of the rice-starch solutions. This enzyme is activated by Cl^- ions, inhibited by EDTA and does not hydrolyse disaccharides.

Amylase B is responsible for only a small fraction of the total amylase activity of the mucosa, but accounts for about half of the amylase activity of the washed particles. It has the properties of a glucamylase (γ-amylase; for a review see Larner, 1960). Glucose is the primary reaction product of the action of a glucamylase on starch. Glucamylases are exoamylases, hydrolysing the terminal α-(1→4)-glucosidic linkages at the non-reducing end of starch chains. During its action the viscosity of a starch solution decreases very slowly. Amylase B also hydrolyses maltose to glucose at approximately the same rate as starch is hydrolysed. It is responsible for about half of the maltase activity of the mucosal preparations.

The physiological function of the intestinal glucamylase cannot be stated at present. Glucamylases have been demonstrated in mammalian liver (Glock, 1936; Torres & Olavarria, 1961; Rosenfeld & Popova, 1962; Lejeune, Thinès-Sempoux & Hers, 1963) and muscle (Torres & Olavarria, 1961; Hers, 1963).

SUMMARY

1. Rat small-intestinal mucosa contains two amylases, one an endoamylase (α-amylase) and one an exoamylase (glucamylase, γ-amylase).

2. The endoamylase accounts for the major part of the total amylase activity of the mucosa. It is chiefly present in a soluble form in mucosal homogenates and has no maltase activity.

3. The glucamylase is chiefly particle-bound in mucosal homogenates. It can be solubilized by papain-digestion of the particles. This enzyme hydrolyses maltose at approximately the same rate as starch. It accounts for about half of the total maltase activity of the mucosa. It has no isomaltase or invertase activity.

This investigation was supported by grants from the Swedish Medical Research Council. D. L. T. was the holder of a R. Samuel McLaughlin Travelling Fellowship. Mrs A. Karstensson and Miss K. Klang are thanked for their skilful technical assistance.

REFERENCES

Dahlqvist, A. (1959). *Acta chem. scand.* **13**, 945.
Dahlqvist, A. (1960). *Acta chem. scand.* **14**, 1797.
Dahlqvist, A. (1961*a*). *Biochem. J.* **78**, 282.
Dahlqvist, A. (1961*b*). *Biochem. J.* **80**, 547.
Dahlqvist, A. (1963). *Biochem. J.* **86**, 72.
Eggstein, M. & Kreutz, F. H. (1955). *Klin. Wschr.* **33**, 879.
Glock, G. E. (1936). *Biochem. J.* **30**, 2313.
Hers, H. G. (1963). *Biochem. J.* **86**, 11.
Larner, J. (1960). In *The Enzymes*, vol. 4, p. 369. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Lejeune, N., Thinès-Sempoux, D. & Hers, H. G. (1963). *Biochem. J.* **86**, 16.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McGeachin, R. L. & Ford, N. J., jun. (1959). *Amer. J. Physiol.* **196**, 972.
McGeachin, R. L., Gleason, J. R. & Adams, N. R. (1958). *Arch. Biochem. Biophys.* **75**, 403.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Rosenfeld, E. L. & Popova, J. A. (1962). *Bull. Soc. Chim. biol., Paris*, **44**, 129.
Stein, E. A. & Fischer, E. H. (1958). *J. biol. Chem.* **232**, 867.
Sumner, J. B. (1924). *J. biol. Chem.* **62**, 287.
Torres, H. N. & Olavarria, J. M. (1961). *Acta physiol. lat.-amer.* **11**, 95.
Weill, C. E. & Hanke, P. (1962). *Analyt. Chem.* **34**, 1736.

Biochem. J. (1963) **89**, 277

Measurement of Synthesis Rates of Liver-Produced Plasma Proteins

By A. S. McFARLANE
National Institute for Medical Research, London, N.W. 7

(*Received* 8 *March* 1963)

Hitherto rates of synthesis of plasma proteins have been inferred from measurements of catabolism in man and animals in protein equilibrium, the important clinical problem of measuring differences between synthesis and catabolic rates in unbalanced states being as yet unresolved. The problem of measuring protein-synthesis rates absolutely, as in nearly all tracer problems, is that of measuring the specific activities of intracellular precursor amino acids. If a mean value for these

is known (say $P\,\mu$c/g.) over an interval t during which new protein containing M g. of precursor and $R\,\mu$c are produced, then $R = P \times M$ and the synthesis rate, M/t, is equal to R/Pt. Hippuric acid has been proposed as an externally available indicator of the specific activity of liver intracellular glycine for this purpose, and Weissman, Tschudy, Bacchus & Eubanks (1961) have suggested that this method can be improved by resolving the complex specific-activity curves for urinary hippuric acid into three exponential components.

A different approach became possible after Delluva & Wilson's (1946) demonstration that the injection of $^{14}CO_2$ leads to protein labelling predominantly in the guanidine carbon of arginine. Carbon dioxide penetrates with great rapidity into cells, and condenses almost instantaneously in the liver with ammonia and ornithine to produce arginine. Because of the close metabolic relationship of arginine and urea, Swick (1958) suggested that urea emerging from the liver cell might be used as an indicator of the prevailing specific radioactivity of the guanidine carbon of intracellular arginine. His method of exploiting this possibility was to give rats a diet containing a constant amount of calcium [^{14}C]carbonate for 4–8 days and to compare the final specific activities of urea carbon and of liver-protein guanidine carbon, the former reaching maximal values in the first day. Such a method, involving substantial labelling of much of the body protein, is clearly not usable in investigations of human plasma protein or tissue protein.

The present paper describes in detail results obtained with alternative single-injection procedures that have been briefly reported (McFarlane, 1963*a*). When [^{14}C]carbonate is injected only a minute proportion of the ^{14}C is incorporated into arginine of new liver-produced plasma proteins, whereas much larger proportions of ^{14}C are so used when given in the form of [6-^{14}C]arginine. In spite of the higher efficiency of utilization of the amino acid, however, the balance of advantage, especially for clinical use, is shown to lie with carbonate.

MATERIALS

Totally labelled [^{14}C]*arginine and* [^{14}C]*carbonate.* These were obtained from The Radiochemical Centre, Amersham, Bucks., the latter in sterile solution at 5–25 mc/m-mole.

[6-^{14}C]*Arginine.* This was synthesized in 60% yield, starting from $Ba^{14}CO_3$, by Turba & Leismann's (1953) method. The amino acid was purified by recrystallization as the flavianate, and its purity demonstrated by paper chromatography and radioautography. As a precautionary measure against the presence of traces of ^{14}C-labelled urea, the amino acid was incubated with urea and urease before injection.

Iodine-labelled proteins. Crude rabbit albumin and fibrinogen were prepared by fractionation of plasma with ammonium sulphate and labelled with isotopic iodine, albumin with ^{131}I and fibrinogen with ^{125}I, by methods described by McFarlane (1958, 1963*b*). Both preparations were then mixed with an excess of inactive crude rabbit γ-globulin and refractionated with ammonium sulphate. The efficiency of this procedure for removing contaminating labelled γ-globulin was confirmed by paper electrophoresis and by scanning the strips for distribution of radioactivity.

Human albumin was prepared by chromatography on carboxymethylcellulose and labelled with ^{131}I as described by Cohen, Freeman & McFarlane (1961).

Animals. Male Sandylop rabbits of the strain bred in this Institute and weighing 2·8–3·5 kg. were used. When iodine-labelled proteins were injected their drinking water contained 0·005% of sodium iodide to decrease thyroid uptake of the isotope.

METHODS

Serum fractionation. In the preliminary experiments described below samples of rabbit serum were treated with 10 vol. of 1% (w/v) trichloroacetic acid in ethanol (Delaville, Delaville & Delaville, 1954) to precipitate total globulins, and the supernatants were dried *in vacuo* to recover crude albumins which were extracted with ether and ethanol. Alternatively, when purer γ-globulins were required the serum samples were precipitated with 18% (w/v) sodium sulphate (Kekwick, 1940) and centrifuged. Total 'albumins' in the supernatants were precipitated with trichloroacetic acid, dialysed till salt-free and dried. The crude γ-globulin precipitates after reprecipitation at 15% (w/v) sodium sulphate concentration, were dialysed first salt-free and then against phosphate buffer, pH 7·5 and I 0·0175, and passed through diethylaminoethylcellulose columns (Levy & Sober, 1960). Paper electrophoresis confirmed that these were free of other proteins.

With serum samples from normal humans who received [6-^{14}C]arginine the sodium sulphate procedure was used but without the final diethylaminoethylcellulose treatment of the γ-globulins.

