Studies on Excretion of Urea by the Toad Kidney.* (32181)

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Several studies have been reported on the mechanism of excretion of urea by the frog kidney. Attention was focused on this animal by the findings, several years ago, that the clearance of urea was several times higher than the glomerular filtration rate(1), indicating an active secretion of urea. Several lines of evidence, both on urea formation and urea excretion, suggest that a relationship might exist between active transport of urea and one or more of the ureogenic enzymes, particularly arginase. Information on formation of urea in the kidney indicated that: a) Renal homogenates form urea from endogenous material, at a rate higher than the liver, mostly from uric acid(2,3). *In vivo* renal formation of urea could not contribute more than 7% of the urinary urea, originating mostly from arginine hydrolysis (4). b) Arginase activity is higher in the kidney than in the liver(3), the level being among the highest described for this enzyme(5), and the other enzymes of the ornithine cycle are absent(3,6). c) Arginase activity is higher in the dorsal than in the ventral part of the kidney(7). d) The activity of urea biosynthetic enzymes increased sharply at stage XX of development of the tadpole(6). On the other hand several findings on urea excretion allow a correlation with the biochemical data indicated above. They are: a) *In vivo* hydrolysis of arginine has a maximum which is far below the rates *in vitro*(8). b) The active secretion of urea occurs in the proximal tubules, present mostly in the dorsal part of the kidney(9). c) It is at stage XX of development of the tadpole that the renal tubules start to have the capacity to secrete urea actively(6).

If arginase is involved in the active transport of urea, we could expect that in those animals which have not developed such a mechanism, renal arginase activity should be much lower or absent. In search of this information, the mechanism of excretion of urea and the level of the ornithine cycle enzymes in liver and kidney of *Bufo arenarum* were studied. In keeping with the view that arginase plays a role in active transport of urea, it will be shown that in *B. arenarum* a passive reabsorption of urea coexists with a low arginase concentration.

Little is known about excretion of urea in amphibian species other than *Rana catesbiana*. The main purpose of this study was to determine the mechanisms and sites of formation and excretion of urea in the toad.

Methods. Adult south american toads, *B. arenarum,* weighing between 100 and 250 g, were anesthetized by immersion in methane tricainesulphonate (2 g per liter) and operated on by a modification of the technique of Uranga and Sawyer(10), which consisted in cannulating both mesonephric ducts and the left femoral artery by a dorsal approach, and the left musculocutaneous vein through a dorsolateral incision. Three to twenty-four hours later the animals, fully awake, were placed in water in individual cubicles. They were primed, per 100 g of weight, with 4 μc of ^{14}C labelled urea of specific activity of 1 μc/μmol and 125 mg of inulin, diluted in isotonic saline. Immediately afterward a constant infusion was started through the musculacutaneous vein, by means of which 0.55 μc of ^{14}C urea and 11.5 mg of inulin per 100 g weight were injected per hour in a volume of 0.45 ml/hr. Blood samples were obtained at the beginning and end of each urinary collection period from the femoral artery. Urine flow was determined

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gravimetrically. The specific activity of urea (4 animals) and the level of inulin were stable in plasma after 30-60 minutes of infusion. After a minimum of one hour of perfusion one to 6 urine collections were made, lasting 10 to 40 minutes each. By collecting the urine from the mesonephric ducts we avoided the possibility of equilibration of urea in the bladder between urine and plasma.

Chemical urea was measured according to the technique of Fawcett and Scott(11). Inulin was measured by the method of Rolf *et al* (12). Total nitrogen was determined by a modified micro-Kjeldahl technique(13). Urea ^{14}C was measured in a low background gas-flow counter. We assume that all the radioactivity measured is in the form of ^{14}C labelled urea. This is probably a safe assumption to make, since at least in *R. catesbiana,* infused with ^{14}C labelled urea, all plasmatic and urinary radioactivity was in the form of urea, as determined experimentally by enzymatic separation(4). Boiling of tissue slices was done according to the technique of Appelboom *et al*(14).

