Studies on an antineoplastic fraction from human urine

Characterization of the major protein in this fraction

Nathan H. SLOANE,*§ W. R. LYNN,* R. M. MACLEOD,* E. P. K. HADE,* Raveendran POTTATHIL† and Andreas P. KYRIAZIS‡

*Department of Biochemistry, University of Tennessee College of Medicine, Memphis, TN 38163; †Department of Pediatric Research, University of Maryland School of Medicine, Baltimore, MD 21201; and ‡Department of Pathology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, U.S.A.

A fraction has been isolated from human urine which exhibits antiproliferative activity against human tumour cell lines without affecting the growth of several normal diploid cell lines or tumour cells of mouse or hamster origin. The major protein present in this fraction has been characterized and tentatively designated antineoplastic urinary protein (ANUP). An $s_{20,w}^0$ value of 3.69 S was obtained by sedimentation velocity analysis, and a subunit molecular mass of 16300 Da was obtained by sedimentation equilibrium and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Centrifugation data also indicated that the protein self-associates. The amino acid analysis of ANUP was consistent with its low pI (4.2) as determined by chromatofocusing analysis. Furthermore, the amino acid composition exhibited some features similar to collagen, as shown by high levels of proline and glycine, the absence of cysteine, and the presence of low levels of hydroxyproline.

INTRODUCTION

Normal human urine has been shown to contain a number of biologically active proteins, including urokinase (54000 Da; Lorand & Condit, 1965; White *et al.*, 1966) and erythropoietin (38000–48000 Da; Goldwasser & Kung, 1971; Schooley & Garcia, 1962, 1965). Thus, these biologically active materials can pass through the kidney. Furthermore, Sloane *et al.* (1982) reported that normal human urine also contains an antineoplastic protein that exhibited antitumour activity directed against human tumour cell lines (lung, pancreas, cervix, breast, melanoma and leukaemia cells) without affecting the growth of several normal human diploid cell lines or tumour cell lines of mouse or hamster origin.

It is the purpose of the present paper to describe the isolation of a protein fraction from human urine that showed growth-inhibitory activity against a variety of human tumour cell lines, and to present data on the chemical and biological properties of this protein, designated the antineoplastic urinary protein (ANUP).

EXPERIMENTAL PROCEDURES

Absorption of ANUP on Florisil

Initial isolation studies were performed on 24 h urine samples of healthy, non-smoking males. Urine samples for large-scale isolation of ANUP consisted of 24 h voided urine samples from hospitalized patients; the urine was collected in the presence of boric acid and butyl-*p*-hydroxybenzoic acid. ANUP from the two sources appeared to be identical.

ANUP was adsorbed from the urine by Florisil (magnesium silicate granules; Florisil Co., Berkley Springs, WV, U.S.A.). The Florisil was first freed of fines

by repeated washes with water and then treated with ascorbic acid to adjust the pH to 3–4. The Florisil granules were then placed into cotton jersey packets (6 in × 7 in; 15.2 cm × 17.8 cm); the upper packet contained 15/30 mesh Florisil (about 250 g) and the lower packet contained 30/60 mesh Florisil (about 250 g). These stacked packets were utilized to absorb the ANUP contained in 200 litres of pooled human urine. The urine was allowed to flow (at 20 litres/h) through these stacked packets from a separatory funnel that was moved continually by hand to cover the entire area of the Florisil; usually 40 litres of urine was adsorbed at one time at 22 °C. The packets were refrigerated until the next pool of urine was treated.

