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Proteins of Human Urine. I. Concentration and Analysis by Two-Dimensional Electrophoresis

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We briefly review the origins of urinary proteins, as these are now understood, and present a scheme for their separations and evaluation, the "ISO-DALT" system of two-dimensional electrophoresis. Some results are illustrated and discussed.

Additional Keyphrases: *ISO-DALT system · data-reduction system · urine analysis as a diagnostic aid · Molecular Anatomy program · origins of proteins in urine · spot identification, quantitation*

Human urine contains many different proteins, all in trace amounts, and evaluation of some of them is diagnostically useful. Two-dimensional electrophoretic methods have been developed for resolving complex mixtures (*1–3*); thus it has become of interest to examine in detail the urinary proteins that normally are present, and those that vary, appear, or disappear in disease.

The development of methods for routinely concentrating urinary proteins has been previously described (*4*), and further developments are recorded in this series. In preliminary studies, several hundred urinary proteins have been resolved (*5*), but only a few identified.

This initial paper is intended to serve several functions. First, the diversity of urinary proteins is discussed, to indicate why their detailed analysis is of interest and importance in clinical chemistry. Second, the method of two-dimensional electrophoresis of urinary proteins is described in sufficient detail to show why it offers an improvement in resolution by one or more orders of magnitude over previous methods. Third, we present results of studies done during development of the system, to illustrate both reproducibility of the results obtainable and a few of the variations observed between samples from different individuals. For maximal usefulness, it is important to identify as many as possible of the spots that appear in a pattern. The final section of the paper is therefore concerned with techniques to be used in spot identification, quantitation, and data reduction. This work lays the foundation for subsequent papers, which will be concerned with the identification and diagnostic importance of individual spots.

Types of Proteins Found in Urine

We have distinguished eight classes of proteins in human urine (*4*), which are reviewed here.

1. Normal plasma proteins, or fragments of them: More than 31 of the proteins appearing in plasma have also been identified in human urine (*6*). The chief interest in their study relates to the detection and differentiation of renal and tubular disease (*7–9*). Probably all of the proteins present in normal plasma will be found in normal urine if sufficiently sensitive methods are used. Of those proteins in urine that have a relative molecular mass (M_r) of less than about 40 000, it is of interest to distinguish those that are excreted unchanged from those that are either modified plasma proteins or are split products of them. In normal plasma, only trace amounts of proteins with M_r of less than 40 000 are present. These pass through the glomerulus and are then largely reabsorbed, and possibly catabolized, in the renal tubules (*10*). In renal tubular disease, the concentration of these trace proteins in urine is greatly increased (*8*). Among them have been identified retinol binding protein (*11–14*), α_1-microglobulin (*15–18*), β_2-microglobulin (*19–21*), fibrinogen split products (*22*), fragments of factor B (*23*), a protein co-isolated with β_2-microglobulin (*24*), and IgG light chains (*25*).

2. Proteins released into the urine from the kidney itself: Of the proteins of renal origin that are normally found in urine, some appear to be unique to the kidney, others are in common with other organs. The Tamm–Horsfall protein (or uromucoid) (*26*) originates in the kidney tubules and is composed of monomers with an M_r of approximately 79 000, assembled into aggregates with masses ranging from 7 to 28 million daltons or greater (*27*). It is the predominant protein of urinary casts (*28*) and forms a part of the organic matrix of kidney stones (*29, 30*). An increase in the excretion rate of this protein is an early sign of kidney rejection (*31*). Normal urine also contains basement-membrane antigens of epithelial and mesenchymal cells of all organs (*33*). The kidney alkaline phosphatase (EC 3.1.3.1) excreted into the urine differs from prostatic alkaline phosphatase, but is antigenically similar to intestinal alkaline phosphatase (*34*). Gamma-glutamyltransferase (EC 2.3.2.2) exhibits greater activity in the kidney than in other tissues (*35*), and its activity in urine is two- to fourfold that of serum (*36*), suggesting that the enzyme leaks from the kidney into the urine, where it may be important in amino acid reabsorption (*37*). The origin of this and other enzymes in urine is difficult to determine definitively. For example, lysosomal enzymes are always excreted in the same ratio in normal human urine, largely by epithelial cells of the proximal tubules (*38*). However, in glomerulonephritis they may also be released by polymorphonuclear leukocytes (*39*). Additional proteins of kidney origin are urokallikrein (*40, 41*) and erythropoietin (*42, 43*). Most of the 30 enzymes that have been detected in urine probably originate in the kidney (*44–46*).

3. Proteins reaching the urine after its formation in the kidney may arise from the surface epithelium of the urogenital tract, from the male accessory glands, and from vaginal secretions that may contaminate urine. As a result of prostatic secretion, the amount of acid phosphatase (EC 3.1.3.2) in urine from men is twice that in urine from women (*47*), but its presence in urine from women shows that it is not solely of prostatic origin. Twelve nonplasma antigens derived from the ureter, bladder, urethra, and prostate have been detected in

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rat urine (*48*). Evidently necrosis in any part of the urogenital system (including the kidney) will result in leakage of tissue proteins, including enzymes, into the urine (*44–46*).

4. Proteins leaked from tissues (either normally or as a result of disease) outside the urogenital tract: Ultracentrifugal analyses of undenatured soluble proteins from tissues (*49*) show that a large proportion of them are small enough to pass through the glomerulus and hence might be expected to appear in urine. In response to a wide variety of types of injury, cells may become more permeable and may shed or leak proteins (*50*). From the viewpoint of urinary protein classification, it is not always clear which proteins should be considered "plasma proteins" (i.e., have some necessary function in plasma) and which are adventitious tissue proteins whose absence from plasma is without effect. The tissue proteins of greatest interest are those that ordinarily are undetectable or are normally present only in trace amounts in plasma or urine, but which increase markedly as a result of disease or injury. However, the initial problem is to identify and characterize tissue proteins normally present. Halbert and associates have described 10 antigens in normal human urine, some of which were common to the submaxillary gland, pancreas, heart, liver, and kidneys (*51, 52*). How these proteins reached the urine is not yet clear, and studies on the clearance of labeled tissue proteins would be most instructive.

In experimental animals, most of the proteins of low molecular mass appear to be of other than plasma origin (*53*), and presumably offer many interesting examples of histuria (i.e., of tissue proteins in urine) in response to experimental manipulation, which offers leads for human studies. These examples include excretion of a collagen derivative in normal rat urine (*54*) and liver-specific antigens appearing in urine during hepatic necrosis produced by CCl_4, thioacetamide, urethane, sodium tannate, beryllium, or cadmium (*55, 56*). Even very slight tissue injury appears to produce measurable histuria. Thus, anethesia of the rat with either pentobarbital or diethyl ether causes excretion of liver-specific antigens for several days (*57*), suggesting, as stressed by Boss et al. (*50*), that injury can be detected by the presence of these antigens in urine even before injury is histologically evident. After x-irradiation in rats, a mucoproteinuria is observed that is indicative of injury to the ground substance of connective tissue (*58*). In both toxic and ischemic testicular necrosis, specific testicular antigens appear in the urine that are not normally detectable by immunodiffusion methods (*55*). Myoglobinuria in man may be produced by damage to either the myocardium or to skeletal muscle (*59*); beta-trace protein reportedly increases in urine after injury to brain tissue (*60*). Many additional examples of tissue proteins in human urine have been described (*50*). Blood group A and B substances (*61, 62*) and transplantation antigens (*63*) have been reported as constituents of normal urine, and a protein containing fucose has been reported to be excreted in schizophrenia (*64*).

