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Effects of intra-animal nephron heterogeneity on studies of glomerular dynamics

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Effects of intra-animal nephron heterogeneity on studies of glomerular dynamics. Quintuplicate determinations of the parameters measured in studies of glomerular dynamics revealed that the intra-animal coefficients of variation for Bowman's space and star vessel pressures, nephron filtration rate, and filtration fractions were 54 to 72% larger than the corresponding interanimal coefficients of variation; those for glomerular capillary pressure were more nearly equal. With a net efferent filtration pressure (ΔP_E) of 10.6 ± SEM 1.9 mm Hg, the rats were far from filtration pressure equilibrium and the calculated ultrafiltration coefficient (K_f) of 2.1 ± SEM 0.2 nl/min · mm Hg was lower than in many other studies. Statistical analysis revealed that the precision of estimates of both the measured and the derived parameters in glomerular dynamic studies is affected appreciably by ignoring the intra-animal effect. The importance of the intra-animal variance in glomerular dynamic studies is greatest when only one or two samples of each measured parameter are obtained in every rat (k = 1 or 2) and least when k is large. Triplicate sampling provides combined SEMs that are not greatly larger than those obtained with k = 5, however, and offers the greatest economy in studies of glomerular dynamics. The number of animals required to provide values with ΔP_E and K_f that are within ± 20% of the "true" values is rather large.

Effets de l'hétérogénéité néphronique intra-animale sur les études de la dynamique glomérulaire. Des déterminations en quintuple des paramètres mesurés dans les études de la dynamique glomérulaire ont révélé que les coefficients de variation intra-animale pour les pressions dans l'espace de Bowman et les vaisseaux étoilés, le débit de filtration glomérulaire et les fractions de filtration étaient 54 à 74% plus grandes que les coefficients de variation inter-animale correspondants; ceux de la pression capillaire glomérulaire étaient plus proches. Avec une pression de filtration effèrente nette (ΔP_E) de 10,6 ± SEM 1,9 mm Hg, les rats étaient loin d'une pression de filtration en équilibre, et le coefficient d'ultrafiltration calculé (K_f) de 2,1 ± SEM 0,2 nl/min · mm Hg était plus faible que lors de nombreuses autres études. L'analyse statistique a révélé que la précision des paramètres mesurés et déduits des études de la dynamique glomérulaire est affectée de façon appréciable en ignorant l'effet intra-animal. L'importance de la variance intra-animale dans les études de la dynamique glomérulaire est très grande lorsque seulement un ou deux échantillons de chaque paramètre mesuré sont obtenus chez chaque rat (k = 1 ou 2), et moindre lorsque k est élevé. Un échantillonage triple offre cependant des SEM combinés qui ne sont pas beaucoup plus grands que ceux obtenus pour k = 5, cependant, et offre la plus forte économie lors des mesures de la dynamique glomérulaire. Le nombre d'animaux nécessaires pour obtenir des valeurs avec Δ_E et K_f entre ± 20% des valeurs "vraies" est assez grand.

Studies of glomerular dynamics entail measurements of glomerular capillary (P_g), star vessel (P_{star}), and proximal tubule or Bowman's space (P_{BS}) pressures together with the single nephron filtration fraction (SNFF) and filtration rate (SNGFR). Because of technical restrictions, it has been customary to measure each parameter in a different nephron within a given kidney, the mean parameter value being derived from a limited number of outer cortical nephrons with the supposition that those sampled will provide reasonably representative values for the superficial nephron population as a whole. The precision of such estimates in each of a series of animals, however, depends on the degree of intrarenal variation of the several measured parameters and on the number of sampled nephrons included in the mean. The present study was undertaken to determine the degree of internephron heterogeneity in the various measured parameters and to assess the impact of such heterogeneity on estimates of pre- (R_A) and postglomerular (R_E) resistances, net- (ΔP_{net}) and end-capillary (ΔP_E) effective filtration pressures, afferent (GBF_A), and efferent (GBF_E) glomerular blood flows and the ultrafiltration coefficient (K_f) in studies of the glomerular dynamics.

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Methods

Studies were performed on female Munich-Wistar rats weighing 167 to 183 g. Rats A to E were obtained from TIMCO, (Houston, Texas, USA) while rats F to K were purchased from Simonsen Labs (Gilroy, California, USA). Neither strain showed evidence of spontaneous hydronephrosis or other renal anomaly. The animals had free access to rat chow (Purina®, Ralston Purina, St. Louis, Missouri, USA) and water until anesthetized for study with sodium pentobarbital, 50 mg/kg body wt i.p. A PE10 femoral arterial catheter was placed for constant monitoring of blood pressure, a tracheostomy tube was inserted, and a femoral vein was cannulated for the infusion of fluids. The left kidney was exposed through an abdominal incision, placed in a holder (Lucite®) and covered with a layer of moistened paper tissue. Cool setting, 1% agarose (LSA, Litex, Denmark) in 140 mM saline was poured over the kidney at 37 to 38°C to minimize respiratory and pulsatile movement during micropuncture. When set, the agarose and the tissue paper layer on the kidney surface were removed leaving the rest of the kidney surrounded by agarose. The kidney surface was covered with 140 mM saline warmed to 37°C and 1 ml of isotonic saline was infused intravenously.

