IOWA STATE UNIVERSITY
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

1953

Response of Lactobacillus arabinosus to p-fluorophenylalanine

Robert Swain Baker
Iowa State College

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

 Part of the Biochemistry Commons

Recommended Citation
Baker, Robert Swain, "Response of Lactobacillus arabinosus to p-fluorophenylalanine " (1953). *Retrospective Theses and Dissertations*. 13084.
https://lib.dr.iastate.edu/rtd/13084

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

www.manaraa.com

NOTE TO USERS

This reproduction is the best copy available.

UMI®

RESPONSE OF *LACTOBACILLUS ARABINOSUS* TO p-FLUOROPHENYLALANINE

16

by

Robert Swain Baker

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

IOWA STATE LIBRARY

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1953

UMI Number: DP12302

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform DP12302

Copyright 2005 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

QP551
B176r
c.1

TABLE OF CONTENTS

T10942

INTRODUCTION

The study of metabolism with analogues of the metabolites has helped in the elucidation of biological processes. Investigations of phenylalanine metabolism have been assisted by several analogues, one of which is p-fluorophenylalanine. Studies by Atkinson[1] showed p-fluorophenylalanine to be a potent antimetabolite for phenylalanine in Lactobacillus arabinosus. In this organism at limiting concentrations of phenylalanine, subinhibitory quantities of the analogue were found to increase the amount of growth as compared to controls lacking the analogue.

This thesis reports studies made with Lactobacillus arabinosus to ascertain if p-fluorophenylalanine is incorporated into proteins under conditions in which this compound causes increased growth of the organism. The amino acid composition was investigated since a hypothetical explanation for the increased growth was the substitution of p-fluorophenylalanine for phenylalanine in the proteins. If p-fluorophenylalanine, an amino acid not known to occur naturally, is incorporated into proteins of Lactobacillus arabinosus, then the amino acid composition of the proteins is altered.

Investigations of alteration in amino acid composition of microorganisms, blood proteins, and eggs have been made.

The workers who studied the alteration of amino acid composition of microorganisms using natural amino acids concluded that alterations occurred in the amino acid composition of the organisms. The change in the amino acid composition of the organisms may have resulted from alterations in the amino acid sequence in the proteins or from changes in the amounts of proteins containing the amino acids. The ambiguity provided by these results may be circumvented by studies of incorporation of amino acid analogues into proteins.

Attention has been focused on the adsorption to proteins of natural, chemically altered, or isotopically labelled amino acids without incorporation into peptide linkages. This thesis reports special procedures instituted to remove free amino acids which might lead to erroneous conclusions if present.

REVIEW OF THE LITERATURE

In the early part of this century, a few investigators were interested in the change in amino acid composition of microorganisms in relation to environmental changes. Although the methods of analysis for amino acids were poor in those days, the conclusion that amino acid composition had been altered has been verified with more refined techniques. Today, we are more cognizant of factors which might lead to false conclusions concerning changes in amino acid composition of proteins. Such misleading factors are (a) an apparent incorporation into proteins due to an adsorption of the amino acid to the protein, and (b) the inability to distinguish between changes in amino acid sequence in proteins and changes in the amounts of proteins containing amino acids. The above criticisms are concerned with natural amino acids; however, in recent years unnatural amino acids which are labelled with chemical groups have been used in studies of this type. Although unnatural amino acids overcome the disadvantage listed in (b); the consequence of (a) still exists as well as (c) breakdown products which might be reused in natural amino acids, and (d) incorporation of the labelled group into the natural amino acid already in peptides by exchange reactions. Factors (a), (b), and (c) apply in cases where natural amino

acids are labelled with isotopes; and (b), (c), and (d) apply in cases where chemical analogues are labelled with isotopes. These factors are considered in the evaluation of the literature to be reviewed. First, reports involving the use of natural amino acids to alter protein amino acid composition will be reviewed. The reports using unnatural amino acids to alter protein amino acid composition will be considered second.

Abderhalden and Rona[2] used Aspergillus niger to study the effect of environmental influences on the amino acid composition of the organism. Although variation of the nitrogen source did result in differences in amino acid composition, the difference was not large enough to be significant; therefore, they did not claim an alteration of the amino acid composition of the organism. Precautions were not taken to remove adsorbed amino acids or to distinguish between the amino acid composition and the protein containing the amino acids. A few years later, Tamura[3] studied the changes in amino acid composition of two bacteria, Mycobacterium tuberculosis and Mycobacterium lacticola. He used the dry cells as a mass; consequently, his results should be considered with criteria (a) and (b) in mind. He found that the nitrogen content remained the same whether the organism was grown on medium containing albumin or on a well-defined medium of components of simple

structure. He reported small differences in the amino acid composition when the organisms were grown on the two types of medium.

Rosedale[4,5] made a study of the amino acid composition of normal and pathological flesh of animals fed on known diets. The proteins were precipitated by boiling in water. Although the proteins were precipitated twice by this method, removal of adsorbed amino acids could be questioned. Rosedale concluded that the normal muscle tissue does not vary to any appreciable extent in the various animals, or in different tissues of the same animal provided the animal secures normal protein in the diet.

In 1939, Dirr[6] administered arginine both orally and intravenously to human subjects. He reported that the arginine content changed from 5.42 per cent to 8.15 per cent in blood serum proteins when given either as 3 grams intravenously or 5 grams orally. Block[7] failed to confirm Dirr's results. Block found that the ratios of histidine, arginine, and lysine remained constant. He determined the arginine by precipitation with silver after coagulating the serum proteins by heating. Repeating by the method of Dirr, i.e. alcohol precipitation of the proteins, Block confirmed his own results obtained by heat coagulation of the protein. Block pointed out the fact that the experiment was carried out on only a few subjects casts doubt on the results in

either of the reports.

Block and Bolling[8] determined the essential amino acids in ten commercially available yeasts. The amino acids were determined on a fat-free residue after hydrolysis by microbiological assay. Here again the adsorption of amino acids to proteins was not ruled out, but the workers concluded that the amino acid content of Saccharomyces cerevisiae was constant though it might vary depending upon the variety of yeast and the nutritive medium. Camien et al.[9] reached the same conclusion as a result of their experiments with Lactobacilli. Estimations of the amino acid content were made on the whole cells. Their conclusion that the amino acid composition varies with the nutrient media is subject to the same criticisms outlined before.

Stokes and Gunness[10] made an extensive report on the amino acid composition of microorganisms with respect to changes in the medium, age of culture, and aeration. All amino acid determinations were made after hydrolysis of washed and dried cells. Penicillium notatum varied in the contents of lysine, isoleucine, methionine, threonine, phenylalanine, and tryptophan (all less) while valine, histidine, arginine, and leucine were greater when the culture was aerated. Bacillus subtilis showed no change in its composition under similar conditions. Differences were

noted when Streptomyces griseus was grown with additional glucose. In the presence of glucose, the content of arginine and histidine decreased, while larger amounts of lysine, leucine, and threonine were found. These workers concluded that the amino acid composition remained constant when the organisms were cultivated in the same medium, but that the composition may vary with medium, aeration, and age of cells. Freeland and Gale[11] in a similar study included comparisons between Gram-positive and Gram-negative organisms. Free and bound amino acids were differentiated by washing one portion of the cells in boiling water for 20 minutes before analysis. Freeland and Gale reported that a greater correction for free amino acids was necessary for Gram-positive species, the amino acid composition was fixed and characteristic of the organism, and the amino acid composition did not change under widely varying cultural conditions.

Csonka et al.[12] studied the methionine and cystine content of chicken eggs. They were aware that the protein balance of the eggs might change rather than the amino acid content of the individual proteins; thus, they compared the ratio of concentration of the two amino acids. The hens were fed diets of varying protein content. The whole egg including the shell was subjected to acid hydrolysis before the amino acids were estimated. Increase in the methionine

content was not always followed by an increase in cystine, and the reverse was true, i.e. an increase in cystine was not always followed by methionine increase. Addition of free cystine and methionine to the diet did not increase the content of amino acids in the proteins of the eggs. Csonka[13] reported that free cystine and methionine could not be detected in the eggs. On a high protein diet the nitrogen increased 44 per cent. The bound cystine-methionine ratio in egg whites of young and old pullets on both high and low protein diets did not change. In the egg yolk, the ratio increased from .55 on a low-protein diet to .62 on a high-protein diet in young pullets. A decrease from .75 on low-protein diet to .63 on a high-protein diet in old pullets was found. Csonka and Jones[14] reported that the amino acid composition of egg proteins varied both with genetical lineage and dietary protein. The facts that free cystine and methionine were not found in the eggs and that the ratio of bound cystine to methionine did not remain constant offers the best evidence that the amino acid composition of the egg yolk may be altered. This does not, however, indicate an alteration of the amino acid composition of the proteins.

Work[15] characterized the free and bound amino acids in Corynebacterium diphtheriae and determined the gross effect of variations in the culture medium on the bound amino acid

content. The free amino acids were separated by extraction with ethyl alcohol of the wet-washed cells. Since the cells were not cytolyzed, the complete removal of free amino acids might be questioned. She concluded that on three different types of medium the amino acid composition remained constant; however, when extra glutamic acid was present in the medium, it was depleted to a greater degree. The glutamic acid content of the cell residues increased under these conditions.

The results of these workers constitute inconclusive evidence that the amino acid composition of a protein may be changed, in view of the previously mentioned interferences, e.g. adsorption and the inability to distinguish between changes in amino acid sequence in the protein and the amounts of protein. More recently, the use of amino acid analogues has been applied to clarify the problem. Although precautions must be taken when using these unnatural analogues, the main advantage is that one does not have to distinguish between changes in the amino acid content of proteins and the amounts of protein containing the amino acid. Other precautions have been mentioned in the first paragraph of this section.

About twenty years ago, Nelson et al.[16] reported that selenium was assimilated by plants in certain western plain states. The assimilated selenium was toxic to animals

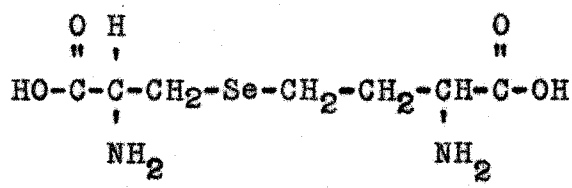
grazing on these plains. Robinson[17] determined selenium in wheat and found 10 to 12 parts per million. Ninety parts per million were found associated with the wheat gluten. Studies of non-toxic wheat failed to show the presence of selenium. Franke[18] conducted feeding experiments with rats and reported a variation in tolerance to toxic grains. Tests were made with selenium in various forms by Franke and Potter[19]. Metallic selenium was not toxic to rats, sodium selenide depressed the growth, and sodium selenite and sodium selenate were toxic. Franke and Painter[20] reported that a hydrolyzate of the wheat gluten was toxic, but when the hydrolyzate was treated with barium carbonate and mercuric chloride, the hydrolyzate lost its toxicity. In another report by Franke and Painter[21], the proteins of animals contained selenium when fed toxic protein. Attempts to remove the selenium by solvents for inorganic selenium failed, as did attempts to deposit it from protein hydrolyzates electrolytically. The workers concluded that the selenium was bound to an organic radical.

