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The Fractionation of Bovine Serum Proteins by Electrophoresis-Convection

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Introduction

An electrophoretic adaptation of the principles of the Clusius column for the fractionation of proteins in solution has been described and tested by Nielsen and Kirkwood.[1,2] Fractionation occurs in a narrow vertical convection channel between two semi-permeable membranes, connecting an upper and a lower reservoir. Separation depends upon the superposition of differential horizontal electrophoretic transport of the components on vertical convective transport of the solution as a whole. The vertical convective transport is controlled by the horizontal density gradient produced by the electrophoretic migration of the proteins across the channel.

An electrophoresis-convection apparatus of improved design has recently been described by Cann, Kirkwood, Brown and Plescia[3] and successfully used to fractionate diphtheria antitoxin pseudoglobulin. The new apparatus has the advantages of being more easily assembled and of yielding results of greater reproducibility than the original apparatus of Nielsen and Kirkwood. With the aid of the new cell and operating procedures presently to be described, the method of electrophoresis-convection promises to be an important technique for protein fractionation, both on a large and small scale.

If the isoelectric points of the components of a protein mixture are sufficiently different, the most effective fractionation procedure consists in successively immobilizing the components at their respective isoelectric points and withdrawal of the mobile components from the top reservoir of the apparatus by electrophoresis-convection. Under representative operating conditions the mobile components can be almost completely removed from 50 ml. of a 4% protein solution in times of the order of ten to thirty hours. Complete exhaust of the mobile components from the top reservoir, although closely approached under ideal conditions, is sometimes inhibited by various disturbing factors. The most important disturbing factors appear to be osmotic transport of solvent from the exterior buffer solution into the cell and the establishment of a stationary state before exhaust when the mobilities of some of the components are of opposite sign at the operating *p*H. Optimum operating conditions must therefore 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 the bovine serum proteins by the isoelectric procedure, reported in the present article.

Experimental

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 7 electrophoretic components as illustrated in Fig. 2a. The nomenclature adopted in labeling the electrophoretically separable components is also given in Fig. 2a. This nomenclature appears to be fairly consistent with that used in the literature.[4,5] The electrophoretic patterns of bovine serum reveal the presence of a component of mobility 2.4–2.6 $\times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. Since it is perhaps not justifiable to resolve this component, its contribution to the electrophoretic distribution has been included with that of the β_2-globulin. However, in the case of the top and bottom fractions of fractionations V and VI, it was possible to resolve this component, designated as β_3-globulin, by the method of Pedersen. The mobilities and relative concentrations of the electrophoretic components of the serum are presented in Table I. These data were obtained in barbital buffer, *p*H 8.7 and ionic strength 0.1.

TABLE I

THE MOBILITIES ($-\mu \times 10^5$) AND RELATIVE CONCENTRATIONS OF THE COMPONENTS OF BOVINE SERUM

	Albumin	α_1	α_2	β_1	β_2	γ_1	γ_2
Mobility	6.52	5.76	4.62	3.86	3.08	1.97	1.28
Relative concn.	43	3	13	3	23	8	7

Electrophoretic Analysis.—The moving boundary technique of Tiselius[6] as modified by Longsworth[7] was used in the electrophoretic analysis. Electrolysis of the protein solution in barbital buffer, *p*H 8.7 and ionic strength 0.1, was allowed to proceed for four 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.[8]

For the purpose of determining the total protein concentration of a solution an arbitary factor relating 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.[9] Electro-

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

(2) L. E. Nielsen and J. G. Kirkwood, THIS JOURNAL, **68**, 181 (1941).

(3) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. Plescia, *ibid.*, **71**, 1630 (1949).

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

(5) E. L. Hess and H. F. Deutsch, THIS JOURNAL, **70**, 84 (1948).

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

(7) L. G. Longsworh, *Chem. Rev.*, **30**, 323 (1942).

(8) L. G. Longsworth and D. A. MacInnes, THIS JOURNAL, **62**, 705 (1940).

(9) F. C. Koch and T. L. McMeekin, *ibid.*, **46**, 2066 (1924).

