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Review

Biomedical relevance of two-dimensional protein mapping

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ABSTRACT

State-of-the-art and future perspectives are discussed for the application of two-dimensional protein maps to basic medical research and routine clinical chemistry problems. Despite the technical advances that allow effective processing of a large number of samples and the refinement of devices and procedures for image analysis, at present two-dimensional maps are mostly confined to research purposes, *i.e.* to the inventory of normal constituents of body fluids and tissues on the one hand, and to qualitative–quantitative alterations of some protein spots in a number of instances (genetic, degenerative, infectious or xenobiotic diseases) on the other. It is hoped that in some instances a single primarily affected component will be able to be identified and then specifically tested (for instance by immunological means) as a diagnostic marker, but complex pathological patterns would still require the analysis of a large number of peptides at the resolution level only afforded by two dimensions. Further simplification of the protocols, for example with ready-made gels, and data reduction systems might then allow the application of the technique to be extended to general clinical laboratories.

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LIST OF ABBREVIATIONS

C%	Relative concentration of the cross-linker in a polyacrylamide gel
CAs	Carrier ampholytes for isoelectric focusing
1-D, 2-D	One- or two-dimensional electrophoretic separation
DTT	Dithiothreitol
IEF	Isoelectric focusing
IPGs	Immobilized pH gradients
IgG, IgM	Immunoglobulin class G or M
ISO-DALT	Sequence of ISOelectric focusing and electrophoresis in presence of SDS [which fractionates proteins according to size (DALTon)] [3,4]
M_r	Relative molecular mass
PAGE	Polyacrylamide gel elelectrophoresis
p*I*	Isoelectric point
SDS	Sodium dodecyl sulfate
SD_x	Standard deviation along the x (*i.e.* p*I*) axis
β-SH	β-Mercaptoethanol
T%	Total monomer concentration in a polyacrylamide gel

1. TWO-DIMENSIONAL ELECTROPHORETIC SEPARATIONS: THE TECHNIQUE

Two-dimensional (2-D) electrophoretic techniques are meant to optimize resolution of all proteins in complex samples and to gather relevant information about every individual component. These concepts work best when the protein mixture is subjected to a sequence of fractionation procedures based on completely unrelated principles that are at the same time able to measure some molecular parameters of the constituent peptides. That is why, although an endless series of combinations could be devised (and a number of them are from time to time applied to the resolution of specific analytical problems), 2-D electrophoresis is nowadays synonymous with isoelectric focusing (IEF) under denaturing conditions (urea and a non-ionic or zwitterionic detergent in the gel, and a reducing agent in the sample) followed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). The electric field is applied in the second step at right angles to the direction of the first migration; as a result, individual peptides are resolved in a pI–M_r (isoelectric point–relative molecular mass) plane.

A few variants of this basic procedure exist, involving both the nature of the IEF medium and the geometrical arrangement during the different steps. For the former, the option is between IEF in the presence of carrier ampholytes (CAs) and immobilized pH gradients (IPGs); for the latter, both the first and second steps of 2-D electrophoresis (1st and the 2nd d) separation can be performed either in a horizontal or in a vertical set-up.

Detailed procedures allowing high-resolution 2-D electrophoresis were first described in 1975 by O'Farrell [1] and by Klose [2]. In 1978 optimized instrumentation and protocols for large-scale operation were devised by Anderson and Anderson [3,4]. The Materials and methods sections of the above papers are still most often referred to in the current literature. The Anderson group is releasing updated versions of a manual describing the "operation of the ISO-DALT system" [5]; for a "practical approach" to 2-D separations, the chapter by Rickwood *et al.* in ref. 6 may prove very useful. Dunn and co-workers [7–9] collected in a very comprehensive review a large body of experience with different technical problems, while a book by Celis and Bravo [10] deals with "methods and applications". Further discussion on all aspects of 2-D separations can be found (among serial publications) in two issues of *Clinical Chemistry* [11,12] and one volume of the *Annals of the New York Academy* of *Sciences* [13] containing the proceedings of international meetings.

2. BIOMEDICAL APPLICATIONS: WHAT TWO-DIMENSIONAL SEPARATIONS CANNOT DO

After the paragraph above as a short reminder on the basics of the 2-D technique and a summary of the general literature, let us move into the main topic. This should mean, in keeping with the subject of this issue "—Electromigration techniques of clinical importance"— onto the clinical applications of 2-D separations. Two US patents have been filed, one dealing with "early detection of infectious mononucleosis" [14], the other with the "diagnosis of Creutzfeldt–Jakob disease by electrophoresis of cerebrospinal fluid proteins" [15] —both through 2-D protein mapping. This could suggest that experience in the field is so advanced that this analytical procedure has become routine practice and that there is room enough for the commercial exploitation of some especially interesting findings. Unfortunately, this is not the case. Why not? In comparison with most current procedures in clinical chemistry, 2-D electrophoresis is expensive, labour-intensive and time-consuming; result evaluation requires some rather sophisticated equipment, and only in a few cases does general agreement exist about the relationship between any specific finding and a given pathology. Moreover, notwithstanding all of its possibilities (see below), the technique falls short of resolving all proteins in a biological sample.

The main problem comes from the balance between resolution and sensitivity. As the level of sensitivity of a given general detection method is increased, the set of the most abundant protein spots one is able to visualize keeps increasing.

However, no refinement in the detection methods will alleviate the problems of a sample containing components whose concentrations vary over several orders of magnitude. To bring the less abundant components above the detection limit, and still resolve and correctly quantitate all spots, one would need (a) to have large enough samples, (b) to be able to run enough material without overloading the gels, (c) to apply a detection system with a very wide dynamic range and (d) to increase the resolution of the system. On the contrary (point a), many biological samples (*e.g.* needle biopsies) are very minute in size (point b), the loading capacity of CA-IEF (and of SDS-PAGE) is limited, while fractionating the mixture into different portions to improve specificity increases the protocol variables as well as the time and labour involved. Moreover (point c), the range of linearity for most detection systems, and, in addition, for the densitometric devices used in spot quantitation, is limited. A partial solution could be to take serial autoradiographic plates from radioactive samples with longer and longer exposures. However (point d), a few spots could still be characterized by very similar molecular parameters: only very large (and very unpractical) gel formats would help in this respect.

Are the above requirements too demanding? Is the overall picture too pessimistic? Well, not quite. Serum is one of the most easily accessible biological samples and —in principle— one of the most informative because, through secretion, shedding or leakage, it contains proteins from all tissues and organs, except the central nervous system. In a search for early markers of disease, serum samples have been collected –within the JANUS project [16]– at one- to three-year intervals from tens of thousands people to be retrospectively investigated in case a pathological condition should develop in one of the donors. However, in a few hundreds of precancer sera analyzed, Jellum *et al.* [17] did not observe any systematic variation from control samples; more disappointing, known cancer markers, such as carcinoembryonic antigen, could not be detected. Even in cancer patients, only an aspecific involvement of the immune system was made evident by an altered pattern of the immunoglobulin G (IgG) spots [18]. Serum is thus a case in point for what we have just mentioned: disease does not always involve dramatic changes in the protein make-up of a biological sample, and very minor components —whether odd housekeeping enzymes or critical regulatory proteins— are likely to escape detection under the standard conditions of analysis.

