ANALYTICAL BIOCHEMISTRY 95, 48–61 (1979)

Analytical Techniques for Cell Fractions

XXV. Concentration and Two-Dimensional Electrophoretic Analysis of Human Urinary Proteins[1,2,3]

N. G. ANDERSON, N. L. ANDERSON, S. L. TOLLAKSEN, H. HAHN,[4] F. GIERE,[5] AND J. EDWARDS

Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

Received October 1978

The diagnostic and experimental possibilities inherent in the analysis of human urinary proteins have not been realized largely because rapid and efficient concentration methods have not been available, and because the resolution of analytical methods used to study them was low. In addition, the view has been widely held that little new or useful information was to be obtained from the analysis of proteins in urine.

In this paper, we examine two methods for concentrating urinary proteins sufficiently for analysis by high resolution two-dimensional (2-D) electrophoresis (1) using the ISO–DALT[6] system (2–6). In orienting studies by two of us (NLA and NGA) (7), it was found that a large number of proteins are seen in 2-D gels of urine protein concentrates, and that unusual spots appeared in the urines of burn, muscular dystrophy, and some cancer patients. A minority of these have the spot locations of serum proteins, suggesting that many urinary proteins remain to be described. We therefore initiated a systematic series of studies directed toward the development of methods and systems that would be useful clinically for analyzing urinary proteins.

We distinguish eight classes of proteins in human urine: (i) plasma proteins and their derivatives, (ii) kidney proteins including the Tamm–Horsfall protein, (iii) proteins released from other portions of the urinary tract including the male accessory glands, (iv) proteins leaked from tissues that reach the urine via the circulation and may be useful indicators of tissue injury, (v) hormones or other signal substances whose function depends in part on rapid removal from the circulation so that their concentration varies rapidly with rate of release, (vi) substances produced by the products of conception, (vii) tumor-associated proteins,

[1] This paper is dedicated to the memory of Dr. Alvin Nason.

[2] The submitted manuscript has been authored by a contractor of the U. S. Government under contract No. W-31-109-ENG-38. The U. S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

[3] Work supported by the U. S. Department of Energy, by Grant RD-43 of the American Cancer Society, and Grant 1376 of the North Atlantic Treaty Organization.

[4] Present address: Medical University of South Carolina, Charleston, S. C.

[5] Present address: Department of Biology, Lake Forest College, Lake Forest, Ill.

[6] "The term ISO–DALT is used to indicate briefly that the separation is based on ISOelectric focusing in one dimension and is proportional to molecular weight in the second, molecular weight being expressed in DALTons." (2).

0003-2697/79/070048-14$02.00/0

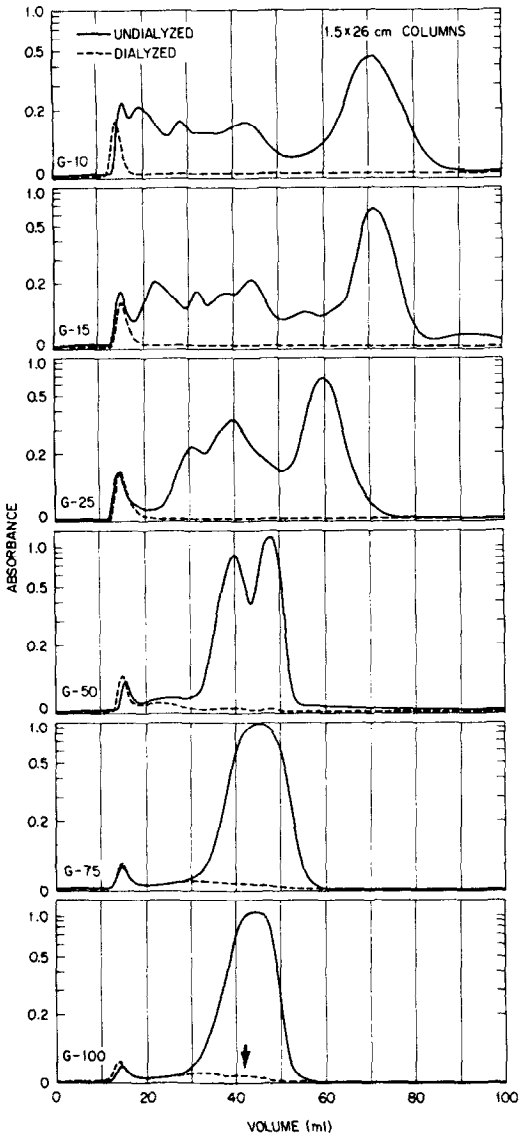

FIG. 1. Dialyzed and undialyzed aliquots from a single sample of human urine chromatographed on six different grades of Sephadex. The column volume is 41.6 ml. Analysis was done by the Folin–biuret reaction. In undialyzed samples there is no sharp separation of protein from dialyzable interfering substances. Considerable material is retarded by more highly crosslinked gels and elutes in greater than 1 column volume (indicated by arrow).

and (viii) products of bacterial or viral infection. Examples of each class are known and are not reviewed here. Rather, we consider the preparative and analytical problems that have thus far limited chemical studies on urinary proteins.

URINARY PROTEIN MOLECULAR WEIGHT RANGE

Normal adults excrete approximately 50–70 g of solids per day in the urine, including approximately 50–120 mg of protein (depending in part on how the protein is measured). To equal the protein concentration of serum, normal urine must be concentrated by a factor of approximately 1000 (8–9). Since protein may constitute as little as 0.1% of the solids present, purification relative to dry mass must also be by a factor of up to 1000.

The very low protein concentration in urine and the presence of low molecular weight substances that interfere with many protein tests have made it difficult to determine the concentration of protein in fresh urine. However, precise measurements of the protein initially present are essential for quantitative evaluation of protein concentration methods. The spectrum of protein and peptide molecular weights appears to be almost continuous, ranging from the large Tamm–Horsfall protein and serum proteins down through small peptides, and extending to amino acids (10). An additional problem therefore arises if the concentration methods used for urinary proteins do not match the analytical method employed. Two-dimensional electrophoresis using the ISO–DALT system does not detect peptides much smaller than 6000 daltons, and the gels are plugged by aggregates larger than about 10^6 daltons. These numbers define the spectrum seen on the 2-D gels. Chemical methods for estimating protein content "see" a much wider range. Thus, protein loss during concentration would be overestimated. We have therefore examined the question of the reality of the continuous urinary protein molecular weight spectrum. At some later time, the question of losses during concentration of proteins seen on 2-D gels will be examined.

Unconcentrated dialyzed and undialyzed urine samples were chromatographed on several grades of Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.) and monitored using the Folin–biuret reaction. The results are shown in Fig. 1. Examination of the patterns shows that no clean separation of protein (assuming the reactive material retained in the dialysis sac is protein) from lower molecular weight material was obtained with any of these materials. This finding is consistent with the view that a continuous spectrum of molecular weights is present, at least down to the cut-off point for the dialysis tubing used.

