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Factors Affecting the *in Vitro* Stability of ^{14}C -Urea in the Urine of Rats

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To assess possible factors affecting the degradative reaction of urea (ureolysis) catalyzed by urease, the *in vitro* stability of urea in rat urine was investigated by changing the reaction temperature and pH or amount of added urease or by masking the reaction mixture from the atmosphere. The fraction of the radioactivity spiked as ^{14}C -urea which was decomposed to $^{14}\text{CO}_2$ in rat urine at 15 and 25°C for 24 h was only about 1.5 and 3.3%, respectively, while that at 35°C was as much as 42%. The activation energy for this reaction was estimated to be approximately 25 kcal/mol. Added urease enhanced the degradation of ^{14}C -urea, the initial apparent rate constant was proportional to the amount of the added enzyme (0.01 to 5.0 U/ml) to yield an approximate catalytic constant of 1.7×10^{-2} ml/U/h. When urease was added at 5.0 U/ml, the highest degree of ureolysis was observed at neutral to very slightly alkaline pH. Covering the surface of the reaction mixture of rat urine with a layer of liquid paraffin appeared to depress almost completely the capture of radioactivity as $^{14}\text{CO}_2$. It is, therefore, suggested that two precautions, *i.e.*, keeping the urine isolated from the atmosphere and keeping the metabolic cage at a temperature below 25°C, should be taken in disposition experiments with urea which involve the measurement of expiratory excretion of radioactivity.

Keywords—urea; ureolysis; *in vitro* degradation; effect of temperature; effect of pH; effect of urease; catalytic effect; expiratory excretion; urinary excretion; urea disposition in rats

Although it had been considered until the middle of this century that urea is the end-product in the metabolism of endogenous nitrogenous compounds and is thus metabolically inert both in man and in experimental animals, there were several reports that intestinal microfloral urease could substantially decompose urea to carbon dioxide and ammonia in man,¹⁻³⁾ rats⁴⁾ and mice.^{5,6)} In our previous report, large proportions of the dose, approximately 12 and 37%, were recovered in expired air when ^{14}C -urea was administered intravenously and orally to rats, respectively.⁷⁾ However, considerable fluctuation, which resulted in approximately 20 to 40% coefficients of variation, was observed in any plot of cumulative expiration (% of the dose) following oral administration. This suggested that there was a large variation from animal to animal in the degradative reaction of urea (ureolysis) catalyzed by urease, which probably occurs in the ileocecal portion of rats. Bacterial sources of urease activity in the gut of mammals have recently been elucidated by Varel *et al.*⁸⁾ and Suzuki *et al.*⁹⁾ However, the effect of urease activity on the ureolysis in biological materials or fluids other than the intestinal contents from conventional (nongerm-free) animals which contain microflora producing urease (*Peptostreptococcus productus*,⁸⁾ *Eubacterium aerofaciens*, *Fusobacterium varium*, *Peptococcus prevotti*⁹⁾ or *Lactobacillus fermenti*¹⁰⁾) has not been investigated. In an experiment where urea is excreted as such or as a metabolite (end-product) into the urine or the feces, if the urine, feces and expired air samples are collected simultaneously in a conventional metabolic cage which permits separate collection of these samples but is so arranged that both urine and feces beakers are open to the expiration air line, a long collection interval such as 12 or 24 h might produce an overestimation of the expiratory excretion resulting from trapping of additional carbon dioxide derived from ureolysis due to possible bacterial contamination in the

urine. There have been no detailed reports describing the *in vitro* stability of urea in any biological medium, such as urine.

Therefore, the purpose of the present investigation was to examine the effects of temperature, pH, added urease and anaerobic state on the stability of ^{14}C -urea spiked in control urine samples from rats, in order to standardize methods of collecting each excretion sample in disposition experiments with urea.

Materials and Methods

Materials— ^{14}C -Urea was purchased from New England Nuclear, Boston, Mass., U.S.A. The specific radioactivity was 8.9 mCi/mmol and the radiochemical purity was more than 99%. Ethanolamine (Katayama Chemicals Co., Ltd., Nagoya, Japan) used for trapping $^{14}\text{CO}_2$ was of liquid scintillation grade. Soluene-350 (Packard Instrument Co., Downers Grove, Ill., U.S.A.) was used for solubilization of fecal samples. Urease (Jack bean, Type III, 2550 U/ml) was purchased from Sigma Chemical Co., St. Louis, Miss., U.S.A. All other chemicals were of analytical grade and were used without further purification.

