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KINETICS AND INHIBITION OF MEMBRANE-BOUND CARBONIC ANHYDRASE FROM CANINE RENAL CORTEX *

GAUTAM SANYAL, NESS I. PESSAH and THOMAS H. MAREN

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Box J-267, JHM Health Center, Gainesville, FL 32610 (U.S.A.)

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Summary

The membrane-bound carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) in the canine renal cortex has been characterized in terms of its CO_2 hydration kinetics and inhibition by sulfonamides and inorganic anions. Comparing these properties with those of the renal cytoplasmic and the human red cell B and C isozymes, it appears that the membrane enzyme is quite different from the soluble carbonic anhydrases. The turnover number of the particulate enzyme is about 3-times lower than that of the cytoplasmic enzyme. The membrane-bound enzyme is also different from its cytoplasmic counterpart in being more resistant against inhibition, particularly against Cl^-. Microsomes from the renal cortex were purified to yield luminal and anti-luminal fractions. Carbonic anhydrase activity was found in both. The luminal and anti-luminal carbonic anhydrases appeared similar in terms of their kinetic properties and susceptibility to inhibition.

Introduction

The kidney contains membrane-bound carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) which constitutes about 3—5% of the total carbonic anhydrase activity of the cortex [1—5]. Free-flow electrophoresis and gradient-centrifugation techniques have been used to separate the crude kidney microsomes into brush-border and basal-lateral membranes, and carbonic anhydrase

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

activity has been found in both [4,5]. The membrane-bound enzyme from human kidney is reported to have a high molecular weight, a higher resistance against denaturation, a different amino-acid composition and a different immunochemistry compared with the renal cytoplasmic enzyme [3,6], the latter being similar to the human red cell C enzyme. Maren and Ellison [2] showed that microsomal carbonic anhydrase in dog kidney is somewhat more resistant to inhibition by certain sulfonamides than its cytoplasmic counterpart. However, the critical kinetic parameters, i.e., the turnover number and K_m value of this membrane-bound enzyme have not been reported. We now report kinetic properties and inhibition pattern of this enzyme in the canine microsomes and in the purified luminal and anti-luminal fractions, and compare those with the renal cytoplasmic and the well-characterized human red-cell carbonic anhydrase isozymes.

Materials and Methods

Membrane preparations. Mongrel dog kidneys were excised and then thoroughly perfused, with 0.25 M sucrose, through the renal artery. The cytosol and the crude microsomes were obtained from the renal cortex homogenate by a centrifugation scheme described by Kinsella et al. [7]. The crude microsomes were then thoroughly washed with Tris-Hepes-mannitol buffer, pH 7.1, until the washing was absolutely free of carbonic anhydrase activity, as determined by CO_2 hydration. The Tris-Hepes-mannitol buffer contained 25 mM Hepes and 100 mM mannitol, adjusted to pH 7.1 with Tris. The luminal and anti-luminal fractions were obtained from the washed microsomes by using a selective precipitation technique [7].

Sulfonamide drugs. These were obtained from Lederle Laboratories, Pearl River, New York. Their properties have been described by Maren [13].

Enzyme activity. CO_2 hydration activity of carbonic anhydrase in kidney fractions was determined as described by Maren and Couto [8]. Enzyme activity is expressed in enzyme units (U), where 1 U indicates the amount of carbonic anhydrase that doubles the uncatalyzed CO_2 hydration rate at 0°C (using 100% CO_2 and barbital buffer).

Determination of total protein in all fractions was carried out by the method of Lowry et al. [9].

Inhibition studies. Enzyme inhibition was studied by following the effects of carbonic anhydrase inhibitors on the catalyzed CO_2 hydration rate at 0.5°C. The cytoplasmic or the microsomal fraction was mixed with the appropriate concentrations of inhibitors in the presence of 100% CO_2 (72 mM) and the initial rate was measured by the changing pH method, using barbital buffer [8].

