Isotopic Studies of Urea Metabolism in Rabbits

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1. The half-life of [^{15}N]urea was found to be significantly longer than that of [^{14}C]urea injected at the same time, the differences being due to endogenous catabolism of urea, which is accompanied by little or no reutilization of ^{14}C but is approx. 20% for ^{15}N . [^{15}N]Urea therefore appears to be valueless as an indicator of nitrogen metabolism unless the extents of endogenous catabolism of urea and of fractional reutilization of ^{15}N can be separately estimated. 2. Though measurements of the radioactivity of expired $^{14}\text{CO}_2$ confirmed the existence of considerable urea catabolism these could not be used for quantitative assessments. 3. Alternative graphical methods based on [^{14}C]urea specific activities in plasma and urine samples were used to calculate the fraction of urea production that is excreted. Values by the two methods were in good agreement and showed that some animals excrete less than half the urea that they produce. 4. Specific activity differences between simultaneous samples of urinary and plasma urea reflect the presence of a pool of urea in the kidney that is not in equilibrium with the body urea pool. Calculations indicate the presence of urea in the kidney that in some cases may represent as much as 15% of the body pool, and in two animals in which post-mortem renal analyses were performed the masses of urea found agreed closely with the calculated values. 5. A model for urea metabolism is proposed that includes this pool in the excretory pathway. The related theory is shown to be adequate to explain the shape of the specific activity curves of urinary urea from the time of injection and the constant delay of the specific activity of urinary urea, relative to that of plasma urea, that is observed after a short preliminary equilibration period. 6. The body urea pool was calculated from the activity retained at 1.5 hr. by excluding renal activity and the corrected specific activity of plasma urea at the same time. The urea pool was calculated to be distributed at the plasma concentration in a substantially smaller water volume than that found by injecting tritiated water in five animals. Reasons for this are discussed. 7. Urea synthesis rates calculated from the pool values are in close agreement with rates calculated from the mass of urea recovered in the urine and the fraction of newly synthesized urea that is excreted.

After the administration of [^{15}N]urea, labelled ammonia and protein appear in the carcass of rats (Schoenheimer, 1942; Bloch, 1946), and the injection of [^{14}C]urea results in labelling of expired carbon dioxide (Leifer, Roth & Hempelmann, 1948). These observations clearly indicate that endogenous decomposition of urea proceeds in living animals.

Extensive earlier investigations suggest that most of the urease in the body is probably confined to bacteria. These urea-splitting micro-organisms are located in different sites in various species, e.g. in the intestines in rats (Chao & Tarver, 1953) and mice (Dintzis, 1953), and in the mouth, nasopharynx and trachea in cats (Kornberg, Davies & Wood, 1953*a, b*,

1954*a*). Much less is known about urease activities in the tissues. Low values were found in liver and kidney slices (Fitzgerald, 1950; Chao & Tarver, 1953) and relatively more significant ones in the gastric mucosa (Luck, 1924; Fitzgerald, 1946; Kornberg & Davies, 1955). Gastric production possibly helps to elevate blood ammonia concentrations in patients with uraemia (Webster & Gabuzda, 1959; Lieber & Lefevre, 1959) and cirrhosis (Rappoport & Kern, 1963), but whether gastric urease is a genuine body enzyme (Conway, Fitzgerald, McGeeney & Geoghegan, 1959) or a bacterial product (Kornberg, Davies & Wood, 1954*b*) remains an open question.

Endogenous catabolism of urea implies that the rate of urea excretion is not the same as that of urea

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production, the former being frequently much lower (Walser & Bodenlos, 1957). Walser & Bodenlos (1959) described a method of measuring the production rate that is based on [^{14}C]urea specific activities of [^{15}N]urea enrichments in body water and urine. Hitherto little of practical value has emerged from quantitative considerations of urea metabolism, and the subject appears to have been neglected. Methods that have recently been proposed for measuring synthesis rates of liver-produced plasma proteins *in vivo* (McFarlane, 1963*a,b*; Reeve, Pearson & Martz, 1963), however, require accurate data on urea synthesis, and the investigations reported below were undertaken partly to obtain this information in rabbits, and as part of a wider study of isotopic methods of measuring metabolic rates in general.

A preliminary communication has already appeared (Koj, Regoeczi, Irons & McFarlane, 1964).

MATERIALS

Animals. Male Sandylop rabbits (2.23–3.47 kg. body wt.), bred at the National Institute for Medical Research, were used. The animals were kept separately in metabolic cages receiving a standard pelleted diet (Short & Gammage, 1959) and were given water *ad lib*.

Labelled compounds. [^{14}C]Urea (specific activity 115 $\mu\text{C}/\text{mg.}$ of compound), $\text{Na}_2^{51}\text{CrO}_4$ (82 $\mu\text{C}/\mu\text{g.}$ of Cr) and tritiated water were obtained from The Radiochemical Centre, Amersham, Bucks. [^{15}N]Urea of enrichment 29.4 atoms % ^{15}N was supplied by the Office National Industriel de l'Azote, France.

METHODS

Injections. [^{15}N]Urea was dispensed by weighing the dry substance on a microbalance, and solutions of [^{14}C]urea were also subdivided by micro-weighing. When both ^{14}C and ^{15}N were used the urea preparations were injected together. From a portion of the urea solution duplicate standard dilutions were made and used to determine the ^{14}C or ^{15}N content/ml. To a known volume of the remainder a trace amount of Na^{131}I was added and the solution was counted in a well-type scintillation counter that was insensitive to the soft β -radiation of ^{14}C . After injection, the washings from the syringe, needle and vial were counted and the proportion of the original dose of urea that was injected was calculated from the two γ -radiation counts, the washings representing 4–15% of the uncorrected dose. Tritiated water injected was estimated by the difference in weight of the syringe before and after injection. Doses normally used were 2–6 μC of [^{14}C]urea and 12–20 mg. of [^{15}N]urea.

General procedure. As a rule, experiments were started by emptying and washing out the bladder of the animals with a soft rubber catheter. Injections were made intravenously and blood samples were collected in test tubes containing 250–500 i.u. of dry heparin over periods of 6–10 hr. Quantitative catheterizations were performed 2–3 min. before each blood sample was withdrawn.