Collection of Rat Urine used for the Preparation of Reaction Mixtures—Male Wistar rats (250–350 g) were individually housed in metabolic cages (KN-646, Natsume, Tokyo, Japan) which were equipped to collect urine and feces separately. Control urine was collected under a layer of liquid paraffin (10 ml) in a conical flask over 12 h without any dosing of urea. Immediately before each *in vitro* experiment, the individual urine samples from three rats were always pooled to prepare freshly a reaction mixture containing 3.0 μg /ml of endogenous urea by appropriate dilution with distilled water.

Effect of Temperature on the Stability of ^{14}C -Urea in Rat Urine—The apparatus for collecting $^{14}\text{CO}_2$ derived from ^{14}C -urea which was spiked in the control urine sample is schematically shown in Fig. 1. This is essentially similar to the system devised by Lauterburg and Bircher¹¹⁾ to collect exhaled gas. A reaction chamber (A) was connected to a row of gas wash bottles (B and C). The first bottle (B) contained 100 ml of concentrated sulfuric acid to retain the exhaled water vapor and the second (C) contained 100 ml of a mixture of ethanolamine and methanol (1:2, v/v) which was used for capturing CO_2 gas and was changed periodically. For quantitative collection of the gas in the reaction chamber, air was drawn at a rate of approximately 0.5 l/min. All of these settings are very similar to those used in a special metabolic cage (KN-450, Natsume, Tokyo, Japan) equipped to collect urine, feces and expiratory air separately, that was employed in our previous⁷⁾ and present studies on urea disposition in rats. Ten or 30 ml of the control rat urine spiked with 1 μCi of ^{14}C -urea (70 μg or 5.0 mg as urea) was placed in the reaction chamber (A).

The reaction temperature was regulated by leaving the chamber (A) in a constant temperature and humidity cabinet, Koitotoron (Ohnishi Netsugaku Kogyo-sho, Nagoya, Japan), which was set at 15, 25 or 35°C ($\pm 0.5^\circ\text{C}$). The pH of the reaction mixture was not adjusted but was measured before and after the 24 h reaction in this experiment. In a preliminary experiment that was carried out with 5.0 mg of urea in 30 ml of the control urine at 35°C, another bottle which contained 100 ml of the mixture of ethanolamine and methanol was connected to bottle (C) in order to examine whether bottle (C) could capture essentially all of the exhaled $^{14}\text{CO}_2$. Since it was verified that more than 99% of the exhaled radioactivity was trapped in the bottle (C), a further gas wash bottle containing the mixture of ethanolamine and methanol was not used in subsequent experiments. To avoid an excessive rise in pH of the reaction mixture due to the production of ammonia, all the *in vitro* experiments were carried out with the addition of relatively small amount of urea (70 μg in 10 ml of the urine) so that the initial concentration of total (endogenous plus added) urea was 10.0 μg /ml.

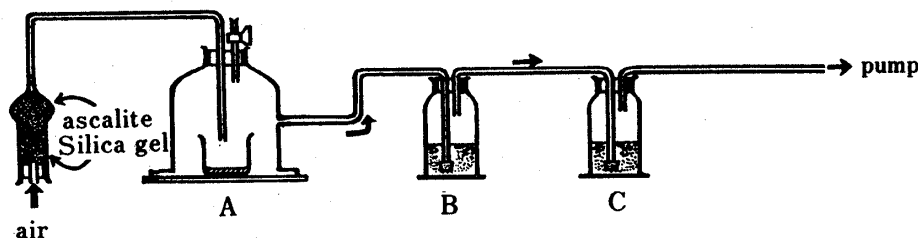


Fig. 1. Schematic Illustration of the Recovery System for Radioactive Carbon Dioxide derived by *in Vitro* Reaction from ^{14}C -Urea spiked in Rat Urine

- A: reaction chamber possessing a urine beaker.
- B: gas wash bottle containing 100 ml of concentrated H_2SO_4 .
- C: gas wash bottle containing 100 ml of ethanolamine-methanol (1:2, v/v) mixture which was changed periodically.
- Air flow rate: approximately 0.5 l/min.

