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Microbiological Studies on the Vitamin B6 Antagonist Found in Flaxseed

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Fungus cultures may have varied effects on the growth of other propinquous fungi. These effects include:

1. A strong antagonism such as was shown by *Emericellopsis humicola* against *P. graminicolum.* Lockwood (7) reported *Streptomyces spp.* demonstrated this type of antagonism to soil fungi.

2. A debilitating effect in which the mycelium continues growth but at a reduced rate. In association with several species in plates, *P. graminicolum* mycelium continued growth at a reduced rate. Griffin (3) reported similar effects.

3. Inhibition of fungus spore germination.

4. Unusual development of sporangia. An unusually large number of sporangia were produced by *P. graminicolum* grown on the plates with *Rhizoctonia solani.*

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Microbiological Studies on the Vitamin B_6 Antagonist Found in Flaxseed[1]

JOHN L. TJOSTEM

Abstract: Extensive investigations were conducted on the development of a microbiological assay of a vitamin B_6 antagonist found in flaxseed. It is the only known naturally occurring inhibitor of vitamin B_6. By means of a screening procedure, ten microorganisms were found to be inhibited by preparations of the B_6 antagonist. One of these, *Bacillus polymyxa,* was investigated more fully. Correlations of the *B. polymyxa* and live chick assays were excellent. All column chromatographic fractions of inhibitor which reduced Tollen's reagent inhibited *B. polymyxa.*

Vitamin B_6 does not competitively reverse the bacterial inhibition. Inhibition of *B. polymyxa* by high levels of Vitamin B_6 and the B_6 antagonist may suggest that the antagonist is an analogue of the vitamin.

[1] This paper is a portion of the author's Master of Science thesis written under suggestion and guidance of his major professor, Dr. Jesse L. Parson, Department of Bacteriology, North Dakota State University, Fargo, North Dakota, 1962. The investigation was entirely supported by a research grant from the National Institute of Health.

Chromatography with ethanol-acetone and acetone alone coupled with the bacterial assay, revealed that the acetone containing solvents, unlike the usual amino acid solvents, moved the hydrolyzed inhibitor near the solvent front. Since ninhydrin positive material remained near the origin, an excellent separation of the inhibitor from these impurities was achieved. The acetone solvents, therefore, offer considerable promise for use in the isolation and purification of the B_6 antagonist.

Introduction

The only known naturally occurring antagonist of vitamin B_6 was found by Kratzer (2) in flaxseed meal. Growth inhibition of young chicks was reversed by the incorporation of pyridoxine into their diet. Schlamb *et al.* (6) found the inhibitor to be contained in the cotyledons of flaxseed. They separated the cotyledon from the hull by saturating the hull with water and crushing the seeds in a roller mill. The cotyledons were removed by floation with petroleum ether. Olsgaard (5) achieved very effective extraction of the inhibitor from the cotyledons with 70% aqueous ethanol. Magill (3) succeeded in separating the inhibitor from much of the impurities by adsorption and elution from the cation resin, Amberlite IR-120 and the anion resin, Amberlite IRA-00. On the bases of these results Magill concluded that the inhibitor was amphoteric in nature. Farely (1) accomplished further purification by means of the ion exchange resin Amberlite IR-120 and Cellex E, and chromatography with a cellulose column.

The need for a rapid assay which could be carried out with small amounts of the inhibitor to facilitate isolation and characterization has been clearly recognized by those who have worked on this project. The purification procedures are time consuming and yield only relatively small amounts of concentrated inhibitor which are often used up in an assay with chicks. For this reason only a small number of birds, usually two or three, are used to assay a given preparation of inhibitor. The development of a microbiological assay has therefore been highly desirable.

