THE FRACTIONATION OF SERUM PROTEINS BY ELECTROPHORESIS-CONVECTION

Thesis by

Raymond Allen Brown

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1949

Abstract

A new improved electrophoresis-convection apparatus for the fractionation of proteins is described. The potentialities of the apparatus are demonstrated in the partial separation of bovine serum proteins and in the sub-fractionation of bovine γ-globulin, Fraction II obtained by alcohol-salt precipitation. A fraction containing 96% γ-globulin has been isolated from bovine serum. Bovine γ-globulin has been separated into eight fractions with mobilities ranging from -1.25×10^{-5} to -2.25×10^{-5} and isoelectric points extending from pH 7.31 to pH 5.74.

An extension of the theory of reversible boundary spreading in the electrophoresis of proteins is presented. The theory has been applied to the characterization of the γ-globulin fractions.

Table of Contents

P A R T I

The Fractionation of Proteins by Electrophoresis-Convection

Description of an Improved Apparatus and Its Use in the Fractionation of Bovine Serum Proteins

Part 1

The Fractionation of Proteins by Electrophoresis-Convection Description of an Improved Apparatus and Its Use in the Fractionation of Bovine Serum Proteins

Introduction

Hardy (1) and Mellanby (2) were two of the first investigators to put the fractionation of proteins upon a rational basis. The former introduced the concept of isoelectric point and the latter that of ionic strength. The "salting out" techniques of fractionating proteins were developed by a number of investigators during the period 1905 to 1930. However, the work was handicapped by lack of adequate methods for the characterization of the fractions.

The development of the electrophoresis apparatus by Tiselius (3) provided a convenient method for protein characterization and stimulated other workers in their efforts to isolate the proteins of blood plasma.

Tiselius (3) used the new electrophoresis apparatus not only for analytical work but also for the preparation of electrophoretically homogeneous proteins. His method has some disadvantages. Only small quantities may be recovered and the separation of two proteins with small mobility differences is quite difficult.

Svensson (4) has reviewed the methods of preparative electrophoresis. In general electrophoretic methods have not been as widely used for protein fractionation as the "salting out" methods.

Cohn and coworkers at the Harvard Medical School have used a complex system of ethanol, salt, and buffers at 0°C to -5°C to separate human plasma into fractions. The separations are accomplished by making use of the solubility differences of the proteins in various solvent systems.

Recently Edsall (5) has made a comprehensive review of the work in volume III of "Advances in Protein Chemistry". There has been a very large number of papers published by the group and only a list of the more important papers will be given (6).

The people at the Harvard Medical School have been able to separate human plasma into several relatively homogeneous components by the use of their ethanol-salt system of fractionation. These proteins migrate as a single peak in the electrophoresis apparatus in alkaline buffers. Nevertheless, the fractions do not in general satisfy all of the criteria necessary for a homogeneous preparation, i. e., homogeneity in the ultracentrifuge, absence of reversible boundary spreading in the electrophoresis apparatus, and a solubility which is independent of the amount of solid phase present.

In 1941 Kirkwood (7) suggested a new method for the fractionation of proteins. The method is a modification of that used in the separation of isotopes by thermal diffusion in a Clusius column. It was proposed that differential electrophoretic transport of proteins be superimposed upon vertical convective transport in a Clusius column. Separation should take place on the basis of mobility differences.

The method was tested experimentally by Nielsen and Kirkwood (8) several years later. Artificial mixtures of hemoglobin with serum albumin and azo-ovalbumin were partially separated and transport experiments were carried out with single components. The experiments showed that the density gradients caused by electrophoretic transport were much larger than those induced by thermal diffusion. As a result the use of thermal gradients in the apparatus was discarded.

Results obtained by Nielsen and Kirkwood (8) indicated that the new

method, electrophoresis-convection, should be of practical value in the separation of electrophoretic components from protein mixtures.

The apparatus used consisted essentially of two glass reservoirs connected by a narrow annular channel formed by concentric sausage casings. An electric field was produced by the passage of a current through the buffer in which the apparatus was immersed.

Since the membranes in the apparatus were not adequately supported, they had a tendency to sag with a consequent change in the dimensions of the channel. As a result the fractionations were not entirely reproducible.

It is the purpose of this thesis to describe a new and improved apparatus designed to eliminate the disadvantages of the older apparatus and to present the results of some representative fractionations made with the new apparatus.

As it is now used the fractionation scheme may be described briefly as follows. Two reservoirs connected by a vertical channel, of width sufficiently small to ensure laminar flow, contain a solution of the proteins to be fractionated. Upon application of a horizontal electric field, differential transport of the mobile components across the channel takes place, producing a horizontal density gradient depending upon the composition gradients. Under the action of gravity, the density gradient induces convective circulation in the channel with a velocity distribution qualitatively similar to that of the Clusius column. The result of the superposition of the horizontal electrophoretic transport and vertical transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending upon their mobilities, with a relative enrichment of the top reservoir with respect to the slow compon-

ents. The mathematical theory of the transport has been worked out by Professor Kirkwood and will be presented in a future article. It has been used in the design of the improved fractionation cell to be described here.

In order to avoid contamination of the solution by electrolysis products, the walls of the convection channel are constructed of semi-permeable membranes, separated from the electrodes by buffer solution. The electric field across the channel is maintained by the electric current carried by the ions of the buffer electrolyte, to which the membranes are permeable. The exterior buffer solution is replenished by a circulation system at a rate sufficient to prevent the electrolysis products from reaching the membranes.

Electrophoresis-convection is related to the Pauli (9) electro-decantation effect, which has been used by Pauli and Stamberger (10) to concentrate colloidal solutions and by Gutfreund (11) to stratify proteins solutions. It appears that the method bears roughly the same relation to the Pauli electro-decantation effect that the Clusius column bears to the Soret effect.

Depending upon the pH of the operation employed, fractionation of a heterogeneous protein may be effected by two modes of operation. In the first method the pH of the solution is such that all the components are either on the alkaline or acid side of their isoelectric points. Under these conditions the components are differentially transported out of the upper and into the lower reservoir, the fractionation depending upon the difference in mobilities of the constituent proteins. The optimum separation of components is obtained by so choosing the operating time that half of the major component is transported out of the upper reservoir.

In the alternative method of operation one of the constituents of the heterogeneous protein is immobilized by operating at its isoelectric point. The mobile components are transported out of the upper and into the lower reservoir, leaving the immobilized component in the upper reservoir.

The successive separation of the components of a protein mixture can be effected by the isoelectric procedure as follows. The component with the most alkaline or acid isoelectric point is first separated from the others in several successive stages by operating at its isoelectric point. The composite of the top cuts of these stages is further processed to purify the desired constituents. The bottom cut of the last of these stages is a concentrate of the mobile components. The procedure is repeated until the mixture has been resolved.

The first method of operation, in which the pH of operation is on either the alkaline or acid side of the isoelectric points of all the components, has been used in a preliminary fractionation of ovalbumin carried out at Cornell University (12). Electrophoretic analysis of the material at a concentration of 1 g/100 ml in phosphate buffer pH 6.8 and ionic strength 0.1 showed that there are two components A_1 and A_2 with mobilities -5.90×10^{-5} and -5.13×10^{-5} respectively. The relative concentration of A_2 was found to be 22%. After a three stage fractionation carried out in phosphate buffer pH 6.8 and ionic strength 0.05 the relative concentration of A_2, the slower moving component, was found to have been increased to 31%.

In a series of exploratory runs made at Cornell University (12) and continued at the Institute, Horse Diphtheria Antitoxin Pseudoglobulin* was used to test the isoelectric method of fractionation. Electrophoretic analysis of the material in barbital buffer pH 8.6 and ionic strength 0.1 at a concentration of 2 g/100 ml yielded 8% α-globulin (mobility -3.72×10^{-5} cm^2 $volt^{-1}$ sec^{-1}), 56% β_2-globulin (-2.44×10^{-5}), and 36% γ-globulin (-1.10×10^{-5}). In phosphate buffer ionic strength 0.1, the γ-globulin was isoelectric at pH 6.54. A two fold increase of the relative γ-globulin concentration was achieved by means of a three stage fractionation operating at the isoelectric point of γ-globulin, pH 6.5, in phosphate buffer ionic strength 0.05. The final top cut had a relative concentration of 74% γ-globulin.

On the basis of the work outlined above it was concluded that the isoelectric procedure is the best method for obtaining electrophoretically homogeneous proteins. Consequently, all the results reported in the thesis were obtained by isoelectric procedure.

Complete exhaust of the mobile components, although closely approached under ideal conditions, is sometimes inhibited by disturbing factors. If the operating pH is so chosen that there are both positive and negative mobilities, a stationary state will be reached before all the mobile components are removed from the top reservoir. A theory of the electrophoresis-convection column valid for values of the parameter, $a\mu E/D$, small relative to unity has been developed by Professor Kirkwood. It shows that when the stationary state is reached, the mean isoelectric

---

* The department of physical chemistry, Harvard Medical School kindly supplied the Horse Diphtheria Antitoxin Pseudoglobulin. This material was prepared at the Massachusetts Antitoxin Laboratory.

point of the top fraction will be the same as the pH of the buffer in the apparatus. Osmotic transport of solvent from the exterior buffer solution into the cell occurs in those runs in which there are mobilities of both signs. The osmotic transport is an increasing function of the field strength and protein concentration and a decreasing function of the ionic strength. The influx of water through the membranes reinforces upward convection and counteracts downward convection, thereby decreasing the rate of transport out of the upper reservoir. Although theory predicts only a slight dependence on initial protein concentration, efficiencies of separation have been observed to be markedly lower at lower protein concentrations. Also, in disagreement with theory, it has been found that poorer separations are obtained with high field strengths than with field strengths below 2.5 volts/cm. Although a thorough investigation has not been made, it is surmised that the decreased fractionation efficiencies obtained at high field strengths and low protein concentrations may be due to the destruction of laminar flow in the channel. In general, optimum operating conditions must be determined by pilot fractionations.

Blood serum which is a mixture of at least six proteins with appreciable differences in isoelectric points is well suited for a test of the isoelectric fractionation procedure. The potentialities of the method of electrophoresis-convection and the effectiveness of the new fractionation unit are well illustrated by the partial separation of bovine serum proteins accomplished by the isoelectric procedure and the results are reported in Part I of the thesis.

Construction of the Electrophoresis-Convection Apparatus

The apparatus described here may be considered as being composed of five principal parts: 1) the fractionation cell consisting of a cell block, two face plates, and semi-permeable membranes; 2) the box housing the fractionation cell and electrodes; 3) the electrode assemblies; 4) the buffer circulating system and 5) the power pack. On assembly the cell consists schematically of a narrow vertical channel connecting upper and lower reservoir. That portion of the channel effective in fractionation is formed by the rectangular space between two sheets of semi-permeable membrane. The cell is immersed in buffer solution to within about an inch above the bottom of the upper reservoir. The electrode assemblies are placed in the box on opposite sides of the cell. During operation electrolysis occurs which tends to change the pH of the buffer solution. To counteract this, buffer is circulated vertically around the cell.

The material used in the construction of the fractionation cell and its housing must meet certain requirements. It must be electrically non-conducting, exhibit no swelling when in contact with aqueous solutions, possess dimensional stability, and be readily machined with high precision. Although there is something to be desired in its machine-ability, lucite appears to be the material most suitable for this purpose. Consequently, it was chosen as the construction material. Where necessary the lucite was cemented with Dupont Cement H-94.

The Fractionation Cell is shown to scale in Plates 1 and 2. The cell block, Plate 1, consists of an upper and lower reservoir between which is a vertical rectangular slot. The reservoirs are connected to this central slot by means of narrow vertical channels passing through

the body of the block. The capacities of the upper and lower reservoirs are 100 and 50 ml. respectively. Both reservoirs are supplied with valves for sampling, and the top reservoir is open to the atmosphere. A recess is milled around the periphery of the rectangular slot on both faces of the cell block. The cell block is supplied with two small legs.

Both face plates, Plate 2, are of identical design. The face plate is essentially a frame which fits into the recess around the periphery of the central slot of the cell block. Around the inner periphery of the back side of the frame is a shoulder constructed so as to fit into the central slot of the cell block. The face plates clamp the sheets of semi-permeable membranes in place against the bottom of the recess. Since the membranes form the face-walls of the effective portion of the channel, the height of the shoulder controls the channel wall separation. In the cell described here the wall separation is 0.037". A 1/64" clearance is allowed between the sides of the shoulder and the central slot and also between the sides of the frame and the recess. The face plate is supplied with two longitudinal ribs, which act as supports for the membranes. The one face plate and the cell block are drilled to receive brass machine screws*, the holes in the face plate being counterbored. The other face plate is drilled and tapped for the screws. The holes in the face plates correspond to those in the cell block.

The effective portion of the channel connecting the upper and lower reservoirs is formed as follows: Visking Corporation cellulose sausage casing, No. 133, is soaked overnight in distilled water. Sheets of semi-

---

*Since the heads and tips of the screws must be electrically insulated and since, in any case, metal screws distort the electric field across the channel walls, the use of natural paper base bakelite is recommended.

permeable membrane are made by cutting along one edge of the casing and unfolding. The wet membranes are applied to both faces of the cell block and pressed along the bottom of the peripheral recess of the central slot by insertion of the face plates into the recess. This produces a gasketed seal. One edge of the plates is bolted snugly to the block by means of three screws. The membranes are now stretched and the free edge of the plates forced back into the recess, exercising care to keep the membranes taut. The remaining screws are put into place, all the screws being tightened sufficiently to prevent leakage. The excess membrane is removed and the heads and tips of the screws electrically insulated with Fenox, supplied by the Bakelite Corporation. In order to prevent drying out of the membranes the cell is filled with distilled water and stored in a container of water.

The Box which houses the fractionation cell and the electrode assemblies is shown to scale in Plate 4. The inside dimensions are such that a snug fit is obtained between the sides of the box and the edges of the cell block. This minimizes loss of electric field by leakage around the cell. The capacity of the box is sufficient to allow the cell to be surrounded by about 2½ liters of buffer solution. The box is supplied with buffer inlet and outlet tubes.

The Electrode Assemblies are also shown to scale in Plate 3. Originally each electrode assembly consisted of three graphite rods mounted in a lucite holder. However, contamination of the solutions by the deterioration of the anode and the inhomogeneity of the electrical field produced by the arrangement warranted changing to the platinum electrodes described here. Each assembly consists of 2 mil platinum foil mounted in a lucite frame. The dimensions of the platinum foil correspond to

those of the inner periphery of the face plates. The dimensions of the frames are such that the strips of platinum and the effective channel of the cell are aligned when the cell and electrode assemblies are housed in the box. Each frame is supplied with a binding post, contact with the platinum foil being made with No. 18 platinum wire. Since the platinum occludes large quantities of electrolytic hydrogen, it is advisable to alternate the polarity of the electrodes from run to run.