Studies on the content of selenium in humin formation during acid hydrolysis[22] indicated more selenium was present in the humin after longer periods of hydrolysis. Hydrolysis with hydriodic acid removed selenium from the protein with little humin formation. The selenium analogue of cysteine was dismissed from consideration when Painter

and Franke found no increase of selenium in the humin during acid hydrolysis in the presence of stannous chloride. Cysteine decomposes under such treatment. Painter and Franke[23] removed the selenium from hydrolyzates with bases or strong oxidizing reagents. Alkaline hydrolysis[24] of wheat gluten in the presence of lead resulted in the formation of lead selenide as well as lead sulfide from cystine. After ashing toxic protein, Horn et al.[25] reported the selenium was not as toxic. This was suggestive of an organic selenium compound. Jones and his coworkers[26] divided a partial hydrolyzate obtained by mild peptic digest into four fractions. Two of these fractions contained neither selenium nor cystine. In the other two fractions, both selenium and cystine were found.

In 1940, Horn and Jones[27] isolated a compound from wheat and from Astragalus pectinatus each grown on soil containing selenium. The compound with an empirical formula of $C_{21}H_{44}N_6Se_2SO_{12}$ gave a strong ninhydrin reaction and all of its nitrogen was amino nitrogen. Its solubility was characteristic of amino acids and difficultly soluble in water like cystine. Using a simple formula, $C_7H_{14}N_2O_4Se$, Horn and Jones proposed a structure. Later these same workers[29] isolated a sulfur containing amino acid, lanthionine, from human hair, chicken feathers, and lactalbumin by boiling the protein with 2 per cent sodium carbonate

solution followed by acid hydrolysis with 20 per cent



hydrochloric acid. If the γ-methylene group is removed in the above formula to make a symmetrical molecule and the selenium replaced with sulfur, the result is lanthionine. The removal of the methylene group reduces the equivalent weight from 135 to 127.5. The experimental value obtained was 130.5.

Moxon[30] studied the toxicity of selenocystine and found it to be very toxic. It was seven times as toxic as β,β'-diselenodipropionic acid. A dose of 8.4 milligrams of selenocystine per kilogram of body weight killed 75 per cent of the rats in two days. Moxon et al.[31] later reported the D-form of selenocystine to be one-third as toxic as the L-form.

The results of these investigations have shown that a selenium-containing amino acid, probably analogous to a sulfur-containing amino acid, is formed by plants growing on soils containing large amounts of selenium. Apparently, it is incorporated into the plant proteins; however, these workers failed to rule out adsorption of the amino acid to

the protein.

Using another amino acid analogue, Dyer[32], in 1938, attempted to substitute ethionine for methionine in the diet of the rat in order to alter the amino acid composition of the proteins. The rats failed to grow, but the inhibition was reversed by the addition of methionine.

Levine and Tarver[33] fed rats ethionine labelled in the ethyl group with radioactive carbon 14. These workers isolated radioactive proteins from the liver, kidney, and spleen. The radioactive ethyl group of ethionine was identified as the S-ethyl isothiourea picrate from a protein hydrolyzate, after separation from methionine by recrystallization. Although Levine and Tarver recognized two other possible explanatory mechanisms, i.e. exchange of methyl groups in proteins or peptides for ethyl groups in free ethionine and ethylation of homocysteine in peptide linkage with the ethyl group from free ethionine, they considered these improbable. The reasons given were that no corresponding reactions have been described for methionine, and the belief that amino acids once in the form of peptides do not undergo any structural modifications. The fact that the ethyl groups were labile, however, was shown by Stekol and Weiss[34]. These latter workers showed that choline could alleviate the growth inhibition due to ethionine in the rat. Also, either antipode of ethionine was active in

causing inhibition which could be reversed by either antipode of methionine. This suggested to them that the inhibition was a function, at least in part, of triethyl choline instead of ethionine in protein synthesis. Confirmation was obtained[35] by the isolation of triethyl choline and ethyl creatinine labelled with the radioactive ethyl group administered as ethionine.

Levine and Tarver[33] attempted to avoid erroneous conclusions due to adsorbed amino acids and to radioactive amino acids synthesized from catabolic products of ethionine. They tried to exclude adsorption effects by washing the precipitated protein with monothioethylene glycol. Control experiments with plasma proteins indicated that nearly all of the radioactive ethionine could be washed from the plasma proteins with monothioethylene glycol. The residual radioactivity in the protein depended upon the original concentration of the amino acid. Similar results were obtained by Borsook et al.[36] in their studies on tissue homogenates. Using radioactive amino acids, they found that washing with trichloroacetic acid failed to remove amino acids which were bound to the protein by complexes with metal ions, such as cobalt and manganese. Complete removal was accomplished by treating the protein with ninhydrin.

In experiments with histone proteins, Brunish and Luck[37] reported radioactive amino acids that were adsorbed

and could not be removed by washing with trichloroacetic acid, by dialysis or by reaction with ninhydrin. The amount of adsorption was dependent upon time and temperature of incubation. These groups of workers concluded that the only way to be certain an amino acid was incorporated into proteins was to identify the amino acid in peptides from partial hydrolysis of the protein.

Smith and Schlenk[38,39] have isolated adenine thiomethyl ribose and 5-thiomethyl ribose from yeast. Saccharomyces cerevisiae and Torulopsis utilis were the best producers of these compounds. The amount of the compounds formed was dependent upon the concentration of methionine. A wide variety of yeasts and bacteria was found to produce thiomethyladenosine. The rabbit produced 0.3 micromoles of thiomethyladenosine per gram of liver[40]. Schlenk and Tillotson[41] reported that 5-ethylthioadenosine was formed from DL-ethionine by yeasts. The amount of the ethyl analogue formed was about half the amount of the methyl compound synthesized under similar conditions. The formation of ethyl mercaptan from acetaldehyde and hydrogen sulfide by yeasts is known, and the utilization of ethyl mercaptan to form the 5-ethyl thioadenosine in yeast has been reported by Schlenk[42]. From these experiments it seems that ethionine may not be as foreign to the biological functions of organisms as was previously believed. Under special

conditions its biosynthesis may take place. The metabolic products of ethionine could contaminate preparations of proteins and result in erroneous conclusions regarding the amino acid composition of the proteins.

Very little is known about biological reactions of amino acids in the form of peptides; however, the work of Schoenheimer and his associates[43] with radioactive isotopes in amino acids showed that proteins are in a dynamic state. Although these workers were unable to differentiate between reactions occurring while the amino acids were in proteins and reactions occurring before incorporation into the proteins, their results showed that amino acids undergo reactions continually. Sizer[44] has shown that tyrosyl residues in proteins are oxidized by tyrosinase. This indicates that the assumption made by Levine and Tarver concerning the improbability of reactions of amino acids in proteins is not valid. Although reports were not found in the literature concerning transmethylation reactions of methionine in peptide linkage, such reactions may not be dismissed.

Levine and Fopeano[45] reported additional evidence for incorporation of ethionine into rat liver proteins by calculations of liver protein as per cent of body weight. Earlier workers found that the amount of liver protein depended upon the amount of protein in the diet. In Levine and Fopeano's experiments, rats fed ethionine in their diet

showed a higher percentage of liver protein than control rats fed a diet without ethionine. The results according to their interpretations supported the evidence for incorporation of the analogue into proteins of the rat.

Fluorinated derivatives of phenylalanine and tyrosine have been used to study metabolism of these amino acids for many years. Kraft[46], in 1936, reported that 3-fluorotyrosine antagonized the effect of thyroxine on tadpoles. Litzka[47] reported the same phenomenon and later reported the use of 3-fluorotyrosine in treating hyperthyroidism. Boyer et al.[48] extended the study of fluoro derivatives of tyrosine in relation to basal metabolic rate of the rat. 3-Fluorotyrosine was the most toxic, followed by 3-fluorophenylalanine, 3,5-difluorotyrosine, and 3-fluoro-5-iodotyrosine. None of these analogues affected the basal metabolic rate nor were useful for its control. Mitchell and Niemann[49] tested 3-fluoro derivatives of phenylalanine and tyrosine on Neurospora crassa. Competitive inhibition was reported with each of the compounds tested. 3-Fluorophenylalanine was the most effective inhibitor of those studied.

Melvin[50] and Atkinson[1] found that p-fluorophenylalanine inhibited growth of Lactobacillus arabinosus. Atkinson reported that phenylalanine was the only aromatic amino acid to reverse the inhibition. The extent of inhibition varied with the concentration of inhibitor, but at limited con-

centrations of phenylalanine, subinhibitory amounts of p-fluorophenylalanine increased the total amount of growth. Atkinson, Melvin, and Fox[51] offered three hypothetical explanations for the increased growth: p-fluorophenylalanine released a complex set of antagonisms which resulted in increased growth, was converted to phenylalanine, or was substituted in some functions of phenylalanine and not in others. Preliminary evidence for incorporation of p-fluorophenylalanine into proteins of Lactobacillus arabinosus was reported by Baker[52]. Adsorption of the analogue was not eliminated, although it was recognized as a possible source of interference.

No other reports are known of similar supplementation of phenylalanine by fluorophenylalanines, but many have reported inhibition by fluorophenylalanines. p-Fluorophenylalanine inhibited growth of Pseudomonas aeruginosa[53], rats[54], Escherichia coli[56]; lowered the blood pressure in the dog[55]; inhibited the action of dopa decarboxylase[55]; and inhibited protein synthesis in Saccharomyces cerevisiae[57] and heart tissue cultures[58].

In the studies on protein synthesis in S. cerevisiae, the assumption was made that an amino acid pool occurred inside the cell. When p-fluorophenylalanine was added to the medium, not only was the incorporation of phenylalanine inhibited by the fluoro analogue, but also the uptake of

other amino acids was inhibited. This was also true in the experiments with heart tissue cultures; however, inhibition did not occur when phenylalanine was obtained from the proteins of the chicken embryo. This suggested to the investigators that the amino acid in peptide linkage was not converted to the free amino acid before incorporation into the tissue of the heart.

MATERIALS AND METHODS

Lactobacillus arabinosus I and mutant strains derived from it were used in these investigations. The original strain was obtained from the American Type Culture Collection (#8014). The strains were transferred monthly and carried in stabs of peptone yeast-extract agar medium. The composition of the medium is shown in Table 1. After an incubation period of 24 hours, the cultures were stored in a refrigerator at 2° C.