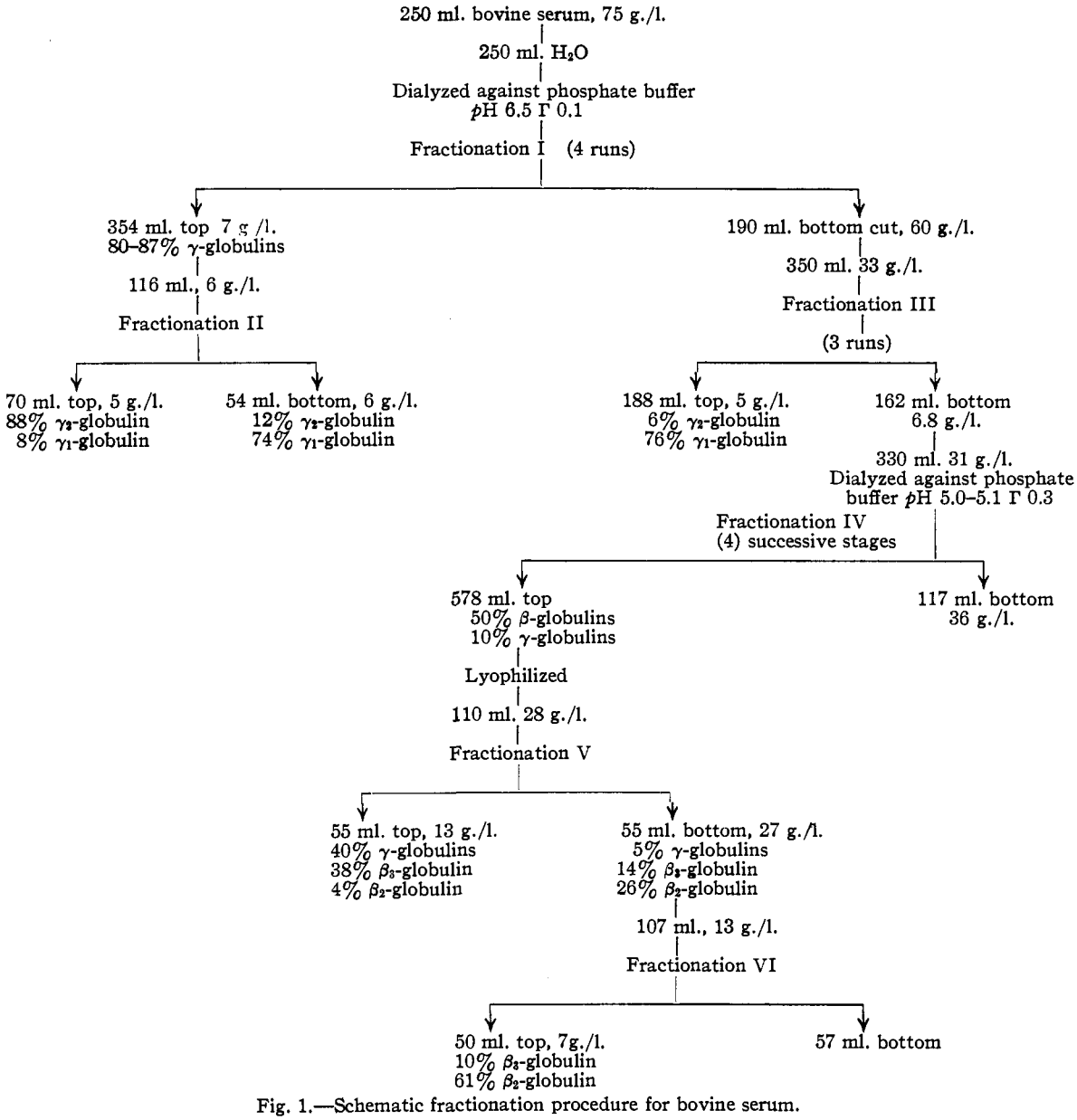

Fig. 1.—Schematic fractionation procedure for bovine serum.

phoretic analysis of a protein solution could be effected to about ±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 Pedersen.[10] The conditions of electrophoresis were such that departures of the apparent distributions from the true distributions of electrophoretic components were negligible compared to the changes in distribution effected by fractionation.[11]

