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THE METABOLISM OF THE BRANCHED-CHAIN AMINO ACIDS IN THE RAT

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by

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ABSTRACT OF Ph.D THESIS

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This thesis is primarily concerned with the adaptation of leucine oxidation in rats that are either fasting or have been fed protein restricted diets. It also includes the adaptation of valine oxidation in rats fed a protein-free diet for periods of up to 3 weeks.

The initial experiments were concerned with resolving the differences of opinion which hold in contention the ability of the malnourished rat to adapt to a low-protein diet and in particular to conserve the branched-chain amino acids. Much of the disagreement was later found to be due to the choice of the radio-active label for measurement of $^{14}CO_2$ output following a tracer dose of the labelled branched-chain amino acid (B.C.A). Parallel studies on the first two enzymes concerned with the catabolism of the B.C.As were also carried out in liver and gastrocnemius muscle to understand better the control mechanism and chief site of oxidation. The preliminary studies demonstrated dehydrogenase activity in skeletal muscle as well as in liver. It was the first demonstration of this enzyme activity in rat skeletal muscle. Moreover, the degree of enzyme adaptation led to the hypothesis that skeletal muscle was the chief site of both oxidation and its control. Further studies were carried out to observe the effects of feeding a protein-free diet to rats of varying ages for periods of 1, 2 or 3 weeks. Enzyme activities and oxidation were measured. This gave further support to the original hypothesis. Subsequently a more accurate measure of skeletal muscle leucine oxidation was developed. By using the method of

a constant tail vein infusion of a radioactive tracer *in vivo* it was possible to estimate accurately the total body flux of leucine, together with total body protein turnover and rates of leucine oxidation. Similarly, by giving a constant infusion of the same radioactive tracer to the perfused hind-limb of an identically treated rat, it was possible to arrive at a more accurate measure of the contribution of skeletal muscle to total body leucine oxidation.

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INTRODUCTION

The initial impetus for this work came from the long-standing interest of this Unit in protein energy malnutrition in children (Waterlow, et al 1960; Waterlow & Alleyne, 1971). The work was primarily concerned with examining the metabolic and biochemical changes produced by protein malnutrition. Protein malnutrition, when it reaches the stage of clinical illness, represents a breakdown of adaptive mechanisms (Waterlow, 1968). Thus, as Waterlow & Alleyne (1971) pointed out, progress in detecting and preventing protein malnutrition depends ultimately upon a better understanding of adaptive changes at the level of both the whole organism and the cell.

There is a wide clinical spectrum of malnutrition from the predominantly oedematous child with Kwashiorkor to the wasted marasmic child. In attempts to understand these extreme forms of malnutrition and the intermediate stages of the disease more attention is now being paid to the significance of fundamental biochemical changes. Definition of those changes which represent breakdown of the adaptive mechanisms is clearly important (Waterlow, 1968).

Animals are able to survive long periods on diets in which protein is reduced or omitted (Mendes & Waterlow, 1958). This capacity to survive suggests the presence of mechanisms for adaptation which limit the effects of altered nutrient intake on metabolism and thus on the composition of the body. The process of adaptation may need to be

distinguished from the responsiveness of the body to short-term changes in nutrient supply. This distinction relates not just to the time-period over which adjustments occur in relation, for example, to food supply, but perhaps also to the mechanisms which are brought into play. Thus there is a great deal of evidence to suggest that very short term responses occur in the hepatic metabolism of amino-acids in the few hours after a meal. In the rat an influx of amino acids leads to a very rapid increase in ornithine - δ - transaminase; the enzyme returns promptly to the pre-feeding levels within 24 hours (Kaplan & Pitot, 1970). However, if protein is withheld from a rat's diet for a period of days, then further changes in hepatic protein metabolism occur which tend to maintain body stores of protein even though the capacity to respond to a sudden inflow of amino acids is reduced (Schimke, 1962). Whereas the immediate response in ornithine - δ - transaminase is probably stimulated *directly* by the *concentration* of amino acids in the tissues, the slower adaptive response may depend not only on the level of an amino acid but also on the circulating level of insulin or other hormones. These examples are given to illustrate the problem of distinguishing between responsiveness and adaptation; no claim is made, however, that there is an absolute distinction between the two processes. Moreover, it is probable that adaptation includes not only changes in hormonal pattern, but also longer term effects on body composition.

The processes of adaptation obviously depend on the nature of the change in diet and perhaps on whether a deficiency in body stores or a change in function has already developed. Thus if the animal is deficient in protein but receives a diet adequate in all respects, other than protein, one would expect the adaptation to be concerned

primarily with the conservation of body protein. This somewhat teleological argument is borne out experimentally; rats or Man fed a low protein diet show an initial rapid decline in the excretion of urinary nitrogen, followed by a slower decrease until the nitrogen output reaches a steady minimum or 'endogenous' level (Munro, 1964). This low protein diet leads to a series of changes in tissue protein metabolism which vary considerably from one organ to another. In short-term experiments, very labile organs, such as liver, pancreas and small intestine are major contributors (Munro, 1964) to the excretion of N in the urine, whereas in more prolonged experiments muscle becomes a major source of protein (Mendes & Waterlow, 1958; Waterlow & Stephen, 1966). Eventually, the proportion of the initial protein lost from muscle may approximate or exceed the percentage lost from liver and other more labile tissues.

The eventual effect of the adaptation is a redistribution of body protein. The major loss is borne by muscle and skin (in the rat), while the protein content of essential tissues such as brain, myocardium and kidneys, is relatively well preserved. Hormones probably play an important part in bringing about this redistribution.

Starvation involves different metabolic processes of great importance in relation to amino acid and protein metabolism (Cahill, 1971). There is a need for glucose as a substrate for certain tissues, eg. brain, which is generated by gluconeogenesis from amino acids (Harper, 1965). Thus in starvation the amino acids serve an additional function to that involved in protein metabolism per se, and there is a greater breakdown and a higher rate of nitrogen excretion in the urine than occurs in the protein depleted animal (Schimke, 1962).

(i) Branched-chain amino acids in malnutrition

One aspect of the adaptive mechanisms which has been investigated in this thesis is the metabolism of branched-chain amino acids and the relevance of this approach to malnutrition must first be considered. In 1963, Holt and his colleagues first showed that the total amounts of free amino acids in the plasma of malnourished children are one half of the normal value and that there was a distorted pattern of individual amino acids in children with Kwashiorkor. The levels of the branched-chain amino acids were markedly reduced whereas the concentrations of lysine, histidine and phenylalanine were little changed. In contrast, there was a rise in the concentrations of some non-essential amino acids. So consistent did this finding appear to be that Whitehead (1964) developed a simple test for protein deficiency by assessing the degree of distortion and expressing the results as a ratio of selected non-essential to essential amino acids in the plasma (the N:E ratio). Thus the greater the distortion the higher the N:E ratio (Whitehead, 1969). There is general agreement (Whitehead & Dean, 1964; McLaren, 1965; Widdowson & Whitehead, 1964) that the amino acid ratio is usually elevated in patients with Kwashiorkor, but not in all malnourished children. An infection or a diet low in calories reduce the distortion and return the ratio towards normal. The intake of protein immediately before the test also proved to be important since the ratio returned to normal within 1-2 days of refeeding the child suffering from Kwashiorkor (Ityorah et al, 1965). This suggested that the amino acid ratio is more representative of the extent to which protein intake is limiting than of the state of protein deficiency, per se. A simple state of protein depletion of body tissues could not be the key factor in causing

distorted amino acid levels, since marasmic children have a marked fall in the protein content of the body but the amino acid ratio remains normal. Conversely, a distorted amino acid ratio can be produced in healthy adults after a short period of 2-3 days on a low protein diet, before any appreciable loss of body protein occurs (Alleyne & Picou, 1971). Nevertheless, the ratio has been used in field studies to detect marginal disturbances in protein metabolism. Thus it seems not unreasonable to think of changes in branched-chain amino acids as either significant markers of the disease process or as intimately involved in the progression of the disease.

(v) Adaptation of the Branched-chain amino acids to protein depletion

Most studies on adaptive enzyme changes have been made on liver. However, both in man and in the rat the amino acids whose concentrations in plasma and tissues are most decreased in protein deficiency are the branched-chain amino acids (BCA s), particularly valine (Whitehead & Dean, 1964). According to Miller (1962), the extra-hepatic tissues are as capable as the liver of oxidising leucine whilst the findings of Mortimore (1970) suggest that valine is not oxidised at all in the liver. The lowered levels of BCA s in plasma seen in protein depletion suggest an inability to conserve these amino acids (Table 1). However, McFarlane & Von Holt (1969 a) showed a decreased oxidation of DL[2^{14}C] leucine in the rat (in vivo) fed a protein deficient diet for eight weeks. In view of these findings it seemed of interest to investigate in more detail the adaptive capacity of the enzymes which catabolise branched-chain amino acids, not only in liver but also in extra-hepatic tissues (skeletal muscle). In comparison with liver, very little is knownabout adaptive changes in muscle enzymes!

TABLE I

Amino Acid levels in plasma of rats fed diets of different NDp:E ratio[1] and the observed N:E ratio[2] in each group of rats.

DIET	0.10 NDp:E	0.035 NDp:E (plasma amino acid: nmoles/ml plasma)	0.00 NDp:E
AMINO ACID			
ASPARTATE	32.8	49.3	73.7
THREONINE	90.2	43.6	65.6
SERINE	313.0	437.0	654.0
ASPARAGINE	70.0	49.8	43.8
GLUTAMATE	225.0	244.0	275.0
GLUTAMINE	675.0	781.0	901.0
GLYCINE	236.0	320.0	472.3
ALANINE	840.0	802.0	523.0
VALINE	233.0	163.0	94.7
ISOLEUCINE	93.9	64.5	39.2
LEUCINE	148.0	115.2	81.3
TYROSINE	84.4	30.8	18.8
PHENYLALANINE	48.7	42.3	39.4
METHIONINE	118.0	68.2	20.2
HISTIDINE	86.0	99.1	130.0
ARGININE	65.9	107.0	76.7
LYSINE	824.0	410.0	362.0
All rats weighed 65g			
Whitehead's N:E ratio	2.78	4.89	7.87

[1] NDp:E — Ratio of energy supplied by utilizable protein : total metabolizable energy.

[2] Whitehead N:E ratio — Ratio of defined non-essential amino acid : essential amino acids.

Non Ess:	ALANINE	Ess:	LEUCINE
	GLYCINE		ISOLEUCINE
	SERINE		PHENYLALANINE
	GLUTAMATE		VALINE
	GLUTAMINE		TYROSINE
			THREONINE
			METHIONINE

(iii) Degradation of Branched-chain amino acids

The metabolic pathways from the BCA s to their final products acetyl CoA, acetoacetate and succinyl CoA have long been elucidated (Meister, 1965). The steps involved in the conversion of leucine, for example, have been identified by isotopic experiments and more recently by enzymatic studies. All the BCA s initially follow a similar pattern, i.e. transamination to their respective α - oxo acid, followed by irreversible oxidative decarboxylation to the corresponding acyl CoA derivative. The remaining steps are analogous to those of fatty acid oxidation. The decarboxylation step is not reversible; this is compatible with the inability of animals to synthesise the BCA s from intermediates other than the analogous α-oxo acids. The scheme (Scheme I) for the degradation of leucine illustrates the well-known ketogenic properties of this amino acid. Although the degradation of valine initially proceeds by a series of reactions similar to those involved in leucine catabolism (Scheme I) the end products are quite different, since valine is glycogenic (Rose et al, 1942). Isoleucine is ketogenic under certain conditions, but under others leads to the formation of carbohydrate. The significance of the BCA s for the synthesis of cholesterol has also been established (Symposium on Cholesterol Metabolism, 1955).

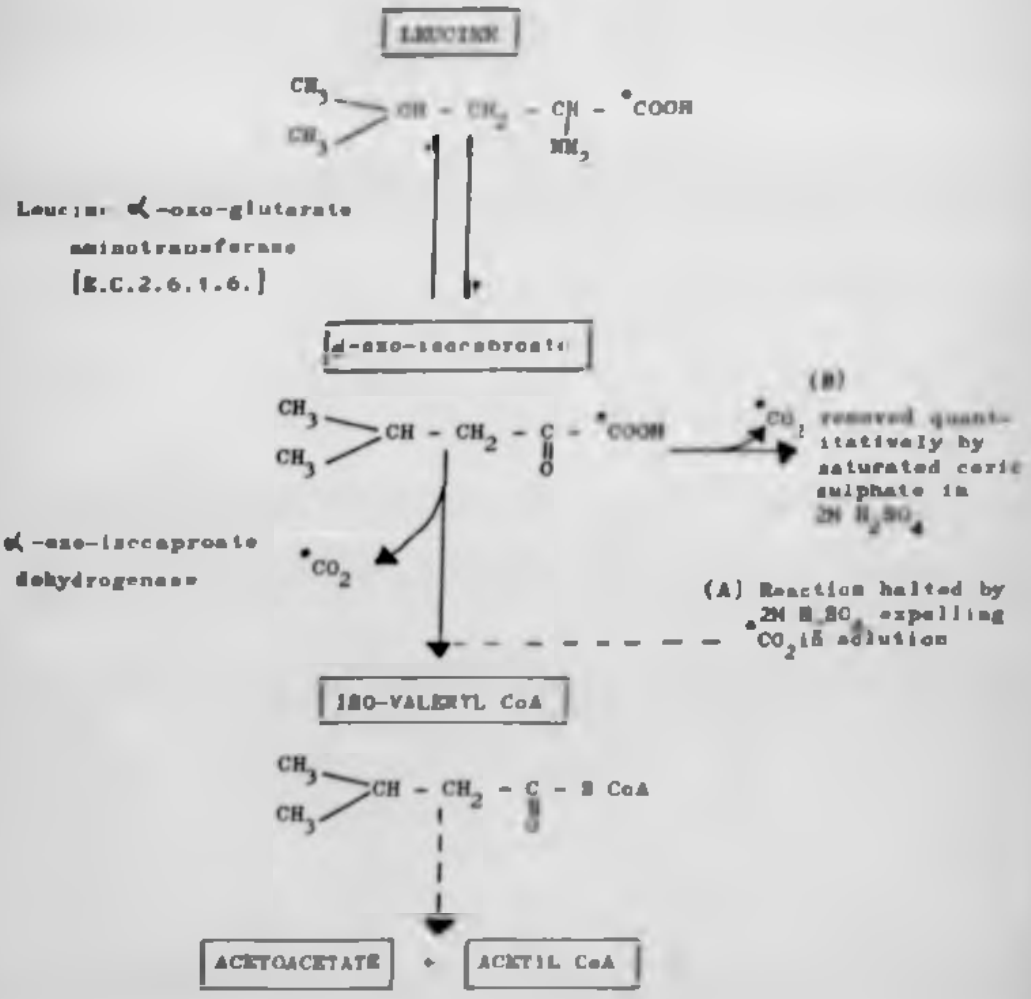
(iv) Site of BCA Degradation

The BCA s have long been considered unusual among the essential amino acids in that catabolism was presumed to occur predominantly in extra-hepatic tissue. This view was supported by results obtained by liver perfusion studies and on the eviscerated surviving rat (Miller, 1962). Miller showed that the extra-hepatic tissues of the

SECTION II B (i)

SCHEME I

Reactions involving the radioactively labelled carboxyl group in L-[1-^{14}C] leucine



eviscerated surviving rat could oxidise DL[1-^{14}C] leucine as effectively as the isolated perfused liver. There are however, several objections to his experimental protocol. The rats were starved for 16–18 hours before the operation and diluted blood was used as the perfusate. Therefore the liver enzymes may have adapted to starvation conditions. Furthermore, no allowance was made for differences in intra cellular specific radio-activity that might arise through the administration of the same amount of DL[1-^{14}C] leucine in preparations of very different weights (eg. liver vs eviscerated rat). This makes it difficult to draw any firm conclusions from the data, except that liver and extra-hepatic tissues do oxidise DL[1-^{14}C] leucine.

(v) Oxidation of BCA s by muscle

Several groups of workers had suggested that muscle might be the main site of BCA oxidation before this thesis was begun. Their evidence was based on the demonstration of $^{14}CO_2$ production from radioactively labelled leucine in isolated perfused heart (Clarke, 1957) or incubated diaphragm (Manchester, 1965).

Young (1970) also pointed out that skeletal muscle has the highest total BCA transaminase activity of all tissues in the rat, being approximately 100 times the amount of enzyme in the liver. He concluded that the catabolism of the BCA s took place mainly in muscle. Elwyn's (1970) work supports this view; for most amino acids the greater part of the load absorbed into the portal vein following a protein meal is metabolised in the liver and the amounts transferred to the peripheral blood are small; the BCA s are exceptions in that a larger proportion of them pass into the general circulation, presumably

being transaminated in muscle and kidney.

In spite of these studies, until the present thesis was begun no quantitative evidence had been produced to support the hypothesis that skeletal muscle was the major site for BCA oxidation. Moreover, quantitative measurements of total body leucine oxidation and of leucine oxidation by individual organs had not been attempted. Although a great deal of work had been carried out on the BCA transaminases and dehydrogenases, the issue was confused by the diversity of choice of the tissues or animals studied. A short review of the knowledge up to that time will illustrate this point.

(vi) Aminotransferases

Ichihara et al (1966) and Taylor & Jenkins (1966) first isolated and characterised a specific branched-chain aminotransferase [E.C.2.6.1.6.] in Hog Heart. Subsequently, three types of aminotransferases (isozymes I, II and III) were reported in various tissues of the rat and hog (Aki et al, 1968; Aki et al 1969; Ogawa et al, 1970). These isozymes could be distinguished either by DEAE cellulose chromatography or by immunological techniques. The properties of these enzymes are summarized in Table 2.

In the hog there is no evidence of isozyme II (Aki, Tokojima & Ichihara, 1969) but isozymes I and III are widely distributed. Ogawa et al, (1970) have shown that isozyme III is only found in the supernatant fraction of hepatoma cells. In the rat, Aki, Ogawa & Ichihara (1968) showed that isozyme I was distributed evenly between the supernatant and mitochondrial fractions of the liver. It transaminated all three BCA s and had a relatively low km. Isozyme II, however, was found exclusively in rat liver, mainly in the mitochondrial fraction

TABLE 2

MICHAELIS CONSTANTS OF BRANCHED-CHAIN AMINO TRANSFERASES & DEHYDROGENASES

	Donor Amino Acid	Source	km(mM)	pH	Reference
General branched-chain aminotransferase	Leucine	Pig Heart	3.8	8.6	Ichihara & Koyama (1966)
	Valine	Pig Heart	11.0		
	Isoleucine	Pig Heart	3.8		
Isozyme I	Leucine	Rat Liver	0.75	8.2	Aki, Ogawa & Ichihara (1968)
	Valine	Rat Liver	4.30		
	Isoleucine	Rat Liver	0.84		
Isozyme II	Leucine	Rat Liver	25.0	8.7	Aki, Ogawa & Ichihara (1968)
	Valine	Rat Liver	-		
	Isoleucine	Rat Liver	-		
Isozyme III	Leucine	Hog Brain	0.56	8.0	Aki, Yokojima & Ichihara (1969)
	Valine	Hog Brain	1.40		
	Isoleucine	Hog Brain	0.67		
BCA Aminotransferase	Leucine	Rat Liver	1.70	7.0	Present work
	Leucine	Rat Muscle	0.37		
BCA Dehydrogenase	Leucine	Rat Liver	0.17	7.0	Present work
	Leucine	Rat Muscle	0.17		
	Leucine	Rat Liver	0.20	6.8	Wellmeier & Harper (1970)

and had a high km for leucine; the other BCA s showed little or no activity with the enzyme. Induction of isozyme II was observed mainly in the supernatant fraction. Isozyme III was almost exclusively in the brain. Isozymes I and III have quite similar properties and can transaminate all three branched-chain amino acids at approximately equal rates. The km of isozyme I for valine is considerably higher than for leucine and isoleucine, the values for which are about the same (Aki et al, 1968). Krebs (1972) has discussed the principle of control of BCA degradation through km, as first suggested with special reference to amino acid metabolism by Mallette, Exton & Park (1969). Thus, if the km values are in general high compared to concentrations of free amino acids, then any increase in amino acid concentration in blood and tissues automatically causes an increased rate of amino acid degradation. However, in illustrating this point, Krebs gave km values for the isozyme I found in hog heart. He then proceeded to study the effects of a protein-free diet on rat liver transaminases, which elsewhere have been reported as having low km values (Aki et al, 1968). No reports are available to suggest that rat skeletal muscle transaminases have the same km values as those described in hog heart. In fact, in the present studies (Table 2) the km value for leucine was 0.37mM in rat skeletal muscle; this is an order of magnitude lower than the values taken by Krebs to represent skeletal muscle transaminases in the rat. However, the principle remains, that control by km is a "fine control" mechanism. There is an additional "coarse control" brought about by adaptive adjustments of the enzyme capacity, through variations either in the rate of enzyme synthesis or in the rate of enzyme degradation.

Studies on the adaptation of branched-chain aminotransferases have been almost exclusively in rat liver and kidney. Shirai et al, (1971) found that isozyme II was more responsive to induction than isozyme I in liver. Isozyme II had a shorter half life and was rapidly induced by cortisol, high protein feeding, and gluconeogenic conditions. Isozyme I in liver was not affected by any of these. The kidney isozyme I was induced only after continuous administration of cortisol over 7 days; hypophysectomy also induced the enzyme whilst adrenalectomy decreased it. Since McFarlane & Von Holt (1969 b) had shown a greater proportion of their enzyme preparation in liver to be in the mitochondrial fraction, they were probably studying isozyme II. However, although they showed that low protein feeding induced an adaptive response, this was only observed in the mitochondrial and not in the supernatant fraction.

Mimura et al (1968) studied the transaminases in rat skeletal muscle, as well as liver and kidney. Their measurements showing induction in both muscle and liver transaminase activities by a protein-free diet were in direct conflict with the reduced enzyme activities of liver BCA transaminases in protein depleted rats reported by McFarlane & Von Holt (1969 b). Mimura et al also found that after administration of hydrocortisone for 3 days, BCA transaminase activity was elevated in both liver and muscle. The activity of BCA transaminase was greater in muscle than liver. These workers did not distinguish which isozyme was being studied but since the preparation was equally reactive with all three BCA's this would suggest that they were in fact looking at Isozyme I. Again this is in conflict with the work of Shirai & Ichihara (1971) who found no effect of cortisol on isozyme I in rat liver; they did show that kidney BCA transaminase (isozyme I) was induced but only after several days

administration of cortisol.

The picture, therefore, at the time when this work was begun, was rather confused. Perfusion experiments suggested that BCA s are mostly oxidised in extrahepatic tissues. Nevertheless, the majority of studies on the activity of BCA transaminases (the enzymes which initiate oxidation) had been made on liver. A protein-depleted diet was found to cause an adaptive fall in one of the liver enzymes (isozyme II, McFarlane & Von Holt, 1969 b) whilst a protein-free diet was found to cause an increase in the liver enzyme (isozyme I, Mimura et al, 1968).

The conflicting evidence reported above showed an obvious need to investigate the BCA transaminases in several tissues, particularly since there was direct conflict not only on the reported effects of protein-depletion on BCA oxidation in vivo but also on the manner of adaptation of the transaminases in rat tissues.

(VII) Dehydrogenases

Transamination of the three BCA s leads to the following α-oxo acids on which the dehydrogenases react :-

leucine → α-oxo-isocaproate (αKIC)

valine → α-oxo-isovalerate (αKIV)

isoleucine → α-oxo-methylvalerate (αKMV)

Danner & Bowden (1966) demonstrated separate BCA dehydrogenases in intact rat liver mitochondria . A preliminary note reported the existence of separate dehydrogenase mechanisms also in human and bovine leucocytes (Goodde, Hüfner et al, 1967). Connelly et al (1968) then isolated a partially purified enzyme from bovine liver cytoplasm inactive towards αKIV but active with αKIC and αKMV. This was

tentatively called an αKIC; αKMV dehydrogenase. The dual specificity of this preparation could be accounted for in two ways. Either it contained two different enzyme complexes that were purified in parallel, or there existed one enzyme complex which had both activities. Bowden & Connelly (1968) were able to demonstrate a single enzyme complex by physical, chemical and kinetic treatments. They also demonstrated separate αKIV dehydrogenase activity almost exclusively located in the mitochondria. The former single enzyme complex was distributed equally in both supernatant and mitochondrial fractions of bovine liver. Wolhueter & Harper (1970) were unable to distinguish separate dehydrogenases in the rat liver mitochondria as attempts to purify the enzyme were rather unsuccessful. Earlier work by McFarlane & Van Holt (1969 b) also demonstrated BCA dehydrogenase activity in rat liver mitochondria, but because of the lack of evidence of distinctive BCA oxo acid dehydrogenases at that time, they were reluctant to attribute the activity to a specific enzyme. Nevertheless, in view of more recent work we may conclude that they were actually measuring the dehydrogenase in rat liver mitrochondria. Interestingly, their work suggests the possibility of two separate enzymes located in the mitochondria and cytoplasm.

Both Connelly et al (1968) and Wolhueter and Harper (1970) looked at dehydrogenase distribution in various tissues of the rat and other animals. In the rat, the greater part of the activity was to be found in the liver and kidney while in beef the activities were more evenly distributed throughout liver, kidney, heart and skeletal muscle. At the time this thesis was begun (1972) no activity of the dehydrogenase had been found in rat skeletal muscle.

