Journal of Chromatography, 320 (1985) 1–14
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,285

UNIFIED VIEW OF MOVING BOUNDARY ELECTROPHORESIS: PRACTICAL IMPLICATIONS (PLENARY LECTURE)

ANDREAS CHRAMBACH
National Institutes of Health, Bethesda, MD 20205 (U.S.A.)

SUMMARY

The physical conditions under which steady-state moving boundaries across strongly or weakly acidic and basic constituents arise, and the properties of these boundaries, can be predicted for any desired pH and range of net mobilities between leading and trailing constituents by theoretical treatments of Svensson (1943), Longsworth (1945), Schumacher (1964), Martin (1970) and Bier (1983) and their scientific offspring. These theoretical treatments are phrased in at least three different mathematical languages and have been differentially labeled as moving boundary electrophoresis, isotachophoresis, displacement electrophoresis, omegaphoresis, disc electrophoresis and multiphasic zone electrophoresis, among others, which obscures their essential equivalence. In practice, this identity, in spite of the terminological differences, should allow for an interconvertible application of these moving boundary theories and the computer programs of Routs (1971), Jovin (1973), Schafer-Nielsen (1981) and Mosher (1983) to (1) the prediction of steady-state phase compositions, pHs and ionic strengths, net mobility ranges of compounds capable of either migrating within, or in the trailing phase behind, a system of sequential moving boundaries and the dimensions of steady state phases in such a system; (2) separations in which optimal resolution depends on the exploitation of molecular size differences on a restrictive gel, as well as those in which optimal resolution is obtained in free solution; (3) separations of compounds migrating within moving boundaries from those migrating in their trailing phases; (4) preparative techniques and apparati with load capacities large enough to contemplate the isolation of proteins in the 0.1–1.0 kg range; (5) separations in moving as well as arrested sequential boundary systems.

INTRODUCTION

Steady-state moving boundaries have substantially enhanced the resolving power of electrophoretic separation procedures in biochemistry through at least four mechanisms: (a) their concentrating capacity, freeing electrophoresis of any limitations in the degree of sample dilution, with provision of uniformly thin starting zones for separations[1–3]; (b) the possibility to partition two or more species to be separated between the moving boundary and the trailing phase[2]; (c) their extraordinarily high load capacity for macromolecules[4]; (d) the possibility to predict pH gradients across

0021-9673/85/$03.30

sequential moving boundaries, and the possibility to arrest them[5,6]. Most of these areas of usefulness of moving boundaries have, however, remained untapped by biochemists at large. This is largely the fault of methodologists of electrophoresis, *i.e.* it is our fault, for three reasons. (i) We have created confusion with regard to the method's choice by giving the impression that moving boundary electrophoresis, displacement electrophoresis, isotachophoresis, omegaphoresis, disc electrophoresis, etc., are different separation methods when in fact they are the same[7]. Even electrofocusing appears to be nothing but a special case of moving boundary electrophoresis[5,6], as will be discussed below. (ii) The theoretical unity of the theory of moving boundaries[7] has been disguised through autistic use of multiple mathematical languages, *viz.* those of Svensson[8]. Longsworth[9], Alberty[10] and Dole[11], of Schumacher[12], of Martin and Everaerts[13,14], of Schafer-Nielsen and Svendsen[15] and of Bier *et al.*[16]. (iii) The computer programs of Routs[17]. Jovin[18], Schafer-Nielsen[15] and Mosher[16] are not sufficiently "user friendly" to fullfill the most important function of computer programs, *i.e.* to make the results of complex theory operationally available to the user in a fully explicit form, without any need for understanding or manipulating the underlying theory.

The purpose of this report is to serve as a stimulus to methodologists, theoreticians and computer programmers to make moving boundary electrophoresis more widely understood and useful for biochemistry by eliminating illusory barriers of communication created by schismatic terminologies and mathematical languages and by creating more explicit and easy-to-use computer programs by which the operative conditions of moving boundary systems can be regulated and which allow for simulation to replace arbitrary and tedious experimental selection of separation conditions.

PHYSICAL CONDITIONS WITHIN STEADY-STATE MOVING BOUNDARIES AND TRAILING PHASES

The previous section listed four areas of application (a–d) of moving boundary electrophoresis in biochemistry, both analytical and preparative. All of these applications require a concise knowledge of the pH, ionic strength and electrolyte composition to which the species of interest is exposed during separation, as well as of the net mobilities of trailing and leading electrolyte constituents which set the limits within which the species of interest is included into the moving boundary or system of sequential moving boundaries, or is excluded from these and confined to the trailing phase (the "stacking limits"). The reason why a user of moving boundary systems needs to know operative pHs and ionic strengths, electrolyte milieu and "stacking limits" concisely is in part related to the scientific method, and in part to operational requirements. The scientific method applied to separations would require *a priori* that one knows the physical conditions under which the separation takes place. But even if one foregoes this requirement of "good science", it is impossible to manipulate rationally, or to optimize for one's purposes, a moving boundary system unless one knows its properties exactly. Furthermore, it is highly advantageous from an operational viewpoint to know these properties numerically.

When separation occurs in the trailing phase, and the moving boundary is used merely as a marker for the calculation of relative mobilities of the zones, or as a device to syphon off contaminants from the resolving phase, an exact knowledge of

the separation milieu at the steady state can be provided readily by the theory of moving boundaries and the programs which utilize it. When separation occurs between an unknown migrating within a system of sequential moving boundaries at the steady state, and electrolyte constituents migrating either ahead of or behind that unknown boundary within boundaries of their own, the exact physical conditions are only known for the electrolyte constituents with known net mobilities and pK values. The conditions under which the unknown finds itself during separation must be deduced from the properties of the leading and trailing phases and are assumed to have intermediate values between those. However, not all relevant parameters vary monotonically within a system of steady-state sequential moving boundaries: *e.g.* pH gradients across boundaries of electrolytes with near-identical pKs differing in net mobilities such as homologous proteins exhibit inverse pH gradients at the steady state (Fig. 7 of ref. 6).

