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John Bienenstock, Thomas B. Tomasi Jr.

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Research Article

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Secretory γA in Normal Urine

JOHN BIENENSTOCK and THOMAS B. TOMASI, JR.

From the Rheumatic Disease Unit, Department of Medicine, State University of New York at Buffalo and The Buffalo General Hospital, Buffalo, New York

ABSTRACT The physicochemical nature of γA was investigated in normal male and female urine concentrated approximately 1000 times. Sucrose density gradient ultracentrifugation and Sephadex G-200 chromatography revealed that urinary γA has sedimentation properties intermediate between 19S and 7S molecules. Isolation of urinary γA by DE 52 chromatography free of other immunoglobulins with subsequent antigenic analysis showed that the urinary γA-molecule is antigenically indistinguishable from the γA-molecules found in other external secretions and has a corrected sedimentation coefficient of 11.8S. In addition, like other secretory γA-molecules and unlike serum polymeric γA, urinary γA resisted mild reductive measures with 0.1M β-mercaptoethanol. Free or unattached secretory "piece" was found in all normal urines tested and in agammaglobulinemic urine. Secretory "piece" antigenic determinants were also found in ureteric urine. The average daily excretion of urinary γA was 1.1 mg. The maximum excretion of urinary 7S γG per 24 hr was approximately 3 mg.

INTRODUCTION

A major part of the urinary γ-globulin has been found to consist of subunits or fragments of immunoglobulins in the form of L chains and Fc and F'c fragments (1–6). Glomerular filtration appears to be the primary route of excretion of circulating serum γ-globulin fragments such as L chains and Fc fragments (1, 4). We have, however, recently found evidence that the F'c may be derived at least in large part from urinary Fc by proteolysis (6). In addition to these fragments and subunits native 7S γG has been shown to exist in small amounts in normal urine by Franklin (3), and a high molecular weight form of γA has been described by Tomasi and Zigelbaum (7) and Turner and Rowe (8). However, it is generally accepted that 19S γM is not a normal constituent of urine, and H chain fragments of γM have not been identified in normal urine.

This report is concerned with an investigation into the nature of γA in normal urine. It was found that γA constitutes a significant fraction of the total urinary imunoglobulins and that in addition, urinary γA is primarily of high molecular weight and indistinguishable from the secretory form associated with certain other external secretions (9).

METHODS

Specimens. 24-hr urine samples were collected from healthy male and female volunteers and preserved in 0.1% sodium azide. None of the females were menstruating at the time of the collection. Samples were kept at 4°C overnight and then filtered on Whatman No. 1 paper. Urine was concentrated by negative pressure dialysis at 28 inches of mercury in Visking[1] 8/32 membranes. All samples were concentrated about 1000 times and centrifuged at 10,000 rpm in the cold before storing at −20°C until used. No 24-hr specimens of urine contained more than 80 mg of protein by Folin (10) or biuret (11) reactions.

Antisera. Antisera were prepared in rabbits and goats by immunization with antigens in complete Freund's adjuvant. Anti-L chain antisera were obtained by immunization with papain Fab fragments of γG isolated by starch block electrophoresis and subsequent gel filtra-

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Address requests for reprints to Dr. John Bienenstock, Department of Medicine, State University of New York at Buffalo, 100 High Street, Buffalo, N. Y. 14203.

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[1] Union Carbide Corp., Visking Div., Chicago, Ill.

tion on Sephadex [2] G-100. This antiserum showed both κ- and λ-chain reactivity. γ-chain–specific antisera were obtained by immunization with the papain Fc fragment. Trace contamination of the Fc preparation with Fab occurred so that this antiserum was absorbed with Bence Jones proteins of κ- and λ-types to produce a specific antiserum that reacted only with γG-, and Fc and F'c (12) fragments. γA- and γM-antisera were commercial preparations [3] or were prepared by immunization with purified immunoglobulin preparations and made specific by suitable absorption with γG and L chains. An antiserum which reacted only with γA–secretory "piece" was made in the burro by immunization with human colostral γA (kindly donated by Dr. J. Bellanti) or by immunization of rabbits with colostral or salivary γA. These antisera were absorbed with Cohn Fraction II and a euglobulin fraction of a macroglobulinemic human serum and reacted only with γA and secretory "piece." The antiserum when absorbed with concentrated normal human serum reacted only with secretory "piece." Both of these absorbed antisera reacted in addition with a macromolecular component present in saliva and colostrum in trace amounts and found in urine in larger quantities. This additional component, which has not been fully characterized, has an alpha mobility on electrophoresis and is not found in normal human sera.[4]

