Spectrophotometric Determination of Pyridoxal and Pyridoxal 5'-Phosphate with 3-Methyl-2-benzothiazolone Hydrazone Hydrochloride, and their Selective Assay

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A spectrophotometric method with 3-methyl-2-benzothiazolone hydrazone hydrochloride was developed for the determination of pyridoxal and pyridoxal 5'-phosphate, and for the selective determination of each in the presence of the other. Pyridoxal and pyridoxal 5'-phosphate react with the reagent to yield the azine derivatives, which give characteristic absorption spectra. The highest extinction values are obtained when pyridoxal and pyridoxal 5'-phosphate are incubated at pH values of about 3.4 and 8.0 respectively; their maxima are at 430 nm. (ϵ 2.74×10^4) and 380 nm. (ϵ 2.24×10^4) respectively. The azine of pyridoxal is only slightly soluble under the neutral and alkaline conditions, whereas that of pyridoxal 5'-phosphate is substantially insoluble in the acid pH range. This difference in solubility of the azines made possible the selective determination of pyridoxal and pyridoxal 5'-phosphate. α -Oxoglutarate and pyruvate are among the substances shown not to interfere with the assay of pyridoxal; their derivatives absorb appreciably only at wavelengths below 420 nm. For the assay of pyridoxal 5'-phosphate in the presence of these compounds measurement at 390 nm. is necessary.

Among several forms of naturally occurring vitamin B₆, pyridoxal and pyridoxal 5'-phosphate function as the most active forms for a variety of chemical and enzymic reactions, as reviewed by Guirard & Snell (1964) and Meister (1965). A number of chemical, microbiological and enzymological methods have been presented for the determination of these compounds (Storvick, Benson, Edwards & Woodring, 1964). However, few procedures are available for the selective determination of each in the presence of the other. Wada & Snell (1961) have devised a method for the determination of pyridoxal and pyridoxal 5'-phosphate with phenylhydrazine. The reaction at 0° under acidic conditions enabled pyridoxal 5'-phosphate to be specifically determined in the presence of pyridoxal, because of the very low reactivity of the latter. This method, however, is not easily applicable to the micro determination of pyridoxal 5'-phosphate in the presence of excess of pyridoxal.

Sawicki, Hauser, Stanley & Elbert (1961) developed a sensitive colorimetric method for the determination of aliphatic aldehydes with MBTH.* Paz *et al.* (1965) reported that MBTH reacts with various carbonyl compounds to yield the azine or 'osazine' derivatives, and that these derivatives have characteristic spectra that permit their

identification. Recently a sensitive and simple spectrophotometric method for the determination of α -oxo acids, D-amino acids and D-amino acid oxidase activity with MBTH has been established (Soda, 1967, 1968).

We now present a spectrophotometric method with MBTH for the determination of pyridoxal and pyridoxal 5'-phosphate, and for their selective determination.

MATERIALS AND METHODS

Chemicals. MBTH and sodium pyruvate were purchased from Aldrich Chemical Co. Inc., Milwaukee, U.S.A. Pyridoxal hydrochloride, pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate, obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., were chromatographically purified by the procedure of Peterson & Sober (1954). L-Amino acids, glycine and α -oxoglutaric acid were products of Kyowa Hakko Kogyo Co., Tokyo, Japan. D-Amino acids were obtained from Ajinomoto Co., Tokyo, Japan. The other chemicals were A.R. grade.

Spectrophotometry. Absorption spectra were taken with a Shimadzu MPS-50L recording spectrophotometer (Shimadzu Co., Kyoto, Japan) with a 1.0 cm. light-path.

Assay procedures. The standard procedures finally developed are given as follows.

(A) Determination of pyridoxal. The standard reaction mixture, unless otherwise stated, consisted of 1.0 ml. of pyridoxal hydrochloride (0.01–0.2 μ mole), 2.0 ml. of 0.6 M-sodium acetate-acetic acid buffer, pH 3.4, and

* Abbreviation: MBTH, 3-methyl-2-benzothiazolone hydrazone hydrochloride.