Deproteinizing plasma. Plasma samples (2–3 ml.) were first brought to pH 4–5 with 0.076 N- H_2SO_4 . Usually an

Fig. 1. Modified flask used to recover $^{14}\text{CO}_2$ from urea after urease action *in vacuo*. The side limbs are connected spirally to the neck in such a way that the urease solution in one and then the citric acid-tungstic acid in the other can be delivered directly into the bulb by tilting and rotating.

equal volume was required, and this was followed by the addition of the same volume of the sodium tungstate precipitant [2 ml. of 10% (w/v) sodium tungstate + 10 ml. of 0.076 N- H_2SO_4 + 10 ml. of water]. The mixture was brought to 100° on the water bath and the protein coagulum removed by centrifugation. A measured volume of the supernatant solution equivalent to a known portion of the precipitation mixture and hence of the original plasma was then transferred to the main bulb of the reaction vessel illustrated in Fig. 1 of McFarlane (1963*b*) and treated with urease as described there.

Measurement of plasma [^{14}C]urea and [^{15}N]urea specific activities. (a) ^{14}C only. The deproteinized plasmas were vacuum-concentrated to about 1 ml. with a water pump. One side arm of the reaction vessel received 1 ml. of a solution containing 1 mg. of urease (Sigma Chemical Co., St Louis, Mo., U.S.A.) in 1 ml. of a solution containing 0.14 g. of sodium pyrophosphate and 0.02 g. of orthophosphoric acid.

The other side arm received 1 ml. of a solution made by mixing equal volumes of 4M-citric acid and the tungstate precipitant. For each batch of urease carbon blank estimations were performed and used to correct specific activities of urea in plasma and urine.

(b) ^{14}C followed by ^{15}N . Since residues in this case were to be made alkaline to liberate ammonia the concentration of pyrophosphate in the urease solution was decreased to 5% of the above value and carbonate-free 0.1N-NaOH was used to adjust the pH to 7 for urease incubation. The volume of the citric acid-tungstic acid mixture was also decreased to 0.5 ml. After recovery of $^{14}\text{CO}_2$ *in vacuo* as described by McFarlane (1963b), the three-way tap with 'cold finger' was replaced with one of the type shown in Fig. 1 coupled to a receiving flask containing 0.5 ml. of 0.5N- H_2SO_4 . Potassium metaborate solution [2 ml. of 69% (w/v)] was placed in the side arm that previously contained urease and, after being cooled, the system was evacuated with an oil pump. With the tap closed the metaborate was tipped into the mixture, which was left to stand at 25° for 1 hr. to allow the liberated ammonia to diffuse over into the H_2SO_4 . Almost complete transfer of ammonia was achieved by vacuum-distillation of nearly all the water with the receiving flask at -76°. The transfer of water was reversed to reduce the volume of ammonium sulphate solution to about 1 ml. before it was transferred to a Rittenberg tube (Sprinson & Rittenberg, 1949). Nitrogen was liberated from ammonium sulphate by oxidation with alkaline sodium hypobromite solution.

Measurement of urinary [^{14}C]urea and [^{15}N]urea specific activities. (a) ^{14}C only. A 0.3 ml. sample of urine was brought to pH 2 and concentrated almost to dryness on the water pump to remove any carbonate that might be present. The subsequent procedure for measuring ^{14}C specific activities was the same as that described for deproteinized plasmas.

(b) ^{15}N only. A 1 ml. sample of urine was brought to pH 6 and passed through a column (3 cm. $\times$ 1 cm.) of Zeo-Karb 225 (The Permutit Co. Ltd., London, W.4) to remove free ammonia. The urea was washed out of the column with about 3 ml. of water and collected in a reaction vessel (fitted with a side arm), which was connected to a flask containing 0.5 ml. of 0.5N- H_2SO_4 , as shown in Fig. 1. The urea was incubated with urease, and the ammonia released from the solution and transferred to the H_2SO_4 as described for the deproteinized plasmas. However, since the concentration of urea in urine is at least ten times that in plasma the ammonia was allowed to diffuse into the H_2SO_4 for only 5–10 min. after the addition of metaborate solution.

Measurement of plasma ^3H specific activities. Blood samples were taken daily for 8–10 days after injecting HTO. Whole blood samples (1.5–2 ml.) were placed in one limb of a 'two-finger' flask and the water was vacuum-distilled over into the other where it was condensed at -76°.

Radioactivity counting. After measuring the mass of $^{14}\text{CO}_2$ in a constant-volume manometer as described by McFarlane (1963b), the gas was condensed with the aid of liquid nitrogen into counting vials containing the liquid scintillator (Woeller, 1961). The HTO samples were weighed into counting vials containing a mixture of dioxan-naphthalene-2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Werbin, Chaikoff & Imada, 1959), with 0.4 g. of water and 9.5 ml. of scintillator solution. The standard was a gravimetric dilution of HTO with ion-

exchange water and counting was in a Packard Tri-Carb liquid scintillation spectrometer (model 314 EX).

Mass spectrometry. The Rittenberg tubes containing the ammonium sulphate and hypobromite solutions were evacuated to 10^{-3} mm. Hg, the solutions being warmed to expel dissolved gases. After oxidation of ammonia, the tubes were immersed in ethanol-solid CO_2 mixture and the nitrogen was led into a 60° Nier-type mass spectrometer tube. The abundance of ^{15}N was calculated from measurements of the intensities of the peaks at mass numbers 28 and 29 by using the equations given by Rittenberg (1946).

Recovery of expired $^{14}\text{CO}_2$. The rabbit was placed in a sealed glass-fronted chamber with continuous recirculation by fan of the air through one half of a duplicated CO_2 absorber. The chamber contained 144 l. of air, of which approx. 30 l./min. was circulated through the absorber. Preliminary tests without a rabbit showed the CO_2 concentration in the chamber to fall exponentially with a half-life of 10 min. Each of the CO_2 absorbers contained 60 g. of soda-lime and was used for 1 hr. Fresh air was introduced into the cage through KOH and soda-lime traps. The CO_2 was liberated from the soda-lime with 50% (w/v) HCl and trapped in two Drechsel bottles each containing 100 ml. of 3N-NaOH. When this was complete portions of the NaOH were treated in the reaction vessel shown in Fig. 1, CO_2 being liberated with 4M-citric acid from the carbonate after establishing a vacuum. Further treatment of the samples was as described for deproteinized plasmas.