Elution of ANUP from Florisil

The contents of each packet were removed and placed in separate 2 litre plastic beakers that were held in ice. The granules from each Florisil packet were washed with cold water followed by 500 ml of cold 15% (v/v) acetone at pH 9.0–9.3 (NH₄OH was used to adjust the pH). Elution of ANUP from the acetone-washed Florisil was accomplished by the addition of 500 ml of cold acetone/glycerol/water (3:6:11, by vol.) to the 15/30 mesh Florisil, the pH was adjusted to 9.0–9.3 and the mixture gently stirred for about 5 min. The eluate was then added to the 30/60 mesh Florisil and the pH was readjusted to 9.0–9.3; after gentle stirring at alkaline pH, the eluate was decanted from the settled Florisil and adjusted to pH 7.0–7.4 with cold 2M-HCl. This elution procedure was repeated twice. The combined neutralized eluates were concentrated to about 100 ml at 4 °C in an Amicon Diaflo filtration apparatus utilizing a UM 20 membrane (Amicon Corp. Danvers, MA, U.S.A.). This

Abbreviation used: ANUP, antineoplastic urinary protein.

§ To whom reprint requests should be addressed.

membrane has a nominal cut-off of 20000 Da. Thus, the concentrate should contain molecules predominantly of molecular mass greater than 20000 Da. The concentrated eluate was dialysed against cold water and, after centrifugation, the clear eluate was lyophilized. This fraction was designated the Florisil eluate; the yield was approx. 10 mg/litre of urine.

Assay to screen antineoplastic activity of human urinary proteins

The antineoplastic activity of each urinary protein fraction was assayed by determining its antiproliferative activity. The growth inhibition assay was performed using the human cervical carcinoma, HeLa, cell line. HeLa cells were grown in minimum essential medium supplemented with 10% (v/v) fetal bovine serum and penicillin and streptomycin. The cells, in 5 ml volumes (10^5 cells), were seeded in 25 cm² flasks. The cultures were inoculated with various doses (10–500 µg/ml) of each urinary protein fraction. After incubation for 96–144 h at 37 °C in CO₂/air (1:19), the number of viable cells in each flask was determined by counting the trypsin-released cells. Control flasks showed $(1.8\text{--}2.0) \times 10^6$ cells after the incubation period.

The amount of protein per ml of medium required to inhibit the growth of HeLa cells by 50% relative to control cells was defined as 1 unit of activity. The Florisil eluate had a specific activity of 5 units/mg.

Protein determinations

Relative protein concentrations were determined spectrophotometrically at 280 nm, using an assumed specific absorption of 1 litre·g⁻¹·cm⁻¹. Protein concentration was also determined by dry weight.

Chromatofocusing separation of the antineoplastic activity after purification by Sephacryl S-200 chromatography

A sample (40 mg) of the biologically active Sephacryl S-200 protein fraction (fractions 27–31; Fig. 1) was dissolved in 3 ml of 0.025 M-imidazole/HCl buffer (pH 7.4). Chromatofocusing was accomplished by the use of a Pharmacia chromatofocusing column with Polybuffer (Pharmacia, 1982). Fractions (3 ml) were collected at a flow rate of 22 ml/h. The pH of each fraction was determined and each fraction was screened for antineoplastic activity as described above.

Purification of ANUP to electrophoretic homogeneity

Batches (3 g) of the Florisil eluate were dissolved in 60 ml of phosphate-buffered saline without calcium (PBSA) containing 0.5 M-NaCl and filtered through a YM 30 Amicon Diaflo membrane. This membrane has a nominal cut-off of 30000 Da. Thus, the filtrate should contain molecules predominantly of approx. 30000 Da or less. The filtrate was dialysed against water and lyophilized. The lyophilized ANUP fraction (1.5 g) was dissolved in 30 ml of 0.1 M-Tris/acetate/0.02 M-magnesium acetate, pH 7.24, and subjected to Sephacryl S-300 chromatography (5 cm × 90 cm column equilibrated with the same buffer at 4 °C). The flow rate was 23 ml/h and fractions of 11.5 ml volume were collected and tested for antineoplastic activity. The active fractions were pooled, dialysed and lyophilized (yield was 300 mg). The protein was then dissolved in the Tris/magnesium acetate buffer containing 0.05 M-KCl and another chromatographic

separation was done on a Sephacryl S-200 column (2.5 cm × 90 cm). The antitumour fractions (5.6 ml/fraction) were located by assay, pooled, dialysed, and lyophilized (the yield was 100 mg). This biologically active ANUP fraction was rechromatographed on the Sephacryl S-200 column as described above and 5.6 ml fractions were collected. The pooled antineoplastic fractions were dialysed and lyophilized (the yield was 60 mg).

