THE PROTEINS OF NORMAL URINE*

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(RECEIVED FOR PUBLICATION JULY 8, 1957)

It has been known since the end of the last century that normal urine contains traces of proteins from both blood and urinary tract (Mörner, 1895); yet many of the textbooks have ignored this, as well as the fact that pathological proteinuria must merge imperceptibly with the normal. One protein apparently arising from the urinary tract has been clearly defined, namely the mucoprotein intensively studied by Tamm and Horsfall (1952).

Electrophoresis was first used to study the nature of the proteins of normal urine by Rigas and Heller (1951). They used the moving boundary method after first concentrating 72-hour specimens of urine by ultrafiltration. The normal daily excretion of protein was found to be about 40 mg., and to consist of components with the same range of mobilities as those found in blood. Unlike blood, however, the globulin components were less well defined, exceeded the albumin in quantity, and were mainly α globulins rather than β and γ globulins. Their findings, which were strikingly uniform and similar in both sexes, have since been confirmed by two other groups of American workers, whose measurements of daily output vary from about 25 to 90 mg. (Boyce, Garvey, and Norfleet, 1954; McGarry, Sehon, and Rose, 1955).

These authors were unable to distinguish clearly between the proteins arising from the blood and those coming from the urinary tract. Here an attempt has been made to do this as well as to identify the individual proteins more clearly after their electrophoretic separation, by using an immunochemical technique in which rabbit antisera prepared against the serum proteins and against the urinary tract proteins respectively precipitate proteins from the two sources separately. The use of this method to study primarily the proteins arising from the blood is described below.

On testing unconcentrated normal urines immunologically by the agar gel diffusion tech-

nique (Ouchterlony, 1949), it has always been possible to demonstrate the presence of albumin, but the other proteins are usually present in too small amounts to be detected unless the urine is first concentrated.

Previously described methods of concentration have not proved entirely satisfactory (McGarry *et al.*, 1955). The essentials of any such method are that it should not denature the proteins, that it should be simple to perform, capable of the continuous concentration of large volumes of urine, and fast enough to concentrate a 24-hour specimen in, say, 48 hours. A method is described here based on the use of a large filtering area, which appears to satisfy these criteria.

Having concentrated the urine proteins it is possible to analyse them by the immuno-electrophoretic method of Grabar and Williams (1953, 1955). This technique was first introduced to us by Dr. Gell, who was kind enough to show us the details of his modifications (1955). Briefly, the proteins of a mixture are first separated in agar by the physical process of electrophoresis and then identified biochemically by precipitating them with their homologous antibodies.

Methods and Materials

Urines Examined.—Pooled urine from normal male adults was collected, and also individual samples from four normal men, four normal women, and four normal boys aged between 3 and 9. All the adults were members of the laboratory staff. The samples were not taken by catheter, but simple precautions were taken to avoid contamination. Urines from the males, apart from the pooled sample, were mid-stream specimens. All samples were preserved with sodium azide (1 g. per litre) until their colloids were concentrated by ultrafiltration.

Concentration of Urine Proteins.—Small quantities of protein solutions can be concentrated satisfactorily by dialysis against concentrated dextran or polyvidone solutions, and this might be the most convenient method for the investigation of cases of gross proteinuria.

*Based on a paper read at the meeting of the Association of Clinical Pathologists at Torquay on April 12, 1957.

It was, however, necessary to concentrate normal urine about 1,000 times, and for this purpose the following technique was evolved.

Approximately 6 ft. of dialysis* tubing (A. Gallenkamp and Co. Ltd. "visking") are encased in "tubegauz" (Scholl). This is done by first sliding the "tubegauz" on to the handles of a long Spencer Wells forceps. One end of the dialysis tubing is gripped by the forceps and the "tubegauz" slid on. A short tapered piece of glass tubing is then pushed into one end; this, with the tubing and gauze wrapped round it, is inserted into the rubber bung, tapered end first. (The inner end of the glass tube is covered with a short piece of rubber tubing to prevent damage to the dialysis tubing while doing this.) The remainder

*Tubing of $\frac{1}{8}$ in. flat width was used. For smaller quantities $\frac{1}{16}$ in. tubing can be used and does not require support by "tubegauz."

FIG. 1.—Ultrafiltration apparatus.

