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Phosphocellulose chromatography of human hemoglobins

Phosphate compounds are known to bind to a number of proteins including hemoglobins. HUISMAN and co-workers demonstrated a differential binding of inorganic phosphates to two adult chicken hemoglobins[1]. More recently, it has been found that Hb A, Hb S, and Hb F have different binding affinities for 2,3-diphosphoglyceric acid[2–4].

Since differences in affinities of hemoglobins for phosphate compounds exist, it seemed highly probable that phosphocellulose could be used for resolving various components of human hemoglobins. This paper, therefore, presents results of studies in which phosphocellulose was used both as an ion-exchange resin and as a stationary phosphate phase for column chromatography of human hemoglobins. Phosphocellulose chromatography separates the major hemoglobins into three subcomponents and separates Hb A_2 from Hb C.

Experimental

Materials. Batches of phosphocellulose from several sources were tried, the most satisfactory being Cellex-P, control No. B-1040, obtained from the Bio-Rad Co., Richmond, Calif. This particular lot was made several years ago and is no longer available. Lots bearing control Nos. 5170, 7414, and 9050 were tried but gave broad bands with poor separation. The same resin with control No. 9185, made recently, is satisfactory but is not as good as the B-1040 batch. To use this technique will require searching for a good batch of resin.

The elution gradient was made with a varigrad of two chambers with mixing in the first chamber by a magnetic stirrer. All buffer solutions were made from Tris citrate and contained 100 mg/l of KCN. The buffer solution used to equilibrate the resin and as a starting buffer was 0.005 *M* in Tris and was adjusted to pH 6.7 with saturated citric acid just before adding the last few milliliters of water in diluting to volume. The terminal buffer was at a pH of 8.2 and 0.01 *M* in Tris. At pH 6.7, Tris is about 97% ionized and at 8.2 it is only 50% ionized. Thus, the concentration of ionized Tris which could compete with cationic hemoglobins for phosphate sites is almost the same in both the starting and the final buffer. Column chromatography was carried out on minicolumns (2 × 150 mm) as previously described[5].

The hemolysates studied contained Hb A, Hbs A and S, Hbs S and C, Hbs A and C, Hb C from patients with C-thalassemia, cord blood hemoglobin, and Hb A and β chain Hb I. Some of the hemolysates were preserved at −46° before use; other samples were examined fresh.

Procedure. As recommended by the manufacturer, the phosphocellulose was prepared by equilibrating the dry resin with a large excess of buffer. The resin was allowed to settle for 15 min and the "fines" removed. This procedure was repeated. The resin was then mixed with a small volume of buffer and poured into columns as previously described[5]. The columns were equilibrated with buffer for at least 30 min before the hemolysates were applied.

The gradient was made by a two-chambered varigrad containing 150 ml for each column to be run. The gradient stream was split three ways to produce identical

gradients which were transferred by a peristaltic pump to each of three columns at a flow rate of about 16 ml/h. Phosphocellulose does not have the capacity of DEAE-cellulose and only 1.5 mg of Hb could be applied to each phosphocellulose column. Most of the resins considered to be unsatisfactory had less capacity and almost the length of the column was required to hold the same amount of hemoglobin. As a consequence, poor separations with very wide bands were obtained. The column effluent was monitored by determining its absorbance at intervals of 1 min with flow-through cuvettes in a Gilford Model 2000 spectrophotometer. The effluent was usually collected in fractional volumes of 4 ml. Fractions that contained each of the separated hemoglobins were pooled and quantitated as the product of the volume and the absorbance. The separated components were also quantitated by integration of the area under the curve of absorbance vs. time.

Results and discussion

The results of phosphocellulose chromatography are presented in Figs. 1–8. Unfortunately, not all chromatograms showed the same high quality of separation.

