Micropuncture study of concentration and fate of albumin in rat nephron

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LEBER, PAUL D., AND DONALD J. MARSH. Micropuncture study of concentration and fate of albumin in rat nephron. Am. J. Physiol. 219(2): 358-363. 1970.—Albumin concentration in various segments of the rat nephron was determined by analyzing free-flow micropuncture samples with a radioimmunoassay. The average proximal tubular fluid concentration was 3.3 mg/100 ml but fluctuated over several milligrams, probably reflecting heterogeneity in glomerular permeability or tubular reabsorption or both. The heterogeneity was sufficient to preclude the use of an albumin-versus-inulin TF/P regression to determine whether there is albumin reabsorption in the proximal tubule. Variation in albumin concentration was also present in the distal tubule, but the average concentration increase between the proximal and distal tubule can be explained by assuming no protein transport occurred in the intervening segments of the nephron. Finally, the data suggest that protein may be reabsorbed in the collecting ducts. Several inconsistencies in current views about protein reabsorption by pinocytosis are discussed in light of the micropuncture data.

radioimmunoassay; albumin transport; pinocytosis

CURRENT BELIEFS about the fate of protein filtered at the renal glomerulus contain a paradox. When proteins that can be easily identified by histochemical, ultrastructural, or radioautographic techniques are followed, they appear to be reabsorbed almost exclusively by a pinocytotic mechanism in the proximal tubule. Some examples are hemoglobin (1, 4), horseradish peroxidase (5), ferritin (11), radiiodinated albumin (10), and lysozyme (7). Pinocytosis, as currently conceived, involves the nonselective transfer of bulk quantities of tubular fluid into the cell. A droplet of fluid is enclosed in a new vacuole formed from plasma membrane invaginating at the luminal surface of the tubular epithelial cell (13). Since we are obliged to retain the concept that inulin is a nonresorbable solute (8), the volume of tubular fluid removed by pinocytosis must be a very small fraction of the total proximal tubule volume flow. However, several studies based on the intravenous administration and subsequent recovery of labeled proteins from kidney homogenates suggest that almost all the filtered protein is reabsorbed (12, 14, 15). To account for this degree of protein reabsorption by pinocytosis would require that almost all the proximal tubular fluid is at one time or another within a pinocytotic vacuole. This conclusion is at odds with what is known about the behavior of inulin and the route of water reabsorption (8).

The paradox may be more apparent than real. Its satisfactory resolution requires that the magnitude of the pinocytotic process be known. Although this cannot be directly measured, the maximum pinocytotic volume flow necessary for protein reabsorption could be determined if the protein concentration in the glomerular filtrate, tubular fluid, and urine were known. Moreover, if significant protein reabsorption does occur, a comparison of the behavior of protein and inulin should allow us to determine whether pinocytosis, as conceived, is an exclusive or even significant route of protein reabsorption.

The application of free-flow micropuncture to the current problem has so far been limited by the lack of a specific, sensitive assay for protein and by the problem of sample contamination. Sensitivity of the assay system proved a stumbling block for Dirks, Clapp, and Berliner (3), who assayed 38 proximal tubular fluid micropuncture samples for albumin with a semiquantitative immunoprecipitin-gel technique (3). Although as little as 2 mg/100 ml of albumin could be detected in a sample of 250 nl, no albumin was found in about half the samples analyzed, and they were unable to draw any conclusions about the average albumin concentration in the proximal tubule. More recently, two groups have indicated in preliminary reports that assays of sufficient sensitivity exist (2, 16). No micropuncture study, however, has satisfactorily established that the protein measured is derived from tubular fluid and is not a contaminant. Visual control is an inadequate precaution because the micropipette passes through the relatively protein-rich interstitial tissue before it reaches the tubular lumen. Any vascular or lymphatic injury in the area, as produced by stripping of the pseudocapsule, will leak protein-laden fluid into the region traversed by the pipette.

A single nanoliter of interstitial fluid with a protein concentration of 1000 mg/100 ml distributed in a protein-free tubular fluid sample of 100 nl would yield on analysis a concentration of 10 mg/100 ml. This figure is comparable to the levels reported by Dirks et al. (3) and by two other groups in preliminary studies (2, 16).