FIG. 2.—Enlarged drawing of portion A-B of ultrafiltration apparatus in Fig. 1.

of the gauze is wetted and pulled out over the dialysis tubing until it is fully extended. The tubing and gauze are then coiled up and placed in the aspirator, the free end being clipped between the neck and the bung (Figs. 1 and 2).

The reservoir containing the urine is connected to the glass tube and suction applied to the jar so that the tubing gradually fills with urine. Hot molten "vaseline" is meanwhile poured over the bung to form an airtight seal. The suction is stopped when a negative pressure of approximately 60 cm. of mercury has been reached. The apparatus is then left on the bench to filter; at intervals the filtrate is drawn off and the reservoir refilled with urine until the whole sample has been processed. In this way very large quantities of urine can be filtered if the vacuum is occasionally renewed.

When only a little urine is left in the tubing the free end is withdrawn leaving a single U of tubing containing all the concentrate, which is then reduced to the desired final volume. During this stage barbiturate buffer pH 8.6 is poured into the bottom of the aspirator so that the concentrate is dialysed against this buffer during its final concentration.

The brown viscous concentrate is removed by cutting the tube on both sides of the fluid, removing the gauze, placing the tube in a centrifuge tube with one end turned back over the rim, and centrifuging.

These concentrates were preserved by adding streptomycin and penicillin each to a concentration of 100 units per ml.

Preparation of Antisera.—Adjuvants were used to stimulate antibody production (Kidd, 1955), rabbits being injected intramuscularly weekly for six to 10 weeks with 2 or 3 ml. of a mixture of :

- 1 part protein mixture (10-20 mg. per ml.).
- 1 part "arlacel A" ("mannide mono-oleate"; Honeywill and Stein Ltd.)
- 3 parts "wyrol CX" (an "esso" light paraffin oil) to which is added 5 mg. per ml. killed dried *Mycobacterium tuberculosis*.

They were bled 10 days after completion of this course.

When further supplies of antiserum were required, the rabbits were re-stimulated by intraperitoneal followed by intravenous injections, as recommended by Wootton (1950). The antisera were preserved with streptomycin and penicillin each at a concentration of 100 units per ml.; sodium azide was found not to diffuse fast enough to prevent the growth of some strains of *B. subtilis* in the agar plates.

In this way three types of antisera were prepared :

(1) *Anti-human-serum-proteins.*—This antiserum was prepared by injection of normal human serum (about 25 samples pooled), and is obviously an excellent tool for investigating those urine proteins which are related to plasma proteins, since it will ignore all other urine proteins. The particular antiserum used here is capable of revealing at least 17 protein fractions in normal human serum, namely, pre-albumin (ρ), albumin, X, three α_1 globulins, six α_2 globulins, five β globulins, and γ globulin (cf. Williams and Grabar, 1955). This nomenclature is somewhat arbitrary, as some of the fractions occupy intermediate positions between the main groups. At the optimum ratio 13 can usually be distinguished : two α globulins are faint, X is obscured by the albumin, and the pre-albumin was only visible when excess antiserum was used.

(2) *Anti-human-urine-proteins.*—This antiserum, prepared by injection of normal urine colloids concentrated as above, may contain antibodies to high molecular weight polysaccharides as well as to proteins. When it is absorbed with normal human serum to remove antibodies to serum proteins, an antiserum specifically against urinary tract colloids is obtained.

(3) *Anti-Tamm-Horsfall-mucoprotein.*—This antiserum was prepared by injecting this protein which had been salted out from normal urine (Tamm and Horsfall, 1952). It was found to be contaminated with some anti-albumin, so this was removed before use by absorbing it with a little normal serum.

Immuno-electrophoretic Analysis.—In this method a drop of the protein mixture to be analysed is placed in a hole cut in a rectangular plate of agar about 3 mm. thick, and a potential difference applied across the plate for several hours, so that the different proteins separate as in paper electrophoresis. Then,

instead of staining them, antiserum is placed in a trough cut in the agar parallel to the direction of the current and about $\frac{1}{2}$ cm. to one side of the row of proteins already separated electrophoretically. The plate is incubated for about 48 hours during which time the separated proteins and the antiserum diffuse towards one another and form arcs of precipitate wherever a protein meets its homologous antibody.