Serum is again a troublesome sample because it contains only a few very abundant proteins, and almost 50% albumin. The selective removal of the latter by immunoadsorption finds a practical limitation in the cost and low capacity of such affinity columns. On the other hand, dye-affinity chromatography on immobilized Cibacron Blue [19] —a very popular substitute for immunodepletion— is by no means specific for albumin [20]. By developing this finding, fifty fractions bound more and more strongly by the dye were obtained from serum and analyzed by 2-D electrophoresis. As the third "dimension", or separation principle. *i.e.* affinity, was independent of charge and mass of the proteins, the overall

resolution could be greatly increased [21]. Hochstrasser *et al.* [22] have recently proposed a two-step procedure by which human sera are subjected to preparative IEF prior to 2-D analysis. In this procedure, some very abundant proteins (*e.g.* albumin and transferrin) can be eliminated by being confined to their p*I* values and all adjacent fractions analyzed in a 2-D map. It is thus possible to load much higher serum volumes, to the extent that even the least represented species become visible. The advantage of using a focusing technique for prefactionation is that the relative position of the spots in the 2-D map does not change; in addition, the residual CAs in the sample are not detrimental to 2-D analysis. By this procedure, Hochstrasser *et al.* [22] were able to detect two new lipoprotein-associated proteins, named NA1 and NA2, believed to be present in sera at only 0.05 mg/ml concentration. By using this prefractionation procedure, it was possible to load up to 20–40 μg of NA1 and NA2, *i.e.* enough for amino terminal sequence. By a search in a DNA sequence database, the NA1 and NA2 polypeptides were found to be two chains of a protein called SP 40/40, a potent inhibitor of the complement system [23]. A few successful examples, however, of runs with grossly overloaded gels are reported, when the prevailing spots move away from the bulk of the other components. This is the case for instance of the separation of whole hemolysates, in which globin chains are of higher p*I* and lower M_r than most red blood cell proteins [24].

Still more problems are to be solved with cells. Solid tissue samples need to be carefully dissected in order to remove any connective tissue. Not surprisingly, in fact, it can be shown (for the heart muscle [25]) that the protein pattern shifts as the percentage fibrousness of the samples increases. Again, it may be difficult to meet this condition with one-shot specimens like biopsies. As will be seen in what follows, most work on cells has in fact been done on lymphocytes or fibroblasts, easily available and readily purified as homogeneous populations.

Any difference in a pathological sample is always to be referred to a "control" pattern, which should include qualitative and quantitative information both on the common protein forms and on their polymorphic variants. This database is being collected for the aforementioned cell types [26], but major practical difficulties are to be faced with the *in vivo* collection of other normal tissue samples. Only the assessment of the reliability of autopsy specimens may help solve this problem [27]. Of course, the database on each pathological condition should also be wide enough to prove that the observed variations are neither coincidental nor secondary aspecific effects [28]. In some cancers, with an ample resection around the affected area, both the pathological sample and some normal tissue become available from the same patient, which makes the above comparisons easier [29,30].

3. WHAT TWO-DIMENSIONAL SEPARATIONS CAN DO

The above list of limitations or cautions or "proviso" was compiled to explain why 2-D protein mapping is still far from routine in the laboratory. But this

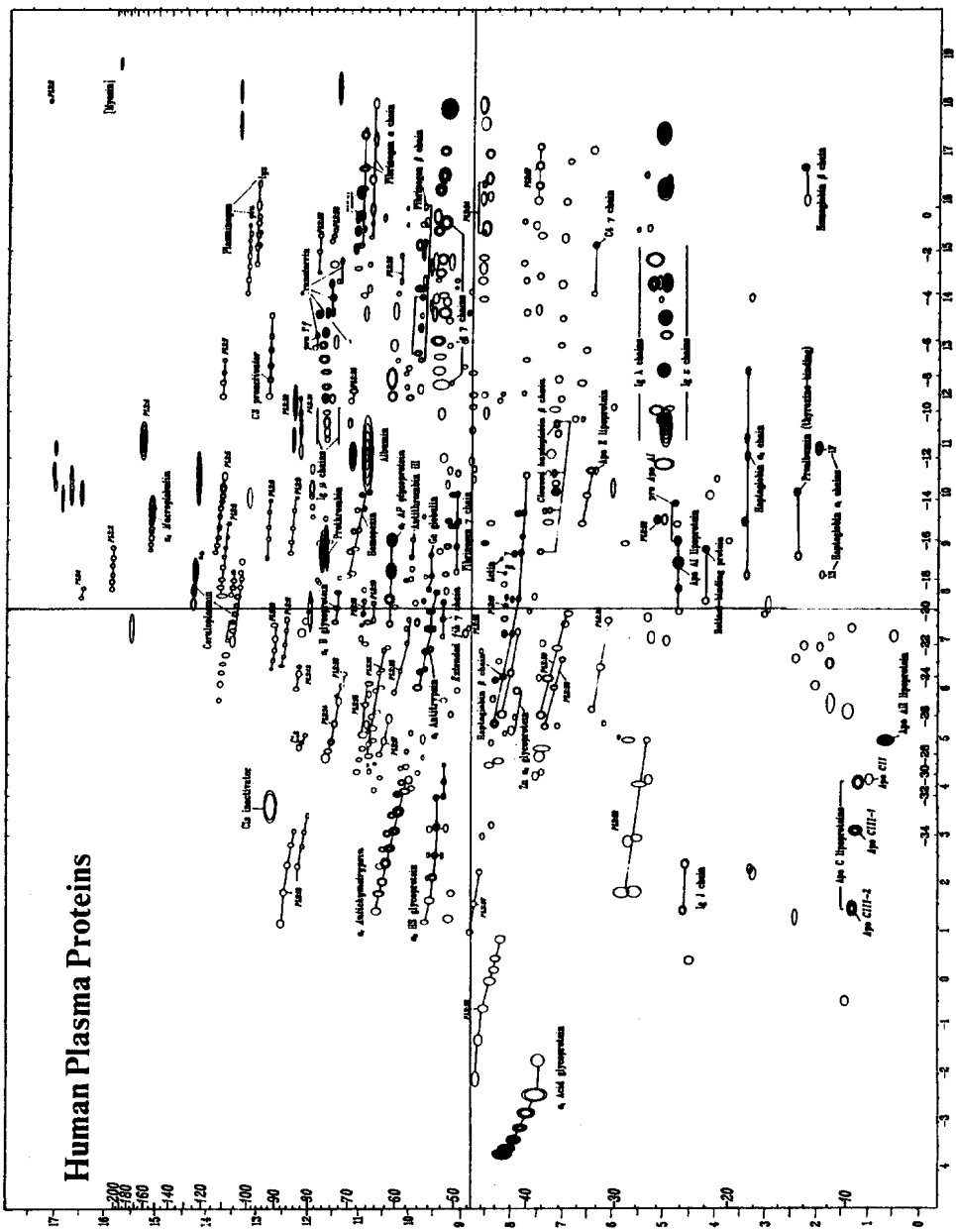

Fig. 1. Standard human plasma protein map constructed via computer analysis (from ref. 50 with permission).

technique —when all the limitations or cautions or "proviso" are taken into account— is a powerful research tool that may help clarify a number of biological problems. The main fields of application related to clinical chemistry have been already reviewed in a number of papers [17,18,31–39]. We will give examples of the following uses:

(a) Inventory of the protein make-up in all normal tissues.
(b) Detection of the protein markers for genetic diseases.
(c) For degenerative diseases.
(d) For infectious diseases.
(e) For toxic diseases.

3.1. Protein indexing in normal tissues and body fluids

The proposal for a systematic analysis of all normal protein constituents of the human body by 2-D electrophoresis was made by Anderson and Anderson in 1979 [40] under the title "Human Protein Index" [41,42]. It was the first example of a global approach to the solution of biological problems through the enumeration (and quantitative evaluation) of tissue (or body fluid) components in a number of physiological and pathological conditions. A similar procedure was then adopted by Jellum [43] for low-M_r metabolites to be analyzed by the combination of gas chromatography and mass spectrometry. A more ambitious proposal of investigation along the same lines eventually became the "Human Genome" project [44]. The Human Protein Index and Human Genome projects partly parallel and partly complement one another. With the former, as already discussed, it becomes more and more difficult to get information about proteins contained in smaller and smaller amounts, while the copy number of most genes equals unity. The sequencing of the non-translated chromosome region gives some hints about regulatory structures, while the quantitation of the mature proteins in each sample describes the outcome of the overall regulatory processes (specificity for a given stage during ontogenesis, for a given tissue, for a given hormone stimulation, etc.). The sequencing of DNA-coding regions offers complete information about amino acid composition, size and surface charge of a polypeptide, and in some cases allows some inference about three dimensional structure and function; 2-D separation should be complemented by blotting and sequencing [45] or by complex metabolic labeling procedures [46] in order to match the above results. From a technical point of view, the protein samples —contrary to genomic or DNA libraries— are directly and unequivocally available without special processing.

