- Cama, H. R., Dalvi, P. D., Morton, R. A., Salah, M. K., Steinberg, G. R. & Stubbs, A. L. (1952). *Biochem. J.* **52**, 535.
- Dmitrovsky, A. A. (1961). *Biokhimiya*, **26**, 126.
- Dowling, J. E. & Wald, G. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 587.
- Farrar, K. R., Hamlet, J. C., Henbest, H. B. & Jones, E. R. H. (1952a). *J. chem. Soc.* p. 2657.
- Farrar, K. R., Hamlet, J. C., Henbest, H. B. & Jones, E. R. H. (1952b). *J. chem. Soc.* p. 2666.
- Futterman, S. (1962). *J. biol. Chem.* **237**, 677.
- Futterman, S. & Saslaw, L. D. (1961). *J. biol. Chem.* **236**, 1652.
- Gillam, A. E., Heilbron, I. M., Jones, W. E. & Lederer, E. (1938). *Biochem. J.* **32**, 405.
- Haas, E. (1944). *J. biol. Chem.* **155**, 321.
- Harris, P. L. (1960). *Vitam. & Horm.* **18**, 367.
- Jensen, J. L., Shantz, E. M., Embree, N. D., Cawley, J. D. & Harris, P. L. (1943). *J. biol. Chem.* **149**, 473.
- Jungalwala, F. B. & Cama, H. R. (1962). *J. Chromat.* **8**, 535.
- Kofer, M. & Rubin, S. H. (1960). *Vitam. & Horm.* **18**, 315.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mahadevan, S., Murthy, S. K. & Ganguly, J. (1962). *Biochem. J.* **85**, 326.
- Mahler, H. R., Mackler, B. & Green, D. E. (1954). *J. biol. Chem.* **210**, 465.
- Malathi, P., Subba Rao, K., Seshadri Sastry, P. & Ganguly, J. (1963). *Biochem. J.* **87**, 305.
- Moore, T. (1957). *Vitamin A*, pp. 283, 284. Amsterdam: Elsevier.
- Murphy, J. B. & Kies, M. W. (1960). *Biochim. biophys. Acta*, **45**, 382.
- Rajagopalan, K. V., Fridovich, I. & Handler, P. (1962). *J. biol. Chem.* **237**, 922.
- Redfearn, E. R. (1960). *Arch. Biochem. Biophys.* **91**, 226.
- Shantz, E. M. (1948). *Science*, **108**, 417.
- Sundaresan, P. R. & Cama, H. R. (1961). *Brit. J. Nutr.* **15**, 225.
- Thompson, J. N., Howell, J. McC. & Pitt, G. A. J. (1961a). *Biochem. J.* **80**, 16p.
- Thompson, J. N., Howell, J. McC. & Pitt, G. A. J. (1961b). *Biochem. J.* **80**, 25p.
- Van Dorp, D. A. & Arens, J. F. (1946a). *Nature, Lond.*, **157**, 190.
- Van Dorp, D. A. & Arens, J. F. (1946b). *Nature, Lond.*, **158**, 60.
- Waddell, W. J. (1956). *J. Lab. clin. Med.* **48**, 311.
- Zachman, R. D. & Olson, J. A. (1961). *J. biol. Chem.* **236**, 2309.

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A Comparison of the Electrophoretic Characteristics of the Human Normal and Sickle Erythrocyte

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The process of sickling in which red cells from sickle-cell-anaemia or sickle-cell-trait subjects lose their biconcave discoid form and become crescent-shaped is now thought to be primarily due to the properties of an abnormal haemoglobin (haemoglobin S) within the cell. Haemoglobin S synthesis *in vivo* is probably under the control of a single gene (the sickle-cell gene) which has undergone mutation (Neel, 1949). Ingram (1957) has shown that the genetically conferred difference between normal and sickle-cell haemoglobin resides in the replacement of glutamic acid by valine in a peptide moiety of the sickle-cell globin, which also accounts for the difference in mobilities of the haemoglobins by paper electro-

phoresis. Secondary physicochemical differences between normal and sickle cells have been reported in the literature. The net exchange of Cs^+ , K^+ and Na^+ ions increases in the order: normal cell, promiscocyte and sickle cell (Tosteson, Carlsen & Dunham, 1955; Tosteson, 1955). Prankerd (1955) has reported a decrease in [^{32}P]orthophosphate uptake by sickle cells and a diminution in the concentration of adenosine triphosphate. The reduced glutathione (GSH) content of sickle cells is also significantly greater than that in normal cells. Prolonged dialysis against physiological saline will remove the GSH from the red cells and render them incapable of sickling (Schneider, Levin & Haggard, 1949; Kass, Ingbar, Harris & Ley, 1951). Electrophoretic studies on the plasma and serum proteins in sickle-cell anaemia have

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revealed abnormal patterns with often a reversal of the albumin:globulin ratio (Fenichel, Watson & Eirich, 1950).

