APPLICATION OF ELECTROPHORESIS-CONVECTION TO THE FRACTIONATION OF BOVINE γ-GLOBULIN

By JOHN R. CANN, RAYMOND A. BROWN, AND JOHN G. KIRKWOOD

(From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena)*

(Received for publication, May 14, 1949)

A method of fractionation of proteins in solution in which an electrophoretic adaptation of the principles of the Clusius column is utilized was suggested by Kirkwood in 1941 (1) and tested experimentally by Nielsen and Kirkwood (2) several years later. Recently an electrophoresis-convection apparatus of improved design has been described and successfully used in the fractionation of the pseudoglobulin of horse diphtheria antitoxin (3) and bovine serum proteins (4).

Fractionation occurs in a narrow vertical channel between two semipermeable membranes, connecting an upper and 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. The result of the superposition of horizontal electrophoretic transport and vertical convective transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to the slow components and the bottom reservoir with respect to the fast components.

The separation of a protein mixture possessing discrete mobility and isoelectric point spectra, *e.g.* serum, into its constituent proteins is accomplished by successive immobilization of the components at their respective isoelectric points and transport of the mobile components from the top reservoir of the apparatus.

In the case of a protein which migrates as a single boundary in an electric field but possesses a specified mobility distribution as revealed by reversible electrophoretic boundary spreading, *e.g.* γ-globulin, fractionation is accomplished by means of a modified isoelectric procedure in which the fractionation is carried out at a pH displaced by an arbitrary amount from the mean isoelectric point of the heterogeneous protein. Transport in the apparatus leads to a redistribution of the protein ions, such that the fractions withdrawn from the top and bottom reservoirs possess mobility dis-

* Contribution No. 1293.

tribution differing from that of the original protein. Fractions possessing different mean mobilities and isoelectric points are obtained by proper choice of the pH. Transport proceeds to a stationary state, in which the top fraction is isoelectric at the operating pH.

Recently the authors (5) reported the results of two stages of fractionation of bovine γ-globulin prepared by ethanol precipitation, Fraction II of bovine plasma. By the modified isoelectric procedure described above, γ-globulin was separated into four fractions of different mean mobilities and isoelectric points. The γ-globulin was fractionated in Stage I at pH 6.7, several tenths of a pH unit removed from its mean isoelectric point. The top and bottom fractions resulting from this stage are designated as Fraction A and Fraction B, respectively. Fraction B served as the starting material for Stage II, which was also carried out at pH 6.7. The top and bottom fractions of this stage are designated as Fraction C and Fraction D,

TABLE I

Electrophoretic Properties of γ-Globulin Fractions

Fraction	$-10^5 \times \bar{u}$*	Isoelectric point†	$10^8 \times \beta$	$-10^5 \times \frac{\Delta\bar{u}}{\Delta pH}$
Original γ-globulin	1.73	~6.5	0.67‡	
A	1.35	7.03	0.65	0.74
B	1.98–2.12			
C	1.63	6.47	0.67	1.3
D	2.20	6.01	0.65	0.78

* In barbital buffer, pH 8.7, and ionic strength 0.1.

† In cacodylate buffer (0.08 N sodium chloride-0.02 N sodium cacodylate).

‡ Non-Gaussian mobility distribution.

respectively. The electrophoretic properties of these fractions are summarized in Table I, where $\bar{u}$ is the mean mobility (cm.^{2} sec.$^{-1}$ volt^{-1}) at pH 8.7, β the standard deviation of the mobility distribution in the neighborhood of the isoelectric point, and $\Delta\bar{u}/\Delta pH$ the slope of the mobility-pH curve in the neighborhood of the isoelectric point. The fractionation of γ-globulin has now been carried through two more stages, Stages III and IV, and four additional fractions obtained.

