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CROSSED IMMUNOELECTROPHORESIS OF ALPHA-1-ANTITRYPSIN
USING THIN LAYER ISOELECTROFOCUSING IN
POLYACRYLAMIDE AS THE FIRST DIMENSION

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By

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ABSTRACT

CROSSED IMMUNOELECTROPHORESIS OF ALPHA-1-ANTITRYPSIN USING THIN LAYER ISOELECTROFOCUSING IN POLYACRYLAMIDE AS THE FIRST DIMENSION

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A two dimensional electrophoretic system, using high voltage thin layer isoelectrofocusing in polyacrylamide and crossed immunoelectrophoresis in agarose, has been combined using a laying on technique. By laying on, all the associated problems due to electroendosmosis differences between the two media are circumvented. This system provided increased resolution of the patterns associated with the polymorphic expression of alpha-1-antitrypsin (α_1-AT). The system involved focusing 12.5 µg of α_1-AT from human serum on a 3.5-5.0 pH gradient across a 24 cm plate. Four millimeter wide polyacrylamide strips were next incised, inverted, and transferred to the surface of agarose just cathodal to agarose containing 3.0% (v/v) antibody to α_1-AT. Crossed immunoelectrophoresis was then carried out. The distribution of peaks was found to be similar to that seen using acid starch gel in the first dimension. A new zone just cathodal to M6 was observed.

TO KATHY

Your love and encouragement have made
this all bearable

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INTRODUCTION

Alpha-1-antitrypsin (α_1-AT), quantitatively the most important inhibitor of proteolytic enzymes in human serum, is a protein which demonstrates a high degree of polymorphism. The first indications of chemical heterogeneity were given by Laurell and Eriksson in 1963.[102] Since the development of an acid starch gel electrophoretic phenotyping technique in 1965[39] and aided by crossed immunoelectrophoresis[52,99] into agarose containing antibody specific to α_1-AT, by 1974 twenty-three different alleles had been described.[27]

With the advent of the study of α_1-AT with thin layer isoelectrofocusing (TLIEF) in polyacrylamide gels,[4,6,7,92] the resolution has been greatly enhanced. Due to the increased resolution, the number of alleles described has increased to 25.[160] In addition, subtypes of alleles have now been described.[49,57,86,93] It seems reasonable that the increased resolution offered by TLIEF should be maintained through a crossed immunoelectrophoresis step. Attempts have been reported combining the two techniques.[7,107] They have not proven satisfactory because the resolution offered by TLIEF has not been preserved. The major problem is that they involved the molding in of the polyacrylamide,

which has an extremely low cathodal electroendosmosis (EEO), into agarose, which has measurably significant EEO.

Such a technique will be important in defining the precise role of a deficiency of this protein with various pathological states.

Inhibitors in Human Plasma

Alpha-1-antitrypsin is a glycoprotein in human sera that inhibits several serine proteases. Normal concentration in human sera is about 220-380 mg/dl. Under various physiological conditions including inflammation and pregnancy its level increases considerably.

Heimburger[67] lists six different protease inhibitors well characterized in human sera (Table 1).

Table 1. Protease inhibitors in human serum

		Normals (mg/dl)
α_1-Antitrypsin	α_1-AT	180-280
α_1-Antichymotrypsin	α_1-X	30-60
Inter-α-trypsin inhibitor	IαI	20-70
Antithrombin III	AT III	22-39
C1-Inactivator	C1 INA	15-35
α_2-Macroglobulin	α_2-M	150-350 males 175-420 females

With the exception of α_1-X, which is specific for chymotrypsin, these inhibitors are capable of inactivating several

proteases. Alpha-1-antitrypsin and α_2-M are present in highest concentration. Both have a wide inhibitory capacity against serine proteases including trypsin, chymotrypsin, plasmin, kallikrein, elastase, and thrombin. Probably the most pathophysiologically important are α_1-AT and derivatives of IαI, because they have the specific ability to neutralize the elastolytic proteases of leukocytes. These enzymes are mediators of inflammation; they attach basal membranes and are probably responsible for the destruction characterizing pulmonary emphysema.

Serine Proteases

The serine proteases are a class of proteolytic enzymes characterized by the presence of a uniquely reactive serine residue. Two families have been well studied, trypsin and subtilisn. The trypsin family includes trypsin, chymotrypsin, elastase, collagenase, thrombin, plasmin, and the proteolytic enzymes of the complement system.

The serine proteases specifically bind the tetrahedral transition state-like complex characteristic of acyl transfer reactions. All of the known covalent inhibitors of the serine proteases form tetrahedral adducts with similar geometry.

The binding template is made up of a number of elements acting together:[90]

(1) an antiparallel β-binding site for the acylating polypeptide chain of the substrate

(2) specific side chain binding sites dependent on the enzyme

(3) a less specific leaving group site

(4) a site for hydrogen bonding to the tetrahedral oxyanion

(5) the reactive serine for covalent binding to the substrate's carbonyl carbon atom.

The charge relay system, consisting of a histidine imidazole nitrogen hydrogen bonded to an aspartic acid carboxylate oxygen, can also be regarded as aiding the other imidazole nitrogen at the active site in binding a proton. In the reaction sequence, this facilitates the process of proton transfer between nucleophiles, i.e., between the serine and the leaving group of the substrate.

Protease Mechanism

The hydrolysis of the peptide bond is initiated when the hydroxyl of the action site serine attacks the carbonyl of the substrate. Simultaneously, the proton of the hydroxyl is transferred to the nearby histidine and the serine hydroxyl oxygen forms a covalent bond with the carbonyl carbon. The substrate's carbonyl oxygen assumes at least an incipient negative charge and the carbonyl bond angles change from a planar sp^2 to a sp^3 tetrahedral configuration. This tetrahedral adduct is stabilized by both the covalent bonding to the serine oxygen and by hydrogen bonding between the carbonyl oxygen and other proton donor group on the enzyme. The proton delivered to the histidine is transferred to the peptidyl -NH- of the leaving group of the substrate. Another proton is "recoiled" from the other side of the histidine,

transferring the proton to the aspartate carboxylate, the third catalytically important amino acid at the active site.

The peptide bond is broken after the nitrogen in the substrate receives the proton from the histidine. The leaving group is then free to break away, taking a proton from the enzyme. The acyl part of the substrate has now formed an acyl-enzyme intermediate. The carbonyl is now reverted to a planar configuration, hydrogen bonding to the carbonyl oxygen is broken, but the covalent bond to the serine oxygen remains intact.

Subsequent deacylation of the acyl-enzyme is the reverse of the acylation process with the leaving group replaced by water or other nucleophile. The hydroxyl group of water forms another tetrahedral intermediate with the acyl enzyme. The proton is transferred to the serine with breaking of the covalent bond between substrate and enzyme. The serine protease molecule has now resumed its original state and is ready to hydrolyze another peptide bond.

Mechanism of Inhibition

Hixson and Laskowski[70] presented evidence that trypsin binds to soybean trypsin inhibitor (STI) in a manner similar to the acylation of the enzyme with its substrate. Trypsin associates with either virgin (Arg-64-Ile bond intact) or with modified (Arg-64-Ile bond cleaved) STI to form a stable complex. Both the carboxyl end of the Arg and the amino end of the Ile are necessary for the inhibitory

function. In the modified inhibitor the resultant two chains are held together by a disulfide bridge. The mechanism entails the establishment of an equilibrium between native inhibitor, enzyme substrate complex, and the modified inhibitor with the peptide bond hydrolyzed.

$$T + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^* \rightleftharpoons T + I^*$$

where T is trypsin, I and I* are virgin and modified inhibitors, respectively, and L and L* are loose, noncovalent, Michaelis-Menton type complexes between trypsin and virgin and modified inhibitors, respectively. The values of Km and Km* are of the order of 10^{-5} M and independent of pH above 4.5. Since dissociation of trypsin inhibitor complexes is less than 10^{-5} M at pH above 4.5, the stable complex (C) predominates above pH 4.5.

In an earlier paper, Ozawa and Laskowski[132] argue that the reactive center need not necessarily require an arginine in the reactive site. Since trypsin is also specific for cleavage adjacent to lysine, such bonds should not be ruled out of consideration. Lysyl substitutions do not affect the trypsin inhibiting activity of STI and of chicken ovomucoid. However, lysyl substitution destroys the trypsin inhibiting activity of pancreatic trypsin inhibitor, lima bean trypsin inhibitor and turkey ovomucoid. X-ray crystallography by Ruhlmann et al.[143] using bovine trypsin and bovine pancreatic trypsin inhibitor, showed the complex to be a tetrahedral adduct with a covalent bond

between the carbonyl carbon of Lys-151 of the inhibitor and the gamma oxygen of the active site serine of the enzyme.

Mechanism of Inhibition by α_1-AT

Few studies have investigated the inhibition of trypsin by α_1-AT. Studies at low pH, which were used to study other antiproteases, cannot be done because α_1-AT is inactivated at low pH.[59] Under dispute is which of the two amino acids is critical in the active site of α_1-AT. Cohen[22] treated the inhibitor with phenylglyoxal hydrate under conditions that modified the arginine residues; the result was blocking of the inhibitory action of α_1-AT on trypsin. However, investigations by Busby and Gan[16] suggest that the reagent also modifies lysyl residues on the α_1-AT. Heimburger et al.[68] modified lysyl residues with maleic anhydride to give a polymaleoyl derivative; the result was a loss of inhibition of trypsin.

Cohen[22] suggested the proteases might combine with α_1-AT, similar to other substrates; however, one of the intermediates was stable. Subsequent investigations have proposed two somewhat opposing hypotheses. Moroi and Yamaski[126] concluded that the α_1-AT-trypsin complex was an acyl intermediate of trypsin through a new carboxyl terminal residue, probably an arginine or lysine and the γ-oxygen of Ser-183 of the enzyme. Johnson and Travis[87] found that by releasing the complex with the nucleophile benzamide hydrochloride, the released fragment of α_1-AT had an amino

terminal threonine and a carboxy terminal Ser-Lys dipeptide similar to that found in native purified inhibitor. They concluded that trypsin cleaves α_1-AT at a threonine near the amino terminal end to activate the inhibitor and then becomes bound by an unknown mechanism to a carboxyl group at a different site of the α_1-AT. Cohen et al.[23] proposed a hypothesis explaining data from both investigations. They found that a Lys-Thr bond is cleaved during base-catalyzed disruption of the complex and that a new carboxyl terminal lysyl residue is formed. Cohen et al.[24] disrupted the complex using ^{18}OH labeled base. The new carboxyl terminal lysyl residue became labeled with ^{18}O. This conforms to the known distribution of oxygens which occurs during the base-catalyzed hydrolysis of acyl esters, which would occur if the α_1-AT-trypsin complex were an acyl ester or if an acyl intermediate formed during the base-catalyzed hydrolysis of a tetrahedral adduct. They then hypothesized that trypsin binds to a Lys-Thr bond near the amino terminal end of the α_1-AT. The bond is probably analogous to that usually formed between trypsin and its substrates. Since there are no intrachain disulfide bonds at the amino terminal end in α_1-AT, a tetrahedral complex is most likely.

Biochemical Characterization

Alpha-1-antitrypsin is a glycoprotein with a carbohydrate moiety of 12%, consisting of galactose, mannose, acetylhexosamine, N-acetylneuraminic acid, and fucose. The

amino acid composition does not show any unusual features except for relatively high concentrations of aspartate (9.8%), glutamate (12.9%), and leucine (9.9%). The protein contains two moles of cysteine per mole, which would give rise to one disulfide bridge.[94] Reported molecular weights range from 45,000 to 54,000 daltons. Glaser,[58] using ultracentrifugation, reported a molecular weight of 52,100 daltons. Recently, Chan et al.[18] reported the complete amino acid sequence of a CNBr-fragment of 109 amino acid residues.

Genetics

Laurell and Eriksson,[102] on routine examination of serum electrophoretic patterns, noted some with a low alpha-one band. Further examination revealed low levels of α_1-AT. Laurell[100] noted slower electrophoretic mobility of the alpha-one fraction in individuals with low levels. Acid starch gel electrophoresis, thin layer isoelectrofocusing (TLIEF), and crossed immunoelectrophoresis (all to be discussed in detail in the literature review) have all been used to study the genetics of α_1-AT. Most observations are consistent with the interpretation that low values are caused by homozygosity for a "deficiency" gene, intermediate values by heterozygosity, and normal values by homozygosity for the "normal" gene. Hence, the normal Pi-M phenotype will usually have a normal serum level of the protein, the extreme deficiency Pi-Z phenotype will

have levels of about 10-15% of normal, and the heterozygous Pi-MZ phenotype will have intermediate levels of α_1-AT.

