charge is determined by reactions unrelated to structural changes, such as hydrolysis of amide groups to yield free carboxyl. Further study will be required to identify the nature of the degradation process.

Nature of the Cross-linking Process.—The cross-linking process may involve any of the reactions which have been postulated in the setting of keratin fibers, *e. g.*, the combination of amino groups with carboxyl groups or with cysteine residues.[25] The evidence of Lundgren[26] that condensation between side-chain amino and carboxyl groups of proteins occurs at 165° in the absence of moisture is suggestive in interpreting the cross-linking of dry fibrin film at 170°.

Summary

1. Fibrin film modified by exposure, at a moisture content of 20%, to steam at 121°, has a higher water-equilibrated fibrin content, a correspondingly lower opacity and higher tensile strength, lower swelling in acid and alkali, lower permeability, greater resistance to enzymatic digestion, and higher affinity for dyes, than unmodified film. The changes in physical properties may be attributed to cross-linking.

2. When the treatment is carried out at a moisture content of 70%, structural breakdown predominates over cross-linking.

3. Prolonged heating of steam-treated fibrin film at moderate temperatures results in increased water imbibition, decreased tensile strength, decreased resistance to tryptic digestion, decreased swelling in acid, increased swelling in alkali, decreased affinity for acid dye, and increased affinity for basic dye. The rate of change increases with the moisture content and also with the temperature, corresponding to an activation energy of 24 kcal.

4. These changes indicate structural breakdown accompanied by either destruction of basic groups or creation of acidic groups.

(25) Speakman and Hirst, *J. Soc. Dyers Colourists*, **59**, 124 (1943).
(26) Lundgren, personal communication.

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Preparation and Properties of Serum and Plasma Proteins. XI. Quantitative Interpretation of Electrophoretic Schlieren Diagrams of Normal Human Plasma Proteins[1,2]

BY S. H. ARMSTRONG, JR.,[3] M. J. E. BUDKA AND K. C. MORRISON

Introduction

In a little over a decade since the introduction of the electrophoresis apparatus of Tiselius,[4] a considerable body of measurements of the distribution of electrophoretic components in normal human plasma has appeared. The earlier studies carried out in the main at *p*H values between 7.0 and 8.0, and at ionic strengths between 0.1 and 0.2, yielded values in good agreement.

Observations, both in this and in other laboratories,[5] had begun to give evidence, at the time of the initiation of the program of large-scale plasma fractionation, for systematic variations in the apparent distribution of the electrophoretic components of the plasma proteins as a function of conditions of electrophoresis in neutral and alkaline *p*H range. Because electrophoretic analyses assumed increasing importance both in the control of purity and yield of the products of plasma fractionation[6,7] and in the characterization of pathological plasma proteins, a systematic study of certain of the variables influencing apparent distributions has been carried out. In this study, findings on artificial mixtures of known composition made from purified proteins of known electrophoretic characteristics have been used to aid in the interpretation of data obtained under parallel conditions of electrophoresis on the more complex natural mixtures presented by plasma and its fractions. The results have been considered in the light of recent theoretical treatments.

To convert schlieren diagram data (given in terms of ratio of refractive index increment of a given component to total refractive index increment of the mixture) to a basis of weight of dried protein or of protein nitrogen, conversion factors have been calculated from independent measurements of refractive index increments of whole plasma and certain of its fractions.[8] In that valid use of refractive index increments requires the

(1) This work has been carried out in part under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This is Number 56 in the series "Studies on Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) Welch Fellow in Internal Medicine of the National Research Council.

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

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

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

(7) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(8) Details of refractive index increment measurements will be presented in a separate communication in this series.

average value for a component as it migrates in the electric field, electrophoretic separations of albumin and γ-globulin have been carried out under conditions used in routine analytical electrophoresis.[7] The conversion factors based on refractive index measurements of these preparations are compared with those based on the chemically separated preparations. The magnitudes of the revisions entailed are illustrated in detail in the estimation of the albumin content of normal pooled human plasma and of certain pathological plasmas. Moreover, the revisions emerging from the study of conditions of electrophoresis together with those from differences in refractive index increment are applied to the uncorrected electrophoretic distribution data given in a previous communication of this series[7] in an attempt to approximate the quantitative limitations of the electrophoretic method in following the chemical fractionation of such a system as normal human plasma.

Experimental

Materials.—Aliquots of plasma were obtained from pools collected by the American Red Cross for fractionation at the Plasma Fractionation Laboratory of the Department of Physical Chemistry of Harvard Medical School.

The methods of preparation of purified protein fractions are presented elsewhere in this series of papers.[7-11] On electrophoretic analysis at *p*H 8.6 and ionic strength 0.1, the albumin was entirely free from globulin, and the γ-globulin, β-globulin, α_2-globulin and fibrinogen were free from albumin.

Electrophoretic Analyses.[12]—In electrophoretic analyses carried out in the apparatus of Tiselius,[4] his long cell, as described by Longsworth,[5] was used. Schlieren diagrams were obtained by the cylindrical lens method of Philpot.[13] In standard runs a straight edge schlieren diagram was employed. To check the agreement between the straight edge and the slit diaphragm with parallel edges, photographs were taken of a selected series of runs under both conditions. The results proved indistinguishable within the limits of error of the method.

Analysis of Schlieren Diagrams.—Photographs of schlieren diagrams of both ascending and descending boundaries, five times magnified, were projected on a glass screen and traced on coördinate paper. Resolution into components was carried out by the method of Pedersen.[14] In this report, tabulated values for each component represent the averages of measurements of ascending and descending boundaries, between which no systematic differences were observed.

In the resolution of schlieren diagrams of the proteins of plasma and its fractions where separation of components is rarely complete and where the shapes of the peaks themselves rarely approach ideal Gauss curves, there is a strong element of the arbitrary. This element is at its least in the resolution of artificial mixtures of purified proteins of sharply defined and widely differing mobilities (Fig. 1); it is at its greatest in the resolution of such schlieren diagrams as are illustrated in Fig. 5 (Fraction II + III). Whereas the diagrams of whole plasma (Fig. 4) and many of its fractions[15] are intermediate between these extremes, the role of conditions of fractionation in effecting differences in the apparent electrophoretic distribution of the various fractions has demanded uniformity

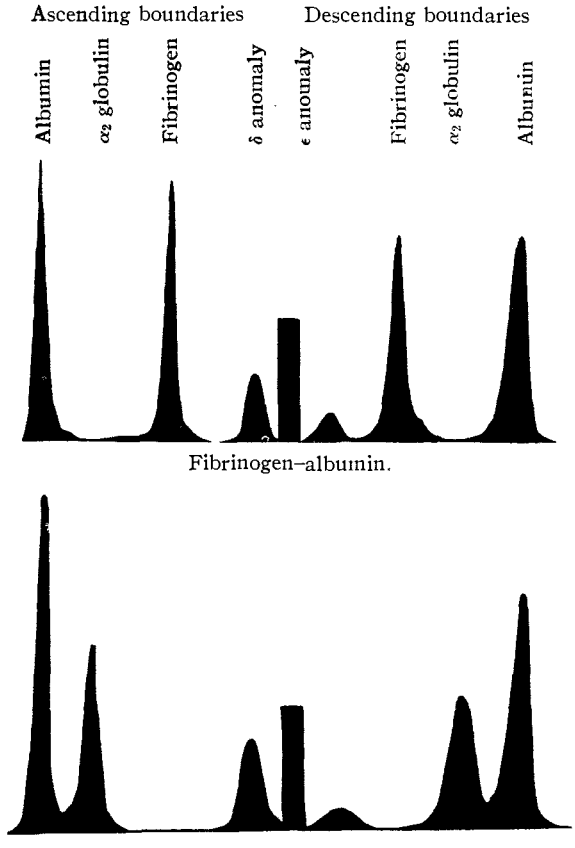


Fig. 1.—Electrophoretic analyses of one to one mixtures of purified proteins. Protein approximately 2%, barbiturate buffer *p*H 8.6, Γ/2 = 0.05.

(8a) E. J. Cohn and W. L. Hughes, Jr., in preparation.

(9) We are indebted to Dr. Jules D. Porsche of the Armour Laboratories for bovine γ-globulin (used in preference to human because the higher mobility leads to expectation of better resolution from boundary anomalies than can be attained with the human material).

(10) J. D. Ferry and P. R. Morrison, in preparation.

(11) J. L. Oncley, L. E. Strong and collaborators, in preparation.

(12) We acknowledge with gratitude the assistance of Dr. A. Sparrow, Mrs. M. Y. Donath, Miss W. Prince, Mrs. E. A. Bering and Miss M. Hasson in carrying out many of the analyses both in this study and in the electrophoretic control of plasma fractionation in the Harvard Pilot Plant.

(13) J. S. L. Philpot, *Nature*, **144**, 283 (1938).