To compare monitoring of gel columns with the Folin–biuret reaction and by uv absorption, the column monitoring system shown diagrammatically in Fig. 2 was constructed. Results obtained with dialyzed urine fractionated on P-series Bio-Gels (Bio-Rad Laboratories, Richmond, Calif.) are shown in Fig. 3. As expected, a large amount of low molecular weight material interfering with the Folin–biuret reaction is present in urine. However, the interference is much less than is the case with uv monitoring. When unconcentrated urine was compared with an aliquot of the same sample that had been dialyzed, the results shown in Fig. 4 were obtained. Under the conditions used, uv absorption was more sensitive to protein. Hence, for monitoring gel filtration columns used in studies on concentration methods, we have employed only uv absorption (ISCO Model UA-5 absorbance monitor). Note that very little protein appears to be lost during dialysis.

These orienting studies suggest that gel chromatography on P-2 or P-4 Bio-Gel may provide a suitable method for separating human urinary proteins analytically and also support the view that an almost continuous molecular weight spectrum is present.

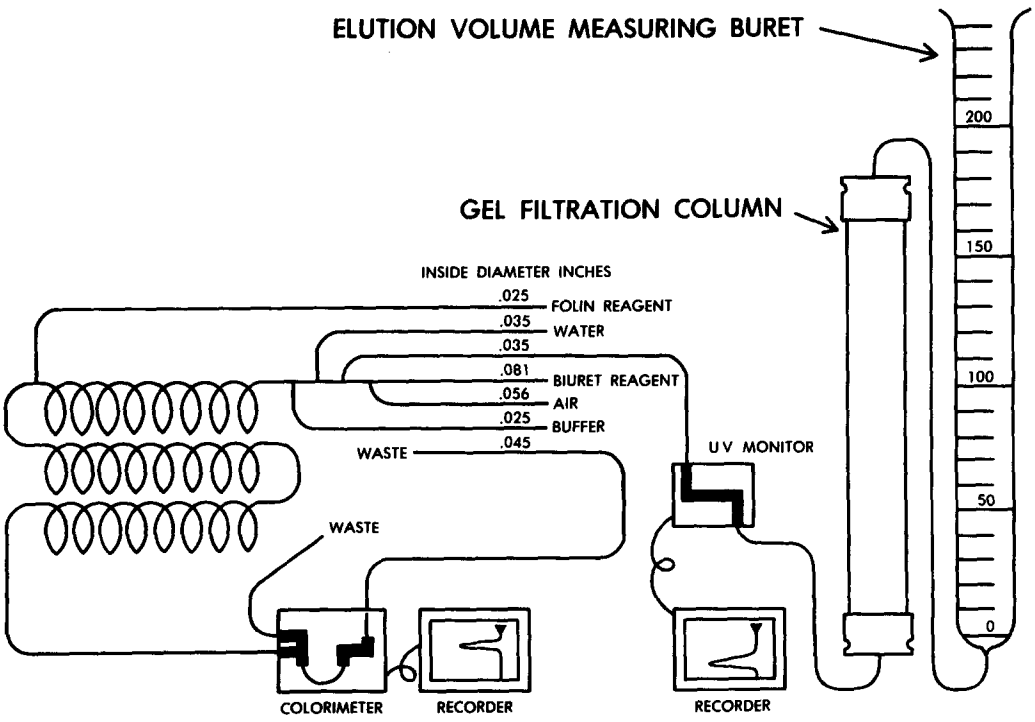

FIG. 2. Schematic diagram of monitoring system for measuring protein in column effluents using the Folin–biuret reaction and ultraviolet absorption at 280 nm.

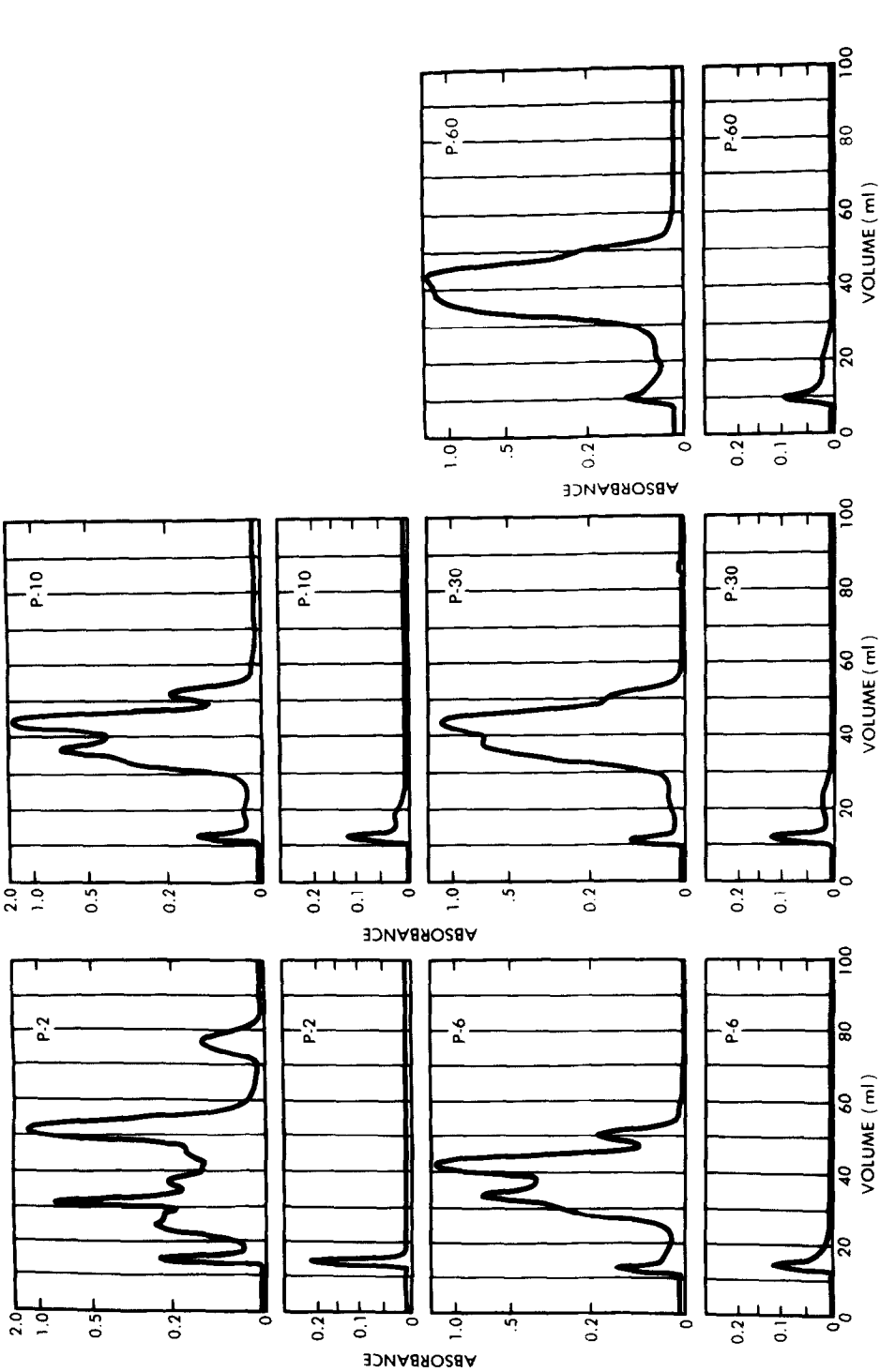

FIG. 3. Dialyzed urine samples chromatographed on P-series Bio-Gels with both uv absorbance and Folin–biuret analysis done for each experiment. The upper chart of each pair shows uv absorbance, the lower chart the Folin–biuret results. Note that uv absorbance is more subject to interference by small amounts of undialyzed material than is the Folin–biuret analysis. P-2 Bio-Gel appears to provide the best separation for total protein determination but cuts the molecular weight spectrum lower than necessary for analysis by two-dimensional electrophoresis.