The detailed technique used was substantially that of Grabar and Williams (1955) and Gell (1955) apart from the following small modifications :

A 0.8% solution of New Zealand agar (Davis Gelatine Company) was used, clarified by filtering while hot through a bed of "hyflo super cell" (cf. Feinberg, 1956); barbiturate buffer in the solid state was then added to it to give a pH of 8.6 and an ionic strength of 0.05 (10.3 g. of sodium diethylbarbiturate and 1.84 g. of diethylbarbituric acid per litre). To prevent the growth of bacteria and moulds, sodium azide was added to a concentration of 1 in 1,000 and the agar finally sterilized at 5 lb. pressure for 15 minutes.

The apparatus was made entirely of perspex, the platinum wire electrodes being contained in separate compartments connected to the inner buffer compartments by glass U-tubes containing barbiturate agar. A direct current supply at 200 to 250 volts was used and the current adjusted to about 30 milliamps for an agar plate measuring $6\frac{1}{2} \times 4\frac{1}{4}$ in., by varying the number of barbiturate agar bridges.

The results can be recorded photographically by direct contact printing, or the agar sheet with its lines of precipitate may be preserved by drying and staining with azocarmine as described by Uriel and Grabar (1956).

It was found more convenient to dry the agar sheet on stretched cellophane than on glass, and, after staining, to differentiate by washing in tap water rather than dilute acetic acid-glycerol solution.

The dried agar sheet on its cellophane can be cut out and filed or it can be mounted as a lantern slide. If projected on a screen details are particularly clearly seen.

When using this immuno-electrophoretic technique certain limitations of the method must always be borne in mind :

(1) The relative densities of the lines are no indication of the concentrations of the different proteins in the mixture. They depend on the response of the inoculated rabbit, and each rabbit behaves differently. This means that each antiserum must be regarded as a different reagent. It does not, however, prevent one comparing two protein mixtures, provided that each is examined with the same antiserum.

(2) The number of lines formed represents the minimum number of proteins in the mixture, as the rabbit may not have made antibodies to all of them.

(3) It is often impossible to arrange the quantities of protein mixture and antiserum used, so that all components of the mixture have antigen/antibody

ratios suitable for the formation of clear lines of precipitate. This means that several proportions of protein mixture and antiserum should always be used.

Results

Ultrafiltration Technique.—In order to make certain that the ultrafiltration did not in itself alter the proteins, some normal human serum was diluted 2,000 times with Ringer solution, and after reconcentration to its original volume was compared with an untreated sample of the same serum.

Nitrogen estimations of the reconcentrated and untreated sera by the micro-Kjeldahl method showed that there had been no appreciable loss on or through the membrane, and comparison of the samples by immuno-electrophoresis showed that the protein composition was almost unchanged qualitatively. The results obtained are reproduced in Fig. 3, which is a direct print from a dried plate stained with azocarmine. When a similar plate was stained for fat with Sudan black, it was found that the β -lipoprotein, although present in the reconcentrated serum, formed a line of precipitate which was more hazy than that formed by the untreated serum. Possibly the dilution had caused some degree of denaturation. (Both specimens were stored under similar conditions in the refrigerator at 2 to 4° C. and not frozen.) It seems, therefore, that the ultrafiltration process

FIG. 3.—Comparison between normal human serum (cup 2) and the same serum diluted 2,000-fold and reconcentrated by ultrafiltration (cup 1). The centre trough contained antiserum against normal human serum proteins. (Direct print from dried agar plate stained with azocarmine.)

FIG. 4.—Analyses with antiserum against normal human serum proteins: Left: Pooled normal serum (S). Centre: Normal urine colloids (U). Right: A mixture of pooled normal serum and normal urine colloids (S+U). (Direct print after staining with azocarmine.)

causes no important qualitative change, although the loss of some minor component on or through the membrane cannot be excluded.

Naked-eye Appearance of Concentrated Normal Urine Colloids.—The depth of the brown colour and the viscosity varied from one subject to another. Some samples formed precipitates on standing; the supernatant was then used for analysis. Others were too viscous for a precipitate to settle. Microscopically such precipitates consisted of mucoid material with a few crystals, including calcium oxalate and a variable number of epithelial cells, polymorphs, and erythrocytes (see Rofe, 1955).