Although steady-state parameters even for systems of any number of trailing constituents are easily computed by programs such as the one by Routs, most users of moving boundary electrophoresis remain ignorant of at least some of the physical properties of those systems. This is not due to a failure of the programs of Routs, Jovin, Schafer-Nielsen and Mosher to compute at least some of the key operative parameters of moving boundary systems, but to the inadequate availability and "user-friendliness" of those programs.

Of the four programs, only the Jovin program is commercially available, *viz.* at nominal cost from the US National Technical Information Service (Fig. 1).[19] More important, a representative computer output describing moving boundary systems across the entire pH range is available in either microfiche or magnetic tape form (Fig. 1). Nonetheless, even that nominal availability is frequently frustrated in practice by the fact that the distributing governmental agency suffers from bureaucratic inefficiency, resulting in delayed, incomplete and sometimes even lost orders. Even

Moving Boundary Electrophoresis Program of T. M. Jovin and Buffer Systems Output Available from the National Technical Information Service (NTIS)*

	Polarity	°C	Format	PB No.	$	Format	PB No.	$
(1.) Buffer systems 1-691	+	0	Tape	196085	97.50	Microfiche	259309	8.75
(2.) Buffer systems 692-1578	+	25	Tape	196086	97.50	Microfiche	259310	10.00
(3.) Buffer systems 1579-2969	-	0	Tape	196087	157.50	Microfiche	259311	13.00
(4.) Buffer systems 2970-4269	-	25	Tape	196088	157.50	Microfiche	259312	13.00
(5.) Instructions for (1)-(4)			Book	196089	4.00			
(6.) Systems catalog			Book	196090	7.75			
(7.) Instructions for (6)			Book	196091	3.50			
(8.) Program for which (1)-(4) were computed			Tape	196092	150.00			
(9.) Program for retrieval of any of the systems (1)-(4)			Tape	203016	150.00			

*NTIS, US Department of Commerce, Springfield VA 22161, USA

Fig. 1. Catalogue for purchase of the moving boundary electrophoresis program of T. M. Jovin[18] and a model output generated by it[19] which provides 4269 appropriate buffer systems.

more seriously, the Jovin program available at the cost of magnetic minitape is written in Fortran, vintage 1970, and must be adapted to the format and language requirements of each particular computer. Even if the "model output" of nearly 5000 moving boundary systems in microfiche form is used in laboratory practice, the program is required if one wishes to obtain the detailed physico-chemical description illustrated in Fig. 2 for "subsystems" with altered trailing ion net mobilities within

SYSTEM NUMBER

DATE = 02/02/79 COMPUTER SYSTEM NUMBER = chr 18.2 x 4.0.
POLARITY = + (MIGRATION TOWARD CATHODE) TEMPERATURE = 0 DEG. C.

CONSTITUENT 1 = NO. 1 , ALANINE
CONSTITUENT 2 = NO. 4 , PYRIDINE
CONSTITUENT 3 = NO. 97 , POTASSIUM +
CONSTITUENT 6 = NO. 16 , FORMIC ACID

PHASES

	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0400	0.1601		0.0659		
C2			0.1996		0.0821	
C3						0.1082
C6	0.0077	0.0209	0.0604	0.0975	0.1137	0.1398
THETA	0.192	0.130	0.303	1.480	1.385	1.292
PHI(1)	0.076	0.060		0.228		
PHI(2)			0.300		0.962	
PHI(3)						1.000
PHI(6)	0.395	0.458	0.993	0.154	0.695	0.774
RM(1)	0.046	0.036		0.137		
RM(2)			0.258		0.827	
RM(3)						1.490
RM(6)	-0.450	-0.522	-1.132	-0.176	-0.792	-0.882
PH	3.55	3.67	5.87	3.00	4.10	4.27
ION.STR.	0.0030	0.0096	0.0600	0.0150	0.0790	0.1082
SIGMA	0.511	1.605	11.571	2.520	15.247	27.461
KAPPA	132.	399.	2660.	616.	3449.	6061.
NU	0.089	0.022	0.022	0.054	0.054	0.054
BV	0.011	0.033	0.098	0.056	0.062	0.056

RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
ALANINE	GM	14.26			2.35
PYRIDINE	GM		6.32		
1N KOH	ML			43.28	
FORMIC ACID	GM	0.96	1.11	2.57	1.79
H20 TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		3.61	5.58	4.24	2.95
KAPPA(25 DEG.C.)		710.	4720.	10827.	1089.

Fig. 2. Representative page from the moving boundary electrophoresis buffer systems output[19] generated by the program of T. M. Jovin[18]. Top panel: physico-chemical description of the buffer system. (i) Moving boundary with low trailing ion net mobility designed for stacking: leading phase = BETA, trailing phase = ZETA. (ii) Moving boundary with high trailing ion net mobility designed for unstacking: leading phase = GAMMA, trailing phase = PI. (iii) Constituent designations: 1 = trailing ion; 2 = leading ion in case (i); 3 = leading ion in case (ii); 6 = common ion. (iv) Other terms: KAPPA = specific conductance (μmho/cm); BV = buffer value. Bottom panel: recipes for preparing the trailing phase (upper buffer); leading phase BETA (upper or stacking gel buffer); leading phase GAMMA (lower or resolving gel buffer). The lower buffer is defined elsewhere in the output (not shown) as 0.05 *M* common ion, 0.0625 *M* counterion.

each of the numbered buffer systems. Thus, if the program is not operative in the laboratory, one is confined to the values of pH and trailing ion net mobilities selected under the arbitrary input conditions used to generate the model output.