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

(15) Certain of these diagrams are illustrated in Figs. 1 and 2 of a previous communication from this Laboratory.[4]

TABLE I

DEVIATIONS OF APPARENT ELECTROPHORETIC DISTRIBUTIONS FROM KNOWN RELATIVE CONCENTRATIONS IN ARTIFICIAL MIXTURES OF PURIFIED PROTEINS

Expt. no.	Conditions of electrophoresis: pH	Conditions of electrophoresis: Γ/2	Conditions of electrophoresis: Protein, g./100 cc.	Known albumin concn., %	Apparent electrophoretic albumin concn., %	Dev. of apparent electrophoretic from known albumin concn., %	Protein components
1	8.6[a]	0.05	2.0	9.8[d]	9.4	−0.4	Albumin fibrinogen
2	8.6[a]	.05	2.0	89.3[d]	90.2	+0.9	
3	8.6[a]	.05	2.0	50.8[d]	53.0	+2.2	
4	8.6[a]	.2	1.0	50.8[d]	51.4	+0.6	
5	8.6[a]	.05	3.0	50[e]	52	+2	Albumin γ-globulin (bovine)
6	8.6[a]	.1	2.0	50[e]	52	+2	
7	8.6[a]	.1	1.0	50[e]	52	+1	
8	7.7[b]	.2	2.5	51[f]	56	+5	Albumin β-globulin
9	8.6[c]	.1	1.2	34[d]	42	+6	
10	8.6[c]	.1	1.3	66[d]	74	+8	
11	8.6[a]	.1	2.0	31[e]	38	+7	
12	8.6[a]	.1	2.0	65[e]	71	+5	
13	8.6[a]	.2	2.0	65[e]	68	+3	
14	8.6[a]	.2	1.0	65[e]	67	+2	
15	8.6[a]	.05	2.5	51[d]	58	+7	Albumin α2-globulin
16	8.6[a]	.05	1.5	51[d]	56	+5	
17	8.6[a]	.05	1.0	51[d]	55	+4	
18	8.6[a]	.3	1.0	51[d]	51	0	

[a] Sodium diethylbarbiturate. [b] Potassium phosphate. [c] Sodium diethylbarbiturate and citrate. [d] Determined by refractive increment measurements. [e] Determined by both refractive increment measurements and protein and non-protein nitrogen analyses. [f] Determined by nitrogen analysis.

of convention in their resolution in the various laboratories that have collaborated in the electrophoretic control of large-scale processing.[16]

Results of Electrophoretic Analyses. I. Artificial Mixtures

The Effects of Protein Concentration and Ionic Strength on the Distribution of Components in Mixtures of Known Composition.[17]—Four types of mixtures have been employed: namely, fibrinogen–albumin, γ-globulin(bovine)–albumin, β-globulin–albumin, and α_2-globulin–albumin. Of these, the first yielded resolution sufficiently clean-cut to permit reproducible measurement of peaks within somewhat less than ±1% of the diagram area (Fig. 1); measurements of the latter two proved reproducible within about ±1%. In the instance of the slow moving γ-globulin, the incomplete resolution from the delta and epsilon anomalies (despite good separation from albumin) precluded as good reproducibility within these limits.[9]

In fibrinogen–albumin mixtures of ratio nine to one or one to nine, the discrepancy between relative concentrations and apparent electrophoretic distributions, even at ionic strength 0.05 and 2% protein, proved below the error in measurement of component areas (Table I, experiments 1 and 2). At ratio approximately one to one between protein components, the apparent albumin contribution to the diagram exceeded the known relative concentration by a small but significant deviation (Table I, experiment 3). Quadrupling the ionic strength and halving the protein resulted in disappearance of the deviation (Table I, experiment 4). Deviations of parallel magnitude were encountered in γ-globulin–albumin mixtures (Table I, experiments 5–7).

In a mixture of albumin with components closer to it in mobility, deviations of apparent albumin contributions to the schlieren diagrams from known relative concentrations were much more striking (Fig. 1). Thus, in a series of four experiments on β-globulin–albumin mixtures (Table I, experiments 8–11), in which both phosphate buffer at *p*H 7.7, ionic strength 0.2, and barbiturate buffer at *p*H 8.6, ionic strength 0.1, were utilized, the albumin contribution to the schlieren diagram was between 5 and 8% in excess of the known composition of the mixture as determined by measurement of protein nitrogen, protein refractive increment, or both.

In both the β-globulin–albumin and α_2-globulin albumin mixtures (Table I, experiments 12–14 and 15–18, respectively) these considerable devia-

(16) In setting up the conventions used in this Laboratory, we wish to acknowledge the assistance of Dr. J. L. Oncley and Dr. L. E. Strong, of the Harvard Medical School, and Dr. J. W. Williams, of the University of Wisconsin. We also wish to express our appreciation to Dr. B. F. Chow, of the Squibb Institute of Medical Research, Dr. W. W. Davis, of the Lilly Research Laboratories, and to Dr. E. Jameson and Dr. J. M. Luck, of Stanford University, for the mutual exchange of electrophoretic plates which assisted in the standardization of diagram photography, projection and resolution.

(17) We wish to acknowledge the collaboration of Dr. C. Riley, whose unpublished observations (made in this Laboratory before the initiation of large-scale plasma fractionation) on the effect of ionic strength on distribution of components of certain albumin fractions in acetate buffers led to this study of artificial mixtures.

tions appeared to become minimal and the apparent electrophoretic distribution closely approached the true relative concentrations under conditions of high ionic strength and low protein concentrations.

Consideration of Findings on Artificial Mixtures in Terms of Recent Theoretical Treatments.—Svensson[18,19] has published theoretical and experimental considerations for the existence, in the region of a boundary of a given electrophoretic component, of concentration gradients of other protein components. Dole's[20] recent and ingenious treatment of moving boundaries in systems formed by strong electrolytes likewise leads, when applied to buffered protein mixtures, to a prediction of "foreign colloid gradients."

Both the Svensson and Dole treatments of these "foreign colloid gradients" have, for mixtures of proteins on the alkaline side of their isoelectric point, the same sign as the concentration gradient of the component on which they are superimposed, and can be shown to increase with increasing protein concentration, and to decrease with increasing ionic strength and with increasing difference between mobility of the component of the boundary in question and the mobility of the component whose gradients may be superimposed. Their magnitude is also a function of the characteristics of the buffer and other ions present, notably increasing with increasing mobilities of ions carrying charge identical in sign with that of the protein net charge.

It likewise may be shown from both theories that buffer gradients, experimentally demonstrated by Longsworth and MacInnes[21] in single-component systems to be superimposed and opposite in sign to protein gradients, are not, as is customarily assumed, proportional to the change in protein concentration at each boundary expressed in refractometric units (which will be the same for proteins of differing mobility), but are proportional to the change in protein concentration expressed in electrical equivalents, which will differ widely for proteins of widely differing mobility. With diminishing epsilon boundary anomalies as a result of increasing ionic strength or decreasing protein concentration, such superimposed buffer gradients may be expected to become sufficiently small as to be unmeasurable in an electrophoretic schlieren diagram.

The theoretical deviations for the artificial mixtures in sodium diethylbarbiturate buffer alone or with chloride, when calculated by the equations of Dole and plotted against the ratio of protein concentration to ionic strength[22,23] (Fig. 2), fall on straight lines which arise from the origin; thus, with decreasing values of this ratio the deviations approach zero. These experimental deviations, of the proper sign, are somewhat greater than the theoretical deviations. This qualitative agreement is as satisfactory as can be expected in the light of the arbitrary nature of many of the assumptions used in the application of the theory to the data.[25] The desirability of carrying out

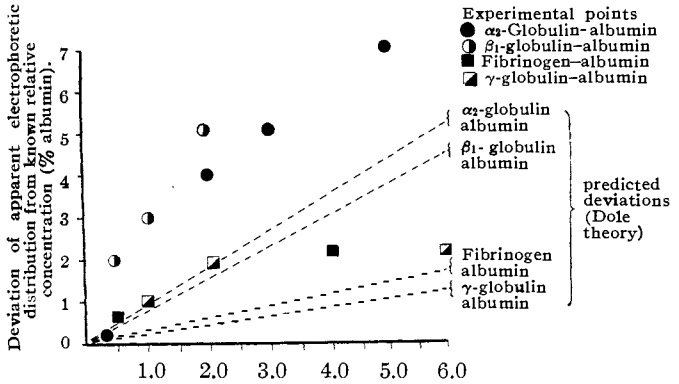


Fig. 2.

(18) H. Svensson, *Arkiv. Kemi. Mineral. Geol.*, **17A**, No. 14 (1943).

(19) H. Svensson, *ibid.*, **22A**, No. 10 (1946).

(20) V. P. Dole, THIS JOURNAL, **67**, 1119 (1945).

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

(22) We are indebted to Dr. J. L. Oncley for the original suggestion of this plot on an empirical basis.

(23) Svensson's treatment leads to the same relations. At higher values of the ratio of protein concentration to ionic strength, the larger deviations seen in the α_2-globulin–albumin and β-globulin–albumin mixtures as compared with fibrinogen–albumin and γ-globulin–albumin mixtures are consistent with the theoretical effect of the differences between mobilities of the various slower protein components and the mobility of albumin.

Cooper,[24] using mixtures of horse globulin of unspecified mobility and bovine albumin, has failed to find deviations between apparent electrophoretic and known relative albumin concentrations at a wide range of protein concentrations and ionic strengths. It is possible that the difference in mobilities between the protein components was sufficient to render deviations minimal.

