LYSINE METABOLISM AND SLAFRAMINE BIOGENESIS IN *RHIZOCTONIA LEGUMINICOLA*

BY

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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A. Introduction

Since 1947 there have appeared reports of excessive salivation in cattle consuming certain forages. The reports increased to such an extent that in 1958 a research program dealing with these forages was undertaken by Byers and Broquist (1,2). They reported that these "slobber forages" were usually red clover and caused excessive salivation whether in the form of pasture, hay or silage. This excessive salivation caused a large decrease in the uptake of feed and hence a large decrease in the production of milk. In 1959, O'Dell, *et al.* (3) reported a severe case of this disease in Missouri in which death of the cattle was often the end result. They found the toxic material responsible for the disease to be organic in nature, slow in losing its activity in solution and soluble in both water and chloroform. Byers and Broquist found that hot water extracts of "slobber forages" could cause excessive salivation in guinea pigs. The above results pointed to the conclusion that the toxic material might be an alkaloid.

Smalley *et al.* (4) and Crump *et al.*(5), studying the problem in Wisconsin, found that the forage was again usually red clover. They discovered that all their forages contained a microscopic dark-brown fungus which they presently isolated in pure culture and identified as *Rhizoctonia leguminicola*. Gough and Elliot (6) identified the fungus as the cause of Black-patch disease of red clover. Smalley produced excessive salivation in guinea pigs force-fed with mycelia grown on extracts of red clover hay.

Similar salivation was caused when mycelia were introduced into the rumen of dairy cows. The fungus was found to excrete no toxic material during its growth.

Aust et al.(7,8) isolated the toxic material and identified it structurally as 1-acetoxy-8-aminooctahydroindolizine. The name "slaframine" was proposed for the compound. The initial work on the biosynthesis of slaframine revealed incorporation of labeled lysine into slaframine. In fact, all six carbons of lysine were incorporated into slaframine. Aust's findings indicated the relevancy of lysine metabolism to the biosynthesis of slaframine in R. leguminicola. The exact role of lysine metabolism is now being investigated in various laboratories. Before discussing the information available concerning the role of lysine in the biosynthesis of slaframine, a review of the two lysine pathways known in organisms is appropriate.

It is indeed seldom that two distinct pathways are known that lead to the synthesis of a common molecule, but such is the case with lysine. In certain lower fungi, bacteria, algae and higher plants lysine is synthesized from pyruvate and aspartate with diaminopimelic acid as a direct precursor. In certain lower and higher fungi and in Euglenids, lysine is synthesized from α-ketoglutaric acid and acetate with α-aminoadipic acid as a direct precursor.

Diaminopimelic acid was first isolated by Work (9) from Corynebacterium diphtheriae and later shown to be present in many bacteria and other microorganisms. Structural considerations of diaminopimelic acid presented it as a possible precursor for lysine. This possibility was strengthened by reports of Dewey and Work (10) of a constitutive enzyme in E. coli which decarboxylated diaminopimelic acid. Certain lysine-requiring mutants were

shown to accumulate diaminopimelic acid and did not exhibit diaminopimelic acid decarboxylase activity. Also a requirement for lysine was seen to parallel a loss in diaminopimelic acid decarboxylase activity in certain mutants. These findings pointed to diaminopimelic acid as a direct precursor of lysine in E. coli.

Radioactivity experiments revealed a competition between aspartate and glucose for four of the carbon atoms of diaminopimelic acid and of lysine (11). Gilvarg reported the accumulation of a compound which he identified as N-succinyl-L-α, ε-diaminopimelic acid in a diaminopimelic acid-requiring mutant of E. coli (12,13). N-succinyl diaminopimelate deacylase activity was discovered in organisms that could synthesize diaminopimelic acid but not in a mutant that accumulated N-succinyl-L-α,ε-diaminopimelic acid.

Gilvarg's E. coli mutant was shown also to accumulate N-succinyl-ε-keto-L-α-aminopimelic acid (14). Transaminase activity that interconverted reversibly N-succinyl-diaminopimelate and α-ketoglutarate was discovered in both mutant and wild strains (15). A preparation of N-succinyldiaminopimelate-glutamate transaminase was shown to be different from the other transaminases in E. coli. This supported the belief that N-succinyl-ε-keto-L-α-aminopimelic acid is a precursor of diaminopimelic acid and the conversion of N-succinyl-diaminopimelate to the keto acid is not a side reaction.

The conversion of aspartic acid β-semialdehyde and pyruvate to N-succinyl-ε-keto-L-α-aminopimelic acid has been shown. The above data suggest a condensation of aspartic acid β-semialdehyde and pyruvate to yield 2,3-dihydrodipicolinic acid whose reduction with TPN further yields Δ^1-piperideine-2, 6-dicarboxylic acid. Succinylation yields N-succinyl-ε-keto-L-α-aminopimelic acid which gives L-α,ε-diaminopimelic acid upon transamination

and deacylation.

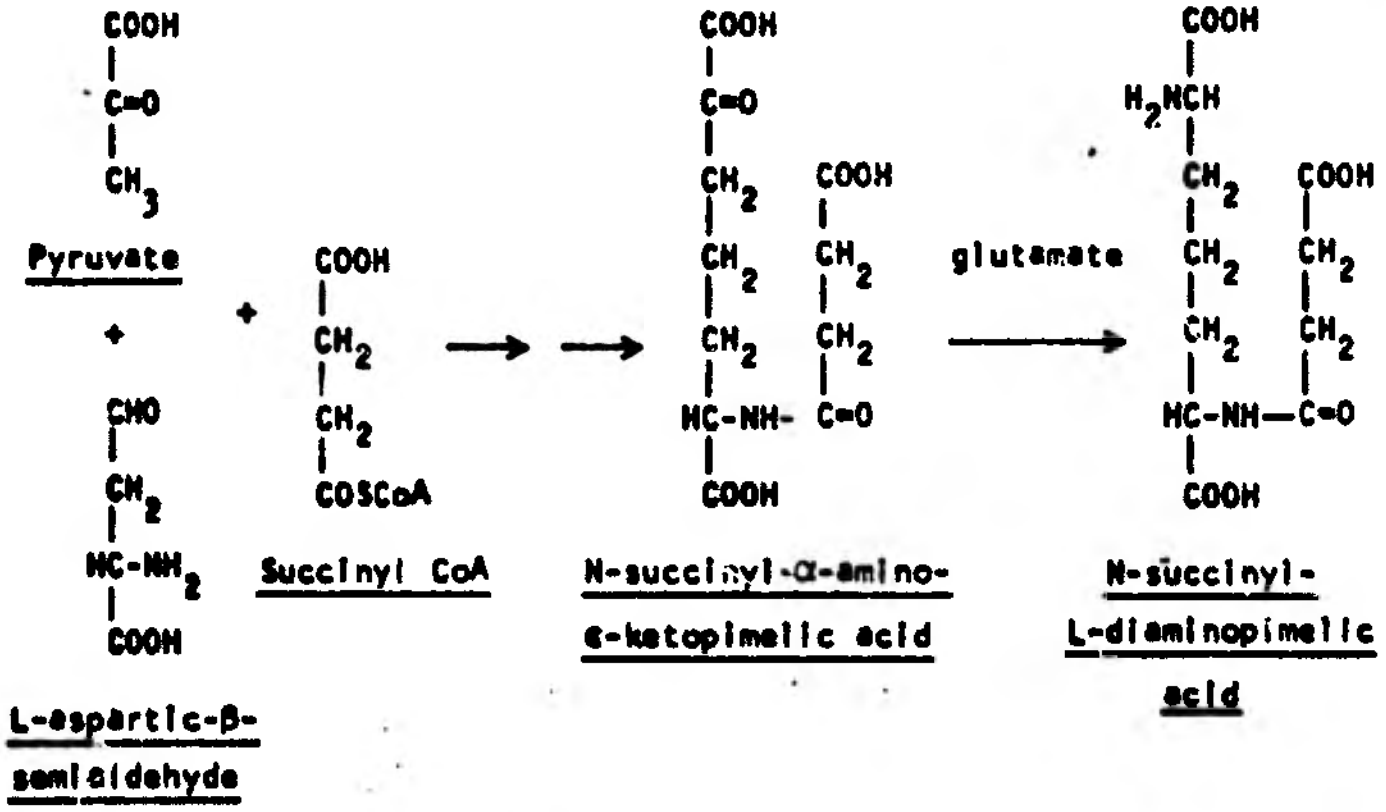
Partially purified diaminopimelic acid decarboxylase was shown inactive toward L-diaminopimelic acid and L-lysine (16, 17). The decarboxylase has demonstrated a high specificity for the mesoform of α,ε-diaminopimelic acid, indicating this isomer to be the immediate precursor of lysine. Conversion of L-α,ε-diaminopimelic acid to the meso-form is catalyzed by a specific epimerase (18). The diaminopimelic acid pathway is summarized in Figure 1.