Table 1. Composition of the Medium Used for Stock Cultures

Constituent	Amount
Bacto-peptone	0.8 g.
yeast extract	0.2 "
sodium acetate	0.5 "
glucose	1.0 "
agar	1.2 "
salt solution A*	0.5 ml.
salt solution B*	0.5 "
water	100 "

*See Table 2.

The mutants used were L. arabinosus PT_1, which does not require exogenous tyrosine and phenylalanine, and L. arabinosus F, which grows without an extended lag phase at levels of p-fluorophenylalanine which inhibit the parent strain for 40 hours. The strain PT_1 was reported by Atkinson[1] and strain F by Baker[52]. Lactobacillus brevis (ATCC #8287) was used for assay purposes.

The inoculum was prepared from the stab cultures by transferring to a broth medium a portion of the cells. The broth medium had the same composition as the stab culture medium except for omission of agar. The broth cultures were incubated at 36° C for 18 to 24 hours after which the cells were centrifuged, the medium decanted, and the cells suspended in 5 ml. of sterile 0.9 per cent sodium chloride solution. After centrifugation and resuspension of the cells in the sodium chloride solution, a dilution of 1 to 50 was made with sterile sodium chloride solution. This suspension was added to 10 ml. tubes of liquid medium and to medium used for plate assays by means of a sterile pipette.

The synthetic basal medium used for the test was essentially that of Kuiken et al.[59] The medium composition is given in Table 2. For plate assay medium, one and three-fourths per cent purified agar was added to the medium. The agar was washed with water and acetone to remove trace

Table 2. Basal Medium

(Composition of one liter of double-strength medium.)

Constituent	Amount
L-arginine·HCl*	400 mg.
DL-alanine	400 "
DL-aspartic acid	800 "
L-cystine	400 "
DL-glutamic acid·H_2O	800 "
L-histidine·HCl	400 "
DL-isoleucine	400 "
DL-leucine	400 "
L-lysine·HCl	400 "
DL-phenylalanine	400 "
L-proline	400 "
DL-serine	400 "
DL-tryptophan	400 "
DL-methionine	400 "
DL-tyrosine	400 "
DL-threonine	400 "
DL-valine	400 "
glucose	40 g.
sodium acetate	15 "
adenine sulfate	10 mg.
guanine·HCl	10 "
uracil	10 "
vitamin concentrate	10 ml.
salt solution A	10 "
salt solution B	10 "
water	1000 "

*Amino acid isomer prefixes used in this thesis refer to configuration rather than rotation, and should be construed as small capitals in the official system for such designations (Crane[60]).

Table 2. (Continued)

Constituent	Amount
Vitamin concentrate	
thiamin·HCl	2 mg.
pyridoxin·HCl	2 "
calcium pantothenate	2 "
riboflavin	4 "
nicotinic acid	8 "
folic acid	100 g.
p-aminobenzoic acid	10 "
biotin	8 "
water	100 ml.
Salt solution A	
KH_2PO_4	25 g.
K_2HPO_4	25 "
water	250 ml.
Salt solution B	
$MgSO_4 \cdot 7H_2O$	10.0 g.
NaCl	0.5 "
$FeSO_4 \cdot 7H_2O$	0.5 "
$MnSO_4 \cdot 4H_2O$	1.5 "
water	250 ml.

quantities of amino acids. The amino acids were obtained from commercial sources and recrystallized when necessary. The p-fluorophenylalanine was prepared from p-fluorotoluene by conversion first to the aldehyde[61] and then by means of the azlactone procedure[62] to the amino acid.

The medium was prepared at twice the final concentration with appropriate amino acids omitted. The p-fluorophenylalanine concentration to be tested was placed in the culture tube and diluted to 5 ml. Five ml. of the double strength medium were added to give a final volume of 10 ml. The tubes were plugged with cotton and autoclaved for 15 to 20 minutes at 15 pounds pressure per square inch of steam. The tubes were incubated at 36° to 37° C in an air incubator equipped with a fan.

Turbidity as a measurement of growth in the tube cultures was determined with the Coleman Universal Spectrophotometer model 11 at a wavelength of 575 millimicrons. In this way, periodic readings during growth were made. The measurements were read as per cent transmission, which is related to optical density. The number of cells is nearly proportional to optical density[63]. The culture tubes were Pyrex, 11 millimeters in diameter and 150 millimeters long. They were optically matched by filling each tube with a dilute solution of copper sulfate. The tubes were matched to a standard tube and marked with an etched line to

coincide with a mark on the tube carrier. In this way, the same position in the carrier could be obtained each time.

Medium for the plate assays was prepared at full strength and autoclaved in the same manner as above. When the medium had cooled to just above the solidification temperature, it was inoculated with the appropriate organism, and 15 ml. were transferred to each plate with a sterile pipette. After solidification, the filter paper disks were placed on the surface, and the plates were incubated as above. The area of the zone of inhibition was calculated from the diameter. The diameter was measured to the nearest millimeter after a 24 hour incubation period.

The paper chromatograms were run on Whatman No. 1 filter paper. For amino acid determination, the papers were seven and one-half by nine and one-eighth inches. A 5 pound jar with a screw cap was used for the chamber. A cylinder was made of paper by stapling two opposite edges together. The edges were not allowed to touch since this caused uneven advancement of the solvent front. The samples were placed 1 inch apart on a base line which was 1 inch from the bottom of the paper. The samples were applied with either a melting point capillary tube or a 5 microliter pipette. After the solvent front had reached the top of the paper, the cylinder was removed, dried, and replaced in the chamber for the solvent to ascend to the top

again. This was repeated again so that the solvent advanced to the top of the paper three times, and good separation of p-fluorophenylalanine from the remainder of the amino acids resulted. The solvent was tertiary-butyl alcohol, methyl ethyl ketone, and water. The proportions were 4:4:1.5 by volume. For qualitative results the amino acids were located by spraying with 0.2 per cent ninhydrin in 95 per cent ethanol or acetone and drying in the oven at 100° C.

For quantitative estimations of amino acids on paper chromatograms, standard samples of the desired amino acid were applied to the paper. After developing with the solvent, the paper was dipped in 0.2 per cent ninhydrin in acetone. The dipping allowed an even application of the solution. The chromatograms were air dried at room temperature. Estimation was made by comparison of the color intensity of the unknown sample with the intensity of the known samples.

For separation of peptides by paper chromatography, filter paper four and one-half by sixteen inches was used. Descending technique was employed in a glass chamber. A stainless steel support was placed in the chamber to hold the glass trough containing the solvent. The solvent system was the same as mentioned above. A beaker of the solvent was placed in the bottom of the chamber to help

saturate the air space. The chromatograms were developed with 0.2 per cent ninhydrin as before.

The filter paper disks for bioassay were cut from the paper chromatograms with a number 8 cork borer. The substance was located by a companion chromatogram of the substance to be assayed. The companion chromatogram was sprayed with ninhydrin to locate the substance, and the corresponding area was cut from the paper chromatogram for assay.

Microbiological assay for phenylalanine was used to estimate the phenylalanine in some of the samples. L. brevis was the assay organism. Since it was necessary to separate phenylalanine from its fluoro analogue, the samples were chromatographed, and the area cut out of the chromatograms as indicated above. The filter paper containing the sample was placed in a tube and 10 ml. of medium lacking phenylalanine was added. After sterilization and inoculation as indicated, the tubes were incubated for 72 hours. A standard curve with graded amounts of phenylalanine added to the medium was used to estimate the phenylalanine in the sample. The samples and standard curve were run in duplicate, and the acid produced by growth was titrated with .050 N sodium hydroxide at the end of the incubation period. Bromthymol blue was the indicator for the titration.

EXPERIMENTAL

In experiments reported previously by Baker[52] on the utilization of p-fluorophenylalanine by L. arabinosus, the inability to exclude adsorption of the p-fluorophenylalanine to the protein of the organism was recognized. To overcome this limitation, the cytolyzate was treated with phenylisothiocyanate in aqueous pyridine according to Edman[64] to block the free amino groups, both terminal on proteins and on free amino acids. Phenylthiohydantoins of the amino acids were formed when the sample was hydrolyzed with acid. The phenylthiohydantoins do not form colored substances with the ninhydrin reagent for amino acids.

Experiment 1

Thirty liters of synthetic medium containing 39.6 milligrams of DL-phenylalanine and 98.9 milligrams of DL-p-fluorophenylalanine were inoculated with L. arabinosus I and incubated for 48 hours. Growth of the organism was followed on the spectrophotometer in 10 ml. tubes containing the same medium. More growth occurred in the presence of p-fluorophenylalanine than in controls with the analogue absent (see Table 3). The cells were centrifuged and suspended in one liter of physiological saline solution.

After standing overnight, the cells were centrifuged, resuspended in saline solution, and centrifuged again. The cells were cytolyzed in a mortar by grinding with sand in the presence of ether. The ether was separated from the cytolyzate and washed twice with 50 ml. of distilled water.

Table 3. The Growth of L. arabinosus I in the Presence of p-Fluorophenylalanine

(Values are per cent transmission; averages of quadruplicate 10 ml. cultures; DL-phenylalanine present in .08 micromoles per tube.)

Ratio of p-fluorophenylalanine to phenylalanine	Incubation Time (Hours)			
	16	24	31	48
0	59	56	54	53
2.5	55	50	46	45

The cytolyzate was taken up with 100 ml. of distilled water, and the sand was washed with 100 ml. of distilled water. All of the water used in washing the sand and ether were combined with the cytolyzate. The total volume was 300 ml.

Two one-half milliliter aliquots of the cytolyzate were used for the determination of nitrogen by micro-Kjeldahl. One hundred and fifty-five milligrams of nitro-

gen per 100 ml. of cytolyzate were found. To calculate the amount of phenylisothiocyanate to add to the cytolyzate, one-half of the nitrogen present was assumed to be free amino nitrogen. In addition, a nineteenfold excess, as suggested in the procedure of Hurst[65], was added based upon the nitrogen calculation. In this experiment, 300 ml. of pyridine was added to the cytolyzate, and the solution made alkaline to bromthymol blue by the addition of sodium hydroxide. After the addition of 50 grams of phenylisothiocyanate, the solution was placed in the incubator at 37° C. The mixture was kept alkaline by the periodic addition of base. When the consumption of base ceased, the mixture was removed from the incubator, and the solvent was distilled under reduced pressure.

