Journal of Chromatography, 418 (1987) 145–185
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3622

REVIEW

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS

MICHAEL J. DUNN

Jerry Lewis Muscle Research Centre, Royal Postgraduate Medical School, Ducane Road, London W12 0HS (U.K.)

(First received November 11th, 1986; revised manuscript received December 8th, 1986)

CONTENTS

0378-4347/87/$03.50

1. INTRODUCTION

Electrophoretic procedures constitute a powerful battery of techniques for the characterisation and analysis of proteins. One-dimensional (1-D) methods of polyacrylamide gel electrophoresis (PAGE) can at best resolve about 100 protein species, a resolution capacity inadequate for the analysis of complex protein mixtures such as represented by samples of whole cells and tissues. A combination of two different electrophoretic procedures can increase this resolving power by several orders of magnitude, especially if the two techniques separate proteins on the basis of independent physicochemical properties.

The first two-dimensional (2-D) electrophoretic separations can be attributed to Smithies and Poulik [1] who demonstrated the improved separation of serum proteins which could be achieved using a 2-D combination of paper and starch gel electophoresis. As innovations in electrophoretic technology occurred, such as the use of polyacrylamide as a separation matrix [2], so new 2-D procedures were also developed. Thus, the use of different polyacrylamide gel concentrations was exploited for 2-D separations [3,4].

An advance of major importance to the development of 2-D technology occurred with the use of isoelectric focusing (IEF) techniques. The first-dimension separation could now be based on the charge properties of the proteins and could be used in conjunction with single concentration [5,6] or gradient [7,8] polyacryl-

amide gels. When IEF was coupled with a second-dimension separation in polyacrylamide gels containing the anionic detergent sodium dodecyl sulphate (SDS), a technique capable of separating proteins on the basis of molecular weight, it formed a 2-D method which resolved proteins according to two truly independent parameters, i.e. charge and size [9]. For this 2-D methodology to be applicable to a wide range of samples with differing solubility properties, modified IEF procedures had to be developed. The most important of these modifications to the IEF system was the inclusion of urea in the gels [10–12] and the use of a mixture of non-ionic detergent (e.g. Triton X-100) and 8 *M* urea [13,14].

By 1975 a system of 2-D PAGE had evolved which allowed separation of a variety of complex protein mixtures on the basis of charge (IEF dimension) followed by size (SDS-PAGE dimension). This technique could be applied to the high-resolution analysis of whole cell and tissue proteins [15–17]. The technique was optimised for the separation of *Escherichia coli* proteins by O'Farrell [18] and this 2-D PAGE method has formed the basis for most of the subsequent developments in 2-D PAGE technology which will be described in this review.

2. METHODOLOGY

2.1. Sample preparation

There is no single method of sample preparation which can be used universally for 2-D PAGE due to the diverse nature of samples subjected to 2-D analysis. Samples of soluble proteins, such as body fluids or cell and tissue extracts, can be used directly for 2-D PAGE, but if they are dilute they will require concentration prior to 2-D analysis. In contrast, samples of solid tissues, circulating cells or cells grown in tissue culture must be disrupted and solubilised before analysis by 2-D PAGE. Whatever method of sample preparation is used, it is essential to minimise the effects of chemical modification and degradation of proteins during sample preparation for 2-D PAGE. The 2-D system is very sensitive to charge modifications (less so to changes in molecular weight) which can result in artefactual multiple spots from a single polypeptide on 2-D maps. Some types of sample can be rich in proteases so that samples should be handled rapidly and kept cold at all times. Protease inhibitors can be added, but they are probably best avoided as they can result in protein charge modifications making them unsuitable for use in 2-D PAGE. Urea is usually added to samples for 2-D analysis. These samples should not be heated as substantial charge heterogeneity can be introduced due to carbamylation of the proteins by isocyanate formed by decomposition of urea.

2.1.1. Samples of body fluids

Samples of body fluids, such as serum, plasma, urine, cerebrospinal fluid (CSF), seminal fluid, sweat, tear fluid, amniotic fluid, aqueous humour from the eye and blister fluid, have all been successfully subjected to 2-D analysis. Although such samples can be stored frozen at −20°C to −70°C prior to processing for 2-D PAGE, this can result in minor changes in 2-D patterns [19]. It is, therefore,

generally preferable to process samples as rapidly as possible. In particular, seminal plasma is very sensitive to proteolytic modification [20].

Serum and plasma samples are generally suitable for direct analysis by 2-D PAGE, requiring only the addition of sample dissociation buffer (see Section 2.2). However, most other body fluid samples have a low total protein concentration and contain high levels of salts which would interfere with the IEF dimension of 2-D PAGE. Thus, prior to 2-D analysis, such samples must be dialysed to remove salts and then concentrated by lyophilisation or precipitation. The detection method to be used to visualise the final 2-D protein map should also be considered at this stage. For example, very sensitive detection methods such as silver staining can be used to advantage as excessive concentration and high sample loadings can be avoided. Using this approach, CSF samples can be successfully separated by concentrating them four-fold by dialysis against 10% polyethylene glycol and applying 15-μl samples to the gels [21].

Proteins of urine samples have been analysed extensively by 2-D PAGE. Urine may contain particulate matter and should be centrifuged at 2000 *g* for 15 min prior to further processing. Urinary proteins, present at about 0.1 g/l in healthy adults, must be separated from the large amount of non-protein components, largely salts, prior to 2-D PAGE. Various methods have been used to remove these components, such as ion-exchange chromatography, ultrafiltration, dialysis and lyophilisation. These procedures have been assessed in detail by Anderson and co-workers [22–24]. They have developed two major methods: (a) dialysis followed by lyophilisation and (b) gel permeation chromatography followed by lyophilisation The latter technique has been found generally to be the most practical and Tracy and Young [25] have recommended a modified procedure based on this approach.

Marshall et al. [26] have successfully detected over 600 polypeptides in silver-stained 2-D maps of unconcentrated, unprocessed human urine, but commented that the resolution obtained was inferior to that using desalted samples. These workers have also obtained satisfactory 2-D maps of unconcentrated CSF and amniotic fluid samples [27]. A caveat concerning the use of lyophilisation is that it can result in protein modifications such as deamidation of asparagine and glutamine residues or oxidation of cysteine residues [18].

Similar methods of sample preparation can be used for other body fluids, except that extensive dialysis is usually unnecessary. For example, salivary proteins can be processed for 2-D PAGE by dialysis and concentration using a centrifugal concentrator [28]. Samples which do not require dialysis can be conveniently concentrated using apparatus such as a Minicon concentrator (Amicon) [26]. Another method which can be used for sample concentration is precipitation with acetone. We [29] found that precipitation of 100 μl of sample with 1 ml cold acetone for 1 h, followed by centrifugation and storage at $-20°C$, resulted in no qualitative loss of protein spots, but possible quantitative losses were not excluded. Acids such as trichloroacetic acid (TCA) can also be used but there is a risk of protein modification and the resultant precipitates can be difficult to redissolve. Immunoprecipitates can be similarly analysed, although the current popularity of protein blotting techniques has diminished the requirement for such analysis.

2.1.2. Solid tissue samples

Various procedures have been described for 2-D analysis of solid tissues, but most methods involve disruption of tissue in the presence of solubilisation buffer. Large pieces of tissue should first be broken mechanically, while frozen if possible, into smaller pieces. The tissue fragments are then homogenized using a device such as the Ultra-Turrax or Polytron. Care should be taken to minimise heating and foaming during homogenisation. Thus, detergent can be added after homogenisation [19] or a suitable antifoam reagent included in the solubilisation mixture. Many tissues contain substantial protease activity which can be inactivated using protease inhibitors or an alkaline pH [25].

The availability of sensitive detection procedures such as silver staining has made it possible to carry out 2-D PAGE analysis of very small amounts of tissue, even single cryostat sections of frozen biopsy material. This approach has the advantages that (i) less tissue is used, (ii) morphological changes can be monitored by histological, histochemical and immunocytochemical analysis of serial sections and (iii) a much wider range of tissue samples should be available for analysis [29]. Cryostat sections can be collected on a cooled fine brush or probe and placed in solubilising buffer or can be applied to the hydrophilic surface of GelBond squares and scraped off into solubilisation buffer [30]. It is also possible to radiolabel such cryostat sections prior to solubilisation using [^{14}C]iodoacetamide [31,32] or by reductive methylation with [^{3}H]sodium borohydride or [^{14}C]formaldehyde [33]. Procedures based on reductive methylation have been shown not to result in charge heterogeneity but this is always a potential hazard with post-synthetic labelling procedures [34].

2.1.3. Cultured cells

Cells grown in tissue culture can be prepared for 2-D analysis without any special treatment simply by direct lysis in the culture plate or well. The medium should be aspirated carefully, the cell layer washed with phosphate-buffered saline (PBS) or an isotonic sucrose solution to reduce contamination with medium proteins (this step is not essential) and lysed by the addition of a small amount of solubilisation buffer. Samples should be stored at $-70°$C. Nucleic acids present in small samples will not interfere with the first-dimension IEF separation, but large quantities of cells should be treated with DNAse and RNAse [35,36].

2.2. Sample solubilisation

Ideally, the sample solubilisation procedure used for 2-D PAGE should result in the disruption of all non-covalently bonded protein complexes and aggregates to form a mixture of individual polypeptides. Incomplete disruption can result in the simultaneous existence of a protein in an aggregated and a single polypeptide state which can cause artefactual spots on 2-D maps [37]. Thus, solubility and disaggregation are crucial factors in 2-D PAGE.

The most commonly used solubilisation procedure is that described by O'Farrell [18] using a combination of 2% (w/v) of the non-ionic detergent NP-40 and 9 *M* urea. This combination of reagents is generally satisfactory for the majority

of samples but it does not always result in disaggregation of all protein complexes or allow entry of all proteins into the first-dimension IEF gels, particularly for more difficult samples such as histone [38], ribosomal [18] and membrane [39] proteins.

SDS disrupts the majority of protein interactions [40], but the anionic nature of this detergent makes it unsuitable for use in IEF gels. However, O'Farrell [18] found that SDS could be used to presolubilise samples for 2-D PAGE without adverse effects. This procedure was developed further by Ames and Nikaido [39] specifically for use with membrane proteins. In this method samples are solubilised initially in SDS, followed by the addition of urea and high concentrations of NP-40 to compete with SDS. Careful control of the ratios of SDS to protein (1:3) and SDS to NP-40 (1:8) is essential to maximise solubilisation while minimising the adverse effects of SDS on the IEF dimension. There have been reports of difficulties with this method (e.g. ref. 41), but it generally works satisfactorily provided that the amount of SDS applied to the IEF gels is low. However, it does result in some loss of resolution in the acidic (anodic) region of 2-D maps which may be due to interaction of sulphate with ampholytes. It must be remembered when using this solubilisation procedure that any free SDS will form mixed micelles with NP-40 which will migrate to the anode during IEF [18]. Even small amounts of SDS can remove all free detergent from the IEF gel [42]. Proteins will consequently be exposed to an evironment containing little or no detergent which can result in their precipitation.

A variety of alternative procedures of sample solubilisation for 2-D PAGE have been reported and are reviewed in [37]. These methods have found application to specific types of sample and have not gained acceptance as general solubilisation methods for 2-D PAGE. For example, membrane proteins can be effectively solubilised by an NP-40–urea mixture under alkaline conditions (pH 10.3) by the inclusion of potassium carbonate [43]. Deoxycholate has been used to selectively solubilise those microsomal proteins not solubilised by SDS [44]. Dockham et al. [45] have recently reported improved solubilisation of erythrocyte membrane proteins using a modified lysis buffer containing 9 *M* urea, 6.4% (w/v) Triton X-100, 205 m*M* L-lysine (free base), 5 m*M* dithiothreitol (DTT) and 15 m*M* sodium thioglycolate. Interestingly, the detergent Triton X-114 can be used to fractionate integral membrane proteins by a temperature-induced phase separation [46,47] and this technique has been used for 2-D PAGE of leukocyte membrane proteins [48].

Zwitterionic, sulphobetaine (SB)-type detergents are effective solubilising agents [49,50] and are compatible with IEF. However, they are not suitable for use in 2-D PAGE as they precipitate in the presence of 8 *M* urea and are ineffective when used alone [42]. However, the zwitterionic detergent 3-[(cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS) was found to be effective in 2-D separations of microsomal proteins [51] and we routinely use an urea–CHAPS mixture for sample solubilisation prior to 2-D PAGE [52,53].

In spite of the various solubilisation procedures which have been described, there is still a need for improved techniques as incomplete entry of sample proteins into first-dimension IEF gels is a commonly observed feature of 2-D PAGE.

Although many detergents have been investigated, surprisingly little attention has been devoted to alternative denaturants, as an increase in denaturing potential should disrupt more protein interactions. Lastly, if urea could be replaced by alternative reagents this would overcome problems associated with protein charge modifications due to carbamylation.

2.3. The first dimension

Although alternative procedures are possible, IEF under denaturing conditions in the presence of both urea and non-ionic or zwitterionic detergent is normally the preferred first-dimension procedure for 2-D PAGE.

2.3.1. IEF gel composition

2.3.1.1. Gel matrix. Polyacrylamide is the gel matrix normally chosen for the IEF dimension of 2-D PAGE. In order to minimise molecular sieving during IEF, weak (3–5% T*) gels are used but the migration of high-molecular-mass proteins can still be impeded by such a matrix. High levels (50–60% C*) of N,N′-methylenebisacrylamide (Bis) cross-linker can be used to generate gels of very high porosity, but these gels are not suitable for 2-D PAGE as they are hydrophobic and mechanically unstable [54,55].

Agarose is a matrix with potential benefits for the IEF dimension of 2-D PAGE due to its capacity for focusing high-molecular-mass proteins. Hirabayashi [56] has described a 2-D system using agarose IEF in the presence of 6 *M* urea. However, this methodology has not proved popular due to problems associated with high levels of urea in agarose gels [37,57] and pH gradient instability during agarose IEF [58,59].

2.3.1.2. Urea and detergents. IEF gels for 2-D PAGE normally contain both 8 *M* urea and non-ionic or zwitterionic detergent. Non-ionic detergents (Triton X-100, NP-40) are usually included at a level of 2% (w/v), although this can be reduced to 0.5% (w/v) without any adverse effect on protein resolution [60]. The zwitterionic detergent CHAPS, which can have advantages for sample solubility [51], can also be used at a level of 0.5% (w/v) in first-dimension IEF gels [52,53]. The presence of 8 *M* urea in the IEF gels necessitates the use of extended focusing times to attain equilibrium due to an increase in both viscosity effects [37] and the Stokes radius of proteins after denaturation by urea [57,61].

2.3.1.3. Synthetic carrier ampholytes. The most important components present in IEF gels are the synthetic carrier ampholytes which generate the pH gradient on which the quality of the separation depends. Several alternative methods are available for the synthesis of carrier ampholytes, reviewed in refs. 37, 55 and 62. Thus, commercial preparations from different suppliers (Ampholine from LKB, Pharmalyte from Pharmacia, Servalyte from Serva, Resolyte from BDH) will contain different ampholyte species. It is, therefore, not surprising that the various commercial ampholytes can give better resolution in different regions of the

*T(%) = total gel concentration (acrylamide plus Bis) (g per 100 ml); C (%) = cross-linking agent concentration (g per 100 ml) × 100% T.

pH profile in IEF gels [42]. In addition, maximisation of the number of ampholyte species in the gel by mixing different commercial ampholyte preparations can enhance resolution of 2-D PAGE [60,63]. It is even possible by blending various wide and narrow pH range ampholytes to manipulate the shape of the pH gradient generated within the IEF gel to match the distribution of proteins [60]. However, this type of gradient engineering is somewhat empirical.