Certain results have suggested an alternative pathway for lysine biosynthesis not involving diaminopimelic acid, but it appears unlikely at the present time that such a pathway exists.

The relationship between α-aminoadipic acid and lysine biosynthesis was first discovered by Borsook et al. (19, 20) who showed that lysine could be converted to α-aminoadipic acid by mammalian tissue. A lysine-requiring Neurospora mutant was isolated that was able to use α-aminoadipic acid in place of lysine (21). Radioactivity experiments showed that all of the label in ^{14}C-α-aminoadipic acid was incorporated into lysine by mutants that could grow on either lysine or α-aminoadipic acid (22). This suggested quite strongly that α-aminoadipic acid was a precursor of lysine in Neurospora. Certain lysine-requiring mutants of the yeast, Ophiostoma multiannulatum, were shown to grow on DL-α-aminoadipic acid, L-α-aminoadipic acid, and α-ketoadipic acid (23).

Radioactivity experiments have indicated the carboxyl group of acetate to be entirely responsible for the ε carbon atom and the carboxyl carbon atom of lysine in Neurospora and yeast with the methyl carbon atom responsible for the other carbon atoms of lysine (24-28). A plausible mechanism is the condensation of acetate with α-ketoglutarate to give homocitric

Figure 1. The diaminopimelic acid pathway of lysine biosynthesis.


Pyruvate
+
L-aspartic-β-semialdehyde
Succinyl CoA
N-succinyl-α-amino-ε-ketopimelic acid
glutamate
N-succinyl-L-diaminopimelic acid
— succinate
L-diaminopimelic acid
meso-DAP
—CO2
L-lysine

acid which yields homoisocitrate, oxaloglutarate and α-ketoadipate by analogy with the Krebs cycle (27, 28).

Conversion of acetate and α-ketoglutarate to α-ketoadipate has been demonstrated (29). Formation of homocitrate from acetate and α-ketoglutarate (30) and its conversion to α-ketoadipate has been shown (31). The step from α-ketoadipate to α-aminoadipate involves a straight-forward transamination reaction.

Radioactive studies in yeast have shown that α-aminoadipic acid is incorporated into lysine having the same specific activity as the initial α-aminoadipic acid (32, 33). α-Aminoadipic acid-δ-semialdehyde has been suggested as an intermediate between α-aminoadipic acid and lysine, with some evidence for its formation in animal tissues. The conversion of α-aminoadipic acid to its δ-semialdehyde has been observed with an enzyme fraction from yeast (34). Successful exchange reactions with labeled pyrophosphate suggest, with the above observations, that α-aminoadipic acid is converted to α-aminoadipyl-δ-adenylate which is then reduced to the semialdehyde. The mechanism is still not known for sure, but the aldehyde appears quite definitely to be a precursor of lysine.

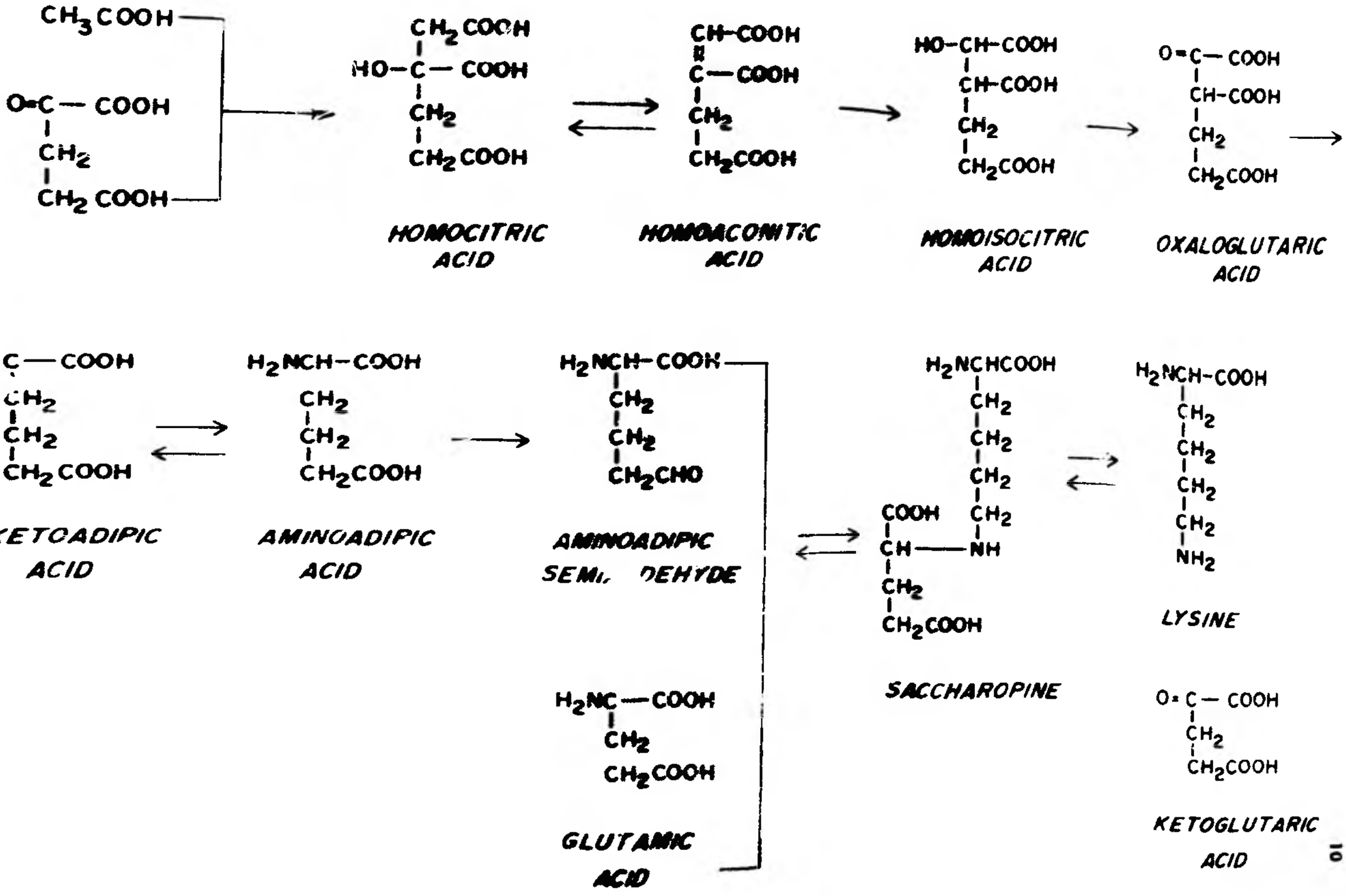
The discovery of saccharopine in yeast by Kjaer and Larsen (35) solved the problem of the conversion of α-aminoadipic acid-δ-semialdehyde to lysine. Kuo et al. (36) reported that labeled α-aminoadipic acid was converted to labeled saccharopine in yeast cells. The formation of saccharopine involves the condensation of the semialdehyde with glutamate, followed by reduction with DPNH (39). Saccharopine is oxidatively cleaved to α-ketoglutarate and lysine by means of the enzyme saccharopine dehydrogenase, requiring DPN as the oxidant (40). The aminoadipic acid pathway is

summarized in Figure 2.