The Buffer Circulating System was designed to counteract the change in pH of the solutions due to the accumulation of electrolysis products about the electrodes during operation. A buffer flow of about 30 liters an hour is sufficient to maintain the pH of the solution constant to within 0.1 of a pH unit at a current density of 2.5 amps. per $dm.^2$. Circulation is by gravity flow. The buffer flows from a twelve liter aspirator bottle into the box housing the cell and electrodes. It is then circulated vertically in the box so as to pass down the front and up the back of the cell, the small legs of the cell block permitting the flow of the solution underneath the cell. The circulated buffer is discharged into a twelve liter flask. A centrifugal pump periodically pumps the circulated buffer back into the aspirator bottle*. The pump is activated as follows. One branch of a mercury manometer is inserted into the side of the aspirator bottle so as to measure the hydrostatic pressure. A flexible rubber sack filled with air and sealed onto the end of the manometer tube separates the buffer solution from the mercury. When

---

*It is desirable to operate under conditions of field strength and current density which do not lead to electrolytic decomposition of the buffer anions. Under these conditions, the original pH of the buffer solution is restored on mixing, and the buffer may be recycled in the circulation system without replenishment.

the hydrostatic pressure decreases to some predetermined value, the pump is activated through an electronic relay (13) by means of a system of three platinum contacts in the open branch of the manometer. The relay is self-energized through one of the contacts thus preventing short cycling of the pump.

The use of silver, silver chloride electrodes, which would eliminate the necessity for buffer circulation, has been considered.

The Power Pack consists of a potentiometer connected across a 110 volt direct current line. A 35 ohm slide wire resistor, maximum capacity of 5 amps., is used for the potentiometer. The voltage drop across the cell and the electrolyzing current are measured with a standard voltmeter and ammeter.

Operation of the Electrophoresis-Convection Apparatus

The apparatus is operated as follows. The lower reservoir and channel of the cell are filled with the buffered protein solution to be fractionated, exercising care to eliminate all air bubbles from the channel. The cell is immersed in buffer solution contained in the box to within about one inch above the bottom of the upper reservoir. The remainder of the protein solution is now put into the upper reservoir. A total volume of 110 to 150 ml. of protein solution may be used depending upon whether or not one wishes to fill the upper reservoir to capacity. The electrode assemblies are placed in the box on either side of the cell making certain that they are parallel to the cell-faces. By making one electrode positive and the other negative a relatively homogeneous electric field can be set up across the channel containing the protein solutions. Buffer is now circulated vertically around the cell. The

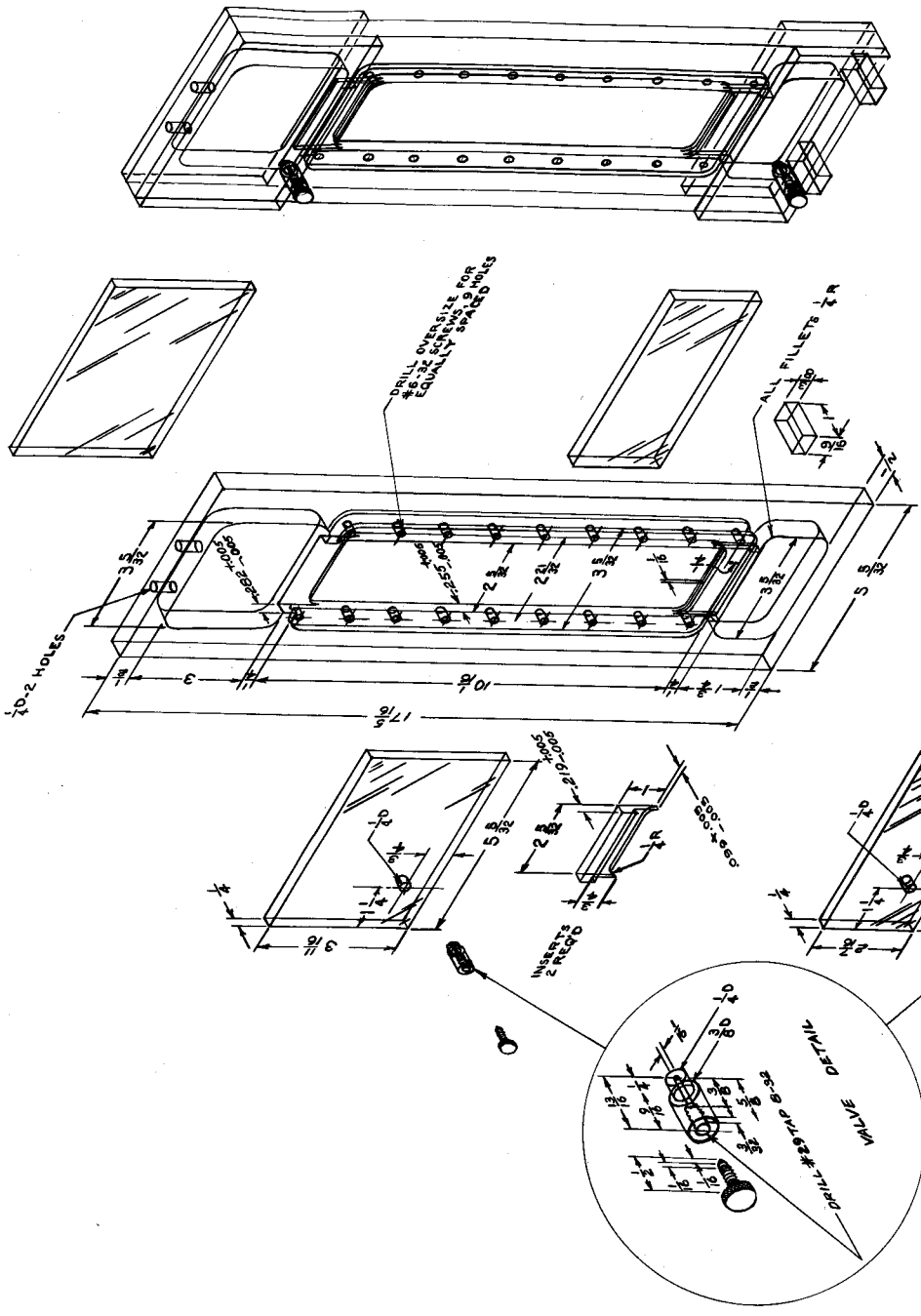
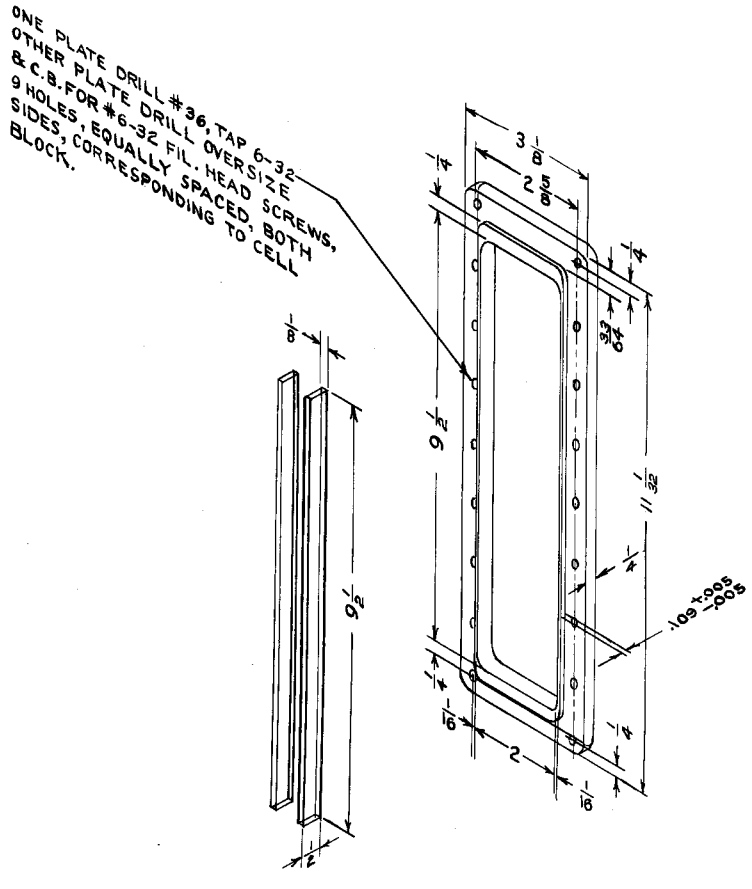
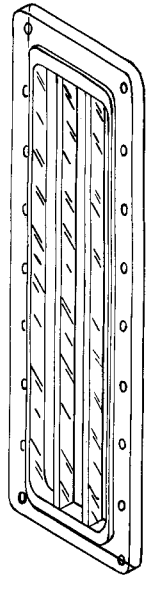


PLATE I

CELL BLOCK ASSEMBLY, ELECTROPHORESIS CONVECTION APPARATUS

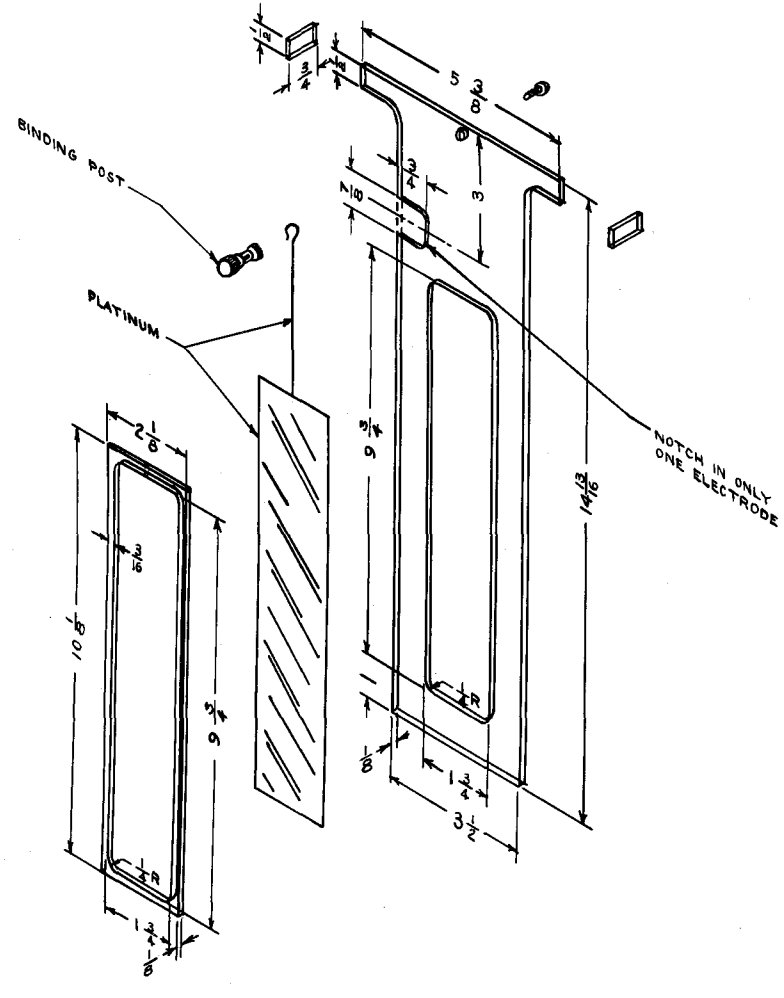


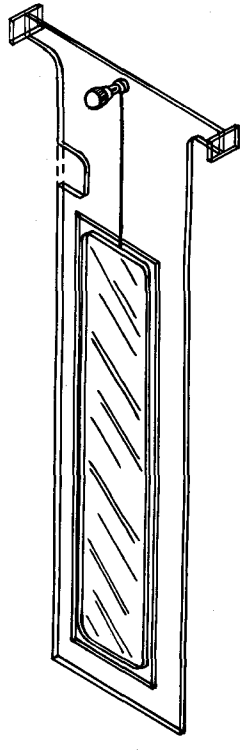


ASSEMBLED FACE PLATE
2 REQ'D.

JOINTS CEMENTED DUPONT NO. H-78 CEMENT
MATERIAL: LUCITE

PLATE 2
FACE PLATE ASSEMBLY, ELECTROPHORESIS CONVECTION APPARATUS



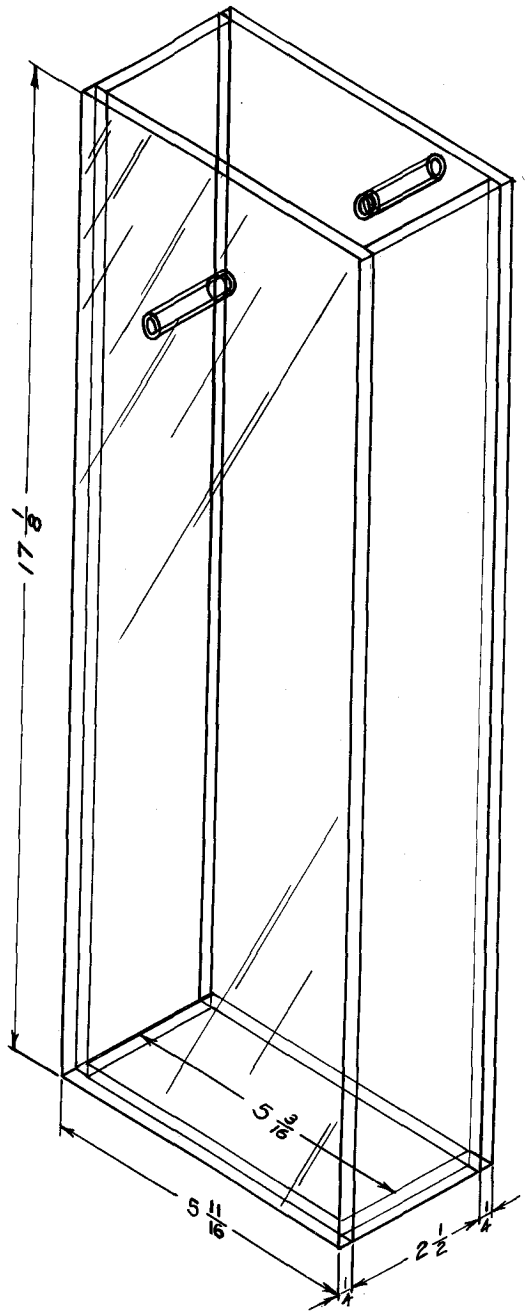


ASSEMBLED ELECTRODE
2 REQ'D.

MATERIAL: LUCITE, UNLESS OTHERWISE SPECIFIED.
ALL JOINTS CEMENTED, DUPONT NO. H-78 CEMENT.

PLATE 3

ELECTRODE ASSEMBLY, ELECTROPHORESIS
CONVECTION APPARATUS



MATERIAL: LUCITE
JOINTS CEMENTED DUPONT NO. H-78 CEMENT

PLATE 4
CASE, ELECTROPHORESIS
CONVECTION APPARATUS

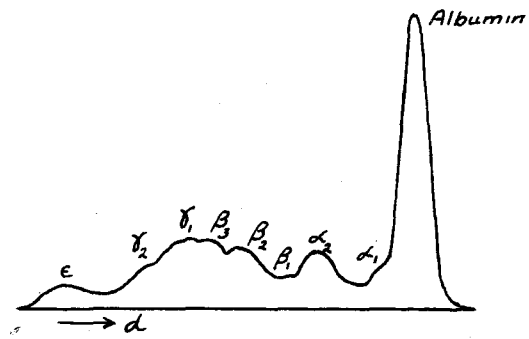
temperature is regulated in a constant temperature coldroom operating at 4°C. More than one fractionation may be conducted at the same time by cascading the desired number of units.

The nominal field strength in the solution is determined from measurements of the specific conductance of the solutions and from the average current density.

At the conclusion of the fractionation the top fraction is withdrawn from the upper reservoir by means of a long hypodermic needle and syringe. This operation is performed while the cell is still in the box and surrounded by buffer solution. The bottom fraction, which includes the solution from both the lower reservoir and the channel, is drawn off through the lower reservoir valve.