Method

Screening Microorganisms for Assay

A screening procedure was used to determine if any of a number of microorganisms could be used for assay of the vitamin B_6 antagonist. Forty-two species were grown initially on nutrient agar slants at their optimum temperatures. The cultures were used to inoculate slants of a completely synthetic medium. This medium which is hereafter called the arginine assay medium is shown below.

	grams/liter		grams/liter
Glucose	10.0	dl-Alanine	0.2
NH_4Cl	3.0	l-Arginine	0.25
KH_2PO_4	0.06	l-Aspargine	0.4
$K_2HPO_4 \cdot 3\ H_2O$	0.06	l-Aspartic acid	0.1
$MgSO_4 \cdot 7\ H_2O$	0.20	l-Cystine	0.05
$FeSO_4 \cdot 7\ H_2O$	0.01	l-Glutamic	0.3
$MnSO_4 \cdot H_2O$	0.02	Glycine	0.1
NaCl	0.01	l-Histidine	0.06
Adenine sulfate	0.01	dl-Isoleucine	0.25
Guanine hydrochloride	0.01	dl-Leucine	0.25
Uracil	0.01	l-Lysine	0.25
Xanthine	0.01	dl-Methionine	0.1
Calcium pantothenate	0.0005	dl-Phenyalanine	0.1
Nicotinic acid	0.001	l-Proline	0.1
P-aminobenzoic acid	0.001	dl-Serine	0.05
Thiamine hydrochloride	0.0005	dl-Threonine	0.2
Folic acid	0.00001	dl-Tryptophan	0.04
Biotin	0.000001	l-Tryrosine	0.1
Ion agar (Consolidated Laboratories)	10.0	dl-Valine	0.25

Slant cultures for the inocula, were prepared from the arginine assay medium to which was added 0.1 μg pyridoxine per ml of medium and potassium citrate to give a final concentration of 0.016 M. The pH was adjusted to 6.8.

Twenty-four hour cultures were suspended in 5 ml of saline and inoculated by making one streak, with a loop of the culture suspension, across the surface of arginine assay medium in square plates. Four to six streaks, each of a different culture, were plated on a single petri plate. Those organisms which would not grow on the assay medium were streaked on plates containing nutrient agar.

The inhibitor preparations used for the screening were an IR-120 eluate and a more highly purified preparation from a Dowex 1 formate column. These two preparations and many others in the work which follows were provided by the Department of Agricultural Biochemistry, North Dakota State University. The activity of the IR-120 eluate was such that 0.5 ml would affect but not kill a chick. Five tenths ml of the Dowex 1 preparation contained one chick LD.

The zones of inhibition were measured after approximately a 24 hr. incubation period at the temperature appropriate for the organism.

Bacillus Polymyxa *as an Assay Organism*

Assay procedure. A soil isolate was found in the screening experiments which was inhibited by the B_6 antagonist. This isolate was later characterized and identified as *Bacillus polymyxa.*

A stock culture of the organism was maintained on nutrient agar slants, but the inoculum for inhibition studies was taken from slants as previously described. A culture from a slant was suspended in 5ml of sterile distilled water diluted to a reading of five on a Klett-Summerson colorimeter with a No. 54 green filter and used for spray inoculation of the medium in petri plates.

Assay disks were dipped into the inhibitor preparation and two or three disks were placed on each plate. The plates were stored at 10°C for 5 to 7 hr. prior to incubation to allow the inhibitor to diffuse into the arginine assay medium containing a 1% concentration of Difco agar. The plates were then incubated at 30° C for 18 to 30 hr.

Correlation of bacterial and chick assays. *Bacillus polymyxa* was tested with several different inhibitors which are listed in the results section. The preparations contained varied amounts of chick inhibitor and were from various stages in the purification process.

Paper chromatography and bioassay of inhibitor. A hydrolyzed Dowex 1 acetate fraction, supplied by the Department of Agriculture Biochemistry, North Dakota State University, was used for paper chromatography and bioassay. The dry weight of the sample was 15 mg/ml.

Chromatograms of Whatman No. 1 filter paper, each 1.5 inch wide, were spotted across the top with 40 μl of inhibitor per inch. Four solvents were made up specifically for this chromatography, and included ethanol and/or acetone because of the known solubilities of the inhibitor in these chemicals. These four solvents were: 1) ethanol-acetone (1:9, v/v); 2) ethanol-acetone (3:7, v/v); 3) ethanol alone; and 4) acetone alone. As a control, the amino acid solvent, butanol-acetic acid-water (4:1:1, v/v), was also used. Each chromatogram was divided into three ½-inch strips. One strip was cut up and the sections placed on the agar for bacterial assay. One of the two remaining strips was tested with Tollen's reagent, the other was developed with ninhydrin. An eluate of IR-120 was also chromatographed with solvents 2) and 4).

