Human Glomerular Basement Membrane. Selective Solubilization with Chaotropes and Chemical and Immunologic Characterization of Its Components†

Hans Marquardt,*‡ Curtis B. Wilson, and Frank J. Dixon

ABSTRACT: About 5% of the cases of human glomerulonephritis are caused by antibodies which react with antigen(s) in the glomerular basement membrane (GBM). To permit elucidation of these antigen(s) human GBM was solubilized with chaotropes. Extraction with 3 M KCl resulted in the solubilization of 8.7%, 3.5 M KBr 25.0%, and 0.4 M sodium trichloroacetate 9.5% of the total protein of the GBM. The resultant GBM proteins were analyzed by sodium dodecyl sulfate gel electrophoresis in polyacrylamide to compare their subunit patterns and to determine their molecular sizes; in addition, they were immunochemically characterized with a rabbit anti-GBM antiserum. At least four distinct GBM antigens were detectable in each extract; however, their concentrations varied with the chaotrope used. The electrophoretic protein subunit patterns of each of the three chaotropic extracts of GBM had three prominent bands and at least 7–19 less intense bands with an apparent molecular size range of 28,000 and 94,000 daltons. The chaotropic extracts were antigenically reactive with serum Ig and Ig eluted from kidneys of patients with anti-GBM nephritis, as demonstrated by a radioimmune assay. This assay provided a relative quantitation of the chaotrope-solubilized antigen(s).

Antibodies which react with antigen(s) present in glomerular basement membrane (GBM)[1] cause roughly 5% of human glomerulonephritides (Wilson and Dixon, 1973). The present studies were intended to define chemically and immunologically the nature of this nephritogenic antigen(s) (NAg) in order to gain a better understanding of the immunopathogenesis of this disease.

To isolate the human NAg we undertook the following: (1) to obtain GBM and to evaluate its purity by chemical and ultrastructural analysis, (2) to solubilize GBM and to obtain the NAg with a minimal concentration of contaminant nonantigenic GBM proteins, and (3) to purify and to characterize chemically the NAg that specifically reacts with human anti-GBM antibodies.

A series of procedures has been introduced to solubilize GBM: urea in the presence or absence of disulfide cleaving compounds (Kefalides, 1972), proteolytic digestion with enzymes of unrestricted (Kefalides and Winzler, 1966; Spiro, 1967) or restricted specificity (Kefalides, 1972; Huang and Kalant, 1968; Marquardt *et al.*, 1973), and detergents in the presence of mercaptoethanol (Hudson and Spiro, 1972; Myers and Bartlett, 1972). Although these methods release soluble protein from GBM, they are nonselective, releasing materials which are chemically extremely complex. The present work is concerned with the more selective solubilization of GBM by chaotropes. Anti-GBM antibodies eluted from a patient's kidneys were used as a biological tool for detecting and quantitating the NAg.

Materials and Methods

Preparation of Glomeruli. The isolation procedure for glomeruli is based on the method of Krakower and Greenspon (1951) as modified by Spiro (1967). Normal human kidneys were obtained at autopsy, then frozen; the cortex was removed and sliced thin with a razor blade; the medulla was discarded. Portions of cortex (50 g) were then gently forced through a 115 mesh stainless steel sieve (pore size 0.124 mm) and washed with cold PBS (0.01 M sodium phosphate buffer - 0.15 M NaCl, pH 7.2). The glomerular suspension of 200 g of cortex which passed through the sieve was poured on a 60 mesh sieve (pore size 0.246 mm) placed above a 150 mesh sieve (pore size 0.104 mm). The sieves were washed with approximately 10 l. of ice-cold PBS in the same sequence, until the material retained on the 150 mesh sieve was free of cells and small tissue fragments and consisted essentially of glomeruli when examined under the microscope. The glomeruli on the 150 mesh sieve were gently recovered as a suspension in PBS and centrifuged at 500*g* at 4° for 15 min, and the glomerular pellet was frozen for future sonic disruption. The material retained on the 60 mesh sieve consisting primarily of large tissue fragments was discarded.

Preparation of GBM. Basement membrane was obtained from isolated glomeruli by ultrasonic disruption. Glomerular pellets derived from 2000 g of kidney cortex were suspended in 80 ml of 1 M NaCl in a 100-ml Rosett cooling cell (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) and immersed in an alcohol–water mixture at −10° to maintain a temperature of 7° or less in the sonication cell. A Branson Sonifier with a 0.5-in. stainless steel probe was used (Model S125, Heat Systems-Ultrasonics, Plainview, N. Y.). The sonicator was set at power output No. 6 and used in 1-min bursts for a total of 6–8 min. Cooling time was allowed between each sonic burst. The extent of glomerular disruption was followed microscopically. The sonicated material was repeatedly

† From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received May 1, 1973.* This is publication number 698 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. Supported by U. S. Public Health Service Grant AI-07007, Atomic Energy Commission Contract AT(04-3)-410, and U. S. Public Health Service Contract PH 43-68-621.

‡ Recipient of Fellowship from the Kidney Foundation of Southern California.

