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ELECTROPHORESIS

Theory, Techniques, and Biochemical and Clinical Applications

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chapter have been used widely in this context and are finding ever-increasing applications.

Immuno-electrophoretic techniques have proved to be of particular value in taxonomy and studies of evolutionary relationships between proteins (e.g. Adair *et al.* 1978). The demonstration of relatedness or otherwise by immunological identity or cross-reactivity has also proved useful in studies comparing normal and mutant membranes and proteins (e.g. Bigelis and Burridge 1978) and of normal and pathogenic cell constituents. A major application of the methods described in this chapter lies in forensic work for the identification of blood stains and other biological tissues and fluids, detection of toxins, etc. In the clinical field tissue typing and matching exploit these immunochemical techniques. They have also proved valuable for the analysis of foods, for example to demonstrate the adulteration of meat products with milk, egg, or vegetable protein (Kluge-Wilm 1978) or to show the presence of casein in heated foods (Klostermeyer and Offt 1978).

9

ISO-ELECTRIC FOCUSING

Iso-electric focusing (IEF) is a method specifically intended for the fractionation of molecular species differing only in net charge. Thus, since separation is not due to any molecular size or 'molecular sieving' effect during electrophoretic transport through the medium, optimum resolution is theoretically obtained in a gel where $T = 0$ per cent. It is therefore performed in essentially non-sieving media, such as in free solution with a density gradient, polyacrylamide gels of high porosity (low T), or in granular beds (e.g. Sephadex).

IEF is an analytical method capable of very high resolution, particularly when shallow, immobilized pH gradients are used, and can separate macromolecules differing in isoelectric point by as little as only 0·001 pH unit. It is also capable of being scaled up for small-scale preparative separations of up to about 1 g whilst still retaining good resolution (0·01–0·02 pI units). In the preparative mode it has often been regarded as useful in one of the final stages of purification regimes, but there is a strong argument for including it at a relatively early stage and quickly removing impurities that might interfere with other methods. With improvements in both materials and techniques, there is no doubt that IEF has justifiably increased greatly in popularity in recent years and looks set for still greater exploitation in future.

9.1. Iso-electric focusing: background and principles

IEF can be regarded as electrophoresis within a pH gradient. Macromolecules therefore migrate through the gradient as long as they retain a net positive or negative charge until they reach the point in the pH gradient which corresponds to their iso-electric point, where the net molecular charge will be zero, and migration ceases. The pH gradient is established with the aid of a mixture of low-molecular-weight amphoteric substances, or ampholytes. If the cathode of an electrolytic cell is placed in a solution of a strong base, the anode in strong acid, and the space between is filled with ampholyte solution, then the ampholytes will carry a net positive charge in the area close to the anode and a net negative charge near the cathode. Thus when the current is switched on they will be repelled by the electrodes and will move into the central region, each ampholyte species becoming stationary where the pH of the surrounding electrolyte equals the iso-electric point of that particular ampholyte (Haglund 1967). The most basic ampholyte thus stays closest to the basic cathodic solution and

the other ampholytes arrange themselves in order of their iso-electric points, ending with the most acidic closest to the acid solution at the anode. The resulting pH gradient is then at equilibrium since any displacement of an ampholyte molecule will result in the formation of a net charge on the molecule causing it to migrate back to its iso-electric point again under the influence of the applied electric field. Provided that mixing (e.g. convection currents etc.) is prevented the pH gradient will remain stable for a considerable time.

It will be evident that the production of a good smooth pH gradient depends largely on the properties of the ampholytes used. As defined by Svensson (1961) these should have the following properties.

(1) Good buffering capacity so that they can define the pH at their iso-electric points, even in the presence of high-molecular-weight ampholytes such as proteins which may be present in the same zone.
(2) Good conductivity at their iso-electric points in order to maintain electrical conductivity in the absence of other electrolytes.
(3) Low molecular weight in order to be readily separated from the macromolecules being studied.
(4) A composition different from the macromolecules so that they do not interfere with assay procedures.
(5) They should not react with or denature the macromolecules being fractionated.

Mixtures of many amphoteric substances such as amino acids and peptides and some amphoteric and non-amphoteric buffer components can act as suitable ampholytes (e.g. Chrambach and Nguyen 1977). The 47-component buffer mixture developed by Cuono and Chapo (1982) is available commercially (as Poly/sep 47 from Polysciences Ltd, 24 Low Farm Place, Moulton Park, Northampton, NN3 1HY, England) and such mixtures, as well as being less expensive than conventional commercial ampholytes, may have advantages if complex formation between ampholytes and sample constituents occurs. However, the great majority of iso-electric focusing experiments are performed nowadays with the aid of commercial ampholyte mixtures. The first of these, and still the most widely used, is marketed by LKB Produkter AB under the brand name Ampholines™. They consist of synthetic mixtures of polyaminopolycarboxylic acids with molecular weights mostly in the region 300–900 (Bosisio, Snyder, and Righetti 1981). Any high-molecular-weight ampholytes are undesirable because in preparative work they may not be readily separated from protein (Baumann and Chrambach 1975), and in analytical IEF gels they could result in artefactual band patterns through binding to and staining with protein dyes (Otavsky and Drysdale 1976). More recently other products have been introduced which contain sulphonic or phosphonic acid groupings in addition to the amino and carboxylic acid groups. These products (Servalyts™, Serva-Feinbiochemica GmbH; Biolytes™, Bio-Rad Laboratories; Pharmalytes™, Pharmacia AB) have recently been compared with the Ampholines (Fawcett 1977; Gelsema, de Ligny, and van der Veen 1979*b*; Låås and Olsson 1981*b*) and shown to have similar performance.

There were a number of experiments in the first half of this century which with the benefit of hindsight can be said to have relevance to IEF, but it was not until the work of Kolin in 1954–1955 and Svensson in 1961–1962 that the principle was shown to be both theoretically and practically sound.

Contributions relating to the theoretical basis of IEF which are scattered through the earlier literature have been collected together and presented in a concise form by Rilbe (1973), and his treatment is given below. It is derived from the simple dissociation theory of electrolytes. Considering an ampholyte HA, the cation of which is therefore H_2A^+ and the anion A^-, in aqueous solution the mass action equations apply so that

$$\frac{[H^+]\,[HA]}{[H_2A^+]} = K_1 \tag{9.1}$$

and

$$\frac{[H^+]\,[A^-]}{[HA]} = K_2. \tag{9.2}$$

We now introduce Rilbe's notation: $h = [H^+]$; $pH = -\log h$; C_+, concentration of H_2A^+; C_-, concentration of A^-, C_0, concentration of uncharged HA and zwitterionic H^+A^- (since as long as it is undissociated it does not matter whether it is uncharged or not); $C = C_+ + C_- + C_0$, total ampholyte concentration. Equations (9.1) and (9.2) then become

$$hC_0 = f_+ K_1 C_+ \tag{9.3}$$

and

$$hC_- = C_0(K_2/f_-) \tag{9.4}$$

where f_+ and f_- are the activity coefficients of H_2A^+ and A^- respectively and the uncharged species is assumed to have an activity coefficient of unity. As a further simplification one can take

$$f_+ K_1 = K_1' \tag{9.5}$$

and

$$K_2/f_- = K_2' \tag{9.6}$$

electric points otherwise a deep conductivity minimum would occur about the neutral point which might result in local overheating. At more extreme pH values (below 4 or above 10) the conductivity contributions of H^+ and OH^- ions are dominant and the conductivity of ampholytes becomes less important. The degree of ionization of an ampholyte is

$$\alpha = \frac{C_+ + C_-}{C} = \frac{h^2 + K_1'K_2'}{h^2 + hK_1' + K_1'K_2'} \tag{9.23}$$

This has a value of unity at both very low and very high pH values, and has a minimum at the iso-ionic point which can be found by combining (9.13) and (9.23):

$$\alpha_i = \frac{1}{1 + (K_1'/4K_2')^{1/2}}. \tag{9.24}$$

The conductivity contribution of an iso-ionic ampholyte is proportional to α_i, so it can be seen that ampholytes with a large pK' difference have a low conductivity in the iso-ionic state. This applies to all neutral natural amino acids which are therefore not useful as carrier ampholytes (Rilbe 1973). As pK' decreases, the degree of ionization increases to a limiting value of 0·5 which is reached for the smallest pK' difference compatible with (9.22). Comparison of (9.21) and (9.24) gives

$$b_i = 4\alpha_i \tag{9.25}$$

and this shows that a high degree of ionization (a good conductivity) is accompanied by a good buffering capacity and *vice versa*.

In practice most carrier ampholytes in general use are polyaminopolycarboxylic acids and hence have a number of ionizable groups at the isoionic point, so that (9.22) no longer holds and α_i does not have an upper limit of 0·5 (for large molecules, such as proteins, with many ionizable groups α_i may approach unity in spite of the fact that the net charge is zero). These facts are actually favourable for the practical application of IEF because a polyvalent ampholyte may therefore have a higher conductivity and a better buffering capacity at the iso-ionic point than a simple bivalent one.

Electrolysis of a mixture of carrier ampholytes gives rise to a smooth and stable pH gradient, the extent and shape of which is determined by a choice of ampholytes and the proportions which are iso-electric within a given pH region. Practical mixtures include as many different ampholyte

species as possible because if the number of species is too small the gradient becomes a series of steps with one pH plateau for each ampholyte species. The pH gradient is always positive all the way from anode to cathode, i.e. the pH increases monotonically in the direction of the current. Although only an empirical relationship, this law of pH monotony (Rilbe 1973) appears always to hold during steady state electrolysis and no local negative pH gradients are ever found. If a complete separation of ampholyte zones occurred there would be regions of water in between them and the local pH would vary between 7 and the various iso-electric points, which would violate this law of pH monotony, so in practice it is found that ampholyte zones overlap and the pH changes smoothly from one iso-electric point to the next.

The next most significant landmark was in 1966 when Vesterberg synthesized artificial mixtures of ampholytes capable of giving good smooth pH gradients. This earlier work has been covered by a number of reviews (e.g. Haglund 1967, 1971; Vesterberg 1971; Rilbe 1973, 1977; Kolin 1977) and will not be discussed further here.

