YALE JOURNAL OF BIOLOGY AND MEDICINE 45, 233–239 (1972)

Technical Problems Associated with Collection of Distal Tubular Fluid in the Rat[1]

ROMULO E. COLINDRES[2] and CLAUDE LECHENE[3]

Department of Medicine, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514, and Department of Physiology, University of Sherbrooke, School of Medicine, Sherbrooke, Quebec

Received for publication 19 November 1971

Studies from several laboratories have shown that tubular fluid collected from the second half of the distal convolution of the rat usually achieves osmotic equilibrium with interstitial fluid in the presence of ADH(1,4,12). These observations have recently been questioned by Lechene, Morel, Guinnebault, and de Rouffignac who suggested that osmotic equilibration might occur only in the cortical collecting ducts(6).

The site of osmotic equilibration in the cortex is of little significance with regard to the function of the countercurrent mechanism; however, the apparent discrepancies in results raise questions about the accuracy and reliability of determinations of individual solute concentrations in rat distal tubular fluid. Studies were carried out independently in Sherbrooke (C. Lechene in collaboration with D. Roy) and in Chapel Hill (R. E. Colindres in collaboration with R. Kramp, M. E. M. Allison, and C. W. Gottschalk) to determine the osmolality of distal tubular fluid in the rat with methods that might detect and minimize artifactual changes in tubular fluid osmolality.

METHODS

Chapel Hill Study

The left kidney of male Wistar rats weighing 200–300 gm was prepared for

[1] The Chapel Hill study was supported by a grant-in-aid from the American Heart Association, and by Grant HE-02334 from the National Institutes of Health.

[2] Dr. Colindres was an International Postdoctoral Research Fellow of the NIH, No. 1 F05 TW-1580 01.

[3] Present address: Department of Physiology, Harvard Medical School, Boston, Massachusetts.

micropuncture; several levels of urine flow were produced by the infusion of 0.9% or 2.5% NaCl at appropriate rates.

Distal convolutions were identified as early, middle, or late by the iv injection of lissamine green and punctured with pipettes filled with mineral oil stained with Sudan black and having an external tip diameter of 6–7 μm.

Three types of tubular fluid samples were collected.

Small samples. A distal tubule was punctured and a droplet of oil one tubular diameter in length was introduced into the lumen to determine the position of the pipette tip and to assess the direction of tubular flow. After the oil droplet was swept away tubular fluid was collected without an oil block at a very slow rate for a period of 10–15 sec, so that only part of the fluid flowing past the pipette tip was collected. The volume of tubular fluid collected was of the order of 0.1 nliter.

Collections with "sudden influx." These were sudden, almost instantaneous collections resulting from the application of strong negative pressure to the pipette. The volume of tubular fluid collected was a fraction of a nanoliter. These collections were specifically designed to accelerate the linear velocity of tubular flow.

Large samples. These samples were obtained in the conventional manner. For this purpose an oil block approximately five tubular diameters in length was introduced into the lumen and tubular fluid was collected at a rate such that the oil block was maintained just distal to the pipette tip in as constant a position as possible. Excessive suction and changes in tubular diameter were avoided. These collections lasted an average of 2 min and the volume of tubular fluid collected, when measured, varied from 5–15 nliters.

In some of these experiments distal tubules with at least two loops on the surface were identified by the injection of lissamine green into a proximal convolution. Intratubular pressure was measured in the early convolution by the servonulling system of Wiederhielm *et al.*(11) as modified by Intaglietta *et al.*(5) while tubular fluid was being collected from the late distal convolution.

In a separate group of rats the puncture sites were identified by microdissection and the osmolality of the distal tubular fluid was correlated with the anatomical site of puncture. The osmolality of plasma and tubular fluid was determined by the microcryoscopic method of Ramsay and Brown(9).

RESULTS

The reliability of the small samples was tested by comparing the osmolality of re-collected small samples with that of the original small collection in hydropenia and under conditions of osmotic diuresis. The correlation of results was excellent along the distal tubule, with most of the observations falling on or close to the line of identity. In only a few of the re-collections did the osmolality of the first sample differ from that of the second by more than 10%. Intratubular pressure was measured upstream from the collection pipette during some of the

small sample collections. In most instances there was no change from the baseline precollection pressure. The remaining collections were associated with very small decreases in intratubular pressure.