Protein catalogues for simple systems like *Escherichia coli* [47] and HeLa cells [48] have long been implemented. For human proteins, the Anderson group did publish a number of papers on body fluids (serum [49,50], Fig. 1; urine [51–53]; saliva [54]; milk [55]; seminal plasma [56]), blood cells (leukocytes [57,58]; red blood cells [24]; platelets [59]) and muscle [60–62] (a more exhaustive list of refer-

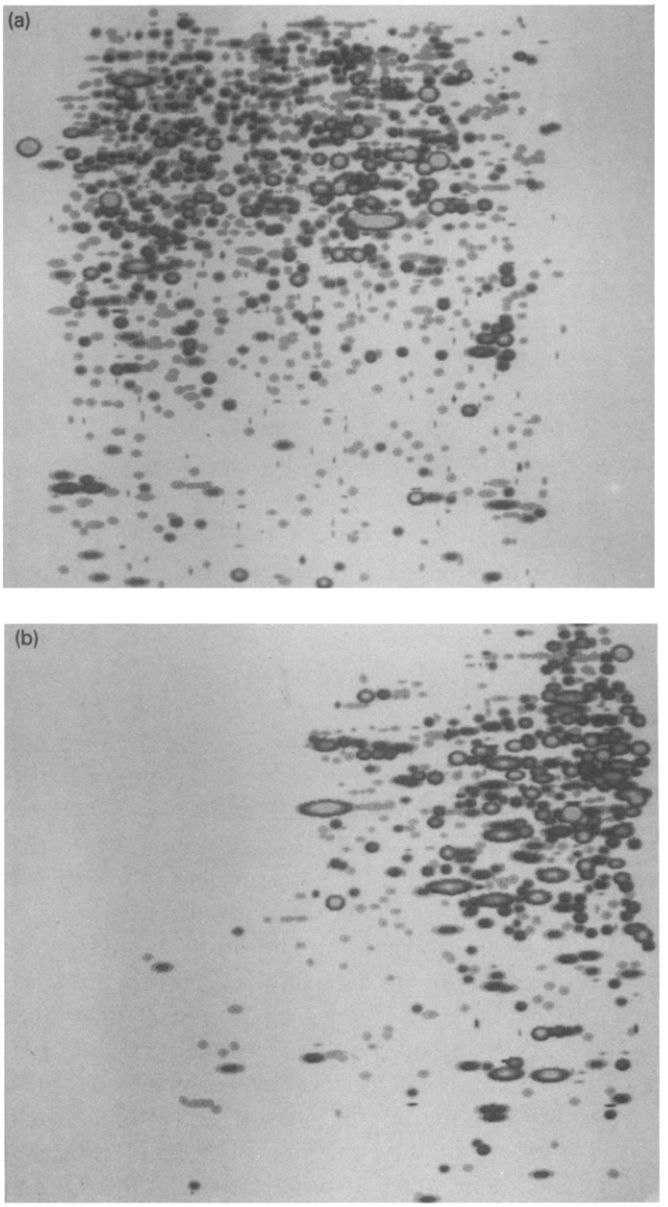

Fig. 2. Synthetic image of a 2-D fluorogram of [^{35}S]methionine-labeled proteins from human epithelial amnion cells. (a) ISO-DALT, (b) BASO-DALT (*i.e.*, non-equilibrium migration with anodic application) (From ref. 161 with permission).

ences may be found in ref. 5). Further topics were covered by other authors, for instance cerebrospinal fluid [63–65], brain [66–68], lymphocytes [69], keratinocytes [70], urine [71], chorionic villi [72], milk [73], uterine [74] and prostatic [75]

fluids and pancreatic juice [76]. Very comprehensive databases on human (transformed amnion cells, Fig. 2 [77] and embryonal lung fibroblasts [78]) as well as murine [79] cell lines have recently been presented.

In order to have complete information about a given system, both the "average" values for its parameters and their "standard deviations" should be known. For the spots in a protein map, this corresponds not only to quantitative variations but also to shifts along the p*I* axis, corresponding to peptide polymorphism and caused by amino acid mutation. Besides its interest in the framework of the molecular anatomy approach we are outlining in this section, the issue of average heterozygosity within a population is of great importance in relationship to genetics and evolution. While reviewing a number of data, Goldman and Merril [80] reported that "the amount of cellular protein heterozygosity observable by 2-D electrophoresis appears to be one third to one half the amount of heterozygosity of allozyme loci studied by 1-D electrophoresis". The figures vary from less than 1% [81, 82] to 2.4% [83] or 3.05% [84] in lymphocytes to 2.55% [85] in platelets, in comparison with 5–6% for serum proteins. It has been questioned whether this outcome is related to a different sampling in either case (total *versus* soluble cytoplasmic protein) and to a different set of structural constraints for membrane components, or to the different analysis medium (separation of native proteins under individually optimized pH conditions *versus* run of the denatured polypeptides over a constant-slope pH gradient). In this respect, it has been shown that in some cases even amino acid mutations not involving charged species can be detected under denaturing conditions [86], but the charge difference may be very minute [87] and the resolution afforded by a wide pH gradient is then insufficient for their resolution.

Special attention to spot quantitative variation in non-mutated samples was given by Kuick *et al.* [88] in order to design a proper strategy for the detection of null variants. Other research themes related to the above topic are the application of 2-D separation to chemical mutagenesis studies [89] and to the estimation of somatic mutation rates (in cultured human lymphoblastoid cells) [90].

3.2. Studies of inborn errors of metabolism and genetic diseases

We can include in this section examples of increasing complexity, for which the application of 2-D electrophoresis ranges from risk assessment for a given condition to a research tool addressing the pathogenetic mechanisms of a genetic disorder.

The simplest situation is illustrated by the correlation between one of the polymorphic forms of apoprotein E (apoE-D) and hyperlipoproteinemia type III. ApoE-D is unable to bind the specific hepatic lipoprotein receptors; as a result the plasma levels of cholesterol and triglyceride increase in the homozygous carriers, who are thus at risk for premature atherosclerosis. 2-D analysis is in this case just a means to improve the resolution of the various isoforms [91–94].

A more complex case is exemplified by the Lesch–Nyhan syndrome, a severe neurological condition for which the underlying molecular defect is known. Among the 400 components analysed from erythrocytes and from phytohaemagglutinin-stimulated lymphocytes, the spots corresponding to hypoxanthine phosphoribosyltransferase showed the same p*I* and M_r as control samples, but their concentration was greatly reduced (by *ca.* 65%). In contrast, the activity of adenine phosphoribosyltransferase was 2.5 times higher than normal. Overall, five polypeptide spots were increased and six were decreased compared with controls [95,96]. From this example, a few guidelines may be derived: when the defective gene is expressed in all tissues, the most convenient and easily available sample may be selected (compare with the opposite example of haemoglobinopathies); for the vast majority of neurological diseases the sample selected is cerebrospinal fluid: Guillain–Barré syndrome [97], Parkinson's disease [98], schizophrenia [99] and Alzheimer's disease [100]. Although one single primary defect is involved, the pathology is often pleiomorphic: in the case of the Lesch–Nyhan syndrome, eleven proteins are quantitatively influenced, demonstrating a complex rearrangement of the impaired purine metabolism besides the expected compensatory overproduction of adenine phosphoribosyltransferase for a shunt pathway.

