AN ELECTROPHORETIC STUDY OF BOVINE PLASMA ALBUMIN—METHYL ORANGE COMPLEX AT 25°C.

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Summary

Kern's micro-electrophoresis apparatus has been employed to study the interaction between bovine plasma albumin ($1 \cdot 49 \times 10^{-4}$ M) and methyl orange ($1 \cdot 53$—$25 \cdot 0 \times 10^{-4}$ M) at the laboratory temperature (25° C.) using acetate buffer of ionic strength 0·05 and pH 5·57. The dissociation constant of the protein-dye complex has been found to be $3 \cdot 6 \times 10^{-4}$ while the maximum number of dye molecules combined per molecule of protein is 20. In contrast to observations given in literature, the electrophoretic patterns do not show any asymmetry in the complex boundary at higher dye concentrations.

Studies on the complex formation between bovine plasma albumin and methyl orange have come into great prominence on account of the simplicity of the system for developing the theory of complex formation. It has been recently shown that a study of these complexes will enable one to differentiate between the various proteins (Klotz *et al.*, 1952). There has been a diversity of opinion regarding the temperature effect on the nature of complex formation between bovine plasma albumin and methyl orange. Klotz *et al.* (1949) have found that with an increase in temperature, the dissociation constant increases while the number of the dye molecules in the complex decreases. However, when the protein forms a complex with drugs of sulphonamide (Davis, 1943) or sodium dodecyl sulphate (Putnam *et al.*, 1945), it is reported that there is very little change with temperature, in the dissociation constant as well as in the amount of anion present in the complex. Briggs *et al.* (1950, 1952) have recently employed the electrophoretic data on bovine serum albumin-methyl orange complex at 4° C. to determine the dissociation constant and the maximum amount of dye that can combine with one molecule of the protein. They have noticed a growing asymmetry in the descending limb with an increase in the concentration of the dye. Preliminary experiments carried out in this laboratory at 25° C. did not show any such asymmetry. It was, therefore, considered desirable to study the electrophoretic behaviour of the complex at 25° C. to know the influence of temperature on the dissociation constant and on the composition of the complex. Electrophoretic studies at elevated temperatures provide the additional advantage of employing higher concentrations of the dye for the complex formation.

EXPERIMENTAL

Reagents.—A sample of A.R. *p*-methyl orange was used to prepare the stock solution of the dye. In order to test the purity of the dye, a weighed amount of the substance was ignited and the ash treated with sulphuric acid. The sodium sulphate thus obtained was about 99% of the theoretical value, indicating that the dye was quite pure (Hunter, 1925).

Crystallised bovine plasma albumin (BPA) was kindly supplied by Armour & Co. The stock solution (10·0%) in distilled water was preserved in a frozen state when not in use.

A stock solution of acetate buffer of ionic strength, 0·9 was prepared by employing reagent grade chemicals. The stock solution was diluted to give a final ionic strength of 0·05 and pH 5·57.

Electrophoresis.—The electrophoresis was carried out in Kern's micro-electrophoresis apparatus (Lotmer, 1951; Cohly *et al.*, 1953) at the laboratory temperature. Since the apparatus was not provided with the thermostatic control, a correction was applied for the temperature in calculating the mobilities. In each experiment, the final temperature of the solution in the cell was noted *in situ* by employing a multiple copper-constantan thermocouple and the average value of the initial and final temperature (which did not differ by more than 2°) was taken for calculating the mobilities. The cell system used in this instrument works on the closed-electrode system described by Longsworth and MacInnes (1939). In absence of the thermostatic control, it was noticed that fluctuations in the ambient temperature would alter the volume of the solution in the closed limb, resulting in the movement of the boundary by purely thermal effects. This defect was avoided by converting the cell system into an open-limb arrangement employed by Tiselius (1937). It was possible to form sharp boundaries (Fig. 1 B) by injecting minute quantities of the buffer into the descending limb by a fine capillary. Silver/silver chloride electrodes and potential gradient of 5·0 volts/cm. were used in all the experiments. The electrophoresis was carried out till the patterns traversed the entire length of the cell. The initial and two more successive patterns at an interval of 30 minutes were photographed to calculate the distribution and mobilities of the patterns.