Molar enzyme concentration. The molar concentrations of carbonic anhydrase in the kidney fractions were determined by specific titration of this enzyme with powerful sulfonamide inhibitors. This method yields, simultaneously, the molar enzyme concentration (E) and the dissociation constant (K_i) of the enzyme inhibitor complex. The equation involved is the following;

$$\frac{I_0}{i} = K_i(1 - i)^{-1} + E$$

where I_0 is the total inhibitor concentration and i is fractional inhibition. A plot of I_0/i vs. $(1-i)^{-1}$, known as the Easson-Stedman plot [10], yields E as the ordinal intercept and K_i as the slope. The application of this method to carbonic anhydrase inhibition has been described by Maren et al. [11]. Inhibition data were obtained at 0.5°C. The enzyme and the inhibitor were pre-equilibrated in presence of the substrate.

Kinetic method. Michaelis-Menten kinetics for CO_2 hydration were studied at 0.5°C (±0.1°C) by using the changing pH method of Maren and Couto [8]. 7 mM barbital buffer were used and the mean pH (the pH at which the buffer was half-titrated) was 7.5. K_m and V were obtained by a weighted least-squares analysis of initial rate data for four or five different CO_2 concentrations, assuming a constant absolute error in rate determinations [12]. The turnover number (k_{cat}) is V/E, E being obtained from the Easson-Stedman determination [10].

Detergent solubilization of membranes. Solubilization of the crude microsomal fraction was achieved by using 1% Triton X-100 in Tris-Hepes-mannitol buffer, pH 7.1. The microsomal suspension was centrifuged and the pellet was homogenized with Triton. This homogenate was spun at 47 000 × g for 30 min and the supernatant contained carbonic anhydrase activity from partially solubilized microsomes.

Results

Enzymic distribution

Table I shows the distribution of carbonic anhydrase activity in the cytosol and microsomal fractions of dog kidney. In terms of enzyme units/mg of total protein, the enzyme activity of the crude microsomes was about 14% that of the cytosol. When the microsomes were further purified to lumen and anti-lumen, carbonic anhydrase activity was found in both fractions. An enrichment of about 3-fold in enzyme activity was observed in the luminal fraction, relative to the crude microsomes, after purification.

Kinetics

Determination of enzyme concentration. Fig. 1 shows our Easson-Stedman determination of the enzyme concentration. For the cytoplasmic enzyme, we used 2-*O*-chlorophenylthiadiazole-5-sulfonamide [13] or chlorzolamide as the

TABLE I

DISTRIBUTION OF CARBONIC ANHYDRASE ACTIVITY IN DOG KIDNEY FRACTIONS (MEAN ± S.D.)

Data for cytosol and crude microsomes are from three dogs. Data for lumen and anti-lumen are from two dogs.

Fraction	Enzyme activity (U/mg protein)
Cytosol	12.1 ± 3.6
Crude microsomes	1.73 ± 0.8
Lumen	2.9 ± 0.2
Anti-lumen	0.90 ± 0.21