Effect of Added Urease on the Stability of ^{14}C -Urea in Rat Urine—Jack bean urease was initially dissolved to give 0.02 to 10.0 U/ml in the control urine which was collected and prepared freshly. One part (5 ml) of the above urine was mixed with the control urine that was spiked with 1 μCi of ^{14}C -urea (70 μg as urea) to make a reaction mixture (10 ml) containing urease at 0.01, 0.1, 1.0, 2.5 or 5.0 U/ml. The reaction conditions were the same as described earlier except that the temperature was kept at 25°C in all cases.

Effect of pH and Masking of the Reaction Mixture on Ureolysis in Rat Urine—The initial pH of both the spiked urine and the urine containing urease was adjusted to 6.0, 7.4 or 9.1 by adding a trace amount of 2N HCl or 2N NaOH solution. The reaction mixture (10 ml) containing 1 μCi of ^{14}C -urea (100 μg as total urea) and 50 U of urease at each pH value as specified above was incubated at 25°C; all other experimental conditions were the same as described before.

The effect of masking the reaction mixture on the recovery of $^{14}\text{CO}_2$ from rat urine was also examined under the same reaction conditions at pH 7.4 except that 10 ml (approximately 5 mm in thickness) of liquid paraffin was layered onto the urine reaction mixture.

Effect of Covering the Urine Sample with Liquid Paraffin on the Expiratory Excretion of Radioactivity ($^{14}\text{CO}_2$) following Intravenous Administration of ^{14}C -Urea to Rats—Three male Wistar rats (213–245 g) were given ^{14}C -urea (10 μCi , 0.675 mg/kg) intravenously and housed individually in metabolic cages (KN-450) equipped to capture $^{14}\text{CO}_2$ in a mixture of ethanolamine and methanol (1:2, v/v) as well as to collect the urine and feces separately. Ten ml of liquid paraffin (approximately 5 mm in thickness) was placed in the urine beaker beforehand. Expiratory excretion of the radioactivity was measured periodically up to 24 h following the administration. Solid fecal pellets were treated in the same manner as described in our previous report⁷⁾ to obtain a pasty sample.

Radioactivity Measurement—In all the experiments except the last one, the radioactive material balance was ascertained by measuring both cumulative recovery as $^{14}\text{CO}_2$ and remaining radioactivity in the reaction mixtures. An aliquot (500 μl) of the ethanolamine-methanol mixture or of 10- to 50-fold diluted urine (100 μl) at 24 h was used for the radioactivity counting. All samples were directly determined with 10 ml of toluene-Triton X-100 liquid scintillator (PPO 5 g, POPOP 300 mg, toluene 700 ml, Triton X-100 300ml), except for the fecal paste, which was solubilized by adding 1 ml of Soluene-350 before mixing it with the scintillator. The radioactivity was determined in a Mark II liquid scintillation spectrometer (Nuclear-Chicago Corporation, Des Plaines, Ill., U.S.A.). The counting efficiency was automatically determined by the ^{133}Ba external standard ratio method and cpm was converted to dpm.

Results and Discussion

Effect of Temperature on the Stability ^{14}C -Urea in Rat Urine

The fractions of spiked radioactivity which remained as ^{14}C -urea in rat urine at three different reaction temperatures are shown in Fig. 2. The loss of radioactivity in the reaction mixture corresponded exactly with the cumulative recovery as $^{14}\text{CO}_2$ in a mixture of

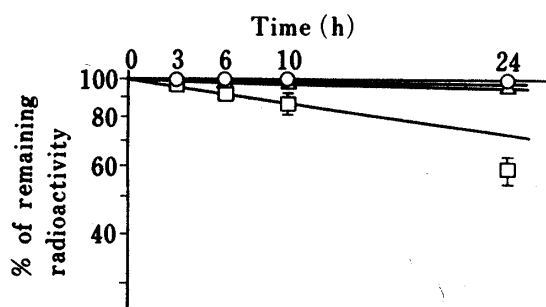


Fig. 2. Effect of Reaction Temperature on the Stability of ^{14}C -Urea in Rat Urine

○, 15°C; △, 25°C; □, 35°C.

