A Ciba Foundation Symposium

PAPER ELECTROPHORESIS

Editors for the Ciba Foundation

G. E. W. WOLSTENHOLME, O.B.E., M.A., M.B., B.Ch.

and

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PREFACE

THE Ciba Foundation, London, is an educational and scientific charity founded by a Trust Deed made in 1947. Its distinguished Trustees, who are wholly responsible for its administration, are The Rt. Hon. Lord Adrian, O.M., F.R.S.; The Rt. Hon. Lord Beveridge, K.C.B., F.B.A.; The Hon. Sir George Lloyd-Jacob; and Mr. Raymond Needham, Q.C. The financial support is provided by the world-wide chemical and pharmaceutical firm which has its headquarters in Basle, Switzerland.

The Ciba Foundation forms an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information. It was opened by Sir Henry Dale, O.M., F.R.S., in June 1949.

In the first seven years, in addition to many part-day discussions, there have been 40 international symposia, each lasting two to four days, attended by outstanding workers from many countries. Other symposia are planned at the rate of five or six a year.

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument. They have also been the occasion for reference to much published and unpublished work throughout the world. The proceedings are issued in full, with only the minimum of editing, in order to pass on to a far wider audience the benefits of these meetings. It is hoped that readers will not only gain information and inspiration from this report, but will also feel that they share in these frank and friendly discussions.

It was Professor E. L. Durrum who first suggested, through Professor E. J. King, that it would be helpful to have an informal meeting of people particularly interested in the

technique and use of paper electrophoresis. He hoped that discussions would lead to the employment of methods allowing a much better basis of comparison between findings in different laboratories.

Although the programme of symposia for 1955 at the Ciba Foundation had already been arranged, the Director decided to fit in an extra meeting on this subject, on an even more informal basis than usual, not only in the hope of advancing and clarifying the technique, but also, by holding the symposium at the end of July, to assist some of the members from overseas to attend on the same journey the International Congress of Biochemistry in Brussels immediately afterwards.

In the arrangement of the programme, the Director received invaluable help from Prof. C. E. Dent, Prof. E. J. King, Dr. D. J. R. Laurence, Prof. N. H. Martin and Prof. F. L. Warren, though responsibility for the invitations to members remained, as usual, wholly with the Director.

It was a great encouragement beforehand and at the symposium to have Dr. A. J. P. Martin as Chairman.

The publication of the proceedings has been delayed mainly because they had to await their turn after those of six other symposia held at the Foundation earlier in the same year, but it is hoped that they have not lost in value and interest in the interval. The assistance of Mr. Rivers and Mr. John Rivers of J. & A. Churchill, Limited, has again been of the utmost help, and it is a pleasure to record here the happiness of the Editors in this continuous working relationship.

List of those participating in or attending the Symposium on "Paper Electrophoresis" 27th–29th July, 1955

G. H. Beaven	National Institute for Medical Research, Mill Hill, London
E. M. Crook	Dept. of Biochemistry, University College, London
C. E. Dent	University College Hospital Medical School, London
E. L. Durrum	Dept. of Pharmacology, Stanford University School of Medicine, San Francisco, California
G. T. Franglen	Dept. of Chemical Pathology, St. George's Hospital Medical School, London
W. Grassmann	Lab. for Protein and Leather Chemistry, Max-Planck-Institut, Regensburg, Germany
W. P. Jencks	Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Mass.
B. Kickhöfen	Dr. A. Wander Forschungs Institut, Säckingen, Germany
A. A. Kinnear	Institute of Pathology, University of Pretoria, South Africa
H. Laurell	Institute of Biochemistry, University of Uppsala, Sweden
D. J. R. Laurence	Postgraduate Medical School, London
M. Lederer	Faculté des Sciences, Institut de Radium, Arcueil (Seine), France
H. J. McDonald	Dept. of Biochemistry, Stritch School of Medicine, Loyola University, Chicago, Illinois
A. J. P. Martin	National Institute for Medical Research, Mill Hill; now at Boreham Wood, Hertfordshire
N. H. Martin	Dept. of Chemical Pathology, St. George's Hospital Medical School, London
C. J. O. R. Morris	Dept. of Experimental Biochemistry, London Hospital Medical College
V. R. Srinivasan	Pharmacology Institute, University of Mainz, Germany; now at Dept. of Pharmacology, Central Drug Research Institute, Lucknow, India