Aspergillus nidulans mutants blocked between α-aminoadipic acid and lysine have been shown to accumulate L-pipecolic acid when grown in growth-limiting amounts of lysine (37). When these mutants are grown on optimal amounts of lysine no accumulation of pipecolic acid can be observed. Further experiments using o-aminobenzaldehyde suggested a precursor of pipecolic acid, namely Δ^1-piperideine-2-carboxylic acid. However, the possibility that Δ^1-piperideine-6-carboxylic acid was the precursor cannot be entirely excluded. Pipecolic acid is however not on the direct biosynthetic pathway of lysine; although rather pipecolic acid is believed to be a product of lysine catabolism in rats (41), Neurospora (42) and germinating seeds of several plants (43).

Lysine biosynthesis in Rhizoctonia leguminicola has been postulated to occur by the aminoadipic acid pathway. Support for this pathway comes from the presence of the aminoadipic acid pathway in other members of the Ascomycete family. The discovery of saccharopine dehydrogenase in Rhizoctonia leguminicola by Chao and Broquist (unpublished results) supports this hypothesis in that saccharopine dehydrogenase has been in general shown to be a marker of the aminoadipic pathway (38). Further support comes from the experiments of Snyder and Broquist (unpublished results) who showed that the label from lysine-1-^{14}C and lysine-6-^{14}C incorporated into slaframine could be diluted with pipecolate. Further experiments (unpublished results) have revealed incorporation of the label from pipecolate-^{14}COOH and aminoadipate-1-^{14}C into slaframine, with pipecolate being incorporated to a greater extent than aminoadipate. Hence it is quite probable that the aminoadipic acid pathway exists in R. leguminicola.

Figure 2. The aminoadipic acid pathway of lysine biosynthesis.


CH3COOH
O=C—COOH
CH2
CH2COOH
HOMOCITRIC ACID
HOMOACONITIC ACID
HOMOISOCITRIC ACID
OXALOGLUTARIC ACID
KETOADIPIC ACID
AMINOADIPIC ACID
AMINOADIPIC SEMIALDEHYDE
GLUTAMIC ACID
SACCHAROPINE
LYSINE
KETOGLUTARIC ACID

The main purpose of this thesis is to attempt to establish without a doubt the presence of the aminoadipic acid pathway in R. leguminicola. D, L-α-aminoadipate-6-^{14}C will be supplied to the fungus. After a period of growth the lysine of the organism will be isolated and analyzed for radioactivity and for biological activity. The occurrence of biologically active, labeled lysine will provide sufficient proof for the aminoadipic acid pathway in R. leguminicola. As stated above, the slaframine will become labeled upon the addition of labeled α-aminoadipate. The labeled slaframine will be isolated to compare the extent of incorporation with previous experiments of this laboratory.

B. Experimental and Results

1. Incorporation of D, L-α-aminoadipate-6-^{14}C into Lysine

Growth of Rhizoctonia leguminicola with D, L-α-aminoadipate-6-^{14}C

R. leguminicola was grown in Roux bottles on the hay infusion medium supplemented with casamino acids used by Aust (44). Inoculation and maintenance of cultures was also that of Aust (44). D,L-α-aminoadipate-6-^{14}C was obtained from Cyclo Chemical Company.

In the first of two experiments, 11.3 x 10^6 dpm D, L-α-aminoadipate-6-^{14}C was passed through a sterilized Millipore filter and injected aseptically into the medium of a 15 day old R. leguminicola culture. Growth was continued at room temperature for approximately four days. In the second experiment, 55.9 x 10^6 dpm D, L-α-aminoadipate-6-^{14}C was distributed between eight bottles of hay medium. The bottles were autoclaved and inoculated according to Aust (44). Growth was for approximately 30 days at

room temperature. In both cases at the end of the growth period, the mycelia were separated from the hay medium and squeezed of any excess medium.

In the first experiment, the medium was collected (270 ml), and a .05 ml aliquot counted for radioactivity in a Beckman DPM 100 liquid scintillation counter using an automatic external standard. The liquid scintillator used was 0.3% PPO, 20% ethanol in toluene. 15 ml was used for counting purposes. The medium contained 3.77×10^6 dpm, indicating that 7.53×10^6 dpm (approximately 67% of the injected counts) were actually absorbed into the mycelium.

Preparation of Hydrolysate

The mycelia isolated were, in each experiment, homogenized with 95% ethanol (approximately 5-6 volumes per gram of mycelia) in a Waring blendor for about five minutes. The homogenate was filtered through a Soxhlet thimble and then extracted in a Soxhlet extractor for approximately 48 hours at boiling. The ethanol extracts were used for the isolation of slaframine which will be discussed in a later section.

The extracted mycelia were dried and refluxed at boiling in 6 N HCl for approximately 24 hours. 100 ml of 6 N HCl were used in the first experiment, while 200 ml were used in the second. The crude hydrolysate was cooled and filtered through Whatman no. 1 paper. Water was added to the filtered hydrolysate and flash evaporated. This was repeated three to four times to remove excess HCl. The hydrolysate residue was diluted with water and mixed with activated charcoal. The suspension was filtered through Whatman no. 1 filter paper to obtain a clear hydrolysate. The hydrolysate was then reduced to a smaller, workable volume by flash evaporation.

Each hydrolysate was assayed for total amino acids relative to leucine

by the method of Moore and Stein (45). The first experiment yielded approximately 400 mg (36 ml) of amino acids, while the second experiment yielded approximately 3200 mg (250 ml). 0.1 ml aliquots were taken from each hydrolysate and counted in the Beckman DPM 100 scintillation counter as before. The hydrolysate from experiment one had 3.41×10^6 dpm or about 30% of the total injected counts, while the hydrolysate from experiment two had 7.56×10^6 dpm or about 14% of the total injected counts. Here total injected counts refer to the counts supplied to the medium and not the counts actually incorporated into the mycelia.

Isolation of Lysine

The lysine was separated from the "purified" cell hydrolysate by chromatography on a column of Aminex-MS cation exchange resin (obtained from Bio-Rad Laboratories) according to the procedure of Kirkpatrick and Anderson (46). The buffer used for elution was pH 4.26. Its compostion was as follows: 2.66% citric acid, 1.56% NaOH, 1.54% (by volume) concentrated HCl, 0.01% (by volume) octanoic acid and 0.1% Brij-35. Glass distilled water was used to make up the buffer to the required volumn. The column of Aminex-MS resin, 12 mm x 175 mm, was packed in sections of one-third. For each section the resin was allowed to settle and then packed with pressure. The column was washed with buffer each time before use. The hydrolysate sample, about 2 ml in size, was introduced and allowed to wash on the column. An additional 10 ml of buffer was washed through the column before elution was begun. An approximate flow rate of 30 ml per hour was maintained. The column was run at room temperature with 2 ml fractions being collected. Under such conditions as the above, lysine will come off the column in the range of fractions 80-90 (47). The neutral and acidic amino acids will

come off early, around fractions 7-30 (47). The other basic amino acids will be retained longer by the column and will come off at various times after fraction 30, depending on the charge at pH 4.26.

In the first experiment 20 ml of the total 36 ml hydrolysate was introduced onto the column. A flow rate of 32 ml per hour was maintained, with 130 2 ml fractions being collected. 0.1 ml aliquots from each fraction were counted as before. 0.2 ml aliquots were used for the ninhydrin assay of Moore and Stein (45). The resulting radioactivity and ninhydrin elution patterns are given in Figure 3. The radioactivity observed was in fractions 13-19 and in fractions 80-105. The radioactivity in fractions 80-105 was 93,000 dpm or 1.68×10^6 dpm for the total hydrolysate. This gives a per cent incorporation of ^{14}C from D, L-α-aminoadipate into lysine of 14.9%, neglecting the fact that approximately one-third of the injected counts remained in the medium. The counts in fractions 13-19 were probably due to residual D, L-α-aminoadipate-6-^{14}C.

