YALE JOURNAL OF BIOLOGY AND MEDICINE 45, 289–298 (1972)

Examination of the Gertz Technique as Applied to the Proximal Tubule of the Rat Kidney*

EDWARD J. WEINMAN,† ROBERT J. HARDY,
MICHAEL KASHGARIAN,‡ and JOHN P. HAYSLETT§

Departments of Internal Medicine, Pediatrics, Pathology, and Biometrics,
Yale University School of Medicine, New Haven, Connecticut 06510

Received for publication

The shrinking-drop micropuncture technique was introduced by Gertz in 1963 as a means of estimating the net reabsorption of tubular fluid from the proximal convolution under *in vivo* conditions(1). Although this method does not provide an absolute measurement of net fluid efflux, since the actual exposed surface area of the tubular epithelial is unknown, it has served as a useful method of comparing the relative effects of the various factors involved in the regulation of this transport process. It is not surprising that recent reports have demonstrated that this technique, like other methods for measuring biological processes, is influenced by a number of methodological variables(2,3).

Recent studies have critically reexamined the split-droplet method in an attempt to standardize the technique and to identify those methodological factors which influence the estimation of the reabsorptive half-time ($t_{1/2}$)(4,5). In one report, the measurement of the reabsorptive half-time was shown to have a coefficient of variation as low as any other method currently used to determine net sodium efflux, when calculated under conditions in which the mechanical application of the method was standardized, the influence of the initial droplet length was controlled, and bias and error in making the actual measurements were minimized(5).

In this report we have summarized our evaluation of the method and offer suggestions for its practical application as a reliable and comparative estimator of net fluid reabsorption in the proximal tubule of the rat kidney.

* Supported by USPHS Grants TIAM 5015, HE 13647-01A1, HE 00834, 5-T01-GM-0047, and the American Heart Association.

† Current address: USAF Hospital, Tinker Air Force Base, Oklahoma City, OK 73145.

‡ Dr. Kashgarian is the recipient of a Public Health Service Career Development Award K3-HE 13683.

§ Dr. Hayslett is an Established Investigator of the American Heart Association.

METHODS

Male Sprague–Dawley rats were anesthetized with Inactin (100 mg/kg body weight) intraperitoneally and prepared for micropuncture as previously described(6). The reabsorptive half-time ($t_{1/2}$) of isotonic saline was measured by time-sequence photomicrography as described by Gertz(1). Proximal tubules were punctured with double-barreled micropipets, containing castor oil stained with Sudan black dye in one arm and isotonic saline (150 m*M* NaCl per liter) in the other. The following details were carefully controlled to maintain uniformity: (1) only straight portions of proximal tubules were included for study; (2) the oil blocks proximal and distal to the saline droplet were of sufficient length to insure stability of the oil column and prevent movement of the column during reabsorption of the saline droplets; (3) the initial droplet length of the saline droplet was between 2 and 3 tubular diameters (60–90 μm); (4) the droplet was placed "upstream" to the point of puncture.

Photographs of the shrinking droplets were taken through an American Optical Stereo Microscope at a magnification of 75 times with a Nikon F 35-mm camera (Nikon Inc., Garden City, NY) at time intervals preset with a Nikon Intervalometer, Model NC-1 (Nikon Inc.). Photographs were taken at intervals of 0, 3.9, 6.9, 9.0, 12.9, and 15.9 sec. All photographs were taken on Kodak Tri-X black and white film (Kodak, Rochester, NY) and developed in ACU-1 film developer diluted 1 to 5 with water (Acufine Inc., Chicago, Illinois) at 75°F for 8 min.

Enlargements were made in a Simmon Omega enlarger (Simmon Bros., Inc., Long Island City, NY) with the Kodak enlarging lens, Ektar F-45, 75 mm (Kodak, Rochester, NY) on Kodak Kodabromide F4 single-weight paper. The magnification of 3.5 times was maintained constant through all experiments. The enlargements were developed in Dektol developer, diluted 1 to 1 with water, (Kodak Inc.). All reagents for the enlarging process were maintained at a temperature of 68° $\pm$ 2° F. The prints were glossed in Flexogloss (Gaf, New York, NY) for 5 min and dried in a Lott Rotomaster Print Dryer (Lott Corp., Jamestown, NY).

