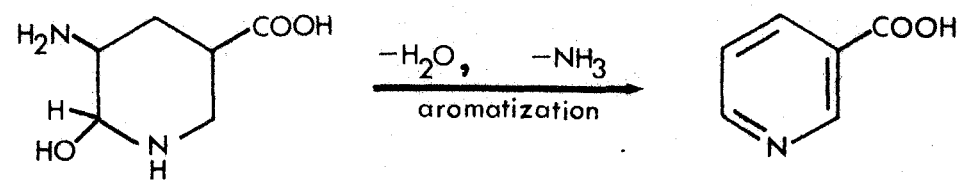
A possible biosynthetic scheme for nicotinic acid starting from γ-methylene glutamic acid



γ-Methyleneglutamic acid

γ-Methyleneglutamide





Nicotinic acid

2. LITERATURE REVIEW

2.1 Relationship between nicotinic acid and *Nicotiana* alkaloids

About 45 years ago, Trier[8] proposed nicotinic acid as a likely precursor of the pyridine ring in the tobacco plant. At that time there had been only one reported isolation of nicotinic acid from a plant source. Robinson[9] raised strong objection to the proposed relation between nicotinic acid and nicotine on chemical grounds. The objections involved:

(i) The inertness of the 3-position of the pyridine ring towards nucleophiles

(ii) The absence of a recorded case of displacement of the carboxyl group of nicotinic acid.

It was over thirty years later that Trier's hypothesis received support from experimental evidence. Dawson and co-workers[10] administered nicotinic acids, specifically labelled with tritium at positions 2, 4, 5 and 6 to tobacco root cultures and demonstrated the retention of hydrogen atoms in positions 2, 4 and 5 during the transformation to nicotine. However, they noted that the hydrogen atom at position 6 is lost. Using the same experimental system, Dawson had previously shown[11] that the ^{14}C label is not transferred from the carboxyl carbon of nicotinic acid to nicotine. Tso and Jeffrey[12] were able to show that ^{15}N labelled nicotinic acid was incorporated into nicotine in *N. rustica* and also that almost all the ^{15}N excess was in

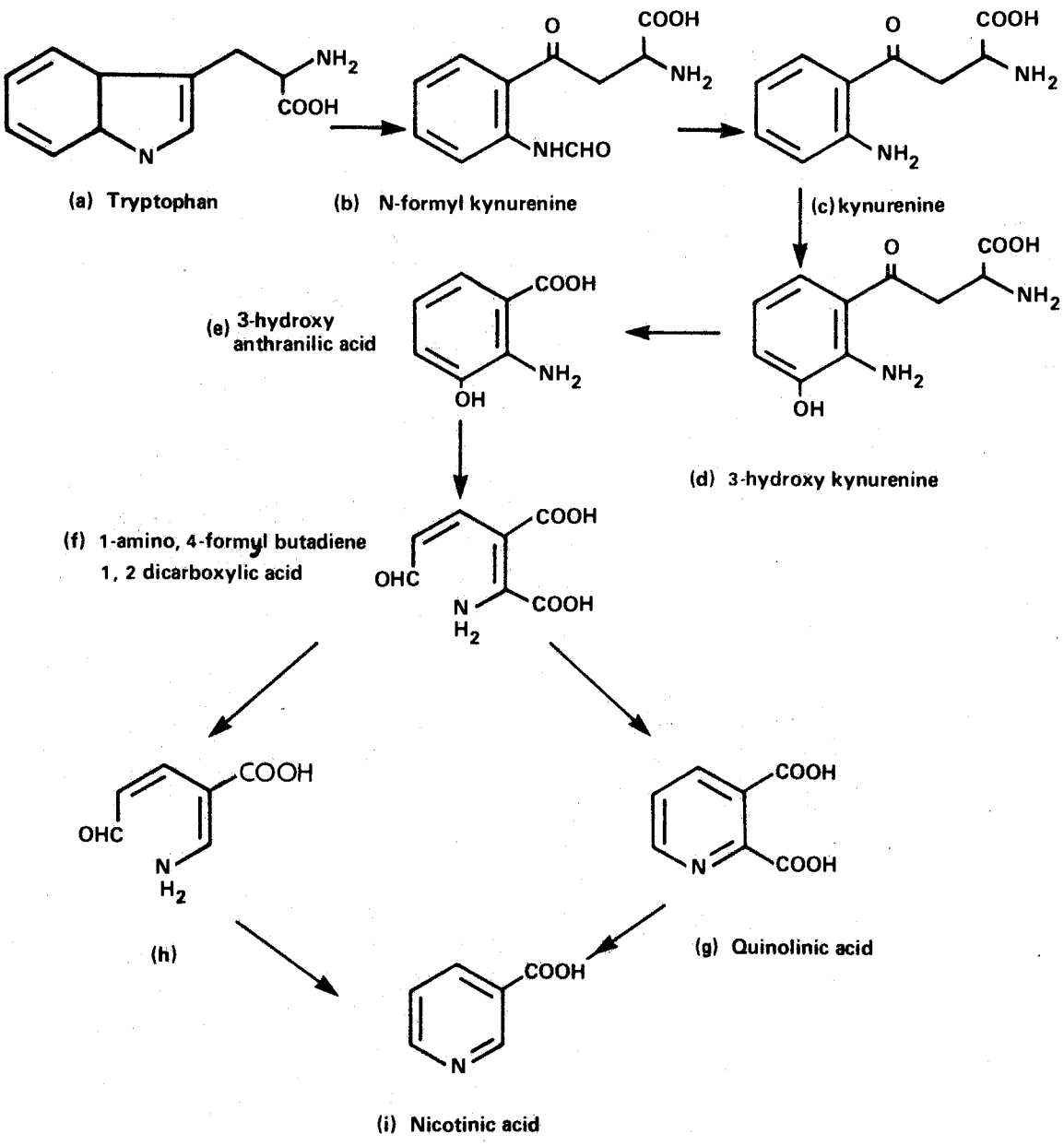
the pyridine ring. These experimental data have been rationalized in the following manner. Since the hydrogen atoms in the positions 2, 4 and 5 of nicotinic acid are retained during its incorporation, it is likely that the carbon atoms in those positions are also retained. To postulate otherwise would require virtually quantitative removal from and restoration to these positions, of the hydrogen isotope as used in Dawson's experiments. It was also suggested that the carbon atom in position 3 is retained owing to the complex nature of bonding at that site. These results led to the conclusion that the pyridine ring of nicotinic acid condenses with the second heterocyclic ring at carbon-3 accompanied by a decarboxylation and also a loss of hydrogen at carbon 6. Possible pathways and mechanisms involved in this condensation have been speculated.

2.2 Nicotinic acid biosynthesis

The fundamental observation made by Elvehjem and his colleagues that tryptophan (Figure 5a) is converted to nicotinic acid in mammalian tissues seemed to explain why symptoms of nicotinic acid deficiency in man could be relieved by the administration of tryptophan. The in vivo conversion of tryptophan into nicotinic acid is shown in Figure 5 and is fully discussed by Goodwin[14]. Tryptophan

FIGURE 5

Tryptophan degradation pathway for nicotinic acid biosynthesis


(a) Tryptophan
(b) N-formyl kynurenine
(c) kynurenine
(d) 3-hydroxy kynurenine
(e) 3-hydroxy anthranilic acid
(f) 1-amino, 4-formyl butadiene 1, 2 dicarboxylic acid
(g) Quinolinic acid
(h)
(i) Nicotinic acid

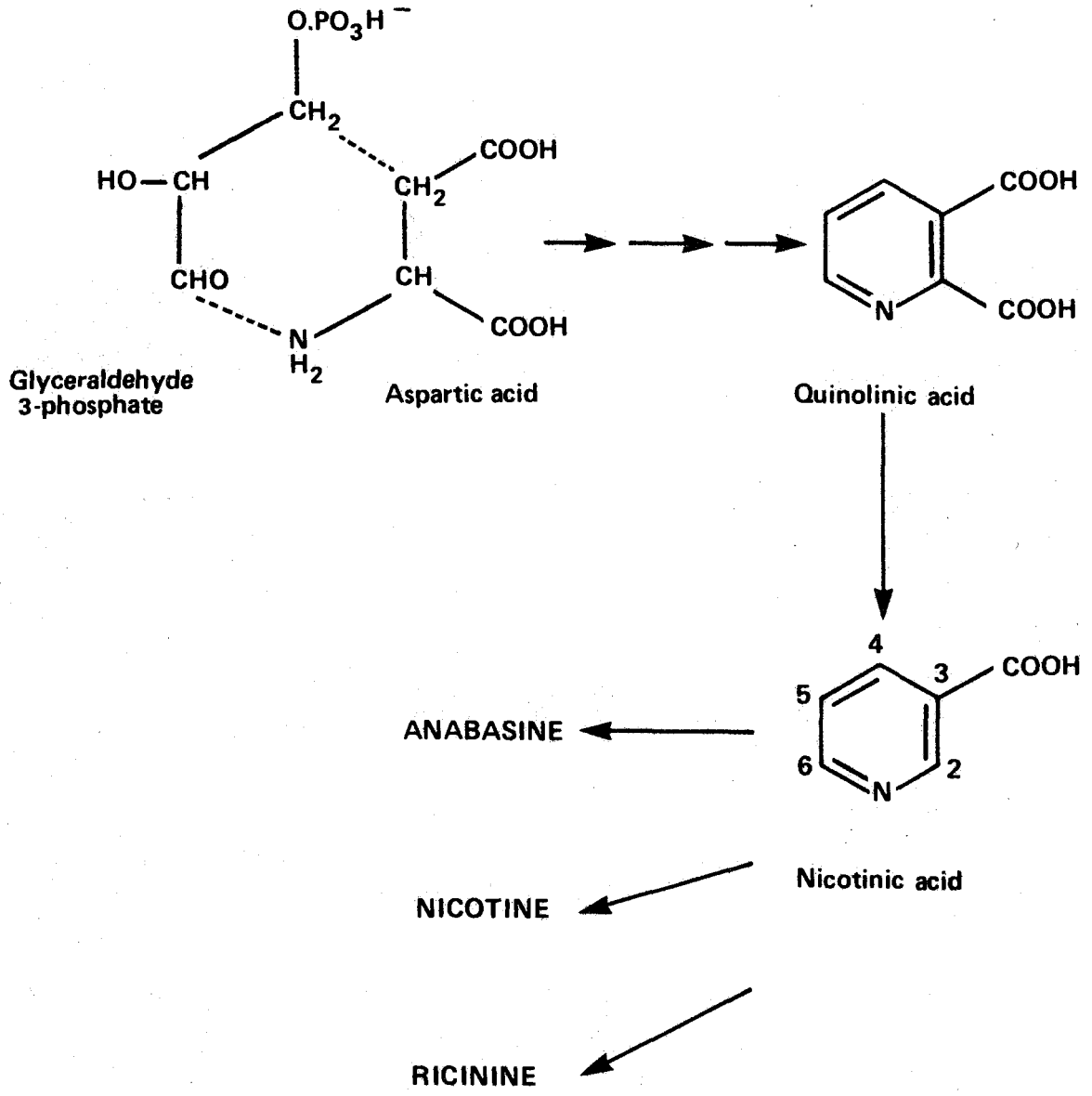
is first converted to N-formyl kynurenine (5b) oxidatively by an iron-porphyrin enzyme system; it then loses its formyl group to give kynurenine (5c). This is hydroxylated to 3-hydroxy kynurenine (5d), the reaction requiring NADPH and then degraded to 3-hydroxy anthranilic acid (5e) by a pyridoxal dependent enzyme. Enzymatic oxidation of this yields 1-amino-4-formylbutadiene-1,2-dicarboxylic acid (5f), which is then converted to nicotinic acid probably via quinolinic acid (5g).

It is generally agreed at present that in mammals, chicks, some fungi and selected bacteria, nicotinic acid biosynthesis proceeds via this pathway. In fact, for some time, this pathway was assumed to be the only one operative in microorganisms. Subsequently, a pathway involving a 3 carbon unit (glycerol or its equivalent) and a 4 carbon dicarboxylic acid (probably aspartic acid) has been implicated in nicotinic acid biosynthesis of some micro-organisms (Figure 6).

Evidence from isotopic experiments suggests the possible absence of the tryptophan degradation pathway for nicotinic acid biosynthesis in higher plants, at least in the plants studied. ^{14}C labelled tryptophan as well as 3-hydroxy anthranilic acid were not incorporated into nicotinic acid in germinating whole corn seeds as well as

FIGURE 6

Glyceraldehyde-aspartic acid pathway for nicotinic acid biosynthesis



Glyceraldehyde 3-phosphate

Aspartic acid

Quinolinic acid

Nicotinic acid

excised soybean leaves. Most of the evidence suggests the glyceraldehyde-aspartate pathway or a related one to be present for the nicotinic acid biosynthesis in Nicotiana species studied.

Byerrum and his colleagues[15] exposed soil grown N. glutinosa and N. rustica plants to $^{14}CO_2$, and when radio-labelled nicotine was isolated and degraded, observed that carbon atoms 4, 5 and 6 were labelled essentially equally, as were carbons 2 and 3. This suggested the possible occurrence of two immediate precursors, one a two-carbon unit and the other a three-carbon unit. As suggested earlier, carbon atoms 2 and 3 were thought to arise from a four-carbon dicarboxylic acid, either succinic or aspartic acid, based on the following experimental evidence:

(i) Acetate 2-^{14}C showed equal incorporation of the label at carbons 2 and 3 in both nicotine[16] and anabasine.[17]

(ii) Succinate 2,3-^{14}C as precursor, showed almost equal incorporation at carbons 2 and 3 of the pyridine ring, with negligible incorporation at carbons 4, 5 and 6.[16]

(iii) Aspartate 3-^{14}C and malate 3-^{14}C administered to N. rustica plants showed almost exclusive incorporation of label into carbons 2 and 3 of the

pyridine ring of nicotine with approximately 60% at C-3 and 40% at C-2.[18]

Observation (iii) above suggested that an unsymmetrical intermediate related to aspartic acid could be the immediate precursor, rather than a symmetrical one such as succinic acid. As pointed out by Byerrum et al.[19], whether or not aspartic acid is the actual immediate precursor for carbons 2 and 3 is open to question. With tobacco and castor plants, when the feedings were over long time periods, no excess incorporation of ^{15}N label was observed with aspartic acid. This was suggested to be due to an equilibration of the isotopic nitrogen atoms with the ammonia pool. Acetate, succinate, malate and aspartate all serve as precursors to carbons 2 and 3, a feature which is explained by involving the Kreb's Tricarboxylic Acid (TCA) Cycle through which all these are interconvertible.

