The Measurement of Synthesis Rates of Albumin and Fibrinogen in Rabbits

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1. A formula is proposed for calculating fractional synthesis rates of liver-produced plasma proteins that dispenses with urinary information or information about the size of the urea pool in the body or the fraction of urea that is endogenously catabolized. 2. Synthesis rates obtained for albumin and fibrinogen agreed well with corresponding catabolic rates for the ^{131}I -labelled proteins except in two of the fibrinogen measurements. 3. Significant reutilization of ^{14}C occurs in some animals after [^{14}C]carbonate injections, giving rise to errors in the calculation of protein synthesis rates. These can best be avoided by using results obtained by injecting [^{13}C]urea simultaneously. [^{15}N]urea is shown not to be satisfactory for this purpose.

A method has been proposed for measuring the synthesis rates of liver-produced plasma proteins by reference to the specific activity of the guanidine carbon of arginine in the protein after injecting [^{14}C]carbonate (McFarlane, 1963*a,b*). The formula used for calculating the fractional rate of albumin synthesis was based on the assumption that all urea is excreted as such. If significant amounts are endogenously catabolized, as they can be in rabbits (Regoezi, Irons, Koj & McFarlane, 1965), a modified formula is required and the following is suggested:

Fractional synthesis rate of albumin

= fractional synthesis rate of urea

$$\times \frac{\text{sp. activity of guanidine C of albumin at time } t}{\text{sp. activity of urea C at time } t}$$

This statement, that the ratio of the fractional synthesis rates of two products of the same precursor is also the ratio of their specific activities at the same time, could only be true of a closed system. Since neither the plasma protein pool nor the body urea pool is closed, hypothetical values at zero time were obtained for both and procedures for doing this are described.

MATERIALS

Animals. Only male rabbits of the Mill Hill (Sandylop) strain were used. Many of them had already been used for the investigations of urea metabolism described by Regoezi *et al.* (1965), and are referred to below by the same alpha-

betical letter. A standard pelleted diet and water *ad lib.* were given, but when iodine-labelled proteins were injected the drinking water contained 0.005% NaI and 0.45% NaCl.

Labelled compounds. The $\text{Na}_2^{14}\text{CO}_3$ in sterile solution (specific activity 5–25 mc/m-mole) and [^{14}C]urea (specific activity 115 $\mu\text{C}/\text{mg}$. of compound) were obtained from The Radiochemical Centre, Amersham, Bucks.

[^{13}C]Urea was synthesized from K^{13}CN by oxidation with permanganate in alkaline solution to cyanate (Gall & Lehmann, 1928). The cyanate was recrystallized from ethanol and was converted into urea by evaporating to dryness with ammonium sulphate on a steam bath (Mann & Saunders, 1952). The urea was recrystallized from ethanol, the ^{13}C yield being 30%.

METHODS

Injections, sampling, deproteinizing plasma and measuring ^{14}C and ^{15}N specific activities. These were all as described by Regoezi *et al.* (1965).

Measurements of plasma ^{13}C . These were made on CO_2 samples representing approx. 0.2 mg. of carbon prepared as described for $^{14}\text{CO}_2$ measurements. Since the Toepler pump of the mass spectrometer has a large dead-space the whole sample containing both $^{14}\text{CO}_2$ and $^{13}\text{CO}_2$ was first introduced into the pump, and after ^{13}C enrichment measurement over 99% was recovered and used for measuring ^{14}C specific activities.

CALCULATIONS

The protein synthesis rate (% of plasma pool/day) is given by:

$$k \times \frac{\text{sp. activity of protein guanidine C at } t_0}{\text{sp. activity of urea C at } t_0}$$

where k is 1.02 times (for reasons given in the Discussion section) the observed slope of the curve of plasma urea specific activities in % per day. Specific activities of protein

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guanidine carbon at t_0 were obtained by increasing the observed values at 6 hr. by 18% for fibrinogens and 28% for albumins to allow for labelled proteins lost from the plasma in this interval by catabolism and by diffusion. The validity of these corrections is discussed below. The specific activity of urea carbon at t_0 was obtained by extrapolating the specific activity curve of plasma urea to zero time.

