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The metabolism of ^{14}C-labelled essential amino acids given by intragastric or intravenous infusion to rats on normal and protein-free diets

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1. Leucine, lysine, valine and phenylalanine uniformly labelled with ^{14}C were given by intragastric or intravenous infusion for 4 h, after an overnight fast, to rats which had been fed on normal or protein-free diets for 20 d. Expired CO_2 was collected during the infusion. At the end of the infusion the rats were killed and radioactivity was measured in liver, muscle, skin, gastrointestinal tract and the remaining carcass.

2. There were no significant differences between the results obtained with different amino acids.

3. When the two routes of infusion were compared, there was no difference in the output of $^{14}CO_2$, expressed as a proportion of the administered radioactivity. With intragastric infusion a slightly larger proportion of radioactivity was recovered from liver and gastrointestinal tract and significantly less from the skin.

4. When the rats adapted to a protein-free diet were compared with those on the control diet, there was again no difference in the proportion of dose oxidized to CO_2. In the rats on the protein-free diet significantly more of the dose was recovered in the liver and gastrointestinal tract, and less in the skin.

5. The rats on the protein-free diet excreted more CO_2 per kg body-weight, with a lower specific radioactivity.

6. The results show no indication of adaptive changes in the oxidation of essential amino acids, but the evidence is not conclusive. Under these experimental conditions the output of $^{14}CO_2$ is not a valid measure of the extent of amino acid oxidation. The difficulties of interpretation are discussed.

In our earlier work we stressed the importance of economy and recycling of nitrogen in the mechanism of adaptation to low protein intakes (Stephen, 1968; Waterlow, 1968). Later it was suggested (Neale, 1971) that the limiting factor may in fact be the animal's ability to reduce the oxidation of the carbon skeletons of the essential amino acids, particularly the branched-chain amino acids, which are metabolized preferentially by muscle (Miller, 1962).

A number of studies provide suggestive evidence of adaptive changes in amino acid oxidation. Yoshida, Leung, Rogers & Harper (1966) fed [U-^{14}C]threonine to rats on a diet with an amino acid imbalance in which threonine was limiting. Compared with controls, there was a reduction in the output of $^{14}CO_2$ which, however, was not statistically significant. Yamashita & Ashida (1969) gave [U-^{14}C]lysine intraperitoneally to rats on a lysine-free diet and found a significant reduction in $^{14}CO_2$ output. McFarlane & von Holt (1969) found a marked decrease in oxidative degradation of [^{14}C]leucine and [^{14}C]phenylalanine given intraperitoneally to rats on a diet

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containing 20 g casein/kg, compared to rats fed on a commercial high-protein diet (270 g/kg), but no change in $^{14}CO_2$ output from alanine or glutamate. Soliman & Harper (1971) demonstrated the reverse effect: an increase in the production of $^{14}CO_2$ after a test dose of [^{14}C]lysine in rats adapted to a high-protein diet. In our own experiments (Neale, 1971) a single dose of amino acid, uniformly labelled with ^{14}C, was given by the intragastric (IG) route to rats which had been fasted for 16 h. In protein-deficient animals the output of $^{14}CO_2$ was less than in controls when the tracer was a mixture of amino acids (algal hydrolysate) or lysine, but there was no reduction with valine or leucine. Moreover, no decrease in output of $^{14}CO_2$ was found with any of the amino acids if they were given intravenously (IV), when it may be supposed that the peripheral tissues rather than the liver have first access to them. Subsequent experiments on eviscerated rats (Neale, 1972) showed that in animals which had been maintained on a protein-free diet there was no reduction in $^{14}CO_2$ output either from [^{14}C]valine or from mixed amino acids uniformly labelled with ^{14}C. These experiments taken together suggested that the ability to adapt may depend mainly, if not entirely, on mechanisms which operate in the liver.

Although we have referred to the reduction in output of $^{14}CO_2$ as an adaptive change, it must be recognized that this output does not give any direct indication of the amount of amino acid oxidized, because the specific radioactivity (SR) at the site of oxidation is not known: in other words, there is dilution to an unknown extent with CO_2 from other sources. If, however, we accept that the two main routes of disposal of amino acids are by synthesis to protein and by oxidation, neglecting other quantitatively less important metabolic pathways, then the proportion of label appearing as $^{14}CO_2$ should give an indication of the relative flow along the pathway of oxidation, compared with that of synthesis to protein.

All the experiments which have been quoted were made with single injections of labelled amino acids. The disadvantage of this method is that the results may be affected by short-term fluctuations in pool sizes and rates of protein synthesis and breakdown. We believe that continuous infusion of tracer over a period of several hours probably gives a more accurate picture. Waterlow & Stephen (1968) showed that after IV infusion of [^{14}C]lysine for 6 h the relative SR of liver and muscle protein correlated well with the relative rates of protein synthesis in the two tissues, calculated from the SR of the free amino acid precursor at plateau. This relationship, which may seem obvious, does not necessarily hold after single injections because of very rapid changes in the SR of the precursor pool.

