Review

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Creatinine and HMH (5-hydroxy-1-methylhydantoin, NZ-419) as intrinsic hydroxyl radical scavengers

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ABSTRACT: Creatinine (Crn) is one of the main intrinsic hydroxyl radical (•OH) scavengers and an ideal one for healthy or normal mammals, although this fact has not yet become widely accepted. Our results from urinary data estimated that ca. 0.4-0.6% of Crn is used daily to scavenge •OH in normal mammals [ca. 50 μmole and ca. 400 pmole of •OH in healthy subjects and normal rats, respectively]. In human subjects, Crn reacts non-enzymatically with •OH to form creatol (CTL: 5-hydroxycreatinine) and demethylcreatinine (DMC) in a one to one ratio, and CTL partially decomposes to methylguanidine (MG). And so, the scavenged mole of •OH by Crn is nearly equal to their molar total sum (CTL + MG + DMC) or 2 × (CTL + MG). The molar ratio of (scavenged •OH)/Crn in healthy subjects and normal rats are 4.4 and 6.0 mmole/mole, respectively, *i.e.* almost similar, but in patients with chronic kidney disease (CKD) the ratio increases up to ca. 60 mmole/mole in proportion to the severity of CKD. Since the level of Crn might not be enough to scavenge all •OH, and MG starts accumulating as a uremic toxin, Crn is not really the ideal scavenger. 5-Hydroxy-1-methylhydantoin (HMH, NZ-419), a Crn metabolite, is another antioxidant, having •OH scavenging ability, and has been shown to inhibit the progression of CKD in rats in stead of Crn, if sufficient amounts are given orally.

Keywords: Creatinine, HMH (NZ-419), intrinsic antioxidant, creatol, methylguanidine, demethylcreatinine

1. Introduction

Creatinine (Crn) is one of the main intrinsic hydroxyl radical (•OH) scavengers (*1-8*), but this simple fact has not yet become widely accepted. One main reason might be an incorrect old belief that Crn has been taught to be an end-metabolite in mammals. However, this belief is not true. Therefore, we first introduced a common dual oxidative metabolic pathway of Crn in mammals (Figure 1) (*3,9-11*), *via* creatol (CTL: 5-hydroxycreatinine, 2-amino-4-hydroxy-3-methyl-4*H*-imidazol-5-one, CAS: 133882-98-1) (Figure 1) (*3,9-17*), and 5-hydroxy-1-methylhydantoin (HMH, NZ-419, 5-hydroxy-1-methylimidazolidine-2,4-dione, CAS: 84210-26-4) (Figure 1) (*3,9-12,18,19*). In this review, we add a further bypass venue, from Crn to demethylcreatinine (DMC, glycocyamidine: GA, 2-amino-1,4-dihydroimidazol-5-one, CAS: 503-86-6) (Figure 1) (*20*) in addition to the above recognized pathways.

The chemistry of Crn has advanced dramatically since Ienaga *et al*. isolated HMH from inflamed rabbit skin tissues in 1987 (*21*) thanks to new bioorganic technologies such as high performance liquid chromatography (HPLC) purification, instrumental and spectroscopic analyses and so on. Before that, there were hundreds of mixed right and wrong reports on the chemistry and/or biochemistry of Crn. However, in seeking more knowledge of the properties of the purely synthetic authentic samples of Crn metabolites [HMH (*19*), CTL (*20*), creatone-A (CTO-A, 2-amino-1-methyl-4,5-imidazoledione, CAS: 115012-08-3) (Figure 1) (*13*), creatone-B (CTO-B, *N*-(*N'*-methylamidino)oxamic acid, CAS: 51093-33-5) (Figure 1) (*13*), DMC (*20*) and so on], we looked at their physicochemical properties in order to understand their reactions.

Giovannetti *et al*. indicated that methylguanidine (MG, CAS: 471-29-4) might be one of the uremic toxins (*22,23*), and that the classical analytical values of MG using pre-treatment with activated charcoal were too high. The formation of MG by charcoal-catalyzed oxidation of Crn was significant and gave false readings (*24*). It was recognized that Crn and creatine (Cr) could be oxidized to MG *via* creatone (CTO) (*25,26*), but they did not know the precise mechanisms of the oxidation. It was thought that such oxidations were mere chemical reactions before Aoyagi and Nagase indicated that the non-enzymatic reaction of Crn into MG occurred also *in*

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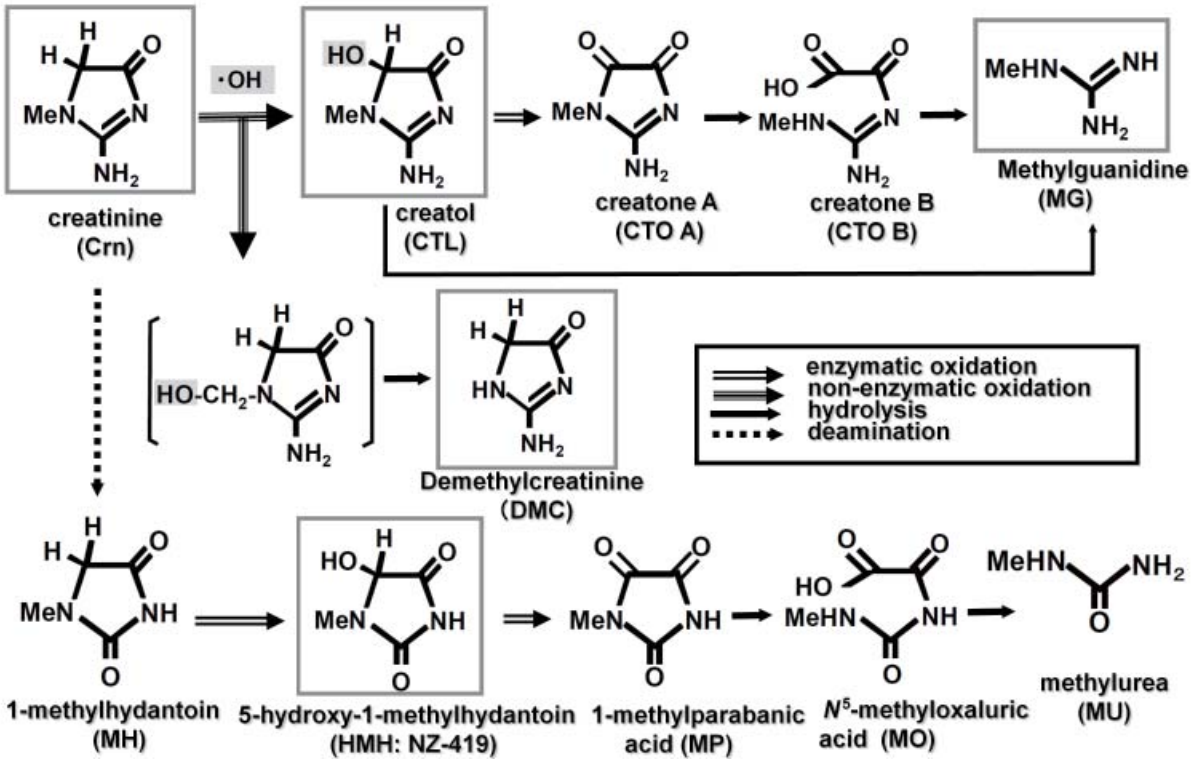


Figure 1. Metabolic pathways of Crn and three key metabolites, HMH, CTL and DMC. The compounds enclosed in boxes were detectable in the sera of CKD patients at stages 3, 4 and 5.