Electrophoretic analyses

Purity of ANUP at various steps in the fractionation procedure was determined by polyacrylamide-gel electrophoresis under both native and denaturing conditions as described by Laemmli (1970).

Antineoplastic analysis of ANUP fractions after SDS/polyacrylamide-gel electrophoresis

In each of two lanes (2 mm × 1 cm) were placed 2 mg of ANUP (Sephacryl S-200 eluate) and the protein was subjected to SDS/polyacrylamide-gel electrophoresis in an 8% gel slab (15 cm × 15 cm × 2 mm thick). After electrophoresis at 30 mA for 16 h one lane was cut from the slab and sliced (2 mm slices) and the material from each slice was eluted with 200 µl of phosphate-buffered saline without calcium; 10–20 µl of each eluate was used to assay for biological activity in HeLa cell monolayers. These cells were grown in 2 cm²–24 multiwell plates in minimum essential medium containing 2% (v/v) foetal bovine serum utilizing approx. 2×10^4 cells/well as inoculum. Four wells were used to assay each level of each gel slice. The other lane was stained with Coomassie Blue and the separated proteins were compared with standard molecular mass markers.

Ultracentrifuge studies

Sedimentation equilibrium. The molecular mass was determined by sedimentation equilibrium using interference optics and the classical low-speed method (Chervenka, 1969) with a Beckman (Spinco) model E analytical ultracentrifuge. An An-H titanium rotor was used together with a standard cell which was equipped with sapphire windows and a 12 mm double-sector synthetic boundary centrepiece. The ANUP sample was dissolved in phosphate-buffered saline. Temperature was controlled at 20 °C. The synthetic boundary run to determine the initial ANUP concentration was done with the same sample and cell (Chervenka, 1969). A partial specific volume of 0.71 ml/g for ANUP was calculated from the amino acid composition (Schachman, 1957). Solution density was calculated from the protein concentration, partial specific volume of ANUP, and the density of the solvent (Chervenka, 1969). Solvent density was determined by pycnometry. Photographic plates were read on a Nikon Shadowgraph (model 6). The sedimentation equilibrium experiment to determine molecular mass was carried out on a sample of the same ANUP preparation which gave a single band of 16 000 Da in SDS gel electrophoresis. The protein concentration was 2.3 mg/ml in phosphate-buffered saline, the rotor speed was 12590 rev./min and the temperature was 20.0 °C.

Sedimentation velocity. The sedimentation coefficient was determined by sedimentation velocity experiments, using Schlieren optics. An An-D rotor was used with the

Fig. 1. ANUP activity in Sephacryl S-200 chromatographic fractions

Florisil eluate, (300 mg), was dissolved in 6 ml of cold (4 °C) 0.1 M-Tris/acetate buffer containing 0.02 M-magnesium acetate at pH 7.24. This solution was applied to a Sephacryl S-200 column (2.5 cm × 90 cm) that had been equilibrated with the buffer; all operations were conducted at 4 °C. A flow rate of 23 ml/h was used to elute the protein peaks; 11.5 ml volumes were collected individually by means of an automatic fraction collector and the relative absorbance at 280 nm was monitored by passing the eluate through a flow cell in a Beckman Acta II spectrophotometer/recorder system. The antineoplastic activity of each fraction was assessed by determining the growth inhibition activity of 0.5–1.0 ml sterile filtered samples of each fraction.

Model E analytical ultracentrifuge with a standard double-sector cell and sapphire windows. ANUP samples in the concentration range 1–10 mg/ml were prepared in phosphate-buffered saline. The observed sedimentation coefficients were corrected to 20 °C for the effects of the viscosity and density of the buffer according to standard procedure (Chervenka, 1969). The photographic plates were read on a Nikon Shadowgraph (model 6).

