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PROTEIN METABOLISM IN DIABETES

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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E.C. Albertse

Abstract of Ph.D. Thesis

PROTEIN METABOLISM IN DIABETES

The rate of whole body protein turnover was measured in uncontrolled diabetic patients by [^{15}N]glycine administration. Before insulin treatment commenced, protein synthesis and breakdown rates were within the normal range despite hyperglycaemia and a negative N-balance. Paradoxically, insulin treatment resulted in a decrease in protein synthesis and an improvement in negative N-balance. The latter was thought to result from the even larger decrease in breakdown.

Whole body protein turnover was therefore investigated in the streptozotocin diabetic rat. [^{14}C]leucine was administered by constant infusion and excretion of the label was determined in expired CO_2 . Protein oxidation was dramatically increased by diabetes and reduced to near normal values by pre-treatment of the rats with insulin. In contrast to the protein synthesis results in diabetic patients, whole body protein synthesis in these rats were significantly decreased by diabetes and normalized by insulin.

However, much larger decreases were observed in parallel studies on protein synthesis in individual rat tissues. Tissue protein synthesis was measured by constant infusion of [^{14}C]tyrosine in control and diabetic rats. The gastrocnemius muscle showed a pronounced decline in fractional synthesis rate, but no change was detected in kidney protein synthesis rate despite a state of hypertrophy as reflected by increased protein mass. Liver protein synthesis appeared to be unaffected by diabetes, and subsequently

a more appropriate method was used for measuring protein synthesis in rapidly turning over tissue like liver.

Incorporation of label was measured 10 minutes after injecting a large dose of [^3H]phenylalanine. Protein synthesis rates were determined in liver, kidney and five muscles of control (diabetic rats treated with insulin) and diabetic (insulin withdrawn) groups. In all five muscles protein synthesis was decreased by insulin withdrawal, with the most rapid and pronounced decline being shown by the gastrocnemius. Again the kidney appeared to be unaffected. Protein breakdown rates, calculated from the changes in protein mass at the time of synthesis measurement, increased progressively in all five muscles with the duration of diabetes. With this method it was demonstrated that diabetes does reduce the overall rate of liver protein synthesis and similarly that of albumin synthesis. Total liver breakdown rates were elevated after one day of insulin withdrawal and thereafter dropped to below control values.

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SECTION 1INTRODUCTION

Severe muscle wasting and a negative nitrogen balance are only some of the metabolic abnormalities associated with the marked hyperglycaemia of uncontrolled diabetes. This disease was described centuries ago as "a melting down of the flesh into the urine" and it is this protein catabolic aspect of diabetes that is the concern of this investigation.

The interrelationship between protein metabolism and the essential anabolic role of insulin was illustrated soon after the discovery of this hormone in 1922 (Banting & Best, 1922). This was shown by the dramatic doubling in body weight and of apparent muscle mass in previously untreated juvenile diabetics within days after commencement of insulin therapy.

Subsequent work by Atchley et al. (1933), Butler et al. (1947) and Danowsky et al (1949) illustrated clearly that in the uncontrolled diabetic, a rapid deterioration of nitrogen balance takes place with severe losses in the ketotic state. Re-establishment of insulin therapy would correct the abnormalities in carbohydrate metabolism within 24 hours, whereas normalization of the negative nitrogen balance required a few weeks.

These effects were confirmed by Leutscher (1942), who demonstrated that insulin not only promoted positive nitrogen balance, but also reduced the elevated plasma amino nitrogen levels in these untreated diabetic patients. Nabarro et al. (1952), reporting on patients recovering from severe diabetic ketosis, showed cumulative negative nitrogen balances of 39 grams over 8 to 12 days, despite total dietary

intakes of 77 grams of nitrogen over this period. They predicted that normalization of nitrogen balance probably required several weeks. This was confirmed by Walsh et al. (1976) who used a neutron activation technique to follow the time course of nitrogen and potassium retention in newly diagnosed diabetics starting insulin therapy. Again, restoration of normal cellular potassium and nitrogen levels required up to 6 - 8 weeks.

The true mechanism of this negative nitrogen balance of uncontrolled diabetes in man has yet to be defined. The essential anabolic function of insulin has been demonstrated in diabetic rats in vivo and with tissues from diabetic rats incubated or perfused in vitro. All support the view that insulin promotes protein synthesis and inhibits protein breakdown (Pain, 1980).

In diabetic humans, only one preliminary study on protein metabolism has been reported (Waterlow et al., 1977). By using techniques which measure whole body protein turnover in man, the overall rate at which protein is synthesized or degraded can be defined. Furthermore, with this method the process primarily responsible for the diabetic nitrogen imbalance can be identified in greater detail. Waterlow et al. (1977) measured whole body protein turnover with [¹⁵N]-glycine in two diabetic patients before and after insulin treatment. In both subjects the rate of synthesis was depressed in the untreated state, and as predicted from the known anabolic effects of insulin, protein synthesis was enhanced by insulin therapy.

It appears therefore that an effective insulin treatment regime results in improvement of both carbohydrate and protein abnormalities present in severe diabetes. However, on a day-to-day basis, fluctuation in diabetic control, despite strict insulin and dietary regimes, has been a cause of great concern. Improved diabetic control is generally accepted to be directly related to retardation or even prevention of

the onset of development of microvascular complications (Hollman & Turner, 1977; Tchobroutsky, 1978; Molnar et al., 1979). The value of conventional criteria for measuring diabetic control, i.e. blood and urinary glucose levels, has been questioned by findings such as the prevalence of stunted growth or even "diabetic dwarfism" in juvenile diabetics (Tattersall & Pike, 1973). Alberti (1973) summarized this dilemma by stating that measurement of a single parameter such as plasma glucose can give only partial information on a multi-metabolite, multisystem disease state.

The possibly detrimental effect of longstanding abnormalities in protein metabolism in diabetes and its relationship to adequate control has yet to be clarified. It has been suggested that most well-controlled diabetics on insulin probably have subnormal levels of many amino acids for at least some part of each day, a state which could be potentially harmful (Carlsten et al., 1966). In fact, the elevation of plasma branched-chain amino acids in uncontrolled diabetes, which may represent an abnormality of peripheral protein metabolism, could possibly be used in conjunction with plasma glucose to monitor the adequacy of insulin control (Berger et al., 1978).

In 1938, Mirsky made the following statement: "An examination of the prevailing views concerning the influence of insulin on protein metabolism suggests that this hormone exerts a paradoxical action". In the discussion that follows on data published in the 40 years since 1938, it will be shown that our knowledge to date can only confirm Mirsky's view.

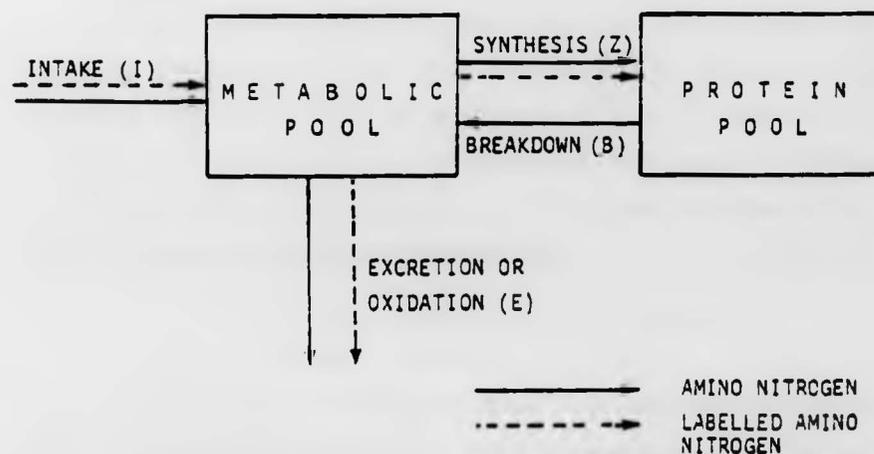
The aim of the present study was therefore to investigate the effect of diabetes and subsequent insulin therapy on protein metabolism in humans and animals, with more exact identification of the effects on protein synthesis, breakdown and oxidation. To accomplish these aims, several different methods were employed, each chosen for a specific

purpose or advantage. This will be illustrated in the following discussion of methods that are available for measurement of protein turnover in humans and animals.

