15. Studies on Erythrocyte Hemins. II

Separation of Erythrocyte Hemins by Paper Chromatography

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It was previously reported¹⁾ that erythrocyte hemin sample was separated into some components by thin-layer chromatography using new solvent system. Successive experiment of paper chromatography for erythrocyte hemin sample was carried out to confirm the results of the previous paper.

This paper describes that erythrocyte hemin sample could be separated into some hemin components by paper chromatography using the solvent system.

Materials and method. *Hemin samples.* Recrystallized human hemin sample was prepared by the Fischer method¹⁾ in our Laboratory. Three hemin samples were commercially prepared from Sigma Chemical Co. (equine, crystalline, type III, lot no. 46B-0050, and bovine, crystalline, type I, lot no. H62B-85-2) and Nutritional Biochemical Co. (without presentation of source, recrystallized, control no. 6475). These hemin samples were stored usually in a brown desiccator containing silica-gel at 5°C until use. About 0.5 mg of hemin sample was freshly dissolved into 1 ml of redistilled acetone and centrifuged for one minute at 2,000 r.p.m. to exclude sediment. Supernatant solution was submitted to the paper chromatography.

Paper chromatography. The solvent system used was chloro-benzene-acetic acid (100:8, by vol.). The benzidine reagent for the detection of hemin components was a freshly prepared mixture of 1% benzidine in absolute methyl alcohol (15 ml), pyridine (0.3 ml), glacial acetic acid (3 ml), water (10 ml), and 30% hydrogen peroxide (0.2 ml). The chromatographic analysis were performed by ascending technique using Whatman No. 1 filter paper strips (2×40 cm). The sample solutions were applied to the starting line located at 4.5 cm apart from one end of the strips. The strips were then dried at room temperature and suspended in a sealed glass cylinder. The strip was remained in the cylinder until the front of the solvent arrived in a distance of 25–30 cm from the starting line. The strips were taken out from the cylinder and

dried at room temperature before spraying the benzidine reagent.

Results and discussion. All the hemin samples tested were separated into four fractions by the paper chromatographic analysis. The result is principally identical to that in the previous report.¹⁾ These four fractions were (1.) middle components (M) located in the middle part, (2.) non-migrating components (O) observed on the starting line, (3.) slow-migrating components (S) distributed between the area of M and O, and (4.) fast migrating components (F). The chromatographic patterns of M-, O- and S- components in all the hemin samples were similar, whereas appreciable differences were found in F. This result concerning F-components was akin to that obtained by the previous study.¹⁾ The migrating distances of the hemin components on the paper chromatograms could be controlled by concentration of the acetic acid in the solvent system.¹⁾ The separation of the hemin components, especially for S, on the paper chromatogram was somewhat incomplete in comparison with the thin-layer chromatographic separation.

Paper chromatographic studies of erythrocyte hemin have been already reported. Chu *et al.*²⁾ used two solvent systems, 2,6-lutidine-water (3.3:2.7, by vol.) and water-n-propyl alcohol-pyridine (5.5:0.1; 0.4, by vol.). Morrison *et al.*³⁾ used four different solvent systems. The first solvent was composed of 2 ml of 99% formic acid, 10 ml of hexane, and 88 ml of chloroform. The second was 4% iso-propyl alcohol in benzene saturated with picric acid. The other

Fig. 1. Paper chromatograms of erythrocyte hemin samples on Whatman No. 1 stained with benzidine reagent; the samples in acetone were developed with chlorobenzene-acetic acid (100:8, by vol.) at 18°C for 3 hrs.

(1) hemin sample (equine, type III, Sigma, (2) hemin sample (bovine, type I, Sigma), (3) hemin sample (N.B.C.), (4) hemin sample (human, prepared in our laboratory).

Rf value of M was 0.44.

two were 2,6-lutidine-water (5.5:4.5, by vol.) and pyridine-iso-propyl alcohol-8 N ammonium hydroxide (1:2:2, by vol.). Both of the above papers described that the spot of hemin was single through application of various solvent systems.

The findings of heterogeneity of the erythrocyte hemin on paper chromatogram have been reported by Morrison *et al.*,^{4),5)} Toalson *et al.*,^{6),7)} and Lee *et al.*^{8),9)} Morrison *et al.*⁴⁾ demonstrated first the presence of one major and three other components. Toalson *et al.*⁷⁾ showed the separation of three components using chloroform solvent. Lee *et al.*⁹⁾ reported also the separation of hemin into three components by the aid of using water-n-propyl alcohol-pyridine solvent system of Chu *et al.*²⁾

For the purpose of separation of hemin samples into multiple components by the paper chromatography, the solvent system used by the present author is more effective than those of other solvent systems. The demonstration of multiple components in erythrocyte hemin sample, observed on the paper chromatogram, is similar to the result obtained by thin-layer chromatographic study.¹⁾

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