Fractionation of Bovine Serum

Material.- Blood from a Hereford cow served as the starting material. Serum was prepared by stirring the blood to remove the fibrinogen followed by centrifugation to remove the cells. Analysis of this material yielded 8 electrophoretic components as illustrated in Figure 3. The nomenclature adopted in labeling the electrophoretically separable components is given in Figure 1. This nomenclature appears to be consistent with that used in the literature except that β_3-globulin is sometimes included in the γ-globulin (14). Hess and Deutsch (15) have designated this component merely as β-globulin. The mobilities and relative concentrations of the electrophoretic components of the serum are presented in Table I. These data were obtained in barbital buffer, pH 8.7 and ionic strength 0.1



Designation of the components of bovine serum.

Figure 1.

Table I

The mobilities ($-u \times 10^5$) and relative concentrations of the components of bovine serum.

	Albumin	α_1	α_2	β_1	β_2	β_3	γ_1	γ_2
Mobility	6.52	5.76	4.62	3.86	3.08	2.44	1.97	1.28
Relative Concentration	43	3	13	3	14	9	8	7

Electrophoretic Analysis.- The moving boundary technique of Tiselius (3) as modified by Longsworth (16) was used in the electrophoretic analysis. Electrolysis of the protein solution in barbital buffer, pH 8.7 and ionic strength 0.1, was allowed to preceed for 4 hours with a field strength of 4 volts/cm. Mobilities were calculated from measurements of the displacement from the initial boundary of the constituent boundaries as suggested by Longsworth and MacInnes (17).

For the purpose of determining the total protein concentration of a solution an arbitrary factor relating the concentration to area units was obtained by planimetric integration of the tracings of enlarged electrophoretic diagrams of solutions of known bovine serum albumin concentrations. The concentrations of the standard solutions were determined by the method of Koch and McMeekin (18). Electrophoretic analysis of a protein solution could be effected to about $\pm 1\%$.

The apparent concentrations of the electrophoretic components of the serum and its fractions were determined from the electrophoretic patterns by finding the ratio, in each case, of the component area to the total area, exclusive of the ϵ boundary. The areas were measured on projected tracings of the descending patterns with a planimeter, resolution into components being carried out by the method of Pederson (19). The conditions of electrophoresis were such that departure of the apparent distribution from the true distribution of electrophoretic components

were negligible compared to the changes in distribution effected by fractionation (20).

Experimental Results

Separation of the γ-globulin fractions from the serum was carried out in phosphate buffer, pH 6.5 and ionic strength 0.1. The isoelectric points of the γ-globulins appeared to be close to this pH. The other components of the serum migrated towards the anode on electrophoresis at 6.5. To effect separation of the β-globulins runs were carried out at pH 5.0-5.1 and ionic strength of 0.3. The scheme used in the fractionation is shown diagramatically in Figure 2. The electrophoretic patterns of Figures 3, 4, and 5 follow the course of fractionation. The γ-globulins were separated from the serum and purified in Fractionations I, II and III. β-globulin fractions were obtained in Fractionations IV, V and VI.

γ-globulin Fractions.- The separation of crude γ-globulin fractions was effected in Fractionation I. Since the γ-globulins were practically immobilized by operating near their isoelectric points, only the albumin, α- and β-globulins were transported out of the upper and into the lower reservoir. Four runs were made in order to study the influence of field strength, duration of electrolysis, and volume of solution being fractionated upon the efficiency of separation.* In all cases the initial concen-

---

* Osmosis, increasing the volume of protein solution, occurred during these runs. The volume of osmoid depended upon the conditions of fractionation. Thus, in Run 1 the volume of protein solution was increased by 6% due to osmosis, while in Run 2 the volume of osmoid was 39% of the initial volume. It was found that osmosis increases with increasing field strength and increasing protein concentration and decreases with increasing ionic strength. It is desirable to minimize osmosis, since the passage of water through the

250 ml. bovine serum. 7.5 g/100 ml.

250 ml. H_2O

Dialyzed against phosphate buffer, pH 6.5, ionic strength 0.1

FRACTIONATION I (4 runs)

354 ml. top cut, 0.7 g/100 ml.
80-87% γ-globulins

190 ml. bottom cut, 6.0 g/100 ml.

350 ml. 3.3 g/100 ml.

116 ml., 0.6 g/100 ml.

FRACTIONATION II

70 ml. top cut, 0.5 g/100 ml.
88% γ_2-globulin
8% γ_1-globulin

54 ml. bottom cut, 0.6 g/100 ml.
12% γ_2-globulin
74% γ_1-globulin

FRACTIONATION III (3 runs)

188 ml. top cut, 0.5 g/100 ml.
6% γ_2-globulin
76% γ_1-globulin

162 ml. bottom cut, 6.8 g/100 ml.

330 ml. 3.1 g/100 ml.
Dialyzed against phosphate buffer pH 5.0-5.1 ionic strength 0.3

FRACTIONATION IV (4 successive stages)

578 ml. top cut
~50% β-globulins
~10% γ-globulins

Lyophilized
110 ml. 2.8 g/100 ml.

117 ml. bottom cut,
3.6 g/100 ml.

FRACTIONATION V

55 ml. top cut, 1.3 g/100 ml.
40% γ-globulins
38% β_3-globulin
4% β_2-globulin

55 ml. bottom cut, 2.7 g/100 ml
5% γ-globulins
14% β_3-globulin
26% β_2-globulin

107 ml. 1.3 g/100 ml.

FRACTIONATION VI

50 ml. top cut, 0.7 g/100 ml.
10% β_3-globulin
62% β_2-globulin

57 ml. bottom cut

Figure 2

Schematic Fractionation Procedure Bovine Serum

tration of the protein solution was 3.7 g protein/100 ml. of solution. The resultant data are presented in Table 2, where

E = nominal field strength in volts/cm.

t = duration of run in hours.

V_i = initial volume of protein solution in ml.

c_i = initial concentration of protein solution in g. protein/100 ml. solution.

v_0 = volume of osmoid in ml.

f_t = top separation factor.

The efficiency of separation is expressed in terms of the top separation factor, which is defined by the relation

$$f_t = \frac{x_i}{x_i^0} \cdot \frac{1 - x_i^0}{1 - x_i} \quad ; \quad x_i = \frac{c_i}{\sum_K c_K} \qquad (1)$$

X^0_i and X_i are the initial and final ratios of the concentration of the immobilized component to total protein concentration in the top reservoir.

The initial separations yielded γ-globulin fractions, the compositions of which compare favorably with that of the crude γ-globulin fraction obtained by Hess and Deutsch (15) using ethanol precipitation. Thus, the top fraction of Run 4 had the composition 86% γ-globulins, 13% β-globulins, and 1% albumin, while the γ-globulins separated in crude form by ethanol precipitation were of composition 85% γ-globulins and 15% β-globulins. Figures 3a and 3b represent the electrophoretic patterns of the original serum and a representative crude γ-globulin

---

membranes into the channel counteracts the downward convection current and reinforces the upward convection current, thereby decreasing the efficiency of separation.

Table 2

(a) Conditions for separation of γ-globulin fractions.

Fractionation	Run	E volts/cm	t hrs.	V_i ml.	c_i g/100 ml.	v_o ml.	f_t
I	1	3.1	14	110	3.7	7	23
	2	6.1	14	115	3.7	45	23
	3	3.1	24½	117	3.7	12	32
	4	3.1	27	155	3.7	9	33
II		3.1	24	116	0.6	8	6
III	Composite of 3 runs	3.1	15	350	3.3		46

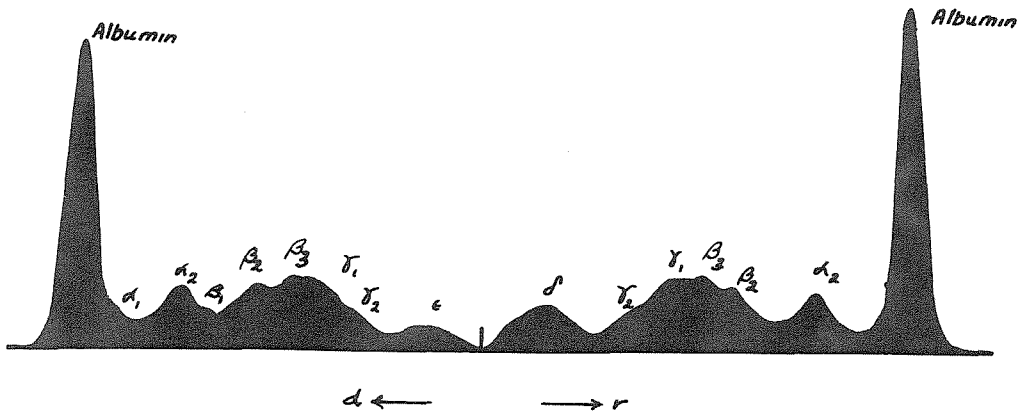
(b) Distribution of electrophoretic components into fractions.

Fractionation	Run	Reservoir	Albumin	α_1	α_2	β_1	β_2	β_3	γ_1	γ	γ_2
			percent of fraction								
Serum			43	3	13	3	14	9	8		7
I	1	top	1		3		8	8	67		13
	2	top	2		1	1	8	8		80	
	3	top	1				6	8		85	
	4	top	1				5	8	63		23
I	Composite 1,2,3,4	bottom	49	2	15	3	14	8	6		3
II		top					4		8		88
		bottom	4				4	6	74		12
III	Composite of 3 runs	top	6	2	2	3	3	2	76		6
		bottom	53	3	16	3	12	7	4		2

fraction, respectively. Partial separation of γ_1-globulin and γ_2-globulin was also effected in these initial fractionations. The relative concentrations of γ_1- and γ_2-globulin in the serum were 8% and 7%, respectively. The top fraction of Run 1 analyzed 67% γ_1-globulin and 13% γ_2-globulin, and the top fraction of Run 4, 63% γ_1-globulin and 23% γ_2-globulin. Electrophoretic resolution of the γ-globulin peak was not obtained on analysis of the solutions from the top reservoir of Runs 2 and 3.

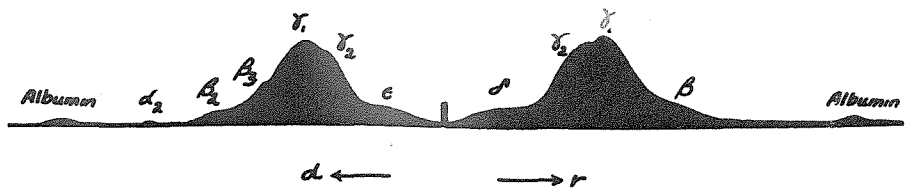
Removal of the albumin, α-globulins and β-globulins from a composite of the initial γ-globulin fractions obtained in Runs 1, 2 and 3 was accomplished in Fractionation II. Electrophoretic patterns of the resulting top and bottom cut are shown in Figure 4. Analysis of the solution in the top reservoir yielded 96% γ-globulins and 4% β-globulins. Considerable separation of γ_1- and γ_2-globulin was also effected in this experiment. Referring to Table 2, it will be noted that the relative concentrations of γ_1- and γ_2-globulin in the upper reservoir were 8% and 88%, respectively, while in the lower reservoir they were 74% and 12%, respectively. The mobilities of the γ_1- and γ_2-globulin under consideration are -1.9×10^{-5} and -1.2×10^{-5} cm^2 sec^{-1} $volt^{-1}$, respectively. These are slightly lower than the mobilities reported by Hess and Deutsch (15) for their fractions which correspond.

Although the calculation of a separation factor for this fractionation is complicated by the transport of γ_1-globulin, it appears that the efficiency of the fractionation was markedly lower than those obtained in Fractionation I. The only difference between Run 1 of Fractionation I and Fractionation II was that the concentration of the protein solution in the former run was 3.7 g/100 ml. as compared to 0.6 g/100 ml. in the



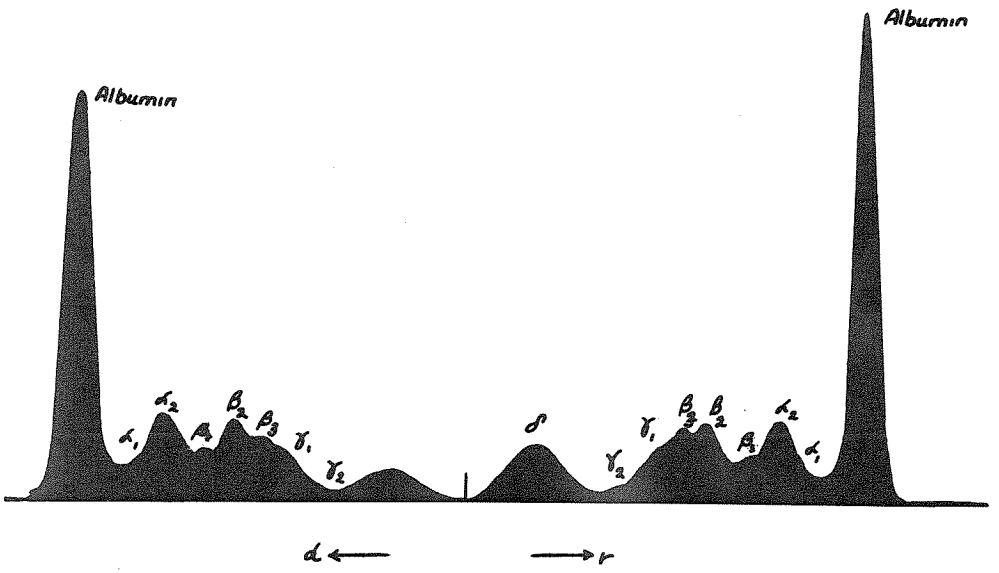
Original Bovine Serum

Figure 3 a.



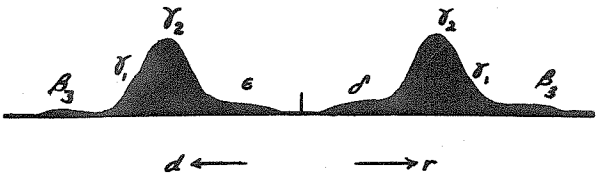
Bovine Serum
Top Cut Fractionation I, Run 1

Figure 3 b.

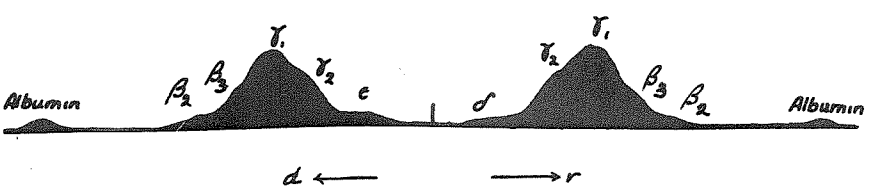


Bovine Serum
Bottom Cut Fractionation III

Figure 3 c.



Bovine Serum
Top Cut Fractionation II



Bovine Serum
Bottom Cut Fractionation II

Figure 4.

latter run. The influence of the concentration upon operating efficiency was confirmed by the results of two runs made to effect separation of crude γ-globulin fractions from serum. Except for concentration, the operating conditions of these runs were identical with those used in Run 1. It was found that the separation factor was 23 for an initial concentration of 2.9 g/100 ml. and only 7 for an initial concentration of 0.8 g/100 ml.