1.1 PROTEIN TURNOVER METHODOLOGY

1.1.1 Introduction

In muscle wasting diseases such as diabetes and starvation, nitrogen balance studies can provide only limited information. The negative nitrogen balance of these physiological states conventionally implies an enhanced protein breakdown rate, but this is not necessarily true. Certainly nitrogen excretion data alone cannot determine the exact nature of protein metabolism. The negative nitrogen balance results not only from an imbalance between dietary input and nitrogen excretion, but also from an imbalance between protein synthesis and protein breakdown (Waterlow et al., 1978a). The relationship between nitrogen balance and protein turnover is illustrated in the following simplified model as first suggested by Sprinson and Rittenberg (1949).



Protein turnover is defined as a continuous dynamic state maintained by ongoing synthesis and breakdown of tissue proteins. Measurement of the rates of these two processes is possible by introducing an isotopically labelled amino acid into the metabolic pool and following the transfer of label between the pools. Three different approaches have been used. Firstly, the rate of disappearance from the metabolic or free amino acid pool would indicate rates of synthesis and oxidation. This method has largely been used in studies with humans and large animals where tissue sampling is difficult. Secondly, uptake of label and incorporation into the protein pool would give an estimate of protein synthesis, a method suitable only if tissue samples can be taken. Thirdly, after incorporation into protein, subsequent disappearance from the protein pool or rate of loss of label would be a measure of the rate of protein breakdown.

These three approaches will be discussed. Reasons will be given why some methods were chosen and others rejected. Detailed reviews on protein turnover methodology has recently been published by Waterlow et al (1978a) and Garlick (1979).

1.1.2 Measurement of whole body protein turnover

The rate at which amino nitrogen enters the metabolic pool (by intake or breakdown) or leaves from the pool (by synthesis or oxidation) is termed flux (Q). If the amino nitrogen (^{14}C or ^{15}N amino acids) is introduced into this pool at a constant rate, the specific activity of the ^{14}C amino acid or the abundance of ^{15}N reaches a plateau value (S_{max}). Flux can then be calculated from

$$Q = \frac{i^*}{S_{\text{max}}} \quad \text{eqn. (1)}$$

where i^* is the rate of infusion of label. If measurements are made over a relatively short period, it can be assumed that no label will

re-enter the metabolic pool from protein breakdown. If the rate of food intake (I) is known and that of amino acid oxidation and excretion (E), rates of synthesis (Z) and breakdown (B) can be calculated from flux by

$$Q = Z + E = B + I \quad \text{eqn. (2)}$$

Either ^{14}C amino acids or stable isotopes of ^{15}N labelled amino acids can be used to obtain Q.

^{14}C Amino acids: With constant infusion of a ^{14}C amino acid, a plateau will be reached in the specific activity of free amino acid in plasma (S) and in the production of $^{14}\text{CO}_2$ (e). In man infused with [^{14}C]leucine, this occurs within 8 hours (Golden & Waterlow, 1977) and in the rat within 2 hours (Sketcher, 1976). If it is assumed that the specific activity in the plasma is the same as that in the metabolic pool of the model, then flux can be obtained from equation 1.

When a carboxyl labelled amino acid such as L-[^{14}C]leucine is infused, leucine enters the oxidative pathway in addition to protein synthesis. The early decarboxylation step removes the ^{14}C label (Adibi, 1976) and re-utilization is prevented by release of the ^{14}C label directly into the bicarbonate pool, from which it is excreted in expired air as $^{14}\text{CO}_2$. In man approximately 20% of the label will be retained in the body (James et al., 1976), but in the rat the label is quantitatively excreted (Sketcher, 1976). The production of $^{14}\text{CO}_2$ is therefore an estimate of oxidation of the ^{14}C amino acid (E). Protein synthesis can therefore be calculated from equation 2 as flux (Q) minus oxidation (E). Similarly if the intake of leucine from food (I) is known, the rate of protein breakdown can be determined as flux minus intake.

The use of ^{14}C amino acids for measuring whole body protein turnover has the advantage of less cost in isotope and of less time

required to reach a plateau in specific activity. However, additional equipment for collection of $^{14}\text{CO}_2$ is needed and the use of a ventilated hood or Douglas bag in patients who are ill adds extra complications. This method of constant infusion of L-[^{14}C]leucine was therefore used in estimating whole body protein turnover only in diabetic rats. An additional advantage was the measurement of whole body oxidation rates in diabetic and insulin treated diabetic rats, information that was not available at the time.

^{15}N labelled amino acids: In humans the use of radioactively labelled ^{14}C amino acids are ethically limited to patients beyond child-bearing age. Stable isotopes, i.e. ^{15}N amino acids, can however be used in a similar way as ^{14}C amino acids, since with a constant infusion the excretion of ^{15}N in urine will also reach a plateau. Since urinary N is a product only of oxidation of amino acids in the metabolic pool, this plateau will be the same as that in the metabolic pool and can be used to calculate flux.

The excretion of ^{15}N in urine can be measured in either urea or ammonia as end product. Measurement with urinary urea as end product was rejected in this study since a plateau of ^{15}N abundance is reached only after 2-3 days in adults (Steffee et al., 1976). This long period was obviously not suitable for measurement of rapid changes in protein turnover which one would expect to find in the acute phases of diabetes. In contrast, a more rapid plateau is reached with ammonia as end product and measurements can be completed within 12 hours (Waterlow et al., 1978a). This was the end product measurement chosen in the present study. [^{15}N]glycine was the amino acid used since it is easily available at a reasonable price.

The measurement of protein turnover with [^{15}N]glycine and estimation of ^{15}N enrichment of urinary ammonia requires consideration of the

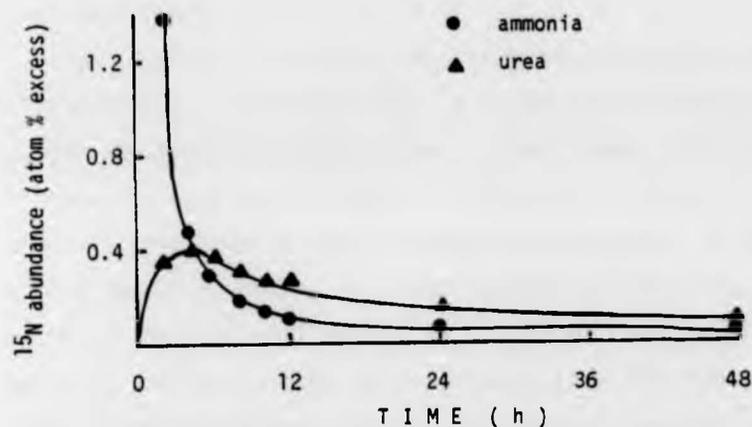
following assumptions (Waterlow et al., 1978a) :

(a) The metabolism of the tracer amino acid must reflect that of the total amino nitrogen, meaning that the proportion of the dose which is excreted as ^{15}N in the end product equals the proportion of flux which is excreted in the end-product. Given that the isotope must not contribute preferentially to the production of the end product, glycine is probably not the best amino acid to use, because it has been suggested to contribute to the formation of urinary ammonia (Pitts & Pilkington, 1966), but there is no evidence that this invalidates the answer. However, availability and relative cost has determined the general use of [^{15}N]glycine.

(b) The synthesis of the end product and the synthesis of protein must occur from the same precursor pool. In the case of urea, the end product reflects liver urea synthesis and in the case of ammonia, synthesis in the kidney, mainly from glutamine. In practice, ammonia seems to give similar values to urea and to values obtained by [^{14}C]-leucine infusion (Golden & Waterlow, 1977; Garlick et al., (1980a). Even when different rates were obtained using ammonia and urea as end products, both end products indicated the same difference between groups of subjects.