Initial concentration of urea was 10.0 $\mu\text{g}/\text{ml}$ urine. Urine pH: initial, 8.00–8.02; final, 8.18–8.21.

Each point is the mean value \pm S.D. for three experiments.

Each regression line was fitted for the data of the first 10 h.

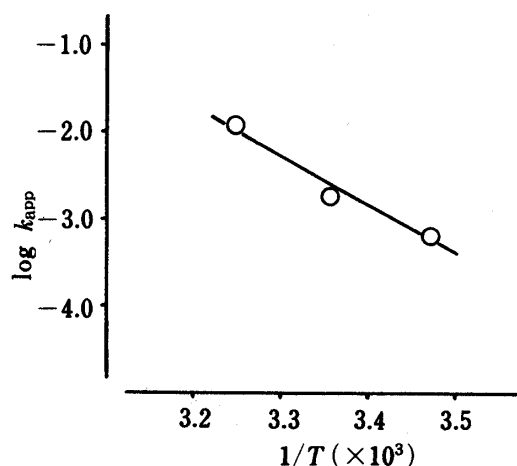


Fig. 3. A plot of $\log k_{\text{app}}$ against $1/T$ for the Decomposition of ^{14}C -Urea in Rat Urine

Regression line: $Y = 15.75 - 5470 X$ ($r = 0.979$, $p < 0.05$), where Y is $\log k_{\text{app}}$ and X is $1/T$.

The apparent rate constant, k_{app} , is expressed as the mean value in h^{-1} . See Fig. 2 for further details.

ethanolamine and methanol. In the control urine, urea was found to be considerably stable at temperatures below 25°C, since the fraction which was decomposed to $^{14}\text{CO}_2$ in 24 h was only about 1.5 and 3.3% at 15 and 25°C, respectively. In contrast, when the reaction temperature was raised to 35°C, that fraction increased to approximately 42% for the same period. The reaction mixture of urine which was kept at 35°C became distinctly turbid within several hours, indicating the possible growth of bacteria which might have urease-like activity able to catalyze the degradative reaction of urea, ureolysis. Since the same possibility existed at both 15 and 25°C, the effect of temperature on the degradation kinetics of urea in rat urine was estimated by calculating the apparent first-order rate constants from the remaining radioactivities during the first 10 h.

A plot of log mean apparent first-order rate constant (k_{app}) against the reciprocal of the absolute temperature ($1/T$) is shown in Fig. 3. There was a fairly good correlation ($r=0.979$, $p<0.05$) between these two parameters. The activation energy for this reaction was estimated to be about 25 kcal/mol and the frequency factor to be $5.6 \times 10^{15}/\text{h}$. These results imply that urea is relatively stable in rat urine even when it is excreted as an end-product, if the collection of the urine is performed at a temperature below 25°C.

Effect of Added Urease on the Stability of ^{14}C -Urea in Rat Urine

Though it has been suggested by van Slyke and Archibald that 1 to 5% addition of egg albumin to urease solution might overcome the difficulties in maintaining the enzyme stability,¹²⁾ egg albumin was not employed in the present reaction mixture in order to keep the conditions simple and as similar to the *in vivo* situation as possible.

Figure 4 shows the effect of added urease on the stability ^{14}C -urea in rat urine at 25°C. The freshly collected urine (control urine) from rats was estimated to contain a negligible quantity of urease (less than 0.01 U/ml), since addition of commercial urease in this amount to the control urine did not significantly affect the stability of urea as assessed by comparing Fig. 4 with Fig. 2 (at 25°C). As shown in Fig. 4, the degradation of urea was accelerated with increase of urease concentration. Although the reaction was carried out at 25°C in order to obtain an appropriate velocity of the reaction and to avoid the heat-induced

