Gluconeogenesis from threonine in normal and diabetic rats

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(Received 12 March 1984/Accepted 16 August 1984)

1. L-[U-¹⁴C]Threonine was infused at a steady rate to non-anaesthetized rats starved for 1 or 3 days and to diabetic rats starved for 1 day. 2. The rates of turnover of threonine, calculated from the equilibrium specific radioactivity (SA) of plasma threonine, were 5.79 ± 1.00 , 11.67 ± 1.43 and $13.35 \pm 1.85 \mu\text{mol/min per kg body wt.}$ in 1-day-starved, 3-day-starved and diabetic rats respectively. 3. The calculated turnover rate of threonine agreed well with the rate expected from the rate of protein turnover reported in the literature. 4. The equilibrium SA of plasma alanine was 5.1–9.8% of that of threonine in the three groups of rats. 5. The equilibrium SA of glucose was 1.42 and 2.90% of that of threonine in 1-day- and 3-day-starved rats respectively. 6. From the non-equilibrium SA of glucose, it is estimated that a higher percentage of ¹⁴C atoms is transferred from threonine to glucose in diabetic than in non-diabetic rats. 7. In spite of increases in gluconeogenesis from threonine in long-starved or diabetic rats, we conclude that threonine remains a minor contributor to plasma glucose. Since it is an essential amino acid, its turnover and contribution to the formation of plasma glucose is an index of catabolism and gluconeogenesis from tissue protein.

Many precursors of plasma glucose are products of glucose metabolism in extrahepatic tissues. These precursors are released into the circulation, and are carried to the liver to be converted into glucose. In this way glucogenic cycles, such as the Cori and the glucose–alanine cycles, arise. Evidently, such cycles do not contribute to ‘new’ glucose which, in the post-absorptive or starved state, is from the glucogenic amino acids of tissue protein. The true contribution of these amino acids to the formation of glucose has not been quantitatively assessed.

The purpose of the present experiments was to estimate glucose formation from an essential amino acid which cannot be formed from glucose and therefore cannot be part of any metabolic cycle. In the post-absorptive state or during starvation the rate of release of an essential amino acid such as valine or threonine into the circulation is an index of the rate at which extrahepatic protein stores are turned over. Glucose formation from such an amino acid consequently allows the estimation of the extent to which tissue protein contributes to gluconeogenesis. In the experiments reported below, L-threonine was selected as an

essential and glucogenic amino acid for this purpose.

Materials and methods

Animals

Experiments were carried out on non-anaesthetized male Sprague–Dawley rats. At 3–7 days before the experiments, a plastic cannula (Clay–Adams PE-50) was introduced into the carotid artery and the jugular vein as described by Popovic & Popovic (1960). The animals had access to water and food pellets (26% protein, 9% fat, 62% carbohydrate, 3% fibre, on a dry-weight basis) and were housed at 19–21°C with a 12-h light (07:00–19:00 h) 12 h-dark cycle. Food, but not water, was withdrawn either 1 or 3 days before the experiment. Diabetes was produced by the intravenous injection of alloxan (40 mg/kg) dissolved in 0.1 M-acetate buffer, pH 4.4. The rats were used 72 h after the injection, when the concentration of glucose in their plasma ranged between 20.7 and 36.9 $\mu\text{mol/ml}$.

Experimental design

A 10 $\mu\text{Ci/ml}$ solution of L-[U-¹⁴C]threonine was infused into the jugular vein at a rate of

Abbreviation used: SA, specific radioactivity.