RESULTS

Survey of the antineoplastic activities of ANUP

The Florisil eluate showed ANUP activity which could be destroyed by trypsin. Therefore, we attributed the activity to a protein or peptide and proceeded on that basis. The results of an initial survey of the growth inhibitory activity of the Florisil eluate are shown in Table 1. Nine human tumour cell lines were studied; the Florisil eluate inhibited the growth of each cell line. The sensitivity of each cell line to the Florisil eluate was different. The hamster tumour cell line was not affected (Table 1).

The partially purified Sephacryl S-200 eluate fraction (Fig. 1) showed 57% inhibition of growth of the HeLa cell line at a concentration of 10 µg/ml (Fig. 2). On the other hand, a 500 µg/ml concentration of the Sephacryl S-200 product did not inhibit the growth of human diploid lines WI38 and Hf54 and mouse tumour lines L929 and S180. Electrophoretically homogeneous (SDS/polyacrylamide-gel electrophoresis) ANUP caused a 50% inhibition of the cell line CALU-6 at a concentration of 40 µg/ml.

ANUP activity in Sephacryl S-200 fractions

The Sephacryl S-200 chromatographic separation of the proteins contained in the Florisil eluate and the amount of antineoplastic activity present in each fraction are shown in Fig. 1. Most activity was found in fractions 27–31. The total protein in these pooled fractions was

Table 1. Antineoplastic activities of ANUP (Florisil eluate) assayed against human tumour cell lines and hamster neoplastic cell line (growth inhibition assay)

The cells were grown in 25 cm² Falcon flasks at 37° in an atmosphere of air/CO₂ (19:1). The pancreas tumor cell line SW 1990 was grown in air in the absence of CO₂. The growth inhibition assays were performed as described in the text.

Tumour cell line	Fraction of cell number compared with control			
	ANUP (µg/ml) . . . 80	160	320	640
Human cell lines				
Breast (BT20)	0.10	—	—	—
Breast (MCF7)	0.44	0.18	—	—
Lung (ECL-3)	—	0.45	0.27	—
Lung (CALU-6)	—	0.27*	—	0.09
Melanoma (KHM-1)	—	0.10	—	—
Bladder (RT-4)	1.0	1.0	—	0.02
Pancreas (Capan-1)	0.77	—	0.30	—
Pancreas (SW 1990)	1.0	0.80	0.40	—
Cervix (HeLa)	—	0.25	0.10	—
Hamster cell line				
Hamster neoplastic cell line (PANC/3)	1.0	1.0	—	1.0
*2.40 µg/ml

Fig. 2. Comparative antineoplastic potencies on HeLa cells of the Florisil eluate and the chromatographically purified Sephacryl S-200 fraction

The method of assay is described in the text. □, Florisil eluate; ▨, S-200 fraction.

12 mg; the total activity was 360 units. This is equivalent to a yield of about 30 units/mg. As shown in Fig. 2, the Sephacryl S-200 pooled fractions had 20 times the specific activity of the Florisil eluate.

Chromatofocusing analysis of the Sephacryl S-200 fractions

The pooled active fractions from the Sephacryl S-200 fractionation (Fig. 1) were chromatofocused (Pharmacia, 1982). The active ANUP (5 mg) was eluted between 155 and 195 ml with a pI of approx. 4.2 (Fig. 3). This material showed a single protein band upon SDS/polyacrylamide-gel electrophoresis that was equivalent to that shown in Fig. 6 and in the Fig. 3 insert. The chromatofocused material had only 20% of the original specific activity. However, after heating a 1 mg/ml solution (in phosphate-buffered saline without calcium) for 5 h at 60 °C there was an increase in specific activity. Whereas 10 µg of the Sephacryl S-200 fraction/ml of medium was required for 50% inhibition of growth of HeLa cells, the heated chromatofocus fraction showed 50% inhibition of growth at 4 µg/ml of medium.

Possibly related to the above is the observation that some enzymes have been shown to be partially inactivated upon passage through the chromatofocusing matrix (B. Britten, personal communication).

