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EFFECT OF HYDRAZINES ON VITAMIN B_6 LEVELS IN THE MOUSE BRAIN

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FOREWORD

This study was initiated by the Biomedical Laboratory of the Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio. The research was conducted by the Institute of Chemical Biology, University of San Francisco, San Francisco, California 94117, under Contract No. AF 33(615)-2332. Kenneth C. Back, PhD, Chief, Toxicology Branch, Toxic Hazards Division, Biomedical Laboratory, was the contract monitor for the Aerospace Medical Research Laboratories. The work was performed in support of Project 6302, "Toxic Hazards of Propellants and Materials," and Task 630202, "Pharmacology-Biochemistry." This study was started in March 1965 and completed in February 1966.

The authors wish to express their appreciation to Mr. George Ledin, Jr., for his help in experimental design and statistical evaluation of data. The authors also desire to express their indebtedness to Mr. Eric Gustavson, Mr. Mars Davis, and Miss Janice Johnson for their excellent laboratory help.

This technical report has been reviewed and is approved.

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ABSTRACT

The effects of administered 1,1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH) on vitamin B_6 levels in mouse brain have been studied. Separation of the B6 group (pyridoxol, pyridoxal, pyridoxamine, and the respective 5-phosphates) by means of paper chromatography revealed that the R_F values obtained are dependent upon the pH of the developing solvent. To obtain the time lag just prior to convulsions induced by UDMH and MMH, a dose-lag time study was conducted; included were pyridoxal (and its 5-phosphate) hydrazones of UDMH and MMH. Graphs of log dose vs lag time are given. The bioassay procedure, though not completed, permits detection of some of the B_6 congeners to a limit of 0.5 nanograms.

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SECTION I

INTRODUCTION

For some time substituted hydrazines have been known to be convulsant agents. Dieke (ref 1) found that thiosemicarbazide at levels of 10-30 mg/kg caused fatal convulsions in all species tested. The degree of toxicity is dependent upon the substituent group: semicarbazide required a dose of 250 mg/kg for activity and hence was not as toxic as thiosemicarbazide (ref 2). Papers have been appearing regularly on the convulsant activity of other substituted hydrazines, especially those with alkyl groups. Among these is the rocket propellant 1,1-dimethyl hydrazine (UDMH), which is also toxic to a variety of species of animals (ref 3). Common characteristics of these toxic agents are that they induce convulsions prior to death and that there is a definite time lapse after administration of the agent until the onset of the seizure. The latter phenomenon can be quantitatively expressed. A straight line was approximated by plotting log of dose vs time lapse (lag time) prior to onset of first seizure (ref 4).

Antidotes for these induced convulsions have also been studied. These include the carbonyls (ref 4), phenobarbital (ref 5), pyridoxamine (ref 6), and pyridoxol (refs 7, 8, 9).

In studying the mechanism of convulsant action of hydrazones, brain levels of γ-aminobutyric acid (GABA) were measured. There is uncertainty as to the effect of semicarbazide and thiosemicarbazide on these GABA levels. The GABA values were reported lowered according to one study (ref 10), and elevated according to another (ref 11).

A theory of action of the convulsant hydrazines was proposed by Snell and coworkers (ref 12). The theory is that the hydrazines combine with pyridoxal to form hydrazone derivatives which are kinase inhibitors; these prevent the phosphorylation of pyridoxol and pyridoxal. The B_6-5-phosphates are essential enzyme cofactors for many brain enzymes which include decarboxylases and amine oxidases.

The members of the vitamin B_6 complex are not equally effective as protective agents. Moreover, a pyridoxol antagonist, 4-deoxypyridoxol, increases the toxicity of at least one hydrazine, phenelzine (ref 13).

Because of the possible relationship between the alkyl hydrazines and pyridoxol, a study was inaugurated in our laboratories to determine the effect of these alkylated hydrazines on the B_6 content of the rodent brain.

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This study required the following phases of investigation: separation of vitamin B_6 complex members (including the 5-phosphate and a mixture of vitamin B_6 from mouse brain) by paper chromatography; in vivo studies of hydrazine and B_6-hydrazone induced convulsions; development of an ultrasensitive quantitative bioassay method for B_6 detection using neurospora; and the verification of our neurospora method with an ultrasensitive quantitative bioassay method for B_6 detection using yeast.

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SECTION II

SEPARATION OF VITAMIN B_6 COMPLEX (INCLUDING THE 5-PHOSPHATES AND A MIXTURE OF VITAMIN B_6 FROM MOUSE BRAIN) BY PAPER CHROMATOGRAPHY

INTRODUCTION

Resolution, detection, and the acceptability of the chromatographed B_6 congeners to the microorganisms involved in the bioautographic procedure are the major problems in this phase of experimentation.

Resolution is dependent upon a number of parameters including solvent system, pH and temperature, and will vary accordingly if one of these parameters is not kept constant. Other experimenters noted that R_F values of the B_6 complex varied from run to run (ref 15) but the suggestion that this variation might have been pH dependent was not made. To achieve optimum separation, we studied the variation of R_F values of the vitamin B_6 group as a function of the pH of a solvent system at constant temperature.

Detection had to be achieved so as not to alter the B_6 congeners and thus inactivate them for the ultrasensitive bioautographic procedure to which the chromatographed strips were later subjected. To detect the components of vitamin B_6 complex, the following developers were investigated: 10% silver nitrate, 10% ferric chloride (separately, and silver followed by ferric), Ehrlich's reagent, and 2,6-dichlorophenolindophenol followed by a counterspray of ammonium hydroxide vapors. Finally, UV light, both short- and long-wave, was used. The long-wave UV light proved to be the most satisfactory detector for this work. The 3660 A lamp for exciting fluorescence in the B_6 complex is simple to use, is sensitive enough, and will not alter the B_6 components for future bioautographic procedures.

Various solvent systems were investigated. These included: n-amyl alcohol; acetone:water; n-amyl alcohol:pyridine:water; n-butanol:water; and n-butanol:pyridine:water. The latter solvent system was chosen since it achieved good separation and did not appreciably interfere with the microorganisms used in the bioassay procedure.

All six B_6 congeners were used: pyridoxol, pyridoxal, pyridoxamine, and their corresponding 5-phosphates. Brains from hydrazine-convulsed and normal mice were also tested for B_6 content via paper chromatography. All data were subjected to statistical analysis.

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METHODS

The solvent used was pyridine:butanol:water (1:2:saturated). The pH of the solvent was adjusted with either HCl or NH_4OH and verified with the aid of pHydrion paper (low range buffer). Twenty-five double strips of Whatman No. 1 chromatographic paper were used for each member of the group; this paper was chosen because it had the most uniform structure of the several types of paper examined, thus giving the least variability in the background absorption (ref 16). A specific member of the B_6 complex was applied three times and the successive spots were dried completely; each composite spot contained 10 µg B_6. All spots were identified by fluorescence under ultraviolet light at 3660 A; the original spot was outlined with pencil. The key followed was: Strips A - pH 5; Strips B - pH 6; Strips C - pH 7; Strips D - pH 8; and Strips E - pH 9. The pH was checked and readjusted when necessary before each run, and the solvent inside each hydrometer jar was changed to avoid contamination with previous compounds. The spotted strips were placed inside the hydrometer jars containing the solvent at the corresponding pH, and set for saturation (15 min). They were then dipped into the solvent and left inside the jars for 1 hour, removed and dried with a heat gun; the entire operation was performed in a darkened laboratory and the chromatographic process took place in a dark chamber. The spot was located with a UV lamp (3660 A). For pyridoxal, fluorescence was greatly enhanced with NH_4OH (conc.) vapor. On the other hand, the fluorescence of pyridoxol and pyridoxamine was slightly inhibited by this treatment. The above method was followed with both individual components and mixtures of components of B_6.

Rodent brains were prepared in the following manner: Mice were sacrificed by cervical fracture and the entire brain removed. The brains were immediately frozen solid to stop all enzymatic action. Homogenization was carried out with teflon-glass pestle and tube at 5000 rpm for 5 minutes. To avoid the frictional heat of homogenization, the tube was chilled with dry ice-acetone during the process. The homogenization solvent was the same as the extraction solvent (pyridine: butanol:water; 1:2:saturated). The brain homogenate was then centrifuged at 4500 rpm for 30 minutes. Individual brain preparations were used for the chromatographic analysis and approximately 20 lambda of supernatant brain homogenate was applied to each chromatographic strip. Each detected spot contained approximately 0.1 µg B_6.

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RESULTS

All R_F values were computed for each pH. Table I gives the mean R_F plus or minus one standard deviation. Analysis of the data was carried out with the aid of the Y-test. * The Y-test was developed as part of this project and is reported in Appendix I. The results of the chromatography work are shown in figure 1; for each result the confidence bands are also given in figures 2, 3 and 4. The R_F values obtained from chromatography of mouse brain are given in tables II and III.

