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The use of carbamylated charge standards for testing batches of ampholytes used in two-dimensional electrophoresis

A method of testing batches of ampholytes is presented. By using carbamylated charge standards to co-electrophorese with the protein sample in the first-dimension isoelectric focusing gel, one can monitor, after running and staining the second-dimension sodium dodecyl sulfate (SDS) slab gel, the continuity of the pH gradient. Charge standards can also be used to check the reproducibility of the pH gradient among batches of ampholytes and to modify the new batch with a small amount of a narrow range ampholyte to assure reproducibility of experiments. Ampholytes for comparison were obtained from three major manufacturers.

1 Introduction

High-resolution two-dimensional acrylamide gel electrophoresis, using isoelectric focusing in the first dimension and electrophoresis in the presence of SDS in the second [1-7], is the highest resolution analytical method currently available for protein separation. To construct a Human Protein Index [8], it is important to be able to define the positions of individual proteins in two-dimensional electrophoresis patterns with a precision comparable to the resolving power of the system. Because pH measurements along isoelectric focusing (first dimension) gels, or molecular mass standards along the edges of second-dimension acrylamide slab gels fail to provide satisfactory precision, internal standards for both charge [9-10] and molecular mass [11-12] have been developed. Since single charge changes in proteins are generally detectable [13], a mixture of sequentially carbamylated proteins gives a row or train of discrete spots in the isoelectric focusing dimension [9].

An integral part of the two-dimensional electrophoresis technique is the use of ampholytes to form the gradient in the isoelectric focusing gel on which the proteins are to be separated. However, the manufacture of ampholytes does not appear to be a precisely controlled process, and discontinuities in gels (gaps or crowded areas) have been repeatedly oberserved [14-16]. If reproducible patterns are to be obtained, ampholytes must be standardized, and it will be necessary to develop methods for evaluating and intercomparing different batches of ampholytes in an objective manner. In this paper we describe the use of internal charge standards prepared from creatine kinase (CK) to evaluate ampholytes and to demonstrate differences between batches from different sources. CK was chosen as a model because it can be obtained commercially in a highly purified form and the native form has a relatively high isoelectric point.

2 Materials and methods

Rabbit muscle CK (E.C. 2.7.3.2) was obtained from Sigma (St. Louis, Missouri). Carbamylated CK standards, prepared as previously described [9, 10], were loaded on the top (cathodal end) of the isoelectric focusing gels in an ISO apparatus [6] either alone or with a solubilized protein sample for co-electrophoresis with the standards. The native form of the CK appears toward the cathodal (basic) end of the gel together with a train of spots extending toward the anodal (acidic) end, which is always to the left. Ampholytes for comparison were obtained from LKB (Bromma, Sweden), Pharmacia (Uppsala, Sweden), and Serva Feinbiochemica (Heidelberg, West Germany). The second-dimensional separation was run in the DALT slab gel ap-

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Abbreviations: SDS: Sodium dodecyl sulfate; CK: Creatine kinase

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paratus, and proteins were detected using Coomassie Blue staining [7].

3 Results

Fig. 1 compares the two-dimensional electrophoresis patterns of carbamylated CK charge standards using wide range ampholytes prepared by three companies (LKB, Serva Feinbiochemica, and Pharmacia). Thirty-three spots are consistently resolved regardless of the ampholyte preparation used. The vertical streaking seen at the acid end of the train is presumably due to altered SDS binding [9]. Although all three ampholyte preparations cover the pH range 3–10, different spot patterns are obtained. Because each CK spot is separated from its neighboring spot by a single charge unit [9], the distribution of spots in the carbamylation train gives an indication of the evenness of the pH gradient in the isoelectric focusing dimension. Some ampholyte preparations produce pH gradients with nonlinear areas (for example, Fig. 1 A and 1 B), whereas other preparations produce gradients that are even throughout (Fig. 1 C). Carbamylated charge standards show the differences in ampholyte pr tions clearly.