Techniques

Ouchterlony and immunoelectrophoretic analyses were performed as previously described (13). Analytical ultracentrifugation was carried out with a Spinco model E ultracentrifuge at 52,640 rpm and 20°C with double sector cells. Sedimentation coefficients were calculated at infinite dilution from plots of $s_{20,w}$ against concentration. Density gradient ultracentrifugation with 10–40% sucrose gradients were performed according to methods outlined before (7).

Gel filtration and chromatography. Concentrated urine samples were subjected to gel filtration on Sephadex G-200 in 0.85% saline. Chromatography on DE 52 (Whatman) [5] with stepwise elution was performed as previously described (14). Protein concentrations were measured by absorption at 280 mμ in a Zeiss spectrophotometer, and by the Lowry modification of the Folin-Ciocalteau method.

Immunoglobulin quantitation. Quantitative immunoglobulin determination was performed by the radial diffusion technique (7) with goat antisera specific for γA and a rabbit anti–γ-chain for γG. Standard calibration curves were constructed with 7S serum γA and a sample of colostral 11S γA obtained as previously described (9). The standard curve obtained by use of the 11S γA was used for quantitation of urinary γA. A calibration curve for γG was prepared with 7S γG isolated from normal serum by starch block electrophoresis and diethylaminoethyl (DEAE) chromatography. All standards were tested for homogeneity by analytical ultracentrifugation (at a concentration of at least 10 mg/ml), disc electrophoresis, and by immunoelectrophoresis and Ouchterlony analysis with several potent anti-whole and anti–γ-globulin antisera.

RESULTS

Several investigators have demonstrated the efficacy of the method used for concentration of urinary protein samples (15, 16). Some doubt has, however, been expressed as to the percentage recovery of higher molecular weight proteins by the method (17). Serum was accordingly diluted 1000 times with saline and concentrated in 8/32 Visking membranes. Full recovery of γG, γA, and γM was demonstrated after concentration, within the experimental error of this method (approximately 10%). The Visking membranes were demonstrated to retain egg white lysozyme (mol wt 14–18,000) within the bag. Urinary filtrate lyophilized and redissolved to a concentration of 1000 times the original showed no immunological reactivity with an antiserum against whole human serum. Quantitative data relating to the recovery of free secretory "piece" were not obtained. Fig. 1 shows a typical Ouchterlony pattern with an antisecretory γA-antiserum that reacts with both secretory "piece" and γA. The additional urinary macromolecular component referred to earlier is seen closest to the antigen well. Free or unattached secretory "piece" diffuses ahead of the whole γA-molecule and is closest to the antiserum well. A similar pattern is seen with both female and male urine. Because the free "piece" diffuses rapidly and removes "piece"-specific antibodies there is no spurring of the secretory γA over the serum γA. It was necessary, therefore, in order to study the immunological relationships of urinary γA to that of serum as well as other secretions, to further purify the urinary γA-molecule and to separate it from unattached secretory "piece." A 10 day collection of urine was concentrated. (The 24 hr-samples were individually pooled and processed.) A total volume of 15 ml of concentrated urine was placed on a column of Sephadex G-200, 4 × 130 cm and eluted with 0.14 M saline. The elution pattern is seen in Fig. 2. γA (α-chain determinants) was eluted in the second half of the first protein peak (pool 1). The reaction of the eluate fractions with a specific anti-"piece" anti-

[2] Pharmacia Fine Chemicals Inc., Piscataway, N. J.

[3] Hyland Laboratories, Los Angeles, Calif.

[4] Bienenstock, J., and T. B. Tomasi. Unpublished observations.

[5] R. A. Reeve Angel, Clifton, N. J.