In the second experiment 2.5 ml of the total 250 ml hydrolysate was introduced onto the column. A flow rate of 28 ml per hour was maintained, with 125 2 ml fractions being collected. Again 0.1 ml aliquots were used for counting and 0.2 ml aliquots for the ninhydrin assay. The resulting radioactivity and ninhydrin elution patterns are given in Figure 4. The radioactivity observed was mainly in fractions 84-103, the expected region of lysine. Some activity was found in the early neutral and acidic fractions. It was not as distinct as in the first experiment, probably due to the fact that incubation was 30 days instead of four days.

The radioactivity in fractions 84-103 was 60,600 dpm or 6.06×10^6 dpm for the total hydrolysate. This yields a per cent incorporation of ^{14}C from D, L-α-aminoadipate into lysine of 10.8%, again neglecting the fact that a sizeable amount of the injected counts remained in the medium. The

Figure 3. The elution of amino acids from a mycelial hydrolysate of Rhizoctonia leguminicola exposed to D,L-α-aminoadipate-6-^{14}C, experiment 1.

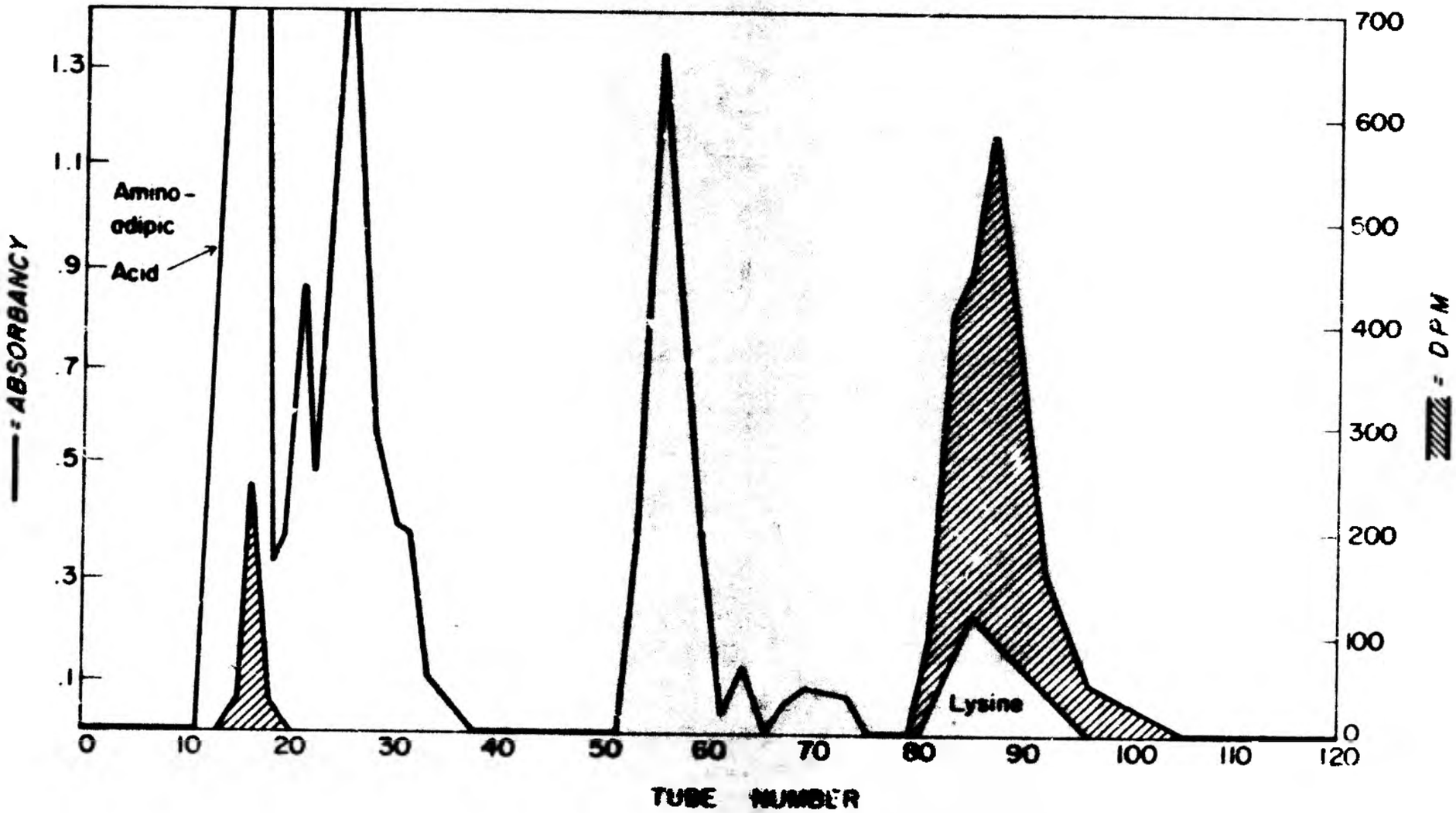
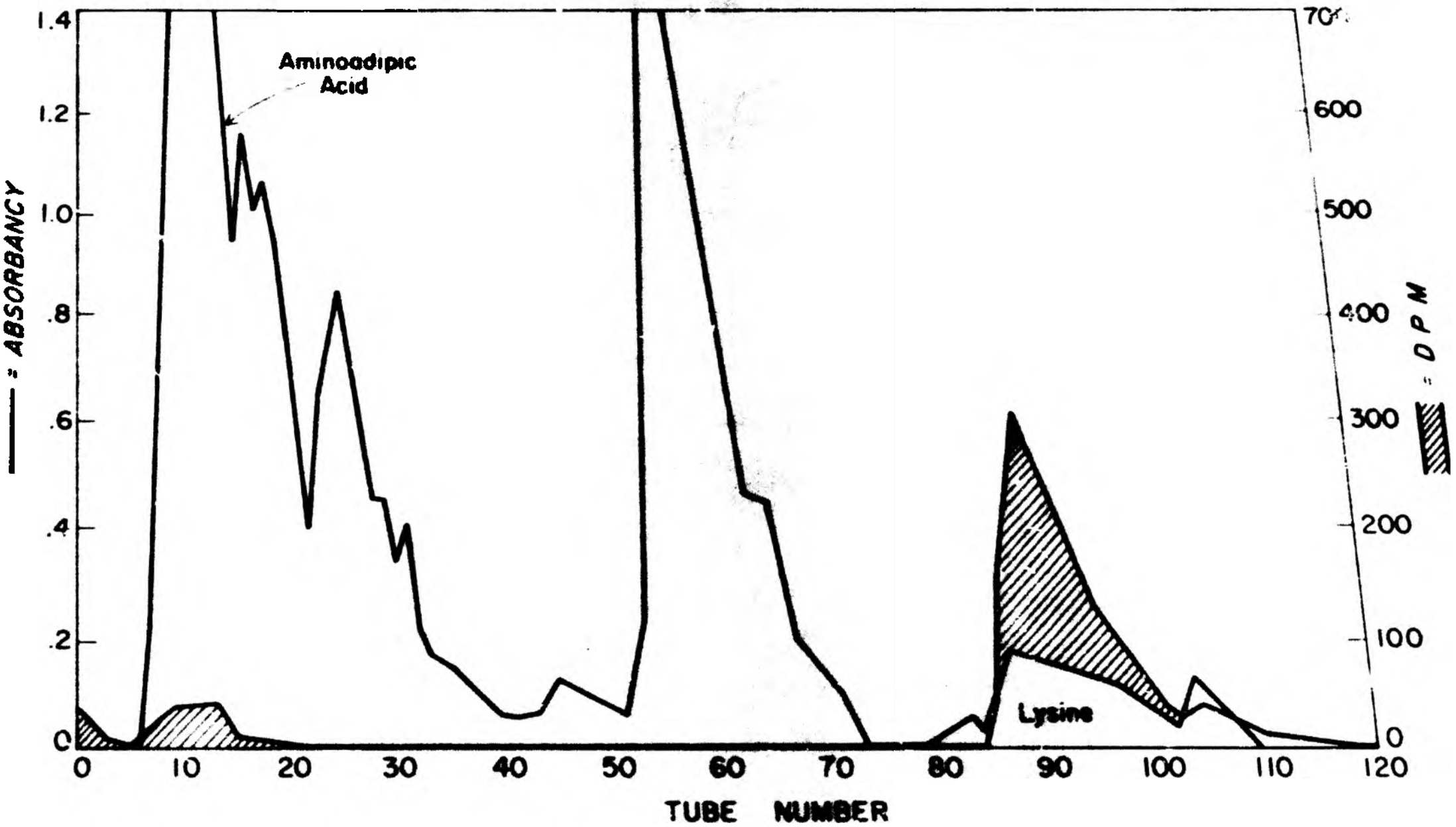

