A MICRO-ELECTROPHORETIC STUDY OF THE COMPLEX FORMATION OF BOVINE PLASMA ALBUMIN (B.P.A.) AND RIBOSE NUCLEIC ACID (R.N.A.)

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Summary

The complex formation of B.P.A./R.N.A. system has been studied at various pH, ionic strengths and concentration ratios. It has been found that complex is formed only below pH 5·60 and $\mu = \cdot 1$. The ionic strength is critical for the formation of the complex as only two boundaries are obtained in both the limbs at $\cdot 02\,\mu$ while at $\cdot 05\,\mu$ three boundaries are obtained in the descending limb and two in the ascending limb consistently. The results have been discussed by modified Jordan's equation to explain the occurrence of third boundary in the descending limb. Calculations made by the method of Briggs *et al.* have failed to yield the value of reaction constants n and k. Similar calculations with data of Goldwasser and Putnam have given the value of $n = \cdot 024$ and $k = \cdot 26 \times 10^{-5}$. The data of Kirkwood also fails to give any constants.

The complex formation between proteins and nucleic acids has been the subject of intense study in recent years (Jordan *et al.*, 1953). Earlier studies of protein-nucleic acid interaction were mostly confined to precipitation reactions, but in the last two decades electrophoretic technique has been widely used. Stenhagen and Teorell (1939) were first to study the complex formation of human serum albumin and thymus nucleic acid by electrophoresis. From their studies carried out within a certain pH and ionic strength range, they concluded that a single soluble complex was formed towards the alkaline side of the isoelectric point, and was pH and ionic strength dependent. Longsworth and MacInnes (1942) dealt with a model system of ovalbumin and yeast nucleic acid and gave a detailed explanation for complex formation. Their study revealed that the rate of electrophoretic separation was comparable to the rate of complex formation and the constants of the reaction could not, therefore, be calculated by mobility and area data. However, recently Briggs and Smith (1950) have calculated the equilibrium constant of the reaction from the data given by Longsworth *et al.* (1942). Goldwasser and Putnam (1950) reported the results of complex formation of bovine serum albumin and calf thymus nucleic acid and found that the nature of the reaction was completely different from that of ovalbumin and yeast nucleic

acid. They failed to calculate the equilibrium constant of this reaction by the method given by Briggs *et al.* (1950) and Alberty *et al.* (1950). However, these studies lead to the conclusion that the nature of the reaction is electrostatic. Kirkwood *et al.* (1952) have also studied the interaction of bovine serum albumin and deoxyribose nucleic acid by electrophoresis and electrophoretic convection. Their data though obtained at a slightly different pH is contrary from that reported by Putnam *et al.* (1950). They conclude that the rate of equilibrium of the complex is neither very slow nor very rapid but is intermediate between the two extremes.

In view of the fact that the studies reported uptil now are more or less qualitative and conflicting, the present study was, therefore, undertaken on a similar model system of bovine plasma albumin and ribose nucleic acid, which has not yet been investigated. The results obtained show a marked difference from other systems reported so far and indicate the presence of two complexes.

EXPERIMENTAL

The protein samples employed were Fraction V (Lot No. M 11902) from bovine plasma and crystalline bovine plasma albumin (Lot No. R 370295 B) kindly supplied by Armour Laboratories, U.S.A., The electrophoresis showed no difference between the mobility and homogeneity of the two samples under the conditions studied. A value of 69000 was used for the molecular weight of the protein for calculation of the molarity of the protein solution (F. Karush, 1954).

The R.N.A. used was a Hoffman La Roche product. It was purified by repeated deproteinization for three times with chloroform-amyl alcohol (4:1) mixture and was reprecipitated by ethyl alcohol according to the method described by Chargaff *et al.* (1948). This purified preparation was homogeneous as shown by a single boundary on electrophoresis, except at pH 8·0 when two boundaries were obtained. A value of 15800 was used for the molecular weight of the nucleic acid for calculation of the molarity of the nucleic acid solution [Gulland *et al.* (1944)].

A 10% solution of bovine plasma albumin was prepared and frozen at − 5° C. It was diluted to the required concentration. As the nucleic acid is insoluble in water, it was dissolved in respective buffers and diluted according to the required concentration.

All buffers employed were prepared from reagent grade chemicals. The complex formation of bovine plasma albumin and ribose nucleic acid was studied in acetate and phosphate buffers of pH 4·5, 4·9, 5·6 and pH 6·45 and 8·00 respectively. The acetate buffers were having the following composition:—

pH 4·5 (·05 MNaAc*—·075 MHAc);

pH 4·9 (·05 MNaAc—·025 MHAc);

pH 5·6 (·05 MNaAc—·005 MHAc).

* Ac = acetate.

The phosphate buffers were having the following composition:—

pH 6·45 (·0218 M NaH_2PO_4—·0094M Na_2HPO_4);

pH 8·00 (·0008M NaH_2PO_4—·0164 MNa_2HPO_4).

All pH values were checked by glass electrode (Beckman pH meter—H-2) and quinhydrone electrode at room temperature. It was found that there was no appreciable change in pH on diluting from ·5 M to ·01 M and therefore, the same concentrated buffer was diluted for changing ionic strength. The conductivities were measured in a Leeds and Northurp Conductivity cell at room temperature and a temperature coefficient of 2% was applied for obtaining the conductivity at the required temperature. The complexes were prepared by mixing the bovine plasma albumin and ribose nucleic acid in the required proportion and diluting accordingly. Usually 2 c.c. of the mixture was enough for duplicate electrophoretic analysis. The mixtures were then dialysed at room temperature (Reiner and Fenichel, 1948) for 4–6 hours with atleast one change of buffer employing the cellophane tubing, obtained from A. Gallenkemp & Co. The solutions thus