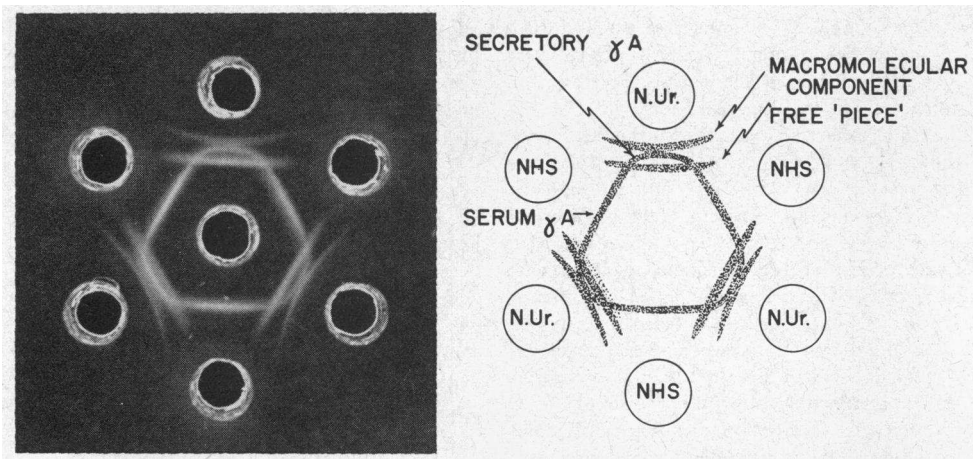

FIGURE 1 Ouchterlony plate showing antigenic character of urinary γA and secretory "piece" in urine. The central well contains an antiserum against colostral 11S secretory γA absorbed with Cohn Fraction II. The top well contained male urine, and the bottom urine wells were filled with female urine. *N.Ur.*, normal urine; *NHS*, normal human serum.

serum was biphasic: first in the same tubes as the α-chain reactivity (pool 1), second in a position corresponding to the end of the "7S peak" and the beginning of the albumin peak (pool 2). By antigenic analysis the first part of this biphasic reaction was subsequently shown to be due to secretory "piece" that is present as part of the 11S secretory γA-molecule and the second to unattached or free secretory "piece." The last and largest peak contains primarily urinary glycopeptides having a sedimentation coefficient of less than 1S. The additional macromolecular component with which the antisecretory antisera react (Fig. 2) is eluted in the ascending portion of the first peak.

When the γA-reactive material present in pool 1 was concentrated and rechromatographed on Sephadex G-200 it was again eluted in a position ahead of the 7S γG, but in a larger volume than the blue dextran marker used for measuring the void volume of the column (Fig. 3). γM was not detected with an anti-μ-chain antiserum in any of the samples. Analytical ultracentrifugation of pool 1 showed a trace component with a sedimentation coefficient of 19S, a second component at 10.7S, and a major component of 3.3S. This pattern was

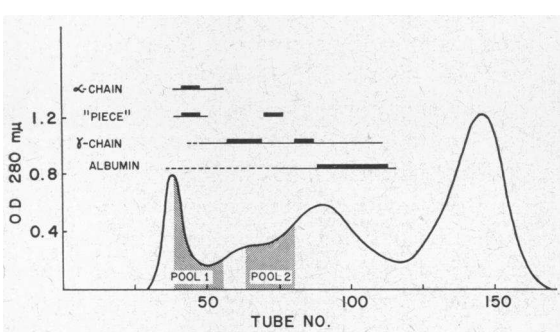

FIGURE 2 Gel filtration showing the elution pattern of a 10 day pool of urine on Sephadex G-200. The distributions in the chromatographic fractions of the various urinary components are shown by the horizontal black bars. Pool 1 contained all of the γA-reactive material. "Piece" determinants were present in both pool 1 and pool 2.

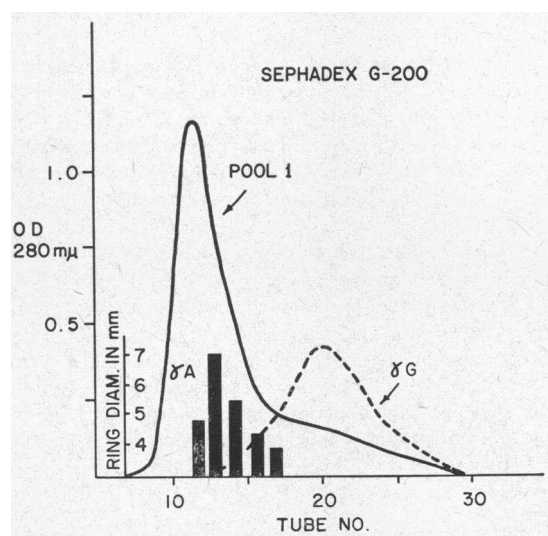

FIGURE 3 Rechromatography of pool 1 from Fig. 2 on Sephadex G-200. γA (solid curve) has an elution position intermediate between the void volume and a γG (dashed curve) marker.

