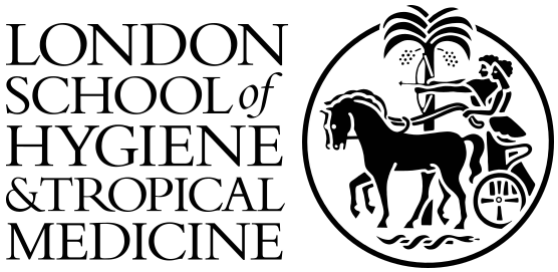

PROTEIN SYNTHESIS IN THE LIVER AND SMALL INTESTINE OF THE RAT

A thesis submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine

by

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ABSTRACT

This thesis, presented in four sections, is concerned with the regulation of protein metabolism in the liver and small intestine of the rat.

Section 1. A method was developed which allows for the measurement of rates of protein synthesis in tissues such as liver and intestine which synthesise and degrade protein very rapidly. The method employed a flooding amount of (^{14}C)leucine (100μmoles per 100g body weight) injected intravenously with measurement of the rate of incorporation of labelled leucine into protein over the following 10 minutes. Several experiments were performed to show that protein synthesis, per se, was unaltered by the flooding amount of leucine.

Section 2. Employing the method described in Section 1, protein synthesis was measured in the liver and the individual components of the gastro-intestinal tract and in the whole animal. These measurements demonstrated the importance of these rapidly turning over tissues to overall protein metabolism in the whole animal.

Section 3. Nutritional and hormonal perturbations leading to the loss of body protein were investigated. Rates of protein synthesis in the liver and small intestine have been examined under three conditions; namely, starvation, dietary protein-deprivation and diabetes. Each of these conditions caused a loss of protein from the liver and a lowered rate of synthesis but by different intracellular mechanisms. In the intestine the synthesis of protein was depressed by starvation and protein-deprivation but maintained in diabetes.

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Section 1. A method was developed which allows for the measurement of rates of protein synthesis in tissues such as liver and intestine which synthesise and degrade protein very rapidly. The method employed a flooding amount of (^{14}C)leucine (100μmoles per 100g body weight) injected intravenously with measurement of the rate of incorporation of labelled leucine into protein over the following 10 minutes. Several experiments were performed to show that protein synthesis, per se, was unaltered by the flooding amount of leucine.

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Section 4. The role of leucine as a regulator of protein synthesis was examined. Protein synthesis was measured using a flooding amount of (^{3}H)phenylalanine in the presence and absence of 100μmoles of unlabelled leucine. The impact of leucine on protein synthesis in liver, intestine and muscle of fed, starved and protein-deprived animals appeared to be negligible in vivo, inspite of many reports that leucine stimulated protein synthesis in vitro.

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THE PROCESS OF PROTEIN TURNOVER

Protein turnover describes the process of continuing renewal of body protein. This dynamic condition whereby body protein is continually degraded and resynthesised, even in the absence of a change in protein mass, was conclusively demonstrated in the 1930's by Schoenheimer et al. (1939) using isotopically labelled tyrosine. The failure of all of the ^{15}N to appear in urinary urea following the administration of ^{15}N tyrosine to non-growing animals suggested that even without the requirement of growth, dietary amino acids were continually being incorporated into protein. Further, since the protein mass was not changing, protein must be continually degraded at the same rate. Although apparently inefficient in that energy is required both for synthesis and degradation, the value of continual protein turnover is most apparent when an animal is adapting to changing conditions. Changes in the amount of protein which is accumulating or disappearing can be brought about by altering the rate at which protein is synthesised or the rate at which protein is degraded or by altering both rates of synthesis and degradation. This ability to alter rates of synthesis and degradation and hence the potential for adaptation exists at several levels for any organism. Adaptation can take place at the level of individual proteins, at the tissue level and for the animal as a whole. The concept of adaptation is perhaps clarified by considering a few examples.

At the level of individual proteins, particularly enzymes, the concept of adaptation can be simply demonstrated for it is here that the correlation between a change in environment and a corresponding change

in the amount of protein seems most direct. Changes in enzymes in the liver in response to nutrient intake have been particularly well-defined and arginase (EC.3.5.3.1.), studied extensively by Schimke (1970), illustrates several adaptive mechanisms. A two-fold increase in the amount and the activity of the enzyme was found in the liver of rats which were changed from a diet containing 8% (wt/wt) protein to fasting conditions. This change in the amount of enzyme was shown to be due to a decrease in the rate at which the protein was degraded. In addition, adaptation from a diet containing 70% protein to an 8% protein-containing diet involved a 40% reduction in the amount of enzyme present. In this case the change in total enzyme was brought about by a decrease in the total amount of enzyme which was synthesised. The transition to the new level of enzyme was facilitated by a transient increase in the proportion of the enzyme which was degraded. After the transition to a new level of enzyme was complete, the proportion of enzyme degraded returned to the original level but the amount of enzyme remained lower due to the decreased amount which was synthesised.

Similarly whole tissues exhibit an overall rate of protein turnover with the rate of synthesis equal to the rate of degradation under conditions where the protein mass is not changing. However, in response to changes in environment rates of synthesis and degradation in the whole tissue may be altered. Garlick et al. (1973) have examined changes in the rate of protein synthesis in liver and gastrocnemius muscle in response to feeding. Rats were trained to consume the entire day's ration in a 4-hour period so changes in synthesis could be assessed in precisely-defined intervals after the meal. In the gastrocnemius muscle the rate of protein synthesis rose after eating and declined substantially in the period following the missed meal. In contrast, no such changes in the rate of protein synthesis were reported

for the liver and the authors suggest that a change in the rate of protein degradation accounted for the 25% increase in protein mass observed by Millward et al. (1974).

Conditions which alter the rates of protein synthesis and degradation in the whole animal have not been as extensively investigated as those which produce changes in turnover rate in either single enzymes or individual tissues. However, in instances such as growth, synthesis must exceed degradation; while in net catabolic states, such as starvation, the rate of protein degradation must be greater than the rate at which protein is being synthesised. Lo and Millward (1977) have estimated that in a young rat (which would be growing at 5-10% per day) an amount of protein equivalent to 27% of the total protein mass was synthesised each day. They have also shown that the rate of protein synthesis was sensitive to changing conditions. After one day of fasting the rate of synthesis was 37% lower than in the fed condition. Thus protein turnover in the whole animal, as for single proteins and individual tissues, provides for adaptation in at least two ways. Rates of synthesis well in excess of those which would be required for the observed increases in the amount of protein allow changes in mass to occur at an accelerated rate. In addition, since synthesis and degradation can be altered independently or simultaneously, there are several ways in which protein mass can be regulated.

Of the many conditions where regulation of protein mass in response to changes in the environment might be studied, the response of tissues such as liver and intestine seems to be particularly apt since they must respond rapidly and repeatedly to changes in the environment associated with feeding. The importance of liver and gut to the study of regulation of protein metabolism is convincingly illustrated by the experiments of Elwyn (described in Elwyn, 1970) which provide an indication of both the magnitude and the complexity of the response of

these tissues to the intake of nutrients. In these experiments dogs were fed a horsemeat diet once a day and the net uptake or output of amino acids from liver and gut was assessed from an estimate of blood flow and the differences in amino acid concentrations across each tissue. In the gut (i.e. stomach, pancreas, small intestine and some of the large intestine) there was a net output of amino acids for the entire 24 hours following a meal. In fact, the total output was higher than the amount present in the diet. The net output of amino acids from the gut, which continued throughout the day, indicated hydrolysis of protein which had been synthesised in the gut. Under normal, steady-state conditions, this output cannot exceed the amount present in the diet as this would result in a continual loss of protein from the gut. Therefore, this result may reflect inaccuracies of the measurement (particularly of blood flow) or possibly the effect of trauma produced by the experimental procedure. This study suggests that the majority of amino acids present in the diet are quickly absorbed by the gut and released into the hepatic portal vein without substantial modification in composition. A small proportion are retained within the gut, presumably as protein which is subsequently hydrolysed.

Unlike the gut the composition of the amino acids leaving the liver was considerably different from that which entered. There was net output only for the branched-chain amino acids (i.e. leucine, isoleucine and valine). For most amino acids there was net uptake by the liver so that

> "the liver acts as a buffer between the digestive tract and the rest of the organism with respect to most amino acids, preventing almost all effects of diurnal events associated with ingestion from reaching peripheral tissues" (Elwyn, 1970, p533)

Amino acids taken up by the liver can either be metabolised to urea or

incorporated into protein. Because some of the protein synthesised by the liver is secreted in the form of plasma proteins, some amino acids will enter the circulation in this way. Elwyn has assessed the relative proportions of the various ways in which the liver disposes of amino acids in the 12hr following a meal to be: 57% converted to urea, 23% entering the circulation unchanged, 6% entering the circulation in the form of plasma proteins and 14% retained in the liver. Unless the protein mass of the liver was increasing these amino acids which were initially incorporated into liver proteins must be released into the circulation by subsequent hydrolysis.

Elwyn's work demonstrates the importance of liver and intestine in the regulation of protein metabolism. Not only are there demonstrable changes in protein mass, particularly in the liver, but these tissues also modulate the nutrient stimulus for the rest of the animal. Consequently, the majority of the work presented here has concentrated on the response of these tissues to conditions such as feeding, starvation, protein-deprivation and diabetes in which the environment around the tissues has been altered. These studies have been carried out in the intact animal so that the responses which were observed are those which normally occur in consort with changes in the rest of the animal. Changes in the rates of protein synthesis in response to varying conditions provides a first step toward understanding the way in which protein metabolism is regulated. Consequently, much of this work has concentrated on developing and elucidating methods for measuring the rates of protein synthesis in tissues such as liver and intestine.

SECTION I. METHODS FOR MEASURING PROTEIN TURNOVER

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C. DEVELOPMENT OF A METHOD TO MEASURE PROTEIN SYNTHESIS IN LIVER AND GUT

1. Methods
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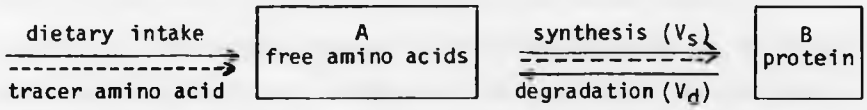
I. METHODS FOR MEASURING PROTEIN TURNOVER

Most methods for the measurement of protein synthesis or degradation in individual tissues in the intact animal have relied on the simplifying assumption that protein is a single pool which is synthesised from one, homogenous pool of free amino acids. This two-pool model is shown in Figure I.1. Amino acids can enter the free pool from protein degradation or from dietary intake and are removed from the free pool by protein synthesis.

Figure I.1



The rate of protein synthesis (V_s) is the rate of transfer of amino acid from the free pool to the protein pool, while the rate of protein degradation (V_d) is the rate of transfer of amino acid from the protein back into the free pool. These rates which represent the transfer of mass in a given time have been called "absolute rates" by Waterlow et al. (1978). Rates of synthesis and degradation can also be expressed as fractions of the protein pool (k_s and k_d, units: time^{-1}). Defining k_s and k_d in this way is slightly confusing since "k" is usually used for rate constants of first order reactions where the transfer of material is a constant fraction of the pool from which it is being transferred. k_s differs from the more conventional use of "k" in that it is defined in terms of the pool into which the material is being transferred (i.e., the protein pool). k_s and k_d are not constants in the sense of never varying, although they are assumed to

be constant over the time of measurement and no assumption about the order of the reactions of protein synthesis and degradation is implied. The advantages of this concept of fractional rates are that it simplifies the mathematical treatment of data on rates of synthesis and degradation (see Waterlow et al., 1978), that it can be calculated directly from measurements of specific radioactivity and also that, in physiological terms, it is a measure of the rate at which protein is renewing itself.

Most estimates of the rates of protein synthesis and degradation have been made using radioactivity labelled amino acids as tracers for the movement of amino acids. The labelled amino acid is introduced into the free pool and mixes with the unlabelled amino acid. Intuitively, the rate of change in the amount of label in the protein pool should be equal to the amount of label entering the pool via protein synthesis minus the amount of label which is leaving the pool via protein degradation. This relationship is expressed in the equation (Waterlow et al., 1978),

$$\frac{d\,C}{d\,t} = v_s\,S_A - v_d\,S_B \qquad \text{equation I.1}$$

The amount of label which enters the protein pool is the product of the amino acid which is entering (v_s) and the amount of label per amino acid in that pool, i.e. the specific radioactivity (S_A). The amount of label which is being lost from the protein pool is equal to the amount of amino acid which is leaving (v_d) times the specific radioactivity of the amino acid in the protein (S_B). If S_A is considerably larger than S_B then label will accumulate in the protein pool; conversely, if S_B is considerably larger than S_A then label will be disappearing from the protein pool. In general, measurements of the rate of protein degradation are made some time after the introduction of the label when

$S_B >> S_A$ and label is being lost from the protein pool. Measurements of the rate of protein synthesis are made soon after the label is introduced when $S_A >> S_B$ and label is accumulating in the protein pool.

As defined in the introduction protein turnover consists of the processes of protein synthesis and degradation. In the steady-state (i.e. when protein mass is not changing) the rate of synthesis must equal the rate of degradation and measurement of either process would give an indication of the turnover of the tissue. In the non-steady state, where protein mass is changing, the two processes of synthesis and degradation must be considered independently. However, since the change in protein mass is a consequence of the balance between synthesis and degradation if two of the three variables (synthesis, degradation and change in mass) are measured, then the change in the third can be inferred. The choice of whether to measure synthesis or degradation is a practical one,since measurements of synthesis and degradation rely on different assumptions. In the discussion which follows the assumptions inherent in several methods will be examined with particular reference to their validity for tissues such as liver and intestine.

I A. DECAY OF LABELLED PROTEIN

Measurements of the rate of protein degradation from the decay of labelled protein is, in principle, quite simple. A labelled amino acid, introduced into the free amino acid pool, is incorporated into protein. The specific radioactivity of the free pool drops as the labelled amino acid is transferred out of the pool via protein synthesis and unlabelled amino acid enters the pool from protein degradation and from the diet ($S_A \longrightarrow 0$). If S_A is assumed to be zero then equation I.1 can be simplified to

$$\frac{d\ C}{d\ t} = -v_d\ S_B \qquad \text{I.2}$$

S_B is, by definition, the amount of label in the protein pool (C) divided by the mass of the protein pool (m_B). Substituting into equation I.2 yields

$$\frac{d\ C}{d\ t} = -\frac{v_d C}{m_B} \qquad \text{I.3}$$

v_d/m_B has been defined as k_d so substituting and integrating equation I.3 with respect to time (t) produces

$$\frac{C_t}{C_o} = e^{-k_d t} \qquad \text{I.4}$$

In other words the total radioactivity in the protein pool falls exponentially. In practice k_d is usually determined graphically from a semi-log plot of total radioactivity vs time which produces a straight line with slope, k_d. The two assumptions which have been made are that S_A is zero and that protein degradation is random with respect to label.

1. The problem of recycling of label

Failure to meet the first assumption (i.e., that $S_A = 0$) results in the problem of recycling. For most amino acids the specific radioactivity of the free pool falls rapidly after injection of a tracer amount of amino acid (see for example Morgan and Peters, 1971) but then it slows down because label is being returned to the free amino acid pool from the degradation of labelled protein. Reincorporation of labelled amino acids means that label is lost from protein at a reduced rate and the rate of degradation which is obtained will be an underestimate of the true rate. In order to overcome the problem of recycling of label, Swick (1958) suggested using arginine labelled in the guanidino carbon [(6-^{14}C)arginine] to label liver protein. He argued that the label released from protein would not be reincorporated because the guanidino carbon for arginine is continually replaced by unlabelled CO_2 during the formation of urea. Although arginine is reincorporated, the label is not recycled. Subsequently McFarlane (1963) and Swick and Ip (1974) have shown that there is less recycling of label of the guanidino carbon of arginine is labelled in vivo by injecting $NaH^{14}CO_3$ than if (6-^{14}C)arginine is given. From the change in the total radioactivity in protein measured over 5d Swick and Ip (1974) reported values for k_d of 14% per day when guanidino-labelled arginine was given compared to k_d of 28% per day when bicarbonate was given. Differences like this between labelled arginine and labelled bicarbonate have been explained by McFarlane (1963) to be a consequence of the difference in specific radioactivity of the arginine which is incorporated into protein of different tissues. If labelled arginine is given the specific radioactivity of arginine will be highest in tissues without the urea cycle (e.g.

muscle). When these proteins are degraded the labelled arginine is recycled to the liver and hence label is reincorporated. If bicarbonate is given, proteins in tissues other than the liver will not be very highly labelled and consequently label will not recycle from these tissues back to the liver. It has also been shown by Millward, (1971) and Swick and Ip, (1974) that isolation of arginine was not necessary since all amino acids labelled by $NaH^{14}CO_3$ were shown not to be recycled in the liver. In tissues other than liver the absence of urea cycle enzymes precludes the use of $H^{14}CO_3$-labelling of the guanidino groups of arginine. Other amino acids containing carbon atoms which exchange with the bicarbonate pool have been used to minimise recycling. For example, Millward (1971) has found that glutamate and aspartate (which become labelled in the carboxyl carbon) were not recycled to an appreciable extent in skeletal muscle.

The magnitude of the problem of recycling of labelled amino acids in intestine has been demonstrated by James (reported in Alpers and Kinzie, 1973) by comparison of the loss of label from protein following injection of (^{3}H)leucine and $H^{14}CO_3$. After 4 days there was twice as much ^{3}H remaining in the protein as ^{14}C. Although suggesting that $H^{14}CO_3$ was preferential to (^{3}H)leucine, the fact that 10% of the $H^{14}CO_3$ was still present after the entire population of cells had been replaced (see further in this section) indicates that even $H^{14}CO_3$ is recycled to some extent. Consequently, the values for k_d of 24-35% per day reported by Muramatsu et al. (1963) based on the decay of (^{35}S)-methionine are likely to be underestimates of the true rate.

2. <u>The problem of non-random loss of label</u>

The second assumption implicit in the prece ding mathematical

analysis is that label is lost from protein in a random way, that is, that any protein molecule has an equal chance of being degraded. If the specific radioactivity of the protein which is degraded is not the same as the specific radioactivity of the remaining protein, then the loss of label will not be a simple exponential. Non-exponential decay is often encountered when mixtures of proteins with different turnover rates are studied (Millward, 1971; Swick and Ip, 1974; Miehe, 1976). Those proteins which have the fastest rates of turnover are initially the most highly labelled. Label is lost from these proteins most quickly so that in time they become the least labelled. Loss of label from the highly labelled, faster turning over proteins, therefore produces a more rapid fall in the total radioactivity. However, as the experiment continues these proteins make less contribution to the loss of label and a rate appropriate to the slower turning over proteins is observed. If the decay curve is analysed as a single exponential then the turnover rate which is obtained will depend on the portion of the curve which is considered or, in other words, the time period. Waterlow et al. (1978) have demonstrated the magnitude of this problem by analysing different portions of the decay curve of $H^{14}CO_3$ labelled liver proteins presented by Swick and Ip (1974). Their analysis is shown below.

t (days)	apparent k_d (% per day)
1	77
3	55
6	41
12	30
30	17

Measurements of decay made over inappropriately long periods of time represent the turnover rate of only the slowest turning over

proteins. Consequently results such as those reported by Miller et al. (1978) of 19% per day in liver and 17% per day in the gut in mice labelled with (^{3}H)glutamate are serious under-estimates of the average turnover rate for all proteins in these tissues. Garlick et al. (1976) have suggested three alternative methods for analysing decay curves from proteins with different rates of turnover to obtain a mean rate of turnover for the tissue. Briefly these suggestions were as follows :

(1) Treatment of the decay curve as the composite of a number of single exponential curves each representing a group of proteins. Three exponentials were used to describe the data of Swick and Ip (1974). Each exponential was solved for the k_d for that fraction as well as the relative mass of that fraction and the weighted, mean k_d was then calculated to be 41.3% per day.

(2) Calculation of k_d from the maximum specific radioactivity of the protein divided by the total area under the curve. This required that the specific radioactivity was measured for at least 21 days. k_d calculated in this way was 42.8% per day.

(3) Analysis of the decay curve as a single exponential at the time interval which is representative of the mean for the tissue. Values for k_d were calculated from simulated mixtures of proteins with different turnover rates and a range of values of k_d were generated by altering the time interval used in the calculation. The k_d value which most resembled the true k_d was found when the interval for calculation was between 3 and 4 times the mean half-life for the mixture. In practice the authors suggested using the time interval when the specific radioactivity had decayed to 10% of its peak value. For liver this meant that the calculation was based on the interval 0 - 6 days and the mean k_d was equal to 40% per day. Therefore, decay curves from mixtures of proteins can be analysed, at least in liver, though the

minimum time required for such a measurement is 6 days.

Non-random loss of label from protein is even more of a problem in intestine than in liver. To understand why this is so an appreciation of morphology and the nature of cell replacement in the tissue is necessary (for review see Creamer, 1974). Unlike most other tissues the cells of the intestinal epithelium, or mucosa, undergo continuous replacement. The production of new cells is confined to areas known as crypts which are located at the base of projections, or villi, produced by the convoluted nature of the luminal surface of the intestine. Cells migrate from the crypt along the villus undergoing considerable morphological and biochemical differentiation (Nordström, 1969; Riechen, 1970 and Weiser, 1973) and finally are exfoliated from the top of the villus. Consequently, along the villus there is a gradient of cell age which is reflected in the enzymes which are located in the different regions. Weiser (1973) developed a method for serially removing cells from the luminal surface of the intestine and demonstrated a gradient from crypt to villus for the enzyme, thymidine kinase (EC.2.7.1.2.1.) and a gradient from villus to crypt for enzymes such as alkaline phosphatase (EC.3.1.3.1.) and the disaccharidases. Estimates for the life-span, i.e. the time taken between cell division and exfoliation, for mucosal cells in rats and mice range between 41-72 hours (reviewed by Cairnie et al., 1965; Gleeson et al., 1972). The fractional turnover rates corresponding to these values would be 33-59% per day. This means that the entire population of cells in the mucosa changes every 2-3 days. Because of the rapidity of cell replacement, decay measurements for protein turnover lasting several days must be interpreted with caution since the population of cells being studied will have changed during the course of measurement.

In addition to protein which is synthesised for the replacement of

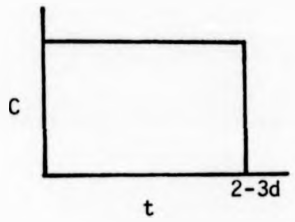
cells, there is also the potential for turnover (synthesis and subsequent degradation)within the cells as they migrate up the villus, i.e. intracellular protein turnover. Studies on the nature of intracellular turnover in intestinal cells have produced conflicting results. Autoradiographic localisation of (^{35}S)methionine in the intestine of rats four hours after a subcutaneous injection reported by Leblond et al. (1957) indicated that the crypt was the most highly labelled, although there was some labelling along the villus. Twenty-four hours after injection the area of maximum labelling appeared further up the villus and by thirty-six hours the area of maximum labelling was at the villus tip. Using autoradiography to assess the rate at which label disappeared from various regions of the intestinal mucosa of mice given an intraperitoneal injection of (^{3}H)leucine Lipkin and Quastler (1962) concluded that in the crypt region the loss of label ($t_{\frac{1}{2}}$ = 20hr equivalent to k_d = 83% per day) was consistent with the rate of cell production, indicating that there was little or no intracellular turnover in these cells. In the villus, although the rate at which label was lost was consistent with a loss derived from the turnover of cells ($t_{\frac{1}{2}}$ = 20 hr), the amount of label in the villus was 50% lower than would have been predicted without assuming intracellular turnover. The authors suggested that intracellular protein turnover was not occurring to any appreciable extent in rapidly proliferating cells like those in the crypt, but that in cells along the villus there was some intracellular turnover and/or secretion of labelled protein. Further evidence for some incorporation of labelled amino acids into protein all along the villus is provided by the experiments of other workers. Shorter and Creamer (1962) found that intraperitoneal injection of (^{3}H)-methionine into mice produced a pattern of incorporation similar to that reported by Leblond et al. (1957) but including a high degree of

labelling in the cells at the villus tip. Correlation of methionine incorporation with (^{3}H)uridine suggested that the majority of RNA was also being synthesised in the crypt but that there was also substantial synthesis in the villus tip region and to a lesster extent along the villus. Das and Gray (1969) analysed longitudinal sections of rat intestine after intraperitoneal injection of (^{14}C)leucine and (^{3}H)-thymidine. Their results indicated that although incorporation of (^{3}H)thymidine fell from crypt to villus, incorporation of (^{14}C)leucine continued at about 30% of the crypt value along the villus. However, they did not report any increase in incorporation at the villus tip. Subsequently, the work of Alpers (1972) has suggested that experiments using radiolabelled tracers to determine the extent to which protein is being synthesised in different regions of intestinal mucosa must be interpreted with caution since the labelling pattern of the cells will depend on the route of administration of the label. Alpers compared the incorporation of (^{14}C)leucine administered intraluminally with (^{3}H)leucine administered intravenously and concluded that every level of the villus synthesised protein to the same extent but that normally intraluminal amino acids (unlabelled in those experiments where the tracer was given intraperitoneally) were used preferentially by the villus while intravenous amino acids were used preferentially by cells in the crypt.

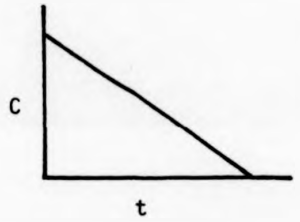
This question of whether proteins in the crypt are labelled preferentially or whether proteins are uniformly labelled from crypt to villus is central to measurements of protein degradation which are made using a method based on the decay of label. Four hypothetical decay curves are shown in Figure I.2 representing the consequences of incorporation into crypt cells only (a and d) or equal incorporation into all cells from crypt to villus (b and c) with subsequent intra-

Figure I.2

Hypothetical curves for the loss of label (C) from protein with time (t), assuming no reincorporation of labelled amino acids and the same k_d for all proteins in the tissue. Both C and t are expressed on a linear scale.



a. incorporation of label into protein in crypt cells only; no intracellular degradation



b. uniform incorporation into protein from the crypt to villus; no intracellular degradation

c. uniform incorporation from crypt to villus; intracellular degradation

d. incorporation of label into crypt cells; intracellular degradation

cellular degradation (c and d) or not (a and b). The interpretation of experimental curves will be further complicated by the problems of reincorporation of labelled amino acids and heterogeneity of turnover rates of different proteins which have already been discussed. One further problem arises from the determination of total radioactivity (C) in intestine. To obtain accurate values for total radioactivity the separation of the mucosa from the underlying serosa must be entirely quantitative.

3. Summary

In summary, measurements of protein degradation based on the decay of labelled protein are very difficult to make with accuracy in tissues such as liver and intestine. For reliable estimates two conditions must be met.

(1). Recycling of label into protein from the reincorporation of labelled amino acids released from the degradation of protein must be minimal. In liver, this can be accomplished by using $H^{14}CO_3$ to label the guanidino group of arginine, so that, when labelled arginine is released from protein the labelled carbon is exchanged for an unlabelled one (via the urea cycle) before the amino acid is reincorporated into protein. In intestine even $H^{14}CO_3$ labelling does not appear to eliminate recycling.

(2). Analysis of the experimental decay curve must take into account the complex nature of the curve produced from a heterogenous mixture of proteins turning over at different rates. To accomplish this the shape of the curve must be accurately determined and the measurement carried on sufficiently long to ensure that 90% of the intital radioactivity has been lost. In liver the minimum time period to meet this

requirement is six days. Assessment of acute changes such as those arising from the ingestion of nutrients is, therefore, impossible. In intestine complicated decay curves arise not only from heterogeneity of the proteins being measured but also from the fact that label may be preferentially incorporated into crypt cells while a large component of protein degradation is occurring at the villus tip where whole cells are being lost.

Because of the problems of recycling and heterogeneity direct measurements of the rate of protein degradation cannot be considered reliable. At this time it appears more satisfactory to measure changes in protein mass and changes in protein synthesis and then to infer the changes in protein degradation which would be necessary to account for the observations.

I B. INCORPORATION OF LABEL INTO PROTEIN

Measurements of protein synthesis are based on assessing the rate at which labelled amino acids are incorporated into protein. The same equation relating the rate of change of the label in protein with the specific radioactivity of the amino acid in the free protein pools is used whether incorporation or loss of label is actually measured, i.e.

$$\frac{dC}{dt} = v_s S_A - v_d S_B \qquad \text{I.1}$$

If steady-state conditions are assumed then the mass of the protein pool is constant* and the equation can be divided by m,

$$\frac{d\left(\frac{C}{m}\right)}{dt} = \frac{v_s}{m} S_A - \frac{v_d}{m} S_B \qquad \text{I.5}$$

By definition $\frac{C}{m}$ is S_B and $\frac{v_s}{m}$ is k_s so these substitutions can be made and also since steady-state conditions were assumed, k_s is equivalent to k_d and equation I.5 can be simplified to

$$\frac{dS_B}{dt} = k_s (S_A - S_B) \qquad \text{I.6}$$

or in integrated form :

$$S_{B(t)} = k_s \left[{}^{t}\!\int_0 S_A dt - {}^{t}\!\int_0 S_B dt \right] \qquad \text{I.7}$$

Solution of this equation is determined by the way in which the isotope is given. When the isotope is given as a single injection, S_A rises initially and then declines, so that it is often easier to solve equation I.7 graphically by making use of the fact that the two

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*It can be shown mathematically that this equation is valid even if m is a variable (Waterlow et al., 1978, p.217).

integrals represent the areas under the curves for S_A vs t and S_B vs t. When the isotope is given by constant infusion, S_A rises to a constant (plateau) value and equation I.6 can be integrated to yield

$$S_{B(t)} = S_A(1 - e^{-k_s t}) \qquad \text{I.8}$$

In this case k_s can be determined from the values of S_A and S_B at the end of the infusion with some assumptions about the nature of the time course for the rise in S_A (see Garlick and Marshall, 1972 and Garlick, 1978). Both single injection and constant infusion have been used to measure liver protein synthesis and these results will be discussed in some detail as well as the applicability of the two methods to measurements in intestine, but common to both methods is the problem of which pool of amino acid which can be measured best represents the specific radioactivity in the pool which is the precursor for protein synthesis (A in Figure I.1).