The carbamylated standards can also be used to moni effectiveness of the pH gradient used for isoelectric foc so that mixtures of narrow and wide range ampholyt be used in obtaining improved protein separations. In the patterns obtained when Servalyt pH 4–9 (Fig. 2 A) 3–10 (Fig. 2 C) were used alone or in combination wi narrow range preparations are shown. When the wide ampholytes were used alone, the CK charge stan showed a more uneven pH gradient (Fig. 2 A and 2 C) when the narrow range preparations were added (Fi and 2 D). Further, the spots on the gradient obtained pH 4–9 ampholytes (Fig. 2 A) were shown to be together than those obtained with the pH 3–10 ampl (Fig. 2 C). When the pH gradient was modified by n Servalyts pH 4–9 (88 %) and pH 6–7 (12 %), the gr was extended across the gel (Fig. 1 B), but spots at the end of the train were not well-resolved. Addition of pI ampholyte (10 %) to the pH 3–10 preparation (

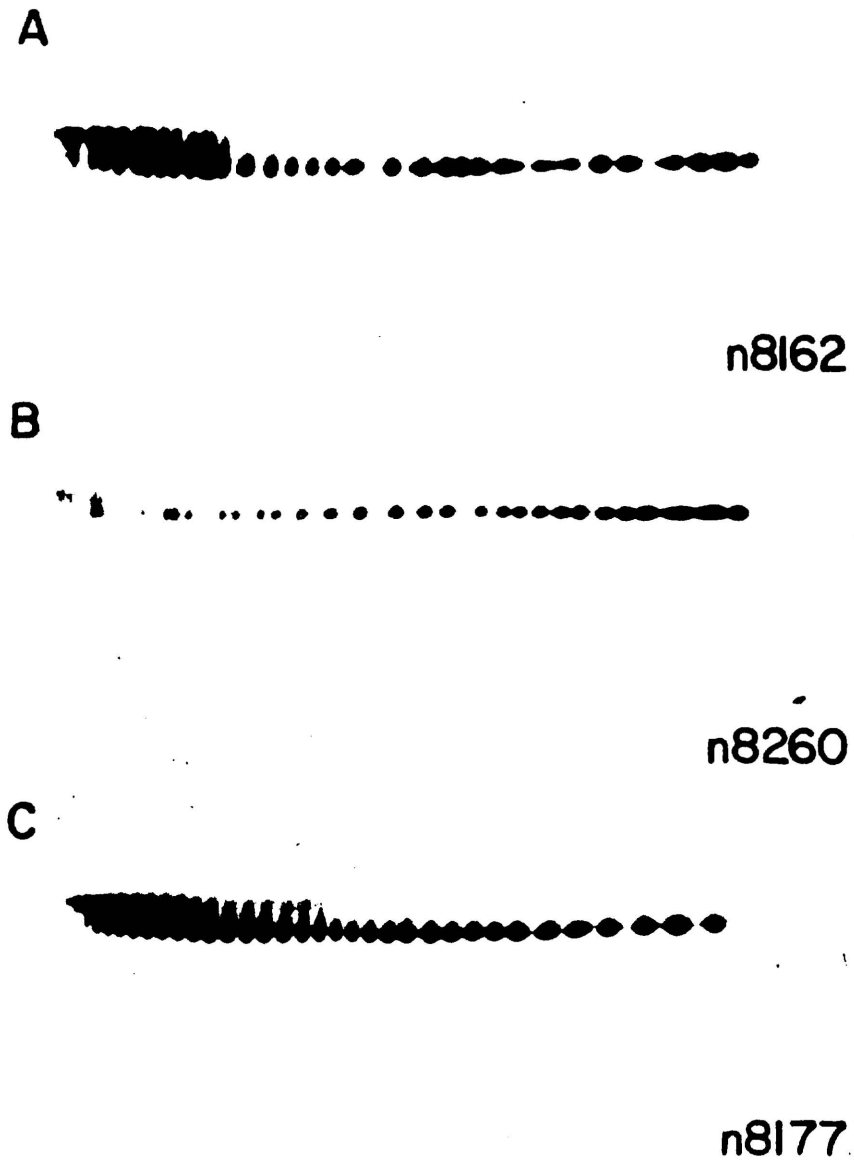

Figure 1. Sections from two-dimensional showing CK trains run in three batche ampholytes produced by different cher companies. (A) LKB pH 3.5–10 ampholyte Servalyt ISO-DALT grade pH 3–10 amph (Serva Feinbiochemica); (C) Pharmalyte 3–10 ampholyte (Pharmacia). Gels are orie with acidic side to the left and basic side t right.