2.3.2. Rod IEF gels

In the procedure devised by O'Farrell [18] cylindrical IEF gels cast in glass tubes were used and this has continued to be the most popular system for the first dimension of 2-D PAGE. The tubes normally used have an internal diameter of between 1 and 3 mm and can be cut to any appropriate length (typically between 12 and 20 cm). Such rod IEF gels are easy to prepare (for a suitable method see ref. 36) but care must be taken to ensure that all the gels are of exactly the same length to minimise variability in the final 2-D maps which complicates analysis. An approach to this problem is to prepare batches of IEF gels and several devices to achieve this have been described [36,64,65]. IEF gels formed in glass tubes are usually focused in apparatus optimal for standard gel electrophoresis rather than IEF. Such apparatus has major disadvantages for IEF including (i) poor cooling necessitating long focusing times as high field strengths cannot be used and (ii) large electrolyte volumes resulting in increased pH gradient drift and loss of ampholytes and proteins from the gels [55].

Rather weak electrolyte solutions (e.g. 20 m*M* sodium hydroxide catholyte, 10 m*M* orthophosphoric acid anolyte) are normally used for rod IEF gels. The gels are usually prefocused at 200 V for 15 min, 300 V for 30 min and finally 400 V for 60 min (total 600 V h). Samples are then applied using a suitable microsyringe to the top of the IEF gels and under a thin layer (ca. 10 μl) of buffer containing urea, detergent and ampholytes to protect proteins from the extreme pH of the electrolyte solution. The gels are then run for the appropriate time (see Section 2.3.5), after which the gels are carefully removed from the tubes by injecting water around the gels or by application of air pressure. Apparatus has been described [65] for ejecting simultaneously twenty gels from their tubes and for subsequently loading them directly on to second-dimension SDS slab gels [66].

The standard 2-D PAGE system using cylindrical IEF gels is undoubtedly capable of producing good high-resolution 2-D protein patterns (Fig. 1). However, a significant disadvantage of this technique is that pH gradients in the first dimension rarely extend above 8 with concomitant loss of basic proteins from the 2-D patterns. This phenomenon is a result of pH gradient instability due to electroendosmotic effects. Teatment of the inner walls of glass tubes used for IEF with methylcellulose can reduce electroendosmotic effects. In addition, pH gradient stability can also be improved by the use of alternative electrolyte solutions [67,68]. Using this approach we [60] have obtained pH gradients which extended to pH 10 in 2-D separations of [^{35}S]methionine-labelled skin fibroblast proteins. Cathodic proteins were represented in the 2-D patterns (Fig. 2), but these spots formed streaks rather than discrete spots even if extended focusing times (20 000 V h) were used.

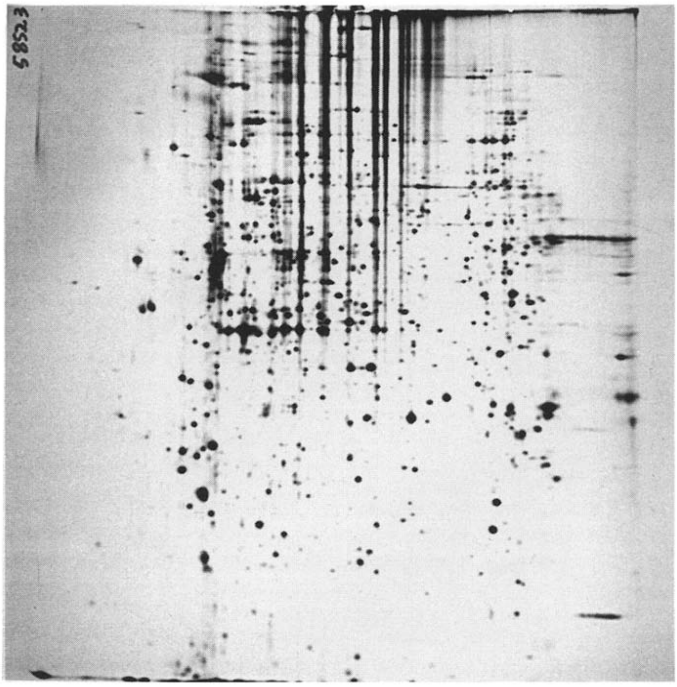

Fig. 1. Fluorograph of a 2-D separation of [^{35}S]methionine-labelled human skin fibroblast proteins using standard rod-gel IEF in the first dimension. Sample analysed by Protein Databases Inc.

2.3.3. Horizontal flat-bed IEF gels

In contrast to the rod IEF gels used in most 2-D PAGE procedures, horizontal flat-bed gels are almost universally used for one-dimensional IEF separations. A significant advantage of flat-bed apparatus is that it is designed specifically for IEF procedures so that small electrolyte volumes can be used and the electrodes can be positioned close to the ends of the gels in order to minimise pH gradient drift and loss of proteins from the gels [55]. The most important advantage of this methodology for 2-D PAGE is that flat-bed IEF gels are capable of resolving basic proteins into discretely separated components rather than poorly resolved, streaked zones. Initial attempts to adapt flat-bed IEF to 2-D separations had rather limited success, probably due to the thick gels which were used [15,69,70]. However, rather more successful procedures have been developed recently by taking advantage of thin [60] and ultrathin [71,72] IEF gels.

We have developed a procedure [52,60,73] in which 0.5 mm thick first-dimension IEF gel slabs are cast on a plastic support in a simple glass cassette fitted with a silicon rubber gasket. The IEF gel on its plastic support must be capable of withstanding the handling procedures involved in equilibration after IEF and transfer to the second SDS-PAGE dimension. We found that polyacrylamide IEF gels can be reliably bound to GelBond PAG sheets in the presence of both 8 *M* urea and 0.5% (w/v) NP-40 or CHAPS provided that the catalyst concentration is adjusted [52]. After polymerization the IEF gel is placed on a suitable flat-bed apparatus and the upper surface of the gel is covered with a plastic sheet, con-

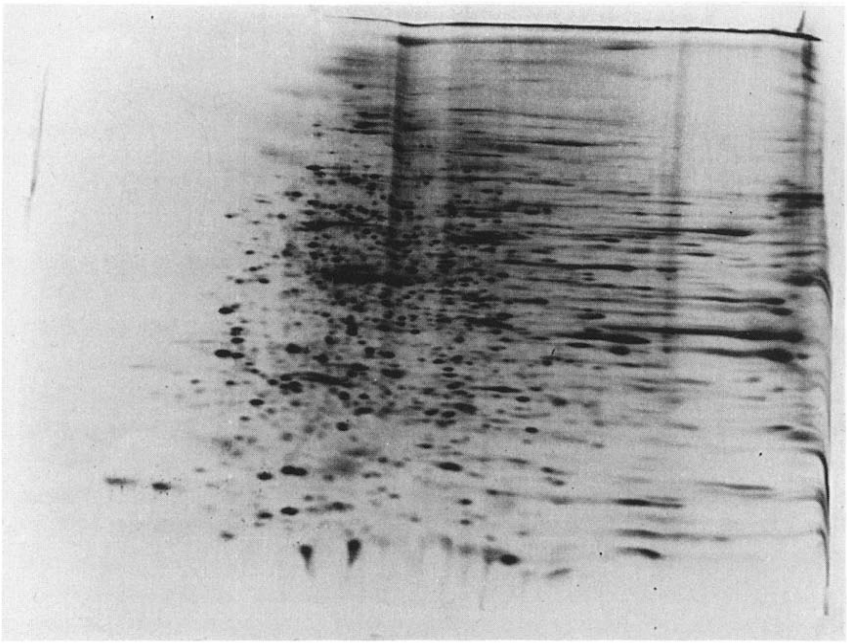

Fig. 2. Autoradiograph of a 2-D separation of [^{35}S]methionine-labelled human skin fibroblast proteins using extended pH gradient rod-gel IEF in the first dimension. Taken with permission from ref. 60.

taining punched-out holes for sample application, to protect the gel from atmospheric effects, dessication and crystallisation of urea. Small electrolyte volumes can be used for flat-bed IEF, but they must be stronger than those used for rod-gel IEF. We use four layers of glass fibre paper strips [74] as wicks and our recommended electrolytes are 1 *M* sodium hydroxide (catholyte) and 1 *M* oxalic acid (anolyte). The gels are run at 15°C as at lower temperatures there is a risk of crystallisation of urea within the gel. A major advantage of flat-bed IEF is the great flexibility in choice of sample application site, thus avoiding problems associated with sample application under conditions of extreme pH. Methods of sample application are discussed in Section 2.3.4 and running conditions in Section 2.3.5.

After IEF the cover sheet is removed and the gel covered with Saranwrap food wrapping film. For non-radioactive samples, individual sample tracks are cut using a template of lane markings made during gel preparation. If they are not to be run in the second dimension immediately, the strips should be frozen rapidly and stored at −70°C. In the case of radioactive samples, the wrapped gel can be placed in direct contact with a sheet of X-ray film and exposed overnight (or longer) at −70°C. The resulting autoradiograph is then used as a guide for cutting off the individual sample tracks.

Flat-bed IEF gels do suffer from some disadvantages for 2-D PAGE. Rod IEF gels have a significantly greater sample loading capacity and are more tolerant to the presence of salts. Flat-bed gels can be rather easily overloaded resulting in

Fig. 3. Autoradiograph of a 2-D separation of [^{35}S]methionine-labelled human skin fibroblast proteins using flat-bed IEF in the first dimension.

distorted protein zones. It has been suggested that flat-bed IEF gels are more prone to skewing of protein zones, but distorted bands can occur using both rod and flat-bed systems [75]. Skewing is, therefore, dependent more on the running conditions employed than the nature of the gel system per se [76]. A typical 2-D separation of [^{35}S]methionine-labelled skin fibroblast proteins using our flat-bed IEF procedure in the first dimension is shown in Fig. 3.

2.3.4. Sample application

There is little choice of sample application site in the case of rod IEF gels as the sample must be applied directly to the top of the gel, i.e. at the cathode or anode. It is generally recommended that, after prefocusing, samples are loaded using a microsyringe to the top of the IEF gels under a layer of solution containing urea and ampholytes to protect sample proteins from the extreme pH of the electrolyte solution. It is theoretically possible to incorporate the sample throughout the gel by including it in the gel solution before polymerisation, but this procedure is not recommended in practice because of the potential of urea, catalysts and heat of polymerisation to modify sample proteins.

In contrast, a notable advantage of a flat-bed IEF system is that the sample can be applied at any desired position along the IEF gel. Various methods of sample application can be employed, but one disadvantage of flat-bed systems is that they will not tolerate such large sample volumes as rod-gel systems. Very

small samples can be applied directly to the gel surface as droplets, but for larger volumes alternative procedures must be used. Sample wells can be formed as indentations in the gel during polymerization using appropriately shaped pieces (e.g. Dymo tape) glued to one surface of the casting cassette [55]. However, this method can lead to distortion of protein bands due to disturbance of the electrical field during IEF. Alternatively, samples can be absorbed to applicators from such materials as filter paper, cellulose acetate, plastic sponge or dessicated polyacrylamide, but it should be noted that some proteins interact strongly with these substances and are not readily eluted during IEF. Better sample application devices are narrow silicon rubber (1 mm thick) strips (available commercially from Desaga) containing holes (for 10-μl samples) or slits (for 20-μl samples) which are simply placed directly on the surface of the gel. Gels can be covered with a plastic sheet to protect them from the effects of the atmosphere, dessication and urea crystallization during IEF [52]. In this case, slots (ca. 10×3 mm) can be punched in the cover sheet to facilitate sample application.

2.3.5. Focusing time

IEF is an equilibrium technique as when proteins reach the position in the pH gradient corresponding to their isoelectric point (p*I*) they carry no net charge and stop migrating. In most 2-D PAGE studies, little attention has been devoted to ascertaining whether equilibrium has been attained. Focusing times are normally reported in terms of a product of voltage and time (V h) [18,65]. However, V h can only be used meaningfully for inter-gel comparisons if the other parameters for the IEF gels are defined. In particular, gel length often varies and it is important to note that the V h product required to attain equilibrium varies with the square of inter-electrode gel length (l) [77]. We have, therefore, suggested [37] that V h l^{-2} is a better unit for describing IEF running conditions. Interestingly, using this parameter we have calculated [37] that IEF running conditions used in a series of published 2-D PAGE procedures varied from as few as 12.9 V h l^{-2} to as many as 80 V h l^{-2}.

It is possible to determine whether equilibration has been reached during IEF by testing whether separated components are coincident when pairs of samples are migrated from the anode and cathode or by constancy of the pattern over long focusing times [75,78]. In studies of human skin fibroblast proteins we found that as many as 87 V h l^{-2} were required for protein coincidence of samples migrated from the anode and cathode [42]. However, equilibration conditions must be ascertained for the particular type of sample under investigation. It should also be remembered that, as a consequence of viscosity effects, long focusing times are required for gels containing urea and the time required to achieve equilibration can be dependent on molecular mass so that problems can be experienced with samples containing components of high molecular mass. Although equilibration conditions during IEF are important for reproducibility and resolution of small charge differences between proteins, it must be stressed that the quality of final 2-D separations is more important than equilibrium per se. Thus, non-equilibrium IEF techniques of 2-D PAGE (see Section 2.3.8) can be used to advantage but the reproducibility of such gels is often difficult to control.

2.3.6. Estimation of pH gradients

The pH gradient obtained during the IEF dimension must be calibrated accurately if p*I* is to be used in characterisation of proteins on 2-D maps. It is often recommended that pH gradients be determined by slicing the IEF gel transversely into small sections and eluting in water or 8 *M* urea. The conductivity of this solution can be raised using 10 m*M* potassium chloride [39]. However, the presence of urea at a high, and often unknown, concentration renders pH measurement difficult as urea decreases the activity coefficient of H^+ ions [79]. The use of a correction factor has been suggested [79], but this is complicated as the magnitude of the urea effect is pH-dependent, so that the correction factor to be applied increases with increasing pH [80,81].

On flat-bed IEF gels, pH gradients can be estimated directly using a flat membrane electrode. This procedure is difficult, but not impossible [82], with rod IEF gels. Naturally, this method is also subject to urea effects and an additional complication is that the temperature of focusing and pH measurement should be the same.

The alternative stategy for calibrating pH gradients is to use a series of marker proteins. These proteins can be known components of the sample being analysed, thereby forming internal standards [36,83,84], or added constituents of known p*I* [85]. Most kits of p*I* markers available commercially are not suitable for use in 2-D PAGE as the p*I* values have only been established under native conditions. The set of acetylated cytochrome *c* markers from Calbiochem can be used with the additional advantage that the IEF run can be monitored visually as these markers are coloured. At present, the best method for calibrating pH gradients is undoubtedly the use of carbamylated charge standards [86–88] generated by heating a suitable protein in the presence of urea for varying lengths of time. The loss of a free amino group below pH 8.5 results in a unit change in charge, so that in final 2-D maps a horizontal row or train of spots of constant mass and spaced apart by 0.1 pH units is observed. The precise number of spots generated is dependent on protein amino acid composition. Commercial preparations of carbamylated proteins for use as charge standards in 2-D PAGE are now available commercially from Pharmacia under the tradename Carbamalyte.