EXPERIMENTAL

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

Electrophoretic Analysis—The moving boundary technique of Tiselius (6) as modified by Longsworth (7) was used in the electrophoretic analysis. Mobilities were determined by electrophoresis of a 1 per cent protein solu-

tion in barbital buffer, pH 8.7, and ionic strength 0.1, at a field strength of about 8 volts per cm. for 2 hours. Mobilities were calculated in accordance with the suggestions of Longsworth and MacInnes (8). Isoelectric points were determined in cacodylate buffer (0.08 N sodium chloride-0.02 N sodium cacodylate).

Experiments in boundary spreading were carried out on 0.5 per cent solutions of the γ-globulin fractions equilibrated against cacodylate buffer. They were performed at the average isoelectric points of the proteins. The power consumption in these experiments did not exceed 0.015 watt per ml. Curves of the refractive index gradient were recorded photographically on Eastman Kodak Company CTC plates by the cylindrical lens schlieren technique. A diagonal knife-edge brought in from below the optical axis was used in the optical system.

It is a result of the theory of reversible boundary spreading, to be published elsewhere, that the mobility distribution of an inhomogeneous protein, $q(u)$, can be expressed in terms of the moments of the refractive index gradient curve taken about the centroidal axis by means of an infinite series, Equation 1. D is the diffusion constant, and E the electric field strength.

$$q(u) = \frac{1}{\sqrt{2\pi}\beta} e^{-u^2/2\beta^2} \left\{ 1 + \sum_{j=3}^{\infty} \frac{C_j}{j!} (-i)^j \alpha^j H_j(iu\sigma/El_E\beta^2\alpha) \right\}$$
$$\beta^2 = (\sigma^2 - \sigma_0^2 - 2Dt_E)/E^2 t_E^2 \qquad (1)$$
$$\alpha = \sqrt{1 - 2(\sigma/\beta E l_E)^2}$$

σ_0 and σ^2 are the second moments of the gradient curves at the moment of application and at time t_E after application of the electric field. β is the standard deviation of the mobility distribution. H_j is the jth Hermite polynomial. The coefficients C_j are related to the higher moments, $\overline{x^n}$, of the gradient curve, *e.g.*,

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

If the gradient curves are Gaussian in form, *i.e.* the mobility distribution is Gaussian, β is identical to the heterogeneity constant h of Alberty (9). Deviations from a Gaussian distribution of mobilities are given by the third and higher moments of the gradient curves. For proteins possessing either Gaussian or non-Gaussian mobility distribution, β may be calculated from Equation 2. D^* is the apparent diffusion constant calculated from the second moments of the gradient curves during electrophoresis. A plot of

$$D^* = (\sigma^2 - \sigma_0^2)/2t_E = D + (E^2\beta^2/2)t_E \qquad (2)$$

D^* *versus* t_E is a straight line which extrapolates back to the normal diffusion constant at zero time. β may be calculated from the slope $\beta^2E^2/2$. The standard deviation of the mobility distributions are tabulated as β. The distribution may be assumed to be Gaussian unless otherwise specified.

Fractionations—The details of construction and operation of the electrophoresis-convection apparatus employed in this investigation have previously been described. Metal screws in the apparatus originally described have been replaced by plastic screws. Paper base bakelite 10-32 screws have been used with some success. However, it has been found that screws made with du Pont nylon, FM-10001, are more satisfactory. A photograph of one of the fractionation units is shown in Fig. 1. The