1. The precursor pool

The necessity for defining the specific radioactivity of the amino acid which is used for protein synthesis is obvious from consideration of the model discussed earlier (Figure I.1). If two conditions are to be compared by injecting the same amount of tracer amino acid into the final amino acid pool, the difference in incorporation will reflect changes in protein synthesis only if the specific radioactivity of the precursor is the same for both conditions. The seriousness of measuring incorporation while ignoring changes in the specific radioactivity of the precursor amino acid has been demonstrated experimentally. Hirschfield and Kern (1969) gave (^{3}H)leucine to control and protein-deprived rats and measured the difference in incorporation into protein

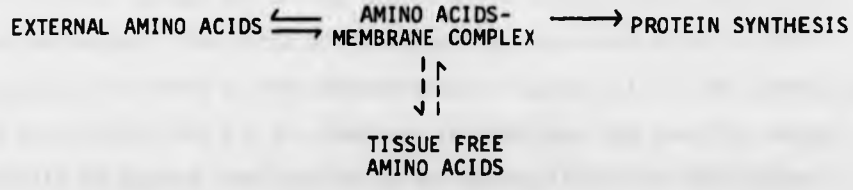
of the jejunum. Their results suggested a rate of synthesis in protein-deprived rats that was more than ten times that in control rats. This seemed most unlikely and the authors attributed the difference in incorporation to differences in the specific radioactivity of the precursor amino acid rather than to differences in the rate of synthesis. Direct comparison of incorporation of a labelled tracer with a measured rate of synthesis which was not influenced by problems of defining the precursor specific radioactivity has been made by Scornik (1974). He showed that after hepatectomy regenerating mouse liver incorporated leucine given in tracer amounts at a rate which was 60% higher than the rate in control mice. When protein synthesis was actually measured the rate in regenerating liver was only about 10% higher than the rate in control liver. Clearly experiments which rely only on the incorporation of labelled amino acids into protein without consideration of possible changes in the specific radioactivity of the precursor can give erroneous results.

Unfortunately defining the site of amino acids used directly for protein synthesis has not proven an easy task. The problem has been approached in a number of ways in systems which include incubated cells, incubated tissues, perfused organs and intact animals (for reviews of the subject see Fern, 1975 and Waterlow et al., 1978). Conclusions from these studies are quite divergent but they can be broadly grouped into experiments which would suggest that amino acids from an extracellular pool (from the medium or from plasma) are used directly for protein synthesis and experiments which suggest that amino acids entering the cell equilibrate with the amino acids already present and that protein is synthesised from this intracellular pool. No attempt will be made to discuss exhaustively all the work which has been done on the location of the amino acid used for protein synthesis but rather experiments will

be discussed which illustrate the different experimental approaches which have been used.

a. extracellular source

Early studies which supported an extracellular source of amino acids as the precursor for protein synthesis did so from an analysis of the kinetics of incorporation of labelled amino acids into protein. Linear incorporation has been taken as evidence that the precursor pool for protein synthesis was not within the cell since the specific radioactivity of intracellular amino acids could be shown to be rising with time. Incorporation from a pool with rising specific radioactivity would show evidence of a slower rate initially, the absence of such a lag has been taken as support for an extracellular precursor for protein synthesis. For example, Halvorson and Cohen (1958) demonstrated that incorporation of (^{14}C)phenylalanine into yeast cells was linear despite an increase in the amount of isotope in the acid soluble pool of the cells. Adding 10^{-2}M valine to the incubation medium substantially inhibited the uptake of (^{14}C)phenylalanine into the intracellular pool but did not appreciably alter the incorporation into protein. To explain their observations the authors proposed the following model in which amino acids from the medium are incorporated into protein without substantial equilibration with the tissue free amino acids.



Linear incorporation of label into protein has also been demonstrated in mammalian tissues incubated *in vitro*. Kipnis et al. (1961) studied the appearance of (^{14}C)proline in the intracellular amino acid pool and tissue proteins of incubated rat diaphragm. The accumulation of isotope into protein was linear despite the fact that the amount of isotope in the intracellular amino acid pool (claimed to be equivalent to the specific radioactivity since the amount of intracellular proline was shown to be unchanged) rose exponentially. A theoretical plot of the protein specific radioactivity based on the observed kinetics of labelling in the intracellular pool predicted that if proteins were being synthesised from the intracellular pool, then the incorporation of labelled proline into protein would be non-linear.

In the study of Kipnis et al. (1961), as in many others, the radioactivity in the intracellular pool was determined by correcting the radioactivity in the tissue homogenate for the amount of label in the extracellular space. The specific radioactivity of extracellular amino acid was assumed to be the same as the incubation medium. However, Hider et al. (1971) were able to show that the specific radioactivity in the extracellular pool was appreciably different from that in the incubation medium. Using a technique reported by Guidotti et al. (1964) extracellular amino acids were released from incubated extensor digitorum longus muscle by placing the tissue in cold (2^{o}C) Krebs-Ringer bicarbonate buffer, pH 7.4. Amino acids from the extracellular space were rapidly washed out of the tissue while intracellular amino acids were unchanged. The ratio of concentrations of amino acids in the extracellular fluid to the concentration of amino acid in the medium was 2.4 for glycine and 1.2 for leucine; in addition, the specific radioactivity of glycine and leucine in the extracellular was only about one-third of the specific radioactivity of the medium. This technique

for removing the extracellular amino acid has the further advantage that the specific radioactivity of intracellular amino acids can be measured directly. In the incubated muscle preparation used, the specific radioactivity of intracellular amino acid rose much more slowly than the extracellular. Linear incorporation of label into protein was demonstrated with both (^{14}C)glycine and (^{14}C)leucine which led the authors to conclude that the precursor for protein synthesis was approximated by the extracellular pool.

An extracellular source of amino acids for protein synthesis has also been suggested by Fern et al. (1971) from studies on incubated small intestine. Segments of rat small intestine were incubated for 10 min in a medium containing (^{14}C)leucine and then transferred to a medium containing (^{3}H)leucine. The specific radioactivity of intracellular leucine was determined from the specific radioactivity of leucine in the tissue homogenate corrected for the extracellular leucine which it contained (extracellular fluid was determined using inulin and the specific radioactivity was assumed to be the same as that in the incubation medium). After the transfer from the ^{14}C-containing medium to the ^{3}H-containing medium the specific radioactivity of intracellular leucine was decreasing with respect to ^{14}C and increasing with respect to ^{3}H. Theoretical plots for the incorporation of label into protein using the experimental values for extracellular and intracellular leucine specific radioactivities were similar to those reported by Kipnis et al. (1961) and Hider et al. (1971). The assumption of an extracellular source for protein synthesis produced a theoretical plot for incorporation which was more linear and more nearly approximated the experimental results.

Since amino acids are bound to transfer RNA before being incorporated into protein, the specific radioactivity of amino acids in

the precursor pool can be determined directly by measuring the specific radioactivity of amino-acyl t-RNA. Van Venrooij et al. (1974) measured leucyl t-RNA under conditions in which the specific radioactivity of the extracellular and intracellular leucine was quite different. HeLa cells grown with (^{3}H)leucine were pulse-labelled with (^{14}C)leucine and the specific radioactivity of leucine was determined in the intracellular, extracellular and t-RNA-bound pools from the ratio of $^{14}C/^{3}H$. The specific radioactivity of leucine bound to t-RNA reached the specific radioactivity of the medium within minutes while that of intracellular leucine was only 50% of that in the medium. In other experiments, incorporation of label into protein was shown to be linear despite a slow rise in intracellular leucine specific radioactivity. Also valine (10mM) was shown to inhibit transport of labelled leucine into the intracellular pool but not to inhibit incorporation into protein. Clearly, for these rapidly growing cells in culture, amino acids are preferentially incorporated from the medium rather than from the intracellular pool.

In vivo measurements of leucyl t-RNA in rat heart made by Martin et al. (1977) also suggest that the precursor for protein synthesis is extracellular. Fifteen minutes after intravenous administration of (^{3}H)leucine, the specific radioactivity of leucine in plasma and bound to t-RNA was similar, while the leucine specific radioactivity in the intracellular (total acid-soluble) pool was 30-48% lower.

b. intracellular source

One of the earliest studies which suggested an intracellular location of amino acids for protein synthesis was that reported by Loftfield and Harris in 1956 within the context of an experiment to determine whether protein was synthesised only from free amino acids or whether peptide fragments could be incorporated directly. ^{14}C-labelled

valine, isoleucine and leucine were infused into rats continually over three days to ensure that the specific radioactivity of these amino acids was constant. An iron compound was then injected to stimulate synthesis of the liver protein, ferritin. The specific radioactivity of the three amino acids from hydrolysed ferritin was compared with the specific radioactivity of the free amino acids from the liver. For all three amino acids the specific radioactivities were in good agreement and the authors concluded that ferritin was synthesised from free amino acids. Further comparison of the specific radioactivity of the free amino acid with the specific radioactivity of the infused amino acid led the authors to conclude that more than half of the amino acids in the free pool are derived from endogenous protein.

Several subsequent studies have relied on differential labelling of the extracellular and intracellular amino acid pools. Comparison could then be made between the specific radioactivity in the protein and the specific radioactivity in either of the two possible precursor pools. Alpers and Thier (1972) infused (^{14}C)leucine intravenously into rats at the same time as (^{3}H)leucine of the same concentration was perfused through the lumen of the jejunum. The ratio of $^{3}H/^{14}C$ was then compared for leucine in protein, in the acid-soluble pool of the tissue, in luminal fluid and in the blood. The ratio of $^{3}H/^{14}C$ in the protein was the same as that for acid-soluble leucine and quite different from either the plasma or luminal fluid. An <u>in vitro</u> study was also reported which, in contrast to those of Fern et al. (1971), suggested that intracellular amino acids were used for protein synthesis. Following pre-incubation of intestinal slices with unlabelled glycine, incorporation of (^{14}C)-glycine into protein showed a lag such as would be expected if the label were equilibrating with the intracellular pool.

The method of differential labelling used by Alpers and Thier

(1972) is obviously applicable only to tissues like the gut where labelled amino acids can be introduced by two different routes. For other tissues Fern and Garlick (1974) devised an experiment whereby rats were infused with one amino acid which could be converted into another labelled amino acid within the cell. The two amino acids used were glycine and serine. One amino acid was infused continuously over six hours. The specific radioactivity of this amino acid reached a constant value in the plasma and a constant, though lower, value in the tissues. Intracellular formation of the other amino acid resulted in a specific radioactivity within the tissue which was much higher than that in the plasma. The experiment was carried out with infusion of either labelled glycine or labelled serine with analysis of the specific radioactivity of free glycine and serine from plasma and from tissues and also the specific radioactivity of both amino acids in tissue protein. The assumption was made that calculation of the rate of protein synthesis from the true precursor pool should produce the same result whichever amino acid was used for the calculation. Rates of synthesis from infused glycine and de novo generated serine as well as infused serine and de novo generated glycine were compared in a number of tissues. Synthesis rates for all tissues calculated from the free amino acid in the tissue were in much better agreement than those calculated from the specific radioactivity of the amino acids in plasma.

Comparison of synthesis rate derived from the incorporation of different amino acids has also been used by Li et al. (1973) in small pieces of rat diaphragm incubated in vitro. The incorporation of (^{3}H) leucine was assessed in tissue which had been incubated with different concentrations of (^{14}C)tyrosine. Tissue was also incubated with (^{3}H) leucine and (^{14}C)tyrosine and then transferred to a medium containing (^{3}H)leucine and unlabelled tyrosine. In both instances the specific radioactivity of labelled tyrosine within the cell would be different

to that in the medium. Comparison of incorporation of tyrosine with the incorporation of leucine gave compatible results only if the specific radioactivity of intracellular tyrosine was used in the calculation. (The specific radioactivity of intracellular tyrosine was calculated from the total tissue corrected for extracellular specific radioactivity using inulin to assess extracellular fluid and assuming tyrosine specific radioactivity to be the same as the medium). Interestingly, at the lowest tyrosine concentration (0.01mM) tyrosine incorporation did not appear to be related to the specific radioactivity of intracellular tyrosine. Although this might have been due to a different precursor at low extracellular concentrations as suggested by Mortimore et al. (1972), the authors suggest the possibility that the result was due to an incorrect assessment of the specific radioactivity in the extracellular fluid; the difference between the extracellular fluid and the medium being greatest for the lowest concentration of tyrosine.

Direct assessment of the specific radioactivity of amino acids for protein synthesis is possible from measurement of amino-acyl t-RNA specific radioactivity and Henshaw et al. (1971) has used this approach to support an intracellular precursor pool. A large (non-tracer) amount of labelled lysine (75μmoles) was injected intraperitoneally into 100g rats so that there would be little difference in specific radioactivity between lysine in the plasma and in the tissue. Twenty minutes after injection the specific radioactivity of lysine bound to tRNA in the liver was found to be equivalent to the specific radioactivity of free lysine in the tissue. Although this study would support the concept of an intracellular precursor, it must be borne in mind that the experiment was designed to minimise the difference between possible precursors and the specific radioactivity of free lysine within the

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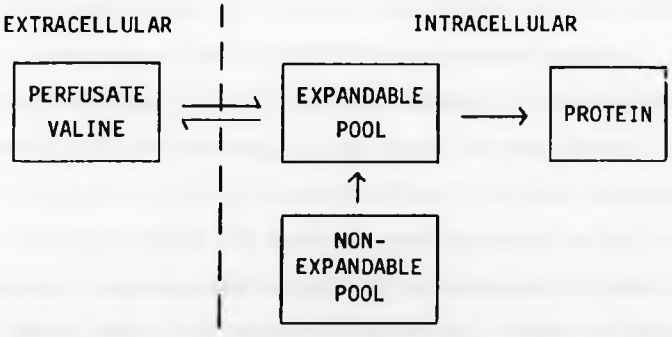
liver was 85% of the specific radioactivity of lysine in the plasma.

c. compartmented, intracellular source

In light of the conflicting conclusions from experiments designed to locate the source of amino acids for protein synthesis the concept of a separate intracellular pool, not necessarily represented by the total intracellular pool, is a very appealing one. The following model was proposed by Mortimore et al. (1972) from studies on valine incorporation into protein in perfused rat liver.



Support for this model was derived from a number of experiments.

(1). Intracellular valine concentration was determined for perfusate concentrations from 0.3 to 15mM. Extrapolation of the plot of extracellular vs intracellular concentrations indicated that in the absence of any extracellular valine, the intracellular concentration would be 0.4mM. At concentrations above this non-expandable pool, the intracellular valine concentration varied directly with the extracellular valine.

(2). The specific radioactivity of intracellular valine was compared with the specific radioactivity of extracellular valine at different levels of extracellular valine. With low extracellular concentrations,

approximately 0.3mM, the specific radioactivity of intracellular valine was only 40% of the extracellular suggesting that most of the intracellular valine was derived from protein degradation. At higher concentrations the specific radioactivity of intracellular valine was closer to that in the perfusate suggesting that the expandable pool contributed appreciably to the overall intracellular specific radioactivity.
(3). Rates of protein synthesis were calculated from valine incorporation into protein at extracellular valine concentrations between 0.3mM - 10mM. Calculations of the rate of protein synthesis using the average intracellular specific radioactivity were highest at the lowest concentration of extracellular valine, falling exponentially until at ~ 5mM valine the calculated rates became constant. Rates of synthesis calculated from the specific radioactivity of extracellular valine showed just the reverse, being lowest for the lowest level of valine. A constant rate of synthesis for all valine concentrations could be calculated from the specific radioactivity of the expandable pool assuming a non-expandable pool of approximately 0.34mM which received amino acids from protein degradation. These unlabelled amino acids were added to the expandable pool but not equilibrating with it.
(4). The calculated rates of synthesis were compared with a measured rate of protein synthesis. Protein synthesis was also measured by perfusing livers from animals which had been injected with (^{14})valine. When valine was not present in the perfusate, the rate at which label was released was a function of the rate of protein degradation minus the rate at which label was reincorporated via protein synthesis. When 15mM valine was then added to the medium reincorporation of label should be prevented and the rate of release of label should be due only to protein degradation. From a comparison of the two rates, a value for protein synthesis was obtained which was equal to that calculated in

experiment 3.

(5). Subsequently Ward and Mortimore (1978) have obtained experimental evidence in perfused livers which suggested that the non-expandable pool was located within lysosomes.

Evidence for a compartmented intracellular pool has also been provided by studies on the specific radioactivity of amino acids bound to t-RNA. Airhart et al. (1974) measured valyl t-RNA in the livers of rats following interperitoneal injection of (^{3}H)valine. The specific radioactivity of t-RNA-bound valine was found to be intermediate between the specific radioactivity of extracellular and intracellular valine. The specific radioactivity of extracellular valine was derived from a weighted mean of valine in hepatic portal plasma and in plasma obtained from the heart. The specific radioactivity of intracellular valine was derived by correcting the tissue homogenate for the extracellular space (Cl^- space) and the calculated specific radioactivity of extracellular valine. Vidrich et al. (1977) have extended the study to changes in specific radioactivity induced at various times after ingestion of a meal. Following feeding the specific radioactivity of intracellular valine became nearer to the specific radioactivity of extracellular valine, but at all times the specific radioactivity of t-RNA-bound valine remained between the intracellular and the extracellular. The authors proposed a model in which the precursor pool was located within the cell membrane and received amino acids from both the intracellular and extracellular pools. Heterogenicity of the t-RNA pool has also been suggested from studies on the kinetics of (^{3}H)-valine incorporation and release from cultured hepatoma cells. Hod and Hershko (1976) have suggested compartmentation of the amino acyl t-RNA pool with one pool in the membrane using external amino acids and one pool in the cytoplasm using intracellular amino acids.

Although the details of the location of the precursor pool(s) are somewhat different in the models discussed, the concept of a compartmented, intracellular pool is complex enough to explain how in some situations the specific radioactivity of the precursor could resemble the specific radioactivity of extracellular amino acids while at other times the specific radioactivity of the intracellular pool might be a closer approximation.

In conclusion, the problem of defining the site of amino acids used for protein synthesis remains both an important one and a complex one. Many apparently similar studies have come to different conclusions. Some general criticisms can be made. Cultured cells, for example, may not be representative of cells within an organism. In general *in vitro* studies are open to questions about the viability of the system: often viability has not been independently assessed. Incubated tissues are often in a net catabolic state, diffusion may not be adequate for uniform access of all cells to the medium (van Venrooij et al., 1972) and finally tissues are composed of heterogeneous cell types which may respond differently to *in vitro* conditions. *In vivo* experiments also have problems. The turnover rates of the metabolic pools involved are very rapid. Mortimore et al. (1972) has suggested that the expandable pool of valine in the liver turns over with a half-life of about one minute and Airhart et al. (1974) postulated a half-life of about one second for the charged t-RNA pool. Obviously, sampling such a system, before it alters, is technically very demanding. However, such criticisms are probably not sufficient to account for the diversity of results. It seems much more likely that there is not simply *a* precursor pool but rather each tissue and each amino acid has a pool from which protein is synthesised. In experiments where these pools are isotopically labelled, the specific radioactivity might be

nearer the intracellular pool or nearer the extracellular pool depending on the amino acid, the tissue, and the concentration of amino acid which is present. Indeed evidence has also been reported that different proteins are synthesised from different precursor pools (Ilan and Singer, 1975 and Fern and Garlick, 1976).

Defining the specific radioactivity in the precursor pool is a problem for all methods of measuring protein synthesis based on the rate of incorporation of labelled amino acids. In the discussion which follows three types of incorporation studies are discussed - single injection of tracer amounts of labelled amino acid, constant infusion of tracer amounts and single injection of large, non-tracer amounts of labelled amino acids. When labelled amino acids are used in tracer amounts either as a single injection or as a constant infusion, the differences in specific radioactivity of the amino acid in different pools can be quite large and arguments about which pool represents the true precursor are very important. The third alternative uses labelled amino acids in large, non-tracer amounts to minimise the differences in specific radioactivity in various pools.

2. Single injection, tracer amounts

One of the earliest attempts to measure protein synthesis using a single injection of a tracer amount of labelled amino acid was that reported by Lajtha et al. in 1957. They injected (^{14}C)lysine into mice and determined the changes in the specific radioactivity of free lysine in plasma, muscle, liver and brain in groups of animals killed 2 - 60 min after injection. Although the authors were primarily interested in the development of a method which would be used to measure the rate of protein synthesis in brain, the results which they obtained for liver

are interesting. The specific radioactivity of lysine in plasma and in the liver intracellular pool (tissue homogenate corrected for the labelled lysine present in blood located within the tissue) rose initially and then declined rapidly. By 20 minutes the specific radioactivity of lysine in plasma was approximately 10% of the 2 minute value and free lysine within the liver was approximately 25% of the peak value. Because of the rapid changes in specific radioactivity, groups of animals at 7 time points were used to define the curve for changes in specific radioactivity with time. Protein synthesis was calculated using the differential equation,

$$\frac{d S_B}{d t} = k_s (S_A - S_B) ,$$

(equation I.6, see p. 29). $^{d S_B}/_{d t}$ was determined graphically from the gradient of the S_B vs t curve and the specific radioactivity of intracellular lysine at time t was used at the precursor, S_A. In adult mice the calculated synthesis rates for liver were as follows: 77% per day, if the 2 min values were used; 56% per day, if the 5 min values were used and 27% per day, if the 10 min values were used. The authors suggest that the results indicate heterogeneity of turnover rates but as Waterlow et al. (1978) have pointed out this is an unlikely explanation at such early time points. Instead, Waterlow et al. (1978) suggest that the discrepancy arises from an inability to define the correct specific radioactivity in the precursor pool. Recalculating the data using the specific radioactivity of lysine in the plasma as the precursor results in apparent turnover rates which are the largest for the 5 min time point and smallest for the 2 min (87 and 50% per day, respectively). At 3 min the specific radioactivity was the same for both plasma and intracellular lysine and the calculated value for protein synthesis was 58% per day.

Haider and Tarver (1969) used a similar approach to study the rate of protein synthesis in the liver. A tracer amount of (^{14}C)lysine was injected into adult rats and the specific radioactivity of lysine was determined in plasma, in the liver intracellular pool (tissue homogenate corrected for the blood which it contained) and in liver protein for several time points after injection. Their study differed from Lajtha et al. (1957) in that measurements were made over two hours so that labelled proteins which were synthesised and secreted were not included in the measurement. The curves for changes in free lysine specific radioactivity were similar to those reported by Lajtha et al. (1957). Lysine in protein rose for the first sixty minutes and then remained constant. The rate of protein synthesis was calculated from the same equation used in the study of Lajtha et al. (1957) but in an integrated form (equation I.7)

$$S_B(t) = k_s\left[\int_0^t S_A dt - \int_0^t S_B dt\right] .$$

The integrals were solved graphically since they represent the areas under the curves for S_A vs t and S_B vs t. In addition the authors included an indication of the standard error of the mean for the specific radioactivity-time points. This information emphasises one of the most severe limitations of a method of this type. For some points the range of one standard error of the mean was equivalent to 50% of the actual mean. Because of the variability among animals and the rapid change in specific radioactivity the calculation of areas enclosed by the specific radioactivity-time curve was based on the mean of 4 - 6 animals at six different time points. The reported value for liver synthesis in rats fed a 27% casein diet was 17.1mg/hr/200g body wt. (equivalent to a fractional synthesis rate of about 40% per day).

By measuring incorporation of a tracer amount of labelled leucine over a much shorter time interval (16 min) Morgan and Peters (1971) were able to simplify the calculation of protein synthesis. Over this time interval the specific radioactivity of leucine in protein, which might be degraded and returned to the free leucine pool, was assumed to be negligible and so the second integral in the above equation was omitted. Also, because incorporation was measured for such a short time, the rate of protein synthesis reported was the total (secreted + retained protein) for the liver. This rate was 6.7mg leucine per g liver per hour which is 92.1% per day expressed as a fractional rate. Peters and Peters (1972) using the same method extended the study to other types of rats. Their rates for liver synthesis in control rats were comparable to the earlier study (92% per day). Liver protein synthesis was also measured at night when the animals were feeding. This rate was 25% higher (116% per day) than the rate measured 12 hours earlier but due to the lack of precision in the method these two values could not be shown to be significantly different ($0.1 < p < 0.2$).

In conclusion, measurements of protein synthesis based on a single injection of tracer amounts of amino acids are possible but such measurements require that the time course for the change in the precursor specific radioactivity be precisely defined. This requires rather large numbers of animals and must be done for each experimental condition which is to be investigated. Further, although these studies used the intracellular amino acid pool for their estimates of protein synthesis, the evidence for alternative precursors means that this choice is somewhat arbitrary.

3. Constant infusion

The infusion of labelled amino acids over three days was used by Loftfield and Harris (1956) to demonstrate that protein was synthesised from free amino acids rather than from incorporation of proteins or peptides. Their study and that of Gan and Jeffay (1967) established that the specific radioactivity of free amino acids could be maintained at a constant (plateau) value and that the plateau specific radioactivity of the amino acid in the intracellular pool of a tissue reflected the relative contributions of amino acids from plasma and amino acids from the degradation of protein.

The use of constant infusion for measuring protein synthesis relies on the fact that if S_A is constant then equation I.6, i.e.

$$\frac{dS_B}{dt} = k_s (S_A - S_B),$$

can be integrated to yield equation I.8,

$$S_{B(t)} = S_A (1 - e^{-k_s t})$$

In practise S_A does not rise instantaneously to a constant value but Waterlow and Stephen (1967 and 1968) have shown that the rise in specific radioactivity can be approximated by an exponential expression. The rate constant for this exponential was determined experimentally for plasma (Waterlow and Stephen, 1967) and the rate constant for the rise in intracellular lysine was approximated from the rate of turnover of protein in that tissue and the ratio of protein-bound lysine to free lysine (Waterlow and Stephen, 1968). Garlick et al. (1973) modified the expression for the rate of rise of intracellular specific radioactivity to take account of the slow rise in specific radioactivity in plasma found with some amino acids (e.g. glycine). Different tissues

and different amino acids require different approximations. Garlick (1978) has provided a comprehensive review for determining the most appropriate approximation in a variety of situations and also for estimating the magnitude of the error which is introduced from making these approximations.

Constant infusion of (^{14}C)tyrosine for six hours was used by Garlick (1972) to measure the rate of protein synthesis in a number of tissues. In the liver (unlike muscle) the specific radioactivity of free tyrosine (total acid-soluble tyrosine from the homogenate) was only about 50% of the specific radioactivity of tyrosine in the plasma at the end of the infusion. Consequently, the rate of protein synthesis was calculated from both of the possible precursors. With free tyrosine in the tissue, the fractional synthesis rate was 59% per day. If, on the other hand, the specific radioactivity of tyrosine in plasma was used as the precursor, the calculated rate of synthesis was 21% per day. Because the measurement was made over 6 hours proteins, such as albumin which are synthesised in the liver but retained for only a short time (e.g. 16 minutes, Peters and Peters, 1972) before being secreted, will not make a significant contribution to the incorporation of label into protein. Secreted protein may account for as much as 35% of the total protein synthesised by the liver (Pain et al., 1978a).

The specific radioactivity of free tyrosine in the jejunum was even lower compared to the plasma so the synthesis rates calculated from tissue homogenate, 218% per day, and from plasma, 50% per day, were even more divergent. Clearly in tissues where the rate of turnover is very high and the specific radioactivity in the tissue is very different from the specific radioactivity in the plasma, the choice of which amino acid to use as the precursor becomes very important. The results from Fern and Garlick (1974) already discussed (p. 37) suggested that for most

tissues the acid-soluble pool of amino acid represented the specific radioactivity of the true precursor. In the jejunum, although in each experiment the rate of synthesis calculated from the infused amino acid agreed with the rate calculated from the *in vivo* labelled amino acid, the synthesis rate calculated from infused glycine (and *in vivo* labelled serine) was about 60% per day, while the result from infused serine (and *in vivo* labelled glycine) was about 130%/d. It seems unlikely that the large difference between these two rates is a consequence of the different amino acids which were used to assess the rate of protein synthesis. Real differences in rates of synthesis between different groups of control rats cannot be ruled out, although the magnitude of the difference would seem to argue against this. It is, perhaps, more probable to conclude that the presence of food in the intestine provided a source of unlabelled protein which affected the measurement.

