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Tetraethylammonium as a Probe for Estimating Renal Plasma Flow in Unanesthetized Rats[1] (40497)

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Renal clearances are usually calculated from simultaneously measured levels of urinary excretion rate and plasma concentrations of the substance under study. The formula for renal clearance is: C = UV/P, in which U is the concentration of the substance in urine, *P* is the concentration in plasma, and *V* is the volume of urine formed per unit time. When a substance is eliminated from the body almost exclusively by being excreted into urine in an unchanged form, renal clearance also represents the mean total body clearance. According to pharmacokinetic theory, the mean total body clearance is: C = Dose iv/AUC, in which AUC represents the area under the plasma disappearance curve, from the time of injection until $t = \infty$.

It is frequently desirable to estimate not only the glomerular filtration rate but also the renal plasma flow in assessing kidney function. Inulin clearance is recognized as a valid measure of glomerular filtration rate "in all animals from fish to man" (1) and thus has been used in our experiments. Although the clearance of p-aminohippurate (PAH) is widely used as an estimate of renal plasma flow (2), we have found that the mean total body clearance of plasma radioactivity after administration of glycyl-1-[^{14}C]labeled PAH in the conscious rat, calculated as Dose iv/AUC, is a satisfactory estimate of renal plasma flow only if measurements are restricted to the first few hours after iv injection.

This paper describes an alternative method of estimating renal plasma flow in the unanesthetized rat by measuring the mean total body clearance of [^{14}C]labeled tetraethylammonium (TEA) bromide, a commercially available compound that is rapidly and completely excreted unchanged into urine. The method does not require catheterization of the urinary bladder or of blood vessels and is, therefore, suitable for use in the unanesthetized rat.

Methods. Experiments were performed on male, Sprague-Dawley rats (Taconic Farms), weighing 150–225 g. Commercial chow and water were available *ad libitum*.

Chemicals and isotopes. TEA bromide was purchased from Aldrich Chemical Co. Inulin and PAH (sodium salt) were purchased from the Sigma Chemical Co. *p*-Acetamidoacetylhippuric acid was prepared from PAH and acetic anhydride, as described by Newman *et al.* (3). *p*-Acetamidohippuric acid and PAH were identified by thin layer chromatography on microcrystalline cellulose (Avicel) plates with a solvent system of *n*-butanol:acetic acid: water (25:4:10).

The following radiolabeled compounds were purchased from New England Nuclear: glycyl-1-[^{14}C]labeled PAH (44.3 mCi/mmol); methoxy-[^{3}H]inulin (186.8 mCi/g); methoxy-[^{14}C]inulin (5.075 mCi/g); (1-^{14}C)-TEA bromide (3.0 and 9.5 mCi/mmol).

Body clearance determinations. Rats were injected in the tail vein with [^{14}C]methoxyinulin, [^{3}H]methoxyinulin, [^{14}C]TEA bromide, or [^{14}C]PAH. The radiolabeled compounds, as purchased, were diluted with the unlabeled compounds so that the desired dose, in an injection volume of 1.5 ml/kg, contained about 10 μCi/rat for [^{14}C]labeled compounds and about 50 μCi/rat for tritium labeled compounds. Some rats received both [^{3}H]methoxyinulin and [^{14}C]TEA in the same injection. Blood samples were obtained from the retro-orbital sinus at 8 or more of the following intervals after injection of the labeled compounds: 3, 5, 10, 20, 40, 90, 180, 240, 360, 1440, 1800 and 2880 min. In most

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rats, the earliest sample was taken at 5 min.

Since TEA ion and inulin are not metabolized to an appreciable degree, their concentrations were determined from the total tritium or [^{14}C]radioactivity in plasma. A suitable aliquot of plasma (0.05–0.5 ml) was placed in a counting vial. Five times the plasma volume of NCS solubilizer (Amersham/Searle) was added to the vial, which was then placed in an oven at 50° for 30 min. Fifteen ml of a solution containing 2,5-*bis*-2-(5-*t*-butylbenzoxazoyl) thiopene (BBOT) was added to each vial and the radioactivity was determined in a liquid scintillation counter. The composition (percentage by weight) of the BBOT solution was: 52.15, toluene; 39.35, methylcellosolve; 8.10, naphthalene; and 0.40, BBOT. The same procedure was also used to determine hippurate-derived radioactivity.