Preparation of the complex.—The complex was prepared by the dialysis equilibrium technique of Klotz *et al.* (1946) and subjected to electrophoretic analysis on the lines worked out by Briggs *et al.* (1950, 1952) and Marvin and Alberty (1950). In Group I experiments, known amounts of the protein and the dye solutions were mixed and the mixture dialysed against the buffer containing an equivalent amount of dye employing cellophane tubing. The dialysis was carried out for 72 hours at 4° C. as reported by Klotz *et al.* (1946). The solutions were then surrounded with water at room temperature for 2–3 hours and subjected to electrophoresis, employing the dialysed solution in the electrophoretic cell and the dialysate as the supernatant liquid in the electrode limbs. The electrophoretic patterns

showed a sharpening in the descending limb and a spreading in the ascending limb (Fig. 1 A). Since this difference in patterns is attributed to the conductance or the pH gradient, between the dialysate and dialysed solution, the period of dialysis at 4° was extended to 7 days. Even this modification could not eliminate the spreading and the sharpening phenomenon. Similar results were obtained when the dialysis was carried out according to Brigg's procedure, where the protein solution (free from the dye) was dialysed against the dye solution. Reiner and Fenichel (1948) found that a period of 2 hours of dialysis was sufficient for the attainment of equilibrium at 24° C., when the protein solution was kept mechanically stirred within and without the dialysis bag. The determination of the conductivity of the dialysate and the protein-dye solution in the present work, also confirmed that a period of 2 hours was sufficient to eliminate the conductivity differences in the Reiner and Fenichel's method (1948). Even then, the spreading and sharpening could not be eliminated. This effect was, however, eliminated completely when the period of dialysis was extended to 4 hours. The complexes studied in this work were, therefore, dialysed for a period of 4 hours at the laboratory temperature. Since the dialysate and the dialysed solution in this group of experiments have the same dye concentration, the protein-dye complex does not dissociate on electrophoresis and gives rise to a single boundary in either of the limbs. The mobility ($\bar{U}_p$) of this boundary (Tiselius, 1930) corresponds to the constituent mobility of the protein complex (Fig. 2, Group I experiments).

In Group II experiments, the protein solution was mixed with a known amount of the dye solution and the complex so formed was subjected to electrophoresis against pure buffer. This procedure gave rise to δ- and ε-boundaries as observed by Briggs *et al.* (1950). This phenomenon persisted even when electrophoresis was carried out, after 24 hours of mixing the two solutions. The δ- and ε-boundaries are caused by stationary concentration gradients. The dialysis of the complex in presence of the dye-buffer dialysate (Group I experiments), can easily eliminate the weak δ- and ε-concentration gradients and was, therefore, employed as the underlying liquid in the cell in Group II experiments, while the overlying solution was pure buffer of the same ionic strength and pH as that of the cell liquid. By this modification, it was possible to eliminate practically the δ- and ε-boundaries as shown in Fig. 2 (Group II experiments).

Results

Table I comprises the electrophoretic analysis of bovine plasma albumin-methyl orange complex at 25° C. The protein solution in these experiments had a concentration of about $1{\cdot}4 \times 10^{-4}$ M (Mol. Wt. = 67,000), while the dye concentration varied from $1{\cdot}5 \times 10^{-4}$ M to $25{\cdot}0 \times 10^{-4}$ M, the latter value being the saturated concentration of methyl orange at 25° C. in the buffer.

The typical electrophoretic patterns obtained in either group of the experiments are given in Fig. 2. Fig. 3 gives a plot of $\bar{U}_p$ against r (the number of dye molecules per protein molecule) in the entire concentration range of the dye. It can be

TABLE I

Electrophoretic analysis of bovine plasma albumin-methyl orange complex at 25° C. in acetate buffer of pH 5·57 and $\mu = 0{\cdot}05$

Expt. No.	Protein concentration $\times 10^4$ M $[P_0]$	Dye concentration $\times 10^4$ M $[A_0]$	Total number of fringes					Mobilities corrected to 25° C. $\times 10^5$ cm.^{2} volts^{-1} sec.$^{-1}$				
			Asc. limb	Desc. limb	Boundary F_3	Protein from complex F_1	Dye protein complex F_2	U_p*	$\bar{U}_p$*	$\bar{U}_A$*	U_A*	U_A†
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
1	1·34	1·5	27	28	0	18	10	5·50	6·42	7·99	..	..
2	1·34	3·0	26	28	0	15	13	5·46	6·75	8·53	..	..
3	1·45	5·0	28	31	0	13	18	5·56	7·40	9·24	..	..
4	1·49	10·0	29	30	0	10	23	5·41	8·62	10·21	..	17·40
5	1·49	10·0	29	33	0	10	23	5·50	8·53	10·13	..	17·13
6	1·49	15·0	30	35	0	10	25	5·48	8·94	11·04	..	17·08
7	1·49	20·0	31	36	0	8	28	5·59	9·40	11·64	..	17·12
8	1·49	25·0	32	39	0	9	30	5·28	9·92	11·02	..	17·23
9	1·25	..	26	26	0	..	..	5·68	..	..	..	..
10	..	25·0	5‡	5	0	..	..	..	..	..	16·48	..

* Values from descending limb only.

† Mobilities of the dye in the ascending limb in Group II experiments.

‡ Saturated solution of only methyl orange at 25° C.