Carbon atoms 4, 5 and 6 are suggested to be derived from glyceraldehyde, based on the following experimental evidence.

(i) Both carbon atoms 4 and 6 of the pyridine ring of nicotine are equally labelled when glycerol 1-^{14}C is administered to N. rustica.[20]

(ii) Carbon 5 is heavily labelled when glycerol 2-^{14}C is used.[20]

(iii) Glyceraldehyde 3-^{14}C administered to the same experimental system showed that over half of the ^{14}C incorporated into nicotine was in position 4 of the pyridine ring while the remainder was almost equally distributed among carbons 2, 3 and 6.[21]

The lack of randomization of the label in positions 4, 5 and 6 indicates that a non-symmetrical intermediate condenses with the C-2 unit to form the pyridine ring. Further, the incorporation of glyceraldehyde 3-^{14}C with a lower dilution than labelled glycerol was taken to suggest that the former is a more immediate precursor than the latter. The appearance of the label on carbons 2 and 3 was thought to be due to glyceraldehyde being metabolized through glycolysis to acetyl CoA and then being converted to succinate (or aspartate) via the TCA cycle.

Thus it appears as if the problem of the origin of nicotinic acid in higher plants is fully solved, with the glyceraldehyde-aspartate pathway being the most likely and probably the only one operative. However, as pointed out by Byerrum[19], there are some results that cannot be easily fitted into this picture. The most apparent are from the group consisting of alanine, β-alanine and propionic acid which show a labelling pattern distinctly different from that shown for any other compound when incorporated into nicotine or ricinine. Most experimental evidence suggest

that the 2-pyridone ring of ricinine (Figure 1.d) is also derived from nicotinic acid via the glyceraldehyde-aspartate pathway. Marion and co-workers[22] thought it would be of interest to find out whether the simpler compounds and amino acids (which are interconvertible with other precursors via the TCA cycle) could also act as precursors for ricinine. When β-alanine 2-^{14}C and propionic acid 2-^{14}C were administered to Ricinus communis plants and ricinine isolated, carbon atom 3 contained almost twice the activity of carbon 2, the labelling pattern being quite different from that observed with acetate and succinate feeding. Thus it seems that neither β-alanine nor propionate is converted to a four-carbon dicarboxylic acid prior to incorporation. At the same time, the high radiochemical yield of alkaloid given by β-alanine suggest that it acts as an immediate precursor. Therefore the biosynthetic pathway through which β-alanine is incorporated into ricinine must be very distinct from that involving acetate.

Although these results have more or less been overlooked, they may be of some significance. It suggests that although the glyceraldehyde-aspartate pathway is quite likely, it need not be the only one operating in all higher plants. Further, it has been shown[23] that in the yeast Saccharomyces cerevisiae, incorporation of radio-

activity occurs from ^{14}C-aspartate and ^{14}C-glutamate into nicotinic acid under anaerobic conditions, but when grown aerobically, the transfer of the label was markedly reduced. On the other hand, a reverse effect was observed with tryptophan and 3-hydroxy anthranilic acid, where the transfer of radioactivity was observed under aerobic conditions which was reduced under anaerobic conditions. Although it has not been established whether the two pathways do exist simultaneously to the same extent under for example, low oxygen pressure, this clearly indicates that two different pathways can exist in the same organism and the establishment of one does not exclude the possibility of another one existing.

3. EXPERIMENTAL

3.1 Growth of Nicotiana glauca plants

N. glauca plants were grown in soil containing pots in a growth chamber under controlled day length and temperature conditions. The plants continued in vegetative growth when the day length was 11 hours. A daytime temperature of 27° C and a night temperature of 13° C were suitable for optimum growth of these plants. They were continuously supplied with a nutrient solution of the following composition:[24]

$Ca(NO_3)_2.4H_2O$	3×10^{-3} M
KNO_3	2×10^{-3} M
$MgSO_4.7H_2O$	2×10^{-3} M
KH_2PO_4	2×10^{-3} M

The following compounds were supplied in microquantities:

	$mg.L^{-1}$
H_3BO_3	2.5
$MnCl_2.4H_2O$	1.8
$ZnSO_4.H_2O$	0.14
Fe(EDTA)	5.0
$CuSO_4.5H_2O$	0.08
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.16

Day temperatures less than 21° C had an adverse effect on the growths of the plants, especially the leaves. The leaves remained small, developed pale green or yellow

spots and were also found to get infected rather easily. Further, the alkaloid content was diminished greatly; indeed anabasine was then present only in trace quantities.

3.2 Labeled compounds

The following ^{14}C-labeled compounds were used for this study:

(i) [U-^{14}C] Nicotinic acid

(ii) γ-Methylene D,L-[2-^{14}C] glutamic acid

(iii) Sodium [1-^{14}C] pyruvate

(iv) Sodium (2-^{14}C] pyruvate

(v) Sodium (3-^{14}C] pyruvate

(vi) L-[U-^{14}C] Aspartic acid

Compounds (iii) to (vi) were purchased from New England Nuclear, Canada, and stored at -10° C until used. γ-methylene glutamic acid was custom synthesized by NEN, Boston, Massachusettes. The radiochemical purity of this sample was determined by two-way ascending chromatography and autoradiography. A 10 μℓ aliquot of an aqueous solution (containing 1 mg. of the compound in 1 ml.) was spotted on a Whatmann 3 MM paper (8.5" x 8.5") and developed for 6 hrs. in 80% aqueous phenol. (R_f = 0.5) The paper was dried and run in propionic acid-n-butanol-water (2:2:1) for 3 hrs., at right angles to the original direction (R_f = 0.38). The chromatogram was used for autoradiography with

No Screen X-ray film and then sprayed with the colour reagent: 0.1% ninhydrin in butanol saturated water, with 1% glacial acetic acid. The X-ray film showed a single dark spot corresponding to the single coloured spot on the paper chromatogram. This spot also corresponded to the standard (non-radioactive) γ-methylene glutamic acid spot run in the same solvent.

Ring labeled nicotinic acid was not available commercially and was obtained by oxidation of biosynthetic nicotine. For this purpose, three young pot-grown N. tabacum plants were exposed to approximately 1.3 mCi of $^{14}CO_2$ for 1 day, using the method described below, and then transferred to a normal atmosphere and allowed to grow for one week. Nicotine was then isolated from the leaves, oxidised to nicotinic acid and purified by the method given below. The sample was considered to be uniformly labeled.

3.3 Administration of labeled compounds

The leaves used in all the feeding experiments were at the same stage of growth, approximately 1 1/2 to 2 months old. Preliminary exploratory experiments showed that leaves of this age showed the highest incorporation of the label from $^{14}CO_2$ into anabasine.

3.3.1 $^{14}CO_2$ feeding

An excised leaf, with the cut end of its petiole in distilled water, was placed in an illuminated glass chamber and a closed system was set up as shown in Figure 7. A known weight of $Ba^{14}CO_3$ was placed as shown and the $^{14}CO_2$ was generated by injecting 1 ml of 10% perchloric acid. The pump was subsequently started to maintain a constant circulation of air within the system and the decrease in radioactivity was monitored using a Geiger-Müller counter. When the chamber showed a 99.5% assimilation of $^{14}CO_2$, the system was exhausted into an NaOH solution to trap the excess $^{14}CO_2$. The leaf was returned to a normal atmosphere, kept illuminated for a further 5 hrs., and then kept in the dark overnight until anabasine was extracted the following day.

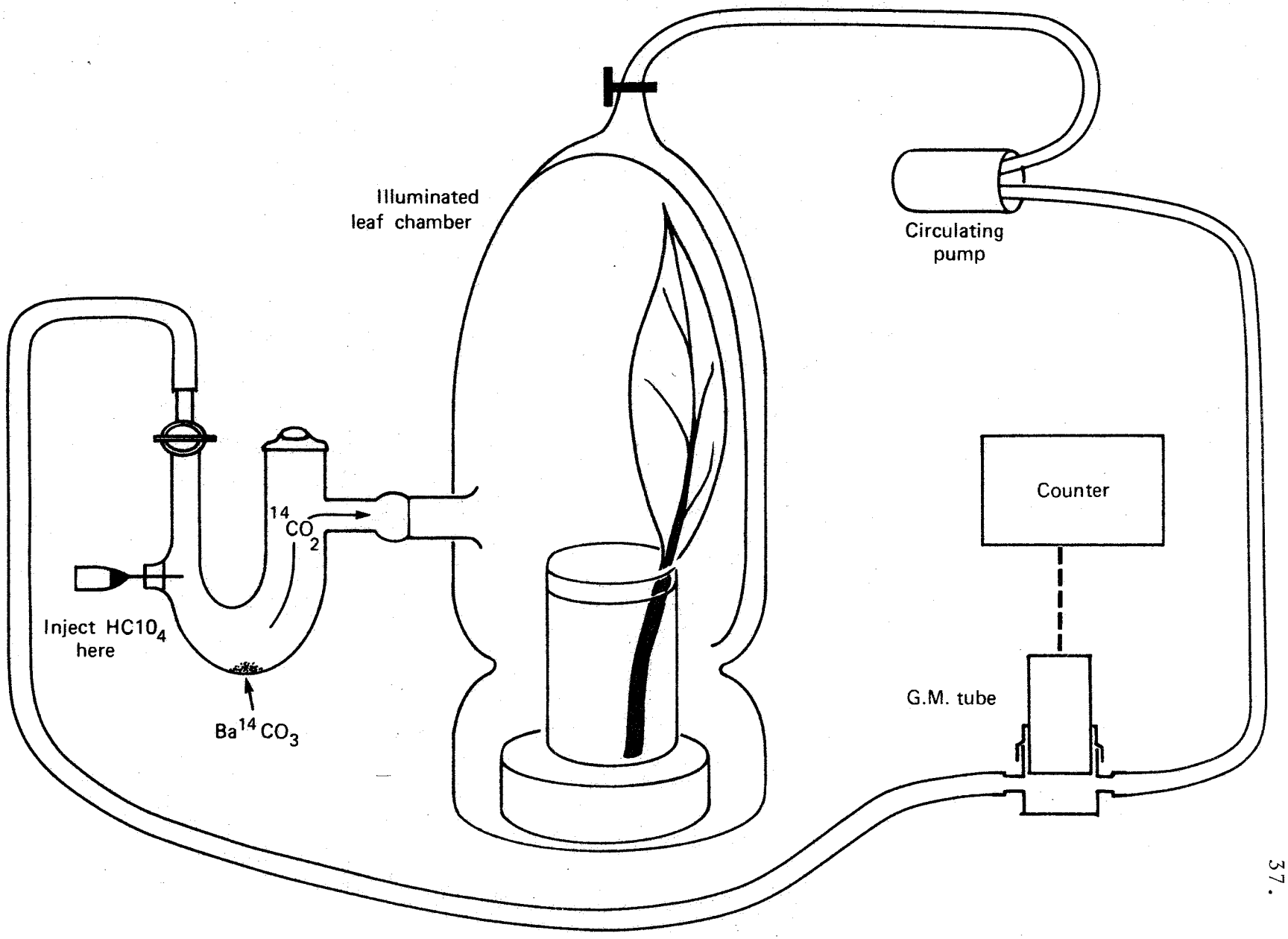
3.3.2 Feeding of other labelled compounds

Each of the other radioactive compounds was administered to an excised leaf through the cut end of the petiole, via the transpirational stream. The feeding procedure was the same for all compounds.

The leaf was carefully detached from the plant, making sure that no air bubbles entered the transpirational stream. With the cut end of the petiole in distilled water, the leaf was illuminated for approximately 1 hr. A known

FIGURE 7

Apparatus for $^{14}CO_2$ feeding


Illuminated
leaf chamber
Circulating
pump
Counter
G.M. tube
$^{14}CO_2$
Inject $HC10_4$
here
$Ba^{14}CO_3$

amount of the compound to be fed was dissolved in 300 µℓ of distilled water and the leaf was transferred into this solution. When almost all the solution was sucked up into the leaf, a further 300 µℓ of water was added. This was repeated several times until the remaining solution showed less than 0.5% of the total activity supplied (approximately 2-3 hrs.). The leaf was then transferred back to distilled water and illumination was continued for a total of 10 hrs., after which it was left in the dark, overnight until anabasine was extracted the following day.

[U-^{14}C] Aspartic acid was obtained as a solution in 0.01 N hydrochloric acid (50 µCi in 500 µℓ) and therefore had to be treated in a different way. A 50 µℓ aliquot of this solution was pipetted out into a 5 ml. pear-shaped flask, 200 µℓ of distilled water added and then dried in a vacuum. This process was repeated (5 x 200 µℓ water) to ensure complete removal of HCl. The remaining solid was dissolved in 500 µℓ of water, from which a 5 µℓ aliquot was pipetted out for radioactivity determination, and the balance administered to the leaf as described above.

