STUDIES ON VITAMIN B6-REQUIRING MUTANTS OF *NEUROSPORA CRASSA*

Thesis by

Bernard S Strauss

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1950

ACKNOWLEDGMENT

This work could not have been done without the aid and guidance of Dr. N. H. Horowitz. I also want to thank Dr. Horowitz for his critical reading of this manuscript; Dr. H. K. Mitchell for much useful advice and constructive criticism; and Dr. William J. Robbins of the New York Botanical Garden for his suggestions on the interpretation of data bearing on nitrate reduction. Finally I want to express my indebtedness to the National Cancer Institute, U.S.P.H.S., for financial assistance and to the staff of the California Institute of Technology for helping make this work possible.

ABSTRACT

1. Mutants of Neurospora crassa requiring vitamin B6 have been investigated.
2. As reported by Stokes et al. (1943) certain of these mutants will grow in the absence of added vitamin B6 if the pH of the medium is above 6 and if ammonium ion is present.
3. The requirement for high pH and ammonium ion has been found to be due to a requirement for free ammonia in the medium. The role of pH is to determine the concentration of free ammonia.
4. Ammonia is specific for the initiation of growth of the pH sensitive mutants in the absence of added vitamin B6.
5. When grown in the presence of sufficient ammonia the pH sensitive mutants are able to synthesize vitamin B6.
6. The net rate of vitamin B6 synthesis is lower in the pH sensitive mutants than in wild type.
7. A glutamic-alanine transaminase has been found in Neurospora. The activity of this enzyme is lower in the mycelium of the pH sensitive mutants grown without added vitamin B6 than in wild type mycelium.
8. Resting mycelium of a pH sensitive mutant will destroy more vitamin B6 in a given period than resting mycelium of a mutant which requires vitamin B6 under all conditions tested.
9. The pH sensitive mutant is inhibited by methionine. This inhibition is overcome by added ammonia, vitamin B6 or sulfanilamide and is competitively overcome by threonine.

10. When either mutant or wild type is grown on a mixture of nitrate and ammonium as a nitrogen source at pH 7 ammonium is assimilated before nitrate.

11. Nitrate can be reduced by pH sensitive mutants grown in the absence of vitamin B6 but the product of reduction cannot be used for growth.

12. It is likely that the lower net rate of B6 synthesis in the pH sensitive mutants is due to a higher rate of vitamin B6 destruction than is shown by wild type.

13. A hypothesis to explain the specificity of ammonia has been developed. According to this idea ammonia in high concentration (relative to wild type requirements) prevents the destruction of vitamin B6.

TABLE OF CONTENTS

I.	Introduction		1
II.	Review of Vitamin B6 Group		2
III.	Genetics		12
IV.	Media		16
V.	Pigment Production		20
VI.	"pH Sensitivity" of the Vitamin B6-Requiring Mutants		20
VII.	Vitamin B6 and its Metabolism in the Vitamin B6-Requiring Mutants of Neurospora		32
	A. Activity of the Forms of Vitamin B6	32	
	B. The Form of Vitamin B6 in the Mutants	36	
	C. Transaminase Activity	47	
	D. Vitamin B6 Balance Studies	50	
VIII.	The Specificity of Ammonia in the Replacement of Vitamin B6		55
IX.	Nitrate Reduction		70
X.	Conclusion and Discussion		78
	A. Statement of the Hypothesis	78	
	B. Interpretation of the Course of Nitrate Reduction in pH Sensitive Mutants	80	
	C. Quantitative Test of the Hypothesis of an Increased Rate of Vitamin B6 Destruction in pH Sensitive Mutants	82	
	D. Qualitative Application of the Hypothesis	90	
	E. The Role of Ammonia	93	
	F. Nature of the Mutation	94	
BIBLIOGRAPHY			96

I. INTRODUCTION

One of the first biochemical mutants reported by Beadle and Tatum (1941) was a strain of Neurospora sitophila requiring pyridoxine. Later Stokes, Foster and Woodward (1943) while attempting to use this strain as an assay organism for vitamin B6, found that it could grow in the absence of the vitamin, pyridoxine, in the presence of ammonium salts at pH values above 6. These investigators also showed that this pyridoxineless mutant was stimulated by thiamine.

Since these investigations certain other vitamin B6-requiring mutants of Neurospora have been isolated. Some of these are similar in their growth requirements to that investigated by Stokes et al., while others apparently have an absolute need for vitamin B6, requiring it for the initiation of growth under all conditions so far tested.

This investigation was begun in an effort to answer the following questions:

1. What is the mechanism of the pH effect?
2. What additional evidence can be gained from these mutants about the relationship between the vitamin B6 group and nitrogen metabolism?
3. Can anything be learned from these mutants about the route of biosynthesis of the vitamin B6 group?

Some information bearing on the first two questions has been obtained. The route of vitamin B6 biosynthesis remains a complete mystery.

Since much of the work to be described depends on an understanding of the peculiarities of the vitamin B6 group, especially the technique of assay, it has been thought desirable to include a review of some work on this group of vitamins.

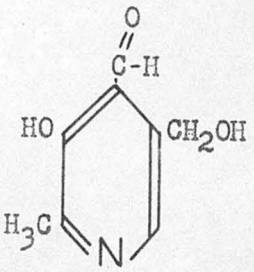
II. REVIEW OF THE VITAMIN B6 GROUP

Vitamin B6 is ubiquitous in its distribution--it has been found in all living organisms so far tested, with some notable exceptions (Holden, Furman and Snell, 1949).

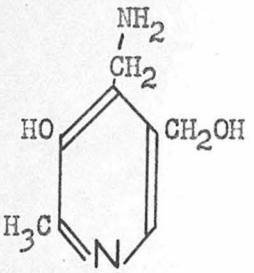
At least five compounds exist with vitamin B6 activity for one organism or another. Pyridoxine (3 hydroxy- 4,5 di (hydroxymethyl) 2 methyl pyridine) has been isolated from rice bran concentrate (Keresztesy and Stevens, 1938; Gyorgy, 1938). The other forms may be considered as derivatives of this compound. They are pyridoxal, pyridoxamine, pyridoxal phosphate and pyridoxamine phosphate (Fig. 1). These compounds are heat, acid and alkali stable but are destroyed by visible light (Hochberg et al., 1944b; Cunningham and Snell, 1945). The position of the phosphate in the phosphorylated forms has not yet been fixed (Umbreit and Gunsalus, 1949). Pyridoxine is the only member of this series so far isolated. The other forms have been characterized by analogy. That is, they have been prepared from pyridoxine chemically and shown to have B6 activity under conditions where pyridoxine itself is inactive. This activity may be initiation of the growth of certain micro-organisms or duplication of the coenzymatic properties of certain

Fig. 1

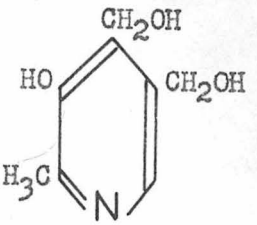
Forms and Derivatives of Vitamin B6



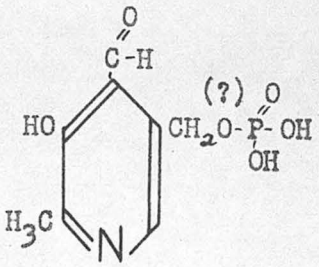
Pyridoxal



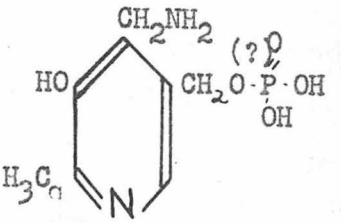
Pyridoxamine



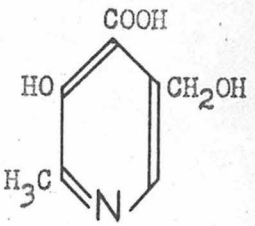
Pyridoxine



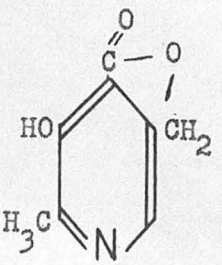
Pyridoxal Phosphate



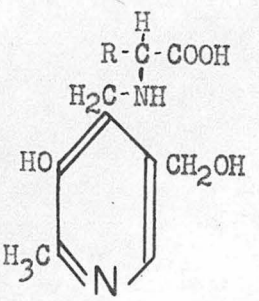
Pyridoxamine Phosphate



Pyridoxic Acid



Pyridoxic Acid Lactone



Pyridoxal-amino acid

extracts. For the nutrition of mammals all of the non-phosphorylated B6 compounds have activity and it is only by the use of microorganisms that separations in the biological activity of the group have been made (Table I).

In the following "vitamin B6" or "B6" will be used to indicate all compounds having the biological activity ascribed to this group. When used without further designation however, vitamin B6 will be considered to refer to the three forms: pyridoxine, pyridoxal and pyridoxamine, structural formulas of which are given above. Pyridoxine will be used only to designate this one form of the B6 group.

A member of the vitamin B6 group, pyridoxal phosphate, serves as the coenzyme for the transaminases (Lichstein, Gunsalus and Umbreit, 1945), for the bacterial decarboxylases (Umbreit, Bellamy and Gunsalus, 1945), for the enzyme forming tryptophan from serine and indole (Umbreit, Wood and Gunsalus, 1946) and for the bacterial enzyme system breaking tryptophan down to ammonia, pyruvic acid and indole (Wood, Gunsalus and Umbreit, 1947). The B6 group is essential for the synthesis of histidine, lysine, threonine, alanine, arginine, phenylalanine, tyrosine, (Lyman, 1947; Stokes and Gunness, 1945; Broquist and Snell, 1949) and for the formation of D - from L -alanine (Holden, Furman and Snell, 1949). The group has been implicated in the synthesis of nicotinic acid from tryptophan (Reid, Lepkovsky, Bonner and Tatum, 1944). Nutritional studies have shown that the B6 group is involved in the metabolism of sulfur amino acids (Cereceda et al., 1948; Speck and Pitt, 1947)

Table I

Requirements by Organisms for Different

Forms of the Vitamin B6 Group

Organism	Growth Response — Form of B6				
	Pyridoxine	Pyridoxal	Pyridoxamine	Pyridoxal phosphate	Pyridoxamine phosphate
Saccharomyces carlsbergensis	+	+	+	0	0
Neurospora crassa	+	+	+		
Streptococcus fecalis R	+*	+	+	-	+
Lactobacillus casei	-	+	-	-	-
Lactobacillus helveticus	-	-	-	+	+

* 1/5000 activity of other forms - approximate

and a connection has been demonstrated between this group and the unsaturated fatty acids (Quackenbush et al., 1942; Sarma et al., 1947).

No good chemical method exists for the determination of the vitamin B6 group, although several have been suggested (Bird, Vandenbelt and Emmet, 1942; Bina, Thomas and Brown, 1944; Hochberg, Melnick and Oser, 1944a; Brown, Bina and Thomas, 1945; Ormsby, Fisher and Schlenk, 1947; Scudi, 1947). These are either non-specific or not sensitive enough. Recourse must therefore be made to biological assay methods and the difficulty in devising a satisfactory assay has led to much of the progress in this field.

In 1942 Snell and collaborators, while attempting to devise an assay for vitamin B6 using lactic acid bacteria, were struck by a series of inconsistencies in their results. Tests of natural products with lactic acid bacteria showed activities corresponding to a greater pyridoxine content than could be demonstrated using rat or yeast bio-assay methods (Snell, Guirard and Williams, 1942). This led these workers to postulate the existence of an active metabolite "pseudopyridoxine", producible from pyridoxine.

"Pseudopyridoxine" has been shown by synthesis to be two substances, pyridoxal and pyridoxamine (Harris, Heyl and Folkers, 1944). Pyridoxine heated with ammonia showed "pseudo-pyridoxine" activity as did pyridoxine treated with a variety of oxidising agents (Snell, 1944).

The separate determination of each of the components of the vitamin B6 group has been worked out by Snell and collaborators.

As shown in Table I, some organisms use efficiently all three forms of the vitamin group, while others use two or only one form of the vitamin. These organisms may then be used to obtain values for the total B6 content and for the concentration of each of the forms. The methods can detect less than a milligamma of B6. Values for pyridoxine itself must always be considered suspect since they are in general low and must be obtained as a result of two difference equations (Rabinowitz and Snell, 1948). Indeed, one of the interesting points to come out of the assays made so far is that the values for pyridoxine itself are low in most of the biological materials tested. Only in rice bran and cereal grains in general is there an appreciable amount of this form (Rabinowitz and Snell, 1948). It is therefore in a sense quite accidental that pyridoxine should have been selected as "vitamin B6"--an accident determined by the material selected for the isolation of B6.

Vitamin B6 does not occur in a "free" state in living organisms. Treatment either by enzymatic digestion or acid hydrolysis has been found necessary for the liberation of nutritionally active extracts. A preliminary extraction procedure is necessary before any assay can be performed (Rabinowitz and Snell, 1947).

It is now believed that most of the bound forms consist of pyridoxal phosphate and pyridoxamine phosphate although some other forms of binding may occur (Snell, 1950). It is a peculiarity of these two compounds that they are more easily hydrolyzed at

pH values of pH 1 - 2 than in more concentrated acid. This peculiarity had led to the postulation of an "acid labile" form of B6 before it was recognized (Melnick, Hochberg, Himes and Oser, 1945). It has also been shown that pyridoxamine phosphate is somewhat more resistant to hydrolysis than is the corresponding pyridoxal phosphate and that pyridoxamine phosphate is active for Streptococcus fecalis R but not for Saccharomyces carlsbergensis.

The primary problem in any assay is one of extraction of vitamin B6 in a form that can be utilized by the assay organism. Recommended procedures call for autoclaving the sample in 0.055 N acid for one hour. Certain materials are resistant and in some cases the extraction must be continued for up to 5 hours. Satisfactory methods of extraction for different materials can only be determined by individual experimentation. Assays must be performed in dim light.

As yet no satisfactory method exists for the determination of the five forms of vitamin B6 in the presence of one another. Rabinowitz (personal communication, 1949) has suggested a possible scheme. Alkaline hydrolysis liberates unphosphorylated forms of B6 (Snell, 1945a) and does not cleave the phosphates. Vitamin B6 bound as phosphate can be liberated by acid hydrolysis. The difference between these two values determined with Lactobacillus casei would represent pyridoxal phosphate (Rabinowitz, Mondy and Snell, 1948). The determination of pyridoxamine phosphate has not been worked out but might be accomplished by destroying pyridoxal (and pyridoxal phosphate?) in a water or alkaline extract with

alkali and acetone and using Lactobacillus helveticus as an assay organism (McNutt and Snell, 1950). This method has not been studied experimentally.

Certain forms of lactic acid bacteria that ordinarily require vitamin B6 can be grown without the vitamin in a complex medium (Bellamy and Gunsalus, 1945). This medium contains a variety of amino acids and streptogenin. D -Alanine must be included. It was first suspected that D -alanine might be a precursor of vitamin B6, but it has now been shown that the vitamin is involved in the conversion of L -alanine to D -alanine. It is interesting that detectable amounts of B6 cannot be extracted from cells grown in this type of medium (Holden, Furman, and Snell, 1949). It is not possible to state that B6 is absent, but it is certainly present in quantities far below the capacity of assay methods to detect with any accuracy.

The amino acid decarboxylases which are known to require pyridoxal phosphate as a coenzyme cannot be demonstrated in organisms grown on the above medium (Lichstein, Gunsalus and Umbreit, 1945). Extracts of these cells can be activated by addition of pyridoxal phosphate with resumption of enzymatic activity. This evidence suggests that the following hypothesis may not be valid in all cases: If an organism requiring a growth factor grows in a medium not containing the factor, then it is synthesizing the factor.

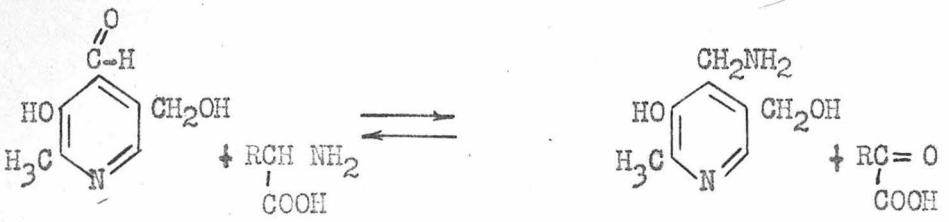
Gale has studied the effect on enzymatic activity of decreasing the quantity of vitamin B6 in the medium in which cells

are grown (Gale, 1948). He finds that as the concentration of vitamin B6 is lowered, there is first no change, then the decarboxylase activity disappears but it is not till lower values are reached that transaminase activity disappears.

Experiments involving vitamin B6 in the synthesis of the variety of amino acids listed above have in the main been carried out by making nutritional studies of a variety of lactic acid bacteria. The method is in general to supply a complex medium, each component of which is required. Vitamin B6 is added and single amino acids withdrawn. Or conversely B6 may be withdrawn from a medium and a mixture of substances added to counteract the withdrawal (Stokes and Gunnes, 1945; Lyman et al., 1947). Experiments of this type are sufficient to define a relationship but are not sufficient to define the mechanism of action.

A similar experimental technique is available with mammals. Animals are placed on a diet lacking vitamin B6 and amino acids are added. Increased toxicity of an amino acid over control animals has been assumed to show a relationship between the amino acid and B6. A connection between B6 and methionine has been established in this way (Cerecedo, Foy and De Renzo, 1948). Or, abnormal metabolites may be isolated from the urine of B6 deficient animals fed an amino acid. B6 has been shown in this way to be involved in tryptophan metabolism since xanthurenic acid is excreted following tryptophan administration. Furthermore, B6 deficient rats cannot metabolise this compound while normal animals are able to convert it to other substances (Reid et al., 1944). More directly, B6 may be shown to cause the excretion of nicotinic acid in the urine (Schweigert and Pearson, 1947).

Although it is unnecessary to review the enzyme work, some mention of the mechanism of coenzyme action in one case may be helpful. Snell (1945b) had postulated that the B6 group would serve as transaminase coenzymes on the basis of his finding that amino acids and pyridoxal reacted in vitro to give pyridoxamine and keto acids:



Later work appeared to bear out this suggestion (Schlenk and Snell, 1945; Green et al., 1945; Umbreit, O'Kane and Gunsalus, 1946) and there is no doubt that pyridoxal phosphate does serve as a cotransaminase. More recent work has indicated that Snell's mechanism may not be correct (Umbreit, O'Kane and Gunsalus, 1948). Pure transaminase preparations can be obtained from pig-heart that are not activated by pyridoxamine phosphate, although pyridoxal phosphate is active. A similar situation exists for the decarboxylases (Umbreit, O'Kane and Gunsalus, 1946). What has apparently happened in both these cases is that in the course of purification, contaminating enzymes that can transform the amine to the aldehyde form of B6 have been removed. According to Snell's idea both forms of B6 should have served as cotransaminases.

Pyridoxal phosphate is the only member of the B6 group for which a definite coenzyme function is known. Yet pyridoxamine seems to be quantitatively the most common form of the B6 group

(Rabinowitz and Snell, 1948). Furthermore, cases exist in which pyridoxamine phosphate is more active nutritionally than is the pyridoxal compound. Evidence of this sort indicates that the coenzymatic functions of the B6 group are far from completely elucidated.

III. GENETICS

Mutant strains used in these studies are indicated in Table II. Genetic investigations reported by Houlahan, Beadle and Calhoun (1949) have indicated that the three strains worked with are single gene mutants fairly close together in the same linkage group (Table III). Tests of 44602 and 37803, and 44204 and 37803 have suggested that the pairs mentioned are not alleles.

Crosses were made in test tubes on Westergaards medium (Westergaard and Mitchell, 1947) containing pyridoxine. The cultures were allowed to age until perithecia had matured and the tube was covered with a black coat of ascospores. At this time a suspension was made of a portion of the ascospore layer and plated on an agar minimal petri plate. Washed purified agar was used, the minimal was Fries salt solution plus sucrose and biotin (Beadle and Tatum, 1945). The plates were activated for half an hour at 60° C and then incubated at 25° C. At 24 hours they were observed under a dissecting microscope. Wild type spores treated in this way show a characteristic long germination tube after 24 hours while mutant spores, in the absence of added growth substance

Table II

Vitamin B6-Requiring Mutants of

Neurospora Worked With

Strain No.	Species	Treatment by which obtained	Parents	pH sensitivity
299 (used by Stokes)	sitophila	x-rays	SA x sa	+
37803	crassa	ultra violet	1A x 25a	-
44204	crassa	ultra violet	4A x 25a	+
44602	crassa	ultra violet	4A x 25a	+

Table III

Linkage Data for Vitamin B6 Requiring

Mutants of Neurospora crassa

(from Houlahan, Beadle and Calhoun, 1949)

Strain No.	Linkage Group	Number of Crosses	Number of Asci	Centromere Distance
37803	D	10	262	10.3
44204	D	4	80	10.5
44602	D	4	70	11.4

Table IV

Test of 37803, 44204 and 44602 to

Determine Possible Non Allelism

Cross	Number of Spores Counted	Number Germinating	Number Appearing as Wild Type	Plate Covered after 3 Days
44602a x 44602A	146	33	1	(-)
37803a x 37803A	26	0	0	(-)
37803a x 44602A	55	43	2	+
37803a x 44204A			too overgrown to count	+

show an abortive tube. Adopting a long germ tube after 24 hours incubation as a criterion of wild type growth, an estimate was made of the number of wild types produced in the crosses made. The plate was then reincubated without having been opened and observed at three days.