Standard curve of inhibition of Bacillus polymyxa. The response of *B. polymyxa* to graded amounts of the inhibitor was determined by measuring the diameter of the zones of inhibition. Three assay disks (¼-inch diameter) for each dilution were placed on separate plates. Immediately after placing the disk on the surface of the medium, 10 μl of an inhibitor preparation was pipetted onto it. The plates were spray inoculated, incubated 24 hr. at 30°C, and the zones of inhibition measured.

Effect of B_6 on reversal of inhibition of Bacillus polymyxa. Assay disks were saturated with a 10 and 100 mg solution of pyridoxine. The disks containing B_6 were placed edge to edge with disks saturated with the hydrolyzed Dowex 1 fraction previously described. In other experiments various forms of B_6 were added to the assay medium. Pyridoxine, pyridoxamine, pyridoxal, pyridoxal phosphate and pyridoxine phosphate (Nutritional Biochemicals Co.) were incorporated into separate plates at level of 0.1, 3.0, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100.0 μg/ml of medium. The inhibitor preparation was pipetted onto disks and the plates inoculated, incubated and read as before.

Results

Of the forty-two organisms assayed, ten were inhibited. The extent to which the organisms were inhibited as well as other factors are shown in Table 1. Because of excellent inhibition of the soil isolate A-12, which also grew well on the arginine assay medium, it was chosen for further investigation.

Table 1. Sensitivity of microorganisms to the inhibitor

Organism	Inhibitor preparation	Medium	Background growth	Inhibition zone in mm
1. *A. aceti*	Dowex 1 and IR-120	ar*	light	30
2. *A. vinelandii*	Dowex 1	ar	moderate	16
3. *B. brevis*	Dowex 1	na**	moderate	21
4. *E. freundii*	Dowex 1 and IR-120	ar	moderate	18
5. *G. tetragena*	Dowex 1	na	moderate	15
6. *M. lysodeikticus*	Dowex 1	na	moderate	20
7. *P. vulgaris*	Dowex 1	na	moderate	12
8. *R. rubrum*	Dowex 1	na	moderate	17
9. Soil isolate A-12 (*B. polymyxa*)	Dowex 1 and IR-120	ar	moderate	30
10. Air contaminant	Dowex 1	na	moderate	15

* Arginine assay medium.
** Nutrient agar for assay.

Modification of the Assay Medium for us with Bacillus polmyxa

Several factors concerning the arginine assay medium were varied. Plates containing 1.0% Difco agar had 31 mm zones of inhibition compared with 25 mm on plates containing 1.5 and 2.0% agar. Plates with Ion agar at 2.0% concentration had inhibition zones of 18 mm. Neither citrate nor phosphate buffers, used in the arginine medium, interfered with the inhibition. The pH of the medium, varying from 6.2 to 7.4, had little effect on the amount of inhibition produced by a given preparation. Glutamic acid was found to be essential for growth of the organism but otherwise it did not affect the assay. A storage period of 5 to 7 hr. in the cold room, to allow diffusion of the inhibitor was

found to improve the sensitivity of the assay and to give larger zones of inhibition. The optimum incubation temperature for assay was 30°C. Eight min. of ultraviolet irradiation was found to improve the assay sensivity. An inhibitor preparation producing a zone 14 mm wide on unirradiated medium produced a zone 34 mm wide on irradiated medium. Medium subjected to 10 mm or longer periods of irradiation did not support satisfactory background growth of *B. polymyxa*. Medium irradiated 5 min. was not as sensitive for inhibitor assay as that irradiated 8 min.