Amino-
adipic
Acid
Lysine
1.3
1.1
.9
.7
.5
.3
.1
0
10
20
30
40
50
60
70
80
90
100
110
120
700
600
500
400
300
200
100
0
= ABSORBANCY
= DPM
TUBE NUMBER

Figure 4. The elution of amino acids from a mycelial hydrolysate of Rhizoctonia leguminicola exposed to D, L-α-aminoadipate-6-^{14}C, experiment 2.


Aminoadipic Acid
Lysine
1.4
1.2
1.0
.8
.6
.4
.2
0
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
TUBE NUMBER
= ABSORBANCY
= DPM

results of the two incorporation studies are summarized in Table I.

Proof for labeled, biologically active lysine

Electrophoresis

Electrophoresis was selected as one of the means for identifying the isolated "lysine" as lysine. Because of the nature of the isolation procedure for lysine (i.e. the use of sodium citrate buffer), it was desirable to remove the sodium citrate from the isolated "lysine" before proceeding with the electrophoresis. Otherwise, the spots would be broadened due to the high concentration of ions at the point of application. A desalting technique utilizing Dowex 50W-X8 (200-400 mesh, spheres, purchased from J. T. Baker Chemical Co.) was employed to remove the sodium citrate. 2 ml of wet Dowex 50W-X8 was placed in a disposable pipette to form a small column. The column was washed with about 15 ml of 4N HCl. Distilled water was washed through the column until the eluate was of the same pH as the distilled water. The sample was placed on the column and then washed with approximately 15 ml of distilled water to remove the citrate anion. The column was washed with about 15 ml of 1N HCl to remove the sodium cation. Finally, 15 ml of 4N HCl was applied to the column, eluating off the lysine relatively free of sodium citrate. The lysine solution was diluted with water and flash evaporated. This was repeated three or four times so as to remove the HCl. Such a procedure was applied to both isolations of lysine.

Fractions 82-90 of experiment one were combined and a small sample desalted for electrophoretic purposes. Fractions 86-97 of experiment two were combined and reduced in volume by flash evaporation until sodium citrate precipitated out. The water solution was removed with a disposable

TAbLE 1

Incorporation of D, L-α-aminoadipate-6-^{14}C into lysine and slaframine in *Rhizoctonia leguminicola*

	Experiment 1	Experiment 2
Number of mycelia mats	1	8
Age of mycelial mats when injected (days)	15	0
Period of growth (days)	4	30
Injected dpm of D,L-α-aminoadipate-6-^{14}C	11.30×10^6	55.90×10^6
Dpm in hydrolysate	3.41×10^6	7.56×10^6
Dpm recovered in lysine	1.68×10^6	6.06×10^6
Per cent incorporation into lysine	14.9%	10.8%
Dpm recovered in slaframine	9.02×10^3	5.83×10^4
Per cent incorporation into slaframine	0.08%	0.10%

pipette from the precipitated sodium citrate and applied to the Dowex 50W-X8 column. The lysine was eluted off with 4N HCl as described previously. The HCl was removed and the remaining residue diluted to 15.0 ml. This solution was used for both electrophoresis and a microbiological assay (to be described later).

Paper electrophoresis was carried out in a .23M sodium acetate buffer pH 4.15 at 320 volts for approximately 3.5 hours using a Beckman Paper Electrophoresis Cell (Durrum Type). In the first experiment the combined fractions 82-90, fraction 16, a lysine standard, and a α-aminoadipate standard were subjected to electrophoresis. In the second experiment the combined fractions 86-97 and a lysine standard were run. In both cases the strips were dipped in 0.3% ninhydrin in acetone and heated in a 100°C oven for approximately one minute. In experiment one fractions 82-90 showed only one spot, that corresponding to a lysine standard. Fractions 16 showed two large spots upon development, one corresponding to an α-aminoadipate standard. The other spot did not move from the origin, indicating neutral substances. In experiment two the lysine fractions 86-97 again showed only one spot, that corresponding to a lysine standard. Figure 5 (b and c) shows a schematic representation of an electrophoretic strip of fractions 86-97 of experiment two, accompanied by a lysine standard strip.

Radioactivity Scans

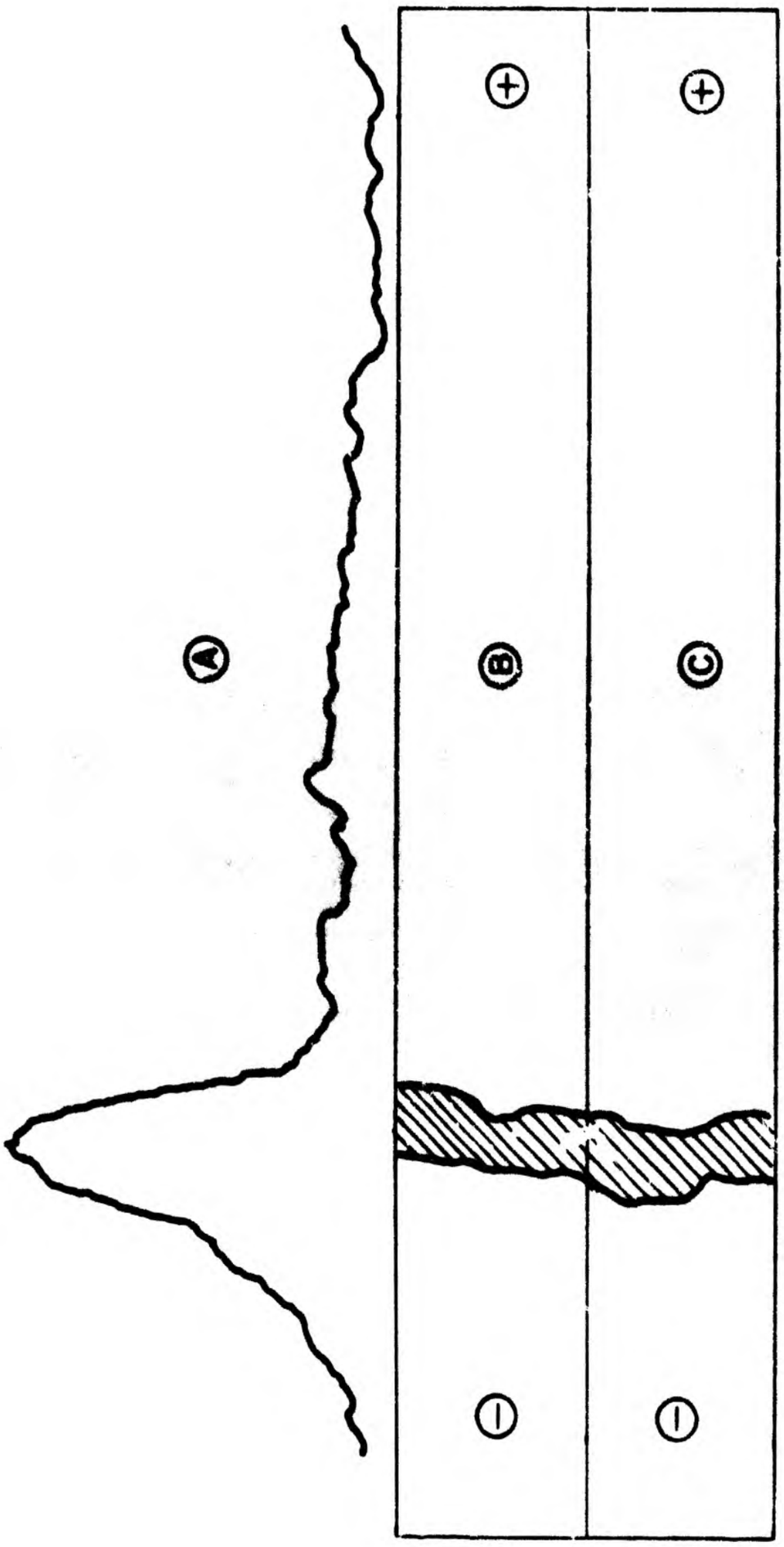
The electrophoretic strips of fractions 82-90 and fraction 16 were scanned for radioactivity using a Vanguard Chromatogram Scanner. The "lysine" spot was found to be radioactive, while the "α-aminoadipate" spot (fraction 16) was not. This was due to the high ratio of amino acids to

Figure 5. Radioactive scan (^{14}C) of lysine isolated from Rhizoctonia leguminicola.