*Ledin, G., Jr., W. R. Gustavson, and A. Furst, "Quality Testing of Chromatographic Data with the Aid of a Statistical Criterion," J. Chrom., V. 22 (1966) 376-380.

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TABLE I

pH DEPENDENCE OF R_F VALUES OF B_6 COMPOUNDS

pH	PYRIDOXOL	PYRIDOXAL	PYRIDOXAMINE
5	0.79±0.01	0.71±0.02	0.25±0.05
	0.79±0.02	0.15±0.01	0.08±0.03 ($5PO_4$)
6	0.79±0.01	0.75±0.01	0.33±0.02
	0.80±0.02	0.16±0.01	0.07±0.03 ($5PO_4$)
7	0.76±0.02	0.64±0.03	0.48±0.03
	0.76±0.02	0.10±0.02	0.05±0.01 ($5PO_4$)
8	0.71±0.01	0.60±0.02	0.53±0.05
	0.73±0.02	0.10±0.01	0.07±0.01 ($5PO_4$)
9	0.57±0.03	0.51±0.01	0.49±0.03
	0.64±0.03	0.11±0.02	0.06±0.01 ($5PO_4$)

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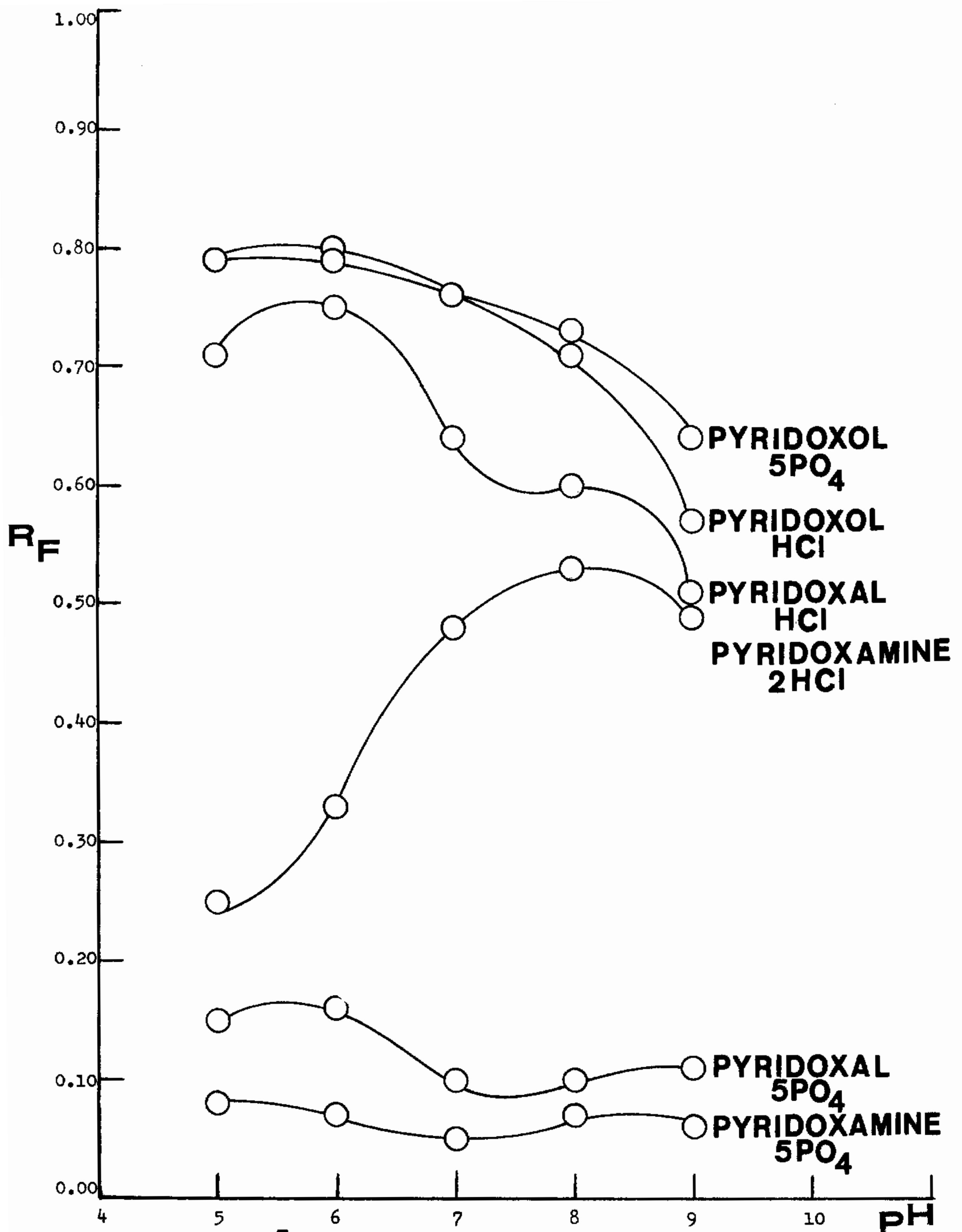

FIGURE 1 pH DEPENDENCE OF R_F OF B_6

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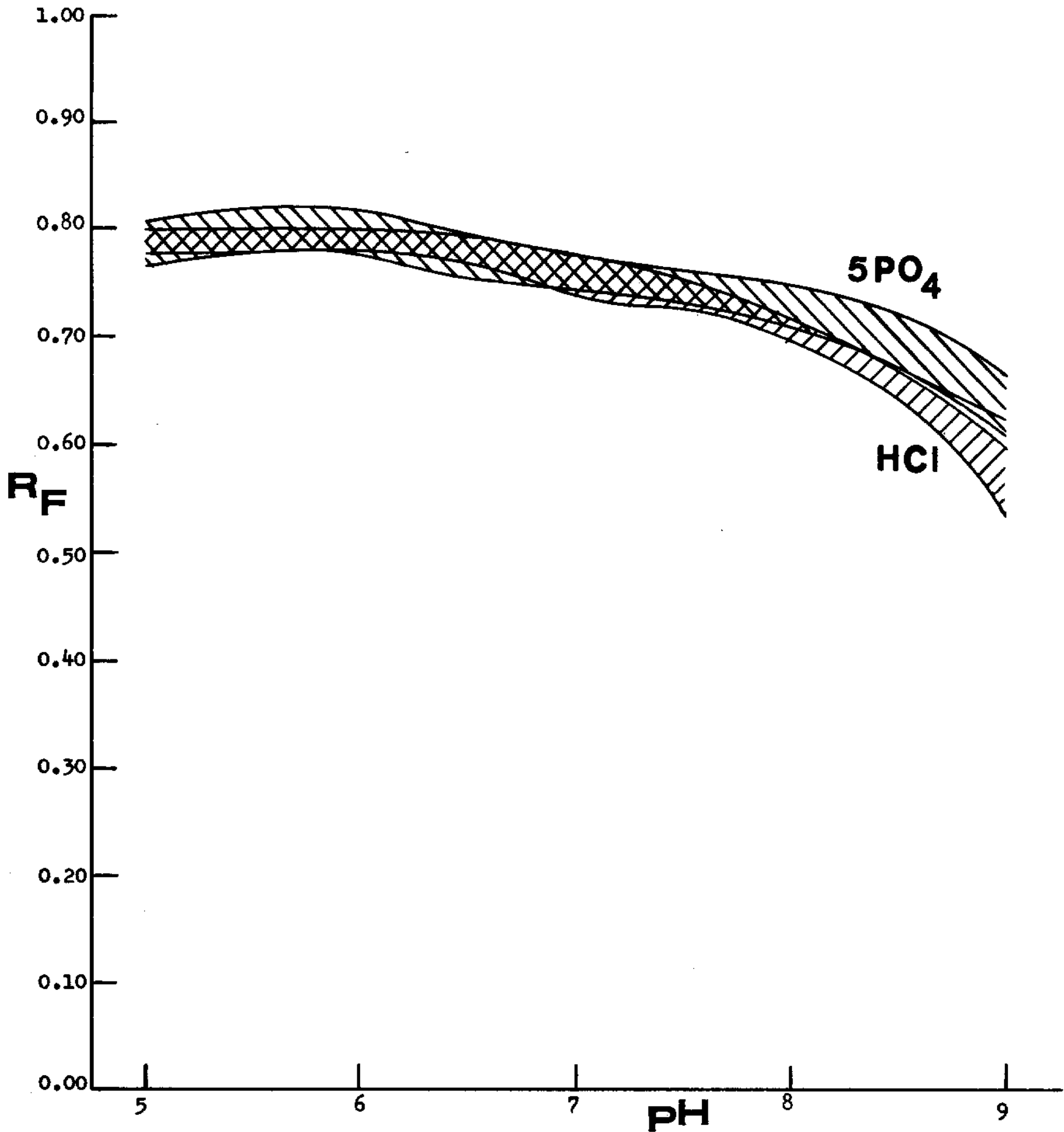