The possibility exists that any given concentration method may lose some protein selectively. For this reason, we have developed two methods in parallel and will continue to examine others that appear promising.

CONCENTRATION USING DIALYSIS AND LYOPHILIZATION

Urinary proteins have been concentrated by a variety of techniques including precipitation (11); absorption on ion exchangers, charcoal, or bentonite (12); ultrafiltration using hydrostatic or colloid osmotic pressure (13,14); pervaporation, or lyophilization (15). We have examined nearly all available methods and have chosen lyophilization combined with dialysis, and gel exclusion combined with centrifugation as the two methods that appear to have greatest promise at present.

In all studies, urine was initially centrifuged in 50-ml plastic tubes at 1500 rpm for 15 min in the Beckman TJ-6 centrifuge and the sediments were discarded.

Because whole urine contains too high a concentration of urea and salts to be lyophilized directly, preliminary dialysis is required. However, exhaustive dialysis may result in insufficient dry mass to ensure efficient retention of protein in the flask during lyophilization (16). The traditional view is that dialysis is best done using large volumes of external solution and rapid stirring (17). If dialysis is to be used with large numbers of urine samples, then the use of large volumes and many stirrers will be costly and space consuming. We have therefore examined the possibility that dialysis could be effectively done in relatively small 1-liter cylinders, with the dialysis sac confined to the upper ⅜ of the cylinder. The rationale behind this arrangement is that as solids diffuse out, the fluid immediately outside the sac increases in density and convects to the bottom of the cylinder. An analogous process produces convection inside the sac, with depleted liquid next to the inner wall rising to the top. In theory, this process of autoconvection should produce a more complete dialysis for a given volume of external solution than is obtained with constant stirring because the external volume is not homogenous.

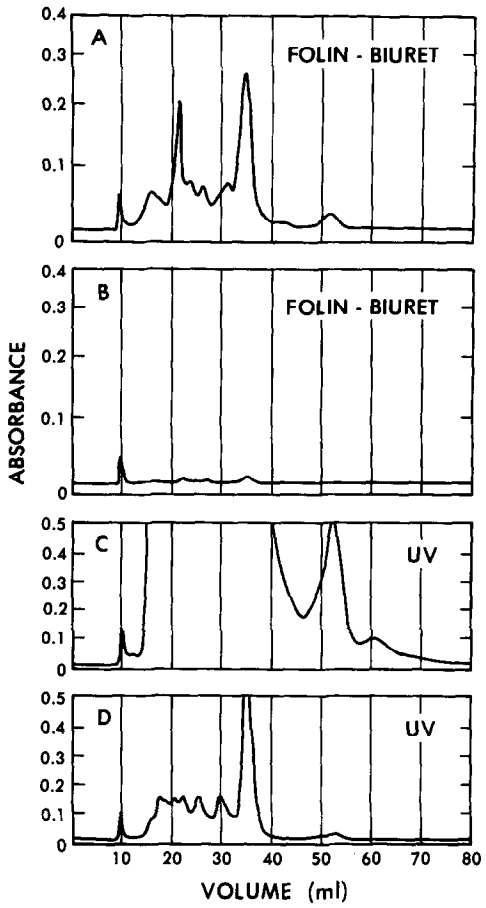

FIG. 4. Comparison of different aliquots of the same urine sample before and after dialysis in Visking ⅜₂ tubing (obtained from Union Carbide Corp., Chicago, Ill. in 1500-ft rolls). (A) Before dialysis, Folin–biuret analysis. (B) After dialysis, Folin–biuret analysis. (C) Before dialysis, uv absorbance monitoring. (D) After dialysis, uv absorbance monitoring.

We have examined autoconvection dialysis using the compact arrangement shown in Fig. 5. Each vessel contains 1 liter of cold distilled water which is not stirred mechanically. One hundred milliliter samples of urine are placed in presoaked 290-cm lengths of Visking ⅜₂ dialysis tubing (Union Car-

bide, in 1500-ft rolls) using the filling funnel shown in Fig. 5. The ends are closed with plastic clips (closure No. 132736, Spectrum Medical Industries, Los Angeles). The tubing is folded four times so that its folded length in the cylinder is approximately 15 cm, and it is held at the top of the cylinder by a No. 13 rubber stopper which clamps the free end of the tubing. Samples were recovered after dialysis for varying periods of time, and the specific gravity at 23°C was determined using a 25-ml pycnometer (Kimble No. 15123, Owens-Illinois, Inc., Toledo, Ohio) which includes a thermometer. The results are shown in Fig. 6, which also shows a control urine sample dialyzed against 3 liters of cold distilled water with constant stirring for 3 h. The loss of mass in a cylinder containing 1 liter of water with no stirring is almost exactly equal to the mass loss during dialysis against 3 liters with stirring.

From the results shown in Fig. 6, it is concluded that dialysis for 3 h in one cylinder followed by an additional 3 h in a second is sufficient in preparation for lyophilization. Approximately 7% of the dry mass is left after this procedure. If one dialysis period is to be extended overnight, it should probably be the second. Using two 12-place racks of tubes containing chilled water, 12 samples can be conveniently processed per day.

The dialysis tubing used will pass protein in the 6000 M_r range (17) but at a very slow rate. Dialysis in the studies reported here is not to equilibrium and during 6 h very little

