Albumin Synthesis by the Perfused Rat Liver

A COMPARISON OF METHODS WITH SPECIAL REFERENCE TO THE EFFECT OF DIETARY PROTEIN DEPRIVATION

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1. Isolated perfused rat livers were used to study synthesis of albumin after donors had been fed on normal or protein-free diets. 2. Methods of determining the liver's ability to produce albumin included incorporation of [^{14}C]carbonate, [^3H]lysine and [^{14}C]arginine, as well as a direct method based on a heterologous perfusing system of rat erythrocytes and rabbit plasma. 3. Livers from protein-deprived rats were found to form extremely little urea and not to incorporate $^{14}\text{CO}_2$ into [^{14}C]urea, but they were capable of producing [^{14}C]urea from [^{14}C]arginine and of incorporating the latter and [^3H]lysine into albumin. 4. By immunological means these livers were found to synthesize less albumin than normal, but their ability was only slightly impaired when related to body weight or liver weight. 5. These findings are consistent with a block in urea-cycle enzymes with relative integrity of arginase activity and of amino acid activation.

Experiments *in vivo* have demonstrated that the hypoalbuminaemia which is characteristic of protein malnutrition results primarily from a decreased rate of synthesis of this protein (Kirsch, Frith, Black & Hoffenberg, 1968; James & Hay, 1968). The relative importance of the factors responsible for this decrease is not easily determined *in vivo* because of the complex metabolic changes that take place during such malnutrition. Experimental use of the isolated perfused liver system appears to offer an opportunity to study these factors independently, provided that a reliable method for determination of the rate of synthesis of albumin is available.

In the present work with the perfused rat liver we have compared different methods for measurement of the rate of synthesis of albumin. Unexpectedly, a [^{14}C]carbonate method based on the principles proposed by McFarlane (1963) and Reeve, Pearson & Martz (1963), which has been used extensively *in vivo*, was found to be inapplicable when livers taken from protein-deprived animals were perfused.

MATERIALS AND METHODS

Liver perfusion. This was carried out with the technique and apparatus described by Cohen & Gordon (1958) and Gordon (1962). Liver and blood donors were rats of the

hooded strain maintained at the National Institute for Medical Research on a normal diet, or in some experiments on a non-protein diet for 16–23 days (for details of diet see Hoffenberg, Gordon, Black & Louis, 1970). Neither group of rats was starved before perfusion, which was continued for a minimum of 4 h.

In some cases perfusion was carried out with a heterologous system consisting of rat erythrocytes washed twice with saline before suspension in the appropriate volume of rabbit plasma (New Zealand White, National Institute for Medical Research stock, maintained on a normal diet). Animals were always bled on the morning of the perfusion.

Rate of albumin synthesis. This was measured by two methods. The first was based on the [^{14}C]carbonate method referred to above. Sodium [^{14}C]carbonate (The Radiochemical Centre, Amersham, Bucks., U.K.; specific radioactivity 20–50 mCi/mmol) was fed into the perfusion system at a constant rate (about 30 $\mu\text{Ci/h}$) and blood samples were taken at frequent intervals for determination of albumin and urea concentration, [^{14}C]urea radioactivity and haematocrit. At the end of the perfusion period a large sample was withdrawn for assay in duplicate of the C-6 specific radioactivity of the albumin arginine. Synthesis of albumin was derived from the equation formulated by Reeve & McKinley (1970):

$$A_s(O, T) = a_s(O, T) [U_s(O, T)/u_s(O, T)]$$

where $A_s(O, T)$ = mg of C-6 of albumin arginine formed during time O, T ; $a_s(O, T)$ = d.p.m. of C-6 albumin [^{14}C]arginine formed during O, T ; $U_s(O, T)$ = mg of urea C formed during O, T ; $u_s(O, T)$ = d.p.m. of urea ^{14}C formed during O, T .

Methods of analysis have been described in full in several publications (McFarlane, 1963; Koj & McFarlane, 1968; Tavill, Craigie & Rosenoer, 1968; Regoezi, Irons,

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Koj & McFarlane, 1965) and no major modifications were introduced in this study. Urea-synthesis rate was obtained from serial assay of urea concentration (by the method of Fawcett & Scott, 1960) and the known perfusate pool volume. All values were adjusted to allow for samples removed and fluid infused during the perfusion.