FIGURE 2 PYRIDOXOL CONFIDENCE BANDS

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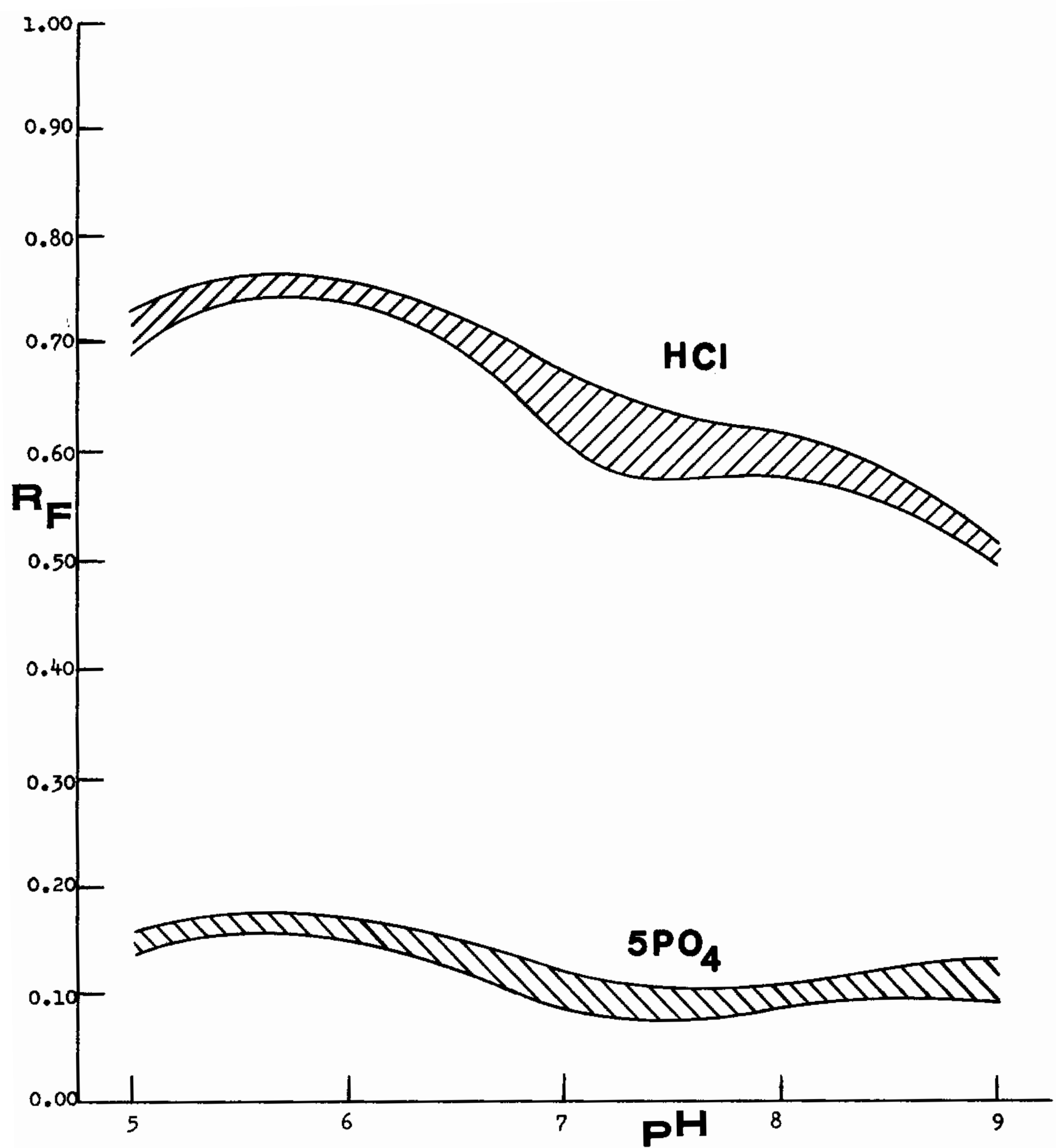

FIGURE 3 PYRIDOXAL CONFIDENCE BANDS

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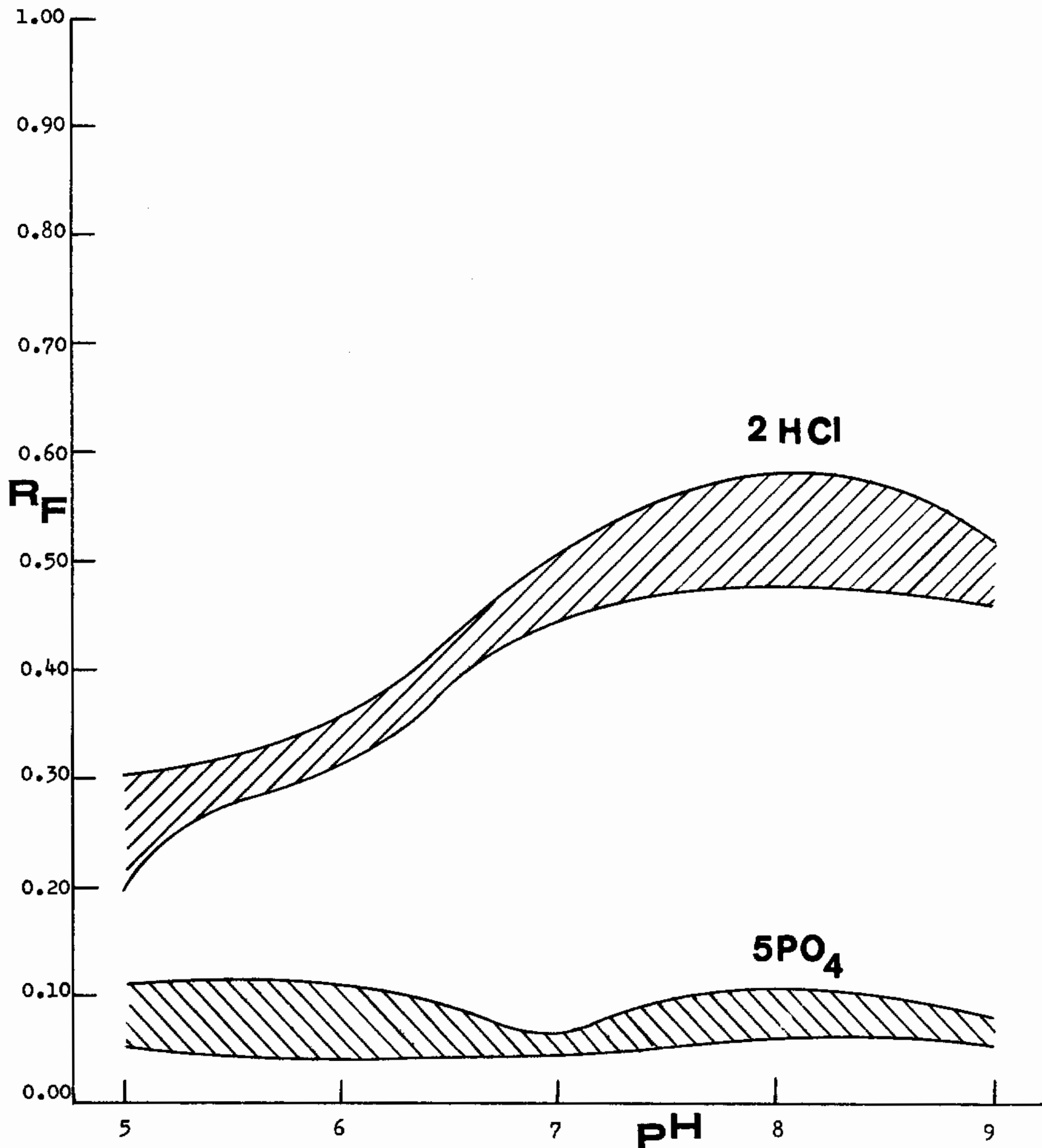

FIGURE 4 PYRIDOXAMINE CONFIDENCE BANDS

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TABLE II

pH DEPENDENCE OF R_F VALUES OF VITAMIN B_6 IN MOUSE BRAIN

pH	Control[a]	UDMH Convulsed[b]	MMH Convulsed[c]
5	0.87±0.03	0.98±0.01	0.75±0.03
	0.07±0.01	0.27±0.02	0.17±0.06
6	0.90±0.01	0.95±0.02	0.79±0.04
	0.07±0.02	0.14±0.02	0.10±0.03
7	0.90±0.02	0.95±0.02	0.76±0.04
	0.07±0.02	0.11±0.02	0.08±0.02
8	0.94±0.03	0.95±0.02	0.78±0.03
	0.09±0.01	0.10±0.03	0.11±0.02
9	0.94±0.02	0.95±0.01	0.84±0.04
	0.08±0.03	0.12±0.02	0.10±0.03

a. Brains from non-convulsed saline-injected animals: Control
b. UDMH: Unsymmetricaldimethylhydrazine (150 mg/kg)
c. MMH: Monomethylhydrazine (50 mg/kg)

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CONCLUSIONS AND DISCUSSION

Chromatography of mixed vitamin B_6 revealed that only four of the six compounds were separable at any one pH. Explanation for this occurrence can follow both statistical and chemical arguments. The statistical argument involves the difficulty of separation of the hydrochloride and the phosphate of pyridoxol. The R_F values of these two compounds are very close (possible hydrolysis) and their confidence bands overlap, with the only possibility of separation occurring at pH 9. The chemical argument enters in the difficulty of identifying the compound with the lowest R_F value (R_F = 0.10 approx.); the phosphates tend to remain at this level, the pyridoxamine phosphate with a tendency to be lower, and the pyridoxal phosphate with a tendency to be just above the R_F = 0.10. For example, when the mixed components of vitamin B_6 were chromatographed, the lowest R_F value noted was 0.12, while the lowest R_F values obtained from chromatography of individual components of vitamin B_6 were those of pyridoxamine phosphate ($R_F = 0.07 \pm 0.03$) and pyridoxal phosphate ($R_F = 0.16 \pm 0.01$).