2.3.7. Disadvantages of synthetic carrier ampholytes

Several problems are associated with the use of synthetic carrier ampholytes in the first-dimension IEF gels. There can be considerable batch-to-batch variability of ampholyte preparations as the procedures used for their synthesis result in complex, rather ill-defined mixtures whose composition is difficult to control reproducibly. This variability can result in irreproducibility of 2-D separations, thereby complicating the task of 2-D gel analysis. Blending of different preparations of synthetic carrier ampholytes can be used to engineer non-linear pH gradients [60]. However, this is an empirical process whose results can be unpredictable and difficult to control reproducibly. Another potential problem is that proteins can interact with synthetic carrier ampholytes [89], but there are very few reports of this phenomenon being observed in practice (reviewed in ref. 55).

The major disadvantage of synthetic carrier ampholytes for IEF in polyacryl-

amide gels is that the inherently high electroendosmosis in this system results in severe cathodic drift with concomitant loss of basic proteins. This problem reaches dramatic proportions in the rod-gel IEF system where pH gradients rarely extend above 8. The stability of pH gradients can be improved by the use of alternative electrolytes [68] and by treating the walls of the glass gel tubes with methylcellulose to mask their charge and so reduce electroendosmosis [90]. Using these strategies for rod-gel IEF in 2-D PAGE we have obtained pH gradients which, measured in the presence of urea, extended to 10.5, but the cathodic proteins were poorly resolved and formed streaks rather than discrete spots [60]. In contrast, using a flat-bed IEF system for 2-D PAGE, where pH gradients normally can extend to 10, we found that basic proteins were resolved as discrete spots [60]. The improved resolution of basic proteins may be attributable to the flexibility in sample application site in the flat-bed system where samples can be loaded away from the regions of extreme pH unavoidable in rod-gel IEF [37]. However, the flat-bed system ony ameliorates, but does not completely overcome, the problems associated with the use of synthetic carrier ampholytes and there is, therefore, a need for alternative approaches to the first dimension of 2-D PAGE.

2.3.8. Non-equilibrium pH gradient electrophoresis

One approach to the problem of resolution of basic proteins in 2-D PAGE was developed by O'Farrell et al. [91] and is known as non-equilibrium pH gradient electrophoresis (NEPHGE). In this procedure, samples are applied at the acidic end of rod gels which are electrophoresed for a relatively short time, typically 800–3000 V h (5–21 V h l^{-2}) [37]. Thus, proteins are separated on the basis of both charge and size in the presence of a rapidly forming pH gradient. Reproducibility of such transient state focusing is difficult to control and is sensitive to experimental conditions, carrier ampholytes, focusing time, gel length and sample composition [37]. Therefore, in comparative studies considerable care should be taken to ensure that samples are electrophoresed under identical conditions.

A major disadvantage of this approach is that two different types of first-dimension gel are necessary for the analysis of each sample. In contrast, the flat-bed IEF method we use as the first dimension of 2-D PAGE is able to resolve both acidic and basic proteins in a single equilibrium procedure. However it should be pointed out that non-equilibrium and equilibrium methods can resolve different types of charge mutation [92]. Neutral amino acid substitutions will not affect the equilibrium position of a protein, but may alter its mobility in a non-equilibrium system. The two procedures could, therefore, yield complementary information for some samples.

2.3.9. Buffer isoelectric focusing

Simple, even non-amphoteric buffers, can be used to generate pH gradients for IEF [93,94]. A mixture containing 47 components suitable for analytical IEF has been developed [74] and is available commercially from Polysciences under the tradename PolySep 47. This mixture can generate broad pH gradients with reasonably even conductivity and stability. The advantages claimed for this system [95] (defined chemical properties, increased reproducibility, relatively low cost,

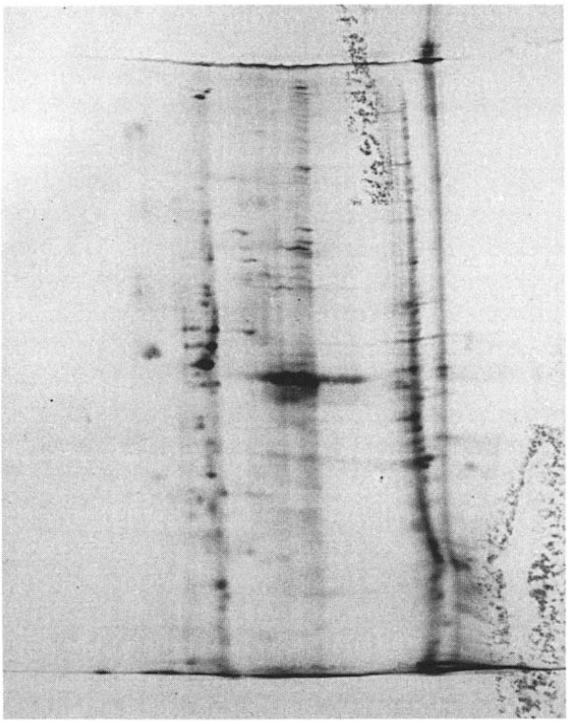

Fig. 4. Autoradiograph of a 2-D separation of [^{35}S]methionine-labelled human skin fibroblast proteins using buffer IEF in the first dimension.

ease of pH gradient engineering, minimal interactions with proteins) appear ideal for 2-D PAGE. This prompted us to test the suitability of buffer IEF using PolySep 47 for the first dimension in 2-D PAGE [53,96].

A typical separation of [^{35}S]methionine-labelled human skin fibroblast proteins using 11% (w/v) PolySep 47 in flat-bed IEF gels containing 8 *M* urea and 0.5% (w/v) CHAPS for the first dimension is shown in Fig. 4. Separations using this technique were markedly inferior to those obtained with conventional 2-D PAGE (cf. Fig. 3). Using the conventional procedure protein spots were distributed over the entire gel, whereas using buffer IEF the proteins were concentrated into four major zones in the IEF dimension.

1-D and diagonal IEF gel techniques were used to investigate the buffer IEF system in more detail [96]. It was concluded that in buffer IEF, proteins were confined to certain regions of the gels and appeared to be concentrated at interfaces. Mixtures of synthetic carrier ampholytes and low concentrations (2%) of PolySep 47 produced improved 2-D separations, with pH gradients extending into the extreme pH ranges [96]. However, buffer IEF alone appears to be a very unpromising approach to overcoming the problems associated with the use of synthetic carrier ampholytes for 2-D PAGE.

2.3.10. Immobilised pH gradients

The use of the Immobiline reagents (LKB) to generate immobilised pH gradients (IPGs) appears to be a more promising approach to an alternative first-

dimension procedure for 2-D PAGE. Only the basic essentials of this technology and those aspects relevant to 2-D PAGE can be discussed here. For a more detailed discussion of IPG technology the reader is referred to some recent reviews [55,97–99].

The Immobilines are a series of seven substituted acrylamide derivatives with different p*K* values. IPG IEF gels are made by generating a gradient of the appropriate Immobiline solutions, so that during polymerisation the buffering groups forming the pH gradient are covalently attached and immobilised via vinyl bonds to the polyacrylamide backbone. This immobilisation results in the elimination of pH gradient drift, but not electroendosmosis, making H gradients reproducible and infinitely stable. These properties are ideal for use in the first dimension of 2-D PAGE.

IPGs were originally optimised for the generation of narrow and ultra-narrow pH gradients. These are not generally suitable for 2-D applications as they will not resolve complex protein mixtures. The formation of extended pH gradients is complicated by the necessity of mixing several buffering species. Computer programs have been developed to overcome this problem [100,101], so that pH 4–10 gradients suited to 2-D PAGE can now be generated [102]. It has also proved possible to generate non-linear IPGs of any desired shape [103,104]. Thus, pH gradient engineering can be used to match the shape of the pH gradient to the distribution of protein p*I*s, thereby optimising resolution during IEF.

Immobiline gels have been cast in capillary tubes for 2-D PAGE [105], but it is simpler to use slab gels cast on suitable supports (e.g. GelBond PAG) in a flat-bed IEF system. Any apparatus capable of generating a controlled linear gradient can be used to form IPGs. The simplest option is a two-chamber gradient mixer [99,106] and an interesting modification of this apparatus has been published recently [107]. The most reproducible and flexible approach is to use a microcomputer-controlled stepmotor-driven high-precision burette system [108,109] which is available commercially from Desaga. An additional advantage of this apparatus is that it can be used to generate non-linear IPGs of any user-defined shape. For example, localised flattening of the pH gradient can be used to selectively improve resolution.

In the standard procedure [106] IPG gels are polymerised at 50°C for 1 h as under these conditions incorporation of all seven Immobiline species into IPG gels is optimal [110]. However, these conditions are not ideal for wide pH gradients as the p*K* 8.5 and p*K* 9.3 species are very unstable at elevated temperatures under alkaline conditions [111]. To overcome this problem, Pietta et al. [111] recommend control of the pH of the gel mixture by titration to pH 7.5–8. An alternative strategy advocated by Altland and Rossmann [112] is to titrate stock Immobilines to pH 6.8 with either 1 *M* Tris (acidic Immobilines) or 1 *M* phosphoric acid (basic Immobilines). The reagents are then diluted to 0.25 *M* with 0.1 *M* Tris phosphate, pH 6.8 and stored at −70°C. This procedure allows polymerisation of IPG gels in neutral buffer at ambient temperature.

IPG gels cannot be used immediately for IEF, but must first be washed to remove salts, catalysts and unpolymerised Immobilines. The gel swells during this process so that it must be reduced to its original weight prior to IEF [98]. This

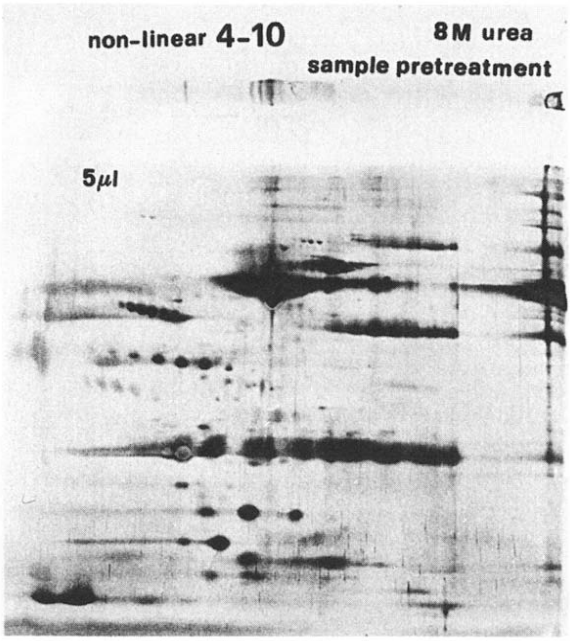

Fig. 5. Silver-stained separation of normal human serum proteins using non-linear pH 4 to 10 IPG IEF in the first dimension. Taken with permission from ref. 103.

process is time-consuming and difficult to control. It is better to use a rehydratable gel system [113,114] in which gels, after polymerisation, are washed, dried and stored for future use. IPG gels can be rehydrated in a large volume of the required solution (e.g. 8 *M* urea) under weight control [113,114]. Alternatively, a simpler, faster and more reproducible approach is to use volume to control the rehydration process [112,115]. In this method the dry gel is reassembled in a cassette of the same dimensions as that in which it was cast and reswollen in a controlled volume of the appropriate solution.

Initial applications of IPG technology to 2-D PAGE concentrated on samples of soluble proteins, particularly serum [103,116,117]. Many of the conditions used in this procedure have been examined in detail [118] and an example of a 2-D separation of serum using this technique is shown in Fig. 5. IPG gels have been used by Görg and co-workers [119–122] for 2-D separations of soluble protein extracts of legume seeds. In these studies narrow and ultra-narrow IPGs were used in combination with thin, horizontal gradient SDS-PAGE gels for the second dimension. Successful 2-D maps of myosin light chains have also been obtained using IPGs showing additional isoforms to those found by conventional 2-D PAGE [123].

In the studies described above, the IPG gels contained urea. However, many samples for 2-D analysis contain multimeric and/or insoluble proteins which require the presence of both urea and non-ionic or zwitterionic detergent to obtain satisfactory 2-D separations [37]. Unfortunately, polyacrylamide gels reswell very inefficiently in the presence of both these reagents [114]. We have found

using a cassette system that gels never reswell fully in the presence of both 8 *M* urea and 0.5% Triton X-100 or CHAPS [53,124]. To overcome this problem we recommend including 0.5% detergent in the IPG gels when they are cast and in all subsequent washing solutions used prior to drying [125].

Although narrow IPGs function well, it is clear that problems are associated with the use of wide pH gradients. These problems include: (i) slow entry of sample proteins into the gel, (ii) lateral band spreading, (iii) prolonged focusing times and (iv) increased electroendosmosis. These problems can be attributed to the inherently low conductivity of the Immobiline system. Improved separations can be achieved by the addition of low concentrations, typically 0.5% (w/v), of synthetic carrier ampholytes of the appropriate range to the solution used for gel rehydration [112,126,127]. This technique is called hybrid isoelectric focusing [112] or mixed carrier ampholyte–IPG IEF [128]. This approach has proved beneficial in 2-D separations of human skin fibroblast proteins [124,125,129], yeast proteins [130] and bacterial membrane proteins [128].

A particular problem that has been encountered using IPG IEF gels for 2-D PAGE is vertical streaking on the 2-D maps [53,120,122,125,129]. This phenomenon is due to the presence of fixed charges on the Immobiline matrix, resulting in increased electroendosmosis in the region of contact between the first-dimension IPG gel and the second-dimension SDS-PAGE gel [121,131]. Consequently, the migration of proteins from the first to the second dimension is disturbed resulting in streaking. IPG strips must be equilibrated for extended periods (30 min) before application to the second dimension. An increase in the pH of the equilibration buffer from the canonical pH 6.8 to pH 8.6 decreases, but does not abolish, vertical streaking [118,125,129]. Görg et al. [130] have devised a strategy aimed at reducing electroendosmotic effects. Glycerol was included at 15% (w/v) in the rehydration solution for the IPG gels and the equilibration buffer contained 6 *M* urea and 30% (w/v) glycerol. This technique resulted in good 2-D maps of yeast [130] and pea [132] (Fig. 6) proteins.

The approach of Righetti and co-workers [133,134] to the problem of streaking has been to use an equilibration protocol based on that originally devised by Jäckle [135] and used by Görg et al. [72] for 2-D PAGE. In this procedure the first-dimension IPG gel strip is fixed for 1 h in 12% (v/v) acetic acid, 50% (v/v) methanol and then equilibrated in Tris buffer pH 8.8 prior to application to the SDS-PAGE dimension. This technique appears to work satisfactorily, but it seems highly likely that some proteins may remain insolubilised within the IPG strip after fixation, resulting in their loss from the final 2-D maps.

IPG technology is of great potential value for 2-D PAGE, particularly with regard to the increased reproducibility that can be achieved [136]. Acceptable 2-D maps of complex protein mixtures are now being produced. However, the problems associated with elution and transfer between the IPG IEF and the SDS-PAGE dimensions must be effectively solved before IPG technology is likely to be established as the standard first-dimension procedure for 2-D PAGE.