(a) - radioactive scan (^{14}C) of electrophoretic strip of isolated lysine.

(b) - electrophoretic strip of isolated lysine from R. leguminicola (fractions 86-97 of experiment two.)

(c) - electrophoretic strip of a lysine standard.


+
+
A
B
C
1
1

radioactivity in fraction 16. The chromatogram would have to have been extremely overloaded to get any trace of activity. The strips of fractions 86-97 were also scanned and again the single spot was radioactive. Figure 5 (a and b) shows a comparison of the radioactivity scan versus the electrophoretic strip of fractions 86-97 of experiment two.

Microbiological Assay using Leuconostoc mesenteroides

Further proof for the isolated substance being lysine would be to show that it could support the growth of a lysine-requiring organism. For this purpose, a Bacto lysine assay medium (manufactured by Difco Laboratories) was selected. The test organism used was Leuconostoc mesenteroides P-60, ATCC #8042, which has an absolute growth requirement for L-lysine and is the recommended organism for lysine using the above mentioned medium.

Preparation of media, assay tubes, inoculum for assay, stock cultures and a standard growth curve were that suggested by Difco (48). A L-lysine stock solution, 150 µg/ml, was used instead of the suggested 60 µg/ml L-lysine solution in preparing the standard growth curve. Four different aliquots of the 15 ml solution (fractions 86-97 of experiment two), 0.3, 1.0, 2.0, and 3.0 ml were selected for assaying. The assay tubes were incubated at 37° C for approximately 34 hours and growth determined turbidimetrically at 660 mµ in a Bausch and Lomb Spectronic 20. The microbiological assay yielded 46 µg/ml for the 15 ml desalted "lysine" fraction of experiment two.

2. Incorporation of D, L-α-aminoadipate-6-^{14}C into siaframine

In the two α-aminoadipate experiments the ethanol extracts of the mycelia were treated with lead subacetate to remove organic compounds and the siaframine, by partitioning between water and chloroform, was isolated as a

yellow dipicrate salt, melting point 183-184° C, upon addition of saturated picric acid. The purification scheme followed was that used by Aust (44). The dipicrate was collected by centrifugation at three-fourths maximum speed in an International Clinical Centrifuge. The supernatant was removed and the dipicrate resuspended in water. The suspension was centrifuged as before and the supernatant removed. The yellow residue was dried in a vacuum oven overnight at 60° C and 300 mm Hg.

The siaframine dipicrate was weighed and a fraction of the sample dissolved in acetone. Aliquots were placed on planchets, dried, and counted in a Nuclear Chicago Planchet Counter (efficiency of 42.9%). In experiment one 8.6 mg of "cold" siaframine dipicrate were added to 1.5 ml of 1N HCl and extracted four times with 2 ml aliquots of ether to remove the picric acid. The resulting aqueous solution was added to the ethanol extract of the mycelium in order to provide carrier for the labeled siaframine, since the yield of siaframine from one mycelial mat is only 1-2 mg. However, upon isolation of the siaframine dipicrate, only 1.5 mg was obtained, indicating that the carrier probably decomposed. 1.4 mg of the sample was dissolved in 1.4 ml of acetone and a 0.1 ml aliquot dried and counted as described above. 277 cpm were obtained for 0.1 mg of the isolated siaframine, while background was 19 cpm. Correcting for the efficiency of the instrument and background, the isolated siaframine contained 9.02×10^3 dpm, .08% of the injected counts.

In the second experiment no carrier was added since eight mycelial mats were used instead of one and the attempt previously at using carrier apparently had failed. 5.3 mg of siaframine was isolated and dissolved in

10.0 ml of acetone. A 0.1 ml aliquot was counted in the Nuclear Chicago Planchet Counter as before. 289 cpm were obtained for 5.3×10^{-2} mg of slaframine while background was 39 cpm. Correcting for the efficiency of the instrument and background, the isolated slaframine contained 5.83×10^{4} dpm, .10% of the injected counts. The results of the two experiments are summarized in the lower portion of Table I.

C. Discussion

^{14}C label from D, L-α-aminoadipate went into both lysine and slaframine, Table I. In the first experiment 14.9% of the injected label was incorporated into lysine, while .08% was incorporated into slaframine. If correction is made for the counts remaining in the medium, then 22.3% of the injected label went into lysine. In the second experiment 10.8% of the injected label was incorporated into lysine, while .10% was incorporated into slaframine. This distribution of radioactivity derived from aminoadipate relative to lysine and slaframine undoubtedly reflects the priority of the cell for lysine for metabolic purposes. The function of slaframine in A. leguminicola metabolism is unknown; the possibility exists that slaframine represents a very minor end product of lysine catabolism in this mold.

The incorporation rates would be doubled if only the L or D form of α-aminoadipate were absorbed into the mycelium. The stereospecificity of the transport of α-aminoadipate into the cell is unfortunately not presently known. Specificity could also reside in the aminoadipic acid pathway in that only one form of α-aminoadipate is metabolized. There always exists the possibility of a racemase that could convert the D and L forms if the enzymes of the aminoadipic acid pathway would take only one form of

α-aminoadipate. Much could be hypothesized.

The electrophoresis of the fractions from the Aminex-MS column in the expected region of lysine revealed only one spot, that corresponding to a lysine standard. The electrophoretic strips of the isolated lysine showed radioactivity only for the spots corresponding to the lysine standards. The microbiological assay revealed the isolated lysine to be biologically active, approximately .69 mg of biological active lysine in the 15 ml desalted fractions of experiment two. .99 mg of lysine were initially placed on the Dowex 50W-X8 column (43,600 dpm) and .70 mg recovered as determined by the radioactivity of the 15 ml desalted fraction (31,000 dpm). Approximately 1.4 mg of lysine were in the fractions 85-102 of experiment two. This value of lysine would indicate the organism is synthesizing approximately 17 mg of lysine per mycelial mat. As previously stated, the yield of slaframine is approximately one mg per mycelial mat (0.3 mg of the free base). It is apparent that one part of slaframine is made per 57 parts of lysine. Therefore, a fine point of control in the aminoadipate pathway must exist relative to lysine and slaframine production.

The findings of Snyder and Broquist showed that lysine -1-^{14}C, lysine-6-^{14}C, aminoadipate-1-^{14}C, and pipecolate-^{14}COOH were incorporated into slaframine. Unlabeled pipecolate markedly reduced the incorporation of the radioactive metabolites into slaframine. The work of Aspen (unpublished results) revealed that the α-amino group of lysine is retained as the bridge nitrogen in slaframine. These findings plus the results of this thesis and the work now in progress in this laboratory suggest that α-aminoadipic acid-δ-semialdehyde is a branch point leading to the biogenesis of slaframine

or lysine in the metabolism of R. leguminicola. A proposed pathway for the biogenesis of slaframine originating from the aminoadipate pathway of lysine biosynthesis is given in Figure 6.