Although these difficulties do arise, four compounds are easily separable at any one time: pyridoxol, pyridoxal hydrochloride, pyridoxamine hydrochloride, and the phosphate of either pyridoxal or pyridoxamine. A medium with a specific pH may be selected to insure separation of desired B_6 compounds (e.g., pyridoxol hydrochloride and pyridoxol phosphate at pH 9).

Our experimental observations confirm the theory that the area of the chromatographed spot decreases with increase of pH of the solvent system (ref 16). Thus, starting with an average area of the original spot of 2.4 cm^2, the chromatographed spot at pH 5 acquired an average area of 9.3 cm^2, while at pH 9 the average area of the chromatographed spot was 6.0 cm^2. A relationship can be established between the area of the chromatographed spot and the pH:

$$\text{Area} = \frac{C}{pH}$$ (where C is a constant inherent to the pH)

The problem of separation was investigated with a probabilistic hypothesis (ref 17), and estimated confidences were from 95% to 99% for pH 5, 6, and 7; the confidence limits dropped considerably at pH 9.

Chromatography of mouse brain revealed only two spots that could be correlated with B_6 components (a special run at pH 7.5 with concentrated brain fluid produced five spots (see table III)). Using R_F criteria, some spots were discarded since the R_F values obtained did not correspond to any one of the R_F values of specific B_6 compounds. Chromatography of brains of convulsed mice produced similar results. R_F values of some hydrazine compounds are shown in table IV for reference.

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TABLE III

R_F VALUES OF VITAMIN B_6 COMPOUNDS IN MOUSE BRAIN AT pH 7.5

0.81±0.04	(Pyridoxol)
0.70±0.01	(Pyridoxal HCl)
0.53±0.02	(Pyridoxamine 2HCl)
0.39±0.03	(Pyridoxal $5PO_4$)
0.15±0.02	(Pyridoxamine $5PO_4$)

TABLE IV

R_F VALUES OF HYDRAZINES (AT pH 6)

Hydrazines	R_F Values
Thiosemicarbazide (TSC)	0.60
Isonicotinic Hydrazide (INH)	0.58
Unsymmetricaldimethylhydrazine (UDMH)	0.43
Monomethylhydrazine (MMH)	0.39

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SECTION III

IN VIVO STUDIES OF HYDRAZINE AND B_6-HYDRAZONE INDUCED CONVULSIONS

INTRODUCTION

A comparative study of the convulsant effects of monomethylhydrazine (MMH), 1,1-dimethylhydrazine (UDMH), and their respective pyridoxal (B_6-Al) and pyridoxal-5-phosphate (B_6-Al-5PO_4) hydrazones was made. Emphasis was placed on the relationship between the dose of the convulsant and the relative times of both onset of the first seizure and of death; toxicity was investigated also.

METHODS

The sources for the hydrazines were: UDMH (K & K Laboratories, Plainview, New York, and Eastman Organic Chemicals, Rochester, New York); MMH (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin).

The hydrazones were all prepared by dissolving the calculated amount of pyridoxal or pyridoxal-5-phosphate in water, and then heating the solution to 50 C. An equimolar amount of the hydrazine dissolved in about 5 ml of water was then added. The temperature was kept at 50 C for 1 to 2 hrs and then the solutions were cooled to 0 C. The crystals that appeared were separated by filtration. Melting points are similar to those reported by Wiley (ref 18).

CONVULSION WORK

Various doses of convulsant agents were administered to Swiss-Albino mice (average weight 22$\pm$2 g) that were weighed just prior to injection. The agents were dissolved or suspended in normal saline and made up to a concentration so that the ml volume of vehicle administered intraperitoneally was equal to: weight of mouse in grams/100. Dose levels ranged from 2.5 to 100 mg/kg, depending upon the convulsant agent.

The animals were housed individually in large cylindrical cages (dimensions: 8" diameter x 7" height). Experiments were carried out in a quiet laboratory with subdued light; care was taken in order to make certain that a convulsing mouse would not trigger any other mouse into convulsions.

Five animals were tested at each dose level. The convulsigenic agent was administered to each animal at 1 minute intervals. The time of onset of first and subsequent convulsions was recorded, although some mice remained in a state of continuous seizures. Time of death was also noted. Saline controls were used.

RESULTS

The data on the synthetic hydrazones are given in table V. The lag times noted after administration of convulsigenic agent and prior to first seizure are summarized in figures 7 and 8.

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The animals were continuously observed after injection. During the preconvulsant stages, the mice became hyperactive. They exhibited virtually continuous movement about the cage (appeared to be seeking food). Just prior to the onset of seizures, the mice appeared occasionally to drift into sleep and remain in a state of mild depression, easily aroused by the smallest noise. Typically, the first grand mal seizure was preceded by smaller (lighter) seizures. Grand mal seizures sometimes occurred without complex prodoma and in this event the animal, which was sitting or walking about the cage, suddenly fell unconscious and immediately progressed into a violent clonic or tonic seizure. The usual major seizure, however, seemed to be merely an extension and intensification of the petit mal. Clonic pattern occasionally persisted, became progressively more violent, then subsided (clonic twitching). The clonic pattern frequently phased into a strong tonic form, which continued through clonic seizures or progressively decreased in severity (semicomatose between seizures). Death usually came with opisthotonos and general body rigidity. Other symptoms noted were paralysis of extremities (especially in hydrazone convulsions), a drift from mydriasis into miosis and of exophthalmos into enophthalmos, hyperventilation, piloerection and apparent Straub tail.

CONCLUSIONS

MMH convulsions were much more violent in nature than those of UDMH. The time of first convulsion was shorter for MMH than for UDMH. The hydrazones lag times were shorter than those of the hydrazines.

Mortality was studied, and the hydrazones were found more toxic than the hydrazines.

This is in accord with results of Dubnick (ref 13), who administered pyridoxal simultaneously with some aromatic substituted hydrazines, or their respective hydrazones. The average time of death in relation to the dose is shown in figures 9 and 10. The percentages mortality vs dose are given in figures 5 and 6.

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TABLE V

SYNTHETIC HYDRAZONES

Vitamin B_6	Hydrazine	Recrystallizing Solvent	Melting Point	R_F Value
Pyridoxal HCl	TSC	Et OH - H_2O	233 d	0.53
Pyridoxal HCl	UDMH	Et OH - H_2O	122 d	0.42
Pyridoxal HCl	MMH	Benzene pet-ether	170 d	0.39
Pyridoxal $5PO_4$	MMH	Benzene pet-ether	d	0.17

d: decomposes

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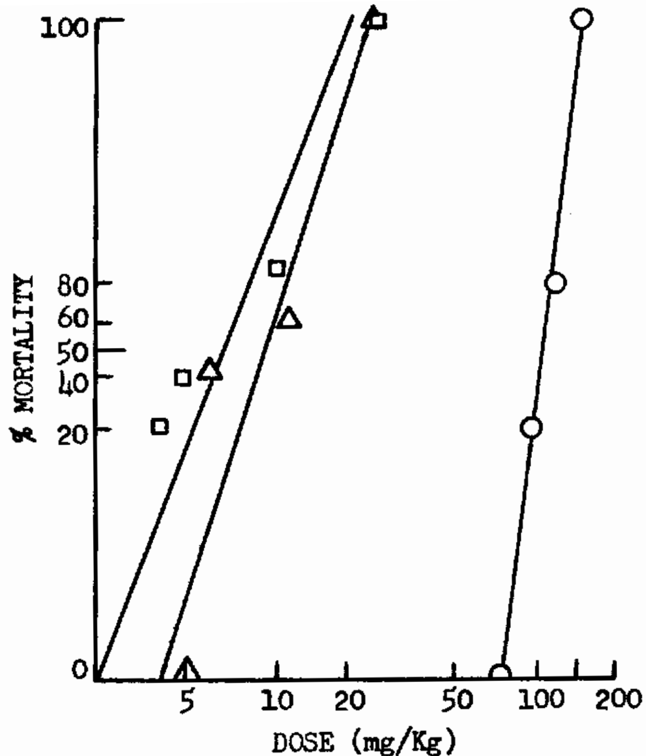