Plasma fractionation. This was by ammonium sulphate precipitation, and twice-precipitated fibrinogens were clotted with thrombin and the clots treated with ether and ethanol before drying, all as described by McFarlane (1963*b*). Albumins were precipitated from the supernatants after 50% saturation with ammonium sulphate by adding minimal amounts of trichloroacetic acid and centrifuged down. They were redissolved by stirring with dilute alkali to obtain a final pH between 7 and 8, and reprecipitated with ammonium sulphate and trichloroacetic acid. This was followed by final dialysis against water and drying.

In one experiment in which [6-^{14}C]arginine was injected into a rabbit that had significant plasma concentrations of ^{131}I-labelled albumin and ^{125}I-labelled fibrinogen, it was possible to use these radioactivities at each stage of the ammonium sulphate precipitation procedure as a guide to protein recoveries and to cross-contamination of albumin with fibrinogen, and vice versa. To conserve ^{125}I-labelled fibrinogen from the small but highly radioactive plasma samples available in this case, 6 mg. of unlabelled carrier fibrinogen was added after the first precipitation. After two precipitations both albumin and fibrinogen products

received crude unlabelled γ-globulin at least 10 times in excess of any of the radioactive protein suspected to be present, and were precipitated once more. Fibrinogens were finally clotted, and albumins, which contained significant amounts of unlabelled γ-globulin, were dialysed and dried. The ^{14}C radioactivities of these proteins in the original plasma were calculated from the ^{14}C:iodine radioactivity ratios in the dry proteins and the corresponding iodine radioactivities/ml. of plasma.

Protein hydrolysis and recovery of arginine. Dried proteins were hydrolysed with 6N-HCl (400 ml./g.) for 20 hr. at 110° in sealed tubes, and the excess of acid was removed under reduced pressure in a rotary evaporator. Neutralization was by adding an aqueous suspension of De-acidite FF (CO_3^{2-} form) (obtained from The Permutit Co., Gunnersbury Avenue, London, W. 4) until CO_2 bubbles ceased to be formed. The resin was filtered off or, if more than 200 mg. of protein had been hydrolysed, the suspension was poured on to a column of the same resin comprising equal quantities of Cl^- and OH^- forms. In the latter case, by washing the column with water nearly pure arginine was eluted.

When pure arginine was required to compare specific activities of guanidine and total arginine carbons after giving totally labelled arginine, the flavianate was crystallized from the crude hydrolysate and recrystallized after dissolving in dilute ammonia. The amino acid recovered by extraction of the warm acidified flavianate solution with pentan-1-ol–benzene (1:1, v/v) was diluted stoicheiometrically with 6 times its weight of unlabelled carrier arginine before dividing and proceeding to total combustion of one part and treatment with arginase of the other.

Total combustion of proteins. When [6-^{14}C]arginine was given to rabbits over 90% of the total radioactivity that subsequently appeared in albumin and fibrinogen was found in the guanidine carbon of arginine, and the amino acid residues after treating the hydrolysates with arginase and urease contained only 3% of the total radioactivity. For this reason total combustion of these proteins was used in some experiments with the amino acid. The dried proteins were weighed into platinum boats and burnt in an oxygen bomb, and the purified $^{14}CO_2$ was transferred to a constant-volume manometer as described by McFarlane & Murray (1963).

Liberation of $^{14}CO_2$. Total hydrolysates, or arginines from these, were incubated at 37° with 1–3 mg. of arginase (20 μM units/mg.; Sigma Chemical Co., St Louis, Mo., U.S.A.) preactivated as follows. A 0·1% solution of the enzyme, which had been dialysed to remove traces of urea, was made 0·05M with respect to manganous sulphate and to sodium maleate at pH 7·00, and incubated for 4 hr. at 37°. After overnight incubation the solutions were acidified with a few drops of 4M-citric acid and concentrated on a boiling-water bath to 2–3 ml. Urease treatment was carried out in the vacuum vessel shown in Fig. 1. One side limb contained 1 ml. of a solution of 100 mg. of Sigma urease in 100 ml. of a solution containing 14 g. of sodium pyrophosphate and 2 g. of orthophosphoric acid. Some batches of the enzyme contained traces of carbonate which were removed by preliminary acidification to pH 5 and evacuation on the water pump. The other side limb received 1 ml. of a solution containing equal volumes of citric acid (4M) and tungstic acid [2 ml. of 10% (w/v) sodium tungstate plus 7·6 ml. of 0·1N-HCl plus 12·4 ml. of water].

The vessel was evacuated for 1–2 min., during which time almost all the air was displaced by water vapour. With the tap closed and the pump disconnected the urease solution was tipped in, and after 1 hr. at 25° this was followed by the citric acid–tungstic acid mixture. The centre bulb was now cooled in liquid nitrogen to condense CO_2, and as a result of the vacuum produced the side limbs, which were maintained at room temperature or warmed in a current of hot air, rapidly dried out. The vacuum flask containing the liquid nitrogen was now transferred to the cold-finger and the centre bulb immersed in ethanol–solid CO_2 mixture. Dry CO_2 now passed over into the cold-finger. To recover CO_2 trapped in the ice the contents of the centre bulb were melted and refrozen with the tap closed, and the transfer operation was repeated. Recovery of CO_2 was 99% and the gas was finally transferred to a constant-volume manometer for pressure measurement.

Radioactivities in plasma and urinary urea. Urine samples were acidified and concentrated to remove CO_2 before being treated with urease in the vacuum flask shown in Fig. 1. Serum samples for urea determinations were dialysed extensively to recover total urea, and plasma samples were deproteinized by acidifying with N-H_2SO_4

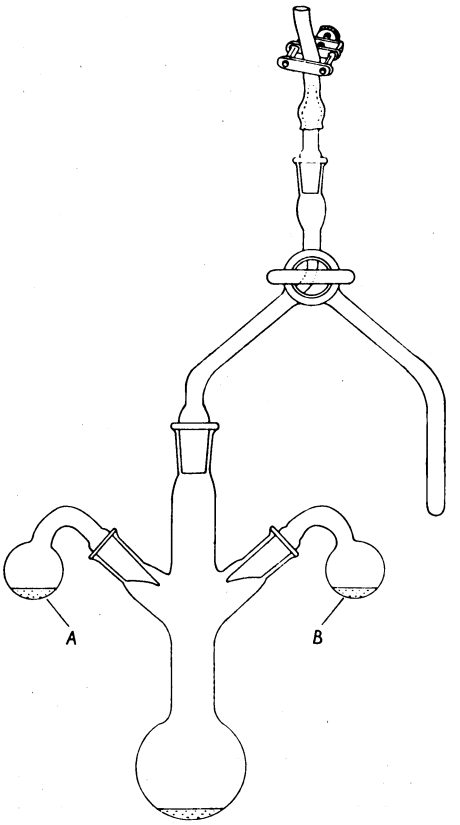

Fig. 1. Vacuum-flask arrangement used to recover $^{14}CO_2$ from protein hydrolysates after treatment with arginase, and from plasma and urine samples. Experimental details are given in the text. *A*, Citric acid–tungstic acid; *B*, urease.

and adding an equal volume of the stock tungstic acid solution used in the citric acid–tungstic acid mixture. After being brought to 100° in a boiling-water bath the protein precipitate was recovered by centrifuging, and a measured volume of the supernatant transferred to the vacuum flask for urease treatment. With small (0·15 ml.) rabbit-plasma samples 3 mg. of unlabelled carrier urea was added beforehand.

Counting. The $^{14}CO_2$ from the manometer was transferred to a proportional Geiger counter (Bradley, Holloway & McFarlane, 1954) or condensed on to 2 ml. of phenethylamine frozen at the temperature of liquid nitrogen (Woeller, 1961). The phenethylamine flask was disconnected and warmed, and the contents were washed out with 16 ml. of a solution containing 0·5% of 2,5-diphenyloxazole and 0·05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (both supplied by the Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) in anhydrous toluene into a vial for counting in the Packard Tri-Carb Scintillator.

The ^{131}I and ^{125}I radioactivities were counted in a well-type sodium iodide-crystal scintillator. Generally 3 ml. of solution was counted, but, in one rabbit experiment in which it was essential to obtain the closest possible correlation between iodine and carbon radioactivities, ^{131}I and ^{125}I values were measured on the dry proteins in pointed tubes before these were quantitatively transferred to platinum boats for combustion. In this case up to 0·2% of the iodine radioactivities appeared in the final $^{14}CO_2$ samples, and their small contributions to the ^{14}C counts were estimated in the second channel of the Tri-Carb Scintillator and subtracted. After the counting of the protein solutions these were used for biuret measurements and results were expressed as disintegrations/min./mg. of total protein. Total body ^{131}I radioactivities of rabbits were measured in a ring of six Geiger tubers as described by Campbell, Cuthbertson, Matthews & McFarlane (1956).