3.4 Extraction and isolation of anabasine

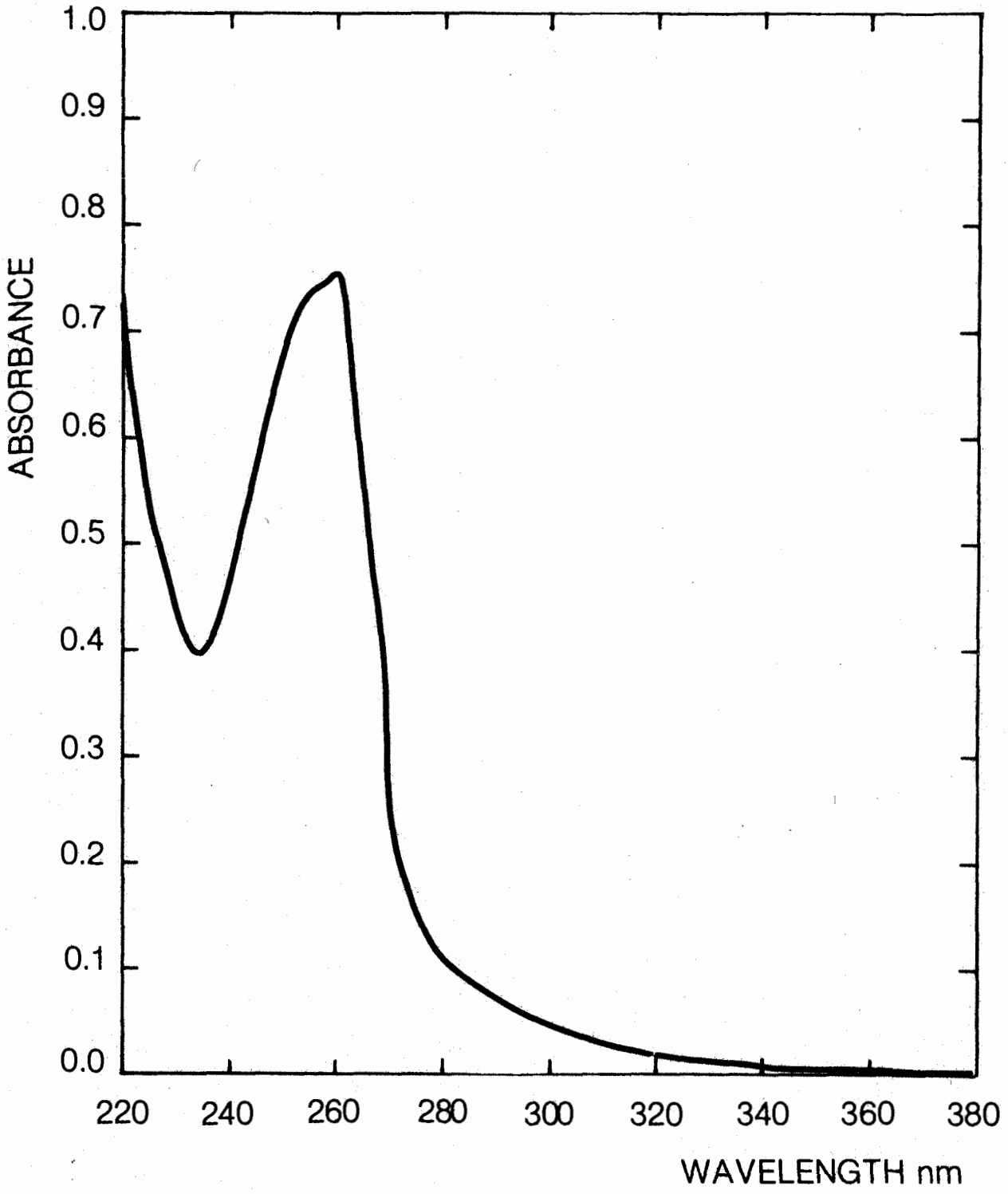
The test leaf was ground in 20 ml. of 80% ethanol at room temperature and the extract centrifuged to remove

the debris. The supernatant was removed and the debris re-extracted and centrifuged. An aliquot of the combined supernatants was used to determine the total radioactivity of the extract. The rest (around 40 ml.) was diluted with 15 ml of water and reduced to 35 ml in vacuo at room temperature. This was extracted with 5 x 20 ml petroleum ether (30° - 60°) to remove chlorophylls and other pigments.

The aqueous solution remaining after the removal of pigments was concentrated down to 3 - 4 ml. in vacuo, made basic (pH = 10.5) with dilute NaOH solution, and extracted continuously with ether for 72 hrs. The ether extract was dried with anhydrous $MgSO_4$ and concentrated in a flash evaporator. This was applied as a band on Whatmann 3 MM chromatography paper (9" x 22"; pretreated with 0.1 M., pH 6.5 phosphate buffer) and developed (descending) in a solution containing 100 ml. n-butanol and 20 ml. 0.1 M, pH 6.5 phosphate buffer for 40 hrs. A standard solution of anabasine in ethanol was spotted as reference. The band corresponding to standard anabasine was detected under UV light, removed and eluted with methanol. The methanolic solution was evaporated to a known volume and the anabasine concentration determined spectrophotometrically. A typical UV spectrum is shown in Figure 8. (λ_{max} = 261 nm; log ε_{max} = 3.46) This solution was concentrated to a

FIGURE 8

UV absorption spectrum of
anabasine in methanol


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
220
240
260
280
300
320
340
360
380
WAVELENGTH nm

smaller volume and 1 ml removed for counting. The specific activity of anabasine extracted was determined from the above results.

3.5 Oxidation of anabasine

The methanolic anabasine solution was evaporated to remove the solvent, 10 ml of water added and then evaporated again in vacuo to remove the last traces of alcohol. The residual anabasine was dissolved in 10 ml of water and a known amount of standard anabasine (5 µℓ or 10 µℓ) was added. Twenty mg. NaOH and 120 mg $KMnO_4$ were added and the solution was heated on a steam bath for 18 hrs. with occasional mixing. A 37% formaldehyde solution was added dropwise to the cooled reaction mixture until the permanganate colour was discharged. The resulting brown suspension was digested on a steam bath and filtered under suction. The solid residue was resuspended in 5 ml water, heated to boiling and filtered. The combined filtrate was acidified (pH = 1 or 2) with conc. HCl warmed to remove CO_2 and then reduced almost to dryness in vacuo. The residue was dissolved in about 3 ml. of water and a dilute solution of NaOH added until the pH reached 4.5 to 5.0. The resulting solution was taken to dryness under a stream of dry air. The residual salts were sublimed at 160°C/0.1 torr pressure, after the addition of a known quantity of carrier nicotinic acid. The sublimate was dissolved in methanol, diluted to a known volume and the

nicotinic acid concentration determined spectrophotometrically. A typical UV absorption spectrum is given in Figure 9. [λ_{max} = 262 nm.; log ε_{max} = 3.42. The amount of nicotinic acid obtained from anabasine oxidation was evaluated by subtracting the amount of carrier added from the total nicotinic acid in the sublimate. This was possible since there is complete recovery of the carrier during sublimation.

The methanolic solution was then concentrated down to 1 ml and used for radioactivity determination.

3.6 Pool size determination

The pool size within the leaf of each compound administered as a substrate, was determined by an isotope dilution technique. A 1 1/2-to-2-month old leaf was used in each case. Four separate leaf extractions were carried out for the four substrates: nicotinic acid, aspartic acid, γ-methylene glutamic acid and pyruvic acid. In each case, a known amount of radioactive compound was added at the initial stage of extraction. The specific activity of each compound extracted was compared with that of the labeled compound added, in order to determine the dilution due to the inherent pool. The pool size was estimated in each case using the following relationship:

$$M_u = \left(\frac{S.A._0}{S.A._1} - 1 \right) M_o$$

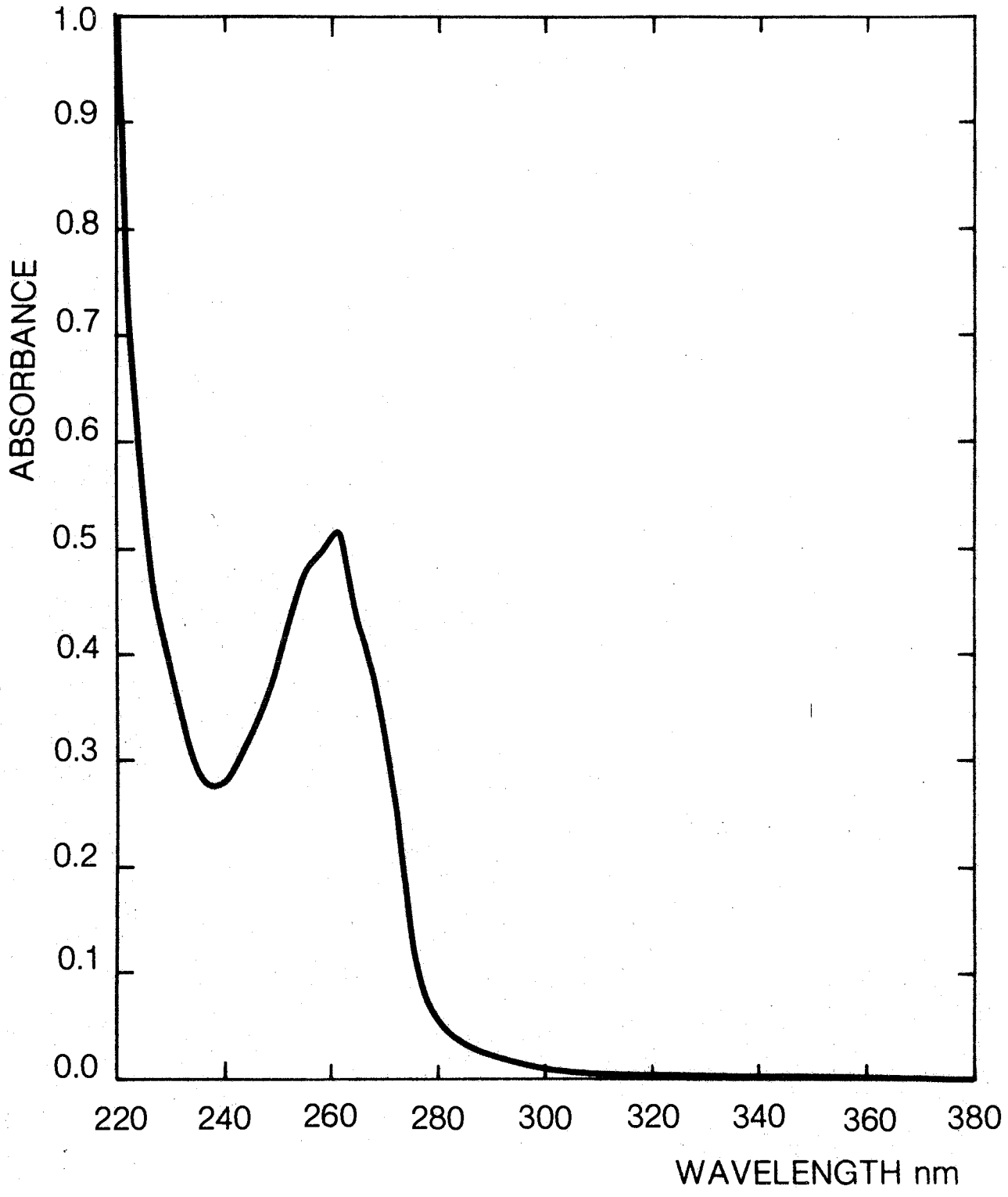
where

M_u = Unknown amount of compound in leaf (pool size)

M_o = Amount of tracer added

FIGURE 9

UV absorption spectrum of
nicotinic acid in methanol


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
220
240
260
280
300
320
340
360
380
WAVELENGTH nm

$S.A._0$ = Specific activity of tracer added

$S.A._1$ = Specific activity of compound isolated

3.6.1 Nicotinic acid

The leaf was extracted with 2 x 20 ml 80% ethanol and the debris centrifuged down. A known amount of nicotinic acid - ^{14}C (U) was dissolved in the combined supernatant, diluted with 15 ml. of water and the volume reduced to 35 ml at room temperature in vacuo. The pigments were removed by extraction with 5 x 15 ml petroleum ether (30° - 60°) and the aqueous solution concentrated. The concentrate was applied as bands on two 9" x 21" Whatmann 3 MM paper along with a standard nicotinic acid solution and chromatographed (descending) for 15 1/2 hrs. in solvent 1, the upper phase of a mixture of n-butanol, glacial acetic acid and distilled water (25:6:25).[25] The papers were examined under UV light, and the dark bands corresponding to standard nicotinic acid (R_f = 0.748) were cut off, cut into small pieces and eluted with 3 x 30 ml boiling methanol. The combined methanol eluate was filtered through a sintered glass funnel to remove paper fibres, concentrated down and reapplied as a band alongside standard nicotinic acid on a 9" x 21" Whatmann 3 MM paper. This was developed for 10 hrs., descending in solvent 2, a mixture of 95% ethanol and 58% ammonium hydroxide (95:5).[25]

The band corresponding to standard nicotinic acid (R_f = 0.516) was eluted as before in methanol, made upto a known volume and the concentration of nicotinic acid determined spectrophotometrically. The total activity was determined by liquid scintillation counting of the above solution and the specific activity calculated.