A number of workers (including Buchanan, 1961 and Harney et al., 1976) have assessed the rate of protein turnover from the incorporation of labelled amino acids into protein by feeding a diet containing a labelled amino acid. Harney et al. (1976) verified that the result from feeding labelled amino acids was the same as that derived from a constant, intravenous infusion of labelled amino acid. The results for individual tissues were in good agreement with those of Garlick (1972), Garlick et al. (1973) and Fern and Garlick (1974) but the method is subject to the same problems as the method of constant infusion in rapidly turning over tissues such as liver and jejunum.

With these tissues the choice of precursor can make as much as a four-fold difference to the estimate of the rate of synthesis (see p. 48). Given the divergence of results of experiments designed to

locate the actual precursor for protein synthesis, choosing either the plasma or the tissue free pool as the precursor introduces a degree of uncertainty into calculated rate of synthesis. In addition to the problem of defining the specific radioactivity of the precursor amino acids, measurements of synthesis made over six hours are further complicated because the degree of labelling in the protein pool becomes significant. Garlick (1972) has reported values for tyrosine in the protein of the jejunum which are almost 50% of the values for free tyrosine in the tissue. This degree of labelling means that the measured specific radioactivity of tyrosine in protein (S_B) must take account of label which has been lost through protein degradation. In tissues like gut where protein degradation is not randon with respect to label, accurate correction for loss of label may not be possible. In addition to affecting the estimate of S_B, label which leaves the protein pool re-enters the free pool so that the specific radioactivity in the free pool (S_A) may not be maintained at a constant value but continues to rise. The time course for changes in the specific radioactivity of the precursor must then be defined from groups of animals at different time points and one of the advantages of the constant infusion method, that of calculating a synthesis rate in each individual animal, is lost.

4. Single injection, large amounts

One way of minimising the difference in specific radioactivity between free amino acids in the plasma and within the tissue is to inject a large amount of labelled amino acid. If the amount of injected amino acid is considerably larger than the free amino acid pool of the animal, then all compartments of the free amino acid pool

will be flooded and the specific radioactivity within the compartments should be close to the specific radioactivity of the amino acid which was injected. The fractional synthesis rate, k_s, can easily be calculated from equation I.6 (p. 29),

$$\frac{dS_B}{dt} = k_s (S_A - S_B),$$

S_B is negligible compared to S_A so the right side of the equation simplifies to $k_s S_A$. dS_B/dt when both t and S_B are 0 at the beginning of the interval being considered is simply S_B/t and the equation becomes

$$k_s = \frac{S_B}{S_A t} \qquad I.9$$

Single injection of a large amount of labelled amino acid was used by Henshaw et al. (1971) to measure the rates of protein synthesis in several tissues in young, growing rats. A large amount of labelled lysine (75μmoles of (^{14}C)lysine per animal) was injected intraperitoneally. Determinations of plasma and intracellular lysine (tissue homogenate corrected for plasma lysine) specific radioactivities 10 and 20 minutes after injection indicated that in the liver flooding had been quite effective since the specific radioactivity of lysine was 88% of the specific radioactivity of lysine in the plasma. The specific radioactivity of plasma lysine fell about 12% between 10 and 20 minutes but the authors used the 20 min value as representative of the entire 30 minute interval. Although this would under-estimate the specific radioactivity the authors argue that this slight under-estimate would compensate for the slight over-estimate due to the assumption that the rise in specific radioactivity was instantaneous following injection. Evidence that protein synthesis was not altered by this large amount of lysine was provided by the observation that similar rates of synthesis

were obtained with 75μmoles and 150μmoles of lysine. The specific radioactivity of t-RNA was shown to be comparable to the specific radioactivity of intracellular lysine (i.e., the specific radioactivity of lysine from the two sources differed by less than 10%) so the rate of protein synthesis was calculated from the specific radioactivity of intracellular lysine. Since incorporation was assessed 20 min after the injection of isotope, the synthesis rate of the liver which was measured included the synthesis of both secreted and non-secreted liver protein. The value obtained for the liver was 73% per day.

A single injection of a massive amount of labelled amino acid has also been used to study the rate of protein synthesis in brain. Dunlop et al. (1975) gave intraperitoneal injections of even larger amounts of amino acids than those used by Henshaw et al. (1971). With 1.5mmoles (1-^{14}C)valine/100g body weight the radioactivity of acid-soluble valine was 90% of the specific radioactivity of the injected valine and this high specific radioactivity was maintained for 2 - 3 hr. A number of experiments were used to prove that the large amount of valine was not influencing the rate of protein synthesis. Comparable rates of incorporation were obtained with 1 - 2mmoles (^{14}C)lysine and 1mmole (^{14}C)histidine per 100g body weight. Rates of synthesis obtained with tracer amounts of labelled tyrosine, lysine and histidine (the time-course for the changes in acid-soluble amino acid was used to determine the mean specific radioactivity of the precursor and the rate of synthesis was measured over the interval 12 - 30 min) also agreed well with the rate of synthesis measured with the large amount of valine. Rates of protein synthesis in rat brain, 15% per day, and mouse brain, 17% per day, were in good agreement with published values obtained with other methods (Oja, 1967 and Garlick and Marshall, 1972).

Injection of a large amount of labelled amino acid ensures that the specific radioactivity of the possible precursor pools is similar but the specific radioactivity of the precursor must still be measured. Scornik (1974) has devised a method of measuring protein synthesis which is not dependent on an assessment of the specific radioactivity of the precursor amino acid. Groups of mice were injected intravenously with (^{14}C)leucine in varying amounts from 33 to 286μmoles leucine per 100g body weight and killed 5 min after injection. The amount of radioactivity in liver protein was determined and then the reciprocal of the specific radioactivity of liver protein was plotted against the reciprocal of the amount of leucine which was injected. Plotting the data in this way allows for the extrapolation of (amount injected)$^{-1}$ to infinite amount. Since at an infinite amount the specific radioactivity in the precursor pool would be equal to the specific radioactivity of the leucine which was injected, the incorporation at this point can be used to calculate the rate of synthesis using the specific radioactivity of the injected amino acid as the specific radioactivity of the precursor. At infinite amount the incorporation of leucine into liver protein after 5 minutes was 5.2×10^3 dpm per mg RNA. If one assumes that liver protein is 8.75% leucine (Morgan and Peters, 1971) and uses the given value for the amount of RNA relative to protein, then the corresponding fractional synthesis rate is 137% per day. Confirmation that large amounts of leucine were not affecting the rate of protein synthesis was provided by the observation that the incorporation of (^{3}H)lysine injected 4 min after 143μmoles of unlabelled leucine/100g body weight was the same as incorporation of (^{3}H)lysine without a prior injection of unlabelled leucine.

By using a massive amount of labelled amino acid to minimise

problems of precursor specific radioactivity and by measuring incorporation over a short period, most of the problems of measuring protein synthesis in tissues with rapid turnover can be overcome. The method of Scornik (1974) is especially appealing as no measurements of the specific radioactivity of the precursor are necessary.

I C. DEVELOPMENT OF A METHOD FOR MEASURING PROTEIN SYNTHESIS IN LIVER AND INTESTINE

Of the methods described in the previous section those involving the single injection of a large amount of labelled amino acid seemed most likely to provide reliable estimates for the rate of protein synthesis in tissues such as liver and gut. The large amount of isotope minimises the problem of accurately defining the precursor specific radioactivity and the short duration of the measurement (5 - 20 minutes) ensures that the labelling of the protein pool is sufficiently low that loss of label from protein does not occur. This experimental section describes a number of experiments which developed and validated a method for measuring protein synthesis in liver and intestine in control rats based on the incorporation of labelled leucine following the injection of a large amount of the labelled amino acid. This section begins with a description of the methods which were used throughout these experiments and are common to experiments described in subsequent sections. The details of additional methods have been included in the sections describing the experiments in which they have been employed.

1. Methods

Animals: Male Wistar albino rats were obtained from Charles River (Margate, Kent) at approximately 50g body weight. Rats were individually caged in plastic cages with wire bottoms and maintained on a 12hr light, 12hr dark schedule at a temperature of 25^{o}C. Control rats were fed *ad libidium* with free access to water. Three different control diets

have been used (see Tables I.1 and I.2): a cubed, breeding diet purchased from Oxoid Limited (Basingstoke, Hants.) containing 23% (w/w) crude protein and two powdered diets based on casein. The powdered diets were used in studies where some aspect of the diet was altered in the experimental group.

Formulation of the powdered diet was based on a cubed diet described by Payne and Stewart (1972) where casein and maize starch were varied to produce diets which differed in protein content but which were isocaloric. To compare different diets the authors defined a NDpE ratio which related the utilizable protein to the total energy content of the diet. Commercial diets which were 20% (w/w) crude protein were assessed to be about 9% NDpE, but the authors suggested increasing the NDpE ratio to 10% to obtain maximum growth and breeding performance. The 12% (w/w) powdered diet was based on this recommendation and contained 10% of the total energy as casein with methionine added to improve the utilization of protein. Subsequently, growth rates with the 12% (w/w) powdered diet were found to be lower than with oxoid so the 12% (w/w) diet was replaced with a powdered diet containing 20% (w/w)*.

Injection procedure: Experiments were routinely carried out when the rats had reached approximately 100g body weight. All experiments were done between 9.30 am and 12.30 pm to minimise the effect of diurnal variations. Unanaesthetised animals were restrained during injection by being wrapped in a tea towel in such a way that only the tail was exposed. Intravenous injections were made into a lateral tail vein using a 26 gauge needle attached to a 1 ml disposal syringe with polythene tubing (0.40mm bore from Portex Limited, Hythe, Kent). The vein could be made more visible by warming the tail in warm water or

*The designation 12% (w/w) and 20% (w/w) express the weight of protein as a percent of metabolizable constituents of the diet.

Table I.1

Composition of Oxoid breeding diet (Oxoid Ltd., Basingstoke, UK)

Crude protein (maize, wheat, soya, fish) % by weight	23
Oil, % by weight	4
Fibre, % by weight	3.5

Vitamins

A IU/kg	50,000
D_3 IU/kg	3,000
E mg/kg	60
B_1 (thiamin) mg/kg	6
B_2 (riboflavin) mg/kg	10
B_{12} (cyanocobalamine) mg/kg	0.035
niacin mg/kg	10
choline chloride mg/kg	1,500
folic acid mg/kg	1
biotin mg/kg	0.250
C (ascorbic acid) mg/kg	400
K (menadione) mg/kg	20

Minerals

Ca g/kg	8.1
P g/kg	6.5
NaCl g/kg	2.3
Mg g/kg	1.8
Fe g/kg	0.1
Mn mg/kg	102.6
Cu mg/kg	25.1
Zn mg/kg	95.5
I mg/kg	1.5
Co mg/kg	3.0

Table I.2

Composition of powdered diet

	g per 100g of metabolizable constituents	
	12% (w/w)	20% (w/w)
Casein	12	20
Corn oil	5.5	5.5
Maize starch + glucose	82	75
L-Methionine	0.10	0.10

Solka Flock (BP Nutrition) was added at 10g per 100g of metabolizable constituents,

Vitamins (BP Nutrition, to specification)

A IU/kg	200,000
D_3 IU/kg	2,200
E mg/kg	110
B_1 (thiamine) mg/kg	22
B_2 (riboflavin) mg/kg	20
B_6 (pyridoxine hydrochloride) mg/kg	100
B_{12} (cyanocobalamin) mg/kg	30
niacin mg/kg	100
choline chloride mg/kg	1,650
folic acid mg/kg	2
para-aminobenzoic acid mg/kg	110
biotin mg/kg	0.44
C (ascorbic acid) mg/kg	990
calcium pantothenate mg/kg	70
K (menadione) mg/kg	50
inositol mg/kg	110

Minerals (ICN Nutritional Biochemicals, Cleveland, Ohio, USA after Bernhart and Tomarelli, 1966)

Calcium carbonate g/kg	0.84
Calcium phosphate g/kg	29.4
Citric acid mg/kg	91
Cupric citrate · $2\frac{1}{2}$ H_2O mg/kg	18
Dipotassium phosphate g/kg	3.2
Ferric citrate · 5 H_2O g/kg	0.223
Magnesium oxide g/kg	1.0
Manganese citrate g/kg	0.334
Potassium iodide mg/kg	0.4
Potassium sulphate g/kg	2.72
Sodium chloride g/kg	1.2
Disodium phosphate g/kg	0.856
Zinc citrate · 2 H_2O mg/kg	53

rubbing the tail with xylene.

Injection solutions: Radiolabelled amino acids were purchased from the Radiochemical Centre (Amersham, Bucks.) at the following specific radioactivities: L-(3,4-^{3}H)valine, 2 Ci/mmole; L-(4-^{3}H)phenylalanine, 10 - 25 Ci/mmole; L-(1-^{14}C)leucine, 50 mCi/mmole; L-(U-^{14}C)lysine, 10 mCi/mmole and L-(U-^{14}C)glycine, 10 mCi/mmole. Unlabelled amino acids were purchased from Sigma London Limited (Poole, Dorset). The volumes which were injected were varied so that each animal received the stated amount per 100g body weight over 15 sec.

(^{3}H)valine	300µmoles and 100µCi L-(3,4-^{3}H)valine in 1 ml water
	150µmoles and 50µCi L-(3,4-^{3}H)valine, 0.5 ml
	60µmoles and 50µCi L-(3,4-^{3}H)valine in 1 ml 0.9% NaCl
	30µmoles and 25µCi L-(3,4-^{3}H)valine, 0.5 ml
(^{3}H)phenylalanine	150µmoles and 65µCi L-(4-^{3}H)phenylalanine in 1 ml water
	75µmoles and 32.5µCi L-(4-^{3}H)phenylalanine, 0.5 ml
	30µmoles and 13µCi L-(4-^{3}H)phenylalanine in 1ml 0.9% NaCl
	15µmoles and 6.5µCi L-(4-^{3}H)phenylalanine, 0.5 ml
(^{14}C)leucine	100µmoles and 10µCi L-(1-^{14}C)leucine in water
(^{14}C)lysine	negligible amount and 2µCi L-(U-^{14}C)lysine in 0.9% NaCl
(^{14}C)glycine	negligible amount and 2µCi L-(U-^{14}C)glycine in 0.9% NaCl

Tissue preparation: Animals were killed by decapitation and blood was collected in heparinised tubes. The liver and the proximal 25% of the small intestine were quickly removed and placed in beakers containing ice-cold water. The time taken between the death of the animal and the

cooling of the tissues was 30 - 40 sec for the liver and 60 - 80 sec for small intestine. After chilling the liver was blotted, weighed and transferred to liquid N_2. The small intestine was flushed with cold saline using a 16 gauge radiopaque teflon catheter attached to a 20ml syringe. Measurements of the length of intestine were made by stretching with a standard weight (3.5g crocodile clip). It was then transferred to a cold, glass plate, slit longitudinally and scraped with the edge of a microscope slide. The layer removed by scraping was designated mucosa, weighed and transferred to a 15ml polypropylene tube. Liquid N_2 was then poured into the tube to freeze the tissue. The layer which remained after scraping was designated serosa and was similarly weighed and frozen in a polypropylene tube.

A system of tissue preparation has been designated to incorporate assays for tissue composition along with the determination of the specific radioactivity of the labelled amino acid. Frozen tissue (~ 0.5g liver, entire mucosa and serosa) was defrosted on ice in the presence of 5ml of 2% (w/v) perchloric acid in a 15ml plastic tube. A glass rod was used to homogenise the tissue and the homogenate was then centrifuged. The supernatant, which contained the free amino acids, was neutralised with 1M KOH. The solution was kept on ice to precipitate as much $KClO_4$ as possible. In some cases this solution could be used directly for determination of specific radioactivity but in other cases the solution was concentrated by freeze-drying.

The pellet from perchloric acid precipitation was washed 3 times with cold 2% $HClO_4$ and then solubilised in 0.3N NaOH (9ml distilled water followed by 1 ml 3N NaOH) incubated at 37^{o}C for 1 hour. Aliquots were removed for the determination of protein, RNA and DNA and the remaining 6ml of solution was reprecipitated with 2.4ml of 20% (w/v) $HClO_4$. Following another wash in 2% $HClO_4$ the pellet was transferred

to a pyrex tube with a teflon-lined screw cap (Sovirel, Levailois-Perret, France) and hydrolysed overnight in 5ml of 6N HCl at 110°C. The protein hydrolysate was then used for the determination of amino acid specific radioactivity.

Protein determination: Protein was assayed with a modification of the method of Lowry et al. (1951) as described by Munro and Fleck (1969). The 0.3N NaOH solution containing solubilized protein was diluted with 0.1N NaOH so that 0.5ml would contain 100 - 200µg of protein (approximately 1 : 30). This solution and a further 1 : 1 dilution were assayed with bovine serum albumin (fraction V, Sigma London Limited, Poole, Dorset) in 0.1N NaOH which had been incubated at 37°C for 30 min as a standard. 0.5ml of sample in 0.1N NaOH was combined with 2.5ml of copper-carbonate reagent (1 ml 1% $CuSO_4.5H_2O$ + 1 ml 2% Na tartrate, mixed and added to 100ml of 2% Na_2CO_3 in 0.1N NaOH) and allowed to stand for 10 min. 0.5ml Folin-Ciocalteu reagent (1 : 1 dilution of 2N reagent purchased from Sigma London Limited) was added with mixing and allowed to stand for 30 minutes. Absorbance was measured with a Gilford spectrophotometer at 750nm against a 0.1N NaOH-reagent blank. The Folin-Ciocalteu reagent was added to successive tubes at an interval of 20 sec so that each sample could be read 30 min after the addition of the reagent.

RNA determination: RNA content was determined from UV absorbance at 260nm. A correction for the interference from peptide material which absorbed at 260nm was made from the absorbance of the solution at 232nm and the extinction coefficients for solutions of RNA and protein from rat liver at 260 and 232nm (Munro and Fleck, 1969). To 1ml of 0.3N NaOH solution containing solubilized tissue was added 0.4ml of 20% (w/v)

$HClO_4$. The solution was kept on ice for about 10 min and then centrifuged. The UV absorbance of the supernatant at 260 and 232nm was measured using a Gilford spectrophotometer with 2% (w/v) $HClO_4$ as a blank. The concentration of RNA in the solution was calculated from the formula: mg RNA/ml = 10.53 (3.4 Absorbance 260nm - 1.44 Absorbance 232nm). The values of the extinction coefficients are those obtained by Munro and Fleck (1969) in rat liver for RNA-phosphorous and 10.53 converts RNA-P to RNA. This method for determining RNA has been compared with a method based on the colorimetric reaction of ribose (orcinol method, Munro and Fleck, 1969) and a method for the determination of RNA from UV absorbance (32µg/ml = 1 absorbance unit at 260nm) corrected for peptide material (1µg/ml peptide = 0.001 absorbance units at 260nm) where the amount of peptide material was estimated directly with the modified method of Lowry et al. (1951) described above. The values of RNA in rat liver and small intestine obtained with the three methods differed by less than 5%.

DNA determination: DNA was determined by the colorimetric reaction of diphenylamine with deoxyribose as described by Giles and Myers (1965). 2ml of 0.3N NaOH solution was reprecipitated with 0.8ml 20% (w/v) $HClO_4$ and the pellet washed in 2% (w/v) $HClO_4$. DNA was hydrolysed by adding 1ml 10% (w/v) $HClO_4$ and incubating at 70°C for 30 min. (The strength of acid and time of incubation were experimentally determined as those necessary to produce optimum hydrolysis with minimum destruction.) Approximately 100µg of hydrolysed DNA in a volume of 2ml of 10% $HClO_4$ was assayed with a 4% solution of diphenylalanine in Analar glacial acetic acid. 0.1ml of 0.16% solution of acetaldehyde was added to each tube and the samples were incubated overnight at 30°C. The absorbance at 595nm was compared with standard calf thymus DNA (Sigma London

Limited) which had been solubilised in NaOH, precipitated with perchloric acid and hydrolysed at 70°C.

Specific radioactivity: Amino acid specific radioactivity was determined with a Locarte amino acid analyser (London). The effluent from the column was divided with a Gilson peristaltic pump so that a known proportion (usually about 20%) went through the ninhydrin reaction of the analyser for quantitation of the amount of amino acid and the remainder was collected in a fraction collector (approximately 6 min fractions, 2.5ml volume) for determination of the amount of radioactivity. The programme which was developed was suitable for determining the specific radioactivity of any of the amino acids used, i.e. valine, leucine or phenylalanine. The column was 23 x 0.9cm containing a cation exchange resin (6 - 8μbeads, 8% cross-linked). Samples were loaded in a pH 2.2 buffer and then eluted with pH 3.60, 0.2M Na^+ buffer at 60°C for 4h. (The composition of the buffers and other reagents are given in Appendix 1.) Resolution was improved by beginning the programme at 25°C and changing to 60°C after 5 min. Quantitation of the amount of amino acid was done from the area of each peak using norleucine which was added with each sample as a standard.

Radioactivity was measured with a Nuclear Chicago Delta 3000 liquid scintillation counter using a Triton X-100/xylene based scintillator (Fricke, 1975). Quenching of samples was determined from an external standard and corrected with a quench curve. Counting was continued until 10^4 counts had accumulated (± 1%).

Statistics: Standard deviation, standard error of the mean and Student's t test for the significance of the difference between two means were done as described by Moroney (1951).

2. Results and discussion

a. Incorporation at infinite dose

Of the two methods which employed large amounts of labelled amino acid to measure protein synthesis, the method described by Scornik (1974) had one advantage over the method described by Henshaw et al. (1971) and that was that the problem of precursor specific radioactivity could be avoided entirely. Incorporation was measured with different amounts of labelled amino acid of the same specific radioactivity and protein synthesis was calculated from the extrapolated incorporation at infinite dose (derived from a plot of incorporation^{-1} vs amount injected^{-1}). At infinite dose the specific radioactivity of the precursor amino acid will be equal to the specific radioactivity of the injected amino acid. All that needs to be measured is the incorporation into protein at different levels of amino acid and the specific radioactivity of the amino acid which is injected.

In addition to control animals (12% (w/w) diet), protein-deprived (10 days on protein-free diet) animals were included in this study. Because levels of branched-chain amino acids had been reported to be lower than in control animals (Morgan and Peters, 1971) inclusion of this group would validate the method for conditions where the levels of endogenous amino acids differed. Groups of animals were injected with four amounts of (^{3}H)valine: 300, 150, 60 and 30 µmoles per 100g body weight. Two different injection solutions were used to ensure a reasonable amount of labelling in all groups. 10 min after injection, the animals were killed and tissues were removed as described in the Methods section. Subsequently, incorporation was measured and corrected for the difference in valine specific radioactivity between

the injection solutions. Table I.3 shows the incorporation of (^{3}H)-valine into protein for the jejunal mucosa. The reciprocal of incorporation was plotted against the reciprocal of the amount injected in Figure I.3. The results for control animals did produce a straight line so the intercept for infinite dose could be obtained and used to calculate the fractional rate of protein synthesis from the equation $k_s = S_B/S_A t$ (see p. 51). The specific radioactivity of the precursor amino acids (S_A) at infinite dose is the same as the specific radioactivity of the injection solution which was determined to be 7.906×10^5 dpm/μmole valine. The specific radioactivity of the amino acid in protein (S_A) was assessed from the extrapolated incorporation into protein (dpm/μg protein) and the valine content of protein which was estimated to be 5.3%*. In control animals the intercept at infinite dose was 0.416 corresponding to 2.404 dpm/μg protein. Therefore, the fractional synthesis rate was calculated to be 97 % per day.

In the protein-free group the plot of incorporation^{-1} vs amount injected^{-1} was not a straight line so no intercept could be determined. The shape of the curve indicates higher incorporation for the lower doses of injected amino acid relative to the incorporation of higher non-tracer amounts of amino acid. Such a situation might arise if the amino acid were incorporated directly from the plasma at lower doses of injected amino acid rather than mixing with the intracellular pool. The difference between control and protein-deprived animals might be explained by assuming that incorporation from plasma occurs only at

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* The valine content of protein in the jejunal mucosa was determined by dividing the incorporation per mg of protein by the actual valine specific radioactivity measured on an amino acid analyser in 8 samples of hydrolysed protein.

Table I.3

The incorporation of (^{3}H) valine into protein of the jejunal mucosa of control and protein-deprived rats

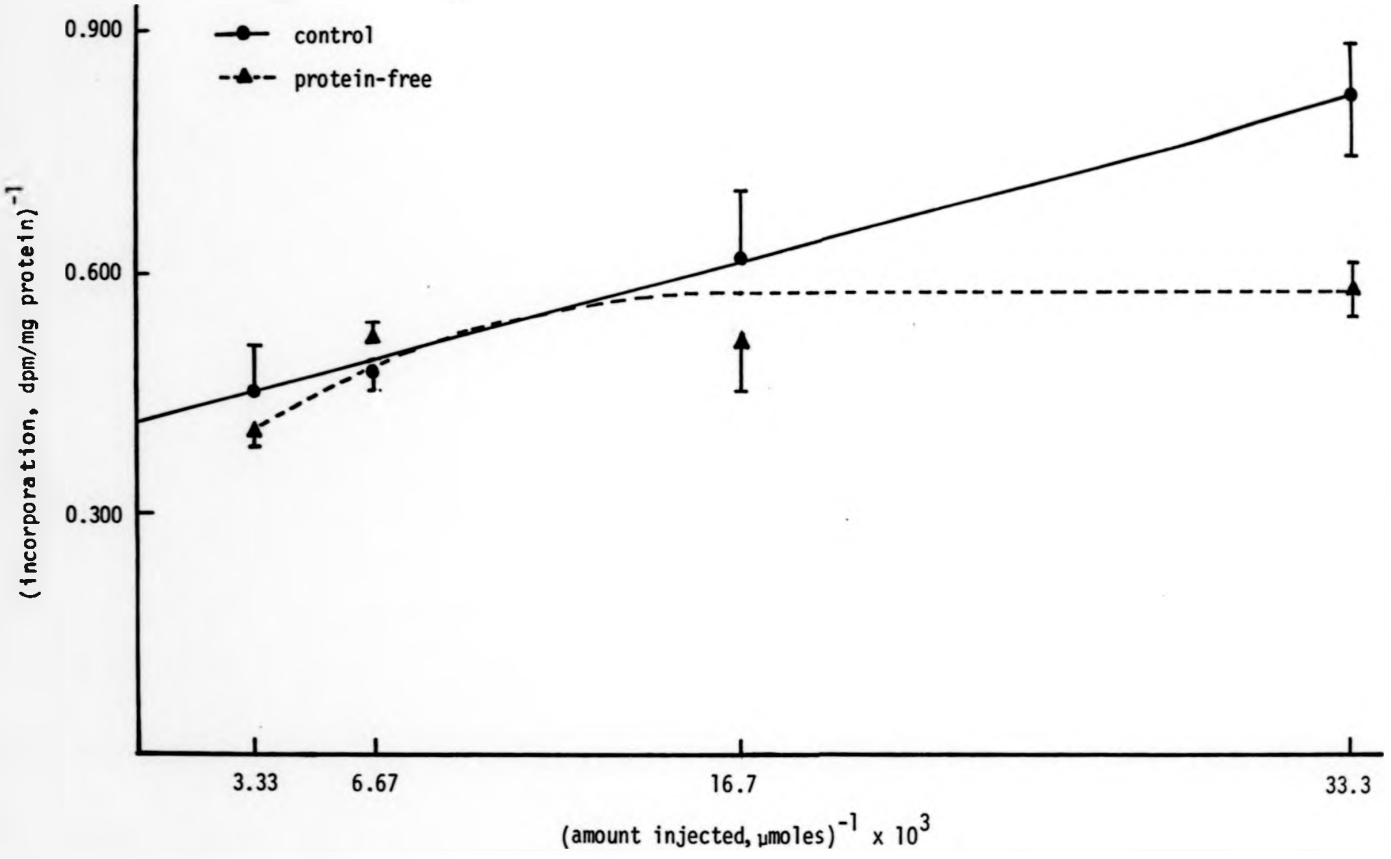
Control rats

amt. injected (µmol/100g bw)	amt. injected^{-1} (µmol^{-1} x 10^3)	incorporation (dpm/µg pro ± SEM)	incorporation^{-1} (µg pro/dpm ± SEM)
300	3.33	2.262 ± 0.22	0.458 ± 0.052
150	6.67	2.086 ± 0.10	0.482 ± 0.024
60	16.7	1.655 ± 0.22	0.623 ± 0.079
30	33.3	1.237 ± 0.11	0.819 ± 0.065

Protein-deprived rats

amt. injected (µmol/100g bw)	amt. injected^{-1} (µmol^{-1} x 10^3)	incorporation (dpm/µg pro ± SEM)	incorporation^{-1} (µg pro/dpm ± SEM)
300	3.33	2.467 ± 0.114	0.408 ± 0.018
150	6.67	1.930 ± 0.071	0.520 ± 0.020
60	16.7	1.973 ± 0.241	0.524 ± 0.072
30	33.3	1.734 ± 0.102	0.581 ± 0.036

Figure I.3 (Amount injected)$^{-1}$ vs (incorporation)$^{-1}$ of (^{3}H)valine into jejunal mucosa of control and protein-deprived rats



very low levels of amino acids and the higher endogenous levels in the control group were sufficient to prevent incorporation of this type. Although control animals yield a linear reciprocal plot and Scornik found similar kinetics for leucine incorporation into mouse liver in both control animals and in animals recovering from partial hepatectomy (Scornik, 1974), this does not seem to be the case for all amino acids and all tissues.

