STUDIES ON THE BIOSYNTHESIS OF LYCOMARASMIN

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Chemistry

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SIMON FRASER UNIVERSITY

June, 1970

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ABSTRACT

Some ^{14}C - labelled amino acids were administered over a 24 hour period to 2 month old cultures of *F. lycopersici* and the incorporation and distribution of ^{14}C - labelled compounds examined in both the culture medium and mycelial protein. The constituents of the culture medium and the amino acids in the mycelial protein hydrolysate were separated by ion-exchange resin columns and paper chromatography.

Lycomarasmin was detected as its cyclic derivative (Substance J) in the acidic amino acid fraction. Present investigations indicate that ^{14}C - labelled α- and β- alanine, aspartic acid, glycine and serine were not incorporated into lycomarasmin over a 48 hour period in amounts which could be detected by the methods used.

It was found that in cultures which were 2 months old, the ^{14}C - amino acids were apparently exclusively utilized as a carbon source by the fungus. Under these conditions no incorporation of the administered ^{14}C - labelled amino acids into the mycelial protein was detected. However, it was found that active metabolism was taking place in cultures 6 days old, as indicated

by the incorporation of aspartic acid - 4 - ^{14}C into the mycelial protein. It was concluded that transformation of amino acids was occurring only during the active growth period of the fungus, and thereafter the amino acids were catabolized and utilized in the respiratory cycle.

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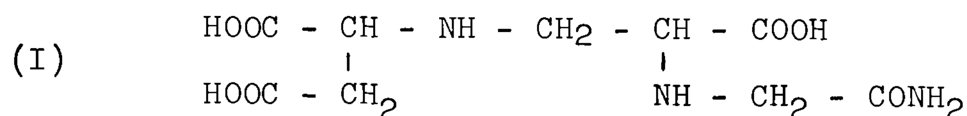
ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Professor A.M. Unrau for his continued guidance, inspiration, and assistance. Acknowledgement gratefully is made by the author to Mrs. T. McDaniel for the growing of cultures for this work, and to Miss B. DuPlessis for typing the thesis. The financial assistance from the Department of Chemistry, Simon Fraser University, and from the National Research Council of Canada is also gratefully acknowledged.

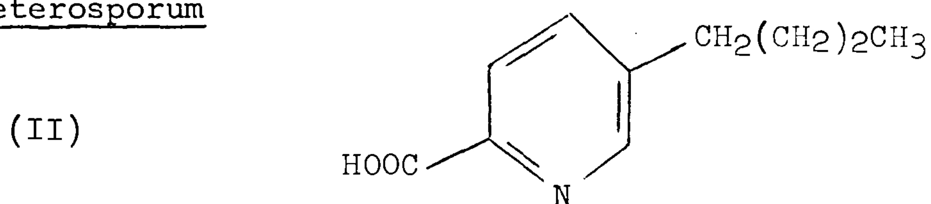
INTRODUCTION

Various strains of *Fusarium*, a fungus belonging to the *Fungi Imperfecti* family, produce wilt diseases in tomato, flax, cotton, watermelon, cowpea, potato, celery, and many other important crops.

Fusarium oxysporum f. lycopersici, causing a wilt disease of tomato plants, is known to produce three wilt inducing toxins in artificial cultures. These toxins bear no chemical relationship with one another. Thus, lycomarasmin (I), has an acyclic alkaloid type structure with a molecular weight of 277.



Fusaric acid,II, the second toxin, is 5-n-butylpicolinic acid, a pyridine base alkaloid, with a molecular weight of 179. It was first isolated by Yabuta *et al.* (1934) from the culture filtrates of *Fusarium heterosporum*



The third toxin, vasinfuscarin (Gaumann, 1953) has not yet been completely purified and like fusaric acid

is not specific to *F. lycopersici* but is also produced by *Gibberella fujikuroi*.

The biogenesis of fusaric acid has been studied by Hill *et al.* (1966) and Vining (1968), and has been found to be a condensation of aspartate and polyacetate. While considerable work has been done on the pathologic and toxic actions of lycomarasmin, there is apparently no published literature on its biogenetic origin.

The structure of lycomarasmin would indicate that its precursors should be found amongst the amino acids. It may be postulated, based on its overall structure, that these may be aspartic acid or fumaric acid, α-alanine or β-alanine and the amides of glycine or acetate, depending on how the molecule is subdivided into its apparent structural units (Fig. 1). The above suggestions find their basis in the fact that on hydrolysis, lycomarasmin gives rise to aspartic acid, pyruvic acid and glycine. A study of a possible biosynthetic pathway could therefore consist of supplying the suggested precursors labelled with ^{14}C, followed hopefully by degradation of the resulting natural product and finally determining the position of the radioactive

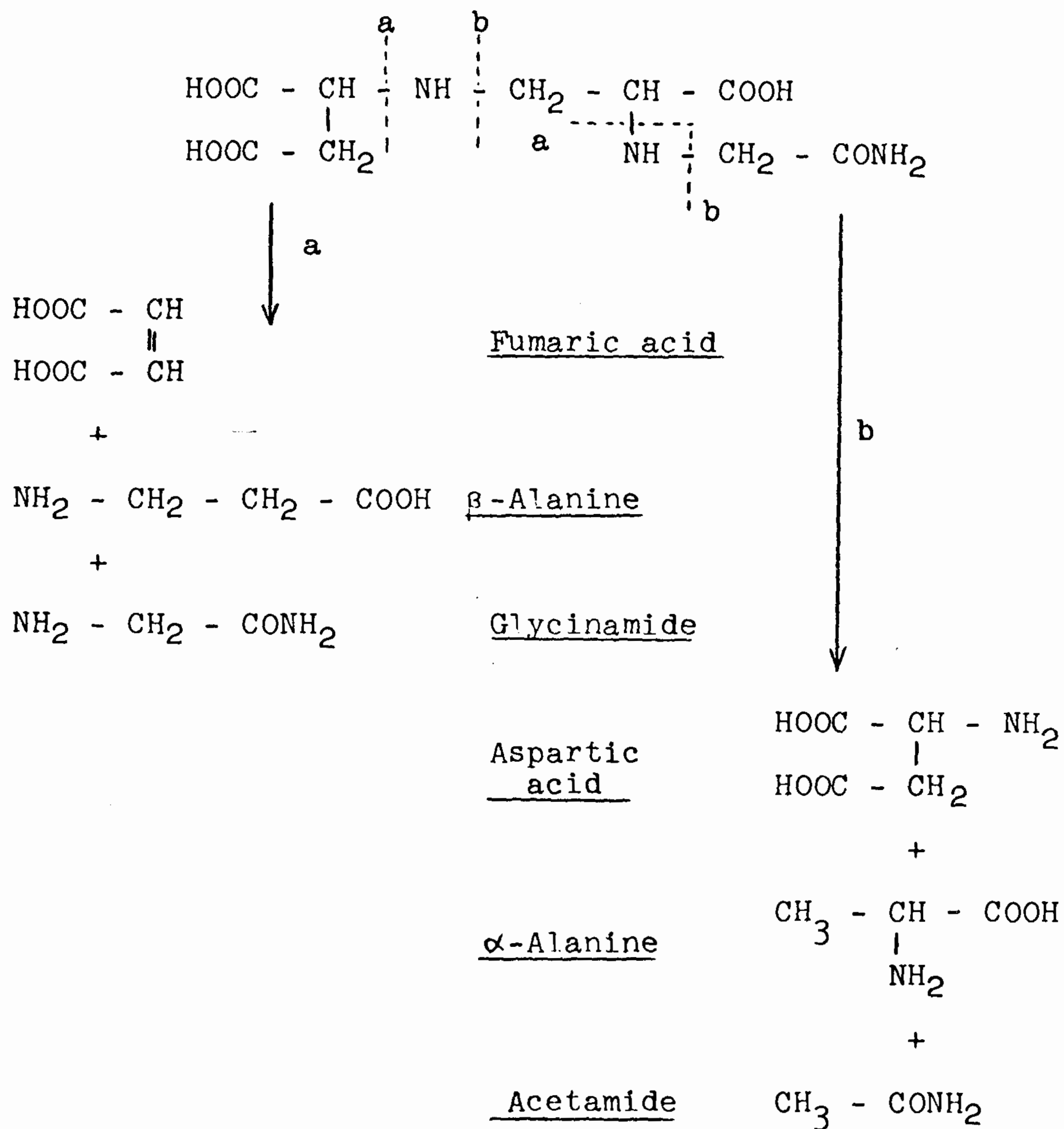


Diagram to show how lycomarasmin can be subdivided into its apparent structural units.

Fig.1

carbon atoms.