Fagerhol and Gedde-Dahl[40] proposed a genetic model with multiple autosomal co-dominant alleles at one locus. The locus is designated Pi for protease inhibitor. Individual alleles are designated Pi^M, Pi^Z, etc. An example of a heterozygous genotype would be Pi^M/Pi^Z, with a resulting phenotype Pi-MZ. Pi-M is the most common allele with a frequency of about 0.9. Other alleles are designated by letters of the alphabet, the position in the alphabet giving an approximation of the electrophoretic mobility of a particular variant. To date, the following alleles have been reported: B, C, D, E, E_2, F, G, I, L, M, M_L, M_B, N, P, S, V, W, W_2, X, Y, Y_2 and Z.[86] Gedde-Dahl et al.[55] reported linkage between the Gm and the Pi loci.

Rare cases of a null allele have been reported.[117,148] Within the limit of sensitivity, no α_1-AT could be detected in these two patients. As determined by the individual families, these findings were thought to be incompatible with co-dominant inheritance. These individuals were designated as null alleles (Pi^-) and relatives were assigned genotypes consistent with this allele. The genotype Pi^M/Pi^- would give a Pi-M- and would have α_1-AT levels about one-half of what would be expected for the normal Pi-M. Martin et al.[116] described a patient with an α_1-AT level of 5 µg/ml. They were able to detect this extreme low amount by using radiolabeled specific antibody. Using these same antibodies for crossed immunoelectrophoresis, they were

able to demonstrate a pattern identical to that of a normal Pi-M. The existence of this null allele is therefore controversial.

Recent work indicates that the M allele may be further divided into a number of subtypes. The designation is subtypes because the major allele products show just a slightly altered mobility, usually discernible only on TLIEF using narrow pH gradients. Johnson[86] reported M_{Lamb} and $M_{Baldwin}$ subtypes with acid starch gel electrophoresis. Kueppers[93] reported a heterozygous subtype Pi-MM_I. Frants and Eriksson[49] and Harada et al.[66] all reported M_1M_2 subtypes. Genz et al.[57] further divided the subtypes into the homozygous types Ma, Mb, and Mc and the heterozygotes into Mab, Mac, and Mbc. There is considerable overlap in the different author terminologies of these subtypes. A standard nomenclature has yet to be determined by workers in this field.*

Biochemical Abnormalities of Variants

The interest is in variants that are associated with a lower than normal concentration of α_1-AT, specifically the Z, S, and null alleles. The biochemical differences for all the different alleles have not been elucidated. Yoshida et al.[161] determined two amino acid substitutions in the Z versus the M protein. Glutamate was substituted

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*At the time of this writing (July 1978), a conference is being held in Paris, France, to decide on a nomenclature.

for by a lysine and another glutamate substituted for by a glutamine. The sialic acid content was also decreased by 26%, accounting for the slower electrophoretic mobility. None of the usual carbohydrate linking amino acids, aspargine, threonine, or serine, was substituted. Therefore, the low sialic acid content may be due to a conformational change introduced by the other two amino acid substitutions. Yoshida et al.[160] determined the difference of the S protein to be one amino acid substitution of a glutamate by a valine.

The lower amount of sialic acid on the Z protein is significant in elucidating the pathophysiology of diseases associated with a deficiency of serum α_1-AT. The liver is the only known site of synthesis. Sharp[144] found periodic acid-Schiff (PAS) positive inclusion bodies in the periportal areas of hepatocytes in Pi-Z patients with severe deficiency. Electron micrographs indicated the material to be located in the rough endoplasmic reticulum. Using fluorescent antibody against α_1-AT, he demonstrated this material to be antigenically related to serum α_1-AT. Eriksson and Larsson[34] purified and partially characterized material from PAS inclusion bodies from liver of Pi-Z patients. They noted no immunological differences between serum and this hepatic α_1-AT using three different immunological techniques. The material had a marked tendency to aggregate, had no sialic acid residues, and had the same molecular weight on SDS electrophoresis. These results suggest the material to be an asialo form of α_1-AT.

Possible mechanisms for the low serum α_1-AT in individuals with the Pi-Z phenotype or other variants are: 1) less secretion, 2) decreased rate of production, and 3) increased rate of removal.

The accumulation of the asialolated α_1-AT in the rough endoplasmic reticulum suggests the first mechanism. Sialic acid is generally accepted as important for the transport of glycoproteins from the cell. Bell and Carrell[14] postulated that the incomplete addition of sialic acid causes the protein to accumulate intracellularly with only small amounts escaping by passive diffusion.

Kuhlenschmidt et al.[97] reported a deficiency of the Golgi membrane sialyltransferase. Such a deficiency could prevent sialylation. As mentioned, the low sialic acid content may be due to a conformational change introduced by the two amino acid substitutions.

Reduction in the rate of synthesis is frequently associated with variant proteins and enzymes; thus, the second alternative should not be ruled out.

Morell et al.[124] demonstrated asialo proteins to be rapidly eliminated from the circulation. Pricer and Ashwell[137] demonstrated that asialo proteins bound preferentially to the membrane fractions of liver cells. Thus, low serum concentration and the accumulation of the Z protein in hepatocytes could be explained on this basis.

Physiological Function

The precise physiological function of α_1-AT is not known. Nor is it known why there is an increase in certain physiological and pathological conditions. Alpha-1-antitrypsin is an acute phase reactant protein. Many of the conditions in which the serum trypsin inhibitory capacity (STIC) is raised involve inflammation. Increases have been noted in gout, rheumatoid arthritis, diabetes mellitus, gastric disease, cirrhosis, hepatitis, jaundice, acute pancreatitis, renal disease, cardiac disease, cancer, myocardial infarction, and with bacterial infection.[30] Ganrot and Bjerre[53] noted doubling of base levels of α_1-AT as a normal feature of pregnancy. This increase was correlated with levels of estrogen hormones when Laurel et al.[103] observed that estrogen-progestin oral contraceptives elevated levels of α_1-AT. Lieberman and Mittman[110] confirmed this elevation but noted that patients homozygous for the Z protein did not show any increase of STIC. Heterozygous patients doubled their intermediate STIC levels, suggesting that the single Pi^M gene alone responds to the hormone. Kueppers[91] noted similar increases after intravenous injections of typhoid vaccine.

Recent work by Arora et al.[10] suggests that α_1-AT plays an immunoregulatory role in the suppression of antigen dependent B-cell response without affecting adherent or T-cells. Work is presently in progress on this campus to determine the mode of action of α_1-AT on cellular proteases.

Association with COPD

Eriksson[32] reported the association of a marked reduction in serum α_1-AT with pulmonary emphysema. The disease was unusual in that the first symptoms were noted at an early age with the onset usually occurring below 40 years of age in 60% of his population and 50 years of age in 90%. In his group of 33 patients, 23 had definite evidence of chronic obstructive pulmonary disease (COPD). The patients did not have frequent episodes of recurrent pulmonary infection. The incidence of primary emphysema was about 50%. The most noticeable abnormality was a symmetric decrease in the peripheral vasculature which was most prominent in the lower lung fields. There is general agreement that the homozygous deficiency state lends itself to a significantly higher risk of developing COPD.[65]

Compared to other types of emphysema, the basal predilection is the most striking feature. This basal predilection is important for two reasons. It represents an important diagnostic tool and it fits a theory of pathogenesis.

Pathogenesis of α_1-AT Deficiency

The major factor in COPD in patients with a severe α_1-AT deficiency seems to be a loss of elastic recoil. Black et al.[15] reported the loss of elastic recoil to be responsible for the decreased expiratory flow rates. In these patients the mechanical properties of the lung were similar to papain-induced emphysema. The ratio of collagen to elastin in normal individuals is 3.4:1.[144] In patients

with α_1-AT deficiency and advanced emphysema, this ratio increases to 4.9:1. These data indicate a decreased pulmonary elastin in emphysema.

Human leukocyte proteases can degrade elastin,[80] collagen,[105] basement membrane and arterial wall,[75] all constituents of pulmonary tissue. Janoff and Scherer[79] demonstrated human polymorphonuclear leukocytes (PMNs) to contain large amounts of elastase. Macrophages also have a lysosomal elastase-like esterase.[81,158] Lieberman and Gawad[109] isolated two proteases from purulent sputum, a "labile" protease and a "stable" protease, which they believed to be a leukocyte elastase. They demonstrated proteolysis of lung tissue with this purulent sputum. Both proteases were shown to be inhibited by serum α_1-AT. The correlation between the degree of inhibition of the leukoproteases and the serum trypsin inhibitory capacity indicated that individuals with an inherited deficiency of α_1-AT are also deficient in leukoprotease inhibitors. Janoff[77] demonstrated human PMN elastase to be inhibited by α_1-AT. Ohlsson and Olsson[130] demonstrated that two neutral granulocytic collagenases are also inhibited by α_1-AT.

The general working hypothesis revolves around a protease-protease inhibitor imbalance in emphysema. This hypothesis rests with the assumption that proteolytic enzyme activity may exceed proteolytic inhibitory activity in the lung. The hypothesis is supported by experimental

models of emphysema which can be produced by intratracheal instillation or aerosolization of proteolytic enzymes. Papain has been used experimentally to produce emphysema in hamsters.[60,134] Mass et al.[118] instilled human PMN homogenates to produce emphysematous lesions in dogs. Elastic fiber destruction was noted with these emphysema models. In view of the ability of the enzyme to degrade elastic fibers and basement membrane at neutral pH, elastase was considered by Janoff[76] as a possible mediator of lung damage in emphysema.

Janoff et al.[82] instilled purified PMN elastase into dog lung. They found that elastase, acting alone, was capable of rapidly inducing emphysematous lesions. They demonstrated with immunohistochemical techniques that the elastase was localized in close proximity to lung elastic fibers. Ultrastructural immunohistochemical techniques demonstrated that the elastase penetrated into intercellular regions of the connective tissue interstitium and attached to interstitial elastic fibers. These studies were done *in vitro* and *in vivo*. Other PMN proteolytic enzymes cannot be excluded. The generalized destruction of alveolar elements suggests that alveolar glycoproteins and basement membrane may also be susceptible.

Wilson et al.[159] demonstrated that sequestered granulocytes damaged the capillary membrane of the lung. The mechanism probably involved microsomal proteases because degranulation was usually present. Discontinuity of the alveolar capillary membrane is considered to be the earliest

morphological sign of emphysema. Welch et al.[156] assumed this process led to an accelerated development of COPD in α_1-AT deficiency. Heinmann and Fishman[69] demonstrated filtration of granulocytes in pulmonary capillaries to be a continuous normal process. The greater perfusion of and, hence, sequestration of granulocytes in the lower lobe compared with the upper could explain the greater extent of tissue destruction seen in the lower zones in α_1-AT deficiency.

The protease-protease inhibitor imbalance theory of emphysema suggests that proteolysis of lung connective tissue can result either from a deficiency of a protease inhibitor or from a protease overload. The above cited models of a protease overload are examples of the latter. Familial emphysema associated with α_1-AT deficiency is an important form of the former.

Intermediate Deficiency Controversy with COPD

The prevalence of homozygotes Pi-Z in the general population is only about 0.1 to 0.2%; hence, homozygosity will account for only a very small proportion of patients with COPD. Of greater importance to the etiology and pathogenesis of COPD is the potential significance of the heterozygous state. Frequency estimates of the heterozygous state are estimated between 5 and 14% of the general population.[65,121]

There is considerable controversy regarding the clinical importance of intermediate levels of α_1-AT. In analogy, the

assumption could be made that the heterozygote usually has some dysfunction that is only fully developed in the deficient homozygote. Lieberman[108] and Lieberman et al.,[112] using the STIC assay, identified heterozygotes in 17 and 18%, respectively, in COPD populations. This frequency was higher than the 4.7% found in control populations. He concluded that heterozygosity predisposes to COPD. In contrast, Larson et al.,[98] also using the STIC assay, reported 8.0% heterozygotes in a chest clinic population and 15.2% in their control population. These data refute the hypothesis of intermediate deficiency as a cause of COPD. Kueppers et al.,[95] using antigen-antibody crossed immunoelectrophoresis, reported 25.5% heterozygotes in a COPD population and 11.7% in their control group. The higher percentages reflect the increased sensitivity offered by crossed immunoelectrophoresis. About half of the patients classified as heterozygous are missed if only STIC levels are assayed.