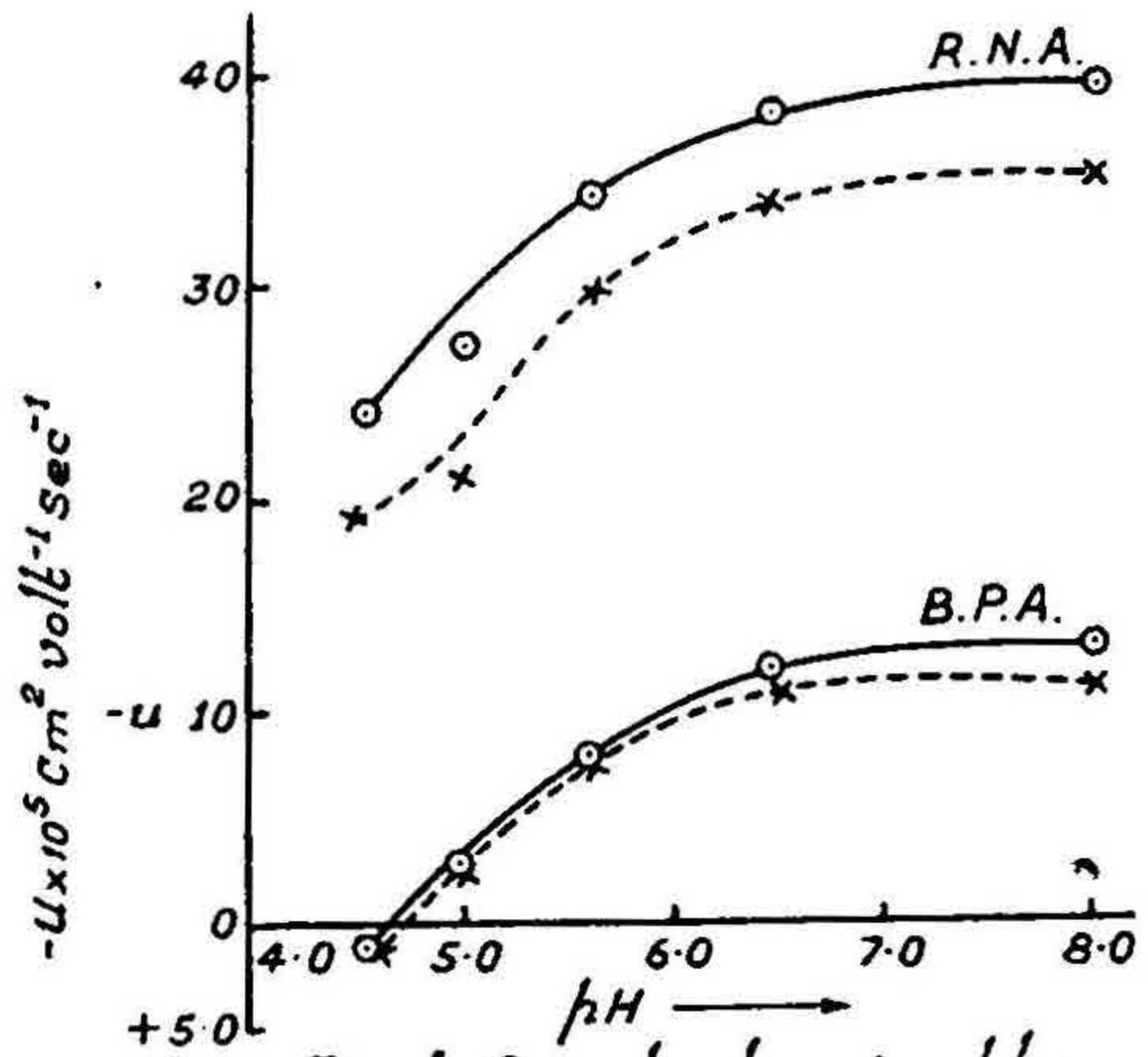

Fig. 1. Graph showing the Variation of mobility of B.P.A. and R.N.A. with change in pH
⊙ Ascending limb
× Descending limb

obtained were either immediately analysed electrophoretically or were frozen at — 5° C. and then brought to room temperature before analysing. No difference was observed in either case. In some solutions (Table II) where a precipitate was obtained on keeping the mixture for some time, a clear supernatant was obtained by centrifugation, and analysed.

The solutions thus prepared were analysed at room temperature in Kern's Microelectrophoretic apparatus (W. Lotmar, 1951; M. A. Cohly *et al.*, 1953). 1·00% B.P.A. gives 30 fringes and 1·00% R.N.A. gives 26 fringes. They were in agreement with the refractive index increment of protein and nucleic acid obtained by Abbey's Refractometer (Refractive index increment for 1% B.P.A. = 0·0018 and for 1% Ribose Nucleic Acid = 0·0015). The cell had a capacity of 0·4 c.c. and was filled according to the technique described by Longsworth *et al.* (1940). A fresh buffer from stock solution was used for analysis. A voltage gradient of 2–5 volts was employed in the study. Ordinary dry cells were used as source of D.C. supply. All mobilities have been measured by the method given by Longsworth

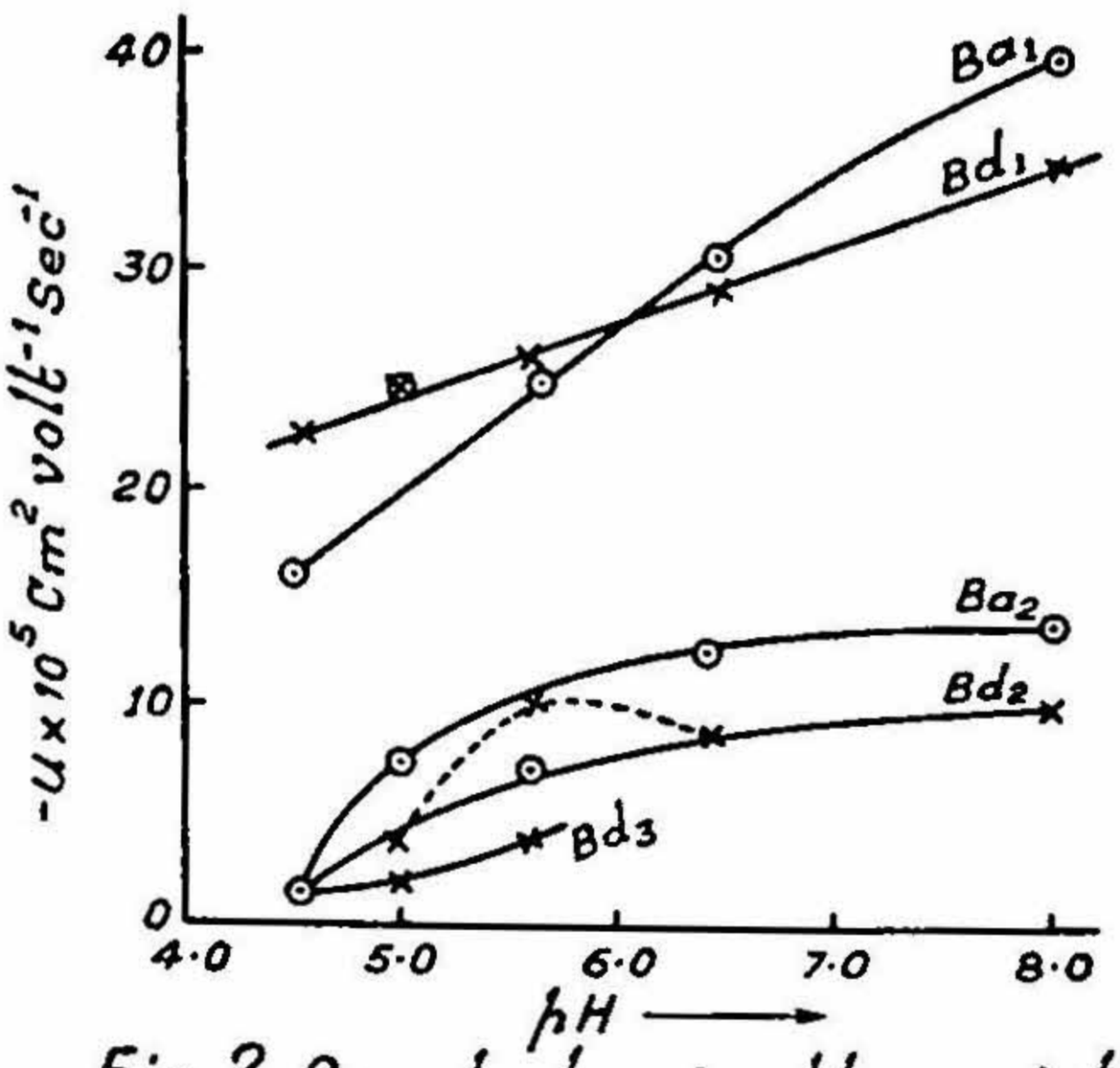

Fig. 2. Graph showing the variation of mobility of the complex (B.P.A./R.N.A.) with change in pH

⊙ Ascending

× Descending

B_d^1, B_d^2 & B_d^3 denote first, second & third Descending boundaries respectively

B_a^1 and B_a^2 denote first & second Ascending boundaries respectively

(1940). The mobilities were calculated at room temperature and were brought to 25° C. by a viscosity correction as given by Johnson and Shooter (1948). Usually two exposures were taken for the same moving boundary and mobilities for both were calculated.