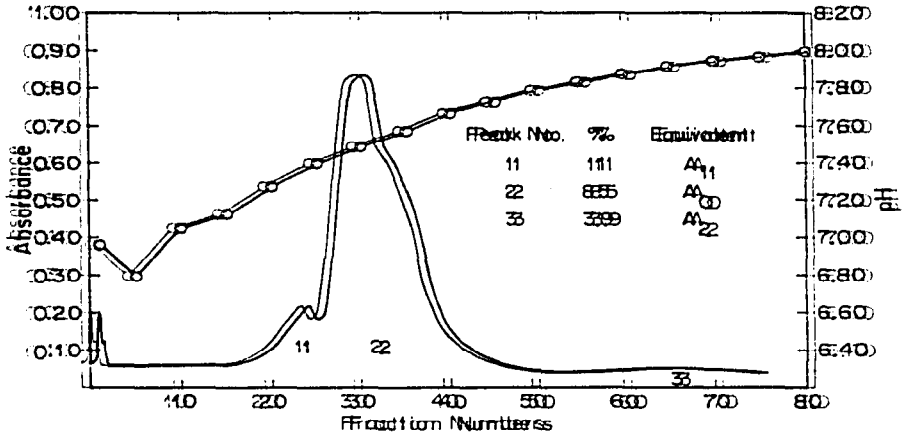

Fig. 1. Phosphocellulose chromatography of a hemolysate containing Hb A. The components are numbered in order of elution. The identification system is arbitrary in that individual numbers are assigned to peaks when it seemed desirable to discuss the component as a whole. See text for detail.

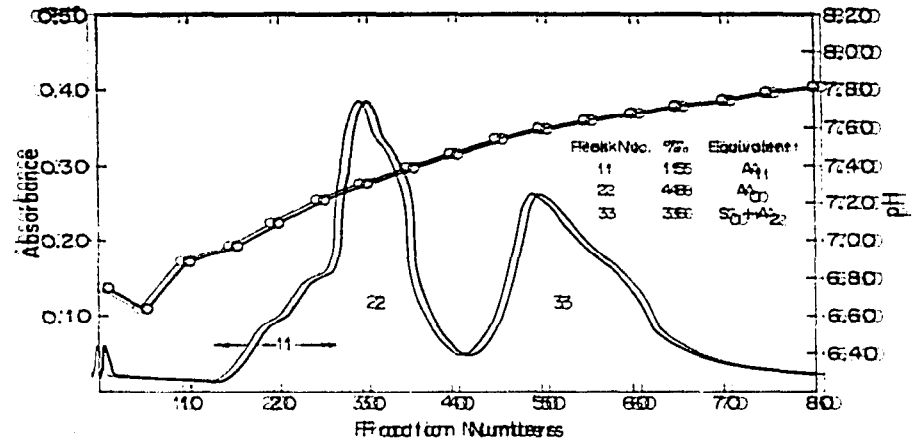

Fig. 2. Phosphocellulose chromatography of a hemolysate containing Hbs A and S. For numbering system see Fig. 1.

An explanation of this inconsistency probably can be found in an inspection of the buffer gradients. The differences in gradient probably were caused by variability in the speed of the magnetic stirring bars, resulting in differences in the pressures in the bottom of the mixing chamber.

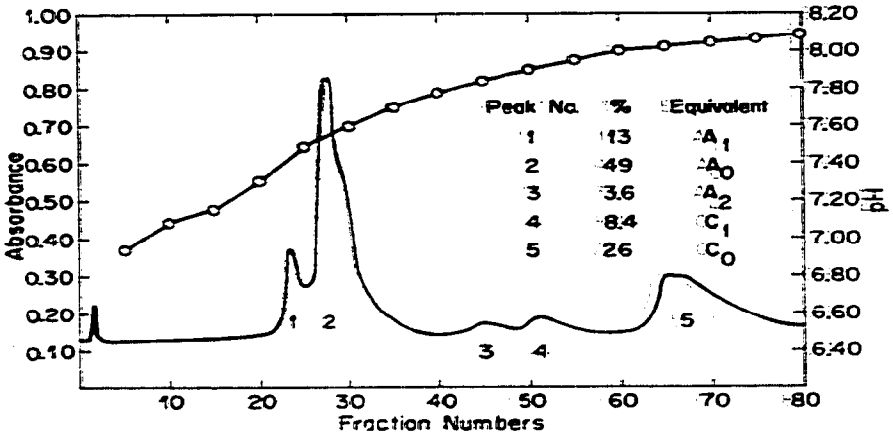

Fig. 3. Phosphocellulose chromatography of a hemolysate containing Hbs A and C. For numbering system see Fig. 1.

