*The Separation of α-Glycerophosphoric Acid from its β-Isomer by Paper Chromatography**

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(Received June 24, 1956)

Introduction

Chemical configurations of naturally occurring phosphatides or phosphatidic acids have been questioned for a long time, the difficulty being due to the reversible migration or hydrolysis of the phosphoryl group during the course of isolation or hydrolytic decomposition in the determination of the structures. Recently, Baer and co-workers have shown for a number of naturally occurring phosphatides that they possess the α-structure and L-configuration[1]. The L-α-configuration of lecithins was confirmed later by Long and Maguire[2]. In addition to the reversible migration of the phosphoryl group, difficulty arises because there has been developed no simple and adequate method of direct determination or identification of β-glycerophosphoric acid (β-GPA), which is less stable than α-GPA.

For either identification or quantitative determination of the α and β-isomers, the following methods have been used: the determination of their rates of hydrolysis by enzymes[3], the isolation of barium α-glycerophosphate (GP) and the barium nitrate complex of barium β-GP[4], the periodate oxidation method[5,6], and the determination of inorganic phosphoric acid derived from oxidative and hydrolytic decomposition[7]. All of these methods are time consuming and do not afford direct identification or determination of the β-isomer. Therefore, simple methods which would give this information would have useful applications.

Although a number of papers dealing

* This work was presented, in part, before the Eighth Annual Meeting of The Chemical Society of Japan, April, 1955.

1) E. Baer and M. Kates, *J. Am. Chem. Soc.*, **70**, 1394 (1948); *J. Biol. Chem.*, **175**, 79 (1948); *J. Am. Chem. Soc.*, **72**, 942 (1950); *J. Biol. Chem.*, **185**, 615 (1950); E. Baer, H. C. Stancer and I. A. Korman, ibid., **200**, 251 (1952); E. Baer, J. Maurukas and M. Russell, *J. Am. Chem. Soc.*, **74**, 152 (1952); E. Baer and H. C. Stancer, ibid., **75**, 4510 (1953); E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 25 (1954); ibid., **212**, 39 (1954); E. Baer, D. Buchnea and A. G. Newcombe, *J. Am. Chem. Soc.*, **78**, 232 (1956).

2) C. Long and M. F. Maguire, *Biochem. J.*, **57**, 223 (1954).

3) H. D. Kay and E. R. Lee, *J. Biol. Chem.*, **91**, 135 (1931).

4) P. Karrer and B. P. Benz, *Helv. Chim. Acta*, **10**, 87 (1927).

5) F. Rapoport, I. Reifer and H. Weinmann, *Mikrochim. Acta*, **1**, 290 (1937).

6) P. Fleury and J. Courtois, *Bull. soc. chim.*, **8**, 397 (1941).

7) C. F. Burmaster, *J. Biol. Chem.*, **164**, 233 (1946).

with the chromatographic separation of organic esters of phosphoric acid of chemical and biological interest have appeared elsewhere, none of these have applied specifically to the problem of separating the glycerophosphates. Khym[8] attained a certain degree of separation of the isomers on an anion exchanger column, but it was not quite complete.

Conditions to be used for chromatography should be selected to eliminate hydrolysis and isomerization reactions. It has been shown that alkaline rather than acidic conditions are preferable[9,10] in order to avoid the occurrence of these two reactions in the case of the glycerophosphates.

We have studied the behaviors of α-GPA and β-GPA, and their alkaline metal and ammonium salts on alumina impregnated paper and achieved a complete separation of the two isomers as sodium salts, by using methanol-ammonia (60 : 5) as a developing solvent. Since it is known that alumina gives rise to secondary reactions in the presence of alkali during the course of chromatography[11], it would be necessary to examine if the compounds under consideration have undergone any chemical changes such as hydrolysis or migration of the phosphoryl group. It has been demonstrated that no hydrolysis or migration of the phosphoryl group takes place during the course of the chromatography. The system presented utilizes the ion exchange property of alumina and exploits differences of the ionic attractive forces which might be exhibited between a cation and anions of different acidities[12].

Experimental

Alumina Impregnated Paper.—Toyo filter paper No. 50 (2×40 cm.) was used. The paper was prepared according to Bush's method[13], except that a twenty-four hour washing time and a forty-eight hour exposure time to ammonia vapor were maintained. The paper prepared in this manner showed pH 8 and had adsorbed an average of 8 per cent by weight of alumina. These conditions should be maintained in order to obtain compact spots of the glycerophosphoric acids. The paper remained sensitive for four to five days when kept in an air-tight container.