RESULTS

The reaction has been studied from three different aspects to throw light on the mechanism and the groups responsible for the complex formation.

1. *Influence of pH.*—The following pH values were employed to study the complex of fixed composition, pure B.P.A. and pure R.N.A. respectively, pH : 4·5, 4·9, 5·6, 6·45 and 8·0. The results have been shown graphically in Figs. 1 and 2. It can be seen that R.N.A. gives anodic mobility in this pH range, and the value becomes constant at the higher pH values. A single boundary is obtained for R.N.A. at all pH values, except at pH 8·0, where a second boundary becomes apparent with the course of electrophoresis. A similar behaviour has been obtained in the case of B.P.A. except that it shows inhomogeneous boundaries while undergoing cathodic migration at pH 4·55. In case of B.P.A./R.N.A. mixture, two boundaries were obtained in either of the limb at all pH values except at pH 4·9 and 5·6. The first boundary corresponds in mobility to free nucleic acid and the slow boundary has a mobility equal to that of free protein. It is further clear from number of fringes in the two boundaries that the complex is fully dissociated into free nucleic acid and free protein. At pH 4·9 and 5·6, however, two boundaries are obtained in the ascending limb, and the descending limb gives rise to three boundaries. The mobilities as well as fringe distribution between the boundaries show complex formation in this pH range, and point out

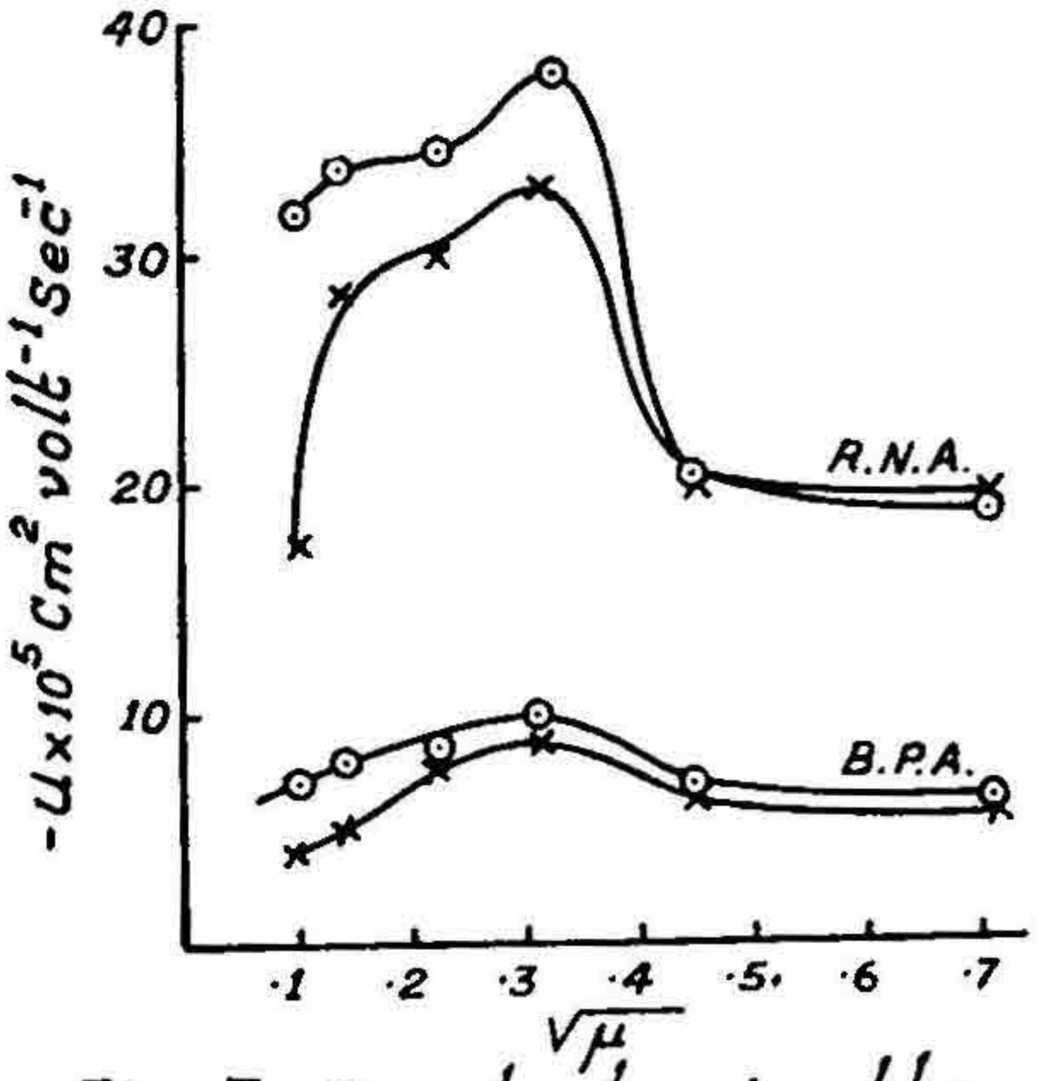

Fig. 3. Graph showing the variation of mobility with change in ionic strength
⊙ Ascending
× Descending

the presence of two discrete complexes. This study also confirms the electrostatic nature of the complexes.

Influence of ionic strength.—A systematic study of the effect of ionic strength on the electrophoretic behaviour of pure B.P.A., pure R.N.A. and B.P.A./R.N.A. complex has been conducted at ·01, ·02, ·05, ·10, ·20 and ·50 ionic strength alues. The results have been presented graphically in Figs. 3 and 4.

It can be seen that a maxima is obtained in all cases which clearly brings forth the fact that buffer ions either combine or effectively block the active sites on B.P.A. and R.N.A. as well as on B.P.A./R.N.A. complex. A single boundary is obtained in the case of both pure B.P.A. and R.N.A. at all ionic strengths against the abnormal behaviour of B.P.A./R.N.A. complex. The complex gives two boundaries in the ascending limb and three boundaries in the descending limb at $\mu = \cdot 01$, $\cdot 02$ and $\cdot 05$, whose mobilities and fringe distribution show complex formation. The higher ionic strengths of $\cdot 10$, $\cdot 20$ and $\cdot 50$ prevent complex formation and give rise to only two boundaries in both the limbs, whose mobilities and fringe distribution show complete dissociation into free protein and free nucleic acid. The fringes were spread out and diffuse at the lowest ionic strength $\cdot 01$ and accordingly the complex formation was confined to the ionic strengths of $\cdot 02$ and $\cdot 05$ to elucidate the nature of complex formation. Similar observations have been reported for bovine serum albumin-calf thymus nucleic acid and ovalbumin-yeast nucleic acid systems, which show extreme dependence upon pH and ionic strength of the buffer (Goldwasser and Putnam, 1950; Longsworth and MacInnes, 1942).