0.8 ml. of aq. 0.14% MBTH solution, freshly prepared or stored at -20° until use. Water was substituted for pyridoxal hydrochloride in a blank. Incubation was carried out at 50° for 30 min.; the reaction mixture was then cooled to

room temperature and the E_{430} was measured against a blank. The amount of pyridoxal in the test solution was determined from a previously prepared calibration curve.

(B) Determination of pyridoxal 5'-phosphate. The standard reaction mixture contained 1.0 ml. of pyridoxal 5'-phosphate (0.01–0.2 μ mole), 2.0 ml. of 0.6 M potassium phosphate buffer, pH 8.0, and 0.8 ml. of 0.1% MBTH solution. After incubation as described above, the E_{380} was measured.

(C) Determination of pyridoxal in the presence of pyridoxal 5'-phosphate. In addition to pyridoxal hydrochloride pyridoxal 5'-phosphate (less than one-tenth of the amount of pyridoxal hydrochloride) was contained in a reaction mixture similar to that described in procedure A. After incubation as described above, the mixture was centrifuged at about 1500g for 5 min. and the E_{430} of the clear supernatant was measured.

(D) Determination of pyridoxal 5'-phosphate in the presence of pyridoxal. The reaction mixture containing pyridoxal 5'-phosphate and pyridoxal was incubated as described in procedure B, and centrifuged at 1500g for 5 min. to remove the precipitate formed. The E_{380} of the supernatant was measured.

Fig. 1. Absorption spectra of MBTH derivatives of pyridoxal and pyridoxal 5'-phosphate. The experimental details are given in the Materials and Methods section. Curve A, pyridoxal (0.1 μ mole) at pH 3.4; curve B, pyridoxal 5'-phosphate (0.1 μ mole) at pH 8.0; curve C, pyridoxal at pH 8.0 (supernatant); curve D, pyridoxal 5'-phosphate at pH 3.4 (supernatant).

RESULTS AND DISCUSSION

Effect of pH. The absorption spectra of MBTH derivatives of pyridoxal and pyridoxal 5'-phosphate obtained under the standard conditions are shown in Fig. 1 (curves A and B). The MBTH derivatives of pyridoxal and of pyridoxal 5'-phosphate exhibit

Table 1. *Effect of pH on the absorption peaks of azine compounds of pyridoxal and pyridoxal 5'-phosphate*

The reactions were carried out by procedures A (for pyridoxal azine) and C (for pyridoxal 5'-phosphate azine) as described in the Materials and Methods section, except for the pH of the reaction systems. Numbers in parentheses indicate the approximate positions of absorption maxima of supernatant solutions from reaction mixtures, because of broadness of the peaks.

Buffer	pH	Pyridoxal azine			Pyridoxal 5'-phosphate azine		
		Ppt. formation	$\lambda_{\max.}$ (nm.)	E	Ppt. formation	$\lambda_{\max.}$ (nm.)	E
Sodium acetate-acetic acid	1.5		430	0.232	+	(430)	0.150
	2.5		430	0.522	+	(428)	0.080
	3.0		430	0.656	+	(428)	0.062
	3.4		430	0.720	+	(428)	0.050
	3.6		430	0.720	+	(428)	0.048
	3.8		430	0.664	+	(428)	0.046
	4.0	+	(427)	0.210	+	(420)	0.040
	5.0	+	(420)	0.178	+	395	0.182
Sodium acetate-HCl	3.0		430	0.600	+	(428)	0.073
	3.4		430	0.680	+	(428)	0.085
Potassium phosphate	5.8	+	(380)	0.022		388	0.450
	6.5	+	(380)	0.023		382	0.518
	7.0	+	(380)	0.019		382	0.530
	7.5	+	(380)	0.017		380	0.532
	7.8	+	(380)	0.017		380	0.590
	8.0	+	(380)	0.017		380	0.590
	8.2	+	(380)	0.022		380	0.529
Glycine-NaOH	8.2	+	(380)	0.022		380	0.522
	8.6	+	(360)	0.028		380	0.500
	10.0	+	(360)	0.089		380	0.450

absorption maxima at 430nm. (at pH 3.4) and at 380nm. (at pH 8.0) respectively. These MBTH derivatives, by analogy with the reaction products of other aldehydes reported by Paz *et al.* (1965) and by Hauser & Kolar (1968), are probably pyridoxal-3-methyl-2-benzothiazolyl azine and pyridoxal 5'-phosphate-3-methyl-2-benzothiazolyl azine, formed by the coupling of an aldehyde group of the vitamin B₆ compounds with the reagent.

