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Review

Electrophoresis of mutant proteins in inherited diseases

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ABSTRACT

The electrophoretic approaches for detection of mutant proteins in inherited diseases are briefly reviewed and discussed. Mutation of a protein, known to be associated with a specific inherited disease, is detected by immunoblotting, immunoprecipitation or enzyme staining, combined with various electrophoretic techniques. Some instrumental and technological devices for two-dimensional electrophoresis have been reported for the screening of mutant proteins in diseases of currently unknown etiology.

CONTENTS

1. INTRODUCTION

Electrophoresis is one of the most important laboratory procedures for the analysis of mutant proteins in inherited diseases. They are analysed, characterized or searched for in two different ways; first, for the diseases with known specific genetic abnormalities; and second, for the diseases without information about their genetic mutations. If the pathogenesis of a disease has already been revealed at the level of the gene, or at least at the level of the protein, analysis of

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the mutant protein is directed to further characterization individually and applied to its diagnosis, prevention and treatment. Various forms of electrophoretic technique have been used for this purpose.

There are far more inherited diseases in which no information is available about the structural or metabolic abnormalities in each patient. Recent advances in molecular genetics have made it possible to approach the problem of these diseases of unknown etiology by genetic linkage analysis at the molecular level, using polymorphic DNA probes. Once linkage to a certain probe is established, then the gene responsible for this disease is searched for, cloned and characterized. This technique, based on "reverse genetics" [1], has proved useful in many hereditary diseases. The well known case of Duchenne muscular dystrophy research has given the most glorious and fruitful result, which revealed the presence of a unique and huge protein (dystrophin) [2], and further detailed structural and functional analyses are currently in progress.

However, another screening system for detection of mutant proteins, two-dimensional electrophoresis, has been tried in some laboratories, although it is not yet fully developed for routine analysis of hereditary diseases. This approach is promising and expected to become a sensitive and useful method of analysis in this field.

This paper is not a comprehensive review of the electrophoretic analysis of hereditary diseases in general, and therefore deals with only some examples of electrophoretic separation of normal and mutant proteins, mainly on the basis of the author's personal data on lysosomal diseases and other inherited diseases.

2. GENETIC POLYMORPHISM

A gene coding for a protein does not always have the same DNA sequence on each chromosome among apparently normal individuals. This genetic diversity first became apparent when the electrophoretic technique was applied to the detection of structurally diverse molecular forms in a single enzyme, without laborious purification procedures. In fact, many proteins exist in two or more forms in the human population [3]. They are the products of multiple genes (alleles) at the same genetic locus coding for the same protein. Different combinations of two alleles give rise to various electrophoretic protein patterns (polymorphism) in a population. These variations have been used as markers for population genetics, but more attention has been focused recently on DNA polymorphism, rather than protein polymorphism, by the discovery of restriction fragment length polymorphisms (RFLPs) [4]. This genetic variation is associated with human individuality without significant effects on physiological conditions, but often associated with phenotypic variation affecting susceptibility to certain diseases. At present, no basic mechanism of functional variation has been revealed in genetic polymorphism at the levels of proteins or genes. Electrophoresis is expected to contribute further to investigations of these "missense" mutations.

TABLE 1

HUMAN INTRACELLULAR AND EXTRACELLULAR PROTEINS WITH ELECTROPHORETICALLY DETECTABLE POLYMORPHISMS (MODIFIED FROM REF. 5)

Plasma proteins

α_1-Acid glycoprotein, α_1-antitrypsin, ceruloplasmin, cholinesterase, complement (C2, C3, C4, C6), β_2-glycoprotein I, haptoglobin α chain, pancreatic amylase, properdin factor II, transferrin, vitamin D-binding protein.

Erythrocyte enzymes

Acid phosphatase 1, adenosine deaminase, adenylate kinase, carbonic anhydrase 2, esterase D, galactose-1-uridyl transferase, glucose-6-phosphate dehydrogenase, glutamic-pyruvic transaminase, glutathione peroxidase, gluthathione reductase, glyoxalase I, NADPH-dependent diaphorase, peptidase (A, C, D), phosphoglucomutase (1, 2), phosphogluconate dehydrogenase, uridine monophosphate kinase.