(24) G. R. Cooper, *J. Biol. Chem.*, **158**, 727 (1945).

(25) In estimating the $V\sigma$ products for the boundaries of the two-component mixtures (Dole,[20] equation 33), and in calculating component concentration ratios across them (Dole,[20] equation 31) (whose summations define the deviations of apparent electrophoretic from true relative proportions), the following values for *relative* mobilities have been used:

Sodium	+22.3	α_2-Globulin	− 4.0
γ-Globulin (bovine)	− 1.7	Albumin	− 6.0
Fibrinogen	− 2.0	Diethylbarbiturate	− 9.5
β_1-Globulin	− 3.7	(Chloride	−36)

The rectilinear character of the plots of theoretical deviations depends on omission to consider the high negative mobility of the chloride ion used (because of low solubility of sodium diethylbarbiturate) in attaining high ionic strengths. Should this be taken into account, the theoretical deviation would be slightly increased in the region of low values of the protein-ionic strength ratio; this shift again would fall close to the limits of diagram measurement.

Concentrations, in terms of electrical equivalents of the protein components, have been assumed independent of ionic strength. The values for albumin have been taken directly from the net charge per gram as given by titration measurements in 0.15 sodium chloride (35×10^{-5} moles/gram at *p*H 8.6); for the α_2-globulin, the β_1-globulin, the fibrinogen and the γ-globulin (whose titration curves are not yet available), net charges per gram have been arbitrarily assigned the same ratio with that of albumin as obtained between their respective relative mobilities and the relative mobility of albumin.

electrophoretic analyses at as high ionic strength and as low protein concentration as limitation of resolution of planimetric analyses permits is thus emphasized by both theoretical considerations and by the fact that in the artificial mixtures here reported apparent electrophoretic distributions appear to approach actual distributions under these circumstances.

The Effect of Caprylate and Thiocyanate Ions on Distribution and Mobilities of the Components of Albumin–Lipoprotein Mixtures of Known Composition.—Qualitative agreement between theory and experiment is by no means proof that the factors treated by Svensson and Dole are solely operative in yielding deviations. It is possible that specific and selective interactions between protein components and buffer ions (which, if on an electrostatic basis, will tend to decrease with increasing ionic strength) may play a role.

That such an interaction between the diethylbarbiturate ion and proteins in the artificial mixtures detailed in the last section is not of the order of magnitude susceptible of quantitative analysis by schlieren diagram area measurement is indicated by the good agreement of specific refractive index increment determination made following dialysis against sodium diethylbarbiturate or sodium chloride (under conditions chosen to minimize the Donnan effect).[8]

For analysis of interaction between ions of size comparable to sodium diethylbarbiturate and proteins, methods based on refractive increments, particularly schlieren diagram area measurements, are relatively insensitive. Thus, in the two-component mixtures described in the previous sections, an increase in the albumin peak area of 2% (corresponding to a deviation of 1%) would require selective "binding" of the order of some 7 moles of diethylbarbiturate per mole of albumin. Changes in electrophoretic mobility (by reason of increase in the net charge of albumin by some 25%) are likely to be far more sensitive than changes in peak areas in detecting selective interaction of this degree.

In mixtures where the relative mobilities of the components are sufficiently separated, conditions yielding small but unequal changes in mobility will not affect the apparent electrophoretic distributions. If, however, as obtains in plasma and many of its fractions, the components are quite close in mobility, conditions yielding unequal changes in interaction between buffer ions and the proteins may be capable of shifting apparent electrophoretic distributions by selective acceleration or retardation of adjacent components, thus changing the degreeof overlapping or separation of peaks.

This phenomenon has been demonstrated in an artificial mixture of crystalline human albumin and a lipoprotein which, as separated from plasma, has shown an average mobility in sodium diethylbarbiturate buffer at *p*H 8.6 and ionic strength 0.1 almost identical with albumin. The mixture, unlike the well-separated two-component mixtures of the previous section (Fig. 1), presents a single asymmetrical component under these conditions (Fig. 3).

The range of mobilities of the separate components and of the mixture, both when the anion is solely diethylbarbiturate, and when one-half the anions are either thiocyanate[26] or caprylate, are presented in Table II. Under the latter circumstances, whereas the mobility of the albumin component has not changed, there has appeared a faster component in amount approximating the relative proportion of the lipoprotein in the mixture. Its mobility corresponds with the average mobility of the asymmetrical lipoprotein peak when run under the same conditions in the absence of albumin.

TABLE II

DISTRIBUTIONS BY MOBILITY RANGES OF ALBUMIN AND LIPOPROTEIN IV-1,1, RUN SEPARATELY AND IN ARTIFICIAL MIXTURE IN DIETHYLBARBITURATE (*p*H 8.6) ALONE AND IN PRESENCE OF CAPRYLATE AND THIOCYANATE (TOTAL IONIC STRENGTH 0.1)

	Conditions of electrophoresis					
	Diethylbarbiturate 0.1 *M*		Diethylbarbiturate 0.05 *M*, caprylate 0.05 *M*		Diethylbarbiturate 0.05 *M* thiocyanate 0.05 *M*	
	$-u \times 10^5$		$-u \times 10^5$		$-u \times 10^5$	
Materials	5.7–6.4, %	7.0–7.7, %	5.7–6.4, %	7.0–7.7, %	5.7–6.4, %	7.0–7.7, %
Albumin	100[a]	0	100[a]	0	100	0
Lipoprotein IV-1,1	85	15	15	85	15	85
Mixture albumin 75%–lipoprotein IV-1,1, 25%	95	5	80	20	80	20

[a] Although caprylate effected no change in albumin mobility under these conditions, the increases in mobility observed at *p*H 7.7 in phosphate buffer by Ballou, Boyer and Luck[33] have been qualitatively confirmed in this Laboratory.

These findings emphasize the importance of the natural properties of the proteins to be studied in addition to the properties of buffer ions.[27] Thus, the extensive recent electrophoretic studies on the

(26) We are indebted to Dr. George Scatchard for suggesting the use of thiocyanate in this study.

(27) The striking difference in degree of interaction between barbiturate (molecular weight 184), caprylate (molecular weight 143) and the lipoprotein IV-1,1, and the similarity of behavior of caprylate and thiocyanate (molecular weight 58) make it clear that properties in addition to molecular weight, stressed by Svensson, are of the first order of importance in determining interaction between identically charged smaller ions and proteins.

Chiefly because of the difficulty in achieving precise definition of the relationship between charge amd mobility,[28] electrophoretic measurements are not ideally adapted for quantitative analysis of interactions of this type. The interaction between thiocyanate and related anions and proteins of several classes is currently under investigation using more precise analytical methods by one of us (S. H. A., Jr.) in collaboration with Dr. George Scatchard.

(28) H. Mueller, in "Proteins, Amino Acids, and Peptides," by E. J. Cohn and J. T. Edsall, Reinhold Publishing Corporation, New York, N. Y., 1943, chap. 25.

strong protein–anionic detergent interactions[29-32] and the interaction of protein with a series of salts of the lower fatty acids[33] used albumin, either of egg or animal origin, as the protein. If the interaction of these groups of substances (which have in common a charged group attached to varying types and sizes of non-polar residues) involves non-polar regions on the protein molecule, the probability exists that proteins which in the natural state are associated with non-polar materials, may display even stronger interaction with a given type of anion than the albumins, which are comparatively free of non-polar material.

For analytical electrophoresis of a system whose proteins fall into several classes in terms of their natural associations with other substances, optimum conditions may involve the use of buffer ions capable of both high and low degrees of specific interaction. In the instance of plasma and its fractions, choice of conditions, though to some extent predictable on the basis of considerations of molecular structure and of behavior of artificial mixtures, has in general required systematic study of the individual materials.

II. Plasma and Plasma Fractions

Mobilities[34] of Chief Components under Standard Conditions for Plasma Fractionation Control.—The electrophoretic components of plasma, from the standpoint of molecular properties and biological functions, are in general gross mixtures.[6,7,35] The value of electrophoretic control as a quantitative first approximation in plasma fractionation has depended on the finding of a fair degree of correspondence of characteristic mobilities for components in plasma and its chief fractions under standard conditions of analysis. The standard buffers which have proved most serviceable in fractionation control have been potassium phosphate at *p*H 7.7, ionic strength 0.2, and Longsworth's[5] sodium diethylbarbiturate, *p*H 8.6, ionic strength 0.1. Mobilities in the former buffer were in agreement with previous values published from this Laboratory.[36]

The most recent fractionations have been followed mainly in the sodium diethylbarbiturate buffer. In Table III are given the ranges of identifying mobilities for the principal electrophoretic components as determined in plasma, in the chief fractions, and in certain of the more purified subfractions. Notes on specific conditions relevant to the various analyses are appended to Table III.

By the data of Table III, the electrophoretic components may be divided into two general classes. The first, comprising albumin, lipoid-poor α_2-globulins and β-globulins, fibrinogen and the γ-globulins, displays mobility ranges fairly insensitive either to the protein environment and/or to conditions and timing of storage; the second, comprising chiefly the lipoid-rich α- and β-globulins, displays mobility ranges quite sensitive to these factors, thus overlapping in various degrees with mobility ranges of the first class. Further studies may well reveal products of intermediate properties.