The role of oxygen concentration, pH, changes in viscosity of the blood and the dependency of the sickling phenomenon on the intracellular concentration of haemoglobin S have been reviewed by Harris (1959) and Allison (1957).

Most of the published work on the phenomenon of sickling has emphasized the role of the abnormal haemoglobin. Dervichian, Fournet, Guinier & Ponder (1952) have suggested that the sickling phenomenon involves co-precipitation of the haemoglobin with the lipids of the membrane, the major proportion of haemoglobin not being involved in the reaction, which would account for the absence of differences in the specific heat, heat of compression (Ponder, 1955) and X-ray-scattering patterns (Dervichian *et al.* 1952). Further, the change in water content of less than 1% on deoxygenation (Tosteson *et al.* 1955) is very small for a process such as crystallization of the haemoglobin. Ponder (1951) has observed the sickling of stroma derived from sickle cells and of sickling processes occurring in fragments of cells that have been disrupted by heat. In the present paper the electrophoretic behaviour of the peripheral regions of sickle cells is compared with those of the normal erythrocyte. Abramson (1929), who examined three cases of sickle-cell anaemia by cell electrophoresis in 66.7 mM-phosphate at pH 7.35, reported that the velocities were practically normal, despite extreme variations in size, shape and haemoglobin content. Observations by Seaman & Pethica (1957) have confirmed those of Abramson (1929). In the present paper some of the values obtained previously for the charge-reversal concentrations of various cations are amended, and the results reinterpreted in the light of recent studies on the ultrastructure of the normal human erythrocyte.

MATERIALS AND METHODS

Samples of sickle-cell-anaemia blood were obtained by venepuncture from hospital patients, and sickle-cell-trait blood from U.S.A.F. personnel in England and from four trait cases found in a survey of coloured students at the University of Cambridge. Normal blood was obtained from donors of differing phenotypes. The bloods were taken into acid citrate-dextrose medium, or defibrinated, or allowed to clot. Specimens were stored at 4° and used up to 30 days after collection.

Blood smears from all anaemia or trait specimens were examined microscopically for initial sickling under a coverslip sealed with petroleum jelly. Sickling was induced by the addition of 1 drop (about 0.04 ml.) of iso-osmotic sodium dithionite (2.1%, w/v) in oxygen-free distilled water to whole blood (0.05 ml.). The resultant mixture was examined for sickling under an unsealed coverslip. The

results of the slide tests were checked by paper electrophoresis of the haemolysed cells against known haemoglobin A and S standards.

The red cells were freed from plasma or serum by washing four times in aq. 0.145M-sodium chloride solution made 0.3 mM with respect to sodium bicarbonate, pH 7.2±0.2, (termed 'standard saline'), by using 25 vol. of the standard saline to 1 vol. of the packed red cells (5 min. at 2000g) per wash. The resultant red cells are termed 'standard washed red cells' and were made up to a 10% (v/v) stock suspension in standard saline. Some standard washed red cells were washed once more in 14.5 mM-sodium chloride in 4.5% (w/v) sorbitol made 0.3 mM with respect to sodium bicarbonate. Solutions of aq. 0.145N-hydrochloric acid and 0.145N-sodium hydroxide were prepared for use in changing the pH of a given suspension of erythrocytes. After the cells had been washed in the medium of lower ionic strength they were examined immediately. The metal salts were of AnalaR grade with the exception of the lanthanum chloride and were used as solutions of the chlorides or nitrates in unbuffered aq. 0.145M-sodium chloride solution. It is not possible to adjust the pH of many metal salt solutions to neutral pH without complex changes occurring (Ahrlund, Hietanen & Sillén, 1954; Hietanen, 1954). These solutions were used therefore at their natural pH which ranged from 4.5 to 6.5, except some of the cupric chloride and uranyl nitrate solutions which had pH values as low as 3.

The water used in all experiments was distilled twice from a still of Pyrex glassware and used within 24 hr. of preparation. All glassware was cleaned with chromic acid-sulphuric acid.

