[CONTRIBUTION FROM THE DEPARTMENTS OF MEDICINE OF THE MASSACHUSETTS GENERAL HOSPITAL AND HARVARD MEDICAL SCHOOL]

The Effect of Ionic Strength and Protein Concentration in the Electrophoretic Analysis of Human Plasma[1,2]

BY G. E. PERLMANN AND D. KAUFMAN

In a recent paper the use of sodium diethylbarbiturate of *p*H 8.6 and 0.1 ionic strength was recommended for the electrophoretic analysis of human plasma and serum.[3] In this buffer certain discrepancies between the descending and ascending patterns are minimized. Moreover a new component, designated as α_1-globulin which migrates with a mobility between that of albumin and that of α_2-globulin, is resolved from the albumin.

While using sodium diethylbarbiturate for the characterization of pathological sera and plasmas, some anomalies were noticed which were believed to be caused by interaction of the protein constituents. Since it is known that interaction can be reduced by increasing the ionic strength of the buffer, the influence of the salt concentrations on the apparent electrophoretic distribution of proteins in normal human plasma was investigated. After these experiments had been started, an article by Svensson[4] came to our attention in which the errors obtained in the electrophoretic analysis of serum in a phosphate buffer of *p*H 7.7 at various ionic strengths are discussed. It therefore seemed of interest to compare the effects obtained in diethylbarbiturate buffer with the ones in phosphate. The results of these investigations are presented in this report.

Experimental

Plasma for analysis was obtained by mixing 10 volumes of blood with 1 volume of a 4% citrate solution. The red cells were allowed to settle and the supernatant plasma decanted. Usually three or four different samples of normal plasma were pooled and analyzed for protein. The Pregl micro-Kjeldahl method was used for the determination of total nitrogen and of the non-protein nitrogen on a tungstic acid filtrate.

The plasma was diluted with buffer to the desired protein nitrogen concentration and dialyzed at 5° for three days with daily change of buffer. The buffer solution used was 0.1 *N* sodium diethylbarbiturate at *p*H 8.6 and 0.1 ionic strength. In some cases sodium chloride was added to obtain a higher ionic strength. The concentrations of the buffers were checked by conductivity measurements.

The electrophoretic experiments were performed at 1.5° in the apparatus described by Tiselius,[5] equipped with the Schlieren scanning device of Longsworth.[6] A single section cell of 11.0 ml. capacity was used.

The concentrations of the electrophoretically separable components were calculated from the planimetric measurements of enlarged tracings of the diagrams. The method suggested by Tiselius and Kabat[7] was used to allocate the area to each peak.[8] Several tracings were made of each diagram. The average variation in the area of the albumin component between such tracings was ±0.5%.

Results

Influence of Change in Ionic Strength.— A considerable portion of this research has been directed toward determining the variation in apparent relative concentrations of electrophoretic components with variation in ionic strength. The experiments were done with plasma diluted to a nitrogen content of 3.0 mg. per ml., corresponding to 1.87% protein, assuming a nitrogen factor of 6.25. During dialysis some dilution of the samples occurred, but the variations in concentration, as estimated from the final refractive index pattern, were so small as to be negligible. The buffer system, which had a *p*H of 8.6 at 25°, contained 0.1 *N* sodium diethylbarbiturate, 0.02 *N* diethylbarbituric acid, and sodium chloride in amounts varying from 0.0 to 0.3 *N*. The addition of sodium chloride had no significant effect on the *p*H of the buffer. Typical electrophoretic patterns of plasma obtained at ionic strength 0.1 and 0.2, respectively, are shown in Fig. 1. The patterns do not show marked differences. There is, however, a slight decrease in the areas under the δ and ϵ peaks at the higher ionic strength; moreover the β boundary anomaly usually found in the descending pattern is reduced under those conditions[9] and there is a somewhat higher degree of symmetry between the descending and ascending diagrams.

Table I shows the apparent distribution of the protein components which was obtained in four

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(3) Longsworth, *Chem. Rev.*, **30**, 323 (1942).