The experiments reported here, we believe, overcome many of the difficulties discussed. We have developed a specific and sensitive radioimmunoassay for rat albumin that can detect as little as 750 pg of the protein in a volume of 150 nl. We have accounted for and quantitated the degree of contamination of our samples by performing control micropunctures with what are best characterized...
as "ersatz samples." Finally, we have compared the fate of albumin and inulin within the nephron in order to gain further insights into the magnitude, site, and mechanism of tubular protein reabsorption.

**METHODS**

Experiments were performed with a total of 11 female Lewis rats. Two different types of experimental protocols were employed and are outlined below.

*Standard experiment.* A 200- to 250-g rat, fasted the previous night, was anesthetized with inactin (100 mg/kg body wt) given intraperitoneally. A double-lumen polyethylene catheter was inserted into a jugular vein and the left kidney was exposed through a flank incision and prepared with the usual techniques used in this laboratory for micro-puncture (8, 9). A priming dose of 50 μCi inulin-14COOH (New England Nuclear Corp.) dissolved in isotonic saline was given, followed by a sustaining infusion of 152 μCi/liter. Approximate tubule location in anticipation of micro-puncture was determined by administering a .05-ml bolus of a 10 g/100 ml lissamine green solution intravenously while observing the renal surfaces. Micropunctures were performed with siliconized borosilicate glass pipettes, 8 μ outside diameter, filled with colored mineral oil. Because contamination of the specimen with blood would render the albumin measurement useless, the following precautions were routinely followed: a tubule segment was punctured only if its long axis was parallel to the pipette, and only if its top surface was free of blood capillaries; the pipette entered the tubule at an angle of 10° or less; tubular fluid was collected against a slight positive pressure applied to the pipette; the tubule diameter was not less than that of adjacent tubules; by gross visual inspection, the true capsule remained in place to isolate the punctured tubule from fluids accumulating on the kidney's surface; and areas of the surface soiled with blood were avoided. Both proximal and distal tubules were sampled; the collection periods were the same duration for both. At the end of a collection period, the pipette containing the sample was withdrawn from the tubule into the oil layer bathing the renal surface, where the pipette tip was sealed with a small plug of mineral oil on a siliconized glass plate and examined at ×100 magnification for the presence of erythrocytes.

Aliquots of the sample were transferred in siliconized constricted pipettes of known volume for inulin and albumin assay. Aliquots taken for inulin analysis were expelled directly into scintillation vials containing the scintillation cocktail (toluene, 1 liter; Spectrafluor (New England Nuclear), 42 ml; BioSolv 3 (Beckman Instruments), 15 ml). Sample aliquots for albumin determination were handled as follows: .1 ml of sterile rabbit serum (Pel-Freeze) was deposited from an automatic pipette with a disposable plastic tip onto a 3 x 3 cm square of Parafilm. Under microscopic control and with the aid of a micro-manipulator the transfer pipette containing the sample was lowered until its tip dipped into the drop of rabbit serum. The contents of the pipette were then slowly expelled into the drop of serum until oil from behind the sample appeared. Care was taken to avoid bubbling air through the serum droplet. The entire droplet, now containing the sample aliquot, was then aspirated into the automatic pipette using the original pipette tip. To insure adequate mixing, the droplet was redeposited and reaspirated several times before it was finally transferred to the tube used for radioimmunoassay.

*Ersatz sample protocol.* The routine followed was essentially the same as that just described except that inulin was not administered. In addition to the regular micropuncture to obtain tubule fluid samples, micropunctures were also performed with pipettes that contained approximately 200 nl of 0.02 M phosphate-buffered (pH 7.4) normal saline. The tip of these pipettes was blocked with about 0.1 ml of mineral oil. Once within the lumen of the tubule, the oil plug was expelled, and the pipette kept in place for the length of the average sample collection period (approx 10 min). Care was taken to avoid the loss of PBS or the aspiration of tubular fluid. At the end of the pseudo-collection period the pipette was withdrawn and its contents assayed. The investigator performing the assays did not know whether a specimen was a tubular fluid or ersatz sample.

Inulin (carboxyl-14C) was counted in a Nuclear Chicago liquid scintillation spectrometer. The samples, varying in volume from 10 nl to 1 μl, were delivered from siliconized and calibrated transfer pipettes into the scintillation cocktail. After mixing, the vials were counted as soon as possible. Data were rejected unless the observed count rate was three or more times the background count rate. The counting procedure employed a channel ratio method to estimate quenching, which proved for the sample size used to be essentially the same for inulin (carboxyl-14C) whether in serum, tubular fluid, or urine.