2.4. Equilibration between dimensions

First-dimension IEF gels are usually equilibrated in Tris buffer, pH 6.8, containing SDS under reducing conditions before application to second-dimension

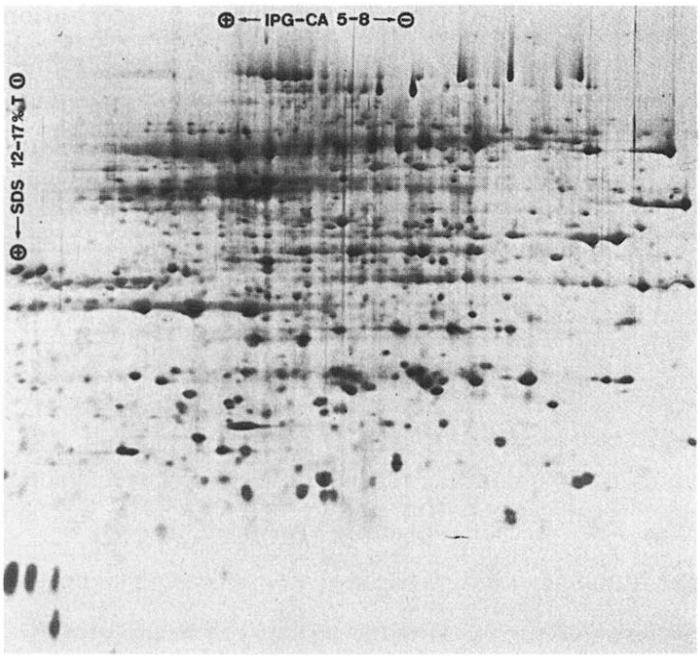

Fig. 6. Silver-stained 2-D separation of pea (*Pisum sativum*, Wyola) proteins using linear pH 5 to 8 IPG IEF in the first dimension. Taken with permission from ref. 132.

SDS-PAGE gels. The main function of this step is to allow proteins to interact fully with SDS so that they migrate properly in the SDS dimension. Thus, equilibration time can be an important parameter. Rod IEF gels are normally equilibrated for 30–40 min [18], while thin-layer slab IEF gels require only 5–10 min [60] and ultrathin IEF gels only 1–2 min [71]. The two latter procedures are advantageous in that band broadening due to protein diffusion is minimised. Indeed, O'Farrell [18] found that in 30 min band width increased by 40% and, perhaps more importantly, considerable loss of protein (5–50%) from the gels can occur [18,36,60,68]. This has prompted some investigators to omit the equilibration step [41,137], but this can result in streaking of high-molecular-mass proteins [18]. Particular problems of streaking have been encountered using IPG IEF gels, necessitating extended (30 min) equilibration times and the use of special conditions (see Section 2.3.10).

2.5. Transfer between dimensions

After equilibration, first-dimension IEF gels must be applied to the second-dimension SDS gels. Good contact at the interface between the two gels is essential if good 2-D separations are to be obtained. Using rod IEF gels, it is usual to cement the first-dimension gel in place with buffered agarose [18]. However, agarose preparations can contain impurities which result in the appearance of artefacts when sensitive detection methods (e.g. silver staining) are used [138]. IEF gels can be fixed in place using rapidly polymerising polyacrylamide [41,

139], but caution should be exercised as the heat generated during polymerisation can denature the proteins. As a final alternative, Garrels [35] applied IEF gels directly to the top of SDS slab gels without using any cementing agent as he found that during electrophoresis the IEF gels became bonded to the surface of the SDS gels. Whatever method is used, care should be taken to avoid stretching of IEF gels during application to the second dimension as this can cause band skewing and distortion of 2-D maps, thereby complicating the task of pattern matching.

When a flat-bed IEF system is used there is little risk of inducing stretching artefacts as the gels are firmly bonded to a flexible plastic support. If the second-dimension gels are to be run in a vertical system, the space at the top of the SDS gels should be filled with equilibration buffer and the IEF strips simply slid into place. Good contact between the two gels is essential and entrapment of air bubbles should be avoided. The strips can be fixed in place with buffered agarose, but this step is not essential [60]. If horizontal SDS slab gels are to be used in the second dimension, the sample strip should be placed, with the gel layer facing downwards, into a preformed trench in the SDS gel [71,72]. The latter method does not work well for IPG IEF gels, and Görg et al. [121] have found it preferable to use a simple laying-on technique avoiding the use of an application trench.

2.6. The second dimension

2.6.1. Gel preparation

Optimal resolution of proteins in 2-D PAGE depends upon the components of a complex protein mixture being distributed evenly across both dimensions [18]. Unfortunately, a survey of molecular weight and p*I* values for a series of proteins shows that they tend to be clustered around a mean value [140]. Thus, for most complex protein mixtures, there is no single polyacrylamide concentration for SDS-PAGE which will produce a uniform distribution of spots in 2-D PAGE.

One approach to this problem, which was used in the original O'Farrell [18] procedure, is to use gradient polyacrylamide gels for the SDS-PAGE dimension. Such gels have the capacity to resolve proteins with a wide range of molecular weights. It should be stressed that the optimum gradient shape for a particular protein mixture under investigation must be established if resolution of that sample is to be maximised. It is often advantageous to flatten the mid-region of the polyacrylamide gradient to maximise resolution in the region of the gradient where the majority of proteins are located. Where fewer proteins exist, usually in the regions of extreme high and low molecular weight, steeper gradients can be employed [42]. The shape of gradient to be used for a particular protein mixture has usually been determined empirically. However, it is possible by statistical analysis of protein distibution and using weighting factors to generate an optimal gradient shape for IEF or SDS-PAGE [1-3].

An alternative approach to using gradient polyacrylamide SDS-PAGE, advocated by Garrels [35], is to use for each sample a series of SDS gels of different polyacrylamide concentrations. This results in a set of 2-D maps, each one resolving different regions of the molecular-weight spectrum. A series of five different gel concentrations is usually recommended (7.5, 9, 12, 15 and 18%) [36] and

this approach is often used in conjunction with a series of first-dimension gels of different pH ranges. This approach is simple, reproducible and produces good results, but the major disadvantage is that a series of 2-D patterns must be concatenated to produce a single 2-D map representative of the total components in that sample. This problem can be readily overcome using an appropriate computer analysis system [141] (see Section 2.10), but is difficult to achieve visually.

The discontinuous buffer system of Laemmli [142] is almost universally used for the SDS-PAGE dimension in 2-D PAGE. Gels of a single polyacrylamide concentration can be readily prepared using simple, standard procedures but it is advantageous to cast gels in batches, rather than singly, as this improves the reproducibility of 2-D maps. The preparation of gradient polyacrylamide SDS gels is more complicated due to the requirement to generate a gradient of the desired shape. The simplest approach is to use a two-chamber gradient mixer of fixed shape [72,143–145]. Such devices are easy to use, commercially available and produce reproducible gradients, but they are not readily adaptable to generating gradients of different shapes. A simple two-chamber device which can generate user-defined gradients has been described by the Anderson group [146,147]. A greater degree of control and reproducibility can be achieved using an electronic gradient former such as the LKB Ultrograd [148]. We routinely use the latter device to cast batches of polyacrylamide gradient SDS gels for 2-D PAGE [29,60]. However, this electromechanical device is rather expensive and somewhat limited in versatility. Microcomputer-controlled systems, such as that devised by Altland and Altland [108], are more versatile and less expensive. In this system a series of stepmotor-driven burettes, controlled by a microcomputer, is used to generate the required polyacrylamide gradient. A commercial version of this apparatus, using less expensive proportional pumps rather than stepmotor-driven burettes, is available from Desaga.

2.6.2. Stacking

A large-pore stacking gel cast on top of the constant percentage or gradient polyacrylamide gel is normally used with the discontinuous buffer system for SDS-PAGE [142]. The advantage of this procedure is that proteins from the IEF gel are concentrated into narrow starting zones. This step is used in most 2-D PAGE procedures, but Garrels [35] omitted it on the basis that protein zones within the IEF gels are already concentrated and the non-restrictive IEF gel itself acts to concentrate proteins. It should be noted that the nature of stacking is somewhat modified in the presence of SDS and it is not strictly necessary to have a discontinuity in pH as unstacking will occur by the change in gel concentration alone [149].

2.6.3. Gel size

The resolution capacity of SDS-PAGE can be inceased by a simple increase in the length of the separating gel [150] and an alteration in this parameter alone can substantially improve resolution by 2-D PAGE [151]. Resolution of the IEF dimension can also be improved by increasing gel length or by an increase in voltage gradient [62]. The latter alternative can only be adopted if adequate

cooling of the IEF system is available to compensate for the increased Joule heating. This can be achieved relatively easily with a flat-bed apparatus fitted with a Peltier cooling device [62] used in conjunction with ultrathin IEF gels. For example, voltage gradients of up to 700 V cm^{-1} have been used in conjunction with 125 μm thick IEF gels [62]. In contrast, the cooling facilities available with most rod-gel IEF systems are rudimentary or non-existent, thereby limiting field stengths to about 50 V cm^{-1}

Young and co-workers [77,152] have pioneered the use of large gel areas for 2-D PAGE in a technique using 32-cm IEF gels and 41-cm SDS-PAGE gels. This technique of "Giant Gels" gives a six-fold increase in the gel area available for separation. The increase in resolution capacity of this 2-D system can be illustrated by studies of rat thymus cells [153] and human Hep cells [154] where, respectively, 5000 and 10 000 protein species were detected. Additional advantages of this system are that there is a 100 fold increase in sample loading capacity and autoradiographic spreading is reduced [152,153].

At the other extreme, other investigators have developed microscale systems for 2-D PAGE [139] in some cases producing 2-D gels as small as postage stamps [155]. Such microscale systems undoubtedly have advantages of reduced run times, brief visualization procedures and low cost, but the potential resolution capacity of such systems must be degraded by the reduced distances between spots.

2.6.4. Molecular mass standards

For comparison between 2-D gel patterns it is essential to have a series of molecular mass markers to calibrate the SDS-PAGE dimension. These markers are usually mixtures of pure proteins of known molecular mass which are electrophoresed along one edge of the SDS gels. Several kits of such marker proteins, both radioactively labelled and non-radioactive, are available commercially. Giometti et al. [156] recommend the use of rat whole heart homogenate which produces a complex series of 80 bands, ranging from 300 to 10 000 molecular mass, which can be used to calibrate the gels. Using the foregoing procedures, separate markers must be used to calibrate the first-dimension IEF gels (see Section 2.3.6.). In an alternative approach, bacteriophage T4 coat proteins [157] and fluorescent, dansylated proteins [158] have been used as markers for both p*I* and molecular mass in 2-D PAGE.

2.7. Fixation

After 2-D PAGE gels are usually fixed before detection of the separated proteins. This step serves both to immobilise the proteins as insoluble precipitates within the gels and to wash out non-protein components which can interfere with subsequent staining [159]. Many published 2-D procedures recommend a methanol–acetic acid–water mixture for fixation, but some low-molecular-mass proteins are not adequately fixed using this method especially in the presence of detergents. The best general fixative is TCA which is most efficient at 20% (w/v). Sulphosalicylic acid (10–20%, w/v) or mixtures of TCA and sulphosalicylic acid

(10%, w/v, of each) can be used [169] but these methods are often not as efficient as TCA alone [159].

2.8. Detection methods

Only a brief review of the most important staining and detection methods can be given here, and for a more detailed account the reader should consult ref. 161.

2.8.1. Coomassie Brilliant Blue

Coomassie Brilliant Blue (CB) R-250 is the most popular general protein stain for 2-D gels. Staining is usually carried out with 0.2% (w/v) CB R-250 in methanol–acetic acid (45:10, v/v) followed by removal of excess dye with the same acid–alcohol solution. An alternative strategy [162] which was used in the original O'Farrell [18] 2-D procedure is to use CB dissolved in TCA in which it has low solubility. The dimethylated form of the dye, CB G-250, can be used to advantage here as its diminished solubility permits its use as a colloidal dispersion. The colloidally dispersed dye does not penetrate the gel, permitting rapid staining of proteins without the development of background staining [163].

The standard CB staining procedures are relatively insensitive, being capable of detecting about 0.5 μg protein per cm^2 of gel [161]. Neuhoff et al. [164] have recently reinvestigated CB staining procedures. In a list of over 600 modifications, they developed an optimal staining procedure using CB G-250 in perchloric acid in the presence of ammonium sulphate which is claimed to be able to detect as little as 0.7 ng protein per mm^2 of gel.

2.8.2. Fluorescent methods

Fluorescent stains for proteins are very sensitive but have not proved popular due to the requirement for ultraviolet illumination for visualisation. These stains can be used post-electrophoretically, e.g. 1-anilinonaphthalene-8-sulphonate [165,166], or pre-electrophoretically by reacting the proteins with the fluorescent component prior to electrophoresis. Reagents which have been used in the latter approach are listed in ref. 167. The compound 2-methoxy-2,4-diphenyl-3(2H)-furanone is of particular interest as it can detect as little as 1 ng of protein and has a linear response over the range 1–500 ng [168]. However, it must be remembered that such pre-electrophoretic staining procedures can cause protein charge modifications resulting in anomalous 2-D separation patterns.

2.8.3. Silver staining

The ability of silver to develop images was discovered in the mid-17th century. This property was first exploited in photography, followed closely by its use in histological procedures. Recently, silver staining methods have been developed to detect proteins separated by PAGE. This was achieved firstly by employing procedures based on histological techniques [169,170], then by photochemical methods [21,171] and finally by photodevelopment procedures [172,173]. Numerous silver staining procedures have been developed in the seven years since the introduction of this methodology for the detection of proteins separated by

PAGE [34,161,174,175]. These various techniques are claimed to be between 20 and 200 times more sensitive than CB R-250, i.e. about 0.05–0.10 ng/mm^2.

The precise mechanism leading to the formation of a visible silver–protein complex has not been fully clarified [34,161], but all staining methods involve the reduction of ionic silver to its metallic form. It has been proposed that silver cations complex with protein amino groups in an alkaline environment and with cysteine and methionine sulphur residues [176]. However, more recently Gersten et al. [177] have implicated 3-dimensional (3-D) protein structure and, therefore, the steric presentation of reactive moieties in 3-D space as being of most consequence and proposed that other factors such as amino acid composition are of secondary importance.

Certain problems specific to 2-D PAGE have been encountered in silver staining. The use of 2-mercaptoethanol in equilibration buffers can result in the appearance of small, vertical streaks [19,178]. Horizontal bands across the entire 2-D gel in the 50 000–68 000 molecular weight region have been attributed to 2-mercaptoethanol [179,180]. Agarose used for cementing first-dimension IEF gels to second-dimension SDS-PAGE gels can be contaminated with peptides and so cause increased background staining [138]. Silver staining can severely quench the detection of radioactivity both by autoradiographic and fluorographic procedures and by scintillation counting [181,182]. This effect is particularly serious for the detection of ^{3}H. In addition caution should be exercised in the interpretation of protein staining patterns as certain proteins, e.g. calmodulin [183], fail to respond to single-step silver staining.

2.8.4. Radioactive detection methods

2.8.4.1. Radiolabelling methods. Proteins can be radiolabelled (i) synthetically by incorporation of single amino acids (usually methionine or leucine) or amino acid mixtures, (ii) post-synthetically (e.g. methylation with [^{3}H]borohydride [184]) or (iii) with a radioactive stain (e.g. [^{59}Fe]ferrous bathophenanthroline [185]). The most commonly used isotopes used in conjunction with gel electrophoresis are ^{3}H, ^{14}C, ^{35}S, ^{32}P and ^{125}I. In addition, techniques exist for labelling specific groups of proteins, e.g. $^{32}PO_4$ for phosphorproteins and [^{3}H]glucosamine for glycoproteins. Synthetic incorporation procedures should not introduce artefacts but synthetic procedures such as iodination can result in charge modification [33]. However, reductive methylation with [^{3}H]borohydride has been found not to affect protein migration [33]. Recently a method has been described for the use of a sulphur-labelling agent (SLR, Amersham), a [^{35}S]methionine-derived reagent for radiolabelling proteins prior to 2-D PAGE, but this method can result in some charge heterogeneity [186]. Background staining can be a problem with radiolabelling procedures, for example as a result of iodination of contaminants in acrylamide [187] or arising from neutron-activated components [188].