These findings suggest that the small samples are reliable, give reproducible results, are associated with minimal changes in intratubular pressure, and are therefore representative of the condition of tubular fluid in undisturbed tubules. The osmolality of samples collected with other methods was thus compared with this standard.

To evaluate the effect of differing collection techniques, the osmolality of the small sample was compared with that of samples collected with sudden influx from the same puncture site. In two instances the osmolality of the latter sample was higher, but in the majority of re-collections the osmolality of the samples collected with sudden influx was 10% or more lower than that of the small sample. In some sudden influx collections the osmolality of the samples was similar to that of the small samples. Since decreases in intratubular pressure always occurred proximal to the collection site during re-collection, the latter finding was presumably related to an admixture of fluid of varying osmolality.

The osmolality of small collections was next compared to the osmolality of large samples re-collected proximal to an oil block. In a few instances the large sample osmolalities were 10% or more higher than that of the small samples, indicating that reflux had taken place. In approximately 50% of the collections, however, the osmolality of the large samples was 10% or more lower than that of the small samples. Intratubular pressure was measured during some of the large collections to try to correlate changes in pressure during the collection with changes in osmolality. Although no such correlation was found, fluctuations in pressure were always noted, indicating that the rate of collection was not uniform during the sampling period. It is thus probable that the mean pressure during the collection did not reflect the degree of acceleration of flow during the actual sampling periods.

Believing that the small samples represent the best measure of *in situ* undisturbed conditions, this technique was employed for collections in order to correlate the osmolality of distal tubular fluid samples with the anatomical site of puncture under conditions of hydropenia and osmotic diuresis. In both conditions there was significant osmotic equilibration along the distal convolution. In hydropenia most of the samples collected from the second half of the distal tubule were isosmotic or nearly so, while during osmotic diuresis approximately 50% of the samples from the second half were hypoosmotic.

Osmolalities were also determined in a small number of Saclay Wistar rats generously provided by Drs. F. Morel and C. de Rouffignac. During hydropenia the tubular fluid osmolality in these animals increased significantly from the first half of the distal tubule to the second half. It was observed, however, that more samples collected from the late distal tubule in Saclay rats were hypoosmotic than in the Chapel Hill Wistar rats under the same conditions. The mean F/P osmolality in the second half of the distal convolution was significantly lower in the Saclay animals, suggesting that there might be a strain differ-

ence with regard to the degree of osmotic equilibration achieved in the distal tubule.

METHODS

Sherbrooke Study

Experiments were performed on male Wistar rats weighing an average of 203 gm that were thirsted for 48 hr and maintained in hydropenia. The left kidney was prepared for micropuncture and tubular fluid was collected from distal tubules with oil-filled pipettes having an external tip diameter of 6 μm. Tubular fluid samples were collected with three techniques. Technique A: These samples were collected proximal to an oil block two to three tubular diameters in length over a short time (mean duration: 76.4 $\pm$ 8.7 SD sec) at a rate such that the oil block was maintained just distal to the pipette tip in as constant a position as possible. The mean volume of tubular fluid collected was 1.12 $\pm$ 0.12 SD nliter. Technique B: These samples were collected during free flow, after injection of a fraction of a nanoliter of a solution containing tritiated inulin. For this purpose a distal tubule was punctured with an oil-filled pipette having a known amount of the marked solution at the tip. The tritiated inulin was injected and after a period averaging 10 sec, tubular fluid was collected without an oil block. The mean duration of these collections was 77.3 $\pm$ 9.2 sec and the mean volume of tubular fluid collected was 1.1 $\pm$ 0.36 nliter. Technique C: These samples were collected proximal to an oil block after microinjection of a small volume of a solution containing tritiated inulin. Once the inulin was injected, an oil block two to three tubular diameters in length was placed in the lumen and after an average of 5.06 sec tubular fluid was collected as in Technique A. The mean duration of these collections was 66.7 $\pm$ 4.9 sec and the mean volume collected was 2.01 $\pm$ 0.2 nliter.