To exclude the possibility that the problem was due to peculiar compartmentation of the valine pool, the method was used with a different amino acid. Phenylalanine was chosen for this study because it was soluble enough to allow for large amounts of amino acids to be given in acceptable volumes and because the free phenylalanine pool is relatively small (Munro, 1970). (^{3}H)Phenylalanine was injected at 150, 75, 30 and 15µmoles per 100g body weight into control rats (oxoid diet). L-(4-^{3}H)Phenylalanine was used with the intention that when phenylalanine was converted to tyrosine the label would be lost and, consequently, although tyrosine was formed from the labelled phenylalanine, it would be unlabelled.

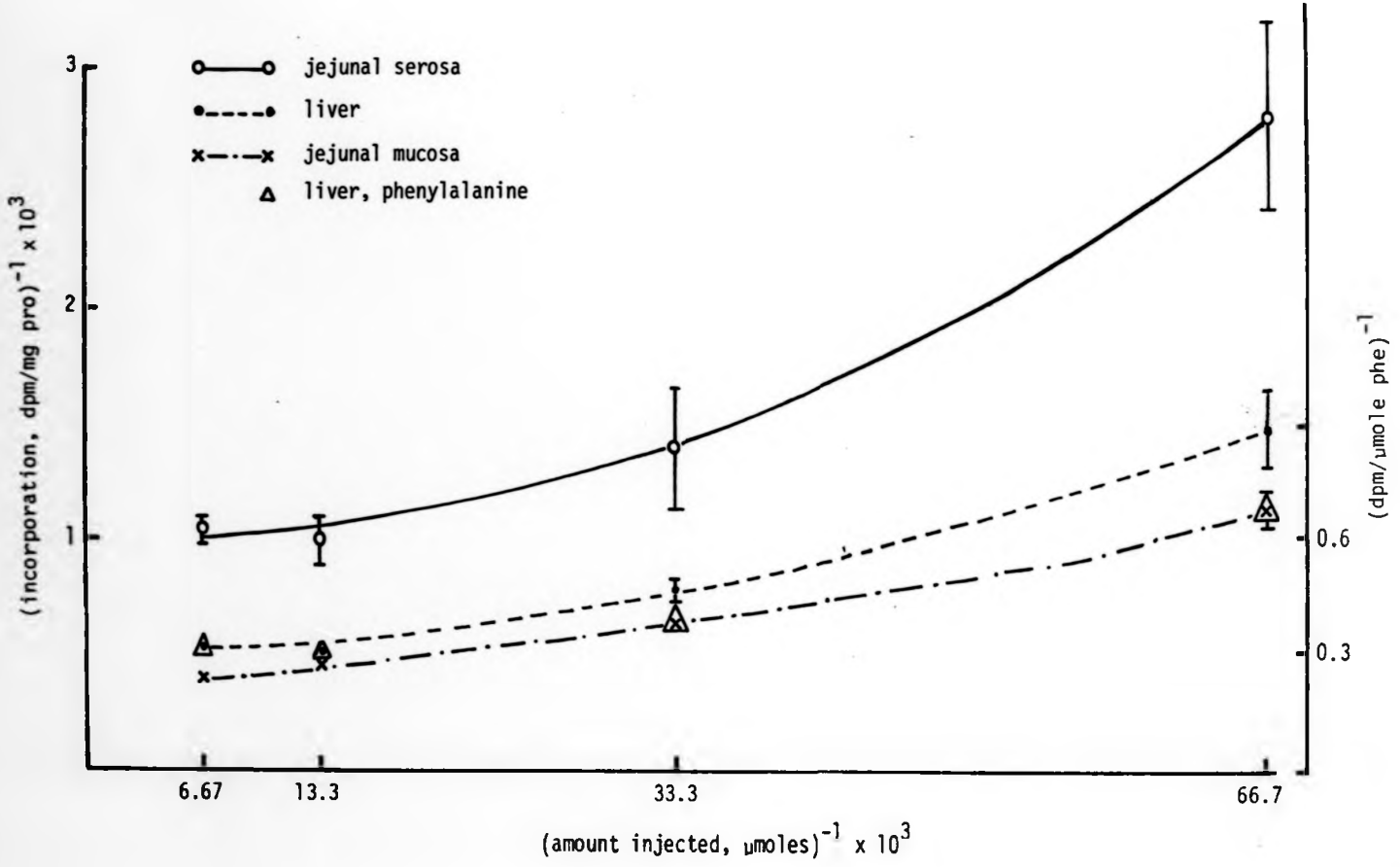
The incorporation of label into protein for three tissues, jejunal mucosa, jejunal serosa and liver is shown in Table I.4. Figure I.4 is a double reciprocal plot of incorporation^{-1} vs amount injected^{-1} of this data. In none of the three tissues did this type of plot produce a straight line. As with valine, the assumption had been made that all of the label in protein was due to incorporation of the injected amino acid. But because of the non-linearity of incorporation and the known conversion of phenylalanine to tyrosine, this assumption was checked by measuring the specific radioactivity of both free tyrosine and free phenylalanine in the tissues of rats receiving 150µmoles of (^{3}H)phenylalanine. In the liver the specific radioactivity of free tyrosine was

Table I.4

Incorporation of (^{3}H)phenylalanine into the protein of jejunal mucosa, jejunal serosa and liver in control rats

amt. injected (μmol)	incorporation (dpm/mg protein ± SEM) jejunal mucosa	jejunal serosa	liver	liver (dpm/μmol phe ± SEM)
150	2364 ± 111	961 ± 44	1824 ± 109	3165 ± 271
75	2155 ± 112	1016 ± 102	1902 ± 137	3210 ± 195
30	1509 ± 90	720 ± 14	1274 ± 43	2521 ± 144
15	908 ± 65	368 ± 29	718 ± 75	1529 ± 165

Figure I.4 (Amount injected)$^{-1}$ vs (incorporation)$^{-1}$ of (^{3}H)phenylalanine into jejunal mucosa, serosa and liver of control rats



about 90% of the specific radioactivity of free phenylalanine at 10 minutes while in the jejunal mucosa and serosa, free tyrosine was about 50% of the specific radioactivity of free phenylalanine. If both labelled phenylalanine and labelled tyrosine were incorporated at the higher levels of injected phenylalanine, it might explain the enhanced incorporation at the largest amount of injected amino acid. To eliminate any incorporation due to tyrosine, the specific radioactivity of phenylalanine in protein was determined and was used for the graph of incorporation^{-1} vs amount injected^{-1} (Δ symbols, Figure I.4). Even when the phenylalaine specific radioactivities were used, a straight line was still not obtained.

Neither the injection of labelled valine nor labelled phenylalanine followed the kinetics of simple isotope-dilution. This is perhaps not surprising in light of the many experiments which have been done to define the precursor pool for protein synthesis (see pp. 30-43). The difference in specific radioactivity of the amino acids of the plasma and those within the tissue is, itself, evidence of compartmentation and could well explain why for some amino acids and some tissues the free amino acid pool does not behave as though it were a single, homogeneous pool. Because of the lack of linearity obtained with valine and phenylalanine, the method of extrapolating incorporation to infinite dose was abandoned and the alternative method of injecting a large amount of amino acid and measuring its specific radioactivity in the acid-soluble pool and in protein (Henshaw et al., 1971) was investigated.

b. Incorporation of a massive amount of leucine

Henshaw et al. (1971) injected rats intraperitoneally with 75μmoles (^{14}C)lysine per 100g body weight and then followed the changes in the

specific radioactivity of free lysine in plasma, liver and muscle for 20 min. They concluded that the specific radioactivity at 20 min could be used as the mean value for the interval 0-20 min because the drop in specific radioactivity was small and would compensate for the small error due to the rise in specific radioactivity immediately after injection. In attempting to use this approach to measure the rate of protein synthesis in rat liver and intestine a number of modifications were made.

Firstly, leucine was used rather than lysine. Lysine has a larger free pool (Munro, 1970), and so theoretically a larger amount would need to be injected to achieve the same degree of flooding. It was hoped that 100μmoles of leucine per 100g body weight, which was the maximum amount which could be injected in a volume of 1 ml, would be even more effective in raising the specific radioactivity of the free amino acid to that of the injection solution and maintaining it at a constant level over the time of measurement. (^{14}C)Leucine with only carbon-1 labelled was used so that label which was removed from the amino acid would be given off as $^{14}CO_2$ and not transferred to other compounds. Assessment of the specific radioactivity of the free and protein-bound amino acid was to be made using an amino acid analyser and leucine had an additional advantage in that the programme for separating leucine was relatively short and quite insensitive to interference by other amino acids.

In addition to changing the amino acid which was injected, the route of administration was also changed. Although intraperitoneal injections are quickly absorbed into the circulation, variation in the rate of absorption among different animals would introduce variation in the measured synthesis rate. An intravenous injection would avoid this potential for variation in the specific radioactivity of the amino acid in the circulation around the gut.

The time dependent changes in specific radioactivity of free leucine in the plasma, liver and jejunal mucosa following the intravenous injection of 100μmoles of L(1-^{14}C)leucine per 100g body weight in control rats (12% (w/w) diet) are shown in Figure I.5. Unlike the nearly constant specific radioactivity of lysine reported by Henshaw et al. (1971), the specific radioactivity of leucine fell quite rapidly in plasma and even more dramatically in the liver and the jejunal mucosa. Twenty minutes after the injection of isotope the specific radioactivity of free leucine in the liver was only about 50% of the value at two minutes. The fall in specific radioactivity of the free amino acid is dependent on the rate of entry of label from the plasma and the turnover rate of the free amino acid pool. The turnover of the free amino acid pool depends on the rate at which unlabelled amino acid from protein degradation enters the pool and the size of the pool which is being diluted by unlabelled amino acid. The more rapid decline in specific radioactivity of leucine compared with lysine could be due to differences in either the rate of entry of label or the turnover of the free amino acid pool. The fact that lysine was given intraperitoneally might mean that label was more slowly absorbed and therefore continued to enter the plasma pool over a longer time. The rate of protein degradation will be the same for both amino acids but the dilution effect that this unlabelled amino acid has on specific radioactivity will be greater for a smaller free pool.

Even though the specific radioactivity of free leucine was changing the change was linear and so the mean specific radioactivity over a given time interval was easily determined. From the specific radioactivity of leucine in protein and free leucine in the tissue homogenate the fractional rate of protein synthesis, k_s, was calculated using equation I.9, $k_s = S_B/S_A t$ (see p. 51). Table I.5 illustrates the range

of values for k_s which result from using various time intervals for calculation. $S_{A(o)}$ is the specific radioactivity of free leucine at time zero and this value was determined by extrapolation from Figure I.5. $\bar{S}_A t$ is the arithmetic mean of the specific radioactivity at the end of the time interval and the value of $S_{A(o)}$ multiplied by the length of the interval in minutes. k_s has been calculated for each value of the specific radioactivity of leucine in protein using the group average $\bar{S}_A t$ and the range of values is indicated by the standard deviation.

Calculating k_s in this way ignores the error in the determination of the intercept $S_{A(o)}$. However, in practice the error of $S_{A(o)}$ will be negligible. Extrapolation assumes a linear fall in specific radioactivity at the same rate as the measured time points. Since the rise in protein specific radioactivity was linear (Figure I.6) it is unlikely that the change in S_A between 0 and 2 minutes was significantly different from the extrapolated value. In addition the specific radioactivity of leucine at 2 min was 80 - 90% of the specific radioactivity in the injection solution and $S_{A(o)}$ must be between the 2 min value and the injection solution. The calculated SD also under-estimated the actual variation because the free leucine specific radioactivity which was used in the calculation was the average value for the group. If $\bar{S}_A$ were calculated from each ten minute value and $S_{A(o)}$, and this value used in conjunction with the protein value (S_B) for each animal, the range of values would be a better estimate of the actual variation.

Alternatively, k_s could be calculated from the two groups of animals by measuring the change in specific radioactivity in the protein and the precursor between two time points. This calculation for the interval 2 - 10 min gave almost the same result as the calculation using the extrapolated value for S_A (see Table I.5), but assessment of error

Figure I.5 Changes in specific radioactivity of free leucine (dpm/nmole ± SD, n=4) in plasma, liver and jejunal mucosa following the injection of 100μmoles (1-^{14}C)leucine per 100g body weight

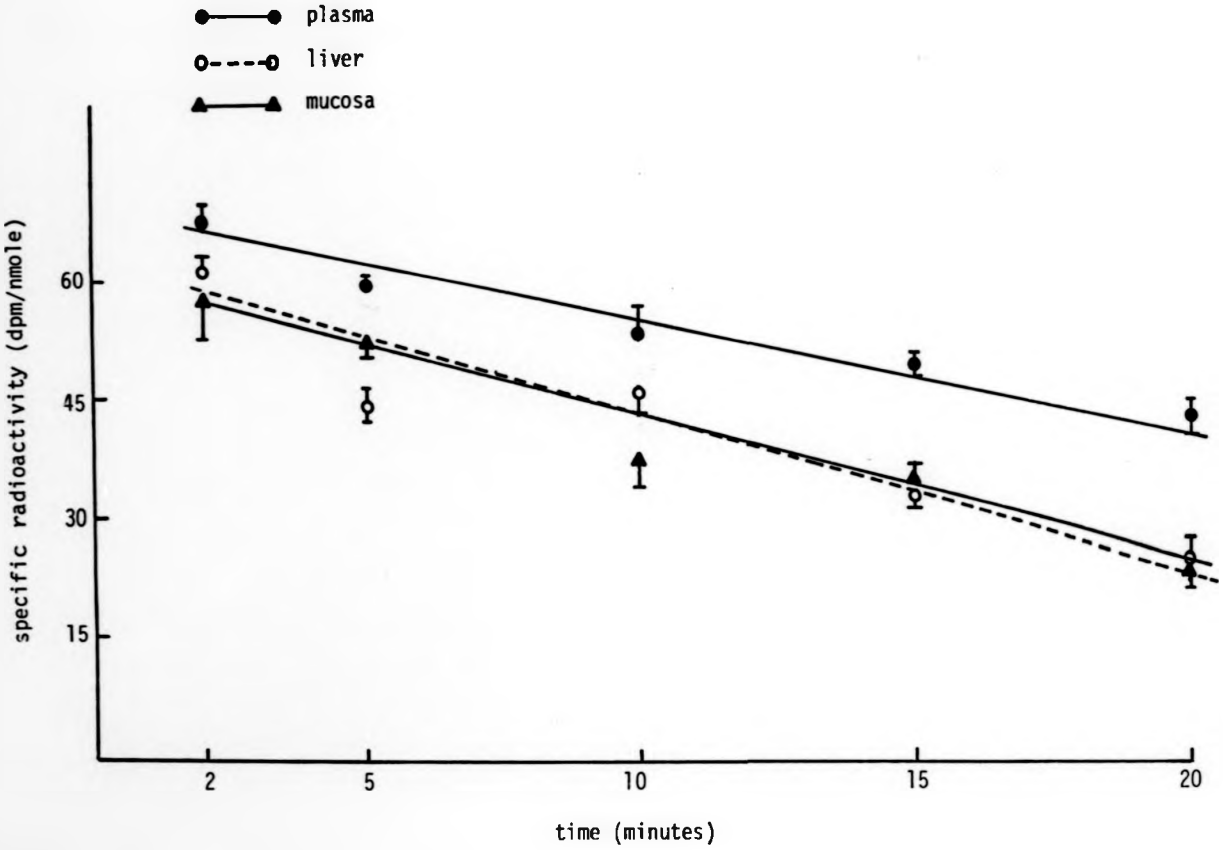


Table I.5

The rate of protein synthesis in liver and jejunal mucosa from the incorporation of 100μmoles of L-(1-^{14}C)leucine per 100g body weight

LIVER

time interval (min)	$\bar{S}_A t$ tissue	$\bar{S}_A t$ plasma	k_s (%/d ± SD) tissue	k_s (%/d ± SD) plasma
0 - 5	290	330	90.4 ± 6.2	79.4 ± 5.5
0 - 10	528	624	78.8 ± 6.5	66.7 ± 5.5
0 - 15	720	883	72.9 ± 5.7	59.5 ± 4.6
0 - 20	866	1106	70.7 ± 6.2	55.4 ± 4.8
2 - 10	407	487	67.8	56.8

JEJUNAL MUCOSA

time interval (min)	$\bar{S}_A t$ tissue	$\bar{S}_A t$ plasma	k_s (%/d ± SD) tissue	k_s (%/d ± SD) plasma
0 - 5	283	330	95.7 ± 5.1	82.0 ± 4.4
0 - 10	519	624	98.7 ± 6.3	82.1 ± 5.2
0 - 15	712	883	112.4 ± 3.8	90.7 ± 3.1
0 - 20	859	1106	110.8 ± 0.5	86.1 ± 0.4
2 - 10	402	487	96.0	79.2

is much more difficult since k_s is determined from the means of two variables (S_B and S_A) at two time points (2 and 10 min). Determining the specific radioactivity of leucine in protein after two minutes of incorporation is also difficult to do accurately without adding a very large amount of isotope.

The values of k_s derived using time intervals from 5 - 20 min were not sufficiently different to suggest that any one interval was better than the others. Two opposing factors must be considered. The longer the interval of measurement the easier it is to assess the specific radioactivity of leucine in protein accurately but care must be taken not to have an interval which is sufficiently long that newly synthesised plasma proteins will be secreted from the liver. Consequently an interval of 10 min has been chosen for subsequent experiments.

Therefore, the procedure for estimating the rate of protein synthesis which has been adopted was to kill groups of animals at two time points (at 2 and 10 min) and to use the free leucine specific radioactivity in all animals to estimate $S_{A(o)}$ for the entire group ($S_{A(o)}$ was determined independently for each control group and for each group of experimental animals). The synthesis rate was calculated for each ten-minute animal using $S_{A(o)}$ from the group and the specific radioactivity of free and protein-bound leucine from the individual animal. The range of variation for a group of animals (SD or SEM) was indicated by the range of values of k_s.

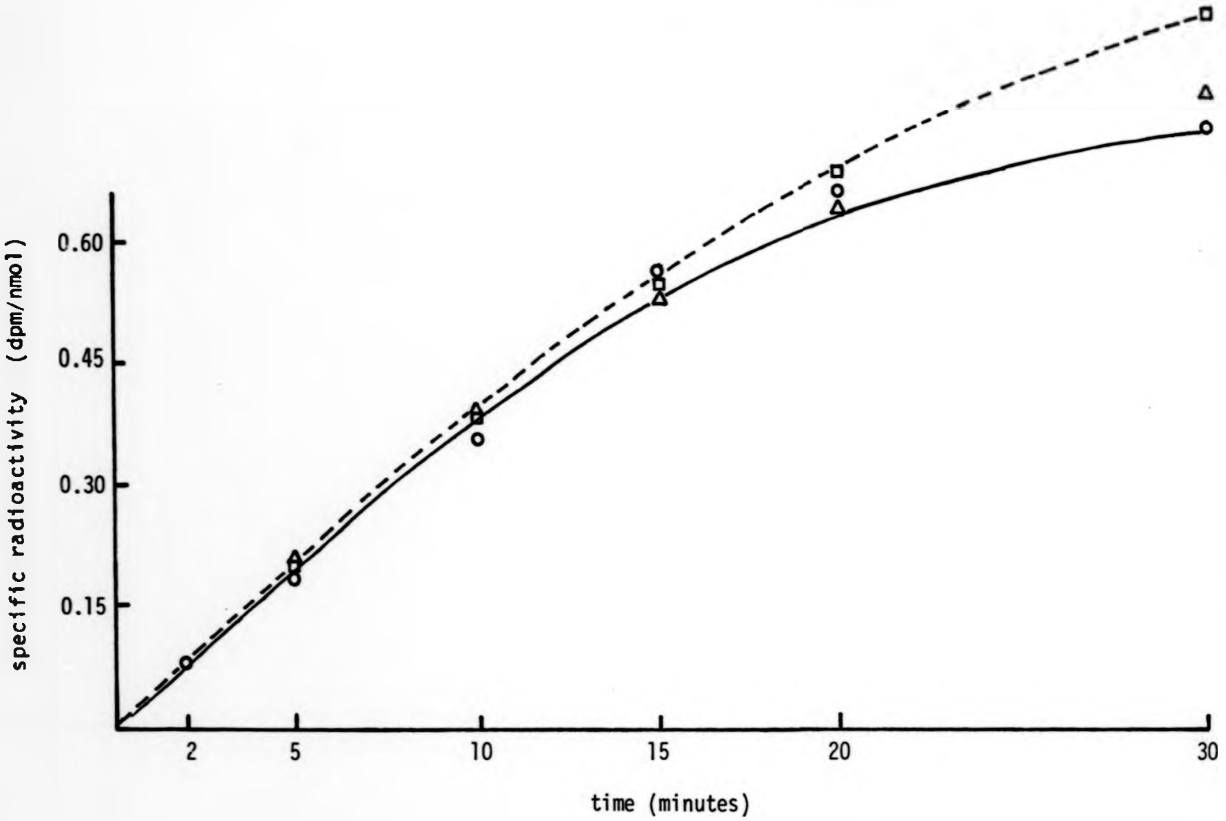
The injection of 100μmoles of (^{14}C)leucine has brought the specific radioactivity of the free amino acid of liver and jejunal mucosa much nearer to each other than when tracer amounts of amino acid were used and consequently the rates of synthesis calculated from either precursor were much nearer. In both tissues the synthesis rate calculated using

plasma leucine over the interval 0 - 10 min was only 15% lower than the synthesis rate calculated from tissue free leucine (Table I.5). This was quite an improvement over the fourfold difference in gut synthesis rates which was observed using the method of constant infusion (see p. 48). The rate of synthesis in liver calculated from the specific radioactivity of tissue leucine (79%/d) compares favorably with other reported values for the rate in rat liver (Henshaw et al., 1971, 78%/d; Peters and Peters, 1972, 92 - 116%/d). Although reliable estimates of the rate of protein synthesis in intestinal mucosa are not available for comparison, a rate of 100 - 110% per day does not seem unreasonable. As described earlier (p. 23), protein synthesis in the intestine can be divided into components for the production of new cells, synthesis (and degradation) of intracellular protein, and the synthesis of secreted protein. Production of new cells requires a fractional rate of protein synthesis of about 60%/d (assuming a cell life span of 41h as reported by Gleeson et al., 1972). Intracellular turnover is implied from the studies of Alpers (1972) showing incorporation of labelled amino acids into cells all along the villus. Alpers and Kinzie (1973) argue that since the cells do not become any larger as they move from crypt to villus, protein must be degraded intracellularly all along the villus. The heterogeneity of turnover rates between homogenate ($t_{\frac{1}{2}}$ = 31h) and brush-border proteins ($t_{\frac{1}{2}}$ = 18h) found by James et al. (1971) also supports the concept of intracellular turnover. However, the demonstration of the loss of labelled protein does not preclude the possibility that label is lost via the secretion of protein rather than through intracellular degradation. As Garlick (1980) has pointed out, differentiating between secretion and intracellular degradation is quite difficult.

As illustrated by Figure I.5 the specific radioactivity of leucine in the tissue fell much more rapidly than the specific radioactivity of leucine in the plasma. This difference between the two possible precursors can be used to assess which precursor would generate the pattern of incorporation of leucine into protein which was actually observed. The specific radioactivity of leucine in the protein of the jejunal mucosa is shown in Figure I.6 along with theoretical curves for S_B calculated using the plasma or the tissue amino acid as the precursor. A synthesis rate of 107%/d was assumed for calculation of S_B using tissue leucine as the precursor and a rate of 89%/d was assumed for calculation of S_B using plasma leucine as the precursor. These rates, which were considered to be reasonable estimates of the rate of synthesis over the time of incorporation, were based on values of S_B and S_A at 10 min derived from the graphs in Figures I.5 and I.6. Over the first twenty minutes the three curves for S_B (Figure I.6) are indistinguishable, but after thirty minutes the specific radioactivity of leucine in the plasma would predict a higher level of incorporation than was actually observed. Hence, although the difference in specific radioactivity between plasma and the tissue was not very large following the injection of a large amount of amino acid, a choice of precursor did need to be made and it was felt that the specific radioactivity of leucine in the tissue was the better estimate of the specific radioactivity of the precursor.

Using a large amount of labelled amino acid to measure protein synthesis minimised problems of precursor specific radioactivity but because this amount of amino acid is very unphysiological it was necessary to show that the rate of protein synthesis was not altered by this technique. Proving that leucine did not affect protein synthesis was rather difficult. Since there were no other reliable

Figure I.6 Comparison of observed specific radioactivity of leucine in protein of jejunal mucosa (○) with calculated specific radioactivity of leucine in protein with plasma (□) or tissue (△) free amino acid as precursor



methods available, the simplest approach involving the incorporation of tracer amounts of other labelled amino acids in the presence and absence of 100μmoles of leucine was attempted. The results of three experiments of this type are shown in Table I.6. In experiment 1 (^{14}C)lysine was injected with or without 100μmoles per 100g body weight of unlabelled leucine and incorporation into protein was measured for ten minutes after injection. The difference in incorporation between lysine alone and lysine plus leucine was not statistically different (using Student's t test) for either the liver or the jejunal mucosa, but in both cases the incorporation of label was depressed in the presence of leucine. This might have been due to an inhibition of the rate of protein synthesis but it was also possible that protein synthesis was unaltered but that the transport of (^{14}C)lysine into the cell was inhibited in the presence of leucine. Christensen (1969), in describing systems of transport for amino acids, discussed one way in which leucine might inhibit the uptake of lysine. Leucine could be transported by the Ly^+ system, which is normally thought to transport amino acids with a positive charge, if it were transported with a Na^+ ion. The amount of leucine present in these animals might be sufficiently large to reduce the transport of (^{14}C)lysine.

In an attempt to reduce competitive transport, experiment 2 was designed so that the 100μmoles of leucine were given two minutes before the (^{14}C)lysine and incorporation into protein was measured for 8 minutes after the label was given. The results for incorporation of (^{14}C)lysine with and without leucine are also shown in Table I.6. The difference between incorporation with leucine and incorporation without leucine again was not statistically significant; however, unlike experiment 1, the incorporation was higher in those animals which received leucine prior to the injection of labelled lysine. It seemed

Table I.6

Incorporation (dpm/mg protein ± SEM, n=5) of tracer amino acids in the presence and absence of 100μmoles of unlabelled leucine

		LIVER		JEJUNAL MUCOSA		MUSCLE	
		tracer alone	tracer + leucine	tracer alone	tracer + leucine	tracer alone	tracer + leucine
Exp. 1.	Leucine + (^{14}C)lysine	148 ± 12	123 ± 19	448 ± 27	401 ± 21	-	-
Exp. 2.	Leucine, then (^{14}C)lysine	105 ± 9	130 ± 8	141 ± 8	182 ± 26	-	-
Exp. 3.	Leucine + (^{14}C)glycine	456 ± 27	403 ± 40	553 ± 44	524 ± 53	47.6 ± 3.1	44.3 ± 3.1

unlikely that this difference in incorporation could be due to an alteration in the rate of protein synthesis especially as incorporation was decreased in experiment 1. Again, the more likely explanation would seem to be that leucine was affecting lysine transport. Christensen (1969) has demonstrated that lysine can be transported via the L system which exhibits counter-transport whereby an amino acid within a cell is exchanged with one outside. In experiment 2 where the cells had been preloaded with leucine counter-transport of unlabelled leucine for labelled lysine might explain the enhanced uptake of (^{14}C)-lysine. In the third experiment (^{14}C)glycine was given with or without unlabelled leucine because glycine uptake is primarily by the A system which does not transport leucine and does not exhibit counter-transport. For all three tissues the incorporation with and without leucine was much closer than either experiment 1 or 2. These three experiments seem to illustrate the problems of inferring changes in rates of protein synthesis from changes in incorporation of tracer amounts of labelled amino acid. Taken all together, however, they do support the conclusion that although leucine in large amounts can affect transport of other amino acids, it does not affect the rate of protein synthesis. Additional experiments which support this conclusion are discussed in section IV.

3. Conclusion

In concluding this section on the development of a method for measuring protein synthesis in liver and intestine, it might be valuable to examine how this method involving the injection of a large amount of labelled leucine meets the criteria set out earlier as necessary for a reliable estimate of protein synthesis. The problem of precursor

specific radioactivity, though not completely eliminated, has been reduced considerably. Because the specific radioactivity of leucine in the two potential precursor pools has become more nearly the same, the potential for error introduced by choosing the wrong precursor has been considerably lessened. This seems particularly important in light of the published studies on the location of the precursor pool. These seem to suggest that there is not a single precursor pool, but that different amino acids and different tissues have different precursor pools. Also, the location of the precursor pool might not be fixed, even for a single amino acid, but might vary with experimental conditions.

In addition to minimising problems of defining precursor specific radioactivity, the large amount of leucine with ten minutes of incorporation eliminates problems which arise from the degradation of labelled protein. Even in tissues like intestine with very high rates of turnover, the specific radioactivity of leucine in protein at ten minutes was only about 1% of the specific radioactivity of free leucine. This means there will not be any significant loss of label once it has been incorporated, so the specific radioactivity of leucine in protein will accurately reflect the rate of synthesis. So it seems that this method does minimise both problems of precursor and problems of recycling of label.