Leukocyte enzymes

Aconitase (soluble), cytidine deaminase, α-L-fucosidase, α-glucosidase, hexokinase 3, mitochondrial glutamic-oxalacetic transaminase, mitochondrial malic enzyme, phosphoglucomutase 3.

It should be noted, however, that these polymorphisms are not associated or related to each single gene diseases.

A list of well known extracellular (plasma) and intracellular proteins with electrophoretically detectable polymorphisms is shown in Table 1. These proteins are detected by conventional protein staining methods or by enzyme staining.

3. QUANTITATIVE AND QUALITATIVE VARIATIONS OF MUTANT PROTEINS IN INHERITED DISEASES

Mutant proteins are detected either by staining of the protein molecules or by monitoring of enzyme activities on the electrophoretic gel. The conventional protein staining methods, such as Coomassie Brilliant Blue and silver stain, are not used so often at present for two reasons: specificity and sensitivity. Generally it is difficult to detect a protein spot that is specifically lost or newly expressed in a certain inherited disease. The amount of gene expression is often too low to be detected, or the protein in question is easily hidden and overlooked among other adjacent or overlapping proteins on the gel, if it is stained only by conventional non-specific staining methods.

Two specific methods to detect a protein have been used to overcome these technical drawbacks; immunochemical techniques and functional monitoring. Protein molecules are detected immunologically, with specific antibodies, after electrophoretic separation (immunoblotting), or they are separated first by immunoprecipitation and then detected on the electrophoresis gel by conventional staining or by more sensitive methods, such as autoradiography and fluoro-

graphy. The proteins can also be monitored on the gel by enzymic staining, if they are not denatured during the protein preparation procedures.

In many inherited diseases, quantitative changes of mutant proteins were observed by electrophoresis and other protein analyses; the amount expressed by the mutant gene is decreased or completely absent. Enzyme staining or other methods for the detection of protein functions are generally more sensitive than the staining of protein molecules.

3.1. Immunoblotting

Recently, immunochemical techniques have been used more than conventional protein staining methods for the analysis of inherited diseases. Immunoblotting is specific and sensitive, but a specific antiserum is necessary for the detection of a protein in this procedure.

We have been using this technique to identify some proteins associated with inborn metabolic diseases in humans: α-glucosidase [6–9], β-hexososaminidase A [10], β-galactosidase [11] and proteolipid protein [12]. The proteins were extracted in 10 m*M* sodium phosphate (pH 6.5), containing 150 m*M* NaCl, and separated as glycoprotein with concanavalin A–Sepharose for α-glucosidase [8] or precipitated with trichloroacetic acid for β-galactosidase [11], subjected to polyacrylamide gel electrophoreis (PAGE) in the presence of sodium dodecyl sulphate (SDS) [13], and then electroblotted to a nitrocellulose membrane [14]. The protein spots were visualized with specific polyclonal antibodies.

One of the typical pictures is shown in Fig. 1. A male patient died of cardiomyopathy of unknown origin at 15 years of age, and the α-glucosidase activity was reduced to 14% of the control mean toward glycogen. The total amount of the enzyme protein was somewhat decreased in fibroblasts, but no qualitative abnormalities were found. The Michaelis constant (K_M) of this enzyme was remarkably high for maltose and glycogen, but not for artificial substrates. This case was reported as a K_M mutant glucogenosis II [8].

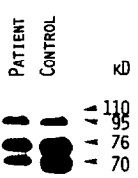

Fig. 1. Immunoblotting of α-glucosidase [8]. Glycoproteins from fibroblasts, separated with concanavalin A–Sepharose, were electrophoresed in 10% polyacrylamide gel in the presence of SDS. After blotting to nitrocellulose, the enzyme protein was visualized with a specific antibody in combination with [^{125}I]protein A.