S. CHR. SOMMERFELT	Central Laboratory, Drammen Hospital, Norway
H. SVENSSON	LKB-Produkter Fabriksaktiebolag, Stockholm
J. DE WAEL	Medical Chemistry Laboratory, Rijks University, Utrecht; now at Biochemical Laboratory, Medical Department, University Hospital, Catharijnesingel, Utrecht
F. L. WARREN	Dept. of Biochemistry, London Hospital Medical College
J. C. WHITE	Dept. of Haematology, Postgraduate Medical School, London

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CHAIRMAN'S OPENING REMARKS

A. J. P. Martin

I should like to begin by saying how grateful we all are to the Ciba Foundation and to Dr. Wolstenholme and his staff for their most efficient organization.

I do not propose to take even my allotted time over introducing the speakers here. I gather that I have been chosen as Chairman principally because I have published nothing on this subject and may, therefore, be held less likely to have a bias than perhaps others. I should like to say however that I started playing with this method in 1942, but I got rather busy on other subjects and never developed it. I can see now just how much I missed by dropping things too soon.

GENERAL METHODS OF PAPER ELECTROPHORESIS WITH EXAMPLES OF ITS USE IN MEDICAL AND BIOCHEMICAL PROBLEMS

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TISELIUS (1), in his classic works on electrophoresis, has shown that differences in electrophoretic mobility make an exact analysis of mixtures of substances possible. According to Tiselius, protein mixtures lend themselves particularly well to electrophoretic analysis and, among them, blood serum and blood plasma, when examined by this novel method, have led to important new findings for clinical application.

However, Tiselius's classical methods require extraordinarily complicated apparatus and outstanding experimental skill and are, therefore, hardly practicable in the daily routine of clinics and scientific or technical laboratories. Moreover, these methods are better suited, generally speaking, for substances with high molecular weight than for those of low molecular weight, and for analysis rather than for a preparative separation of complicated mixtures of substances. For this reason paper electrophoresis and some similar procedures also employing porous supporting media as anti-convection agents are a useful compliment to classical electrophoresis. The simple and convenient procedure, the reduced amount of substance required, and the possibility of a simplified combination with specific colour reactions, with biological and serological tests and with the application of radioactive isotopes are only a few of the favourable features of this method which, though relatively new, is already widely used.

Electrophoretic separation of dyeing agents and snake toxins on a filter strip soaked in an electrolyte solution was first described by König (1937) and Klobusitzky and König (1939) (2), but their work did not attract much attention. Then, following up the work of Tiselius, this simple method was further developed, 1949–1950, almost simultaneously and independently of each other, by Th. Wieland and Fischer (3), and by Turba and Enenkel (4) in Germany, and by Durrum (5) in the U.S.A., for microseparation of proteins and amino acids and, immediately afterwards, by Grassmann and co-workers (6) and others (7) into a quantitative method, initially for serum analyses. Meanwhile about 4,000 reports have been published, all dealing with the methods of paper electrophoresis and its manifold applications in a large number of different fields. I cannot even attempt in this short introduction to give you a comprehensive report on the subject, and I should like to restrict myself to reporting on the general development, touching upon special features or difficulties in the various methods, and giving you a few typical examples of application.