The net molecular charge on a protein, glycoprotein, or lipoprotein is determined by the content on the one hand of acidic groups (carboxyl groups of aspartic, glutamic, or uronic acids or, less commonly, phosphate and sulphate groupings) and on the other hand of basic moieties such as the amino and guanidino groupings of lysine and arginine. Thus most of these macromolecules have iso-electric points somewhere within the pH range 3–11, and in a pH gradient will migrate to their iso-electric points where they will focus in sharp bands. A number of important consequences follow from this. Firstly, as well as separating a number of proteins in a mixture, the iso-electric points of each of them can be very simply determined merely by measuring the pH in the gradient at the position of the focused band. The iso-electric point pI is an important parameter in the characterization of a macromolecule and it is most readily determined by IEF since other methods are more time consuming and require much more material. Secondly, since a protein will migrate from any point in the gradient to its pI the position of sample application is unimportant and often quite dilute sample solutions can be used. For example, in a density-gradient column, sucrose and ampholytes can be added to a dilute sample solution which is then used directly for making up the gradient. Since the focused band only occupies a small volume a concentration factor of a hundred-fold or more is easily achieved in this way. Thirdly, electrophoretic migration virtually ceases at the pI so that the band pattern stabilizes and, within limits, becomes insensitive to experimental variables such as time and applied voltage. Band patterns and measured pI values are therefore highly reproducible. Furthermore, the displacement of any ampholyte molecule, including a sample protein molecule, from its pI induces a net

molecular charge of appropriate sign so that the molecule returns to its iso-electric point. Therefore unlike all other electrophoretic techniques there is no band spreading due to diffusion.

The protein pI measured by IEF is very close to the true iso-ionic point since the ionic strength within the medium of the pH gradient is very low (Vesterberg and Svensson 1966). Resolution of the method is excellent and often exceeds that obtainable in other electrophoretic methods. When unknown mixtures are being examined it is usual to use ampholytes giving a pH gradient with a wide range (e.g. pH 3–10), but once the region of particular interest has been identified narrow-range ampholyte mixtures covering 2 or 3 pH units or less can be used. Resolution in the resulting shallow gradients is often such that two macromolecular species differing in pI by only 0·02 of a unit can be clearly distinguished.

The theoretical basis of resolving power between focused zones in IEF has been summarized by Rilbe (1973) and is based on the fact that the differential equation for IEF represents a balance between electrical and diffusional mass transport so that

$$CuE = D\frac{dC}{dx}$$

where C is the protein concentration, u its mobility in $cm^2\ V^{-1}s^{-1}$, D its diffusion coefficient in cm^2s^{-1}, and E the field strength in $V\ cm^{-1}$ Focused zones are narrow and the field strength within a zone can be taken to be constant so u can be regarded as a linear function of the linear displacement x within the column. Therefore

$$u = -fx \tag{9.27}$$

where f is a proportionality factor. Equation (9.26) can then be written as:

$$dC/C = (Ef/D)x\,dx \tag{9.28}$$

Since E, f and D can be regarded as constants, integration gives

$$C = C(0)\exp(-fEx^2)/2D \tag{9.29}$$

where $C(0)$ is the integration constant (the local concentration maximum at the level where the protein is iso-electric). This expresses a gaussian concentration distribution with inflection points x_i, at

$$x_i = \pm(D/fE)^{1/2} \tag{9.30}$$

The proportionality factor f can be written as a derivative:

$$f = -\frac{du}{dx} = -\frac{du}{d(pH)}\frac{d(pH)}{dx} \tag{9.31}$$

Substituting this in eqn (9.30) we obtain

$$x_i = \pm\frac{D}{-E\,(du/d(pH))\,(d(pH)/dx)} \tag{9.32}$$

Considering now two closely spaced zones, the pH difference ΔpH between them is determined by their separation Δx in the pH gradient:

$$\Delta pH = \Delta x\frac{d(pH)}{dx} \tag{9.33}$$

The separation at which two closely spaced gaussian zones become clearly seen as partly resolved zones is proportional to the inflection points and therefore

$$\Delta x = Kx_i \tag{9.34}$$
$$\Delta(pH) = Kx_i \tag{9.35}$$

where K is a constant which depends upon the precise criteria used to define the point of resolution but is usually given the value $K = 3$. Combining (9.32) with (9.35) gives

$$\Delta(pH) = K\left\{\frac{D(d(pH)/dx)}{-E(du/d(pH))}\right\}^{1/2} \tag{9.36}$$

From this it can be seen that good resolution is aided by a low diffusion coefficient and a high mobility slope ($du/d(pH)$) at the iso-electric point.

In general, proteins satisfy these criteria, but whether peptides can be focused satisfactorily depends chiefly upon the mobility slope at their iso-electric points (Rilbe 1973). Good resolution is also achieved by using a high field strength and a shallow pH gradient, both of these being factors which can be varied by the experimenter within the limits imposed by his apparatus.

The overall resolution achieved does of course also depend upon the subsequent handling and analysis of the focused sample. As soon as the

current is switched off diffusion spreading can occur, so it is important to measure the pH gradient and collect the separated components immediately. With density-gradient columns there is inevitably a slight loss in resolution during the draining or pumping out of the gradient solution and the very acts of collecting fractions or of slicing up gels containing focused gradients are of necessity accompanied by some loss in separating power.

It should be remembered that pI values are temperature dependent, usually decreasing with increasing temperature, so that the pH of fractions or gel slices should be measured at the same temperature as that at which the IEF experiment was performed. This temperature should be stated whenever results are presented (Vesterberg 1971). Many workers have reported pI values taken from measurements of the pH gradient recorded at 20 or 25 °C following IEF at 4 °C. Fredriksson (1977; 1978) has shown that, while pI values for both proteins and ampholytes are temperature dependent, the shifts need not necessarily coincide and errors as high as 0·2 pH may arise, especially for some weakly acidic proteins.

9.2. Iso-electric focusing in density gradients

There are a number of descriptions of small density-gradient stabilized IEF columns of only a few ml volume (e.g. Fawcett 1977; Leaback 1977; Jackson and Russell 1984) and even IEF in capillary tubes of a few μl volume (e.g. Bispink and Neuhoff 1977). However, by far the most widely used types of apparatus are the commercially available 110 ml and 440 ml columns marketed by LKB Produkter AB. Both these columns are of the same basic design, shown diagrammatically in Fig. 9.1, and they are suitable for either analytical or small-scale preparative work. The design is almost identical to the original column design (Vesterberg and Svensson 1966), and the continued popularity of density-gradient IEF with these columns is a tribute to the soundness of this early work.

(a) *Sample loading capacity*

The major factors influencing the sample load-carrying ability in density gradient IEF experiments are the solubility of the protein of interest at its pI, the buffering capacity of the ampholytes at their pIs, and the carrying capacity of the density gradient (Winter and Karlsson 1976).

For analytical purposes Vesterberg (1971) recommends 5 mg or less of protein in a zone in the 110 ml column and up to 25 mg for preparative purposes. The use of a steep pH gradient (wide pH range ampholytes) gives sharp concentrated focused zones, but if zones were less sharp average protein concentrations in the zones would be lower so that more material could be focused without exceeding the solubility limit or the local

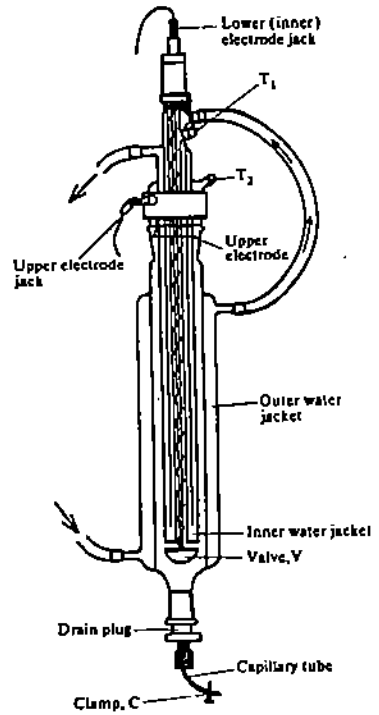

FIG. 9.1. LKB Model 8100 isoelectric focusing column. Arrows show the direction of flow of the cooling water through the outer and then the inner water jackets. The shaded area is the region occupied by the sample and pH gradient; below this and surrounding the inner electrode is the dense electrode solution and above it is the light (upper) electrode solution. Reproduced by kind permission of LKB Produkter AB.

density within the density gradient. While the degree of focusing can be regulated to some extent by changes in the applied voltage, the best way of obtaining relatively broad zones without losing resolution is to use narrow-range ampholytes which give a shallow pH gradient.

the gradient have different viscosities a more constant and controllable flow rate will be achieved if the gradient is pumped out. The pumping rate should be adjusted to 60–100 ml h^{-1}. The column effluent can be passed through a flow cell for monitoring the u.v. absorbance (usually at 280 nm for proteins). Fractions of 1 ml should be collected from the 110 ml column, the collector tube size being such that the pH of each fraction can be measured with a pH meter fitted with a suitable combination electrode. Since it is temperature dependent (Fredriksson 1978), the measurement of the pH gradient in this way should be made at the same temperature as that used for the column run. Uptake of atmospheric CO_2, particularly by fractions in the alkaline region, can cause considerable errors, so pH measurements should be made as soon as possible after collection of the fractions. Delincée and Radola (1977, 1978) for example have reported that a fraction of pH 9·1 falls to 8·8 after standing in air for 16 h and at higher pH errors would be even greater.

If there is heavy precipitate formation in the lower part of the column, emptying from the top by pumping dense sucrose solution into the bottom of the column may be advantageous. Alternatively the gradient can be collected stepwise from the top by pumping out small portions each about 5 mm deep.

For many subsequent analyses the presence of ampholytes and density gradient materials in fractions is not harmful, but in some cases their removal is necessary. This is usually achieved by dialysis, ion-exchange chromatography, collection of the protein by salting out, precipitation, or gel filtration (which should be performed at high ionic strength to reduce possible electrostatic interactions between ampholytes and the protein). If the gel filtration is performed in a volatile buffer (e.g. 0·5 M acetic acid or ammonium hydrogen carbonate) a salt-free sample can be obtained by simple lyophilization.

Traces of ampholytes in protein samples isolated after IEF can be detected by thin-layer chromatography, the protein being applied to a cellulose TLC plate which is developed in a solvent of 10 per cent trichloroacetic acid (TCA), dried and sprayed with ninhydrin (Bloomster and Watson 1981). Proteins do not move from the point of application but ampholytes give a diffuse ninhydrin-positive zone (R_f > 0·5) and as little as 1·25 μg can be detected.

9.3. Iso-electric focusing in polyacrylamide

Polyacrylamide gel is a useful anti-convective medium for IEF experiments and possesses a number of advantages over density gradients in columns, particularly in analytical work. Among the major advantages are the following.

(1) It is much more rapid.
(2) A large number of samples can be run at the same time and compared, particularly if a slab gel system is used (this contrasts with at best 1 sample per 24 h with a gradient column).
(3) Only very simple equipment is needed and this can also be used for other gel electrophoretic techniques.
(4) Compared with the column method only small amounts of expensive ampholytes are needed.

(5) Very small samples consisting of only a few micrograms can be analysed.
(6) It can readily be used for one of the dimensions of two-dimensional separation systems, e.g. for protein mapping or for a subsequent immuno-electrophoretic step.