An example of multifactorial inheritance, *i.e.* of the overall effect of polygenes and environmental factors, is exemplified by systemic lupus erythematosus. The 2-D patterns of serum and lymphocyte proteins from inactive lupus patients, their relatives and normal controls were compared. Two variant polypeptides were detected in lymphocytes and one in serum of 66–82% of the patients, 40–61% of the relatives and 20–36% of the controls [101]. The three variant proteins were observed at once in about 50% of the affected individuals, and it can be concluded they correlate with the disease. The identification of these proteins may thus be the basis for the elucidation of the genetic factors contributing to the development of systemic lupus erythematosus. These remarks can be extended to a number of other genetic disorders, such as ataxia–telangiectasia [102], Down's syndrome [103,104] or Joseph disease [105], in which it is difficult to discriminate —among the observed variations in protein pattern— between primary, specific disease effects and compensatory rearrangements.

3.3. Cancer

We have already mentioned that hopes of an early diagnosis of the disease through the screening of serum samples have been dashed [17]. Indeed, with the obvious exception of myeloma [106], it does not seem that the affected tissues secrete any specific abnormal product in the blood circulation at a concentration high enough to be detected by any general staining method. 2-D electrophoresis on the other hand, was able to detect in the urine of patients with prostatic cancer a specific marker, corresponding to a normal prostate consituent released after transition to malignancy [52].

Some work was done on solid cancers [29,30,107] (with the precautions already mentioned), but the largest investigations were devoted to leukemias and lymphomas, in which the affected cells can be repeatedly sampled from each patient and easily purified. Preliminary work included a thorough investigation of the normal protein pattern for the different blood cell sets (lymphocyte, monocyte, granulocyte) [58] and subsets (B and T lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes) [68]. Furthermore, the growth stimulation of normal lymphocytes by a number of effectors was studied as a model system for the abnormally controlled growth of malignant cells (mitogen stimulation by concanavalin A [108] and phytohaemagglutinin [109], heat shock [110]). In well differentiated leukemias, when almost pure B (chronic lymphocytic leukemia) or T cells (cutaneous T-cell leukemia) are present, the overall protein pattern very closely corresponds to that of normal lymphocytes separated by a fluoresence activated cell sorter [111]. Chronic lymphocytic leukemia and acute myelogenous leukemia cells differ, either qualitatively or quantitatively, in their content of 54 proteins, and can be readily distinguished by automatic computer analysis [112]. Surface molecules in chronic lymphocytic leukemia lymphocytes were analyzed in detail [113]: the surface immunoglobulins (IgM) were found to be monoclonal, in keeping with the hypothesis of a clonal origin for cancer cells. As for acute lymphoblastoid leukemia, twelve spots were detected that could distinguish between the major subgroups of the disease [114]. One of them (L3) was not expressed in the leukemic cells of infants, while it could be detected in older children [115]. A phosphorylated polypeptide, designated p18, was found in much higher concentrations in all acute leukemic blast cells (irrespective of lineage or differentiation stage) than in resting or proliferating non-leukemic lymphoid cells [116]. The cloning of the cDNA for p18 revealed that this gene is highly conserved among several animal species; its sequence has little homology with any previously characterized protein, but the gene itself seems to be a member of a partially homologous gene family [117].

Of prognostic value if extended from murine fibrosarcoma cell lines to human solid tumours is the finding [118] of specific markers for spontaneous metastasis. A polypeptide (Hi:2) was found to be expressed by all clones with high incidence of lung metastases; another (Lo:6) is reproducibly observed in weakly metastatic clones.

3.4. From spots to probes

The example just mentioned is paradigmatic of the procedures which could, and should, lead from a qualitative–quantitative alteration of the 2-D protein map to an understanding of the background biological events. One possibility is to probe at the DNA level: the protein, recovered from the gel slab by blotting, is partially sequenced, and this information is used for the synthesis of an appropriate polynucleotide, which is used for the identification of the complementary

sequence in a cDNA or genomic DNA library. An alternative pathway may be addressed at the protein level: the polypeptide is used to raise monoclonal antibodies, which in turn can be applied for a larger scale purification (and further characterization) of the protein by immunoadsorption. This procedure was for instance exemplified by Tracy *et al.* [119] with the characterization of a protein (PC30), eventually identified as α_1-microglobulin, that had been observed in high concentration in sera of uremic patients.

3.5. Infectious diseases

2-D maps are mostly applied for the characterization of the different strains of the parasites, for instance *Leishmania* [120] or *Treponema pallidum* subsp. *pallidum* [121]. But once a protein is demonstrated to be typical of a given pathogen it can be purified and used for the development of an immunological test: this was for instance the case of a protein (NG8.4) from *Neisseria gonorrhoeae* [122]. Still another possibility is the research for viral [123] or virally induced proteins [124]. This was most successfully done for the detection of infectious mononucleosis: two proteins appear at much higher concentrations in infected than in control leukocytes. One of them (Inmono:2) seems to be a T-cell marker and its concentration to correlate with the increase of the T-cell population brought about by the disease; the synthesis of the other (Inmono:1) is stimulated by Epstein–Barr virus infection. The usefulness of this finding rests on the earlier appearance of the altered protein pattern in comparison with a positive result with the classical horse erythrocyte agglutination by the specific heterophile antibody.

3.6. Xenobiotic exposure

2-D electrophoresis was also applied for the monitoring of toxicity tests on cell [125] and animal models [126–131] in order to gather the most detailed information about the site of aggression of the different chemicals. Some applications were made also to environment and occupational toxicology, for instance with the survey by Marshall *et al.* [132] on the urinary proteins of workers exposed to cadmium for many years: low-M_r proteins increased in concentration in these samples [132].

4. WHAT TWO DIMENSIOAL SEPARATIONS COULD DO IN THE FUTURE: PERSPECTIVES ON THE ROUTINE USE OF 2-D MAPS

Wider application of 2-D maps, mostly within a clinical chemistry laboratory, seems to require (a) simple and reliable protocols, (b) the availability of ready-made gels and of (semi)automated equipment and (c) reproducible results for easier evaluation. The last point is in fact the most critical requirement. 2-D maps based on CA-IEF were often blamed for inter- and even intrabatch variability of

spot position (although different reports may give very different figures for SD_x [133–135]!). This drawback results from the influence of at least three negative factors: (a) mechanical stress of the first-dimension gel; (b) gradient instability with time; (c) interference from added buffers.

Geometrical distortion is a serious —though trivial— cause of variability. Most usually, the first dimension is run on 1- to 1.5-mm-thin gel rods, 16 cm in length, cast with a soft acrylamide matrix, *e.g.* $T = 3.75\%$, $C = 3\%$. In an experiment with coloured proteins we measured the position of the bands after IEF (with the gel still resting within the glass tube) and compared it with the results after SDS and staining: SD_x was found to be twice as large after the second than after the first dimension [136]. That is one reason why it was proposed to run the IEF step on horizontal slabs, either in the presence of CAs [137] or with IPGs [138] (Figs. 3 and 4). Moreover, in the latter case, gels in capillary tubes give a lot of trouble and little advantage over conventional IEF. Not only do they require a relatively complex set-up to be cast [136, 139], but the (forced [140]) inclusion of CAs causes a pronounced water transport from the alkaline region of the gel [136] —which nullifies the advantage of spot reproducibility afforded by IPGs. In fact, when the first dimension is run in strips supported by a plastic backing, *i.e.* GelBond PAG foil, and on a pure IPG matrix, the observed variability of spot position barely exceeds the experimental error, *i.e.* 0.6 mm [141] (a figure one order of magnitude lower than what is reported for CA-IEF [134]).

