Effect of Endotoxin on Plasma Albumin and Fibrinogen Synthesis Rates in Rabbits as Measured by the [^{14}C]Carbonate Method

By A. KOJ AND A. S. MCFARLANE

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 24 November 1967)

1. Rates of synthesis of plasma albumin and fibrinogen were measured by the [^{14}C]carbonate method in normal rabbits and in animals that received a single intravenous injection of *Shigella* endotoxin 14–48 hr. earlier. 2. The accuracy of the method was improved by introducing refinements into procedures for measuring ^{14}C radioactivities associated with both urea and proteins that are lost from the plasma during the synthesis interval. 3. The synthesis interval (time between injecting carbonate and measuring specific radioactivities of protein guanidine carbon in plasma) can be shortened with advantage to 3–4 hr. 4. Injection of endotoxin markedly decreased the fractional rate of loss in the first few hours of injected radioiodine-labelled fibrinogen and to a smaller extent of similarly labelled albumin from the plasma. The absolute rate of synthesis of fibrinogen was increased in endotoxin-treated rabbits by more than 400% compared with normal animals, and the rate of synthesis of albumin was increased by about 60%.

Regoecki, Henley, Holloway & McFarlane (1963) observed changes in the replacement rate of injected iodine-labelled fibrinogen in rabbits after repeated injections of a pyrogen (endotoxin) and suggested that the rate of synthesis of this protein had been increased while its fractional catabolic rate remained unaltered. A direct method, such as the [^{14}C]carbonate method, of measuring rates of synthesis of liver-produced plasma proteins in a short-term experiment ought to give a quantitative measure of the changes caused by endotoxin, and the results of applying this method are given below.

The [^{14}C]carbonate method depends on comparing the synthesis rate of the protein with that of urea, the ratio being proportional to the total ^{14}C radioactivities incorporated into the guanidine carbon of protein arginine and into urea carbon in a given time (the 'synthesis interval'). The history of the development of the method has been reviewed by McFarlane (1963a), who also discussed methods of measuring the proportions of ^{14}C -labelled protein and urea molecules that escape for one reason or another from the sampling or plasma compartment during the synthesis interval.

During the present series of experiments it became evident that measured protein losses in normal rabbits were greater than previously estimated values (McFarlane, Irons, Koj & Regoecki, 1965). The new values presented below are based on measurements made after injecting independently (iodine-) labelled proteins and [^{13}C]urea along with the [^{14}C]carbonate.

METHODS

Endotoxin injections. Rabbits used in the experiments (adult males, Sandylop or New Zealand White) received a single intravenous injection of 1 ml. of standard pyrogen (Humphrey & Bangham, 1959) solution in 5% (w/v) glucose (purified O-somatic antigen of *Shigella dysenteriae*, Division of Biological Standards, National Institute for Medical Research). The amount of endotoxin injected varied from 10 to 50 μg . (3–16 μg /kg. body wt.), but in most experiments was 20 μg . Two out of the ten rabbits injected with endotoxin (25 and 50 μg .) died within 48 hr. and all the others showed loss of appetite, respiratory distress, pyrexia and increased concentration of plasma urea, and in some animals the plasma was strongly lipaemic. Synthesis rates of albumin and fibrinogen were measured 14–48 hr. after injection of endotoxin.

Labelled compounds. (a) [^{13}C]Urea. This was prepared by the high-pressure condensation of $^{13}\text{CO}_2$ and NH_3 (Bryan, 1947) as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949).

(b) [^{14}C]Carbonate. This was supplied (as $\text{Na}_2^{14}\text{CO}_3$, specific radioactivity 5mc/m-mole) by The Radiochemical Centre, Amersham, Bucks.

(c) ^{125}I - and ^{131}I -labelled proteins. Rabbit albumin was prepared by removing the globulins with saturated $(\text{NH}_4)_2\text{SO}_4$ from citrated rabbit plasma as described by McFarlane (1963a). After exhaustive dialysis against distilled water the solution of albumin was stored frozen in ampoules each containing 10 mg. of protein in 0.2 ml. At labelling with ^{125}I or ^{131}I carrier iodine was supplied as ICl as described by McFarlane (1964) and in such amount that 50–60% incorporation was equivalent to 1 atom of iodine/mol. of albumin.

Fibrinogen was prepared and labelled with ^{125}I or ^{131}I as described by McFarlane (1963b).