Resolution is even higher than with density gradient columns since gels can be treated (e.g. fixed, stained, etc.) immediately after focusing with no time-consuming fraction-collection step during which mixing and diffusion can occur (Fig. 9.2). Proteins differing in pI by only 0·0025 of a pH unit have been resolved (Allen, Hurley, and Talamo 1974). The major disadvantage is that in preparative work it is not so easy to recover the focused protein zones, which are recovered readily and almost quantitatively from density-gradient columns. Also in polyacrylamide gel isoelectric focusing (PAGIF) pH gradient drift may be much more severe (see Section 9.3(e)) than in density gradients, where this phenomenon is usually imperceptible.

(a) *Gel composition and apparatus*

Unlike other electrophoretic applications of polyacrylamide gel in which molecular sieving plays an important role in the separation, in PAGIF any sieving effect actively slows down the attainment of the final focused band pattern and hence should be avoided. Thus highly porous gels should be used, and in the case of polyacrylamide gels with T = 5 per cent and C = 3–5 per cent are generally suitable and have quite good mechanical properties. Better mechanical properties can be obtained by using gels with T = 7 per cent and C = 4 per cent, but these should only be used for proteins with molecular weights below about 100 000. For very large proteins gels with T = 3·5 per cent or less are indicated and agarose (Section 9.4) or composite polyacrylamide–agarose gels (Section 6.3) can also be used. Baumann and Chrambach (1976*a*) advocate a stacking-gel type of composition with low T (typically 5 per cent) and high percentage cross-linking (e.g. 15 per cent) but with DATD used in place of Bis. However, it was subsequently shown (Bosisio *et al.* 1980) that when DATD is used as a cross-linker large amounts (up to 80–90 per cent) of the DATD remains unpolymerized in the final gel. This explains many of the curious and undesirable properties sometimes reported with DATD cross-linked gels. In view of the possibility that the gels may contain relatively large amounts of toxic monomer making them potentially unsafe to handle, it would seem that the use of DATD should be avoided for all gel applications. Bis cross-linked gels with T = 5–7 per cent and C = 3–5 per cent do in fact still retain some ability to hinder the migration of even very small molecules and the adoption of formulations similar to those used in

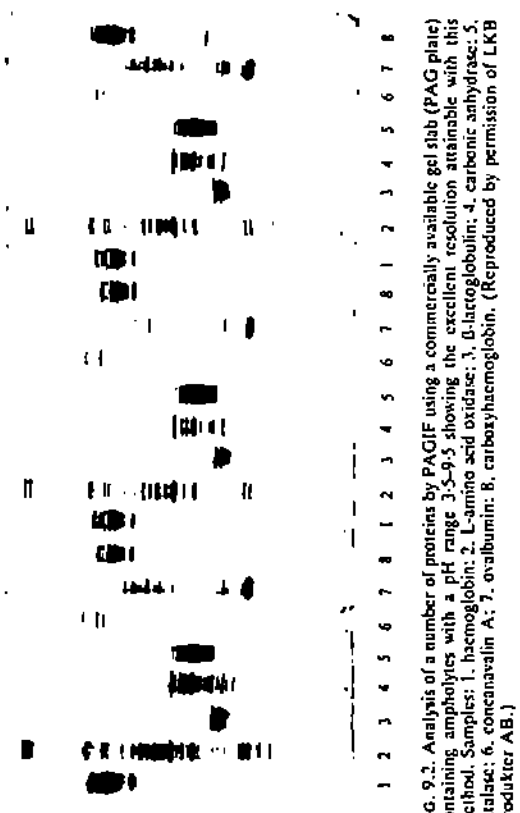

FIG. 9.2. Analysis of a number of proteins by PAGIF using a commercially available gel slab (PAG plate) containing ampholytes with a pH range 3·5–9·5 showing the excellent resolution attainable with this method. Samples: 1, haemoglobin; 2, L-amino acid oxidase; 3, β-lactoglobulin; 4, carbonic anhydrase; 5, catalase; 6, concanavalin A; 7, ovalbumin; 8, carboxyhaemoglobin. (Reproduced by permission of LKB Produkter AB.)

stacking gels in PAGE does seem to offer some advantages. If T is kept constant (and relatively low) pore size increases as C is increased above 5 per cent, and with $T = 6$ per cent gels Bisisio *et al.* (1980) found that using the protein ferritin (MW = 440 000) gels became essentially non-retarding when C reached 30 per cent and running times were halved by comparison with $C = 3$–5 per cent gels. In $T = 6$ per cent and $C = 20$ per cent gels cross-linked with Bis, about 20 per cent of the Bis remained as monomer after 1 h, but after 'ageing' overnight this fell to less than 4 per cent (no such ageing effect was seen with DATD gels). Gels with $C = 30$ per cent are brittle and difficult to handle so Bosisio *et al.* (1980) recommend casting the gels on silanized glass plates to which they adhere and this overcomes most handling problems. For most purposes when very low sieving effects are necessary gels with $T = 5$–7 per cent and $C = 15$–20 per cent probably represent a reasonably good compromize.

Gel preparation is very similar to conventional PAGE procedures (Chapter 2), except that the required amounts of acrylamide and Bis or other cross-linker are dissolved not in buffer but in H_2O or better, in 10 per cent sucrose or sorbitol (see below). TEMED is usually added, although Riley and Coleman (1968) suggest that it can be ommitted as components of the ampholyte mixture can perform the same function. Sufficient ampholyte solution to give a final concentration of 1 per cent is then mixed in and the polymerization catalyst added. Specially prepared narrow-range ampholytes and separators (Sections 9.2(e) and 9.2(f)) can be incorporated if required. Either chemical polymerization with ammonium persulphate or photopolymerization with riboflavin can be used, and the quantities of catalyst and TEMED can be as described earlier (Section 2.6). However, Pharmacia workers (Låås, Olsson and Soderberg 1980) advocate omission of TEMED and careful deaeration of acrylamide solutions which then permits very low levels of ammonium persulphate to be used (200 μl of a solution containing 22·8 mg ml^{-1} for every 30 ml of gel mixture). Deionization of acrylamide solutions before catalyst addition with a mixed-bed ion-exchange resin (Amberlite MB-1 or similar) was also claimed to be beneficial. When very acidic ampholytes (e.g. pH range 2·5–4) are used with conventional amounts of TEMED and persulphate (and no deaeration step), it may be necessary to add a small amount of sodium sulphite to achieve polymerization in a reasonable length of time (see Section 2.7). For very alkaline ranges (e.g. pH 7–10) photopolymerization may be unsatisfactory. Many workers (e.g. Chrambach *et al.* 1973; Karlsson, Davies, Öhman, and Andersson 1973) advocate the addition of 10–12·5 per cent glycerol, sucrose, or sorbitol to the gel mixture to improve mechanical stability and wall adherence of the gel in glass tubes. It also greatly reduces pH gradient drift, so its use is to be recommended. Additions of urea also increase viscosity and have similar beneficial effects.

Uncharged thiol-protecting reagents and high levels of urea (e.g. up to 8 M) to cause protein dissociation can be incorporated in PAGIF gels without difficulty.

The apparatus used for PAGIF is identical to that used for PAGE. The simple and inexpensive cylindrical gel rod or vertical or horizontal slab systems can be used, and are set up as described in Chapter 2. All systems have their particular merits and their devotees, but the most widely used are the simple rod systems, which are popular for the first dimension of two-dimensional protein mapping (see Chapter 11), and the horizontal slab configuration. Since the focused bands become almost stationary in PAGIF, electrodecantation and difficulties with uneven cooling are not encountered. Consequently this is a technique in which either a simple design of horizontal slab apparatus or more sophisticated forms such as the LKB 2117 Multiphor and 2217 Ultraphor, the Shandon Model 600, the Desaphor or Mediphor from Desaga, and the Pharmacia FBE 3000 Flat Bed Apparatus possess a number of advantages. These include (1) a ready and direct comparison of a number of samples run under identical conditions, (2) simple handling of a single slab instead of a number of gel rods, (3) the ability to vary the length and thickness of the gel slab, (4) ready access to the gel slab so that the sample can be positioned anywhere, either before or after establishing the pH gradient, (5) simple measurement of the pH gradient using a microelectrode touched on to the gel surface, and (6) easy measurement of the voltage gradient if desired using voltage probes. Premanufactured gel slabs suitable for use on horizontal apparatus and containing ampholytes with various pH ranges are available commercially (e.g. LKB, Serva, etc.).

Manufacturers of horizontal slab gel apparatus generally include all the necessary accessories in terms of gel moulds, stands, clamps, instructions, etc. required for the preparation of PAGIF slabs. Naturally the details differ from one manufacturer to the next, but there is a clear trend towards thinner gels and those of 2–3 mm thickness have now been largely superceded as the 'standard' size by 0·5–1·0-mm-thick gels. Since for a given voltage and gel composition current is proportional to cross-sectional area, it follows that reducing gel thickness reduces current and hence heat generation. Cooling is also usually more efficient with thin gels, so that for both reasons they can be run at much higher voltages (field strengths) than thicker gels, which greatly improves resolution and also gives faster separation times. Staining and destaining are also more rapid and of course less reagents, including expensive ampholytes, are used so the use of thin gels has considerable advantages. Thin gels are relatively easily torn or distorted so it is usual practice to support the gel either on a plastic film (e.g. LKB PAG Moulding Sheet or cellophane) or on a silanized glass plate. For this the clean glass plate to be used in the moulding chamber is

dipped for a few minutes in a 0·1 per cent solution of Silane A-174 in acetone and then allowed to air dry leaving a film of silane molecules on the plate (Bosisio *et al.* 1980). These contain reactive double bonds so the polyacrylamide gel is chemically bound to the glass plate and washing with steel wool is required if the glass is to be reused. The silane solution is stable for several months if kept dry at 4 °C in the dark.

In a typical arrangement for 1 mm thick gels (such as the Pharmacia system) the mould is assembled as shown in Fig. 9.3. The glass plate on which the gel will eventually be supported is laid flat and, if the above silanization is not used, it is wetted with a few drops of water and a piece of PAG Moulding Sheet applied. Air bubbles trapped between the sheet and the glass are removed by rolling with a rubber roller. The spacer gasket is placed in position followed by the top glass plate. If required, the inside face of this can be lightly coated with silicone fluid to prevent the gel sticking when the mould is disassembled. The mould is then clamped together, placed in a vertical position, and filled with the gel mixture using a syringe fitted with a relatively fine needle by lifting a small portion of the gasket specifically made with a small flap for the purpose. With other designs a U-shaped gasket along three sides of the mould is used and the fourth side sealed with adhesive tape after filling. A typical recipe for 30 ml of a gel mixture giving a $T = 5$ per cent, $C = 4$ per cent gel is shown in Table 9.3. The acrylamide, Bis and glycerol (or 3 g sucrose for acid pH ranges or 3 g sorbitol for alkaline pH ranges) are dissolved in H_2O and stirred for a few minutes with about 0·3 g of mixed bed ion-exchange resin (e.g. Ambertite MB-1) to remove traces of acrylic acid which can give rise to excessive gradient drift. If stock solutions of acrylamide and Bis are routinely made up and kept for a period of time, as is commonly the case, ion-exchange resin should be present in the stock bottle, but if solutions are freshly prepared it may be possible to omit ion-exchange treatment altogether. The gel mixture is then deaerated with a water pump, the ampholytes, TEMED and persulphate added and the mixture placed in the gel mould. Due to the toxicity of acrylamide and Bis monomers (see p. 9) gloves should be worn and care taken to avoid skin contact throughout these manipulations. If polymerization is uneven it

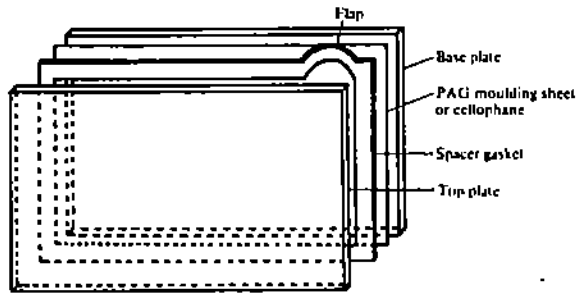

Fig 9.3. PAGIF gel mould assembly.

