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CHROMATOGRAPHY OF PHOSPHATE ESTERS

Part I. Separation by Paper Chromatography

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Abstract. Phosphate diesters have been separated by paper chromatography on Whatman paper No. 1 by means of *n*-butanol : 4% boric acid (86 : 14) as an irrigation solvent system, at 25°. R_f values of the esters in pure form as well as in mixtures have been reported.

In view of the vital role played by the phosphate esters in physiological processes, these esters have been a subject of extensive study¹⁻³ during the recent years. Phosphate esters in natural systems usually occur in the form of mixtures. Similarly, when an attempt is made to prepare simple phosphate esters which act as model compounds for the naturally occurring substances, usually a mixture of the esters is obtained. It, therefore, becomes necessary for a detailed study of phosphate esters to develop and standardize methods for their separation and identification. In addition to the other routine analytical methods, chromatography is one such tool which can be of great help in the separation and analysis of phosphate esters. Although small variations in conditions can change the results altogether, an attempt has been made to establish the conditions which give the best results.

Since the separation of amino acids⁴ by paper chromatography, this technique has been extensively used in other classes of organic compounds. Hettler⁵ has given an account of the standard paper chromatographic method for the separation of inorganic phosphates. Such an attempt has been made here for the separation of simple organic phosphate esters. Any particular method of paper chromatography can be considered serviceable provided that a sharp separation of the substances is obtained (a minimum difference of 0.055 in R_f values is necessary if the spots are not to overlap) and if under constant conditions R_f values remain the same within a fixed limit of error. In the case of phosphorus compounds it is often necessary to allow greater variation of R_f values (0.05 instead of 0.02)⁶. Hanes and Isherwood⁷ separated sugar phosphates by paper chromatography. Guided by their success it was decided to try the technique of paper chromatography to resolve the mixture of simple phosphate diesters. Although the phosphate diesters were prepared⁸ to make a study

of their reactivity, it was during their preparation that an idea of their chromatographic study was adopted. In the beginning, the conditions for paper chromatography were standardized on the following phosphate diesters and then under the same conditions, different mixtures prepared from the same esters were attempted to be resolved: 4-nitrophenyl methyl phosphate (A), di-2-nitrophenyl phosphate (B), di-2-nitro-4-acetylphenyl phosphate (C), di-3-nitrophenyl phosphate (D), di-2, 4-dinitrophenyl phosphate (E), di-4-nitrophenyl phosphate (F), 2-nitro-4-acetylphenyl methyl phosphate (G), di-2-nitro-4-chlorophenyl phosphate (H), and di-4-methoxyphenyl phosphate (I).

In all the seven mixtures, *i.e.* (1) A + F + H, (2) B + E + H, (3) A + B + D, (4) A + B + E + H, (5) A + B + F + H, (6) A + B + D + F + H, and (7) A + B + D + F + G + H were prepared from these esters and then resolved by paper chromatography.

From the list of the phosphate diesters it can be seen that some esters are quite different from the others while some are structurally so close to each other that they not only have the same functional groups but also the same molecular formula. Therefore, the chromatographic conditions established for the separation of these esters can be considered as generally applicable to this class of compounds.

Chromatographic Paper. Whatman paper No. 1 was found to be the most satisfactory for this type of chromatography. Treatment of the paper with other solvents such as methanol or acetic acid did not give better results. Therefore, Whatman paper No. 1 was used as such.

Preparation of Samples. Solutions of the phosphate diesters for spotting on the paper were prepared in acetone, and the substances were applied on the starting point on a line which was situated at a distance of about 4.0 cm from the immersed end of

the paper and the spots were about 2.5 cm apart. The concentrations of the samples showed a marked effect on the R_f values. Generally, the R_f values increased with decreasing concentrations but in some cases the R_f values decreased with decreasing concentrations. It was also observed that the relative R_f values also changed with the change in concentration of the sample, e.g. di-4-nitrophenyl phosphate showed higher R_f values than that of 2-nitro-4-acetylphenyl methyl phosphate at low concentration of the samples but the reverse was observed at higher concentrations. Therefore, the solutions of phosphate diesters with fixed concentration, were prepared in acetone for spotting on the chromatogram, and fresh solutions were prepared every time. It was also noticed that in many cases, the R_f value of a pure compound was slightly different from that of the same compound in mixture. This small difference is not unexpected.⁹

Selection of Solvent. In the beginning of our study solvents used by Hanes and Isherwood⁷ in the study of sugar phosphates, were tried but very high R_f values (greater than 0.9) were obtained for most of the esters. Then a wide range of acidic, neutral and basic solvent systems in varying proportions of the components was tried. The solvents used were generally of reagent grade but were purified when felt necessary. Fresh solvents were used every time. The chromatographic chamber was saturated¹⁰ with the solvent vapours before use, by placing the solvent into it and leaving for 16 hr with its lid on. The irrigation period was 10 hr to irrigate 3/4th of the paper.