5. Hormones or other signal substances: Human choriogonadotropin was first isolated from pregnancy urine (*65*), and serves as a prototype for hormone-isolation studies. All of the known pituitary protein hormones appear to have been detected in urine (*66*), although questions have recently been raised concerning prolactin (*67*). The large amount of proteinaceous material of medium and low molecular mass that is present in urine very probably includes many active principles that remain to be described.

6. Substances released by the products of conception: Five major antigens have been described in human placental tissue (*68*), three of which appear in both the serum and urine of pregnant women (*69*). Whether the numerous phase-specific autoantigens of the fetus (*70*) reach the mother's urine during normal pregnancy remains to be determined.

7. Tumor-associated substances: Several antigens of possible clinical interest have been described in the urine of cancer patients, in addition to placental antigens (*71*) and alpha-fetoprotein (*72*). These include carcinoembryonic antigen (*73*), an antigen that apparently is specific for bladder cancer (*74*), and a melanoma-specific protein (*75, 76*). A cationic leukocyte antigen, and lysozyme, have been reported in the urine of patients with chronic myelocytic leukemia (*77*). The most systematic approach to the problem of indicators in cancer patients' urine has been by Rudman and associates, who described a series of candidate indicators (*78–86*).

8. Products of bacterial or viral infection: Bacteria and intact infectious virions in urine have been widely studied. However, little attention has been paid to the possibility of detecting in urine so-called "early proteins" appearing in cells after viral infection, capsid antigens synthesized in excess, or bacterial or mycotic antigens resulting either from systemic or urogenital tract infections. It is of interest that virally infected cells have been reported to synthesize some virally coded proteins of quite low molecular mass (*87*), which would be expected to appear in urine as a result of infection.

Strategy For Analysis

The number of different proteins that may occur in urine appears from this brief review to be quite large, but the total mass of protein present is normally quite small relative to the volume of urine. The amount of protein excreted per day by normal adults is usually given as <150 mg per 24 h (*88, 89*), and depends greatly on the analytical method used. When the non-dialyzable components are concentrated and the total nitrogen determined and corrected for hexosamine and sialic acid nitrogen content, a better figure of 200 mg per 24 h is obtained (*90, 91*). Any strategy for resolving urinary proteins must therefore be concerned initially with urinary protein concentration and with the possibility of accompanying protein degradation and selective losses. Once satisfactory concentration methods are available, fractionation may be undertaken by classical methods aimed at isolation of one protein after another without a clear picture of how many are actually present. The methods used may be based on physical properties or on antigenicity. We have previously described general methods for fractionation based on the sequential preparation of immunoabsorptive columns to remove first major and then successively more minor antigens from complex mixtures, by rapid recycling affinity chromatography (*92, 93*). An alternative and preferable approach is to emphasize very-high-resolution analytical methods, which quite literally "see" most of the components present. The resolution sought should, in theory, allow a thousand or more proteins or protein subunits to be resolved, while the sensitivity should ultimately match that available with radioimmunoassays. If such resolution can be achieved and a very large number of different proteins seen, then variations related to disease may be studied, identifications made, and the entities of greatest interest isolated.

High-Resolution Two-Dimensional Electrophoresis

Currently, the two analytical techniques with the highest resolution for proteins are isoelectric focusing (IEF) in the presence of urea and electrophoresis in sodium dodecyl sulfate (SDS), both performed in microporous acrylamide gels. The two separations depend on characteristics that are independent of each other. Isoelectric focusing in high concentrations of urea, neutral detergents, and reducing agents reflects the ratio of acidic and basic groups on largely unfolded single peptide chains, which in turn reflects the amino acid composition (*1*). SDS electrophoresis, for quite interesting reasons, separates on the basis of chain length or molecular mass. When SDS coats a peptide chain, it gives the chain a strong negative charge (*94*), and to the extent that this charge is

uniformly distributed, the charge is proportional to length (or mass). In free electrophoresis all particles having the same ratio of surface charge to mass have the same mobility regardless of size; therefore, all proteins should move at the same (or nearly the same) rate. However, in a microporous acrylamide gel, large particles are physically entangled and retarded more than small ones, so that the separation is proportional to molecular mass and, to a much smaller degree, dependent on shape. The separations are highly reproducibile but are subject to a number of artifacts. For example, the carbohydrate in glycoprotein contributes additional mass but does not bind SDS and may cause physical retardation leading to an overestimation of the true mass (protein + carbohydrate). The separations observed with proteins of low molecular mass also tend to be anomalous (*95, 96*).

Both IEF and SDS electrophoresis can resolve approximately 100 proteins in practice, and probably 200 or more with additional development. Either of these methods alone is not sufficient. Even if one-dimensional electrophoresis could resolve all of the proteins of urine, it would not solve our problem, which is to determine the tissue from which each protein originated. This requires that we be able to resolve tissue protein mixtures that are more complex. To identify the origin of a protein in urine (e.g., as being from brain), it is necessary to resolve proteins from the tissue in question sufficiently to demonstrate the presence or absence of the particular protein. The human genome is thought to code for 30 000 to 50 000 proteins or protein subunits (i.e., protein gene products), and 10% or fewer of these appear in any one cell type. Obviously, analytical methods that will resolve 100–200 proteins are insufficient for this task. If, however, IEF and SDS electrophoresis are combined in a two-dimensional analysis (*1–3*), then the resolution becomes, both in theory and in practice, the product of the separate resolutions—i.e., 10 000 for present systems, with approximately 40 000 thought possible. This stunning fact opens up for the first time the possibility of elucidating both a molecular anatomy and a molecular pathology of man.

Two-dimensional electrophoresis was first proposed by Smithies and Poulik in 1956 (*97*); they used different pH's in the two dimensions. Stegeman (*98*) first adapted IEF and SDS electrophoresis for two-dimensional use in 1970. The present very high resolution is almost entirely owing to the work of O'Farrell (*1*). Our goal has been to develop the method into a clinically useful system with standards for both molecular mass and charge (isoelectric point), to develop general methods for spot identifications (enzyme activity, etc.), to solve the difficult problems associated with data reduction and data management, and to begin to write a molecular anatomy of body fluids, tissues, cells, and subcellular particulates in a rational manner. In this paper, we are interested in the proteins of urine, an easily obtainable sample not requiring invasive methods. This work, however, is also the point of entry into more difficult problems of tissue and cell analysis. [Note that we have previously prepared maps of human plasma (*99*), erythrocyte lysate (*100*), and muscle (*101*) proteins.]

Urine Concentration

To evaluate concentration methods, it is desirable to know how much protein is initially present. Various methods have been devised for determining protein concentration in urine, all of which have characteristic problems. These stem from the very low concentration of protein normally present, from the presence of substances that interfere with sensitive tests such as the Folin reaction, and from the apparently continuous molecular weight spectrum of the proteins present. This spectrum extends from aggregates of high molecular mass such as the Tamm–Horsfall protein, through serum proteins, to a variety of low-molecular-mass proteins and small peptides, and on in an apparently uninterrupted spectrum to tri- and dipeptides and amino acids (*102, 103*). Different concentration methods cut this spectrum at different places. For example, methods involving precipitation of proteins would only be sensitive to proteins precipitated by the methods used, and these may not include all very small proteins, or in some cases even uromucoid. The two-dimensional electrophoresis techniques used here map proteins ranging in molecular mass from approximately 200 000 daltons to about 10 000. The amount of protein in that molecular-mass range in urine may be determined at the outset by dialysis and lyophilization so that losses during preparation can be evaluated (*90*). Unfortunately, no method for routinely and rapidly making this analysis on unconcentrated samples is currently available.