It is a well established fact that lycomarasmin can be isolated only from culture filtrates which are at least 2 to 4 months old. By this time, the fungus is well past its active growth period and lysis of the mycelium is well established. Gaumann (1957), however, reports that lycomarasmin can be demonstrated in the mycelium of F. lycopersici as early as the seventh day when the mycelium is in the log phase of growth. These facts suggest that lycomarasmin is either an endocellular metabolite which is excreted into the culture medium later in the growth cycle, or it is a product of mycelial protein breakdown. These characteristics are in contrast to those found for fusaric acid which has been shown by Sanwall (1956) to be a product of active metabolism.

The problem therefore arises of obtaining specific incorporation of ^{14}C - amino acids into lycomarasmin which is presumably formed in the early stages of growth but can only be isolated in significant quantities at a later period. This might be overcome by administration of ^{14}C - amino acids during the period of active metabolism and growth. The distinct possibility,

however, exists that if such a procedure is employed, a high degree of "scrambling" of the label may be observed due to metabolism of the amino acids in their more characteristic metabolic pathways.

The object of this study is, therefore, to examine the metabolism of some amino acids in cultures of *F. lycopersici* which were usually two months old, followed by a search for possible incorporation and distribution of the ^{14}C - label in various metabolites (eg. derived amino acids, etc.) and also its possible distribution in lycomarasmin.

It has been demonstrated by Gaumann *et al.* (1950) that the amount of toxin produced by this fungus in artificial cultures varies with the particular strain used. For this investigation three different strains of the fungus were obtained. The amount of toxin produced was assayed by paper chromatography of the culture filtrate of each strain. By visual comparison of the intensity and size of the ninhydrin positive areas it was possible to ascertain which strain gave the maximum yield of lycomarasmin. All the succeeding metabolic studies were consequently carried out on the one particular strain which appeared to be most efficient in the production of the toxin.

LITERATURE SURVEY

The existence of amino acids in the culture filtrates and mycelial protein of F. lycopersici has long been recognized. Qualitative studies of the amino acids using chromatographic techniques have been made by Fluck and Richle (1955) and a quantitative study by Sandhua (1959). Venkata Ram (1959) also reported the occurrence of free amino acids in the culture filtrates of numerous species of Fusarium.

All the commonly occurring amino acids were found present which indicates that the fungus can effectively synthesis all its amino acids from certain inorganic sources of nitrogen, primarily ammonia, and that among the most important mechanisms for the incorporation of this nitrogen into organic structures are those provided by the enzyme systems, glutamic acid dehydrogenase and aspartase. The formation of glutamic acid and aspartic acid in this way occurs at the expense of α-ketoglutaric acid and oxaloacetic acid which are themselves participants in the respiratory system, commonly known as the tricarboxylic acid cycle (TCA). It is known that Fusarium contain a considerable pool of free amino acids (Mehadevan, 1966) among which glutamic acid, aspartic acid, and also alanine predominate. The latter amino acid may

possibly arise from pyruvic acid in a manner analogous to the formation of glutamic and aspartic acid.

A great many studies are available which have a bearing on the synthesis of amino acids (Meister, 1965). On the one hand are the results of biochemical studies using mutants of *Neurospora*, *Aspergillus*, a few other fungi and yeasts; on the other are the studies in which compounds labelled with ^{15}N and ^{14}C have been fed and the amino acids and proteins later isolated and identified. ^{14}C - Labelled amino acids were found in the cultures of *Candida utilis* growing on a medium of ^{14}C - fructose (Roberts *et al*., 1955). ^{14}C - 2 - Acetate was supplied to uredospores of *Uromyces phaseoli* and *Puccinia helianthi* and, shortly after, isotopically labelled glutamate, aspartate and alanine were detected in both and, in addition, lysine in the latter species (Staples *et al*., 1961). Studies such as these, on the biosynthetic processes in germinating spores leaves little doubt that the products of carbohydrate catabolism provide skeletal material for the amino acids.

In general the amino acids, constituting the proteins of micro-organisms after growth on a radioactive source, fall into several distinct families according to their degree of labelling with the radioactive isotope

(Abelson and Vogel, 1955; Abelson *et al.*, 1953; McQuillen and Roberts, 1954). These groups comprise: (1) the glutamic acid family, consisting of glutamic acid, proline and arginine; (2) the aspartic acid family, containing aspartic acid, mathionine, threonine, and isoleucine; (3) lysine; (4) the pyruvic acid family, including α-alanine, valine, and leucine; (5) histidine (Levy and Coon, 1954; Ames *et al.*, 1953); (6) the serine family, namely serine, glycine and cysteine; and (7) the aromatic family consisting of phenylalanine, tyrosine, and tryptophan (Tatum and Perkins, 1950). (Fig. 2).

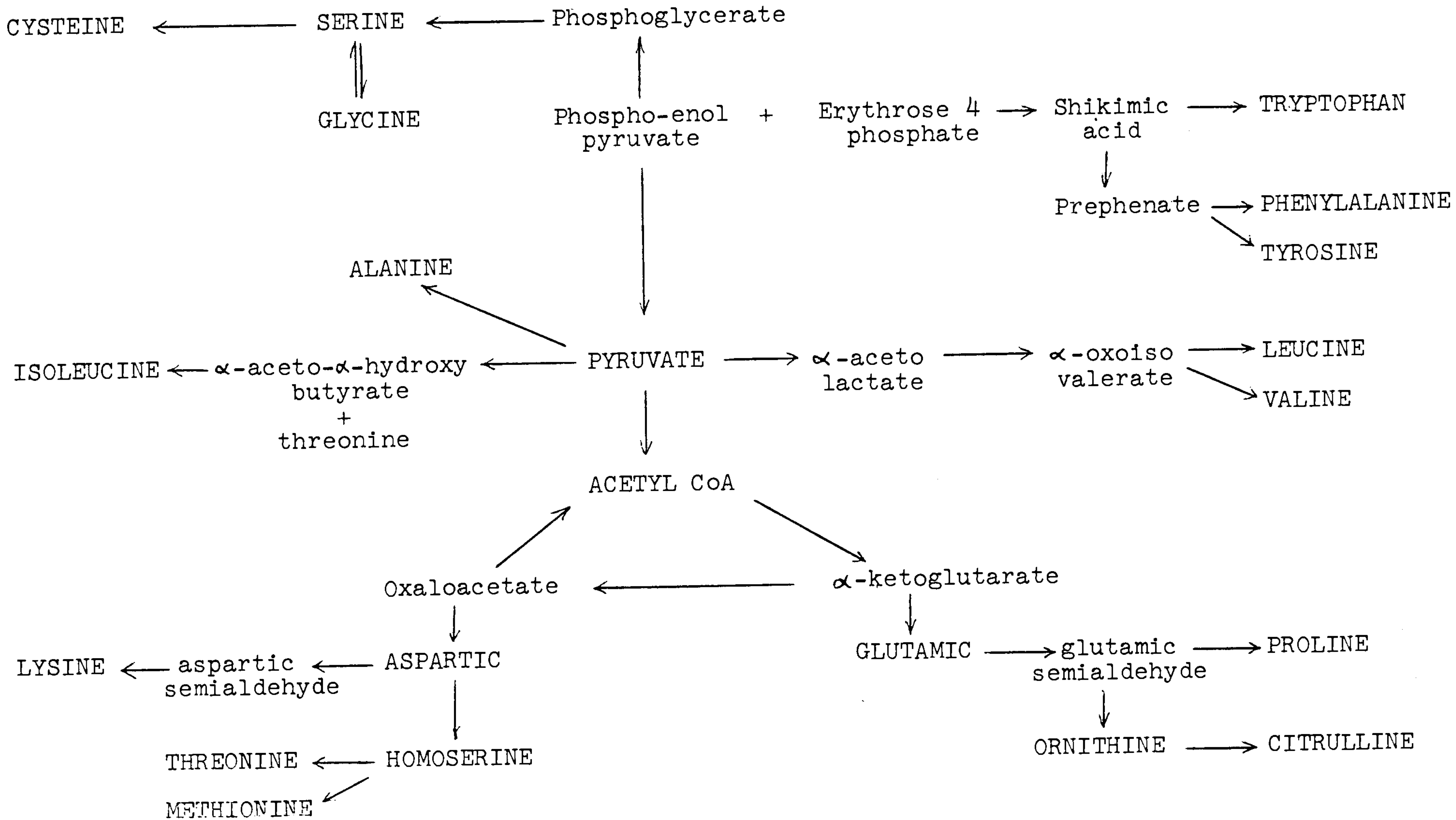
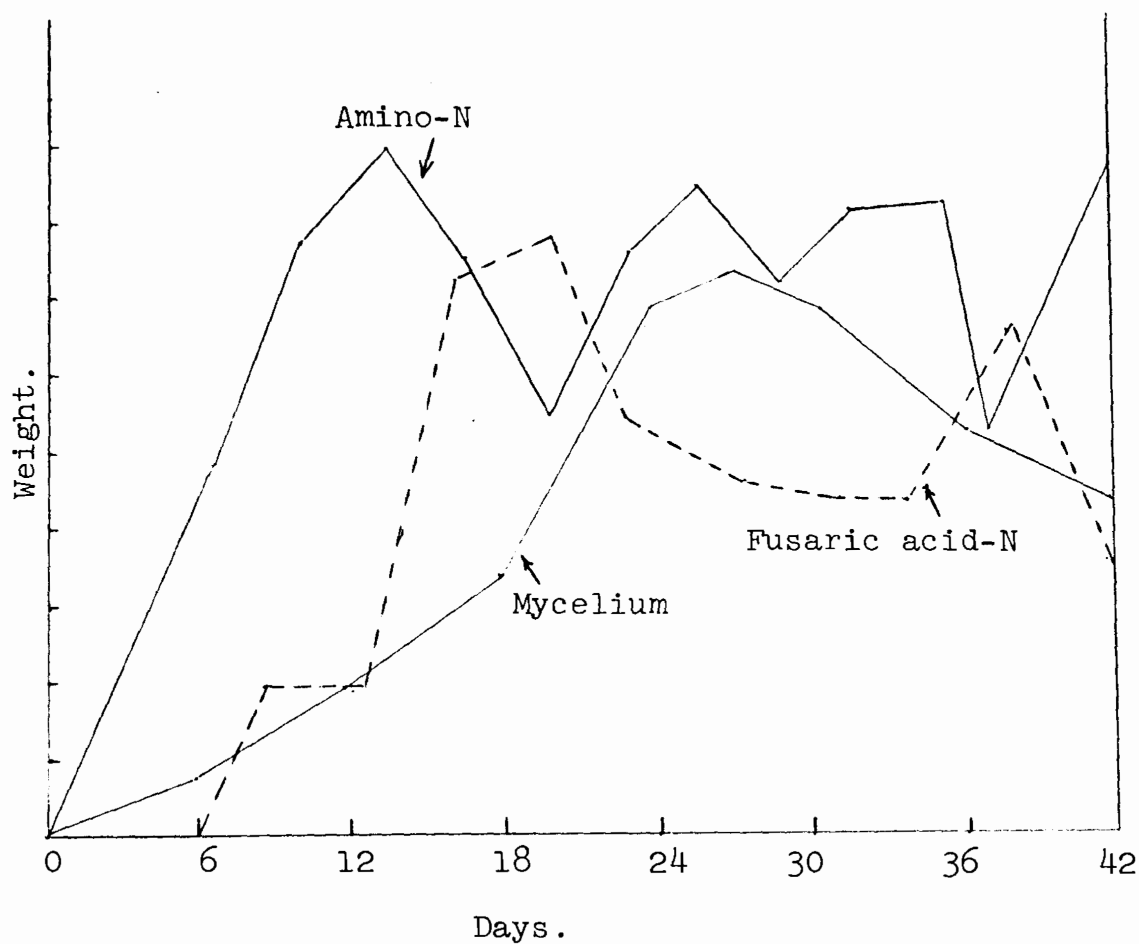


