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Detection of Unique Tumor-Associated Proteins in the Urine of Patients with Transitional Cell Carcinoma by High Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis

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DETECTION OF UNIQUE TUMOR-ASSOCIATED PROTEINS IN
THE URINE OF PATIENTS WITH TRANSITIONAL CELL
CARCINOMA BY HIGH RESOLUTION TWO-DIMENSIONAL POLYACRYLAMIDE
GEL ELECTROPHORESIS

by

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ABSTRACT

DETECTION OF UNIQUE TUMOR-ASSOCIATED PROTEINS IN THE URINE OF PATIENTS WITH TRANSITIONAL CELL CARCINOMA BY HIGH RESOLUTION TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Robert David Lehman
Old Dominion University, 1979
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A two-dimensional polyacrylamide gel electrophoretic technique was developed for the detection of unique tumor-associated proteins in urine of patients with transitional cell carcinoma (TCC) of the urinary bladder. This technique yielded high resolution of polypeptides in the urine of cancer patients. Urine was chosen as a source of tumor-associated components because the location of the tumor should release already solubilized tumor substances into the urine.

Urinary proteins were concentrated and then analyzed by the two-dimensional electrophoretic technique using isoelectric focusing in the first dimension and sodium dodecyl sulfate- polyacrylamide gel electrophoresis in the second dimension. Ten transitional cell carcinoma patients, ten renal cell carcinoma patients, two benign bladder patients, two benign kidney patients, and seven normal donors were included in this study. Urinary proteins that were associated with transitional cell carcinoma and not found in normal urine or urine from patients with benign diseases are described.

DEDICATION

To my parents Alfred William and Gladys Cansino Lehman, my brothers Laurence and Richard and to my sister Nancy.

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LIST OF ABBREVIATIONS

BB=benign bladder
BIS=N,N', methylene bisacrylamide
BK=benign kidney
 β -ME=beta mercaptoethanol
IEF=isoelectric focusing
N=normal patient
PAGE=polyacrylamide gel electrophoresis
PAGE/SDS=polyacrylamide gel electrophoresis supplemented with sodium dodecyl sulfate
pI=isoelectric point
PMSF=phenylmethanesulfonylfluoride
RCC=renal cell carcinoma (kidney cancer)
SDS=sodium dodecyl sulfate
TAA=tumor-associated antigen
TCC=transitional cell carcinoma (bladder cancer)
TEMED=N,N,N',N'-tetra-methylethylenediamine
Tris=tris (hydroxymethyl) aminomethane
TSA=tumor-specific antigen

CHAPTER I

INTRODUCTION

One of the principle problems associated with an effective treatment of human cancer is the failure to detect a neoplasm in the earliest stages of development. Failure to diagnose the presence of the neoplasm early usually results in metastasis (spreading of the cancer to secondary sites throughout the body). Should the malignant growth be detected at its primary organ site, the chances for remission following therapeutic treatment increase dramatically.

The field of immunology offers one of the greatest potentials for improving the chance of early detection of neoplastic growth using specific and sensitive immunoassays. To develop a sensitive and specific immunodiagnostic test, a component, namely a tumor-associated antigen (TAA) or antibody must be detected, characterized, and highly purified. Tumors actively growing in vivo produce recognizable histopathologic tumor-associated antigens that may be tested biologically. As tumors grow, more TAA is released into the surrounding medium. TAAs have been described for most human tumors, including bladder tumors by the use of in vitro immunological tests. Nevertheless, none of the TAAs have been sufficiently purified or characterized to meet the criteria for use in a diagnostic test.

The task of purifying a tumor-associated antigen is a difficult one and involves several problems that must be overcome: (1) there is no standard source for TAA. Extracts have been prepared using fresh surgical specimens, primary and metastatic tumor tissue, cultured human tumor cells, serum

components, and urine from cancer patients. However, the preparation of antigen from these sources results in marked variability in specific immunological activity from specimen to specimen. (2) The antigens must be in soluble form for immunoassays. Such solubilization procedures may either inactivate the antigen or fail to extract the tumor antigens. (3) The crude extracts contain a wide variety of "normal" and fetal antigens in addition to the TAA, thereby, making detection and purification of the latter extremely difficult. (4) It is difficult to determine if the test is measuring a specific reaction against tumor antigen, viral antigen, or normal antigens. Finally (5), urine has proteins of normal origin as well as proteins from pathologic changes. Urine; however, should be a source of already solubilized unique components associated with genitourinary tract cancer.

Because protein components soluble in the urine seem to be closely related to pathologic changes in the urogenital system, many attempts have been made to analyze, purify and identify urinary polypeptides (i.e. esterases, glycoproteins, etc.). Results from immunologic testing further suggest the presence of tumor antigen in urine from cancer patients. Therefore, it is tempting to speculate that the urine from patients with bladder cancer contain cancer related substances, possibly TAA.

In an attempt to enhance the identification of unique proteins in the urine of cancer patients, a two-dimensional polyacrylamide gel electrophoretic technique was used in this study. This procedure employs isoelectric focusing in the first dimension and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in the second dimension. Approximately 125-150 polypeptides may be resolved using this technique employing protein-specific stains. The protein-stained gels are then observed for any unique "spots" in the separated bladder cancer patient urine. Polypeptides seen in transitional cell carcinoma patients but not observed in normal, benign kidney, benign bladder, or renal cell

carcinoma patients are discussed and characterized according to isoelectric points, molecular weights, and concentrations in the urine sample. The two-dimensional profile may, therefore, be used as a means of characterizing unique urinary proteins present in urine from patients with bladder cancer based on overall pattern differences and/or similarities.

CHAPTER II

REVIEW OF THE LITERATURE

Biology Of Urogenital Cancer

In the past five years, great strides have resulted in increased control and maintenance of genitourinary tumors. Transitional cell carcinoma (TCC) or bladder cancer is responsible for approximately 4% of new cancer cases per year in the United States population with an occurrence of 13 cases/100,000 population. This highly invasive malignancy ranks second to prostatic cancer as the most frequent urogenital tumor and occurs most commonly in the male with an approximate threefold greater incidence in males than in females (110). The life history and aggressiveness of the tumor is quite diverse and unpredictable sometimes recurring quite frequently in some patients and rarely recurring in other cancer patients (32). As with renal cell carcinoma (RCC), TCC first demonstrates itself by gross or microscopic hematuria (22, 85, 110). To date, cystoscopy is the definitive diagnostic procedure (33). Increased local thickening of the bladder wall may be seen at the primary site of bladder carcinoma (38).

The "stage" of the cancer reflects the aggressive nature and localization of the tumor within various organs of the body. The stage is very important in the complete diagnosis of the cancer patient. The Jewett staging for TCC (59) is shown in Table 1 and the staging for RCC (55) is demonstrated in Table 2. A neoplastic tumor confined to the dome of the bladder (4 cm or less) and classified as stage O, A, or B may be eliminated fairly easily by surgery. If the neoplasm has not metastasized to the lymph nodes, cure is possible (22). Appendix A shows diagrammatically the advancement of the neoplasm into the bladder wall.

TABLE 1
STAGING OF TRANSITIONAL CELL CARCINOMA TUMOR INVASION

STAGE	TUMOR INVASION
O	Pre-invasion. Carcinoma <u>in situ</u> in a flat or papillary form which does not penetrate the basement membrane
A	Neoplasm penetrates basement membrane into submucosa but does not invade the muscle
B	Infiltration of the superficial muscle
C	Infiltration of deep muscle and perivascular fat
D1	Extension into adjoining organs (e.g. pelvic side wall, prostate, vagina, lower anterior abdominal wall)
D2	Metastasis to nodes and viscera

From Jewett: J. of Urol. 55:366-372 (1946).

TABLE 2
STAGING OF RENAL CELL CARCINOMA TUMOR INVASION

STAGE	TUMOR INVASION	FIVE YEAR SURVIVAL RATE
A1	Tumor is encapsulated No renal parenchyma invasion	85%
A2	Tumor capsule broken Renal parenchyma invasion No lymphatic or venous invasion Renal capsule intact	60-70%
B	Kidney capsule invasion Perirenal fascia invasion Perinephric fat invasion Microscopic lymphatic and venous invasion	50%
C1	Invasion of perinephric fascia Involvement of renal vein or inferior vena cava	30%
C2	Gross lymphatic invasion	20%
D	Distant metastasis usually to lung bone or brain	10%

From: Holland J.M. Cancer 32:1030-1042 (1973).

Appendix B demonstrates the staging of RCC. Radical cystectomy followed by radiation therapy is the choice of initial treatment for tumors of high stage (stage C and D) cancers. Carcinoma in situ is treated by radiation therapy pre-operatively (110). Catalona (18) warns against extensive operations or radiation therapy because this treatment may result in depression of immunological reactivity to the invading tumor. He reports impaired host immunocompetence in 100% of patients with stage C and D tumors and 27% impairment in stage A and B tumors.

Malignancy is the ability of cells to grow progressively in vivo and lends itself to the possibility of metastasis. The transformation of normal cells into neoplastic cells is accompanied by major biological alterations. Changes include the appearance of soluble antigens that previously were not present in normal cells (1, 15, 43, 61, 65, 79, 80). Urinary tract neoplasms detected in the past by the appearance of cancer cells in the urine or in the vagina (16, 70, 84) have led to the use of the routine "PAP smear" which detects neoplastic cells in the urogenital tract. This method of detection, although diagnostic, is not the preferred method as cancer cells are sometimes not discovered until metastasis has occurred.

Immune Response to Tumors

The immune system is an extremely complex array of molecular and cellular functions that are conceptionally distinct. The cell-mediated immune system is derived from thymus-processed lymphocytes (T-cell) whereas the humoral immune system lymphocytes (B-cell) are thought to originate from bone-marrow stem cells. The immune system may be manipulated to the benefit of the cancer patient (122). There is evidence that the T-and B-lymphocytes must act together following appropriate antigenic stimulation to fully express the immune response needed to defend the body against bladder cancer (49, 83, 90).

Antigens may be of normal origin termed histocompatibility antigens (HL-A) such as species-specific, tissue-specific, and organ-specific antigens; or may be viral, bacterial or tumor-specific antigens (102). The human body elicits an immunological response to antigens foreign to itself by increasing immunoglobulin levels, and by activating the T-and B-cell populations. As the tumor size and invasive properties increase, immunocompetence decreases to the point that the body is overcome by the tumor mass; therefore, monitoring of immunological components, namely TAA or tumor-associated antibodies, in cancer patients is extremely important during the course of cancer treatment. Recognizable histopathologic TAAs are produced by the actively growing neoplasm and therefore the larger the tumor size, the more cells elicit the TAA (40, 61). Reducing tumor size yields an increased anti-TCC cell-mediated immunity during administration of Bacille Calmette-Guérin, an adjuvant used to increase the responsiveness of the immune system to the tumor (50).

Urinary Proteins

Detection of unique components in complex biological fluids such as urine is a major technical problem. Urine was chosen as a source of unique proteins associated with bladder cancer because the location of the tumor within the bladder should release already soluble tumor components into the urine. Antigens, being specific cell-surface immunological determinants, are shed into the surrounding medium; therefore, the urine should be a natural reservoir of these tumor components. Several reports describe the atypical nature of the urine of cancer patients compared with the urine of normal patients (14, 22, 46, 91, 109, 113, 127). The urine of normal patients contains 60-80 mg/liter of colloids, of which 60% are proteins and is termed "physiological proteinuria" (53, 54). Thirty-one proteins have been isolated in the urine that may also be found in the blood of normal patients (11). These proteins include polypeptides such as

histocompatibility antigens (97), esterases and other enzymes (31, 53, 123), and glycoproteins (30). Grant describes twelve non-plasma proteins which originate in the kidney, ureter, urinary bladder, urethra, and prostate (45). Attention has been specifically focused on the significance of non-plasma tissue proteins in the urine excretion called "histuria" (5) of which the kidney and bladder are the most direct and obvious sources (46).

Detection Of Protein Abnormalities In Urine Of Cancer Patients

Cancer patients have abnormal excretion of polypeptides both quantitatively and qualitatively as compared to the protein content in the urine of normal patients (14, 109, 112). The content of proteins in the urine changes with the growth and development of particular neoplasms. Rudman, et. al. (109) have reported increased excretion of polypeptides between the range 12,000 - 50,000 daltons in patients with advanced neoplasms and have attributed these proteins to the tumor tissue rather than to abnormal tubular reabsorption or defective permeability of an impaired glomerulus. They suggested that the increased excretion of these proteins was responsible for pathological proteinuria. Definite correlations between damage in the urogenital system and molecular size of proteins excreted is well established. The excretion of high molecular weight proteins (e.g. large amounts of albumin, and transferrin) are characteristic of glomerular damage whereas tubular dysfunctions are associated with the elimination of small amounts of albumin and low molecular weight molecules (e.g. immunoglobulin light chains, lysozyme, β -2 microglobulin) (10, 29, 87, 88, 99, 112). Although the protein content and protein profile are variable with each urogenital disorder, the proteinuria often is caused by hemoglobin in the urine. Hemoglobin is the most common indication of cancer of the kidney, renal pelvis, ureter (68), bladder (39), or prostate (21). Gross hematuria is reported in over 50% of all RCC cases. Diseases in neighboring organs can cause hematuria by

the invasion of urinary structures (e.g. cancer of the uterus, vagina, or colon).

Detection of Tumor Components

The detection of TAA is potentially the most reliable approach to immunodiagnosis of a malignancy. In 1965, Gold and Freeman discovered an antigen that was present in colon carcinoma and in the fetal colon but absent in normal adult tissue (42). This antigen was termed the carcinoembryonic antigen (CEA) and was later found to be present in numerous malignant tumors and not specifically in colon carcinoma as originally believed (47). Since the advent of the concept of TAA, many immunological in vitro tests have been developed to measure the activity of the TAA (41, 140, 142). Immunological tests such as leukocyte migration inhibition and leukocyte adherence inhibition (6, 61, 111, 140, 142) measure the cell-mediated immunity of cancer patients blood leukocytes in response to the presence of a foreign antigen. Leukocytes from bladder cancer patients are cytotoxic to neoplastic bladder cells from their own (autologous) tumor and from other patient's tumors (allogeneic) (17) due to surface antigens.

Extracts containing TAAs have been isolated from fresh surgical specimens (71, 81, 82), metastatic tumor tissue (27, 142), serum components (52, 66, 104, 121, 140) and urine (109, 120). Hollinshead (56) has described the partial purification of a 40,000 dalton TAA unique to TCC patients by gel filtration and polyacrylamide gel electrophoresis. Rabbit antisera specific for transitional cell carcinoma has been prepared using TAA patients with this malignancy (44) and a partially-purified TAA was characterized and tested. Wright and Sieg (141) have isolated a partially purified bladder cancer-associated antigen from tumor cell extracts by gel filtration.

Review Of Electrophoresis

Many systems have been employed to evaluate and monitor the urinary

excretion (i.e. characteristic proteins of cancer) in patients with renal and bladder cancer. A technique widely used to evaluate complex biological fluids is electrophoresis. Electrophoresis, which is the movement of electrically charged colloid particles under the influence of an applied electrical potential, has undergone numerous revisions and modifications to enhance the resolving power of this method of protein separation. Proteins, at an appropriate buffered pH, have either a net negative or net positive charge; therefore, they can be made to move toward either the cathode (for positively charged proteins) or the anode (for negatively charged proteins) by applied current.

In 1937, Teselius performed the first crude attempts at electrophoresis and separated serum globulins into α , β , and γ components by "moving boundary" electrophoresis (124). He later refined electrophoresis and introduced zone electrophoresis concurrent with the development of paper electrophoresis. Many support media were tried (e.g. gelatin) until Smithies introduced starch gel as a medium for support (116-118) and separated serum proteins as well as other protein mixtures. Four years later, Davis and Ornstein (25) introduced preliminary results of polyacrylamide gel electrophoresis. Smithies had already introduced the concept of molecular sieving in starch gels; however, Raymond showed conclusively that molecular sieving yielded sharper zones in polyacrylamide gels than in starch gels (93). Raymond further modified the electrophoretic procedure by introducing a vertical gel slab (94) and varied the concentration of the acrylamide monomer to achieve a desired porosity within the gel matrix (96). Slater (115) modified the concept of molecular sieving by varying the gel concentration and introduced the concept of a gel concentration gradient where the pore size of the gel is constantly decreasing in the direction of migration of the proteins. Wright (133) described a gradient "differential disc electrophoresis" in polyacrylamide gels which further refined the molecular siev-

ing effect of the gel.

Isoelectric Focusing

Further developments in electrophoresis were pioneered in polyacrylamide gels with the advent of isoelectric focusing. Isoelectric focusing, also called electrofocusing, has been a successful tool of protein separation that has undergone various modifications. Isoelectric focusing is a means of separation in which proteins are separated by electrophoresis in a starch or polyacrylamide gel containing a pH gradient linearly distributed over the length of the gel. Proteins migrate through the pH gradient until they reach a characteristic point, the isoelectric point, (pI) or pH where the proteins bear no net charge; therefore they do not migrate under an electrical influence. The idea of separating molecules by electrofocusing has been known for a long time. In 1912, Ikeda and Suzuki, two Japanese scientists, were granted a patent for a method of producing glutamic acid from plant proteins by electrofocusing (58). Though unrefined, the Japanese scientists laid down the primitive methods of isoelectric focusing that later were modified by Williams and Waterman (131) who used a 14-chambered apparatus. Gel rod disc isoelectric focusing electrophoresis has been described (19, 48, 126, 143, 144) and involves the movement of macromolecules in a pH gradient to a region of their isoelectric pH value (119). The pH gradient is produced by applying a voltage to a mixture of ampholytes (amino-carboxylic acids possessing a range of isoelectric points and ranging in molecular weight from 300-600 daltons) (48). Preliminary isoelectric focusing was performed in a sucrose density gradient for preparative fractionation of protein samples (23, 128). The reagents used for sucrose gradients were expensive and the procedure was slow. Therefore, polyacrylamide gels were introduced to stabilize the pH gradients.