(4) Svensson, *Ark. Kemi, Mineralogi og Geologi*, **17A**, Nr. 14 (1943).

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

(6) Longsworth, THIS JOURNAL, **61**, 529 (1939).

(7) Tiselius and Kabat, *J. Exptl. Med.*, **69**, 119 (1939).

(8) Some of the diagrams have been evaluated also by following the procedure of Pedersen (Pedersen in Svedberg and Pedersen "The Ultracentrifuge," Oxford University Press, London, 1940, p. 296). The variations between these two methods were slight and would not affect the interpretation of the results. A detailed comparison of the two methods of evaluation of electrophoretic diagrams has recently been given by Longsworth.[3]

(9) An observation made by Dr. Longsworth, private communication.

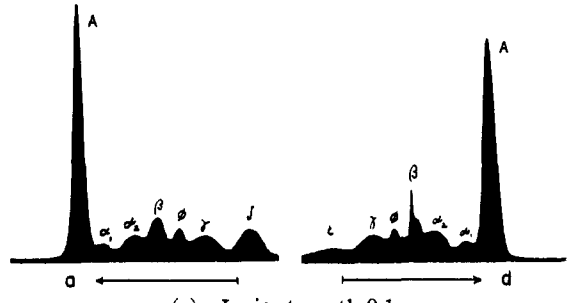
TABLE I

ELECTROPHORETIC DISTRIBUTION OF PROTEINS IN A 1.87% SOLUTION OF NORMAL HUMAN PLASMA IN SODIUM DIETHYLBARBITURATE BUFFER OF *p*H 8.6 AT VARIOUS IONIC STRENGTHS[a]

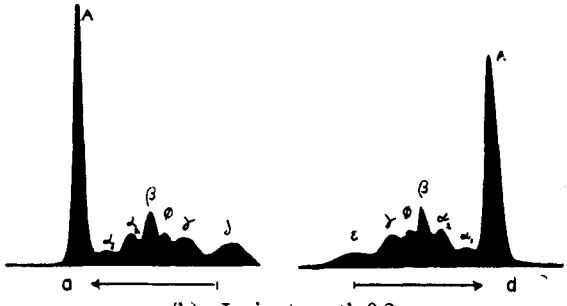
Buffer composition	Ionic strength	Concentration in per cent. as Albumin	Globulins α_1	α_2	β	ϕ	γ
0.1 *N* NaV	0.1	57.8	4.3	9.3	13.0	5.8	9.8
0.02 *N* HV		57.8	4.0	9.4	13.2	5.9	9.7
0.1 *N* NaV							
0.02 *N* HV	0.15	56.5	4.4	9.8	12.7	5.8	10.8
0.05 *N* NaCl		56.2	4.1	8.6	13.8	5.4	11.9
0.1 *N* NaV							
0.02 *N* HV	0.2	54.8	4.2	9.8	13.0	5.9	12.3
0.1 *N* NaCl		54.8	4.3	9.6	13.3	5.8	12.2
0.1 *N* NaV							
0.02 *N* HV	0.3	54.6	4.2	9.7	13.4	6.0	12.1
0.2 *N* NaCl		54.8	4.0	9.1	13.9	6.1	12.1

[a] The upper figures in each case represent those obtained from the descending patterns.