Pyridoxal and pyridoxal 5'-phosphate were allowed to react with MBTH in buffers of various pH values. With pyridoxal the highest extinction was obtained by incubation in sodium acetate-acetic acid buffer, pH 3.4 or 3.6 (Table 1). The absorption maximum (430nm.) did not shift on varying the pH from 1.0 to 3.8. The reaction of pyridoxal with MBTH at pH values above 4.0 resulted in the formation of light-yellow precipitates. The supernatants obtained by centrifugation had negligibly small absorption spectra, one of which is shown in Fig. 1 (curve C). With pyridoxal 5'-phosphate, however, the highest extinction was obtained by incubation at pH 7.8 or 8.0, and the absorption maximum was at 380nm. The MBTH derivative of pyridoxal 5'-phosphate was slightly soluble at pH values below 4.0, and the resultant supernatant showed an extinction several per cent of that of pyridoxal azine obtained under the same conditions (curve D in Fig. 1, and Table 1). Thus the optimum pH values for the determination of pyridoxal and pyridoxal 5'-phosphate were about 3.4 and 8.0 respectively. Sodium acetate-acetic acid buffer, pH 3.4, was adopted in the standard mixture, since the colour intensity of the pyridoxal-MBTH derivative was greater than when sodium acetate-hydrochloric acid buffer was used. Substitution of phthalate

buffer for sodium acetate-acetic acid buffer led to the formation of a precipitate. Potassium phosphate buffer, pH 8.0, was employed for the determination of pyridoxal 5'-phosphate, although satisfactory results were also obtained with glycine-sodium hydroxide buffer and veronal buffer.

Effect of concentration of buffer. Since the extinctions of the azines derived from pyridoxal and pyridoxal 5'-phosphate are influenced by pH, use of the higher concentration of buffer is advantageous in maintaining the optimum pH. The effects of various concentrations of sodium acetate-acetic acid buffer, pH 3.4, and potassium phosphate buffer, pH 8.0, on the colour intensities of pyridoxal azine and pyridoxal 5'-phosphate azine respectively were investigated. No appreciable change in the extinctions and the absorption maxima of both the azines occurred in the buffer concentration range 0.1–0.7 M; 2ml. of 0.6 M buffer was employed in the standard reaction mixtures.

Effect of concentration of MBTH. The relationship between concentration of MBTH and the extinction of the azines is presented in Table 2. The extinction of the azine derivative from pyridoxal 5'-phosphate was not affected by the concentration of MBTH used in the 0.012–0.2% range, but a higher concentration of MBTH than 0.1% was required to obtain the maximum colour intensity of pyridoxal azine under the conditions employed; 0.8ml. of 0.14% and 0.8ml. of 0.1% MBTH solution were used in the standard reaction mixtures for the determination of pyridoxal and pyridoxal 5'-phosphate respectively.

Effect of incubation time. When pyridoxal was allowed to react with MBTH, maximum colour intensity was obtained within 160min. at 25° and within 25min. at 50° (Fig. 2). The final colour intensity was stable for at least 5hr. at 50°. However, the reaction of pyridoxal 5'-phosphate with

Table 2. *Effect of concentration of MBTH on the colour intensities*

The reactions were carried out by procedures A (for pyridoxal azine) and B (for pyridoxal 5'-phosphate azine) as described in the Materials and Methods section, except for the concentrations of MBTH.