The route by which the tracer amino acid enters the pool may also be important. It is not known whether the body handles amino acids entering from the gut via the portal bloodstream in the same way as it handles those entering the systemic circulation from breakdown of body protein. Picou & Taylor-Roberts (1969), in comparisons on two children, found little difference in calculated rates of total nitrogen turnover when ^{15}N-labelled amino acids were given by IG or IV infusion. However, Alpers (1972) has recently suggested that amino acids given into the lumen of the intestine become preferentially incorporated into intestinal mucosal protein, compared with amino acids given by the intraperitoneal route.

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The purpose of this work was to get more information about the extent to which the rat can adapt to protein depletion by reducing the oxidation of the carbon skeletons of essential amino acids. Measurements of recovery of label in different tissues and in the whole body were made both as a check on the recovery of $^{14}CO_2$, and to see if there were differences in the distribution of label in the body. The SR of liver and muscle proteins were determined as a guide to the rates of protein synthesis in these tissues. A second objective was to mimic the portal and systemic presentation of amino acids to the tissues by using two routes of infusion. Thus comparisons were made of the effects of three variables: the nature of the infused amino acid (valine, leucine, lysine or phenylalanine), the route of infusion (IG or IV) and the state of protein nutrition of the animal (normal and protein-free diet).

EXPERIMENTAL

Animals and diets

Male black- and white-hooded rats were obtained from commercial suppliers at 21 d old. One group of animals was immediately given the stock (HP) diet (Oxoid, pasteurized breeding diet, protein content 205 g/kg; Oxoid Ltd, London SE1). The other group was given a protein-free (PF) diet as described by Flores, Sierralta & Monckeberg (1970). Both groups of animals were maintained on these diets for 18–20 d and were fasted overnight before being infused.

Methods of infusion

For IG infusion in conscious rats a small polyethylene cannula (Portex Std Nylon) was carefully manipulated into the oesophagus and the tip pushed down to the level of the stomach. After this the rats were quickly anaesthetized with diethyl ether, and the cannula tip protruding from the mouth was brought out through the cheek through a small incision and tied into place with a cotton ligature. After recovery from anaesthesia the rat was placed in an air-tight glass tube 35–45 mm diameter and 120–150 mm long, depending on the size of the rat. Both ends of the glass tube were fitted with air-tight rubber bungs through which passed glass tubes; at one end there was also a small hole for the polyethylene cannula. Carbon dioxide-free air was drawn over the rats by a filter pump and the expired air was bubbled through two tubes in series containing 50 ml 2 M-KOH. Complete extraction of CO_2 from the expired air was tested by bubbling it through a solution of $Ba(OH)_2$ (50 g/l) immediately after the two solutions of KOH. The polyethylene cannula was led out through a hole in the rubber bung; the hole was sealed with Plasticine, and the cannula then attached to a needle on a previously weighed syringe which formed part of a continuous infusion pump (Scientific & Research Instruments Ltd, Croydon, Surrey).

IV infusion was performed by a tail-vein infusion with the rat placed inside the glass tube, the tail being held in position so as to protrude from a hole in the rubber bung. The fitting from a 20 gauge disposable needle was dissolved off with chloroform and a length of polyethylene tubing (0·4 mm internal diameter) was attached. This was then flushed through with distilled-water. The rat tail was placed in warm water

for about 1 min, cleaned with xylene and the prepared needle was then inserted into a lateral vein and held firmly with adhesive tape as described by Garlick & Marshall (1972). An initial rapid backflow of blood down the cannula invariably signalled a successful penetration of the vein. The cannula was attached to a previously weighed syringe in a continuous infusion pump.

Amino acids used were [U-^{14}C]-labelled L-lysine, L-valine, L-leucine and L-phenylalanine, of specific activity 10 mCi/mmol, obtained from the Radiochemical Centre, Amersham, Buckinghamshire. The ^{14}C-labelled amino acids were diluted with saline (9 g/l) to give a solution containing 5 μCi/ml and non-radioactive amino acid was added to give a final concentration of 5 mM. Infusion of amino acids was continued for 4 h at a rate of 0·7 ml/h. During the course of the infusion, at 1, 2, 3 and 4 h intervals from the starting time, the two KOH collection solutions were replaced with fresh KOH. At each of these four time-intervals, the two 50 ml solutions were mixed and a portion of the final 100 ml taken for measurement of the amount and specific activity of CO_2.

Preparation of tissues

At the end of the 4 h infusion the cannula was removed, the rat immediately anaesthetized with diethyl ether and killed by bleeding. Collections of this blood were sometimes made in distilled-water, which was diluted and counted for radioactivity. Samples of liver and of muscle from the hind-limb were then quickly removed, homogenized in 3 ml trichloroacetic acid (TCA; 100 g/l) in a ground-glass homogenizer and the supernatant fraction was separated by centrifugation. The supernatant fraction was then shaken twice with diethyl ether to remove the TCA and a sample taken for counting of ^{14}C radioactivity. The precipitated protein was washed twice with TCA and then taken up in 1 M-KOH. This solution was warmed and 100 μl was diluted to 10 ml for estimation of protein content and radioactivity.

After removal of the liver and muscle samples, the whole liver, whole gastro-intestinal tract including the spleen, the whole skin and the remaining 'carcass' were weighed and placed in 2 M-KOH (100 ml for the organs and 400 ml for the carcass). After they had been weighed and had stood overnight, the solutions were heated to boiling until the tissues were dissolved. They were then made up to the original volume, diluted twice, and samples taken for measurement of radioactivity and protein content.