experiments when the ionic strength was varied from 0.1 to 0.3. The results are expressed as the ratio of the area ascribed to each component to the total area exclusive of the δ and ϵ boundaries. The compositions of the descending as well as of the ascending patterns are given. The most striking feature is a decrease in the apparent albumin concentration from 57.8%, obtained at 0.1 ionic strength, to 54.8% in the buffer of 0.2 ionic strength. No significant changes were found to occur in the α_1-, α_2-, β-globulin and fibrinogen. However, the observed increase in the concentration of the slowest moving component, the γ-globulin, exceeds the errors due to variable degrees of resolution and to the arbitrary allocation of area to this component. Increasing the salt concentration above 0.2 ionic strength does not alter the apparent distribution. At 0.4 ionic strength, however, greater variation among the globulin components was noticed. In this experiment the potential gradient had to be reduced considerably to avoid convection effects. Thus the experiment had to be carried out over a period of sixteen hours during which time the effect of diffusion became noticeable. The separation of the globulins was less complete. The results indicate that a limiting value in the apparent relative concentration of albumin is reached in a buffer medium of 0.1 *N* sodium diethylbarbiturate, 0.02 *N* diethylbarbituric acid, and 0.1 *N* sodium chloride when the protein concentration is 3.0 mg. nitrogen per ml.



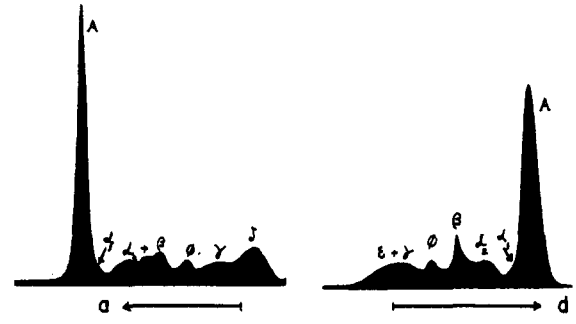
(a) Ionic strength 0.1.



(b) Ionic strength 0.2.

Fig. 1.—Electrophoretic patterns of a 1.87% solution of normal human plasma in sodium diethylbarbiturate buffer of *p*H 8.6 and at: (a) ionic strength 0.1 after electrophoresis for 13,020 seconds at 5.21 volts per centimeter; (b) ionic strength 0.2 after electrophoresis for 16,200 seconds at 4.09 volts per centimeter.

Anomalies at Low Salt Concentration.—In Fig. 2, diagrams are reproduced which were obtained with the same plasma concentration as that used for the experiment illustrated in Fig. 1a. The buffer medium, however, consisted of 0.05 *N* sodium diethylbarbiturate and 0.01 *N* diethylbarbituric acid. This pattern is of interest in that it shows an asymmetry between the α_2- and β-globulin portions of the descending and ascending patterns. Since the α_1-globulin was not resolved from the albumin and no separation of the γ and ϵ boundaries was obtained, a comparison of the composition with that computed from Figure 1a is impossible. An increase in the ionic strength to 0.1 by addition of 0.05 *N* sodium chloride resulted in a pattern identical with that of Fig. 1a.



Ionic strength 0.05.

Fig. 2.—Electrophoretic patterns of a 1.87% solution of normal human plasma in sodium diethylbarbiturate buffer of *p*H 8.6 and at ionic strength 0.05 for 13,500 seconds at 4.03 volts per centimeter.

Influence of Change in Protein Concentration.—Since it had been shown[10] that boundary anomalies, such as the δ and ϵ effect, can be de-

(10) Longsworth and MacInnes, THIS JOURNAL, 62, 705 (1940).

pressed to a certain extent by reducing the protein concentration, it seemed of interest to compare measurements in which the buffer composition was kept constant and the protein concentration varied from 0.9 to 2.5%. In Table II the complete analysis of these experiments is given. A comparison with the values listed in Table I shows that the effects observed in both series of experiments are of the same magnitude.

TABLE II

ELECTROPHORETIC DISTRIBUTION OF PROTEINS IN NORMAL HUMAN PLASMA IN SODIUM DIETHYLBARBITURATE BUFFER OF *p*H 8.6, AT IONIC STRENGTH 0.1 AND VARIOUS PROTEIN CONCENTRATIONS[a]

Mg. nitrogen per ml.	Concentration in per cent. as					
		Globulins				
	Albumin	α_1	α_2	β	ϕ	γ
4.0	58.4	4.2	9.5	12.3	5.8	9.8
	58.1	4.2	9.3	12.9	5.8	9.7
3.0	57.8	4.3	9.3	13.0	5.8	9.8
	57.8	4.0	9.4	13.2	5.9	9.7
2.25	56.4	4.3	9.4	13.3	5.9	10.7
	56.3	4.1	9.5	13.2	6.2	10.7
1.5	54.6	4.3	9.4	14.2	6.1	11.4
	54.6	4.3	9.2	14.2	6.5	11.2

[a] The upper figures in each case represent those obtained from the descending pattern.