PAH concentrations were also determined, as described by Bauer *et al.* (4) by a modification of the method developed by Bratton and Marshall (5) for sulfanilamide.

For each rat, the plasma concentrations of the compound were plotted on semi-logarithmic paper against time. The data for each rat were fitted, if possible, to a two or three compartment model, represented by equations:

$$P = A_o e^{-\alpha t} + B_o e^{-\beta t}$$

and

$$P = A_o e^{-\alpha t} + B_o e^{-\beta t} + C_o e^{-\gamma t}$$

AUC was calculated as $A_o/\alpha + B_o/\beta$ for the two-compartment model, and $A_o/\alpha + B_o/\beta + C_o/\gamma$ for the three-compartment model.

Results are expressed as mean values ±SE, with the number of rats indicated in parentheses.

Results. Problems with PAH clearances. The values of plasma radioactivity measured after three hr following injection of small doses (1–25 mg/kg iv) of [^{14}C]PAH were higher than the values at 90 and 120 min (Fig. 1). Although the height of the "hump" was less than 3% of the initial plasma PAH level, it had a marked effect on the estimate of AUC and thus on the estimate of the clearance. When plasma levels of PAH after a dose of 125 mg/kg iv were measured by a colorimetric method (4), however, no

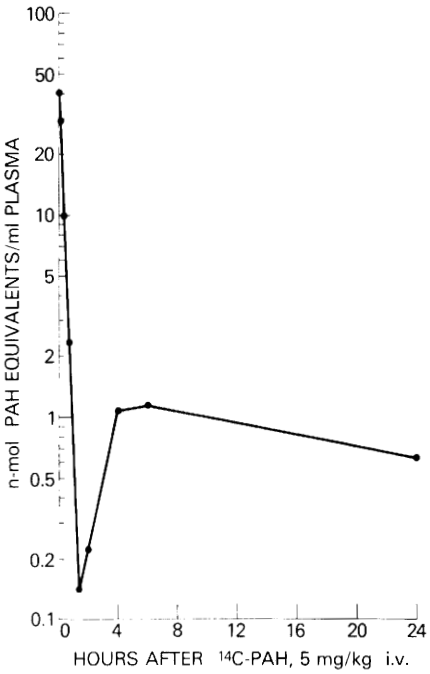

FIG. 1. Plasma concentrations of radioactivity in a rat plotted against time after an iv injection of [^{14}C]PAH (5 mg/kg).

"hump" was observed (Fig. 2). Instead, values could be fitted to a biexponential model and the clearance could be calculated as Dose iv/AUC, in which AUC was calculated as $A_o/\alpha + B_o/\beta$. The absence of a "hump" in the plasma disappearance curve of PAH, measured colorimetrically, suggested that the "hump" was not PAH, but metabolites of PAH. The finding that deproteinization of the plasma with 0.6 M perchloric acid decreased the size of the "hump" about 50% raised the possibility that [^{14}C]PAH was hydrolyzed to unlabeled *p*-aminobenzoic acid and to 1-[^{14}C]glycine and that the glycine was incorporated into plasma proteins. In addition, it also seemed possible that a part of the [^{14}C]PAH might be acetylated to *p*-acetamidohippuric acid, a reaction that occurs in man, but not in dog (3). The formation of *p*-acetamidohippuric acid could contribute to the "hump" in the plasma disappearance curves of [^{14}C]PAH radioactivity if its elimination were considerably slower than PAH.