Failure to use moving boundary electrophoresis quantitatively and numerically hinges mostly on the widespread confusion between setting and operative phases and their properties. A practical computer output must distinguish and specify clearly what the pH, conductance and leading ion net mobility is prior to initiation of the current, and what values pH, conductance and trailing ion net mobility assume after passage of the moving boundary. Among the four programs, only the output format of the Jovin program (Fig. 2) is sufficiently explicit in this regard. It presents under each buffer system number the physical properties existing in the two phases across single moving boundaries for sets of two values of the trailing and leading ion net mobility —one low one for the purpose of stacking*, one high one for the purpose of unstacking. It describes their physical properties numerically, clearly distinguishing the properties of the setting phases (prior to initiation of the current) from those arising after passage of the moving boundary. Nonetheless, even this output is handicapped by its limitation to single moving boundaries, *i.e.* failure to consider multiple sequential moving boundaries in the manner of the Routs program (Fig. 3). The output format is also burdened by the terminological complexity inherited from the Longsworth–Alberty–Dole theory of moving boundaries with greek letters and alternative numbers designating the phases, and numerical distinction between trailing, leading and common constituents in the various phases. The output is also made unnecessarily complex by its fixation on the particular experimental setup of disc electrophoresis, *i.e.* its organization into sets of "stacking gels" and "unstacking gels". We have therefore introduced recently a simplified format for moving boundary systems which avoids this complexity and should provide the user in numerical fashion with the most important physical parameters needed to understand and use at least a small number of selected buffer systems in the four ways (a–d) alluded to in the Introduction. This format should allow the user to regulate at will the trailing ion net mobility so as to stack or unstack the species of interest and to regulate its migration rate relative to the displacement rate of the moving boundary at least within a narrow selection of tabulated buffer systems[20].

In contrast to the other theoretical treatments and programs, the most recent one of Bier *et al.*[16] appears capable of dealing with the transient states of moving boundaries as well as the steady state. Avoiding a number of simplifying assumptions, such as the neglect of diffusion or protons and hydroxyl ions, it is the most rigorous treatment to date but also the most complex one, requiring great amounts of computer time that have limited the simulations based on this program reported to date to a few simple model systems.

---

* The term "stacking"[1] denotes migration of a charged compound within the moving boundary between leading and trailing buffer constituent; "unstacking" correspondingly denotes migration in the phase behind the moving boundary the physical conditions of which are generated by passage of the moving boundary. Since the charged compound migrating between leading and trailing buffer constituents is setting up a moving boundary of its own and thus a system of sequential multiple moving boundaries, stacking can also be defined simply by migration as a moving boundary. In fact, the term is a pictorial one likening a system of sequential moving boundaries to a stack of coins.

CONSTITUENT DATA

	(PK/MOBILITY)1	(PK/MOBILITY)2	(PK/MOBILITY)3			
COUNTERION						
TRIS -BASE	8.84	9.51	0	0	0	0
SEQUENCE						
MES	6.41	10.21	0	0	0	0
ACES	7.34	10.90	0	0	0	0
TES	7.96	9.51	0	0	0	0
TRICINE	8.00	7.42	0	0	0	0
BICINE	8.74	16.01	0	0	0	0
GLYGLY	8.94	13.46	0	0	0	0
ASPNH	9.00	11.00	0	0	0	0
TAUR	9.70	14.15	0	0	0	0
GLY	10.46	17.17	0	0	0	0
GABA	11.33	12.76	0	0	0	0

PHS START .4000E+01
STEP .5000E+00
FINAL .8000E+01
C-I.ST ION .5000E-01

ION	PH	C-ION	M-MOB	C-COUNTER	CONDUCT
MES	4.00	.5000E-01	.04	.9377E-04	.1821E-04
ACES	4.45	.5322E-01	.01	.3348E-04	.6483E-05
TES	4.79	.4683E-01	.01	.1521E-04	.2943E-05
TRICINE	5.16	.3700E-01	.00	.5360E-05	.1230E-05
BICINE	5.08	.7593E-01	.00	.8351E-05	.1615E-05
GLYGLY	5.21	.6478E-01	.00	.5995E-05	.1159E-05
ASPNH	5.27	.5643E-01	.00	.5140E-05	.9939E-06
TAUR	5.58	.6777E-01	.00	.2575E-05	.4976E-06
GLY	5.93	.8052E-01	.00	.1205E-05	.2327E-06
GABA	6.42	.5604E-01	.00	.3780E-06	.7281E-07

PMAX = -.2455E-04

MES	4.50	.5000E-01	.12	.5761E-03	.1615E-04
ACES	4.97	.5353E-01	.05	.2171E-03	.6039E-05
TES	5.28	.4719E-01	.02	.9228E-04	.2559E-05
TRICINE	5.61	.3732E-01	.01	.3530E-04	.9773E-06
BICINE	5.80	.7052E-01	.02	.8649E-04	.2397E-05
GLYGLY	5.81	.6532E-01	.01	.4687E-04	.1298E-05
ASPNH	5.81	.5691E-01	.01	.3513E-04	.9724E-06
TAUR	6.21	.6837E-01	.00	.2157E-04	.5960E-06
GLY	6.78	.8124E-01	.00	.1684E-04	.4624E-06
GABA	7.10	.5920E-01	.00	.3615E-05	.9828E-07