The possibility of including in the IPG matrix a number of additives makes them a more flexible separation support than conventional IEF. CAs may prove useful in this context as solubilization aids [142,143], however, at high concentra-

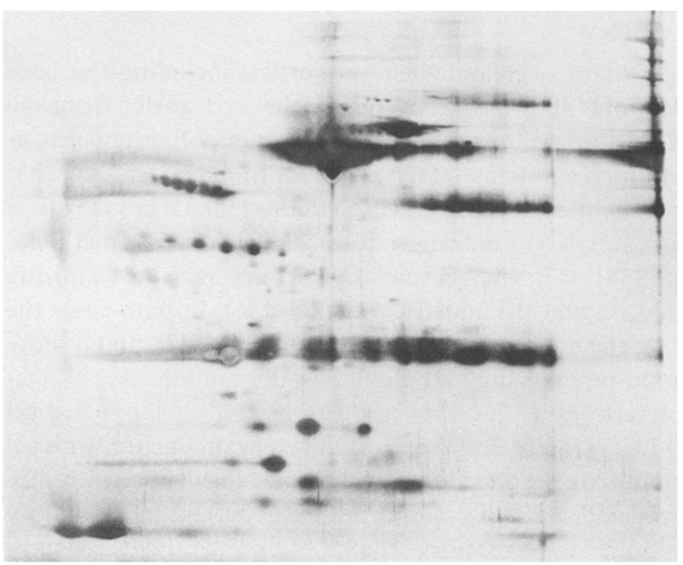

Fig. 3. Example of IPG-DALT run on serum proteins, on a 4–10 non-linear gradient. (from ref. 145).

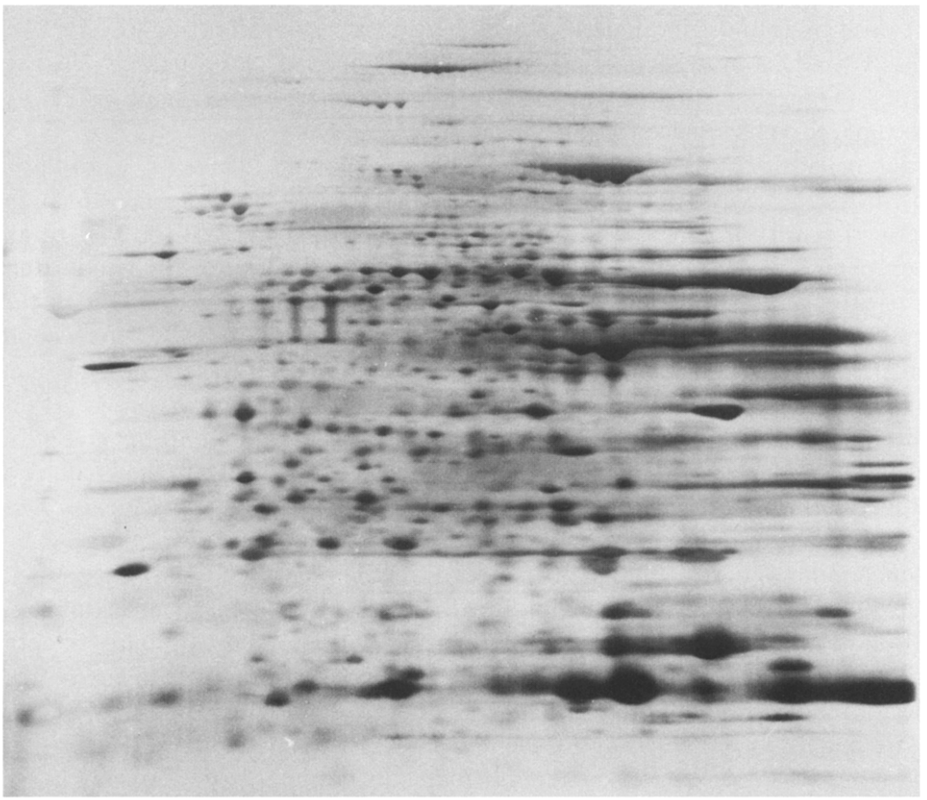

Fig. 4. Example of IPG-DALT on human heart specimen (front wall of the left ventricle in a patient with dilatative myocardiopathy) (from ref. 25).

tion their buffering power takes over and their properties, including the ones causing the "cathodic drift" [144], prevail. Although a better transfer from the first strip is obtained with "dilute" IPG matrices [145], the Immobiline concentration should always be kept high enough, and CA concentration low enough, that the former contributes most of the buffering power to the system. Of course this is true for any buffer at high enough concentration: for instance β-mercaptoethanol either in the sample [145–148] or in the gel matrix (to counteract the oxidizing power of both the IPG matrix and the added CAs) [149–151]. In both cases the additive has some effect on the course of the pH gradient [146,147], and a more rational solution appears to be reducing and alkylating the sample.

Another reason for preferring the first dimension to be run on supported gel strips is the ease and rapidity of interfacing to the SDS gel in comparison with gel rod handling. We prefer for the second dimension the classical vertical set-up with a polyacrylamide gradient and a discontinuous buffer system, on which we embed the gel strip within an excess of agarose [145] —this step takes seconds. However, equally good results are obtained with no pH (but only acrylamide

concentration) discontinuity, and no lateral spreading is observed when the gel strip in inserted above the SDS slab without physical connection [152]. The interfacing between first and second dimensions when both are run in parallel is most straightforward: the IEF strip is simply overlaid to the SDS slab, gel side down. However, with IPGs care should be taken to reduce the electroendoosmotic flow, and the focusing strips need to be laden with glycerol and urea at high concentration and are to be removed once the transfer is completed, in order to avoid serious pattern distortion [151].

We feel that the horizontal–vertical sequence is the easiest, requires standard apparatus and takes little room and equipment when many gels are run in parallel; it is self-correcting against the electroendosmotic flow (the gel cannot possibly ever dry out and burn); the SDS slabs are better protected from dust; with the classical thickness of 1.5 mm, they are sturdy enough not to require a mechanical support and are easily stained in large numbers in a single box, without the risk of mirror deposition on the plastic backing when silver nitrate is used. Moreover the spots are usually closer to the ideal rounded shape than when the horizontal–horizontal sequence is chosen.

On the other hand, the second most important factor we have mentioned for the spreading of the technique is the availability of precast gels. The choice of the companies is here almost totally in favour of the horizontal–horizontal set-up. Ready-made polyacrylamide gradient gels for the vertical tanks are still in the catalogues, but the advertized systems are thin gels to be run in the same horizontal apparatus as for IEF, with thick strips containing concentrated buffer instead of large electrodic compartments. Ready-made gels for CA-IEF have long been available, but they have a short shelf-life and, mostly, they do not include (nor are easily equilibrated with) the classical denaturing additives, urea and detergents. On the contrary, IPG strips are sold as dry gels, and may be reswollen at will in any appropriate native or denaturing medium.

Most companies have chosen the sizes of the gels to be run in the apparatuses they commercialize such that they may easily fit the standard photographic film sheets, for easy handling when autoradiographic or fluorographic procedures are required. In order to increase resolution, as large a format as possible would be helpful, and a new standard of 40 × 40 cm has in fact been proposed [153]. However, for practical reasons, the first attempt at automatization with applications to virtually every electrophoretic technique including 2-D separation (the Phast System, Pharmacia-LKB Biotechnology, Uppsala, Sweden) took the opposite direction, preferring speed of operation over resolution.

5. CONCLUSIONS

It seems likely that, although combinations of a number of simple techniques may be proposed, for instance cellulose acetate electrophoresis followed by SDS in the Phast System [154], the classical combination of focusing under denaturing

conditions and SDS-PAGE will remain the most popular. The demonstrated superiority of IPGs in terms of reproducibility of the results should shift with time the choice of the focusing procedure, and the commercial availability of precast gels should make the technique available even to inexperienced or infrequent users. It seems likely that the same consumables will be made available for the "micro" scale within a short time, which would give the extra bonus of a fast operation and an automatic staining step.

A number of computer systems for spot quantitation, pattern matching and database construction of 2-D images have been described [155–159]. Appel *et al.* [160] have recently proposed an "expert" version of their package (MELANIE) able to diagnose automatically a few (at the moment) selected diseases from the information obtained by analyzing a 2-D map. By classifying some typical spots, the system can recognize if new biopsies are from lung cancer, gastrointestinal tract cancer, breast cancer or sarcoma. Clearly, the potential of having expert systems which recognize the pattern of expression of multiple genes and which blindly diagnose cancer condition and possibly tissue origin is very exciting and encouraging.