Fig.2 Relation of amino acids to TCA cycle intermediates.

Studies on the biogenesis of fusaric acid in *F. lycopersici* by Sanwall (1956) and in *G. fujikuroi* by Sandhua (1959) showed a quantitative relationship of α-amino nitrogen with fusaric acid production. Sandhua used the isotopic competition technique of Roberts *et al.*, (1955) to determine which of the commonly occuring amino acids contribute their carbon to the fusaric acid. This involved the feeding of inactive amino acids along with radioactive glucose to the fungus. A resulting decrease in the specific radioactivity of fusaric acid was taken as a measure of the contribution of carbon from the respective metabolite to fusaric acid during its biosynthesis by *G. fujikuroi*. The amino acids which were found to participate in the biosynthesis were then supplied in labelled form to the fungus in order to study the conversion or incorporation of their radioactivity. The results showed that α-alanine and serine were particularly efficient donors of carbon to fusaric acid in which conversion of their specific activity was about 28 percent. Thus, the consideration of α-alanine and serine as near precursors of fusaric acid was strongly suggested.

Sanwall investigated the relations of α-amino nitrogen and fusaric acid contents of still cultures of

F. lycopersici over a period of six weeks. It was found that amino acids were produced in the medium from the beginning of the growth period, reached a peak at the end of of 12 days, and except for a decrease between the 13th to 21st day, and again between the 33rd and 39th day, were always present at the peak level. The disappearing amino acids were most likely reutilized for protein synthesis as has been reported for micro-organisms in general (Foster, 1949). In the same study, Sanwall reports that the dry weight of the mycelium increased steadily till the 25th day of growth. A gradual decrease thereafter indicated autolysis (Fig. 3).



Fusaric acid and amino nitrogen content, and dry weight of the mycelium of F.lycopersici.

Fig.3

To demonstrate the metabolic products of the fungi in host plants, the technique of "tagging" the pathogen with a suitable radioisotope has been used. Wheeler (1952), using ^{14}C - labelled glucose, was able to tag effectively five different fungi at a concentration of 30 μC/ml. He observed at this level of radioactivity that some of the conidia were rendered non-viable, but the fungus did not lose its virulence. Kern (1954) showed that the mycelium of F. lycopersici could be safely tagged with radioactive glycine at a concentration of 90 μC/ml. after a growth period of 7 to 12 days. After 24 hours growth in a medium containing glycine - 2 - ^{14}C, the mycelial protein was found to contain small amounts of ^{14}C - labelled aspartic acid and glutamic acid with larger amounts of labelled serine, glycine, leucine and valine.

There are many examples in the literature of biogenetic studies on fungal metabolites using ^{14}C - amino acids. One major field which has received much attention is that of the ergot alkaloids which can be produced in mycelial cultures. Different strains have different requirements but, in general, the highest yield of alkaloids was achieved in still cultures in the late growth phase when the polyols, carbohydrates and

lipids were maximal or just declining (Taber and Vining, 1961; Vining and Taber, 1963). During a study of the nutritional requirements for ergot alkaloid production by *Claviceps purpurea* it was observed that addition of L - tryptophan to the medium substantially increased the yield. When tryptophan - β - ^{14}C was added to cultures, radioactivity was incorporated efficiently into alkaloids of both the clavine and lysergic acid types, and it was concluded that tryptophan served as a precursor of the ergoline ring. Mevalonic acid serves as a source of the other carbons (Taylor and Ramstad, 1960).

These and various other techniques using radioactive tracers have been useful in elucidating the pathways involved in fungal metabolism and have permitted a greater insight into the steps involved in the processes of biosynthesis.

EXPERIMENTAL METHODS AND RESULTS

1. Conditions for Growth of Fungus.

Three strains of *Fusarium oxysporum F. lycopersici* used in these studies were obtained.

- Strain no. VRS 27 from Dr. Pepin, Canadian Department of Agriculture, U.B.C.
- Strain no. 9848 from the American Type Culture Collection.
- Strain no. 5414 from Dr. S. Naef-Roth, Institut fur Spezielle Botanik der Eidgenossischen Technischen Hochschule, Zurich.

Stock cultures were maintained on potato-dextrose agar slants.

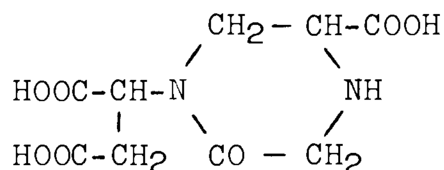
Inoculum was prepared by transferring the mycelium to 2 litre Erlenmeyer flasks containing 500 ml. of modified Richards' medium and maintaining in still culture at 25°C in the dark.

Modified Richards' medium has the following composition; glucose 5.0 g., ammonium nitrate 1.0 g., potassium phosphate 0.5 g., magnesium sulphate 0.25 g., ferric chloride 0.002 g. in 100 ml. of distilled water.

2. Chromatographic Examination of Culture Filtrate

Four cultures of each strain were worked up at the end of 3 months growth by the method suggested by Hardegger _et al._, (1963) for the isolation of lycomarasmin. The resulting solution was chromatographed on Whatman no.1 paper using pyridine: acetic acid: water (50:35:15v/v) for 10 to 12 hours. Dried chromatograms were sprayed with 0.25% ninhydrin in ethanol and developed in an oven at 105°C for 15 minutes.