PMAX = -.8852E-05

MES	5.00	.5000E-01	.38	.1863E-02	.3708E-04
ACES	5.85	.5335E-01	.34	.1681E-02	.3330E-04
TES	5.77	.4814E-01	.06	.3066E-03	.5902E-05
TRICINE	5.89	.3819E-01	.01	.7344E-04	.1406E-05
BICINE	7.99	.6303E-01	2.39	.1074E-01	.2322E-03
GLYGLY	7.98	.5886E-01	1.33	.6596E-02	.1286E-03
ASPNH	7.72	.5518E-01	.57	.2940E-02	.5673E-04
TAUR	8.64	.5989E-01	1.14	.7898E-02	.1106E-03
GLY	9.24	.6360E-01	.98	.1279E-01	.9480E-04
GABA	9.72	.4829E-01	.31	.1056E-01	.2992E-04

PMAX = .8628E-05

MES	5.50	.5000E-01	1.12	.5477E-02	.1047E-03
ACES	6.52	.5178E-01	1.43	.6844E-02	.1342E-03
TES	6.85	.4848E-01	.69	.3544E-02	.6441E-04
TRICINE	6.15	.4068E-01	.03	.1421E-03	.2434E-05
BICINE	8.18	.6083E-01	3.44	.1589E-01	.3216E-03
GLYGLY	8.24	.5680E-01	2.25	.1189E-01	.2105E-03
ASPNH	8.17	.5325E-01	1.50	.8358E-02	.1403E-03
TAUR	8.82	.5778E-01	1.65	.1315E-01	.1541E-03
GLY	9.34	.6133E-01	1.20	.1789E-01	.1126E-03
GABA	9.83	.4622E-01	.39	.1593E-01	.3654E-04

PMAX = .7592E-05

MES	6.00	.5000E-01	2.86	.1402E-01	.2666E-03
ACES	6.97	.5160E-01	3.25	.1557E-01	.3027E-03
TES	7.47	.4831E-01	2.32	.1229E-01	.2163E-03
TRICINE	7.80	.4232E-01	1.02	.6343E-02	.9499E-04
BICINE	8.38	.6059E-01	4.84	.2460E-01	.4509E-03
GLYGLY	8.47	.5657E-01	3.43	.2061E-01	.3197E-03
ASPNH	8.47	.5304E-01	2.63	.1710E-01	.2450E-03
TAUR	8.98	.5755E-01	2.26	.2188E-01	.2107E-03
GLY	9.44	.6107E-01	1.50	.2660E-01	.1394E-03
GABA	9.93	.4593E-01	.49	.2471E-01	.4572E-04

PMAX = .4592E-05

Fig. 3. Input and output of the program of Routs[17] describing some physical properties of sequentia moving boundary systems. Fortran adaptation by P. J. Svendsen.

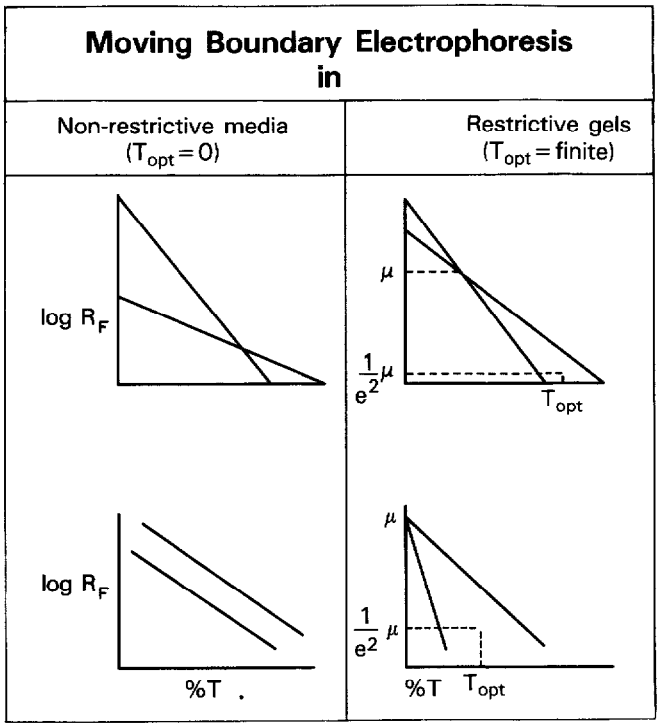

Fig. 4. Optimally resolving gel concentrations in four types of possible separation problem analyzed by Ferguson plot. Left panel: separation problems where $T_{opt} = 0$, indicating the use of liquid or non-restrictive" gel media and separation within the stack, or selective stacking and unstacking. Right panel: separation problems where $T_{opt} \neq 0$, indicating gel electrophoresis in the trailing phase, with or without selective stacking or unstacking.

MOVING BOUNDARY ELECTROPOHORESIS ON GELS *VS.* LIQUID MEDIA

To exploit the size and shape differences as well as the net charge differences among molecular species optimally, gel electrophoresis must be conducted at a particular gel concentration (designated as T_{opt}) which is mathematically defined for each pair of species which is to be separated[21] (Fig. 4). Experimentally, it is gel electrophoresis at several gel concentrations, followed by construction of the Ferguson plot (log R_F vs. $\%T$) and calculation of T_{opt} which determines objectively whether a particular component pair is optimally separated at $\%T = 0$, either in liquid media or "non-restrictive gels"* (Fig. 4, left panel), or at a finite optimal gel concentration (Fig. 4, right panel). Thus, the selection of an optimal technique and apparatus for separation in either liquid or gel media cannot be made arbitrarily without risk of sacrificing resolving power.