Preparation of electrophoretically homogeneous ANUP

In order to prepare biologically active electrophoretically homogeneous ANUP fraction (Fig. 4) it was necessary to carry out the following. (a) Filtration of the Florisil eluate (in the presence of 0.5 M-NaCl) through a YM 30 Diaflo membrane. This filtration allowed 25–50% of the total protein and about 75% of the antineoplastic activity to pass through the membrane with a 1.5-fold increase in specific activity. (b) Chromatography of the filtrate on a Sephacryl S-300 column. (c) Chromatography of the active Sephacryl S-300 fraction on a Sephacryl S-200 column. The antineoplastic protein was located in peak 4 (Fig. 5); rechromatography of this active material on the Sephacryl S-200 column yielded essentially homogeneous ANUP (Fig. 5, insert). The yield was about 200 µg/litre of urine with a 30% recovery of ANUP

Fig. 3. Chromatofocus analysis of the Sephacryl S-200 fraction (Fig. 1, fractions 27–31) and correlation of the protein fractions eluting at different pH values with ANUP activity

The techniques are described in the text. Lane A shows protein markers (chymotrypsinogen and ribonuclease). Lane B shows the protein in the peak (fractions 155 ml–195 ml); the protein eluted in this fraction showed biological activity as described in the text. The other peaks that showed absorbance at 280 nm were inactive.

	Protein (mg)	Activity (units)	Specific activity (units/mg)
Urine (300 litres)			
↓			
Florisil eluate	3000	15 000	5
↓			
YM30 Diaflo membrane filtrate in the presence of 0.5 M-NaCl	1500	11 400	7.7
↓			
Sephacryl S-300 chromatography	900	9000	10
↓			
Sephacryl S-200 chromatography	120	4500	30
↓			
Sephacryl S-200 rechromatography	60	4500	77

Fig. 4. Flow sheet and recovery table for the preparation of electrophoretically homogeneous ANUP from 300 litres of urine

g. 5. Preparation of electrophoretically homogenous ANUP by Sephacryl S-200 chromatography of the ANUP prepared from the Amicon YM30 Diaflo filtrate after Sephacryl S-300 chromatography

The insert represents the Sephacryl S-200 rechromatography of peak 4. The procedures and conditions of chromatography are the same as described in the legend to Fig. 1 except that 4.6 ml fractions were collected.

activity and each unit was equivalent to 13 μg of ANUP; this fractionation represented a 15-fold purification based on the protein/unit in the Florisil eluate (200 μg /unit). However, since urine contains approx. 100 mg of protein/litre, the total protein purification is in the order of 500-fold. The apparent molecular mass of the homogeneous protein fraction was approx. 28 000 Da as determined by Sephacryl S-200 gel filtration in comparison with markers of known molecular mass. The biologically active ANUP preparations were shown by both exclusion chromatographic techniques and chromatofocusing to yield the same homogeneous preparation upon SDS/polyacrylamide-gel electrophoresis (Figs. 3 and 6).

Sedimentation velocity and equilibrium studies

Centrifugation studies were done using the electrophoretically homogeneous ANUP fraction described above. A plot of $s_{20,w}$ versus concentration was extrapolated to $c = 0$ to give $s_{20,w}^0 = 3.69 \text{ S}$ and $s_{20,w} = s_{20,w}^0 - 0.324 c$.

The sedimentation equilibrium results, a graph of the log of concentration versus the square of the radial

distance from the centre of rotation at equilibrium, showed a curvilinear relationship. One interpretation of such data is that a complex mixture is present. We think this interpretation does not apply to the ANUP study because other experiments indicate that ANUP is a homogeneous substance but one which tends to aggregate. For example, the active fraction from isoelectric focusing (Fig. 3) is homogeneous with regard to charge and is also homogeneous in size when run on SDS/polyacrylamide-gel electrophoresis (Fig. 6). Solution properties (see below) indicate that ANUP is probably in a monomer-polymer equilibrium and it is this interpretation we choose for the sedimentation equilibrium results. The tangent to the curve at the meniscus radial position had a slope corresponding to a molecular mass of 16 300 Da; this represents the minimum molecular mass. The tangent to the curve at the bottom of the sample cell had a slope corresponding to a molecular mass of 77 500 Da. Thus, ANUP in solution appears to be a mixture of monomers (16 300 Da) and various polymers of this basic unit up to approximately a