The more recent studies to evaluate the susceptibility of the MZ are basically of four types:

(1) physiological, radionuclide, and pathological exam of symptomatic Pi-MZ's compared with Pi-M subjects

(2) the distribution of Pi phenotypes in patients with COPD and in a control group

(3) pulmonary function of asymptomatic Pi-MZ's, usually found as relatives of patients with COPD, compared with Pi-M controls

(4) pulmonary function and/or symptoms in a large population.

Kanner et al.[88] conducted a study of the first type. They concluded that heterozygosity was an important factor in the development of COPD. The remarkable result from their study was that, by using regional ^{133}Xe ventilation, perfusion scans and statistical analysis, they could classify Pi-M, Pi-MZ and Pi-Z into three separate pulmonary dysfunction groups. This indicated that lung disease in the heterozygote was different than that found in the deficient patient. In the second type of study Cox et al.[29] concluded that there was an increased risk of COPD in the Pi-MZ but not in the Pi-MS. They pointed out the extreme importance of accurate phenotyping procedures when assessing the relative risk of the heterozygote. Eriksson et al.,[35] using a consecutive series of autopsies, concluded that heterozygosity predisposed to both liver and lung disease but did not influence the survival rate. It should be noted that phenotyping could not be done in this study. Deficiencies were inferred by the presence of PAS positive granules in the liver. Using the third type of study, Mittman et al.[122] noted the presence of lung parenchymal disease was seen more frequently in the relatives with intermediate STIC levels than in relatives with a normal STIC. However, a high percentage of the relatives were smokers. These investigators were the first to postulate the role of external irritants as having a synergistic role in the development of COPD in the heterozygote. Aronson et al.,[9] using a selected population of young asymptomatic heterozygotes who were not smokers,

gave preliminary evidence that all asymptomatic Pi-MZ's have evidence of small airway disease. Studies of the fourth type in large unselected populations[135,152] revealed no evidence that the Pi-MZ phenotype puts the person at an increased risk for the development of COPD.

The differences noted in the last two types of studies suggest that studies done on Pi-MZ groups made up of relatives of patients with COPD may be detecting other familial factors that, in conjunction with a moderate degree of α_1-AT deficiency, could lead to COPD. Galston et al.[51] studied the relationship between a familial variation of leukocyte lysosomal protease and α_1-AT. What they found was that normal elastase-like esterase and leukoprotease activities appeared to be associated with an unfavorable clinical course in patients with intermediate or low STIC. Low activity was associated with a favorable course in those patients with intermediate or low STIC's. Taylor and Kueppers[149] clouded this conclusion by electrophoresing granulocytic lysosomal extracts at pH 8.6. Three mobilities of elastase were noted--fast, intermediate, and slow. The esterase activity was lowest in the slow electrophoretic category. Patients with COPD had a four-fold prevalence of this slower mobility elastase. These observations are the opposite of what would be expected if the only determinant in the proteolysis theory were an increase in protease concentration. The authors suggested a potential mechanism to explain this dilemma. The existence of structurally and/or functionally altered elastase that is

incompletely inhibited by α_1-AT, or an abnormal elastase and abnormal α_1-AT may contribute to an abnormal interaction between protease and protease inhibitor. Results from both these studies imply that further investigations are necessary.

Hepatic Disease

The first hint of a relationship between childhood liver disease and a deficiency of α_1-AT was noted by Sharp et al.[145] When surveying serum protein electrophoretic patterns, the first serum with a low alpha-globulin band was that of a child with hepatic cirrhosis. This child and a brother were shown to be α_1-AT deficient. Ten unrelated infants of the Pi-Z phenotype were reported who had hepatitis or cirrhosis of the liver. Other causes of hepatic cirrhosis were excluded. The disease starts with hepatosplenomegaly, elevated bilirubin, alkaline phosphatase and transaminases. During the following six months, bilirubin returns to normal but alkaline phosphatase, transaminases, cholesterol and triglycerides remain moderately elevated. Histologic findings are cholestasis and periportal fibrosis. Only about 10-20% of Pi-Z infants develop the disease.[1] All with the disease show signs of a slowly progressive cirrhosis even though cholestasis diminishes. Heterozygous infants apparently have no clinical manifestation of the disease.

In the adult, cirrhosis is a well established hepatic manifestation of α_1-AT deficiency. It does not differ from

cirrhosis from other causes. Cirrhosis may be seen associated with emphysema[12,25] or may be the only manifestation of a deficiency.[17,74]

In both children and adults, the major abnormality is a deposit of an amorphous substance in the parenchymal liver cell. Sharp[144] was the first to describe these deposits. They were best seen in the cytoplasm of a variable number of hepatocytes, after saliva or diastase digestion as PAS positive globules. The material was identified as similar to α_1-AT by fluorescent tagged anti-α_1-AT. This accumulated material is an asialated form of α_1-AT, and possible mechanisms for its accumulation have already been discussed in this manuscript. Electron microscopy located this material in the lumen of the rough endoplasmic reticulum, which was markedly dilated. No globules were observed in the Golgi apparatus. The number of globules varied between hepatocytes. Ishak et al.[74] and Lieberman et al.[111] demonstrated the globule-containing cells to be randomly distributed through the lobule, but more abundant in periportal areas and around hepatic veins. A periportal distribution is evident in livers with portal fibrosis or cirrhosis, the globule-containing cells being adjacent to collagen septa. With fibrosis or cirrhosis, necrosis was also observed. Aagenaes et al.[2] noted the globules only in the parenchymal cells, not in the Kupffer cells. They also estimated that between 20-60% of the hepatocytes contained the globules and that the percentage was higher in the homozygous deficient than the

heterozygous patient. In adult patients, the specific lesions have been identified in the heterozygous Pi-FZ,[45,46] Pi-SZ[17,111,133] and Pi-MZ[111] as well as the Pi-Z phenotype.

The association between cirrhosis and a heterozygous deficiency of α_1-AT may be only a fortuitous one,[48,125] or there may be a definite correlation as argued by Palmer et al.[133] and Triger et al.[150] As with the early studies of the heterozygote and COPD, the discrepancies arise because definitive phenotyping procedures were not used or were not available. The synergistic role of alcohol and other xenobiotics cannot be ruled out.

There is no obvious relationship between the accumulation of α_1-AT in the hepatocyte and hepatic damage. It is not known why a decrease in serum α_1-AT and/or its accumulation in the liver may lead to hepatocellular necrosis, fibrosis, or cirrhosis. The hypothesis of a protease-protease inhibitor imbalance has not successfully been applied to the liver. Gans[54] suggested leukocyte or Kupffer cell proteases could be responsible for the damage when serum concentrations are low. Lieberman et al.[111] proposed that increased vulnerability results from the globules of α_1-AT in the hepatocytes.

Neonatal Respiratory Distress Syndrome (RDS)

Evans et al.[36] observed that 12 of 14 infants with RDS had low STIC and elastase inhibitory capacity. They noted that levels returned to normal when the infant improved. Mathis et al.[119] observed that in a series of

34 Pi-M infants half had significantly lower serum levels of α_1-AT. The severity of RDS was inversely proportional to the serum levels of α_1-AT. With improvement, levels returned to normal. The lung hyaline membranes stained with fluorescein-labeled antibody to α_1-AT. The initial low levels can probably be explained by absorption to the membrane. Levels return to normal with disappearance of the membrane.

Rheumatoid Arthritis

Cox and Huber[28] reported a significantly higher incidence in adults of heterozygotes having classical or definite rheumatoid arthritis. Children with juvenile rheumatoid arthritis had no difference in their Pi phenotypes. Since elastase and other proteolytic enzymes, released from leukocytes in the synovial fluid, are known to attack cartilage,[78] inadequate amounts of the major proteolytic inhibitor could be hypothesized as having a role in intensifying the cartilage destruction.

Pancreatic Fibrosis

Freeman et al.[50] presented a case report of pancreatic fibrosis in a Pi-Z woman who also had emphysema. A search for other causes of the fibrosis yielded negative results. They then speculated on whether α_1-AT plays a role in protecting the pancreas.

Celiac Disease

Walker-Smith and Andrews,[151] on examination of 13 children with untreated celiac disease, found one child with severe deficiency of α_1-AT and four with intermediate levels. In another group of 15 children on a gluten free diet with the same disease, five had intermediate levels of α_1-AT. Greenwald et al.[61] reported a patient with severe deficiency having emphysema, cirrhosis and intestinal mucosal atrophy.

Asthma

Katz et al.[89] screened a population of children with severe atopic bronchial asthma. They found the α_1-AT levels and phenotypes to be similar to a control group. However, one difference was noted. The children with steroid-dependent severe asthma had a higher incidence of heterozygosity for the Z protein than did the non-steroid dependent and the control groups.

Sex Chromosome Mosaicism or Trisomy 21

Aarskog and Fagerhol[3] suggested a causal link between sex chromosomal mosaicism and severe or heterozygous variants of α_1-AT. Kueppers et al.[96] found a significant increase of heterozygotes in a small population with sex chromosome mosaicism. Fineman et al.[47] also suggested heterozygosity as an etiological factor in trisomy 21. The question of a possible role of α_1-AT in cell division has not been investigated.

Periodontal Disease

Allen and Spicer[5] reported an apparent interaction of Pi variants with the ABO blood system. The Pi-M_2 with an A or B blood type is more susceptible to periodontal disease, while Pi-M_2 with an O blood type is more refractory toward the disease than the normal Pi-M.

LITERATURE REVIEW

Various electrophoretic procedures over the last 15 years have played an integral role in the study of α_1-AT, especially in elucidating the genetics of the protein.

Laurell and Eriksson,[102] after screening 1500 paper gel electrophoretic patterns from diseased patients, noted five patients with the α_1 band missing. Agar gel electrophoresis showed no band sharpening of the α_1 zone. The α_1 lipoprotein and orosomucoid levels were normal. Three of their five patients were noted to have widespread pulmonary lesions. The sister of one of the patients had the same lung disease. In addition, one patient had a history of rheumatoid arthritis.

The suggestion of a link with a deficiency of the α_1 zones and the pulmonary lesions prompted Eriksson[32] to further investigate one of the families. The propositus had dyspnea at age 34 which steadily progressed. At age 36, the diagnosis of emphysema was made. Agar gel electrophoresis revealed an absence of the α_1 zone in both this patient and a sister who was also diagnosed as having emphysema. A brother, who had died earlier of complications of emphysema, was presumed to have had an absence of the α_1 zone. The electrophoretic distribution of the

antitryptic activity, determined by using DL-arginine p-nitroanilide hydrochloride as a substrate, was determined after separation in agar. Alpha-1-antitrypsin levels were determined by subtracting the α_2-macroglobulin activity from the total antitryptic activity.

Eriksson found three levels of α_1-AT in this family: normal, 60% and 10% of normal. Combined with the agar gel electrophoresis information, he concluded the deficiency and emphysema correlation to be familial. The deficient woman married a normal man and had four children with 60% of α_1-AT. These data indicated a recessive mode of inheritance.

Laurell[99] introduced and gave experimental details of crossed immunoelectrophoresis. The technique involves separation of protein in one direction followed by immunoprecipitation against antibody at right angles to the first direction. In this first demonstration, Laurell used agarose gel electrophoresis of whole human sera at pH 8.6 as the first dimension. A strip of agarose was then cut along the axis of migration and set into a trough of equal dimension in agarose containing antibody to whole human sera. In this technique, current is then passed through the antibody containing agarose. The antigens (different proteins in the sera) are forced into the antibody containing agarose. They migrate until the zone of equivalence is reached, where they precipitate as antigen-antibody complexes. Hence, the size of an antigen peak is

proportional to the antigen concentration and inversely proportional to the amount of antibody in the agarose.

The crossed immunoelectrophoresis technique was used by Axelsson and Laurell[11] to give the first indication of the electrophoretic polymorphism of α_1-AT. Using agarose gel electrophoresis as the first dimension followed by crossed immunoelectrophoresis in the second against an antibody specific for α_1-AT, they came up with three zone patterns, i.e., normal, slow and double. This distribution of zones and the presence of normal and deficient STIC led the authors to conclude that the phenotypes could be explained by different combinations of three allelomorphic, autosomal genes for α_1-AT.