[1] Abbreviations used are: GBM, glomerular basement membrane; C-S-GBM, collagenase-solubilized GBM; NAg, nephritogenic antigen (s); Ig, immunoglobulin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; aGBM, rabbit anti-GBM antiserum.

centrifuged at 210*g* for 2 min to separate the basement membrane from insufficiently disrupted debris and washed five times with ice-cold 1 M NaCl at 1600*g* for 10 min, followed by five washes with ice-cold distilled water to remove salt. The resultant GBM was suspended in distilled water and kept frozen. An aliquot was lyophilized and then dried for several days at room temperature in a desiccator containing anhydrous calcium sulfate (Drierite) and phosphorus pentoxide to determine the amount of dry GBM.

Electron Microscopy. The basement membranes were sampled after washing with distilled water, fixed in 1% osmic acid, embedded in Vestopal W, and stained with uranyl acetate and lead citrate.

Extraction of Soluble GBM Antigens. DIGESTION WITH COLLAGENASE (C-S-GBM). GBM was rendered soluble by prolonged incubation with purified collagenase from *Clostridium histolyticum* according to the method previously described (Marquardt *et al.*, 1973). Collagenase digestion and dialysis effectively solubilized over 98% of the GBM.

EXTRACTION WITH CHAOTROPIC AGENTS. Effects of Chaotropic Concentrations on the Yield of Solubilized GBM. GBM (10 mg/ml) suspended in water was preincubated at 34° for 15 min. At time zero a concentrated solution (*e.g.*, 4 M sodium trichloroacetate in 0.05 M Tris-HCl buffer (pH 8.0, 8 M LiBr) or crystalline (*e.g.*, KCl, KBr) chaotropic agents were added to the desired molarity and incubated with constant stirring. After 24 hr, the reaction mixture was clarified by centrifugation at 105,000*g* for 2 hr at 4°, and the amount of solubilized material determined. The supernatants were then dialyzed against TBS (0.05 M Tris-HCl buffer–0.14 M NaCl, pH 8.0), and the volume was reduced by pressure filtration (Amicon Corp., Lexington, Mass., type UM-10).

Factors Influencing the Yield of Solubilized GBM. (1) Time and Temperature. Suspensions of GBM and chaotropic agents were prepared as in the above paragraph, incubated with constant stirring at 34 or 11°. At various time intervals, aliquots of the reaction mixture were taken, clarified by centrifugation, and the amount of solubilized material was determined.

(2) pH. GBM was incubated with constant stirring at 34° for 24 hr in solutions containing 3.5 M KBr and either 0.02 M sodium acetate buffer (pH 3.8–6.2) or 0.02 M Tris-HCl buffer (pH 6.6–9.2). The residue was separated by centrifugation and the amount of solubilized material in the supernatant was determined.

Sodium Dodecyl Sulfate Gel Electrophoresis. Extracts obtained by treatment of GBM with chaotropic agents were dialyzed against water, lyophilized and subjected to sodium dodecyl sulfate gel electrophoresis on polyacrylamide according to the method of Weber and Osborn (1969). Samples containing 100 μg of protein were dissolved and incubated in 0.001 M sodium phosphate buffer (pH 7.0), containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 1 hr at 37°. Acrylamide gels (10%) containing 0.2% methylenebisacrylamide were used. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid overnight and stained with Coomassie Brilliant Blue.

Double Diffusion Analyses. Ouchterlony analyses were performed in 0.6% agarose according to methods previously described (Tan and Kunkel, 1966). Petri dishes (100 × 15 mm) were filled with 25 ml of 0.6% agarose in PBS with 0.1% sodium azide added. The wells were 8 mm in diameter and spaced 4 mm apart.

Preparation of Antisera. ANTISERUM TO GBM (aGBM). The antiserum to human GBM was prepared in rabbits by repeated injection of particulate GBM. The antigen was incorporated into complete Freund's adjuvant (Arlacel–Bayol F–PBS containing 50 mg of GBM/ml and 4 mg of *Mycobacterium tuberculosis* H37RA/ml, 1.5:8.5:10). Initially, animals (2.5- to 3.5-kg New Zealand white rabbits) were injected with 0.5 ml of the emulsion into each footpad; subsequent injections of 0.5 ml at four separate subcutaneous sites were made on days 14 and 28. Animals were bled and antisera were harvested 10 days after the last injection.

The antiserum was absorbed with human plasma and fractionated at 50% saturation with ammonium sulfate (Marquardt *et al.*, 1973).

ABSORPTION EXPERIMENTS. To characterize the chaotropic extracts immunochemically, aGBM was absorbed with the 3 M KCl extract, 3.5 M KBr extract, and 0.4 sodium trichloroacetate extract. These absorption experiments were performed as follows: 0.1 ml of KCl extract (219 μg of protein), 0.1 ml of KBr extract (233 μg of protein), and 0.1 ml of sodium trichloroacetate extract (215 μg of protein) were added to 0.2 ml of aGBM in separate experiments, incubated for 0.5 hr at 37°, followed by incubation at 4° overnight, and centrifuged (2100*g*, 20 min, 4°) prior to testing by double-diffusion experiments.

