Evaluation of "True" Creatinine Clearance in Rats Reveals Extensive Renal Secretion

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The renal clearance of endogenous creatinine is widely used to assess glomerular filtration rate (GFR) and renal function in animal investigations. The objective of the present investigation was to evaluate the extent of renal secretion of endogenous creatinine in rats and the effect of probenecid, the classical inhibitor of organic anion transport, on creatinine clearance. Ten female Lewis rats received ^{3}H-inulin (5-μCi i.v. bolus followed by 5 μCi/hr) throughout a 6-hr period. Three hours after initiation of the inulin infusion, probenecid was administered (92.4-mg/kg i.v. bolus followed by 0.59 mg/min/kg). Steady-state serum concentrations of about 500 μg/ml probenecid were achieved. Renal clearance was assessed between 1 and 3 hr (control) and between 4 and 6 hr (probenecid treatment). A preliminary study in seven rats demonstrated no time-dependent change in inulin or creatinine clearance between these two study intervals. Creatinine clearances were determined by an alkaline picrate assay which incorporated Fuller's earth (Lloyd reagent) to remove interfering noncreatinine chromogens from serum samples and these values were compared with those using a nonspecific picrate assay. "True" clearance ratios of creatinine to inulin (CL_{cr}/CL_{in}) were greater than unity (2.33 ± 0.83, mean ± SD) and were significantly decreased after probenecid treatment (1.26 ± 0.28, $P < 0.01$). Probenecid had no effect on GFR, as assessed by inulin clearance. Using the nonspecific picrate assay, CL_{cr}/CL_{in} was 1.12 ± 0.41, which was not significantly different from unity and which decreased to 0.53 ± 0.12 after probenecid treatment. Therefore, creatinine undergoes extensive renal secretion in female Lewis rats.

KEY WORDS: probenecid; creatinine clearance; glomerular filtration rate (GFR); renal function; rats; creatinine assay.

INTRODUCTION

Creatinine clearance is extensively used in both clinical and animal studies as a measure of glomerular filtration rate (GFR). Nevertheless, it is well recognized that approximately 10 to 40% of the clearance of creatinine in humans is due to active tubular secretion (1,2) so that estimates of creatinine clearance would overestimate GFR as determined by inulin clearance. The fact that creatinine clearance values tend to be similar to the values of GFR estimated by other means (inulin, EDTA) is often the result of the nonspecific assay used to quantitate serum creatinine concentrations (1,3). The lack of specificity of the Jaffé-picric acid reaction has been known ever since it was introduced (3). The presence of noncreatinine chromogens in serum samples results in an apparent serum creatinine value as much as 36% higher, with a mean value 18% higher, than the true creatinine concentration in human serum. Therefore, because of the presence of these two opposing but unrelated effects (active secretion and nonspecific assay), creatinine clearance values in normal subjects tend to reflect GFR (2).

Less is known concerning the contribution of active tubular secretion to the renal clearance of creatinine in rats, as well as the influence of the nonspecificity of the picric acid assay on the creatinine clearance determination (4). At physiological serum creatinine concentrations in the rat, investigators have reported clearance ratios of creatinine to inulin (CL_{cr}/CL_{in}) of unity or less than unity (5–8). Therefore, there is no evidence in the literature to suggest the presence of creatinine renal secretion at physiological serum creatinine concentrations. However, following the administration of exogenous creatinine, i.e., at elevated serum creatinine concentrations, creatinine undergoes renal secretion in rats (9–12), a process which can be inhibited by probenecid (10). The influence of the nonspecificity of the Jaffé-picric acid assay on endogenous creatinine clearance values has not been assessed in rats; however, Meyer *et al.* (4) have shown that rat serum creatinine concentrations, determined by an HPLC method, are approximately one-half the value determined by assays using a nonspecific Jaffé-picric acid reaction. If this is true, then the calculated creatinine clearance values, based on creatinine concentrations quantitated by a nonspecific picric acid assay, would represent large underestimations of the true values in rats.