Quantitative Analyses of Total Protein in Normal Urine.—Nitrogen estimations carried out on the colloid concentrates from the 24-hour specimens of two of the adult male subjects gave figures of 51 and 61 mg. protein respectively, on the assumption that 1 mg. nitrogen is equivalent to 6.25 mg. protein. As these results are within the range found by previous workers both by this and by the surface tension method (Tárnoky, 1951), no further estimations were made.

Immuno-electrophoretic Analyses.—These were done using the following antisera, whose preparation is described above:

(1) *Antiserum Prepared Against Normal Human Serum Proteins.*—All the urine samples gave

essentially similar results when analysed with this antiserum. There were differences in detail, but these were no more marked between samples from males and females than between samples from subjects of the same sex. Fig. 4 shows an example of such an analysis, in which a sample of concentrated adult male urine is compared with pooled normal human serum. It will be seen that eight out of the 13 protein fractions demonstrable in serum are also demonstrable in this urine, namely, albumin, four α globulins, two β globulins, and γ globulin. Not only do the urine concentrate and serum produce a similar pattern of lines when they are analysed separately but when mixed together the lines of precipitate from the two sources are seen to coincide. This confirms that the precipitin lines obtained with the urine represent substances identical electrophoretically and immunologically with the proteins of normal serum. Further, when the antiserum trough was arranged to form a rectangle around the separated urine and serum proteins, the albumin

FIG. 5.—The immunological identity of the albumin, α_1 globulin and γ globulin fractions from urine (U) and serum (S). The antiserum was contained in a rectangular trough enclosing the area shown in the photograph. (Direct print after staining with azocarmine.)

and γ globulin lines from urine and serum respectively were found to join, showing, as on an ordinary agar diffusion plate, that they are identical antigenically (Fig. 5). By suitable arrangements other fractions can be made to behave similarly.

One fraction of the β globulins, the β lipoprotein, has not been demonstrated in urine. Staining the dried agar plate with Sudan black for lipid shows a conspicuous β lipoprotein fraction in the serum but no corresponding band in the urine. Most of the α globulins of serum can be demonstrated in urine, but their concentrations compared with those of the other proteins do not appear to be any greater in urine than in serum. This suggests that the " α globulins," which Rigas and Heller found to predominate on moving boundary electrophoresis of normal urine protein, do not arise from the blood.

Two common but inconstant anomalies of the urine immuno-electrophoretic pattern are tailing of the albumin line and duplication of the γ globulin line. The cause of the former is not clear: the latter is presumably due to partial breakdown of the γ globulin fraction in the urine. Neither phenomenon was seen in the serum re-concentrated after 2,000-fold dilution, so that they cannot be due to dilution alone, but are presumably due to interaction with other substances sometimes present in the urine. It is interesting that so few of the proteins in urine appear to be altered by such substances.

There is almost always some pigmented material left unmoved in the hole into which the urine concentrate was placed before electrophoresis, especially if the sample is very viscous. If the urine becomes infected, all discrete lines may disappear; and, even if stored sterile, the lines tend to become hazy after a week in the refrigerator.

(2) *The Same Antiserum After Absorption with Urine Colloids.*—This absorbed antiserum, although now producing no precipitin lines when tested against urine concentrates, was found to show six lines with normal human serum, namely, X, two α globulins, and three β globulins. In other words these six protein fractions, which are present in normal serum, are absent or nearly absent from normal urine. By staining with Sudan black it was confirmed that one of these six protein fractions was β lipoprotein (Fig. 6).

(3) *Antisera Prepared Against Human Urine Colloids.*—Two rabbits were used for the preparation of antisera against normal human urine colloids and both produced potent antisera as judged by the content of anti-albumin etc.; yet on testing

FIG. 6.—Inability of the kidney to excrete β lipoprotein. In trough: antiserum (against normal human serum proteins) absorbed with normal urine colloids. In cup (S): Normal human serum. (Direct print after staining with Sudan black to show the β -lipoprotein fraction. The five other lines present before staining are no longer clear.)

them against normal human serum, some of the plasma protein antibodies were found to be missing, including that against the β lipoprotein fraction. This is further evidence for its absence from normal urine.