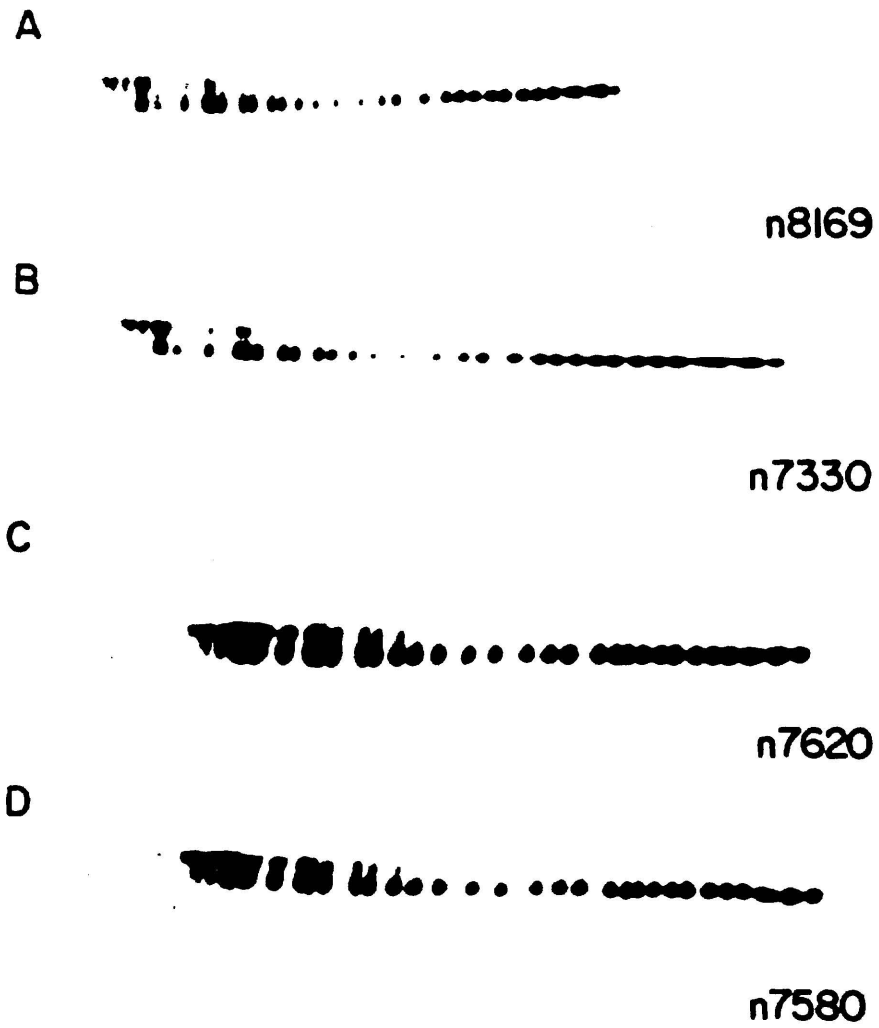

Figure 2. Sections from two-dimensional gels showing the complete carbamylation trains of creatine kinase (CK) run in various ampholytes on the first dimension isoelectric focusing gel. (A) Servalyt pH 4–9; (B) 88 % Servalyt pH 4–9 and 12 % Servalyt pH 6–7; (C) Servalyt pH 3–10; (D) 90 % Servalyt pH 3–10 and 10 % Servalyt pH 5–8.

produced a slightly more extended gradient with the basic end of the train more evenly spread (Fig. 2 D) than when the pH 3–10 preparation was used alone (Fig. 2 C).

The problem of achieving an optimum pH gradient is further complicated by the proteins in the sample which also act as ampholytes and may distort the pH gradient. When very small radiolabeled samples are used and proteins are detected by autoradiography, the protein effect on the pH gradient is lessened. However, when high protein loading is required so that staining can be used for detection, the ampholytes used must be chosen to compensate for sample protein effects. This problem is accentuated when the sample contains an excess of one protein, as in the case of albumin in serum.

In Fig. 3, a human peripheral blood lymphocyte preparation was analyzed together with carbamylated CK charge standards, using the three ampholyte preparations described in Fig. 1. The lymphocyte proteins were radiolabeled with ^{35}S-methionine so the protein loading was very small. The standards are numbered as previously described [9, 10] and actin (a) is at postition –17.5 as previously reported [9]. In the LKB preparation of this sample (Fig. 3 A), a large space occurs to the left of spot 18 which effectively compresses the pH gradient between positions –1 to –17. A larger gap appears in the train than is shown in Fig. 1 A, in which the CK charges were run with this ampholyte, but without the added lymphocyte proteins. The Servalyt (Fig. 3 B) and the Pharmalyte (Fig. 3 C) preparations run with lymphocytes give a more evenly spaced protein pattern, similar to those produced in the absence of sample protein (Fig. 1 B and 1 C).