- Lycomarasmin is relatively unstable in aqueous solution and readily cyclises to form Substance J (III) during the extraction procedure.



(III)

- Lycomarasmin (Rf. 0.19 in solvent pyridine: acetic acid: water) reacts with ninhydrin to give a blue colour.
- Substance J(Rf. 0.29 in solvent pyridine: acetic acid: water) reacts with ninhydrin to give a yellow colour.

It was therefore necessary to consider the colour intensity of areas corresponding to both compounds in order

to ascertain the best strain of fungus for the metabolic studies. Strain no. 9848 from Zurich was found to give the greatest yield of lycomarasmin and was therefore used for all succeeding metabolic studies.

3. Radioisotopic Techniques

a) Radioactive materials and administration.

The following ^{14}C - labelled amino acids were obtained from the International Chemical and Nuclear Corporation, California, and New England Nuclear, Massachusetts:

DL - alanine - 1 - ^{14}C, DL - aspartic acid - 4 - ^{14}C, glycine - 2 - ^{14}C, β - alanine - 1 - ^{14}C, and serine - U - ^{14}C.

An aqueous solution of each amino acid was prepared in a standard volumetric flask and sterilized for 30 minutes at 130°C. The solutions were subsequently stored in polyethylene vials at -10°C. The purity of each amino acid was checked before use by paper chromatography and strip counted as described in Section (b), page 19.

Aliquots (1μC) of the appropriate amino acid solution were administered to each culture which had been growing for 2 months using a sterilized hypodermic syringe fitted with a 12 inch needle. The solution containing the ^{14}C label was injected into the culture medium below the mycelial pad.

b) Detection of ^{14}C - labelled compounds.

The technique used for detection of the radioactive areas of the paper chromatograms was similar to that described by Wang and Jones (1959). After drying the paper chromatogram (Whatman no. 1), strips (4 cm. in width) from each chromatogram were cut into 2.5 cm. sections, and each of these inserted into a scintillation vial. To each vial was added 20 ml. of toluene cocktail and the activity determined in the usual way using a wide ^{14}C - isoset window. In cases where radioactive areas were close together, the paper strip was cut into 1 cm. sections and counted as described. The toluene cocktail consisted of 4 g. of 2.5-diphenyloxazole (PPO) and 50 mg. of p - bis 2 - (5 phenyloxazolyl) - benzene (POPOP) per litre of toluene. The counting efficiency obtained using this method was found to be 55 - 60 %.

c) Estimation of $^{14}CO_2$.

After 24 and 48 hour periods, the carbon dioxide evolved by the 2 month old organism was assayed by flushing the culture flask with carbon dioxide free nitrogen and passing the exit gas through 5 ml. of ethanolamine. The trap was removed and the solution diluted to a measured volume. A 0.5 ml. aliquot of this ethanolamine solution was counted in a toluene/PPO cocktail containing methyl cellosolve (15:3) (18 ml.).

The results are shown in Table I.

TABLE I.

Percentage of label evolved as $^{14}CO_2$.

Feeding period	^{14}C-labelled amino acid				
	α-Alanine	Aspartic acid	Glycine	Serine	β-Alanine
24 hours	4.3	1.0	0.3	1.2	0.1
48 hours	10.8	2.5	0.5	1.8	0.3

4. Chromatographic Techniques.

Direct paper chromatography of the culture filtrate or a concentrate thereof gave poor separation of the constituent amino acids. Thus, for quantitative estimation of these compounds, the removal of sugars and salts of the nutrient medium was found to be indespensible. The amphoteric nature of amino acids has been widely used for their adsorption on either cation or anion exchange resins followed by gradient buffer elution.

The fungal extracts were fractionated by use of Dowex 50W(x8) and Dowex 1(x10) resins into basic, acidic and neutral amino acids, organic acids and sugars as described below. Each fraction was evaporated *in vacuo* to dryness and the residue dissolved in 1 ml. of water, and stored at -10°C. A 0.1 ml. aliquot of each fraction was counted using a cocktail consisting of 100 g. of naphthalene and 5 g. PPO per litre of dioxane.

The components of the amino acid fractions were separated by paper chromatography (Whatman no. 1) on a 24 cm. wide strip using a solvent composed of phenol, methanol, water (35:50:15v/v/v) in the ascending direction. The chromatograms were dried at room temperature. In order to facilitate complete evaporation of phenol, the chromatograms were aerated for two to three days till the

odour of phenol was no longer apparent. A 4 cm. wide strip was cut from the chromatograms and the radioactive areas were located as described above. The respective radioactive areas were eluted with hot water from the remaining portion of the chromatograms. The eluates were concentrated *in vacuo* and each fraction chromatographed in the ascending direction in a second solvent composed of butan - 1 - ol, acetone, 14.7*M* ammonia, water (40:40:8:20v/v). A third solvent consisting of phenol, water was found to be useful for the separation of aspartic acid and glutamic acid.

Dried chromatograms were sprayed with 0.25% ninhydrin solution in ethanol followed by heating in an oven at 105°C for 15 minutes. Reference compounds were run with the unknown substances.

a) Ion Exchange Resins and Preparation of Columns.

Dowex 50W(x8)(H^+) 200-400 mesh and Dowex 1(x10)(Cl^-) 200-400 mesh were obtained from J.T. Baker Chemical Co., N.J. The heavy particles were removed from the commercial resin by suspending the resin in 2 volumes of water, stirring and decanting immediately. This resulted in a more closely packed column with less chance of channeling. The fine resin particles were removed by allowing the resin suspension to stand until the majority of the resin beads had settled. Decanting of the upper turbid liquid removed fine particles and facilitated an increased liquid flow rate through the column.

Ordinarily, the resin was suspended in 2 volumes of 2N hydrochloric acid, heated to 100°C (with continuous stirring to prevent bumping), and then allowed to cool for ½ hour. The yellow supernatant liquid was decanted and the procedure repeated after which time the supernatant liquid was clear. This yellow material usually appeared after the resin was stored for 2 months and interferred with further analysis by paper chromatography. Washing the stored resin with water before use readily removed the coloured substance. Typically, the resin was poured into glass columns,

allowed to settle and converted to the appropriate ionic charge by irrigating with electrolyte in the usual way. Liquid was maintained above the resin at all times.

Dowex 50W(x8)(H^+) resin.

To insure that the Dowex 50W(x8) cation resin was fully in the H^+ form, 2 litres of 2N hydrochloric acid per 450 g. of resin were allowed to slowly pass through the column bed. The resin was then washed with water until neutral and stored (5°C) until use under a layer of water to a maximum of 3 months.

Dowex 1(x10)(formate) resin.

Following the treatment with boiling hydrochloric acid, about 450 g. of Dowex 1(x10) resin was converted to the formate form by allowing 3 litres of 3M sodium formate to slowly pass through the column bed. One litre of 0.1N formic acid was subsequently allowed to pass through the column, followed by a water wash until neutral. The resin was stored at 5°C until use.

Dowex 1(x10)(acetate) resin.

When weakly adsorbed anions were separated (e.g., glutamate and aspartate) from the other amino acids,

Dowex 1(x10) was converted to the acetate form using sodium acetate and acetic acid in the manner described in the previous section. The freshly charged resin was stored at 5°C until use.

Preparation of the ion-exchange columns.

The ion-exchange columns were prepared using 1x20 cm. (internal diameter) glass columns in the usual manner. The resin was packed with slight air pressure to give a column height of 10 cm. and the upper liquid decanted. The resin bed was further compacted with air pressure. Liquid was maintained above the resin bed at all times.

b) Extraction of Free Amino Acids from the Culture Medium.

The culture was filtered through several layers of cheesecloth and the culture filtrate evaporated to near dryness (about 10 mls.). A portion (2 ml.) of the concentrated solution was added to the Dowex 50W(x8)(H^+) column. The non-adhering substances (neutral and acidic material) were washed through the column with 35 ml. of water while the basic substances were adsorbed by the resin. The columns were arranged in series such that the sample liquid and wash water from the Dowex 50W(x8) (H^+) column immediately passed into the Dowex 1(x10) (formate) resin bed. The non-adsorbed substances (neutral compounds) were washed through the Dowex 1 column with 35 ml. of water while the acidic substances were adsorbed by this resin.

Elution of basic substances from Dowex 50W(x8) (H^+) resin.

The basic substances (amino acids and nucleic acid bases) were eluted from the Dowex 50W(x8)(H^+) column with varying concentrations of hydrochloric acid. Elution with 50 ml. of 2<u>N</u> acid removed the acidic and neutral amino acids but not the basic amino acids. Subsequent

washing with 10N or concentrated hydrochloric acid (6N HCl was unsatisfactory) dislodged the basic amino acids. The latter washing also regenerated the column for similar subsequent use. Excess mineral acid was removed by thorough washing with distilled water.