Finally where a comparison of results obtained with other methods was possible, as with the rate of protein synthesis in liver, then the estimates of protein synthesis which were obtained agreed well with reported values. Therefore, this method has been employed to quantitate the contribution of the rate of protein synthesis of the liver and the gastrointestinal tract to the rate of whole-body protein synthesis. The method has also been employed to study changes in the rates of

protein synthesis in response to altered nutritional and hormonal states.

SECTION II. CONTRIBUTION OF LIVER AND GASTROINTESTINAL TRACT TO WHOLE-BODY PROTEIN SYNTHESIS

A. METHODS

B. RESULTS AND DISCUSSION

II. CONTRIBUTION OF LIVER AND GASTROINTESTINAL TRACT TO WHOLE-BODY PROTEIN SYNTHESIS

The rapid rates of protein synthesis which were obtained in liver and intestine suggested that these two tissues might constitute a significant component of protein synthesis in the whole animal. In order to assess the relative contributions made by different tissues to the overall rate of synthesis it is necessary to consider the absolute rate of synthesis, that is, the fractional rate of protein synthesis (the proportion of the protein pool synthesised each day) multiplied by the amount of protein contained within the tissue. Waterlow et al. (1978) have attempted to proportion the absolute rate of protein synthesis among the various organs of the rat. Liver and 'other viscera' appeared to account for 30% of the protein synthesised by the whole animal. Their estimate was based on the results of several studies. The fractional synthesis rate was assessed by constant infusion of (^{14}C)tyrosine over 6 hours and consequently underestimates the synthesis rate by an amount equal to the synthesis of plasma proteins. The rate of synthesis of 'other viscera' was assessed from the specific radioactivity of lysine in viscera relative to other tissues, three days after an injection of a tracer amount of (^{14}C)lysine. No attempt was made to assess the specific radioactivity of free lysine which was incorporated and no attempt was made to prevent the recycling of label. The rate of protein synthesis in the whole animal was assumed to be the same as the total turnover rate (flux) of free tyrosine in the body estimated from the specific radioactivity of tyrosine in the plasma. As the authors comment this will underestimate the rate of synthesis because the specific radioactivity of tyrosine in plasma was taken as

representative of the specific radioactivity at the site of synthesis. Although the specific radioactivity of the amino acid at the site of synthesis is not known for certain, it probably lies between the specific radioactivity of the amino acid in the plasma and in the tissues (Mortimore et al., 1972). On the other hand, tyrosine flux will overestimate the rate of synthesis because no correction was made for the proportion of tyrosine which leaves the plasma pool for oxidation and pathways other than synthesis. Assuming the two errors are of similar magnitude, the two should cancel each other. However, a more direct assessment of the rate of synthesis in the whole animal and the contribution of the rapidly turning over tissues to the overall rate of synthesis was possible using the incorporation of leucine into protein following an injection of a large amount of labelled leucine. The comparison of the rate of synthesis in individual tissues with the whole body could then be made where the rates of synthesis had been measured under identical conditions using the same technique.

II A. METHODS

Most of the methods used have already been described in section I (p. 55). Two groups of 10 control animals (20% (w/w) diet) were used. One group was used to measure rates of synthesis in individual tissues and one group was used to measure whole-body protein synthesis. All animals were injected with 100μmoles of (1-^{14}C)leucine per 100g body weight with 10μCi per animal where tissue synthesis rates were measured and 30μCi per animal where whole-body synthesis was measured. As before, the liver and gastrointestinal tract were quickly removed and cooled in ice-cold water. The carcass was immediately frozen by crushing between a heavy metal weight and a metal plate, both cooled in acetone/solid CO_2, and then transferred to liquid N_2. In those animals where whole-body synthesis was measured, the tissues were blotted, weighed and frozen in liquid N_2.

Tissues or whole carcasses were ground to a fine powder with a mortar and pestle containing liquid N_2. Portions (1 - 5g) of the powder were then precipitated in 2% (w/v) $HClO_4$. RNA, protein and the specific radioactivity of leucine in protein and in the acid-soluble supernatant were measured as described previously. The rate of protein synthesis was calculated from each 10 min animal using the specific radioactivity of free and protein-bound leucine and the value $S_{A(o)}$. The specific radioactivity of leucine at 0 min ($S_{A(o)}$) was extrapolated from the values of groups of animals killed 2 and 10 min after injection. The equation used, $k_s = S_B / S_A t$, has also been described in section I (p. 77).

II B. RESULTS AND DISCUSSION

The rates of protein synthesis obtained in the whole animal, liver, stomach, small and large intestine are shown in Table II.1. The fractional rate of synthesis (% per day) is the percent of the protein pool which was synthesised each day. In the whole body the fractional synthesis rate, 34%/d, was considerably lower than in any of the individual tissues which were measured. This, no doubt, reflects the contribution of tissues with much slower rates of synthesis to the rate for the whole animal. Waterlow et al. (1978) have reported that skeletal muscle contains 40% of whole-body protein and has a fractional rate of synthesis of about 14%/d in rats of this size.

The actual amount of protein which was synthesised by the whole animal per day can be obtained by multiplying the fractional rate of synthesis by the protein content of the rat. In these animals the amount of protein synthesised per day was 3.1g. The value agrees quite well with the value of 4g protein/d reported by Waterlow et al. (1978) from the constant infusion of (^{14}C)tyrosine despite the theoretical objections to the method using tyrosine. Two modifications of the method of constant infusion have been used to overcome the theoretical objections and to obtain more accurate values for the rate of protein synthesis in the whole animal. In one modification, used by Albertse et al. (1979), oxidation has been measured and used to correct flux. In another modification, Lo and Millward (1977) measured the rate of synthesis from the incorporation of tyrosine into whole-body protein and the specific radioactivity of acid-soluble tyrosine at the end of six hours. The results from both studies compare well with the value obtained using the single injection technique.

Table II.1

Protein content and the rate of protein synthesis for individual tissues and the whole body of the rat

Fractional rates of synthesis (%/d) were calculated from groups of 5 animals killed 2 and 10 minutes after injection of 100μmoles L-(1-^{14}C)leucine per 100g body weight. Total rates of protein synthesis (mg protein/day) were calculated by multiplying k_s by the corresponding protein content. Results are means ± SD.

	Protein content	Protein synthesis		
	(g)	(%/d)	(mg protein/d)	(% of whole body)
Whole body	9.30 ± 0.17	33.6 ± 2.9	3130 ± 410	100
Liver	0.70 ± 0.11	105.4 ± 2.5	743 ± 120	24
Stomach	0.05 ± 0.01	73.9 ± 1.2	34 ± 6	1
Small intestine	0.46 ± 0.05	103.4 ± 6.4	477 ± 58	15
Large intestine	0.12 ± 0.02	62.1 ± 10.3	78 ± 22	3

Albertse et al. (1979) infused L-(1-^{14}C)leucine and used the amount of $^{14}CO_2$ expired to correct the flux value for the leucine which was oxidised. Flux was calculated from the specific radioactivity of leucine in the plasma and the value obtained for synthesis was 1.46 µmoles of leucine/min in 100g rats. To convert their value into terms which can be compared with this experiment it was necessary to estimate the amount of leucine in rat protein. This determination was made by quantitating the amount of leucine present compared to the amount of protein in a 0.1N NaOH solution of powder from whole rat. The amount of leucine was measured on an amino acid analyser after hydrolysis of the protein in 6N HCl. Norleucine was added to correct for loss of leucine in hydrolysis. The mean of six determination was 6.91% ± 0.36 (SD). Using this value for the leucine content of rat protein, 1.46µmoles of leucine/min can be converted into 4g protein/d per 100g body weight.

Lo and Millward (1977) obtained a value for the fractional rate of protein synthesis of 27%/d in rats of similar size. The agreement with 34% per day is quite reasonable. One would expect that the rate of synthesis measured over 6 hours would be slower than a rate of synthesis measured over 10 min. In six hours very rapidly turning over proteins will have turned over several times so that the amount of label contained in these proteins will no longer be strictly proportional to the rate of synthesis.

The values for synthesis rates in individual tissues are interesting. The rate of synthesis in liver (105%/d) was higher than in the previous experiment using the single injection of leucine. The rate of synthesis for the small intestine (103%/d) includes both the mucosa and the more slowly turning over serosa (see section III). It is quite probable that the rate of synthesis in mucosa alone in these rats would also have been higher than that measured in the previous

experiment. This variation may be similar to that reported by other workers for different control groups (Henshaw et al., 1971 and Garlick et al., 1975) but it is also possible that the higher rates of synthesis in liver and intestine in this experiment reflect the higher protein content of the diet (20% (w/w) diet) used in this experiment compared to the previous one.

The rate of protein synthesis in the large intestine (62%/d) was considerably slower than the rate of synthesis in the small intestine. Leblond et al. (1957) and Altman (1974) have reported a gradient in the rate at which cells turnover along the length of the intestine and the slower rate of protein synthesis in the large intestine probably reflects, in part, this slower rate of the turnover of cells. The rate of protein synthesis in stomach (74%/d) was similar to that of the large intestine and the rate was much higher than the rates of synthesis reported for other muscular tissues such as heart, 17%/d, and gastrocnemius muscle, 14%/d (Garlick et al., 1975).

An assessment of the contribution of each tissue to the overall rate of protein synthesis in the whole animal is shown in the final column of Table II.1. Although the tissues which were measured in this experiment comprise only 14% of whole-body protein, the total amount of protein synthesised per day in liver, stomach, small and large intestine was equal to 43% of the total for the whole body.

SECTION III. RATES OF PROTEIN SYNTHESIS UNDER CONDITIONS OF PROTEIN LOSS

A. STARVATION

1. Introduction

   a. Changes in protein mass

   b. Changes in protein synthesis in liver

   c. Changes in protein synthesis in intestine

2. Methods

3. Results and discussion

   a. Changes in protein mass
   b. Changes in protein synthesis in liver
   c. Changes in protein synthesis in intestine

B. PROTEIN-DEPRIVATION

1. Introduction

   a. Changes in protein mass
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C. DIABETES

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III. RATES OF PROTEIN SYNTHESIS UNDER CONDITIONS OF PROTEIN LOSS

Comprehension of the regulation of protein metabolism involves an understanding of the processes by which an animal gains or loses protein in response to changes in environment. The total effect of the individual components of regulation of protein metabolism can be expressed by the nitrogen balance; that is, the relationship between the amount of nitrogen ingested and the amount of nitrogen which is excreted. Alterations in nitrogen excretion in response to alterations in nitrogen intake and, consequently, the relative constancy of protein mass have been known for a long time (a review of early studies is contained in Neuberger and Richards, 1964). With more severe alterations in nutrient intake or alterations in the hormonal environment, negative nitrogen balance, i.e. net protein loss, can be induced. Several conditions in which protein is lost from the body are discussed by Munro (1964). In particular three conditions representing three different types of deprivation are relevant to this work: complete lack of nutrient intake, or starvation; lack of a specific nutrient, as in protein-deprivation, and lack of the hormone, insulin, in animals made diabetic with streptozotocin.

Loss of protein from a tissue indicates that the rate at which protein is synthesized is lower than the rate at which protein is being degraded. There are, however, several ways in which this situation might arise (see Introduction). The rate of synthesis could fall with no change in the rate of degradation, the rate of degradation could be increased with no change in the rate of synthesis or synthesis and degradation might both change but in such a way that the rate of degradation still exceeded the rate of synthesis. As discussed earlier

(pp. 27-28) the rate of protein degradation is extremely difficult to measure reliably, especially in a tissue such as intestine which has a complex turnover of cells in addition to very rapid protein turnover. However, measuring the rate of protein synthesis provides the first step in understanding the regulation of protein metabolism. With subsequent measurements of changes in protein content, changes in protein degradation can be inferred.

This section is organised with subsections on starvation, protein-deprivation and diabetes. Each subsection begins with an introduction including an assessment of relevant studies from other workers, followed by a discussion of the experimental work outlining the results and relating them to possible mechanisms of regulation.

III A. STARVATION

1. Introduction

a. Changes in protein mass

Within a context of whole body protein loss, individual tissues do not respond uniformly. Variations are observed both in the amount of protein which disappears and the rate at which protein is lost. In starvation protein is lost rapidly from liver and intestine and more slowly from other tissues (Addis et al., 1936; Ju and Nasset, 1959). Losses of about 13% from liver have been observed following a one-day fast (Peters and Peters, 1972 and Garlick et al., 1975). Protein content of the total intestine also declined with two days of fasting in a study reported by McManus and Isselbacher (1970). The protein content of muscle (specifically gastrocnemius muscle), on the other hand, was unchanged even after a two-day fast (Garlick et al., 1975). With longer periods of starvation the pattern changes and the protein content of the liver does not decline further while the protein content of muscle begins to decrease (Hagen and Scow, 1957; Millward et al., 1976; Garlick et al., 1978). Obviously during longer periods of deprivation muscle protein must ultimately become the source of amino acids because of its proportionally larger mass.

b. Changes in protein synthesis of the liver

Changes in the rate of protein synthesis in the liver of starved animals have been reported by a number of authors. Although not

a direct measure of protein synthesis, the assessment of polysome profiles has been used to infer changes in protein synthesis. In this type of study a decrease in the rate of protein synthesis is inferred from a decrease in the proportion of active polyribosomes (ribosomes attached to messenger-RNA) to inactive, monomeric ribosomes. A review of the changes in polysome profiles which accompany several states of nutrient deprivation has been provided by Munro et al. (1975). The effect of starvation on polysome profiles can be viewed as an extension of the change which has been demonstrated in association with the normal, diurnal cycle of feeding and fasting. Fishman et al. (1969) showed that the proportion of polysomes to total ribosomes increased from 50% to 73% in association with eating (darkness).

Consideration of in vivo studies where protein synthesis was measured directly (and where the specific radioactivity of the precursor has been measured) yields a somewhat confused picture. Decreased protein synthesis has been reported by Henshaw et al. (1971) using a flooding amount of (^{14}C)lysine. Interestingly, other studies have found the rate of synthesis in liver to be unaltered by fasting. Peters and Peters (1972) observed no change in synthesis following an 18-hour fast when synthesis was measured from the incorporation of a tracer amount of (^{14}C)leucine over 16 minutes. Using continuous infusion of labelled amino acids, Waterlow and Stephen (1968) and Garlick et al. (1975) observed no change in the rate of protein synthesis up to 2 days after eating. It must be recalled, however, that the continuous infusion method measures only the synthesis of proteins which are not secreted and therefore remain in the liver. The measurement of Peters and Peters (1972) over 16 minutes was of the total synthesis of the liver, including both secreted and non-secreted protein. The authors also measured the synthesis of albumin as a proportion of

the total synthesis by comparing the counts in immunoprecipitated albumin with the counts incorporated into the total liver. Albumin synthesis was shown to be depressed by 40% after the 18h-fast. Because the fall in albumin synthesis was only from 11% of total synthesis to 6.6%, the effect on total synthesis was not large enough to be detected. It is possible that changes in polysome profiles observed by Fishman et al. (1969) and others (reviewed by Munro et al., 1975) provide a more sensitive measure of changes in synthesis than the method of Peters and Peters (1972) or that changes in polysome profiles primarily reflect changes in the synthesis of secreted proteins, which are not measured by the constant infusion method.

However, a study of Pain et al. (1978a), using a similar method of immunoprecipitation of albumin to Peters and Peters (1972) has demonstrated no change in the ratio of albumin to total liver synthesis. Pain et al. (1978a) also measured albumin synthesis (as a % of total), from the appearance of label in the plasma up to 2 hours after an injection of labelled amino acid and found it was unchanged after a two-day fast. In addition to using younger rats (100g vs 200g), Pain et al. (1978a) assessed the radioactivity in albumin and in total liver protein 10 minutes after an injection of a flooding amount of labelled leucine (100 moles (^{14}C)leucine per 100g body weight). Use of a flooding amount of labelled amino acid should overcome errors which might arise if the amino acids at the site of albumin synthesis were of a different specific radioactivity than those at the site of synthesis of other liver proteins, as has been suggested by Fern and Garlick (1976).

Therefore, although a number of studies have investigated the effect of starvation on the rate of protein synthesis in the liver, the situation is far from clear. The loss of protein mass could be accounted for by decreased synthesis, as reported by Henshaw et al. (1971) and

suggested from studies of polysome profiles (Munro et al., 1975). On the other hand, if protein synthesis is unaltered (Waterlow and Stephen, 1968 and Garlick et al., 1973, 1975) then protein degradation must have been increased to account for losses of protein mass. Support for regulation of protein mass through changes in protein degradation has been provided by studies in perfused liver which demonstrated that the rate of protein degradation, unlike the rate of protein synthesis, was sensitive to nutrient supply (Woodside and Mortimore, 1972).

c. Changes in protein synthesis in intestine

Changes in protein synthesis in the intestine in response to starvation are potentially even more complex than those in the liver. In addition to secreted and non-secreted proteins a component of protein synthesis in intestinal mucosa is necessary for the production of new cells. Measurements of protein synthesis in intestine which take account of the specific radioactivity of the precursor amino acid have not been made. A decrease in cell proliferation in response to starvation, however, has been reported by many (Diller and Blauch, 1946; Hooper and Blair, 1958; Brown et al., 1963; Altman, 1972; Hopper et al., 1972 and Löhrs et al., 1974). Both Brown et al. (1963) and Löhrs et al. (1974) reported decreases in the production of new cells of as much as 50%. A decrease in the fractional rate of protein synthesis would therefore be anticipated due to the slower proliferation of cells. In addition, if the loss of protein mass were also due to decreased synthesis (rather than increased degradation) an even larger fall in protein synthesis should be observed.

Owing to the conflicting conclusions for the changes in protein synthesis in the liver and the lack of reliable estimates for the

changes in synthesis in intestine with starvation, the problem has been investigated.

2. Methods

The effect of starvation on protein synthesis in liver and intestine was measured using a flooding amount of (^{14}C)leucine as described in Section I. The effect of two days' starvation was examined in two groups of rats. One group received a cubed diet (oxoid, 23% crude protein), the other received the 12% (w/w) diet (p. 58) until the rats weighed approximately 100g. At that weight the control animals were killed and the experimental group were kept a further two days without food but with free access to water.

3. Results and discussion

a. Changes in protein mass

The body weight and tissue composition for control and 48hr-fasted rats are shown in Table III.1. Similar changes occurr ed with starvation whether the rats were previously fed on oxoid or the 12% (w/w) diet. About 30% of body weight was lost, although about 10% of that loss was due to the contents of the gut rather than body mass. Liver weight decreased by about 50%, with decreases in protein and RNA of about 25%, and there was no change in the DNA content. This would suggest that the number of cells had not changed but that each cell was smaller. The results for the mucosa are expressed as mg per cm of length. Quantitative removal of the entire intestine was technically difficult and changes expressed per cm are thought to indicate changes in the

functional unit of the intestine. RNA and protein were both decreased by 30% but DNA was more resistant to the effect of starvation. As with the liver, this would indicate that the cells were smaller in the intestine of starved rats.

The jejunal mucosa of the oxoid-fed group was larger than the mucosa from the 12% (w/w)-fed group, containing more protein, RNA and DNA. This comparatively larger mucosa, in addition to a faster growth rate (data not shown), in the oxoid-fed group seemed to suggest that the 12% (w/w) diet was less adequate than oxoid. The conclusion of Payne and Stewart (1972) that 10% NDpE was an optimum diet was made on rats receiving the 10% NDpE diet in a pelleted form and it is possible that when the diet is presented in a powdered form that the protein: energy ratio has to be somewhat higher.

b. Changes in protein synthesis in liver

Rates of protein synthesis, as both fractional rates and absolute rates, for fed and starved rats are shown in Table III.2. The fractional rate of protein synthesis was 87% per day in both fed groups. After two days of starvation liver protein synthesis was lower than in the fed animals but the magnitude of the change was different in the two fasted groups. In rats which had been fed oxoid prior to starvation, liver synthesis was decreased by 30%, considerably more than the 10% drop in k_s observed when the rats had been maintained on the 12% (w/w) diet prior to starvation. Interestingly, it appears that prior adaptation to the less adequate diet allowed the fractional rate of synthesis in the liver to be maintained at a higher level under the stress of acute starvation. However, comparing the two fasted groups on the basis of changes in absolute synthesis, that is, the amount of

Table III.1

Body weight and tissue composition in control and two-day starved rats. Values are mean ± SEM, n=10.

	Oxoid fed	48h starved	12% (w/w) fed	48hr starved
Body wt (g)	102 ± 4	74 ± 3[a]	120 ± 3	89 ± 2[a]
Liver (mg per 100g initial body wt)				
Weight	4540 ± 80	2380 ± 110[a]	4310 ± 90	2560 ± 50[a]
Protein	602 ± 34	487 ± 35[b]	611 ± 26	408 ± 18[a]
RNA	34.2 ± 1.9	22.2 ± 1.2[a]	35.5 ± 1.5	26.6 ± 0.77[a]
DNA	7.5 ± 0.4	7.1 ± 0.2[ns]	10.1 ± 0.3	10.4 ± 0.4[ns]
Jejunal mucosa (mg per cm)				
Weight	28.5 ± 1.0	19.0 ± 1.0[a]	23.4 ± 1.5	19.2 ± 0.8[b]
Protein	2.88 ± 0.14	2.04 ± 0.10[a]	2.62 ± 0.13	2.01 ± 0.11[c]
RNA	0.258 ± 0.012	0.153 ± 0.010[a]	0.194 ± 0.015	0.137 ± 0.009[c]
DNA	0.132 ± 0.007	0.113 ± 0.005[b]	0.103 ± 0.007	0.0966 ± 0.006[ns]

Significance of the difference from control assessed by Student's t test

a $p < 0.001$

b $0.02 < p < 0.05$

c $0.001 < p < 0.01$

protein synthesised by the liver per day, indicates that both groups were decreased by about 38%.

Because the changes in fractional rate were different in the two fasted groups, this study suggests that starvation should not be regarded as a single phenomenon but rather should be viewed in conjunction with the dietary state of the animals before the onset of fasting. The study of Garlick et al. (1975), for example, was on rats fed a 10% NDpE diet prior to starvation and, consequently, the changes in synthesis would be expected to be of smaller magnitude than those reported by Henshaw et al. (1971).

A decrease in the fractional rate of synthesis could be brought about either by a decrease in the synthesis of all proteins or it could involve a change in the rate of synthesis of some proteins relative to others. In the liver, studies of differential effects on the synthesis of different proteins have involved looking at the response of secreted protein, particularly albumin, in comparison to the overall response. Although a selective decrease in albumin synthesis, relative to the rest of liver proteins, has been reported by Peters and Peters (1972), the study carried out in the oxoid fed and fasted groups (Table III.2) reported by Pain et al. (1978a) would suggest that the changes in albumin synthesis were parallel to the changes in total liver synthesis. Further, because there was no selective change in the synthesis of albumin in these animals, the decrease in total synthesis must have been accompanied by a decrease in the synthesis of non-secreted liver protein, contrary to the findings of Waterlow and Stephen (1968) and Garlick et al. (1975).

Table III.2

The effect of two days of starvation on the rate of protein synthesis in liver and jejunal mucosa. Results are means ± SEM, n=5.

	Oxoid fed	48h. starved	12% (w/w) fed	48h. starved
Liver				
fractional synthesis rate (%/d)	87 ± 3	62 ± 7[a]	87 ± 2	80 ± 3[ns]
absolute synthesis rate (mg/100g initial body wt/d)	564 ± 51	342 ± 74[b]	557 ± 30	357 ± 19[b]
RNA/protein (mg/mg)	5.94 ± 0.16	4.63 ± 0.15[b]	5.70 ± 0.43	6.28 ± 0.13[ns]
synthesis/RNA (mg protein synthesised/mg RNA)	15.5 ± 0.90	15.2 ± 1.5[ns]	15.5 ± 1.0	12.7 ± 0.5[d]
Jejunal mucosa				
fractional synthesis rate (%/d)	136 ± 7	111 ± 6[a]	118 ± 3	89 ± 4[b]
absolute synthesis rate (mg/cm/d)	3.66 ± 0.26	2.30 ± 0.16[c]	3.31 ± 0.18	1.84 ± 0.24[e]
RNA/protein (mg/mg)	9.02 ± 0.42	7.45 ± 0.25[a]	7.37 ± 0.33	6.92 ± 0.52[ns]
synthesis/RNA (mg protein synthesised/mg RNA)	14.1 ± 2.3	14.7 ± 1.6[ns]	16.74 ± 1.07	13.78 ± 1.7[ns]

p values assessed by Student's t test

a $0.01 < p < 0.02$

b $0.001 < p$

c $0.001 < p < 0.01$

d $0.02 < p < 0.05$

e $p \simeq 0.001$

Changes in liver protein synthesis at the subcellular level

The information about the regulation of protein metabolism which can be provided by *in vivo* studies is somewhat limited. An assessment of changes in the amount of RNA relative to changes in rates of protein synthesis can indicate whether a change in fractional synthesis rate has been brought about by a change in the number of ribosomes or by a change in the amount of protein synthesised per ribosome (the 'activity' of RNA, Henshaw et al., 1971 and Millward et al., 1973). In addition studies of polysome profiles have also been used to infer changes in the processes of initiation and elongation of polypeptide chains which then give rise to altered rates of protein synthesis.

The processes of polypeptide formation have recently been reviewed by Pain and Clemens (1980). Basically, initiation involves the binding of monomeric ribosomes to messenger-RNA to form polyribosomes. Chain elongation is the movement of the ribosome along the messenger with the addition of amino acids to the polypeptide chain, followed by chain termination, which is not thought to be limiting *in vivo* (Henshaw, 1980). The proportion of monomeric ribosomes (monomers) to polyribosomes provides an indication of the relative rates of initiation and elongation, since initiation determines the rate at which polysomes are formed from monomers and the rate of elongation determines how quickly polysomes will return to monomers.

Starvation has been shown to affect the number of ribosomes, the activity of ribosomes and the relative proportions of monomers to polyribosomes. Because the number of ribosomes and their activity are inter-related, they will be discussed together followed by a discussion of the changes in initiation and elongation induced by starvation.

Because most of the RNA in the cell is present as ribosomal RNA,

changes in the total RNA of cells reflect changes in the number of ribosomes (Henshaw, 1980). As shown in Table III.2 the RNA to protein ratio for the two-day starved group is significantly lower than the oxoid-fed group, suggesting that a decrease in the number of ribosomes has occurred. This decrease in RNA relative to protein is sufficient to account for the decrease in the fractional synthesis rate, indicated by the fact that the amount of protein synthesised per unit RNA was unchanged. In contrast, the 12% (w/w) fed and starved groups showed no decrease in the RNA to protein ratio and, consequently, the decrease in synthesis rate was brought about by a decrease in the amount of protein synthesised per unit of RNA.

Reduced synthesis brought about by both a loss of RNA (Munro et al. 1953 and Hirsch and Hiatt, 1966) and a decrease in the rate of synthesis per ribosome (Henshaw et al., 1971) has been reported for the liver of fasted animals. Henshaw (1980) has reviewed what is known about the mechanism for changes in synthesis and has suggested that a fall in activity occurs as an animal is adapting to changing conditions because the decline in synthesis is more rapid than the decline in ribosome content. With time, the ribosome content also falls and the activity of the ribosome returns to normal. Seen in this context the oxoid-fed and then fasted group has adapted to starvation by two days while the 12% (w/w) fed and then fasted group is still in the process of adapting.

Changes in polysome profiles suggest a way by which changes in the amount of protein synthesised per unit RNA in response to starvation are brought about. An increase in monomeric ribosomes and, consequently, a decrease in initiation relative to elongation in the liver of fasted rats has been reported by several groups (Fleck et al., 1965; Webb et al., 1966; Wilson and Hoagland, 1967; Staehelin et al., 1967 and Henshaw et al., 1971). An increase in the proportion of ribosomes present as monomers does not mean that only initiation has been inhibited but rather that the inhibition of initiation was greater than the inhibition

of elongation. The rate of elongation has been estimated from the synthesis rate per active ribosome (e.g. the synthesis rate per unit of RNA in polysomes) by Henshaw et al. (1971) in the liver of rats which had been starved for 3 days and was found to be slower than in control rats.

Protein degradation

Although a decrease in protein synthesis in response to starvation has been demonstrated, concomitant changes in protein degradation cannot be ignored. Large changes in liver protein mass following a meal (Millward et al., 1974) without changes in the rate of protein synthesis (Garlick et al., 1973) suggest that normally changes in protein degradation might be the important site of regulation. Although plasma insulin levels are lower in starvation (Rannels et al., 1977) and the amino acid concentration of portal blood will certainly be lower in the fasted animal, studies with perfused livers have failed to demonstrate any effect of amino acids (Woodside and Mortimore, 1972; Peavy and Hansen, 1976) or insulin (Mortimore and Mondon, 1970) on protein synthesis; but rather, have shown that both amino acids and insulin inhibit the rate of protein degradation. It is possible that the observed effects on protein synthesis in response to starvation are secondary to effects on protein degradation. Studies of sequential changes in protein mass and rate of total protein synthesis, measured from the incorporation of a flooding amount of labelled amino acid over a short time interval might provide additional insight into the regulation of protein mass.