Table 1. Stage of chronic kidney disease

Stage	Description	Clinical GFR*,*** (mL/min/1.73 m^2)	Relative GFR/GFR_0	Rat GFR** (mL/min/kg)
1	Kidney damage with normal or ↑GFR	≥ 90		
2	Kidney damage with mild ↓GFR	60-89		
3	Moderate ↓GFR	30-59	0.30-0.59	1.7-3.3
4	Severe ↓GFR	15-29	0.15-0.29	0.9-1.6
5	Kidney failure	< 15 (or dialysis)	< 0.15	< 0.9

* Clinical GFR_0, GFR of normal subjects, has been reported to be about 100 mL/min/1.73 m^2.
** Rat GFR_0, GFR of normal rats, has been reported to be about ca. 5.7 mL/min/kg.
*** The National Kidney Foundation. K/DQI Clinical practice guidelines on chronic kidney disease (2002).
We classified CKD stages of rats based on rat GFR. Ienaga & Yokozawa (2010).

vivo, and they recognized that the main reactive oxygen species (ROS) in this oxidation was the •OH radical, at least in hepatocytes (*27-29*). Since then Yokozawa *et al*. indicated that the precursor of MG *in vivo* was truly Crn (*30*), and we hypothesized a new pathway *via* CTL which had been just isolated from the urine of creatininemia patients (*12*). We soon proved the hypothesis that a dual metabolic pathway of Crn *via* CTL or HMH was common in mammals by using a ^{13}C-labeling technique (*9*), and began to investigate the precise metabolic pathways of Crn which are discussed below.

For the precise determination of MG in the serum of human subjects, we needed to take special sampling precautions. For example, abstention from sake (rice wine) on the previous day is recommended, because acetylagmatine in sake interferes with the analyses (*31*).

Crn has been taught as being a key compound in monitoring kidney function for chronic kidney disease (CKD) patients. This is even now true, and recently serum Crn (sCrn) is widely used to calculate the estimated glomerular filtration rate (eGFR) (*32-34*). As shown below, up to ca. 10% of Crn is catabolized daily *via* HMH, CTL, and probably DMC in diabetic (DM) and/or CKD patients. The problems of catabolism as well as excretion for CKD patients might be a kind of warning bell to use sCrn. However, the estimation of GFR is based on the correlation between the GFR and the sCrn value itself. Regardless of such problems, eGFR seems to remain important. Since we have classified rats into five stages of CKD as well, we can now use the data in Table 1 to define the extent of kidney damage in human patients and in rats (*6*).

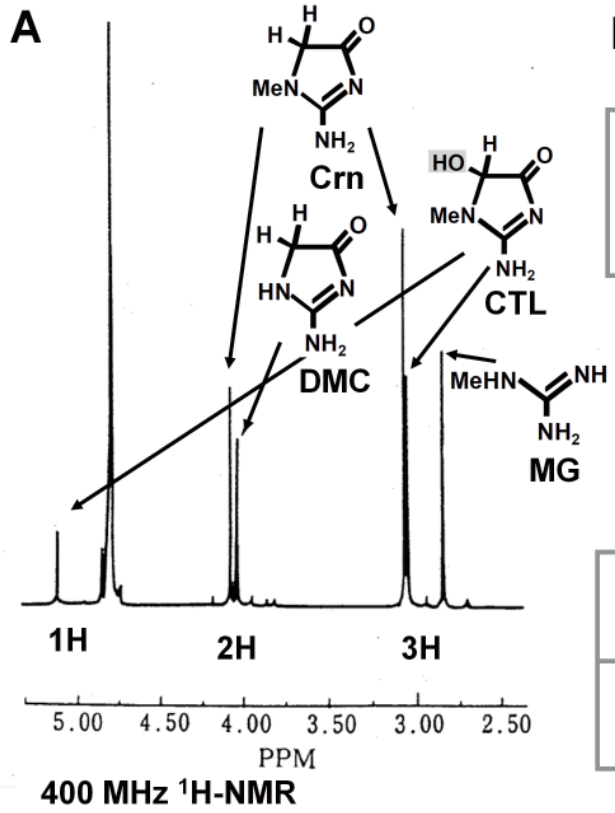
Principally, we explain in this review how Crn reacts with •OH radicals and acts as an •OH radical scavenger. We revised the correct structures of CTO-A and CTO-B (*13*), and proposed the •OH related oxidation mechanism of Crn *via* CTL, CTO-A, CTO-B to MG (Figure 2B). Our *in vivo* Crn pathway, including the pathway to DMC, has been already reproduced in cell-free *in vitro* test tube experiments under Fenton's reaction conditions (*15*). By reviewing clinical data, we not only showed that Crn seems to be a good intrinsic •OH scavenger, but it also allowed us to estimate how many moles of •OH might be scavenged daily in normal rats and healthy human subjects. For CKD patients, however, Crn cannot now be considered as the ideal compound (*35*), because CTL and MG as well as Crn start to accumulate inside bodies. We want to show here that the Crn might not be enough to scavenge all of the •OH radicals present, although up to ca. 3% of Crn it seems to be used to scavenge •OH radicals in CKD patients just before therapy for end-stage renal disease (ESRD) (*7,8*).

Last we want to introduce HMH as another, and possibly, alternative intrinsic antioxidant (*3,10,36-39*). Intrinsic levels of HMH increase in mammals (*8,10*) in DM and CKD patients and orally administered HMH has prevented the progression of CKD at stages 3-5 in rats (*3,5,6,40,41*). We believe that HMH could act as a 'self-defense' substance.

2. Mammalian metabolic pathways of Crn

About twenty years ago, Ienaga *et al*. isolated HMH (*20*) as a novel rice germination regulator together with MH (1-methylhydantoin, 1-methylimidazolidine-2,4-dione, CAS: 616-04-6) (Figure 1) from inflamed rabbit skin tissues but not from normal skin tissues. Others isolated MH as a rice germination regulator from some anaerobic microbes (*42*), but not together with HMH. The Crn metabolic pathway to sarcosine *via* MH in anaerobic microbes had been well clarified (*11*). We soon proved that once MH (formed probably under anaerobic conditions) is absorbed into aerobic mammalian bodies, it is converted by oxidation readily to HMH which is then metabolized to methylurea (MU: CAS: 623-59-6) (Figure 1) (*18,19*).

Analogically-based thinking about chemical structures led us to the isolation of CTL, together with HMH, from urine of creatininemia patients (*12*). Crn reacts non-enzymatically, at first with •OH radicals to give CTL or DMC, and then CTL is further enzymatically oxidized to CTO-A which is hydrolyzed to MG *via* CTO-B (Figures 1 and 2B). Under slow reaction conditions [ex. Crn (20 mM), $K_3Fe(CN)_6$ (0.1 mM), 3% H_2O_2 (100 mL), 25ºC, 2 days], only peaks of Crn, CTL and DMC were detectable in ^{1}H-NMR spectra (Figure 2A) and HPLC chromatograms. In contrast, under faster reaction conditions [ex. Crn



B Formation of CTL, MG & DMC

Crn → CTL → CTO-A → CTO-B → MG
↓ ·OH — oxidation
DMC — hydrolysis

C Oxidation of CTL into MG

	Non-enymatic	Vitamin C synthase	*L*-2-hydroxyl acid oxidase
Rat liver	○	○	
Rat kidney	○		○
Human Liver	○		
Human kidney	○		○

Figure 2. Fenton's reaction of Crn. A: Proton-NMR spectrum of the reaction mixture. **B:** Reaction scheme observed in the reaction mixture. **C:** Enzymatic and non-enzymatic conversion of CTL to MG.

