Separation and identification of pyridoxal and pyridoxal-5-phosphate by paper chromatography

SILIPRANDI, SILIPRANDI AND LIS studied the electrophoretic separation of vitamin B_6 compounds[1] and data on the paper chromatography of these compounds has been given by other workers[2–5]. On the whole, however, the paper chromatographic separation of these compounds has, so far, been little investigated, possibly because the methods for locating them on the paper are either insensitive or complicated. The classical method is biological and depends on their ability to promote growth of certain micro-organisms[2]. Another detection method depends on reaction with the phosphate group in pyridoxal-5-phosphate and pyridoxamin-phosphate. All vitamin B_6 compounds can be developed by diazotization as well. These methods cannot be used for the demonstration of less than μg amounts of these agents. When present on the paper in high concentrations (5–10 μg) all B_6 compounds give a spontaneous fluorescence in the U.V. region after chromatography in certain solvent systems.

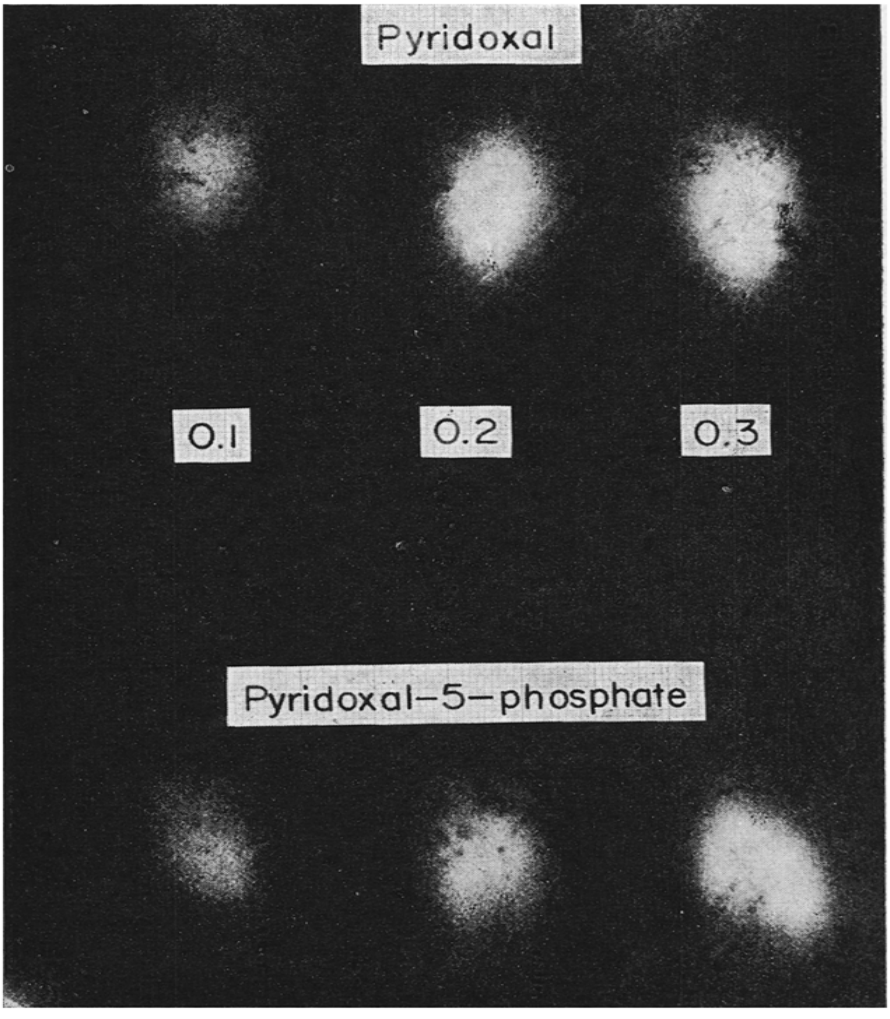

Fig. 1. Separation of mixtures of pyridoxal and pyridoxal-5-phosphate by paper chromatography in *n*-butanol saturated with water for 8 h. The amount of each compound is given in μg. The photograph was taken in U.V. light using Gevaert Scopix G film, with an alkaline solution of picric acid serving as a yellow filter, the exposure time was 40 sec.