Measurement of free $^{14}CO_2$ in tissues

In a few experiments the dissolved $^{14}CO_3^{2-}$ in the KOH solutions was measured by putting 2 ml of the alkaline solutions of liver and carcass into the outer well of a 25 ml Erlenmeyer flask fitted with a centre well. Into this was put a roll of filter-paper to which was added 0·2 ml phenylethylamine–toluene–methanol 2:1:1 (by vol.). The flask was then sealed with an air-tight rubber cap and 0·5 ml concentrated HCl added to the outer well with a syringe. The CO_2 released was collected for 30 min in the centre well. After absorption was complete the filter-paper with the contents of the centre well were washed out with 200 μl methanol and placed in 10 ml scintillation fluid (Bray, 1960).

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Table 1. *Weights of whole body, carcass and liver of weanling rats given the control diet (205 g protein/kg) and the protein-free diet for 18–22 d, and killed after a 16 h fast*

(Mean values with their standard errors for sixteen animals/group)

Tissue	Wt of tissue (g) Control	Protein-free
Whole body	80·0 ± 3·7	30·9 ± 2·6
Carcass	52·0 ± 3·4	20·1 ± 1·7
Liver	2·86 ± 0·16	1·16 ± 0·12

Chemical and radioactive measurements

The CO_2 content of KOH solutions was measured gravimetrically after precipitation with $Ba(OH)_2$. The protein content of the alkaline solutions of tissues was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) after 100-fold dilution.

The radioactivity of these solutions was measured after mixing 200 μl with 200 μl hyamine hydroxide (1 M in methanol) (Packard Instrument Company, Illinois, USA) and adding 10 ml scintillation fluid. Counts were measured in a Packard Tricarb liquid scintillation spectrometer model 2420 in the ^{14}C window. The channels ratio method was used for quench correction. A quench curve was constructed with different volumes of 1 M-KOH–hyamine hydroxide 1:1 (v/v) in 10 ml scintillation fluid containing a known number of disintegrations per min as *n*-[^{14}C]hexadecane (Radiochemical Centre, Amersham). Most tissue samples were counted with an efficiency of about 70%. The infusion solutions were counted after appropriate dilution as 1 ml in 10 ml scintillation fluid, at an efficiency of 80%.

The radioactivity of $^{14}CO_2$ in expired air was measured by pipetting 200 μl of the KOH solution into 10 ml scintillation fluid containing 40 mg finely divided silica/ml (Aerosil; Bush Beach, London). The counting efficiency of these samples was 75%.

RESULTS AND DISCUSSION

Body- and tissue weights

Table 1 shows the mean body-weights of sixteen rats/diet together with liver and carcass weights at the time of killing. The sixteen rats used for the results in Table 1 were a representative sample of the total number of rats/diet (thirty on the HP diet and twenty-eight on the PF diet) used in this study. Only sixteen rats are included, since records of body- and tissue weights were only kept after several infusions had been completed.

Total recovery of radioactivity, tissue distribution and output of labelled CO_2

The detailed results are shown in Table 2 and summarized in Tables 2, 4–6. Table 2 shows that, in addition to the effects of the three factors studied, nature of amino acid infused, route of infusion and diet, there were in some instances significant inter-

Table 2. *Percentage recovery of ^{14}C in whole animal, liver, gastrointestinal tract, carcass, skin and expired CO_2 after intravenous (IV) and intragastric (IG) infusions of labelled amino acids for 4 h into rats on a normal (HP) or a protein-free (PF) diet*

Route	Diet	Amino acid	Whole animal	Liver	Gastro-intestinal tract	Carcass	Skin	Expired CO_2
IV	HP	Valine	88·1	11·8	20·8	31·7	7·6	16·2
IV	HP	Leucine	97·3	8·8	14·6	38·4	23·6	11·9
IV	HP	Lysine	85·7	8·5	12·1	33·7	15·4	16·1
IV	HP	Phenylalanine	85·6	12·0	16·3	28·4	14·9	8·8
IV	PF	Valine	93·8	10·7	21·8	37·9	6·9	16·4
IV	PF	Leucine	93·8	12·2	23·8	32·8	5·9	19·2
IV	PF	Lysine	88·5	11·6	16·2	41·6	8·7	10·5
IV	PF	Phenylalanine	91·7	14·5	20·5	35·3	7·1	14·0
IG	HP	Valine	89·1	9·8	20·1	29·7	10·7	18·8
IG	HP	Leucine	79·0	6·7	26·2	23·9	7·9	14·3
IG	HP	Lysine	81·5	9·3	18·9	30·2	7·2	15·9
IG	HP	Phenylalanine	85·6	14·6	21·0	33·6	7·5	14·1
IG	PF	Valine	91·1	12·5	20·9	34·6	4·6	18·5
IG	PF	Leucine	96·8	15·0	28·5	32·6	5·1	15·7
IG	PF	Lysine	93·8	13·8	23·9	39·2	4·7	12·2
IG	PF	Phenylalanine	86·9	16·2	17·9	35·7	4·9	12·5
Mean			89·3	11·8	20·2	33·7	8·9	14·7
SE of mean			2·74	0·88	2·06	1·98	1·25	1·78

actions between the factors. Most of these interactions, however, cannot be easily interpreted in physiological terms.