The material taken from the bottom reservoir of Fractionation I was further processed in Fractionation III to remove γ-globulins. The data are presented in Table 2. Analysis of the solution withdrawn from the top reservoir yielded 76% γ_1-globulin and 6% γ_2-globulin. The solution taken from the bottom reservoir contained only 6% γ-globulins, Figure 3. Thus, the concentration of γ-globulins in the serum was reduced by about 60% in two successive stages of fractionation. Further fractionation would have eventually removed all of the γ-globulins, but for the purpose of this investigation that was not warranted.

β-globulin Fractions.- Subsequent to the separation of γ-globulin fractions, crude β-globulin fractions were separated from the serum by fractionation at pH 5.0-5.1 and ionic strength 0.3. Two further stages of fractionation served to purify these crude fractions. The pertinent data are presented in Table 3. The efficiency of separation of the β_2 and β_3-globulin fraction is expressed in terms of the top separation factor, f_t, defined by a relation analogous to Eqn. 1.

At pH 5.0-5.1 the β_2- and β_3-globulin appeared to be close to their isoelectric points. On electrophoresis the mobile serum proteins were transported out of the upper and into the lower reservoir, leaving the β-globulins in the top reservoir. Under the same conditions of

field strength and duration of electrolysis, the operating efficiency in this instance was less than in the case of the separation of the γ-globulins. There were two reasons for this. First, the mobilities of the migrating components were less than in the case of the γ-globulin fractionations, thus decreasing the rate of horizontal electrophoretic transport. Also, at the operating pH the mobilities of the residual γ-globulins in the serum were opposite in sign to those of albumin, α-globulins and β_1-globulin. The horizontal electrophoretic transport of a component counter to that of other mobile components decreases the horizontal density gradient across the channel, thereby decreasing the convective velocity. Indeed, a steady state should eventually be established after which no further change in the composition of the solutions in the reservoirs will occur.

The separation of crude β-globulin fractions was effected by four successive stages of fractionation, Stages a, b, c, and d of Fractionation IV. The material from the bottom reservoir of Fractionation III served as the starting material for Stage a; the bottom fraction of Stage a was refractionated in Stage b; etc. Each stage represents a composite of two or three runs, the starting material and conditions of operation being the same in each case. The electrophoretic pattern of a representative crude β-globulin fraction is shown in Figure 5a. The "purest" of these crude fractions had the composition 20% albumin, 20% α-globulins, 2% β_1-, 34% β_2-, 20% β_3-globulin, and 4% γ-globulins. Electrophoretic analysis of the material obtained from the bottom reservoir of Stage d yielded no γ-globulin and only 6% β_2- and β_3-globulin, Figure 5b. Thus four successive stages of fractionation brought about a 73% reduction of the relative concentration of β_2- and β_3-globulin. Nitrogen

determinations, made on the initial solution and the solutions obtained from the upper and lower reservoir of Stages c and d, yielded the following material balance:

Total protein fractionated	4.8 g
" protein in composite of top fractions from Stages c and d	0.8 g
" protein in lower reservoir of Stage d	4.2 g
" protein recovered	5.0 g

After adjusting the concentration by lyophilizing, the composite of the crude β-globulin fractions served as starting material for Fractionation V. The results of this fractionation are quite interesting. Analysis of the material in the top reservoir yielded 42% β_2- and β_3-globulin and 40% γ-globulins. The bottom reservoir contained 40% β_2- and β_3-globulin and only 5% γ-globulins. Apparently the γ-globulins, which had mobilities opposite in sign to the other mobile components, migrated into the upward convection current and were concentrated in the top reservoir. Considerable separation of β_2- and β_3-globulin was also achieved. The top reservoir contained 4% β_2- and 38% β_3-globulin, and the bottom reservoir 26% β_2- and 14% β_3-globulin.

Finally, the solution taken from the bottom reservoir in Fractionation V was diluted and reprocessed in Fractionation VI. Analysis showed that the resulting top fraction contained 72% β-globulins. The electrophoretic pattern of this material is shown in Figure 5c. Further purification of the β-globulins was not warranted for the purpose of this investigation.

Table 3

(a) Conditions for separation of β-globulin fractions.

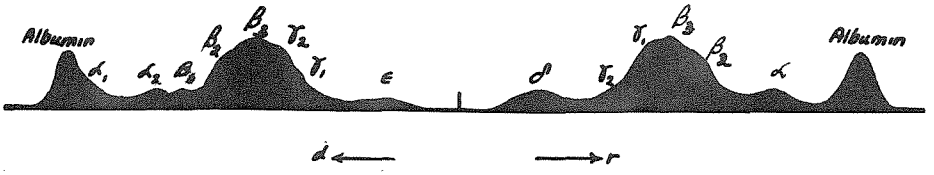
Fractionation	Stage	E Volts/cm	t hrs.	c_i g/100 ml.	f_t
IV	a	2.3	29	3.1	4.5
	b	3.5	31	2.9	6.8
	c	3.5	29	2.1	
	d	3.5	27		
V		2.3	30	2.8	
VI		2.3	30	1.3	3.4

(b) Distribution of electrophoretic components into fractions.

Fractionation	Stage	Fraction	Albumin	α_1	α_2	β_1	β_2	β_3	γ
			per cent of fraction						
Serum			43	3	13	3	14	8	16
IV	a	Top	15	8	9	4	28	24	12
		Bottom	61	3	17	2	9	5	4
	b	Top	20	7	13	2	34	20	4
		Bottom	65	3	16	4	8	3	1
	Composite c and d	Top	28	9	21	3	24	11	4
	d	Bottom	70	4	17	3	6[b]		
V		Top	6		12[a]		4	38	40
		Bottom	29	5	18	3	26	14	5
VI		Top	10		14[a]		62	10	4
		Bottom	39	5	22	1	24	4	5

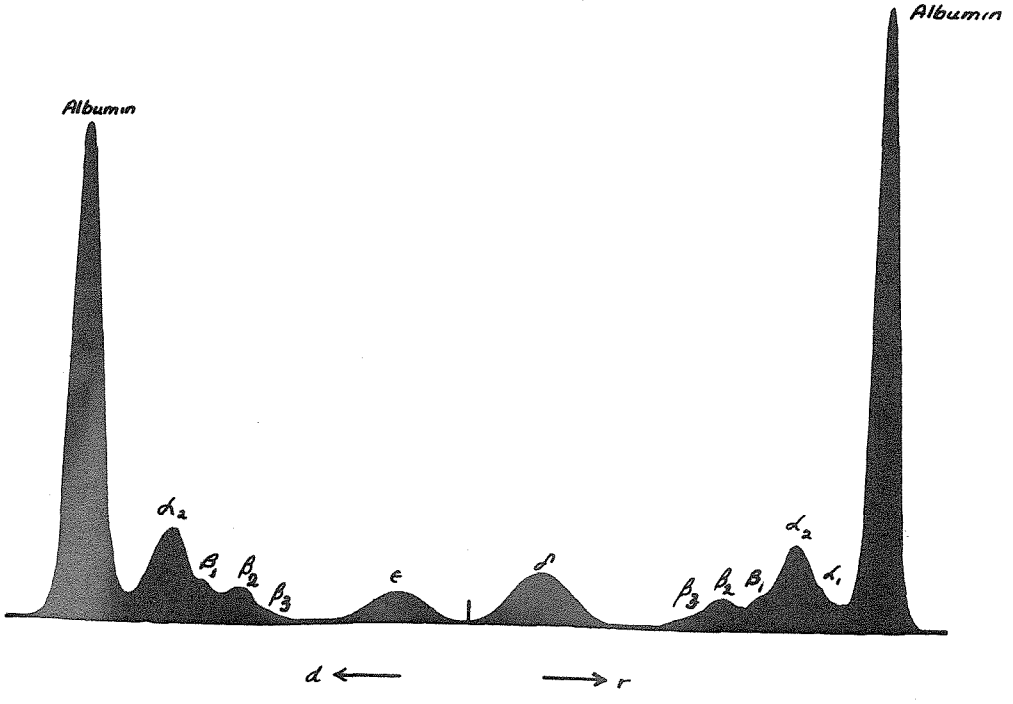
a Includes α_1 and β_1

b Per cent β_2 and β_3



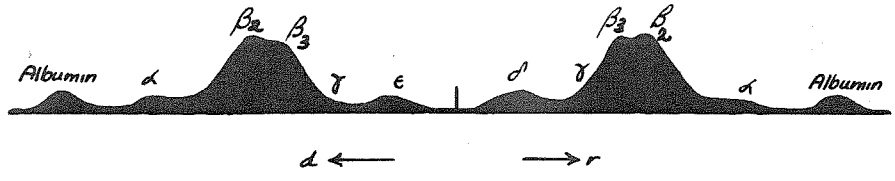
Bovine Serum
Top Cut Fractionation IV, Run a

Figure 5 a.



Bovine Serum
Bottom Cut Fractionation IV, Run d

Figure 5 b.



Bovine Serum
Top Cut Fractionation VI

Figure 5 c.

Discussion

A theoretical analysis of transport in the electrophoresis convection column has been worked out by Professor Kirkwood for the case in which the parameter $\frac{a\mu E}{D} < 1$. a is the wall separation in the channel of the apparatus. It allows the calculation of the time of exhaust, θ, of a protein of mobility u, diffusion constant D, and initial concentration C_0 from a top reservoir of volume V. In a solvent of density ρ_0 viscosity coefficient η_0 and at a field strength E, one obtains

$$\theta = \frac{10^{-4} K V D}{h b l \mu^2 E^2}$$

$$h = \left(\frac{2 \eta_0 D l}{\alpha \rho_0 C_0 g} \right)^{1/4} \qquad (2)$$

where b is the channel width, l the channel length, K is an apparatus constant of the order of magnitude of one hour, g is the acceleration of gravity, and α/ρ_0 is the density increment produced by one gram of protein per 100 ml. of solution. The fraction transported in time t is a function of t/θ.

From Eqn. (2), it is seen that in an isoelectric fractionation of specified duration, the top separation factor for the immobilized component should increase with increasing field strength and mobility of the mobile component and remain relatively insensitive to the initial protein concentration. Although Eqn. (2) is qualitatively confirmed by a number of the experimental fractionations reported here and may be used for preliminary estimates of suitable operating conditions, certain disturbing factors play a role which make it undesirable to work at very high field strengths or at very low protein concentrations.

There appear to be three principal disturbing influences. The first is osmotic transport of solvent into the channel from the external buffer solution. This effect is reduced by decreasing the field strength and increasing the ionic strength. The second is the establishment of a stationary state before complete exhaust of the mobile components from the top reservoir. This effect occurs only when some of the mobile components have mobilities of opposite sign. When operating conditions cannot be chosen to avoid mobilities of opposite sign, some sacrifice in separation efficiency must be accepted. The third disturbing effect is the destruction of laminar flow in the channel, which can be inhibited by increasing the viscosity of the solution or by decreasing the field strength and the channel wall separation.

In general, it is necessary to determine optimum operating conditions, minimizing the effect of the disturbing factors by pilot fractionations. However, except in unusual cases, separation factors of a satisfactory magnitude are attainable under conditions far removed from the optimum.

The high efficiencies of separation and the large quantities of material fractionated in a single run, without sacrifice of "purity" of the fractions, coupled with the ease of the manipulations and economy of time promise to make electrophoresis convection a valuable tool for the fractionation of naturally occurring protein mixtures. This method should supplement the ethanol fractionation of biological tissues and fluids as carried out by Cohn, et al. Thus, fine separations of the plasma fractions obtained by alcohol precipitation could be readily effected by electrophoresis convection.

References

1. W. B. Hardy, J Physiol 33, 251 (1905)

2. J. Mellanby, J Physiol 33, 338 (1905)

3a. A. Tiselius, Nova Acta Regia Soc Sci Upsaliensis IV, No. 4 (1930)

b. A. Tiselius, Trans Faraday Soc 33, 524 (1937)

4. H. Svensson, "Advances in Protein Chemistry" Vol IV, 251 (1948) Academic Press Inc, New York, N. Y.

5. J. T. Edsall, "Advances in Protein Chemistry" Vol III, 383 (1947) Academic Press Inc, New York, N. Y.

6a. E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell, and W. L. Hughes, J Am Chem Soc 62,3386 (1940)

b. T. L. McMeekin, J Am Chem Soc 62, 3393 (1940)

c. E. J. Cohn, J. A. Luetscher Jr, J. L. Oncley, S. H. Armstrong Jr, and B. D. Davis, J Am Chem Soc 62, 3396 (1940)

d. E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes Jr, and S. H. Armstrong Jr, J Clin Invest 23, 417 (1944)

e. J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron, and P. M. Gross Jr, J Am Chem Soc 71, 541 (1949)

7. J. G. Kirkwood, J Chem Phys 9, 878 (1941)

8. L. E. Nielsen and J. G. Kirkwood, J Am Chem Soc 68, 181 (1946)

9. M. Adolf and W. Pauli, Biochem Z, 152, 360 (1924)

10. P. Stamberger, J. Colloid Sci., 1, 93 (1946)

11. H. Gutfreund, Biochem J., 37, 186 (1943)

12. O. J. Plescia, Thesis, Cornell University, Ithica, New York, (1947)

13. R. C. Hawes, Ind and Eng Chem, Anal Ed., 11, 222 (1939)

14. K. R. Hogness, J. W. Giffee and V. L. Koenig, Arch Biochem., 10, 281 (1946)

15. E. L. Hess and H. F. Deutsch, J Am Chem Soc 70, 84 (1948)

16. L. G. Longsworth, Chem Rev., 30, 323 (1942)

17. L. G. Longsworth and D. A. MacInnes, J Am Chem Soc, 62, 705 (1940)

18. F. C. Koch and T. L. McMeekin, J Am Chem Soc, 46, 2066 (1924)

19. T. Svedberg and K. O. Pedersen, "The Ultracentrifuge", Oxford University Press, London. 1940, page 296

20a. L. G. Longsworth, J Phys and Colloid Chem. 51, 171 (1947)

b. S. H. Armstrong Jr., M. J. Budka, and K. C. Morrison, J Am Chem Soc, 69, 416 (1947)

Part II

The Fractionation of Bovine γ-globulin by Electrophoresis-Convection

Part II

The Fractionation of Bovine γ-globulin by Electrophoresis-Convection

It was suggested in Part I that electrophoresis-convection should supplement the ethanol fractionation of biological tissues and fluids as carried out by Cohn and coworkers (1). Thus sub-fractionation of the plasma fractions obtained by alcohol precipitation should be readily accomplished by electrophoresis-convection.

An example of such a sub-fractionation is the fractionation of bovine γ-globulin prepared by ethanol precipitation, Fraction II of bovine plasma, and is the subject of Part II of the thesis. γ-globulin was chosen because of its known heterogeneity and its immunological importance.

In the case of a protein, which migrates as a single boundary in an electric field but has a specified mobility distribution as revealed by reversible electrophoretic boundary spreading, fractionation is accomplished by means of a modification of the isoelectric procedure described in Part I. In the modified procedure, the pH of fractionation is chosen 0.2 to 1.0 pH unit removed from the average isoelectric point of the protein. Choice of the pH of operation is governed by the heterogeneity of the starting material and properties desired in the two resulting fractions. As a result of convective electrophoresis, there occurs a redistribution of the protein ions in the apparatus such that the fractions withdrawn from the top and bottom reservoir possess mobility distributions differing from that of the original protein. In runs of long duration it has been found that the mean isoelectric point of the top fraction is approximately the same as the pH of the buffer in which the fractionation has been

performed as predicted by the theory of stationary states in the electrophoresis-convection apparatus. A mathematical theory of transport in the electrophoresis-convection channel worked out by Professor Kirkwood for the case in which the parameter $\frac{a\mu E}{D} < 1$*, predicts that a stationary state will be reached in the apparatus in those runs in which there are both negative and positive mobilities. When the stationary state is reached, the mean isoelectric point of the top fraction will be equal to the pH of the buffer in the apparatus.

