Metabolism of Leucine in Protein-Calorie-Deficient Rats

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1. Protein-calorie-depleted rats exhibit a decreased ability to oxidize leucine as compared with control animals. 2. The block in degradation of L-leucine is due to decreased activities of L-leucine transaminase and D-amino acid oxidase associated with liver mitochondria. 3. Cytoplasmic L-leucine transaminase in liver is not affected by the protein depletion. 4. The specific activities of the mitochondrial enzymes can be increased by a single oral dose of 1g. of Bacto-Peptone (meat hydrolysate). The effect of Bacto-Peptone can be inhibited by prior administration of dosages of 10mg. of puromycin/100g. body wt., suggesting that the increased enzyme specific activities may be due to new synthesis of enzyme protein.

The overall oxidative degradation of two essential amino acids (leucine and phenylalanine) is markedly decreased in rats fed on a low-protein diet whereas that of two non-essential amino acids (glutamate and alanine) is virtually unaffected. The decreased degradation of leucine is due to a block in the catabolic pathway at some stage before the formation of 3-methylbutyryl-CoA (isovaleryl-CoA) (McFarlane & Holt, 1969).

In animal tissues amino acids are oxidatively deaminated to their respective α -oxo acid analogues, the L-isomers by the action of a transaminase in the presence of an amino group acceptor and the D-isomers by the action of D-amino acid oxidase (Meister, 1965a). The α -oxo acids are then decarboxylated to give the next lower homologous fatty acid. It is doubtful whether any other pathway contributes significantly to the degradation of leucine in animal tissues. A leucine decarboxylase has been found in plants and micro-organisms, but not in animals (Meister, 1965b). Although an L-amino acid oxidase has been purified from rat kidney, its activity is very low, and it is unlikely that this enzyme plays a major role in the conversion of amino acids into α -oxo acids (Krebs, 1964; Meister, 1965b).

Previous observations (McFarlane & Holt, 1969) showed that rats deprived of protein exhibit an increased urinary excretion of 4-methyl-2-oxo-pentanoate (α -oxoisocaproate) and a decreased urinary excretion of leucine. This suggested that the primary block in leucine catabolism occurs at the

stage of decarboxylation of 4-methyl-2-oxopentanoate.

To gain further insight into the observed changes of leucine degradation, the activities of the enzymes concerned had to be determined. Existing procedures for the determination of enzymic transamination involve either measurement of the transfer of an amino group to an acceptor α -oxo acid or measurement of the α -oxo acid formed from the amino acid (Bergmeyer & Bernt, 1963). Both these methods require separation of the reaction products from the reactants. However, losses incurred during the separation procedures impose limitations on the accuracy, sensitivity and reproducibility of the techniques. Other methods, such as measurement of glutamic semialdehyde formed by α -transamination of ornithine (Peraino & Pitot, 1963), are based on physicochemical properties peculiar to the reactants and are applicable for these transamination reactions only. Therefore a rapid sensitive routine method had to be developed for the simultaneous determination of α -oxo acid decarboxylase and transaminase (or D-amino acid oxidase) activities in crude tissue preparations.

MATERIALS AND METHODS

Chemicals. Labelled compounds were obtained from The Radiochemical Centre (Amersham, Bucks.) or from Calbiochem (Los Angeles, Calif., U.S.A.), or were prepared from starting materials obtained from one of these sources. All other chemicals, unless otherwise stated, were A.R. grade or equivalent and were obtained from various commercial sources.

Animals and diets. Animals, diets and feeding regimen were as described in the preceding paper (McFarlane & Holt, 1969). The term 'protein-depleted rat' refers to an

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animal that had been fed on the protein-deficient diet for 8 weeks.

Measurement of radioactivity. All determinations of radioactivity were conducted with a Packard Tri-Carb model 314E liquid-scintillation spectrometer. The scintillator system used contained 0.3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 5.0 g. of 2,5-diphenyloxazole in 1 l. of toluene.

Tissue preparations. Animals, kept without food for 14 hr. overnight, were killed by decapitation and the livers were excised, chilled and weighed. Mitochondria were prepared, by the method of Hogeboom (1955), from a 10% (w/v) liver homogenate in 0.25 M-sucrose, pH 7.4. Mitochondria-free supernatant fractions were obtained by centrifuging 10 ml. portions of the liver homogenates at 15000 g at 0–3° for 15 min. in a Sorvall RC-2B refrigerated centrifuge.