Measurement of ornithine cycle enzymes. The activity of ornithine transcarbamoylase (carbamoyl phosphate-L-ornithine carbamoyltransferase, EC 2.1, 3.3), argininosuccinate synthetase [L-citrulline-L-aspartate ligase (AMP) EC 6.3, 4.5], argininosuccinase (L-argininosuccinate arginine-lyase, EC 4.3, 2.1) and arginase (L-arginine ureohydrolase, EC 3.5, 3.1) were determined according to a modification of the method of Brown and Cohen(15).

Liver and kidney were homogenized in a Potter-Elvehjem all-glass homogenizer with 9 volumes of either water or 0.1% cetyltrimethylammonium bromide and centrifuged twice at 4000 G, for 15 minutes, at 4°C. With the exception of the arginine-synthetase system, and the argininosuccinate cleavage enzyme in which the first supernatant was used, the enzyme extract for the other enzyme assays contained equal parts of the first and second supernatant, having therefore a dilution of 1/20 with respect to the original tissue. The reactions were stopped by addition of 0.5 M perchloric acid to a final volume of 7 ml except for the argininosuccinate cleavage enzyme in which 0.2 ml of 5 M perchloric acid was used instead. The blanks were done with either boiled enzyme or with addition of perchloric acid prior to addition of the substrate. Carbamoyl phosphate synthetase‡ was measured in water homogenates extracted twice as described by Brown and Cohen, but incubated in a different medium, containing 50 μmoles of ammonium chloride, 100 μmoles of potassium bicarbonate, 5 μmoles of L-acetyl-glutamic acid, 5 μmoles of ATP, 10 μmoles of magnesium sulphate, 5 μmoles of L-ornithine, 50 μmoles of Tris Buffer pH 7.4, 150 units of partially purified ornithine transcarbamoylase, and 0.45 ml of supernatant diluted 1/16, in a final volume of one ml. Rat liver ornithine transcarbamoylase was purified by a modification of the technique of Burnett and Cohen(16) and tested to demonstrate the lack of carbamoyl phosphate synthetase activity. Ornithine transcarbamoylase was determined in water instead of CTB extracts and the incubation medium contained 20 μmoles of L-ornithine, 20 μmoles of dilithium carbamoylphosphate, 90 μmoles of glycylglycine buffer pH = 8.4, and 0.2 ml of the extract mixture, in a final volume of 2 ml. The length of incubation was 15 minutes. Argininosuccinate synthetase and argininosuccinase were determined as a single activity (arginine-synthetase system) in CTB extracts. The incubation medium contained 5 μmoles of $MgSO_4$, 5 μmoles of ATP (disodium salt), 5 μmoles of L-citrulline, 5 μmoles of L-aspartic acid, 50 μmoles of phosphate buffer pH = 7.3 and an excess of beef liver arginase (4 units; 1 unit equal to the amount which forms 1 μmole of urea per minute); and 0.4 ml of the tissue extract in a volume of 1 ml. Incubation time was 60 minutes. The argininosuccinate cleavage enzyme was assayed in a medium containing 2 μmoles of argininosuccinate pH = 7.0; 50 μmoles of potassium phosphate buffer, pH = 7.3; excess beef liver arginase and 0.4 ml of the first supernatant, in a final volume of 1 ml. Incubation was for 20 minutes. The reaction was stopped by addition of 0.2 ml of 5 M perchloric acid. Arginase assays were

‡ Carbamoyl phosphate synthetase has not been numbered by the Commission on Enzymes.

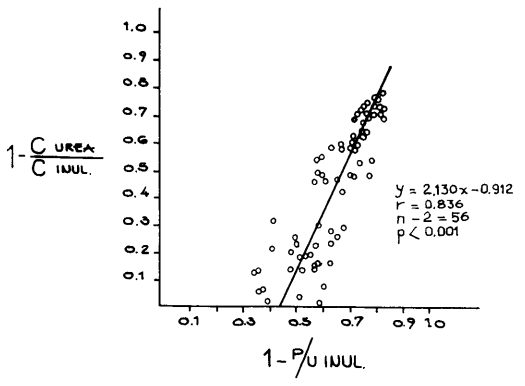

FIG. 1. Relationship between urea reabsorption and water reabsorption. $1-\frac{C\ urea}{C\ inul}$ = fraction of filtered urea reabsorbed. 1—P/U inul = fraction of filtered water reabsorbed.