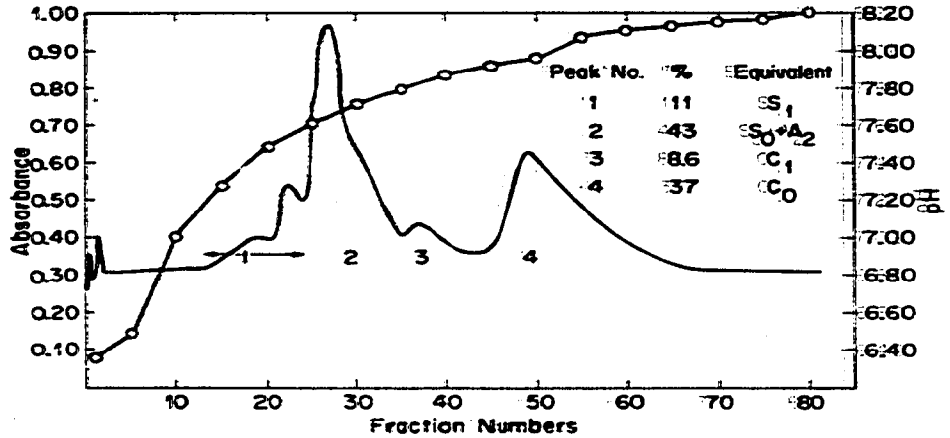

Fig. 4. Phosphocellulose chromatography of a hemolysate containing Hbs S and C. For numbering system see Fig. 1.

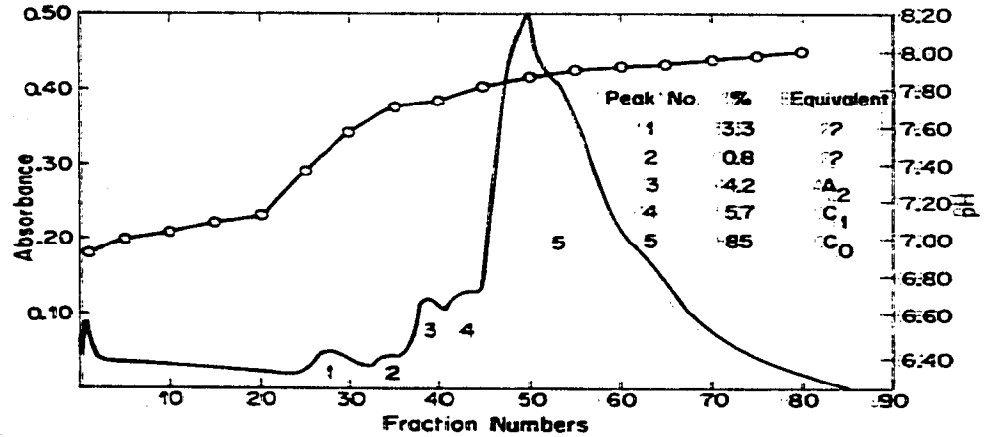

Fig. 5. Phosphocellulose chromatography of a fresh hemolysate from a person with Hb C-thalassemia. For numbering system see Fig. 1.