Measurements of droplet length were taken directly from the photomicrographs with dividers and expressed in microns by comparison with a stage micrometer photographed and enlarged in the same manner. In these studies, the droplet length (h) was measured as the distance between the opposing meniscal

TABLE 1

MEASUREMENTS OF THE DROPLET LENGTH IN MICRONS AT THREE TIME PERIODS FOR FOUR SHRINKING DROPS

Shrinking drop	Initial length ($h(0)$)	$h(3.9)$ (μm)	$h(6.9)$ (μm)	$h(9.9)$ (μm)
1	90	72	51	39
2	63	48	36	24
3	72	60	42	36
4	63	51	39	30

surfaces. Measurements of the droplet lengths were performed without knowledge of the experimental situation.

RESULTS

The relationship between the initial droplet length and the reabsorptive half-time is shown graphically in Fig. 1. Fifty-four droplets from nine hydropenic animals are plotted as a scatter diagram. The regression line of the reabsorptive half-time versus initial droplet length [$h(0)$] is $t_{1/2} = (0.01)h(0) + 9.95$. The slope of the regression equation is 0.01 ± 0.04 (slope $\pm$ standard deviation of the slope). As demonstrated in this figure, within the range of 60–90 μm changes in the initial droplet length do not influence the determination of the $t_{1/2}$ value. In these experiments $t_{1/2}$ was determined from the relationship between the logarithm of the percentage of the initial droplet length remaining at each time interval and the time elapsed since the initial observation as discussed below.

Computer Calculations of the Reabsorptive Half-Times

Gertz suggested that in the rat there is a linear relationship between the logarithm of the volume remaining in the split droplet and time(1). From this formulation, the following is derived:

$$\ln \frac{h(t)}{h(0)} = -K \frac{2}{r} t, \tag{1}$$

expressed in terms of reabsorptive rate per unit length of tubule in mm^3/mm/sec, where $h(0)$ = the initial droplet length, $h(t)$ = the length of the oil droplet at time t, t = the time elapsed since the initial observation, r = the tubular radius, and K = the rate of flow through the tubular wall per unit surface area per unit time.

In order to estimate the slope, $-\frac{2}{r}K$, of the linear regression line in Eq (1), we use a weighted least-squares estimate where the standard deviation of the ob-

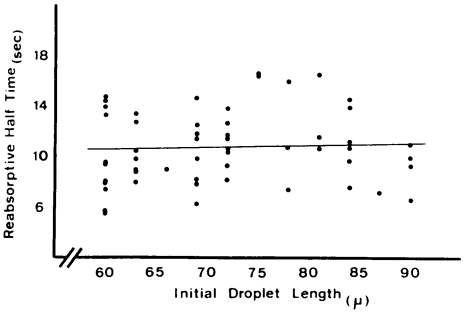

FIG. 1. A scattergram of 54 determinations of the reabsorptive half-time from nine hydropenic control rats. The reabsorptive half-time is plotted as a function of the initial droplet length. The regression line is not significantly different from zero.

servations at time t is proportional to t. Using this procedure, $(1/t)\ \ln[h(t)/h(0)]$ may be considered to be a measure of the slope; hence for each shrinking droplet

$$(1/p)\ \Sigma\ (1/t_i)\ \ln[h(t_i)/h(0)] = b \tag{2}$$

where b is an estimate of the slope, $t_i (i=1, \ldots\ldots, p)$ is the time elapsed since the initial observation, and p is the number of measurements of $h(t)/h(0)$ in the summation.

The average slope for a group of n shrinking-drops is

$$\text{average slope} = (1/n) \sum_{j=1}^{n} b_j \tag{3}$$

and the standard deviation of the average slope is given by

$$\left(\frac{1}{n(n-1)} \sum_{j=1}^{n} [b_j - \text{average slope}]^2 \right)^{1/2} \tag{4}$$

Table 1 gives the values observed in four illustrative shrinking drops in terms of the length of the saline droplet at time t = 0, 3.9, 6.9, and 9.9 sec. In Table 2 the slope of the regression line for each sequence of measurements is computed using Eq. (2).

