Endogenous loss of leucine and methionine in adult male rats

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1. The fractional rate of loss of ^{14}C and body-weight was measured in adult male rats after giving ^{14}C-labelled methionine or leucine and maintaining rats for 30 d on a low-protein or a specific methionine + cystine-free diet: carcasses were then analysed for protein and fat ^{14}C radioactivity.

2. The fractional loss of $^{14}CO_2$ from [^{14}C]methionine or [^{14}C]leucine between day 20 and day 30 was always greater than the fractional loss of body-weight.

3. Carcass protein ^{14}C radioactivity after giving [^{14}C]leucine was higher than after giving [^{14}C]methionine, but fat ^{14}C radioactivity after either ^{14}C-labelled amino acid was only a small proportion of the total body ^{14}C radioactivity.

4. After correction of the fractional loss of $^{14}CO_2$ for urinary ^{14}C loss, but not body-weight loss, absolute amino acid loss was calculated using published values for methionine and leucine content of rats.

5. The best estimates of endogenous amino acid loss obtained using 1-^{14}C-labelled amino acids, expressed as mg/kg body-weight$^{0.75}$ per day were leucine 79, methionine 38.

Said & Hegsted (1970) have pointed out the difficulty of determining the maintenance requirement of the rat for amino acids which are highly reutilized, such as lysine. In principle the maintenance requirement should represent the amount of endogenous amino acid lost by oxidation. The difficulty in measuring it arises from the fact that the animal can adapt to a low intake by reducing the extent of oxidation. This capacity for adaptation applies not only to the oxidative enzymes in the liver but also to the branched-chain amino acid dehydrogenases in muscle (Reeds, 1974; Sketcher, Fern & James, 1974; Sketcher & James, 1974).

In earlier experiments on young rats (Neale & Waterlow, 1974*b*) an attempt was made to measure the endogenous oxidation of leucine and lysine after radioactive labelling. Two kinds of measurements were made: first, the rate of loss of label from the body was determined by carcass analysis at 20 and 30 d after injection; the second measurement was of the rate of loss of label as $^{14}CO_2$. The two methods gave similar results. For valid conclusions to be drawn the label must be distributed as uniformly as possible throughout all body proteins, because otherwise the specific radioactivity (SR) at the site of oxidation is indeterminate and it is not possible to calculate the rate of amino acid loss from the rate of loss of label. It was found that reasonable uniformity of labelling was achieved after 20 d.

It is difficult to formulate the correct diet for measurement of the maintenance requirement. If the dietary intake of the particular amino acid being studied is too high, the amount oxidized will be greater than the requirement for maintenance. If it is too low, body-weight is lost and maintenance by definition will not be achieved. Our approach to this problem is exactly the same as when the maintenance requirement for total protein is determined by measurement of the endogenous or obligatory nitrogen loss on a protein-free diet in which all other nutrients, including energy, are provided in adequate amounts. On such a diet there is inevitably some loss of body-weight. Nevertheless, provided that the limiting factor is N, it has been generally accepted that the N loss under these conditions is a measure of the maintenance requirement (FAO/WHO, 1965). It is true that the requirement in terms of any protein is apparently greater than the obligatory N loss, but this seems to be because of inefficient utilization of the protein and does not alter the principle. Therefore we consider it legitimate to equate the endogenous loss of an amino acid by oxidation with the requirement of that amino acid for maintenance.

In these experiments with ^{14}C-labelled amino acids we have to consider effects which may result from the location of the label. One of the amino acids used in our earlier work (Neale & Waterlow, 1974*b*) was L-[U-^{14}C]leucine. Leucine is a ketogenic amino acid and therefore it is possible that when it is deaminated, some of the labelled carbon atoms may be incorporated in fat and retained in the body. If so, the rate of loss of ^{14}C as CO_2 would not be a true measure of the rate of oxidation of leucine. On the other hand, if only the carboxyl-C is labelled, the sole labelled compound formed will be $^{14}CO_2$, since the first step in the oxidation of leucine is irreversible decarboxylation (Sketcher *et al.* 1974). Therefore in the present experiments we have compared results obtained with [1-^{14}C]- and [U-^{14}C]leucine. We wished also to determine the endogenous rate of oxidation of methionine. Benevenga & Harper (1970) found in short-term experiments with [^{14}C]methionine that much more $^{14}CO_2$ was produced from [carboxyl-^{14}C]- than from [methyl-^{14}C]methionine. With [methyl-^{14}C]methionine a large proportion of the label was retained in phospholipids and other compounds. It therefore seemed of interest to compare the rate of ^{14}C loss from these two forms of labelled methionine in experiments continued for longer periods.