Trypsin was a crystalline salt-free specimen obtained from Armour Laboratories Ltd. The neuraminidase was obtained from Behringwerke A.-G. in the form of an aqueous solution of purified material isolated from *Vibrio cholerae* filtrate. Treatment of the red cells with neuraminidase and trypsin was carried out as has been previously described (Cook, Heard & Seaman, 1961; Seaman & Heard, 1960).

The electrophoretic mobilities were obtained by use of a horizontal cylindrical electrophoresis cell (Bangham Flemans, Heard & Seaman, 1958; Seaman & Heard, 1961). The rate of migration of the red cells under a known potential gradient was observed directly by the microscope. A minimum of ten red cells was observed per mobility determination and the maximum variation in the rate of migration from one cell to another was about 15%. The electrophoretic mobilities of the red cells in the various metal salt solutions were constant for the period between 5 and 15 min. after their addition to the electrolytic media provided that the pH was greater than 4. The natural pH of any of the metal salt solutions was not altered by more than 0.3 pH unit by the addition of red cells to give the requisite 0.05% (v/v) suspension for examination by cell electrophoresis. All systems were in equilibrium with the atmosphere unless specified otherwise.

Deoxygenation of red-cell suspensions in aqueous salt solutions was effected by evacuation of a glass chamber by means of a rotary vacuum pump. The red-cell suspension occupied about one-fifth of the internal volume of the chamber which was evacuated four times followed by the admission of oxygen-free nitrogen to approximately atmospheric pressure after each evacuation. The deoxy-

genated cell suspensions gave a negative pyrogallol test, indicating the almost complete absence of dissolved oxygen. Before the chamber of the electrophoresis apparatus was filled anaerobically with deoxygenated suspensions it was flushed out with pure nitrogen. Sickie cells could be observed in the electrophoretic chamber when deoxygenated suspensions of the appropriate cells were examined.

Sphering of red cells was produced by the passage of 2% (v/v) suspensions of either normal erythrocytes or promeniscocytes in aq. 0.145M-sodium chloride solutions over Ballotini (approx. 0.5 mm. spheres) in a glass column (90 cm. $\times$ 1.0 cm. diam.). The pH of the suspensions was adjusted to 8-9 with aq. 0.145M-sodium hydroxide solution before passage of the red-cell suspension over the Ballotini. Sickie cells under these conditions haemolysed rapidly and could not be studied.

RESULTS

The promeniscocytes and sickie cells show the same behaviour as normal erythrocytes with regard to distribution in the rate of migration of individual cells, effect of anticoagulants and storage as whole blood at 4°. Sickie cells show greater haemolysis during washing and their stability towards changes in pH is also less. The results for the sickie cells are based on average values for blood drawn from about ten persons.

Fig. 1 shows the pH-mobility relationships in aq. 0.145M-sodium chloride solution for oxygenated and deoxygenated sickie cells, with the relationship obtained for normal erythrocytes shown as the broken line. The mobility varies with pH in a manner indicating the presence of one or more monobasic acid groups of closely similar pK effective at the electrophoretic slip plane. The pK of the surface group(s) is about 2.5.

The charge-reversal spectra for promeniscocytes (oxygenated suspension), sickie cells (deoxygenated suspension) and normal erythrocytes (oxygenated suspension) are given in Fig. 2. The values for the charge-reversal concentrations of the various

cations have been obtained directly from plots of the logarithm of the cation concentration in the presence of a constant concentration of Na^+ ions (0.145 g.ion/l.) versus electrophoretic mobility and where necessary extrapolation of the plots to zero electrophoretic mobility. The charge reversal concentrations for the alkaline-earth cations are given in the upper ranks, the other bivalent cations in the middle ranks, and the ter- and quadri-valent ions in the lower ranks.

Table 1 gives the electrophoretic mobilities of normal and sickie cells examined at pH 7 in either standard saline or 14.5 mM-NaCl in aq. 4.5% sorbitol before and after treatment with neuraminidase or trypsin. The standard deviation is approx. $0.05 \mu\text{sec.}/v/\text{cm.}$ based on 50 individual readings for the normal and sickie cells in a medium of ionic strength 0.145 g.ion/l.

No differences were detectable by cell electrophoresis between normal erythrocytes and sickie cells with regard to the adsorption of haemoglobin and serum proteins at ionic strengths 0.0145 and 0.145 g.ion/l.

The electrophoretic mobilities of normal erythrocytes and promeniscocytes spherized by passage through a column of Ballotini were found to be the same as those for normal biconcave discoid erythrocytes.