The objectives of the present investigation were (i) to compare creatinine clearances in female Lewis rats using nonspecific and specific (modified by the use of Fuller's earth) picric acid assays for creatinine, (ii) to determine if endogenous creatinine undergoes active renal secretion by determining the creatinine to inulin clearance ratio, and (iii) to examine the influence of probenecid, the classical inhibitor of organic anion transport, on the renal clearance of endogenous creatinine and on GFR (inulin clearance) in rats.

MATERIALS AND METHODS

Materials. The following reagents were used in the study: creatinine standard solution, 15 mg/dl (Sigma Diagnostics, St. Louis, MO); Lloyd reagent (Fuller's earth or hydrated aluminum silicate, J. T. Baker Chem. Co., Phillipsburg, NJ); sodium tungstate (Merck and Co. Inc., Rahway, NJ); picric acid (Allied Chemical, Morristown, NJ); probenecid (Sigma Chemical Co., St. Louis, MO); sulfamethazine (USP); [^{3}H-methoxy]inulin (New England Nuclear, Dupont Co., Chadds Ford, PA); and Biodegradable Counting Scintillant (Amersham Corp., Arlington Heights, IL).

Probenecid bolus injection and infusion solutions were prepared by initially dissolving probenecid in 0.1 *N* NaOH and adjusting the pH to 7.4 with dilute HCl. The solution was then diluted to the appropriate volume with saline. Control solutions used in the investigation consisted of the probenecid solution vehicle. The alkaline picrate solution used in the creatinine assay was prepared by mixing 10 parts picric acid (12 g/L) with 35 parts 0.2 *N* NaOH.

Study Design. Ten female Lewis rats weighing between 219 and 247 g (Charles River, Wilmington, MA) had right jugular vein, right carotid artery, and bladder cannulas im-

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planted under light ether anesthesia 1 day prior to the study day. Each study was 6 hr in duration and consisted of consecutive control and probenecid-treatment periods. A preliminary study in seven additional female Lewis rats examined the possibility of time-dependent alterations in GFR over the 6-hr study interval. ^{3}H-Inulin was administered as a 5-μCi bolus injection followed by an infusion of 5 μCi/hr over the 6-hr period, and its renal clearance determined between 1 and 3 hr and between 4 and 6 hr. No significant time-dependent alterations in the serum concentrations, urinary excretion rates, or renal clearance values of inulin or creatinine were observed.

On the study day, at time zero, rats received a bolus injection of the control solution (9.24 ml/kg) and 5 μCi ^{3}H-inulin (0.3 ml) through the jugular vein cannula followed by a constant-rate infusion of control solution (1.24 ml/hr) containing ^{3}H-inulin (5 μCi/hr). Urine was collected from 1 to 3 hr and blood samples were obtained from the carotid artery at 1, 2, and 3 hr (0.3, 0.4, and 0.3 ml, respectively). After the blood and urine collection at 3 hr, rats received a bolus dose of probenecid (92.4 mg/kg, 10 mg/ml) via the jugular vein and the infusion was switched to that of probenecid (0.59 mg/min/kg) containing ^{3}H-inulin (5 μCi/hr). The bolus injection and infusion rate of probenecid were designed to achieve mean serum concentrations of about 425 μg/ml based on pharmacokinetic parameters for a 100-mg/kg dose of probenecid in rats (13). After a 1-hr equilibration period, urine was collected between 4 and 6 hr and arterial blood samples were obtained at 4, 5, and 6 hr (0.3, 0.4, and 0.3 ml, respectively).