carried in CTB extracts; the incubation medium contained 50 μmoles of a Na glycinate buffer, pH = 9.5, 0.5 μmol of Cl_2 Mn, 85 μmoles of L-arginine, 0.3 ml of the supernatant mixture, in a volume of 1 ml. Incubation time was 30 minutes. The product of the first two assay systems, citrulline, and of the latter two, urea, were measured according to the method of Archibald(17). These procedures were used on rat liver homogenates before applying them to homogenates of liver and kidney of *B. arenarum* and the values obtained coincided with those of the literature(5). Both analytical and incubation duplicates were performed in all cases. Units are defined as 1 μmol of product per minute per gram of wet tissue.

Results. To determine the manner in which the renal tubules of the toad handle urea, the renal excretion of urea and inulin was studied. A total of 58 clearance periods of ^{14}C urea and inulin was determined in 12 animals. These results, expressed as fraction of filtered water reabsorbed *vs* fraction of filtered urea reabsorbed, are plotted in Fig. 1, where the regression line is also indicated. The correlation between the 2 parameters was positive ($r = 0.836$, $p<0.001$). It can be seen that 48% of water is reabsorbed before urea starts to move along. The concentration of chemical urea in the plasma, measured in 6 animals, was very high and varied beween 4 and 40 mM. Knowing the plasmatic concentration of ^{14}C urea and assuming a volume of distribution of urea of 70% of body weight, the total amount of ^{14}C urea present in the animal at the end of the experiment was calculated. This amount of ^{14}C urea plus the amount eliminated in the urine accounted for about 80% of the injected urea. The remaining 20% must have been eliminated by a route other than the kidney.

To determine whether any correlation could be drawn between the level of ornithine cycle enzymes and the renal excretion of urea, their levels were determined in homogenates of kidney and liver. Table I presents these data. Activity of carbamoyl phosphate synthetase in the kidney could not be detected. The concentrations of the other 4 enzymes were well below those of the liver. The level of argininosuccinate synthetase was, in relation to the others, the one closest to the hepatic level. In 2 animals, not included in the Table, the level of the argininosuccinate cleavage enzyme was determined in liver and kidney. The values obtained in the liver were 1.48 and 1.68 μmoles/min/g of wet tissue and in the kidney 1.09 and 1.2 μmoles/min/g of wet tissue, this enzyme being therefore in excess with respect to the argininosuccinate synthetase enzyme. The level of carbamoyl phosphate synthetase in the liver was relatively large compared with that of the other enzymes. The hepatic level of the arginine synthetase system indicates that argininosucci-

TABLE I. Level of the Ornithine Cycle Enzymes in Liver and Kidney of *Bufo arenarum*.

	CPS	OTC	ASS	Arginase
Liver	4.39 ± 1.5 (4)	88 ± 1.43 (3)	.88 ± .09 (5)	88 ± 5 (3)
Kidney	B.L.D. (3)	.72 ± .17 (3)	.26 ± .17 (3)	1.92 ± .35 (3)

The level is expressed as the mean ± standard deviation in units where 1 unit = 1 μmol of product per minute per g of wet tissue. The numbers in parentheses indicate the number of animals studied.

CPS: Carbamoyl phosphate synthetase. OTC: Ornithine transcarbamoylase. ASS: Arginine synthetase system. B.L.D.: Below level of detection.

TABLE II. Urinary Excretion of Nitrogen in *Bufo arenarum*.

Toad No.	Ureter	Total nitrogen (mg/h/kg)	Urea nitrogen (% of total)	Ammonia nitrogen (% of total)
13	Left	27.8	60.4	14.1
	Right	26.2	65.4	14.6
14	Left	16.7	74.4	17.5
	Right	18.8	77.3	18.4
15	Left	20.5	82.3	18.3
	Right	24.2	75.7	17.1
16	Left	1.69	84.0	16.1
	Right	1.52	87.2	17.0

nate synthetase is the rate-limiting enzyme, as is the case in other systems, and is, under the conditions assayed, near the minimum required for the maximal rate of urea excretion (20 μmoles/hr).