Catabolic rates. Semilogarithmic plots of plasma albumin ^{131}I specific activities were resolved graphically into two components with slopes b_1 and b_2 and y -axis intercepts c_1 and c_2 . The catabolic rate (K_{12}) is given by $b_1 b_2 / (c_1 b_2 + c_2 b_1)$ (Matthews, 1957). In experiments with ^{131}I -labelled fibrinogen total urinary activities excreted every 24 hr. were divided by the average fraction of the injected dose present in the plasma at the same time. Rates obtained by the two methods have been shown to be the same (McFarlane, 1963c).

EXPERIMENTAL

Protein synthesis rates. Four rabbits each received 200 μC of ^{14}C carbonate by intravenous injection several days after ^{14}C urea experiments when ^{14}C activities in their body urea had declined to insignificant levels. Two animals (E and F) also received 15.7 and 11.5 mg. of ^{15}N urea respectively. Blood samples (about 5 ml.) were taken into heparin at about hourly intervals for 6 hr., when a terminal 15 ml. sample was taken into citrate for measurements of protein specific activities. Heparin samples were used for ^{14}C urea and ^{15}N urea determinations.

All four animals received ^{131}I -labelled proteins at different earlier times as part of a study of protein catabolic rates. Rabbits A and B received ^{131}I -labelled albumin 9 weeks earlier, and rabbits A, B and F received ^{131}I -labelled fibrinogen 6 months earlier and rabbit F 5 weeks earlier. In these experiments small heparin-treated plasma samples were obtained at intervals over 2 weeks, and in the fibrinogen experiments urines were collected daily from metabolic cages.

Reutilization of ^{14}C . To investigate to what extent this occurs in ^{14}C carbonate experiments eight rabbits were injected first with a mixture of 5 μC of carrier-free ^{14}C urea and 10–15 mg. of ^{15}N urea, and later with similar amounts

of ^{15}N urea and 200 μC of ^{14}C carbonate. Two rabbits were also injected with 8 and 14 mg. of ^{13}C urea and 200 μC of ^{14}C carbonate. Heparin-treated plasma samples were obtained at intervals for 6–8 hr. and used for urea specific activity and ^{13}C and ^{15}N enrichment measurements.

RESULTS

Protein synthesis rates. Results in Table 1 show that synthesis rates for ^{14}C -labelled albumin varied between 19.7 and 27.7% per day and for ^{14}C -labelled fibrinogen between 30.1 and 36.8% per day. Agreement between synthesis rates for the ^{14}C -labelled proteins and catabolic rates for the ^{131}I -labelled proteins is considered satisfactory for albumin and for two of the four fibrinogen experiments.

Reutilization of ^{14}C . In four animals in Table 2 (D, E, F and I) ^{15}N urea slopes were significantly unchanged in the two experiments, but the slope ratios ($k^{15}\text{N}/k^{14}\text{C}$) increased from 0.69 to 0.84 in the ^{14}C urea experiments to 0.76 to 0.99 in the ^{14}C carbonate ones. In the four animals in which the ^{15}N urea slopes changed between experiments slope ratios increased from 0.68 to 0.84 in the ^{14}C urea experiments to 0.86 to 0.95 in the ^{14}C carbonate ones. ^{13}C Urea half-lives in both experiments were 6–7% shorter than of ^{14}C urea produced endogenously from ^{14}C carbonate.

DISCUSSION

To avoid confusing consequences of endogenous urea catabolism in determining protein synthesis rates by the guanidine carbon method, Koj, Regoezi, Irons & McFarlane (1964) recommended calculating urea synthesis rates from the product of the mass of urea carbon in the body and the slope

Table 1. *Synthesis rates of ^{14}C -labelled albumin and fibrinogen and catabolic rates of the ^{131}I -labelled proteins in four rabbits*

The specific activity of protein guanidine carbon (columns 3 and 4) is corrected for 18% loss of fibrinogen and 28% loss of albumin from the plasma in the first 6 hr. The synthesis rates (columns 5 and 6) are obtained from plasma urea specific activity slope (column 1) $\times$ specific activity of protein guanidine carbon at t_0 (columns 3 or 4) $\times$ $1.02 \times 24/\text{plasma urea specific activity at } t_0$ (column 2).