Apparent Distributions[37] of Electrophoretic Components of Pooled Normal Human Plasma under Standard Conditions for Plasma Fractionation Control.—Because Longsworth,[5] working with diethylbarbiturate buffer at *p*H 8.6, described a component between albumin and α-globulin which had failed to separate in the lower *p*H range of the buffers used in earlier analyses, a systematic study has been made of the apparent distributions of normal plasma pools in potassium phosphate buffer at *p*H 7.7, ionic strength 0.2, and in sodium diethylbarbiturate buffer, *p*H 8.6, ionic strength 0.1.

Results for the phosphate buffer on 8 pools did not differ from the average values for individual normal plasmas previously published from this Laboratory.[36]

In Table IV are presented analyses in the sodium diethylbarbiturate buffer of the same 8 pools, together with 12 additional pools obtained over a period of over 2 years. The values for the contribution of the γ-globulin and fibrinogen components under these conditions deviate only slightly from the values obtained at *p*H 7.7 in phosphate buffer. The α_1-globulins, comprising about 5% of the total area of the schlieren diagram, together with a decrease of some 2% in the β-globulins, with a corresponding increase in α_2-globulins, yield a value for total α-globulins of approximately 14%. The average albumin value, namely, 55.2%, lies below that obtained at *p*H 7.7 in phosphate by an amount equivalent to the contribution of the α_1-globulins.

Whereas agreement with these distribution data is in general good, Dole[38] and Deutsch and Goodloe,[39] at slightly higher protein concentration-ionic strength ratios in diethylbarbiturate buffer of *p*H 8.6, have reported apparent albumin con-

(29) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **144**, 183 (1943).

(30) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).

(31) H. Neurath and F. W. Putnam, *J. Biol. Chem.*, **160**, 397 (1945).

(32) F. W. Putnam and H. Neurath, *ibid.*, **160**, 239 (1945).

(33) G. A. Ballou, B. D. Boyer and J. M. Luck, *ibid.*, **159**, 111 (1945).

(34) Mobilities have been calculated from descending boundary positions, employing conductivity measurements at 0° on the buffers against which proteins have been dialyzed. The arbitrary factors in such calculations are discussed by Svensson.[19] The reproducibility of resultant values has been in general adequate for fractionation control.

(35) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Chem.*, in press.

(36) J. A. Luetscher, Jr., *J. Clin. Investigation*, **20**, 99 (1941).

(37) The apparent distributions for the chief plasma fractions have been presented in paper IV of this series, Tables III, IV and IX.[7] Distributions of subfractions will be given in further papers detailing methods of separation and other properties.

(38) V. P. Dole, *J. Clin. Investigation*, **23**, 708 (1944).

(39) H. F. Deutsch and M. B. Goodloe, *J. Biol. Chem.*, **161**, 1 (1945).

TABLE III

OBSERVED RANGES OF MOBILITIES ($-u \times 10^5$) USED FOR IDENTIFICATION OF ELECTROPHORETIC COMPONENTS IN PLASMA AND PLASMA FRACTIONS (SODIUM DIETHYLBARBITURATE BUFFER pH 8.6, $\Gamma/2 = 0.1$)

Material	Electrophoretic components: Albumins	α_1-Globulins	α_2-Globulins	β_1-Globulins	β_2-Globulins	Fibrinogen	γ-Globulins
Plasma[a]	5.7–6.2	4.6–5.1	3.6–4.1	2.8–3.2	2.5–2.8	1.7–2.3	0.8–1.3
Chief Fractions							
Fraction I[b,c]	5.8–6.2	.....	3.6–4.3	2.6–3.2		1.7–2.2	.5–1.0
Fraction II + III[a,d,e,j]	5.9–6.4	4.9–5.4	3.9–4.5	3.1–3.8	2.4–3.0	1.7–2.2	.7–1.2
Fraction IV (methods 1–5)[d,e,f,g]	5.9–6.4	4.8–5.4	3.8–4.3	2.7–3.1	2.4–2.8	1.7–2.3	.8–1.3
Fraction IV-1 (method 6)[d,e,f,g]	5.8–6.3	4.8–5.3	4.0–5.5	2.7–3.9		.....	.9–1.2
Fraction IV-4 (method 6)	5.7–6.3	4.7–5.3	3.8–4.3	2.6–3.0	.....	1.6–1.9	.....
Fraction V	5.8–6.2	4.8–5.3	3.7–4.2	2.8–3.4	.....	.....	.....
Fraction VI	5.8–6.2	4.8–5.2	3.7–4.2	.....	.....	.....	.....
Subfractions							
Fraction I-1 (purified fibrinogen)[b,c]	.....	.....	.....	2.5–3.2		1.7–2.2	0.8–1.3
Fraction II-1,2 (purified γ-globulin)	.....	.....	.....	.....	1.8–2.3	.....	.6–1.0
Fraction II-3 (purified γ-globulin)	6.0–7.0	.....	.....	2.2–3.5		.....	.6–1.2
Fraction III-0 (β_1-lipoprotein)[g,i]	5.9–6.3	4.9–5.4		3.9–4.3	2.2–2.5	.....	.8–1.2
Fraction III-0,2 ("x" lipoprotein)[i]	.....	.....	.....	4.1–5.6	.....	.....	.....
Fraction III-2 (prothrombin)[j]	5.7–6.3	4.4–5.5		3.2–3.7	1.9–2.6	.....	0.7–1.2
Fraction III-1 (isoagglutinins)	5.9–6.4	4.8–5.5	3.9–4.3	3.1–3.7	2.1–2.8	.....	.8–1.2
Fraction IV-1,1W (α_1-lipoprotein)[f,h]	.....	6.1–7.5	4.4–5.6	2.7–3.7		.....	.....
Fraction IV-6,2 (purified α_2-globulin)	.....	.....	3.8–4.2	.....	.....	.....	.....
Crystalline albumin	5.8–6.2	.....	.....	.....	.....	.....	.....

[a] Resolution of β_1- and β_2-globulins is not sufficiently clean-cut to give reproducible apparent distributions. [b] For satisfactory resolution of fibrinogen and the adjacent slow-moving β-globulin peaks, the duration of electrophoresis at a potential gradient of 6 to 8 volts per cm. approximates four to five hours. Whereas under these conditions albumin and α-globulin boundaries will be out of the field, agreement between fibrinogen determinations by electrophoresis and by clottable nitrogen is close. (See also J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *J. Clin. Investigation*, **23**, 557 (1944), Table II and ref. 7, Table IX, footnote *a*.) [c] Fibrinogen has an extremely high temperature coefficient of solubility between 0 and 5°. To avoid precipitation in the cell in runs of material high in fibrinogen, it is advisable to carry out analyses at approximately 8° under continuous observation in order that boundary disturbances due to thermal convection currents may be forestalled when necessary by lowering the field strength. [d] Occasionally in Fraction II + III and certain of its subfractions, and uniformly in Fraction IV (methods 1–5) and Fraction IV-1 (method 6),[7] a small component (absent from the schlieren diagram of whole plasma) separates from the chief albumin peak; its mobility varies from -7.0 to -7.5×10^{-5}. It has been included with the albumins in distribution data. [e] Speed and maintenance of low temperatures are particularly essential in preparing alcohol-containing samples of Fractions II + III, IV, and the subfractions high in lipoids for analysis. When dialyses are set up immediately following separation of fractions, a very small portion of the material present in the precipitate paste of Fraction IV (methods 1–5) or IV-1 (method 6)[7] is insoluble in routine buffer and can be removed by high-speed centrifugation at 0°. With increasing periods of storage, the amount of insoluble material increases as does the spread of the individual component peaks, thus rendering quantitative interpretation of analyses increasingly difficult. [f] Part of the component migrating in this fraction with mobility characteristic of albumin, either as in plasma or as crystallized, differs strikingly from chemically separated albumin both with respect to lipoid content and solubility characteristics. [g] In solutions of lipoproteins whose turbidity is sufficient to interfere with analysis, clarification can generally be effected by filtration through a #8 Seitz pad. If such filtration is carried out under comparable conditions in following successive fractionations, the distribution data, although subject to correction for selective adsorption of proteins on the filter material, have proved useful for comparative purposes. [h] It is possible to store this fraction either in aqueous solution sterilized by Seitz filtration or as dried from the frozen state. Under the latter circumstances, mobilities have in general been unaffected by periods of storage up to one year. In the former, mobilities have been found to increase by as much as -1×10^{-5} sq. cm./volts/sec. in a period of 1 month. This behavior is in contrast with materials low in lipoid, *e. g.*, Fraction II, Fraction III-1, Fraction V, and crystalline albumin, whose mobilities would appear to be independent of storage conditions under ordinary circumstances. [i] Separated ultracentrifugally from Fraction III-0 by Dr. J. L. Oncley. Whereas mobility in the parent material is consistent with β_1-globulin, highest mobility for the purified fraction was observed when this fraction was added in artificial mixture to lipoprotein IV-1,1. When added to plasma in artificial mixture, it appeared to migrate chiefly with the α_2-globulins (mobility -4.1×10^{-5}). Further studies on factors affecting mobility are in progress. [j] Resolution of α_1- and α_2-globulins is not sufficiently clean-cut for reproducible distributions. [k] The effect of thiocyanate on the mobility of Fraction IV-1,1 would appear to a first approximation independent of mobility changes consequent to storage. (Mobility determinations ($-u \times 10^5$) at pH 8.6, $\Gamma/2 = 0.1$, after one year at 0°.)