Patterns of human seminal fluid with added CK standard are shown in Fig. 4 to illustrate a sample with high protein content, necessitated because seminal fluid proteins must be visualized by Coomassie Blue staining. The sample protein altered the pH gradient when the Pharmalyte preparation was used (Fig. 4 A), as demonstrated by the elongation of the basic end of the CK charge standard train (compared with Fig. 1 C). The protein in this sample had little effect, however, on the LKB gradient (compare Fig. 4 B with Fig. 1 A), in contrast to the lymphocyte sample (Fig. 3 A). These seminal fluid patterns are reproducible and will be described in detail in a subsequent publication [17].

Fig. 5 shows human plasma run with Pharmalyte pH 3–10 together with CK standards. This pattern illustrates the distorting effect that an excess of one protein can have on the pH gradient. There are large gaps in the CK train below serum albumin that were not present when the standards were analyzed alone (Fig. 1 C) or with small amounts of lymphocyte protein (Fig. 3 C).

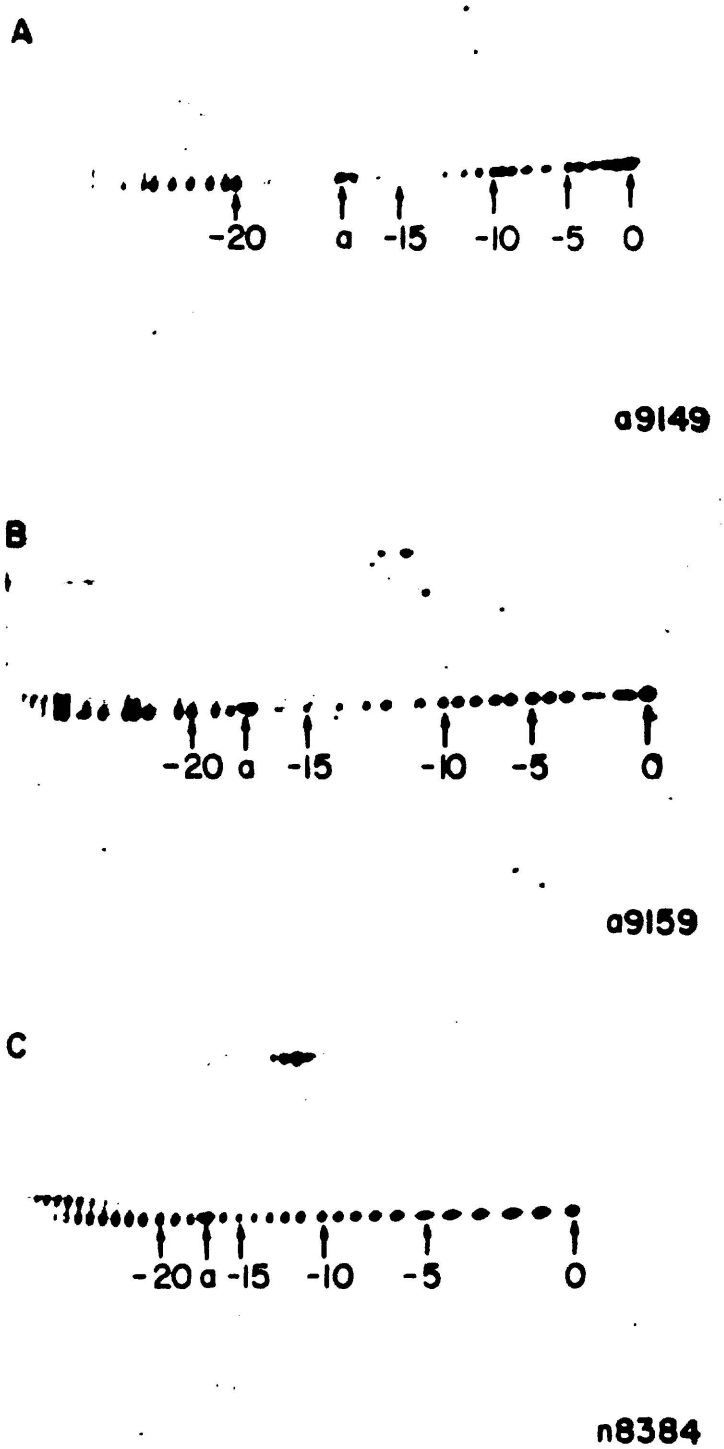

Figure 3. Sections from two-dimensional gels of human peripheral lymphocytes co-electrophoresed with CK carbamylation train standards and stained with Coomassie Blue so only a few lymphocyte spots appear to show differences in separation using different ampholytes. (A) LKB pH 3.5–10; (B) Servalyt ISO-DALT grade pH 3–10; (C) Pharmalyte pH 3–10. In all cases actin (a) is located at charge –17.5.