TABLE 9.3
Typical gel formulation required for PAGIF slab gels

Acrylamide	1·44 g
Bis	60 mg
Glycerol	3·0 ml
H_2O	to 27·8 ml
Ampholytes	2·0 ml
TEMED	10 µl
Ammonium persulphate (35 mg ml^{-1})	200 µl

may prove beneficial to flush the mould with nitrogen before adding the gel mixture.

With this type of apparatus it is very easy to make gels of almost any thickness by simply using gaskets of different thicknesses. While Neuhoff and Radola and their co-workers have been particularly active in the very successful development of ultra-thin gels of 50 or 100 µm thickness (see Section 12.1), most of the advantages of using thin gels can be gained with slightly thicker gels which are rather easier to prepare and handle. A procedure first introduced by Görg, Postel, and Westermeier (1978) has since become quite widely used. For this the gasket in an arrangement such as that described above (they used LKB Multiphor equipment) is replaced by a 'home-made' gasket cut from one, two, or three layers of Parafilm (50 cm sheet) around three sides of the mould. This gives gels of 0·12, 0·24, or 0·36 mm thickness respectively. They used gels mounted on LKB PAG Moulding sheet (applied to one glass plate as above) or cellophane which was soaked in distilled water for a few minutes, carefully spread wet on the glass plate, and dried. As it dries out it straightens into a wrinkle-free layer. In order to fill the mould two paper clips are inserted into the open side in order to make room for inserting a syringe needle and the mould about half filled with gel mixture. The paper clips are withdrawn and the clamps holding the mould together then squeeze the glass plates towards each other causing the gel mixture to spread and fill the entire mould. After polymerization they recommend that the mould is cooled for a few minutes, before opening by inserting a small spatula or knife and gently twisting it. A thin film of silicone liquid applied beforehand to the top plate to aid separation of the glass without tearing the gel may be particularly helpful with such thin gels. Commercial equipment for preparing thin gels with thicknesses ranging from 0·1 mm to 0·5 mm is now available (e.g. LKB Ultro Mould No. 2217-200). Some indication of the improved resolution obtained with thin gels, as opposed to those 1 mm thick, can be gauged from Fig. 9.4.

(b) *Sample application and focusing*

The sample should be relatively salt free (see Section 9.2(h)) as excessive amounts of salt lead to poor separations with 'wavy' bands. When the gel rod system is used it is better if the sample is dissolved in a solution of 1 per cent ampholytes containing 10 per cent sucrose and then layered on top of the gels as for PAGIF. This is then carefully overlaid with a further 5–10 mm depth of a solution of 1 per cent ampholytes containing 5 per cent sucrose, before filling the upper electrode

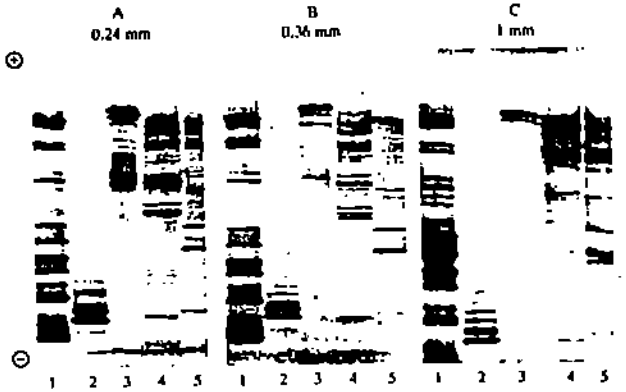

FIG 9.4. The influence of gel thickness on the resolution obtainable during separation of proteins by PAGIF on T = 7 per cent, C = 2·5 per cent gels using Servalyt pH 2–11 range ampholytes followed by Coomassie Brilliant Blue G-250 staining. (A) Gel of 0·24 mm thickness, (B) 0·36-mm-thick gel and (C) 1·0-mm-thick gel. Lane (1) standard proteins, (2) trypsin, (3) soyabean lipoxidase, (4) and (5) legume seed proteins. (Reproduced from Görg, Postel and Westermeier (1978) by permission of the authors and publishers.)

chamber, to protect the sample from the extreme pH of the electrode solution. With the horizontal slab apparatus the sample can either be applied to wells preformed in the slab, or more usually is impregnated into small pieces of filter paper laid directly on the surface of the slab.

With the apparatus for gel rods the electrode chambers are filled with 0·5 M NaOH, ethanolamine, or ethylenediamine in the cathode compartment and 0·5 M H_2SO_4 or H_3PO_4 in the anode compartment, but 1 per cent ampholytes of suitably acidic or basic pH ranges can also be used for either or both electrode solutions and produce less extreme pH changes at the ends of the gel. Sponge or paper strips impregnated with these solutions are usually used in horizontal slab apparatus. Pharmacia Fine Chemicals AB recommend the use of 0·04 M glutamic acid in the anode compartment when acidic pH ranges are being investigated (0·1 M H_2SO_4 for the most acidic pH 2·5–5 range), 0·2 M HEPES when alkaline ranges are being used and 0·04 M aspartic acid for the broad pH 3–10 range. The cathode solution suggested is 1 M NaOH for alkaline pH ranges and pH 3–10, 0·2 M L-histidine for ranges below pH 6·5 and 0·2 M NaOH for the pH 5–8 range.

Various electrical conditions have been employed with gel rods, but the choice is not usually critical. Wellner and Hayes (1973) used rods 100 mm long and applied 120 V for 9 h, whereas Chrambach *et al.* (1973) used various times and voltages up to 800 V. Wrigley (1971) advocated that the voltage should be increased gradually up to a maximum of about 400 V with 65-mm-long rods and the current should not

exceed 2 mA per rod. With salt-free samples the gradient formed in about 15 min under these conditions while focusing of proteins could take up to 2 h. When a cooled horizontal slab apparatus is used with 1–2 mm thick gels a power of up to 40–60 W can be applied, which means initial voltages in the region of 200–400 V or more and final voltages in the region of at least 1000–1200 V, so that typical running times are 2 h or less.

PAGIF gels are often run with coolant circulating at 4–10 °C, although cold water at about 15 °C is generally adequate, and indeed is to be preferred if the gel contains high concentrations of urea (to inhibit aggregation or to encourage dissociation of protein complexes) as this may crystallize out at low temperatures. Since resolution and zone sharpness are improved by a steep voltage gradient (see p. 249) it is preferable to use the highest voltage commensurate with the cooling capability of the apparatus, and voltages of up to 3000 V (averaging about 300 V cm^{-1}) have been claimed to be advantageous by Lläs *et al.* (1980). However, resolution only varies according to the square root of the field strength (eqn 9.36) so very high voltages are not obligatory. With thin gels high voltages can be applied initially and increased more rapidly than when relatively thick gels are employed, so separation times can be reduced to 1 h or less.

It is often convenient to have a visual marker of the progress of the analysis and for this we have found it most useful to apply samples of haemoglobin (or any other coloured protein) to the end sample positions on the PAGIF slab. If further samples of haemoglobin (pI ≈ 6·8) are also applied in a matching position on the opposite side of the slab the two samples will migrate towards one another and when focusing is complete they should coalesce. Making allowance for the fact that haemoglobin is a relatively small protein which may reach equilibrium before some of the larger proteins that may be present in the sample, focusing should be continued for a short time after this before switching off. Cleeve and Tua (1984) have used bovine serum albumin prestained with bromocresol green (pI ≈ 4·6) as a visual marker in the same way.

The most common problem associated with PAGIF is the occurrence of distortions in the pH gradient. This produces wavy bands in the focused pattern and can be caused by poor electrical contact between electrodes and electrode strips, variations in gel slab thickness, uneven wetting of electrode strips, and insufficient cooling leading to local hot spots. The use of incorrect or inappropriate electrode solutions can also give rise to such distortions. The most common cause however is the presence of too much salt in the gel or in the samples. It is important therefore not to use excessive amounts of catalyst for gel polymerization and if necessary samples should be desalted by gel filtration or dialysis against water or ampholyte solution. With thin gels polymerized onto plastic backing sheets it is particularly easy to overcome problems caused by residual polymerization catalysts by simple washing procedures (Eckerzall and Connor, 1984). The gels can be prepared with acrylamide and Bis dissolved in H_2O or buffer and any of the catalyst systems described for PAGE gels (Chapter 2). Buffer and catalyst salts are washed out by immersing in several changes of H_2O and the gels are then transferred to a solution of ampholytes in H_2O and allowed to equilibrate.

Some biologically sensitive samples and enzymes are relatively unstable at the low ionic strengths prevailing in IEF separations, particularly at pH values close to their isoelectric points, and in these cases it may be beneficial to pre-run the PAGIF slab for 30–60 min, so that the pH gradient is established before the samples are applied and residence times can be kept to a minimum.

(c) *Determination of the pH gradient*

After focusing, the gel rods are sliced into pieces 2·5–5·0 mm long and each slice is chopped up or homogenized in a small volume (e.g. 1·0 ml) of deionized water. The pH is then measured directly with a pH meter. This same method can be used with pieces cut from a gel slab, but in this case it is quicker and more simple to measure the pH directly using a surface electrode (e.g. Ingold Type 403–30–M8 or similar). Since pH values are to some extent temperature dependent it is important that these measurements are made at the same temperature as that used during the IEF separation. Measurements at 4 °C give lower pI values than those at 25 °C and differences can be substantial, being about 0·1 pH unit at pI = 4, and of 0·5 pH unit or more above pI = 9. Fredriksson (1978) has published tables enabling appropriate corrections to be made for this temperature effect but the addition of agents such as sucrose, urea, glycerol, sorbitol, etc., can also alter the pK of ionizable groups, so it is a good rule always to measure pH (pI) values under precisely the same conditions as occur during the actual separation.