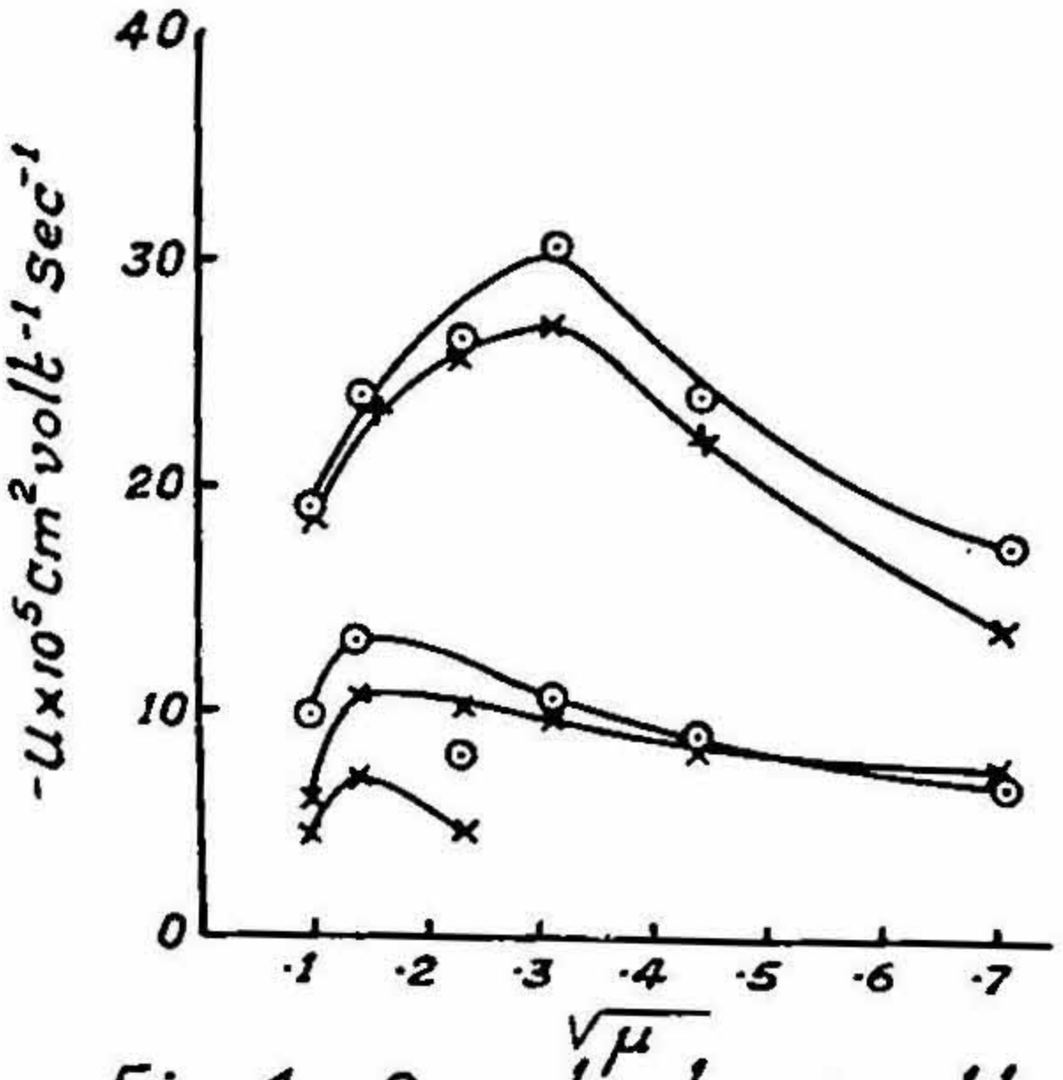

Fig. 4. Graph showing the variation of mobility of the B.P.A./R.N.A. mixture with ionic strength
⊙ Ascending limb
× Descending limb

Influence of B.P.A./R.N.A. mixing ratio.—The study has been confined to two ionic strengths of $\mu = \cdot 02$ and $\mu = \cdot 05$ as explained above and the B.P.A./R.N.A. molar ratio has been varied over a wide concentration range. The results have been given in Tables I and II.

TABLE I

Electrophoretic analysis of mixtures of bovine plasma albumin and ribose nucleic acid in ·02 M acetate buffer of pH 5·60 at 25° C.

Experiment Number	Composition		Mobility $\times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$					Relative number of fringes in various boundaries				
			Ascending			Descending		Ascending			Descending	
	B.P.A. $\times 10^4$ M.	R.N.A. $\times 10^4$ M.	A2	A1	D3	D2	D1	A2	A1	D3	D2	D1
1–1383	1·39	..	7·53	..	6·15	..	..	29	..	28	..	..
2–1028	2·70	0·63	11·95	23·52	..	9·16	18·32	55	1	..	55	3
3–1039	2·84	1·27	12·30	25·54	..	9·41	20·48	60	3	..	57	6
4–1049	1·31	1·27	13·78	28·86	..	9·84	22·45	28	3	..	27	5
5–1058	1·45	3·16	14·47	29·75	..	10·38	23·48	15	18	..	10	24
6–947	0·87	3·80	13·48	24·23	7·13	10·75	23·78	25	11	9	13	10
7–1066	0·58	4·89	16·40	32·50	..	10·93	24·68	28	13	..	25	16
8–1062	0·29	6·33	18·93	33·37	..	11·40	25·38	21	9	..	7	24
9–1021	..	6·15	..	33·37	..	..	24·55	..	25	..	..	24

TABLE II

Electrophoretic analysis of mixtures of bovine plasma albumin and ribose nucleic acid in ·05 M acetate buffer of pH 5·60 at 25° C.