Three tests were made using the following crosses: 44602A x 37803a, 44204A x 37803a, 44602A x 44602a, 37803A x 37803a. In all of the tests crosses of the pH sensitive by pH non-sensitive strains gave growth sufficient to cover the plate after three days. No growth was obtained in the control crosses of 37803 x 37803 or 44602 x 44602. In one test the wild type appearing spores were counted after 24 hours as described above (Table IV). Control crosses of 44602 x 44602 showed 33 spores germinating out of 146 counted with one spore showing a long germination tube appearing after 24 hours. However no growth visible by direct observation could be seen after 3 days on this plate. In none of the above experiments were the presumed wild types subcultured on minimal medium.

Heterocaryon formation has been observed between 44204A and 37803A but not between 44602A and 37803A or 44204A and 44602A. Slants of minimal agar were inoculated with the pairs indicated and compared after three days' incubation at 25° C with control tubes inoculated with one strain alone and with controls inoculated onto medium containing vitamin B6. Good growth was obtained only in those tubes containing vitamin B6 or inoculated with both 44204A and 37803A. The supposed heterocaryon has been successfully transferred to minimal medium.

The experiments suggest that the pH sensitive and non-sensitive strains are not alleles. With the techniques that have been used, it is impossible to rule out the possibility that back mutation in one of the ascospores or in one of the conidia used for the heterocaryon tests is the explanation for the growth obtained. The only method of rigorously showing that the strains 44602 and 37803 are not alleles would be the standard method of obtaining asci from crosses and after dissecting the asci in order, testing cultures derived from each of the ascospores for their vitamin B6 requirement. If it could be shown that 44204 and 37803 form a true heterocaryon this would be satisfactory evidence that these two strains are not alleles. One method of demonstrating true heterocaryon formation would be the isolation of hyphal tips from a culture of the heterocaryon. If a true heterocaryon is formed the tips will give rise to three types of cultures, heterocaryotic, mutant type A and mutant type B in a ratio depending on the relative frequency of the different types of nuclei in the heterocaryon and on the number of nuclei per tip.

The mutant strains used for these studies are different from 299 of Neurospora sitophila used by Stokes et al. (1943) but the gross nutritional characteristics of all "pH sensitive" vitamin B6-requiring mutants appear to be similar (Table V).

IV. Media

Several modifications of standard Fries minimal medium have been used (Table VI). For control of pH values at about pH 7 a M/15

Table V

Nitrogen Sources Serving for the Initiation

of Growth of pH Sensitive Mutants*

Compound	pH 5.6		pH 7.0	
	0 B6	+ B6	0 B6	+ B6
NH_4^+ salts	0	+	+	+
Nitrates	0	+	0	+
Amino Acids	0	+	0	+
Others: i.e. urea, amines	0	+	0	+

*(Adapted from Stokes et al)

Table VI

Minimal Media Used

I. M/15 Phosphate

KH_2PO_4 - 9.1 gm
$MgSO_4$ - 0.5 gm
$CaCl_2$ - 0.1 gm
NaCl - 0.1 gm

Trace Elements

B (as a salt)		.01 mg
Cu	"	0.10 mg
Fe	"	0.20 mg
Mn	"	0.02 mg
Mo	"	0.02 mg
Zn	"	2.00 mg

Sucrose - 20 gm
Biotin - 5 gammas

Water - 1 liter

N source as desired
Adjust with KOH to
desired pH

II. Potassium Tartrate

$K_2C_4H_4O_6 \cdot \frac{1}{2}H_2O$ - 6.38 gm
KH_2PO_4 - 1.0 gm
$MgSO_4$ - 0.5 gm
$CaCl_2$ - 0.1 gm
NaCl - 0.1 gm

Trace Elements - as in I

Biotin - 5 gammas
Sucrose - 20 gm

Water - 1 liter

N source as desired
pH $\cong$ 5.6

III. Basal Medium for Yeast bioassay (after Johnson)

Per 500 ml double strength medium

Sucrose	- 50 gm
KH_2PO_4	- 550 mg
KCl	- 425 mg
$CaCl_2 \cdot 2H_2O$	- 125 mg
$MgSO_4$	- 125 mg
$FeCl_3$	- 2.5 mg
$MnSO_4$	- 2.5 mg

Citric Acid	- 1.0 gm
Potassium Citrate ($K_3C_6H_5O_7 \cdot H_2O$)	- 5.0 gm
Casein - 10% Acid Hydrolyzed	- 40 ml
Thiamin	- 250 gammas
Inositol	- 25 mg
Biotin	- 10 gammas
Ca pantothenate	- 2.5 mg
Nicotinic Acid	- 2.5 mg

phosphate medium has been used (TableVI). (Potassium salts have been used throughout these studies.) For studies at the pH of Fries minimal, potassium tartrate has been substituted for ammonium tartrate. Ammonium and nitrate nitrogen have in general been supplied in separate compounds.

A precipitate is formed on autoclaving M/15 phosphate medium at pH values around 7. As growth proceeds this gradually disappears. It is probable that this is a mixture of calcium ammonium, and calcium magnesium phosphates which would be formed and precipitated at these pH values. Removal of the precipitate allows growth to proceed but the dry weight produced is diminished.

Growth studies have been made by measuring the dry weight of mycelium produced in 20 ml of medium. 125 ml Erlenmeyer flasks are used and are incubated in a darkened room at a constant temperature of 25° C. Weights from duplicate cultures have been averaged in all the data. A standard growth period of 72 hours has been used except where otherwise noted.

At the beginning of these studies experiments were performed in which the nitrogenous components of the medium were autoclaved separately and added after cooling to the rest of the medium. No significant difference in response to vitamin B6 or to nitrogen source was obtained by this procedure. In a similar manner sugar was autoclaved separately with no significant differences noted.

V. PIGMENT PRODUCTION

All the vitamin B6 mutants worked with excrete a yellow pigment to the medium when conditions for growth are less than optimal. This pigment is produced when these strains are grown on less than half maximal B6 and also on limiting nitrogen. A growth period of four days may be required to permit observation of the color. It has been possible to extract this pigment at pH 3 into butanol and to return it to the water phase by extraction of the butanol layer with pH 7 phosphate buffer. The absorption spectrum has been determined with the Beckman spectrophotometer. A steady decrease in absorption throughout the range of 250 to 350 millimicrons is obtained when the spectrum is determined in acid or neutral solution. In 0.1 N NaOH a broad absorption band is noted with a peak at about 330 mμ . No other characterization of the pigment has been made.

VI. "pH sensitivity" of the Vitamin B6-Requiring Mutants

Although Stokes et al. (1943) had found that the pyridoxinless mutant of N. sitophila could grow without added vitamin B6 at higher pH values, this growth could only be initiated on a medium containing ammonium salts as a nitrogen source. In preliminary growth experiments with 44602 of N. crassa I noticed that the growth obtained in a three day period was a function of

the amount of ammonium ion after a threshold value had been reached (Fig. 2) and was also a function of the hydrogen ion concentration (Fig. 3).

Growth of 44602 in the absence of added vitamin B6 is therefore a function of both ammonium and hydrogen ion concentration. Together both of these quantities uniquely determine the concentration of free ammonia in a solution, since:

$$NH_3 + H_2O \rightleftharpoons NH_4OH \rightleftharpoons NH_4^+ + OH^-$$

can be considered

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

and

$$[NH_3] = K \frac{[NH_4^+][OH^-]}{[H_2O]}$$

or, for a water solution since

$$[H_2O] \cong K'$$

and

$$[OH^-] = K_w/[H^+]$$

therefore

$$[NH_3] = \frac{KK_w}{K'} \frac{[NH_4^+]}{[H^+]} = K'' \frac{[NH_4^+]}{[H^+]}$$

It was therefore decided to determine whether ammonia might not be the determinant for mutant growth without added vitamin B6.

To test the hypothesis media were set up at various pH values with varying amounts of ammonium nitrogen. Uninoculated flasks of media were analyzed for ammonia and the pH values taken

Fig. 2

Growth of 44602 as a Function of the

Concentration of $(NH_4)_2SO_4$ N

Growth in dry wt after 72 hours at 25° C in M/15 phosphate minimal pH 7.0

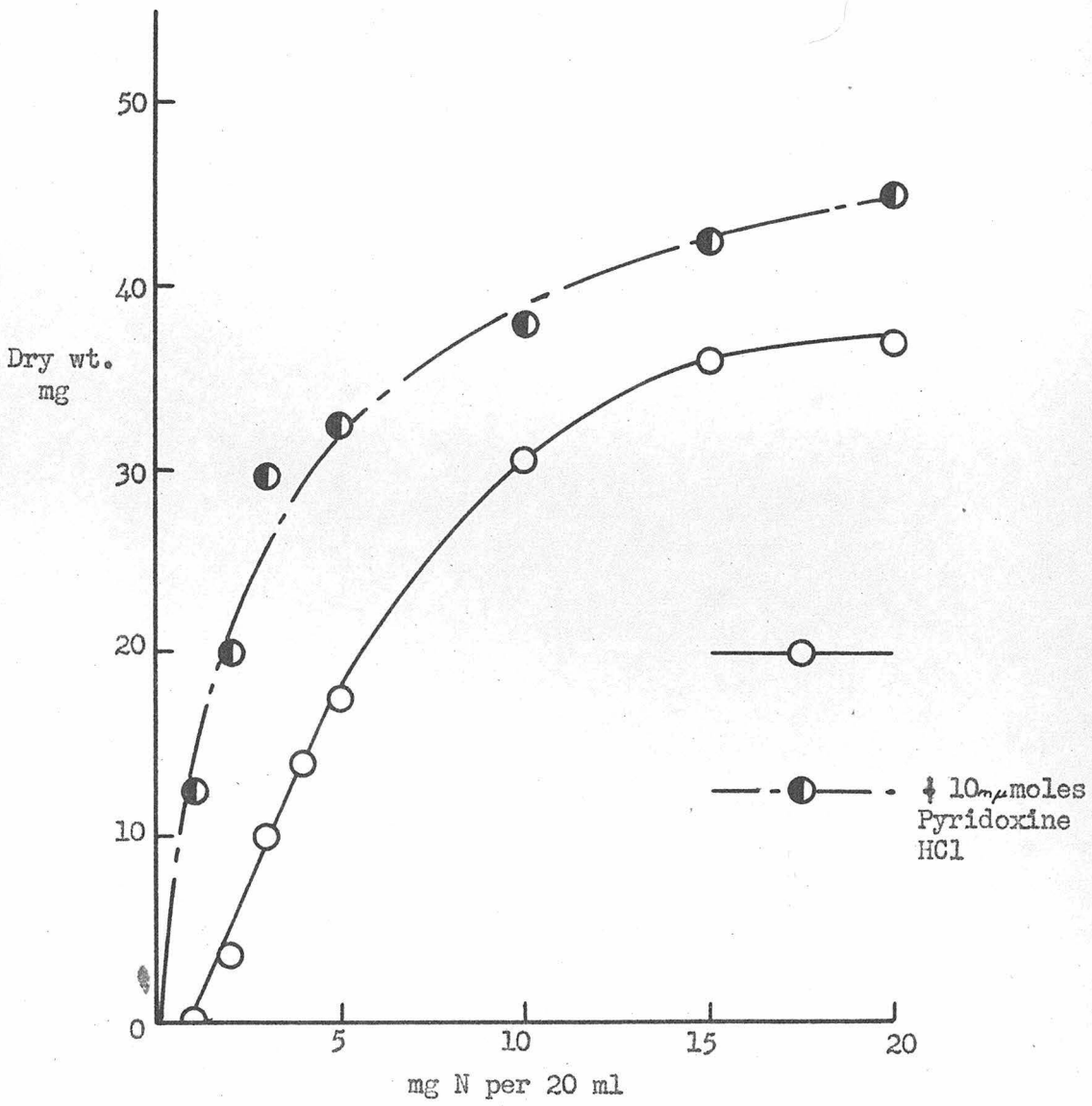


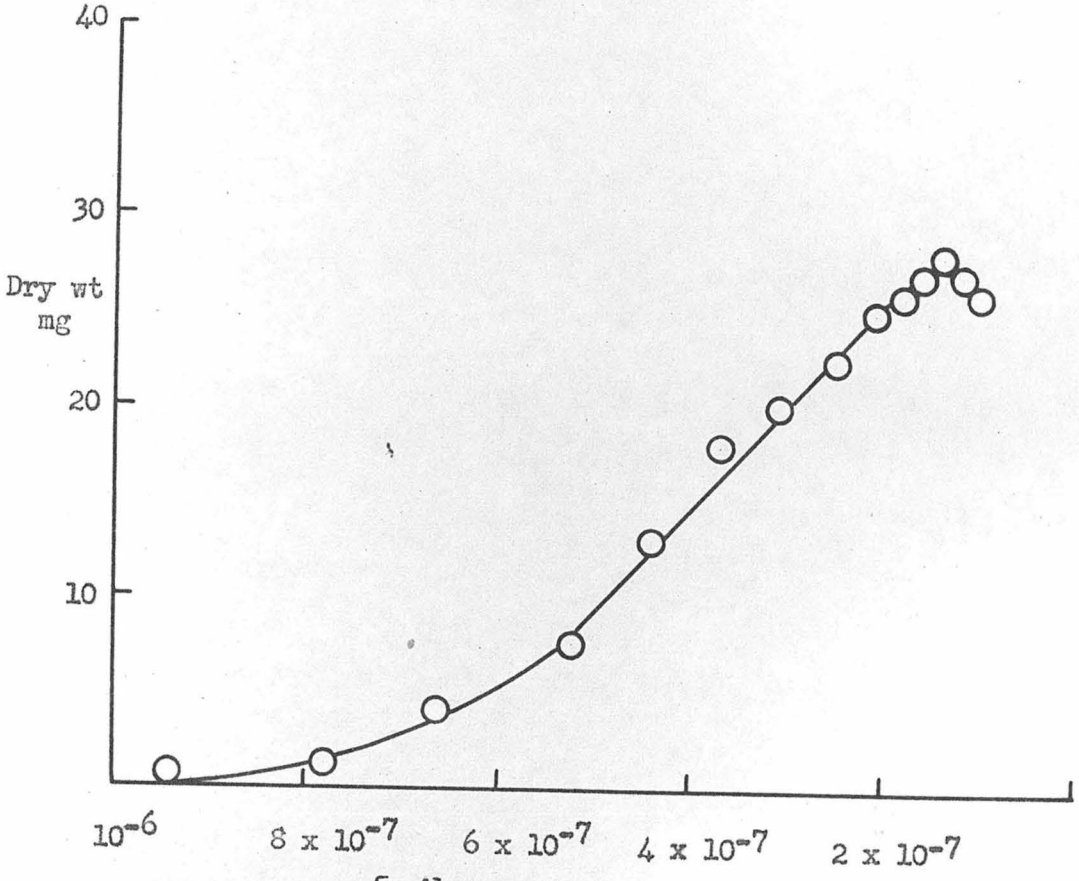
Fig. 3

Growth of 44602 as a Function of

$[H^+]$ of the Medium

Medium: M/15 Phosphate

N source: 1 gm $(NH_4)NO_3$ per liter + 5 gm Ammonium Tartrate



$$\frac{[H^+]_f + [H^+]_i}{2}$$

$[H^+]_f$ = final hydrogen ion concentration

$[H^+]_i$ = initial hydrogen ion concentration

with the Beckman glass electrode. The precaution of analysis for the determination of ammonium nitrogen is necessary since autoclaving at pH values near 7 causes a loss of ammonia from the medium (Table VII). The pH of the medium after autoclaving was taken for the calculation of $[H^+]$. The constant used for calculation of ammonia concentration changes but little with ionic strength (Riemann, Neuss and Naimann, 1942) and this factor was therefore neglected.

High ammonium concentration permits growth of 44602 to proceed at pH values below those obtainable with the ordinary concentration of nitrogen present in minimal medium. It has been concluded as a result of the correlation between growth and ammonia concentration (Fig. 4) that the requirement for growth without added vitamin B6 is a requirement for a relatively high amount of free ammonia in the medium.

The high concentration of phosphate does not affect these results. Similar pH-Growth curves are obtained on a variety of buffers (i.e., succinate, phthalate, malate). Phosphate has been used because it is the most efficient in the range of interest.

Wild-type growth appears to be independent of the ammonia concentration (Fig. 5a) and is a function only of the total nitrogen (Fig. 5b). Calculated either on the basis of total nitrogen or of ammonia, the nitrogen requirement for the initiation of growth appears to be at least eight times greater for the mutant, 44602, than for wild type 4A or for the mutant in the presence of added vitamin B6 (Fig. 2). A threshold concentration

Table VII

Effect of Autoclaving and Shaking on

Nitrogen Composition of Medium

Medium = M/15 Potassium Phosphate
M/15 Potassium Tartrate
2 % Sucrose
Biotin and trace elements as in minimal

20 ml per flask + $(NH_4)_2SO_4$ N as shown

pH before autoclaving	N before autoclaving	N after autoclaving	N after autoclaving and shaking 4 hrs. at 25° C
4.60	2.46	2.53	2.53
5.98	2.46	2.47	2.48
6.52	2.36	2.35	2.33
7.00	2.36	2.19	2.17

Values averages of 2

N as mg/ml x 20 ml

Autoclaving 15 minutes at 15 p.s.i.