Fractionation.—The details of construction and operation of the electrophoresis-convection apparatus employed in this investigation are described in the accompanying paper.[3] The fractionation cell consists of a narrow vertical channel connecting upper and lower reservoirs. The channel is formed by the space between two sheets of semipermeable membrane. The cell containing the protein solution to be fractionated was immersed in buffer solution and a homogeneous electric field applied across the channel by means of external platinum electrodes. The nominal field strength was calculated from measurements of the conductivity of the solutions and the average current density. Electrolysis products were prevented from reaching the membranes by circulation of the external buffer solution. Fractionations were carried out in a constant temperature cold room operating at 4°.

Samples withdrawn from the upper and lower reservoirs at the conclusion of the run were dialyzed against distilled water to remove buffer salts. The concentrations of the solutions were adjusted by lyophilization and dilu-

(10) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940, p. 296.

(11) (a) L. G. Longsworth, *J. Phys. and Colloid Chem.*, **51**, 171 (1947); (b) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947).

tion. The solutions were then dialyzed against barbital buffer, *p*H 8.7 and ionic strength 0.1, for seventy-two hours with three changes of buffer and analyzed electrophoretically.

The serum and its fractions were prepared for fractionation by dialyzing against distilled water to remove salts and other dialyzable materials. After adjusting the concentrations, the solutions were brought to the desired *p*H by dialysis against buffer for seventy-two hours with three changes of buffer.

Experimental Results

Separation of the γ-globulin fractions from the serum was carried out in phosphate buffer, *p*H 6.5 and ionic strength 0.1. The isoelectric points of the γ-globulins appeared to be close to this *p*H. The other components of the serum migrated toward the anode on electrophoresis at *p*H 6.5. To effect separation of the β-globulins, runs were carried out at *p*H 5.0–5.1 and ionic strength of 0.3. The scheme used in the fractionation is shown diagrammatically in Fig. 1. The electrophoretic patterns of Figs. 2, 3, and 4 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.

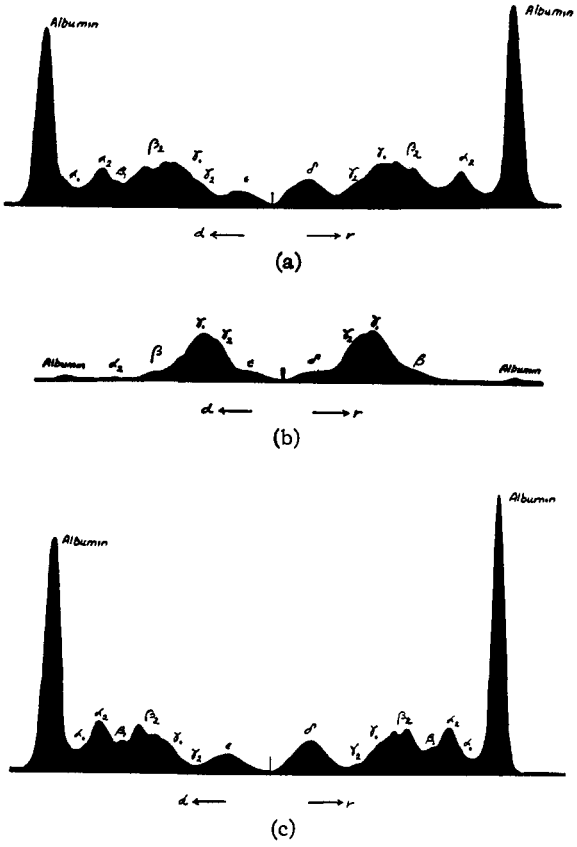

Fig. 2.—Electrophoretic patterns of bovine serum fractions: (a) original bovine serum; (b) top cut, fractionation I, run 1; (c) bottom cut, fractionation III.