3.6.2 Pyruvic acid

The method used here was adapted from El Hawary and Thompson's procedure[26] for the separation and estimation of blood keto acids. The relative instability of pyruvic acid necessitates its extraction as a derivative, in this case the 2,4-dinitrophenylhydrazone. A known amount of ^{14}C-labeled sodium pyruvate was added into the tissue grinder containing 20 ml of 2N hydrochloric acid, and the test leaf was ground in this solution. The extract was centrifuged and the supernatant added to 40 ml. of a solution of 2,4-dinitrophenylhydrazine in 2 N HCl (2 mg/ml). The debris was reextracted with a further 20 ml portion of 2N. HCl, centrifuged and the supernatant added to the above solution. The combined extracts (in 2,4-dinitrophenylhydrazine) were incubated for 45 min. at 38° C, extracted with 5 x 25 ml of ethyl acetate, and the combined organic layers (in the form of an emulsion) were centrifuged for 20 min. at 2500 rev. min^{-1}. The upper

organic phase was pipetted out and the lower aqueous phase reextracted with fresh ethyl acetate (4 x 10 ml.). This was centrifuged as before and the organic layer combined with the previous ethyl acetate extract. The combined extracts were concentrated down to approximately 40 ml. and then extracted with 6 x 15 ml of 10% Na_2CO_3 solution. The combined Na_2CO_3 extracts were washed once with 10 ml ethyl acetate to remove any trace of unreacted 2,4-dinitrophenylhydrazine and then acidified dropwise with ice-cold conc. HCl until no evolution of CO_2 could be observed. The hydrazones were reextracted into ethyl acetate (6 x 20 ml), dried with anhydrous $MgSO_4$ and the solvent evaporated in vacuo. The solid residue was dissolved in a few drops of phosphate buffer (0.1 M; pH = 7.2) and applied as a band on 8" x 8" Whatmann 3 MM chromatographic paper with standard pyruvic acid - 2,4-dinitrophenylhydrazone on either side. The chromatogram was developed ascendingly for 4 1/2 hrs. in a mixture of n-butanol, ethanol, 0.5N ammonium hydroxide (7:1:2). The band corresponding to the standard compound (R_f = 0.35) was cut off, eluted with ethyl acetate, the solvent evaporated and rechromatographed as described previously. The paper was autoradiographed and the radioactive band cut off, cut into small pieces and

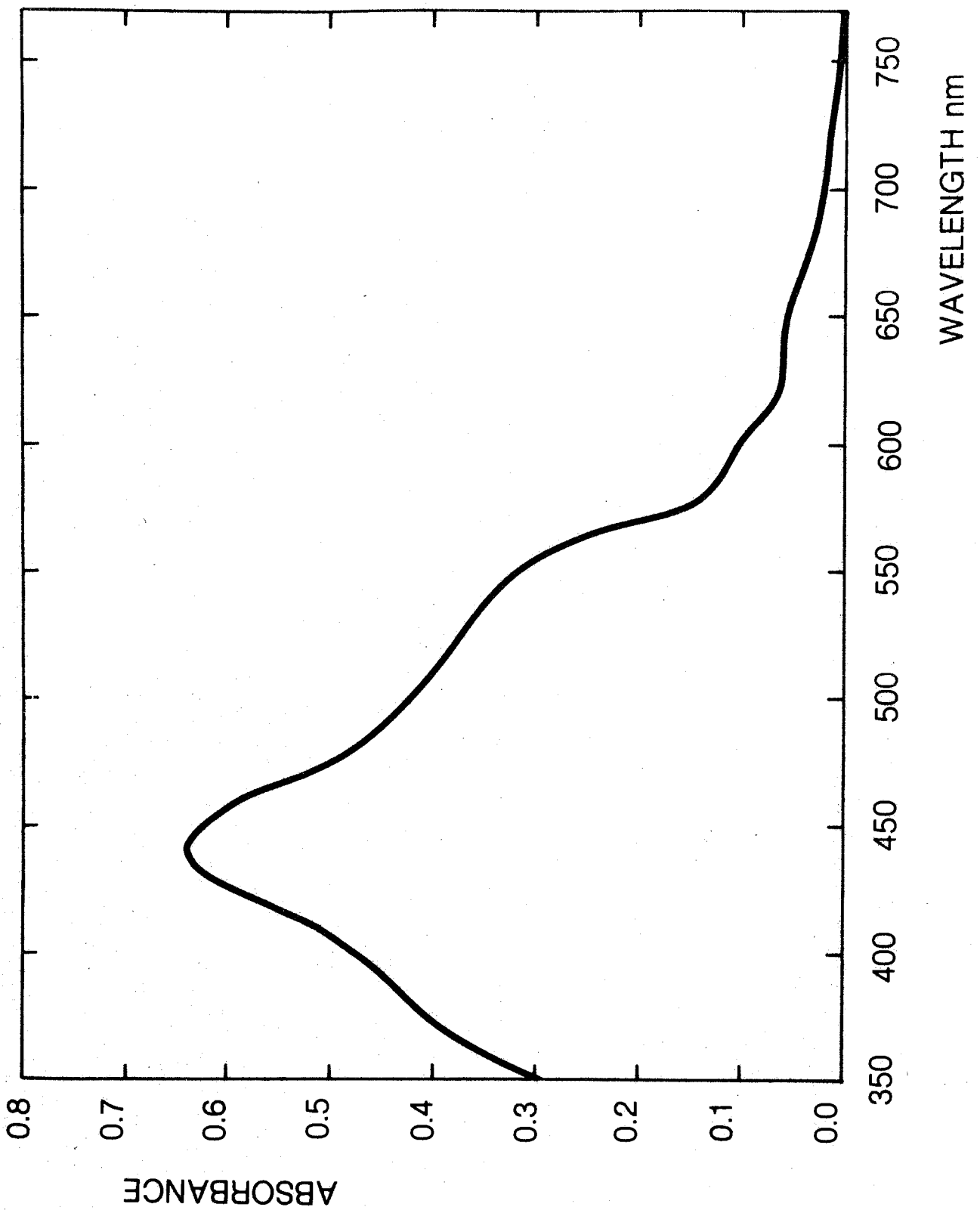
placed in a 15 ml sintered glass funnel connected to a filter tube. Three ml of 10% Na_2CO_3 solution were added and the paper was allowed to disintegrate. The mixture was then filtered under suction. Two ml. of 2 N. NaOH solution were added to the residue on the funnel, stirred and filtered. This was repeated with a further 2 ml of NaOH solution and the combined filtrate diluted to 10 ml with distilled water. A known aliquot of this solution was used for radioactivity determination. The visible absorption spectrum of sodium pyruvate - 2,4-dinitrophenylhydrazone in strongly basic medium shows a λ_{max} at 442 nm. (Figure 10), which was used to estimate its concentration in the extract. A standard curve of A_{442} vs. concentration of the synthetic standard compound was made and at each concentration, a visible spectrum was recorded to ensure the presence of the absorbance maximum at λ = 442 nm. From these results, the specific activity of pyruvic acid extracted was computed and subsequently used for the estimation of the pool-size.

Preparation of pyruvic acid - 2,4-dinitrophenylhydrazone

Two hundred and fifty mg. of sodium pyruvate were dissolved in 5 ml of water and the solution combined with 75 ml of a saturated solution of 2,4 dinitrophenylhydrazine in 2 N. HCl. The mixture was stirred and allowed to

FIGURE 10

Visible absorption spectrum of pyruvic acid-2,4-dinitrophenylhydrazone in strongly basic solution


ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
350
400
450
500
550
600
650
700
750
WAVELENGTH nm

stand for 30 min. The yellow solid was filtered under suction and recrystallised twice from hot glacial acetic acid: m. pt. = 217° C - 218° C[26][Found: C = 40.2%: H = 3.0%; N = 20.7%. Calculated for $C_9H_8O_6N_4$: C = 40.9%; H = 3.0%; N = 20.9%]

3.6.3 Aspartic acid and γ-methylene glutamic acid

The pool sizes of the two amino acids under study were evaluated separately using the same experimental procedure. In each case, the leaf was ground in 2 x 20 ml 80% ethanol, debris centrifuged out and a known amount of radioactive amino acid mixed with the supernatant. The pigments were removed with 30° - 60° petroleum ether as described previously, the aqueous portion concentrated and applied as a band on a 8" x 8" Whatmann 3 MM chromatographic paper. The chromatogram was developed ascendingly in 80% aqueous phenol for 6 hrs, dried and autoradiographed in order to locate the radioactive band. The band was cut off, cut into small pieces, placed in a 60 ml sintered glass funnel connected to a buchner flask and disintegrated with 30 ml. distilled water. The extract was filtered by suction and the process repeated with 2 x 20 ml of water. The combined water extracts were freeze-dried, the residue dissolved in a few drops of water and rechromatographed in 80% aqueous phenol. The radioactive band was

eluted in the manner described previously, after locating it by autoradiography. The contents of this band were next chromatographed in a second solvent, a mixture of butyric acid, butanol and water (2:2:1) for 4 hrs. The radioactive band was located by autoradiography and the amino acid eluted as before. The eluate was concentrated to a known volume and an aliquot removed for radioactivity measurements. The remaining solution was freeze-dried, dissolved in 1 ml water and its concentration determined colorimetrically as given below.

The procedure used for the estimation of the amino acid concentration was a modified ninhydrin colorimetric analysis developed by Rosen.[27] The 1-ml. sample obtained after chromatographic purification was treated in the same way as 1 ml samples of known concentrations from which a standard curve was made.

Reagents used:

(a) Cyanide-acetate buffer

(i) An NaCN solution was prepared by dissolving 4.9 mg in 100 ml distilled water.

(ii) An acetate buffer solution was prepared by dissolving 27 g. sodium acetate trihydrate in 20 ml of distilled water, adding 5 ml glacial acetic acid and diluting the solution to 75 ml.

One ml of the cyanide solution (i) was diluted to 50 ml with acetate buffer solution (ii).

(b) 3% Ninhydrin solution

Three g. of ninhydrin were dissolved in ethylene glycol monomethyl ether (methyl cellosolve) and the volume adjusted to 100 ml.

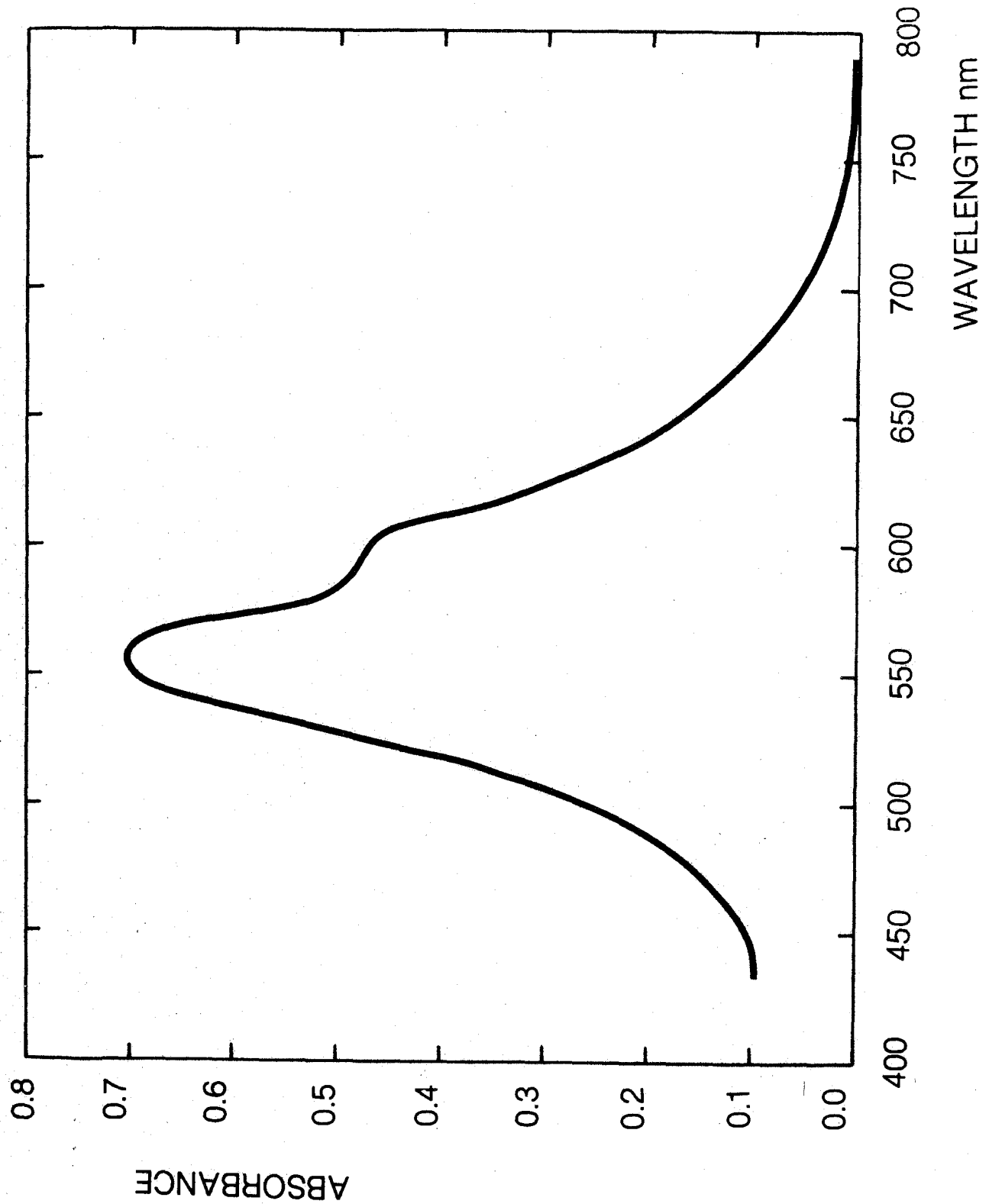
(c) Diluent

This consisted of a solution containing equal volumes of distilled water and isopropyl alcohol.

To 1 ml solution of amino acid, 0.5 ml. of the cyanide-acetate buffer and 0.5 ml of ninydrin solution were added and the mixture was heated in a stoppered tube for 15 min. in a water bath at 100° C. Immediately after removal from the bath, 5 ml of the diluent were added, mixed thoroughly with a Vortex mixer and allowed to cool to room temperature. Each solution was diluted to 10 ml with the diluent and the absorbance determined at λ = 570 nm. All amino acids (except proline and hydroxyproline) show a characteristic absorption band with a λ_{max} at 570 nm. (Figure 11). The concentration of the amino acid in the final chromatographically-pure extract was determined by comparison with the corresponding standard curve of A_{570} vs. amino acid concentration.

FIGURE 11

Visible absorption spectrum of
aspartic acid-ninhydrin complex
in cyanide-acetate buffer: pH 5.0 to 5.2


ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
650
700
750
800
WAVELENGTH nm

3.7 Radioactivity determinations

In all leaf extractions, the radioactivity of the initial alcohol extract as well as that of the aqueous solution left after the removal of pigments were determined using a Nuclear Chicago model 8775 counter operating with an external detector (Geiger-Müller end window type). Twenty or forty-minute counts were recorded and the counting errors were determined at a 95% confidence level using the following relationships:

$$\pm r_{b+s} = \frac{1.96\sqrt{N_{b+s}}}{T_1}$$

$$\pm r_{b} = \frac{1.96\sqrt{N_{b}}}{T_2}$$

$$\pm r_{s} = \sqrt{(r_{b+s})^2 + (r_b)^2}$$

where: r_s, r_b and r_{b+s} are the counting errors of sample only, background only and background plus sample respectively,

N_b and N_{b+s} are the total counts of background alone and background plus sample respectively,

T_1 and T_2 are the total counting times for sample plus background and background alone respectively.