Experimental procedure. A normal or endotoxin-treated rabbit was injected intravenously with 10–15 mg. of [^{13}C]urea (51.1 atom % excess) and 30 min. later with a mixture of approx. 200 μC of [^{14}C]carbonate, 5 μC of [^{131}I]labelled albumin and 50 μC of [^{125}I]labelled fibrinogen. Blood samples (1.5 ml.) were taken from the marginal ear vein into heparinized test tubes at intervals during the 6 hr. after the injection. Then 0.5 ml. of plasma from each sample was diluted with 0.5 ml. of an unlabelled urea solution containing 0.09 mg. of urea carbon, and [^{125}I] and [^{131}I] radioactivities were measured in a glass-stoppered centrifuge tube in a well-type scintillation counter with optimum pulse-height-analyser settings for counting [^{125}I] and [^{131}I] separately. No [^{125}I] counts came through in the [^{131}I] channel and 18.1% of the [^{131}I] counts came through in the [^{125}I] channel.

After the counting the plasmas were deproteinized with tungstic acid, and urea in the supernatant was decomposed by urease as described by Regoezci, Irons, Koj & McFarlane (1965). The whole of the labelled CO_2 from urea was used to fill the Toepler pump of the mass spectrometer and after measurement of the ^{13}C enrichment at least 98% of the sample was recovered and condensed into the triethylamine scintillator mixture for ^{14}C counting in a Packard Tri-Carb liquid-scintillation spectrometer as described by McFarlane (1963a).

The urea concentration in plasma was measured independently by a colorimetric procedure (Fawcett & Scott, 1960) and ^{14}C specific radioactivities and ^{13}C enrichments of urea carbon were then corrected for dilution with carrier urea. The plasma sample that was diluted 100-fold for urea estimation was also used for measuring total proteins by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard, thus enabling [^{125}I] and [^{131}I] radioactivities to be expressed as counts/min./mg. of total plasma protein.

A 10 ml. blood sample was taken at 5 hr. after the [^{14}C]carbonate injection and used for isolating albumin and fibrinogen. In some experiments in which the specific radioactivities of carbon in guanidine groups in protein were plotted against time 5–6 ml. samples of blood were taken at approx. 45, 90, 135, 180, 270 and 360 min. Albumin and fibrinogen were isolated from plasma by conventional precipitation with $(\text{NH}_4)_2\text{SO}_4$, contamination of the albumins with 3–4% of β -globulin being evident after polyacrylamide-gel electrophoresis. Fibrinogen was clotted with thrombin, and the clot washed thoroughly with 0.9% NaCl solution and water and then dried. After hydrolysis of the proteins with 6N-HCl arginine was isolated by using an ion-exchange resin (De-Acidite FF) in the HCO_3^- form and then treated with arginase followed by urease (McFarlane, 1963a). The radioactivity of $^{14}\text{CO}_2$ deriving from the guanidine carbon was measured in combination with triethylamine by scintillation counting.

When only small amounts of fibrin were available for hydrolysis carrier urea was added before liberation of guanidine carbon and a correction was applied based on the colorimetric determination of urea in a portion of the sample after treatment with arginase. This enabled volumes of plasmas for protein fractionation to be kept down to 2–3 ml. Fibrinogen concentrations were measured in 0.2 ml. samples of EDTA-treated plasmas that were clotted by adding 5 units of thrombin (Leo Pharmaceutical Products, Copenhagen, Denmark) in 1 ml. of sodium

phosphate buffer, pH 6.1 (Jacobsson, 1955). After 2 hr. the clot was wound on to a thin glass rod, washed with 0.9% NaCl solution and dissolved by heating in 3 ml. of N-NaOH for 5 min., and the amount of protein was measured by the method of Lowry *et al.* (1951). Albumin concentration in plasma was calculated from the [^{131}I] specific radioactivities of recovered albumin samples and the counts/min./ml. in the first sample of plasma, and the same principle was used to estimate the total volume of plasma *in vivo*.

THEORETICAL BASIS OF CALCULATIONS

The following simplified formula has been proposed (McFarlane *et al.* 1965) for calculating fractional protein synthesis rates by using the [^{14}C]carbonate method:

$$\text{Protein F.S.R.} = \text{urea F.S.R.} \times \frac{\text{H.S.R. of guanidine C}}{\text{H.S.R. of urea C}}$$

where F.S.R. stands for fractional synthesis rate and H.S.R. for hypothetical specific radioactivity.

After an injection of [^{13}C]urea the fractional synthesis rate of urea is best obtained from the slope of the time curve of [^{13}C]urea enrichments in the plasma multiplied by 1.02 to allow for higher enrichments in the extravascular water than in the plasma (Regoezci *et al.* 1965). The hypothetical specific radioactivities are defined as values that would have been obtained if all the ^{14}C -labelled protein produced in the synthesis interval t had been retained in the plasma and all the [^{14}C]urea in the body water. They were formerly and perhaps misleadingly referred to as specific activities at t_0 since the method of estimating one of them (urea) was by extrapolating the specific radioactivity–time curve to zero time. Extrapolation does not give an exact result for urea losses, however, since the slow arrival of ^{14}C -labelled molecules in the plasma is not taken into account. Also, in some animals the slope of the curve is influenced by continuing synthesis of [^{14}C]urea. More accurate methods are described below for calculating both ^{14}C -labelled protein and [^{14}C]urea radioactivities that have been lost from the relevant compartment by catabolism, diffusion or excretion during the synthesis interval.

