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REVIEW

APPLICATIONS OF POLYACRYLAMIDE GEL ELECTROPHORESIS AND POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING IN CLINICAL CHEMISTRY

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CONTENTS

1. BACKGROUND

Fractionation of plasma proteins has had two major goals. The first has been to understand the function and structure of blood plasma, body fluids, and tissue proteins, and the second to use this knowledge in diagnosis and therapy in the field of clinical medicine. These two areas of endeavor began with the first efforts of Howe [1] in 1921 to separate plasma proteins by means of salting-out techniques. This was followed by the development of free-boundary electrophoresis by Tiselius [2] and of paper zone electrophoresis by Konig [3] in 1937, followed by the improvements of Durrum [4]. This led to the first practical use of boundary and zone electrophoresis in both research and clinical laboratories. The development of cellulose acetate electrophoresis by Kohn [5] in 1957 provided a practical, simple method of serum protein separation for the clinical laboratory with a resultant proliferation of use and development that continues to grow, even today. These techniques, however, as routinely practiced resolve only 5—10 of hundreds of plasma protein components present.

The immunoelectrophoretic techniques of Grabar and Williams [6] combining immunodiffusion and agar gel electrophoresis provided a method where 20—30 fractions could readily be demonstrated, thus, markedly expanding the qualitative information obtainable with procedures that could readily be carried out by clinical laboratory personnel.

A number of excellent books and monographs have been published which fully describe the plasma protein changes in many disease states. The reader is referred especially to the more comprehensive works in this area [7—12] for background reading.

The development of high-resolution starch gel electrophoresis by Smithies in 1955 [13] and the moving boundary buffer system on starch gel by Poulik in 1957 [14], followed a year later by the development of the zymogram technique by Hunter and Burstone [15] for characterizing multiple molecular forms of enzymes, provided a method of greater resolution for in-depth qualitative studies of plasma and tissue proteins, and enzymes. Because of technical complexity these procedures became more useful in research areas and did not find ready clinical applications. They have been utilized widely by geneticists, and have provided much of the basis for the further studies on genetic polymorphism. These advances were followed by the development of polyacrylamide gel electrophoresis first reported by Raymond and Weintraub in 1959 [16].

The purpose of this chapter is to present an overview of the application

of polyacrylamide gel systems in the field of clinical medicine. Toward this aim the material is separated into the areas of polyacrylamide gel electrophoresis (PAGE) and polyacrylamide gel isoelectric focusing (PAGIF). Material in each section is presented in terms of the familiar serum protein patterns, followed by other body fluids and tissue proteins. Histochemical procedures for the enzymes of clinical importance are similar in both PAGE and PAGIF and thus are combined and presented in tabular form.

2. PROTEIN SEPARATION IN POLYACRYLAMIDE GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL

As noted previously, polyacrylamide gel electrophoresis was introduced by Raymond and Weintraub in 1959 [16]. Ornstein and Davis in 1962 [17] greatly advanced resolution in acrylamide with the development of the disc technique. They utilized a discontinuous buffer and pH system designed to produce initial zone stacking between a leading and trailing buffer ion which resulted in a concentration effect and narrow starting zones. The procedure has been employed both in the originally described gel cylinders and on flat gel slabs. This system is hereafter designated as system one. Allen et al. [18] developed a discontinuous buffer and ionic strength system at a continuous pH in flat slab pore gradient gels which produced thin starting zones without a sample or stacking gel to provide greater flexibility, particularly for isoenzyme studies. This system is hereafter designated as system two. These two systems have been widely used for both clinical and research applications. Computer derived discontinuous buffer systems to produce selective stacking and unstacking were developed by Jovin [19, 20] to provide an optimal resolution in the separation of components of similar size and charge. Separation based directly on molecular weight differences was developed by Maizel [21], who treated samples with sodium dodecyl sulfate (SDS), to produce a unit charge on each of the macromolecules present, allowing them to separate in continuous or gradient pore size gels based solely on molecular weight.

The first serum patterns in PAGE [16, 17] had the initial effect of implying that it was a high-resolution technique. However, the resolution of multicomponent systems, containing several hundred components, such as serum, remains unsolved with this technique. Improvements between 1969 and 1973 with various multiphasic buffer systems [18—20, 22], gel gradient strategies and the theoretical considerations developed, particularly those presented by Robard and Chrambach [23] and Kapadia et al. [24] clearly demonstrate that PAGE is limited in its resolving capability to some 30—35 serum proteins.

The development of synthetic polyamino carboxylic ampholytes by Vesterberg in 1967 [25] and polyamino sulfonic and carboxylic ampholytes by Pogacar and Jarecki [26] in 1974 and Grubhofer [27] in 1972 provided a new approach to protein separation, that of isoelectric focusing. This procedure is, for all practical purposes, an electrophoretic equilibrium method for separating proteins according to their isoelectric point and offers unique advantages for both preparative and analytical methods. By utilizing various two-

dimensional gel methods further resolution may be obtained. For example, by coupling isoelectric focusing in the first dimension with SDS electrophoresis in the second dimension O'Farrell [28] was able to increase resolution of the plasma proteins, identifying up to 1000 radioactively labelled serum proteins in a single specimen. Obviously, the ability to increase resolution of a mixture of proteins by over two orders of magnitude in recent years has not as yet even been partially exploited in the field of clinical medicine. Such an information explosion has perhaps, rather led to considerable perplexity and a wait-and-see attitude by many clinical laboratory workers to determine the practical potential of this tool in clinical diagnosis and therapy control.

Current literature seems to indicate that as PAGE replaced starch gel electrophoresis, PAGIF has already replaced PAGE in many applications as the method of choice for the separation and isolation of complex mixtures of proteins.

For clinical applications it should be stressed that no single set of conditions or procedures has yet been found optimal for all protein and isozyme separations. The potential use of PAGE must be considered and compared with the more recent developments with PAGIF, to select an optimal system for a given task.

For both methods, a rigorous standardization of all steps involved in the practical performance of the technique is a major prerequisite for utilization of their full potential as diagnostic tools. It is essential to adopt a standard set of conditions for sample preparation and handling, gel purification, polymerization, separation conditions, staining, and quantitative evaluation. Pattern deviations due to methodological effects must be minimized to achieve an acceptable level of reliability and reproducibility. A brief review of some of the more pertinent points is, therefore, appropriate before presenting specific applications of these methods in clinical medicine.

3. STANDARDIZATION OF SAMPLE COLLECTION AND HANDLING

Blood sample collection and handling procedures in each hospital are usually processed on a strict routine and this area might not seem to warrant comment; however, it should be emphasized that the higher the resolution techniques, the more minor alterations resulting from handling and processing inconsistencies will be magnified, leading to greater variation in results. Many of the tests most applicable to these techniques, thus, must be handled as non-routine, special chemistries. Precautions must be taken to assure that the sample arrives at the laboratory as soon as possible after collection.

Serum should be drawn from the blood samples immediately after clotting and the clot may be best allowed to contract in the cold. Plasma for enzymes, clotting factors, and lipoproteins should be obtained from chilled blood samples and run as soon as possible, or stored frozen at $-70°$, with the exception of samples for lipoprotein analysis, which may be stored at $4°$ for not more than 48 h. On the other hand, hemoglobin samples may be treated with potassium cyanide and glycerol and held at $-20°$ for subsequent analysis for periods of two to three weeks without affecting separation patterns quali-

tatively or quantitatively. Urine may be concentrated by freeze dialysis and then stored at —70°. Long-term storage of samples for future reference, or for comparative serial studies, should be made in aliquots to avoid repeated freezing and thawing and again held at —70° or lower.

PAGE systems employing electrochemical conditions where the sample is photopolymerized in a sample gel at pH 6.8 may result in the denaturation of isoenzymes [29] or provide a final pH environment below the isoelectric point for some of the protein macromolecules of interest. In the latter instance, these will be trapped in the opaque sample gel and not migrate. Lactic dehydrogenase isoenzyme 5 is a prime example of this problem. Maintaining the sample prior to and during the initial phase of electrophoresis at pH 9.0 may be necessary not only to assure that the majority of proteins are above their p*I* but also to prevent precipitation of proteins in complex mixtures such as tissue extracts and body fluids [30]. PAGE system two lends itself to this requirement since the system operates at a continuous pH of 9.0 and requires no stacking or sample gel. Adjustment of sample pH to 9.0 prior to PAGIF also may be made and will not affect resultant resolutions.

4. STANDARDIZATION OF APPARATUS AND OPERATIONS

The usual clinical requirement for the ability to process a number of samples and controls simultaneously favors the choice of equipment which minimizes the risk of apparatus-inherent complex manipulations. Equipment designed to produce gel slabs offers a number of advantages over that designed for separation in cylindrical gels, or gel rods.

(i) On flat slabs many samples, e.g., 12—30 with PAGE apparatus and up to 60 with PAGIF systems, can be applied side by side and separated under identical conditions. Standards may be included on the same gel for exact side-by-side comparison and to act as internal control checks.

(ii) Flat slabs, as normally used, vary from 0.5 mm to 3 mm thick and have more efficient dissipation of the Joule heat produced during separation than the usually employed 5—6 mm diameter cylindrical or rod gels. (The use of capillary tube gels is not considered in this context since they have a very limited, practical application in the clinical laboratory.)

(iii) Due to their rectangular cross-section, flat gels are better evaluated by quantitative microdensitometry with a much decreased risk of optical artifacts.

(iv) Flat gels allow two-dimensional analysis where a combination of techniques such as cellulose acetate—PAGE or PAGIF, or PAGIF—SDS may be used for expanded resolution in analytical studies and in addition these dry more easily for storage or autoradiography.