The calculations were performed on an Olivetti Programma 101 programmed to determine the slope for each shrinking drop and the average slope for the group of droplets using Eq. (3). These computations can be made on any calculator capable of computing natural logarithms.

The $t_{1/2}$ is calculated using the average slope from the relationship

$$t_{1/2} = \frac{\ln(1/2)}{\text{average slope}} = \frac{-0.69315}{\text{average slope}}$$

DISCUSSION

The split-droplet micropuncture technique has been widely used as a method of examining the net efflux of tubular fluid from the proximal segment of the nephron. It is the only *in vivo* method available in which the transport function of the tubular epithelia can be examined in isolation from the influences of filtered load and the velocity of tubular flow. The accuracy and reproducibility of the method is dependent upon reducing the errors inherent in the method and in minimizing the error of observer bias in the determination of the rates of reabsorption under various physiological conditions. In an effort to reduce inherent sources of variability in comparing measurements made in different animals, the mechanical application of the method was standardized in the present study. Punctures were made "upstream toward the glomerulus" with a long proximal block to insure stability of the oil column and to negate the effects of the pressure created by glomerular filtration; only straight portions of the proximal tubules were used to reduce the influence of inapparent nephron tortuosity.

TABLE 2

CALCULATION OF SLOPE OF THE REGRESSION FOR THE FOUR SHRINKING DROPS GIVEN IN TABLE 1 SHOWING INTERMEDIATE COMPUTATIONS (EQ. 2) AND AVERAGE SLOPE FOR THE GROUP (EQ. 3)[a]

Shrinking drop	$t_1 = 3.9$ sec			$t_2 = 6.9$ sec			$t_3 = 9.9$ sec			Slope
	$\frac{h(3.9)}{h(0)}$	$\frac{1}{3.9}$	$\ln\left[\frac{h(3.9)}{h(0)}\right]$	$\frac{h(6.9)}{h(0)}$	$\frac{1}{6.9}$	$\ln\left[\frac{h(6.9)}{h(0)}\right]$	$\frac{h(9.9)}{h(0)}$	$\frac{1}{9.9}$	$\ln\left[\frac{h(9.9)}{h(0)}\right]$	
1	.80		−0.057	.57		−0.081	.43		−0.085	−0.075
2	.76		−0.070	.57		−0.081	.38		−0.098	−0.083
3	.83		−0.048	.58		−0.079	.50		−0.070	−0.066
4	.81		−0.054	.62		−0.069	.48		−0.074	−0.066

[a] Average slope $= \frac{1}{4}(-0.075 - 0.083 - 0.066 - 0.066) = -0.0725$.

In describing this technique, Gertz assumed that the rate of reabsorption remained constant with time throughout the entire period of shrinkage and was independent of the initial droplet length(1). Others have observed, however, that the initial droplet length does influence the estimation of the $t_{1/2}$ value and that the relationship between the logarithm of the percentage of the initial droplet length remaining and time becomes increasingly curvilinear after the first portion of the saline droplet is absorbed(2–5).

It should be noted that in reports evaluating these relationships, there has been significant variation in both the methodology and species of animal employed. Steinhausen showed, in the rat, that initial droplet lengths of 90 μm and longer had no influence on the $t_{1/2}$ while at droplet lengths less than 90 μm, the $t_{1/2}$ was progressively shortened(2). Levinsky and associates demonstrated, in rats, a linear relationship over a wide range of initial droplet lengths (50–150 μm) with considerable scatter of values(3). In an another report, Nakajima and coworkers studied the relationship between these variables in split-droplet micropuncture experiments in the dog(4). Within the range of 75–125 μm, no relationship between the initial droplet length and the $t_{1/2}$ was found, although an influence was demonstrated when lengths less than 75 μm and greater than 125 μm were compared. They noted, however, that if the total length is taken as the distance between the base of the meniscus of the oil column, a correlation between the $t_{1/2}$ and the initial droplet length was not observed. In a recent study Gyory(5) examined the relationship between droplet length and time and noted that it became increasingly linear when a correction factor was applied to the measured intrameniscal length. In his report a correction for the meniscus was made by adding the width of the tubule (the diameter) to the droplet length, measured as the distance between the oil menisci. From Gyory's data it may be noted that when a larger correction is applied (i.e., 4/3 times the tubular diameter), the correlation between the initial droplet and the $t_{1/2}$ was reduced even further.