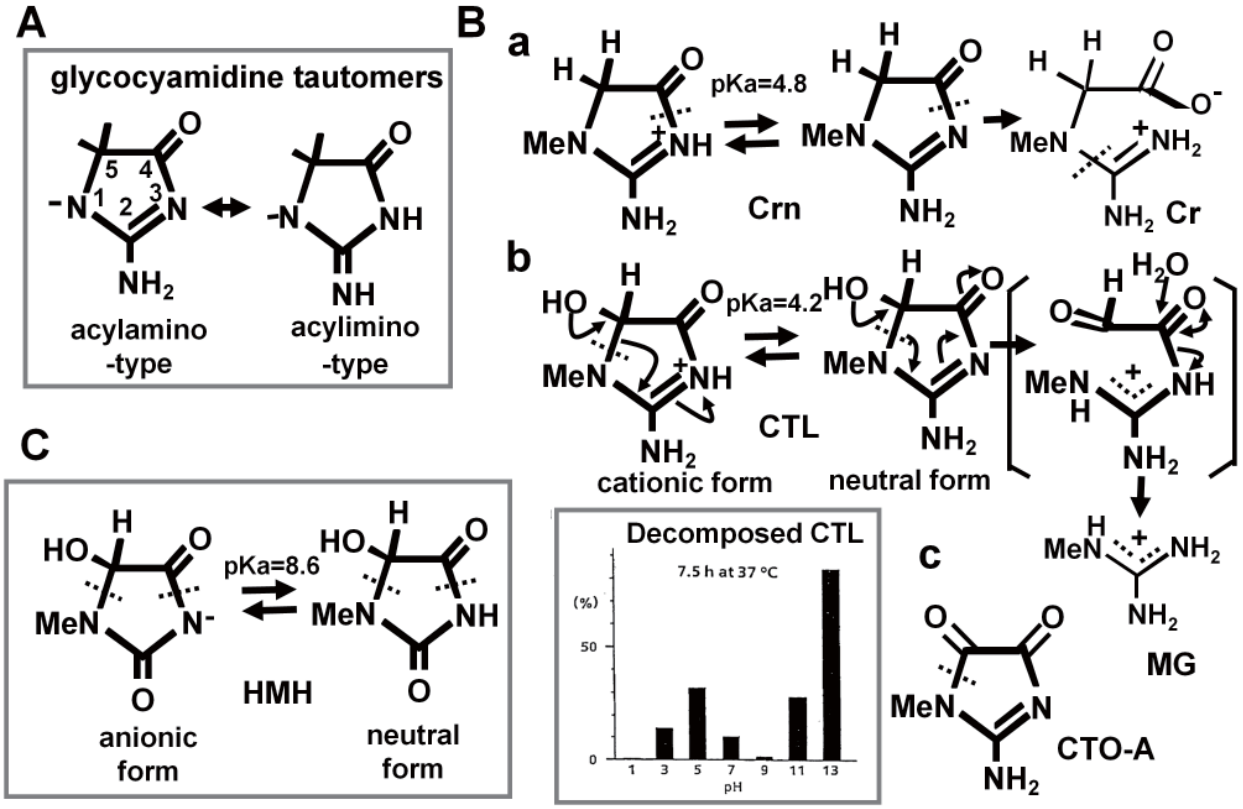


Figure 3. Physicochemical properties of Crn derivatives. A: Glycocyamidine tautomers. **B:** Ring opening reaction: **a**. Crn; **b**. CTL; **c**. CTO-A. **C:** Ionization of HMH.

(20 mM), $FeCl_3$•$6H_2O$ (10 mM), 1% H_2O_2 (100 mL), 25ºC, 3 h], CTO-A and CTO-B were also detectable in addition to Crn, CTL and DMC in ^{1}H-NMR spectra and HPLC chromatograms (*15*). Once CTL is formed, an alternative ring-opening reaction of CTL by hydrolysis can also readily occur to give MG directly (Figures 1, 2B and 3Bb), as mentioned above. Although CTO-A and CTO-B could not be detected in physiological fluids, we thought that enzymatic oxidation can occur even *in vivo*. In fact, we isolated two kinds of MG synthases (enzymes that oxidize CTL to CTO-A) from rat liver and rat kidney (Figure 2C): *viz*, vitamin C synthase (L-gulono-γ-lactone oxidase) (*43*) and L-2-hydroxyl acid oxidase (*44,45*), respectively. The concentrations of MG both in physiological fluids and tissues in rats were higher than those of CTL, whereas CTL was predominant in human sera. We think that the difference might be explained by the presence or absence of vitamin C synthase.

We herein revise our *in vivo* pathways by addition of a pathway from Crn into DMC (Figure 1) even *in vivo*, because we can measure DMC in human sera of CKD patients and find that 1.3 mole of serum DMC (sDMC) was formed with one mole of serum CTL (sCTL) (Figure 4B), *i.e.* molar ratio of DMC to CTL was roughly one. This result indicated that this conversion *in vivo* should be a non-enzymatic oxidation reaction with •OH radicals, too.

3. Physicochemical properties of Crn and Crn metabolites; tautomerism, ionization and hydrolysis

We have found that there is some misunderstanding about the chemistry of Crn. For example, even though Kenyon *et al*. clearly reported tautomeric preferences for Crn in 1971 (*46*), the wrong tautomer (acylimino type: Figure 3A) of Crn is still written in biochemical textbooks and papers instead of the correct one (acylamino type: Figure 3A). Without exact knowledge of the physicochemical properties of Crn, it is hard to fully understand its chemical or biological reactions. Better sense can be made if the acylamino tautomer for Crn is used.

Our CTL structure as the acylamino form (*1,8,12,47*) was recently supported also by Krawczyk (*48*), who detected CTL as the intermediate from Crn to MG in oxidation with activated charcoal.

Another consideration which should be taken into account is the ionization of Crn and its metabolites in order to understand their reactions in solution. Ionization constants give us very valuable information about the ionic species involved. We know the corresponding ionization constants (pKa values) of our substances, *viz* Crn = 4.8 (basic pK), CTL = 4.2 (basic pK), DMC = 4.8 (basic pK), MG = 13.4 (basic pK), HMH = 8.6 (acidic pK). These values indicate that MG is a strong base and exists in an acidic (cation) form at neutral pH (around 7.0). However, glycocyamidine derivatives