Assay of enzyme activities. Amino acids are oxidatively deaminated and decarboxylated according to the general reaction:

Amino acids labelled in the carboxyl group will result in labelled α -oxo acids. Subsequent decarboxylation by a decarboxylase will yield $^{14}\text{CO}_2$. Therefore the amount of $^{14}\text{CO}_2$ plus any labelled α -oxo acid not decarboxylated will be a measure of the transaminase or D-amino acid oxidase activity. α -Oxo acids undergo quantitative decarboxylation in the presence of $\text{Ce}(\text{SO}_4)_2$ (Meister, 1952). At a given substrate concentration the enzymically produced $^{14}\text{CO}_2$ from amino acids labelled in the carboxyl group reflects the decarboxylase activity and the sum of enzymically liberated plus $\text{Ce}(\text{SO}_4)_2$ -liberated $^{14}\text{CO}_2$ the transaminase activity (with L-leucine as substrate) or D-amino acid oxidase (with D-leucine as substrate).

The apparatus used was a 25 ml. wide-mouthed round-bottomed centrifuge tube stoppered with a 'needle-puncture' rubber cap, of the type used in preparation of solutions for hypodermic injection. At the top of the tube, suspended from the stopper, was a 2 ml. plastic beaker containing a strip of Whatman no. 4 filter paper (0.8 cm. $\times$ 8.0 cm.), accordion-pleated to fit inside the beaker and moistened with 0.2 ml. of 1.0 M-Hyaminate hydroxide in methanol (Rapkin, 1961). The incubation system (total volume 2.0 ml.), containing [^{14}C]leucine, the necessary cofactors and tissue preparation, was placed in the bottom of the centrifuge tube, which was then stoppered and incubated in a Warburg apparatus, with continuous shaking at 37°, for 1 hr. The enzymic reaction in the incubation mixture was stopped by injection of 0.5 ml. of 10 N- H_2SO_4 to liberate enzymically produced $^{14}\text{CO}_2$. Incubation was continued for a further 1 hr. to ensure that all $^{14}\text{CO}_2$ in the incubation mixture was released from solution and trapped in the Hyamine. The plastic beaker was removed, the filter-paper strip transferred to a counting vial containing 10 ml. of toluene scintillator and any Hyamine

remaining in the beaker rinsed into the counting vial with two 0.5 ml. lots of ethanol. The radioactivity of the sample was then measured to determine the amount of $^{14}\text{CO}_2$ produced.

The incubation tube was centrifuged to remove precipitated protein and a 1.0 ml. sample of the supernatant was incubated as above for 1 hr. with 3.0 ml. of saturated $\text{Ce}(\text{SO}_4)_2$ in 4 N- H_2SO_4 for quantitative decarboxylation of carboxyl-group-labelled α -oxo acid. The $^{14}\text{CO}_2$ arising from this treatment was trapped in Hyamine and its radioactivity measured as above to determine the amount of 4-methyl-2-oxopentanoate produced by the tissue preparation. The particular conditions of the reaction were determined with known quantities of sodium 4-methyl-2-oxo[1- ^{14}C]pentanoate.

Determination of DNA and protein. The DNA content of liver homogenates was determined by the method of Schneider (1957). The protein concentration in tissue preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Re-feeding experiments. Animals were kept without food overnight and, between 8.00 a.m. and 9.00 a.m. the following morning, were given either 75 mg. of DL-leucine or 1 g. of Bacto-Peptone (meat hydrolysate) in 2.5 ml. of water by oral intubation. Animals were killed 3 hr. later and enzyme activities were assayed. Some animals were given 10 mg. of puromycin/100 g. body wt. (Pitot & Peraino, 1963), by intraperitoneal injection, 1 hr. before the Bacto-Peptone was administered.

Statistical tests. The significance of the difference between the means of two sets of values was determined with the *t* test for small samples (Bancroft, 1965).

RESULTS

Enzymes of leucine catabolism. Initial experiments revealed that (a) a boiled homogenate of liver showed no leucine-catabolic activity and (b) the utilization of leucine was linear with respect to time and homogenate concentration. This established that the $^{14}\text{CO}_2$ and 4-methyl-2-oxo-[1- ^{14}C]pentanoate produced during the incubation were the result of enzyme activity.