The second method of measuring albumin synthesis was based on a direct determination of albumin produced by the rat liver during perfusion with heterologous (rabbit) plasma. By preliminary wash-out of the liver with 20–25 ml of the rat erythrocyte–rabbit plasma mixture, the total amount of rat albumin in the system at the start of perfusion could be decreased to less than 20mg, thus facilitating measurement of the small quantities subsequently synthesized. The rate of synthesis was derived from serial determination of rat albumin concentration by a radial immunodiffusion technique (see below) and the known volume of plasma in the system at the time of sampling; corrections were applied for plasma removed during the perfusion.

Albumin synthesis has been expressed as mg/h for each perfusion and also as per mg/h per 100g rat. Because the livers could not be weighed before perfusion, which was started with the organ *in situ*, measurements were made in a group of 24 comparable normal rats and 15 rats fed on a non-protein diet for 18 days. These liver weights (4.1 and 4.6% of total body weight respectively) were used to derive the 'assumed' values of Table 1.

Amino acid incorporation into albumin. In these studies [³H]lysine (L-[4,5-³H]lysine monohydrochloride, 510 μ Ci/mmol) or [¹⁴C]arginine (L-[guanidino-¹⁴C]arginine monohydrochloride, 15–25 μ Ci/mmol), obtained from The Radiochemical Centre, was infused into the system at a constant rate (about 8 μ Ci/h for lysine, 2 μ Ci/h for arginine). Serial plasma samples were treated with 10% trichloroacetic acid and albumin was extracted from the precipitate with acetone–water (4:1, v/v) (Schwert, 1957). Extracts were freeze-dried and weighed portions (1–2mg) were assayed for radioactivity.

Hepatic intracellular lysine specific radioactivity. In these experiments [³H]lysine was infused as described above. After 30 and 60 min in separate experiments the

perfusion was stopped and the livers were immediately perfused with at least 10ml of saline before homogenization by hand in 5 vol. of 1% picric acid. After centrifugation the supernatant was passed through Dowex 1 (X2; Cl⁻ form) to remove picric acid, the amino acids being eluted with 0.02M-HCl (Stein & Moore, 1954). The solution thus obtained was evaporated to dryness twice after further addition of water. The amino acids were dissolved in 1.0ml of water and lysine was separated by passage through a Zeo-Karb 225 (H⁺ form) column as described by Koj (1968), after which its concentration in the eluate was determined with ninhydrin. Finally a portion was taken up in Bio-Solv BBS-3 (Beckman Instrument Inc.), to which scintillation fluid [toluene–1,4-bis-(5-phenyloxazol-2-yl)benzene–2,5-diphenyloxazole] was added to assay radioactivity.

Determination of total albumin concentration. This was carried out in duplicate by the acid–alcohol method of Fernandez, Sobel & Goldenberg (1966). To allow calculation of albumin pool size, the haematocrit was measured at the beginning and end of each experiment.

Determination of rat albumin concentration by immunodiffusion. Antisera to rat albumin, prepared in rabbits or goats, were kindly supplied by Dr D. A. Darcy and Dr R. Lane. Specificity of the antisera was checked by immunoelectrophoresis against whole rat serum.

After incorporation into agar-gel plates these antisera were used for measurement of rat albumin concentration by the radial immunodiffusion method of Mancini, Carbonara & Heremans (1965). A four-point standard curve was prepared for each set of assays and all measurements were made in duplicate.

Measurement of radioactivity. Methods for the isolation of the carbon of urea and of the guanidine residue of albumin arginine have previously been described (McFarlane, 1963). After volumetric assay the CO₂ was trapped in phenylethylamine under liquid N₂, mixed with toluene–1,4-bis-(5-phenyloxazol-2-yl)benzene–2,5-diphenyloxazole scintillation fluid and the radioactivity was counted in a Beckman liquid scintillation counter LS 200 B for 10–20 min. Count rates varied between 3 $\times$ 10³ and 8 $\times$ 10³ c.p.m.

Table 1. *Rates of albumin synthesis in perfusions of rat liver*

Livers were from rats fed on a normal diet (NL) or on a non-protein diet (OPL) with normal blood (NB), rat or rabbit plasma being used as indicated. ¹⁴CO₂ method, carbonate method; Imm. diff. method, immunodiffusion method with heterologous plasma system. Liver weights were assumed as described in the Materials and Methods section. The number of perfusions in each group is indicated in parenthesis.