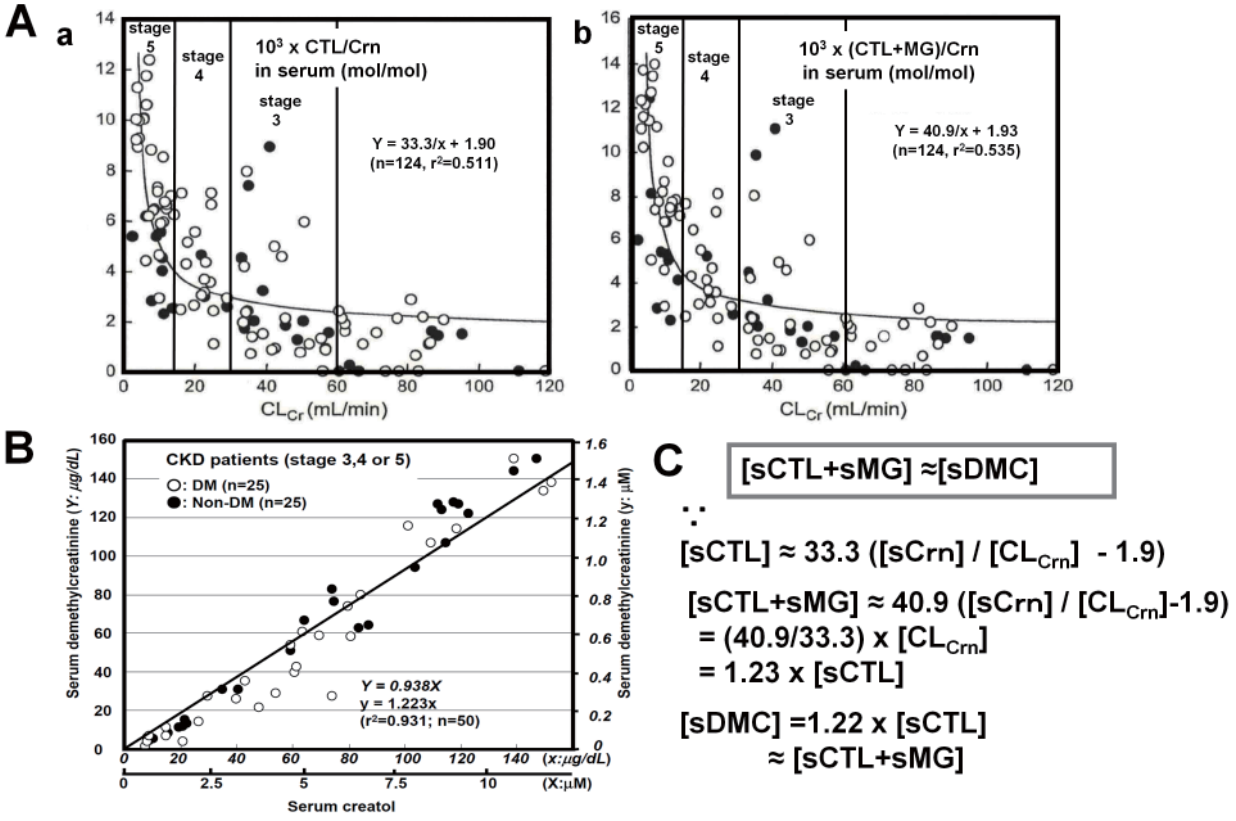


Figure 4. Correlationship between molar ratios: (CTL + MG)/Crn, CTL/Crn and DMC/Crn. A: Relationship between CL_{Cr} and (CTL + MG)/Crn or CTL/Crn in serum. **B:** Relationship between CTL/Crn and DMC/Crn in serum. **C:** Relationship between (CTL + MG)/Crn and DMC/Crn in human subjects.

(Figure 3A), Crn, CTL and DMC, exist as neutral forms (non-ionized species) in blood and tissues. In contrast, in acidic urine a proportion of a corresponding acidic form of glycocyamidine derivatives are observed. Since the ring-opening reaction of CTL by hydrolysis showed a local maximum value at pH 5 (enclosed decomposed CTL in Figure 3Bb) (*47*), a contribution of the acidic form cannot be ignored (Figure 3Bb).

The cleavage bond of Crn is the N3-C4 bond (Figure 3Ba). We can explain that CTL cleaves selectively at the N1-C5 bond (Figure 3Bb) (*3,15,47*), although some people might feel that something may be wrong. However, when we consider the correct acylamino tautomer and the non- or ionized forms together with the effect of a hydroxyl group at position C-5, a cleaving bond other than the N1-C5 bond is unacceptable. The cleavage bond of CTO-A can be explained differently, simply by high steric hinderance around the N1-C5 bond compared with that around the N3-C4 bond (Figure 3Bc).

One mystery which was confusing was that previously no peak was seen for CTL in physiological fluids observed even using sensitive HPLC techniques. The reason is simple: although CTL exists in mammals in a concentration at least equimolar to that of MG, the detectability of cyclic guanidino compounds such as Crn and CTL is very poor (three digits) in comparison with non-cyclic guanidino compounds such as MG in conventional HPLC methods using fluorogenic agents such as 9,10-phenanthrenequinone (PQ) (*49*). A simple solution involved the addition of an alkaline hydrolysis step between column separation and the reaction with PQ (*47,50*): we were thus able to increase the detectability of CTL 50-fold to overcome the problem.

HMH is an acidic substance (pKa is 8.6) (*21*), although fairly weaker than acetic acid (pKa 4.8). It is recognized that the anionic form of acetic acid is predominant under physiological conditions (pH around 7.0). In contrast, the neutral form of HMH should be predominant (97.5%) at pH 7.0 (Figure 3C).

4. Background of our estimation

4.1. *Detection of •OH radicals*

Because the •OH radical is so reactive, its existence cannot be directly monitored and an indirect biomarker of the •OH radical would be useful for patients with various diseases. In order to estimate the amount of •OH radicals, the •OH adducts or reaction products with •OH have to be measured as •OH biomarkers. For example, one of the most frequently used biomarkers for •OH radicals are 8-hydroxyguanine (8-OHG) or 8-hydroxydeoxyguanosine (8-OHdG); •OH

adducts of guanine or deoxyguanosine (dG) are formed, respectively. Their use is limited because they show only the amount of •OH radicals formed inside nuclei and mitochondria. Before determination of 8-OHG and 8-OHdG in urine, several reaction steps with 8-OHdG containing nucleotides must occur. It should be noted that the reported daily excreted 8-OHdG in urine of healthy subject is fairly low: 8-OHdG/Crn: 8.4 ± 2.7 μg/g (n = 25) (*51*); 1.86 ± 1.09 μmole/mole (n = 67) (*52*). If we assume urinary Crn (real range from 500 to 2,000 mg/day) to be roughly one gram, then the 8-OHdG amount would be ca. 10 μg (ca. 35 nmole). This result shows at the same time that dG scavenges ca. 35 nmoles of •OH radicals daily. Therefore, our estimation (see below) that the daily scavenged amount of •OH radicals by Crn is ca. 50-500 μmoles in healthy subjects and severe CKD patients; making it shockingly high.

4.2. *Classification of CKD into 5 stages for human subjects and rats*

The normal glomerular filtration rate (GFR_0) for human healthy subjects and rats had been reported to be ca. 100 mL/min/1.73 m^2 (*53*) and ca. 5.7 mL/min/kg (*54*), respectively, so the relative value of the glomerular filtration rate (GFR), GFR/GFR_0 can be calculated. As reported earlier (*6*), we classified the CKD of rats into 5 stages like those in humans (*32,55*). Table 1 shows a description of the 5 stages of CKD in human subjects and rats (*6*). In the case of rats, if the GFR had not been measured, we can estimate the eGFR by using our correlation equation below (y: GFR/GFR_0 = GFR/5.7; x: sCrn mg/dL) (*6*):

$$y = 0.47/x - 0.035 \ (n = 12: R^2 = 0.97)$$

5. *In vitro* and *in vivo* reaction of Crn with •OH radicals

5.1. *Cell-free in vitro reaction of Crn with •OH radicals*

We can now follow the bio-mimetic reactions of Crn with •OH radicals generated in a cell-free *in vitro* Fenton reaction. In the nuclear magnetic resonance (NMR) tube, we can follow the reactions even without purification (Figures 2A and 2B) (*15*). Both NMR spectra and HPLC charts indicated that the oxidation of Crn with •OH radicals gave at first CTL or DMC and then CTL was further oxidized to CTO-A which is hydrolyzed to MG *via* CTO-B (Figures 1 and 2B). Direct hydrolysis of CTL to MG was also indicated (Figures 1 and 2B). All these reactions also occur *in vivo* except for the step from CTL to CTO-A in which this oxidation is enzymatic instead of non-enzymatic (*43-45*).

5.2. *In vitro reaction of Crn with •OH radicals in cultured cells*

Since CTL is an •OH radical adduct of Crn, and MG is the product of CTL, the molar sum (CTL + MG) equals the corresponding moles of •OH radicals reacted with Crn. In isolated rat hepatocytes, Crn is converted by •OH radicals to CTL and MG, and the conversion is enhanced by stimulation with puromycin aminonucleoside (*39,56,57*). The contribution of protein kinase C activation has been clearly shown, by its translocation from cytosol to membrane, not only in •OH radical generation but also in formation of CTL and MG (*57*).

5.3. *Evidence of the in vivo reaction of Crn with •OH radicals*

Aoyagi and Nagase have already shown that the non-enzymatic reaction of Crn with •OH radicals provided MG (*27-29*). We have shown that •OH radicals play a crucial role in the first *in vivo* step, Crn→CTL, but not in the following next step, CTL→MG (*15-17*). Dimethylthiourea, an •OH radical scavenger, selectively inhibited the first step (*17*).

