corresponding -1-phosphate ester. This can be explained by the unfavorable equilibrium[27] between the 1- and 6-phosphates and the removal of N-acetylglucosamine-6-phosphate from the reaction mixture by enzymes which convert it to other hexose phosphates.[11]

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MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, EMORY UNIVERSITY]

The Formation of Pyridoxal and Pyridoxal 5-Phosphate Hydrazones[1]

BY RONALD G. WIEGAND

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Specific reaction rates for the formation of pyridoxal 5-phosphate hydrazones from pyridoxal 5-phosphate and a series of hydrazides have been determined, as well as the experimental activation energies for the reactions. The acid dissociation constants of the hydrazides are shown to be linearly related to the specific reaction rates. Data for the reaction of hydrazides with pyridoxal are also given.

Hydrazides have been shown to inhibit several enzyme systems dependent on pyridoxal 5-phosphate (PLP) as a coenzyme.[2-4] The mechanism of this inhibition has been postulated to be the formation of a hydrazone with pyridoxal[5,6] or pyridoxal 5-phosphate.[3]

Vilter and co-workers[5] have suggested that the increased excretion of vitamin B_6 activity in humans following chronic treatment with isonicotinyl hydrazide (INH) is due to excretion of pyridoxal isonicotinyl hydrazone. They further suggest that this complex is formed directly from the interaction of INH and pyridoxal. However, these authors present no evidence to support their suggestion. Williams and Abdulian[6] demonstrated pyridoxal semicarbazone in the urine of semicarbazide treated dogs, which is the only instance, to the author's knowledge, that a pyridoxal hydrazone has been demonstrated to be formed *in vivo*. They conclude that convulsant hydrazides, including INH, combine with pyridoxal to form the corresponding pyridoxal hydrazone.

Davison[3] suggests that INH combines with the phosphorylated form of pyridoxal, and presents experimental activation energies for the reaction of INH with PLP in the presence and absence of B_6-requiring enzymes (16 and 14 kcal./mole, respectively). This similarity of energies of activation is considered by Davison as evidence supporting the initial interaction of INH with pyridoxal phosphate, forming the phosphorylated hydrazone.

The present study was undertaken to clarify the reaction between a series of convulsant hydrazides and both pyridoxal and pyridoxal 5-phosphate. Any correlation between the physicochemical characteristics of the reactions and the convulsant activity of the hydrazides[7] would be of interest. The relative rates of reaction with both forms of vitamin B_6, as well as the experimental activation energies of the reactions, would be of value in defining more precisely the mechanism of the inhibition of enzymatic reactions by hydrazides.

Experimental

Dissociation Constants of Hydrazides.—The acid dissociation constants of thiosemicarbazide (TSC), thiocarbohydrazide (TCH), semicarbazide (SC) and carbohydrazide (CH) were determined at 25.0 ± 0.3° by titration with 0.0984 *N* HCl. A Beckman model G *p*H meter was used to measure *p*H. Two and a half millimoles of hydrazide was dissolved in 25 ml. of redistilled water for titration, except in the case of thiocarbohydrazide where 50 ml. of water was used to allow solution. pK_a values were taken as the points of half neutralization. Without corrections for ionic strength, the pK_a values obtained are reported in Table I. The pK_a of semicarbazide determined under these conditions agrees well with the value of 3.68 reported by Bartlett.[8]

Specific Reaction Rates for Formation of Hydrazones of Pyridoxal and Pyridoxal 5-Phosphate.—All reactions were run in 0.05 *M* phosphate buffer, *p*H 7.4, under conditions of constant total acidity.[9] The reaction was followed spectrophotometrically[3] in a Beckman model DU spectrophotometer equipped with thermospacers for temperature regulation of the cell compartment. The compartment temperature was constant within 0.5°. The increase in optical density of the solutions was measured at a wave length chosen to give a large difference in molar extinction coefficient between pyridoxal or pyridoxal-5-phosphate (PLP) and the corresponding hydrazone. The wave lengths used were 288 mμ for the formation of PLP semicarbazone (PLPSC) and pyridoxal and PLP carbohydrazones (PLCH and PLPCH), 306 mμ for PLP thiocarbohydrazone (PLPTCH), 315 mμ for PLP thiosemicarbazone (PLPTSC), and 330 mμ for the formation of PLP isonicotinyl hydrazone (PLPINH). The ultraviolet absorption spectra of free PLP and the PLP hydrazones are given in Fig. 1. The spectra were obtained using a Beckman model DK-2 ratio recording spectrophotometer.