Table 1 gives the intracellular distribution of the enzymes deaminating and decarboxylating D- and L-leucine. The activities related to unit fresh weight of liver show that approx. 30% of the L-leucine catabolism occurs in the supernatant and 70% in the mitochondria. Over 90% of D-leucine oxidation occurs in the mitochondrial fraction. The remainder, which was found in the supernatant, can probably be attributed to contamination of the latter by mitochondrial fragments. In a separate experiment, with 1 mM-sodium 4-methyl-2-oxo[1- ^{14}C]pentanoate (50 000 disintegrations/min.) in place of labelled leucine, it was determined that over 90% of the 4-methyl-2-oxopentanoate-decarboxylating activity is found in the mitochondria also.

Maximal 4-methyl-2-oxopentanoate-decarb-oxylating activity is achieved when the incubation

Table 1. *Intracellular distribution of leucine-catabolic activity in mitochondrial and supernatant fractions of liver from control rats*

The incubation system contained: 1.0mM-D- or -L-[1-¹⁴C]leucine (100000disintegrations/min.), 1.0mM-sodium α -oxoglutarate, 25mM-Sørensen's phosphate buffer, pH7.4, and mitochondria or supernatant equivalent to 100mg. of fresh tissue. Values given are the means of duplicate determinations. The variation from any quoted mean does not exceed $\pm 2.1\%$.

Liver fraction	Leucine degraded (m μ moles/hr./mg. of protein)					
	D-Leucine			L-Leucine		
	To CO ₂ (decarboxylase)	To α -oxo acid	Total (oxidase)	To CO ₂ (decarboxylase)	To α -oxo acid	Total (transaminase)
Mitochondria	42.00	0.96	42.96	7.72	Nil	7.72
Supernatant	0.18	1.22	1.40	0.22	2.72	2.94

Table 2. *Effect of added α -oxoglutarate on the degradation of leucine by mitochondrial and supernatant fractions of liver from control rats*

The incubation system contained 1.0mM-D-[1-¹⁴C]leucine (50000disintegrations/min.) or -L-[1-¹⁴C]leucine (100000disintegrations/min.), 1.0mM-sodium α -oxoglutarate, 0.2mM-thiamine pyrophosphate, 0.1mM-CoA, 2.0mM-MgCl₂, 50mM-KCl, 1.0mM-ATP (dipotassium salt), 0.1mM-cytochrome *c*, 25mM-Sørensen's phosphate buffer, pH7.4, and mitochondria or supernatant equivalent to 100mg. of fresh tissue. When α -oxoglutarate was omitted, the incubation system was made up to volume with 0.25M-sucrose, pH7.4. Results are the means of duplicates. The variation from any quoted mean does not exceed $\pm 3.2\%$.

Liver fraction	α -Oxoglutarate	Leucine degraded (m μ moles/hr./mg. of protein)					
		D-Leucine			L-Leucine		
		To CO ₂ (decarboxylase)	To α -oxo acid	Total (oxidase)	To CO ₂ (decarboxylase)	To α -oxo acid	Total (trans- aminase)
Mitochondria	Omitted	47.40	1.18	48.58	3.16	Nil	3.16
Mitochondria	Added	45.90	1.20	47.10	8.05	Nil	8.05
Supernatant	Omitted				0.16	Nil	0.16
Supernatant	Added				0.23	2.81	3.04

system contains 0.2mM-thiamine pyrophosphate, 2.0mM-Mg²⁺, 0.1mM-CoA and 1.0mM-ATP. Addition of NAD⁺ or lipoic acid or both did not significantly influence the reaction.

L-Leucine utilization is enhanced by the presence of α -oxoglutarate in the incubation system, whereas D-leucine catabolism is not (see Table 2). This indicates the operation of a transaminase in the catabolism of the former, whereas that of the latter is probably catalysed by D-amino acid oxidase.

Substrate saturation for the transamination of L-leucine in mitochondria with excess of α -oxoglutarate present is not reached at 20mM, at which concentration the amino acid becomes insoluble in the test system. α -Oxoglutarate saturation is reached at 10mM. The D-amino acid oxidase, similarly, cannot be saturated with D-leucine up to a concentration of 18mM. The mitochondrial

4-methyl-2-oxopentanoate-decarboxylating enzyme(s), in contrast with the deaminating enzymes, operates at much lower substrate saturation, between 0.5 and 1.0mM. The reaction sequence could therefore not be measured at saturation concentrations of the amino acids, but only at such substrate concentration that allowed a linear reaction rate during the incubation period.