Independent of these observations, Fagerhol and Braend,[39] using a horizontal discontinuous acid starch gel electrophoretic procedure at pH 4.95, revealed a pattern of three or four "prealbumin" bands of varying strength. These patterns were found in five different combinations. The "prealbumins" for the majority of the patterns appeared as a three band distribution of "one weak in front of two relatively heavy bands." This pattern was designated MM for medium mobility. A slower moving pattern of three bands was designated as SS for slow mobility. Four zone patterns of lower staining intensity which seemed to be a combination of the MM and either SS or a faster mobility were designated as MS, FS, and FM. They did not observe an FF pattern. The authors proposed a genetic theory of three codominant alleles which they

called Pr^F, Pr^M and Pr^S. The MM pattern was found in 96% of 390 randomly selected donors.

Fagerhol and Laurell[41] then joined forces, exchanged sera and determined that Fagerhol's "prealbumin" and Laurell's α_1-AT were identical. By using discontinuous acid starch gel in the first dimension and crossed immunoelectrophoresis, they described six zones attributed to α_1-AT. The original three band pattern was identified as bands 2, 3 and 4. One more anodal band and two more cathodal bands were demonstrated by the sensitive immunoprecipitation step. All the homozygous patterns except the ZZ had the same six zone pattern. Only three zones were seen with the ZZ, probably because of the low serum concentration associated with the ZZ. Heterozygote patterns fit the pattern of one allele product superimposed on another, with the major zones showing about half the staining intensity.

The terminology of Pr (prealbumin) was replaced by Pi (protease inhibitor) in this paper. Pi was chosen for α_1-AT because it is the major protease inhibitor in human serum and trypsin is only one protease that it will inhibit. In his review article, Fagerhol[37] recognized that the Pi system consisted of at least seven codominant alleles. The seven known alleles at that time were Pi^F, Pi^I, Pi^M, Pi^S, Pi^V, Pi^X, and Pi^Z. At that time there were no known exceptions to the codominant mode of inheritance.

Fagerhol[38] modified the acid starch gel electrophoresis procedure and was able, in addition to the six zones, to

demonstrate two more zones in the homozygous phenotype after crossed immunoelectrophoresis. The two new zones were labeled 3 and 5 and were close to and in front of the two major zones now labeled zones 4 and 6. The relative protein content of each zone, 1 through 8, was (in percent): 2.5, 14.4, 5.3, 40.9, 3.7, 33.6, 2.8 and 2.4. The three major zones 2, 4 and 6 accounted for 89% of the total α_1-AT in each of the homozygous phenotypes. The exception was again the ZZ, where zones 2 and 4 contained relatively more protein and zone 6 relatively less. These quantitative results are questionable because the height of each peak was the only measured parameter. No attempt was made to estimate the area under each peak. Also, the heights were compared with a standard curve obtained by using "rocket" immunoelectrophoresis[101] to quantitate the α_1-AT. The crossed immunoelectrophoresis is a modification of "rocket" immunoelectrophoresis. With "rocket" immunoelectrophoresis the area under the peak is not critical to quantitation of the peaks. As would be predicted by earlier experimentation, the distribution of zones from each allele was maintained in the heterozygous sera.

Laurell and Persson[104] developed what they considered to be a simpler method of Pi phenotyping. The system consisted of agarose gel electrophoresis at pH 5.15 followed by crossed immunoelectrophoresis. To reduce the EEO of the agarose and to sharpen the zones of the α_1 region, linear acrylamide was incorporated into the agarose. The

system mandated that an internal reference solution be used. They used a carbamylated immunoglobulin L chain of the lambda type. Also, because the stain was poor in the first dimension, it was imperative that a crossed immunoelectrophoresis be performed with every sample. The homozygous phenotype was resolved into only five zones in this system.

With acid starch gel electrophoresis and crossed immunoelectrophoresis techniques, 23 alleles had been described.[27]

The isoelectric point range of α_1-AT is about 4.2 to 4.7; albumin is about pH 4.9. Also, the heterogeneity of α_1-AT is only seen at acid pH. Thus, thin layer isoelectric focusing[106] should have the capability of separating the Pi phenotypes. Three separate reports utilizing acid pH isoelectrofocusing on thin layer polyacrylamide gels were published at about the same time.[4,7,92] The experimental protocols were similar in all three papers, and each utilized a pH gradient approximately 3.5 to 5.0. Densitometry by Allen et al.[4] showed a distribution of peaks similar to that obtained by acid starch gel, followed by crossed immunoelectrophoresis.[38] The high resolution obtained by TLIEF and the sensitivity of Coomassie Blue dye allowed the majority of phenotypes to be ascertained simply by this one dimensional procedure.

An explanation for the microheterogeneity had not been offered up to this point. Because the heterogeneity was similar with TLIEF, the microheterogeneity was attributed

to charge alone. Mega and Yoshida[123] separated the components by preparative starch gel electrophoresis and DEAE-ion exchange. The anodal components contained more sialic acid per molecule than the cathodal components. The molecular size of each component was identical.

The increased resolution with TLIEF is expected since proteins are maximally resolved when focusing is completed. In contrast, with the acid starch gel procedure, proteins will diffuse with time once they begin to unstack following passage of the moving boundary.

Within the time span of about two years the number of recognized alleles increased to 25 and described phenotypes to about 40.[136] Since the protein is inherited as an autosomal codominant allele, the combinations of possible phenotypes including the homozygous phenotypes is 325. As an aid in documenting new possible phenotypic expressions, some authors attempted to develop a crossed immunoelectrofocusing technique from the polyacrylamide gel.

Arnaud et al.[6] first used the technique to document that the area around the zones was indeed α_1-AT. The resolution was poor, as even the two major zones could not be resolved.

Lebas[107] and Arnaud et al.[7] used the crossed immunoelectrofocusing technique to study the genetic polymorphism of the protein. The resolution was poor in that the resolving power offered by the TLIEF was lost in the immunoprecipitation step.

The problem with these attempts is that they involve molding in of charge free polyacrylamide which has virtually no EEO into agarose which has significantly measurable EEO. Briefly, two phenomena occur when this is attempted. A buffer flow will emerge on the anodal side of the polyacrylamide strip and the cathodal side of the strip will dry, breaking the electrical current. The buffer flow will cause loss of resolution, and the breakage of electrical current will distort the field strength in the agarose.

Numerous investigators have attempted to reduce the EEO of the agarose. Reports include charge modification of the agarose,[63,64] alkali treatment,[115] treatment with an anion-exchange resin,[138] purification and the addition of a nonionic water soluble polymer,[84] purification and augmentation of viscosity,[85,155] and mixing of polyacrylamide into the agarose.[146] In addition, methods have also been described using technically tedious and time consuming modification of the crossed immunoelectrophoresis. Loft[114] used a procedure for crossed-line immunoelectrophoresis that involved washing the polyacrylamide with barbital buffer, using low voltage for 35 hours, removing the polyacrylamide strip, and finally filling in the gap with agarose. Groc and Jendry[62] described a special chamber for performing the crossed immunoelectrophoresis.

Another method of solving the problem was offered by Soderholm and Smyth.[147] They introduced a simple technique of laying on the polyacrylamide for crossed

immunoelectrofocusing. With this method, proteins on the surface of the acrylamide are transported by diffusion and electrophoresis into the agarose gel. The field strength in the agarose is little affected by surface application of the acrylamide gel.

One last attempt was made by Arnaud et al.[8] to mold the polyacrylamide into agarose which had also been mixed with polyacrylamide to reduce the EEO. The polyacrylamide strip was laid on a glass plate, agarose with anti-α_1-AT was poured up to the strip, and then the whole plate was overlaid with the agarose-polyacrylamide solution. This was a technically demanding technique that mandated all procedures to be run at 4 C in order to further reduce problems from EEO. Again, the resolution was not preserved through the second dimension.

OBJECTIVES

The objectives of this research were to:

1. Develop a method that would combine crossed immunoelectrophoresis with TLIEF and not lose resolution.
2. Use this method to study the microheterogeneity of alpha-1-antitrypsin.
3. Offer the method so that it could be used to study the microheterogeneity of other proteins.

MATERIALS AND METHODS

Human Serum

The majority of human sera received in this laboratory are from relatives of patients presented at the Chest Clinic at Ingham Medical Center in Lansing, Michigan. We are currently screening relatives of these patients, searching for young asymptomatic, non-smoking individuals who are Pi-MZ. Whole blood is collected by venipuncture, allowed to clot, and serum taken off after centrifugation. Serum was stored at -20 C until analysis was performed. Occasionally, sera obtained from other clinical laboratories are sent to our laboratory for phenotyping.

Phenotyping

Alpha-1-antitrypsin phenotypes are determined by culling information from three independent procedures.

A. Thin Layer Isoelectrofocusing (TLIEF)

Thin layer isoelectrofocusing was accomplished by modifications of the methods of Allen et al.[4] and Kueppers.[92] Acrylamide, N,N' methylene bisacrylamide, ammonium persulfate (Ames Co., Elkhart, IN) and sucrose (Fisher Scientific Co., Fair Lawn, NJ) were used to make the gel. Ampholines pH 3.5-5.0 (LKB Products Inc., Bromma, Sweden) were increased

to 2.4% (w/v) and 1 M glycine (Sigma Chemical Co., St. Louis, MO) was used for the cathode strip. The percent of crosslinking (C)[71] was increased to 5%. N,N,N',N'-tetramethylethylene diamine (TEMED) was not used since the ampholytes themselves contain tertiary amino groups to catalyze the polymerization. The gel was prefocused for 30 minutes at a constant voltage of 500 to eliminate the persulfate. Focusing was accomplished with an LKB 2117 Multiphor Basic Unit and LKB 2130 D.C. Power Supply (LKB Products Inc., Bromma, Sweden) at 4 C with cooling supplied at 10 L/min with a Lauda K-2/RD (Brinkman Instruments Inc., Westbury, NY) temperature regulator. Maximum power and amperage were set at 30 watts and 30 milliamperes, respectively. Maximum voltage was set at 850. With this system, α_1-AT will migrate anodal to the majority of other serum proteins into a series of distinct reproducible zones.

Major zones with TLIEF at pH 3.5-5.0 are generally sufficient to determine the phenotype. Figure 1 demonstrates some of the phenotypic expressions of α_1-AT. Pi-M will migrate as two major zones, M4 and M6. Minor zones M7 and M8 are noted cathodal to M6; M2 is located anodal to M4. Since the protein is inherited as an autosomal codominant allele, one would expect the heterozygote to show allele products from both loci. The Pi-MZ shows the allele products of both the M and the Z loci. With the Pi-MZ, the major zone Z4 is located just cathodal to M7 and Z6 just cathodal to M8. Because of the low concentration donated from the Z loci, these are the only Z zones generally noted.

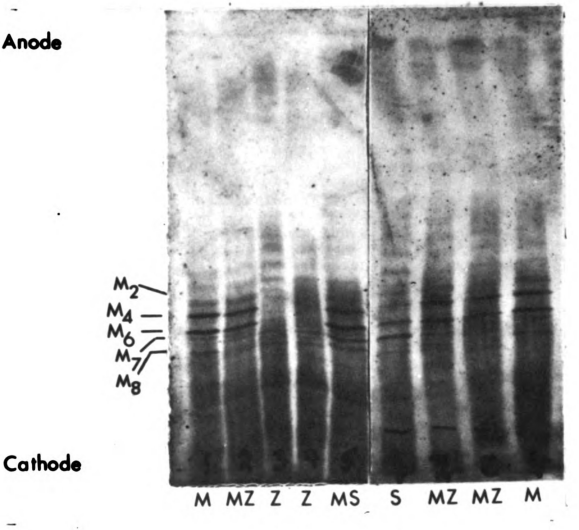

Figure 1. Analytical TLIEF of α_1-AT at pH 3.5-5.0.

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The Pi-Z is obvious by its cathodal mobility and absence of zones in the region where one sees the major M zones. The major zones Z4 and Z6 are located as noted with the Pi-MZ. The Pi-MS has as its main feature the major S6 zone between the minor M zones M7 and M8. The S4 zone is buried under the M6 zone. The Pi-S has the same distribution as a Pi-M, but the whole banding pattern is shifted cathodally.