Experiment Number	Composition		Mobility $\times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$					Relative number of fringes in various boundaries				
			Ascending			Descending		Ascending			Descending	
	B.P.A. $\times 10^4$ M.	R.N.A. $\times 10^4$ M.	A2	A1	D3	D2	D1	A2	A1	D3	D2	D1
1–1267	1·45	..	7·93	..	..	7·85	..	29	..	..	30	..
2–1274	1·31	0·63	9·04	28·09	6·61	..	17·6	28	1	26	..	4
3–901	1·26	1·27	5·56	24·74	4·04	10·29	24·76	28	3	19	7	6
4–672	1·45	1·52	5·68	25·25	4·17	9·97	24·61	32	5	22	8	7
5–858	1·26	2·53	7·14	24·67	4·27	10·28	26·02	31	6	7	20	9
6–679	1·45	3·04	6·98	25·27	4·34	10·39	26·65	33	9	8	20	15
7–917	1·31	6·33	7·79	25·45	4·60	10·23	25·60	30	19	11	16	27
8–1279	0·73	3·16	10·93	26·56	5·68	9·7	24·24	17	12	9	7	12
9–866	0·58	5·89	8·32	25·45	5·17	10·32	25·82	15	21	11	4	21
10–1286	0·47	4·43	11·69	26·22	6·80	9·28	26·68	18	10	5	5	16
11–689	0·73	7·59	10·52	25·25	6·15	9·85	25·27	20	27	6	7	31
12–697	0·36	7·59	10·71	26·22	6·75	16·45	25·97	13	24	5	4	30
13–854	0·19	4·94	11·42	26·72	9·07	17·86	27·61	7	17	5	2	17
14–821	..	4·62	..	34·61	..	..	29·98	..	21	..	..	19

Experiments have been repeated in some cases and these values have also been inserted in the above tables to show the consistency of different values. There is a marked difference in the electrophoretic behaviour of the complex at the two ionic strengths. The complex dissociates into two boundaries in both the limbs at $\mu = \cdot 02$ except at B.P.A./R.N.A. gram weight concentration ratio of 1 : 1, where the descending limb gives rise to three boundaries. Typical electrophoretic patterns at this ionic strength are given in Fig. 9 (*a*).

Fig. 5. Graph showing the plot of r/A against r of the B.P.A/R.N.A. complex at $\mu = \cdot 05$ $pH = 5 \cdot 6$
⊙ plot of r_1/A_1 against r_1
× plot of r_2/A_2 against r_2

The third boundary was only obtained at this concentration ratio and was consistently present in duplicate experiments. This boundary was obtained from the slow moving boundary after prolonged electrophoresis. It can be seen from the electrophoretic analysis in Table I that the data supports the assumption of a single dissociable complex of B.P.A./R.N.A. at this ionic strength. As a first approximation the results have been calculated by equations derived by Smith and Briggs (1950) and the resulting values of r and $r/[A]$ have been plotted in Fig. 6. The results have been represented by Langmuir's adsorption isotherm as follows:—

$$\frac{r}{[A]} = \frac{n}{k} - \frac{r}{k} \qquad (1)$$

where r = moles of bound R.N.A. per molecule of protein

[A] = free R.N.A. molar concentration

k = dissociation constant

n = limiting value of r (A) $\rightarrow \infty$ or maximum number of binding sites.

The value of [A] has been computed from the following equation:—

$$[A] = [A_0] \frac{\bar{u}_A - \bar{v}_P}{u_A - \bar{u}_P} \qquad (2)$$

where $[A_0]$ = total initial molar concentration of R.N.A.

$\bar{u}_A$ = Constituent mobility of nucleic acid computed from the mobility of the first boundary in the descending limb.

$\bar{u}_P$ = Constituent mobility of protein, computed from the slow boundary in the ascending limb (Fig. 9 *a*).

u_A = Mobility of free nucleic acid, computed from mobilities of free R.N.A. experiments.

A straight line has been drawn to join these points (Fig. 6). Similarly Fig. 9 *b* gives some of the electrophoretic patterns for B.P.A./R.N.A. mixtures at $\mu = \cdot 05$. The ascending limb shows only two boundaries, but the descending limb gives rise to three boundaries (except at low B.P.A./R.N.A. ratio) having characteristic mobilities and area distribution. It has been pictured that the following reaction takes place in the descending limb:

$$P + \nu R \rightleftarrows PR_\nu \rightleftarrows P' + P'R_\nu \qquad (3)$$

where PR_ν and $P'R_\nu$ represent two discrete B.P.A./R.N.A. complexes. It has been further assumed that the complex PR'_ν is only obtained in ascending limb, and further it does not undergo any dissociation on electrophoresis. The results have been calculated on the above hypothesis by equations (1) and (2) and plotted in Fig. 5.

DISCUSSION

It has been shown in the present study that B.P.A./R.N.A. complex formation takes place within a narrow range of pH (4·5–5·6) and ionic strength (·01–·10) as observed by other workers (P. Doty *et al.*, 1952; Kirkwood, *et al.*, 1952). The mobilities of B.P.A. reported above are in agreement with the results obtained by other workers (Longsworth *et al.*, 1949) except for the inconsistent variation in the value of mobility with ionic strength (Fig. 3). Longsworth *et al.* (1949), Alberty (1949), Velick (1949) and Tiselius and Svensson (1940), have reported the data relating the variation of mobility with ionic strength on different pure proteins. Tiselius and Svensson (1940) have studied in detail the effect of ionic strength on mobility of ovalbumin at pH 7·1, and have reported a regular increase in

mobility with decreasing ionic strength, thereby extrapolated the mobility at zero ionic strength. They have explained this consistent variation in mobility on the basis of Debye-Huckle-Henry theory. Other workers (reference cited above) have studied the mobility at only two ionic strengths of ·01 and ·10 to find out the change in isoelectric point of the protein and correlate the change to the effective charge on the protein molecule. However, an inconsistent variation in the mobility of bovine serum albumin has been reported by Paul Putzeys *et al.* (1940). Their results show the presence of a maxima at pH 5·74 and 5·99. In the absence of other studies in literature on B.P.A., it may be stated that the net charge on B.P.A. at a certain pH depends on the effective concentration of the buffer ions.