(v) Less preparation time is needed for multisample analysis and for both PAGE and PAGIF prepackaged gels of various types and pH ranges are now commercially available.

5. STANDARDIZATION OF GEL FORMATION AND SEPARATION CONDITIONS

A number of aspects for the control required to achieve reproducible gels

prepared "in-house" have been reported [31]. Reagent purification and polymerization conditions have been reported by Allen et al. [29] and Chrambach and Robard [32]. Catalysts and polymer cross-linking concentration effects have been discussed by Watkins and Miller [33].

Constant power application provides the most efficient and controllable form of power to effect separations in both methods. However, the high voltage gradients required to focus proteins optimally in PAGIF are well beyond the capabilities of earlier 500V constant power supplies and require a device similar to that recently developed by LKB capable of delivering 2000 V.

Normally, PAGE and PAGIF systems utilize glass plates as a support system for the polyacrylamide gel slabs, thus limiting heat transfer due to the characteristics of glass. Cooling efficiency can be increased over 200-fold by substituting ceramic plates such as beryllium oxide (Berlox K-150) for glass to improve heat transfer as described by Allen et al. [34]. Shorter run times and higher voltage gradients may thus be achieved without heat denaturation of labile proteins.

6. POLYACRYLAMIDE GEL ELECTROPHORESIS: APPLICATIONS IN CLINICAL MEDICINE

6.1. Plasma proteins

Both systems one and two have been extensively utilized to study plasma protein changes effected by a variety of diseases and physiological stresses. Hoffmeister [35] has standardized method one and has studied over 10,000 patient samples with the technique. Densitometric traces of serum proteins separable by the two methods are shown for orientation and comparative purposes in Figs. 1 and 2, and the proteins separable and quantifiable by these methods that are important in disease in Table 1.

6.1.1. Immunoglobulins

A number of reports has been published concerning the differentiation of the myeloma proteins and Waldenströms macroglobulinemia [36—39]. Normally, it requires considerable experience to detect unequivocally abnormal proteins in the immunoglobulin region, particularly if the immunoprotein concentration is in the same range as neighboring normal proteins, or if the patient belongs to an unusual haptoglobin type. It has been demonstrated also with method one by post-electrophoretic immunoprecipitation that certain immunoglobulin components with an isoelectric point above 8.5 remain in the turbid stacking gel [39], which is generally discarded. Therefore, with this technique a myeloma protein could be missed, which is an intolerable risk for the clinical chemist. Use of a clear sample gel, or system two, obviates this problem and even those proteins which migrate cathodically at pH 9.0 are well defined in the clear cap gel region [40]. On the other hand, the concentrating feature of PAGE, with both systems one and two, allows the detection of very low paraprotein concentrations, particularly in type 1—1 haptoglobin serum, and has detected paraproteinemia in cases with a

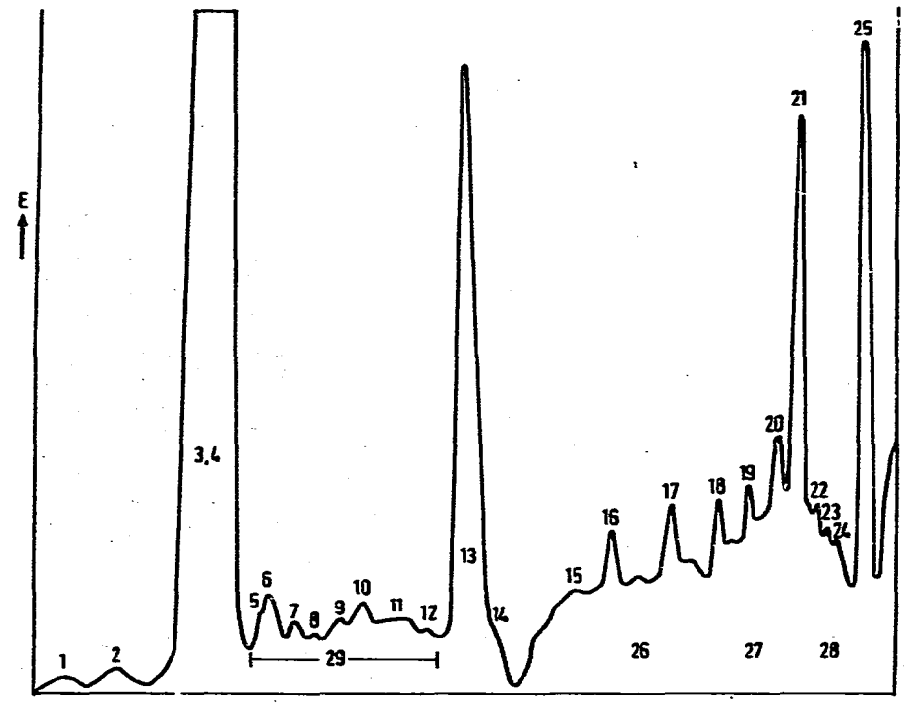

Fig. 1. A densitometric trace of the most important serum proteins separated by PAGE system one. 1, Prealbumin; 2, acid α_1-glycoprotein; 3, albumin; 4, α_1-antitrypsin; 5,7, Gc-globulins; 6, α_1 HS-glycoprotein, α_1-antichymotrypsin; 8, unknown; 9, unknown; 10, ceruloplasmin; 11, unknown; 12, hemopexin; 13, transferrin; 14, inter-α_1 trypsin inhibitor; 15, β_1-A-globulin; 16—19, haptoglobin polymers type 2—2; 20, β-glycoprotein; 21, α_2-macroglobulin; 22—24, haptoglobin polymers type 2—2; 25, β-lipoprotein; 26, I_gA; 27, I_gG; 28, I_gM; 29, α_1-lipoprotein. (After Hoffmeister [35].)

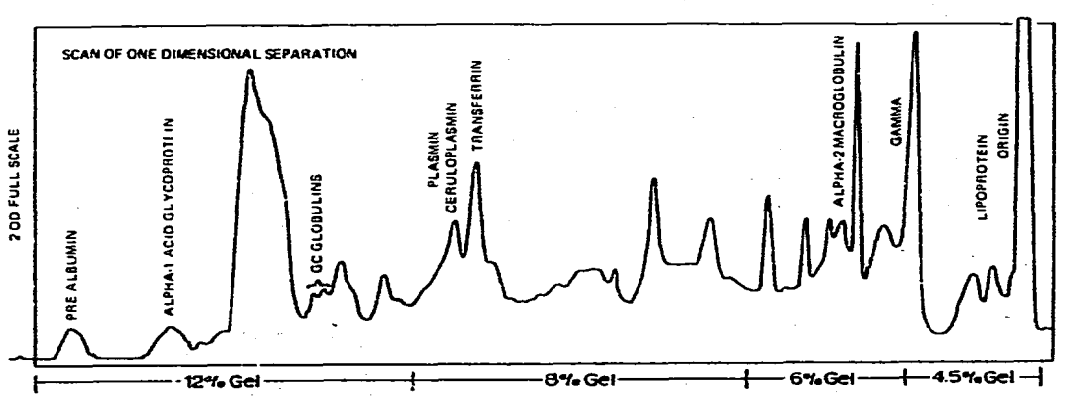

Fig. 2. A densitometric trace of a 2—2 haptoglobin-type serum separated by system two on 4.5—6—8—12% pore-size gradient at a continuous pH of 9.0 using a discontinuous sulfate—borate buffer system. The schematic of this separation is shown in Fig. 8.

HUMAN SERUM PROTEINS SEPARATED BY PAGE

Protein	Biological function	Importance in diagnosis
Acid		Increase: neoplasms, inflammations, rheumatoid arthritis
α_1-Glycoprotein	?	Decrease: liver diseases
Prealbumin	Thyroxine-binding globulin, retinol-binding globulin	Decrease: liver diseases
Albumin	Osmot. function, protein pool, binding of ions, dyes, etc.	Decrease: kidney diseases, neoplasms, liver diseases, chron. inflammations
α_1-Antitrypsin	Proteinase-inhibitor	Increase: inflammations liver diseases
Gc-Globulin	Three genetic types	Decrease: liver diseases
Ceruloplasmin	Copper-binding globulin, oxidase	Increase: pregnancy, neoplasms Decrease: Morbus Wilson
C-Reactive protein	Phagocytosis promoting activity	Increase: inflammatory conditions
Hemopexin	Heme-binding	Decrease: hemolytic anemias
Transferrin	Iron-binding	Decrease: neoplasms, inflammations, paraproteinemias, nephrosis
β_1 A-Globulin	Complement-factor	Increase: infections, toxoplasmosis Decrease: autoimmune diseases
Haptoglobins	Binding of hemoglobin three genetic types	Increase: inflammations, neoplasms, infections, PCP, nephrosis
Type 1—1 Type 2—1 Type 2—2		Decrease: liver diseases
α_2-Macroglobulin	Proteinase-inhibitor	Increase: liver diseases, diabetes, nephrosis
α-Lipoprotein HDL	Transportation of cholesterol	Increase: lipid metabolism disturbances Decrease: liver diseases, Tangier disease
Pre-β-lipoprotein LDL	Transportation of lipids	Increase: type-3,4 hyperlipoproteinemia
β-Lipoprotein LDL	Transportation of lipids	Increase: nephrosis, type-2 hyperlipoproteinemia Decrease: liver diseases
Chylomicron VLDL		Increase: type-1, hyperlipoproteinemia
γA-Globulin	Antibody	Increase: liver diseases, chron. infections, PCP Decrease: paraproteinemias, antibody deficiency syndrome
γG-Globulin	Antibody	Increase: liver diseases, chron. infections, myeloma, PCP Decrease: paraproteinemias, antibody deficiency syndrome

clinical myeloma suspicion, but where a normal immunoelectrophoretic pattern was present [35]. In the hands of a skilled person it is probably the most reliable zone electrophoretic method for early detection of the paraproteinemias. The distinct protein peaks may also reveal whether monoclonal or polyclonal paraproteins are involved.