0.027 ml/min. Four blood samples were withdrawn from the carotid artery, at 60, 90, 120 and 150 min from the beginning of the infusion in non-diabetic rats and at 90, 120, 150 and 180 min in diabetic animals. Immediately on withdrawal, the blood samples were centrifuged at 15600g for 2 min and the plasma was separated for analysis for glucose, alanine and threonine. In a second series of experiments on similarly cannulated and prepared rats, a primed infusion of D-[3-³H]glucose was given (10 μ Ci priming dose followed by 0.1 μ Ci/min) intravenously. Samples were taken at 60, 90, 120 and 150 min after the injection of the priming dose in all experiments on both diabetic and non-diabetic animals. In these experiments only the concentration and the ³H SA of glucose was determined. The mean weights of the animals are shown in Tables 1 and 3.

Chemical analysis

The concentration of glucose was determined from 0.01 ml of plasma with a Beckman II Glucose Analyzer. The ³H SA of glucose was determined as described by Hetenyi & Mak (1970). The ¹⁴C SA of glucose was determined as described by Chiasson *et al.* (1974).

SA values of threonine and alanine were determined in protein-free plasma. To 250 μ l samples of plasma, norleucine (500 nmol in 250 μ l of 1 mM-HCl) was added as an internal standard. To this mixture 500 μ l of 20% (w/v) sulphosalicylic acid was then added to precipitate protein. After centrifugation for 5 min at 5000g, amino acids were separated from sulphosalicylate and other acidic components by treatment with Dowex 50WX8 (H⁺ form). Bound amino acids were eluted from the resin with excess 2% ammonia. Ammonia was removed by freeze-drying. Asparagine and glutamine, which were not resolved from threonine and serine on the Dowex ion-exchange column used for the amino acid analysis, were removed from the amino acid mixtures by hydrolysis in 6M-HCl for 4 h at 106°C in sealed evacuated tubes. After evaporation of HCl under vacuum, the samples were dissolved in a known volume of 1 mM-HCl and samples were taken for amino acid analysis, scintillation counting and separation of radioactive components by t.l.c. The amounts of individual amino acids were determined with a Varian Vista 54 Liquid Chromatograph modified for amino acid analysis (Klapper, 1982) and the amounts of radioactivity were determined in a Nuclear-Chicago liquid-scintillation counter. The proportions of radioactivity contributed by alanine and threonine were calculated by determining the proportion of radioactivity co-migrating with these amino acids on t.l.c.. This was done with 10 μ l samples on NM300 plates (Analtech, Wil-

mington, DE, U.S.A.) by ascending development in 2-methylpropan-2-ol / butan-2-one / acetone / methanol / water / NH₃ (sp.gr. 0.88) (40:20:20:1:14:5, by vol.). This system provided a wide separation of threonine (*R_F* 0.49) and alanine (*R_F* 0.14). Plates were overrun for 2 h to provide optimal separation. The areas corresponding to [¹⁴C]threonine and [¹⁴C]alanine were located by an Actigraph III scanner (Nuclear-Chicago) and scraped into counting vials. The samples were digested overnight with 0.5 ml of Scintigest (Fisher) and were counted for radioactivity 16–18 h later in 10 ml of Scintiverse (Fisher) in a liquid-scintillation spectrometer (Nuclear-Chicago, Mark II). Counts were converted into d.p.m. by external standardization with [¹⁴C]toluene.

From these determinations the SA values of threonine and alanine in plasma were computed. Their concentrations in plasma were calculated by using the recovery ratio of norleucine, as determined by amino acid analysis.

Calculations

Steady-state turnover rates (*R_T*) of threonine and, in the second series of experiments, of glucose were calculated as the ratio of tracer infusion (*R**, as d.p.m./min) and the equilibrium SA (d.p.m./mg of C). The fraction of alanine arising from circulating threonine was calculated as the ratio of equilibrium SA(alanine) to SA(threonine). An analogous ratio was used to calculate the fraction of glucose arising from threonine; however, the equilibrium SA(glucose) was estimated from the curve of SA(glucose) versus time by an iterative method, assuming that the asymptote is approached as $SA(t) = SA(1 - e^{-kt})$. This calculation was not carried out in the experiments on diabetic rats because the excessive S.D. of the calculated asymptote, owing to the steep rise of the SA(*t*) curve over the entire length of the experiment, led to an uncertain estimate. The mean fraction of glucose arising from threonine multiplied by the mean *R_T*(glucose) as calculated from the second series of experiments on identically treated rats yields the estimated rate of transfer of C atoms from the threonine to the glucose pool. The fraction of threonine turnover converted into glucose was calculated as the ratio of the rate of transfer of C atoms from threonine to glucose divided by *R_T*(threonine).