Measurement of the urea content of the kidneys. The animal's blood was labelled by injecting autologous erythrocytes tagged with ^{51}Cr by the method of Mollison & Veall (1955). On the next day [^{14}C]urea was injected and samples were collected for 4–5 hr. in the way normally followed for obtaining information on the renal delay time and excretion rate of urea. The animal was then killed by injecting an overdose of pentobarbitone sodium. The kidneys were removed immediately and split longitudinally in two, each half being quickly separated into cortex, inner and outer medulla, which were readily distinguishable by macroscopical examination.

All parts were weighed on the microbalance, one set being dried for 48 hr. at 105° for estimating water content, and the other being used for analysis. The latter samples were ground up with sand and a solution of saponin to complete the destruction of erythrocytes. After centrifuging at 26000g for 2 hr. the supernatant was removed and portions were counted in duplicate to estimate the ^{51}Cr activity. The volume of supernatant trapped in the sediment was calculated from the loss in weight of the sediment during drying and the similarly measured water content of the supernatant. Portions of the supernatant were also deproteinized and analysed for urea as described above for plasma samples. Values for whole kidneys were calculated on the basis of the weight relationship of corresponding parts in the half-kidneys and assuming their content of water, urea and trapped blood to be the same. The ^{51}Cr activities in the kidney sections were converted into blood volumes by reference to the activity in a terminal blood sample.

CALCULATIONS

Fractions of urea production excreted and catabolized. Two graphical procedures, which are illustrated in Fig. 2, were used. In one, which is based on that of Walser & Bodenlos

Fig. 2. Illustration of two graphical methods used to evaluate urinary activities. In method A (a) the activity that accumulated in the urine up to time t is plotted against the activity calculated to have left the body in the time $t - t_d$ according to the slope of the plasma exponential. In method B (b) the activity found in the urine plus the activity calculated to be present in the kidney at time t is plotted against the activity calculated to have left the body in the same time. E.E., 'Excess excretion'.

(1959), the fraction of injected [^{14}C]urea activity that was collected in the urine up to time t was plotted against the activity calculated to have left the body water in time $t - t_d$ at a constant rate. This rate, k , is equal to the terminal slope of the specific activity curve of plasma urea, and t_d (the delay time) is the interval between times at which urea specific activities in urine and plasma are the same, as is illustrated in Figs. 3 and 6. The second procedure is based on a 'mixing' renal urea pool theory, which is given in the Appendix (Charlwood, 1965), to explain the higher specific activities observed in urinary than in plasma urea at the same time. In this case activities collected in the urine at t , to which were added activities calculated to be present in this renal pool also at time t , were plotted against $1 - e^{-kt}$.

The term 'excess excretion' is applied arbitrarily to both ordinates when the abscissae are zero. When both abscissae are 1.00 the ordinates are assumed to represent the fraction of injected activity that was excreted.

Body-water urea pool. This was calculated by assuming that all the urea activity retained in the body (excluding activity in the kidney) at 1.5 hr. was distributed with a mean urea specific activity 2% higher than in plasma urea at the same time. As explained in the Discussion section this is to allow for a persistent specific activity difference between intra- and extra-vascular urea.

Delay time (t_d). This time was used by Walser & Bodenlos (1959) to indicate the interval between times at which urea specific activities were the same in urine and plasma, and values were determined graphically as shown in Figs. 3 and 6. Because of the exponential nature of the excretion process, urinary urea specific activities must be plotted at times (t') that correspond to mean integrated specific activities in the plasma:

$$t' = \{\ln[k(t_2 - t_1)/(e^{-kt_1} - e^{-kt_2})]\}/b_1$$

where t_1 and t_2 are the times at which consecutive urine samples were collected. A convenient procedure that we have used to apply this formula, and that gives the same results as the one used by Walser & Bodenlos (1959), is as follows. Activity that leaves the plasma between consecutive plasma samples (i.e. at t_1 and t_2) is calculated and divided by the mg. of urea carbon excreted in the same interval. A time is then located on the plasma specific activity curve at which urea of this mean integrated specific activity would have to be hypothetically excreted to produce urinary urea of the specific activity observed, and urinary urea specific activities were then plotted at this time.

Renal urea pool. This was calculated as the product of urea excretion rate in mg./hr. and delay time (t_d) in hr., a relationship that follows from the theories underlying both graphical procedures.

Urea synthesis rates. These were calculated in two ways. Values based on the urea pool (cf. Table 3) were obtained by multiplying the body-water urea pool (again excluding renal urea) by k , which was increased by 2% to allow for net transfer of activity from the extravascular to the vascular compartment. Values based on the excretion rate were obtained by multiplying observed rates of urinary urea excretion in mg./hr. by the reciprocal of the fraction of urea production that was excreted.

Reutilization of ^{15}N . The difference between [^{15}N]urea and [^{14}C]urea synthesis rates in mg./hr. gives the amount of urea synthesized/hr. by reutilization of urea nitrogen. This rate is also alternatively expressed in Table 1 as a fraction of the true urea catabolic rate measured with ^{14}C .

EXPERIMENTAL

[^{14}C]Urea and [^{15}N]urea in the same animal. Seven rabbits were injected with a mixture of [^{14}C]urea and [^{15}N]urea and synthesis rates were calculated from both kinds of isotopic data.

Urea diffusion from the bladder. [^{14}C]Urea, without carrier and in 20 ml. of 0.9% NaCl solution, was introduced through a rubber catheter into the emptied bladders of four rabbits and blood samples were taken at intervals for estimating urea specific activities. The total radioactivity that had diffused into the urea pool was assessed by plotting these specific activities and extrapolating to zero time. The activity thus obtained was then multiplied by the mass of urea in the body pool as measured in a previous experiment.

A more successful experimental arrangement for achieving the same result proved to be to inject [^{14}C]urea intravenously and to observe plasma specific activities with and without catheterization. This was done in two rabbits.