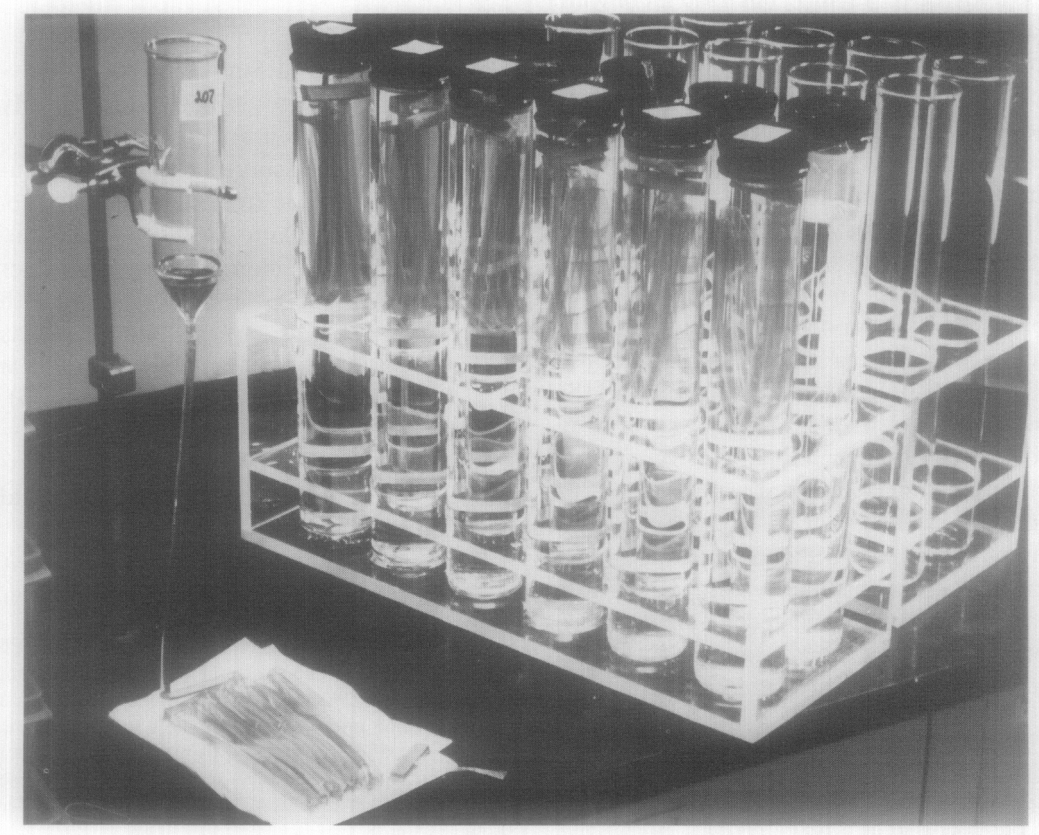

FIG. 5. One-liter cylinders used for dialysis of 100-ml fresh urine samples in $^{8}/_{32}$ Visking tubing. Note that dialysis tubing is confined in upper portions of cylinders. Funnel used to fill dialysis tubing is shown on left. Cylinders are 6.5 cm o.d. and 40.5 cm long.

material to be seen in 2-D gels would be lost.

The dialyzed urine is shell frozen in a modified large conical-tipped lyophilization flask[7] as shown in Fig. 7. After lyophilization, the protein is scraped into the tip of the flask and dissolved in 2 ml of distilled water. The recovered protein is then filtered through P-4 Bio-Gel (100–200 mesh) in a disposable 1 × 30 cm column[8] having a bed volume of 20 ml. Gel bed washing and elution were with distilled water at 5°C. At each step the column was allowed to drain until the meniscus was even with the top of the gel, i.e., to run dry. With the mesh and column sizes used, no air enters the gel. To remove low molecular weight substances, the reconstituted lyophilized urine was then carefully placed on top of the column which was allowed to run dry. Then 4.5 ml of water was added to move the protein front to the bottom of the column. Small conical lyophilization flasks (shown in Fig. 7) were then placed under each column, and an additional 3 ml of water was added to the top of the column to displace the protein. When elution was complete, the flask contents were frozen in the conical portion of the small flasks (not shell frozen) and lyophilized. This procedure restricts the protein to a small area in the flask. Salts interfere with isoelectric focusing in the ISO system. Any remaining salt is therefore removed by resuspending the lyophilized protein in 2 ml of distilled water and dialyzing it for at least 1 h in No. 3 Spectropor tubing (Spectrum Medical Industries, Los Angeles) against distilled water. The dialyzed protein is then relyophilized in a small conical bottom flask as before. Using a small stainless-steel spatula, the dried protein is removed to either 1.5-ml or 400-μl polyethylene microfuge tubes[9] and briefly centrifuged to give a dry pellet of protein. The protein was dissolved in 60 μl of a solution containing 8 M urea, 2% NP40[10] detergent (Particle Data Laboratories, Elmhurst, Ill.), and 1% mercaptoethanol [larger volumes (100–250 μl) are necessary with many pathological samples]. Then 8 μl was added of a solution containing 0.05 M CHES, 2% sodium dodecyl sulfate (SDS), 1% dithiothreitol, and 10% glycerol. Dissolution is aided by stirring with a stainless-steel wire, 15 gauge, with a rounded and polished end.

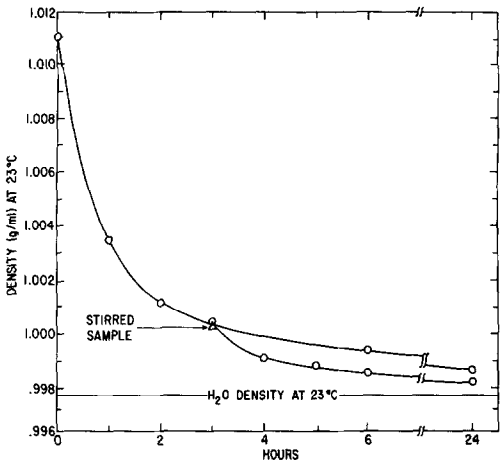

FIG. 6. Change of specific gravity of urine with dialysis time in cylinders shown in Fig. 5. The lower curve from 3 h on shows data obtained from samples moved to second cylinder after 3 h dialysis in the first cylinder. For comparison, the specific gravity is given of a sample dialyzed 3 h against 3 liters of water with constant stirring.

In some instances, high molecular weight materials remain undissociated and give a

[7] Large and small lyophilization flasks were made from 600- and 40-ml Virtis lyophilization flasks (Catalog Nos. F118 and F122), available from Virtis Corporation, Gardiner, N. Y. 12525.

[8] Catalog No. 737-2250; 1 × 30-cm glass barrel econo-columns, Bio-Rad Laboratories, Richmond, Calif. 94804.

[9] Polyethylene microfuge tubes, Catalog No. 314326 available from Beckman Instruments, Spinco Division, Palo Alto, Calif. 94304. Tubes from other sources do not have same internal shape at the bottom to fit the stainless-steel homogenizer pestle used here and do not withstand the high centrifugal forces used when these tubes are cut off and used in the rotor and adapter shown in Fig. 8.

[10] Abbreviations used: NP40, Nonidet-P40; SDS, Sodium dodecyl sulfate; CHES, Cyclohexylaminomethane Sulfonic acid.