Elution of acidic substances from Dowex 1(x10) (formate) resin.

The acidic substances (mainly organic acids) were eluted from the Dowex 1(x10)(formate) column with 60 ml. of 8N formic acid. As above, the column was simultaneously regenerated and washing with water until neutral left the resin bed ready for further use.

Separation of acidic and neutral amino acids.

The solution containing neutral and acidic amino acids was evaporated to dryness to remove the mineral acid, the residue dissolved in water and the constituents separated by passage through a Dowex 1(x10)(acetate) column. This resin adsorbed the acidic amino acids while the neutral amino acids were washed through with 35 ml. of water. The acidic amino acids were eluted with 60 ml. of 8N formic acid.

The results are shown in Table II.

TABLE II.

Fractions from culture filtrates after a 24 hour feeding period.

Fraction	^{14}C-labelled amino acid. α-Alanine	Aspartic acid	Glycine	Serine
Sugars	<0.5	<0.5	<0.5	<0.5
Organic acids	<0.5	0.9	1.0	<0.5
Amino acids				
Basics	<0.5	<0.5	<0.5	<0.5
Neutrals	16	<0.5	30	20
Acidics	<0.5	12	0.6	0.6

Figures are expressed as percentages of total ^{14}C-activity administered.

c) Extraction of Free Amino Acids from the Mycelium.

The mycelium from the cultures was harvested, washed thoroughly, excess water removed on a Buchner funnel and quickly mixed with 120 ml. of absolute ethanol and 30 ml. of water. The mycelium was reduced to a paste in an homogenizer and stored in the cold (5°C) for a few hours. After centrifugation, the ethanol was evaporated under vacuum and the aqueous solution extracted three times with an equal volume of chloroform to remove fatty material (e.g. glycerides, etc.). The aqueous layer which contained the amino acids, was evaporated to dryness. The residue was dissolved in 2 ml. of water, filtered through cotton, and chromatographed on the Dowex 50W(x8)(H^+) and Dowex 1(x10)(acetate) resin columns.

The results are discussed on page 33.

d) Hydrolysis of the mycelium.

The mycelial solids from the ethanol-water extraction was suspended in 100 ml. 6N hydrochloric acid and heated in a sealed tube for 24 hours. After filtering, the hydrochloric acid was removed by evaporation under vacuum and the residue dissolved in 30 ml. of water. This solution was extracted with chloroform as before and the aqueous layer chromatographed on the Dowex 50W(x8)(H^+) and Dowex 1(x10)(acetate) resin columns.

The results are discussed on page 33.

DISCUSSION

The catabolism of carbohydrates usually involves three stages.

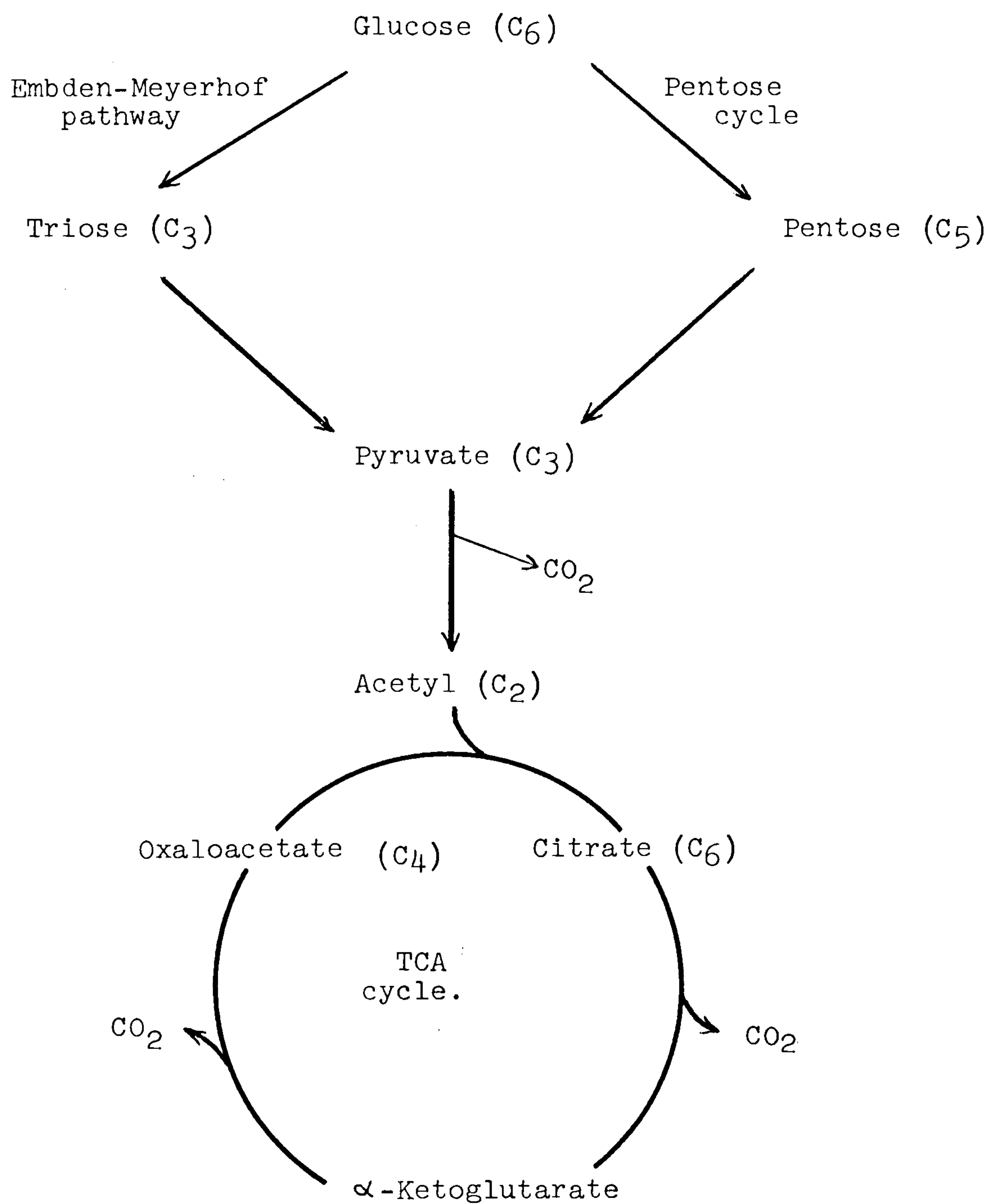
- Stage I, the carbohydrates are converted, if necessary, to an appropriate form, such as hexose, and phosphorylated.
- Stage II, degradation proceeds from a 6-C compound to 3- or 2-C compounds by various routes.
- Stage III, the degradation is completed to 1-C compounds, notably CO_2, and intermediates are available for anabolic processes.

It is a well established fact that there are several different metabolic pathways by which sugars can be fermentated by micro-organisms. By studies of the occurrence of enzymes and intermediates and of radioisotopic distributions in CO_2 or intermediates after supplying ^{14}C- glucose in variously labelled forms, it has proved possible to infer the presence and contribution of the Embden-Meyerhof-Parnas (EMP) and the Pentose Phosphate (PP) pathways in _Fusarium._ Heath _et al._ (1956) on the basis of ^{14}C data has suggested that, in _Fusarium_

lini, under anaerobic conditions, about 83% of the glucose was metabolized via the EMP scheme and about 17% via the PP cycle.

The majority of energy available in hexose is made available in the final stage of the catabolism of carbohydrates, in which pyruvate is dissimulated, usually aerobically, via the TCA and glyoxylate cycles with the formation of a variety of di- and tri-carboxylic acids which can act as intermediates for synthesis or, in certain conditions, accumulate and remain stored and metabolically inactive (Fig. 4).

In the experiments described herein, the ^{14}C - amino acids were in every case catabolized relatively quickly by the culture. No other amino acid containing ^{14}C was detected in the culture filtrate or existed in the mycelium besides that which was administered. There was no incorporation of the ^{14}C - amino acids into the mycelial protein. When the experiments were repeated by administering the ^{14}C - amino acids over a longer period of time (48 hours), similar results were obtained. This indicates that transformation of the amino acids was not taking place over a 48 hour period in cultures over 2 months old. However, the fungus was actively respiring



Degradation of glucose.
Fig.4

and utilized the amino acids as a source of energy as evidenced by the evolution of labelled carbon dioxide. For this to occur, the amino acids were most likely catabolized to intermediates or closely related intermediates, involved in the respiratory system (TCA cycle) of the micro-organism. As mentioned in the literature survey (page 6) several amino acids are synthesized directly from intermediates of the TCA cycle, and their catabolism is known to take place in a reversible direction, e.g. oxidative conversion of the amino acid to a keto acid. The catabolism of the individual amino acids is discussed in the following sections.