Two-Dimensional Electrophoresis

Two-dimensional starch gel electrophoresis was first reported by Smithies and Poulik (117) for separating proteins of human serum. In 1958, they combined two zone electrophoretic methods (filter paper and starch gel) in the form of two-dimensional zone electrophoresis and separated, and to some extent identified, various subfractions of classical components that Tiselius described in his separation technique. Raymond (95) introduced acrylamide as a support medium in two-dimensional electrophoresis. This technique was basically that of Smithies and Poulik; however, this means of separation of serum yielded more polypeptides. It was not until 1969 that a two-dimensional polyacrylamide gradient system was used in a slab gel in which the concentration of the polyacrylamide increased progressively along the length of the gel slab. Two-dimensional electrophoresis of proteins yielded numerous polypeptides; however, because separation was based on the same parameter in both dimensions (molecular weight and charge properties), the proteins were distributed along a diagonal instead of being randomly distributed with the gel area.

With the concept of two-dimensional electrophoresis established but not yet fully developed, Kenrick and Margolis (60) described a two-dimensional system in which isoelectric focusing was performed in the first dimension followed by electrophoresis in a second dimension with a gradient polyacrylamide slab gel (4.5%-26% polyacrylamide). Because of the size-limiting features of the gradient slab gel, this combined two-dimensional system more effectively separated the proteins than either isoelectric focusing or polyacrylamide gel electrophoresis (130). O'Farrell further improved upon the above two-dimensional system by adding sodium dodecyl sulfate, an ionic detergent to the second-dimensional gel mixture; therefore, charge differences of each protein are minimized due to the net negative charge provided by the SDS (77, 78). Two-

dimensional electrophoresis has also been documented for the separation of tissue and serum components (2, 3, 4, 23, 60, 91, 117, 118, 134, 135, 138).

Wilson (132), and Anderson and Anderson (2) reported increased resolution and reproducibility of two-dimensional gel profiles by adding SDS and β -ME to the protein samples to be separated so that maximal solubilization could be achieved.

No two-dimensional electrophoretic technique for urine using IEF in the first dimension and PAGE/SDS in the second dimension has been reported; however, electrophoresis of urine from patients with renal disease has been documented. Pires, et. al. (91) have reported a one-dimensional SDS/polyacrylamide tube gel electrophoretic technique for patients with renal disease. They showed that patients with glomerular diseases excreted high molecular weight proteins; whereas, patients with tubular damage had an abundance of low molecular weight proteins in their urine. Vesterberg and Nise (127) have performed slab gel isoelectric focusing on urinary proteins from workers occupationally exposed to cadmium poisoning. They showed differential electrophoretic profile of the glomerular and tubular malfunctions of the exposed and unexposed workers. Nery, et. al. (76) performed disc electrophoresis on urine of patients with bladder cancer and showed CEA activity on partially purified fractions. They described multi-component aggregates present in the urine with molecular weights ranging from less than 1000 to several million daltons.

Since electrophoresis has developed into a useful tool for the characterization of complex biological fluids, it is advantageous to incorporate IEF and polyacrylamide gel electrophoresis into the study of proteins in the urine of cancer patients in hopes that a unique component specific to carcinoma of the bladder may be found and isolated. When the component is isolated, an immunological diagnostic test can be developed and used for the early detection of this malignancy.

CHAPTER III

MATERIALS AND METHODS

Urinary Protein Extraction

Twenty-four hour urine specimens were obtained from Dr. Paul Schellhammer, Department of Urology of the Eastern Virginia Medical School, and from Dr. James Reid of the Portsmouth Naval Hospital Department of Special Hematology. All cancer patients were from the Tidewater Virginia area and were histologically diagnosed and staged as having renal cell carcinoma (RCC) or transitional cell carcinoma (TCC). As controls, urine samples were obtained from patients with benign bladder (BB), and benign kidney (BK) tumors. Individual and "pooled" urine specimens from normal employees in the Department of Microbiology & Immunology at the Eastern Virginia Medical School were obtained and used as controls. Table 3 is a list of urine samples employed in this study. Sodium azide (NaN_3) was added to each urine sample to a final concentration 0.02% (w/v) to inhibit bacterial growth. This concentration of azide was maintained throughout the preparation procedure. The urine was concentrated 100-150 fold using a modification of the procedure of Reisfeld (98) for isolating urinary histocompatibility (HL-A) antigens. All procedures involving urinary protein preparations were carried out at 4°C to minimize proteolytic activity.

The pH of the crude untreated urines ranged from 4.25 to 7.50. Each urine was adjusted to a pH of 8.0 by addition of a 1M Tris-HCl, pH 9.0 buffer to the urine. The precipitate that often resulted was removed by centrifugation at 3000 x g for 30 min at 4°C and the precipitate discarded. The resulting supernatant

Table 3
List of Cancer Patient And Control Urines Used In Study

<u>Patient</u>	<u>Age</u>	<u>Race</u>	<u>Sex</u>	<u>Protein Yield (mg/liter)</u>	<u>Diagnosis</u>	<u>Stage</u>	<u>Comments</u>
E.A.	76	B	M	103	TCC	D	paraplegic/neurogenic bladder
G.B.	66	W	M	67	BB		
I.B.	68	W	M	10	RCC	C	
W.B.	71	W	M	150	TCC	D	Grade III
P.B.	19	W	M	62	N		
R.B.	64	B	M	150	RCC	D	Metastasis to rt. lung
S.B.	67	W	F	90	BK		radical neprectomy
A.D.	64	W	M	172	RCC	D	also has cancer of prostate
C.D.	58	W	M	63	RCC	C-D	Stage A
J.D.	54	W	M	92	RCC	A	rt. renal mass
B.G.	76	W	F	176	TCC	O	hematuria, post op.
J.G.	48	W	M	15	TCC	D	metastasis to spine
P.G.	40	W	M	93	BK		paraplegic
A.H.	68	B	F	19	RCC	C	
R.J.	69	W	M	11	RCC	B	
W.J.	75	W	M	43	TCC	B	Grade II-III, hematuria
B.L.	25	W	M	40	N		
J.L.	38	W	M	69	RCC	A	hematuria
K.M.	56	W	F	170	RCC	D	metastasis to lung, skin, abdomen
L.M.	18	W	F	27	N		
J.O.	56	B	M	170	RCC	A-B	metastasis to lung, liver mass
L.P.	72	B	F	64	BB		quadraplegic/neurogenic bladder
P.P.	42	W	M	158	TCC	A	Grade III
G.R.	54	W	M	56	TCC	unstaged	Grade II
G.S.	70	W	F	346	TCC	B	Grade II, hematuria
I.S.	23	W	F	5	N		
K.S.	35	W	M	13	N		
A.T.	63	W	M	29	RCC	B	
W.T.	56	W	M	433	TCC	B	Grade II-III, hematuria
E.U.	19	W	M	23	N		
L.W.	74	W	F	185	TCC	B	Grade III

BB=Benign Bladder, BK=Benign Kidney, N=Normal, RCC=Renal Cell Carcinoma, TCC=Transitional Cell Carcinoma

was filtered through Whatman #2 filter paper under reverse pressure at 15 lb/in². Solid ammonium sulfate was added in increments of 100 g with constant stirring at 4°C until a saturation point of 80% (560 g/liter) was obtained. The precipitate was harvested by centrifuging at 20,000 x g for 20 min at 4°C. The supernatant was discarded and the precipitated proteins were collected and transferred to a dialysis bag with a molecular weight cutoff of 12,000 daltons. The dialysis bag was then placed into one liter of sterile phosphate buffered saline, pH 7.3 (PBS-Solution W: Appendix D) supplemented with 0.02% (w/v) NaN₃ and placed in a refrigerator at 4°C. The PBS was changed once a day for three days or until all of the proteins became resuspended in solution. The presence of sulfate ions was tested for by adding a few drops of 10% BaCl₂ solution to the dialysate. If no white precipitate was seen, indicating the absence of sulfate ions, the proteins were dialyzed against two changes of sterile PBS at 4°C without NaN₃ and further concentrated to 10 ml by ultrafiltration in a sterile Amicon unit using a UM5 ultrafiltration membrane having a molecular weight cutoff of 5000 daltons. The ultrafiltrate was centrifuged at 150,000 x g at 4°C for one hour, the supernatant sterilized by membrane filtration using a sterile 0.45 micron membrane filter, and the sterile urinary proteins were placed into sterile serum bottles. Protein concentration was determined by the method of Lowry (67) and urine samples stored at -70°C for later use.

Preparation Of Urinary Sample

Basically, the preparation of the concentrated urinary proteins was similar to that of Wilson (132), and of Anderson and Anderson (3). Sodium dodecyl sulfate and β-mercaptoethanol were added to the urinary proteins prior to electrophoresis so that the final concentration of each reagent was 0.1% and 5%, respectively. It was extremely important to keep this mixture on ice during preparation to prevent protein degradation (132).

First-Dimensional Separation

Isoelectric focusing was performed by developing a linear pH gradient with a polyacrylamide matrix and allowing the charged macromolecules to migrate through the matrix until reaching a point in the gel where the net charge of each polypeptide equaled zero ($\text{pH}=\text{pI}$). Isoelectric focusing was carried out in glass tubes (150 mm x 3 mm i.d.) that were thoroughly cleaned with 7X laboratory detergent, soaked overnight in a 3% (v/v) solution of Photoflo (Eastman-Kodak Co.) and oven-dried. Three tube gels were prepared for each urine sample in each run. Two of the tube gels were stained to determine the reproducibility in the first dimension; the third tube was used for electrophoresis in a second dimension. The tubes were marked 115 mm from the bottom, sealed at the bottom with Parafilm, and placed in a level gel tube holder. It was important to keep the length of the gel constant in all runs due to the fact that variability in the gel length produced a variable pH gradient. To make 10 ml of isoelectric focusing mixture (enough for 10 tubes), the recipe in Table 4 was followed consisting of a mixture of 9M urea, 7% acrylamide and 2% ampholines. The urea was completely dissolved by warming to 37°C at which time 15 μl of ammonium persulfate solution (Solution C: Appendix D) and 10 μl of TEMED were added and the mixture quickly but carefully loaded into the glass tubes by use of a long Pasteur pipette. Care was taken not to trap any bubbles during the filling of the tubes. The tubes were filled to the 115 mm mark and gently overlayed with gel overlay solution (Solution D: Appendix D). The discrete interface between the top of the gel and the overlay solution should be seen within 20 min after overlaying. Full polymerization was accomplished within one hour after the addition of TEMED. After one hour, the gel overlay solution was aspirated and replaced with 20 μl of lysis solution (Solution F: Appendix D). After one hour, the lysis solution was aspirated, the Parafilm removed and tubes placed in a

TABLE 4

ISOELECTRIC FOCUSING MIXTURE

5.5 g of ultra pure urea
1.35 ml of IEF acrylamide (Solution A: Appendix D)
2 ml of Nonidet P-40 (Solution B: Appendix D)
1.9 ml of Abbott Co. sterile distilled water
0.4 ml of Biolyte ampholine, pH range 5 to 7
0.1 ml of Biolyte ampholine, pH range 3 to 10

standard tube gel electrophoretic chamber (Bio-Rad Model 155) equipped to hold 3 mm i.d. tubes. The tubes were aligned so that the top of the gel was positioned in the upper reservoir chamber. The lower reservoir was filled with 0.01M H_3PO_4 (Solution G: Appendix D) and bubbles were removed from under the gel bottom so electrical contact could be made. Twenty μl of fresh lysis solution (Solution F: Appendix D) was added to the gel top and overlayed gently with 0.02M NaOH (Solution H: Appendix D). The upper reservoir was refilled and the gels were then pre-run at room temperature to develop the pH gradient. The schedule for pre-electrophoresis was as follows: 15 min at 200 volts, 30 min at 300 volts, and 30 min at 400 volts (regardless of the number of tubes). During this time, the SDS and β -ME were added to the urinary proteins which were kept on ice to prepare them for electrophoresis. After the pre-electrophoresis run was complete, the power was turned off, the upper reservoir emptied, and the overlays aspirated from the top of the gel. After sample preparation was completed, the protein samples (400 μg of protein) were loaded onto the top of the gel by use of a Hamilton microsyringe. The samples were then overlayed with 10 μl of sample overlay solution (Solution I: Appendix D). The overlay was carefully overlayed with 0.02M NaOH solution so as not to "bomb" the interface of the protein sample. The upper chamber was filled with sodium hydroxide solution. The gels were electrophoresed at room temperature overnight at 500 volts, increased to 700 volts in the morning and run for one hour at this voltage. After this time, the voltage was increased to 800 volts and continued for one additional hour. The product of volts and the time (in hours) totaled 10,000 volt-hours.

Once the first-dimensional electrophoretic run was complete, the gels were removed by first rimming the inside of the IEF tube with a syringe filled with distilled water and equipped with a 21 gauge x 1½ in needle. The needle was

inserted between the gel tube wall and the water gently forced into the space between the gel and tube wall to reduce friction. A 20 ml syringe was connected to the cathode end of the tube via a short piece of Tygon tubing and the gel was forced out by slight pressure. Once the gel was extruded, the negative end of the gel was marked by stabbing it with a 24 gauge needle dipped in India ink.

Equilibration Of The IEF Gel For Two-Dimensional Electrophoresis

The IEF gel was equilibrated by placing the gel into a (6 mm x 150 mm test tube with 15 ml of SDS sample buffer (Solution P: Appendix D). The tube was sealed with Parafilm and gently shaken at room temperature for 45 min. This equilibration was necessary because the proteins that now have no net charge (due to immobilization at a well defined pI) must be given a charge if they are to move in a second electrophoretic system.

Staining Of The First-Dimensional Gel

Two of the three triplicate IEF gels were stained by first fixing the proteins into the polyacrylamide matrix by placing the IEF gel into 15 ml of fixing solution (Solution J: Appendix D) and shaking at room temperature for one hour. The fixing solution was replaced with 15 ml of staining solution (Solution K: Appendix D) and the gels incubated at 56°C for 45 min. The gels were then destained with Solution L (Appendix D) for 18 hours followed by replacement with fresh destaining solution. Destaining was continued for an additional 18 hours until the background between the protein bands was clear. The destaining solution was decanted and replaced with IEF preserving solution (Solution V: Appendix D) to preserve the gel and enhance the protein bands. Gels were stored at 4°C until photographed or scanned by a spectrophotometer.

Measurement Of The pH Gradient

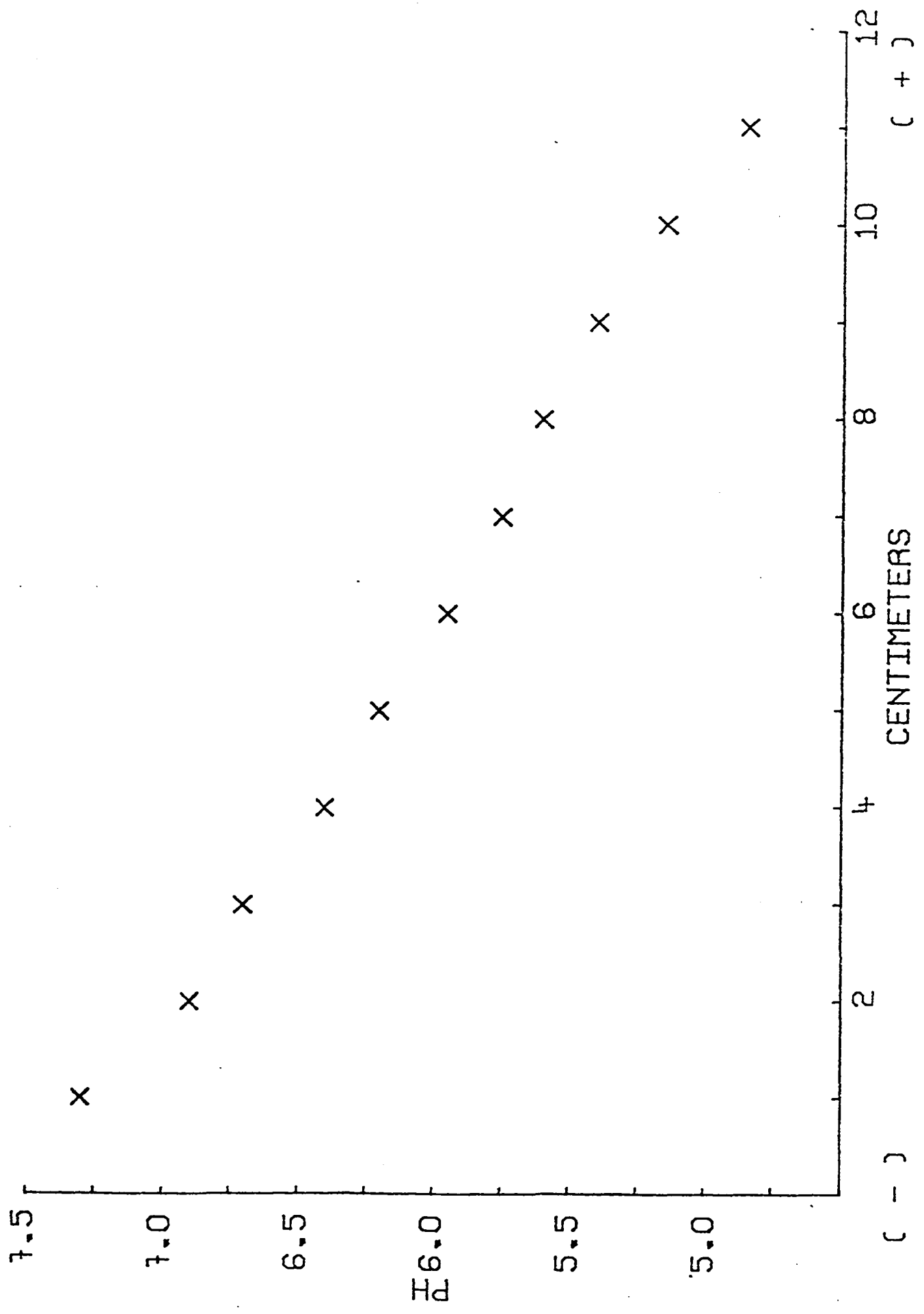
An IEF gel without protein (blank gel) was extruded from the tube after

electrophoresis (as described above) and cut into 10 mm sections with a scalpel. Each section was put into a 13 mm x 100 mm test tube with 2 ml of Abbott Co. distilled water and shaken at room temperature for one hour. The pH of each tube was measured and plotted as a function of centimeters of gel versus pH. A typical pH gradient using the indicated percentages of Biolyte ampholines is shown in Figure 1.

