APPLICATIONS OF A NEW METHOD OF DESCENDING DENSITY GRADIENT ELECTROPHORESIS

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INTRODUCTION

The problems encountered in descending density gradient electrophoresis, such as sample insertion, droplet sedimentation, stability of the moving zone, etc., are of sufficient magnitude that this method of analyzing charged macromolecules is infrequently used. When successfully applied, however, this method offers efficient and rapid separation as well as complete recovery of all fractions, to permit direct quantitative analysis of a multicomponent system. This aspect is of considerable importance

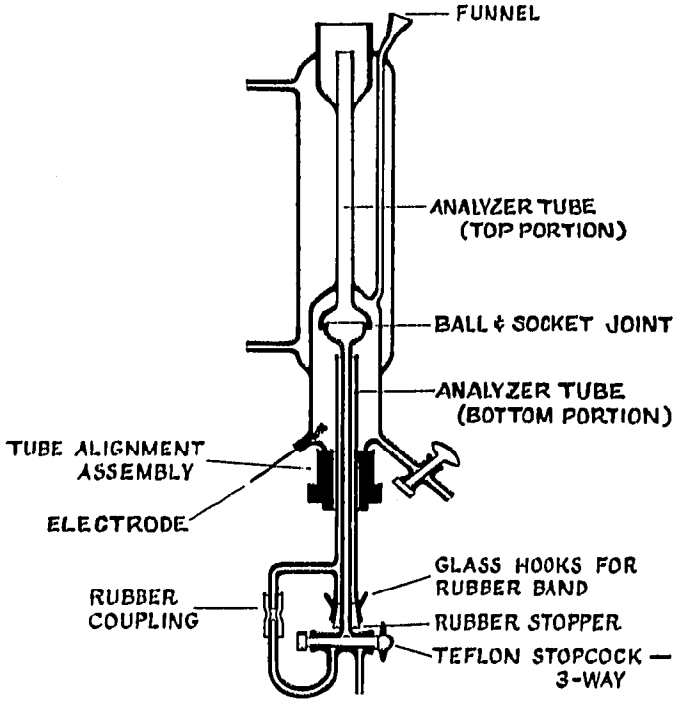

Fig. 1. The modified column is shown with the tube alignment assembly in place and the ball and socket joint closed. Modifications introduced into the glass work of the lower section of the column are: (1) Insertion of the lower portion of the analyzer tube through a rubber stopper (the tube is broken at the level where it enters the ball joint, the stopper is installed and the break is then reconstituted); addition of glass hooks, and a rubber band around the three-way stopcock to secure the rubber stopper and yet retain its flexibility. (2) Replacement of glass stopcocks by teflon. (3) Insertion of a rubber coupling as diagramed, to eliminate rigidity.

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in enzyme studies when one wants to assess both the extent of purity of a sample and the activities of the different fractions.

A complete technique for descending sucrose density gradient electrophoresis was developed after introducing several refinements into the column of BERG AND BEELER[1]. These included mechanical alterations and innovations to the column itself and the construction of a gradient making and sample insertion device. By applying the technique of sample insertion developed by SVENSSON[2] and the use of a

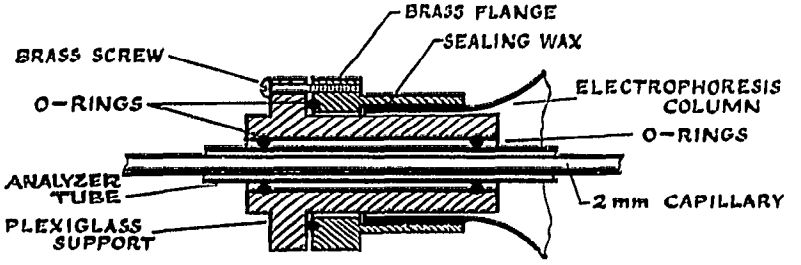

Fig. 2. The tube alignment assembly drawn approximately to half scale. The effect of this design is that the bottom portion of the analyzer tube is permitted to ride in a channel supported by two "O rings". In addition to acting as support, these "O rings" prevent the electrolyte from draining out of the column. They fit tightly around the diameter of the analyzer tube and are kept in place by grooves in the adjacent plexiglass. An additional "O ring" seal is used at the junction of the brass flange and the plexiglass support. To seal the tube alignment assembly to the column, the lower portion of the analyzer tube including the plexiglass support is separated from the brass flange. The electrophoresis column is chucked in a glass lathe and the brass flange cemented to it by flowing a sealing wax in between the surfaces while heating gently. Before the wax has cooled, the plexiglass support is secured to the brass flange by brass machine screws and the ball and socket joint is closed to ensure that alignment is maintained.