The calculated $t_{1/2}$ for a shrinking droplet is an algebraic function of $h(t) + ad$, where $h(t)$ is the droplet length, (d) is the diameter of the droplet, and $a(a)$ is the value used for the correction of the meniscus. If (a) is taken to be zero, Eq. (2) may be expanded in the following manner:

$$b = \frac{1}{p} \Sigma \left(\frac{1}{t_i}\right) \ln \left(\frac{h(t_i)}{h(0)}\right) = \frac{1}{p} \Sigma \left(\frac{1}{t_i}\right) [\ln h(t_i) - \ln h(0)]$$

$$= \frac{1}{p} \Sigma \left(\frac{1}{t_i}\right) \ln h(t_i) - \ln h(0) \frac{1}{p} \Sigma t_i.$$

The slope (b) is, therefore, a linear function of the logarithm of the initial droplet length [ln $h(0)$]. Since the $t_{1/2}$ is a simple function of the slope, it is not surprising that $t_{1/2}$ is correlated with the initial droplet length. If the above computations are repeated with $h(t)$ replaced by $h(t) + ad$, the slope would then be a linear function of ln$[h(0) + ad]$. It can thus be expected that $t_{1/2}$ computed in this way would be correlated with $h(0) + ad$. Accordingly, the statistical tests

of significance on the correlations between the $t_{1/2}$ and the initial droplet length $h(0)$ presented in the Gyory paper are difficult to interpret since the correlations are made on comparisons between the initial droplet length and a function of the initial droplet length, namely, the $t_{1/2}$.

In addition to using a "correction factor" to compensate for differences in initial droplet length it is also possible to control this variable by standardizing the initial length of droplets examined. Under the conditions employed in the present study, no relationship between the reabsorptive half-time and the initial droplet length was observed when the initial droplet length was limited to the range of 60–90 μm, conveniently approximated as two to three times the width of the oil-filled tubule. The droplet length was measured as the distance between the opposing menisci and no correction was made for the area around the meniscus. Regardless of the possible influence of the initial droplet length over a wide range of values on the estimation of the reabsorptive half-time ($t_{1/2}$), use of a limited range of initial droplet lengths offers an alternative method of avoiding this possible source of variability. It is likely that use of other limited ranges of initial droplet length than employed in this study would serve equally well in this respect.

The mechanism responsible for the possible influence of the initial droplet length on $t_{1/2}$ is uncertain. One possible explanation is that the rate of reabsorption is not constant with time as originally suggested by Gertz(1). If the rate of reabsorption is not constant under the conditions of stopped flow but decreases proportionately with increasing initial droplet length (Appendix, Paragraph A), then the slope of the regression line becomes a decreasing function of the initial length. This implies that the $t_{1/2}$ would be longer for large initial lengths than for shorter initial lengths. Alternatively, a decrease in the rate of flow across the tubular wall may be an inherent property of the transport system under conditions of stopped flow (Appendix, Paragraph B). In this case the rate of change of K (the flow through the tubular wall) would be proportional to the initial length, and a larger change in K would be predicted with increasing initial droplet lengths associated with a prolongation of the $t_{1/2}$. When the rate of change is small, the linear relationship predicted by Gertz would be approached. Such an alteration in the rates of reabsorption may provide an explanation for the influence of the initial droplet length on the $t_{1/2}$ as well as an explanation for the observed curvilinear relationship between the remaining length and time.