Total recovery of ^{14}C

The total recovery in the whole animal (Table 2) ranged from 81·5 to 97% of the dose (means for each group of three or four rats). Most of the label unaccounted for was probably lost in the blood. In a few instances blood was collected as completely as possible, and was found to contain about 5% of the dose. A small proportion may also have been lost in the urine, which was not collected (cf. Aguilar, Harper & Benevenga, 1972).

The recovery was not affected by the route of infusion, but it was significantly lower in the rats on the HP diet. This may be because food protein in the gastrointestinal tract reduced the absorption of the administered amino acid.

Comparison of different amino acids

The nature of the amino acid infused had some influence on the distribution of radioactivity, although in general it was less important than that of the other two factors (Table 3). The largest effect was in the liver, because of the high uptake of phenylalanine (Table 4). There were also significant but smaller differences in the incorporation in the gastrointestinal tract (Table 4) and in skin (Table 4). It is of interest that a large output of $^{14}CO_2$ was given by those amino acids (valine and leucine) which are oxidized mainly by peripheral tissues. $^{14}CO_2$ derived from lysine was, however, also high in the HP group.

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Table 3. *Significance level of variance ratios in respect of ^{14}C radioactivity recovered in various tissues and in expired CO_2 in rats infused by two routes, intravenous or intragastric (A), with four different amino acids (B) and maintained on two different diets, normal or protein-free (C)*

	Total recovery	Liver	Gastro-intestinal tract	Carcass	Skin	Expired CO_2
A Route	NS	*	***	***	***	NS
B Amino acid	NS	***	**	*	**	**
C Diet	***	***	**	***	***	NS
A v. B	NS	NS	*	NS	***	NS
B v. C	NS	*	NS	NS	**	**
A v. C	*	*	NS	*	***	NS
A v. B v. C	*	NS	NS	*	***	NS

NS, not significant.

* $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

Table 4. *Comparison of the percentage recovery of ^{14}C in whole animal, in different tissues and in expired CO_2 after infusing [U-^{14}C]valine, -leucine, -lysine and -phenylalanine for 4 h into rats on two diets (normal and protein-free) by two routes (intravenous and intragastric)*

	Whole animal	Liver	Gastro-intestinal tract	Carcass	Skin	Expired CO_2
Valine	90·5	11·2	20·9	33·5	7·5	17·5
Leucine	91·7	10·7	23·3	31·9	10·6	15·3
Lysine	87·4	10·8	17·8	36·1	9·0	13·7
Phenylalanine	87·4	14·3	18·9	33·2	8·6	12·4
SE of means	1·37	0·44	1·03	0·99	0·62	0·89

For each amino acid in each tissue, the mean value for both dietary groups and both routes of infusion.

Comparison of IV and IG infusion

The total recovery rates were not significantly different with the two routes of infusion (Table 5).

There were small differences in the tissue distribution of the label. As might be expected, with IG infusion relatively more was taken up by the gastrointestinal tract, less by the peripheral tissues. Except in the liver, the differences were more marked in the rats on the HP diet. The biggest difference was in the uptake by the skin, which in both dietary groups was about 100% more in the rats receiving the IV infusion. This illustrates the important part played by the skin in the protein metabolism of the rat. In this animal the skin contains about 30% of total body protein, and much of this protein is rather labile (Waterlow & Stephen, 1966).

Although many of the differences shown in Table 5 are statistically significant, except for the values for skin, they are not large. This point is of practical importance in relation to measurements of total protein turnover or amino acid flux by the method of constant amino acid infusion (Waterlow & Stephen, 1967, 1968). If amino acids entering by the portal blood are handled differently from those derived from the breakdown of tissue protein and entering the peripheral circulation, then, whatever

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Table 5. *Comparison of the percentage recovery of ^{14}C in different tissues and in expired CO_2 after infusion of labelled amino acids into rats for 4 h by two routes (intravenous (IV) and intragastric (IG))*

	Whole animal	Liver	Gastro-intestinal tract	Carcass	Skin	Expired CO_2
IV	90·3	11·3	18·3	35·7	11·3	15·3
IG	88·0	12·3	22·2	31·7	6·6	14·2
SE	0·97	0·31	0·73	0·70	0·44	0·63

Each value represents the mean of results obtained with all four amino acids ([^{14}C]valine, -leucine, -lysine and -phenylalanine) in both dietary groups (normal and protein-free).

Table 6. *Comparison of the percentage recovery of ^{14}C in different tissues and in expired CO_2 after infusion of labelled amino acids for 4 h into rats on two dietary regimens: normal (HP) and protein-free (PF)*

	Whole animal	Liver	Gastro-intestinal tract	Carcass	Skin	Expired CO_2
HP	86·2	10·2 (11·8)	18·8 (21·8)	31·2 (36·2)	11·9 (13·8)	14·5 (16·8)
PF	92·1	13·3 (14·4)	21·7 (23·6)	36·2 (39·3)	6·0 (6·5)	14·9 (16·0)
SE	0·97	0·31	0·73	0·70	0·44	0·63

Each value representst he mean of results obtained with four amino acids ([^{14}C]valine, -leucine, -lysine and -phenylalanine) by two routes of infusion (intravenous and intragastric).