Fig. 4

Growth of 44602 as a Function of

Calculated Initial $[NH_3]$

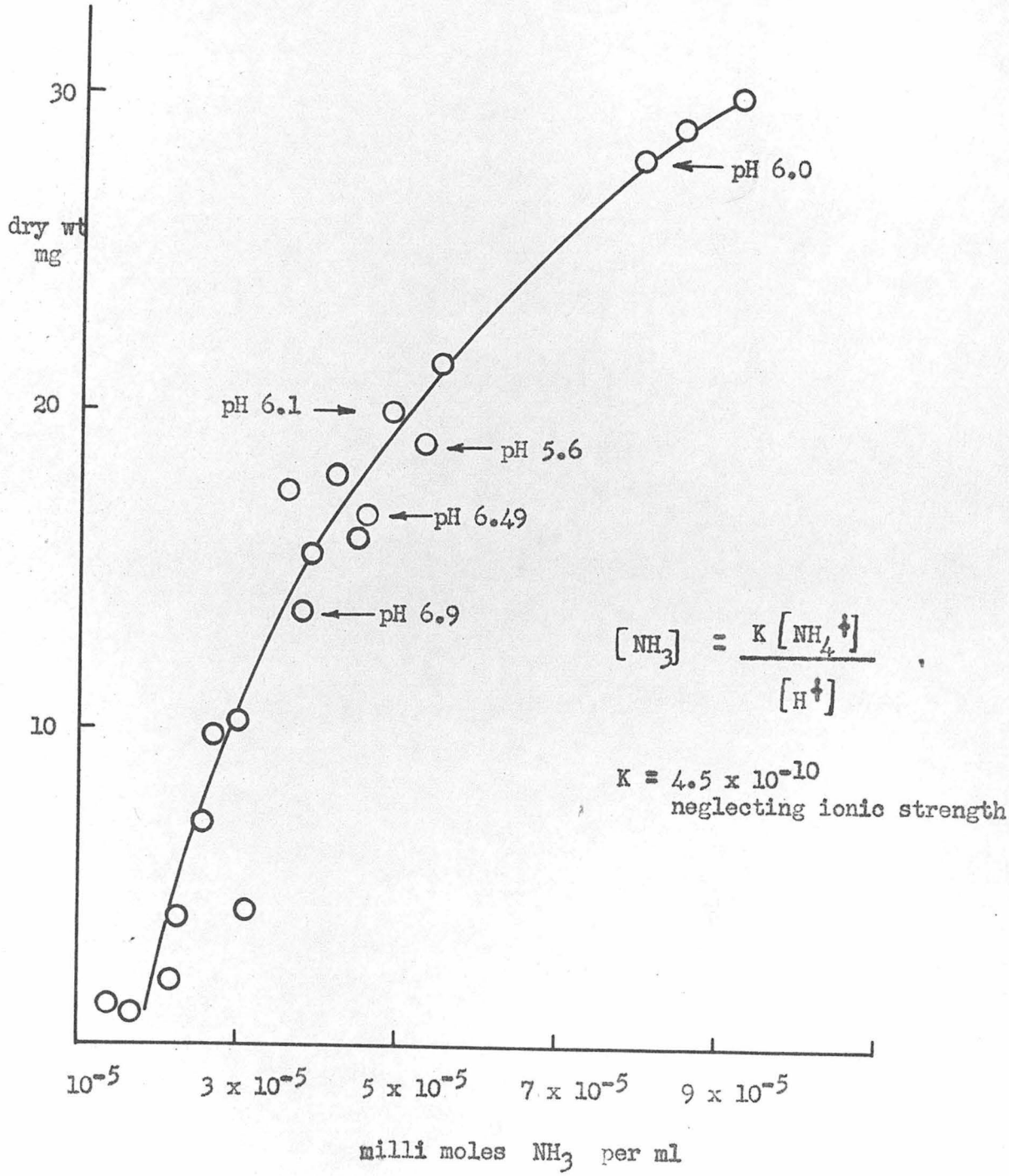


Fig. 5a Non relation of Wild Type 4A Growth to the Concentration of NH_3

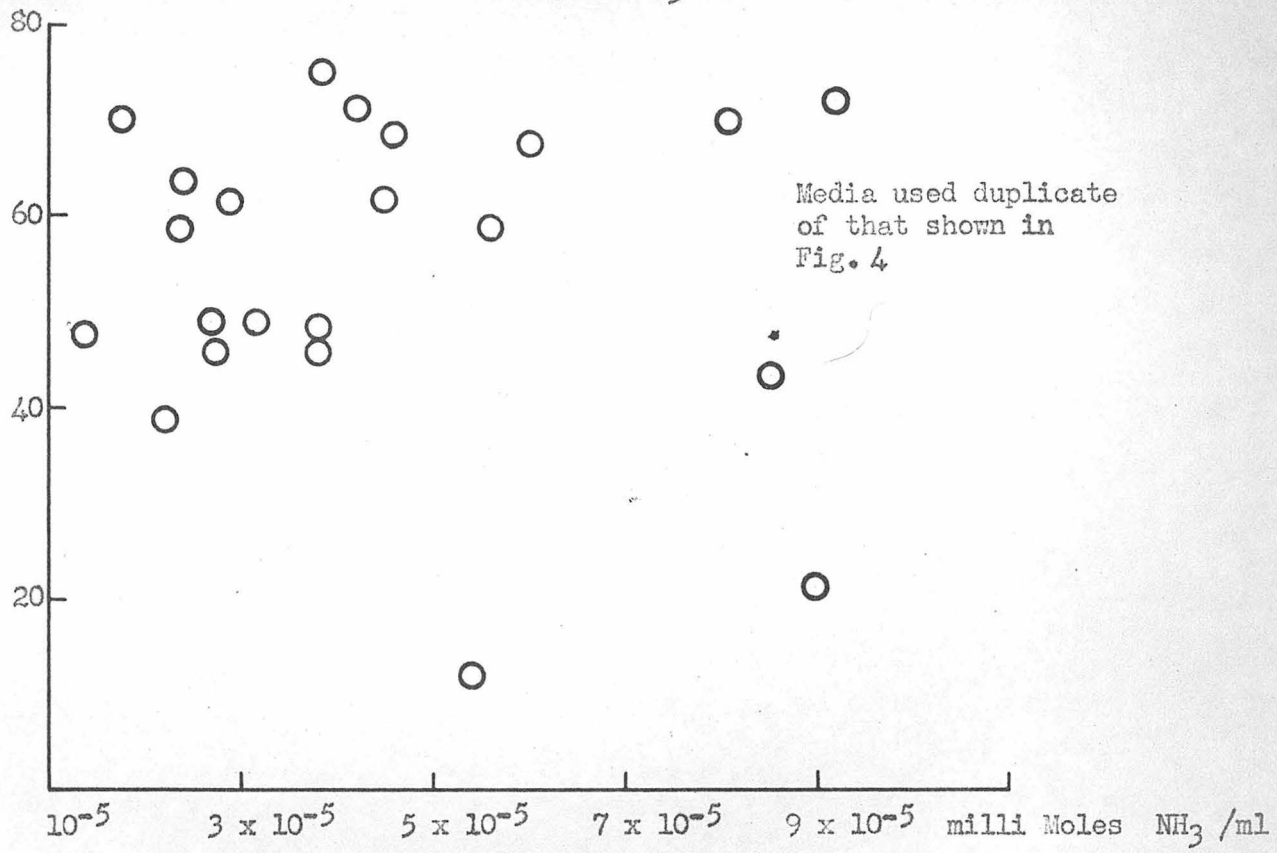
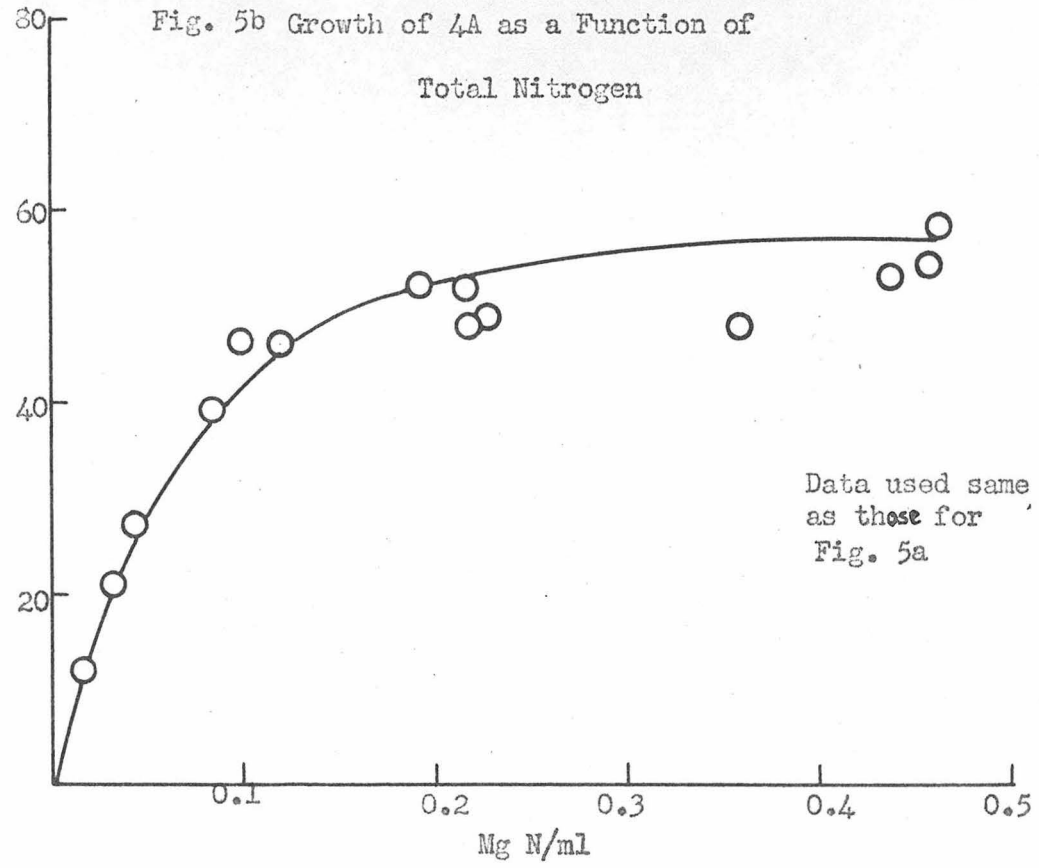


Fig. 5b Growth of 4A as a Function of Total Nitrogen



of ammonium appears to be necessary for 44602 and this threshold is absent and the absolute requirement is diminished when vitamin B6 is added.

Although this conclusion has been tested quantitatively only with 44602 there is little doubt that it applies equally to 44204 or to Stokes 299. In these cases also, there is a requirement concomitantly for high pH and ammonium ion in order for growth to proceed without added vitamin B6.

The rate of growth of the mutant in the absence of added vitamin B6 is closely dependent on the concentration of ammonia in the medium. In the presence of vitamin B6 the mutant shows the same rate dependence on ammonia as does wild type. (Fig. 6). This experiment provides confirmatory evidence that nitrogen is limiting for mutant growth under conditions where wild type is not limited.

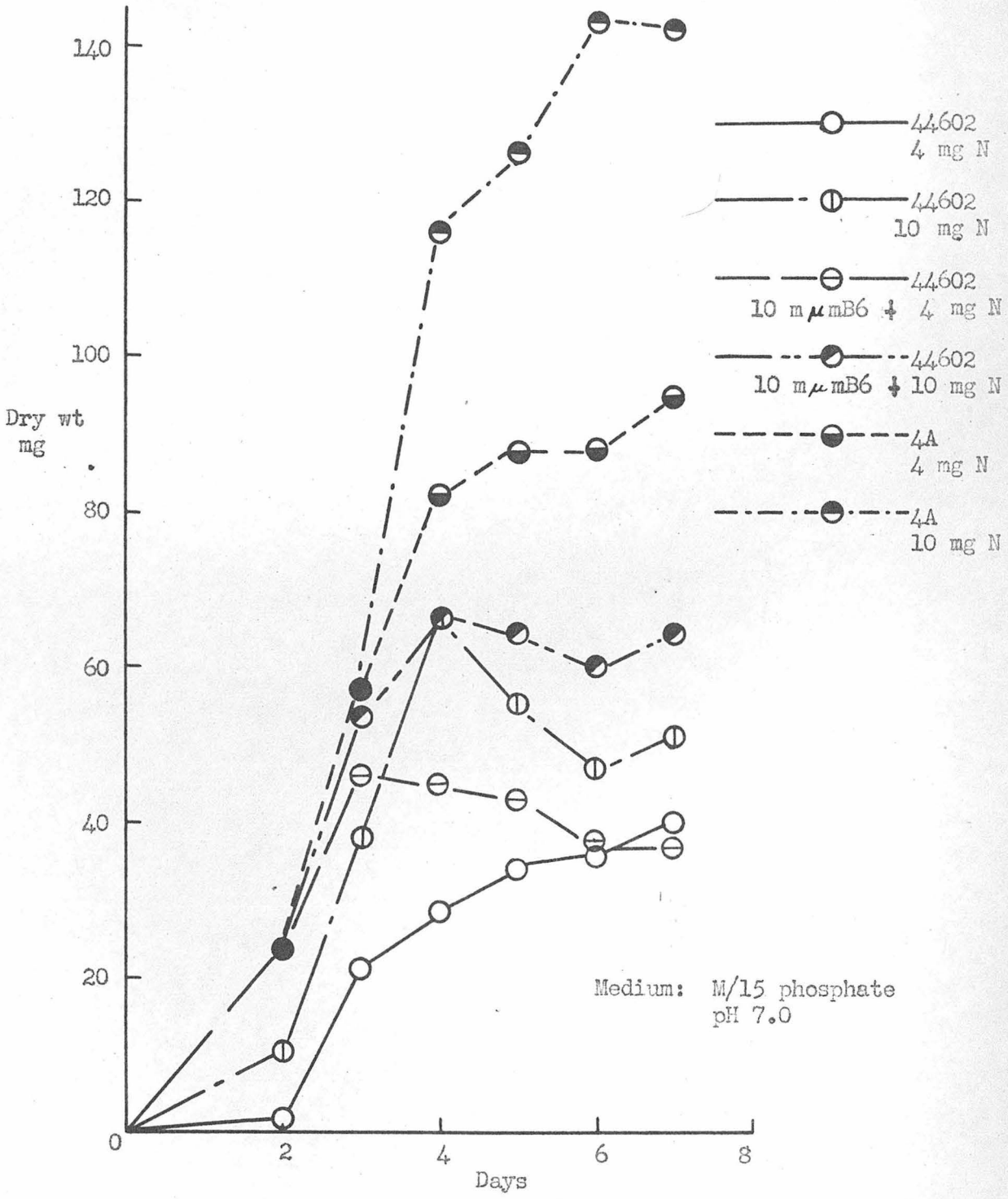
The total crop of mutant mycelium obtainable in the absence of added vitamin B6 approaches that of the mutant in the presence of vitamin B6. However it has not been possible to make the total crop of mutant mycelium approach that of wild type 4A grown with the same medium.

Action by weakly ionized substances has been demonstrated before. A set of phenomena analogous to the one described here was investigated by van Dam (1918). This investigator, while studying the effect of hydrogen ion and lactate ion as factors in the killing of lactic acid bacteria came to the conclusion that these factors were related and that the effective agent was

Fig. 6

Increase in Dry Weight with Time

as Affected by NH_4^+ Concentration



undissociated lactic acid. The presumption is that the uncharged molecule can permeate the cell more readily.

Evidence bearing on this point has been obtained by Clark and Shive (1934). These workers were able to show that tomato roots are able to absorb ammonia more readily at high pH values.

Similar observations have been made with Neurospora. Both 4A and 44602 were grown on M/15 phosphate medium at pH 7 for three days at 25° C. Medium for the growth of 4A contained one mg of ammonium sulfate nitrogen per 20 ml, that for 44602 contained 6 mg of nitrogen. At the end of this growth period the mycelium was removed and placed in a phosphate-tartrate-sugar medium overnight in order to exhaust the supply of free nitrogenous compounds in the mycelium. Standard sterile technique was employed throughout this and all similar experiments. The mycelium was then transferred to a medium containing phosphate, sucrose, tartrate, biotin and trace elements with ammonium sulfate as shown (Tables VII, VIII). The flasks were shaken for four hours and the amount of ammonia remaining in the medium determined.

More ammonia disappears at high pH values and in all cases this amount is greatly increased by pyridoxine. This latter effect may not be specific since the addition of thiamine results in a significant rise in absorption. The thiamin effect may be related to its known ability to stimulate the growth of the mutant. Even though the specificity of the B6 effect is not proven it is interesting to note that this vitamin group has been implicated

Table VIII

Uptake of Ammonia by Neurospora

Strain	Addition	pH	dry wt.	$N_{original}$	N_{final}	$\frac{N_{orig} - N_{final}}{\text{dry wt.}} \times 10^2$
44602	None	4.62	24.6	2.50	2.28	0.895
		5.80	24.0	2.46	2.20	1.08
		6.68	23.4	2.31	2.02	1.26
44602	10 mμ moles Pyridoxine HCl	4.62	24.0	2.50	2.23	1.12
		5.80	23.0	2.46	2.15	1.35
		6.68	22.8	2.31	1.97	1.49
44602	10 mμ moles Thiamin HCl	4.62	25.2	2.50	2.24	1.03
		5.80	20.6	2.46	2.20	1.26
		6.68	19.6	2.31	2.05	1.32
4A	none	4.62	26.8	2.50	2.27	0.86
		5.80	20.0	2.46	2.23	1.15
		6.68	19.2	2.31	2.01	1.56

Dry weight in mg
N in mg/ml x 20 ml
Medium as in Table VII

in processes of amino acid synthesis. If such a process occurs here it would be one point at which B6 and ammonia cooperate in a reaction sequence.

High pH undoubtedly has many non-specific effects on Neurospora. An increase in the formation of conidia is generally noted at the higher pH values. At pH values of about 7 nitrate becomes a poor nitrogen source. Certain strains of wild type show decreased growth at the higher pH values and the amount of B6 excreted to the medium by wild type 4A goes down. Different strains may differ in their tolerance to higher pH values. Notwithstanding the general effects it is felt that the important role of pH for these "pH sensitive" mutants lies solely in the determination of the free ammonia concentration.

VII. Vitamin B6 and its Metabolism in the Vitamin B6-requiring Mutants of Neurospora

A. Activity of the Forms of Vitamin B6

Each of the vitamin B6-requiring strains worked with responds about equally well to the three common forms of the vitamin B6 group, pyridoxal, pyridoxamine and pyridoxine. The behavior is similar to that of No. 299 of N. sitophila (Snell and Rannefeld, 1945). Growth experiments were performed on potassium tartrate minimal using potassium nitrate or ammonium sulfate as nitrogen sources. Nitrogen was added to give a concentration of 1 mg per ml. The B6 compound tested was filter

sterilized and added to the rest of the cooled, previously autoclaved medium in dim light.

The three forms have practically identical activity when ammonium is a nitrogen source. Pyridoxine is slightly better for the production of dry weight on nitrate nitrogen but the differences are not great (Fig. 7).

Other derivatives of the vitamin B6 group have been tested for ability to initiate growth. Several pyridoxal-amino acid compounds* (Fig. 1) have been tested according to the method of Snell and Rabinowitz (1948) with results essentially similar to theirs. The compounds have at best 1 % of the activity of pyridoxine on either nitrate or ammonium minimal. Pyridoxic acid lactone (Fig. 1) prepared by the oxidation of pyridoxine as described by Huff and Perlzweig (1944) gave a similar low activity of about 2 % that of pyridoxine.

Stokes et al. (1943) had reported that thiamine was stimulatory to 299 of N. sitophila. A similar effect has been noted in both pH sensitive and non-sensitive mutants. Under certain conditions one mole of thiamine is more effective in producing a growth response than an additional mole of vitamin B6 (Fig. 8). Mungate (1946) has reported that 25 gammas of thiamin hydrochloride increase the crop of mycelium obtained from a wild type culture by about 10 %. The thiamin effect on the mutants is shown by a fraction of a gamma of thiamin and under these conditions wild types 1A, 4A and 25a are not stimulated. These mutants

---

*Merck synthetic obtained from Dr. Karl Folkers

Fig. 7

Growth of 44602 with Different Forms

of Vitamin B6

Medium: Potassium tartrate minimal. KNO_3 as N source. pH 5.6

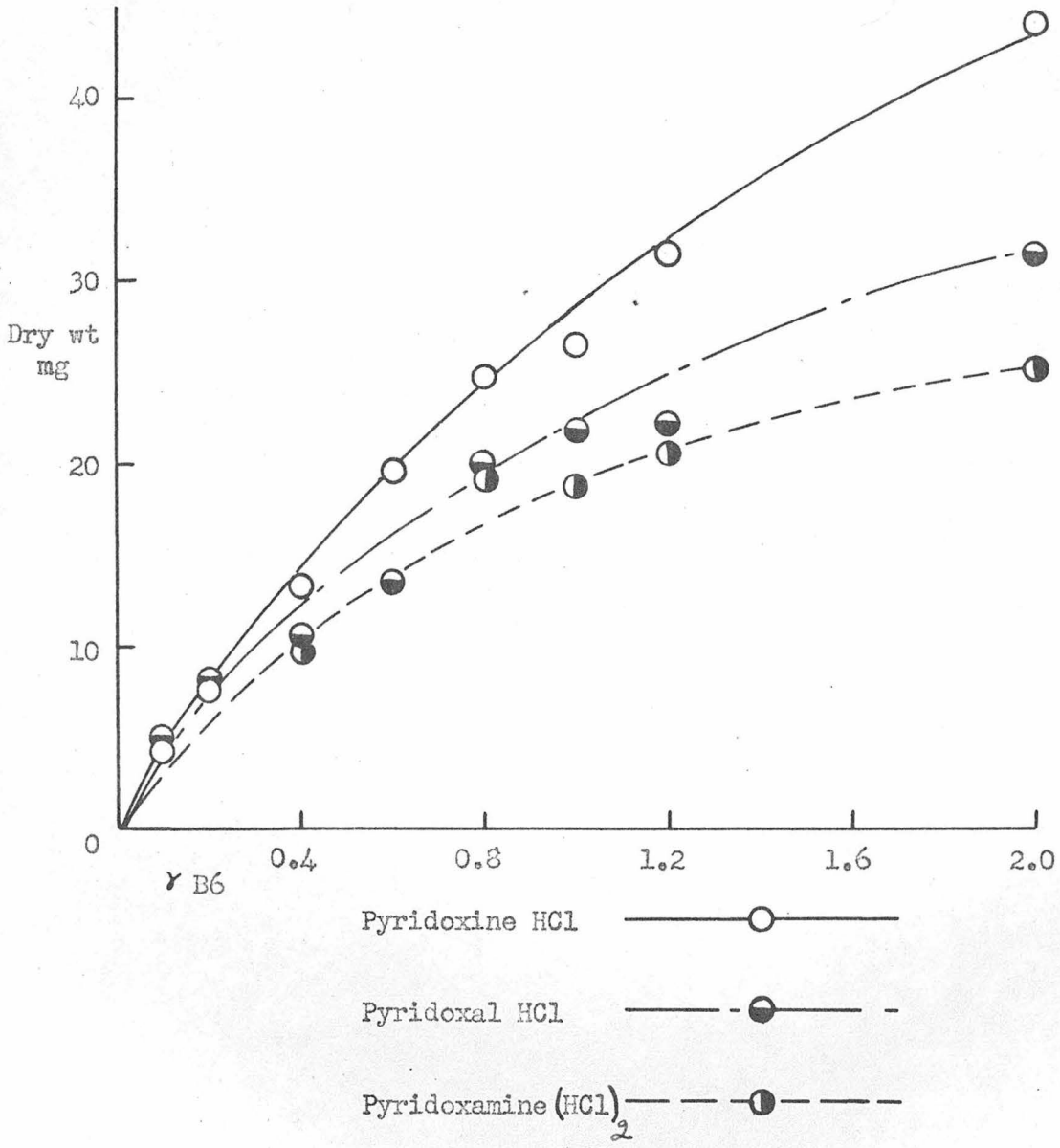
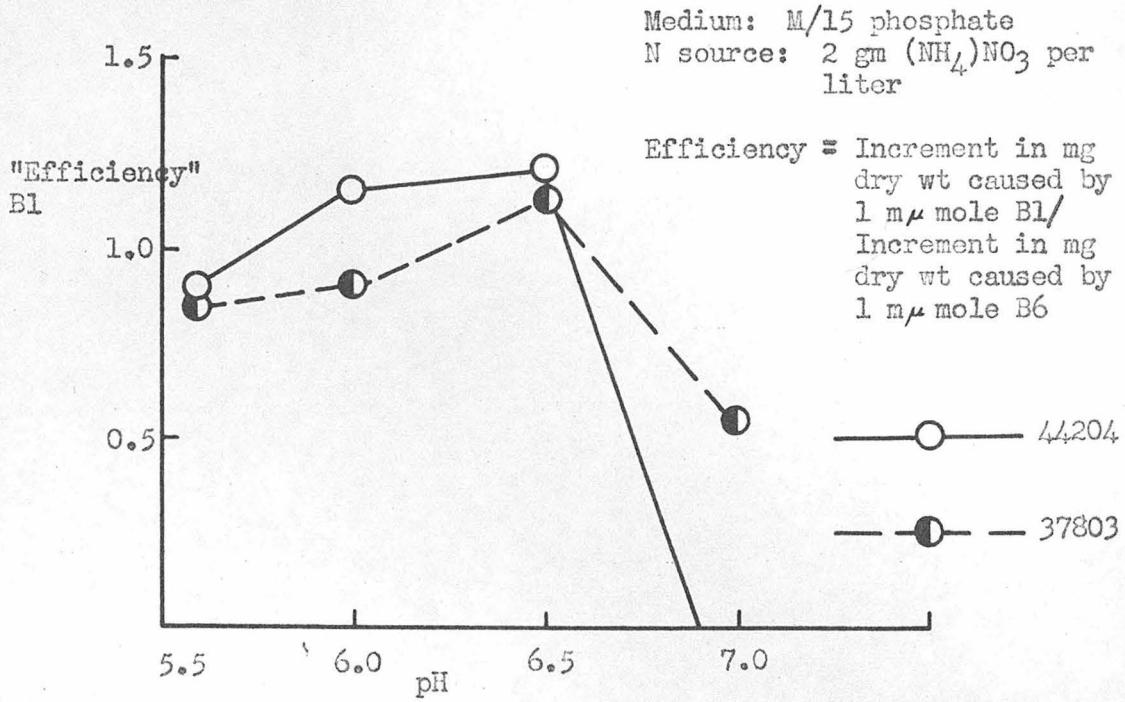
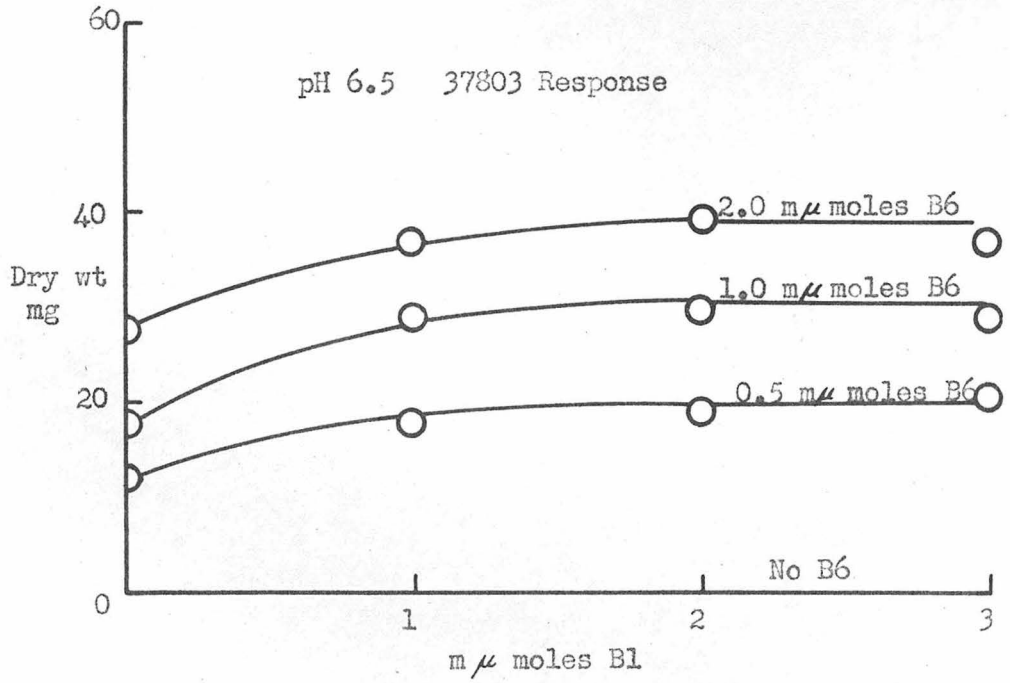


Fig. 8

Effect of Thiamin on Vitamin B6 Requiring Mutants of Neurospora





therefore suffer also from a partial thiamine deficiency.