Protein determination on concentrated urine gives different results when different methods are used, for example, when Coomassie Blue binding or the biuret or Folin methods are compared. A large proportion of urinary proteins are glycoproteins, and these methods are not sensitive to carbohydrate. Our objective is the development of an analytical system for quantitating large numbers of different proteins, and only when it is completed will it be possible to evaluate losses during preparation in terms of each specific protein. We have adopted the strategy of developing one or two concentration methods that allow a large number of proteins to be seen. Variations in concentration methods can then be evaluated once the entire system is functional. We have developed two methods for urine protein concentration, which give essentially similar results (*4*), and the simpler of these is used here. It involves preliminary dialysis, lyophilization, and gel filtration over P-4 Biogel (Bio-Rad Labs., Richmond, CA 94804), followed by lyophilization of the excluded volume peak in a tared flask to allow the weight of the recovered protein to be determined. The protein is then dissolved in a mixture of 8 mol/L urea, NP-40 nonionic detergent, and mercaptoethanol as previously described (*1–3*). We used 10- to 20-μL samples containing 1 to 2 mg of protein per analysis. This procedure removes most of the Tamm–Horsfall protein, which adheres to gel-filtration materials (*27*); globulins not soluble in distilled water would also tend to be lost.

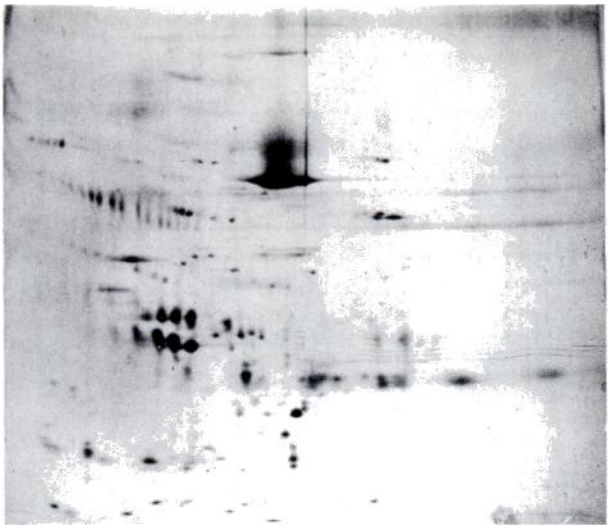
The ISO-DALT System

Because one objective of this initial paper is to demonstrate the usefulness of two-dimensional mapping of human urinary proteins, presentation of many of the technical details is delayed for inclusion in subsequent papers. Because the separation depends on *Iso*electric focusing and on molecular mass estimation (expressed in *Dalt*ons), the name "ISO-DALT" has been applied to the system (*2, 3*).

The ISO system: First-dimensional separations are done in banks of 20 1.5-mm (i.d.) glass tubes in a device that allows all 20 gels to be cast, loaded, and run in parallel. Numerous small modifications of the ISO system previously described (*2*) have been made, but the operating principles remain the same. Over 100 gels may be cast and samples loaded by one operator in a morning. Extrusion of the gels manually, with a small syringe, is uneven and may lead to gel breakage; hence this operation is now done semi-automatically with use of a peristaltic pump. Prototype systems for automatic sample loading are undergoing evaluation.

The DALT system: The second-dimension separations are run on slabs cast between 18 × 18 cm glass plates essentially as previously described (*3*). The casting apparatus has been modified to allow 20 to 80 slabs to be cast simultaneously. Electrophoresis is done in DALT tanks that have a capacity of 10 slabs, and as many as 10 tanks (100 slabs gels) are run in parallel.

Fixing, staining, destaining, and photography: Post-



Gel No. N2134

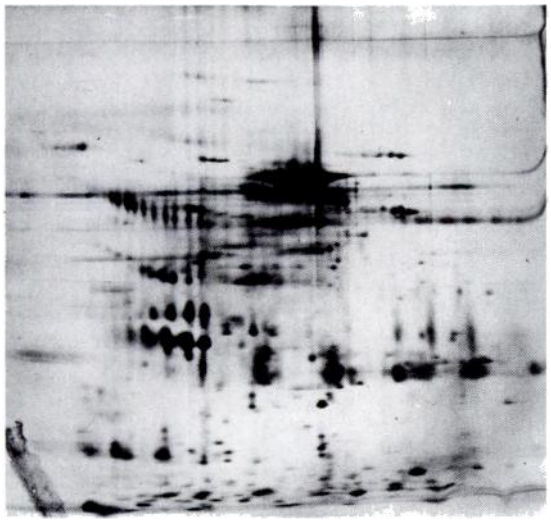
Fig. 1. Two-dimensional electrophoretic pattern of 1 mg of urinary proteins from a normal premenopausal woman

electrophoretic treatment of gels is as previously described (*3*) except that photographic processing is now more closely controlled; film development is carried out with close attention to temperature and by using nitrogen-burst agitation for 2 s at 20-s intervals. Prints are made on high-contrast paper developed by the stabilization process.

Orientation of patterns: Pictured patterns are oriented so that molecular mass increases in the vertical direction while the pH in the isoelectric focusing dimension increases from left to right, in keeping with the conventions of Cartesian coordinates. (Note that some authors publish figures reversed right to left from this convention.) The pH is not recorded on the charts published here because it cannot be determined with precision on the small IEF gels and precisely related to final spot position. Use of internal IEF standards will be illustrated in subsequent papers.

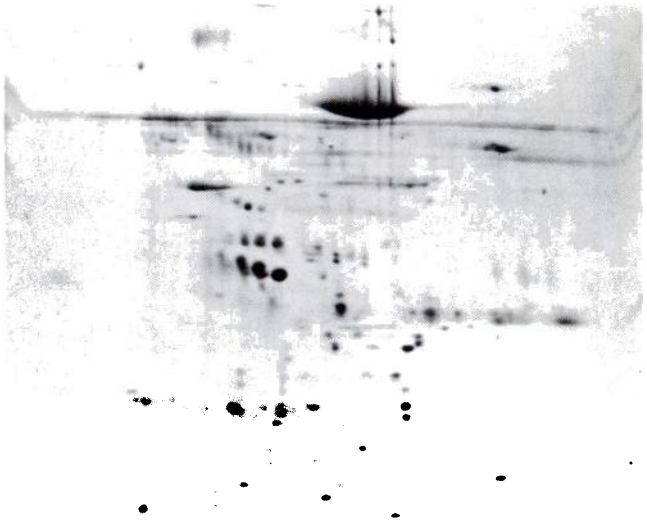
Two-Dimensional Analysis of Urinary Proteins

The results obtained with urine samples from normal adults are shown in Figures 1–4. For comparison, Figure 5 is a diagram of a map of the two-dimensional pattern of human plasma and identifications (*99*). The urine patterns are samples from normal pre- and postmenopausal women, and from 27- and 59-year-old normal men. Sex-associated differences



