and 3 that a significant amount of radioactive phosphorus is incorporated into the myelin-enriched fraction obtained from the brain of adult rats. However, the method of preparation of the myelin-enriched brain fraction described above is necessarily crude and the criteria for homogeneity, both chemical and microscopic, are only qualitative. To this extent therefore these experiments have failed to give a clear-cut answer and further investigations with more refined methods will be necessary to determine whether or not myelin lipids and other brain structural constituents are completely inert in the adult animal.

SUMMARY

1. Radioactive phosphate and sulphate have been injected into neonatal rats.

2. Most of the persistent radioactivity is found in the lipids of the brain mitochondria, but this fraction also probably contains myelin.

3. In other experiments radioactive phosphorus as phosphate was injected into neonatal and adult rats and a myelin-enriched fraction isolated from the brain.

4. Relatively more radioactive lipid was found in the myelin-enriched fraction from the brains of neonatal rats than in the rest of the brain lipid.

5. In contrast less radioactive phospholipid was obtained from the myelin-enriched fraction from adult rats than from the remaining brain lipid.

This work has been supported by a grant from the Medical Research Council. We are also grateful to Professor G. Payling Wright, in whose laboratory this work was begun.

REFERENCES

- Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
 Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.
 Chou, J. T. Y. & Meek, G. A. (1958). *Quart. J. micr. Sci.* **99**, 278.
 Davison, A. N. (1961). *Biochem. J.* **78**, 28 p.
 Davison, A. N. & Dobbing, J. (1959). *Biochem. J.* **73**, 701.
 Davison, A. N. & Dobbing, J. (1960a). *Biochem. J.* **75**, 565.
 Davison, A. N. & Dobbing, J. (1960b). *Biochem. J.* **75**, 571.
 Davison, A. N., Morgan, R. S., Wajda, M. & Payling Wright, G. (1959). *J. Neurochem.* **4**, 360.
 Folch, J. P., Lees, M. & Sloane-Stanley, G. (1957). *J. biol. Chem.* **226**, 497.
 Korey, S. R. (1957). In *The Metabolism of the Nervous System*, p. 87. Ed. by Richter, D. London: Pergamon Press Ltd.
 LeBaron, F. N. & Folch, J. P. (1959). *J. Neurochem.* **4**, 1.
 Long, C. & Staples, D. A. (1961). *Biochem. J.* **78**, 179.
 Petersen, V. P. & Schou, M. (1955). *Acta physiol. scand.* **33**, 309.
 Samson, F. E., Balfour, W. M. & Jacobs, R. J. (1960). *Amer. J. Physiol.* **199**, 693.
 Whittaker, V. P. (1959). *Biochem. J.* **72**, 694.

Biochem. J. (1961) **81**, 12

An Electrophoretic Investigation of Interactions between Bovine Plasma Albumin and Charged Dextran Derivatives

BY T. E. THOMPSON* AND W. M. McKERNAN†

Department of Experimental Pathology, University of Birmingham and Medical Research Council Industrial Injuries and Burns Unit, Birmingham Accident Hospital

(Received 15 February 1961)

The formation of soluble complexes through the interactions of macromolecules bearing net charges of the same sign is a phenomenon of considerable interest. Particular examples of this type of complex may be found in the antigen-antibody system (e.g. Singer & Campbell, 1955a, b, c; Singer, Eggman & Campbell, 1955), the polymerization of acid insulin (Oncley, Ellenbogen, Gitlin & Gurd, 1952; Doty & Myers, 1953), the polymerization of

α -chymotrypsin (Kegeles & Rao, 1958), the interaction between nucleic acids and proteins (Goldwasser & Putnam, 1950) and the interactions of plasma proteins with charged polysaccharides (Walton, 1952; Noguchi, 1956; Bernfeld, Donahue & Berkowitz, 1957; Sasaki & Noguchi, 1959).

The work reported in this paper is an investigation, with free-boundary electrophoresis, of the effect of variations of pH, ionic strength and molecular size on the formation of soluble complexes between bovine plasma albumin and synthetic derivatives of dextran. Two types of dextran derivatives have been employed: a sulphate ester and an ether-linked diethylaminoethyl derivative. Interactions between the bovine plasma albumin

* Present address: Department of Physiological Chemistry, John Hopkins School of Medicine, Baltimore 5, Maryland.

† Present address: Biochemistry Department, British Glues and Chemicals Ltd., 123 Grange Road, London, S.E. 1.

and the dextran sulphate derivatives have been studied in the pH range above the isoelectric point of the albumin, and interactions between this protein and the amine dextrans have been studied below the albumin isoelectric point.

EXPERIMENTAL

Materials

The properties of the sulphate and diethylaminoethyl (DEAE) derivatives of dextran used in this study are summarized in Table 1. The native dextran, a glucose polymer, was partially hydrolysed with acid and the hydrolysate fractionated with acetone to yield a series of dextrans differing in mean molecular weight. Selected acetone fractions were then converted either into the sulphate ester (Ricketts, 1952) or into the DEAE derivatives (McKernan & Ricketts, 1959, 1960). The mean molecular weights of the parent dextran fractions were determined from intrinsic viscosity measurements, with the equation of Wales, Marshall & Weissberg (1953). From these values and the sulphur or nitrogen content of the substituted dextrans, the mean molecular weights of the derivatives were obtained (McKernan, 1958.)

At I 0.10, 20°, the sulphate esters have a titration constant of 2.0 and the DEAE derivatives a titration constant of 8.8. Both types of dextran derivative are stable under all conditions employed in this study.

Two lots (BA-0170 and BE-1370) of Armour crystalline bovine plasma albumin were used in this study. Both preparations behaved electrophoretically as homogeneous solutes except in the range pH 3.5–4.5. In this pH range two components were observed (cf. Aoki & Foster, 1956). The two preparations were examined in the Spinco analytical ultracentrifuge at pH 7.4, I 0.10, 5.0°. Lot BA-0170 contained about 5% of a fast component just visible as a shoulder on the main peak. Lot BE-1370, however, showed only a trace of a similar component. In the interaction studies reported in this paper both albumin preparations were indistinguishable.

Sodium salts of the following buffer ions were used in appropriate pH ranges; glycinate, acetate, mono- and dibasic phosphates and diethylbarbiturate (veronal). The pH of each buffer was measured with a glass electrode at 20°. Buffer ionic strengths are reported as 1. mole⁻¹. To minimize specific ion effects on either the albumin–polyelectrolyte interactions (Noguchi, 1960) or the electrophoretic behaviour of the macromolecules, all buffer systems contained sodium chloride. In most cases the buffer salt contributed 0.05 to the ionic strength, the remainder being contributed by sodium chloride. Buffer conductivities were measured at 0°, with a Mullard conductivity bridge. All mobilities reported refer to this temperature.