Interference in pH measurements from absorption of atmospheric CO_2 has already been referred to in Section 9.2(j) and this is potentially more troublesome in gel IEF techniques, particulary in horizontal gel slabs or granular flat beds (Section 9.5). If this is a cause of difficulty then the experiments can be performed in a nitrogen atmosphere, as for example in the PAGIF studies on histones by Valkonen and Piha (1980), and some designs of commercial equipment (e.g. LKB 2217 Ultrophor) have provision for this. If use of a CO_2-free atmosphere is not practical, or simply as a more convenient alternative, a number of marker proteins of known pI can be focused on the same gel slab. The pI of the unknown is then determined from a 'standard plot', with extrapolation if necessary. Some suitable marker proteins which are commercially available in high purity are shown in Table 9.4. IEF calibration kits of mixtures of marker proteins of known pI are now available commercially (e.g. Pharmacia Fine Chemicals AB). Very extensive tables giving pI values and molecular weights of over 1200 different proteins have been published (Righetti and Caravaggio 1976; Righetti, Tudor, and Ek 1981).

Among the advantages of ITP, particularly analytical ITP in capillary tubes, are rapid analysis times, high sensitivity and reproducibility, and good resolution combined with easy quantitative measurements. Difficulties include assessing whether steady state stacking has been achieved, the formation of mixed zones, the number of factors to be taken into account for optimizing the separation, establishing the identities of particular zones or peaks, and the choice of suitable spacer ions. In the field of protein chemistry ITP has the advantages over IEF that proteins and enzymes are not necessarily exposed to environments of very low ionic strength or concentrated at their iso-electric points (which may lead to precipitation or inactivation). Unfortunately, however, few individual spacer ions exist in the mobility range of proteins, and the application of highly complex spacers such as Ampholines which contain 500 or more constituents delays attainment of the steady state and also usually results only in mixed zone separations (Nguyen and Chrambach 1979). Therefore until better spacer ions are found or produced it seems unlikely that ITP will be applied as extensively as IEF and other gel electrophoretic methods, at least within the field of macromolecular separations.

When samples contain small molecules of interest or are mixtures of large and small molecules, even at this relatively early stage of development the use of ITP may provide information which is more difficult to obtain by other methods. Good examples of this are its use in studies of the periodate oxidation of carbohydrates where quantitative measurements can be made even with limited amounts of material (Honda, Wakasa, Terao, and Kakehi 1979; Oku, Hirotsune, and Shigeta 1979), in measuring the concentrations in blood serum of metabolites such as uric acid (Verheggen *et al.* 1980) or of drugs such as valproate (Mikkers *et al.* 1980), the concentrations of citrate (Tschöpe and Ritz 1980) or of purines and pyrimidines (Oerlemans *et al.* 1981) in urine, and in studies on the composition of venoms (Einarsson and Moberg 1981). Using the known pK_a and absolute mobility (m_0) values of all buffer constituents and the sample ions Hirokawa *et al.* (1984) have been able to assess separation conditions for anaesthetic metabolites in urine by computer simulation, the results being in good agreement with observed experimental separations.

11

TWO-DIMENSIONAL ELECTROPHORESIS

Two-dimensional (2D) peptide maps (fingerprints) have been accepted and used for many years in studies involving the structure of proteins or their modification. In a similar way 2D macromolecular maps of intact proteins, nucleic acids, or polysaccharides can be most useful for the characterization of tissues, biological fluids, extracts of tissues and organs, or any other unknown mixture containing these constituents. They are also useful, or potentially useful, for identification purposes (e.g. taxonomy, forensic work, etc), for studying genetic variation and relationships, for the detection of stages in cellular differentiation and studies of growth cycles, for the examination of pathological states and the diagnosis of disease, and for many other purposes.

Nearly all the methods described in previous chapters can be regarded as single dimension macromolecular maps, and under the most favourable circumstances perhaps as many as 50–60 different components can be resolved, particularly when polyacrylamide gradient gels or IEF gels are used. However, it is more usual for resolution to be limited to 30–40 components because in most biological systems the various components are usually present in widely differing concentrations. This means that it is often not possible to apply sufficient sample for the detection of minor components without grossly overloading the major constituents. It may also be difficult to detect a minor component if it is very close to a major one. These considerations also apply to 2D maps, but here the problems associated with the reproducibility of band patterns (between samples, between experiments, and interlaboratory variation) and identification of individual components become much more demanding because by using high resolution steps for both dimensions it is possible to resolve several thousand protein zones on a single gel. Not surprisingly therefore many recent developments in 2D mapping have been related to the acquisition and handling of data.

The literature contains a great many different two-dimensional (2D) electrophoresis procedures, separating molecules on the basis of various different parameters and aimed at solving a wide variety of problems. A number of these methods have already been referred to in earlier chapters (e.g. transverse gradient gel electrophoresis in Chapter 4, peptide mapping in Chapter 5, many of the immunoelectrophoretic methods described in Chapter 8, etc), while others are discussed in Chapter 12 (e.g. methods on paper, cellulose acetate, thin-layers, etc.). Rightly or wrongly, few of these are usually thought of as 2D macromolecular maps and this term is usually

buffer containing 10 per cent glycerol, 0·1 per cent SDS, and 0·001 per cent Bromophenol Blue is applied across the top of the gel slab. With the standard 11 cm wide gel, this corresponds to about 0·6 ml of proteinase solution and appropriate concentrations were about 0·1 $\mu g\ ml^{-1}$ for papain (25 units mg^{-1}), 0·3 $\mu g\ ml^{-1}$ for Staphylococcus aureus V8 protease (560 casein units mg^{-1}) or 1·25 $\mu g\ ml^{-1}$ for α-chymotrypsin. Electrophoresis was performed at 25 V for 15 min and then 75 V until the Bromophenol Blue reached a position 2·5 mm above the start of the resolving gel. The power was then switched off and the slab allowed to stand for 30 min to permit proteolytic digestion of sample proteins within the sample stack. After this, electrophoresis was resumed in the usual way to separate the peptides generated. Slabs were stained with Coomassie Blue R250 or by silver staining. Dansylation (or presumably any other fluorescent labelling) of the initial proteins was found to be very helpful in that it enabled the progress of digestion and separation of peptides to be very easily monitored by simple u.v. illumination. A very similar approach to peptide mapping was used by Lonsdale-Eccles *et al.* (1981) in which protein-containing sample strips from the first stage SDS–PAGE, in a T = 8·75 per cent slab, were equilibrated with 70 per cent formic acid, incubated with 5 per cent cyanogen bromide in 70 per cent formic acid for 1 h under N_2 and washed repeatedly with 10 per cent acetic acid to remove excess cyanogen bromide. This gel was then reequilibrated with Laemmli (1970) stacking gel buffer, before being applied across the top of the stacking gel for a second stage SDS–PAGE separation of the peptides generated, using a T = 10–15 per cent polyacrylamide gradient slab gel.

As can be seen therefore many different types of two-stage electrophoretic separations can be devised to suit various separation problems, but in essence the two stages are no different to the one-dimensional procedures described in other chapters. Not surprisingly, the apparatus used and methods for staining and destaining, flourescent or radiolabelling, scanning, autoradiography, data handling, etc., are the same as those used in one-dimensional procedures.

11.2 Two-dimensional (2D) separations: General considerations and apparatus

Two-dimensional maps can be prepared using virtually any combination of the one-dimensional methods already described. Clearly, if the method used for the second dimension separates components on a basis similar to that of the first-dimensional separation (e.g. PAGE at two different T or pH values), the components will tend to be clustered about the diagonal and resolution will be less than optimal. In general, the best maps are obtained when the basis for separation is different in the two dimensions (e.g. mainly on a size basis in one dimension and according to charge in the other), since this results in a more even distribution of components over the surface of the map. Fractionation on the basis of molecular charge differences can be achieved with IEF or ITP, particularly when these are conducted in relatively non-restrictive media. Electrophoresis on paper, cellulose acetate, agarose gels, composite polyacrylamide–agarose gels, and highly cross-linked ($C > 15$ per cent) polyacrylamide gels of very low T (minimum pore size is attained with 5 per cent cross-linking so that gels with high C are actually less restrictive than gels with C = 2–10 per cent) also give separations in which the molecular charge factor predominates. Molecular size differences are important in PAGE performed in gels of high concentration T, in gels with a polyacrylamide concentration gradient (gradient gels), and particularly in SDS–PAGE. Some degree of separation due to differences in hydrophobicity (Section 5.9(e)) can be achieved by incorporating a non-ionic detergent such as Triton X-100 and urea into the gel, since this binds to and retards hydrophobic proteins (Hoffman and Dowben 1978*b*; Fernandes, Nardi, and Franklin 1978).

A frequent approach to the preparation of 2D maps involves running the first-dimensional separation in a cylindrical gel rod of 3–6 mm diameter. The gel rod is then placed across the top of a slab gel for the second-dimensional separation at right angles to the first. The rod is sealed in place with either a little agarose gel or polyacrylamide gel made up in the appropriate buffer. With most designs of vertical slab gel apparatus the positioning of the gel rod across the top is relatively simple, especially since it is not necessary for the rod to be precisely aligned over the slab, but if desired special adapters to aid positioning can be made (e.g. Hoffman and Dowben 1978*a*). If a multiphasic buffer system is to be employed for the second dimension, the gel rod should if possible be equilibrated by washing in stacking gel buffer and should be embedded in stacking gel. This should also extend below the rod so that components migrate through 10–20 mm of stacking gel before entering the separation gel phase.

If continuous buffer systems are used in the second dimension, the diameter of the gel rod from the first dimension should not be greatly different from the thickness of the gel slab. For example with 6 mm diameter gel rods, 3 mm thick slabs give satisfactory results but when 1 mm slabs are used patterns are more blurred (Hoffman and Dowben, 1978*a*). With multiphasic buffer systems greater size differences can be tolerated owing to the zone-sharpening effects. Thin (1 mm) slabs are more readily dried for subsequent storage or autoradiography than thick ones and often give higher resolution due to greater permissible voltage gradients and shorter running times because of the more efficient dissipation of heat, but they are a little more difficult to handle.

Very often however a gel slab configuration is used for the first dimension separation as well as the second. In this case strips of the first dimension gel are cut out and inserted across the top of the second dimension slab in much the same way as if rod gels were used. When a continuous buffer system is used for the second dimension this sample gel slice (or indeed gel rod when one of them is used) should preferably be

soaked in 10-fold diluted buffer for a few minutes to reduce its conductivity and aid zone sharpening (see p. 80), while for discontinuous systems stacking gel buffer should be used. For best resolution a relatively narrow (i.e. 1–3 mm wide) strip of gel should be cut out of the first dimension gel, although if zone sharpening or stacking is adequate this is not a very critical point. It is usual for the strip to be of the same thickness as the second dimension gel (it will often have been run in the same apparatus), so the strip is simply inserted between the glass plates and pushed down either onto the surface of the gel (for continuous buffer systems) or to within 10–20 mm of the surface (for multiphasic systems), and sealed in place with 1 per cent agarose in diluted buffer for the former case or with stacking gel in the latter. This is slightly different from the rod gel arrangement where it is normal for the rod to be placed across the top of the glass plates forming the gel mould rather than being pushed down between them. Indeed many workers advocate the bevelling of the tops of the plates inwards so that the gel rods rest in a trough.