(VIII) Adaptation in Oxidation of BCA s

Before 1972 only a few workers had studied adaptation in BCA oxidation. McFarlane & Von Holt (1969 a) demonstrated a steady decrease in the oxidation of DL[$2^{14}C$] leucine in rats given a diet containing 20g protein/kg for eight weeks. This decline accounted for a 75% fall in oxidation from week two to week eight. Neale (1972), however, was unable to show any adaptation in the oxidation of U-^{14}C labelled branched-chain amino acids in rats given a diet containing 10g protein/kg for 2 weeks. Both these investigations may be criticised because of the choice of label; this will be discussed in detail at a later stage.

Further studies by McFarlane & Von Holt (1969 b) were undertaken to investigate the site of control of this oxidation. They concluded that there was a block in the decarboxylation of leucine with a reduction in the mitochondrial transaminase and D-amino acid oxidase in liver. Their work also included measurements of the BCA → α KIC dehydrogenase, despite their unwillingness to recognise this. They showed the possibility of two separate enzymes located in the mitochondrial and cytoplasmic fractions from liver homogenates; the mitochondrial enzyme was decreased in protein depleted rats but the cytoplasmic enzyme remained unaltered. The reduced mitochondrial enzyme activity led to an increase in α - oxo-isocaproic acid (α KIC) in the medium. This was reflected in vivo by an increased urinary output of the branched-chain α oxo acid. Despite this evidence, it was difficult for them to suggest an absolute decrease in dehydrogenase enzyme activity since they found no significant difference in the oxidation of labelled α KIC by rat liver mitochondria from control or protein depleted rats.

No other workers at that time had attempted to correlate adaptation in BCA oxidation in vivo with parallel studies on enzyme adaptation. For example, Mimura et al (1968) only studied the aminotransferases in liver, kidney and muscle of rats fed a 0% or 75% protein diet for 10 days. Wolhueter & Harper (1970), on the other hand, looked only at the adaptation of the liver mitochondrial dehydrogenase enzyme in rats fed 0, 9, 18, 30, 50 and 80% casein diets for 5 days. Thus, there was no attempt to relate the adaptation of either enzyme to the rate of oxidation of BCA in vivo, and there was a lack of studies of skeletal muscle. The present thesis set out to investigate the existence and activity of BCA dehydrogenases in skeletal muscle, and to study any adaptation in BCA oxidation due to protein depletion or starvation together with measurements of the aminotransferases and dehydrogenases under the same conditions. Because extra-hepatic tissues were thought to be chiefly responsible for leucine oxidation (Miller, 1962) and because perfused livers gave a net continuous release of BCA s, leading to their accumulation in the medium (Bloxham, 1971), the studies were planned to include measurements of both liver and skeletal muscle enzymes. Conclusions drawn from these enzyme studies in the early part of the work led to the involvement of the perfused rat hind-limb preparation. Thus quantitative measurements of leucine oxidation could be obtained in a more physiological preparation of skeletal muscle. Furthermore, by using the constant infusion technique developed by Waterlow & Stephen (1968) and described more recently by Garlick & Marshall (1972), a quantitative measure of whole body BCA oxidation could be obtained. This made it possible to assess the contribution of skeletal muscle to oxidation of BCA s in the whole body. The constant infusion method also allows us to measure both whole body protein turnover and amino

acid flux. Similar data can be obtained from the perfused hind-limb.

(iv) The Perfused Hind-Limb Preparation

The hind-limb preparation to be described is a modification of techniques employed by Jefferson, Koehler & Morgan (1972) and Ruderman, Houghton & Hems (1971). This approach offers a number of advantages for studying the control of skeletal muscle metabolism under a variety of well-defined situations. 1) The preparation consists mainly of muscle; 2) substrates and hormones are delivered to the cells by the normal capillary bed; 3) the preparation remains in a good physiological state during perfusion for periods up to 3 hours, as judged by several criteria; 4) large samples of skeletal muscle and perfusate can be obtained rapidly and with ease for estimating enzyme activities, metabolic intermediates or substrate levels.

The major disadvantage of the preparation is the inclusion of adipose tissue, connective tissue, skin and bone. Objections which might arise because of their possible contribution to the oxidation of BCA s are partly overcome by the routine procedure of removing the adipose tissue overlying the psoas muscles, tying off the tail and ligaturing the major vessels to the skin. The skin is left on the limb to minimise reduction in hind-limb temperature and evaporative losses from the exposed tissues.

SUMMARY

Although in recent years the metabolic pathways of the BCA s have been elucidated (Meister, 1965) the chief sites of oxidation and the mechanisms involved in adaptation have yet to be investigated in detail.

Conditions such as Maple Syrup Urine disease (McKenzie & Woolf, 1959) and Jamaican Vomiting Sickness (Tanaka et al, 1972) have led us to recognise blocks in the oxidative pathway as possible candidates for control points in the metabolism of the BCA s. Certainly studies on malnourished rats (McFarlane & Von Holt, 1969 a & b) have shown the body's ability to bring about a net reduct on in the oxidati on of these essential amino acids.

Since there is widespread agreement that muscles can oxidise leucine to CO_2 (Manchester, 1965; Young, 1970) it seemed paradoxical that the BCA dehydrogenase had not been demonstrated in this tissue (Volbuster & Harper, 1970) at the time when this thesis was begun. Moreover, many workers were still pointing to the liver as the chief organ in which the BCA's are oxidised when evidence was accumulating to suggest that muscle was the major site (Miller, 1962; Young, 1970; Elwyn, 1970; Bloxam, 1971). With a modification of the method of McFarlane & Von Holt (1969 b) and with gentler homogenisati on of muscle tissue, ~~a~~ KIC dehydrogenase activity was observed in the gastrocnemius, extensor digitorum longus (EDL), soleus and plantaris muscles of the rat (Table 3). Furthermore, as discussed in section II C because of the rapidity with which the muscle dehydrogenase activity adapted in times of dietary stress, before any adaptation was observed in the liver enzymes, it was necessary to use the hind-limb preparation to study the mechanisms involved in adaptation and to quantitate the contribution of skeletal muscle to the oxidation of leucine in the whole body. In parallel experiments, total body leucine oxidation was determined in vivo by the constant intravenous infusion of a tracer dose of radioactive L [$1^{14}C$] - leucine.

TABLE 3

Enzyme activities in different muscles of the rat given a 10% NDp:E diet. L-leucine α-oxoglutarate aminotransferase [EC.2.6.1.6] and α-oxo-isocaproic acid dehydrogenase (αKIC) activities. (μmoles leucine / g wet wt / hr)

Muscle type	(Rat wt) (g)	Leucine : α-oxo-glutarate aminotransferase	αKIC dehydrogenase
Extensor digitorum longus (EDL)	(70)	15.79	0.79
Soleus	(70)	15.88	0.99
Plantaris	(70)	17.74	1.51

SECTION II

THE EFFECT OF DIET ON OXIDATION OF THE BRANCHED CHAIN AMINO ACIDS.

A The effect of low-protein feeding and starvation on the oxidation of DL- and L-isomers of leucine in vivo.

(i) INTRODUCTION

The rate of leucine oxidation measured *in vivo* by monitoring the excretion of $^{14}CO_2$ after a single intraperitoneal tracer dose of DL[2-^{14}C] leucine, has been shown to decrease in adult rats fed a low-protein diet (20 g casein/kg) for 8 weeks (McFarlane & Von Holt, 1969 a). The authors suggested that the decline in oxidation rate resulted from a block in the decarboxylation of α-oxo-isocaproic acid in response to a reduced activity of the leucine: α-oxo-glutarate aminotransferase enzyme. This was confirmed by enzymatic assays of mitochondrial fractions prepared from the liver (McFarlane & Von Holt, 1969 b).

However, Neale (1971), working with uniformly labelled ^{14}C amino acids in the L-isomer form only, failed to demonstrate any conservation of either leucine or valine when injected intravenously or intragastrically into protein-depleted weanling rats. Because of these discrepancies both in methodology and results, L[1-^{14}C] -leucine was chosen to study the effects of protein depletion on leucine oxidation *in vivo*. This choice, discussed extensively in Section IV(ii), also allowed a comparison with the *in vitro* data. Measurements were also made with [DL 1-^{14}C] leucine because of the possible involvement of the D-amino acid oxidase [E.C.1.4.3.3] and to document the degree to which the differences between the findings of McFarlane & Von Holt and Neale might result from the choice of label.

A (ii) ANIMALS AND DIETS

Female hooded rats (Animal Suppliers (London) Ltd) weighing 35-40g were housed three to a cage and allowed free access to a powdered diet which contained (g/kg) :- Casein (Casumen : Prideaux Milk Foods, Evercreech, Somerset) 109; maize starch (Corn Products Ltd, Manchester) 426; dextrinised starch (Corn Products) 272; solka floc (cellulose) (Johnson, Jorgensen & Wettre Ltd, London) 91; arachis oil, 45; mineral salt, 45; B-vitamin mixture, 10; fat soluble vitamin supplement, 0.9 and L-methionine (Sigma Chemicals Ltd, London) 0.9. See also Table 4.

Details of the mineral salt mixture, the B-vitamin mixture and fat-soluble vitamin supplement are given by Payne & Stewart (1972). The ratio of energy supplied by utilizable protein to the total metabolizable energy (NDp:E ratio) was 0.10(control di-t) and maintained growth at the rate of 3.0g/d.
A low-protein (L.P) diet was designed to maintain the animals at a constant weight over a 2 week feeding period; the appropriate NDp:E ratio for this was found to be 0.01. This diet was the same as the control (N.P) diet except that it contained 32.9g casein/kg, the difference being replaced by an equivalent weight of maize starch (Table 4).

A (iii) MATERIALS AND METHOD

All radioactive materials were obtained from the Radiochemical Centre, Amersham, Bucks. Specific radioactivities of amino acids were: L[1-^{14}C]-leucine (60 mCi/mmol), L[U-^{14}C]-leucine (10mCi/mmol) and DL[1-^{14}C]leucine (10 mCi/mmol).

SECTION IIA(ii) TABLE 4

COMPOSITION OF EXPERIMENTAL DIETS

	High Protein Control (H.P) (0.10 NDp:E) (g)	Low Protein (L.P) (0.035 NDp:E) (g)	Zero Protein (0.00 NDp:E) (g)
Maize Starch	2345	2773	2950
Dextrinised Starch	1500	1500	1500
Casein	600	175	-
"Solka Floc" (cellulose)	500	500	500
Arachis oil	250	250	250
J & F salt mixture	250	250	250
Vitamin B group	55	55	55
L-methionine	5.0	2.2	-
Fat-soluble vitamins	5.0	5.0	5.0
Total	5510	5510	

2,5 Diphenyloxazole was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks.

Amino acids were from Sigma Chemicals, London.

All other reagents were from British Drug Houses, Poole, Dorset.

Measurement of Catabolism of ^{14}C-labelled Branched Chain Amino Acids IN VIVO by Pulse Intragastric Dosage.

Animals were allowed free access to food and water until 5 hrs before testing. In this way, the stomach would be empty before the experiments began. The oxidation rates of various ^{14}C-labelled branched-chain amino acids were then assessed by the intragastric injection of the label in a solution of NaCl (9g/l, pH7.0 μCi per 100g body-weight) with the appropriate branched chain amino acid as carrier (1 μmol L-leucine/100g body-weight).

Accurate measurements of syringe weights before and after injection were used to determine the amount of label given to each animal. The animal was then placed immediately within a sealed glass container and expired $^{14}CO_2$ was trapped by drawing the expired air through three tubes in series, each containing 40ml 4N-NaOH.

Trapping was considered to be complete, because the third tube contained less than 1% of the trapped label. Collections were made for 3 hours because McFarlane & Von Holt (1969) observed that the percentage of the dose excreted as $^{14}CO_2$ reaches a plateau by 3 hours. Aguilar, Harper & Rameyenga(1972) also observed the constant production of $^{14}CO_2$ after the 3-hour time period with many of the amino acids. Valine, however, showed a slight decline in $^{14}CO_2$ production after this period which was thought to indicate a limited supply or increased demand

in other metabolic pathways.

The NaOH from the 3 tubes was pooled and 1 ml sample acidified with 2 ml 2M.H_2SO_4 in a Marie-Flask. Evolved $^{14}CO_2$ was trapped on a filter paper soaked with 0.25 ml phenylethylamine in a centre well. The filter paper was then counted with 13 ml of a 3:10 methanol/toluene mixture containing 0.4% P.P.O (2, 5 diphenyloxazole) in a Packard 2420 liquid scintillation counter.

A (iv) RESULTS AND DISCUSSION

In vivo oxidation of DL[$1^{14}C$] leucine was greater than that of L[$1^{14}C$] leucine in all groups except the animals on the control diet who were fasted for 48 hrs. Fasting produced a marked increase in the evolution of $^{14}CO_2$ except in the rats on the control diet tested with DL[$1^{14}C$] leucine, and fasting of the protein-depleted animals restored the output almost to the levels found in the control rats without fasting (Table 5). Oxidation of both DL [$1^{14}C$] leucine and L [$1^{14}C$] leucine was reduced in the animals given the low-protein diet.

The reduction in DL-leucine oxidation with low-protein feeding, seen by McFarlane & Von Holt (1969 a) in adult rats, has been confirmed with young growing animals. The use of the L-isomer has also shown that the results reflect changes in the normal leucine catabolic pathway and not simply alterations in the activity of D-amino acid oxidase E.C.1.4.3.3. The observations made by Neale (1971) with U^{14}C labelled leucine and valine in which he failed to find evidence of adaptation, may have been due to several points in his experimental procedure. Firstly, we do not know whether the diets were isocalorie and hence the animals could have been exhibiting

TABLE 5

In vivo oxidation of [1-^{14}C]-leucine using either the DL- or L-isomer in rats given a high- or low-protein diet or fasted.

(Mean values with standard errors where given)

Isomer	No. of rats in group	EDp:E ratio of diet*	Whether fasted for 48hr	Evolved $^{14}CO_2$ (% of dose given)
DL	2	10	-	31.3
	2	10	+	27.0
	3	3.5	-	11.1 ± 0.8 a
	3	3.5	+	22.0 ± 3.2 a
L	3	10	-	15.9 ± 0.3 bc
	3	10	+	29.0 ± 3.2 c
	3	3.5	-	4.0 ± 1.0 b
	2	3.5	+	10.9

Statistical comparisons of groups : values marked with the same letter differ significantly at the following levels : a, $P < 0.01$; b, $P < 0.001$; c, $P < 0.02$.

* Percentage of total metabolizable energy supplied by utilizable protein.

TABLE 3

In vivo oxidation of [1-^{14}C]-leucine using either the DL- or L-isomer in rats given a high- or low-protein diet or fasted.

(Mean values with standard errors where given)

Isomer	No. of rats in group	NDp:E ratio of diet*	Whether fasted for 48hr	Evolved $^{14}CO_2$ (% of dose given)
DL	2	10	-	31.3
	2	10	+	27.0
	3	3.5	-	11.1 ± 0.8 a
	3	3.5	+	22.0 ± 3.2 a
L	3	10	-	15.9 ± 0.3 bc
	3	10	+	29.0 ± 3.2 c
	3	3.5	-	4.0 ± 1.0 b
	2	3.5	+	10.9

Statistical comparison of groups : values marked with the same letter differ significantly at the following levels : a, $P<0.01$; b, $P<0.001$; c, $P<0.02$.

* Percentage of total metabolisable energy supplied by utilisable protein.

the pattern of oxidation seen in fasting rats (Table 5). Secondly, $^{14}CO_2$ excretion was measured after only 1 hour, at which time constant production of $^{14}CO_2$ has not been attained (Aguilar, et al. 1972). Finally, the use of $U^{14}C$ labelled branched-chain amino acids for this particular study is in question and will be discussed elsewhere (Section IV (ii)).

B Measurement of Branched-Chain Amino Acid α-oxo-glutarate aminotransferase E.C.2.6.1.6. and α-oxo-isocaproate dehydrogenase in liver and muscle tissues.

(i) Principles

The principles of the assay are shown in Scheme I. Amino acids labelled in the carboxyl group will result in labelled α-oxo acids. Subsequent decarboxylation by the dehydrogenase will yield $^{14}CO_2$. Therefore the amount of $^{14}CO_2$ plus any labelled α-oxo acid not decarboxylated will be a measure of the transaminase activity. In the presence of ceric sulphate (Meister, 1952), α-oxo-acids undergo quantitative decarboxylation. At a given substrate concentration the enzymically produced $^{14}CO_2$ from amino acids labelled in the carboxyl group reflects the dehydrogenase activity (A) and the sum of the enzymically liberated plus ceric sulphate liberated $^{14}CO_2$ (A + B) reflects the aminotransferase activity. These principles, first described by McFarlane & Von Holt (1969 b), allow the separate estimation of the activities of the α-oxo acid dehydrogenases and aminotransferases.

Leucine and valine α-oxo-glutarate aminotransferase activities were measured in both liver and muscle by a modification of the assay system described by McFarlane & Von Holt (1969 b). Both

liver and muscle were homogenised at 4°C by hand in a Dual glass homogenizer. Hand homogenisation was necessary because preliminary work showed that dehydrogenase activity was destroyed if more vigorous techniques were used. For example, Wolhueter, et al (1970) were unable to demonstrate significant amounts of the dehydrogenase activity in skeletal muscle despite the findings of several workers showing that leucine was oxidised in the peripheral tissues (Miller, 1962; Manchester, 1970). In their investigation on dehydrogenase activity the Polytron was used, which is known to disrupt several enzyme complexes. As the dehydrogenase is thought to be complexed to several cofactors (Connelly, Danner & Bowden, 1968) and located on the outer wall of the inner mitochondrial membrane (Johnson & Connelly, 1972) it was important to investigate other methods of homogenisation and re-examine the possibility of dehydrogenase activity in skeletal muscle.

B (ii) Optimum assay conditions were assessed for both aminotransferase and dehydrogenase activity in liver and muscle, with respect to their pH maxima, substrate concentration of leucine or valine, and α-oxo-glutarate and cofactor requirements (Figs. 1-4). For muscle final concentrations of 10mmol leucine/ℓ and 15mmol α-oxo-glutarate/ℓ were used at pH 7.0 in 25 mM Sorensen's phosphate buffer (disodium hydrogen phosphate, 25mmol/ℓ, adjusted to pH 7.0); for liver the same system was used except that the final concentration of α-oxo-glutarate was 10mmol/ℓ.

In the first stage of the assay (Scheme I) the $^{14}CO_2$ evolved (A) was taken as a measure of the dehydrogenase activity of the crude homogenate system. Counts were proportional to homogenate concentration

and linear for the 60 min. period of assay at 37°C. Allowance was made for the non-specific evolution of $^{14}CO_2$ from L[1^{14}C] leucine on the addition of 2M-H_2SO_4 at the end of the incubation by routinely incubating blanks and subtracting this from the experimental values. After centrifugation of the incubation mixture at 3000g for 10 minutes, 1 ml of the supernatant fraction was assayed for residual ^{14}C α-oxo-acid by chemical decarboxylation with saturated ceric sulphate in 2M-H_2SO_4. Any non-specific evolution of $^{14}CO_2$ from the action of saturated ceric sulphate on L[1^{14}C] -leucine was again routinely measured in blanks at this stage. In summary, then, the pre-assay mixture containing L[1^{14}C] leucine, α-oxo-glutarate and Sorensen's phosphate buffer at pH 7.0 was pre-incubated in a Warie flask at 37°C for 5 min. The reaction was then started by the addition of crude homogenate and halted 60 min. later by injecting 2 ml 2M H_2SO_4 through the rubber cap of the flask. $^{14}CO_2$ evolved was trapped in 0.25 ml phenylethylamine soaked in filter paper in a centre well. After 1 hr. the contents of the centre well were transferred to a scintillation vial and counted in the manner described in Section IIA (iii). The incubation mixture was then assayed as described above for residual ^{14}C α-oxo-acid and $^{14}CO_2$ trapped and counted in a similar manner as before.

RESULTS AND DISCUSSION

B (iii) Determination of optimum assay conditions for leucine amino-transferase and α-KIC dehydrogenase activity in liver and gastrocnemius muscle.

The decarboxylation of α-oxo-isocaproate is analogous to the conversion of pyruvate to acetyl CoA (Meister, 1965 a). The cofactors required in the pyruvic decarboxylase reaction are TPP, CoASH, Mg^{++},

NAD^+ and lipoic acid. McFarlane & Von Holt (1969 b) investigated these cofactor requirements in liver mitochondrial preparations and found that whilst NAD^+ or lipoic acid or both did not significantly influence the reaction, 2.0 mM Mg^{++}, 0.1 mM CoA, 1.0 mM ATP and 0.2 mM TPP did. Since I was using a crude homogenate, it was necessary to re-investigate these requirements.

The results are presented in Fig. 1, showing that addition of all or individual cofactors did not enhance dehydrogenase activity. One can only postulate that Mg^{++}, CoA and ATP in the crude homogenate were present in sufficient concentration to induce maximal activity. Lipoic acid is known to be bound firmly to enzyme protein and will not be removed during homogenization (Reed, 1960).

Pyridoxal 5' phosphate (Braunstein, 1960) and FAD (Reinert, 1960) are known to be cofactors for the aminotransferase. However, these cofactors are also firmly bound to the enzyme and the requirements were not studied. α-oxo-glutarate is known to transaminate readily with L-leucine (Meister, 1965 b) and the substrate enhancement of aminotransferase activity by α-oxo-glutarate (2 kg) was studied, together with the substrate saturation effect of L-leucine. Fig. 2 shows that in liver at 2 kg 10 mM, liver aminotransferase activity was still increasing with 15 mM leucine. As it was possible to achieve saturation of the enzymes with respect to kg concentration at 10 mM, an arbitrary decision was made for the assay medium to contain 10 mM L-leucine and 10 mM α-oxo-glutarate in Sorensen's phosphate buffer (25.0 mM) at pH 7.0. This concentration of leucine was also chosen in the gastrocnemius assay because it corresponded to the concentration for maximum activity found in muscle preparations and the increment in activity with concentrations

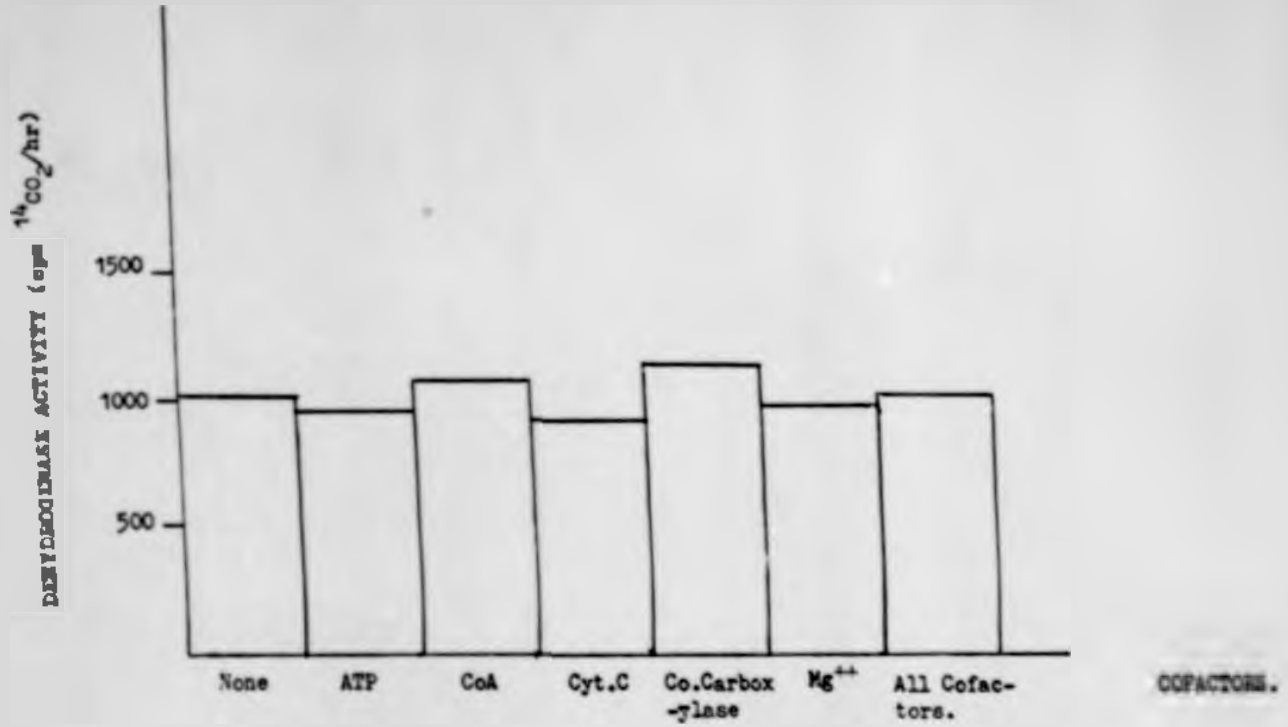


FIG. 1. Determination of co-factor requirements for αKIC dehydrogenase activity in liver. Final concentrations in 3.0 ml:-1 in 10 Homogenate (1.0 ml); leucine 10 mM (0.9 ml); akg 10 mM (0.1 ml); Cofactors in Sorensen's buffer pH 7.0; ATP 1.0 mM; CoA 0.1 mM; Co-carboxylase 0.2 mM; Cytochrome C, 0.1 ml and Mg^{++} 2.0 mM.