Albumin analysis was performed with a radioimmunoassay we developed. The general theory of radioimmunoassay has been described on many occasions. In brief, if a fixed quantity of antibody and radiolabeled antigen are allowed to react with unlabeled antigen, the amount of labeled antigen bound by the antibody is found to be a function of the total amount of antigen present. This relationship may be used to construct a standard curve.

The success of a radioimmunoassay, therefore, depends on a method of separating free and antibody-bound antigen. Many methods are available but the different solubilities of the free and antibody-bound rat albumin in 40% (NH4)2SO4 proved more than adequate to produce a reliable assay system.

Anti-rat albumin antibody was prepared by giving male albino rabbits initial immunizing intramuscular injections of an emulsion containing 1 ml of 500 mg/100 ml rat albumin (fraction V, Pentex, Inc.) and 1 ml of Freund's complete adjuvant (Difco). At 2-week intervals thereafter, 5 mg of rat albumin were given intravenously for 3 months. Following a rest period of 2 months the course was repeated. When a serum containing antibody of sufficiently high affinity for use in the immunoassay was obtained, the rabbit was exsanguinated and the serum separated

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1 Abbreviations for composition of solutions commonly used in this paper are: PBS - phosphate-buffered normal saline; 1/10 RS PBS, 1/40 RS PBS = 1/10 and 1/40 dilutions of sterile rabbit serum in PBS.
by standard techniques. The serum was stored frozen with 1/10,000 Merthiolate added as a preservative. By actual test the affinity of these sera was more than 100-fold greater for rat albumin than for rat gamma globulin.

Rat albumin (fraction V, Pentex, Inc.) was iodinated with a modification of the method of Hunter and Greenwood (6). Two micrograms of rat serum albumin were reacted with 2 mc of 125I (Cambridge Nuclear Corp.) as the sodium iodide and 48 μg of Chloramine T (Eastman Chemicals) in a total volume of 40 μl at pH 7.4 (sodium phosphate buffer) for 3 min at room temperature. The reaction was stopped with an excess (120 μg) of Na metabisulfite. The iodinated protein was diluted several hundred-fold with ½ rabbit serum in phosphate-buffered saline. Excess iodide was removed by batch treatment with an insoluble anion-exchange resin (Iobeads, New England Reagent Laboratories). The final product was stored at 4°C with 0.01% Merthiolate over Iobeads. The radioiodinated albumin was used within 3 days of its preparation.

Immediately prior to use in the radioimmunoassay, concentrated (NH₄)₂SO₄ was added to the 125I stock to produce a concentration of 50%. The solution was kept in an ice water bath for 30 min and then centrifuged to remove any insoluble material. The supernatant was next diluted in 1/40 RS-PBS to reduce the (NH₄)₂SO₄ concentration to less than 1%, and this "preprecipitated" solution was used as the source of radioiodinated albumin for the assay.

In the usual assay the sequence described next was followed.

To 10 x 75-mm plastic test tubes in an ice water bath were added, in sequence, 0.2 ml of phosphate-buffered saline, 0.1 ml of sterile rabbit serum containing the albumin standard or micropuncture sample, 0.1 ml of appropriately diluted and "preprecipitated" 125I rat serum albumin solution, and 0.1 ml of a dilution of rabbit anti-rat albumin antibody. After the addition of the antibody to start the reaction, the tubes were rapidly stirred with a vortex mixer and then incubated at 37°C for 30 min. The tubes were then transferred to a cold room (4°C) for a period of 36 hr. The reaction was stopped by the addition with rapid mixing of sufficient 80% (NH₄)₂SO₄ to produce a final concentration of 40%. Following incubation for 30 min in an ice water mixture, the tubes were spun at 16,000 rpm (4°C) for 30 min. After centrifugation the supernatant was aspirated and discarded. The entire tube containing the precipitate was then counted automatically on a gamma ray spectrometer (Nuclear Chicago).

The actual dilution of antibody in 1/10 RS-PBS and the dilution of rat albumin standards and 125I rat albumin in 1/40 RS-PBS were done empirically so as to adjust the useful range of the assay. In some instances, different size aliquots of sample were used to extend the working range of the assay and a subsequent volume correction was applied to the data.