METHODS

Animals and diets

Male Wistar rats weighing 200–300 g were used, five for each treatment. They were housed in cages with raised wire-mesh bottoms, 2–3 rats/cage. All rats were initially given a low-protein (39 g protein/kg) basal diet (Table 1) for a period of at least 7 d. At 10.00 hours without a prior fast, the rats were given either [1-^{14}C]- or [U-^{14}C] leucine, or [1-^{14}C]- or [methyl-^{14}C]methionine, by stomach tube. CO_2 was collected for the next 2 h to determine the initial loss of ^{14}C activity as $^{14}CO_2$. All the rats were then maintained on the basal diet for the next 17 d, during which CO_2 was collected for 2–4 h at regular daily intervals. At 17 d the rats who had received either form of [^{14}C]leucine were changed to a lower-protein diet (15·6 g protein/kg, Table 1) and

Table 1. *Composition (g/kg) of the basal, low-protein and cystine and methionine-free diets given to rats*

Ingredient	Basal	Low-protein	Cystine and methionine-free
Casein	50	20	—
Arachis oil	50	50	50
Dextrinized starch	300	300	300
Maize starch	538	568	538
Salt mixture*	50	50	50
Vitamin mixture*	11	11	11
DL-methionine	1·0	1·0	—
Amino acid mixture†	—	—	51

* Composition as described by Payne & Stewart (1972).

† Contained L-amino acids in the following amounts (g/kg diet): valine 3·48, arginine HCl 1·91, histidine HCl monohydrate 2·13, isoleucine 2·75, threonine 2·64, serine 2·18, proline 2·18, leucine 4·00, phenylalanine 3·48, tyrosine 1·15, tryptophan 0·94, lysine HCl 5·19, glycine 4·77, glutamic acid 14·3.

those labelled with either form of [^{14}C]methionine were changed to a cystine- and methionine-free diet (Table 1). CO_2 was again collected for 2–4 h/d until day 30, when the animals were killed and carcasses analysed for ^{14}C radioactivity. Labelled amino acids of specific activity 62 and 56·4 mCi/mmol were obtained from the Radiochemical Centre Ltd, Amersham, Bucks., and diluted with water to give a solution containing 5 μCi/ml. No carrier amino acid was added. The volume of solution given was 1·5 ml, containing 7·5 μCi radioactivity.

Analyses

Rats were killed by chloroform inhalation and dried to constant weight in a hot-air oven at 105° for 24–48 h. The dry weight was measured. The carcass was then broken into small pieces and petroleum ether (b.pt 40–60°) added and left overnight. Fat was extracted in a Soxhlet apparatus, with many washings of the carcass residue. The fat was then dried in a vacuum oven and weighed. The remaining fat-free carcass was dissolved overnight in 500 ml 2 M-KOH, boiled to dissolve all resistant tissues and diluted to 1 l with distilled water. Respiratory CO_2 was collected as previously described in 50 ml 2 M-KOH (Neale & Waterlow, 1974*b*). Each day's urine was collected on large sheets of filter paper (Whatman No. 1) which were extracted in KOH and water.

Samples of carcass fat, protein and urine were prepared for counting by combustion of known amounts in a Packard Tri-carb Sample Oxidizer, model 306 (Packard Instrument Company, Downers Grove, Illinois, USA). The resulting sample was counted in a Tracerlab liquid scintillation counter (ICN Pharmaceuticals (UK) Ltd, Surrey). All other chemical and radioactivity estimations were those previously described (Neale & Waterlow, 1974*a*).