Rabbit	Plasma urea		Sp. activity of protein guanidine C at t_0		Synthesis rate of ^{14}C - labelled protein		Catabolic rate of ^{131}I - labelled protein	
	Sp. activity slope (100k)	Sp. activity at t_0						
	(%/hr.)	($\mu\text{C/g.}$)	Albumin ($\mu\text{C/g.}$)	Fibrinogen ($\mu\text{C/g.}$)	Albumin ($\mu\text{C/g.}$)	Fibrinogen ($\mu\text{C/g.}$)	Albumin ($\mu\text{C/g.}$)	Fibrinogen ($\mu\text{C/g.}$)
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
A	21.8	23.0	1.08	1.43	25.0	33.2	22.4	40.6
B	23.9	23.5	0.79	1.21	19.7	30.1	18.9	41.8
E	18.9	18.1	1.18	1.54	27.7	36.2	—	36.0
F	15.0	18.8	1.33	1.89	25.9	36.8	—	34.0

Table 2. Comparison of slopes of curves of plasma urea ^{14}C specific activities and urea ^{15}N and ^{13}C isotopic enrichments

Each rabbit received [^{15}N]urea twice, on the first occasion along with [^{14}C]urea and on the second with [^{14}C]carbonate, from which it produced [^{14}C]urea by endogenous synthesis. Two other rabbits received a mixture of [^{14}C]carbonate and [^{13}C]urea.

Rabbit	[^{14}C]Urea and [^{15}N]urea injected			[^{14}C]Carbonate and [^{15}N]urea injected			[^{14}C]Carbonate and [^{13}C]urea injected		
	k [^{14}C]urea	k [^{15}N]urea	$k^{15}\text{N}/k^{14}\text{C}$	k [^{14}C]urea	k [^{15}N]urea	$k^{15}\text{N}/k^{14}\text{C}$	$k^{14}\text{C}$	$k^{13}\text{C}$	$k^{13}\text{C}/k^{14}\text{C}$
A	0.238	0.163	0.68	0.147	0.127	0.86	—	—	—
C	0.167	0.140	0.84	0.141	0.125	0.89	—	—	—
D	0.190	0.145	0.76	0.189	0.144	0.76	—	—	—
E	0.243	0.180	0.74	0.189	0.187	0.99	—	—	—
F	0.203	0.140	0.69	0.150	0.138	0.92	—	—	—
G	0.226	0.191	0.84	0.163	0.155	0.95	—	—	—
H	0.277	0.227	0.82	0.169	0.161	0.95	—	—	—
I	0.133	0.110	0.84	0.126	0.111	0.88	—	—	—
77	—	—	—	—	—	—	0.134	0.142	1.06
63	—	—	—	—	—	—	0.182	0.195	1.07

of the plasma urea specific activity curve, and substituting these for measured excretion rates as was done by Regoeczi *et al.* (1965). However, as was shown there, some uncertainty surrounds urea pool values by any of the available methods and a considerable advantage in using the formula now proposed is that the size of the urea pool is not required. It is also convenient that urines need not be collected and analysed.

By definition, activity appearing in urea or protein guanidine carbon must be equal to the product of the absolute synthesis rate of urea or protein guanidine carbon and the mean specific activity of the common precursor, in this case of the guanidine carbon of intracellular arginine. Eliminating the precursor specific activity, we have:

Synthesis rate of protein guanidine C

Synthesis rate of urea C

$$= \frac{\text{Total activity in protein guanidine C at } t}{\text{Total activity in urea C at } t}$$

Dividing both sides of this equation arbitrarily by the sizes of urea and protein guanidine carbon pools, then:

Fractional synthesis rate of albumin

Fractional synthesis rate of urea

$$= \frac{\text{Sp. activity in protein guanidine C at } t}{\text{Sp. activity in urea C at } t}$$

and if the system is assumed to be closed the specific activities at t can be replaced by maximum specific activities. In practice, however, losses both of labelled urea and of labelled protein occur during

the interval of measurement. By extrapolating the curve of plasma urea specific activities to zero time a satisfactory hypothetical maximum value for [^{14}C]urea specific activity before such losses have time to occur was obtained, but this procedure could not be applied to the protein specific activity curves since these showed no terminal exponentials inside the experimental period. Instead, *ad hoc* allowances of 28% for albumin and 18% for fibrinogen were made for losses of these proteins from the plasma by catabolism and by diffusion in 6–7 hr. These were based on analyses of results from six rabbits that received ^{131}I -labelled fibrinogen and four that received ^{131}I -labelled albumin, and from which several plasma samples were obtained in the first 6 hr. An allowance of 10% was made for the slower rate of arrival of the ^{14}C -labelled molecules in the plasma, as proposed by McFarlane (1963*b*). The use of average allowances in this way is unsatisfactory since considerable individual variation was evident in the animals studied, and this has also been seen with ^{131}I -labelled albumin in humans (Walker, Ross & Hammond, 1960). It would clearly have been better to inject iodine-labelled proteins along with the [^{14}C]carbonate.

The above losses are assumed to have been made good immediately in the animal by replacement with unlabelled proteins. Contrary to earlier practice no allowance was made for losses due to blood sampling since these are believed not to have been replaced in the experimental interval. Especially with fibrinogen, protein synthesis rates probably increase soon after blood loss (Miller, Hanavan, Titthasiri & Chowdhury, 1964). However, in view of the delay associated with delivery of newly synthesized molecules into the plasma it was thought

that specific activities of circulating proteins were unlikely to be significantly affected within 6 hr. of removing a series of blood samples that amounted in all to less than 15% of the blood.

The slope of the urea specific activity curve, which is the remaining component in the synthesis formula, should correspond with the true synthesis rate of urea expressed as a fraction of the body pool. Observed values were therefore increased by 2%, as explained by Regoezi *et al.* (1965), to allow for net transfer of labelled molecules from extravascular to plasma compartments, and errors in making this small allowance are obviously unlikely to be significant. Also, in spite of numerous possibilities for reverse transfer of urea from kidney and bladder pools after [^{14}C]urea injection, plasma slopes modified in this way were found by the same authors to correspond closely to synthesis rates calculated from observed excretion rates and fractions of urea production excreted.

In the [^{14}C]carbonate experiments, however, the use of observed plasma slopes in this way could be seriously misleading if synthesis of [^{14}C]urea were to continue at significant rates during the periods beginning at 1.5 hr. over which these slopes were measured. An indirect earlier observation had suggested that synthesis is effectively complete at 1.5 hr., namely that the half-life of ^{14}C -labelled albumin in the 4 weeks after an injection of [^{14}C]carbonate was the same as that of simultaneously injected ^{131}I -labelled albumin (McFarlane, 1963b). It was reasoned then that if synthesis of [^{14}C]guanidine carbon of albumin had ended the same must also be true of [^{14}C]urea carbon.

To investigate this point in greater detail rabbits E and F were given [^{15}N]urea along with [^{14}C]carbonate but the longer observed half-lives of [^{15}N]urea than of [^{14}C]urea could have been due as much to reutilization of ^{15}N as to continuing synthesis of [^{14}C]urea from [^{14}C]carbonate. A series of eight rabbits, including E and F, was therefore injected with both [^{14}C]urea and [^{15}N]urea and on another occasion with [^{14}C]carbonate and [^{15}N]urea, with the half-life results shown in Table 2. Unfortunately, only in half the series was the half-life of [^{15}N]urea the same in both experiments. In these [^{14}C]urea half-lives were always much shorter than [^{15}N]urea values, and the differences are significantly greater when [^{14}C]urea rather than [^{14}C]carbonate was given. The longer half-lives of endogenously produced [^{14}C]urea were consistent with approx. 10% recycling of [^{14}C]carbonate. However, in two of the animals differences were large and in the other two only small. In the other four experiments [^{15}N]urea half-lives changed so that the animals could not be regarded as synthesizing urea at the same rate in both experiments. Nevertheless, slope ratios (0.68–0.84) in the [^{14}C]urea experiments were

again significantly smaller than in the corresponding [^{14}C]carbonate ones (0.86–0.95).