(4) *Antisera Specific for Urinary Tract Colloids.*—Analyses have also been carried out with the antisera prepared against urine colloids and against the Tamm-Horsfall mucoprotein after serum protein antibodies had been removed from them by absorption with normal human serum. They reveal the presence of several proteins in the urine of normal men and women which do not occur in the blood, the principal one being the Tamm-Horsfall mucoprotein. Fig. 7 shows the precipitin line obtained with the antiserum against this mucoprotein. It will be seen that its

mobility by this technique is much less than when purified and measured by moving boundary electrophoresis (Perlmann, Tamm, and Horsfall, 1952). The precipitin line extends over the whole α globulin range. There seems little doubt that it

FIG. 7.—Mobility of Tamm-Horsfall mucoprotein. Left trough: Antiserum against human serum proteins. Middle trough: Empty. Right trough: Antiserum against Tamm-Horsfall mucoprotein. Serum in left cup (S). Urine colloids in right cup (U). (Direct print of unstained plate.)

must be the cause of the blurred pattern and the apparent increase in α globulins when normal urine proteins are examined by moving boundary or paper electrophoresis. The elongated appearance of the line may be due to absorption of the mucoprotein on to the agar or to the mucoprotein being a mixture of molecular species of varying mobility but similar antigenic properties, cf., the γ globulin fraction. It is hoped to settle this point and to report further details of the urinary tract proteins at a later date.

Discussion

Normal urine evidently contains small quantities of many different proteins. The majority are electrophoretically and immunochemically identical with the plasma protein fractions, and so presumably arise from the blood. Others, among which the mucoprotein described by Tamm and Horsfall is probably the most abundant, have not been found in plasma and so presumably arise from the urinary tract. Several previous authors have suspected that this mucoprotein must come from the urinary tract; the present findings provide clear evidence in favour of this view.

The presence of plasma protein fractions in normal urine, especially the globulins, would be difficult to explain on the older view that the normal glomerulus is impervious to albumin or larger molecules (unless they are foreign). During the last few years, however, it has seemed increasingly probable that the glomerular capillaries are similar to other capillaries and produce a filtrate containing about 15 to 20 mg. of protein per 100 ml. similar to C.S.F. and other biological filtrates (Rather, 1952). Almost all this protein, amounting to about 30 g. daily, must be reabsorbed by the tubule cells; it is then probably broken down to amino-acids by an enzyme system of their mitochondria (Oliver, MacDowell, and Lee, 1954; Sellers, 1956). On this theory the plasma proteins in normal urine are easily explained as proteins from the glomerular filtrate which have not been completely reabsorbed by the tubules.

The proportions of these proteins excreted will depend on the characteristics of tubular reabsorption as well as on the degree of glomerular permeability. Sellers (1956) assumed that the reversed A/G ratio of normal urine found by Rigas and Heller meant that the tubules must reabsorb albumin more completely than the globulins; the present findings, however, suggest that this assumption is unnecessary. the low A/G ratio being due to the presence of urinary tract protein. In the nephrotic syndrome, in which the interfering effect of urinary tract protein is dwarfed by

large quantities of excreted plasma proteins, Hardwicke and Squire (1955) found that, when their patients were given infusions of human serum albumin, there were increases in the clearances of the four electrophoretic globulin fractions as well as the expected increase in the albumin clearance, all five in definite proportions to one another, suggesting that the tubules reabsorb all proteins without selection up to their maximum capacity (threshold). As they point out, if in fact the tubules do reabsorb the different types of protein without selection, the proportions of plasma proteins excreted in the urine will mirror those in the glomerular filtrate, and the study of urine and serum proteins will provide a simple way of assessing the permeability of both normal and diseased glomerular capillaries.

For example, it should be possible to deduce some information about normal glomerular permeability from the present immuno-electrophoretic analyses by comparing the proteins excreted by the kidney with those not excreted, and seeing if the results are consistent with information available about capillary permeability in general. The glomerular capillaries differ from others in being covered by the visceral layer of Bowman's capsule, but this layer probably only acts as a support, for by electron microscopy both this epithelial layer and the endothelium appear to have pores large enough to allow any plasma protein molecule through, the real filtering medium apparently being the continuous basement membrane between them (Pease, 1955). In any case, whether or not the capillaries are covered by an epithelial layer as in the glomerulus or choroid plexus, all the evidence suggests that the ease with which protein molecules pass through capillary membranes depends primarily on the relative size of the molecules (see Yoffey and Courtice, 1956). The explanation of this on the "pore" theory of capillary permeability does not necessarily imply that the pores vary in size. Pappenheimer (1953) and Pappenheimer, Renkin, and Borrero (1951) pointed out that even if the pores are all the same size there will not be a clear-cut size of molecule which will just pass completely through the pores or not pass through at all. The more nearly the diameter of a molecule approaches that of the pore, the more will its passage tend to be restricted, until it is too large to pass at all.