Area method of estimating [^{14}C]urea losses. Advantage is taken of available plasma [^{13}C]urea data, and the assumption is made that equilibration between plasma and extravascular urea is so rapid that newly synthesized [^{14}C]urea can be regarded as almost instantaneously distributed throughout the body urea pool. In such a situation and because [^{13}C]urea curves were effectively single exponentials [^{14}C]urea radioactivity lost was equal to the area under the [^{14}C]urea total radioactivity curve $\times k$ ($k = 0.693/\text{half-life of } ^{13}\text{C} \text{ urea}$).

It is more convenient to measure ^{14}C specific radioactivities and these were plotted against time on linear graph paper (cf. Fig. 1). The area under each ^{14}C specific radioactivity curve was then measured up to time t with a planimeter and used along with the slope of the [^{13}C]urea curve to calculate [^{14}C]urea lost. The hypothetical specific radioactivity of [^{14}C]urea at t was calculated by increasing the measured plasma value at this time in proportion to the ratio of lost to retained radioactivity at the time. Potential errors associated with substituting specific for total activities are discussed later.

Method for estimating ^{14}C -labelled protein losses. ^{14}C -labelled proteins are also delivered gradually into the

Fig. 1. Curve of [¹⁴C]urea specific radioactivities (●—●) in animal no. 3 compared with arbitrarily positioned curve of [¹³C]urea enrichments (—) in plasma.

plasma, but the reasons why losses arise are more complex. Not only are the proteins catabolized, but albumin exchanges progressively with at least two extravascular pools of significant sizes and exchange rates, compared with a single rapidly exchanging one for urea. In such a situation the essential conditions for applying the area method as used for urea are not satisfied.

A computer method of estimating losses into the two postulated extravascular pools may be used. The approximate sizes and exchange rates of the pools must first be estimated and for this purpose graphical resolution of iodine-labelled protein curves was used followed by pool parameter calculations by the procedure of Matthews (1957), assuming a mammillary system with two reversibly exchanging extravascular protein pools and unidirectional catabolic losses occurring from the central compartment only. With these parameters the pool system was then recreated electronically in a Pace TR48 analogue computer and the experimentally determined time curve of [6-¹⁴C]-arginine specific radioactivities in protein simulated in the central plasma compartment. The computer was then used to plot time curves of protein radioactivities in the satellite pools and of catabolic losses, and these are shown in Fig. 2. Total losses and hence hypothetical specific radioactivities of protein were then calculated (cf. Table 3).

A digital programme was also prepared for use in conjunction with an Atlas computer. ¹⁴C specific radioactivities were read off the smoothed protein [6-¹⁴C]arginine curve at 10 min. intervals and presented to the computer along with the protein pool parameters, so that personal errors associated with simulating these curves in the analogue computer disappeared. The method was more accurate as well as more convenient once the programme had been prepared and tested.

Fig. 2. Typical analogue-computer curves from one experiment (cf. also Table 3). Measured ¹⁴C radioactivities of guanidine carbon in plasma albumin are shown as a smoothed curve (—). The three derived curves showing relative radioactivities were produced by computer from pool parameters derived by graphical analysis of ¹³¹I-labelled albumin radioactivities in the plasma. ---, Rapidly exchanging extravascular pool of albumin equivalent to 10.7% of the plasma albumin; actual radioactivities in this pool are one-tenth of the values shown., Slowly exchanging extravascular pool of albumin equivalent to 170% of the plasma pool. -.-.-, Losses of radioactivity by catabolism of albumin; actual values are one-tenth of those shown.

However, inspection of total radioactivity curves derived from analogue (cf. Fig. 2) or digital computers revealed two consistent features that justified the subsequent use of a simpler approximate procedure not requiring the use of a computer. First, total ¹⁴C radioactivities in the smaller protein pool were observed to be almost invariably maximal within 200 min. and thereafter could be calculated from the plasma radioactivity at the same time by applying a pool-size factor and neglecting small differences in specific radioactivity between the pools. Secondly, radioactivity that left the larger extravascular pool in intervals within 300 min. represented such a small proportion of the radioactivity remaining in this pool that the latter could be calculated with satisfactory accuracy by an area method, namely by multiplying integrated [6-¹⁴C]-arginine radioactivities of plasma proteins by the fractional transfer rate of the relevant protein from the plasma into this pool.