	No SCN	NaSCN 0.05 *M*
Dry	5.7	6.9
Sterile solution	7.0	7.8

tributions of slightly higher average values (approximately 60%) on normal individual human plasmas totaling 18. A similar albumin value emerges from Moore's[40] study of 15 individual human sera at unspecified protein concentration-

(40) D. H. Moore, *J. Biol. Chem.*, 21 (1945).

TABLE IV

DISTRIBUTION OF COMPONENTS IN ELECTROPHORETIC SCHLIEREN DIAGRAMS OF NORMAL HUMAN PLASMA POOLS ANALYZED IN SODIUM DIETHYLBARBITURATE BUFFER *p*H 8.6

Pool	Albumins, %	Total α-globulins, %	β-Globulins, %	γ-Globulins, %	Fibrinogen, %	α1-Globulins, %
70	57	14	13	10	6	6
71	55	15	13	10	7	5
72	57	14	12	10	7	5
73	54	14	14	11	7	5
74	54	15	14	11	6	6
75	54	13	14	12	7	5
82	55	14	14	11	6	5
83	55	14	14	11	6	5
84	55	13	14	11	7	5
91A	55	14	13	11	7	5
92A	56	14	13	11	6	6
93A	54	15	13	12	6	6
95A	55	14	13	12	6	6
97	55	14	13	12	6	5
106	55	13	14	11	7	5
107	55	14	13	11	7	5
108	56	14	14	10	6	5
SPA	55	13	14	11	7	5
SPB	56	16	12	10	6	5
162	55	13	14	11	7	5
Average values, plasma pools	55.2	14.0	13.4	11.0	6.5	5.3
Standard dev.	1.3	0.8	1.6	0.7	0.6	0.5
Coeff. of variation	2	6	12	6	9	9

ionic strength ratio. Agreement of our distribution data with the findings of Perlmann and Kaufman[41] on small normal human plasma pools under exactly parallel conditions is close in every respect.

Effect of Mobility Variations with Conditions of Electrophoresis on Apparent Distributions of Components.—Being in general inhomogeneous with respect to form of titration curve and capacity for selective interaction with polar and non-polar materials, the various molecular species comprising an electrophoretic component can exhibit varying rates of change of mobility as a function of *p*H, type of ions in the environment and ionic strength.

The validity of a quantitative second approximation in the electrophoretic control of plasma fractionation depends in good part on the empirical appraisal of the effects of such factors on apparent electrophoretic distributions.

Effect of *p*H.—Experiments carried out in several buffers at a variety of *p*H ranges[42] have confirmed the finding of Longsworth[5] that most uniform separation of plasma components is attained at *p*H 8.6 using diethylbarbiturate.

That the separation of α_1-globulin is chiefly a differential *p*H effect is supported by the failure to note separation in buffer below *p*H 8.0.[44]

Below is presented a comparison of the mobilities of the chief components of plasma at identical ionic strength (0.2) and protein concentration in phosphate (*p*H 7.7) and diethylbarbiturate (*p*H 8.6) buffers. While components at the ends of the diagram move slightly faster, the intermediate α_2- and β-globulins move *more slowly* at the higher *p*H than at the lower. This relationship suggests the dependence of distributions in the standard buffers on factors other than *p*H alone.

	$-u \times 10^5$					
*p*H	Albumins	α1-Globulins	α2-Globulins	β-Globulins	Fibrinogen	γ-Globulins
7.7	5.0	No sepn.	3.7	2.7	1.9	1.0
8.6	5.1	4.0	3.0	2.4	1.9	1.2

Effects of Caprylate and Thiocyanate Ions.—The influence of mobility shifts of small segments of the protein population distribution without gross changes in mobility of the chief components is emphasized in comparison of schlieren diagrams of a single plasma pool run at identical protein concentration, *p*H and ionic strength in the presence of thiocyanate or caprylate (Fig. 4) and in standard diethylbarbiturate buffer. The mobilities of the chief components are approximately identical. Under the former circumstances, the area of the β-globulin component, long known in the natural state to be associated with lipoids, is strikingly smaller, and the component next faster in mobility (α_2-globulin) is larger by the same amount than the re-

(41) G. E. Perlmann and D. Kaufman, THIS JOURNAL, **67**, 368 (1945).

(42) **Borate Buffers** *p*H 9.1, $\Gamma/2 = 0.1$—Despite greater separation of mobilities of chief components, the globulin peaks, in exhibiting variable degrees of overlapping, rendered reproducible resolutions difficult. Separation of α_1-globulin from albumin was in general sharp on the ascending side, in part owing to an unusually sharp albumin peak. On the descending side the albumin peak was far wider than seen in either standard buffer. Average of mobilities and apparent distributions for pools of normal human plasma run at 2% protein are given below. When run at 1% protein under identical conditions, the apparent contribution of albumin was 55%; resolution of other components was not satisfactory.

	Albumins	α1-Globulins	α2-Globulins	β-Globulins	Fibrinogen	γ-Globulins
$-u \times 10^5$	6.8	5.5	4.4	3.6	2.4	1.3
Distribution (%)	61	7	5	12	7	8

Glycine Buffer *p*H 9.1, $\Gamma/2 = 0.1$.—The disadvantages encountered in borate at identical *p*H and ionic strength appeared in exaggerated form.

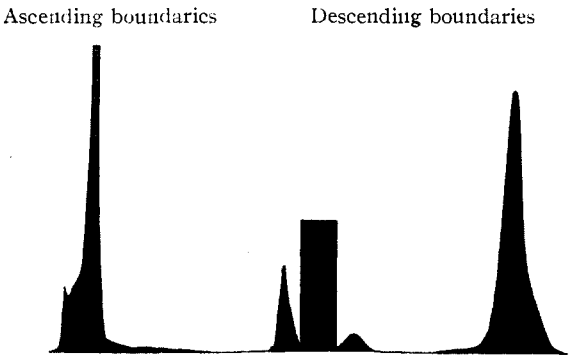
Acetate Buffers.—Extensive observations on the apparent distributions of the 3 components which separate on prolonged electrophoresis in crystalline bovine albumin at *p*H 4.0 have yielded no correlation with the solubility properties of the several crystalline fractions as determined by Hughes and Cohn. These studies have not been extended to the 2 components of human albumin (first described by Luetscher[43]) although the electrophoretic homogeneity of the faster of these components has been established by one of us (S. H. A., Jr.) in collaboration with Dr. C. Riley by fractionation in the separatory electrophoretic cell.

(43) J. A. Luetscher, THIS JOURNAL, **61**, 2888 (1939).

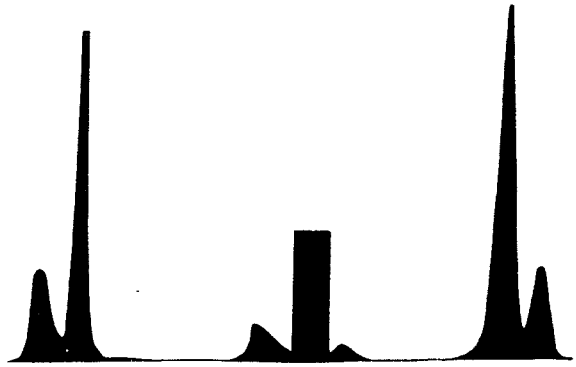
(44) On prolonged electrophoresis in diethylbarbiturate buffer, *p*H 8.6, the ascending boundary, both in plasma and in crystalline albumin, has been observed to resolve into two peaks. Such splitting has been interpreted by Svensson[19] as suggesting selective interaction between buffer ions and a fraction of the protein component.

spective areas of these components in standard buffer.[45] Distributions of the other components show no significant differences.

Purified fractions, believed to consist of natural mixtures of lipoid-rich and lipoid-poor proteins which are close to each other in mobility under standard conditions, have given diagrams similar to those obtained for artificial lipoprotein–albumin mixtures in the presence of thiocyanate and caprylate (Fig. 3). The interpretation of apparent distributions will require a more quantitative understanding of the interactions of ions of this type with purified lipoproteins than is now available.