In diabetes an additional problem arises in measurement of ^{15}N abundance in ammonia as end product, namely the development of keto-acidosis in the severely uncontrolled diabetic. This shift in acid-base balance does influence ammonia production by the kidney, but it is not known how increased ammonia production will affect ^{15}N excretion. This problem was avoided by selecting only non-ketoacidotic patients. In fact, the data of Waterlow et al. Ch.17(1978a) showed no apparent change in total ammonia excretion in diabetic patients before and after insulin therapy.

In the study to be presented, [^{15}N]glycine was given as a single oral dose and not by constant infusion, since this required the least involvement from the patient. Instead of a plateau, the ^{15}N abundance in urinary ammonia falls rapidly to very low values, as shown in this figure from Waterlow et al. (1978a) :



Time course of excretion of ^{15}N in ammonia and urea after a single dose of [^{15}N]glycine

A time (t) can be defined as the point when the isotope has been effectively eliminated from the metabolic pool, but before protein breakdown occurs and label re-enters the pool. From the figure it can be seen that this point occurs at about 9-12 hours. The figure also shows the complications involved in using urea as end product, since a similar point is less easily detected. Since the turnover of the urea pool is very slow, excretion of label is delayed and t is difficult to define.

With the single oral dose, flux is then calculated from the following equation, which is analogous with equation (1) used to

calculate flux from constant infusion,
i.e.

$$Q = \frac{d^*}{\int_0^t S \cdot dt}$$

where d^* is the oral dose of isotope and the integral is the area under the curve for ^{15}N abundance in urinary ammonia against time. The integral is computed from one or more urine collections between times zero and t .

Two further points have to be considered with administering $[^{15}\text{N}]$ glycine as a single oral dose, i.e. the size of the dose and whether the isotope is given before or after a meal. The size of the $[^{15}\text{N}]$ glycine dose has been shown to effect the excretion of label and hence the calculated rates of synthesis and breakdown. With influx of a large amount of isotope, i.e. more than 300mg $[^{15}\text{N}]$ glycine, a high proportion of the label will be excreted, since the label enters the metabolic pool as a single non-tracer amino acid (Waterlow et al., 1978b). Administration of the labelled amino acid with or without food has also been shown to be important. If the isotope is given with a meal, absorption will delay entry of label into the metabolic pool, thus minimizing the large dose effect. In addition, there is also a direct effect of food intake on the rate of protein synthesis (Garlick et al., 1980a). Hence it is important that the rate of food intake at the time of measurement is accurately controlled. In the present study, 100 mg $[^{15}\text{N}]$ glycine was given as a single oral dose and the time of administration, i.e. before or after a meal was tested and modified in several protocols.

1.1.3 Measurement of the rates of protein synthesis in individual tissues

An alternative method of determining protein synthesis is by

measurement of incorporation of label into the protein pool. Since this method requires removal and analysis of tissues, this approach has been more commonly used in animal experiments, although the use of muscle biopsies for protein synthesis measurements in humans has been reported (Lundholm & Schersten, 1975; Halliday & McKeran, 1975).

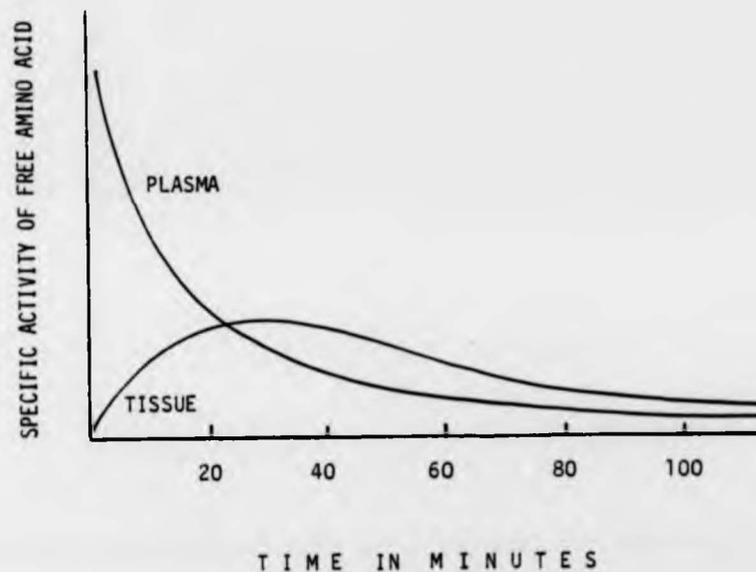
Two measurements need to be made on each tissue, i.e. of the amount of label incorporated into protein and secondly of the specific activity of the free amino acid at the site of protein synthesis. The direct precursor of protein synthesis, i.e. the aminoacyl-tRNA can be measured as shown by Airhart et al. (1974) and Khairallah & Mortimore (1976) in liver, but this is technically difficult. For routine use specific activity of the free amino acids in the tissue (intracellular pool) has been determined. Sometimes that in the plasma (extracellular pool) has also been used. However, both precursor pools present problems in interpretation of results, since the actual precursor pool is probably compartmented (Mortimore et al., 1972). It is probably wise to measure the specific activity in both pools and interpret the results with the possibility of either under- or over-estimating the actual rate of synthesis (Garlick et al., 1973).

In the present investigation of protein synthesis in individual tissues of diabetic rats, two methods were employed which minimized the errors involved in choice of the precursor pool, i.e. constant infusion of a tracer dose of labelled amino acid (Section 6) or by single injection of a non-tracer dose of labelled amino acid (Section 7).

1.1.3.1 Constant infusion of a tracer dose of labelled amino acid

If a tracer amount of a labelled amino acid is given as a single injection, the specific activity of the free amino acid in plasma falls rapidly to very low values. The intracellular specific activity

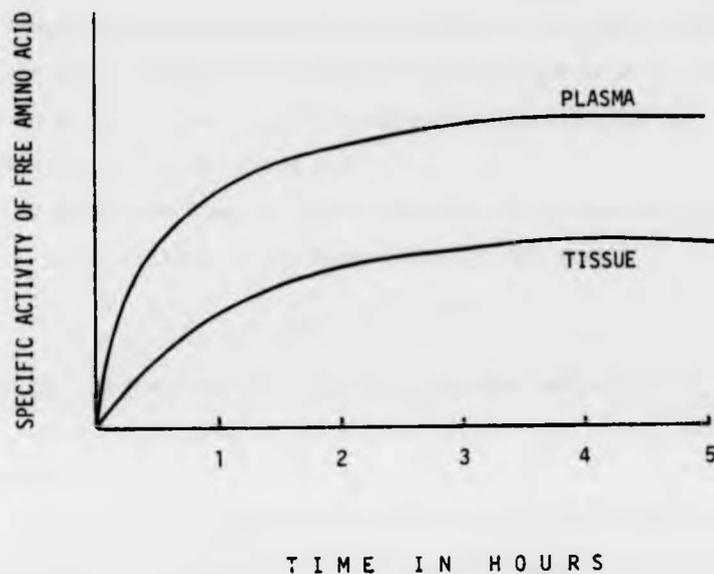
initially rises, but then also declines as shown in the following figure :



From: Garlick (1979)

To determine the time course of these changes, groups of animals would have to be sacrificed at timed intervals, making this a tedious and expensive approach.

The changes in specific activity can be more accurately defined with constant infusion of the tracer labelled amino acid. This is illustrated in the following figure :



From: Garlick (1979)

With continuous administration of the isotope, the specific activity of the free amino acid pool in plasma rapidly rises to a plateau. A plateau is also reached in specific activity of free amino acids in the tissue, but this plateau is lower and takes longer to be reached. The advantage of this method is that a single measurement at the end of the infusion can define the entire time course of specific activity (Waterlow & Stephen, 1968). Continuous administration of the isotope can be achieved by giving the label as part of an agar gel diet, (Harney et al., 1976), but more consistent results are obtained by constant intravenous infusion, the method employed in this study (Section 6).