Values in parentheses were obtained by expressing the recovery as per cent of total amount of radioactivity recovered.

the route of infusion, calculation of total flux cannot be accurate. The present results, however, suggest that errors caused in this way are not likely to be large. This is in agreement with the conclusion of Picou & Taylor-Roberts (1969), who compared the turnover rates of [^{15}N]glycine given by the IG and IV routes in two children, and found no significant difference.

There was no difference between the two routes in the proportion of label oxidized. It had been expected from the previous work of Neale (1971), in which labelled amino acids were given in a single dose, that with amino acids such as valine and leucine, which are oxidized mainly by peripheral tissues, a difference would be found between IV and IG infusion. It seems that a continuous infusion minimizes any differences caused by the route of administration.

Comparison of normal and protein-free diets

As was expected from previous work (Waterlow, 1959), a larger proportion of the isotope was found in the liver and gastrointestinal tract in the rats on the PF diet, although the differences are not great (Table 6). Rather surprisingly, the recovery of label from the carcass was also greater in the depleted rats. Since uptake into muscle protein was lower (see below) the higher uptake in the carcass must probably be attributed to the fraction which is not muscle, termed 'residue' in previous work (Waterlow & Stephen, 1966); this fraction contains about 15% of total body N, and has a high rate of turnover.

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Table 7. *Percentage recovery of ^{14}C radioactivity in the protein fraction of liver and muscle tissue after intragastric (IG) and after intravenous (IV) infusion of [^{14}C]amino acids into rats on a normal (HP) or a protein-free (PF) diet*

(Mean values with their standard errors; number of samples in parentheses)

		Per cent total radioactivity recovered in protein			
		IG infusion		IV infusion	
[^{14}C]amino acid	Diet	Liver	Muscle	Liver	Muscle
Phenylalanine	HP	94·9 ± 0·5 (4)	59·3 ± 0·4 (4)	92·6 ± 0·6 (4)	64·3 ± 1·6 (4)
	PF	92·0 ± 0·6 (4)	61·8 ± 0·5 (4)	94·3 ± 0·7 (3)	67·4 ± 2·4 (3)
Leucine	HP	93·7 ± 0·7 (4)	68·5 ± 0·5 (4)	90·4 ± 0·8 (3)	70·3 ± 1·2 (3)
	PF	93·7 ± 0·8 (4)	43·0 ± 0·8 (4)	94·4 ± 1·3 (3)	56·3 ± 1·6 (3)
Valine	HP	83·2 ± 1·2 (4)	35·2 ± 0·9 (4)	85·8 ± 2·3 (3)	40·6 ± 2·1 (3)
	PF	88·2 ± 0·7 (4)	39·3 ± 1·2 (4)	93·1 ± 1·9 (3)	36·2 ± 2·3 (3)
Lysine	HP	73·2 ± 2·2 (4)	27·6 ± 3·4 (4)	67·1 ± 3·4 (4)	25·6 ± 1·9 (4)
	PF	79·4 ± 1·2 (4)	18·3 ± 2·7 (4)	73·2 ± 2·9 (3)	22·8 ± 2·3 (3)

The largest difference in uptake between animals given the two diets was found in the skin. This again indicates the metabolic importance of this tissue in the rat.

The total recovery was less in the rats on the HP diet. If the amount of radioactivity in each tissue is expressed as a percentage of the mean total amount recovered, the differences noted above are reduced, but they are still significant.

With both routes of infusion the previous diet had no effect on the proportion of infused radioactivity which appeared as $^{14}CO_2$. These results therefore differ from those obtained by us (Neale, 1971) and by others (McFarlane & von Holt, 1969; Soliman & Harper, 1971) after single injections of labelled amino acids. The interpretation of this is discussed in more detail below.

Distribution of label between protein and non-protein fractions

When a labelled amino acid is given by single injection and the animal is killed 1 h or more afterwards, very little radioactivity is found in the free amino acid fraction. With a constant infusion the situation is different, because the free amino acid pool is continuously receiving the tracer. It was therefore of interest to determine the distribution of label between protein-bound and free amino acid. Results for muscle and liver are shown in Table 7. The proportion of the total counts in the tissue that was recovered in protein was much lower in muscle than in liver, reflecting the lower rate of synthesis.

There was no evident influence of either diet or route of infusion. However, in both tissues there were clear differences between the results with different amino acids. These probably reflect differences in the relative pool sizes of free and protein-bound amino acid. From the values given by Young (1970) for the free amino acid content of muscle and by Mitchell (1959) for the amino acid composition of muscle protein, the following values are found for the ratios, free : bound amino acid; leucine $1·1 \times 10^{-3}$, phenylalanine $1·4 \times 10^{-3}$, valine $1·6 \times 10^{-3}$, lysine $4·5 \times 10^{-3}$. Thus the free amino acid pool is relatively much larger for lysine.

