*Separation of α-Glycerophosphate from Its β-Isomer by Column Chromatography**

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It has been reported previously from our laboratory that sodium glycerophosphates can be resolved completely into the α and β-isomers on the alumina impregnated paper with methanol-ammonia (60 : 5) as a developing solvent and that no hydrolysis or migration of the phosphoryl group seems to occur, as far as the sensitivity of the test employed is concerned[1]. The purpose of application of this method to column chromatography is twofold: firstly, to obtain pure samples of both isomers on a milligram level for biochemical interest and secondly, to give an absolute experimental evidence to support our previous results on hydrolysis and migration of the phosphoryl group. For the latter, the samples obtained by chromatography were analyzed according to Burmaster's method[2], which affords the determination of the inorganic phosphate, α and β-glycerophosphates in a mixture of the three by spectrophotometrical means on the color developed by the reduction of the phosphomolybdate with stannous chloride.

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1) C. Urakami and Y. Kakutani, This Bulletin, **30**, 21 (1957).

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

Experimental

Apparatus.—The chromatographic tube used was of the size 10 mm. in diameter and 150 mm. in length, provided with an inner joint connection with a sealed-in fritted glass disk and a stop-cock.

Spectrophotometric measurements were carried out with Shimazu Model DF-II with a filter No. 634. All measurements were taken within the range of its maximum sensitivity, 0.1-0.3 extinction.

Samples and Reagents.—The solutions for analysis of the samples, α-1, α-2 and β-3 as indicated in Table I, were prepared so that each ml. contained approximately 20 μg. of phosphorus. Assay of the solutions was carried out by taking one-half of this quantity.

The solutions of the following reagents were prepared according to the specifications given by Burmaster; anhydrous sodium sulfite (reagent grade of Wako Pure Chemicals Co.), ammonium molybdate (the first grade of Daito Chemical Co.), stannous chloride (special grade of Yashima Chemical Co.), and sodium periodate (Marukawa Chemical Co.).

Bromothymol blue, 0.1% solution, was used for detection of the zone of the glycerophosphoric acids.

Adsorbent.—One part by weight of alumina for chromatography (200 mesh, Yashima Chemical Co.) and nine parts by weight of cellulose powder (No. 50 of Toyo-filter paper Co.) were mixed thoroughly with a sufficient quantity of the mixed solvent, methanol-ammonia (60 : 5), and the mixture allowed to mercerate for ten hours. During the last two hours the mixture was stirred vigorously with a magnetic stirrer. About one gram of the slurry was poured, all at once, into the chromatographic tube and after the adsorbent had settled, the column was packed firmly with an additional quantity of the solvent, about 100 ml., by weighting at the top and applying suction at the base of the column to give 24-26 mm. in length.

Chromatographic Technique.—When about one ml. of the solvent was remaining over the surface of the column, a suspension of 2 mg. of the sample or the mixed sample containing one mg. each of the isomers in 5 ml. of the solvent mixture was applied and adsorption of the solute was allowed to take place for about one hour, with the stop-cock being left open and without application of suction. When a few mm. of the solvent were remaining over the surface of the column, a furthere amount of the solvent was added. Exposure of the column to air at this point should be strictly avoided, otherwise the adsorption of the solute is so strong that the zone will not move. The rate of flow was maintained at one ml. per min. by applying suction. The percolate was collected in a 100 ml. fraction up to a total volume of 1 l. The presence of the solute, the α-glycerophosphate, was found in the fraction collected between 850 and 950 ml. of the percolate. By applying the indicator, we have observed that the glycerophosphate advanced down the column in a clean-cut narrow zone.

Preparation of Solutions for Analysis.—A 100 ml. fraction containing the α-glycerophosphate was evaporated almost to dryness on a water bath at a low temperature, below 45°C, a small amount of distilled water added to the residue, any insoluble material found removed by filtration, and rinsed several times with fresh water to insure quantitative recovery of the phosphate. The filtrate and washings were combined and made up to 6 ml. in an accurately graduated tube. An aliquot of 3 ml. was used for analysis of α-glycerophosphoric acid and 1.5 ml. each for the β-isomer and inorganic phosphate.

The β-isomer was recovered in the following manner: First, the zone of the adsorbed β-isomer was located, after about 1 l. of the percolate had been collected, by passing a solution of a few drops of bromothymol blue indicator in the solvent and by drying the column somewhat with application of vacuum. An excessive drying should be avoided, or the detection of the zone would not be possible. A distinct yellow zone was found within 5 mm. from the surface of the column, the rest being colored blue green. Therefore, after extruding the somewhat wet column from the tube, a section was cut 5 mm. from the upper end of the adsorbent. It was extracted three times with a 5 ml. portion of a weak alkaline solution, pH 8, and any insoluble material removed by centrifugation. The supernant liquid obtained was evaporated to a small volume and made up to 6 ml. with distilled water. An aliquot of 3 ml. was used for analysis of the α- and 1.5 ml. each for the β-isomer and inorganic phosphorus.