The diffusion of urea in the reverse direction, i.e. from the body urea pool into the bladder, was investigated in one animal by ligating the ureters just below the level of the kidneys before injecting [^{14}C]urea. Then 1 hr. was allowed

for the [^{14}C]urea to diffuse into the bladder, which contained a solution of unlabelled urea corresponding in volume, urea mass and concentration to a 4 hr. urine sample from a normal rabbit.

Urea distribution in the kidneys. Two rabbits were injected with [^{14}C]urea and ^{51}Cr -labelled erythrocytes and killed 4–5 hr. later to permit urea and water measurements in both kidneys as described in the Methods section.

Urea space and total body water volume. Masses of body water and distribution spaces of urea were compared in five rabbits by using [^{14}C]urea and HTO at different times.

Urea synthesis and excretion rates. Ten normal rabbits were injected with [^{14}C]urea and small blood samples were taken at regular intervals. Quantitative urine collections were by catheter at intervals over 8 hr. and both synthesis and excretion rates of urea were calculated.

RESULTS

Comparison of [^{14}C]urea and [^{15}N]urea in the same animal. Results of all the experiments are shown in Table 1 and curves from a typical one in Fig. 3. The ^{14}C specific activities and ^{15}N enrichments were both constantly higher after 30 min. in the urine than in the plasma, urine/plasma ratios being slightly greater for ^{14}C than for ^{15}N . Measurements with ^{15}N gave substantially longer half-lives than with ^{14}C , and no linear correlation could be found between urea catabolic rates on the one hand and amounts of recycling of nitrogen on the other.

Urea exchange between body urea pool and bladder. When the first blood sample was taken 10 min. after

introducing [^{14}C]urea into the bladder radioactivity was present in significant amounts, and extrapolation of several values obtained in the first hour indicated that approx. 5% of the dose had passed from the bladder into the body urea pool in this time. On the other hand, only 0.01% was transferred in 1 hr. in the opposite direction in an animal with ligated ureters that received [^{14}C]urea intravenously. This suggested that reabsorption of labelled urea from the bladder might be decreased by frequent catheterization, and such a consequence was observed in the experiment illustrated in Fig. 4. The abrupt change of slope coinciding with the onset of catheterizations suggests that the synthesis rate for urea was increased apparently by 14.5%, whereas no change occurred in plasma urea concentration or in urea output. In another similarly

Fig. 3. Specific activities in urinary (Δ , $\triangle$) and plasma ($\bullet$, $\circ$) urea after injecting [^{14}C]urea (Δ , $\circ$) and [^{15}N]urea ($\bullet$, $\bullet$) together into a normal rabbit. The urinary specific activities are greater than plasma ones at the same time with both ^{14}C and ^{15}N . Since urine and plasma curves with the same isotope are parallel this can also be described by means of an apparent delay interval (t_d) between times at which urine and plasma specific activities are equal. The half-life of [^{15}N]urea specific activity (3.05 hr.) is longer than that of [^{14}C]urea (2.50 hr.).

Table 1. *Reincorporation of urea nitrogen into newly synthesized urea*

The values in column 1 were obtained from slopes of terminal plasma specific activity exponentials. For the values in column 2 synthesis rates are given by urea pool $\times k$. The values in column 3 are given by dividing the difference in synthesis rates (column 2) by the urea catabolism rate (mg./hr.) measured with ^{14}C . Rabbit 7 (2.93 kg.) was investigated: (a) under normal conditions; (b) 6 days later, at the end of a course of treatment over 40 hr. with neomycin sulphate (4×0.5 g. by stomach tube); (c) 31 days after treatment with neomycin.

Rabbit	$k^{15}\text{N}$ $k^{14}\text{C}$	Difference in synthesis rates (mg./hr.)	Reutilization rate of urea N (% of [^{14}C]urea catabolism rate)
	(1)	(2)	(3)
1	0.84	5.5	32.2
2	0.82	8.5	51.2
3	0.84	6.9	43.1
4	0.74	11.3	69.7
5	0.81	10.5	68.1
6	0.69	12.0	88.8
7 (a)	0.77	7.3	38.4
(b)	0.91	2.9	22.8
(c)	0.89	3.5	17.7

Fig. 4. [^{14}C]urea specific activities in the plasma and urine of a rabbit after injecting [^{14}C]urea and starting catheterizations at 4 hr. Δ , Urine specific activities; $\circ$, plasma specific activities.

Table 2. Masses and activities of urea in the kidney in relation to delay time and excretion rate of urea

	Rabbit M64				Rabbit M68			
	Right kidney		Left kidney		Right kidney		Left kidney	
	Urine		Plasma		Urine		Plasma	
	Outer	Inner	Outer	Inner	Outer	Inner	Outer	Inner
[¹⁴ C]Urea injected (μ C)	Cortex	medulla	Cortex	medulla	Cortex	medulla	Cortex	medulla
Time of death (hr.)	1.480	1.529	1.575	2.696	1.129	0.943	1.327	1.638
Delay time (hr.)	0.337	1.438	0.319	5.650	0.299	0.925	0.309	3.410
Urea excretion rate (mg. of C/hr.)	5.80	6.50	5.82	6.50	5.53	6.14	5.39	6.27
	0.248	0.058	0.342	0.014	0.167	0.038	0.280	0.007
Urea concentration (mg. of C/ml.)								
[¹⁴ C]Urea specific activity (μ C/g.)								
Urea content (mg. of C)								
Urea concentration (mg. of C/ml.)								
¹⁴ C specific activity of urea (μ C/g.)								
Vol. of trapped blood† (ml.)								

* By extrapolating the urinary specific activity curve to the time of death.

† By using ⁵¹Cr-labelled erythrocytes.

treated rabbit the synthesis rate appeared to be increased by 12% over a 5 hr. catheterization interval compared with an earlier experiment without catheterizations.

Distribution of urea in the kidneys. Results in Table 2 show that the total renal urea in one rabbit was 13.71 mg. and in the other 8.52 mg. Less than 1% of this is attributable to error arising from trapped blood. The amounts of urea found agree closely with the product of delay time (0.50 and 0.31 hr.) and urea excretion rate (30.4 and 26.6 mg. of carbon/hr.) in each animal, as is required by both theories [cf. the Appendix (Charlwood, 1965)]. Specific activities were slightly lower in the renal cortex than in the plasma at the same time, an observation for which no explanation is available.