The activity which was unaccounted for in the labelling experiments was presumably lost from unstable intermediates (e.g. pyruvic acid) during the extraction procedure on the ion-exchange columns and from volatile intermediates during the evaporation procedure. Paper chromatography of the culture filtrate before extraction and strip counting after drying the chromatogram for 1 hour, showed a large proportion of the total activity occurred near the solvent front. After drying the chromatogram over a period of 8 hours, this activity had almost disappeared, which indicated the evaporation of a volatile compound from the paper. However, this was not examined in any further detail.

Alanine and Aspartic Acid

The only known pathway for the degradation of α-alanine is by transamination to yield pyruvic acid. Pyruvate is ordinarily subsequently oxidised to CO_2 by means of the tricarboxylic acid cycle.

Aspartic acid is similarly related to the TCA cycle. By loss of the amino group, aspartic acid is converted to either oxaloacetic acid or fumaric acid, both of which are integral members of the TCA cycle and subsequent degradation of each can be explained in terms of the operation of the TCA cycle (Fig. 5).

α-Alanine and aspartic acid were administered as mixtures of their D- and L-isomers and the fungus apparently utilizes both forms as carbon sources. In general, micro-organisms exhibit specificity for the L-isomer of a particular amino acid. However, in many bacterial and mold systems there appears to be good evidence for distinct enzymes for the L- and D-isomers of the amino acids, e.g. Brewer's yeast generally deaminates only the L-amino acids with the exception of glutamic acid, aspartic acid, and asparagine of which both isomers are utilized. At least two mechanisms may be envisaged which might account for the facile biological degradation of both isomers. One rests in the possibility

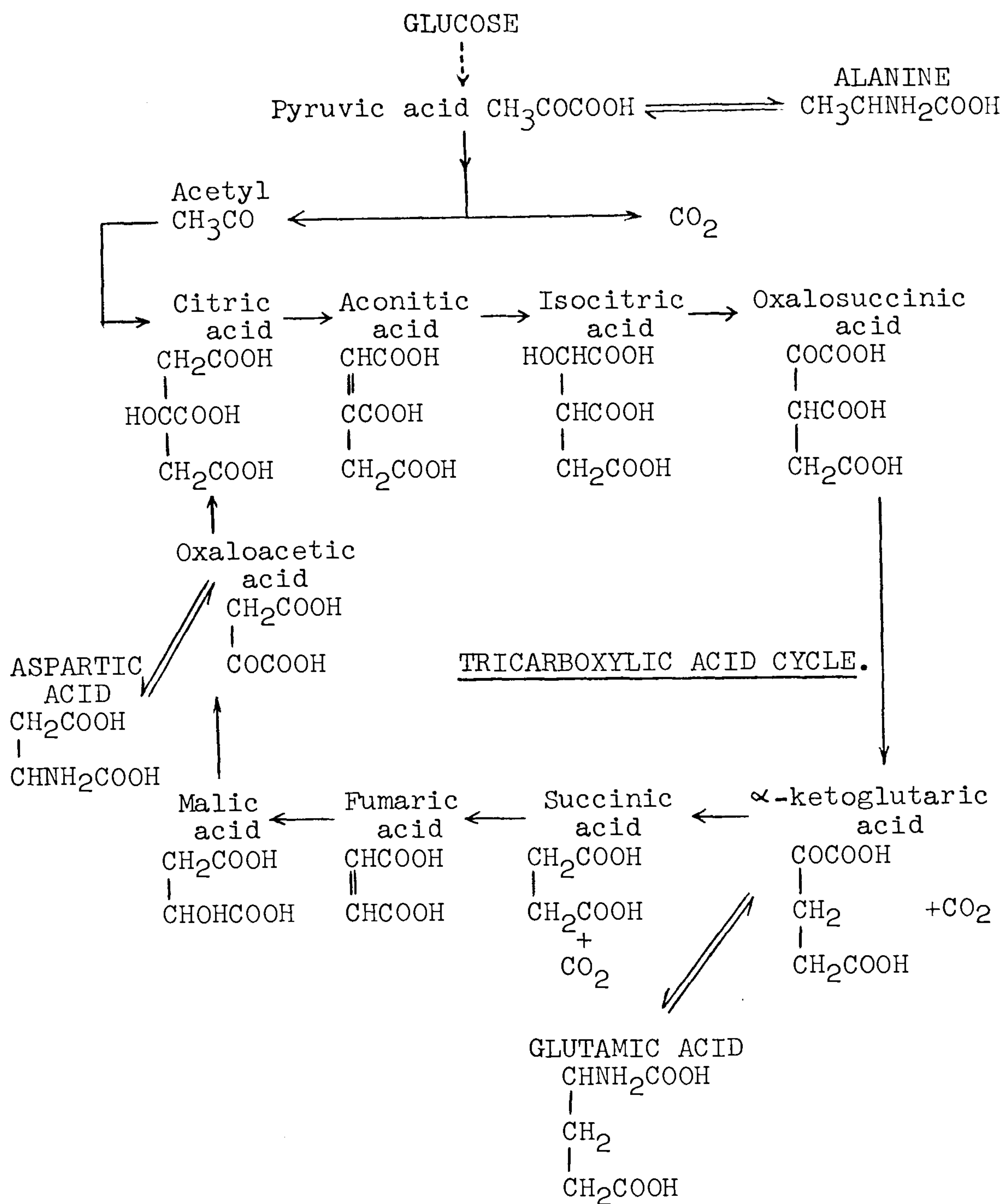


Fig.5

that an enzyme system is available which has a characteristic equilibrium favouring degradation instead of synthesis of the D-isomer. Another possibility may lie in the absence of absolute specificity of some enzymes for only the one antipode.

Biosynthesis of Lysine

In a separate experiment with cultures which were 7 days old, it was found that aspartic acid - 4 - ^{14}C was readily incorporated into mycelial protein over a 24 hour period. On hydrolysis of the mycelium, labelled aspartic acid, glutamic acid, threonine and lysine were detected.

Two distinct pathways are known that lead to the synthesis of lysine. In certain fungi, e.g. *Neurospora*, and algae, the carbon skeleton of lysine arises from acetate and α-ketoglutarate by a biosynthetic sequence that includes α-aminoadipic acid. The other pathway has been found in bacteria, higher plants, blue-green and green algae, and certain fungi, e.g. *E.coli*, in which the lysine carbon chain is synthesized from pyruvate and aspartate and α, ϵ- diaminopimelic acid is a key intermediate (Fig. 6). Vogal (1959) has surveyed the pathways of lysine biosynthesis in many bacteria, algae, and plants; a summary of his observations is given in Table III. Thus far, no

LYSINE BIOSYNTHESIS.

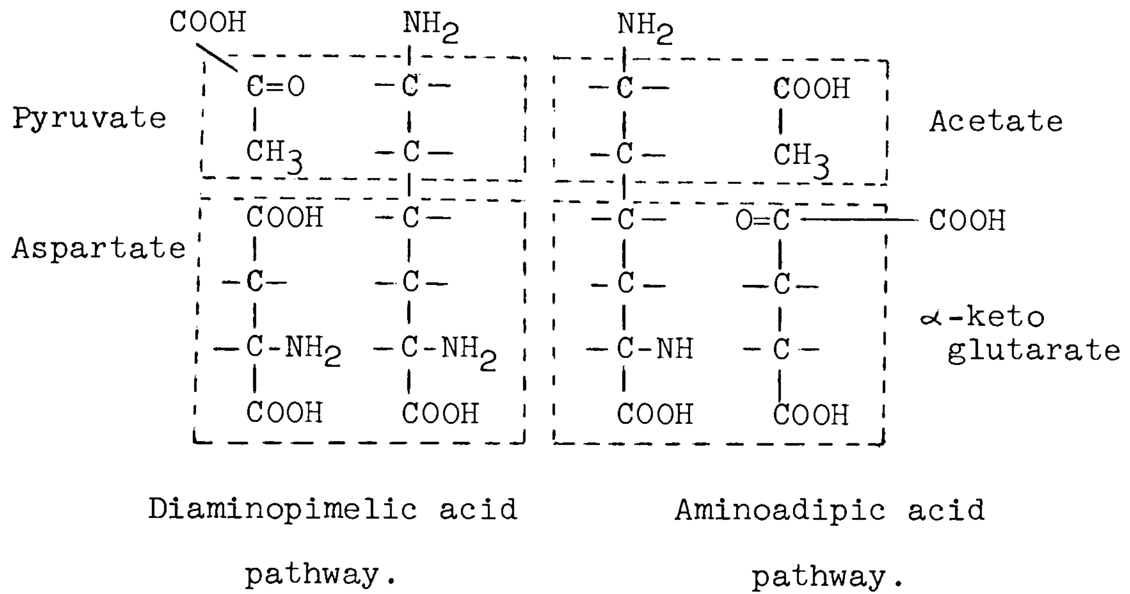


Fig.6

TABLE III

α, ϵ-Diaminopimelic acid pathway		α-Aminoadipic acid pathway
	Bacteria	
Pseudomonads Eubacteria Actinomycetes		
	Lower fungi	
Hyphochytriales Saprolegniales Leptomitales		Chytrids Blastocladiales Mucorales
	Higher fungi	
		Ascomycetes Basidiomycetes
	Green organisms	
Green algae Ferns Flowering plants		Euglenids