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REFERENCES

1 P. H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007–4021.
2 J. Klose, *Humangenetik*, 26 (1975) 231–243.
3 N. G. Anderson and N. L. Anderson, *Anal. Biochem.*, 85 (1978) 331–340.
4 N. L. Anderson and N. G. Anderson, *Anal. Biochem.*, 85 (1978) 341–354.
5 L. Anderson, *Two-Dimensional Electrophoresis: Operation of the ISO-DALT system*, Large Scale Biology Press, Washington, DC, 1988.
6 B. D. Hanes and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, Oxford University Press., Oxford, 1990.
7 M. J. Dunn and A. H. M. Burghes, *Electrophoresis*, 4 (1983) 97–116.
8 M. J. Dunn and A. H. M. Burghes, *Electrophoresis*, 4 (1983) 173–189.
9 M. J. Dunn, *J. Chromatogr.*, 418 (1987) 145–185.
10 J. E. Celis and R. Bravo (Editors), *Two-Dimensional Electrophoresis of Proteins: Methods and Applications*, Academic Press, Orlando, FL, 1984.
11 *Clin. Chem.*, 28 (1982) 737–1092.
12 *Clin. Chem.*, 30 (1984) 1897–2108.
13 *Ann. N. Y. Acad. Sci.*, 428 (1984) whole volume.
14 K. E. Willard, *U.S. Pat. Appl.*, US 406,830 (1983).
15 M. G. Harrington, C. R. Merril, D. M. Asher and D. C. Gajdusek, *U.S. Pat. Appl.*, US 67,420 (1987).
16 O. Torgensen, *Excerpta Med.*, 275 (1973) 121–122.
17 E. Jellum, A. K. Thorsrud, M. H. Vatn, I. A. Grimstad, I. Brennhovd, K. M. Tveit and A. Pihl, *Ann. N. Y. Acad. Sci.*, 428 (1984) 173–185.

18 R. P. Tracy and D. S. Young, in B. D. Hanes and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, Oxford University Press, Oxford, 1990, pp. 193–240.
19 J. Travis and R. Pannell, *Clin. Chim. Acta*, 49 (1973) 49–52.
20 E. Gianazza and Ph. Arnaud, *Biochem. J.*, 201 (1982) 129–136.
21 Ph. Arnaud and D. W. Sammons, unpublished results.
22 A. C. Hochstrasser, R. W. James, D. Pometta and D. F. Hochstrasser, *Appl. Theor. Electr.*, 1 (1990) in press.
23 D. F. Hochstrasser, R. D. Appel, R. Vargas, R. Perrier, F. Ravier, M. Funk, C. Pellegrini, A. F. Muller and J. R. Scherr, in preparation.
24 J. J. Edwards, N. G. Anderson, S. L. Nance and N. L. Anderson, *Blood*, 53 (1979) 1121–1132.
25 E. Gianazza, L. Osio, G. Grazioli, S. Astrua-Testori, P. G. Righetti, R. Accinni, I. Renoldi and A. Repossini, *Clin. Chem.*, 32 (1987) 2011–2018.
26 J. E. Celis, G. P. Ratz, P. Madsen, B. Gesser, J. B. Lauridsen, K. P. Brogaard Hansen, S. Kwee, H. Holm Rasmussen, H. V. Nielsen, D. Crüger, B. Basse, H. Leffers, B. Honoré, O. Møller and A. Celis, *Electrophoresis*, 10 (1989) 76–115.
27 T. O. Brock and D. L. McIlwain, *Neurochem. Pathol.*, 3 (1985) 1–14.
28 N. L. Anderson, C. S. Giometti, M. A. Gemmell and M. Macy, *Ann. N. Y. Acad. Sci.*, 428 (1984) 135–143.
29 R. P. Tracy, L. E. Wold, R. M. Currie and D. S. Young, *Clin. Chem.*, 28 (1982) 915–919.
30 A. K. Thorsrud, M. H. Vatn and E. Jellum, *Clin. Chem.*, 28 (1982) 884–889.
31 E. Jellum and A. K. Thorsrud, *Clin. Chem.*, 28 (1982) 876–883.
32 D. Young and R. P. Tracy, *Electrophoresis*, 4 (1983) 117–121.
33 R. P. Tracy and N. L. Anderson, *Clin. Lab. Annu.*, 2 (1983) 101–130.
34 R. P. Tracy and D. Young, *J. Clin. Lab. Autom.*, 3 (1984) 235–243.
35 D. Goldman and C. R. Merril, in J. E. Celis and R. Bravo (Editors), *Two-Dimensional Electrophoresis of Proteins: Methods and Applications*, Academic Press, Orlando, FL, 1984, pp. 241–258.
36 R. P. Tracy, D. S. Young, J. A. Katzmann and R. J. Jenny, *Ann. N. Y. Acad. Sci.*, 428 (1984) 145–157.
37 D. S. Young, R. P. Tracy and A. T. Endler, *Bull. Mol. Biol. Med.*, 10 (1985) 373–386.
38 E. Jellum and A. K. Thorsrud, *Scan J. Clin. Lab. Invest.*, 46 (1986) (Suppl. 184) 71–76.
39 S. Hanash, *Adv. Electrophor.*, 2 (1988) 341–384.
40 N. G. Anderson and N. L. Anderson, *Behring Inst. Mitt.*, 63 (1979) 169–210.
41 N. G. Anderson and N. L. Anderson, *J. Am. Med. Ass.*, 246 (1981) 2621.
42 N. G. Anderson and N. L. Anderson, *Clin. Chem.*, 28 (1982) 739–748.
43 E. Jellum, *Trends Anal. Chem.*, 1 (1981) 12–16.
44 R. Dulbecco, *Science*, 231 (1986) 1055–1056.
45 *Electrophoresis*, 11 (1990) 517–593.
46 G. I. Latter, E. Metz, S. Burbeck and J. Leavitt, *Electrophoresis*, 4 (1983) 122–126.
47 R. A. VanBogelen, M. E. Hutton and F. C. Neidhardt, *Electrophoresis*, 11 (1990) 1131–1166.
48 R. Bravo and J. E. Celis, in J. E. Celis and R. Bravo (Editors), *Two-Dimensional Electrophoresis of Proteins: Methods and Applications*, Academic Press, Orlando, FL, 1984, pp. 445–476.
49 N. L. Anderson and N. G. Anderson, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 5421–5424.
50 N. L. Anderson, R. P. Tracy and N. G. Anderson, in F. Putman (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, Orlando, FL, 2nd ed., 1984, pp. 221–270.
51 N. G. Anderson, N. L. Anderson and S. L. Tollaksen, *Clin. Chem.*, 25 (1979) 1199–1210.
52 J. J. Edwards, N. G. Anderson, S. L. Tollaksen, A. G. Von Eschenbach and J. J. Guevara, *Clin. Chem.*, 28 (1982) 160–163.
53 J. J. Edwards, S. L. Tollaksen and N. G. Anderson, *Clin. Chem.*, 28 (1982) 941–948.
54 C. S. Giometti and N. G. Anderson, in B. J. Radola (Editor), *Electrophoresis '79*, Walter de Gruyter, Berlin, 1980, pp. 395–404.
55 N. G. Anderson, M. T. Powers and S. L. Tollaksen, *Clin. Chem.*, 28 (1982) 1045–1055.