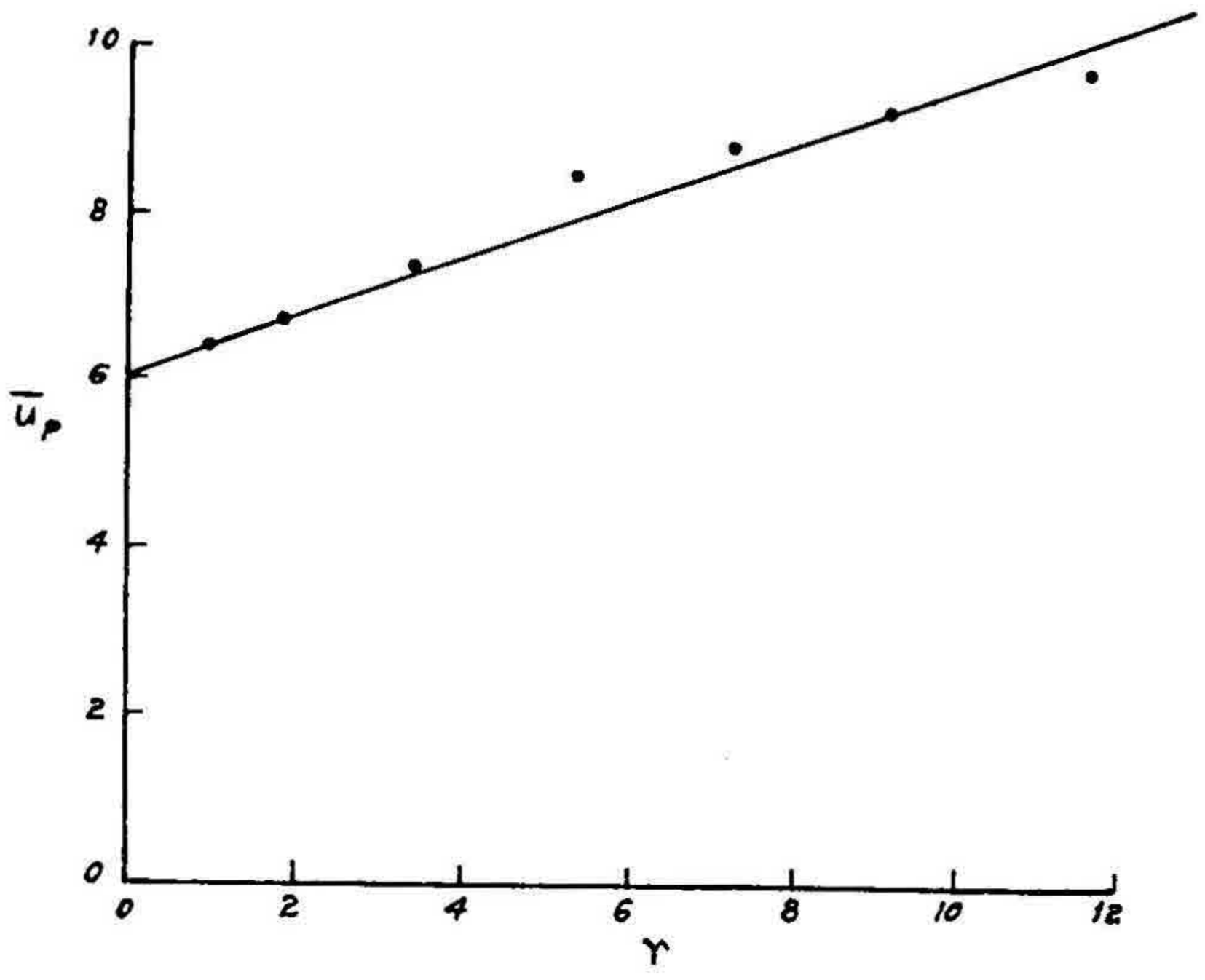


FIG. 3. Values of r calculated from Equation (3) against U_p.

seen from the linear relationship that each additional anion of methyl orange increases the constituent mobility of protein by a constant value of $0{\cdot}21 \times 10^{-5}$ cm.^{2} volts^{-1} sec.$^{-1}$ as against a value of $0{\cdot}13 \times 10^{-5}$ reported by Briggs *et al.* (1950) at 4°C. Fig. I B gives the typical electrophoretic patterns obtained in experiment 6 (Table I) at various time intervals of electrophoresis. Fig. I B also indicates the various boundaries obtained in Group II experiments when the electrophoresis is carried out for 30 minutes (B_1 and B_2). The fast moving boundary (LM) corresponds to the free dye (mobility = U_A), while the slow one (NO) corresponds to the protein dye complex in the ascending limb (constituent mobility = $\bar{U}_p$). Similarly, the fast moving boundary (QR) of the descending limb B_2 corresponds to the dye-protein complex (constituent mobility = $\bar{U}_A$) while the slow one (PQ) corresponds to the pure protein (mobility = U_p). The mobilities U_A, $\bar{U}_p$, $\bar{U}_A$ are obtained by taking the mean position of the fringes at various intervals.