The accuracy of this simpler method depends critically on the synthesis interval chosen, and fortunately the favourable intervals, namely those between 200 and 300 min., are ideal for other reasons. An important practical advantage of the method is that information about the final catabolic half-life is not required, only the slope of the iodine-labelled protein curve in the interval between 1 and 6 hr. after the injection. Iodine-labelled protein curves in the first 6 hr. could be resolved almost invariably into two components, the intercepts and slopes of which were then

Table 1. [^{13}C]- and [^{14}C]-urea data from normal and endotoxin-treated rabbits, including calculations of [^{14}C]-urea losses at 200 and 300 min. by alternative methods

Animal nos. 1-5 were normal rabbits, and animal nos. 9-14 and 7 were treated with endotoxin. Slopes of plasma urea curves, columns (3) and (4), were calculated from half-lives according to the formula:

$$\text{Slope} = \frac{0.693}{t_{1/2} \text{ (days)}}$$

Hypothetical [^{14}C]-urea specific radioactivities were calculated from:

$$\text{Measured sp. radioactivity at } t \times \frac{\text{lost + retained radioactivity at } t}{\text{retained radioactivity at } t}$$

Values in columns (5), (11) and (12) were obtained from the formulae:

$$(5) = \frac{(3)-(4)}{(3)} \times 100; (11) = \frac{(8)-(9)}{(9)} \times 100; (12) = \frac{(8)-(10)}{(10)} \times 100$$

Animal no.	Concn. of urea (mg. of urea C/ml. of plasma)	Slopes of plasma urea curves				[¹⁴ C]Urea sp. radioactivity		Hypothetical [¹⁴ C]urea sp. radioactivity (μc/g. of urea C)					
		[¹³ C]Urea (3)	[¹⁴ C]Urea (4)	Difference (%) (5)	Time of max. (min.) (6)	Max. value (μc/g. of urea C) (7)	By extra-polation to t ₀ (8)	By area method		Difference (%)			
								200 min.	300 min.	200 min.	300 min.		
(1)	(2)							(9)	(10)	(11)	(12)		
1	0.08	5.87	5.12	12.8	65	13.06	17.23	17.75	18.03	-2.9	-4.4		
2	0.10	3.57	3.57	0.0	80	15.20	20.45	19.20	19.20	6.5	6.5		
3	0.08	6.05	4.99	17.5	45	22.57	30.63	31.37	32.95	-2.4	-7.0		
4	0.07	6.05	6.24	-3.1	60	18.02	24.77	22.97	22.96	7.8	7.9		
5	0.06	6.05	5.25	13.2	70	14.87	24.55	23.44	24.15	4.7	1.7		
9	0.14	4.25	3.33	22.4	100	11.26	13.74	15.00	15.78	-8.4	-12.9		
10	0.15	3.07	2.35	23.5	100	8.41	6.13	6.80	7.51	-9.9	-18.4		
11	0.14	3.84	4.16	-8.3	80	8.56	11.13	10.36	10.36	7.4	7.4		
12	0.10	4.34	2.81	35.3	90	15.54	19.60	21.63	22.75	-9.4	-13.8		
13	0.10	4.64	4.54	2.2	90	19.14	27.93	25.71	25.71	8.6	8.6		
14	0.14	2.10	1.92	8.6	90	6.44	7.30	7.32	7.32	-0.3	-0.3		
7	0.07	4.64	4.54	2.2	90	23.20	31.62	29.86	29.95	5.9	5.6		

treated mathematically according to Matthews's (1957) equations for a hypothetical model comprising a small extravascular pool exchanging reversibly with the plasma pool and an infinitely large one into which radioactivity is transferred from the plasma without reverse flow. Results for total protein losses after 200 min. obtained with this method were lower than those given by the digital computer, but by less than 8%.

RESULTS AND DISCUSSION

Fractional synthesis rates of urea. The results in Table 1 confirm the importance of using the slope of the specific activity curve of injected [^{13}C]urea rather than that of endogenously produced [^{14}C]urea in the protein synthesis rate formula as was recommended by McFarlane *et al.* (1965). As shown, differences between the alternative slopes could affect protein synthesis rates by up to 17.5% in normal animals and by up to 35% in endotoxin-treated animals. These differences presumably reflect mainly continuing synthesis of urea from $^{14}\text{CO}_2$. Reutilization of $^{14}\text{CO}_2$ was significant in six out of the 12 experiments recorded in Table 1 without any reason for its erratic incidence being apparent. Clearly it is best to rely on the slope of injected [^{13}C]urea. Alternatively, a preliminary experiment with [^{14}C]urea to determine the parameters of urea synthesis and excretion can be carried out a few days in advance of the protein synthesis measurement with [^{14}C]carbonate, but this involves an assumption about the day-to-day stability of these parameters that should first be verified.