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Table 8. *Relative specific radioactivities (SR) of muscle and liver protein after intragastric (IG) and intravenous (IV) infusion of [14C]amino acids into rats on a normal (HP) or protein-free (PF) diet*

(Mean values with their standard errors; number of rats in parentheses)

		Relative SR (counts/min per mg muscle protein / counts/min per mg liver protein)	
Amino acid	Diet	IG infusion	IV infusion
Phenylalanine	HP	0·057 ± 0·018 (4)	0·106 ± 0·019 (4)
	PF	0·048 ± 0·005 (4)	0·056 ± 0·003 (3)
Leucine	HP	0·120 ± 0·010 (4)	0·124 ± 0·015 (3)
	PF	0·039 ± 0·011 (4)	0·044 ± 0·007 (3)
Valine	HP	0·060 ± 0·002 (4)	0·087 ± 0·003 (3)
	PF	0·037 ± 0·008 (4)	0·063 ± 0·008 (3)
Lysine	HP	0·054 ± 0·008 (4)	0·167 ± 0·007 (4)
	PF	0·026 ± 0·004 (4)	0·033 ± 0·004 (3)
Mean ± SE	HP	0·073 ± 0·009 (16)	0·123 ± 0·010 (14)
	PF	0·038 ± 0·004 (16)	0·049 ± 0·004 (12)

It is apparent from these values that, when labelled amino acids are given by infusion, because of the slow uptake by muscle the crude distribution of label in tissues does not accurately reflect the extent of incorporation into protein.

In a few experiments with [14C]phenylalanine, measurements were made of the recovery of label from the tissues as $^{14}CO_2$. In the control rats 2·0% of the total counts in the carcass and 0·24% of those in liver were recovered as $^{14}CO_2$; in rats on the PF diet the corresponding values were 1·0% of carcass counts and 0·54% of liver counts. Since these are very small proportions of the total radioactivity in the tissues, further measurements of this kind were not made.

SR of liver and muscle protein

In all instances the relative activity (SR of muscle protein ÷ SR of liver protein) was somewhat higher with IV than with IG infusions and for the HP group the mean difference was significant, although the difference for leucine was not (Table 8). The mean values for the PF group were not significantly different. The higher relative activity with IV infusion is consistent with the differences in distribution of label shown in Table 5.

By comparison with uptake in liver, uptake into muscle protein was greatly reduced in the rats given the PF diet. It was shown in previous work (Waterlow & Stephen, 1968) that, with a constant infusion of labelled amino acid, the relative SR of liver and muscle protein correlates well with the relative rates of synthesis in the two tissues, calculated from the SR of the precursor at plateau. Thus, the present results are in agreement with many previous observations (Waterlow & Stephen, 1968; Millward, 1970*b*) that protein depletion reduces the rate of protein synthesis in muscle more than in liver. These results, however, only allow comparisons between relative rates of synthesis in different tissues of the same animal.

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Output of respiratory CO_2

The total amounts of expired CO_2 collected during the 4 h IG infusions are shown in Table 9. With some IV infusions leakage of air around the tail was suspected, so that some CO_2 from the room air would have been drawn into the KOH solutions. This would not affect the recovery of radioactivity, but it would cause errors in SR and amounts of CO_2 expired. These results have therefore been discarded.

The output per kg body-weight was on average 35% greater in the rats on a PF diet. This is consistent with the findings of Miller & Payne (1962) that rats given a low-protein diet required more energy to maintain body-weight, and produced more CO_2, than rats on a diet of normal protein content. The mechanism of this effect is unknown, but it must be taken into account in the interpretation of measurements of $^{14}CO_2$ output under different dietary conditions.

SR of respiratory CO_2

The results obtained with the four amino acids given by IG infusion, corrected for dose and body-weight, are shown in Figs 1 and 2. With IV infusions, values were available only for lysine and leucine. When lysine was infused the specific activity of $^{14}CO_2$ and its time-course were almost the same whether the infusion was IG or IV. With leucine the SR of $^{14}CO_2$ was much lower after IV than after IG infusion, but for the reasons given above this could be an artifact.

Two points require comment. With all the amino acids except leucine the SR was lower in the rats on the PF diet. This follows from the fact that the total output of CO_2 was greater, whereas the proportion of the radioactive dose appearing as $^{14}CO_2$ was the same. We have no explanation for the anomalous results with leucine.

Secondly, it was surprising that the SR did not approach a plateau in 4 h, although the results of Ford & Reilly (1969) for amino acid infusions in sheep show that the SR of respiratory CO_2 had reached a plateau between 3 and 4 h. Millward (1970*a*) labelled the carbonate pool in rats with $^{14}CO_3^{2-}$ and found that it turns over with a half-life of about 12 min. The reactions of amino acid oxidation obviously introduce a considerable delay, whatever the route by which the amino acid is given. After single IG injections of uniformly-labelled amino acid the SR of respiratory CO_2 was found to reach a peak after about 1 h, and then to decay with a half-life of the order of 1·5 h (R. J. Neale, unpublished results). From these results one would not expect constant infusion to produce a plateau SR in 4 h.