Second-Dimensional Separation

Separation in the second dimension was accomplished by PAGE/SDS electrophoresis using a Bio-Rad Model 220 slab gel electrophoresis chamber. A polyacrylamide gradient system of 10%-20% polyacrylamide supplemented with 0.1% SDS was employed in this study. A notched beveled plate (180 mm x 140 mm) was used to facilitate acceptance of the first-dimensional gel into the second-dimensional system (Figure 2). The glass plates were thoroughly cleaned with 70% (v/v) isopropanol solution, washed in 7X laboratory detergent, rinsed in distilled water, soaked overnight in a 6% (v/v) Photoflo solution (Eastman-Kodak Co.) and oven dried. The plates were assembled with Bio-Rad PVC spacers (140 mm long x 20 mm wide x 1.5 mm) in order to form a gel slab of 120 mm x 160 mm x 1.5 mm thick. Figure 3 shows the apparatus with the beveled-notched plate set up so the bevel was toward the outside of the apparatus. The rectangular gel mold was clamped onto the apparatus tightly and the bottom of the mold was sealed with a greased gasket. For pouring the gel gradient, a standard two-chambered gradient mixer (Buchler Scientific Co., Fort Lee, N.J.) was assembled as shown in Figure 4. The electrophoresis chamber was then placed on a table leveler prior to pouring. The gel solutions for 10% and 20% polyacrylamide (0.1% SDS, 0.375M Tris-HCl, pH 8.8) are presented in Table 5. Both solutions were kept chilled during preparation of the gel slab.

Figure 1. Determination of pH values for the first-dimensional polyacrylamide electrophoretic separation. Percentages of ampholines (Table 2) and conditions of electrophoresis are presented in the text. Cathode is toward the left of the graph.



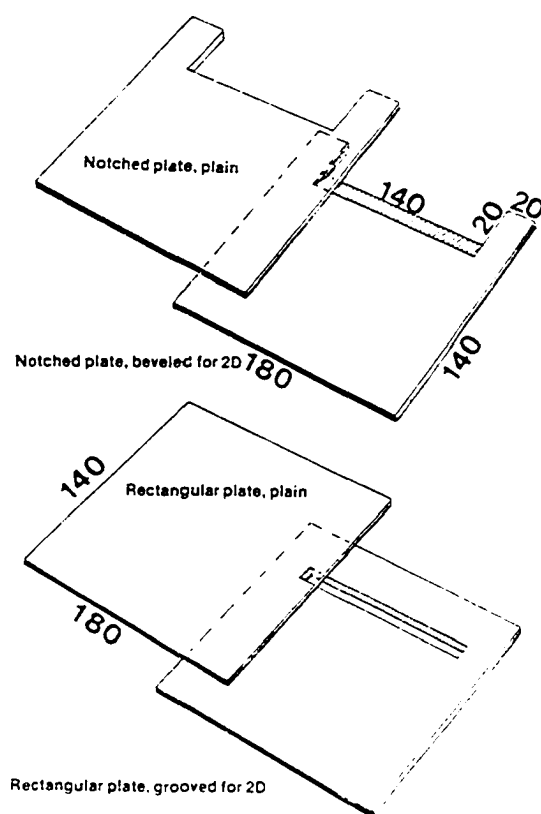


Figure 2. Glass Plates used for the second-dimensional polyacrylamide separation. Dimensions are in mm. From: Bio-Rad Laboratories, April, 1978 Price List, p.126.

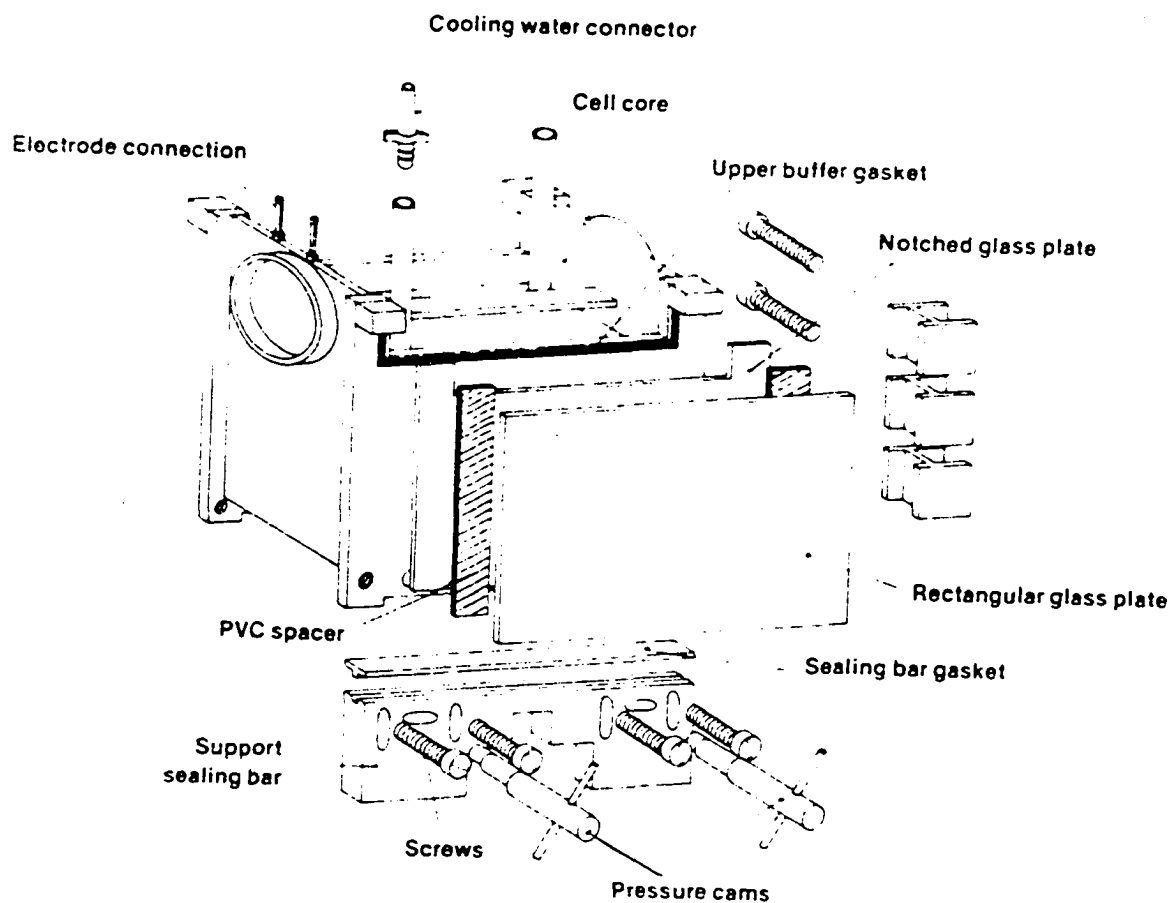


Figure 3. Apparatus used in second-dimensional polyacrylamide gel electrophoresis. Glass plates (Figure 2) are clamped to the apparatus for gradient polyacrylamide electrophoresis. From: Bio-Rad Laboratories, April, 1978 Price List, p.127.

Figure 4. Representative drawings of the apparatus used to pour two-dimensional gradient polyacrylamide slab gels. Gradient mixing chambers (Chamber A for 10% polyacrylamide solution, Chamber B for 20% solution) are 15 mm i.d. x 115 mm and are attached to a ring stand. C is a stopcock to regulate the flow between chambers. Outflow regulation (D); electric stirring motor, E (for mixing the 10% and 20% solutions); drainage assembly (F); mixture level (a and d), 12 ml mark. The T-Connector outflow assemblage (H) consists of two butterfly infusion needles (19 gauge x 7/8 in) (n). The needles are attached to the gel mold so that the bevel needle touched the inside of each PVC spacer.

Gel Mold: The mold (L) is held vertically by the electrophoretic cell. Exploded view shows assemblage of gel mold as seen in Figure 2. PVC spacer (g) is 1.5 mm thick. Dimensions of gel mold: Outer- 180 mm x 140 mm x 10.5 mm. Inner-160 mm x 120 mm x 1.5 mm. From: Wright, G.L., Am. J. Clin. Path. 57(2):173-185 (1972).

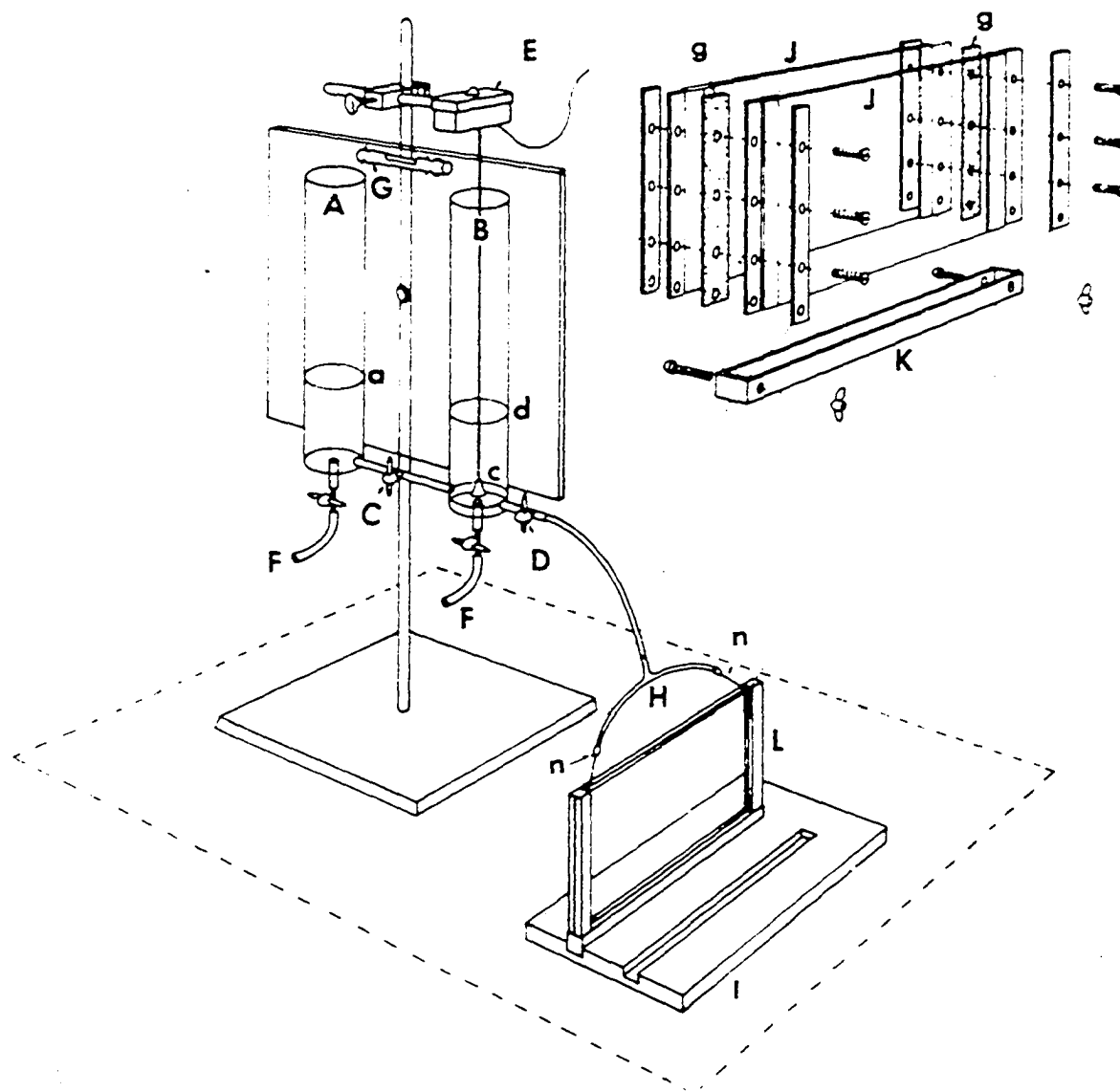


TABLE 5
SOLUTIONS FOR TWO-DIMENSIONAL GRADIENT GELS

<u>Light Solution (10% polyacrylamide)</u>	<u>Heavy Solution (20% polyacrylamide)</u>
3 ml of lower gel buffer (Solution N: Appendix D)	3 ml of lower gel buffer (Solution N: Appendix D)
4 ml of acrylamide (Solution M: Appendix D)	8 ml of acrylamide (Solution M: Appendix D)
5 ml of Abbott Co. distilled water	1 ml of Abbott Co. distilled water
60 μ l of ammonium persulfate (Solution C: Appendix D)	40 μ l of ammonium persulfate (Solution C:Appendix D)
6 μ l of TEMED	6 μ l of TEMED
	1.2 g of sucrose

A total volume of 24 ml was necessary to produce a slab 160 mm long by 120 mm high and 1.5 mm thick. This volume filled the apparatus to a level 20 mm below the bevel of the notched plate. To avoid bubble formation, the solid sucrose was brought into solution by lightly vortexing prior to the addition of the lower gel buffer. The 10% acrylamide solution was poured into the back chamber (Chamber A:Figure 4) of the gradient mixer and the 20% acrylamide solution was poured into chamber B as shown in Figure 4 keeping the stopcock (C) between the two chambers closed. The magnetic stirrer was turned on and three ml of the 20% acrylamide solution was allowed to flow by gravitational force into the Tygon tubing. After three ml had been displaced from the outlet chamber, the stopcock between the two chambers (C) of the gradient mixer was opened. Optimal flow rate was 3 ml/min and did not exceed 5 ml/min. The flow rate was regulated so that "bombing" of the acrylamide between the glass plates did not occur. Once pouring was complete, 5 ml of distilled water was gently overlayed so that a straight top formed and to prevent drying out of the gel top. Polymerization was complete within one hour after which the water was removed from the top of the slab gel and replaced with 5 ml of lower gel buffer (Solution N:Appendix D) diluted 1:4 with distilled water. The gel was left overnight at room temperature.

The following day, the stacking gel (4.5% acrylamide, 0.1% SDS, 0.125M Tris-HCl, pH 6.8) sufficient for two slab gels as shown in Table 6 was prepared. The diluted lower gel buffer solution was removed from the top of the slab gel and the gel top carefully blotted dry with bibulous paper. After the addition of the ammonium persulfate and the TEMED, the solution was added between the glass plates and filled to the base of the notch in the beveled plate. The solution was carefully overlayed with distilled water to prevent drying out. Polymerization occurred within one hour.

TABLE 6
TWO-DIMENSIONAL STACKING GEL

1.5 ml of acrylamide (Solution M:Appendix D)
2.5 ml of upper gel buffer (Solution O:Appendix D)
6 ml of Abbott Co. distilled water
30 μ l of ammonium persulfate (Solution C:Appendix D)
10 μ l of TEMED

Loading Of The Isoelectric Focusing Gel Onto The Second Dimension

After the IEF had been equilibrated in the SDS sample buffer (Solution P:Appendix D) for 45 min, it was ready to be subjected to electrophoresis in the second dimension. The water covering the stacking gel was removed and the gel top blotted dry with bibulous paper. An agarose solution (Solution Q: Appendix D) was used to hold the IEF gel in place between the beveled and rectangular plates (Figure 5). This solution also aided in avoiding the mixing of protein bands when they began to migrate into the slab gel. The agarose solution was heated in a boiling water bath prior to use. The IEF gel was removed from the SDS sample buffer and placed on a piece of Parafilm 100 mm x 130 mm. The gel was blotted dry of excess SDS sample buffer. One ml of melted agarose solution was poured along the top of the stacking gel and the Parafilm was used to quickly transfer the cylindrical gel onto the melted agarose in the notch (Figure 5). The negative end (India ink stab) was to the left when loading the cylindrical gel into the notch in order to keep all two-dimensional patterns consistent. The negative end of the gel was placed 10 mm from the left PVC spacer and was never flushed up against the spacer. The gel was then overlayed with 1-2 ml of agarose solution and the agarose was allowed to solidify.

Electrophoresis In The Second Dimension

Once the agarose solution had solidified, there was a space of 15 mm between the anodic end of the cylindrical gel tube and the right PVC spacer. A spatula was used to cut a well into the agarose 10 mm long and 5 mm deep. This agarose cube was discarded so that the "mixed buffered molecular weight standards" (Appendix E) could be added. The bottom pressure bar of the electrophoresis apparatus was removed and the gel was placed into the electrophoretic tank. Four liters of running buffer (Solution R:Appendix D) was

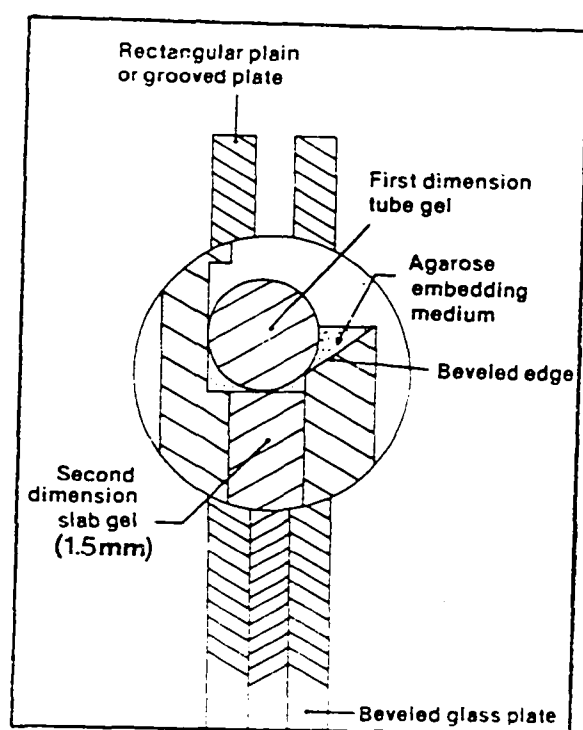


Figure 5. Diagrammatic cross-sectional representation of the first-dimensional cylindrical gel embedded into agarose medium in between the two glass plates for separation in the second dimension. From: Bio-Rad Laboratories, April, 1978 Price List, p.125.

prepared prior to use and poured into the tank and upper chamber. Bubbles were removed from the bottom of the space between the glass plates by streaming running buffer between the glass plates using a long Pasteur pipette crooked at the end. The electrophoretic unit was placed in a refrigerator at 4°C and 100 µl of the "mixed buffered molecular weight standards" (See Appendix E) were added to each well cut into the agarose layer. This gave a total protein content of 2.5 µg/each molecular weight marker. Gels were run at 6 milliamps/gel (constant current) for 20 hours. Running time varied with the percent of acrylamide used in these experiments; however, this schedule was sufficient for a 10%-20% polyacrylamide gradient.