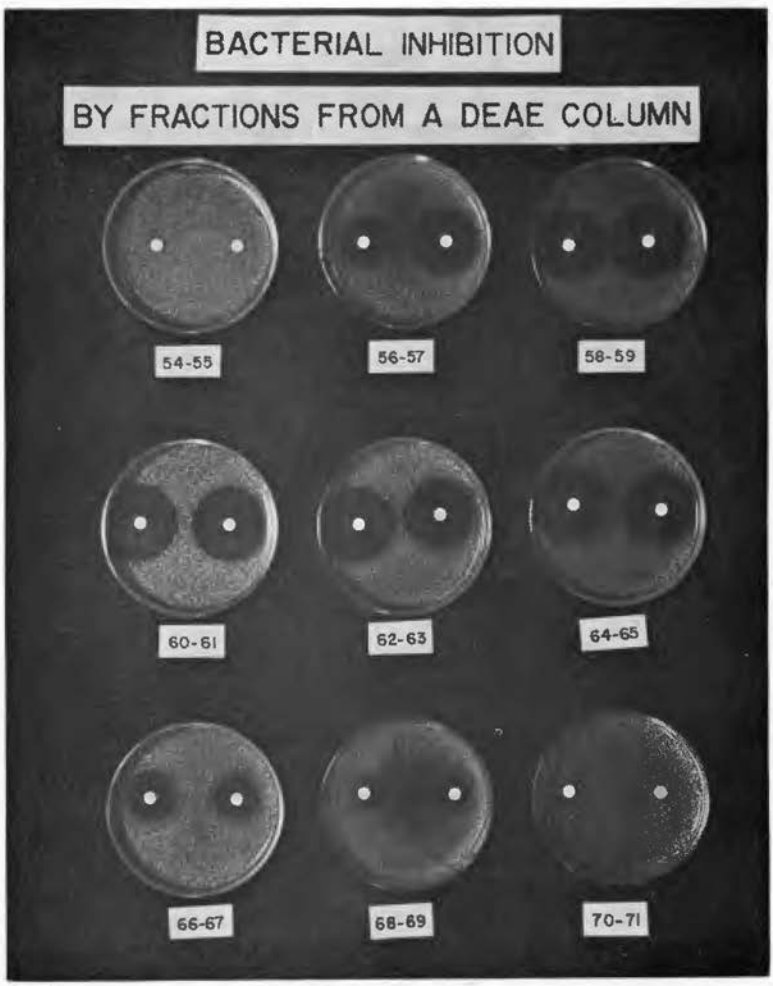

Figure 1. *B. polymyxa* assay of hydrolyzed inhibitor put through a DEAE column (fractions provided by Lamoureux, Department of Agricultural Biochemistry, North Dakota State University)

Correlation of Bacterial and Chick Assays

The chick activity and microbiological activity gave perfect correlation in twenty-six of thirty cases, the four exceptions could not be reassayed due to insufficient quantities of inhibitor. In all subsequent assays complete bacterial and chick correlation was observed.

Farley (1) reported that compounds that reduce Tollen's reagent were present in all physiologically active preparations and absent in all preparations which gave no chick inhibition. This same correlation was true for the bacterial assay.

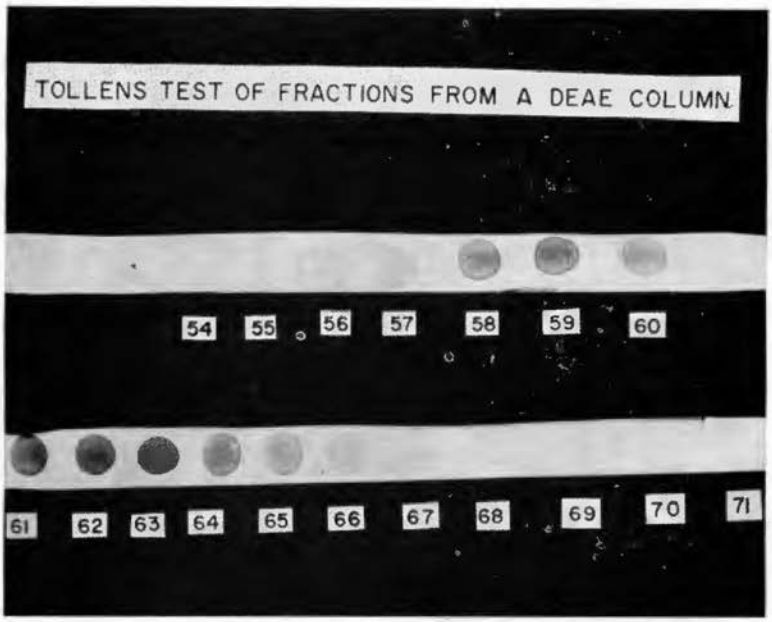

Figure 2. Tollen's test on the same DEAE fractions used to demonstrate bacterial inhibition in Figure 1 (Tollen's test by Lamoureux, Department of Agricultural Biochemistry, North Dakota State University)