fluorometer as an analyzer, a routine method of zonal electrophoretic analysis has been developed which permits breakage-free and highly reproducible electrophoresis. This technique overcomes several practical problems of earlier electrophoretic procedures, including leakage and breakage, and permits the method to yield reproducible and analytic data.

CONSTRUCTION OF THE ELECTROPHORESIS COLUMN AND GRADIENT DEVICE

A. Mechanical design of the column

The major mechanical improvements and innovations on the column described by BERG AND BEELER[1] are the construction of a tube alignment assembly and redesign of the glass work of the lower section (see Figs. 1 and 2).

The tube alignment assembly permits the glass tubing to run within two "O rings" which prevent leakage at the bottom of the column and permit the ball and socket joint to be broken and resealed gently, preventing convection and other mechanical disturbances to the gradient. The friction of the "O rings" is sufficient to hold the lower tubing rigidly in position after the ball and socket joint is broken. It also holds the ball and socket joint closed. The "O ring" seal between the brass flange (cemented to the column) and the plexiglass support is held by brass machine screws which permits the whole lower portion of the electrophoresis tube to be removed for cleaning.

Three functionally important additions—the rubber coupling, the glass hooks

and rubber band, and the rubber stopper with the capillary tube running through—permit the column to be used without breakage by introducing flexibility into an otherwise solid glass structure. The teflon three-way stopcock obviates grease at this point and permits smoother regulation of flow.

B. Automatic gradient making and sample insertion device

The automatic gradient making and sample insertion device is illustrated in Fig. 3. Several features of the design of this apparatus should be emphasized as they are important to its proper functioning to obtain analytic data. The mechanical emphasis of the design is on flexibility and it is in this capacity that the rubber connections on the stopcock and the ball and socket joints on the capillary tubing serve. When connection is made with the column, the rubber stopper in the mixing chamber can be pushed down slightly to bring the liquid in the sample chamber to the end of its tubing to avoid air bubbles. The relative positioning and arrangement of the constituents has been found to be the most convenient for the necessary mechanical manipulations.

The use of compressed air instead of pumps to force the solution into the column greatly reduces the cost of the operation and simplifies the procedure. Many combinations of capillary size, geometry, filling pressures and drainage rates were experimented with before settling on the described experimental procedure.

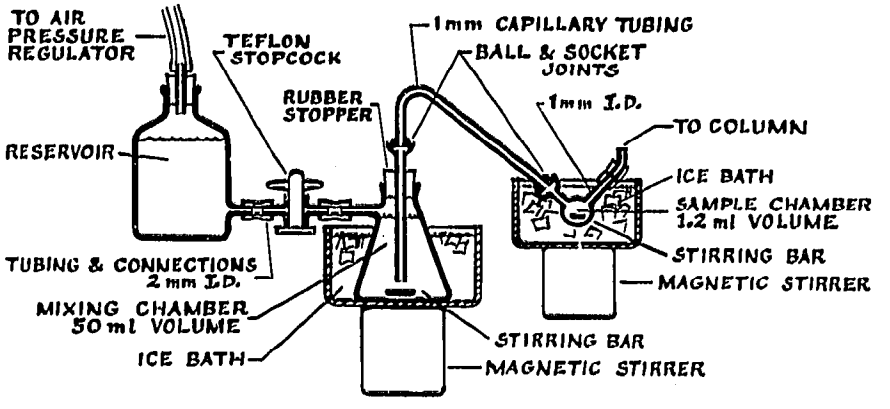

Fig. 3. The automatic gradient making and sample insertion device is represented as it is used during filling of the column. The volumes and dimensions of the components are given in the text. The maintenance of the two vessels in ice baths during the filling procedure is essential. (Refer to Section C of EXPERIMENTAL.)