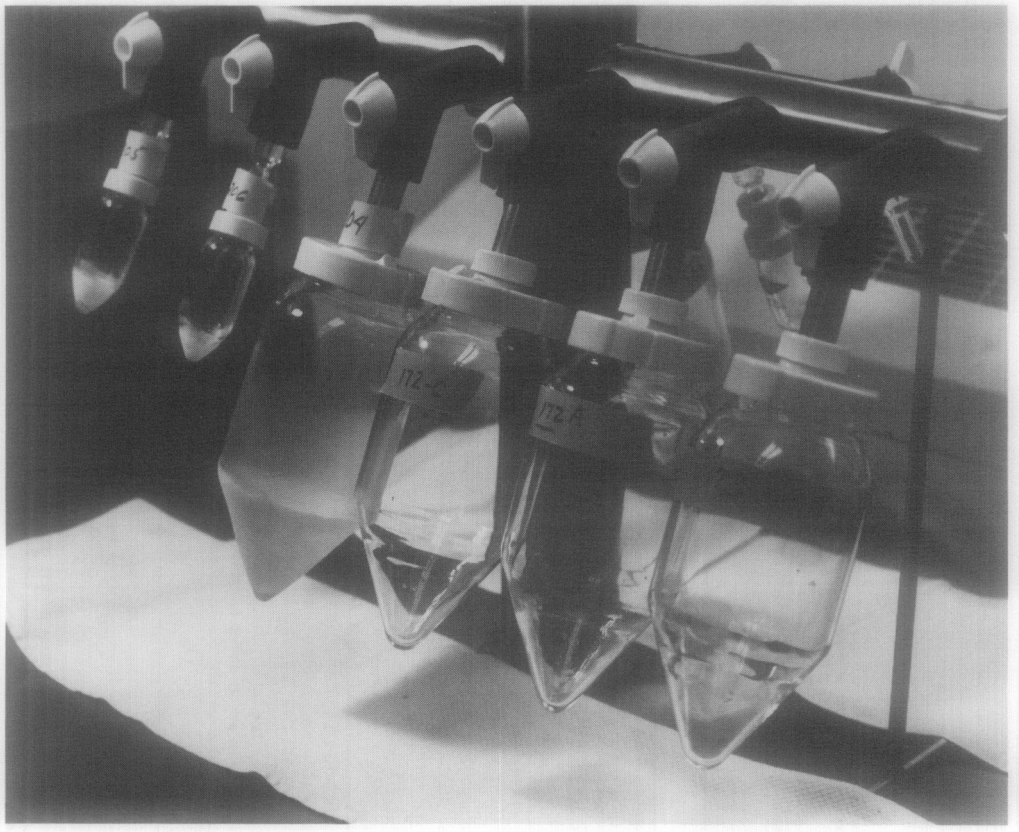

FIG. 7. Large (right) and small (left) conical lyophilization flasks used for urine protein samples. Vessels were made by attaching upper portion of funnels to Vir-Tis lyophilization flasks and drawing off the funnel stems. The original volume of large vessels was 600 ml and of the small vessel was 40 ml.

viscous sample that may clog acrylamide gels used for isoelectric focusing. These viscous samples are therefore sucked up into narrow fluorocarbon tubes and transferred to the 75-μl centrifuge tubes[9] shown in Fig. 8. The tubes are then placed in polycarbonate adapters which hold all the tubes at the same radius in a horizontal position and are centrifuged for 30 min at 20°C in a Beckman VTi 50 vertical tube rotor ($\omega^2 t = 36.80 \times 10^7$). This procedure sediments particles 26 S and larger. The K factor for this tube in the rotor described is 9.6 at 50,000 rpm (where $K = tS$, and S = sedimentation coefficient and t = time in hours). The supernatant is then analyzed using the ISO–DALT techniques previously described (2,3). This centrifugal system solves the problem in two-dimensional electrophoresis of cells and tissues of the removal of DNA and other very high molecular weight substances that otherwise plug acrylamide gels.

CONCENTRATION WITH P-6 BIO-GEL

When a dry hydrophilic gel such as P-6 Bio-Gel is added to an aqueous solution containing salts and protein, the water and salts rapidly permeate the swelling gel, leaving the protein and a fraction of the water and salts in the excluded volume. In a suitable centrifugal system, this excluded volume may be centrifuged away and recovered (18). The efficiency of this technique depends on how much the gel swells, on the size of the beads, and on the completeness of exclusion of the proteins desired. Gels

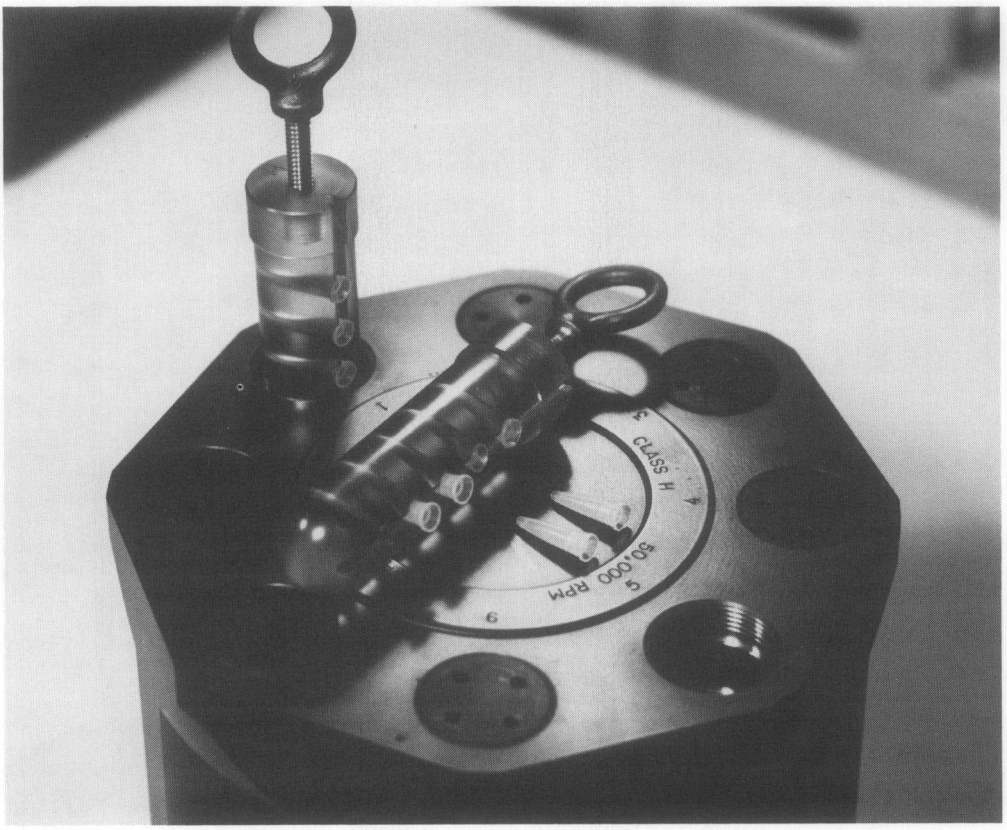

FIG. 8. Adapters and small centrifuge tubes for spinning 75-μl volumes at speeds up to 50,000 rpm and 210,000g maximum in VTi 50 vertical tube rotor. Centrifuge tubes are made by cutting off 400-μl polyethylene microfuge tubes. Adapters are of polycarbonate, density 1.2 g/cc. The threaded steel withdrawing rings shown are used to remove adapters from rotor at end of run.

with very small pores swell rapidly, but do not take up much water. More open gels take up larger amounts of water, reach equilibrium more slowly, and may be penetrated by desired protein species. The bead size affects the efficiency of centrifugal drainage of the void volume, small beads requiring a higher centrifugal force. As a compromise between these factors, we have chosen to use P-6 Bio-Gel, 50–100 mesh. One gram of dry gel swells in distilled water to give a packed volume of 5 ml after low speed centrifugation. The gel is regenerated after use by extensive washing with distilled water, followed by dehydration in absolute methanol. The gel is then dried in a vacuum oven at 36°C and reused.

Eighteen grams of dry P-6 Bio-Gel was placed in a 250-ml polyethylene beaker, and 100 ml of fresh centrifuged urine was added. The slurry was stirred briefly, and transferred with the aid of a stainless-steel spatula to a 90-mm plastic Buchner funnel[11] containing two pieces of Whatman No. 1 filter paper. The tips of the funnels were cut off to allow them to fit tightly into 250-ml disposable plastic beakers,[12] and the filter–beaker combinations were placed in modified Beckman TJ-6 centrifuge adapters as shown in Fig. 9. After centrifugation at 2000 rpm at 10° for 5 min, an average of 32 ml

[11] Nalge 4280 polypropylene Buchner funnels, for 70-mm filter paper (large) and 42.5-mm filter paper (small) available from Scientific Products, McGaw Park, Ill. 60085.