Fig. 5 DOSE-MORTALITY CURVE, UDMH COMPOUNDS

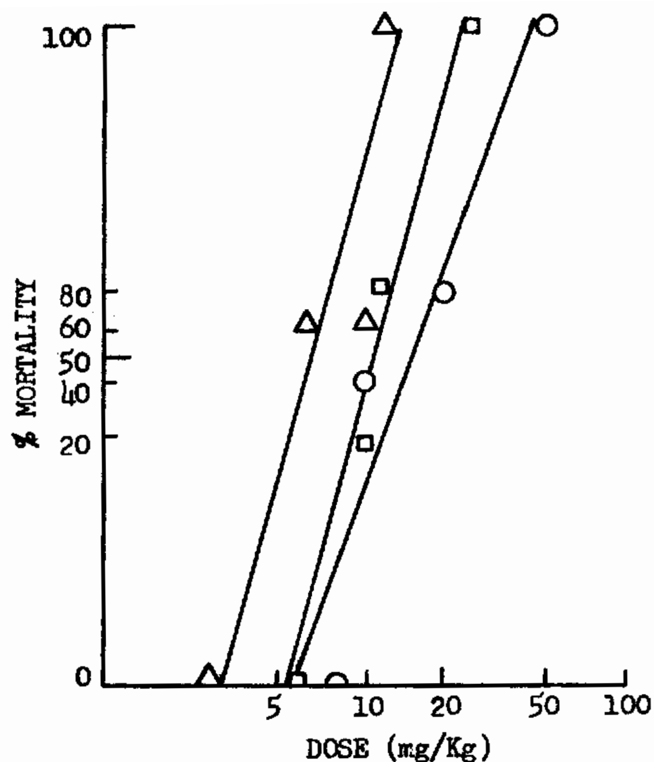

Fig. 6 DOSE-MORTALITY CURVE, MMH COMPOUNDS

LEGEND FOR FIGURES 5-10	
○	Hydrazine (UDMH or MMH)
□	B_6-AL-Hydrazone
△	B_6-AL-5PO_4-Hydrazone
	Each point represents the average of 5 mice.

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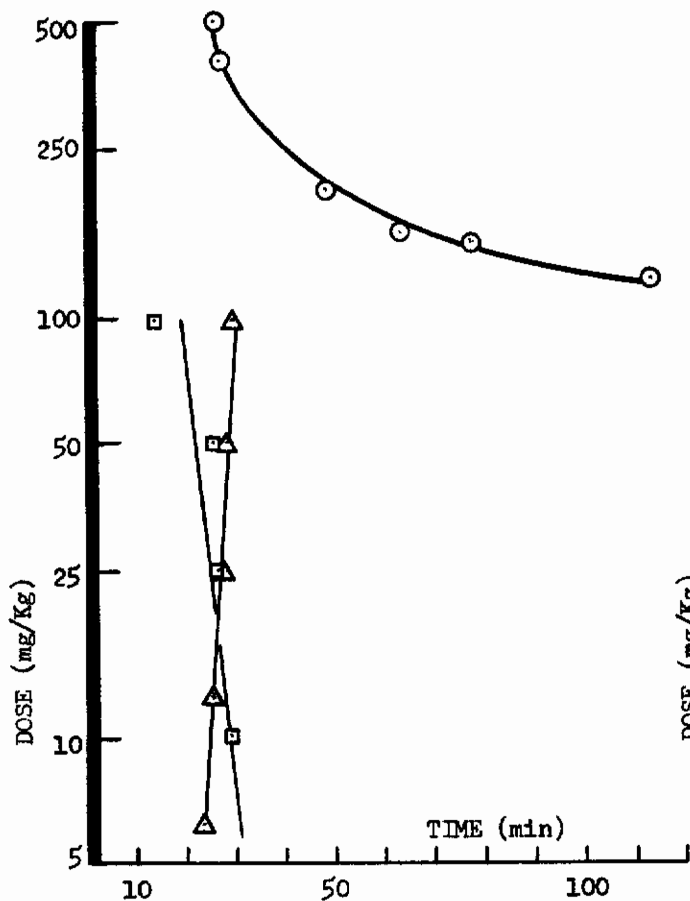

Fig. 7 CONVULSION TIME, UDMH COMPOUNDS

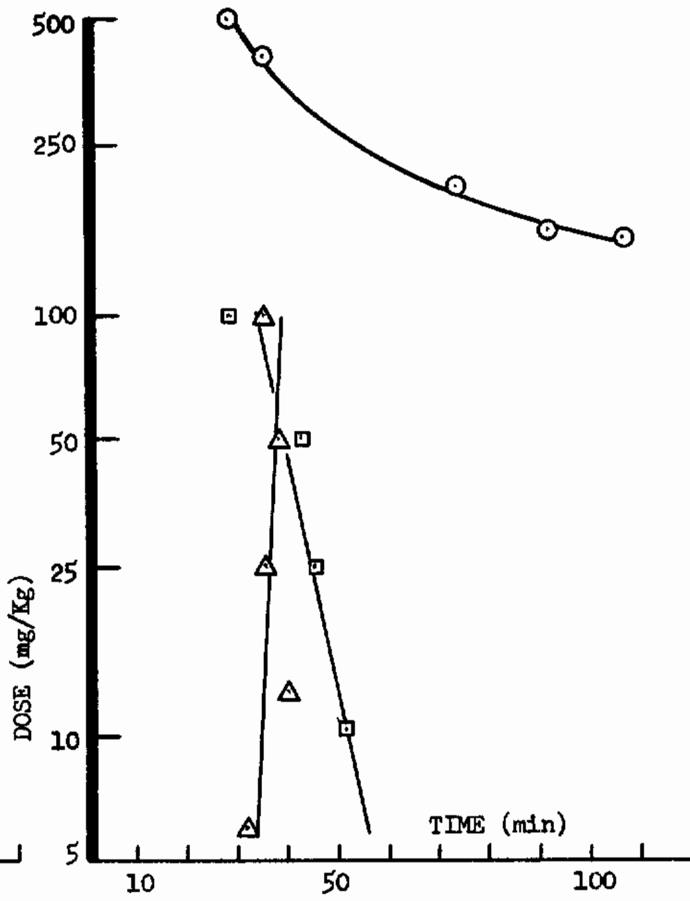

Fig. 9 DEATH TIME, UDMH COMPOUNDS

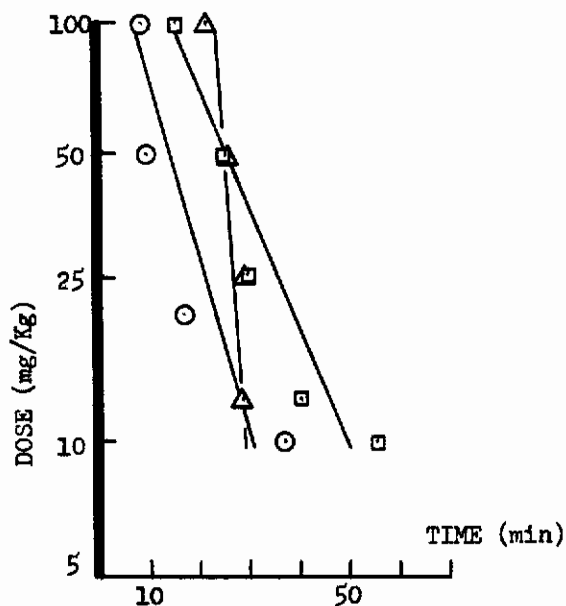

Fig. 8 CONVULSION TIME, MMH COMPOUNDS

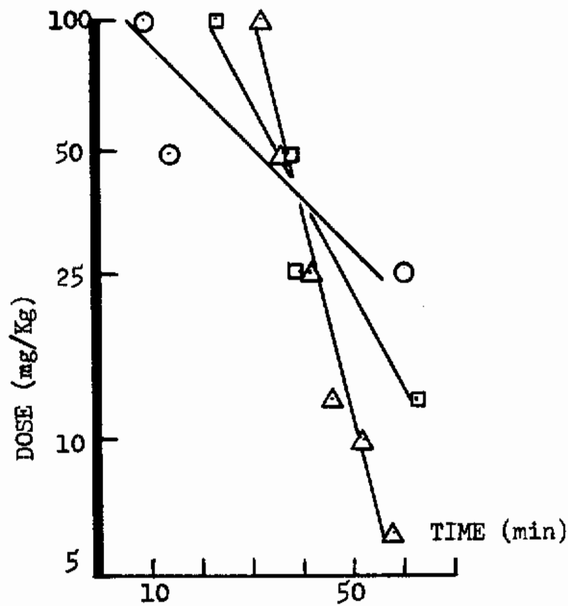

Fig. 10 DEATH TIME, MMH COMPOUNDS

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SECTION IV

DEVELOPMENT OF AN ULTRASENSITIVE QUANTITATIVE BIOASSAY METHOD FOR B_6 DETECTION USING NEUROSPORA

INTRODUCTION

Brain extracts containing B_6 were chromatographed on paper strips as described in Section II - Methods. The strips were then dried and placed in sterile culture tubes that contained an agar deficient in B_6. The agar also contained spores from a strain of B_6-dependent Neurospora. The culture tubes were incubated and mycelia were removed. The amount of growth was gravimetrically determined and compared with a standard.