To assess these possibilities, two rats were injected with 1 mg/kg (47 μCi/rat) of the [^{14}C]labeled compound and blood samples

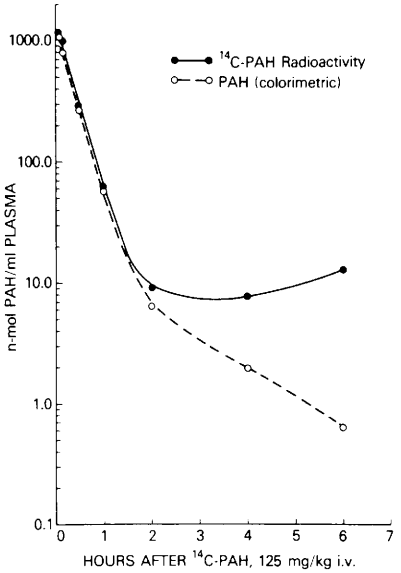

FIG. 2. Plasma concentrations of radioactivity (solid line) and PAH concentrations, determined colorimetrically, in a rat plotted against time after an iv injection of [^{14}C]PAH (125 mg/kg) iv.

were obtained at 5, 10, 20, 40, 90, 180, 300 and 360 min thereafter. The plasma disappearance curves for PAH-derived radioactivity in these rats confirmed the appearance of the "hump". Aliquots of the 360 min plasma samples and of the pooled 5, 10, 20 and 40 min samples were spotted on TLC plates and developed with butanol:acetic acid:water (25:4:10). Using a Packard radiochromatogram scanner to detect the radioactive peaks, we found that the pooled early samples gave a broad two-humped peak, with R_f values corresponding approximately to PAH (0.60–0.65) and p-acetamidohippurate (0.72–0.82). Unfortunately, the radioactivity in the 6-hr sample was too low to assay by the scanner. With the hope of obtaining better resolution, developed chromatograms of the 6 hour samples were divided into 22 zones. Each zone was scraped into a counting vial. Water (0.1 ml) and BBOT (15 ml) were added and the radioactivity was counted. Almost all the radioactivity was associated with two peaks; one peak was at the origin and presumably represented glycine that was incorporated into protein and the other had a value of 0.18 which was nearly identical to that of glycine in our system (0.20). There was also a much smaller peak which migrated with the solvent front. No radioactivity was detected at R_f values corresponding to PAH or *p*-acetamidohippuric acid. It therefore seems probable that most of the "hump" of radioactivity is due to glycine and the glycine incorporated into plasma proteins.

Since the "hump" did not contain detectable PAH, we ignored values of [^{14}C]PAH derived radioactivity measured after 3 hr in calculating PAH clearances (Table I). The mean values of PAH clearance, thus calculated, in 17 rats which received doses of 1–125 mg/kg iv was 26.4 ± 1.52 ml kg^{-1} min^{-1}. However, this value will be in error to the extent that a part of the clearance represents a metabolic clearance on the one hand and the area under the curve of acetamidohippuric acid contributes to the total area under the curve on the other hand.

Clearance of TEA. Since renal plasma flow can be measured with any compound which is extracted almost completely in one passage through the kidney, we have investigated the clearance of TEA for this purpose. TEA is excreted in man and in dog at rates which are "in the range of substances almost completely cleared from the renal arterial blood" (6). The plasma disappearance curve for [^{14}C]-TEA radioactivity in the rat is a smooth curve (Fig. 3), with no suggestion of a "hump". Plasma levels of [^{14}C]TEA radioactivity can

TABLE I. MEAN TOTAL BODY CLEARANCES[a] IN UNANESTHETIZED RATS OF INULIN, PAH, AND TEA.

Compound	Dose	Label	Clearance
	mg kg^{-1} iv		*ml kg^{-1} min^{-1}*
Inulin	25	[^{14}C]methoxy	6.5 ± 0.31 (4)
Inulin[b]	5	[^{3}H]methoxy	5.9 ± 0.18 (21)
PAH	1	[^{14}C]glycyl	22.2 ± 2.04 (3)
PAH	5	[^{14}C]glycyl	29.4 ± 2.79 (4)
PAH	25	[^{14}C]glycyl	29.3 ± 1.50 (6)
PAH	125	None	22.0 ± 2.02 (4)
PAH	1–125	[^{14}C]glycyl or none	26.4 ± 1.52 (17)
TEA	0.3–20[c]	1-^{14}C	27.6 ± 0.92 (28)

[a] Clearances were calculated as Dose iv/AUC. AUC was calculated as $A_o/\alpha + B_o/\beta + C_o/\gamma$ for data fitted to a triexponential curve (inulin and TEA) and $A_o/\alpha + B_o/\beta$ for PAH. In calculating PAH clearances, plasma radioactivity values at 4 hr and later were not used.