Concn. of MBTH (%)	Relative extinction	
	Pyridoxal azine (at 430nm.)	Pyridoxal 5'-phosphate azine (at 380nm.)
0.012	28	100
0.024	46	99
0.048	69	102
0.070	90	100
0.100	100	100
0.120	100	102
0.140	100	100
0.200	100	100

Fig. 2. Colour development as a function of incubation time and temperature. Pyridoxal (0.1 μmole) was allowed to react with MBTH at 50° (●) or 25° (○) under the conditions of procedure A described in the Materials and Methods section.

MBTH proceeded extremely rapidly (Fig. 3, curve A), the extinction reaching a maximum within 2 min. even at 25°. The colour was stable for at least 12 hr. at 25° and for at least 3 hr. at 50°. The much faster reaction of pyridoxal 5'-phosphate than of pyridoxal probably reflects the fact that pyridoxal primarily exists in the form of a hemiacetal under acidic conditions, as mentioned previously (Metzler & Snell, 1955; Wada & Snell, 1961).

Range of usefulness of the method. Plots of extinction against the amount of pyridoxal and pyridoxal 5'-phosphate in the range 0.01–0.2 μ mole were linear. The molecular extinction coefficients of pyridoxal azine and pyridoxal 5'-phosphate azine were found to be 2.74×10^4 and 2.24×10^4 respectively under the standard conditions. In a series of 15 assays on different samples of pyridoxal or pyridoxal 5'-phosphate the precision range was $\pm 3\%$.

Selective determination of pyridoxal and pyridoxal 5'-phosphate. As described above, both pyridoxal and pyridoxal 5'-phosphate react with MBTH to produce the azines quantitatively under the appropriate optimum conditions. When pyridoxal, for the determination of which the optimum pH is about 3.4, is incubated with MBTH at the alkaline pH, the azine compound formed is substantially insoluble. On the other hand, pyridoxal 5'-phosphate, whose determination is carried out at pH 8.0, reacts with the reagent at the acid pH to yield the slightly soluble azine. This difference in solubility of the azines permits the selective determination of pyridoxal and pyridoxal 5'-phosphate. Pyridoxal 5'-phosphate was specifically determined by procedure D in the presence of as well as in the absence of pyridoxal. Since pyridoxal 5'-phosphate azine is not completely insoluble at pH 3.4, its presence

affects the determination of pyridoxal. The colour intensity of pyridoxal azine (0.1 μ mole) was increased by about 7% by the addition of the same amount of pyridoxal 5'-phosphate, but if the amount of pyridoxal 5'-phosphate is less than one-tenth of that of pyridoxal its influence is negligible.

The method of Wada & Snell (1961) for the selective determination of pyridoxal 5'-phosphate in the presence of pyridoxal is based on the difference in reactivity with phenylhydrazine. As shown in Fig. 3, pyridoxal 5'-phosphate reacted much faster with MBTH than pyridoxal. This difference in the reaction rate also enables pyridoxal 5'-phosphate to be selectively determined in the presence of pyridoxal. When the extinction of pyridoxal 5'-phosphate azine formed at 25° was measured within 120–180 sec. of incubation, pyridoxal 5'-phosphate could be determined without serious interference from pyridoxal (curve C in Fig. 3), especially if the amount of pyridoxal also present was less than one-fifth of that of pyridoxal 5'-phosphate.

Interfering substances. None of pyridoxine, pyridoxamine, pyridoxamine 5'-phosphate, D- and L-alanine, D- and L-glutamic acid, glycine, D- and L-lysine, L-methionine, D- and L-phenylalanine, glutaric acid, citric acid, succinic acid, sodium chloride, potassium chloride, ammonium sulphate and magnesium chloride (1.0 μ mole) interfered with the assay of pyridoxal and pyridoxal 5'-phosphate. As reported previously (Paz *et al.* 1965; Soda, 1967), various α -oxo acids react with MBTH to yield the azines, which give characteristic u.v.-absorption spectra. When α -oxoglutaric acid was incubated with MBTH at pH 3.4 under the conditions of

Fig. 3. Colour development as a function of incubation time. Pyridoxal 5'-phosphate (0.1 μ mole) (curve A) or pyridoxal (0.1 μ mole) (curve B) was allowed to react with MBTH at 25° under the conditions of procedure B as described in the Materials and Methods section. For curve C, 0.1 μ mole of pyridoxal 5'-phosphate was allowed to react in the presence of the same amount of pyridoxal.