Whereas in whole body protein measurements synthesis was defined as the absolute rate of grams protein synthesized per unit time (e.g.

g protein per day), in single tissues this will be expressed as a fractional synthesis rate (k_s). This is the absolute rate divided by the amount of protein present and represents the proportion or percentage of the protein content of that tissue which is replaced per unit time (e.g. % per day). Fractional synthesis rates are then calculated in the following way :

Waterlow and Stephen (1968) showed that the rise to plateau in plasma can be fitted to the exponential of the form

$$S_p = S_{p \max} (1 - e^{-\lambda_p t}) \quad (4)$$

where S_p is the specific activity of plasma amino acid, $S_{p \max}$ the value of S_p at plateau, t the period of infusion and λ_p the rate constant.

In tissues the specific activity of the free amino acid, which is assumed to be the precursor for protein synthesis, also rises to a plateau. This plateau is lower than that in plasma because of dilution by unlabelled amino acids from protein breakdown. The rate constant (λ_t) describing the time course to plateau in tissue is dependent on the type of tissue and the amino acid infused. With tyrosine as amino acid, λ_t in liver can be taken to equal λ_p .

In liver and kidney, tissues with rapid rates of protein turnover, the specific activity of free amino acid (S_t) is given by :

$$S_t = S_{t \max} (1 - e^{-\lambda_t t}) \quad (5)$$

where λ_t is the rate constant for the rise of specific activity to plateau and $S_{t \max}$ the plateau value of S_t .

In skeletal and cardiac muscle, where the appropriate value of λ_t is Rk_s , the time course is given by a different function :

$$S_t = S_{t \max} (1 - e^{-Rk_s t}) \quad (6)$$

where R is the ratio of protein-bound to free amino acid content of the tissue and k_s the fractional rate of protein synthesis.

Incorporation of the free amino acid into protein, expressed as the specific activity of amino acid in the protein (S_B), is given by the equation (Zilversmit, 1960) :

$$\frac{dS_B}{dt} = k_s (S_i - S_B) \quad (7)$$

This equation is then solved, using the expressions shown above (equations 5 and 6) for S_i . This gives the fractional rate of protein synthesis, expressed as a function of the ratio of specific activities of free and protein-bound amino acid (S_B/S_i) at the end of the infusion, i.e. for liver and kidney :

$$\frac{S_B}{S_i} = \frac{\lambda_p}{(\lambda_p - k_s)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-\lambda_p t})} - \frac{k_s}{(\lambda_p - k_s)} \quad (8)$$

and for skeletal and cardiac muscle :

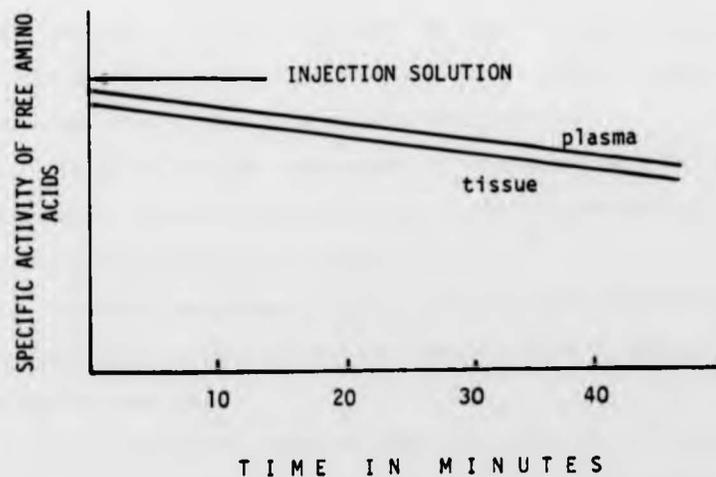
$$\frac{S_B}{S_i} = \frac{R}{R - 1} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-Rk_s t})} - \frac{1}{(R - 1)} \quad (9)$$

These last two equations are then solved graphically for k_s by plotting curves of k_s versus S_B/S_i at fixed values for t , λ_p or R . Values for k_s are then obtained from corresponding experimental values of S_B/S_i (Garlick et al., 1973).

In this study (see Section 6) [^{14}C]tyrosine was used since it rises rapidly to plateau in both plasma and tissues (Garlick et al., 1973) and an enzymatic assay was available for determining the specific activity (Garlick & Marshall, 1972).

1.1.3.2 Single injection of non-tracer amounts of labelled amino acid

Henshaw et al. (1971) demonstrated that when a dose of labelled amino acid large enough to flood the endogenous free amino acid pool was injected, the specific activity of the amino acid rose rapidly to a value close to that in plasma. Over the following 20 minutes they found that these specific activities remained essentially constant. McNurlan et al. (1979) did observe a slow decline in specific activity over this time, but the decline was linear, as is shown in the following figure :



From: Garlick (1979)

The mean specific activity of the intracellular amino acid during the first 10 minutes (\bar{S}_A) can be obtained by making measurements in

groups of animals killed at 2 and 10 minutes after injection. The fractional synthesis rate k_s can then be calculated from the equation :

$$k_s = \frac{S_B}{\bar{S}_A \cdot t} \times 100$$

where S_B is the specific activity of the free amino acid in protein at 10 minutes, \bar{S}_A the mean specific activity of the amino acid in the precursor pool between 0 and 10 minutes and t , time expressed in days. The value for \bar{S}_A was obtained by plotting the decline back to zero. For the value of S_A at zero it is assumed that the rate of decline between zero and 2 minutes is equal to the rate between 2 and 10 minutes.

Despite the larger numbers of rats required by this technique, there are three major advantages :

- (a) the small difference between the specific activities in tissue and plasma minimizes the errors involved in the correct choice of a precursor pool as the site of protein synthesis.
- (b) the short time of measurement of incorporation eliminates the problems of re-utilization of amino acids from proteolysis in tissues with rapid turnover such as gut and liver.
- (c) synthesis measurements during the 10 minute incorporation will include not only that of cellular hepatic proteins but also of the secretory proteins.

This method was therefore employed in Section 7 to estimate changes in protein synthesis rates during the very early stages of diabetes.

1.1.4 Measurement of the rates of protein breakdown in individual tissues

1.1.4.1 Decay of labelled amino acids

In theory it is possible to measure decay or loss of label from

the protein pool. The decay of total label in the protein then estimates the fractional rate of breakdown and decay of specific activity, the fractional rate of synthesis. In practice however, the interpretation of decay curves is difficult because of the following two problems :

(a) Recycling of the label: Since only 20% of amino acids are actually oxidized and excreted, recycling or reutilization of the remaining 80% for new protein synthesis will obscure measurement of the actual loss of label (Waterlow et al., Ch. 11, 1978a). This is a particular problem in measuring decay in tissues with slow turnover such as muscle, which continue to incorporate label released from tissues with more rapid turnover. Recycling can be reduced by using labels which are rapidly converted and excreted, e.g. [6-¹⁴C]arginine in liver (Swick, 1958; Schimke, 1964) and NaH¹⁴CO₃ in liver and muscle (Millward, 1970).

(b) Decay in mixed proteins: In mixed proteins from whole tissues decay is not exponential. Only in single purified proteins will label be lost exponentially and decay be linear when plotted on a semi-log axis. It is possible to analyse decay curves in heterogeneous tissues, but this requires measurement over very long periods of several days (Garlick et al., 1976). This approach is clearly impractical for measurements of rapid changes in the diabetic state.

For studying the turnover rates of individual enzymes in tissues, Arias et al. (1969) introduced the double label technique. This method involves injecting [¹⁴C]leucine a few days before sacrifice and [³H]-leucine a few hours before the rat is killed. The ³H activity is then a measure of the initial labelling and the ³H/¹⁴C ratio the rate of decay. This technique is particularly useful for comparing turnover rates of different proteins in the same tissue. However one of the major assumptions is that a steady state must be maintained when the two labels are injected, which is obviously difficult in diabetes.

Dice et al. (1978) therefore had to modify the double label method to investigate the rate of intracellular protein degradation in liver and muscle of diabetic rats. They injected the [^{14}C]leucine into an insulin treated diabetic rat, then withdrew insulin for three days and allowed the ^{14}C label to degrade in vivo before sacrifice. The [^3H]leucine was injected into a normal animal which was killed after 4 hours. However, even with this modification, the double label method can give only information about the relative turnover rates of one or two isolated proteins and it is difficult to extrapolate from these results to the rate of protein breakdown in a tissue in general.