The second-order reaction for the formation of pyridoxal or PLP hydrazones was made to conform to first-order kinetics by use of a 200-fold molar excess of hydrazide. Two ml. of 0.10 m*M* pyridoxal (Nutr. Biochem. Corp.) or PLP (Calif. Found. for Biochem. Res., 100 ± 3% pure) was mixed with 2.0 ml. of 20 m*M* hydrazide at the start of the reaction, both solutions having been brought to temperature equilibrium before mixing. Optical density readings were taken at 30 second to 5 minute intervals, depending on the speed of the reaction, until the reaction was about three-quarters complete. Readings were then taken until constant. A 10 m*M* hydrazide blank in 0.05 *M* phosphate buffer was used. Optical density readings for 0.05 m*M* pyridoxal or PLP in 0.05 *M* phosphate buffer (OD_0) were taken at the wave lengths used for the rate de-

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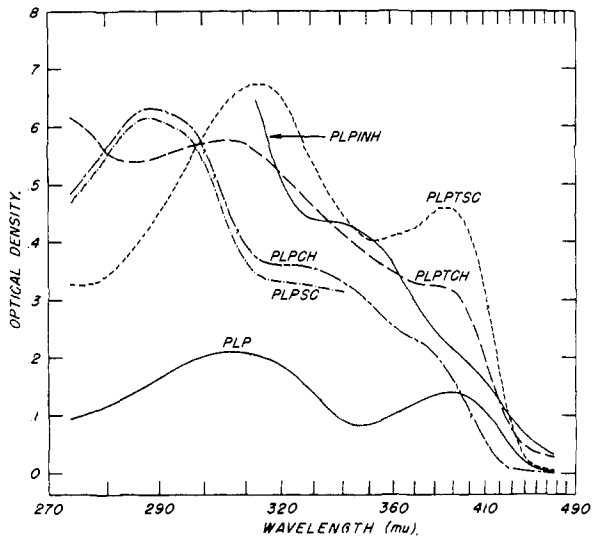

Fig. 1.—Absorption spectra of PLP and PLP hydrazones, 0.05 mM, in 0.05 M phosphate buffer, pH 7.4. Abbreviations indicate the hydrazone involved.

terminations, and the fraction reacted (x) was calculated as $x = (OD_t - OD_0)/(OD_f - OD_0)$, where OD_t = optical density at the time of determination and OD_f = final optical density. Specific reaction rates (k) were determined under the above conditions over the temperature range 12 to 37°. The specific reaction rates are reported in Fig. 2 as the natural logarithms of k at the reciprocal of the absolute temperature at which they were determined.

Experimental Activation Energies.—Experimental activation energies were obtained for the reaction between PLP and carbohydrazide, semicarbazide, thiocarbohydrazide, isonicotinyl hydrazide and thiosemicarbazide. The values were determined from the slope of the least squares regression line calculated from the data of Fig. 2 by means of the Arrhenius equation.[10] The energy of activation for the reaction between pyridoxal and carbohydrazide was also determined.

Results and Discussion

The specific reaction rates for the formation of PLP hydrazones and pyridoxal carbohydrazone at 25°, as obtained from the least squares regression lines of the Arrhenius plots in Fig. 2, are listed in Table I.