METHODS

Subculture procedure. Homemade neurospora culture medium* (16.25 g) was placed in 250 ml of distilled water which was heated to boiling until all of the culture media dissolved. Ten ml of medium was poured into each of 25 culture tubes which were then autoclaved at 16 psi for 15 min at 121 C. The tubes were slanted and cooled at 5 C until the media jelled. Then the tubes were streaked with Neurospora 299 (Neurospora Sitophila ATCC 9276, manufactured by Difco Laboratories, Inc.) by sterile technique and incubated at 30 C for 48 hours.

As a check on the extent of growth of our subculture, the following criterion was developed. A culture should show sufficient growth after 72 hours of incubation at 30 C so that a 50 ml flask containing 30 ml of culture agar was nearly filled. If this condition was not met, the culture agar was considered deficient in some essential nutrient such as a trace element or a vitamin. It was expected that the assay medium plus B_6 also should show good growth in 72 hours of incubation at 30 C.

Assay procedure. Difco Dehydrated Pyridoxal Assay Medium (5 g) was mixed with 100 ml of distilled water in a 250 ml Erlenmeyer flask. The mixture was heated to just below boiling point and stirred until a uniform solution was reached. The solution was allowed to cool and was autoclaved for 30 min at 15 psi at 121 C. Pyrex (32 x 200 mm) culture tubes stoppered with cotton were autoclaved in the usual manner. Meanwhile, 10 ml of normal saline was inoculated with a loop of Neurospora Sitophila ATCC 9276 and the test tube was shaken until a good suspension was obtained. This mixture was poured into the Erlenmeyer flask containing the assay medium and swirled. The autoclaved culture tubes were chilled in a refrigerator, and 20 ml of Neurospora inoculated assay medium was poured into each tube.

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*Commercial Neurospora culture medium did not prove satisfactory in producing a reliable 48 hr subculture. The Homemade Neurospora culture medium developed for this work is given in Appendix II.

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After sterilizing the chromatographic strips for 1 hr at 70 C they were placed in the culture tubes so that each strip would become saturated with the media. The strips were incubated for five days at 26 C; at the end of 5 days the strips containing mycelia were removed from the tubes. A very sharp cork bore (1 cm diameter) was used to cut uniform circles of the mycelia for gravimetric analysis. Blanks were cut with the same tool and from the same strip so that by difference an accurate net weight of mycelia could be obtained. The samples and blanks were dried in a vacuum desiccator for 24 hrs, and then weighed.

RESULTS

To demonstrate the Neurospora Bioassay Method for B_6 detection, sets of 10 chromatographic strips were spotted with an amount of vitamin B_6 varying from 5×10^{-7} µg B_6 per spot to 5 µg B_6 per spot. Two strips were left unspotted to serve as controls. The B_6 congeners tested were pyridoxal, pyridoxol, and pyridoxal 5-phosphate. The results are summarized in table VI and in figure 11.

TABLE VI

QUANTITATIVE DETECTION OF VITAMIN B_6 WITH NEUROSPORA

	Weight of Mycelia (mg)		
µg/spot	B_6 Al $5PO_4$	B_6 Al HCl	B_6 Ol HCl
5	7.0	4.0	1.5
5×10^{-1}	2.5	1.5	0.5
5×10^{-2}	1.0	0.5	0.25
5×10^{-3}	0.5	0.25	-
5×10^{-4}	0.25	-	-
5×10^{-5}	-	-	-

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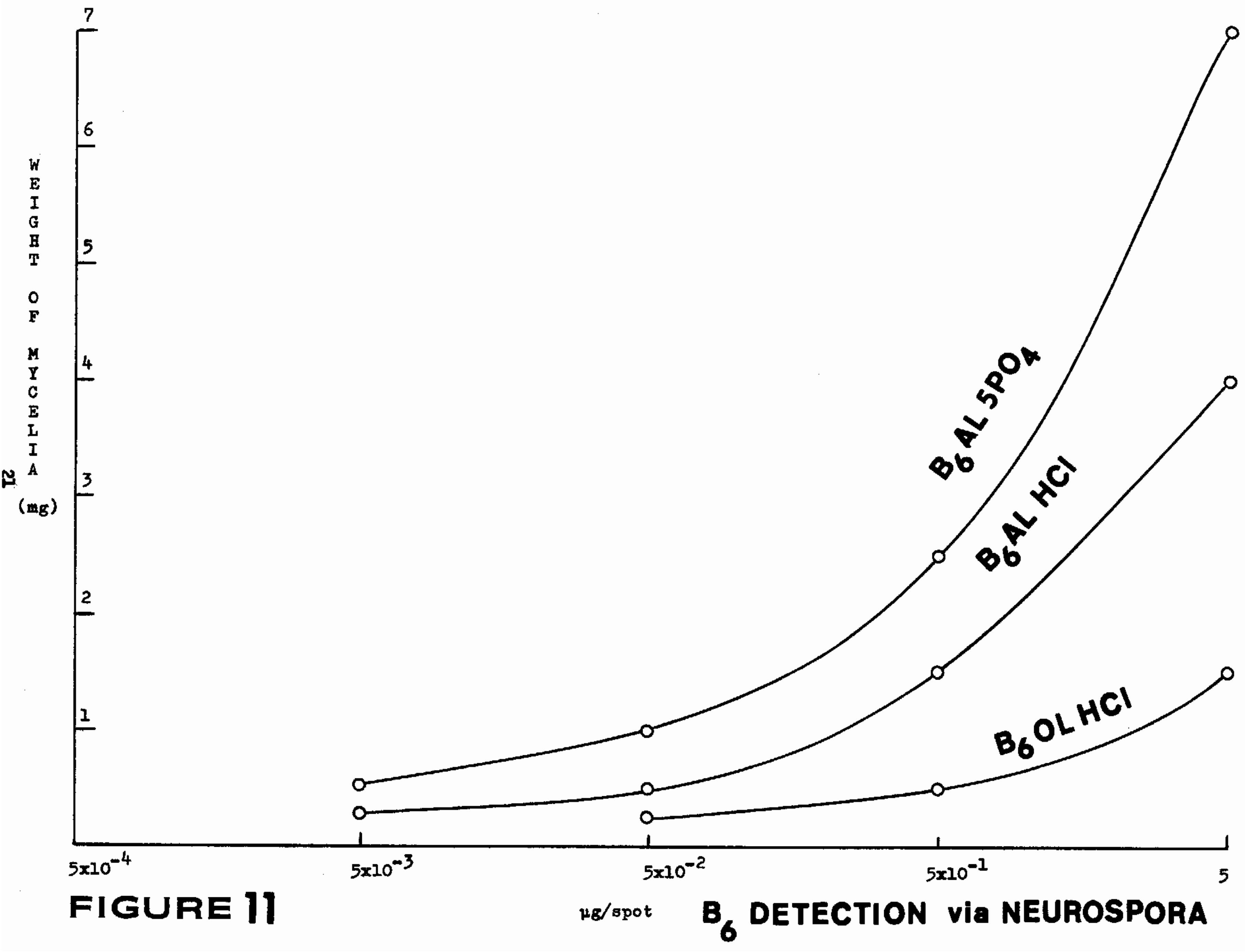

FIGURE 11 B_6 DETECTION via NEUROSPORA

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CONCLUSIONS AND DISCUSSION

A number of problems arose at the start of this phase of experimentation, and more difficulties were encountered as the research progressed. First, it was necessary to obtain a strain of neurospora that would yield reproducible results after successive subcultures. After much trial and error experimentation with various sources, we found that American Type Culture Collection neurospora was best suited for this work. Commercial culture media was not satisfactory in producing a "standardized" rate of growth for the subcultures. A homemade neurospora culture medium was developed, tested and found satisfactory for this work. A method for testing the standardized rate of growth was also developed.

Another problem involved contamination. The medium we employed in B_6 assays did not favor the growth of bacteria and this rarely presented a problem. The most annoying types of contamination we found were from fast growing molds which could obscure the entire surface of a chromatographic strip in a few days. In general, however, the contamination problems have been overcome and we are able to detect B_6 congeners at a concentration of 5 nanograms per spot.