Synthesis formulae. The equation used in calculating synthesis rates is based on the expression given above, namely $P = R_p/M_p = R_u/M_u$, where 'p' and 'u' refer respectively to the guanidine carbons of protein and of urea, both assumed to arise directly from the guanidine carbon of arginine. Thus M_p, the mass of guanidine carbon of new protein, and hence the mass of new albumin produced in the interval t, is available if radioactivities appearing in the same interval in the form of new urea and new protein and the mass of new urea carbon are measured. The fundamental tracer principle involved, namely that the ratio of mass to radioactivity in products of the same precursor is constant, was suggested for use in this connexion by Reeve, Pearson & Martz (1963). It is in some respects complementary to the device proposed by Weissman *et al.* (1961) for circumventing the difficulty of isolating the precursor, namely by measuring the parameters of two fragments of a product (in their case two labelled amino acids in the same protein). In both cases, since total rather than specific activities were used, difficulties inherent in relying on results from small labile precursor pools to which Koch (1962) has drawn general attention appear to be avoided.

The relationship $M_u/R_u = M_p/R_p$ may be modified arbitrarily by dividing above and below on the protein side by the mass of guanidine carbon present in the total albumin of the plasma. If it is assumed that the mass of new urea carbon (M_u) is equal to the mass of urea carbon excreted in the same time, then the fraction of plasma albumin newly synthesized/day, or the fractional synthesis rate, becomes equal to:

$$\text{Specific activity of guanidine carbon of albumin at time } t \times \frac{\text{urea carbon excreted in time } t}{\text{total urea activity appearing in time } t} \times \frac{24}{t}$$

The specific activity of the guanidine carbon of protein referred to is the value that would have been measured if all the labelled protein had been retained in the plasma. However, some is catabolized and some escapes into the extravascular space, so that adjustments have to be made to the measured values of the specific activities.

EXPERIMENTAL

Investigations with [^{14}C]*arginine*

Preliminary experiments. Two 3 kg. rabbits were injected with 15 μc of [6-^{14}C]arginine, and 10 ml. blood samples were taken at various times. Crude albumin and γ-globulin fractions were prepared and the specific activities of guanidine carbon measured in each case.

Another 3·3 kg. rabbit received 50 μc of totally labelled [^{14}C]arginine, and 6 ml. blood samples were taken at 2, 4 and 7 hr., and then daily for a week. In this case only albumins were isolated and hydrolysed.

Two normal adult humans each received 50 μc of [6-^{14}C]-arginine together with 50 μc of ^{131}I-labelled human albumin. Blood samples (15 ml.) were taken, six in the first 24 hr., and 24 spread over the next 10 weeks. Serum samples were fractionated with ammonium sulphate into albumins and γ-globulins, which were hydrolysed and their arginines isolated as the flavianates.

Measurements of synthesis rates. ^{131}I-labelled albumin (60 μc) and ^{125}I-labelled fibrinogen (78·5 μc) were injected together into a 3·3 kg. rabbit, and small blood samples were taken at intervals for a week to determine the catabolic rates. The initial rates of disappearance of both labelled proteins from the plasma were also studied by using several small blood samples taken in the first 5 hr. Starting on the fourth day and continuing during the next part of the experiment, the animal received 200 mg. of neomycin daily by stomach tube to decrease bacterial digestion of urea in the gut (Walser & Bodenlos, 1959; Levenson, Crowley, Horowitz & Malm, 1959).

The animal was now catheterized, the bladder washed out and 50 μc of [6-^{14}C]arginine immediately injected into an ear vein. Blood samples (0·7 ml.) were taken from the other ear every 10 min. for 40 min. Over the next 5 hr. four 6 ml. blood samples were taken, in each case immediately after catheterization. The plasma so removed was replaced with unlabelled plasma given in two injections so that the animal remained in approximate protein balance. Then 3 weeks after this experiment 17·5 μc of [^{14}C]urea was injected into the same rabbit, and blood samples were taken over 5 hr. to enable the animal's urea-pool factor and renal clearance rate to be measured.

Investigations with [^{14}C]*carbonate*

Rabbit experiment. A 3·03 kg. rabbit received 5 ml. of plasma from a rabbit that had been injected with ^{131}I-labelled albumin 50 hr. earlier in order to 'screen out' any rapidly catabolized labelled protein. Sodium [^{14}C]carbonate

Table 1. *Specific activities of guanidine carbon in crude albumin and γ-globulin fractions of rabbit serum at intervals after the injection of* 15 μc *of* [6-^{14}C]*arginine*

Experimental details are given in the text.

	Time of bleeding (hr.)	Sp. activity of guanidine carbon (μc/g.)		Ratio of sp. activity of guanidine carbon of γ-globulin to that of albumin
		Albumin	γ-Globulin	
Rabbit 1	6	1·1	5·72	5·2
Rabbit 1	22·5	0·93	3·81	4·1
Rabbit 2	37	1·13	5·81	5·14
Rabbit 2	216	0·42	3·02	7·2

(500 μc) was added to the serum before injection. Four 0·6 ml. blood samples were taken at 10 min. intervals, a 1 ml. sample at 1 hr. and five 6 ml. ones in the next 21 hr. and every second day for 18 days. Serum samples were counted for ^{131}I radioactivities and then dialysed to recover ureas. Proteins left behind were fractionated with sodium sulphate to recover total albumins and γ-globulins. Urine samples required for measurements of synthesis rate were collected at 1, 2·5 and 6·8 hr.

Human experiments. Five adult males each received 100–200 μc of [^{14}C]carbonate. Four were normal and one (B.R.E.) was a diabetic of long standing not suspected to have an abnormality affecting his plasma proteins. One (F.R.E.) drank a suspension of $Ba^{14}CO_3$ in water immediately on wakening and rested in bed for the next 20 min. The others were injected intravenously with sodium [^{14}C]-carbonate after a light non-protein breakfast. Blood samples (40 ml.) were taken into citrate at approx. 4 hr. and in two cases also at approx. 6 hr., and at the same time 10 ml. was taken into heparin. Urine was voided at the time these samples were taken. The larger samples were fractionated with ammonium sulphate and the smaller heparin ones were used for measurements of plasma urea.

One subject (F.R.E.) received ^{131}I-labelled human albumin 14 days before the ^{14}C-labelling experiment, and another (G.O.R.) 6 years before, and their catabolic rates were measured as described by Cohen *et al.* (1961).

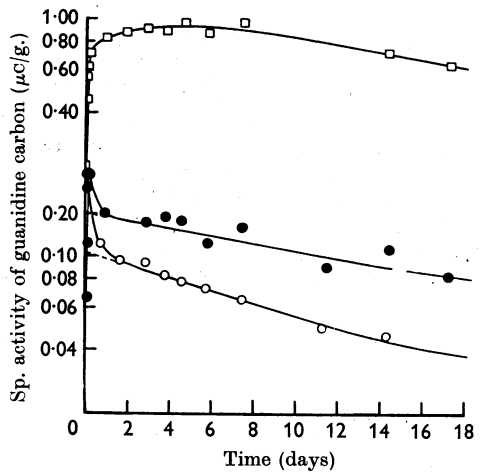

Fig. 2. Specific radioactivities of guanidine carbon of plasma proteins after the injection of 50 μc of [6-^{14}C]-arginine into a normal adult human. Experimental details are given in the text. The values for ^{14}C-labelled γ-globulin (□) are much higher than those for ^{14}C-labelled albumin (●), and there are marked differences between the curves for ^{14}C-labelled albumin (●) ($t_{\frac{1}{2}}$ = 450 hr.) and for ^{131}I-labelled albumin (○) ($t_{\frac{1}{2}}$ = 265 hr.) due to ^{14}C reutilization.

RESULTS

Preliminary experiments. Results of the first few experiments are shown in Table 1 and reveal much higher radioactivities in guanidine carbon of γ-globulins than in that of crude albumins. That this was due to higher dilution of the injected labelled guanidine carbon of [6-^{14}C]arginine at the site of albumin synthesis was shown in the next experiment by injecting totally labelled [^{14}C]-arginine. In all albumins in this experiment specific activities of total carbon or arginine were 8 ± 1·5 times as high as that of guanidine carbon in the same arginine, the rather large random variation being attributed to variable contamination of albumins with γ-globulins.

Results of the final experiment of this series were very similar in both subjects and confirmed the above effect in humans. Fig. 2 shows that after the first few hours the specific activities of the guanidine carbon of arginine were throughout 7–8 times as great in γ-globulins as in albumins at the same time. The broad plateau on the curve of γ-globulin had already been observed in specific-activity curves for human γ-globulin when other labelled amino acids were injected, and taken to reflect much greater recycling of amino acids within reticuloendothelial cells producing γ-globulins than within liver cells producing albumins (McFarlane, 1961). However, recycling of guanidine carbon of arginine in liver cells is evidently not negligible, as was shown by the much longer half-life of the ^{14}C than of the ^{131}I label of albumin in this experiment. The latter value (265 hr.) is within the range for normal humans found by Cohen *et al.* (1961) with this label and is believed to represent the true catabolic half-life of the protein.