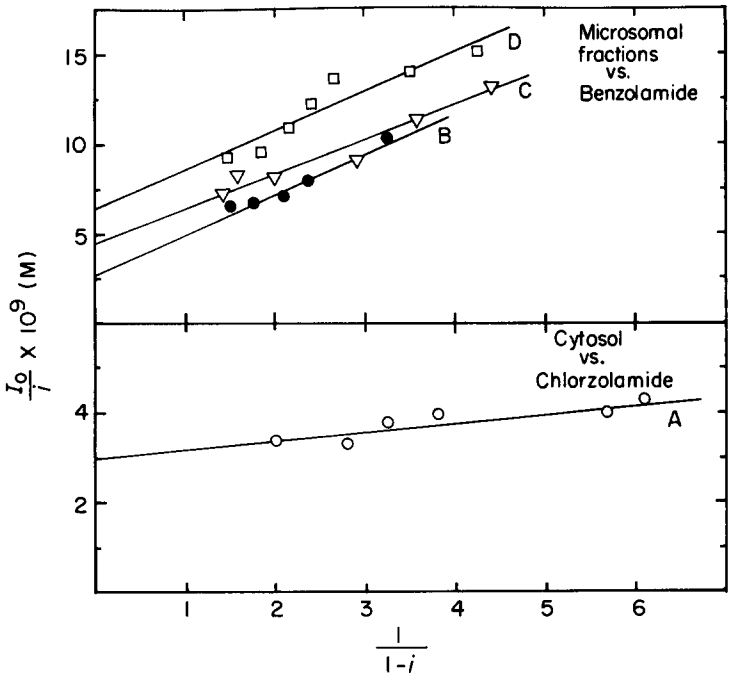

Fig. 1. Typical Easson-Stedman plots for inhibition of canine renal carbonic anhydrases CO_2 hydration reaction at 0.5° C, using 72 mM CO_2 and 7 mM barbital buffer. Enzyme, CO_2 and inhibitor are incubated for 1 min and reaction started by addition of buffer. A. Cytoplasmic enzyme vs. chlorzolamide (2-O-chlorphenylthiadiazole-5-sulfonamide), U = 2.0. B. Crude microsomes vs. benzolamide (2-benzenesulfonamido-1,3,4,thiadiazole-5-sulfonamide), U = 0.93. C. Anti-lumen vs. benzolamide, U = 1.4. D. Lumen vs. benzolamide, U = 1.7.

inhibitor. This was done because chlorzolamide is one of the strongest known inhibitors of human red cell C enzyme and requires very little time (less than 1 min) to achieve equilibration with the enzyme. An E_0 value of $1.5 \cdot 10^{-9}$ M per enzyme unit and a K_i value of $3 \cdot 10^{-10}$ M were obtained for dog renal cytosol. This E_0/U value is close to that known for human carbonic anhydrase C form [8]. Chlorzolamide was found to be almost 100-fold less active towards microsomal carbonic anhydrase confirming an earlier report [2], and consequently was not used for an accurate determination of enzyme concentration in this case. For the microsomal fractions, 2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide [11] or benzolamide was used in the Easson-Stedman titrations. Benzolamide required only 1 min for equilibration with the membrane-bound enzyme, in contrast to 4 min required for the cytoplasmic enzyme. This long incubation time requirement made benzolamide unsuitable for Easson-Stedman titration of the cytosol enzyme. The Easson-Stedman plots for benzolamide inhibition of crude microsomal, luminal and anti-luminal carbonic anhydrase are very similar to one another yielding a K_i value close to $2 \cdot 10^{-9}$ M in each case. The E_0/U values were $2.8 \cdot 10^{-9}$ M, $3.8 \cdot 10^{-9}$ M and $3.1 \cdot 10^{-9}$ M, respectively for the crude microsomes, lumen and anti-lumen.

K_m and k_{cat}. Fig. 2 illustrates the Lineweaver-Burk plots for CO_2 hydration by carbonic anhydrases in cytoplasmic and microsomal fractions. Table II gives