[b] These rats also received a simultaneous dose of TEA bromide. See Fig. 4.

[c] See Fig. 4 for the doses in various experiments. Twenty-one of these rats also received inulin, 5 mg/kg iv, in the same injection as TEA bromide.

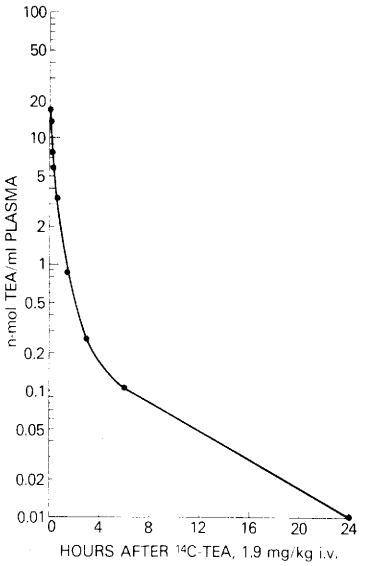

FIG. 3. Plasma concentrations of radioactivity in a rat after iv injection of [^{14}C]TEA (1.9 mg/kg).

be fitted satisfactorily to the tri-exponential equation, $P = A_o e^{-\alpha t} + B_o e^{-\beta t} + C_o e^{-\gamma t}$. Unlike PAH, AUC for the plasma disappearance curve of TEA can be calculated readily for each rat and the clearance of TEA can be calculated from the relationship, C = Dose iv/AUC.

The clearance values for TEA in our experiments (Fig. 3, Table I) are about the same as those for a dose (125 mg/kg) of PAH which is large enough to permit measurement of plasma levels by a specific colorimetric method (Table I). TEA clearances are also approximately the same as [^{14}C]PAH radioactivity clearances, provided that PAH radioactivity data for 4 hr and later are not used in the calculation. We have not measured clearances higher than those for TEA with any other compound. Thus, our data support the hypothesis that the clearance of TEA provides at least as good an estimate of renal plasma flow in this species as does the clearance of PAH.

Inulin clearance. It is often desirable to measure glomerular filtration rate at the same time as renal plasma flow. In groups of rats which received [^{3}H]methoxyinulin (5 mg/kg iv) in the same injection with a dose of [^{14}C]TEA (0.3–20.0 mg base/kg, Fig. 4), there were no significant differences in the clearance of inulin. The mean value of inulin clearance in the 21 rats which also received a dose of TEA was 5.9 ± 0.18 ml kg^{-1} min^{-1}. This value should be compared with another experiment in which four rats received only ^{14}C-methoxyinulin (25 mg/kg iv); the clearance in this experiment was 6.5 ± 0.31 ml kg^{-1} min^{-1}. Apparently, the glomerular filtration rate, as estimated by the clearance of inulin, was not significantly affected by the simultaneous administration of TEA.

Filtration fraction. The filtration fraction is defined as the ratio of the glomerular filtration rate to the total renal plasma flow. If one accepts the clearance of TEA as an estimate of renal plasma flow and the clearance of inulin as an estimate of the glomerular filtration rate, then the filtration fraction in the 21 rats which received both inulin and TEA iv simultaneously varied from 0.15 to 0.30, with a mean value of 0.21 ± 0.009.

Discussion. The usual method of measuring renal clearance (C = UV/P) in animals and in man would not involve difficulties from the mechanisms responsible for the "hump" in our experiments with PAH because the experimental procedures are restricted to short intervals of 10–60 min, the plasma PAH concentrations are kept constant by using an iv infusion, and the rate of PAH excretion is measured directly.