In all these experiments it was not possible to decide precisely how much more actively labelled the guanidine carbon of γ-globulin was than that of the corresponding albumin because of the relatively crude nature of the albumins studied. It was evident that small amounts of γ-globulin in the albumin fractions could bring about large increases of specific activity in them, and the wide spread of experimental values for ^{14}C-labelled albumin about the mean in Fig. 2 is attributed to this. Clearly, if accurate measurements of albumin synthesis were to be possible much more rigorous purification of albumins was required, or alternatively some device for circumventing the effects of contamination with γ-globulins. It was decided in the next experiment to include fibrinogen in the synthesis measurements because of the readiness with which this liver-produced plasma protein can be isolated from small plasma samples in a relatively pure state, and also to wash all radioactive fibrinogens and albumins with unlabelled γ-globulin to minimize contamination effects on their specific activities.

Synthesis measurements with [6-^{14}C]*arginine.* Fig. 3 shows time curves of specific activities of guanidine carbons of albumin, fibrinogen and γ-globulin in this experiment. Maximum specific activities were reached in 6 hr. and values lay close to the smoothed curves, supporting the evidence of paper electrophoresis and radioautography that in this case there was no cross-contamination between radioactive γ-globulins and liver-produced proteins. The specific activities of γ-globulin were at first 8 and later 11·5 times the corresponding calculated values for albumin.

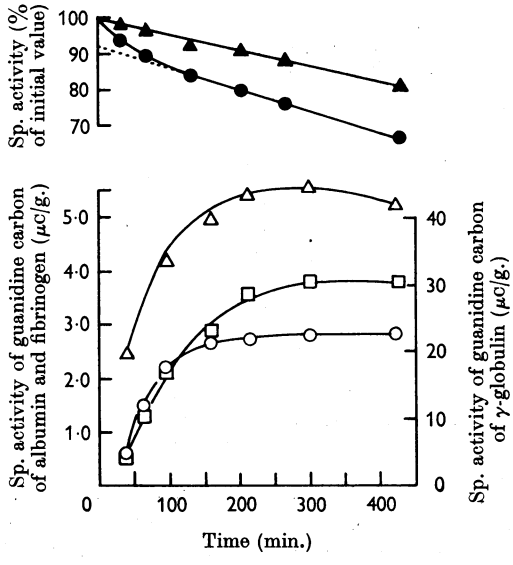

Fig. 3. Specific radioactivities of guanidine carbon of plasma proteins after the injection of 50 μc of [6-^{14}C]-arginine into a rabbit. Experimental details are given in the text. The values for ^{14}C-labelled γ-globulin (□) are many times greater than those for ^{14}C-labelled albumin (○). Values for ^{14}C-labelled fibrinogen (△) are also shown. ^{125}I-labelled fibrinogen (▲) and ^{131}I-labelled albumin (●) were injected a week earlier to measure the rates of loss of these proteins from the plasma in the first few hours, and their catabolic rates in the week preceding the ^{14}C experiment.

Results shown in Table 2 concern albumin and fibrinogen synthesis during four intervals each beginning with the injection and ending with a catheterization which enabled the mass of urea excreted and the ^{14}C radioactivities associated with it to be determined for each interval. Most of the labelled urea was retained in the body water, the amounts being calculated from radioactivities of plasma urea at the end of the interval in question and a factor representing the ratio of the radioactivity of total urea to that of plasma urea. The determination of this factor was the main objective in the supplementary experiment with [^{14}C]urea in which the total radioactivity retained as urea after equilibration was found to be 18·5 times the urea radioactivity in the plasma at the same time, or $18{\cdot}5 \times 130 = 2405$ times the radioactivity in 1 ml. of this animal's plasma (cf. Fig. 4).

Total radioactivities appearing in proteins must include for this purpose radioactivities removed in plasma samples and radioactivities lost by catabolism and by diffusion into the extravascular compartment. Estimates of radioactivities lost by catabolism and diffusion [columns (6) and (9) in Table 2] were based on measurements of the disappearance of the iodine-labelled proteins in the preliminary experiment (cf. Fig. 3). They required modification in the first two intervals, however, during which ^{14}C-labelled albumins and ^{14}C-labelled fibrinogens were rapidly accumulating, whereas in the iodine experiment the labelled proteins were steadily declining in concentration from the time of injection. The modification consisted in estimating the mean concentrations of the ^{14}C-labelled proteins from areas under the ^{14}C-specific-activity curves and applying corresponding iodine disappearance rates to them. ^{125}I-labelled fibrinogen left the plasma at a steady rate of 3·1 %/hr. The rate for ^{131}I-labelled albumin was generally somewhat faster. This particular rabbit showed a more rapid rate for albumin in the first 1·5 hr., a phenomenon which we have not seen with other albumins in rabbits and which suggests the presence of approx. 7 % of rapidly catabolized denatured protein. The assumption was made that only the steady rate established after 1·5 hr. applied to the ^{14}C-labelled protein.

Maximum values for synthesis rates were obtained in 4·9 hr. No labelled protein appeared

Table 2. *Calculation of the synthesis rates of albumin and of fibrinogen after the injection of 50 μc of [6-^{14}C]arginine into a rabbit*

Experimental details are given in the text. The calculations were made as follows. (4): Urea ^{14}C radioactivity/ml. of plasma × urea space factor (i.e. 2405). (6) and (9): Protein ^{14}C radioactivities/ml. of plasma × plasma vol. (130) + radioactivities removed in previous samples. (7) and (10): Protein ^{14}C radioactivities estimated from iodine data to have been catabolized or to have passed into the extravascular space (see the text). (12): $\frac{(2)\times(8)}{(5)\times(1)}\times 222$ (i.e. mg. of albumin containing 1 mg. of guanidine carbon). (13): $\frac{(12)\times 24}{52}$, where 5200 = total plasma albumin in mg. (14): $\frac{(2)\times(11)}{(5)\times(1)}\times 180$ (i.e. mg. of fibrinogen containing 1 mg. of guanidine carbon). (15): $\frac{(14)\times 24}{6\cdot5}$, where 650 = total plasma fibrinogen in mg.

Interval from injection (hr.)	Urinary urea carbon	Radioactivity of urea (μc)			Radioactivity of albumin (μmc)			Radioactivity of fibrinogen (μmc)			Albumin synthesis rate		Fibrinogen synthesis rate	
												Fractional		Fractional
	(mg.)	In urine (3)	Retained (4)	Total (5)	In plasma (6)	Lost (7)	Total (8)	In plasma (9)	Lost (10)	Total (11)	mg./hr. (12)	(%) (13)	mg./hr. (14)	(%) (15)
(1)	(2)													
2·12	48	3·14	13·29	16·42	66·6	4·86	71·46	17·71	0·71	18·42	21·4	9·9	4·47	16·5
3·50	81	5·11	13·02	18·13	69·6	10·06	79·66	18·82	1·46	20·28	22·6	10·4	4·66	17·2
4·90	119	6·97	12·75	19·72	73·0	13·96	86·96	19·61	2·32	21·93	23·8	11·0	4·86	18·0
6·90	163	8·70	11·39	20·09	76·1	18·19	94·29	19·36	3·52	22·88	24·6	11·36	4·85	17·9

in the plasma in the first 30 min. during which time much of the [^{14}C]urea had already been delivered into the body water. This urea was diluted with the body pool of urea which in this animal, as shown by the results of the supplementary experiment in which [^{14}C]urea was injected (Fig. 4), was turning over with a half-life of 3·0 hr. At this rate of replacement only one-third of the [^{14}C]urea in the body water at 2·17 hr. should have been present at 6·9 hr. However, 85% was found [column (4)], confirming that substantial new production of urea took place after 2·17 hr.

Values for fractional synthesis rates after 4·9 hr. in columns (13) and (15) of Table 2 are to be compared with 13·8% for ^{131}I-labelled albumin and 23% for ^{125}-labelled fibrinogen in the same animal. The latter values were the same whether calculated from urinary or total body radioactivities of albumin or from slopes of plasma-radioactivity curves taken in conjunction with intravascular:extravascular distribution ratios (McFarlane, 1963*c*).

Synthesis measurements with [^{14}C]*carbonate.* Results from the single rabbit experiment are shown in Figs. 5 and 6.

Comparison of Figs. 3 and 5 shows that whereas albumin was maximally labelled at 3–4 hr. in the plasmas of both rabbits (which were of comparable weight) the maximum specific activity of guanidine carbon of arginine in albumin was 2·8 μc/g. for 50 μc of [6-^{14}C]arginine injected and 4·3 μc/g. for 500 μc of [^{14}C]carbonate injected. The efficiency of labelling was therefore much lower when [^{14}C]carbonate was used. A second striking difference was that, with [^{14}C]carbonate, specific activities of γ-globulin in this particular carbon were about one-half those of albumin.