Staining And Destaining Of The Second-Dimensional Gel

Once the electrophoretic run was terminated, the power supply was turned off and the plates were unclamped from the electrophoretic apparatus. The PVC strips were removed using a laboratory spatula, and the gel plates pried apart by constant pressure. The gel was stained at room temperature for one hour in staining solution (Solution T:Appendix D). Following staining, the gel was destained in several changes of destaining solution (Solution U:Appendix D) until the background had a slight tint of blue to enhance the contrast. After destaining was complete, the gels were placed in a Ziplock "baggie" with 7 ml of perservative (Solution V:Appendix D) and stored in a refrigerator at 4°C.

Photography

Both first-and second-dimensional gels were photographed by using a Nikon-F camera with Tri-X Pan (ASA 32) black and white 35 mm film. The first-dimensional gels were photographed in the tubes with perservative (Solution V:Appendix D). Second-dimensional gels were photographed after the stacking gel was cut from the separating gel with a laboratory spatula. A conventional x-

ray viewing box was placed under the gels during photography. The f stop was set at 8 and the shutter speed was at 1/60 sec for the first- and second-dimensional gels.

Gel Density Scanning

First-dimensional gels were scanned on a Beckman Acta C III Spectrophotometer at 580 nm using a tungsten lamp. A 0.1 mm slit was inserted, the band width was 2 mm and the slit width was 0.9 mm. Attenuators were inserted into the spectrophotometer to reduce background noise. Gels were scanned at 1.5 cm/min.

CHAPTER IV

RESULTS

Comparative Urinary Protein Excretion

Comparative analysis of the urinary protein excretion of normal donors to that of cancer patients revealed that the patients with a urogenital dysfunction (a benign or malignant kidney or bladder) demonstrated elevated protein concentrations in the urine. The protein concentration of normal urine ranged from 5 mg/l to 62 mg/l (Table 3) with an average yield of 28 mg/l for normal donors. TCC patients, RCC patients, BK patients and BB patients showed elevated urinary protein concentrations of 166 mg/l, 95 mg/l, 92 mg/l, and 66 mg/l, respectively. TCC patients usually had six times more precipitate protein per liter than did normal donors. Forty percent of TCC patients and 20% of RCC patients demonstrated hematuria or red blood cells in the urinary sediments. None of the normal donors, BB, or BK patients demonstrated blood in the urine.

First-Dimensional Separation

Densitometric scans of the first-dimensional electrophoregrams of the urine from cancer patients, benign patients, and normal donors are presented in figures 6-11. A gel scan of the isoelectric focusing gel of a TCC patient's (Stage A-patient P.P.) urinary proteins is presented in figure 8 which illustrates the problem of some proteins being masked by the diffuse albumin band (pH 6.00-6.50). Proteins that are seen only in TCC profiles in the second-dimensional gel slabs (discussed below) are masked by albumin; however, one unique urinary protein associated with TCC was observed outside the area that albumin occupies in the first-dimensional gel. This protein is shown in figure 8 as the second

darkly-stained band from the cathodic end (right side) of the IEF gel. This protein (to be discussed) has a pI from 6.95-7.50.

Many variations in protein band patterns that are optically faint in the first-dimensional gels are revealed in the densitometric gel scans of the isoelectric focusing gels. The variations are believed to be associated with the random excretion of urinary proteins, both quantitatively and qualitatively, as well as minor variations in the genetic makeup of each patient. In the densitometric scans of the first-dimension, similarities in the pI of certain proteins are consistent (e.g. there is a peak at all times in the pH range 6.25-6.30 corresponding to the pI of albumin). The variations make it difficult to find consistency in proteins in decreased concentrations in the urine.

Routinely, no proteolytic inhibitor was added to the urine samples; however, to eliminate the possibility of proteolytic degradation of urinary proteins phenylmethylsulfonylfluoride (PMSF), a serine esterase inhibitor (35) was added to two random urine samples. Urine samples from K.M. (a RCC patient) and W.B. (a TCC patient) were divided in half and the proteins precipitated with ammonium sulfate. One-half of each urine was not treated while the other half was treated in the presence of 1mM PMSF (35). This concentration of PMSF was kept constant throughout the protein precipitation procedure. Densitometric gel scans of the IEF gel of the urine of a RCC patient (K.M.) are shown in figures 6 and 7. Figure 6 shows the electrophoretic profile of the sample treated in the presence of PMSF (densitometric gel scan-figure 6) whereas figure 7 (densitometric gel scan) shows the urine sample from this patient that was concentrated without the addition of PMSF. The first-dimensional gels appeared to be similar (figure 12). Most importantly, proteolytic activity was believed not to have occurred because no "extra spots" were seen in the second-dimensional electrophoregram of the urine not treated with PMSF (figure 24).

The first-dimensional (isoelectric focusing) electrophoregrams are presented in figures 12, 13, and 14. Figure 12 shows the reproducibility of an identical urine sample treated routinely with and without the addition of a protease inhibitor. Figure 13 shows the reproducibility of samples run under similar conditions; whereas, Figure 14 shows the reproducibility of samples run under similar conditions. The resolution of the first-dimensional gels was limited compared to the two-dimensional polyacrylamide gel electrophoregrams (described below) because separation in the first dimension was based on only one separation parameter (VIZ the net electrical charge of the proteins). Some urinary proteins present in low concentrations appeared to be "masked" by proteins in high concentrations or having the same pI.

Second-Dimensional Electrophoresis

The two-dimensional gels were analyzed by comparing the patterns on top of an x-ray viewing box. With few exceptions, the similarities of the gel patterns were generally excellent. Similar protein patterns were observed for each of the different types of urine samples examined. The higher molecular weight proteins were pore-limited in the upper portion of the gel (10%-15% polyacrylamide); whereas, the proteins with a lower molecular weight migrated further toward the bottom of the gel (15%-20% polyacrylamide). Molecular weight markers are shown separated on the right side of the two-dimensional gel profiles (figures 15-25).

Albumin was the largest spot in the two-dimensional gel patterns; and was in the greatest concentration in most all urines tested. Elevated levels of albumin were observed in the two-dimensional profile in one of the two BK patients studied. The absence of an albumin spot in the two-dimensional profiles of patients A.H. (RCC), R.B. (RCC), G.B. (BB) and P.G. (BK) was observed. Furthermore, these patients lacked all high molecular weight proteins greater than

60,000 daltons. On the other hand, 30% of the RCC patients (patients A.D., K.M., and J.O.) and 10% of the TCC patients (patient G.S.) had protein patterns completely devoid of low molecular weight polypeptides smaller than 45,000 daltons. Normal donors demonstrated polypeptides ranging from 11,000 to 85,000 daltons. Patients with benign diseases (patients G.B. and P.G.) had "smearing" of low molecular weight proteins (10,000 to 45,000 daltons) in the two-dimensional profile which was assumed to be evidence of denaturation.

All proteins that appeared to be associated with bladder malignancy had a molecular weight range from 35,000 to 75,000 daltons. A two-dimensional gel electrophoregram of the urinary proteins of patient L.W. (TCC-Stage B) is presented in figure 15. This gel shows four proteins, in section B1 and C1 of the representative diagram (upper figure) that were observed in 80% of all TCC patients and were not observed in any RCC, BB, BK or normal urine sample. These four proteins were distributed linearly above the albumin and therefore, appear to have a molecular weight range between 70,000 to 75,000 daltons. The pI of these proteins ranged from 6.05-6.25 and always appeared as a tetramer.

The two-dimensional electrophoregram of the urinary proteins of patient E.A. (TCC-Stage D) is presented in figure 16. A unique polypeptide spot was seen in this profile in section B3 (upper figure) that migrated to a point between the 45,000 and 53,000 dalton molecular weight range, at approximately 50,000 daltons. This protein was more "basic" than the four proteins mentioned above. The pI of the polypeptide was between 6.30-6.40 and was present in 6 out of the 10 TCC patient two-dimensional patterns. It was possible that the other four TCC patient profiles had this protein, but the concentration in the urine may have been below detectable levels. None of the RCC, BK, BB, or normal urines demonstrated the presence of this protein in the urine.

A two-dimensional profile of TCC patient G.S. (stage B) is presented in figure 17. A unique protein spot was observed in section A3 of the two-

dimensional pattern (upper figure). This protein was seen in 100% of all TCC patients studied and was undetectable in all RCC, BB, BK, and normal urine samples. The protein has an apparent molecular weight from 35,000-40,000 daltons with a diffuse pI ranging from 6.95-7.50. This spot was much larger (i.e. in higher concentration) in urines from patients with advanced neoplastic progression. For example, Stage B TCC patients had a higher concentration of this protein in their urine than did a Stage O patient.

A two-dimensional profile of patient W.J. (Stage B-TCC) is shown in figure 18. A 68,000 dalton protein doublet in section B2 (upper figure) was seen in 40% of all TCC patient urines. These proteins have a pI from 6.50-6.75 and were always present as a dimer. The doublet was not seen in any RCC, BB, BK, or normal urine sample.

A two-dimensional profile of the urine of patient B.G. (TCC-Stage O) is shown in figure 19. Two proteins approximately 53,000 daltons which were detected in both TCC and RCC patients are shown in section C3 (upper figure). The more basic polypeptide (left spot) electrofocused at pH 6.10-6.20 whereas the more acidic protein (right spot) focused at pH 5.90-6.00 in the first-dimensional gels. These proteins were absent from normal donor urines and from patients with BB and BK diseases. These proteins stained more intensely in TCC patient urine electrophoregrams suggesting an increased concentration in TCC urines than in RCC urine samples.

The two-dimensional profile of the pooled normal samples from six age and sex-matched normal donors is shown in figure 20. The unique urinary proteins described above were not detected in the normal urine electrophoregrams. As described above, these unique proteins were also absent from electrophoregrams of patients with RCC (Figure 21), BK (Figure 22), and BB (Figure 23) regardless of the stage of disease.

The unique proteins regarded as associated with transitional cell carcinoma are summarized in a composite two-dimensional map (figure 25). Albumin and the molecular weight markers were added for orientation purposes only. The ten urinary proteins shown in this figure are considered to be possible candidates as tumor-associated proteins in transitional cell carcinoma. The ten proteins ranged in isoelectric point from 6.05-7.50 and in the molecular range from 35,000 to 75,000 daltons.

Figure 6. Densitometric gel scan of the isoelectric focusing gel of the urine of patient K.M. (RCC-Stage D). This gel was treated with the addition of PMSF. Cathode is toward the right side.

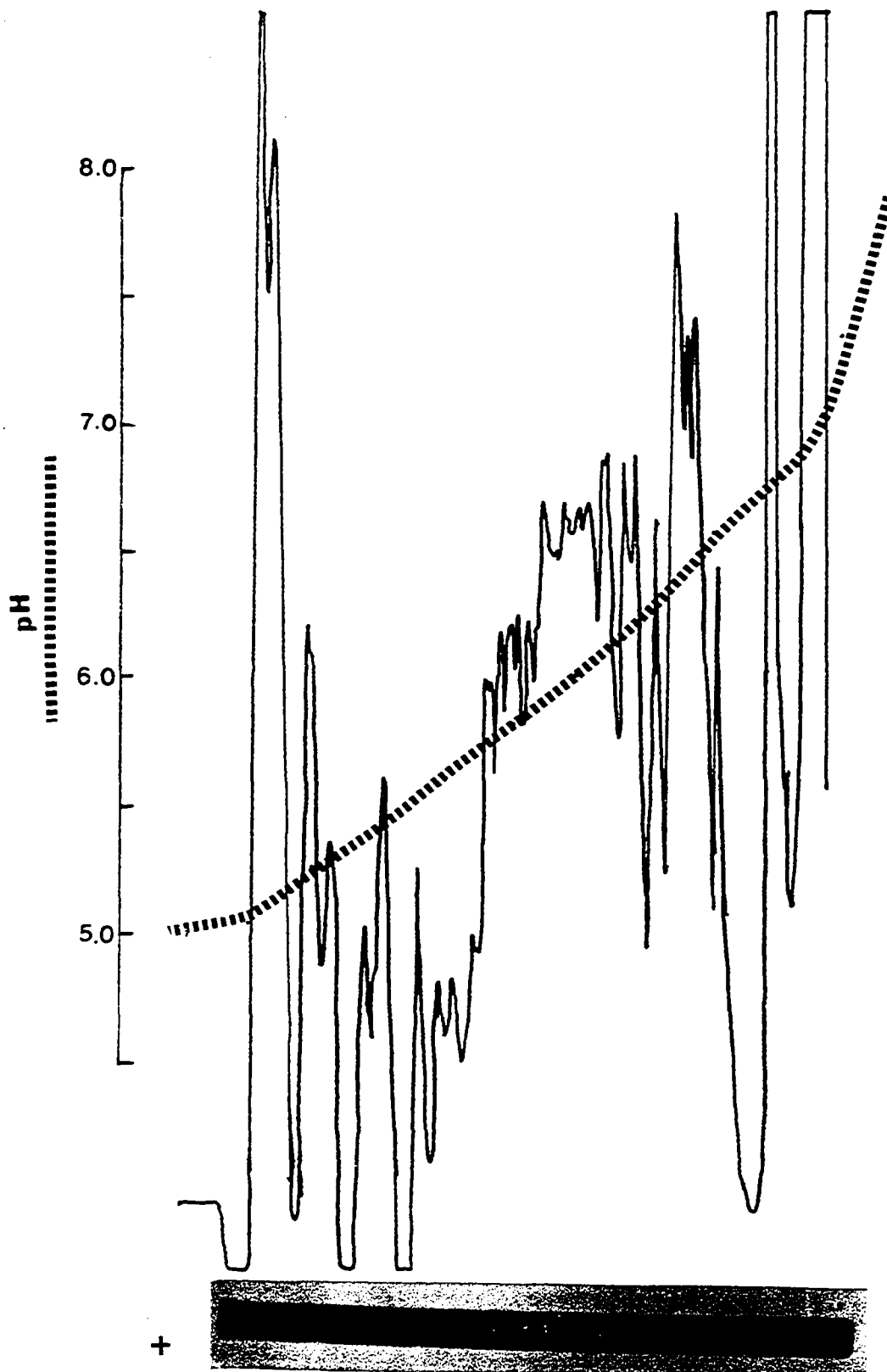


Figure 7. Densitometric gel scan of the isoelectric focusing gel of the urine of patient K.M. (RCC-Stage D). This gel was treated without the addition of PMSF. Cathode is toward the right side.

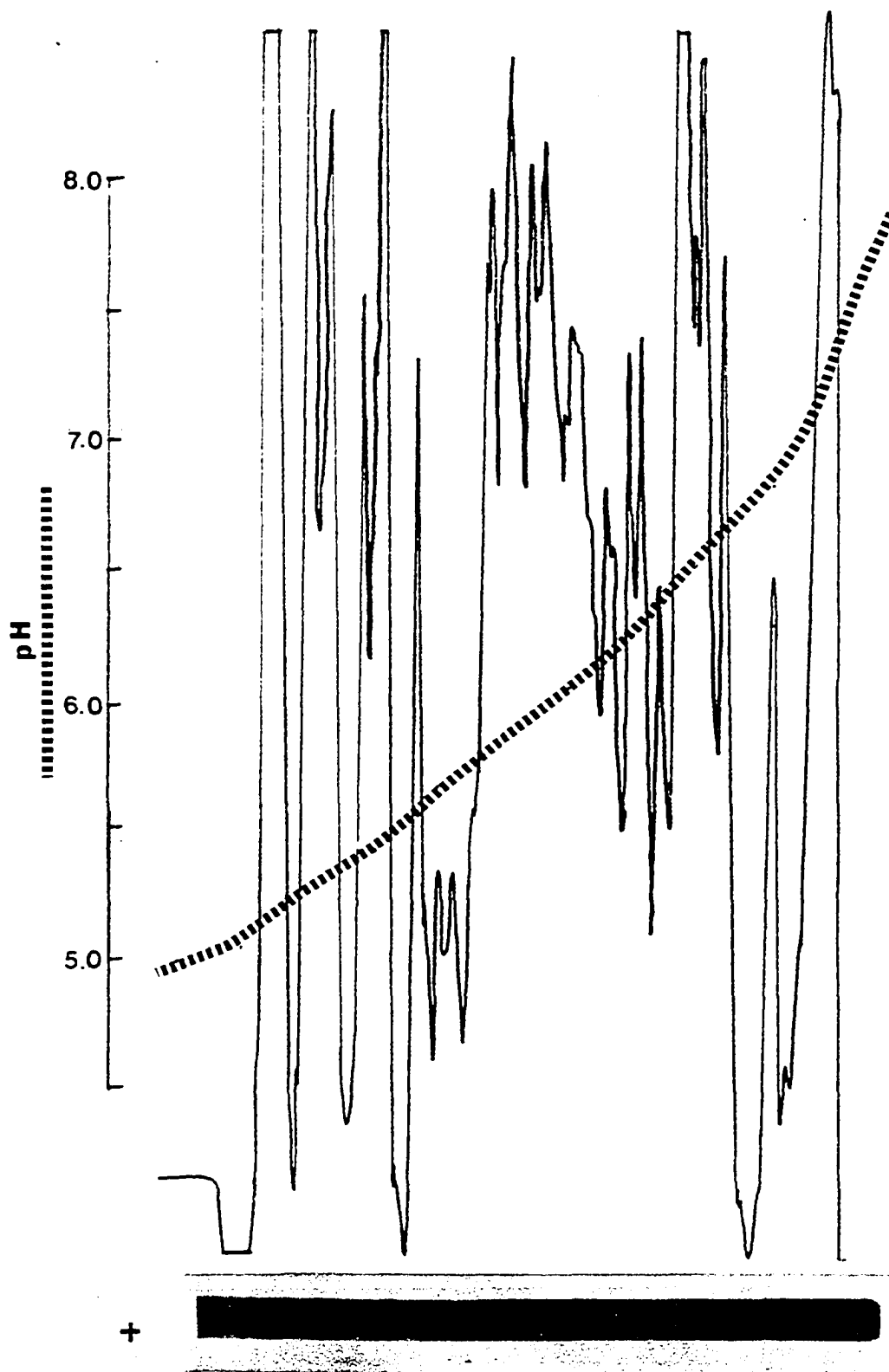


Figure 8. Densitometric gel scan of the isoelectric-focusing gel of the urine of patient P.P. (TCC-Stage A). Cathode is toward the right side.