Methods

Electrophoresis experiments were performed with a free-boundary Tiselius apparatus manufactured by Hilger and Watts Ltd., London. The instrument was equipped with a cylindrical lens, schlieren optical system employing a half-plane diagonal diaphragm (Philpot, 1938). The diaphragm was fixed at an angle of 25° throughout the course of this study. Standard 11 ml. cells were used. In addition to photographs of the starting boundary, at least two photographs of both the descending and ascending cell limbs were taken during the course of an experiment. Measurements of the mobilities and areas were made from enlarged ($\times 2.5$) tracings of the photographs. Unless specifically stated otherwise, all mobilities were calculated from descending boundary data.

Solutions were prepared by adding a measured volume of buffer to weighed amounts of bovine plasma albumin and the dextran derivative. Concentrations of bovine plasma albumin and the dextran derivatives are reported as g./100 ml. of solvent. In preparation for an electrophoresis experiment, 15 ml. of solution was dialysed against 1.5 l. of buffer with stirring at 4.0° for 16–20 hr. Because of the difficulty in estimating the concentration of either the dextran amine or the dextran sulphate in the presence of protein, no attempt was made to determine the concentrations of the interactants after dialysis. However, tests made by determining bovine plasma albumin concentration spectrophotometrically before and after dialysis indicated that no significant concentration change occurred provided that the cellophan bag was tightly tied and carefully emptied after dialysis.

RESULTS

Examination of separate interactants

In order to characterize the electrophoretic behaviour and to establish the relative refractive-index increments of the individual interactants, experiments were carried out on the single components over the range of pH, concentration and ionic strength employed in the interaction studies.

The descending limb mobility of bovine plasma albumin at I 0.10, 0°, as a function of pH, is plotted in Fig. 1. Results obtained in this study are shown and, for comparison, literature values obtained under similar, but not identical, conditions are included.

Measurements carried out at I 0.05 and 0.20, at selected pH values, indicated that the mobility of bovine plasma albumin was essentially independent of ionic strength over this range in the buffer systems employed.

Table 1. *Properties of dextran derivatives*

Preparation	Sulphur (%)	Nitrogen (%)	$[\eta]$ of parent dextran	Mean mol. wt. of derivative	No. of charges per glucose unit
Dextran sulphate E-3	18.45	—	0.10	2.7×10^4	2.3
Dextran sulphate D-6	18.60	—	0.32	2.7×10^6	2.3
Dextran amine BD-9	—	4.0	0.10	1.6×10^4	0.8
Dextran amine BD-2	—	3.3	0.32	1.5×10^6	0.6

Fig. 1. Electrophoretic mobility of bovine plasma albumin (Armour crystalline). Δ , Singer & Campbell (1955b), mobility referred to 1.3°, I 0.1; $\circ$, $\bullet$, Aoki & Foster (1956), mobility referred to 0°, I 0.10; $\square$, $\blacksquare$, this paper, mobility referred to 0°, I 0.10. $\circ$, $\bullet$ and $\square$, $\blacksquare$ designate the mobilities of the two components observed in the range pH 3.5–4.5.

Fig. 2. Electrophoresis schlieren patterns. The vertical arrows mark the positions of the initial boundaries. (a) Dextran sulphate E-3, 1.50 g./100 ml., pH 7.35, I 0.10; field strength, 2.89 v cm.⁻¹, 81 min.; u , -12.0×10^{-5} cm.² v⁻¹ sec.⁻¹ (b) Dextran amine BD-2, 1.00 g./100 ml., pH 3.45, I 0.10; field strength, 4.52 v cm.⁻¹, 85 min.; u , $+8.0 \times 10^{-5}$ cm.² v⁻¹ sec.⁻¹.

The relative refractive-index increment, expressed in arbitrary area units/unit concentration of bovine plasma albumin, was determined from the average of the total areas under the schlieren curves in both cell limbs, and was found to be 277 ± 4 . Within the limits of error of the determination, the relative refractive-index increments were found to be independent of bovine plasma albumin concentration, ionic strength and pH over the range of conditions employed in this study.

The electrophoretic behaviour of the dextran sulphates was investigated in the range from pH 5.4 to 8.5 and at I 0.10 and 0.20. The two dextran sulphate preparations behaved very similarly under these conditions. Each preparation migrated as a single asymmetric peak which had a weight-average mobility, for the E-3 preparation, of $(-11.9 \pm 0.2) \times 10^{-5}$ cm.² v⁻¹ sec.⁻¹, and for the D-6 preparation $(-11.3 \pm 0.2) \times 10^{-5}$ cm.² v⁻¹ sec.⁻¹. The mobility was independent of the pH and ionic strength, but the shapes of both descending and ascending boundaries were dependent to some extent upon these two parameters. This dependence probably reflects interactions between the dextran sulphate and the specific buffer ions. Schlieren patterns are shown in Fig. 2a for the E-3 dextran sulphate at pH 7.35, I 0.20.

The number of squares of area/unit concentration for the E-3 dextran sulphate was found to be 166 ± 3 , and, for the D-6 preparation, 159 ± 3 . Under the conditions employed in this study the relative refractive-index increments proved to be independent of pH, ionic strength and dextran sulphate concentration.

Exhaustive dialysis at 4° of a concentrated dextran sulphate solution against a limited volume of buffer indicated that if dialysable components were present they represented less than 3% of the total dextran sulphate. In these tests the concentration of dextran sulphate outside the dialysis bag was estimated by means of the metachromasia exhibited by toluidine blue in the presence of dextran sulphate (Walton & Ricketts, 1954).

Both dextran amines gave rise to a single moving boundary, which was very slightly skewed. The weight-average mobility was independent of pH over the range from 2.5 to 4.5 and independent of ionic strength in the interval from 0.05 to 0.20. The mobilities determined from descending limb data were: BD-9 $(8.18 \pm 0.3) \times 10^{-5}$ cm.² v⁻¹ sec.⁻¹, and BD-2 $(8.05 \pm 0.3) \times 10^{-5}$ cm.² v⁻¹ sec.⁻¹. For the BD-9 preparation the number of squares of area/unit concentration of dextran amine was 239 ± 2 , and for BD-2 preparation, 244 ± 2 . Both of these values, within the limits of error, proved to be independent of concentration, pH and ionic strength. Schlieren patterns of BD-2 at pH 3.45, I 0.10, are shown in Fig. 2b.

Interaction studies

Systems containing both bovine plasma albumin and dextran sulphate as solute components were examined electrophoretically in the pH interval from 5.4 to 8.5 for evidence of interactions between the macromolecular species. A parallel investigation was carried out with mixtures of bovine plasma albumin and dextran amine in the range pH 2.4–4.6.