Elution of Anti-GBM Antibodies from Diseased Human Kidneys. Anti-GBM antibodies were eluted from the kidneys of patients with anti-GBM nephritis by the acid–citrate technique described previously (Lerner *et al.*, 1967).

Radioimmune Assay of NAg in Soluble Chaotropic Extracts of GBM. NAg was assayed by a double antibody technique as described previously (Marquardt *et al.*, 1973). The quantitation of NAg in chaotropic extracts was based on the ability of NAg to inhibit the binding of [^{125}I]C-S-GBM. An amount of anti-GBM antibodies (renal eluates) sufficient to bind 50% of the total reactive [^{125}I]C-S-GBM was used.

The inhibition of binding of NAg in [^{125}I]C-S-GBM by increasing amounts of added NAg in C-S-GBM, KCl extract, KBr extract, sodium trichloroacetate extract was determined in the following way. Serial twofold dilutions of C-S-GBM or chaotropic extracts covering the range from approximately 0.1- to 100-μg total protein, 0.5-μg total protein [^{125}I]C-S-GBM, and 1 μg of eluted anti-GBM antibody were incubated in a reaction volume of 180 μl for 15 hr at 4°. IgG-bound [^{125}I]C-S-GBM was then precipitated with rabbit anti-human IgG. Total precipitation was monitored by the inclusion of ^{131}I-labeled human IgG in each reaction. The washed precipitate was counted in a dual-channel sodium iodide scintillation counter, and the per cent of [^{125}I]C-S-GBM precipitate was calculated using the amount of control precipitate by the secondary antibody alone.

Quantitative Determinations. Total phosphorus was analyzed by the ascorbic acid method (Chen *et al.*, 1956) after hydrolysis of the membranes with nitric acid (Baginski *et al.*, 1967).

Samples for amino acid analyses were hydrolyzed at 110° for 24 hr in constant-boiling 6 N HCl. Analyses were carried out by Dr. Edward J. Miller as previously described (Miller, 1972).

Total protein was determined by Lowry's method (Lowry *et al.*, 1951) using human complement (C3) as a standard.

Results

Preparation of GBM. The isolated human glomeruli showed a high degree of purity when examined under the microscope (Figure 1) and contained less than 0.5% tubular fragments

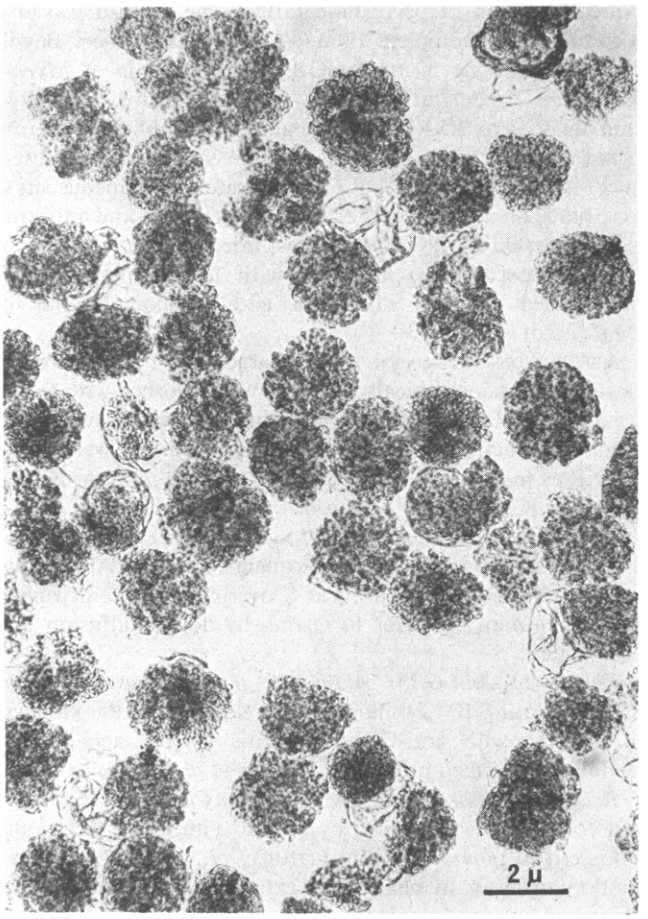

FIGURE 1: Photomicrograph of isolated human glomeruli. Light microscopy: ×55.