The ^{14}C specific activities of protein declined rapidly after reaching maximum values (cf. Fig. 6). With ^{14}C-labelled albumin the subsequent course of the curve followed closely that of ^{131}I-labelled albumin. This behaviour, which contrasts with that found after the injection of [^{14}C]arginine, implies

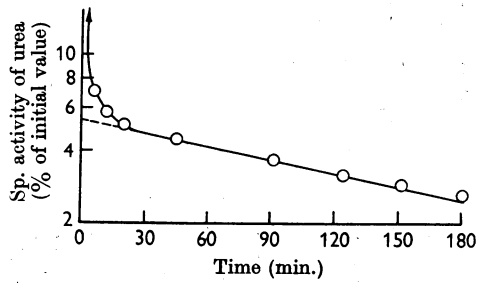

Fig. 4. After the experiment illustrated in Fig. 3 the rabbit was injected with [^{14}C]urea to measure the body urea factor. The half-life value was 3 hr. Experimental details are given in the text.

almost complete absence of reutilization after a few hours. This situation is also suggested by the finding that radioactivities of plasma [^{14}C]urea declined after 1·5 hr. with a half-life of 3 hr. (cf. Fig. 5), which may be close to the value characteristic of urea excretion after a single injection of this substance into normal rabbits (cf. Fig. 4).

Since the rate of disappearance of ^{131}I-labelled albumin in the first 5 hr. of this experiment was the same as in the previous one, percentage additions, necessary to allow for loss of albumin radioactivity by catabolism and diffusion in the two intervals (2·5 and 6·8 hr.) chosen for synthesis measurements, were obtained from the curve for ^{131}I-labelled albumin in Fig. 3 in conjunction with the curve for ^{14}C-labelled albumin in Fig. 5, and amounted to 9·1 % at 2·5 hr. and an additional 12 % at 6·8 hr. At these times the measured specific activities of albumin guanidine carbon were 4·35 and 3·60 μc/g. When these values were increased by the above percentages, and by 3·8 % at 2·5 hr. and an additional 2·5 % at 6·8 hr. due to radioactivities withdrawn in plasma samples, they became 4·91 and 4·58 μc/g. Theoretically, in the absence of reutilization after 2·5 hr. they should have been the same. The rabbit had excreted 0·0675 g. of urea carbon at 2·5 hr. and an additional 0·123 g. at 6·8 hr., and total urea radioactivity was 12·2 μc whether obtained from the sum of excreted and retained activities at 1, 2·5 or 6·8 hr. Thus the fractional synthesis rate of albumin was:

$$\frac{0{\cdot}0675 \times 4{\cdot}91 \times 2{\cdot}40}{12{\cdot}2 \times 2{\cdot}5} = 26\,\%/\text{day}$$

measured in the first 2·5 hr., and:

$$\frac{0{\cdot}19 \times 4{\cdot}58 \times 240}{12{\cdot}2 \times 6{\cdot}8} = 25{\cdot}2\,\%/\text{day}$$

measured in the first 6·8 hr.

The catabolic rate (cf. Fig. 6) obtained from excreted ^{131}I radioactivities in the next 28 days was 24 %/day. Alternatively, the plasma ^{131}I half-life of 180 hr. corresponded to a decay rate of 9·24 % of total albumin/day, and since extrapolation of the plasma exponential indicated approx. 35 % of the total albumin to be intravascular this gave 26·7 % of the plasma albumin catabolized/day, both values in good agreement with the values from the synthesis of ^{14}C-labelled albumin.

Results of the human investigations are presented in Table 3. Values for the excretion of urea carbon during the synthesis intervals corresponded

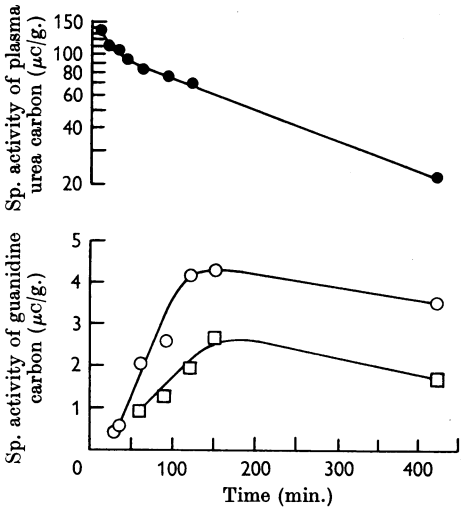

Fig. 5. Specific radioactivities of the guanidine carbon of plasma proteins and of plasma urea after the injection of 500 μc of $NaH^{14}CO_3$ into a rabbit. Experimental details are given in the text. The values for ^{14}C-labelled γ-globulin (□) are about one-half of those for ^{14}C-labelled albumin (○). The values for [^{14}C]urea (●) ($t_{\frac{1}{2}}$ = 3 hr.) are shown in the upper curve.

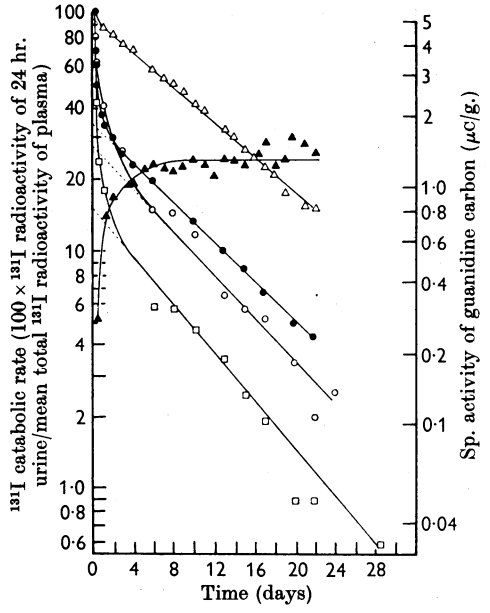

Fig. 6. After the experiment illustrated in Fig. 5 the rabbit was injected with ^{131}I-labelled albumin. Experimental details are given in the text. The values of specific radioactivity are: □, ^{14}C-labelled γ-globulin ($t_{\frac{1}{2}}$ = 150 hr.); ○, ^{14}C-labelled albumin ($t_{\frac{1}{2}}$ = 175 hr.); ●, ^{131}I-labelled albumin ($t_{\frac{1}{2}}$ = 180 hr.); △, total body ^{131}I ($t_{\frac{1}{2}}$ = 195 hr.). To obtain catabolic rate values (▲) the ^{131}I radioactivities of 24 hr. urines were expressed aspercentages of mean total ^{131}I radioactivities in the plasma on the same day. The similarity of the half-lives of ^{14}C-labelled albumin and of ^{131}I-labelled albumin suggests that ^{14}C reutilization was absent.

Table 3. *Calculation of synthesis rates of albumin and of fibrinogen after the administration of* 100–200 μc *of* [^{14}C]*carbonate to five adult humans (radioactivities refer to a uniform hypothetical dose of* 100 μc)

Experimental and other details are given in the text.

Subject	G.O.R.	F.R.E.	B.A.T.	B.R.I.		B.R.E.	
Age (years)	47	39	28	52		27	
Body weight (kg.)	76	67	78	63		64·5	
Excretion of urea carbon (g./day)	4·39	4·54	3·25	5·04		6·05	
Synthesis interval (t) (hr.)	3·5	3·5	4·33	4·13	5·90	4·40	6·60
Radioactivity of plasma urea ($\mu\mu$c/ml.)	10·4	10·4	7·3	28·7	28·0	19·2	19·5
Radioactivity of urea retained* (μc)	0·43	0·40	0·36	0·958	0·935	0·73	0·74
Radioactivity of urea excreted (μc)	0·098	0·094	0·180	0·490	0·560	0·40	0·54
Radioactivity of urea retained + urea excreted (μc)	0·528	0·494	0·540	1·448	1·495	1·13	1·28
Specific radioactivity of guanidine carbon of albumin (μc/g.)	0·0149	0·0121	0·0153	0·0377	0·0343	0·0165	0·0159
Specific radioactivity of guanidine carbon of fibrinogen (μc/g.)	—	0·0301	0·0473	0·0580	0·0763	0·0290	0·0320
Albumin synthesis rate† (%/day)	13·6	12·2	10·14	14·4	13·3	9·68	8·65
Fibrinogen synthesis rate† (%/day)	—	29·3	14·4	22·2	29·5	17·0	17·4

* Radioactivity of urea/ml. of plasma × body water volume (i.e. body water × factor that varies with age between 0·62 and 0·52 in the range 20–60 years).