Hydrostatic pressures were measured with a servonull capacitance device (David Smith, Chapel Hill, North Carolina, USA)

using 1 to 2 μ O.D. micropipettes filled with 2 M NaCl, their tips platinized [1] to permit visualization on insertion into the kidney. (In vitro testing established that platinization does not influence the pressure values obtained). The pipette holders were attached to a micromanipulator (Leitz) by way of a stepping microdrive (DKI, Tujunga, California, USA) that permitted adjustment of the pipette tip by remote triggering, micron by micron. Pipette insertion was performed at ×128 to 320 magnification. Placement of the pipette tip was aided by the tone wave of an auditory voltage-controlled oscillator, driven by the recorder's output, which obviated the need for visual observation of the recorder until a reasonably stable oscillation pattern was heard. P_{BS} was measured as the pressure within Bowman's space that remained constant over a 30-sec period or longer. The electronically derived mean pressure was recorded to the nearest 0.5 mm Hg. The pipette was then advanced until its tip was seen to reach the glomerular tuft and adjusted until a promising or stable pulsatile recording of glomerular capillary pressure (P_g) was obtained. Final adjustment was achieved with the stepping microdrive. Pressures were accepted only if the wave form was clean and uniform and, complex by complex, remained stable for at least 1 min. Mean values were read to the nearest 1 mm Hg. The experiment was aborted if four or more acceptable P_g measurements were not obtained within 45 min. P_{star} was measured to the nearest 0.5 mm Hg in five star vessels of each rat. Measurements of both SNGFR and SNFF could be completed usually within the succeeding 70 min; only rats C and H required an additional 3 and 7 min, respectively.

Single nephron filtration rate (SNGFR) was measured with carbon 14-labeled inulin (New England Nuclear Corp., Cambridge, Massachusetts, USA) as described in detail elsewhere [2]. The rats received a priming dose of 40 μCi of inulin in 1 ml 140 mM NaCl; plasma inulin activity was maintained by continuous infusion of the same solution at 0.0375 ml/min. Five measurements of SNGFR were obtained in each rat.

After SNGFR measurements were completed, the inulin infusion was replaced by saline alone. ^{125}I-labeled crystalline bovine serum albumin (CBSA) was prepared and injected for estimates of SNFF, as described previously [3]. ^{125}I-CBSA (5 to 10 μl, ~100 μCi) was added to 1 ml of plasma drawn from a donor animal, mixed and injected intra-arterially into each rat. Heparin, 100 IU, was injected intravenously in 0.5 ml 140 mM saline. Star vessel blood collections were begun approximately 15 min later using 12 to 14 μ O.D. acid-washed, siliconized glass micropipettes filled with silicone oil (Dow 200, Contour Chemical Co., Woburn, Massachusetts, USA). A droplet of silicone was injected before blood collection to assure proper placement of the pipet, and collections were made at a rate that permitted an ongoing flow of blood into the radiating capillaries to avoid the possibility of retrograde collection. The handling of star vessel and arterial blood samples for the estimation of filtration fraction were as described in detail previously [3]. Quintuplicate measurements of ^{125}I activity in individual samples of arterial blood in a previous study provided a mean coefficient of variation of 2.2 SEM ± 0.3% [4].

The colloid osmotic pressure (COP_A) of duplicate 15 to 25 μl arterial plasma samples was measured by direct osmometry (Instrumentation for Physiology and Medicine, Model 3A) as described elsewhere [4]. Paired values for COP rarely differed by greater than 0.5 cm H_2O (0.37 mm Hg); the mean difference in paired values was 0.2 mm Hg. Efferent arteriolar oncotic pressure was determined from COP_A and SNFF according to the equation [4]:

$$COP_E = COP_A\,(\overline{I_E^*/I_A^*})^{1.475}$$

where I* is the ^{125}I activity in arterial (A) and star vessel plasma (E), respectively. The equation provides results indistinguishable [4] from those predicted by the Landis and Pappenheimer equations [5] relating protein concentration to COP.

Calculations

Single nephron glomerular filtration rate (SNGFR) was determined as:

$$SNGFR = (TF/P)_{In} \cdot V \qquad (1)$$

where $(TF/P)_{In}$ is the tubule fluid:plasma inulin concentration ratio and V is the tubule fluid collection rate.

Single nephron filtration fraction (SNFF) was determined as:

$$SNFF = 1 - \overline{I_A^*/I_E^*} \qquad (2)$$

Afferent arteriolar plasma flow (GPF_A) for each rat was estimated from mean filtration rate ($\overline{SNGFR}$) and mean filtration fraction ($1 - \overline{I_A^*/I_E^*}$) as:

$$GPF_A = \overline{SNGFR/SNFF} = \overline{SNGFR}\,/(1 - \overline{I_A^*/I_E^*}) \qquad (3)$$

and afferent glomerular blood flow rate was calculated as:

$$GBF_A = GPF_A/(1 - \overline{Hct_A})$$
$$= \overline{SNGFR}\,/\,(1 - \overline{I_A^*/I_E^*})\,(1 - \overline{Hct_A}) \qquad (4)$$

where $\overline{Hct_A}$ is the mean hematocrit value of arterial blood which was assumed to be equal to that of blood entering the afferent arteriole.

Efferent arteriolar blood flow rate of individual animals was determined as:

$$GBF_E = GBF_A - \overline{SNGFR}$$
$$= \overline{SNGFR}/[(1 - (1 - \overline{I_A^*/I_E^*})\,(1 - \overline{Hct_A}) - \overline{SNGFR}] \qquad (5)$$

Preglomerular resistance, R_A, the sum of all renal resistances proximal to the glomerular capillary and expressed as millimeters of mercury, minutes per nanoliters, was derived from:

$$R_A = (MAP - \overline{P}_g)/GBF_A$$
$$= (\overline{MAP} - \overline{P}_g)\,(1 - \overline{I_A^*/I_E^*})\,(1 - \overline{Hct_A})/\overline{SNGFR} \qquad (6)$$

where $\overline{P}_g$ is the mean of glomerular capillary hydrostatic pressure values for that rat. Values were expressed $\times 10^{10}$ dyne sec cm^{-5} when multiplied by 7.982.

Efferent arteriolar resistance, R_E, was estimated from:

$$R_E = (\overline{P}_g - \overline{P}_{star})/GBF_E$$
$$= (\overline{P}_g - \overline{P}_{star})/\overline{SNGFR}\,[(1 - \overline{I_A^*/I_E^*})^{-1}\,(1 - \overline{Hct_A})^{-1} - 1] \qquad (7)$$

where $\overline{P}_{star}$ is the mean of star vessel hydrostatic pressure values. The data obtained here showed a large residual net efferent filtration pressure (see **Results**). The axial profile of net pressure change in the capillary was found by network modeling [6] to be essentially linear, as also found in the study of

Arendshorst and Gottschalk [7]. Accordingly, the ultrafiltration coefficient, K_f was estimated for each animal as:

$$K_f = \overline{SNGFR}/\overline{\Delta P}_{net}$$
$$= \overline{SNGFR}/[\overline{P}_g - \overline{P}_{BS} - 0.5\,(\overline{COP}_A + \overline{COP}_E)] \quad (8)$$

where $\overline{COP}$ is the intra-animal mean of plasma oncotic pressure, $\overline{\Delta P}_{net}$ is mean net effective filtration pressure and $\overline{P}_{BS}$ is the mean of hydrostatic pressure values in Bowman's space.