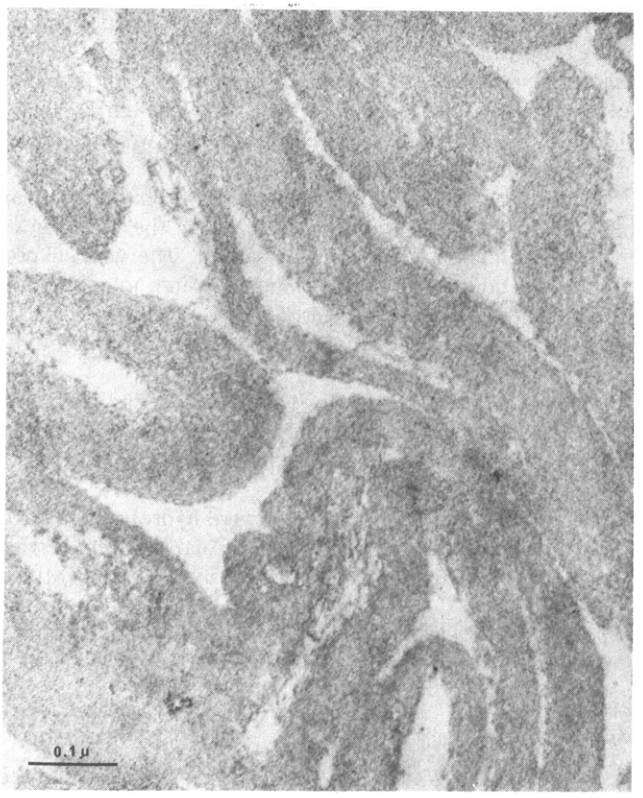

FIGURE 2: Electron micrograph of isolated human glomerular basement membrane (taken by Dr. Giorgio Tonietti). ×117,450.

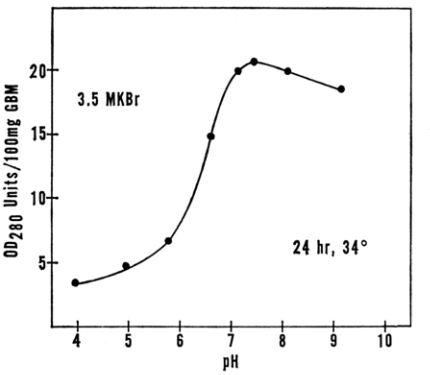

FIGURE 3: Effect of pH on the solubilization of human GBM with 3.5 M KBr at 34° for 24 hr.

and less than 4% Bowman's capsules/100 glomeruli. The purity of GBM obtained from the isolated glomeruli after ultrasonic disruption was evaluated by chemical analysis and by ultrastructure analysis with the electron microscope. The membranes appeared amorphous, similar to those seen in intact glomeruli. Cell fragments were not detectable (Figure 2). The average total phosphorus was 0.04%, indicating negligible quantities of DNA, RNA, and phospholipids. The yield of dried GBM was, on the average, 200 mg/1000 g of kidney cortex.

Extraction of GBM with Chaotropic Agents. EFFECTS OF CHAOTROPIC CONCENTRATIONS ON THE YIELD OF SOLUBILIZED GBM. Previous studies have shown that human GBM can be rendered soluble by extraction with chaotropic agents (Marquardt and Wilson, 1972). For example, about 9.8% of the GBM can be solubilized with 3.5 M KBr in one extraction step. Thus, an extensive effort was made to develop a simple, reproducible, and selective extraction method which yielded sufficiently large amounts of soluble basement membrane protein which were antigenically reactive with patients' eluted anti-GBM antibodies. Three chaotropes (Cl^-, Br^-, CCl_3COO^-) differing in their relative chaotropic potency have been used.

KCl. Experiments to determine the optimal extraction conditions revealed that 3 M KCl solubilized more GBM proteins than either 0.5,1.0, or 2.0 M. Treatment of GBM with 3 M KCl at neutral pH and 34° for 24 hr solubilized 3% of the total protein of the dry membrane.

KBr. Similar results have been obtained for the solubilization of GBM with KBr. The amount of GBM solubilized by KBr depended on the concentration of the chaotrope used and reached its maximum with 3.5 M KBr. Extraction of GBM with 3.5 M KBr for 24 hr at 34° rendered 18 OD units (280 nm)/100 mg of GBM soluble or 9.8% of the total protein of the dry membrane.

Sodium Trichloroacetate. It has been demonstrated that haloacetates destabilize biomembranes and multicomponent systems (Hanstein *et al.*, 1971). Their water structure breaking properties are similar to those of inorganic chaotropes such as Br^- or Cl^-. The order of potency of these chaotropic ions is generally $CCl_3COO^- \gg Br^- > Cl^-$. Trichloroacetate, at pH 8.0, was used to study the effect of a strong chaotrope on the solubilization of GBM. The amount of solubilized material was optimal at a 0.4 M concentration of the chaotrope and then slightly decreased at higher concentrations of sodium trichloroacetate, reflecting an irreversible denaturation of GBM proteins. Treatment of GBM with 0.4 M sodium trichloroacetate, at pH 8.0, for 24 hr at 34° solubilized 9.4 OD

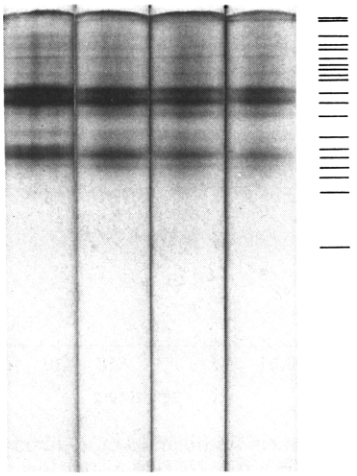

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of human GBM protein subunits on polyacrylamide. Gels contained GBM materials solubilized with KBr at different concentrations for 24 hr at 34°.