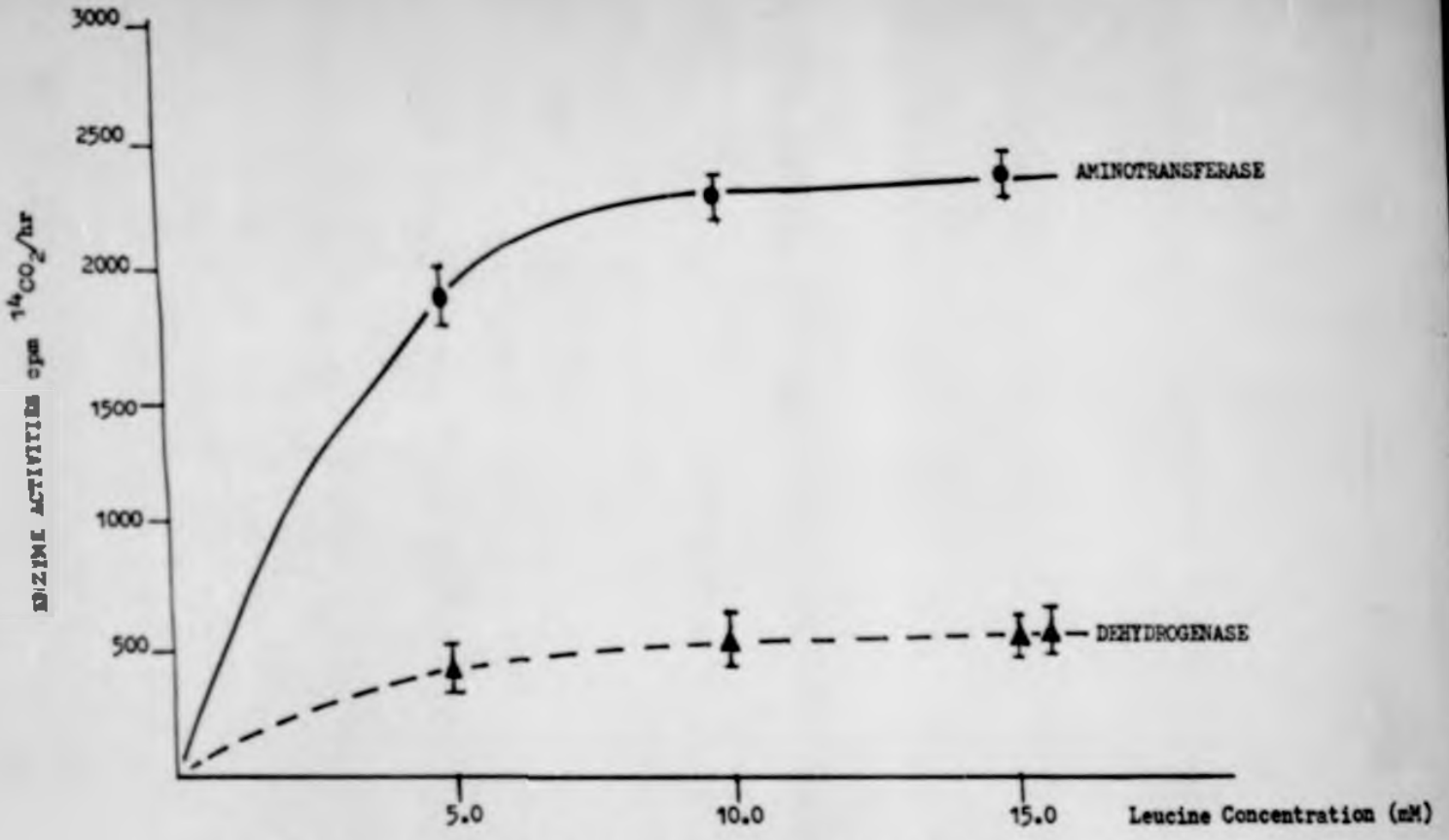


FIG. 2. The determination of maximum substrate concentration for leucine aminotransferase and αKIC dehydrogenase activity in liver. The assay employed a 1 in 10 homogenate (1.0 ml); leucine 0.9 ml; akg 10 mM (0.1 ml) and Sorensen's phosphate buffer, pH 7.0 (1.0 ml).

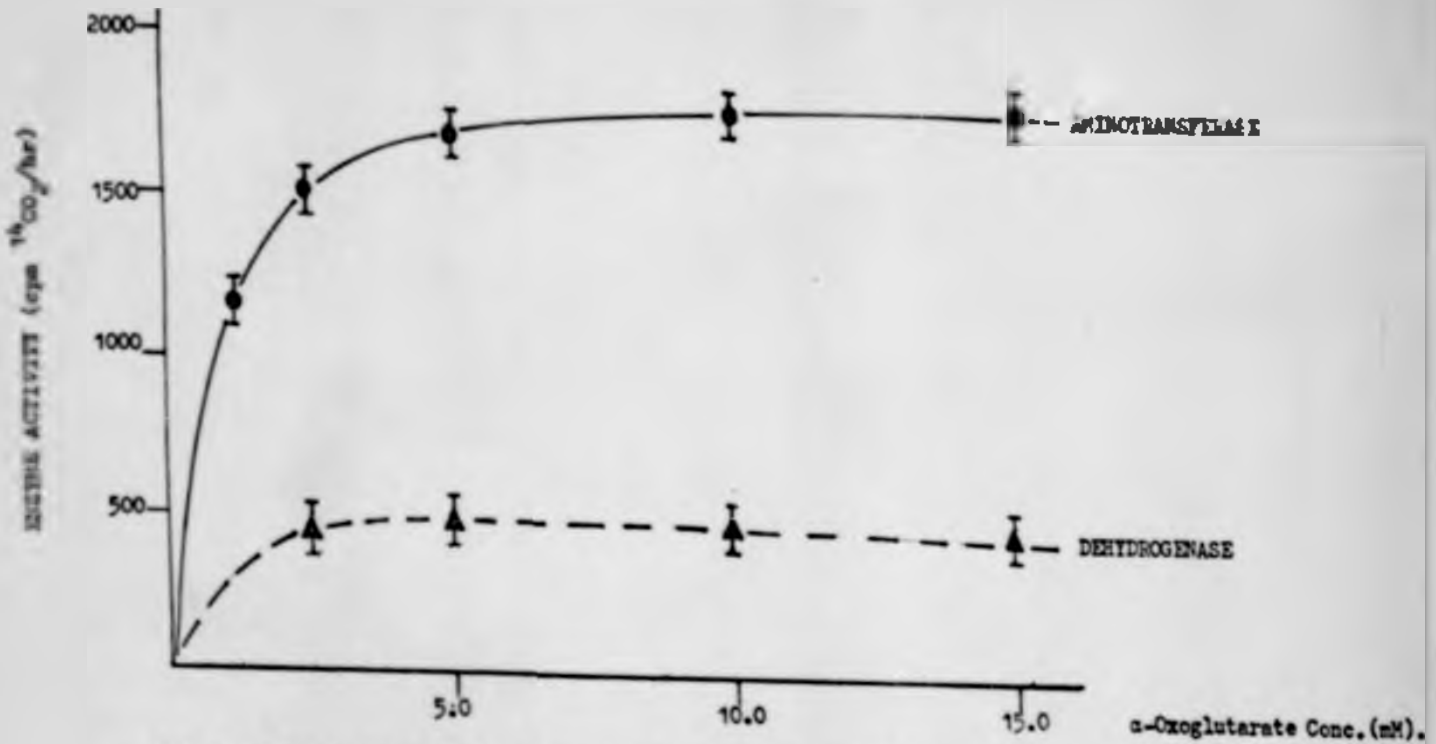


FIG. 3. Determination of the maximum concentration of akg for both liver leucine α-oxoglutarate aminotransferase and αKIC dehydrogenase activity.
1 in 10 homogenate (1.0 ml) Leucine 10 mM (0.9 ml); akg (0.1 ml); Sorensen's phosphate buffer pH 7.0 (1.0 ml).

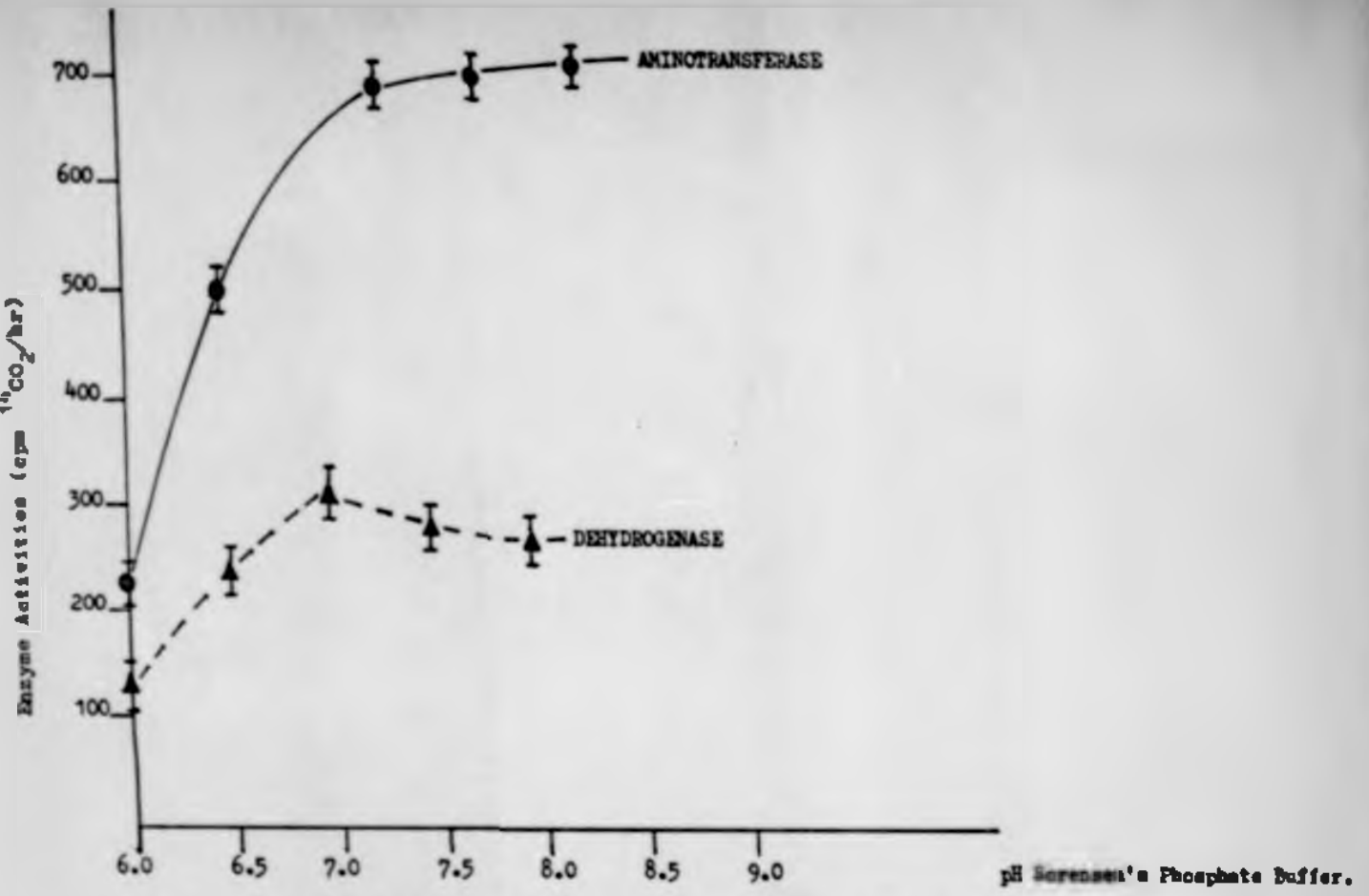


FIG. 4. pH optima for liver leucine αoxoglutarate aminotransferase and α KIC dehydrogenase activity. The assay consisted of a Sorensen's phosphate buffer (pH 7) 1.0 ml; a 1 in 10 homogenate 10 mN (1.0 ml); leucine 10 mN (0.9 ml) and akg 10 mN (0.1 ml).

of leucine above 10 mM was very small (fig.2). However, it was necessary to use 15 mM αKG to obtain maximal activity in muscle.

Initial experiments on boiled homogenate showed no catabolic activity. However, in the crude homogenate, catabolism of L[1-^{14}C]-leucine was both linear with time over a period of 60 min. and linear with respect to concentration of the homogenate. This established that production of $^{14}CO_2$ and of αKIC was the result of enzyme activity.

C (i) The Effect of Low-Protein Feeding on Leucine Catabolic Enzymes in Liver and Gastrocnemius Muscle.

McFarlane & Von Holt (1969 b) suggested a block in the decarboxylation of αKIC as a result of reduced αKIC dehydrogenase activity in liver mitochondrial preparations from rats fed 20g protein/kg for 8 weeks. A similar fall in the dehydrogenase activities for all three BCA's has also been demonstrated in liver homogenates from animals given a diet containing 90g protein/kg when compared with the response to a diet containing 800g protein/kg (Volbuster & Harper, 1970). Since oxidation of L[1-^{14}C] leucine was reduced in rats fed a low-protein diet (Table 5) the effects of the diet on activities of both the aminotransferase and dehydrogenase in liver and muscle were studied.

C (ii) IN VITRO oxidation in Cell-free extracts

Measurements were made with L[1-^{14}C] leucine in tracer doses on tissues from rats maintained on the same dietary regimes as described in Section IIA(i). Only animals previously given the diet with an NDpiE ratio of 0.10 were fasted. Liver and

gastrocnemius muscles were removed, frozen with solid CO_2 and stored at $-15^{\circ}C$ for subsequent assay. Preliminary experiments revealed no deterioration in enzyme activities during the storage period. Leucine α-oxo-glutarate aminotransferase E.C. 2.6.1.6. and αKIC-dehydrogenase activities were measured in both liver and muscle by the modified method used by McFarlane & Von Holt (1969 b) as described previously in Section IIB(ii).

C (iii) RESULTS

Assay of the activity of the first two enzymes in the oxidative pathway of leucine (Table 6) showed them to be present not only in liver but also in the gastrocnemius muscle. Dehydrogenase activity was clearly demonstrated in the gastrocnemius muscle of animals on all three dietary regimens, and was present in greater activity/mg protein than that found in liver.

Muscle aminotransferase activity was even higher and was approximately ten times as great per mg protein as that in liver. Thus the ratio, aminotransferase activity : α-oxo-acid dehydrogenase activity was much higher in muscle. The enzymes in liver and muscle differed not only in their levels of activity but also in their response to dietary stress. Low-protein feeding produced a rise in muscle aminotransferase activity but a fall in dehydrogenase activity to less than half the control value. In contrast, liver aminotransferase and dehydrogenase activities were not changed appreciably. Thus *in vivo* conservation of injected L[1-^{14}C]leucine (Table 5) was occurring without any change in liver dehydrogenase activity, which hitherto has been held responsible for the reduced oxidation of α-oxo-isocaproic acid in protein depleted animals (McFarlane & Von Holt, 1969a,b)

SECTION IIC(iii)

TABLE 6

Leucine α-oxoglutarate aminotransferase and α-oxo isocaproate dehydrogenase activities of liver and muscle homogenates in rats either given a control or a low protein diet or fasted for 48 hr.

(Mean values with their standard errors for six rats per group)

Tissue	NDp:E ratio of diet*	Whether fasted for 48hr	Activity of enzymes (n mol leucine or α-oxo acid oxidized/mg protein per hr)	
			Leucine α-oxoglutarate aminotransferase	α-oxo isocaproate dehydrogenase
Muscle	10.0	-	69.6 ± 3.33 c	2.15 ± 0.20 a, b
Muscle	10.0	+	72.3 ± 2.31 d	1.28 ± 0.17 b
Muscle	3.5	-	86.8 ± 2.84 c, d	0.91 ± 0.07 a
Liver	10.0	-	6.5 ± 0.60	0.68 ± 0.07 e
Liver	10.0	+	7.8 ± 0.44	0.99 ± 0.07 e, f
Liver	3.5	-	6.6 ± 0.44	0.65 ± 0.04 f

Statistical comparison of groups: values marked with the same letter differ significantly at the following levels:-

a, $P < 0.01$; b, c, d, e and f, $P < 0.01$.

*[illegible] of energy supplied by utilisable protein : total metabolizable protein.

The experiments with fasted rats indicated that changes in enzyme activity (Table 6) could occur rapidly, for within 48 hr, muscle dehydrogenase activity had fallen markedly. In contrast, liver dehydrogenase activity rose.

D (i) The possible role of age in determining the response of animals to protein-free feeding.

The initial experiments on leucine oxidation in vivo showed that 65g growing rats had the ability to reduce their oxidation of leucine when growth was arrested on a low protein diet (0.035 NDpiE). Neale (1971) observed that weanling rats given a protein-free diet were unable to conserve either leucine or valine when L[U^{14}C]-leucine or L[U^{14}C]-valine was given as a pulse dose intragastrically or intravenously. Further studies (Neale, 1972) with eviscerated and control adult rats fed a protein-free diet failed to show any adaptation in oxidation by the peripheral tissues. Again the difficulties in the interpretation of these experiments are dealt with in Section IV(ii). Because of these discrepancies, a further assessment was made of the ability of weanling rats to reduce the catabolism of L[1^{14}C]-leucine and valine on a protein-free diet, and the results were compared with those in older animals. Furthermore, the experimental design included assessment of liver and muscle enzyme activities in rats at different stages of development. Thus, it was possible to relate any changes in enzyme activity to the ability to reduce the catabolism of leucine and valine.

D (ii) ANIMALS AND DIETS

Three different groups of female hooded Lister rats were used to investigate the oxidation of leucine and valine and the

adaptation in the catabolic enzymes of these two branched-chain amino acids in animals of different ages when given a protein-free (PF) diet for 1, 2 or 3 weeks. The first group was obtained at weaning and immediately fed ad libitum a PF diet. Two other groups of rats were fed initially on control diet from weaning in which the ratio of energy supplied by utilizable protein to total metabolizable energy (NDp:E) was 0.10. When one group reached a weight of approximately 85.0g the animals were given a PF diet for 1, 2 or 3 weeks. The last group was maintained on the 0.10 NDp:E diet until the rats had reached an average weight of 200g when they too were given the PF diet for 1, 2 or 3 weeks.

Control animals received an adequate protein intake, i.e. 0.10 NDp:E, throughout and were assessed at weights of 35, 85 and 200g body weights. All the animals studied were fed until 5 hr before the experiment for reasons which have been discussed (Section IIA(iii)). The rats were given an intragastric injection of a tracer dose of L[1-^{14}C]leucine or L[1-^{14}C]valine (1μCi/kg body weight in a solution of 9g NaCl/l with 10 μmol leucine/kg body wt as carrier - similarly for valine) and placed immediately in a sealed vessel through which air was drawn at constant rate. The CO_2 was trapped as described in Section IIA(iii). Oxidation during the first 3 hr. was expressed as the percentage of the labelled dose expired as $^{14}CO_2$.

D (iii) ENZYME ASSAYS

Aminotransferase and dehydrogenase activities were measured *in vitro* in muscle and liver preparations from rats given the same diets as those used for the assessment of oxidation *in vivo*. Groups of rats were killed by decapitation at weekly intervals

and their livers and gastrocnemius muscles were excised and quickly frozen on solid CO_2. Four enzyme activities were measured in both tissues : L-leucine α-oxo-glutarate aminotransferase (α-KIC), L-valine α-oxo-glutarate aminotransferase E.C.2.6.1.6. and α-oxo-isovalerate dehydrogenase (α-KIV). For the assay a weighed amount (500g) of liver or muscle tissue was homogenised by hand in ice-cold Sorensen's phosphate buffer (25 mmole/l) pH 7.0 to give a 1:10 (w/v) homogenate. Enzyme activities were determined by the methods described previously (Section IIB(ii)).

D (iv) RESULTS

Table 7 shows the proportion of labelled leucine and valine excreted as CO_2 by the three groups of animals. Weanling and 200g animals excreted a similar proportion of label. Equivalent amounts of $^{14}CO_2$ were evolved from labelled leucine and valine in these animals given a normal diet and the individual values within groups were very consistent.

The PF diet led to a decrease in oxidation rates in all three age groups. The response to a PF diet was rapid, since the greatest decrease in $^{14}CO_2$ excretion occurred within the first week; thereafter further decreases in the catabolism of either L[1-^{14}C]-leucine or L[1-^{14}C]-valine were small (Table 7). There was a tendency for the youngest group of animals to have the greatest decrease in $^{14}CO_2$ output, eg. there was a 59% decrease in leucine oxidation in the weanling rats compared with a decrease of 37% in the mature group. The adaptation in valine oxidation seemed less effective than that for leucine, with higher excretion rates of $^{14}CO_2$ from L[1-^{14}C]valine in rats on the protein-free diet.

SECTION IID(iv)

TABLE 7

In vivo oxidation of L[1-^{14}C] leucine and L[1-^{14}C] valine (1.0 μCi/100g body wt) in rats on control (0.10 NDp:E^{+}) and protein free (0.00 NDp:E^{+}) diets. $^{14}CO_2$ excretion in 3 hours expressed as a percentage of labelled dose given.

Group and initial rat weight	Time on 0.0 NDp:E diet (weeks)	Leucine	Valine
WEANING			
35g	0	15.0 ± 0.9(3)	13.4 ± 1.4(3)
	1	7.0 ± 1.4(6)*	9.3 ± 1.7(3)*
	2	6.2 ± 0.9(6)*	8.3 ± 0.4(3)*
	3	6.2 ± 0.9(5)*	7.9 ± 1.1(5)*
GROWING			
85g	0	15.4 ± 4.2(3)	14.9 ± 3.1(3)
	1	9.6 ± 1.4(6)*	7.9 ± 1.5(6)*
	2	6.5 ± 2.2(6)*	8.4 ± 1.0(6)*
	3	7.4 ± 2.1(6)*	9.1 ± 1.2(5)*
ADULT			
200g	0	14.3 ± 0.8(3)	15.9 ± 1.5(3)
	1	8.8 ± 0.9(3)*	11.2 ± 1.7(3)*
	2	9.0 ± 2.0(3)*	10.7 ± 2.2(3)*
	3	9.0 ± 0.3(3)*	———

Means ± S.D.

Figures in parenthesis indicate the number of observations

* Results differ significantly ($P<0.02$) from the control value for the group.

+ Ratio of energy supplied by utilisable protein to total metabolisable protein.

Enzyme Activities

The enzyme activities show a significant trend in decreasing with age (Tables 8 and 9). This is true for both the leucine and valine catabolic enzymes in liver and muscle tissues. This may be functionally important in maintaining a relatively constant fraction of the leucine turnover being oxidized, i.e. approximately 15%. Thus a decrease in specific activity of the enzyme would offset the total enzyme activity due to a net body weight gain with age. The results contradict Neale's (1972) work inasmuch as protein-free feeding brought about an adaptive response.

Enzyme Activity in Liver

Results obtained from determinations of enzyme activities suggest that in the control animals the weanling rats had higher aminotransferase and dehydrogenase activities (Table 8). The dehydrogenase activity relating to leucine catabolism decreased in all animals within 1 week and a further reduction in liver oxidation capacity occurred as the PF regimen continued. As with the *in vivo* measurements of leucine oxidation, the decrease in enzyme activity was most marked in the weanling rats. The liver activities of KIV dehydrogenase showed a marked reduction only in the weanling group.

In contrast to the decrease in dehydrogenase activities, the aminotransferase levels in liver increased in rats given a PF diet (Table 8). Large increases were often seen, eg. in L-valine : α-oxo-glutarate aminotransferase in the younger animals. A further difference between the response in amino transferase and dehydrogenase activities was the transient

nature of the increase in most groups; frequently the highest level was seen after 1 or 2 weeks and by the end of 3 weeks aminotransferase levels were often almost the same as the initial control activities. Thus, after 1-2 weeks on a PF diet the ratio, aminotransferase:dehydrogenase activity in the homogenates had changed markedly, with an increase in the value from 2-3 in the control period to 5-22.

Enzyme Activities in Muscle (Gastrocnemius).

Measurements of enzyme activities in homogenates of muscle (Table 9) confirmed the earlier work (Table 6) that there was a higher aminotransferase:dehydrogenase ratio in this tissue than in liver. There were similar changes in the weanling and growing rats given the PF diet. Again, there was a definite decrease in dehydrogenase activities after 1 week, with further reduction by the end of 3 weeks on the PF diet. In the oldest group of animals there was a significant decrease in activity but, as in the liver, the change was much less than in the younger animals. Thus, although the initial activity in muscle of the KEC dehydrogenase was much higher in the younger animals than in the mature (200 g) rats, once adaptation had occurred, the younger animals showed a greater reduction in activity than those in the oldest group. The aminotransferase activities rose to high levels in the early phase of feeding on a PF diet in all three groups but returned towards normal by the end of the feeding periods.