56 J. J. Edwards, S. L. Tollaksen and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1340–1355.
57 K. E. Willard and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1327–1334.
58 M. A. Gemmell and N. L. Anderson, *Clin. Chem.*, 28 (1982) 1062–1066.
59 C. S. Giometti and N. G. Anderson, *Clin. Chem.*, 30 (1984) 2078–2083.
60 C. S. Giometti, N. G. Anderson and N. L. Anderson, *Clin. Chem.*, 25 (1979) 1877–1884.
61 C. S. Giometti, M. Bárány, M. J. Danon and N. G. Anderson, *Clin. Chem.*, 26 (1980) 1152–1155.
62 C. S. Giometti and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1918–1921.
63 D. Goldman, C. R. Merril and M. H. Ebert, *Clin. Chem.*, 26 (1980) 1317–1322.
64 M. J. Walsh, L. Limos and W. Tourtellotte, *J. Neurochem.*, 43 (1984) 1277–1285.
65 F. Wiederkehr, A. Ogilvie and D. J. Vonderschmitt, *J. Neurochem.*, 49 (1987) 363–372.
66 D. E. Comings, *Clin. Chem.*, 28 (1982) 782–789.
67 D. E. Comings, N. G. Carraway and A. Pekkula-Flagan, *Clin. Chem.*, 28 (1982) 790–797.
68 R. K. Narayan, W. E. Heydorn, G. J. Creed, P. L. Kornblith and D. M. Jacobowitz, *Clin. Chem.*, 30 (1984) 1989–1995.
69 K. E. Willard-Gallo, D. W. Houck and M. R. Loken, *Eur. J. Immunol.*, 18 (1988) 1453–1461.
70 J. E. Celis, D. Crüger, J. Kiil, K. Dejgaard, J. B. Lauridsen, G. P. Ratz, B. Basse, A. Celis, H. H. Rasmussen, G. Bauw and J. Vandekerckhove, *Electrophoresis*, 11 (1990) 242–254.
71 J. G. Guevara, Jr., B. Herbert and B. Martin, *Electrophoresis*, 6 (1985) 613–619.
72 P. L. Trnka, E. Pergament and N. G. Anderson, *Clin. Chem.*, 30 (1984) 2040–2042.
73 M. F. Goldfarb, M. S. Savadove and J. A. Inman, *Electrophoresis*, 10 (1989) 67–70.
74 D. T. MacLaughlin, N. F. Santoro and H. H. Bauer, *Biol. Reprod.*, 34 (1986) 579–585.
75 Y. C. Tsai, H. H. Harrison, C. Lee, J. A. Daufeldt, L. Oliver and J. T. Grayhack, *Clin. Chem.*, 30 (1984) 2026–2030.
76 G. A. Scheele, *Excerpta Medica*, 642 (1984) 89–94.
77 J. E. Celis, B. Gesser, H. H. Rasmussen, P. Madsen, H. Leffers, K. Dejgaard, B. Honore, E. Olsen, G. Ratz, J. B. Lauridsen, B. Basse, S. Mouritzen, M. Hellerup, A. Anderson, E. Walbum, A. Celis, G. Bauw, M. Puype, J. Van Damme and J. Vandekerckhove, *Electrophoresis*, 11 (1990) 989–1071.
78 J. E. Celis, K. Dejgaard, P. Madsen, H. Leffers, B. Gesser, B. Honore, H. H. Rasmussen, E. Olsen, J. B. Lauridsen, G. Ratz, S. Mouritzen, B. Basse, M. Hellerup, A. Celis, M. Puype, J. Van Damme and J. Vandekerckhove, *Electrophoresis*, 11 (1990) 1072–1113.
79 J. I. Garrels, B. R. Franza, C. Chang and G. Latter, *Electrophoresis*, 11 (1990) 1114–1130.
80 D. Goldman and C. R. Merril, *Ann. N. Y. Acad. Sci.*, 428 (1984) 186–199.
81 E. H. McConkey, B. J. Taylor and D. Phan, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 6500–6504.
82 K. E. Walton, D. Styer and E. I. Gruenstein, *J. Biol. Chem.*, 254 (1979) 7951–7960.
83 D. Goldman and C. R. Merril, *Am. J. Hum. Genet.*, 35 (1983) 827–837.
84 S. M. Hanash, L. J. Baier, D. Welch, R. Kuick and M. Galteau, *Am. J. Hum. Genet.*, 39 (1986) 317–328.
85 S. Hanash, J. V. Neel, L. J. Baier, B. B. Rosenblum, W. Niezgoda and D. Markel, *Am. J. Hum. Genet.*, 38 (1986) 352–360.
86 P. Comi, B. Giglioni, S. Ottolenghi, A. M. Gianni, G. Ricco, U. Mazza, G. Saglio, C. Camaschella, P. G. Pich, E. Gianazza and P. G. Righetti, *Biochem. Biophys. Res. Commun.*, 87 (1989) 1–8.
86 K. Altland and A. Banzhoff, *Electrophoresis*, 7 (1986) 529–533.
88 R. D. Kuick, S. M. Hanash and J. R. Strahler, *Electrophoresis*, 9 (1988) 192–198.
89 S. M. Hanash, E. H. Y. Chu, R. Kuick, M. Skolnick, J. Neel, J. Strahler, S. Pivirotto and W. Niezgoda, *Proteins: Struct., Funct., Genet.*, 2 (1987) 13–19.
90 E. H. Y. Chu, M. Boehnke, S. M. Hanash, R. D. Kuick, B. J. Lamb, J. V. Neel, W. Niezgoda, S. Pivirotto and G. Sundling, *Genetics*, 119 (1988) 693–703.
91 V. I. Zannis and J. L. Breslow, *Biochemistry*, 256 (1981) 9077–9081.
92 V. I. Zannis, P. W. Just and J. L. Breslow, *Am. J. Hum. Genet.*, 33 (1981) 11–24.
93 G. Utermann, A. Steinmetz and W. Weber, *Hum. Genet.*, 60 (1982) 344–351.
94 J. M. Ordovas, L. Litwack-Klein, P. W. F. Wilson, M. M. Schaefer and E. J. Schaefer, *J. Lipid Res.*, 28 (1987) 371–380.