As an internal check on the composition of each solution and on the planimetry, the average of the measured total ascending and total descending areas (including anomalies) was compared with the expected total area calculated from the refractometric data and known concentrations of interactants. In the interaction studies with dextran sulphate the difference between total measured area and total calculated area averaged over all experiments was 2.7%, with a maximum difference of 6.2%. The error varied from 0 to 3.8% with a mean error of 1.8% in the interaction studies involving dextran amine. Formation of insoluble interaction products was not observed under any set of conditions employed in this work.

Electrophoretic evidence for interaction between macromolecular solutes is obtained if the schlieren patterns of the moving-boundary systems in one or both cell limbs do not represent the simple sum of the corresponding patterns obtained upon electrophoresis of the individual components under similar conditions (Alberty, 1953). Strict additivity is not observed even if no interaction between solutes occurs. These deviations are usually small compared with the deviations resulting from interaction and are discussed below.

When non-additivity of the system is attributed exclusively to solute interaction, a convenient empirical measure of the 'degree of interaction' (α) is defined by

$$\alpha = 1 - F_2/f_2. \quad (1)$$

Here F_2 is the apparent refractometric fraction of free interactant comprising the slowest moving boundary in the limb, and f_2 is the true refractometric fraction of this interactant in the system.

Although in this paper only values of α will be reported, an analogous measure of interaction, $\beta = 1 - F_1/f_1$, based on the apparent refractometric fraction of dextran derivative in the fastest migrating boundary in the ascending limb, can also be defined. The subscripts 1 and 2 designate the dextran derivative and bovine plasma albumin respectively.

The apparent refractometric fraction, F_i , is defined by

$$F_i = A_i/\Sigma A_i. \quad (2)$$

Here A_i is the area in arbitrary units under the schlieren curve of the i th moving boundary and ΣA_i is the total area under all moving boundaries in the

cell limb. The true refractometric fraction f_i is given by

$$f_i = R_i C_i / \Sigma R_i C_i. \quad (3)$$

Here R_i denotes the relative refractive-index increment of the i th interactant measured in arbitrary area units per unit concentration. C_i is the concentration of this interactant.

In all systems studied the mobility of bovine plasma albumin was less than the mobility of the dextran derivative and thus less than the mobility of any complex species. Therefore free bovine plasma albumin in the equilibrium mixture and free bovine plasma albumin produced by re-equilibration within the moving-boundary system should appear as the slowest moving boundary in the descending limb. If interaction occurs between the bovine plasma albumin and the dextran derivative, the apparent refractometric fraction of bovine plasma albumin across this slowest descending boundary will be less than the known true refractometric fraction of bovine plasma albumin in the system. This will be the case whether re-equilibration occurs within the boundaries or not. Thus if no interaction occurs, $\alpha = 0$, but if all bovine plasma albumin is held in complex and no re-equilibration occurs, then $\alpha = 1$. It is evident that α is a complicated function of the concentration of interactants, the number of complex species and the rates of complex dissociation and formation. It is also evident that if re-equilibration occurs, then α cannot be related simply to the concentration of free bovine plasma albumin in the equilibrium mixture.

If α is to be a measure of interaction, then it is necessary that the slowest descending boundary be entirely free of the dextran derivative, i.e. that it be composed of bovine plasma albumin only. Similarly, if β is to be a measure of interaction, then the fastest ascending boundary must be free of bovine plasma albumin. A series of isolation experiments carried out with a split cell (Tiselius, 1937) indicated that these conditions obtained. (The design of this type of cell makes it possible to isolate the slowest moving component in the descending limb, and the fastest moving component in the ascending limb.) A summary of the results obtained in two typical isolation experiments is given in Table 2.

The results of the studies of the bovine plasma albumin–dextran sulphate interaction are presented in Tables 3–5. These Tables list the conditions of each experiment, the true refractometric fraction f_2 and the apparent descending limb refractometric fraction F_2 for bovine plasma albumin, the measured value of α , and the calculated value of α referred to in the Discussion and the mobilities of the fast boundary and the bovine plasma albumin boundary.

Table 3 illustrates the dependence of the measured α on pH. α , essentially zero at pH 8.50, rises

Table 2. *Results of a typical isolation experiment*

Expt. no.	Composition of buffer	pH	<i>I</i>	Composition of equil. solution		Composition of isolated fraction		Cell limb
				BPA	E-3	BPA*	E-3†	
				(g./100 ml.)	(g./100 ml.)	(g./100 ml.)	(g./100 ml.)	
41	Phosphate-NaCl	7.32	0.10	1.00	1.50	0.263	<0.002	Desc.
42	Phosphate-NaCl	7.30	0.10	1.53	1.01	<0.005	0.23	Asc.

* BPA concentrations determined spectrophotometrically at 280 mμ, $E_{1\text{cm}}^{1\%}$ 6.55.
† E-3 concentration determined by fibrinogen-clotting assay.

Table 3. *Bovine plasma albumin-dextran sulphate interaction as a function of pH*

Ionic strength 0.10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran sulphate (g./100 ml.)		BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm. ³ v ⁻¹ sec. ⁻¹)	
		E-3	D-6						Fast	BPA
44	8.50	0.84	—	1.50	0.738	0.756	-0.02	0.068	10.6	5.62
15	7.40	0.42	—	1.52	0.850	0.616	0.28	0.032	12.1	4.80
26	6.40	0.43	—	1.50	0.847	0.311	0.63	0.011	9.7	4.23
45	8.50	—	0.98	1.53	0.720	0.740	-0.02	0.092	10.6	5.63
39	7.35	—	0.49	1.50	0.835	0.636	0.24	0.048	11.5	4.53
42	6.40	—	0.51	1.50	0.829	0.382	0.54	0.018	8.5	3.66
68	5.85	—	0.51	1.41*	0.832	0.151	0.82	<0.01	7.15	3.47
70	5.40	—	0.53	1.49*	0.831	0	1.00	<0.01	5.90	—

* BPA lot BE-1370.