All samples for these countings were prepared on concentric copper planchets.

Radioactivity of the other solutions was determined using a Beckmann LS - 250 liquid scintillation system. All counts were taken with the window settings shown in Figure 12. All samples were counted for 100 min or to a 0.3% error (2σ), to minimize the counting error. The ^{14}C count was obtained from the B channel reading, since no ^{3}H was present in any of the samples. The $dis.min^{-1}$ in each case was evaluated from the $counts.min^{-1}$ of B channel, external standard ratio S and background $counts.min.^{-1}$, using a prewritten computer programme for the conditions used.[28] The cocktail used throughout these experiments had the following composition:

	$g.L^{-1}$
2,5-diphenyl oxazole (PPO)	7.0
p-bis[2-(phenyl oxazolyl)]-benzene	0.3
Naphthalene	100.0

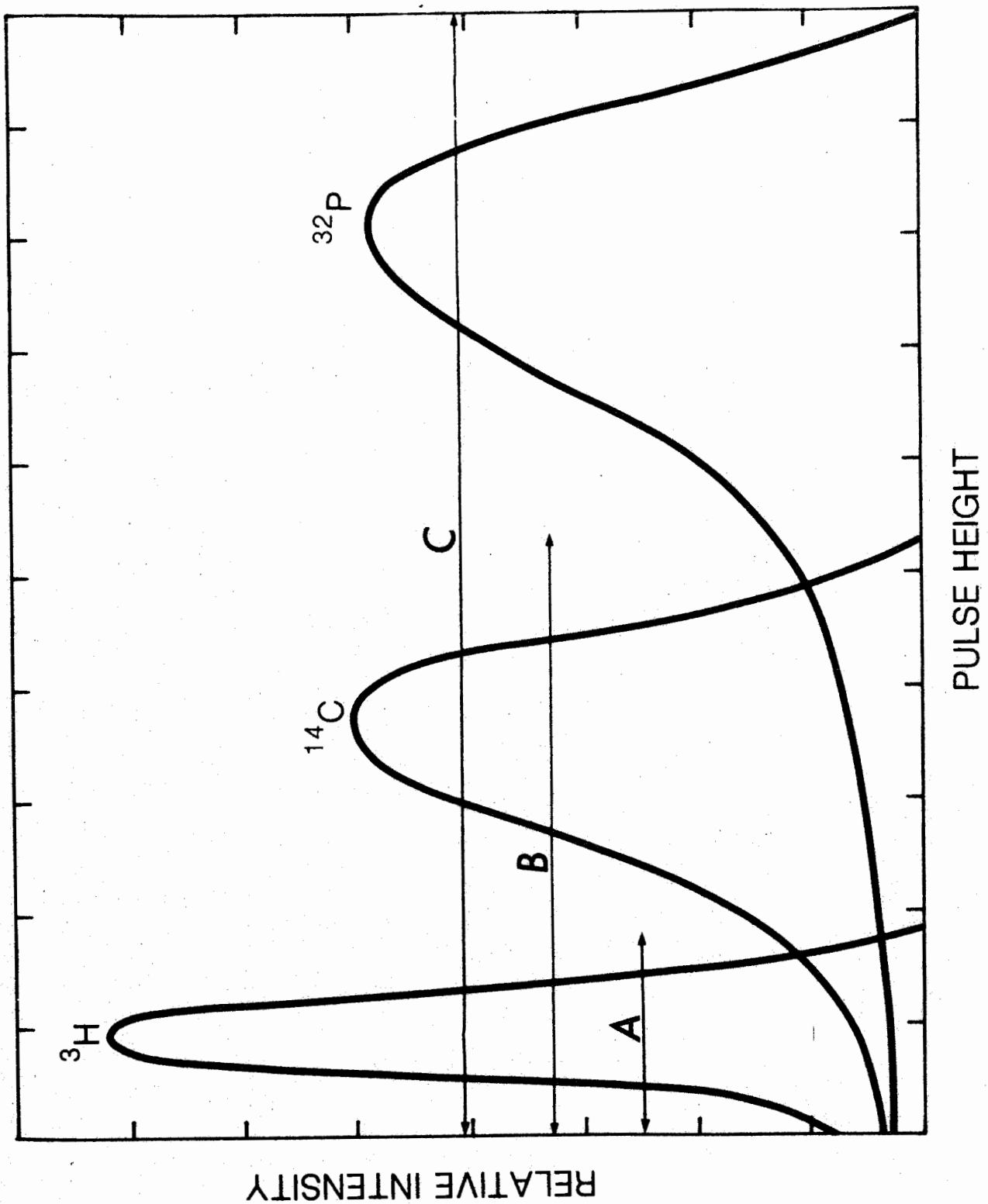
The solution was made in scintillation-quality dioxane. Each vial was first counted with 20 ml. of cocktail to obtain the background count for the specific sample. Thereafter, 19 ml of the same solution were counted with 1 ml of sample.

3.8 Absorbance measurements

All UV-visible spectra were recorded on a Cary 17 model recording spectrophotometer. The A_{max} values for pool size determinations were measured using a Varian-

FIGURE 12

Typical spectra of relative intensity vs pulse height for the three isotopes ^{3}H, ^{14}C and ^{32}P showing the window settings for channels A, B and C.


RELATIVE INTENSITY
PULSE HEIGHT
^{3}H
^{14}C
^{32}P
A
B
C

Techtron model 635 uv-visible spectrophotometer.

3.9 Autoradiography

The chromatogram on which the radioactive region is to be located, was marked at the four corners with a radioactive ink. Kodak No-Screen X-ray film was cut into the size of the chromatogram and exposed in an X-ray cassette for a sufficient length of time. The film was then developed, alligned with the chromatogram using the four marker spots and the region corresponding to the dark band on the film was cut off.

4. RESULTS AND DISCUSSION

4.1 Feeding of radiolabeled compounds

Table I gives the quantities, the specific activities and the corresponding total activity of each substrate administered. It could be seen that approximately 5 μCi of total activity was fed in each case, except for nicotinic acid. Due to the low specific activity of the biosynthetically prepared nicotinic acid, it would have been necessary to introduce over 25 mg of material into a single leaf in order to administer 5 μCi. This amount would not only have upset the normal metabolism of the leaf, but also would have had a toxic effect.

The radioactivity measurements of the initial extracts of the six feeding experiments are given in Tables II to VII. The rest of the results and the important information obtained from them are presented in the form of the following equations:

(i) The total amount of anabasine extracted (M_a) after the feeding of the labeled compound is given by:

$$M_a \text{ (mmoles)} = \frac{A_{261} \cdot V_a}{\varepsilon_a \cdot 1} \qquad (1)$$

where A_{261} = Absorbance at λ = 261 nm.

V_a = Total (effective) volume of extract (ml)

ε_a = Molar extinction coefficient at λ = 261 nm. (2884 $cm^2 . mole^{-1}$)

	Substrate	Specific Activity $dis.min^{-1} mM^{-1}$	Quantity Fed mg	Total Radio-activity Fed μCi
1.	[U-^{14}C] Nicotinic Acid m. wt. = 123	4.6×10^{7}	0.985	0.17
2.	[U-^{14}C] Aspartic Acid m. wt. = 133	4.5×10^{11}	3.8×10^{-3}	5.73
3.	γ-Methylene [2-^{14}C] glutamic acid m. wt. = 159	1.2×10^{9}	1.485	5.13
4.	Sodium [1-^{14}C] pyruvate m. wt. = 110	1.9×10^{10}	7.5×10^{-2}	5.79
5.	Sodium [2-^{14}C] pyruvate	8.3×10^{9}	0.185	6.20
6.	Sodium [3-^{14}C] pyruvate	4.0×10^{10}	3.0×10^{-2}	4.91

TABLE I. Specific activity, quantity fed and the total radioactivity administered of each substrate.

1 = Path length of UV cell (1 cm)

(ii) Specific activity of anabasine extracted (S_a) is given by:

$$S_a \ (dis.min^{-1} . \ mmole^{-1}) = \frac{D_a . V_t}{V_u . M_a} \qquad (2)$$

where D_a = Radioactivity of sample counted ($dis.min^{-1}$)

V_u = Volume of sample used for counting (ml)

V_t = Total volume of extract before counting (ml)

(iii) The total amount of nicotinic acid obtained by the oxidation of anabasine extracted from the leaf (M_n) is given by:

$$M_n \ (mmoles) = \left(\frac{A_{262} . V_n}{\varepsilon_n . 1} - \frac{W_n}{MW_n}\right)\left(\frac{M_a . MW_a}{M_a . MW_a + \rho_a V'_a}\right) \qquad (3)$$

where A_{262} = Absorbance at $\lambda = 262$ nm

V_n = Total (effective) volume of nicotinic acid solution used for A_{262} measurement (ml)

ε_n = Molar extinction coefficient at $\lambda = 262$ nm. ($2630 \ cm^2.mole^{-1}$)

1 = Path length

W_n = Weight of nictotinic acid added prior to sublimation (mg)

MW_n = Molecular weight of nicotinic acid (123.1)

M_a = Total anabasine extract from (1) (mmoles)

MW_a = Molecular weight of anabasine (162)

V'_a = Volume of standard anabasine added prior to oxidation (μℓ)

ρ_a = Density of anabasine (1.045)

(iv) The specific activity of nicotinic acid obtained from the leaf (S_n) is given by:

$$S_n \ (dis.min^{-1}.\ mmole^{-1}) = \frac{D_n}{M_n} \qquad (4)$$

where D_n = Total radioactivity of nicotinic acid ($dis.min^{-1}$)

M_n obtained from (3)

4.1.1 [U-^{14}C] Nicotinic acid feeding

Table II gives the radioactivity measurements of the initial extracts.

From equation (1) above,

$$M_a = \frac{0.97 \times 50}{2884} = 1.7 \times 10^{-2} \text{ mmoles}$$

From equation (2)

$$S_a = \frac{11813 \times 5.0}{1.0 \times 1.7 \times 10^{-2}} = 3.5 \times 10^{6} \ dis.min^{-1}.\ mmole^{-1}$$

From equation (3)

$$M_n = \left(\frac{1.495 \times 25.0}{2630} - \frac{1.685}{123}\right)\left(\frac{1.7 \times 10^{-2} \times 162}{1.7 \times 10^{-2} \times 162 + 1.045 \times 5}\right)$$

Extract	Volume for Counting µl	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate counts.min^{-1}	Total Radioactivity in extract counts.min^{-1}
80% Ethanol	250	51	20	1243	411	41 ± 4	8364
Petroleum ether (30° - 60°)	500	16	20	640	402	12 ± 3	384
Aqueous extract left after removal of pigments	250	39	20	1426	420	50 ± 4	7800

TABLE II. Radioactivity determinations of initial extracts. [U-^{14}C] Nicotinic acid feeding.

$= 1.8 \times 10^{-4}$ mmoles.

From equation (4)

$$S_n = \frac{618}{1.8 \times 10^{-4}}$$

$$= 3.4 \times 10^{6} \text{ dis.min}^{-1}.\text{mmole}^{-1}$$

4.1.2 [U-^{14}C] Aspartic acid feeding

Table III gives the radioactivity measurements of the initial extracts.

From equation (1) above,

$$M_a = \frac{0.630 \times 100}{2884}$$

$$= 2.2 \times 10^{-2} \text{ mmoles.}$$

From equation (2),

$$S_a = \frac{554 \times 19.8}{2.2 \times 10^{-2}}$$

$$= 5.0 \times 10^{5} \text{ dis.min}^{-1}.\text{ mmole}^{-1}$$

From equation (3),

$$M_n = \left(\frac{0.690 \times 41.67}{2630} - \frac{0.735}{123}\right)\left(\frac{2.2\text{x}10^{-2}\text{x}162}{2.2\text{x}10^{-2}\text{x}162+1.045\text{x}5}\right)$$

$$= 2.0 \times 10^{-3} \text{ mmole.}$$

From equation (4),

$$S_n = \frac{900}{2.0\text{x}10^{-3}}$$

Extract	Volume for Counting µl	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate counts.min^{-1}	Total Radioactivity in extract counts.min^{-1}
80% Ethanol	100	40	20	3681	241	172 ± 6	68,800
Petroleum ether (30° - 60°)	500	11	40	2160	440	43 ± 6	946
Aqueous extract left after removal of pigments	100	42	20	3503	286	161 ± 6	67,620

TABLE III. Radioactivity determination of initial extracts. [U-^{14}C] Aspartic acid feeding.

$$= 4.5 \times 10^{5} \text{ dis.min}^{-1} \text{. mmole}^{-1}$$

4.1.3 γ-Methylene [2-^{14}C] glutamic acid feeding

Table IV gives the radioactivity measurements of the initial extracts.

From equation (1),

$$M_a = \frac{0.76 \times 50}{2884}$$

$$= 1.3 \times 10^{-2} \text{ mmoles}$$

From equation (2),

$$S_a = \frac{50 \times 12.8}{0.5 \times 1.3 \times 10^{-2}}$$

$$= 9.8 \times 10^{4} \text{ dis.min}^{-1} \text{. mmole}^{-1}$$

From equation (3),

$$M_n = \left(\frac{0.58 \times 50}{2630} - \frac{0.755}{123}\right)\left(\frac{1.3\times10^{-2} \times 162}{1.3\times10^{-2}\times162+1.045\times}\right)$$

$$= 1.4 \times 10^{-3} \text{ mmole}$$

From equation (4),

$$S_n = \frac{135}{1.4 \times 10^{-3}}$$

$$= 9.6 \times 10^{4} \text{ dis.min}^{-1} \text{. mmole}^{-1}$$

4.1.4 Sodium [1-^{14}C] pyruvate feeding

Table V gives the radioactivity measurements of the initial extracts.

Extract	Volume for Counting µℓ	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate $counts.min^{-1}$	Total Radioactivity in extract $counts.min^{-1}$
80% Ethanol	100	39	20	11620	306	566 ± 10	220740
Petroleum ether (30° - 60°)	250	16	40	4280	443	107 ± 3	6848
Aqueous extract left after removal of pigments	100	34	20	12365	261	618 ± 11	205700

TABLE IV. Radioactivity determinations of initial extracts. γ-methylene glutamic acid feeding.