ΔP_E, efferent capillary net filtration pressure, was calculated as:

$$\Delta P_E = \overline{P}_g - \overline{P}_{BS} - \overline{COP}_E \quad (9)$$

The simplified equations shown above were used for determination of interanimal means and variances. The expanded equations (that is, those using the constituent elements measured in glomerular dynamic studies) were required for estimates of intra-animal variances in ΔP_{net}, ΔP_E, K_f, GBF_A, GBF_E, R_A, and R_E with regard to the uncertainty in the measured means (see below).

Statistical analysis

With S_i^2 denoting the intra-animal variance of any of the measured parameters P_g, P_{star}, P_{BS}, SNGFR, and SNFF, the intra-animal variance, V_i, for the derived parameter means was established from the following equations whose justification is given in the **Appendix**:

$$V(\Delta P_E) = S_{P_g}^2 + S_{P_{BS}}^2 + S_{P_{star}}^2 \quad (10)$$

$$V(\Delta P_{net}) = S_{P_g}^2 + S_{P_{BS}}^2 + 0.25S_{COP_A}^2 + 0.25S_{COP_E}^2 \quad (11)$$

$$V(K_f) = [\overline{GFR}/(\overline{P}_g - \overline{P}_{BS} - 0.5\overline{COP}_A - 0.5\overline{COP}_E)]^2 \quad (12)$$
$$\cdot [S_{GFR}^2/\overline{GFR}^2 + V(\Delta P_{net})/(\overline{P}_g - \overline{P}_{BS} - 0.5\overline{COP}_A - 0.5\overline{COP}_E)]^2$$

$$V(GPF_A) = [(\overline{GFR} \cdot \overline{C}_E)/(\overline{C}_E - \overline{C}_A)]^2 \cdot [(\overline{GFR}^2 \cdot S_{C_E}^2 \quad (13)$$
$$+ \overline{C}_E^2 \cdot S_{GFR}^2 + S_{GFR}^2 \cdot S_{C_E}^2)/(\overline{GFR} \cdot \overline{C}_E)^2$$
$$+ (S_{C_E}^2 + S_{C_A}^2)/(\overline{C}_E - \overline{C}_A)^2 - 2\,\overline{GFR} \cdot S_{C_E}^2/\overline{GFR} \cdot \overline{C}_E(\overline{C}_E - \overline{C}_A)]$$

$$V(GBF_A) = [(\overline{GFR} \cdot \overline{C}_E)/(\overline{C}_E - \overline{C}_A)(1 - \overline{Hct}_A)]^2 \quad (14)$$
$$\cdot [V(\overline{GPF}_A)(\overline{C}_E - \overline{C}_A)^2/(\overline{GFR} \cdot \overline{C}_E)^2 + S_{Hct}^2/(1 - \overline{Hct}_A)^2]$$

$$V(GBF_E) = V(GBF_A) + S_{GFR}^2 - 2S_{GFR}^2[\overline{C}_E/(\overline{C}_E - \overline{C}_A) \quad (15)$$
$$(1 - \overline{Hct}_A) - S_{C_E}^2(1 - \overline{Hct}_A)/((\overline{C}_E - \overline{C}_A)(1 - \overline{Hct}_A))^2$$
$$+ \{(\overline{C}_E - \overline{C}_A)^2 \cdot S_{Hct}^2 + ((1 - \overline{Hct}_A)^2 + S_{Hct}^2))(S_{C_E}^2 + S_{C_A}^2)\}$$
$$\cdot \overline{C}_E\,((\overline{C}_E - \overline{C}_A)(1 - \overline{Hct}_A))^3]$$

$$V(R_A) = [(\overline{MAP} - \overline{P}_g)/GBF_A]^2 \quad (16)$$
$$\cdot [(S_{MAP}^2 + S_{P_g}^2)/(\overline{MAP} - \overline{P}_g)^2 + V(GBF_A)/GBF_A^2]$$

$$V(R_E) = [(\overline{P}_g - \overline{P}_{star})/GBF_E]^2 \quad (17)$$
$$\cdot [(S_{P_g}^2 + S_{P_{star}}^2)/(\overline{P}_g - \overline{P}_{star})^2 + V(GBF_E)/GBF_E^2]$$

(The protein concentrations $\overline{C}_A$ and $\overline{C}_E$ in Eqs. 13 to 15 are calculated from $\overline{COP}_A$ and $\overline{COP}_E$ using the Landis and Pappenheimer equation [5] and are presented in this fashion to be of use to other investigators who determine filtration directly from C_A and C_E.)

Representing the ith intra-animal variance calculated from the formulas above for a particular parameter by $V(A_i)$, the pooled intra-animal variance, S_w^2, for n animals is:

$$S_w^2 = [V(A_1)(k_1 - 1) + V(A_2)(k_2 - 1) \ldots \quad (18)$$
$$+ V(A_n)(k_n - 1)]/(N - n)$$

where k is the number of samples per rat, n is the number of rats in the series and $N = \Sigma k$. Where k is constant:

$$S_w^2 = [(V(A_1) + V(A_2) + \ldots V(A_n)]/n \quad (19)$$

The overall variance of the estimate, S_T^2, which incorporates both the inter-animal variance, S_A^2, and the mean of intra-animal variances for the parameter in a series of rats is expressed as [8]:

$$S_T^2 = S_A^2 + S_w^2/k)/n \quad (20)$$

Because each parameter was estimated from the mean value of n animals, the central limit theory applies and the distribution of the estimated value is approximated reasonably by a normal distribution with the above variance, S_T^2. Thus, the 95% confidence interval for a parameter was determined using the mean of n animal values as the mean value ± 1.95 S_T. To be 95% confident that the mean value determined from a series of n animals is within ± d unit of the true parameter value, n and k must be sufficiently large so that S_T^2 is $\leq (d/1.96)^2$. Hence, the minimal sample size, n, required for a given k to reflect the calculated mean value within ± d unit of the true value with 95% confidence was approximated by rearrangement as:

$$n = 3.84\,(S_A^2 + S_w^2/k)/d^2 \quad (21)$$

Means are presented with the standard deviation (SD) to provide estimates of the variation in the population or with the standard error of the mean (SEM) as a measure of the precision of estimates.