Sample Solutions.—The solutions of sodium α-*, sodium β-**, barium α-*, and barium β-glycerophosphates*** were prepared by dissolving a weighed amount of the respective salt in distilled water so that each 10 μl. of the solution contained 250 μg. of the free acid. For the preparation of a solution of a mixture of the sodium salts of the α- and β-isomers, 104 mg. each of the salts were dissolved in 1 cc. of distilled water so that each 30 μl. of the solution contained 250 μg. of the free acids. Barium α-GP was converted to the ammonium salt by interacting with ammonium sulfate, and sodium β-GP to the free acid by adding 1N hydrochloric acid. The solutions of these two compounds were prepared so that each 30 μl. of the solution contained 250 μg. of the free acid.

Chromatographic Technique.—The sample solution was applied at 3 mm. from one end of the paper (after removing 1.5 cm. from both ends of the air-dried paper) with a number of applications under air-drying at 25-30° so that the dried spot was 0.6-0.8 mm. in diameter. The temperature specified here is an important factor, and must be adhered to since the solute is strongly adsorbed at a higher temperature and the spot will not move at all. The paper was equilibrated in a closed tower containing a mixture of methanol and ammonia (60 : 5) for four hours and developed for 2.5 hr. at 11-14°C by the descending method. After airdrying, the paper was sprayed with bromothymol blue indicator, whereupon the acidic spot appeared in a distinct yellow color and a cation, when present, in a blue against a grass-green backgound. This method of identification is much easier than the one commonly used[14] for the organic phosphates. The chromatogram so obtained remained unchanged for a considerable length of time, more than a year, if kept in an air-tight container.

Although we used a sample size of 250 μg. for each spot in order to make a distinct observation of the color, the method was found to be sensitive to an amount less than 60 μg. The Rf values are not affected by varying the time of pre-exposure to the solvent vapor but are somewhat affected by varying temperature.

Tests for Hydrolysis and Migration of the Phosphoryl Group.—The alumina impregnated paper was cut in a length a few mm. longer than

8) Private communication.
9) M. C. Bailly, *Compt. rend.*, **206**, 1902 (1938).
10) M. C. Bailly, *Bull. soc. chim.*, **9**, 314, 340 (1942).
11) E. Lederer and M. Lederer, "Chromatography", Elsevier Publishing Co., New York, N. Y. (1954), pp. 276-9.
12) W. Kiessling, *Biochem. Z.*, **273**, 103 (1934).
13) I. E. Bush, *Nature*, **166**, 445 (1950).

* The sample was prepared from the corresponding barium salt (C. Urakami and Y. Kakutani, Repts. Sci. Living, Osaka City Univ., Series D, No. 1, 3 (1953)) by removing the barium ion as the sulfate, by adding 1N sodium hydroxide to the ammonium salt formed, by precipitating the salt with alcohol, and finally by removing any excess of alkali present by repeated washing with alcohol.

** The sample was obtained from Delta Chemical Works and was reported to be 98 per cent pure.

*** The sample was prepared from the corresponding sodium salt by converting first to the ammonium salt with ammonium oxalate and then to the barium salt with barium chloride, and by precipitating the latter salt from its concentrated aqueous solution by adding a mixture of alcohol and ether. It was purified by preparing its barium nitrate complex.

14) F. A. Isherwood and C. S. Hanes, *Nature*, **164**, 1107 (1949).

the length corresponding to the Rf value of the glycerophosphoric acids and one end was cut in a zigzag manner. Five hundred μg. each of the α and β-isomers in the free acid forms were chromatographed on a separate sheet of paper under the conditions employed previously and the solvent allowed to drip from the irregularly cut end into a small collector. Several similar runs were made with each acid so that at least 10mg. of the sample were collected. The removal of the acid from the paper took a little over 2.5 hr.; thereafter the paper showed no acid spot remaining when sprayed with the indicator. The liquid collected was concentrated at room temperature under reduced pressure and the residue converted to the barium salt in both cases. In these samples no inorganic phosphorous was detected. The barium salt of the α-compound did not give any insoluble precipitate with barium nitrate, but barium β-GP did give an insoluble precipitate. Both samples were chromatographed on fresh papers and their Rf values were found to be identical with those previously found. The sodium salt prepared from the liquid collected from the chromatogram of barium β-GP was applied on a fresh paper and developed as described above. The acid spot was detected only at the position of the application.

To test whether or not the unmoved spot of sodium β-GP had undergone any chemical changes, the salt was applied on a small piece of the paper cut in a length of about 7 mm. from the starting line and eluted for four hours to collect the sodium ion and a further three hours to collect ammonium β-GP. Within the respective time, any alkaline or acid spot disappeared from the paper. The second fraction collected was applied on a fresh paper and chromatographed. The Rf value found for this sample was identical with that observed for either its free acid or barium salt, 0.46.