The extent of gluconeogenesis from threonine was also assessed as the sum of amount of radioactivity (d.p.m.) present in the glucose pool at *t* = 150 min and the estimated amount of radioactivity eliminated therefrom over 150 min. This sum divided by the amount of radioactive threonine (d.p.m.) infused over 150 min (90×10^6 d.p.m.) equals the fraction of radioactivity (d.p.m.) in-

fused as threonine entering the glucose pool. Algebraically:

$$F = \frac{SA(t)M + K \cdot M \cdot \overline{SA} \cdot \Delta t}{R^*(\text{threonine}) \cdot \Delta t} \quad (1)$$

where F = the fraction of d.p.m. incorporated into circulating glucose, M = glucose pool (mg of C) estimated as $0.25 \times (\text{body wt., in g}) \times (\text{plasma glucose concn., in mg of C/ml})$, $K = R_T/M$ calculated from the second series of experiments, $SA(t)$ = the SA of plasma glucose (d.p.m./mg of C) at $t = 150$ min, $\overline{SA}$ = the mean SA of plasma glucose between $t = 0$ and $t = 150$ min, calculated by integration of the SA-versus-time curve, $\Delta t = 150$ min and (t) refers to $t = 150$ min.

Materials

L-[U- ^{14}C]Threonine (SA 226 Ci/mol) was purchased from Amersham International, Amersham, Bucks, U.K., and D-[3- ^3H]glucose (SA 10.8 Ci/mmol) from New England Nuclear Corp., Boston, MA, U.S.A. All other chemicals were obtained from Fisher Scientific, Toronto, Canada, and were of the highest purity available.

Results

The ^{14}C SA values of threonine, alanine and glucose during the infusion of [^{14}C]threonine in rats starved for 1 or 3 days and in diabetic animals starved for 1 day are shown in Fig. 1. A steady SA of threonine and alanine in the plasma was reached or closely approximated in all three groups of animals. This was not the case for glucose.

The plasma concentrations of threonine, alanine and glucose in the three groups of rats are shown in Table 1, together with the mean R_T of threonine calculated from the curve for SA/R^* versus time shown in Fig. 1. The equilibrium SA was calculated as the mean SA observed at and after 120 min. The mean plasma concentration of threonine is significantly lower in diabetic than in either group of non-diabetic rats ($t = 4.31$, $P < 0.01$ for the difference between the 1-day-starved and diabetic rats; $t = 5.13$, $P < 0.01$ between 3-day-starved and diabetic rats). The turnover rate of threonine is significantly higher in the 3-day-starved or diabetic groups compared with 1-day-starved animals ($t = 3.53$, $P < 0.01$, and $t = 3.77$, $P < 0.01$ respectively). The mean plasma concentration of alanine was lower in diabetic than in non-diabetic 1-day-starved rats ($t = 2.76$, $P < 0.05$), but the mean value in 3-day-starved rats did not differ significantly from that for either of the other groups.