EXPERIMENTAL

A. Buffer designations and preparation

A variety of simple buffer systems was tried and found to work well. However, TRIS–HCl limited the growth of contaminating microorganisms, as well as serving as a good buffer. It was therefore used for all of the following experiments.

The buffered sucrose solution for the bottom reservoir of the column and for the reservoir of the gradient maker is made by dissolving 550 g of reagent grade sucrose in 1 l of 0.005 *M* TRIS-HCl at pH 7.6 (made by bringing 10.0 ml of 1 *M* TRIS and 8 ml of 1 *M* HCl to 1 l). The buffered sucrose solution thus prepared is designated 55 %

buffered sucrose. A dilution of the 55 % buffered sucrose solution is made by bringing one volume of the sucrose up to ten volumes with 0.005 *M* TRIS–HCl pH 7.6 and designated 5.5 % buffered sucrose. This 5.5 % buffered sucrose is used in the mixing chamber of the gradient device. 0.005 *M* TRIS–HCl pH 7.6 is used in the top reservoir of the column.

B. Preparation of the electrodes and column for use

The electrodes are made from silver wire according to the method of BERG AND BEELER[1]. They are plated with silver chloride by making them the anode in a 0.1 *N* HCl solution using a platinum wire as the cathode. A current of 10–30 mA is used until the electrodes are dark grey in color[3].

One-half of the ball and socket joint is lightly coated with Dow Corning silicone high vacuum grease and the bottom portion of the electrophoresis tube is inserted and secured by machine screws.

The electrodes are inserted through rubber stoppers and placed in position in their respective reservoirs. Glass hooks must be fused onto the circumference of the nipple of the bottom reservoir through which the electrode enters and the bottom electrode and stopper secured by a strong rubber band to prevent leakage. The top rubber stopper has the electrode inserted through it on one side to the bottom of the reservoir. Through the other side a long stemmed 50 ml separation flask is inserted to the bottom of the reservoir.

After the electrodes are positioned and secured the bottom electrode is covered with saturated saline, by about 1 1/2 in. The top electrode is covered with buffer to 1 in. below the top of the electrophoresis tube. The ball and socket joint of the electrophoresis tube must now be closed and secured by pressing it into union and rotating it to ensure that the thin coating of silicone makes a watertight seal. 55 % buffered sucrose is added to the bottom reservoir to 1/2 in. above the level of the electrophoresis tube. This is done at room temperature to keep the viscosity low and prevent bubbles from getting trapped during the filling procedure. The pump to the refrigerated water bath (regulated at 7°) is now turned on and the apparatus is allowed several hours to equilibrate.

C. The electrophoretic run

To begin, the reservoir of the gradient making device is filled with the 55 % buffered sucrose solution, the mixing chamber with 50 ml of the 5.5 % buffered sucrose solution. To the sample chamber is added 0.2 ml of the sample, followed by 1.0 ml of the 5.5 % buffered sucrose solution (any other combinations of solutions which retain this composition of sucrose and buffer can be used). The assembly is arranged as shown in Fig. 3 and the mixing and sample chambers are immersed in ice water as illustrated. The magnetic stirrers should be turned on and the solutions allowed to reach thermal equilibrium. Meanwhile 10–15 ml of the buffer is added to the electrophoresis column itself and 5 ml of this is subsequently drained off through the stopcock to clear the apparatus of air bubbles which would have disturbed the sample zone and gradient. The automatic gradient making and sample insertion device should be rigidly positioned beneath the column so that connection can be easily made. The mixing and sample chambers must always remain in ice water. The stopcock can now be opened between the reservoir and mixing chamber and the air

pressure regulated at 1/2 lb. The rubber stopper in the mixing chamber can then be pressed down to force material from the sample chamber to the edge of its tube being careful not to let it overflow. Connection is now made to the column by pressing the tubing from the sample chamber over the nipple of the teflon three-way stopcock of the electrophoresis column.