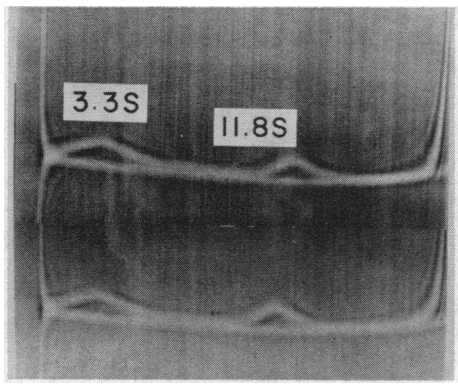

FIGURE 4 Analytical ultracentrifugation pattern of a purified urinary γA fraction after DE 52 chromatography, at 52,640 rpm, 56 min after full speed was attained. The upper frame is before and the lower frame is after incubation with 0.1 M β mercaptoethanol. The $s^{\circ}_{20,w}$ of urinary γA was 11.8S. The second more slowly sedimenting 3.3S component is a urinary-specific component of unknown composition.

found consistently with six different urinary samples. The nature of the 3.3S component is unclear from our experiments. From the elution position on a Sephadex G-200 column and the sedimentation characteristics the 3.3S material is assumed to be constituted by highly asymmetric molecules. Its composition is uncertain and fractions containing this component (free of γA) do not react with antisera against whole human serum or with any of the secretory antisera used in this study.

All of the γA-reactive material obtained from gel filtration (pool 1) was concentrated, dialyzed overnight against 0.01 M phosphate buffer, pH 7.4, and chromatographed on DE 52. Subsequent elution with 0.1 M phosphate, pH 6.0, produced a fraction rich in γA. Double diffusion in agar with an anti-whole human serum revealed a γA precipitin arc and an additional component that was present in trace amounts and could not be detected on immunoelectrophoresis. Final recovery of γA as compared to the original urine sample before gel filtration was approximately 20%. After concentration, this specimen was subjected to analytical ultracentrifugation, the results of which are shown in Fig. 4. Two major components are recognized: one having a sedimentation coefficient ($s^{\circ}_{20,w}$) of 11.8S and the other of 3.3S. The addition of 0.1 M β-mercaptoethanol did not appreciably alter the sedimentation coefficient of γA as shown in the lower half of Fig. 4.

Urinary γA purified as described was then compared to normal human serum, 11S colostral γA, and whole concentrated saliva with antisecretory γA-antiserum as shown in Fig. 5. Urinary γA is antigenically identical with salivary and colostral γA, serum γA being deficient to all three. Reac-

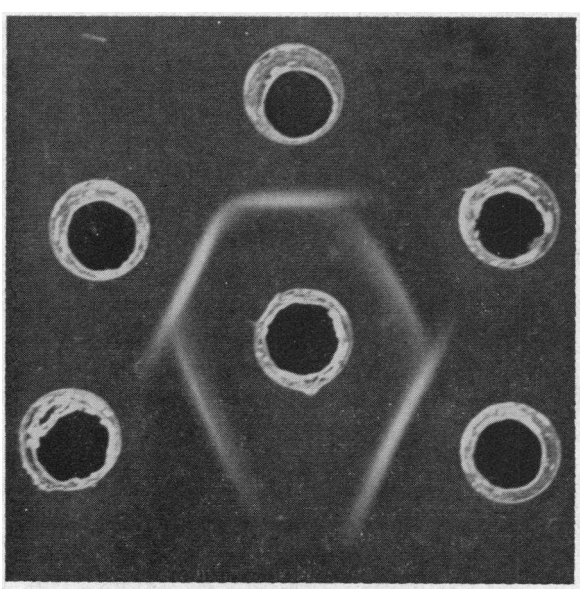

FIGURE 5 Ouchterlony plate showing antigenic relationship of γA from normal human serum (*NHS*), colostrum (*Col.*), urine (*γAu*) and saliva (*Sal.*). The central well contains an antisecretory γA-antiserum (*anti-Col.γA*) that reacts with both secretory "piece" and γA–H chain determinants.

tions of identity were also shown among those secretory molecules sharing "piece" determinants using a specific antisecretory "piece" antiserum. Immunoelectrophoresis of the purified γA-fraction reveals precipitin arcs of the same mobility as serum γA with both an antiserum 7S γA and with secretory "piece" antisera (Fig. 6); this finding confirms the presence of secretory "piece" attached to the γA-molecule.