4 Discussion

This paper has chosen for illustration a few highlights that have developed from the approximately 600 gels with CK carbamylation train standards that we have run to date, most of them co-electrophoresed with protein samples. We have shown that charge standards show differences among batches of ampholytes. It has become obvious that the gradients obtained by using ampholytes for isoelectric focusing must be studied and adjusted for each investigator's particular use and for each protein sample to be separated. The fact that these pH gradients can be so easily monitored with charge standards makes much easier the task of determining the gradient and deciding upon the appropriate mixture of wide range and narrow range ampholytes. Charge standards also can be used to check the reproducibility of the pH gradient from one batch of a manufacturer's ampholyte to the next, and to adjust the new batch with some narrow range mix to assure reproducibility of experimental results. There is an evident need to extend charge standards to cover a wider pH range, and also to cover a range of molecular masses (*i. e.*, for the standards to appear at different levels in the

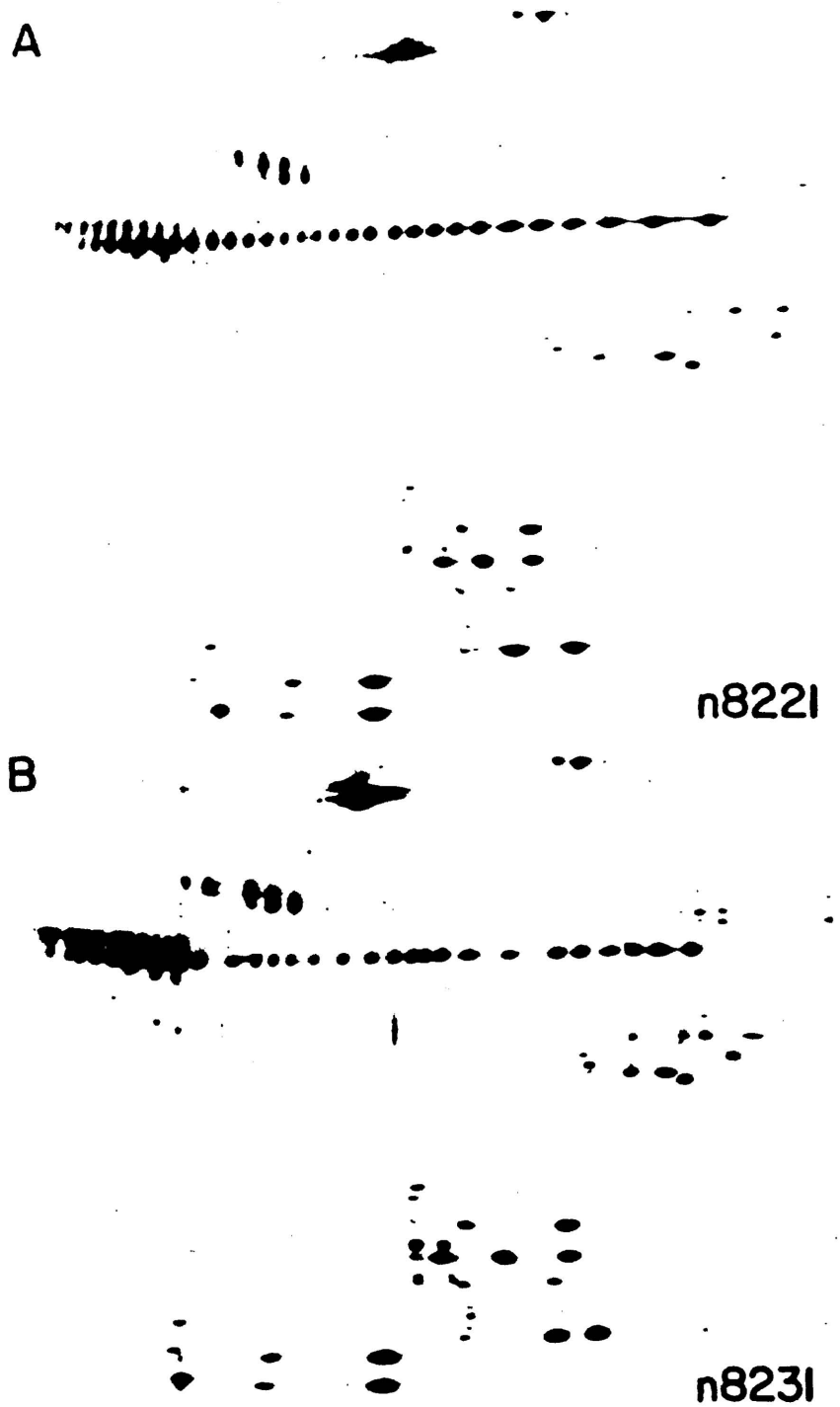

Figure 4. Sections from two-dimensional gels of human seminal fluid run with CK standards with two different kinds of ampholytes used in the first dimension focusing gels. (A) Pharmalyte pH 3–10; (B) LKB pH 3.5–10.

mass dimension); it may not always be possible to position spots accurately in all parts of the gel by reference to a single charge train. The problem of accurate assignment of pH to each spot in the CK carbamylated charge train and in other charge trains, and the problem of positional assignments relative to standards after nonequilibrium pH gradient electrophoresis [18, 19] will be discussed in a subsequent paper.

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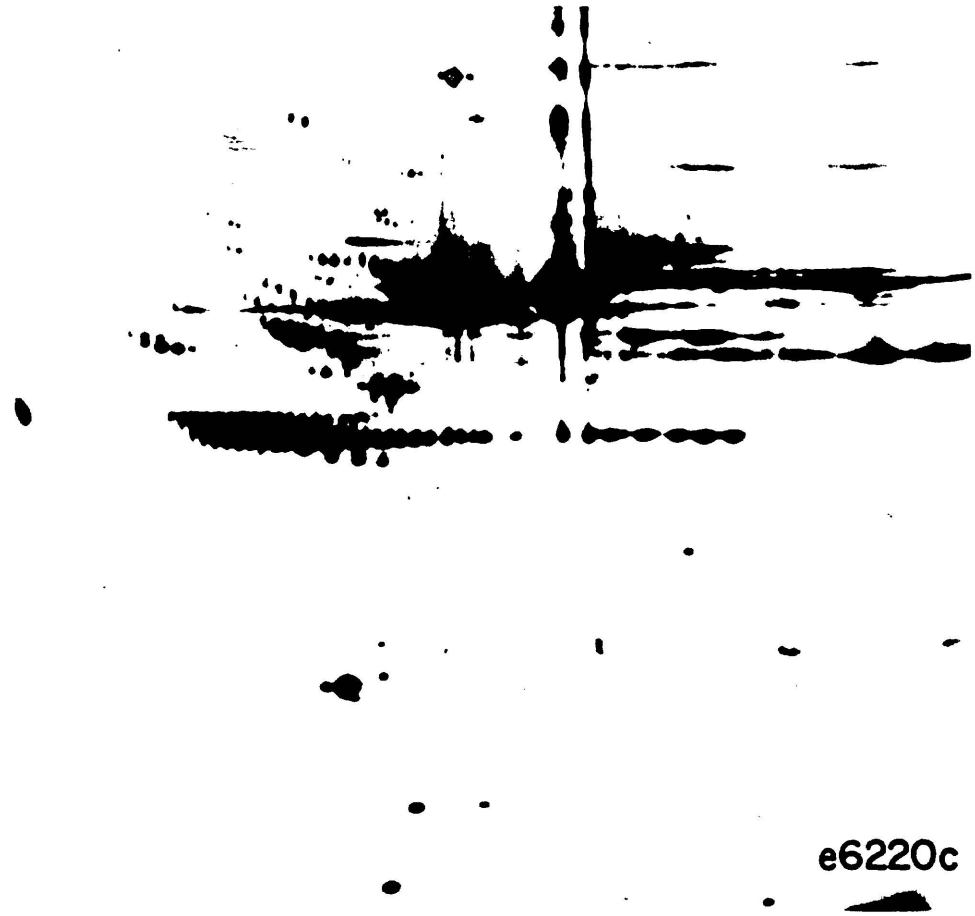

Figure 5. Two-dimensional electrophoretic pattern of normal human blood plasma from a 21-year-old male co-electrophoresed with CK charge standards. Pharmalyte pH 3–10 ampholyte was used in the ISO gel.

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