Estimation of [^{14}C]urea radioactivities. Estimates of urea hypothetical specific radioactivities by the previously recommended procedure of extrapolating to zero time are shown in Table 1 for comparison with estimates by the more exact area method. Extrapolation should provide overestimates of true values, whereas hypothetical specific radioactivities both higher and lower than those given by the area method were obtained. It appears that various degrees of reutilization of $^{14}\text{CO}_2$ are the main factor responsible. In experiments with no apparent reutilization (animal nos. 2, 4, 7, 11 and 13) extrapolation gave 5.6–8.6% higher hypothetical specific radioactivities compared with the values obtained by the area method. On the other hand, extrapolation values were lower in experiments with significant reutilization (animal nos. 3, 9, 10 and 12), but the difference increased predictably with the length of the synthesis interval. In the presence of moderate reutilization (animal nos. 1, 5 and 14), where [^{13}C]urea and [^{14}C]urea slopes differed by 8–13%, errors were mutually compensating so that area and extrapolation procedures gave effectively the same hypothetical specific radioactivities.

The practical inference is that when hypothetical

Fig. 3. Comparison of plasma curves of screened and unscreened albumin labelled with ^{131}I and ^{125}I respectively.

specific radioactivities of urea obtained by extrapolation instead of by the area method are used a random error of $\pm 10\%$ can be introduced into protein synthesis rates.

Estimation of ^{14}C -labelled protein radioactivities. Since the procedure used to estimate ^{14}C -labelled protein losses is based on rates of loss of injected radioiodine-labelled proteins it is important that the latter should behave in a closely similar fashion to the ^{14}C -labelled protein. To verify this for albumin a rabbit was injected with a mixture of the ^{131}I -labelled albumin and the same protein labelled with ^{125}I and biologically 'screened' (McFarlane, 1956) in another animal for 24 hr. As shown in Fig. 3, the two isotopes were eliminated at exactly the same rate during the first 6 hr., and we conclude that the ^{131}I -labelled albumin contained no unusually rapidly eliminated component the presence of which might result in erroneous conclusions about extravascular pool sizes. The same is true of ^{131}I -labelled fibrinogen, as was shown in earlier work in which the behaviours of ^{14}C -labelled fibrinogen and ^{131}I -labelled fibrinogen were directly compared in the same rabbit (McFarlane, 1963b).

Table 2 shows intercepts and half-lives derived from plasma ^{125}I or ^{131}I radioactivities in normal and endotoxin-treated animals. Distributions of ^{14}C -labelled albumin radioactivities shown in Table 3 were estimated at different times in the same experiment by the alternative procedures described. Values for total losses by the two methods agree for synthesis intervals longer than 200 min. Since the alternative estimates did not differ in five animals by

Table 2. *Behaviour of radioiodine-labelled proteins in normal and endotoxin-treated rabbits*

Time (hr.) indicates the interval between injection of endotoxin and radioiodine-labelled protein. Intercept (%) indicates the relative radioactivity in plasma extrapolated to t_0 . S, Mill Hill strain of Sandylps; NZ, New Zealand White.

Animal no.	Endotoxin		Animal type	Albumin		Fibrinogen	
	Amount (μ g.)	Time (hr.)		Intercept (%)	Half-life (hr.)	Intercept (%)	Half-life (hr.)
1	—	—	S	90	10.0	100	10.8
2	—	—	S	80*	14.8*	95	12.0
3	—	—	S	86	10.4	100	12.2
4	—	—	S	80*	12.0*	95	6.5
5	—	—	NZ	80	17.5	—	—
6	—	—	S	84	10.0	92	8.8
7	—	—	S	83	10.0	91	10.5
8	—	—	NZ	82	8.0	88	8.6
Mean	...	...	...	83	11.6	94.5	9.9
9	50	14	NZ	94	8.5	100	20.0
10	10	14	NZ	95	8.5	100	17.5
11	25	14	NZ	90	12.0	—	—
12	20	24	S	84	14.9	96	16.7
13	20	41	S	100	8.9	100	11.2
14	20	48	NZ	86	14.3	93	20.0
7	20	48	S	90	12.2	93	15.2
15	20	24	S	100	8.9	100	11.2
Mean	...	...	...	92	11.0	97.5	16.0

* Approximate values due to almost continuous curvature of specific radioactivity curves in the first 6 hr.

Table 3. *^{14}C -labelled albumin total radioactivities in extravascular pools and lost by catabolism in animal no. 3 at various times as estimated by alternative area and computer methods*

Radioactivities in pool 2 were lost from the plasma by catabolism of albumin and values in pools 3 and 4 were lost by diffusion of the labelled protein into the corresponding extravascular compartments.