SECTION IID(iv)

TABLE 8

Liver enzyme activities in rats given a protein-free (PF) diet: L-leucine and L-valine α-oxoglutarate aminotransferase and α-oxo isocaproic acid (α-KIC) and α-oxoisovaleric acid (α-KIV) dehydrogenase activities (n mol amino acid or α-oxo-acid oxidised/ mg protein per hr.)

(Mean values with their standard errors for groups of four rats)

Group	Mean Body Weight	Time on PF diet (weeks)	LEUCINE				VALINE			
			α-oxoglutarate amino transferase		α-KIC		α-oxoglutarate amino transferase		α-KIV	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
WEANING	35g	0	8.6	0.8	3.5	0.45	5.5	0.7	2.8	0.7
		1	14.5	3.5	2.2	0.60	18.0*	3.5	1.9	0.25
		2	12.2*	0.7	2.2	0.45	22.3*	0.5	1.2	0.10
		3	10.9	1.05	0.8*	0.20	-	-	0.4*	0.05
GROWING	85g	0	7.0	0.6	1.8	0.1	3.0	0.35	0.9	0.05
		1	12.1*	0.9	1.3	0.2	21.0*	0.50	0.9	0.35
		2	-	-	-	-	-	-	-	-
		3	8.9*	0.45	0.6*	0.1	4.9*	0.25	1.2*	0.05
ADULT	200g	0	7.8	0.6	1.2	0.065	3.1	0.65	1.6	0.1
		1	10.4	0.9	1.0	0.06	7.1*	0.60	1.4	0.075
		2	6.6	0.7	0.8*	0.045	6.1*	0.60	1.1*	0.045
		3	6.3	0.15	1.0*	0.04	3.3	0.35	1.2*	0.04

* Values differ significantly from the value for the control group ($P<0.05$)

SECTION IID(iv)

TABLE 9

Gastrocnemius muscle enzyme activities in rats given a protein free (PF) diet :- L-leucine and L-valine α-oxogluterate aminotransferase and α-oxo-isocaproic acid (α-KIC) and α-oxoisovaleric acid (α-KIV) dehydrogenase activities (n moles leucine or valine or the corresponding α-oxo acid oxidised/mg protein/hr).

(Mean values with standard errors for group of four animals)

Group	Mean Body Weight	Time on PF diet (weeks)	LEUCINE				VALINE			
			α-oxoglutarate amino transferase		α-KIC		α-oxoglutarate amino transferase		α-KIV	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
WEANING	35g	0	43.2	4.75	4.0	0.6	44.1	4.95	3.4	0.65
		1	110.8*	2.55	2.3	0.6	94.8*	6.05	2.7	0.10
		2	85.4*	6.2	2.6	0.7	56.0	1.9	0.5*	0.10
		3	60.3*	4.7	1.6*	0.25	-	-	0.6*	0.05
GROWING	85g	0	91.3	2.7	3.9	0.3	43.6	4.75	2.6	0.15
		1	91.7*	5.65	2.5	0.2	58.7*	2.80	2.2	0.50
		2	-	-	-	-	-	-	-	-
		3	65.9*	3.9	2.3*	0.5	46.7	4.05	1.5*	0.35
ADULT		0	40.6	4.85	1.2	0.04	35.3	1.5	1.1	0.06
		1	70.1*	9.7	1.0	0.065	44.8*	2.15	0.6*	0.04
		2	61.2*	3.8	0.8*	0.04	41.3*	0.8	0.7*	0.10
		3	56.2*	5.9	0.9*	0.06	38.8	1.3	0.9*	0.04

* Values differ significantly from the value for the control group ($P < 0.05$)

SUMMARY OF SECTION II

Oxidation of leucine *in vivo* was reduced in animals given a low protein diet. Fasting, however, caused an increase in the output of $^{14}CO_2$ from L[1-^{14}C] leucine given intragastrically. Measurements of both leucine α-oxoglutarate aminotransferase and α-oxo-isocaproate dehydrogenase activities in animals fed the control and low protein diets were carried out. Dehydrogenase activity was demonstrated in skeletal muscle homogenates, although other workers had failed to show this (Volbueter & Harper, 1970). Furthermore, the low-protein diet led to a fall in muscle dehydrogenase activity but increased liver dehydrogenase activity. Aminotransferase activity in muscle rose in the low-protein and fasted animals but the activity in liver was unchanged.

This work was carried out on weanling rats and contradicted the work of Neale (1971). He observed that weanling rats given a protein-free diet were unable to conserve either leucine or valine as judged by output of $^{14}CO_2$. One reason for this difference may be that Neale used uniformly labelled amino acids. The second series of experiments were designed to make further assessments of the ability of weanling rats to reduce the catabolism of L[1-^{14}C]-leucine and L[1-^{14}C]-valine on a protein-free rather than a low-protein diet, and the results were compared with those in older animals. Essentially the same results were obtained, showing that protein restriction in the diet led to a reduction in $^{14}CO_2$ output from the radioactively labelled branched-chain amino acids.

The results in Table 6 (Section IIC(iii)) confirmed Young's (1970) observations that the greatest total B.C.A. aminotransferase activity was located in skeletal muscle. Moreover, one can calculate the total

dehydrogenase capacity of both liver and skeletal muscle from the data in Table 6 (Section IIC(iii)) and the reported tissue distribution of dehydrogenase activity (Volbueter & Harper, 1970). Liver accounts for only 2% of the body's dehydrogenase activity, with <0.05% for brain and <0.05% for kidney; their combined capacity is insufficient to account for leucine oxidation *in vivo*. If muscle protein is assumed to be 50% of total body protein, then muscle has the highest calculated total dehydrogenase activity. The supposition, therefore, is that muscle must play an important part in the regulation of branched-chain amino acid oxidation.

With this as a working hypothesis, the second phase of the work was begun. A hind-limb preparation was developed along similar lines to those described by Ruderman et al (1971) and Jefferson et al (1972), since this was essentially a more physiological *in vitro* skeletal muscle preparation than a homogenate. By constant infusion of labelled amino acid into the perfusion fluid it was possible to determine quantitatively the oxidation of leucine by skeletal muscle in the hind-limb preparation. With the same technique *in vivo*, as described by Waterlow & Stephen (1968) and later by Garlick & Marshall (1972), a quantitative estimate of whole body leucine oxidation could also be obtained. Muscle's contribution to total body oxidation of leucine could therefore be estimated more accurately than had previously been done (Manchester, 1965). Moreover, the constant infusion method offered the opportunity to study protein turnover under conditions of dietary stress both *in vivo* and in the perfused hind-limb.

SECTION III

LEUCINE TURNOVER IN THE WHOLE BODY AND IN THE PERFUSED HIND LIMB

A. Estimation of $^{14}CO_2$ retention in the bi carbonate pool.

In order to measure the oxidation of leucine, it is necessary first to know whether the $^{14}CO_2$ formed is quantitatively excreted. Recently it has been shown that 20% of an infused dose of $NaH^{14}CO_3$ was being retained in man (James, Garlick, Sender & Waterlow, 1976) and that 80% was excreted as $^{14}CO_2$. Therefore, it was essential, if accurate oxidation rates were to be measured, to estimate the retention, if any, of $^{14}CO_2$. To do this, a tracer dose of $NaH^{14}CO_3$ was infused constantly into the tail vein of a rat and $^{14}CO_2$ output monitored on the assumption that when I [$1^{14}C$] leucine is decarboxylated, the $^{14}CO_2$ gains direct access to the bi carbonate pool.

Constant infusion of $NaH^{14}CO_3$ in vivo

Female hooded rats weighing approximately 170g were fed ad libitum 0.10 NDp:E powdered diet for one week and were then placed individually in glass cylinders as described in Section IIIB(ii). The tail vein was cannulated (Section IIIB(ii)) and $NaH^{14}CO_3$ (1.0 μCi/ml in 0.9% saline) infused at a flow rate of 0.48 ml/hr. CO_2-free air was pumped over the animal (400 ml/min) and the effluent gases bubbled through three sequential hyamine hydroxide/ethanol traps. $^{14}CO_2$ production was monitored for 3 hours to estimate the amount of labelled $^{14}CO_2$ retained within the rat tissues and the half-life of the bicarbonate pool.

RESULTS

Fig.5 shows that $^{14}CO_2$ output reached plateau specific radioactivity

Fig 5

Output of $^{14}CO_2$ during constant infusion by tail vein of $NaH^{14}CO_2$ (3.0 μCi / ml in 0.9% saline) into three rats (180 g body wt.)

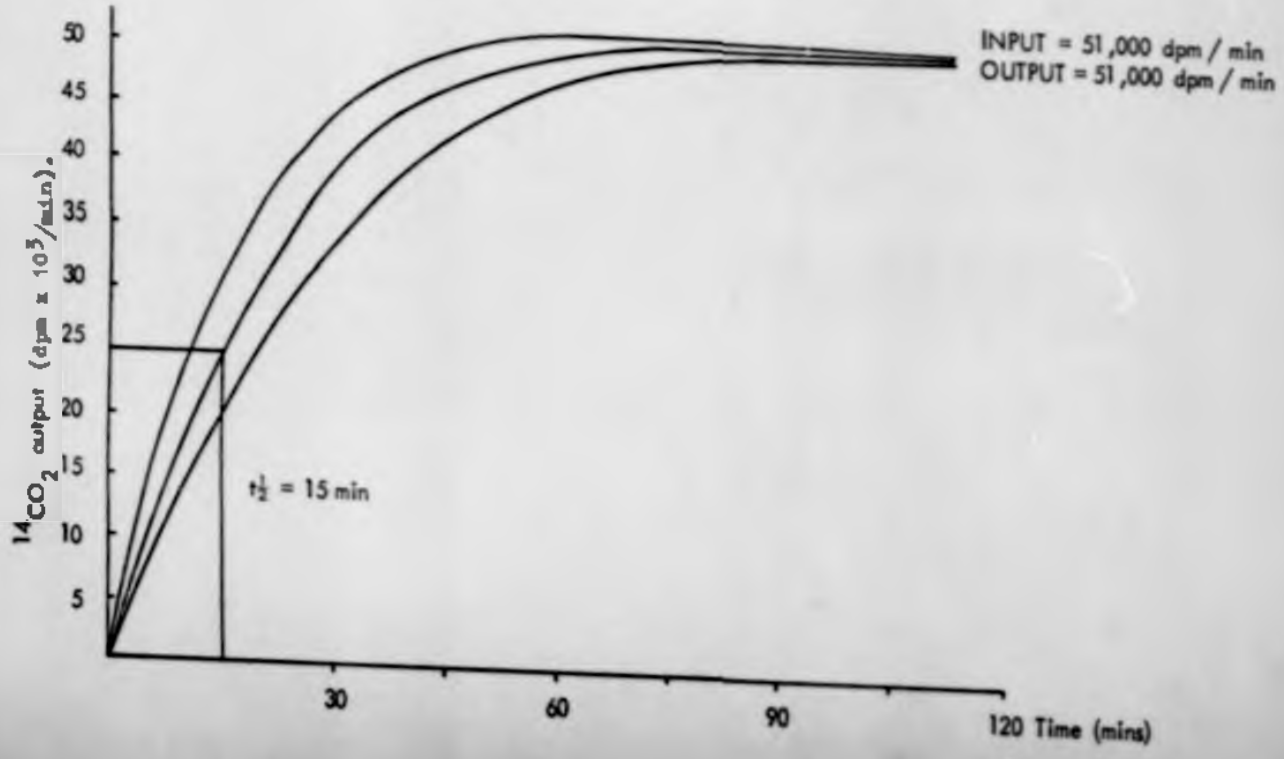
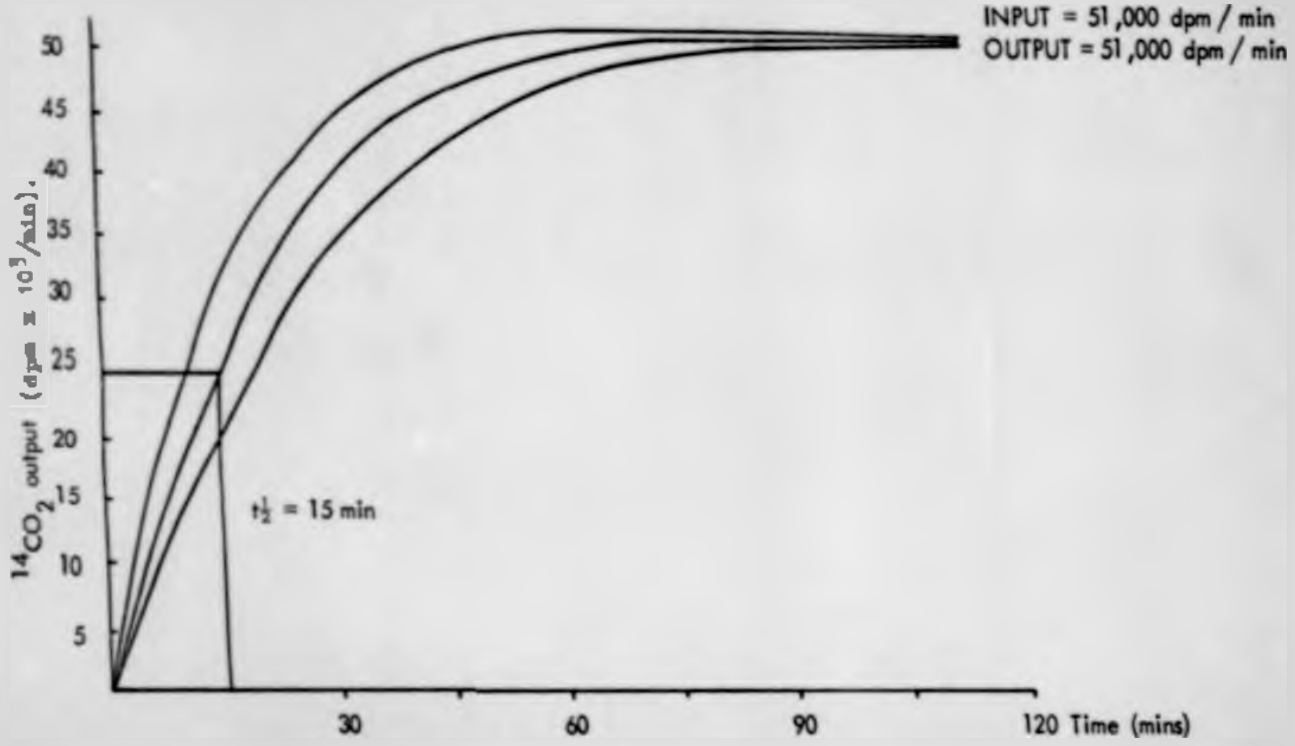


Fig 5

Output of $^{14}CO_2$ during constant infusion by tail vein of $NaH^{14}CO_2$ (3.0 μCi / ml in 0.9% saline) into three rats (180 g body wt.)



within 60 minutes and that the calculated half-life of the bicarbonate pool is 15 minutes. This is in agreement with the estimate made by Millward (1970) of 12½ minutes. Furthermore, the figure shows that there is negligible retention of $^{14}CO_2$ in the bicarbonate pool unlike the findings of James, Garlick, Bender and Waterlow in man, where 20% was retained. Therefore, no correction was necessary in estimating absolute oxidation rates in subsequent studies involving the constant infusion of L[1-^{14}C]-leucine, on the assumption that the $^{14}CO_2$ decarboxylated immediately enters the bicarbonate pool.

B (i) LEUCINE TURNOVER IN THE WHOLE RAT IN VIVO

Earlier experiments both in this laboratory (Section II) and in others (McFarlane & Van Holt,1969a; Neale 1971, 1972) have estimated the rate of B.CA oxidation with an intragastric, intraperitoneal or intravenous pulse dose of radioactive tracer amino acid . Unless large quantities of cold amino acid are added the *precursor specific radioactivity in the various tissues will be* changing rapidly. Therefore, the constant intravenous infusion technique described by Garlick & Marshall (1972) was employed to measure more accurately the rates of leucine oxidation in vivo. With infusion of a constant tracer dose of L[1-^{14}C] -leucine the precursor pools for oxidation and protein synthesis (intra-cellular pools) reached plateau specific radioactivity within 2 hrs (Fig.8), and the level was maintained to the end of the infusion. Similar results have been obtained with the infusion of a number of different amino acids (Gan & Jeffrey, 1967; Waterlow & Stephen, 1968; Garlick & Marshall, 1972; Seta et al, 1973). In liver the plateau SA of free leucine reached approximately 50% of that of plasma whilst in

the gastrocnemius muscle it was in excess of 70% (fig.8). The fact that the specific radioactivity of the free leucine at plateau was different in the plasma and the various tissues is indicative of effective compartmentation. One reason for the lower specific radioactivity in the tissue is dilution of the intracellular pool by unlabelled amino acids (leucine) derived from proteolysis (Gan & Jeffay, 1967; Waterlow & Stephen, 1968).

B (ii) METHODS

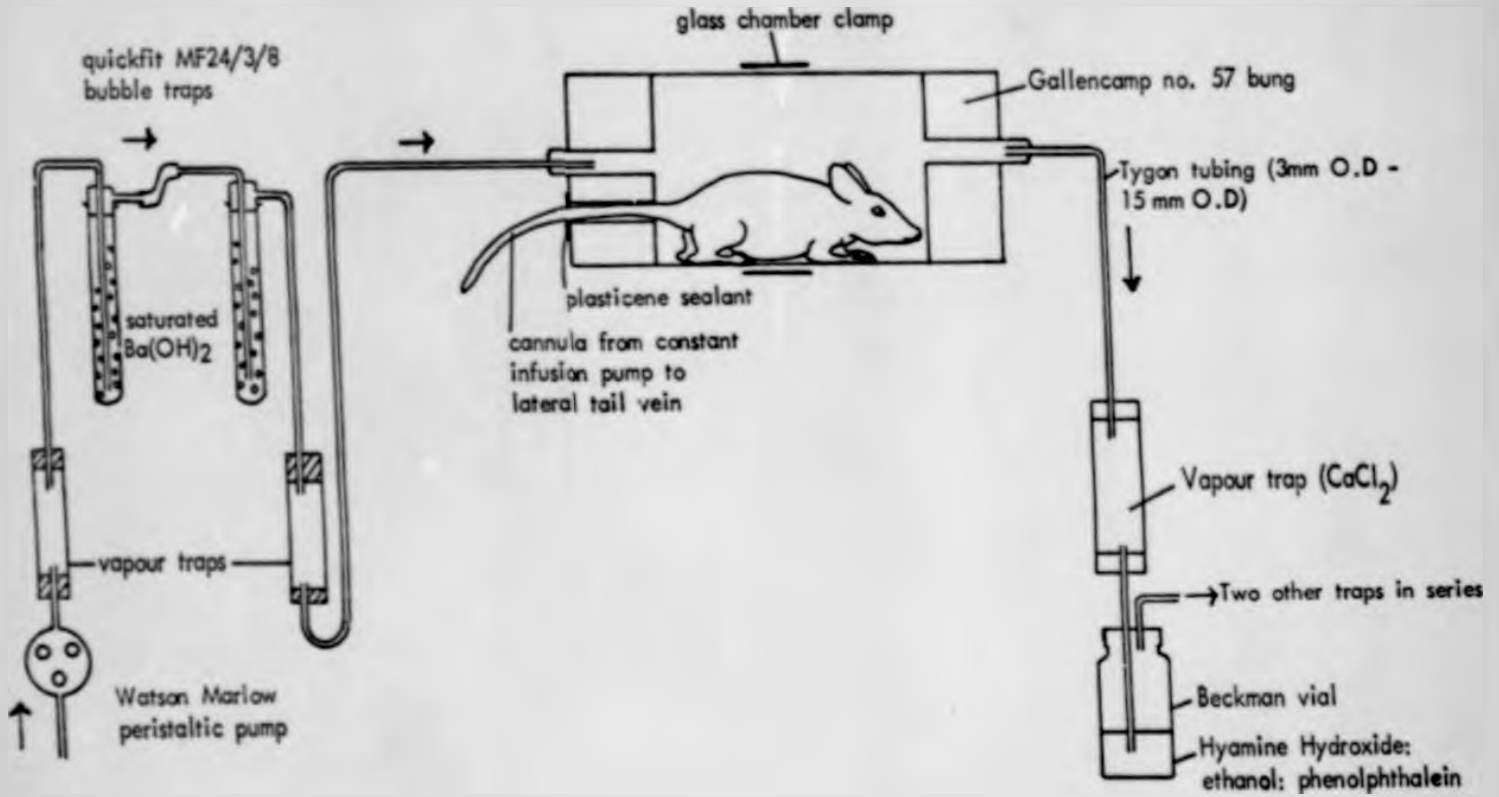
(a) Rates of oxidation

In order to determine rates of leucine oxidation the following experimental protocol was used. One group of female hooded rats (Animal Suppliers (London) Ltd) weighing approximately 170g were fed ad libitum for one week on a powdered diet (Net dietary protein : energy ratio, 0.10 NDp:E) which maintains normal growth rates. A group of rats was then infused via the tail vein with a tracer dose of L[1-^{14}C]-leucine (5.0 μCi/ml in 0.9% saline) continuously for 6 hours (0.48 ml/hr). Another group was fed a protein free powdered diet for one week and then infused in a similar manner. The protein-free diet was iso-energetic with the 0.10NDp:E diet (see Table 4).

The animals were 'trained' for 2 days before the infusion of labelled amino acid to sit quietly in a glass cylinder, stoppered at either end, but ventilated adequately (see fig.6). This procedure acclimatised the rats sufficiently for them to sit happily throughout the 6 hour infusion periods while $^{14}CO_2$ output was being monitored. The tail of the rat was passed through a small hole in one of the rubber stoppers and fixed in place with adhesive tape to prevent it being retracted into the tube by the

FIG 6

APPARATUS FOR THE COLLECTION OF CO_2 DURING INFUSIONS IN VIVO



rat when the tail vein was cannulated (method described in Section IIIB(ii)). CO_2-free air was pumped through the cylinder at a rate of 400 ml/min and then through three sequential traps of hyamine /ethanol mixture with phenolphthalein indicator. 1mmole CO_2 was considered to have been trapped when the indicator changed from pink to colourless as it had then reacted with 1 m mole of hyamine hydroxide in the first vial. The time taken to trap 1 m mole of CO_2 was recorded throughout the experiment. Thus both the total amount of CO_2 produced/hr and the specific radioactivity of the CO_2 could be determined. 10ml toluene : PPO (2, 5 dinitrophenyloxazole, 4g/l toluene) was then added to the vial and immediately counted. The efficiency of counting was 7[illegible].

Apparatus for $^{14}CO_2$ collection from L[1-^{14}C]-leucine infusion

Air was pumped at a rate of 400 ml/min through $CaCl_2$ to remove water vapour and thence to sequential 20ml volumes of saturated $Ba(OH)_2$ in Quickfit bubble traps (MF 24/3/8) to remove CO_2. The CO_2 free air then passed again over $CaCl_2$ into the glass cylindrical chamber (fig.6). The cylinder (25cm x 6cm I.D) was stoppered at either end with Gallenkamp No.57 rubber bungs. The inlet bung had 2 bore holes (8mm. I.D), one for the passage of incoming CO_2-free air and the other for the animal's tail to pass through. The outlet bung had one 8mm bore hole which led by the connecting Tygon tubing (3mm O.D x 1.5mm. I.D) to a further water vapour trap and thence to a vial containing 3 ml of a 2:1 hyamine hydroxide:ethanol mixture with phenolphthalein as indicator. Two more vapour traps and vials were connected in series to the first trap to check CO_2 trapping efficiency. Trapping in the

first vial was about 98% efficient. Preliminary work involving the constant infusion of $NaH^{14}CO_3$ showed that there were no leaks in the system, especially where the tail passed through the bungs (Section IIIA).

Rates of $^{14}CO_2$ production were calculated as dpm $^{14}CO_2$ evolved per minute of infusion. By constantly monitoring the output of $^{14}CO_2$ throughout a six hour period, the rate of rise to plateau specific radioactivity could be measured. From these data it was possible to estimate not only total body leucine flux but also the flux through the oxidative pathway.

(b) Rates of protein synthesis

Female hooded rats (Animal Suppliers (London) Ltd) were obtained at 170g and fed ad libitum a powdered diet (0.10 NDp:E) for one week. Another group of similar rats were fed a protein-free powdered diet (0.00 NDp:E) for one week. Animals from both groups were then infused via the tail vein with a tracer dose of [^{14}C] leucine in the manner described below. Animals were infused for 2 or 4 hours at which times they were sacrificed by decapitation. Mixed venous blood was rapidly spun and a known volume of plasma added to 2ml of cold 3.0% sulpho-salic ylic acid (S.S.A) to precipitate protein. Samples of both liver and gastrocnemius muscle were also rapidly taken, homogenized in cold SSA and stored at -18°c. Measurements of free and bound leucine specific radioactivities were carried out on a Locarte amino acid analyser fitted with a column effluent stream splitter (Fern & Garlick, 1973). Fractions were counted in 10ml of 0.4% PPO (2,5 dimitro phenyloxamole in a mixture of toluene:triton X-100 (2:1) at an efficiency of 85-90%. Results for free and

bound leucine specific radioactivity at 6 hours were obtained from tissues removed from animals used to determine oxidation rates.