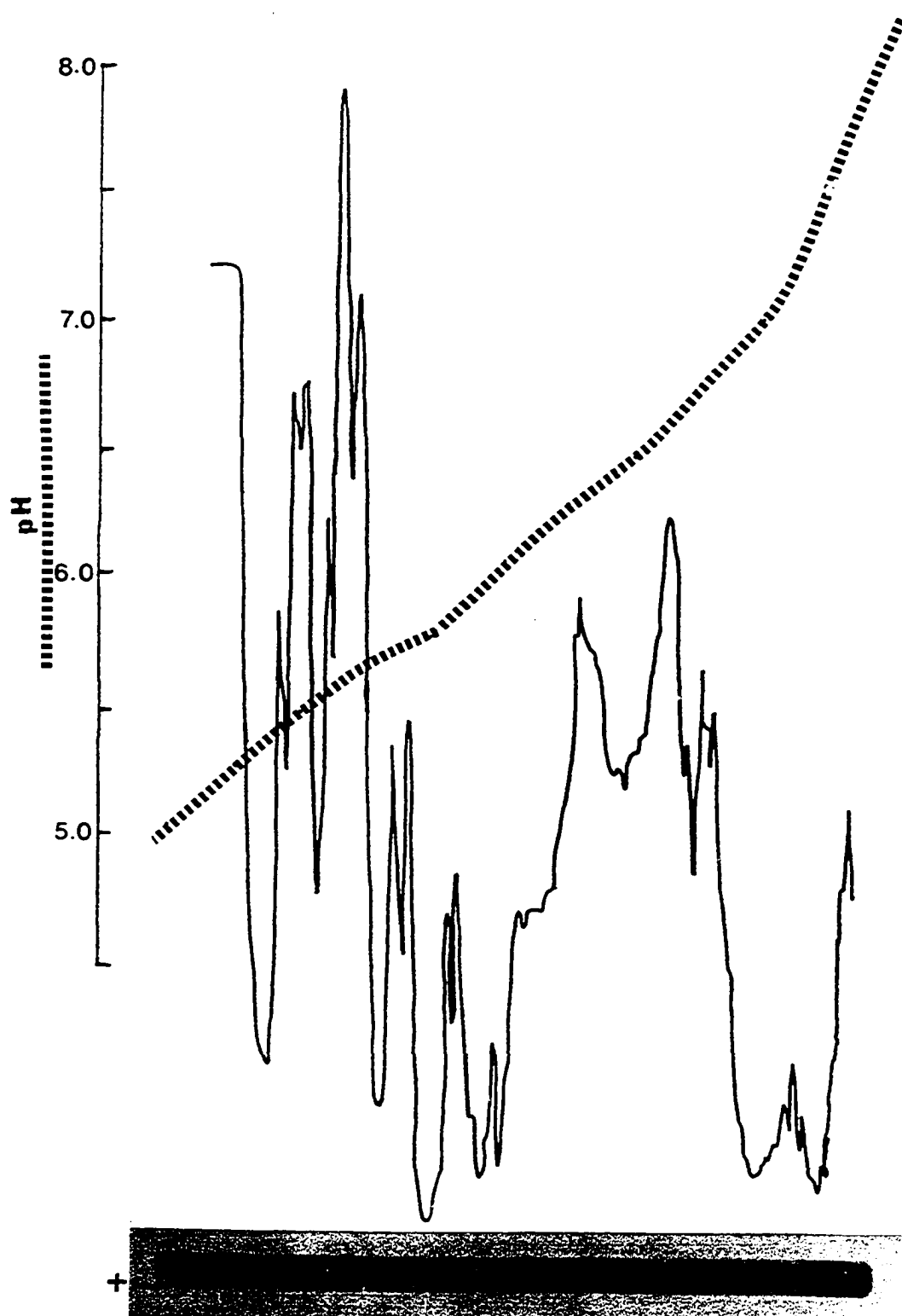
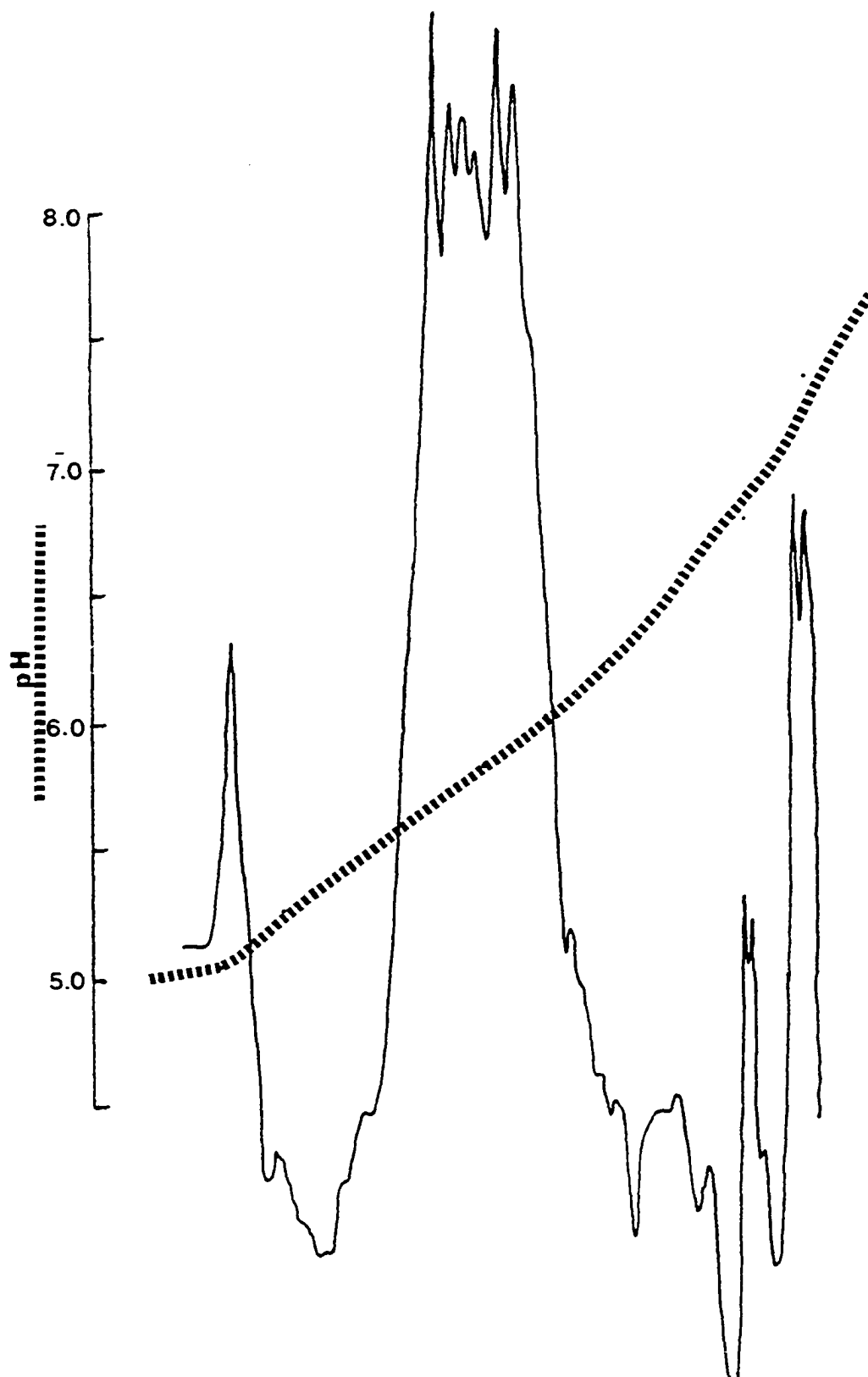


Figure 9. Densitometric gel scan of the isoelectric focusing gel of the urine of patient L.P. (BB). Cathode is toward the right side.



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Figure 10. Densitometric gel scan of the isoelectric focusing gel of the urine of patient P.G. (BK). Cathode is toward the right side.

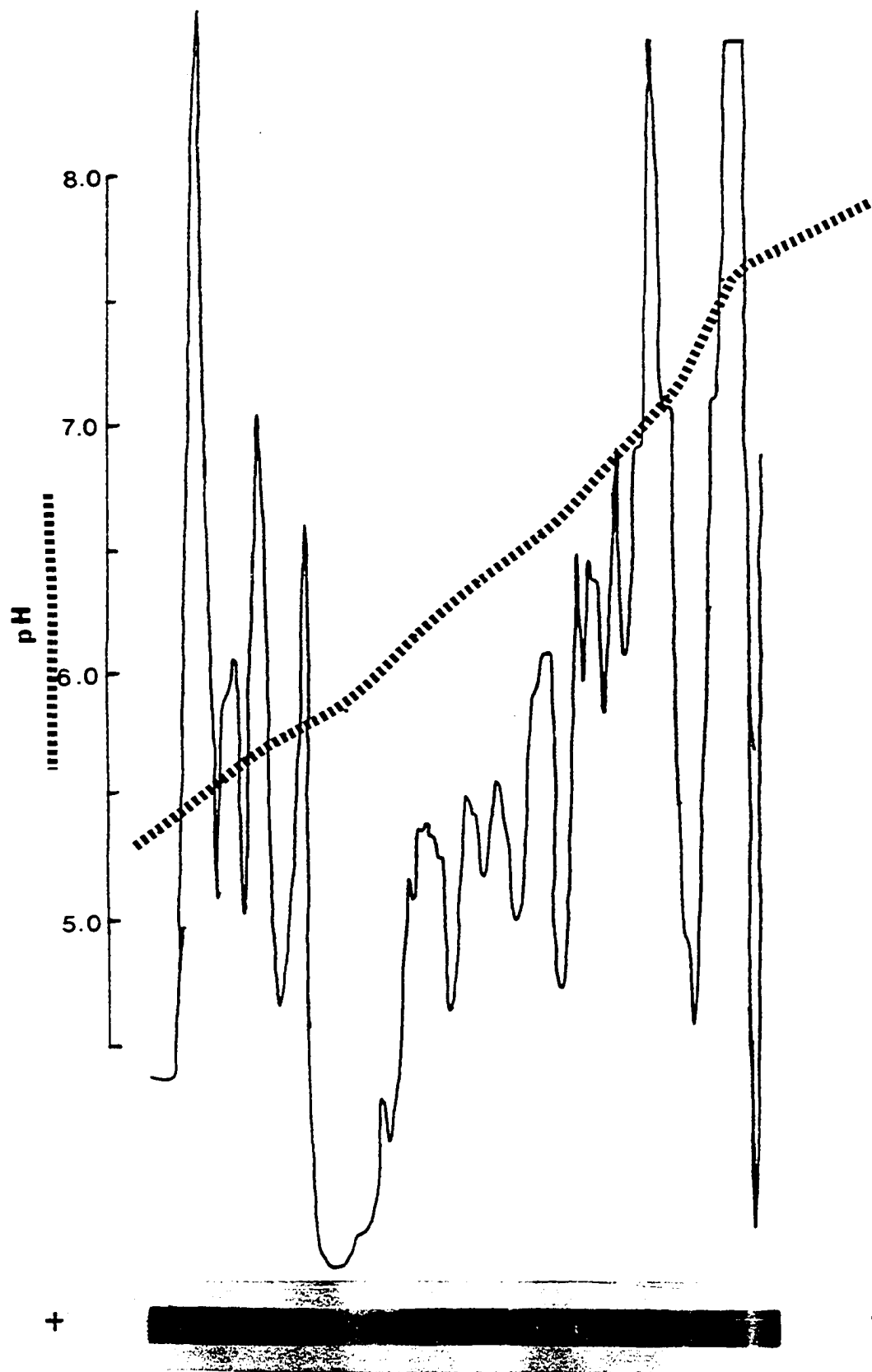


Figure 11. Densitometric gel scan of the isoelectric focusing gel of the urine of a normal donor (L.M.). Cathode is toward the right side.

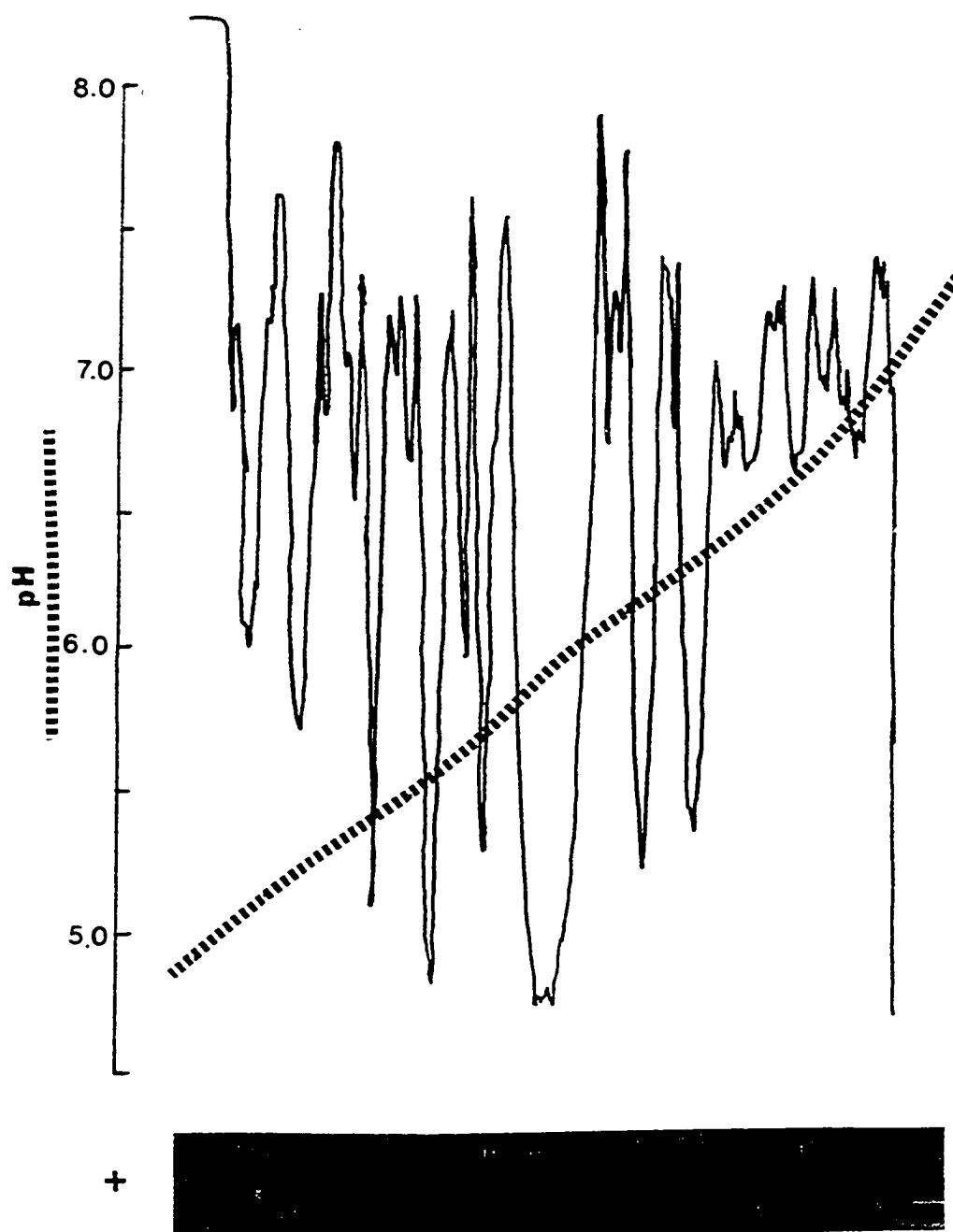


Figure 12. Isoelectric focusing gels of the urine of RCC patient K.M. This figure demonstrates the reproducibility between PMSF-treated (left gel) and PMSF-untreated (right gel) urine samples. Cathode is at the top of the gel.

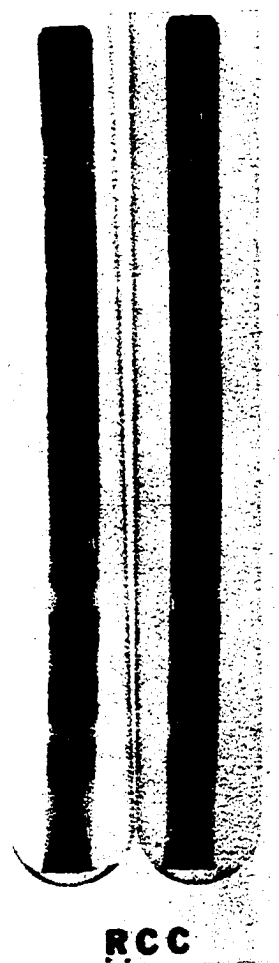


Figure 13. Isoelectric focusing gels demonstrating the reproducibility within the urines of pathologic and normal donors. From left to right: patient J.D. (RCC); patient L.P. (BB); normal donor (K.S.); patient P.G. (BK); and patient B.G. (TCC). Cathode is toward the top of the gel.