Using the modified isoelectric procedure γ-globulin has been separated into a number of fractions with different mean mobilities and isoelectric points.

Other workers have separated γ-globulin into sub-fractions by the use of the alcohol-salt precipitation method. Oncley and coworkers (2) have obtained three sub-fractions from Fraction II of human plasma with different isoionic points and immunological properties. Since it has not been possible to find electrophoretic data for the fractions, it is difficult to compare them with the fractions obtained by electrophoresis-convection. Smith (3) has separated "γ-globulin" and "T-component" from fraction II-III of bovine plasma. Deutsch and coworkers (4) have isolated two fractions of γ-globulin from human plasma and the plasma of a number of animals. Their fractions, which they name γ_1 and γ_2, correspond roughly to Smith's "T-component" and "γ-globulin", respectively, and to Fractions A and B, respectively, reported here.

---

* $\underline{a}$ is the wall separation in the apparatus, E the field strength, D the diffusion constant of the protein to be ffactionated, and u the mobility of the protein at the pH of operation.

Experimental

Material: Two separate lots of γ-globulin were obtained from Armour* and used in the fractionations. The first lot was used for fractionations 1 through 5 in Table I and had a mobility of -1.73×10^{-5} cm^2 volt^{-1} sec^{-1} at a concentration of 1% in barbital buffer pH 8.7 and ionic strength 0.1. Referring to Figure 2, it will be seen that in this buffer the γ-globulin migrated as a single peak during electrophoresis. A second lot had a mobility which was considerably lower, -1.51×10^{-5}.

Electrophoretic Analysis: The moving boundary techniques of Tiselius (5) as modified by Longsworth (6) were used in the electrophoretic analysis. Mobilities were determined by electrophoresis of a 1% protein solution in barbital buffer pH 8.7 and ionic strength 0.1 either at a field strength of 4 volts/cm for 4 hours or at a field strength of 8 volts/cm for 2 hours. Mobilities were calculated in accordance with the suggestions of Longsworth and MacInnes (7). With a few exceptions, mentioned later, mobilities were checked by running in opposite channels of the electrophoresis apparatus or a hydrostatic leak test was made before the start of the run. Because of the care used in obtaining the mobilities, it is believed that 0.1×10^{-5} is a significant mobility difference.

Boundary spreading experiments were carried out on 0.5% solutions of γ-globulin and its fractions equilibrated against cacodylate buffer (0.08N NaCl-0.02N Na cacodylate). These experiments were performed at the average isoelectric points of the proteins. The power consumption in boundary spreading experiments did not exceed 0.015 watts/cc. The refractive index gradient curves were recorded photographically on East-

---

* Armour Laboratories, Armour and Company, Chicago, Illinois, kindly supplied the bovine γ-globulin, Fraction II of bovine plasma.

man Kodak Co CTC plates using both the schlieren scanning technique of Longsworth and the cylindrical lens technique. In the cylindrical lens method a diagonal knife edge brought in from below the optical axis was used in the optical system. The standard deviations of the mobility distributions calculated by both methods agreed to within 5%. In all cases, the standard deviation of the mobility distribution, β, is tabulated for the proteins. For gaussian distributions of mobility, β reduces to the heterogeneity constant, h, of Alberty (8). (see Part III of thesis)

Results

The fractionation of γ-globulin was carried out in four stages. The first stage of fractionation consisted of a separation of the γ-globulin into two major components and was accomplished with 100-120 ml. of a 2-3% γ-globulin solution equilibrated against phosphate buffer pH 6.7 and ionic strength 0.1. This pH was several tenths of a pH unit removed from the average isoelectric point of the first lot of γ-globulin. Eight runs were made with the first lot of globulin in order to study the influence of field strength and duration of electrolysis upon the efficiency of fractionation*. Table I, Runs 1 through 5, presents the results of a representative series of fractionations, where

$\mathbb{E}$ = nominal field strength in volt/cm.

t = duration of run in hours.

C_i = initial concentration in grams protein/100 ml.

---

* Osmotic transport of solvent into the channel from the exterior buffer solution increased the volume of protein solution during these runs. The rate of influx of solvent was found to be an increasing function of the field strength. Thus, at field strength of 3.1-2.6 volts/cm the rate of influx was 0.9 ml/hour, at 1.6 volts/cm- 0.4 ml/hour, and at 1.0 volt/cm- negligible.

$\bar{u}$ = mobility (cm^2 $volt^{-1}$ sec^{-1}) at pH 8.7 in barbital buffer

I.P.= isoelectric point in cacodylate buffer
(0.08 N NaCl- 0.02 N Na cacodylate)

With the exception of Run 1 these initial separations all yielded the same top fraction, designated Fraction A. Fraction A had a mobility of -1.35×10^{-5} and an isoelectric point of 7.03, about 0.5 pH unit greater than the mean isoelectric point of γ-globulin of lot one. This fraction corresponds roughly to the bovine γ_2-globulin of Hess and Deutsch (4), reported to have a mobility of -1.25×10^{-5}. The electrophoretic pattern of Fraction A is presented along with that of the original globulin in Figure 2.

The top fraction resulting from Run 1 had a mobility of -1.52×10^{-5} which is significantly greater than that of Fraction A. The isoelectric point of this fraction was approximately the same as that of Fraction A. It appears that the optimum conditions for fractionation are 1.0 to 2.6 volts/cm at operating times 118 and 42 hours respectively. A number of routine fractionations of γ-globulin, employing a field strength of 1.6 volts/cm and an operating time of about 48 hours, have been carried out in this laboratory. The results obtained in these runs have shown that fractionations accomplished by electrophoresis-convection are very reproducible.

The bottom fractions, Fraction B, resulting from the first stage of fractionation had mobilities ranging from -1.98 to -2.12×10^{-5}. This fraction appears to correspond to the bovine γ_1-globulin of Hess and Deutsch (4), which has a mobility of -2.1×10^{-5}.

With one exception, material balances were obtained to within 5% for all the experiments with γ-globulin in which nitrogen analyses were made. Analyses were not made in all the routine runs. In one experiment

Table I

First Stage of the Fractionation of Bovine γ-globulin

a) Experimental conditions

Run	pH	E(volts/cm)	t (hours)	Initial Concentration g/100 ml.
1	6.7	3.1	24	3.0
2	6.7	2.6	47	3.2
3	6.7	1.6	42	2.4
4	6.7	1.6	77	2.4
5	6.7	1.6	118	2.8
6	6.7	1.6	52	

b) Properties of fractions

Run	Fraction	Yield(g protein)	$-10^5 x/\bar{x}$	I.P.	$10^5 x/\beta$
	1st lot γ-globulin		1.73	~6.5	0.67
	2nd lot γ-globulin		1.51	6.75	
1	Top		1.52		0.64
	Bottom		2.10		
2	Top	1.4	1.36		0.62
	Bottom	1.6	2.07		
3	Top	1.1	1.33		0.67
	Bottom	1.4	2.12		
4	Top	1.4	1.33	7.03	0.66
	Bottom	1.4	2.02		
5	Top	1.	1.38		
	Bottom	1	1.98		
6	Top		1.52		
	Bottom		1.49		

the temperature of the cold room rose well above 4°C due to refrigeration breakdown. Because of Joule heating some denaturization occurred in the channel resulting in a material balance of only 25%.

Before the further sub-fractionation of Fractions A and B could be carried out, it was necessary to make a series of routine fractionations for the purpose of accumulating quantities of Fractions A and B. The first lot of γ-globulin having been exhausted, a second lot was procured and used in some of the fractionations. Run 6 of Table I is a representative fractionation carried out with the second lot of γ-globulin in which 110 ml of a 3% solution equilibrated against phosphate buffer pH 6.7 and ionic strength 0.1 was used. It will be noted that the top and bottom cuts have mobilities which are almost identical with the starting material.

The run was made before the material had been characterized on the assumption that the first and second lot of γ-globulin were identical. Upon characterization, it was discovered that the average isoelectric po point of the second lot was the same as the operating pH, 6.7. For the case in which the average isoelectric point of the starting material is equal to the operating pH, theory predicts that a stationary state will be established almost immediately and that very little separation will be accomplished. This has been confirmed by experiment. It must be emphasized that a rather complete electrophoretic characterization of a material is a necessary prerequisite to successful fractionation by electrophoresis-convection.

The second and third stages of fractionation consisted of the further separation of Fraction B. Stage two was accomplished at a pH several tenths more basic than that of the average isoelectric point of

Fraction B. The pH of operation in Stage three was eight tenths more acid than that of the isoelectric point for Fraction B. Fraction A served as the starting material in Stage four. Figure 1 is a schematic representation of all the fractionations.

Table II summarizes the experimental conditions for the Fractionations of Stages two through four. Table III presents the results of the fractionations. The symbols used are the same as in Table I. $d\bar{\mu}/d\,pH$ is the slope of the mobility-pH curve calculated from data obtained in isoelectric point determination on the assumption that the curve may be approximated by a straight line in the neighborhood of the isoelectric point.

Electrophoretic patterns obtained in barbital buffer, pH 8.7, will be found in Figure 2 for all fractions of Stages one and two. Patterns of fractions from Stages three and four will be found in Figure 3. The straight line at the bottom of the pattern represents the initial boundary.

Fractionation in Stage two, Run 7, was carried out with a composite of Fractions B equilibrated against phosphate buffer, pH 6.7 and ionic strength 0.1. The resulting top fraction, Fraction C, had a mobility -1.63×10^{-5} and an isoelectric point, pH 6.47. Its properties are quite similar to the original starting material. The mobilities used for the isoelectric point determination were not checked. However, it is believed that the isoelectric point is correct to several hundredths of a pH unit, although the slope of the mobility-pH curve tabulated in Table III may be in considerable error. The bottom fraction, Fraction D, had a mobility -2.20×10^{-5}, and an isoelectric point, pH 6.01, about 0.5 pH unit lower than the average isoelectric point of γ-globulin from the first lot.

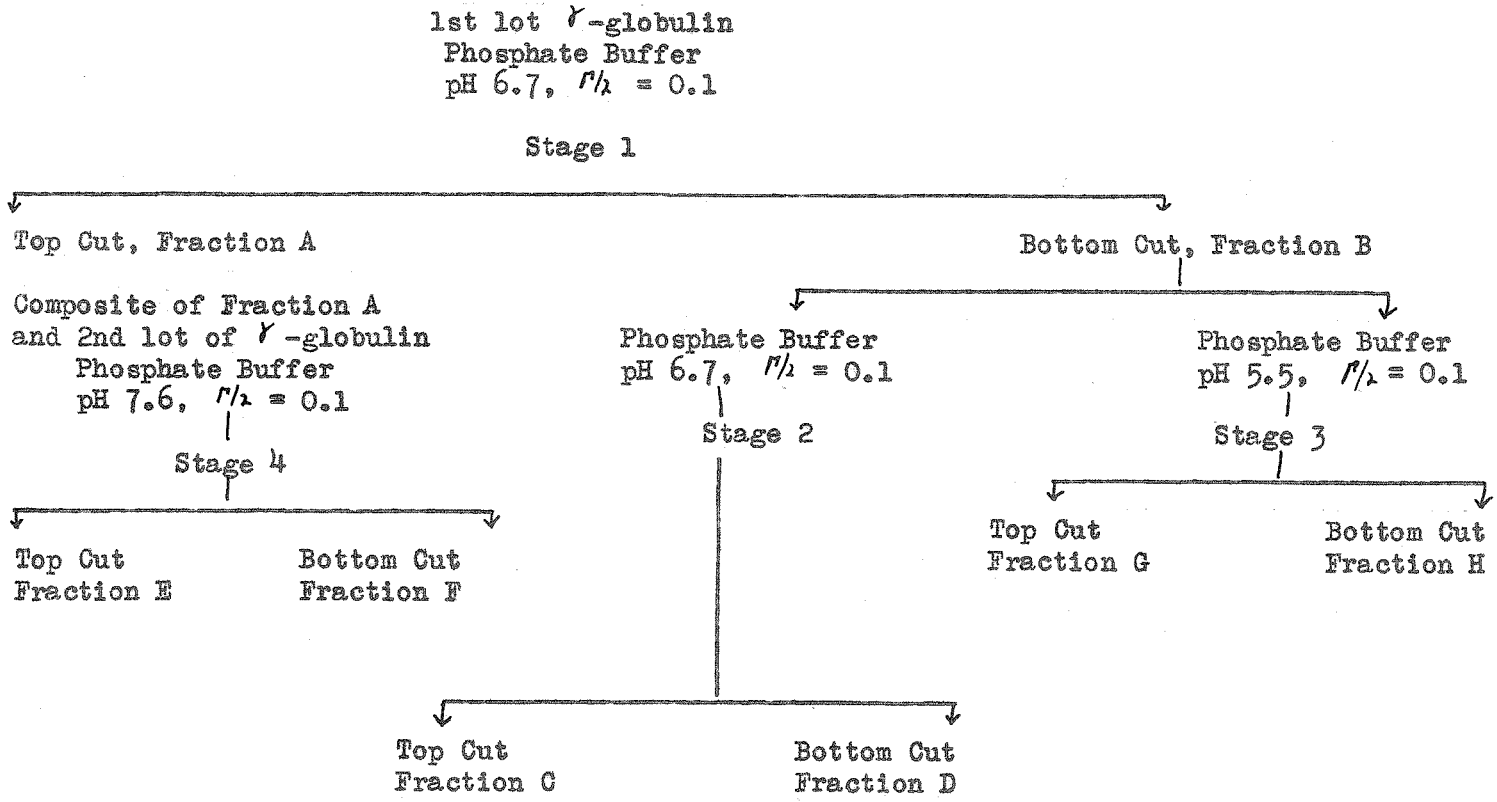


Figure 1, Schematic Fractionating Procedure

Table II

Experimental Conditions for Stages Two, Three, and Four

Run	pH	E (volts/cm)	t (hours)	Initial Concentration (g/100 ml.)
7	6.7	1.6	48	2
8	5.5	1.6	52	2.3
9	7.6	1.7	52	2.8
10	7.6	1.7	53	2.4

Table III

Run	Fraction	Yield (g/protein)	$-10^5 x_\alpha$	$-10^5 x \Delta\bar{\mu}/\Delta$pH	I.P.	$10^5 x_\beta$
	1st lot γ-globulin		1.73		6.5	0.67*
4	Top (A)	1.4	1.33	0.74	7.03	0.66
	Bottom (B)	1.4	2.02			
7	Top (C)	1.0	1.63	1.3	6.47	0.67
	Bottom (D)	1.7	2.20	0.78	6.01	0.65
8	Initial Material		1.91	0.76	6.29	0.75
	Top (G)	0.6	2.25	0.64	5.74	0.63*
	Bottom (H)	2.3	1.81	0.81	6.41	0.77*
9	Top (E)	1.1	1.24	0.35	7.31	
	Bottom (F)	2.0	1.78	0.89	6.51	0.59
10	Top (E)	1.2	1.25			0.55
	Bottom (F)	1.5	1.69			

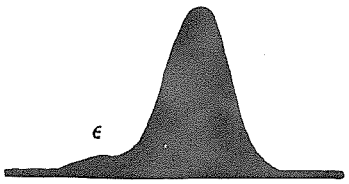
The first lot of γ-globulin having been exhausted, a similar material was prepared by making a composite of Fraction B and γ-globulin with mobility, -1.51×10^{-5}. Sufficient Fraction B for the third stage of fractionation was prepared by fractionating the material in phosphate buffer, pH 6.7 and ionic strength 0.1. The bottom fraction, Fraction B, had a mobility, -1.91×10^{-5}, and an isoelectric point, pH 6.29.