Clearly the use of decay rates as measurement of protein breakdown presents major complications in investigating disease states such as diabetes. Most probably decay methods should be reserved for measurements on single purified proteins and not for measuring breakdown in heterogeneous whole tissue proteins. In the present study protein breakdown in diabetes was estimated by two methods of indirect measurement.

1.1.4.2 Indirect estimation of rates of protein breakdown

(a) Changes in protein mass: Fractional rates of protein breakdown (k_d) are inferred by the difference between measured rates of fractional synthesis (k_s) and the fractional rate of growth of protein mass (k_g) i.e.

$$k_d = k_s - k_g$$

The amount of growth of protein mass in the tissue is measured over a period of several days. k_g is then derived from the gradient of the growth curve divided by the protein mass of the tissue at the day that k_s was measured.

This time scale of difference between measurements of k_s and k_g implies that this method of deriving breakdown is subject to some

errors. In muscle, where k_s was measured by 6 hour constant infusion and k_g over several days, the effect of diurnal variations in muscle protein mass was shown to be small enough not to invalidate the interpretation of the data (Garlick et al., 1973). This method has been successfully used for estimating rates of breakdown in muscle (Turner & Garlick, 1974; Garlick et al., 1975; Millward et al., 1975, 1976).

In liver an additional correction must be made if k_s includes synthesis of both cellular and secretory proteins (e.g. with the large dose method), since synthesis of the secreted proteins contribute very little to hepatic protein mass. Thus hepatic breakdown rates should be derived from the difference between growth and synthesis of only cellular proteins. Synthesis rates of cellular proteins can be estimated from the difference between that of total hepatic proteins and synthesis of albumin and other secreted plasma proteins. This was the approach used in Section 7 where total hepatic protein synthesis was measured by a large dose of [3 H]phenylalanine. An alternative method was used by Scornik and co-workers to measure the synthesis rates of cellular and secretory proteins. Total protein synthesis was measured by label incorporation over 5 minutes only. The export protein component was estimated from the proportion of incorporated label which was lost between 7 and 180 minutes, further incorporation being prevented by inhibition of protein synthesis by injection of pactamycin at 5 minutes (Scornik & Botbol, 1976; Conde & Scornik, 1976).

(b) Excretion of 3-methyl histidine: Measurement of the rate of excretion of 3-methyl histidine is a non-isotopic approach to estimate muscle protein breakdown which eliminates all the problems of label recycling. 3-Methyl histidine is present in actin of all skeletal muscles and in myosin of white muscle. Synthesis takes place by addition of a methyl group to histidine after formation of the actin and myosin

peptide chains. When muscle protein is degraded, the 3-methyl histidine is quantitatively excreted without reutilization. Young et al. (1973) and Bilmazes et al. (1978b) have suggested that the measurement of 3-methyl histidine in urine can provide an in vivo index of the rate of skeletal muscle protein breakdown. This method has not to date been used to measure whole body muscle protein breakdown in diabetic humans, but it has been employed in studies of starvation (Young et al., 1973; Wassner et al., 1977; Ogata et al., 1978), in burn patients (Bilmazes et al., 1978a) and in rats on protein free diets (Haverberg et al., 1975; Omstedt et al. 1978). It has also been employed as an index of protein breakdown in perfused hindquarters of diabetic rats (Hansen et al., 1977).

In the present investigation, rates of 3-methyl histidine excretion were measured in urine of diabetic patients before and after insulin treatment (Section 3).

1.2 CHARACTERISTIC FEATURES OF DIABETIC ANIMAL MODELS

In the published data available on the effect of diabetes on protein turnover in animals, various methods were used to induce diabetes. In the ideal situation the animal model should exhibit the same metabolic abnormalities of human diabetes. The abnormalities present in each model are however dependent on the genetic, physical or chemical interventions that were employed to induce the diabetic state.

1.2.1 Genetic diabetogenic factors

Spontaneous diabetes occurs genetically in many animal species and is associated with metabolic abnormalities of hyperglycaemia, hyperinsulinaemia and obesity. However, because of species-specific differences in the severity of these abnormalities, the genetic strain of animal must be chosen with care to enable eventual comparison with a specific type of human diabetes. The different strains cover a wide

range of abnormalities, e.g. the 'fatty' rat with normoglycaemia despite hyperinsulinaemia; the Chinese hamster with severe ketosis, hyperglycaemia and hypoinsulinaemia and the New Zealand obese mice that are hyperglycaemic but do not develop ketosis or insulin deficiency (Herberg & Coleman, 1977). However, in some species, spontaneous progression towards normal glucose homeostasis can occur.

1.2.2 Dietary and chemical diabetogenic factors

Dietary factors have long been suspected of being one of the causes of the increase seen in diabetes in Western society. High carbohydrate diets or high fat-low carbohydrate diets are known to increase insulin requirements in animals (Trout et al., 1978). The duration of the dietary treatment can reduce this animal model from a transitory to a permanent form of diabetes (Somogyi & Gruenfield, 1962).

Temporary hyperglycaemia and glycosuria can be induced by frequent administration of high doses of somatotropin, prolactin, estrogen, the adrenal steroids or growth hormone (Williams, 1974). The injection of guinea-pig anti-insulin serum causes an acute but transient form of diabetes, due to immediate degranulation of the β -cells and binding or neutralization of endogenous insulin by the antibodies. However, repeated injections are required to maintain the diabetic state (Wright, 1959, 1960; Armin et al., 1960).

Permanent diabetes will result from removal of more than 80% of the pancreas, but this obviously decreases both insulin and glucagon levels. Thus pancreatectomy as an animal model of diabetes is probably of historical importance only.

Chemical destruction of the β -cell can be achieved by injecting either alloxan or streptozotocin. Until the 1960's, alloxan was commonly used to induce experimental diabetes. Alloxan causes immediate necrosis of the β -cells and atrophy of the Islets of

Langerhans, leaving the acinar tissue intact (Lukens, 1948). Within 24 hours hyperglycaemia and glycosuria develops and in later stages ketosis with highly elevated circulating concentrations of free fatty acids and ketones (Mansford & Opie, 1968).

In contrast to alloxan, streptozotocin causes hyperglycaemia and glycosuria without ketosis. In the present study streptozotocin was chosen as diabetogenic agent since the clinical population of diabetics that were to be investigated were of normal weight, non-ketotic and insulin-dependent.

1.2.3 Streptozotocin

Streptozotocin (N-methylnitroso-carbamylglucosamine) is derived from Streptomyces achromogenes and has antibacterial, carcinogenic and cytotoxic properties. In the rat, streptozotocin will selectively destroy pancreatic β -cells, with the α -anomeric form significantly more effective in binding at the receptor site on the β -cell surface (Rossini et al., 1977). Rossini and co-workers have suggested that it is this unknown α to β anomer ratio which may be the cause of variation in streptozotocin potency.

Streptozotocin causes a triphasic blood glucose response within 24 hours of injection. Initially there is a drug related but non-specific hyperglycaemia, followed by marked hypoglycaemia resulting from the release of insulin from necrotized β -cells. After 24 hours, diabetes with constant and severe hyperglycaemia and glycosuria will be present (Junod et al., 1969). The diabetogenic action of streptozotocin can be prevented by pre-treatment of the rat with nicotinamide. This protection is probably due to a prevention of development of reduced cellular NAD concentrations which generally follows injection with streptozotocin. This emphasizes the crucial role of NAD in the normal function of the pancreatic β -cell (Schein & Bates, 1968; Golden et al., 1971).

The diabetogenic action of streptozotocin can be directly related to dosage and age of the animal, with young rats markedly more resistant to the drug (Turner & Heard, 1972; Portha et al., 1974). Ketonuria can only be produced with very high doses but this also invariably leads to an early death. Therefore, by manipulating the dosage, it is possible to produce diabetes of graded severity (Schein et al., 1971).