3.2. Immunoprecipitation–electrophoresis–fluorography

Recently, this method has been used frequently in our laboratory for the analysis of various lysosomal enzymes: β-galactosidase [15–18], α-glucosidase [19–21] and protective protein/carboxypeptidase [15,22]. This procedure is particularly useful for the analysis of the intracellular turnover of newly synthesized proteins [23,24]. Fibroblasts are cultured and pulse-labelled with [^{3}H]leucine in the leucine-free Eagle's minimum essential medium (MEM) containing dialysed 5% fetal calf serum for a few hours, and the culture is further continued in the same medium containing unlabelled leucine (pulse-chase study). The cells are then subjected to immunoprecipitation and electrophoresis, and the protein spots are visualized by fluorography.

In a typical experiment with human β-galactosidase-deficient fibroblasts, an abnormally large precursor enzyme was detected in one of the infantile G_{M1}-gangliosidosis patients (Fig. 2). Human β-galactosidase appears normally as an $84 \cdot 10^3$ relative molecular mass (M_r) precursor, and then processed via an $88 \cdot 10^3$ intermediate to a $64 \cdot 10^3$ mature enzyme, which remains for at least 20 h of the chase period. But the mature form of this enzyme was not detected in any of the G_{M1}-gangliosidosis patients, although the precursors of apparently normal molecular mass were present in most of them. One case (No. 2) in Fig. 2 showed an abnormal precursor molecule, which was calculated at $86 \cdot 10^3$ in molecular mass, instead of $84 \cdot 10^3$. Further gene analysis of this case revealed a 165-nucleotide duplication in the β-galactosidase genome, producing an abnormally large mRNA in lymphoblastoid cells (Yoshida *et al.*, unpublished results). The protein data were thus well correlated with the DNA–RNA data in this case.

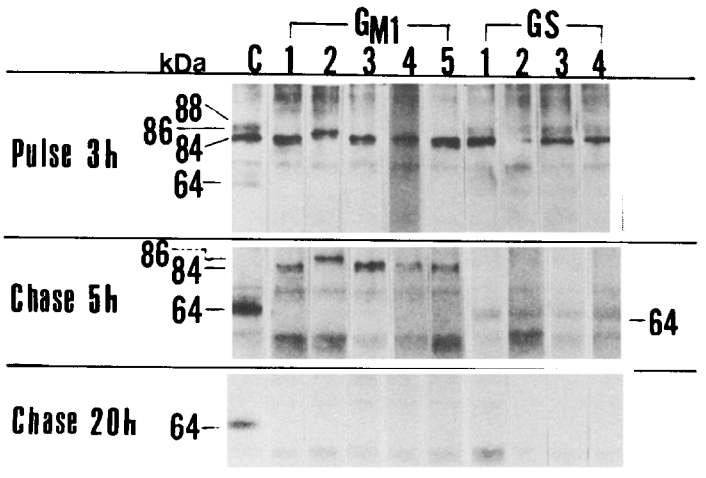

Fig. 2. Pulse-chase analysis of fibroblast β-galactosidase labelled with [^{3}H]leucine [16]. The cells were pulse-labelled for 3 h, and then chased up to 20 h. G_{M1} = G_{M1}-gangliosidosis; GS = galactosialidosis; C = control cells. The case number is at the top of each lane.