Filling of the column can now begin by slightly opening the three-way stopcock of the column to permit entry of the material from the sample chamber holding the air pressure at the same 1/2 lb. Should any faster filling be required, this should be regulated by the stopcock and not by increasing the pressure as bubbles may be forced into the column to disturb the sample zone. As the sample is being inserted and the gradient made, the column should be visually inspected at the level of the ball and socket joint for uniformity of change of refractive index. If the gradient is properly made, the solution here will look perfectly homogeneous showing smooth change of refractive index. Should convection be present, this will be evidenced by non-continuous change of refractive index and the column must be refilled. Convection frequently arises from non-uniformity of temperature of the solution leaving the mixing chamber. This can be controlled by slowing down the filling rate, but we have not found it necessary to extend this beyond one hour. Maintenance of the temperature of the water circulating through the column at 7° and an ice water bath for the sample and mixing chambers allows smooth sample and gradient insertion by maintaining the correct density relationships as the sample and gradient are put on to the column.

The column is filled within 1/4 in. of the top and then brought to the top by using the other channel in the three-way stopcock. This permits slow hydrostatic equilibration between the lower column reservoir and the electrophoresis tube before the ball and socket joint is broken. Saturated NaCl is now added to the bottom of the top reservoir by opening the stopcock of the previously described separation flask until the level of the buffer rises to a height about 1 in. above the top of the electrophoresis tube. Continuity is maintained meanwhile between the lower reservoir and the electrophoresis tube through the three-way stopcock. This accomplished, the ball and socket joint is broken by a gentle twisting motion and pulled down slightly. Small changes in the refractive index occur near the ball and socket joint when this is done but cause no difficulty. The tube alignment assembly permits this operation to be accomplished smoothly and reproducibly as the outer tube rides on two "O rings" which are lightly lubricated with Dow Corning high vacuum grease.

At this point the current can be turned on and the experimental voltage applied. For our buffer it is found that a current of 4–7 mA can be expected for a voltage of 400. For deviations from these buffer conditions, a difference in the power dissipated can be expected and the conditions of temperature, voltage, sucrose gradient, strength of the sucrose solutions, etc., may not yield stable gradients or stable stationary and moving sample zones. The requisite conditions may be found experimentally. It is suggested that one should put the sample on without applying a voltage and drain it off at several intervals of time to show stability of the initial zone. After initial stability of the sample zone has been demonstrated, electrophoresis of a homogeneous sample at a constant concentration, followed by drainage at several intervals of time will demonstrate a stability of the moving zone.

At the completion of the electrophoresis, the ball and socket joint must first be resealed by gently rotating the glass tubing through the tube alignment assembly and