B. The Form of Vitamin B6 in the Mutants

Since the three forms of the B6 group are practically equivalent it does not appear that the mutation to pH sensitivity has altered the capacity of the mutants to transform one form of vitamin B6 into another. It was however necessary to demonstrate that vitamin B6 could be synthesized by the pH sensitive mutants and to show that the B6 synthesized was qualitatively identical with that produced by wild type. This point was felt to be of considerable importance since one interpretation of the growth requirements in the absence of added vitamin B6 was the synthesis by the mutant of a new form of the vitamin, useful for ammonia utilization but inactive for nitrate reduction.

Stokes et al. (1943) had attempted to demonstrate that the pH sensitive mutants could produce vitamin B6. They showed that extracts of the pyridoxinless strain of N. sitophila act like pyridoxine in stimulating the growth both of their mutant at lower pH values and Lactobacillus casei. These extracts were made by autoclaving the mycelium in 1 N HCl for one hour.

Since the time of their investigation it has been shown that organisms requiring vitamin B6 can grow in the absence of the growth factor providing a complex medium is supplied (cf. above). It was therefore necessary to show that the growth stimulation they obtained was actually due to the vitamin and not to a complex mixture of amino acids extracted by the acid autoclaving. To

demonstrate the presence of vitamin B6 itself it was felt that extracts should be prepared that stimulated growth but did not contain sufficient nitrogen to serve as a nitrogen source.

Strains 44602 and 4A were grown in carboys under forced aeration in M/15 phosphate medium at pH 7. At the end of a three day growth period the mycelium was harvested and extracted by autolysis (Fig. 9). This crude extract had B6 activity for 44602 (Table IX). The crude extract was then purified (Fig. 9) by following some of the preliminary steps used in the isolation of vitamin B6 (Gyorgy, 1938). The activity of the purified extracts leaves no doubt that a substance with complete B6 activity is produced by the pH sensitive mutant grown in the absence of added vitamin B6 (Table IX).

It is realized that the method of extraction by autolysis is a poor one since one has no control over the reactions occurring during this period. However, similar active preparations have been obtained following extraction by autoclaving with 0.055 N acid. This latter method of extraction has been used for the assay experiments reported below. Preparations obtained by acid autoclaving show vitamin B6 activity for the non pH sensitive mutant 37803 and for Saccharomyces carlsbergensis ATCC 4228 as well as for 44602.

Further indication of the qualitative identity of B6 in both mutants and wild type has been obtained by chromatographing extracts of 4A and 44602 obtained by acid autoclaving. An ascending butanol-water partition was used on filter paper strips. Consecutive one cm strips were then cut and extracted with minimal medium. These extracts were tested for vitamin B6 activity using 44602 as

Fig. 9

Steps in the Purification of Vitamin B6

from 44602 and 4A mycelium

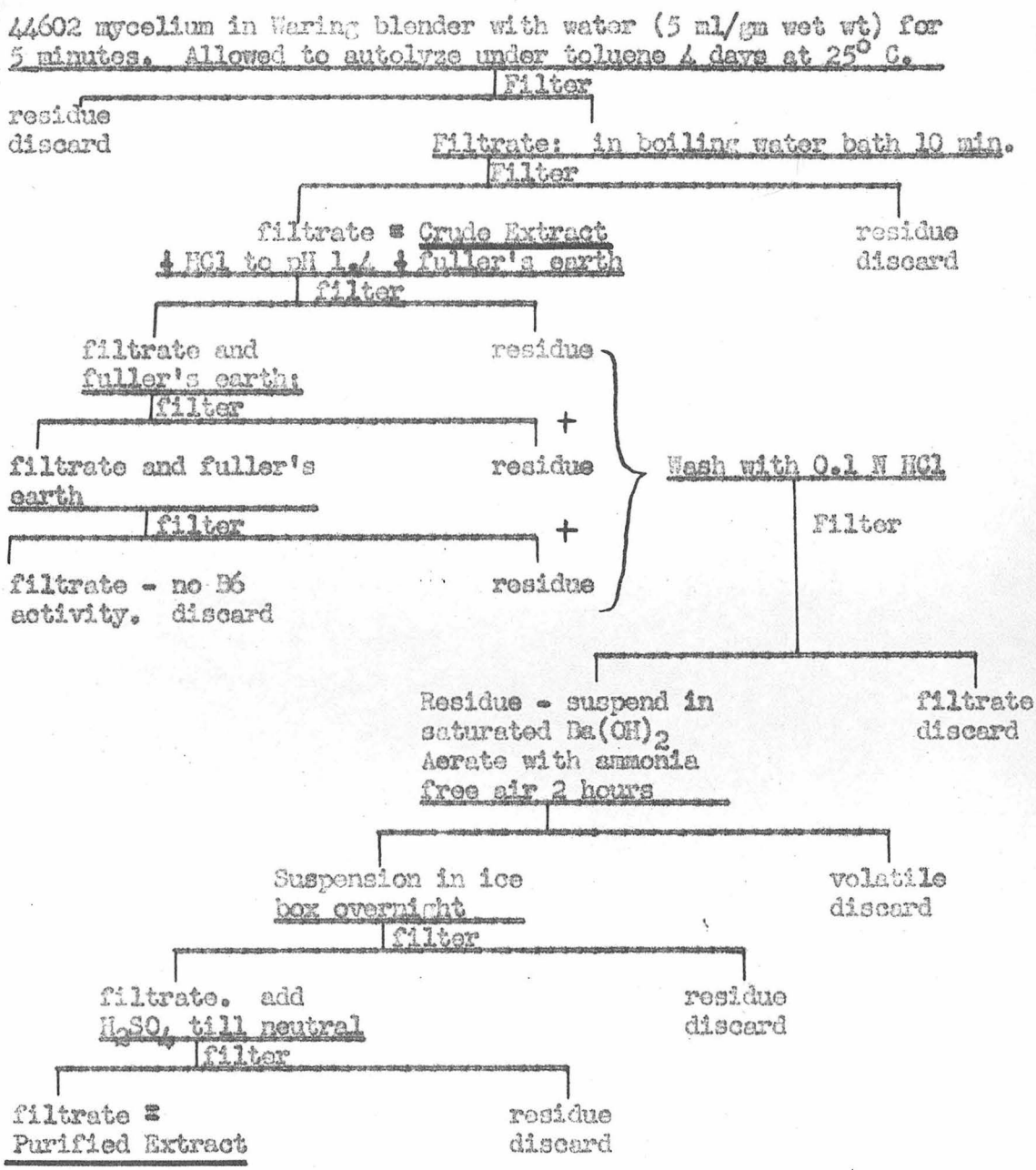


Table IX

Vitamin B6 Activity for 44602 of Crude and Purified Extracts of 4A and 44602 grown without added Vitamin B6

Recorded growth (mg dry wt) of 44602 after 3 days at 25° C on potassium tartrate minimal with no added vitamin B6 + nitrogen source as shown.

I. Crude Extracts - 1 ml represents 200 mg starting material

Extract of 44602 139 g				4A - 220 gms wet wt			
ml extract							
1½	1	½	N source	1½	1	½	0
26.5	20.4	9.5	none	23.8	18.2	11.9	0
46.9	36.0	24.0	$(NH_4)_2SO_4$	54.2	46.4	32.8	0
35.9	27.2	15.6	KNO_3	42.3	33.2	18.6	0

II. Purified Extracts

Extract of 44602 g				4A			
ml extract							
1	½	¼	N source	1	½	¼	0
6.2	3.6	2.4	none	6.2	3.7	2.7	0
58.0	42.8	24.8	$(NH_4)_2SO_4$	64.8	49.5	28.5	0
33.1	19.6	12.0	KNO_3	60.5	27.8	15.3	0

an assay organism. Growth was estimated visually. One narrow and one broad band were obtained with both wild type and mutant extracts. These bands correspond roughly in their position to pyridoxal, pyridoxamine and pyridoxine when a mixture of the three was chromatographed separately (Table X). Because of the limitations of the technique used these results can be considered as only suggestive.

In order to investigate possible quantitative differences between the strains it was decided to use Saccharomyces carlsbergensis ATCC 4228 as an assay organism. A yeast bioassay method was used for the following reasons:

1. The yeast method is more sensitive (to 1 milligamma) and faster (16 hours) than is the Neurospora method.
2. More work has been done using this organism so that the limits and specificity of the assay are better understood.
3. It has been found that the pH sensitive mutant is inhibited by methionine (cf below). It would be difficult to eliminate the possibility of such inhibition in the assay of a complex extract.

The assay method of Atkin et al. (1943) as modified by Johnson (1948) has been used. The basal medium for this assay is listed in Table VI. Samples are added to 5 ml double strength medium, the volume is brought to 9 ml and the medium is steamed ten minutes. Inoculum is prepared by making a suspension of a 24 hour culture in water to about 90 % transmission at 650 mμ .

Table X

Paper Chromatography of the Vitamin B6 Group

Chromatographed	Tube No.											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Pyridoxine HCl	±	-	±	-	-	±	±	+	±	+	++	
2. Pyridoxal HCl	-	±	±	-	-	-	-	+	±	++	+	
3. Pyridoxamine HCl	++	+++	+++	++	-	-	+	-	-	-	±	±
1 + 2 + 3	+	++	+++	+	±	±	±	±	++	++		
4A Extract	±	+	±	±	±	±	+	±	+	+	++	
4A Extract + 1 + 2 + 3	+	++	++	±	±	±	±	+	+	+++	++	
44602 Extract	+	+	+	±	±	±	±	±	++	+	±	±
44602 Extract + 1 + 2 + 3	+	++	+++	+	±	±	±	±	+	++	+++	+

NOTE: Extract of mycelium by drying in hot air stream at 80° C. Powdering. Extract with 10 ml water at 80° C 5 minutes. Filter.

Mycelium grown at pH 7.0 - no added B6
318 mg 4A; 327 mg 44602

\- = no growth
± = trace
\+ = growth
++ = good growth

One ml of inoculum is added to the cooled medium and the whole is incubated at 30° C for 16 - 18 hours. The assay is "fixed" by steaming and read turbidimetrically at 650 mμ. Results are calculated by comparison with a standard curve of turbidity produced as a function of milligammas of pyridoxine hydrochloride. Values are calculated as the hydrochloride since extraction is with HCl and the neutralization is carried to about pH 4.5.

Extracts are assayed at several concentrations to ascertain an absence of drift. To be valid a bioassay must not show a regular increase or decrease in the assay value per ml as the total amount of extract to be tested is increased. Such a regular variation in assay values is termed drift and may be due to the presence of inhibitory substances or to stimulatory materials other than the one assayed for (Snell, 1948). Drift does occur in certain assays of undiluted Neurospora medium. In these cases values have been corrected by extrapolation to zero concentration of extract. When this has been done the corrected values have been indicated in the tables as showing drift.

One interpretation of the data relating to the growth of the pH sensitive mutant is that some labile form of vitamin B6 is produced which is inactive in vivo but which is decomposed by the extraction treatment to liberate active B6. This hypothesis was partially tested by comparing the ratios of B6 obtained after a "gentle" and "harsh" method of extraction for both wild type and a pH sensitive and non-sensitive mutant. The expectation was that if the hypothesis of a labile inactive form of B6 were correct,

the ratio

$$\frac{\text{B6 extracted by "gentle" procedure}}{\text{B6 extracted by "harsh" procedure}}$$

would be much lower in the pH sensitive mutants than in either wild type or the pH non-sensitive mutants since loss of the inactive form would be activated by the gentler extraction method.

Lyophilized, powdered mycelium of 44602, 44204, 25a and 4A grown in the absence of vitamin B6 and of 44602 and 37803 grown in the presence of vitamin B6 was prepared. The mycelium grown in the presence of B6 was prepared by transferring mycelium which had been grown for three days in the presence of 25 gammas of pyridoxine hydrochloride to flasks containing a medium with no nitrogen source and allowing them to stand for 24 hours alone or with 10 gammas of pyridoxine hydrochloride added.

To obtain values for "gentle" extraction, extracts were prepared by suspending a sample of mycelium in water, immersing in a boiling water bath for five minutes and filtering. In the case of 37803 and 44602 grown in the presence of B6 the immersion in boiling water was omitted although the assay medium containing the sample was steamed in all cases. Samples were suspended in 0.055 N HCl and autoclaved at 15 p.s.i. to obtain values after "harsh" extraction. In no case was there a significant difference in the ratios obtained (Table XI).

The results of these experiments indicate that the vitamin B6 content of wild type and mutant strains is qualitatively identical within the limits of the experimental technique.

Table XI

B6 Content of Mycelium after

Harsh and Gentle Extraction

I.

Strain	B6 (γ/g) extractable with water	B6 after acid autoclaving			Ratio water extractable / acid extractable		
		20 min	40 min	80 min	20 min	40 min	80 min
44	9.8	12.0	18.7	18.1	.82	.53	.54
25a	13.1	19.9	30.2	24.9	.66	.43	.53
44602	5.2	8.2	9.2	11.6	.63	.57	.45
44204	4.1	6.5	9.2	9.3	.63	.45	.44

II. a. Mycelial pads grown in presence of B6. Washed. Transferred to medium with no N source, no B6 for 24 hours.

Strain	1 B6 extractable with water	2 B6 after 60 min autoclaving	Ratio 1/2
44602	2.3	8.8	.26
37803	1.8	7.2	.25

b. As in (a) but transferred to medium with 10 γ B6 (pyridoxine HCl); no N source.

44602	5.8	14.9	.39
37803	4.4	11.5	.38

Parent wild types have a higher B6 content per gram dry weight of mycelium than do the pH sensitive mutants (Table XI). This is indicative of differences in the net rate of B6 synthesis since the slow growing wild type 25a, which produced less dry weight in the growth period than the pH sensitive mutants, nonetheless had a higher B6 content than the fast growing wild type 4A. The results are not interpretable on the basis of different "biological ages". The rate of growth for 25a is presumably limited by other factors. Since all the assays so far performed have indicated a B6 content for the mutant 44602 of only 50 to 60 % that of wild type it is felt that they permit the conclusion that the net rate of B6 synthesis is lower in pH sensitive mutants than in wild type. No distinction can be made in this type of experiment between a lowered rate of synthesis and an enhanced rate of destruction.

It will be recalled that Stokes et al. (1943) had come to the conclusion that the pH sensitive mutant they worked with contained as much vitamin B6 per gram dry weight as wild type. These investigators used a method that does not extract all of the vitamin present. Since their investigation it has been shown that in a one hour period 0.055 N acid extracts more B6 than does more concentrated acid (Table XII). They used 1 N HCl. It is possible that the differences are due to differences in the net rate of vitamin B6 synthesis for different strains. The data on which they base their conclusion indicate about a 20 % difference between wild type and the mutant they worked with.

Table XII

Effect of Acid Autoclaving on

Extraction of Vitamin B6

Results as γ Pyridoxine HCl per gram dry weight of mycelium

Extraction Method	Strain			
	4A	25a	44602	44204
0.055 N HCl 1 hr. at 15 p.s.i.	18.6	19.4	11.9	9.2
0.5 N HCl 1 hr. at 15 p.s.i.	---	14.5	8.0	5.7
0.055 N HCl 5 hrs. at 15 p.s.i.	21.4	22.0	13.4	10.4
0.5 N HCl 5 hrs. at 15 p.s.i.	---	16.5	11.9	8.7

C. Transaminase Activity

Since the intracellular B6 concentration of the pH sensitive mutants is lower than that of wild types, one might expect to find a lowered activity of enzymes having a vitamin B6 derivative as a coenzyme. This has been demonstrated for the case of the glutamic-alanine transaminase.

The transaminases have been reported as having wide distribution in nature. (Braunstein, 1939; Braunstein and Kritzman, 1937; Lichstein and Cohen, 1945; Leonard and Burris, 1947). Pyridoxal phosphate has also been shown to be the coenzyme of this enzyme group (Green et al., 1945; Umbreit, O'Kane and Gunsalus, 1948). A convenient colorimetric method for the estimation of pyruvic acid has been described by Green et al. (1945) and glutamic acid can be detected chromatographically by a phenol-water partition on filter paper followed by development with ninhydrin. It was therefore decided to investigate the reaction

alanine + alpha keto glutaric acid $\rightleftharpoons$ pyruvic acid + glutamic acid

in Neurospora. The enzyme is present (Table XIII) and its activity can be demonstrated with whole mycelial pads, ground mycelium or with a fraction soluble in water obtained from lyophilized ground mycelium. The Neurospora enzyme is activated by pyridoxal phosphate* (Table XIV).

---

* Merck synthetic obtained from Dr. H. K. Mitchell

Table XIII

TRANSAMINASE ACTIVITY IN NEUROSPORA

Contents of Mixture	Strain	Time shaken hours	μ moles pyruvic produced	Glutamic acid detected	Mycelium mg dry wt	$\frac{\mu \text{ moles pyruvic}}{\text{mg dry wt} \times \text{hours}}$
Mycelium in 20 ml pH 7.0 M/15 phosphate buffer	4A	4	0	--	3.3	0
	44602	4	0	--	4.3	0
Mycelium + 60 μ moles α keto-glutarate in 20 ml pH 7 buffer	4A	4	1.0	0	3.0	0.08
	44602	4	0.5	0	3.3	0.04
Mycelium + 600 μ moles DL α alanine in 20 ml pH 7 buffer	4A	4	1.7	0	3.0	0.14
	44602	4	1.7	0	3.4	0.13
Mycelium + 600 μ moles DL α alanine + 60 μ moles α ketoglutarate in 20 ml pH 7 buffer	4A	4	10.3	+	2.3	1.12
	44602	4	8.2	+ (weak)	2.6	0.79
Heat inactivated mycelium + 600 μ moles alanine + 60 μ moles α ketoglutarate in 20 ml pH 7 buffer	4A	4	1.2	--	4.2	0.07
	44602	4	0	---	3.9	0

Mycelium grown 3 days at 25° C on 20 ml M/15 phosphate minimal pH 7 containing 0.1 % sucrose and 20 mg $(NH_4)_2SO_4$ N

Glutamic acid production checked chromatographically on paper using a phenol-water partition and ninhydrin development. Values are averages of duplicates.

Table XIV

Activation of the Glutamic Alanine Transaminase

by Pyridoxal Phosphate*

Enzyme preparation = soluble fraction after 44602 and 4A grown 3½ days at pH 7.0, ground while frozen and allowed to stand in water (toluene added) 3 hours.