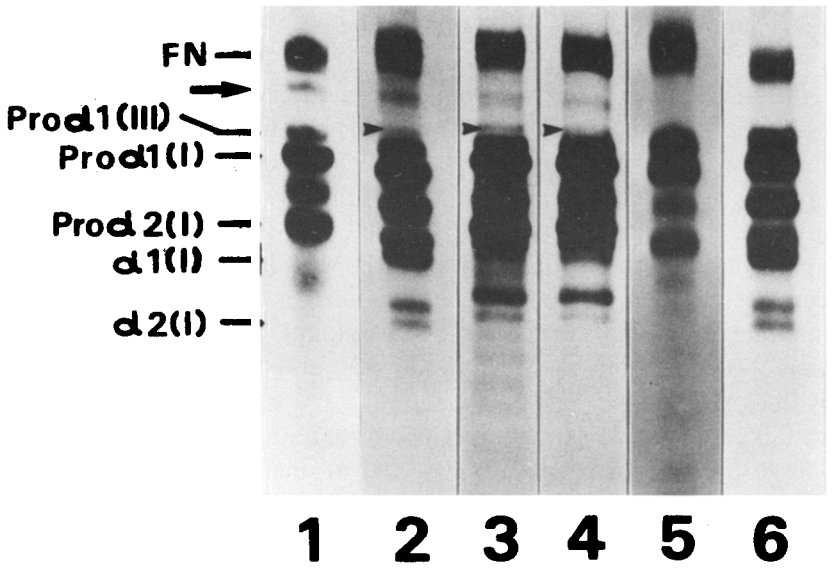

Fig. 3. Electrophoresis of ^{3}H-labelled fibroblast collagens (from ref. 26, with permission of the author). Proteins were reduced with 2-mercaptoethanol and separated in 5% polyacrylamide gel. Lanes 1–5 fibroblasts from Marfan syndrome patients; lane 6 control fibroblasts. In lanes 1–4, an additional protein band was observed (arrow). Another band (arrowhead) was also detected in lanes 2–4.

On the other hand, the cells from patients with galactosialidosis, a β-galactosidase deficiency disease due to a defect of another functional protein [25], showed normal synthesis of β-galactosidase protein, which was degraded rapidly within a few hours (Fig. 2; GS).

Another example is shown of the immunoprecipitation–electrophoresis of an abnormal collagen in Marfan syndrome patients [26]. In this experiment, collagens labelled with [^{3}H]proline were secreted from fibroblasts into the culture medium and treated as described above. An abnormal band of $185 \cdot 10^{3}$ was detected (Fig 3; arrow) and identified as α1(IV) collagen, which is not expressed normally in dermal fibroblasts. Its pathogenetic significance is not known at present, and this collagen may not be a mutant protein in a strict sense. However, abnormal expression may be associated with the genetic abnormality in this disease. Collagen abnormalities have been reported also in osteogenesis imperfecta and Ehlers–Danlos syndrome in similar experiments [27].

3.3. Enzyme staining

Enzyme proteins are detected for their activities after separation by electrophoresis or electrofocusing. The enzyme molecules should not be denatured in this analysis. The staining method varies for each enzyme. We use the following

separation/visualization methods for β-galactosidase [28] and β-hexosaminidase [29–31]. Initially, we used chromogenic substrates (*p*-nitrophenyl derivatives) to detect enzyme activities, but later fluorogenic substrates became commercially available, which are naturally more sensitive. We started with starch gel electrophoresis, but cellulose acetate electrophoresis was generally more useful, convenient and easy to handle for routine clinical analysis.

Hepatic β-galactosidase isozymes were separated by starch gel electrophoresis [32], and the residual amount of isozyme A was found to be variable in G_{M1}-gangliosidosis patients (Fig. 4). After electrophoresis, the gel was overlaid with a filter paper that had been presoaked in the substrate (4-methylumbelliferyl α-galactoside) solution at pH 5.0, incubated for 30–60 min, and visualized under a UV lamp. These results (heterogeneous appearance of the isozyme A) were not related to clinical manifestations of these patients [28].