Total body water volumes. Rabbits A, B, D, E and F, which were injected with tritiated water some weeks after the [¹⁴C]urea experiments referred to in Table 3, gave water volumes which were 68, 69, 65, 69 and 69% respectively of their body weights.

Synthesis and excretion rates of urea and fractions excreted. Results from the ten rabbits that received [¹⁴C]urea, one of them before and after neomycin treatment, are given in Table 3. Although all animals were on the same diet the excretion rates of urea carbon varied between 8.2 and 38.4 mg./hr. Delay times also varied widely, from 0.33 to 1.05 hr. Such wide variations were surprising in a closely inbred strain of rabbits housed in almost identical conditions. 'Excess excretions' calculated by both methods also showed wide variations that were correlated to some extent with renal delay times (cf. Fig. 5) and with fractions excreted (columns 9 and 11 in Table 3). The latter values agreed well by the two methods.

Measurements of expired ¹⁴CO₂. The few measurements made after injecting [¹⁴C]urea showed that the maximum rate of expiration was not reached until 2 hr. (cf. Fig. 6). In the experiment illustrated calculations based on fitted plasma and urine curves showed that 34% of the synthesized urea was being catabolized. An expiration curve of ¹⁴CO₂ activity was then calculated to correspond to a situation in which 95% of the activity was presumed to be liberated and to appear immediately in the expired air. This is shown by the broken line and it is evident that expiration, in addition to being seriously delayed, is only 60–70% complete. Because of these limitations no further measurements of expired ¹⁴CO₂ were carried out.

Urea space. Urea carbon pool values were used to calculate urea spaces by assuming the urea in the pool to be distributed in a water volume at the same urea concentration as in the plasma water. These volumes, expressed in column 13 of Table 3 as fractions of the body weight, varied from 43 to 64%. Values for rabbits A, B, D, E and F were 52, 50, 48,

Table 3. *Synthesis and excretion rates of urea in ten rabbits*

Rabbit	Body wt. (kg.)	Injected activity (μ c)	Plasma specific activity slope (100k) (%/hr.)	Urea carbon excretion (mg./hr.)	Urine/ plasma specific activity ratio	Calc. renal urea carbon pool (mg.)	Procedure A				Procedure B				Urea carbon space (% of body wt.)	Urea carbon synthesis rate (mg./hr.)	
							Urine/ plasma delay (t_d) (hr.)	Excess excretion, intercept (%)	Fraction of urea production excreted (%)	Excess excretion, intercept (%)	Fraction of urea production excreted (%)	Urea carbon pool (mg.)	Excess excretion, intercept (%)	Fraction of urea production excreted (%)		From pool	From excretion rate
A _u	3.08	2.28	22.2	25.0	1.05	26.2	(5)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)		
B _u	3.15	2.39	27.7	38.4	0.83	24.2	1.26	16.0	0.62	12.5	0.64	171	52	38.6	39.7		
C _{u1}	2.99	5.17	19.1	12.3	1.06	4.1	1.18	7.0	0.71	7.5	0.70	189	50	47.6	54.5		
C _{u2}	2.99	5.38	17.8	17.8	0.83	14.7	0.83	4.5	0.42	1.5	0.39	153	46	29.8	29.6		
D _u	2.76	5.26	15.6	15.8	0.71	11.2	1.14	15.5	0.57	7.5	0.57	162	43	29.5	31.2		
E _u	3.47	6.23	24.3	27.5	0.42	17.6	1.10	6.5	0.55	6.0	0.54	181	48	28.8	29.0		
F _u	3.06	5.99	20.3	25.4	0.70	17.8	1.10	8.5	0.65	6.0	0.65	162	56	40.2	42.3		
G _u	2.61	5.10	22.6	28.1	0.60	16.9	1.13	8.5	0.89	10.0	0.63	173	50	35.8	38.5		
H _u	3.40	5.02	27.7	30.0	0.42	12.6	1.15	5.0	0.67	4.0	0.67	187	64	43.0	42.0		
I _u	2.70	4.61	13.1	14.7	0.94	13.8	1.12	8.5	0.64	7.0	0.64	159	60	45.0	46.9		
J _u	2.23	5.71	28.3	8.2	0.58	4.75	1.16	5.5	0.41	5.5	0.42	206	49	27.6	35.4		
								6.0	0.36	4.0	0.31	85	48	24.5	24.5		

The calculated renal urea carbon pool (column 7) was obtained from the urea carbon excretion (column 4) $\times t_d$ (column 5). The values for procedure A (columns 8 and 9) were obtained from a plot of cumulative urinary activities (y) against $x = 1 - e^{-k(t-t_d)}$, the 'excess excretion' intercept (column 8) being the activity excreted when $x = 0$ and the fraction of urea production excreted (column 9) being that when $x = 1.00$ (Walser & Bodenlos, 1959). The values for procedure B (columns 10 and 11) were obtained from a plot of cumulative urinary and renal activities against $1 - e^{-kx}$, the 'excess excretion' intercept (column 10) being the activity excreted and present in the kidney when $x = 0$ and the fraction of urea production excreted (column 11) being that when $x = 1.00$ [see the text and the Appendix (Charlwood, 1945)]. The urea carbon pool (column 12) was obtained as described in the text by assuming that all the activity retained in the body at 1.5 hr. was distributed in a urea pool with a specific activity 2% greater than that of plasma urea at 1.5 hr.; the activity retained at this time was obtained from a plot of urine + kidney activities against time, the value read off at 1.5 hr. then being divided by the fraction of urea production excreted. The urea carbon space (column 13) was obtained from $100 \times$ urea carbon pool (column 12)/urea carbon concentration in body water $\times$ body wt. The urea carbon synthesis rate from the pool (column 14) was obtained from $1.02 \times$ urea carbon pool (column 12) $\times$ plasma specific activity slope (column 3)/100, where the factor 1.02 is included to allow for net return of activity from the extravascular water (see the text). The urea carbon synthesis rate from the excretion rate (column 15) was obtained from urea carbon excretion (column 4)/fraction of urea production excreted (mean of columns 9 and 11).