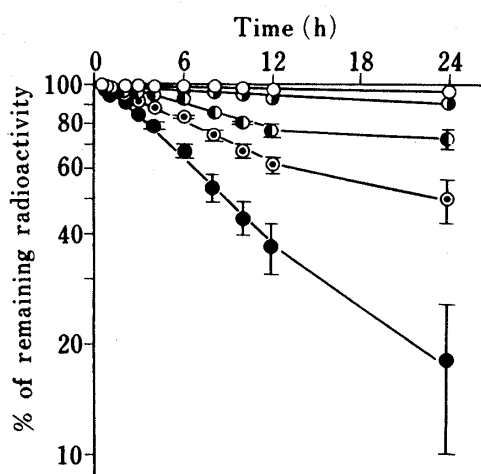


Fig. 4. Effect of Added Urease on the Stability of ^{14}C -Urea in Rat Urine at 25°C

○, 0.01 U/ml; □, 0.1 U/ml; △, 1.0 U/ml; ◐, 2.5 U/ml; ●, 5.0 U/ml.

Urine pH: initial, 7.98–8.01; final, 8.15–8.22.

Each point is the mean value \pm S.D. for three experiments.

See Fig. 2 for further details.

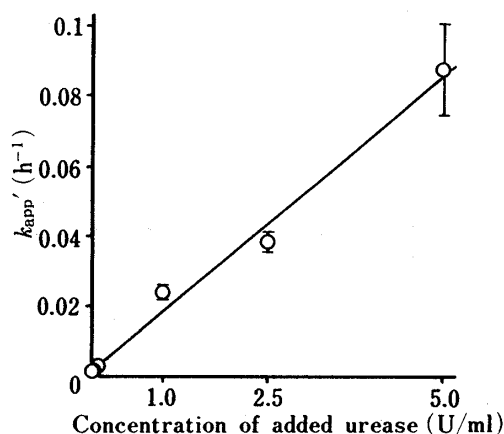


Fig. 5. Relationship between Initial Apparent Rate Constant (k_{app}) for Ureolysis in Rat Urine and Initial Concentration of Added Urease

Regression line: $Y=0.0017+0.168 X$ ($r=0.995$, $p<0.001$), where Y is k_{app} and X is the initial concentration of added urease.

Each point is the mean value \pm S.D. for three experiments.

See Fig. 4 for further details.

inactivation of added urease as far as possible, the slope of the plot of remaining % radioactivity against time seemed to be reduced in the second 12 h as compared with the first 12 h. From the finding that this slowdown phenomenon tended to be more distinct with smaller addition of urease than with the larger addition, it is supposed that the retardation in the reaction may be due to inactivation of the urease by aging and/or change in reaction pH. Thus, it was considered that the effect of added urease should be estimated from the initial rate constant obtained in the first 12 h. Figure 5 represents the relationship between the initial apparent rate constant (k_{app}') and the initial concentration of added urease, indicating the existence of significant catalytic effect of urease. The catalytic constant in this reaction was calculated to be approximately 1.7×10^{-2} ml/U/h from the equation $k_{app}' = k_{app} + k_c$ (AU), where k_{app} is the apparent rate constant without any added urease, namely the rate constant obtained when the urease concentration (AU) is zero (Fig. 2 or 3 at 25°C), and k_c is the catalytic constant (slope in Fig. 5).

Effect of pH on the Urease Activity for Ureolysis in Rat Urine

Since it has been reported that the optimal pH for Jack bean urease employed at relatively high concentrations is around pH 7.0 to 7.5,¹²⁾ the activity of added commercial urease (Jack bean, Type III) in the experiments of Fig. 4, where the initial pH value was approximately 8.0, might be considered to be slightly less than maximum. However, the pH-dependency of urease activity for ureolysis in rat urine has never been investigated. Cumulative production of $^{14}\text{CO}_2$ derived from ^{14}C -urea by ureolysis in the weakly acidic to weakly alkaline pH regions for 12 h is summarized in Table I. A pH-profile of k_{app}' estimated from the reac-

TABLE I. Effect of Initial pH on the Cumulative Production of $^{14}\text{CO}_2$ by Ureolysis in Rat Urine Spiked with ^{14}C -Urea (1 μCi , 10.0 μg as Urea/ml) and Urease (5.0 U/ml) for 12 h

Initial pH	$^{14}\text{CO}_2$ recovery (% of Spiked radioactivity)	
	Without L. P. ^{a)} layer (n=3)	With L. P. layer (n=3)
6.00	25.3 \pm 3.1 ^{b)}	— ^{c)}
7.40	78.9 \pm 6.2	0.010 \pm 0.009
8.00	62.9 \pm 4.8 ^{d)}	0.011 \pm 0.007
9.10	42.8 \pm 3.2	—

a) Liquid paraffin.

b) Mean value \pm S. D.

c) Not measured.

d) From the results in Fig. 4.