Summary

In summary, the rate of protein synthesis in the liver of starved animals was decreased compared to the rate of synthesis in well-fed controls. Decreased synthesis was accompanied by a loss of ribosomes (in the oxoid fed and then starved group) and by a decrease in the amount of protein synthesised per ribosome (in the 12% (w/w)-fed and then starved group. Decreased synthesis per unit RNA is consistent with polysome profiles showing increased monomers relative to polysomes observed by others. Such changes in polysome profiles suggest that the rate of initiation relative to elongation has been decreased, while studies by Henshaw et al. (1971) suggest that the rate of elongation is also lower in the liver of starved rats.

c. Changes in protein synthesis in intestine

The fractional rate of protein synthesis was decreased in both the oxoid-fed then fasted and the 12% (w/w)-fed then fasted group (Table III.2). Interestingly, the rate of protein synthesis was higher in control animals fed oxoid than in the group receiving the 12% (w/w) diet so that after starvation, which decreased the rate of synthesis 20-25%, the rate of synthesis in the group which had been fed oxoid prior to fasting remained higher. In addition to a reduction in the fractional rate of synthesis, the amount of protein in the mucosa was reduced by fasting so the absolute rate of synthesis (mg per cm per day) had declined by about 40% in both groups.

Like protein synthesis in the liver there are distinct components of protein synthesis in intestinal mucosa. Not only are proteins recycled intracellularly and synthesised for secretion but protein is also synthesised for the replacement of cells. Therefore it would be

of interest to know if all three components change proportionately or not. Unfortunately measurements of total synthesis cannot differentiate among the three components of synthesis and techniques for immunoprecipitation of a characteristic, secreted protein, like albumin, are not available for the intestine. One can only look at the magnitude of the decrease in synthesis rate (20-25%) in conjunction with changes in cell turnover observed by others. In Section I (p. 23) the rate of protein synthesis required for the production of new cells was calculated to be about 60%/d. If the drop in protein synthesis observed in starvation were only due to a slower production of new cells, cell turnover would have to be twice as long in starved animals. A change of this magnitude is not unrealistic. Both Brown et al. (1963) and Löhrs et al. (1974) have reported changes in cell turnover of this magnitude in the intestine of mice that had been starved for two days. This means simply that it is possible that the decreased protein synthesis was due to decreased cell production. It is also possible that all components of protein synthesis are depressed, as is the case of liver.

The reduction in fractional synthesis rate in the jejunal mucosa from the group fed oxoid before fasting could be entirely accounted for by the drop in RNA to protein ratio with no observed change in the amount of protein synthesised per unit RNA, a situation similar to that in the liver of the oxoid-fed and then starved group. In the 12% (w/w)-fed and then fasted group, however, both the RNA to protein ratio and the synthesis per unit RNA were reduced and contributed to the reduction in protein synthesis.

Studies to elucidate the mechanism for changes in the rate of protein synthesis in intestine have not been done. Analysis of polysome profiles such as have been done in the liver has not been attempted in the intestine partly, perhaps, due to problems with digestive enzymes.

Possible regulators of protein synthesis including hormonal changes, changes in luminal and plasma amino acids, alterations in motility or damage to cells due to the passage of food through the intestine have not been sufficiently correlated with changes in the rates of protein synthesis to determine control mechanisms. What has been demonstrated is that some component of diet must influence protein synthesis in the jejunal mucosa since the rate of synthesis was higher in oxoid-fed controls than in those receiving the 12% (w/w)-powdered diet. In addition, two days of fasting depressed the rate of synthesis in both groups.

III B. PROTEIN-DEPRIVATION

1. Introduction

a. Change in protein mass

In contrast to starvation where amino acids from degraded protein are converted into glucose (Felig et al., 1969), in protein-deprivation energy is provided from dietary sources so that the effects associated with lack of protein specifically can be studied. The pattern of protein loss from individual tissues is somewhat different in that protein is lost from liver but the loss of protein from muscle is much less severe than in complete starvation (Henry et al., 1953 and Mendes and Waterlow, 1958). In the study of Garlick et al. (1975) the protein content of muscle (quadriceps) was not significantly different from the control value even after nine days of protein deprivation. After one day of protein deprivation, the liver protein mass was reduced by about 13%. Protein was lost, but at a slower rate, over the subsequent nine days so that the protein content of the liver was reduced by about 25%.

The intestine from animals maintained on protein-deficient diets does not undergo the rapid loss of protein shown by tissues such as liver (Ju and Nasset, 1959 and Platt et al., 1964). In a study of Hirschfield and Kern (1969) the amount of protein in the mucosa of the intestine was greater in control rats than in rats maintained for three weeks on a protein-free diet, but when the amount of protein was expressed per 100g body weight there was no difference between controls

and protein-deprived animals. The jejunal muscosa has been shown to be more resistant than the ileal mucosa to atrophy induced by a protein-free diet (Hill et al., 1968). Hirschfield and Kern (1969) have postulated that mucosal protein was preserved because of the endogenous protein (from intestinal secretions and cell exfoliation) which was present within the lumen of the intestine. Although the intestine could not carry on 'eating itself' indefinitely, such a mechanism might provide for short-term protection.

b. Changes in protein synthesis in liver

Since the rate of loss of protein from liver and intestine differs in response to protein-deprivation, it is interesting to see how changes in protein synthesis and degradation differ between the two tissues. As in starvation, changes in protein synthesis in liver, in response to protein-deprivation, must be considered in relation to the two components of synthesis, i.e. synthesis of hepatic proteins and synthesis of secreted proteins such as albumin. The synthesis of protein which remains in the liver has been measured by the method of continuous infusion and both Waterlow and Stephen (1968) and Garlick et al. (1975) have reported that this rate was increased when animals were maintainedon a protein-free diet. In the study of Garlick et al. (1975) the fractional rate of protein synthesis in the liver rose from 53% per day to 94% per day following nine days of protein deprivation. Similarly the rate of protein degradation (calculated from the synthesis rate and the rate of change of protein mass) increased from 47% per day at the beginning of the experiment to 94% per day after 9 days. Rates of degradation in excess of synthesis were reported for day 1, 2 and 3 of protein deprivation. Increased degradation has also been

observed in livers perfused without amino acids (Woodside and Mortimore, 1972 and Peavy and Hansen, 1976). When amino acids were supplied, the rate of protein synthesis was unaltered but the rate of protein degradation was significantly decreased.

Haider and Tarver (1969) have also reported an increase in the rate of protein synthesis in the liver in response to a protein-free diet. Synthesis was measured by injecting a tracer dose of (^{14}C)lysine and determining the changes in the specific radioactivity of tissue free (corrected for contamination by plasma lysine) and protein-bound lysine over 2 hrs. The time interval chosen makes interpretation of the results a little difficult since it was not short enough to include all of the synthesis of plasma proteins nor long enough to ensure that none of the label in protein was contained in plasma proteins. The reported rates of synthesis, however, agreed quite well with those of Garlick et al. (1975). The rates of synthesis reported were 17.1mg per hour per 200g body weight for rats on a normal (27% protein) diet and 26.2mg per hour per 200g body weight for rats fed a protein-free diet for 5-7 weeks. The data can be recalculated as fractional synthesis rates of 44% per day for controls and 72% per day in the protein-deprived group.

A similar method of employing a tracer amount of (^{14}C)leucine and monitoring the changes in leucine specific radioactivity was used by Morgan and Peters (1971). Unlike Haider and Tarver (1969), however, they measured the incorporation of label into protein over only sixteen minutes so that the rate of synthesis obtained represented the total synthesis of the liver. In contrast to the studies of non-secreted liver protein synthesis, their study showed a decrease in total synthesis following 10 days of protein deprivation from 6.70 to 4.72mg protein per g of liver per hour. The corresponding fractional synthesis rates are

92% per day and 69% per day. Changes of a similar magnitude have been reported in protein-deprived mice. Using a flooding dose of (^{14}C)-leucine and measuring incorporation over 5 min, Conde and Scornik (1976) reported changes in the fractional rate of total liver synthesis from 68% per day in control mice to 49% per day in mice maintained for six days on a protein-free diet. The results of studies on total liver proteins would seem to indicate that the rate of synthesis was reduced substantially by dietary protein deprivation unlike the studies on non-secreted liver proteins, which indicated that synthesis was either unchanged or increased.

The difference between the two types of measurement is of course the synthesis of secreted proteins. Of the proteins secreted from the liver, albumin is the largest single component, contributing 10-20% of the total synthesis (Peters and Peters, 1972 and Pain et al., 1978a). A number of workers have examined the response of albumin synthesis to dietary protein deprivation. Morgan and Peters (1971) measured albumin synthesis both from incorporation of label into immunoprecipitable albumin within the liver over 16 min and from the secretion of labelled albumin into the plasma up to 128 min after the injection of labelled leucine. Albumin synthesis as a proportion of total liver synthesis was 11% and dropped to 6% of total following 10 days of protein-deprivation. Pain et al. (1978b) using the same technique of immunoprecipitation, but a flooding dose of isotope, reported similar changes in albumin synthesis from 15% of total to 8% of total liver synthesis after 9 days of protein-deprivation. Haider and Tarver (1969) reported an even greater reduction in albumin synthesis, which in their study was reduced from 44% of total synthesis to 14%. A decrease in albumin synthesis in protein-deprivation has also been reported by Jeejeebhoy et al. (1973). Albumin synthesis was measured by isolating labelled albumin from the plasma

and was shown to be only about 25% of the control value after three days of protein-deprivation. This estimate of synthesis was made on circulating albumin and therefore would include a fall in synthesis brought about by both a decline in liver mass and a decrease in total liver synthesis. Although the change in liver mass was not reported, changes of about 25% have been reported by others (e.g. Garlick et al., 1975). The decrease in albumin synthesis not brought about by the loss of liver mass must have been about 50%.

Changes in albumin synthesis of about 50% are still not enough to account for the differences in conclusions between those studies which measured non-secreted protein synthesis and those which measured total liver synthesis. The interpretation of the way in which the changes in liver mass has been brought about is, of course, totally dependent on whether synthesis has changed or not.

c. Changes in protein synthesis in intestine

In the intestine where protein mass is rather resistent to dietary protein-deprivation, rates of protein synthesis might be maintained at control levels. However, care must be taken in interpreting studies of incorporation with tracer amounts of labelled amino acids. In protein-deprivation the plasma levels of many essential amino acids are decreased (Morgan and Peters, 1971). Injection of a tracer amount of labelled amino acid into a smaller pool will result in free amino acids of higher specific radioactivity than in control animals, where the unlabelled pool of amino acids is larger. Hencein protein-deprivation, higher incorporation as reported by Waterlow (1959), Lipkin and Quastler (1962), Stenram (1962), Muramatsu et al. (1963) and Hirschfield and Kern (1969) does not necessarily reflect a higher rate of synthesis.

The lack of dietary amino acids also increases the recycling of amino acids, which would influence the reliability of measurement of protein degradation by methods based on the decay of label. Both Lipkin and Quastler (1962) and Muramatsu et al. (1963) have shown that although incorporation of labelled amino acids into the intestine was higher in protein-deprived than in control animals, label was lost more slowly from the intestine of animals maintained for 2-3 weeks on a protein-free diet. Rather than indicating a reduced rate of degradation it seems more likely that these results are due to enhanced recycling of labelled amino acids. Increased synthesis and reduced degradation would suggest that the protein mass of the intestine should be increasing in protein-deprivation, a condition which has not been reported by any of the above studies.

Studies on changes in turnover of cells in the intestine in response to protein-deprivation are perhaps more reliable than estimates of protein turnover using tracer amounts of labelled amino acids. Lipkin and Quastler (1962) suggested that cell turnover, that is both the proliferation of new cells and the rate of migration of cells along the villus, was not grossly altered in mice maintained on a protein-free diet for two weeks. Hopper et al. (1972) investigated the response in rat intestine to various periods of protein-deprivation. A summary of their results appears below :

Period of deprivation	Cell-cycle time	Migration rate
3 weeks	longer	increased
7 weeks	longer	normal
11 weeks	longer	decreased

The actual turnover rate of cells in the intestine is a function of both parameters; that is, the time taken for the replication of new cells

(cell-cycle time) and the rate of which cells migrate along the villus (or migration rate, measured as the % of villus traversed in a given time). Although cell turnover rates were not reported, changes in cell-cycle times were larger than the changes in migration rate and so cell turnover was probably decreased in these animals.

The effect of protein-deprivation on protein synthesis in intestine has not been investigated with a method which, in light of current thinking, would seem to be reliable. Moreover, the effect of protein-deprivation on liver synthesis, although more thoroughly investigated, is still somewhat ambiguous. Consequently the effect on protein synthesis of the lack of dietary protein in the presence of an otherwise adequate diet has been investigated with a flooding amount of (^{14}C)leucine.

2. Methods

The effect of protein-deprivation and diabetes on protein synthesis was assessed in a single experiment. In this study animals were maintained on a 20% (w/w) diet. One group of control animals was killed on day 0 (body weight ~100g), the same time as the diabetic group received 13mg of streptozotocin (Sigma (London) Limited, Poole, Dorset) in 0.3ml, 0.05M citrate buffer, pH 4.5, mixed just prior to intravenous injection (carried out under ether anaesthetic) and the protein-deprived group was given a diet in which maize starch had been substituted for casein. On day 8 the rate of protein synthesis was measured in the protein-deprived group, followed by the second control group on day 9 and the diabetic group on day 10.

The level of glucose in the blood of the diabetic group was assessed from 20μl of blood removed from the end of the tail. The assay using

the glucose oxidase method of Werner et al. (1970) from a kit supplied by Boehringer Corporation was as follows: 20µl of blood + 1ml uranyl acetate, centrifuged. 50µl supernatant + 2.5ml glucose oxidase. After 35 min, the absorbance at 420nm was measured against 50µl (0.0046mg) of standard glucose solution which had been treated in the same way.

3. Results and discussion

a. Changes in protein mass

The changes in body weight and tissue composition in rats maintained for 8 days on a powdered diet devoid of protein are shown in Table III.3. Two control groups are also shown for comparison. 'Day 0' controls were killed at the same time as the protein-deprived group was transferred from a diet containing 20% (w/w) to a diet containing 0% (w/w). The 'day 9' control group was maintained on the 20% (w/w) diet for nine days longer than the day 0 group. The data for liver are expressed per 100g initial body weight so the day 9 control group represents the normal growth in rats of similar age to the protein-deprived group. The data for intestine, in mg/cm, do not include all of the growth since increase in length is not included. Rats maintained for 8 days on a protein-free diet not only failed to grow but actually lost about 25% of their original body weight. The livers of these animals contained substantially less protein (40% less) and RNA (35% less) than the day 0 controls. The DNA content was slightly higher than the day 0 controls but still less than controls of the same age. DNA content seems, therefore, to be the least sensitive to protein deprivation.

Table III.3

The effect of dietary protein-deprivation on body weight and tissue composition. Results are expressed as means ± SEM, n=10.

	Control day 0	Control day 9	Protein-free day 8
Body weight (g)	104 ± 2	169 ± 3	72 ± 2[d]
Liver (mg/100g initial body weight)			
weight	5000 ± 55	9510 ± 119[a]	3560 ± 97[d]
protein	838 ± 15	1641 ± 28[a]	483 ± 16[d]
RNA	40.7 ± 0.6	76.8 ± 1.3[a]	26.5 ± 1.1[d]
DNA	9.88 ± 0.15	17.36 ± 0.40[a]	12.02 ± 0.5[d,e]
Jejunal mucosa (mg/cm)			
weight	37.4 ± 1.5	43.3 ± 1.8[c]	20.5 ± 0.9[d]
protein	5.27 ± 0.19	6.23 ± 0.17[b]	2.77 ± 0.14[d]
RNA	0.319 ± 0.010	0.360 ± 0.009[b]	0.166 ± 0.007[d]
DNA	0.149 ± 0.005	0.168 ± 0.004[b]	0.113 ± 0.004[d]
Jejunal serosa (mg/cm)			
weight	10.83 ± 0.36	12.49 ± 0.33[b]	7.50 ± 0.34[d]
protein	1.55 ± 0.05	1.66 ± 0.04[ns]	1.05 ± 0.03[d]
RNA	0.0479 ± 0.0025	0.0567 ± 0.0025[c]	0.0223 ± 0.002[d]
DNA	0.0345 ± 0.0014	0.0299 ± 0.0012[c]	0.0276 ± 0.001[d]

p values assessed by Student's t test :

a difference between day 0 and day 9 controls, $p < 0.001$
b difference between day 0 and day 9 controls, $0.001 < p < 0.01$
c difference between day 0 and day 9 controls, $0.02 < p < 0.05$
d difference between day 0 control and protein-free, $p < 0.001$
e difference between day 9 control and protein-free, $p < 0.001$

The loss of RNA from the liver of protein-deprived rats has been investigated by Munro. In a review of several studies (Munro, 1968) he concluded that RNA degradation was accelerated intitially in the absence of amino acids and that in particular the amino acid, tryptophan, was necessary to prevent this. After 1-2 days the rate of RNA degradation slowed down. In a more recent study Lewis and Winick (1979) have studied the turnover of ribosomal RNA in the liver of rats maintained on a low protein (6% casein) diet and have shown that the decrease in cytoplasmic RNA was not due to a change in synthesis or cytoplasmic degradation but rather was due to an increase in the amount of RNA which was 'wasted', i.e. RNA degraded in the nucleus.

Changes in composition of two components of the jejunum, the mucosa and the serosa, were assessed separately. In both the mucosa and the serosa substantial amounts of protein (35 - 45%) and RNA (50%) had been lost. Unlike the liver, the DNA was also lower (20%) in the protein-deprived animals than the day 0 controls.

b. Changes in protein synthesis in liver

The fractional rates of protein synthesis in the liver of rats maintained on a protein-free diet for 8 days are presented in Table III.4 along with the rates of synthesis in the two control groups. The two control groups differ in that the day 9 group was maintained on a 20% (w/w) diet 9 days longer than the day 0 group. In the older group the rate of synthesis was somewhat lower which might represent a developmental change. Comparing the rate of synthesis in the protein-deprived group with the day 9 control group indicates that synthesis had declined by 30%, a decrease similar to that induced by the 48hr fasting of well-fed control animals. This result would support the

conclusions of Morgan and Peters (1971) and Conde and Scornik (1976) that total liver synthesis was depressed by dietary protein-deprivation for 6 - 10 days. In order to compare these results with the studies made by Waterlow and Stephen (1968) and Garlick et al. (1975) using the constant infusion method, some estimate of the changes in plasma protein synthesis must be made. Most measurements of the changes in albumin synthesis in protein-deprived rats suggest that a decrease of about 50% can be expected (Morgan and Peters, 1971; Jeejeebhoy et al., 1973 and Pain et al., 1978b). If plasma proteins account for about 30-40% of total liver synthesis (Pain et al., 1978a) and are assumed to be decreased by an amount similar to albumin with dietary protein deprivation* the following calculation can be made :

	k_s	Secreted protein	Non-secreted protein
Control	97%/d	34%/d	63%/d
Protein-deprived	68%/d	12%/d	56%/d

The calculated drop in the synthesis of non-secreted liver protein is about 10%. This is slightly lower than the drop in total synthesis of the liver in this study and very different from the increase in the synthesis of non-secreted liver protein measured by the method of constant infusion.

Studies which were discussed in section I (pp. 64-71) on the

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* This may be an over-estimate of the change in synthesis of secreted protein. Studies of Pain (Unpublished) suggest that the synthesis of plasma proteins other than albumin is not decreased in protein-deprived rats.

Table III.4

The effect of dietary protein-deprivation on the rate of protein synthesis in liver, jejunal mucosa and jejunal serosa. Results are means ± SEM, n = 5

	control (day 0)	control (day 9)	protein-free (day 8)
LIVER			
fractional synthesis rate (%/d)	106 ± 4	97 ± 2[a]	68 ± 2[d]
absolute synthesis rate (mg/100g initial body wt/d)	885 ± 53	1618 ± 64[b]	336 ± 28[e]
RNA/protein (mg/mg)	4.86 ± 0.056	4.68 ± 0.043[a]	5.49 ± 0.11[f]
synthesis/RNA (mg protein synthesised/mg RNA)	21.6 ± 0.8	20.9 ± 0.6[a]	12.4 ± 0.6[d]
JEJUNAL MUCOSA			
fractional synthesis rate (%/d)	164 ± 5	143 ± 3[b]	112 ± 4[d]
absolute synthesis rate (mg/cm/d)	8.42 ± 0.56	8.68 ± 0.64[a]	2.99 ± 0.25[e]
RNA/protein (mg/mg)	6.07 ± 0.91	5.97 ± 0.87[a]	6.04 ± 0.17[h]
synthesis/RNA (mg protein synthesised/mg RNA)	26.4 ± 0.8	24.4 ± 1.0[a]	18.6 ± 0.2[d]
JEJUNAL SEROSA			
fractional synthesis rate (%/d)	59 ± 5	69 ± 3[a]	44 ± 2[g]
absolute synthesis rate (mg/cm/d)	0.896 ± 0.066	1.162 ± 0.077[c]	0.479 ± 0.34[e]
RNA/protein (mg/mg)	3.07 ± 0.09	3.40 ± 0.10[a]	2.10 ± 0.16[f]
synthesis/RNA (mg protein synthesised/mg RNA)	20.0 ± 1.8	20.1 ± 1.2[a]	20.8 ± 0.7[h]

p values assessed by Student's t test

a difference between day 0 and day 9 controls, not significant
b difference between day 0 and day 9 controls, $p < 0.001$
c difference between day 0 and day 9 controls, $0.025 < p < 0.05$
d difference between day 9 control and protein-free, $p < 0.001$
e difference between day 0 control and protein-free, $p < 0.001$
f difference between day 0 control and protein-free, $0.001 < p < 0.01$
g difference between day 0 control and protein-free, $0.025 < p < 0.05$
h difference between either control group and protein-free, not significant

incorporation of varying amounts of (^{3}H)valine and (^{3}H)phenylalanine into various tissues of the rat may suggest a possible reason for the differing conclusions from studies with a flooding amount of isotope and those using tracer amounts of amino acids. Graphs of incorporation with varying amounts of amino acid (plotted as incorporation^{-1} vs dose^{-1}) were very non-linear, particularly in tissues from animals which had been maintained on a protein-free diet. This non-linearity suggests that the introduced label was not being diluted by a single homogeneous pool but rather that label was being distributed into a non-homogeneous pool, and the pattern of distribution was influenced by the amount of amino acid given. An apparent increase in protein synthesis in the liver would be observed if the pool which provided amino acids for protein synthesis shifted from nearer the intracellular pool in the normal, fed state, to nearer the extracellular pool under conditions of protein-deprivation. Although the argument for such a shift is purely theoretical, the results of measurements of protein synthesis made with tracer amounts of amino acids must be interpreted with caution because of potential problems in assessing the specific radioactivity of the precursor.

In addition to the fall in the fractional rate of synthesis, Table III.4 also illustrates the additional effect of the loss of protein from the liver on the total amount of protein produced by the tissue. The absolute rate of synthesis compared to the day 0 controls was depressed by 60%.

Changes in liver protein synthesis at the subcellular level

As with starvation changes in the subcellular processes of protein synthesis can be inferred from calculations relating the changes in the amount of RNA relative to the changes in the rate of protein synthesis

and also from studies on the distribution of the various forms of ribosomal RNA, i.e. polysome profiles.

In protein deprivation the loss of RNA (i.e. ribosomes) was parallel to the loss of protein (Table III.4). Synthesis per RNA was about 40% lower in the protein-deprived group than in the control group and similar changes have been reported by Morgan and Peters (1971) and Conde and Scornik (1976). A decrease in RNA activity suggests a change in the proportion of active ribosomal RNA to inactive r-RNA. Indirect evidence for such a change has been provided by studies of polysome profiles.

Reviewing the results of several studies on the effects of amino acids Munro (1968) has reported a shift in the distribution of polysomes from heavier aggregates (active) to lighter (inactive) ones in the liver of rats which had been fasted for 18h which was reversed when a mixture of amino acids was given 24h before the animals were killed. In particular tryptophan was necessary to reverse the effect. Moreover, the effect on polysomes produced by an imbalanced amino acid mixture was demonstrable even if the animals had been treated with actinomycin D. As actinomycin D inhibits the transcription of DNA, it can be concluded that the shift from inactive to active ribosomes occurs without the synthesis of RNA, and is, therefore, not mediated by a general change in the synthesis of messenger-RNA.

Polysome profiles have also been studied by Gaetani et al. (1972) in the livers of rats maintained for 30d on a protein-free diet. The proportion of free ribosomes was found to be increased from 19 to 41% of total r-RNA and membrane-bound ribosomes were decreased from 81 to 59%. Free ribosomes were reported to contain 18% monomers and dimers in the control group and up to 48% in the protein-deprived group. This increase in monomers and dimers from about 3% of the total in fed rats to about 20% in the protein-deprived group could account for some of

the decrease in the amount of protein synthesised per RNA. Since the proportion of active free ribosomes remained about the same (15-20% of the total) the authors conclude that the source of inactive ribosomes was the disaggregation of polysomes bound to membranes. Secreted proteins such as albumin and possibly other proteins which are modified in some way after synthesis or proteins which become incorporated into membranes or organelles are synthesised on membrane-bound polysomes (Shore and Tata, 1977). The disaggregation of membrane-bound polysomes could explain the observed decrease in albumin synthesis relative to total liver synthesis. Pain et al. (1978b) have demonstrated that these changes in albumin synthesis are correlated with changes in the concentration of messenger RNA for albumin.

In addition to the specific effect on the synthesis of proteins synthesised on membrane-bound polysomes such as albumin, the changes in polysome profiles also reflect a decrease in the rate of initiation relative to the rate of elongation. As with starvation, however, decreased initiation could be accompanied by a smaller decrease in the rate of elongation. In fact this has been suggested by Gaetani et al. (1972) and demonstrated in HeLa cells (Vaughan and Hansen, 1973), ascites cells (van Venrooij et al., 1970) and liver slices (Clemens, 1972) deprived of amino acids.

Protein degradation

A decrease in the rate of liver protein synthesis has been established, but whether or not the rate of protein degradation has also changed cannot be inferred from this study. In order to assess changes in protein degradation precisely it is necessary to measure the protein mass of liver at several different times to determine the

rate at which the mass is changing. Conde and Scornik (1976) have made such a study and they have concluded that the primary effect of dietary amino acids in the mice which were refed after 6 days of protein deprivation was the suppression of protein degradation. Total liver synthesis was increased by about 20% while protein degradation decreased from about 40% per day to 0% per day. The studies of Woodside and Mortimore (1972) and Peavy and Hansen (1976) discussed earlier also suggest that amino acids affect protein degradation rather than synthesis. Some caution must be used in interpreting the results from perfusion studies since the rates of synthesis which are measured are often lower than the in vivo rates. The fractional synthesis rate calculated from Woodside and Mortimore (1972) is approximately 35% per day, so that, failure to demonstrate an effect on synthesis may be due to the insensitivity of a rate which was already depressed.

It is important to point out that the effect of amino acid supply on protein metabolism has been looked at in two ways which may not be equivalent. Regulation may well be different in long-term adaptation to the lack of amino acids than in the acute situation such as refeeding or providing amino acids to a liver by perfusion. In the acute situation the effect of amino acids seems to be primarily to decrease protein degradation. Lack of amino acids over longer periods, such as in the present study, is accompanied by a decrease in protein synthesis. Protein degradation must also be decreased in this situation because the protein mass of the liver is hardly changing after nine days of protein-deprivation (Garlick et al., 1975).

Summary

In summary, both RNA and protein were lost from the liver of rats maintained on a protein-free diet. The loss of protein mass in the

liver was accompanied by a 30% fall in the fractional rate of protein synthesis with a selective decrease in the rate of albumin synthesis relative to total liver synthesis. This fall in synthesis was not due to a loss of RNA relative to protein but rather was due to a decrease in the amount of protein synthesised per unit of RNA. Evidence from polysome profiles would suggest an increase in inactive forms of ribosomal RNA was accompanied by a decrease in membrane-bound polysomes (such as synthesise albumin). Studies demonstrating decreased rates of elongation in protein-deprived rats would suggest that both initiation and to a lesser extent, elongation, are decreased.

c. Changes in protein synthesis in intestine

Both the mucosa and the serosa of the jejunum showed a marked decrease in the fractional rate of protein synthesis (Table III.4) with dietary protein deprivation. This result contradicts many previous studies which had shown an increased incorporation of labelled amino acids in the intestine of protein-deprived animals (Waterlow, 1959; Lipkin and Quastler, 1962; Stenram, 1962; Muramatsu et al., 1963 and Hirschfield and Kern, 1969). The difference between this study and the others is that the measurements reported here have taken into account the specific radioactivity of the amino acid which has been incorporated. As discussed in the introduction to this section, if the pool of free amino acid is smaller in the protein-deprived animal then injection of the same amount of isotope will result in higher specific radioactivity in the amino acid which is incorporated, and an apparently higher rate of synthesis. Morgan and Peters (1971) have shown that the plasma levels of branched-chain amino acids were lower in protein-deprived rats. Confirmation of the difference in size of the

free amino acid pool can be gained from examining the initial fall in specific radioactivity of the flooding amount of leucine. The difference in specific radioactivity between the leucine which was injected and the specific radioactivity of leucine at time 0 gives an indication of the size of the entire endogenous pool of unlabelled amino acid.