Reaction mixture

0.2 M Phosphate pH 7.3	1.0 ml
0.2 M Ketoglutarate	0.4 ml
M DL α alanine	0.4 ml
Enzyme preparation	0.4 ml
Pyridoxal phosphate*(when added) 10^{-4}	0.1 ml
4A Enzyme	26.1 mg/ml
44602 Enzyme	25.9 mg/ml

Time shaking at 25° C = 2 hours

Values as $\frac{\mu \text{ mol pyruvic}}{\text{dry wt enzyme x time (hrs)}}$ averages of duplicates

Reaction Mixture	Extract of	
	4A	44602
1. alanine + enzyme	0.011	0.004
2. alanine + α ketoglutarate + enzyme	0.018	0.011
3. alanine + α ketoglutarate + enzyme + pyridoxal phosphate	0.048	0.068

* Merck synthetic

For this problem the main question has been *in vivo* activity. Therefore experiments to compare wild type and mutant activities were performed using resting mycelial pads grown three days at pH 7 with 0.1 % sucrose and washed with distilled water before immersion in the reaction mixture. In four separate experiments (Table XV) the pH sensitive mutant showed an average of 57 % of the activity of one of its parent wild types. This compares with a B6 content for the mutant grown in the absence of vitamin B6 at pH 7.0 of about 65 % that of the same parent wild type 4A.

Because of the coincidence in the results shown in Table XV it is felt that there is a significantly lower transaminase activity in the pH sensitive mutant than in wild type.

D. Vitamin B6 Balance Studies

The non pH sensitive mutant 37803 invariably grows more in 72 hours on a given amount of vitamin B6 than does the pH sensitive mutant 44602 (Fig.10). One might have expected that the situation would be reversed. Since the pH sensitive mutants are able to synthesise vitamin B6, albeit at a lower net rate than wild type, one might have expected that they should be able to produce more growth on a given amount of vitamin B6 added to the medium than the pH non-sensitive strains.

In an attempt to further clarify the situation a vitamin B6 balance study was carried out on the pH sensitive strain 44602,

Table XV

Glutamic-Alanine Transaminase Activity of 4A

and 44602 Resting Mycelium

Alanine + α ketoglutaric acid ⟶ Pyruvic Acid + Glutamic Acid

Reaction mixture contains 60 μ moles α ketoglutaric acid (8.75 mg), 600 μ moles DL α alanine (54.4 mg) in M/15 phosphate buffer pH 7.0 made to 20 ml. Reaction stopped after shaking at 25° C by removing mycelial pad and immersing reaction mixture in boiling water bath for 5 minutes. Glutamic acid production has been checked chromatographically.

Values given are $\frac{\mu \text{ moles pyruvic produced}}{\text{mg dry wt} \times \text{time (hrs)}}$ (average of two determinations)

obtained by subtracting the value for (mycelium + DL α alanine) from the pyruvic acid produced by the total system (alanine + α ketoglutarate + mycelium). Controls were also run containing (α ketoglutaric acid + mycelium), mycelium alone, reactants alone (individually and combined), and the complete system with heat inactivated mycelium. In no case were the control values greater than 1/5 the value for the complete system.

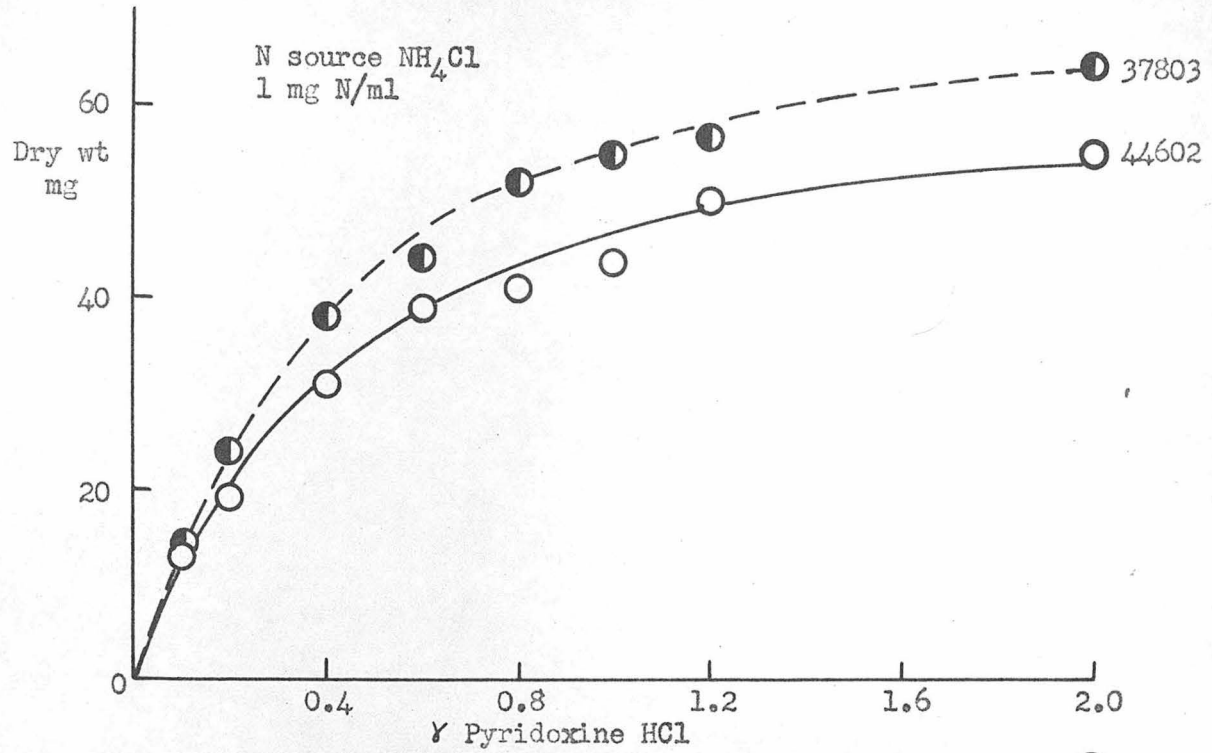
Expt.	Strain	Time	Values	% 4A Value
1	4A	3½	0.74	
	44602	3½	0.33	45
2	4A	4½	0.68	
	44602	4½	0.47	69
3	4A	4	0.98	
	44602	4	0.66	67
4*	4A	3	0.38	
	44602	3	0.17	45
			Ave.	57 %

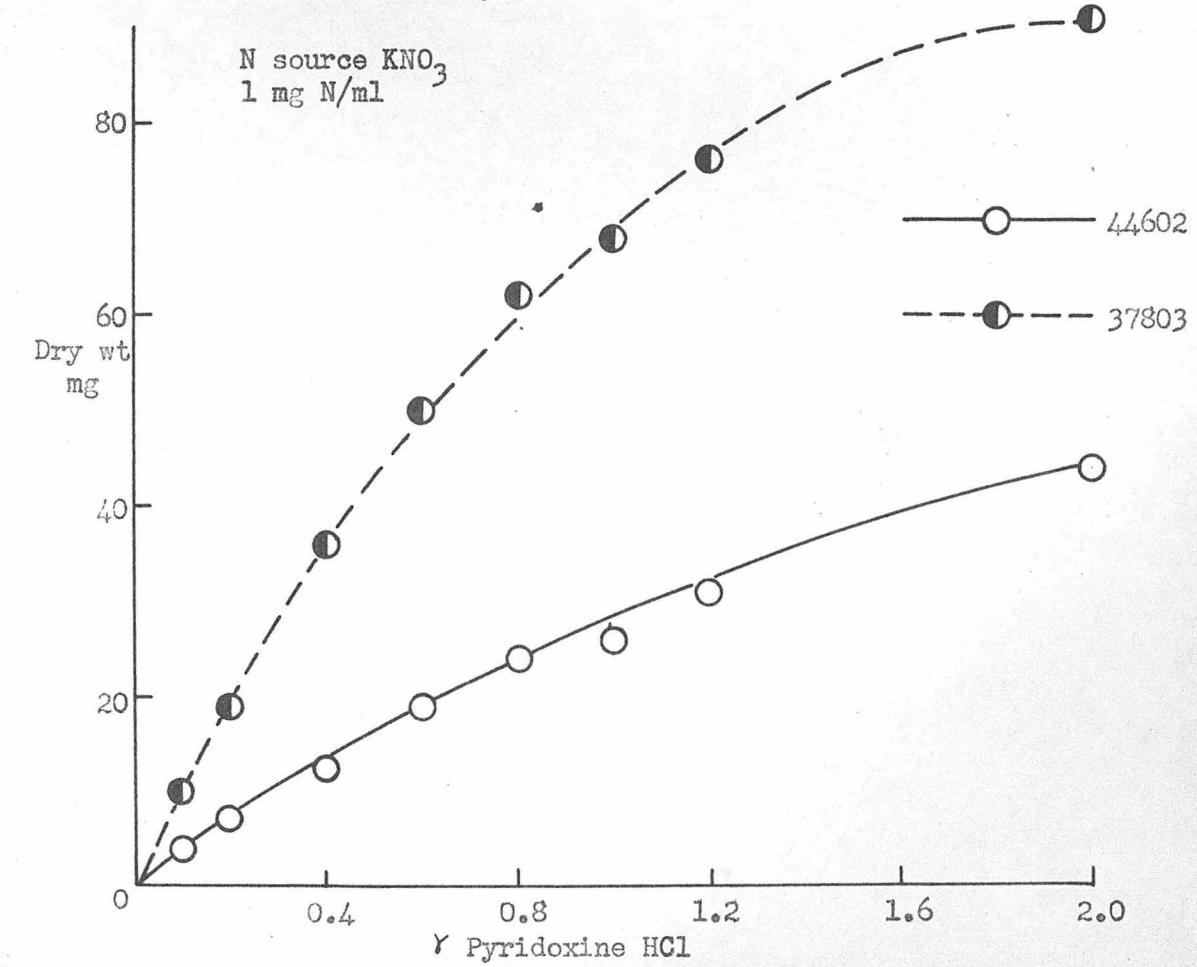
*Notes: average dry weight ≅ 3 mg
Expt. 4--total volume 5 ml
15 μ moles α ketoglutarate
150 μ moles dl α alanine

Fig. 10

Response of Vitamin B6 Requiring Mutants to Pyridoxine

Medium: Potassium tartrate minimal pH 5.6





Inoculum--8 day cultures

the pH non-sensitive strain 37803, and wild type 4A. In these experiments a known amount of vitamin B6 was added to a culture and growth was allowed to proceed for four days. The medium was then collected, filtered and the mycelium lyophilized.

The following determinations were made: (1) total acid extractable B6 in the mycelium, (2) free B6 in the medium, (3) total B6 in the medium following acid hydrolysis. The difference between (2) and (3) represents the amount of phosphorylated or otherwise combined B6 in the medium. As a measure of the net amount of vitamin B6 destruction the amount of vitamin B6 not recovered [amount B6 added - (1) + (3)] has been used. In all cases uninoculated flasks containing vitamin B6 have been incubated and assayed to determine that B6 has not been destroyed by the experimental procedure.

No net synthesis of vitamin B6 by 37803 could be demonstrated (Table XVI). No net synthesis of B6 by 44602 was observed except at pH 7 on ammonium as a nitrogen source. On nitrate at pH 5.6 the net quantity of B6 destroyed appeared greater for 44602 than for 37803. In all the experiments wild type shows a net synthesis of vitamin B6 much of which appears in the medium.

In order to eliminate the complicating effects of growth in these experiments non-growing cultures were next used. These were obtained by growing 44602 and 37803 in Fernbach culture flasks at pH 5.6. 500 ml of potassium tartrate minimal with ammonium sulfate as a nitrogen source and 25 gammas of B6 added (as pyridoxine hydrochloride) wereused in each flask. Cultures were incubated at

Table XVI

Growing Culture Balance Experiments with Vitamin B6

I. Grown with KNO_3 as N source, pH 5.6
10 γ pyridoxine HCl added, 10 γ recovered in blank.
All values averages of two separate assays of same sample.

1 Strain	2 dry wt. mg.	3 B6 γ/gm mycelium	4 Total B6 in mycelium	5 B6 in medium free	6 B6 in medium after acid hydrolysis	7 B6 not accounted for 10 - (4+6)	8 Net B6 destroyed mg x 10^3
44602	395	7.15	2.82	2.69	3.37	3.8	9.6
37803	680	6.23	4.23	1.69	2.21	3.6	5.3
4A	956	38.9	37.2	≅ 25	≅ 41	net synthesis	
				Drift			

II. Grown with $(NH_4)_2SO_4$ as N source, pH 5.6
10 γ B6 added as above

44602	484	8.50	4.12	0.34	1.34	4.54	9.4
37803	622	6.08	3.78	0.36	.62	5.60	9.0
4A	1436	38.6	55.5	≅ 29	≅ 13	net synthesis	
				Drift			

III. Grown with $(NH_4)_2SO_4$ as N source (1/2 mg/ml), pH 7.1
10 γ B6 added as above

44602	470	12.5	5.88	4.89	5.78	net synthesis	
37803	647	4.8	3.10	2.33	2.95	3.95	6.1
4A	493	18.0	8.88	14.6	12.7	net synthesis	

25° C for three days. At the end of this growth period the medium was removed from the flasks and the mycelium was washed once with 500 ml of sterile distilled water. This was then replaced with 500 ml of a solution containing per liter: 5 grams sucrose, 6.4 grams potassium tartrate and 0.5 grams mono-potassium phosphate. For both 44602 and 37803 one flask received ten gamma of pyridoxine hydrochloride while the other served as a control. These flasks were then incubated for 24 hours at 25° C after which assays were made on both mycelium and medium (Table XVII).

In this experiment 44602 has destroyed or inactivated about twice as much B6 per mg dry weight as has 37803. Since both cultures were rapidly growing at three days the effect is not due to differences in the biological ages of the two mycelial pads. The data indicate that B6 is destroyed more rapidly in 44602 than in 37803 under the conditions of this experiment.

VIII. The Specificity of Ammonia in the Replacement of Vitamin B6

As a result of a number of trials of different nitrogen sources it appears that ammonia is specific for the initiation of growth of the pH sensitive mutants in the absence of added vitamin B6. Casein hydrolysate and most of the amino acids have been tested at both pH 5.6 and pH 7 (Table XVIII). Various amino acid mixtures have also been tested including those found by Lyman et al. (1947) to replace vitamin B6 for Lactobacillus arabinosus. None of these

Table XVII

Non-Growing Culture Balance Experiment with Vitamin B6

1 Strain and Contents	2 dry wt. mg.	3 B6 in mycelium γ/gm	4 Total γB6 in mycelium	5 B6 in medium-free	6 B6 in medium after acid hydrolysis	7 B6 not accounted for 10 -(4+6)	8 Net B6 destroyed γ per mg x 10^3
44602 no B6	471	8.75	4.12	0.14	1.7		
44602 + B6	506	14.9	7.54	1.00	4.33		
Difference	+ 35	6.1	3.42	0.86	2.6	3.98	8.0
37803 no B6	712	7.18	5.1	0.03	0.5		
37803 + B6	704	11.5	8.1	1.97	4.58		
Difference	- 8	4.3	3.0	1.94	4.08	2.92	4.2

Table XVIII

Substances Tested as Nitrogen Sources for the Initiation of Growth of 44602 in the Absence of Added Vitamin B6

NH_4^+	+
NO_3^-	-
NO_2^-	-
Amino Acids	
Alanine	-
Arginine	-
Aspartic acid	-
Asparagine	-
α Amino butyric acid	-
Glutamine*	+
Glutamic acid	-
Histidine	-
Lysine	-
Isoleucine	-
Methionine	-
Serine	-
Threonine	-
Tryptophan	-
Casein Hydrolysate	-
Various other mixtures	-

Others	
Amides	
Acetamide	-
Amino fumaric diamide*	+
Oxalacetic diamide	-
Urea*	-
Amines	
Methylamine	-
Dimethylamine	-
Choline	-
Benzidine	-
p Amino phenol	-
Pyridine	-
Pyruvic Oxime	-

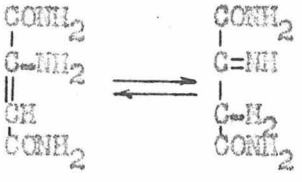
*Filtered sterile

substances initiates growth although they can be used as nitrogen sourcesin the presence of added B6. Glutamine and amino fumaric diamide are able to initiate growth in the absence of added vitamin B6 but here a spontaneous breakdown to free ammonia in the medium is the most likely explanation.

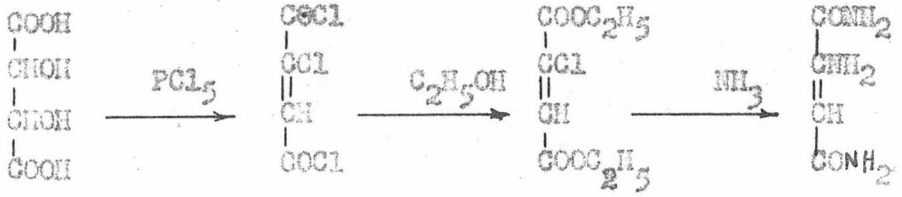
Glutamine is required in high enough concentration for liberation of the amide nitrogen as ammonia to be sufficient to account for the growth produced. Since a three day incubation period is required spontaneous hydrolysis in the medium employed (M/15 phosphate) would probably occur. Further testing showed that the glutamine sample used contained free ammonium ion.

Amino fumaric diamide was tested at the suggestion of Dr. H. K. Mitchell. Although it at first looked promising as a substance capable of initiating growth of 4402, later experiments indicated that its action was due to breakdown to ammonia.

This compound is interesting since it can be formally considered as possessing a free imino group; i.e.

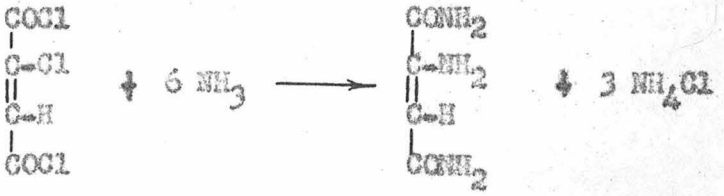


It was first prepared by Perkin (1886) using the following sequence of reactions:



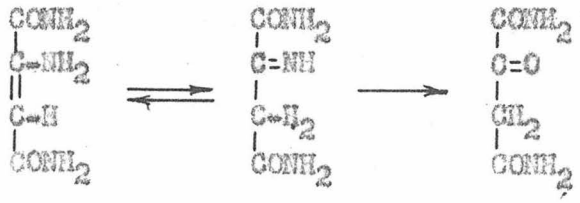
Tartaric acid — Chlorofumaric chloride — Ethyl chloro-fumarate — Amino fumaric diamide

A modification of this method suggested by Dr. H. K. Mitchell was used in the present experiments. Chlorofumaric chloride was reacted directly with cold concentrated aqueous ammonia by dropping the chloride slowly into the ammonia solution. The brown diamide settled out after completion of the reaction and was collected



by filtration and recrystallised from water. (Nitrogen found by Kjehldahl digestion, 32.1 %; theoretical 32.5 %. Decomposition point 176 - 186° C; reported, greater than 180°.) The product appeared similar to that prepared by Perkin's method. This method gives low yields but was adopted because of its simplicity and speed. (Yield from 38 grams of tartaric acid, 4.1 gm; theoretical 40 grams)

Oxalacetic diamide was prepared by dissolving one gram of amino fumaric diamide in 5 ml of 1 N sulfuric acid by boiling. The solution was placed in the ice box until white crystals separated after which the material was collected and recrystallized from water. (Decomposition point, 176 - 184° C; reported 180° C. Yield, 0.2 gm; theoretical 0.87 g)



Oxalacetic diamide did not initiate growth of 44602 in the absence of added vitamin B6 although it was able to serve as a nitrogen source for wild type. Amino fumaric diamide initiated growth in M/15 phosphate medium at pH 7 and in potassium tartrate minimal at pH 5.6 but in this latter case the pH was found to have risen to pH 6.7 by the conclusion of the experiment.