Analyses were performed on a computer (IBM 370).

Results

The individual mean values ± SD of SNGFR, P_g, P_{BS}, and P_{star}, generally measured in quintuplicate (but with only four measurements of P_g in three animals), are shown in Table 1. The overall means of intra-animal mean values ($N = 11$) were: SNGFR 29.7 ± SEM 0.6 nl/min; SNFF, 0.288 ± 0.010; P_g, 49.2 ± 1.1 mm Hg; P_{BS}, 12.0 ± 0.2 mm Hg; and P_{star}, 12.6 ± 0.2 mm Hg. No differences in measured mean values were found between Timco and Simonsen rats ($P > 0.2$ or higher for all but SNGFR, $P > 0.05$). They are thus considered a single population for statistical purposes. Duplicate measurements of P_g in nine glomeruli differed, on average, by 1.8 ± SEM 0.4 mm Hg, a value significantly different from 0 ($P < 0.001$). No correlation was found between the mean SNGFR values of the 11 animals and $\overline{P}_g$, $\overline{P}_{star}$, or $\overline{P}_{BS}$ ($P > 0.2$ or higher). $\overline{P}_g$ for the individual rats did not correlate with $\overline{P}_{BS}$ or $\overline{P}_{star}$ ($P > 0.4$). The intra-animal coefficients of variation (SD/mean) for SNFF and SNGFR averaged 0.188 ± SEM 0.013 and 0.119 ± 0.010, respectively ($N = 11$). P_g, P_{BS}, and P_{star} showed rather less intra-animal variability, their coefficients of variation averaging 0.069 ± SEM 0.007, 0.087 ± 0.008 and 0.091 ± 0.006, respectively. The interanimal coefficients of variation for these same parameters were: P_g 0.072; P_{BS}, 0.051; P_{star}, 0.054; SNGFR,

Table 1. Intra-animal means and SDs of measured parameters in individual rats

Rat	MAP	COP_A	COP_E	P_g *mm Hg*	P_{BS}	P_{star}	SNGFR *nl/min*	SNFF
A	126	13.3 ± 1.6	22.7 ± 2.8	54.0 ± 2.9	11.6 ± 1.2	12.5 ± 1.0	29.6 ± 2.7	0.30 ± 0.04
B	114	15.4 ± 1.0	24.1 ± 2.5	56.0 ± 2.7	12.6 ± 0.8	12.6 ± 0.8	30.1 ± 2.5	0.26 ± 0.06
C	119	15.2 ± 0.6	31.9 ± 3.4	46.0 ± 2.1	13.0 ± 1.5	13.6 ± 1.5	33.7 ± 3.5	0.34 ± 0.04
D	111	15.3 ± 0.2	26.4 ± 4.5	47.5 ± 3.9	12.2 ± 1.2	12.9 ± 1.4	31.0 ± 4.7	0.27 ± 0.05
E	117	15.3 ± 0.5	27.2 ± 3.2	45.0 ± 4.5	12.0 ± 1.3	13.2 ± 1.4	30.5 ± 2.7	0.32 ± 0.05
F	122	16.1 ± 0.5	29.9 ± 3.2	47.0 ± 4.8	12.6 ± 1.6	12.5 ± 0.8	30.1 ± 2.8	0.32 ± 0.07
G	115	16.2 ± 0.5	28.7 ± 4.9	46.4 ± 4.5	11.7 ± 0.9	13.2 ± 1.5	27.6 ± 4.5	0.31 ± 0.07
H	118	17.2 ± 0.8	25.6 ± 2.2	50.2 ± 2.3	10.8 ± 0.6	11.9 ± 0.9	27.3 ± 3.4	0.24 ± 0.05
I	123	14.7 ± 0.8	23.4 ± 2.1	49.8 ± 3.1	12.0 ± 1.2	12.5 ± 1.4	29.0 ± 3.0	0.26 ± 0.05
J	111	18.2 ± 0.7	28.4 ± 3.5	47.5 ± 3.1	11.5 ± 0.4	11.1 ± 1.1	31.5 ± 5.3	0.27 ± 0.05
K	112	14.4 ± 1.3	25.0 ± 5.0	45.3 ± 3.0	12.4 ± 1.0	12.9 ± 1.0	26.4 ± 3.7	0.29 ± 0.06
Mean ± SD of individual means (N = 11)		15.6 ± 1.4	26.7 ± 2.9	49.2 ± 3.6	12.0 ± 0.6	12.6 ± 0.9	29.7 ± 2.1	0.29 ± 0.03

Abbreviations: MAP, mean arterial pressure; COP_A, colloid osmotic pressure; COP_E, efferent arteriolar oncotic pressure; P_g, glomerular capillary pressure; P_{BS}, pressure of Bowman's space; P_{star}, pressure of star vessel; SNGFR, single nephron glomerular filtration rate; SNFF, single nephron filtration fraction.