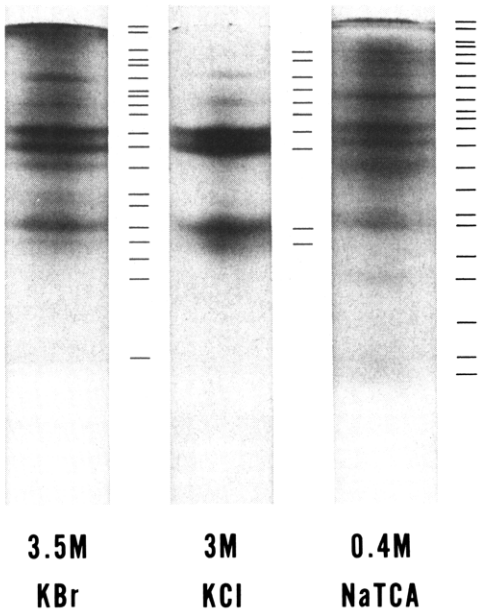

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis of human GBM protein subunits on polyacrylamide. Gels contained GBM materials solubilized with chaotropic agents under optimal conditions (34°, 48 hr).

units (280 nm)/100 mg of GBM or 5.1% of the total protein of the dry membrane and was therefore less effective at its optimal concentration than a weaker chaotrope such as Br^-. That the difference in the solubilization of GBM by KBr and sodium trichloroacetate was not caused by the different cations has been ascertained by using LiBr as chaotropic salt. It was found that solubilization with LiBr is as efficient as KBr.

OTHER FACTORS INFLUENCING THE YIELD OF SOLUBILIZED GBM. Our experiments demonstrated that extraction of GBM with 3.5 M KBr solubilized more protein than either 3 M KCl or 0.4 M sodium trichloroacetate. To improve the extraction method with 3.5 M KBr further, we have studied factors influencing the yield of solubilized material.

Time and Temperature. At the optimal concentration of the chaotropic agent, the amount of solubilized GBM was affected by the temperature of the reaction mixture and the extraction time. The solubilization at 34° in the presence of 3.5 M KBr was more effective than at lower temperatures (*e.g.*, 11°), and a 48-hr extraction time yielded a maximum of solubilized material.

pH. Coulombic forces can contribute significantly to the stability of a membrane. Since chaotropes are neutral salts, their ionization would not be affected by pH changes. Therefore, any pH effect on the chaotropic induced destabilization of a membrane reflects a contribution of electrostatic forces for its stability.

The effect of pH on the yield of solubilized GBM is shown in Figure 3. The solubilization of GBM in the presence of 3.5 M KBr is most extensive at pH 7.3, becomes markedly inhibited at lower pH values but less at higher pH values.

Treatment of GBM with 0.4 M sodium trichloroacetate (pH 8.0) and 34° resulted in the extraction of 9.5% of the total protein, with 3.5 M KBr (pH 7.3) and 34° solubilized 25% of the total protein and 3.0 M KCl at 34° was capable of solubilizing 8.7% of the total protein.

Proteins in the Soluble Extracts of GBM. SODIUM DODECYL SULFATE GEL ELECTROPHORESIS. Figure 4 shows the electrophoretic protein subunit patterns obtained from GBM solubilized with 1, 2, 3, and 3.5 M KBr. Comparison of the gels showed no differences in the distribution of protein in molecular weight nor in the stained intensity of the bands. Similar results but different subunit patterns have been obtained with KCl and sodium trichloroacetate extracts. However, as seen in Figure 5, the subunit patterns of the three different chaotropic extracts of GBM showed differences in the distribution of protein in molecular weight. Each extract had three prominent bands and at least 7–19 less intense bands with an apparent molecular size range of 28,000–94,000 daltons. These molecular weights were estimated according to Weber and Osborn (1969) from their relative mobilities on polyacrylamide gels as compared to marker proteins and interpolating the molecular weights from plots of log molecular weight *vs.* relative mobility. They should be considered to be only approximations.

DOUBLE-DIFFUSION ANALYSES. For the immunochemical characterization of the antigens in chaotropic extracts obtained from GBM, a rabbit anti-GBM antiserum (aGBM) was used. Gel diffusion analysis revealed the presence of at least four different GBM antigens in the various extracts, as shown in Figure 6A.

These four antigens were present in KCl extract, KBr extract, and sodium trichloroacetate extract, since the reactivity of aGBM to these four antigens could be completely absorbed in antigen excess by any one of these extracts. However, the concentrations of at least one of the four antigens appeared to be less in the KCl extract than in the others. This could be demonstrated by absorbing aGBM partly with KCl extract (I), KBr extract (II), and sodium trichloroacetate extract (III) and testing these absorbed antisera by Ouchterlony analysis in separate experiments using KCl extract, KBr extract, and sodium trichloroacetate extract as antigenic extracts. Precipitin lines observed between partially absorbed aGBM antisera (I, II, and III) and chaotropic extracts are illustrated in Figure 6B–D and show two reactive antibodies in the KCl-absorbed serum and only one line in the others. That the different chaotropes do not cause characteristic alterations in the antigens is suggested by the lack of spurring in Figure 6A. This is seen more clearly in Figure 6B–E where there is an apparent identity of one of the antigens in all three extracts