TABLE I

R_F VALUES AND FLUORESCENCE REACTIONS OF VITAMIN B_6 COMPOUNDS

*Compounds**	*R_F values in solvents***:*					*Comments*
	I	*II*	*III*	*IV*	*V*	
Pyridoxine	0.48	0.55	0.42	0.75	0.41	weak fluorescence after chromatography in solvents II, III and V.
Pyridoxamine	0.09	0.10	0.30	0.42	0.27	blueish fluorescence after chromatography in solvents II and V.
Pyridoxal	0.78 0.42	0.58	0.39	0.76	0.40	strong greenish fluorescence after chromatography and subsequent treatment with semicarbazide.
Pyridoxic acid	0.61	0.50	0.65	0.73	0.74	blueish fluorescence after chromatography in solvents II, IV and V.
Pyridoxal-5-phosphate	0.70	0.13	0.02	0.63	0	strong greenish fluorescence after chromatography and subsequent treatment with semicarbazide.

* Pyridoxine hydrochloride and pyridoxamine dihydrochloride were furnished by Hoffmann-La Roche, Basel. Pyridoxal-5-phosphate and 4-pyridoxic acid were obtained from L. Light & Co., England. Pyridoxal hydrochloride was delivered by Sigma, U.S.A.

** Solvents: I = *n*-Butanol saturated with *N* hydrochloric acid. II = *n*-Butanol saturated with water. III = *n*-Butanol saturated with *N* ammonia. IV = Methanol–butanol–benzene–water (2:1:1:1). V = Isopropanol–ammonia–water (20:1:2). Chromatography was performed descending in solvent I, ascending in all the other solvents.

The intensity and colour of the fluorescence varies markedly with the solvent system used as seen in Table I.

It was observed in this laboratory that the reaction of semicarbazide with pyridoxal and pyridoxal-5-phosphate resulted in products which gave intense greenish fluorescence under test tube conditions and also on a paper chromatogram. The former reaction has been developed into a sensitive analytical method for the measurement of very small amounts of pyridoxal-5-phosphate. The details of this method will be given elsewhere.

The compounds were applied on filter papers (Whatman No. 1) in amounts ranging from 0.05–1 μg in the case of pyridoxal and pyridoxal-5-phosphate and 1–5 μg of other vitamin B_6 metabolites. Chromatography was performed in different solvents in the dark for approximately 16 h at room temperature. The paper chromatograms were dried at about 65°. When sprayed with a solution of 0.1 *M* semicarbazide in a mixture of 0.1 *M* tris buffer, pH 9, and ethanol (2:3), the spots containing pyridoxal and pyridoxal-5-phosphate emitted an intense greenish fluorescence in U.V. light (Sterisol UV-lamp, Original Hanau, applied with a filter absorbing visible light, Jena Farb- und Filter-glas, UG 1). The fluorescence was found to be stable for weeks. Less than 0.1 μg of each compound could be detected. The sensitivity of the reaction is illustrated in Fig. 1. The R_F values and the details of the spontaneous fluorescence reactions in different solvents are given in Table I.

The routine procedure when viewing the paper chromatograms was as follows:

(1) Observation of spontaneous U.V. fluorescence.

(2) Semicarbazide treatment and registration of spots containing pyridoxal and pyridoxal-5-phosphate.

(3) Diazotization by the Pauly reagent.

The semicarbazide reaction seems to be a simple and sensitive method for the identification of pyridoxal and pyridoxal-5-phosphate on paper chromatograms. Adaptations of this technique for the study of biological material is in progress.

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[1] N. SILIPRANDI, D. SILIPRANDI AND H. LIS, *Biochim. Biophys. Acta*, 14 (1954) 212.
[2] W. A. WINSTEIN AND E. EIGEN, *Proc. Soc. Exptl. Biol. Med.*, 67 (1948) 513.
[3] A. PUSZTAI, *Magy. Kem. Folyoirat*, 60 (1954) 323.
[4] H. E. WADE AND D. M. MORGAN, *Biochem. J.*, 60 (1955) 264.
[5] A. BONAVITA, *Arch. Biochem. Biophys.*, 88 (1960) 366.

Received May 6th, 1963