## Variation of $R_F$ of vitamin $B_6$ group with pH*

The $R_F$ values of some members of the vitamin $B_6$ complex have been reported to vary from run to run[1]. A reinvestigation of these variable results in our laboratory revealed that the $R_F$ values of this group are a function of pH of the developing solvent at constant temperature. All six members were tested: pyridoxol, pyridoxamine, pyridoxal, and their corresponding 5-phosphates. All data were subjected to statistical analysis.

*Methods and materials*

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 had the most uniform structure of several, thus giving the least variability in the background absorption[2]. Single compounds, dissolved in water, were applied three times, and the successive spots were dried completely; each composite spot contained 10 $\mu$g of $B_6$. Origins were visualized by fluorescence under U.V. light at 3660Å and outlined.

The pH was checked and adjusted when necessary before each run, and the solvent inside each hydrometer jar was changed to avoid contamination with previous compounds. In a darkened laboratory, the strips were saturated for fifteen minutes, then dipped and kept in contact with the solvent for one hour, removed, and dried with a heat gun. The pH range tested was from 5.0 to 9.0.

The spot was located with the aid of the U.V. light. For pyridoxal, fluorescence was greatly enhanced by exposure of the strip to $NH_4OH$ (conc.) vapor, not so for pyridoxol and pyridoxamine; fluorescence in these two compounds was, in fact, slightly inhibited. This method was followed with both individual components and mixtures of components of $B_6$.

*Results and discussion*

$R_F$ values were computed for each pH. Table I gives the mean $R_F \pm$ one standard deviation. Analysis of the data was carried out with the aid of the $Y$-test[3]. These results are charted on Figs. 1, 2, 3 and 4.

Four of the six components of the $B_6$ group can be separated by paper chromatography at any one pH. The separation of pyridoxol from its 5-phosphate can be accomplished only at pH 9; this may be due to hydrolysis of the phosphate at other acidities.

It is difficult to distinguish the phosphate of pyridoxal from that of pyridoxamine. When a mixture of the $B_6$ group was chromatographed at any pH, the minimum $R_F$ found was 0.12, which is a value in between the $R_F$ values of the 5-phosphates of pyridoxamine and pyridoxal. In spite of this difficulty, it is always possible to separate pyridoxol, pyridoxal hydrochloride, pyridoxamine hydrochloride, and either the phosphate of pyridoxal or pyridoxamine.

Our experimental observations confirm the report that the area of the chromatographed spot decreases with increase of pH of the solvent system[4]. Thus, starting

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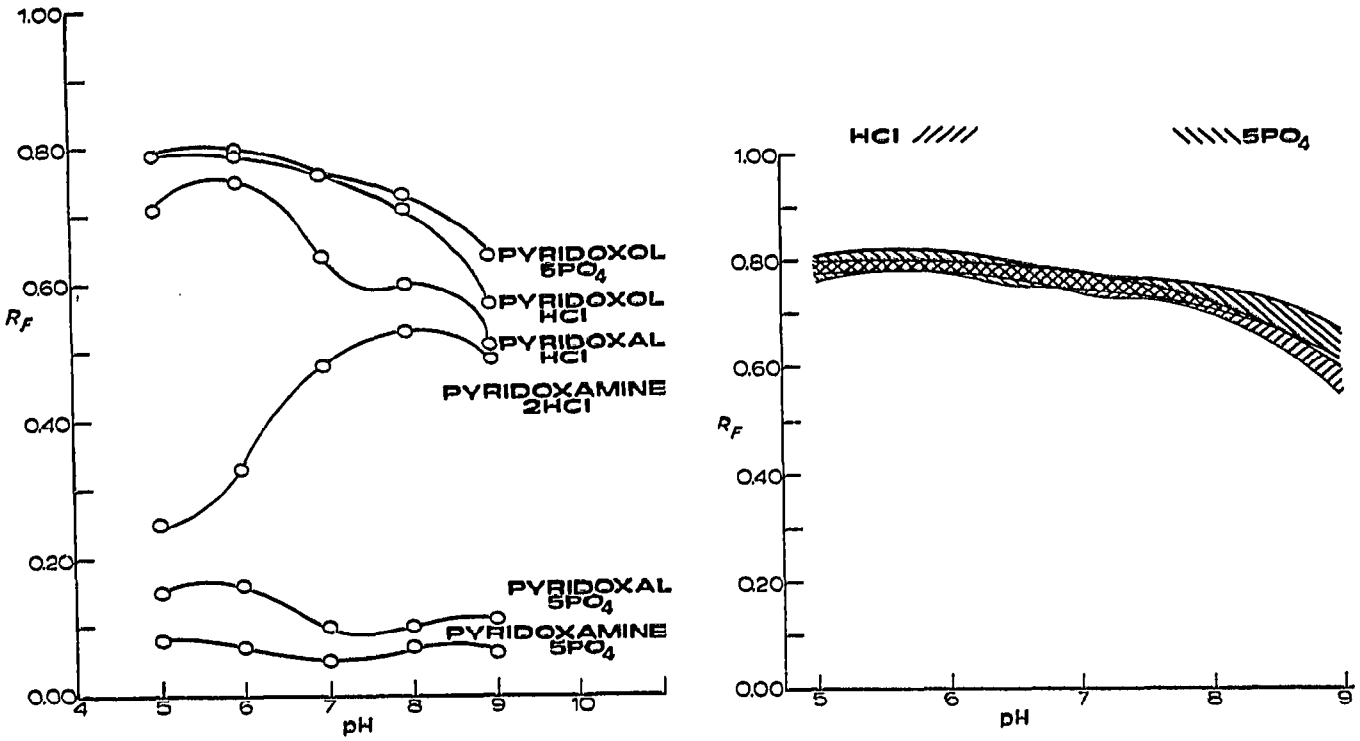