B. Alpha-1-antitrypsin

The radial immunodiffusion (RID) method of Fahey and McKelvey[42] was used to quantitate the α_1-AT. Quantiplate (Kallestad, Chaska, MN) immunodiffusion plates were used. Five µl of serum and standards were pipetted into the wells. At 18 hours, the precipitin rings were measured with a PEAK (Meloy Laboratories Inc., Springfield, VA) magnifying comparator capable of measuring to one-tenth millimeter. The log of the concentration was plotted against the diameter of the precipitin rings and a best line fit determined. Unknowns were extrapolated from the graph. Normals, determined by the manufacturer, were given as 220-380 mg/dl.

C. Serum trypsin inhibitory capacity (STIC)

Total STIC was measured by a modification of the method of Homer et al.[72] The 3X crystallized trypsin (Worthington Biochemicals, Freehold, NJ) was standardized by the method of Chase and Shaw.[19] The assay was performed by incubating differing amounts of serum with trypsin and estimating the

inhibition of the trypsin's ability to hydrolyze the synthetic substrate benzoyl arginine ethyl ester (BAEE) (Sigma Chemical Co., St. Louis, MO). The reaction was followed at 253 nm with an ACTA III spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Normals, determined in this laboratory, were 0.65-0.94 mg of trypsin inhibited per ml of serum.

The quantitative estimate of α_1-AT and STIC were used as adjunct information in determining the phenotypes. Table 2 gives the levels of various phenotypes as found in our laboratory. By assuming the Pi-M to be 100% of normal,

Table 2. α_1-AT and STIC levels in sera of various phenotypes

Phenotype	No.	α_1-AT mg/dl	SD	% Normal	STIC Try. inhibited per ml serum	SD	% Normal
M	102	310	82	100	.88	.17	100
MZ	23	157	52	51	.57	.09	65
Z	5	61	16	20	.26	.01	30
MS	7	266	117	86	.80	.09	91
FM	2	235/235	---	---	.78/QNS	---	---
S	1	92	---	---	.44	---	---

the α_1-AT and STIC levels could be calculated as a percent of normal and correlated with different phenotypes. Generally, the Pi-MZ will have levels of α_1-AT approximately 50-55% of

normal. The homozygous severe deficient Pi-Z had levels of approximately 20% of normal. The Pi-MS had levels approximately 90% of normal. Only two Pi-FM's and one Pi-S have been seen thus far. Data were insufficient for meaningful calculations.

All three results (TLIEF, STIC, α_1-AT) were used in final determination of the Pi phenotype.

Crossed Immunoelectrophoresis (CIEP)

A. TLIEF across the length

The technique was a modification of focusing across the width used to establish the Pi phenotypes. The production of the gel was essentially the same. However, to facilitate handling of the gel for the second dimension, a sheet of clear acetate transparency film 3M type 383 (3M Co., St. Paul, MN) was fitted between the rubber gasket and one side of the glass mold.

The gel was placed acetate film side down on the cooling block of the Multiphor and prefocused along the length for 30 minutes at a constant voltage of 500.

Fresh human sera were incubated with 1% mercaptoethanol for 20 minutes at room temperature. The concentration of the α_1-AT was then adjusted to 0.5 mg/ml with 12.0% (w/v) sucrose. Approximately 25 µl was then placed on the gel surface by means of a 10 x 10 mm piece of Whatman 3MM filter paper (Whatman, Inc., Clifton, NJ) about 15 mm from the cathode.

Focusing was accomplished by the application of 1960 volts (80 volts/cm) at equilibrium with a maximum power setting of 30 watts (0.56 mW/mm^3). The cooling block remained at 4 C with a closed system temperature regulator at 10 l/min. After 30 minutes, the paper tabs were removed and the focusing allowed to continue for 11.5 hours.

B. Transfer technique

After 12 hours for TLIEF in the first dimension, the area between approximately 4 and 14 cm from the cathode was separated from the rest of the gel by using a rectangular steel blade as a guide and slicing the gel with a fine-tipped scalpel blade. With the template on the cooling block and the rectangular steel blade as guides, 4 mm wide strips were then sliced along the axis of migration, inverted and transferred to the antibody-free area of the agarose, 2 mm cathodal to the junction of the antibody containing agarose.

C. Immunoelectrophoresis

One percent Indubiose A 45 agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) was prepared with barbital buffer pH 8.6, ionic strength 0.02. Twelve milliliters of agarose was poured onto an 84 x 94 mm glass plate to a thickness of 1.5 mm and allowed to cool. The gel was then sliced 25 mm from one of the 84 mm edges, and the majority of the gel (69 x 84 mm) was discarded. A 30 µl/ml solution of monospecific antibody to α_1-AT (Behring Diagnostics, Sommerville, NJ) made with 8 ml of

agarose cooled to between 50 and 55 C was then poured onto the remaining portion of the plate. The antibody-free agarose was then subsequently oriented as the cathode.

Plates were immunoelectrophoresed at 17.5 volts/cm for two hours at 25 C using the buffer for previous agarose preparation. The plates were then pressed, washed, and stained with Coomassie Brilliant Blue R-250 according to established immunoelectrophoretic procedures.[153]

D. Quantitation of immunoprecipitin zones

Clarke and Freeman[21] demonstrated that antigen amounts can be quantitated by measuring the area enclosed under the immunoprecipitate. Weeke[154] suggested the best method of estimating the area to be the product of the height times the width measured at the point which bisects the height. Measurements were made with the same comparator used for the RID. Total area was calculated as the sum of the area under each individual peak from a separate allele and each peak was expressed as a percentage of the total.

pH Determination

Determinations of pH were made at 1 cm intervals directly on the gels with an Ingold Model 6020 (Ingold Electrode Inc., Lexington, MA) surface electrode. The electrode and pH buffers were calibrated and kept at the same temperature as the gel, 4 C.

Joule Heating

The average Joule heating was estimated by the equation

$$\text{cal/sec} = \frac{\text{voltage} \cdot \text{amperage}}{4.185} \quad \text{(ref. 120)}$$

Voltage and amperage were read directly off the power supply during the course of a TLIEF experiment. The heating was expressed as cal/sec/cm^3 by dividing the cal/sec by the volume of the gel.

RESULTS

TLIEF Across the Length

It was initially determined that the length of migration of the α_1-AT across the width of the gel was too short to give the maximal resolution with the immunoprecipitation step. Thus we first defined parameters for focusing along the 24 cm length of the thin layer gel. Parameters for analytical TLIEF across the width of a 24.0 x 11.0 x 0.2 cm gel, pH 3.5-5.0, have been well defined. Since the basic gel dimensions were to remain the same, the power settings of wattage and amperage were not altered. Maximum allowable power was left at 30 watts (0.56 mW/mm^3) and maximum amperage left at 30 mA. The average heat generated per unit of gel, expressed as $cal/sec/cm^3$, was shown in Figure 2A. This was a typical result of an electrofocusing experiment across the width of the gel. The maximum allowable voltage was set at 80 volts/cm.

The $cal/sec/cm^3$ upon application of power started at about 0.11 and quickly rose to just under 0.14, where it stayed for about an hour. During the last 1 1/2 hours of focusing, the $cal/sec/cm^3$ dropped slightly to about 0.135. With an application of 1960 volts across the length (80 volts/cm), the $cal/sec/cm^3$ started out at about 0.14,

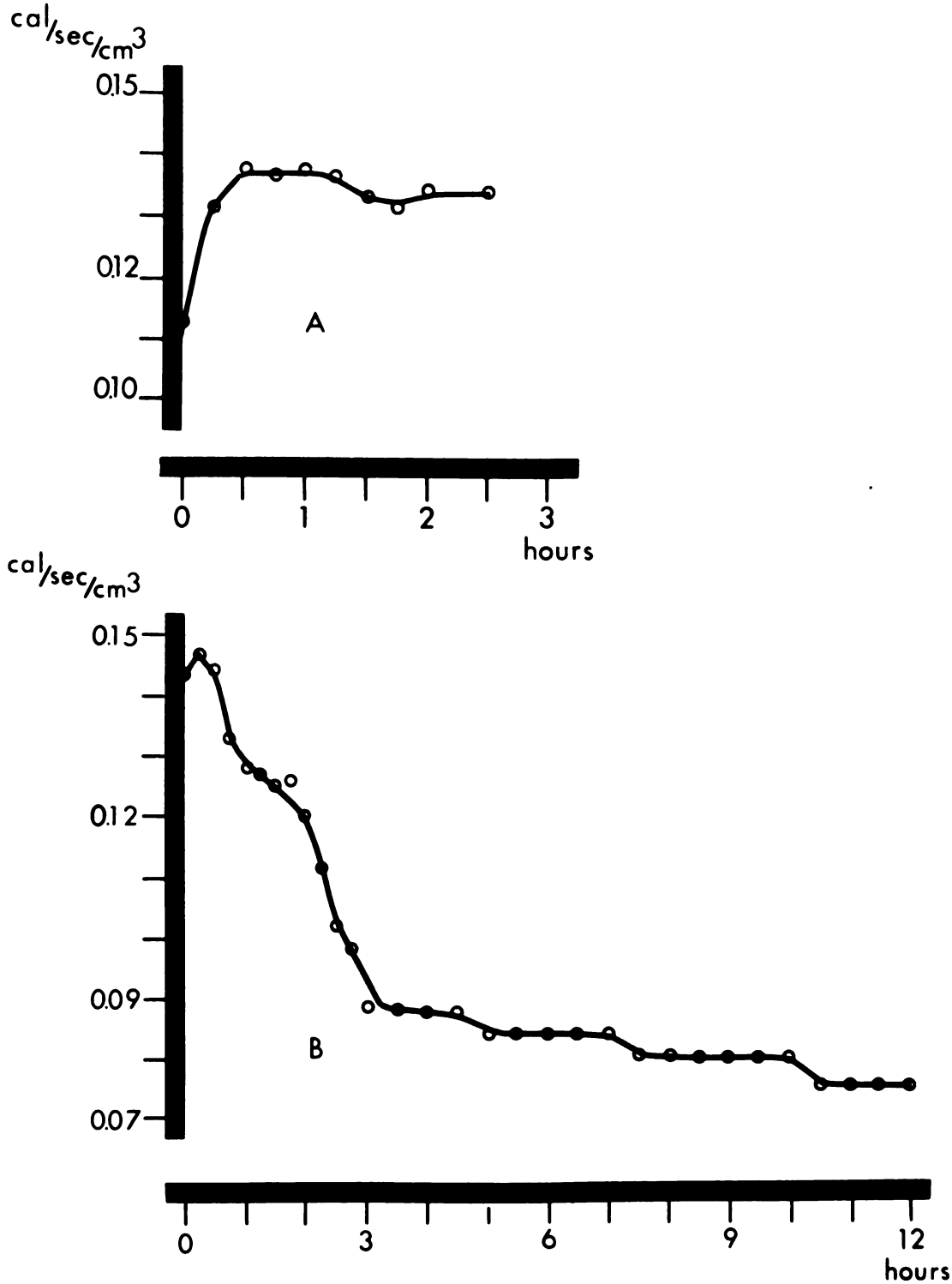

Figure 2. Joule heating of TLIEF gels.

quickly rose to just under 0.15, but by three hours had dropped to 0.09 (Figure 2B). During the next nine hours the cal/sec/cm^3 slowly dropped to about 0.08. The Joule heating started out higher when focusing along the length but quickly dropped to levels below that observed when focusing across the width. The gel was kept at 4 C with a closed system circulating regulator at 10 l/min.

The relationship between voltage and amperage during 12 hours of focusing along the length is given in Figure 3. Voltage started at 1140 volts and by 2 1/2 hours had reached the maximum allowable voltage of 1960 volts, where it remained until completion of the experiment. Meanwhile, amperage started out near its maximum of 30 mA and within approximately three hours fell to 10 mA. During the next nine hours the milliamperage slowly decreased to 8.5.

The distance in millimeters covered by the α_1-AT from the most anodal to cathodal zones of a Pi-M was measured along the axis of migration over different times allowed for focusing. Results are given in Table 3.