DISCUSSION

Only those components of the ultrastructure of the cell that contribute to the electrokinetic charge at the slip plane are detectable by electrophoretic methods. Electrophoretic examination of the promeniscocyte and sickie cell has not revealed any significant differences from the normal erythrocyte with regard to mobility, or its modification by enzymes, adsorption of proteins, or interfacial ion-redistribution phenomena. Most of the investigations were carried out in aq. 0.145M-sodium chloride solution, so that the mobility is relatively insensitive to changes in the ionic strength. The charge-reversal concentrations for the various cations are higher in the presence of aq. 0.145M-sodium chloride solution (Seaman & Pethica, 1957) and consequently less sensitive to variations in the number of red cells/ml. of suspending fluid. The adsorption of the products of haemolysis was also minimized by using standard saline (Heard & Seaman, 1960) rather than a medium of lower ionic strength. The leakage of inorganic ions from the red cells after their addition to the metal salt solutions is a complicating factor particularly with electrolytic solutions of low pH, e.g. the release of phosphate ions from the red cells will render UO_2^{2+} ion-binding studies unreliable. Many of the cations are reversibly bound and their effect on the electrophoretic mobility can be eliminated by

Fig. 1. The pH-mobility relationships in standard saline for oxygenated sickle-cell-anaemia cells (□), oxygenated sickle-cell-trait cells (○) and deoxygenated sickle-cell-anaemia cells (■). The relationship for normal human oxygenated erythrocytes is shown as a broken line.

Fig. 2. Cation-charge-reversal spectra for (a) human normal erythrocytes, (b) promenisococytes (oxygenated), and (c) sickle cells (deoxygenated) in standard saline.

Table 1. *Electrophoretic mobilities of normal and sickle erythrocytes in media of ionic strength 0.145 and 0.0145 g.ion/l. after standard washing and also after treatment with neuraminidase or trypsin*

Experimental details are given in the text.

Ionic strength	...	...	Mobility (μ /sec./v/cm.)			
			Normal erythrocyte		Sickle erythrocyte	
			0.145	0.0145	0.145	0.0145
Treatment						
Control			1.08	2.78	1.07	2.75
Neuraminidase			0.25	1.23	0.25	—
Trypsin			0.78	2.40	0.80	—

washing the red cells in standard saline. However, copper salt and uranyl salt solutions show only partial reversibility in this respect over the range of concentrations used in the ion-binding studies. Sickle cells haemolyse more readily than normal red cells so that examination of such cells, particularly a few hours after a standard wash, reveals a greater sensitivity towards certain ions such as Cu^{2+} . Although such differences may be minimized by rewashing the cells, the greater loss of ions from sickle cells is reflected in somewhat lower

charge-reversal concentrations for certain cations (see Fig. 2), although the order of the ions in the charge-reversal spectrum remains the same as that for the normal erythrocyte.

The charge-reversal spectra reported for red cells by Seaman & Pethica (1957) have been corrected since they were obtained on cells that had probably been inadequately washed. Further, the electrophoresis chamber used during these determinations was subsequently shown to be defective for at least some of the measurements. However, the

only charge-reversal concentrations significantly different from those reported previously are those for the UO_2^{2+} and Th^{4+} ions on both normal and sickle cells and the Cu^{2+} ions on normal cells. There is no significant difference between the charge-reversal concentrations for Cu^{2+} ions for normal and sickle erythrocytes provided that measurements are made immediately after the cells have been washed. The sensitivity to Cu^{2+} ions previously reported for sickle cells 30 days after collection was probably a reflexion of the rapid breakdown of some of the cells leading to a high concentration of haemolysate in the suspending medium sensitive to Cu^{2+} ion-binding.

The cation spectra of the human normal and sickle erythrocytes can now be reinterpreted in the light of recent studies on the ultrastructure of the normal human erythrocyte. The spectra are similar to those reported for sheep erythrocytes by Bangham, Pethica & Seaman (1958), and for mouse erythrocytes by Bangham & Pethica (1959).

The normal human erythrocyte behaves as a macropolyanion at an ionic strength of 0.145 g.ion/l. (Seaman & Heard, 1960). This conclusion is based on the absence of any change in mobility after treatment of the cells with aqueous solution of formaldehyde, acetaldehyde or tosyl chloride, coupled with a lack of anion specificity (i.e. the mobility is the same in equivalent concentrations of CNS^- , I^- , Br^- and Cl^- ions) and an absence of a positive branch to the pH-mobility relationship for red cells at high ionic strength. The action of neuraminidase, together with supporting evidence based on the action of proteolytic enzymes, indicates that the dominant ionogenic species at the surface of the human erythrocyte is the carboxyl group of a bound *N*-acetylated neuraminic acid (Cook *et al.* 1961; Seaman & Uhlenbruck, 1963).