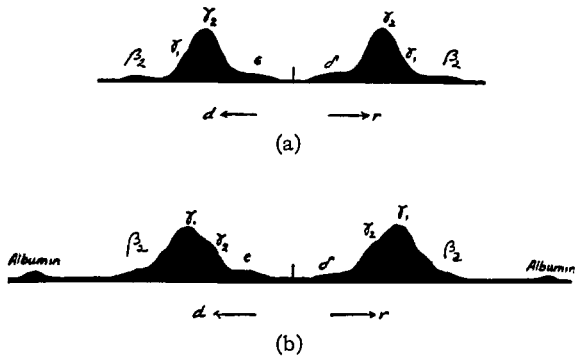

Fig. 3.—Electrophoretic patterns of bovine serum fractions: (a) top cut, fractionation II; (b) bottom cut fractionation II.

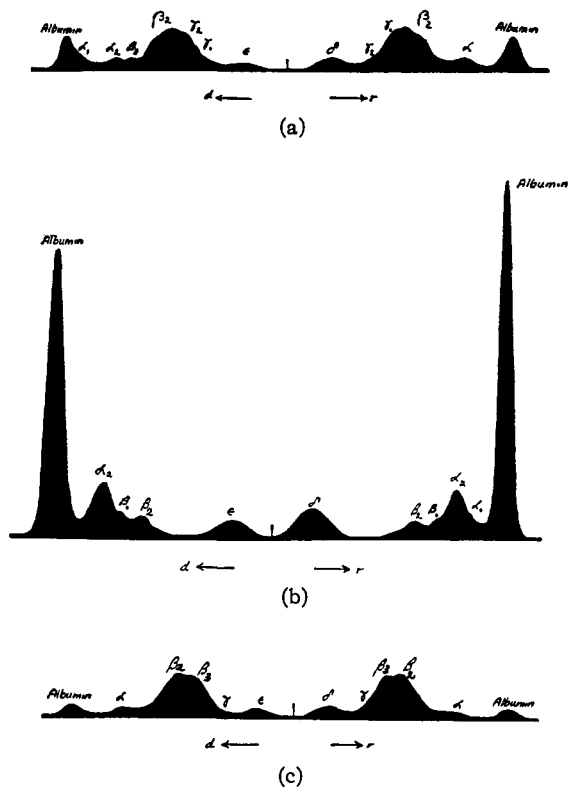

Fig. 4.—Electrophoretic patterns of bovine serum fractions: (a) top cut, fractionation IV, run a; (b) bottom cut, fractionation IV, run d; (c) top cut, fractionation VI.

β-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 frac-

tionated upon the efficiency of separation.[12] In all cases the initial concentration of the protein solution was 3.7 g. protein/100 ml. of solution. The resultant data are presented in Table II, 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.

$$f_t = \frac{x_i}{x_i^0}\frac{1 - X_i^0}{1 - X_i} \qquad x_i = \frac{c_i}{\sum_k c_k} \qquad (1)$$

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

TABLE II

(a) CONDITIONS FOR SEPARATION OF γ-GLOBULIN FRACTIONS

Fractionation	Run	E, v/cm.	t, hr.	v_i, ml.	c_i, g./100 ml.	v_0, ml.	f_t
I	1	3.1	14	110	3.7	7	23
	2	6.1	14	115	3.7	45	24
	3	3.1	24.5	117	3.7	12	35
	4	3.1	27	155	3.7	9	35
II		3.1	24	116	0.6	8	5.1
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	γ_1	γ	γ_1
			% of fraction							
Serum			43	3	13	3	23	8		7
I	1	Top	1		3		16	67		13
	2	Top	1		1	1	16		81	
	3	Top	1				13		86	
	4	Top	1				13	63		23
I	Composite 1, 2, 3, 4	Bottom	49	2	15	3	22	6		3
II		Top					4	8		88
		Bottom	4				10	74		12
III	Composite of 3 runs	Top	6	2	2	3	5	76		6
		Bottom	53	3	16	2	20	4		2

(12) 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 membranes into the channel counteracts the downward convection current and re-inforces the upward convection current, thereby decreasing the efficiency of separation.

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[5] 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 was of composition 85% γ-globulins and 15% β-globulins. Figures 2a and 2b represent the electrophoretic patterns of the original serum and a representative crude γ-globulin fraction, respectively. Partial separation of γ_1- 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 Fig. 3. 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 II, 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[5] 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 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 latter run. The influence of 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 frac-

tionation III to remove γ-globulins. The data are presented in Table II. 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, Fig. 2c. 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 *p*H 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 III. The efficiency of separation of the β-globulin fraction is expressed in terms of the top separation factor, f_t, defined by a relation analogous to eqn. 1.