Figure 14. Isoelectric focusing gels demonstrating the similarities as well as variations between urine samples of patients with pathologic urines and normal donor urines. From left to right: patient G.B. (BB); patient P.G. (BK); patient G.R. (TCC); patient A.H. (RCC) and normal donor (I.S.). Cathode is toward the top of the gel.



BB BK TCC RCC NORMAL

Figure 15. Second dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient L.W. (TCC-Stage B). A diagrammatic representation (top) of the gel slab is divided into sections. Four proteins are seen in sections B1 and C1. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first dimensional gel is to the left of the gel slab.

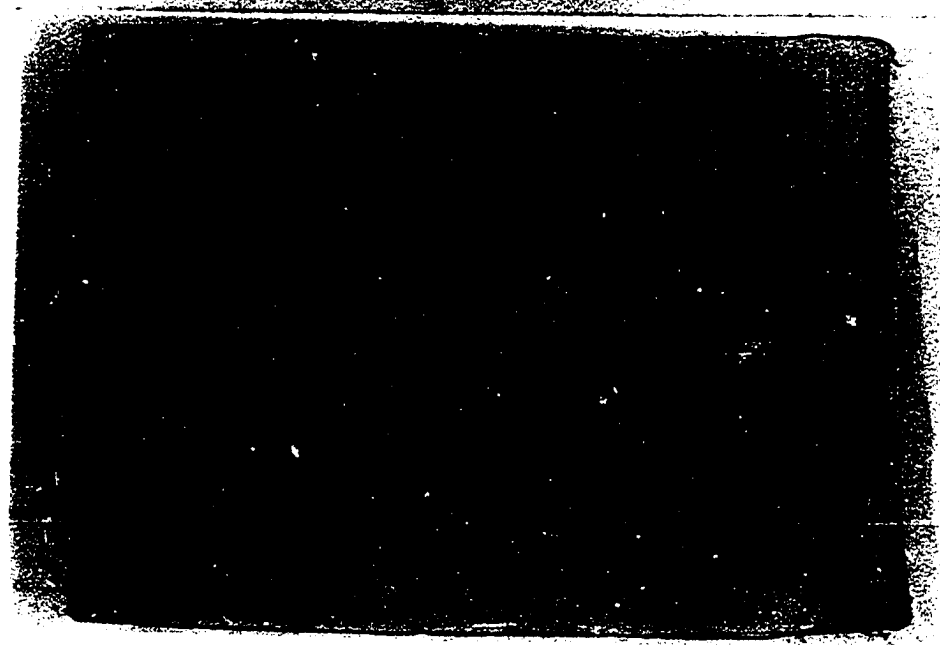
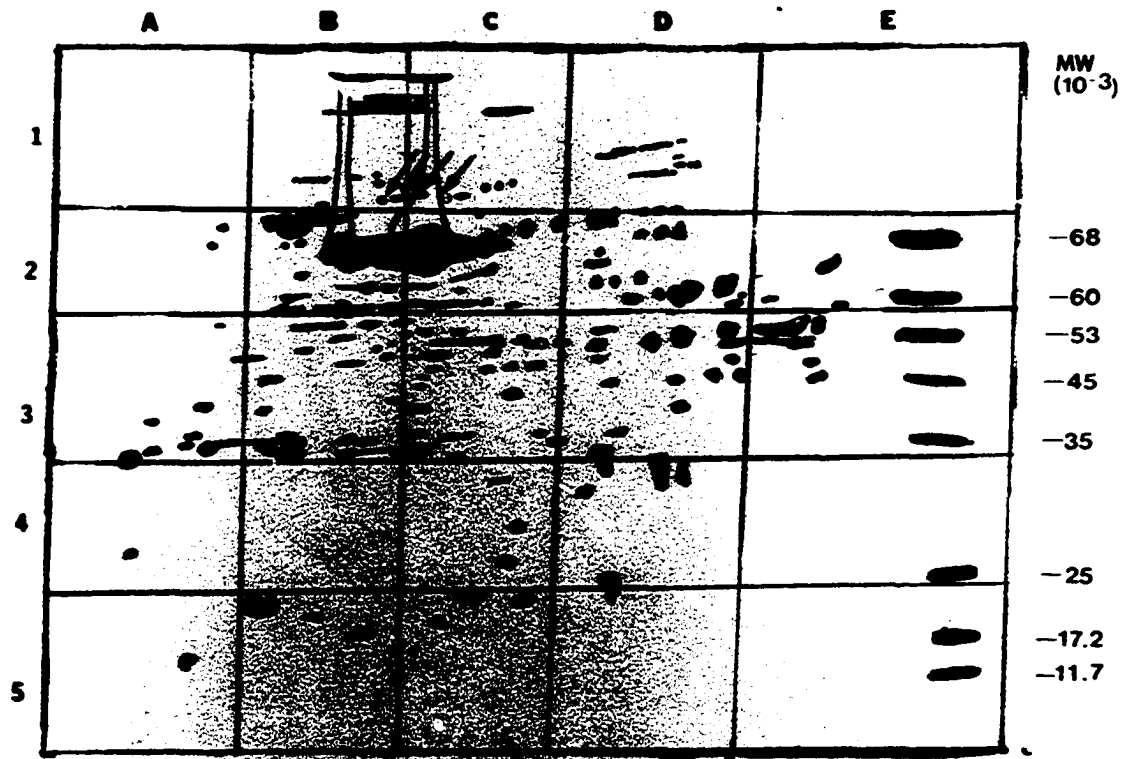


Figure 16. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient E.A. (TCC-Stage D). A diagrammatic representation (top) of the gel slab is divided into sections. One protein is seen in section B3. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first dimensional gel is to the left of the gel slab.

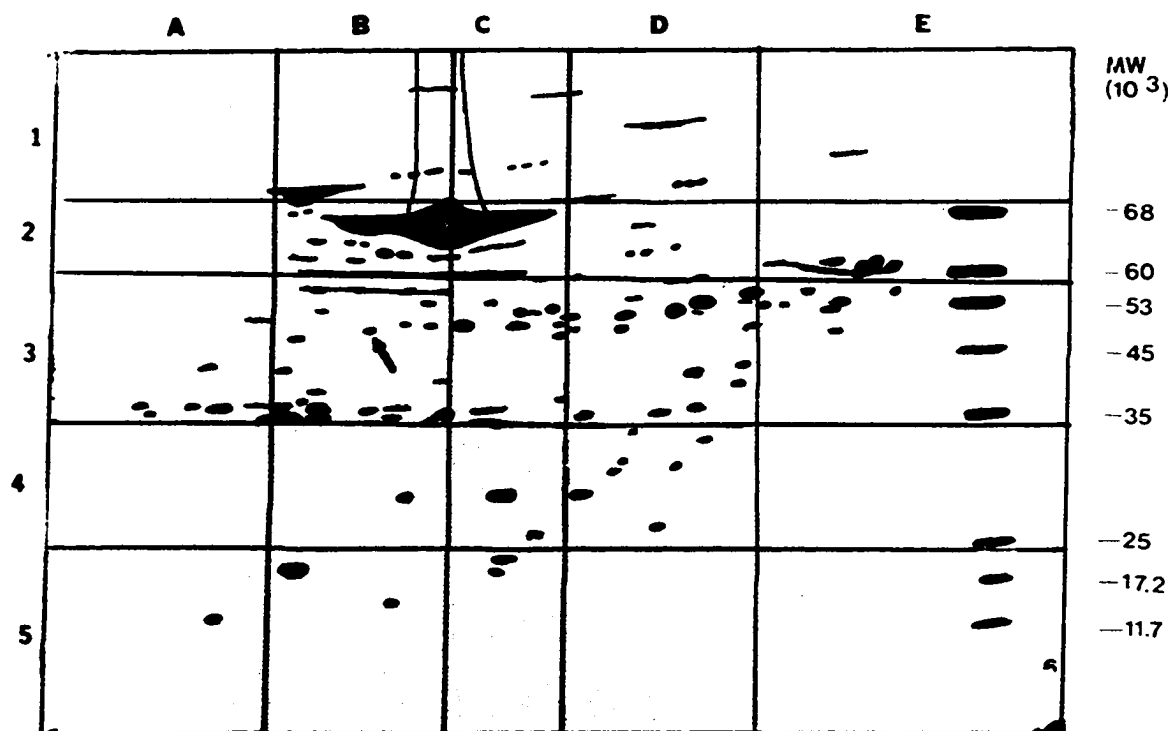


Figure 17. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient G.S. (TCC-Stage B). A diagrammatic representation (top) of the gel slab is divided into sections. One protein is seen in section A-3. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first-dimensional gel is to the left of the gel slab.

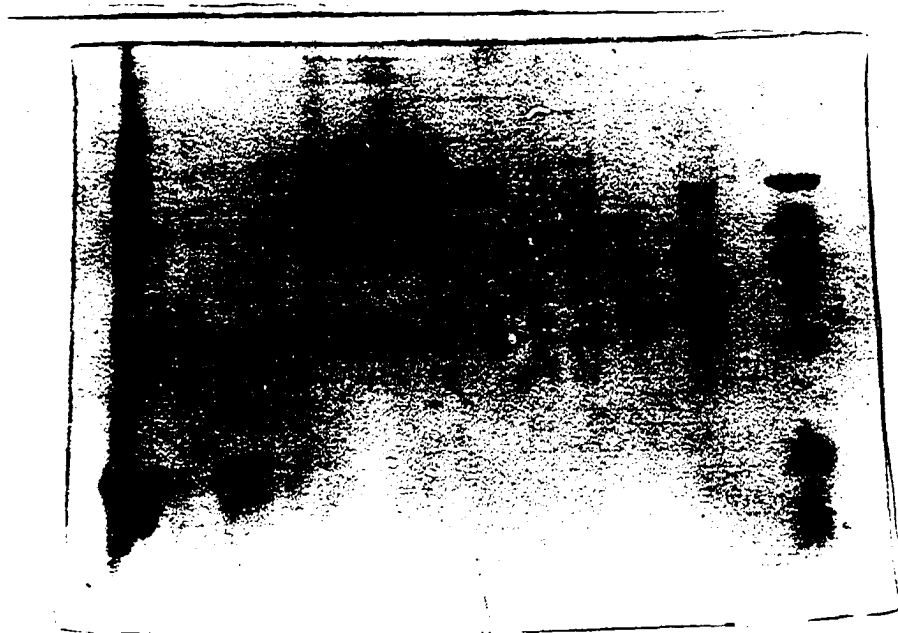
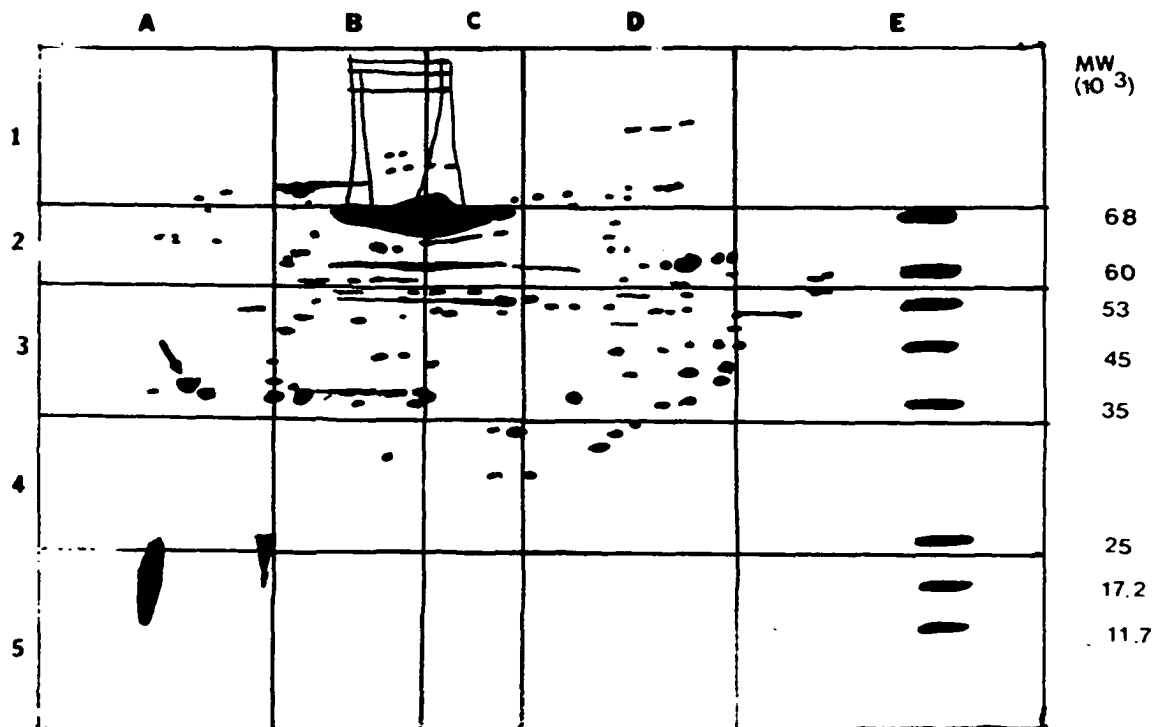


Figure 18. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient W.J. (TCC-Stage B). A diagrammatic representation (top) of the gel slab is divided into sections. Two proteins are seen in section B2. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first dimensional gel is to the left of the gel slab.

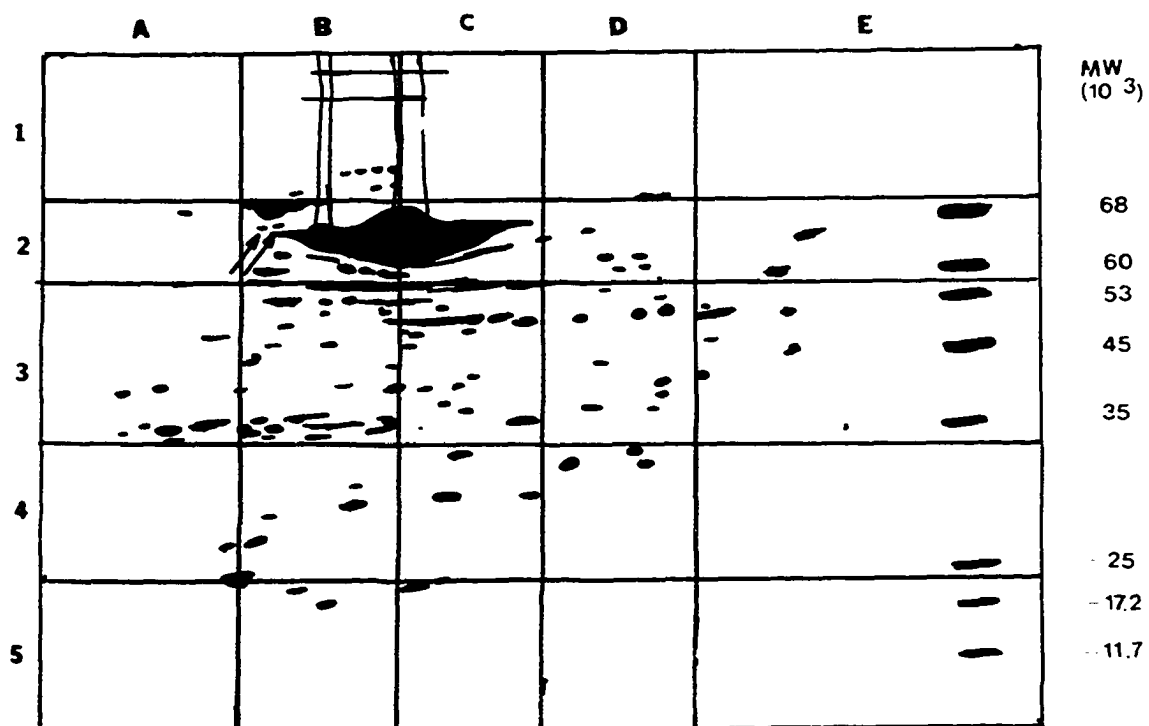


Figure 19. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient B.G. (TCC-Stage B). A diagrammatic representation (top) of the gel slab is divided into sections. Two proteins are seen in section B3. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first dimensional gel is to the left of the gel slab.

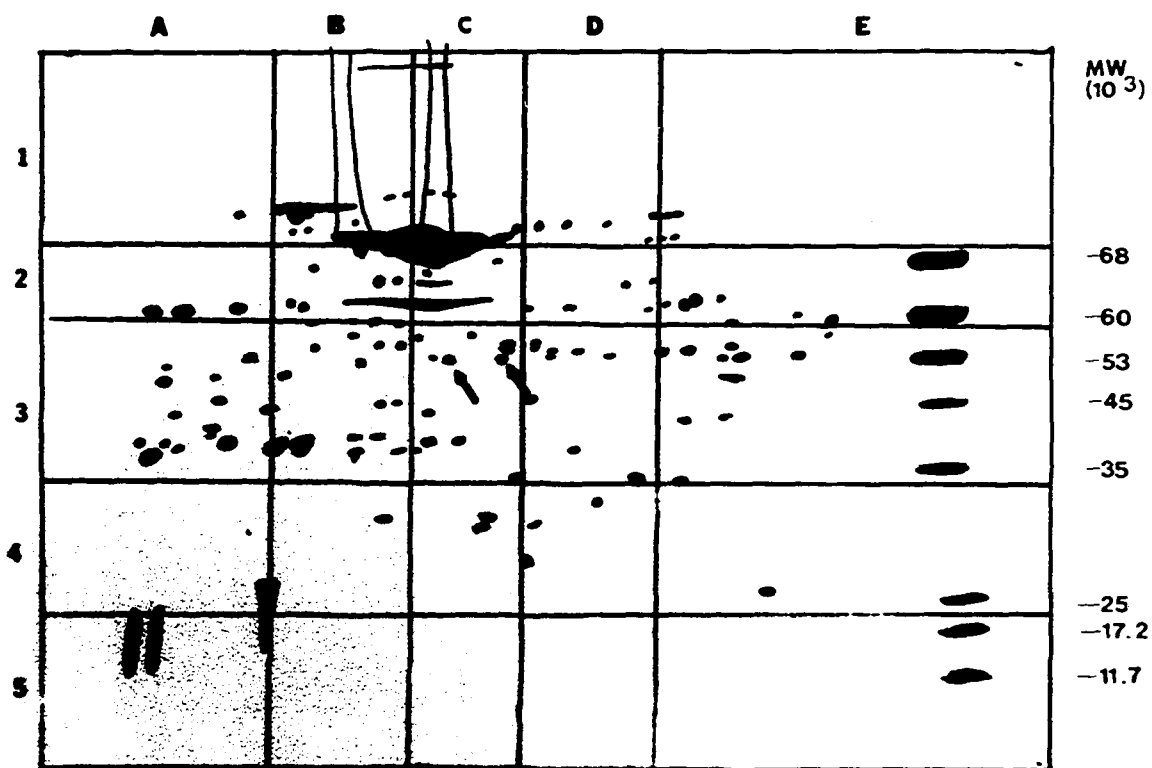


Figure 20. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of six normal donors. A diagrammatic representation (top) of the gel slab is divided into sections. Notice the absence of the 10 unique proteins associated with TCC. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first-dimensional gel is to the left of the gel slab.