Fig. 5. Values for 'excess excretion' plotted against delay times in the same rabbit. The data are taken from Table 3. ○—○, Procedure A; △—△, procedure B.

Fig. 6. Rate of expired $^{14}\text{CO}_2$ activity (□, actual; X, theoretical) in relation to plasma (○) (half-life 3.40 hr.; $k=0.203$) and urinary (△) ^{14}C urea specific activities at various times after injecting ^{14}C urea. The broken curve represents a theoretical rate of expiration based on 34% urea catabolism and immediate expiration of 95% of the activity liberated by this catabolism.

56 and 50% respectively, which are all substantially lower than corresponding values referred to above for body water measured with tritium. Urea synthesis rates calculated from pool or excretion rate data were significantly the same.

Effect of neomycin. The effect of neomycin treatment on rabbit C was to increase the fraction of urea production excreted from 0.4 to 0.57. In addition the renal pool was increased from 4.1 to 14.7 mg. of urea carbon and values for 'excess excretion' increased almost proportionately.

DISCUSSION

A difference in the half-lives of ^{15}N urea and ^{14}C urea in the body water was expected, but not

to the extent observed. Since equilibrium conditions were established in the plasma after half an hour it appears that the time required for urea nitrogen, liberated presumably in the intestine, to reappear as circulating newly synthesized urea must be short. These observations, which confirm the findings of Walser & Bodenlos (1959), must greatly restrict the usefulness of ^{15}N urea as a tracer in quantitative biology as proposed by San Pietro & Rittenberg (1953) and others. It is apparent from Fig. 3 and Table 1 that rates of synthesis of urea will be seriously underestimated when calculated from ^{15}N data. For this reason the metabolic data in Table 3 and our conclusions about these rates are based solely on the use of ^{14}C urea.

Walser, George & Bodenlos (1954) described a mass-spectrometric method for estimating the extent of recycling of the ^{15}N label by analysing the statistical distribution of $^{15}\text{N}_2$, $^{14}\text{N}_2$ and $^{15}\text{N}^{14}\text{N}$ molecules in urea samples. The procedure used in the present work, namely injecting a mixture of ^{14}C urea and ^{15}N urea, provides an alternative and perhaps more convenient way of obtaining the same information. This is based on the assumption that both ^{14}C urea and ^{15}N urea are distributed in the same space and that the reutilization rate of the ^{14}C label is small. Evidence favouring the latter assumption is given by McFarlane, Irons, Koj & Regoeczi, 1965. Whereas Walser & Bodenlos (1959) found between 3 and 12 hr. after injecting ^{15}N urea that 15% of the ^{15}N urea present in the plasma of a normal human was due to recycling, our calculations (cf. column 1 in Table 1) suggest a corresponding mean value of 21% for normal rabbits.

In the hope that recycling of urea nitrogen could be eliminated by abolishing endogenous catabolism of urea, neomycin was administered to a few animals. However, as indicated by the representative result in Table 1, treatment with large doses for several days before and during the experiment only partly achieved this result. The fraction of urea production catabolized was diminished to about 60% of the normal value, and the persistence of catabolism was confirmed qualitatively by collecting and measuring $^{14}\text{CO}_2$ in the expired air. A similar result is evident with rabbit C in Table 3, the fraction catabolized being decreased from 60 to 43% by neomycin treatment. The drug in our hands appeared to be less effective in suppressing catabolism of urea in rabbits than it was when Walser & Bodenlos (1959) used it in humans. This could be due to a relatively higher gastric urease activity in rabbits that is known to be unaffected by neomycin (Belding & Kern, 1963).

The observation that approx. 5% of ^{14}C urea injected into the bladder was absorbed into the body water in 1 hr. is in keeping with the results of Levinsky & Berliner (1959a), who demonstrated

during the passage of an artificial urine from the pelvis through the urethra of dogs at low flow rates that the urea concentration fell by 15%, and by larger amounts when the urine was allowed to pool in the bladder. They also demonstrated that movement of solutes across the bladder wall was in the direction of the concentration gradient. Both kinds of experiment are difficult to interpret quantitatively and do not give a clear picture of the probable situation in the intact animal. In addition, the value of 5% of bladder urea reabsorbed in 1 hr. may be an underestimate since carrier urea was not used and the high concentration of urea normally found in the bladder may help to promote its reabsorption.

Urea arriving in the bladder has a specific activity that may be up to 26% higher than in the plasma at the same time. With a difference of 10% calculations show that if 30% of this urea was returned continuously to the body water the slope of the plasma specific activity curve would be decreased by approx. 15%. Changes of this order were observed in two experiments (one of which is shown in Fig. 4), when hourly catheterizations were started after a period of 4 hr. during which urine accumulated in the bladder. Since measurements of urea synthesis rates described in the present work were performed with approximately hourly catheterizations it is reasonable to conclude that values a few per cent higher might have been obtained if some form of continuous collection of urine had been carried out. As the phenomenon depends on numerous individual factors including delay time, urine flow rate, urea concentration differences between plasma and urine, permeability conditions and possibly even the degree of bladder distension, it is not possible to generalize. It is evident, however, that prolonged periods of urine retention should be avoided in making measurements of this kind.

Chinard & Enns (1955) injected [^{14}C]urea along with creatinine into the renal artery of dogs and observed that excretion of the labelled urea was delayed relative to that of creatinine. Their investigations were confined to the first 15 min. after the injection and could not give quantitative information about the delay. It was left for Walser & Bodenlos (1959) to attempt an exact description of the phenomenon as they observed it in humans. At times longer than 1 hr. after injecting [^{14}C]urea they found that specific activities were constantly 10–20% higher in the urine than in the plasma at the same time and that the difference (t_d) between times at which urea specific activities in the urine and plasma were the same was about 30–45 min. They proceeded to allow for this difference by comparing activities collected in the urine up to time t with activities that they calculated from the slope of the terminal plasma exponential (k) to have left

the body water up to $t - t_d$. A difficulty associated with their procedure is to give a meaning to activity that is actually collected in the urine in d min. When the above procedure was applied to data in Table 3 the results suggested that a maximum of 3.5–16% more of the injected activity appeared in the urine in the early stages of the experiment than had apparently left the body water in the same time at the k rate. This fraction of injected activity, or the so-called 'excess excretion', was presumably excreted by virtue of the very high specific activities of plasma urea in the pre-equilibrium period, and if so would depend on the rate of loss of urea from the plasma into the extravascular compartments relative to that into the urine.