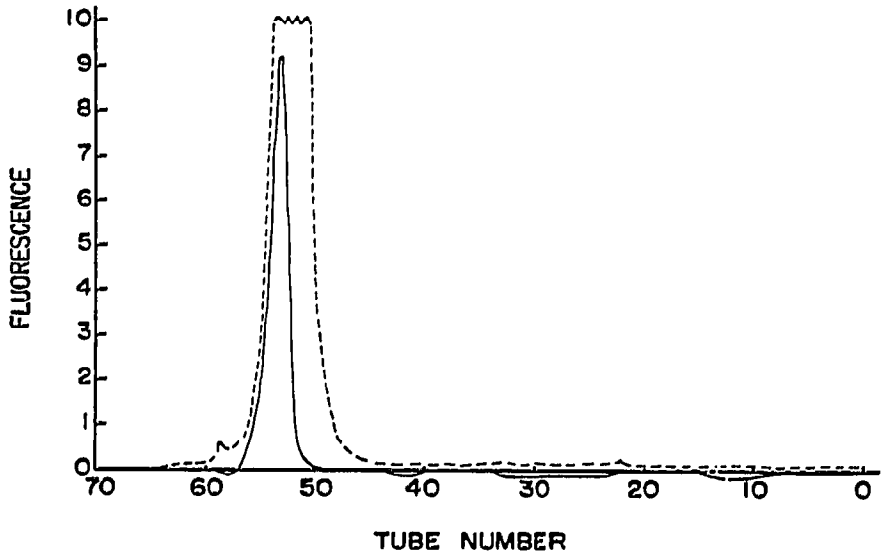

Fig. 4. Control runs demonstrating the initial distribution of the sample on the column are shown. (———) Chymotrypsinogen. Sample chamber composition: 1 ml chymotrypsinogen (1.61 mg/ml in 0.005 *M* TRIS–HCl pH 7.6); 0.1 ml 55 % sucrose; the remainder of the sample chamber is filled with 0.005 *M* TRIS–HCl pH 7.6. Filling pressure: 2 lb. Filling time: 30 min. Elution pressure: 1 lb. Fluorometer aperture: 30. Fluorometer filters: 7–37, 7–60. (- - - -) Beef heart lactic dehydrogenase. Sample chamber composition: 0.8 ml lactic dehydrogenase (5 mg) in 0.005 *M* TRIS–HCl pH 7.6; 0.1 ml 55 % sucrose; the remainder of the sample chamber is filled with 0.005 *M* TRIS–HCl pH 7.6. Filling Pressure: 2 lb. Elution pressure: 1 lb.

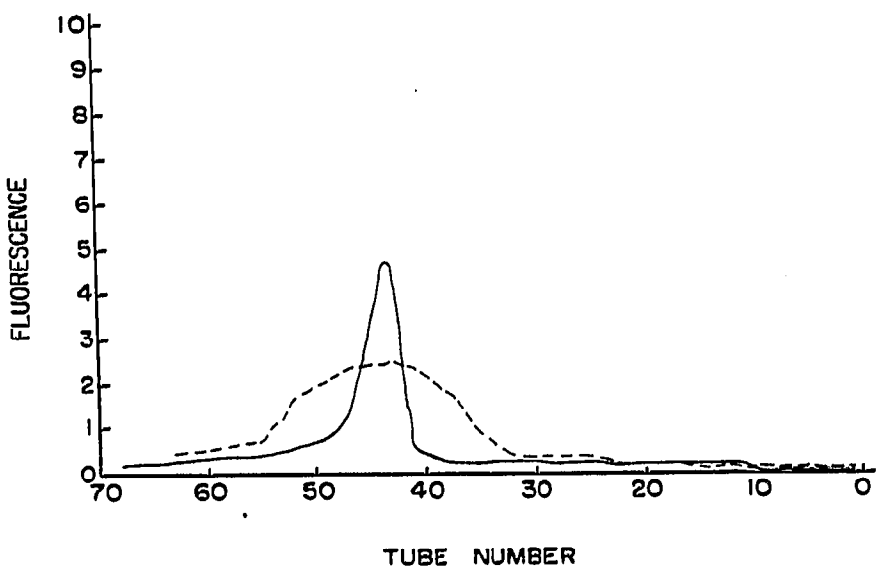

Fig. 5. Chymotrypsinogen at two different concentrations is electrophoresed under standard conditions and eluted. This graph demonstrates that non-ideal distribution of protein throughout the sample zone results from overloading. (———) Chymotrypsinogen. Sample chamber composition: 2.3 mg solid chymotrypsinogen dissolved in 1.2 ml 5.5 % buffered sucrose. Fluorometer aperture: 3. (- - - -) Chymotrypsinogen. Sample chamber composition: 20 mg solid chymotrypsinogen dissolved in 5.5 % buffered sucrose. Fluorometer aperture: 10. Fluorometer filters: 7–37, 10 % neutral density.

pressing it into union with its counterpart. The tube alignment assembly allows this to be done smoothly and reproducibly. Next, the excess buffer forming the bridge above the electrophoresis tube is removed with a pipette.