6.1.2. β-Globulins

β_1 A-Globulin, or C_3, may be found to be decreased in autoimmune diseases, such as lupus erythematosis, and increased in infections, especially toxoplasmosis. These changes may be seen most readily in 1—1 haptoglobin types and/or by staining with PAS. Levels may be verified with immunoelectrodiffusion techniques [41].

Transferrin is very apparent and well separated from ceruloplasmin in system one and two although their relative positions are not the same (see Figs. 1 and 2). Decreases are seen in this protein in nephrosis and malignant neoplasms, especially bronchial carcinoma [35] and in various inflammatory diseases. Quantification of this protein is readily made by densitometric techniques with an average value of 0.222 ± 0.022 g/dl and a reference range of 0.178—0.266 g/dl.

The β_2 lipoproteins may be separated readily in both systems one and two following prestaining with Sudan black B. Both the pre-β, very low density lipoprotein (VLDL) and the β, low density lipoprotein (LDL) may be quantified on these systems. In system one the chylomicron region remains in the opaque sample gel while in system two it will penetrate into the first millimeter of the 3.0% separating gel allowing microdensitometric quantification. The rapid separation time 30—35 min, with visualization during the separation, makes this a very useful and relatively simple technique for the differentiation of the familial hyperlipoproteinemia types 2a, 2b, and 3, and in differentiating type 4 from type 5 [42]. Fig. 3 shows a typical series of patterns obtained using system two.

Hemopexin in system one normally appears adjacent to transferrin, and anodally to ceruloplasmin in system two. While this protein appears as a minor component in serum its decrease, like that of the haptoglobulins, is apparent in hemolytic anemias.

6.1.3. α_2-Globulins

α_2-Macroglobulin, which contains about 5% of the proteinase-inhibiting activity of the serum, is also important in binding hormones. It is strongly increased in liver diseases, diabetes, and in nephrotic syndrome and its clear separation and location in relation to γ-globulin allows ready differentiation of Waldenströms macroglobulinemia from myeloma.

Ceruloplasmin, a copper binding α_2-globulin is markedly increased in malignant neoplasms and in pregnancy, while it is decreased in Wilson's disease. Ceruloplasmin is more readily separable as a discrete zone in system two, employing a gel pore size gradient, than in system one, using a continuous gel pore size.

The haptoglobins are particularly well separated by PAGE techniques. The addition of hemoglobin to a serum sample readily identifies haptoglobin

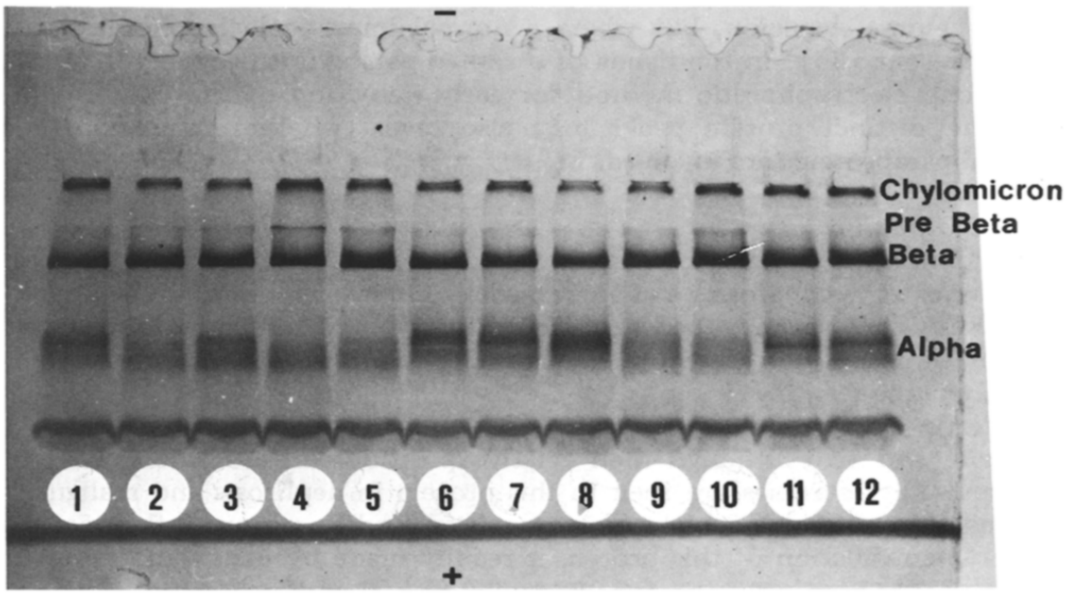

Fig. 3. Separation of pre-stained serum lipoproteins stained with Sudan black B. The separation was carried out in system two on a 3—6—9% T-pore-size gradient at a continuous pH of 9.0 using a discontinuous citrate—borate buffer system. Sample 6, 7, and 8, are female as indicated by the increase HDL level. Samples 1, 2, 3, and 11 show the four typical HDL types seen with this method and sample 4 indicates an increased pre-β band. Albumin is marked with the tracking dye added to the cathodical buffer.

types without staining, although benzidine-peroxide staining as shown in Fig. 4 may be carried out to enhance visualization. Hoffmeister [35] found that haptoglobins were markedly elevated in over 90 confirmed cases of bronchial carcinoma. He also found that tumors in a progressive stage demonstrated a progressive decrease of transferrin and γ-globulin, while tumors of the female genitalia, the stomach, pancreas, and intestine showed less significant change in the haptoglobin levels. Haptoglobins were found to be elevated in primary chronic polyarthritis, the nephrotic syndrome, and toxoplasmosis. In contrast to neoplasms and chronic inflammatory processes, transferrin will remain normal and the γ-globulins increase in the sera of patients with PCP and toxoplasmosis. The latter also show an increase in β_1 A-globulin.

6.1.4. α_1-Globulins

The α_1 high-density lipoproteins (HDL) may be prestained with Sudan black B and separated with both PAGE systems one and two. In system two polymorphisms in the HDL are apparent as in the increased HDL levels in females as illustrated in Fig. 3. In Tangier disease HDL bands are absent. The Gc globulins show three distinct genetic types on both systems one and two. While these proteins are more of an aid to identification in forensic medicine, they do increase in liver diseases. The α_1-acid glycoprotein (orosomucoid) is also well resolved on both PAGE systems and is increased in inflammatory conditions, rheumatoid arthritis and malignant neoplasms.

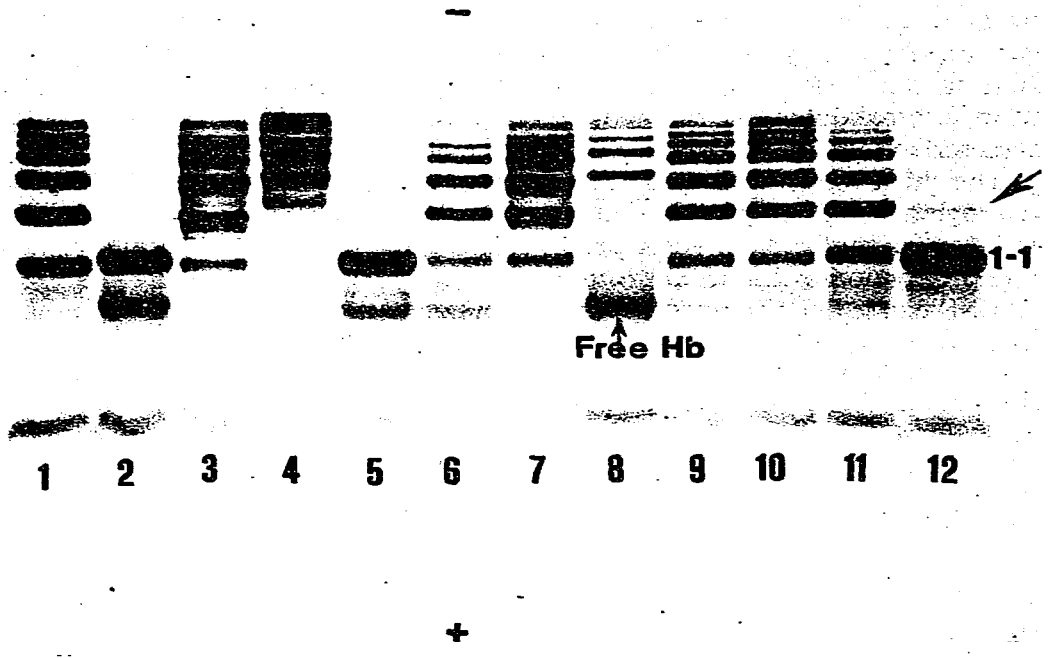

Fig. 4. Serum mixed with hemoglobin and separated on system two in a 4.5—6—8—12% T-pore-size gradient at a continuous pH of 9.0 in a discontinuous citrate—borate buffer system. The separation was stained with benzidine-peroxide. Samples 1, 3, 6, 7, 9, 10, and 11 are haptoglobin type 2—1, samples 4 and 8 are haptoglobin types 2—2, and samples 2, 5, and 12 are haptoglobin types 1—1. The pipette tip was purposely not rinsed or changed between samples 11 and 12 to show sensitivity of carry over of the major 2—2 haptoglobin marked by arrow. Fig. 4 with permission of Ortec.