Extract	Volume for Counting µℓ	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate counts.min^{-1}	Total Radioactivity in extract counts.min^{-1}
80% Ethanol	200	39	20	7702	701	350 ± 9	68250
Petroleum ether (30° - 60°)	300	11	40	6962	1411	139 ± 4	5097
Aqueous extract left after removal of pigments	150	30	20	6761	523	312 ± 8	62400

TABLE V. Radioactivity determinations of the initial extracts. Sodium [1-^{14}C] pyruvate feeding.

From equation (1),

$$M_a = \frac{0.70 \times 125}{2884}$$

$$= 3.0 \times 10^{-2} \text{ mmoles.}$$

From equation (2),

$$S_a = \frac{192 \times 15}{1 \times 3.0 \times 10^{-2}}$$

$$= 9.6 \times 10^{4} \text{ dis.min}^{-1}\text{.mmole}^{-1}$$

From equation (3),

$$M_n = \left(\frac{0.460 \times 125}{2630} - \frac{0.40[illegible]}{12[illegible]}\right)\left(\frac{3.0 \times 10^{-2} \times 162}{3.0 \times 10^{-2} \times 162 + 1.045 \times 10}\right)$$

$$= 5.9 \times 10^{-3} \text{ mmole}$$

From equation (4),

$$S_n = \frac{541}{5.9 \times 10^{-3}}$$

$$= 9.2 \times 10^{4} \text{ dis.min}^{-1}\text{. mmole}^{-1}$$

4.1.5 Sodium [2-^{14}C] pyruvate feeding

Table VI gives the radioactive measurements of the initial extracts.

From equation (1)

$$M_a = \frac{0.755 \times 250}{2884}$$

$$= 6.5 \times 10^{-2} \text{ mmole.}$$

Extract	Volume for Counting µl	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate counts.min^{-1}	Total Radioactivity in extract counts.min^{-1}
80% Ethanol	100	42	20	6166	181	299 ± 8	125580
Petroleum ether (30° - 60°)	300	25	40	2403	397	50 ± 3	4167
Aqueous extract left after removal of pigments	100	21	20	10481	258	511 ± 10	107310

TABLE VI. Radioactivity determinations of the initial extracts. Sodium [2-^{14}C] pyruvate feeding.

From equation (2),

$$S_a = \frac{322 \times 27}{0.5 \times 6.5 \times 10^{-2}}$$

$$= 2.7 \times 10^{5} \text{ dis.min}^{-1}. \text{ mmole}^{-1}$$

From equation (3),

$$M_n = \left(\frac{0.730 \times 125}{2630} - \frac{1.430}{123}\right)\left(\frac{6.5 \times 10^{-2} \times 162}{6.5 \times 10^{-2} \times 162 + 1.045 \times 10}\right)$$

$$= 1.2 \times 10^{-2} \text{ mmole}$$

From equation (4),

$$S_n = \frac{2424}{1.2 \times 10^{-2}}$$

$$= 2.0 \times 10^{5} \text{ dis.min}^{-1}. \text{ mmole}^{-1}$$

4.1.6 Sodium [3-^{14}C] pyruvate feeding

Table VII gives the radioactivity measurements of the initial extracts.

From equation (1),

$$M_a = \frac{0.410 \times 50}{2884}$$

$$= 7.1 \times 10^{-3} \text{ mmole}$$

From equation (2),

$$S_a = \frac{1421 \times 10}{1 \times 7.1 \times 10^{-3}}$$

$$= 2.0 \times 10^{6} \text{ dis.min}^{-1}. \text{mmole}^{-1}$$

Extract	Volume for Counting µl	Total Volume ml	Counting Time min.	Sample + Background Counts	Background Counts	Sample Count Rate $counts.min^{-1}$	Total Radioactivity in extract $counts.min^{-1}$
80% Ethanol	100	46	20	9841	902	447 ± 10	205620
Petroleum ether (30° - 60°)	300	15	20	19940	823	956 ± 14	47800
Aqueous extract left after removal of pigments	100	45	20	7382	831	328 ± 9	147600

TABLE VII. Radioactivity determinations of the initial extracts. Sodium [3-^{14}C] pyruvate feeding.

From equation (3),

$$M_n = \left(\frac{0.565 \times 100}{2630} - \frac{0.330}{123}\right)\left(\frac{7.1 \times 10^{-3} \times 162}{7.1\times10^{-3}\times162+1.045\times10}\right)$$

$$= 1.9 \times 10^{-3} \text{ mmole.}$$

From equation (4),

$$S_n = \frac{3230}{1.9 \times 10^{-3}} = 1.7 \times 10^{6} \text{ dis.min}^{-1}. \text{ mmole}^{-1}$$

4.2 Pool-size determination

The pool-sizes of the four substrates, nicotinic acid, aspartic acid, γ-methylene glutamic acid and sodium pyruvate were estimated using the following relationship:

$$M_u = \left(\frac{S.A._0}{S.A._1} - 1\right) M_0 \qquad (5)$$

where M_u = Pool size

M_0 = Amount of tracer added

$S.A._0$ = Specific activity of tracer added

$S.A._1$ = Specific activity of compound isolated

4.2.1 Nicotinic acid

The total amount of nicotinic acid in the leaf extract N_e is given by:

$$N_e \text{ (mmoles)} = \frac{A_{262} \cdot V_e}{\varepsilon_n}$$

where V_e = Total volume of extract

A_{262} = Absorbance of this solution at λ = 262 nm.

ε_n = Molar extinction coefficient at λ = 262 nm.

From the results,

$$N_e = \frac{0.58 \times 25}{2630}$$

$$= 5.5 \times 10^{-3} \text{ mmole.}$$

The specific activity ($S.A._1$) is given by:

$$(\text{dis.min}^{-1}\ S.A._1\ \text{mmole}^{-1}) = \frac{D_e \cdot V_e}{V_c \cdot N_e}$$

where D_e = Radioactivity of sample counted (dis.min^{-1}.)

V_c = Volume used for counting

From the results

$$S.A._1 = \frac{8147 \times 25}{1 \times 5.5 \times 10^{-3}} = 3.7 \times 10^7 \text{ dis.min}^{-1}.\text{mmole}^{-1}$$

Using equation (5) above

$$\text{Pool size } (M_u) = \left(\frac{4.6 \times 10^7}{3.7 \times 10^7} - 1\right) \quad 0.755$$

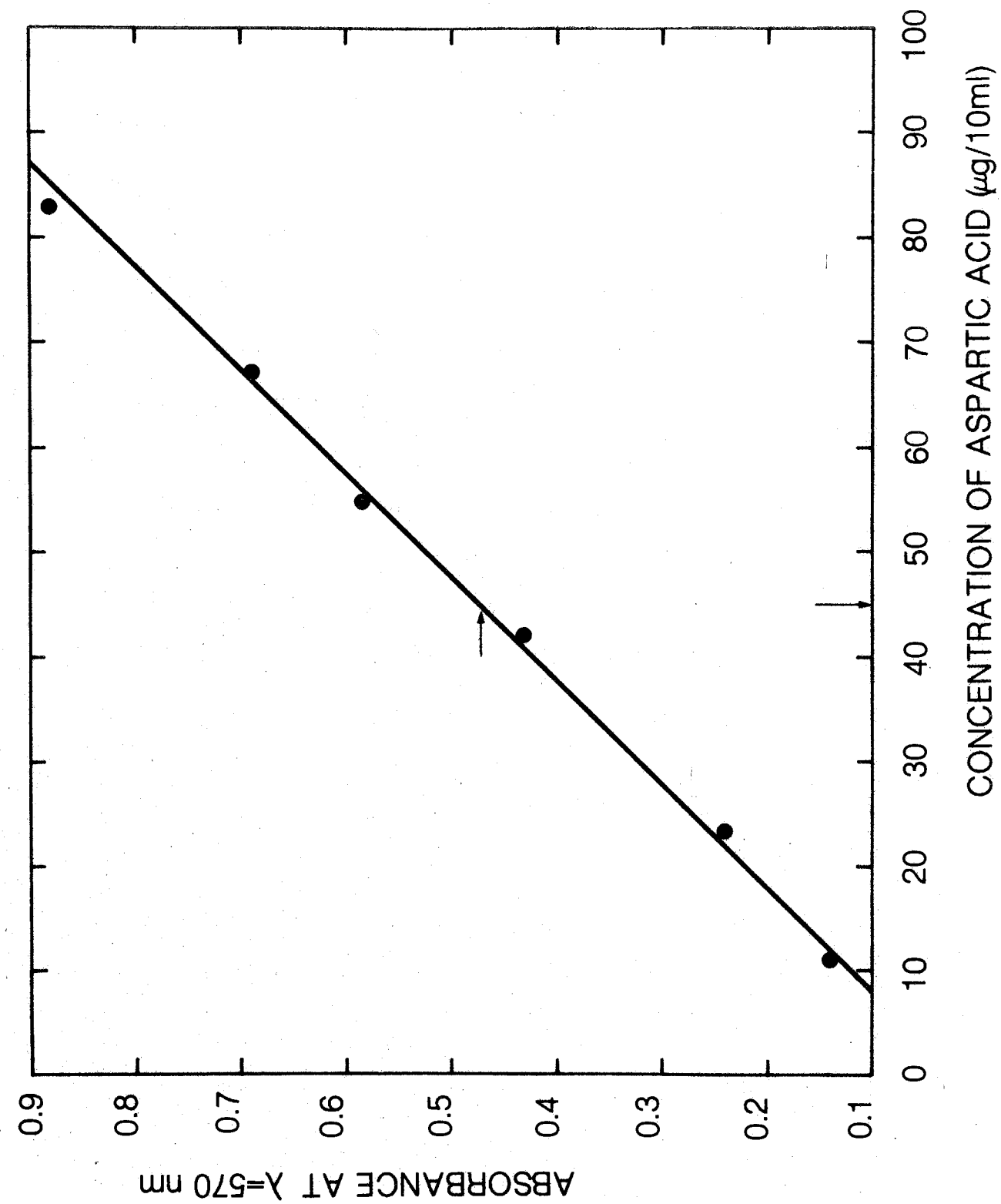
$$= 0.184 \text{ mg.}$$

4.2.2 Aspartic acid

Figure 13 shows the standard curve for the ninhydrin-aspartic acid reaction product. An absorbance of 0.470 corresponded to 45 µg aspartic acid in the sample.

FIGURE 13

Standard curve for the ninhydrin complex with aspartic acid
Absorbance at λ = 570 nm vs concentration of aspartic acid


ABSORBANCE AT λ=570 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
CONCENTRATION OF ASPARTIC ACID (μg/10ml)

The concentration of the amino acid was calculated in the following manner.

$$\text{Total radioactivity of extract} = \frac{D_e \cdot V_e}{V_c}$$

where D_c ($dis.min^{-1}$) = Radioactivity of sample counted

V_e (ml) = Total volume of extract

V_c (ml) = Volume used for counting

$$\text{Total amount of amino acid in the extract (m.mole)} = \frac{W}{MW} \cdot \frac{V_e}{(V_e - V_c)}$$

where W (mg) = Weight of amino acid from standard curve

MW = Molecular weight of amino acid

$$\text{Specific activity of amino acid in extract } (dis.min^{-1}.m.mole^{-1}) \quad \frac{D_e.MW.(V_e-V_c)}{W \cdot V_c} \qquad (6)$$

Using equation (6) above, the Specific activity $S.A._1$ of aspartic acid is given by:

$$S.A._1 = \frac{1311 \times 133.1 \times (50.0-0.1)}{0.045 \times 0.1}$$

$$= 1.9 \times 10^{9} \; dis.min^{-1}.mmole^{-1}$$

From equation (5) above:

$$\text{Pool size } (M_u) = \left(\frac{4.5 \times 10^{11}}{1.9 \times 10^{9}} - 1 \right) 0.00165$$

$$= 0.389 \text{ mg}$$

4.2.3 γ-methylene glutamic acid

Figure 14 shows the standard curve for the ninhydrin-γ-methylene glutamic acid reaction product.

Using equation (5) above

$$S.A._1 = \frac{65372 \times 159 \times (5-1)}{0.035 \times 1}$$

$$= 1.2 \times 10^{9} \text{ dis.min}^{-1} . \text{ mmole}^{-1}$$

From equation (5) above

$$\text{Pool size } (M_u) = \left(\frac{1.2 \times 10^{9}}{1.2 \times 10^{9}} - 1 \right) 0.270$$

$$= \text{Nil.}$$

4.2.4 Sodium pyruvate

Figure 15 shows the standard curve for pyruvic acid - 2,4,dinitrophenylhydrazone in basic solution.