In the arrangement (8) developed in our laboratory (*see* Svensson, this Symposium, p. 96, Fig. 3) the filter paper strip utilized for separation is located in a moist glass chamber with its ends dipping into the electrolyte, a method first used in the very simple arrangement of Th. Wieland (9). In this form the apparatus is in use even today for routine analyses. The electrodes are surrounded by a baffle system, and the electrode vessels are divided into two parts lest either dissolved or gaseous electrolytic products should reach the strip. In the Durrum arrangement (5) (*see* Svensson, this Symposium, p. 95, Fig. 2) the strip has been placed over a glass rod in the shape of an inverted V. The particular traits of this apparatus will be dealt with later on in this Symposium. In the apparatus designed by Cremer and Tiselius (7), finally, the filter paper strip is covered by a glass plate, placed in a trough filled with toluene (*see also* Svensson, this Symposium, p. 101, Fig. 9). The principle of this arrangement is found again later in the

various arrangements for high voltage electrophoresis; it is an easy way of removing the heat produced and reduces to a great extent evaporation of the electrolyte liquid from the strip, but there are some other disadvantages.

Also, in apparatus containing a moisture-saturated vessel, evaporation can be reduced to a minimum provided the field strength is kept at a moderate level (4–5 v. per cm.), the buffer solutions are not too concentrated and a relatively low temperature is maintained. Under such conditions the conductivity of the strip hardly ever rises in the course of the experiment, and the migration velocity remains practically constant during the greater part of the electrolysis period (8). The separation of serum under these conditions requires approximately twelve hours. When appropriate buffer systems are employed and a field strength of 10–12 v. is maintained, satisfactory results for serum separation may be obtained within 3–4 hours with no special cooling devices (10).

Some interesting details can be seen in an apparatus designed by McDonald (11). The strip is led over a number of prismatic supports, made of some porous material, which draw up liquid from the glass vessels and supply the strip with it at the same rate as evaporation takes place. Any evaporated liquid is thus constantly replaced at several spots all along the strip, and the undesirable wick flow from the strip ends can be greatly reduced. The other suggestion of McDonald's is to fill the vessel with hydrogen instead of air, because the thermal conductivity is approximately 13–14 times greater (12).

The exact determination of electrophoretic mobility in paper electrophoresis is known to be a somewhat difficult problem. On the electrophoretic movement is superimposed the flow of liquid due to evaporation and electro-osmosis. But there is another important factor: in contrast to electrophoresis in a homogeneous medium the particles do not migrate on a linear, but on a somewhat winding, path. In reality they have, therefore, to travel a longer path in order to proceed a given distance over the strip. The course of the

field lines is not linear either, but curved, and the local field strength is, therefore, not really comparable with that in homogeneous medium. Kunkel and Tiselius (13) have examined the theoretical angle of the problem and they indicate a way to determine the electrophoretic mobility almost exactly.

There is one more point that should be mentioned here. The paths of the migrating particles are not equal in length for each of the particles but are statistically scattered. This causes an additional dispersion, which results in a certain widening or blurring of the zones being superimposed on the pure thermodiffusion. In contrast to thermodiffusion this dispersion is not dependent on the length of the experiment but on the length of the migration path and on the properties, both morphological and adsorptive, of the filter paper.

Unstained substances, such as proteins or amino acids, must be stained after the separation so as to enable a quantitative determination to be made. In the case of amino acids this is done by means of ninhydrin. Proteins are stained with anionic dyes, such as bromophenol blue or, as we suggest, amidoschwarz. For quantitative analysis the strip (after Cremer and Tiselius, 7) may be cut in pieces of 0·5 cm. width, and the dyestuff content of each is then determined photometrically after elution. A much simpler procedure, almost universally applied today, is one worked out by us; the strip is made transparent by embedding it in a medium whose refractive index is equal to that of the cellulose fibre, and is moved, millimeter by millimeter, past an illuminated slit, thereby measuring extinction by means of a photoelectric cell. The results obtained are almost identical with those obtained by Tiselius's method. Automatic scanners have been developed in Germany and, as far as I know, in the U.S.A.