The residue was suspended in 100 ml. of distilled water, which was then removed by distillation under reduced pressure. The residue was suspended in 200 ml. of distilled water, and 200 ml. of concentrated hydrochloric acid was added. After refluxing for 24 hours, the acid was distilled off under reduced pressure. The residue was suspended in 100 ml. of distilled water and taken to dryness again. This residue was suspended in 250 ml. of ether and 200 ml. of distilled water by shaking. The ether was separated and extracted with two 50 ml. portions of distilled water. The aqueous extract was added to the residue. Two

and one-half grams of Norite A, which had been activated by washing in 5 per cent acetic acid (Partridge[66]), was added to the aqueous suspension of the mixture. Thorough mixing was accomplished with the aid of a mechanical shaker. A portion of the material was lost during the shaking by splashing when a stopper was accidently removed. Shaking was continued for a total period of 4 hours. The charcoal was filtered off and eluted with 250 ml. of 20 per cent acetic acid solution containing 5 per cent phenol. A low speed stirring motor was used for mixing the suspended contents. The charcoal was filtered off after a period of 3 hours, and the filtrate extracted with 300 ml. of ether to remove the excess phenol. The ether extracts were washed with 100 ml. of distilled water. These aqueous extracts were added to the filtrate, and the solution was taken to dryness by distillation under reduced pressure.

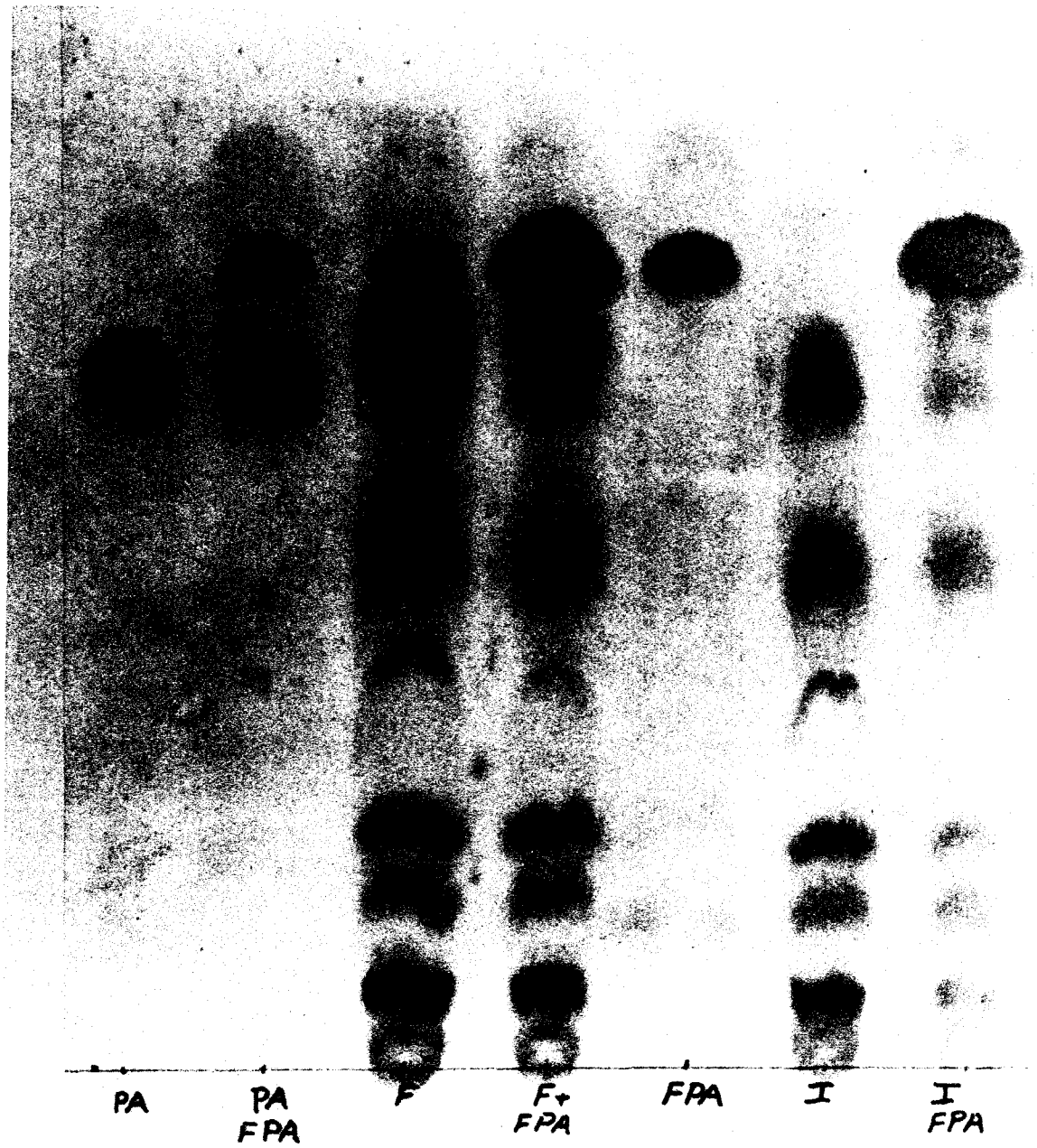
The residue was suspended in 50 ml. of water and transferred to a centrifuge tube. After centrifugation, the supernatant was poured off, and the residue washed again with distilled water. The supernatants were combined and concentrated under reduced pressure. The combined solid material was centrifuged and washed with hot distilled water. The pH was adjusted to approximately 6 with 0.2 N sodium hydroxide solution. The solution was used for chromatographic analysis. It was labelled "F".

Experiment 2

A cytolyzate of L. arabinosus cells grown on complete synthetic medium without p-fluorophenylalanine was used as a control for the above experiment. Three different controls were set up. In one, 8 milligrams of p-fluorophenylalanine was added to the cytolyzate before treatment with phenylisothiocyanate. The purpose was to ascertain if the phenylisothiocyanate blocked the free amino acids. This control is referred to as control "G". In the second control, referred to as "H", 8 milligrams of p-fluorophenylalanine was added after phenylisothiocyanate treatment. The addition was made after the excess phenylisothiocyanate had been destroyed by acid. The purpose of this control was to insure that any p-fluorophenylalanine in the acid hydrolyzate could be detected by the method employed. The third control, labelled "I", was processed like the other controls, except p-fluorophenylalanine was not added to the cytolyzate at any time. This control would indicate if there were any ninhydrin reacting substances with the same R_f value as p-fluorophenylalanine. The controls were processed in the same way as outlined above.

The results of the chromatographic analysis of samples "F" and "I" are shown in Figure 1. Controls of p-fluorophenylalanine (FPA) and phenylalanine (PA) are included.

Figure 1. Chromatographic separation of protein hydrolyzate of L. arabinosus I. (Left to right) Phenylalanine; phenylalanine and p-fluorophenylalanine; sample F (hydrolyzate of L. arabinosus I grown in the presence of p-fluorophenylalanine under stimulatory conditions); sample F and p-fluorophenylalanine; p-fluorophenylalanine; sample I (hydrolyzate of L. arabinosus I grown in a complete medium without p-fluorophenylalanine); sample I and p-fluorophenylalanine.



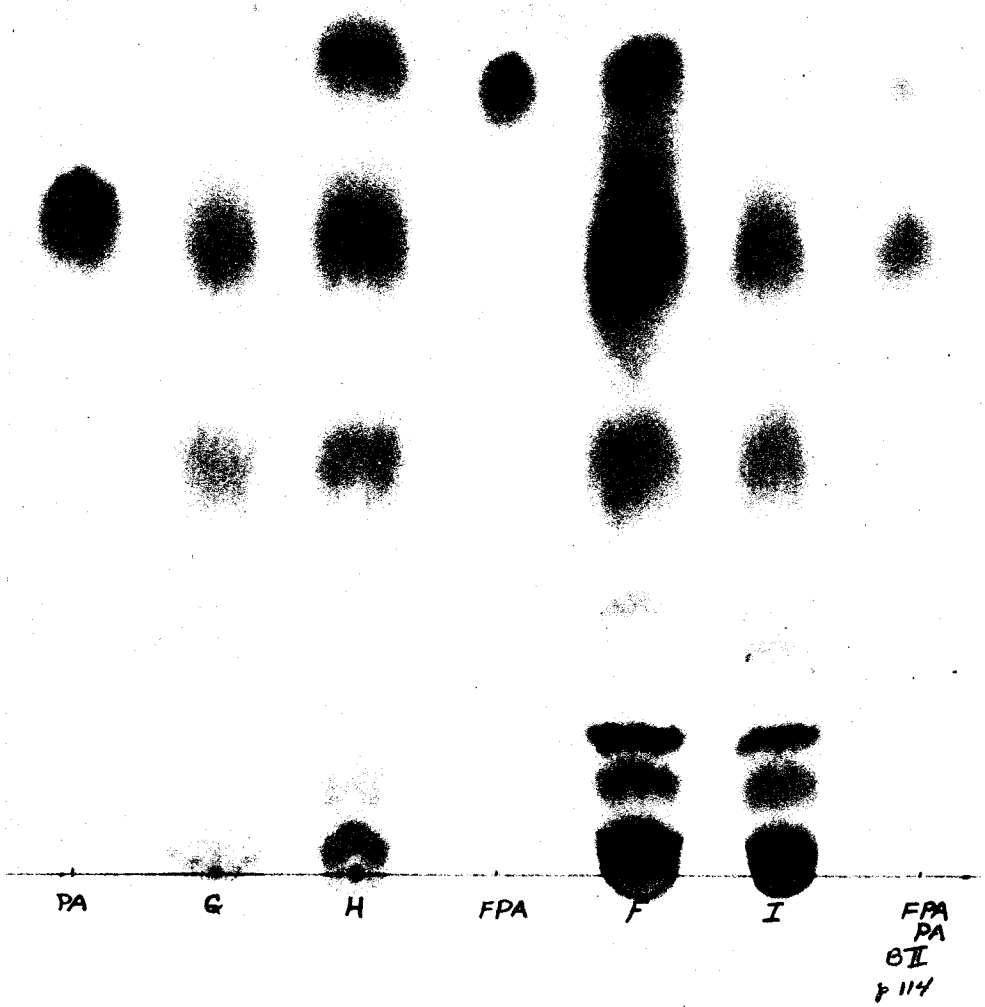
Book II p104

An amino acid spot with the same R_f value as p-fluorophenylalanine was present in sample "F" and not in sample "I". Addition of p-fluorophenylalanine to sample "F" (F and FPA) produced an enlargement of the spot attributed to p-fluorophenylalanine without a change in the R_f value. Addition of p-fluorophenylalanine to sample "I" produced a spot where none existed before.

Figure 2 is a reproduction of the chromatogram of samples "F", "G", "H", and "I". Sample "G" and "I" did not have spots with the same R_f value as p-fluorophenylalanine, while sample "F" and "H" did have spots with the same R_f value as p-fluorophenylalanine. The p-fluorophenylalanine added in sample "G" appeared to have been blocked by the phenylisothiocyanate treatment. In sample "H", the p-fluorophenylalanine added after the phenylisothiocyanate treatment appeared in the chromatogram but at a lower concentration than was anticipated.