Table 4. *Bovine plasma albumin-dextran sulphate interaction as a function of ionic strength*

Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	<i>I</i> (l.mole ⁻¹)	E-3 (g./100 ml.)	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm. ³ v ⁻¹ sec. ⁻¹)	
									Fast	BPA
15	7.40	0.10	0.42	1.52	0.850	0.616	0.28	0.032	12.1	4.80
13	7.40	0.10	1.26	1.52	0.656	0.381	0.42	0.062	12.0	4.10
23	7.35	0.20	0.42	1.51	0.850	0.834	0.02	0.012	12.2	4.71
22	7.35	0.20	1.24	1.51	0.658	0.634	0.04	0.034	11.7	4.15

Table 5. *Bovine plasma albumin-dextran sulphate interaction as a function of interactant concentrations*

Ionic strength 0.10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran sulphate (g./100 ml.)		BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm. ³ v ⁻¹ sec. ⁻¹)	
		E-3	D-6						Fast	BPA
85	7.35	0.43	—	0.52*	0.668	0.505	0.24	0.018	13.6	4.90
84	7.35	0.44	—	1.00*	0.789	0.640	0.19	0.022	13.3	5.00
15	7.40	0.42	—	1.52	0.850	0.616	0.28	0.032	12.1	4.80
29	7.35	0.84	—	1.49	0.740	0.484	0.35	0.058	12.2	4.40
51	7.35	1.27	—	0.20	0.197	0.132	0.33	0.041	11.5	3.80
34	7.32	1.27	—	0.50	0.383	0.231	0.40	0.058	12.3	4.07
33	7.32	1.27	—	1.01	0.557	0.342	0.38	0.072	11.9	4.00
13	7.40	1.26	—	1.52	0.656	0.381	0.42	0.062	12.0	4.10
26	6.40	0.43	—	1.50	0.847	0.311	0.63	0.011	9.7	4.23
25	6.40	1.24	—	1.50	0.656	0.264	0.60	<0.01	10.5	3.50
77	5.85	—	0.18	1.42*	0.934	0.434	0.53	<0.01	5.20	3.31
68	5.85	—	0.50	1.41*	0.832	0.151	0.82	<0.01	7.15	3.47
76	5.40	—	0.19	1.42*	0.929	0.073	0.92	<0.01	3.53	2.33
70	5.40	—	0.53	1.49*	0.831	0	1.00	<0.01	5.90	—

* BPA lot BE-1370.

steadily to the maximum value of 1.0 at pH 5.40. The electrophoresis patterns obtained at pH 8.50 were almost exactly the sum of schlieren patterns obtained for the individual interactants. At pH 7.4 two moving boundaries were evident in the descending limb, but three moving boundaries developed in the ascending limb. However, at pH 6.40 the number of boundaries in the two limbs were reversed; three developed in the descending limb, and two developed in the ascending limb. At pH 5.85 only one ascending boundary developed, and this boundary migrated with a mobility about one-half of that which would be expected for free dextran sulphate. In the descending limb two boundaries were observed at this pH. At pH 5.40 only one moving boundary was observed in each cell limb. The descending boundary at this pH had a mobility midway between the mobilities of D-6 and bovine plasma albumin. Except for certain details in the ascending patterns E-3 and D-6 were indistinguishable in their interaction with bovine plasma albumin. The schlieren patterns of ascending and descending cell limbs for five conditions of interaction are reproduced in Fig. 3.

Fig. 3. Electrophoresis schlieren patterns illustrating the interactions between bovine plasma albumin and dextran sulphate as a function of pH at I 0.10. The vertical arrows mark the positions of the initial boundaries. (a) pH 7.4, 4.84 v cm.⁻¹; bovine plasma albumin, 1.52 g./100 ml.; E-3, 0.42 g./100 ml.; 85 min. (b) pH 7.3, 4.72 v cm.⁻¹; bovine plasma albumin, 1.50 g./100 ml.; D-6, 0.49 g./100 ml.; 90 min. (c) pH 6.40, 6.25 v cm.⁻¹; bovine plasma albumin, 1.50 g./100 ml.; D-6, 0.51 g./100 ml.; 70 min. (d) pH 5.85, 5.99 v cm.⁻¹; bovine plasma albumin, 1.41 g./100 ml.; D-6, 0.50 g./100 ml.; 65 min. (e) pH 5.40, 5.58 v cm.⁻¹; bovine plasma albumin, 1.49 g./100 ml.; D-6, 0.53 g./100 ml.; 65 min.

Table 4 summarizes the results of four experiments carried out at pH 7.4, but at two different ionic strengths (I 0.10 and 0.20). It appears from this limited set of data that the interaction decreases sharply with increasing ionic strength. The schlieren patterns obtained at I 0.20 are very closely the sum of the schlieren patterns obtained under these conditions for the individual interactants.

The variation of the degree of interaction with concentration of interactant is illustrated by the data assembled in Table 5.

Tables 6 and 7 summarize the electrophoresis results for the bovine plasma albumin-dextran amine systems.

DISCUSSION

Two types of deviations might be expected in systems containing two interacting solute components: (a) the number of moving boundaries in one or both cell limbs is either greater or less than two; (b) the apparent descending limb refractometric fraction of the slower-moving interactant is less than the true refractometric fraction, and the apparent ascending limb refractometric fraction of the faster-migrating interactant is less than the true refractometric fractions. It is important to examine these assumptions to determine whether observed deviations from simple additivity can in fact be attributed solely to macromolecular interaction in the systems investigated.

Observed deviations of type (a) can normally only be attributed to interaction between solutes. Consequently, deviations of this type constitute the strongest evidence for the existence of such interactions. It must be pointed out, however, that in buffer solvents of the type used in this work (composed of the buffer plus sodium chloride) a rapidly moving boundary which does not correspond to a macromolecular component might possibly be observed. In the buffer system used above pH 3.5, such a boundary would be expected to have a mobility between that of Cl⁻ ion and the buffer anion (Svensson, 1946). Since in these systems the mobilities of the Cl⁻ ion and the buffer anion were greater than the mobility of the dextran derivative, the mobility of a spurious boundary would be greater than the mobility of the dextran derivative. In no experiment was a boundary observed to have a mobility greater than that of the dextran derivative.

With the glycine-sodium chloride buffer systems used at pH 2.4 and 3.5, a spurious boundary would be expected to have a mobility less than that of the dextran amine. However, in the experiments in these buffers only two moving boundaries were observed. Therefore in the experiments in which more than two moving boundaries were observed the existence of additional boundaries must

Table 6. *Bovine plasma albumin-dextran amine interaction as a function of pH*

Ionic strength 0.10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran amine (g./100 ml.)		BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$10^5 u$ (cm. ² v ⁻¹ sec. ⁻¹)	
		BD-9	BD-2						Fast	BPA
72	2.40	1.01	—	0.98*	0.530	0.484	0.09	—	8.15	7.97
66	3.50	1.00	—	1.00*	0.537	0.478	0.11	0.13	8.08	4.72
54	4.05	1.02	—	1.02	0.524	0.473	0.10	0.07	8.51	0.82
56	4.55	1.01	—	0.99	0.519	0.526	-0.01	-0.01	8.44	-0.86
73	2.40	—	1.00	1.01*	0.534	0.353	0.34	—	8.10	7.80
40	3.50	—	1.00	1.00	0.518	0.422	0.18	0.23	8.06	4.92
75	4.55	—	1.00	1.01*	0.534	0.532	0	-0.01	7.97	-0.90

* BPA lot BE-1370.