II Possible Terminal Step in Slaframine Biogenesis

A. Introduction

Aust prepared deacetylslaframine by alkaline hydrolysis of slaframine and observed that deacetylslaframine was devoid of physiological activity (44).

This implied that a key step in the biogenesis of slaframine was formation of the acetate ester. It seems likely that such acetylation might be the terminal step of slaframine biosynthesis. In this respect the acetylation

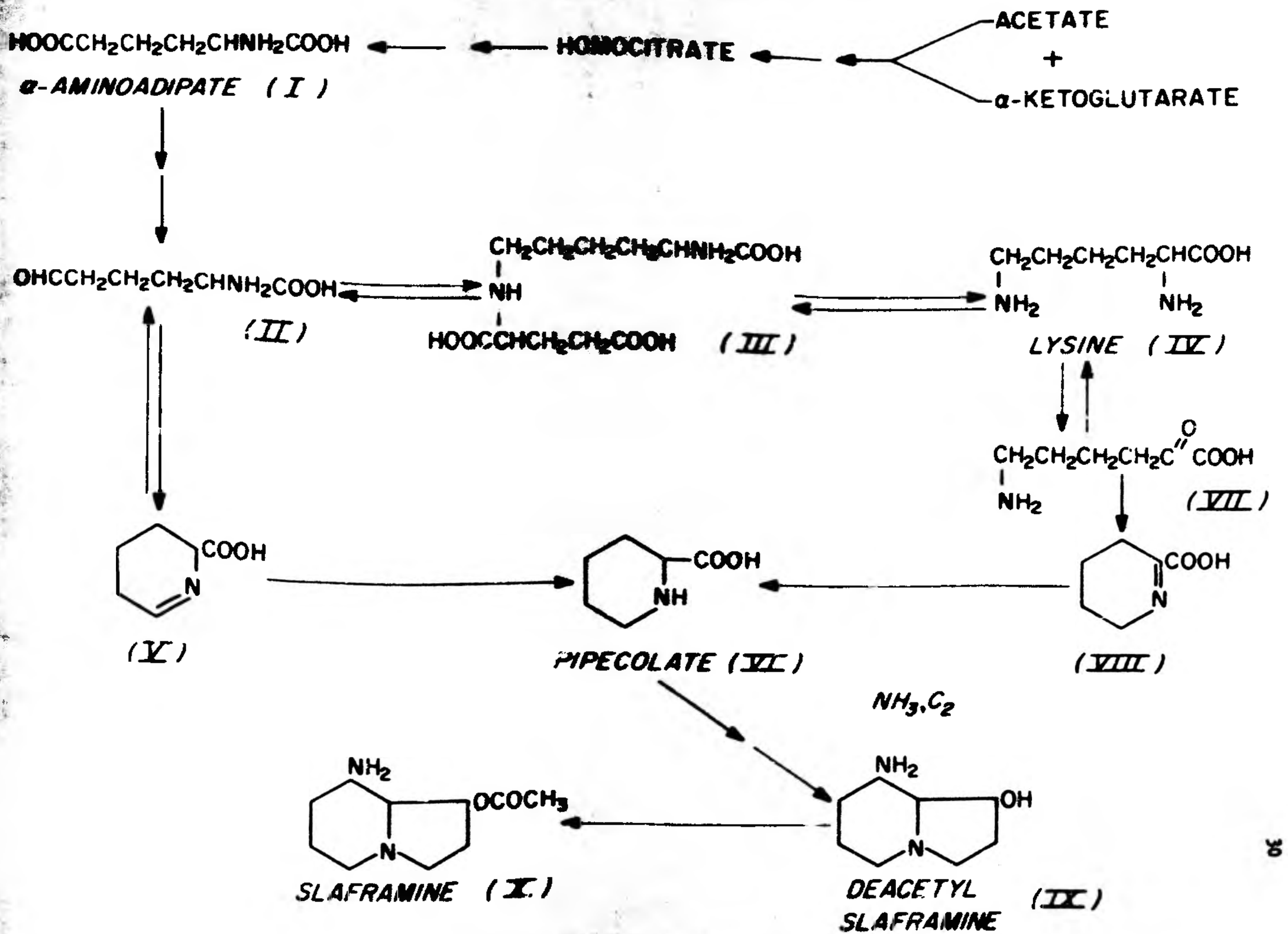
Deacetylslaframine ——————> Slaframine

of choline to give acetylcholine is a prime example of the importance of an acetate ester for parasympathomimetic activity and served as the impetus for work to be described.

Choline ——————> Acetylcholine

Therefore, an effort was made to see if evidence could be obtained for deacetylslaframine as an immediate precursor for slaframine. Because of the access to ^{3}H-slaframine of high specific activity and the chemical evidence for the simple acetylation of deacetylslaframine to yield slaframine, ^{3}H-slaframine would be subjected to base hydrolysis to yield deacetylslaframine. ^{3}H-deacetylslaframine would be injected into growing cultures of R. leguminicola; growth would be permitted for various times; and the slaframine isolated and counted for radioactivity. A high degree of incorporation of the label would point to deacetylslaframine as being an immediate precursor

Figure 6. A proposed pathway for the biogenesis of slaframine.


HOOCCH2CH2CH2CHNH2COOH
α-AMINOADIPATE (I)
HOMOCITRATE
ACETATE
+
α-KETOGLUTARATE
OHCCH2CH2CH2CHNH2COOH
(II)
CH2CH2CH2CH2CHNH2COOH
NH
HOOCCHCH2CH2COOH
(III)
CH2CH2CH2CH2CHCOOH
NH2
NH2
LYSINE (IV)
CH2CH2CH2CH2C
O
COOH
NH2
(VII)
COOH
N
(V)
COOH
NH
PIPECOLATE (VI)
COOH
N
(VIII)
NH3, C2
NH2
OCOCH3
N
SLAFRAMINE (X)
NH2
OH
N
DEACETYL SLAFRAMINE
(IX)

of slaframine, and serve as a basis for more detailed study.

B. Experimental and Results

Base Hydrolysis of Slaframine to Deacetylslaframine

^{3}H-slaframine dipicrate was obtained from New England Nuclear who performed the Wilzbach tritium exchange with slaframine isolated in this laboratory. ^{3}H-slaframine dipicrate was dissolved in 20 ml of 0.1N HCl and extracted three to four times with an equal volume of ether until all the picric acid was removed from the aqueous layer. The aqueous layer was removed and added to 4.4 ml of 6N NaOH to make a 1N NaOH solution. The solution was incubated between 40 and 50° C for approximately 20-30 minutes depending on the particular experiment. These ranges were chosen from earlier experiments dealing with the length and temperature of hydrolysis as followed chromatographically. 1N NaOH was suggested by Aust (44).

The aqueous solution was extracted three times with an equal volume of chloroform. The chloroform solutions were combined, dried by passage through anhydrous sodium sulfate, and bubbled with dry HCl from a reaction vessel of concentrated sulfuric acid and sodium chloride. The chloroform was evaporated under reduced pressure using a flash evaporator. 5.0 ml of chloroform was added to the residue.

Chromatography of Deacetylslaframine

Approximately 60 µl of the chloroform solution was spotted (approximately 30 µl per spot) on Whatman no. 1 paper and developed in a butanol: acetic acid: water system (4:1:1) for approximately nine hours. The chromatograms were sprayed with a 0.3% ninhydrin in ethanol solution and developed in a 100° C oven for approximately two minutes.

Previous experiments conducted concerning the chromatographic behavior of slaframine and deacetylslaframine showed excellent separation of the two compounds in the butanol:acetic acid:water system. Figure 7 shows a schematic representation of a chromatogram of deacetylslaframine and slaframine. The R_f value (relative to the solvent front) of slaframine was usually .48, while the R_f value of deacetylslaframine was usually .33. The R_f values of deacetylslaframine had a tendency to change somewhat depending on how old the solvent system was (i.e. an R_f value of .26 occurred quite frequently), but the two compounds were still well separated.