2.8.4.2. Autoradiography. Radiolabelled proteins separated by 2-D PAGE are most frequently detected by autoradiography. Usually gels are dried, then placed in direct contact with a suitable X-ray film and exposed for the appropriate time. Sequential exposures for different times are often necessary due to the wide range

of protein abundances in most complex protein samples. Wet gels can be used for autoradiography but the film must be protected by interposing a barrier (e.g. by wrapping the gel in Saranwrap plastic film). Direct autoradiography is relatively efficient for isotopes such as ^{14}C, ^{35}S, ^{125}I or ^{32}P but ^{3}H is very inefficiently detected as its low-energy β-emissions cannot penetrate the gel matrix.

2.8.4.3. Fluorography. The inefficiency of detection of weak β-emitters (e.g. ^{3}H) by autoradiography prompted the development of a more sensitive method of scintillation autoradiography termed fluorography [189]. In this method the fixed wet gel is impregnated with a scintillant, usually 2,5-diphenyloxazole (PPO), so that low-energy β-emissions excite the fluor molecules to emit photons which can form an image on blue-sensitive X-ray film. Dimethylsulphoxide (DMSO) is the normal solvent used for impregnation of gels with PPO [189], but other solvents (e.g. glacial acetic acid [190]) or water-soluble fluors (e.g. sodium salicylate [191]) can be employed. The use of a low temperature during the fluorographic exposure increases sensitivity, a twelve-fold increase for ^{3}H and a nine-fold increase for ^{14}C and ^{35}S being obtained at $-70\,^{\circ}C$. Pre-exposure of the X-ray film to a brief flash of light (ca. 1 ms) greatly increases the sensitivity of the fluorographic process two- to three-fold and results in a greater range in linearity of the film response [192,193]. Unfortunately fluorographic procedures cause spreading of spots compared with conventional autoradiography resulting in a decrease in effective resolution [193,194].

2.8.4.4. Dual-isotope techniques. The comparison of protein patterns produced by 2-D PAGE is simplified using a dual-labelling technique in which the two protein samples to be compared are labelled with different isotopes (e.g. ^{3}H and $^{14}C/^{35}S$) and co-electrophoresed on the same 2-D gel. A subsequent fluorographic step will detect proteins labelled with ^{3}H, ^{14}C or ^{35}S while a separate autoradiographic procedure will only detect stronger β-emissions (^{14}C or ^{35}S). The patterns from the two samples are then interpreted subtractively [194–196]. An alternative procedure uses a combination of β-(^{35}S) and γ-(^{75}Se) emitting isotopes incorporated in methionine analogues [197]. A fluorographic step is used to detect both isotopes, while a second autoradiographic exposure with an exposed X-ray film interposed between the gel and the film is used to detect only the γ-emitter (^{75}Se).

2.8.4.5. Non-autoradiographic methods. Radiolabelled proteins, located on a 2-D gel by staining or autoradiography, can be excised and counted by liquid scintillation spectrometry. However, this procedure is too laborious for routine use.

A multi-wire proportional counter has been used to quantitate β-emissions from 2-D gels [198] but although this method is very rapid and sensitive, resolution is limited to 2 mm. Other techniques for rapid electronic recording of β-emissions from 2-D gels have been described [199–201], but these procedures require further development to increase their resolution and their ability to differentiate between different isotopes.

2.9. Identification and characterization of proteins

2-D PAGE is an excellent procedure for separating complex protein mixtures, the components of which can be quantitated and characterized in terms of p*I* and

molecular mass. However, the technique itself does not yield directly any further information on the physicochemical nature or functional properties of the separated proteins. Thus additional techniques providing a third dimension are required for such characterization.

2.9.1. Cell subfractionation

Information can be derived from 2-D maps of total cell or tissue proteins by identifying groups of proteins associated with particular cellular compartments, e.g. nuclei, mitochondria, microsomes, plasma membranes, cytoplasm and cytoskeleton.

2.9.2. Co-electrophoresis with standard proteins

There is an extensive panel of proteins which have been purified and characterized. These can be used in co-electrophoresis experiments to identify particular components in complex protein mixtures. An example of this is for total *Escherichia coli* proteins where the identities of 136 components have been established [202,203].

2.9.3. Specific staining and affinity

Specific affinities of proteins such as dyes, cofactors, vitamins, neurotransmitters, hormones and specific inhibitors can be used to identify proteins on 2-D gels [204]. Staining methods have also been developed for the detection of specific classes of polypeptides [167].

2.9.4. Immunological procedures: "Western blotting"

The unique specificity combined with the high affinity of antibodies raised against protein antigens make these molecules sensitive reagents for protein analysis. Such antibodies can be used to immunoprecipitate particular proteins which can then be analysed by 2-D PAGE. Alternatively a protein mixture can be depleted of a particular component(s) by immunodeletion [62,205].

Antibodies can be used to precipitate proteins directly in 2-D gels (immunofixation) but the application of this procedure is limited in polyacrylamide gels due to the slow diffusion of immunoglobulin molecules in this matrix. This problem has been overcome by transferring (blotting) the separated proteins from the gels onto the surface of a thin substrate such as nitrocellulose membranes, where they can readily interact with antibodies. This technique, based on DNA blotting techniques originated by Southern [206], is popularly known as Western blotting [207]. Protein transfer can be achieved by capillary [208], contact-diffusion [207] or vacuum [209] techniques, but more rapid and efficient transfer is achieved if the proteins are electrophoretically removed from the gel to the support by application of an electric field perpendicular to the plane of the 2-D gel. This technique based on the method of Towbin et al. [210] is known as electroblotting and is reviewed in refs. 211–215.

Various types of filters are suitable for Western blotting but nitrocellulose is generally the most popular substrate as it does not require derivatisation before use and is compatible with general protein stains such as Amido Black. Sensitive

staining of protein patterns transferred to nitrocellulose can be achieved with Indian ink [216] and colloidal gold or silver particles [217,218], particularly after alkali pretreatment [219]. Positively charged nylon membranes have a greater binding capacity than nitrocellulose and can be used successfully for immunostaining but until recently there was no satisfactory general protein staining procedure. However, sensitive staining is now possible on nylon membranes either (i) using a cationic cacodylate iron colloid followed by treatment with potassium ferrocyanide [220] or (ii) by in situ biotinylation of the blotted proteins followed by reaction with enzyme-conjugated avidin and an appropriate substrate.

After proteins have been transferred to nitrocellulose all the unoccupied potential binding sites on the blot must be blocked before probing with a specific ligand. Serum albumin [210] or gelatin [221] is often used for this purpose, but these proteins are not always non-reactive during subsequent probing, particularly using lectins due to the presence of glycoproteins in the blocking reagent. Alternatively, a dilute solution of the non-ionic detergent polyethylene sorbitan monolaurate (Tween 20) can be used [222] but certain proteins can be displaced from nitrocellulose by such detergent treatment.

After blocking, the blot can be reacted with the specific antibody or ligand. The primary antibody can be labelled for direct visualization, but indirect sandwich techniques using a second antibody are more sensitive. The second antibody can be fluorescently labelled (e.g. fluorescein isothiocyanate), radiolabelled (usually with ^{125}I) or conjugated to an enzyme (e.g. horseradish peroxidase, alkaline phosphatase, β-galactosidase or glucose oxidase). Fluorescent methods require ultraviolet (UV) illumination and radiolabel procedures require a delaying autoradiographic step, so that the methods dependent on enzyme activity are becoming increasingly popular. *Staphylococcus aureus* protein A which binds specifically to the Fc regions of immunoglobulin G (IgG) can be used in some cases in place of the secondary antibody.

Recently even more sensitive techniques have been developed for visualization. One approach is the use of gold-labelled secondary antibodies which have the added advantages that they are visible (red colour) without further development [223,224] and sensitivity can be increased by silver enhancement. The other approach exploits the specificity of the interaction between the small-molecular-weight vitamin biotin and the protein avidin. Many biomolecules (antibodies, lectins) can be readily conjugated with biotin and used as the second reagent in blot probing. The blots are then visualized in a third step using avidin conjugated with a suitable enzyme. Even greater sensitivity can be achieved using preformed complexes of a biotinylated enzyme with avidin as many enzyme molecules are present in such complexes.

2.9.5. Amino acid analysis, peptide mapping and protein sequencing

Amino acid composition data can be obtained directly on 2-D gels if parallel cultures of the same cell line are radiolabelled with a different amino acid. Latter et al. [225] used twenty different amino acids and from quantitation of the autoradiographs of the 2-D gels from each of the twenty cultures were able to deter-

mine the amino acid composition of some proteins. However, in this technique it must be assumed that labelling is uniform and interconversion between amino acids is minimal. The other approach is to use a microscale chemical amino acid analysis method on protein spots punched out from 2-D gels [226]. Peptide mapping can also be carried out on spots cut out of 2-D gels [227]. The procedures that can be used are either 1-D peptide mapping based on the method of Cleveland et al. [228], modified to improve resolution [229] or a 2-D fingerprinting procedure [187,230].

The original automated Edman degradation method of protein sequencing [231] required several mg of protein. However sensitivity in the subnanomole range is required for the direct sequencing of proteins isolated from 2-D gels. The recently developed gas-phase sequenator can produce useful sequence information from as little as 10–20 pmol of protein (0.5–1 μg for a 50 000 molecular weight) [232]. Aebersold et al. [233,234] have recently devised a procedure for the isolation of proteins from SDS-PAGE [233], IPG IEF [234] and 2-D PAGE [233] gels. In this method Whatman GF/C or GF/F glass fibre filter paper is activated by etching with trifluoroacetic acid and then used for electrophoretic transfer (electroblotting) of proteins from gels. The proteins are immobilised on the glass fibre sheets by ionic interactions or by covalent attachment. This technique can be applied to a variety of proteins with no apparent restriction due to size, charge and solubility properties of the proteins. The transferred proteins are then detected with CB or fluorescent staining with 3,3′-dipentyloxacarbocyanine iodide. After detection, the protein bands or spots are cut out and inserted directly into the gas-phase sequenator, with the piece of glass fibre acting as a support for the protein during sequencing. Protein in the range 50–150 pmol can be sequenced and extended runs obtained due to improved stepwise yields and reduced backgrounds. This methodology seems now to have matured to a state where it can be applied as a third dimension to 2-D PAGE.

2.10. Quantitative analysis of 2-D patterns

The object of experiments using 2-D PAGE is often to resolve complex protein mixtures. This results in complicated 2-D patterns from which only a limited amount of information can be extracted by visual inspection. Thus there is a need for automated computer analysis systems to extract qualitative and quantitative information from individual gels to provide for pattern matching between gels and to construct databases for different types of samples. An in depth review of this topic is outside the scope of this chapter so that only a few brief comments will be made here and for further details the interested reader is referred to refs. 34, 141 and 235–243.

The essential steps in the analysis of 2-D gel patterns have been described by Dunn and Burghes [34] and include data acquisition, calibration, filtering or noise reduction, spot detection, spot quantitation, pattern matching and database construction. Most systems have used scanning densitometers or vidicon cameras for digitisation of raw 2-D gel images but increasing use is now being made of laser densitometers [244] and charge coupled devices (CCDs, essentially solid

state vidicon cameras made in the form of arrays of phototransistors). Most of the computer systems which have been designed for 2-D analysis have used large and expensive minicomputer systems and even custom-built hardware. However, recent advances in microprocessor technology combined with the availability of cheap memory and mass storage devices has now made it a practical possibility to perform 2-D gel analysis on the present generation of microcomputers. For example, the analysis software developed by Garrels et al. [141] has been implemented on a Masscomp microcomputer system and is available commercially as PDQUEST (Protein Databases) [245]. An additional advantage of this system and the ELSIE III software package of Miller et al. [242] is that they are written in the C programming language and operate under the UNIX (AT&T) operating system. They are, therefore, at least in theory, largely machine-independent and should be relatively readily portable to a variety of mini- and micro-computer systems using the UNIX operating system. This problem of software portablity has also recently been addressed using the RATFOR programming language to develop 2-D gel analysis as part of a general image processing system (known as GIPSY) [246].

The first generation of analysis systems for 2-D gels has used traditional serial or pipeline computers capable only of performing a single operation at a time. Recent developments in massively parallel computers have provided a computer architecture which should significantly increase the speed and efficiency of 2-D gel analysis and provide a novel framework within which to develop second-generation 2-D gel analysis algorithms [247,248]. Possible new strategies could include the application of Markov random field probabalistic theory using simulated annealing and iterated conditional node techniques [248] and artificial intelligence systems [247,249,250].

3. BIOMEDICAL APPLICATIONS

The majority of human diseases, both inherited and acquired, result in alterations of the biochemical composition of cells and/or body fluids. The detection of such changes requires the availability of suitable techniques for multicomponent analysis [251]. Techniques such as gas chromatography and mass spectrometry are suitable for low-molecular-weight metabolites but the development of 2-D PAGE procedures has allowed this approach to be extended to the multicomponent analysis of proteins. This technique can be used both as a tool in investigations into the causes of disease and also as a clinical test for the disease state.

3.1. Studies of body fluids

Serum and plasma samples are generally suitable for direct analysis by 2-D PAGE. In contrast most other body fluids have a low total protein concentration and contain high levels of salts which can interfere with the IEF dimension of 2-D PAGE. The latter group of samples must be specially processed prior to 2-D separation (see Section 2.1.1).

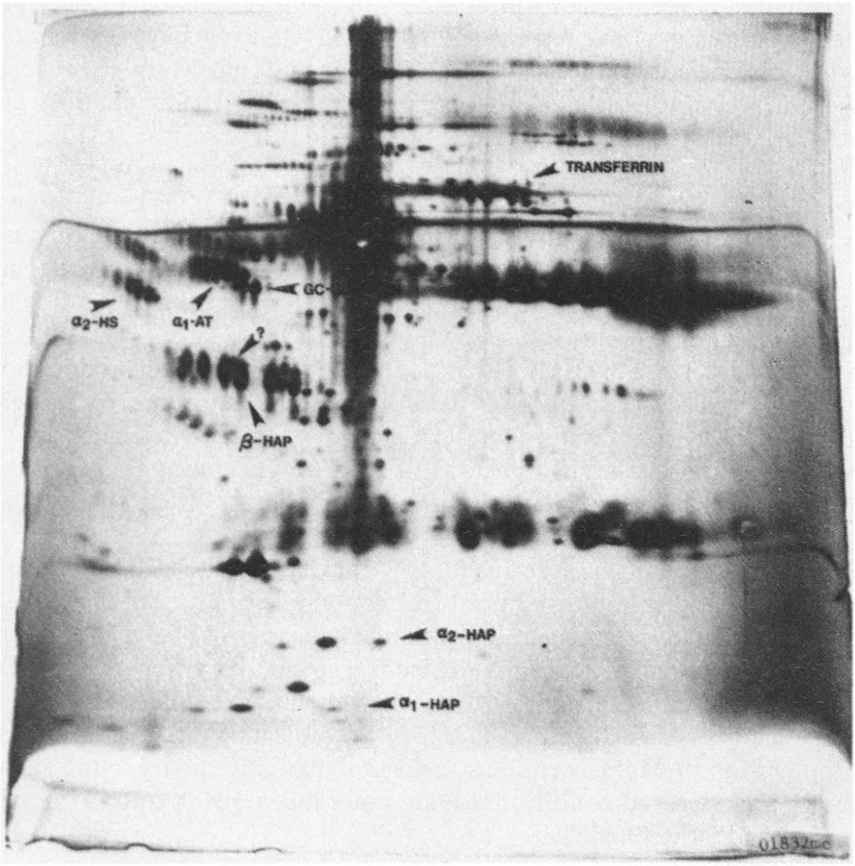

Fig. 7. Silver-stained separation of normal human serum proteins. Taken with permission from ref. 19.