The existence of two arginases that differ electrophoretically and immunologically has been determined in several ureotelic and uricotelic species(18). It was therefore of interest to compare the Km from renal and hepatic arginase of this animal. The Km of hepatic arginase in 4 animals was $33.7 \times 10^{-3} \pm$ st. dev. 7.1×10^{-3} M, and the Km of renal arginase in 6 animals was $16.1 \times 10^{-3} \pm$ st. dev. 5.4×10^{-3} M. This difference between the affinity for the substrate of hepatic and renal arginase is much smaller than the one observed by Soberon *et al* for the different arginases they studied. Although Km values alone are not enough evidence to allow a definite conclusion, the Km observed here for both arginases is not far from the Km described for the hepatic arginase of ureotelic animals, suggesting that the enzyme from both organs is not different.

To see whether the hepatic enzyme level could account for all of the excreted urea, the rate of urinary excretion as well as the percentage of urinary nitrogen which was in the form of urea were determined. These data, shown in Table II indicate that most of the excreted nitrogen was in the form of urea, having an average of 76% $\pm$ st. dev. 8.7.

To determine whether any fraction of the urinary urea was formed in the kidney, the specific activity of urea was determined in the urine and plasma of 4 animals (previously included in Fig. 1). If a substantial amount of urea is formed in the kidney and passes into the urine its specific activity in the urine would be lower than that in the plasma. The results of one typical experiment, shown in Table III, indicate that this is not the case. As can be seen, the U/P count/U/P urea was not different from unity; its average in the 4 animals was 1.01 $\pm$ st. dev. 0.09.

The concentration of urea in the kidney was determined in 3 animals. The slices from each kidney were boiled immediately after the end of the experiment, within 4 minutes of killing the animal, and the concentration of chemical and labelled urea were measured in the supernatant. The concentration in tissue water was calculated on the assumption that the fresh tissue contained 80% water. The results are indicated in Table IV. It can be seen that urea accumulates in tissue water up to 3 times the plasma concentration. These values are well below those found in the kidney of *R. catesbiana,* which secretes urea actively, and approach those found when active secretion is inhibited with 2,4-DNP(8). In one animal (No. 9) the tissue water/plasma ratio was higher for chemical than for labelled urea. This might be due either to endogenous urea formation or to incomplete equilibration of ^{14}C urea with tissue urea.

Discussion. Clearances of urea are lower than those of inulin, and the correlation between urea and water reabsorption indicates that in the toad urea diffuses passively from lumen to blood. The comparatively larger reduction of the amount of tubular water would produce an increase in urea concentration in tubular lumen which may eventually be higher that the urea concentration in the cells. On this basis, urea may passively diffuse down the concentration gradient. The plasmatic

TABLE III. Determination of Fraction of Excreted Urea Formed in the Kidney.

Ureter	Time,* min	U/P inul.	GFR, ml/h/kg	Plasma urea, mM	C of chemical urea, ml/h/kg	C of ^{14}C labelled urea / GFR	U/P count / U/P chemical urea
Left	137-153	3.02	13.24	3.78	4.95	.41	1.09
	153-174	4.10	8.77	3.78	2.39	.25	.92
	174-192	3.90	8.42	3.67	2.46	.27	.96
Right	137-153	4.39	12.56	3.78	3.52	.30	1.08
	153-174	5.61	7.42	3.78	1.57	.21	1.01
	174-192	4.34	5.40	3.67	1.70	.36	1.15

* In the time column, starting time coincides with administration of the priming dose of inulin and ^{14}C labelled urea, immediately followed by beginning of constant perfusion.
GFR: Glomerular filtration rate; C: Clearance.

concentration of chemical urea, which can be as high as 40 mM, is well above the one measured in the bullfrog. This might be related to the different environment inhabited by these species. In contrast to *R. catesbiana,* the *B. arenarum* does not live in a completely aquatic environment and its high plasmatic concentration of urea might play an asmotic role in the conservation of water.