Synthesis interval (min.)		^{14}C -labelled albumin radioactivity (% of maximum value in pool 1)				
		100	150	200	250	300
By computer	Pool 1	88.5	99.0	94.0	90.5	88.5
	Pool 2	0.63	1.48	2.32	3.13	3.91
	Pool 3	3.46	6.43	7.99	8.62	8.84
	Pool 4	5.87	13.46	20.68	27.19	33.19
	Total losses	10.0	21.4	31.0	38.9	45.9
By area method	Pool 3	—	—	10.0	9.7	9.5
	Pool 4	5.68	13.3	21.1	28.4	35.5
	Total losses	—	—	31.1	38.1	45.0

more than 8% from each other, and since these differences were decreased to less than half in final estimates of protein synthesis rates, it was concluded that the accuracy of the simpler area method was adequate for present purposes. Values for protein hypothetical specific radioactivities in Table 4 and for fractional synthesis rates of albumin and fibrinogen in Table 5 are all based on estimates of ^{14}C -labelled protein losses by the approximate area method.

Both methods of computing losses require ^{131}I or ^{125}I data and time curves of ^{14}C -labelled protein radioactivities in the plasma that were available in ten experiments with seven normal and three endotoxin-treated rabbits. As shown in Fig. 4 the general shapes of the ^{14}C radioactivity curves were similar in all animals for one protein and indeed only after the times of the maxima did they differ for albumin and fibrinogen, indicating that the time constants of delivery of ^{14}C -labelled proteins

Table 4. Hypothetical specific radioactivities of guanidine carbon in albumin and fibrinogen at different times after injection of [¹⁴C]carbonate

Values are expressed as percentages of plasma specific radioactivities at the end of the interval.

Animal no.	Albumin					Fibrinogen				
	200 min.	250 min.	300 min.	Observed max. sp. radioactivity (μC/g. of guanidine C)	Time of max. (min.)	200 min.	250 min.	300 min.	Observed max. sp. radioactivity (μC/g. of guanidine C)	Time of max. (min.)
1	129.6	137.3	144.6	0.635	135	114.6	119.6	123.9	0.924	300
2	132.3	142.9	148.5	0.896	135	120.1	126.5	131.5	1.267	135
3	133.1	142.1	150.9	0.867	135	120.1	126.5	129.8	1.755	135
6	139.4	147.7	156.2	0.871	120	130.6	139.7	147.1	1.360	120
6	125.6	131.1	137.5	0.608	180	117.2	120.9	124.6	1.509	270
4	129.3	135.6	142.0	0.737	300	127.9	135.1	139.6	1.257	300
8	137.5	145.5	155.0	0.313	135	130.1	137.5	142.2	0.676	135
Mean ...	132.4	140.3	147.8	—	167	122.9	129.4	134.1	—	199

Fig. 4. Measured [¹⁴C]guanidine specific radioactivities in plasma proteins at different times. Experimental points are for albumin and were derived from ten rabbits (seven normal and three endotoxin-treated). The continuous lines indicate upper and lower limits within which nearly all the points fell. Corresponding fibrinogen specific radioactivities, which are not plotted, fell within the limits shown by the broken lines.

by the liver are much the same in all animals and independent of the protein.

Hypothetical specific radioactivities of protein guanidine carbon for three synthesis intervals in the experiments with seven normal rabbits are shown in Table 4, from which it is evident that measured plasma values at 300 min. have to be increased on an average by 47.8% for albumin and 34.1% for fibrinogen to obtain hypothetical values. In view of the general similarity in the shapes of ¹⁴C radioactivity curves individual variations in ¹⁴C-labelled protein losses that are evident in Table 4

Fig. 5. Losses of radioiodine-labelled albumin and fibrinogen from the plasma compared with calculated losses of the [¹⁴C]guanidine-labelled proteins. (a) 300 min.; (b) 200 min. Linear regression equations:

Albumin: $Y_1 = 11.2 + 0.635 X$
 $Y_2 = 18.4 + 0.415 X$

Fibrinogen: $Y_3 = 6.9 + 0.848 X$
 $Y_4 = 9.9 + 0.632 X$

must be due mainly to differences in extravascular pool sizes and in protein transfer rates between pools.

In Fig. 5 are plotted measured losses of ¹²⁵I or ¹³¹I radioactivity as percentages of the initial plasma values against calculated ¹⁴C-labelled protein losses in the same interval as percentages of

measured [^{14}C]guanidine specific radioactivities in albumin and fibrinogen at the end of the interval. A striking correlation is evident between ^{125}I or ^{131}I and ^{14}C losses, the calculated coefficients (Snedecor, 1956) at 200 and 300 min. respectively being 0.941 and 0.937 for albumin and 0.936 and 0.975 for fibrinogen. Data from the experiments with three endotoxin-treated rabbits are included in this Fig. 5, since ^{14}C -labelled protein losses in these animals were also proportional to radioiodine-labelled protein losses in the same animal, though they are significantly below the average for normal rabbits. Clearly a great deal of effort and several blood samples can be dispensed with by using regression equations or appropriate graphs, but coefficients for other proteins and animal species should be determined in separate experiments.