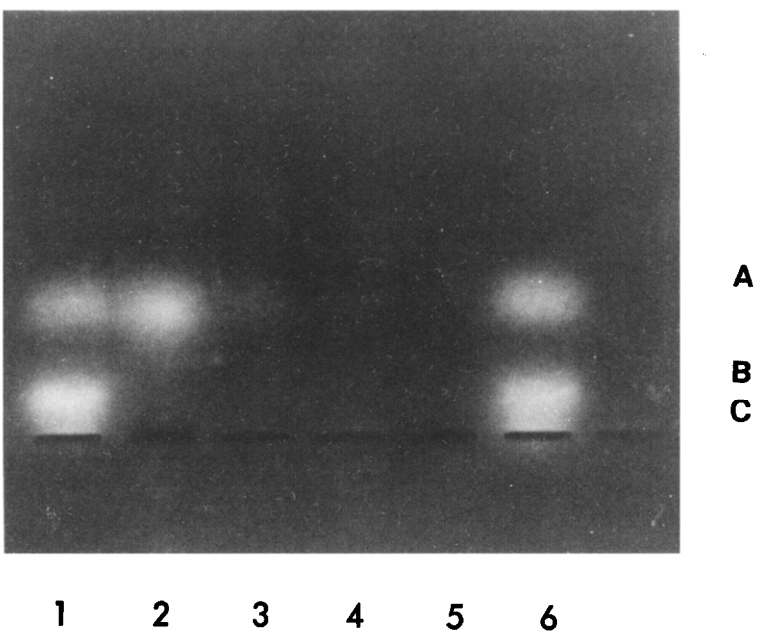

Fig. 4. Starch gel electrophoresis of hepatic β-galactosidase [28]. The enzyme activity was stained with 4-methylumbelliferyl β-galactoside as substrate. One fast-moving spot (A) and two slow-moving spots (B and C) were observed. Lanes 1 and 6 controls; lanes 2–5. G_{M1}-gangliosidosis. A large A spot was detected at lane 2 and a faint A spot at lane 3.

Essentially the same procedure was performed for the β-hexosaminidase separation on a cellulose acetate membrane [29,30]. This enzyme was separated into two spots, designated A and B, and three different patterns were observed in the classical Tay–Sachs disease, juvenile G_{M2}-gangliosidosis, and Sandhoff disease (Fig. 5). β-Hexosaminidase A was completely deficient in Tay–Sachs disease and apparently normal in Sandhoff disease [29].

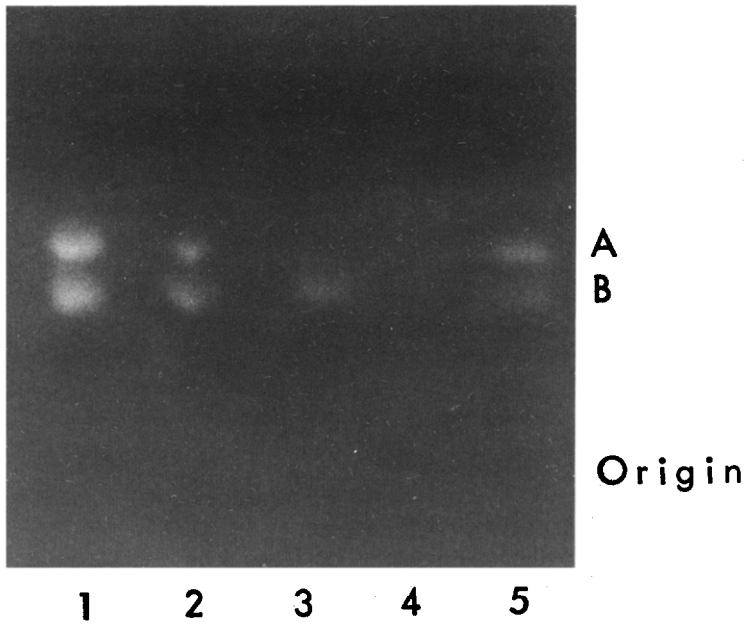

Fig. 5. Cellulose acetate electrophoresis of hepatic β-hexosaminidase [29]. The enzyme activity was stained with 4-methylumbelliferyl N-acetyl-β-glucosaminide as substrate. Two bands, designated A and B, were observed in control tissues. Lanes 1 and 5, controls; lane 2, juvenile G_{M2}-gangliosidosis (band A decreased); lane 3, Tay–Sachs disease (band A deficient); lane 4, Sandhoff disease (both bands A and B deficient).

For quantitative analysis, these spots were cut out, extracted in glycine buffer (pH 10–11), and measured by fluorometry. The enzyme diagnosis of Tay–Sachs disease was made possible by this procedure of quantitative β-hexosaminidase A assays [31], and applied to the carrier diagnosis in the family members of the patients (Fig. 6).