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SECTION V

DEVELOPMENT OF AN ULTRASENSITIVE QUANTITATIVE BIOASSAY METHOD FOR B_6 DETECTION USING YEAST

INTRODUCTION

As a means of checking our neurospora method, a yeast assay was begun. This study was carried out to formulate another accurate method of quantitative pyridoxal detection. A colorimeter was used to determine the extent of yeast growth in a 0.9% saline solution at different concentrations of pyridoxal. Such growth is dependent upon, and directly proportional to, the concentration of pyridoxal.

METHODS

The colorimeter was calibrated by correlating a known weight concentration of Baker's Yeast with colorimeter readings.

The yeast strain Saccharomyces Carlsbergensis (culture 4228) was carried on Difco Malt Agar slants. These stock slants were incubated at 30 C for 24 hrs and stored in the refrigerator for not more than two weeks.

To prepare the inoculum for assay, fresh slants were streaked and incubated for 24 hrs. Some fresh growth was then removed with a sterile wire loop and suspended in 10 ml of sterile 0.9% saline in a colorimeter tube. Using the colorimeter, the yeast concentration was adjusted to 1 mg moist yeast per ml of saline and was ready for use.

Five ml of basal pyridoxal-free medium and the solution of the pyridoxal were placed in a series of test tubes (total volume 9 ml per tube). The tubes were then autoclaved for 15 min at 15 psi, cooled, inoculated with 1 ml of yeast suspension, and incubated at 30 C for 18 hours. During the incubation, the yeast suspension was agitated in a Fisher-Kahn shaker to insure an equally dense growth. The amount of growth was then determined in the colorimeter.

RESULTS

The results are summarized in figure 12.

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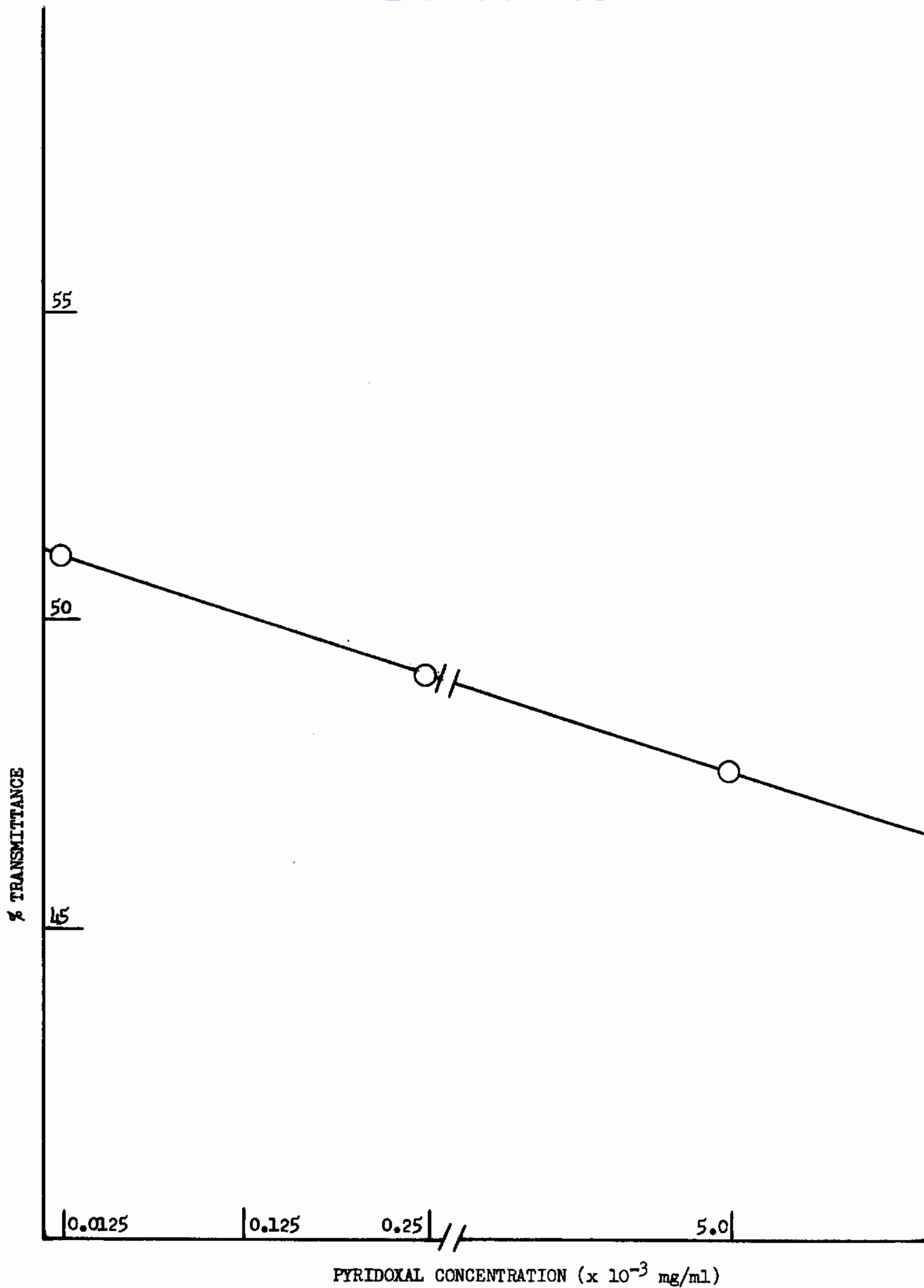

FIGURE 12 **YEAST DETECTION METHOD**

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CONCLUSION

To date, the feasibility of the above mentioned detection method has been explored. Preliminary calibration curves have been run, and preliminary microquantitative experiments with pyridoxal have been performed. We have been able to detect pyridoxal as shown on the yeast detection (figure 12) by this method down to concentrations of 12.5 nanograms. With refinements of the technique, we expect to be able to detect B_6 compounds in the range of 0.5 nanograms.

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APPENDIX I

QUALITY TESTING OF CHROMATOGRAPHIC DATA WITH THE AID OF A STATISTICAL CRITERION

INTRODUCTION

An exhaustive mathematical treatment of the chromatographic process, even if of high theoretical interest, is usually of no practical value for the practicing chemist. The interpretative mathematics of biology and chemistry may provide a reasonably close explanation of the phenomena involved, but are generally inaccessible tools for the biologist or chemist who need to analyze the experimental data on hand and obtain an answer of validity. Many good theories (refs 19, 20) have been written and chromatography has been presented as a convolution process, or a Poisson process (ref 21), but again, almost no literature is available on the practical aspects of evaluating the data upon completion of the experiment (ref 22).

The criterion here derived is the result of the authors' work on the statistics of the chromatography of the vitamin B_6 in which it was desirable to establish a simple test for reproducibility. This criterion is a fast test for dispersion (% error) that provides a narrow confidence band and in many cases will prove to be easier to use, quicker, and better than the very well known and often misused t-Student's and Chi-Square tests.

THEORY

Although the R_F of a specific compound at a fixed pH should be a constant value, in actual practice these figures vary from experiment to experiment. This variation is bounded:

$$0 \leq x_k \leq 1 \qquad (1)$$

where x_k is the k-th R_F value.

The expression x_k , of course, can be applied to all R_F values, which naturally will have bounds within the closed interval $[0,1]$.

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For statistical purposes, it is convenient to normalize the x_k by defining a new variable, the variability ratio:

$$Y_K = \frac{x_k}{X} \tag{2}$$

where X is the mean R_F value. One of the common forms of the doefficient of dispersion is:

$$d = \frac{s}{X} \tag{3}$$

$$d = \sqrt{\frac{\sum_{k=1}^{N} (Y_k - 1)^2}{N-1}} \tag{4}$$

where s is the standard deviation of the sample, and N is the number of readings.

Optimum reproducibility (replicability) conditions call for a maximum coefficient of dispersion, the magnitude of which will depend upon the difficulty of the separations. The researcher who needs to check the reproducibility of the experimental data may obviously use formulas (3) or (4); but, generally speaking, this is a tedious and time-consuming process. A much quicker way of checking if the coefficient of dispersion is within the established interval is the "Y-test".

The Y-Test

In order to have $d \leq d_o$, it is necessary to have:*

$$Y_{max} = \frac{x_{max}}{X} \leq 1 + d_o \quad \text{and} \quad Y_{min} = \frac{x_{min}}{X} \geq 1 - d_o$$

Proof:

Formula (4) may be rewritten in the following approximate form:

---

*Necessary, but not sufficient. The speed of this test is obtained at the expense of some accuracy.