In draining the column, consideration should be given to the direction of the sucrose gradient. The greatest density is toward the bottom, and thus stability of the developed zones will be maintained if drainage through the detector maintains this gradient. For an analyzer, all of the following experiments used the G.K. Turner Fluorometer model 111 with 1 ml quartz flow-through cell, a Baird Atomic 280 mμ interference filter as primary filter and Dow Corning 7–37 or 7–60 filters as secondary

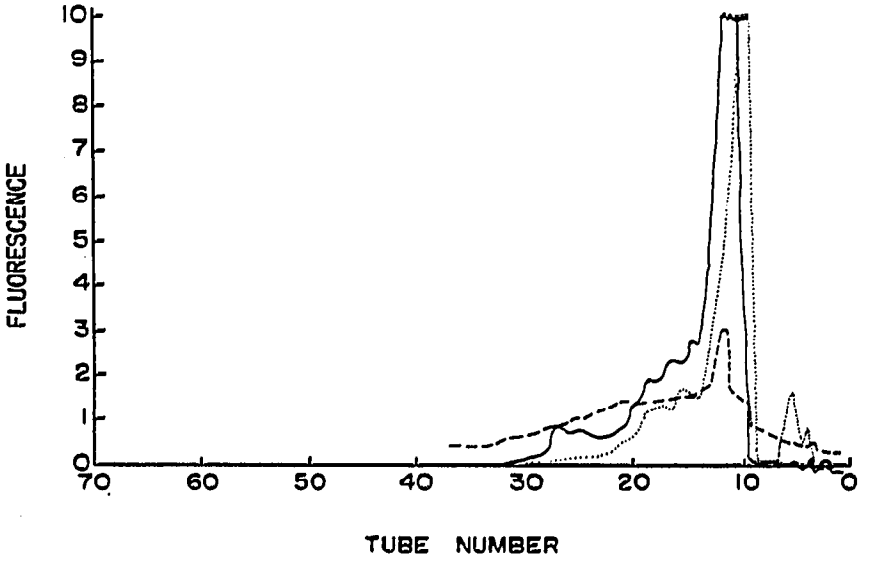

Fig. 6. These three experiments show that heterogeneity of a commercial preparation of lactic dehydrogenase is clearly demonstrable on this column; that partial purification of lactic dehydrogenase on hydroxyapatite is reflected by its electrophoretic profile; and that stability of the moving sample zone is achieved at low protein concentrations. (- - - - - -) Commercial beef heart lactic dehydrogenase. Sample chamber composition: 0.8 ml lactic dehydrogenase (5 mg) in 0.005 *M* TRIS-HCl pH 7.6; 0.1 ml 55 % buffered sucrose; the remainder of the sample chamber is filled with 0.005 *M* TRIS–HCl pH 7.6. Filling Pressure: 2 lb. Elution pressure: 1 lb. Voltage: 810. Current 11–22 mA. Time: 6 h. Fluorometer aperture: 1. Fluorometer filters: 7–37, 7–60. (———) Beef heart lactic dehydrogenase after partial purification on hydroxyapatite. 5.3 mg sample. Electrophoresis time: 12 h. (. . . . .) Beef heart lactic dehydrogenase after partial purification on hydroxyapatite. 5 mg sample. Electrophoresis time: 6 h.

filters. The column was eluted through the fluorometer under a regulated 1/2 lb. of air pressure. A Texas Instruments Recorder is driven by the fluorometer and the event marker is driven by a fraction collector collecting nineteen drops per tube.

When running a new sample, preliminary experiments should be performed to determine the shape of the sample zone so that one can properly adjust the slit of the light source to give a good full scale reading as well as obtaining the band width, shape and volume element which contains the sample.

For a given material, it is suggested that a series of experiments be done using increasing times of electrophoresis, followed by immediate drainage. These experiments will be important to establish the stability of the moving zone as well as giving practical information on the shape and rate of movement of the sample zone.

MATERIALS AND METHODS

α-Chymotrypsinogen (CGC 762, chromatographically pure), and beef heart lactic dehydrogenase were purchased from Worthington Biochemical Corporation. Reagent grade chemicals were used throughout.

Alkaline treatment of α-chymotrypsinogen was performed by slowly increasing the pH of a solution from 7.6 to 10.3 with 0.1 *N* NaOH and holding it there for differing lengths of time depending on the preparation. Preparations at room temperature were returned within 1 min to pH 7.6 with 0.1 *N* HCl, while preparations treated in the cold were held at pH 10.3 for 1 h in a pH stat using 0.1 *N* NaOH as titrant before returning to pH 7.6.