	Mean wt. of rat (g)	Mean wt. of liver (assumed) (g)	Rates of albumin synthesis (means with ranges in parentheses)			
			¹⁴ CO ₂ method		Imm. diff. method	
			(mg/h per 100g rat)		(mg/h per 100g rat)	
			(mg/h)	(mg/h per 100g rat)	(mg/h)	(mg/h per 100g rat)
NL+NB (rat) (11 perfusions)	320	13.1	5.4 (2.5–7.6)	1.7 (0.9–2.5)	—	—
NL+NB (rabbit) (6 perfusions)	350	14.3	5.0 (2.7–8.3)	1.4 (0.7–2.8)	4.0 (2.6–4.6)	1.1 (0.8–1.3)
OPL+NB (5 perfusions)	157	7.2	—	—	1.4 (0.9–1.7)	0.9 (0.6–1.1)

Portions of freeze-dried albumin fractions for determination of ^3H or ^{14}C after [^3H]lysine or [^{14}C]arginine infusion were dissolved in 0.1M-NaOH and Aerosil-1,4-bis-(5-phenyloxazol-2-yl)benzene-2,5-diphenyloxazole scintillator was added before assay (Sargent & Campbell, 1965). Radioactivity of intracellular lysine was assayed as described above. Under these conditions counting efficiency was approx. 32% for ^{14}C and 25% for ^3H , based on the use of standards of these amino acids made up in precisely the same way.

Design of experiments. Rates of albumin synthesis were measured in three different ways: incorporation of [^{14}C]carbonate and of [^3H]lysine and by means of the radial immunodiffusion method of Mancini *et al.* (1965). With these methods comparisons were made of production of albumin by livers taken from normal and protein-deprived rats. Further comparative studies were made of the appearance of [^{14}C]urea after addition of [^{14}C]arginine to the perfusion system, and of hepatic intracellular lysine specific radioactivity after addition of [^3H]lysine.

RESULTS

In 11 separate experiments with normal livers perfused with blood taken from rats fed on a normal diet, the mean rate of albumin synthesis by the [^{14}C]carbonate method was 5.4mg/h or 1.7mg/h per 100g rat (mean rat wt. 320g; mean liver weight was assumed to be 4.1% of the total body weight, or 13.1g, see the Materials and Methods section; Table 1).

Table 1 also shows the results of six perfusions with the heterologous plasma system. By the [^{14}C]carbonate method albumin synthesis by livers taken from rats fed on a normal diet was slightly lower than was found with control homologous plasma perfusions, but this difference was not

significant. Thus the presence of rabbit plasma did not appear to impair the ability of rat liver to synthesize albumin. Direct quantitative measurement by the immunodiffusion technique gave lower values, which again were not significantly different from controls ($P > 0.1$).

Difficulty was experienced with the use of [^{14}C]carbonate in the perfusion of livers taken from protein-deprived rats. Total urea synthesis in this situation was so low that the method proved inapplicable. Whereas normal rat livers under perfusion conditions produced 50–150 μmol of urea/h, only one of nine of these livers produced more than 15 μmol over a 4–5h perfusion period, and in the remainder a linear rate of synthesis could not be obtained. Fig. 1 shows the slope of urea carbon specific radioactivity after infusion of $^{14}\text{CO}_2$ into perfusion systems with normal livers. Livers from protein-deprived animals were able to incorporate extremely little ^{14}C into urea or albumin arginine, and no comparable slopes could be obtained. Yet Fig. 2 shows that these livers were capable of

Fig. 1. Incorporation of $^{14}\text{CO}_2$ into urea by normal rat livers after infusion of [^{14}C]carbonate (20–50 mCi/mmol). Total urea production by these livers ranged from 50 to 150 μmol /h. Livers taken from rats fed on non-protein diets produced no measurable urea and failed to form [^{14}C]urea after [^{14}C]carbonate infusion.

Fig. 2. Formation of [^{14}C]urea after infusion of [^{14}C]arginine (sp. radioactivity 15–25 μCi /mmol). ●, Liver from rat fed on a normal diet; ○, two livers from rats fed on a non-protein diet. Perfusions were conducted under identical conditions.