Therefore, the chromatograms would reveal the extent of hydrolysis of slaframine to deacetylslaframine. Of the three experiments performed, all three produced chromatograms like figure 7a, except for a slight downward tailing in the third experiment.

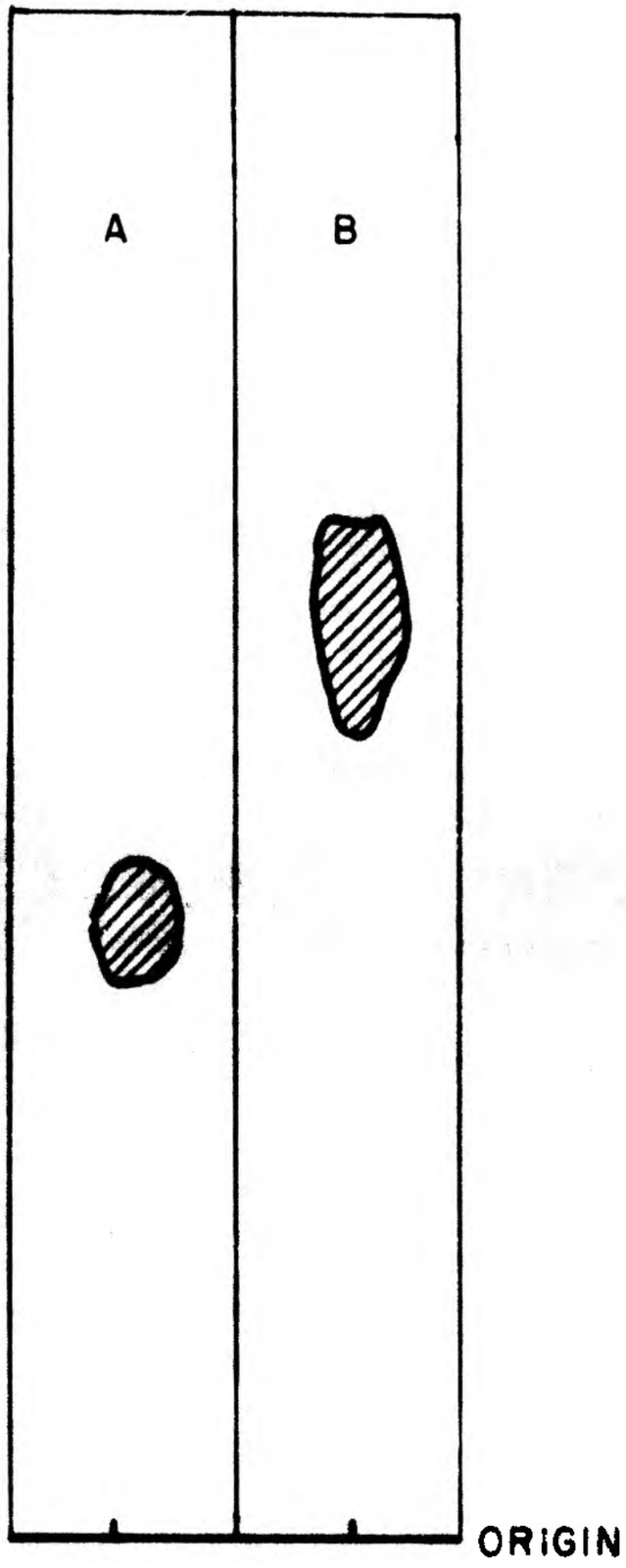
Injection and Growth of Mycelia

The chloroform solution was again flash evaporated under reduced pressure and diluted with 31.0 ml of water. The aqueous solution was passed through a sterilized Millipore filter and injected aseptically (approximately 3 ml per bottle) into 10 bottles of growing mycelia. A 0.1 ml aliquot was set aside and counted in a Beckman DPM 100 as before to determine the total injected counts. The mycelia were allowed to grow at room temperature and the slaframine isolated as previously described. The slaframine was weighed and a fraction of the sample dissolved in 0.1N HCl. The solution was extracted three times with an equal volume of ether. The aqueous phase was removed and its pH adjusted to approximately 10.5 with 1N NaOH. The aqueous solution was extracted three times with an equal volume of

Figure 7. Chromatography of siaframine and deacetylsiaframine in a butanol:acetic acid:water system (4:1:1).

(a) - Chromatogram of deacetylsiaframine.

(b) - Chromatogram of siaframine.


A
B
ORIGIN

chloroform. The chloroform layers were placed in a scintillation vial and approximately 30 µl spotted on Whatman no. 1 paper which was chromatographed and developed as before. The chloroform solution was dried over nitrogen by heating on a hot plate and then counted in the Beckman DPM 100 as before. The siaframine chromatographs of the first two experiments revealed only one spot like that in figure 7b. The chromatogram of the third experiment showed two spots (R_f values of .51 and .34) corresponding supposedly to siaframine and deacetylsiaframine, with the siaframine spot the darker of the two.

The first experiment, using 10 7 day-old mycelia and 7.5 day incorporation period, yielded 3.2% incorporation of the total injected counts into siaframine. The second experiment, using 10 14 day-old mycelia and a 6 day incorporation period, yielded .14% incorporation into siaframine. The third experiment, using 10 3 day-old pads and a 16 day incorporation period, yielded .57% incorporation into siaframine. The summary of the data of the three experiments is given in Table 2.

C. Discussion

The degrees of incorporation into siaframine of 3.2%, .14%, and .57% are not indicative of an immediate precursor to siaframine. This however does not by any means exclude it as a possible candidate. There seems to be no overwhelming permeability problem of deacetylsiaframine into the mycelia. In experiment two 3.9×10^6 dpm of the injected 5.73×10^6 dpm (68%) remained in the medium, but even considering this, only .43% of the counts absorbed into the mycelia were incorporated into siaframine.

Even though the counts are absorbed into the mycelia to a sufficient extent, the question arises whether the conversion of deacetylsiaframine

TABLE 2

Incorporation of ^{3}H-deacetylsiatramine into siaframine

	Experiment Number		
	1	2	3
Number of mycelial mats	10	10	10
Age of mycelial mats when injected (days)	7	14	3
Period of growth (days)	7.5	6	16
Injected dpm as ^{3}H-deacetylsiaframine	3.98×10^{5}	5.73×10^{6}	3.15×10^{6}
Dpms recovered in siaframine	1.26×10^{4}	7.95×10^{3}	1.81×10^{4}
Per cent incorporation into siaframine	3.2%	0.14%	0.57%

to slaframine is compartmentalized and the ^{3}H-deacetylslaframine is not hampered in some way in getting into the slaframine pathway.

One could question the nature of the substance injected into the mycelia, since only chromatographic evidence was used for identification of substrate. Aust (44) isolated crystalline deacetylslaframine after treating slaframine with 1N NaOH momentarily in hot water and obtained IR, nmr, and mass spectographic evidence for deacetylslaframine. The disappearance of a spot known to be slaframine and the appearance of another spot on alkaline hydrolysis would suggest the spot to be deacetylslaframine.

The results certainly do not give unequivocal evidence of deacetylslaframine as an immediate precursor of slaframine, but this possibility cannot be strictly ruled out.

III Summary

This thesis concerned a study of certain aspects of lysine metabolism and slaframine biogenesis in Rhizoctonia leguminicola. Slaframine, 1-acetoxy-8-aminooctahydroindolizine, is a parasympathomimetic alkaloid, in which the piperidine ring was known to arise from lysine metabolism of the mold. It is shown by appropriate radioisotopic techniques that the label from D, L-α-aminoadipate-6-^{14}C was incorporated into the lysine of the cell proteins of the mold as well as into slaframine. These experiments established that the organism synthesized lysine via the aminoadipic acid pathway and that slaframine is derived from a branch point of this pathway.

In preliminary studies aimed at the possible elucidation of the terminal step in the biosynthesis of slaframine, the incorporation of ^{3}H-deacetylslaframine into slaframine was studied in growing cultures of R. leguminicola.

Icola. However, the data were not conclusive that deacetylsiaframine is the immediate precursor of siaframine.

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