The above procedure, which depends on using [^{15}N]urea as an indicator of urea synthesis rates, is not entirely satisfactory in view of the large element of catabolism and reutilization that previous studies have shown to be inherent in [^{15}N]urea slopes. Clearly, it would be advantageous to use [^{13}C]urea, the slope of which (according to earlier experience with [^{14}C]urea) should be sensibly free of reutilization effects. This was realized towards the end of this work and only two rabbits were injected with both [^{13}C]urea and [^{14}C]carbonate. The results, however, indicate that small amounts of [^{14}C]urea in fact continue to be synthesized for at least a few hours after the period of intensive radioactive synthesis that immediately follows [^{14}C]carbonate injections. To obtain the highest accuracy in the calculation of protein synthesis rates suitable allowance for this should be made, and the use of [^{13}C]urea slopes along with [^{14}C]urea specific activities at t_0 in the protein synthesis formula is recommended.

Extrahepatic synthesis of urea may also contribute to observed values for [^{14}C]urea specific activity slopes. This could be brought about by the action of extrahepatic arginase, which according to Roberts (1949) is present in mouse skin and according to Davies, Defalco, Shander, Kopelman & Kiyasu (1961) is present in brain, although some observers do not regard either as a significant site of urea production. It is also probable that intestinal coliform bacteria release urea (Roberts, Abelson, Cowie, Bolton & Britten, 1955), and indeed this may be a major contributory source to the observed differences in [^{13}C]urea and [^{14}C]urea half-lives in a [^{14}C]carbonate experiment. In general, extrahepatic urea could conceivably be either unlabelled or labelled from carbonate to almost any extent. Insofar as this urea contributes activity to the urea pool any effect that it may have will be eliminated by using the half-life of injected [^{13}C]urea, as suggested in the synthesis formula. No correspondingly simple device is available for dealing with effects due to unlabelled urea production outside the liver, the slope of the [^{13}C]urea curve reflecting the fractional synthesis rate of urea from any source whatsoever. Therefore protein synthesis rates determined by the method can be in error in proportion to the extent of any extrahepatic synthesis of unlabelled urea that is in progress at the time of the [^{14}C]carbonate experiment.

A final question concerns the general feasibility of assessing the accuracy of the suggested [^{14}C]carbonate method by comparing synthesis rates of ^{14}C -labelled proteins with corresponding catabolic rates of the ^{131}I -labelled proteins, as has already been attempted by McFarlane (1963b) and by Reeve,

Pearson & Martz (1963) with [6- ^{14}C]arginine. Essentially two different things are being measured: a short-term protein synthesis rate that is greatly dependent on the steady rate of urea synthesis being maintained over 5–6 hr., and a long-term catabolic rate that assumes an equilibrium between synthesis and catabolic rates of plasma proteins over a 14-day period. Since little is known about diurnal and other fluctuations in both rates, it is perhaps too much to hope for better agreement than is evident in Table 1. In addition the possibility that disturbances incidental to injections and removal of blood samples may affect the short-term ^{14}C measurements in particular has to be considered. Nevertheless, it seems important to try to establish a relationship between the two rates, and the attempt to do so described in the present paper cannot be considered as final. It is patently advisable to inject [^{13}C]urea along with [^{14}C]carbonate and to determine losses of newly ^{14}C -labelled proteins by injecting iodine-labelled ones at the same time, a procedure that will require simultaneous use of alternative iodine isotopes in protein combination. It is possible, for instance, that the close agreement shown in Table 1 between albumin synthesis and catabolic rates was to some extent fortuitous, a prolongation of the half-life of [^{14}C]urea due to reutilization being counter-balanced by an equivalent effect due to extrahepatic synthesis of unlabelled urea.

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