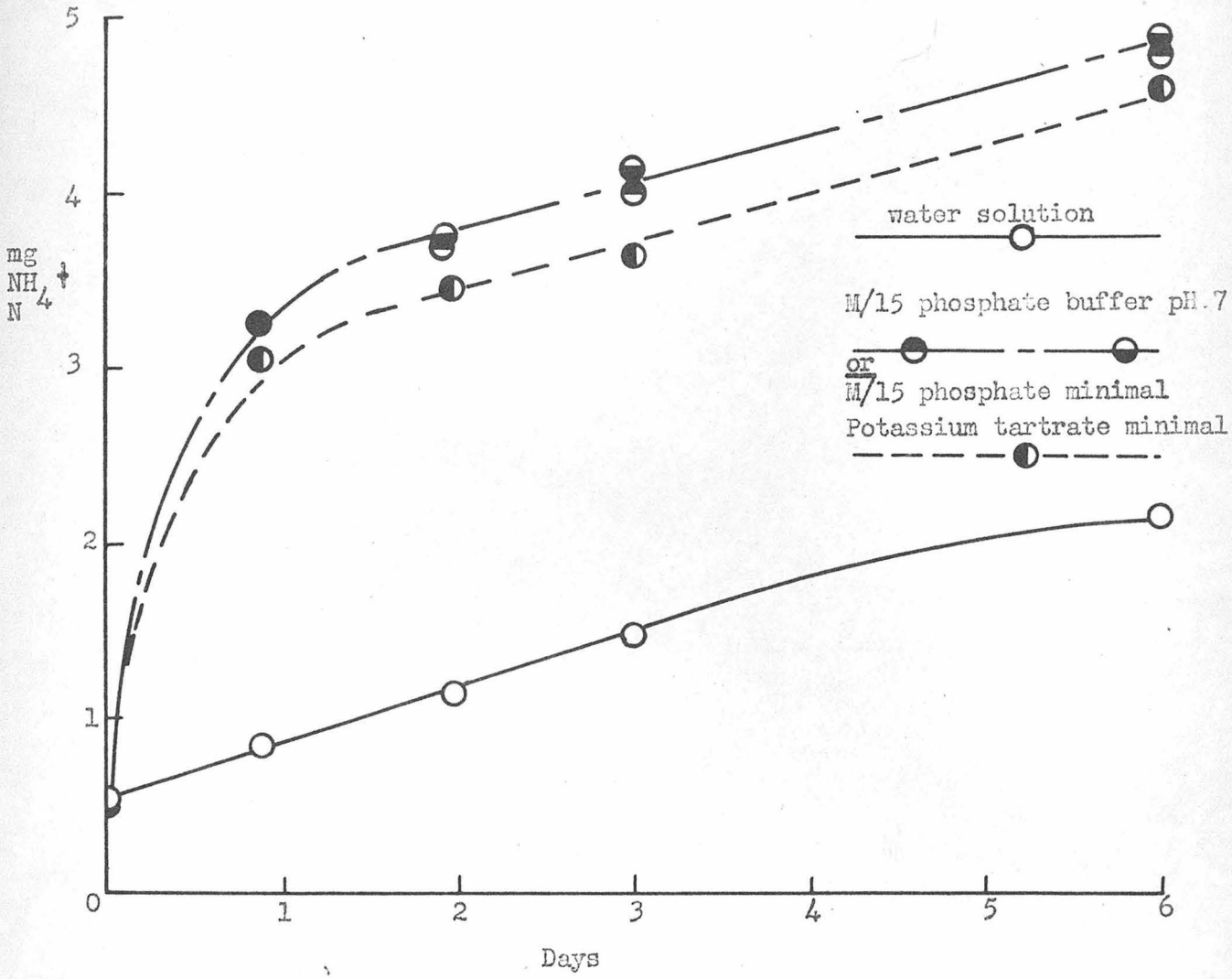
Although amino fumaric diamide dissolved in water after standing three days did not give a test for ammonia with Nessler's solution (a white precipitate is formed with this reagent) it was decided to determine whether the constituents of the medium might not catalyse a decomposition. Ammonia was determined by aeration into standard acid after adding saturated potassium carbonate solution. The zero day values represent the amount of hydrolysis caused by the method of analysis (Fig. 11). It is therefore clear that the constituents of the medium catalyze decomposition of the compound in a short enough time to make ammonia available in sufficient concentration for the initiation of growth of 44602.

While no compound yet tested has served as a nitrogen source for the initiation of growth in the absence of vitamin B6, several amino acids have been able to increase the rate of growth of 44602 in the presence of a limited amount of ammonium nitrogen. It is of course possible that it is not the nitrogen of these compounds that is being used but rather the carbon skelpton. Or the compound may be directly assimilated <u>in toto</u>. These possibilities are admitted and a decrease in amino groups along with the disappearance of ammonium nitrogen would have to be shown to satisfy

Fig. 11

Spontaneous Decomposition of Amino Fumaric Diamide

Media contain 10 mg Compound Nitrogen per 20 ml



these objections. Since nitrogen is known to be a limiting factor in these experiments and there is a large amount of carbon in the medium as sucrose these objections may not be valid.

There are three classes of amino acids with respect to their effect on the mutant growing in the absence of added vitamin B6:

1. Stimulatory: alanine, asparagine, cysteine, glutamic acid, isoleucine
2. Slightly stimulatory or no effect: aspartic acid, histidine, leucine, lysine, phenylalanine proline, serine, valine
3. Inhibitory: arginine, methionine

All of these amino acids are stimulatory when vitamin B6 is added to the medium.

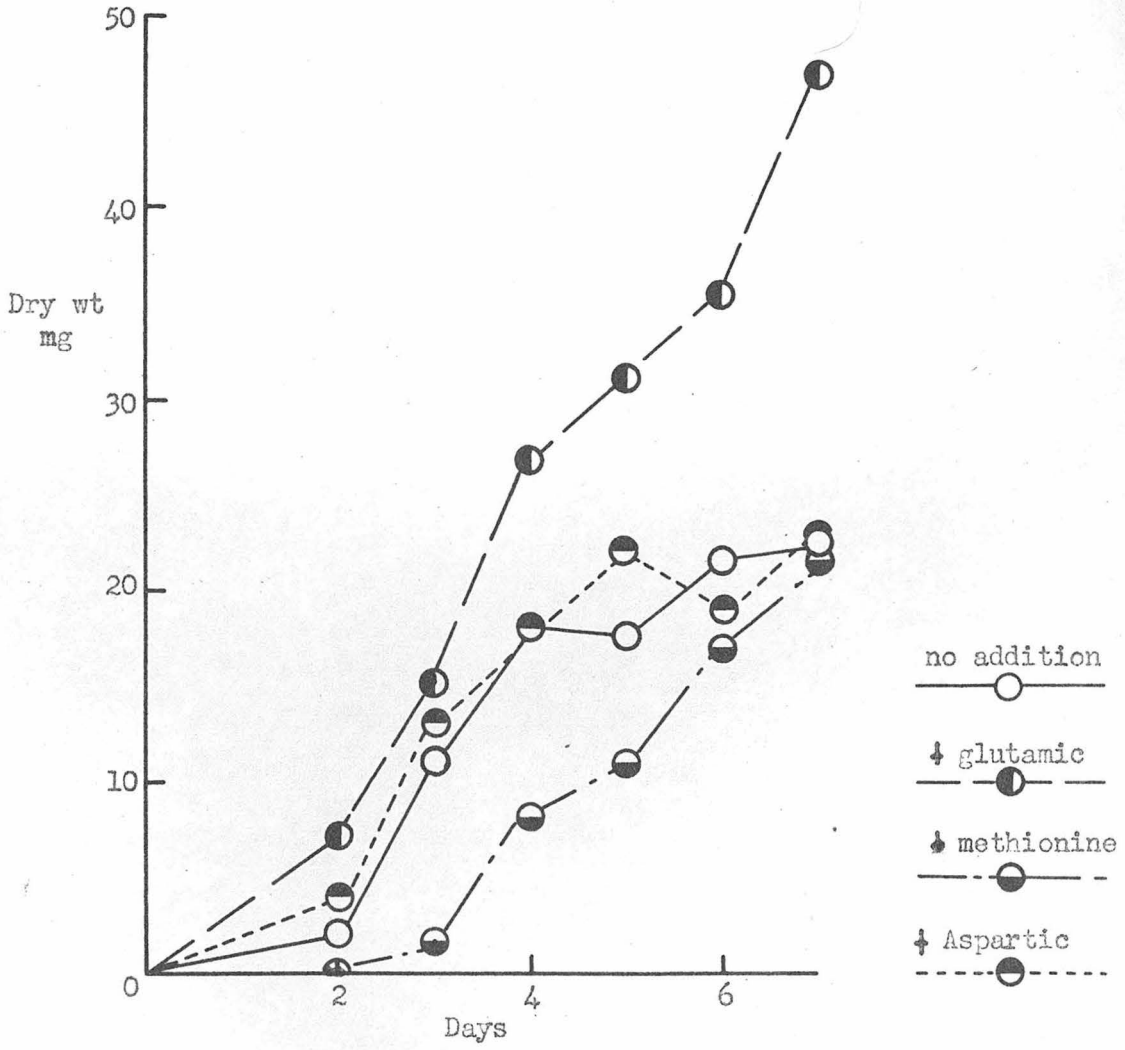
These data were obtained by measuring dry weight of mycelium as a function of time (Fig. 12). M/15 phosphate minimal at pH 7.0 was used as the medium. This contained approximately 3 mg ammonium sulfate nitrogen per 20 ml. The particular amino acid tested was added to give an additional 5 mg of nitrogen per 20 ml.

Ammonia appears to be indispensible notwithstanding the increased growth rates in the presence of particular amino acids. In one experiment pads of mycelium were grown to about 6 mg dry weight in media containing ammonia and asparagine. They were then rinsed with sterile distilled water and were transferred to fresh media at pH 7 containing in one case asparagine as a nitrogen source

Fig. 12

Effect of Three Amino Acids on Growth of 44602

with Low NH_4^+ N and no Added Vitamin B6



Medium: M/15 Phosphate
pH 7.0 3 mg $(NH_4)_2SO_4$ N per 20 ml

and in the other ammonia. The cultures transferred to asparagine medium failed to increase in dry weight although the controls (ammonium medium) continued to grow.

The non-stimulatory effect of some of the amino acids as well as the stimulatory effect of others may be related to the pH of the medium. Thus, cysteine in moderately low concentration is toxic to wild type at pH 5.6 whereas at pH 7.0 it is stimulatory to 4A and to 44602 grown in the presence and absence of added vitamin B6* (Table XIX). Cysteine was added as the hydrochloride without neutralization. The mechanism of this effect is not known, although the toxicity of cysteine HCl may be due to a lowering of the pH of the medium.

The inhibitory action of arginine is apparently related to the high concentration present since up to 6 mg of arginine per 20 ml has no inhibitory effect. The case of methionine is more interesting.

Both D- and L-methionine are inhibitory, as little as 0.4 mg per 20 ml being effective. In no case has it been possible to decrease the amount of growth to zero by increasing the amount of methionine. Furthermore the inhibition is overcome as the culture ages (Fig. 12) either by "adaptation" or destruction of

---

*After autoclaving solutions containing higher concentrations of cysteine hydrochloride at pH 7 a crystalline precipitate (probably cystine) is seen in the medium, some of which may eventually be occluded on the smaller mycelial pads giving them a jewel-like appearance.

Table XIX

Effect of pH on the Toxicity of Cysteine

pH	Strain	mg cysteine HCl					
		0	1	2	10	20	50
5.5	4A	106	94.6	99	88.5	29.3	17.9
7.0	4A	106	98	104	102	116	137
5.5	44602 + 2 γ B6	67	75	71	77	18.4	0
7.0	44602 + 2 γ B6	88	88	87	87	102	134
7.0	44602	56.7	62.5	61.5	55.5	67.5	78.2

Medium: M/15 tartrate

M/15 phosphate

10 mg $(NH_4)_2SO_4$ -

N/20 ml

the methionine present during the growth period. Methionine is stimulatory in the presence of added vitamin B6 and is stimulatory when 44602 is grown in the absence of added vitamin B6 but in the presence of relatively large amounts of ammonia (Table XX). In this case, as the ammonium concentration is increased methionine becomes first non-inhibitory and then at higher ammonium concentrations stimulatory.

Methionine inhibition is competitively overcome by threonine.(Table XXI). Sulfanilamide also overcomes methionine inhibition (Table XXII) and at higher concentrations of sulfanilamide, the sulfanilamide inhibition is reduced by the presence of methionine as has been demonstrated for wild type (Zalokar, 1950). Methionine-threonine relationships have been demonstrated with other mutants of Neurospora (Teas, Horowitz and Fling, 1948; Zalokar, 1950). The mechanism of the action for 44602 is not known.

As a result of these experiments it is believed that only ammonia in high concentration can serve as a nitrogen source for the initiation of growth of the pH sensitive mutants in the absence of added vitamin B6. It will be recalled that what appears to be a threshold concentration of ammonia is required (Fig. 2, 3, 4) and that 44602 requires more ammonium nitrogen than wild type. The only other substances able to initiate growth either have B6 activity or are able to spontaneously break down to ammonia. Those substances which merely stimulate growth may also act by breakdown to ammonia.

Table XX

Relationship between Nitrogen level and

Methionine Inhibition of 44602

Medium: M/15 phosphate pH 7.0

mg NH_4^+ N	mg DL - methionine					
	0	0.5	1.0	1.5	2.0	2.5
3	15.8	10.5	7.0	6.0	6.5	7.3
4	22.5	16.0	14.9	17.0	15.8	15.5
5	30.8	23.7	23.0	24.5	22.4	22.8
7.5	40.5	39.2	36.1	36.1	35.0	34.9
10	44.7	49.5	50.1	48.2	46.6	48.9
15	54.1	60.2	58.6	58.7	59.7	61.2

Table XXI

Inhibition of 44602 by Methionine

and its Reversal by Threonine

Medium: M/15 phosphate pH 7.0: 4 mg $(NH_4)_2SO_4$ N/20 ml

mg dry wt after 3 days at 25° C recorded

mg DL Threonine	mg L Methionine					
	0	0.4	0.6	0.8	1.0	1.2
0	13.9	6.1	4.9	3.9	4.2	4.0
1	15.5	5.6	3.8	3.1	4.0	4.0
2	14.4	11.2	8.2	5.5	6.6	1.9
3	15.3	15.3	15.4	9.2	8.2	3.6
4	15.5	17.7	16.2	15.6	11.6	5.6
5	15.1	18.5	15.6	11.8	11.2	3.0

Table XXII

Sulfanilamide and Methionine

Inhibition of 44602

Sulfanilamide molarity (medium)	mg DL-Methionine				
	0	0.5	1.0	1.5	2.0
0	23.4	16.9	16.1	17.0	16.2
5×10^{-5}	17.1	24.3	24.6	23.4	22.5
10^{-4}	6.6	14.8	18.2	17.8	19.4

The failure of various amino acids and other easily decomposable compounds like urea to initiate growth may be due to the inability of the organism to decompose them rapidly enough to supply the high ammonia concentration needed.

IX. Nitrate Reduction

Considerable attention has been devoted to the question of nitrate reduction by 44602. Growth of this mutant is not initiated by nitrate in the absence of added vitamin B6, nor does the presence of nitrate increase the crop of mycelium over a 21 day period from a medium containing a limiting amount of ammonia. In the presence of added vitamin B6, nitrate is used as a source of nitrogen. Nitrate is not used as readily by 44602 in the presence of vitamin B6 as is ammonia, although the pH non-sensitive mutant 37803 can grow more on nitrate than on ammonium ion at pH 5.6 in a three day growth period.

The data at first glance appeared to indicate a role for vitamin B6 in the reduction of nitrate. The argument may be summarized as follows: In the presence of vitamin B6 nitrate may be utilized as a nitrogen source by 44602; in the absence of added vitamin growth can proceed on ammonia but not on nitrate. Furthermore, although certain amino acids are able to stimulate growth when ammonium ion is present, nitrate is not able to increase the crop of dry weight no matter how long the growth period. Therefore vitamin B6 must be involved in the process of nitrate reduction and

this process does not proceed to a significant extent in 44602 growing in the absence of added vitamin B6 because of the lowered intracellular concentration of B6 under these conditions.

This argument has turned out to be fallacious. The present interpretation of the data is that vitamin B6 is involved in the utilization of the products of nitrate reduction, but is not necessarily involved in the reduction process itself.

To study this question it was necessary to determine what happens to ammonia and nitrate in cultures of 44602 grown in the absence of added vitamin B6. Parallel cultures were set up in M/15 phosphate medium starting at pH 7. The first set contained approximately 3 mg of ammonium sulfate nitrogen per 20 ml while the second set contained in addition 5 mg of potassium nitrate nitrogen. (The high concentration of potassium in these experiments is probably not a factor since identical growth curves have been obtained with a M/15 phosphate medium made with sodium salts and containing as the potassium source only 0.1 g of potassium chloride per liter.) Under the conditions of these experiments it has been found that nitrate is reduced but not utilized for growth.

Ammonium nitrogen in the medium has been determined by making the medium alkaline with potasium carbonate followed by aeration into standard acid and back titration with alkali. Nitrate nitrogen has been determined by reduction to ammonia with Devardas' alloy and potassium hydroxide at room temperature (Borsook and Dubnoff, 1939). Two hours was used for the period of reduction and aeration.

$$4\ Zn + NO_3^- + 7\ OH^- + 6\ H_2O \longrightarrow 4\ Zn(OH)_4^= + NH_3$$

$$8\ Al + 3\ NO_3^- + 5\ OH^- + 18\ H_2O \longrightarrow 8\ Al(OH)_4^- + 3\ NH_3$$

This method is ambiguous insofar as it records all compounds reducible to ammonia, or hydrolyzable to ammonia with potassium hydroxide and not hydrolyzable with potassium carbonate. For example, nitrite and hydroxylamine would be included in the "nitrate" values.

Total reduced nitrogen in the medium has been determined by a standard Kjeldahl method using a selenium-potassium sulfate catalyst with subsequent aeration into acid and Nesslerisation or steam distillation into acid and back titration. This method has been found satisfactory only for nutrient solutions not containing nitrate. The presence of sucrose in the medium is sufficient to cause the reduction of about 40 % of any nitrate present (Table XXIII) making difficult a definitive determination in such cases.

Methods have been worked out for total nitrogen including nitrate (Furman, 1939). Salicylic acid in sulfuric acid is added to a solid sample containing nitrate. The nitric acid released nitrates the salicylic acid. The resulting nitro-compound is then reduced with sodium thiosulfate to amino nitrogen after which a standard procedure determines total nitrogen. This method would presumably not permit the determination of nitrite and any other intermediate reduction products that might be present. There is no rapid wet combustion method for the determination of total nitrogen in solutions containing nitrogen-oxygen compounds along with large quantities of organic material. This determination has therefore not been attempted.

Table XXIII

Reduction of Nitrate in a Kjeldahl Digestion with H_2SO_4

Contents	mg NH_3 N found
a. 1 mg KNO_3 N	0
b. 2% sucrose - 1 ml	0.02
c. 1 mg KNO_3 N + 1 ml 2% sucrose	0.39 0.41

approximate conversion

$$NO_3^- \longrightarrow NH_3 \qquad 40\%$$

Total mycelial nitrogen has been determined by digestion with sulfuric acid and hydrogen peroxide followed by direct Nesslerization. It has been found that in a nitrate medium nitrate does not accumulate in the mycelium so that the limitations discussed previously do not hold.

Wild type growing in a medium containing ammonium and nitrate starting at pH 7 apparently utilizes all the ammonium present and then assimilates the nitrate (Fig. 13). Most of the nitrogen enters the mycelium, after which a part may be returned to the medium, perhaps by autolysis of the older cells. The dry weight continues to increase long after all ammonium or nitrate nitrogen has been assimilated. Behavior of this type has been reported for other fungi (Foster, 1949).

The behavior of 44602 is significantly different (Fig. 14). All the free ammonia disappears but at no time can most of it be detected in the mycelium. This ammonia nitrogen occurs in a combined reduced state in the medium. After all the free ammonia is assimilated nitrate begins to disappear. Some of the products of nitrate assimilation apparently appear in the mycelium while the remainder may be in the medium as in the previous case. Notwithstanding this additional reduced nitrogen in the medium and mycelium there is no increase in dry weight over control cultures on medium containing only ammonium nitrogen. Although total reducible nitrogen remains constant until the disappearance of ammonium ion, nitrite (determined by the sulfanilic acid--