Gel No. N2363

Fig. 2. Pattern of urinary proteins from a normal postmenopausal woman



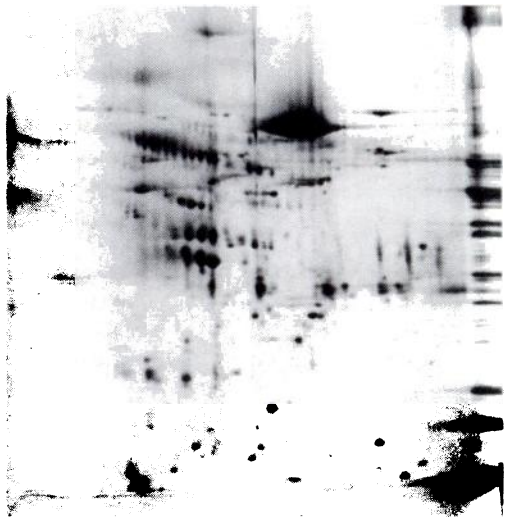
Gel No. N2057

Fig. 3. Pattern of urinary proteins from a 27-year-old normal man

are small and will be discussed in detail in subsequent papers. To facilitate comparisons, a map of the spots found in Figure 4 is included as Figure 6, in which landmark areas are indicated in large blocks. Landmark area I includes serum albumin (based on its known presence and by reference to the map in Figure 5).

Landmark area II appears to contain three proteins and charge modifications of them. Figure 7 shows enlargements of this area from analyses of urine from four individuals. From previous studies, it appears that horizontal rows of evenly spaced spots usually represent charge modifications of one protein owing to deamidation or to variations in sialation. On close examination, vertical streaking is often found to consist of closely spaced dots in vertical array. It is most often due to variations in the amount of carbohydrate added after translation. When charged groups such as neuraminic (sialic) acid residues are added to carbohydrates, negative charge increases with molecular mass. This produces a row of spots that slope upward to the left. In Figure 7, a small amount of vertical streaking is seen, but the main effect is ascribable to modifications of charge.

Landmark area II presents interesting problems, because it is not apparent how many different proteins may comprise



Gel No. N2921

Fig. 4. Pattern of urinary proteins from a 59-year-old normal man

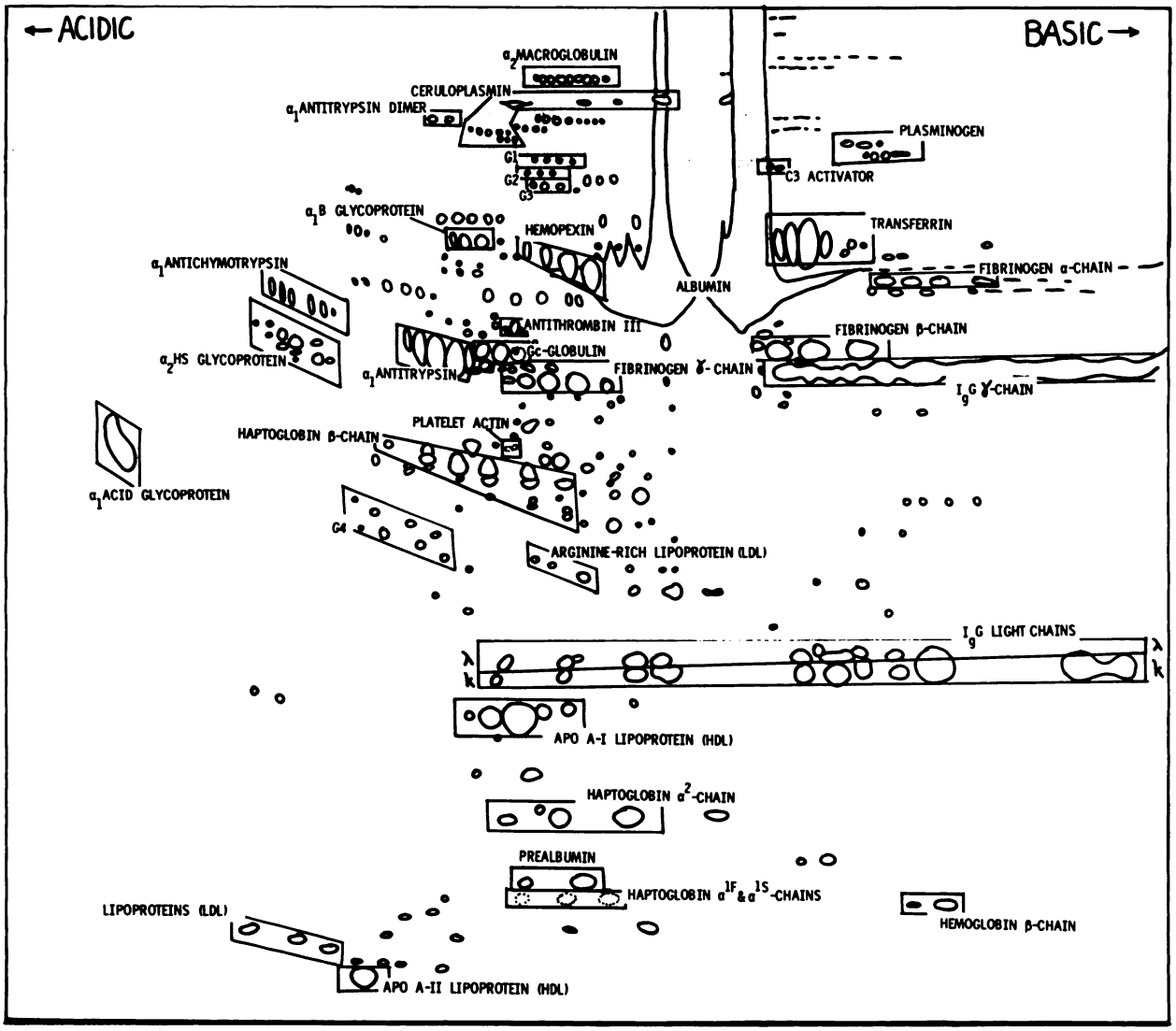


Fig. 5. Diagram of the pattern of human plasma proteins
Adapted from Anderson, N. L., and Anderson, N. G., *Proc. Natl. Acad. Sci. USA* **74**, 5421–5425 (1977).

the central row. Spots *1–6* may represent charge modifications of one protein gene product; however, in other instances where this is the case, an even gradation in spot intensity is seen. Here, spot *2* is intensified in panels *B* and *D* as compared with panels *A* and *C*, and this may be due to superimposition of a different protein gene product at position 2. The spots numbered 7–12 represent a more usual charge modification series. Whether spots *13* and *14* are related remains to be ascertained. The spot series in landmark area II illustrate the importance of examining many urines, to see which spots are related and which vary independently, and the importance of using several different methods for spot identifications as described subsequently.

Figure 8 shows landmark area III, which includes the transferrin cluster *1* (identified by mapping immunoprecipitates prepared by using antisera to transferrin mixed with concentrated human urinary protein, and by comparison with the map in Figure 5); an unidentified cluster (*2* in Figure 8), which may possibly include amylase; and IgG heavy chains indicated by *3* (identified by comparison with plasma).