The concentration of arginase in the kidney of an animal which reabsorbs urea passively is around 1.92 μmoles/min/g of tissue, while that of *R. catesbiana* which secretes urea actively is around 1000 μmoles/min/g of tissue. This fact indirectly supports the findings which suggest the participation of this enzyme in the active transport of urea. The physiological significance of the presence of ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinase in the kidney of *B. arenarum* and its absence in *R. catesbiana*(3,6) is not apparent.

The similarity of the specific activity of urea in the urine and plasma of *B. arenarum* indicates that no significant part of urinary urea is formed in the kidney of this species. The *in vitro* levels of renal arginase activity in *R. catesbiana* are much greater than the *in vivo* levels(8) suggesting that part of the arginase activity observed *in vitro* is due to a different enzyme which could be involved in the process of active transport of urea. The demonstration of two different arginases in the liver of several species(18) is in line with this possibility. In *B. arenarum,* the Km of renal arginase does not differ significantly from that of hepatic arginase, suggesting that these enzymes are not different.

TABLE IV. Urea Accumulation in the Kidney.

Toad No.	Ureter	Tissue water/plasma Urea	Count
9	Left	3.60	1.25
	Right	3.14	1.26
10	Left	1.13	1.04
	Right	1.25	1.13
16	Left	1.77	1.57
	Right	1.87	1.79

Summary. Clearances of ^{14}C labelled urea, chemical urea and inulin, were determined under steady-state conditions in the toad *Bufo arenarum.* Clearances of urea were always lower than those of inulin indicating that urea is reabsorbed. In contrast with *Rana catesbiana,* the kidney of *B. arenarum* has a very low level of arginase (1.92 μmoles/min/g of wet tissue), and all the other enzymes of the ornithine cycle are present, with the exception of carbamoyl phosphate synthetase. It is demonstrated that no substantial amount of urinary urea is formed in the kidney of this species. The low level of arginase, in a species which reabsorbs urea passively, strengthens the hypothesis that active transport of urea is related to renal arginase activity.

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A Toxic Effect of 2-Thiouracil on Pyrimidine Metabolism.* (32182)

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2-Thiouracil, an antithyroid compound, is also an inhibitor in pathways of nucleic acid metabolism(1,2). It has been reported that the antithyroid effect results from an interference with the iodination of thyroxine precursors rather than as a result of any effect on nucleic acid metabolism(2,3,4).

The effect of 2-thiouracil on nucleic acid metabolism in animals seems to result in an inhibition of growth and development. This inhibition is suggested because the compound inhibits plant protein and RNA synthesis, has an antiviral effect against numerous plant and animal viruses, and interferes with the development of sea urchin eggs(5,6,7,8). The present study presents evidence for a precise effect of 2-thiouracil on nucleic acid metabolism detected by following growth response in an organism while nucleic acid metabolism was influenced by the presence of 2-thiouracil. *Escherichia coli* was chosen as the organism for study to eliminate thyroid effects. An *E. coli* auxotroph requiring uracil or orotic acid for growth was used to determine the effect of 2-thiouracil on utilization of uracil and orotic acid.

Materials and methods. E. coli cells were grown at 37° without aeration in a minimal salt medium containing 14.0 g K_2HPO_4, 6.0 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, and 10 g glucose in 1000 ml. Uracil (3.9×10^{-4} M) was added to the medium to maintain the uracil-requiring *E. coli* auxotroph. The two *E. coli* organisms used in these experiments were isolated as revertants from an *E. coli* auxotroph obtained from Dr. Norman Durham, Oklahoma State University. One organism required uracil or orotic acid for growth and was designated as the uracil-requiring (Ura^-) *E. coli* strain. The uracil requirement was then eliminated from the Ura^- strain, and the resulting organism requiring no uracil addition to the minimal salt medium for growth was designated as the non-uracil-requiring (Ura^+) *E. coli* strain. L-aspartic acid, DL-ureidosuccinic acid, and dihydro-L-orotic acid would not support the growth of the Ura^- strain. Biochemical compounds used in these studies were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth of the organism was followed by

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