Table 7. *Bovine plasma albumin-dextran amine interaction as a function of ionic strength and interactant concentration*

pH 3.50. Descending cell limb. BPA, Bovine plasma albumin.

Expt. no.	I (l.mole ⁻¹)	BD-2 (g./100 ml.)	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$10^5 u$ (cm. ² v ⁻¹ sec. ⁻¹)	
								Fast	BPA
48	0.05	0.50	1.50	0.764	0.696	0.09	—	7.80	5.00
49	0.05	1.50	0.52	0.271	0.231	0.15	—	7.46	3.75
18	0.05	1.50	1.51	0.519	0.416	0.20	—	7.27	3.76
37	0.10	0.49	1.01	0.689	0.630	0.08	0.11	8.05	5.38
40	0.10	1.00	1.00	0.518	0.422	0.18	0.23	8.06	4.92
47	0.10	1.51	1.00	0.415	0.324	0.22	0.25	7.50	4.18
21	0.20	0.50	1.02	0.686	0.647	0.06	0.06	8.00	5.60
20	0.20	1.00	0.50	0.349	0.351	0	0.10	7.90	5.60
30	0.20	1.01	1.02	0.519	0.532	-0.02	0.06	7.80	5.45

be attributed to interaction. In the bovine plasma albumin-dextran sulphate experiments deviations of type (a) were observed in all systems having values of $\alpha > 0.10$. However, in the bovine plasma albumin-dextran amine systems evidence of this kind for interaction was not obtained since two ascending and two descending moving boundaries were observed in all experiments.

While deviations of type (b) would be expected to occur in interacting systems, the observation of this sort of deviation does not by itself prove macromolecular interaction. The apparent and true refractometric fractions cannot, in general, be expected to be equal, even in simple systems in which there is no interaction between components. Deviations in the apparent refractometric fractions in a non-interacting system are for the most part due to the fact that small gradients of components which do not disappear in a given moving boundary are superimposed on the gradient of the component which does disappear. As a result, the apparent refractometric fractions have different values in the ascending and descending limbs, and are functions of the ratio of total protein concentration to buffer salt concentration (Svensson, 1943; Armstrong, Budka & Morrison, 1947; Longworth, 1947; Nichol & Gosting, 1958). In many protein systems the

deviations in apparent refractometric fractions seem to decrease as this ratio is decreased. Also, the deviations are less in systems in which large differences exist between the mobilities of the protein components (Svensson, Benjaminsson & Brattsten, 1949).

Quantitative estimates of the magnitude of type (b) deviations expected in the absence of interaction cannot be made from the moving-boundary theory for weak electrolytes (Svensson, 1948; Alberty & Nichol, 1948; Alberty, 1950). However, several authors (Longworth, 1947, 1959; Armstrong *et al.* 1947; Alberty, 1948; Cann, 1949; Brown & Timasheff, 1959) have utilized the moving-boundary theory for strong electrolytes (Dole, 1945; Svensson, 1946) with considerable success in discussing electrophoretic behaviour in protein systems. The series solutions for the Dole equations recently obtained by Nichol & Gosting (1958) have considerably simplified the calculation of apparent refractometric fractions with the theory for strong electrolytes. These equations have been used to calculate the values of α which would be expected in the absence of interaction in the bovine plasma albumin-dextran derivative systems studied.

Although, in principle, apparent refractometric fractions can be calculated following Nichol &

Gosting (1958) for systems containing more than one supporting electrolyte, the calculations are very cumbersome. In order to simplify the calculations all ions other than the macromolecular ions in the systems studied have been taken to be K^+ and Cl^- ions. It is not likely that this simplification is the source of any significant error in the calculation. To carry out these calculations it is necessary to know the equivalent concentrations and the equivalent refractions of both the bovine plasma albumin and the dextran derivative under the conditions of a particular experiment. The equivalent concentration C_i of the i th component is given by the following relation:

$$C_i = 10p_i \left[\frac{Z_i}{M_i} \right]. \quad (4)$$

Here p_i is the concentration (g./100 ml. of soln.), Z_i is the net charge per mole in protonic units and M_i is the molecular weight of the i th component. The equivalent refraction k_i may be obtained from the specific refraction k_i for the i th component by

$$k_i = \frac{k_i}{10} \left[\frac{M_i}{Z_i} \right]. \quad (5)$$

For the bovine plasma albumin at pH 7.4 a net charge of -22 was estimated from the titration data of Tanford, Swanson & Shore (1955), with allowance being made for Cl^- ion-binding. The effective net charge on the bovine plasma albumin at some other pH was then assumed to be proportional to the electrophoretic mobility at that pH (Brown & Timasheff, 1959). The molecular weight of the bovine plasma albumin was taken to be 6.9×10^4 and specific refraction was taken as 0.00186 (g. dl. $^{-1}$) $^{-1}$ (Armstrong *et al.* 1947).

The charge Z appearing in equations (4) and (5) is the charge associated with the electrokinetic unit in solution. Because a portion of the gegen-ion atmosphere migrates with the macromolecule, the effective charge of the electrokinetic unit will be less than the net charge on the macromolecular ion as estimated from titration data. With the bovine plasma albumin, this difference is probably small because of the relatively small net charge on the protein, and no significant error results from estimating Z on the basis of titration data. However, such cannot be expected to be the case with the dextran sulphates and dextran amines. Polyelectrolytes of charge densities comparable with the dextran derivatives have been shown to bind as much as 90% of the gegen-ion atmosphere into the kinetic unit (Huizenga, Grieger & Wall, 1950; Mathieson & McLaren, 1956; Botré, Crescenzi, Liquori & Mele, 1959). Clearly the effective charge on the dextran derivatives cannot be calculated from the sulphur or nitrogen content. For the purpose of the calculations based on the Nichol-

Gosting equations, and in the absence of more detailed information, it has been assumed that the charge/mass ratio (Z/M) of the dextran derivative is proportional to the electrophoretic mobility, and that the proportionality constant is the same for both bovine plasma albumin and the dextran derivative. The value of Z/M for the dextran derivative was then estimated from the following equation:

$$\left[\frac{Z}{M} \right]_1 = \frac{u_1}{u_2} \left[\frac{Z}{M} \right]_2. \quad (6)$$

Here u is mobility, and the subscripts 1 and 2 refer to the dextran derivative and bovine plasma albumin respectively. The values of $(Z/M)_2$ and u_2 used were those obtained at pH 7.4. Values of equivalent refractions for the dextran derivatives were calculated from the relative refractive-index increments for the derivatives and for bovine plasma albumin given in the preceding section and from the specific refraction for bovine plasma albumin.