The specific activity of pyruvate in the extract ($S.A._1$) is given by:

$$\underset{(\text{dis.min}^{-1} . \text{ mmole}^{-1})}{S.A._1} = \frac{D_e . V_e . MW}{V_e . W}$$

FIGURE 14

Standard curve for the ninhydrin complex with γ-methylene glutamic acid
Absorbance at λ = 570 nm vs concentration of amino acid

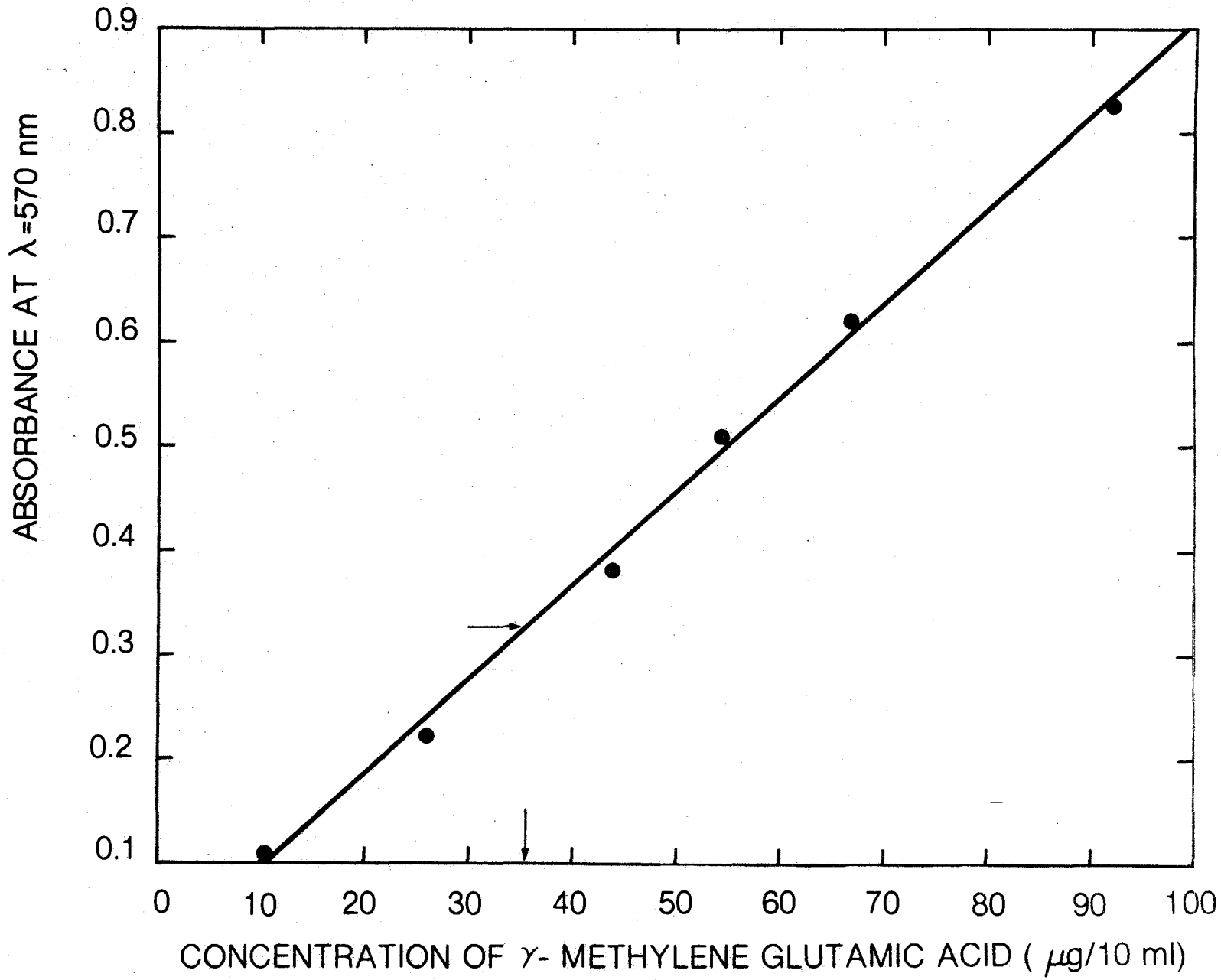
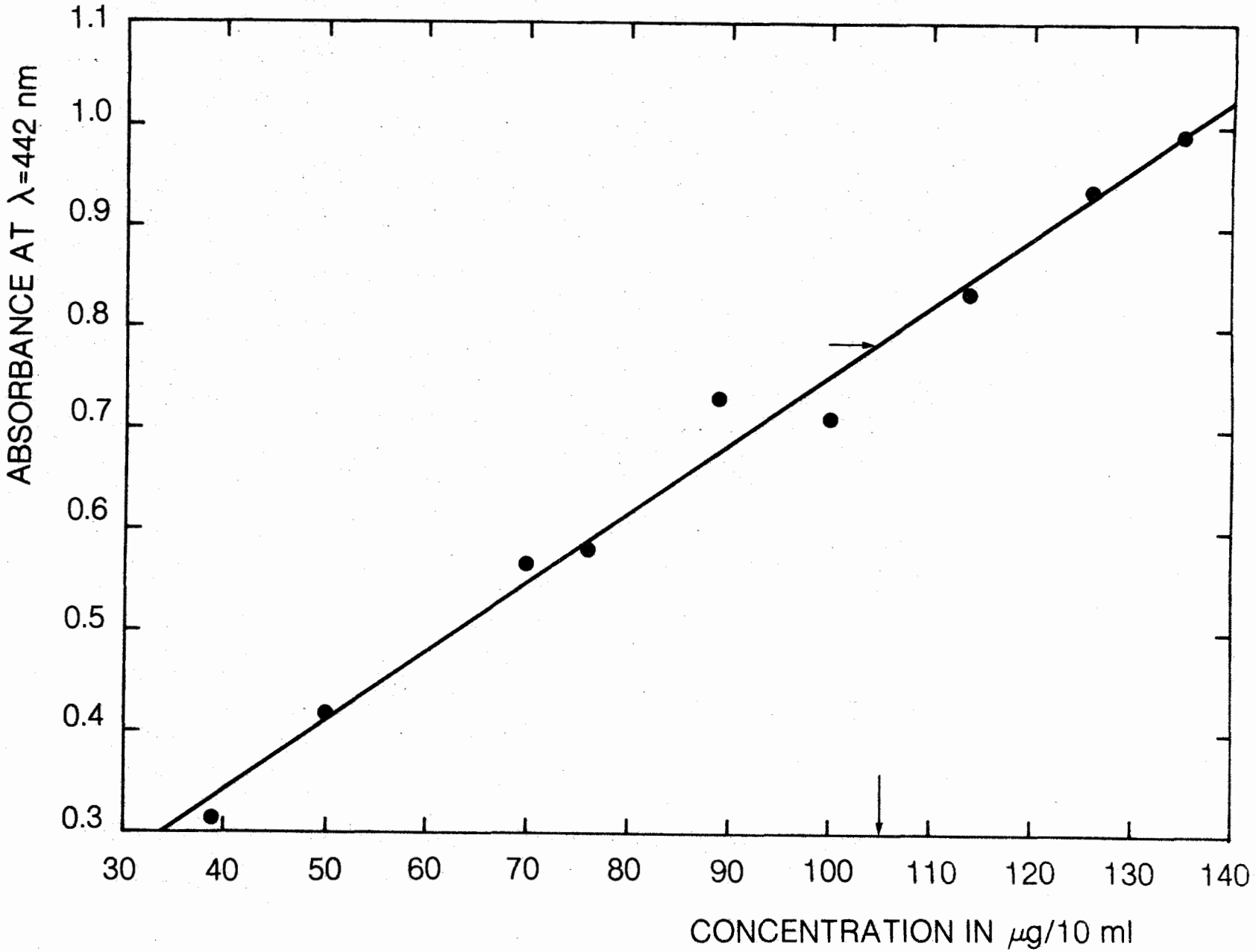

ABSORBANCE AT λ=570 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
CONCENTRATION OF γ- METHYLENE GLUTAMIC ACID (μg/10 ml)

FIGURE 15

Standard curve for pyruvic acid-2,4 dinitrophenylhydrazone Absorbance at λ = 442 nm vs concentration of hydrazone


ABSORBANCE AT λ=442 nm
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
30
40
50
60
70
80
90
100
110
120
130
140
CONCENTRATION IN μg/10 ml

where D_e (dis.min^{-1}) = Radioactivitity of sample counted

V_e (ml) = Total volume of extract

V_c (ml) = Volume used for counting

W (mg) = Weight of the hydrazone from the standard curve

MW = Molecular weight of the hydrazone

From the results,

$$S.A._1 = \frac{31764 \times 10 \times 268}{0.1 \times 0.105}$$

$$= 8.1 \times 10^9 \text{ dis.min}^{-1} . \text{ mmole}^{-1}$$

From equation (5) above,

$$\text{Pool size } (M_u) = \left(\frac{1.9 \times 10^{10}}{8.1 \times 10^9} - 1 \right) 0.050$$

$$= 0.067 \text{ mg}$$

It should be noted that uniform feeding conditions were employed throughout the series of experiments with different substrates and different leaves. As such, any difference in the amount of substrate incorporated may be due to a difference in utilization, including anabasine biosynthesis.

The criterion used in this study to determine the immediacy of a precursor was the "dilution factor". In a radiolabeled-substrate feeding experiment, the dilution factor is the ratio of the specific activity of the substrate to the specific activity of the product isolated. By comparing the dilution values, one can determine the relative efficiency with which each substrate is incorporated into a particular end product. With certain assumptions, it also gives an idea of how far a precursor is from the final product in a biosynthetic pathway. One assumes, e.g., that any dilution of the activity of the substrate initially or along the pathway to the product is due to non-radioactive material already present. This means that the specific activity of the substrate administered is not the important value in estimating the dilution factor. One has to evaluate the specific activity of each substrate within the leaf, after feeding but before incorporation into the product. For this

purpose, one must know the pool size of each.

As described previously, the pool sizes were determined by an isotope dilution method. The advantage in this is that one need not worry about the efficiency of the extraction procedure used; i.e. it is not important whether all the compound was extracted or not. Instead, one has to make sure that the compound isolated is pure. The pool size of a compound within an organism could vary depending on a number of factors, an important one being its age. Therefore, the leaves used for pool-size determinations were approximately of the same maturity (as judged by blade area) as those used for feeding experiments; i.e. 1 1/2 to 2 months old. It is assumed that the pool-sizes determined for each substrate corresponded to the pool sizes at the time of feeding radio-labeled compounds. It is also assumed that all of the radioactive compound administered would find its way to a common pool and get diluted uniformly, before "compartmentation" into different pools (if it occurs) supplying different biosynthetic pathways.

The specific activity of the "pool" of each substrate ($S.A._p$) was evaluated using the following relationship:

$$(dis.min^{-1}.m.mole^{-1}) \quad S.A._p = S.A._f \cdot \frac{W_f}{W_f+W_p} \qquad (7)$$

where $S.A._f$ = Specific activity of substrate fed ($dis.min^{-1}.m.mole^{-1}$)

W_f = Amount of substrate fed (mg)

W_p = Pool size (mg)

Using the $S.A._p$ values, the "dilution factors" (D.F) were determined as:

$$D.F. = \frac{S.A._p}{S.A._n} \quad (8)$$

where $S.A._n$ = Specific activity of nicotinic acid isolated

The results are summarized in Table VIII.

The dilution factor of 11.5 for nicotinic acid is almost three orders of magnitude smaller than that of any other precursor tried. This shows both that the leaves of Nicotiana glauca could use nicotinic acid for anabasine biosynthesis and that in this system it is probably utilized without degradation of the ring. The dilution value of 11.5 could be taken as an indication that there is only a single pool between nicotinic acid and anabasine; probably an activation pool but the major portion of the the dilution value may be due to the presynthesized non-radioactive anabasine stored within the leaf which would

TABLE VIII

Specific activity of each substrate at the pool and the corresponding dilution factor values.

Substrate	Specific Activity of ^{14}C substrate administered $dis.min^{-1}.mmole^{-1}$	Quantity Used mg	Pool Size mg	Specific Activity of Substrate at the Pool $dis.min^{-1}.mmole^{-1}$	Specific Activity of Nicotinic Acid from Anabasine of the leaf $dis.min^{-1}.mmole^{-1}$	Dilution Factor
Nicotinic acid - ^{14}C (U)	4.6×10^{7}	0.985	0.184	3.9×10^{7}	3.4×10^{6}	11.5
Aspartic acid - ^{14}C (U)	4.5×10^{11}	0.004	0.389	4.6×10^{9}	4.5×10^{5}	1.0×10^{4}
γ-methylene glutamic acid 2 - ^{14}C	1.2×10^{9}	1.485	--	1.2×10^{9}	9.6×10^{4}	1.3×10^{4}
Sodium pyruvate 1 - ^{14}C	1.9×10^{10}	0.075	0.067	1.0×10^{10}	9.2×10^{4}	1.1×10^{5}
Sodium pyruvate 2 - ^{14}C	8.3×10^{9}	0.185	0.067	6.1×10^{9}	2.0×10^{5}	3.1×10^{4}
Sodium pyruvate 3 - ^{14}C	4.0×10^{10}	0.030	0.067	1.2×10^{10}	1.7×10^{6}	7.1×10^{3}

dilute that synthesized from radioactive substrate. The specific activity of anabasine extracted (hence that of nicotinic acid by its oxidation) would therefore be reduced causing a corresponding increase in the dilution factor.

Preliminary feeding experiments with $^{14}CO_2$ gave significant radioactivity in the nicotinic acid isolated, suggesting that the leaf could make its own nicotinic acid and need not be transported from another part of the plant.

Table VIII shows that γ-methylene glutamic acid and sodium [2-^{14}C] pyruvate have slightly higher dilution factor values than that of aspartic acid, whereas sodium [3-^{14}C] pyruvate has a slightly lower value. The similar dilution values of aspartate, γ-methylene glutamate and pyruvate imply that each is approximately as efficient as the other in serving as a precursor for nicotinic acid. This observation can be interpreted in two ways, in view of the suggested glyceraldehyde - aspartate pathway for nicotinic acid biogenesis

(i) Aspartate is not the immediate precursor for nicotinic acid, i.e. aspartate does not undergo a direct condensation with another compound to provide the required carbons and nitrogen of nicotinic acid.

(ii) Aspartate may be a direct precursor but there could well be other pathways involving other precursors such as pyruvate or a derivative of γ-methylene glutamate.

It is difficult to argue conclusively, for or against either of the above possibilities with the results presented here, but this clearly opens up a new aspect of the problem; i.e. it is necessary to look into other compounds as possible direct precursors for nicotinic acid, in order to understand this biosynthesis fully.

One of the more significant results presented here is the value of the dilution factor for γ-methylene glutamic acid, compared to that of aspartic acid. It was mentioned in Chapter 1, that γ-methylene glutamate was tried since it was a possible precursor for nicotinic acid as opposed to the glyceraldehyde-aspartate condensation. This compound was suggested to provide all the carbons, including the carboxyl carbon for nicotinic acid. If this were the case, we would have expected to get a much lower dilution factor for this when compared with aspartate. This is especially so when one considers the fact that aspartate is a common compound within the plant system and also an intermediate in several biosynthetic schemes, whereas γ-methylene glutamate is not.

In addition to that, we could not detect any free γ-methyleneglutamate within the leaf. Thus one would not argue strongly the possibility of this compound undergoing a cyclization to produce nicotinic acid. Yet one cannot exclude the possibility of this compound serving as a direct precursor since a high turnover rate would make it impossible for one to detect its presence within the leaf.