6. Estimation of scavenged amount of •OH radicals by Crn

Theoretically, the daily scavenged amount of •OH radicals by Crn is nearly equal to the daily urinary molar sum of CTL, MG and DMC, because CTO-A and CTO-B could not be detected in physiological fluids. In this review, the concentration of substances is depicted within square brackets [ ], and s and u before the abbreviation of the names of substances mean the substance in serum and urine, respectively. There is a good correlation between [sDMC] and [sCTL] as shown in Figure 4B (*20*), *i.e.*

$$[sDMC\ (\mu M)] = 1.22 \times [sCTL\ (\mu M)]$$

In Figures 4Aa and 4Ab, both [sCTL (μM)]/[sCrn (μM)] and [sCTL (μM) + sMG (μM)]/[sCrn (μM)] were correlated with Crn clearance [CL_{Crn} (mL/min)]. Therefore,

[sCTL (μM) + sMG (μM)] ≈ 1.23 × [sCTL (μM)] ≈ [sDMC (μM)] (Figure 4C).

In other words, the total sum (sCTL (mole) + sMG (mole) + sDMC (mole)) in an arbitrary amount of serum is likely to be equal to 2 × (sCTL (mole) + sMG (mole)).

Both molar ratios (CTL+MG)/Crn in serum and urine were shown to be almost equal (*4*), although the ratio CTL/Crn would not be steady, because the ratio MG/CTL increased during excretion from blood into urine. Among amounts of urinary CTL, MG and DMC (uCTL, uMG and uDMC), we assumed that uDMC

(mole) might be also nearly equal to (uCTL (mole) + uMG (mole)), although we do not have data on uDMC (mole). Therefore, (uCTL (mole) + uMG (mole) + uDMC (mole)) might be equal to 2 × (uCTL (mole) + uMG (mole)). The double amounts of the obtained absolute molar concentration of (CTL + MG) in 24 h urine were thought to be the estimated scavenged moles of •OH radicals.

6.1. *Estimation of daily •OH radical scavenging capacity of Crn in normal mammals*

The daily urinary molar sum of (uCTL + uMG) was calculated based on reported values (*1,9*). There seemed to be a great difference between values of healthy human subjects and normal rats (ca. 25 μmole/day and ca. 200 pmole/day, respectively) (Figure 5A). However, it is noteworthy that mean values of the corresponding molar ratios (uCTL + uMG)/uCrn were similar: 1.73 mmole/mole and 2.93 mmole/mole, respectively (Figure 5B) (*1*). These values seem to be reproducible, because the control subjects in another clinical report gave almost the same value (1.77 mmole/mole) (*2*). This fact might suggest that if we determined the muscle corrections, then oxidative stress related to •OH radicals in normal animals does not show a crucial species difference, although oxidative stress in rats seems to be on the higher side compared to those in human subjects.

For calculation of the molar ratio (uCTL + uMG)/uCrn, spot urine instead of 24 h urine can also be used (*4*) (Figure 5B). As stated above, (scavenged •OH radicals)/Crn can be calculated to be equal to the molar ratio 2 × (uCTL + uMG)/uCrn.

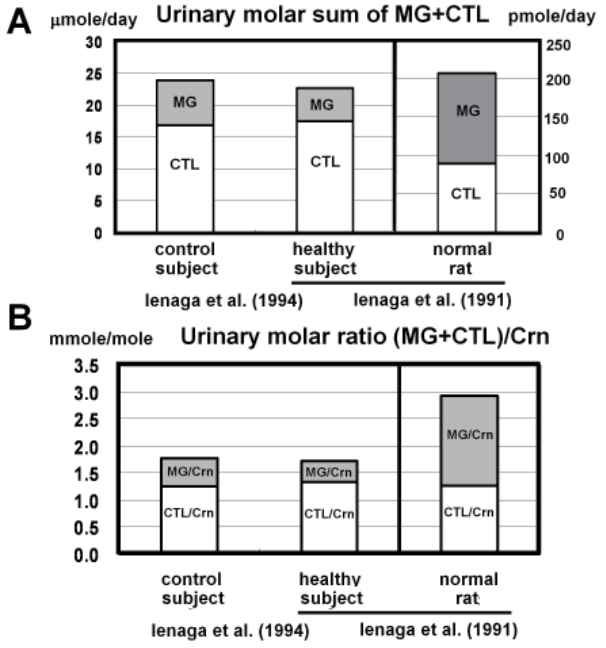


Figure 5. Estimation for amount of •OH radicals scavenged with Crn in mammalian bodies with CKD: based on daily urinary molar sum, MG + CTL (A) and urinary molar ratio, (MG + CTL)/Crn (B). *Ref.* Ienaga *et al*. (1991); Ienaga *et al*. (1994).

Healthy subjects and normal rats daily use ca. 0.4 and 0.6% of Crn, respectively, in order to scavenge •OH radicals. The scavenged amount of •OH radicals can be estimated to be ca. 50 μmole/day and ca. 400 pmole/day, respectively.

Two reports on daily excreted values of CTL and MG in healthy human subjects with normal kidney function gave similar values: the first are 17.4 and 5.3 μmole, respectively, for healthy subjects (*9*); and the second are 16.8 and 7.1 μmole, respectively, for normal subjects (*1*) (Figure 5A). Despite the conversion of CTL into MG during kidney excretion, CTL remained still dominant (Figure 5A).

Daily excreted mean moles of CTL and MG for normal rats were 78 and 118 pmole, respectively, making MG the main metabolite of Crn for normal rats. In the conversion of CTL into MG, there are a few pathways. Although non-enzymatic hydrolysis and MG synthase in kidney (*44,45*) seemed to play a common important role in mammals, the presence of vitamin C synthase in rat liver (*43*) and its absence in human beings is likely to be one reason for the difference between human subjects and rats.

6.2. *Estimation of daily •OH radical scavenging capacity of Crn in CKD mammals*

The molar ratio (CTL + MG)/Crn in serum and urine is correlated well with sCrn values (*1,50,58*). It was thought that sMG increases in proportion to sCrn according to a simple equation, but we have shown that the correlation followed a quadric equation (*1,50,58*). Since the molar ratios, sCTL/sCrn and sMG/sCrn increase in proportion to sCrn (x: M) according to the simple equation, then sCTL and sMG should theoretically increase according to a quadric equation. Based on the reported data (*1,58*), we recalculated the molar equivalents and obtained new equations. Y: (CTL + MG)/Crn mmole/mole; Z: (CTL + MG) μM; X: Crn μM; X': Crn: mg/dL (Figure 6).

For DM patients with CKD:

$$Y = 0.00627X - 0.0142$$
or $Y = 0.71X' - 0.61$ (the 1st equation) (Figure 6A)

$$Z = 0.00627X^2 - 0.0142X$$
or $Y = 0.71X'^2 - 0.61X'$ (the 2nd equation) (Figure 6B)

It should be noted that if we treat the serum specimens from CKD patients at stages 3 and 4 carefully, sMG is frequently not detectable, whereas sCTL can be measured easily since it is relatively quite high (Figure 6C) (*1,57*).