The question of whether this simple method is quantitatively exact has been widely discussed and examined from all angles. The planimetric integration is, strictly speaking, only permissible if there is a linear relation between the dyestuff density and extinction, i.e. if Beer's law is applicable

mutatis mutandis. It can be proved by experiment that this condition also holds true for dyes which are distributed in a transparent foil (14). Nevertheless, certain other conditions must be fulfilled if correct numerial values are to be achieved. The dye must—this can easily be shown theoretically—be absolutely homogeneously distributed over the entire width of the strip, the substance must be applied in an entirely uniform line across the strip at the beginning of the experiment, and it must have migrated during the electrophoresis in an entirely linear front. Figs. 1a and 1b show the linear relation between the area integral of the Gauss curves obtained and the amount of albumin (14).

A quantitative comparison of the numerical values established for the various protein fractions is further dependent on the condition that an equal amount of dye, for example amidoschwarz, is taken up by the various protein fractions. This condition is only approximately fulfilled for serum proteins; it is, however, known to be better for amidoschwarz than for any other anionic dyes (14). For all dyes the binding capacity of albumin is slightly higher than that of the various globulins. The fact that nearly correct values are, nevertheless, generally obtained is due to a fortunate compensation of experimental errors. The not absolutely linear calibration curves are an important factor in this respect (8, 14).

As far as I can see there is an absolute need for making the strip transparent before measuring in order to obtain correct results, for on the non-transparent strip the optical conditions are not clear, Beer's law has no validity, the calibration curves are bent, and usually too low values are obtained for albumins and too high values for globulins (14, 15). Other details, for instance the drawing of the baseline, will be discussed in a later paper.

Conformity with the numerical values (Table I) obtained by means of the classical method is satisfactory (8, 16, cf. 17). The mean deviations caused by the method itself are surprisingly low (Table II) (8, 17).