When pool 2 from the first Sephadex G-200 gel filtration (Fig. 2) containing free "piece" was concentrated, dialyzed against the starting buffer, and eluted on DE 52, most of the "piece" reactive material was eluted with 0.05 M phosphate buffer, pH 6.0. However, a small amount of "piece" reactive material was eluted with the lowest ionic strength buffer used (0.01 moles/liter, pH 7.4) together with 7S γG. Immunoelectrophoresis of this peak developed with anti-γ-chain and "piece" specific antisera reveals a single arc in the β region with the characteristic mobility of free or unattached secretory "piece." Free "piece" eluted with 0.05 M buffer, pH 6.0, is seen in the lower half of Fig. 7. This finding indicates that secretory "piece" is not attached to γG in the urine. The mobility and character of the free "piece" arc are entirely similar to that found in agammaglobulinemic urine and saliva and that obtained with a "piece" preparation isolated by reduction and alkylation from colostral γA (18).

Sucrose density gradient ultracentrifugation of six whole concentrated normal urines was performed together with normal human serum. The results are graphically shown in Fig. 8 with one typical sample. Quantitation of γA was carried out by the radial diffusion technique. Urinary γA was shown to have a sedimentation position intermediate between that occupied by the 7S and 19S immunoglobulins of serum in keeping with the evidence for an 11S secretory type of urinary γA.

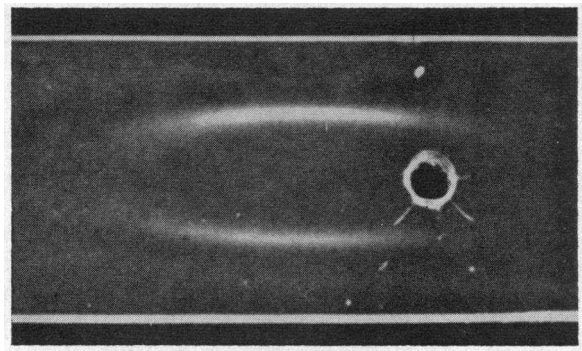

FIGURE 6 Immunoelectrophoretic pattern of a urinary γA preparation showing precipitin arcs of comparable mobility with both anti-α-chain (upper trough) and antisecretory (lower trough) antisera. The antisecretory antisera have been absorbed with normal serum and are "piece" specific. The anode is to the right.

In order to investigate whether the urinary γA was derived from the renal tract higher than the bladder five samples of ureteric urine were obtained during ureteric catheterization and tested by double diffusion in agar after concentration. Secretory "piece" determinants were demonstrated in all these samples. However, all samples contained large amounts of serum proteins including serum type γA and γG. The patients from whom samples were obtained had moderate or gross renal disease. Contamination of ureteric samples with serum proteins from trauma or indirect admixture with serum proteins through glomerular leakage

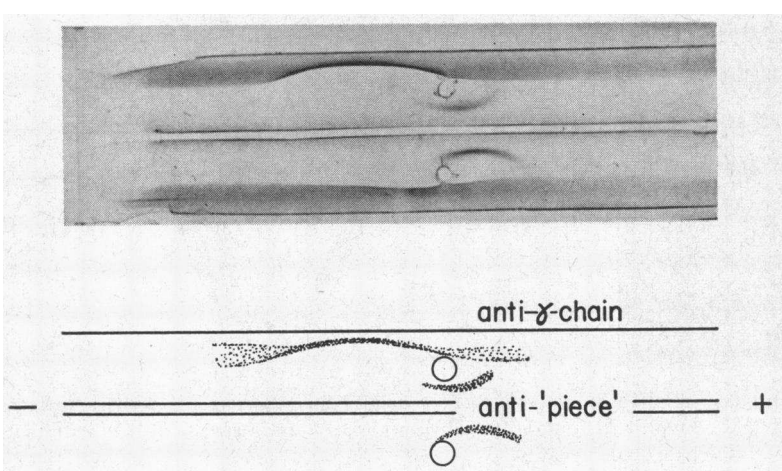

FIGURE 7 Stained immunoelectrophoretic pattern of fractions obtained with two separate buffers on DE 52. Starting sample was the urinary "piece"-reactive material, not bound to γA (pool 2, Fig. 2). Upper well contains material eluted with 0.01 M phosphate buffer, pH 7.4; lower well contains eluate obtained with 0.05 M phosphate buffer, pH 6.0. γG and free "piece" arcs of differing mobilities are seen, indicating that "piece" is not attached to urinary γG.