Fig. 13

Uptake of Nitrogen from the Medium by Wild Type 4A

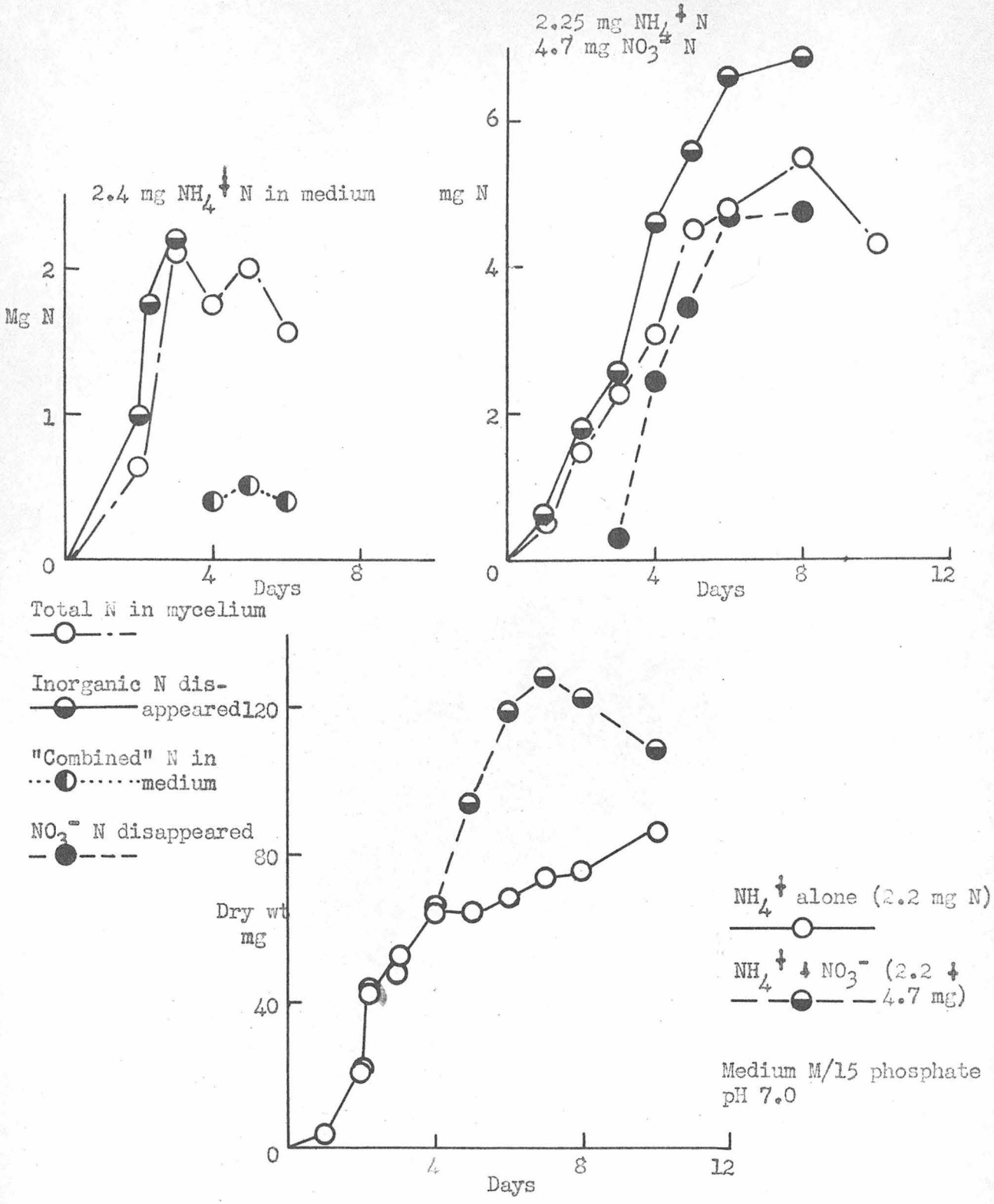
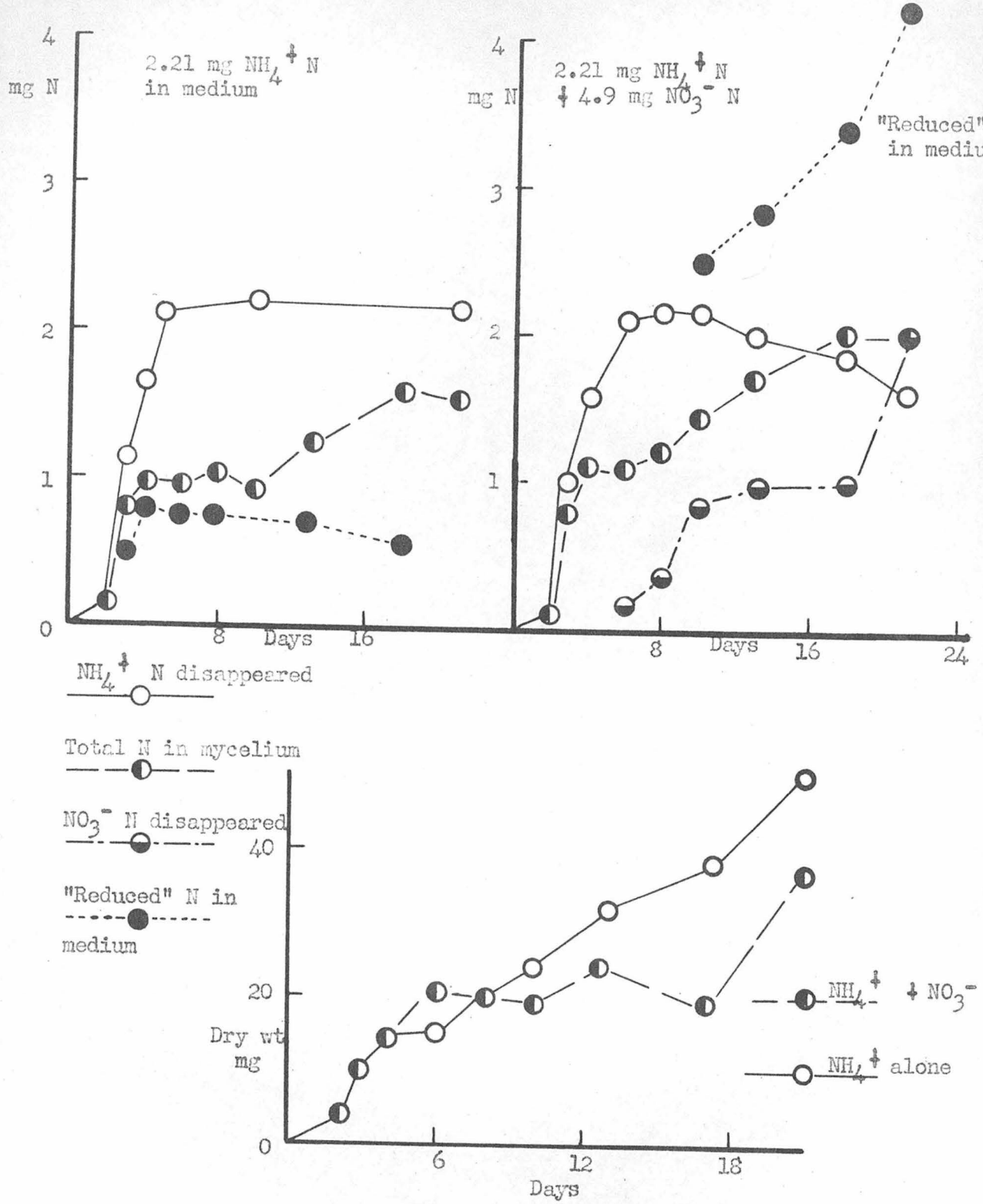


Fig. 14

Uptake of Nitrogen from the Medium by 44602



alpha naphthylamine diazotization test) appears in small quantities before the disappearance of ammonium ion. Nitrite does not appear in cultures grown on ammonium ion alone.

The apparent inability of the pH sensitive mutant to utilize nitrate can be explained on the basis of these observations. It is not necessary to assume, as had been previously supposed, that these mutants are unable to reduce nitrate. The data show that the pH sensitive mutant 44602 grown in the absence of added vitamin B6 can reduce nitrate but only after the initial disappearance of ammonia from the culture medium. Wild type behaves similarly, as shown above. The product(s) of nitrate reduction under these conditions apparently cannot be used for growth of 44602. Indeed, it appears that these products may be inhibitory (Fig. 14). Attempts to detect amino acids in medium from which nitrate has disappeared have been successful (positive ninhydrin tests and chromatographic separation) but the quantity has been too small to permit isolation.

One interpretation of the data relating to nitrate reduction by 44602 is that reduction must proceed along an organic pathway in the mutant strains, since if ammonia were formed from nitrate the mutant would be expected to show increased growth.

Another interpretation is as follows: It has been shown that all the ammonia is assimilated before reducible nitrogen begins to decrease. Previous experiments have shown that the utilization of various substances for growth of 44602 in the absence of added vitamin B6 is possible only in the presence of initially high concentrations of ammonia. However nitrate does not begin

to be reduced until all the ammonia has disappeared. It is possible that even if ammonia were produced as the product of nitrate reduction it would not accumulate in sufficient quantity to cause a growth stimulation and would be diverted into other paths of synthesis. The products of such synthesis might not be expected to lead to increased mycelial growth under the experimental conditions.

X. Conclusion and Discussion

A. Statement of the Hypothesis

The following facts have been established:

1. The net rate of vitamin B6 synthesis is lower in the pH sensitive mutants growing in the absence of added vitamin B6 than in wild type.
2. Ammonia is specific for the initiation of growth of the pH sensitive mutants in the absence of added vitamin B6. The pH sensitive mutant requires more nitrogen for growth in the absence of added vitamin B6 than does wild type or the same mutant grown with added vitamin B6.
3. Nitrate can be reduced by the pH sensitive mutants grown in the absence of vitamin B6 but the product(s) of nitrate reduction are not utilized for growth.
4. The pH non-sensitive mutant 37803 does not show any net synthesis of vitamin B6. Resting cells of the

pH sensitive mutant 44602 destroy about twice as much vitamin B6 as do cells of 37803 in the same period of time.

Two general hypotheses have been considered as explanations of the data:

(a) The rate of synthesis of vitamin B6 is lower in the pH sensitive mutants than in wild type. Large quantities of ammonia increase the rate of vitamin B6 synthesis in the pH sensitive mutant so that it approaches the rate of wild type B6 synthesis.

(b) The rate of vitamin B6 synthesis is the same in both mutant and wild type. However, the rate of vitamin B6 destruction is greater in the pH sensitive mutants than in wild type. This greater rate of destruction creates a net requirement for vitamin B6. Ammonia in high concentration is able to reduce the rate of vitamin B6 destruction.

Hypothesis (b) is qualitatively able to explain the data so far obtained. As will be shown below it does not quantitatively account for some of the data. Hypothesis (a) is unable to account for the fact that resting cells of the pH sensitive mutant 44602 destroy twice as much vitamin B6 as do cells of the pH non-sensitive mutant 37803 in the same period of time. Except where noted both hypotheses explain the data in a similar manner.

B. Interpretation of the Course of Nitrate Reduction in pH Sensitive Mutants

It is possible to interpret the lack of ability of the pH sensitive mutants grown without added vitamin B6 to use the products of nitrate reduction in the following way:

Nitrate reduction does not begin before free ammonia has been removed from the system (cf. above). On the basis of either hypothesis (a) or (b) it is evident that by the time most of the ammonia has been removed from the system the cells of 44602 grown in the absence of vitamin B6 will be suffering from a net vitamin B6 deficiency. (Hypothesis (a)--as ammonia is removed the rate of B6 synthesis drops and the net B6 in the cells diminishes since there is a natural rate of B6 destruction; hypothesis (b)--as ammonia is removed the rate of vitamin B6 destruction increases, diminishing the net B6 in the cells.)

Armstrong, Feldott and Lardy (1950) have shown that the percentage of dietary nitrogen incorporated into the body is lower in vitamin B6 deficient rats than in control animals. A similar phenomenon is encountered here. All the products of ammonia assimilation can initially be accounted for in wild type mycelium (Fig. 13). However the mutant, although able to assimilate ammonia is unable to incorporate the products of ammonia assimilation into its mycelium and excretes them into the medium. This observation is in agreement with the idea that 44602 suffers from a net deficiency of vitamin B6 once ammonia has been removed from the medium.

When all the ammonia present has been assimilated nitrate is reduced. Vitamin B6 is necessary for the utilization for growth of the products of reduction whatever the initial reduced form may be. However, by the time nitrate begins to be reduced the mycelium is suffering from a vitamin B6 deficiency. The product(s) of reduction are therefore not utilised.

Ammonia is able to induce a net synthesis of vitamin B6. However, a threshold concentration of ammonia is required. If ammonia were produced as a product of nitrate reduction it would be produced at a relatively slow rate (Fig. 14). It is possible that as soon as it appeared it would be assimilated by the normal mechanisms (i.e., glutamic acid production) and would not accumulate in the system. Therefore, even if ammonia were produced from nitrate it would not be present in large enough quantity to induce the net vitamin B6 synthesis needed to permit growth. The product(s) of the assimilation of nitrate might then be transformed by processes of shunt metabolism (Foster, 1949) into other compounds.

Increase in dry weight of the mutant and wild type continues after the disappearance of ammonium ion from the system. Mycelial nitrogen may also increase in the mutant. It is known (cf. above) that certain organisms requiring vitamin B6 are able to grow without the vitamin in a complex medium. It is possible that in the presence of ammonia the substances necessary for growth in the absence of intracellular vitamin B6 are synthesized in sufficient amounts so that after the disappearance of free ammonia

(and consequently vitamin B6) growth occurs at the expense of the previously synthesized material. Nitrate reduction would not lead to the formation of this variety of substances since by the time the reduction occurs, ammonia and therefore B6 have disappeared and the cells have lost their capacity to transform the initial products of reduction. On the basis of the above hypothesis it should be possible to find an artificial mixture of substances that will initiate mutant growth in the absence of vitamin B6 or ammonia. Such a mixture has not yet been found. It is also true that the percentage of nitrogen in the mycelium decreases as the culture ages. Part of the increase in dry weight therefore occurs by increase in the proportion of non-nitrogenous material.

C. Quantitative Test of the Hypothesis of an Increased Rate of Vitamin B6 Destruction in pH Sensitive Mutants

The hypothesis that vitamin B6 is destroyed more rapidly in the pH sensitive mutants than in wild type depends on the data obtained in the vitamin B6 balance studies. Two types of balance experiments have been performed, one using growing cultures and the other using resting cells. At the suggestion of Dr. H. K. Horowitz a method was developed to test the compatability of the data obtained in the two types of experiment. I should like to thank Dr. Horowitz for his aid in developing the equations.

The present calculations are based on the assumption that growth is exponential; that is, if N is the dry weight of

mycelium, then at any time t

$$N = N_0 e^{kt}$$ where N_0 is the initial dry weight.

This assumption has been tested using the only data available, those shown in Fig. 6. If the assumption is correct then a plot of the logarithm of the dry weight produced as a function of time should be linear since:

$$\log_{10} N = k t \log_{10} e + \log_{10} N_0$$

and the slope of the line so obtained can be used to calculate k. Such a plot is shown in Fig. 15.

It is further assumed that in the presence of vitamin B6 the value of k is the same for both mutants and wild type. This assumption must be somewhat in error since the dry weight of cultures of the mutants and wild types used is never the same after four days growth. A linear plot is also obtained using the data of Fig. 6 when the cube root of the dry weight is plotted as a function of time. The calculations below have been made on the basis of an exponential type of growth but the results are similar using any growth function so long as the growth of both wild type and the mutants can be expressed by the same type of function.

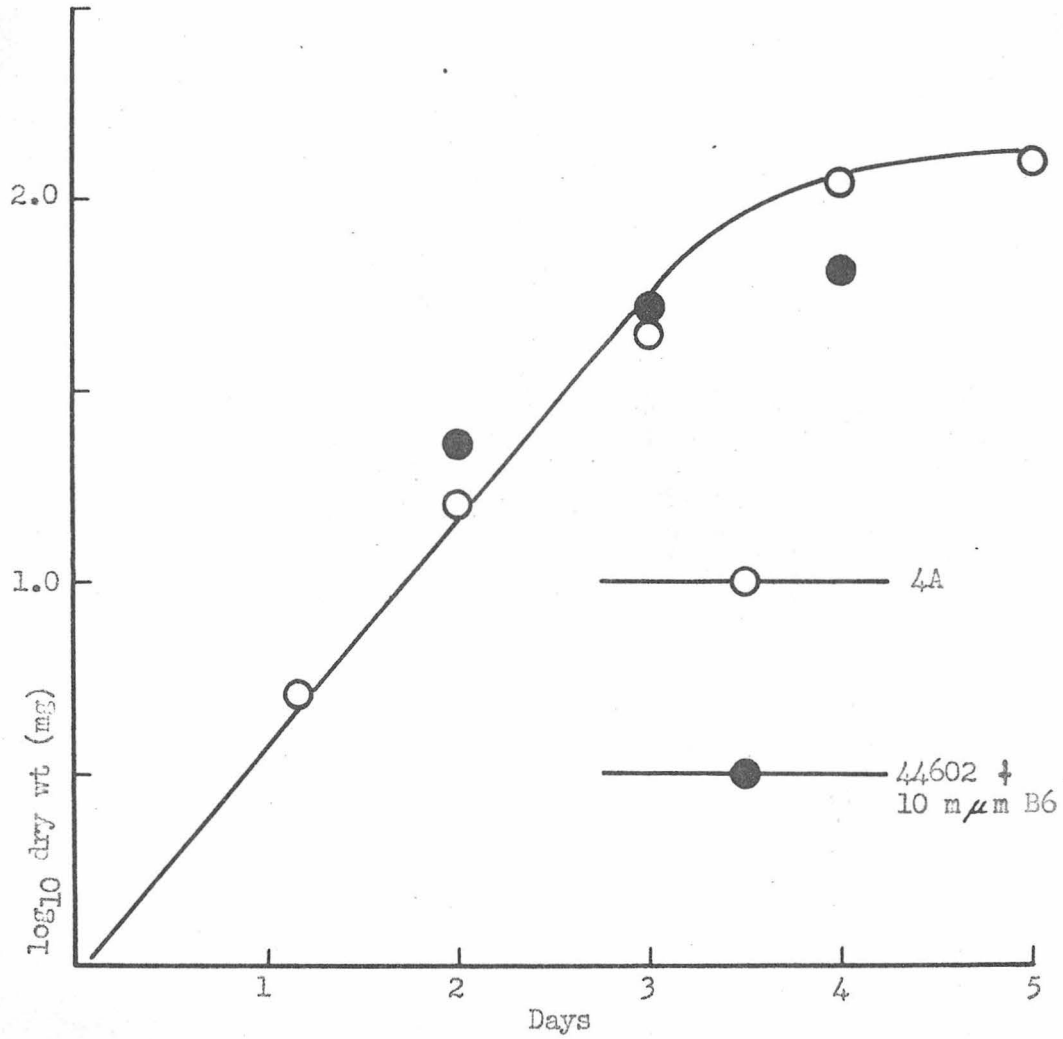
In these calculations the rate of B6 synthesis $\frac{d B_6}{d t}$ is assumed to be proportional to the amount of cell material present at any one time; i.e.

$$\frac{d B_6}{dt} = K N = K N_0 e^{kt} .$$

Fig. 15

Exponential Growth of Neurospora

Data as in Fig. 6



Medium: M/15 Phosphate pH: 7 Note: Dry wt after one day from separate experiment.

N source: $(NH_4)_2SO_4$.

10 mg N/20 ml

Similarly the rate of B6 destruction $\frac{d B_d}{dt}$ is assumed proportional to the amount of cell material;

$$\frac{d B_d}{dt} = K' N = K' N_o e^{kt} .$$

Now the net rate of B6 synthesis $\frac{d B_n}{dt}$ will be equal to the difference of these two quantities:

$$\frac{d B_n}{dt} = \frac{d B_s}{dt} - \frac{d B_d}{dt}$$

or from the above

$$\frac{d B_n}{dt} = K N_o e^{kt} - K' N_o e^{kt}$$

which gives, on integration between the limits set by the final and initial states of a culture,

$$B_{n_f} - B_{n_o} = (N_o e^{kt} - N_o) \frac{K - K'}{k} .$$

But $N = N_o e^{kt}$;

therefore

$$\frac{B_{n_f} - B_{n_i}}{N - N_o} = \frac{K - K'}{k}$$

or

$$\frac{\Delta B_n}{\Delta N} = \frac{K}{k} - \frac{K'}{k}$$

from our assumption

$$d B_s = K N_o e^{kt} \, dt$$

therefore after integration

$$\frac{\Delta B_s}{\Delta N} = \frac{K}{k}$$

Similarly it can be shown that

$$\frac{\Delta B_d}{\Delta N} = \frac{K'}{k} \quad ;$$

therefore

$$\frac{\Delta B_a}{\Delta N} = \frac{\Delta B_g}{\Delta N} - \frac{\Delta B_d}{\Delta N} \quad .$$

For non-growing cultures we assume that K and k equal 0; therefore

$$- \frac{d B_a}{dt} = K' N_o = \frac{d B_d}{dt}$$

where N_o is the weight of the resting mycelium and

$$\frac{\Delta B_d}{N_o} = K' t \quad .$$

K' according to our assumptions is the same for growing and non-growing cultures.

Two non-growing cultures (a) and (b) can then be compared with growing cultures as to their rates of 86 destruction since

$$\frac{\left[\frac{\Delta B_d}{N_o}\right]_a}{\left[\frac{\Delta B_d}{N_o}\right]_b} = \frac{K'_a t}{K'_b t}$$

and

$$K' = \frac{\Delta B_d}{\Delta N} k \quad ;$$

therefore at the same time t

$$\frac{\left[\frac{\Delta B_d}{N_o}\right]_a}{\left[\frac{\Delta B_d}{N_o}\right]_b} = \frac{\left[\frac{\Delta B_d}{\Delta N}\right]_a k}{\left[\frac{\Delta B_d}{\Delta N}\right]_b k} \quad .$$

These equations can be used in a quantitative test of the data shown in Tables XVI and XVII.

Assume that wild type 4A and the mutant 37803 have the same value of K' and k; that is

$$\left[\frac{\Delta B_d}{\Delta N}\right]_{4A} = \left[\frac{\Delta B_d}{\Delta N}\right]_{37803}$$

but that 37803 synthesizes no vitamin B6. Then since

$$\frac{\Delta B_n}{\Delta N} = \frac{\Delta B_s}{\Delta N} - \frac{\Delta B_d}{\Delta N}$$

for 4A on nitrate at pH 5.6 (Table XVI).

$$\frac{41 + 37 - 10}{.956} = \frac{\Delta B_s}{\Delta N} - \frac{3.6}{0.68}$$

$$\frac{\Delta B_s}{\Delta N} = 76 \text{ gammas/g}.$$

Now assuming on the basis of our hypothesis that 44602 and 4A have the same value of K and k; that is

$$\left[\frac{\Delta B_s}{\Delta N}\right]_{4A} = \left[\frac{\Delta B_s}{\Delta N}\right]_{44602}$$

then for 44602 on nitrate at pH 5.6

$$-\frac{3.8}{.395} = 76 - \frac{\Delta B_d}{\Delta N}$$

$$\frac{\Delta B_d}{\Delta N} = 86 \text{ gammas/g}$$

A series of these calculations has been tabulated in Table XXIV.