95 C. R. Merril and D. Goldman, *Clin. Chem.*, 28 (1982) 1015–1020.
96 C. R. Merril, D. Goldman and M. Ebert, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 6471–6475.
97 M. G. Harrington, C. R. Merril, D. M. Asher and D. C. Gajdusek, *N. Engl. J. Med.*, 315 (1986) 279–281.
98 M. G. Harrington and C. R. Merril, *Clin. Chem.*, 30 (1984) 1933–1937.
99 M. G. Harrington, C. R. Merril and E. F. Torrey, *Clin. Chem.*, 31 (1985) 722–726.
100 L. E. Townsend, J. Gilroy, P. Lewitt, D. E. Wolfe, N. Pomara, J. Weintraub and D. Reitz, *Neurochem. Pathol.*, 6 (1987) 213–218.
101 F. Morito, A. Ohta, H. Kaneoka, T. Nagayoshi, M. Hilda and M. Yamaguchi, *Clin. Chem.* 34 (1988) 700–704.
102 J. P. Murnane and R. B. Painter, *Biochemistry*, 22 (1983) 1217–1222.
103 J. Weil and C. Epstein, *Am. J. Hum. Genet.*, 7 (1979) 478–483.
104 J. Klose, E. Zeinckle and K. Sperling, *Clin. Chem.*, 29 (1982) 987–992.
105 R. N. Rosenberg, L. Thomas, F. Baskin, J. Kilpatrick, C. Bay and W. L. Nyhan, *Neurology*, 29 (1979) 917–922.
106 R. P. Tracy, R. A. Kyle and D. S. Young, *Hum. Pathol.*, 15 (1984) 122–129.
107 P. J. Wirth, *Electrophoresis*, 10 (1989) 543–554.
108 K. E. Willard and N. L. Anderson, in B. Radola (Editor), *Electrophoresis '79*, Walter de Gruyter, Berlin, 1980, pp. 415–424.
109 E. P. Lester, P. Lemkin, L. Lipkin and H. L. Cooper, *J. Immunol.*, 126 (1981) 1428–1434.
110 N. L. Anderson, C. S. Giometti, M. A. Gemmell, S. L. Nance and N. G. Anderson, *Clin. Chem.*, 28 (1982) 1084–1092.
111 N. L. Anderson, J. C. Wiltsie, C. Y. Li, K. E. Willard-Gallo, R. P. Tracy, D. S. Young, M. T. Powers and N. G. Anderson, *Clin. Chem.*, 29 (1983) 762–767.
112 E. P. Lester, P. F. Lemkin, J. F. Lowery and L. E. Lipkin, *Electrophoresis*, 3 (1982) 364–375.
113 K. E. Willard-Gallo, Y. Humblet and M. Symann, *Clin. Chem*, 30 (1984) 2069–2077.
114 S. M. Hanash, L. J. Baier, L. McCurry and S. A. Schwartz, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 807–811.
115 S. M. Hanash, R. Kuick, J. Strahler, B. Richardson, G. Reaman, L. Stoolman, C. Hanson, D. Nichols and H. J. Tueche, *Blood*, 73 (1989) 527–532.
116 S. M. Hanash, J. R. Strahler, R. Kuick, E. H. Y. Chu and D. Nichols, *J. Biol. Chem.* 263 (1988) 12 813–12 815.
117 X. X. Zhu, K. Kozarsky, J. R. Strahler, C. Eckerskorn, F. Lottspeich, R. Melhem, J. Lowe, D. Fox, S. M. Hanash and G. F. Atweh, *J. Biol. Chem.*, 264 (1989) 14 556–14 560.
118 I. A. Grimstad, A. K. Thorsrud and E. Jellum, *Cancer Res.*, 48 (1988) 572–577.
119 R. P. Tracy, J. A. Katzmann, T. K. Kimlinger, G. A. Hurst and D. S. Young, *J. Immunol. Methods*, 65 (1983) 97–107.
120 N. G. Saravia, M. A. Gemmell, S. L. Nance and N. L. Anderson, *Clin. Chem.*, 30 (1984) 2048–2052.
121 S. J. Norris, J. F. Alderete, N. H. Axelsen, M. J. Bailey, S. A. Bakerzander, J. B. Baseman, P. J. Bassdorf, R. E. Baughn and A. Cockayne, *Electrophoresis*, 8 (1987) 77–92.
122 P. Jackson, V. F. Urwin, M. R. Torrance and J. A. Carmen, *Electrophoresis*, 10 (1989) 456–463.
123 M. A. Churchill and R. J. Radloff, *J. Virol.*, 37 (1981) 1103–1106.
124 K. E. Willard, *Clin. Chem.*, 28 (1982) 1031–1135.
125 E. Jellum, A. K. Thorsrud and F. W. Karasek, *Anal. Chem.*, 55 (1983) 2340–2344.
126 T. Manabe, T. Okuyama, A. Suzuki and A. Shigematsu, *J. Chromatogr.*, 225 (1981) 65–71.
127 T. Marshall and O. Vesterberg, *Electrophoresis*, 4 (1983) 363–366.
128 N. L. Anderson, M. Swanson, F. A. Giere, S. Tollaksen, A. Gemmell, S. Nance and N. G. Anderson, *Electrophoresis*, 7 (1986) 44–48.
129 T. Kadofuku and T. Sato, *J. Chromatogr.*, 419 (1987) 315–322.
130 N. L. Anderson, F. A. Giere, S. L. Nance, M. A. Gemmell, S. L. Tollaksen and N. G. Anderson, *Fundam. Appl. Toxicol.*, 8 (1987) 39–50.

131 G. Zastrow, S. Günther, W. Postel, A. Görg, H. A. Diehl and E. H. J. M. Jansen, *Electrophoresis*, 11 (1990) 655–657.
132 T. Marshall, K. M. Williams and O. Vesterberg, *Electrophoresis*, 6 (1985) 47–52.
133 P. M. Hurley, N. Catsimpoolas and G. N. Wogan, in N. Catsimpoolas (Editor), *Electrophoresis '78*, Elsevier, Amsterdam, 1978, pp. 283–296.
134 E. Fosslien, R. Prasad and J. Stastny, *Electrophoresis*, 5 (1984) 102–109.
135 J. Taylor, N. L. Anderson and N. G. Anderson, *Electrophoresis*, 4 (1983) 338–346.
136 E. Gianazza, G. Tedesco, N. Cattò, L. Bontempi and P. G. Righetti, *Electrophoresis*, 9 (1988) 1972–182.
137 A. H. M. Burghes, M. J. Dunn and V. Dubowitz, *Electrophoresis*, 3 (1982) 354–363.
138 B. Bjellqvist, K. Ek, P. G. Righetti, E. Gianazza, A. Görg, R. Westermeier and W. Postel, *J. Biochem. Biophys. Methods*, 6 (1982) 317–339.
139 D. Hochstrasser, V. Augsburger, M. Funk, R. Appel, C. Pellegrini and A. L. Muller, *Electrophoresis*, 7 (1986) 505–511.
140 E. Gianazza, A. Osnaghi, L. Bontempi, P. G. Righetti and F. Celentano, *Appl. Theor. Electrophoresis*, 1 (1989) 155–159.
141 E. Gianazza, S. Astrua-Testori, P. Caccia, P. Giacon, L. Quaglia, and P. G. Righetti, *Electrophoresis*, 7 (1986) 76–83.
142 M. A. Rimpiläinen and P. G. Righetti, *Electrophoresis*, 6 (1985) 419–422.
143 P.K. Sinha and P. G. Righetti, *J. Biochem. Biophys. Methods*, 12 (1986) 289–297.
144 H. Rilbe, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, Walter de Gruyter, Berlin, 1977, pp. 35–50.
145 E. Gianazza, S. Astrua-Testori, P. Giacon and P. G. Righetti, *Electrophoresis*, 6 (1985) 332–339.
146 P. G. Righetti, G. Tudor and E. Gianazza, *J. Biochem. Biophys. Methods*, 6 (1982) 219–227.
147 E. Gianazza, P. De'Ponti, C. Ettori, C. Magenes and P. G. Righetti, *Electrophoresis*, submitted for publication.
148 P. G. Righetti, M. Chiari, E. Casale and C. Chiesa, *Appl. Theor. Electrophoresis*, 1 (1989) 115–121.
149 G. Cossu, M. G. Pirastru, M. Satta, M. Chiari, C. Chiesa and P. G. Righetti, *J. Chromatogr.*, 475 (1989) 283–292.
150 K. Altland and U. Rossmann, *Electrophoresis*, 6 (1985) 314–325.
151 J. R. Strahler, S. M. Hanash, L. Somerlot, W. Postel and A. Görg, *Electrophoresis*, 8 (1987) 165–173.
152 P. K. Sinha, personal communication.
153 D. A. Young, *Clin. Chem.*, 30 (1984) 2104–2108.
154 A. Lapin, F. Gabl and H. Kopsa, *Electrophoresis*, 10 (1989) 589–595.
155 A. D. Olson and M. J. Miller, *Anal. Biochem.*, 169 (1989) 49–70.
156 J. I. Garrels, *J. Biol. Chem.*, 254 (1979) 7961–7977.
157 N. L. Anderson, J. Taylor, A. E. Scandora, B. P. Coulter and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1807–1820.
158 P. R. Lemkin and L. E. Lipkin, in A. Geisow and C. Barrett (Editors), *Computing in Biological Science*, Elsevier, Amsterdam, 1983, pp. 181–231.
159 P. Vincens, N. Paris, J. Pujol, C. Gaboriaud, T. Rabilloud, J. Pennetier, P. Matherat and P. Tarroux, *Electrophoresis*, 7 (1986) 347–356.
160 R. Appel, M. Funk, C. Pellegrini, D. Hochstrasser and A. F. Muler, in P. Magnenat and G. Thalman (Editors), *Computer-Generated Images: The State of the Art*, Springer, Berlin, 1985, pp. 118–124.
161 J. E. Celis, G. P. Ratz, A. Celis, P. Madsen, B. Gessen, S. Kwell, N. V. Nielsen, H. Ydel, H. B. Lauridsen and B. Basse, *Leukemia*, 2 (1988) 561–601.