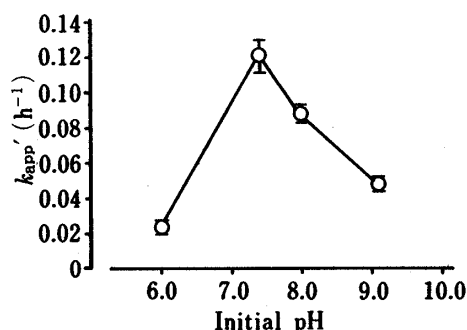


Fig. 6. A pH- k_{app}' Profile of the Ureolysis Catalyzed by Urease in Rat Urine at 25°C

Initial concentrations of urea and urease were 10.0 $\mu\text{g}/\text{ml}$ and 5.0 U/ml urine, respectively. Each point is the mean value \pm S.D. for three experiments.

tion time course in the presence of urease at 5 U/ml and at 25°C is shown in Fig. 6. The largest value for k_{app}' was obtained at around pH 7.4, indicating that the greatest stability and thus the highest activity of the urease should also be exhibited in the neutral to very slightly alkaline pH region in rat urine.

Effect of Covering the Reaction Mixture or Excreted Urine with Liquid Paraffin on Collection of $^{14}\text{CO}_2$

As shown in Table I, almost no radioactivity was recovered as $^{14}\text{CO}_2$ derived from ^{14}C -urea when the urine reaction mixture was incubated at 25°C in the presence of 5 U/ml of urease but in the absence of any contact of the mixture with atmospheric air (excluded by complete coverage of the solution with a layer of liquid paraffin). Thus it is recommended to use a bar-

rier such as liquid paraffin or silicone oil over urine samples in order to avoid an overestimation of expired radioactivity.

In addition, when the urine samples were collected under liquid paraffin from rats that had received intravenously 10 μ Ci of 14 C-urea (0.675 mg/kg as urea), and had been housed in metabolic cages kept at 23 to 24°C, the cumulative expiratory excretion of radioactivity was not significantly different from the value obtained previously under the same experimental conditions except for omission of the liquid paraffin barrier,⁷⁾ as shown in Table II. There

TABLE II. Cumulative Urinary, Fecal and Expiratory Excretions of Radioactivity in 24 h following Intravenous Administration of 14 C-Urea (10 μ Ci, 0.675 mg as Urea/kg) to Rats

Biological materials	Recovery (% of dose)	
	With L. P. ^{a)} layer (n=3)	Without L. P. layer (n=4) ^{b)}
Urine	81.8 \pm 3.01 ^{c)}	82.7 \pm 2.87
Feces	0.41 \pm 0.11	0.31 \pm 0.13
Expired air	12.0 \pm 0.98	12.1 \pm 1.70
Total	94.2 \pm 2.89	95.1 \pm 2.76

a) Liquid paraffin.

b) Reference 7).

c) Mean value \pm S. D.

were also no significant differences in the urinary and fecal excretion data. This means that the previous experiments in which the rats had been housed in the same metabolic cages as that in the present work (equipped to collect the expired air and kept at 23 to 24°C) did not overestimate the cumulative expiratory excretion of radioactivity. However, even a small rise in the temperature to above 25°C (at which the metabolic cages are maintained) would lead to some overestimation of the expiratory excretion of the radioactivity. It may therefore be concluded that the precautions of keeping the urine isolated from the atmosphere and keeping the metabolic cage at an appropriate temperature such as 20 to 25°C must be taken in experiments involving measurement of expiratory excretion of radioactivity, and in which urea is excreted as an end-product or in disposition experiments where the urea is administered.

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