Run 8, the third stage of fractionation, was accomplished with 108 ml. of a 2.3% solution equilibrated against phosphate buffer, pH 5.5 and ionic strength 0.1. The mobility, -2.25×10^{-5}, of the resulting top fraction, Fraction G, was only slightly greater than that of Fraction D. However, its isoelectric point, pH 5.74, was three tenths of a pH unit lower than that of Fraction D. The bottom fraction, Fraction H, had a mobility of -1.81×10^{-5} and an isoelectric point, pH 6.41.

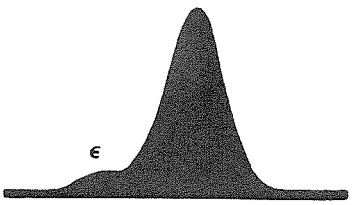
The initial material for Runs 9 and 10 consisted of a composite of Fraction A and γ-globulin with mobility -1.51×10^{-5}. Two separate runs were made using 110 ml. of a 2-3% solution equilibrated against phosphate buffer pH 7.6 and ionic strength 0.1. The resulting top fraction, Fraction E, had a mobility of -1.25×10^{-5}, and an isoelectric point, pH 7.31. Although its mobility was only 0.1×10^{-5} lower than that of Fraction A, there was a difference of 0.3 of a pH unit in their isoelectric points. Fraction F, the bottom fraction had a mobility and isoelectric point of -1.74×10^{-5} and pH 6.51 respectively.

Discussion

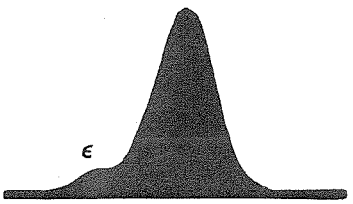
It is theoretically possible to obtain a complete representation of the mobility distribution of a heterogeneous protein in terms of the second and higher moments of the refractive-index gradient curves obtained in boundary spreading experiments. (see Part III) However, the



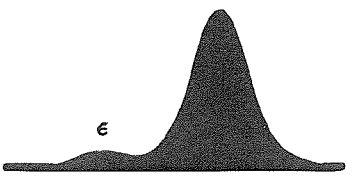
γ-globulin



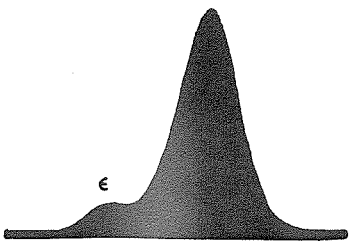
Fraction C



Fraction A



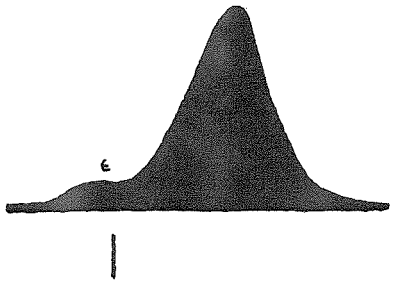
Fraction D



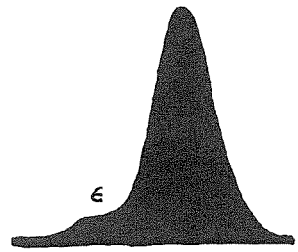
Fraction B

Electrophoretic Patterns pH 8.7

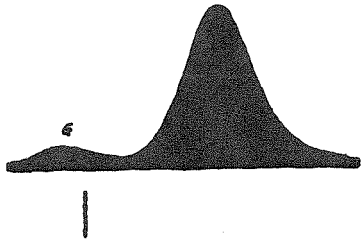
Figure 2.



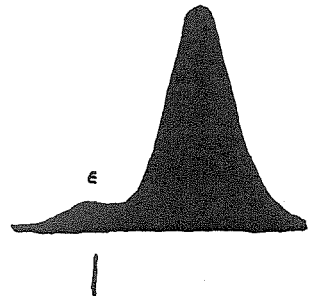
Initial Material
Stage 3



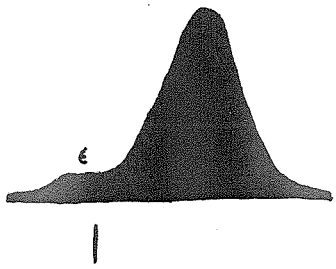
Fraction E



Fraction G



Fraction F



Fraction H

Electrophoretic Patterns pH 8.7

Figure 3.

precision and accuracy involved in the calculation of third and higher moments do not in general warrant inclusion of those terms defining departures from the gaussian in the expression for the mobility distribution. As a first approximation the mobility distribution may be adequately represented as a gaussian probability function whose standard deviation is taken as that of the actual mobility distribution. The standard deviations of the mobility distributions for all the γ-globulin fractions have been included in Table III. Although all the distributions have been approximated by a gaussian probability function, a distinction has been made between fractions with gaussian and non-gaussian mobility distributions. β in Table III for fractions of the latter type has been marked with an asterisk. A necessary condition for a gaussian mobility distribution is that the apparent diffusion constant, determined from the half-widths at the inflection point of boundary spreading refractive index gradient curves, plot as a straight line against time. This test has proven the most sensitive of several tests and has been used to distinguish between the two types of mobility distributions.

Theory predicts that fractionation of a heterogeneous protein with a gaussian mobility distribution will yield two fractions having gaussian mobility distributions with the same standard deviation as the original. This seems to be the case for those fractionations performed at a pH only several tenths of a pH unit removed from the average isoelectric point. Fractions A, C, and D, obtained by fractionating at a pH not far removed from the average isoelectric point, all have gaussian distributions with standard deviations approximately equal to that of the original material.

Results of those fractionations accomplished at a pH 0.6 to 0.8 of a pH unit removed from the average isoelectric point are not consistent with the theoretical predictions. In Run 8, fractionation of a material having a gaussian mobility distribution yielded two non-gaussian fractions. Although the top and bottom fractions of Runs 9 and 10 had gaussian mobility distributions, the standard deviation of the distribution for the top fraction was lower than that of the starting material.

Disagreement of the results of Runs 8, 9, and 10 with theory is probably due to two effects. One, fractions which are gaussian at the isoelectric point may not be thus at other pH. Although the starting material for Run 8 had a gaussian mobility distribution in the neighborhood of the average isoelectric point, it will be seen that the electrophoretic pattern, obtained in barbital buffer pH 8.7 and presented in Figure 3, was non-gaussian and skewed. Differences in the dependence of mobility upon pH for the components in the fraction undoubtedly are responsible for the skewing. With this in mind, it should be noted that $\Delta \bar{u}/\Delta$ pH observed for Fractions G and E are significantly lower than that for the other fractions. Second, the approximation of the mobility distribution by a gaussian probability function is probably a poor one for that part of the mobility distribution far removed from the average isoelectric point.

Experience gained in the fractionation of γ-globulin indicates that the most desireable method of fractionating a protein migrating as a single boundary in an electric field but possessing a specified mobility distribution is to operate at a pH about eight tenths of a pH unit removed from the average isoelectric point rather than only two or three tenths of a pH unit removed. There are three advantages to operating at a pH farther removed from the mean isoelectric point.

One, more homogeneous top fractions may be obtained. Two, there is less tendency to produce a number of similar fractions when repeated fractionation is employed. Three, there is more economy of material since the major portion of the material is concentrated in the more heterogeneous bottom fraction ready for the next fractionation. Of course, the material may be first separated into two major components by operating only slightly removed from the isoelectric point if desired.

γ-globulin may be considered to consist of only three fractions, A, C, and D. The mobility distribution of each fraction at pH 6.5 has been normalized to an area corresponding to its weight fraction of the γ-globulin and plotted in Figure 4. The solid curve in Figure 4 represents the sum of the three distributions. In Figure 5 this composite curve is compared* with the gaussian probability function which represents to a first approximation the mobility distribution of the first lot of γ-globulin. The mobility distribution of γ-globulin is normalized to unit area. It will be noted that the agreement between the two curves is good.

Since some of the fractions are not independent fractions but are linear combinations of other fractions, it is impossible to draw similar graphs for all the fractions. However, the mobility distributions for Fractions C, D, E, F, G, and H have been approximated by gaussian probability functions normalized to an area corresponding to their weight fraction of the γ-globulin and plotted in Figure 6. Obviously, the sum of the distributions will not be the same as that of the original.

* In comparing the distributions the assumptions that β is independent of pH and concentration in the range of interest and that the components all have the same dependence of mobility upon pH has been made.

In all the analytical work with γ-globulin it has been assumed that the material possessed a continuous rather than a discrete spectrum of mobilities. The only justification for this assumption is that it lends itself to mathematical simplification and yields results which are not inconsistent with experiment.

γ-globulin has been separated into eight fractions with mobilities ranging from -1.25×10^{-5} to -2.25×10^{-5} and isoelectric points extending from pH 7.31 to pH 5.74. Fractions C, F, and H all have isoelectric points and mobilities close to that of γ-globulin itself and it is difficult to distinguish one from the other on the basis of electrophoretic characterization. Nevertheless, they represent different center cuts isolated from γ-globulin. There are subtle differences among them. The original γ-globulin had a symmetrical non-gaussian mobility distribution. Fraction H had a non-symmetrical mobility distribution. Fractions C and F both possessed gaussian mobility distributions. Other methods of characterization should reveal greater differences among these fractions.

$\Delta\bar{u}/\Delta$pH for Fraction E and G is significantly lower than the values obtained for the other fractions. This difference in the dependence of mobility upon pH implies either a difference in chemical structure or a difference in the specific adsorption of buffer ions. Chemical characterization of the fractions and a study of their interaction with buffer salts would be desireable.

The results of the investigation presented in Part II, this thesis, illustrates the ease with which a heterogeneous protein can be fractionated by electrophoresis-convection. It is obvious that it is possible to obtain a very large number of fractions from heterogeneous proteins. The large quantities of material that can be fractionated and the reproducibility

of the fractionation promise to make electrophoresis-convection a valuable tool in the sub-fractionation of the plasma fractions obtained by ethanol precipitation.

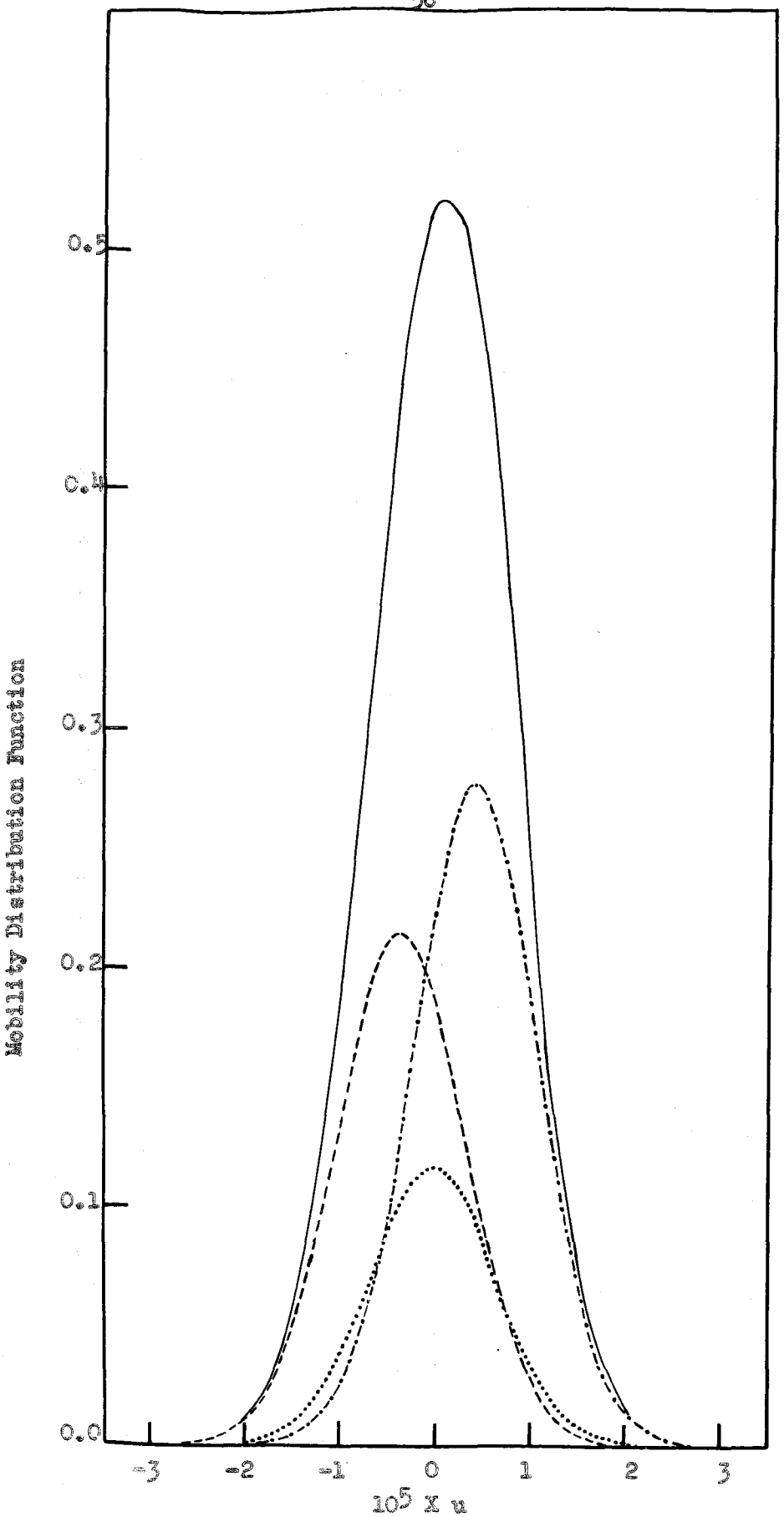

Mobility Distribution Function
0.5
0.4
0.3
0.2
0.1
0.0
-3
-2
-1
0
1
2
3
10^5 x u

Figure 4.