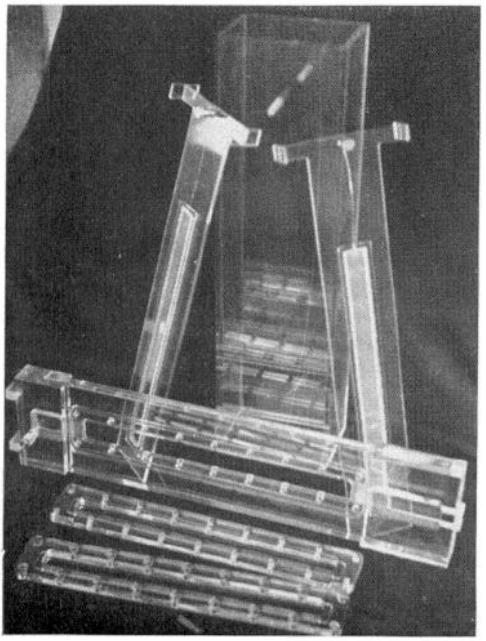

FIG. 1. Electrophoresis-convection apparatus

fractionation cell consists of a narrow vertical channel connecting upper and lower reservoirs.[1] The channel is formed by the space between two

[1] The volumes of the top and bottom reservoirs of the apparatus used in this investigation were 100 and 50 ml., respectively. Recently a small fractionation cell, Fig. 1, with top and bottom reservoir capacities of 15 and 10 ml., respectively, has been constructed and successfully used to separate γ-globulin from rabbit serum. The channel wall separation and the length of the channel in this apparatus are the same as in the one described previously. The width of the channel and the platinum electrodes are one-third that specified previously; the longitudinal ribs of the face plates have been eliminated. Except for these changes the dimensions of the cell are essentially the same as in the larger apparatus. In this apparatus, the 6-32 brass machine screws used to bolt the face plates to the cell block have been replaced by 10-32 paper base bakelite screws, thus eliminating the necessity for electrically insulating the heads and tips of the screws as well as the distortion of the electric field across the channel by the metal screws.

sheets of semipermeable membrane. The cell containing a solution of the protein to be fractionated is immersed in buffer solution and a homogeneous electric field applied across the channel by means of external platinum electrodes. Electrolysis products are prevented from reaching the membranes by circulation of the external buffer solution. Fractionations are carried out in a constant temperature cold room at 4°.

γ-Globulin fractions withdrawn from the top and bottom reservoirs at the conclusion of the runs were filtered, dialyzed first against distilled water and then dilute saline solution at 4° to remove buffer salts, and finally lyophilized. The dried protein preparations dissolved readily to form clear and stable solutions at their isoelectric points. In a few cases it was necessary to filter off a small amount of suspended material. Solutions of the various fractions were equilibrated against barbital or cacodylate buffer and analyzed electrophoretically.

Results

A composite of Fraction A and γ-globulin served as the starting material for Stage III.[2] This γ-globulin, which had a mobility of -1.51×10^{-5} and an isoelectric point of 6.75, was from a different batch from that used in Stage I. Stage III was carried out in phosphate buffer, pH 7.6, which was about 0.6 pH unit on the alkaline side of the mean isoelectric point of the starting material. The fractionation was carried out in duplicate. The pertinent data are presented in Table II.

The electrophoretic properties of the resulting top cut, Fraction E, are quite striking. The mobility of Fraction E was -1.24×10^{-5}. The mean isoelectric point was found to be 7.31, about 0.8 pH unit greater than that of the original γ-globulin (-1.73×10^{-5}). Furthermore, the heterogeneity constant was significantly lower than the values found for other top fractions, and $\Delta\bar{u}/\Delta pH$ was considerably lower than the values for the other fractions. The material withdrawn from the bottom reservoir in Stage III is designated Fraction F. Fraction F, obtained in Run 1, had a mobility of -1.78×10^{-5} and an isoelectric point of 6.51. The mobility of Fraction F obtained in Run 2 was -1.69×10^{-5}.

The top and bottom fractions of Run 2 were recombined in proportion to their relative concentrations in the starting material. The mobility and heterogeneity constant of this composite were -1.45×10^{-5} and 0.59×10^{-5}, respectively, which are to be compared with the calculated values of -1.54×10^{-5} and 0.67×10^{-5}.