Results and Discussion

By applying 2 mg. of the pure sample of barium α-glycerophosphate or sodium β-glycerophosphate to a column consisting of a mixture of alumina and cellulose (1 : 9) 25 mm. in length and by developing with methanol-ammonia (60 : 5) as a solvent, 85 % of the former was recovered in the 100 ml. fraction collected between 850 and 950 ml. of the percolate and 35 % of the latter in the extract of a section cut 5 mm. from the column surface, as shown in Table II. When a mixture of the sodium salts of the two isomers was applied, 53–56 % of the α and 20–30 % of the β were recovered. No inorganic phosphorus was detected in all these samples recovered and also no β-glycerophosphoric acid was found to be present in the fraction containing the α-isomer or the α in the extract containing the β-isomer, as shown in Table II.

We have also succeeded in separating the isomers by applying a 5 mg. load on the column of the same length but its retention volume was found to be less

TABLE I

ANALYSES OF GLYCEROPHOSPHATE SAMPLES

Sample No.	Diln. factor	Extinction			P, μg. found		Total P μg. found**		Total P, μg. calcd.	Purity %	
		Total	α	β*	α	β	α	β		α	β
α—1	2	0.108	0.108	0.000	4.1	0.0	8.2	0.0	10	82	00
	〃	0.203	0.203	0.000	4.3	0.0	8.6	0.0	10	86	00
	〃	0.202	0.202	0.000	4.25	0.0	8.5	0.0	10	85	00
α—2	〃	0.226	0.169	0.057	3.75	1.3	7.5	2.6	10	75	26
	〃	0.213	0.169	0.044	3.75	1.0	7.5	2.0	10	75	20
	〃	0.223	0.174	0.049	3.85	1.1	7.7	2.2	10	77	22
β—3	〃	0.230	0.000	0.230	0.0	4.8	0.0	9.6	10	00	96
	〃	0.230	0.000	0.230	0.0	4.8	0.0	9.6	10	00	96
	〃	0.228	0.000	0.228	0.0	4.75	0.0	9.5	10	00	95

* Obtained by subtracting extinction of α from that of total.

** Obtained by multiplying P, μg. found directly from the extinction by the dilution factor, 2.

In all these samples no inorganic phosphorous was detected.

α-1, Barium α-glycerophosphate[3]; α-2, Disodium α-glycerophosphate. $5.5H_2O$ (Wako Pure Chemicals Co.); β-3, Disodium β-glycerophosphate. $5.5H_2O$ (Yashima Chemical Co.).

TABLE II

ANALYSES OF GLYCEROPHOSPHATES OBTAINED BY CHROMATOGRAPHIC SEPARATION

Sample No.	Diln. factor	Extinction			P found μg.		Total P found, μg.		Total P in Sample, μg.		Recovery %	
		Total	α	β	α	β	α	β	α	β	α	β
α—1	14	0.234	0.234	0.000	10.35	0.00	144.9	00.0	171.7	00.0	84	00
β—3	6	0.216	0.000	0.216	0.00	9.6	0.0	57.6	0.0	163.0		35
Mixt. of												
α—2	8	0.103	0.103	0.000	4.2	0.0	33.6	0.0	63.4	20.3	53	
β—2	4	0.124	0.000	0.124	0.0	5.09	0.0	20.4		102.0		20
Mixt. of												
α—2	4	0.109	0.109	0.000	8.9	0.0	35.6	0.0	63.4	20.3	56	
β—3	4	0.188	0.000	0.188	0.0	7.6	0.0	30.5		102.0		30

A 2 mg. sample of the sodium salt was used for the individual run; one mg. each was used for the mixed sample. The α-sample was collected in a 100 cc. fraction taken between 850–950 ml. of the percolate: the β-sample was extracted from the adsorbent cut 5 mm. from the surface of the column. No inorganic phosphorus was detected in all these samples.

than 50 ml. and the recovery of the α less than 20%, the major portion being found in the first 100 ml. This indicates that a 5 mg. load is close to the saturation point for the capacity of the amount of the adsorbent employed. We have also attempted to increase the capacity of the column by increasing the column length but encountered some difficulty in obtaining a uniformly packed column, due to difference in density of the two components of the adsorbent used.

These results show, therefore, that a mixture of a milligram each of the sodium salts of α and β-glycerophosphoric acids can be resolved completely on the column chromatographic system of this nature and provide a conclusive evidence for our previous observation that no hydrolysis or migration of the phosphoryl group occurs during the course of the chromatography.

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3) C. Urakami and Y. Kakutani, *Repts. Sci. Living, Osaka City Univ., Series D*, No. 1, 3 (1953).