Interpretation of measurements of $^{14}CO_2$ output

The present experiments have shown no difference between the protein-depleted and control rats in the proportion of labelled amino acid which was oxidized; the results are therefore in conflict with previous work by us and others, in which the tracer was given by single injection (McFarlane & von Holt, 1969; Neale, 1971). The observations of McFarlane & von Holt (1969) are particularly striking. After injection of labelled leucine or phenylalanine there was a very large difference in the output of

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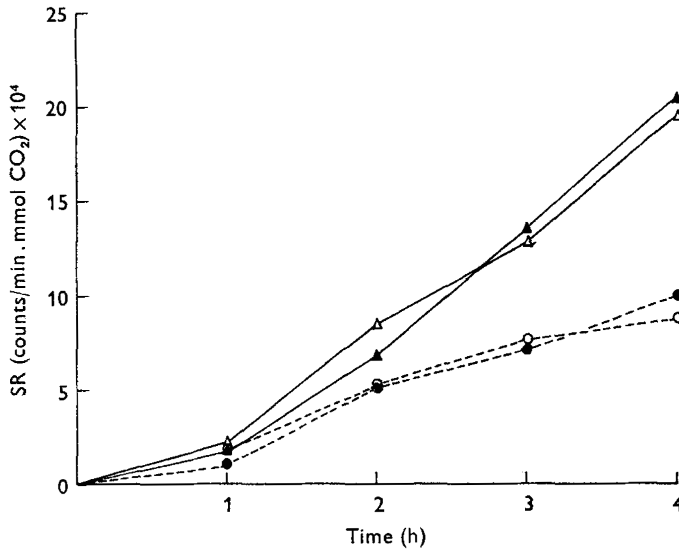

Fig. 1. Specific radioactivity (SR) of respiratory CO_2 during intragastric infusion of labelled amino acids into rats on a normal (HP) or a protein-free (PF) diet. ▲, [^{14}C]Phenylalanine, HP; ●, [^{14}C]phenylalanine, PF; △, [^{14}C]lysine, HP; ○, [^{14}C]lysine, PF.

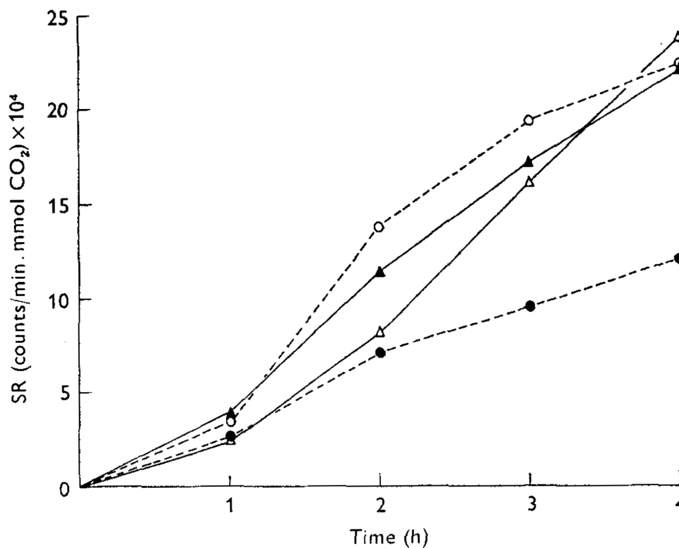

Fig. 2. Specific radioactivity (SR) of respiratory CO_2 during intragastric infusion of labelled amino acids into rats on a normal (HP) or a protein-free (PF) diet. ▲, [^{14}C]Valine, HP; ●, [^{14}C]valine, PF; △, [^{14}C]leucine, HP; ○, [^{14}C]leucine, PF.

$^{14}CO_2$ between control rats and those on a low-protein diet. Their results with leucine are not strictly comparable with ours, because the tracer they used was DL-[2-^{14}C]-leucine, whereas we used L-[U-^{14}C]leucine. In their control rats receiving a 270 g protein/kg diet the cumulative excretion of ^{14}C after 3 h was 40 % of the dose, which is very much more than that found in the present experiments (Table 9). Clearly the

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Table 9. *Total CO_2 excretion by rats on a normal (HP) or protein-free (PF) diet during 4 h intragastric (IG) or intravenous (IV) infusions of amino acids*

(Mean values with their standard errors; number of animals in parentheses)

Amino acids	Excretion (mmol CO_2/kg body-wt) HP	PF
	IG infusion	
Phenylalanine (4)	379 ± 20	585 ± 64
Valine (4)	420 ± 15	616 ± 17
Lysine (4)	392 ± 15	576 ± 59
Leucine (4)	307 ± 38	430 ± 101
	IV infusion	
Lysine (4)	408 ± 16	(3) 556 ± 54
Leucine (3)	500 ± 42	(3) 440 ± 51

metabolism of the 2-C atom is not representative of that of the carbon skeleton of leucine as a whole. With phenylalanine they, like us, used the [U-^{14}C]-labelled compound; the output after 3 h was 10% of the dose in the controls, and in the protein-deficient rats zero.