Paper Chromotography and Bioassay of Inhibitor

A hydrolyzed Dowex 1 fraction was chromographed with five solvents, four of which were made up specifically for this chromotography. An amino acid solvent was used as a control. It was found that the hydrolyzed inhibitor was separated from the ninhydrin positive material present in the sample by acetone alone or ethanol-acetone (1:9 or 3:7 v/v) solvents (Table 2). With these solvents the ninhydin positive materials remained close to the origin while the inhibitor was found near the solvent front. Neither the inhibitor nor the ninhydrin positive materials of the unhydrolyzed IR-120 eluate moved appreciably from the origin with either the acetone alone or the ethanol-acetone solvents.

Table 2. Paper Chromatography of Inhibitor (fractions 59, Figure 1)

Solvent	Microbiological assay R_f	Ninydrin R_f	Tollen's R_f
1. Ethanol-acetone (1:9,v/v)	0.84	0.01	0.84
2. Ethanol-acetone (1:7,v/v)	0.83	0.03	0.83
3. Ethanol alone	0.18	0.07 & 0.20	0.18
4. Acetone alone	0.86	0.00	0.86
5. Butanol-acetic-acid-water (4:1:1,v/v)	0.44	0.27, 0.40 & 0.53	0.44

Standard Curve of Inhibition of Bacillus polymyxa.

The linear response of *B. polymyxa* to a logrithmic increase in the concentration of the inhibitor is shown in Figure 3.

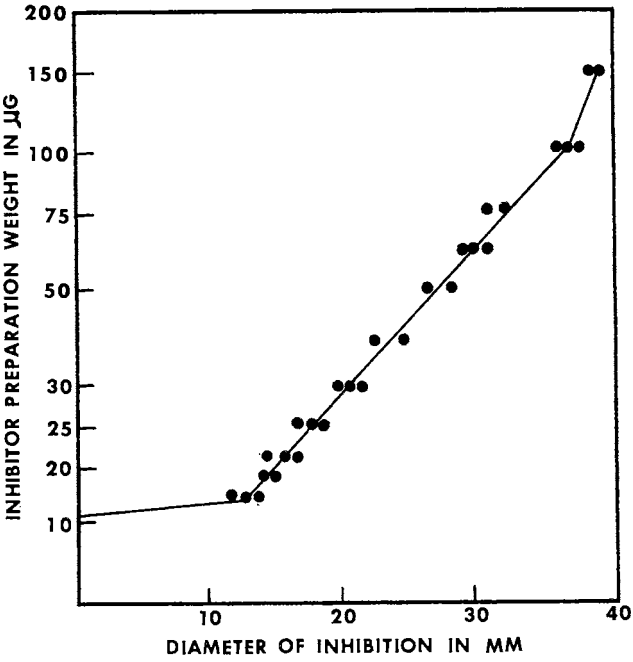

Figure 3. Standard curve of inhibition of *B. polymyxa* by hydrolyzed DEAE inhibitor preparation, fraction 59 of Figure 1, plotted on semi-log scale, and zone diameters shown include the 6.35 mm diameter of assay disk.

Effect of B_6 *on Reversal of Inhibition of* Bacillus polymyxa

Pyridoxine, pyridoxal and pyridoxamine added singly to the arginine assay medium at 0.1, 3.0, 5.0 and 10.0 µg/ml caused growth stimulation. The zones of inhibition on these plates ranged from 18 to 21 mm. The control plates with no added vitamin B_6 had zone diameters of 21 to 24 mm. The three forms appeared equal with regard to their effect on zones of inhibition.