*p*H 8.6, sodium diethylbarbiturate, Γ/2 = 0.1

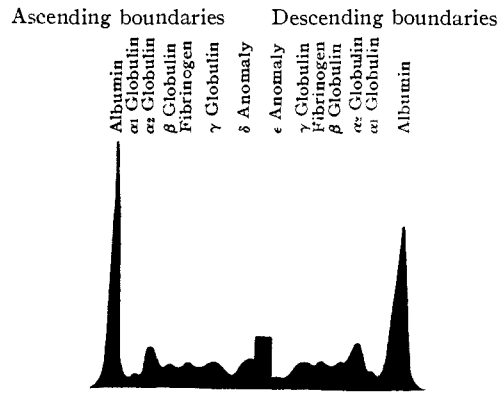


*p*H 8.6, sodium caprylate, Γ/2 = 0.05; sodium diethylbarbiturate, Γ/2 = 0.05

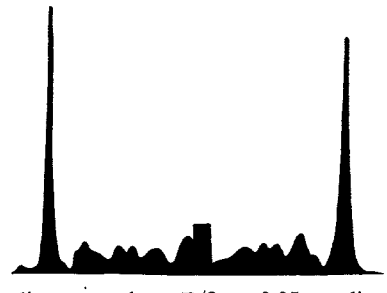
Fig. 3.—Effect of caprylate ion on separation of mixture of proportions {albumin 75% / lipoprotein IV-1,1, 25%

Effects of Ionic Strength and Protein Concentration.—The chief components of plasma and its fractions, while varying in absolute mobilities in a fashion consistent with previous reports and with the findings in mixtures, have shown no variations in relative mobilities or in their spread sufficient to give overlaps when

(45) A small component (in area less than 2% of the diagram) was occasionally observed in the presence of caprylate ahead of the albumin peak. It was most prominent on the ascending side. Whether it represents a very small amount of lipoprotein separable from the albumins under these conditions, or whether it is a false boundary (of the type intermediate in mobility between the buffer anions[19]) is undetermined.



*p*H 8.6, sodium diethylbarbiturate, Γ/2 = 0.04; sodium thiocyanate, Γ/2 = 0.06



*p*H 8.6, sodium caprylate, Γ/2 = 0.05; sodium diethylbarbiturate, Γ/2 = 0.05

Fig. 4.—Effect of caprylate and thiocyanate ions on separation of components of normal pooled human plasma.

separation of components has been clean-cut under standard conditions.

In those fractions where such clean-cut separation does not obtain (*e.g.*, Fraction II + III, illustrated in Fig. 5, at extremes of the ionic strength range studied), shifting peak contours (reflecting small mobility changes) are such that variability in resolution of individual diagrams by comparably trained workers has proved too great to permit the evaluation of the influence of the factors treated by Svensson and Dole on apparent distributions.

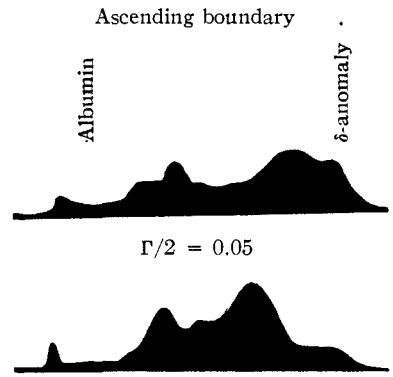


Fig. 5.—Fraction II + III.

The Effects of Protein Concentration and Ionic Strength on the Apparent Distributions of Components of Normal Pooled Human Plasma and its Fractions.—The proteins of a single plasma pool, whose distribution was quite normal under standard conditions, were studied at various protein concentrations and ionic strengths. Results are given in detail in Table V. Whereas differences between individual adjacent values are quite close to the standard deviation of the measurements under routine conditions (in the instance of albumin ±1.3, Table IV), between more widely separated values they are large enough to be of significance.

TABLE V

DISTRIBUTION OF ELECTROPHORETIC COMPONENTS OF PLASMA POOL #170 AS DETERMINED BY ANALYSIS AT SEVERAL PROTEIN CONCENTRATIONS IN SODIUM DIETHYLBARBITURATE OF *p*H 8.6 AND VARYING IONIC STRENGTH

Γ/2	Protein concn., g./100 cc.	Albumins, %	Total α-globulins, %	β-Globulins, %	Fibrinogen, %	γ-Globulins, %	α1-Globulins, %
0.05	2.5	57.9	10.7	14.0	6.3	11.1	4.4
	2.0	57.7	12.5	14.3	6.5	9.0	3.6
	1.0	55.9	13.9	15.0	6.3	8.9	4.2
0.10	2.3	57.3	11.2	14.7	6.7	10.1	4.0
	2.0	55.1	14.2	12.3	7.8	10.6	5.0
	1.5	55.4	13.2	13.6	6.6	11.2	5.4
	1.0	53.7	14.8	14.2	7.0	10.3	5.4
0.20	2.5	53.4	12.3	15.1	6.6	12.6	6.6
	2.0	52.3	14.9	13.8	7.3	11.7	4.7
	1.0	51.4	15.0	14.3	6.9	12.4	5.6
0.30	2.0	51.3	14.4	16.3	5.8	12.2	5.7
	1.5	50.4	15.8	14.8	6.3	12.7	8.3
	1.0	51.0	15.4	14.5	6.7	12.4	6.8

As in the case of artificial mixtures, in general the apparent albumin contribution to the diagram decreases both with decreasing protein concentration and with increasing ionic strength. The effect of protein concentration is less marked at ionic strength 0.3 than at ionic strength 0.1.

Two further changes with increasing ionic strength compensate for decreasing albumin, namely, increases in the apparent contribution of the α-globulins, and in the γ-globulins only slightly greater than the standard deviation of the values as measured under routine conditions. The increase in α_1-globulins at ionic strengths above 0.2 may be ascribed to a small but fairly distinct component intermediate in mobility between α_1-globulin and albumin.

Perlmann and Kaufman,[41] in studying the effects of protein concentration and ionic strength on the schlieren diagrams of small normal human plasma pools at *p*H 8.6, have encountered variations essentially identical in magnitude and direction with those here observed, although our limiting value for albumin concentration at ionic strengths of 0.2 or greater is lower than theirs by approximately 2%.

Apparent Distributions of Plasma Proteins in Terms of the Theoretical Treatment of Dole.—There are presented below the theoretical values for the apparent electrophoretic distribution of a three-component mixture containing albumin, globulins of intermediate mobility (treated as a single component) and γ-globulin at the extremes of the experimental range of protein concentration and ionic strength given for the data on plasma.[46] The theoretical albumin values together with the experimental values (Table V) are plotted against the protein concentration–ionic strength ratio in Fig. 6, analogous to the plot for artificial mixtures in Fig. 2. As in the case of these mixtures, so also in plasma theoretical changes with changing conditions are of the same sign but considerably smaller than those encountered in experiment.

Γ/2	Protein concn., g./100 cc.	Albumins, %	Globulins of intermediate mobility, %	γ-Globulins, %
0.05	2.5	54.4	34.0	11.6
.30	1.0	52.2	35.9	11.9
Actual distribution		52.0	36.0	12.0

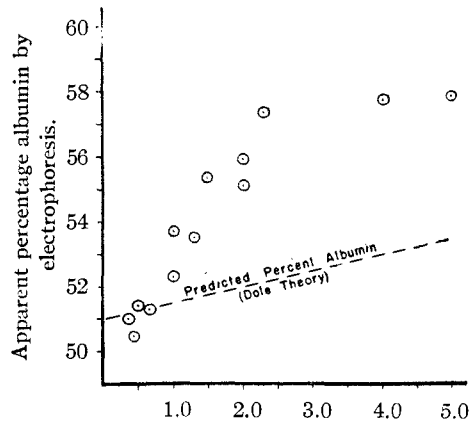


Fig. 6.

At high values of the protein–ionic strength ratio, the theoretical positive deviation of the component of highest mobility (albumin) is not counterbalanced in a mixture of three or more components by an equivalent negative deviation of a component of lowest mobility (γ-globulin). Thus, theoretical considerations give no explanation of the very small apparent selective increase in the γ-globulin content of plasma with decreasing protein concentration and increasing ionic strength suggested by both our data and the data of Perlmann and Kaufman.

(46) Conventions of calculation are those used in artificial mixtures. The following *relative mobilities* have been used for protein components.

Albumins	−6.0
Intermediate globulins	−3.5
γ-Globulins	−1.0

For those fractions in which satisfactory reproducibility (by reason of clear separation) is attainable, shifts have been both comparable to those already given in detail for artificial mixtures of similar electrophoretic characteristics and of approximately the same ratio to theoretically predicted deviations. Theoretical and experimental considerations lead to expectation of greatest deviations of apparent from actual distributions in the following circumstances: (1) multiple components of which one lies at either extreme of the mobility scale and approximates in apparent contributions the sum of the other components; (2) two components, close both in relative mobilities and apparent contributions.

Although, for plasma and its fractions, it is by no means proved that at high ionic strengths and low protein concentrations apparent electrophoretic distributions approach true relative concentrations as closely as in artificial mixtures, the agreement cited provides the basis for the small correction factors applied in a subsequent section to the analyses of plasma and of the various fractions as carried out under the standardized conditions needed for control of uniformity in fractionation.

The Magnitudes of Schlieren Diagram Revisions Consequent upon Differences in Component Refractive Index Increments

Conversion Factors

If W_c, W_m be the concentrations (g./l.) of a component c and of the mixture m containing c, respectively; and if $\Delta n/\Delta W_c$, $\Delta n/\Delta W_m$ be the corresponding specific refractive index increments, the contribution of the component to a true schlieren diagram of the mixture is

$$\frac{\text{Area}_c}{\text{Area}_m} = \frac{W_c(\Delta n/\Delta W_c)}{W_m(\Delta n/\Delta W_m)}$$

Thus, the conversion factor, which, when multiplied by the contribution of the component to the schlieren diagram, yields its relative concentration in the mixture, is the ratio of the refractive index increment of the mixture to that of the component.