The usual standard curve was constructed from duplicate points at a minimum of four albumin concentrations. Although there are several methods of plotting a standard curve, the data over the useful range of the assay can be made to fit a straight line represented by the equation

\[ \text{DPM} = (\text{constant}) \times \text{log (albumin concentration)} \]

The line was fitted by the method of least squares and the sample albumin concentration (interpolated) was determined from the observed radioactivity.

**RESULTS**

Precision of radioimmunoassay for rat albumin. Albumin recoveries were evaluated at two concentrations for 100-nl samples. Samples were taken from droplets of known concentration (w/v) that had been deposited earlier on siliconized glass plates beneath a layer of water-saturated mineral oil. For conditions identical to those in a typical experiment, the mean recovery from 10 samples at a nominal concentration of 1.0 mg/100 ml was 1.1 mg/100 ml with a standard deviation of 0.17 mg. For 6 samples at a nominal concentration of 5 mg/100 ml the mean recovery was 4.5 mg/100 ml with a standard deviation of 0.38 mg.

Comparison of albumin recovery from tubular fluid and ersatz samples. There was a significant difference between the concentration of albumin recovered from proximal tubular fluid and that recovered from pipettes loaded with PBS...
FIG. 3. Albumin concentration plotted as a function of inulin TF/P from same specimen. Different symbols refer to different animals.

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<th>TABLE 1. Percent recovery of albumin from different media</th>
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* Number in parentheses is number of samples analyzed.
† When dilute protein solutions are prepared without carrier proteins, losses due to surface adsorption and denaturation occur. Note that while absolute recovery is low from solutions of rat albumin in PBS the proportional recovery at two different concentrations is the same. This suggests that losses occurred in preparation of solution and not in assay system. ‡ We have no explanation for the greater than 100% recovery.

that has been introduced into proximal tubules. Figure 1 illustrates the difference by comparing the cumulative frequency of samples having a concentration equal to or less than the value of albumin plotted on the abscissa.

While the separation of the two populations is obvious by inspection, this distinction can be made in a more formal manner with the Wilcoxon signed rank test (17). The calculated value for the sum of the signed ranks (4) for 15 randomly paired values is 14. This result indicates that the observed difference between the two types of samples would occur by chance alone less than one time in a hundred.

Concentration of albumin in proximal tubule. The average concentration of albumin in 18 samples was 3.3 mg/100 ml, with a standard deviation of 2.6 mg/100 ml. Figure 2 illustrates that there is no apparent relationship between the inulin TF/P and the albumin concentration. The correlation coefficient relating these measurements is very close to zero (–0.095).

Concentration of albumin in distal tubule and urine. There was a significant increase in the average albumin concentration paralleling the increase in average inulin TF/P between the proximal and distal tubules. As in the proximal tubule, however, we found no significant relationship in the distal tubule between the inulin TF/P and the albumin concentration. Between the distal tubule and the bladder urine, in five animals, the albumin concentration increased less than a factor of two or even fell, while the inulin TF/P rose as much as 35-fold (Table 2, Fig. 3). The possibility that urine denatured, destroyed, or in some way made albumin unreactive in the immunosay was excluded by the efficient recovery of known amounts of albumin incubated with urine at 37 C for 30 min (Table 1).

DISCUSSION

Albumin can enter a micropuncture sample in at least three ways: by contaminating the pipette as it crosses the interstitial space on its way into and out of the tubule, by leaking through the puncture hole during the sample collection, and by filtration at the glomerulus. Since we wish to assess the magnitude of glomerular filtration and tubular transport, it is necessary to show that the first two sources are insignificant. We devised the ersatz sample protocol to evaluate contamination as the pipette moved to and from the tubule. If one accepts that this source of contamination is identical for both tubular fluid and ersatz samples, any observed differences in their albumin concentrations cannot be due to extratubular contamination alone. A possible objection to this experiment is that the observed differences are not due to the presence of albumin in the tubular fluid but to some property of tubular fluid. Specifically, the recovery of albumin from tubular fluid may be more efficient than from PBS. This possibility cannot be directly tested because of the limited amounts of tubular fluid obtainable. Instead, a correction based on actual recoveries of albumin from PBS may be applied to the ersatz sample data. Since the recovery of albumin from PBS was greater than 50% (Table 1), the observed values for the ersatz samples were doubled. Even after applying this maximum correction (see footnote to Table 1) the difference between the ersatz and tubular fluid samples remained significant at the 5% level with the Wilcoxon signed rank test.