Comparison of D- and L-leucine catabolism in control and protein-depleted rats. The leucine-transaminating, leucine-deaminating and 4-methyl-2-oxopentanoate-decarboxylating abilities of control and protein-depleted rats are shown in Tables 3-8. The results are expressed in terms of the DNA content of the liver. Reference to DNA permits direct comparison of enzyme activities/cell, since the liver DNA content is unaffected by changes in dietary protein intake (Mendes & Waterlow, 1958; Mariani, Migliaccio, Spadoni & Ticca, 1966).

Table 3. *Catabolism of L-[1-¹⁴C]leucine by mitochondrial and supernatant fractions of liver from control and protein-depleted rats*

Results are the mean values obtained from six control and four protein-depleted animals. Duplicate measurements were made for each animal. The incubation system contained: 1 mM-L-[1-¹⁴C]leucine (400 000 disintegrations/min.), 10 mM-sodium α -oxoglutarate, 0.2 mM-thiamine pyrophosphate, 0.1 mM-CoA, 2.0 mM-MgCl₂, 50 mM-KCl, 1.0 mM-ATP (dipotassium salt), 0.1 mM-cytochrome c, 25 mM-Sørensen's phosphate buffer, pH 7.4, and mitochondria or supernatant equivalent to 100 mg. of fresh tissue. Significance of differences: $P < 0.001$ for *a-b*, *c-d* and *e-f*; other differences between the two groups of animals are not significant (i.e. $P > 0.05$).

Liver fraction	Animal group	L-Leucine degraded (μ moles/hr./mg. of DNA)		
		To CO ₂ (decarboxylase)	To α -oxo acid	Total (transaminase)
Mitochondria	Control	220.3 ^a	27.1 ^c	247.4 ^e
Mitochondria	Depleted	82.6 ^b	48.9 ^d	131.5 ^f
Supernatant	Control	10.5	74.4	84.9
Supernatant	Depleted	7.0	72.0	79.0

Table 4. *Catabolism of D-[1-¹⁴C]leucine by mitochondria of liver from control and protein-depleted rats*

Results are the mean values obtained from six control and four protein-depleted rats. Duplicate measurements were conducted for each animal. The incubation system was the same as that used in Table 3 except that labelled L-leucine was replaced by 10.0 mM-D-[1-¹⁴C]leucine (400 000 disintegrations/min.), no α -oxoglutarate was added and mitochondria equivalent to only 25 mg. of fresh tissue were used. Significance of differences: $P < 0.01$ for *a-b*; $P < 0.001$ for *c-d* and *e-f*.

Animal group	D-Leucine degraded (μ moles/hr./mg. of DNA)		
	To CO ₂ (decarboxylase)	To α -oxo acid	Total (oxidase)
Control	573.0 ^a	2327 ^c	2900 ^e
Depleted	382.8 ^b	948 ^d	1331 ^f

As shown in Tables 3 and 4, the activities of mitochondrial enzymes for both L- and D-leucine degradation are significantly decreased in protein-depleted animals. This is true for the L-leucine transaminase, the D-leucine oxidase and the 4-methyl-2-oxopentanoate-decarboxylating enzyme(s). The cytoplasmic (supernatant) L-leucine transaminase is, however, not affected.

With L-leucine as substrate (Table 3) the decreased decarboxylation of 4-methyl-2-oxopentanoate is accompanied by an accumulation of α -oxo acid in the system. There is also a marked decrease in L-leucine transamination. To exclude the possibility of only an apparent decrease of transaminase activity owing to a mass-action effect exerted by the

Table 5. *Effect of added 4-methyl-2-oxopentanoate on L-leucine transaminase activity of the mitochondrial fraction of liver from a protein-depleted rat*

Each value is the mean of a duplicate measurement. The variation from any quoted mean does not exceed $\pm 2.4\%$. The composition of the incubation system was the same as in Table 3. Sodium 4-methyl-2-oxopentanoate was added at a final concentration of 5.0 mM.