Analysis of ^{3}H-Inulin, Creatinine, and Probenecid. Radioactivity in serum and urine was determined to calculate the renal clearance of inulin. To quantitate the radioactivity, 10 μl of either serum or urine was mixed with 10 ml of scintillant in a glass scintillation vial. Samples were counted on a Model 1900 CA Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Creatinine serum and urine concentrations were determined using two spectrophotometric assay methods incorporating the Jaffé reaction. The first method was that described by Heinegard and Tiderstrom (14) (Sigma diagnostic kit No. 555); the second method utilized the Jaffé reaction as well but incorporated Fuller's earth (Lloyd reagent) to remove interfering substances (15). This second method was chosen as a specific creatinine assay since it provides similar results as determined by isotope dilution-mass spectrometry, a primary reference method (16). For the second method, serum (100 μl), standard or water (10 μl), 0.15 *M* sodium tungstate (100 μl), and 0.165 *M* sulfuric acid (200 μl) were vortex-mixed for 30 sec, then centrifuged at 12,000*g* for 5 min. A 350-μl aliquot of the supernatant was transferred to a second tube containing 500 μl of Fuller's earth suspension (6 g/L). This tube was vortex-mixed for 30 sec and centrifuged for 5 min. The supernatant was aspirated to waste and 500 μl of the alkaline picrate solution was added to the pellet. The tube was vortex-mixed until the pellet was thoroughly resuspended (approx. 10 sec), allowed to stand for 60 min, then centrifuged for 5 min. The absorbance of the supernatant was measured versus water at 509 nm (Model U-2000 spectrophotometer, Hitachi Instruments Inc., Webster, NY). Urine samples were analyzed in the same manner as serum except that the Fuller's earth suspension was added directly to the mixture of urine, tungstate, and sulfuric acid. Serum standard curves were prepared by adding known amounts of creatinine to serum samples followed by protein precipitation and extraction (with subsequent subtraction of the blank): therefore, any loss of creatinine through incomplete adsorption to Fuller's earth would be similar for the standard curve and experimental samples and would not affect the results (16). However, the adsorption of creatinine to the Fuller's earth used in this investigation appears to be complete since no differences in creatinine concentration were seen for our urine samples when Fuller's earth was or was not used in the assay. The standard curve for serum ranged from 0.063 to 0.750 mg/dl, while that for urine ranged from 0.5 to 3.0 mg/dl. Standard curves prepared in serum and water by adding known amounts of creatinine (0.25 to 1.5 mg/dl) had similar values at each concentration point (after subtraction of the appropriate blanks) and the same slope (differed by 0.25%). Therefore, the presence of endogenous creatinine in the serum standards, which results in greater total creatinine concentrations compared with the serum samples, did not produce any concentration-dependent influence on the serum creatinine assay. For the modified creatinine assay, the intraday coefficient of variation using five serum samples was 3.1% and the interday coefficient of variation over 3 days was 12.0%. All serum and urine samples were assayed on the same day. Probenecid did not interfere with the analysis of creatinine.

Serum concentrations of probenecid at 4, 5, and 6 hr were determined using a modification of a previously described HPLC assay (17). Briefly, serum samples were prepared for analysis by protein precipitation with 1 part serum and 2 parts acetonitrile containing sulfamethazine (25 μg/ml), the internal standard. The mobile phase (0.01 *M* monobasic potassium phosphate, pH 6.0, and acetonitrile, 80:20) was pumped at a flow rate of 1.0 ml/min through a 12.5-cm C_{18} Partisphere column (Whatman Inc., Clifton, NJ). Retention times were 3.7 and 5.4 min for sulfamethazine and probenecid, respectively.

Data Analysis. Inulin clearance was calculated directly from the counts per minute since serum and urine samples were found to be quenched to the same degree. Inulin clearance (CL_{in}) was calculated by the following equation:

$$CL_{in} = \frac{(UF)\ (^3H_u)}{^3H_s}$$

where UF is urine flow (ml/min), ^3H_u is ^{3}H counts in urine per minute, and ^3H_s is the mean ^{3}H counts in serum per minute over the time period of measurement. Renal creatinine clearance was calculated by dividing the urinary excretion rate of creatinine for either the 1 to 3-hr or the 4 to 6-hr period by the mean serum concentration of creatinine over that time period. Data from the different treatment periods were compared using paired *t* tests. Probenecid concentrations at the three collection times were compared using analysis of variance, followed by a Tukeys test. Results are expressed as mean ± SD.

RESULTS

In female Lewis rats, the mean control CL_{cr}/CL_{in} ratio was 2.33 (±0.83, SD) and ranged from 1.65 to 4.10 (Fig. 1).