Table XXIV

Medium	4A		44602		37803	
	$\frac{\Delta B_2}{\Delta N}$	$\frac{\Delta B_1}{\Delta N}$	$\frac{\Delta B_2}{\Delta N}$	$\frac{\Delta B_1}{\Delta N}$	$\frac{\Delta B_2}{\Delta N}$	$\frac{\Delta B_1}{\Delta N}$
Potassium tartrate pH 5.6. Nitrate as N source. 1/2 mg N per ml	76	5.3	76	86	0	5.3
Potassium tartrate pH 5.6. Ammonium as N source. 1/2 mg N per ml	61	9.0	61	70.4	0	9.0
M/15 phosphate pH 7.1. Ammonium as N source. 1/2 mg N per ml	33.5	6.1	33.5	30	0	6.1

Values as γ/g

With these values we may calculate an expected ratio of

$$\frac{\left[\frac{\Delta B_d}{N_o}\right]_{44602}}{\left[\frac{\Delta B_d}{N_o}\right]_{37803}}$$ for resting cells. Values of $\frac{\Delta B_d}{\Delta N}$ for an ammonium medium at pH 5.6 have been used for the comparison since the resting cells were prepared by growing mycelium under these conditions.

$$\frac{\left[\frac{\Delta B_d}{N_o}\right]_{44602}}{\left[\frac{\Delta B_d}{N_o}\right]_{37803}} = \frac{\left[\frac{\Delta B_d}{\Delta N}\right]_{44602}}{\left[\frac{\Delta B_d}{\Delta N}\right]_{37803}} = \frac{70.4}{9.0} \cong \frac{8}{1}$$

The actual values found were (Table XVII)

$$\frac{\left[\frac{\Delta B_d}{N_o}\right]_{44602}}{\left[\frac{\Delta B_d}{N_o}\right]_{37803}} = \frac{8.0}{4.2} \cong \frac{2}{1}$$

These calculations then do not provide support for the hypothesis insofar as a comparison between growing and non-growing balance experiments does not show agreement between calculated and expected values. The calculations do show internal consistency within the growing culture experiments by showing a decrease in B6 destruction by 44602 as the ammonia concentration is increased, while 37803 destruction of B6 remains practically constant (Table XXIV). They also show that it is not necessary to assume a greater amount of B6 synthesis by 44602 at pH 7 than at pH 5.6 to explain the data.

The difference between the calculated and experimental values of the ratio $\left[\frac{\Delta B_d}{N_o}\right]_{44602} \Big/ \left[\frac{\Delta B_d}{N_o}\right]_{37803}$ may lie in the assumptions used in deriving these relationships. The values of k may not be the same for 44, 44602 and 37803. A direct determination of this constant for growth in Fernbachs would be desirable since k enters into the ratios that are compared and has been assumed to be equal for the three strains.

Any synthesis of vitamin B6 in the resting cells of 44602 would change the results in the desired direction. It is also possible that B6 is destroyed only in the course of metabolism. Since no appreciable nitrogen is added to the non-growing cultures the amount of vitamin B6 destroyed might be lessened. In view of the uncertainty in the values of k the results of these calculations are not considered lethal for the hypothesis although they cannot be considered to support it.

D. Qualitative Application of the Hypothesis

Several facts not explicable on the basis of the hypothesis of a lowered rate of B6 synthesis can be easily fitted to the hypothesis of an increased rate of vitamin B6 destruction in the pH sensitive mutants as compared to wild type.

It has been shown that 37803, a pH non-sensitive mutant invariably grows more in a 72 hour period on a given amount of vitamin B6 than does 44602, a pH sensitive mutant (Fig. 10)

If the pH sensitive mutant destroyed B6 at the same rate as 37803 or if it showed a net B6 destruction less than that of 37803 the growth response of the two mutants would be at least the same. The greater growth by the pH non-sensitive mutant can only be explained on the basis of a higher rate of destruction of B6 by the pH sensitive mutant.

Another pH sensitive mutant, 44204, has been tested for its response to vitamin B6. When nitrate is a nitrogen source 37803 grows more in 72 hours on a given amount of vitamin B6 than does 44204. On ammonium nitrogen at pH 5.6, however, 44204 grows slightly more in 72 hours than does 37803 when vitamin B6 is added.

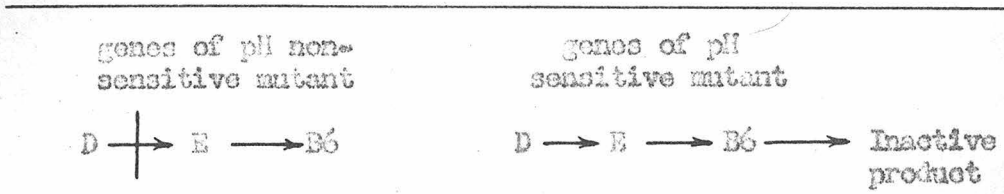
This may be explained by assuming that the rate of vitamin B6 destruction is not as high in 44204 as in 44602. Therefore, the addition of an amount of ammonia not sufficient to cause 44602 to respond to B6 better than 37803 (that is, insufficient to make the net rate of B6 destruction less in 44602 than in 37803) is sufficient to affect 44204 so that it grows better than 37803.

It has been shown that 44204 and 37803 will form a heterocaryon while 44602 and 37803 will not. Heterocaryons are formed by the fusion of the mycelium of two strains. The resulting mycelium contains nuclei derived from each of the strains which fused. For biochemical mutants the effect is pictured as follows:

genes of (1)	genes of (2)
A → B	A → B
D → E	D → E

If B and E are necessary for growth the heterocaryotic mycelium will be able to synthesize them and grow although either of the strains taken separately will not be able to grow.

The pH sensitive and non-sensitive strains present a different picture in a heterocaryon.



Formation of a heterocaryon between a pH sensitive and non-sensitive mutant may increase the total rate of B6 synthesis if the effect of the pH sensitive mutant is to provide an excess of E. However a heterocaryon would grow on minimal medium only if the rate of B6 destruction characteristic of the pH sensitive mutant were not so large as to counteract the increase in B6 synthesis. 44204 does form a heterocaryon with 37803 and we have assumed above that it does not destroy B6 as fast as 44602 which does not form a heterocaryon with 37803.

This hypothesis of a mutation causing a greater rate of destruction of a required substance is most similar in nature to one propounded by Van Overbeek (1935) to explain the dwarf mutant of corn nana. He presented evidence indicating that the dwarf character is due to an increase in the rate of auxin destruction in the mutant as compared to wild type.

E. The Role of Ammonia

Ammonia in high concentration is specific for the initiation of growth of the pH sensitive mutants in the absence of added vitamin B6. On the basis of the provisional hypothesis ammonia could act either by increasing the rate of B6 synthesis or by preventing B6 destruction.

The hypothesis that ammonia acts by increasing the rate of B6 synthesis seems unnecessarily complicated. If the provisional hypothesis is correct the mutation has increased the activity of some enzyme or enzyme system destroying vitamin B6. If ammonia were assumed to increase the rate of B6 synthesis this would require assuming that two enzyme systems had been changed by the mutation since ammonia is not required for wild type synthesis of B6; indeed the net synthesis of vitamin B6 by 4A is greater with nitrate as a nitrogen source than with ammonium ion (Tables XVI and XXIV).

A formal mechanism for the action of ammonia is the following:

$$A \longrightarrow B \longrightarrow \longrightarrow B6 \underset{\longrightarrow}{\overset{NH_3}{\longleftarrow}} P$$

That is, as a result of the mutation B6 is converted to some product P which is inactive as vitamin B6 for Neurospora or for Saccharomyces. In the presence of excess ammonia this reaction can be reversed.

F. Nature of the Mutation

The reaction leading to B6 destruction may be a reaction present in wild type and occurring at an increased rate in the mutant or it may be a new reaction. On the basis of the above formulation if the reaction occurs normally, then one would expect to find that the pH non-sensitive mutant 37803 would grow more on a medium containing ammonium as a nitrogen source than on a medium containing nitrate when vitamin B6 is limiting since more B6 would be destroyed on a nitrate medium. Experimentally it is found that 37803 grows more in a three day period on ammonium than on nitrate when up to 0.4 gamma of pyridoxine hydrochloride is supplied in 20 ml but at higher concentration of B6 37803 grows more on the nitrate medium. It has also been shown (Table XVI, XXIV) that 37803 destroys more B6 on an ammonium medium than on a nitrate medium. Unless this determination is in error it would seem that the reaction leading to B6 destruction in 44602 would have to be essentially a new reaction.

Foster (1949) has presented an interesting discussion of "shunt metabolism" which bears on this point. He points out that every loss of function must be compensated by an apparent gain of a new function. There are present in fungi a variety of enzymes some of which are generally saturated while normally others remain far from saturation. Preventing the action of one of the saturated enzymes will result in a shunting of substrate into generally unused channels with the production of a new metabolite.

An example of this for Neurospora would be the production of orotic acid by certain pyrimidineless mutants. The vitamin B6-requiring mutants grown on a limited amount of B6 also have a new function: the production of a yellow pigment.

It is possible that a mutation leading to the blocking of some reaction might have the effect of increasing the rate of vitamin B6 destruction. This could occur by making available some substrate needed for the inactivation reaction or by increasing the activity of the enzyme system inactivating B6 by lowering the concentration of an inhibitor naturally present. More directly of course, an enzyme itself might be changed so that it had a higher turnover number or a new specificity. Any suggestion as to a detailed mechanism is of course extremely tenuous.

On the basis of the provisional hypothesis ammonium ion is specific because it is the only substance that can provide a sufficient quantity of ammonia inside the cell to protect the available vitamin B6. Substances convertible to ammonia do not yield ammonia in high enough concentration to prevent a greater rate of inactivation than synthesis. Therefore they are not able to initiate growth, although certain of these substances may stimulate growth in the presence of ammonia. Vitamin B6 itself is able to initiate growth of pH sensitive mutants since it raises the B6 level high enough to overcome the effects of the inactivating reaction.

BIBLIOGRAPHY

Armstrong, K. L., Foldott, G. and Lardy, H. A. 1950
Relationship of Vitamin B6 to the Metabolism of D-Amino Acids
Proc. Soc. Expt. Biol. Med. 73, 159

Atkin, L., Schultz, A.S., Williams, W. W. and Frey, C. N. 1943
Yeast Microbiological Methods for the Determination of Vitamins: Pyridoxine
Ind. Eng. Chem., Anal. Ed. 15, 141

Beadle, G. W. and Tatum, E. L. 1941
Genetic Control of Biochemical Reactions in Neurospora
Proc. Natl. Acad. Sci., U.S. 27, 499

Beadle, G. W. and Tatum, E. L. 1945
Neurospora II. Methods of Producing and Detecting Mutations Concerned with Nutritional Requirements
Am. J. Bot. 32, 678

Bellamy, W. D. and Gunsalus, I. C. 1945
Tyrosine Decarboxylase II. Pyridoxine Deficient Medium for Apo Enzyme Production
J. Bact. 50, 95

Bina, A. F., Thomas, J. M. and Droum, E. B. 1944
The Determination of Vitamin B6 (Pyridoxine) in Foods
J. Biol. Chem. 148, 111

Bird, O. D., Vandenbelt, J. M. and Emmet, M. M. 1942
Adaptation of the Scudi Colorimetric Method for Pyridoxine
J. Biol. Chem. 142, 317

Borsook, H. and Dubnoff, J. W. 1939
Methods for the Determination of Submicro Quantities of Total Nitrogen, Ammonia, Amino Nitrogen, Amides, Peptides, Adenylic Acid and Nitrates
J. Biol. Chem. 131, 163

Braunstein, A. E. 1939
Die Enzymatische Umaminierung der Aminosäuren und ihre Physiologische Bedeutung
Enzymologia 7, 25

Braunstein, A. E. and Kritzman, M. G. 1937
Über den Ab- und Aufbau von Aminosäuren Durch Umaminierung
Enzymologia 2, 129

Broquist, H. P. and Snell, E. E. 1949
Studies of the Mechanism of Histidine Synthesis in Lactic Acid Bacteria
J. Biol. Chem. 180, 59

Brown, E. B., Bina, A. F. and Thomas, J. M. 1945
The Use of Diazotized p-Amino Acetophenone in the Determination of Vitamin B6 (Pyridoxine)
J. Biol. Chem. 158, 455

Cerecedo, L. P., Foy, J. R. and DeRenzo, E. C. 1948
Protein Intake and Vitamin B6 Deficiency in the Rat. II. The Effect of Supplementing a Low-Protein Pyridoxine Deficient Diet with Cystine or with Methionine
Arch. Biochem. 17, 397

Clark, H. E. and Shive, J. W. 1934
The Influence of the pH of a Culture Solution on the Rates of Absorption of Ammonium and Nitrate Nitrogen by the Tomato Plant
Soil. Sci. 37, 203, 459

Cunningham, E. and Snell, E. E. 1945
The Vitamin B6 Group VI. Comparative Stability of Pyridoxine, Pyridoxamine and Pyridoxal
J. Biol. Chem. 158, 491

Foster, J. W. 1949
Chemical Activities of Fungi
New York, Academic Press

Furman, N. H. 1939
Scott's Standard Methods of Chemical Analysis, Vol. I 5th Edition
New York, D. van Nostrand

Gale, E. F. 1948
The Chemical Activities of Bacteria
London, Univ. Tutorial Press

Green, D. E. Leloir, L. F., and Nocito, V. 1945
Transaminases
J. Biol. Chem. 161, 559

Gyorgy, P. 1938
Crystalline Vitamin B6
J. Am. Chem. Soc. 60, 983

Harris, S. A., Heyl, D. and Folkers, K. 1944
The Vitamin B6 Group II. The Structure and Synthesis of Pyridoxamine and Pyridoxal
J. Am. Chem. Soc. 66, 2088

Hochberg, M., Melnick, D. and Oser, B. L. 1944a
Chemical Determination of Pyridoxine in Biological Materials and Pharmaceutical Products. The Multiple Nature of Vitamin B6.
J. Biol. Chem. 155, 109

Hochberg, M., Melnick, D., and Oser, B. L., 1944b
On the Stability of Pyridoxine
J. Biol. Chem. 155, 129

Holden, J. T., Furman, C., and Snell, E. E. 1949
The Vitamin B6 Group XVI. D-Alanine and the Vitamin B6 Content of Microorganisms
J. Biol. Chem. 178, 789

Houlahan, M. B., Beadle, G. W., and Calhoun, H. G. 1949
Linkage Studies with Biochemical Mutants of Neurospora crassa
Genetics 34, 493

Huff, J. W., and Perlzweig, W. A. 1944
A Product of Oxidative Metabolism of Pyridoxine, 2-Methyl-3-Hydroxy-4-Carboxy-5-Hydroxy-Methyl Pyridine. (4 Pyridoxic Acid)
J. Biol. Chem. 155, 345

Hungate, F. P. 1946
The Biochemical Genetics of a Mutant of Neurospora crassa Requiring Serine or Glycine
Thesis, Stanford University, 1946

Johnson, D. C. 1948
Methods of Vitamin Determination
Minneapolis, Burgess Publ. Co.

Keresztesy, J. C. and Stevens, J. R. 1938
Crystalline Vitamin B6
Proc. Soc. Expt. Biol. Med. 38, 64

Leonard, M. J. K. and Burris, R. H. 1947
A Survey of Transaminases in Plants
J. Biol. Chem. 170, 701

Lichstein, H. C. and Cohen, P. P. 1945
Transamination in Bacteria
J. Biol. Chem. 157, 85

Lichstein, H. C., Gunsalus, I. C. and Umbreit, W. W. 1945
Function of the Vitamin B6 Group: Pyridoxal Phosphate (Codecarboxylase) in Transamination
J. Biol. Chem. 161, 311

Lyman, C. M., Moseley, O., Wood, S., Butler, B. and Hale, F. 1947
Some Chemical Factors which Influence the Amino Acid requirements of the Lactic Acid Bacteria
J. Biol. Chem. 167, 177

McNutt, W. S. and Snell, E. E. 1950
Pyridoxal Phosphate and Pyridoxamine Phosphate as Growth Factors for Lactic Acid Bacteria
J. Biol. Chem. 182, 557

Melnick, D., Hochberg, M., Himes, H. W. and Oser, B. L. 1945
The Multiple Nature of Vitamin B6: Critique of Methods for the Determination of the Complex and its Components
J. Biol. Chem. 160, 1

Ormsby, A. A., Fisher, A. and Schlenk, F. 1947
Note on the Colorimetric Determination of Pyridoxine, Pyridoxal and Pyridoxamine
Arch. Biochem. 12, 79

Perkin, W. H. 1888
Chlorofumaric and Chloromaleic Acids and the Magnetic Rotatory Power of some of their Derivatives
J. Chem. Soc. (London) 53, 695

Quackenbush, F. W., Kummerow, F. A., Platz, B. R. and Steenbock, H. 1942
Linoleic Acid, Pyridoxine and Pantothenic Acid in Rat Dermatitis
J. Nutrition 24, 225

Rabinowitz, J. C. 1949
Personal Communication

Rabinowitz, J. C., Mondy, N. I. and Snell, E. E. 1948
Vitamin B6 Group XIII. An Improved Procedure for Determination of Pyridoxal with Lactobacillus Casei
J. Biol. Chem. 175, 147

Rabinowitz, J. C., and Snell, E. E. 1947
Extraction Procedures for the Microbiological Determination of Vitamin B6
Anal. Chem. 19, 277

Rabinowitz, J. C. and Snell, E. E. 1948
Vitamin B6 Group XIV. Distribution of Pyridoxal, Pyridoxamine and Pyridoxine in some Natural Products
J. Biol. Chem. 176, 1157

Reid, D. F., Lepkovsky, S., Bonner, D. and Tatum, E. L. 1944
The Intermediary Metabolism of Tryptophane in Pyridoxine Deficient Rats
J. Biol. Chem. 155, 299

Riemann, W., Neuss, J. D. and Naimann, B. 1942
Quantitative Analysis
New York, McGraw Hill

Sarma, P. S., Snell, E. E. and Elvehjem, C. A. 1947
The Bioassay of Vitamin B6 in Natural Materials
J. Nutrition 33, 121

Schlenk, F. and Snell, E. E. 1945
Vitamin B6 and Transamination
J. Biol. Chem. 157, 425

Schweigert, B. S. and Pearson, P. B. 1947
Effect of Vitamin B6 Deficiency on the Ability of Rats and Mice to Convert Tryptophane to N'-Methyl-nicotinamide and Nicotinic Acid
J. Biol. Chem. 168, 555

Scudi, J. V. 1947
The Assay of Vitamin B6 by Means of Physico-Chemical Methods
Biolog. Symp. XII, 240

Snell, E. E. 1944
The Vitamin B6 Group I. Formation of Additional Members from Pyridoxine and Evidence Concerning Their Structure
J. Am. Chem. Soc. 66, 2082

Snell, E. E. 1945a
The Vitamin B6 Group IV. Evidence for the Occurrence of Pyridoxamine and Pyridoxal in Natural Products
J. Biol. Chem. 157, 491

Snell, E. E. 1945b
The Vitamin B6 Group V. The Reversible Interconversion of Pyridoxal and Pyridoxamine by Transamination Reactions
J. Am. Chem. Soc. 67, 194

Snell, E. E. 1948
Use of Microorganisms for Assay of Vitamins
Physiol Rev. 28, 255

Snell, E. E. 1950
Microbiological Methods in Vitamin Research
Vitamin Methods, P. Gyorgy, Editor
New York, Academic Press

Snell, E. E., Guirard, B. M. and Williams, R. J. 1942
Occurrence in Natural Products of a Physiologically Active Metabolite of Pyridoxine
J. Biol. Chem. 143, 519

Snell, E. E. and Rabinowitz, J. C. 1948
The Microbiological Activity of Pyridoxylamine Acids
J. Am. Chem. Soc. 70, 3432

Snell, E. E. and Rannefeld, A. N. 1945
The Vitamin B6 Group III, The Vitamin Activity of Pyridoxal and Pyridoxamine for Various Organisms
J. Biol. Chem. 157, 475

Spoek, M. L., and Pitt, D. A. 1947
A Relationship between Cystine and Pyridoxal or Pyridoxamine in the Nutrition of Certain Bacteria
Science 106, 420

Stokes, J. L., Foster, J. W. and Woodward, C. R., Jr. 1943
Synthesis of Pyridoxin by a "Pyridoxinless" X-ray Mutant of Neurospora sitophila
Arch. Biochem. 2, 235

Stokes, J. L. and Gunness, M. 1945
Pyridoxamine and the Synthesis of Amino Acids by Lactobacilli
Science 101, 43

Teas, H. J., Horowitz, N. H. and Fling, M. 1948
Homoserine as a Precursor of Threonine and Methionine in Neurospora
J. Biol. Chem. 172, 651

Umbreit, W. W., Bellamy, W. D. and Gunsalus, I. C. 1945
The Function of Pyridoxine Derivatives: A Comparison of Natural and Synthetic Codecarboxylase
Arch. Biochem. 7, 185

Umbreit, W. W. and Gunsalus, I. C. 1949
Codecarboxylase not Pyridoxal 3-Phosphate
J. Biol. Chem. 172, 279

Umbreit, W. W., O'Kane, D. J. and Gunsalus, I. C. 1946
Mechanism of Pyridoxal Phosphate Function in Bacterial Transamination
J. Bact. 51, 576

Umbreit, W. W., O'Kane, D. J. and Gunsalus, I. C. 1948
Function of the Vitamin B6 Group: Mechanism of Transamination

Umbreit, W. W., Wood, W. A. and Gunsalus, I. C. 1946
The Activity of Pyridoxal Phosphate in Tryptophan Formation by Cell-Free Enzyme Preparations
J. Biol. Chem. 165, 731

van Dam, W. 1918
Uber den Einfluss der Milchsäure auf die Milchsäuregärung
Biochemische Z. 87, 107

van Overbeek, J. 1935
The Growth Hormone and the Dwarf Type of Growth in Corn
Proc. Natl. Acad. Sci. (U.S.) 21, 292

Westergaard, M. and Mitchell, H. K. 1947
Neurospora V. A Synthetic Medium Favoring Sexual Reproduction
Am. J. Bot. 34, 573

Wood, W. A., Gunsalus, I. C. and Umbreit, W. W. 1947
Function of Pyridoxal Phosphate: Resolution and Purification of the Tryptophanase Enzyme of Escherichia Coli
J. Biol. Chem. 170, 313

Zalokar, M. 1950
The Sulfonamide Requiring Mutant of Neurospora: Threonine-Methionine Antagonism
Unpublished