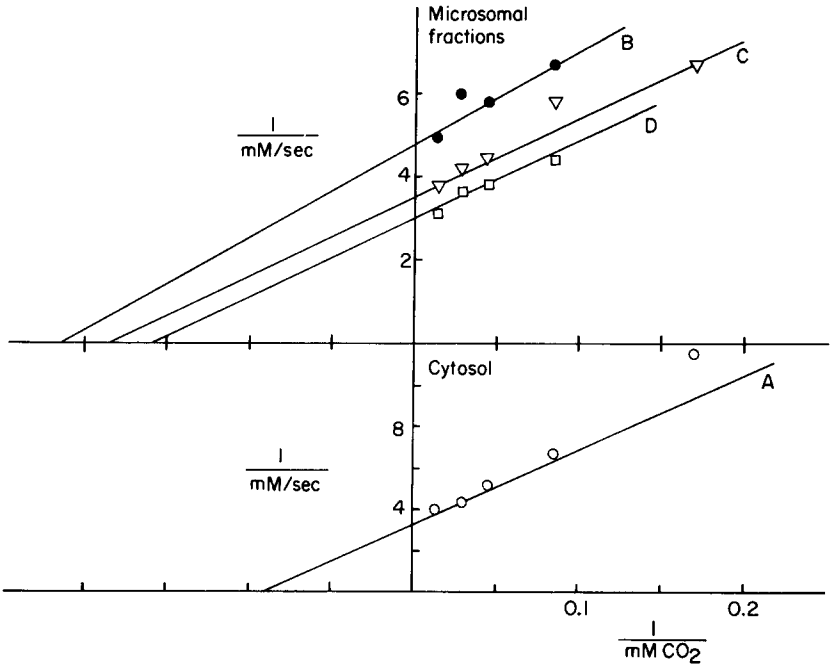

Fig. 2. Double-reciprocal plots of initial enzymic rates vs. CO_2 concentrations for canine renal carbonic anhydrase at 0.5°C and mean pH 7.5. Uncatalyzed rate constant is 0.0025 s^{-1}. The solid lines are drawn by weighted least-squares, assuming a constant absolute error in rate determinations. A. Cytoplasmic enzyme, $E = 1.8 \cdot 10^{-9}$ M. B. Crude microsomes, $E = 3.1 \cdot 10^{-9}$ M. C. Anti-lumen, $E = 4.3 \cdot 10^{-9}$ M. D. Lumen, $E = 6.5 \cdot 10^{-9}$ M. Enzyme concentrations are determined by the method given in Fig. 1.

the K_m and k_{cat} values for these fractions and compares the data with the kinetic constants for human red cell B and C isozymes. The luminal and anti-luminal fractions of the microsomes yielded K_m and k_{cat} values comparable with crude microsomes. The cytoplasmic enzyme yields a 3-fold higher k_{cat} ($1.63 \cdot 10^5$ s^{-1}) and a 2—3-fold higher K_m (13 mM) compared to the microsomes. When these data are compared with those for human red cell enzymes obtained under identical experimental conditions, the cytoplasmic enzyme appears to have a K_m value similar to and a k_{cat} value slightly lower than those

TABLE II

KINETIC COMPARISON OF HUMAN RED CELL AND CANINE RENAL CARBONIC ANHYDRASE ISOZYMES

All data are for CO_2 hydration at 0°C and pH 7.5.

	n	k_{cat} ($s^{-1} \cdot 10^{-4}$)	K_m (mM)
Human red blood cell			
C	3	23.6 ± 0.8	10.5 ± 1.3
B	6	2.92 ± 0.24	7.1 ± 2.5
Canine kidney			
Cytosol	3	16.3 ± 0.85	13.6 ± 2.9
Crude microsomes	1	6.7	4.7
Lumen	2	5.0	4.2 ± 1.5
Anti-lumen	1	6.6	6.3

TABLE III

Ki VALUES ($M \cdot 10^8$) OF SULFONAMIDE INHIBITORS AGAINST CANINE RENAL CARBONIC ANHYDRASES COMPARED TO HUMAN RED CELL B AND C ISOZYMES

Data are for CO_2 hydration at 0°C and pH 7.5. For sulfanilamide, acetolzamide and methazolamide, $K_i = I_{50}$. For the more powerful inhibitors benzolamide and chlorzolamide, the K_i value was calculated either from Easson-Stedman plots or by using the relation $K_i = I_{50} - 1/2 \cdot E_0$. HCAC, human carbonic anhydrase C form; HCAB, human carbonic anhydrase B form.

Inhibitor	HCAC	HCAB	Cytosol	Crude microsomes	Lumen	Anti-lumen
Sulfanilamide	200	5000	720	2100	2100	2500
Methazolamide	1.0	1.0	2.2	10	13	13
Acetazolamide	1.0	20	0.9	12	15	18
Benzolamide	0.04	0.2	0.08	0.15	0.25	0.19
Chlorzolamide	0.04	0.1	0.03	2.9	3.3	1.4
Ethoxzolamide	0.2	0.2	0.6	3.7	—	—

of the C enzyme. The renal microsomal fractions have K_m values comparable with human B enzyme and turnover numbers about 2-fold higher than that of the B enzyme.