It will be apparent from the above that the apparatus used for 2D separations is the same as that described in Chapter 2. Since slab gel apparatus is obligatory for the second dimension, and quite often used for the first dimension as well, the smaller types of power supply are not really adequate and units should be capable of 500 V and a minimum of 150 mA, but preferably of 250 mA or more.

11.3 High resolution two-dimensional (2D) procedures

Since virtually any electrophoretic method except those performed in free solution can be employed for either dimension a great variety of 2D separation methods are possible and most of them have indeed been tried at one time or another. As stated above sample components are spread over a 2D map surface most evenly when the basis of separation is different for each dimension, and clearly for the highest possible overall resolution methods of high resolution should be chosen for each step. Since no high resolution method separating purely on the basis of differences in hydrophobicity exists at the present time, the choice is narrowed to size fractionation in one dimension and charge fractionation in the other. The method of highest resolution currently available for the former is SDS–PAGE (including SDS–PGGE) and for the latter is IEF in polyacrylamide gels (PAGIF) or agarose gels (AGIF).

While this combination of separation methods was used at quite an early stage in the development of 2D macromolecular mapping it was the elegant work of O'Farrell (1975) that really demonstrated the full capabilities of this approach. He was able to resolve about 1100 different proteins from lysed *Escherichia coli* cells on a single 2D map (Fig. 11.1) and suggested

FIG. 11.1 Two-dimensional mapping of *E. coli* proteins. Approximately 10 μg (in 25 μl) of ^{14}C-labelled proteins (about 100 000 counts min^{-1}) were separated by IEF in the first dimension (left to right) and by SDS–PAGE on a 9.25–14.4 per cent exponential acrylamide concentration gradient gel in the second dimension (run top to bottom) followed by autoradiography with an 825 h exposure time. Over 1000 individual spots could be counted on the original autoradiogram. (Reproduced from O'Farrell (1975) by permission of the author and publishers.)

that the maximum resolution capability may be as high as 5000 different proteins. Apart from meticulous attention to detail, major reasons for the advance in resolution obtained by O'Farrell compared to earlier workers included the use of samples labelled with ^{14}C or ^{35}S to high specific activity, and the use of thin (0·8 mm) gel slabs for the second dimension which could then be dried down easily before autoradiography. This means that labelled zones in the gel are physically very close to the emulsion of the autoradiographic film, so reducing the area of film exposed to the radiation from any one zone and keeping loss of resolution to a minimum. Autoradiography was able to detect protein zones corresponding to one part in 10^7 of the sample (usually 1–20 μg was applied initially, as higher loads caused zone spreading, although up to 100 μg could be applied). Coomassie Blue R250 staining was about three orders of magnitude less sensitive (about 0·01 μg in an individual spot) and only about 400 *E. coli* proteins could be detected. Since the procedure was intended for the total analysis of proteins, denaturing agents capable of solubilizing most proteins were present in both dimensions, urea and non-ionic detergent for the first (IEF) dimension and SDS for the second. The system does of course enable both the isoelectric point (pI) and molecular weight (MW) of any particular zone to be measured, but since the system is denaturing this will of course be the subunit pI and MW for any multimeric protein rather than the native values.

Nucleic acids can interfere with IEF separation by interacting with basic proteins and ampholytes and while this is not usually serious, it may be beneficial to remove them by selective precipitation or extraction, isopycnic centrifugation, ion-exchange chromatography, or by digestion with nucleases. O'Farrell used the last of these methods, and suspended centrifuge pellets of *E. coli* cells in 100 µl of 0·01 M tris–HCl buffer pH 7·4 containing 5 mM $MgCl_2$ and pancreatic RNase (50 $\mu g\ ml^{-1}$), sonicated them, added DNase to a level equivalent to 50 $\mu g\ ml^{-1}$, and allowed the mixture to stand on ice for 5 min. He then added solid urea to 9 M and diluted the mixture 1:1 with a lysis solution of 9·5 M urea containing 2 per cent Nonidet P-40, 5 per cent 2-mercaptoethanol, and 2 per cent Ampholines (made up of 1·6 per cent pH range 5–7 and 0·4 per cent pH 3–10). Samples should be run immediately or frozen until required. Ideally 25 µl samples should be applied to the first dimension gel and should contain at least 100 000 cpm and less than 40 µg protein.

For the first dimension O'Farrell (1975) used gel rods prepared in glass tubes 130 mm long and 2·5 mm in diameter. The constituents required to make 10 ml of gel mixture are shown in Table 11.1 and these (less the TEMED) were mixed in a flask, deaerated for 1 min, the TEMED added, and the solution loaded into the tubes to within 5 mm of the top. The gel mixture was overlaid with a little 8 M urea solution. After about 1 h (the gels should have polymerized well before this) the urea solution was replaced by a little lysis solution and the gels allowed to stand for a further hour or more. Small pieces of dialysis membrane were then fixed over the lower ends of the tubes with rubber bands and the tubes placed in the electrophoresis apparatus. Fresh portions (~20 µl) of lysis solution were added to the tops of the tubes which were then filled with 0.02 M NaOH and the apparatus

TABLE 11.1
Constituents for the first dimension PAGIF gel (O'Farrell system)

Constituent	Amount
Urea	5.5 g
30 per cent acrylamide solution: 28.38 per cent (w/v) acrylamide + 1·62 per cent Bis in H_2O	1.33 ml
Nonidet P-40 (10 per cent w/v in H_2O)	2·0 ml
Ampholines, pH 5–7	0.4 ml
Ampholines, pH 3–10	0.1 ml
H_2O	1.97 ml
Ammonium persulphate (10 per cent in H_2O)	10 µl
TEMED	7 µl

filled with buffers, using 0·01 M H_3PO_4 in the lower reservoir and degassed (to remove CO_2) 0·02 M NaOH in the upper reservoir. Gels were then prerun at 200 V for 15 min, 300 V for 30 min and finally 400 V for 30 min. The power was switched off and the upper buffer reservoir emptied and lysis buffer and NaOH removed from the top of the gel. The sample was then applied, overlaid with 10 µl of 9 M urea containing 0·8 per cent pH 5–7 Ampholines and 0·2 per cent of the pH 3–10 range. The tube was carefully filled with 0·02 M NaOH and the buffer reservoir also filled with this. Gels were run at 400 V for 12 h followed by 800 V for 1 h (or at least 5000 volt-hours). The gels were pushed out of the glass tubes by pressure on a syringe connected to the tube with a length of plastic tubing and then shaken for 30–120 min in 5 ml of second dimension SDS sample buffer.

For the second dimension O'Farrell (1975) used the buffer system of Laemmli (1970), so for slabs of uniform concentration running gel the recipe shown in Table 5.4 is used. The amounts of acrylamide and Bis used for the running gel could be varied easily if desired and O'Farrell used gels between T = 5 per cent and T = 22·5 per cent. He obtained better results using an exponential concave concentration gradient, generally of T = 9–15 or 10–14 per cent. To prepare these he used a simple two-chambered gradient mixer where the volume of the front chamber was kept constant by plugging with a stopper (see p.106). Glycerol was added to 70 per cent to the heavy acrylamide solution to increase its density. O'Farrell degassed both the light and heavy solutions and halved the level of catalysts shown in Table 5.4. The running gel mixture was pumped into the gel mould until about 25 mm from the top, then overlaid with H_2O and allowed to polymerize. The overlaying H_2O was removed and replaced with running gel buffer diluted four-fold and the gel stored overnight. The gel mould was then filled with stacking gel mixture and the top covered with a plastic strip, taking care to trap no air bubbles underneath, in order to ensure a flat top surface to the gel. When the stacking gel has polymerized the plastic strip is removed and the first dimension gel laid along the top of it and sealed in place with 1 per cent agarose gel. This is made by warming the agarose to 80 °C in SDS sample buffer (0·0625 M tris-HCl pH 6·8 containing 2·3 per cent SDS, 5 per cent 2-mercaptoethanol, and 10 per cent glycerol). After about 5 min to allow the agarose to gel the apparatus is filled with buffer, 40–50 µl of 0·1 per cent Bromophenol Blue added to the upper reservoir as tracking dye, and gels then subjected to electrophoresis until this dye reached the bottom of the

gel. O'Farrell used a constant current of 20 mA with his slabs which were 134 mm wide, 146 mm long, and 0·8 mm thick and electrophoresis took about 5 h.

The two major ways in which sample proteins can be lost in the O'Farrell procedure are in the IEF stage where some proteins may not enter the gel (the additions of urea and Nonidet P-40 are designed to keep this to a minimum) or may migrate off the basic end (e.g. basic proteins such as histones, ribosomal proteins, etc.) and in the equilibration of the IEF gel prior to running on the SDS gel. The problem of basic proteins is discussed below. Depending on the identity of the protein and duration of equilibration losses resulting from this cause can be 5–25 per cent. This can be eliminated by omitting the equilibration altogether, although it may result in slight streaking in the SDS dimension. To minimize this O'Farrell (1975) recommends that the stacking gel of the SDS slab be increased from 25 mm in depth to 50 mm and that gels are run at 20 mA initially with 2 per cent SDS in the upper reservoir buffer instead of the usual 0·1 per cent. After about 20 min this buffer is replaced with the usual 0·1 per cent SDS buffer.

Since its original publication O'Farrell's technique for 2D analysis has been very widely followed, often with some minor modifications to suit the particular sample being examined. Thus the concentration and particularly the pH range of Ampholytes chosen for the first dimension should be adjusted to give a good distribution of components along the gel, and urea and non-ionic detergent concentrations can also be modified depending upon the samples or even omitted altogether if desired. On most occasions a series of preliminary experiments will be needed to establish the best conditions. The presence of urea generally unfolds sample proteins however and facilitates the binding of the maximum amount of SDS during equilibration before the second dimension, so that prolonged incubation or heating with SDS, as routinely used in one-dimensional SDS–PAGE experiments, is not necessary. Some workers (e.g. Burghes *et al.* 1982) have used slab gels for the first dimension PAGIF separation and applied slices to the top of the second dimension slab. Modifications to the size and acrylamide concentration of the SDS slab are also often made. Ho *et al.* (1979) used small commercially available gradient gel slabs (Gradipore) for the second dimension for example, whilst Johnson (1982) used slabs of almost double length (305 mm), running gel buffer adjusted to pH 8·3, not the usual 8·8, and a T = 10–20 per cent gradient which he claimed gave improved resolution, particularly of small MW proteins, when unequilibrated IEF gels were employed. Factors affecting the quality and resolution of 2D protein maps have been discussed by a number of workers (e.g. Tracy *et al.* 1982*b*; Burghes *et al.* 1982; Duncan and Hershey 1984), as a result of which it appears that it is probably beneficial to reduce the concentration of acrylamide used for the IEF gel to T = 3·5 per cent, to run the IEF gel at 800–1000 V for at least 10 000 volt/hours (but probably not more than about 15 000 volt/hours), and to increase the electrolyte concentrations in the buffer chambers. Concentrations of 1 M NaOH and 1 M H_3PO_4 or more have been used but Duncan and Hershey (1984) found little further improvement when the catholyte was increased above 50 mM in NaOH (which appeared to be optimal) or with more than 25 mM H_3PO_4 for the anolyte. Increasing the concentration of one electrode solution but not the other shifts the pH gradient up or down, but if it is necessary to do this it is probably preferable to extend the pH gradient by altering the composition, by adding constituents such as arginine or lysine to the catholyte and aspartic or glutamic acids to the anolyte, or to prepare appropriate cocktails of ampholytes.