Tail Vein Infusion of Labelled Amino Acid

A 20 gauge disposable hypodermic needle was separated by dissolving its fittings in chloroform and inserted into a length of narrow bore polythene tubing (0.4 mm I.D). The other end of the tubing was fixed to a hypodermic syringe containing physiological saline. The rat was then wrapped in a hand-towel to restrict its movement during the infusion period. Its tail was left free to be inserted into warm water for a few minutes both to clean it and to increase the blood flow. The tail was then cleansed with xylotol which also makes the vessels dilate. The needle was inserted into a lateral vein and firmly held in place with adhesive tape. The needle and tubing were cleared of blood by injecting approximately 0.1ml of saline. The cannula was then attached to the syringe portion of a continuous infusion pump. A solution of L [1-^{14}C] leucine in physiological saline was infused at a rate of 0.48ml/hr (5.0μCi/ml prepared by dissolving solid L [1-^{14}C]-leucine of specific radioactivity 60mCi/mmol in saline without any carrier).

B (iii) LEUCINE OXIDATION RATES IN VIVO

CALCULATION - If one assumes that following decarboxylation of L [1-^{14}C]-leucine $^{14}CO_2$ gains direct access to the bicarbonate pool then the oxidation rate for leucine in the whole rat is given by the following equation when plateau specific radioactivity of $^{14}CO_2$ output has been reached.

$$E = \frac{SR\ ^{14}CO_2 \text{ at plateau (dpm/min)}}{SR \text{ free leucine in muscle ICF (dpm/}\mu\text{mole)}}$$

This calculation is based on the assumption that oxidation occurs in a pool in which the SR of free leucine is equal or similar to that is muscle. It gives rates as μmoles/min but in Table 10 the oxidation rates have been expressed as μmoles/hr/180g rat in order to obtain a better comparison between control and rats fed a protein-free diet.

RESULTS

Fig.7 shows the rise to plateau in $^{14}CO_2$ specific radioactivity which is attained in little over 2 hrs in the well-fed group and after a little longer in the protein-free group. The specific radioactivity of free leucine in the gastrocnemius muscle (Table 10) was taken as a representative average of the precursor specific radioactivity of all tissues in which oxidation of leucine occurs. Since the SR in the precursor pool (GASTROCNEMIUS ICF) and in the pool from which $^{14}CO_2$ was derived (HCO_3^-) had reached plateau by 2 hours and since plateau was maintained for up to 6 hours (fig.8) it was possible to estimate the rate of leucine oxidation from these two values. Because of the difference in mean body weight for the two groups, figures were adjusted so that results were expressed per 180g body weight. As can be seen from Table 10, the output of $^{14}CO_2$ (dpm/min) at plateau specific radioactivity in the well-fed group was greater than in the group fed the protein free diet. When rates of oxidation are determined, with adjustments for the specific radioactivity of the gastrocnemius muscle ICF pool, then there is a reduction from 29.90 μmoles/hr/180g body wt. in the well fed group to 20.50 μmoles/hr/180g body wt. in the protein-free group (Table 10).

TABLE 10

ABSOLUTE OXIDATION RATES OF L[1-^{14}C]leucine IN VIVO (μmoles/hr/180g rat)

Effect of protein-free feeding

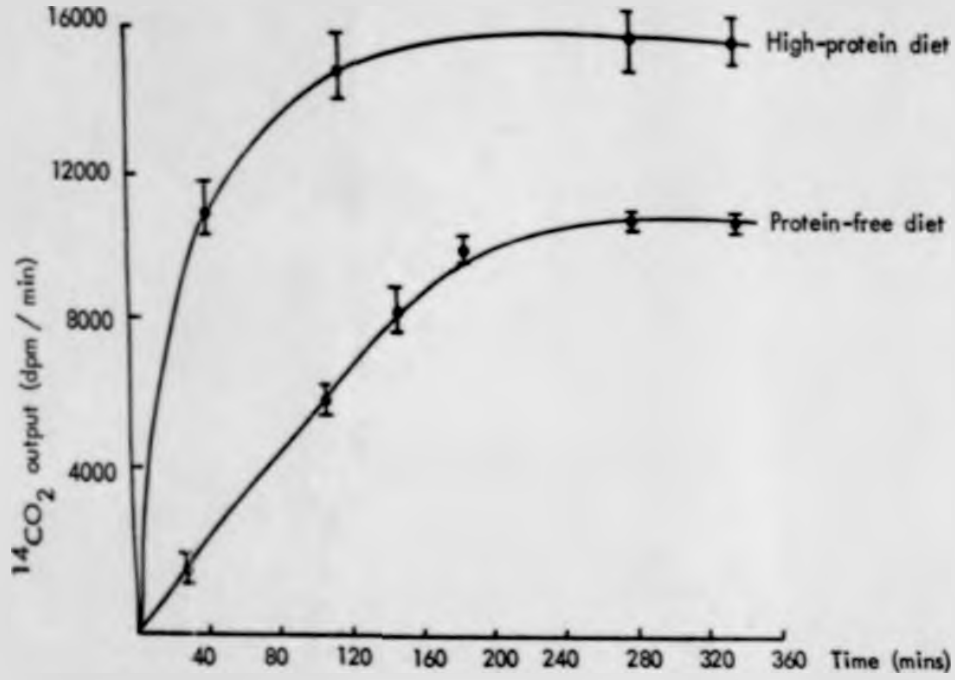
Diet %NDp:E	No. Observations	Rat Weight (g)	$^{14}CO_2$ output dpm/min infusion per 180g rat	L[1-^{14}C] leucine input (dpm/min)	SR of 1-C leucine (Gastrocnemius) (dpm/μmole leucine)	Leucine Oxidation Rate (μmoles/hr/180g ra
10	(3)	185 ± 11.4	15 727 ± 1489	91 000	30 800 ± 3260	29.90 ± 4.1
0	(3)	165 ± 4.5	10 083 ± 1559	91 000	32 110 ± 4450	*20.50 ± 1.9

Figures given are Means ± S.D.

*Differs significantly from control value $p < 0.01$

Fig 7

Output of $^{14}CO_2$ (dpm / min) during constant infusion of a tracer dose of L(1 ^{14}C)-leucine (5.0 μCi/ ml at 0.48 ml / hr) in vivo in rats fed either an adequate protein diet (10% NDpcE) or a protein-free diet



B (iv) RATES OF PROTEIN SYNTHESIS

CALCULATION - The method of determining rates of protein synthesis by infusion of [^{14}C] lysine (Waterlow & Stephen, 1968) has been modified by Garlick, Millward & James, (1973) so that any amino acid may be used.

We assume that the precursor SR for protein synthesis is that of the total intracellular free amino acid, although the problem of intracellular compartmentation may introduce errors. The ideal would be to measure the SR of the amino acid bound to t-RNA, but in practice this is very difficult.

The basic equation for calculating the rate of protein synthesis in a tissue from the SR of intracellular free amino acid as precursor is:

$$\frac{dSR}{dt} = K_s (S_i - S_p), \text{ where } S_i \text{ and } S_p \qquad \text{equ.(1)}$$

are the SRs of intracellular amino acid and of protein, and Ks is the fractional rate of protein synthesis.

S_i takes some time to reach plateau, and, therefore, in order to calculate S_p, some information is needed about the time course of S_i. This information can to some extent be obtained from measurements on plasma. Waterlow & Stephen (1967) showed that during infusion of [^{14}C] lysine the SR of plasma lysine rose to plateau by a pathway approximating to a single exponential :

$$Sp = S_{p\ max} (1-e^{-\lambda pt}). \qquad \text{equ.(2)}$$

where Sp = the SR of plasma lysine at any time t;

Sp_{max} = the plateau SR of plasma lysine

λp is a rate constant

λp for lysine was shown to be from 12-24 days^{-1}

(Waterlow & Stephen, 1967) and for tyrosine 80 $days^{-1}$ (Garlick et al, 1973). In the present work, λp for leucine was found to be 33 $days^{-1}$.

Similarly, the time-course of SR of the free amino acid in the tissue (Si) can, without significant error, be expressed as a single exponential : $Si = Simax\ (1-e^{-\lambda it})$ equ.(5)
The problem is to determine the value of λi. This question has been discussed in detail by Garlick et al, 1975). From their conclusions it was considered that under the conditions of the present experiments it would be appropriate to take $i = \lambda p = 33\ days^{-1}$. This approximation is justified when the ratio of protein bound to free amino acid is large, as is the case with leucine in muscle and liver. Substituting eqn. (5) into eqn. (1), taking $\lambda i = \lambda p$, and integrating, gives:

$$\frac{S_2}{S_1} = \frac{\lambda p}{\lambda p - K_s} \quad \frac{(1-e^{-K_s t})}{1-e^{-\lambda p t}} - \frac{K_s}{\lambda p - K_s} \qquad \text{equ.(4)}$$

- an equation originally derived by Swick (1958). This equation can be solved graphically for K_s, and enables the rate of protein synthesis in liver and muscle to be determined from the experimentally measured SRs of leucine in the protein and free amino acid pool of the tissue at the end of the infusion. Accurate determination of λ_i is unnecessary, since large variation in its value result in only small variations in K_s (Waterlow & Stephen, 1968). K_s, the fractional rate of synthesis, is expressed as the % of protein mass replaced each day.

B (v) RESULTS

(a) Effect of protein-free feeding on muscle synthesis rates

Plateau SR of free leucine was attained within 2 hours of starting the infusion in both plasma and gastrocnemius muscle ICF (fig.8). The plateau value was maintained over a 6 hr period (Table 11) and incorporation into skeletal muscle protein was linear. Feeding a protein-fee diet led to an increase in plasma SR of free leucine. This is consistent with observations made by Garlick, Millward, James & Waterlow (1975). At the same time protein-synthesis in the gastrocnemius muscle was reduced from 9.3% D^{-1} to 6.0% D^{-1}. The synthesis rates observed in gastrocnemius were similar to rates of protein synthesis estimated in the same muscle of 100g Wistar rats but infused with $U^{14}C$-tyrosine (Garlick et al, 1973).

(b) Liver synthesis rates in vivo

As with the SR of muscle ICF, the specific radioactivity of intracellular free leucine in liver reached a plateau by 2 hours which was maintained up to the end of the 6 hour infusion (fig.8). Feeding a 0% NDpiE diet led to a small but significant rise in the plateau SR in liver ICF. This is consistant with data of Garlick et al (1975). Protein synthesis was increased from 50% D^{-1} to 121.0% D^{-1} on feeding the protein-free diet for 7 days (Table 12). Garlick et al (1975) found that liver protein synthesis rates increased in 100g Wistar rats from approximately 50% D^{-1} to 94% D^{-1} by the 9th day of protein-free feeding. Their results were obtained by the constant infusion of $U^{14}C$ tyrosine.

Fig 8

Specific Radioactivity (SR) of L(1 ^{14}C)-leucine in plasma, liver ICF and gastrocnemius muscle ICF in rats (≈180 g) fed 10% NDpr.E or 0% NDpr.E diet for 1 week

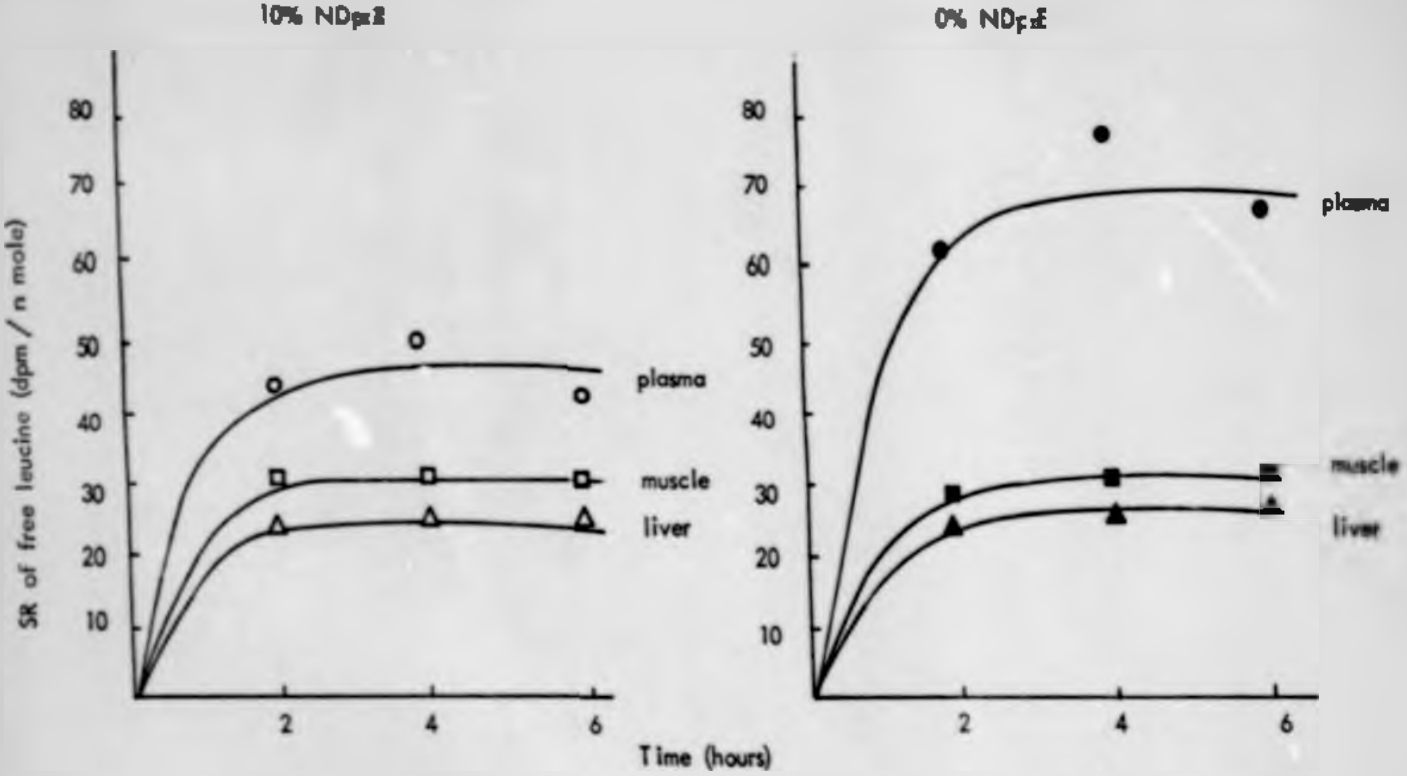


TABLE 11 MUSCLE PROTEIN SYNTHESIS RATES IN VIVO :- Specific radioactivity of leucine in plasma, gastrocnemius ICF and muscle protein in rats fed either 10% NDp:E or 0% NDp:E diet for one week and then infused. Fractional synthesis rates of muscle protein are calculated as described in the text. Rats were infused with a tracer dose of L-[1-^{14}C]leucine for 2, 4 and 6 hr at a rate of 0.48 ml/hr (5.0 μCi/ml in 0.9% saline)

TIME (hours)	Dietary Group NDp:E %	LEUCINE SPECIFIC RADIOACTIVITY (dpm/nmole leucine) Plasma (S_p)	Gastroc ICF (S_i)	Gastroc Protein (S_p)	Muscle Protein Synthesis Rate (% D^{-1})	
2	10	43.4 ± 2.6	32.7 ± 2.1	0.192 ± 0.075	10.00	9.32 ± 0.61
4	10	50.2 ± 9.8	30.9 ± 2.9	0.371 ± 0.140	8.81	9.32 ± 0.61
6	10	40.4 ± 7.4	29.3 ± 3.6	0.580 ± 0.100	9.15	9.32 ± 0.61
2	0	61.7 ± 13.6	30.5 ± 2.4	0.120 ± 0.048	6.69	*5.97 ± 0.64
4	0	77.2 ± 9.5	31.8 ± 2.5	0.249 ± 0.053	5.74	*5.97 ± 0.64
6	0	63.7 ± 10.3	32.1 ± 3.7	0.385 ± 0.050	5.48	*5.97 ± 0.64

Means ± S.D.

*Differs significantly from control muscle protein synthesis rate $p<0.01$.

TABLE 12 LIVER PROTEIN SYNTHESIS RATES IN VIVO :- Specific radioactivity of leucine in plasma, liver ICP and liver protein in rats fed either 10% NDp:E or 0% NDp:E diet for one week. Fractional liver protein synthesis rates were calculated as described in Section IIIB(iv). Rats were infused with a tracer dose of l-[1^{14}C]leucine for 2, 4 or 6 hours at a rate of 0.48 ml/hr (5.0 μCi/ml in 0.9% saline).

TIME (hour)	Dietary Group NDp:E %	LEUCINE SPECIFIC RADIOACTIVITY (dpm/nmole leucine) Plasma	Liver ICP	Liver Protein	Liver Protein Synthesis Rate (% D^{-1})	
2	10	43.4 ± 2.6	24.9 ± 2.5	0.79 ± 0.27	54.92	49.77 ± 4.6
4	10	50.2 ± 9.8	25.1 ± 2.0	1.53 ± 0.09	45.84	49.77 ± 4.6
6	10	40.4 ± 7.4	25.4 ± 1.9	2.57 ± 0.29	48.54	49.77 ± 4.6
2	0	61.7 ± 13.6	29.0 ± 5.1	2.14 ± 0.27	131.03	121.92 ± 8.7*
4	0	77.2 ± 9.5	29.6 ± 1.8	4.37 ± 0.09	116.60	121.92 ± 8.7*
6	0	63.7 ± 10.3	29.9 ± 2.7	6.68 ± 0.40	115.12	121.92 ± 8.7*

Means ± S.D.

*Differs significantly from control liver protein synthesis rate $p < 0.01$.

B (vi) TOTAL LEUCINE FLUX IN VIVO

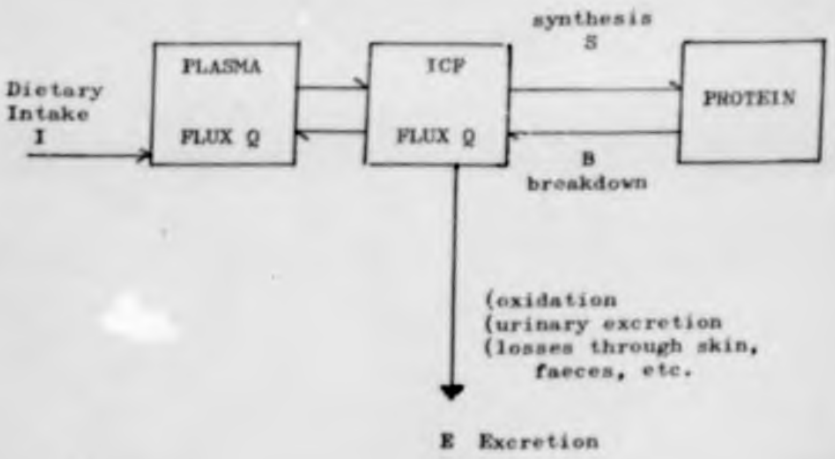
As Waterlow & Stephen (1968) showed, the rate of amino acid flux can be estimated by the constant infusion of a labelled amino acid. The flux is defined as the inflow to, or the outflow from a hypothetical amino acid pool, such that flux Q = E + S = I + B (see fig.9).

When measurements are made on plasma one can obtain one estimate of flux on the assumption that the plasma represents the precursor pool. At plateau, with constant SR of the infused amino acid, the rate of entry and exist of amino acid must be equal. Therefore $d^* = Qp.SR$, where d^* is the rate of infusion of isotope, Qp the flux, and SR the plateau specific radioactivity. The value so obtained, Qp, under-estimates the true flux, and hence the synthesis rate, because no account is taken of recycling of amino acids within the cell. Nevertheless, this method is adequate for comparative purposes, and has been used for measurements of total protein turnover both in man (O'Keefe, Sender & James, 1974; James, Sender, Garlick & Waterlow - 1974) and in rats (Waterlow & Stephen, 1967; Garlick , Millward, James & Waterlow, 1975).

However, we know that the precursor pool is not homongeneous (Fern & Garlick, 1974). Theoretically, a better estimate of the true flux would be obtained from the weighted mean SR at plateau of intracellular free leucine in the whole body. This was not considered practicable, and therefore in the present experiments the plateau SR of free leucine in the gastrocnemius muscle was taken as representative of the SR in the body pool as a whole. This seemed reasonable, since muscle represents the largest fraction of body tissue. Thus we obtain 2 estimates of flux,

SECTION III B(vi)

FIG.9



from the SR of leucine in plasma and in gastrocnemius.

The rate of leucine oxidation (E in fig.9) is calculated by dividing the output of $^{14}CO_2$ (dpm/hr) by the appropriate plateau SR (plasma or gastrocnemius). The excretion of label in urine, sweat and faeces was not measured. Previous experiments (Section IIIA) with constant infusion of $NaH^{14}CO_3$ showed that there was negligible retention of $^{14}CO_2$ in the bicarbonate pool.

The data on rates of flux and oxidation obtained in this way are presented in Table 13.

These results can be converted to rates of protein turnover if it is assumed that 534 μmoles leucine are contained in 1g protein (Fern, 1975 - PhD Thesis). Furthermore, if 20% of body weight is protein then the fractional synthesis rate of protein in the whole body is approximately 18% D^{-1} in the well fed 180g rat. This rate agrees quite well with that obtained by Garlick et al (1973) of 40g/kg body wt/day estimated by the infusion of $U^{14}C$ tyrosine. James (1972) reported similar results with the infusion of both ^{14}C lysine and ^{14}C glycine.

B(vii) DISCUSSION

If we consider the estimates derived from measurements on plasma (Table 13A), then on changing from a 10% NDpE intake to a 0% NDpE diet leads not only to a reduced total body flux but also to a reduction in total body synthesis and oxidation of leucine. The breakdown rate shows very little variation. O'Keefe et al (1974) also observed that in patients who had undergone elective surgery, synthesis rates and oxidation rates determined in this manner decreased, but breakdown rates were unaltered. These

TABLE 13 FLUX RATES OF LEUCINE IN THE WHOLE RAT, determined :

A. from the specific radioactivity of free leucine in plasma.

B. from the average for each group of rats of the specific radioactivity of free leucine in gastrocnemius ICP

(μmoles / Day / 180g body wt)

DIET %NDp:E	Total Body Synthesis Rate 'S'	+	Total Body Oxidation Rate 'E'	=	Total Leucine Input (diet)* 'I'	+	Total Body Breakdown Rate 'B'	=	Total Body Flux Rate Q
(A)									
10	2397	+	536	=	1134	+	1799	=	2933
0	1713	+	227	=	0	+	1940	=	1940
(B)									
10	3555	+	766	=	1134	+	3097	=	4231
0	3678	+	486	=	0	+	4164	=	4164

* Input estimated from measurements of food intake.

measurements were based on plasma plateau values and on the assumption that "free leucine within tissues equilibrates with plasma rapidly enough to form a single free leucine pool". The results in Table 13 show that these assumptions have to be questioned. When the plateau SR of free leucine in gastrocnemius is taken as representative of that of free leucine in the whole body, then the interpretation of the results is entirely different. On feeding a protein-free diet (Table 13B) the total flux does not alter, nor does the synthesis rate. The oxidation rate is decreased while the breakdown rate is increased (Table 13B).

What is the reason for this discrepancy between the two methods of estimating flux and the values derived from it? The discrepancy arises from the fact that the deficient diet changes the relationship between the specific radioactivities in plasma and muscle. The plateau SR's are shown in Table 14.

On the protein-free diet the plasma SR is higher, and hence the flux is less. However, the muscle SR is the same, and hence the ratio $\frac{\text{plateau SR in muscle (SRm)}}{\text{plateau SR in plasma (SRp)}}$ is reduced from 70% in the rats on the normal diet to about 50% on the protein free diet. This means that there is more internal recycling of amino acid. The degree of recycling, R; i.e. the proportion of amino acid derived from protein breakdown which is taken up again into protein within the cell is given by the relationship derived by Auh & Waterlow (1970):

$$R = 1 - \frac{SRm}{SRp}$$

Therefore, the conclusions to be drawn from Table 14 seem to be that on the protein free diet the 'plasma' flux, representing the exchange of amino acid between tissues, is reduced, but

TABLE 14

Specific radioactivity of free leucine in plasma (Sp) and gastrocnemius muscle ICF (Si) in rats fed either a 10% NDp:E or 0% NDp:E diet for one week and the ratio of Si/Sp *in vivo*.

	Specific radioactivity (dpm/nmole leucine)		
%NDp:E	PLASMA (Sp)	MUSCLE (Si)	Si/Sp
10	44.7	31.0	0.69
0	67.5	31.5	0.47

recycling within the tissues is increased. How these changes are brought about needs further work. Moreover, the conclusion can only be tentative, because it may not be correct to take the SR in gastrocnemius as representative of the whole body.

C (i) LEUCINE TURNOVER IN THE PERFUSED HIND-LIMB

The hind-limb perfusion technique used in the present work will be described in detail as it was a modification of the methods described by Ruderman et al (1971) and Jefferson et al (1972). Preliminary measurements showed that 80% by weight of the hind-limb was attributable to skeletal muscle and connective tissue, 19% to skin and tail and the remaining 3% to bone. As the major vessels to the skin were tied and the tail constricted by a tight ligature, the preparation was essentially a skeletal muscle perfusion.