Table 2. Influence of the number of determinations of each of the measured parameters per rat on SEs of mean values

	COP_A	COP_E	P_g	P_{BS}	P_{star}	SNGFR	SNFF
			mm Hg			*nl/min*	
SEM of individual Means (±)	0.44	0.86	1.07	0.19	0.21	0.64	0.010
Combined SEM (±)							
k = 5	0.43	0.98	1.17	0.24	0.26	0.80	0.012
k[a] = 10	0.43	0.93	1.12	0.22	0.24	0.71	0.011
k[a] = 3	0.45	1.06	1.23	0.27	0.29	0.90	0.014
k[a] = 1	0.49	1.37	1.27	0.39	0.42	1.27	0.019

Abbreviations: COP_A, colloid osmotic pressure; COP_E, efferent arteriolar oncotic pressure; P_g, glomerular capillary pressure; P_{BS}, pressure of Bowman's space; P_{star}, pressure of star vessel; SNGFR, single nephron glomerular filtration rate; SNFF, single nephron filtration fraction.
[a] The value was estimated assuming 10, 3, or 1 samples (k) of each parameter taken in the same series of 11 rats with the intra-animal and interanimal variances obtained with k = 5.

0.072; and SNFF 0.111, values which, with the exception of P_g, are significantly lower than the corresponding intra-animal coefficients of variation ($P < 0.001$). The intra- and interanimal coefficients of variation in both P_g and COP_E were not significantly different ($P > 0.2$). The mean inter- and intra-animal coefficients of variation for the several measured parameters were not different in the two rat substrains ($P > 0.10$ or higher).

The interanimal and combined SEMs of the measured parameters (the latter obtained from Eq. *20* of **Methods**) are shown in Table 2 together with the combined SEM values expected assuming the same degree of internephron heterogeneity if only 3 (k = 3), 10 (k = 10), or a single sample (k = 1) had been obtained in each rat. With k = 1, the combined SEMs for P_{BS}, P_{star}, SNGFR, and SNFF are ~ 60 percent larger than when k = 5 and approximately twice the interanimal SEM. When k = 3, the SEMs are only some 5 to 17% higher than when k = 5 whose values, in turn, are some 4 to 10% higher than with k = 10. Although only 11 and 16 animals, respectively, suffice to provide a mean that is within ± 20% of a "true" mean for ΔP_{net} and K_f at the stated confidence level with k = 5, 38 rats are required to obtain this degree of precision for estimates of ΔP_E. Fifty-five, 16, and 28 animals would be needed to provide means for ΔP, ΔP_{net}, and K_f within ± 20% of their "true" values at the 95% confidence level when k = 1. By contrast, the numbers of rats required with k = 3 are not greatly different from those needed to provide the same degree of precision with k = 5.

As a corollary and again using Eq. *21* of **Methods**, five samples of each measured parameter in 11 rats were found to provide 95% confidence intervals for R_A, R_E, GBF_A, GBF_E, and GPF_A within ± 11% of their respective means. The confidence intervals for $\overline{\Delta P}_{net}$, $\overline{\Delta P}_E$, and $\overline{K}_f$, by contrast, were far wider (± 20, ± 37, and ± 24% of their means, respectively.) Had a smaller number of primary measurements been taken in this same series of animals, it is estimated that the 95% confidence interval for the estimated values would be widened even more. At k = 1 and N = 11, for example, the 95% confidence limits for $\overline{K}_f$, $\overline{\Delta P}_E$, and $\overline{\Delta P}_{net}$ are estimated at ± 31.6, ± 44.6, and ± 24.1% of the mean while those for blood and plasma flows and individual vascular resistances would range from ± 15.8 to ± 18% about the mean.

Discussion

The glomerular ultrafiltration coefficient (K_f), Bowman's space pressure (P_{BS}) and the pre- (R_A) and postglomerular (R_E) resistances constitute the only established independent intrare-

nal determinants of glomerular filtration. With P_{BS} and the extrarenal determinants of filtration held constant, any perturbation which changes SNGFR must do so by its effects on R_A, R_E, or K_f. Unfortunately, these three cardinal parameters cannot be measured directly but instead are estimated from the measured values for SNGFR, SNFF, P_g, P_{BS}, and P_{star} which, together with measurements of colloid osmotic pressure, also provide for the determination of mean (ΔP_{net}) and end capillary (ΔP_E) net filtration pressure and glomerular blood flow (GBF_A). As may be seen from Eqs. 5 to 7 of **Methods**, some of the derived estimates are calculated from measurements of five or more independent parameters. The precision and accuracy of the means of these measured and derived terms in a series of rats are influenced by both the intra- and the interanimal variances although, to date, little attention has been paid to the influence of intra-animal functional heterogeneity on such calculations. The present study thus was udnertaken to assess the degree of internephron heterogeneity in the measured parameters of glomerular dynamics in two substrains of Munich-Wistar rats and to determine the effect of that heterogeneity on subsequent estimates of the derived parameters.

The primary data obtained here are quite similar to those reported by Arendshorst and Gottschalk [7] for euvolemic rats and by Tucker and Blantz [9] using hydropenic rats. Our mean value for P_g is somewhat higher and SNFF is rather lower than those found in a number of studies of hydropenic rats (for example, [10, 11]) however. With MAP, P_{star}, P_{BS}, and SNGFR values essentially the same as in those reports, the differences in P_g, SNFF, and the correspondingly lower COP_E translate into comparable departures in GPF_A, GBF_A, GBF_E, R_A, and R_E. The mean K_f is notably lower than that in many (for example, [12]), but not all [7, 13], reports in the literature. As found in the study of Arendshorst and Gottschalk [7], a large residual net filtration pressure left the animals far from filtration pressure equilibrium. How much these differences between studies relate to sample size, animal preparation, technical considerations, or the use of separate Munich-Wistar substrains is unclear. So-called "Boston" rats were reported by Arendshorst and Gottschalk [7] to differ in several respects from their "Chapel Hill" colony, but the two substrains examined here exhibited no statistically significant differences either in means or in the degree of functional heterogeneity of any parameter. It may be noted, however, that we are comparing only small numbers of rats in each substrain. The degree of internephron heterogeneity in SNGFR and SNFF found here in TIMCO rats was distinctly less than that found by us in the same substrain in a previous study (mean intra-animal coefficients of variation 0.20 and 0.25, respectively [3]). Many published reports have made no mention of the number of measurements (except for SNGFR) obtained in each rat, making the possible contribution of differing sample size hard to assess. Those studies that do provide two or three values (and sometimes more) of one or more measured parameters per rat [7, 9, 14] do, however, show heterogeneity that is comparable to or greater than that which we have found here. We are not aware of any other study that provides five or more measurements of all the remaining parameters but, obtaining three to five measurements in each of five normal rats, Blantz [14] recorded a mean intra-animal coefficient of variation for P_g of 0.11 that is quite similar to that of 0.07 ± SEM 0.007 determined here. With relatively small coefficients of variation for P_{BS} and P_{star}, it seems that the degree of internephron heterogeneity found in each of the measured terms in the present study, although substantial, is not atypically large. It is noteworthy, therefore, that the mean intra-animal CVs for P_{BS}, P_{star}, SNGFR, and SNFF obtained with five measurements per rat were some 54 to 72% higher than the corresponding interanimal coefficients of variation (CV) while those for P_g and COP_E were more nearly equal to the interanimal CVs. Intra-animal variation in the several measured parameters thus was of a magnitude that would exert a distinct influence on the precision of estimates of means although it has been customary in the past to derive means and SEs without regard to the effect of intra-animal functional heterogeneity.