Fig. 3. Incorporation of [^3H]lysine into albumin, expressed as d.p.m./mg of albumin. ●, Livers from rats fed on a normal diet (22 perfusions); ○, livers from rats fed on non-protein diet (seven perfusions). Mean values $\pm$ s.e.m. are shown. Specific radioactivity of lysine: 510 $\mu\text{Ci}/\text{mmol}$.

producing [^{14}C]urea after infusion of [^{14}C]arginine, at a rate that was at least as rapid as that found with a liver taken from a rat fed on a normal diet. To examine this anomaly more closely, the immunodiffusion method was applied to these livers and Table 1 includes the results by this technique. Total albumin synthesis was much decreased, ranging from 0.9 to 1.7 (mean 1.4) mg/h in five experiments (significance of difference from control livers, $P < 0.001$). This difference, however, is no longer statistically significant when albumin synthesis is expressed in terms of total body- or liver-weight ($P > 0.1$), for the protein-deprived rats weighed less (mean 157g) and their liver weights were proportionately decreased (assumed to be 4.6% of the total body weight or 7.2g, see the Materials and Methods section).

With [^3H]lysine a different picture was seen. Fig. 3 shows the incorporation of this amino acid into albumin. Although a wide range was found

Table 2. Specific radioactivity of intracellular lysine of perfused liver taken from rats fed on a normal or protein-free diet

Perfusions were carried out under identical conditions for 30 and 60 min in four separate experiments. Details are given in the Materials and Methods section.

Diet	Intracellular lysine (c.p.m./ μg)	
	30 min	60 min
Normal	109	313
Protein-free	106	276

with 22 normal rat livers, significantly greater rates of incorporation occurred in seven livers from protein-deprived animals ($0.02 < P < 0.05$). A check was made on whether or not this apparently greater rate of incorporation of lysine into albumin was due to a higher intracellular lysine specific radioactivity in the livers of protein-deprived rats. Table 2 shows there was no difference between these livers and controls, suggesting that a normal or even enhanced rate of lysine incorporation after protein-deprivation does occur.

DISCUSSION

The decreased rate of synthesis of albumin that occurs in protein malnutrition has in the past been measured by indirect methods based on the behaviour of radioactive iodine-labelled molecules (Picou & Waterlow, 1962; Cohen & Hansen, 1962; Freeman & Gordon, 1964; Hoffenberg, Black & Brock, 1966; James & Hay, 1968). Attempts to measure rates of albumin synthesis by more direct methods have proved to be difficult *in vivo*. Thus interpretation of ^{14}C -labelled amino acid incorporation requires accurate knowledge of precursor pool specific radioactivity. The [^{14}C]carbonate method of McFarlane (1963) and Reeve *et al.* (1963) was designed to overcome this problem by comparing two products of a common precursor pool, but doubts must be expressed about its universal applicability (see below).

With the perfused liver, however, a direct method may be used that cannot be applied *in vivo*. Thus, after perfusion with heterologous blood, precipitation by means of an antiserum provides information about the rate of synthesis of a protein, which does not depend on any assumption or correction factor. John & Miller (1969) have exploited this method most vigorously and report synthesis rates for albumin that are approximately 50% of those obtaining *in vivo*, even after full supplementation of their perfusion system with amino acids, growth hormone, insulin and cortisol.

An earlier study by Gordon & Mutschler (1965)

reported similar low values for albumin production by the perfused liver, based on the use of $^{14}\text{CO}_2$ and on a revised value from a previous publication in which rat livers had been perfused with a human plasma system (Gordon & Humphrey, 1960). The same limitation of synthesis rates has been found in the present study, in which the mean value for livers of control animals (mean weight 320 g) was 5.4 mg/h. This may be compared with 11.7 mg/h per 300 g rat in the study of the intact animal by Kirsch *et al.* (1968).

The possible limitation of albumin synthesis by the isolated perfused liver, compared with that found *in vivo*, might reasonably be attributed to a diminished amino acid supply, for Jefferson & Korner (1969) were able to stimulate protein production under similar conditions by supplying amino acids in concentrations up to ten times those found in normal rat plasma. John & Miller (1969), however, failed to demonstrate normal albumin synthesis despite amino acid supplements of an equally generous degree. The imperfect behaviour of the system *in vitro* does not entirely detract from its usefulness for comparative studies.