By infusion L[^{14}C] leucine and measuring $^{14}CO_2$ output and the SRs of free leucine in the intracellular free pool of gastrocnemius and in protein it was possible to estimate rates of both leucine oxidation and protein synthesis. Thus the contribution of skeletal muscle to total body leucine oxidation and protein synthesis could be determined.

C (ii) THE PERFUSED HIND-LIMB PREPARATION

The operative procedure involved ligation of superficial vessels to the abdominal wall and skin of the hemicorpus followed by pelvic evisceration, ligation of major branches of the great vessels and finally, cannulation of the aorta. The liver was then excised at the level of the posterior vena cava and the blood allowed to drain free from the transected hemicorpus.

Details of the operation were as follows:-

Rats were anaesthetized by intraperitoneal injection of phenobarbitone(Pulsrin : 100 U/100g body wt).

The base of the rat's tail was then ligated and a mid-line incision made in the abdominal wall from the pubic symphysis to the xyphoid process. The incision was extended laterally towards the kidneys. The superficial epigastric arteries (1) to the abdominal wall together with the saphenous branch (2) and the superficial vessels to the skin (3) were ligated on both sides (fig.10). After this the inferior mesenteric artery (4) and part of the descending colon (5) were ligated. The colon was pulled forward; the ovarian (6), uterine, pubic epigastric trunks, bladder and uterus (7) were ligated and the whole reproductive tract and bladder dissected out.

Next, ligatures were placed round the suprarenal and renal vessels (8) and the kidneys removed. The coeliac and superior mesenteric vessels (9) were also ligated. An incision was then made into the thorax and a loose ligature placed round the descending aorta above the level of the diaphragm. The aorta was clamped above the loose ligature and an incision made in its wall, into which the canula (blunt ended needle : serum size '0') was inserted and tied firmly in place. The perfusion was begun (10 ml/min) and the Spencer Wells clamp was removed immediately. The liver was quickly excised above the posterior vena cava and the animal transected above the level of the aortic cannula. The fat pads overlying the psoas muscles were then carefully dissected out. The perfusate was allowed to flush the residual rat blood from the hemicorpus for a period of 4-5 minutes. The preparation was then transferred to a stainless

FIG 10

HIND LIMB PREPARATION

Ligatures of the rat hind-limb preparation
(for details see text)

steel perfusion box and the perfusate recycled. The entire operative procedure lasted approximately 8–10 minutes.

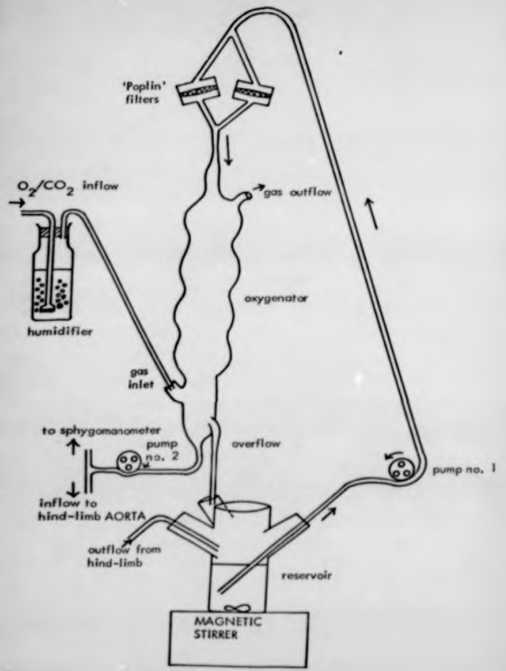
Perfusion Apparatus

A schematic flow chart of the 'Perfusion Apparatus' is set out in fig.11. Essentially the apparatus is similar in design to that described by Ruderman, Houghton & Hems (1971), consisting of two pumps (Watson-Marlow M.H. flow inducers), a convoluted glass oxygen chamber, reservoir, sphygmo-manometer and animal trough. They were all connected with tygon tubing. Sterilised 'Poplin' cotton material was used in the filter.

The perfusate reservoir had an approximate capacity of 50ml and in the bottom was placed a small magnet. This was rotated during recycling of the perfusate by the magnetic stirrer placed under the reservoir. The reservoir had three outlets. From one, the perfusate was drawn by pump No.1 and pumped via the filter to the top of the convoluted glass oxygen chamber over whose surface the perfusate filmed. Humidified O_2 : CO_2 (95/5) flowed counter-current to the perfusate at a known fixed rate. It was possible to collect the gas leaving the top of the chamber in order to estimate $^{14}CO_2$. The perfusate pooling at the bottom of the chamber was able to overflow back to the reservoir by the 2nd reservoir opening or it could be pumped (No.2) via the sphygmomanometer (to measure in line pressure of the perfusate) to the hind-limb in a closed stainless steel animal trough. The perfusate flow rate (10ml/min) into the hind-limb was kept constant by pump No.2.

The animal trough was constructed of stainless steel (length 17.5cm; width 10cm; depth 5.2cm) with a central dull angled depression for easy collection of perfusate draining from the

FIG 11 PERFUSION APPARATUS



vena cava of the perfused hemicorpus. The outlet from the end wall of the trough led back to the third opening of the reservoir. The cannula passed through a small hole above this outlet and was inserted into the aorta of the preparation. The limb rested on a stainless steel gauze which fitted in the trough, leaving a space below in which the perfusate collected and passed out of the trough to the reservoir. Both the gauze and trough were made to slope slightly for gravity collection of the perfusate. The tray was sealed with a transparent perspex lid which was kept closed during perfusions to minimise evaporative losses of water from the exposed parts of the hind-quarters and of $^{14}CO_2$ from the perfusate.

Preparation of the Perfusion Medium

A modified krebs-Henseleit bicarbonate buffer containing bovine albumin and aged human erythrocytes constitutes the basic medium. Substrates and hormones are added to the basic medium as desired. The bicarbonate buffer contains the following salts in mM concentrations; NaCl, 118.5; KCl, 4.75; $CaCl_2 \cdot 6H_2O$, 1.08; $MgSO_4 \cdot 7H_2O$, 1.20; KH_2PO_4, 1.2 and $NaHCO_3$, 25.0. The buffer must be prepared fresh each day from stock solutions of the individual components. Stock solutions are made up in the following concentrations (g/l); NaCl, 138.5; KCl, 35.4; $CaCl_2.6H_2O$, 55.6; $MgSO_4.7H_2O$, 29.4; KH_2PO_4, 16.3 and $NaHCO_3$, 42.0. The buffer is prepared by mixing the following proportions (in ml) from the stock and making up to 1 litre with double deionized water; NaCl, 50; KCl, 10; $CaCl_2$, 10; $MgSO_4$, 10; KH_2PO_4, 10. This mixture is gassed with 95% O_2 : 5% CO_2 for 15 minutes at 0° to 4°C prior to adding the $NaHCO_3$ (50 ml). This

lowers the pH and prevents precipitation of calcium bicarbonate. The buffer is gassed for a further 10 min. 18mg/l tyrosine and 0.3ml (stock) 1 M pyruvate were added before the perfusate was made up. Tyrosine was added at this stage because owing to its insolubility it is not possible to prepare it in a stock solution of essential amino acids.

Human aged blood (21 days old) was centrifuged at 2500 rpm for 15 min at 4°C in an MSE centrifuge and both plasma and leucocytes aspirated by pasteur pipette from the top of the erythrocytes. The erythrocytes were then washed three times with the Krebs-Henseleit bicarbonate buffer, each time the supernatant having been removed after centrifugation.

The perfusate was then prepared from the washed erythrocytes (90 ml), bovine serum albumin (60 ml of 15% solutation, w/v), non-essential amino acids (0.8 ml), insulin (0.4 ml stock) and glucose (1.0 ml of 20% solution, w/v) together with heparin (0.2 ml of 1000 U/ml solution). The volume was made up to 200 ml with Krebs-Henseleit bicarbonate buffer so that the final concentration of albumin was 4.5%; glucose 5.55 mM and of amino acid as in Table 15.

Stock solutions required for preparation of this complete medium were as follows:-

(1) A mixed solution of essential amino acids; details are listed in Table 15.

(2) A mixed solution of non-essential amino acids; details in Table 15.

(3) 20% glucose solution; 20.0g D-glucose was dissolved in bicarbonate buffer and made up to 100ml.

TABLE 15

CONCENTRATION OF AMINO ACID MIXTURES FOR HIND-LIMB PERFUSIONS

a) Non-Essential Amino Acid Mixture = 2 x Plasma

	Mol.Wt.	Conc. in Stock Solution (M)	Conc. in Perfusate (mM)
ALANINE	89	0.225	0.90
ASPARTATE	113	0.025	0.10
ASPARAGINE	150	0.025	0.10
CYSTEIN	121	0.025	0.10
GLUTAMATE	147	0.100	0.40
GLUTAMINE	146	0.100	0.40
GLYCINE	75	0.125	0.50
PROLINE	115	0.125	0.50
SERINE	105	0.100	0.40

b) Essential Amino Acid Mixture

ARGININE	174	0.100	0.20
HISTIDINE	209	0.050	0.10
ISOLEUCINE	131	0.050	0.10
LEUCINE	131	0.050	0.10
LYSINE	146	0.175	0.35
METHIONINE	149	0.050	0.10
PHENYLALANINE	165	0.050	0.10
THREONINE	119	0.125	0.25
TRYPTOPHAN	204	0.050	0.10
TYROSINE	-	-	(0.10)
VALINE	117	0.010	0.20

(4) 250 mUnit/ml insulin solution.

(5) Bovine serum albumin solution; 66.0g of bovine serum albumin (Pentex Fraction V, Research Division, Miles Laboratories Ltd) was dissolved in 240 ml bicarbonate buffer. When the albumin was dissolved 13.2 ml of N-NaOH (1 ml of N-NaOH to 5g of Albumin) was added to neutralize residual fatty acids. This solution was dialysed against three changes of bicarbonate buffer for 1 to 2 days using Visking tubing (Gallenkamp 36 x 32 mm). Dialysed albumin solution was diluted with bicarbonate buffer to 440 ml to give a final concentration of 15g% (w/v).

C (iii) VIABILITY OF THE HIND-LIMB PREPARATION

Some of the most sensitive indicators of the viability of the perfused hind-limb are potassium efflux, ATP and creatine phosphate concentration in skeletal muscle, tissue water content, oxygen uptake and visual appearance (Ruderman (Thesis) 1972); Jefferson et al (1972). In a control perfused hind-limb, Ruderman showed that the mean K^+ level at the end of the 39 minute perfusion was almost the same as at the start. Insulin (12.5 mU/ml) caused a net uptake of K^+ which was detectable after 5 minutes. This was not due to movement of potassium into red cells since this uptake was not observed in recycled perfusate alone.

However, when the perfusion period was extended to 120 minutes, Ruderman found that perfusate potassium tended to rise after the first 65 minutes. He suggested that this may have been due to red cell haemolysis which occurs in all perfusions, and a fall in perfusate pH due to lactate accumulation. Ruderman

showed a significant increase in perfusate potassium when the pH of the medium was set between 7.1 - 7.2 and estimated that if the entire perfusate potassium had resulted from tissue leakage, less than 5.0% of total tissue potassium would have been lost.

Measurement of perfusate potassium in the present work showed that the concentration in the perfusate rose from 3.2 mEq/l to 5.6 mEq/l over a 2 hour period. This output was similar to the figure obtained by Ruderman. The initial uptake of potassium in the first 65 minute period observed by Ruderman would certainly have been aided by the high concentration of insulin in the perfusate (12.5 mU/ml), which is known to enhance the movement of K^+ from plasma to tissues (Mahler, et al, 1968). The present results, however, were obtained with only 0.25 mU/ml of insulin in the perfusate.

The water content of muscle indicated the integrity of the normal osmotic balance between the intra and extra-cellular fluid and plasma. In preparations in which K^+ balance was within the normal range, the water content of the gastrocnemius muscle averaged 73.0%. Jefferson et al (1972) reported a water content of 74.8% for the same muscle under similar conditions (Table 16).

The appearance of the hind-limb provides a simple check on viability. When the blood flow to the limb is inadequate (i.e. 6.0 ml/min) the feet are cyanotic and distinctly blue in appearance compared with the pink colour of the normal rat. Almost all the preparations showed this normal pink colour.

TABLE 10

PROPERTIES OF THE PERFUSED HIND-LIMB PREPARATION

CRITERIA	IN VIVO	Ruderman et al (1971)	Jefferson et al (1972)	observed in present work
Perfusion time (min)	-	120	180	120
Water Content (ml/100g)				
(1) Psoas	74.0 ± 0.6	-	74.5 ± 0.5	73.6 ± 0.4
(2) Gastrocnemius	73.4 ± 0.6	-	74.8 ± 0.4	73.0 ± 0.5
ATP in muscle (μmoles/g)	6.02 ± 0.19	5.2 ± 0.3	6.14 ± 0.15	-
Creatine phosphate (μmoles/g)	15.04 ± 0.29	15.10 ± 0.60	14.58 ± 0.58	-
Insulin (mU/ml)	0.05	12.5	25.0	0.25
K^+ 0-120 [mEq/l]	-	1.0	-	2.4
Perfused Flowrate (ml/min)	-	8.0	12.0	10.0
Perfusion pressure (mmHg)	-	-	137 ± 4	90 ± 10
Protein Synthesis rate in Gastrocnemius ($\%D^{-1}$)				
(1) 10% NDp:E diet	9.32 ± 0.61	-	-	8.85 ± 2.35
(2) 0% NDp:E diet	5.97 ± 0.64	-	-	4.69 ± 2.06

Protein synthesis rates in the hind-limb were also found to be comparable to rates of skeletal muscle protein synthesis in the whole animal. This parameter is certainly a good indicator of limb viability. For rapidity of determining the viability of a limb, the continuous monitoring of $^{14}CO_2$ (released during the catabolism of L[1-^{14}C]leucine)was frequently used. Initial studies suggested that the preparation was viable for 2-3 hours but with experience gained one could only guarantee a viable preparation for at least 2 hours. After this period of time, the pressure on the perfusate input side of the hind-limb began to rise slowly, thus interfering with the oncotic pressure between plasma and muscle cells and causing oedema. Haemolysis and hence K^+ leakage became a problem and the pH of the perfusate would begin to fall from pH 7.4 and approach pH 7.1. It was therefore decided that the longest experimental preparations should only last 2 hours. This proved to be a disadvantage as plateau specific radioactivity of $^{14}CO_2$ output had not been obtained in 2 hours (fig.12). Work could obviously be carried out to increase the length of time for which the preparation is viable, thus giving greater accuracy in estimating absolute oxidation rates. With experience it was possible to reject those preparations which would not have survived a 2 hour perfusion. The visual appearance would immediately indicate any onset of anoxia or oedema and the arterial pressure was a sensitive indicator of viability, as was the $^{14}CO_2$ output, which could be measured immediately.

Although not all the parameters which have been used as a check by other workers were examined, I believe, with a certain degree of confidence, that the present results are based on preparations as viable as those reported in the literature. The finding of synthesis rates in the hind-limb preparation comparable to those in the whole animal adds credibility to this assumption (Table 16).

C (iv) METHODS

Female hooded rats (Animal Suppliers (London) Ltd) weighing approximately 170g were fed ad libitum for one week on a powdered diet (0.10 hDp1E) which maintains normal growth rates. A group of rats was then used for the hind-limb preparation as described previously (Section IIIC(ii)). Once recycling of the perfusate had commenced, a tracer dose of L[1-^{14}C]leucine (5.0 μCi/ml) was constantly infused at a rate of 0.48 ml/hr into the reservoir (fig.11). The constituents of the perfusate were similar to those described previously (Section IIIC(ii) except that the amino acid concentrations were increased above those normally found in plasma in order to compensate for amounts removed by net protein synthesis and oxidation. The amino acid composition of the perfusate was also varied according to the previous dietary state of the animal. Normal plasma concentrations of amino acids in the rat were taken from the results obtained in this laboratory (Table 1). Amino acids were added to the perfusion medium to give the following concentrations in terms of normal rat plasma (1) Rats on a normal diet : non essential amino acids 2.5 x plasma, essential amino acid, 2.0 x plasma

(10% NDp:E, High a'a). (2) Rats obtained at 170g and fed ad libitum the protein-free diet for one week; non essential amino acids 3.5 x plasma, essential amino acids 0.75 x plasma (0% NDp:E, Low a'a).

Insulin was added to the medium (250 μU/ml) only in the preparations from rats on the normal diet (10% NDp:E). These concentrations of amino acids were chosen in order to maintain both protein synthesis rates and amino acid concentrations at similar levels to those found in vivo. Preparations of hind-limbs from animals fed the normal and protein free diets were also perfused in an identical manner but the concentrations of amino acids in the medium were exchanged between the groups to determine the possible role of amino acid concentration in the regulation of leucine oxidation and/or protein synthesis. The four groups perfused were thus designated :-

GROUP

1 10% NDp:E, High a'a.

2 10% NDp:E, Low a'a.

3 0% NDp:E, High a'a.

4 0% NDp:E, Low a'a.

Insulin was also infused (70 μU/hr) into all groups of hind-limbs to compensate for losses due to adhesion of the insulin to the glass wall of the lung and reservoir and possible degradation by the perfused hind-limb. O_2/CO_2 (95:5) was used to gas the perfusate at 300 ml/min and was then bubbled through 3 ml of a 2:1 mixture of hyamine hydroxide/ethanol with phenolphthalein was an indicator (Kaihara & Wagner, 1968). Immediately the hyamine had been neutralised a new vial was

substituted. A second bubble trap - in series with the first - showed that trapping of $^{14}CO_2$ in the first vial was 98% efficient. The time taken to trap 1 mmole of CO_2 (1 MOLE Hyamine reacts with 1 MOLE of CO_2) was recorded throughout the experiment. The SR of the CO_2 could thus be determined. 10 ml toluene : PPO (2, 5 dinitrophenyloxanole, 4.0g/l toluene) was added to the vials which were counted in a Beckman Liquid Scintillation counter (Model LS-150). With the external standard ratio method, efficiency of counting was 75%. Samples of plasma were taken at regular intervals; at the end of a 2 hour perfusion plasma and gastrocnemius samples were taken rapidly for estimation of SR of free and protein bound leucine by ion-exchange chromatography on a Locarte amino acid analyser fitted with a column effluent stream splitter (Fern & Garlick, 1973). Fractions were counted in 10 ml of 0.4% PPO in a mixture of toluene : triton - X-100 (2:1) at an efficiency of 85 - 90%.

c (v) Oxidation of leucine in the hind-limb.

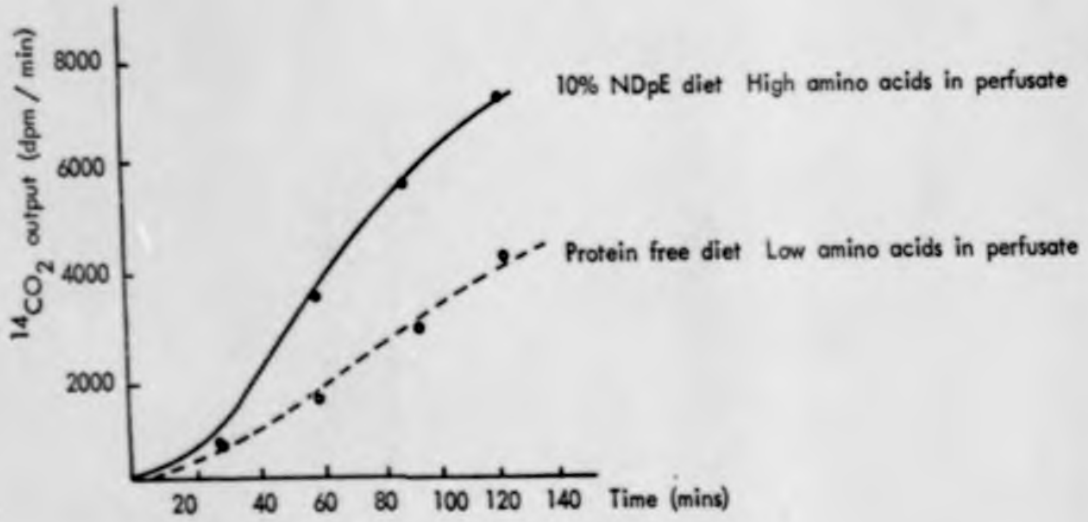
Rates of $^{14}CO_2$ production were calculated as dpm $^{14}CO_2$ evolved per minute of infusion time. In order to estimate the absolute rate of oxidation of leucine by the hind-limb and the rate of synthesis of skeletal muscle, the SR of leucine in the precursor pool was taken to be that of free leucine in the gastrocnemius muscle.

RESULTS - Estimation of Leucine Oxidation in the Perfused Hind-Limb.

Fig.12 shows the rise in $^{14}CO_2$ output in the perfused hind-limb. The rate of $^{14}CO_2$ output was still rising at the end of the 2

Fig 12

Output of $^{14}CO_2$ (dpm / min) during constant infusion of a tracer dose of L(1^{14}c)-leucine (5.0 μCi / ml at 0.48 ml / hr) in perfused hind-limb of rats fed either an adequate (10% NDpE) or a protein-free diet for 1 week



hour period. This reflects the relatively large volume of the perfusate, which must increase the time needed to reach plateau specific radioactivity for both the precursor (free leucine) and for the product of oxidation.

The rate of $^{14}CO_2$ output is a measure of the rate of leucine oxidation. However, absolute rates of leucine oxidation can only be determined if the specific radioactivity of the precursor is known. For the perfusion experiments, rates of oxidation are calculated from the maximum rate of $^{14}CO_2$ output at the end of the perfusion and the SR of free leucine in the gastrocnemius muscle at that time. Since plateau $^{14}CO_2$ output had not been reached (fig.12) in the hind-limb, these calculations will give an underestimate of the true oxidation rate by muscle.

In 2 rats (group 2) fed 10% NDp:E diet, the output of $^{14}CO_2$ (dpm/min) from the hind-limb apparently increased when the conditions in the perfusate were altered to simulate plasma from rats fed a 0% diet (Table 17). For this, the essential amino acids were lowered, the non-essentials increased and only that amount of insulin infused necessary to compensate for normal losses due to adhesion to glass etc. The apparent increase in $^{14}CO_2$ output, however, disappeared when the SR of the free intracellular leucine was taken into account (Table 17,) so that in absolute rates of oxidation there is no significant difference between the 10% NDp:E groups perfused with either High or Low amino acids. Feeding a 0% NDp:E diet and perfusing with High a'as amino acids

TABLE 17.

Rates of leucine oxidation in the perfused hind-limb: effect of protein-free feeding and variation in the amino acid concentration of the perfusate. Means ± SD.

GROUP (No. in group)	DIET (%CHp:E)	AMINO ACID CONCENTRATION	MUSCLE WEIGHT (g)	SA OF MUSCLE ICP (dpm/nmole)	$^{14}CO_2$ OUTPUT (dpm x 10^3/min)	RATE OF LEUCINE OXIDATION (nmoles/hr) Total	per g muscle	per 81g/100g rat
1 (5)	10	high	54.2 ± 2.4	90 ± 24	7.0 ± 0.6	4.85 ± 1.8	0.10 ± 0.02	7.34 ± 2.1
2 (2)	10	low	50.1	144	11.6	4.82	0.10	7.80
3 (6)	0	high	49.9 ± 4.0	105 ± 36	6.4 ± 0.7	3.99 ± 1.6	0.08 ± 0.03	6.59 ± 1.7
	0	low	55.3 ± 3.1	122 ± 23	4.0 ± 0.4	2.03 ± 0.6	0.04 ± 0.01	2.96 ± 0.7

* differs significantly from Group 1 : $p > 0.05$.

\+ perfusate contained 80.4 x 10^3 dpm/min L-^{14}C - leucine.

(group 3) led to a fall in absolute oxidation which was not statistically significant. (Table 17). If, however, the limbs were perfused with Low amino acids, the output of $^{14}CO_2$ decreased despite an increase in specific radioactivity of intracellular free leucine (Table 17,). The absolute rate of oxidation was reduced quite significantly from 1.99 μmoles/hr to 2.01 μmoles/hr on altering the levels of amino acid and insulin. This reduction fits in with the previous work carried out in vivo and in vitro.

The contribution of muscle to total body leucine oxidation may be estimated from the data in Table 17 if several assumptions are made. This is considered in Section IIID. Skeletal muscle, which forms approximately 80% of the hind-limb preparation, was assumed to be the sole contributor to leucine oxidation by the hind-limb. Possible contributions by bone, connective and nerve tissue were not considered although *recently Buse, Jurasinic & Reid (1975) have published* data on the oxidation of branched-chain amino acids by nerve, muscle and aorta. Since adipose tissue is also known to oxidise leucine (Rosenthal et al, 1974) the major fat pads overlying the psoas muscles were removed during the surgical preparation. The vessels supplying the skin were also ligated to minimize any oxidation by this tissue. The success of these measures is borne out by the results on protein synthesis in the hind-limb, in the following section.