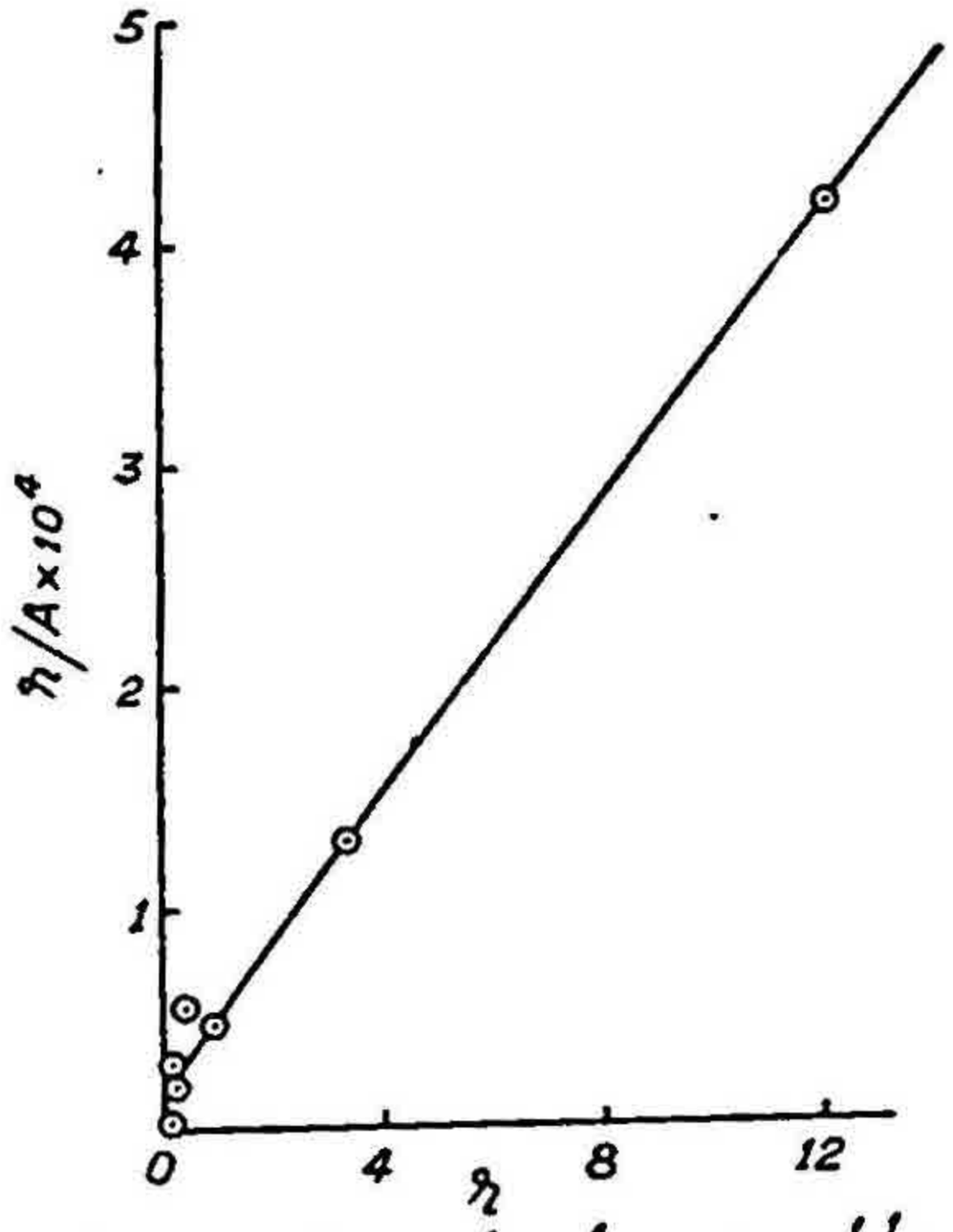

Fig.6. Graph showing the plot η against η/A of the values of μ = ·02 pH = 5·6

A similar study with R.N.A. shows that the variation of mobility follows a maxima. Cohn (1942), Delcambe (1950), Volkin *et al.* (1951) and Watanbe (1950, 1951) have reported the results of electrophoretic analysis on R.N.A. at different pH and ionic strength values. No work has been reported on R.N.A. at different ionic strengths and accordingly the present study leads to additional information for characterising R.N.A. The results can be explained on the basis of studies reported by Vora *et al.* (1941) on colloidal electrolytes. The observation does not confirm the extensive studies on D.N.A., reported by the Gulland school (1949).

The B.P.A./R.N.A. complex behaves differently at ·05 and ·02 ionic strengths. The presence of third boundary in the descending limb has been taken as a definite evidence for the formation of a second complex whose dissociation

constant is greater than the rate of electrophoretic separation. Such a hypothetical case has been discussed by Longsworth *et al.* (1942) under category V and as shown by Alberty (1950) can be studied by electrophoresis to evaluate n and k.

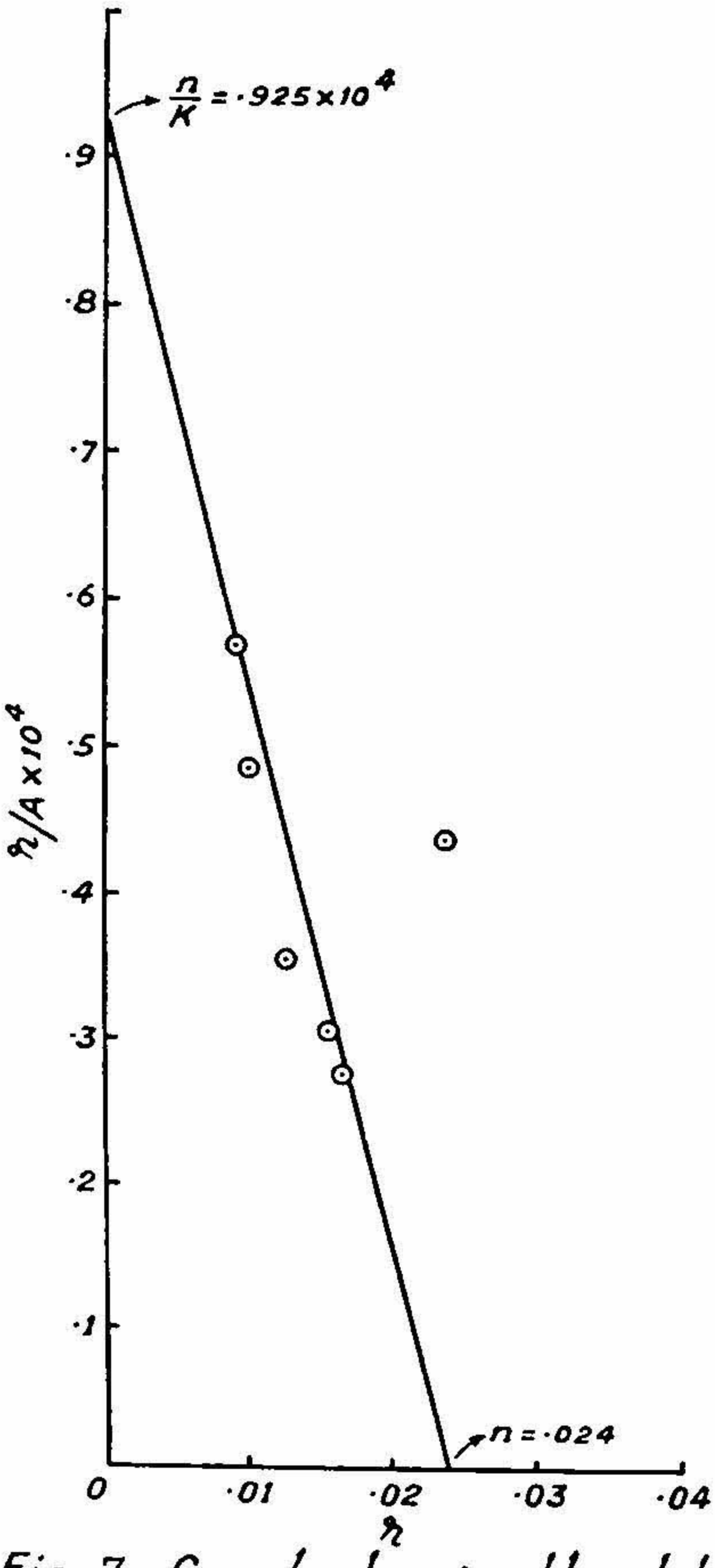

Fig. 7. Graph showing the plot of r and r/A from Goldwasser and Putnam's data

The results have been calculated as a first approximation and given in Figs. 5 and 6. The figures show that very little dissociation of the complex takes place and as an extreme case the extrapolated value of n becomes negative. A more exact

extrapolation may be obtained by conducting more experiments at low initial mixing ratios. P. Doty *et al.* (1952), however, have reported their studies on B.S.A.–D.N.A. complex formation by light scattering and have given a value of $n = 11 \pm 2$ for B.P.A.