	SR of leucine (dpm/μmole)	Estimated endogenous leucine (μmoles/animal)
Injection solution	1.48×10^5	
Control	0.98×10^5	51.5
Protein-free	1.19×10^5	24.4

The calculation suggests that measuring incorporation of a tracer amount of amino acid without correcting for the specific radioactivity of the free amino acid might produce an error of nearly two-fold.

The decrease in fractional synthesis rate in mucosa was similar in magnitude to that produced by 2 days' starvation. Studies on the turnover rate of cells in the intestine of protein-deprived animals would suggest that cell turnover was probably decreased but not as dramatically as in starvation (Lipkin and Quastler, 1962 and Hopper et al., 1972). Consequently a part of the decrease in the fractional rate of protein synthesis probably reflected the decreased cell production while another portion reflected a decrease in intracellular turnover.

The presence of nutrients in the intestine has been suggested as a regulatory factor in gut metabolism including the turnover of cells (for review see Alpers and Kinzie, 1973). This study (Table III.4) and the two control groups in the starvation study (Table III.2) would suggest that the nature of the nutrients consumed was important and that both protein synthesis and intestinal mass were influenced by the

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amount of protein consumed [the quantity of food eaten is similar in control and protein-deprived rats (Quartey-Papafio, unpublished observations)]. The changes in fractional synthesis rate and protein mass are reflected in the large decreases in absolute synthesis rate in both mucosa (decreased about 60%) and serosa (decreased about 50%).

Table III.4 also provides some information on the way in which the fall in synthesis rate (i.e. fractional rate) has been brought about. In the mucosa RNA and protein were lost in parallel, so the decreased synthesis must have been due to a decrease in the amount of protein which was synthesised per unit of RNA. This situation is similar to that in the liver and it is possible that some of the same kind of changes in ribosomal-RNA are occurring in the mucosa, although such changes have not been investigated.

In the jejunal serosa the alternative change has occurred. The preferential loss of RNA relative to protein is sufficient to account for the decrease in synthesis and the amount of protein synthesised per unit of RNA was unaltered. It is interesting to contrast the response of the serosa, which is essentially smooth muscle, with the response of skeletal muscle to dietary protein deprivation. In studies of Millward (reported by Waterlow et al., 1978) including protein-deprivation, starvation, protein-restriction and energy-restriction decreased synthesis in skeletal muscle was accompanied by a decrease in RNA to protein ratio as well as a decrease in the amount of protein synthesised per RNA.

III C. DIABETES

1. Introduction

a. Changes in protein mass

A third condition in which there is loss of body protein is diabetes (Manchester, 1970a). Following the destruction of the insulin-producing cells of the pancreas with either alloxan or streptozotocin, growth is impaired but not all tissues are affected to the same degree. Substantial amounts of muscle are lost; liver and the intestine, particularly the mucosa are actually increased (Jervis and Levin , 1966 and Schedl and Wilson, 1971a). Miller et al. (1977) reported increases of 20% in the dry weight of the mucosa from diabetic rats compared to control values. Maintenance of the intestine may be partially due to increased food intake which accompanies diabetes (Booth, 1972), although Miller et al. (1977) reported a 10% increase in the dry weight of the intestine even when food was restricted to the level of control animals. This increase in mass of the mucosa was also associated with an increased absorption of nutrients (Olsen and Rosenberg, 1970; Schedl and Wilson, 1971b and Lal and Schedl, 1974). Diabetes is, therefore, particularly interesting because protein is being lost from the whole body despite an adequate intake of nutrients. In addition, unlike starvation and protein-deprivation where the changes in individual tissues were similar in nature, though differing in the severity and the rapidity of the change; in diabetes different tissues actually respond in quite dissimilar ways.

b. Changes in protein synthesis in liver

Changes in protein synthesis in diabetes tend to reflect the changes in protein mass. Loss of muscle protein accompanied by a 40% decrease in protein synthesis has been reported in alloxan diabetes by Hay and Waterlow (1967). Pain and Garlick (1974) have reported a 70% decrease in protein synthesis in the gastrocnemius muscle of rats treated with streptozotocin. In contrast, no change in the rate of protein synthesis, measured with the continuous infusion of (^{14}C)tyrosine, was observed in the liver. An insensitivity of liver protein synthesis to insulin has also been demonstrated in perfused liver (Mortimore and Mondon, 1970). Pain and Garlick (1974) have also compared control and diabetic rats after an overnight fast to eliminate differences between the two groups which might be due to hyperphagia in the diabetic group. The same agreement between control and diabetic was observed whether both groups were fed or fasted.

As with starvation and protein-deprivation, changes in the synthesis of secreted and non-secreted protein can be considered independently. The study of Pain and Garlick (1974) demonstrated that the synthesis of non-secreted liver protein was unaltered by diabetes. Measurement of albumin synthesis by immunoprecipitation of labelled albumin from the liver of diabetic animals has shown that it was decreased by 25% in streptozotocin-treated animals (Pain et al., 1978c). An even larger decrease of about 60% has been reported by Peavy et al. (1978) in perfused livers from alloxan-diabetic rats.

c. Changes in protein synthesis in intestine

Protein synthesis in the intestine of diabetic animals has not been

measured. An increase in protein mass brought about by hyperphagia would suggest altered synthesis or degradation as the mass was increasing. However, if the mass then stabilised at a higher value, synthesis and degradation rates could return to their original values. Alteration in cell proliferation might also be expected to produce changes in the rate of protein synthesis. The study of Miller et al. (1977) on the kinetics of cell turnover in the intestine of diabetic rats indicated that cell proliferation (measured by the number of cells labelled with (^{3}H)thymidine per crypt), migration rate (cell positions per hour) and the number of cells per villus were all increased by about 8% compared to non-diabetic animals. Consequently, despite the increase in the number of cells, cell life-span remained unchanged by diabetes. Although the authors also reported a two-fold increase in (^{3}H)thymidine incorporation per crypt, it seems unlikely that this was due to a two-fold increase in the rate of DNA synthesis, as suggested by the authors, since cell life-span was unaltered.

2. Methods

Methods for this study are discussed with the study on protein-deprivation.

3. Results and discussion

a. Changes in protein mass

The development of diabetes in the rat following streptozotocin injection seemed to have several phases. The initial phase, lasting 1-2 days, was charaterised by anorexia with quite severe weight loss.

Gradually the animals began to eat and weight loss stopped. The food intake, 19g/d, during this phase (about day 5) was about the same as the control group but higher when expressed on a per body weight basis. By day 10 the intake in the diabetic group was about 31g/d and the animals were slowly recovering the initial weight loss. It was during the third phase that the experiment to measure the rate of protein synthesis was done.

The changes in body weight and tissue composition which were observed are outlined in Table III.5 along with the data from the same two control groups as in the protein-deprivation experiment. On day 10 the livers of the diabetic animals contained about 25% more protein and 20% more DNA than the day 0 control group, but the amount of RNA was about 12% lower. It would appear that RNA was the most sensitive to the effect of diabetes, though protein, RNA and DNA levels were all below the day 9 control levels.

In the jejunal mucosa the reverse was true. Protein and RNA levels were actually higher than the day 9 control group. The DNA level, however, was not increased to the same extent, suggesting that the number of cells was not increased but rather the cells were larger. The jejunal serosa was not demonstrably different in the diabetic group or either of the two control groups. In contrast, the loss of other forms of muscle, notably skeletal muscle, have been reported in other studies on diabetes (Munro, 1964; Manchester, 1970b).

b. Changes in protein synthesis in liver

Previous studies on the rate of protein synthesis in diabetic animals had shown no change in the synthesis rate of non-secreted liver protein (Hay and Waterlow, 1967 and Pain and Garlick, 1974) but a decrease in

Table III.5

The effect of streptozotocin-induced diabetes on body weight and tissue composition. Results are expressed as means ± SEM, n=10

	Control day 0	Control day 9	Diabetic day 10
Body weight (g)	104 ± 2	169 ± 3[a]	93 ± 3[d]
Liver (mg/100g initial body weight)			
weight	5000 ± 55	9510 ± 119[a]	4980 ± 250[e]
protein	838 ± 15	1641 ± 28[a]	1052 ± 69[d]
RNA	40.7 ± 0.6	76.8 ± 1.3[a]	35.6 ± 2.1[f]
DNA	9.88 ± 0.15	17.36 ± 0.40[a]	11.9 ± 0.6[d]
Jejunal mucosa (mg/cm)			
weight	37.4 ± 1.5	43.3 ± 1.8[c]	49.9 ± 2.7[g]
protein	5.27 ± 0.19	6.23 ± 0.17[b]	7.02 ± 0.4[g]
RNA	0.319 ± 0.010	0.360 ± 0.009[b]	0.416 ± 0.03[g]
DNA	0.149 ± 0.005	0.168 ± 0.004[b]	0.151 ± 0.002[h]
Jejunal serosa (mg/cm)			
weight	10.83 ± 0.36	12.49 ± 0.33[b]	13.50 ± 0.89[g]
protein	1.55 ± 0.05	1.66 ± 0.04[ns]	1.68 ± 0.10[g]
RNA	0.0479 ± 0.0025	0.0567 ± 0.0025[e]	0.0584 ± 0.005[g]
DNA	0.0345 ± 0.0014	0.0299 ± 0.0012[e]	0.0296 ± 0.001[g]

p values assessed by Student's t test

a difference between day 0 and day 9 controls, $p < 0.001$
b difference between day 0 and day 9 controls, $0.001 < p < 0.01$
c difference between day 0 and day 9 controls, $0.025 < p < 0.05$
d difference between day 0 control and diabetic, $0.001 < p < 0.01$
e difference between day 0 control and diabetic, not significant
f difference between day 0 control and diabetic, $0.025 < p < 0.05$
g difference between day 9 control and diabetic, not significant
h difference between day 9 control and diabetic, $0.001 < p < 0.01$

the synthesis of albumin as a proportion of total synthesis (Pain et al., 1978c and Peavy et al., 1978). The anticipated result for total liver synthesis was a decrease of about 10% (assuming albumin synthesis decreased from 12% of total to 8% with similar changes in other plasma proteins). The observed drop in synthesis rate of nearly 50% (see Table III.6) was quite surprising.

Aspects of this study and that of Pain and Garlick (1974) which might account for the different conclusions must be considered. It is difficult to compare the severity of the diabetic state between this study and that of Pain and Garlick (1974) but such a difference might influence the degree to which liver synthesis was depressed. In a preliminary study where synthesis was measured 5 days after streptozotocin injection with a flooding dose of leucine in animals which were less diabetic (plasma glucose ~250 mg/100ml), liver synthesis was depressed about 20% (from 82%/d down to 64%/d). The animals in the study reported here were extremely diabetic (plasma glucose ~500 mg/100ml) and represented the survivors of a much larger group which were injected with streptozotocin. If the animals in the study of Pain and Garlick (1974) were less diabetic it might explain, in part, why no changes in the synthesis of non-secreted liver protein was observed.

In addition to a possible difference in the state of the animals which were studied there are two differences in methodology between this study and that of Pain and Garlick (1974) which might give rise to different values for the rates of synthesis. The first involves the problem of precursor specific radioactivity and the second difference involves the time of incorporation which was used. Problems in assessing the specific radioactivity of the precursor are more likely with the method of constant infusion because of the difference in the specific radioactivity of tyrosine within the tissue and in the plasma. It was

Table III.6

The effect of streptozotocin-induced diabetes on the rate of protein synthesis in liver, jejunal mucosa and jejunal serosa. Results are means ± SEM (n = 5)

	control (day 0)	control (day 9)	diabetic (day 10)
LIVER			
fractional synthesis rate (%/d)	106 ± 4	97 ± 2[a]	52 ± 4[e]
absolute synthesis rate (mg/100g initial body wt/d)	885 ± 53	1618 ± 64[b]	545 ± 42[f]
RNA/protein (mg/mg)	4.86 ± 0.056	4.68 ± 0.043[a]	3.40 ± 0.13[e]
synthesis/RNA (mg protein/mg RNA)	21.6 ± 0.8	20.9 ± 0.6[a]	15.3 ± 0.7[g]
JEJUNAL MUCOSA			
fractional synthesis rate (%/d)	164 ± 5	143 ± 3[b]	140 ± 10[d]
absolute synthesis rate (mg/cm/d)	8.42 ± 0.56	8.68 ± 0.64[a]	9.76 ± 0.75[d]
RNA/protein (mg/mg)	6.07 ± 0.09	5.97 ± 0.09[a]	5.95 ± 0.09[d]
synthesis/RNA (mg protein/mg RNA)	26.4 ± 0.8	24.4 ± 1.0[a]	23.5 ± 9.6[d]
JEJUNAL SEROSA			
fractional synthesis rate (%/d)	59 ± 5	69 ± 3[a]	69 ± 4[d]
absolute synthesis rate (mg/cm/d)	0.90 ± 0.07	1.16 ± 0.08[c]	1.15 ± 0.09[d]
RNA/protein (mg/mg)	3.07 ± 0.09	3.40 ± 0.01[a]	3.47 ± 0.14[d]
synthesis/RNA (mg protein/mg RNA)	20.0 ± 1.8	20.1 ± 1.2[a]	20.4 ± 1.0[d]

p values assessed by Student's t test

a difference between day 0 and day 9 controls, not significant
b difference between day 0 and day 9 controls, $p < 0.001$
c difference between day 0 and day 9 controls, $0.025 < p < 0.05$
d difference between day 9 controls and diabetic, not significant
e difference between day 9 controls and diabetic, $p < 0.001$
f difference between day 0 control and diabetic, $0.001 < p < 0.01$
g difference between day 9 control and diabetic, $0.001 < p < 0.01$

argued in the discussion on protein-deprivation, for example, that a change in the size of the free amino acid pool provided the potential for a change in the pool, from tissue to plasma, which supplied the tyrosine for protein synthesis. Such a potential also exists in diabetes where the free amino acid pool is larger than in control animals. Increased plasma levels of amino acids have been reported by Clark et al. (1968), Bloxham (1972) and Blackshear and Alberti (1974) and perhaps more relevant for the liver, per se, amino acids in the hepatic portal circulation may well be increased by hyperphagia.

The second difference in methodology, that involving the length of incorporation period, means that the spectrum of proteins measured by the two techniques may be slightly different. Proteins which turnover very rapidly will not contain label in proportion to their synthesis by the end of six hours (Waterlow et al., 1978) and, consequently, those proteins measured by constant infusion will not be identical to those measured by ten minutes of incorporation.

One other study in which liver protein synthesis was shown to be decreased by diabetes was that of Peavy et al. (1978). Protein synthesis was measured in perfused livers over 90 minutes and was decreased about 12% two days after alloxan injection and about 40% three days after injection. Measured in this way protein synthesis will primarily, although not entirely, reflect the synthesis of non-secreted hepatic protein.

The large decrease in fractional synthesis rate in the study reported here is responsible for the observed drop in absolute synthesis (Table III.6) since the protein content of the liver (Table III.5) was maintained. This is quite different to starvation and protein-deprivation where a drop in the amount of protein synthesised per day was the result of both a decrease in the fractional rate of synthesis and a loss of protein mass. Because protein mass has been

maintained, accompanied by decreased synthesis, the rate of protein degradation must also have fallen by a similar amount. Decreased degradation occurred inspite of a lack of insulin, contrary to what one would expect from the study of Mortimore and Mondon (1970). However, it is possible that the effect of a large influx of amino acids due to hyperphagia was sufficient to depress degradation as suggested by Woodside and Mortimore (1972).

Changes in protein synthesis at the subcellular level

Calculations based on this study and studies of others which have looked at subcellular changes which accompany diabetes would also suggest that the rate of synthesis was depressed. The calculation of RNA to protein ratio (Table III.6) implies that the number of ribosomes in the liver has decreased by about 25%. This decrease is not sufficient, however, to account for all the observed drop in synthesis and the synthesis per unit of RNA (i.e. per ribosome) has also dropped by about 25%.

Studies on polysome profiles, reflecting changes in ribosomal RNA, would also suggest that the amount of protein synthesised per RNA was decreased by diabetes. A decrease in the proportion of polyribosomes with a reduced capacity of isolated ribosomes to incorporate labelled amino acids in vitro has been demonstrated (Korner, 1961; Tragl and Reaven, 1971). Reduced polysome content (Tragl and Reaven, 1972) could be reversed by treating diabetic animals with insulin and amino acids (Clemens and Pain, 1974). In addition Pain et al. (1974) have demonstrated a selective decrease (of about 17%) in membrane-bound polysomes, though in vitro incubation of preparations of free and bound polyribosomes indicated that both populations had decreased in activity

by about the same amount (25-30%). One might expect that membrane-bound polysomes were decreased in diabetes in conjunction with the observed decrease in albumin synthesis.

More recently the changes in polysome profiles in the liver of diabetic rats have been reinvestigated with methods which ensure greater recovery of RNA. Peavy et al. (1979) have demonstrated that the distribution of total ribosomes from the liver of alloxan-diabetic rats was the same as in control rats despite a decrease in the synthesis of non-secreted liver protein (measured in perfused liver, Peavy et al., 1978) of about 12%. Because polysome profiles only reflect changes in initiation relative to elongation, it is possible that a decrease in synthesis was brought about by reductions in both initiation and elongation. Measurements of rates of elongation (see Jefferson, 1980) did not support this hypothesis, however. The discrepancy between studies showing decreased rates of protein synthesis and those showing no alterations in total polysomes has not been satisfactorily explained.

The mechanism for the alteration in albumin synthesis is much more thoroughly understood. Peavy et al. (1979) have studied polysomes which were synthesising albumin with a labelled antibody for albumin. The relative number of polysomes synthesising albumin was found to be decreased in the same porportion as the decrease in albumin synthesis (i.e. decreased 50-60%, Peavy et al., 1978). This decrease in polysomes making albumin was shown to be due to a decrease in the amount of messenger-RNA specific for albumin (Peavy et al., 1978). Moreover, the decrease in albumin message was reversed when the animals were treated with insulin (Peavy et al., 1978).

Summary

In diabetes the fractional rate of protein synthesis was decreased by about 50%. This drop in synthesis which was larger than either starvation or protein-deprivation was brought about by both a decrease in the number of ribosomes and a decrease in the amount of protein synthesised per ribosome. The work of others has suggested that the decrease in synthesis was due, in part, to a selective decrease in albumin synthesis. Because synthesis was decreased without a change in protein mass, protein degradation must also be depressed by diabetes. The differences in results reported by different investigators may be partially due to differences in methodology and partially due to differences in the state of the animals used. In this context, the influence of dietary intake to moderate the effects on protein metabolism induced by diabetes may be particularly important.

Changes in protein synthesis in intestine

The results for the synthesis rates of both jejunal mucosa and jejunal serosa from diabetic animals are shown in Table III.6. In neither tissue was the fractional rate of protein synthesis altered. For mucosa this is perhaps not surprising in light of the increased food intake and the fact that tissue protein and RNA were both maintained. For serosa, the maintenance of synthesis rate in diabetes suggests that serosa is much less sensitive to the lack of insulin than skeletal muscles or heart (Pain and Garlick, 1974; Jefferson et al., 1972, 1974, 1977; Flaim and Jefferson, 1979 and Chua et al., 1979b).

The slight hypertrophy of the jejunal mucosa is reflected in the slight (and not statistically significant) increase in the absolute

synthesis rate. Moreover, as the hypertrophy was brought about by similar increase in both protein and RNA, the RNA to protein ratio (Table III.6) is virtually the same in the diabetic group and in the two control groups for both tissues. With no changes in synthesis rate or RNA to protein, the amount of protein synthesised per unit RNA was not changed in either muscosa or serosa.

Although it is possible that the increased food intake had a compensatory effect on protein synthesis which might otherwise have been decreased, the preliminary study which measured the rate of synthesis 5 days after streptozotocin injection (see p. 137) when the animals were less hyperphagic also failed to demonstrate any change in the rate of protein synthesis. The effect of diabetes on protein synthesis in intestine is very different from the effect of starvation and protein-deprivation and suggests that regulation of protein metabolism in the intestine may be much more responsive to changes in nutrient intake than to changes in hormonal environment; unlike the liver, where synthesis appears to be regulated both by nutrient intake and hormonal levels.

SECTION IV. LEUCINE AND THE REGULATION OF PROTEIN METABOLISM

A. METHODS

B. RESULTS AND DISCUSSION

1. Use of a massive amount of phenylalanine to measure synthesis

2. The effect of 100μmoles of leucine on protein synthesis

IV. LEUCINE AND THE REGULATION OF PROTEIN METABOLISM

In this final section the role of leucine as a regulator of protein synthesis will be examined. Quite distinct from the role of dietary amino acids in influencing protein synthesis, which was discussed in the previous section, a specific stimulatory role for the single amino acid, leucine, has been suggested by a number of workers, particularly for protein synthesis in muscle. These studies will be discussed in some detail because in addition to their contribution to an understanding of the regulation of protein metabolism, such studies might cast doubt on the validity of a method which relies on injecting massive amounts of leucine.

Several *in vitro* systems have been used to investigate the possibility that leucine regulates protein turnover in muscle. In 1975 Buse and Reid reported that the incorporation of (^{14}C)lysine into protein in incubated rat diaphragm was stimulated by preincubation for 1 hour with 0.3mM leucine. Stimulation was not observed with either valine or isoleucine but could be demonstrated with leucine whether the rat had been fed or fasted prior to the experiment. An effect of leucine on the transport of labelled lysine (rather than protein synthesis) was dismissed since the incorporation of label into protein from (1-^{14}C)acetate was also stimulated by the presence of leucine. Additional experiments demonstrated enhanced incorporation of (^{3}H)tyrosine into protein even though the specific radioactivity of tyrosine (from total acid soluble counts and the amount of tyrosine) was unchanged. In addition to suggesting that leucine increased protein synthesis, the authors also suggested that leucine decreased the degradation of protein. This conclusion was supported by the fact that the concentration of

free lysine in the tissue was 30% lower in those tissues which had been incubated with leucine, even if cycloheximide was also included in the incubation medium. Subsequent experiments (Buse and Weigand, 1977) have shown that the effect of leucine could not be duplicated with metabolites of leucine.

Fulks et al. (1975) have also demonstrated enhanced protein synthesis and reduced protein degradation in incubated rat diaphragm in the presence of leucine. In this study pieces of diaphragm weighing about 30mg were incubated with 10mM glucose and mixtures of amino acids at plasma levels (after Mallette et al., 1969) and at five times plasma levels. Protein synthesis was measured from the incorporation of (^{14}C)-tyrosine into protein and the specific radioactivity of free tyrosine in the tissues (corrected for extracellular tyrosine using inulin space). Labelled tyrosine was present in the medium at 0.1mM. In the diaphragm from fed rats incubated with 5 x plasma levels of the branched-chain amino acids (leucine, isoleucine and valine) the rate of protein synthesis was increased 24%. This increase was comparable to the amount of stimulation produced by a mixture of all amino acids at 5 x plasma levels. Protein degradation, measured from the amount of tyrosine released into the medium from tissue incubated with 0.5mM cycloheximide, was decreased 26% when all amino acids were present and 18% with only the branched-chain amino acids. The effect of leucine, valine and isoleucine individually was assessed in tissue incubated from hypophysectomised rats. A mixture of all three amino acids increased synthesis by 47% and decreased degradation by 28%. Leucine alone increased synthesis 25%; isoleucine + valine increased synthesis 16%. Protein degradation was decreased 25% by leucine alone and 22% by isoleucine + valine. This experiment suggested, in contrast to the finding of Buse and Reid (1975), that the effect of the branched-chain

amino acids on synthesis was additive. Moreover, leucine alone or isoleucine + valine brought about the full suppression of degradation. The lack of any effect on degradation by other oxidisable substrates (β-hydroxybutyrate or octanoic acid) suggested that the effect of leucine was not due to an effect on energy supply alone.

Another muscle preparation, that of the perfused hemicorpus, has been used by Li and Jefferson (1978) to demonstrate the effect of leucine on protein turnover. Protein synthesis was measured in the hemicorpus of 48-hour fasted rats, from the incorporation of (^{14}C)phenylalanine into protein. The specific radioactivity of intracellular phenylalanine was maintained at a constant level by the addition of 0.4mM phenylalanine to the perfusate. Addition of plasma levels of other amino acids (after Jefferson et al., 1977) did not alter synthesis significantly, but if amino acids were added at 5 x plasma levels, synthesis was enhanced by 43%. 5 times plasma levels of just the branched-chain amino acids also stimulated synthesis by 25%. Ten times plasma levels of the branched-chain amino acids produced the same degree of stimulation as 5 times plasma levels of all amino acids. Leucine alone, at ten times plasma levels, also produced a 25% increase in protein synthesis. Glucose was not added to the perfusate, but additional experiments with glucose and palmitate confirmed that the effect of leucine was not just the result of providing an energy source. On the basis of their studies on the amount of ribosomal RNA present as 40S subunits the authors suggested that leucine, like insulin, stimulates protein synthesis by preventing the formation of a block in initiation.

The effect of leucine on protein degradation was also assessed by Li and Jefferson (1978). Proteolysis was measured both from the change in specific radioactivity of phenylalanine in the perfusate and from the release of phenylalanine in the presence of 100μM cycloheximide. Although the rates of degradation measured with cycloheximide were

slightly smaller than those calculated from the release of phenylalanine, in both cases protein degradation was reduced by about 30%.

In addition to the experiments on 48 hour fasted rats, Li and Jefferson (1978) also studied young, fed rats and older, fasted rats. Young, fed rats also showed an increase in synthesis with 5 times plasma levels of leucine, isoleucine and valine but a smaller (12%) decrease in protein degradation. Older (230g) fasted rats showed no effect of 5 times plasma levels of branched-chain amino acids on synthesis or degradation.

Similar experiments with perfused heart have shown that branched-chain amino acids at five times plasma levels enhanced protein synthesis as much as all amino acids at 5 times plasma levels (Rannels et al., 1974). Rannels and co-workers (1974) have demonstrated that, unlike diaphragm and hemicorpus, the effect on protein synthesis was not unique to leucine, but was also brought about by oxidisable non-carbohydrate substances: lactate, pyruvate, acetoacetate, β-hydroxybutyrate, palmitate and oleate. Chua et al. (1979a) have extended the study to the individual branched-chain amino acid and their degradation products. They have shown a stimulation of synthesis with leucine but not with isoleucine or valine (1mM). The transamination product of leucine, α-ketoisocaproate, enchanced synthesis at 1mM while the decarboxylation products of leucine (isovalerate) and valine (isobutyrate) affected synthesis at concentrations of 10mM. The effect of branched-chain amino acids and their metabolites on protein degradation was also assessed from the release of phenylalanine into the perfusate in the presence of 0.02mM cycloheximide. In addition to 1mM leucine, several metabolites of branched-chain amino acids decreased protein degradation, including: α-ketoisocaproate, isovalerate, isobutyrate and tiglic acid (the CoA derivative of tiglic acid is an oxidation product of isoleucine). Reduced proteolysis was also

observed with acetoacetate,acetate and propionate. Although some metabolites such as α-ketoisocaproate, isovalerate, isobutyrate and possibly acetoacetate increased the intracellular concentration of leucine, others did not. The authors speculate that the mechanism for enchanced synthesis might be mediated through glucose-6-phosphate which has been shown to stimulate polypeptide chain initiation in reticulocytes (Lenz et al., 1978 and Ernst et al., 1978). Although the levels of citric acid cycle intermediates have not been measured, the authors argue that they might be elevated as a consequence of branched-chain amino acid oxidation. These in turn might inhibit glycolysis, thereby raising levels of glucose-6-phosphate.

Perhaps even more interesting than in vitro studies with muscle preparations are studies which have assessed the role of leucine in regulating protein metabolism in vivo. In studies in the intact animals the ability of leucine to stimulate insulin secretion (Floyd et al., 1963 and Fajans et al., 1967) must be borne in mind. Buse et al. (1979) injected both insulin and glucose along with branched-chain amino acids and measured polysome profiles in psoas muscle from 4-day starved rats. Insulin was injected in order to isolate a direct effect of leucine on protein synthesis from a more indirect mediated effect. An increase in polysomes relative to ribosomal subunits and monomers was observed 1-2 hours after the intraperitoneal injection of 200μmoles of leucine, isoleucine and valine (2.9-fold increase) and a similar effect (2.5-fold increase) with 200μmoles of leucine alone.

Two relevant studies on the effect of leucine on protein metabolism have also been done in man. Sapir and Walser (1977) demonstrated improved nitrogen balance in patients who were infused with the ketoacid analogues of branched-chain amino acids. Sherwin (1978) has demonstrated a similar improvement in nitrogen balance in fasting, obese man receiving

leucine intravenously. Leucine was given as a priming dose and then by constant infusion (in amounts to produce plasma levels about 350mM, which is about the level of leucine in plasma following a protein meal) to subjects after 3 days and 4 weeks of fasting. In both instances nitrogen balance was improved 25-30% on the day in which leucine was infused. No change in urinary 3-methyl histidine (an index of degradation of muscle protein) was observed, suggesting that the improved nitrogen balance was brought about by enhanced protein synthesis. The effect of leucine on protein metabolism was shown not to be mediated through insulin or glucagon since plasma levels of both hormones remained unchanged by leucine infusion. In subjects who had been fasted for 4 weeks, leucine infusions were accompanied by elevated levels of plasma glucose and reduced glucose utilisation (assessed from (^{3}H)glucose turnover).