Calculations

The fractional rate of loss of ^{14}C radioactivity was calculated as described previously (Neale & Waterlow, 1974*b*), i.e. from the mean daily output of $^{14}CO_2$ collected over

Table 2. *Body-weight changes during days 0–17 and 17–30 after giving either [1-^{14}C]- or [U-^{14}C]leucine or [1-^{14}C] or [methyl-^{14}C]methionine to adult male rats*

Amino acid	Initial body-wt (g)	Change in body-wt (% initial body-wt/d) days 0–17	days 17–30
[1-^{14}C]leucine	223	+0·39	−0·27
[U-^{14}C]leucine	227	+0·25	−0·86
[1-^{14}C]methionine	265	+0·19	−0·62
[methyl-^{14}C]methionine	278	+0·20	−1·02

Table 3. *Percentage initial loss of [1-^{14}C]- and [U-^{14}C]leucine and [1-^{14}C]- and [methyl-^{14}C]methionine as $^{14}CO_2$ and CO_2 specific radioactivity (SR) 2 h after intragastric administration of the labelled amino acid to rats*

(Mean values with their standard errors: no. of rats in parentheses)

Amino acid	^{14}C dose lost as $^{14}CO_2$ (%)	CO_2 SR (disintegrations/min per mmol CO_2/per kg body-wt × 10^3)
[1-^{14}C]leucine	2·96 ± 0·43(5)	69·1 ± 11·2(5)
[U-^{14}C]leucine	2·66 ± 0·26(5)	52·1 ± 5·2(5)
[1-^{14}C]methionine	9·13 ± 1·20(5)	203·9 ± 29·2(5)
[methyl-^{14}C]methionine	2·37 ± 0·17(5)	47·7 ± 6·9(5)

several days between day 20 and day 30, divided by the mean amount of radioactivity remaining in the body protein at the end of 30 d.

The absolute rate of amino acid loss was calculated as the fractional rate of loss × the total amount of amino acid in the body. For leucine the total body content was taken from measurements in our previous study (Neale & Waterlow, 1974*b*); for methionine it was taken from measurements published by FAO (1970).

RESULTS

Body-weight and food intake

The basal diet containing 50 g casein/kg (Table 1) was adequate to support a slow rate of growth in these adult rats, as shown in Table 2. In the two groups of rats which received [^{14}C]leucine the diet was changed on day 17 to one containing 20 g casein/kg. Weight could not be maintained on this diet: the mean rate of loss was 0·56 % of body-weight/d (Table 2). In rats which received [^{14}C]methionine the change to a diet devoid of methionine + cystine produced a slightly greater mean body-weight loss (0·81 %/d).

The change of diet also caused a decrease in food intake, either on the same day or on the next day. In the rats which received [^{14}C]leucine the mean food intake on days 16, 17, 18, 19 and 20 was 73, 62·5, 67, 74 and 67·5 g/kg body-weight/d. In the rats which received methionine the mean intake decreased from 65 g/kg body-weight per d on day 17 to 47, 46, 56 g/kg per d on day 18, 19 and 20 respectively. This greater,

Table 4. *Percentage loss of ^{14}C radioactivity as $^{14}CO_2$ following doses of [1-^{14}C]- and [U-^{14}C]leucine and [1-^{14}C]- and [methyl-^{14}C]methionine prior to and after changing the basal diet to a low-protein or amino acid-free diet at day 17 of the experiment*

(Mean values with their standard errors: no. of rats in parentheses)

	$^{14}CO_2$ loss (% original ^{14}C dose/d)								
Day ...	13	15	17	19	21	23	25	27	29
[1-^{14}C]leucine	0·592	—	0·541	—	0·492	—	0·394	0·454	0·450
SE	0·04(5)		0·05		0·04		0·02	0·02	0·02
[U-^{14}C]leucine	—	0·721	0·640	—	0·608	—	0·539	0·525	0·552
SE		0·03(5)	0·04		0·05		0·05	0·05	0·04
[1-^{14}C]methionine	—	0·487	0·329	0·263	—	0·396	0·396	—	0·367
SE		0·07(5)	0·06	0·01		0·02	0·01		0·04
[methyl-^{14}C]methionine	—	0·454	0·360	0·530	—	0·360	0·444	—	0·376
SE		0·01(5)	0·02	0·02		0·04	0·04		0·03

sustained decrease in intake is consistent with the somewhat greater decrease in body-weight.