6.1.5. Albumin

Albumin is not separated in either system from the α_1 proteinase inhibitors (α_1 Pi) unless the gel monomer concentration exceeds 22%. The 50,000 dalton α_1 Pi migrates in routinely used gel strengths with the forward edge of the albumin increasing the albumin value by 3—4% in normal individuals. In Pi-deficient individuals or in normal individuals with inflammatory processes where α_1 Pi is elevated, albumin variations of 5—6% may be due solely to the α_1 overlap. Decreases seen in albumin in kidney diseases, neoplasms, liver disease, and chronic inflammation normally will be readily apparent, although not as a true reflection of the albumin actually present. Prealbumin is resolved well by both systems one and two and shows a concurrent decrease with albumin in liver diseases.

6.2. Proteins of other body fluids

6.2.1. Cerebrospinal fluid

The concentrating effect of both systems one and two is of particular advantage for the analysis of cerebrospinal fluid (CSF) as compared with other zone electrophoretic methods. A clear cut decision can be made with PAGE to determine whether elevated protein levels in the CSF are caused by a

disturbance of the blood—CSF barrier, or by synthesis of proteins within the central nervous system. Disturbance of that barrier will produce an approximation of the CSF pattern to that of the serum pattern. A pattern of CSF showing a breakdown of the blood—brain barrier or blood contamination is characterized by the following alterations [39]:

(i) The high molecular weight proteins are markedly increased, i.e. the α_2-macroglobulin, β-lipoprotein and haptoglobin polymers.

(ii) The low molecular weight proteins like prealbumin constitute a smaller relative proportion of the total protein.

(iii) CSF bands like the third post-albumin became undetectable.

Those diseases, where new proteins are synthesized in the central nervous system become detectable by CSF electrophoretic analysis. They produce a protein profile different from the patient's serum. These include neurosyphilis, multiple sclerosis, subacute sclerosing leucoencephalitis, infectious meningitis, brain tumors, intervertebral disk profusion, cerebral infarction, and Bence-Jones proteins [43, 44].

Takeoka et al. [43] have divided the PAGE CSF-protein pattern into 6 zones based on mobility. These are the prealbumin region, albumin region, the post-albumin region divided into two zones between albumin and transferrin, a post-transferrin zone and a γ-globulin zone. Utilizing this technique, and staining with Amido Schwarz 10-B, normal values of CSF of the prealbumin zone were 10.97 ± 2.31%, albumin zone 40.74 ± 5.72%, A_1 zone 5.25 ± 0.72%, A_1 zone 8.27 ± 1.24%, transferrin 10.82 ± 2.18%, post-transferrin 7.94 ± 1.93% and γ zone 16.05 ± 2.49%.

Several authors have also described the abnormal and pathological CSF protein patterns [45—48] and Papadopoulos and Suter [49] have reported that PAGE furnished useful supplementary information for the differential diagnosis of neuropathies (encephalitis, meningitis, Gullain-Barre syndrome, etc.). Weiss et al. [37] have shown the difference between α_2 and α_{1a} myeloma and macroglobulinemia (Waldenström) using system one.

6.2.2. Urine

Generally urinary proteins need to be concentrated for PAGE, for example by ammonium sulfate precipitation [50], concentration by dialysis, ultrafiltration, lyophilization or benzoic acid adsorption [51]. Anderson [52], on the other hand, has found that freeze dialysis is an excellent way to concentrate urine in order to preserve enzyme activity and to minimize protein denaturation.

Ammonium persulfate precipitation has been employed by Adams-Mayne and Jirgensons [53] to extract Bence-Jones proteins which they then investigated by the use of the acid PAGE at pH 4.3.

Pesce et al. [54] have employed SDS PAGE on concentrated urine samples to differentiate the proteinurias of glomerular and tubular origins based on the molecular weight of the proteins present. Glomerular proteinuria is characterized by the presence of albumin and plasma proteins of a molecular weight larger than albumin, while tubular proteinuria is characterized by albumin and proteins of molecular weights below albumin. Urinary chorionic gonadotropin has been analyzed in urine and followed during the course

of pregnancy by a number of investigators [55—57]. Both chorionic gonadotropin and leuteinizing hormone have been studied by Kaplan et al. [58].

6.2.3. Saliva

Up to 21 different proteins have been identified in parotid and submaxillary saliva by PAGE using system one [59—62]. Differences observed among individuals were found to be due to biological and genetic variation. However, within individuals good reproducibility was found. Salivary amylase may be analyzed by system one [63] and the method is particularly favorable when compared with starch gel, since acrylamide is not hydrolyzed by amylases.

6.2.4. Exudates, transudates, and other body fluids

While a number of studies investigating other body fluids in pathological conditions have been reported using PAGE, these methods have not apparently found any widespread use in the field of clinical chemistry. System one has been used to study synovial, ovarian, and follicular cysts, and ascites in carcinomatous peritonitis [64—66]. System one has also been used to differentiate radicular cysts from granulomas in root canal fluids [67].

6.3. Tissue proteins

Tissue proteins have not been studied extensively by PAGE for clinical purposes, however characteristic patterns of different ferritins in human HeLa and KB cells have been described by Richter [68, 69]. Structural proteins of amyloid fibrils in human amyloides livers have been described [70,71] as has thyroglobin from congenital goitre [72], while Sykes et al. [73] have studied the collagens of human dermis.

A number of enzyme and isoenzyme studies have been made also on a variety of tissue and cell extracts, and on body fluids. These will be listed separately in tabular form for both PAGE and PAGIF.

7. ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL: APPLICATIONS

7.1. Plasma proteins

The theory and fundamental aspects of isoelectric focusing have been reviewed by a number of authors [74—76] and have been particularly well presented in a comprehensive study by Righetti and Drysdale [77], to which the interested reader is referred.

Isoelectric focusing in polyacrylamide gel is beginning to experience considerable interest and use as a clinical tool. The method has the high-resolution capacity that PAGE seemed to promise, but in the light of experience was found to lack. The lag between research development and routine clinical application with this technique is perhaps to be expected as with any other new technique. While apparatus costs like those for PAGE are considerably higher than for agar gel and cellulose acetate systems, they are well below those of a number of routine instruments normally found in the clinical chem-

istry laboratory. Additional complications with PAGIF are certainly present in terms of interpretation and assessment of patterns. Analysis of serum, for example, by electrophoresis on cellulose acetate or agar gels can reveal 10—12 components and three times this on various PAGE systems. However, in PAGIF the pattern complexity may increase five-fold on extended pH range gels. The interpretation and quantification of this many components would at first appear to present a formidable task, but use of this technique with narrow pH range ampholytes, immunological, and enzyme techniques simplifies the problem.

Some 50 protein components are revealed in the serum as shown in the schematic presentation of a two-dimensional cellulose acetate—PAGIF pH 3—10 gel of a 1—1 haptoglobin type illustrated in Fig. 5. Expansion of the region between pH 3.5 and 5.0 indicates that eight components are present in the α_1 region characteristic of a Pi MM phenotype. A comparative schematic presentation of a two-dimensional cellulose acetate—PAGIF gel of serum from a 2—2 haptoglobin type is shown in Fig. 6. Similarly, many other classical serum components may be optimally resolved using such a scheme. The normal γ-globulins, for example, are best resolved on a pH gradient of 5.5—7.5.

Since the review of Latner in 1975 on PAGIF as a clinical tool [78] numerous advances have been made in the area of clinical pathology and methodology. Banding positions, or isoelectric points, of an ever-increasing number of clinically important proteins have now been identified. Likewise, a number of reports of multiple molecular forms of enzymes from serum, CSF, urine, saliva, and tissues have been published, providing a foundation for the cataloging by isoelectric point of clinically important body fluid and tissue enzymes and proteins.

Two important advantages of PAGIF on horizontal flat slabs as compared to PAGE have allowed, and hold great promise in the area of the identification and quantification of protein components. The first is the previously mentioned ability to separate large numbers of samples side by side. The second is that the focused sample components are presented at the surface of the gel allowing the application of two recent techniques which greatly expand the analytical potential of this technique.

The immunoprint technique developed by Arnaud et al. [79], primarily to study the α_1 Pi system, allows separated sample components to be identified immunologically directly from the gel without resorting to a more complex crossed immunoelectrophoresis procedure. The immunoprint technique has been used also to study transferrin and haptoglobin heterogeneity [80]. Basically, the immunoprint is accomplished by soaking a cellulose acetate strip in appropriately diluted specific antibody and then laying it on the focused gel. A glass plate is applied to sandwich the cellulose acetate under slight pressure against the gel. After ½ h the plate is removed and the strip placed in phosphate-buffered saline for several changes over 24 h to wash off unprecipitated proteins. The strip is then stained with Coomassie RB250 and the mirror-image bands reflect the insoluble, specific antigen—antibody complex as shown in Fig. 7. Thus, any of the many serum proteins for which