Substitution of specific for total radioactivities. The fundamental relationship on which the synthesis method is based concerns total ^{14}C radioactivities incorporated into protein and urea, and the convenient substitution of specific radioactivities that provides protein fractional synthesis rates is only strictly permissible in studying animals in a steady state. In non-steady-state conditions total ^{14}C radioactivities in plasma protein and body water urea are readily measurable at the end of the synthesis interval, but to estimate radioactivity losses during the interval requires more information than was obtained in these experiments. It will be necessary to plot total radioactivities (^{14}C -labelled protein and urea) in the plasma and to apply to the areas under these curves fractional rates of loss based on integrated slopes of ^{131}I -labelled protein or [^{13}C]urea total activity curves over the same interval. It may be advisable also to use exceptionally short synthesis intervals, to minimize the error involved in measuring protein and urea total activity losses.

In the experiments described instability was evident in two groups of rabbits. In one group, which had up to 20% of the total blood removed in the form of samples, plasma urea concentrations increased by up to 25% in 5 hr. This was almost certainly due to a decrease in excretion, since the slope of the [^{13}C]urea enrichment curve was approximately constant. Though the final slope of the [^{13}C]urea total activity curve was 20% less than the initial one, the net effect of applying a total activity rather than a specific activity treatment in this case was to increase the albumin synthesis rate by only 3-4%. In the second group fibrinogen concentrations increased in the plasma by up to 0% in the 5 hr. as a result of endotoxin treatment, and in this case the use of specific activities had resulted in an almost proportionate underestimate of fibrinogen synthesis rate. However, this was insignificant in relation to the magnitude of the

Table 5. *Fractional and absolute synthesis rates of albumin and fibrinogen in rabbits, measured by using two synthesis intervals*

Animal no.		Body wt. (kg.)	Plasma volume (ml.)	Intravascular pool (mg.)		Fractional synthesis rate (% of intravascular pool/day)												Absolute synthesis rate																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
						Albumin						Fibrinogen						Albumin			Fibrinogen																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
						200 min.		300 min.		200 min.		300 min.		(mg./day)		(mg./day)		(mg./kg./day)		(mg./kg./day)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
						A	B	A	B	A	B	A	B	(14)	(15)	(16)	(17)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								

A values were obtained from the formula:

$$[\text{C}^{13}]\text{Urea slope} \times 1.02 \times \frac{\text{hypothetical protein guanidine C sp. activity}}{\text{hypothetical urea C sp. activity}}$$

B values were obtained as described for the A values, but with urea activities reached at $t = 50$ min. compared with protein activities at t when $t = 200$ or 300 min.

changes that were observed to follow endotoxin treatment.

Optimum synthesis interval. Where sufficient experimental data were available synthesis rates were calculated (cf. Table 5) for both 200 and 300 min. intervals and including two experiments (animal nos. 3 and 12) in which reutilization was marked. Average values were calculated only for the longer synthesis interval, since in some experiments only a single large blood sample was taken at 300 min. Differences in the results are small and show no particular trend with time, even in experiments in which [^{14}C]urea clearly continued to arrive in the body water in significant amounts after 200 min. We conclude that it is not necessary to wait for all or nearly all the newly labelled protein and urea to be delivered by the liver, and that at least in the rabbit rates of protein synthesis can and should be measured with intervals as short as 200 min.

The interval of approx. 30 min. between injection of [^{14}C]carbonate and the first appearance of labelled protein in the plasma is presumed to be the time taken to displace a pool of the protein from the liver. If this is correct protein hypothetical specific radioactivities that would have been obtained in the plasma at time t should be compared in the synthesis rate formula with similar urea radioactivities that would have been reached at time $t - 30$ min. To investigate the order of error that might be associated with this procedure alternative extreme situations (A and B in Table 5) were chosen in which radioactivities are compared without any delay-time allowance on the one hand and with one of 50 min. on the other. Results show that the magnitude of the delay time was not important even when 200 min. synthesis interval was used, and indeed the correction may be omitted altogether.

Effects of toxin. Total mean loss of radioiodine-labelled albumin at 300 min. amounted to 38.6 (s.d. ± 2.34)% of the injected radioactivity in normal rabbits, and of fibrinogen to 31.5 (s.d. ± 5.77)%. Corresponding values for endotoxin-treated animals were 34.4 (s.d. ± 4.83)% for albumin and 22.4 (s.d. ± 5.02)% for fibrinogen. Differences between normal and endotoxin-treated rabbits were tested by Student's t test (Snedecor, 1956) and found to be statistically significant, especially for fibrinogen, as indicated by P values (albumin: $P < 0.05$; fibrinogen: $P < 0.005$).