[12] Tripour polypropylene beakers, 250 and 50 ml, available from Scientific Products, Inc.

of excluded-volume concentrate was recovered. Eight grams of dry gel was then added and the process repeated, using a funnel and beaker of the same dimensions. The volume recovered from the second concentration varies between 12–15 ml. It is poured into a small 50-ml plastic beaker and 4 g of dry P-6 Bio-Gel is added with stirring. The slurry is then transferred to a small plastic Buchner funnel[11] (42.5 mm) in which have been placed two sheets of Whatman No. 1 filter paper. The lower portion of this funnel is discarded, and the upper portion set in a 50-ml plastic beaker.[12] The combination fits snugly into a 250-ml plastic beaker which in turn fits into the adapter shown in Fig. 9. The snug fit of all components described is fortunate and accidental. The third concentration step yields a final volume of 7–8 ml. If further concentration of gel exclusion is required, the very small filtration adapter LKB 2117-502 PEGG Elution Columns, LKB Instruments, Rockville, Md. shown in Fig. 9 may be used.

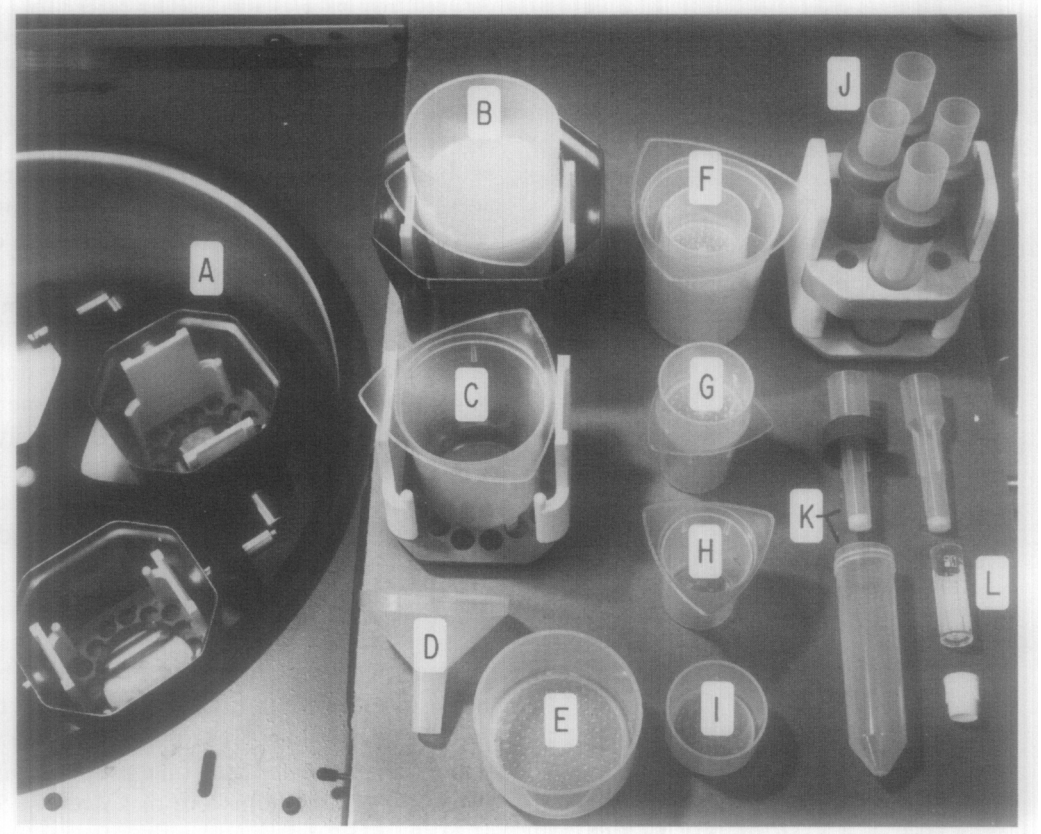

FIG. 9. Three sizes of filters and adapters used to centrifuge void-volume proteins from urine–Bio-Gel P-6 (50–100 mesh) slurrys. (A) TJ-6 centrifuge rotor with modified adapters in place. (B) Plastic Buchner funnel in plastic beaker supported by modified plastic adaptor in stainless-steel centrifuge cup. (C) Receiver beaker in plastic adapter. (D) Bottom portion of plastic Buchner funnel. Note that funnel is cut off to fit beaker. (E) Upper portion of two-piece Buchner funnel. (F) Small Buchner funnel (upper portion only) and small receiver beaker in place which fits into larger beaker such as is illustrated in (C). (G) Upper portion of small two-piece plastic Buchner funnel (I) which fits into small beaker (H). (J) Smallest centrifugal filter system in place in centrifuge adapter, composed of plastic tubes with filters (Part No. 2117-502) available from LKB and plastic centrifuge tube with 16-mm hole in cap as shown in (K). When small volumes are to be collected and frozen directly, the LKB tubes are fitted into 5-ml vials (Catalog No. 486, Walter Sarstedt, Inc., Princeton, N. J.) (L), which in turn are placed in the 50-ml tubes shown in (K).

Because the sample volume obtained after the three-step concentration with the second method is somewhat larger than that obtained with the first dialysis–lyophilization procedure, a larger column has been used for removal of salts and other low molecular weight materials. The column employed is 2.5 × 30 cm loaded with P-4 Bio-Gel (100–200 mesh) and eluted with distilled water at a flow rate of 2 ml/min. The effluent is monitored at 280 nm, and the void volume peak is collected as shown in Fig. 10A. A large fraction of the uv-absorbing material in human urine elutes in a volume greater than the total column volume (see Figs. 1 and 2) and hence appears to interact with the gel. A rather lengthy wash period is therefore required to regenerate the column if washing is done in a forward direction (Figs. 10A and C). As shown in Figs. 10B and D, the wash time may be considerably shortened if the flow direction is reversed.

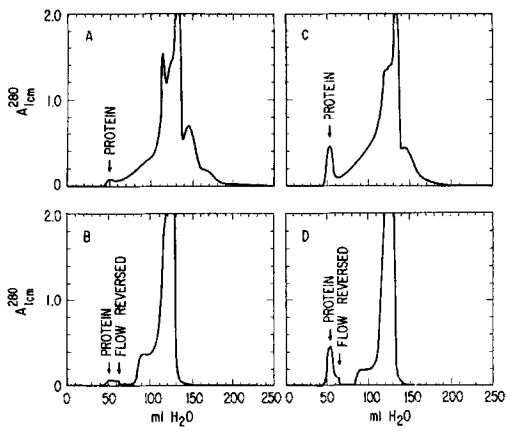

FIG. 10. Gel filtration of unconcentrated and concentrated urine samples on P-4 Bio-Gel, 100–200 mesh, as described in text. (A) Five milliliters of unconcentrated urine used as sample. Distilled water used for elution is pumped in forward direction during entire run. (B) Five milliliters of unconcentrated urine such as sample (A), with flow reversed at time indicated. Note that wash time is diminished with reverse flow washing. (C) Concentrate prepared by P-6 Bio-Gel concentration of 100 ml of the same urine used in (A) and (B), with forward flow during the entire run. (D) Repeat of (C) procedure, but with flow reversed during washing. In all experiments flow was maintained at a constant rate using a peristaltic pump.