Landmark area IV includes four major ascending rows of spots, plus variable minor ones. Row *1* in Figure 9 shows the most molecular mass heterogeneity (vertical streaking) and the greatest charge heterogeneity, with up to 14 charge shifts evident in some instances. Row *2* is quantitatively the most variable, as may be seen by comparing panels *A* and *B* with panel *I* in Figure 9. Row *3* shows little or no slope when compared to the other rows and shows three and sometimes four spots, which may actually be close vertical doublets (see panel *E*). Row *4*, which always contains a very prominent spot on its most basic (right) side, has three or four additional spots, which often occur as vertical triplets. In most urines we have analyzed, a horizontal streak (*5* in Figure 9) having a molecular mass approximating that of actin is seen.

Landmark area V is characterized by rows of IgG light chains (*1* in Figure 10) showing roughly the same distribution of density in a horizontal direction as is found in plasma (compare Figure 5). As expected, the major source of heterogeneity is attributable to charge differences. Two unidentified markers occur (*2* and *3* in Figure 10), while spot *4* is the most basic (right-hand) spot in row *4* in Figure 9.

The greatest heterogeneity is seen in the constituents of low molecular mass, which appear in the lower ¼ of the patterns seen in Figures 1–4. These are quite constant in the same individual, regardless of the time of day that the sample is taken, but careful analysis will probably uncover interesting diurnal variations. Doubtless several interesting signal substances whose function remain to be explored are present in this low-molecular-mass region.

In this initial paper, we are concerned with the reproducibility and complexity of the patterns seen, and with asking whether we now have an analytical system and the techniques required for the systematic identification of spots and for the analysis of changes seen in disease. The results presented thus

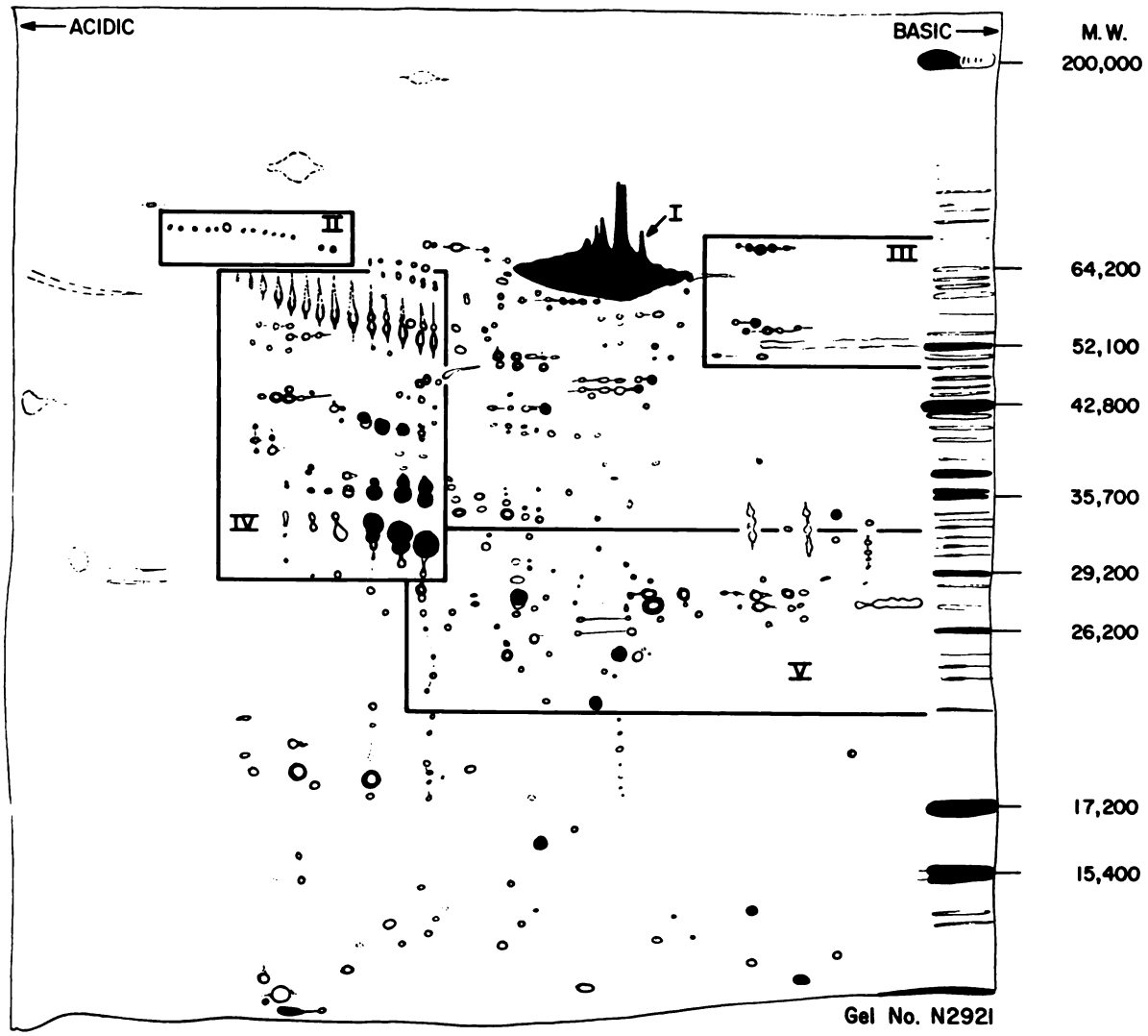


Fig. 6. Diagram of the human urinary proteins drawn from Figure 4

Proteins in higher concentration are shown in black for orientation purposes. Five landmark areas are indicated, four of which are illustrated in detail in area enlargements shown in Figures 7–10 and described in the text. Rat-heart molecular mass standards are shown along right edge

far suggest that resolution sufficient for meaningful studies has been obtained. The question of whether the effort required to do the identifications is justified depends in large measure on the types of results that might be obtained and their importance in patient care and in screening for occult disease. To indicate the types of studies that may be done, we include here two additional patterns showing interesting abnormalities. Figure 11 is from a patient with bladder cancer. It illustrates a central thesis of this work, which is that the study of human disease requires analytical tools whose resolution matches the complexity of the samples (cells and tissues) being analyzed. Quite obviously, the search for reliable indicators among so many candidate spots requires that we be able to "see" and quantitate as many of them as possible.

Figure 12 is the pattern for a urine sample from a person exposed industrially to thorium, and shows the excessive excretion of proteins of low molecular mass characteristic of renal tubular pathology. Without extensive further population studies, it is not possible to relate patterns of this type to the thorium exposure. What is illustrated, however, is that two-dimensional electrophoretic analysis is a potentially powerful tool for studying the early stages of a variety of diseases in human populations exposed to toxic agents. Based on the analysis of over 300 urine samples during the course of developing the analytical systems used here, we conclude that the effort required to identify the major proteins present in these patterns is justified and, therefore, we next consider briefly the methods that will be used.

Locating and Matching Spots

Spot matching: The number of spots seen on two-dimensional "maps," especially of tissues, exceeds the number of known enzymes or other proteins. For some time, therefore, many protein gene products will be identified largely by two-dimensional electrophoresis and by techniques derived from it. The basic problem is to find out whether a certain spot observed in the pattern for the urine from one individual is identical with that observed in a pattern for urine from a second individual, and whether both are the same as a protein found in a specific tissue, such as brain. There are small variations between gels, making position with reference to the coordinates of the gel itself insufficient for description and identification. Hence, several different techniques have been developed to answer the question of whether the spot on one gel is the same as that seen on another—i.e., whether it has the same characteristics in two-dimensional separations.