4. SEARCH FOR UNKNOWN MUTANT PROTEINS

In the majority of inherited diseases, the molecular pathophysiology is not known and efforts have been made to detect mutant genes, mainly by the "reverse genetics" approach. Genetic linkage analysis is a useful technique, but another approach is possible to search for a gene product without any information about its function. Two-dimensional electrophoresis is probably the best candidate at present for the protein screening of this type. It consists of a combination of isoelectric focusing and PAGE, originally developed by Margolis and Kenrick [33] and improved by O'Farrell [34].

It can reveal several hundred protein spots on one gel, but its use for routine analysis has been hampered mainly by three major inherent problems: reproduc-

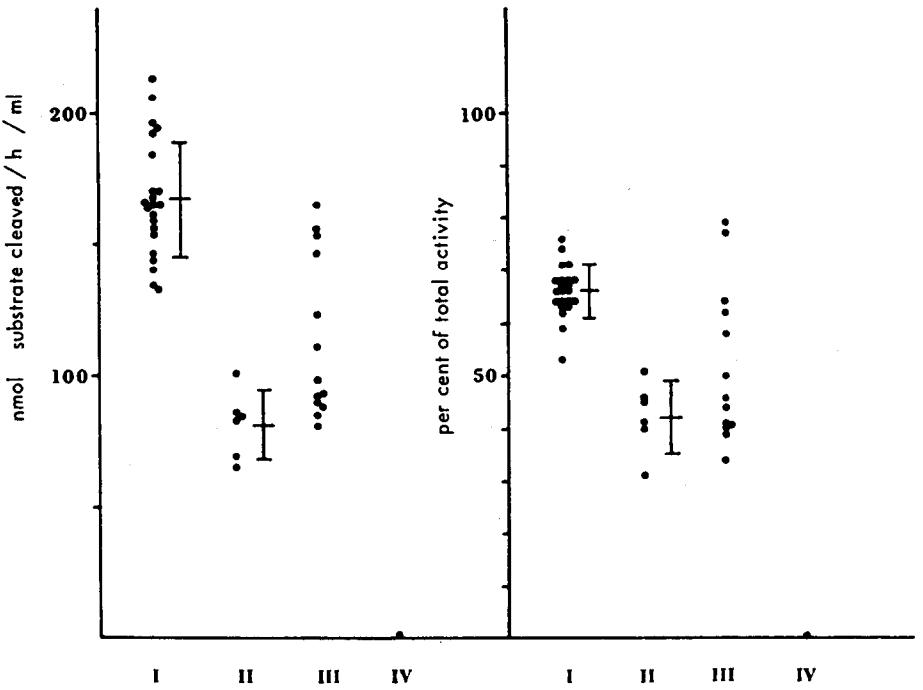

Fig. 6. Quantitative measurements of β-hexosaminidase A activity after separation by electrophoresis on a cellulose acetate membrane [31]. The enzyme activity was assayed with 4-methylumbelliferyl N-acetyl-β-glucosaminide as substrate. Group I, normal controls: groups II, parents of Tay–Sachs disease patients; group III, unaffected family members other than parents; group IV, Tay–Sachs disease patients. The left panel shows the enzyme activity, and the right panel shows the ratio of β-hexosaminidase A to B.

ibility, spot identification and data processing. Various efforts have been made to overcome these drawbacks, including computerized scanning systems such as TYCHO [35], GELLAB [36,37], AUTOMACH [38], HERMeS [39,40], GIPSY [41] and PDQUEST [42]. However, a large computer system is not always available in every clinical or research laboratory. We therefore started to develop a device for semi-automated computer analysis of two-dimensional electrophoresis gels [43].