* The term "non-restrictive" is put in quotation marks in order to emphasize that all gels are restrictive to molecules of all sizes. In a practical sense, however, "non-restrictive" gels are those which allow a maximally charged molecular species to remain stacked in a moving boundary with low trailing ion net mobility [*e.g.* 0.064 relative to Na^+ at pH 10.5 (ref. 23)].

A Ferguson plot for the determination of T_{opt} can be readily obtained from a single electrophoresis experiment at minimally three, optimally seven gel concentrations in a gel tube apparatus[22]. If $T_{opt} = 0$, one has the choice between liquid and "non-restrictive gel" media. Depending on the number of experiments, the need for automated data collection, the available sample size, the requirement of a speedy analysis, the needed preparative load capacity, etc., one may prefer capillary, rotating tube, gel slab or gel tube apparatus. If $T_{opt} \neq 0$, the choice of instrumentation depends on the number of needed analyses and the need for preparative load capacity mainly, and is basically one between the various forms of gel slab and gel tube apparatus.

VARIOUS MODES IN WHICH MOVING BOUNDARY ELECTROPHORESIS CAN BE USED TO MODIFY EITHER CONDITIONS IN THE STACK OR THOSE IN THE TRAILING PHASE FOR THE PURPOSE OF SEPARATION

Moving boundary electrophoresis can be used either in gel or in liquid media to effect separations in three different ways: (a) by concentrating the sample, in practical independence of sample volume and without labor input, as a micron-thin starting zone of the resolving phase; (b) by partitioning between the components stacked in a system of sequential moving boundaries and those migrating behind it, either at the concentration stage or at the resolving stage; and (c) by providing a reference zone in the resolving phase for the measurement and the regulation of R_F values.

(a) The benefit of automatic sample concentration for biological species of either small or large size and the importance of a uniformly thin starting zone for electrophoretic or chromatographic separations, independently of sample volume, is self-evident. Operationally such concentration merely involves the application of the sample onto the surface of a leading phase of at least the same volume as the sample, overlayering it with a solution of trailing constituent and counterion, and the initiation of electrophoresis. To be certain that the species of interest will be concentrated, one needs to tailor the leading and trailing ion net mobilities to the required values. This can only be done by a systematic experiment, usually involving a systematic increase in the trailing ion net mobility at the chosen pH (Fig. 12 of ref. 2) starting with an exceedingly low value (0.050 or less relative to Na^+). The gel concentration used for concentration may be "non-restrictive" if the species of interest migrates slowly relative to the net mobility of the trailing constituent, or it may be restrictive if the species of interest is a rapidly migrating one. "Non-restrictive" media in this context may be dilute and/or highly cross-linked gels[23], or they may be liquid density gradients or simple liquids. Evidently, diffusion of the stacked species increases in the same order, and therefore so does the zone width of the starting zone which enters the resolving phase.

(b) The same systematic variation of trailing ion net mobility that is used to define the condition for concentrating the species of interest can also be used to find the maximal value for the trailing ion net mobility at which the species remains stacked (selective stacking). Or it may be used to find the minimal trailing ion net mobility at which the species unstacks, *i.e.* migrates in the trailing phase behind the moving boundary. In that case, the maximal number of rapidly migrating (relative to the species) contaminants remain concentrated in the moving boundary migrating

ahead of the species of interest (selective unstacking).Again, this operation may employ gel concentrations which decrease the net mobility, or "non-restrictive" media of either the gel or the liquid type.

Selective unstacking of contaminants is equally useful whether separation is designed to proceed inside of a system of sequential moving boundaries (a stack) or whether it is followed by a separation in the trailing phase, *i.e.* under conditions of unstacking of the species of interest in the resolving phase. Selective stacking of contaminants, and selective unstacking of the species of interest, is of course a strategy open only to separations in the trailing phase.

(c) A reference zone is needed for the characterization of components by relative mobility (R_F)*; as the advantage, compared with assignment of absolute mobility values to the zones, that sources of imprecision such as temperature, pH or ionic strength variations between experiments, cancel out to some degree since they tend to equally affect the migration of the reference zone and the zone of the components of interest. Moving boundaries, of all the possible reference zones, have the advantage of extreme zone sharpness which reduces the measurement error, and the further advantage that the reference zone does not widen in proportion to migration distance.

It is important to realize that separations both in the stack and in the trailing phase are moving boundary electrophoresis since in both cases separations are regulated by control of the numerical values of trailing ion net mobilities, separated species are characterized by reference to a moving boundary, and pH and ionic strength is governed in the resolving phase by operation of the moving boundary equation[7].

PREPARATIVE MOVING BOUNDARY ELECTROPHORESIS

The unparalleled load capacity of moving boundary electrophoresis for preparative purposes derives from the regulating functions. The molar concentrations of stacked species are regulated at the same order of molar concentrations as those of any other trailing electrolyte constituents which define the buffer system. Thus, a macromolecule of molecular weight 10,000, stacked at a regulated concentration of 0.01 *M* is concentrated to *ca.* 100 mg/ml. Such enormous values have been substantiated experimentally for proteins[3,4]. Thus, preparative moving boundary electrophoresis gains a maximal load capacity when it is applied to stacked proteins, and uses a gel of the maximal practicable surface area. This has become important recently, through the *in vitro* synthesis of medically important proteins by genetically transformed bacteria at the unprecedented scale of 100 g and above. Among electrophoretic methods, only moving boundary electrophoresis can deal with that amount of protein. In the particular case of human growth hormone (hGH) made from transformed bacteria[24], this has been possible in two different ways: by selective unstacking of hGH at pH 7 in a "non-restrictive" gel, and by selective stacking of hGH at pH 10.5 in a 9% *T* polyacrylamide gel. Combining the two techniques, *ca.* 1 g of

* R_F is defined here as the migration distance (or time) of a species divided by the migration distance (or time) of the moving boundary in front of the species. R_F is a physical constant characteristic of the particular species in the particular milieu in which migration takes place provided that this milieu is constant.