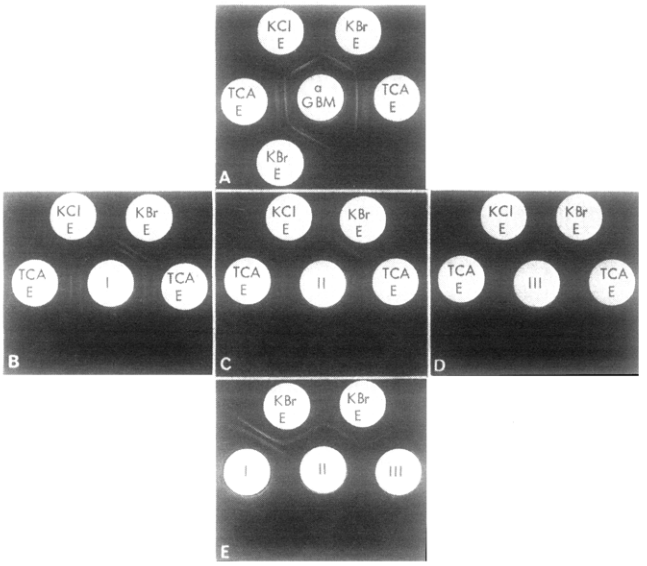

FIGURE 6: Double diffusion study of chaotropic extracts derived from human GBM with a rabbit anti-GBM antiserum (aGBM): (I) aGBM partly absorbed with KCl E (extract); (II) aGBM partly absorbed with KBr E; (III) aGBM partly absorbed with sodium trichloroacetate (NaTCA) E (TCA E = NaTCA E).

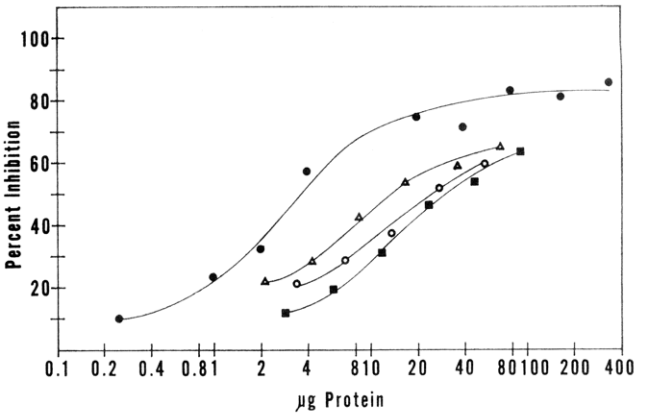

FIGURE 7: Inhibition curves obtained by addition of cold C-S-GBM (●), KBr E (△), KCl E (○), NaTCA E (■) to a constant amount (0.5-μg total protein) of [^{125}I]C-S-GBM, and (1 μg) of renal eluate antibody from patient S.H.

which reacted with the unabsorbed antibody in each absorbed aGBM.

AMINO ACID ANALYSES. The amino acid composition of the peptide material extracted with chaotropes is given in Table I and is compared with the composition of particulate human GBM. Noteworthy for the amino acid composition is the occurrence of substantial amounts of hydroxyproline and hydroxylysine in GBM and the chaotropic extracts, suggesting that collagen must be one of the components. From the data obtained, it can be concluded that the extraction of collagen or "collagen-like" material is favored more by 3 M KCl than 0.4 M sodium trichloroacetate, assuming that the hydroxyproline and hydroxylysine content reflects a measurement of the amount of collagen in a tissue or tissue extract.

TABLE I: Amino Acid Composition of Human GBM and Chaotropic Extracts from Human GBM.

	Residues/1000 Residues			
Amino Acid	GBM	KCl E[a]	KBr E	NaTCA[b] E
3-Hydroxyproline	18.4	0.6	0.4	0.5
4-Hydroxyproline	84.6	96.2	84.3	48.4
Aspartic acid	57.7	54.4	60.8	67.2
Threonine	32.5	25.0	27.5	38.0
Serine	45.7	36.9	39.3	47.3
Glutamic acid	86.7	84.2	91.7	102.8
Proline	79.0	65.5	68.6	76.7
Glycine	225.4	308.4	273.6	207.2
Alanine	53.7	38.9	44.1	57.1
Half-cystine	17.2	10.9	18.1	30.8
Valine	36.6	24.3	34.6	46.6
Methionine	14.6	15.1	13.8	13.9
Isoleucine	33.7	35.2	33.1	31.7
Leucine	66.6	65.4	65.1	72.1
Tyrosine	14.7	10.4	14.8	19.0
Phenylalanine	30.1	28.7	28.6	31.1
Hydroxylysine	29.9	46.6	35.4	23.6
Lysine	14.6	11.2	16.1	20.2
Histidine	16.9	9.9	13.0	18.0
Arginine	41.4	32.2	37.1	48.0

[a] E = extract. [b] NaTCA = sodium trichloroacetate.

The amino acid composition of extracts obtained from GBM by using a strong chaotrope (sodium trichloroacetate) differs from the material obtained from GBM by extraction with a weaker chaotrope (KCl). The former contains less hydroxyproline, hydroxylysine, and glycine but significantly more cystine, aspartic acid, glutamic acid, lysine, arginine, tyrosine, leucine, valine, and alanine, suggesting a favored solubilization of noncollagenous protein rich in polar and hydrophobic amino acids by sodium trichloroacetate.