C (vi) PROTEIN SYNTHESIS RATES IN THE PERFUSED HIND-LIMB

Protein synthesis rates in skeletal muscles of the perfused hind-limb were calculated by the equation given in Section IIIB(iv)

derived by Garlick et al (1973). The pool sizes of free leucine in perfusate and gastrocnemius muscle are shown in Table 18, and the synthesis rates in Table 19. The fractional synthesis rate (FSR) of protein in skeletal muscle of the normal (0.10 NDpiE) hind-limb perfused with High amino acids (Table 19, Group 1) was remarkably similar to that of skeletal muscle protein in vivo (Table 11). Furthermore, when limbs from rats fed the protein-free diet were perfused with Low amino acid levels (group 4) the FSR of protein in gastrocnemius muscle was reduced from 8.85% D^{-1} in the normal limb to 4.69% D^{-1}. Again, this value was similar to the rate observed in vivo in animals fed a protein free diet. This reduction in fractional synthesis rate brought about by protein-deprivation is in agreement with the findings of Garlick et al (1975) carried out in rats in vivo but infused with [$U^{14}C$]-tyrosine. This agreement with results in vivo reinforces the earlier claims that the preparations were viable for at least 2 hours. It is of interest to note that when either nutritional group was infused with Low amino acid levels the protein synthesis rates appeared to decrease (Table 19, groups 2 and 3), but less significantly in the protein-free group (4). This is all the more interesting since recent workers have suggested the possible role of leucine in the regulation of protein synthesis in skeletal muscle (Fulks et al, 1975; Buse & Reid, 1975). However, no claims are made here as there were only 2 observations in the 10% NDpiE, Low amino acid group. The hypothesis will be discussed, however, at a later stage.

TABLE 18 Concentrations of free leucine in perfusate and in intracellular fluid (ICF) of gastrocnemius muscle after 2 hours' perfusion of the hind limb, and in plasma and muscle *in vivo*. Means ± SD

Group perfused	No. in group	Diet (%ED:E)	Amino acid concentration	Leucine pool size (μmoles/ml or μmoles/g. tissue wet weight)		
				Perfusate or plasma	Muscle ICF	Ratio ICF/plasma
1	5	10	high	138.2 ± 25.6	261.5 ± 24.7	1.89 ± 0.5
2	3	10	low	98.0 ± 8.6	242.5 ± 35.1	2.47 ± 0.4
3	5	0	high	125.4 ± 30.1	223.7 ± 29.2	1.78 ± 0.3
4	4	0	low	88.1 ± 19.0	195.7 ± 8.0	2.22 ± 0.5
in vivo						
	7	10	-	104.5 ± 10.4	170.6 ± 15.4	1.63 ± 0.3
	9	0	-	76.7 ± 12.4	160.2 ± 11.7	2.09 ± 0.4

TABLE 19 Protein synthesis rates in the hind limb: effect of protein-free feeding and variation in the amino acid concentration of the perfusate. Means ± SD

Group (no.in group)	Diet (%ND$_p$E)	Amino acid concentration	SR of free and bound L-[^{14}C]-leucine (dpm/nmole leucine)			Protein synthesis rate ($\%d^{-1}$)
			perfusate	gastrocnemius muscle ICF	muscle protein	
1 (4)	10	high	182.8 ± 17.5	89.8 ± 27.5	0.41 ± 0.09	8.85 ± 2.3
2 (2)	10	low	296.2	144.1	0.10	1.46
3 (5)	0	high	200.2 ± 22.3	112.6 ± 35.4	0.29 ± 0.04	5.97 ± 2.4
4 (4)	0	low	213.0 ± 18.9	122.7 ± 23.6	0.33 ± 0.07	4.69 ± 2.1

D CONCLUSIONS

Contribution of muscle to the turnover of leucine in whole body.

Flux rates may also be determined in the hind-limb preparation. Furthermore, if the estimates are corrected for total body muscle mass then a figure can be obtained giving an idea of the contribution of total skeletal muscle to leucine oxidation in the whole body. Again as with the measurements on the whole rat, the calculation of flux and synthesis rates can be made in 2 ways:

A. From the final SR of free leucine in the perfusate.

B. From the final SR of free leucine in muscle.

The results are shown in Table 20. Comparison of the 2 methods of calculation can be made for 2 sets of results, group 1 and 4. In both cases, method A gives much lower flux rates than method B. This again shows the importance of recycling.

Estimates of rates of synthesis (leucine uptake into protein) and of oxidation of leucine, in the total muscle mass of the rat, based on the SR in gastrocnemius, are shown in Table 21. These rates have been calculated on the assumption that skeletal muscle mass is 45% of body weight (Munro, 1969) and that the rats weighed 180g. The values for flux are the same as those shown in Table 20, adjusted for the greater muscle mass in the whole body compared with the hind-limb.

Oxidation

When the conditions found *in vivo* are simulated in the perfusates of limbs from rats fed 10% NDp:E or 0% NDp:E diets for one week (Comparison of groups 1 and 4), then

TABLE 20 Leucine flux rates in the hind-limb preparation: (A) estimated from SR of free leucine in 'plasma' (Table 19) and $^{14}CO_2$ output (Table 17) and (B) estimated from SR of free leucine in gastrocnemius muscle (Table 19) and $^{14}CO_2$ output (Table 17).

A

Group	Diet (%ND_p:E)	Amino acid concentration	Muscle weight (g)	Leucine flux rate in hind limb (μmoles/day)
1	10	high	54.2	633
4	0	low	55.3	543

B

1	10	high	54.2	1275
2	10	low	50.1	804
3	0	high	49.9	1099
4	0	low	55.3	944

leucine oxidation rates in total skeletal muscle are estimated to fall from 166 μmoles leucine/day/180g body wt (group 1) to 69 μmoles/day/180g body wt (group 4) (Table 21). In vivo, the total body leucine oxidation fell from 766 μmoles/day/180g body wt to 486 μmoles/day/180g body wt in identical groups of animals (Table 13). Therefore, the reduction of 97 μmoles/day in the perfused hind-limb preparation (skeletal muscle) represents at least 19% of the fall in leucine oxidation seen in vivo on changing from a 10% NDp:E diet to a 0% NDp:E diet. Since this is an under estimate (Section IIIC(v) of skeletal muscle leucine oxidation, it is evident that muscle is one of the major contributors to the control of leucine oxidation in times of dietary stress. These figures also suggest that in the well fed group skeletal muscle is contributing at least 22% of total body leucine oxidation.

When the hind-limb from well-fed rats is perfused with low amino acids (group 2) the oxidation rate increases slightly (Table 21). If limbs from rats fed 0% NDp:E diet are perfused with high levels of amino acids (group 3) then leucine oxidation rates are greater than when the amino acid concentration of the perfusate is low (group 4).

Synthesis

The estimates shown in Table 21, of the uptake of leucine into total muscle protein, are derived from flux measurements on the hind-limb on the assumption that all [^{14}C]leucine which is not oxidised is taken up into protein. The results are given in terms of μmoles leucine/180g body weight/day. These can be converted into fractional rates of muscle protein synthesis,

TABLE 21 Leucine flux rates in total body muscle estimated on the assumption that skeletal muscle mass is 45% of total body weight. Figures adjusted to 180 g rat.

Group	Diet (%ND_p:E)	Amino acid concentration	Flux rates (μmoles/day/ 180 g rat)		
			Muscle oxidation rate	Muscle protein synthesis rate	Total
1	10	high	166	1739	1905
2	10	low	188	1112	1300
3	0	high	110	1673	1783
4	0	low	69	1313	1382

on the assumption that muscle is 45% of body weight, and contains 20% protein. Thus we have 3 ways of calculating the rate of protein synthesis in muscle.

A. From measurements of labelling of protein and free leucine in gastrocnemius after constant infusion in the whole animal (Section IIIB(iv).

B. From the same measurements, after perfusion of the hind-limb (Section IIIC(vi).

C. From measurements of flux and oxidation in the perfused hind-limb (this section).

Methods B and C, although they are based on the same preparation and although both rely on determination of the SR of free leucine in gastrocnemius, are independant; in B the additional information is obtained from the SR of protein, whereas in C it is obtained from measuring the oxidation.

The results of this comparison are shown in Table 22. The Agreement is reasonable, suggesting that the assumptions on which the various calculations are based are not too much in error.

TABLE 22 Comparison of protein synthesis rates in skeletal muscle estimated (A) *in vivo* (B) in the gastrocnemius muscle of the perfused hind-limb and (C) from flux rates in the hind-limb.

Dietary group (NH_2)	Protein synthesis rate ($\%d^{-1}$)		
	A	B	C
	in vivo	gastrocnemius muscle (hind limb)	total muscle (hind limb)
10	9.32	9.00	8.85
0	5.97	6.80	4.69

SECTION IV

DISCUSSION

The hypothesis that led to this work is that oxidation of the branched-chain amino acids (BCA) may be the factor which limits the ability of an animal to adapt to low protein intakes. The reasoning behind this hypothesis was as follows :-

(a) There is ample evidence that when dietary protein is restricted, there is a greater fall in the plasma concentrations of the BCAs than in the other essential amino acids. This suggests that the mechanisms for removing the BCAs may be more active.

(b) We know that the enzymes in the liver responsible for oxidizing amino acids are very sensitive to the level of dietary protein, so that when protein intake is reduced the activity of the enzymes falls. However, the available evidence suggested that the BCAs are oxidized mainly in muscle and not in liver. Up to now there has been little information about the capacity of muscle enzymes to adapt to variations in protein intake. If these enzymes cannot adapt, this would be an important factor limiting the animal's capacity to economise protein.

The problem has been tackled in two ways; by measurements of the activity of the enzymes responsible for oxidizing the BCAs; and by measurements of the overall rate of oxidation in the whole animal and in the perfused hind-limb as judged by the output of $^{14}CO_2$ from labelled substrates.

(i) Enzymes concerned with the oxidation of BCAs

The first-reversible-step in oxidation is transamination to give an α-oxo acid. The second-irreversible-step is oxidative decarboxylation of the α-oxo acid.

BCA aminotransferase activity

In agreement with most other workers, the highest aminotransferase activity was found in skeletal muscle. Both fasting and low protein feeding led to an increase in the skeletal muscle aminotransferase activity, whilst an increase in the liver enzyme activity was observed only in animals on a low protein diet.

Ichihara, Noda & Ogawa (1973) have suggested that the step involving transamination may be the rate-limiting reaction in the oxidation of leucine, particularly in the liver. This supported Krebs' suggestion (1972) that the aminotransferase could be rate-limiting through Km control. The value often quoted for the Km of leucine is 3.8mM, obtained from measurements on hog-heart (Ichihara & Koyama, 1966). The Km for BCA aminotransferase in rat skeletal muscle has never been published, but the present work gives a value close to 0.4 mM for leucine (Table 2). This is very much closer to the Km value of the dehydrogenase than was previously thought. If the enzyme were to decrease in protein-depletion then this would represent an additional 'course' control over amino oxidation. On the other hand measurements in vitro show that the aminotransferase activity is many times that of the dehydrogenase. If these results hold in vivo the activity of the dehydrogenase must be rate-limiting.

The literature does not agree on the effect of either starvation or protein depletion on leucine aminotransferase activity in rat tissues. McFarlane & Van Holt (1969 b), Krebs (1972) and Adibi et al (1975) have all shown reductions in the enzyme in tissues from protein depleted rats. Moreover, McFarlane &

Von Holt found that adaptation of this enzyme occurred in the mitochondrial fraction. This would agree with Shirai et al (1971) that Enz II (mainly mitochondrial) was more responsive to induction than Enz I (supernatant). Both Adibi et al (1975) and Krebs (1972) found that protein depletion did not affect the aminotransferases in kidney.

On the other hand, Mimura et al (1968) found that a protein-free diet led to increases in aminotransferase activity in muscle, kidney and liver but not in intestine. Reeds (1974) observed only slight increases in the enzyme in skeletal muscle and a marginal decrease in the liver enzyme activity. In the present work, Mimura's observations have been confirmed in rats fed a protein-free diet. However, if rats were fed a low protein diet, just sufficient to maintain body weight, then only muscle aminotransferase increased. Recently, Featherston & Horn (1973) observed no effect on leucine aminotransferase activity in chick skeletal muscle, liver or kidney following 48 hr starvation or a protein-free meal. On the other hand, both in Adibi's work (1975) and in that reported here starvation led to an increase in muscle aminotransferase.

Evidently, therefore, the literature is in a state of confusion. Much of this may arise from the fact that we are dealing with several isozymes (I - III) and that compartmentation of these isozymes may lead to different responses, depending on the 'trigger' agents and 'penetration of compartments'. Ideally, one should study the effects of protein malnutrition on individual isozymes within compartments such as mitochondria and cell cytoplasm. McFarlane's work (1969 b) goes some way to achieving this, but no

comprehensive study has been made up to the present time.

BCA Dehydrogenase

In the present work, skeletal muscle was estimated to have the highest total tissue dehydrogenase activity. Both fasting and protein-free feeding led to a decreased activity of the dehydrogenase enzyme in skeletal muscle. In liver, however, fasting increased the enzyme activity whilst a low protein diet, sufficient to maintain body weight, did not affect the enzyme activity. Branched-chain α -oxo acid dehydrogenase activity has a wide tissue distribution in the rat and other animals. So far, it has been demonstrated in liver, kidney, heart, skeletal muscle, skin, lung, intestine, brain and white blood cells (Connelly et al 1968; Wolhueter & Harper, 1970; Reeds, 1974; Danner et al, 1975). The present work demonstrates the presence of α -oxo isocaproic acid dehydrogenase and α -oxo isovalerate dehydrogenase in rat skeletal muscle. Prior to this most workers were unable to demonstrate any dehydrogenase activity in rat skeletal muscle (Connelly et al, 1968; Wolhueter & Harper, 1970). The failure of Wolhueter & Harper to find this enzyme in muscle may have been the result of their use of the Polytron homogenizer for the preparation of muscle extracts, as my investigations showed that this technique causes a total loss of enzyme activity. Presumably, soni cation disrupts the enzyme complex from the outer face of the inner mitochondrial wall where it is known to be located (Johnson & Connelly, 1972). By using gentler methods of homogenisation it has been possible to show that α-KIC dehydrogenase within the total mass of muscle may be as much as thirty times that found in whole liver. Reeds (1974) also demonstrated α-KIV dehydrogenase activity in rat

skeletal muscle at much the same time. He, too, homogenized the muscle in Duall glass tissue grinders.

Muscle has the greatest total enzyme activity and is considered to be the major site of BCA oxidation. Recently, Beatty et al (1974) reported that there was no difference in the output on $^{14}CO_2$ from labelled leucine in incubated red or white skeletal muscles. However, an exploratory survey of different muscle types demonstrated the possibility of varied distribution of enzyme activity (Table 3). Thus the greatest dehydrogenase activity was found in the plantaris and lower activities in the Extensor digitorum longus (EDL) and soleus muscles. There exists in muscle another system for the deamination of amino acids, other than the aminotransferase-dehydrogenase system already discussed. This is the scheme proposed by Lowenstein (1972) in which a 'purine nucleotide cycle' catalysed by the sequential activity of the enzymes adenylosuccinate synthetase. (E.C.6.3.4.4), adenyle succinate lyase (E.C.4.3.2.2) and AMP deaminase (E.C.3.5.4.6) brings about the deamination of aspartic acid. Operation of the cycle (Tornheim & Lowenstein, 1972) appears to be kinetically linked to glycolytic activity (Tornheim & Lowenstein, 1971). Lowenstein (1972) demonstrated an inverse relationship between the activities of glutamate dehydrogenase and AMP-deaminase in a number of rat tissues.

Turner & Fern (1974) have shown that AMP-deaminase activity is lowest in the soleus, intermediate in the EDL and highest in the plantaris muscle. The present observations of the BCA dehydrogenases seem to follow a similar pattern. Turner et al (1974) also suggested that the reciprocity between the activities of AMP deaminase and glutamate dehydrogenase extended to different

types of skeletal muscle. They showed that protein restriction resulted in decreased AMP-deaminase activity in plantaris and soleus and had no effect in the EDL. Relatively smaller decreases in AMP-deaminase activity were found in soleus and plantaris when a protein-free diet was fed. This response agrees with the observations made earlier, that a protein-free diet leads to a reduction in BCA oxidation, but a low protein diet produces an even greater reduction. Thus it seems that the conservation of branched-chain amino acid oxidation in rats fed either a low protein or protein-free diet is associated with the lowering of both AMP-deaminase and BCA dehydrogenase activities in skeletal muscle.

Fate of the α-oxo acids

In considering the activity of the enzymes *in vivo*, it has to be borne in mind that measurements were made on crude homogenate preparations and may not bear any physiological significance. It is noteworthy that urinary excretion of α-oxo isocaproate is increased in the protein depleted rat (McFarlane & Van Holt, 1969 a). This would suggest a decreased breakdown of this branched-chain α-oxo acid and a consequent increase in its pool size. Reeds (1974) suggested that the α-oxo acid may move from muscle to liver, where further oxidation or reamination could occur (Walser et al, 1974). Excess would presumably be excreted in the urine. There are few reports on the concentration of branched-chain α-oxo acids in plasma (Tanaka et al, 1972) and certainly none which give concentrations in the intra-cellular pool of rat tissues. The *in vitro* enzyme measurements show a transient rise in the BCA aminotransferase activities in both liver and muscle of protein-depleted rats. At the same time,

the dehydrogenase activities were decreasing. Presumably, the production of α-oxo acids might exceed the capacity of the dehydrogenase. Moreover, this enzyme is known to be inhibited by [illegible] (Wolhueter et al, 1970). This in itself might act as a feed forward inhibition of the irreversible oxidation of the BCAs in muscle. In starvation, where increased oxidation is observed, it is only in liver that the dehydrogenase activity is increased, together with the aminotransferase activity. This would seem to support Reed's hypothesis that the α-oxo acid might pass from muscle to liver. However, this needs to be clarified by measurements of plasma and tissue intra-cellular concentrations of α-oxo acid in control, starved and protein depleted rats, since Tanaka et al, (1972) found little or no increase in the α-oxo acids of starved rats.

(ii) THE CHOICE OF LABEL

This thesis is primarily concerned with the oxidation of the branched-chain amino acids and in particular of leucine. There have been differing opinions about the degree of adaptation in BCA oxidation in malnourished rats which have arisen as a result of the use of different labelled forms of leucine and valine.

McFarlane & Von Holt (1969 a) found that $^{14}CO_2$ output from DL-leucine, labelled in the 2C position, was decreased in rats fed a 2% casein diet for eight weeks. Neale (1971, 1972), however, could not detect any reduction in the oxidation of U^{14}C labelled leucine, valine or lysine or of mixed amino acids when given to rats fed a 1% casein diet for 15-17 days. Neale (1971) administered the amino acids either by the intragastric or intravenous

route. He found that protein depletion led to an actual increase in $^{14}CO_2$ output from all amino acids except valine. The route of entry made no significant difference to the output of $^{14}CO_2$ in either the control or protein depleted groups. This was in agreement with work by Picou & Taylor-Roberts (1969), who compared the turnover rates of ^{15}N-glycine by intragastric or intravenous routes in two children, and found no significant difference. Neale concluded that the BCAs in particular could not be conserved in protein depleted rats. His later studies (1972) supported this hypothesis since totally eviscerated rats previously maintained on a protein-free diet, were unable to reduce their oxidation of injected valine or mixed amino acids compared with eviscerated rats previously given a high protein diet. This suggested that the extra-hepatic tissues are unable to adapt in protein-depleted rats.

McFarlane's choice of DL-[2-^{14}C]-leucine (1969 a) may be criticised on two accounts. Firstly, D-amino acid oxidase is known to be present in most tissues (Meister, 1965 a) and any adaptation in response to reduced protein intake may reflect adaptation in the D-amino acid oxidase as well as in the catabolic enzymes involved in the normal pathway of leucine degradation. Indeed, further studies by McFarlane & co-workers (1969 b) did show a reduction in activity of the D-amino acid oxidase in liver. Secondly, with the label in the 2C- position the acetyl CoA moiety in which the label appears could theoretically be syphoned off into fatty acid synthesis, cholesterol synthesis and the TCA cycle. If significant amounts of ^{14}C are retained in these other compounds, measurement of the output of $^{14}CO_2$ will underestimate the true extent of leucine oxidation.

There are similar objections to the uniformly labelled L-leucine or L-valine that Neale used to investigate adaptation of the BCAs in malnourished rats (1971, 1972). After decarboxylation, the remaining C atoms of the carbon skeleton could be shunted via hydroxy-methyl-glutaryl CoA (HMGCoA) into cholesterol production; via acetoacetate into fat metabolism; or via acetyl CoA into the TCA cycle. Again if any of these pathways are active, they might obscure a reduction in the initial irreversible oxidation of leucine at the step when αKIC is decarboxylated.

Reeds (1974) specifically examined the effect of the position of the label by comparing the oxidation of L[U-^{14}C] valine with that of L[1-^{14}C]-valine in rats fed a low protein diet identical to that employed by Neale (1971). Reeds showed that with uniformly labelled valine there was no difference between protein-depleted and control animals in the output of $^{14}CO_2$, either *in vivo* or *in vitro*. The results, therefore, were in agreement with those of Neale (1971). However, when identical groups of rats were given L[1-^{14}C]-valine the excretion of $^{14}CO_2$ was reduced in the low-protein group. This supported my observations at much the same time, but with L[1-^{14}C]-leucine. Reeds argued from the theoretical viewpoint that "if some of the non-carboxylic label in U-^{14}C valine is retained in protein, glucose, fat or cholesterol, then it follows that an estimate of valine catabolism with L[1-^{14}C]-valine will always be higher and should never be lower than an estimate with U-^{14}C-valine". He showed this to be true *in vitro*, but *in vivo* in weanling rats and in protein-depleted rats the estimate of valine catabolism with [1-^{14}C]-valine was lower than with uniformly labelled valine. Two explanations were offered.

1) The decarboxylation of αKIV was not rate-limiting.

2) That $^{14}CO_2$ formed by the decarboxylation of αKIV enters the bicarbonate pool at a slower rate than labelled CO_2 formed in the TCA cycle, or is preferentially reutilized in CO_2 fixation. The bicarbonate pool has been estimated in the rat to turn over with a half-life of 12½ minutes (Millward, 1970). Measurements of the turnover of CO_2 with 1-^{14}C-leucine (Section IIIA) gives almost identical results, suggesting that the carboxyl C does not enter the bicarbonate pool at a slower rate than the other C atoms of the amino acid.

Therefore Reeds' observation remains unexplained.

This thesis was also concerned with measuring quantitatively the rate of leucine catabolism in both the perfused hind-limb and the whole animal. The constant infusion method (Waterlow & Stephen, 1968; Garlick & Marshall, 1972) allows us to do this. The overall equation of the balance of amino acid flow into and out of the plasma compartment is :

$$Q = S + C = B + I$$

(Flux = Synthesis + Oxidation = Breakdown + Input)

The rate of amino acid oxidation (C) can be determined from the rate of excretion of $^{14}CO_2$ at plateau. The proportion of the infused dose excreted as $^{14}CO_2$ gives us the proportion of the flux oxidized. The equation assumes that the only pathways of amino acid utilization are uptake into protein and oxidation. Moreover, as already pointed out, the measurement of $^{14}CO_2$ excretion will only give a correct estimate of oxidation if : (1) no products of oxidation are retained in compounds such as fat, and (2) no $^{14}CO_2$ is retained in or taken up from the

bicarbonate pool.

As we have seen, by using an amino acid labelled only in the 1-C position the first requirement is met since on oxidation the 1-C atom passes directly into the bicarbonate pool. Measurement of the rate of excretion of $^{14}CO_2$ from the bicarbonate pool (Section IIIA) shows that the second problem is also overcome in the rat when we use this form of labelled leucine.

Therefore, in conclusion, theoretical considerations were much in favour of L[1-^{14}C]labelled BCA being used both *in vivo* and *in vitro* throughout the experiments described in this thesis. Initial experiments with DL[1-^{14}C]-leucine were carried out, but Reed's (1974) confirmation of the problems involved in the use of U-^{14}C label made any further investigation with this particular label unnecessary.

(iii) The DL or L-isomer in Measurements of Oxidation

McFarlane & Van Holt (1949 a) observed that 40% of a dose of DL[2-^{14}C]-leucine was excreted as $^{14}CO_2$ in control rats and that this figure was reduced to 10% in protein-depleted rats. Similar results were obtained in the present work when rats fed either a high or a low protein diet were injected intragastrically with a pulse dose of DL[1-^{14}C]leucine. Both groups had higher rates of $^{14}CO_2$ excretion than was obtained with the L-isomer (Table 5).

The higher rates of excretion observed with DL- mixture of isomers may result from the high activity of the D- amino acid oxidase enzyme in liver mitochondria (McFarlane & Van Holt, 1949 b). McFarlane et al found that $^{14}CO_2$ production from D[1-^{14}C]-leucine was twice that of L[1-^{14}C]-leucine and that the specific activity

— of D- amino acid oxidase was approximately 10 fold that of leucine aminotransferase. Further evidence indicating the errors which may be caused by the D-isomer was obtained on investigating the effects of fasting in rats fed high or low protein diets. With DL-leucine we could not detect the increase in oxidation of leucine normally observed with the L-isomer (Section IIA(iv)).

If accurate measurements of $^{14}CO_2$ production are to be made for the purpose of estimating flux rates by the constant infusion method (Waterlow & Stephen, 1968) then it is essential that the L-isomer is used. For these reasons, L[1-^{14}C]leucine was used in all subsequent studies.