	L-Leucine degraded (μ moles/hr./mg. of DNA)		
	To CO ₂ (decarb-oxylase)	To α -oxo acid	Total (trans-aminase)
4-Methyl-2-oxopentanoate			
Omitted	95.7	51.0	146.7
Added	28.0	222.2	250.2

Table 6. *Decarboxylation of 4-methyl-2-oxopentanoate by the mitochondrial fraction of liver from control and protein-depleted rats*

Results are the mean values obtained for three animals in each group. Duplicate measurements were conducted for each animal. The composition of the incubation system was the same as in Table 4 except that D-leucine was replaced by 5.0 mM-sodium 4-methyl-2-oxo[1-¹⁴C]pentanoate (40 000 disintegrations/min.). Values in parentheses denote the ranges of the results.

Animal group	CO ₂ produced (μ moles/hr./mg. of DNA)
Control	1448 (1398-1527)
Depleted	1363 (1329-1387)

accumulated α -oxo acid, liver mitochondria from a protein-depleted animal were incubated with L-[1-¹⁴C]leucine in the presence and absence of

Table 7. *Effect of feeding with DL-leucine or Bacto-Peptide on L-leucine catabolism by the mitochondrial fraction of liver from protein-depleted rats*

Each value is the mean obtained from the number of animals shown in parentheses for each group. Duplicate measurements were made for each animal. The composition of the incubation medium was the same as in Table 3. DL-Leucine (75mg.) or Bacto-Peptide (1g.) was force-fed at 3 hr., and puromycin (10mg./100g.) was injected intraperitoneally at 4 hr., before the rats were killed. Significance of differences: for *a-c*, $P < 0.001$; for *a-d*, $P < 0.05$; for *a-e*, $P < 0.02$; for *b-d*, $P < 0.05$; for *d-e*, $P < 0.01$; for *f-h*, $P < 0.001$; for *f-i*, $P < 0.05$; for *f-j*, $P < 0.01$; for *g-i*, $P = 0.05$; for *k-m*, $P < 0.01$; for *k-o*, $P < 0.02$; for *l-n*, $P < 0.02$; for *n-o*, $P < 0.05$; differences between other pairs, such as *b-c*, *b-e*, *g-h*, *g-j*, *i-j*, *k-n*, *l-m* and *l-o*, are not significant (i.e. $P > 0.05$).

Animal group	Treatment	L-Leucine degraded (mμmoles/hr./mg. of DNA)		
		To CO ₂ (decarboxylase)	To α-oxo acid	Total (trans- aminase)
Control (6)		220.3 ^a	27.1 ^f	247.4 ^b
Depleted (4)		82.6 ^b	48.9 ^g	131.5 ⁱ
Depleted (3)	Leucine-fed	94.6 ^c	54.0 ^h	148.6 ^m
Depleted (3)	Peptide-fed	147.7 ^d	35.5 ⁱ	183.2 ⁿ
Depleted (3)	Puromycin, peptide-fed	110.0 ^e	41.4 ^j	151.4 ^o

Table 8. *Effect of feeding with DL-leucine or Bacto-Peptide on D-leucine catabolism by the mitochondrial fraction of liver from protein-depleted rats*

Each value is the mean obtained from the number of animals shown in parentheses for each group. Duplicate measurements were made for each animal. The composition of the incubation system was the same as in Table 4. Treatment with DL-leucine, Bacto-Peptide and puromycin was the same as indicated in Table 7. Significance of differences: for *a-c*, $P < 0.01$; for *b-e*, $P = 0.05$; for *f-h*, $P < 0.001$; for *f-j*, $P < 0.001$; for *g-i*, $P < 0.01$; for *i-j*, $P < 0.05$; for *k-m*, $P < 0.001$; for *k-o*, $P < 0.01$; for *l-n*, $P < 0.001$; for *n-o*, $P < 0.05$; differences between other pairs, such as *a-d*, *a-e*, *b-c*, *b-d*, *d-e*, *f-i*, *g-h*, *g-j*, *k-n*, *l-m* and *l-o*, are not significant (i.e. $P > 0.05$).