Radioimmune Assay of NAg in Soluble Chaotropic Extracts of GBM. The inhibition curves (Figure 7) obtained by addition of cold C-S-GBM or chaotropic extracts to a constant amount of [^{125}I]C-S-GBM and specific anti-GBM antibodies from a patient's renal eluate demonstrated that the assay was capable of detecting the NAg and of following its isolation and purification from GBM extracts. It showed that the KBr extract was more efficient in inhibiting the precipitation of NAg in [^{125}I]C-S-GBM by anti-GBM antibodies than the KCl or sodium trichloroacetate extracts. Relative inhibition of a specific immunologic reaction by chaotropic extracts is an indirect measurement of the concentration of NAg in these extracts and indicates that this antigen is favorably solubilized by 3.5 M KBr.

Discussion

The antigen(s) that reacts with anti-GBM antibodies in human glomerulonephritis can be isolated only after solubilization of normal human GBM. Previous studies on GBM had demonstrated that collagenase was most effective for the solubilization of the GBM. However, this crude extract proved to be chemically extremely complex although antigenically reactive with anti-GBM antibodies from patients' renal eluates (Marquardt *et al.*, 1973). The present studies have shown that chaotropic agents can be used to solubilize the NAg selectively from GBM.

The mechanism of action of chaotropic agents has been discussed previously (Hatefi and Hanstein,, 1969, 1973). In

brief, most membranes and multicomponent systems are stabilized by hydrophobic interactions among proteins. Other stabilizing forces may be hydrogen bonds, coulombic effects, disulfide bonds, and London dispersion forces. The strength of hydrophobic interaction is directly related to the ordered structure of the surrounding water and to the large entropy decrease involved in transferring apolar groups of membranes from a lipophilic surrounding to water. Chaotropic ions disorder water structure and therefore lower the thermodynamic barrier for the transfer of apolar groups of membranes to the aqueous phase, weaken hydrophobic interactions, and lead to membrane destabilization. Generally, ions with low charge density (*e.g.*, large radius and single, mostly negative charge) are chaotropic.

If coulombic forces contribute significantly to the interaction among proteins in a membrane, then a weakly chaotropic anion with relatively high charge density (*e.g.*, Cl^-) should be more effective for destabilizing this membrane than a strong chaotrope. If both electrostatic and hydrophobic forces contribute to the stabilization of a membrane, then it might be expected that chaotropes with intermediate charge densities (*e.g.*, Br^-) would be more effective in destabilizing the native structure of a membrane than either very weak or very potent chaotropes.

KBr at its optimal concentration is more effective in solubilizing the membrane and in releasing the antigen from the membrane than a very strong chaotrope, such as trichloroacetate, suggesting that coulombic forces contribute significantly to the stabilization of the GBM and to the interaction of NAg with the remaining peptide portion of the basement membrane. The effect of pH on the chaotropic induced destabilization of GBM is in agreement with this assumption. Chaotrope induced resolutions of membranous systems with qualitatively similar time and temperature characteristics have been discussed (Davis and Hatefi, 1969).

Chaotropes only partly solubilized the GBM and released the NAg less efficiently than collagenase. A possible explanation is suggested. The antigen which specifically reacts with anti-GBM antibodies from patients' renal eluates is liberated quantitatively from the remaining residue of the GBM after extraction with chaotropes by digestion with collagenase (H. Marquardt and F. J. Dixon, 1973, unpublished data). Separation of this digest by exclusion chromatography on Bio-Gel A-1.5 m revealed the presence of antigenically reactive material in each fraction. Reduction and alkylation of the high molecular weight fraction resulted in a marked decrease in its size, as demonstrated by exclusion chromatography. The antigenicity of the NAg was, however, destroyed. These experiments clearly indicate that disulfide bonds stabilize the GBM significantly and possibly prevent its complete solubilization by chaotropes.

Analysis of solubilized basement membrane proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed extreme size heterogeneity for the KBr and the sodium trichloroacetate extracts. However, the protein subunit composition of the KCl extract resolved into only ten different components. Varying concentrations of chaotropes yield identical subunit patterns, indicating that the heterogeneity of solubilized GBM proteins was not a result of the extraction method employed.

Extracts of the three chaotropes displayed their own unique protein subunit patterns. However, comparison of the three chaotropic extracts revealed that all the prominent bands and most of the less intense bands had identical relative mobilites, suggesting that these subunits were identical in size and not the product of a random degradation of the peptide portion by either chemical or proteolytic treatment.

Immunochemical analyses of the chaotropic extracts with a rabbit anti-GBM antiserum absorbed with human plasma demonstrated that solubilization of GBM with KCl, KBr, or sodium trichloroacetate under a variety of conditions released at least four distinct antigens which were immunologically identical and present in each extract.

The antigens detected by the heterologous anti-GBM antiserum are localized in the noncollagenous portion of the GBM. This could be demonstrated by Ouchterlony analysis using comparable concentrations of purified human skin collagen which did not react with the antiserum. Cross-reactivity between skin and GBM collagen has been recently established (Rothbard and Watson, 1972).