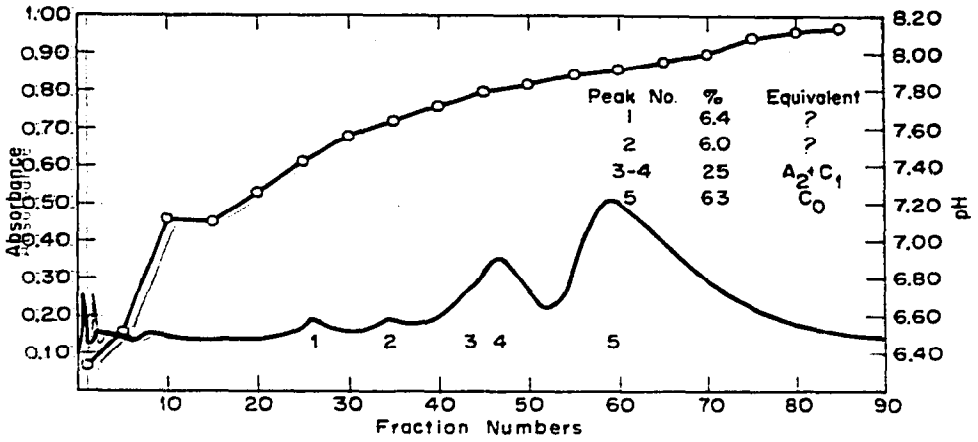

Fig. 6. Phosphocellulose chromatography of a preserved hemolysate from the patient who gave the sample in Fig. 5. For numbering system see Fig. 1.

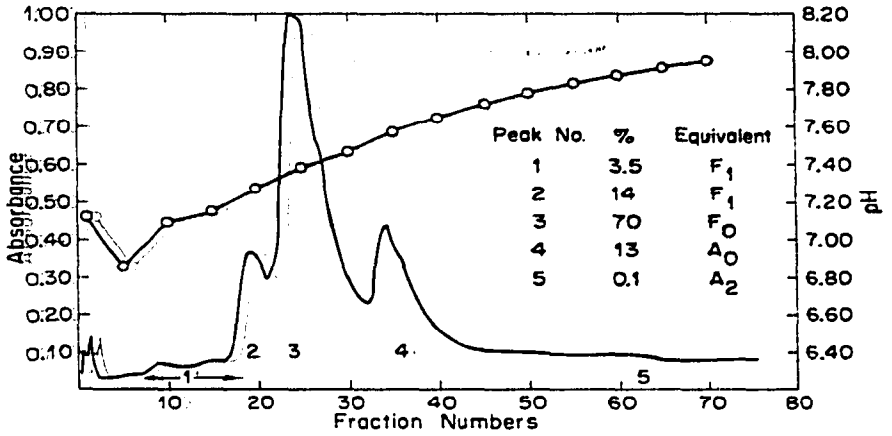

Fig. 7. Phosphocellulose chromatography of a cord blood hemolysate. For numbering system see Fig. 1.

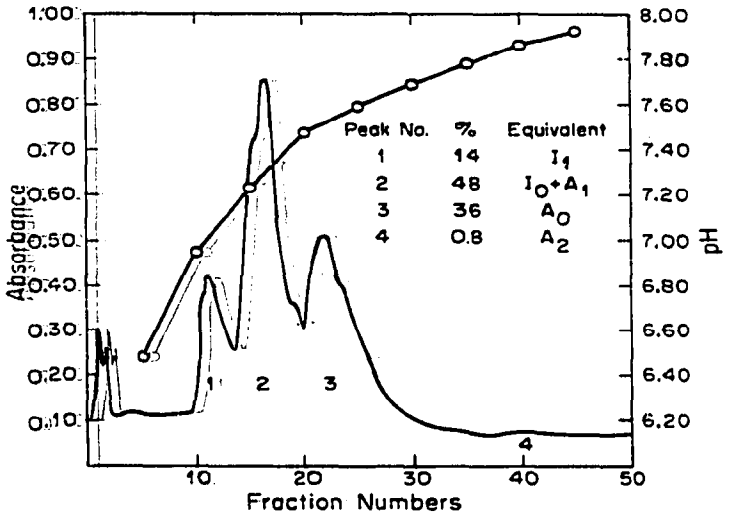

Fig. 8. Phosphocellulose chromatography of a hemolysate containing Hbs A and I. For numbering system see Fig. 1.