If the apparent relative concentrations found for the albumin component are plotted against the starting protein concentration, an approximately linear relationship is obtained. This is illustrated in Fig. 3.

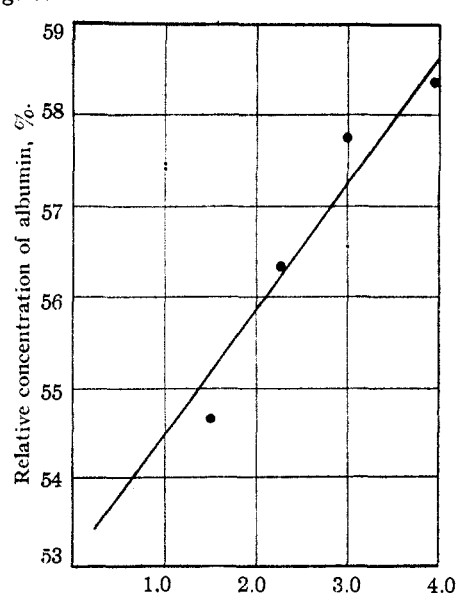


Fig. 3.—Relative concentration of albumin as a function of the protein concentration.

Discussion

The routine procedure in the evaluation of electrophoretic Schlieren diagrams is to compute the concentration of each component from the ratio of its area to the total area exclusive of the δ and ϵ boundaries. Since, however, the electrophoretic patterns represent the change in refractive index due not only to the protein but also to superimposed salt gradients, this procedure involves certain assumptions, as pointed out by Longsworth. Thus it is assumed that the refractive index change caused by the salt is proportional to the protein gradient and that the proportionality factor is the same for all proteins in a given solution.[3] Moreover, since the proteins migrate through a mixture of varying composition caused by the partial separation of the components under the influence of the electric current, an additive gradient due to protein ions other than the one responsible for the boundary will also be superimposed except in two cases: in the fastest moving component on the ascending side, the albumin, and in the slowest on the descending side, the γ-globulin.

Svensson[4] called attention to these superimposed protein gradients. He also predicted variations in the apparent relative concentrations by assuming that the change in concentration is directly proportional to the changes in conductivity of all ion species present and inversely proportional to the mobility of the buffer ions. Using a 4% solution of pig serum in phosphate buffer with ionic strength 0.1 to 0.4, he recorded an apparent decrease in the albumin from 59 to 44% and a simultaneous increase in all globulin fractions.

The results of the experiments on human plasma in a sodium diethylbarbiturate buffer presented in this paper seem to confirm Svensson's findings. The changes in the apparent relative concentration of albumin from about 57% in a buffer of 0.1 ionic strength to 54% at 0.2 ionic strength and the increase in the γ-globulin from 10 to 13% are significant.

In the experiments in which the ionic strength of the buffer was kept constant but the protein concentration altered, changes of the same order of magnitude were observed. A comparison of the values in Table I with those listed in Table II indicates that it is the ratio of protein concentration to ionic strength that influences the apparent distribution of proteins in an electrophoretic diagram.

Since plasma, a protein mixture of at least six electrophoretic components, represents a very complex system, preliminary experiments on an artificial mixture of bovine serum albumin and pseudo-γ-globulin were carried out. An increase in ionic strength from 0.1 to 0.2 in such a solution containing 3.0 mg. protein nitrogen per ml. caused a change in the apparent relative concentration of the albumin from 52.4 to 50.3% and from 47.6 to 49.7% in the γ-globulin.