(iv) Tissues Oxidising Leucine

The ability to transaminate and decarboxylate leucine and the other BCAs is widely distributed. Tissues which can do this include the kidney, liver, heart, muscle and brain (Dawson & Hird, 1967; Clarke, 1957; Buse & Buse, 1967; Johnson, Herring & Field, 1961) as well as human white cells and skin fibroblasts in culture (Mancia, Mutzler & Levitz, 1961). Adipose tissue is also capable of oxidising leucine (Feller & Feist, 1962) and Rosenthal et al (1974) suggested that in humans, adipose tissue was one of the major extra-hepatic sites for leucine metabolism, concerned mainly with the biosynthesis of sterols. Recently, oxidation of leucine has also been observed in sciatic nerves Hertog et al, 1974) and in the isolated aorta of the rat (Buse, Jursinic & Reid, 1975).

Despite the overwhelming demonstration of the widespread ability of tissues to oxidise leucine it has not only been suggested but

also dogmatically stated that skeletal muscle is the major site of leucine oxidation. As discussed earlier, this stems from the work of Miller (1962), who demonstrated that extra hepatic tissues were as capable of oxidising leucine as liver. However, his preparation would also have included skin, adipose tissue, peripheral nerve, brain, lung and kidney as well as skeletal muscle. Manchester (1965), then Meikle & Klain (1972) and Odessey & Goldberg (1972) argued that if the metabolism of diaphragm (the tissue studied) was representative of that of skeletal muscle *in vivo*, then muscle was probably the major site for CO_2 production from leucine, since skeletal muscle comprises approximately 45% of body weight of the rat (Munro, 1969). A more accurate approach to the estimation of oxidation rates *in vivo*, and the isolated perfused hind-limb was attempted in the present work and will be discussed at a later stage. However, in the context of the present discussion it is worth noting the work carried out *in vitro* on the regulation of BCA oxidation in skeletal muscle, diaphragm, heart and liver.

(v) Hormonal Effects on BCA oxidation - Insulin

Manchester (1965) found that when rat diaphragms were incubated in the presence of insulin (100 mU/ml) there was a consistent but small stimulation of oxidation of L[1-^{14}C]leucine. However, neither Meikle & Klain (1972), using 30 mU/ml insulin nor Buse, Biggers & Buse (1972) with 1.0 mU/ml insulin were able to repeat Manchester's observations on incubated diaphragms. Insulin did stimulate $^{14}CO_2$ production from leucine by hearts obtained from rats after a 48 hr fast and perfused without glucose (Buse et al, 1972). This may have represented stimulation of amino acid transport into muscle cells by insulin (Manchester, 1970).

Millward et al (1974) showed that in rats fasted for 72 hours the intracellular concentration of BCAs increased 4-5 times. At the same time the plasma insulin concentration decreased by 46%. Oxidation of BCAs was not measured in these experiments, but it is reasonable to suppose that it was increased in the starved rats. In this case, therefore, an increase in oxidation would be associated not with stimulation by insulin, but with increased availability of free amino acids in the intracellular pool, derived from protein breakdown.

Epinephrine and Glucagon

Buse, Biggers, Drier & Buse (1973) found that stimulation of BCA oxidation by epinephrine in diaphragm and by glucagon in heart was only demonstrable in tissues from fasted rats, and only during incubation or perfusion without glucose or pyruvate. Moreover, the fact that perfusion with 5.5 mM glucose suppressed the stimulation of branched-chain amino acid oxidation by epinephrine or glucagon suggested that hormonal stimulation of BCA oxidation may not occur under physiological conditions. However, hepatic gluconeogenesis from alanine is stimulated by glucagon (Mallette et al, 1969). A cycle involving the branched-chain amino acids may complement the alanine cycle. Under conditions when the hepatic uptake of alanine is stimulated, the hepatic output of BCAs is increased. In muscles, stimulated oxidation of branched-chain amino acids complements the release of alanine, the carbon skeleton of BCAs serving as an energy source for muscle cells and the amino group for the transamination of pyruvate to alanine. It is not suggested that this would be quantitatively important.

Corticosteroids

Glucocorticoids are known to cause protein catabolism in peripheral tissues and thus to cause an increase in the pool size of free amino acids in the liver. Although work has been carried out on the induction by corticosterone of liver and kidney leucine transaminase activity (Shirai & Ichihara, 1971) it is only recently that oxidation of leucine by incubated skeletal muscle has been studied in vitro (Ryan et al, 1975). These workers demonstrated that 24 hr after corticosterone injection into a rat, teased strands of the transversus abdominus muscle on incubation oxidised greater amounts of L-[1-^{14}C]-leucine than controls who had not received corticosteroids. However, in the same group of experiments, Ryan et al (1975) demonstrated that teased strands of muscles from 24 hr fasted rats oxidised less leucine than unfasted controls, which is contrary to the accepted view of the effects of fasting on leucine oxidation in muscle. This work obviously needs repeating before one might conclude that corticosterone has a regulatory role in leucine oxidation during fasting.

(vi) Effect of Fasting on BCA Oxidation

The experiments with animals fasted for 48 hr show that oxidation of leucine is increased in both the groups fed a normal and a low-protein diet. The sequence of events appears to be as follows. Increased amounts of free leucine are liberated in muscle, as a result of reduced protein synthesis and increased protein breakdown (Millward, 1970). Free leucine levels in muscle do rise, but this rise is not progressive, nor very great. This means that the increased production of leucine must be balanced by an increased rate of removal.

Measurements of A-V differences in the forearm during fasting show little increased output of leucine as such. Most of the extra output of amino acids is in the form of alanine and glutamine (Felig et al, 1969). Presumably amino groups from other amino acids, including leucine, are transferred to alanine and glutamine by transamination and by the action of glutamine synthetase. Perfusion experiments (Ruderman & Lund, 1972) showed that addition of leucine to the perfusate led to an increase in alanine and glutamine and that glutamine alone was increased when NH_4Cl (5mM) was added. Alanine synthesis is catalyzed by glutamate-pyruvate aminotransferase and glutamine synthesis by glutamine synthetase. If L-methionine D, L-sulphoximine (a specific inhibitor of glutamine synthetase) was present, the increase in glutamine release induced by leucine was diminished by 50%. Glutamine rather than alanine appeared to be the major vehicle of nitrogen transport from muscle to other tissues.

In these circumstances, one might expect an increase in activity of the enzymes catalysing these reactions - at least in muscle (a) the aminotransferase and (b) the dehydrogenase. Transamination, with the donation of the amino group from leucine to alanine or glutamine (Felig & Wahren, 1971) would be facilitated by the high concentrations of aminotransferase present in muscle (Young, 1970). This transamination seems to occur more readily than is normal, because, despite the net breakdown of muscle protein with the production of more leucine, there is no great accumulation of free leucine within the muscle. The enzyme experiments with fasted animals showed that leucine aminotransferase was increasing in activity in both muscle and liver. In contrast, the activities of liver and kidney leucine aminotransferase

(Wolhueter & Harper, 1970), the first enzyme of the leucine pathway,

are unchanged after fasting.

More recently, Adibi et al (1975) measured leucine aminotransferase activities in liver, skeletal muscle and kidney from starved rats. After 12 hr of starvation both muscle and kidney enzyme activities were slightly reduced. When starvation was prolonged for a full day, leucine aminotransferase increased approximately two-fold in both tissues. A 5-day fast resulted in an additional increase in specific activity of the enzyme in muscle. Throughout the prolonged starvation period, leucine aminotransferase activity remained unaltered in liver. These changes may be functionally important.

The enzyme experiments with fasted animals showed that both liver and muscle α-oxo acid dehydrogenase activities altered significantly within 48 hr (Table 6). Muscle enzyme activity was diminished, whereas liver enzyme activity increased. Formerly, Wolhueter & Harper (1970) showed that the activity of liver α - oxo isocaproate dehydrogenase, the second enzyme in the pathway of leucine catabolism, and the activity of skeletal muscle α-oxo- acid CoA transferase (Williamson et al, 1971), the first enzyme involved in acetoacetate utilisation, increased during fasting. The former agrees with the present observation that α -oxo isocaproate dehydrogenase activity increases during fasting in the liver (Table 6). If the step of decarboxylation were not rate-limiting, then an increased provision of substrate would increase α-oxo acid oxidation despite the lowered dehydrogenase activity seen in muscle. Any limitations in decarboxylation could also allow the excess α-oxo acid to pass from muscle to liver, where the increased

dehydrogenase activity would aid its further oxidation. This would certainly be a mechanism whereby liver could derive energy from the carbon skeleton of branched-chain amino acids present in high concentrations in muscle protein. However, since fasting does not normally lead to a large increase in the plasma concentration of the BCA α-oxo acids (Tanaka et al, 1972) and the capacity of liver oxidising oxo acids is limited, it is possible that muscle plays an important part in the increased oxidation of leucine during fasting.

Clarke (1957) and Manchester (1965) have shown that heart and isolated rat diaphragms were capable of oxidising leucine to give rise to CO_2. Goldberg's (1972) experiments with diaphragm also suggested that the α-oxo acid of leucine is oxidized in muscle and the incubation experiments (Table 6) provide further evidence of the oxidation of leucine to carbon dioxide by muscle. Subsequent work in Goldberg's laboratories (Odessey & Goldberg, 1972; Goldberg & Odessey, 1972) showed that appreciable oxidation of leucine occurred in the dark soleus and pale extensor digitorum longus muscles. Moreover, diaphragm catabolised leucine at rates similar to liver slices but several fold less actively than epididymal fat pad or kidney and brain slices. Oxidation of L[1-^{14}C]-labelled leucine, valine and isoleucine increased three to five fold in the diaphragms of animals fasted for 3 days. Protein synthesis during this period was 50% lower than control values. The fall in amino acid incorporation was evident within one day of fasting, but amino acid oxidation did not increase until the second day. We have previously seen that leucine aminotransferase was increased in muscle (Wolhueter & Harper, 1970; Adibi et al 1975) by day 1 of a fast and that by the second day the α-oxo-

isocaproate dehydrogenase had increased in liver but decreased in muscle (Table 6). The km of the dehydrogenase activity in liver mitochondria has been shown to be 0.2 mmol/l (Wolhueter & Harper, 1970). Since the muscle enzyme has approximately the same km (Table 2), the the concentrations of leucine in muscle are the approximate range in which the oxidation rates will be determined by the leucine concentration. It is unlikely that the intra-cellular pool of amino acid is homogenous so that variations in leucine concentrations greater than those seen for the whole tissue may occur at the precursor site for oxidation. Thus an enhanced absolute rate of oxidation may occur in muscle in starvation despite a fall in the apparent activity of the enzyme assayed in vitro.

(vii) Effect of Feeding Low Protein Diets on BCA Oxidation

Stephen (1968) and Waterlow (1968) have stressed the importance of economy and recycling of nitrogen in the mechanism of adaptation to low protein intakes. Neale (1971) later suggested that the limiting factor may be the animal's ability to reduce the oxidation of the carbon skeleton of essential amino acids. As mentioned earlier, he found that the branched-chain amino acids in particular showed no reduction in oxidation in protein-depleted rats.

However, a number of studies provide evidence of adaptive changes in amino-acid oxidation. 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 a) found a marked reduction in the oxidation of DL [$\alpha^{14}C$] leucine and $U^{14}C$ -phenylalanine given intraperitoneally to rats

on a 2% casein diet. There was no change in the oxidation of the non-essential amino acids alanine or glutamine. The present work has confirmed McFarlane & Von Holt's observations. When rats that had been fed a 3.5% NDp:E diet for 3 weeks were given an intragastric dose of L[1-^{14}C] leucine the percentage of the dose excreted as $^{14}CO_2$ was markedly reduced when compared to controls on a normal diet. At much the same time, Reeds (1974) also confirmed McFarlane's work, using L[1-^{14}C] valine. However, when U-^{14}C valine was used there was no reduction in $^{14}CO_2$ output in protein-depleted rats. This second observation confirmed Neale's work (1972) who later found that even if U^{14}C - leucine, valine, lysine or phenylalanine were constantly infused intravenously for periods of up to 4 hr. there was no difference in the proportion of dose oxidized to CO_2 in rats adapted to a protein-free diet compared with those on a control diet (Neale & Waterlow, 1974 a). More recently, Neale (1975) examined the catabolism of U^{14}C valine in adult male Wistar rats (250-300g) given high casein or low casein diets (250g/kg and 50g/kg). At this protein intake, the body-weight of the latter group was maintained constant for periods of 7 - 9 days. Without prior fasting overnight (as in his previous experiments) the rats were infused with the labelled valine for periods of up to 7 hours, with continuous collection of $^{14}CO_2$ at half-hourly intervals. The provision of maintenance levels of protein now produced an overall reduction in oxidative catabolism of U^{14}C -valine to $^{14}CO_2$. Neale, however, still maintained that a protein-free diet caused a breakdown in the adaptive process, resulting in a high rate of loss of essential amino acids from the body (Neale & Waterlow, 1974 b).

However, in the present work a net reduction in the oxidation of L-[1-^{14}C]-leucine has been observed in rats of all ages fed a protein-free diet. This has been a consistent observation in rats given either a pulse intragastric dose or a constant tail vein infusion; and in the perfused hind-limbs of protein-depleted rats. These observations have been supported by parallel measurements of the first two enzymes in the catabolic pathway for leucine in liver and muscle of control and protein-free fed rats. It may be worth noting that in Neale's earlier work (1974) when no reduction in oxidative catabolism was observed in rats fed the protein-free diet: 1) plateau specific radioactivity of $^{14}CO_2$ output was not attained in 4 hr. infusion; 2) the animals were fasted overnight. In his second set of experiments on rats fed a maintenance diet (Neale, 1975) the rate of $^{14}CO_2$ output did reach a plateau. Moreover, these animals were not fasted overnight, as they had been in his previous experiments. Fasting will tend to obscure the adaptation to low protein diets by increasing the oxidation of the branched-chain amino acids. It seems, therefore, that the discrepancy between the present results and those of Neale can be resolved if attention is paid to the details of the experimental design.

(viii) Oxidation Rates In Vivo

Several workers have given a single dose of ^{14}C amino acid *in vivo*, and expressed the rate of oxidation as the proportion of the dose excreted as $^{14}CO_2$ in the next few hours (McFarlane & Van Holt, 1969 a; Brookes, Owens & Garrisons, 1972; Neale, 1971, 1972). This approach was used in the first part of this work, since it does give useful qualitative information. However, it is not possible to obtain absolute rates of amino acid oxidation

unless the specific activity of the precursor at the site of the oxidation is known. This information is very difficult to obtain when the amino acid is given as a single dose, because the specific activity is changing very rapidly. The constant infusion method (Waterlow & Stephen, 1968; Garlick & Marshall, 1972) overcomes this difficulty, since an equilibrium, i.e. constant specific radioactivity, is attained in plasma and tissue free amino acids, and presumably therefore in the precursor pool for synthesis and amino acid oxidation. Fern & Garlick (1973, 74) showed that the best estimate of the rate of amino acid oxidation is calculated as (radioactivity excreted as $^{14}CO_2$ after plateau SR has been achieved) '/. (SR of free amino acid at the site of oxidation). The SR is always lower in the intracellular pool than in plasma because the labelled amino acid in the intracellular pool is diluted with unlabelled amino acid derived from protein breakdown. Errors will therefore arise if oxidation rates are calculated from the SR plasma; the effect will be to underestimate the rate of oxidation.

The difficulty is that when we are trying to determine the absolute rate of amino acid oxidation in the whole animal, it is not practicable to measure the SR in the intracellular pool of every tissue which may be oxidizing leucine, because as shown in Section IV(iv), leucine is oxidized in many tissues. Since muscle accounts for about 45% of body weight, and is active in oxidation of leucine it seems logical to calculate the rate of oxidation in the whole body from the SR in the intracellular pool of muscle. As shown in Table 14, the SR of free leucine in muscle is approximately 70% of that in plasma. This agrees with the

value found by Garlick, Millward & James (1973) for the average SR of free tyrosine in tissues compared with that in plasma. Thus the use of 1-^{14}C leucine in a constant infusion with calculation based on the SR in muscle, probably offers the most accurate method available of measuring the overall oxidation rate of leucine.

As shown in Table 10, a protein-free diet led to decreased oxidation of leucine in the rat, thus refuting Neale's claim (1971, 1972, 1974) that the rat is incapable of reducing the oxidation rate of BCAs on a low protein diet.

The equation given above may be written in another way :- the total rate of oxidation $E = \frac{e^*}{d^*} . Q$ where $\frac{e^*}{d^*}$ is the proportion of dose excreted at plateau and Q is the total rate of amino acid turnover or flux. ($Q = \frac{d^*}{\text{plateau SR}}$, (Waterlow & Stephen, 1967).

From this it follows that a reduction in the absolute rate of oxidation could be brought about in two ways :-

(1) By a fall in $\frac{e^*}{d^*}$, i.e. in the proportion of the flux which is oxidized.

(2) By a fall in flux, the proportion oxidized remaining constant.

or both factors could be altered.

The decrease in oxidation of leucine is one step towards the reduction of N-excretion which is known to occur in a low protein diet (Waterlow, 1968). Picou & Taylor-Roberts (1969) have shown that the fall in N-excretion is due to a decrease in the proportion of the flux which is oxidized and excreted and not to a decrease in the overall flux. The present work confirms this (Table 13),

but only when flux rates are determined from the SR of the intracellular free amino acid. Recently, Garlick et al (1975) found that tyrosine flux was relatively unchanged in rats fed a protein-free diet for 3 days. However, by day 21 on the diet, the flux was greatly reduced. In the present experiment, leucine flux remained unaltered by day 7 of protein-free feeding. In contrast, the interpretation leading from measurements of the SR plasma amino acid is that both flux and the proportion of flux oxidised are reduced.

(ix) Oxidation Rates in the Hind-Limb

Measurements of leucine oxidation rates in perfused hind-limbs of rats fed a control or protein-free diet confirmed the results obtained both *in vivo* and *in vitro*. As a result of the *in vitro* experiments carried out earlier it was hypothesised that skeletal muscle was the major site of leucine oxidation in the whole animal and that adaptation to dietary protein intake was most significant in this tissue. However, the results with the perfused hind-limb fail to demonstrate this with any degree of certainty because of the difficulties encountered with the preparation. Without further work it was not possible to improve the estimates in the time available.

Preliminary work showed that in the perfused hind-limb with constant infusion of a tracer dose of [1-^{14}C] -leucine, the SR of free leucine in the perfusate rose to a plateau value. This confirmed the predictions based on a mathematical model drawn up by P.J. Garlick, and described in Section IIIB(iv). Furthermore, it was predicted that the SR of free leucine in the gastrocnemius ICF pool should follow quite closely the rise to plateau in the

perfusate. Unfortunately, as may be observed in fig.12, Section IIIC(v), the output of $^{14}CO_2$ had not reached plateau at a time when both the SR of free leucine in the perfusate and ICF were predicted to have done so for reasons discussed in Section IIIC(v). Furthermore, as discussed earlier, the preparation was not maintained much beyond this period although the $^{14}CO_2$ output would be expected to reach plateau by 3 hr. of a constant infusion. Therefore, the estimates of total body muscle leucine oxidation and hind-limb oxidation rates are underestimates. Nevertheless, this method offers the most accurate approach available to determine muscle's contribution to total body leucine oxidation. The calculated leucine oxidation rate in total body skeletal muscle was 7.14 µmoles/hr in 180g rat or approximately 25% of total body tissue leucine oxidation. In preliminary experiments on perfused livers where plateau $^{14}CO_2$ output was achieved during a 3 hr. perfusion, the estimated contribution of liver to total body leucine oxidation was 7%. These experiments have not been presented here. However, it may be concluded that the work with the hind-limb preparation confirms the view that skeletal muscle has a greater capacity than liver to oxidise leucine (Young, 1970). Furthermore, the reduction in leucine oxidation rates seen in perfused hind-limbs of rats fed a protein-free diet would indicate that skeletal muscle plays an important role in reducing total body leucine oxidation rates <u>in vivo</u>.

(x) <u>Leucine as a possible Regulator of Skeletal Muscle Protein Synthesis and Leucine Oxidation</u>

It may be observed from the present work that a reduction in the level of perfusate amino acids led to reduced leucine incorporation

into protein and reduced oxidation to $^{14}CO_2$ (Tables 17 and 19) in both well-fed and protein depleted rats. In vivo, where the concentrations of the essential amino acids, and in particular the BCAs, are known to be reduced under conditions of protein depletion, similar results may be observed (Tables 10 and 11). Unfortunately, the design of the hind-limb experiments does not allow us to state categorically whether it is leucine concentration that regulates the skeletal muscle synthesis rates since insulin was also a variable factor.

The main question arising from the present work is how rates of protein turnover are related to changes in amino acid concentration. Millward et al (in Press) have shown that in skeletal muscle there were increases in most of the free amino acids following feeding which corresponded to increases in RNA and in the rate of protein synthesis (Millward et al, 1973; Garlick et al, 1973). More recently, Garlick et al (1975) have shown that in rats fed a protein-free diet the fall in skeletal muscle synthesis rate after one day is accompanied by a similar fall in the amount of RNA. In the present work (Table 18) feeding a protein-free diet led to a reduction in the intracellular pool size of leucine in the gastrocnemius muscle. However, something of a paradox is found in starvation. Here we observe a greatly reduced rate of synthesis in skeletal muscle accompanied by a decrease in tissue RNA but a significant increase in concentration of tissuesessential amino acids - particularly of methionine and the BCAs (Millward et al 1974). Therefore, it would appear that amino acid concentration is unlikely to be a major controlling factor in skeletal muscle protein synthesis. Moreover, Millward et al (1974) have shown a direct correlation of tissue RNA concentration with that of

plasma insulin, but little correlation with that of the intracellular essential amino acids.

Recently, Fulks et al (1975) described a simple method for measuring the rates of protein synthesis and degradation in isolated rat diaphragm. Tyrosine was chosen for studies of protein turnover, since it rapidly equilibrates between intracellular pools and the medium, it can be measured fluorometrically, and it is neither synthesized nor degraded by diaphragms. Fulks et al found that the addition of amino acids at plasma concentration both promoted protein synthesis and inhibited degradation. Five times normal plasma concentrations of the amino acids had larger effects. The three branched-chain amino acids together stimulated synthesis and reduced degradation, while the remaining plasma amino acids did not affect either process significantly. Thus they surmised that leucine, isoleucine and valine appeared responsible for the effects of plasma amino acids on protein turnover in the muscle. Leucine by itself or isoleucine and valine together, also were able to inhibit protein degradation and promote synthesis. This was followed by a similar report by Buse & Reid (1975) who also studied the incorporation of radioactively labelled precursors into muscle protein in isolated rat hemi-diaphragms. They found that a mixture of the BCAs (0.1mM each) added to the media containing glucose, stimulated the incorporation of ^{14}C lysine into protein. When tested separately, valine was ineffective, isoleucine was inhibitory, but 0.5mM leucine increased the specific radioactivity of muscle protein during the incubation with ^{14}C lysine or ^{14}C acetate in hemi-diaphragms from fed or fasted rats, with or without insulin. Furthermore, during incubation with ^{3}H -tyrosine (0.35mM) the addition of

0.5mM leucine increased the specific radioactivity of muscle proteins, while the specific radioactivity of intracellular free tyrosine remained constant and its concentration decreased, suggesting that leucine promoted protein synthesis. Their hypothesis was that "the concentration of leucine in muscle cells or compartments thereof may play a role in regulating the turnover of muscle proteins and influence the transition to negative nitrogen balance during fasting, uncontrolled diabetes and post-traumatic state. Leucine may play a pivotal role in the protein sparing effect of amino acids". More recently, Millward et al (1976) have produced contrary evidence. They showed that in diabetic, hypophysectomised, starved and glucocorticoid treated rats skeletal muscle protein synthesis was decreased but the concentrations of the BCAs in the pooled supernatants of the combined gastrocnemius and quadriceps muscles were increased. This, they concluded, indicated that the BCAs are unlikely to be involved in the regulation of protein synthesis in vivo.

SUMMARY

The cumulative evidence of *in vitro* hind-limb and *in vivo* observations reinforces McFarlane & Von Holt's (1969 a) findings, that leucine oxidation is reduced in protein depleted animals. Furthermore, this adaptation is maintained in animals deprived of protein but not of energy. However, the mechanism of adaptation is not maintained during starvation where high oxidation rates of the BCAs are observed.

Initial experiments carried out *in vitro* demonstrated for the first time dehydrogenase activity in skeletal muscle which adapted to dietary stress. Since the greatest total enzyme capacity for both the leucine aminotransferase and α-KIC dehydrogenase was estimated to be in skeletal muscle this tissue is proposed as a major site of BCA oxidation.

The hind-limb perfusion experiment fell short of confirming the expected contribution to total body leucine oxidation. However, the measurements of leucine oxidation rates in the hind-limb preparation represent the best available estimates of the contribution of muscle to total body leucine oxidation, despite the difficulties encountered.

The evidence from both the *in vitro* and *in vivo* work would suggest that leucine oxidation is primarily affected by intracellular concentration of the free amino acid and that the 'fine control' is engineered by the first two enzymes in the metabolic pathway. Leucine has recently been put forward as a possible candidate for the regulation of skeletal muscle protein synthesis. This has been discussed in the light of results obtained both in the perfused hind-limb and *in vivo*.

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