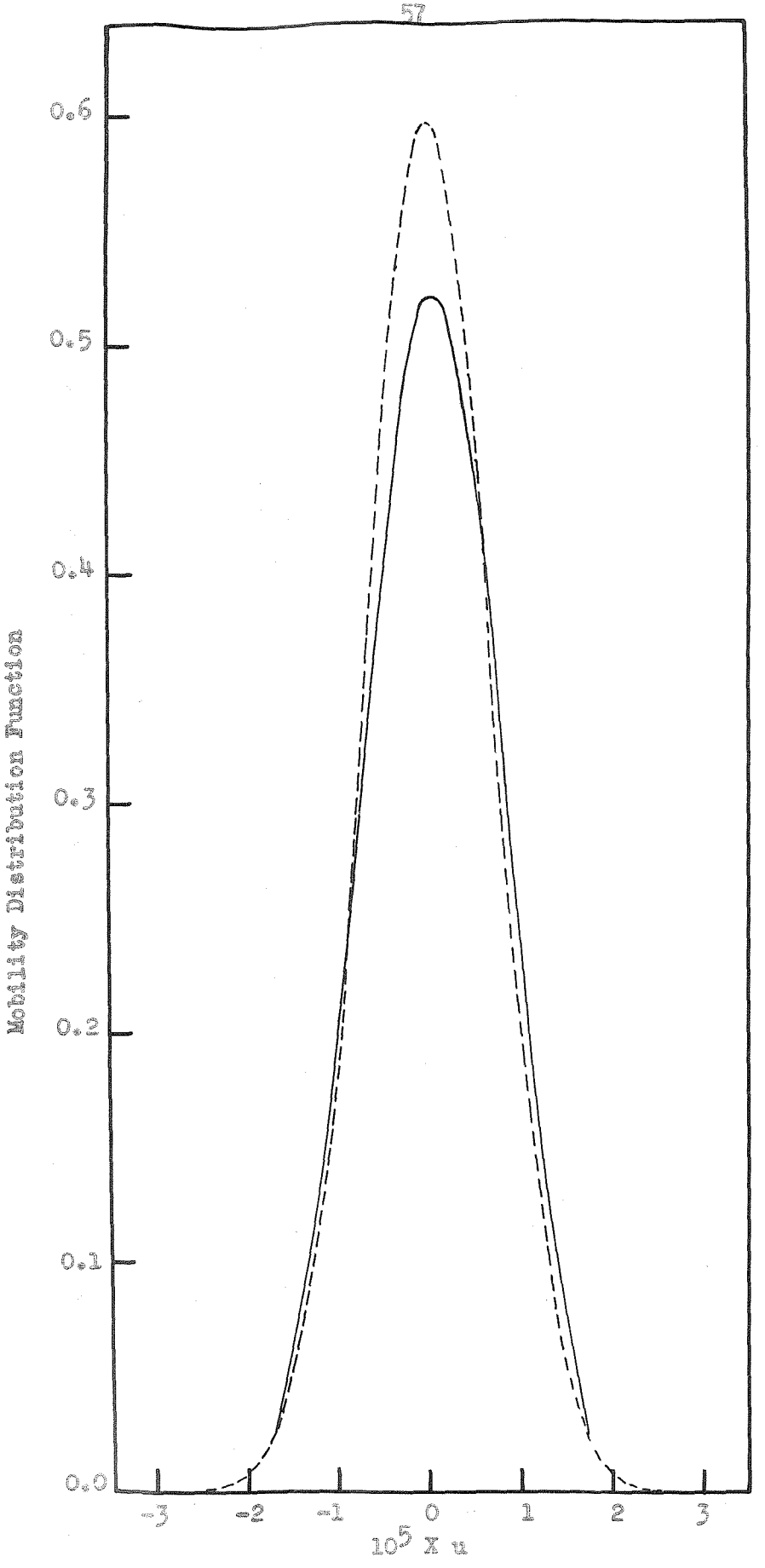

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Mobility Distribution Function
-3
-2
-1
0
1
2
3
10^5 x u

Figure 5.

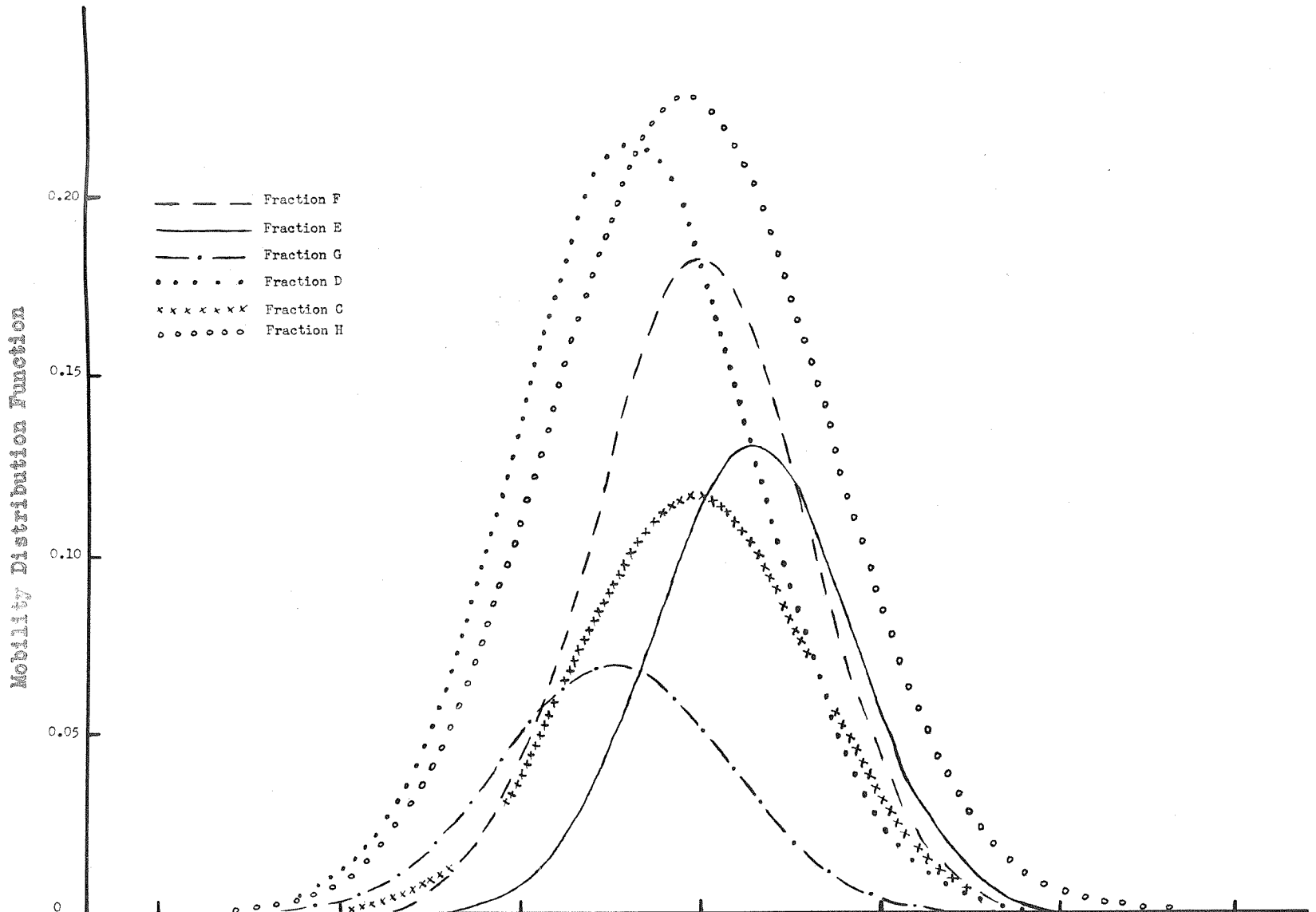

Fraction F
Fraction E
Fraction G
Fraction D
Fraction C
Fraction H
Mobility Distribution Function
0.20
0.15
0.10
0.05
0
-3
-2
-1
0
1
2
3
10^5 x u

Figure 6.

References

1. E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes Jr., and S. H. Armstrong Jr., J Clin Invest 23, 417 (1944)

2. J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron, and P. M. Goss Jr., J Am Chem Soc 71, 541 (1949)

3. E. L. Smith, J Biol Chem 164, 345 (1946)

4a. H. F. Deutsch, L. J. Gosting, R. A. Alberty, and J. W. Williams, J Biol Chem 164, 109 (1946)

b. H. F. Deutsch, R. A. Alberty, and L. J. Gosting, J Biol Chem 165, 21 (1946)

c. J. C. Nichol and H. F. Deutsch, J Am Chem Soc 70, 80 (1948)

d. E. L. Hess and H. F. Deutsch, J Am Chem Soc 70, 84 (1948)

5. A. Tiselius, Trans Faraday Soc., 33, 524 (1937)

6a. L. G. Longsworth, Chem Rev., 30, 323 (1942)

b. L. G. Longsworth, Ind and Eng Chem, Anal Ed., 18, 219 (1946)

7. L. G. Longsworth and D. A. MacInnes, J Am Chem Soc 62, 705 (1940)

8. R. A. Alberty, J Am Chem Soc, 70, 1675 (1948)

Part III

Extension of the Theory of Reversible Electrophoretic Boundary Spreading of Proteins

Part III

Extension of the Theory of Reversible Electrophoretic Boundary Spreading of Proteins

There are two criteria for the electrophoretic homogeneity of a protein. The first is that under the influence of an electric field the protein migrates as a single boundary in buffers of various hydrogen ion concentrations and ionic strengths. The second is that the rate of spreading of the protein boundary under conditions such that convection and anomalous electrical effects are avoided should be no greater than that due to diffusion alone. In the absence of spreading due to convection and conductivity or pH differences, boundary spreading indicates heterogeneity.

The boundary of an inhomogeneous protein is spread simultaneously by diffusion and by the differences in the mobilities of the differently charged ions. Sharp and coworkers (1) have shown that the mobility distribution may be obtained from the refractive-index gradient curves. In particular, they showed that, in the case in which spreading due to diffusion is negligible compared to electrical spreading, the standard deviation of the mobility distribution, H, may be calculated from the relation

$$H = \frac{1}{E} \cdot \frac{\Delta\sigma}{\Delta t} \qquad (1)$$

where $\Delta\sigma/\Delta t$ is the time rate of change of the standard deviation of the gradient and E the electric field strength. H is called a heterogeneity constant.

Recently Alberty and coworkers (2) have considered the case in which diffusion of the protein is not negligible and the mobility distribution may be represented by the gaussian probability function. Assuming that

the diffusion constant, D, is the same for all protein ions, they showed that in this case the refractive index gradient will have a gaussian form. A heterogeneity constant, h, may be calculated from the equation

$$D^* = \frac{\sigma^2 - \sigma_0^2}{2 t_E} = D + \frac{E^2 h^2 t_E}{2} \qquad (2)$$

where σ_0 is the standard deviation of the gradient curve at the moment the field is applied, and σ is the standard deviation after electrophoresis for t_E seconds. The heterogeneity constant, h, is actually the standard deviation of the mobility distribution q (u).

$$q(u) = \frac{1}{h\sqrt{2\pi}} e^{-u^2/2h^2} \qquad (3)$$

The theory of boundary spreading as developed by Alberty and co-workers is limited in that it is applicable only to those proteins possessing a gaussian distribution of mobilities. Furthermore, the theory yields the necessary condition that if the gradient curve is gaussian in form then the mobility distribution is gaussian, but fails to show that this is a sufficient condition. The purpose of this paper is to present a general theory of reversible boundary spreading applicable to both those proteins possessing gaussian mobility distributions and those possessing distributions which deviate from the gaussian.

In applying equation 2 to the interpretation of the results of spreading experiments Alberty and coworkers calculate the apparent diffusion constants from the half-widths at the inflection points of the gradient curves. However, this method of computation is valid only if the gradient curves are gaussian in form. If the curves are non-gaussian

D* calculated in this manner does not plot as a straight line vs. t_E. It is a consequence of the theory presented here that, no matter what form the gradient curves assume, the apparent diffusion constants calculated from the second moments of the gradient curves plot as a straight line vs. the time of electrophoresis. Furthermore, the standard deviation of the mobility distribution may be calculated from the slope of this line.

Theoretical

If the electrophoresis of a heterogeneous protein with a mobility distribution q(u) is carried out under conditions such that convection and anomalous electrical effects are avoided, the refractive index gradient, $\partial n/\partial x$, as a function of height in the electrophoresis cell, x, at time t_D after the formation of the boundary and time t_E after application of the electric field, is given by equation 4. This equation assumes that diffusion is simply superimposed on the electrophoretic migration. D is the diffusion constant which is assumed to be

$$\frac{\partial n}{\partial x} = \frac{(n_1 - n_2)}{2\sqrt{\pi D t_D}} \int_{-\infty}^{\infty} q(u)\, e^{-(x - E t_E u)^2/4 D t_D}\, du \qquad (4)$$

the same for all protein molecules. (n_1-n_2) is the difference in refractive index of the protein solution and the buffer. It is assumed that the refractive index increment is the same for all protein molecules. u is the electrophoretic mobility and E the field strength.

Equation 4 may be transformed into the general form

$$f(x) = a \int_{-\infty}^{\infty} q\left(\frac{u'}{E t_E}\right) h(x - u')\, du' \qquad (5)$$

the solution of which is given by

$$q(u) = 1/2\pi a \int_{-\infty}^{\infty} [F(s)/K(s)]\, e^{iu's} ds \qquad (6)$$

F(s) and K(s) are the Fourier transforms of f(x) and k(x), respectively; and u' is equal to uEt_E.

It is convenient to represent the experimental refractive-index gradient curve in terms of its moments about the centroidal axis by means of a Gram-Charlier series (3)

$$f(x) = \frac{h_1 - h_2}{\sigma\sqrt{2\pi}} e^{-x^2/2\sigma^2} \left[1 + \sum_{K=3}^{\infty} \frac{C_K}{K!} H_K\left(\frac{x}{\sigma}\right) \right] \qquad (7)$$

H_K is the k-th Hermite polynomial; and σ^2 is the second moment of the gradient curve, $\overline{x^2}$. The coefficients c_k are related to the higher moments of the gradient curves, e. g.,

$$C_3 = \overline{x^3}/\sigma^3, \quad C_4 = \overline{x^4}/\sigma^4 - 3$$

Insertion of the Fourier transform of equation 7 into equation 6 and integration yields the mobility distribution

$$g(u) = \frac{1}{\beta\sqrt{2\pi}} e^{-u^2/2\beta^2} \left[1 + \sum_{K=3}^{\infty} \frac{C_K}{K!} (-i)^K \alpha^K H_K\left(\frac{iu\sigma}{Et_E\beta^2\alpha}\right) \right]$$

$$\beta^2 = (\sigma^2 - 2Dt_D)/E^2t_E^2 = (\sigma^2 - \sigma_0^2 - 2Dt_E)/E^2t_E^2$$

$$\alpha = \left[1 - 2(\sigma/\beta E t_E)^2 \right]^{1/2} \qquad (8)$$

It will be noted that the coefficients C_k in the expression for q(u) are the same as those appearing in equation 7. σ_0 is the standard deviation of the gradient curves at the moment the electric field is applied.

From equation 8 one sees immediately that the necessary and sufficient condition for a gaussian distribution of mobilities is a gaussian

refractive-index gradient curve. In this case the standard deviation of the mobility distribution, β, is identical with the heterogeneity constant, h, of Alberty, and coworkers (2). Deviations from a gaussian distribution of mobilities are given by the third and higher moments of the gradient curve.

If spreading of the gradient curve due to diffusion is negligible compared to electrical spreading, β reduces to the heterogeneity constant, H, of Sharp and coworkers (1) for both gaussian and non-gaussian distributions. If diffusion is not negligible, H will decrease with time and approach β asymptotically according to equation 9.

$$H = \sqrt{\beta^2 + 2D/E^2 t_E} \tag{9}$$

For both gaussian and non-gaussian distributions of mobilities may be calculated from the equation

$$D^* = \frac{\sigma^2 - \sigma_0^2}{2t_E} = D + E^2\beta^2/2t_E \tag{10}$$

D* is the "apparent diffusion constant" calculated from the second moment of the experimental gradient curves during the electrophoresis. It is not permissible, of course, to use the half-widths at the inflection point to determine the apparent diffusion constant in the case of a non-gaussian mobility distribution. According to equation 10 a plot of the apparent diffusion constant vs. the time of electrophoresis should yield a straight line, which extrapolates back to the normal diffusion constant at zero time. The standard deviation of the mobility distribution may be calculated from the slope $E^2\beta^2/2$.