The calculated values of α are listed together with the measured values of α in Tables 3-7. For the bovine plasma albumin-dextran sulphate systems (Tables 3-5) the calculated values of α are less than 0.10 in all cases. Under these conditions no evidence of type (a) for interaction was obtained; the calculated and measured values of α are in good agreement (pH 7.4, I 0.20 and pH 8.5, I 0.10). However, in cases where evidence of type (a) interaction was obtained, the measured values of α are at least six times as great as the calculated values.

In the bovine plasma albumin-dextran amine systems there was no evidence of type (a) to suggest the existence of interactions. In addition, comparison of the measured and calculated values of α shows good agreement between the two sets of values. This suggests that essentially no interaction occurs in these systems.

However, evidence of a somewhat different nature was obtained for interaction between bovine plasma albumin and BD-9 at pH 4.05, I 0.10. Under these conditions in a system containing both bovine plasma albumin and BD-9 the slow descending boundary composed only of bovine plasma albumin failed to split in two, as is the case with pure bovine plasma albumin (Aoki & Foster, 1956). Substitution of the parent dextran, from which BD-9 was prepared, for BD-9 in a similar experiment, however, did not prevent formation of the expected double BPA boundary. These observations are illustrated in Fig. 4. It is evident from these experiments that an interaction of some sort occurs between bovine plasma albumin and BD-9 under these conditions. The interaction appears to affect directly the bovine plasma albumin-isomerization reaction. The fact that the two albumin species were not observed in the slow descending boundary

Fig. 4. Electrophoresis schlieren patterns obtained at pH 4.05, I 0.10. The vertical arrows mark the positions of the initial boundaries. (a) Bovine plasma albumin, 1.00 g./100 ml.; field strength 5.23 v cm.⁻¹, 120 min. (b) Bovine plasma albumin, 1.00 g./100 ml. + dextran amine BD-9, 1.00 g./100 ml.; field strength 5.23 v cm.⁻¹, 120 min. (c) Bovine plasma albumin, 1.00 g./100 ml. + dextran, 1.00 g./100 ml.; field strength 5.23 v cm.⁻¹, 150 min.

which is free of BD-9 (at least within the limit of analysis) suggests that the interaction produces an irreversible alteration in the bovine plasma albumin.

Longworth & MacInnes (1942) investigated the interaction between ovalbumin (P) and nucleic acid (N) and described the electrophoretic behaviour which might be expected in five cases of complex formation based on the reaction:

In this equation k_1 and k_{-1} are the rate constants for the forward and reverse reaction respectively and r is the number of moles of nucleic acid reacting with 1 mole of protein.

The electrophoretic behaviour observed in the present research could be expected to be similar to two of the cases described by Longworth & MacInnes. In the fourth case the rate constants k_1 and k_{-1} are of such magnitude that the rate of adjustment of the equilibrium is comparable with the rate of electrophoretic separation of the components. In this system, however, the mobilities and areas of the components would be time-dependent and in no instance did the data reported in this paper show such a time-dependence. Thus it would appear that the behaviour of the present system is only directly analogous to the fifth case described by Longworth & MacInnes. In this case the

rate constants k_1 and k_{-1} are large and of the same order of magnitude, with the result that the equilibrium is adjusted within the moving-boundary system as rapidly as is required by the electrophoretic separation of components. The migration of the faster interactant into solvent in the ascending limb, but into solution in the descending limb, results in the separation of pure fast interactant as the leading boundary in the ascending limb and pure slow interactant as the trailing boundary in the descending limb. Because of interaction the apparent refractometric fractions determined from these two boundaries will be less than the true refractometric fractions of the corresponding interactants. These apparent fractions are, however, larger than would be the case if re-equilibration did not take place, due to the dissociation in each limb of part of the complex. As a direct consequence of re-equilibration there is no boundary which may be identified simply with the complex, and, in addition, the total number of moving boundaries in each limb may not be equal and may be less than, equal to or greater than two. A simple interpretation of the apparent refractometric fractions will therefore not yield the true equilibrium constant for complex formation.

No general theory is available for the detailed interpretation of moving-boundary patterns in interacting systems exhibiting re-equilibration. Theoretical treatments for certain special cases of complex formation have been presented (Smith & Briggs, 1950; Alberty & Marvin, 1950; Field & Ogston, 1955; Gilbert & Jenkins, 1956, 1959; Cann, Kirkwood & Brown, 1957; Kegeles & Rao, 1958; Gilbert, 1959).

The theory of Gilbert & Jenkins is based on the reaction given by equation (7), when $r = 1$. Although the bovine plasma albumin-dextran derivative interactions can in general be expected to be more complicated than this, it does appear that under one set of conditions the bovine plasma albumin-E-3 system (pH 7.4, I 0.10) behaved approximately like one of the systems discussed by Gilbert & Jenkins (1959), in which the complex and the faster interactant have the same mobility; three moving boundaries are predicted for the ascending limb and two for the descending limb. In addition, the concentration of the slower interactant across the trailing descending boundary is shown to be the concentration of this component in the bulk equilibrium mixture. It is therefore possible to calculate the equilibrium constant for complex formation from the descending limb data. Gilbert & Jenkins (1959) also predict for the ascending limb data that a plot of $1/(u_c - u_2)$ against, the reciprocal of total concentration of fast interactant (component 1), should be linear with an intercept equal to $1/(u_1 - u_2)$. Here u_c is the mobility of the second

boundary in the ascending limb, u_1 and u_2 are the mobilities of the fast and slow interactants respectively.

The bovine plasma albumin-E3 system at pH 7.4, I 0.10, did exhibit at all concentrations of interactants three ascending and two descending moving boundaries. In addition, the mobility of the fast descending boundary was equal to that of E-3 (Table 3). This could only occur if the complex and E-3 had the same mobility. The plot suggested by Gilbert & Jenkins of the ascending limb mobility data is presented in Fig. 5. It can be seen that the data are reasonably well fitted by a straight line with intercept 0.088, in good agreement with the predicted value of 0.092. Table 8 lists the equilibrium constant calculated from the descending limb data for each of eight experiments. In these calculations the molecular weights of bovine plasma albumin and E-3 dextran sulphate were taken as 6.9×10^4 g.mole⁻¹ and 2.7×10^4 g.mole⁻¹ respectively. The equilibrium concentration of bovine plasma albumin was calculated from the apparent refractometric fraction of this component, the

Fig. 5. Plot of the ascending mobility function of Gilbert & Jenkins (1959) against the reciprocal of the total concentration of dextran sulphate (fast interactant). The system consists of dextran sulphate, E-3 (interactant 1) and bovine plasma albumin (interactant 2), pH 7.4, I 0.10.

average total measured area and the relative refractive index increment of bovine plasma albumin. Agreement among the values of the equilibrium constant listed in Table 8 is fairly good. It must be noted in this regard that no allowance has been made for the presence of buffer ion and sodium chloride gradients within the moving boundaries. In addition to the deviations in F_2 which have been discussed, it would be expected that these gradients would effect re-equilibration at the moving boundaries, since the interaction is a function of both pH and ionic strength.