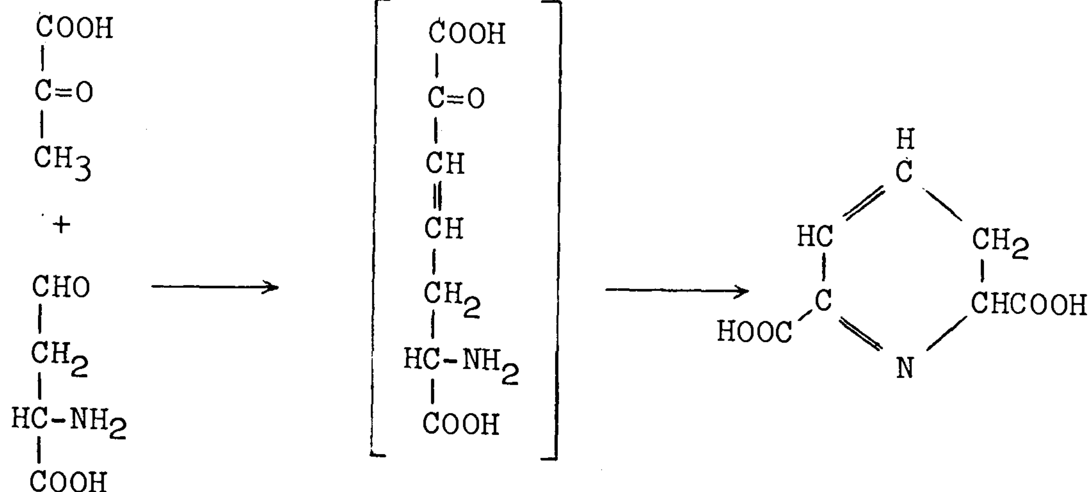
Pathways of lysine biosynthesis in micro-organisms.

evidence has been obtained for the occurrence of both pathways within the same organism.

Thus, the label in aspartic acid - 4 - ^{14}C will only be incorporated into lysine via the diaminopimelic acid pathway. Studies on the occurrence of diaminopimelic acid in various micro-organisms and experiments on the incorporation of labelled acetate and aspartate into lysine have provided data concerning the pathways of lysine biosynthesis (Wood 1949 & 1950). The actual steps, however, of the diaminopimelic acid pathway remain uncertain, but a plausible scheme has been suggested (Fig. 7).

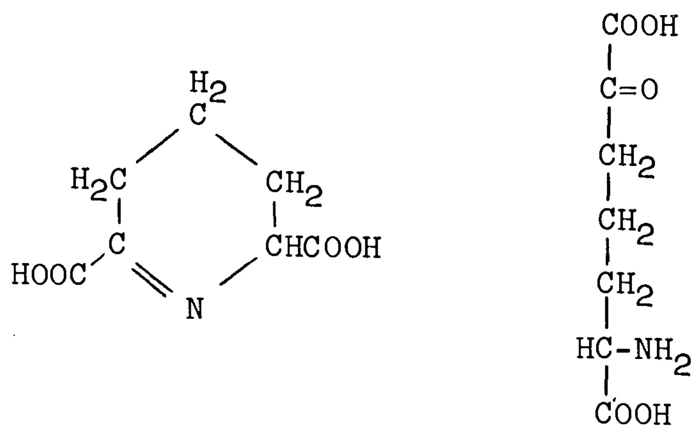
Biosynthesis of Threonine

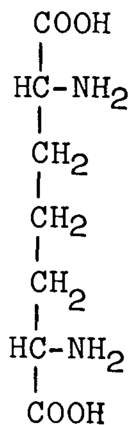
Information concerning the synthesis of threonine has come from studies on several micro-organisms. The first evidence that homoserine was a precursor of threonine arose from investigations of a mutant of *Neurospora crassa* which required both methionine and threonine for growth; homoserine replaced both methionine and threonine for the growth of this mutant (Teas *et al.* 1948). Yeast and *E.coli*, when grown on labelled acetate, produced threonine and aspartate exhibiting similar isotope distributions, Ehrensvard *et al.* (1951). Isotope competition studies were

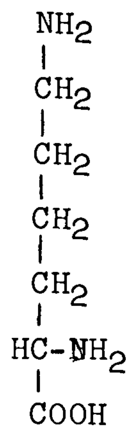


Aspartic
β -semialdehyde

2,3 Dihydrodipicolinic
acid







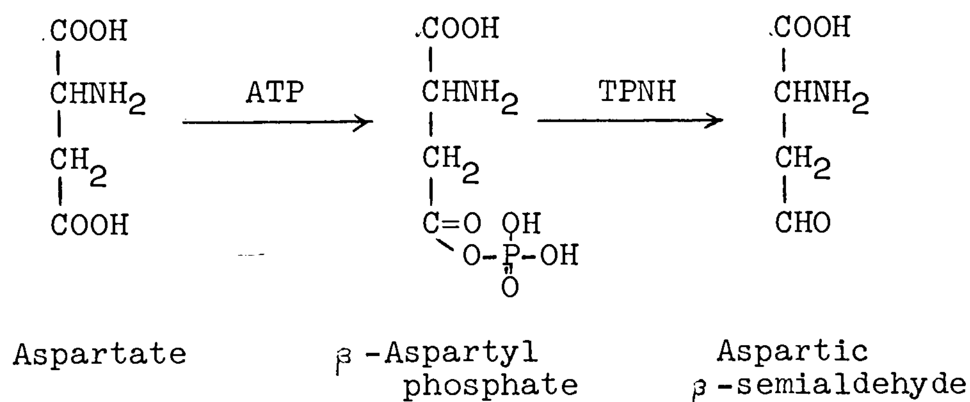
Diaminopimelic acid

Lysine

Fig.7

also consistent with the belief that homoserine was a precursor of threonine (Abelson *et al.* 1952). Further elucidation of the biosynthetic route to threonine was made by Cohen and Hirsch (1954), and Black and Grey (1953), who discovered two new intermediates in the aspartic acid - threonine pathway, namely, β -aspartyl phosphate and aspartic acid β-semialdehyde (Fig. 8).

THREONINE BIOSYNTHESIS.



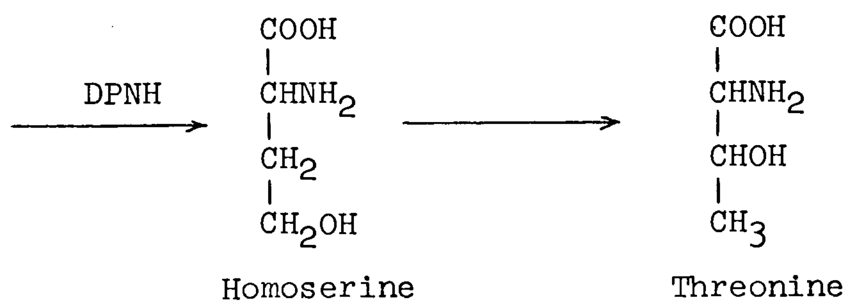


Fig.8

Glycine and serine

The amino acids, glycine and serine, have close metabolic relationships. Three possible pathways for the dissimulation of glycine are:

- Pathway I; condensation of "active" formaldehyde and glycine to form serine. The latter can be hydrolytically deaminated to pyruvic acid.
- Pathway II; transamination to glyoxylic acid and the oxidation of this acid to formate and CO_2 or to oxalic acid.
- Pathway III; condensation with succinyl CoA to form α- amino - β - ketoadipic acid in the glycine-succinate cycle (Fig. 9).

In addition to the conversion of serine to glycine, other possible pathways of serine catabolism have been studied (Elwyn et al., 1956). Reaction I, leading by dehydration and deamination to pyruvate, is essentially a one way reaction, while Reaction II, giving rise to hydroxy pyruvate by either transamination or oxidative deamination, probably occurs in both directions. Reaction III, decarboxylation of serine to ethanolamine, is implied

GLYCINE CATABOLISM.