In small series of rats, means of values should show less variability than single samples taken from the same animals since, under the latter circumstance, extreme values may be sampled purely by chance. In the presence of appreciable variability of values within and between different rats, the overall variance for a series of measurements is given by Eq. *20* of **Methods**. According to that equation, the combined variance (S_T^2) of a parameter mean is twice as large as the interanimal variance (S_A^2) when the intra-animal (S_W^2) and interanimal variances are equal and only a single sample of a parameter is measured in each rat (that is, $S_T^2 = 2S_A^2$). The variance of that parameter mean would then be underestimated by one-half and the SD (that is, $\sqrt{S_T^2}$) underestimated by some 30% if determined without concern for the intra-animal variance. Similarly, with $S_A^2 = S_W^2$, the combined variance for a parameter would be underestimated by 25 and 17%, respectively, even with triplicate and quintuplicate sampling when the term in S_W^2 is neglected. In the present study where the intra-animal variance of every measured parameter (usually based on five measurements) was equal to or larger than the interanimal variance, the estimates of SD of SNGFR, SNFF, P_{star}, P_{BS}, and P_g are indeed 22, 25, 27, 29, and 11% higher, respectively, than those calculated with the intra-animal variance ignored.

Just as internephron heterogeneity affects the precision of estimates for each of the measured parameters, so must it also influence estimates of individual vascular resistances, the ultrafiltration coefficient, net filtration pressures, and blood or plasma flow rates that are derived from those same values. GPF_A, for example, is calculated as: $GPF_A = \overline{SNGFR}/\overline{SNFF}$. With a high degree of internephron functional heterogeneity, however, mean values for SNGFR and SNFF obtained from a relatively small sample in a given rat cannot be considered exact. Instead, the "true" means of both these parameters are presumed to stand somewhere within the limits of ±1.96 SEM of the measured means, a range which in this and other studies is quite wide. Fortunately, the uncertainty in the term $\overline{SNGFR}/\overline{SNFF}$ in a given rat can be accommodated in the estimated variance (V_{GPF_A}) of the quotient as given in Eq. *13* of **Methods** and the SEM of the plasma flow estimate then can be obtained as $\sqrt{V_{GPF_A}/n}$. (The SEM in this case provides a gauge of the precision of the estimate for each rat). Based on five measurements of the two constituent parameters in each of 11 rats, the mean ± interanimal SEM for GPF_A was found in this study to be 104 ± 3.3 nl/min. The mean intra-animal SEM, however, was ± 7.7 nl/min and the combined SEM, therefore, was ± 4.8 nl/min (determined from Eq. *20* of **Methods**). The interanimal SEMs for

Table 3. Interanimal means ± SEM and the combined inter- and intra-animal SEM of estimated parameters

	Mean ± SEM[a]	± Combined SEM[b]				95% Confidence interval[c]
		k = 1	k = 3	k = 5	k = 10	k = 5
ΔP_{net}, *mm Hg*	16.1 ± 1.58	1.98	1.71	1.65	1.60	12.9 to 19.3
ΔP_E, *mm Hg*	10.6 ± 1.87	2.41	2.06	1.99	1.81	6.9 to 14.3
K_f, *nl/min mm Hg*	2.1 ± 0.23	0.33	0.27	0.25	0.24	1.6 to 2.5
GBF_A, *nl/min*	204 ± 6.7	16.5	11.0	9.6	8.3	185 to 222
GBF_E, *nl/min*	174 ± 6.7	16.0	10.7	9.3	8.6	155 to 192
GPF_A, *nl/min*	104 ± 3.4	8.4	5.6	4.8	4.1	95 to 113
R_A, $\times 10^{10}$ *dyne sec cm*$^{-5}$	2.7 ± 0.13	0.23	0.17	0.15	0.14	2.4 to 3.0
R_E, $\times 10^{10}$ *dyne sec cm*$^{-5}$	1.7 ± 0.07	0.14	0.10	0.09	0.08	1.5 to 1.9

Abbreviations: ΔP_{net}, net-capillary effective filtration pressure; ΔP_E, end-capillary effective filtration pressure; K_f, ultrafiltration coefficient; GBF_A, afferent glomerular blood flow; GBF_E, efferent glomerular blood flow; GPF_A, afferent arteriolar plasma flow; R_A, preglomerular resistance; R_E, efferent arteriolar resistance.

[a] Mean ± SEM of individual animal mean value ($N = 11$).

[b] The values were estimated from the combined intra- and interanimal variances. SEM was estimated with k = 1, 3, or 10 provided predicted values assuming 1, 3, or 10 samples (k) were taken in each of the same series of 11 rats.

[c] $\bar{X} \pm 1.96$ combined SEM.