Fibroblasts were used for protein analyses in our system to search for mutant proteins that may have been expressed in this cell species. Each gel was divided into nine areas (Fig. 7), and the spots visualized in each square were numbered in order of molecular mass and isoelectric point (from upper left to lower right). Spots not clearly separated were counted as a spot group. Then the gel was surveyed with an image sensor video camera connected to a high-resolution black-and-white display monitor. Each spot analysis was performed with an image analysis program commercially available (TIAS-100) on a personal computer (Fig. 8). First, the screen was standardized by calculating an average of eight

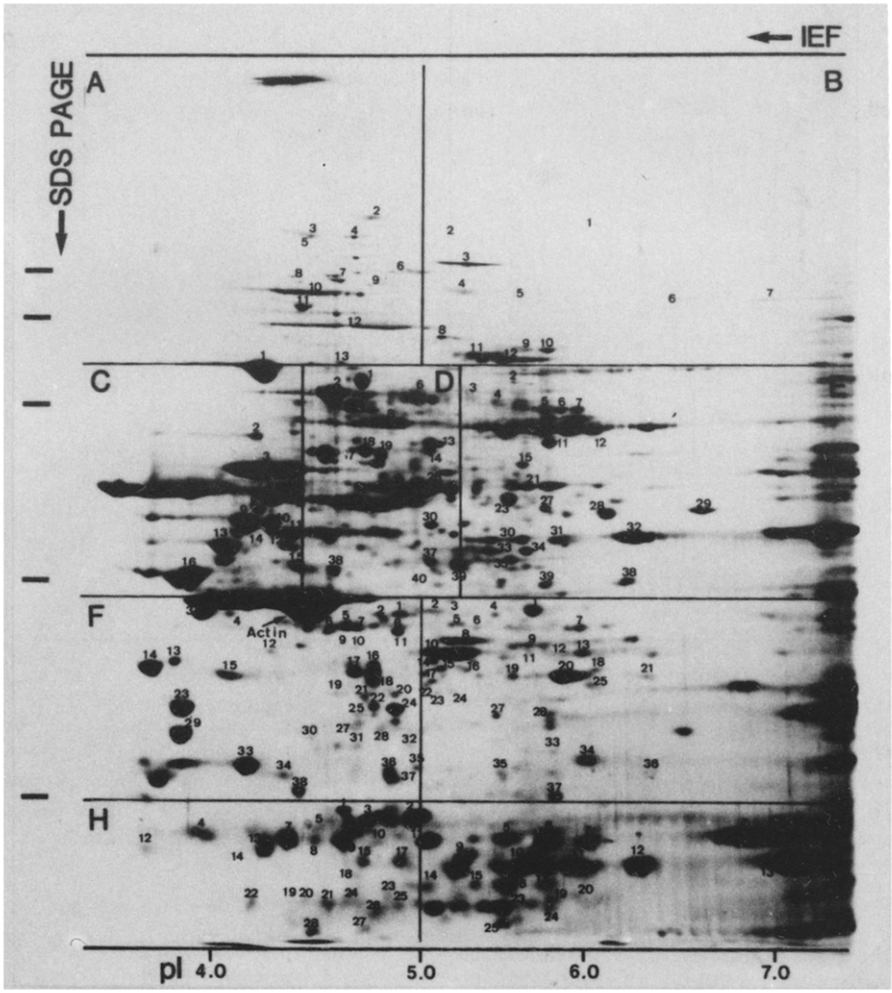

Fig. 7. Two-dimensional electrophoretic pattern of human fibroblast proteins (silver stain; ref. 43). Electrofocusing (IEF) was run with the acidic side on the left and the basic side on the right, and electrophoresis was run vertically. The whole area was divided into nine squares (A–I), and spots in each square were numbered from upper left to lower right. Actin is indicated with an arrow. The marker lines on the left indicate molecular mass values (from top to bottom), 116, 97, 66, 45 and 29 · 10^3 M_r.

recordings to minimize the effect of surrounding light sources. Next, three small square areas without protein spots or any artifactual stains were selected randomly, and the mean of their intensities was calculated as a background value. The spots to be examined on the monitor display were demarcated manually with surrounding squares, their integrated intensity was measured, and the background intensity was subtracted.