Fig. 1. $R_F$ values of vitamin $B_6$ cogeners at various pH.

Fig. 2. Pyridoxol confidence bands.

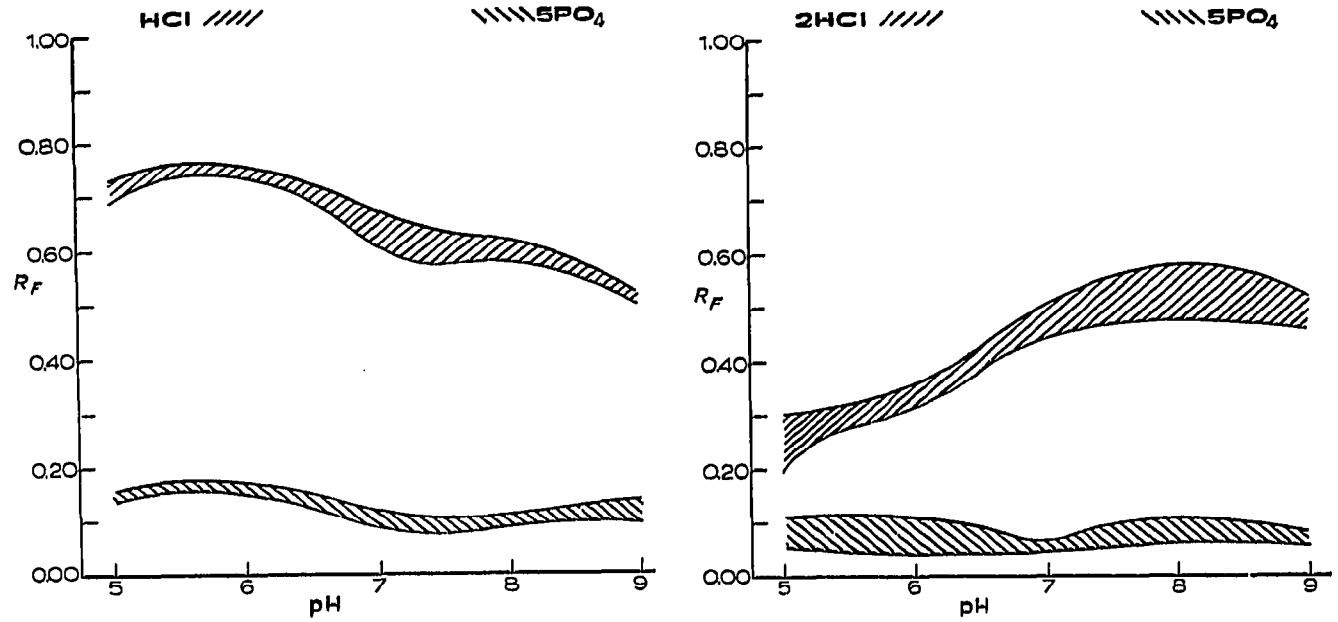


Fig. 3. Pyridoxal confidence bands.

Fig. 4. Pyridoxamine confidence bands.

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 | ($5PO_4$) |

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: area $= C/pH$ (where $C$ is a constant inherent to the pH).

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

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