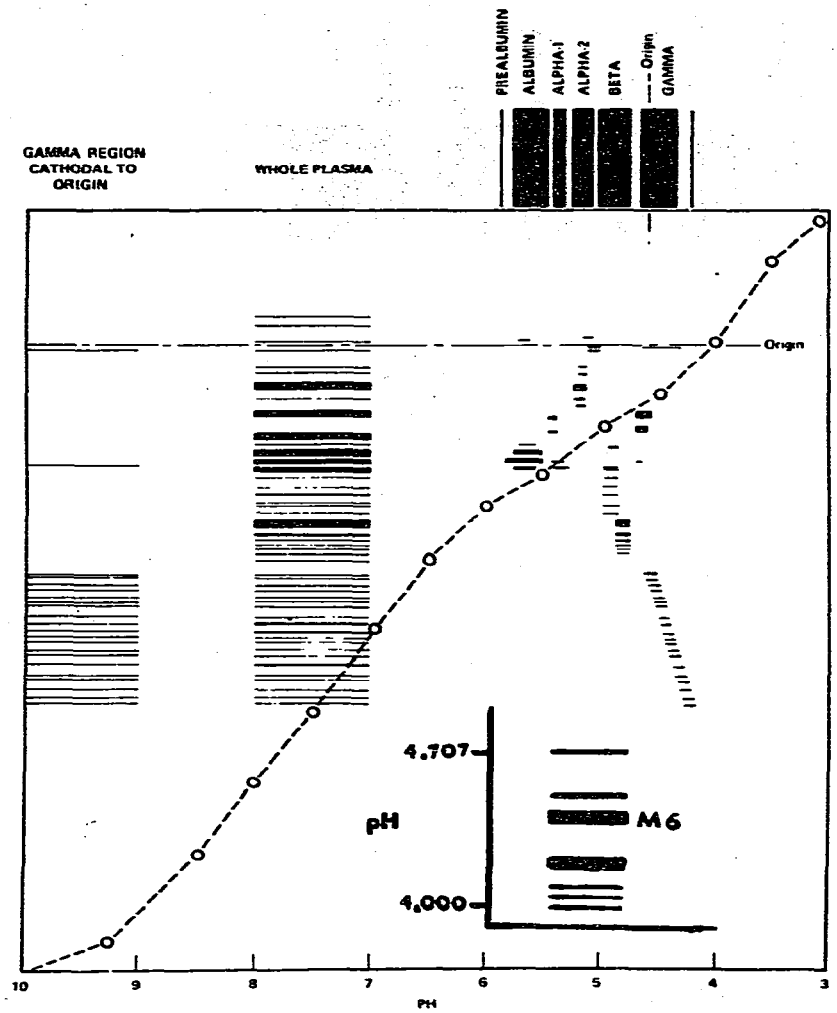

Fig. 5. Schematic separation of serum from a 1—1 haptoglobin type separated in the first dimension on cellulose-acetate and in the second dimension PAGIF on a pH 3.5—10 gradient. The insert in the lower right hand corner is the same serum separated on a narrow range 3.5—5.0 pH gradient indicating a typical MM α_1 Pi phenotype. Coomassie RB250 staining was used on both support media.

specific immunological reagents are available, may be directly analyzed with this technique.

The replicate printing technique developed by Narayanan and Raj [81] has not only the potential of the application just described, but also the ability to perform a number of additional biochemical tests on each separation. This technique consists of taking the focused gel and imprinting it sequentially onto a 1-mm thick agar layer on glass plates. While each print is weaker than the preceding one, up to 5 or 6 prints may be made in practice from a given separation. Thus, proteins, glycoproteins, various isoenzyme tests, and immunoprints can be made from a given gel separation. The exact location

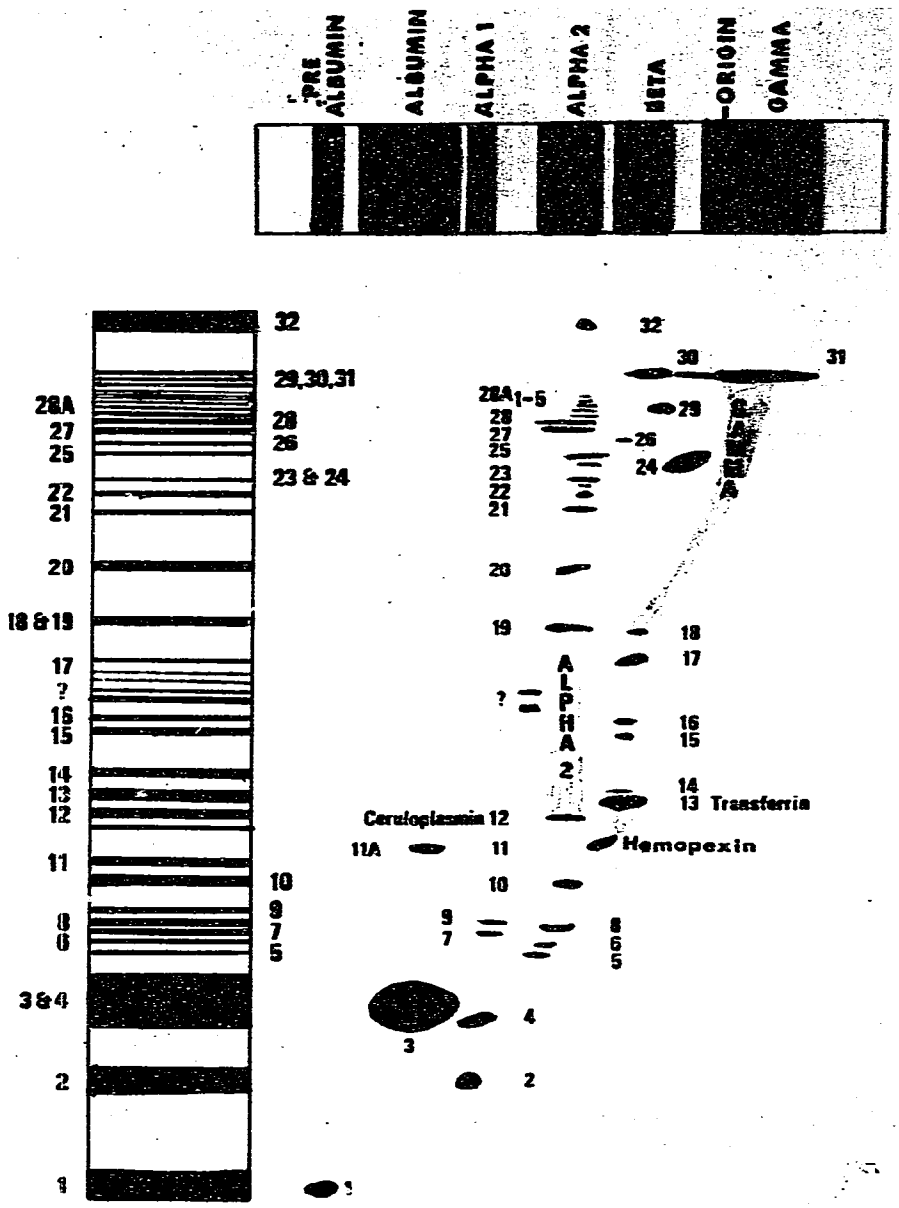

Fig. 6. Schematic separation of serum from a 2—2 haptoglobin type separation in the first dimension on cellulose acetate and in the second dimension on a discontinuous sulfate—borate buffer system in a 4.5—6—8—12% T-pore-gradient gel slab. Note the cloud formation and consequent lack of resolution in both the α_2- and γ-globulin regions. Coomassie RB250 staining was used on both support media. The unidimensional separation on the left is also given in the densitometric trace in Fig. 2. 1, Prealbumin; 2, α_1-acid glycoprotein; 3, albumin; 4, α_1 antitrypsin; 5, 6, 8, α_2-globulin; 7, 9, Gc globulins; 10, unknown; 11, hemopexin; 11a, unknown; 12, ceruloplasmin; 13, transferrin; 14, unidentified β-globulins; 15, 16, β-α_2-globulins; 17, 18, unidentified β-globulins; 19—23, 25, 28A_{1-5}, haptoglobin polymers; 24, β-α-globulin; 26—28, α_2-macroglobulins; 29, unidentified β-globulins; 30, β_1-lipoproteins; 31, I_gM; 32, chylomicron.

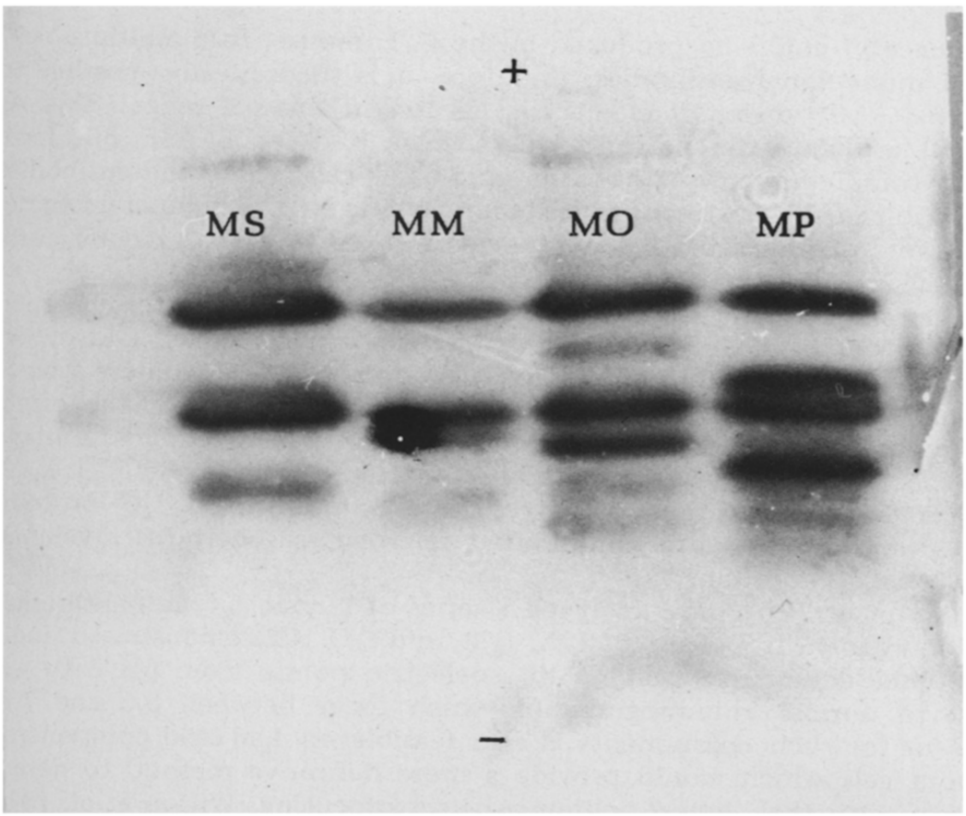

Fig. 7. Immunoprint fixation on cellulose acetate of various α_1 Pi types separated by PAGIF on a pH 3.5—5.0 gradient. The specific immunoprecipitate was stained after washing and fixation with Coomassie RB250.

of each band is the same on all plates allowing multiple characteristics of each component to be determined simultaneously. The development of commercially available thin gels backed on flexible Mylar film by LKB (Rockville, Md., U.S.A.) has provided the critical step necessary to both make printing feasible and allow cross-comparison, since the Mylar-backed master does not shrink or swell during fixation and staining procedures following printing.