Pyridoxine, pyridoxal and pyridoxamine at 15 μg/ml gave a marked reduction in the background growth, but no effect was observed on the zones of inhibition. However, when these three forms were added separately to the assay medium at 20 and 25 μg/ml the zones of inhibition were increased to 25 through 27 mm. The background growth was very light and the plates had to incubated 30 hr. before growth became visible. Levels of 50 to 100 μg/ml completely inhibited the organism. Pyridoxine phosphate and pyridoxal phosphate added to the medium at the same levels as the unphosphorylated forms did not visibly affect the assay.

Discussion

In the search to find an assay microorganism, the screening procedure, applied to forty-two organisms, revealed ten which were inhibited (Table 1). Nine of the ten organisms inhibited were gram negative. The other, *B. polymyxa,* is closely related to the gram negative organisms. It is classified gram variable by Bergey's Manual. The physiology of gram negative organisms is considerably different from that of gram positive bacteria. Some antibiotics are effective against gram positive and not gram negative and vice versa. Of course the two types of bacteria react differently to the gram stain and the cell wall and permeability properties are markedly different. It is therefore not a surprise to find that this inhibition was confined to one of the two groups of bacteria investigated.

Correlation of inhibition of *B. polymyxa* to that of the chick was excellent. Inspection of the data in Table 2 shows that the time required to inhibit a chick was inversely related to the zones of inhibition of the bacteria for those inhibitor preparators with data on time required for inhibition of the chicks.

Because of the key role that glutamic acid plays in amino acid metabolism, its effect on the assay was investigated. *B. polymyxa* could not be cultured on the arginine assay medium if glutamic acid were deleted; however, reducing the concentration of glutamic acid did not affect the assay except to give a lighter background growth. Glutamic acid is a very important amino acid in transamination and decarboxylation reactions in that many other amino acids may be formed from it. The fact that glutamic acid is required by *B. polymyxa* may be important in determining the mode of action of the B_6 antagonist for this bacterium. Further evidence for a possible role of glutamic acid in inhibition by the B_6 antagonist is the chick which shows symptoms of a nervous disorder when given the B_6 inhibitor. McCormik and Snell (4) described certain brain disorders in humans which they attributed to a decrease in gamma-amino-

butyrate caused by deficiency in pyridoxal phosphate. Furthermore the antitubercular drug isoniazid inhibits pyridoxal kinase, thus preventing the formation of pyridoxal phosphate, which in turn may result in decreased levels of gamma-aminobutyric acid. Since *B. polymyxa* is dependent upon glutamic acid it may be that inhibition of a reaction similar to the decarboxylation described above occurs when the inhibitor is present. Gamma-aminobutyric acid, however, neither replaces the requirement of glutamic acid by *B. polymyxa,* nor reverses the bacterial inhibition produced by the B_6 antogonist.

The response of *B. polymyxa* to graded amounts of the hydrolyzed inhibitor, DEAE fraction 59 (Figure 1) was determined. The preparation was concentrated enough to show maximal response. At 100 μg per disk the quantitive response changed as indicated by the increase in slope of the standard curve in Figure 3. The response, however, may have been limited by diffusion. If a longer diffusion period had been allowed, a larger zone would be produced and more inhibitor would have been required to produce a maximal response. The lower threshold at 13 μg dry weight per disk was evident as was the linear response of inhibition (determined by zone diameter to logarithmic increases in the amount of inhibitor. Refinements in the assay medium may lower the threshold of minimal response considerably. The preparation used here may not have been as pure as some other samples previously assayed in which amounts as low as 5 μg gave detectable inhibition. This preparation was the most refined preparation available at the time; also much research had been carried out with this fraction. The activity of the fraction remained consistent over the several week period during which it was used.