Animal group	Treatment	D-Leucine degraded (mμmoles/hr./mg. of DNA)		
		To CO ₂ (decarboxylase)	To α-oxo acid	Total (oxidase)
Control (6)		573.0 ^a	2327 ^f	2900 ^b
Depleted (4)		382.8 ^b	948 ^g	1331 ⁱ
Depleted (3)	Leucine-fed	321.5 ^c	972 ^h	1294 ^m
Depleted (3)	Peptide-fed	483.5 ^d	2095 ⁱ	2568 ⁿ
Depleted (3)	Puromycin, peptide-fed	529.4 ^e	1006 ^j	1536 ^o

added 4-methyl-2-oxopentanoate. The addition of 4-methyl-2-oxopentanoate does not decrease the transamination rate (see Table 5). On the contrary, there is an increase in the rate of leucine degradation for which no satisfactory explanation has been found.

The above suggests that enzymic decarboxylation of 4-methyl-2-oxopentanoate produced endogenously, i.e. from D- or L-leucine, is decreased in the livers of protein-depleted rats. The decarboxylation of added sodium 4-methyl-2-oxo[1-¹⁴C]pentanoate, however, is not diminished (Table 6).

Effect of including leucine or a mixture of amino acids in the diet on the catabolism of leucine in protein-depleted rats. As shown in Tables 7 and 8, feeding protein-depleted animals with DL-leucine

has no significant effect on any of the enzymes concerned with the early stages of leucine catabolism in the liver. Feeding with Bacto-Peptide, however, produces significant increases in the specific activities of all the mitochondrial enzymes. The latter effects can be inhibited by prior administration of puromycin.

DISCUSSION

The metabolic pathway from L-leucine to acetoacetate and acetyl-CoA has long been established (Meister, 1965a). However, until recently, details of the first two steps in the pathway have remained unclear. Although it was known for some time that L-leucine transaminates with

α -oxoglutarate, only recently has a specific enzyme been isolated that catalyses reversible transamination between the three branched-chain amino acids, L-leucine, L-valine and L-isoleucine, and α -oxoglutarate (Taylor & Jenkins, 1966a,b). In addition, in heart muscle and in liver there are two leucine transaminases that are isoenzymes (Ichihara & Koyama, 1966). One of these is localized in the mitochondria and the other in the cytoplasm (Ichihara, Takahashi, Aki & Shirai, 1967). The cytoplasmic activity can be separated into two fractions by DEAE-cellulose chromatography, one of which catalyses the transamination of all three branched-chain amino acids, whereas the other is active only with leucine.

D-Leucine is deaminated by D-amino acid oxidase (Meister, 1965b). This enzyme has been found in the kidney and liver of a large number of mammals (Meister, 1965b; Krebs, 1964), localized in cytoplasmic particles containing also urate oxidase and catalase (de Duve, Wattiaux & Baudhuin, 1962). These particles sediment with the mitochondrial fraction, but can be separated by subfractionation. D-Amino acid transaminases have so far been found only in micro-organisms and it is doubtful whether they exist in mammalian tissues (Meister, 1965b).

The conversion of 4-methyl-2-oxopentanoate into 3-methylbutyryl-CoA is assumed to involve a decarboxylation reaction analogous to the conversion of pyruvate into acetyl-CoA (Meister, 1965a).

In the current investigation, conversion of L-leucine into 4-methyl-2-oxopentanoate was found to be enhanced by the addition of α -oxoglutarate, indicating the operation of transamination. The distribution of enzyme activity between the mitochondrial and supernatant fractions suggests the existence of two enzymes. It would appear that these enzymes correspond to the leucine transaminase isoenzymes described by Ichihara *et al.* (1967). α -Oxoglutarate did not enhance the deamination of D-leucine, suggesting that the conversion of the latter into 4-methyl-2-oxopentanoate is catalysed by D-amino acid oxidase associated with the mitochondrial fraction. As compared with the L-leucine transaminase the D-amino acid oxidase activity is roughly tenfold higher. This is in good agreement with the observation by Greenberg (1961) that a greater amount of ketone bodies is produced from D- or DL-leucine than from the L-isomer.

The decarboxylation of 4-methyl-2-oxopentanoate requires Mg^{2+} , thiamine pyrophosphate and CoA for maximal activity, and is associated with the mitochondrial fraction. These cofactor requirements point to an enzyme similar to the pyruvate oxidase complex. Under the experimental conditions the intramitochondrial NAD^+ con-

centration was obviously sufficiently high, so that added NAD^+ had no stimulatory effect.