7.1.1. Immunoglobulins

PAGIF has been successfully applied in the analysis of the extensive heterogeneity of the immunoglobulins. This technique has been used to study both the heterogeneity resulting from distinct structural genes and that generated by post-synthetic modifications of biosynthetically homogeneous protein. With thin-layer PAGIF Williamson et al. [82] have shown in pH 5.5—7.5 gradient gels, with a resolving power of 0.005 pH units, that there are some 400 theoretical focusing positions for a single antibody band using the method developed by A· 'eh et al. [83]. However, impressive as this resolution may seem, Kreth and 'liamson [84] have determined that a minimum statistical estimate of 8000 m. oclonal antibodies against the haptene 3-nitro-4-hydroxy-

5-iodophenylacetyl could be produced in the C_3H mouse. In a multiple-band spectrum of monoclonal antibodies, therefore, it is theoretically possible to distinguish 5×10^4 different isoelectric spectra in this pH range [82]. As an additional tool in differentiating such spectra Keck et al. [85, 86] have developed autoradiographic methods on thin-layer gels where the antibodies are first immobilized by treatment with sodium sulfate and then glutaraldehyde. The bands were then treated by ^{125}I-labeled antigen and analyzed by autoradiography.

From a more practical clinical viewpoint, PAGIF has been used by Cornell [87] to show that in some instances the method would, like PAGE, detect monoclonal proteins in serum before they were detectable by cellulose acetate or immunoelectrophoresis. Awdeh et al. [88] have shown that the origin of the microheterogeneity from a plasma cell tumor, which produces a single molecular species of I_gG_2, is due to lability after synthesis. Brendel et al. [89] have reported also that non-myelomatous monoclonal I_gG proteins possess the same individuality and limited microheterogeneity as myelomatous monoclonal I_gG proteins.

Trieshmann et al. [90] have analyzed a series of human I_gG autoantibodies and have found them to consist of I_gA, I_gM, and I_gG. All demonstrated single peaks by liquid column focusing with isoelectric points from pH 3 to 4.5 in contrast to normal immunoglobulins which focus between 5.5 and 7.5. Assessing sera for such components is also feasible on I_gM acid components in large pore gels which would provide a more definitive method to detect microheterogeneity than liquid column isoelectrofocusing. Wilson et al. [91]

Their finding of a similar band in one out of twelve normal individuals suggests that the diagnostic value of this band, however, must be viewed with caution since the I_gG acts only as a carrier [92]. Dale et al. [93] have demonstrated that it is possible to distinguish I_gA myelomatosis from I_gG myelomatosis using two-dimensional gel techniques and even the I_gA light chain can be demonstrated in the serum.

Cwynarski et al. [94] have described I_gG paraproteins from patients with various gammopathies, both malignant and benign, and present the possibility that a number of "benign-paraproteinemias" may in fact represent premalignant phases.

Bouman et al. [95] have investigated the microheteroneity of I_gG from plasmacytomas and found the monoclonal I_gGs to display 3 to 13 bands. They also indicated that post-synthetic deamidation was not found to occur spontaneously and consequently is not the cause of the microheterogeneity observed.

7.1.2. β-Globulins

The allotyping of complement components C_4, C_2, C_5, C_6, C_7, and factors B and D in whole serum separated by PAGIF followed by an *in-gel* hemolytic assay has been reported by Hobart and Lachmann [96]. Polymorphisms in C_2 were found to be unlinked to HLA although C_2 deficiency is known to be linked genetically to this system.

Transferrin which migrates as a single zone in PAGE has been shown by Hovanessian and Awdeh [97] to be separable into two zones by isoelectric focusing with isoelectric points of 5.6 and 5.2, respectively. The former is monoferric transferrin and the latter diferric transferrin. Should distinct functions exist for the mono- and diferric transferrin or for the two sites of transferrin then the ratio of Fe-transferrin:Fe_2-transferrin might become a valuable index of serum iron metabolism.

Fibrinogen heterogeneity has been demonstrated by Gaffney [98] and Soria et al. [99], who have also employed PAGIF to study abnormal fibrinogens. Arnesen [100] has employed this technique to the study of fibrinogen degradation and fibrin split products.

The diagnosis of Type 3 hyperlipoproteinemia (Broad-β Disease) is based on the demonstration in fasting plasma of a low-density lipoprotein which in zone electrophoresis migrates in the β region. Godolphin and Stinson [101] have demonstrated a Sudan black B-staining band in untreated serum with an isoelectric point of pH 5.44 which appears to be characteristic of the disease. Utermann et al. [102] have shown that this disease is further characterized by lack of an apo-lipoprotein designated E III and suggest that the disease is the result of an autosomal recessive inheritable trait. These authors have also developed a rapid screening method which does not require prior ultracentrifugation of the serum for such analyses.

7.1.3. α_2-Globulins

The α_2-macroglobin has been studied by Jones et al. [103] in relation to its subunit structure following thiol reduction. Microheterogeneity has been studied by Frenoy and Bourrillon [104], and Ohlson and Skude [105] have demonstrated semi-quantitatively the determination of complexes between various proteases and human α_2-macroglobulins.

Ceruloplasmin may be identified by means of its oxidase activity on *p*-phenylene diamine or by using standard immunological reagents for ceruloplasmin.

The haptoglobins may be separated by PAGIF and identified as to genetic type by the addition of hemoglobin with or without enhancement by benzidine staining. Chappuis-Cellier [80] has used the immunoprint technique to localize the haptoglobins. The potential advantage of PAGIF over PAGE for the study of the haptoglobins would appear to be in elucidating greater microheterogeneity and possible subtypes in conjunction with simplified set-up procedures.

The α_2-HS glycoprotein microheterogeneity has been observed by Hamber et al. [106], who reported an isoelectric point range of 4.7—5.1 which is just outside of the p*I* range of the α_1 proteinase inhibitors but overlaps that of the heterogeneic plasma glycoprotein kininogens. Thus, the α_2-HS glycoproteins may require differentiation from the kininogens by techniques such as the immunoprint method.

7.1.4. α_1-Globulins

The α_1 Pi systems have received considerable interest in the last few years with well over 300 separate reports appearing in the literature. Deficiency states have been associated with a predisposition to emphysema [107], in-

fantile cirrhosis [108], juvenile arthritis [109], non-allergic asthma [110], and other inflammatory diseases. Allen et al. [111] have recently found that the more serious heterozygous deficiency states MZ and MS predispose individuals to periodontal disease marked by erosive bone loss unless meticulous oral hygiene is practiced. Pi typing by PAGIF developed by Allen et al. [112] and Arnaud et al. [113] allows ready differentiation of the 30 identified alleles and some 56 phenotypes presently described. The microheterogenic system first demonstrated on the so-called acid starch gel system of Fagerhol and Laurell [114], is capable of being fully resolved only by PAGIF. Sixty samples can be analyzed per gel slab within 3 h and Pi typing is a more accurate and practical way of detecting heterozygote deficient individuals than radial immunodiffusion (RID) and trypsin inhibitory capacity techniques. Since α_1 Pi is an acute phase reactant, serum levels in heterozygote deficiency states are often raised during infection or in patients treated with steroids, particularly estrogens, and therefore, individuals with deficiency states could present with serum levels in the low normal or normal range. Over 30% of MS Pi types show low normal or normal levels even in the absence of infection or steroid treatment [115], and thus, would be assessed as normal by commonly employed tests to determine serum levels. The microheterogeneity and subgroup analysis possible with PAGIF are shown in Fig. 8.

One-step verification of Pi allele products in the region of isoelectric points from 4.40 to 4.72 may be accomplished by the protease probe techniques of adding trypsin to the serum which clears the Pi bands, since the complexes formed have much higher isoelectric points, as described by Allen et al. [34] and by immunoprint fixation procedure described by Arnaud et al. [79].

Separation of lipoproteins on gel slabs in which whole serum is placed on filter paper tabs results in considerable surface smearing of the VLDL and LDL which do not penetrate the gel. Gel rods containing a 5% monomer concentration have been used by Kostner et al. [116] to separate the lipoproteins with the HDL region showing four distinct bands. Kostner et al. [117], utilizing a modified system employing photopolymerized gel rods containing 23% ethylene glycol, were able to separate 8 bands from prestained sera. While HDL_2 and HDL_3 heterogeneity has been demonstrated by sucrose gradient column studies by Sodhi et al. [118], Blaton et al. [119] and Eggena et al. [120], PAGIF has not been widely used. Removal of VLDL and LDL from serum by heparin and magnesium as described by Burstein and Scholnick [121] will provide samples on which HDL may be separated without resorting to prior density gradient ultracentrifugation. Scanu et al. [122] have studied the HDL in depth by analytical PAGIF and have found that focusing is most valuable in the characterization of products separated and purified by prior chromatographic procedures.