The most likely explanation of the difference between the present results and those obtained after single injection is heterogeneity of the precursor pool (Garlick, Millward & Waterlow, 1974). Initially, after single injection, there is a preferential uptake of radioactivity by liver and viscera, and later redistribution to the peripheral tissues (Waterlow, 1959). From the scanty evidence available in the literature (Henriques, Henriques & Neuberger, 1955; Lajtha, Furst, Gerstein & Waelsch, 1957; Gaetani, Mariani, Spadoni & Tomasi, 1961) it seems probable that for about the 1st hour the SR of the precursor amino acid at the site of oxidation is higher in the viscera than in the periphery, so that for a time the output of $^{14}CO_2$ reflects predominantly the rate of oxidation in the visceral tissues. With a continuous infusion there is a closer approach to uniformity of distribution of isotope in the precursor pool, although even in 4 h isotopic equilibrium was not reached.

One might therefore conclude that the difference between the results with single injections and continuous infusions reflects the fact that any adaptation which occurs takes place mainly in liver and internal organs, and that the continuous infusion, by creating conditions in which relatively more of the $^{14}CO_2$ is derived from peripheral tissues, actually obscures any adaptive change.

If this argument is correct, it follows that after a single injection neither the SR of expired $^{14}CO_2$ nor the proportion of dose excreted as CO_2 reflects accurately the extent of amino acid oxidation in the body pool as a whole. The labelled amino acid is not acting as a true tracer, because it is not uniformly mixed in the body pool. One cannot, therefore, conclude that if, say, 10% of the dose is excreted as $^{14}CO_2$, then 10% of the amino acid flux during the time of observation has gone through the oxidative pathway.

It must be emphasized that in our experiments the aim was to get information about the fate of amino acids in the endogenous pool. Therefore the tracer was given without other amino acids to rats which had been fasted overnight. The situation is,

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however, quite different when the objective of the experiment is to trace the fate of amino acids from the food. Thus Brookes, Owens & Garrigus (1972) gave [U-^{14}C]-lysine to rats fed on diets containing different concentrations of lysine and collected the respiratory CO_2 for 6 h. They calculated the percentage of dietary lysine oxidized from the percentage of injected radioactivity appearing as $^{14}CO_2$. It is an implicit assumption in this experimental design that the labelled amino acid is acting as a tracer for food amino acids only. The label was given by a single intracardiac injection, after which the rat was presumably fasted during the period of CO_2 collection, although this is not stated. One may question whether under these conditions the labelled lysine really acts as a tracer for dietary lysine ingested over the previous period. Empirically, however, the method seemed to give satisfactory results, since estimates of lysine requirements derived in this way agreed with those calculated from growth rates. In similar experiments Aguilar *et al.* (1972) showed that when varying amounts of [^{14}C]methionine were given orally, the proportion of dose oxidized varied linearly with the methionine content of the diet. In these experiments CO_2 was collected for 24 h. The tracer was given with the food, and the rat apparently had access to it throughout the collection period.

It is apparent from this discussion that it is difficult to draw unequivocal conclusions from the output of $^{14}CO_2$ after labelled amino acids have been given. Apart from the theoretical problems, some of the discrepancies between the results of different workers may result from differences in the details of experimental design. Single-injection experiments with uniformly labelled amino acids have given evidence of economy of some amino acids, e.g. lysine (Yamashita & Ashida, 1969; Neale, 1971) and threonine (Yoshida *et al.* 1966) which are oxidized mainly in liver, but not of the branched-chain amino acids, which are oxidized mainly by muscle (Neale, 1971, 1972). Aguilar *et al.* (1972) found that, when young rats were given an amino acid diet equivalent to one containing 180 g casein/kg, in which the indispensable amino acids were individually labelled with ^{14}C, the proportion of radioactivity oxidized to CO_2 varied from one amino acid to another. As these authors point out, this may reflect differences in the metabolic pathways; but it may also reflect differences in the site of oxidation. In the experiments of Aguilar *et al.* (1972), CO_2 was collected over a period of 24 h. It is probable that during the greater part of that time the SR of the free amino acid was not uniform in the different tissues of the body.

A further difficulty is that the results obtained with [1-^{14}C]-labelled amino acids are not the same as when the amino acid is uniformly labelled, because the oxidative pathway for the 1-C atom is different from that of the rest of the carbon skeleton. This problem has been discussed by Reeds (1974).

It is obvious that from the point of view of adaptation it is of little value for the oxidation of one or two essential amino acids to be reduced unless oxidation of all is reduced. The experiments reported here suggest that when there is a general deficiency, as on a protein-free diet, economy of oxidation plays little part in the mechanism of adaptation. However, for the reasons given the question cannot be regarded as settled.

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Conclusions

These experiments lead to four main conclusions. First, under the conditions chosen (rats fasted overnight and infused for 4 h) the route by which the amino acid is presented causes only minor differences in the way in which the infused amino acid is handled. Secondly, it has not been possible to show that protein deficiency causes any reduction in the extent of amino acid oxidation, as judged by the output of labelled CO_2. Therefore we have not been able to confirm the suggestion made by us and others that part of the mechanism of adaptation to low protein intakes is a reduction or economy in oxidation of the carbon skeletons of some essential amino acids. Thirdly, it is clear that interpretation of results on the output of $^{14}CO_2$ after administration of labelled amino acids presents many pitfalls. Finally, it is worth re-emphasizing that in the rat the skin has a significant role in the protein metabolism of the whole body.

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