Higher urea specific activities in urine than in plasma can also be explained by more rapid passage of [^{14}C]urea through the kidney than of unlabelled urea. Though isotopic discrimination of this order is considered on general grounds to be unlikely, the possibility that some part of the observed effects arises in this way cannot be entirely dismissed. It is considered more likely, however, that the phenomenon is intimately associated with the urea-concentrating mechanism. A full discussion of this mechanism is outside the scope of the present paper but the following salient points may be relevant.

By superimposing urea on an osmotic load the volume of urine required for its excretion is substantially decreased (Gamble, McKhann, Butler & Tuthill, 1934; Crawford, Doyle & Probst, 1959; Jaenike, 1960). A urea concentration gradient is also known to exist in serial kidney sections, with values that increase from the cortex to the papillae, (Ullrich & Jarausch, 1956). These and other observations led to the postulate that urea accumulates in the medullary interstitial fluid by passive diffusion from the collecting ducts and is trapped there by capillary countercurrent exchange. The presence of this interstitial urea is believed to help greatly in balancing the osmotic effects of intratubular urea (Berliner, Levinsky, Davidson & Eden, 1958; Levinsky, Davidson & Berliner, 1959; Levinsky & Berliner, 1959b).

An alternative hypothesis due to Schmidt-Nielsen (1958) is based on the countercurrent multiplier theory of Hargitay & Kuhn (1951). This claims, at least in part, an active transport of urea across the medullary tissue, a view which is supported by a demonstration that the kidneys of sheep on a low protein intake have a medullary strip that contains urea at much higher concentration than in the urine (Schmidt-Nielsen & O'Dell, 1959). Continuous recycling of urea between adjacent limbs of the same loop of Henle, which is part of the theory, might partly explain a frontal type of delay, especially if a high proportion of urea is recycled.

Alternatively, urea in a section of the tubules can

be visualized as mixing with the renal pool, a view which is more in keeping with the passive hypothesis of Berliner and his colleagues. This is the basis of the model illustrated in Scheme 1 of the Appendix (Charlwood, 1965), and of part of the accompanying mathematical treatment. This theory appeared to be adequate to explain both the higher specific activities of urinary urea throughout and the shape of the urinary excretion curve in the first $d_{min.}$, and to illustrate it hypothetical curves were derived from typical mean values of the parameters for rabbits (cf. Fig. 1 of the Appendix). The plasma curve is generally similar in shape to experimentally found ones (compare with Figs. 3 and 6), except that the transition between initial and final exponentials is more abrupt. This may reflect a finite experimental time of mixing of the injected material with the plasma, whereas instant mixing is assumed in the model. The calculated curve of urinary specific activities is also similar to the experimental ones, the delay time (t_d) being 29 min.

When b_1 , which is a fraction of the plasma urea pool, was multiplied by the ratio of total urea to urea in the plasma [$1 + (K_{13}/K_{31})$], the value obtained was approx. 2% less than the sum of K_{12} and K_{15} . This indicates the order of error that is involved in the commonly accepted assumption that specific activities are the same for urea in the plasma and in the body water after equilibrium is reached. For this reason a factor of 1.02 was included in the formula used to calculate urea synthesis rates in Table 3. Also, if the c_1 intercept is taken, as is often done, to represent the fraction of total urea present in the plasma, the value obtained was 3.4% higher than the true value given by $K_{31}/(K_{13} + K_{31})$. However, when c_1 is used in this way an allowance is usually also made for 'excess excretion' on the assumption that c_1 is the specific activity of body urea when all retained activity, i.e. injected material less 'excess excretion', is hypothetically uniformly distributed throughout the body pool. If an allowance is made for 'excess excretion' the error in pool size estimation is correspondingly diminished.

Activities in the urine at different times were also calculated, and in spite of 'excess excretion' less was present in the first 2 hr. than was calculated to have left the body water in the same time. This arises from retention of activity in the kidney, and when allowance was made for retained renal activity the 'excess excretion' in the hypothetical average rabbit was found to be 3.5% of the injected dose. Because of mutually compensating effects of 'excess excretion' and retention in the kidney pool a plot of cumulative urine activities against time was almost linear, passing through the origin.

The size of the renal pool was obtained by multiplying the delay time by the urea excretion rate. This relationship is required by the theory under-

lying the frontal delay procedure and, as shown, must also be approximately true to satisfy the mixing pool theory. In the two instances in which post-mortem analyses were carried out the values found for renal urea (cf. Table 2) agreed closely with this product, although urea samples recovered from different sections of the kidney did not always have the same specific activity. No explanation is available for the observation that cortical urea values were slightly lower than plasma ones. Some urea that equilibrates very slowly with the body pool may be present in the cortex, or urea synthesis may take place there, or another substance may be present that liberates carbon dioxide under treatment with urease. Further investigation is required to settle these points, and until the differences are explained the assumption that most of the urea in the kidney is present in a single pool with specific activity equal to that of urinary urea must be regarded as hypothetical.

Values for renal pools varied from 4.1 to as much as 26.2 mg., or from 2 to 15% of the estimated body urea pool. From the ratio of urea masses in the kidney and in the plasma and the specific activity difference between urinary and plasma urea the proportion of injected activity present in the kidney at different times was calculated. Plasma volumes were not measured but from previous experience we know that in rabbits of the particular strain used these were equivalent to 4.0% of the body weight. Mainly according to the size of the pool renal activities varied between 2 and 15% of the injected dose at 1 hr. and at later times declined asymptotically along with plasma activities.