† Synthesis rate expressed as % of plasma protein/day = (100 × specific radioactivity of protein guanidine carbon × urea carbon excreted/day)/(radioactivity of total urea). To allow for protein activities lost by diffusion and catabolism these values were increased by 10% for both proteins when the interval was 3·5–4·4 hr. and by 15% when the interval was 5·9–6·6 hr.

to daily rates that varied between 3·25 and 6·05 g./day. The subjects also varied greatly in their ability to make ^{14}C-labelled urea from [^{14}C]-carbonate. Thus, whereas F.R.E. produced 0·494 μc of [^{14}C]urea from 100 μc of [^{14}C]carbonate, B.R.I. produced about 1·5 μc. Similar wide variations were observed in the radioactivities of plasma urea, and are reflected in the values for the radioactivity of retained urea since these were obtained by multiplying urea radioactivities/ml. of plasma by body water volumes that varied between 33 and 46 l. Specific activities of guanidine carbon of plasma proteins were approximately proportional to total urea radioactivities. Thus B.R.I.'s protein specific activities were approx. 3 times as high as F.R.E.'s for the same amount of radioactivity injected, and yet fractional synthesis rates of albumin and fibrinogen were almost the same in both subjects.

Calculations of synthesis rate again included allowances for albumin and fibrinogen which had been catabolized or lost into the extravascular compartment. These were derived from rabbit data in Table 2 in which it was evident that the following proportions of newly synthesized rabbit proteins were lost at 3·5, 4·9 and 6·9 hr., namely of new albumin 12·5, 16 and 19·3%, and of new fibrinogen 7·2, 10·2 and 15·3%. By using these data and allowing for the fact that the rate of albumin catabolism in humans is generally about one-half that in rabbits (that of fibrinogen being the same in both species), values of 10% lost at 3·5–4·3 hr. and 15% at 5·9–6·6 hr. were calculated for both proteins in human plasma. These are necessarily approximate only and must be affected by individual variations of several kinds. Although fibrinogen is catabolized faster than albumin both in humans and rabbits, its rate of loss from the plasma does not increase quite as much with time in the first 6–7 hr., apparently because of the much smaller extravascular pool (20% of total body fibrinogen).

Albumin synthesis rates varied between 8·65 and 14·4%/day, and fibrinogen values between 14·4 and 29·5%/day. Accuracy was lower with fibrinogen because of the much smaller weights of this protein that were available for hydrolysis and counting. The only reliable comparison that can be made is with G.O.R.'s catabolic rate measured with ^{131}I-labelled albumin 2 weeks before the experiment with ^{14}C-labelled carbonate, namely 10·5%/day compared with 13·6% for his synthesis rate. F.R.E.'s catabolic rate was 9·5% but this was measured 6 years earlier.

DISCUSSION

These experiments started from the assumption, based on results of previous work with rats, that the efficiency of incorporation of carbonate carbon into the guanidine carbon of plasma proteins was so low that the clinical use of carbonate for protein studies could not be envisaged. To raise this efficiency arginine was prepared labelled only in the guanidine carbon, and as anticipated the proportion of this carbon which was incorporated into

plasma albumin was much higher (ten times in rabbits and over 30 times in humans).

However, rabbit and human experiments with this amino acid revealed that the guanidine carbon of γ-globulins was always many times more highly labelled than that of albumins at the same time. Clearly the reason for this must be that γ-globulins are made in cells that have no ability to synthesize arginine whereas albumin and fibrinogen are made in liver cells possessing this capacity. New unlabelled arginine produced in hepatic cells can be assumed to dilute the incoming labelled arginine so that arginine of lower specific activity is presented at the intracellular site of protein synthesis. Since arginine synthesis is believed to be restricted to liver cells this dilution effect may provide a decisive test of whether or not a particular protein is made in the liver.

The presence of much more highly labelled γ-globulin in plasma samples complicates the isolation of the other proteins in sufficiently pure form, which in any case is difficult with small samples. Even fibrinogen, which for these purposes may be clotted out, is not immune from this difficulty since γ-globulin is the principal contaminant after preliminary salting-out of this protein. The procedure finally adopted for dealing with this problem was to add an excess of unlabelled γ-globulin to labelled-fibrinogen and labelled-albumin fractions, and to reprecipitate. In this way both proteins were obtained having no detectable ^{14}C radioactivity other than that attached to the protein itself but containing significant amounts of unlabelled γ-globulin.

Because of the presence of highly purified ^{125}I-labelled fibrinogen and ^{131}I-labelled albumin injected before this particular ^{14}C-labelling experiment began, it was possible to estimate the proportions of albumin and fibrinogen in the original plasmas that had been recovered in the final preparations, and so to correlate their ^{14}C radioactivities with total plasma-albumin and fibrinogen radioactivities that were required for synthesis calculations. The iodine-labelled proteins also provided essential information about catabolic rates and rates of transcapillary transfer of these proteins into the extravascular space which was also required to test the proposed method of measurement of synthesis rate.

The procedure for measuring synthesis consists essentially in combining the ratio of total radioactivities appearing in the same interval in two products (protein guanidine carbon and urea carbon) of the same precursor (guanidine carbon of intracellular arginine) with the measured mass of one of them (urea) in order to obtain the mass of the other which is synthesized in the same interval. Thus it is essentially based on total radioactivities, although in one formula used here a device was introduced whereby specific radioactivities of protein guanidine carbon were conveniently used for calculating fractional synthesis rates.

The method appears to be well adapted in principle for use in studying disturbed metabolic states, since it is theoretically independent of changes affecting catabolism. In practice, however, this is not entirely true, since during the interval required for the synthesis measurement a portion of newly labelled protein is catabolized in addition to some that becomes inaccessible for measurement by passing into the extravascular compartment. Total radioactivities lost for practical purposes in both these ways must be measured, and one way of doing this was to obtain values in the first few hours after injecting iodine-labelled proteins. These findings, however, had to be modified to allow for the abruptly falling concentrations of iodine-labelled molecules compared with the slow accumulation of biosynthetically labelled ones in the ^{14}C-labelling experiment.

Inaccuracies in the assessment of losses of newly labelled proteins increase markedly with time so that the shortest permissible interval should be chosen for carrying out synthesis measurements. This cannot be less than the minimum time required for all newly labelled protein to escape from the liver into the plasma, and if effective periods of intracellular synthesis are prolonged by reutilization of the labelled carbon this minimum interval becomes correspondingly difficult to determine. Estimated rates of synthesis of albumin and fibrinogen in Table 2 increased up to 4·9 hr. and were substantially the same at 6·9 hr., suggesting that by 4·9 hr. all the initially labelled proteins had been discharged into the blood stream.

Because of the extrahepatic origins of normal and pathological γ-globulins there can be no direct connexion between their synthesis and that of urea in the liver, and hence the method cannot be used for measuring synthesis rates of these proteins. Agreement between synthesis rates of albumin and fibrinogen by using ^{14}C-labelling (Table 2) and catabolic rates measured with the iodine-labelled proteins was only fair, and further experiments with [6-^{14}C]arginine are necessary.

It was decided to investigate essentially the same synthesis procedure but with single injections of [^{14}C]carbonate. That γ-globulins were now less highly labelled than liver-produced proteins at the same time was presumably due to the fact that [6-^{14}C]arginine originated from [^{14}C]carbonate inside hepatic cells and was only able to reach γ-globulin-producing cells after being diluted with unlabelled arginine present in the body water. The specific activities of γ-globulin were slightly more than half those of albumin (cf. Fig. 5), a result which suggested that problems of contamination

which arose when the amino acid was injected were no longer serious.

Reutilization of the ^{14}C was observed to occur on a much diminished scale, suggesting a further significant advantage to be had with [^{14}C]carbonate. When ^{14}C-labelled arginine or other essential amino acid is injected at least half goes immediately to the tissues, giving rise to highly labelled intracellular proteins that are later broken down and serve to maintain a significant concentration of the labelled amino acid in the blood stream. [^{14}C]Carbonate, on the other hand, is inefficiently converted into [6-^{14}C]arginine which is then preferentially available for synthesis of liver and plasma proteins (except γ-globulins) before passing into the blood stream. Thus guanidine carbons of tissue proteins are less highly labelled (by a factor of at least 100) when [^{14}C]carbonate is given, and reutilization occurs on a negligible scale. This implies that the simultaneous synthesis of ^{14}C-labelled plasma proteins and ^{14}C-labelled urea occurs as a fairly discrete event effectively lasting possibly not more than a few minutes. The labelled urea is believed to be uniformly distributed throughout the body water within 20 min., whereas the labelled proteins require much longer than this to be fully discharged into the plasma. Subsequently, as shown in the rabbit experiment in Fig. 6, disappearance rates were such as to suggest that unequivocal information about catabolism can also be obtained with the ^{14}C label, whereas this was clearly impossible when the ^{14}C-labelled amino acid was injected (cf. Fig. 2).