Probenecid administration resulted in serum probenecid concentrations at 4, 5, and 6 hr of 455 ± 34, 497 ± 22, and 532 ± 27 μg/ml (mean ± SD, $n = 10$). There were significant increases in probenecid serum concentrations over time, most likely due to the nonlinear kinetics of probenecid (13). Probenecid treatment resulted in a statistically significant decrease in creatinine serum concentrations, the urinary excretion rate of creatinine, the creatinine renal clearance, and the CL_{cr}/CL_{in} ratio (Figs. 1 and 2). There was no alteration of ^{3}H-inulin clearance following probenecid treatment (Fig. 3), suggesting no change in GFR. A graph showing the relationship between the renal clearance values for creatinine and those for inulin during control and probenecid-treatment periods is given in Fig. 4. Creatinine clearance decreased in all animals following probenecid treatment and the values approached the line of unit (CL_{cr}/CL_{in} decreased to 1.26 ± 0.28; $P < 0.01$).

A comparison of serum creatinine concentrations and the CL_{cr}/CL_{in} ratios determined using the picric acid assay, with and without the incorporation of Fuller's earth, is presented in Fig. 5. Higher values for serum creatinine and lower values for creatinine clearance and the CL_{cr}/CL_{in} ratio were obtained when creatinine was analyzed using a picrate assay without Fuller's earth. The creatinine concentration of a serum sample determined in triplicate in the absence of Fuller's earth was more than double that seen following the incorporation of this reagent in the assay (0.53 ± 0.01 mg/dl without Fuller's earth vs 0.22 ± 0.00 mg/dl with Fuller's earth). Urine creatinine concentrations analyzed using the two methods were similar. When samples from the present investigation were assayed using the less specific creatinine procedure, the results were as follows: the CL_{cr}/CL_{in} ratio was not significantly different from unity (1.12 ± 0.41) and probenecid treatment caused a significant decrease in the clearance ratio, to 0.53 ± 0.12 (Fig. 5). These clearance ratios suggest that creatinine undergoes renal reabsorption as well as renal secretion (that is subject to probenecid inhibition). This contrasts with the results of the present investigation when creatinine concentrations were quantitated using a more specific assay.

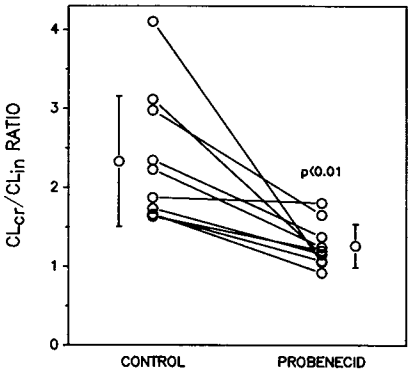

Fig. 1. Clearance ratios of creatinine to inulin from control and probenecid-treatment periods in individual rats. Connected open circles represent the individual clearance ratio values for each rat during the two measurement periods ($n = 10$). The circles with vertical error bars represent the mean and standard deviation of the two measurement periods. A significant decrease in the clearance ratio values was observed with probenecid treatment ($P < 0.01$).

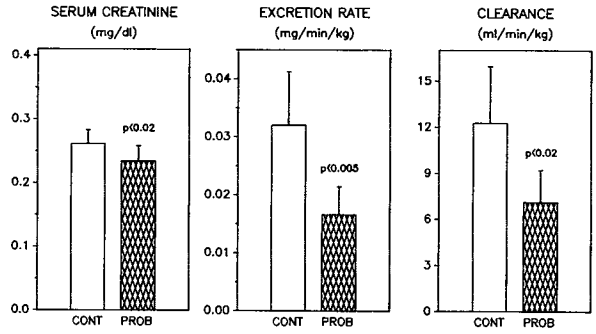

Fig. 2. Serum concentration, urinary excretion rate, and renal clearance of creatinine during control and probenecid-treatment periods. The results are expressed as mean ± SD; $n = 10$.