Blaufox *et al.* (7) have described a method

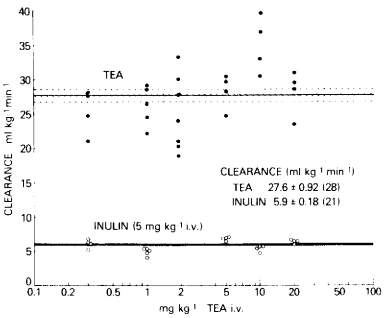

FIG. 4. Mean total body clearance of TEA (0.3–20 mg/kg iv) and inulin (5 mg/kg iv). Each solid circle represents the mean total body clearance of the indicated dose of TEA in one rat. Twenty-one of the 28 rats which received TEA also received inulin (5 mg/kg) in the same injection. The inulin clearances are represented by the open circles. Seven rats received TEA (1.9 mg/kg) without inulin. The upper heavy line represents the mean TEA clearance in 28 rats ± SE (crosshatched area). The lower heavy line represents the mean inulin clearance ± SE (thin lines).

for measuring in the anesthetized rat the clearance of o-iodohippurate ^{125}I. Their calculations are based on a two-compartment model, with body clearances calculated from a formula derived from the relationship C = Dose iv/AUC. They suggest the use of only two blood samples, 10 and 60 min after the iv injection of the iodohippurate. They determined the slope of the alpha and beta phases from a recording of the radioactivity over the head, a procedure which was possible because the rat had been injected with a gamma emitter. Other experiments confirmed that the ratio of radioactivity recorded over the head to that in the plasma was relatively constant. By this method, they obtained a clearance for iodohippurate in 14 normal anesthetized rats of 21.1 ml kg^{-1} min^{-1}. Although this method will be in error to the extent that o-iodohippurate undergoes hydrolysis, and the area under the curve is the sum of the areas of both o-iodohippurate and *o*-iodobenzoate, this value did not differ statistically from PAH clearances in the same rats 1–3 days later, using "20-min urine collection periods and midpoint tail vein blood". Thus the errors theoretically present in their method are probably insignificant. It is of interest that their clearance for *o*-iodohippurate did not differ significantly from the body clearances of a large dose (125 mg/kg) of PAH in our experiments (Table I). Other investigators (8, 9) have also reported PAH clearances in the range of 20–25 ml kg^{-1} min^{-1}.

Neither the PAH nor the TEA method takes into account the extraction ratio in calculating renal plasma flow. Cortney *et al.* (8) reported an average PAH clearance of 24 ml kg^{-1} min^{-1} and a calculated renal plasma flow of 33 ml kg^{-1} min^{-1} "in the 31 clearance periods in which both PAH clearance and extraction ratio were measured." These investigators reported an average value of 0.81 for the extraction ratio of PAH in the rat. The finding that the clearance values of TEA are about the same as those of PAH (Table I) suggests that the extraction ratio of TEA is comparable to that of PAH, a view in accord with that held by other investigators (6, 11).

The existence of two tubular transport mechanisms, one for acids such as PAH and the other for bases such as TEA, has been recognized since the fifties (10, 11). Uptake studies with renal slices have proved that the two transport mechanisms do not interfere with each other (12). Thus, in studying the renal effects of an acidic compound, it may be advantageous to measure renal plasma flow with a base. This view is supported by the findings of McNay *et al.* (13) that the blood acids in experimental uremia in the dog depress the extraction and clearance of PAH without affecting these parameters for TEA.

It is usually accepted that PAH and TEA are secreted only by the proximal tubules (1, 2). The extraction of these substances is reduced by the fraction of blood which perfuses the medulla and papilla and which is cleared of these substances only through glomerular filtration. Although incomplete filtration of a substance may result from binding to plasma protein, the binding of PAH to plasma proteins in the rat has been estimated to be only about 10% (8). Similarly the binding of TEA to plasma proteins is also negligible. Indeed Rennick and Farah (11) were unable to detect any binding of TEA to plasma proteins in the dog.

Objections may be raised to the use of TEA clearance as a measure of renal plasma flow because TEA causes cardiovascular responses (14). However, falls in blood pressure elicited by TEA are relatively short-lasting and can be avoided by using doses which are too small to affect the circulation. In the rat, moreover, as in man and the dog, autoregulation of the total renal blood flow and the glomerular filtration rate has been demonstrated (15–17); this phenomenon refers to the relative constancy of renal blood flow and glomerular filtration rate during marked changes in aortic blood pressure. The relatively constant values of TEA and inulin clearances in our experiments (Fig. 4) suggest that autoregulation was effectively controlling the renal blood flow and the glomerular filtration rate during falls in systemic blood pressure which may have occurred with the larger doses of TEA.