TABLE I

	Spec. react. rate, 25° (min.$^{-1}$)	Exp. act. energy (kcal./mole)	pK_a of hydrazide
PLP thiosemicarbazone	0.11	9.4	2.08
PLP isonicotinyl hydrazone	.27	9.9	
PLP thiocarbohydrazone	.50	9.6	3.26
PLP semicarbazone	.73	6.4	3.70
PLP carbohydrazone	1.68	9.4	4.29
PL carbohydrazone	0.015	17.2	

There is essentially no correlation between the convulsant potency[7] of these hydrazides and the rate of complex formation with pyridoxal 5-phosphate. The more potent thio-compounds react more slowly with PLP than their oxygen analogs, though among the sulfur or oxygen compounds the more potent reacts more rapidly. Since there is no significant difference in time of onset of convulsions in mice with intraperitoneal injection of the CD_{50} of the hydrazides,[7] the lack of correlation between rate of reaction with PLP and CD_{50} was not unexpected.

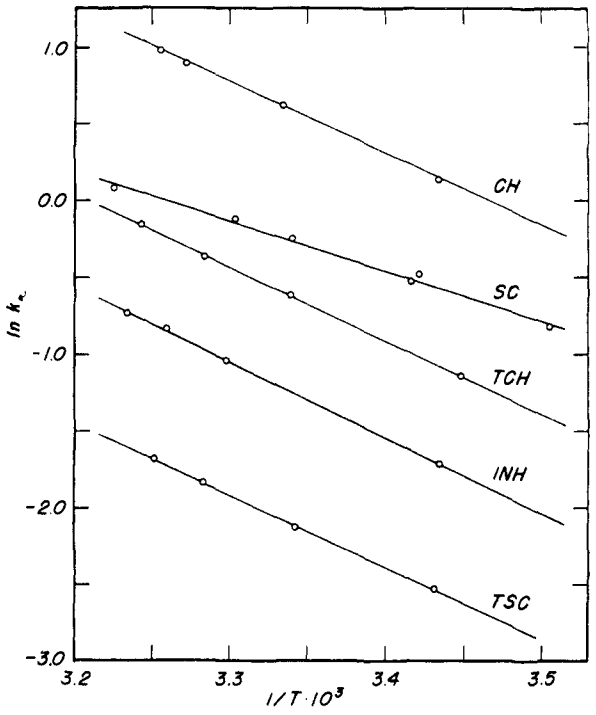

Fig. 2.—Arrhenius plot of specific reaction rates for the reaction of pyridoxal 5-phosphate with the hydrazides shown. 0.05 mM PLP and 10 mM hydrazide in 0.05 M phosphate buffer, pH 7.4. The lines are least squares regression lines calculated for the experimental points shown.

The rate of the acid-catalyzed hydrazone formation[11] with PLP depends on the concentration of the cationic species of the hydrazide. The relation between ln k (25°, pH 7.4) and the pK_a values of the hydrazides TSC, TCH, SC and CH is linear. It appears that the cationic form of the hydrazide, which is the conjugate acid of the uncharged form, is able to catalyze hydrazone formation. Bartlett[8] demonstrated that it is the uncharged form of semicarbazide which reacts with carbonyl groups. At pH 7.4 the fraction of hydrazide in the cationic form is sufficiently small so as to make no observable difference in the concentration of free, uncharged hydrazide, and thus all specific reaction rates were obtained under similar conditions of concentration.

The experimental activation energies for the reaction between pyridoxal 5-phosphate and the hydrazides are collected in Table I. With the exception of PLP semicarbazone, the values are in the range 9.4 to 9.9 kcal./mole. Analysis of the possible variation in slope of the least squares regression line calculated from the experimental values of specific reaction rate shows that these energies of activation are the same within limits of experimental error. PLP semicarbazone has a significantly lower experimental activation energy. However, it should be noted that the rate of for-

(10) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941, p. 2.

(11) E. E. Royals, "Advanced Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1954, p. 664.

mation of PLP semicarbazone is as would be expected from its pK_a value in spite of the experimental activation energy of the reaction.