It is not possible to derive a dependable picture as to the exact nature of these variations on the

basis of the electrophoresis data thus far obtained. It seems probable that they are caused by the changes of the salt and protein gradients superimposed on any given boundary in the mixture. The proportionality of the relative concentration of albumin to the total protein as shown in Fig. 3 is in agreement with the assumption made by Longsworth for superimposed salt gradients.[11]

A similar relationship might perhaps be found for all the variations observed. The results thus indicate that as the ratio of the concentration of protein to that of salt is decreased the relative concentrations, as determined from the ratios of pattern areas, approach their true values. For purposes of accuracy it appears preferable to increase the ionic strength of the buffer medium rather than to reduce the protein concentration as has been pointed out by Svensson.[4] The patterns obtained at low salt concentration (Fig. 2) indicate a different type of phenomenon. An interaction of proteins, usually disregarded in electrophoretic analysis, does exist and becomes apparent with small variations in experimental conditions. It is obvious from the anomalies shown in Fig. 2 that in a mixture, one protein must influence the transference of another protein and therefore affect the electrophoretic separation of components.

(11) Compare footnote on page 336 in reference (3) and Roberts and Kirkwood, THIS JOURNAL, 63, 1373 (1941).

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Summary

The effect of ionic strength and protein concentration on the apparent distribution of the components in the electrophoretic analysis of normal human plasma was investigated. A sodium diethylbarbiturate buffer of *p*H 8.6 was used.

In a 2% protein solution the apparent concentration of albumin decreased from 57 to 54% and the γ-globulin rose from 10 to 13% when the ionic strength was increased from 0.1 to 0.3.

When the ionic strength of the buffer was kept constant and the protein concentration varied, changes of the same order of magnitude were found.

The results indicate that the ratio of the concentration of protein to that of salt influences the apparent distribution of proteins in an electrophoretic diagram. On decrease of this ratio true values for the relative concentrations are approached.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY]

Reaction of Methylene Chloride and other Halide Vapors with Sodium

BY ALFRED SAFFER[1] AND T. W. DAVIS

A study of the reaction of alkyl iodides with metallic sodium[2] at temperatures in the neighborhood of 300° led to a consideration of hydrogen-transfer reactions of the type $CH_2X + {-}R \rightarrow RH + {-}CH_2X$, where —R is an alkyl free radical and X is a halogen atom. It was suggested that some of the $-CH_2X$ radicals would form dihalides, *e. g.*, CH_2X_2, by picking up halogen from the original reactant.[3] These dihalides should themselves enter Wurtz-type reactions and, indeed, Bawn and Milstead[4] have found that the treatment of methylene and ethylene dihalides with sodium vapor gives practically 100% ethylene when nitrogen is the carrier gas and nearly 100% methane or ethane when hydrogen is the carrier. The experiments of Bawn and Milstead were carried out by the dilute flame method under such conditions that the initial reactions were certainly in the gas phase. But despite the clarity and directness of these experiments, we have undertaken a further study of the reaction of sodium with dihalides under conditions more closely resembling those in our earlier investigation and we find totally different products from those reported by Bawn and Milstead.

Procedure.—The procedures employed were essentially identical with those used earlier.[2] The organic halide vapors were allowed to bubble through an excess of sodium liquid either continuously or in batches and the products were removed by a Toepler pump, condensed and separated by isothermal evaporation. Material volatile at −196°, −131° and −78° was removed successively and analyzed in an Orsat type apparatus.

Unreacted sodium was destroyed by methyl alcohol and the "black residues" insoluble in the alcohol were removed by filtration, washed, dried, weighed and analyzed. Sodium chloride was determined by Volhard analysis.

The batch addition of halide vapor was accomplished in two different ways and the composition of the products depended on which of the two

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(2) Saffer and Davis, THIS JOURNAL, 64, 2039 (1942).

(3) West and Schlessinger, *ibid.*, 60, 961 (1938).

(4) Bawn and Milstead, *Trans. Faraday Soc.*, 35, 889 (1938).