DISCUSSION

An ideal marker, whose renal clearance can be used to assess GFR, should be freely filtered at the glomerulus and should not be reabsorbed, secreted, or metabolized by the renal tubule. Inulin appears to meet the criteria of an ideal marker of GFR since no reabsorption or secretion has been observed *in vivo* (18) or *in vitro* in micropuncture studies (19). Creatinine, although extensively used to evaluate the GFR and renal function in clinical and animal investigations, is known to be eliminated by renal tubular secretion in humans (1,2) and this route of elimination appears to become increasingly prevalent with renal dysfunction (1,20). In addition, there is evidence for renal tubular reabsorption (21). Less is known concerning the contribution of renal secretion or reabsorption to the renal clearance of endogenous creatinine in rats.

In the present investigation, at physiological concentrations of creatinine in female Lewis rats, renal secretion of creatinine is evident, as assessed from the CL_{cr}/CL_{in} ratios, which were greater than unity (2.33 ± 0.83). The extent of this secretion appears to be variable, with clearance ratios varying from 1.63 to 4.10. Previous investigators have reported CL_{cr}/CL_{in} ratios greater than unity in the rat, but only following the administration of exogenous creatinine, i.e., at high plasma creatinine concentrations (9–12). The results of the present investigation contrast with those of previous in-

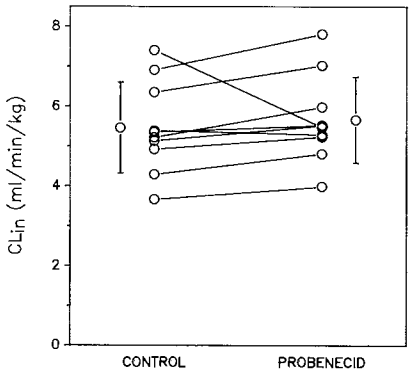

Fig. 3. The renal clearance of ^{3}H-inulin during control and probenecid-treatment periods. Connected open symbols represent the individual clearance values for each rat during the two measurement periods. Circles with vertical error bars represent the overall mean and standard deviation during the two measurement periods.

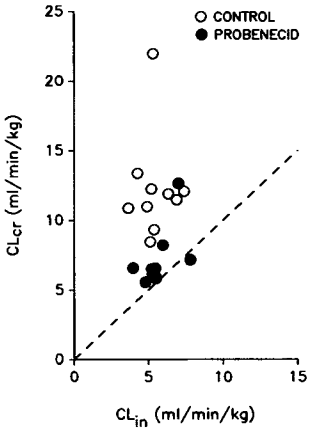

Fig. 4. Relationship between renal clearance values for creatinine and inulin during control and probenecid-treatment periods. ○ and ● represent individual values during the control and probenecid-treatment periods, respectively. The dashed line is the line of unity.

vestigations performed at physiologic serum creatinine concentrations; in these studies clearance ratios of approximately unity or less than unity were reported (5–8). Namnum *et al.* (7) report CL_{cr}/CL_{in} ratios in anesthetized male Fisher rats of 0.5 and in conscious rats of 0.7, and Michael *et al.* (6) report a mean clearance ratio of 0.71 in male and female Wistar rats, suggesting that creatinine undergoes net reabsorption at endogenous creatinine concentrations.

The creatinine clearance values determined in the present investigation may be regarded as "true" clearance values and are higher than those obtained in studies that have utilized a conventional picric acid assay which does not incorporate some technique in the assay to remove the noncreatinine chromogens present in serum samples (1,4). The nonspecificity of the serum creatinine analysis appears to be a greater problem in the quantitation of creatinine in rat and mouse serum samples than seen clinically (4), possibly due to the lower creatinine concentrations present in rodent serum. Using the more specific assay, the serum creatinine values obtained in the present study averaged 0.26 mg/dl for the untreated periods. This value is lower than some reported values (as summarized in Ref. 22) but agrees well with concentrations obtained by HPLC (4,23) and with a Beckman creatinine analyzer (24,25). As a consequence of the lower creatinine serum concentrations obtained with the more specific creatinine assay, creatinine clearance values and CL_{cr}/CL_{in} ratios are greater than those determined with a less specific assay. In the present investigation, when a nonspecific creatinine assay was used, lower CL_{cr}/CL_{in} ratios (1.12 ± 0.41) were obtained. Therefore, the nonspecificity of the serum creatinine assay may explain at least some of the previously reported low creatinine-to-inulin clearance ratios. The CL_{cr}/CL_{in} ratios of greater than unity that have been reported at higher creatinine serum concentrations may be the consequence of decreased interference in the serum creatinine assay at these higher serum concentrations. An alternate explanation for the observed discrepancies may be rat strain differences in the renal handling of creatinine.