The choice of appropriate controls, (e.g. age, weight or pair fed) for evaluation of results obtained from diabetic animals is complicated by the hyperphagia which develops within 4 to 6 days in diabetic rats (Booth, 1972) and insulin treated diabetic rats (Panksepp et al., 1975; Rezek, 1976).

1.2.4 Changes in food intake during diabetes and insulin treatment

Since amino acid supply has been suggested to influence protein synthesis (Munro, 1970), and starvation has been shown to result in decreased liver and gut protein synthesis (McNurlan et al., 1979), it was not unreasonable to expect that an increase in the supply of amino acids could affect protein turnover.

Normal rats have an impressive ability to balance their food intake against energy expenditure. In the normal rat a diurnal feeding pattern is present, with large meals and short intervals during the dark cycle and small meals during the light period (Panksepp, 1973). Appropriate adjustments in food intake take place after long-term force feeding or starvation, with restoration of normal body weight by either hypo- or hyperphagia. This adaptability is absent in rats with experimental lesions of the ventromedial hypothalamus (VMH), implicating the hypothalamus as having a central mediator role in controlling food intake (Stricker, 1978).

The ventromedial nucleus of the hypothalamus is generally accepted

as being the satiety center, having a dominant role in overriding the hunger effect of the hypothalamic lateral nucleus, or feeding center (Rezek, 1976). These hypothalamic centers appear to function primarily as integrators of incoming information via the enteroinsular axis, a concept formulated by Unger & Eisentraut in 1969. The enteroinsular axis concept proposes a peripheral, vagally mediated glucosensitive mechanism associated with the liver, the gastrointestinal tract and peripheral and central glucoreceptors.

Exogenous insulin administered experimentally to normal humans or rats consistently causes hyperphagia. Instead of its normal role as a satiety hormone, insulin now acts as a hunger-inducing hormone. Since exogenous insulin is injected into free-feeding animals generally during normoglycaemia, the effect of insulin is to cause marked hypoglycaemia. This stimulates the glucoreceptors and initiates feeding behaviour in the rat (Briese, 1971; Panksepp et al., 1975; Rezek, 1976).

In diabetes, cellular glucoprivation resulting from the insulin deficiency similarly stimulates glucoreceptors and thus increase food intake (Panksepp & Nance, 1972; Bray, 1974). Experimental diabetes can induce hyperphagia in rats within 5 to 7 days after administration of either alloxan or streptozotocin (Kumaresan & Turner, 1965; Panksepp et al., 1972; Booth, 1972). Food intakes vary from 30 - 50% above that of age and weight controls and has been shown to be strongly correlated with the degree of glycosuria and body weight of the diabetic rat (Sturtevant, 1954).

The hyperphagia of diabetic rats also causes changes in the actual feeding pattern of the rats, with disruption of the nocturnal eating rhythm to that of a constant intake of small meals with short intermeal intervals. Booth (1972), using experimental chambers with photoelectric cells and automated feeders, determined that diabetic rats are

much more likely to partially chew rat pellets and interrupt a meal feeding time.

Hyperphagia of the diabetic rat may be seen as a well controlled, compensatory response to a lack of utilizable fuels. In fact, in 1952 Brodsky showed that if insulin withdrawn diabetic rats were allowed to overeat, their survival could be prolonged despite severe glycosuria, polyuria and ketonuria. Decreased glucose utilization and liver glycogen and fat reserve depletion have all been investigated as possible causes of this hyperphagia. Recently Friedman (1978) demonstrated that diabetic rats systematically increased their food intake as the amount of fat in the diet was reduced, or ate normal amounts of high fat diets. It is interesting to note that the hyperphagia of exogenous insulin is also accompanied by decreased plasma free fatty acid levels.

In these hyperphagic diabetic animals, VMH lesions will reduce food intake, in contrast to its effect on normal rats in causing hyperphagia and overt obesity. This again emphasizes that the overeating of the diabetic animal is probably an active but fruitless attempt to store nutrients concomitant with its depletion of body stores. Furthermore, in the normal rat with VMH lesions, exogenous insulin will not cause hyperphagia as seen in the unoperated animal (Bray, 1974; Panksepp et al. 1975).

In conclusion, in the experimental work to be presented on streptozotocin diabetic and insulin treated diabetic rats, close attention was paid to the degree of hyperphagia. Food intakes were monitored in all experiments. In Section 5 some experiments were performed before the onset of hyperphagia to exclude possible effects on protein turnover.

In the following discussion of published work on the effect of

diabetes on protein synthesis and breakdown, the various diabetic animal models or diabetogenic agents used will be reported in the context of the different effects each could induce.

1.3 THE EFFECT OF INSULIN ON LEVELS OF CIRCULATING AMINO ACIDS

The early work by Mirsky (1938) on nephrectomized dogs and by Lotspeich (1949) on depancreatized dogs, suggested that insulin may stimulate protein synthesis, since they found that the same amino acids that disappeared from the plasma with insulin treatment, could be identified in the protein of skeletal muscle. Insulin release is triggered by intake of a mixed diet, and it is this temporary hyperinsulinaemia which accelerates transport of glucose and of certain amino acids across muscle cell membrane, followed by augmented incorporation of these amino acids into tissue protein (Cahill, 1971, 1976).

In diabetes the plasma levels of amino acids are affected disproportionately. There is an elevation of the concentration of the branched-chain amino acids, i.e. valine, leucine and isoleucine and in contrast, a decrease in levels of alanine in man (Carlsten et al., 1966; Felig et al., 1970; Wahren et al., 1972, 1976) and in animals (Clark et al., 1968; Bloxam, 1972; Blackshear & Alberti, 1974).

The glucose-alanine cycle: The classical work of Felig and co-workers on the effect of diabetes on the circulating levels of amino acids were, at the time, the only detailed published data available on the in vivo relationship of insulin to protein turnover in normal and diabetic man. Felig et al. (1970) suggested that the glucose-alanine cycle (or nitrogen shuttle system) was not only important for hepatic gluconeogenesis, but it offered a non-toxic alternative to ammonia in the transfer of amino nitrogen from muscle to liver. Essentially, in the glucose-alanine cycle glucose is released by the liver and taken up by the muscle. Here it is converted to pyruvate and then by transamination, to alanine.

The alanine is released from muscle, taken up by the liver and re-converted to glucose.

By catheterization of appropriate blood vessels, these workers investigated the flow of amino acids across tissues of the forearm or leg and from the splanchnic bed. They demonstrated changes in the glucose-alanine cycle during the following physiological states :

(a) In normal man in the post-absorptive state: In the fasted state with relatively low insulin levels, the muscle is in negative nitrogen balance and there is a net release of amino acids, with alanine and glutamine accounting for more than 50% of this amount (Pozefsky et al., 1969, 1976). The alanine is taken up by the liver where its carbon chain is converted to glucose and the amino group to urea. The glutamine is also extracted by the splanchnic bed, primarily by the gut, which converts it to alanine for subsequent uptake by liver. The origin of alanine in muscle can be explained only by de novo synthesis, since it exceeds the amount released by proteolysis. The carbon skeleton is derived from either transamination of pyruvate (Pozefsky et al., 1969; Felig et al., 1977) or from other amino acids (Odessey et al. 1974; Garber et al., 1976; Goldstein & Newsholme, 1976). The amino group for alanine synthesis is probably derived from oxidation of the branched-chain amino acids (Goldberg & Odessey, 1972; Odessey et al., 1974).

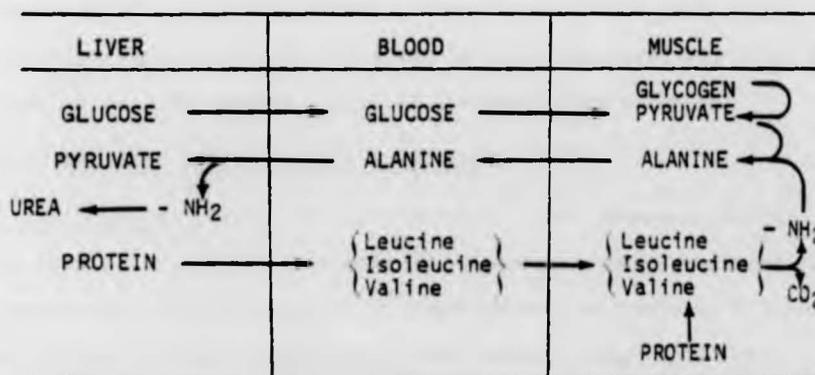
(b) In normal man after a protein meal: Repletion of muscle nitrogen results from firstly a large output of branched-chain amino acids from the splanchnic bed, followed by net uptake by peripheral muscle and synthesis of alanine. The branched-chain amino acids therefore escape hepatic uptake and metabolism (Wahren et al., 1976; Felig et al., 1977).