The molar ratio (CTL + MG)/Crn (Z: mmole/mole) in serum and urine is correlated well also to the inverse

of GFR (or Crn clearance: CL_{Crn}) (W: mL/min) (*4*), *i.e.*

$$Z = 40.9/W + 1.93 \text{ (in serum) (Figure 4Ab)}$$

As shown in Figure 7, the molar ratio (CTL + MG)/Crn in urine increased 10 and 17 times more in CKD patients (*1*) and in rats at stage 5 (*9*), respectively: from 3.0 mmole/mole (CTL/Crn = 1.3, MG/Crn = 1.7 mmole/mole) in the control rats up to 31.3 mmole/mole (CTL/Crn = 12.0, MG/Crn = 19.3 mmole/mole) in

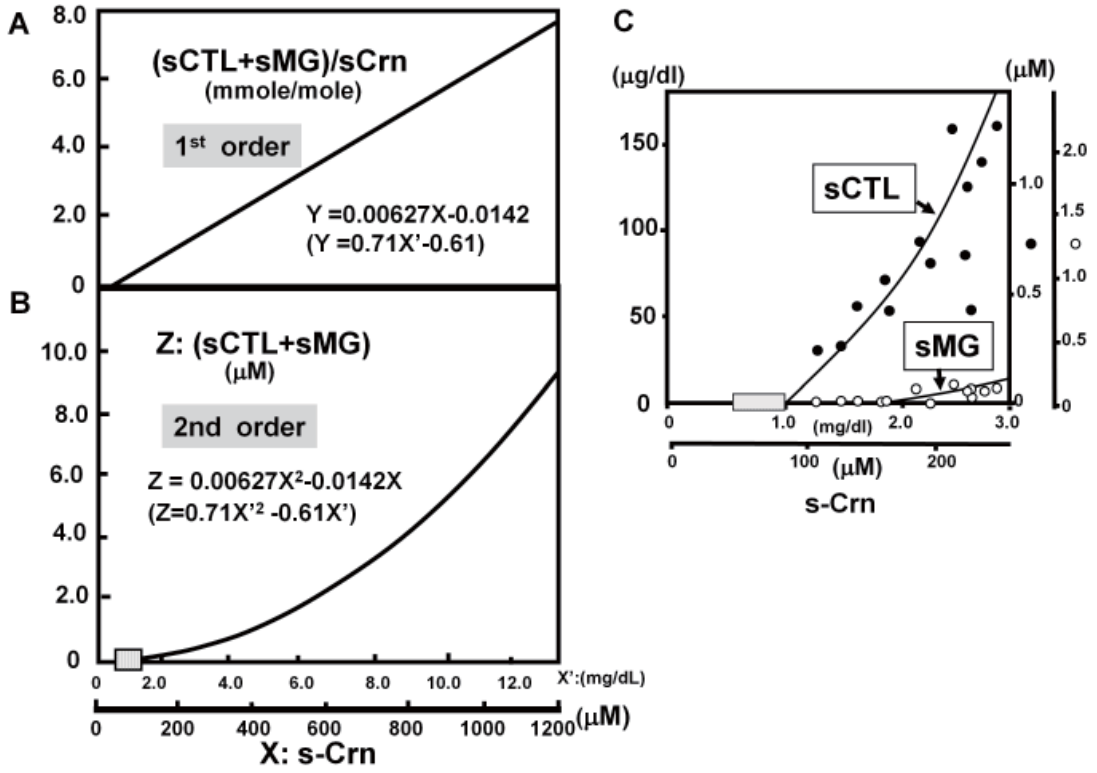


Figure 6. Correlation between Crn and (CTL + MG)/Crn or (CTL + MG). A: 1st order equation of (CTL + MG)/Crn with Crn. **B:** 2nd order equation of (CTL + MG) with Crn. **C:** Correlation between Crn and CTL or MG.

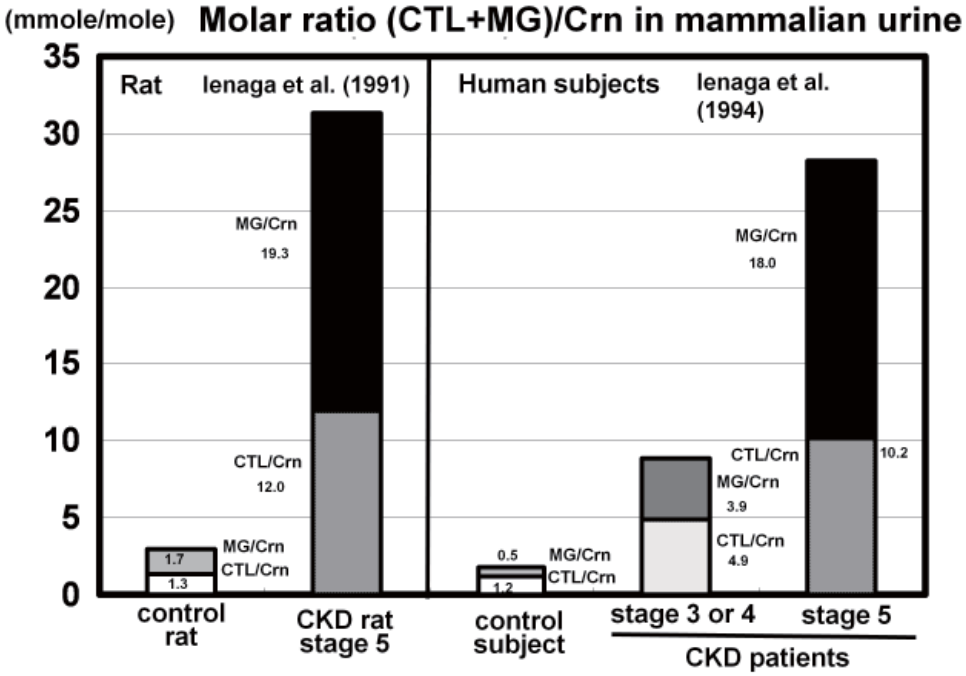


Figure 7. Molar ratio of (CTL + MG)/Crn in mammalian urine with or without CKD.

CKD rats at stage 5; from 1.7 mmole/mole (CTL/Crn = 1.2, MG/Crn = 0.5 mmole/mole) in the control subjects up to 28.2 mmole/mole (CTL/Crn = 10.2, MG/Crn = 18.0 mmole/mole) in CKD patients at stage 5.

We can suppose that molar ratios of (DMC/Crn) and (CTL + MG)/Crn are nearly equal. If so, it should be noted that about up to 6% of Crn was used to scavenge •OH radicals, in other words, one mole of Crn scavenged 60 mmole of •OH radicals in clinical patients or rats with CKD at stage 5, just before ESRD therapy. For CKD patients at stage 3 or 4, the molar ratio (CTL + MG)/Crn had an in-between value, 8.8 mmole/mole (CTL/Crn = 4.9, MG/Crn = 3.9 mmole/mole) when compared with controls (Figure 7).

The GFR values together with MG levels in every 48 h-urine sample in the progression of CKD (produced by adenine-loading) has been reported in previous research (Figure 8) (*54*). If we use the GFR_0 value of 5.65 ± 0.27 mL/min/kg (value at the day 0), each relative renal function GFR/GFR_0 (%) can be calculated and each stage can be estimated (Figure 8A). Thus, a CKD stage is identified for each MG value (Figure 8B). As shown in Figure 8C, urinary MG/Crn values increased inversely with decrease in GFR/GFR_0 (%): from 1.6 mmole/mole (GFR/GFR_0 = 100%) to 27.4 mmole/mole (GFR/GFR_0 = 4%) (Figure 8C). Since, however, we do not have any data about DMC in rats, we dare not estimate the amount of •OH radicals scavenged as DMC.