Whether the urea pool in the kidney which is responsible for storing this activity is properly to be regarded for these purposes as part of, or outside, the total body urea pool is a matter of definition. As the mixing pool theory has been presented the assumption is made that no urea returns from this pool to the body, whereas 50–60% of filtered urea is known to be reabsorbed from the kidney (Effersøe, 1951; Andersen & Nielsen, 1955; Andersen, 1961). However, the main source of reabsorbed plasma filtrate including urea is the convoluted portion of the proximal tubules (Walker, Bott, Oliver & MacDowell, 1941; Lassiter, Gottschalk & Mylle, 1961), and most of this part of the nephron is localized in rabbits in the outer zone of the medulla (Schmidt-Nielsen & O'Dell, 1961). Since the specific activity of urea in this part of the kidney was found to be close to that of urea in the blood (cf. Table 2), the filtered urea that is returned is unlikely to affect the specific activity of urea in compartment 2. Thus K_{12} can be regarded as representing the exchange rate of filtered minus reabsorbed urea. We have therefore elected to deal with the renal pool as a temporary excretory reservoir outside the body pool,

and to combine urinary and kidney activities. Clearly other procedures are possible.

Since the observed slope of plasma urea activities is affected by net transfer of activity from the extravascular to the plasma pool, $k = 1.02 b_1$ was used in the expression $1 - e^{-kt}$, the assumption being that this represents the true net rate of loss of activity from the plasma compartment in the post-equilibrium phase.

Constant fractions of activity leaving the body water that appeared according to this (B) type of plot in the urine and kidney are shown in Table 3 (column 11) and are to be compared with similar values in column 9 derived from the Walser & Bodenlos (1959) (A) type of plot. The close agreement which is evident is hardly surprising, since values were derived graphically essentially at long times in both cases and disturbing effects of renal delay and 'excess excretion' should diminish with time. The two sets of values indicate that 31–71% of the urea synthesized was excreted, the remainder being assumed to have been endogenously catabolized.

Fractions of activity in the urine and kidney obtained graphically when $1 - e^{-kt} = 0$ are shown in Table 3 (column 10) and are both higher and lower than corresponding values in column 8. The approximate proportionality with delay time that is shown by both sets of values in Fig. 5 is surprising since it is difficult to see any reason why 'excess excretion' should depend on renal pool parameters. It is possible that 'excess excretion' could be decreased to insignificant proportions by introducing the [^{14}C]urea sufficiently slowly, since a value of only 3% was given by a rabbit that received the urea subcutaneously. In this experiment the remaining parameters such as synthesis rate and fraction excreted were normal.

From many points of view, however, the interest in 'excess excretion' is academic since it is clearly an artifact of the isotopic technique. The quotient of urea excretion rate, as measured in the urine, and the body urea pool, should give the fractional excretion rate of urea without obligatory recourse to isotopes, and if this is divided by the fraction of urea production that is excreted (obtained, as shown above, by alternative isotopic procedures that are in good agreement) then the fractional synthesis rate should result. The problem with this procedure is first to define and secondly to measure the true urea pool. The possibility of carrying out a satisfactory chemical analysis of all organs and tissues for urea can probably be ruled out, especially for skin and connective tissue. It is, of course, feasible to measure water contents in this way but it may not be assumed that urea is present everywhere at the same concentration as in the plasma. Our findings show that the kidney contains several times

more urea than corresponds to its water content and the plasma urea concentration, and only a negligible fraction of this excess could be attributed to trapped urine.

An alternative isotopic method of measuring the urea pool that is not dependent on 'excess excretion' was therefore used. This depends on choosing a time, namely 1.5 hr., at which renal urea specific activities can be relied on to be constantly higher than plasma ones, and from a plot of urine and kidney activities against time a value was read off at 1.5 hr. In this operation, in which renal urea specific activities were taken to be the same as urinary urea ones, it would not have made much difference to the general result if, for example, a mean of plasma and urinary urea specific activities had been used. This value was divided by the fraction of urea production that is excreted (cf. Table 3) and the result subtracted from the injected dose to obtain the activity retained at 1.5 hr. This activity was then visualized as being distributed in body urea at a mean specific activity that was 2% higher than in plasma urea at the same time. Values so obtained are shown in Table 3 (column 12) and are all lower than corresponding values calculated by the Walser & Bodenlos (1959) procedure. For reasons that are not clear to us the difference in each case corresponds approximately to the size of the renal pool. Values in column 12 were used to calculate the urea synthesis rates shown in column 14. These agree closely with values in column 15 obtained by dividing the observed excretion rate by the fraction of urea production that was excreted. The agreement is particularly striking in those animals in which more than half the urea production was endogenously catabolized, and leaves little doubt about the validity of these estimates of fractions of new urea excreted and mass of urea synthesized/hr. It also suggests, in spite of the numerous possibilities that have been discussed for back diffusion of urea from the bladder and kidney, that observed k values, whether subjected to the 2% correction we have introduced or not, reflect closely the true net rate of loss of urea from the plasma.

If it is assumed that the extrarenal water contains urea at the plasma concentration, then values for the urea pool arrived at as described can be used to calculate the urea fluid space. As shown above, values for this space expressed (cf. column 13) as a fraction of the body weight were much lower than corresponded to body water volumes in the five animals that were also injected with tritiated water. Whereas those based on the urea pool were between 50 and 60% of body weight the range of water volumes was from 65 to 69% of body weight. Somewhat better agreement was obtained when urea pools calculated by the Walser & Bodenlos (1959) procedure were used.

The reason for the discrepancies is not entirely clear. Because of the relatively long half-life of body water it is not believed that complications due to 'excess excretion', such as we have seen to arise with injections of isotopically labelled urea, need be considered with tritiated water. On the other hand, tritium is known to exchange with hydrogen in body constituents, and this is believed to produce a dilution effect equivalent to a water volume of 1-4% of the body weight (Pinson & Langham, 1957). Also, urea is present in the water of the nervous system (Fremont-Smith, Dailey, Merritt, Carroll & Thomas, 1931) and in gastric juice (Kornberg *et al.* 1954b) and possibly in other sites at lower concentrations than in the plasma water, but at slightly higher concentrations in erythrocytes (Ralls, 1943). Obviously, to use body water volumes to calculate the body urea pool some assumptions are necessary about the distribution of urea concentrations and the degree of tritium-hydrogen exchange. This suggests that the isotopically labelled urea dilution procedure used in the present work may be more accurate in spite of uncertainties that still appear to affect the measurement and significance of urea activities in the kidney.

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