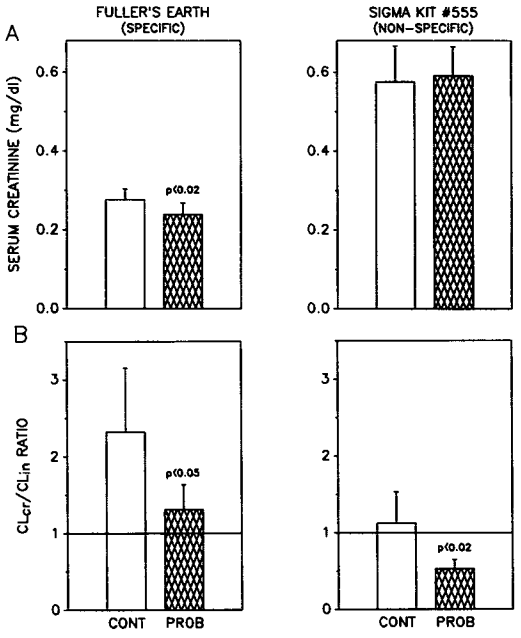

Fig. 5. Comparison of serum creatinine concentrations (A) and creatinine-to-inulin clearance ratios (B) determined by two creatinine assays. Rats were studied during control and probenecid-treatment periods. Serum samples were analyzed using a picric acid method (Sigma diagnostic kit No. 555) and a modified picric acid method which incorporates Fuller's earth to remove interfering noncreatinine chromogens. Urine creatinine concentrations are similar when analyzed by these two methods. The results are expressed as mean ± SD; $n = 10$.

In the present investigation, probenecid, at serum concentrations of about 500 μg/ml, significantly decreased the CL_{cr}/CL_{in} ratio but not the clearance of inulin. Therefore, the decrease in creatinine clearance does not appear to be due to an effect of probenecid on glomerular filtration but, instead, to a probenecid-induced inhibition of creatinine secretion. A lack of effect of probenecid on GFR in rats is in agreement with the results of other investigators (12,26). Fingl (12) and Harvey and Malvin (10) have previously reported that probenecid can decrease the CL_{cr}/CL_{in} ratio in rats but this was examined following the administration of exogenous creatinine (at serum creatinine concentrations between 15 and 120 mg/dl, which are much higher than the endogenous level of 0.26 mg/dl) and not at physiological creatinine concentrations. Creatinine is a weak organic cation, with a pK_a of 4.95 at 15°C (27); however, it has been demonstrated that the renal secretion of creatinine in humans, dogs, and chickens can be inhibited by both organic anions (28–31) and organic cations (28,32,33).

Probenecid treatment also produced a small but significant decrease in the serum concentration of creatinine, despite the fact that its urinary excretion was decreased by approximately 50%. The reason for this is unknown. In our preliminary study, six of the seven rats exhibited a small and nonsignificant decrease in serum creatinine concentration during the second study period, suggesting the possibility of a small time-dependent change in serum creatinine concentrations. Additionally, it is possible the probenecid may exert a direct effect on creatinine formation or distribution.

In conclusion, creatinine clearance is extensively used in animal studies to assess GFR and renal function. "True" endogenous creatinine clearance is substantially greater than

inulin clearance in female Lewis rats, indicating the presence of tubular secretion of creatinine. Probenecid can significantly decrease the ratio of creatinine clearance to inulin clearance in rats *in vivo* without altering the GFR, suggesting that its effect is due to inhibition of the tubular secretion of creatinine.

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