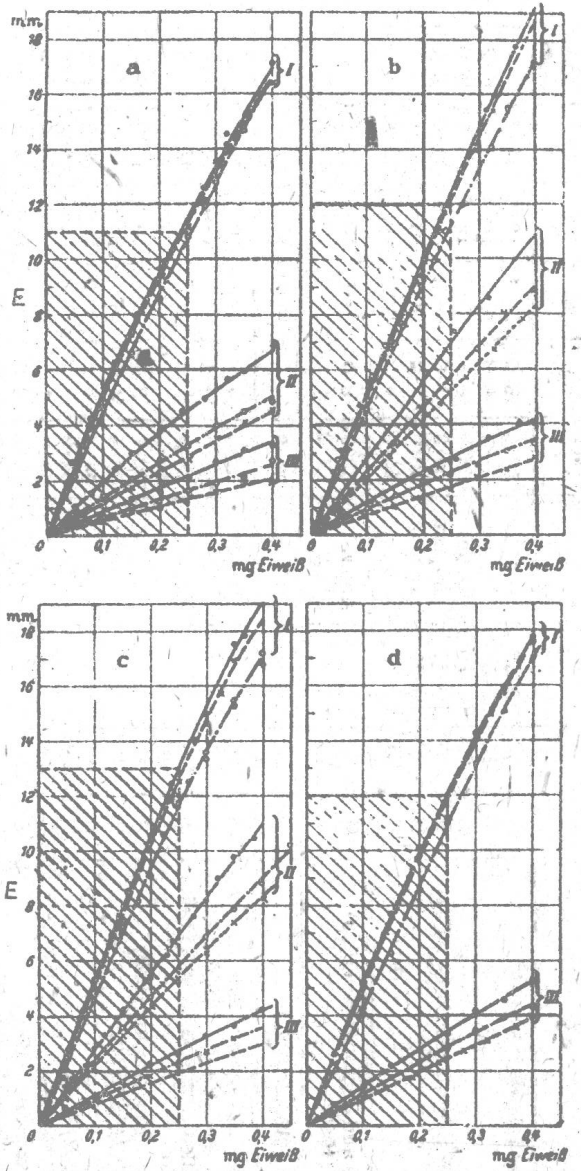

FIG. 1b. Calibration curves for albumin, α- and γ-globulin. (a) Determined by "Elphor H"; (b) determined by "Zusatzgerät zum Eppendorf-Kolorimeter"; (c) determined by "automatic densitometer of Eberle-Edelmann"; (d) determined by "Extinktionsschreiber" of Carl Zeiss. Stained with (i) amidoschwartz 10 B, (ii) azocarmin, (iii) bromophenol blue. .-.-. albumin; x-x-x γ-globulin; o-.-o α-globulin. Ordinate: area integral E.mm (Grassmann and Hannig, 14).

Table I

ANALYSIS OF NORMAL SERA BY CLASSICAL METHODS AND BY PAPER ELECTROPHORESIS (AVERAGE VALUES BY DIFFERENT AUTHORS. AFTER WUNDERLY) (17)

Investigators	*Plasma P / Serum S*	*No.*	*Age in years*	*Electrophoretic Method*	*Relative per cent*					
					Albumin	*Globulin* α_1	α_2	β	γ	Φ *Fibrinogen*
Seibert and Mitarbeiter (1947)	S	43	18–55		53·3	8·0	10·4	13·8	14·2	—
Rafsky, Brill, Stern and Corey (1952)	P	13	3–8	Tiselius Philpott-Svensson	57·7	6·3	10·6	10·3	9·7	5·2
	P	30	9–16		58·8	4·9	9·5	12·6	9·5	5·3
	P	15	19–25		60·3	4·6	7·2	12·1	11·0	5·1
	S	80	18–55		56·0	7·2	8·8	13·1	14·7	—
	S	31	65–95		48·6	8·5	10·2	16·7	15·9	—
Hartmann and Schumacher (1950)	S	130		Antweiler	63·3	6·0	5·8	13·1	11·5	—
Everbeck (1950)	S	20		,,	63·2	9·0		13·7	14·1	—
Schaub and Alder (1951)	S	22		Kern	54·3	8·8	10·6	11·7	14·6	—
Plückthun and Götting (1951)	S	15		Paper	59·0	4·2	8·0	10·6	18·2	—
Grassmann and Hannig (1952) (8)	S	25		,,	61·3	4·1	8·1	11·0	15·5	—
Köiw, Wallenius and Grönwall (1952)	S	10		,,	72·9	1·4	3·5	8·6	13·6	—
Gras (1952)	S	26		,,	66·6	10·1		8·7	14·6	—
Caspani and Bernasconi (1952)	S	11		,,	62·7	3·9	7·5	10·7	14·9	—
Sommerfelt (1952) (16)	S	13		,,	55·4	4·4	8·1	10·0	22·2	—
Goa (1952)	S	17		,,	59·2	3·8	6·0	11·1	19·6	—
Brante (1952)	S	39		,,	64·9	7·6		11·1	16·4	—
Esser, Heinzler and Wild (1953)	S	44		,,	59·0	4·3	7·7	12·5	16·5	—
Antonini and Piva (1953)	S	15		,,	55·7	4·0	7·9	12·6	19·5	—
Knapp and Sieler (1953) (16)	S	8		,,	60·5	4·0	9·5	11·0	15·0	—
Sonnet and Rodhain (1953) (16)	S	13		,,	61·7	4·3	7·5	10·2	16·3	—
Bogdanowicz, Osinski and Stein (1953) (16)	S	25		,,	61·9	4·2	8·0	11·3	14·6	—
		Mean value		P.E.	61·6	3·9	7·3	10·7	16·5	
		Variations			55·4–72·9	1·4–4·4	3·5–9·5	8·6–12·6	13·6–22·2	