	Acetic A.	MES	ACES	MOPS	HEPES	TES	HEPPS	TAPS	Tricine	Bicine	OH-prol	ser	gly
r	-.7900E+00	.4400E+00	.4700E+00	.4300E+00	.2800E+00	.4100E+00	.4300E+00	.4100E+00	.3200E+00	.6900E+00	.7000E+00	.5700E+00	.7400E+00
pK	0.4740E+010	.5000E+000	.5000E+000	.5000E+000	.5000E+000	.5000E+000	.5000E+000	.5000E+000	.2300E+010	.2300E+010	.2300E+010	.1900E+010	.2400E+01
conc (M)	0.1000E+010	.5788E+000	.6162E+000	.5663E+000	.3750E+000	.5411E+000	.5663E+000	.5411E+000	.4267E+000	.8829E+000	.8948E+000	.7590E+000	.9418E+00
pH	0.2370E+010	.3550E+010	.4010E+010	.4051E+010	.4308E+010	.4330E+010	.4546E+010	.4885E+010	.5453E+010	.5521E+010	.6106E+010	.6104E+010	.6431E+01
ϕ	0.4266E-020	.4872E-030	.1586E-030	.1569E-030	.1313E-030	.8644E-040	.5019E-040	.2409E-040	.8275E-050	.3411E-050	.6763E-060	.1066E-050	.3947E-06
$\bar{r}$	-.3370E-02	.2144E-03	.7454E-04	.6746E-04	.3675E-04	.3544E-04	.2158E-04	.9875E-05	.2648E-05	.2353E-05	.6134E-06	.6377E-06	.2921E-06
κ(μmhos/cm)	0.1047E-020	.6657E-040	.2315E-040	.2095E-040	.1141E-040	.1101E-040	.6703E-050	.3067E-050	.8217E-060	.7310E-060	.1903E-060	.1887E-060	.9048E-07
ν(ml/Coulomb)	-.8822E-03	.8822E-03	.8822E-03	.8822E-03	.8822E-03	.8822E-03	.8822E-03	.8822E-03	.8830E-03	.8822E-03	.8834E-03	.8825E-03	.8845E-03
v(cm/day)	-.3267E-05	.3268E-05	.3267E-05	.3267E-05	.3267E-05	.3267E-05	.3267E-05	.3267E-05	.3270E-05	.3267E-05	.3272E-05	.3269E-05	.3270E-05

Fig. 5. Representative computer outpout, using thirteen acidic constituents, of the program of L. M. Hjelmeland[5] providing some physical properties for sequential moving boundary systems arrested by replacing the common ion through proton and hydroxyl ion. The parameter r designates the ionic mobility relative to Na^+, $\bar{r}$ the relative net mobility; ϕ designates the ratio of ionized to non-ionized forms of the constituent; specific conductance, κ, is given in μmho/cm. Boundary displacement is given in terms of v and v.

hGH can be potentially recovered from each run on a gel slab of 50 × 1.8 cm, and proportionately more from more suitable apparatus designs with larger gel surface area.

It should be noted that until recently, high preparative protein loads precluded the attainment of the steady state whenever either the multiplicity of components and/or the load became substantial, in view of the limitations with regard to migration path length and time[4]. The possibility to arrest or mobilize the stack by regulation of the common ion concentration potentially abolishes this problem with regard to migration path length (as does counterflow). It remains true, however, that load and multiplicity are proportional to the time required to reach the steady state. Since the resistance across highly concentrated protein zones is large, this time cannot be shortened by increasing the voltage. Preparative moving boundary separations of proteins remain slow in proportion to load and multiplicity.

MOVING *VS.* ARRESTED SEQUENTIAL BOUNDARIES

The possibility by a very simple steady-state treatment and program to predict the phase compositions and pHs of sequential moving boundary systems[5,6] has provided (a) an understanding of the nature of electrofocusing systems as systems of nearly-arrested multiple sequential boundaries, and (b) a practical method for the design of electrofocusing systems to fit ones needs, using computer simulation rather than experiment.

(a) The hypothesis that stable pH gradient (electrofocusing) systems are nearly-arrested sequential moving boundary systems rests on the realization that boundary displacement is proportional to the concentration of the common ion (counterion) in steady-state moving boundary systems[5,15]. Thus, at zero concentration of the common ion, when solvent protons and hydroxyl ions provide the sole counterions, the displacement rate becomes very small so that the pH gradient across the multiple sequential moving boundaries appears to be standing still (Fig. 5). Since steady-state concentrations are known, each constituent can be added in an amount occupying an equal fraction of the path length between catholyte and anolyte.