A composite of Fraction B and the γ-globulin possessing a mobility of -1.51×10^{-5} appeared to be roughly comparable to the γ-globulin of

[2] Since the starting material for Stage III is a composite of Fraction A and unfractionated γ-globulin, it does not represent a true stage in the fractionation scheme. However, for convenience, we will refer to it as such.

mobility -1.73×10^{-5}. This composite was fractionated under the same conditions as in Stage I. The resulting bottom fraction had a mobility of -1.91×10^{-5}, an isoelectric point of 6.29, and a heterogeneity constant of 0.75×10^{-5}. This material possessed a Gaussian distribution of mobilities at its mean isoelectric point, although at pH 8.7 the electrophoretic pattern was non-Gaussian and skewed. Differences in the dependence of mobility upon pH for the various components of the fractions undoubtedly are responsible for this departure of the mobility distribution from a Gaussian

TABLE II

Fractionation of Bovine γ-Globulin

Experimental Conditions

Stage No.	Run No.	pH	*E*	*t*	*C*
			volts per cm.	*hrs.*	*gm. per 100 ml.*
III	1	7.60	1.7	52½	2.8
	2	7.58	1.7	53½	2.4
IV		5.48	1.6	51½	2.3

Properties of γ-Globulin Fractions

Stage No.	Run No.	Fraction	Yield	$-10^5 \times \bar{u}$*	Isoelectric point†	$10^5 \times \beta$	$-10^5 \times \frac{\Delta\bar{u}}{\Delta pH}$
			gm. protein				
III	1	Top (E)	1.1	1.24	7.31		0.35
		Bottom (F)	2.0	1.78	6.51	0.59	0.89
	2	Top (E)	1.2	1.25		0.55	
		Bottom (F)	1.5	1.69			
IV		Top (G)	0.6	2.25	5.74	0.63‡	0.64
		Bottom (H)	2.3	1.81	6.41	0.77‡	0.81

* In barbital buffer, pH 8.7, and ionic strength 0.1.

† In cacodylate buffer (0.08 N sodium chloride-0.02 N sodium cacodylate).

‡ Non-Gaussian mobility distribution.

one. This fraction, which appeared to be the same as Fraction B, served as the starting material for Stage IV.

The operating pH in Stage IV was 5.5, or 0.8 pH unit on the acid side of the mean isoelectric point of Fraction B. The material withdrawn from the top reservoir, Fraction G, had about the same mobility as Fraction D. However, its isoelectric point was about 0.3 pH unit more acid than that of Fraction D, and its mobility distribution was non-Gaussian. The bottom cut, Fraction H, which had a mobility of -1.81×10^{-5} and an isoelectric

point of 6.41, possessed a skewed non-Gaussian distribution of mobilities with a rather large standard deviation. The calculated mobility and heterogeneity constant for a composite of the two fractions, in proportion to their relative concentrations in the starting material, are -1.91×10^{-5} and 0.76×10^{-5}, respectively. These calculated values are in excellent agreement with the experimental values quoted above for Fraction B.