Inhibition by sulfonamides. Table III gives K_i values of sulfonamide inhibition against the cytoplasmic, crude microsomal, luminal and anti-luminal fractions. The corresponding values for human red cell B and C enzyme are also given for comparison. Data for the crude microsomes, lumen and anti-lumen are nearly identical. These microsomal fractions are more resistant towards inhibition than the cytoplasmic enzyme, the most notable difference being exhibited by chlorzolamide, which is a 100-fold less active towards the microsomal enzyme. For the other sulfonamides used in our study, resistance of the microsomal carbonic anhydrase relative to the cytosol ranges from 3- to 13-fold in agreement with the findings of Wistrand and Kinne [4]. The incubation time requirements for enzyme-inhibitor equilibration were different for the cytosol and the membrane fractions. The microsomal enzyme required 1 min with benzolamide, 2 min with ethoxzolamide and none with the others. The cytoplasmic enzyme required 2 min with acetazolamide, and 4 min with benzolamide and ethoxzolamide, while equilibration was achieved in 1 min with

TABLE IV

COMPARISON OF K_i VALUES (mM) FOR ANION INHIBITION OF CO_2 HYDRATION BY HUMAN RED CELL CARBONIC ANHYDRASES AND DOG KIDNEY FRACTIONS

Data are for CO_2 hydration at 0°C and pH 7.5, using 72 mM CO_2. No incubation of the anionic inhibitors was necessary with any of the fractions. HCAC, human carbonic anhydrase form C; HCAB, human carbonic anhydrase form B.

Inhibitor	HCAC *	HCAB *	Cytosol	Microsomes	Lumen	Anti-lumen
KCNO	0.020	0.0007	0.010	0.042	—	—
KCNS	0.60	0.20	0.40	4.0	—	—
KI or NaI	26	0.30	8.9	110	125	74
NaCl	200	6.0	63	~2000	>500	900

* Data from Ref. 14.

TABLE V

COMPARISON OF CRUDE MICROSOMES AND TITRON-SOLUBILIZED MICROSOMES. KINETIC PARAMETERS AND INHIBITION DATA

Data are for CO_2 hydration at 0°C and pH 7.5, using 72 mM CO_2. None of the inhibitors, except benzolamide, required any measureable incubation time. Benzolamide needed 1 min with crude microsomes and 2 min with solubilized microsomes.

	k_{cat} ($s^{-1} \cdot 10^{-4}$)	K_m (mM)	K_i (mM)									
			Ethoxzolamide	Sulfanilamide	Acetazolamide	Methazolamide	Benzolamide	Chlorzolamide	Cl^-	I^-	CNS^-	CNO^-
Crude microsomes	6.7	4.7	$3.7 \cdot 10^{-5}$	$2.1 \cdot 10^{-2}$	$1.2 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-6}$	$2.9 \cdot 10^{-5}$	2400	110	4.0	$4.2 \cdot 10^{-2}$
Solubilized microsomes	6.6	5.7	$2.8 \cdot 10^{-5}$	$2.0 \cdot 10^{-2}$	$0.7 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	$0.8 \cdot 10^{-6}$	$1.5 \cdot 10^{-5}$	960	35	1.8	$2.8 \cdot 10^{-2}$

chlorzolamide and methazolamide. Some loss in activity of the cytoplasmic enzyme was observed after 4 min of incubation in the presence of substrate CO_2. This was taken into account in calculating the K_i values for benzolamide and ethoxzolamide against cytosol.

Inhibition by anions. Inhibition of the renal carbonic anhydrases by CNO^-, CNS^-, I^- and Cl^- was studied. The I_{50} values obtained against the cytosol and the membrane fractions are given in Table IV, which also includes data reported earlier on human carbonic anhydrase C and B forms [14], for comparison. The anions exhibited significantly greater inhibitory activity towards the cytosol relative to the membrane fractions. The smallest difference was found with KCNO, the strongest inhibitor in this series, the microsomal enzyme being about 3—4-times more resistant than the cytosol. CNS^- and I^- were about 10-fold more active against the cytosol enzyme compared with the microsomes. NaCl at 450 mM did not have an observable effect on the microsomal enzyme, while for the cytoplasmic enzyme an I_{50} value of 63 mM was obtained. It required about 2 M NaCl to produce 50% inhibition of the microsomal carbonic anhydrase. This caused a significant change in the ionic strength of the reaction medium. Incubation of the microsomes with Cl^- for up to 10 min did not increase the inhibitory activity of Cl^-. The anion inhibition data for the cytosol are close to the reported data for human carbonic anhydrase C form [14]. The membrane enzyme is highly resistant to anions and does not resemble either the human B or C enzyme. The B enzyme is more anion sensitive than any of the renal fractions or the C enzyme [14].