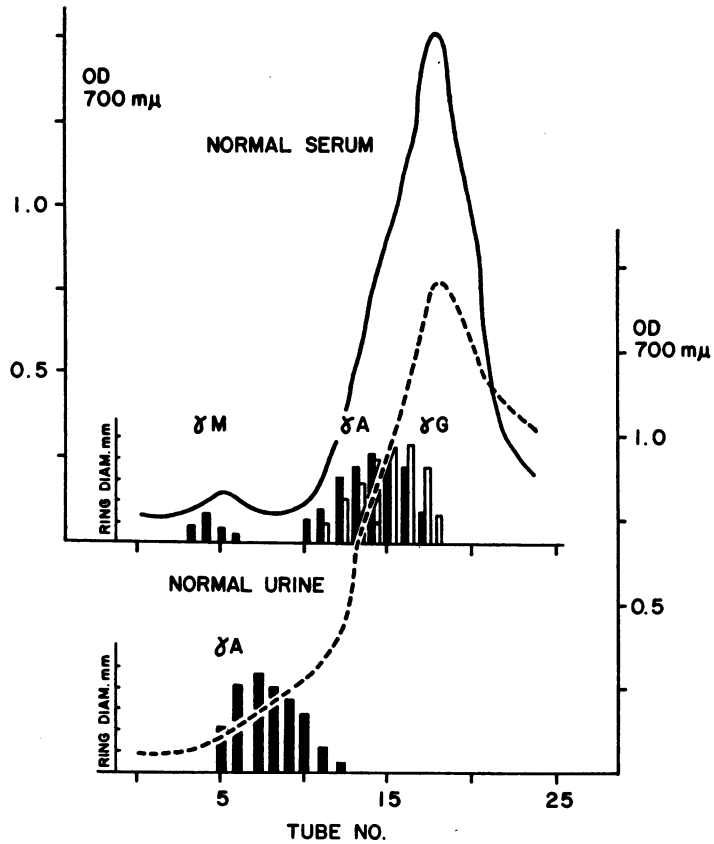

FIGURE 8 Sucrose density gradient (10–40%) ultracentrifugation of serum and urine. Urinary γA (dashed curve) is seen to have an intermediate position between γM (19S) and serum γA and γG(7S). Urinary γG is not shown since reactivity is seen from the 7S region to the top of the gradient because of the presence of heavy chain-related fragments.

were impossible to avoid, and insufficient material was available for fractionation studies. Therefore, no conclusions can be drawn as to whether the "piece" determinants in ureteric urine were attached to the intact 11S secretory molecule, represented free "piece," or both.

Quantitation of γA was performed by the radial diffusion technique on urines from 19 healthy volunteers: 6 females and 13 males. When serum 7S γA was used as standard, the results were one-third to one-fourth lower than when colostral γA was used for calibration of the standard curve. Since, as shown above, the majority of the urinary γA is of the high molecular weight (11S secretory type) the colostral γA calibration curve was used. The results are shown in Table I. Since no subunits or fragments of γA were found in the low molecular weight fractions of the gel filtration experiments, the values obtained for excretion of γA are probably reasonably accurate. The average daily excretion of γA is, therefore, approximately 1.0 mg.