Perdew *et al.* (1983) replaced the non-ionic detergent (Nonidet P-40) used in the first dimension IEF gel with a similar proportion of the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), which they claimed had superior membrane protein solubilizing properties and was particularly effective at disaggregating hydrophobic proteins. It appeared to greatly reduce the streaking sometimes observed in the IEF separation and improve the clarity of the resulting protein maps.

Among the most significant developments in high resolution 2D protein mapping reference must be made to the work of Anderson and Anderson (1977; 1978*a*, *b*) who have described apparatus (termed the ISO-DALT system) for preparing and running a large number of O'Farrell-type gels together. This greatly enhances reproducibility and comparison between the resulting protein maps as well as enabling a very large number of samples to be handled in a short time. A variation (termed BASO-DALT) and giving enhanced resolution of basic proteins by employing NEPHGE in the first dimension (see below) has also been described (Willard *et al.* 1979). The approach makes practical the Molecular Anatomy Program at the Argonne National Laboratory in the USA, the object of which is to be able to fractionate human cells and tissues with the ultimate aim of being able to describe completely the products of human genes and how these vary between individuals and in disease.

Another important development which must be mentioned is the application to 2D mapping of IEF in gels with immobilized pH gradients for the first dimension (Westermeier *et al.* 1983; Gianazza *et al.* 1984). Immobiline IEF gels are described in Section 9.3(h). These are prepared and run on Gel-Bond PAG polyester supports which after the separation are cut into strips which can then be applied across the tops of SDS–PAGE slabs for the second dimension. The use of gels with immobilized pH gradients has a number of advantages compared to conventional PAGIF. The pH range of the gradient can be very clearly defined and the range calculated to give optimum separation of sample constituents. Once formed the gradients are more stable than those formed with ampholytes so that gradients are more reproducible. Resolution is also superior, as is sample load carrying capacity, and the gradient is not disturbed by the

presence of salt ions in the samples or buffers. Owing to the very low conductivity of Immobiline gradients sample zones may be sharpened by increasing the voltage gradient to very high values during the final stages of focusing without causing excessive heating. Against this their preparation is a little more technically demanding and their cost higher. Nevertheless, the advantages, particularly in terms of gradient reproducibility which will undoubtedly facilitate inter-sample and inter-laboratory comparisons will ensure that they become widely used in future for 2D protein mapping as well as for single dimension separations.

Elegant micro-versions of O'Farrell's method have been reported by Rüchel (1977) and by Poehling *et al.* (1980) in which the first-dimension IEF run is performed with gels in small capillary tubes followed by a second-dimension SDS–PAGE step on postage-stamp-size gel slabs with a T = 1–40 or T = 6–25 per cent gradient. The method was applied to the analysis of proteins from single cells (large mollusc neurons) and proteins from plant-parasitic nematodes.

Unfortunately histones and other basic proteins are generally not well separated by O'Farrell's method and the acid–urea–detergent systems described in Section 5.9c may be preferable. In O'Farrell's method they often do not enter the first-dimension IEF gel or if they do they may appear as streaks. This may be due in part to a collapse of the pH gradient at the alkaline end. For example, Giometti, Anderson, and Anderson (1979) report that even with ampholytes of very wide range intended to give a pH range of 2–11 the actual gradient after focusing in a gel was from about pH 3·0 to 8·5. It may also be due to the difficulties of completely removing DNA from histone samples, and this led Sanders, Groppi, and Browning (1980) to develop a procedure for sample preparation in which 0·4 M NaCl was added to the pH 4·8 buffer before nuclease digestion and in which a three-fold excess of protamine was then added, both steps being intended to reduce DNA–histone interactions.

To overcome the difficulties of separating such basic proteins in the first dimension O'Farrell, Goodman, and O'Farrell (1977) introduced the technique of non-equilibrium pH gradient electrofocusing (NEPHGE). For the 2D mapping of proteins using NEPHGE for the first dimension, gel rods identical to those described above for IEF separations are prepared, i.e. gels with T = 4 per cent and C = 5 per cent prepared in 9 M urea, 2 per cent Nonidet NP-40 and 2 per cent ampholytes, usually of wide pH range but narrow-range ones (e.g. pH 6–8) have been used (Sanders *et al.* 1980). NEPHGE is performed in the same way as IEF except that the electrodes are reversed and sample is applied at the acidic end rather than the basic end of the gel and the voltage applied for a shorter time than in IEF. The lower electrode is thus the cathode and the lower reservoir contains base (e.g. 0·02–0·1 M NaOH) and the upper electrode is the anode (e.g. in 0·01–0·05 M H_3PO_4 or H_2SO_4). Samples dissolved in the urea–detergent solution, with or without ampholytes, are applied to the top of the gels and overlaid with more dilute urea solution to protect the proteins in the sample from direct contact with the acid electrode solution which is then added to the upper reservoir. Different groups of workers have used different voltage conditions for the actual separation, ranging from 400 V for 2 h to 500 V for 8 h (O'Farrell *et al.* 1977; Giometti *et al.* 1979; Willard *et al.* 1979; Sanders *et al.* 1980; Horst *et al.* 1980), but the precise conditions will depend on the geometry of the apparatus and in particular the gel length. However, in all cases the combination of time and voltage is much less than that used for IEF (usually 15–40 per cent). Under such conditions the pH gradient may not have reached full equilibrium and certainly most proteins will not have focused at their iso-electric points. Nevertheless, they will be largely separated on the basis of charge differences as in IEF, but it must be remembered that unlike IEF no information can be gained about the true iso-electric points of proteins separated in this way.

Giometti *et al.* (1979) claimed that basic protein samples extracted with SDS-containing solutions could be examined directly by NEPHGE, but other workers were unsuccessful (Horst *et al.* 1980) and it seems possible that the non-ionic detergent Nonidet NP-40 may not always be capable of totally displacing SDS from protein–SDS complexes. Likewise, some groups have reported that acidic proteins in the same sample are not well separated during the NEPHGE analysis of basic proteins, while other groups (e.g. Giometti *et al.* 1979) have attained resolutions nearly as good as in conventional IEF. After separation NEPHGE gels can be stained and treated in the same way as IEF gels. For 2D mapping work of basic proteins, the first-dimension separation by NEPHGE is followed by a second-dimension SDS–PAGE separation in the usual way.

11.4 'Low resolution' two-dimensional (2D) procedures

O'Farrell's method of two-dimensional mapping, with either IEF or NEPHGE in the first dimension, employs strongly denaturing conditions so that all multi-subunit proteins are broken down to their constituent subunits. This can lead to a very large number of separated zones on the final map, particularly if subunits differ and if very complex samples of biological origin are examined.

This complexity has led to various methods of simplifying zone patterns as an aid to their interpretation and to the concept of 'low resolution' 2D protein mapping. Philosophically this is rather interesting because it might be thought of as attempting 'to put the clock back' in the sense that many of the earlier electrophoretic techniques were low resolution of necessity

rather than by design! However there is no doubt that lower resolution methods do still have their place, and may offer significant advantages in terms of technical simplicity, speed (enabling a greater through-put of samples), ease, and simplicity of interpretation and hence less sophisticated and expensive equipment for scanning and data handling. Although this is of course bought at the expense of the amount of information that can be extracted from the results, there may be sufficient for the purpose in hand. A common feature of all the procedures discussed in this section is that either a relatively low resolution method (e.g. agarose gel, starch gel, cellulose acetate, paper electrophoresis, PAGE, etc.) is combined with a relatively high resolution technique (PAGIF, IEF-agarose, SDS–PAGE, PGGE, etc.) or two low resolution stages are employed. In either case the final resolution obtained is lower than would have been the case if high resolution methods had been employed for both dimensions.

At the lowest end of the resolution spectrum combinations between paper, starch gel, cellulose acetate, and agarose gel electrophoresis are nowadays seldom used. Combinations using PAGE for both dimensions were quite widely used in the early days of 2D analysis but are now limited very largely to applications where buffers of very widely different pH are used for the two dimensions or where a denaturant such as urea is present for one dimension but not the other. The 2D-PAGE system of Kaltschmidt and Wittmann (1970), modified by Lastick and McConkey (1976) to facilitate the use of small samples and reduced running times, gives a very good map of ribosomal proteins.

For this (Lastick and McConkey 1976) a first dimension gel rod (5 mm diameter, 115 mm long) of T = 4·1 per cent, C = 3·2 per cent polyacrylamide gel in a buffer of 0·2 M tris, 0·26 M boric acid, and 0·01 M Na_2EDTA pH 8·6 containing 6 M urea (0·2 per cent TEMED and 1·9 mM ammonium persulphate as polymerization catalysts) was used; with the same buffer diluted three-fold in the apparatus. After installing in the apparatus the top of the gel was covered with at least 1·5 cm depth of an overlay of this same buffer diluted 10-fold but containing 8 M urea. The sample itself was dissolved in a portion of 10-fold diluted buffer to which 5 per cent 2-mercaptoethanol had been added, warmed to 60 °C for 10 min, cooled, a little sucrose or more urea added, and carefully layered between the overlay buffer and the top of the gel. The system thus makes use of the zone sharpening effect of lower conductivity zones produced by diluting sample buffers (see p. 80). After electrophoresis towards the cathode at 65 V for 17 h the gels are extruded, soaked for no more than 5 min in 0·35 M acetic acid containing 6 M urea, and 5 per cent 2-mercaptoethanol, and placed across the top of a 170 × 130 × 0·8 mm polyacrylamide gel slab of T = 15·5 per cent, C = 3·0 per cent made up in 0·44 M acetic acid, 0·025 M KOH buffer, pH about 4·6, containing 6 M urea (polymerization catalysts: 0·5 per cent TEMED and 10 mM ammonium persulphate). The first dimension rod gel was sealed in place with 1 per cent agarose made up in the acetic acid gel soaking solution and the second dimension then run towards the cathode at 100 V for 18 h. The system therefore separates the ribosomal proteins not only at two different pH values but also in a relatively non-restrictive gel in the first dimension, so that separation is predominantly according to charge differences, and in a much more restrictive second dimension gel so that size differences then play a greater role. Thus while not entirely according to charge or size differences in either dimension the basis of separation is sufficiently different in the two dimensions to give quite a good distribution of the proteins over the whole surface of the gel map.