Our system does not use sophisticated equipment or machines as in the pro-

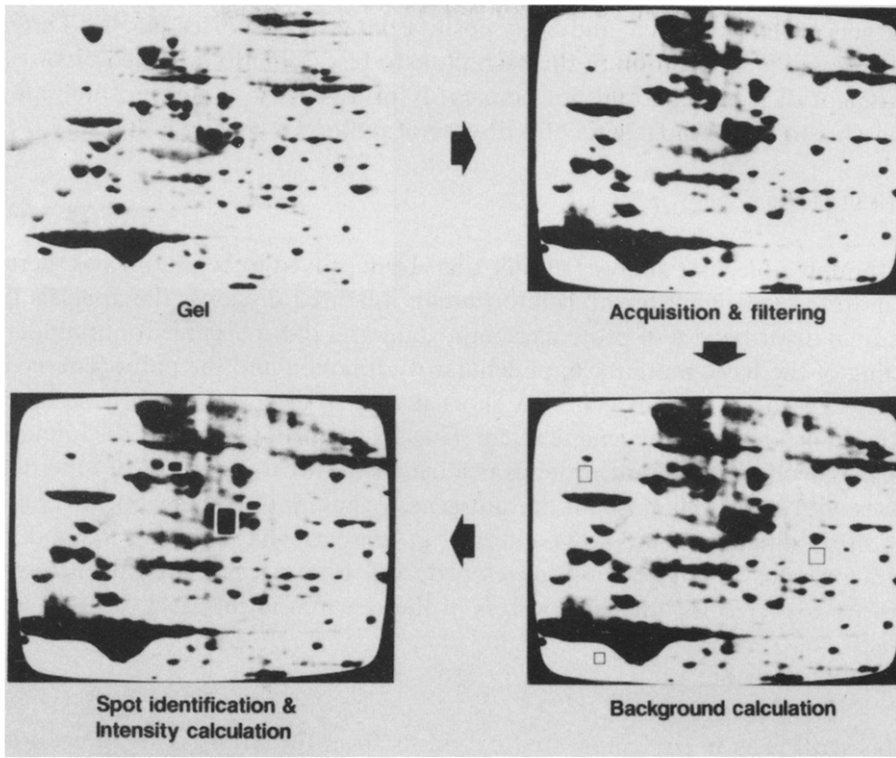

Fig. 8. Procedure for detection and quantitation of spots on the gel shown in Fig. 7 [43]. The stained gel (upper left) was scanned with an image sensor video camera connected to a black-and-white display monitor (upper right). The screen was standardized and filtered by calculating an average of eight recordings. Then, three small square areas without protein spots or any visible artifact stains were selected randomly, and the mean of their intensities was calculated as a background value (lower right). Individual spots were demarcated manually with surrounding squares, their integrated intensities were measured (lower left), and the background intensity was subtracted.

grams referred to above, but we achieved reliable and reproducible results with human fibroblasts although they showed considerable individual variations quantitatively and qualitatively. This system has not achieved routine clinical analysis as yet. There are still various points to improve for practical use, and we have not yet been successful in finding specific protein abnormalities for the several inherited diseases we chose, in which mutant genes may express abnormal proteins in mesodermal cells. However, during the preliminary screening steps, mycoplasma-infected cells were found to express abnormal protein spots, which were cleared off by treatment of the cells with an anti-mycoplasma drug [44]. Further work is in progress on various other inherited diseases.

Two-dimensional electrophoresis is used also for the survey of proteins assigned to specific chromosomes. Downs syndrome (trisomy 21) has been one of

the targets of this approach, and a few positive data were reported [45,46]. This is another possible application of this technique to research into inherited diseases. Assignment of a protein, and not necessarily of a gene, to a chromosome may offer a clue to the pathogenesis of a disease of unknown genetic mutation.

5. CONCLUSIONS

Although molecular analysis of DNA has been proved to be useful, and therefore more prevalent, in research into human inherited diseases, the analysis of functional disturbances of proteins is important and indispensable for an understanding of the basic mechanism of cellular dysfunction and the pathogenesis of diseases caused by mutant genes. A short review of electrophoretic techniques used in the field of clinical genetic research was described. Currently, the immunological detection of mutant proteins is most useful for the analysis of inherited diseases with information about mutant genes or mutant proteins, but there are many other diseases of unknown etiology at present. The screening method of mutant proteins is not yet fully developed, and future technological improvements are expected for further progress of the research in this field.

6. ACKNOWLEDGEMENTS

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