An attempt was made to quantitate urinary γG. However, it is known that in urine fragments of the γG–heavy chain are found, such as the Fc and F'c fragments (2, 5, 6). When radial diffusion quantitative procedures were applied to urine with a specific anti–γ-chain antiserum two standards were compared: 7S γG-globulin and the papain Fc fragment. Since the smaller molecules diffuse faster, larger precipitin rings were found for the same concentration when compared to the native 7S immunoglobulin. Thus, the values obtained by reading ring diameters on the 7S standard revealed higher concentrations than were obtained with the Fc fragment calibration curve and, therefore, they are overestimates of the amount of γG-globulin. Since it is not known whether the fragments represent precursor subunits or breakdown products of the parent molecules, the values for the γG excretion per day which were obtained in this manner must represent a maximum figure. The results are shown in Table I. The average is 3.0 mg/day. Daily indi-

TABLE I

Immunoglobulin Quantitation in Sera and Urines from Healthy Subjects

	Urine				Serum		
	γG	γA	γG/γA		γG	γA	γG/γA
	mg/24 hr	*mg/24 hr*			*mg/ml*	*mg/ml*	
M. R.	2.45	0.88	2.78		9.00	0.58	21.47
A. M.*	3.51	1.09	3.22		12.45	1.12	8.04
L. C.*	1.27	1.23	1.03		9.30	0.77	12.08
K. K.*	2.15	1.08	1.99		10.70	0.49	21.84
D. T.	5.73	1.80	3.18		14.30	1.79	7.99
M. A.*	0.99	0.56	1.77		5.80	1.00	5.80
T. T.	2.39	0.67	3.57		11.20	0.87	12.87
T. B.	4.82	2.05	2.35		13.80	0.90	15.33
D. C.*	3.27	0.76	4.30		15.00	1.26	11.90
J. C.	2.69	0.58	4.64		15.00	1.09	13.76
J. S.	2.90	1.03	2.82				
J. B.	2.33	1.21	1.93				
D. B.*	1.02	1.41	0.72				
R. P.	6.29	1.29	4.86				
G. D.	4.46	1.20	3.72				
A. S.	2.13	1.50	1.42				
R. C.	2.87	0.37	7.76				
A. S.	3.88	1.23	3.15				
A. A.	1.81	1.04	1.74				
Total (n = 19)	59.96	20.98	56.95	(n = 10)	116.55	9.87	131.08
Mean	3.00	1.10	3.00		11.65	0.99	13.11
Variance							
Paired *t*			1.28				28.82
Unpaired *t* (n = 19)			2.71			(n = 10)	28.82
Standard deviation							
Paired *t*			1.13				5.37
Unpaired *t* (n = 19)			1.65			(n = 10)	5.37

Student paired *t* test on 10 paired serum and urine samples, $P < 0.001$.
Unpaired *t* test with 19 urines and 10 sera, $P < 0.001$.
* Females.

vidual ratios of γG to γA were calculated and gave a mean value of 3 : 1 ; this value differs significantly from the values usually found in normal serum. When Student's paired *t* test (19) was used to compare γG/γA ratios on 10 paired serum and urine samples the degree of significance as indicated by *P* was less than 0.001. The unpaired *t* test with 17 degrees of freedom for the 19 urines also showed a *P* value of less than 0.001. It should be emphasized that the serum values for the 10 individuals tested have a greater standard deviation than usual. The mean value of the γG/γA ratio for normal serum if a large enough population is utilized is 6–8 : 1 (20).

DISCUSSION

Urinary γA has been isolated free of other immunoglobulins and found to have a sedimentation coefficient of approximately 11S. It is not dissociated by β-mercaptoethanol as are the γA-polymers found in small amounts in normal serum and large amounts in certain myeloma sera. Urinary γA contains secretory "piece" determinants and seems to be analogous to the secretory γA so far thought to be characteristic of external secretions.

Previous investigators have described the presence of γA in normal urine (8, 21, 22). However, little effort has been made in these studies to char-

acterize the urinary γA-molecule though there has been some evidence reported which our results confirm and extend. Tomasi and Zigelbaum (7) showed on density gradient ultracentrifugation that urinary γA had intermediate sedimentation characteristics (approximately 10S). Turner and Rowe have confirmed (8) that urinary γA occupies an elution position on Sephadex G-200 gel filtration characteristic of an 11S molecule

In certain external secretions the γA-immunoglobulins have been shown to predominate (9, 23) Unique antigenic determinants which confer upon the secretory γA-molecule antigenic specificity have been found to be due to a polypeptide chain referred to as secretory "piece." The role of secretory "piece" in relation to transport and other biological functions is as yet obscure. It has been suggested that γA may be synthesized locally by interstitial plasma cells rather than derived from plasma, and that in transit across the epithelial cells to the glandular lumen, secretory "piece" might be added (9). Supporting evidence for this theory comes from the immunofluorescent studies of salivary glands in which secretory "piece" staining occurred only in epithelial cells, whereas γA was found in plasma cells in the interstitium of the gland (9). Further evidence has been obtained by South, Cooper, Wollheim, Hong, and Good (4) who demonstrated that γA was not found in the saliva of agammaglobulinemic patients, whereas free secretory "piece" was always detected. However, some specific transport of γA from serum to saliva did occur when high serum levels were obtained by infusion.