These data show that at least more than 3% of Crn

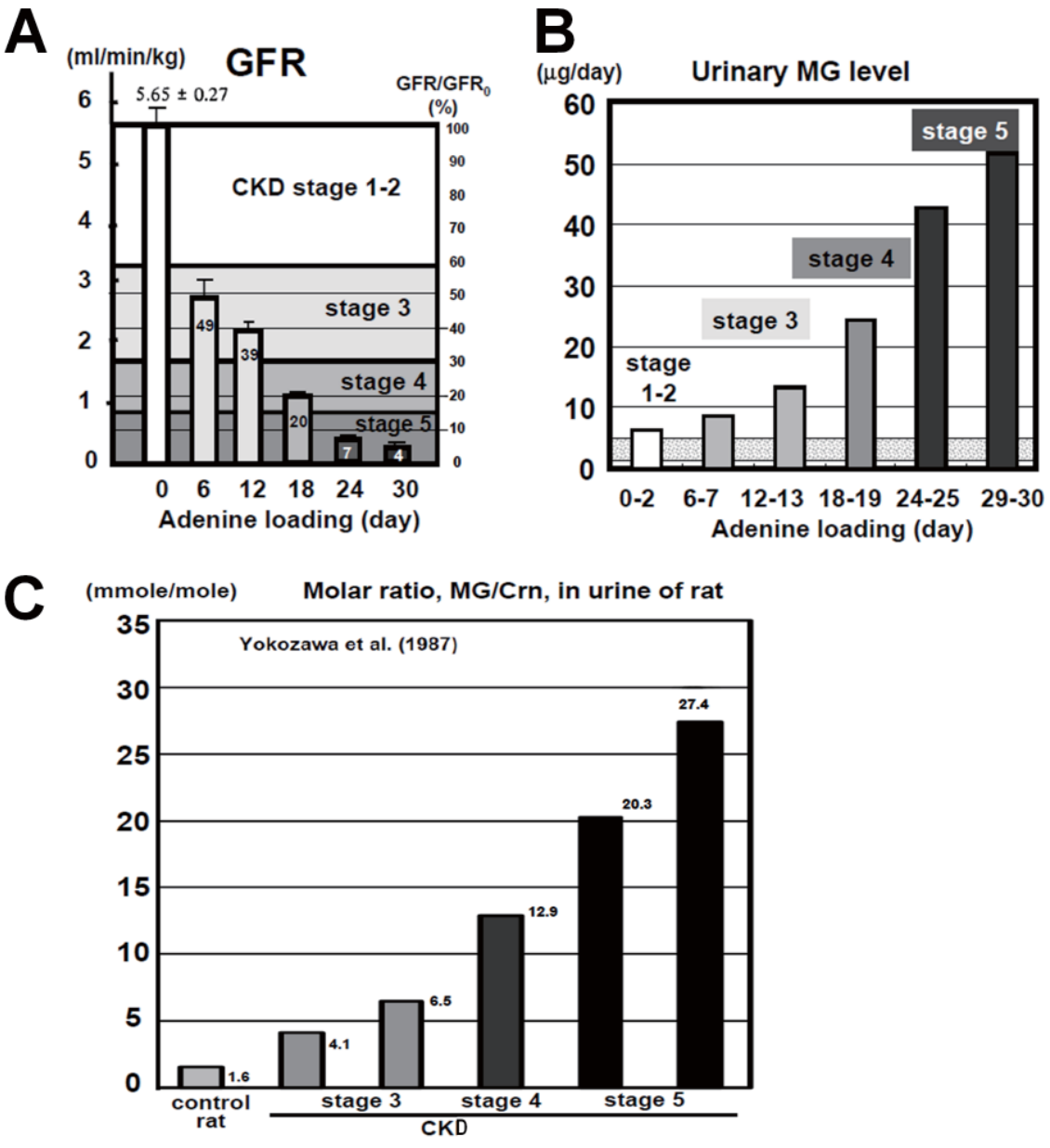


Figure 8. Increase in scavenging amount of •OH radicals with Crn in rats in proportion to the progression of CKD. A: Classification of CKD stage in adenine-loaded rats based on GFR value and its relative GFR percent to normal rats. **B:** Urinary MG level and CKD stage of rats. **C:** Urinary molar ratio, MG/Crn, and CKD stage of rats. *Ref.* Yokozawa *et al*. (1987).

in CKD rats at stage 5 is used in order to scavenge •OH radicals.

7. Presumption of places where Crn scavenges •OH radicals in mammals

7.1. *Places where Crn might scavenge •OH radicals in mammals*

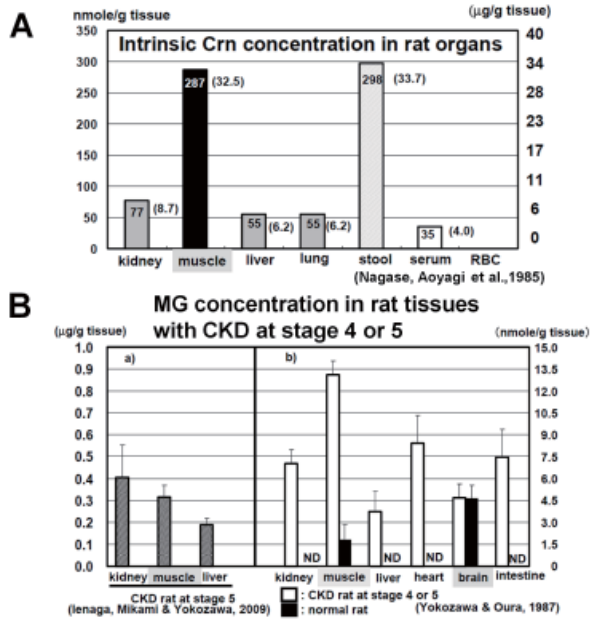


Figure 9. Concentration of Crn and MG in rat organs. A: Intrinsic Crn concentration in organs of normal rats. **B:** MG concentration in organs of rats with and without CKD at stage 4 or 5. *Ref.* Nagase *et al*. (1985); Ienaga *et al*. (2009); Yokozawa and Oura (1987).

Fluids and organs where Crn scavenges •OH radicals in mammals were presumed from the reported Crn levels in rat organs (*28*) and/or the reported MG levels in rat organs induced by Crn injection (*28*) or CKD induction (*5,59*) and from the autoradiogram of ^{14}C-Crn (*60*).

For normal rats, intrinsic Crn levels in kidney, liver and lung were nearly comparable (77, 55, 55 nmole/g tissue, respectively) but in muscle (the main Crn synthesizing organ) (287 nmole/g tissue) it was ca. five times higher (*28*) (Figure 9A).

Autoradiograms of *i.v.* injected ^{14}C-Crn (*60*) had clearly shown that Crn was easily distributed within 5 minutes and rapidly disappeared in kidney, liver, heart and lung. In contrast, ^{14}C-Crn was never absorbed in muscle and brain, where intrinsic Crn is mainly synthesized.

Detectable levels of MG have been observed in normal rat muscle and brain (*59*). In contrast in organs, which receive a fairly high blood supply, such as kidney, liver and heart, MG was readily detectable in rats with CKD at stage 4 or 5 (Figure 9B) (*5,59*). We believe that in those organs where MG was detectable, Crn scavenges •OH radicals.

7.2. *Flow diagrams of Cr and Crn*

We have illustrated the flow of Cr and Crn in mammalian bodies in Figure 10. Cr biosynthesis in mammals is fairly well established (Figure 10A) (*11*), and we illustrate analogously how Crn might be catabolized *via* CTL in mammals (Figure 10B).