The charge-reversal sequence, Co^{2+} , Ni^{2+} , Mn^{2+} ions, reported for both normal and sickle cells by Seaman & Pethica (1957) and confirmed in the present work (Fig. 2), is that reported for carboxyl colloids by Kruyt (1949). The relative positions of UO_2^{2+} and Th^{4+} ions are also those expected for a carboxyl colloid. The results for Ca^{2+} , Ba^{2+} and Sr^{2+} ions were obtained by extrapolation and are not considered to be sufficiently accurate to warrant discussion.

The use of reversal of charge concentrations of ions as a criterion of the type or types of charge group is likely to be unreliable for a number of reasons: (a) the high concentration of electrolyte leads to non-iso-osmotic conditions with attendant disorganization and concomitant leakage of ions from the cell; (b) the reversal of charge concentration for a particular ion will depend on the charge density of the cell; (c) steric effects may play a role, particularly as the charge-reversal concen-

tration is approached; (d) the coexistence of several types of charge groups may result in an apparent abnormal sensitivity towards certain ions.

The approximate *pK* value at an ionic strength of 0.145 g.ion/l. of the ionogenic groups effective at the surface of sickle cells is 2.5, which is the same as that for the normal erythrocyte. The *pK* of *N*-acetylneuraminic acid is 2.6 (Svennerholm, 1956). The surface *pK* is unlikely to arise entirely from a single ionogenic species and there may be minor contributions from other groups or a 'charge effect' arising from the redistribution of ions in the interfacial region.

The action of neuraminidase and trypsin on sickle cells (Table 1) is electrophoretically indistinguishable from that on normal cells. No significant differences therefore have been found between the normal and sickle erythrocyte from the point of view of exterior-surface ionogenic constitution.

SUMMARY

1. It is shown that promesococytes, sickle cells and normal human erythrocytes have similar pH-mobility relationships, the *pK* of the surface groups being about 2.5 in all cases.

2. The cation-charge-reversal spectra and the electrophoretic behaviour of the different types of red cell after treatment with neuraminidase or trypsin do not differ significantly from one another.

3. No differences were detectable by cell electrophoresis between normal erythrocytes and sickle cells with regard to the adsorption of haemoglobin and serum proteins. The electrophoretic mobilities of normal erythrocytes and promesococytes spherized by passage over glass Ballotini were found to be the same as those for the normal biconcave discoid erythrocytes.

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REFERENCES

- Abramson, H. A. (1929). *J. gen. Physiol.* **12**, 711.
- Ahrland, S., Hietanen, S. & Sillén, L. G. (1954). *Acta chem. scand.* **8**, 1907.
- Allison, A. C. (1957). *Biochem. J.* **65**, 212.
- Bangham, A. D., Flemans, R., Heard, D. H. & Seaman, G. V. F. (1958). *Nature, Lond.*, **182**, 642.
- Bangham, A. D. & Pethica, B. A. (1959). *Proc. R. phys. Soc. Edinb.* **28**, 43.