The fact that the interaction between bovine plasma albumin and the dextran sulphates is a function of the ionic strength suggests that the primary force involved is electrostatic. Furthermore, it would seem that electrostatic-attraction forces must be involved in stabilizing the complex, since interaction decreases with increasing ionic strength. These observations suggest that the bonds involved in complex formation are between the negative charges on the dextran sulphate and the residual positive charges on the bovine plasma albumin (cf. Haurowitz, 1950; Sasaki & Noguchi, 1959). It is then probable that two causes operate to produce the observed decrease in interaction at higher pH values: (1) as the pH is raised the net negative charge on the bovine plasma albumin is increased and hence the electrostatic repulsion between the protein and the dextran sulphate is increased (Noguchi, 1956); (2) as the pH is raised the number of positive charges on the protein which are available for interaction is decreased.

Exposure of bovine plasma albumin to dextran amine at pH 4.05 prevents the splitting of the bovine plasma albumin during electrophoresis at this pH, but no other evidence was obtained for bovine plasma albumin-dextran amine interaction. Soluble complexes of the type observed in the bovine plasma albumin-dextran sulphate systems certainly do not form in the bovine plasma albumin-dextran amine systems. This difference may in part be due to the fact that the charge density of the dextran amines is about one-third of

Table 8. *Equilibrium constant for bovine plasma albumin-dextran sulphate interaction*

pH 7.4, I 0.10. Calculated according to Gilbert & Jenkins (1959).

Expt. no.	E-3 (g./100 ml.)	BPA (g./100 ml.)	F_2 (desc.)	Total area in squares	BPA (g./100 ml.; desc.)	$10^{-4} K$ (l.mole ⁻¹)
85	0.43	0.52*	0.505	217	0.396	2.22
13	1.26	1.52	0.381	634	0.922	1.69
29	0.84	1.49	0.484	520	0.961	2.37
15	0.42	1.52	0.616	478	1.125	3.58
33	1.27	1.01	0.342	485	0.658	1.43
34	1.27	0.50	0.231	349	0.308	1.42
51	1.27	0.20	0.132	269	0.135	1.04
84	0.44	1.00*	0.640	359	0.829	1.51

Average 1.91

* BPA lot BE-1370.

that of the dextran sulphates (Table 1). There is, however, considerable evidence to indicate that organic cations, in general, interact much less strongly with plasma albumin than do anions (e.g. Klotz, Gelewitz & Urquhart, 1952; McKernan & Ricketts, 1960). Haurowitz (1936) has suggested that the apparent inability of serum albumin to form strong complexes with basic colloids may be explained by assuming that the CO_2^- groups are located in the interior of the protein molecule and hence are not available for interaction. Klotz *et al.* (1952) have proposed that the CO_2^- groups are unavailable for cation binding because of intramolecular interaction with the hydroxyl groups of neighbouring hydroxyamino acids. It is also possible that the detailed charge configuration on the protein surface is an important parameter in complex formation. For example, clusters of closely grouped positive charges on the bovine plasma albumin could be important in the formation of bovine plasma albumin-anion complexes; the absence of similar clusters of negative charge would then be reflected in the considerably lower affinity of bovine plasma albumin for organic cations.

SUMMARY

1. An electrophoretic investigation of the formation of soluble interaction complexes between bovine plasma albumin and charged dextran derivatives (sulphate esters and diethylaminoethyl ethers) is described. The investigation is limited to conditions of pH such that the net charge on the protein and the charge on the polyelectrolyte have the same signs.

2. In the dextran sulphate systems soluble complex formation is maximal at the isoelectric pH of the protein and decreases with increasing pH. Complexes cannot be detected above pH 8.5. Complex formation decreases with increasing ionic strength. The equilibrium constant for complex formation is calculated from the electrophoresis results under one set of conditions which constitute a special case of the theory of Gilbert & Jenkins.

3. Dextran amine is shown to suppress the albumin isomerization reaction at pH 4.0. Except for this observation, no evidence has been obtained for complex formation between the bovine plasma albumin and the dextran amines.

4. The mobility of bovine plasma albumin at $I\ 0.10$, 0° , is determined as a function of pH.

5. Deviations from simple electrophoretic behaviour expected in the absence of interaction in the systems studied are evaluated with the moving-boundary theory for strong electrolytes.

The authors wish to thank Professor J. R. Squire, Dr K. W. Walton, Dr G. A. Gilbert and Dr C. R. Ricketts for many interesting and helpful discussions during the

course of this work. We wish also to acknowledge our indebtedness to Dr C. R. Ricketts for generously supplying the two dextran sulphate preparations used in this work. One of us (T.E.T.) gratefully acknowledges the support given to him through a Personal Grant by the Medical Research Council.