Fig. 6. SDS/polyacrylamide-gel electrophoretic analysis of homogenous ANUP prepared from Peak 4 (Fig. 5 insert)

The electrophoresis studies were performed with a 10 cm running gel (10% polyacrylamide) and a 0.8 cm stacking gel (5% polyacrylamide). Each analysis utilized the following molecular mass markers: bovine serum albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (20 kDa) and ribonuclease (14 kDa). The procedures used were those described by Laemmli (1970). The applied current was 1 mA/gel tube for 60 min, followed by 2 mA/gel tube for 200 min. The gels were stained [0.4 g of Serva BlueW/litre of 20% (w/v) trichloroacetic acid] for 38 min at 68 °C and then destained overnight in water. The faint band at the top of the running gel occurred at the interface between the stacking and running gels.

pentamer. Since the solution conditions for the sedimentation velocity experiments were approximately the same as for the equilibrium study, the $s_{20,w}^0$ represents the average sedimentation coefficient for a mixture of molecules (monomers, dimers, trimers, etc.).

Solubility and solution properties

A number of interesting solubility and solution properties of ANUP were observed. The solubility of ANUP is a function of concentration, ionic strength of the solvent, the temperature and the length of time of exposure to the selected conditions. In the temperature range 0–20 °C and at concentrations of 5 to about 10 mg/ml, ANUP tends to aggregate and precipitate from solution unless a relatively high ionic strength, e.g. 0.5 M-NaCl, is maintained for the solvent. Resolubilization of lyophilized ANUP requires high ionic strength solvent (0.5 M-NaCl). This bulk property of ANUP solutions is reflected in the observations made on more dilute solutions by means of the analytical ultracentrifuge and by electrophoresis.

The sedimentation equilibrium results reported above showed the presence of a subunit of minimum molecular mass 16300 Da and higher ordered aggregates of this subunit in a solution containing 2.3 mg of ANUP/ml, at 20 °C, in phosphate-buffered saline without calcium (and without added NaCl).

In a solubility experiment ANUP, which precipitated from low-ionic-strength solutions, was first resolubilized

Fig. 7. Polyacrylamide-gel electrophoresis without (a) and with (b) SDS of the water-insoluble ANUP fraction after solubilization in 6 M-guanidinium chloride

The insoluble fraction was prepared from the protein that was eluted in Peak 4 (shown in Fig. 5). In low ionic strength (upon dialysis against water) the biologically active ANUP fraction precipitated from solution and was resolubilized in 6 M-guanidinium chloride at 4 °C; the ANUP fraction was freed from the guanidinium by dialysis against many changes of phosphate-buffered saline at 4 °C. (a) The electrophoresis was performed according to the Laemmli (1970) procedure using a 10% gel. The gel was stained with Coomassie Blue. (b) The protein sample was diluted in sample buffer that contained 2.0 ml of glycerol, 2.5 ml of 0.5 M-Tris/HCl, pH 6.8, 2.0 ml of 10% (w/v) SDS, 1.0 ml of 2-mercaptoethanol and water to 10 ml; the sample was heated rapidly to 90 °C for 2 min and cooled before electrophoresis in a 12% polyacrylamide gel that contained 0.1% SDS; the reservoir buffer also contained 0.1% SDS. Electrophoresis was allowed to proceed until the marker dye had run off the gel. The gel was stained with Coomassie Blue. The elution positions of molecular mass standards are indicated.

by stirring the precipitate in 6 M-guanidinium chloride in the cold for 16 h. The guanidinium chloride was then removed by dialysis in the cold against phosphate-buffered saline without calcium. This ANUP solutions was then analysed by both convention and SDS-containing polyacrylamide-gel electrophoresis. Without SDS the ANUP appeared as a single band (Coomassie Blue staining; Fig. 7a) which retained biological activity. With SDS ANUP gave a single Coomassie Blue band which corresponded to a molecular mass of 16000 Da when

compared with standards in the same system. The guanidinium chloride-solubilized ANUP fraction showed a single protein band on electrophoresis in a 12% gel with SDS; the electrophoresis was terminated after the dye front had been eluted from the gel (Fig. 7b).