In the above method 6 M urea was present in the gels for both dimensions so the conditions were mildly denaturing throughout. Other 2D arrangements with PAGE used for both dimensions may use both denaturing and non-denaturing conditions. Such an approach may have merit in studies of subunit structure and conformation. Using T = 5·1 per cent, C = 2·4 per cent polyacrylamide gels in 0·09 M tris, 0·09 M boric acid, 2·5 mM Na_2EDTA buffer containing 10 per cent glycerol throughout, Schumacher *et al.* (1983) used this approach for the detection of circular viroids and virusoids. The gel for the second dimension was identical to that for the first but included 8 M urea and was also run at 50 °C to ensure denaturing conditions.

More usually however 2D methods within this category make use of a simple, relatively low resolution step coupled with a high resolution method in the other dimension. They may be non-denaturing arrangements, such as PAGE/PGGE as used by White and Ralston (1981) in studies of water-soluble erythrocyte membrane proteins, PAGIF/PAGE as used by Karpetsky *et al.* (1984) in studies of nuclease activity and PAGE/PAGIF as used by Jones, Broadbent, and Gurnsey (1982) for salivary proteins. Denaturing arrangements usually employ SDS or less frequently urea or other detergents in one or both dimensions, e.g. PAGE/SDS–PAGE (White and Ralston 1981; Jones *et al.* 1982), PAGE/SDS–PGGE etc. Considerations of technical simplicity and a desire to use non-denaturing conditions giving relatively uncomplicated maps of proteins in the native state led Felgenhauer (1979) and Felgenhauer and Hagedorn (1980) to examine body fluid proteins using 1 per cent agarose strips run in 0·2 M barbital buffer pH 8·6 in the first dimension followed by PGGE in the second dimension. The agarose strips were placed along the top of the gradient slab and sealed in place with agarose in the usual way. Gradients of T = 3–25 per cent (C = 5 per cent) were used for larger proteins (MW of 30 000 or more) and of T = 20–50 per cent for small proteins down to a MW of about 3000. The gradient gels were prepared in 0·089 M tris–0·082 M boric acid buffer (pH 8·6) containing 2·5 mM EDTA.

The method thus separates predominantly according to charge differences in the first dimension and size differences in the second. This same combination of separation factors was also employed by Lonberg-Holm *et al.* (1982) and Bagley *et al.* (1983) in studies of human plasma proteins, but in this case an agarose gel electrophoresis first dimension with a 0·06 M barbiturate buffer pH 8·6 was followed by SDS–PGGE in a T = 5·5–11 per cent polyacrylamide gradient gel using the Laemmli (1970) system of discontinuous buffers. Slices of the agarose gel were applied directly to the SDS–PAGE slab without any intermediate soaking or equilibration in

order to minimize losses of separated components. In spite of the fact that denaturing conditions were used in the second dimension, it was claimed that most plasma proteins were represented on the final map by single spots and that recoveries were better than with the O'Farrell high resolution procedure, although resolution was not so good and individual spots were less compact so that sensitivity was lower. The O'Farrell type of procedure dissociates many proteins into subunits and has very high resolution so that individual proteins are often split into groups of multiple or overlapping spots, the complexity of the map being increased by genetic variation and by natural microheterogeneity, often due to variations in the carbohydrate portion of glycoproteins for example. Poor migration of basic proteins and low recoveries of large proteins (MW > 10^5) are also problems associated with high resolution methods that Lonberg-Holm *et al.* (1982) claim are partly alleviated by their method, using which they were able to detect at least 60 separate plasma proteins and follow changes in them caused by leukapheresis, plateletpheresis or following snakebite.

11.5 High resolution non-denaturing or partially denaturing 2D procedures

At the present time IEF in either agarose of polyacrylamide gel, is the only high resolution electrophretic method capable of separating macromolecules purely according to charge differences while PGGE and SDS/PAGE or SDS–PGGE are the methods of highest resolution separating by size differences. Thus PAGIF or AGIF combined with a PGGE separation in the other dimension represents the highest resolution 2D system capable of operating under fully non-denaturing conditions. Partially denaturing high resolution 2D systems would thus consist of AGIF or PAGIF combined with SDS–PAGE or SDS–PGGE or of PGGE combined with AGIF or PAGIF performed in the presence of denaturants such as urea, since charged detergents such as SDS interfere seriously with IEF and cannot be used. AGIF, PAGIF, and PGGE can all be performed with high concentrations of glycerol or with non-ionic detergents such as Nonidet P-

FIG 11.2. 2D gel electrophoretic maps of normal human blood serum proteins, separated (A) under non-denaturing conditions by PAGIF in the first (horizontal) dimension and PGGE in the second dimension, and (B) under fully denaturing conditions with PAGIF in the presence of 2 per cent Nonidet P-40 and 9 M urea in the first dimension and SDS–PAGE in the second. (Reproduced from (A) Manabe *et al.* (1982) and (B) Tracy *et al.* (1982) by permission of the authors and publishers. © 1982, Clinical Chemistry.)

40, Triton X-100 etc. present, and these may be useful for solubilizing samples such as membrane proteins and can be considered as providing mildly denaturing or partially denaturing conditions.

Like the O'Farrell (1975) procedure the methods also provide information about the isoelectric point and MW of protein components, which in the case of the totally non-denaturing systems, will be the values for the native proteins and not of subunits. Because of this lack of dissociation, the maps contain many fewer spots than are seen in maps with fully denaturing conditions. This can be seen for example by comparing maps (Fig. 11.2) of human plasma proteins separated by PAGIF/PGGE (Manabe *et al.* 1982) with similar samples examined by the O'Farrell system (e.g. Tracy *et al.* 1982*a*) or of salivary proteins separated by the same two methods (Marshall 1984). In a clinical context at the present time it may therefore be easier to observe changes in proteins associated with disease if non-denaturing 2D mapping is used. However as automatic scanning and data-handling equipment capable of handling the greater complexity of gels run under denaturing conditions becomes more readily available, this advantage will be rapidly eroded and there is no doubt that much more significant information can be obtained from the really high resolution denaturing systems.

11.6 2D separations of nucleic acids

Nucleic acid molecules of all sizes have very similar charge densities at about neutral pH values, so that separations based on charge differences (e.g. AGIF or PAGIF) are seldom satisfactory. This seriously limits the scope for 2D separations which must therefore be based on size or conformation differences. At acid pH values, below about pH 4-5, each of the four different types of base residues making up the RNA or DNA contributes a slightly different charge so that the net charge then depends on both chain length and base composition. The base pairing phenomenon is lost at low pH values and the addition of denaturants such as urea or formamide disrupts tertiary and secondary structure. Working at elevated temperatures of very low pH also has a denaturing effect. In order to spread the nucleic acids in a mixture adequately over the surface of a 2D gel, it is obvious that as with protein mapping the basis of the separation should be as different as possible in the two dimensions.

(a) *RNA*

Many different systems have been used for RNA separations but according to de Wachter and Fiers (1982) most can be grouped into three categories:

'urea shift', 'concentration shift', and a 'pH shift' usually combined with a urea and a concentration shift.

For the urea shift approach PAGE is run in both dimensions under identical conditions of pH (usually between 4·5 and 8·5) and gel concentration but with a denaturing agent such as urea at high concentration (6–9 M) present for one of the dimensions. Discontinuous buffer systems are not usually employed with nucleic acids but sharpening of zones can be achieved by ensuring that the sample has a lower conductivity than the buffer in the gel and apparatus, so that the voltage gradient across the sample is infinitely steeper than elsewhere (see p.80). It is often convenient for the urea to be added for the second dimension so that RNA fragments containing hidden breaks migrate as base-paired complexes in the first dimension but are dissociated in the second. The method has been used to study small RNA fragments of about 13–80 nucleotides in length resulting from RNase T_1 digestion of larger RNA, for the fractionation of tRNA (on T = 16 per cent gels) and mRNA (on T = 6 per cent gels).

The concentration shift method relies on a change in T, all other conditions being the same for both dimensions, so that separation depends upon conformational differences being manifest at one or both gel concentrations. Some zone sharpening is seen as samples migrate from a low T gel strip into a high T environment, so the more dilute gel should be used for the first dimension. The method has been used to fractionate RNA in the size range 80–400 nucleotides.

The third category of a pH shift coupled with both urea and concentration shifts (de Wachter and Fiers 1972) has been the most widely used, particularly for fingerprinting RNase T_1 digests of viral RNA. The first dimension consisted of running a T = 10·3 per cent, C = 0·9 per cent gel containing 7 M urea at pH 3·3–3·5 using 0·025 M citric acid as buffer in both gel and the apparatus, with NaOH to adjust the pH to 3·5 if necessary. The catalyst mixture used to induce polymerization consisted of 0·225 mM $FeSO_4 \cdot 7H_2O$ and 1·5 mM ascorbic acid which are added to the acrylamide and Bis in the citric acid, with H_2O_2 being added to 0·012 per cent (40 µl of 30 per cent H_2O_2 per 100 ml of gel mix) immediately before the gel is poured. Under these conditions subsequent electrophoresis will separate the RNA fragments largely according to base composition and the presence of urea prevents aggregation of complementary sequences (base pairing). Gels are briefly prerun (e.g. 250 V for 1·5 h), samples applied and electrophoresis performed until the Bromophenol Blue or Xylene Cyanol FF tracking dye is close to the bottom of the slab. Strips of this gel are then cut out and either applied in the usual way across the top of a preformed slab of second dimension gel or placed in the gel mould and second dimension gel mixture poured in around it. No equilibration step is required because the pH very rapidly adjusts to that of the second dimension gel slab. This consisted of a T = 20·6 per cent, C = 0·9 per cent slab made up in 0·05 M tris adjusted to pH 8·0 with citric acid, and with 40 µl TEMED and 40 mg of ammonium persulphate per 100 ml of gel mixture as polymerization catalysts. The concentration and pH of buffers used is not critical and can be varied considerably. Steward and Crouch (1981) for example used 0·75 M tris mixed with 0·75 M boric acid and 0·022 M Na_2EDTA which gave a pH of 7·8 for the second dimension. The important point is that it should be neutral or weakly alkaline and without denaturants so that mobility is primarily a function of chain length. Lee and Fowlks (1982) used a slightly modified version of this method for fingerprinting digests of viral RNA (Fig. 11.3).

Most of these methods are rather limited in terms of the size range of RNA that