However, after study of many chromatograms, the presence of three subcomponents of each major hemoglobin fraction becomes evident (Fig. 2, fraction 2; Fig. 4, fraction 2; Fig. 8, fraction 2). Using a gentle gradient, these three major subcomponents are repeatedly and consistently present. Additional minor subcomponents are found irregularly. Although accurate quantitation of the subcomponents is impossible, the second subcomponent of each triad contains the largest quantity of hemoglobin or at least occupies the elution peak. The presence of the third subcomponent is more generally obvious than the first (Fig. 1, fraction 2; Fig. 3, fractions 2 and 5; Fig. 5, fraction 5; Fig. 7, fraction 3).

One possible source of heterogeneity could be the differences in mobility of cyanmethemoglobin and oxyhemoglobin, since these compounds may have different affinities for phosphate. However, chromatography of oxyhemoglobin, 75% oxyhemoglobin plus 25% cyanmethemoglobin, and 25% oxyhemoglobin plus 75% cyanmethemoglobin failed to show any chromatographic differences.

In addition to the heterogeneity noted above, several minor hemoglobin components, corresponding to the so-called electrophoretically fast-moving minor hemoglobins, elute before the major peaks come off the column (*e.g.* Fig. 1, fraction 1; Fig. 2, fraction 1; Fig. 3, fractions 1 and 4). These minor hemoglobins cannot be related to specific components separated by other methods without more data. However, it is interesting to note that an aged, frozen hemolysate from a patient with Hb C-thalassemia (Fig. 6, peak 4) has increased minor hemoglobin components compared to a fresh sample from the same patient (Fig. 5, peak 4). These components probably correspond to fractions found by Huisman *et al.*[6,7] using DEAE-Sephadex and Amberlite IRC 50 chromatography in aging specimens of hemoglobin.

One important characteristic of phosphocellulose chromatography is its ability to separate Hb A_2 from Hb C (Fig. 3, fraction 3; Fig. 5, fraction 3). Unfortunately there is some difficulty in completely separating Hb A_2 from a fast-moving minor component of Hb C so that quantitation is difficult. In the patient with Hb C-thalassemia, Hb A_2 was found to be 4.2% (Fig. 5) while in a patient with homozygous Hb C (not shown in graph form) Hb A_2 amounted to 2.7%. Another patient with Hbs A plus C, had a Hb A_2 level of 3.6% (Fig. 3). Note that in a hemolysate from a normal adult the Hb A_2 was 3.9%.

Dozy and Huisman[8] have reported a method for quantitating Hb A_2 in the presence of Hb C using CM-Sephadex and Tris–maleic acid buffers, but each column takes a week to run. Perhaps phosphocellulose chromatography can be modified, by adjusting the chromatographic conditions, to produce complete separation of these components and allow quantitation of Hb A_2 in the presence of Hb C more rapidly than by CM-Sephadex chromatography. Precise quantitation of Hb A_2 by minicolumn chromatography, however, cannot be expected. Furthermore, phosphocellulose chromatography fails to resolve Hb S and Hb A_2 since Hb A_2 seems to have a mobility quite similar to the third subcomponent of Hb S (Figs. 2 and 4).

It is interesting that the Hb A_2 in cord blood could be quantitated in spite of its small quantity. The Hb A_2 level of a sample of Hbs A plus I (abnormal β chain) (Fig. 8) is probably spuriously low because of the age of the sample.

The chromatographic pattern of cord blood shows that Hb F elutes before Hb A. This pattern is similar to that found with Amberlite IRC 50 chromatography, DEAE-cellulose chromatography and agar gel electrophoresis but unlike the findings

with CMC chromatography, starch gel electrophoresis and polyacrylamide gel electrophoresis. With phosphocellulose chromatography there seem to be multiple (two to three) fast-moving fetal hemoglobins in cord blood samples, whereas by other methods, there seems to be a single fast-moving Hb F.

In summary, phosphocellulose chromatography separates hemoglobins into patterns which indicate much more heterogeneity than found by other chromatographic techniques. The identity and significance of many of these components is yet to be established. Also, phosphocellulose chromatography is one of the few techniques capable of separating Hb A_2 and Hb C.

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