Quantitative estimations of p-fluorophenylalanine in sample "F" were made by biological assay and by ninhydrin color comparisons. For the biological assay, the p-fluorophenylalanine was cut from chromatograms not treated with ninhydrin. These circular disks were placed on the surface of medium inoculated with L. arabinosus PT_1. After an incubation period of 24 hours, the zone of inhibition was measured, and the area was calculated. Table 4 shows the

Figure 2. Chromatographic separation of protein hydrolyzates of controls and of L. arabinosus I grown in the presence of p-fluorophenylalanine. (Left to right) Phenylalanine; sample G (hydrolyzate of L. arabinosus I grown in a complete medium without p-fluorophenylalanine, but p-fluorophenylalanine was added before treatment with phenylisothiocyanate); sample H (hydrolyzate of L. arabinosus I grown in a complete medium without p-fluorophenylalanine, but p-fluorophenylalanine was added after treatment with phenylisothiocyanate); p-fluorophenylalanine; sample F (hydrolyzate of L. arabinosus I grown in the presence of p-fluorophenylalanine under stimulatory conditions); sample I (hydrolyzate of L. arabinosus I grown in a complete medium without p-fluorophenylalanine); p-fluorophenylalanine and phenylalanine.



BII
p 114

results obtained with a 50 microliters sample of "F". Figure 3 is the curve obtained when the area of inhibition is plotted against concentration of DL-p-fluorophenylalanine. The area of 1200 square millimeters corresponded to 7 micrograms of DL-p-fluorophenylalanine.

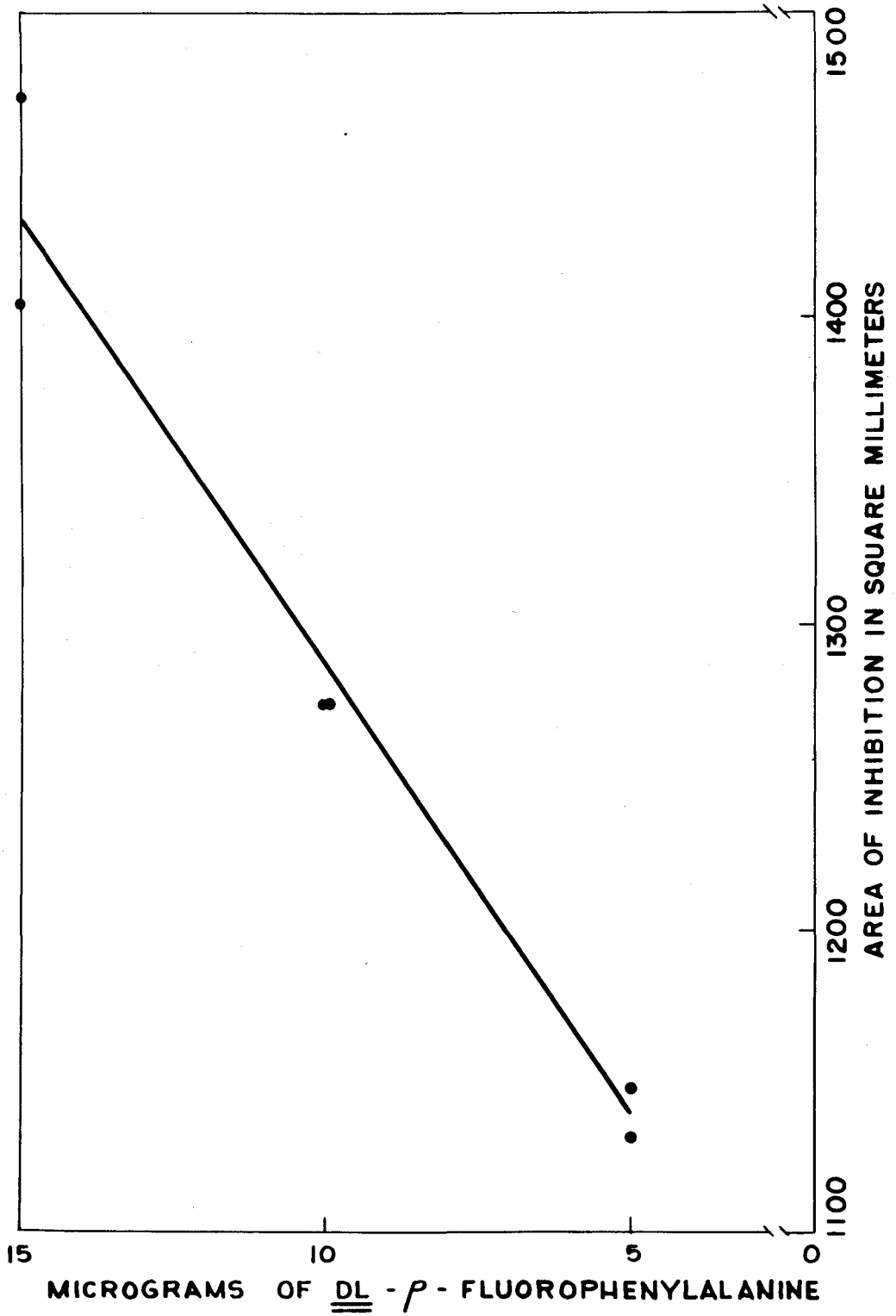
Table 4. Biological Assay of p-Fluorophenylalanine in Sample "F"

Sample	Area of Inhibition (Square millimeters)		
	1	2	Average
5 micrograms DL-p-fluorophenylalanine	1130	1150	1140
10 micrograms DL-p-fluorophenylalanine	1274	1274	1274
15 micrograms DL-p-fluorophenylalanine	1406	1469	1438
"F"			1200

If one assumes the inhibition was due to one antipode, then 3.5 micrograms were present in 50 microliters of sample "F".

The determination of p-fluorophenylalanine in sample "F" was made by visual comparison of the ninhydrin color intensity with standard samples of DL-p-fluorophenylalanine. A chromatogram of 3, 5, and 10 micrograms of DL-p-fluoro-

Figure 3. The area of inhibition as a function of DL-p-fluorophenylalanine concentration.


1500
1400
1300
1200
1100
AREA OF INHIBITION IN SQUARE MILLIMETERS
15
10
5
0
MICROGRAMS OF DL - P - FLUOROPHENYLALANINE

phenylalanine and 50 microliters of sample "F" was run. The color intensity of the p-fluorophenylalanine in "F" was between the intensity of the 3 and 5 microgram samples of DL-p-fluorophenylalanine. This agreed with the value of 3.5 micrograms obtained in the biological assay, and indicated that the inhibition and supplementary responses were due to one and the same stereoisomer.

Quantitative estimations were made for phenylalanine by the ninhydrin color comparison method and by microbiological assay using L. brevis. Five micrograms of phenylalanine per 50 microliters of sample "F" were determined by the color comparison method and 3 micrograms per 50 microliters of sample "F" by microbiological assay.

The biological assay, using L. arabinosus PT_1 for p-fluorophenylalanine, was more sensitive than the ninhydrin reagent. For this reason, sample "G" was tested for p-fluorophenylalanine. A narrow zone of inhibition was present around the filter paper disk. The average area was 401 square millimeters. An estimation of one microgram of p-fluorophenylalanine was made by extrapolation of the curve in Figure 3. This result indicated that perhaps the phenylisothiocyanate treatment did not block all the analogue.

In another experiment with L. arabinosus I grown in 40 liters of synthetic medium, p-fluorophenylalanine was

not detected in the hydrolyzed cytolyzate. Apparently this result was due to an extensive period of time in which the cytolyzate was in contact with the charcoal used to absorb the aromatic amino acids. Destruction of amino acids by oxidation in the presence of charcoal has been reported by Wieland et al.[68] In subsequent experiments, the charcoal absorption procedure was omitted.

Experiment 3

In order to determine the extent of the blocking of free amino groups by the phenylisothiocyanate procedure, two experiments were performed. In the first experiment, 2.21 milligrams of DL-p-fluorophenylalanine was dissolved in 2 ml. of distilled water; 2 ml. of pyridine was added; and the solution was made alkaline to bromthymol blue with 0.2 N sodium hydroxide. Thirty-two and four-tenths milligrams of phenylisothiocyanate was added, and the mixture was placed in the incubator at 37° C for 24 hours. The solvent was removed by distillation, and the residue was washed with 20 ml. of distilled water. After the water was distilled off, 20 ml. of 6 N hydrochloric acid was added to the residue. The mixture was refluxed for 24 hours, and the acid removed by distillation. The residue was washed with several milliliters of distilled water and taken to dryness again. The residue was suspended in 0.3 ml. of distilled

water and 0.4 ml. of .05 N sodium hydroxide. The pH of the solution was approximately 5. Estimation of p-fluorophenylalanine by color comparison indicated that 210 micrograms of p-fluorophenylalanine were not blocked by the phenylisothiocyanate treatment. This was about 10 per cent of the starting material.

Experiment 4

In the second experiment to determine the extent of the blocking of free amino groups by the phenylisothiocyanate procedure, 30 milligrams of p-fluorophenylalanine was added to a cytolyzate of L. arabinosus I which had been grown on a complete synthetic medium. This cytolyzate was treated with phenylisothiocyanate as in the experiment outlined at the beginning of the section. After removal of the acid, the washed residue was extracted twice with 100 ml. of ether. The residue was suspended in 20 ml. of water, and the pH adjusted to 6 with concentrated sodium hydroxide. A black oil, which formed, was removed by ether extraction. The aqueous solution was diluted to 50 ml. in a volumetric flask. Chromatographic analyses for p-fluorophenylalanine all failed to resolve the amino acids in the area in which the analogue occurs. The solution was concentrated to 25 ml. and chromatographed again with the same results. Another

chromatogram which had not been treated with ninhydrin was cut into four sections each three-fourths inch wide. Section 1 began one-fourth inch below the top of the solvent front. The four sections were assayed for p-fluorophenylalanine by biological inhibition assay with L. arabinosus PT_1. A zone of inhibition, 32 millimeters in diameter, occurred on the plate containing sections 3 and 4. This corresponded to approximately 2 micrograms of DL-p-fluorophenylalanine in 10 microliters of the hydrolyzate or 16 per cent recovery. (See Table 6).

In a companion chromatogram, sections 2 and 3 were cut in one piece. The sections were eluted by the method of Consden et al.[67] The eluate was concentrated and chromatographed in the same solvent as before. The area corresponding to the R_f value of p-fluorophenylalanine was cut and assayed with L. arabinosus PT_1. No inhibition occurred.

The results in the preceding experiments indicated that the phenylisothiocyanate treatment did not block completely the free amino acids. In order to circumvent this disadvantage, the cell cytolyzate was hydrolyzed to peptides by partial hydrolysis, and the identification of p-fluorophenylalanine was attempted from the mixture of peptides.