The ratios of equilibrium SA-values are shown in Table 2. The estimated extrapolated value was

Fig. 1. SA (d.p.m./ μg of C) of plasma threonine, alanine and glucose normalized with respect to the rate of infusion of threonine (R^* , d.p.m./min)

Ordinate, SA/R^* ; abscissa, time (min). Mean values $\pm$ S.E.M. are shown: $\blacksquare$, in 1-day-starved rats; $\bullet$, in 3-day-starved rats; $\triangle$, in diabetic rats. Calculated asymptotes for the normalized SA of glucose ($\pm$ S.D.): 1-day-starved group, 0.11 ± 0.0007 ; 3-day-starved group, 0.12 ± 0.005 ; diabetic, $0.3 \pm 1.15[(\text{d.p.m.}/\mu\text{g of C})/R^*]$. Because of the large S.D. in the diabetic group, this estimated asymptote was not used in further calculations.

used for the equilibrium SA(glucose) in non-diabetic rats. Whereas the equilibrium ratio of SA(alanine) to SA(threonine) varies little among the three groups of rats, the SA(glucose)/SA(alanine) ratio is higher after 3 days than after 1 day of starvation.

As calculated from the ratios of SA(threonine)/SA(glucose), 1.42% of the C atoms in circulating glucose originated from threonine in 1-day-starved rats, and 2.9% in 3-day-starved rats.

The turnover rate of glucose in similar groups of rats is shown in Table 3. Combining the data in the Tables: (a) in rats starved for 1 day, $21.3 \mu\text{g}$ of glucose C/min per kg originates from the circulating threonine; on the other hand, 7.9% of the C atoms turned over in the threonine arrive in circulating glucose; (b) in rats starved for 3 days, $41.6 \mu\text{g}$ of glucose C/min per kg arises from circulating threonine, whereas 7.4% of the C atoms turned over as threonine arrive at glucose.

Table 1. Plasma concentrations of threonine, alanine and glucose and the rates of turnover of threonine in starved and diabetic rats

Mean values $\pm$ S.E.M. are shown, for the numbers of rats in parentheses. The turnover rate of threonine was calculated from the composite curves shown in Fig. 1. Equilibrium SA was calculated from SA(*t*) at and after 120 min. *Significantly lower than in 1- or 3-day-starved rats ($P < 0.01$); **significantly lower than in 1-day-starved rats ($P < 0.05$); ***significantly higher than in 1-day-starved rats ($P < 0.01$).

	Plasma concn. (μ mol/ml)			Turnover rate of threonine (μ mol/kg per min)	Body wt. (g)
	Threonine	Alanine	Glucose		
1-day-starved (6)	0.599 ± 0.031	0.789 ± 0.036	6.22 ± 0.39	5.79 ± 1.00	471 ± 22
3-day-starved (6)	0.542 ± 0.014	0.723 ± 0.006	6.78 ± 0.28	$11.7 \pm 1.43^{***}$	437 ± 15
Diabetic (5)	$0.445 \pm 0.012^*$	$0.593 \pm 0.065^{**}$	32.78 ± 4.94	$13.4 \pm 1.85^{***}$	359 ± 19

Table 2. Ratios of equilibrium SA of threonine, alanine and glucose in the plasma

The equilibrium (Equ.) SA of glucose was calculated by extrapolation as described in the Materials and methods section (see also the legend to Fig. 1).

	Equ.SA(glucose)/ Equ.SA(threonine)	Equ.SA(glucose)/ Equ.SA(alanine)	Equ.SA(alanine)/ Equ.SA(threonine)
1-day-starved	1.42	14.5	9.8
3-day-starved	2.90	56.9	5.1
Diabetic	—	—	7.8

Table 3. Plasma concentration and rate of turnover of glucose

Mean values $\pm$ S.E.M. are shown, for the numbers of rats in parentheses.

	Plasma concn. (μ mol/ml)	Turnover rate (μ mol/min per kg)	Body wt. (g)
1-day-starved (8)	6.61 ± 0.17	20.84 ± 0.72	516 ± 24
3-day-starved (5)	6.50 ± 0.17	19.95 ± 0.56	466 ± 15
Diabetic (6)	26.39 ± 2.56	47.51 ± 5.06	371 ± 27

Thus the increased transfer of C atoms from threonine to glucose during prolonged starvation parallels the increase in the turnover rate of threonine.