Loss of $^{14}CO_2$

Table 3 shows the proportion of the initial dose of ^{14}C lost as $^{14}CO_2$ during the first 2 h after the injection. There was no significant difference in the oxidation of [1-^{14}C]- compared to [U-^{14}C]leucine, and the values are similar to those of Sketcher *et al.* (1974). There was, however, a much larger production of $^{14}CO_2$ from [1-^{14}C]- than from [methyl-^{14}C]methionine, in agreement with the findings of Benevenga & Harper (1970). As was found by them, and by us in our previous study (Neale & Waterlow, 1974*b*), the rate of loss of $^{14}CO_2$ was very much greater in the first few hours after the injection than later on (compare Tables 3 and 4).

The SR of the CO_2 put out in the first 2 h is also shown in Table 3. The later time-course of the SR is shown in Fig. 1. By 4 d in the rats receiving [^{14}C]leucine (both forms) the SR had decreased to less than 1 % of the 2 h value. The SR continued to decrease for the next 10 d, but from day 15 onwards the decline almost ceased. In the rats which received [^{14}C]methionine the pattern of change was similar.

In all groups of rats the rate of loss of $^{14}CO_2$, expressed as a percentage of the original dose, was fairly constant from day 17, when the diet was changed, until the end of the experiment on day 30 (Table 4).

Retention of ^{14}C in the body

Table 5 shows the fat and water content of the rats, together with the amounts of radioactivity recovered in protein and fat at the end of the experiment. For each amino acid, the amount of ^{14}C retained in protein was the same regardless of the position of the label. The retention of ^{14}C from [^{14}C]leucine was greater than that from [^{14}C]methionine. With [1-^{14}C]leucine or [1-^{14}C]methionine negligible amounts of radioactivity were found in body fat: 30 d after the injection of [U-^{14}C]leucine 1·07 % of the initial dose of ^{14}C was recovered in fat. This may be compared with 3·8 % of ^{14}C retained in