The thyroxin-binding globulin has been shown by PAGIF to exhibit microheterogeneity similar to the other α_1-globulins. Some 9 bands have been found with PAGIF by Marshall et al. [123], with 4 major and 5 minor bands from purified material obtained from pooled plasma with a p*I* region from 4.2 to 5.2. Although this reported range of isoelectric points overlaps that of the proteinase inhibitors, the low concentration of thyroxin-binding glob-

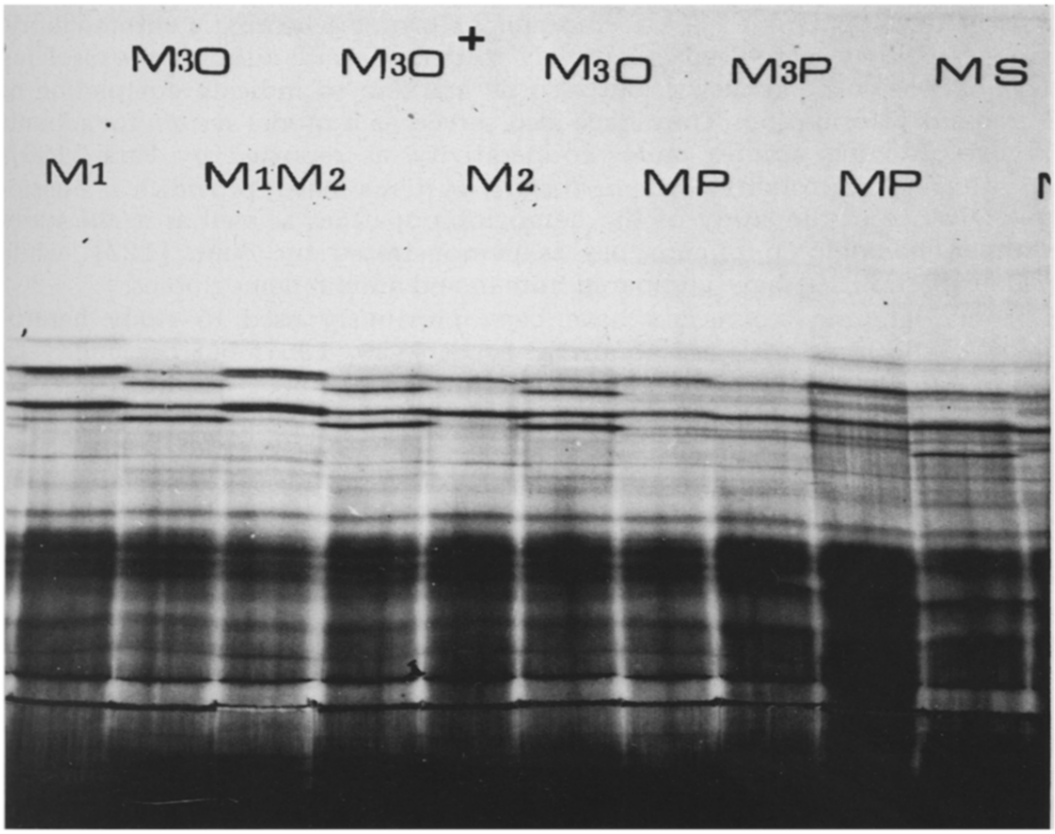

Fig. 8. Serum samples separated by PAGIF on pH gradient 3.5—5.0 on 1-mm-thick gels showing the complexity of M variants in the Pi system as well as the common S allele and the rare O and P allele products.

ulin, 1—2 mg per 100 ml serum, in practice, causes no interference with Pi typing. Detection of the thyroxin-binding globulins by immunoprint techniques may be difficult due to their very low concentration. To date, radiographic techniques applied directly to the gel have not proven satisfactory for localizing these proteins.

α-Foetoprotein normally undetectable within days after birth reappears in the serum of patients with primary liver carcinoma and embryonal carcinoma. It has been isolated and its microheterogeneity studied by PAGIF. Two major α-foetoprotein peaks with isoelectric points of 5.08 and 5.42 have been described by Alpert et al. [124] in both hepatomas and foetal serum. Two components of α-foetoproteins have also been described in the ascites fluid from a patient with primary carcinoma by Sokolov et al. [125] with isoelectric points of 4.78 and 5.20, respectively.

7.2. Hemoglobin

Aside from their clinical importance, the hemoglobins hold a special place in isoelectric focusing. They have played perhaps the most important role in

the early development of the technique. As proteins bearing a chromophoric heme group, they were used extensively to demonstrate not only the resolving power of isoelectric focusing, but also as markers to indicate completion of pH gradient information. They have also served as a model system for subunit exchange, binding studies and cooperativity as reported by Park [126]. High-voltage, controlled low-temperature systems have provided a considerable advance in the study of the hemoglobinopathies as well as in the study of human hemoglobin phenotypes as demonstrated by Bunn [127], while Drysdale et al. [128] have compared human and animal hemoglobins.

Chromatographic techniques have been previously used to study heterogeneity of both normal and abnormal types [129, 130], but the ability to separate rapidly multiple samples by PAGIF on pH 6—8 gradient gels for both quantitative and qualitative studies offers a valuable tool for clinical studies. Jeppsson and Bergland [131] showed that thin-layer PAGIF was able to separate Hb-Malmo from Hb-A, and Hb-F from Fb-F_{11}, which was not possible with previous zone electrophoretic techniques. Another high oxygen affinity hemoglobin with a mutation at B97 and associated with familial erythrocytosis was reported using this technique by Taketa et al. [132]. Monte et al. [133] have recently mapped hemoglobin mutants by PAGIF. Altland [134] has developed a screening process for hemoglobinopathies using a portion of the dried blood samples obtained for PKU screening. He employed silicon rubber strips, with holes punched at intervals to serve as sample wells, into which the samples dissolved in 50 μl of 0.02 *M* KCN were applied in two rows of 48 samples to a single gel. A center anode strip is used with a cathode strip at both the top and bottom of the gel so that 96 samples per run may be analyzed.

Koenig et al. [135] have shown that the measurement of Hb-AIc provides a reliable index of control of carbohydrate metabolism in diabetes, and Trivelli et al. [136] have reported that Hb-AIc is increased two-fold or more in patients with poorly controlled diabetes mellitus. Spicer et al. [137] using high-voltage, constant-power PAGIF have shown that Hb-AIc and the other fast hemoglobins Hb-AIa and Hb-AIb may be separated and accurately quantified by microdensitometry on pH 6—8 gradient gels as illustrated in Fig. 9. This method is rapid with the added advantage that abnormal hemoglobin phenotypes are also detected if present. For pediatric patients, these authors have utilized standard heparinized micro-hematocrit tubes to obtain blood from a finger or ear prick for both Hb-AIc analysis and for Pi typing or other plasma protein or enzymes analyses as may be desired.

7.3. Cerebrospinal fluid

Vesterberg [138] has shown that patients with multiple sclerosis have a relative increase in the CSF of alkaline globulins, i.e. those with an isoelectric point greater than 7.5 at 10°. Delmotte [139] has also reported similar findings. Stibler and Kjellin [140] have demonstrated the focusing patterns in CSF of degenerative neurological diseases. The same authors have also studied the protein patterns in hereditary ataxia, hereditary spastic paraplegia, muscular dystrophy, and spinal muscular atrophies.

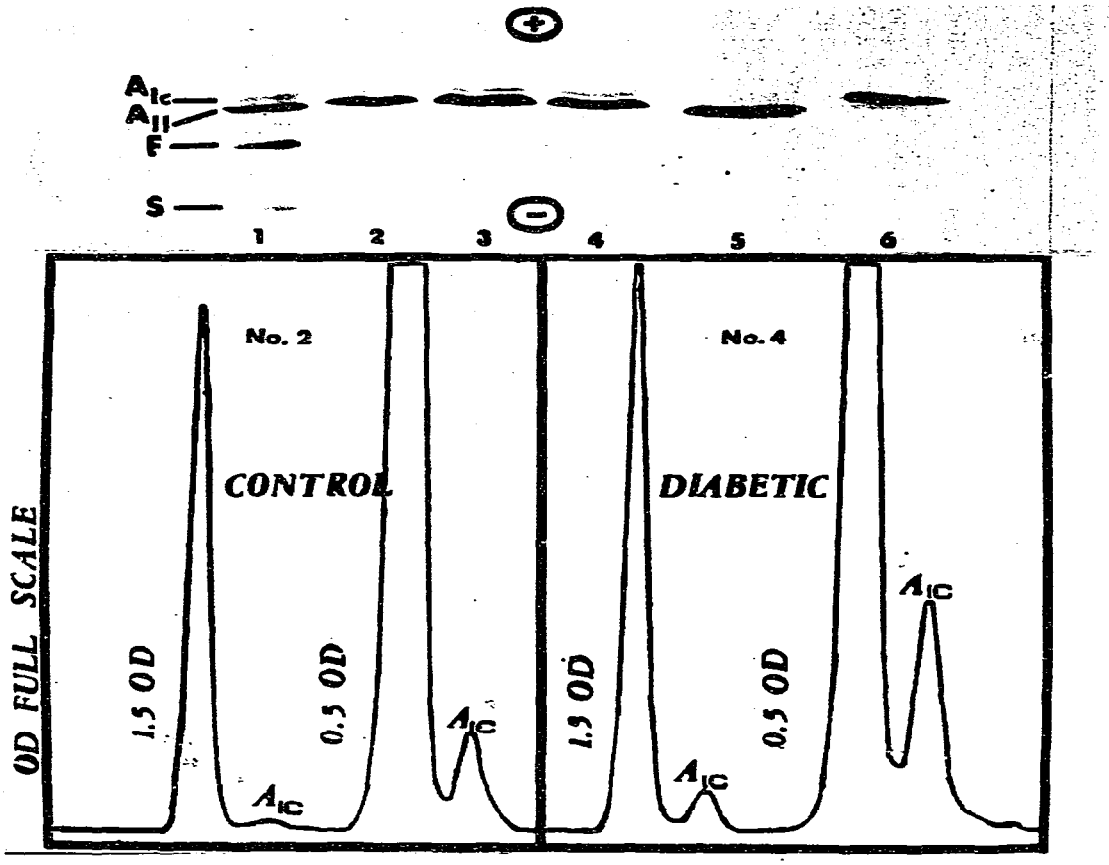

Fig. 9. Separation of hemoglobin by PAGIF on pH 6.0—8.0 gradient. Sample 1 is a mixture of F, S, AII, and A_{Ic}. Sample 2 is from a normal individual, samples 3 and 4 from diabetics. Sample 5 is twice rechromatographed AII from a Biorex column and sample 6 is similarly rechromatographed HbA_{Ic}. Separations are directly fixed in 12.5% TCA and subjected to quantitative microdensitometry as shown below.