Relative concentration figures are applicable to estimation of absolute quantities of a component when the quantities and the refractive increments are expressed in the same units of measure.

Values of Conversion Factors.—In Table VI are given conversion factors for the principal electrophoretic components of schlieren diagrams of plasma and the chief fractions, based on the specific refractive index increments (in terms of weight of dried protein) which are listed opposite each fraction and component.

For the albumin and γ-globulin components the refractive increment values derive from measurements on materials electrophoretically separated from human plasma. The close approach, with increasing purity, of their specific increments to the specific increment of the component separated chemically in course of fractionation is illustrated in the measurements on a group of albumin preparations detailed in Table VII.[47]

TABLE VI

CONVERSION FACTORS: SCHLIEREN DISTRIBUTIONS TO RELATIVE CONCENTRATIONS APPLICABLE TO PROTEIN FRACTIONS MEASURED BY WEIGHT OF DRIED PROTEIN

	$\frac{\Delta n \times 10^4}{\Delta W}\left(\frac{\text{g. protein}}{\text{liter}}\right)$	Electrophoretic components: Albumins	α-Globulins	β-Globulins	Fibrinogen	γ-Globulins
	1.85	1.80	1.76	1.88	1.86	
Pooled plasma	1.83	0.99	1.02	1.04	0.97	0.98
Fraction I	1.86	1.00	1.03	1.06	0.99	0.98
Fraction II + III	1.80	0.97	1.00	1.02	0.96	0.97
Fraction IV-1	1.79	0.97	0.99	1.02	0.95	0.96
Fraction IV-4	1.84	1.00	1.02	1.04	0.98	0.99
Fraction V	1.86	1.00	1.03	1.06	0.99	1.00

For the other components, values represent tentative estimates based on measurements on subfractions.[48]

By reason of the fact that largest observed differences between specific refractive index increments of plasma proteins (in terms of weight of dried protein) fall below 5%, the conversion factors are so close to unity that the consequent revisions of schlieren distributions, with rare exceptions, are close to the magnitude of experimental error of diagram area measurement.

In following the distribution of *total* protein in the various plasma fractions, the usual method has been determination of protein nitrogen. To data in these terms the conversion factors given in Table VI are *not* applicable; conversion factors based on refractive index increments in terms of nitrogen ($\Delta n/\Delta N = \Delta n/\Delta W \times$ nitrogen factor) are required. Such factors will deviate from unity almost in direct proportion to ratios of nitrogen factors. The considerable revisions of electrophoretic distributions entailed in mixtures of components of varying nitrogen content are illustrated in computations of the albumin contribution to protein nitrogen of three plasmas of varying lipemia (Table VIII).

Range of Application of Schlieren Diagram Revisions

If the distribution of protein nitrogen in a group of plasma fractions is multiplied through

(47) The close agreement between the refractive index increments in terms of nitrogen of the purest electrophoretically separated albumin and of crystalline albumin (1.17×10^{-3} and 1.16×10^{-3}, respectively (Table VII) limits the maximum possible lipoprotein (refractive index increment in terms of nitrogen,[20] 1.80×10^{-3}) content of electrophoretically separated albumin to below 3%.

(48) The arbitrary factors in estimation are greatest in the instance of the α- and β-globulins which, in comprising several proteins of differing carbohydrate and lipoid content, show the widest refractive index increments (in terms of weight of dried protein) variations.

TABLE VII

APPARENT ELECTROPHORETIC DISTRIBUTIONS AND REFRACTIVE INDEX INCREMENTS[8] OF ALBUMIN SEPARATED BY ELECTROPHORESIS FROM NORMAL POOLED HUMAN PLASMA[a]

Cell segment sampled (ascending boundary)	Apparent distribution of electrophoretic components, %					$\frac{\Delta n \times 10^4}{\Delta W\left(\frac{\text{g. protein}}{\text{liter}}\right)}$	$\frac{\Delta n \times 10^3}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$
	Albumins	Globulins α	Globulins β	Globulins γ	Fibrinogen		
775 sq. mm. channel area[b]							
Bottom	80	10	7	1	2	1.82	1.21
Middle	93	6	1	..	..	1.83	1.19
Top	96	4	..	..	..	1.83	1.18
75 sq. mm. channel area[b]							
Top	98	2	..	..	..	1.86	1.18
Top	98	2	..	..	..	1.85	1.17
Cryst. albumin[c]	>99	..	..	..	..	1.86	1.16

[a] In separatory electrophoreses, sodium diethylbarbiturate buffer of *p*H 8.6 was employed in order to attain maximum separation of albumin from the α-globulins, particularly from α_1-globulin. [b] Two types of cells were used: an analytical cell of cross section area 75 sq. mm. and a separatory cell of cross section area 775 sq. mm. In the former, a higher degree of purity was achieved than in the latter owing to the fact that dissipation of heat from the 75 sq. mm. cell is sufficiently rapid to permit a potential gradient of 10 volts/cm., which effects as complete separation as the length of the cell permits within 36 hours. The yields, however, from each run were quite small, averaging 40 mg. of protein. Slow diffusion of heat from the 775 sq. mm. cell requires potential gradients below 1.5 volts/cm. Thus, although the average yield for equivalent separation of boundaries was approximately ten times that obtained in the analytical cell, the time required for such separation was over five times as long with consequent increased diffusion. [c] Chemically separated.[8]

TABLE VIII

CONVERSION OF SCHLIEREN ALBUMIN CONTRIBUTIONS TO BASIS OF PROTEIN NITROGEN

Material	Refractive index increments $\frac{\Delta n \times 10^4}{\Delta N\left(\frac{\text{g. protein N}}{\text{liter}}\right)}$	Conversion factor $\frac{\Delta n/\Delta N\text{ (plasma)}}{\Delta n/\Delta N\text{ (albumin)}}$[a]	Albumin contribution to schlieren diagram, %	Albumin contribution to protein nitrogen, %
Pooled normal human plasma proteins	1.24	1.06	51	54
Pathological plasma proteins (moderate lipemia)	1.63	1.39	32	45
Pathological plasma proteins (severe lipemia)	2.07	1.77	9	17

[a] $\Delta r/\Delta N$ (albumin) = 1.16×10^{-4} (*Cf.* Table VII).

by their respective nitrogen factors, the findings of the previous section (Table VI) indicate that apparent schlieren diagram data should be applicable to resultant weight distributions with very little error on the basis of refractive increment. This has been done for normal human plasma, as fractionated by method 6, in paper IV of this series (Table IX).[7] Apparent electrophoretic schlieren distributions under standard conditions (sodium diethylbarbiturate, *p*H 8.6, ionic strength 0.1) were employed.

TABLE IX

DISTRIBUTION (GRAMS PER LITER OF PLASMA) OF ELECTROPHORETIC COMPONENTS IN PLASMA AND TOTAL PLASMA FRACTIONS (METHOD 6) AS ESTIMATED FROM STANDARD AND REVISED SCHLIEREN DATA

	Electrophoretic components					
	Albumins	α-Globulins	β-Globulins	γ-Globulins	Fibrinogen	Total
Standard Distributions. Nitrogen Factor Assumed 6.25						
Plasma	33.8	8.6	9.9	6.8	2.5[a]	61.5
Total, fractions I–VI	32.8	8.7	10.6	6.5	2.9	61.5
Standard Distributions Corrected for Nitrogen Factor Differences						
Plasma	36.3	9.2	10.6	7.2	2.5[a]	65.8
Total, fractions I–VI	32.6	10.1	12.6	7.4	3.1[a]	65.8
Revised Distributions Corrected for Nitrogen Factor and Refractive Increment Differences						
Plasma[b]	34.8	10.1	11.4	8.0	2.5[a]	65.8
Total, fractions I–VI[c]	33.0	9.2	12.7	7.8	3.1[a]	65.8

[a] Distributions of fibrinogen have been treated as described in paper IV of this series,[7] Table IX, note *a*. [b] The distribution for plasma is derived from average values at $\Gamma/2$ = 0.2–0.3 (Table V) converted to basis of weight of dried protein by factors given in Table VI. [c] The chief divergences from distributions under standard conditions are as follows: II + III, an estimated 2–3% increase in γ-globulin contribution over standard conditions is estimation at protein 1%, $\Gamma/2$ = 0.2. IV-1, on the basis of tentative interpretations of runs in the presence of thiocyanate 10 to 20% of the material of average mobility -6.0×10^{-5} is classified as albumin. IV-4, distributions of β-globulin increases by about 5% at expense of other component, at low protein-ionic strength ratios. VI, on the basis of close mobilities of components, the variation of distribution with protein–ionic strength ratio is greatest of all the fractions but is of little significance in this summation owing to its small protein content (1 g. per liter of plasma).

In Table IX, the results (g. protein per 1000 cc. plasma) computed in this fashion for plasma and the sum of the chief fractions are contrasted with (a) corresponding results assuming uniform nitrogen factor (6.25), and (b) corresponding results corrected for varying nitrogen factor and employing apparent electrophoretic distributions obtained under conditions believed to give close approximation to actual relative concentrations and further corrected for specific refractive increment differences.