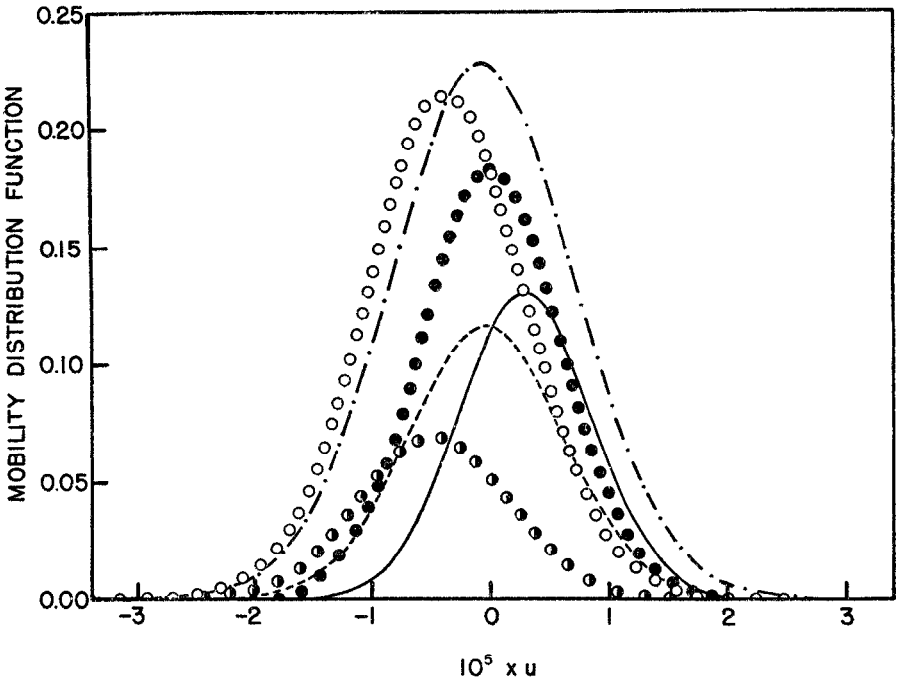

FIG. 2. Mobility distribution functions at pH 6.5 of the six unique fractions of bovine γ-globulin; - -, Fraction C; O, Fraction D; —, Fraction E; ●, Fraction F; ◑, Fraction G; — · —, Fraction H.

DISCUSSION

Bovine γ-globulin has been separated into eight fractions which constitute mean mobility and mean isoelectric point spectra, ranging from -1.25×10^{-5} to -2.25×10^{-5} and from 7.31 to 5.74 respectively. The various fractions can be distinguished from one another by at least two electrophoretic properties. Thus, Fractions C, F, and H have about the same mean isoelectric points but different mean mobilities at pH 8.7 and different mobility distributions at their respective mean isoelectric points. The considerable variation in $\Delta\bar{u}/\Delta$pH among the fractions is probably a reflection

of chemical and structural differences. The mobility distribution[3] of each of the six unique fractions at pH 6.5 has been normalized to an area corresponding to its weight fraction[4] of γ-globulin, -1.73×10^{-5}, and is plotted in Fig. 2. Fractions G and H possessed non-Gaussian mobility distributions; however, as a first approximation, the mobility distribution can be adequately represented as a Gaussian probability function whose standard deviation is taken as that of the actual mobility distribution.

The theory of transport in an electrophoresis-convection channel predicts that the fractionation of a protein possessing a Gaussian mobility distribution, with specified first and second moments, will result in a top fraction which also possesses a Gaussian mobility distribution, having the same second moment as that of the original protein. The first moment will, of course, be different from that of the original protein. The theory also predicts that transport in the apparatus will proceed to a stationary state when the first moment of the mobility distribution of the material in the top reservoir vanishes. Both of these predictions have been approximately realized in these experiments.[5] The mobility distribution of the material

[3] It is assumed that, within the range of concentration and pH considered, the standard deviation of the mobility distributions of the γ-globulin fractions is independent of protein concentration and pH. It is also assumed that the slopes of the mobility-pH curves of all the protein ions present in a given fraction are the same and equal to the $\Delta\bar{u}/\Delta pH$ of the fraction.

[4] The relative concentrations of Fractions E and F were calculated on the assumption that the material fractionated in Stage III was Fraction A and not a composite of Fraction A and γ-globulin of mobility 1.51×10^{-5}.