Amino acid (or carbohydrate) analyses of electrophoretically and immunologically heterogeneous basement membrane extracts provides only limited information as to its composition. However, there were significant and reproducible differences in the contents of cystine and of polar and hydrophobic amino acid residues among the various chaotropic extracts. Assuming that most of the cystine residues in the sodium trichloroacetate extract are involved in disulfide cross-links would explain its extremely complex protein subunit composition on polyacrylamide gels after reduction of disulfide bridges.

Noteworthy for the amino acid composition of the KCl extract is the occurrence of substantial amounts of hydroxyproline, hydroxylysine, and glycine, suggesting that collagen might be one of the major components. On the other hand, the KCl extract contained markedly less proline and alanine, significantly more isoleucine and leucine, cystine, and aromatic amino acids, particularly tyrosine, than interstitial collagens. In addition, the electrophoretic subunit pattern of the KCl extract suggests that its components are smaller in size than isolated α chains of skin collagen. Purification studies are now in progress to determine the homology of "collagen-like" basement membrane peptides with well-characterized CNBr peptides derived from vertebrate skin collagens.

These studies clearly established the presence of soluble antigen(s) in extracts of GBM which specifically reacts with anti-GBM antibodies from a patient's renal eluate, as demonstrated by a radioimmune assay. The specificity of antibodies in such eluates has been established (Lerner *et al.*, 1967; Koffler *et al.*, 1969; McPhaul and Dixon, 1970) and the specificity of this assay has been shown (Marquardt *et al.*, 1973). The radioimmune assay is based on competition of labeled standard antigen (C-S-GBM) with unlabeled test antigen for a fixed number of antibody-combining sites and has proved useful in following the isolation and purification of the NAg.

Acknowledgments

The authors thank Dr. Hanstein and Dr. Hatefi for helpful advice, Miss Janis Tobin and Miss Ricki Hammond for excellent technical assistance, Mrs. Phyllis Minick for editorial advice, Mrs. Phyllis Keim for preparing the manuscript, and Mrs. Pat Wright, Mrs. Karen Prescott, and Mr. Gerry Sandford for preparing the illustrations.

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Methyl 4-Mercaptobutyrimidate as a Cleavable Cross-Linking Reagent and Its Application to the *Escherichia coli* 30S Ribosome†

Robert R. Traut,* Alex Bollen, Tung-Tien Sun, John W. B. Hershey, John Sundberg, and L. Ross Pierce

ABSTRACT: The compound, methyl 4-mercaptobutyrimidate, of which the synthesis is described, has been used to produce disulfide-linked dimers and higher oligomers between neighboring proteins on the intact 30S ribosome from *Escherichia coli*. The imidate function of the reagent is first allowed to react with amino groups on ribosomal proteins, following which the particles modified by the addition of extra SH groups are oxidized under mild conditions to form disulfide cross-links. The formation of new products of molecular weight greater than existing protomeric ribosomal proteins is monitored by gel electrophoresis in the presence of sodium dodecyl sulfate. Upon reduction of the oxidized ribosome or the extracted proteins, the normal protein pattern on gels in sodium dodecyl sulfate is completely restored. Similarly the reduction products, or proteins bearing extra SH groups, retain their characteristic behavior during electrophoresis in buffers containing 8 M urea at pH 8.6 and 4.5. Identification of the specific protein components of purified dimers by two-dimensional slab gel electrophoresis is thus feasible. Possible minor alterations in electrophoretic mobility due to partial ionization of SH groups are completely avoided by alkylation of the proteins with iodoacetamide. The compound thus represents a new cleavable cross-linking reagent which should be applicable not only to the investigation of the topography of ribosomal proteins but also protein–protein interactions in a variety of other biological systems.

Of major importance in current research on the mechanism of protein synthesis and ribosome structure is the determination of the spatial arrangement of ribosomal proteins and the identification of ribosomal binding sites for initiation and elongation factors. Recent work has concentrated on the protein topography of the *Escherichia coli* 30S ribosomal subunit. A variety of methods are being used toward these ends (for a complete review, see Wittmann and Stöffler, 1972).

One of the most direct experimental approaches for the determination of the overall topography of ribosomal proteins as well as the identification of those specific proteins comprising the binding sites for protein factors involved in protein synthesis is the use of protein-specific bifunctional reagents to cross-link either neighboring ribosomal proteins or ribosome-bound factors and ribosomal proteins. At this time bisimido esters (Bickle *et al.*, 1972; Clegg and Hayes, 1972; Lutter *et al.*, 1972) and bismaleimides (Chang and Flasks, 1972; Kurland *et al.*, 1972) have been used to identify certain pairs of 30S ribosomal proteins covalently joined by the reagent employed.

Several approaches are available for the identification of

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616. *Received April 19, 1973.* This work was supported by a grant from the U. S. Public Health Service GM 17924 and the American Cancer Society NP-70. R. R. T. is an Established Investigator of the American Heart Association. A. B. is Chargé de Recherches of the Fonds National de la Recherche Scientifique of Belgium.