That secretory "piece" can be secreted independently of γA is evidenced by the finding of free "piece" in the urine (18) and saliva (24) of patients with agammaglobulinemia. Secretory "piece" antigenic determinants have been demonstrated in ureteric urine in the present study. Whether these represent free "piece," that present as part of the 11S secretory molecule, or both has yet to be determined. Thus, secretory "piece" might be independently secreted somewhere along the upper renal tract, as has been suggested for the Tamm Horsfall protein. In the latter case it is secreted by the distal renal tubular epithelium (25). In the case of ureteric urine serum type γA can coexist with unattached secretory "piece." This suggestion has been confirmed by experiments [6] in which isolated secretory "piece" from agammaglobulinemic urine and also that isolated from reduced and alkylated colostral γA was incubated with serum γA in the presence and absence of reducing agents. Subsequent immunoelectrophoresis did not demonstrate attachment of secretory "piece" to γA. It may be, therefore, that the formation of secretory γA is more complex than the simple production of a secretory "piece" with nonspecific affinity for serum type γA although more work is certainly needed to exclude this possibility, particularly in view of the known ability of γA to complex with various molecules such as albumin (26). It would seem to us, however, that glomerular leakage of serum type γA with subsequent chance association with secretory "piece" is not the most likely explanation to account for production of urinary γA.

Two additional explanations to account for the uniform excretion of unattached secretory "piece" in normal urine may be entertained. It is possible that free "piece" circulates in normal plasma, and selective renal excretion occurs similar to that postulated for free L chains (1, 27). However, secretory "piece" cannot be detected by double diffusion in agar in normal human serum concentrated 30 times.[7] An alternative explanation is that free secretory "piece" is formed from the breakdown of secretory γA by urinary enzymes. This explanation appears unlikely since secretory γA isolated from colostrum or saliva has been shown to be relatively resistant to the action of proteolytic enzymes [8] (18). In addition, no low molecular weight γA-reactive molecules have been detected in concentrated normal urine although degradation into immunologically nonreactive fragments cannot be excluded.

That urinary γA may participate in immune defense mechanisms is suggested by the recent observations of Tourville, Bienenstock, and Tomasi [9] who demonstrated γA-antibodies in normal

[6] Tomasi, T. B., and J. Bienenstock. Unpublished observations.

[7] Bienenstock, J., and T. B. Tomasi. Unpublished observations.

[8] Plaut, A., J. Bienenstock, and T. B. Tomasi. Heat inactivation of gastrointestinal enzymes capable of degrading immunoglobulins. Submitted for publication.

[9] Tourville, D. R., J. Bienenstock, and T. B. Tomasi. Natural antibodies of human serum, saliva, and urine reactive with *Escherichia coli*. Submitted for publication.

urine against *E. coli*. In immunized individuals γA–urinary antibodies have been demonstrated against *S. typhi* (28), polio virus (29), and tetanus toxoid (8). In addition, a γA–rheumatoid factor has been isolated from the urines of patients with high serum titers of rheumatoid factor and a total 24 hr urinary protein excretion of less than 80 mg (30).

It is interesting to note that in 1948 on the basis of experiments with cholera vaccine Burrows and Havens (31) concluded that urinary and fecal antibody were independent of serum antibody and, therefore, not derived from it, at least directly by transudation. A γG/γA ratio which differs from that found in serum has been shown to occur in urine, and because of difficulties in quantitating γG (outlined under Results) it is possible that the γA class of immunoglobulins predominates in the urine as it does in other external secretions. Granted that secretion of γA occurs along the urinary tract; then with the demonstration of "piece" determinants in ureteric urine in this study the upper renal tract might be thought to be the major source. It is still possible, however, that the lower urinary tract participates in the formation of secretory "piece," secretory γA, or both. The possibility that local synthesis of secretory γA occurs somewhere along the urinary tract is presently under active investigation.

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