The fraction (*F*) of the radioactivity (d.p.m.) infused as threonine transferred to glucose during the first 150 min of the infusion as calculated by eqn. (1) was 7.6% in the group of rats starved for 1 day, 5.8% in rats starved for 3 days, and 10.8% in diabetic rats.

Discussion

Assuming that the daily turnover rate of protein in the rat is 25–30 g/kg body wt. per day (reviewed by Cahill *et al.*, 1972) and that on average threonine constitutes 4.4% of tissue protein (Ruderman, 1975), 6.4–7.7 μ mol of threonine/min per kg arising from protein is expected to be turned over. This value is in approximate agreement with our findings based on tracer experiments.

When starvation was prolonged to 3 days, threonine turnover was nearly doubled. A similar increase was observed in diabetic rats. Starvation, and even more, diabetes, increase the rate of net

protein breakdown, and this may be reflected by any increased turnover of threonine. Paradoxically, the net hepatic uptake of threonine was reported to be decreased in anaesthetized rats starved for 44 h, as compared with those starved for 20 h (Remesy *et al.*, 1983).

Threonine in mammals is metabolized by three different pathways. (i) The dehydratase pathway, initiated by threonine dehydratase (EC 4.2.1.16) in the rat (Goldstein *et al.*, 1962). The existence of a specific threonine dehydratase has also been claimed (Dale, 1978). This pathway leads through oxobutyrate to succinate and is therefore gluconic. (ii) An aldolase pathway, catalysed by threonine aldolase (EC 4.1.2.5) leads to acetate and glycine. Since in rats glycine is not glycogenic, except in prolonged starvation (Remesy *et al.*, 1983), this pathway is not expected to contribute to glucose formation in the post-absorptive state. (iii) Mitochondrial threonine dehydrogenase (EC 1.1.1.103) converts threonine into L-amino- β -oxobutyrate, which is metabolized via aminoacetone to methylglyoxal plus ammonia and finally to pyruvate (Green & Elliott, 1964). The activities of threonine dehydratase and threonine aldolase are

located entirely in the liver. Bird & Nunn (1983) estimate 87% of threonine dehydrogenase activity to be hepatic in rats. They demonstrated that the relative contributions of the three pathways to threonine catabolism depend on the nutritional state of the animal. Whereas in fed rats threonine dehydratase accounts for 10% and the dehydrogenase for 87% of catabolism, after 3 days of starvation these values change to 78% and 20% respectively. The aldolase pathway contributes little to threonine catabolism, and its relative contribution is not altered by starvation, glucagon or cortisol.

Bird & Nunn (1983) also conclude that the physiological significance of the dehydratase pathway is in providing threonine carbon for gluconeogenesis, as suggested by Mak & Pitot (1981). Threonine dehydratase activity is increased in glucogenic states such as starvation, high-protein diet (Remesy *et al.*, 1983; Bird & Nunn, 1983), also by treatment with cortisol, and to a lesser extent with glucagon (Bird & Nunn, 1983). In the absence of insulin, dexamethasone or glucagon increased the activity of the enzyme in cultured rat hepatocytes (Mak & Pitot, 1981). The activity of the enzyme has not been studied in diabetic rats, in which gluconeogenesis is expected to be increased significantly above normal.

During prolonged starvation the plasma concentration of threonine has been reported to decrease in rats (Remesy *et al.*, 1983). A similar decrease in the concentration of free threonine in the liver has been described in starved rats (Remesy *et al.*, 1983; Bird & Nunn, 1983) as well as in cortisol- or glucagon-treated rats (Bird & Nunn, 1983). In our experiments the prolongation of starvation from 1 to 3 days caused only a marginal decrease in plasma threonine, but the value was significantly lower in diabetic than in non-diabetic rats starved for 1 day. Because of the high R_T of threonine, however, the plasma clearance rate (R_T /plasma concentration) of threonine was higher in both 3-days-starved and diabetic rats than in normal animals starved for 1 day. This seems to indicate that, when the rate of gluconeogenesis is elevated and threonine dehydratase becomes the principal pathway of catabolism, the metabolic clearance of threonine in the liver, and consequently its plasma clearance in the intact rat, increases.