Experiment 5

The cells of L. arabinosus I were grown and harvested in the same manner as in the experiment on page 28. Table 5 shows that more growth occurred in the preeence of p-fluorophenylalanine. The total amount of growth was more than had been obtained in previous experiments. (See Table

Table 5. The Growth of L. arabinosus I in the Presence of p-Fluorophenylalanine

(Values are per cent transmission; averages of duplicate 10 ml. cultures; DL-phenylalanine present in .08 micromoles per tube.)

Ratio of p-fluoro-phenylalanine to phenylalanine	Incubation Time (Hours)	
	24	32
0	30	31
2.5	24	22

3). After cytolysis, micro-Kjeldahl analysis for nitrogen on two and one-half milliliter aliquots showed an average of 220 milligrams of nitrogen per 100 ml. The total volume was 250 ml. The solvent was distilled from the cytolyzate under reduced pressure. Two-hundred and fifty milliliters of concentrated hydrochloric acid were added to the dried cytoly-

zate, and the mixture placed in the incubator at 37° C for 3 days. The acid was removed by distillation under reduced pressure. The residue was suspended in 100 ml. of distilled water and then taken to dryness. After the residue was transferred to a smaller flask with distilled water, the solvent was removed by distillation. The residue was next transferred to a test tube with hot distilled water. The solution was adjusted to pH 6 with sodium hydroxide before centrifuging the suspended material to the bottom of the tube. The supernatant was decanted into a 50 ml. volumetric flask, and the precipitate was washed four times with 5 ml. of distilled water. The supernatants were combined and extracted with 50 ml. of ether. The ether extract was washed with 5 ml. of distilled water, and all of the aqueous solutions combined and diluted to 50 ml. in the volumetric flask. This solution labelled "Q" was used for the chromatograms.

The free amino acids in Q were blocked with phenylisothiocyanate. A 0.3 ml. sample of Q was treated with 43 milligrams of phenylisothiocyanate dissolved in 0.3 ml. of pyridine. The solution was made alkaline with sodium hydroxide, and by the periodic addition of sodium hydroxide during the period of reaction, the solution was kept alka-

line. The reaction was carried out at 37° C for 12 hours. After the reaction had ceased, the excess phenylisothiocyanate and pyridine were extracted with 3 ml. of benzene. The remaining 0.6 ml. of solution were hydrolyzed by the addition of 0.6 ml. of concentrated hydrochloric acid. The tube was sealed and placed in an oven at 100° C for 15 hours. When cool, the seal on the tube was broken, and the acid was removed by evaporation in a vacuum desiccator. The solution was treated twice with two drops of dilute ammonium hydroxide. After each addition of the ammonium hydroxide, the solution was taken to dryness. The residue was suspended in 0.30 ml. of distilled water. This material was labelled Q_P and is referred to in the description of subsequent experiments as Q_P.

Twenty microliters of Q_P was chromatographed by ascending technique. Figure 4 shows the resultant chromatogram. Due to the high concentration of amino acids in the lower part of the chromatogram, the other amino acids were abnormally displaced, and p-fluorophenylalanine could not be identified in the sample.

In order to determine if any p-fluorophenylalanine was in the sample, a companion chromatogram of 20 microliters of sample Q_P was cut in five sections as shown in Figure 4. Each section was assayed for p-fluorophenylalanine with L. arabinosus PT_1. Zones of inhibition were found around

Figure 4. Chromatographic separation of protein hydrolyzates of L. arabinosus I cytolyzates. (Left to right) Chromatogram is of a 20 microliter sample of Q_P (a complete hydrolyzate of L. arabinosus I grown in the presence of p-fluorophenylalanine and which had been treated with phenylisothiocyanate prior to complete hydrolysis). Upper portion shows strips cut for biological assay and rechromatographing. Q_{PE} (an eluate of area containing p-fluorophenylalanine from a chromatogram of sample Q_P) and 10 micrograms of p-fluorophenylalanine; Q_{PE}; 3, 5, and 10 micrograms of p-fluorophenylalanine.

Table 6. Biological Assay of p-Fluorophenylalanine in Sample Q_P

(Values are area of inhibition in square millimeters.)

Sample	Aliquots 1	2
3 micrograms of DL-p-fluorophenylalanine	1134	1134
5 micrograms of DL-p-fluorophenylalanine	1385	1320
10 micrograms of DL-p-fluorophenylalanine	1735	1809
15 micrograms of DL-p-fluorophenylalanine	2043	2123
Section 1 of Q_P	425	380
Section 2 of Q_P*	1452	855
Section 3 of Q_P*	573	1452
Section 4 of Q_P	Increased	Growth
Section 5 of Q_P	"	"
Sample Q_{PE}	1452	
Sample Q_H**	1809	1661

*Apparently the p-fluorophenylalanine in aliquot 1 advanced farther than in aliquot 2.

**Sample Q_H is not to be confused with sample H in experiment 2, p. 32.

sections 2 and 3. Table 6 shows the results of the bioassay on two aliquots of sample Q_P.

Since it seem likely that the inhibition was due to the L-form, Table 7 gives the amount of L-p-fluorophenylalanine corresponding to the inhibition observed with each

Table 7. Amount of L-p-Fluorophenylalanine in Samples Assayed by Biological Inhibition

(Values are in micrograms.)

Sample		1	2	Average
Q_{P1}		.25	.125	
Q_{P2}*		3.0	1.0	
Q_{P3}*		.50	3.0	
	Total	3.75	4.125	3.9
Q_H		5.25	4.35	4.8
Q_{PE}		3.		3.

*Apparently the p-fluorophenylalanine in aliquot 1 advanced farther than in aliquot 2.

sample. The total inhibition of the sample Q_P corresponded to 3.9 micrograms of L-p-fluorophenylalanine in 20 microliters of hydrolyzate. This represents the amount of non-aminoid p-fluorophenylalanine residue in the peptides.

Evidence that the inhibition present in sections 1, 2, and 3 was due to p-fluorophenylalanine was obtained by the identification of the amino acid in the eluate of sections 2 and 3. The combined area of sections 2 and 3 was eluted from three companion chromatograms of 20 microliters each of sample Q_P. The method of Consden et al.[67] was used for the elution. After evaporating to dryness, the eluate labelled Q_{PE} was dissolved in 60 microliters of distilled water. Figure 4 shows the chromatogram of Q_{PE}. The intensity of the p-fluorophenylalanine spot was estimated at 3 micrograms per 20 microliters of sample Q_{PE} by comparing with color intensities of known amounts of p-fluorophenylalanine. The chromatogram of Q_{PE} and 10 micrograms of p-fluorophenylalanine showed that the spot had the same R_f value as known p-fluorophenylalanine.

Biological assay of Q_{PE} indicated 3 micrograms of the active form to be present. This is shown by the area of inhibition given in Table 6.

The total amount of p-fluorophenylalanine in sample Q was estimated from a total hydrolysis of sample Q. To 0.3 ml. of sample Q, an equal volume of concentrated hydrochloric acid was added. The tube was sealed, and hydrolysis was carried out in the same manner as the hydrolysis of sample Q_P. The removal of acid after hydrolysis and the neutralization of the hydrolyzate was done in the same way

as sample Q_P. The resulting hydrolyzate was suspended in 0.3 ml. of distilled water and labelled Q_H. (Sample Q_H is not to be confused with sample H in Experiment 2, p.32.) Estimation of p-fluorophenylalanine in Q_H by paper chromatography indicated 6 micrograms per 20 microliters of sample Q_H. As shown in Table 7, the biological assay indicated that 4.8 micrograms of L-p-fluorophenylalanine were present in 20 microliters of Q_H. The amount of inhibition in the biological assay is shown in Table 6.

The zones of increased growth around sections 4 and 5 (see Table 6) were probably due to phenylalanine. This would be the approximate area in which to expect the natural amino acid to be since p-fluorophenylalanine occurred in sections 1, 2, and 3, and since p-fluorophenylalanine leads phenylalanine on the chromatograms.

Partial fractionation of the mixture of peptides was accomplished by descending filter paper chromatography. The chromatograms were run on paper 16 inches long with a cleansing tissue stapled to the end to absorb the solvent when it had descended the length of the paper. The chromatograms were run for 15 hours, and on treatment with ninhydrin, they gave a colored strip from the origin to within a few inches of the cleansing tissue. The greatest concentration of ninhydrin reacting substances was in the lower one-half of the chromatogram. In order to determine

if p-fluorophenylalanine was concentrated in any section of the chromatogram in the peptide form, three samples of 20 microliters each were chromatographed as companion strips. The resulting chromatograms were divided into four parts. The section designated as Q_1 was the lower three and one-half inches of the chromatogram. The remainder of the chromatogram was divided into 3 sections three and one-half inches wide and designated as Q_2, Q_3, and Q_4 proceeding from the top of section Q_1 to the end of the paper.

The sections were eluted by the method of Consden et al.[67] A 2 ml. bore capillary tube 13 centimeters long was used in the elution. The total volume of eluate was apporoximately 0.3 ml. The eluates were transferred to Pyrex test tubes of 10 by 75 millimeter size. To each sample, 0.3 ml. of concentrated hydrochloric acid was added. The tubes were sealed and placed in an oven at 100° C for 15 hours. When cool, the seal on each tube was broken, and the contents evaporated to dryness in a vacuum desiccator containing sodium hydroxide pellets. One drop of distilled water was added to each residue. After evaporation to dryness, two drops of dilute ammonium hydroxide were added to each tube. The excess ammonia was removed in the same manner as the acid. After suspending in 5 microliters of distilled water, the total amount of the hydrolyzates was applied to filter paper for chromatographing; likewise, Q_3

Figure 5. Chromatographic separations for locating p-fluorophenylalanine in a partially hydrolyzed sample of L. arabinosus I cytolyzate. (Left to right) Chromatograms of samples of Q_H (a partial hydrolyzate of L. arabinosus I, grown in the presence of p-fluorophenylalanine, which has been fully hydrolyzed without phenylisothiocyanate treatment); Q_{1-H}; 5 and 10 micrograms of p-fluorophenylalanine; Q_{2-H}; Q_{3-H}; and Q_{4-H} (eluates of a chromatogram of Q_H divided into 4 sections).


5
10
FPA

and Q_4 were suspended in 10 microliters of distilled water and chromatographed. Figure 5 shows the chromatograms of the hydrolyzed samples. It was evident that the p-fluorophenylalanine was concentrated in sample Q_3. Approximately 5 micrograms of p-fluorophenylalanine were estimated to be in sample Q_3. Faint spots were also visible in samples Q_4 and Q_H.

Sample Q_H was obtained as indicated on page 53. Since a smaller amount of sample Q_H than Q_3 was applied, only a very faint spot with the same R_f value as p-fluorophenylalanine was present on the chromatogram. The material in sample Q_H corresponded to 5 microliters of sample Q, while Q_3 corresponded to about 40 microliters of sample Q.