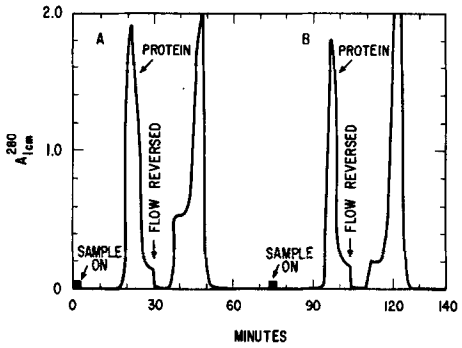

FIG. 11. Elution pattern of P-4 Bio-Gel chromatography of P-6 Bio-Gel concentrate of two urine samples obtained from patients with bladder cancer. Protein peak eluted with pump at constant flow rate. Reverse flow washing done under gravity at a higher flow rate.

A column system with suitable valving to enable forward flow for analysis and reverse flow for regeneration was therefore constructed and used routinely. The use of this system is illustrated in Fig. 11.

The collected peak volume (up to 18 ml) was lyophilized in a large conical-bottom flask, taken up in 2 ml of water for final dialysis in Spectrapor No. 3 tubing (Spectrum Medical Industries), and then relyophilized in small flasks. The sample was then prepared for ISO-DALT analysis as described for the first concentration method.

PROTEOLYTIC DEGRADATION

Small peptides present in urine may be the result of proteolytic degradation due to proteolytic enzymes present in urine. To examine this possibility, the final preparations of lyophilized protein were dissolved in 50 μl of distilled water and either frozen immediately or incubated at 37°C for 1 h. They were then brought to 8 M with dry urea, and to 2% in NP40[9] and 1% mercaptoethanol and analyzed by two-dimensional electrophoresis. No differences were noted between incubated and unincubated samples of normal human urine concentrates. Note that pathological urine samples may contain larger amounts of proteolytic enzymes and behave differently.

CONCENTRATED URINES

Some urines are naturally quite concentrated and give precipitates of urates and phosphates on cooling. In the dialysis–lyophilization method this precipitation is not a problem if the urines are promptly centrifuged at 10°C and then dialyzed. Dialysis appears to remove material faster than it can precipitate under these conditions. Similarly with the second method, few problems occur if it is carried through rapidly. If that is not possible, then the concentration of solutes may be rapidly diminished by mixing the initial 100 ml of urine with 18 g (dry) of P-6 50–100 mesh Bio-Gel which has been equilibrated with distilled water and then centrifuged to remove void volume water. When the mixture of wet gel and urine is centrifuged, the protein is recovered but more than half of the low molecular weight material is left behind in the gel. Further concentration of protein is then carried out as described using dry P-6 Bio-Gel.

ANALYSIS WITH THE ISO–DALT SYSTEM

Two-dimensional electrophoretic analysis using isoelectric focusing in the first dimension and electrophoresis in the presence of SDS in the second was done essentially as previously described (2,3). Numerous small modifications have been made in this system which will be described elsewhere.

Aliquots of the same urine sample from a male donor were concentrated by both methods and the results are shown in Figs. 12A and B. The results are almost identical and superimposable. Comparison of these urine patterns with those of normal human serum or plasma obtained from the same individual using an optical comparator suggests that less than half the spots are identical with those found in plasma.

DISCUSSION

Two methods have been developed for the concentration of human urinary proteins by a factor of over 1000 for analysis by high-resolution two-dimensional electrophoresis. The patterns obtained with the two methods are almost identical. No evidence of proteolytic degradation during incubation of concentrated samples at 37°C for 1 h was seen, suggesting that minimal protein breakdown occurred during concentration. Careful comparison of urine patterns with two-dimensional serum protein patterns from the same individuals suggested that less than half of the urinary proteins are identical with major serum proteins also identified by the ISO–DALT method (Fig. 12). Minor serum proteins, such as growth hormone, follicle stimulating hormone, luteinizing hormone, and prolactin are small enough to pass through the kidney, are in the right molecular weight range to appear on 2-D gels, and are found in a functional state in human urine (19). The kidney concentration factor (providing no specific resorption occurs) and the concentration factors for the concentration methods used here are theoretically sufficient to yield spots visible on 2-D gels stained with Coomassie blue. The identification of spot locations for protein hormones and the locations of the enzymes (20) and antigens known to occur in urine will be discussed in subsequent papers.

A major problem with hemodialysis for treatment of renal failure is the lack of data on the characteristics and functions of substances of intermediate molecular weight (~6000–30,000) These may pass unhindered through normal kidneys, but may increase in concentration in plasma during prolonged dialysis because artificial kidneys have a molecular weight cutoff lower than normal ones.

The thesis underlying this work is that human urinary proteins include a special class of indicators that will be experimentally and clinically useful. If injured cells almost invariably leak protein, a fraction of which is small enough to pass through the kidney (21,22), then tissue-specific indicators of organ damage may be expected to occur in urine. The diagnostic importance