Goldwasser and Putnam have studied the electrophoretic behaviour of D.N.A./B.S.A. system at different pH values, ionic strengths and concentration

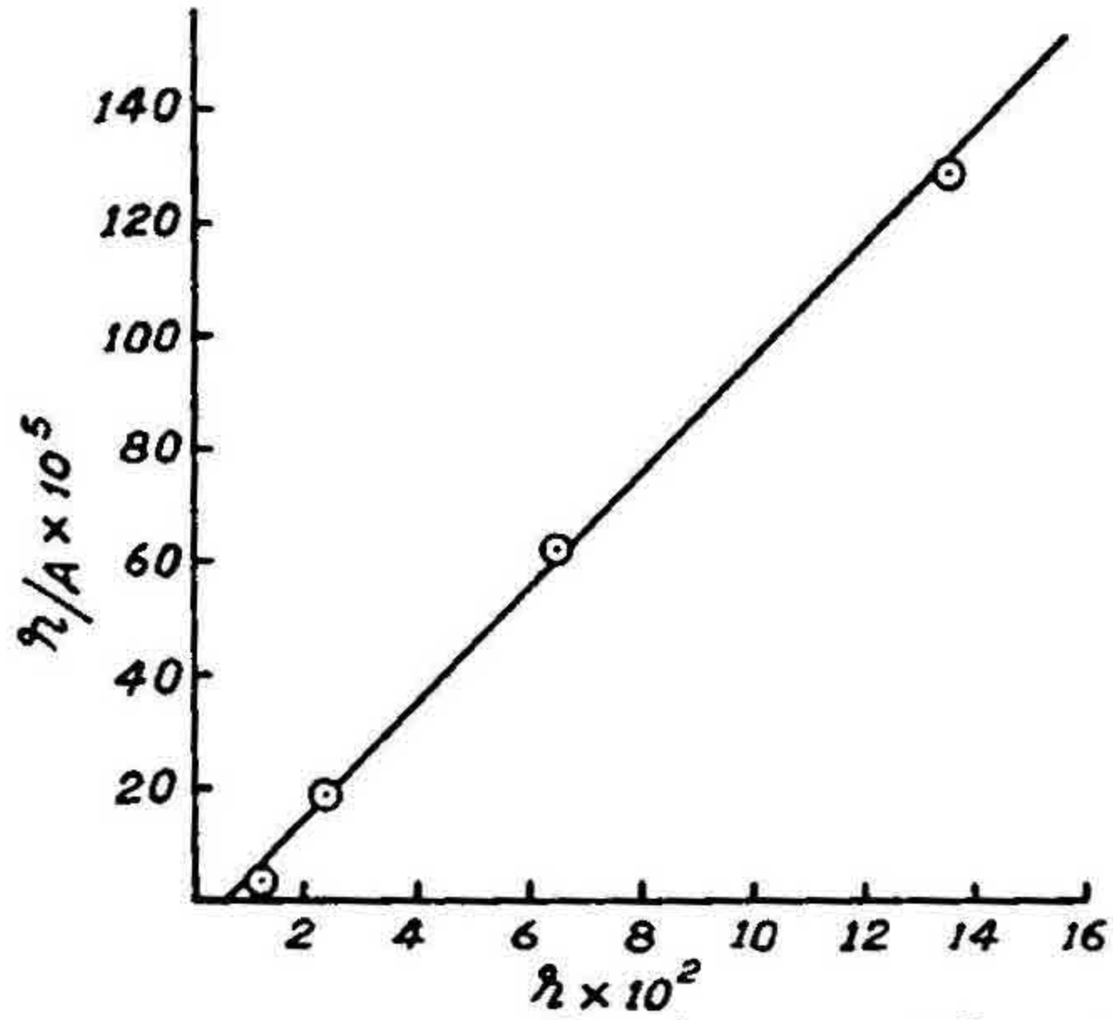

Fig. 8. Graph showing the plot of r against r/A of Kirkwood's data

ratios. They have discussed the enantiographic nature of electrophoretic patterns and have concluded in conformity with our study. They have reported the formation of three boundaries in the ascending limb and two boundaries in the descending limb, but have not given any mobility measurement for the third boundary. They have described this peculiar behaviour in the light of similar studies by Longsworth (1942) for ovamucoid and yeast nucleic acid and by Chargaff *et al.* (1941), on B.S.A. and heparin complex. Goldwasser and Putnam (1950) have further mentioned their inability to calculate the values of n and k on account of non-applicability of their data to Smith and Briggs scheme of analysis. We have recalculated the values of r and $r/[A]$ from Goldwasser and Putnam data and plotted the results in Fig. 7. This gives $n = \cdot 024$ (for D.N.A.) and $k = 0 \cdot 26 \times 10^{-5}$. These values are different from the values reported by Doty. However, an analogous study has been reported on this system (B.S.A./D.N.A.) by Kirkwood *et al.* (1952). They have qualitatively described their data and have not mentioned the molar concentration of protein and nucleic acid. They were able to obtain fairly enantiographic pattern of the boundaries in each limb in their study. Their results are plotted in Fig. 8, which shows a similarity with our data.

Our study points out that B.P.A./R.N.A. complex is different in its behaviour as compared to B.S.A./D.N.A. complex referred to above. The latter complex gives rise to three boundaries in the ascending limb while two in the descending, however, a reverse phenomenon is obtained with B.P.A./R.N.A. complex. A tentative explanation has been offered earlier in this paper.

The following modification of Jordan's (1953) interpretation has been adopted to explain the presence of the third boundary in the descending limb:—

$$P + \nu R \rightleftarrows PR_{\nu} \rightleftarrows P' + P'R_{\nu} \qquad (3)$$

This interpretation is in qualitative agreement with the data given in Tables I and II which clearly shows that the complex does not dissociate in the ascending limb, but it undergoes dissociation in the descending limb. The formation of artificial complexes of protein in nucleic acid has been recently employed by Chargaff *et al.* (1954), for the fractionation of D.N.A. A similar study with R.N.A. will prove extremely useful to show its heterogeneity in various tissues (Markhem *et al.*, 1954).