Behavior of Inorganic Phosphate, Sulfate and Chloride Ions.—Since an inorganic phosphate ion might be formed as a product of hydrolysis during the course of the chromatography and a small amount of $(NH_4)_2SO_4$ or HCl might be present in the sample solutions prepared, it would be necessary to examine the behavior of these ions on the paper. The dilute solutions of NaH_2PO_4, $(NH_4)_2SO_4$, and HCl were subjected to chromatography under the conditions specified above. The phosphate and sulfate ions were found to remain unmoved (B and D in Fig. 2), whereas the chloride ion showed a strong yellow spot at the position of application and a faint one at the Rf value corresponding to that of the α-isomer (C Fig. 2). The yellow spot found at Rf 0.45 with the β-isomer was not, however, due

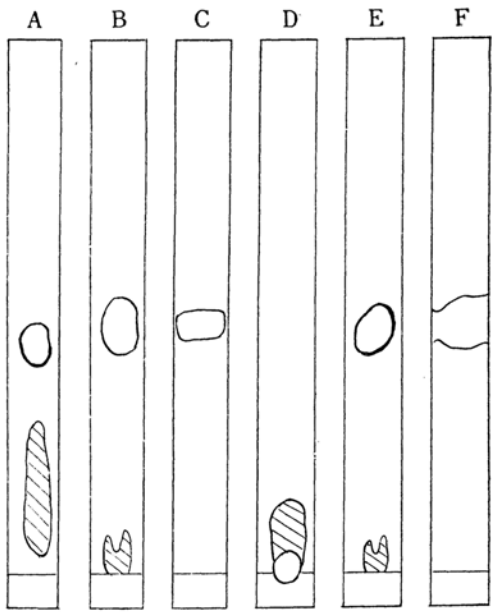

Fig. 1. Reproduction of the paper chromatograms of the glycerophosphates by the descending method. Solvent, methanol-ammonia (60 : 5); 4 hours pre-exposing to the solvent vapor; 2.5 hours developing. The shaded spots represents cations and the unshaded spots anions. A, sodium α-GP; B, barium α-GP; C, ammonium α-GP; D, sodium β-GP; E, barium β-GP; F, β-GP.

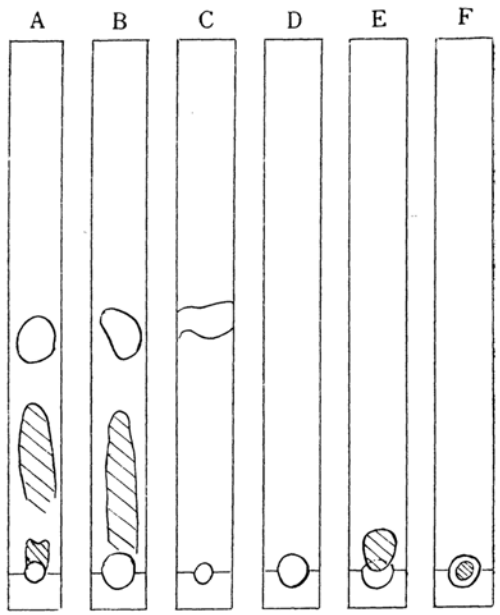

Fig. 2. Reproduction of the paper chromatograms of a mixture of sodium α- and β-GP, inorganic anions and glucose phosphates. The conditions employed are identical with those given for Fig. 1. A, a mixture of sodium α- and β-GP; B, a mixture of sodium α-GP and monosodium phosphate; C, HCl; D, ammonium sulfate; E, potassium glucose 1-phosphate*; F, barium glucose 6-phosphate*.

* We are indebted to Professor Jiro Nikuni of the Osaka University for these samples.

to this chloride ion alone since organic phosphorous was detected in the eluate.

Results

As shown in Table 1, ammonium, barium and sodium salts of α-GPA, barium β-GP and β-GPA showed almost the same Rf value, 0.45-0.46. The Rf value of sodium α-GP was found, however, to be always somewhat lower than that of barium α-GP. Sodium β-GP, on the other hand, remained unmoved, the blue spot of the sodium ion being found just above or covering the upper half of the yellow spot of the acid (D in Fig. 1). A complete separation of α-GPA from β-GPA and from inorganic phosphoric acid was attained (A and B in Fig. 2). No hydrolysis or migration of the Phosphoryl group was found to occur during the course of the chromatography. Results of Burmaster's method on the sample collected by the column method[15] support our present observation.

The compounds which are more acidic than the glycerophosphoric acids, such as glucose 1- and 6-phosphoric acids were examined under the identical conditions. They were found to remain unmoved from the initial position of application.