The number of fringes in each of the boundaries being proportional to the concentration of the respective components, they can be employed to determine the concentration of the respective constituents. A dye concentration of 25×10^{-4} M gives rise to 5 fringes while $1{\cdot}5 \times 10^{-4}$ M protein solution gives 31 fringes against pure buffers. It is thus clear that the presence of dye has comparatively negligible effect on the refractive index of the pure protein solution.

Smith and Briggs (1950) have calculated the dissociation constant and the composition of the protein-dye complex by employing the Langmuir's adsorption isotherm

$$\frac{1}{r} = \frac{K}{n\,[A]} + \frac{1}{n} \qquad (1)$$

where r = moles of bound dye per mole protein,

n = the limiting value of r as $[A] \to \infty$ or the maximum number of binding sites,

K = a dissociation constant of the complex,

$[A_0]$ = total molar concentration of the dye in the solution,

$[A]$ = the molar concentration of the free dye anions,

$[P_0]$ = total molar concentration of the protein.

A plot of $1/r$ against $1/[A]$ gives the values of n and K. The scheme of analysis described by Smith and Briggs (1950) and Alberty and Marvin (1950) makes it possible to obtain values of $[A]$ from the electrophoretic patterns when the values of $[A_0]$ and $[P_0]$ are known. A consideration of the dissociation equilibrium in the descending limb of the II group of experiments has enabled Briggs *et al.* (1950) to calculate the value of $[A]$ by the following equation:

$$[A] = [A_0]\,\frac{\bar{U}_A - U_p'}{\frac{[P_0]}{[P]^c}(U_A - \bar{U}_A) + (\bar{U}_A - U_p')} \qquad (2)$$

where $[P_0]$, $[A]$ and $[A_0]$ refer to the terms explained above,

$[P]^c$ = concentration of protein in the slow moving boundary PQ (Fig. 1 B, B_2).

U_A = mobility of free dye anions,

$\bar{U}_A$ = constituent mobility of dye in presence of protein,

U_p' = apparent mobility of the PQ boundary (Fig. 1 B, B_2).

The ratio $[P]^c/[P_0]$ can be calculated from the concentration gradients of the boundaries in the descending limb as follows:

$$\frac{[P]^c}{[P_0]} = \frac{F_1\left(1 + \frac{F_1}{F_1 + F_2}\right)}{F_1 + F_2 + F_3 - F_4}$$

where F_1 = number of fringes in the PQ boundary
F_2 = number of fringes in the QR boundary } Fig. 1 B, B_2

F_3 = number of fringes in the ϵ – boundary,

F_4 = number of fringes due to total dye calculated from the number of fringes obtained for a standard dye solution.

It has been shown by Alberty and Marvin (1950) that the factor $\frac{[P]^c}{[P_0]}$ can also be calculated by the mobilities of the boundaries in either of the limbs as follows:

$$\frac{[P]^c}{[P_0]} = \frac{\bar{U}_p' - \bar{U}_A}{\frac{k_1}{k_2} U_p - \bar{U}_A}$$

where, k_1 and k_2 are the specific conductance of the solution in the QR and PQ boundaries (Fig. 1 B), the other terms having the significance given above.

The value of $\bar{U}_p'$ can be obtained, however, from the descending limb of Group I experiments, described earlier. It can be shown that the values of [A] can also be obtained by the following relations:

$$[A] = [A_0] \frac{\bar{U}_A - \bar{U}_p}{U_A - \bar{U}_p} \tag{3}$$

$$[A] = \frac{[A_0](\bar{U}_A - U_p)\left(\frac{n-1}{n}\frac{[P]^c}{[P_0]} + \frac{1}{n}\right) - [P_0]\left(1 - \frac{[P]^c}{[P_0]}\right)(\bar{U}_A - U_p)}{(\bar{U}_A - U_p)\left(\frac{n-1}{n}\frac{[P]^c}{[P_0]} + \frac{1}{n}\right) + U_A - \bar{U}_A} \tag{4}$$

Values of [A] calculated from equation 2 or 3 have been used to obtain the values of r in equation 1. Fig. 4 (Curve I), gives a plot of $1/r$ against $1/[A]$ from which we obtain $n = 18{\cdot}2$ and $K = 4{\cdot}32 \times 10^{-4}$ (equation 1). The value of n