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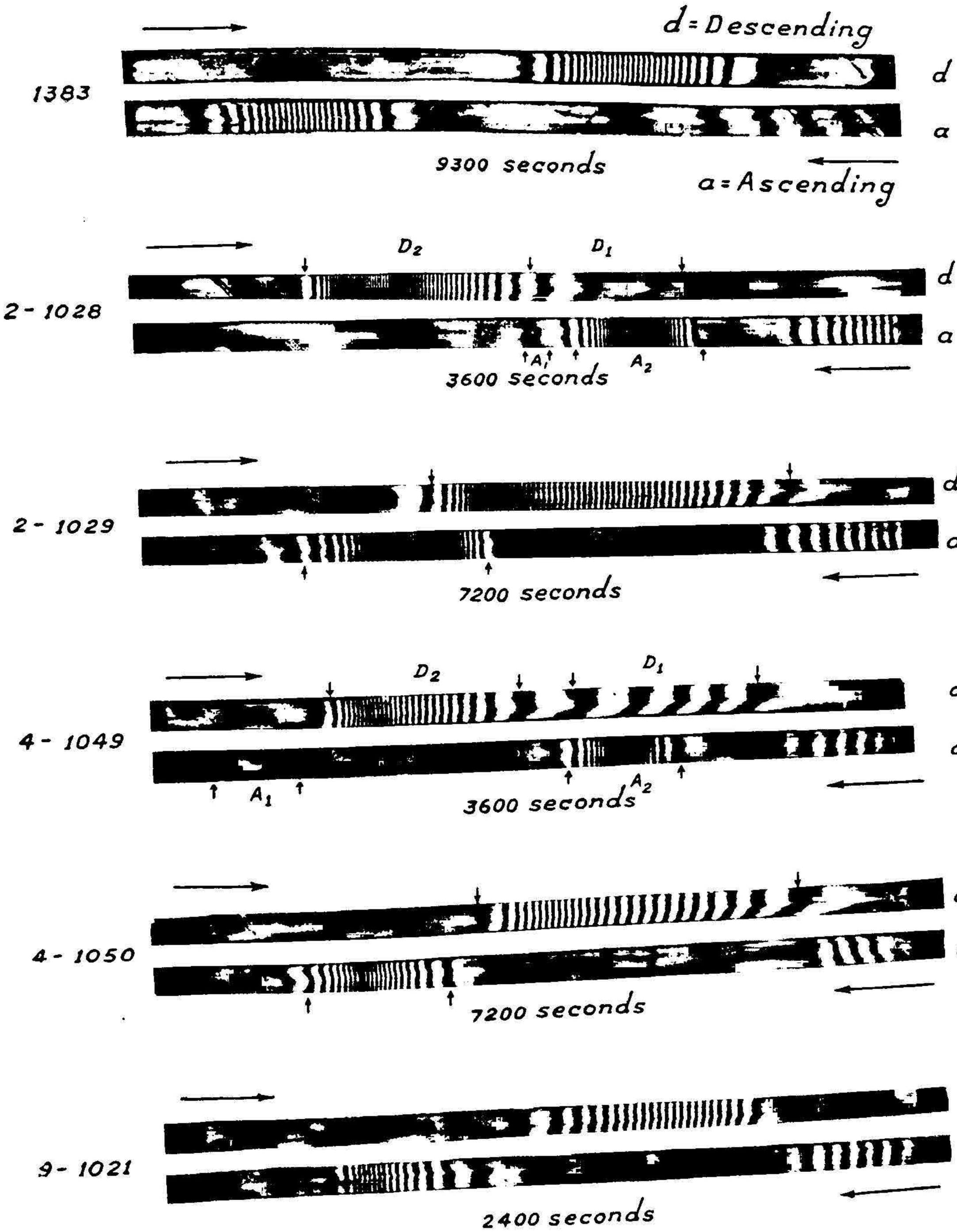

FIG. 9 (*a*). Electrophoretic patterns of B.P.A.— R.N.A. system in Acetate buffer pH = 5·60μ = ·12 at various molar concentrations at a voltage gradient of 2·1 volts per cm.

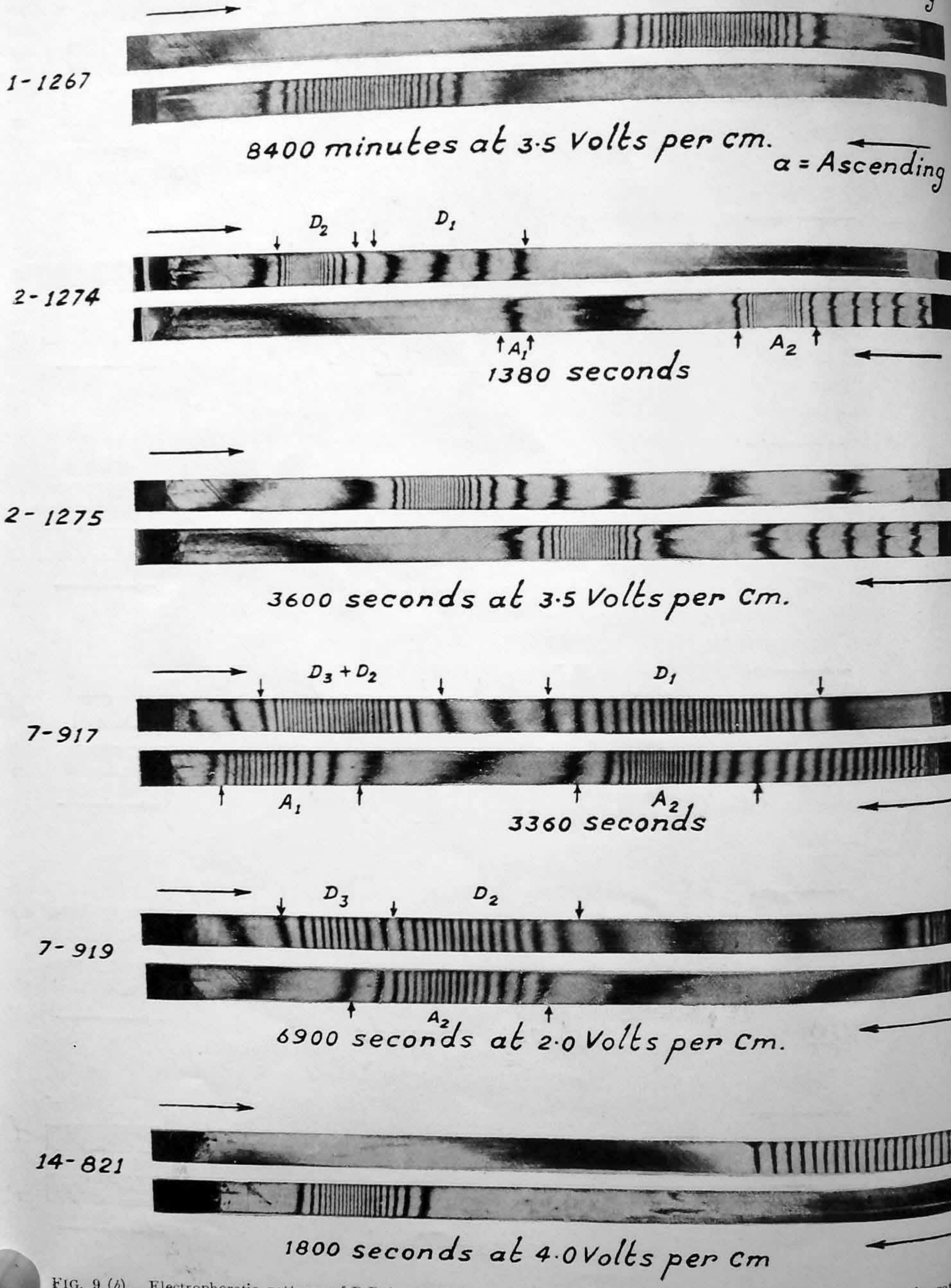

FIG. 9 (*b*). Electrophoretic patterns of B.P.A.—R.N.A. system in Acetate buffer pH = 5·60 μ = ·05 at various molar ratios.

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