Discussion

When the behavior of the anions and cations of the sodium glycerophosphates and the glucose phosphates on the paper are examined, it is seen that dissociation of the salts becomes difficult with increase in acidity of the acid residues (A and D in Fig. 1; E and F in Fig. 2): pKa_1 of α-GPA, β-GPA, glucose 1- and 6-phosphoric acids are 1.40, 1.37[12], 1.10[16] and 0.94[17], respectively. This does not, however, apply to the case of the barium glycerophosphates (B and E in Fig. 1), where the Rf values of both isomers are identical (Table I). The significant difference in Rf value is observed with the sodium salts. In affording the resolution of the isomers, the effect of the sodium ion appears, therefore, to be more pronounced than that of the difference in acidity of the two isomers[18]. Analogous effects have been observed with inorganic salts[19-21]. Of course, further study on this point is indicated, but we consider that there probably exists a stronger ionic attractive force between a strongly basic cation and an anion of a salt, under the conditions specified here, than between a less basic cation and the same anion.

TABLE I

Rf VALUES OF α- AND β-GLYCEROPHOSPHATES

Temp. °C	α			β		
	Ba	Na	NH_4	Ba	Na	Free
12	0.46	0.44	0.46	0.46	0.00	0.45
14		0.42				0.46
		0.45[a]				
15		0.43[b]				

Each of these Rf values represents an average of those obtained from 4 or 5 chromatograms. [a] Four hours of developing time. [b] Obtained from the chromatogram of a mixture of sodium α- and β-glycerophosphates.

The movement of these acid spots is primarily facilitated by the cellulose present, since it has been observed that the acid residue of the glycerophosphates advances with wide zone formation on a plane filter paper strip at a much faster rate than on the alumina impregnated paper; and, furthermore, that it does not move at all when the concentration of alumina is increased by more than 20 per cent by weight on the paper. Thus, with respect to the apparent movement of the ions, the capillary movement through the cellulose fibers is considered to be more effective than the ion exchange mechanisms of alumina.

The compactness of the acid and base spots is perhaps brought about by such ion exchange mechanisms, exerted by the alumina adsorbed on the filter paper, since the spreading of the anions observed on the plane filter paper can be brought under control to a certain extent on the alumina impregnated paper. In the presence of a strong acid, alumina ionizes in the following manner and behaves as a base[22]:

$$Al(OH)_3 \rightarrow Al(OH)_2^+ + OH^-$$

The anion of an organic acid salt is then attracted by the cation of the alumina ionized as indicated. On the other hand, in the presence of a strong base, the alumina ionizes into the following ionic species and behaves as an acid:

$$Al(OH)_3 \rightarrow H^+ + AlO_3H_2$$
$$\rightarrow H^+ + AlO_2^- + H_2O$$

15) Presented before the 9th Annual Meeting of The Chemical Society of Japan, April, 1956.
16) C. F. Cori, S. P. Colowick and G. T. Cori, *J. Biol. Chem.*, **121**, 465 (1937).
17) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **185**, 113 (1927).
18) W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.*, **65**, 2355 (1943).
19) L. Sacconi, *Discuss. Faraday Soc.*, **7**, 173 (1949).
20) M. Shibata, Science (Japan), **19**, 570 (1949).
21) M. Tanaka and M. Shibata, *J. Chem. Soc. Japan*, **71**, 312 (1950).
22) F. P. Treadwell and W. T. Hall, "Analytical Chemistry", Vol. I, John Wiley and Sons, Inc., New York (1937), Ed. 9, pp. 189-192.

The cation dissociated from an organic phosphate is then attracted by the anion derived from the alumina as shown above.

There is, however, another factor to be taken into consideration, the solvent system. If a solvent system is of such a nature that it helps in dissociating salts into their respective ionic species, and also increases the solubilities of the anions formed, their movement may be facilitated. Such may be the case with the glucose phosphates.

When separation by electrophoretic or ion exchange methods alone fails, it may be possible to achieve a better resolution of homologoues or isomeric organic acids with somewhat different acid strengths by selecting an appropriate cation and by chromatographing on this type of adsorbent system.

Summary

The behaviors of α- and β-glycerophosphoric acids, their alkaline metal and ammonium salts on alumina impregnated paper, developed with a mixed solvent of methanol and ammonia (60 : 5), have been described. It has been demonstrated that a mixture of the sodium salts of the two acids may be completely separated in this way without hydrolysis or migration of the phosphoryl group. The method also affords a distinct separation of α-glycerophosphoric acid from a mixture of the inorganic phosphates, the β-glycerophosphates, and the glucose 1- and 6-phosphates; and also of the β-glycerophosphates from a mixture of the inorganic phosphates, the glucose 1- and 6-phosphates. The mechanisms possibly involved have been discussed.

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