Of all the solvent systems tried only two gave satisfactory results, i.e., ethyl acetate : pyridine : water (12 : 5 : 4) and *n*-butanol : 4% boric acid (86 : 14). Although both these solvents gave good separation, the former solvent system gave slightly elongated spots whereas the latter gave compact type of spots with reasonable R_f values for good differentiation. Therefore, this solvent system was used throughout our investigations.

Although with different solvent systems different

patterns in which the order of R_f values varied distinctively were obtained, it was possible, as in the case of sugars,¹¹ to alter the R_f values of the esters without changing their relative order by adjusting the properties of the solvent systems.

Detection of Spots. After irrigation, the paper was removed from the chamber, dried in open air and then subjected to a suitable procedure for the location of the spots. Two methods were tried for the detection of the spots. The first one involved the use of the general ammonium molybdate reagent of Hanes and Isherwood⁷ which is applied as a developer for all types of phosphorus compounds.

The second method is based on the fact that the phosphates show intense uv absorption. So the spots were detected by placing the irrigated and dried chromatogram under the direct uv light from "Mineralight Short Wave UVS-12", and marked with a pencil. All the phosphate diesters except di-4-methoxyphenyl phosphate which did not show any absorption due to reasons beyond our understanding, were easily detected by this method.

The advantage of the first method is that the colour of the spots is so developed that it can be preserved permanently whereas the second method is simple and rapid. The second method was preferred in the present studies. In this case the spots looked dark blue in colour and the filter paper looked light violet under the uv light.

Recently a versatile and sensitive method for the detection of organic phosphates on a paper chromatogram has been developed by Firman and Gray¹² but it was not felt necessary to try this method.

Temperature. As demonstrated by Consden *et al*,⁴ the main factors influencing R_f values like the composition of the solvent system, change with temperature. Therefore, the temperature was kept constant within $\pm 0.5^\circ$.

Results and Comments. The R_f values of the phosphate diesters in the pure form as well as in the mixtures are given in Table 1.

TABLE 1. R_f VALUES OF PHOSPHATE DIESTERS MEASURED BY PAPER CHROMATOGRAPHY ON WHATMAN CHROMATOGRAPHIC PAPER NO. 1, AT 25°, WITH *n*-BUTANOL : 4% BORIC ACID (86 : 14) AS AN IRRIGATING SOLVENT SYSTEM.

Phosphate diesters	Pure		In mixtures					
A 4-Nitrophenyl methyl phosphate	0.33	0.33		0.32	0.33	0.33	0.33	0.33
B Di-2-nitrophenyl phosphate	0.46		0.46		0.46	0.45	0.46	0.45
C Di-2-nitro-4-acetylphenyl phosphate	0.48			0.47				
D Di-3-nitrophenyl phosphate	0.53			0.55			0.53	0.52
E Di-2, 4-di-nitrophenyl phosphate	0.55		0.57		0.55			
F Di-4-nitrophenyl phosphate	0.58	0.59				0.57	0.58	0.57
G 2-nitro-4-acetylphenyl methyl phosphate	0.62							0.62
H Di-2-nitro-4-chlorophenyl phosphate	0.69	0.70	0.70		0.69	0.68	0.69	0.69

During the present investigations, one-way ascending paper chromatographic technique of Williams and Kirby¹³ was applied by which obviously in a mixture containing many components, a complete separation might not be achieved on a strip of small length because some components of the mixture may have very little differentiation or may run to the same position of the chromatogram. This was actually observed during our investigation. In such cases a number of different solvents with different properties can be used so that the components running together in one solvent system will probably separate in another solvent system. Although many one-way chromatograms, each in different solvents, could be compared, yet more illustrative information would be obtained if two solvent systems are used in conjunction for prepping a two-way chromatogram than if the two solvents are used to prepare two separate one-way chromatograms.

Any relationship between the structure or any other property of the phosphate esters and the R_f values could not be ascertained. Nor is it always possible because the slightest variation in the conditions of chromatography, like the composition of the irrigating solvent system, concentration or the amount of the sample itself, temperature, etc., would vary the R_f values significantly.

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