Kinetics and inhibition of detergent solubilized microsomal enzyme. The effects of solubilization of the microsomes, with 1% Triton X-100 at pH 7.1, on the kinetics and inhibition of microsomal carbonic anhydrase are summarized in Table V. Easson-Stedman titration (data not shown) of the Triton-solubilized microsomes against benzolamide yielded an E_0/U value of $2.8 \cdot 10^{-9}$ M, the same as the crude microsomes. The K_i value against benzolamide obtained from the same plot is $0.8 \cdot 10^{-9}$ M, 2.5-fold less compared to crude microsomes. The other sulfonamides that we tested were less discriminatory between the solubilized and the crude membrane-bound enzymes. The turnover number and the K_m for CO_2 hydration kinetics of the detergent-solubilized enzyme were also similar to those of the crude microsomal enzyme. The solubilized microsomal carbonic anhydrase appears to be somewhat more sensitive towards the anions compared to the unsolubilized membrane. Even so, the resistance against Cl^- is remarkable.

Discussion

Our results indicate that the renal cytoplasmic and membrane-bound enzymes are different isoenzymes of carbonic anhydrase. Kinetic similarity of the cytoplasmic enzyme from human kidney and the human red cell C enzyme has been reported by Wistrand and Wahlstrand [15], who obtained identical turnover numbers for CO_2 hydration by these two enzymes at 25°C. We also found cytoplasmic carbonic anhydrase from dog kidney to have k_{cat} and K_m at 0°C values, comparable with the human C enzyme. The turnover number for the microsomal membrane-bound carbonic anhydrase has so far not been

reported, since the molar concentration of the enzyme in a membrane preparation has not been determined. We have determined the molar enzyme concentration in microsomal preparations by titrating the enzyme with a powerful sulfonamide inhibitor (benzolamide). Our k_{cat} and K_m data for the crude microsomal, luminal and anti-luminal enzyme are very similar. The turnover number of this renal particulate carbonic anhydrase appears 3—4-fold lower than the human C enzyme and about 2-fold higher than the B enzyme.

What most distinguished the membrane enzyme from both human B and C isoenzymes were our inhibition data. The sulfonamide drugs differ greatly in their discrimination toward the cytosol and the membrane fractions, from almost none to 100-fold. The resistance of the membrane enzyme towards Cl^- is physiologically interesting, since the membrane is exposed to a higher chloride concentration than the cytoplasmic enzyme. Similar resistance towards Cl^- is exhibited by the shark rectal gland [16], and the avian salt gland and bladder carbonic anhydrases (Swenson, E.R., unpublished data). The Cl^- resistance of the microsomal enzyme did not change appreciably upon incubation of the membranes with Cl^- or upon detergent-solubilization of the membranes. Preliminary studies with the purified microsomal enzyme from human kidney have indicated that it is indeed different from the soluble isozymes of carbonic anhydrase [6].

Although the K_i values reported for microsomes are somewhat lower than those previously used for our physiological calculations (based on the K_i values for the soluble fraction), this does not alter, in any way, the conclusions from such experiments. Depending on the drug, the fractional inhibition at the peak effect would be somewhat less than previously reported; for example, in the case of acetazolamide, where the difference in K_i values is about 10-fold, the fractional inhibition at the peak effect would be 0.998 instead of 0.9998 [18]. For benzolamide, the difference would be even less, since there is only a 3-fold difference in K_i values between microsomes and soluble fractions. The essential point is that the physiological conclusions were drawn from the effects at the peak of the dose-response curve, and independent of the number calculated for fractional inhibition. For acetazolamide this was 10—20 mg/kg. For benzolamide this was 1 mg/kg [18].

Note added in proof (Received October 27th, 1980)

Since submission of this manuscript, we learned that human renal medulla contains only carbonic anhydrase C in cytosol (Wahlstrand, T. and Wistrand, P.J. (1980) Uppsala J. Med. Sci. 85, 7—17). This agrees with observations on the human renal cortex by the same workers [15] and with present data on the whole renal cortex of dog.

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