The best internal agreement between plasma and the sum of its fractions involves the least justifiable conditions of estimation, namely, a constant nitrogen factor of 6.25 (experimental factors vary from 6.10 to 8.40).

It would appear, then, that either uncertainties of diagram resolution (Fractions II + III, IV-1), or labile mobilities of parts of the β and α components[49] can introduce errors as large as those studied in the well-resolved artificial mixtures or introduced by differing nitrogen factors and specific refractive index increments. Moreover, these errors can compensate one another.

From this it follows that whereas the apparently small difference between the first and second approximations for the γ-globulin content of a liter of plasma (1.2 g.) represents a difference of 3,000 final packages in the γ-globulin yield from the large-scale processing of 1,000 liters, the electrophoretic method is not well adapted to the setting of yield standards within these limits. Such standards have been in general reached by analytical methods capable of greater precision on the products of successive fractionations under uniform conditions.

The chief value of the electrophoretic method has been in the indication (by large apparent distribution differences with systematic variation of fractionation conditions, of optimum separations for uniform large-scale processing. For this purpose, its use in first approximation under standard conditions has in general been as satisfactory as in second approximation and far less expensive in time and experiment.

It may emerge that in certain subfractionations of plasma or similar systems susceptible of more accurate resolution, close relative mobilities and apparent distributions may require protein-ionic strength ratios and buffer anion mobilities differing from standard sodium diethylbarbiturate. Both theoretical considerations and mixture studies, however, indicate that deviations of apparent distributions from relative concentrations decrease with increasing electrophoretic homogeneity of the component separated from such a system; thus, in some instances it may prove more practical to estimate actual distributions in a mixture from quantitation of pure subfraction than from systematic variation of conditions of electrophoresis. The latter procedure gains further validity from the absence of any simple correspondence between electrophoretic studies and systematic studies of plasma and its fractions by other physical-chemical methods which can be better interpreted in terms of molecular properties.[35]

The same general considerations apply to the value of detailed refractive increment measurements on subfractions whose further purification is to be undertaken.

A very practical import of the refractive increment revision is in the estimation, in animal or human studies, of variations of concentration of a lipoid-poor plasma component in the presence of significant lipemia.[51] The accurate determinations of such levels is impossible without either simultaneous determinations of plasma specific refractive increments or, as a first approximation, nitrogen factors.[52] In this type of investigation, where relative changes are of principal importance, the errors due to superimposed "foreign" colloid and buffer ion gradients are, under standard conditions, sufficiently small and sufficiently constant to be neglected.

(49) Studies carried out on whole plasma thrice extracted by the method of MacFarlane[50] have shown striking diminutions of β-globulins with corresponding increase of the α_2-globulins sufficient to double their apparent contribution to the diagram. The α_1-globulin component likewise is diminished, and the albumin increased to approximately the same extent.

(50) A. S. MacFarlane, *Nature*, **149**, 439 (1942).

Summary

In artificial two-component mixtures of purified plasma proteins of known electrophoretic properties in sodium diethylbarbiturate at *p*H 8.6, deviations of apparent electrophoretic distributions from known relative concentrations have in general been found to vary inversely with the difference between relative mobilities and with divergence of the ratio of relative concentration of the components from unity, although directly with the ratio of the protein concentration to the ionic strength obtaining during analysis.

Experimental deviations have been consistent both in sign and in sign of variations with estimates based on recent theoretical treatments, but were somewhat greater in magnitude.

Variations of apparent distributions of the electrophoretic components of plasma and of its chief fractions have proved comparable to those found in artificial mixtures of similar electrophoretic characteristics.

In artificial mixtures of purified plasma proteins differing in natural association with lipoids, unequal variations in effect of barbiturate, caprylate and thiocyanate anions on component relative mobilities have yielded striking differences between apparent electrophoretic distributions. Comparable differences in apparent distributions of the α- and β-globulin components in plasma and certain fractions run in the presence of mixtures of these anions have been observed. Analytical electrophoretic data alone have not given a sufficiently quantitative appraisal of strong but differing anion-protein component interactions to permit reliable estimation of unknown relative concentrations from comparison of apparent distributions.

(51) The largest errors are encountered in applying uncorrected schlieren distributions (with falsely low albumin and γ-globulin values) to total protein measurements by methods based on serum specific gravity, also falsely low with high lipemia.

(52) When levels are to be followed as a function of parenterally administered protein, two alternate procedures are possible if the protein and the patient's plasma proteins differ significantly in fat: (a) Quantitative electrophoretic mixture studies as described by Luetscher.[53] This has the added advantage of giving a direct check on *where* in the diagram the added protein migrates. At *p*H 7.7, for example, globin, a basic protein, is not limited in distribution to a single component. (b) Refractive index increment measurements (or as a first approximation, nitrogen factor measurements) on the plasma and the protein to be administered.

(53) J. A. Luetscher, Jr., *J. Clin. Investigation*, **23**, 465 (1944).

The observed mobility ranges for the chief electrophoretic components of plasma and its fractions, as studied under conditions standard for control of large-scale fractionation, fall into two classes: (1) components poor in lipoids, whose characteristic mobilities appear independent of conditions of fractionation, degree of purity and usual conditions and duration of storage; (2) components rich in lipoid whose characteristic mobilities vary with these factors, generally toward increasing negativity, thus yielding overlaps of mobility range with components of the first class.

The small variations of apparent electrophoretic distributions of normal human plasma pools under standard analytical conditions is indicated in a series of analyses carried out over a period of years in potassium phosphate at *p*H 7.7 and sodium diethylbarbiturate at *p*H 8.6. Comparisons with apparent distributions in other buffers less useful for fractionation control are given.

Specific refractive index increments of electrophoretically separated albumin and γ-globulin have been found to approach with increasing electrophoretic homogeneity the increments for these components as separated chemically.

Conversion factors (based on refractive index increments in terms of weight of dried protein) have been computed for the chief electrophoretic components in plasma and the major fractions. When applied, together with corrections based on nitrogen factor differences and on deviations of apparent distributions from true relative concentrations in mixture studies, in revision of schlieren data for a second approximation of the distribution of components in plasma and fractions, the internal consistency of the results has proved little better than when unrevised apparent distributions obtained under standard conditions are used. Two factors appear chiefly responsible: uncertainties in diagram resolutions of components comprising molecular species of considerable mobility spread, together with changes of apparent distributions resulting from slight mobility changes with processing.

The principal value of the electrophoretic method in the control of fractionation has been its use in quantitative first approximation: (a) for revealing gross differences of apparent distributions with variation in conditions of fraction separation, and (b) as a rapid empirical control of uniformity of successive separations under constant conditions.

Specialized indications for the use of corrected electrophoretic diagrams in clinical and biological investigation are given.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

A Photoelectric Instrument for Comparing the Concentrations of Very Dilute Aerosols, and Measuring Low Light Intensities[1]

BY FRANK T. GUCKER, JR., HUGH B. PICKARD[2] AND CHESTER T. O'KONSKI

A study of aerosols and their filtration requires a rapid and sensitive method of measuring aerosol concentration. Under usual working conditions, the most penetrating aerosols, liquids of 0.3 micron (μ) diameter, are used at about 100 micrograms (γ) per liter. Coarser particles impinge upon the filter fibers because they cannot follow the sharply-bending flow lines, while finer particles diffuse to the fibers more rapidly because of their Brownian movement. Smokes much over 100 γ per liter coagulate rapidly and tend to clog the filter. Direct weighing or chemical methods require excessively long test periods. However, the optical effect of smokes, first noted by Michael Faraday,[3] and studied by John Tyndall,[4] yields a rapid and sensitive measure of the concentration of a colloidal system. Richard C. Tolman and Elmer B. Vliet,[5] described a Tyndallmeter employing visual measurement of the light scattered at right angles to the Tyndall beam. They showed that this light is proportional to the concentration of ammonium chloride smoke, from 1000 to 5 γ per liter, the lowest concentration they studied.

Recently, Victor K. LaMer and David Sinclair, working under the National Defense Research Committee at Columbia University, developed an elegant apparatus for observing the light scattered at small forward angles, which is much more intense for particles of 0.3 μ diameter than that scattered at right angles. Dr. Sinclair[6] states that he could detect the light from a dioctyl phthalate (DOP) smoke at 0.001 γ per liter. However, at this concentration the field of view contains so

(1) This paper is based on work done for the Office of Scientific Research and Development under Contract OEMsr-282 with Northwestern University. It was presented before the Division of Physical and Inorganic Chemistry at the meeting of the American Chemical Society at Chicago, in September, 1946. A fuller description of certain phases of the work is contained in a thesis presented to the Graduate School of Northwestern University by Chester T. O'Konski, as partial fulfillment of the requirements for the degree of M.S. in June, 1946.

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(3) M. Faraday, *Proc. Roy. Soc.* (London), **6**, 356 (1857).

(4) J. Tyndall, *Phil. Mag.*, [4] **37**, 384 (1869).

(5) R. C. Tolman and E. B. Vliet, THIS JOURNAL, **41**, 297 (1919).

(6) Private communication.