Pattern matching: Duplicate analyses of a tissue or body fluid give very reproducible results, and constellations of spots are found that are readily recognizable. To compare patterns in detail, one may physically superimpose gels and compare them by sliding one gently over the other, or may superimpose photographic transparencies of the gels (with or without the use of color to distinguish one pattern from the other), or use

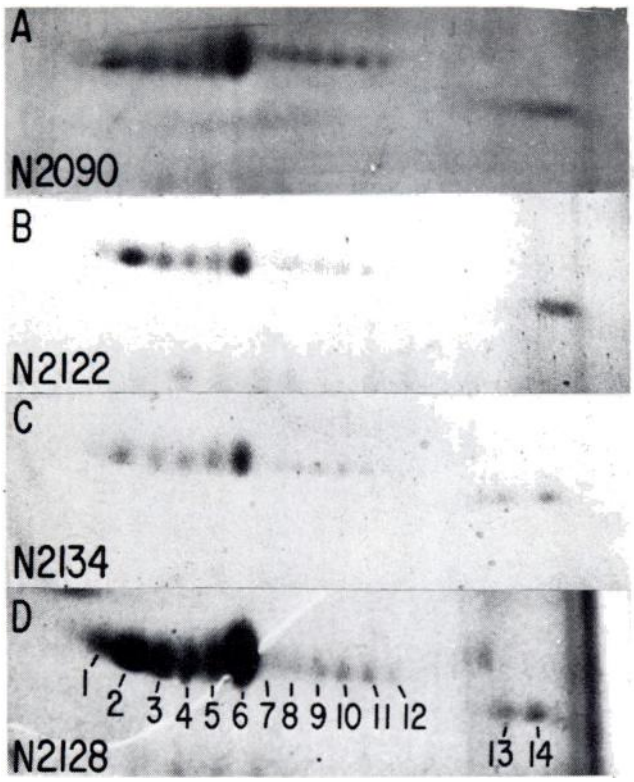


Fig. 7. Enlargements of landmark area II from analyses of four different urines, illustrating both reproducibility and compositional variations observed

Spots *1–6* appear to be charge modifications of one protein, and *7–12* of another. Spots *13* and *14* may be similarly related.

an optical comparator in which the images of two gels (the gels themselves or photographs of them) may be alternately viewed or flickered. An apparatus such as that shown in Figure 13 is used for the latter two methods. Visual pattern matching is useful when only a very few patterns are to be compared, but computerized comparisons are essential for analyzing large numbers of patterns. Note that the precision with which individual spots are tentatively identified is a function of the number of nearby spots; hence, dense constellations allow greater precision of localization.

Co-electrophoresis: The question of which urinary proteins have properties identical with those of plasma proteins may be answered by electrophoresing mixtures and carefully comparing patterns to see which spots are superimposed and which are not. This method can be made remarkably sensitive because spot size is a function of loading (*1*). Hence, if two samples of one protein (one sample radiolabeled during its synthesis and present in trace amounts, and the other unlabeled and present in larger amounts) are electrophoresed together, one will dilute the other if they are identical—i.e., the stained spot will be of the same dimensions as that observed on an autoradiograph. If they are different, but by accident migrate to exactly the same spot on the gel, a very small autoradiographic spot will be seen in the center of the larger stained one. Computerized analysis of three-dimensional plots (x and y coordinates and absorbance) allows additional analytical refinement.

Charge modification: Many proteins exist in two or more forms, owing to post-translational charge modification by (e.g.) sialization, phosphorylation, or deamidation after synthesis of the peptide chain. Two proteins having the same sequence of amino acid residues may thus appear horizontally displaced from each other. Artificial charge modifications may also be used to demonstrate the relationship between two proteins. As previously demonstrated (*104*), deamidation leads to rows of spots, the total number of which will be equal to one more than the number of positive charges originally present. This provides a simple method of demonstrating compositional similarity, because identical proteins should give the same number of spots. Not only is this a method for counting positively charged groups, it is also a reflection of the number of negatively charged groups present, because the spacing of deamidation partials is very small when many carboxyl groups are present but much larger when they are few. Spacing is actually a reflection of the titration curve of a peptide.

Use of standards: Spot positions may be defined with reference to standards for molecular mass and for isoelectric point. These must be internal, to compensate for small distortions in the gels and for nonlinearity of ampholyte-generated pH gradients. Measurement of pH of the isoelectric focusing gel with a microelectrode is instructive, but cannot be precisely related to spot position. Rows of spots produced by deamidation provide internal positional standards, which are convenient and easily used (*104*). Molecular-mass standards have been used along one edge of the slab gel (Figure 4), but do not provide exact positional reference points. Because the first-dimension isoelectric-focusing gels are sealed in place on the slab gels with agarose, the agarose provides a convenient carrier for molecular-mass standards, which then extend all the way across the gel and provide a series of horizontal reference bands (*5*). By combining standards for both charge and molecular mass, very precise localization may be achieved,

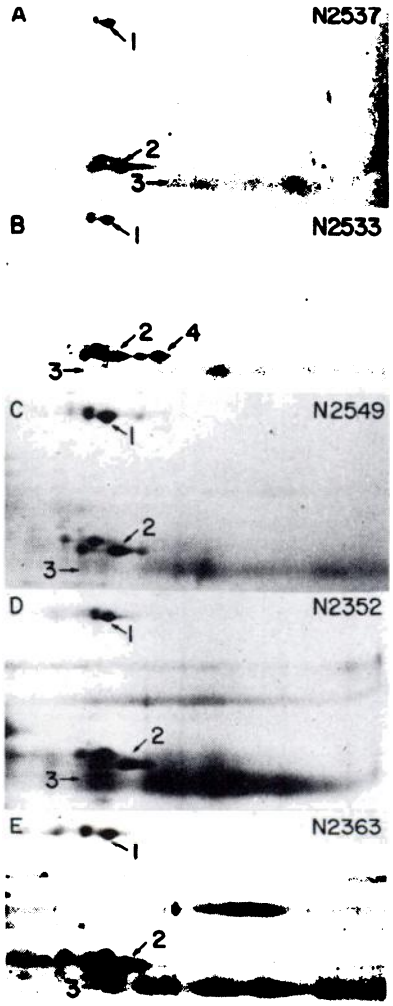


Fig. 8. Enlargements of landmark area III from five different individuals

The cluster around spot *1* has been identified as transferrin (see text); the cluster around spot *2* may be amylase. Indistinct and charge-heterogeneous horizontal streak *3* is composed of IgG heavy chains. Note presence of additional spot *4* in panel B (N2533) which may be a genetic variant