3.1.1. Serum and plasma

The proteins of normal human plasma and serum have been extensively characterised by 2-D PAGE [19,25,68,252,253]. A typical 2-D gel electrophoresis map of normal human serum is shown in Fig. 7. Many of the spots on 2-D maps of serum have been identified and the sort of reference map which can be produced is shown in Fig. 8. It is clear that many serum proteins are represented on these 2-D maps as charge trains which is due to glycosylation. A problem in 2-D analysis of serum proteins is the abundance of particular proteins such as albumin and immunoglobulins which can obscure some of the minor components. One approach to this problem is a deletion technique where affinity chromatography on Affi-Gel Blue and Sepharose-bound protein A can be used to remove albumin and immunoglobulins, respectively [19]. However, it must be remembered that these techniques are not strictly specific and it is quite probable that other components are deleted from the 2-D pattern.

Serum protein patterns have been screened by 2-D PAGE in a variety of clinical syndromes. For example, changes have been observed in 2-D maps of serum pro-

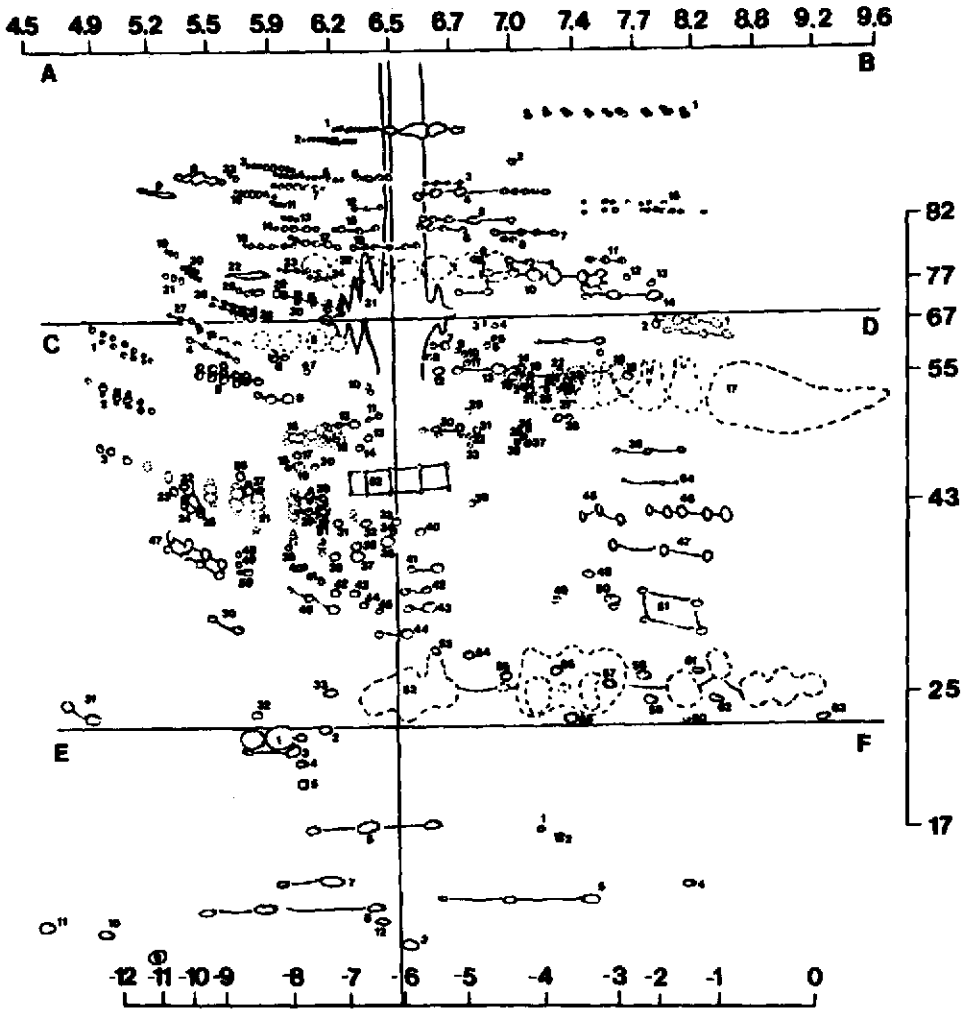

Fig. 8. Reference 2-D map of human serum proteins. Taken with permission from ref. 25.

teins in muscle trauma [25], in myocardial infarction [254], in cases of alcohol abuse [255,256], in patients with fish-eye disease [257] and after chemical exposure [258,259]. The best characterised set of changes in 2-D maps of plasma proteins is in the case of multiple myeloma and other monoclonal gammopathies [251,260,261] where the immunoglobulin heavy and light chains are highly restricted in terms of map location.

3.1.2. Urine proteins

The 2-D analysis of urine proteins is complicated by the low protein concentration (ca. 100 mg/l) necessitating extensive sample concentration (500- to 1000-fold) coupled with procedures for removing salts and other non-protein components. Methods which have been devised to overcome this problem include (i) dialysis and gel permeation chromatography [22,24], (ii) gel exclusion chro-

matography and centrifugation [22], (iii) dialysis against polyethylene glycol [262] and (iv) acidified acetone extraction and gel exclusion chromatography [263]. In contrast, Marshall et al. [26] have been able to obtain successful 2-D maps of unconcentrated samples of urine. These procedures have been used to produce detailed reference 2-D maps of urine proteins and to detect abnormal patterns associated with prostatic adenocarcinoma [264], multiple myeloma [24], muscle disease and damage [265,266], occupational exposure to cadmium [26], rheumatoid arthritis [262] and renal disease [267].

3.1.3. Other body fluids

A variety of other body fluids such as CSF [173,268–270], semen [20], prostatic fluid [271,272], amniotic fluid [273–276], saliva [272,277], sweat [27,28] aqueous humour [278] and suction blister fluid [251,279] have been characterized by 2-D PAGE and some clinical correlates established. These samples have been found to contain tissue-specific proteins in addition to serum components [280].

3.2. Inborn errors of metabolism and genetic diseases

Techniques of modern molecular biology have greatly increased our knowledge and understanding of several inherited human disorders at the gene level. However, in relatively few cases has this approach yielded direct information on the gene products involved in these diseases. This problem can be addressed by exploiting the high-resolution capacity of 2-D PAGE to separate simultaneously the complex protein mixtures represented by whole cells and tissues. Goldman and Merril [281] have identified four major areas where 2-D PAGE can make a contribution to human medical genetics. (i) Defects involving a mutant protein species with altered charge, molecular weight abundance, stability, or processing can be detected. Such a change could, but need not necessarily, represent the primary mutation in a particular genetic disease. (ii) Patterns of secondary metabolic effects can be characterised and these can elucidate disease pathways. (iii) Disease-specific and disease-associated markers can be identified. (iv) Protein polymorphisms can be identified and these can be useful for gene mapping.

In human fibroblasts 2-D PAGE has detected mutations in the β-actin gene following neoplastic transformation [282,283]. In 2-D maps of cultured fibroblasts an extra protein spot has been observed in trisomy 18, although it has not been established whether this is the primarily affected gene product [284].

Disease-associated alterations in 2-D gel patterns have been described for a variety of disorders. These include Duchenne muscular dystrophy [285–287], Ataxia-Telangiectasia [288], Lesch-Nyhan syndrome [289–290], trisomy 21 (Down's syndrome) [291–293], Joseph Disease [294], Tangier disease [295] and cystic fibrosis [296]. In some of these examples it has not yet been established whether the result of the primary mutation or of secondary protein modulations is being observed by 2-D PAGE. Such protein modulations have also been observed in response to non-genetic conditions such as heat shock [297,298], rheumatoid arthritis [299] and hormones [151,300].

3.3. Cancer and malignant transformation

The molecular mechanisms underlying malignant transformation and cancer have not been well elucidated. Several investigations have used 2-D PAGE analysis of normal and malignant tissue to search for changes associated with the disease state. For example, qualitative and quantitative changes in 2-D protein profiles have been characterised in renal carcinoma [25], breast cancer [301,302], hepatoma [303], sarcomas [304] and colonic adenocarcinoma [305,306], but further investigation is essential to establish the specificity of such tumour-associated protein changes.

The alternative approach has been to investigate the process of malignant transformation by 2-D PAGE analysis of protein synthesis in neoplastic human cultured cells. This topic has been recently discussed in detail by Celis et al. [307] and the interested reader is referred to this excellent review. The striking observation from this type of investigation has been that relatively few changes in protein synthesis occur following transformation. The changes which do occur are generally quantitative in nature and reflect alteration in the rate of synthesis, modification and degradation of proteins normally expressed rather than the appearance of new, transformation-specific proteins. The latter event has been reported in virally transformed cells but is not commonly observed in chemically or spontaneously transformed cells. This, as Celis et al. [307] point out, is perhaps surprising considering the major changes in cell morphology, architecture and behaviour that accompany transformation.

4. ACKNOWLEDGEMENTS

I would like to thank Mrs. C. Trand for typing the manuscript and Mrs. K. Davidson for preparing the illustrations. Financial support of the Muscular Dystrophy Group of Great Britain is acknowledged.

5. SUMMARY

The high-resolution capacity of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) makes it an excellent tool for the analysis and characterisation of complex protein mixtures. The evolution of two-dimensional electrophoresis is briefly described. The various steps involved in 2-D PAGE, the identification and characterization of proteins separated by 2-D PAGE and the quantitative and qualitative analysis of 2-D patterns are discussed in detail and some new approaches are described. In the final section a brief outline of some of the biomedical applications of 2-D PAGE to screening of body fluids, genetic diseases, inborn errors of metabolism, cancer and neoplastic transformation are discussed.

REFERENCES

1 O. Smithies and M.D. Poulik, Nature, 177 (1956) 1033.

2 S. Raymond and L. Weintraub, Science, 130 (1959) 711.
3 S. Raymond and M. Nakamichi, Anal. Biochem., 7 (1964) 225.
4 J. Margolis and K.G. Kenrick, Nature, 221 (1969) 1056.
5 G. Dale and A.L. Latner, Clin. Chim. Acta, 24 (1969) 61.
6 V. Macko and H. Stegemann, Hoppe-Seyler's Z. Physiol. Chem., 350 (1969) 917.
7 A.V. Emes, A.L. Latner and J.A. Martin, Clin. Chim. Acta, 64 (1975) 69.
8 A.L. Latner and A.V. Emes, in P.G. Righetti (Editor), Progress in Isoelectric Focusing and Isotachophoresis, North-Holland, Amsterdam, 1975, p 223.
9 H. Stegemann, Angew. Chem., 82 (1970) 640.
10 T. Barret and H.J. Gould, Biochim. Biophys. Acta, 294 (1973) 165.
11 D. Suria and C.C. Liew, Can. J. Biochem., 52 (1974) 1143.
12 A.J. MacGillivray and D. Rickwood, Eur. J. Biochem., 41 (1974) 181.
13 S. Bhakdi, H. Knüfermann and D.F.H. Wallach, in P.G. Rhigetti (Editor), Progress in Isoelectric Focusing and Isotachophoresis, North-Holland, Amsterdam, 1975, p. 281.
14 D.A. Chignell and P.T. Wingfield, Fed. Proc., Fed. Am. Soc. Exp. Biol., 33 (1974) 1283.
15 G. Iborra and J.M. Bühler, Anal. Biochem., 74 (1976) 503.
16 J. Klose, Humangenetik, 26 (1975) 231.
17 J. Klose, in D. Neubert and H.J. Merkes (Editors), New Approaches to the Evaluation of Abnormal Embryonic Development, G. Thieme, Stuttgart, 1975, p. 375.
18 P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007.
19 R.P. Tracy, R.M. Currie and D.S. Young, Clin. Chem., 28 (1982) 890.
20 J.J. Edwards, S.L. Tollaksen and N.G. Anderson, Clin. Chem., 27 (1981) 1335.
21 C.R. Merril, D. Goldman, S.A. Sidman and M.H. Ebert, Science, 211 (1981) 1437.
22 N.G. Anderson, N.L. Anderson, S.L. Tollaksen, H. Hahn, F. Giere and J. Edwards, Anal. Biochem., 95 (1979) 48.
23 N.G. Anderson, N.L. Anderson and S.L. Tollaksen, Clin. Chem., 25 (1979) 1199.
24 J.J. Edwards, S.L. Tollaksen and N.G. Anderson, Clin. Chem., 28 (1982) 941.
25 R.P. Tracy and D.S. Young, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 193.
26 T. Marshall, K.M. Williams and O. Vesterberg, Electrophoresis, 6 (1985) 47.
27 T. Marshall and K.M. Williams, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 523.
28 R.W. Rubin and N.S. Penneys, Anal. Biochem., 131 (1983) 520.
29 M.J. Dunn and A.H.M. Burghes, in M.J. Dunn (Editor), Gel Electrophoresis of Proteins, Wright, Bristol, 1986, p. 203.
30 B.J. Thompson, A.H.M. Burghes, M.J. Dunn and V. Dubowitz, Electrophoresis, 2 (1981) 251.
31 C.S. Giometti and N.G. Anderson, Clin. Chem., 27 (1981) 1918.
32 C.S. Giometti, M.J. Danon and N.G. Anderson, Neurology, 33 (1983) 1152.
33 O. Kuhn and F.H. Wilt, Anal. Biochem., 105 (1980) 274.
34 M.J. Dunn and A.H.M. Burghes, Electrophoresis, 4 (1983) 173.
35 J.I. Garrels, J. Biol. Chem., 254 (1979) 7961.
36 R. Bravo, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL. 1984, p. 3.
37 M.J. Dunn and A.H.M. Burghes, Electrophoresis, 4 (1983) 97.
38 K.E. Willard, C.S. Giometti, N.L. Anderson, T.E. O'Connor and N.G. Anderson, Anal. Biochem., 100 (1979) 289.
39 G.F.L. Ames and K. Nikaido, Biochemistry, 15 (1976) 616.
40 A.L. Shapiro, M.O. Scharff and J.V. Maizel Biochem. Biophys. Res. Commun., 28 (1967) 815.
41 J. Klose and M. Feller, Electrophoresis, 2 (1981) 12.
42 A.H.M. Burghes, M.J. Dunn, H.E. Statham and V. Dubowitz, Electrophoresis, 3 (1982) 185.
43 M.N. Horst, S. Mahaboob, M. Basha, G.A. Baumbach, E.H. Mansfield and R.M. Roberts, Anal. Biochem., 102 (1980) 399.
44 M.A. Kaderbhai and R.B. Freedman, Biochim. Biophys. Acta, 601 (1980) 11.
45 P.A. Dockham, R.C. Steinfeld, C.J. Stryker, S.W. Jones and G.A. Vidaver, Anal. Biochem., 153 (1986) 102.