The second possible source of sample contamination, entrance of albumin through the micropunctate tract while the sample is being collected, is not excluded by the results of the ersatz sample protocol. As that protocol does not involve the collection of tubular fluid, even if contaminating albumin were to enter the tubule, it could only find its way into the pipette by diffusion at its tip. The experimental exclusion of this type of contamination is clearly not possible, for it would require not only the collection of tubular fluid but a prior and independent knowledge of the real tubular fluid albumin concentration.

Although we cannot definitely exclude contamination of this sort, we suggest that it is unlikely to be significant. Since the tubules retained their normal diameters during sampling, it can be assumed that the hydrostatic pressure in the tubule lumen was higher than in the interstitial space. In the event that the sealing of the tubule cell to the pipette wall was not hydraulically intact, the pressure gradient favored flow of fluid from the lumen to the exterior, in the wrong direction for contamination.

Subject to the reservation that the second type of contamination cannot be rigorously excluded, it is probable...
that the average albumin concentration in the proximal tubule of normal Lewis female rats is about 3 mg/100 ml. This result agrees closely with the values obtained by Carone et al. (2) and Van Liew et al. (16); it implies that albumin reabsorption occurs between the proximal tubule and the bladder, since its concentration increased by less than a factor of 10 while the inulin TF/P increased more than 100-fold (Table 2, Fig. 3).

A comparison of the rate of increase in the inulin TF/P and albumin concentration along the proximal tubule has proven to be of little practical value. Carone et al. (2) have argued that protein and water are reabsorbed at the same rate in the proximal tubule. Although this may prove to be true, we are not convinced that their data, based on the apparent constancy of albumin concentration along the proximal tubule, can support this conclusion. We have found that the albumin concentration does not apparently vary systematically with the proximal tubular fluid's inulin TF/P (Fig. 2). This lack of correlation cannot be used to infer that protein and water are reabsorbed at the same rate, since the 95% confidence limits for the slope of our albumin-versus-inulin TF/P regression line includes values consistent with virtually no protein reabsorption (2.67 mg/100 ml) or with protein reabsorption at a rate greater than that of water (3.84 mg/100 ml). The use of regression analysis usually assumes that the independent variable is a known or assigned number, rather than one obtained experimentally. If the experimental uncertainty in the inulin TF/P is taken into account, an even greater range of slopes is possible, and this indeterminacy increases further when the independent variable is merely an estimate of percent proximal tubule length (2). Moreover, the decision about the direction and magnitude of tubular transport of any compound depends ultimately on a knowledge of its concentration in the glomerular filtrate. The variability in the albumin concentration is no less in our early proximal specimens (low inulin TF/P) than in any other region. This reflects that, as expected, the filtrate's albumin concentration does not have a single value. We would argue that the relatively large observed variation in albumin concentration between nephrons makes it difficult to obtain a reliable estimate of the direction or rate of proximal protein transport using the technique of free-flow micropuncture. (We attempted microperfusion of isolated segments of single tubules with solutions of labeled albumin and inulin to overcome this difficulty, but these failed primarily because of losses of high specific activity radioalbumin in the perfusion pipette.)

Our data and those of Carone et al. (2), however, do indicate that protein transport may occur at some site beyond the accessible portions of the distal tubule. The increase in albumin concentration between the distal tubule and the bladder is considerably less than the corresponding increase in inulin TF/P. The excellent recovery of albumin after incubation with fresh rat urine would seem to preclude the possibility that urine destroys albumin or inhibits the immunoassay system. It is thus reasonable to assume that albumin is removed from the tubular fluid, perhaps in the collecting ducts.

Our data have not allowed us to estimate the actual rate at which pinocytosis occurs, but it does allow us to draw an important inference. While most of the filtered protein is resorbed, relatively little or no inulin is. Pinocytosis is therefore either insignificant or it cannot be the simple bulk fluid transport mechanism described in the introduction. It may be that protein is selectively concentrated in the pinocytotic vacuole before it is sealed off from the tubular lumen. Alternatively, significant pinocytosis may not occur under the conditions of our experiments. The pinocytotic route of protein resorption might only occur when there is a high concentration of protein in the ultrafiltrate. If this explanation is correct, a decrease in inulin clearance should be produced by an increase in glomerular permeability to protein.

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