These, therefore, were the enzymes investigated: L-leucine transaminases (cytoplasmic and mitochondrial), D-amino acid oxidase (mitochondrial) and 4-methyl-2-oxopentanoate decarboxylase (mitochondrial). The last-named enzyme is so called tentatively; it is recognized that no enzyme has been described that specifically decarboxylates 4-methyl-2-oxopentanoate and only circumstantial evidence is presented here to suggest that such an enzyme may exist.

Of the enzymes concerned with L-leucine catabolism, only the cytoplasmic leucine transaminase is unaffected by protein depletion. The mitochondrial leucine transaminase and 4-methyl-2-oxopentanoate decarboxylase (when L-leucine is used as substrate) are significantly decreased in activity. The decrease in mitochondrial leucine transaminase activity cannot be explained by a mass-action effect exerted by the accumulated α -oxo acid on the enzyme (Table 5). The accumulation of α -oxo acid in the system suggests that there may also be a genuine decrease in activity of the 4-methyl-2-oxopentanoate decarboxylase. The increase in 4-methyl-2-oxopentanoate excretion in protein-depleted rats (McFarlane & Holt, 1969) supports this view. However, no such decrease was found when 4-methyl-2-oxopentanoate was used as substrate (Table 6). From this and the results in Tables 4 and 3 one may deduce that the rate of α -oxo acid generation is the rate-limiting factor in the 4-methyl-2-oxopentanoate decarboxylase reaction and the decreased activity of decarboxylation in depleted animals is only apparent but does not reflect a decrease in enzyme concentration. However, this would not account for the accumulation of α -oxo acid *in vitro* (Table 3) and the increased excretion (McFarlane & Holt, 1969). A satisfactory answer to this discrepancy has to await further investigation.

D-Amino acid oxidase activity is decreased significantly per cell, in confirmation of the observations of other authors (Knox, Auerbach & Lin, 1956).

The results given in Tables 7 and 8 show that leucine-catabolizing enzymes in protein-depleted animals increase in activity after feeding with Bacto-Peptone. It had been previously observed that tryptophan pyrrolase can be induced by tryptophan (Knox & Mehler, 1951) and that tyrosine- α -oxoglutarate transaminase can be induced by tyrosine (Lin & Knox, 1958). These enzymes operate at the beginning of the respective catabolic pathways. A block in the early stages of degradation of leucine could be located in this investigation, *in vivo* as well as *in vitro*, as occurring before the formation of 3-methylbutyryl-CoA. The

reappearance of leucine transaminase, amino acid oxidase and to a smaller extent 4-methyl-2-oxopentanoate decarboxylase activity after the animals had been fed with Bacto-Peptone strongly suggests that enzyme induction might be the mechanism by which leucine-catabolizing activity is controlled. The fact that the Bacto-Peptone effect can be inhibited by prior administration of puromycin adds weight to this view.

Although the feeding with Bacto-Peptone caused a significant increase in the activity of all the enzymes concerned with the first two steps of leucine catabolism, the activities of the L-leucine-converting enzymes did not return to normal (Table 7). However, the induction of D-leucine degradation by Bacto-Peptone led to a return of D-leucine oxidase activity to normal values (Table 8). The equivocal results on the decarboxylating activity may indicate the presence of more than one enzyme decarboxylating 4-methyl-2-oxopentanoate.

It is difficult to find an explanation for the fact that the Bacto-Peptone effect could not be duplicated by feeding the rats with DL-leucine. It may be that, owing to the slight solubility of leucine, the amount of the L-isomer given was only about one-third of that present in the Bacto-Peptone used and may not have been adequate to produce a detectable increase in enzyme activity.

One point of particular interest, namely the difference in response of the mitochondrial and cytoplasmic leucine transaminases to dietary protein restriction, has emerged from these studies. Differences in responses of the two isoenzymes to various stimuli have also been observed by Ichihara *et al.* (1967). Because there is no 4-methyl-2-oxopentanoate decarboxylase activity in the cytoplasm, leucine cannot be further degraded without the aid of the mitochondrial enzymes. The mitochondrial leucine transaminase, perhaps because of the close proximity to the 4-methyl-2-oxopentanoate decarboxylase, plays the key role in the overall degradation of the amino acid and appears to be controlled independently from the cytoplasmic enzyme.

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