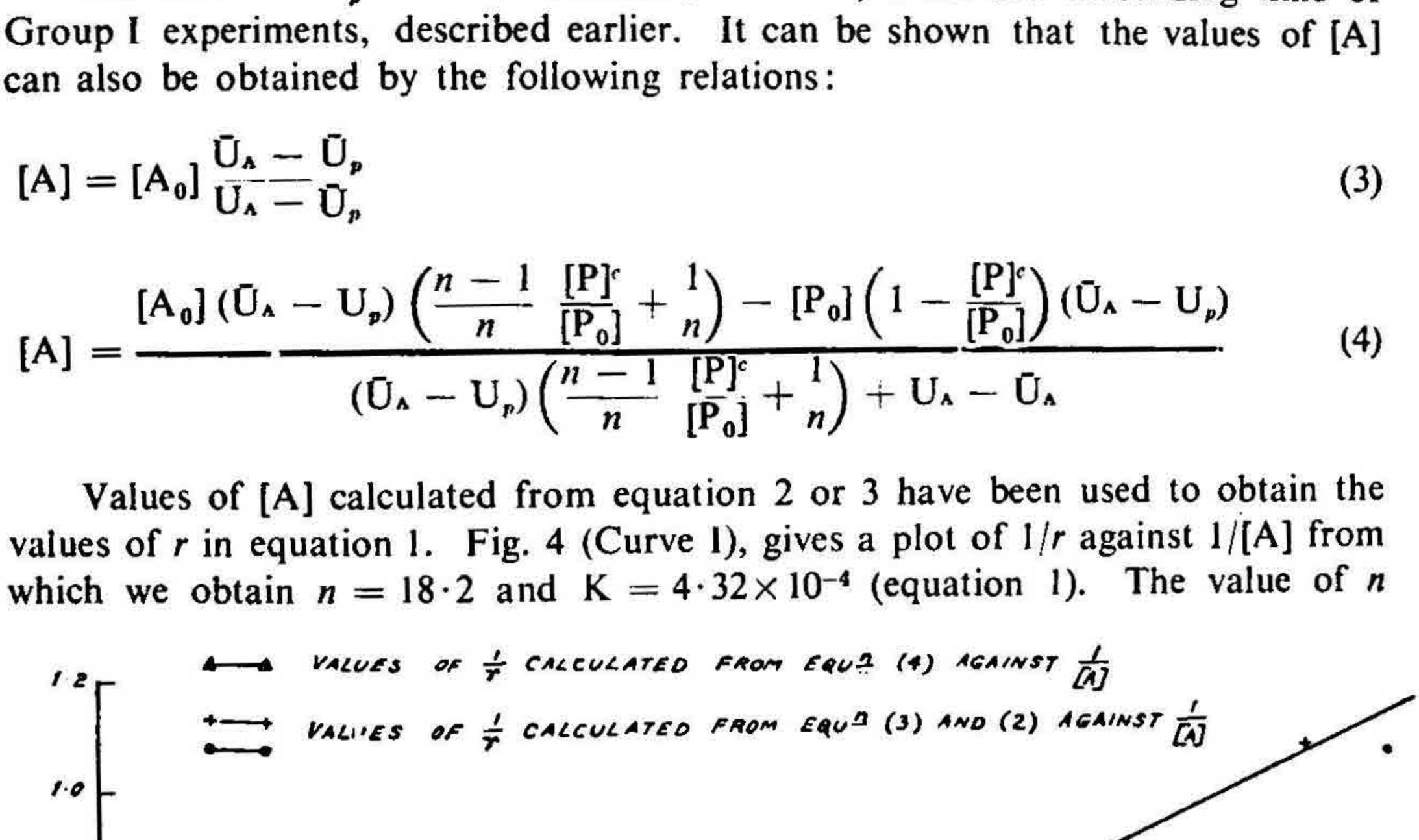


FIG. 4. A plot of $\frac{1}{r}$ against $\frac{1}{[A]}$

has been used to calculate the values of [A] from equation (4) and the new values of 1/[A] and $1/r$ are plotted in curve 2 of the same figure which gives $n = 20{\cdot}0$ and $K = 3{\cdot}62 \times 10^{-4}$. The corresponding values at 4° C. obtained by Briggs *et al.* (1950) are $n = 25{\cdot}2$ and $K = 2{\cdot}92 \times 10^{-4}$. Thus, an increase in temperature from 4° to 25° has slightly decreased the value of n while the value of K has been enhanced.

DISCUSSION

It can be seen from Fig. 2 that there is no asymmetry in the leading peak (BC) of the descending limb of the Group II experiments when the concentration of the dye is increased upto $25{\cdot}0 \times 10^{-4}$ M. But, working at 4° C. Briggs *et al.* (1950, 1952) have reported an asymmetry when the dye concentration is $> 10 \times 10^{-4}$ M. They have also noticed that the change in light absorption by the dye, through this region in the cell follows an unsymmetrical pattern at these concentrations. Such a behaviour of the complex has also been noticed by Putnam and Neurath (1945) in their studies on bovine serum albumin and sodium dodecyl sulphate complexes. They have attributed this asymmetry due to the formation of micelles of sodium dodecyl sulphate. Similar observations have been reported by the comparatively recent studies of BPA–Butyl orange complex by Burkhard *et al.* (1953).