- Bangham, A. D., Pethica, B. A. & Seaman, G. V. F. (1958). *Biochem. J.* **69**, 12.
- Cook, G. M. W., Heard, D. H. & Seaman, G. V. F. (1961). *Nature, Lond.*, **191**, 44.
- Dervichian, D. G., Fournet, G., Guinier, A. & Ponder, E. (1952). *Rev. Hémat.* **7**, 567.
- Fenichel, R. L., Watson, J. & Eirich, F. (1950). *J. clin. Invest.* **29**, 1620.
- Harris, J. W. (1959). *Progr. Haemat.* **2**, 47.
- Heard, D. H. & Seaman, G. V. F. (1960). *J. gen. Physiol.* **43**, 635.
- Hietanen, S. (1954). *Acta chem. scand.* **8**, 1626.
- Ingram, V. M. (1957). *Nature, Lond.*, **180**, 326.
- Kass, E. H., Ingbar, S. H., Harris, J. W. & Ley, A. B. (1951). *J. clin. Invest.* **30**, 652.
- Kruly, H. R. (1949). *Colloid Science*, vol. 2, p. 259. Amsterdam: Elsevier.
- Neel, J. V. (1949). *Science*, **110**, 64.
- Ponder, E. (1951). *C.R. Soc. Biol., Paris*, **145**, 1665.
- Ponder, E. (1955). *J. gen. Physiol.* **38**, 575.
- Pranker, T. A. J. (1955). *Clin. Sci.* **14**, 381.
- Schneider, R. G., Levin, W. C. & Haggard, M. E. (1949). *J. Lab. clin. Med.* **34**, 1249.
- Seaman, G. V. F. & Heard, D. H. (1960). *J. gen. Physiol.* **44**, 251.
- Seaman, G. V. F. & Heard, D. H. (1961). *Blood*, **18**, 599.
- Seaman, G. V. F. & Pethica, B. A. (1957). *Proc. 2nd int. Congr. Surface Activity: Cell/Water Interface*, **4**, 277.
- Seaman, G. V. F. & Uhlenbruck, G. (1963). *Arch. Biochem. Biophys.* **100**, 493.
- Svennerholm, L. (1956). *Acta Soc. med. upsalien.* **61**, 75.
- Tosteson, D. C. (1955). *J. gen. Physiol.* **39**, 55.
- Tosteson, D. C., Carlsen, E. & Dunham, E. T. (1955). *J. gen. Physiol.* **39**, 31.

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Nucleoproteins

2. FRACTIONATION OF DEOXYRIBONUCLEOPROTEINS INTO ACIDIC PROTEINS AND LYSINE-RICH HISTONES ASSOCIATED WITH DEOXYRIBONUCLEIC ACID*

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A study of the action of salts and phenol on mammalian tissues has shown that the ability to separate the DNA from the protein depends on the nature of the salt used (Kirby, 1957). Hydrophilic salts (0.3M) released very little DNA, whereas lipophilic salts released DNA to which some protein remained attached. Salts that had both lipophilic and chelating properties liberated DNA with the minimal amount of residual protein.

Sodium benzoate in combination with phenol released a DNA-protein complex from rat liver, the proportion of protein being dependent on the molarity of the benzoate used (Kirby, 1958). This reaction has been extended to other tissues and compared with the reaction of sodium phenolphthalein diphosphate.

Amino acids were previously estimated (Kirby, 1958) as their Cu^{2+} ion complexes after separation by paper chromatography and paper electrophoresis. The colours tended to fade and the results were not always reproducible. Solvent systems have been found that will separate all the amino acids by two-dimensional paper chromatography, and the ninhydrin products formed complexes with Cd^{2+} ions that were stable for 24 hr. This method of estimating amino acids in nucleoprotein complexes is much more reliable than the previous one.

* Part 1: Kirby (1958).

MATERIALS AND METHODS

Purification of alkoxyethanols and the origin of the transplantable tumours were as described by Kirby (1959).

Estimation of amino acids. Deoxyribonucleoprotein (9–10 mg.) was hydrolysed with 5.8N-HCl (0.5 ml.) in a sealed tube at 110° for 24 hr. The tube was opened, centrifuged to remove insoluble material, the supernatant quantitatively transferred to a small flask (B10 joint) and the HCl removed by rotary evaporation at 40° for 15 min. Acetic acid (20%, v/v) was added several times and evaporated to remove residual HCl. The residue was dissolved in 20% acetic acid (0.1 ml.) and 0.01 or 0.02 ml. was used for each chromatogram (on Whatman no. 1 paper). The solvent for first-dimensional chromatography was butan-2-one-acetic acid-water (14:6:5, by vol.). After upward development for 16 hr. the papers were dried and developed in the second dimension with butan-2-one-2-methylpropan-2-ol-water-aq. NH_3 (sp.gr. 0.88) (60:16:24:3, by vol.). This solvent was made up at least 24 hr. before use, and 2 hr. before chromatography a small beaker containing aq. NH_3 (sp.gr. 0.88) was placed in the tank and then removed when the papers were immersed.

The papers were dried in a fume cupboard for 48 hr. at 20° (drying for a shorter time led to high blank values because of residual NH_3) and dipped in a solution of ninhydrin (0.2%) in acetone. The colours developed during 48 hr. at 20°. The purple spots were cut out and the colours eluted with a mixture of 5 ml. of a solution of CdCl_2 (analytical reagent) (1 g.) in water (5 ml.), redistilled 2-methoxyethanol (95 ml.) and acetic acid (1 ml.). After 1 hr.