The specific reaction rate for the formation of pyridoxal carbohydrazone is markedly less than for the formation of PLPCH (Table I). Similar qualitative results also were obtained with semicarbazide and thiosemicarbazide. The experimental activation energy for the formation of pyridoxal carbohydrazone (PLCH) is almost double that for the formation of the phosphorylated complex. Both the rate of reaction and the experimental activation energy indicate more favorable conditions for the formation of PLPCH than PLCH.

Davison[3] presented evidence that PLP isonicotinyl hydrazone is the initial complex formed in an *in vitro* homogenate preparation, with an experimental activation energy of approximately 16 kcal./mole for the reaction. Davison's value for the energy of activation for the formation of PLP-INH obtained with only PLP and INH present is 14 kcal./mole. This latter value does not correspond very closely to the value of 9.9 kcal./mole presented in Table I. The activation energy for the reaction between pyridoxal and INH has not been determined. However, with pyridoxal and carbohydrazide the activation energy is 17.2 kcal./mole. Reference to Table I shows that the values reported by Davison more closely approximate the value for the experimental activation energy of pyridoxal carbohydrazone than any of the PLP hydrazones.

The urinary excretion of pyridoxal semicarbazone by dogs treated with semicarbazide reported by Williams[6] may be due to dephosphorylation of the initial complex, since high phosphatase activity for PLPSC has been demonstrated in the kidney.[12]

It is suggested that the higher specific reaction rates and lower experimental activation energies favor the formation of hydrazones of pyridoxal 5-phosphate rather than of pyridoxal. Direct demonstration of the pyridoxal 5-phosphate hydrazone complexes *in vivo* will be the subject of a further communication.

(12) R. G. Wiegand, Ph.D. Thesis, Emory University, 1956.

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[CONTRIBUTION FROM THE MALLINCKRODT CHEMICAL LABORATORIES OF HARVARD UNIVERSITY]

The Quantitative Evaluation of the Effect of Hydrogen Bonding on the Strength of Dibasic Acids

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A quantitative measure of the effect of hydrogen bonding on the ionization constants of a dibasic acid can be obtained by comparing the first ionization constant of the dibasic acid with the ionization constant of the corresponding methyl ester. It can thus be established that, for most dibasic acids, the effect of hydrogen bonding on the K_1/K_2 ratio is negligible relative to the electrostatic effect; for maleic acid and for some highly alkylated aliphatic acids, the hydrogen bonding effect is appreciable but not dominant. The conjugate acids of bipyridyl, cited by McDaniel and Brown, do not show evidence of an important hydrogen bonding effect.

Introduction

In a recent article Hunter[2] pointed out that internal hydrogen bonding will increase the ratio of the first to the second ionization constants of maleic and of some other dicarboxylic acids. Subsequently, Brown and his collaborators[3] extended Hunter's qualitative treatment to a large number of additional dibasic acids and suggested that, for certain acids, the hydrogen-bonding effect may be an important one. A method for the quantitative evaluation of the effect of hydrogen bonding on the K_1/K_2 ratio in dibasic acids is here formulated and applied to the examples previously[3] cited; in general, the effect of hydrogen bonding is found to be small. Salicylic acid[4-6] and similar acids where the hydrogen bond is in a ring of six atoms including two double bonds,[7] and some highly alkylated aliphatic acids,[3] are exceptional cases where the effect of hydrogen bonding may be important. For most dibasic acids, a statistical factor of 4 and an electrostatic effect[8-10] are primarily responsible for the values of the K_1/K_2 ratios.

The first ionization constant of phthalic acid is 1.2×10^{-3}, the ionization constant, K_E, of monomethyl phthalate is 0.6×10^{-3}. On statistical grounds alone, the first ionization constant of a dibasic acid should be twice the ionization constant of the corresponding monoester.[11] Since for phthalic acid such is almost exactly the fact, there is very little room for an effect of hydrogen bonding. Presumably the fact that the ring for hydrogen-bonded phthalic acid contains seven members, and the fact that internal hydrogen bonding must compete with external (solvent) hydrogen bonding, are responsible for the experimental finding above.

(1) On leave of absence from Haverford College, Haverford, Penna.

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