When [^{14}C]carbonate is used it should theoretically only be necessary to collect and measure the urea radioactivity in a single early urine sample and in the body water at the same time to know the total radioactivity appearing as urea. If no reutilization occurs subsequent urine radioactivities will merely reflect the renal clearance rate of urea. Until more is known about this, however, it is probably advisable to collect and measure urine samples for some days. In the rabbit experiment illustrated in Fig. 6 this was done for 94 hr., and the accumulated urine radioactivy over this period was 13·6 μc, or 10 % more than the estimated total urea radioactivity at 6·8 hr. Similar increases have been observed in humans, and these could be underestimates since possible additional losses of [^{14}C]-urea by bacterial digestion in the gut cannot be excluded. Only in one rabbit experiment (cf. Table 2) was a bacteriostatic agent used, and the general need for this remains a matter for investigation. Since the ratio of mass to radioactivity of new urea appears in the synthesis formula, losses of [^{14}C]urea from this or any other cause at least will not introduce a proportional error in synthesis values.

An additional reason for collecting and measuring urine samples beyond the interval of synthesis measurement concerns the measurement of the mass of newly formed urea. Only a small proportion of the total body urea is excreted in the synthesis interval, and fluctuations in the volume of the body water could bring about significant temporary retention or over-excretion of urea. It will probably prove to be advantageous to substitute for the measured mass of urea excreted in the synthesis interval a value calculated from the mean urea excretion rate averaged over 24 hr. This may also help to deal with the problem of increases that occur in plasma urea concentrations after protein meals, although clearly meals of this kind should be avoided during the synthesis interval.

Errors in estimating urea radioactivity retained in the body also significantly affect the overall accuracy of the procedure described. Though urea radioactivities/ml. of plasma were measured with sufficiently high accuracy, the use made of arbitrary fractions of body weight (0·62 to 0·52 for adult males in the age range 20–60 years) to estimate body water represents a compromise which is barely satisfactory with normal subjects and which would not be acceptable in investigating patients with metabolic disorders. Even when the volume of body water is exactly known an error will arise from the fact that specific activities of urea must be higher in the extravascular space than in the plasma at the same time, owing to excretion taking place from the latter (McFarlane, 1957). This specific-activity difference has been calculated to introduce an error of 4 % into the measurement of body water by conventional procedures with [^{14}C]urea (McFarlane, Garrow & Waterlow, 1962). For present purposes the most exact procedure is to inject [^{14}C]urea before the synthesis experiment, or at a suitable interval afterwards, and after equilibrium is established to measure radioactivities/ml. of plasma directly and radioactivities of total retained urea by differences. As it happened, in the one experiment in which this was done and which supplied the results in Table 2, the result was almost the same as that obtained by assuming that the [^{14}C]urea radioactivity of the plasma was also distributed at the same concentration throughout a fluid volume equivalent to 70 % of the animal's body weight.

An *ad hoc* allowance of 10 % was made for the proportion of new labelled albumin and fibrinogen which was lost in 4 hr., and 15 % for the proportion of both lost in 6 hr. Since these were partly based on measurements made on rabbits and must in any case be subject to individual variations, they are approximate only. Also, whereas on the basis of results obtained with rabbits 4 hr. was regarded as a long enough interval in which to measure

synthesis rates, when later blood samples were taken from two subjects the specific activity of the guanidine carbon of fibrinogen in one was higher at 6 hr. than at 4 hr. In general new fibrinogen arrives more slowly in the plasma than does albumin, and this result suggests that it may be necessary to allow 6 hr. in humans for all the labelled fibrinogen to leave the liver.

When these several sources of error are taken into consideration, it seems that the degree of agreement obtained here between synthetic and catabolic rates in the few cases in which comparison was possible was as satisfactory as could be expected, and was particularly so in the rabbit experiment with [^{14}C]carbonate. So far as the five normal subjects who also received [^{14}C]carbonate are concerned, it can only be said that the synthesis method gave results that fall within the range of normal values for catabolic rates of albumin in humans to be found in the literature. Though the fact that parameters of urea synthesis varied so widely amongst the five is of some interest in itself, it is of special significance for present purposes that the capacity to incorporate [^{14}C]carbonate into guanidine carbons of albumin and fibrinogen varied also with the capacity to synthesize urea. Thus, whereas the synthesis rate of albumin and the rate of excretion of urea were sensibly the same in F.R.E. and B.R.I. (Table 3), total radioactivity incorporated into urea and into guanidine carbons of albumin were both higher in F.R.E. by the same factor. This factor was approx. 3, when allowance was made for the different amounts of [^{14}C]-carbonate injected. Also, whereas B.A.T. and B.R.E. had similar synthetic rates for albumin and incorporated similar amounts of radioactivity into the protein, B.A.T. was excreting urea at twice the rate of B.R.E. and consequently only half as much radioactivity was incorporated into his urea. These proportionalities can hardly be accidental and appear to confirm the existence of a close association between the synthesis of urea and of the guanidine carbon of arginine in liver-produced plasma proteins. To determine whether this association implies precisely the relationship given by the synthesis formula used here will require further and more exact measurements with both ^{14}C and ^{131}I labels.

An additional important matter to be considered concerns the radiation hazard associated with the injection of [^{14}C]arginine or [^{14}C]carbonate into humans. In several investigations with a variety of ^{14}C-labelled amino acids, including totally labelled arginine, in animals and humans, it was our experience that not more than 1% of the injected radioactivity was incorporated into total γ-globulins, provided that these were not present in abnormally high concentrations, as they are, for instance, in some myeloma patients (cf. McFarlane, 1961). This general conclusion can also be deduced from results of human experiments with [2-^{14}C]-glycine by Berlin, Tolbert & Lawrence (1951), and of dog experiments with [ϵ-^{14}C]lysine by Yuile, O'Dea, Lucas & Whipple (1952). Therefore, when [6-^{14}C]arginine is released from the liver after the injection of [^{14}C]carbonate, it may be assumed that the maximum radioactivity that will be incorporated into guanidine carbons of carcass proteins is unlikely to exceed 100 times that incorporated into γ-globulins.

B.R.I. in Table 3 may be taken as a least favourable subject from the radiation-hazard point of view, since he labelled his albumin with highest efficiency. Of the injected radioactivity 0·02% was incorporated into albumin and a normal proportion (one-sixth or 0·0035%) into γ-globulin. Therefore, up to 0·35% could have been fixed in carcass proteins at the same time. This is not all the protein radioactivity since with [^{14}C]carbonate considerable labelling occurs in carboxyl groups. Greenberg & Winnick (1949) exposed rats continuously for 48 hr. to $^{14}CO_2$ and showed that 28% of the radioactivity found in total liver proteins at the end of this period was present in guanidine carbon of arginine. The remainder was mostly in carboxyl groups of glutamic acid, aspartic acid and alanine. The proportion in arginine could be higher after a single injection of [^{14}C]carbonate and it may be expected to vary among organs. By using 30% as a mean overall value in humans at 24 hr. a maximum of approx. 1% of the injected radioactivity is suggested as being present in carcass proteins at this time, a value which Greenberg & Winnick (1949) found in rats. No evidence has been presented for any comparable amounts of radioactivity persisting in non-protein forms. The larger amounts that rapidly enter the tricarboxylic acid cycle become associated with fats, carbohydrates and other substances which are generally more rapidly turned over than proteins. Gould, Sinex, Rosenberg, Soloman & Hastings (1949) gave single injections of sodium hydrogen [^{14}C]carbonate to rats and found that 86% of the radioactivity was expired and excreted in 1 hr., 93% in 2 hr., and 95% in 4 hr.

It would be more satisfactory if it were possible to confirm this estimated maximum retention value of 1% at 24 hr. in humans by direct measurements. Although equipment for monitoring expired air has been greatly improved it is still extremely difficult to decide to within better than 1–2% how much of a dose of 100 μc of [^{14}C]-carbonate has been expired. It must be remembered that up to 2% may be excreted as urea carbon (cf. B.R.I. in Table 3). When Brues & Buchanan (1948), who used rats in a flushed-chamber type of

apparatus, found several per cent retention after 8 hr., subsequent carcass analysis in some cases indicated only 0·5 %. Instruments for human use which depend on carbon dioxide absorbers, and on masks that are worn intermittently during the period of expiry, are subject to several inaccuracies, and counters for continuously recording radioactivities in expired air are not sensitive enough (Tolbert, Kirk & Baker, 1956; Leroy, Okita, Tocus & Charleston, 1960).

The minimum dose of [^{14}C]carbonate which will suffice for a human measurement is mainly determined by the concentration of fibrinogen in the plasma. A normal adult has approx. 0·5 mg. of guanidine carbon in the fibrinogen contained in 25 ml. of his plasma. After an injection of 100 μc of [^{14}C]carbonate this carbon will contain 15–30 $\mu\mu$c (cf. Table 3) and will give a count rate approximately equal to background in the Tri-Carb Scintillator. The larger mass of guanidine carbon obtainable from albumin in the same sample more than compensates for its lower specific radioactivity. By assuming that a 25 ml. plasma sample will be available and that more than 60 % of the guanidine carbon of fibrinogen will be recovered, 100 μc may be taken as the minimum dose of [^{14}C]carbonate to use for an approximate synthesis measurement.