In the non-diabetic rat, threonine is only a minor contributor to gluconeogenesis. This was also observed in sheep (Egan *et al.*, 1983), where only 0.3% of glucose carbon was derived from threonine and 3% of the carbon atoms that turned over in threonine appeared in plasma glucose. Nevertheless, glucose formation from threonine appeared to be a regulated process, since during the infusion of phlorizin a larger fraction of threonine turnover

was converted into glucose (Egan *et al.*, 1983). In our experiments the prolongation of starvation increased the turnover of threonine, but not the fraction of its C atoms converted into plasma glucose. The observed increase in the contribution of threonine carbon to circulating glucose was in parallel with the increase in the turnover rate of threonine and not due to a preferential conversion of threonine into glucose carbon.

In diabetic rats, a larger fraction of ^{14}C atoms was transferred from threonine to glucose than in the comparable group of non-diabetic rats. Whereas the F values (10.8% and 7.6%) in these two groups differ only little, the F value calculated in diabetic rats grossly underestimates the true rate of transfer of ^{14}C atoms from threonine to glucose. This is because, in diabetic rats, at the time of the termination of the experiment the equilibrium SA of plasma glucose has not even been remotely approximated (see Fig. 1). In the 1-day-starved non-diabetic rats, in which the SA of glucose was at the time of the termination of the experiment only about 6% below its estimated equilibrium value, the calculated F value underestimates the extent of the transfer of ^{14}C atoms to a much smaller degree.

As judged by the SA of plasma alanine, this pool became labelled to a much higher degree than did circulating glucose. This observation argues against the possibility of glycolysis in the extra-hepatic tissues being a major source of ^{14}C atoms in plasma alanine, and by inference pyruvate and lactate. Moreover, in the rat neither threonine dehydratase (Bird & Nunn, 1983) nor threonine dehydrogenase is present in muscle (Green & Elliott, 1964). It is likely that the labelling of plasma alanine is the result of the hepatic catabolism of threonine via the dehydratase pathway, which leads to succinate→oxaloacetate→phosphoenolpyruvate→glucose. Pyruvate, and thus alanine and lactate, then become labelled by the pyruvate kinase reaction. The activity of the latter is decreased in gluconeogenic states, which may account for the higher SA(glucose)/SA(alanine) ratio in 3-day-starved rats (Table 2).

Whereas the estimated rate of transfer of ^{14}C atoms from threonine (via succinate) and other metabolites entering the tricarboxylic acid cycle on their way to glucose serves the purpose of comparison adequately, it underestimates the true contribution of threonine C atoms to glucose. The underestimation is due to the metabolic exchange of C atoms in the hepatic oxaloacetate cycle (Krebs *et al.*, 1966) and was calculated to be by a factor $H = 1.35$ –1.55 in the rat (Hetenyi, 1979, 1982; Hetenyi & Ferrarotto, 1983). Should indeed the contribution of threonine to gluconeogenesis be via the provision of succinate, the factor H is expected

to be applicable to the calculation of the true rate of glucose synthesis. By using this factor, in 1-day-starved rats about 2.2% of circulating glucose can be calculated to arise from threonine. This contribution increases to about 3.9% after prolonged starvation.

We are much obliged to Dr. S. Raman for his advice on curve-fitting. The excellent assistance of Mr. Jack Anthony, Mrs. Cathy Ferrarotto, Miss Mary Byers and Mrs. Mary Masika and the efficient secretarial services of Mrs. Jocelyne Parker-Parent and Mrs. Jany Gavey are gratefully acknowledged. The work was supported by generous grants from the Medical Research Council of Canada (MT-1038 and MT-4106).

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