(b) Considering the substantial labor involved in creating a pH gradient of a particular desired extent and slope, the possibility to computer-simulate pH gradients made up by any number of constituents nearly instantly and to compute, at the same time, their degree of displacement with time, appears important. The practical hold-up to such computations is the need for p*K*s and ionic mobilities of the buffer constituents which constitute the program input. In particular there is a present paucity of values of ionic mobility of monovalent and amphoteric buffers of the Good's buffer type and those with small p*I* − p*K* differences, which are the most capable of conducting at their p*I*s[25], and of values determined at 0°C in general. Experimental pH gradients of the system as a function of time reflect the predicted values only to a rough approximation (Fig. 6). It is not known as yet to what degree this inaccuracy of predicted pH gradients is due to excessive p*I* − p*K* values of the constituents, or to their insufficient multiplicity, or inappropriately low leading ion concentrations or other factors. On the basis of present data it appears that, with constituents of large p*I* − p*K* differences, an increase in multiplicity stabilizes pH gradients and reduces the discrepancy between predicted and experimental values of pH. Possibly, inter-

actions between constituents modify pI and pK values so as to mimic the conductance and buffering capacity of compounds with low pI − pK differences for which no ionic mobility values are known at this time.

In sequential moving boundary systems with negligible displacement rates the regulated net mobility of proteins and buffer constituents alike is close to zero. Thus, although constituents are aligned at the steady state in order of mobility, amphoteric buffers and proteins are also near-isoelectric[30]. The experimental observation of isoelectric alignment of proteins on pH gradients is therefore not counterindicated by the hypothesis that the pH gradients are generated by a moving boundary mechanism. Nor is the theoretical treatment of electrofocusing in terms of a isoelectric alignment of amphoteric species[26] necessarily incompatible with the realization that the isoelectric state is brought about by moving boundary arrest. The possibility to set up pH gradients with mixtures of exclusively non-amphoteric buffers does, however, rule out the possibility that isoelectric alignment is responsible for all of the forms of natural pH gradient formation. Furthermore, since there is no need to assume more than one mechanism by which natural pH gradients form, the hypothesis of a single moving boundary mechanism appears sufficient to explain all the facets of pH gradient electrophoresis at this time.

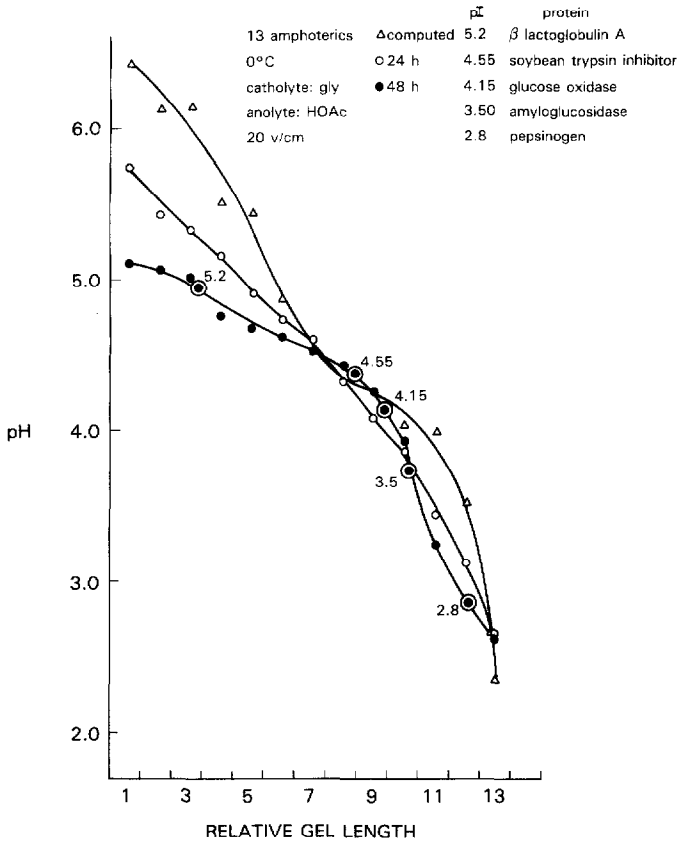

Fig. 6. Computed and experimental pH gradients of the composition given in Fig. 5. 5% T, 15% C_{DATD}, 0°C, 20 V/cm of gel. Data of Zs. Buzás *et al.*[6] and A. Chrambach[30].

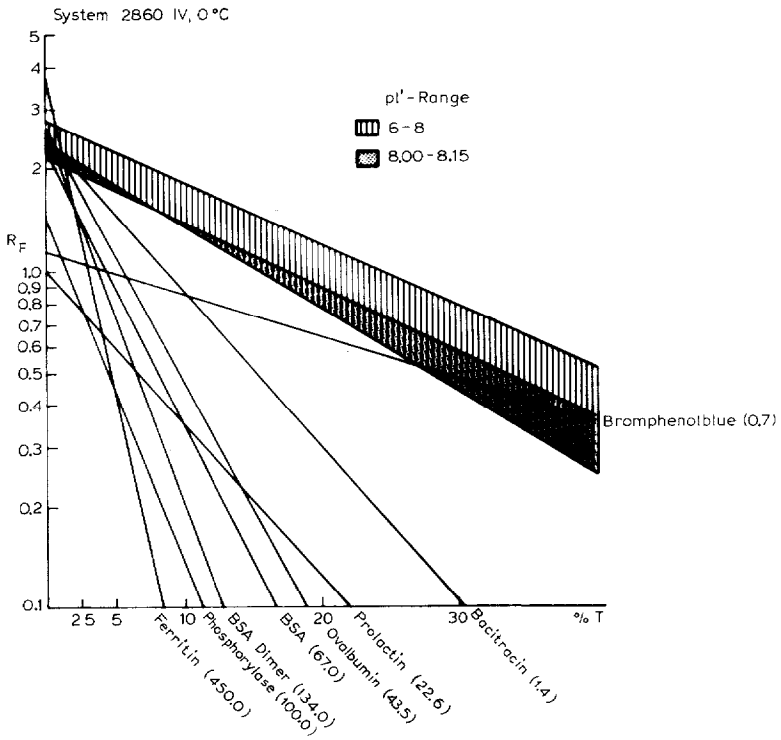

Fig. 7. Ferguson plots of standard proteins and narrow fractions of Ampholine (P. J. Svendsen and A. Chrambach, unpublished data).