Another source of variability and a potential source for subjective bias is the method of determining the rate of reabsorption from the measured droplet lengths. In earlier studies, the percentage of the initial droplet length remaining as time elapses was plotted as a semilogarithmic function of time from a linear regression line approximated by visual inspection. Recent workers have used a calculated least-squares regression line to determine the rates of reabsorption(5). In the present study, we have employed a "weighted" least-squares regression formula for calculating the $t_{1/2}$. With this type of analysis each measurement of the droplet length is weighted as an independent observation in the eventual

calculation of the $t_{1/2}$. The calculations of these linear regressions are readily adaptable to any desk-top computer capable of calculating natural logarithms. This method avoids both the errors associated with manual graphing and the potential errors of subjective bias.

Since the actual surface area available for reabsorption in the proximal tubule cannot be measured by current techniques, it is uncertain whether the use of the Gertz technique provides an accurate estimate of the net efflux of tubular fluid. It is surprising, however, that despite the probable underestimation of the surface area and the other possible sources of error inherent in the method, there is relatively good agreement in the determinations of the fractional reabsorption of tubular fluid in the proximal tubule calculated from the $t_{1/2}$ and the transit time and determination made from the TF/P inulin ratio at the end of the proximal tubule under free-flow conditions(8). The primary value of the Gertz technique, when employed under properly controlled conditions and calculated in the way suggested in this report, is in providing a reliable and accurate method for comparing alterations in net efflux due to the influence of different physiological conditions or the action of factors that effect the transport process. Since the mechanical application of this technique is known to be critically important, it is imperative that standardized methodology be adhered to by investigators using this form of analysis.

SUMMARY

Some of the methodologic factors known to influence the determination of the reabsorptive half-time ($t_{1/2}$) by the Gertz technique, as applied to the proximal tubule of the rat kidney, were examined in the present study. Within the range of initial droplet lengths of 60–90 μm, no influence of the initial droplet length on the $t_{1/2}$ was observed. This limited range of initial droplet lengths offers an alternative method of avoiding the influence of the initial droplet length on the $t_{1/2}$.

The formulas for a "weighted" least-squares regression line to determine the $t_{1/2}$ from the measured data are derived. If reliable and reproducible estimates of net fluid efflux from the proximal tubule are to be obtained by the Gertz technique, strict methodological criteria must be applied, known sources of variability standardized, and observer bias minimized.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mr. Richard Whittaker, the secretarial help of Mrs. Linda Frisco, and the advice and review of this manuscript of Dr. Franklin H. Epstein.

APPENDIX

Two formulations may be constructed that predict the influence of initial droplet length on the half-time of reabsorption. Both models are based on the premise that K, the rate of flow through the tubular wall, is not constant with time as is assumed under the model formulated by Gertz. K may change in proportion to the surface area which has already been used in the reabsorptive process or alternatively the change in K may be an inherent property of the reabsorptive pathway.

A. Suppose K changes in proportion to the surface area which has already been used in the reabsorptive process. Then, the reabsorptive rate at time t may be of the form $A - B[S(0) - S(t)]$, where B is the rate of decrease in the rate of reabsorption per unit of surface area which has already been used in the reabsorptive process. In this model the rate of change $h(t)$ with respect to t is given by

$$\frac{dh(t)}{dt} = -\{(2/r)A - 4\pi B[h(0) - h(t)]\}\, h(t). \tag{5}$$

The solution to the differential equation in (5) is obtained from the integral

$$\int \frac{dx}{x(a + bx)} = -\frac{1}{a} \ln \frac{a + bx}{x},$$

where $a = (2/r)A - 4\pi Bh(0)$ and $b = 4\pi B$.
The particular solution of interest in this problem is

$$\ln \left\{ \frac{h(t)}{h(0)} \left[\frac{1}{1 - 2\pi r(B/A)[h(0) - h(t)]} \right] \right\} = -[(2/r)A - 4\pi Bh(0)]t. \tag{6}$$

When B approaches zero the left side of Eq. (6) approximates $\ln[h(t)/h(0)]$ and the equation can be reduced to

$$\ln \frac{h(t)}{h(0)} \sim -[(2/r)A - 4\pi Bh(0)]t.$$

In this formulation the magnitude of the slope of the regression equation is a decreasing function of the initial length, $h(0)$. This implies that $t_{1/2}$ will be longer for a larger $h(0)$ than for a small $h(0)$.