46 C. Bordier, J. Biol. Chem., 256 (1981) 1604.
47 G. Pryde, Trends Biochem. Sci., 11 (1986) 160.
48 K.E. Willard-Gallo, Y. Humblet and M. Symann, Clin. Chem., 30 (1984) 2069.
49 A. Gonenne and R. Ernst, Anal. Biochem., 87 (1978) 28.
50 L.M. Hjelmeland, D.W. Nebert and A. Chrambach, Anal. Biochem., 95 (1979) 201.
51 G.H Perdew, H.W. Schaup and D.P. Selivonchick, Anal. Biochem., 135 (1983) 453.
52 M.J. Dunn, A.H.M. Burghes, K. Patel, J.A. Witkowski and V. Dubowitz, in V. Neuhoff (Editor), Electrophoresis '84, Verlag Chemie, Weinheim, 1984, p. 281.
53 M.J. Dunn, A.H.M. Burghes and K. Patel, Protides Biol. Fluids, 33 (1985) 479.
54 A. Bianchi Bosisio, C. Lochenstein, C. Snyder, R.S. Snyder and P.G. Righetti, J. Chromatogr., 189 (1980) 317.
55 P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Application, Elsevier, Amsterdam, 1983.
56 T. Hirabayashi, Anal. Biochem., 117 (1981) 443.
57 I. Olsson and T. Låås, J. Chromatogr., 215 (1981) 373.
58 P. Serwer and S.J. Hayes, Electrophoresis, 3 (1982) 80.
59 B.J. Thompson, M.J. Dunn, A.H.M. Burghes and V. Dubowitz, Electrophoresis, 3 (1982) 307.
60 A.H.M. Burghes, M.J. Dunn and V. Dubowitz, Electrophoresis, 3 (1982) 354.
61 I.E. Creighton, J. Mol. Biol., 129 (1979) 235.
62 R.C. Allen, C.A. Saravis and H.R. Maurer, Gel Electrophoresis and Isoelectric Focusing of Proteins, Walter de Gruyter, Berlin, 1984.
63 A.H.M. Burghes, M.J. Dunn, J.A. Witkowski and V. Dubowitz, in D. Stathakos (Editor), Electrophoresis '82, Walter de Gruyter, Berlin, 1983, p. 371.
64 J.I. Garrels, Methods Enzymol., 100 (1983) 411.
65 N.L. Anderson and N.G. Anderson, Anal. Biochem., 85 (1978) 331.
66 R. Ramasamy, S.P. Spragg, M.I. Jones and R. Amess, Electrophoresis, 6 (1985) 43.
67 R. Duncan and J.W.B. Hershey, Anal. Biochem., 138 (1984) 144.
68 R.P. Tracy, R.M. Currie, R.A. Kyle and D.S. Young, Clin. Chem., 28 (1982) 908.
69 M.R. Goldsmith, E.C. Rattner, M. Macy, D. Kochler, S.R. Balikov and G.C. Bock, Anal. Biochem., 99 (1979) 33.
70 R. Rangel-Aldao, J.W. Kupiec and O.M. Rosen, J. Biol. Chem., 254 (1979) 2499.
71 A. Görg, W. Postel, R. Westermeier, E. Gianazza and P.G. Righetti, J. Biochem. Biophys. Methods, 3 (1980) 273.
72 A. Görg, W. Postel and R. Westermeier, in R.C. Allen and P. Arnaud (Editors), Electrophoresis '81, Walter de Gruyter, Berlin, 1981, p. 259.
73 M.J. Dunn, A.H.M. Burghes, J.A. Witkowski and V. Dubowitz., Protides Biol. Fluids, 32 (1985) 973.
74 C.B. Cuono and G.A. Chapo, Electrophoresis, 3 (1982) 65.
75 G.R. Finlayson and A. Chrambach, Anal. Biochem., 40 (1971) 292.
76 M. Jonssen, Electrophoresis, 1 (1980) 141.
77 B.P. Voris and D.A. Young, Anal. Biochem., 104 (1980) 478.
78 H. Delincee and B.J. Radola, Anal. Biochem., 90 (1978) 603.
79 N. Ui, Ann. N.Y. Acad. Sci., 209 (1973) 198.
80 W.J. Gelsema, C.L. de Ligny and N.G. van der Veen, J. Chromatogr., 154 (1978) 161.
81 W.J. Gelsema, C.L. de Ligny and N.G. van der Veen, J. Chromatogr., 171 (1979) 171.
82 B.-E. Chidakel, N.Y. Nguyen and A. Chrambach, Anal. Biochem., 77 (1977) 216.
83 R. Bravo, J. Bellatin and J.E. Celis, Cell Biol. Int. Rep., 5 (1981) 93.
84 R. Bravo and J.E. Celis, Clin. Chem., 28 (1982) 766.
85 K.E. Peters and D.E. Comings, J. Cell Biol., 86 (1980) 135.
86 R.A. Steinberg, P.H. O'Farrell, U. Friedrich and P. Coffino, Cell, 10 (1977) 381.
87 N.L. Anderson and B.J. Hickman, Anal. Biochem., 93 (1979) 312.
88 S.L. Tollaksen, J.J. Edwards and N.G. Anderson, Electrophoresis, 2 (1981) 155.
89 M. Jonssen, S. Fredrikson, M. Jontell and A. Linde, J. Chromatogr., 157 (1978) 235.
90 J.W. Vanderhoff, F.J. Micale and P.H. Krumrine, Sep. Purif. Methods, 6 (1977) 61.
91 P.Z. O'Farrell, M.M. Goodman and P.H. O'Farrell, Methods Cell Biol., 12 (1977) 1133.

92 P.G. Righetti, J. Chromatogr., 173 (1979) 1.
93 N.Y. Nguyen and A. Chrambach, Anal. Biochem., 74 (1976) 145.
94 N.Y. Nguyen and A. Chrambach, Electrophoresis, 1 (1980) 14.
95 C.B. Cuono, G.A. Chapo, A. Chrambach and L.M. Hjelmeland, Electrophoresis, 4 (1983) 404.
96 A.H.M. Burghes, K. Patel and M.J. Dunn, Electrophoresis, 6 (1985) 453.
97 P.G. Righetti, E. Gianazza and B. Bjellqvist, J. Biochem. Biophys. Methods, 8 (1983) 89.
98 P.G. Righetti, J. Chromatogr., 300 (1984) 165.
99 P.G. Righetti, C. Gelfi and E. Gianazza, in M.J. Dunn (Editor), Gel Electrophoresis of Proteins, Wright, Bristol, 1986, p. 141.
100 G. Dossi, F. Celantano, E. Gianazza and P.G. Righetti, J. Biochem. Biophys. Methods, 7 (1983) 123.
101 E. Gianazza, G. Dossi, F. Celantano and P.G. Righetti, J. Biochem. Biophys. Methods, 8 (1983) 109.
102 E. Gianazza, S. Astrua-Testori and P.G. Righetti, Electrophoresis, 6 (1985) 113.
103 E. Gianazza, P. Giacon, B. Sahlin and P.G. Righetti, Electrophoresis, 6 (1985) 53.
104 K. Altland, R. Hackler, A. Banzhoff and A. von Eckardstein, Electrophoresis, 6 (1985) 140.
105 D.F. Hochstrasser, V. Augsburger, M. Funk, R. Appel, C. Pellegrini and A.F. Muller, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 566.
106 LKB Application Note No. 324, LKB Produkter, Bromma, 1984.
107 J.S. Fawcett and A. Chrambach, Electrophoresis, 7 (1986) 260.
108 K. Altland and A. Altland, Electrophoresis, 5 (1984) 143.
109 K. Altland and A. Altland, Clin. Chem., 30 (1984) 2098.
110 P.G. Righetti, K. Ek and B. Bjellqvist, J. Chromatogr., 291 (1984) 31.
111 P. Pietta, E. Pocaterra, A. Fiorino, E. Gianazza and P.G. Righetti, Electrophoresis, 6 (1985) 162.
112 K. Altland and U. Rossmann, Electrophoresis, 6 (1985) 314.
113 E. Gianazza, G. Artoni and P.G. Righetti, Electrophoresis, 4 (1983) 321.
114 C. Gelfi and P.G. Righetti, Electrophoresis, 5 (1984) 257.
115 K. Altland, A. Banzhoff, R. Hackler and U. Rossmann, Electrophoresis, 5 (1984) 379.
116 E. Gianazza and P.G. Righetti, in V. Neuhoff (Editor), Electrophoresis '84, Verlag Chemie, Weinheim, 1984, p. 87.
117 E. Gianazza, A. Frigerio, A. Tagliabue and P.G. Righetti, Electophoresis, 5 (1984) 209.
118 E. Gianazza, S. Astrua-Testori, P. Giacon and P.G. Righetti, Electrophoresis, 6 (1985) 332.
119 A. Görg, W. Postel, R. Westermeier, B. Bjellqvist and K. Ek, in D. Stathakos (Editor), Electrophoresis '82, Walter de Gruyter, Berlin, 1983, p. 354.
120 A. Görg, W. Postel, J. Weser and R. Westermeier, in H. Hirai (Editor), Electrophoresis '83, Walter de Gruyter, Berlin, 1984, p. 525.
121 A. Görg, W. Postel and J. Weser, Protides Biol. Fluids, 33 (1985) 467.
122 R. Westermeier, W. Postel, J. Weser and A. Görg, J. Biochem. Biophys. Methods, 8 (1983) 321.
123 J.-J. Pernelle, P. Chafey, J.-L. Lognonne, P.G. Righetti, A.B. Bosisio and J.P. Wahrmann, Electrophoresis, 7 (1986) 159.
124 M.J. Dunn, K. Patel and A.H.M. Burghes, in M.M. Galteau and G. Siest (Editors), Progrès Récents en Electrophorèse Bidimensionelle, Presses Universitaires, Nancy, 1986, p. 3.
125 M.J. Dunn and K. Patel, Protides Biol. Fluids, 34 (1986) 695.
126 J.S. Fawcett and A. Chrambach, Protides Biol. Fluids, 33 (1985) 439.
127 J.S. Fawcett and A. Chrambach, Electrophoresis, 7 (1986) 266.
128 P.G. Righetti and E. Gianazza, in M.M. Galteau and G. Siest (Editors), Progrès Récents en Electrophorèse Bidimensionelle, Presses Universitaires, Nancy, 1986, p. 11.
129 M.J. Dunn and K. Patel, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 574.
130 A. Görg, W. Postel, S. Günther and J. Weser, Electrophoresis, 6 (1985) 599.
131 A. Görg, W. Postel, S. Günther and J. Weser, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 435.

132 J. Weser, W. Postel, S. Günther and A. Görg, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 607.
133 P.G. Righetti, C. Gelfi and E. Gianazza, Chimica Oggi, March (1986) 55.
134 E. Gianazza, S. Astrua-Testori, P. Caccia, L. Quaglia and P.G. Righetti, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 563.
135 H. Jäckle, Anal. Biochem., 98 (1979) 81.
136 E. Gianazza, S. Astrua-Testori, P. Caccia, P. Giacon, L. Quaglia and P.G. Righetti, Electrophoresis, 7 (1986) 76.
137 E. Rosenmann, C. Kreis, R.G. Thomson, M. Dobbs, J.L. Hamerton and K. Wrogemann, Nature, 298 (1982) 563.
138 R. Amess, J.E. Fox and S.P. Spragg, Electrophoresis, 6 (1985) 186.
139 H.M. Poehling and V. Neuhoff, Electrophoresis, 1 (1980) 90.
140 E. Gianazza and P.G. Righetti, J. Chromatogr., 193 (1980) 1.
141 J.I. Garrels, J.T. Farrar and C.B. Burwell, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 37
142 U.K. Laemmli, Nature, 227 (1970) 680.
143 J. Margolis and K.G. Kenrick, Nature, 214 (1967) 1334.
144 G.M. Rothe and H. Purkhanbaba, Electrophoresis, 3 (1982) 33.
145 G.M. Rothe and W.D. Maurer, in M.J. Dunn (Editor), Gel Electrophoresis of Proteins, Wright, Bristol, 1986, p. 37.
146 N.L. Anderson and N.G. Anderson, Anal. Biochem., 85 (1978) 341.
147 N.G. Anderson and N.L. Anderson, Behring Inst. Mitt., 63 (1979) 169.
148 J.F. Poduslo, Anal. Biochem., 114 (1981) 131.
149 M. Wyckoff, D. Rodbard and A. Chrambach, Anal. Biochem., 78 (1977) 469.
150 D.W. Anderson and C. Peterson, in R.C. Allen and P. Arnaud (Editors), Electrophoresis '81, Walter de Gruyter, Berlin, 1981, p. 41.
151 V.M. Nikodem, B.L. Trus and J.E. Rall, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 4411.
152 D.A. Young, B.P. Voris, E.V. Maytin and T.A. Colbert, Methods Enzymol., 91 (1983) 190.
153 D.A. Young, Clin. Chem., 30 (1984) 2104.
154 J. Klose and E. Zeindl, Clin. Chem., 30 (1984) 2014.
155 R. Rüchel, J. Chromatogr., 132 (1977) 451.
156 C.S. Giometti, N.G. Anderson, S.L. Tollaksen, J.J. Edwards and N.L. Anderson, Anal. Biochem., 102 (1980) 47.
157 P. Kurian, D.M. Gersten, P.V. Suhocki and G. Ledley, Electrophoresis, 2 (1981) 184.
158 B.W. Lubit, Electrophoresis, 5 (1984) 358.
159 M.D. Frey and B.J. Radola, Electrophoresis, 3 (1982) 27.
160 O. Vesterberg, in B.J. Radola (Editor), Electrophoresis '79, Walter de Gruyter, Berlin, 1980, p. 95.
161 C.R. Merril, M.G. Harasewych and M.G. Harrington, in M.J. Dunn (Editor), Gel Electrophoresis of Proteins, Wright, Bristol, 1986, p. 323.
162 A. Chrambach, R.A. Reisfeld, H. Wyckhoff and J. Zaccari, Anal. Biochem., 20 (1967) 150.
163 W. Diezel, G. Koppenschläger and E. Hofmann, Anal. Biochem., 48 (1972) 617.
164 V. Neuhoff, R. Stamm and H. Eibl, Electrophoresis, 6 (1985) 427.
165 B.K. Hartman and S. Udenfriend, Anal. Biochem., 30 (1969) 391.
166 K. Patel, D. Easty and M.J. Dunn, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 662.
167 B.D. Hames and D. Rickwood, Gel Electrophoresis of Proteins: A Practical Approach, IRL Press, London, 1981, p. 249.
168 B.O. Barger, F.C. White, J.L. Pace, D.L. Kemper and W.L. Ragland, Anal. Biochem., 70 (1976) 327.
169 C.R. Merril, C.R. Switzer and M.L. Van Keuren, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 435.
170 R.C. Switzer, C.R. Merril and S. Shifrin, Anal. Biochem., 98 (1979) 231.
171 C.R. Merril, M. Dunau and D. Goldman, Anal. Biochem., 110 (1981) 201.
172 C.R. Merril, M. Harrington and V. Alley, Electrophoresis, 5 (1984) 289.
173 C.R. Merril and M. Harrington, Clin. Chem., 30 (1984) 1938.