In a related electrophoresis experiment, it was possible to demonstrate that active ANUP was recoverable from regions of an SDS/polyacrylamide gel that corresponded to molecular masses of approx 16000 and 32000 Da. ANUP was electrophoresed in the presence of SDS but the sample was not heated in SDS prior to electrophoresis as specified in the published procedure (Laemmli, 1970). Following electrophoresis, the gels were sliced and eluted as previously described and aliquots from each eluted gel slice were assayed for activity. After 96 h incubation the control wells showed approx. 2.5×10^5 cells/well whereas the eluates (20 μ l) from gel slices on the regions corresponding to 16000 and 32000 Da showed a 60–70% reduction in cell numbers per well. All the other gel slice eluates showed cell numbers equal to the control. Assays were performed in triplicate and the cell counts of the triplicate assays agreed within $\pm 15\%$. The presence of biological activity in only two areas of the gel indicated that this was a clean separation of ANUP under the conditions of the experiment.

Amino acid analysis of ANUP

The amino acid analysis of the homogeneous ANUP is shown in Table 2. The presence of 15 aspartic acid residues and 20 glutamic acid residues represents 23% of the 150 amino acid residues of this protein. Thus, although the number of asparagine and glutamine residues are not known it is reasonable that the protein should exhibit a pI of 4.2.

DISCUSSION

A protein fraction exhibiting antiproliferative activity against the HeLa cell line (human cervical tumour cells) has been isolated from human urine and purified to electrophoretic homogeneity. The protein appears to be composed of monomeric units each with a molecular mass of approx. 16000 Da; the protein does not exhibit antiviral activity. The low pI of ANUP is not inconsistent with the high percentage (23%) of aspartic and glutamic acid residues. Furthermore, the protein fraction exhibits some chemical characteristics similar to those of collagens (Bornstein & Sage, 1980) and the C1_q component of complement (Porter & Reid, 1978) as shown by (a) high levels of proline (34 residues) and glycine (38 residues) (these two amino acids account for 48% of the total amino acid content of ANUP); (b) the absence of cysteine; and (c) the presence of hydroxyproline (two residues) in this protein. This protein fraction has been designated the antineoplastic urinary protein (ANUP).

A number of proteins and factors which exhibit antineoplastic activity have been reported. Tumour necrosis factor is a macrophage product that induces the necrosis of an intradermal tumour implant (Carswell *et al.*, 1975). Lymphotoxin, a product of human lymphoblastoid cell line 1788, causes lysis of mouse L-929 fibroblasts (Aggarwal *et al.*, 1983). Lymphotoxin is also an anticarcinogenic factor (Evans & DiPaolo, 1981) as measured by its ability to inhibit chemical- or u.v.-induced transformations of Syrian hamster cells. In addition,

Table 2. Amino acid analysis of the antineoplastic urinary protein

The amino acid analysis of the electrophoretically pure ANUP was performed according to the method described by Cranberg (1984) by high resolution amino acid analysis of amino acid phenylisothiocyanate derivatives utilizing the Beckman h.p.l.c. system with models 110A, 420 and 332. The electrophoretically homogeneous ANUP preparation (1 mg or 100 μ g) was dissolved in 1.00 ml of constant boiling HCl with the addition of 2 μ l of water-saturated phenol. Hydrolysis was performed in an N₂-flushed vial, under vacuum, at 105 °C for 24 h. After hydrolysis the sample was evaporated to dryness under vacuum and taken up in coupling buffer before derivatization. The sample showed trace amounts of hydroxylysine and a small amount (0.4 residues/molecule) of a compound that eluted as phosphoserine but the identity of this compound has not been established. The analysis was kindly performed by Dr. W. E. Jefferson, Department of Biochemistry, University of Tennessee College of Medicine, Memphis, TN. The same hydrolysis method was utilized to determine the lysine content of the sample by the method of Moore & Stein (1963). The analysis was kindly performed by Sister Burcharda Brinkley, Department of Biochemistry, University of Tennessee, College of Medicine, Memphis, TN.