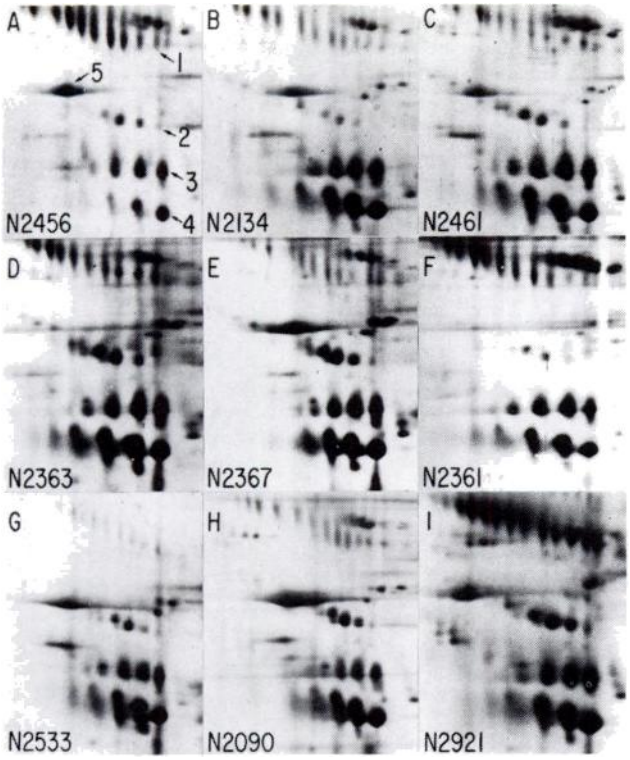


Fig. 9. Landmark area IV showing reproducible presence of four rows of spots labeled *1–4*

Sloping of rows is due to combined molecular weight and charge heterogeneity

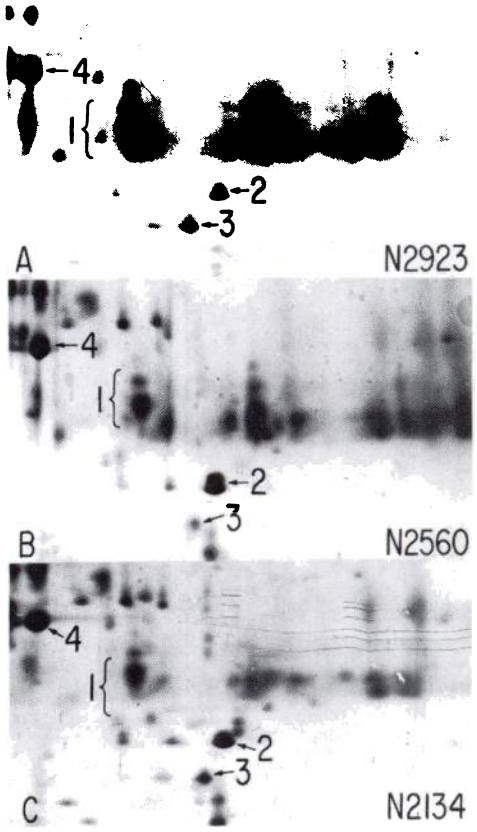


Fig. 10. Landmark area V

Charge-heterogeneous horizontal band *1* is composed of IgG light chains. Spots *2* and *3* are useful identification markers, while the arrow at *4* indicates the prominent most basic spot in row *4* of Figure 9

depending on the resolution of the grid provided by the standards. With heart-muscle proteins as a molecular-mass standard, about 86 such bands may be distinguished.

Subtractive methods: Mixtures to be mapped may first be fractionated to remove some constituents, for example, by heat treatment or by precipitation with ammonium sulfate or alcohol. A procedure that removes a spot from one mixture should also remove it from a second, giving additional indication of identity.

Identification of Spots

Co-electrophoresis: Known pure proteins may be co-electrophoresed with mixtures to associate an activity with given spots. For systematic identifications, this requires the purification of a long series of proteins.

Identifications based on physical data: Where accurately determined SDS molecular masses and isoelectric points (for the analytical methods used, in this case in the presence of urea) are available, these may assist in identification.

Immunochemical identifications: Antibodies specific to a known enzyme or other protein may be used to prepare immunoprecipitates that dissociate in the two-dimensional analysis used here to give light and heavy chains, and the precipitated antigen whose position in the pattern may then be determined (*99*). If the specific antibodies are sufficiently pure, these may be added to a mixture, the mixture centrifuged or the antigen–antibody complex removed with staphylococcal protein A, and the proteins remaining in solution mapped. The antigen in question should then disappear from the pattern. Some antigens are still reactive after two-dimensional separation and will bind antibody. The position of the bound antibody may then be determined, either because it is labeled or because it will further react with labeled counter antisera.

Identification of matching physical properties: Curves for precipitation, heat denaturation, or other inactivation may be prepared that describe the behavior of one enzyme in a mixture. For example, a series of identical dialyzed samples may be heated for the same time interval to a range of increasing temperatures, cooled, centrifuged, and the activity of one or more enzymes determined in all samples. Activity is then plotted vs. temperature to give a heat-denaturation curve (*105*). If the samples obtained through the thermal denaturation range are examined by two-dimensional electrophoresis, one or more spots will be observed to disappear over the range in which activity disappears. These are then candidates for that enzyme. Substrates and cofactors shift heat-denaturation curves. Hence if the procedure is repeated

Fig. 11. Urinary protein pattern for a male patient with bladder cancer, illustrating very marked alterations in pattern and presence of multiple additional spots in comparison with the usual pattern (e.g., Fig. 3)

Gel No. N1925

Fig. 12. Urinary protein patterns seen in a person exposed industrially to thorium

Note predominance of low-molecular-mass proteins suggestive of tubular damage

with a substrate, the enzyme specific for it will give a different precipitation curve. By analyzing this second set of samples obtained in the presence of the substrate, the number of candidate spots may be greatly reduced—in the most favorable case, to the subunit(s) of the enzyme sought. This general approach may be used with any fractionation method, including ion-exchange and gel chromatography.

Genetic identification: When samples containing variants of known enzymes are available (for example, erythrocytes containing a variant enzyme or lacking an enzyme completely), these may be mapped and the absence or displacement of one or more spots noted. Ideally, association of an activity with a spot should depend on more than one of the techniques listed.

Limits of Detection

With use of Coomassie Blue staining, spots of some proteins, such as transferrin, containing as little as 50 ng of protein may be seen. Undoubtedly, more sensitive staining methods will be developed in the future. With radiolabeled proteins and spot detections by autoradiography or fluorography, the range can be extended downward several orders of magnitude; the limits are set by the specific activity of the label, the sensitivity of the photographic method used, and the time allowed for the exposure. Proteins may also be labeled after synthesis by using reagents that do not alter the surface charge, such as ^{14}C-labeled iodoacetamide, which attaches to sulfhydryl groups.

The necessity for computerized data reduction in the studies described here arises from the fact that both the number of spots (protein gene products) and the number of gels (we have run more than 16 000 thus far) are too great to allow visual assimilation of data. Ideally, we require a system that can normalize patterns (i.e., compensate for small distortions) by superimposing them on a standard pattern, and yield a list of abundances (integrated densities) of each protein gene product on each gel. Such data can easily be examined for differences between samples and for correlations of abundance of any spot with external variables such as disease, injury, drug therapy, or age. This approach may yield a list-based diagnosis.

We have constructed a prototype data-reduction system based on a PDP 11/60 (Digital Equipment Co. Inc., Marlboro, MA 01752) with data input from a high-speed densitometer (P-1000 scanner; Optronics International, Chelmsford, MA 01824) (a gel image being digitized as 2 to 4 × 10^6 pixels). The system also incorporates a 512 × 512 resolution color cathode-ray tube display (GMR-27; Grinnell Systems Corp., San Jose, CA 95131) for monitoring image processing and for matching patterns. The first-stage programming, which includes image digitization, smoothing, background subtraction, and initial spot detection is now complete. Fitting of spots with two-dimensional gaussian curves to resolve overlaps is partly complete, as is the program to match resulting spot lists to a reference pattern by using the color cathode-ray tube display. Programs to compute the statistics for each spot and search for correlations will be completed shortly. The processing times required reflect the enormous number of numerical calculations involved in the extraction of hundreds to a thousand or more integrated densities from a 2000 × 2000 point image. Array processors will greatly shorten processing times in the future.