In organs, where Crn is mainly synthesized, such as muscle and brain: the concentration of Crn is

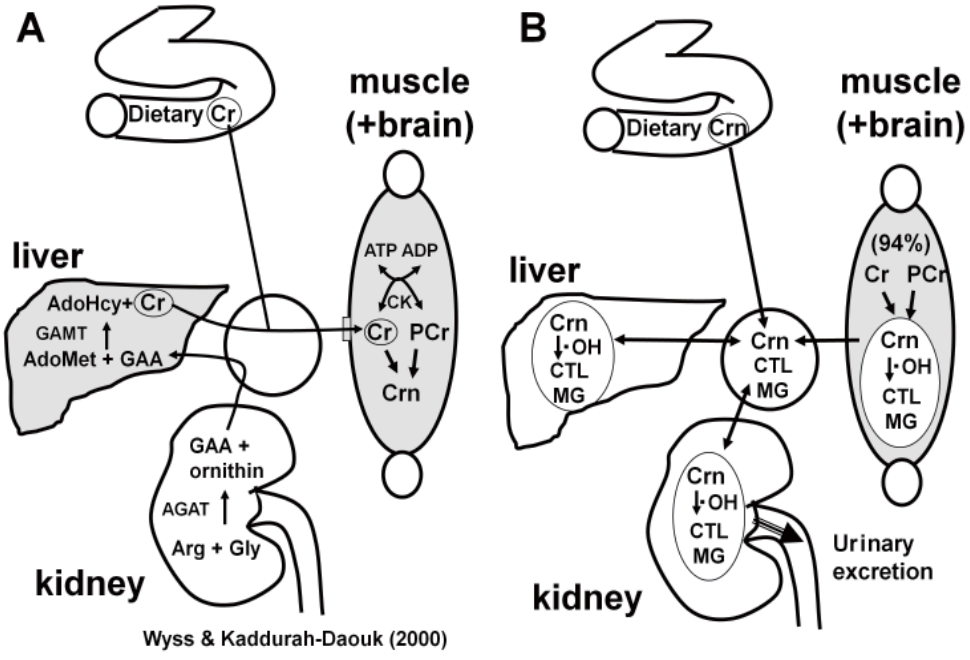


Figure 10. Flow of Cr and Crn in mammalian organs. A: Cr. *Ref.* Wyss and Kaddurah-Daouk (2000). **B:** Crn and its metabolites.

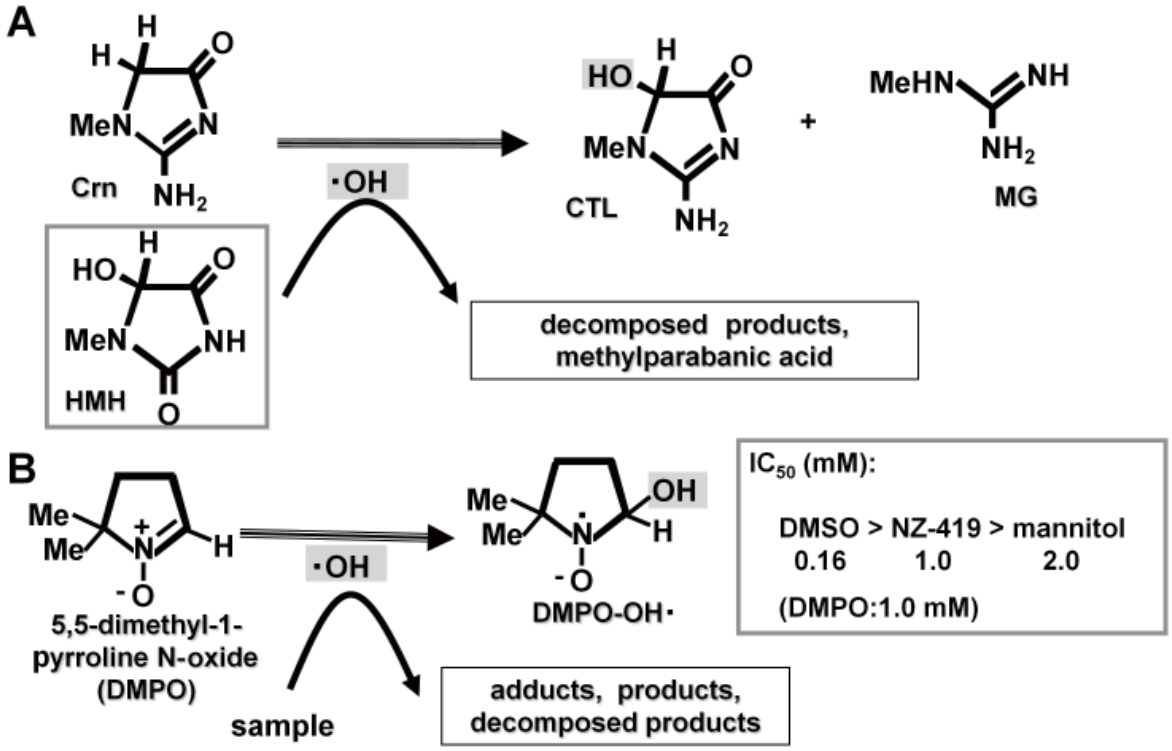


Figure 11. Antioxidant effect of HMH. A: Inhibition of CTL formation from Crn by HMH. **B:** Inhibition of DMPO-OH adduct formation by HMH.

maintained at a high level and MG is detectable not only for rats with CKD at stage 4 or 5, but also for normal rats (Figure 10B). Since the concentration of Crn never increased in those organs on injection of Crn, there seems to be a one-way flow of Crn following the concentration gradient, from muscle to blood flow. Although in contrast, in other organs such as kidney, liver and heart, where Crn is not synthesized, Crn seems to flow both in and out of the organs. In those organs, when Crn levels are elevated and the GFR decreases enough, CTL and MG are accumulated: just like MG in rats with CKD at stage 4 or 5 (Figure 10B).

8. Merits of measurement of 8-OHdG or Crn-related markers such as (CTL + MG)/Crn in mammals

If we want to know the total amount of •OH radicals in mammalian bodies, Crn-related markers are most likely to be more reliable than 8-OHdG markers for urinalysis. The absolute amount of the former is 10^3-fold higher than the latter, and the former markers are measured directly without any further degradation process, whereas the latter is indirect, requiring not only degradation from the nucleotide chain but also excretion from the nucleus or mitochondria in cells into the urine *via* cytoplasm, blood, *etc.*

However, for estimation of DNA damage by •OH radicals inside the nucleus or mitochondria, 8-OHdG is likely to be one of the best markers. Thanks to specific antisera against 8-OHdG we can show the difference in DNA damage between nucleus and mitochondria. Thus, at 8 weeks after the onset of diabetes, the levels of 8-OHdG are significantly increased in mitochondrial DNA (mtDNA) from kidney of diabetic rats but not in nuclear DNA; suggesting the predominant damage of mtDNA (*61*).

If we want to know further •OH radical levels scavenged by Crn in the nucleus and mitochondria as well as in organs, we need specific antibodies against CTL and/or MG. Preliminary results, using such kinds of antisera, have shown specific staining for CTL and MG in brain and kidney.

9. HMH as an alternative intrinsic antioxidant

Since the level of Crn might not be enough to scavenge •OH radicals in mammals with CKD at stages 3, 4 and 5 and MG starts accumulating as a uremic toxin, Crn is no more the ideal scavenger. Alternatively, HMH has been recognized as an another intrinsic antioxidant (*3,10,36-39*). Intrinsic levels of HMH increase in DM and CKD patients (*8,10*). HMH prevents the progression of CKD at stages 3-5 in rats, if enough HMH is given orally (*3,5,6,37-41*) instead of Crn. We believe that HMH might be a kind of 'self-defense' substance.

However, the •OH radical scavenging effect of HMH, tested by the ESR method, is not so very different in comparison to Crn (Figure 11B) (*36-39*): it is stronger than mannitol but weaker than DMSO. The

intrinsic concentration of HMH cannot be higher than Crn, however. Therefore, we think it is hard to explain the mode of action of HMH only by the direct •OH radical scavenging effect. Formation of CTL from Crn both under *in vitro* and *in vivo* conditions is inhibited by HMH (Figure 11A) (*3-7,35,39-41*). We have noticed that its inhibition in cells is more apparent than its effect in a cell-free system and is comparable to DMSO (*39*). We are now investigating further the cellular protective effects of HMH as an anti-oxidant, or some as yet unknown effect, in detail.

When HMH is made to react with •OH radicals, the products are too labile to be detected. Under mild conditions, only methylparabanic acid (Figure 1), an oxidative metabolite of HMH (*18,19*), can be partially monitored. At the same time, a radical of HMH seems much more labile than the radical from Crn. CTL is also an •OH radical scavenger like HMH. However, ESR data suggests that HMH and Crn are stronger than CTL in this respect. These phenomena might explain why HMH seems safe to use as a drug.

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