The molecular dimensions of a few of the better known plasma proteins have been calculated (Hughes, 1954). Albumin, γ globulin, fibrinogen, α lipoprotein, and transferrin are all elongated with short diameters of the order of 35 to 50 Å, whereas β lipoprotein is spherical with a diameter

of 185 Å. Pappenheimer, who studied diffusion rates through the muscle capillary wall in cats, found that his results could be explained by uniform cylindrical pores 60 to 90 Å in diameter. Such pores would stop the β lipoprotein molecule completely, but would allow the other molecules above to pass through in varying amounts according to their size. Our finding that β lipoprotein is not detectable in normal urine is in agreement with this and with the findings of Baudouin, Lewin, and Hillion (1953) and Gavrillesco, Courcon, Hillion, Uriel, Lewin, and Grabar (1955), who investigated the proteins of C.S.F. by paper electrophoresis and immuno-electrophoresis respectively. Morris and Courtice (1955), on the other hand, have found this lipoprotein by paper electrophoresis in the lymph of some animals, especially cholesterol-fed rabbits, so that it can evidently pass through the capillary walls of these animals. Its absence from human urine in the nephrotic syndrome, a disease in which particularly large amounts of this lipoprotein occur in the serum (Hardwicke, 1954), is further evidence that it probably cannot pass through the normal glomerular capillary wall in man, and that the glomerular pores are therefore smaller than 185 Å. The alternative possibilities that it is completely destroyed as it passes down the urinary tract, or is completely reabsorbed by the tubule cells, seem unlikely.

Hardwicke and Squire (1955) have pointed out how, on the hypothesis of non-selective tubular reabsorption, comparison of the excretion of large and small molecule proteins in the urine could be used to study changes of glomerular permeability in disease, so that the relative importance of glomerular leakage and tubular malabsorption in a given case of proteinuria could be deduced from quantitative measurements of the serum and urine proteins. Studying cases of gross proteinuria in which the proportion of urinary tract protein would be too small to affect the results, they found by paper electrophoresis that the clearances of the high molecular weight globulin fractions γ and α_2 were much less than that of the albumin fraction in nephrosis but became relatively higher in acute nephritis, a disease in which gross glomerular damage is known to occur.

It should be possible to apply this principle satisfactorily to less severe cases of proteinuria, if the urine protein fractions are estimated by some method which excludes the interfering effect of urinary tract protein. Uncorrected urine A/G clearance ratios measured by paper electrophoresis are difficult to interpret (Wolvius and Verschure,

1957). It is hoped that measurement of the clearance ratio of albumin to γ globulin by this technique may be more satisfactory, since neither of these fractions overlaps with the urinary tract protein; or the clearance ratio of albumin to some convenient protein fraction of larger molecular weight could be estimated immunologically by such a technique as that recently described by Gell (1957).

Summary

The proteins of normal urine have been concentrated by ultrafiltration under negative pressure and then analysed by the immuno-electrophoretic technique of Grabar and Williams.

By using rabbit antisera raised both against normal human serum and against normal human urine colloids respectively, it has been shown not only that normal urine contains small quantities of many different protein fractions, but also that these proteins are divisible into two groups:

(1) A group of protein fractions which, on the grounds of their apparent identity with the corresponding plasma protein fractions, have probably filtered from the blood through the glomeruli.

Although these protein fractions of urine include most of the protein components of plasma, β lipoprotein and several other fractions present in plasma were not detected in urine. It seems, therefore, that, although the glomerular pores must be much larger than used to be supposed, they are not wide enough to let through every plasma protein.

(2) A smaller group of proteins not demonstrable in blood and presumably arising from the urinary tract; the mucoprotein described by Tamm and Horsfall was found to be a member of this group of urinary tract proteins.

The high proportion of " α globulin" found in normal urine protein by moving boundary or paper electrophoresis is probably not due to differential tubular reabsorption of the plasma proteins but to the presence of this urinary tract protein.

We should like to thank Mr. G. Wright and Miss M. Carpenter for technical assistance and Mr. R. Ross for Figs. 1 and 2.

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