Most of the samples collected with Technique B were re-collection of the samples collected with Technique A. Technique C was employed in a separate group of animals.

When samples were collected with Techniques B and C a volume of tubular fluid of less than 1 nliter was placed in counting fluid containing toluene and Spectrofluor Butyl PBB. The tritium injected and collected was counted in a Nuclear Chicago Mark II liquid scintillation spectrometer.

The recovery of inulin during tubular fluid collections was calculated as follows: $\%\ \text{Recovery} = \frac{H^3 \text{ in collected sample}}{H^3 \text{ in solution injected}} \cdot 100.$

Recoveries of more than 10% of injected inulin were considered to indicate significant reflux during the collection. All the nephrons were filled with Neoprene after the collections and microdissected to determine the site of puncture. The osmolality of plasma and tubular fluid was determined by the microcryoscopic method of Ramsay and Brown(9) using volumes of approximately 75 pliters with appropriate corrections for the size of the samples.

RESULTS

With the three techniques employed during hydropenia, there was significant osmotic equilibration along the distal convolution. Most of the samples collected from the second half were isosmotic.

To exclude the possibility of reflux as an explanation for the high osmolality of late distal samples, the percentage of recovery of injected inulin (presumably reflecting the degree of reflux) was correlated with the F/P osmolality ratio in samples from the first and second half of the distal tubule collected with Techniques B and C. In samples collected from the second half, there was no correlation between the percentage of recovery of inulin and the F/P osmolality ratio ($r = -0.025$). Eight of twenty-five late distal samples collected with Technique B, and seven of eight late distal samples collected with Technique C, were isosmotic in the absence of reflux. Even in those late distal samples where reflux was demonstrated, the F/P osmolality ratio did not deviate much from unity. In the early distal samples the F/P osmolality increased significantly as inulin recovery increased ($r = 0.69$ $P<0.05$).

Techniques B and C were also used for collection of proximal tubular fluid. In all instances the recovery of inulin was negligible, suggesting that reflux may occur less frequently during proximal collections than during distal collections.

DISCUSSION

Using methods that decrease the risk of accelerated flow or reflux or both, the Chapel Hill and Sherbrooke experiments have confirmed the results of previous studies, showing that under conditions of hydropenia, tubular fluid in the rat achieves or approaches osmotic equilibrium in the second half of the distal tubule(1,4,12). Under conditions of osmotic diuresis with 2.5% NaCl, the Chapel Hill results demonstrate that approximately 50% of late distal samples are hypoosmotic, suggesting that the rate of tubular flow may limit the degree of osmotic equilibration. Furthermore, just as there are species differences(2,3) the studies performed on Saclay Wistar rats suggest that there may be hereditary or environmentally induced strain differences with respect to the degree of osmotic equilibration achieved. It is impossible to state whether or not these strain differences explain the apparent discrepancies between the results in this study and those reported by the French workers(6) since in the latter study the punctures were not localized anatomically and as the authors themselves state, it is possible that most of their collections were from early distal tubules.

The Sherbrooke study demonstrates that the isosmotic values obtained are probably not explained by reflux, since in 15 of 33 isosmotic samples from the second half of the distal tubule, inulin recovery was absent or very small, and for the whole group, there was a lack of correlation between the inulin recovery and the osmolality of the sample. The observation that even those late distal samples associated with reflux, had very little deviation from the isosmotic value

may be explained by assuming that reflux occurred only from the cortical collecting ducts or that osmotic equilibration of hyperosmotic fluid occurred during retrograde flow. Alternatively it is possible that in some of these samples there may have been a fortuitous admixture of hyperosmotic and hypoosmotic fluid, yielding an osmolality similar to that of plasma. Of importance was the observation that in the first half of the distal tubule, where tubular fluid is known to be hypoosmotic(1,4,6,12), there was a significant positive correlation between the degree of reflux and the osmolality of the sample.