The other possibility is that γ-methyleneglutamic acid is converted to some other compound which in turn is capable of serving as a direct precursor for nicotinic acid. Yet, if it is metabolized into something else prior to incorporation and it could still be almost as efficient as aspartate in producing nicotinic acid, then it is quite possible that there is some compound other than aspartate that could well be the immediate precursor. Thus there could be the simultaneous occurence of one or more pathways other than the one involving glyceraldehyde and aspartate, at least in N. glauca leaves.

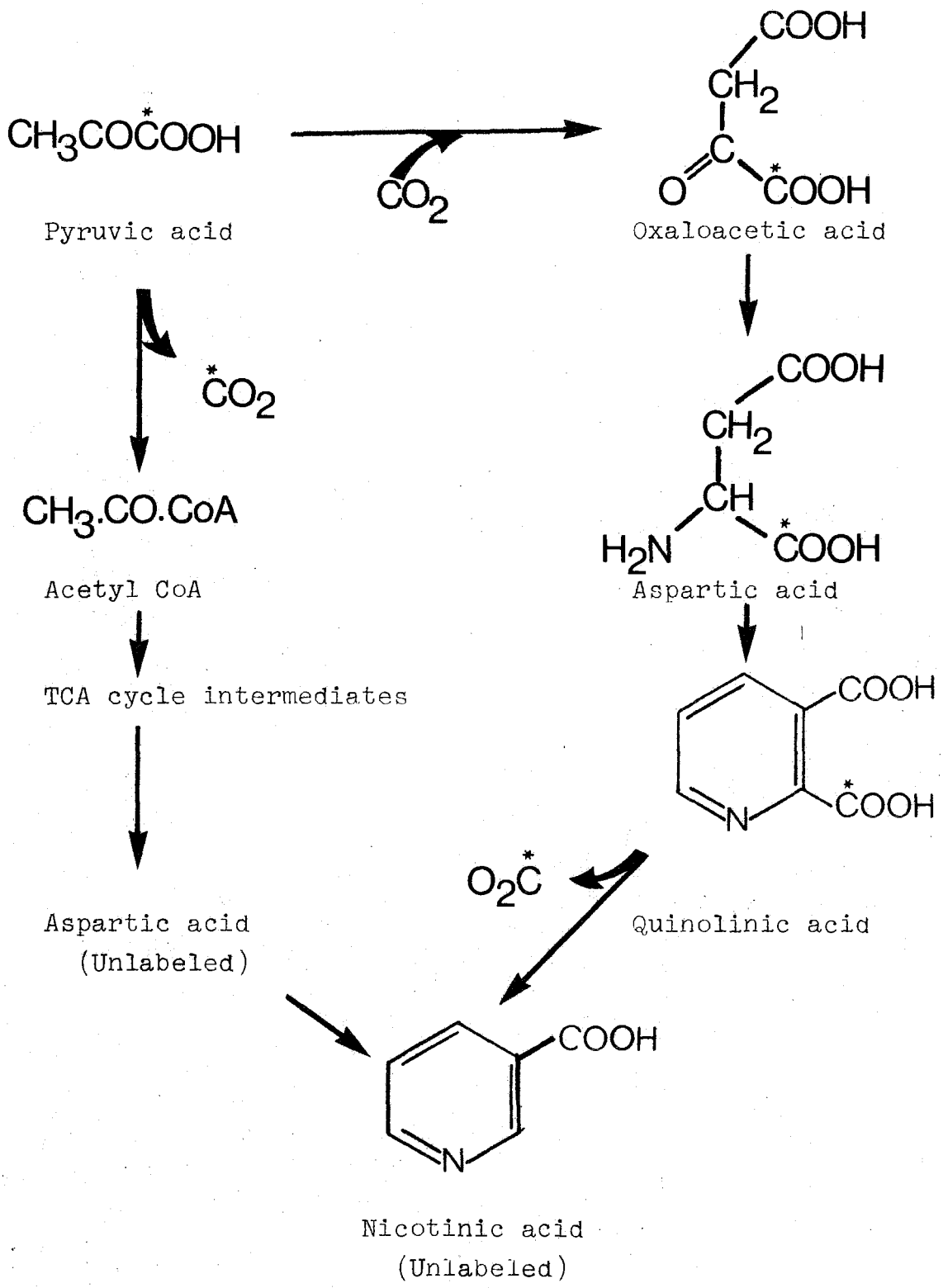
The dilution factors given in Table VIII indicate that pyruvate could be a key intermediate in a second pathway. If pyruvate were to be converted to aspartate either by decarboxylation to acetyl CoA and via the TCA cycle intermediates or by conversion to phosphoenol pyruvate and by carboxylation to oxaloacetate followed by transamination,

prior to incorporation into nicotinic acid, it is unlikely that it would show an almost identical dilution factor value to aspartate. Table VIII also shows that [1-^{14}C] pyruvate is incorporated into nicotinic acid, though with a lower efficiency than aspartate, in N. glauca leaves. This cannot be explained if one assumed that pyruvate is incorporated into the pyridine ring by a prior metabolization to aspartate, since the ^{14}C label would be lost in going to nicotinic acid (Figure 16). But one cannot rule out the possibility of pyruvate being incorporated by its conversion to 3-phosphoglyceraldehyde via the reversal of glycolysis. This would mean that pyruvate would supply the label to the 3-carbon unit that gives rise to carbons 4, 5 and 6 of nicotinic acid and not to the 2-carbon unit giving rise to carbons 2 and 3. But this cannot explain the significantly different dilution values obtained for the three specifically labeled pyruvate feedings since if they were supplying the 3-carbon unit, one would expect similar values. On the other hand, the order of increase of the dilution values for the three pyruvates ($1\text{-}^{14}C > 2\text{-}^{14}C > 3\text{-}^{14}C$) suggests its metabolization via the TCA cycle although as mentioned before, this would not account for the transfer of the label from [1-^{14}C] pyruvate. This seems to suggest that pyruvate itself could be a direct precursor for nicotinic acid in an alternative pathway.

It is also worth mentioning that there are some contradictory results obtained with the substrates used in our

FIGURE 16

The possible alternative pathways for the conversion of pyruvic acid to nicotinic acid via aspartate


CH3COCOOH
CO2
COOH
CH2
C
O
COOH
Pyruvic acid
Oxaloacetic acid
CO2
CH3.CO.CoA
Acetyl CoA
COOH
CH2
CH
H2N
COOH
Aspartic acid
TCA cycle intermediates
COOH
N
COOH
O2C
Aspartic acid
(Unlabeled)
Quinolinic acid
COOH
N
Nicotinic acid
(Unlabeled)

experiments, especially with aspartate. Yang and Waller[29] fed aspartate - 4 - $^{14}C,^{15}N$ to Ricinus plants and observed the incorporation of ^{14}C into the CN group and ^{15}N into the ring nitrogen of ricinine approximately in the ratio of 1:1, suggesting that this compound is incorporated into the product molecule as a unit. On the other hand, Johns and Marion[30] reported that aspartate- 3- ^{14}C feeding gives almost equal labelling at C-2 and C-3 of ricinine with C-7 also being labeled in an overall ratio of 2:2:1. They also observed that aspartate ^{15}N feeding gives the same ^{15}N excess in both the pyridone and nitrile nitrogens. These results suggest the removal of the amino group from aspartate and passage through a symmetrical intermediate such as succinic acid (via the TCA cycle) before incorporation. Similar observations have been made in studies with tobacco plants. If one considers the time periods allowed for metabolism in the above studies, it can be understood that in short term feeding experiments there may well be only partial equilibration of the amine nitrogen with the nitrogen pool and at least some aspartate appears not to be incorporated via a symmetrical intermediate. When the feedings are over longer time periods, the aspartate nitrogen seems to become equilibrated with the ammonia pool and the 2-and 3-carbons get equally labeled by passing through a symmetrical intermediate.

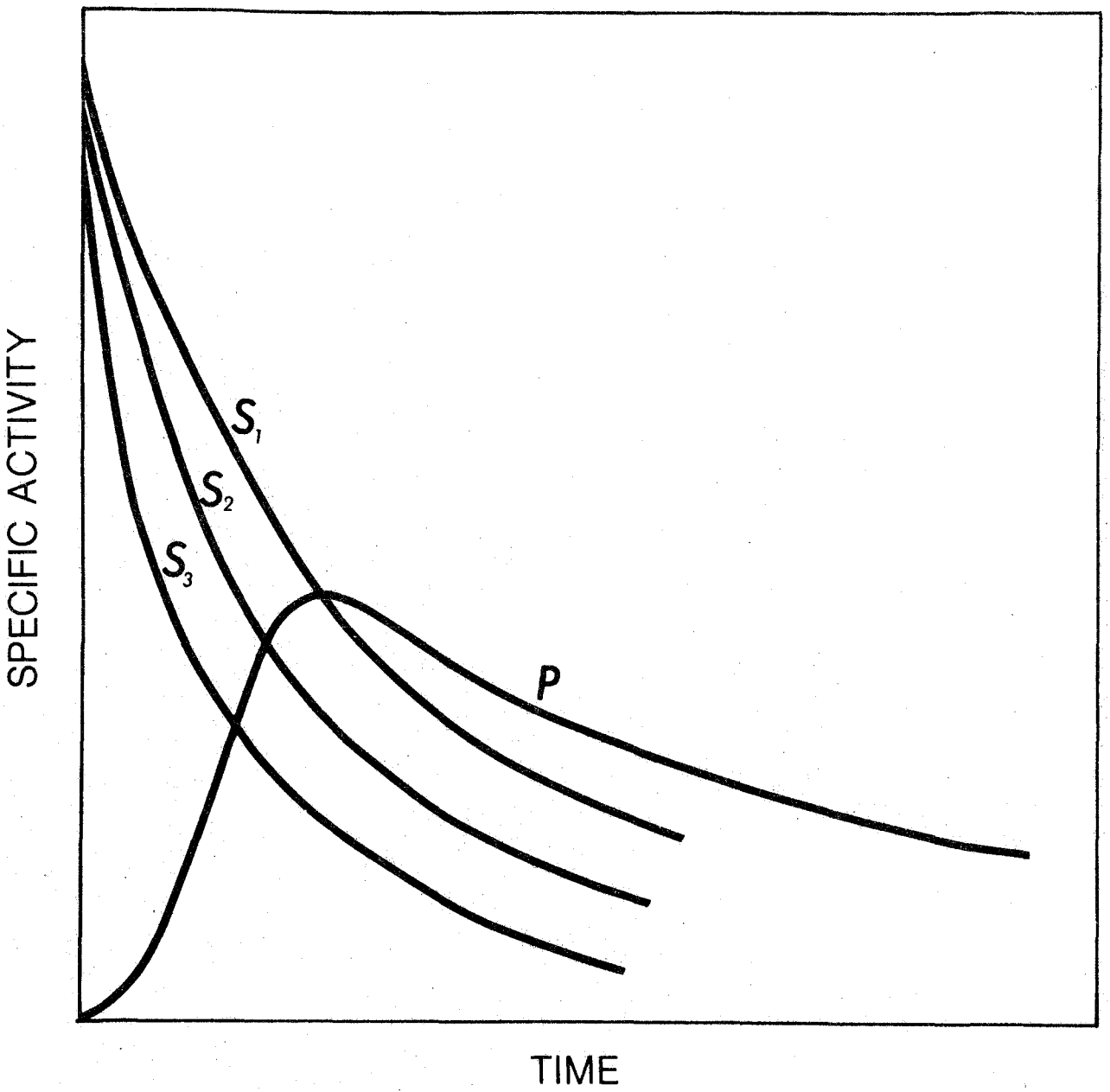
In our experiments, the total feeding time, as well as the total metabolic time were kept constant in order to avoid discrepancies of the above nature and to be able to compare the results obtained with different substrates. But it would be interesting to find out how the specific activities of the substrates vary with time as related to the same variation of the product. If the sequence of the precursors in a biosynthetic pathway could be represented by:

$$S_3 \longrightarrow S_2 \longrightarrow S_1 \longrightarrow P$$

where S_3, S_2, S_1 are precursors and P is the product, the variation of the specific activities with time, of labeled substrates and of the product formed, could be shown by the curves, S_3 , S_2 , S_1 , and P in Figure 17. As would be noticed, the curve for the immediate precursor S_1 cuts the product curve at its maximum point whereas those for S_2 and S_3 do so on the left of the maximum. Thus a study of this nature would give much more information, especially regarding the immediacy of a precursor, than of dilution factor values.

FIGURE 17

Variation of specific activities with time, of labeled precursors and of product formed.


SPECIFIC ACTIVITY
S_1
S_2
S_3
P
TIME

CONCLUSION:

The results presented here indicate that excised leaves of Nicotiana glauca can use nicotinic acid for anabasine biosynthesis and it is by far the most efficient precursor of any one tried here. The low dilution factor of 11.5 suggests that it gives rise to the pyridine ring without any degradation and may be the immediate precursor (possibly in an activated form). γ-Methylene glutamic acid, though not detected within the leaf, was almost as efficient as aspartate in serving as a precursor for nicotinic acid, suggesting that there may be alternative pathways involving precursors other than aspartate and glyceraldehyde. E.g. pyruvate could be a major intermediate in a second pathway as we observe that [2-^{14}C] pyruvate and [3-^{14}C] pyruvate transfer the label into nicotinic acid almost as efficiently as aspartic acid. The observation that the label is transferred from carboxyl labeled pyruvate (even though to a lesser extent) lends further support to such a hypothesis since if it were to be incorporated via aspartate, it would lose the label through decarboxylation. It is possible that pyruvate supplies the label via the 3-carbon unit (3-phosphoglyceraldehyde) by the reversal of glycolysis although this should result in similar dilution values for the three

specifically labeled pyruvates. This is not observed.

This means that the origin of the pyridine ring in Nicotiana alkaloids, at least in the system studied, is still an open problem. The postulated glyceraldehyde-aspartate pathway may well exist but others (e.g. involving pyruvate or γ-methyleneglutamate) are equally possible.

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