Our values of inulin clearance agree with the value of 6 ml kg^{-1} min^{-1} mentioned by Cortney *et al.* (8) for nondiuretic rats. Higher values of inulin clearance, up to about 12 ml kg^{-1} min^{-1}, have been reported by various

investigators (8, 16) in rats loaded with saline iv (isotonic or hypertonic) or water po, to promote diuresis.

The use of [^{14}C]TEA and ^{3}H-inulin permits the simultaneous estimation of glomerular filtration rate, renal plasma flow, and filtration fraction in the same unanesthetized rat.

Summary. Mean total body clearances were measured in unanesthetized rats as Dose iv/AUC in which AUC is the area under the plasma radioactivity disappearance curve for [^{14}C]labeled inulin, PAH, and TEA and also for [^{3}H]inulin. The clearance of inulin radioactivity, either ^{14}C or ^{3}H, was a satisfactory estimate of glomerular filtration rate. The clearance of [^{14}C]radioactivity from labeled TEA was a reliable estimate of renal plasma flow. The renal filtration fraction, which was calculated as the ratio of inulin and TEA clearances, varied from 0.15 to 0.30, with a mean value of 0.21 in the 20 rats in which both clearances were measured simultaneously.

Renal plasma flow and glomerular filtration rate can therefore be estimated in the same unanesthetized rat by the clearances of TEA and inulin, calculated as Dose iv/AUC from the disappearance curves of ^{14}C and ^{3}H plasma radioactivity after a single iv injection containing both [^{14}C]TEA and [^{3}H]methoxyinulin.

1. Pitts, R. F. "Physiology of the Kidneys and Body Fluids." 3rd ed. Year Book Medical Publishers, Chicago (1974).
2. Levinsky, N. G., and Levy, M. "Clearance Techniques in Renal Physiology, Sect. 8, Handbook of Physiology" (Orloff, J. and Berliner, R. W., eds.), p. 103. American Physiological Society, Washington, D.C. (1973).
3. Newman, E., Kattus, A., Genecin, A., Genest, J., Calkins, E., and Murphy, J., Bull. Johns Hopkins Hosp. **84,** 135 (1949).
4. Bauer, J. D., Ackerman, P. G., and Toro, G. "Bray's Clinical Laboratory Methods" 7th ed. Mosby, St. Louis (1968).
5. Bratton, A. C., and Marshall, E. K., Jr., J. Biol. Chem. **128,** 537 (1939).
6. Rennick, B., Moe, G. K., Lyons, R. H., Hobbler, S. W., and Neleigh, R. J., J. Pharmacol. Exp. Therap. **91,** 210 (1947).
7. Blaufox, M. D., Guttmann, R. D., and Merrill, J. P., Amer. J. Physiol. **212,** 629 (1967).
8. Cortney, M. A., Mylle, M., Lassiter, W. E., and Gottschalk, C. W., Amer. J. Physiol. **209,** 1199 (1965).
9. Peters, G., Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol. **235,** 113 (1959).
10. Peters, L., Pharmacol. Rev. **12,** 1 (1960).
11. Rennick, B., and Farah, A., J. Pharmacol. Exp. Ther. **116,** 287 (1956).
12. Farah, A. and Rennick, B., J. Pharmacol. Exp. Ther. **117,** 478 (1956).
13. McNay, J. L., Rosello, S., and Dayton, P. G., Amer. J. Physiol. **230,** 901 (1976).
14. Acheson, G. H., and Moe, G. K., J. Pharmacol. Exp. Ther. **87,** 220 (1946).
15. Kallskog, O., Lindbom, L. O., Ulfendahl, H. R., and Wolgast, M., Acta Physiol. Scand. **94,** 289 (1975).
16. Heller, J., and Horacek, V., Pflugers Arch. **370,** 81 (1977).
17. Arendshorst, W. J., Finn, W. F. and Gottschalk, C. W., Amer. J. Physiol. **228,** 127 (1975).

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