Fig. 4. Absorption spectra of colours derived from pyridoxal, α -oxoglutaric acid, pyruvic acid and mixtures thereof with MBTH. The reactions were carried out by procedure A as described in the Materials and Methods section. Curve A, pyridoxal (0.1 μ mole); curve B, α -oxoglutaric acid (0.1 μ mole); curve C, pyruvic acid (0.1 μ mole); curve D, pyridoxal + α -oxoglutaric acid; curve E, pyridoxal + pyruvic acid.

Fig. 5. Absorption spectra of colours derived from pyridoxal 5'-phosphate, α -oxoglutaric acid, pyruvic acid and mixtures thereof with MBTH. The reactions were carried out by procedure B as described in the Materials and Methods section. Curve A, pyridoxal 5'-phosphate ($0.1 \mu\text{mole}$); curve B, α -oxoglutaric acid ($0.1 \mu\text{mole}$); curve C, pyruvic acid ($0.1 \mu\text{mole}$); curve D, pyridoxal 5'-phosphate + α -oxoglutaric acid; curve E, pyridoxal 5'-phosphate + pyruvic acid.

procedure A, its azine formed exhibited an absorption maximum at 320nm. , but no absorption at 430nm. , where the absorption maximum of the pyridoxal azine was found (Fig. 4). The azine of pyruvic acid also had no absorption at 430nm. As shown in Fig. 4, the presence of α -oxoglutaric acid and pyruvic acid did not influence the absorption of pyridoxal azine at 430nm. Thus pyridoxal can be determined by this method without interference by such α -oxo acids. α -Oxoglutaric acid and pyruvic acid gave rise to small extinctions at 380nm. , but there was no absorption at 390nm.

when they were treated with MBTH at $\text{pH } 8.0$ by procedure B. The α -oxo acids consequently had no influence on the absorption of pyridoxal 5'-phosphate azine at 390nm. , but did so at 380nm. , as shown in Fig. 5. When the extinction of the azine was measured at 390nm. , the effect of α -oxo acids was negligible in the determination of pyridoxal 5'-phosphate, although this caused a slight decrease in the sensitivity.

The spectrophotometric method with MBTH described here is suitable for the selective determination of pyridoxal and pyridoxal 5'-phosphate in a complex system, e.g. biological fluids.

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REFERENCES

- Guirard, B. & Snell, E. E. (1964). In *Comprehensive Biochemistry*, vol. 15, p. 138. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Hauser, T. R. & Kolar, M. A. (1968). *Analyt. Chem.* **40**, 231.
- Meister, A. (1965). *Biochemistry of the Amino Acids*, 2nd ed., p. 375. New York: Academic Press Inc.
- Metzler, D. E. & Snell, E. E. (1955). *J. Amer. chem. Soc.* **77**, 2431.
- Paz, M. A., Blumenfeld, O. O., Rojkind, M., Henson, E., Furfine, C. & Gallop, P. M. (1965). *Arch. Biochem. Biophys.* **109**, 548.
- Peterson, E. A. & Sober, H. A. (1954). *J. Amer. chem. Soc.* **76**, 169.
- Sawicki, E., Hauser, T. R., Stanley, T. W. & Elbert, W. (1961). *Analyt. Chem.* **33**, 93.
- Soda, K. (1967). *Agric. biol. Chem., Tokyo*, **31**, 1054.
- Soda, K. (1968). *Analyt. Biochem.* **25**, 228.
- Storvick, C. A., Benson, E. M., Edwards, M. A. & Woodring, M. J. (1964). *Meth. biochem. Anal.* **12**, 183.
- Wada, H. & Snell, E. E. (1961). *J. biol. Chem.* **236**, 2089.