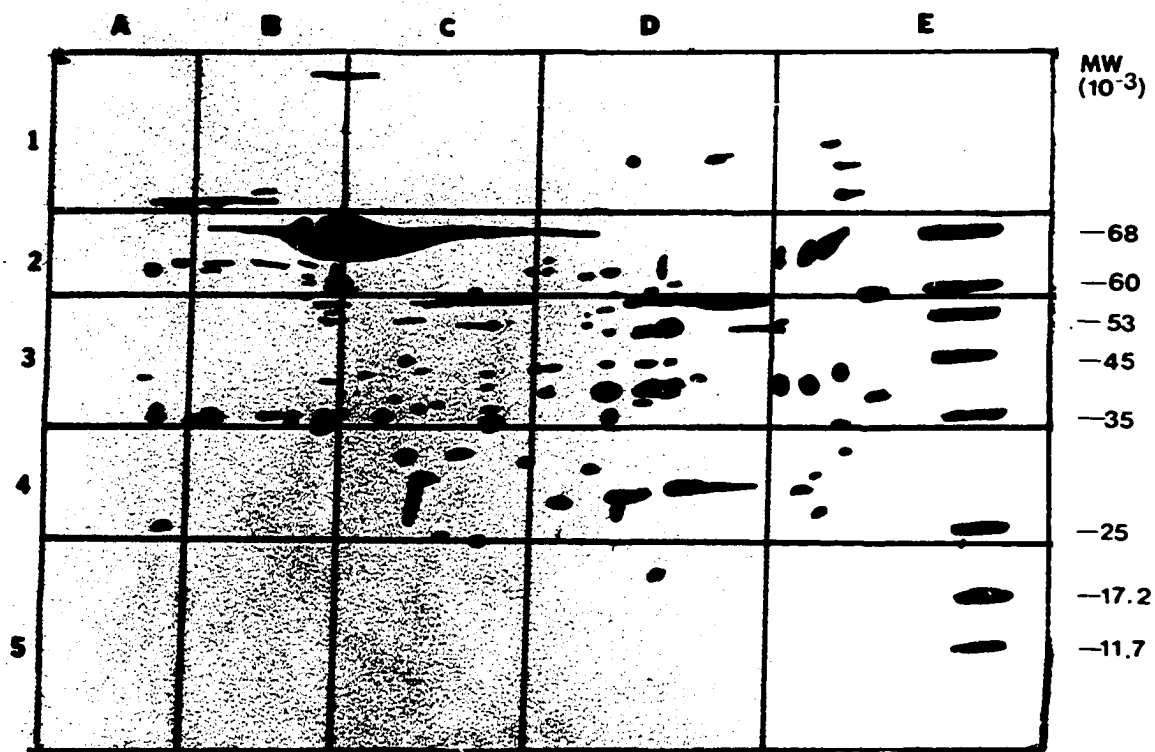


Figure 21. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient K.M. (RCC-Stage D). A diagrammatic representation (top) of the gel slab is divided into sections. Notice the absence of the 10 unique proteins associated with TCC. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first-dimensional gel is to the left of the gel slab.



Figure 22. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient S.B. (BK). A diagrammatic representation (top) of the gel slab is divided into sections. Notice the absence of the 10 unique proteins associated with TCC. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first-dimensional gel is to the left of the gel slab.

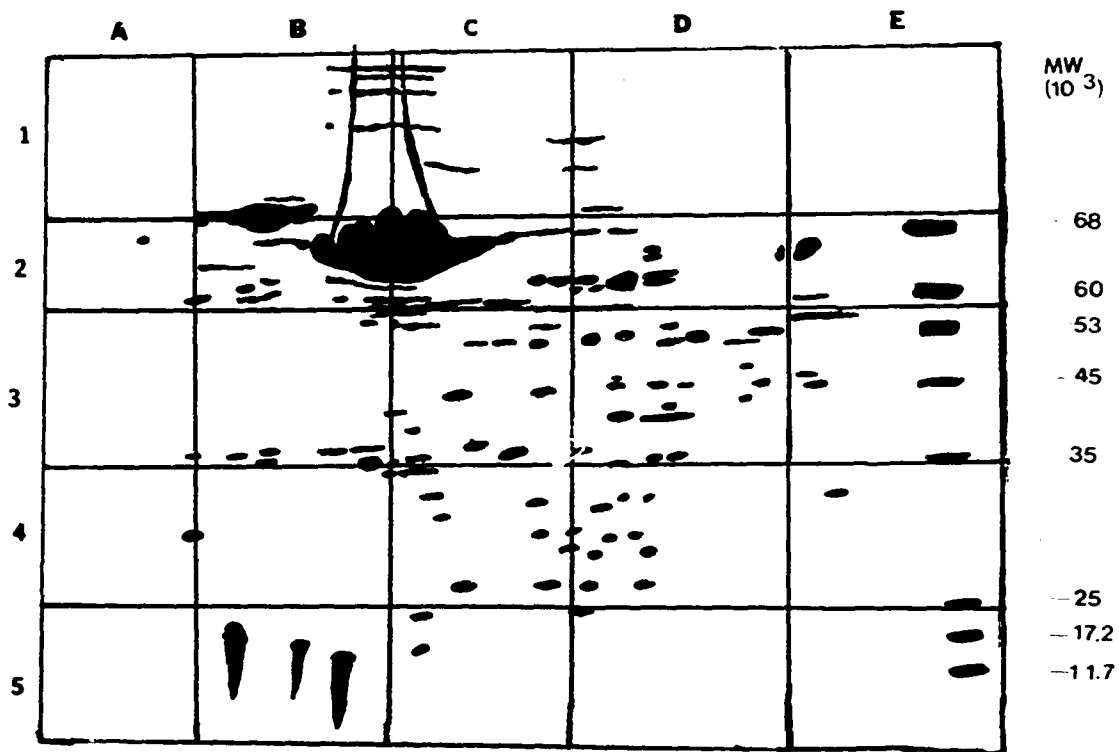


Figure 23. Second-dimensional SDS/polyacrylamide gel electrophoregram of the urine of patient L.P. (BB). A diagrammatic representation (top) of the gel slab is divided into sections. Notice the absence of the 10 unique proteins associated with TCC. A photographic reproduction (bottom) of the gel slab is shown. Molecular weight markers are seen to the right side of the diagram and photograph. The cathode of the first-dimensional gel is to the left of the gel slab.

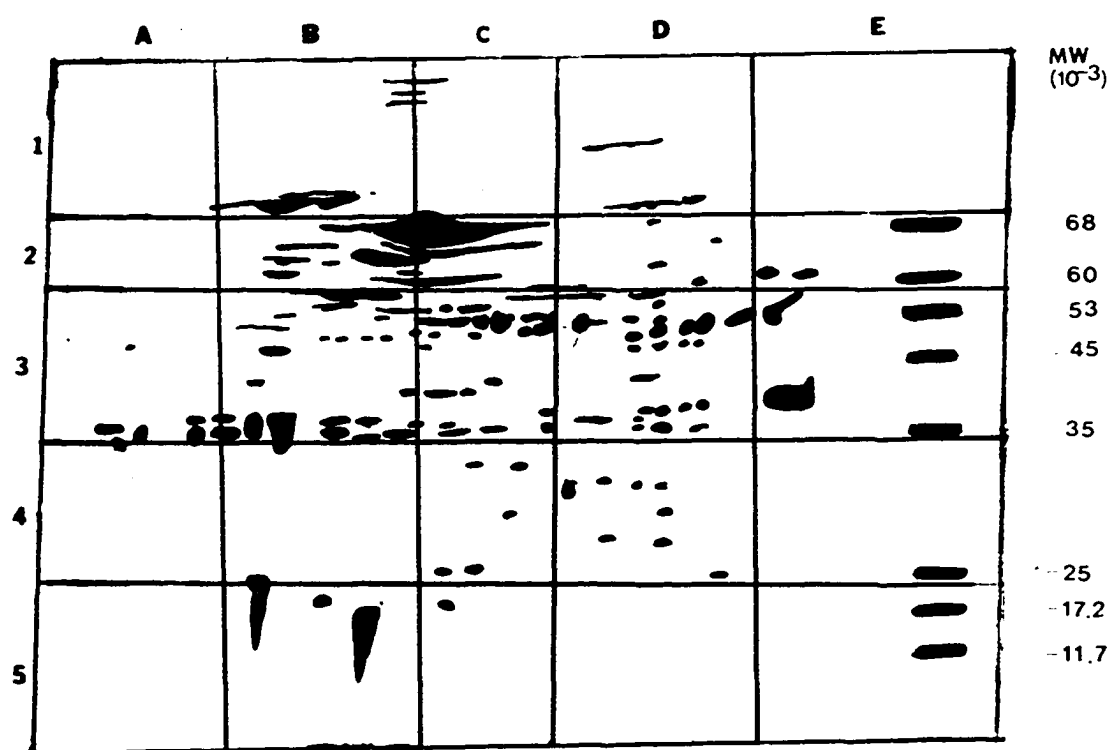
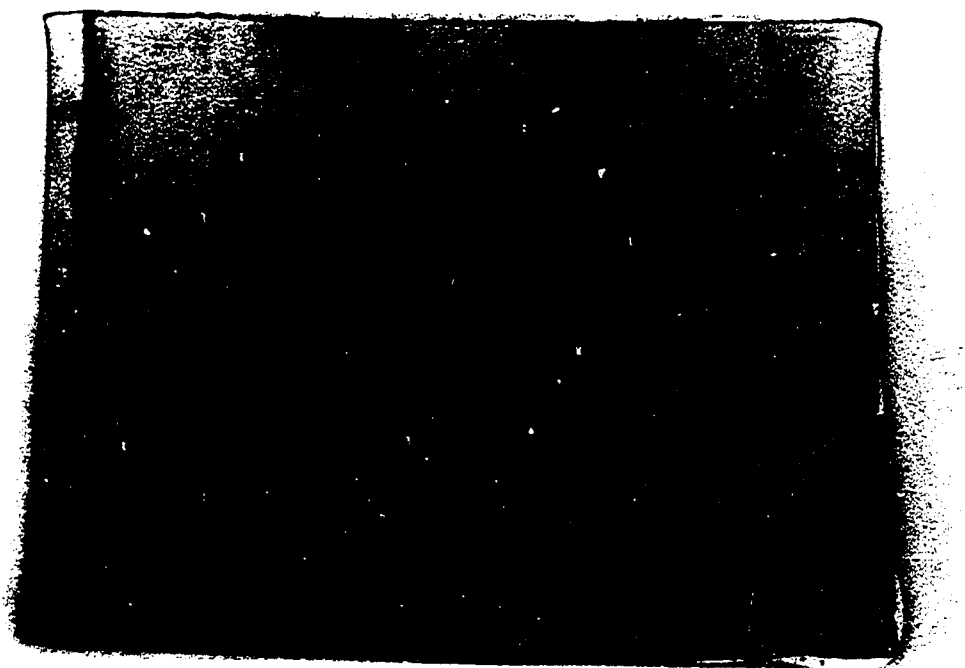


Figure 24. Comparison of the second-dimensional SDS/polyacrylamide gel slabs of the urine of RCC patient K.M. The top figure represents the urine treated with 1mM PMSF; whereas, the bottom photograph is the urine electrophoregram without PMSF. Cathode of the first-dimensional gel is toward the left side of the gel slab.



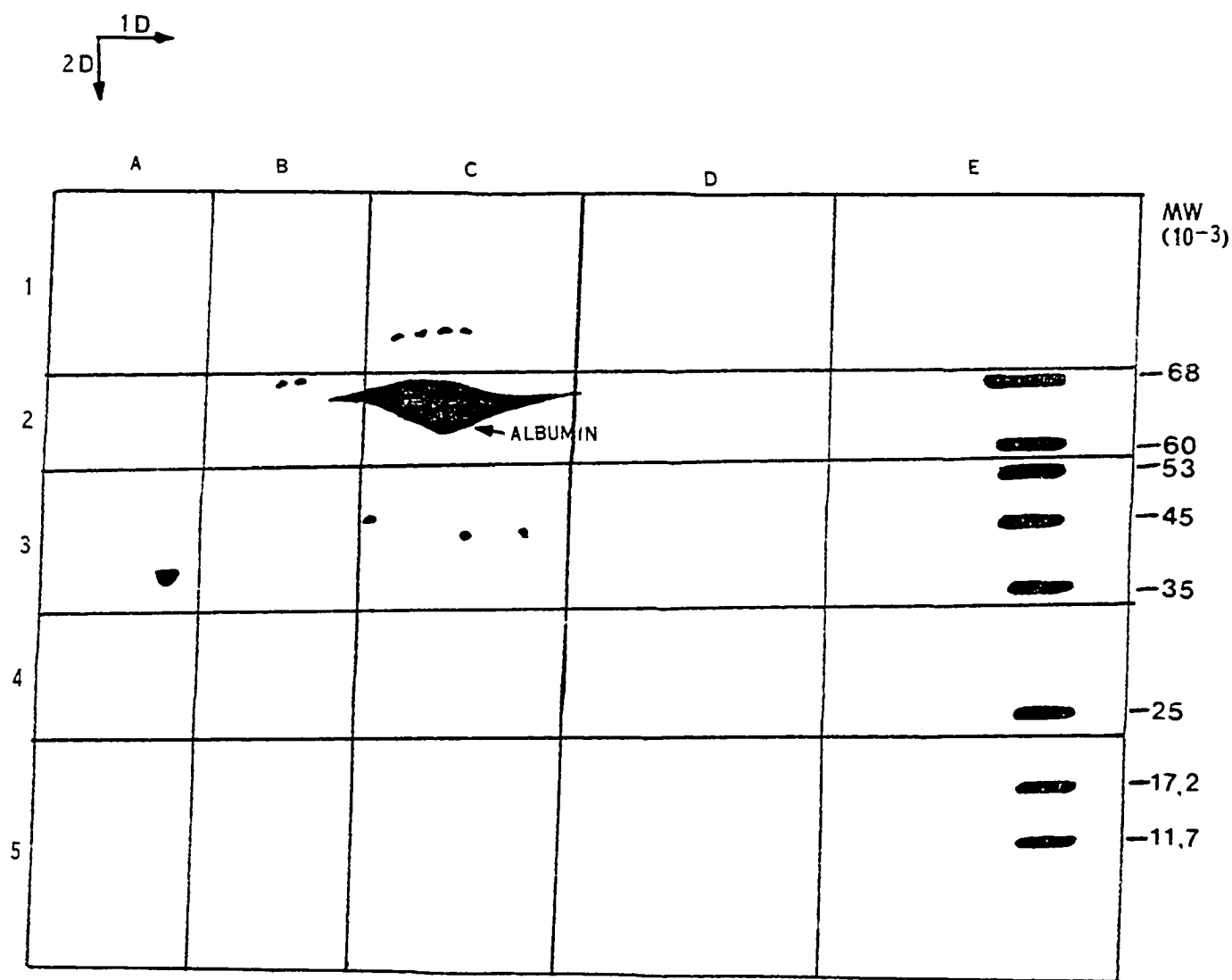


Figure 25. Composite drawing of a two-dimensional electrophoregram showing the location of the unique urinary proteins associated with TCC. Albumin and molecular weight markers are shown for orientation purposes only.

CHAPTER V

DISCUSSION

The purpose of this investigation was to determine if unique tumor components in the urine of patients with transitional cell carcinoma could be detected and characterized (i.e. isoelectric point and molecular weight) by two-dimensional polyacrylamide gel electrophoresis. Urine was chosen as a source of unique tumor proteins because the bladder should release into the urine soluble tumor components associated with malignancy. Because numerous protein components in the urine of renal and bladder patients seem to be closely related to the pathological changes in the genitourinary tract (91, 120), it is plausible to differentiate various renal and bladder disorders and to characterize them by electrophoretic analysis of proteins in the urine. By characterizing these components, purification is possible so that an immunodiagnostic test for early detection of bladder cancer may become possible. The specific assay for the detection of bladder cancer antigens and/or immune complexes can facilitate their isolation. However, as in most malignant disease, the nature of the antigens is unknown.

Urinary proteins were concentrated by ammonium sulfate precipitation and subjected to isoelectric focusing in the first dimension followed by electrophoresis in a second-dimensional system using SDS/polyacrylamide slab gel electrophoresis. One-dimensional isoelectric focusing gels were reproducible in the pH range 4.50-6.50. However, the cathodic end (pH 6.50-7.50) of the isoelectric focusing gel showed a sizable variation among gel patterns. Limited information was acquired from the first-dimensional gels; therefore, two-

dimensional polyacrylamide gel electrophoresis (by using two different separation parameters) made it possible to acquire greater information about the urinary proteins. By varying the concentrations and pH ranges of the ampholines in the first dimension and by creating an optimal polyacrylamide gradient in the second dimension, maximum separation and reproducibility of the gels was achieved. These percentages of ampholine and polyacrylamide were obtained by trial and error. Variability among first-dimensional gels were derived from using different batches of ampholines (36). Differences in normal human antigens, and proteins excreted during neoplastic growth and advance of cancer (i.e. stage) from each patient studied contributed to the variations seen when only one means of separation was employed.