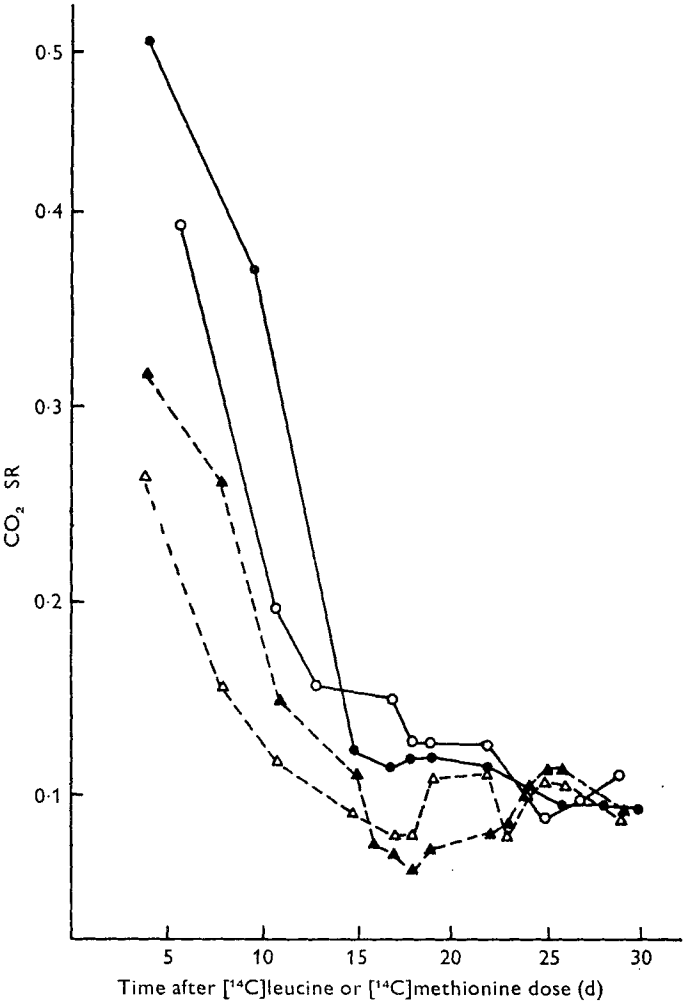

Fig. 1. CO_2 specific radioactivity (SR) (disintegrations/min per mmol CO_2 per kg body-wt × 10^3) after intragastric administration of [1-^{14}C]- or [U-^{14}C]leucine and [1-^{14}C]- or [methyl-^{14}C]methionine and replacement of the basal (50 g casein/kg) diet at day 17 with either a diet of 20 g casein/kg ([^{14}C]leucine group) or a diet with 51 g of a methionine and cystine-free amino acid mixture/kg ([^{14}C]methionine group). ●——●, CO_2 SR with [U-^{14}C]leucine; ○——○, CO_2 SR with [1-^{14}C]leucine; ▲---▲, CO_2 SR with [1-^{14}C]methionine; △---△, CO_2 SR with [methyl-^{14}C]methionine. The animals were killed on day 30.

fat after a 6 h infusion of [U-^{14}C]leucine (Neale, unpublished results). After injection of [methyl-^{14}C]methionine about 2% of the initial dose of ^{14}C was found in fat at the end of the experiment. This represents about 10% of the total amount retained.

Excretion of ^{14}C in urine

Urinary ^{14}C was not measured in the experiments with leucine. Earlier studies (Neale & Waterlow, 1974*b*) suggest that this was a minor route of loss. In those

Table 5. *Body-weight, body composition and ^{14}C radioactivity retained in total body protein and fat of adult male rats 30 d after injection of either* L-[1-^{14}C]- *or* L-[*methyl*-^{14}C]*methionine or* L-[*U*-^{14}C]- *or* L-[1-^{14}C]-*leucine*

(Mean values with their standard errors: no. of rats in parentheses)

	Body-weight (g)		Body composition (g/kg body-weight)				^{14}C retained (% dose given)			
			Water		Fat		Fat		Protein	
Amino acid	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
[1-^{14}C]methionine	234·2	7·8(5)	541	14	110	8	0·1		21·4	1·2
[methyl-^{14}C]methionine	236·9	1·0(5)	515	22	119	14	2·11	0·14	19·2	1·1
[1-^{14}C]leucine	221·8	5·1(5)	637	6·1	95·4	7·5	0		34·4	1·7
[U-^{14}C]leucine	221·3	3·4(5)	608	16	93·9	0·0	1·07	0·12	32·8	1·0

Table 6. *Mean loss of radioactivity as $^{14}CO_2$, fractional rate of loss of ^{14}C radioactivity and fractional body-weight change from 20 to 30 d after giving* [1-^{14}C]- *or* [*U*-^{14}C]*leucine or* [1-^{14}C]- *or* [*methyl*-^{14}C]*methionine to adult male rats*

(Mean values with their standard errors, where given; no. of rats in parentheses)

	$^{14}CO_2$ loss (% original ^{14}C dose/d)		Fractional rate of ^{14}C loss (% dose remaining/d)		Fractional body-wt, change (% mean body wt/d)
Amino acid	Mean	SE	Mean	SE	
[1-^{14}C]leucine	0·447	0·02(5)	1·30	0·06	0·27
[U-^{14}C]leucine	0·558	0·016(5)	1·70	0·06	0·86
[1-^{14}C]methionine	0·394	0·08(6)	1·84	0·04	0·62
[methyl-^{14}C]methionine	0·432	0·024(6)	2·26	0·13	1·02

Table 7. *Corrected fractional rate of loss of ^{14}C radioactivity and absolute loss of leucine and methionine 20–30 d after giving* [1-^{14}C]- *or* [*U*-^{14}C]*leucine or* [1-^{14}C]- *or* [*methyl*-^{14}C]*methionine to adult male rats*

		Amino acid content in whole body (mg/kg body-wt)	Fractional* loss (%/d)	Absolute loss	
Amino acid	Position of label			mg/kg body-wt	mg/kg body-wt$^{0·75}$
Leucine	1-^{14}C	9700	1·45	141	79
	U-^{14}C		1·90	184	104
Methionine	1-^{14}C	3560	1·92	68	38
	methyl-^{14}C		2·71	96	54