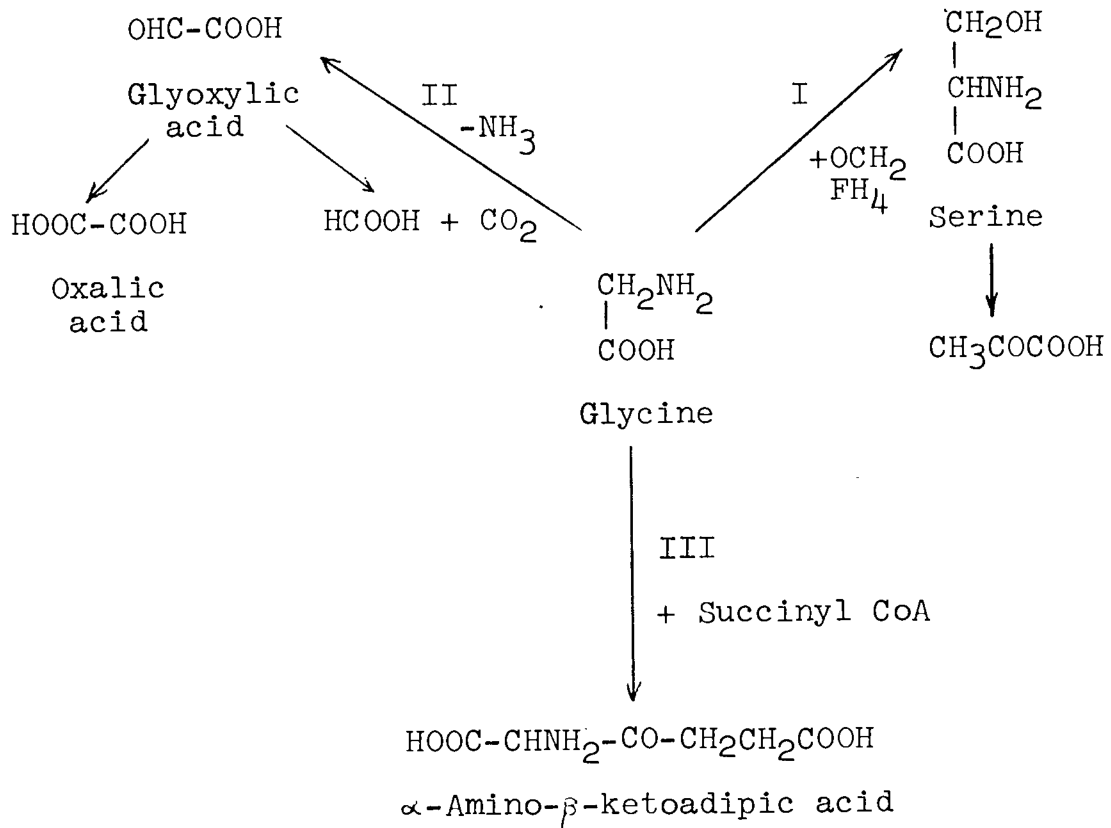


Fig.9

by isotopic studies in animals but apparently has not been assessed in fungi (Fig. 10).

The CO_2 can be derived from serine by two pathways: (1) dealdolisation, followed by oxidation of the folic acid-bound formaldehyde via formate to CO_2, and (2) conversion to pyruvate and subsequent oxidation via the TCA cycle.

A study of the metabolism of serine - U - ^{14}C in cultures which were 7 days old showed that the incorporation of radioactivity into the mycelium was relatively small compared with that obtained for aspartic acid - 4 - ^{14}C. On hydrolysis of the mycelial protein, the ^{14}C - label was found in glycine and serine in approximately the same proportions. The activity on the paper chromatogram using the described method was about a tenth of that obtained when using aspartic acid - 4 - ^{14}C. This suggests that the majority of serine in the mycelial protein is formed from glycine as shown by Kern (1954).

SERINE CATABOLISM.

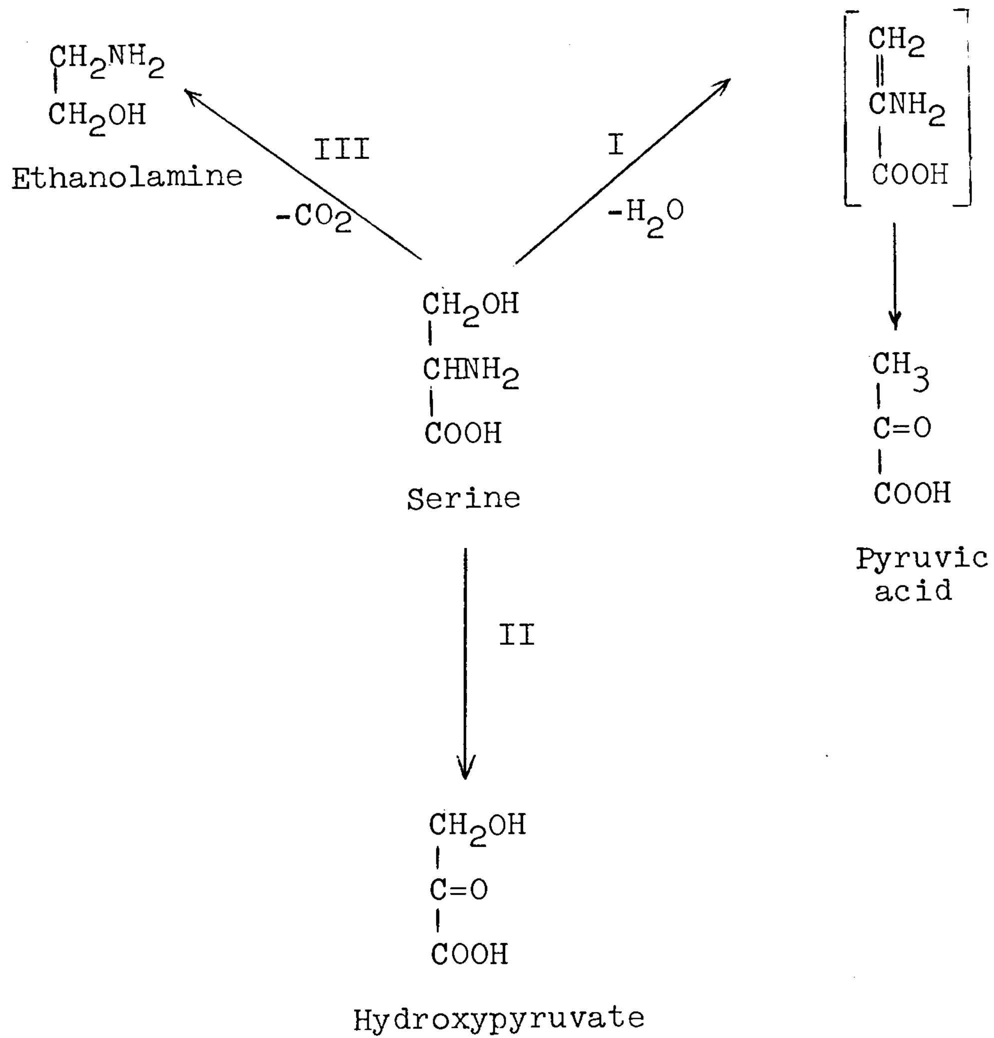


Fig.10.

Substance J, which was a component of the acidic amino acid fraction from the ion-exchange columns, could be chromatographically distinguished from the constituent amino acids.

Solvent system	Rf. of Substance J
Phenol:methanol:H_2O	0.21
Butanol:acetone:H_2O:NH_3	0.002

No activity was detected in Substance J from cultures which were 7 days old or from those 2 months old. This indicated that ^{14}C - labelled α- and β-alanine, aspartic acid, glycine and serine were not incorporated into lycomarasmin over a 24 hour period in amounts which could be detected using the method described. Since relatively small aliquots of ^{14}C - labelled amino acids were administered, it cannot yet be concluded that some of these compounds might not be specifically incorporated at a low level. However, the significance of this observation is that it shows that the origin of lycomarasmin in the fungus must be somewhat different to that of fusaric acid which Sanwall has shown to readily incorporate amino acids, and is probably due to the fact that amino acids are known to

be direct precursors of the pyridine ring. Lycomarasmin has a structure more closely related to the alkaloids than the peptides, and if its formation is via a pathway similar to that of alkaloid biosynthesis, then incorporation of ^{14}C - amino acids may only occur at low levels, about 0.01%, as has been found for many alkaloids. The lower limit of detection was approximately 0.1%, using the methods described herein.

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