[5] For purposes of characterization, the mean isoelectric points and standard deviations of the mobility distributions of the bovine γ-globulin fractions were determined in chloride-cacodylate buffer. This buffer was used because of the desirability of carrying out electrophoretic experiments in buffers containing

Fraction	Isoelectric point		$-10^5 \times (\Delta\bar{u}/\Delta pH)$		$10^5 \times \beta$	
	Chloride-cacodylate	Phosphate	Chloride-cacodylate	Phosphate	Chloride-cacodylate	Phosphate
Unfractionated γ-globulin (-1.51×10^{-5})	6.75	6.33	0.75	0.97	0.61	0.66
A	7.03	6.19	0.74	1.15		
D	6.01	5.73	0.78	1.10		
E	7.31	6.44	0.35	0.92		
G	5.74	5.52	0.64	1.18		

univalent anions. In addition to the electrophoretic characterization of the fractions in chloride-cacodylate buffer, it also appeared desirable to determine isoelectric points and standard deviations of mobility distribution in phosphate buffer, since the fractionations were carried out in this buffer. Since preparation of this manuscript such determinations have been made on several fractions. For purposes of

fractionated in Stage IV was non-Gaussian and skewed at pH values removed from the mean isoelectric point. As a result, the top fraction obtained in this stage exhibited a non-Gaussian mobility distribution at its mean isoelectric point.

Although the bottom fractions from an original Gaussian distribution are not themselves Gaussian, they are not highly skewed. Therefore, we will represent them as Gaussian. The precision with which the moments of the curves of the refractive index gradient can be determined does not justify a more refined treatment.

The results of the fractionation of bovine γ-globulin illustrate the ease with which a heterogeneous protein can be fractionated by electrophoresis-convection. The large quantities of material that can be fractionated in a relatively short time, the ease of the manipulations, and the reproducibility of the fractionations promise to make electrophoresis-convection a valuable method for the subfractionation of the plasma fractions obtained by ethanol precipitation.

SUMMARY

Bovine γ-globulin has been separated into eight fractions which constitute mean mobility and mean isoelectric point spectra ranging from -1.25×10^{-5} to -2.25×10^{-5} at pH 8.7 and from 7.31 to 5.74 respectively.

This investigation demonstrates the applicability of electrophoresis-convection to the subfractionation of the plasma fractions obtained by ethanol precipitation.

---

comparison the resulting data are presented, along with the corresponding data obtained in chloride-cacodylate buffer, in the tabulation. It will be noted that there are rather large differences between the mean isoelectric points in chloride-cacodylate and in phosphate buffer. Although unfractionated γ-globulin has appreciably different isoelectric points in chloride-cacodylate and phosphate buffer, the standard deviations of the mobility distribution at the mean isoelectric point are approximately the same in the two buffers. Although the theoretical isoelectric condition of the top fraction at the operating pH was realized in Stage IV of the fractionation, the data obtained in phosphate buffer reveal that in the case of Stage I and Stage III the fairly good agreement between the theoretical isoelectric condition of the top fraction at the operating pH and the observed isoelectric condition based on measurements in chloride-cacodylate buffer is fortuitous. Obviously, this does not reflect upon the use of chloride-cacodylate buffer for purposes of electrophoretic characterization of the fractions. Failure to realize the isoelectric condition of the top fraction at the operating pH in Stages I and III may have been due to insufficient time of operation for attainment of a stationary state. However, this seems improbable. Theoretically, departure from the isoelectric condition is to be expected if some of the components of the heterogeneous protein produce different density increments per unit weight or possess different diffusion constants. A detailed treatment of this problem cannot be made at this time.

BIBLIOGRAPHY

1. Kirkwood, J. G., *J. Chem. Phys.*, **9**, 878 (1941).
2. Nielsen, L. E., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **68**, 181 (1946).
3. Cann, J. R., Kirkwood, J. G., Brown, R. A., and Plescia, O. J., *J. Am. Chem. Soc.*, **71**, 1603 (1949).
4. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 1609 (1949).
5. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 2687 (1949).
6. Tiselius, A., *Tr. Faraday Soc.*, **33**, 524 (1937).
7. Longsworth, L. G., *Chem. Rev.*, **30**, 323 (1942); *Ind. and Eng. Chem., Anal. Ed.*, **18**, 219 (1946).
8. Longsworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
9. Alberty, R. A., *J. Am. Chem. Soc.*, **70**, 1675 (1948).