* Urinary loss added to loss of $^{14}CO_2$: for [U-^{14}C]leucine, urinary loss taken as 12% of total (Neale & Waterlow, 1974*b*); for [1-^{14}C]leucine, no data–taken to be the same as above; for [1-^{14}]methionine, urinary loss 4% of total present results; and for [methyl-^{14}C] methionine, urinary loss 20% of total present results (see below).

experiments the rate of loss of ^{14}C from the whole body, which will include loss in the urine, was about 12% greater than the loss in expired CO_2 (1·75%/d versus 1·5%/d). In the experiments with [^{14}C]methionine urinary loss of ^{14}C was measured on one day between days 20 and 30. With [1-^{14}C]methionine the urine accounted for 4% of the total daily loss, and with [methyl-^{14}C]methionine it accounted for 20%.

Fractional and absolute rates of amino acid loss

Table 6 shows the mean daily loss of $^{14}CO_2$ between days 20 and 30, expressed as a percentage of the original dose remaining at the end of 30 d. The fractional rate of loss of ^{14}C from methionine was greater than that from leucine. With both amino acids the label in the carboxyl-C gives a somewhat lower rate of loss. No correction has been made for loss of isotope in the urine.

Table 6 also shows the fractional rates of body-weight loss. In Table 7 we have calculated the absolute rates of loss as the product of the fractional rate and the total body amino acid content.

DISCUSSION

The changes in the SR of expired CO_2 (Fig. 1) reflect the changes in the SR of the amino acid at the site of oxidation. The relative constancy found after about 2 weeks agrees with our previous observation, that at this time after a single dose the labelling of tissue proteins has become fairly uniform (Neale & Waterlow, 1974*b*). Aguilar, Harper & Benevenga (1972) reported that after a single injection of a labelled amino acid the SR of expired CO_2 became constant after 24 h. The present results are not in agreement with this: admittedly there is a very large fall in the first few hours, but even after several days the SR is still falling, and decreases by a factor of 4 between 4 and 10 d. Therefore we do not believe that valid conclusions about rates of oxidation can be drawn from short-term experiments with single doses of labelled amino acids.

In interpreting the rates of loss of isotope between days 20 and 30 we have to decide whether or not to take account of the loss of body-weight which followed the change of diet on day 17. This depends on whether the weight loss results from an inadequacy of food, and therefore of energy; or whether the specific amino acid is the limiting factor. In the first instance the weight loss would presumably represent partly loss of lean tissue and partly loss of fat. The loss of lean tissue should be accompanied by a proportional loss of the labelled amino acid. If some fat is lost as well, one would expect the fractional rate of weight loss to be greater than the fractional rate of isotope loss. This did not happen: in all instances the weight loss was less than the isotope loss. Therefore we conclude that the weight loss did not result simply from an energy deficit.

If the limiting factor was the intake of the specific amino acid, we have to explain how the weight loss can be less than the isotope loss. The situation is rather different for the experiments with the two amino acids.

Leucine. The low-protein diet after day 17 provided some leucine, although evidently not an amount adequate for maintenance. In this situation one would expect to find a relation between weight loss and isotope loss such as that depicted diagrammatically in Fig. 2. With intakes of the limiting amino acid between zero and the maintenance requirement M, the rate of isotope loss should be almost constant, reflecting the endogenous oxidation. As intake begins to exceed requirement, the amount of labelled amino acid oxidized will gradually increase. The rate of body-weight loss will be maximal at zero intake of the limiting amino acid; as the intake increases weight loss will become less, and at intakes at and above the maintenance level weight loss will