Many of these endotoxin-treated animals had correspondingly enlarged fibrinogen plasma pools and it is tempting to conclude that absolute rates of loss had remained constant. On closer inspection, however, the results show a degree of spread such that this conclusion cannot be upheld. Atencio, Bailey & Reeve (1965) after studying a population

of apparently normal rabbits with different pool sizes concluded that the fractional transfer rate of fibrinogen was remarkably constant.

The decreased rates of loss of labelled protein probably reflect decreased rates of transfer to the extravascular compartment, and may be the result of the damage to vascular endothelium revealed by electron-microscopic studies (McKay, Margaretten & Csavossy, 1966). It is noteworthy that permeability to fibrinogen, which has a higher molecular weight, should be more affected than that to albumin. This problem seems to need further investigation, however, since Chien, Dellenback & Usami (1965) reported increased lymph/plasma fibrinogen concentration ratios in dogs shortly after injection of a large dose of *Escherichia coli* endotoxin.

The impression that originated in results of earlier studies (Regoezci *et al.* 1963) with ^{131}I -labelled fibrinogen alone, that the synthesis rate of the protein was increased by endotoxin treatment, is amply confirmed. The observed mean increase of 250% in the fractional rate is far in excess of the errors in the methods used and rises to 435% when calculated on a basis of mg. of fibrinogen/kg. body wt./day (Table 5). This increase is perhaps more striking when considered in conjunction with the observation that no change could be detected in the fractional catabolic rate (Regoezci *et al.* 1963). Synthesis rates were almost as high at 14 hr. as at 48 hr., and further experiments are necessary to determine the time curves of the response and the effect of varying the dose of endotoxin.

Acute increases in fibrinogen concentrations have been observed in a variety of clinical conditions without evidence for similar changes affecting albumin (McCathie, Owen & Macpherson, 1966; Weimer & Humelbaugh, 1967). With sublethal doses of toxin and by using more sophisticated methods it is clear that albumin synthesis rates were increased, but to a much smaller extent than fibrinogen ones. The marked response in the latter could be regarded as directly and specifically to a particular stimulus, and the albumin change as possibly secondary to a variety of metabolic or other effects of the endotoxin.

The authors thank Mr G. Dickinson for mass-spectrometer assays and Mrs Wendy Hayward and Mr John Heather for expert technical assistance. They also gratefully acknowledge the expert computer advice and assistance from Mr B. J. Hammond, Mr D. F. C. Iles and Mr J. G. White. A.K. was on leave from the Department of Physiological Chemistry, Medical School, Cracow, Poland.

REFERENCES

- Atencio, A. C., Bailey, H. R. & Reeve, E. B. (1965). *J. clin. Med.* **66**, 1.
Bryan, C. E. (1947). *Atomic Energy Commission Isotopes Division Circular* no. C-8.

- Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 157. New York: John Wiley and Sons Inc.
- Chien, S., Dellenback, R. J. & Usami, S. (1965). *Proc. Soc. exp. Biol., N.Y.*, **118**, 1187.
- Fawcett, J. K. & Scott, J. E. (1960). *J. clin. Path.* **13**, 156.
- Humphrey, J. H. & Bangham, D. R. (1959). *Bull. World Hlth Org.* **20**, 1241.
- Jacobsson, K. (1955). *Scand. J. clin. Lab. Invest.* **7** (Suppl.), 14.
- Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McCathie, U., Owen, J. A. & Macpherson, A. I. (1966). *Scot. med. J.* **11**, 83.
- McFarlane, A. S. (1956). *Biochem. J.* **62**, 135.
- McFarlane, A. S. (1963a). *Biochem. J.* **89**, 277.
- McFarlane, A. S. (1963b). *J. clin. Invest.* **42**, 346.
- McFarlane, A. S. (1964). In *Mammalian Protein Metabolism* vol. 1: *Metabolism of Plasma Proteins*, p. 298. Ed. by Munro, H. N. & Allison, J. B. New York: Academic Press Inc.
- McFarlane, A. S., Irons, L., Koj, A. & Regoeczi, E. (1965). *Biochem. J.* **95**, 536.
- McKay, D. G., Margaretten, W. & Csavossy, I. (1966). *Lab. Invest.* **15**, 1815.
- Matthews, C. M. E. (1957). *Phys. in Med. Biol.* **2**, 36.
- Regoeczi, E., Henley, G. E., Holloway, R. C. & McFarlane, A. S. (1963). *Brit. J. exp. Path.* **44**, 397.
- Regoeczi, E., Irons, L., Koj, A. & McFarlane, A. S. (1965). *Biochem. J.* **95**, 521.
- Snedecor, G. W. (1956). *Statistical Methods*, 5th ed., pp. 46, 122, 160. Ames: Iowa State University Press.
- Weimer, H. E. & Humelbaugh, C. (1967). *Canad. J. Physiol. Pharmacol.* **45**, 241.