Unless exceptions are noted beneath the experimental figures each run was performed using the following conditions: Filling pressure 1/2 lb., pH 7.6, length of run 12 h, temperature 7°, gradient 5.5 % buffered sucrose to 55 % buffered sucrose, voltage 400, current 4–7 mA, Fluorometer aperture 10, primary filter Baird Atomic interference filter 280 mμ, secondary filter Dow Corning 7–37, recorder speed 12 in. per h, event marker denotes 19 drops. Migration is downward towards the cathode for chymotrypsinogen and towards the anode for lactic dehydrogenase.

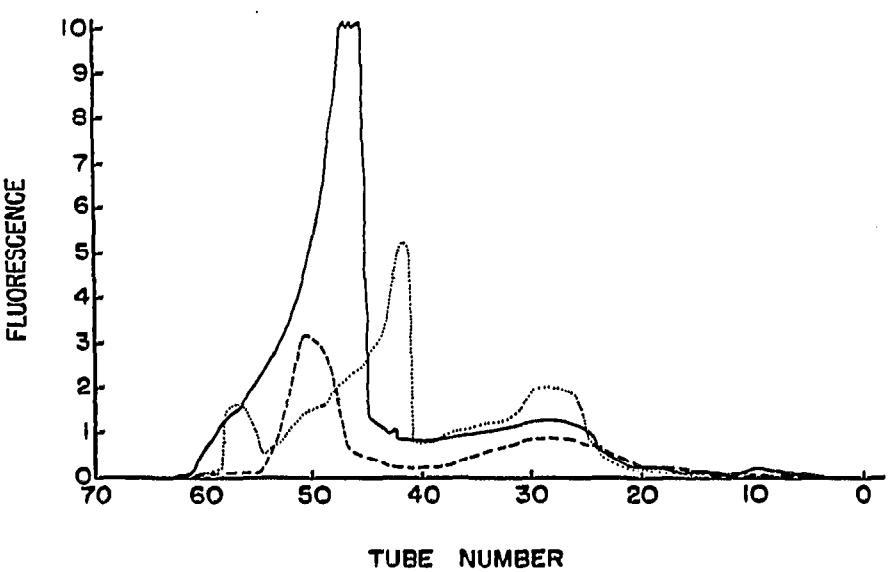

Fig. 7. Electrophoretic profiles of three alkaline treated preparations of chymotrypsinogen are presented. Two of these are the identical sample with respect to alkaline treatment, but an aliquot of the sample was frozen after the alkaline treatment and stored for a week for use in the second experiment; in the first case, 0.1 mg of protein was used; 0.3 mg in the second. The third sample was prepared separately. Heterogeneity of the chymotrypsinogen is noticeable after alkaline treatment. A difference is also observed resulting either from freezing and thawing or from increased concentration. The alkaline treatment was carried out in the manner and concentration described under MATERIALS AND METHODS. (. . . . . .) Chymotrypsinogen. Sample chamber composition: 0.2 ml chymotrypsinogen (0.1 mg) in 0.005 *M* TRIS–HCl pH 7.6; 1 ml 5.5 % buffered sucrose. Fluorometer aperture: 30. (———) Chymotrypsinogen. Sample chamber composition: 0.6 ml chymotrypsinogen (0.3 mg) of previous sample kept frozen one week and thawed; 0.6 ml 5.5 % buffered sucrose. Fluorometer aperture: 30. (- - - - - -) Chymotrypsinogen. Sample chamber composition: 0.2 ml chymotrypsinogen (0.86 mg) in 0.005 *M* TRIS–HCl pH 7.6; 1 ml 5.5 % buffered sucrose. This sample was prepared separately from the other two. Fluorometer aperture: 30.

EXPERIMENTAL RESULTS

The symmetrical distributions of sample on the column shown in Fig. 4 demonstrate that the requirement for initial stability and symmetrical distribution of the sample zone is met. Two different proteins, widely divergent in molecular weight, are viewed at two degrees of resolution for gross and fine amplification of the sample distribution. The absence of any droplet sedimentation or other aberrant deposits of sample is clearly indicated.