(c) Anionic and cationic sequential moving boundaries may, depending on the extent of the pH-range, coexist in an electrofocusing system. These may chemically interact in ways that dissociate rapidly, slowly or not at all in the electric field, depending on the interaction forces and energies. Such interactions have been observed as, for instance, effects on the acidic pH gradient region upon introduction of a basic constituent[27]. Also, since the steady state implies an alignment in order of net mobilities, it is a corollary of the hypothesis that hydroxyl ions in the catholyte, having higher net mobilities than those of any of the other constituents, will continuously pass the entire train of anionic constituents, thereby augmenting pHs and perturbing the steady state. The analogous corollary holds for protons in the anolyte and the cationic train. These considerations suggest a particular lability of pH gradient systems encompassing very high and low pHs.

(d) Analogous simulations of pH gradients and boundary displacement are presently being attempted for the case in which the concentration of the common ion is increased stepwise from zero toward increasing finite values. Under those conditions, the net mobilities of buffer constituents and proteins must necessarily dissociate, ultimately giving rise to contiguous either buffer or protein boundaries. The notion that small molecular weight "spacers" can be found with net mobilities between those of the proteins is a myth for most systems and has been experimentally ruled out at least for "non-restrictive" gels (Fig. 7)[28].

Such systems have nonetheless theoretical interest, since they provide a con-

tinuous transition between conventional "electrofocusing" and "isotachophoretic" systems and thus provide additional evidence for their fundamental identity as moving boundary systems differing only in boundary displacement rate. They may also have specific practical applications in protein separations where zero net mobility is usually associated with isoelectric association and precipitation phenomena which make preparative recovery of proteins from electrofocusing systems hazardous. Such systems with very low displacement rates also avoid the dilemma of preparative moving boundary electrophoresis under conventional conditions where the migration path length and time are limiting the number and concentration of proteins which can be separated at the steady state[4].

REFERENCES

1 L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
2 A. Chrambach, *J. Mol. Cell. Biochem.*, 29 (1980) 23.
3 B. Chen, D. Rodbard and A. Chrambach, *Anal. Biochem.*, 89 (1978) 596.
4 G. Baumann and A. Chrambach, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 732.
5 L. M. Hjelmeland and A. Chrambach, *Electrophoresis*, 4 (1983) 20.
6 Zs. Buzas, L. M. Hjelmeland and A. Chrambach, *Electrophoresis*, 4 (1983) 27.
7 L. M. Hjelmeland and A. Chrambach, *Electrophoresis*, 3 (1982) 9.
8 H. Svensson, *Ark. Kemi. Mineralogi Geologi*, 17A (1943) 1.
9 L. G. Longsworth, *J. Amer. Chem. Soc.*, 67 (1945) 1109.
10 R. A. Alberty, *J. Amer. Chem. Soc.*, 72 (1950) 2361.
11 V. P. Dole, *J. Amer. Chem. Soc.*, 67 (1945) 1119.
12 E. Schumacher and T. Studer, *Helv. Chim. Acta*, 47 (1964) 957.
13 A. J. P. Martin and F. M. Everaerts, *Proc. Roy. Soc. Ser. A*, 316 (1970) 493.
14 F. M. Everaerts, J. L. Beckers and T. P. E. M. Verheggen, *Isotachophoresis: Theory, Instrumentation and Applications*, Elsevier, Amsterdam, Oxford, New York, 1976.
15 C. Schafer-Nielsen and P. J. Svendsen, *Anal. Biochem.*, 114 (1981) 244.
16 M. Bier, O. A. Palusinski, R. A. Mosher and D. A. Saville, *Science*, 219 (1983) 1281.
17 R. J. Routs, *Thesis*, Technische Hogeschool, Eindhoven, Solna Skriv-&Stenograftjaenst, Solna, Sweden, 1971.
18 T. M. Jovin, *Ann. N.Y. Acad. Sci.*, 209 (1973) 477.
19 T. M. Jovin, M. L. Dante and A. Chrambach, *Multiphasic Buffer Systems Outpout*, National Technical Information Service, Springfield, VA, 1970. (For PB numbers, see Fig. 1.)
20 A. Chrambach and T. M. Jovin, *Electrophoresis*, 4 (1983) 190.
21 D. Rodbard and A. Chrambach, in R. C. Allen and H. R. Maurer (Editors), *Electrophoresis and Electrofocusing on Polyacrylamide Gel*, W. de Gruyter, Berlin, New York, 1974, p. 28.
22 A. Chrambach and D. Rodbard, in D. E. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, Information Retrieval Limited Press, London, 1981, p. 93.
23 G. Baumann and A. Chrambach, *Anal. Biochem.*, 70 (1976) 32.
24 G. Kapadia, A. J. S. Jones and A. Chrambach, in preparation.
25 P.-G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier Biomedical Press, Amsterdam, 1983.
26 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456.
27 C. B. Cuono and G. A. Csapo, *Electrophoresis*, 3 (1982) 65.
28 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 94 (1979) 202.
29 N. Y. Nguyen, D. Rodbard, P. J. Svendsen and A. Chrambach, *Anal. Biochem.*, 77 (1977) 39.
30 A. Chrambach, in V. Neuhoff (Editor), *Recent Developments in Buffer Electrofocusing, Electrophoresis '84*,Verlag Chemie, Weinheim, 1984, p. 3.