(c) In diabetes in the post-absorptive state: In severe diabetes the branched-chain amino acid levels are elevated (Clark et al. 1968; Berger et

al., 1978), muscle oxidation of these amino acids increased and plasma alanine decreased (Wahren et al., 1972; Chochinov, 1978). These low alanine levels in diabetes results from enhancement of normal hepatic uptake of alanine, i.e. increased by 45% in the diabetic state (Wahren et al., 1972). Therefore in diabetes, depletion of glycogen stores due to hypoinsulinaemia switches metabolism to gluconeogenesis via enhanced synthesis of alanine and oxidation of branched-chain amino acids.

(d) In diabetes after a protein meal: Diabetics respond to a protein meal with an even more dramatic elevation in plasma branched-chain amino acids, but with only a transient net uptake by the muscle and reduced repletion of muscle nitrogen. In contrast to normal controls, alanine output from the muscles is not decreased, thus reflecting on-going branched-chain amino acid oxidation in the absence of an adequate insulin secretory response to food intake (Sherwin et al., 1976; Wahren et al., 1976). Insulin treatment of the diabetic immediately decreases these elevated branched-chain amino acids levels (Carlsten et al., 1966; Berger et al., 1978).

These findings, i.e. of enhanced oxidation of branched-chain amino acids and of alanine release from muscle has led Odessey et al. (1974) to propose a branched-chain amino acid cycle :



These changes in utilization and oxidation of amino acids brought about by a lack of insulin plus the negative nitrogen balance of uncontrolled diabetes, all suggest that protein turnover itself is altered in either rates of synthesis or breakdown.

1.4 THE EFFECTS OF DIABETES AND INSULIN ON PROTEIN SYNTHESIS

1.4.1 Whole body protein synthesis

To date there appear to be only two studies published on the effect of diabetes on whole body protein synthesis. In 1949 Hoberman used [^{15}N]glycine to measure the rate of protein turnover on alloxan diabetic rats. Although his experiments would appear to indicate that whole body protein synthesis was reduced and rates of protein breakdown increased, the results are difficult to interpret in absolute terms. Not only were the diabetic rats starved for 3 days prior to the 2 day measurement of protein turnover, but this long period of measurement and therefore longer time for recycling of amino acids would also have obscured determination of the actual rate of protein breakdown.

The only study on diabetic subjects is that of Waterlow et al. (1977), who reported some preliminary observations on two diabetic patients where [^{15}N]glycine was used to measure protein synthesis before and after insulin control. They observed that in the pre-insulin period protein synthesis was severely depressed and after 4 to 5 days of insulin therapy, rates of synthesis were enhanced.

1.4.2 Skeletal muscle protein synthesis

In vivo studies: Early work by Forker et al. (1951) demonstrated that protein synthesis was decreased in muscle of eviscerated, depancreatized dogs. By measuring incorporation of [^{35}S]methionine into protein of skeletal muscle after a single injection of the isotope, they found that

incorporation was reduced in the diabetic dog, an effect which could be reversed by treatment of the dogs with insulin. A more rigorous approach was that of Hay & Waterlow (1967) who infused [^{14}C]lysine into alloxan diabetic rats and determined specific activities of the amino acid in plasma and in the free amino acid pools of skeletal muscle. They observed that protein synthesis in muscle of these diabetic rats was decreased by 37%. Much larger reductions in muscle protein synthesis of 60-70% were demonstrated in streptozotocin diabetic rats by Pain & Garlick (1974) and Millward et al. (1976) who used a constant infusion of [^{14}C]tyrosine. Near normal rates of protein synthesis could be achieved by treatment of the rats with insulin (Pain & Garlick, 1974). Both studies revealed a reduction in the RNA to protein ratios and a reduction in the rate of synthesis per unit RNA, suggesting that the fall in synthesis was partly the result of a loss of ribosomes and partly the result of a reduced activity of remaining ribosomes.

Perfusion studies: In perfusions of rat hindquarters from normal animals as used by Ruderman et al. (1971) or the hemicorpus preparation of Jefferson et al. (1974), the rate of protein synthesis declined if these muscles were perfused in the absence of insulin. An increase in the release of amino acids from the muscle into the perfusate could be measured, which could be prevented by addition of insulin. Ruderman et al. (1971) identified these amino acids to be mainly alanine and glutamine, as was shown in in vivo studies of the release of amino acid from the human forearm (Pozefsky et al., 1969). Insulin addition to the perfusate of these normal rat hindquarters had a minimal effect on alanine and glutamine release. In contrast, in diabetic muscle where release of these two amino acids was greatly enhanced, addition of insulin resulted in a marked suppression (Ruderman & Berger, 1974).

Jefferson and his co-workers (1972, 1974, 1977) have investigated the effect of insulin in perfusions of muscle preparations from normal or diabetic rats by measuring not only rates of protein synthesis or breakdown, but also the changes on a subcellular level. In the absence of insulin or in diabetic muscle preparations, rates of protein synthesis decreased in psoas and gastrocnemius muscle of the perfused hemicorpus. This was shown to be associated with development of a block in initiation of the peptide chain, which could be prevented by addition of insulin (Jefferson et al., 1972, 1974). The early work in the 1960's by Wool and co-workers (Wool et al., 1968, 1972), had demonstrated that the decrease in protein synthesis observed in muscle was probably due to defects in polypeptide initiation. This is believed to be the rate-limiting step for translation, which in some tissues is known to be sensitive to insulin (Manchester, 1970). In the studies of Wool and other workers, the activity of ribosomes in tissues was investigated by analysis of polysome profiles, where a decrease in the monomer pool and increase in polysome size would reflect enhanced initiation and rate of protein synthesis (Pain, 1980). On the subcellular level, Wool et al. (1972) could therefore demonstrate that ribosomes of skeletal muscle from diabetic rats were less able to incorporate labelled amino acids in vitro. They observed accumulation of disaggregated polysomes and suggested that ribosomes in diabetic rat muscle were not less efficient, but rather contained smaller proportions of active polysomes. The number of polysomes could be greatly increased by pre-treatment of the diabetic rats with insulin (Wool et al., 1972) or as was shown by Jefferson et al. (1974, 1977), by addition of insulin to the perfusate of rat hemicorpus preparations of normal rats.

The effect of insulin in muscle perfusions could be demonstrated to be specific to muscles with different types of fibres both when starved

rats (Goodman & Ruderman, 1979) and diabetic rats (Flaim & Jefferson, 1979) were used. Muscles containing red fibres have been reported to have more rapid protein synthesis rates than those with more white fibres (Goldberg, 1967b; Millward et al., 1978]. Short (1969) did not find any difference in insulin sensitivity between the two types of muscle. However, Goodman & Ruderman (1979) did show that addition of insulin to perfusates of hindquarters from starved rats increased glucose uptake by extensor digitorum longus muscle (predominantly white fibres), whereas the soleus (predominantly red fibres) was only marginally affected. Similar work has recently been reported by Flaim & Jefferson (1979) in perfused hemicorpus preparations of 48 hour alloxan diabetic rats. Protein synthesis was reduced in both gastrocnemius and psoas (mixed fibre type) and soleus (predominantly red fibres). However, in the psoas, levels of ribosomal subunits were enhanced but not in the soleus, reflecting a resistance in the latter red muscle to development of a block in initiation. Whereas the decrease in synthesis in the gastrocnemius was associated with a decrease in synthesis of protein per RNA unit, the decrease in soleus protein synthesis resulted from the reduced levels of RNA. The addition of insulin caused reaggregation of subunits and increased rates of synthesis in the more white or mixed fibre tissues, but failed to stimulate protein synthesis in the soleus.