REFERENCES

- Alberty, R. A. (1948). *J. chem. Educ.* **25**, 619.
 Alberty, R. A. (1950). *J. Amer. chem. Soc.* **72**, 2361.
 Alberty, R. A. (1953). In *The Proteins*, vol. 1, part A, chapt. 6. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
 Alberty, R. A. & Marvin, H. H., jun. (1950). *J. phys. Colloid Chem.* **54**, 47.
 Alberty, R. A. & Nichol, J. C. (1948). *J. Amer. chem. Soc.* **70**, 2297.
 Aoki, K. & Foster, J. F. (1956). *J. Amer. chem. Soc.* **78**, 3538.
 Armstrong, S. H., jun., Budka, M. J. E. & Morrison, K. C. (1947). *J. Amer. chem. Soc.* **69**, 416.
 Bernfeld, P., Donahue, V. M. & Berkowitz, M. E. (1957). *J. biol. Chem.* **226**, 51.
 Botré, C., Crescenzi, V. L., Liquori, A. M. & Mele, A. (1959). *Trans. Faraday Soc.* **55**, 1975.
 Brown, R. A. & Timasheff, S. N. (1959). In *Electrophoresis*, chapt. 8. Ed. by Bier, M. New York: Academic Press Inc.
 Cann, J. R. (1949). *J. Amer. chem. Soc.* **71**, 907.
 Cann, J. R., Kirkwood, J. G. & Brown, R. W. (1957). *Arch. Biochem. Biophys.* **72**, 37.
 Dole, V. P. (1945). *J. Amer. chem. Soc.* **67**, 1119.
 Doty, P. & Myers, G. E. (1953). *Disc. Faraday Soc.* **13**, 51.
 Field, E. O. & Ogston, A. G. (1955). *Biochem. J.* **60**, 661.
 Gilbert, G. A. (1959). *Proc. Roy. Soc. A*, **250**, 377.
 Gilbert, G. A. & Jenkins, R. C. Ll. (1956). *Nature, Lond.*, **177**, 853.
 Gilbert, G. A. & Jenkins, R. C. Ll. (1959). *Proc. Roy. Soc. A*, **253**, 420.
 Goldwasser, E. & Putnam, F. W. (1950). *J. phys. Colloid Chem.* **54**, 79.
 Haurowitz, F. (1936). *Kolloidzshr.* **74**, 208.
 Haurowitz, F. (1950). In *Chemistry and Biology of Proteins*, p. 186. New York: Academic Press Inc.
 Huizenga, J. R., Grieger, P. F. & Wall, F. T. (1950). *J. Amer. chem. Soc.* **72**, 2636.
 Kegeles, G. & Rao, M. S. N. (1958). *J. Amer. chem. Soc.* **80**, 5724.
 Klotz, I. M., Gelewitz, E. W. & Urquhart, J. H. (1952). *J. Amer. chem. Soc.* **74**, 209.
 Longworth, L. G. (1947). *J. phys. Colloid Chem.* **51**, 171.
 Longworth, L. G. (1959). In *Electrophoresis*, chapt. 3. Ed. by Bier, M. New York: Academic Press Inc.
 Longworth, L. G. & MacInnes, D. A. (1942). *J. gen. Physiol.* **25**, 507.
 McKernan, W. M. (1958). Ph.D. Thesis: University of Birmingham.
 McKernan, W. M. & Ricketts, C. R. (1959). *Chem. & Ind.* p. 1490.
 McKernan, W. M. & Ricketts, C. R. (1960). *Biochem. J.* **76**, 117.
 Mathieson, A. R. & McLaren, J. V. (1956). *J. Chem. Soc.* p. 303.
 Nichol, J. C. & Gosting, L. J. (1958). *J. Amer. chem. Soc.* **80**, 2601.

- Noguchi, H. (1956). *Biochim. biophys. acta*, **22**, 459.
- Noguchi, H. (1960). *J. phys. Chem.* **64**, 185.
- Onley, J. L., Ellenbogen, E., Gitlin, D. & Gurd, F. R. N. (1952). *J. phys. Chem.* **56**, 85.
- Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
- Ricketts, C. R. (1952). *Biochem. J.* **51**, 129.
- Sasaki, S. & Noguchi, H. (1959). *J. gen. Physiol.* **43**, 1.
- Singer, S. J. & Campbell, D. H. (1955a). *J. Amer. chem. Soc.* **77**, 3499.
- Singer, S. J. & Campbell, D. H. (1955b). *J. Amer. chem. Soc.* **77**, 3504.
- Singer, S. J. & Campbell, D. H. (1955c). *J. Amer. chem. Soc.* **77**, 4851.
- Singer, S. J., Eggman, L. & Campbell, D. H. (1955). *J. Amer. chem. Soc.* **77**, 4855.
- Smith, R. F. & Briggs, D. R. (1950). *J. phys. Colloid Chem.* **54**, 33.
- Svensson, H. (1943). *Ark. Kemi Min. Geol.* **17A**, no. 14.
- Svensson, H. (1946). *Ark. Kemi Min. Geol.* **22A**, no. 10.
- Svensson, H. (1948). *Acta chem. scand.* **2**, 841.
- Svensson, H., Benjaminsson, A. & Brattsten, I. (1949). *Acta chem. scand.* **3**, 307.
- Tanford, C., Swanson, S. A. & Shore, W. S. (1955). *J. Amer. chem. Soc.* **77**, 6414.
- Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 514.
- Wales, M., Marshall, P. A. & Weissberg, S. G. (1953). *J. Polym. Sci.* **10**, 229.
- Walton, K. W. (1952). *Brit. J. Pharmacol.* **7**, 370.
- Walton, K. W. & Ricketts, C. R. (1954). *Brit. J. exp. Path.* **35**, 227.

Biochem. J. (1961) **81**, 23

Eugenol Lignin: its Chemical Properties and Significance

By D. E. BLAND

Division of Forest Products, C.S.I.R.O., Melbourne, Australia

(Received 11 January 1961)

Observations by several investigators have shown that many different substances may appear to act as precursors of lignin in growing plants or in artificial systems. Stone (1953) has pointed out that deposition of a radioactive lignin from a labelled precursor is not in itself proof that the supposed precursor has been incorporated into the lignin. The radioactivity may be due merely to co-polymerization.

Schubert, Acerbo & Nord (1957) demonstrated the incorporation of labelled *p*-hydroxyphenyl-pyruvic acid into the lignin of sugar cane. Acerbo, Schubert & Nord (1958) showed that the carboxyl-labelled acid gave an active lignin but that the vanillin produced from it was inactive, thus suggesting that the aromatic ring of the acid was incorporated as such into the lignin. Kratzl & Billek (1959) found that the [3-¹⁴C]-labelled acid, when infused into lignifying spruce tissue, gave an active lignin but that the vanillin produced from it was of very low activity. This appears to be a case of co-polymerization. However, Brown, Wright & Neish (1959) found that the wheat plant could synthesize guaiacyl and syringyl radicals from this precursor.

Artificial lignification of *Elodea* sections in solutions containing peroxide and eugenol has been demonstrated by Siegel (1954). In a system with added peroxidase a lignin was deposited on a filter-paper matrix (Siegel, 1956). The lignin formed was similar to natural lignin in ultimate composition (Siegel, 1957). Higuchi (1957), experimenting with

bamboo-shoot and horseradish enzyme preparations, observed the formation of a lignin-like substance from old, but not from freshly distilled, eugenol. He concluded that the lignin-like substance was formed from coniferaldehyde in the eugenol. On the other hand, Stafford (1960) found that a lignin-like substance was deposited in *Phleum* leaf sections even by freshly distilled eugenol. This 'lignin', although giving a positive phloroglucinol test, differed from the natural lignin or that formed from ferulic acid in not being extractable with 2% sodium hydroxide.

Artificial lignification of oat (*Avena*) coleoptiles, *Elodea* internodes and other tissues in the same medium as used by Siegel in his experiments was studied by Wardrop & Davies (1959) mainly from the morphological point of view, and they concluded from electron micrographs and staining reactions of the lignifying tissue that the artificial lignin was deposited between the cellulose microfibrils in the wall in the same way as natural lignin.

These morphological studies leave unanswered the question of whether the lignin deposited is identical with natural lignin. It has been pointed out by Freudenberg (1958) that no eugenol exists in cambial sap and that formation of artificial lignin from this substance has nothing to do with natural lignification. On the other hand, just as an organic substance may be synthesized by different routes, it is conceivable that a living plant could utilize a number of unnatural precursors to lay down a lignin indistinguishable from its natural lignin. For