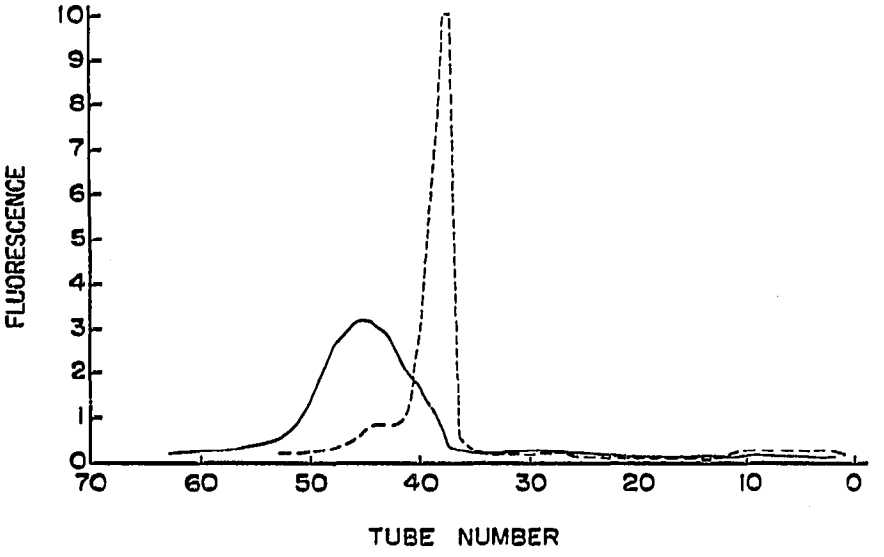

Fig. 8. Two electrophoretic profiles of the same alkaline treated preparation are shown before and after freezing. In contrast to the alkaline preparations of Fig. 7, the protein concentration was increased to 2.5 mg/ml before pH adjustment. Comparison of Fig. 7 and 8 points out differences in the preparations due to concentration and freezing effects; the homogeneity of Fig. 8 is in sharp contrast with the heterogeneity of Fig. 7. (————) Chymotrypsinogen. Sample chamber composition: fill sample chamber with chymotrypsinogen subjected to alkaline treatment at a concentration of 2.46 mg/ml in 5.5% buffered sucrose. Fluorometer aperture: 3. (- - - -) Chymotrypsinogen. Sample chamber composition: fill sample chamber with same chymotrypsinogen preparation as above after freezing overnight. Fluorometer aperture: 3.

Fig. 5 demonstrates the effect of overloading the initial sample zone. There was no indication of instability on visual inspection of the sample zone on the column. However, analysis of the graphs shows that non-ideal electrophoretic movement of the sample is caused by too high a concentration of protein in the initial zone. For a given sample, several of these experiments performed at different concentrations will establish the useful range of this procedure.

That stability of the moving zone is achieved at the proper sample concentration is seen in Fig. 6 where comparison of 6 h and 12 h electrophoretic profiles of lactic dehydrogenase indicates a very stable moving zone (in the 12-h experiment, the sample was initially placed higher on the column, which accounts for the relative positions). Comparison of the two 6-h profiles in this figure draws attention to the increasing electrophoretic homogeneity of lactic dehydrogenase after partial purification on hydroxyapatite. The column thus provides an analytic means of measuring homogeneity as well as a method of purification in such systems.

In Figs. 7 and 8, electrophoretic profiles of chymotrypsinogen subjected to the alkaline treatment in low concentration are presented. The effect of freezing and thawing the same sample after return to neutral pH stands out as does slight variation in the preparation (at room temperature) of separate samples.

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SUMMARY

A complete technique is presented which, with only minor changes determined by the protein in hand, is valuable as an analytic aid in the study of proteins in dilute or concentrated solution.

The results on the changes in chymotrypsinogen on alkaline treatment are seen to be resolved only in dilute solution and the phenomenon was discovered by the use of this method.

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