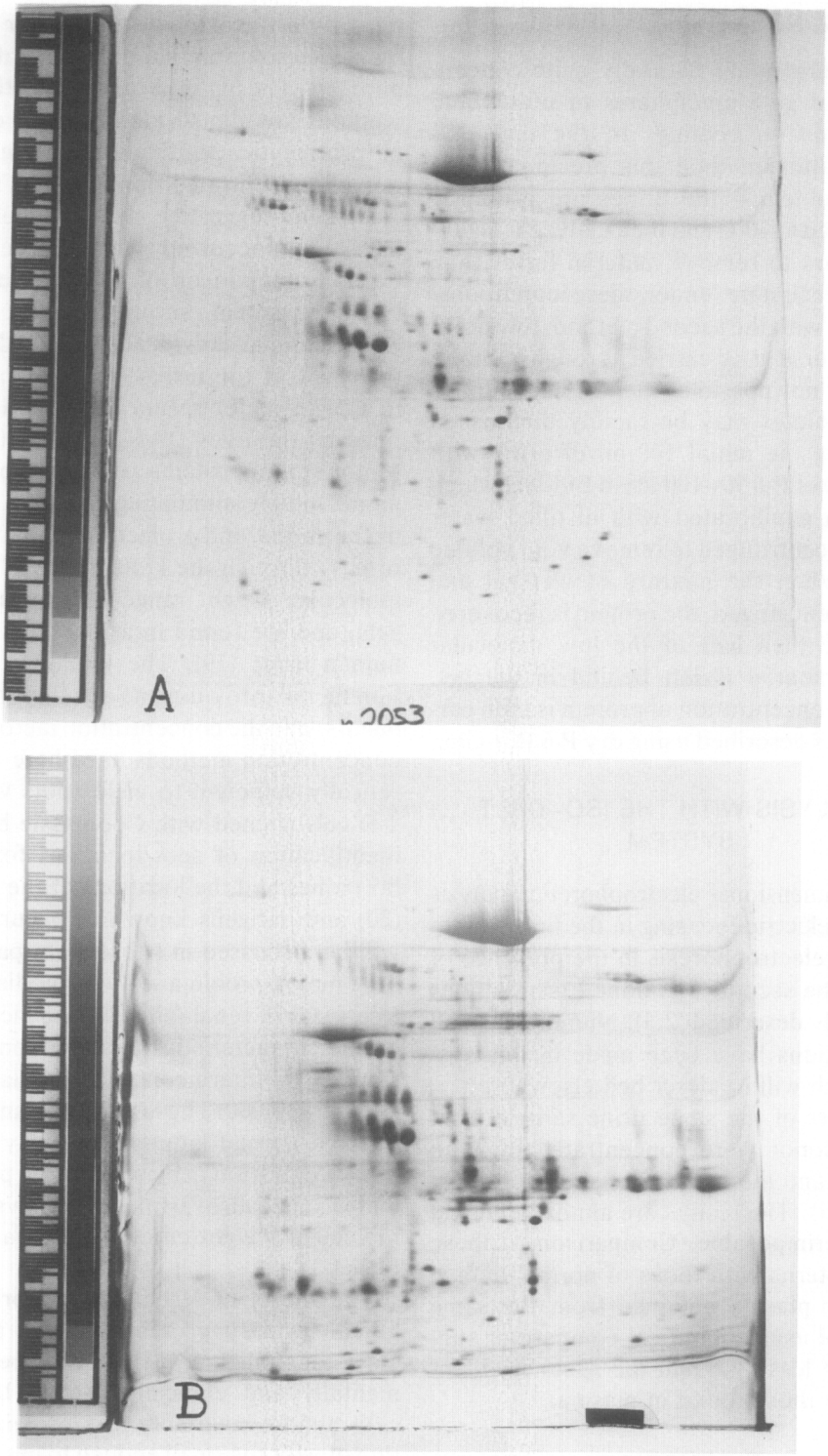

FIG. 12. Analysis of concentrates of normal male urine by high resolution two-dimensional electrophoresis using the ISO–DALT system. (A) Concentrate prepared by dialysis and lyophilization. (B) Concentrate prepared by Bio-Gel P-6 method. Acidic end of isoelectric focusing gel is to the left.

of being able to identify cell or tissue injury and the site where it occurs by a noninvasive method is self-evident. What has been less obvious is that membrane damage and consequent leakage may be a common denominator of chemical, viral, and radiation damage generally. If this is indeed true, the analysis of urinary proteins will become important in studies on the effects of environmental pollutants on exposed human populations, and in the assessment of both early and late effects of exposure to ionizing radiation (23).

The presence of tumor-associated antigens and factors in the urine of cancer patients has been repeatedly reported (24–30) and requires systematic evaluation using the analytical methods described here.

For the study of human protein variants and for attempts to measure the background mutation rate in man (31), it is important to be able to examine as many different human proteins as possible. About one-third of amino acid substitutions results in a charge change that would be detectable on two-dimensional gels. Urinary proteins are an additional and easily obtained protein mixture for human genetic studies.

The major barriers to the environmental effects, cancer, and genetic studies on human urinary proteins listed here have been lack of rapid and efficient concentration methods and lack of sensitive and high resolution analytical techniques for seeing what proteins were present. These barriers no longer exist.

REFERENCES

1. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
2. Anderson, N. G., and Anderson, N. L. (1978) *Anal. Biochem.* **85**, 331–340.
3. Anderson, N. L., and Anderson, N. G. (1978) *Anal. Biochem.* **85**, 341–354.
4. Anderson, N. L., and Anderson, N. G. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5421–5425.
5. Anderson, N. L., Eisler, W. J., and Anderson, N. G. (1979) *Anal. Biochem.*, **91**, 441–445.
6. Anderson, N. L., and Hickman, B. J. (1979) *Anal. Biochem.* **93**, 312–320.
7. Anderson, N. G., and Anderson, N. L. (1979) *Mitteilung Behring Inst.*, in press.
8. Diem, K. (ed.) (1962) Documenta Geigy Scientific Tables, 6th ed., pp. 527–528, Geigy Pharmaceuticals, Ardsley, N. Y.
9. Free, A. H., and Free, H. M. (1975) Urinalysis in Clinical Laboratory Practice, CRC Press, Cleveland, Ohio.
10. Hamilton, P. B., and Lou, M. F. (1972) *Biochem. Med.* **6**, 192–204.
11. McGarry, E., Sehon, A. H., and Rose, B. (1955) *J. Clin. Invest.* **34**, 832–844.
12. Alderson, G., Ward, W. H., and Fevold, H. L. (1955) *J. Biol. Chem.* **157**, 43–58.
13. Miyasato, F., and Pollak, V. E. (1966) *J. Lab. Clin. Med.* **67**, 1036–1043.
14. Berggard, I. (1961) *Clin. Chem. Acta* **6**, 413–429.
15. Carrell, S., and Theilkaes, L. (1973) *Nature (London)* **242**, 609–610.
16. Harris, R. J. C. (1954) Biological Application of Freezing and Drying, Academic Press, New York.
17. Morris, C. J. O. R., and Morris, P. (1976) Separation Methods in Biochemistry, pp. 923–959, Pitman, London.
18. Fischer, L. An Introduction to Gel Chromatography. (1969) North-Holland/American Elsevier, New York.
19. Kutsky, R. J. (1973) Handbook of Vitamins and Hormones. Van Nostrand Reinhold, New York.
20. Wolf, P. L., Williams, D., and Von der Muechll, E. (1973) Practical Clinical Enzymology. pp. 323–341, Wiley, New York.
21. Boss, J. H., Dishon, T., Durst, A., and Rosenman, E. (1973) *Isr. J. Med. Sci.* **9**, 490–508.
22. Greene, E. L., Halbert, S. P., and Pallavicini, J. C. (1971) *Int. Arch. Allergy* **40**, 861–880.
23. D'Addabbo, A., Viterbo, F., and Fanfani, G. (1966) *Strahlentherapie* **130**, 426–431.
24. Gross, S., Galicka, N., Grabarczyk, M., Giannini, M., Burzynski, S., and Stolzmann, Z. (1977) *Clin. Chem.* **23**, 148–149.
25. Okon, E., Rosenman, E., Dishon, T., and Boss, J. H. (1973) *Brit. J. Cancer* **27**, 362–369.
26. Hall, R. R., Lawrence, D. J. R., Darcy, D., Stevens, U., James, J., Roberts, S., and Neville, A. M. (1972) *Brit. Med. J.* **1972**, v. **3**, 609–611.
27. Carrel, S., and Theilkaes, L. (1973) *Nature (London)* **242**, 609–610.
28. Gozzo, J., Daley, M., Schlesinger, R., and Monaco, A. (1974) *Fed. Proc.* **33**, 805.
29. Tischendorf, F. W., Ledderose, G., Wilmanns, W., Tischendorf, M. M., and Tischendorf, G. W. (1972) *Nature* **245**, 379–380.
30. Rudman, D., del Rio, A., and Akgun, S. (1969) *Amer. J. Med.* **46**, 174–187.
31. Neel, J. V., Tiffany, T. O., and Anderson, N. G. (1973) *in* Environmental Chemical Mutagens (Hollaender, A., ed.), Vol. 3, chap. 28, pp. 105–150, Plenum, New York.