It has already been pointed out that an increase in temperature slightly enhances the value of K and diminishes the value of n. Employing dialysis equilibrium technique, Klotz *et al.* (1949) also obtained similar results at a dye concentration of 5×10^{-5} M.

A comparison of the patterns in Fig. 2 further shows that the protein spreading CB is enhanced when present in combination with the dye AB. It can also be seen that an asymmetry is present only in the ascending limb of group I and group II experiments at low dye concentrations ($5{\cdot}0 \times 10^{-4}$ M and lower) and this vanishes as the dye concentration is increased. In the experiments conducted by Briggs *et al.* (1950) on the other hand, there is no asymmetry in the ascending limb at low dye concentrations while an asymmetry is noticed at high dye concentrations only in the descending limb. This divergence in observations at the two temperatures remains to be explained. Even experiments conducted at low potential gradients of about 1·0 volt/cm. gave patterns similar to Fig. 2 and indicated that there were no thermal gradients in the boundaries. It is also interesting to note that in Group II experiments (Fig. 1 B) when the dye concentration is 10×10^{-4} M and higher, a free dye boundary LM is obtained while Smith and Briggs (1950) have reported this boundary at a concentration of 20×10^{-4} M. This may perhaps be due to the low temperature employed by these authors. Reference to Fig. 1 A indicates that enantiographic patterns are not obtained in the ascending and descending limbs when the temperatures of dialysis and electrophoresis are 4° and 25° respectively. When both dialysis and electrophoresis are carried out at 25° however, it is seen (Fig. 2, group I) that symmetrical patterns

FIG. 1 A. Electrophoretic Pattern of a Mixture of Bovine Plasma Albumin Methyl Orange in Group I. Experiment at 5·25 Volts per cm. in Acetate Buffer (pH = 5·57, Ionic Strength = ·05) for 5400 seconds, Dye Concentration = $10{\cdot}0 \times 10^{-4}$M, Bovine Plasma Albumin = $1{\cdot}49 \times 10^{-4}$M, Dialysis carried out at 4° C. for 7 days.