Both studies also confirm that during distal collections it is possible to collect from downstream, even though the collections are made proximal to an oil block and the volume of tubular fluid collected is only of the order of 1 nliter as in the Sherbrooke Study. The fact that retrograde flow was much more frequent during free flow in the latter study (26 of 37 samples collected with Technique B showed significant recoveries of inulin), in no way detracts from the small sample free flow collections performed in the Chapel Hill Study, since in the latter case, the samples collected were approximately 10 times smaller than in the Sherbrooke Study and the rate of collection was also slower, i.e., 0.1 nliter in 15 sec as opposed to 1 nliter in 67 sec.

Using the small samples as a standard for comparison, the Chapel Hill study has demonstrated that it is possible to artifactually lower the osmolality of tubular fluid collected from distal convolutions, probably as a result of an acceleration of the linear velocity of tubular flow in a segment of the nephron which has a relatively low water permeability, even in the presence of ADH(8,10). It is unlikely that the measures usually taken to detect reflux during distal collections would permit one to detect an acceleration of tubular flow velocity. Furthermore visible decreases in tubular diameter are not likely to be apparent in these small tubules which cannot be reduced below a minimal volume. One solution to the problem might be the use of ultramicroanalytical methods that would permit several chemical determinations to be made in a fraction of a nanoliter of fluid. The use of the electron probe for this purpose offers promise(7). Until methods are improved, however, it is suggested that in addition to the measures usually taken to detect reflux, a very small sample of tubular fluid be obtained first for osmolality determinations, whenever larger samples are needed for other chemical measurements. Comparison of the osmolality of both samples might then be useful to exclude the possibility of accelerated flow or reflux during the large collection.

REFERENCES

1. Bank, N., and Aynedjian, H. S., A micropuncture study of the renal concentrating defect of potassium depletion. *Amer. J. Physiol.* **206,** 1347–1354 (1964).
2. Bennett, C. M., Brenner, B. M., and Berliner, R. W., Micropuncture study of nephron function in the Rhesus monkey. *J. Clin. Invest.* **41,** 203–216 (1968).
3. Clapp, J. R., and Robinson, R. R., Osmolality of distal tubular fluid in the dog. *J. Clin. Invest.* **45,** 1847–1853 (1966).

4. Gottschalk, C. W., and Mylle, M., Micropuncture study of the mammalian concentrating mechanism: evidence for the countercurrent hypothesis. *Amer. J. Physiol.* **196,** 927–936 (1959).
5. Intaglietta, M., Pawula, R. F., and Tompkins, W. R., Pressure measurements in the mammalian microvasculature. *Microvas. Res.* **2,** 212–220 (1970) .
6. Lechene, C., Morel, F., Guinnebault, M., and Rouffignac, C. de, Etude par microponction de l'elaboration de l'urine. I. Chez le rat dans differents etats de diurese. *Nephron* **6,** 457–477 (1969).
7. Lechene, C., The use of the electron microprobe to analyze very minute amounts of liquid samples. *National Conference on Electron Probe Analysis 5th* 32A–32C, New York, 1970.
8. Persson, E., Water permeability in rat distal tubules. *Acta Physiol. Scand.* **78,** 364–375 (1970).
9. Ramsay, J. A., and Brown, R. H. J., Simplified apparatus and procedure for freezing point determinations upon small volume of fluid. *J. Sci. Instrum.* **32,** 372–375 (1955).
10. Ullrich, K. J., Rumrich, G., and Fuchs, G., Wasserpermeabilität und transtubulärer Wasserfluss corticaler Nephronoabschnitte bei verschiedenen Diuresezuständen. *Pflugers Archiv. Gesamte Physiol. Menschen Tiere* **280,** 99–119 (1964).
11. Wiederhielm, C., Woodbury, J. W., Kirk, S., and Rushmer, R. F., Pulsatile pressures in the microcirculation of the frog's mesentary. *Amer. J. Physiol.* **207,** 173–176 (1964).
12. Wirz, H., Der osmotische Druck in den corticalen Tubuli der Rattenniere. *Helv. Physiol. Pharmacol. Acta* **14,** 353–362 (1956).