B. Suppose the K changes with time, so that $K(t)$ denotes the rate of flow through the wall per unit surface area per unit time, at time t. Let $V(t)$ be the volume of the tubule occupied by the fluid between the two oil columns at time t and let $S(t)$ be the surface area of the tubule between the two columns of oil at time t. Following Gertz's reasoning we can draw an analogy between the enclosed space between the oil columns and a right cylinder. In this way relationships between $V(t)$, $S(t)$, and $h(t)$ are given by $V(t) = \pi r^2 h(t)$ and $S(t) = 2\pi r h(t)$. The rate of change of $V(t)$ as a function of t becomes

$$\frac{dV(t)}{dt} = -K(t)S(t). \tag{7}$$

Using the relationships between $V(t)$, $S(t)$, and $h(t)$ the differential equation in (7) has a general solution given by

$$\ln h(t) = -(2/r) \int_0^\tau K(\tau) d^t. \tag{8}$$

If $K(t)$ is constant for all values of t, then Eq. (8) reduces to Eq. (1) [see text] which is similar to the result computed by Gertz. Another choice for $K(t)$ which allows the rate of reabsorption to vary with t is $K(t) = Ae^{-ct}$. In this case Eq. (8) reduces to

$$\ln \frac{h(t)}{h(0)} = -(2/r)(A/c)(1 - e^{-ct}), \tag{9}$$

where A is the reabsorption rate at time $t = 0$ and c is the relative rate of change in the reabsorption rate. If c is positive and proportional to $h(0)$, a longer $t_{1/2}$ would be associated with large initial length. As long as c is small, however, Eq. (9) reduces to $\ln[h(t)/h(0)] = -(2/r)At$, which agrees with Gertz's formulation.

The change in the rate of reabsorption with time may also provide an explanation for the progressively curvilinear character of $\ln[h(t)/h(0)]$ with increasing time.

REFERENCES

1. Gertz, K. H. Transtubulare Natriumchloridflüsse und Permeabilität fur Nichtelekrolyte im proximalen und distalen Konvolut det Rattenniere. *Arch. Gesamte Physiol.* **276,** 336–356 (1963).
2. Steinhausen, M., Messungen des tubulare Harnstromes und der tubularen Reabsorption unter erhöhtem Ureterdruck. *Arch. Gesamte Physiol.* **296,** 105–130 (1967).
3. Levinsky, N. G., Lalone, R. C., and Arbus, G. S. The split-drop method and PAH uptake by kidney slices as assay techniques. *in* Regulation of Body Fluid Volumes by the Kidney, pp. 56–66. S. Karger, Basel, Switzerland, 1970.
4. Nakajima, K., Clapp, J. R., and Robinson, R. R. Limitations of the shrinking-drop micropuncture technique. *Amer. J. Physiol.* **219,** 345–357 (1970).
5. Györy, A. Z. Reexamination of the split-droplet method as applied to kidney tubules. *Arch. Gesamte Physiol.* **324,** 328–343 (1971).
6. Weinman, E. J., Kashgarian, M., and Hayslett, J. P. Role of peritubular protein concentration in sodium reabsorption. *Amer. J. Physiol.* **221,** 1251–1258 (1971).
7. Hayslett, J. P., Kashgarian, M., and Epstein, F. H. Changes in proximal and distal tubular reabsorption produced by rapid expansion of the extracellular fluid. *J. Clin. Invest.* **46,** 1254–1263 (1967).
8. Weiner, M. W., Weinman, E. J., Kashgarian, M., and Hayslett, J. P. Accelerated reabsorption in the proximal tubule produced by volume depletion. *J. Clin. Invest.* **50,** 1379–1385 (1971).