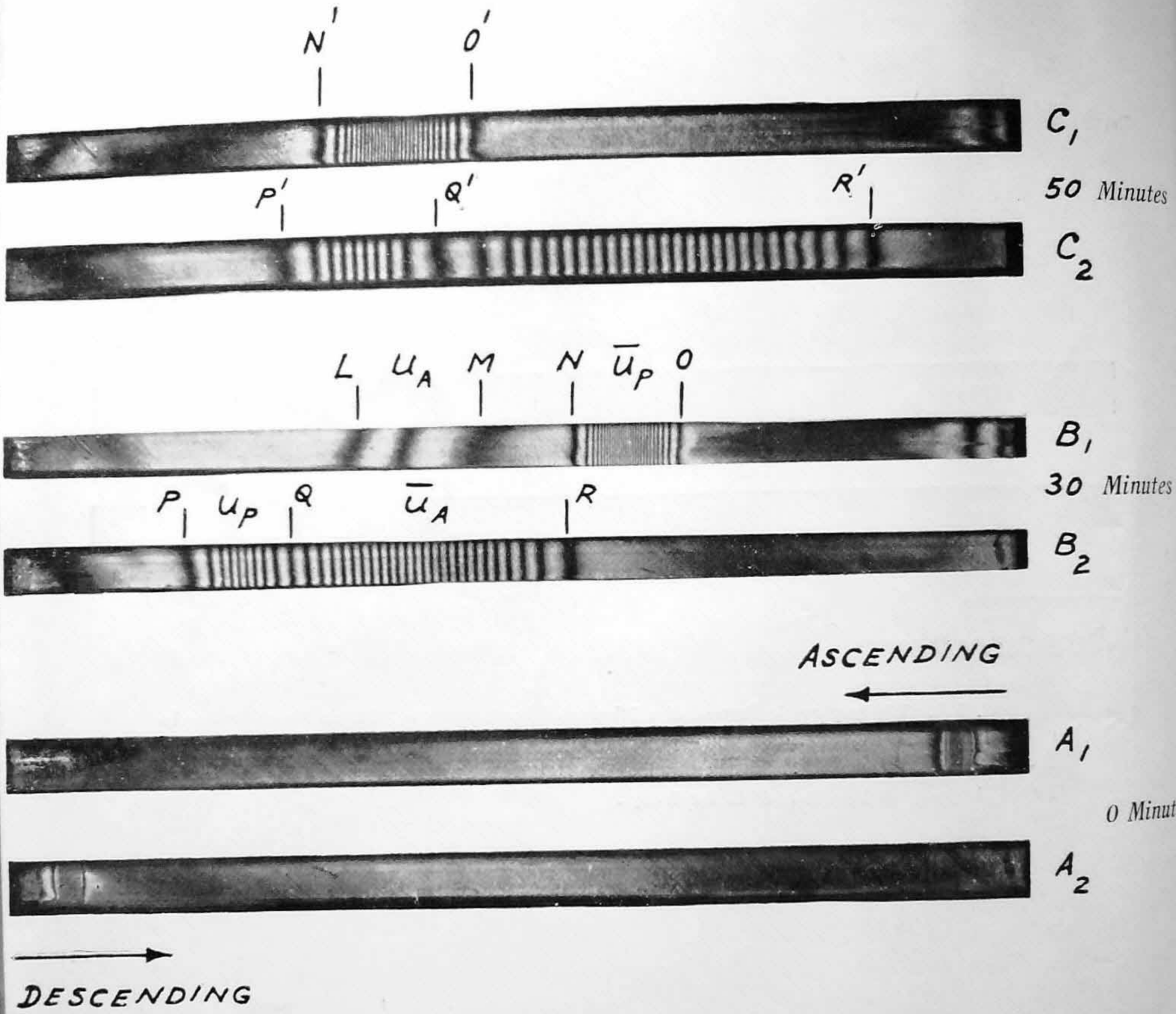


FIG. 1 B. Electrophoretic Patterns of a Mixture of Bovine Plasma Albumin ($1{\cdot}49\times10^{-4}$M) —Methyl Orange ($15{\cdot}0\times10^{-4}$M) at Successive Intervals under 5 Volts/cm. in Group II Experiment. A_1, B_1 and C_1 are for ascending limb, A_2, B_2 and C_2 are for descending limb.