Table 3. Pathlength of α_1-AT versus time of focusing

Focusing (hours)	6	7	9	12	14
Pathlength (mm)	26	30	39	44	57

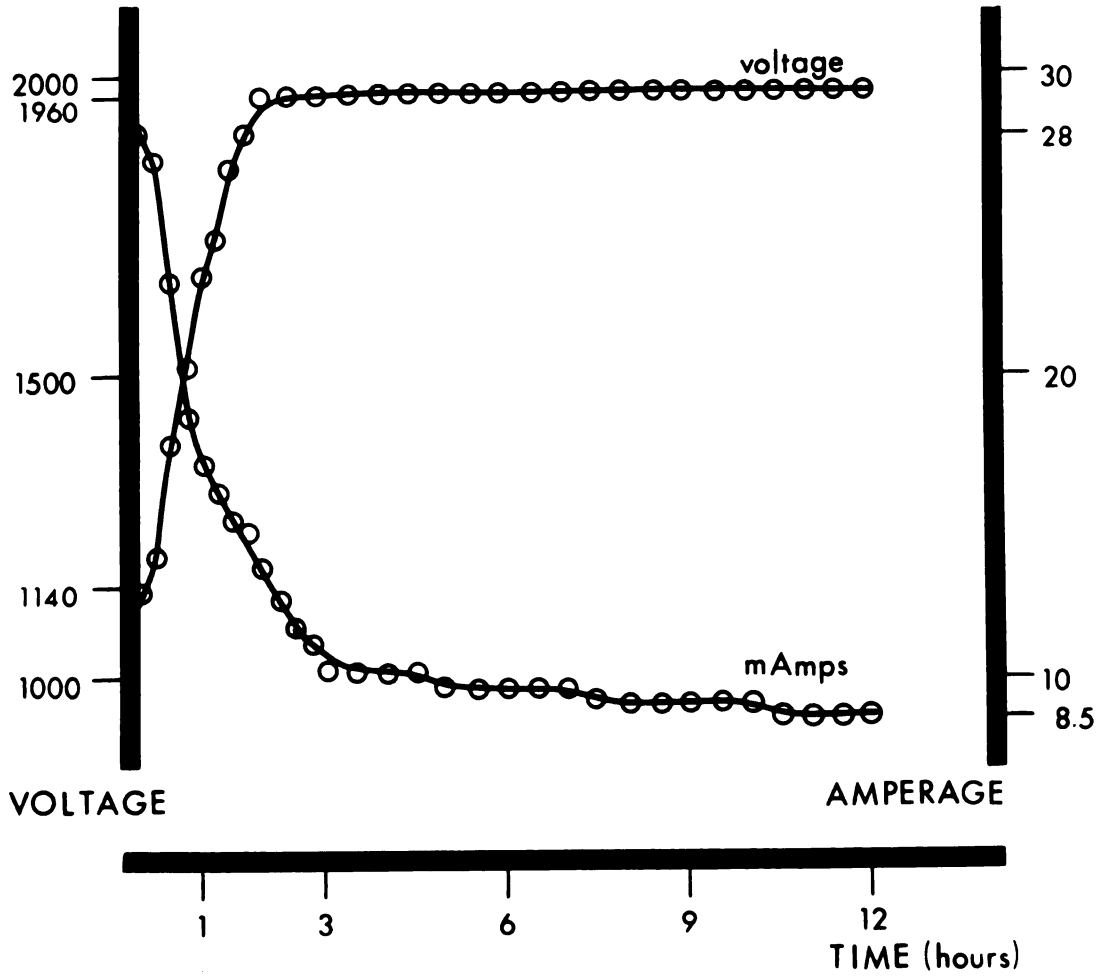

Figure 3. Voltage and amperage during 12 hours of focusing across the length.

The pH of the gel, measured at 1 cm intervals, was measured at two hour intervals, from 2 through 12 hours of focusing. Results are summarized in Figure 4 and Table 4.

Table 4. Least squares analysis of pH gradient formation

Hours	y-intercept	slope	r^2
2	4.68	-0.054	0.7817
4	5.02	-0.068	0.9879
6	5.05	-0.071	0.9749
8	5.03	-0.069	0.9905
10	4.79	-0.057	0.9914
12	4.78	-0.055	0.9953

Least squares and visual analysis of the data indicated the pH gradient to be fully formed across the pH 3.5 to 5.0 gradient at four hours. Also, the linearity, as estimated by the coefficient of determination, slope and the y-intercept, all indicated the gradient to be stable from four through eight hours of focusing. However, at 10 hours the gradient was still linear but the slope and y-intercept indicated the line to be somewhat flattened. The y-intercept changed from about 5.0 to about 4.8. At 12 hours, the gradient was essentially the same as at 10 hours. Since the large molecular weight proteins will move very slowly when close to their pI, to insure maximum focusing we decided to let the focusing continue for 12 hours.

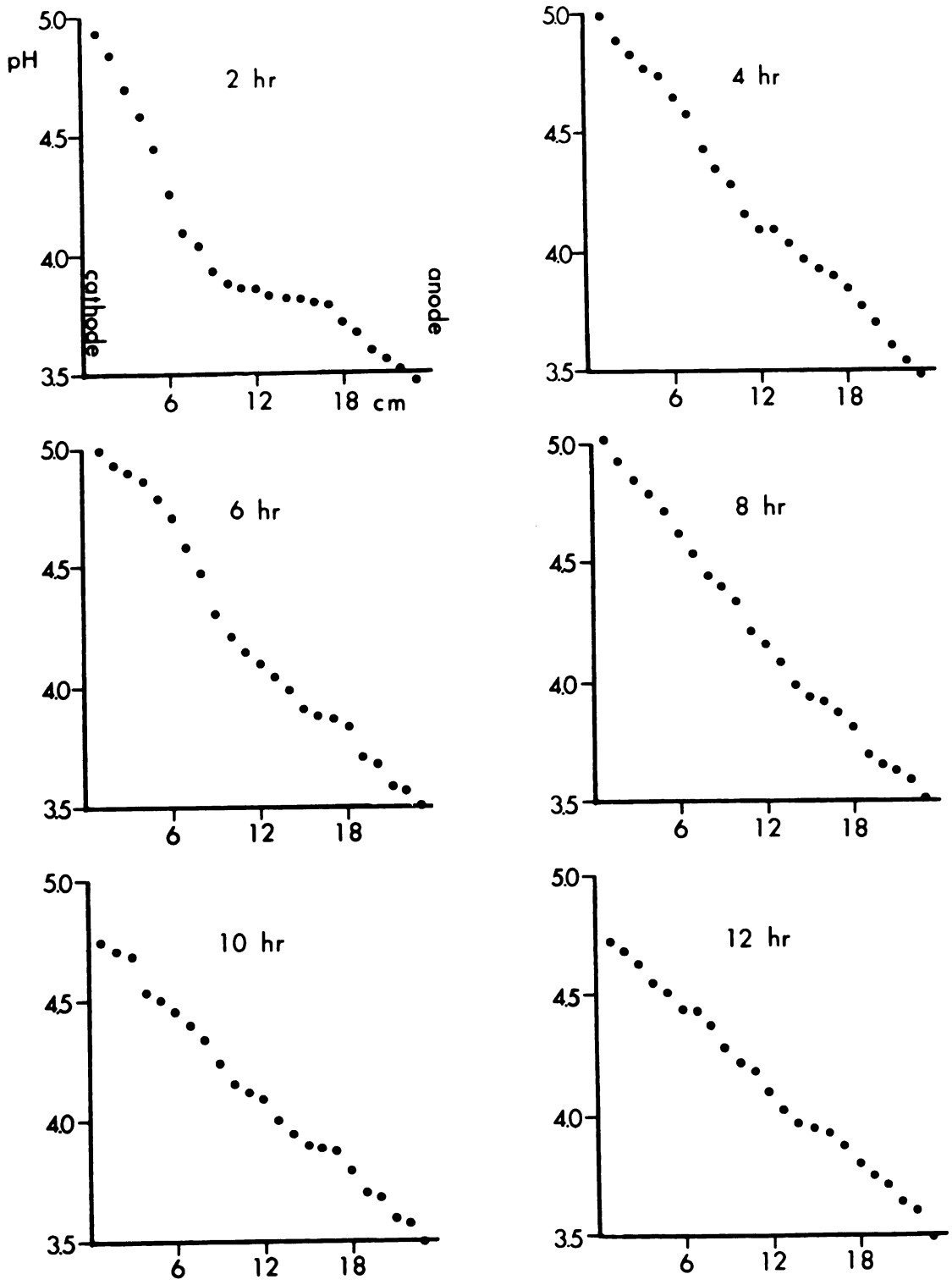

Figure 4. pH gradient formation during 12 hours of focusing across the length.

Crossed Immunoelectrophoresis (CIEP)

Undiluted serum of phenotype Pi-M was focused from the cathode end of the gel (Figure 5A) and from the anode end of the gel (Figure 5B), and each followed by CIEP. The anode was to the left for the first dimension and to the top for the second dimension. The patterns are similar except for the trailing of α_1-AT seen between the pattern and its point of application. This trailing was not obvious with only the one dimensional TLIEF procedure; it was observed only with the sensitive immunoelectrophoresis step.

The Pi-M serum was diluted with 12.0% (w/v) sucrose to give final concentrations of α_1-AT of 0.25, 0.50, 0.75, 1.00, and 1.50 mg/ml. These samples were focused for 12 hours and then cross immunoelectrophoresed (data not presented). Optimal concentrations of α_1-AT by visual observation of the resultant patterns was determined to be 0.5 mg/ml. At this concentration there was no trailing of the protein and yet all known zones were resolved. Height of the major zones M4 and M6 was approximately 15 mm, ideal for quantitation. The staining intensity was adequate. Using a 10 x 10 mm tab size for application of the serum, the tab will absorb approximately 25 µl. The protein load of α_1-AT was then 12.5 µg.

Serum phenotyped as Pi-M was diluted to 0.5 mg/ml of α_1-AT with 12.0% sucrose and CIEP was done. The results are shown in Figure 6. The arrow was used as a point of reference to indicate the mobility of the major zone M6.

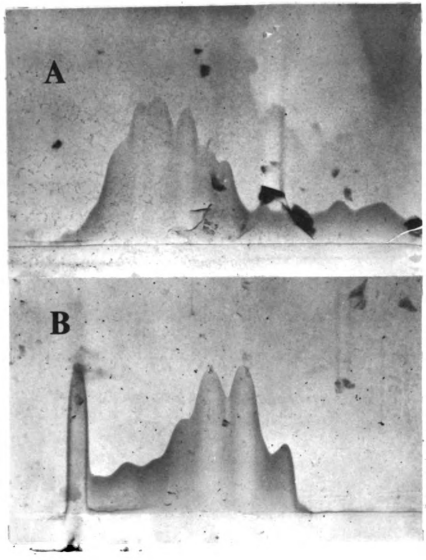

Figure 5. CIEP of undiluted serum phenotyped as Pi-M.

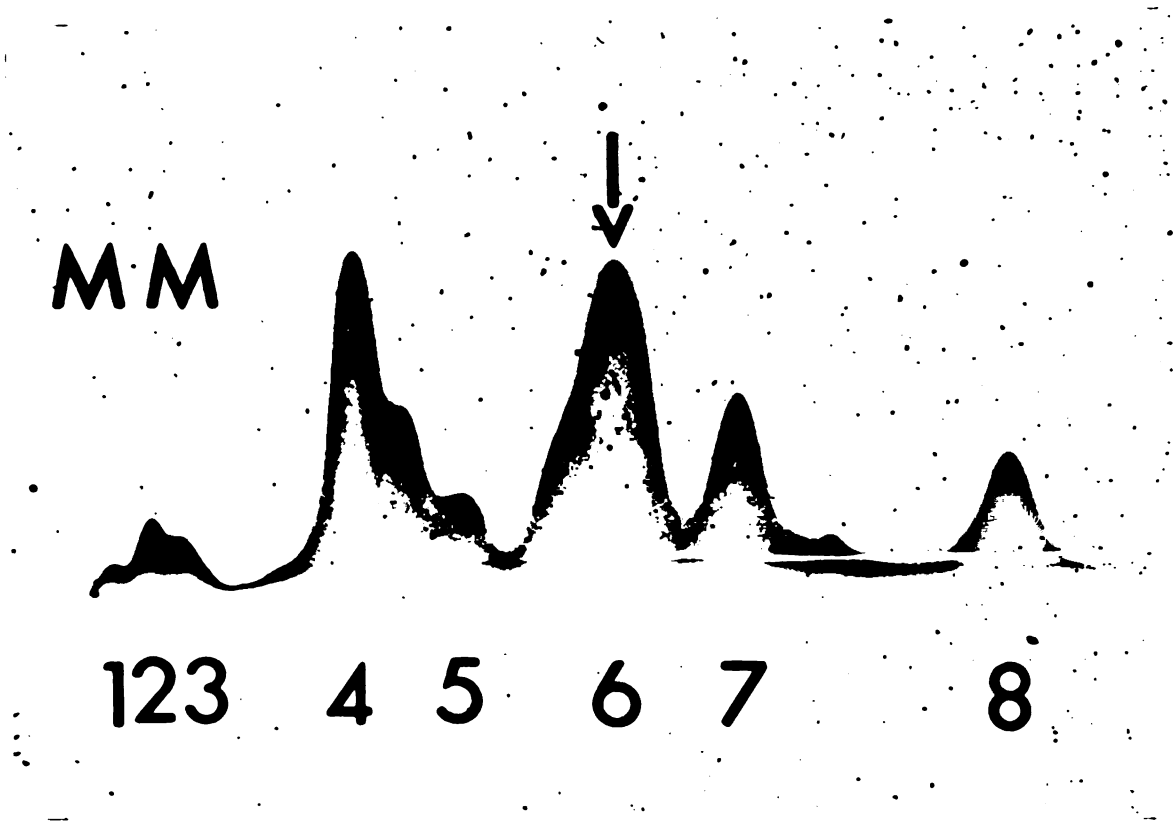

Figure 6. CIEP of serum phenotyped as Pi-M diluted to 0.5 mg/ml.