Experimental

The optical system used in this work was the cylindrical lens schlieren optical system described by Longsworth (4). A diagonal knife edge brought in from below the optical axis was used in the optical system. The refractive-index gradient curves were recorded photographically on Eastman Kodak Co. CTC plates. The photographs were enlarged and traced.

Boundary spreading experiments were carried out with 0.5% protein solution equilibrated against cacodylate buffer (0.08N NaCl - 0.02N Na cacodylate). These experiments were performed at the average isoelectric point of the protein. The power dissipation was less than 0.015 watts/cc.

The bovine γ-globulin (Fraction II of bovine plasma) used in this investigation was kindly supplied by Armour Laboratories, Armour and Company, Chicago, Illinois.

Results and Discussion

If the refractive-index gradient curves of a heterogeneous protein are gaussian in form, i. e., the protein possesses a gaussian mobility distribution, the standard deviation of the mobility distribution, β, is identical with the heterogeneity constant, h, of Alberty and coworkers (2). In this case the apparent diffusion constants may be calculated either from the second moments or the half-widths at the inflection point of the gradient curves. This is well illustrated by electrophoretic spreading experiments performed on Fraction A of Part II. Figure 1 shows that D* plots as a straight line vs. t_E as predicted by equation 10. The line extrapolates back to the diffusion constant observed upon reversal of the current at 240 min. for an equal period of time ($2.3 \times 10^{-7} cm^2 sec^{-1}$). The heterogeneity constant calculated from the slope of the plot is $0.62 \times 10^{-5} cm^2 volt^{-1} sec^{-1}$. It will be noted that the apparent diffusion

constants calculated from the second moments and half-widths at the inflection point of the gradient curves are in excellent agreement.

The interpretation of the results of electrophoretic spreading experiments performed with bovine γ-globulin (Armour Fraction II) affords an illustration of the application of the general theory of reversible boundary spreading to a heterogeneous protein possessing a non-gaussian mobility distribution. In this case it is not permissible to calculate the apparent diffusion constants from the half-widths at the inflection point of the gradient curves. Referring to Fig. 2, it will be noted that the plot of D* calculated from the half-widths at the inflection point vs. t_E is not linear but is concave to the abscissa. A similar result was reported by Anderson and Alberty (2) for the normal γ-globulin of horse plasma. However, the apparent diffusion constants calculated from the second moments of the gradient curves plot as a straight line vs. the time of electrophoresis as predicted by equation 10. The standard deviation of the mobility distribution, β , calculated from the slope of the line is 0.67×10^{-5} cm^2 $volt^{-1}$ sec^{-1}.

The heterogeneity constant, H, of Sharp and coworkers (1) calculated from the second moments of the gradient curves using equation 1 is plotted vs. t_E in Fig. 3. H decreases with time and approaches β asymptotically because diffusion is not negligible. In Fig. 3 the solid curve through the experimental points was calculated from equation 9 using $\beta = 0.67 \times 10^{-5}$ and $D = 2.2 \times 10^{-7}$.

A complete representation of the mobility distribution of the protein entails, of course, the determination of the higher moments of the gradient curves. An attempt was made to compute the third and fourth moments of the gradient curves. However, the precision and accuracy

involved in the computations did not warrant inclusion of those terms defining the departures from the gaussian in the expression for the mobility distribution, equation 8. Using refined experimental techniques, e. g., sharpening of the initial boundary, it might be feasible to determine the higher moments of the gradient curves, particularly if the curves depart markedly from the gaussian.

o Second Moment
x Half Width
D* x 10^7 cm^2 sec^-1
10
8
6
4
2
0
5
10
15
10^3 x T (seconds)

Figure 1.

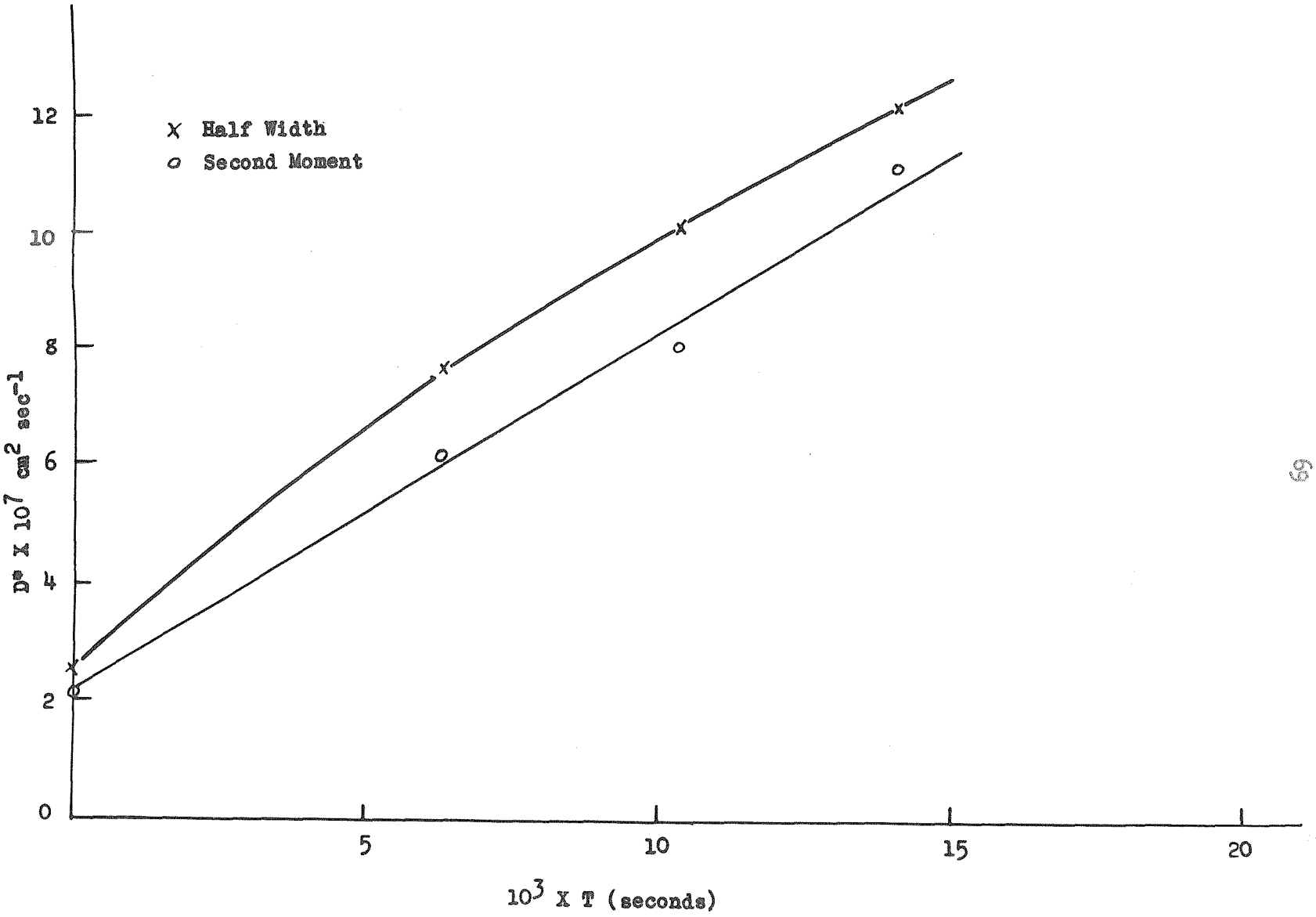

X Half Width
o Second Moment
12
10
8
6
4
2
0
D* x 10^7 cm^2 sec^-1
5
10
15
20
10^3 x T (seconds)

Figure 2.

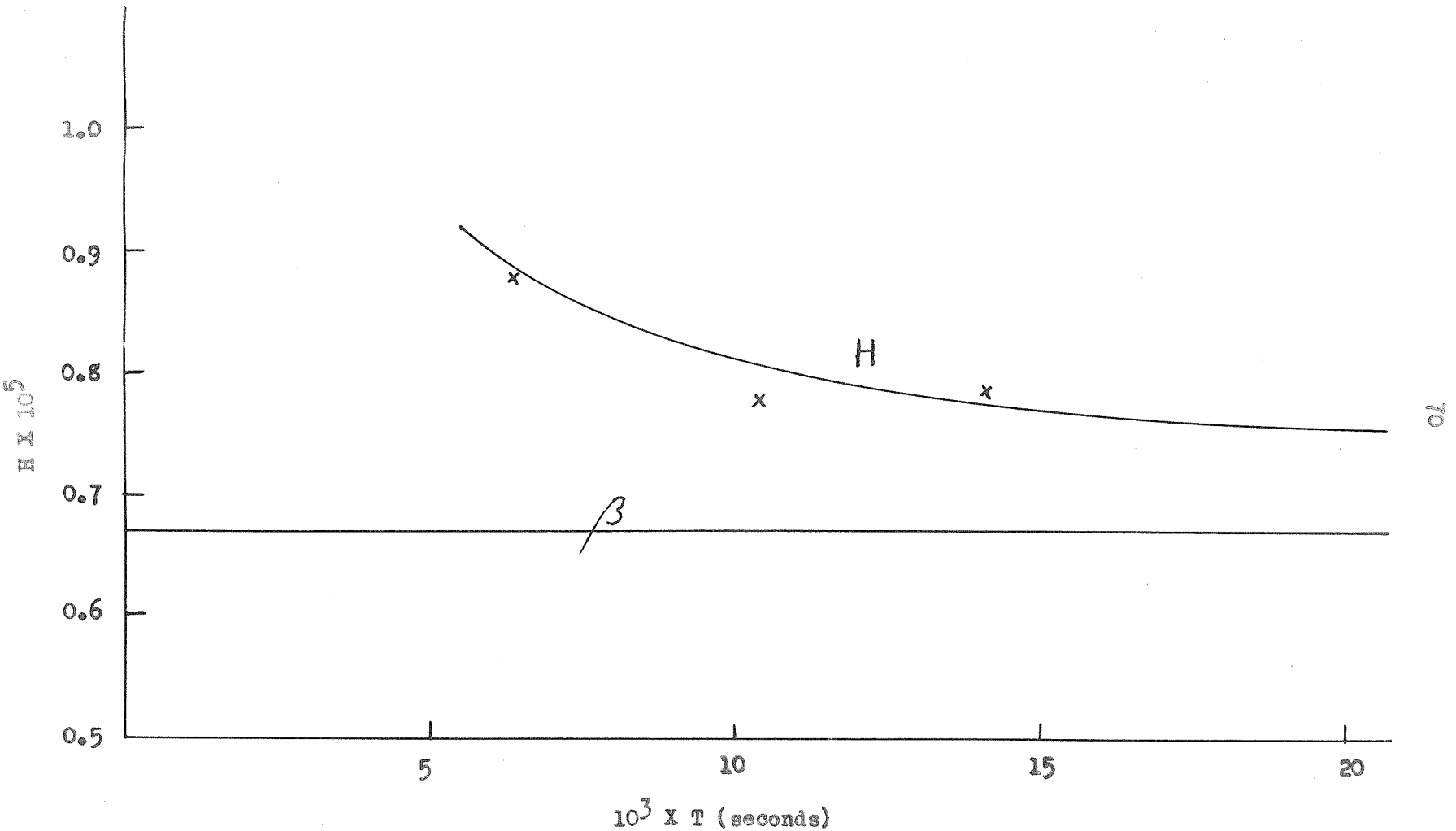


Figure 3.

References

1. D. G. Sharp, M. H. Hebb, A. R. Taylor, and J. W. Beard, J Biol Chem 142, 217 (1942)

2a. R. A. Alberty, E. A. Anderson, and J. W. Williams, J Phys Colloid Chem 52, 217 (1948)

b. R. A. Alberty, J Am Chem Soc 70, 1675 (1948)

c. E. A. Anderson and R. A. Alberty, J Phys Colloid Chem 52, 1345 (1948)

3. H. L. Rietz, "Mathematical Statistics" page 65, The Open Court Publishing Co., LaSalle, Illinois.

4. L. G. Longsworth, Ind. and Eng. Chem, Anal Ed, 18, 219 (1946)

Propositions

1. It should be possible to adapt the coulometric titration method of Myers and Swift (1) to the determination of iron. The procedure would be to first electrolytically reduce the iron to the ferrous state in the titration apparatus and then to quantitatively oxidize the iron with electrolytically generated bromine. An isolated anode would be necessary in the first step and an isolated cathode in the second. 100% current efficiency would not be expected in the first step.

2. Considerable interest has been shown in the possibility of performing electrophoresis experiments in gels because of the short duration of experiments and relatively simple apparatus needed. Electrophoresis in gels also offers the possibility of very high resolution of the components in a heterogeneous protein since diffusion in the short experiment would be negligible. The technique should be used to investigate the mobility distributions of γ-globulin and its fractions.

3. The rate of radioactive exchange between tripositive and quinquepositive antimony in hydrochloric acid solutions should be a function of acid concentration. Furthermore, if the quinquepositive antimony is prepared by oxidation of tripositive antimony in hydrochloric acid, the rate of exchange will be a function of the "age" of the quinquepositive antimony.

4. The titration of reagin developed by Miller and Campbell (2) should be confirmed by the isolation of reagin from the precipitate and confirmation of its presence by clinical tests. Isolation of ovalbumin reagin from the precipitate of ovalbumin, anti-ovalbumin, and reagin may be accomplished in two steps. The precipitate is first solubilized and the ovalbumin removed (3). The antibody may be separated from reagin by

electrophoretic techniques.

5 a) A standardized nomenclature for the electrophoretic components of sera should be adopted. This could be done at some future conference on electrophoresis.

b) The assignment of names such as γ_1 and γ_2 to fractions isolated from serum is to be discouraged. In general, the fractions are not unique or homogeneous. Their properties depend upon the conditions of fractionation. Fractions are best identified by a statement of their physical and chemical characteristics.

6. Emil Smith has stated (chemistry seminar) that in hyperimmune animals the antibody activity spreads out into components other than γ-globulin. Observation of antibody activity in the β and α-globulin fractions may be due to the difficulty of separating γ-globulin from the other fractions in hyperimmune sera. It is suggested that immunologically purified antibody (3) be obtained from immune and hyperimmune animals and the heterogeneity of the preparations be compared by means of boundary spreading experiments.

7. Dielectric studies participated in by the author at U. S. Rubber Co. indicated that polar molecules may be completely dissociated when dissolved in vulcanized rubber. It may be possible to determine whether such molecules are dissociated by studying the infra-red spectra of a substance, known to associate through hydrogen bonding in the pure liquid, when it is dissolved in rubber.

8. Separation of a second lipo-protein phase is probably responsible for the "β-anomaly" observed in the electrophoresis of human and other sera. An attempt should be made to correlate the occurrence in different species of the "β-anomaly" with the amount of lipo-protein in the serum.

9. Performance of electrophoresis experiments in alkaline buffers seems to have become an established tradition. More work should be done in acid buffers. In some cases better resolution of components is known to be possible in acid buffers.

10. The bookstore should be required to keep a stock of the more standard texts on hand. A retail outlet is supposed to be something more than a place to receive orders.

References

1. R. J. Myers and E. H. Swift, J Am Chem Soc 70, 1047 (1948)

2. H. Miller and D. H. Campbell, Ann Allergy 5, 236 (1947)

3. E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry" Charles C. Thomas, Publisher, Springfield, Illinois.