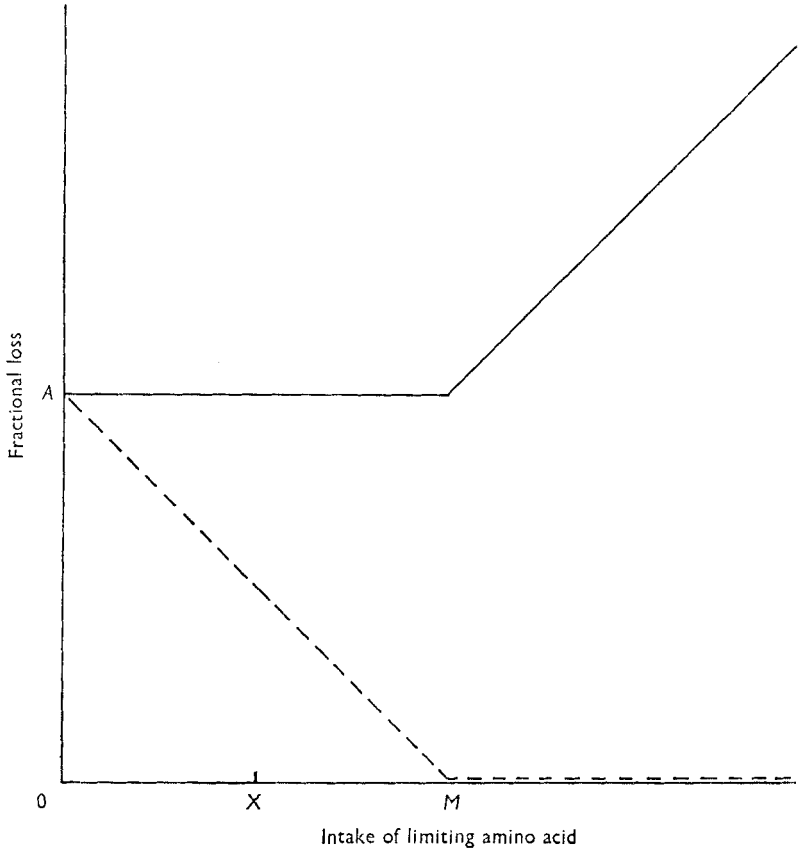

Fig. 2. Relationship between the intake of the limiting amino acid and the fractional loss of weight and $^{14}CO_2$: ——, $^{14}CO_2$ loss; – – –, body-weight loss; *A*, *X*, *M*, see pages 266 and 267.

become zero. Therefore at intermediate points between o and *M*, e.g. at *X*, the rate of weight loss will be less than that of isotope loss.

Methionine. Here the position is different, since the diet was methionine-free. We would therefore expect to find the situation represented by point *A* in Fig. 2, at which fractional rates of weight loss and of isotope loss are the same. This was not in fact found, and we have to explain how isotope loss can be greater than weight loss. Part of the explanation may lie in an observation made some years ago by us in rats (Waterlow, Garrow & Millward, 1969), and by Bressani, Braham, Elias & Balconi (1966) in dogs, that on a low-protein diet the ratio, sulphur: N in the urine is greater than on a high-protein diet. McCance & Widdowson (1960) showed that the ratio S:N in liver is about 1·4 times that in muscle. If, as is probable, the oxidation of methionine occurs mainly in the liver, then the proportion of total body methionine oxidized/d would be greater than the proportion of total body N, and hence body-weight, lost per day. However, this phenomenon only goes half-way towards explaining the excessive rate of isotope loss which also occurs with other amino acids (Neale & Waterlow, 1974*b*).

We conclude, first, that under the conditions of these experiments it is not justifiable to correct the fractional rate of $^{14}CO_2$ loss for the loss in body-weight. Secondly, with both amino acids carboxyl-^{14}C gave the lower rate of loss. This is probably the best estimate of the rate of endogenous amino acid oxidation. It is not unreasonable to suppose that with [U-^{14}C]leucine or [methyl-^{14}C]methionine some of the extra $^{14}CO_2$ is derived from ^{14}C in fat, oxidized in the course of fat turnover.

Our present estimate of the rate of endogenous oxidation of leucine in mature rats, 79 mg/kg body-weight$^{0.75}$ per d (Table 7), is almost identical with the value (80 mg/kg body-weight$^{0.75}$ per d) found previously in young rats (Neale & Waterlow, 1974*b*); it is still much higher than Said & Hegsted's (1970) estimate of the maintenance requirement of 44 mg leucine/kg body-weight$^{0.75}$ per d. On the other hand, our value for rate of endogenous oxidation of methionine (38 mg/kg body-weight$^{0.75}$ per d) is slightly lower than Said & Hegsted's (1970) estimate of 43 mg/kg body-weight$^{0.75}$ per d as the requirement for methionine without cystine.

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