DISCUSSION

Table 8 gives the total amount of p-fluorophenylalanine found in the cytolyzate of L. arabinosus I by paper chromatography and biological assay in Experiment 5. The table includes values for p-fluorophenylalanine substituted on the amino group, e.g. the analogue without a free amino group

Table 8. The Amount of p-Fluorophenylalanine Present in the Cytolyzate of L. arabinosus I Determined by Paper Chromatography and Biological Assay in Experiment 5

	Methods of Analysis	
	Paper Chromatography	Biological Assay
Total p-fluorophenylalanine in cytolyzate	15 milligrams	12 milligrams
p-Fluorophenylalanine substituted on amino group	7.5 "	9.7 "
Per cent of total p-fluorophenylalanine recovered as substituted p-fluorophenylalanine	50 per cent	81 per cent

present in peptides, and the per cent of the total p-fluorophenylalanine represented by the amino-substituted analogue. The results of both methods showed that well over 10 or 16

per cent of the p-fluorophenylalanine was recovered as amino-substituted p-fluorophenylalanine. The amount of the analogue recovered as the amino-substituted amino acid was greater than that which could be attributed to incomplete reaction with phenylisothiocyanate. This indicated that a portion of the p-fluorophenylalanine was incorporated into the proteins of L. arabinosus I.

The difference between the total p-fluorophenylalanine and the p-fluorophenylalanine substituted on the amino group, as shown in Table 8, is probably p-fluorophenylalanine with a free amino group. The difference could be due to free p-fluorophenylalanine or p-fluorophenylalanine bound through its carboxyl group to another amino acid. In either case, the free amino group would react with phenylisothiocyanate; therefore, p-fluorophenylalanine with a free amino group in peptides could not be distinguished from free p-fluorophenylalanine by the methods of analysis which were used.

Although the values obtained by the two methods in Experiment 5 are not in complete agreement, the same qualitative conclusion that p-fluorophenylalanine is incorporated into the proteins of L. arabinosus I can be deduced from each set of data. The value obtained for the total amount of p-fluorophenylalanine by paper chromatography is probably more accurate than the value obtained by inhibition

assay. Diffusion of amino acids on the filter paper sometimes results in a portion remaining on the paper when the disks are cut for inhibition assay. The value obtained by biological assay for amino-substituted p-fluorophenylalanine is probably more accurate than the value obtained by paper chromatography. The samples for biological assay were held in strips cut from a chromatogram and assayed; however, the sample for quantitative paper chromatography was obtained by elution of strips from a chromatogram followed by evaporation to dryness, and then quantitative paper chromatography was employed.

The qualitative results of Experiment 1 with L. arabinosus I, confirming the earlier work reported by Baker[52] when the organism was grown in the presence of p-fluorophenylalanine, indicated that the fluoro analogue of the amino acid was incorporated into peptides of the organism. Quantitative results, on the other hand, indicated that some of the p-fluorophenylalanine which appears to be incorporated may be the free amino acid present in the cytolyzate which was not completely blocked by the phenylisothiocyanate treatment. The biological assay for p-fluorophenylalanine in the control cytolyzate "G" of Experiment 2 gave evidence that the blocking of free amino acids, which in qualitative tests appeared to be complete, was incomplete. Confirmation was obtained with two additional experiments.

In Experiment 3 with the pure amino acid, 10 per cent of the amino acid was recovered as estimated by ninhydrin color intensities. When the amino acid was added to a cytolyzate of L. arabinosus I, grown in the absence of p-fluorophenylalanine as in Experiment 4, 16 per cent of the p-fluorophenylalanine was recovered after treatment with phenylisothiocyanate as estimated by biological assay. The discrepancy between the two experimental results is no larger than would be anticipated in biological assays.

Incomplete blocking of the free amino acid may be attributed to incomplete reaction of phenylisothiocyanate with the amino acid; to hydrolysis of the phenylthiocarbamyl derivative of the amino acid by 6 N hydrochloric acid used for protein hydrolysis; or to adsorption of 10 per cent of the amino acid to cell components in a manner which was unavailable for reaction with phenylisothiocyanate. Adsorption of the amino acid, which could not be removed by ninhydrin reagent as reported by Brunish and Luck[37], probably would be unavailable for reaction with phenylisothiocyanate.

In Experiment 1, the quantitative results for p-fluorophenylalanine and phenylalanine showed 280 micrograms of the fluoro analogue and 400 micrograms of the natural amino acid to be present in the hydrolyzate. These values are minimum values because of losses occurring in

the experimental procedure. The value for phenylalanine was unexpectedly low since 19.8 milligrams of L-phenylalanine were present in the medium. Since phenylalanine was the limiting factor of the amount of growth in the absence of the fluoro analogue, a fuller utilization of the natural amino acid would be expected in the presence of p-fluorophenylalanine.

Activated charcoal, according to Partridge[66], adsorbs aromatic amino acids preferentially; however, the chromatographic results showed approximately nine amino acid spots. Although adsorption and elution of the charcoal may not be quantitative, the destruction of aromatic amino acids by oxidation in the presence of charcoal (Wieland[68]) was probably a source of loss of phenylalanine and p-fluorophenylalanine. Inability to detect p-fluorophenylalanine in the hydrolyzate in Experiment 2, page 41, was due probably to extensive destruction by charcoal. In later experiments, the treatment with charcoal was omitted.

The following limitations in the experimental procedure should be recognized. Methods for estimating p-fluorophenylalanine in protein hydrolyzates were not highly accurate. For example, when cutting the filter paper disks for inhibition assay, the location of the amino acid as determined by companion chromatograms might vary if the rate of flow of the solvent was irregular. The areas of

inhibition were calculated by assuming that the zones were circular.

Difficulties arose in estimating the intensity of p-fluorophenylalanine in samples by the ninhydrin method when the value fell between or outside known concentrations of p-fluorophenylalanine.

Phenylisothiocyanate used to block amino acids with a free amino group also reacts with any p-fluorophenylalanine present in peptides with a free amino group. For this reason, the total amount of p-fluorophenylalanine in the proteins of L. arabinosus I could not be estimated by the phenylisothiocyanate treatment.

Reaction of phenylisothiocyanate with p-fluorophenylalanine was incomplete under the experimental conditions. The small amount of free p-fluorophenylalanine which was present due to the incomplete reaction did not interfere with the qualitative conclusion that p-fluorophenylalanine was incorporated into the proteins of L. arabinosus I in Experiment 5; however, complete removal of all free amino acids would facilitate quantitative conclusions.

In addition to these experimental limitations, evidence for alteration of the amino acid composition of the L. arabinosus I proteins would be more conclusive if p-fluorophenylalanine was identified as a portion of a peptide in which all the other amino acids were known. The

identification of p-fluorophenylalanine by other properties, e.g. melting point of the pure compound and/or of a known derivative, would supplement paper chromatography and inhibition assay methods.

In the experimental procedure reported previously[52], p-fluorophenylalanine was identified only by paper chromatography. Improved methods of chromatography as well as inhibition assay identified p-fluorophenylalanine more conclusively in the experiments reported in this thesis. p-Fluorophenylalanine was estimated quantitatively by the ninhydrin method and by the inhibition assay, while quantitative estimations were not made before. Treatment of the partial hydrolyzate with phenylisothiocyanate to remove free amino acids is preferable to washing the intact cells repeatedly with saline.

The advantages of performing a partial hydrolysis of the cytolyzate of L. arabinosus I, grown under stimulatory conditions, were that the total amount of p-fluorophenylalanine could be determined before as well as after treatment with phenylisothiocyanate, and the partial hydrolysis of the proteins should release any free amino acids trapped by the proteins.

Estimation of the p-fluorophenylalanine in the partial hydrolyzate both before and after phenylisothiocyanate treatment indicated that part of the analogue was

present in the cytolyzate without a free amino group. In the previous report[52], this evidence was not obtained.

Considering the criteria outlined in the Review of Literature (page 3), the experiments reported in this thesis attempt to overcome the effects of adsorbed free amino acids by hydrolyzing the proteins to peptides and treating with phenylisothiocyanate. The use of an unnatural amino acid circumvents the disadvantage with a natural amino acid of having to distinguish between a change in the amount of protein containing the amino acid and a change in the amounts of amino acid in the protein. Although nothing is known about the metabolic breakdown of p-fluorophenylalanine in L. arabinosus I, the possibility of utilizing the products to synthesize an amino acid with the same R_f value and inhibition response in L. arabinosus PT_1, as p-fluorophenylalanine, is remote. The likelihood of transfluoridation from cellular free p-fluorophenylalanine to peptide bound phenylalanine or tyrosine has not been excluded, but the bond strength of organic fluorides makes this seem infeasible.

Indirect evidence indicated that supplementation and inhibition responses of L. arabinosus I with p-fluorophenylalanine were due to the same stereoisomer. Since proteins are composed mainly of amino acids of the L-configuration, it was assumed that the p-fluorophenylalanine

identified in the cell hydrolyzates was of this configuration. In the quantitative estimation of p-fluorophenylalanine, the amount obtained by ninhydrin color comparison was one-half the value obtained by inhibition analysis. This is apparent in sample Q_{PE} of Experiment 5. In Figure 4, sample Q_{PE} showed the same ninhydrin color intensity as 3 micrograms of DL-p-fluorophenylalanine; however, the area of inhibition, as shown in Table 6, was equivalent to 6 micrograms of DL-p-fluorophenylalanine.

Incorporation of p-fluorophenylalanine into the proteins of L. arabinosus I constitutes an alteration of protein synthesis. The amino acid residue sequence of the protein has been altered in a way not controlled by the reproduction mechanisms of the organism. This indicated, therefore, that the amino acid composition of a protein is mutable. The effect of the change in protein structure on the biological function of the protein is unknown. It is conceivable, however, that biological activity of an enzyme, for example, may vary as the amino acid composition deviates from the pattern set by the enzyme reproduction mechanism. The implications of a system allowing variability in amino acid composition of proteins in conjunction with variability in biological function have been discussed by Fox[69]. Fox postulated that molecular variability, in the structure of compounds, such as proteins, which results in

variation of biological activity, may afford a selective advantage to cells with the ability to synthesize compounds with increased activity. In a competitive society, the cells producing compounds with higher biological activity would win the struggle for survival. Fox points out that the families of molecules known to occur naturally such as antibiotics, vitamins, hormones, enzymes, and the inability of chemists to isolate a pure protein may be consequences of molecular variation.