Amino acid	Residues (mol/mol of monomer)
Lys	3.3
His	2.0
Arg	3.8
Hyp	2.0
Asp	15.0
Thr	4.4
Ser	8.0
Glu	20.0
Pro	34.0
Gly	38.0
Ala	7.7
Cys	0.
Val	2.3
Met	1.0
Ile	2.0
Leu	1.2
Tyr	2.0
Phe	0.49
Glucosamine	2.0

Wanebo *et al.* (1984) reported on the antitumor effects of normal human serum factor (Normal Human Globulin Fraction 1) on human tumour cells *in vitro*. This protein (140000 Da) showed a certain degree of specificity towards human tumour cell lines at concentrations of 100–2000 μ g/ml. More recently, Ransom *et al.* (1985) presented data on the anticancer action of an immunological hormone, named leukoregulin. This protein (140000 Da) inhibited the growth of a number of human tumour cell lines without affecting normal human cells.

The relationship of ANUP to these factors remains to be determined. However the species specificity of ANUP could *a priori* exclude a strict identity of ANUP with other lymphotoxins and the tumor necrosis factor of mouse origin.

We wish to acknowledge the excellent technical assistance of Mrs. Lydia H. Davis, Department of Pediatric Research, University of Maryland School of Medicine, with some aspects of this research.

REFERENCES

- Aggarwal, B. B., Moffatt, B. & Harkins, R. N. (1983) Lymphokine workshop, pp. 521–525, Academic Press, New York
- Bornstein, P. & Sage, H. (1980) *Annu. Rev. Biochem.* **49**, 957–1003
- Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. & Williams, B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666–3670
- Chervenka, C. H. (1969) *A Manual of Methods for Analytical Ultracentrifugation*, pp. 17–50, Spinco Division of Beckman Instrument Inc., Palo Alto
- Cranberg, R. R. (1984) *Liquid Chromatogr. Magazine* **2**, 776–781
- Evans, C. H. & DiPaolo, J. A. (1981) *Int. J. Cancer* **27**, 45–49
- Goldwasser, E. & Kung, G. K. H. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 697–698
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lorand, L. & Condit, E. V. (1965) *Biochemistry* **4**, 265–270
- Moore, S. & Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831
- Pharmacia Fine Chemicals Inc. (1982) *Chromatofocusing Procedures*, Pharmacia, Uppsala
- Porter, R. R. & Reid, K. B. M. (1978) *Nature (London)* **275**, 699–704
- Ransom, J. H., Evans, C. H., McCabe, R. P., Heinbaugh, J. A., Chin, M. & Hanna, M. G. (1985) *Cancer Res.* **45**, 851–862
- Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32
- Schooley, J. C. & Garcia, J. F. (1962) *Proc. Soc. Exp. Biol. Med.* **109**, 325–328
- Schooley, J. C. & Garcia, J. F. (1965) *Blood* **26**, 204–207
- Sloane, N. H., Hade, E. P. K., Macleod, R. M. & Pottathil, R. (1982) *Proc. Am. Assoc. Cancer Res.* **23**, 16 (abstr. 64)
- Wanebo, H. J., Green, S., Marshall, D. & Pace, R. C. (1984) *J. Natl. Cancer Inst.* **72**, 545–555
- White, W. F., Barlow, G. H. & Mozen, M. M. (1966) *Biochemistry* **5**, 2160–2169

Received 22 May 1985/16 October 1985; accepted 30 October 1985