GROUP I

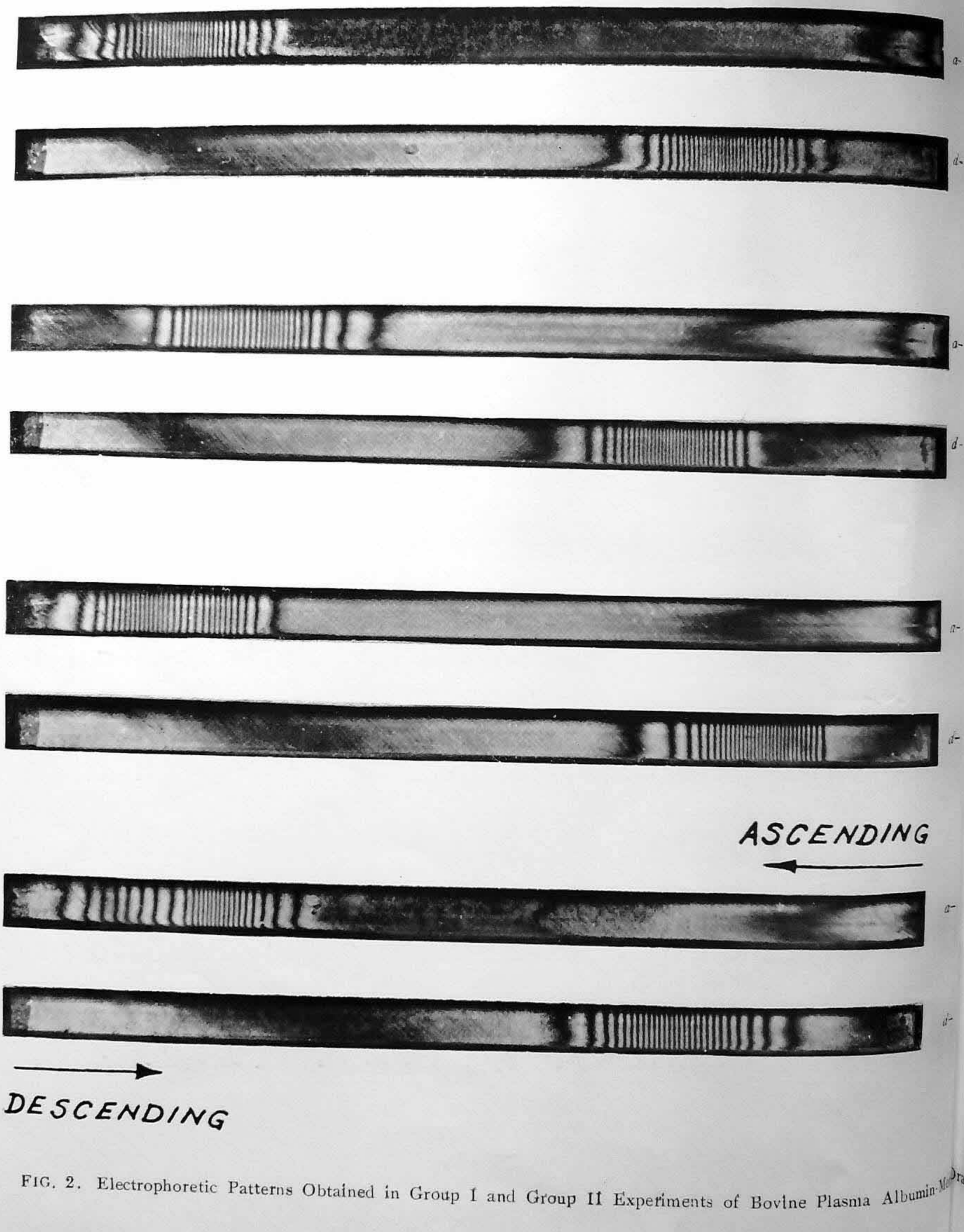
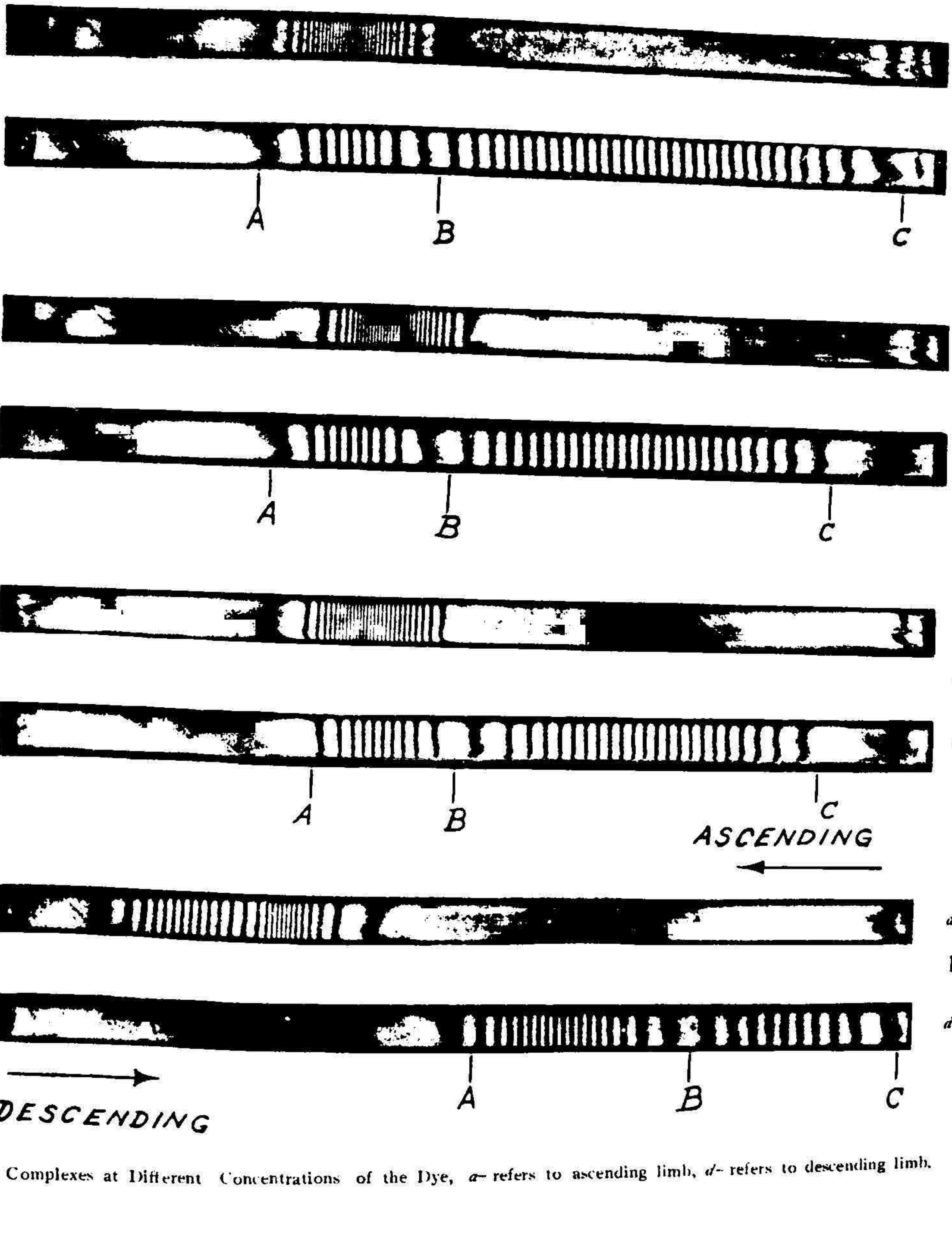


FIG. 2. Electrophoretic Patterns Obtained in Group I and Group II Experiments of Bovine Plasma Albumin-Me Orange C

GROUP II



e Complexes at Different Concentrations of the Dye, *a*– refers to ascending limb, *d*– refers to descending limb.

are obtained in both the limbs. This is evidently due to the changes in the conductivity between the protein solution and the overlying solution brought about by a dissociation of the protein-dye complex at the higher temperature. It is therefore, necessary that the temperature of complex formation and electrophoretic analysis should be the same if ambiguous results and spurious gradients in the electrophoretic pattern have to be avoided.

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