Contrary to these studies which suggested that leucine stimulated protein synthesis, Millward et al. (1976) have argued against a role for leucine in regulating protein metabolism, particularly in muscle, because of the lack of correlation between the level of free leucine and the rate of protein synthesis. For example, leucine concentrations 1.5 to 4 times normal levels were reported for 4 conditions of hormonal or dietary alterations and in all of these conditions the observed rate of protein synthesis was 50% or less of control values. The authors argue that the changes in free amino acid concentrations are the result of (rather than the cause of) changes in dietary amino acid supply, protein synthesis and protein degradation. Large fluctuations in leucine concentrations, in particular, are a consequence of the fact that the free leucine pool is relatively small compared to the amount of leucine in protein. Consequently, changes in protein synthesis and degradation can remove or add proportionately large amounts to the free leucine pool. A lack of correlation between leucine levels and rates

of protein synthesis does not, however, suggest that leucine has no effect on protein synthesis, but rather it indicates that leucine is not the only regulator of protein synthesis. Changes in other factors such as hormones might modulate or even over-ride any effect of leucine.

Therefore, the effect of leucine on protein metabolism has been investigated in tissues of the intact animals. Because a flooding amount of labelled amino acid was thought to be necessary for measuring protein synthesis (see section I), an alternative amino acid was used to measure the rate of synthesis in the presence and absence of added leucine. Labelled phenylalanine was chosen because it is soluble enough to allow for the injection of a large amount in a relatively small volume, and also because the endogenous phenylalanine pool is comparatively small (Munro, 1970) so the flooding procedure should be effective. Another very important advantage of using phenylalanine was the ability to analyse the specific radioactivity of phenylalanine fluorimetrically, thereby, eliminating the time consuming procedure with the amino acid analyser which was used for leucine. The method for analysing the specific radioactivity of phenylalanine which is described in some detail was developed in conjunction with P.J. Garlick and V.R. Preedy.

IV A. METHODS

Animals were maintained as described in section I, control animals received the oxoid diet. The control and starved animals were from the same group of rats, the protein-free animals were a separate group. At the time of measurement the control group weighed 134g and the two-day starved group weighed 102g. The protein-free group weighed 113g at the time of transfer to the protein-free diet and after 9 days of protein-deprivation the mean weight was 90g. The flooding dose of phenylalanine was administered intravenously as 1ml per 100g body weight of 150mM L-(4-^{3}H)phenylalanine (approximately 50μCi) in water or 100mM leucine. Tracer doses of 7.7μCi L-(U-^{14}C)threonine and 38μCi L-(4,5-^{3}H)lysine per ml were given either in 0.9% (w/v) NaCl or 150mM unlabelled phenylalanine. The injection procedure and the removal of tissues was the same as in sections I and III but heart and gastrocnemius muscle were also included.

Tissues were prepared as described in section I with a few modifications. Powder from frozen tissues were precipitated in 3ml of cold 2% (w/v) $HClO_4$ and centrifuged. The supernatant (containing free phenylalanine) was neutralised with 1.5ml of saturated tripotassium citrate which precipitated $KClO_4$ and provided the appropriate pH (about 6.3) for incubation with phenylalanine decarboxylase. Preparation of the solution containing hydrolysed protein in 6M HCl for enzyme incubation involved evaporating to dryness and resuspending the amino acids in 3ml, 0.5M sodium citrate, pH 6.3.

Conversion of phenylalanine to β-phenylethylamine was accomplished by incubating 1ml of supernatant or hydrolysate with 0.5ml of a suspension of L-tyrosine decarboxylase (acetone powder of S.faecalis, type I from

Sigma (London) Limited) at 50°C, overnight. L-tyrosine decarboxylase was found to contain phenylalanine decarboxylase activity and was a less expensive form of the enzyme than L-phenylalanine decarboxylase. Enzyme suspensions were made up in 0.5M citrate, pH 6.3, and contained 0.7 units/ml for supernatants and 1.4 units/ml for hydrolysates in addition to 0.5mg/ml pyridoxal phosphate.

Extraction of β-phenylethylamine (and not tyramine) was accomplished by adding 1ml 3M NaOH and shaking with 10ml n-heptane. Removal of the organic layer was facilitated by freezing the lower, aqueous phase in dry ice/methanol and decanting. β-phenylalanine was then extracted into 4ml 0.01M H_2SO_4 which was frozen in dry ice/methanol so that the organic layer could be decanted. 1-2ml were used to determine radioactivity and 1ml (for free phenylalanine) and 0.02ml (for protein-bound phenylalanine) were assayed for phenylethylamine.

Fluorimetric assay of β-phenylethylamine by a modification of the method of Suzuki and Yagi (1976) involved the addition of 0.5ml 2mM leucylalanine, 1ml 50mM ninhydrin and 2.5ml 1M potassium phosphate, pH 8.0 to 1ml of sample (in 0.001M H_2SO_4). Solutions of peptide, ninhydrin and buffer were all made separately and combined just before use. Only the buffer was stored, other reagents were made each day. Samples and standards containing 0.1 - 1ml 20mM β-phenylethylamine (stored frozen as 1M β-phenylethylamine in water) were incubated at 60° for 1h in the dark and then cooled on ice for 15 min.

The fluorescence, which was very unstable to both heat and light, was measured at 495nm (excitation 390nm) at a fixed interval of 7s after the introduction of sample. Tissue blanks (without enzyme) and enzyme blanks (without tissue) were routinely analysed and did not indicate any interferring fluorescent material. Tissue blanks were also counted to ensure that contaminating radioactivity was not present. A standard

of (^{3}H)phenylalanine which was analysed routinely, indicated a coefficient of variation of 3.2% over 19 determinations.

IV B. RESULTS AND DISCUSSION

1. Use of a massive amount of phenylalanine to measure synthesis

The changes in specific radioactivity of phenylalanine between 2 and 20 minutes after the intravenous injection of 150μmoles (^{3}H)phenylalanine per 100g body weight in plasma, liver, jejunal mucosa and gastrocnemius muscle are shown in Figure IV.1. As with leucine (Figure I.5) the decline in specific radioactivity was linear with time. This enabled synthesis rates to be calculated from groups of animals killed at two and ten minutes as before. The fall in specific radioactivity following phenylalanine injection was not as great as with leucine, presumably because of the larger amount injected (150μmoles rather than 100μmoles) and the smaller endogenous pool of free amino acid (Munro, 1970).

Synthesis rates measured with a massive amount of (^{3}H)phenylalanine are shown in Table IV.1. In calculating these rates an allowance has been made for the time which was taken between the death of the animal and the time when the tissue was placed in ice-cold water. Because the total time for incorporation was only ten minutes, the time between death and the cessation of incorporation was not insignificant, so an assessment of this time interval was undertaken as follows. The incorporation of (^{3}H)valine into protein following a massive injection of labelled valine (300μmoles/100g body weight) was measured at 0.42 and 10 min after administering the isotope. Incorporation at the nominal 0.42 and 10 min, shown in Figure IV.2 for the jejunal mucosa, was extrapolated to the time of zero incorporation. This estimate of the time taken to stop incorporation was 0.75 min. Actual timing of the removal

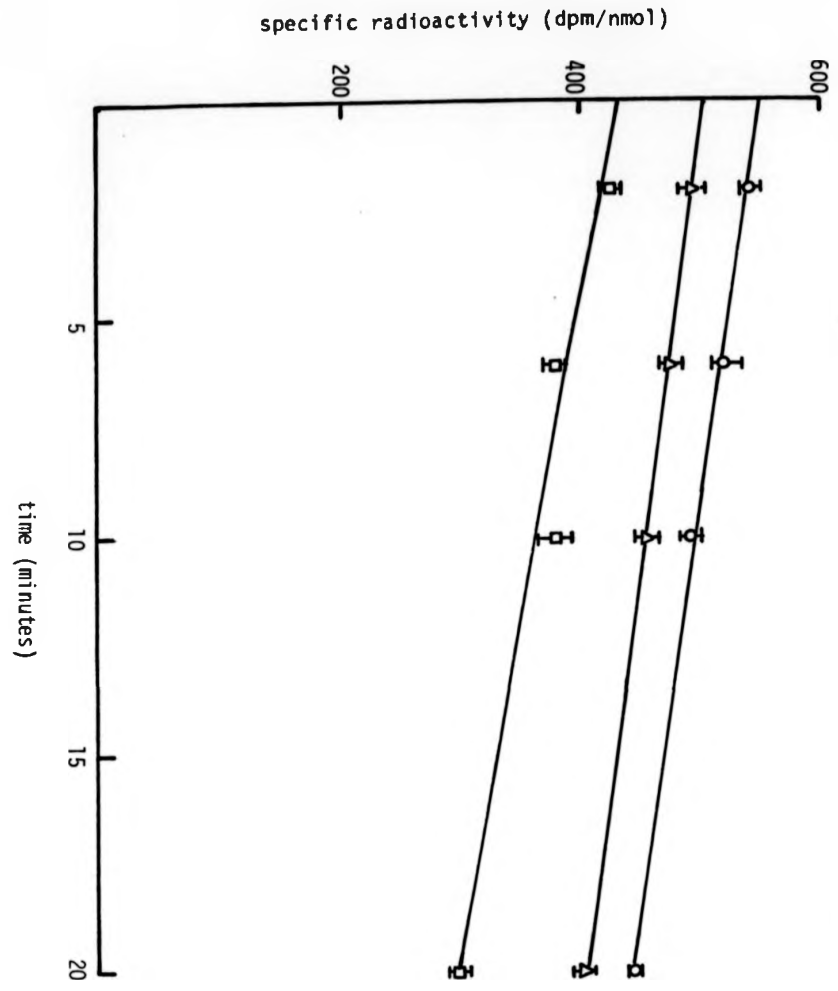

specific radioactivity (dpm/nmol)
200
400
600
5
10
15
20
time (minutes)

Figure IV.1

Figure IV.1

The change in specific radioactivity of phenylalanine in plasma (○), gastrocnemius muscle (△) and jejunal mucosa (□) after injection of 150μmol L-(4-^{3}H)phenylalanine per 100g body wt. Each point represents the mean ± SEM from four animals. The points for liver are superimposable on the points for muscle.

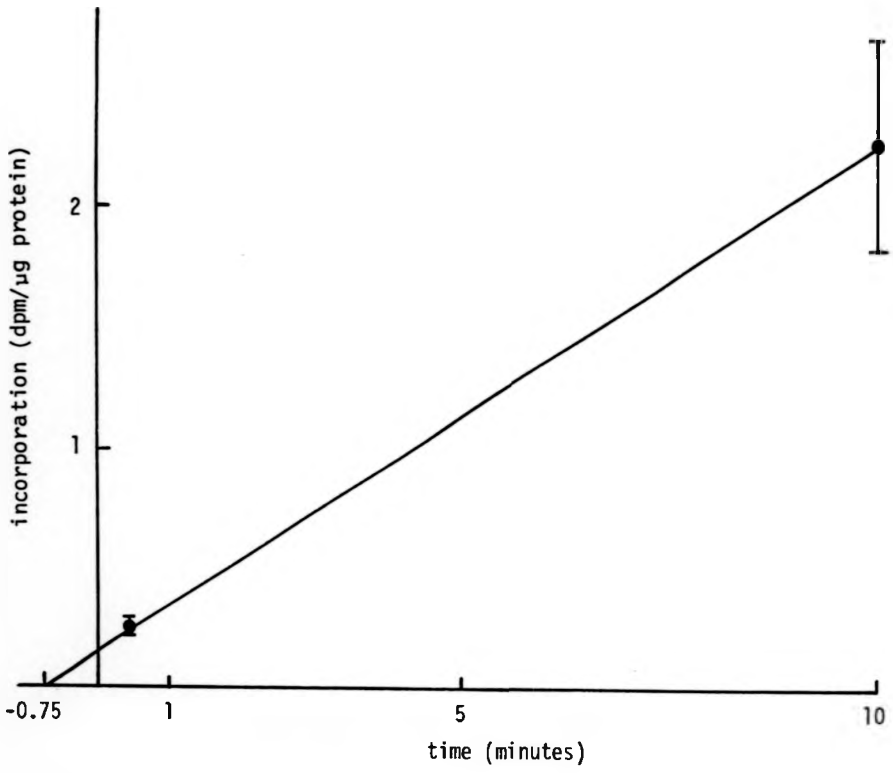
Table IV.1

Fractional synthesis rates in tissues of young male rats

Synthesis rates were assessed from the incorporation of 150μmoles (4-^{3}H)phenylalanine per 100g body weight for 10 minutes after intravenous injection. Results are the mean for a group of 6 animals ± SD.

Tissue	k_s, %/d
Liver	83.3 ± 8.0
Jejunal mucosa	119.2 ± 8.6
Jejunal serosa	51.4 ± 5.1
Gastrocnemius muscle	16.9 ± 1.6

Figure IV.2 Incorporation of (^{3}H)valine (dpm/µg protein ± SD) 0.42 and 10 minutes after injection of 300µmoles (^{3}H)valine per 100g body weight



and chilling of the jejunum made during the experiment had indicated that the procedure took 1.09 minutes. The agreement between the two estimates was quite reasonable and the actual time recorded for each tissue between injection and cooling in ice was used as the time of incorporation in all calculations of k_S. The times taken for the removal of the tissues were 0.75 min for liver, 1.09 min for intestine, 0.42 min for gastrocnemius muscle and 1.48 min for heart. Use of a longer time in the calculation of k_S means that values are smaller than those reported in sections I-III for (^{14}C)leucine incorporation. Except for the time factor the results are in good agreeement. The value for gastrocnemius muscle also compares favorably with values for the rate of protein synthesis derived from a constant infusion of (^{14}C)tyrosine for six hours (Waterlow et al., 1978).

As with the massive amount of leucine, it was important to demonstrate that the massive amount of phenylalanine used to measure protein synthesis was not affecting the rate of synthesis. To demonstrate this, two labelled amino acids, (^{3}H)lysine and (^{14}C)threonine, were injected in tracer amounts with and without 150μmoles of unlabelled phenylalanine. Lysine and threonine were used because they are not throught to share a transport system with phenylalanine or each other (Christensen, 1969). The incorporation of the tracers alone and in the presence of a flooding dose of phenylalanine is shown in Table IV.2. Although transport problems were not eliminated completely (e.g. (^{14}C)-threonine incorporation into liver protein), none of the differences between tracer alone and tracer plus phenylalanine is statistically significant and the results would suggest that this large amount of phenylalanine did not alter protein synthesis. In addition, the studies of other workers have suggested that phenylalanine does not affect the rate of protein synthesis in muscle (Buse and Reid, 1975; Fulks et al.,

Table IV.2

Incorporation of (^{3}H)lysine and (^{14}C)threonine in the presence and absence of 150μmoles of unlabelled phenylalanine per 100g body weight

L-(4,5-^{3}H)lysine and L-(U-^{14}C)threonine were injected intravenously in 1ml of 0.9% (w/v) NaCl or 150mM unlabelled phenylalanine. Incorporation, measured after 10 min, is expressed in dpm/mg RNA ± SEM ($\times 10^{2}$, n = 6). None of the differences between tracer alone and tracer plus phenylalanine is significant ($p < 0.05$).

	(^{3}H)lysine		(^{14}C)threonine	
	tracer alone	tracer + phe	tracer alone	tracer + phe
Liver	700 ± 57	707 ± 32	183 ± 24	238 ± 25
Jejunal mucosa	570 ± 42	594 ± 29	252 ± 29	288 ± 20
Gastrocnemius muscle	231 ± 16	200 ± 21	114 ± 16	108 ± 11

1975; Jefferson et al., 1974) or liver (Woodside and Mortimore, 1972).

Having shown that the flooding amount of phenylalanine did not alter protein synthesis, this method could then be used to study the effect of leucine on protein synthesis in the intact animal.

2. The effect of 100μmoles of leucince on protein synthesis

To examine the ability of leucine to alter protein synthesis 150μmoles of (^{3}H)phenylalanine per 100g body weight were given alone or with 100μmoles of unlabelled leucine per 100g. Control, starved and protein-deprived animals were studied. Starved and protein-deprived animals were included because the stimulatory effect of leucine had been demonstrated in highly catabolic states (e.g. incubated muscles, starving man) and also because they provided states with contrasting levels of plasma amino acids. Plasma levels of branched-chain amino acids are lower in protein-deprived animals (Morgan and Peters, 1971), while in starvation they are increased relative to control levels (Millward et al., 1976). Liver, jejunal mucosa and serosa were studied to determine whether or not the results presented in section III were influenced by the leucine which was used to measure the rate of synthesis. Because the stimulatory effect of leucine has been reported in muscle (Rannels et al., 1974; Buse and Reid, 1975; Fulks et al., 1975; Li and Jefferson, 1978), two types of muscle were also studied; skeletal muscle (gastrocnemius) and cardiac muscle. Synthesis rates in the presence and absence of a large amount of leucine are shown in Table IV.3. The synthesis rate per unit RNA has been calculated (Table IV.4) to check that difference in synthesis rates did not arise because of differences in RNA levels between two groups.

Table IV.3

Fractional synthesis rates (%/d ± SEM, n = 6) in several tissues measured with 150μmoles of (^{3}H)phenylalanine per 100g body weight in the presence and absence of 100μmoles of leucine. None of the differences between leucine and no leucine is statistically significant.

	CONTROL		STARVED		PROTEIN-FREE	
	no leu	leu	no leu	leu	no leu	leu
Liver	86.3 ± 5.6	82.1 ± 2.6	71.8 ± 2.9	73.6 ± 2.1	69.4 ± 4.3	68.6 ± 1.1
Jejunal mucosa	123 ± 4	117 ± 3	92.0 ± 3.2	101 ± 4	94.9 ± 6.0	99.7 ± 1.6
Jejunal serosa	52.1 ± 2.0	56.5 ± 2.8	31.4 ± 1.2	31.2 ± 1.2	(not measured)	
Gastrocnemius	16.9 ± 0.6	18.6 ± 0.9	5.83 ± 0.33	5.76 ± 0.63	4.05 ± 0.65	3.51 ± 0.25
Heart	19.6 ± 0.8	20.1 ± 0.7	11.9 ± 0.7	10.7 ± 0.9	10.3 ± 0.2	10.6 ± 0.4

Table IV.4

Protein synthesised per RNA (mg protein/mg RNA ± SEM, n = 6) in several tissues. Protein synthesis was measured with 150μmoles of (^{3}H)phenylalanine per 100g body weight in the presence and absence of 100μmoles of leucine. None of the differences between + and - leucine is statistically significant

	CONTROL		STARVED		PROTEIN-FREE	
	no leu	leu	no leu	leu	no leu	leu
Liver	17.5 ± 0.7	15.6 ± 0.6	14.5 ± 0.7	15.9 ± 0.7	12.2 ± 0.4	12.1 ± 0.3
Jejunal mucosa	17.9 ± 0.7	16.8 ± 0.5	14.1 ± 0.6	16.0 ± 0.8	12.1 ± 0.8	12.4 ± 0.3
Jejunal serosa	18.2 ± 0.6	21.0 ± 1.5	17.8 ± 0.5	17.6 ± 0.5	(not measured)	
Gastrocnemius	16.7 ± 0.8	18.5 ± 1.2	8.45 ± 0.55	8.57 ± 1.11	10.3 ± 1.4	8.45 ± 0.47
Heart	12.5 ± 0.6	12.5 ± 0.7	9.10 ± 0.42	8.91 ± 0.88	9.26 ± 0.22	9.02 ± 0.38

One explanation for the failure to demonstrate an effect of leucine on protein synthesis is that the ten minutes of incorporation was too short for the effect of leucine to become apparent. Most experiments which have demonstrated an effect of leucine have measured rates of synthesis after incubation with leucine lasting about an hour (e.g. Buse and Reid, 1975; Fulks et al., 1975). Consequently, protein synthesis was measured in several tissues of starved rats one hour after the animals had received an intraperitoneal injection of 100μmoles of unlabelled leucine per 100g body weight. Starved animals were studied since those studies which had reported an effect of leucine had been carried out under conditions of net catabolism (i.e. incubated muscles and starving man). A disadvantage of using starved animals is that because individual animals are affected by starvation to different degrees, the groups are not as homogeneous as one would like for discerning a difference caused by the injection of leucine. The results of this experiment are shown in Table IV.5. In no tissue was the difference in synthesis rate measured after leucine significantly different from rates measured in animals which had not received leucine. The synthesis rates for gastrocnemius muscle are interesting. In the group of animals which received leucine the synthesis rate was 10% higher than in those animals which had not received leucine. However, the amount of protein synthesised per unit RNA was not different in the two groups, suggesting that the observed difference in synthesis was due to a difference in the amount of RNA between the two groups. Reported values for the turnover rate of RNA (Grimble and Millward, 1977) would suggest that an increase in RNA is unlikely to have been produced by the injection of leucine over such a short period of time, but probably reflects a difference which existed between the groups before the experiment was performed.

Table IV.5

The effect of 1 hour's pretratement with 100μmoles of leucine per 100g body weight on protein synthesis in several tissues of starved rats.

Protein synthesis was measured in two-day starved rats with 150μmoles (4-^{3}H)phenylalanine per 100g body weight 1hr after an intraperitoneal injection of 100μmoles of leucine per 100g body weight. Control animals received no injection prior to the measurement of synthesis. Results are expressed as means ± SEM (n = 6) and none of the differences between leucine and no leucine is statistically significant.

	CONTROL		LEUCINE-TREATED	
	k_s	syn/RNA	k_s	syn/RNA
Liver	66.0 ± 2.5	13.7 ± 0.5	67.0 ± 2.3	14.2 ± 0.5
Jejunal mucosa	93.9 ± 2.1	13.0 ± 0.2	88.8 ± 4.1	13.3 ± 0.5
Jejunal serosa	34.6 ± 1.8	19.2 ± 0.8	35.5 ± 2.1	17.7 ± 0.9
Gastrocnemius	4.48 ± 0.42	8.28 ± 1.04	5.00 ± 0.31	8.25 ± 0.57
Heart	12.2 ± 2.0	11.1 ± 2.1	10.1 ± 1.3	8.71 ± 0.93

As has been discussed before (section I, pp. 30-43, section III, pp. 125,139) correct measurement of the specific radioactivity of the amino acids used for protein synthesis is absolutely essential for reliable estimates of synthesis rates. A comparison of the specific radioactivities of free phenylalanine in tissues from all the leucine experiments is presented in Table IV.6. The differences in specific radioactivity, between animals receiving leucine and those which did not, are quite small, suggesting that leucine did not influence the uptake of labelled phenylalanine or the turnover rate of free phenylalanine pool to any appreciable extent. Consequently an effect of leucine on protein synthesis, undetected because of concomitant changes in the specific radioactivity of the precursor can be ruled out.

It would seem, therefore, that the effect of leucine on protein metabolism is a paradox: demonstrable in incubated muscle from the rat and fasting man but not demonstrable by measurement of rates of protein synthesis in tissues of rats in vivo. Despite the fact that leucine has been shown to stimulate the release of insulin (Floyd et al., 1963; Fajans et al., 1967) no change in synthesis rate was observed either over 10 minutes or over the subsequent hour. The first possibility which must be examined is whether the magnitude of change expected with leucine is sufficiently large to be observable in the tissues which were measured in vivo. The improvement in nitrogen balance reported by Sherwin (1978) was about 3g N per day. If turnover is assumed to be about 30g N per day (Waterlow et al., 1978) then the improvement is about 10%. An increase in synthesis distributed equally among all the tissues might be difficult to confirm experimentally, although the data in Tables IV.3 and IV.5 do not suggest even a small increase in all tissues. Moreover, in perfused liver, where the effect of leucine has been specifically studied, the results did not suggest that leucine

Table IV.6

The specific radioactivity of free (^{3}H)phenylalanine ($\bar{S}_A$ calculated over 0 - 10 min, expressed in dpm/nmol ± SEM, n = 6) from several tissues of rats injected with 150μmoles (4-^{3}H)phenylalanine per 100g body weight with or without 100μmoles of unlabelled leucine per 100g body weight

	Control		Starved		Protein-free		Starved	
	no leu	leu	no leu	leu	no leu	leu	no leu	leu, 1h
Liver	381 ± 8	388 ± 3	378 ± 4	384 ± 4	750 ± 1	769 ± 4	361 ± 3	361 ± 3
Jejunal mucosa	321 ± 3	340 ± 4	361 ± 1	359 ± 1	736 ± 3	727 ± 7	334 ± 2	345 ± 3
Jejunal serosa	361 ± 5	361 ± 4	365 ± 3	363 ± 1	(not measured)		298 ± 5	319 ± 6
Gastrocnemius	398 ± 1	388 ± 4	363 ± 5	365 ± 3	764 ± 4	800 ± 3	372 ± 3	376 ± 2
Heart	368 ± 4	380 ± 2	321 ± 5	335 ± 6	738 ± 5	689 ± 7	325 ± 1	343 ± 4

affected protein synthesis at all (Woodside and Mortimore, 1972). If one assumes that the improved nitrogen balance was due to an increase in muscle protein synthesis only, then the effect of leucine should be apparent. Waterlow et al. (1978) have suggested that muscle protein synthesis contributes about 14% of the total synthesis of protein in the rat. If this is the case the synthesis rate in muscle would have to double to produce the observed change in nitrogen balance. Clearly, changes of this magnitude would not go undetected.

In trying to resolve the paradox it is necessary to review the studies discussed earlier. The studies of Sapir and Walser (1977) and Sherwin (1978) are important because they demonstrate an effect of leucine (or the ketoacid analogue of leucine) in the intact animal (man). In these studies the observed effect was an improvement in nitrogen balance, not synthesis, per se. Sherwin's observation (1978) of unaltered 3-methylhistidine excretion would argue against the effect on nitrogen balance being due to reduced degradation of muscle protein, though some question about the interpretation of data on 3-methylhistidine excretion has been raised by Millward et al. (1980). Furthermore, alternative amino acids, sources of nitrogen and sources of energy were not investigated, so there is some doubt about the observed effect being due specifically to leucine.

The in vitro experiments on tissues of the rat have been more thoroughly investigated with alternative amino acids and energy sources, though the results are not completely consistent. Other branched-chain amino acids (valine and isoleucine) were reported to have no effect on protein synthesis in incubated diaphragm (Buse and Reid, 1975) or perfused heart (Chua et al., 1979). By contrast Fulks et al. (1975) reported that neither valine not isoleucine had an additional effect on protein degradation in incubated diaphragm but that both had an additive

effect on protein synthesis. Metabolites of leucine did not have an effect on protein metabolism in diaphragm (Buse and Weigand, 1977) but did have an effect in perfused heart (Chua et al., 1979a). Alternative oxidisable substrates such as β-hydroxybutyrate, palmitate or octonoate had no effect in diaphragm (Fulks et al., 1975) or perfused hemicorpus (Li and Jefferson, 1978) but did have an effect in perfused heart (Rannels et al., 1974; Chua et al., 1979a).

A plausible explanation for contrasting effects of leucine in different tissues *in vitro* is that suggested by Fulks et al. (1975). They have suggested that because leucine, isoleucine and valine are degraded in muscle (Odessey and Goldberg, 1972; Goldberg and Odessey, 1972), and the rate of oxidation of these amino acids can be increased in conditions such as fasting (Goldberg et al., 1974) and diabetes (Odessey et al., 1974), that under certain circumstances these amino acids might become limiting for protein synthesis. In perfused liver the amino acids which have been shown to influence turnover are tryptophan, proline, methionine and phenylalanine (Woodside and Mortimore, 1972); those which are metabolised in liver. Leucine, isoleucine and valine, which are not degraded in liver, also did not seem to affect turnover. A way of reconciling the *in vitro* results and those obtained *in vivo* would then be able to assume that leucine becomes limiting in incubated muscle because it is being rapidly metabolised, but that *in vivo* this situation does not arise either because the amino acid is not as rapidly metabolised or because the degradation of protein in tissues such as liver helps to maintain higher levels of leucine.

The experiments reported here emphasize the importance of studies carried out in the intact animal. Although a system of regulation has been demonstrated *in vitro*, it does not mean that it is necessarily of importance in the whole animal. Studies by Morgan and co-workers

(personal communication) on the perfusion of a working heart tend to support the conclusion that under conditions which are nearer to those *in vivo*, leucine does not have a significant effect on protein synthesis.

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Appendix 1. Buffers used with the amino acid analyser

pH 2.2 Loading buffer, 2 litres :

Citric acid (Analar)	42g
Sodium hydroxide (Analar)	16.8g
Brij 35 (25% w/v)	4ml
Sodium n-octanoate (Analar)	0.2g
Conc. HCl (Analar)	32ml

pH 3.2 0.2M sodium citrate, 10 litres

Citric acid	210g
Sodium carbonate (Analar)	105.5g
Sodium n-octanoate	1g
Conc. HCl	75ml

Degassed under vacuum. Brij 35 added to 0.2%. pH adjusted to 3.60.

Diluent buffer

deionized water containing 0.01% sodium n-octanoate and 0.2% Brij 35.

Ninhydrin reagent

2-methoxy-ethanol (East Anglia)	3125ml
4M sodium acetate pH 5.5 (BDH)	1250ml
Ninhydrin (Analar)	60g
Titanium trichloride dolution (BDH)	2 x 10 ml
Deionized water	625 ml

Appendix 2. Tritosol scintillator (Fricke, 1975)

per litre :	PPO	3g
	triton X-100	257ml
	ethylene glycol	37ml
	absolute ethanol	106ml
	xylene	600ml