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$$d = \sqrt{\frac{N}{N - 1}} \; (Y_{max} - 1) \tag{5a}$$

and, solving for Y_{max}:

$$Y_{max} = 1 + \sqrt{1 - \frac{1}{N}} \, d \tag{5b}$$

where Y_{max} is the maximum variability ratio (or, what is the same, the ratio of the highest allowable reading to the mean) permitted for a given dispersion d. Using the binomial expansion, (5b) may be rewritten as follows:

$$Y_{max} = 1 + (1 - \frac{1}{2N} - \frac{1}{8N^2} - \ldots) \, d \tag{6}$$

Depending on how large N is, one of the following approximate expressions can be used:

$$Y_{max} = 1 + (1 - \frac{1}{2N})d \tag{7a}$$

$$Y_{max} = 1 + d \tag{7b}$$

The same line of reasoning may be applied for Y_{min}. In this case, formulas (7a) and (7b) would be replaced by:

$$Y_{min} = 1 - (1 - \frac{1}{2N})d \tag{7a'}$$

$$Y_{min} = 1 - d \tag{7b'}$$

Naturally, formulas (7) can be used to determine the Y-test for any given dispersion d. Particularly, if $d = d_o$, then, using (7b) and (7b'), $Y_{max} = 1 + d_o$, and $Y_{min} = 1 - d_o$.

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EXAMPLES

To illustrate the theoretical results obtained above, two examples of vitamin B_6-Amine-$5PO_4$ (synthetic compound, and its presence in mouse brain) will be analyzed. This technique of analysis is of course also applicable to pyridoxols and pyridoxals, and, in general, to any R_F data.

A systematic procedure for a statistical analysis of this kind is the following:

1 - Sort the R_F values in ascending or descending order of magnitude. *

2 - Compute the mean R_F value.

3 - Compute all $|X-x_k|$ and, correspondingly, all $|X-x_k|^2$.

4 - From these calculations determine $\dot{s}$, and d .

The above procedure is followed in tables I and II, and then comparisons are drawn by applying the criterion developed in Theory. The two examples show that, if the Y-test is applied, the above procedure is reduced to steps 1 and 2 only:

1 - Sort the R_F values in ascending or descending order of magnitude. *

2 - Compute the mean R_F value.

3 - Obtain an estimate of the coefficient of dispersion by applying the Y-test (for example, $d = \frac{\text{greatest } R_F}{\text{mean } R_F} - 1$), or see if the data falls within established confidence limits (for a desired d).

*This step is not necessary. Its convenience lies in the facts that the distribution of the R_F values around the mean will be appreciated by a glance, and x_{max} and x_{min} will be easiest to pick out.

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TABLE VII

ANALYSIS OF THE R_F VALUES OF VITAMIN B_6-AMINE-5PO_4 (SYNTHETIC COMPOUND)

(Solvent at pH = 6.5)

k	x_k	$\|X-x_k\|$	$\|X-x_k\|^2 \cdot 10^{-6}$
1	0.09	0.03	900
2	0.11	0.01	100
3	0.11	0.01	100
4	0.11	0.01	100
5	0.11	0.01	100
6	0.11	0.01	100
7	0.11	0.01	100
8	0.11	0.01	100
9	0.12	0.00	0
10	0.12	0.00	0
11	0.12	0.00	0
12	0.12	0.00	0
13	0.12	0.00	0
14	0.12	0.00	0
15	0.13	0.01	100
16	0.13	0.01	100
17	0.13	0.01	100
18	0.13	0.01	100
19	0.13	0.01	100
20	0.15	0.03	900

Here N = 20, X = 0.12.
Let us set d = 0.10 = 10%
(expecting 90% of our data to be within one standard deviation from the mean).

If we now apply the Y-test, we will obtain:

$Y_{max} = 0.15/0.12 = 1.25$ and

$Y_{min} = 0.09/0.12 = 0.75$ which gives

$d = Y_{max}-1 = 1-Y_{min} = 25\%$.

Hence some of our data are outside the desired confidence band. Let us assume that x_1 and x_{20} are outside. Then, applying the Y-test again, we obtain:

$Y_{max} = 0.13/0.12 = 1.08$ and

$Y_{min} = 0.11/0.12 = 0.92$ which give

$d = Y_{max}-1 - 1-Y_{min} = 8\%$.

If we were to carry out the usual computations of standard deviation, etc. we would find:

N = 20, X = 0.12, s = 0.01, d = 8%

and for the narrower band:

N = 18, X = 0.12, d = 7%.

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TABLE VIII

ANALYSIS OF R_F VALUES OF VITAMIN B_6-AMINE-5PO_4 FROM MOUSE BRAIN

k	x_k	$X-x_k$	$X-x_k{}^2 . 10^{-6}$
1	0.10	0.04	1600
2	0.11	0.03	900
3	0.12	0.02	400
4	0.13	0.01	100
5	0.13	0.01	100
6	0.13	0.01	100
7	0.13	0.01	100
8	0.13	0.01	100
9	0.13	0.01	100
10	0.13	0.01	100
11	0.13	0.01	100
12	0.13	0.01	100
13	0.13	0.01	100
14	0.14	0.00	0
15	0.14	0.00	0
16	0.14	0.00	0
17	0.14	0.00	0
18	0.15	0.01	100
19	0.15	0.01	100
20	0.15	0.01	100
21	0.16	0.02	400
22	0.18	0.04	1600

Here N = 22, X = 0.14.
Let us again set d = 0.10 = 10%.
If we now apply the Y-test, we will obtain:

Y_{max} = 0.18/0.14 = 1.29 and

Y_{min} = 0.10/0.14 = 0.71 which gives

$d = Y_{max} - 1 = 1 - Y_{min}$ = 29%.

Hence some of our data are outside the desired confidence band. Let us assume that x_1, x_2, x_3, x_{21}, and x_{22} are outside.
Then, applying the Y-test again, we obtain:

Y_{max} = 0.15/0.14 = 1.07 and

Y_{min} = 0.13/0.14 = 0.93 which gives

$d = Y_{max} - 1 = 1 - Y_{min}$ = 7%.

If we were to carry out the usual computations of standard deviation, etc. we would find:

N = 22, X = 0.14, x = 0.02, d = 14%

and for the narrower band,

N = 17, X = 0.14, d = 6%.

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APPENDIX II

HOMEMADE NEUROSPORA CULTURE MEDIUM

Component	Amount	Quantity
Minimal		1000 ml
Biotin	10 μg	
NH_4 Tartarate	5 g	
NH_4 Nitrate	1 g	
KH_2PO_4	1 g	
$MgSO_4 \cdot 7H_2O$	0.5 g	
NaCl	0.1 g	
$CaCl_2$	0.1 g	
Distilled H_2O	1000 ml	
Vitamin Solution		10 ml
Thiamin	100 mg	
D - Riboflavin	30 mg	
Pyridoxol	75 mg	
Ca - Pantothenate	200 mg	
Parabenzoic Acid	5 mg	
Nicotinamide	75 mg	
Choline	200 mg	
Biotin Crystalline	1 mg	
Inositol	1 g	
EtOH H_2O (90%)	1000 ml	
Trace Elements		1 ml
Boric Acid	57 mg	
$ZnCl_2$	4200 mg	
$ZnSO_4$	4960 mg	
$MgSO_4$	72 mg	
$NaMoO_4$	42 mg	
$CuSO_4$	254 mg	
$FeSO_4 \cdot 7H_2O$	990 mg	
Distilled H_2O	1000 ml	
Difco Nutrient Casenate Agar		1 g
Yeast Extract		2.5 g

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APPENDIX III

MATERIALS AND REAGENTS

I. Chromatography

1. Whatman Chromatographic Paper No. 1.

2. Vitamin B_6 manufactured by Nutritional Biochemicals Corporation, Cleveland, Ohio.

II. Convulsions

1. Swiss-Albino mice supplied by Berkeley-Pacific Laboratories, Berkeley, California.

2. UDMH (K & K Laboratories, Plainview, New York and Eastman Organic Chemicals, Rochester, New York).

   MMH (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin).

III. Neurospora

1. Neurospora Sitophila (ATCC 9276) manufactured by Difco Laboratories, Inc., Detroit, Michigan.

2. Neurospora Sitophila (ATCC 9276) manufactured by American Type Culture, Washington, D.C.

3. Bacto Pyridoxine Assay Medium (Control 460530) manufactured by Difco Laboratories, Inc., Detroit, Michigan.

IV. Yeast

1. Yeast manufactured by Standard Brands, Inc., New York, N.Y.

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13. ABSTRACT

The effects of administered 1,1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH) on vitamin B_6 levels in mouse brain have been studied. Separation of the B_6 group (pyridoxol, pyridoxal, pyridoxamine, and the respective 5-phosphates) by means of paper chromatography revealed that the R_F values obtained are dependent upon the pH of the developing solvent. To obtain the time lag just prior to convulsions induced by UDMH and MMH, a dose-lag time study was conducted; included were pyridoxal and (its 5-phosphate) hydrazones of UDMH and MMH. Graphs of log dose vs lag time are given. The bioassay procedure, though not completed, permits detection of some of the B_6 congeners to a limit of 0.5 nanograms.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
1,1-dimethylhydrazine						
Monomethylhydrazine						
Toxic effects						
Vitamin B_6						
Chromatography						
Bioassay						
Mouse brain						

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