In an attempt to minimize variations among gel patterns and to further identify the components in urine, two-dimensional electrophoresis was performed. The criteria for using two-dimensional polyacrylamide gel electrophoresis were (1) reproducibility of the urine gel patterns, (2) sensitivity, (3) discreteness of the protein spots, and (4) information obtainable from the protein pattern.

A problem observed with the two-dimensional systems was streaking of the proteins to the left side (cathodic side) of the two-dimensional electrophoregrams. The streaking was noticed to increase each time the urine sample was frozen and thawed. The addition of SDS and β -ME helped solubilize the aggregates that were seen in the cathodic end of the first-dimensional gels.

Regardless of the limitations and difficulties, the results described in this study have identified ten proteins that are considered to be unique components present in the urine of patients with transitional cell carcinoma. Normal urine contained from 100-150 polypeptides as demonstrated by the the two-dimensional electrophoretic method used in this study. Similarities of normal and pathological urinary proteins were present in the two-dimensional polyacrylamide

gel profiles. By comparing the pI and molecular weight of each stained polypeptide in the polyacrylamide gel slab, unique components in pathological urine were identified. These proteins were not detected in the urine of patients with BK, BB, and RCC or normal donors. The nature of the proteins described are, to date, unknown. An absolute determination of a tumor-associated component in the urine of patients with TCC cannot be ascertained until immunological tests have been performed (e.g. Ouchterlony assays, fluorescent antibody techniques) to precisely determine the specificity. This will require purification of the putative TCC-associated urinary proteins.

Hollinshead, et. al., (56) have reported a partially purified a 40,000 dalton polypeptide in the urine of TCC patients that they describe as a bladder cancer TAA. It is possible that this bladder cancer TAA may be the protein found in section A3 of the upper illustration in figure 17. This protein occurred in greater abundance in patients with advanced stages of TCC. This protein was not seen in any BB, BK, RCC patient or normal donors; whereas, 100% of the TCC patients demonstrated the presence of this protein in their urine. Although the molecular weight of this polypeptide is similar to that described by Hollinshead, the reliability of comparing or identifying proteins based on molecular weights in polyacrylamide gels is limited (129).

Rudman, et. al., (109) describe increased excretion of polypeptides in the urine within the molecular weight range from 12,000-50,000 daltons in patients with advanced neoplastic urogenital disorders. The number and size of the urinary proteins demonstrated in the two-dimensional electrophoregrams of TCC and RCC patients, as well as in the total protein yield (Table 3), confirms this report.

It is evident that the data obtained in this study has some inherent problems associated with the random sample of urines obtained as well as the

analytical procedure used. Until more information and minor improvements on the reproducibility and reliability of protein patterns is obtained, and the interpretation and identification of the individual protein components of urine are refined, one should be cautious in calling proteins found in pathological urine as tumor-associated components associated with a malignancy. Though certain proteins were found in increased amounts in TCC urine and could not be detected in normal, BB, BK, and RCC urines, more sensitive and specific immunological tests for detection will be required before these "unique" proteins can be termed "tumor-associated". Nevertheless, the two-dimensional electrophoregrams may have merit in the initial detection and localization of candidate tumor-associated urinary proteins.

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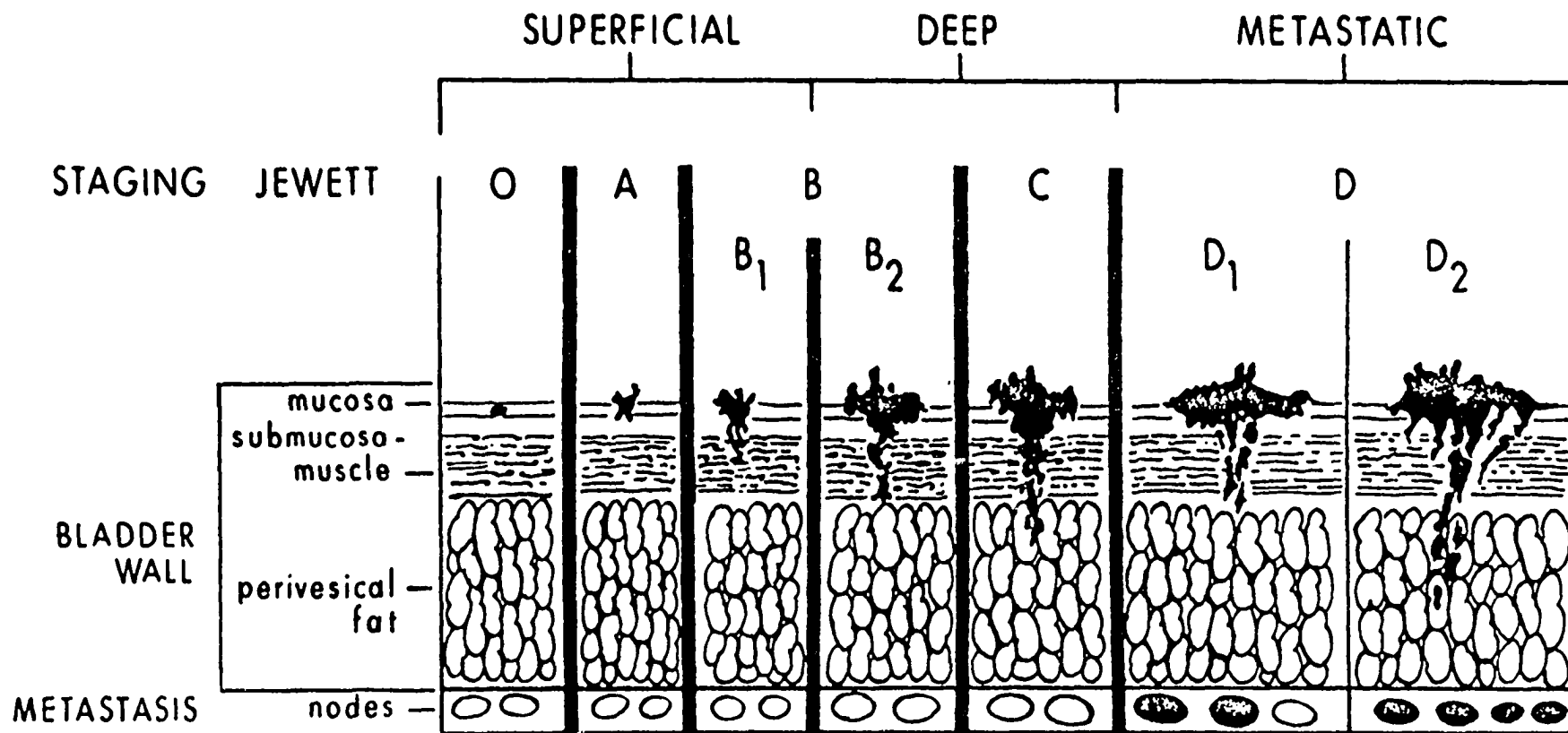
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Appendix A. Jewett Staging of Transitional Cell Carcinoma. From: Jewett, H.J., and Strong, G.H. J. Urology 55: 366-372 (1966).



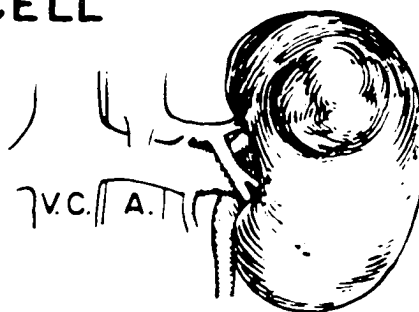
- Stage 0 - confined to superficial mucosa
- A - submucosal infiltration
- B₁ - superficial muscle invasion
- B₂ - deep muscle invasion
- C - perivesical infiltration
- D - spread outside of bladder

Appendix B. Staging of Renal Cell Carcinoma. From: Holland, J.M. Cancer
32: 1030-1042 (1973).

STAGING OF RENAL CELL CARCINOMA

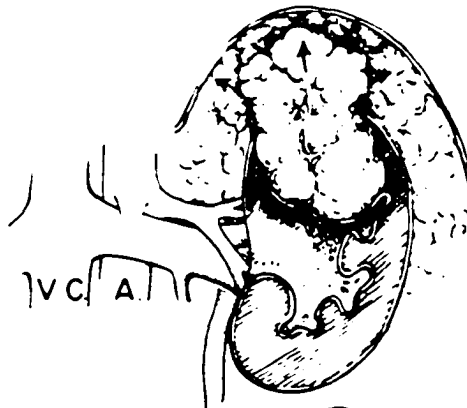
STAGE A

TUMOR WITHIN CAPSULE



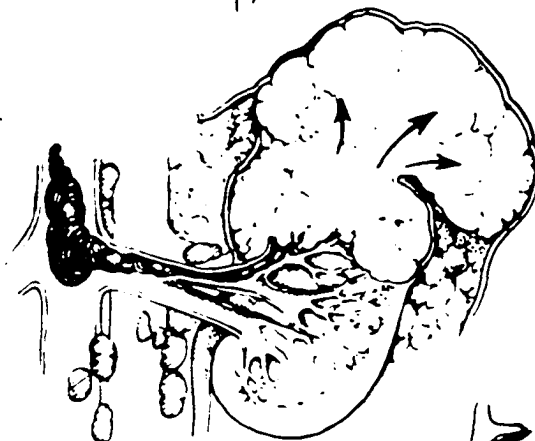
STAGE B

TUMOR INVASION OF PERINEPHRIC FAT (CONFINED TO GEROTA'S FASCIA)



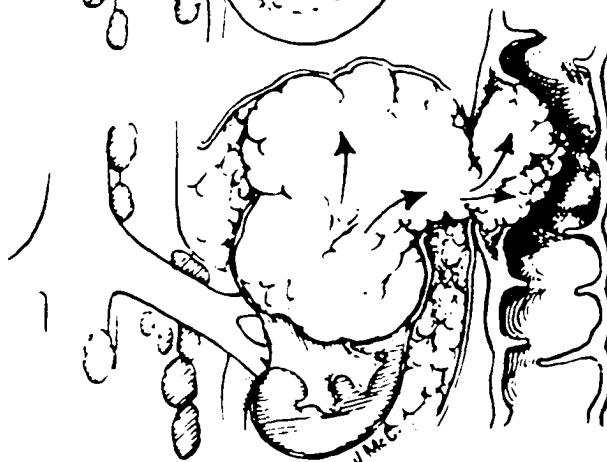
STAGE C

TUMOR INVOLVEMENT OF REGIONAL LYMPH NODES AND/OR RENAL VEIN AND CAVA



STAGE D

ADJACENT ORGANS OR DISTANT METASTASES



APPENDIX C

CHEMICALS

Ampholines were obtained from Bio-Rad Laboratories (Richmond, Calif.) as Biolytes (40% solids) within pH range 5 to 7 and pH range 3 to 10. Acrylamide, N,N' methylene bisacrylamide (BIS) and sodium dodecyl sulfate (SDS) were also purchased from Bio-Rad Laboratories. Nonidet P-40 (NP-40) was purchased from Particle Data Laboratories (Elmhurst, Ill.) Fisher Scientific Co. (Fair Lawn, N.J.) supplied the ammonium persulfate, phosphoric acid, sodium hydroxide, and sulfosalicylic acid. Ultra pure urea was purchased from Schwarz/Mann (Orangeburg, N.Y.) Ammonium sulfate, trichloroacetic acid (TCA), glycerol, sucrose and N,N,N',N'-tetra-methylene diamine (TEMED) were obtained from Baker Chemical Co. (Phillipsburg, N.J.) Methanol was supplied by A.H. Thomas Co. (Philadelphia, Pa.) Mallincrodt (Paris, Ky.) supplied the acetic acid. Sigma Chemical Co. (St. Louis, Mo.) supplied the β -mercaptoethanol (β -ME), phenyl-methylsulfonylfluoride (PMSF) and Coomassie Brilliant Blue R-250 used in these experiments. Agarose (SeaKem) was obtained from Marine Colloids (Rockland, Maine). The following molecular weight markers were used. Molecular weights (M.W.) are given in daltons.

<u>MOLECULAR WEIGHT MARKER</u>	<u>SUPPLIER</u>	<u>MOLECULAR WEIGHT</u>
Bovine Serum Albumin (BSA)	Calbiochem (San Diego, Calif.)	68,000
Catalase	Mann Research (New York, N.Y.)	60,000
L-Glutamic Dehydrogenase	Sigma Chemical (St. Louis, Mo.)	53,000
Ovalbumin	Pharmacia (Piscataway, N.J.)	45,000
Pepsin (Hog Stomach)	Mann Research (New York, N.Y.)	35,000
Chymotrypsinogen A	Pharmacia (Piscataway, N.J.)	25,000
Myoglobin	Mann Research (New York, N.Y.)	17,200
Cytochrome c	Calbiochem (San Diego, Calif.)	11,700

APPENDIX D

REAGENTS, BUFFERS AND SOLUTIONS

- A 30% Acrylamide Stock for IEF Gels: 28.38% (w/v) Acrylamide and 1.62% (w/v) BIS. (Filtered through Whatman #2 filter paper and stored in a brown glass bottle).
- B Stock NP-40 Solution: 10% (w/v) Nonidet P-40 in distilled water. (Filtered through Whatman #2 filter paper).
- C Ammonium Persulfate: a 10% (w/v) solution prepared fresh each week.
- D Gel Overlay Solution: 8M Urea (stored frozen as 1 ml aliquots).
- E SDS Sample Solution: 1% (w/v) SDS in distilled water.
- F Lysis Solution: 9.5M Urea, 2% (w/v) NP-40, 2% Ampholines (Comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10). (Stored as frozen 1 ml aliquots).
- G 1 X Anode Electrode Solution: 0.01M H_3PO_4 (May be prepared as 10 X solution and diluted 1:10 before use).
- H 1 X Cathode Electrode Solution: 0.02M NaOH (may be prepared as a 10 X solution and diluted 1:10 before use).
- I Sample Overlay Solution: 9M Urea, 1% Ampholines (Comprised of 0.8% pH range 5 to 7 and 0.2% pH range 3 to 10). (Stored frozen as 1 ml aliquots).
- J IEF Gel Fixing Solution: 3.5% (w/v) Sulfosalicylic acid, 11.5% (w/v) Trichloroacetic acid, 30% (v/v) Methanol.
- K IEF Gel Staining Solution: 8% (v/v) Acetic Acid, 25% (v/v) Ethanol, 0.2% (w/v) Coomassie Brilliant Blue R-250 (Filtered through Whatman #2 filter paper).
- L IEF Gel Destaining Solution: 8% (v/v) Acetic Acid, 25% (v/v) Ethanol.
- M 30% Acrylamide Stock for SDS Gels: 29.2% (w/v) Acrylamide and 0.8% (w/v) BIS. (Filtered through Whatman #2 filter paper and stored in a brown glass bottle).
- N Lower Gel Buffer: 1.5M Tris-HCl, pH 8.8, and 0.4% (w/v) SDS. (Filtered through Whatman #2 filter paper).
- O Upper Gel Buffer: 0.5M Tris-HCl, pH 6.8, and 0.4% (w/v) SDS. (Filtered through Whatman #2 filter paper).
- P SDS Sample Buffer: 10% (w/v) glycerol, 5% (v/v) β -mercaptoethanol, 2.3% (w/v) SDS and 0.0625M Tris-HCl, pH 6.8. (Filtered through Whatman #2 filter paper).
- Q Agarose Gel: 1 gm of agarose melted in 100 ml of Solution P.
- R SDS Running Buffer: 0.025M Tris base, 0.192M Glycine, and 0.1% (w/v) SDS (prepared prior to use).
- S Buffer For The Preparation Of Molecular Weights: 0.088M Tris-HCl, pH 6.8.
- T Staining Solution for SDS Gels: 25% (w/v) Trichloroacetic acid, 0.2% (w/v) Coomassie Brilliant Blue R-250. (Filtered through Whatman #2 filter paper).
- U Destaining Solution for SDS Gels: 7% (v/v) Acetic Acid.
- V Preservative for IEF and SDS Gels: 5% (v/v) Acetic Acid.
- W Phosphate-Buffered Saline: 0.35% (w/v) KH_2PO_4 , 1.06% (w/v) $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.85% (w/v) NaCl, pH 7.3.

X Buffer for Molecular Weight Standards: 0.088M Tris-HCl, pH 6.7.

Solutions A,B,C,D,M,N,O,P,Q,S,W,X were stored at 4°C.

Solutions G,H,J,K,L,T,U,V were stored at room temperature.

All other solutions stored as indicated.

Solutions A,B,D,E,F,I,M,N,O,P,Q,S should be prepared using Abbott Co. sterile distilled water to maintain a high level of water purity within the gel matrix.

APPENDIX E

PREPARATION OF MOLECULAR WEIGHT STANDARD

Proteins of known molecular weights were included in the protein separation in the second dimension. Molecular weight markers used (Appendix C) were electrophoresed under similar conditions as the samples. To prepare molecular weight standards, the following recipe was prepared in a test tube for each molecular weight standard used.

"MOLECULAR WEIGHT STANDARD STOCK"

5 mg of each molecular weight standard was diluted in 1 ml of 50% (w/v) sucrose.

"BUFFERED MOLECULAR WEIGHT STANDARDS" (200 µg/ml)

40 µl of 5 mg/ml molecular weight standard stock
100 µl of 1% SDS (Solution E: Appendix D)
100 µl of 50% sucrose
50 µl of β-ME
710 µl of 0.088M Tris-HCl, pH 6.7 (Solution X: Appendix D)

This solution (5% β-ME, 0.1% SDS, 0.0625M Tris-HCl, pH 6.7) was boiled for 3 min and frozen as stored aliquots at -70°C.

To facilitate adding the standard to the second dimensional slab gel, the "buffered molecular weight standards" were mixed by pipetting 60 µl of each buffered standard into a test tube marked "mixed buffered molecular weight standards". This tube was frozen at -70°C until adding it to the well cut into the agarose gel used in the second dimension.