Rothschild, Oratz, Mongelli & Schreiber (1968), using a ^{14}C carbonate method, found a 50% fall in albumin production by perfused livers taken from rats starved for 18–24 h before liver extirpation. Gordon (1966) found relatively little decrease (15%) with livers taken from animals fed on a protein-free diet for as long as 7–10 weeks; his studies were based on incorporation of ^{14}C leucine into albumin, and depleted hepatic intracellular leucine pools made interpretation more difficult. In the work reported here normal rates of incorporation of ^3H lysine into albumin were found (Fig. 3). This was verified by direct measurement of hepatic intracellular specific radioactivity of this amino acid. Previous work with protein-deprived animals has demonstrated a constancy of both hepatic and plasma lysine concentrations (Haider & Tarver, 1969; Waterlow & Stephen, 1968; Gan & Jeffay, 1967).

By using a direct method of measurement, total albumin synthesis was found to be decreased in the present study when livers were taken from rats fed on a non-protein diet for 16–23 days. This decrease was not significant when expressed in terms of liver weight or body size. The continued synthesis and release of albumin by these livers at a reasonable rate in relation to size was unexpected in view of the changes in hepatic polyribosome profile, which are known to occur after severe protein restriction (Munro, 1966, 1968; Jefferson & Korner, 1969; Wannemacher, Cooper & Yatvin, 1968; Gaetani, Massotti & Spadoni, 1969). This retention of function may be explained by the resistance to disaggregation of the membrane-bound polyribosomes, which are thought to produce 'export proteins'

including albumin (Wilson & Hoagland, 1967; Wilson, Hill & Hoagland, 1967; Takagi & Ogata, 1968; Redman, 1969; Williams & Ganoza, 1969).

A point of interest to emerge from this study was the low rate of urea synthesis and extremely low rate of incorporation of ^{14}C into urea under the imposed conditions of protein deprivation. Yet when these livers were perfused with ^{14}C arginine, radioactivity rapidly appeared in urea carbon, at a rate at least as high as that found normally (Fig. 2). This finding might partly be explained by the action of arginase (L-arginine ureohydrolase, EC 3.5.3.1) released from haemolysed erythrocytes during perfusion, but this contribution is unlikely to be significant and it is probable that the block to $^{14}\text{CO}_2$ incorporation into urea occurs at an early stage of the urea cycle. Although opinions differ about the particular enzyme that might be rate-limiting in states of protein deprivation, it is generally agreed that the activity of arginase is affected later and to a lesser extent than the four 'earlier' enzymes (Schimke, 1962; Deosthale & Tulpule, 1969). If this is so, our findings might be explained as follows: urea-cycle enzyme activity is decreased (as reflected by insignificant incorporation of carbonate into urea or arginine) with virtual cessation of urea synthesis, but arginase activity is preserved and amino acid activation continues, perhaps at a higher than normal rate.

Our problem of urea-production rates must be reconciled with the experience of Kirsch *et al.* (1968), who were able to measure urea synthesis in intact rats fed on essentially similar non-protein diets. Despite the widespread distribution of arginase in the body, extrahepatic urea production is not likely to explain this difference (Tavill *et al.* 1968). If it existed to a significant degree, the ^{14}C -carbonate method would be invalid since its interpretation depends on homogeneity of the precursor pools for both urea carbon and the arginine C-6 of albumin. It is possible in the intact animal that the extreme muscle wasting of prolonged protein deficiency (Waterlow, 1969) causes the release into the circulation of a stream of amino acids which stimulate the liver to higher rates of urea-cycle activity and urea synthesis than are found with the isolated liver perfusion.

This raises a more fundamental question with regard to the measurement of albumin synthesis. The ^{14}C carbonate method depends on integrity of urea-cycle enzymes for valid interpretation. Our studies indicate that albumin synthesis (measured directly by immunological techniques) can take place under conditions of protein deprivation, when it would not be measurable by a method based on urea-cycle-enzyme function. Although agreement is usually good, care must be taken in those circumstances in which differential inhibition or

induction of enzyme function may occur or where arginine may be supplied by an extrahepatic, non-urea-cycle, source.

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