Discussion

High-resolution two-dimensional electrophoresis has been developed as a tool for research in clinical chemistry and applied to the analysis of human urinary proteins. More than 250 constituents are resolved, nearly all of which remain to be identified. Two-dimensional maps of human urinary proteins lack the sharpness and clarity of maps for plasma or cell proteins, and in addition show vertical streaking. Some of the vertical streaks may be due to small proteins such as the β_2-microglobulin co-isolating protein described in rabbit urine (*24*), which does not stain with Coomassie Blue and does not absorb light at 280 nm.

The preparation method used here is a denaturing one, which therefore has the advantage of producing protein subunits, most of which are protein gene products. This greatly simplifies the problem of finding out what subunits are present. If a protein is a tetramer, and either of two subunit variants may be in any position, then up to five isoenzymes

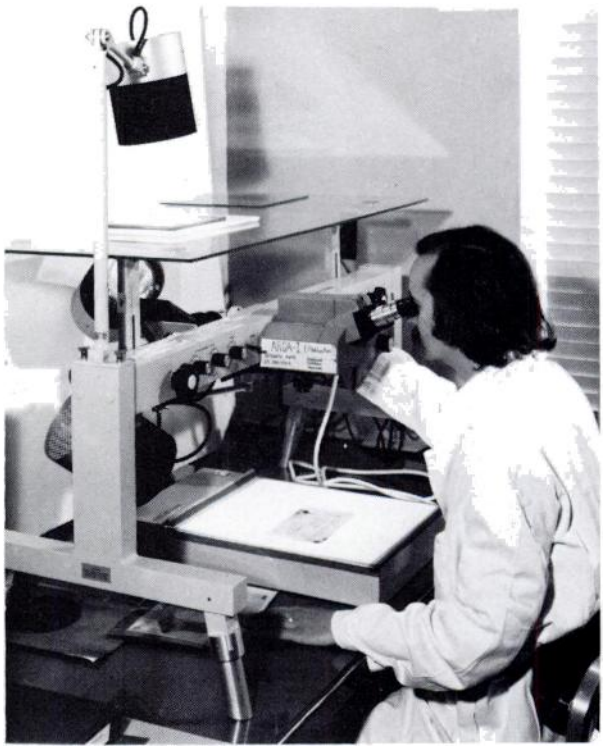


Fig. 13. Optical comparator used to compare two-dimensional electrophoretic patterns by either the flicker technique or by viewing superimposed images in two different colors

may occur. If one looks only at the subunits, as is done here, then only two are seen, but they may vary in their proportions.

Many protein gene products, especially those occurring in plasma or on the surface of cells, are post-translationally modified by the addition of carbohydrates, which may change their molecular mass sufficiently for them to be detected in SDS electrophoresis, or by the addition or removal of charged groups, resulting in heterogeneity detectable by isoelectric focusing. This double heterogeneity has been clearly demonstrated in previous studies on plasma proteins (*99*) and accounts for the "runs" of spots that are horizontal in a few instances, but most often are slanted upward to the left. In most instances, this means that as carbohydrate is added post-translationally to a protein, there is an increased chance for the addition of a small charged group such as neuraminic acid. The in vivo synthesis of carbohydrate side chains is not as precise a process as protein synthesis, the sugar moieties being assembled on a carrier as a chain or tree and then transferred as a unit to a protein. Although incremental increases in SDS molecular mass have been seen, in most instances heterogeneity in carbohydrate assembly results in a vertical smearing of a spot. Many instances of this vertical smearing are seen in urinary proteins as, for example, in Figures 1–4.

To determine which instances of charge heterogeneity are attributable to neuraminic acid, we treated a concentrated preparation of urinary proteins with neuraminidase (EC 3.2.1.18) and analyzed it two-dimensionally. By mixing the original sample with partial and complete neuraminidase digest, it is possible to count the number of neuraminic acid residues (*99, 106*).

The Molecular Anatomy Program, of which this work is a part, is an interdisciplinary program aimed at resolving as many of the discrete molecular constituents of cells as possible. It is not feasible to develop the requisite analytical systems completely and then to determine their usefulness in clinical studies. Rather, development and evaluation in biomedical research must proceed in parallel. The question asked here is simply whether we now have a prototype system that yields new and useful information that in turn justifies continuation and completion of development. This and subsequent papers concerned with spot identifications and with abnormal samples will provide the answers. The initial studies are exploratory, and may identify disease indicators for which competition assays should be developed. Eventually, however, given automation and very rapid computerized data reduction, two-dimensional high-resolution electrophoresis may become a routine tool in clinical chemistry. This possibility raises again the problem of "too much" data: the amount of data collectible in clinical chemistry is theoretically almost limitless. What is lacking is relevant information. We need accurate and specific measurements of the molecular defects underlying very many human diseases. We do not yet have all necessary information for determining these defects. Hence, the problem is not too much data, but rather a lack of the relevant data.

The problem of examining human populations who are at risk owing to chemical or radiation exposure is now a serious one and one that commands much public attention. For historically valid reasons, government policy has been to emphasize measurement of exposure, to minimize exposure as far as possible, and to estimate the extent of the human effects of low-level exposure by extrapolation from animal studies. It has been assumed that no common-denominator methods exist or could be developed that would allow mutagenicity, carcinogenicity, and other cellular toxicity to be detected and measured. With the development of high-resolution two-dimensional electrophoretic techniques this assumption may no longer be true. Approximately one-third of amino acid substitutions (point mutations) produce charge shifts that are detectable as differences in isoelectric focusing position. In addition, major sequence deletions or additions are detectable as changes in the SDS dimension. Carcinogenicity involves altered gene expression and has been too complicated to be studied meaningfully by pre-existing methods that involved the measurement of changes in only a few protein gene products. To find out whether one or a few protein gene products exists that can serve as indicators of carcinogenesis and may possibly also be useful early indicators of cancer, it is again important to be able to detect as many protein gene products as possible. Protein gene product mapping as described here is therefore an essential tool in attempts to understand and detect cancer in human populations.

The problem of toxicity unrelated to genetic damage or to carcinogenicity has been a difficult one to approach experimentally, and it has been assumed that many different measurements would be required to detect different types of injury to various organs. If the thesis that toxicity generally involves cell leakage is true, and if the leaked proteins appear at least in part in urine, then their detection and measurement should provide valuable diagnostic assistance. Lastly, the kidney may be directly injured by toxic agents, or may be indirectly damaged in autoimmune disease, and the resulting changes in the pattern and composition of excreted proteins could be measured.

To evaluate these possibilities, we have developed prototype systems for two-dimensional electrophoretic analysis of urinary proteins that allow up to 10 000 analyses to be made per year. In parallel with further development of these systems, selected human pathological samples are being examined, and the systematic identification of spots carried out. We thus propose gradually to approach a time when small, carefully selected human populations at risk from exposure to environmental pollutants or radiation may be usefully examined.

Subsequent papers will be devoted to brief presentations of this work as it progresses.

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