Approx. 2·2 mc of ^{14}C distributed evenly throughout the tissues of a 70 kg. man and assumed to be permanently retained will deliver 0·1 rem/day (Morgan, 1947). A single injection of 100 μc of [^{14}C]carbonate giving rise to 2 μc which is assumed to be permanently retained after 24 hr. should therefore deliver 0·1 millirems/day or slightly less than that due to cosmic radiation. The relative hazard associated with the use of [^{14}C]carbonate or [6-^{14}C]arginine in adult humans can be assessed as follows. Results already given show that for the same specific radioactivity in guanidine carbons of albumin and fibrinogen it is necessary to use 20 times more radioactivity in the form of [^{14}C]-carbonate. When the ^{14}C-labelled amino acid is given, however, a 40–80-times greater proportion of its radioactivity (up to 80 % compared with 1–2 %) is incorporated into carcass proteins, so that the hazard with [^{14}C]carbonate is substantially less than that with the equivalent radioactivity in the form of the ^{14}C-labelled amino acid.

SUMMARY

1. [6-^{14}C]Arginine was injected into rabbits and humans and guanidine carbon of plasma proteins was liberated enzymically as carbon dioxide. Specific activities of this carbon in γ-globulins were many times higher than in liver-produced proteins at the same time, and marked reutilization of the injected ^{14}C occurred.

2. By 'washing' ^{14}C-labelled albumins and ^{14}C-labelled fibrinogens with unlabelled γ-globulins the accuracy of the measured specific activities of guanidine carbon was improved. These were used in conjunction with the radioactivities of total [^{14}C]urea to calculate synthesis rates of albumin and fibrinogen in the rabbit. Values were somewhat lower than catabolic rates measured over a longer interval with ^{131}I-labelled albumin and ^{125}I-labelled fibrinogen. No significant advantage was apparent in making the synthesis measurement over an interval longer than 4 hr. with albumin and 6 hr. with fibrinogen.

3. When [^{14}C]carbonate was given as a single injection specific activities of the guanidine carbon of γ-globulins were about one-half of those of liver-produced proteins, and reutilization was negligible. In adult humans a maximum of 0·02 % of the injected radioactivity was incorporated into the guanidine carbons of plasma proteins.

4. Fractional synthesis rates were calculated by using a formula which is independent of the arginine content of plasma proteins and of their concentrations in the plasma. Close agreement was obtained between the catabolic rate (in experiments with ^{131}I-labelling) and the synthesis rate (in experiments with ^{14}C-labelling) of albumin in the rabbit, and evidence was obtained favouring a close association of urea synthesis and synthesis of guanidine carbons of albumin and fibrinogen in normal humans.

5. A procedure is described for the use of 100–200 μc of [^{14}C]carbonate for measuring fractional synthesis rates of albumin and fibrinogen in humans, and results of its use are illustrated. Although the dose required for the same degree of labelling of protein is much higher than with [6-^{14}C]arginine, the radiation hazard is less with [^{14}C]carbonate, which also has other advantages.

I am greatly indebted to several colleagues for special services, particularly to Dr J. Walker for guidance during the synthesis of [6-^{14}C]arginine, and to Dr T. Freeman, Dr A. Bruce-Robertson and Dr L. Irons. The skilled assistance of Miss G. Henley and Mr G. Dickinson is gratefully acknowledged.

REFERENCES

Berlin, N. I., Tolbert, B. M. & Lawrence, J. H. (1951). *J. clin. Invest.* **30**, 73.

Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.

Brues, A. M. & Buchanan, D. L. (1948). *Cold Spr. Harb. Symp. quant. Biol.* **13**, 52.

Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. & McFarlane, A. S. (1956). *Int. J. appl. Radiat. Isotopes*, **1**, 66.

Cohen, S., Freeman, T. & McFarlane, A. S. (1961). *Clin. Sci.* **20**, 161.

Delaville, M., Delaville, G. & Delaville, J. (1954). *Ann. pharm. franç.* **12**, 109.
Delluva, A. M. & Wilson, D. W. (1946). *J. biol. Chem.* **166**, 739.
Gould, R. G., Sinex, F. M., Rosenberg, I. N., Soloman, A. K. & Hastings, A. B. (1949). *J. biol. Chem.* **177**, 295.
Greenberg, D. M. & Winnick, T. (1949). *Arch. Biochem.* **21**, 166.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Koch, A. L. (1962). *J. theoret. Biol.* **3**, 283.
LeRoy, G. V., Okita, G. T., Tocus, E. C. & Charleston, D. (1960). *Int. J. appl. Radiat. Isotopes*, **7**, 273.
Levenson, S. M., Crowley, L. V., Horowitz, R. E. & Malm, O. J. (1959). *J. biol. Chem.* **234**, 2061.
Levy, H. B. & Sober, H. A. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 250.
McFarlane, A. S. (1957). *Progr. Biophys.* **7**, 116.
McFarlane, A. S. (1958). *Biochem. J.* **182**, 53.
McFarlane, A. S. (1961). *Proc. 4th. int. Congr. clin. Chem.* p. 1. Edinburgh and London: E. and S. Livingstone Ltd.
McFarlane, A. S. (1963*a*). *Biochem. J.* **87**, 22P.
McFarlane, A. S. (1963*b*). *J. clin. Invest.* **42**, 35.
McFarlane, A. S. (1963*c*). In *Metabolism of Plasma Proteins: Mammalian Protein Metabolism.* Ed. by Munro, H. N. & Allison, J. B. New York: Academic Press Inc. (in the Press).
McFarlane, A. S., Garrow, J. S. & Waterlow, J. C. (1962). *Radioisotopes in Tropical Medicine.* p. 17. Vienna: International Atomic Energy Agency.
McFarlane, A. S. & Murray, K. (1963). *Analyt. Biochem.* (in the Press).
Morgan, K. Z. (1947). *J. phys. Colloid Chem.* **51**, 984.
Reeve, E. B., Pearson, J. R. & Martz, D. C. (1963). *Science*, **139**, 914.
Swick, R. W. (1958). *J. biol. Chem.* **231**, 751.
Tolbert, B. M., Kirk, M. & Baker, E. M. (1956). *Amer. J. Physiol.* **185**, 269.
Turba, F. & Leismann, A. (1953). *Angew. Chem.* **65**, 535.
Walser, M. & Bodenlos, L. J. (1959). *J. clin. Invest.* **38**, 1617.
Weissman, S., Tschudy, D. P., Bacchus, H. & Eubanks, M. (1961). *J. Lab. clin. Med.* **57**, 136.
Woeller, F. H. (1961). *Analyt. Biochem.* **2**, 508.
Yuile, C. L., O'Dea, A. E., Lucas, F. V. & Whipple, G. H. (1952). *J. exp. Med.* **96**, 247.

Biochem. J. (1963) **89**, 290

The Effect of Penicillin, Novobiocin, Streptomycin and Vancomycin on Membrane Synthesis by Protoplasts of *Bacillus megaterium*

By M. D. YUDKIN

Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, Tennis Court Road, Cambridge, and Department of Bacteriology and Immunology, Harvard Medical School, Boston 15, *Mass., U.S.A.**

(*Received* 29 *March* 1963)

There are few antibiotics whose mechanism of action is thoroughly understood. For some, it has been suggested that the lethal action is by interference with the synthesis or the function of the cell membrane; but in general the evidence is insufficient to decide whether the membrane is the primary point of attack of the antibiotic.

Penicillin is thought to act by inhibiting the synthesis of the cell wall in susceptible organisms, and the breakdown of the osmotic barrier which has been observed in penicillin-treated bacteria is generally supposed to be a consequence of this inhibition (for review see Salton, 1960). However, Prestidge & Pardee (1957) reported that inhibitors of protein synthesis largely prevented the leakage of cell contents from organisms exposed to penicillin, and concluded that penicillin caused the formation of an enzyme that attacked the cell membrane. Novobiocin was shown by Brock & Brock (1959) to produce somewhat similar effects in *Escherichia coli*: growing cells treated with the antibiotic lost protein and ribonucleic acid to the medium and suffered an increase in permeability, whereas non-growing organisms were unaffected. It was concluded that novobiocin exerts its bactericidal action by preventing the synthesis of new membrane in growing bacteria. Streptomycin was shown to cause the leakage of nucleotides and amino acids from growing *E. coli* without altering the intracellular concentration of these compounds, and this leakage was attributed to the formation of defective membrane (Anand & Davis, 1960); the hypothesis was supported by the discovery that growth in the presence of streptomycin accelerates the efflux of K^+ ions from the same organism (Dubin & Davis, 1961). Reynolds (1962) has compared the effect of penicillin and vancomycin on *Bacillus megaterium* and *Staphylococcus aureus*. High concentrations of sucrose or salts prevented the inhibition by penicillin of the accumulation of

* Present address.