The inhibition of L. arabinosus I by p-fluorophenylalanine has been reported previously[1]. In view of the results reported in this thesis, it appears that the activity of p-fluorophenylalanine in respect to phenylalanine can be divided into two classes: One, the biological functions in which the analogue inhibits the utilization of phenylalanine, and two, the biological functions in which the analogue substitutes for phenylalanine. A third class might be those reactions of phenylalanine which p-fluorophenylalanine does not affect in any way. Elucidation of the relationship between the first two classes is dependent upon more knowledge of the mechanisms of inhibition and tolerance of p-fluorophenylalanine in L. arabinosus I.

The techniques employed in these experiments and the results of the experiments suggest further studies. p-Fluorophenylalanine may be useful in following the pathway

of free amino acids to their inclusion into proteins. The biological effect of peptides containing p-fluorophenylalanine would be interesting to study as well as the effect of ortho and meta fluorophenylalanine under conditions which cause both stimulation and inhibition of L. arabinosus I by p-fluorophenylalanine. The genetical implications of changes which might take place by the incorporation of the analogue into gene proteins are important and should not be overlooked, especially if an organism can be trained to replace its phenylalanine requirement with p-fluorophenylalanine. The substitution of an amino acid with a chemical analogue as reported in this thesis can be extended to other amino acids as well as to other analogues. The mechanisms of inhibition and tolerance are worthy of study. The results obtained from these experiments may be useful in elucidating mechanisms of protein synthesis and phenylalanine metabolism.

SUMMARY AND CONCLUSIONS

Many attempts to alter the amino acid composition of proteins have been made. Natural, unnatural, and isotopically labelled amino acids have been used in these studies. Early workers overlooked the effects of adsorption of amino acids to proteins. In the studies with natural amino acids, the inability to distinguish between changes in the amino acid composition of the proteins and changes in the amounts of proteins containing the amino acids was a disadvantage. In studies with unnatural amino acids or isotopically labelled amino acids, the incorporation of the labelled group into the natural amino acid already in peptides by exchange reactions and the synthesis of natural amino acids from breakdown products of the unnatural amino acids might lead to erroneous conclusions.

An amino acid containing selenium was isolated from grain grown in soil having large amounts of selenium. The selenium compound appeared to be an analogue of a naturally occurring amino acid containing sulfur. Levine and Tarver[33] reported the incorporation of ethionine into proteins of the liver, spleen, and kidney of the rat. They assumed ethionine to be an unnatural amino acid and devoid of other biological reactions. Stekol and Weiss[35], however, report the formation of triethylcholine and the ethyl analogue of

creatinine from ethionine. Schlenk and Tillotson[41] reported that 5-ethylthioadenosine was formed from ethionine by yeasts, bacteria, and rabbit liver. These reports showed that ethionine may take part in several biological reactions. Levine and Tarver failed to exclude interferences from these reactions.

Atkinson[1] reported p-fluorophenylalanine stimulated the growth of L. arabinosus I. Preliminary evidence of incorporation of this amino acid into the organism's proteins was reported by Baker[52]. The experimental results reported in this thesis led to the conclusion that p-fluorophenylalanine was incorporated into the proteins of L. arabinosus I. p-Fluorophenylalanine was determined by paper chromatography and by biological inhibition of L. arabinosus PT_1. A cytolyzate of L. arabinosus I, grown in the presence of p-fluorophenylalanine, was partially hydrolyzed with concentrated hydrochloric acid. Twelve to 15 milligrams of p-fluorophenylalanine were estimated to be present in the cytolyzate. A portion of the cytolyzate was treated with phenylisothiocyanate and the p-fluorophenylalanine was estimated again. Seven and five-tenths to 9.7 milligrams were estimated to be bound in the proteins in such a way that the amino group of the acid was not free. The recovery of p-fluorophenylalanine was greater than that attributed to incomplete reaction with

phenylisothiocyanate.

The results and limitations of the experiments are discussed. The evidence which indirectly showed that the inhibition and stimulation response was due to L-p-fluorophenylalanine is discussed.

It was concluded that p-fluorophenylalanine can partially substitute for phenylalanine in the proteins of L. arabinosus I, and that the stimulation and inhibition responses were due to the same stereoisomer of p-fluorophenylalanine.

LITERATURE CITED

1. Atkinson, D. E. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library. 1949.

2. Abderhalden, E. and P. Rona. Z. physiol. Chem. 46: 179, 1905.

3. Tamura, S. Z. physiol. Chem. 88: 190, 1913.

4. Rosedale, J. L. Biochem. J. 16: 27, 1922.

5. Rosedale, J. L. Biochem. J. 22: 826, 1928.

6. Dirr, K. Z. physiol. Chem. 260: 65, 1939.

7. Block, R. J. J. Biol. Chem. 133: 71, 1940.

8. Block, R. J. and D. Bolling. Arch. Biochem. 7: 313, 1945.

9. Camien, M. N., A. J. Salle, and M. S. Dunn. Arch. Biochem. 8: 67, 1945.

10. Stokes, J. L. and M. Gunness. J. Bacteriol. 52: 195, 1946.

11. Freeland, J. C. and E. F. Gale. Biochem. J. 41: 135, 1947.

12. Csonka, F. A., C. A. Denton, and S. J. Ringel. J. Biol. Chem. 169: 259, 1947.

13. Csonka, F. A. J. Nutrition. 42: 443, 1950.

14. Csonka, F. A. and M. Jones. J. Nutrition. 46: 531, 1952.

15. Work, E. Biochim. et Biophys. Acta. 3: 400, 1949.

16. Nelson, E. M., A. M. Hurd-Karrer, and W. O. Robinson. Science. 78: 124, 1933.

17. Robinson, W. O. J. Assoc. Offic. Agr. Chemists. 16: 423, 1933.

18. Franke, K. W. J. Nutrition. 8: 597, 1934.

19. Franke, K. W. and V. R. Potter. J. Nutrition. 10: 213, 1935.

20. Franke, K. W. and E. P. Painter. J. Nutrition. 10: 599, 1935.

21. Franke, K. W. and E. P. Painter. Cereal Chem. 13: 67, 1936.

22. Painter, E. P. and K. W. Franke. Cereal Chem. 13: 172, 1936.

23. Painter, E. P. and K. W. Franke. Am. J. Botany. 27: 336, 1940.

24. Painter, E. P. and K. W. Franke. J. Biol. Chem. 134: 577, 1940.

25. Horn, M. J., E. M. Nelson, and D. B. Jones. Cereal Chem. 13: 126, 1936.

26. Jones, D. B., M. J. Horn, and C. E. F. Gersdorff. Cereal Chem. 14: 130, 1937.

27. Horn, M. J. and D. B. Jones. J. Am. Chem. Soc. 62: 234, 1940.

28. Horn, M. J. and D. B. Jones. J. Biol. Chem. 139: 649, 1941.

29. Horn, M. J. and D. B. Jones. J. Biol. Chem. 139: 473, 1941.

30. Moxon, A. L. J. Am. Pharm. Assoc., Sci. Ed. 29: 249, 1940.

31. Moxon, A. L., K. P. DuBois, and R. L. Potter. J. Pharmacol. Exptl. Therap. 72: 184, 1941.

32. Dyer, H. M. J. Biol. Chem. 124: 519, 1938.

33. Levine, M. and H. Tarver. J. Biol. Chem. 192: 835, 1951.

34. Stekol, J. A. and K. Weiss. J. Biol. Chem. 179: 1049, 1949.

35. Stekol, J. A. and K. Weiss. J. Biol. Chem. 185: 577, 1950.

36. Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy. Federation Proc. 8: 489, 1949.

37. Brunish, R. and J. M. Luck. J. Biol. Chem. 197: 869, 1952.

38. Smith, R. L. and F. Schlenk. Arch. Biochem. and Biophys. 38: 159, 1952.

39. Smith, R. L. and F. Schlenk. Arch. Biochem. and Biophys. 38: 167, 1952.

40. Smith, R. L., E. E. Anderson, R. N. Overland, and F. Schlenk. Arch. Biochem. and Biophys. 42: 72, 1953.

41. Schlenk, F. and J. A. Tillotson. J. Biol. Chem. [In Press.]

42. Schlenk, F., Ames, Iowa. [Private Communication.] 1953.

43. Schoenheimer, R. "The dynamic state of body constituents." Cambridge, Harvard University Press. 1942.

44. Sizer, I. W. Advances in Enzymol. 14: 129, 1953.

45. Levine, M. and J. V. Fopeano. J. Biol. Chem. 202: 597, 1953.

46. Kraft, K. Z. physiol. Chem. 245: 58, 1936.

47. Litzka, G. Deut. med. Wochschr. 63: 1037, 1937.

48. Boyer, P. D., R. J. Evans, and P. H. Phillips. J. Pharmacol. Exptl. Therap. 73: 176, 1941.

49. Mitchell, H. K. and C. Niemann. J. Am. Chem. Soc. 69: 1232, 1947.

50. Melvin, S. [Unpublished research.] Iowa State College. 1947.

51. Atkinson, D. E., S. Melvin, and S. W. Fox. Arch. Biochem. and Biophys. 31: 205, 1951.

52. Baker, R. S. Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1951.

53. Martin, G. J. and J. N. Moss. Am. J. Pharm. 121: 169, 1949.

54. Armstrong, M. A. and J. D. Lewis. J. Biol. Chem. 188: 91, 1951.

55. Martin, G. J., R. Brendel, and J. M. Bieler. Exptl. Med. and Surg. 8: 5, 1950.

56. Bergmann, E. S., S. Sieber, and B. E. Volcani. Biochem. J. 54: 1, 1953.

57. Halvorson, H. O. and S. Spiegelman. J. Bacteriol. 64: 207, 1952.

58. Francis, M. D. and T. Winnick. J. Biol. Chem. 202: 273, 1953.

59. Kuiken, K. A., W. H. Norman, C. M. Lyman, F. Hale, and L. Blotter. J. Biol. Chem. 151: 615, 1943.

60. Crane, R. J. A.C.S. Official Reports, Chem. Eng. News. 25: 1364, 1947.

61. Coleman, G. H. and G. E. Honeywell. Org. Syntheses Coll. 2: 89, 1943.

62. Gillespie, H. B. and H. R. Snyder. Org. Syntheses Coll. 2:489, 1943.

63. Toennies, G. and D. L. Gallart. Growth. 13: 7, 1949.

64. Edman, P. Acta Chem. Scand. 4: 277, 1950.

65. Hurst, T. Unpublished M. S. Thesis. Ames, Iowa, Iowa State College Library. 1951.

66. Partridge, S. M. Biochem. J. 44: 521, 1949.

67. Consden, R., A. H. Gordon, and A. J. P. Martin. Biochem. J. 41: 590, 1947.

68. Wieland, H., I. Drishaus, and W. Koschara. Ann. Chem. Justus Liebig. 513: 203, 1934.

69. Fox, S. W. Am. Naturalist. [In Press.]

ACKNOWLEDGEMENT

The author is grateful for the advice and encouragement given by Dr. Sidney W. Fox during the course of this work.