174 C.R. Merril and D. Goldman, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 93.
175 C.R. Merril, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 273.
176 J. Heukeshoven and R. Dernick, Electrophoresis, 6 (1985) 103.
177 D.M. Gersten, P.H. Wolf, R.H. Ledley, L.V. Rodriguez and E.J. Zapolski, Electrophoresis, 7 (1986) 327.
178 T. Marshall and A.L. Latner, Electrophoresis, 2 (1981) 228.
179 J. Guevara, D.A. Johnston, L.S. Ranageli, B.A. Martin, S. Capetillo and L.V. Rodriguez, Electrohoresis, 3 (1982) 197.
180 D. Ochs, Anal. Biochem., 135 (1983) 470.
181 C.R. Merril, D. Goldman and M.L. Van Keuren, Electrophoresis, 3 (1982) 17.
182 M.L. Van Keuren, D. Goldman and C.R. Merril, Anal. Biochem., 116 (1981) 248.
183 C. Rochette-Egly and C. Stussi-Garaud, Electrophoresis, 5 (1984) 285.
184 G.E. Means and R.E. Feeney, Biochemistry, 77 (1968) 2192.
185 E.J. Zapolski, D.M. Gersten and R.S. Ledley, Anal. Biochem., 123 (1982) 325.
186 T. Rabillaud and H. Therre, Electrophoresis, 7 (1986) 49.
187 J.H. Elder, R.A. Pickett, J. Hampton and R.A. Lerner, J. Biol. Chem., 252 (1977) 6510.
188 E.J.Zapolski, F. Padrasky, R.S. Ledley and D.M. Gersten, Electrophoresis, 5 (1984) 354.
189 W.M. Bonner and R.A. Laskey, Eur. J. Biochem., 46 (1974) 83.
190 D.E. Pulleybank and G.M. Booth, J. Biochem. Biophys. Methods, 4 (1981) 339.
191 J.P. Chamberlain, Anal. Biochem., 98 (1979) 132.
192 R.A. Laskey and A.D. Mills, Eur. J. Biochem., 56 (1975) 335.
193 R.A. Laskey, Methods Enzymol., 65 (1980) 363.
194 E.M. McConkey, Anal. Biochem., 96 (1976) 39.
195 K.E. Walton, D. Styer and E.Z. Gruenstein, J. Biol. Chem., 254 (1979) 7951.
196 E.Z. Gruenstein and A.L. Pollard, Anal. Biochem., 76 (1976) 452.
197 R. Lecocq, A. Hepburn and F. Lamy, Anal. Biochem., 127 (1982) 293.
198 B.J. Scott, J.E. Bateman and A.R. Bradwell, Anal. Biochem., 123 (1982) 1.
199 S. Burbeck, Electrophoresis, 4 (1983) 127.
200 J.B. Davidson and A.L. Case, Science, 215 (1982) 1398.
201 J.B. Davidson, in V. Neuhoff (Editor), Electrophoresis '84, Verlag Chemie Weinheim, 1984, p. 235.
202 P.L. Bloch, T.A. Phillips and F.C. Neidhardt, J. Bacteriol., 141 (1980) 1409.
203 T.A. Phillips, P.L. Bloch and F.C. Neidhardt, J. Bacteriol., 144 (1980) 1024.
204 N.G. Anderson and N.L. Anderson, Clin. Chem., 28 (1982) 739.
205 R.C. Allen, P. Arnaud and S.S. Spicer, in R.C. Allen and P. Arnaud (Editors), Electrophoresis '81, Walter de Gruyter, Berlin, 1981, p. 161.
206 E.M. Southern, J. Mol. Biol., 98 (1975) 503.
207 B. Bowen, J. Steinberg, U.K. Laemmli and H. Weintraub, Nucleic Acid Res., 8 (1980) 1.
208 J. Renart, J. Reiser and G.R. Stark, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 3116.
209 M. Peferoen, R. Huybrechts and A. De Loof, FEBS Lett., 145 (1982) 369.
210 H. Towbin, T. Staehelin and J. Gordon, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 4350.
211 K. Gooderham, in J. Walker and W. Gaastra (Editors), Techniques in Molecular Biology, Croom Helm, London, 1983, p. 49
212 J.M. Gershoni and G.E. Palade, Anal. Biochem., 131 (1983) 1.
213 J.M. Gershoni, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim 1986, p. 305.
214 J. Symington, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 127.
215 U. Beisiegel, Electrophoresis, 7 (1986) 1.
216 K. Hancock and V.C.W. Tsang, Anal. Biochem., 133 (1983) 157.
217 M. Moeremans, G. Daneels and J. De Mey, Anal. Biochem., 145 (1985) 315.
218 M. Moeremans, G. Daneels, M. De Raeymaeker and J. De Mey, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 328.

219 C.W. Wrigley and J.H Skerrit, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 291.
220 M. Moeremans, M. De Raeymaeker, G. Daneels and J. De Mey, Anal. Biochem., 153 (1986) 18.
221 C.A. Saravis, Electrophoresis, 5 (1984) 54.
222 B. Batteiger, W.J. Newhall and R.B. Jones, J. Immunol. Methods, 55 (1982) 297.
223 M. Moeremans, G. Daneels, A. Van Dijck, G. Langanger and J. De Mey, J. Immunol. Methods, 74 (1984) 353.
224 G. Daneels, M. Moeremans, M. De Raeymaeker and J. De Mey, J. Immunol. Methods, 89 (1986) 89.
225 G.I. Latter, S. Burbeck, J. Fleming and J. Leavitt, Clin. Chem., 30 (1984) 1925.
226 T. Manabe, K. Kojima, S. Jitzukawa, T. Hoshino and T. Okuyama, Clin. Chem., 28 (1982) 819.
227 S.J. Fey, R. Bravo, P.M. Larsen and J.E. Celis, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 169.
228 D.W. Cleveland, S.G. Fischer, M.W. Kirschner and U.K. Laemmli, J. Biol. Chem., 252 (1977) 1102.
229 S.J. Fey, R. Bravo, P.M. Larsen, J. Bellatin and J.E. Celis, Cell Biol. Int. Rep., 5 (1981) 491.
230 R. Bravo, S.J. Fey, J.V. Small, M.P. Larsen and J.E. Celis, Cell, 25 (1981) 195.
231 P. Edman and G. Begg, Eur. J. Biochem., 1 (1967) 80.
232 R.M. Hewick, M.W. Hunkapiller, L.E. Hood and W.J. Dreyer, J. Biol. Chem., 256 (1981) 7990.
233 R.H. Aebersold, D.B. Taplow, L.E. Hood and S.B.H. Kent, J. Biol. Chem., 261 (1986) 4229.
234 R.H. Aebersold, D.B. Teplow, L.E. Hood and S.B.H. Kent, Protides Biol. Fluids, 34 (1986) 715.
235 S.P. Spragg, R. Amess, M.I. Jones and R. Ramasamy, in M.J. Dunn (Editor), Gel Electrophoresis of Proteins, Wright, Bristol, 1986, p. 363.
236 N.L. Anderson, J. Taylor, A.E. Scandora, B.P. Coulter and N.G. Anderson, Clin. Chem., 27 (1981) 1807.
237 J. Taylor, N.L. Anderson, A.E. Scandora, K.E. Willard and N.G. Anderson, Clin. Chem., 28 (1982) 861.
238 P.E. Lemkin and L.E. Lipkin, Comput. Biomed. Res., 14 (1981) 272.
239 P.F. Lemkin and L.E. Lipkin, Comput. Biomed. Res., 14 (1981) 355.
240 P.F. Lemkin and L.E. Lipkin, Comput. Biomed. Res., 14 (1981) 407.
241 K.-P. Vo, M.J. Miller, E.P. Geiduschek, C. Nielsen, A. Olson and N.-H. Xuong, Anal. Biochem., 112 (1981) 258.
242 M.J. Miller, A.D. Olson and S.S. Thorgeirsson, Electrophoresis, 5 (1984) 297.
243 M.J. Miller, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 539.
244 H. Marsman and R.W. Van Resandt, Electrophoresis, 6 (1985) 242.
245 S.H. Blose, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 552.
246 J.J. Tyson and R.M. Haralick, Electrophoresis, 7 (1986) 107.
247 D.J. Potter and M.M. Skolnick, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 556.
248 K.A. Smith and M.J. Dunn, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 560.
249 P. Vincens, N. Paris, J.-L. Pujol, C. Gaboriaud, T. Rabillaud, J.-L. Pennetier, P. Matherat and P. Tarroux, Electrophoresis, 7 (1986) 347.
250 P. Vincens, Electrophoresis, 7 (1986) 357.
251 E. Jellum and A.K. Thorsrud, Clin. Chem., 28 (1982) 876.
252 N.L. Anderson and N.G. Anderson, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 5421.
253 T. Marshall, K. Williams and O. Vesterberg, Clin. Chem., 30 (1984) 2008.
254 Z.A.R. Gomo, P.M.S. Clark, L.J. Kricka, K. Woods, B. Buckley and T.P. Whitehead, Electrophoresis, 4 (1983) 298.
255 T. Marshall, O. Vesterberg and K.M. Williams, Electrophoresis, 5 (1984) 122.

256 T. Marshall, K.M. Williams and O. Vesterberg, in V. Neuhoff (Editor), Electrophoresis '84, Verlag Chemie, Weinheim, 1984, p. 258.
257 T. Marshall, K.M. Williams, L. Holmquist, L.A. Carlson and O. Vesterberg, Clin. Chem., 31 (1985) 2032.
258 T. Marshall and O. Vesterberg, Electrophoresis, 4 (1983) 363.
259 T. Marshall, K.M. Williams and O. Vesterberg, Electrophoresis, 6 (1985) 392.
260 A.L. Latner, T. Marshall and M. Gambi, Electrophoresis, 1 (1980) 82.
261 R.P. Tracy, R.M. Currie, R.A. Kyle and D.S. Young, Clin Chem., 28 (1982) 900.
262 P.M.S. Clark, L.J. Kricka and T.P. Whitehead, Clin. Chem., 26 (1980) 201.
263 J.G. Guevara, B.H. Herbert and B.A. Martin, Electrophoresis, 6 (1985) 613.
264 J.J. Edwards, N.G. Anderson, S.L. Tollaksen, A.C. von Eschenbach and J. Guevara, Clin. Chem., 28 (1982) 160.
265 N. Frearson, R.D. Taylor and S.V. Perry, Br. Med. J., 282 (1981) 2002.
266 N. Frearson, R.D. Taylor and S.V. Perry, Clin. Sci., 61 (1981) 141.
267 N. Bauer, J. Nagel and J.-E. Franz, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 677.
268 D. Goldman, C.R. Merril and M.H. Ebert, Clin. Chem., 26 (1980) 1317.
269 M. Harrington and C.R. Merril, Clin. Chem., 30 (1984) 1933.
270 M.G. Harrington, C.R. Merril, D. Goldman, X.-H. Xu and D. McFarlin, Electrophoresis, 5 (1984) 236.
271 G.B. Dermer, L.M. Silverman and J.F. Chapman, Clin. Chem., 28 (1982) 759.
272 Y.C. Tsai, H.H. Harrison, C. Lee, J.A. Daufeldt, L. Oliver and J.T. Grayhack, Clin. Chem., 30 (1984) 2026.
273 M.I. Jones, S.P. Spragg and T. Webb, Biol. Neonate, 39 (1981) 171.
274 P. Burdett, J. Lizana, P. Eneroth and K. Bremme, Clin. Chem., 28 (1982) 935.
275 M.I. Jones and S.P. Spragg, Electrophoresis, 4 (1983) 291.
276 K.E. Kronquist, B.F. Crandall and L.G. Cosico, in H. Hirai (Editor), Electrophoresis '83, Walter de Gruyter, Berlin, 1984, p. 229.
277 C.S. Giometti and N.G. Anderson, in B.J. Radola (Editor), Electrophoresis '79, Walter de Gruyter, Berlin, 1980, p. 395.
278 J. Segers, M. Rabaey and R. Van Oye, Electrophoresis, 5 (1984) 48.
279 G. Volden, A.K. Thorsrud, I. Bjornson and E. Jellum, J. Invest. Dermatol., 75 (1980) 421.
280 G.B. Dermer and J.J. Edwards, Electrophoresis, 4 (1983) 212.
281 D. Goldman and C.R. Merril, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 241.
282 J. Leavitt and T.J. Kakunaga, J. Biol. Chem., 255 (1980) 1650.
283 J. Leavitt, D. Goldman, C.R. Merril and T. Kakunaga, Clin. Chem., 28 (1982) 850.
284 I. Willers, S. Singh, H.W. Goedde and J. Klose, Clin. Genet., 20 (1981) 217.
285 K. Patel, J. Guest and M.J. Dunn, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 652.
286 J. Guest, K. Patel and M.J. Dunn, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 658.
287 A.E. Hughes, C.A. Graham, W.H.I. McLean, J. Burn and N.C. Nevin, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 723.
288 J.P. Murnane and R.B. Painter, Biochemistry, 22 (1983) 1217.
289 C.R. Merril, D. Goldman and M. Ebert, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 6471.
290 C.R. Merril and D. Goldman, Clin. Chem., 28 (1982) 1015.
291 J. Weil and C. Epstein, Am. J. Hum. Genet., 7 (1979) 478.
292 M.L. Van Keuren, D. Goldman and C.R. Merril, in R.C. Allen and P. Arnaud (Editors), Electrophoresis '81, Walter de Gruyter, Berlin, 1981, p. 355.
293 J. Klose, E. Zeinckle and K. Sperling, Clin. Chem., 29 (1982) 987.
294 R.N. Rosenberg, L. Thomas, F. Baskin, J. Kirkpatrick, C. Bay and W.L. Nyhan, Neurology, 29 (1979) 917.
295 S. Visvikis, M.F. Dumon, J. Steinmetz, M.M. Galteau, M. Clerc and G. Siest, in M.J. Dunn (Editor), Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, 1986, p. 638.

296 C. Kirkpatrick, R. Lecocq, F. Lamy, V. Defleur, G. Dedobeleer, D. Baran, F. Rodesch and J.E. Dumont, Pediatr. Res., 19 (1985) 1341.
297 N.L. Anderson., C.S. Giometti, M.A. Gemmell, S.L. Nance and N.G. Anderson, Clin. Chem., 28 (1982) 1084.
298 M.J. Miller, N.H. Xuong and E.P. Geiduschek, Proc. Natl. Acad., Sci. U.S.A., 76 (1979) 5222.
299 K.E. Willard, A.K. Thorsrud, E. Munthe and E. Jellum, Clin. Chem., 29 (1982) 1067.
300 J.I. Garrels, Devel. Biol., 73 (1979) 134.
301 J. Stastny, R. Prasad and E. Fosslien, Clin. Chem., 30 (1984) 1914.
302 P. Motte, J.-M. Bidart, J.C. Delarue, E. Comoy, P. Moingeon and C. Bohuon, Clin. Chem., 30 (1984) 1947.
303 P.J. Wirth, T. Benjamin, D.M. Schwartz and S.S. Thorgeirsson, Cancer Res., 46 (1986) 400.
304 J. Forchhammer and H. Bravo-Macdonald, in J.E. Celis and R. Bravo (Editors), Gene Expression in Normal and Transferred Cells, Plenum, New York, 1983, p. 291.
305 R.P. Tracy, L.E. Wold, R.M. Currie and D.S. Young, Clin. Chem., 29 (1982) 915.
306 A.K. Thorsrud, M.H. Vatn and E. Jellum, Clin. Chem., 28 (1982) 884.
307 J.E. Celis, R. Bravo, P.M. Larsen, S.J. Fey, J. Bellatin and A. Celis, in J.E. Celis and R. Bravo (Editors), Two-Dimensional Gel Electrophoresis of Proteins, Academic Press, Orlando, FL, 1984, p. 307.