At this concentration, all eight zones of the Pi-M were seen and there is no longer any trailing of the α_1-AT. However, a new problem was recognized. Zone M4 showed a cathodal asymmetry. Zones M6 and M8 showed an anodal asymmetry. A new "zone" was resolved between M7 and M8.

To overcome these artifacts, the raw serum was first reduced with 2-mercaptoethanol (1.0% w/v) for 20 minutes at room temperature, then adjusted to 0.5 mg/ml with the 12.0% sucrose.

Distribution patterns of α_1-AT were similar to those observed using acid starch gel electrophoresis in the first dimension (Figure 7). The homozygous Pi-M phenotype migrated into a series of eight distinct zones. Using the terminology of Fagerhol,[38] the major contributions were from M4 and M6. Minor zones M1, M2 and M3 were displaced further anodally from M4. Zone M5 was clearly resolved between the two major zones. A new M zone, designated as M6', was consistently noted just cathodal to M6. The most cathodal minor zones M7 and M8 were seen cathodal to this new zone.

Heterozygous phenotypes appeared as one allele product superimposed upon the other (Figure 8). Zones would overlap while others stood out as single distinct zones. In the Pi-MZ (Figure 8A), Z4 and Z5 were just cathodal to M7. Z6 was just cathodal to M8. The most cathodal minor Z zones were barely perceptible and dots were drawn on the photographs over their positions. Zones Z1, Z2 and Z3 were under the area of M5 and M6 and could not be distinguished.

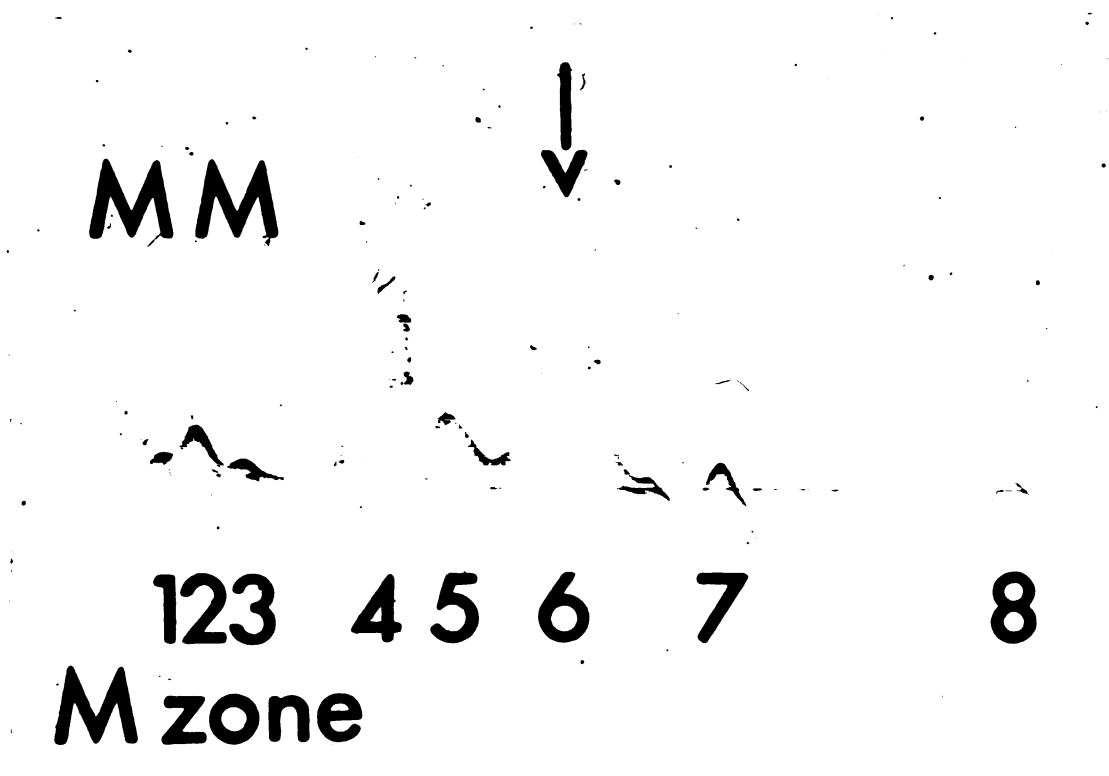

Figure 7. CIEP of a homozygous phenotype Pi-M.

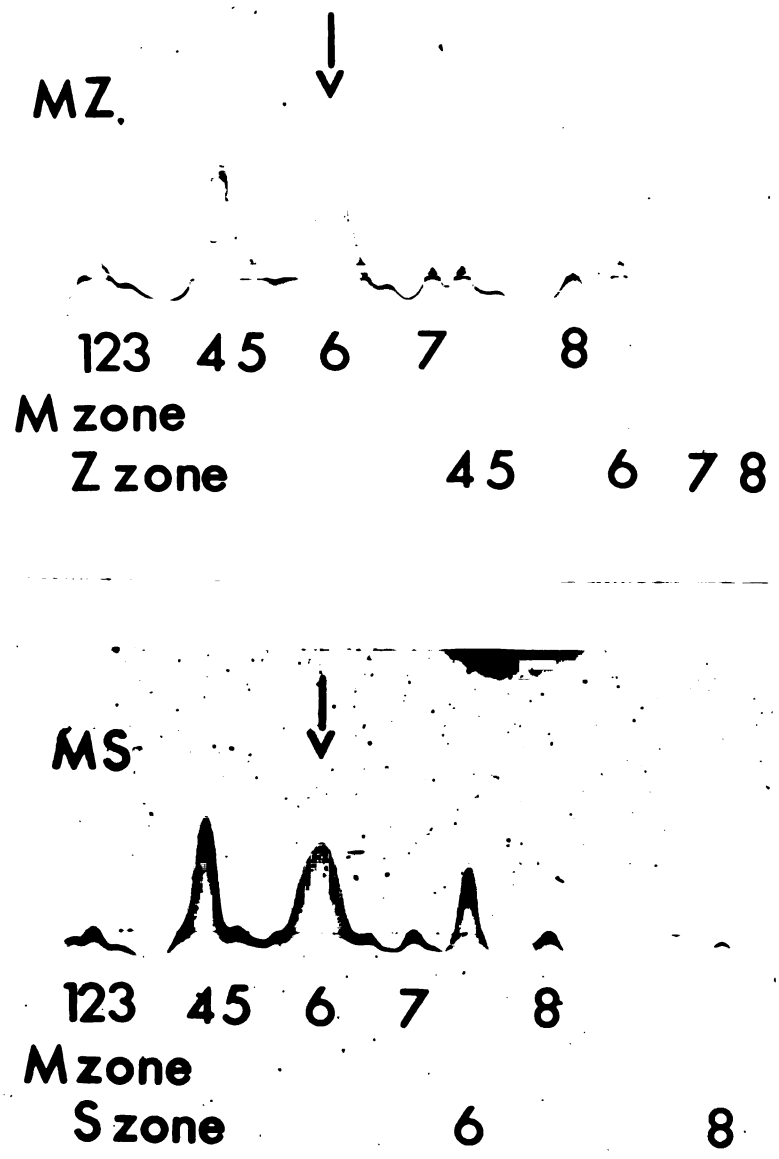

Figure 8. CIEP of heterozygous phenotypes Pi-MZ and Pi-MS.

The Pi-MS phenotype (Figure 8B) had as its main features the major zone S6 between the minor zones M7 and M8. The most cathodal zone S8 was cathodal to M8. The major zone S4 coalesced with the major zone M6.

The Pi-Z phenotype was characterized by its low serum concentration and cathodal mobility. The Pi-Z had two distinct patterns with CIEP. The acute form (Figure 9A) was distinguished from the chronic form (Figure 9B) by the size of the zone Z2. This zone in the acute form was larger than the chronic form. The major zone Z6 did not have the new zone just cathodal to its position. Also, because of the low concentration, the zone Z8 was not observed.

The percentage distributions of α_1-AT among the different Pi zones in the homozygous phenotypes Pi-M and Pi-Z were given in Table 5. In the Pi-M phenotype the

Table 5. Distribution of α_1-AT among zones in different homozygous Pi phenotypes

Phenotype	No.	1 %	2 %	3 %	4 %	5 %	6 %	6' %	7 %	8 %
Pi-M	3	1.5	5.5	2.0	34.4	6.7	39.9	2.2	4.3	3.5
Pi-Z chronic	2	4.4	4.0	1.6	47.0	0.7	38.6	---	3.7	---
Pi-Z acute	1	1.4	19.3	0.5	41.8	0.4	34.6	---	2.0	---

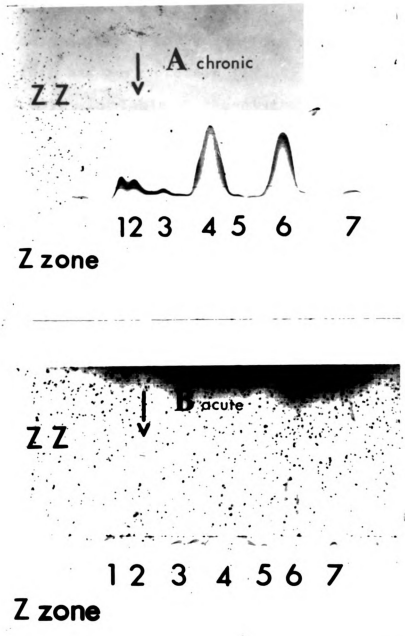

Figure 9. CIEP of acute and chronic forms of the Pi-Z phenotype.

major contribution was from zones M4 and M6. These two zones accounted for 74.3% of the protein. The distribution of the Pi-Z phenotype was similar in that the major zones Z4 and Z6 accounted for the majority of the protein. However, the difference in the amount of protein between Z2 in the chronic form and the acute form was significant. The acute form had more protein (19.3%) than did the chronic (4.0%). In Table 6 the distribution of a Pi-M seen with acid starch gel as the first dimension[38] was compared with TLIEF at pH 3.5-5.0 as the first dimension. Comparison of each separate zone could not be done because of the limited data. However, the overall mean difference, as shown by the Student's t-test, can be thought of as zero, indicating the overall distribution to be the same between the two techniques.

Table 6. Comparison of the Pi-M distribution between acid starch gel and TLIEF

Zone	starch gel %	starch gel SD	TLIEF %	TLIEF SD	d	SD
1	1.0	0.2	1.5	0.6	-0.5	0.4
2	9.6	2.3	5.5	0.6	4.1	1.4
3	5.2	0.6	2.0	1.0	3.2	0.7
4	42.7	1.5	34.4	3.0	8.3	1.9
5	3.4	0.4	6.7	4.1	-3.3	2.4
6	35.4	2.4	39.9	1.0	-4.5	1.5
6'	---	---	2.2	1.4	---	---
7	1.6	0.6	4.3	1.4	-2.7	0.9
8	1.6	0.6	3.5	0.6	-1.9	0.5

$\bar{x}_d = 0.34$

$\bar{s}_d = 1.6$

$t = 0.2145$

DISCUSSION

Electrophoretic mobility has long been used to separate and characterize proteins. The resolving power of conventional electrophoresis is limited because mobility is also a function of composition, pH, and ionic strength of the medium. TLIEF represents a major advance in the science of electrophoretic separation of proteins and other amphoteric substances. The technique is an equilibrium electrophoretic method for segregating amphoteric molecules according to their isoelectric points (pI) in stable pH gradients. Isoelectrofocusing (IEF) has been refined to the point where one can theoretically detect components whose pI differ by as little as 0.01 pH units. The technique is thus the method of choice for examining proteins with charge microheterogeneity such as α_1-AT. The method also can be used as a rigorous test of homogeneity. Because of the separation ability, the technique also lends itself to preparative procedures. Finally, IEF can be used to define the pI of unknown proteins.