Two-dimensional gel techniques have been used by Fossard et al. [141] for a variety of studies of CSF and Latner [142] has reported that in an unusually high proportion of patients suffering from multiple sclerosis an abnormal protein spot was found which had a p*I* slightly more acid than transferrin.

7.4. Urine

Rotbol [143] appears to have first applied isoelectric focusing to human urinary proteins on polyacrylamide gel. He found some 30 protein zones to be present in normal urine. Vesterberg [138] has identified the location of the β_2-microglobulin and found more than 40 protein zones in urine from patients with advanced renal damage. Hall and Vasiljevic [144] have characterized further the β_2-microglobulin and described two homologies, one with

TABLE 2

PAGE AND PAGIF ZYMOGRAM METHODS

Enzyme	Substrate/reaction mixture	PAGE	PAGIF	Source	Application	Reference
Acid phosphatase	α-Naphthol phosphate—Fast Garnett GBC	x	x	White cell	Hairy cell leukemia	160
	4-methyl-umbelliferyl phosphate		x	extract		157
Alkaline phosphatase	α-Naphthol phosphate	x	x	Serum	Lymphatic leukemia, infectious mononucleosis	161
Amylase	Contact print		x	Saliva		162
	overlay Phadebus ^{R}test system (Pharmacia)	x		urine,	Rise in 1 & 2	163
	starch iodine			sera	pancreatic disease injury	
Acid and glucosidase	4-Methylumbelliferyl α-D-Glucopyranoside		x	Muscle, liver	Pompe's disease	164
α-L fucosidase	4-Methylumbelliferyl α-L-Fucopyranoside		x	Liver	Mucopolysaccharidosis F	165 166
Glucose-6-PO_4 dehydrogenase	Glucose-6-phosphate monosodium salt NBT, NADPT, PMS	x	x	Red cells	Genetic variants, hemolytic episodes	167 168
N-Acetyl-β-D-hexosaminidase	α-Naphthol-As-BI-N-acetyl-,3-D-glucosimide—Fast; Garnett GBC salt		x	Serum, tissue, amniotic fluid	Tay-Sachs	169 170 171
β-Galactosidase	4-Methylumbelliferyl N-acetyl-β-galactosamine		x	Liver	Mucopolysaccharidosis Type 1, 2, 3	172
Esterases	α-Naphthol butyrate		x	Skin,	Wound esterase	173
	α-Naphthol acetate Fast Red TR	x		serum,	differentiation of bacterial and viral pneumonias,	174
			x	kidney,	platinate drugs,	175
			x	spleen	histiocytic neoplasms	176
Tyrosinase	L-Dopa (L-3,4 and 1-hydroxyphenylalanine)		x	Melanocytic	Melanoma	156

Peroxidase	DAB-H_2O_2		x	Red cells, uterine fluid	Estrogen-induced growth	177 178
		x				
Creatinine phosphokinase	Creatine PO_4—PMS NBT	x	x	Serum	Myocardial injury	179 180
Lactic dehydrogenase	Lithium lactate	x	x	Serum, red cells, urine	Myocardial injury pyelonephritis	181 182 183
Proteases	Casein agar overlay		x	Tissue cells		184
Pyruvate kinase	Phosphoenol pyruvate NADH, LDH, ADD—MTT		x	Red cells	Hereditary hemolytic anemia	185

a p*I* of 5.3 and the other with a p*I* of 5.7. This protein, which has a molecular weight of 11,500, has been established to be the smaller of the two polypeptides of the HLA antigen and is elevated in the majority of myeloma patients. The highest β_2-microglobulin levels are found in patients with tubular deficiencies and in patients following renal transplantation. Patients afflicted with Balkan nephropathy are a special example of renal tubular disorder in which high levels of β_2-microglobulin are found [144].

Boulton and Huntsman [145] have used both PAGE and PAGIF to distinguish myoglobin from hemoglobin in non-fresh urine of kidney donors where conversion of myoglobin to met-myoglobin makes spectroscopic recognition of this pigment unreliable. Hultberg et al. [146] have studied the isoenzymes of four acid hydrolases in both kidney and urine and have found that there was a predominance of isoenzymes with a low isoelectric point in the urine. In contrast, in kidney tissue extracts, isoenzymes with higher isoelectric points predominated. Kellar et al. [147] have studied the colony stimulating factor from human leukemia urine and identified a glycoprotein demonstrating marked microheterogeneity with isoelectric points between pH 3.3 and 4.1.

7.5. Salivary proteins

Beeley [148] has employed PAGIF in gel rods to separate the salivary proteins and Bennink and Cornell [149] have used the method as a criterion in the purification and partial characterization of four proteins from human parotid saliva. Chisholm et al. [150] have found additional protein bands with acid isoelectric points in the saliva of individuals with Sjögren's syndrome and those with rheumatoid arthritis. Pronk [151] found three different protein type patterns in parotid saliva which were shown by family studies to be phenotypic expressions of one autosomal locus with two co-dominant alleles. These were found to be correlated with α-amylase activity.

7.6. Enzymes

The significance of enzymes present in the plasma, originating from healthy and diseased tissue, as indicators of disease and therapy control is readily apparent in clinical medicine. Implicit in such correlations of enzyme levels with disease is that their elevation or decrease is representative of events occurring in a particular disease state at the cellular level. Alterations in the level of a particular enzyme or isoenzyme may result either from the tissue or from a deficit in clearance. Moreover, genetic and physiological factors can singly, or in combination, alter plasma levels in the absence of disease.

Many enzymes may be analyzed by both methods using suitable histochemical stains, a number of which have been compiled by Raymond [152], Righetti and Drysdale [77], and Maurer [153]. PAGE requires the gel to be pretreated in a sufficiently concentrated buffer to equilibrate the gel to the proper pH for a given reaction. PAGIF requires similar treatment, however, these gels with ampholyte concentrations of 20—50 m*M* make it quite easy, in most cases, to utilize a 100—200 m*M* buffer directly with the substrate

and complexing agents. Wadström and Smith [154] have reported ways to overcome adverse pH conditions in PAGIF gels. Additionally, enzymes may lose activity after PAGIF due to chelation of necessary metal cofactors. For example, Latner et al. [155] have reported 90% loss of activity of alkaline phosphatase unless the zinc cofactor was added back to the reaction mixture.

7.7. Tissue proteins and enzymes

PAGE and PAGIF have not been widely used as clinical tools to study soluble proteins and enzymes obtained from tissues or cells. However, this potential deserves mention as it is certainly within the microanalytical capability of both systems. For example, only a portion of wedge biopsy material is necessary to provide sufficient material for soluble protein extraction. Studies such as the detection of carcino-foetal liver ferritins by Alpert et al. [156]. The multiple molecular forms of tyrosinase in melanomas have been described by Burnett and Seilor [157] and tartrate resistant acid phosphatases from leucocytes in hairy-cell leukemia by Yam et al. [158].

Latner [78] has also shown differences in kidney tissue in renal carcinoma and in the cervical mucosa of pap-smear-positive women. These few examples suggest tissue and cell extracts particurlarly when separated by PAGIF, in combination with replicate print and immunoprint techniques, may in the future play a valuable role in clinical diagnosis.

Some selected clinically significant enzymes which may be qualitatively and quantitatively analyzed with both these techniques are given in Table 2 along with their diagnostic significance and source of origin.

8. OTHER HIGH-RESOLUTION METHODS

Two-dimensional electrophoresis employing PAGIF in the first detection and SDS—PAGE in the second described by O'Farrell [28] provide a more exotic method of high resolution than the earlier PAGIF—PAGE techniques of Latner [142]. This technique deserves special mention, not so much for its present applicability as a routine clinical tool, but for its potential to study gene products at the molecular level. This method has been used by Ivarie et al. [159] to detect missense mutations in structural genes and in monitoring gene expression in response to environmental stimuli.

9. SUMMARY

The use of polyacrylamide gel electrophoresis and polyacrylamide gel isoelectric focusing techniques in the area of clinical medicine is reviewed. The two techniques are compared both to previous electrophoretic techniques and to each other with regard to simplicity of operation and degree of resolution required for a given laboratory task. Plasma, body fluids and tissue proteins, and isozymes that presently may be utilized in clinical diagnosis and clinical research are presented with emphasis attempted toward describing the simplest procedure providing adequate resolution for a given task.

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