Vitamin B_6 did not competively reverse the inhibition of the B_6 antagonist for B. *polymyxa.* In low levels from 0.1 to 10 μg/ml of medium, there was a small reduction of zone diameter, but this may have been due to a general growth stimulation. On the other hand it could have been that the amount of inhibitor was too much to be reversed by vitamin B_6 at the range below 10 μg/ml. Since 30 μg of inhibitor preparation was applied to the disk, and if the inhibitor from the disk diffused into 3 ml of medium, then each ml would contain 10 μg of inhibitor, assuming one hundred per cent purity. Therefore if the molecular weight of the inhibitor were the same as that of vitamin B_6 at a mole for mole ratio, 10 μg/ml of B_6 should have reversed the inhibition. The preparation, however, was by no means pure so less than 10 μg/ml if inhibitor was present and less than this amount of B_6 should have caused reversal of the inhibition. This was not the case; and since higher levels of B_6 inhibited the

bacterium it would appear that inhibition of *B. polymyxa* is noncompetitive with vitamine B_6. Rather its noncompetitive nature may be similar to that of isoniazid discussed above which inhibits the pyridoxal kinase enzyme by forming a hydrazone with the carbonyl group of pyridoxal.

The fact that higher levels (20 μg) of either pyrodoxine, pyridoxal, or pyridoxamine decreased the background growth and increased the zones of inhibition, and that 50 μg caused total inhibition, may be an indication that the inhibitor is an analog of the vitamin and inhibits *B. polymyxa* because of the similarity of its structure to vitamin B_6. Wiseman (7) using everted intestinal sacs reported that pyridoxal, in levels comparable to those used here, inhibited the active transport of glycine and l-histidine. The phyosphorylated forms did not affect the assay with *B. polymyxa.* These forms usually enter the cell less readily which may explain why they had no effect upon the growth and/or inhibition of *B. polymyxa.*

Paper chromatography of the hydrolyzed inhibitor, DEAE fraction 59, was carried out with solvents, so chosen that they could be evaporated readily from the paper, which could then be applied directly to the surface of agar plates for assay. The purpose of the chromatography was to correlate the position of the material on the paper chromatogram, that inhibited the bacteria, to that which reduced Tollen's reagent. Acetone was chosen because of its volatility and ease of removal by drying and because the inhibitor was soluble in aqueous ethanol. Ethanol was also added to acetone to move the inhibitor down the paper chromatogram. It was also considered that as a result of the known relatively insoluble nature of amino acids in acetone that their movement might be limited in solvents containing acetone. Acids and bases were omitted from these solvents because of the difficulty of removing them from the paper chromatograms and because of the possibility of their inhibition of *B. polymyxa* on subsequent assay. Contrary to expectations the inhibitor was found to travel near the solvent front with the solvents acetone-alcohol and acetone alone. This was a most surprising discovery since in all previous research the inhibitor had been found to be insoluble in cetone; however, all prior work on acetone solubility had been on unhydrolyzed inhibitor. From these results it was clear that acid hydrolysis of the inhibitor had markedly changed its solubilities, especially in acetone.

Since the hydrolyzed inhibitor traveled at the solvent front, and the amino acids remained at the origin, with acetone as the solvent, it offered a very promising method of the separation of the inhibitor from the tenacious amino acid impurities. Such a separation was not possible with the usual amino acid solvents.

This important principle for use in purification of the inhibitor was made possible because of the capacity of the bacterial assay to detect very small quantities of the inhibitor on paper chromatograms. The inhibitor, as located by bacterial assay, and the Tollen's reagent for reducing groups were found at the same position on the chromatograms for all solvents used. The identical location of the Tollen's positive material and the microbiologically active component lends support to a similar relationship proposed by Farley (1) for the Tollen's sensitive substances and chick activity; the two relationships considered together provided support for the belief that the inhibitors for *B. polymyxa* and the chick are the same.

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Preliminary Survey of the Algae of Lake Ahquabi

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Abstract: Forty-one genera of algae are reported from Lake Ahquabi, Warren County, Iowa. The most commonly occurring genera are *Cladophora, Spirogyra, Hydrodictyon, Microcystis, Oscillatoria* and *Aphanizomenon.* The latter three contribute to extensive blooms on the lake in late summer and fall.

Lake Ahquabi, located in Warren County, Iowa, is a 130 acre artificial lake occupying parts of sections 14 and 23, R24W, T75N. The lake was established by impoundment in 1935 in an area of the Kansan drift covered by loess and underlain by a bedrock of sandstone and shale. Average depth of the lake is approximately 10 feet with a maximum depth of 22 feet in one area. The shallow littoral zones in the north and south arms