In IEF a stable pH gradient, increasing from anode to cathode, is established by electrolysis of carrier ampholytes in an anti-convective medium. In the absence of an electrical field, the gel bed will have a pH equal to the average

value of all the carrier ampholytes. In the case of pH 3.5-5.0, the average pH will be approximately 4.25. Ampholytes with a pI above 4.25 will have a net positive charge; those below 4.25 will have a net negative charge. With the application of current these amphoteric molecules will migrate according to their surface charge until their net charge is zero, where each will stop and accumulate. The ampholytes, because of their high buffering capacity, will impart to the immediate environment a pH equal to their particular pI. Alpha-1-antitrypsin, with a pI range of about 4.2-4.7, will have a net negative charge when applied at the cathodal end. Hence, it will migrate toward the anode into a region of lower pH. As it does, the protein will lose negative charge and gain positive charge by protonation of its carboxyl and amino functions. Migration will then stop when each of the α_1-AT zones reach their respective pI. Should the protein diffuse from its pI, it will develop a net charge and be repelled back to its pI. Thus, the α_1-AT or any other amphoteric molecule reaches an equilibrium position where it is concentrated into an extremely sharp band.

To understand why acid starch gel electrophoresis and TLIEF at acid pH produce similar distributions, an understanding of the electrochemistry of separation is needed. The acid starch gel uses a discontinuous buffer with an initial gel buffer at pH 4.95 and cathode buffer at pH 6.3. The running pH will be determined by the Kohlraush regulating function, which by direct measurement is pH 7.5.[4] The net

effect is to reduce the net charge of the albumin to below that of the α_1-AT so that the α_1-AT will migrate anodally to the albumin in the sieving starch medium. The α_1-AT has a lower isoelectric point than the other α_1-components, which will focus cathodal to albumin.

We wanted a robust gel that would withstand mechanical transfer and have the smallest median pore radius. Since focusing involves migration of the protein on the surface of the gel and molecular sieving would not play a part in the separation, the smallest theoretical pore radius was deemed ideal. Total acrylamide (T) was adjusted to 5%, and the bis-acrylamide for crosslinking (C) was adjusted to 5%. At 5%, the gel would have the smallest pore radius.[43] The gel was cast onto the plastic film (3M type 383) to facilitate the transfer process.

The critical value in determining the electrical load is the potential voltage drop per cm. Thus, higher potential differences may be tolerated when focusing over a longer gel path. However, with too large a voltage, deleterious effects of Joule heating will result in jagged zones or refractive borders. The average Joule heat generated per unit of gel, expressed as cal/sec/cm^3, was shown in Figure 2A. This was a typical result of an electrofocusing experiment across the width of the gel. The maximum allowable voltage was set at 80 volts/cm. The Joule heating started out higher when focusing along the length but quickly dropped to levels below that observed when focusing across the width. Because the Joule heating dropped to below that

seen when focusing across the width, the application of 1960 volts should not prove detrimental to the resultant protein patterns. The gel was kept at 4 C with a closed system circulating regulator at 10 l/min.

As the ampholytes migrate to their respective pI's, the resistance continually increases and the conductivity continually decreases. With full establishment of the pH gradient, the minimum conductivity is obtained. The current in mA has its maximum value at the beginning because of the even distribution of ampholytes in the gel. Typically, the amperage drops precipitously during the initial stages of the experiment; and when the amperage has reached its minimum value, this indicates that the pH gradient has formed. In general, one would use constant voltage on this type of system. However, with TLIEF the power would drop and the separation process would slow down, impairing the resolution due to the increased diffusion. Constant power, then, is the method of choice for TLIEF. Figure 3 showed the relationship between the amperage and voltage during a typical TLIEF run. Within the limits of the gel, the power will stay constant.

Table 2 showed the pathlength covered by the α_1-AT versus time. With increased time of focusing, the pathlength is increased. These data seem at first to be incompatible with the concept of IEF being a "steady state" method. Two phenomena are associated with IEF over extended periods of time: (1) plateau phenomenon and (2) cathodic drift.[140] The plateau phenomenon is of little consequence

to this result because this is associated with pH ranges over the range of neutrality and is best explained by movement of "amphoteric" water into the neutral region. What is happening can best be explained by the cathodic drift phenomenon.

The cathodic drift has not been fully explained. However, Baumann and Chambach[13] have concluded the major force to be electrophoresis of ampholytes resulting in ampholyte depletion from the gel. The movement takes place "en bloc", especially in the direction of the cathode, probably due to chemical interaction among the ampholyte species. They could not rule out chemical modification of the ampholytes by reaction with acid or base electrode solutions. Also, using ^3H_2O, they determined that EEO could play a major role in the unidirectional drift of the ampholytes.

The resolving power of the technique can be defined as the change in pI with which two proteins can be separated. Righetti and Drysdale[141] derived the following expression for the resolving power:

$$\Delta pI = 3.07\left[\frac{D\ [d(pH)/dx]}{-E\ [dU/d(pH)]}\right]^{1/2}$$

where D = diffusion constant, dU/d(pH) = mobility, d(pH)/dx = slope of pH gradient, and E = field strength. The diffusion constant and mobility can be treated as constants. Then, in order to obtain the best resolving power, the product D[d(pH)/dx] should be as low as possible and E[dU/d(pH)] as

high as possible. By focusing for 12 hours with the maximum voltage of 1960 volts, exploitation of cathodic drift flattens the pH gradient at a high field strength. Thus, we have maximized the resolving power with the commercially available ampholines of pH 3.5-5.0.

Figure 5 demonstrated the necessity of diluting the serum. Adjusting the α_1-AT to 0.5 mg/ml was determined to be the optimum antigen concentration. There was no trailing of the α_1-AT and all the zones were seen with complete resolution intact. Twelve percent sucrose was chosen to dilute the serum because this is the final concentration in the gel itself. Also, at the low pH sucrose will not ionize. Ionization would result in migration and accumulation of positive ions toward the cathode. This would cause a further shortening of the pH gradient at this more basic end.

Antibody concentration in the agarose was determined by the following criteria: height of precipitin peaks, intensity of staining, and the ability to wash out unprecipitated protein.

Figure 6 illustrated another problem. In addition to the eight zones, artifacts have also been introduced. M4 shows a cathodal hump; M6 and M8 both show an anodal hump. A small zone is resolved between M7 and M8. Pierce et al.[136] noted extra cathodal zones of some of their serum after TLIEF and attributed the problem to reactive thiol groups on the protein. Mild reduction accomplished by incubation with 1% mercaptoethanol eliminated the problem.

The technique described herein is important in the study of α_1-AT, a deficiency of which is associated with many pathological states. The majority of phenotypes can be resolved with TLIEF alone. With the approximately 25 alleles, 40 known phenotypes and Pi-M subtypes described thus far, the possibility of new phenotypic expressions on TLIEF is most likely. The distribution with TLIEF in the first dimension, as illustrated in Table 6, is similar to that seen with acid starch gel in the first dimension. Previous attempts at crossed immunoelectrophoresis from polyacrylamide have not, in our estimation, preserved the resolution offered by the first dimension. In none of the reports have all eight zones of a homozygous phenotype been presented. In addition, an immunofixation method has not resolved all eight zones.[8] The plethora of confusing literature on the Pi-M subtypes indicates that the controversy over phenotypes resolved by TLIEF alone is not settled. Preliminary observations from only two samples (data not presented) suggest that the subtype phenotypic differences from the Pi-M allele may be of a quantitative nature. With our system the Pi-M1M2 subtypes show quantitative differences between zones M4 and M5. Also, quantitative differences are noted between M6 and the new zone just cathodal to zone 6. Further investigations with this sensitive two dimensional technique are planned in an attempt to clarify such phenotyping problems.

Allan and Spicer[5] observed chronic and acute forms of the Pi-Z phenotype. The acute form was seen in association

with juvenile hepatic cirrhosis and noted in individuals under 18 years of age. The pattern noted showed Z2 more intensely stained with TLIEF. This darker staining zone was not seen with the chronic form. With the two dimensional procedure, we were able to quantitate the difference in the zone Z2 between these two forms. It is interesting to note that, if these individuals survive the juvenile hepatic cirrhosis, their serum pattern will change to that seen as the chronic form.

The next phase of characterization of α_1-AT will involve the purification with the microheterogeneity of the protein intact. In studying the kinetics, physicochemical and immunologic properties of the protein, there should be a sensitive method to demonstrate that the purified protein is the same as is present in serum with respect to all the zones of α_1-AT. Thin layer isoelectrofocusing alone does not satisfy this criterion since only the major zones are well characterized for phenotyping purposes. Schemes have been presented where the different zones have been separated.[123,128] The crossed immunoelectrophoresis will provide a sensitive technique to determine the homogeneity of the preparation with respect to each zone.

Separately, polyacrylamide and agarose provide ideal matrices for protein separation and immunoprecipitation. Polyacrylamide, because of the virtual absence of charge groups and the option of varying the median pore radius, is the ideal matrix to focus proteins of different isoelectric points using TLIEF. Agarose is the matrix of

choice for immunoprecipitation due to its large pore size and strong mechanical properties at low concentration. Commercially available agarose, because of the associated anionic residues, has significant EEO while polyacrylamide has virtually none.

The tendency of ionically charged molecules making up a gel medium to shift when a voltage is applied across the gel is called electroendosmosis (EEO). Since the gel matrix is physically unable to move, it is the solution within the matrix that actually flows. In agarose, anionic residues of sulfate and/or pyruvate are affixed to the matrix and thus restrained from moving in the electrical field. However, the dissociable cations and their associated water molecules are able to move toward the cathode. The net result is a movement of water toward the cathode.

If two gels, one with substantial cathodal EEO and the other with virtually none, are placed adjacent to each other, contact is made, and an electrical field is applied. One of two phenomena can occur:

1. If the gel with the substantial EEO is placed anodal to the other, a buffer flow will emerge between the gels allowing the protein to flow unevenly, resulting in distortion upon immunoprecipitation.
2. If the gel with the substantial EEO is placed cathodally to the other, it will start to dry on its anodal side, which will lead to a

discontinuation of the electrical current through the gels.

If a polyacrylamide gel strip is inserted into a groove in an agarose gel with EEO, both of the above-mentioned phenomena will occur. A buffer flow will emerge on the anodal side of the strip and the cathodal side of the strip will dry, breaking the electrical current. This is the reason why it has been extremely difficult to obtain precipitin patterns when molding a polyacrylamide strip into agarose which are distinct and reproducible on crossed immunoelectrophoresis.

It became clear that because of the EEO problems, molding in of the polyacrylamide would not work. Since the proteins separated with the TLIEF technique are focused on the surface of the gel, it seemed reasonable that a laying on method could alleviate all of the problems with EEO. This technique is offered as a useful improvement on the method of Soderholm and Smyth[147] as a means of identifying and studying microheterogeneity of many other proteins.

SUMMARY AND CONCLUSIONS

A method has been presented that successfully preserves the resolution offered by TLIEF in polyacrylamide through a crossed immunoelectrophoresis step in agarose. The method involved focusing of α_1-AT across the length of a 24.0 cm gel and exploitation of cathodic drift to get maximal resolution. A laying on technique was then used for the second dimension to circumvent the problems of electroendosmosis between the two media.

The distribution of zones was similar to that seen with acid starch gel electrophoresis. However, with the increased resolution offered by TLIEF, the method should prove useful in elucidating new phenotypic expressions as they are found. The precise role of the various phenotypes with disease has not been elucidated. The method should prove helpful in that role.

The next phase of characterization of α_1-AT will involve the purification with the microheterogeneity of the protein intact. In studying the kinetics, physicochemical and immunologic properties of the α_1-AT, this technique should be useful in demonstrating that the purified product is the same as present in serum.

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