At *p*H 5.0–5.1 the β-globulins 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 are 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 *p*H 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 to 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 Fig. 4a. The "purest" of these crude fractions had the composition 20% albumin, 20% α-globulins, 1% β_1-, 55% β_2-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-globulin, Fig. 4b. Thus, four successive stages of fractionation effected a 73% reduction of the relative concentration of β_2-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.
Total protein in composite of top fractions from stages c and d	0.8 g.
Total protein in lower reservoir of stage d	4.2 g.
Total 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 effected. 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

TABLE III

(a) CONDITIONS FOR SEPARATION OF β-GLOBULIN FRACTIONS

Fractionation	Stage	E, v./cm.	t, hr.	c_i, g./100 ml.	f_t
IV	a	2.3	29	3.1	4.4
	b	3.5	31	2.9	7.3
	c	3.5	29	2.1	
	d	3.5	27		
V		2.3	30	2.8	
VI		2.3	30	1.3	3.7

(b) DISTRIBUTION OF ELECTROPHORETIC COMPONENTS INTO FRACTIONS

Fractionation	Stage	Fraction	Albumin	α_1	α_2	β_1	β_2	β_3	γ
			% of fraction						
Serum			43	3	13	3	23		15
IV	a	Top	16	8	9	3	52		12
		Bottom	61	3	17	1	14		4
	b	Top	20	7	13	1	55		4
		Bottom	65	3	17	4	10		1
	Composite c and d	Top	28	9	20	4	35		4
	d	Bottom	70	4	17	3	6		
V		Top	6		12[a]		4	38	40
		Bottom	29	5	18	3	26	14	5
VI		Top	10		14[a]		61	10	5
		Bottom	39	5	22	1	24	4	5

[a] Includes α_1 and β_1.

that the resulting top fraction contained 71% β-globulins. The electrophoretic pattern of this material is shown in Fig. 4c. Further purification of the β-globulins was not warranted for the purpose of this investigation.

Discussion

A theoretical analysis of transport in the electrophoresis-convection column, to be published elsewhere, allows the calculation of the time of exhaust, θ, of a protein of mobility μ, 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

$$\theta = 10^{-4}KVD/hbl\mu^2E^2$$
$$h = (2\eta_0 Dl/\alpha\rho_0 g C_0)^{1/4} \qquad (2)$$

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 $\alpha\rho_0$ 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 makes 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. Although a thorough investigation has not been made, it is surmised that decreased fractionation efficiency observed at low protein concentrations with correspondingly low viscosities, may be due to this effect.

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.*[13] Thus, fine separations of the plasma fractions obtained by alcohol precipitation could be readily effected by electrophoresis-convection. As an example, the results of a preliminary experiment with bovine γ-globulin obtained by ethanol fractionation indicate that separation into γ_1- and γ_2-globulin can be effected with considerable ease. Thus, about 1.4 g. of pure γ_2-globulin was obtained in a single forty-seven hour run at a field strength of 2.6 volts/cm. and a protein concentration of 3.2 g./100 ml.

Hess and Deutsch[5] report that bovine γ_1- and γ_2-globulins exhibit reversible boundary spreading on electrophoresis, indicating that these proteins are inhomogenous. Alberty, Anderson and Williams[14] have shown that in the case of human γ_2-globulin the mobility distribution may be represented by the Gaussian probability function, leading to a Gaussian distribution of isoelectric points. By taking advantage of the small differences in isoelectric points, it should be possible to separate γ_1- and γ_2-globulin into sub-fractions by electrophoresis-convection. Such a program is now in progress in this Laboratory.

Summary

The partial fractionation of bovine serum proteins has been effected by electrophoresis-convection. γ-Globulins with a purity of 96% and β-globulins with a purity of 71% have been separated from the serum. Furthermore, considerable separation of γ_1- and γ_2-globulin has been obtained. This investigation demonstrates the applicability of electrophoresis-convection as a tool in the fractionation of naturally occurring inhomogeneous proteins.

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