R_A, R_E, GBF_A, GBF_E, ΔP_{net}, ΔP_E, and K_f also were appreciably smaller than the combined SEMs (Table 3). Thus, the use of the interanimal SEM in comparing means of the derived parameters instead of the larger combined values might, at times, lead to the erroneous assumption that truly nonsignificant differences between means are, to the contrary, statistically significantly different. Conversely, the large SEMs derived from the combined variances would tend to conceal differences between means in separate series that might prove to be statistically significant at the 95% confidence level or higher if a sufficiently large number of samples were obtained. The value of the Student t for the significance of difference in unpaired means may be determined as: $t = (\bar{X}_1 - \bar{X}_2) / \sqrt{SEM_1^2 + SEM_2^2}$ (Aspin-Welch approximate t statistic). According to that equation and assuming constancy of the CV in two separate studies where five samples of the measured parameters are obtained in each of 11 rats, we find that we would be unable to detect true differences in K_f that are below ± 50% of our present mean when the SEM is calculated from the combined variance; differences in the mean values for R_A, R_E, and GPF_A (and thus GBF_A and GBF_E) that are within approximately ± 20% of our measured values likewise could not be appreciated at the 95% confidence level. Had we obtained only two measurements of each parameter in seven animals (and some studies appear to have largely used single sampling), we could not have detected true differences smaller than + 35% or −25 to −30% in the mean values for individual vascular resistances and blood flow rates; even a doubling or halving of K_f could not be proven to be significant at the 95% confidence level. This finding is of more than trivial interest since potentially important physiologic effects of physical or pharmacologic manipulation on K_f, R_A, and R_E might then go totally undetected.

The precision of estimate of the mean value for any given parameter is increased as the number of measurements is increased. This may be seen from Eq. *21* of **Methods** which includes the term S_W^2/k. S_W^2 exerts its full effect when k = 1, while the term in S_W^2/k virtually disappears when k is very large, then leaving the combined variance essentially equal to the interanimal variance. The degree of concordance between the interanimal and the combined variances in the present study reflect the fact that five samples of each parameter were measured in every rat (that is, k = 5) and the difference between the two variances would have been much greater if only one or two samples per rat had been obtained instead (see Table 3). While a large sample size is required to improve the precision of estimates of mean values in the face of marked internephron heterogeneity, however, distinct limitations on sampling are imposed by time constraints in the laboratory. Equally restrictive, we have had such great difficulty in obtaining even four measurements of P_g in individual rats with strict criteria for acceptability imposed that the 11 rats that form the basis for this report represent only a very small minority of animals prepared for study. A reasonable compromise between precision and practicality thus must be struck in performing glomerular dynamic studies. As may be seen in Tables 2 and 3, we find that the combined SDs would not have been reduced notably if ten estimates of each parameter had been taken in every rat in the present study, and the SDs obtained assuming three samples of each parameter per rat are not very much higher than those obtained here with k = 5. At any given degree of variance, the SEM is determined as an inverse function of the number of rats used (that is, SD / $\sqrt{n}$), so that the precision sacrificed when three, rather than five, measurements of each parameter are obtained per rat can be offset by increasing the number of rats included for study. Because of time and technical constraints in obtaining large numbers of measurements in each rat, it seems in fact that the greatest economy would be achieved by taking only three measurements of each parameter once the intra-animal variances have been established to be comparable to those found in the present studies. As an example, we estimate from Eq. *21* of **Methods** that 63 animals are required to provide a mean K_f value that is within ± 10% of a given mean at the 95% confidence level when k = 5, but only eight extra rats need be added to the series to provide the same result with k = 3 (Table 4). Similarly, with the degree of internephron heterogeneity found here, 16 rats suffice to describe confidently the mean K_f within ± 20% of its value with k = 5, and 18 rats would satisfy this requirement if three samples of each of the measured parameters were obtained instead. Comparable economy could be expected for ΔP_E, ΔP_{net}, GPF_A, GBF_A, GBF_E, R_A, and R_E with triplicate sampling (Table 4). Even with k = 5, however, it may be noted that the number of animals required to provide

Table 4. Numbers of rats required to provide mean values within a given percentage of error of each derived parameter according to sample size (k)/rat ($P = 0.05$)

	Number of rats required							
	ΔP_E	ΔP_{net}	K_f	GBF_A	GBF_E	GPF_A	R_A	R_E
± 10% Error								
k = 1	219	64	110	28	36	28	33	28
k = 3	161	48	71	13	16	12	17	14
k = 5	149	45	63	10	13	9	14	11
± 15% Error								
k = 1	98	29	49	13	16	13	15	13
k = 3	72	22	32	6	8	6	8	6
k = 5	66	20	28	5	6	4	7	5
± 20% Error								
k = 1	55	16	28	7	9	7	8	7
k = 3	40	12	18	4	4	3	5	4
k = 5	38	11	16	3	3	3	4	3

Abbreviations: P_E, end-capillary effective filtration pressure; P_{net}, net-capillary effective filtration pressure; K_f, ultrafiltration coefficient; GBF_A, afferent glomerular blood flow; GBF_E, efferent glomerular blood flow; GPF_A, afferent arteriolar plasma flow; R_A, preglomerular resistance; R_E, efferent arteriolar resistance.

mean values for ΔP_E and K_f that are within ± 20% of the mean at the 95% confidence level is much larger than that customarily used in micropuncture studies. On the other hand, very ordinary numbers of rats provide this degree of precision for estimates of blood flows and individual vascular resistances whether k = 1, 3, or 5.

In sum, substrains of Munich-Wistar rats used in the present study exhibited a degree of internephron functional heterogeneity in the various measured parameters of glomerular dynamics that was, for the most part, significantly greater than the interanimal variability of mean values. This finding appears consistent with results obtained from multiple samplings of specific parameters in other laboratories [7, 14, 15], but one cannot necessarily extrapolate our results quantitatively to all rat strains, to male rats (the present study was performed exclusively in females) or to volume-expanded animals. Further, we cannot know whether the degree of intra-animal variation found here and in the studies of others also applies in intact, unanesthetized rats. With intra-animal variances generally larger than interanimal variances, under experimental conditions, however, there would seem to be no advantage to the practice of taking very small numbers of measurements of a parameter in a given rat and using those values as "controls" for an equally small number of measurements made in the same animals after experimental manipulation (for example, [11, 12]).

Appendix

Many parameters in nephron function estimated from a sample can be influenced by both interanimal and intra-animal variances. Thus, the accuracy and precision of estimates depend on the number of animals, n, and the number of replications, k, per animal. In practice, each parameter is estimated by calculating the mean value for each animal and then by taking the overall mean of the n animal values.

For a given parameter of interest, let S_A^2 be the interanimal variance based on n animal values and $V(A_i)$, i = 1, 2, . . ., n, be the intra-animal variance for the ith animal based on k replications. S_A^2 can be calculated simply as the sample variance of the n values, each representing the estimate of the parameter for an animal. Because the determinant variables making up the parameter are measured independently from different samples, however, $V(A_i)$ cannot be calculated directly. The intra-animal variances can be approximated closely from the mean and the variance of the measured variables for the animal using appropriate combinations of the formulas given below.

Let X and Y be two variables. If X and Y are independent, the variance of the sum, product and quotient based on the sample means and the sample variances of X and Y can be approximated by the following formulas:

$$V(X + Y) = V(X) + V(Y) \qquad (a1)$$

$$V(XY) = \overline{X}^2V(Y) + \overline{Y}^2V(X) + V(X)V(Y) \qquad (a2)$$

$$V(X/Y) = (\overline{X}/\overline{Y})^2[V(X)/\overline{X}^2 + V(Y)/\overline{Y}^2] \qquad (a3)$$

$$Cov(XY, X) = \overline{Y}V(X) \qquad (a4)$$

The above formulas may be expanded for three or more variables and may be combined to encompass more complex formulas. They are appropriate for the variables in nephron function because, in a given animal, the variables are measured independently and were found here on correlation analysis to be approximately independent.

For simplicity of presentation, the following notations are used to express the measured variables in expressing the approximate intra-animal variances of the eight parameters.

$$P = P_g,\ Q = P_t,\ R = COP_E,\ T = COP_A,\ M = MAP$$
$$X = SNGFR,\ Y = C_A,\ Z = C_E,\ W = Hct_A,\ C = P_c$$

In the following, the sample mean and the variance of P_g, for example, are denoted as $\overline{P}$ and S_p^2, respectively.
Net residual filtration pressure:

$$V(\Delta P_E) = S_P^2 + S_Q^2 + S_R^2$$

Mean net filtration pressure:

$$V(\Delta P_{net}) = S_P^2 + S_Q^2 + 0.25S_T^2 + 0.25S_R^2$$

Afferent plasma flow:

$$V(Q_A) = [\overline{XZ}/(\overline{Z} - \overline{Y})]^2[\overline{X}^2S_Z^2 + \overline{Z}^2S_X^2 + S_X^2S_Z^2)/\overline{XZ})^2 + (S_Z^2 + S_Y^2)/(\overline{Z} - \overline{Y})^2 - 2\overline{X}S_Z^2/\overline{XZ}(\overline{Z} - \overline{Y})]$$

Hydraulic conductivity:

$$V(K_f) = [\overline{X}/(\overline{P} - \overline{Q} - 0.5\overline{T} - 0.5\overline{R})]^2$$
$$[S_X^2/\overline{X}^2 + V(\Delta P)/(\overline{P} - \overline{Q} - 0.5\overline{T} - 0.5\overline{R})^2]$$

Afferent blood flow:

$$V(GBF_A) = (\overline{XZ}/T)^2[V(Q_A)/\{\overline{XZ}/\overline{Z}\text{-}\overline{Y})\}^2 + S_W^2/(1 - \overline{W})^2]$$

where $T = (\overline{Z} - \overline{Y})(1 - \overline{W})$.

Efferent blood flow:

$$V(GBF_E) = V(GBF_A) + S_X^2 - 2S_X^2[\overline{Z}/T - S_Z^2(1 - \overline{W})/T^2$$
$$+ \overline{Z}/T^3\ \{(\overline{Z} - \overline{Y})^2 S_W^2 + ((1 - \overline{W})^2 + S_W^2)(S_Z^2 + S_Y^2)\}]$$

Preglomerular resistance:

$$V(R_A) = [\overline{M} - \overline{P})/\overline{GBF}_A]^2[S_M^2$$
$$+ S_P^2)/(\overline{M} - \overline{P})^2 + V(GBF_A)/\overline{GBF}_A^2]$$

Efferent arteriolar resistance:

$$V(R_E) = [\overline{P} - \overline{C})/\overline{GBF}_E]^2[S_P^2$$
$$+ S_C^2)/(\overline{P} - \overline{C})^2 + V(GBF_E)/\overline{GBF}_E^2]$$

Next let Z_{ij} be the jth measurement on the ith animal in a series of n animals for the parameter Z. It is reasonable to assume that Z_{ij} consists of three independent parts: the true parameter value, U, the inter-animal effect, A_i, and the intra-animal effect W_{ij} as follows:

$$Z_{ij} = U + A_i + W_{ij}$$

Let S_A^2 and S_W^2 denote the sample inter- and intra-animal variances. The total variance of the overall mean based on n animals with k measurements per animal is estimated by:

$$S_T^2 = (S_A^2 + S_W^2/k)n \qquad (a5)$$

Since each parameter is estimated by the mean value of n animals, the central limit theorem applies, and the distribution of the estimated value can be reasonably approximated by a normal distribution with the above variance S_T^2. Thus, the 95% confidence interval for a parameter can be obtained as follows using the mean of n animal values:

$$\text{Mean value} \pm 1.96\ S_T \qquad (a6)$$

To be 95% confident that the mean value determined from a series of n animal is with ±d unit of the true parameter value, n and k must be sufficiently large so that S_T^2 in Eq. (*a5*) must be less than or equal to $(d/1.96)^2$. Using this fact, the minimum sample size, n, required to reflect the calculated mean value within ±d unit of the three values can be approximated by rearrangement. For given k, the required sample size can be determined as:

$$n = 3.84(S_A^2 + S_W^2/k)/d^2 \qquad (a7)$$

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