Buse and Reid (1975) have suggested that leucine may play an essential role in the regulation of protein synthesis. In perfused hemicorpus preparations of fasted rats, promotion of protein synthesis could be brought about by addition of 5 times normal plasma values of a complete amino acid mixture, with the same amount of branched-chain amino acids or with leucine alone (Li & Jefferson, 1970). This effect was associated with a reduction in ribosomal subunits, suggesting

accelerated peptide-chain initiation. Whereas Li & Jefferson (1979) did not find that insulin plus the branched-chain amino acids could further facilitate protein synthesis, Atwell et al. (1977) reported that insulin did have an additive effect on in vivo polysome formation. They suggested that the branched-chain amino acids and specifically leucine were essential requirements for muscles in the fasted state to fully respond to insulin's anabolic effect (Buse et al., 1979).

Studies on incubated muscles: Investigations with incubated muscles from the early fifties and sixties has shown that labelled amino acid incorporation could be stimulated by addition of insulin to the incubation medium (Krahl, 1952, 1953; Sinex et al., 1952; Wool & Manchester 1962; Kurihara & Wool, 1968). This stimulation of amino acid incorporation could be brought about by increasing not only insulin, but also amino acid concentration. Lundholm & Schersten (1975, 1977) demonstrated that a complete mixture of amino acids at plasma levels had a greater effect on incubated human skeletal muscle than insulin. Similar results were obtained by Buse & Reid (1975) and Fulks et al. (1975), who showed that addition of branched-chain amino acids and specifically leucine could promote protein synthesis in incubated diaphragms of normal rats. Fulks et al. (1975) suggested that all three branched-chain amino acids were effective in promoting protein synthesis, whilst Buse & Reid (1975) indicated that the effect was more specific to leucine.

In diabetic rats, where protein synthesis was decreased in incubated diaphragms, the effect of branched-chain amino acids was not limited to leucine only; all three amino acids were effective in stimulating protein synthesis (Buse & Weigand, 1977). Furthermore, in these studies, leucine also inhibited protein degradation. Buse and co-workers therefore suggest that the branched-chain amino acids may have an essential negative feedback control on protein wasting present in

diabetes and so function to counteract the decline in protein synthesis and enhancement of protein degradation (Buse & Weigand, 1977). However, no in vivo data has yet been produced to prove or disprove the regulating role of leucine in protein turnover in the normal, starved or diabetic state.

1.4.3 Cardiac muscle protein synthesis

In vivo studies: The work of Pain & Garlick (1974) is to date the only published information on the in vivo rate of protein synthesis in cardiac muscle from diabetic rats. By constant infusion of [14 C]tyrosine, they showed that rates of protein synthesis were decreased by 44% in streptozotocin diabetic rats. This decline was however less marked than the decrease of 69% in the more white or mixed fibre tissues such as the gastrocnemius. The decrease in RNA to protein ratio that they observed was similarly smaller than that seen in skeletal muscle.

In vitro studies: Perfusion of normal cardiac muscle requires not only insulin, but also high levels of amino acids to initiate and accelerate incorporation of labelled amino acids (Morgan et al., 1971a, b; Chain & Sender, 1973; Sender & Garlick, 1973). The addition of insulin appeared to prevent a block developing in peptide-chain initiation which would take place if normal cardiac muscles were perfused in the absence of insulin. As shown also during perfusion of skeletal muscle, addition of branched-chain amino acids to the perfusate promoted protein synthesis also in cardiac muscle (Rannels et al., 1974).

In contrast to normal heart muscle, early work by Rannels et al. (1970) and Chain & Sender (1973) demonstrated that perfused cardiac muscle from diabetic rats responded quite differently. The rate of protein synthesis was not influenced by the diabetic state and normal numbers of polysomes and subunits were observed in perfused heart muscle of diabetic rats. Since in normal cardiac muscle the action of insulin

in promoting initiation could be replaced by palmitate, Rannels et al. (1970) suggested that in diabetes the increased free fatty acid levels of this catabolic state could substitute for insulin to maintain normal cardiac protein synthesis. This would be a possible explanation for the absence of cardiac muscle atrophy but presence of skeletal muscle wasting observed in uncontrolled diabetes. The incubated aorta of diabetic rats similarly appears to be resistant to changes in protein synthesis; a reduction in [^{14}C]leucine incorporation could only be demonstrated after at least 5 weeks of diabetes (Arnqvist & Dahlkvist, 1979).

However, a more recent publication from the Hershey group (Chua et al., 1979) does report a 37% reduction in rates of protein synthesis in perfused cardiac muscle of alloxan diabetic rats studied after 3 days of insulin withdrawal. This decrease measured in vitro is consistent with the 44% decrease reported by Pain & Garlick (1974) in vivo in 5 day streptozotocin diabetic rats.

1.4.4 Liver protein synthesis

In vivo studies: Both Hay & Waterlow (1967) and Pain & Garlick (1974) reported that in vivo rates of hepatic protein synthesis in diabetic rats were relatively unchanged. Since they used a 6 hour constant infusion method, these results would apply to rates of non-secretory or cellular hepatic proteins only. The plasma proteins would not have been included in the measurement because these proteins are secreted within 15 to 20 minutes after synthesis (Morgan & Peters, 1971). By contrast, in vivo experiments using specific immunoprecipitation techniques demonstrated a 25% reduction in albumin synthesis in streptozotocin diabetic rats (Pain et al., 1978b) and a decrease of 80% in alloxan diabetic rats (Peavy et al., 1978).

In the present study two different methods were used to specifically differentiate between the effect of diabetes on total liver proteins (Section 7) and on cellular proteins only (Section 6).

In vitro studies: Urea production was shown to be enhanced in perfused livers from alloxan diabetic rats (Green & Miller, 1960) or in livers of normal fed rats perfused in the absence of insulin (Mondon & Mortimore, 1967). Since addition of insulin to the perfusate did not appreciably alter protein synthesis but reduced the release of amino acids, the main effect of insulin in liver appears to be an inhibitory action of protein degradation rather than a stimulation of protein synthesis (Mortimore & Neely, 1975).

Whereas in normal rats addition of insulin to the perfusate has little effect on the synthesis of fixed or non-secretory proteins (Mortimore & Mondon, 1970), synthesis of plasma proteins can only be achieved if enough amino acids and insulin are added to maintain a positive nitrogen balance (John & Miller, 1969). In perfusions of diabetic livers, the synthesis of albumin was shown to be decreased (Peavy et al., 1978). They reported that the reduction in albumin synthesis was associated with a fall in albumin mRNA content, which could be restored to normal levels by insulin treatment.

In the liver, polysome changes due to diabetes are less marked than in skeletal muscle, but even so liver ribosomes are less capable of incorporating amino acids into protein (Tragl & Reaven, 1971, 1972). In diabetic rats, disruption of the ultrastructure of the rough endoplasmic reticulum and a fall in the membrane-bound proportion of ribosomes in the liver has been demonstrated (Reaven et al., 1973; Pain et al., 1974; Alford et al., 1975). This ultrastructural damage could be reversed with in vivo insulin therapy (Reaven et al., 1973; Pain et al., 1974). Since the primary site of synthesis of the secretory proteins appears to

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be by ribosomes bound to the membranes of the rough endoplasmic reticulum (Shore & Tata, 1977), it is therefore not surprising that the main effect of insulin and diabetes should be on the synthesis of some of these plasma proteins, as reported by Peavy et al. (1978) for albumin.

In vitro studies with isolated hepatocytes prepared by collagenase digestion showed that incorporation of amino acids into total hepatic protein was reduced in hepatocytes from diabetic rats (Ingebretsen et al., 1972). In vitro addition of insulin to these hepatocytes obtained from diabetic rats could not stimulate synthesis of albumin (Peavy et al., 1978). This is in contrast to the stimulation of albumin synthesis (Dich & Gluud, 1975; Crane & Miller, 1977), and of total liver proteins (Wagle & Sampson, 1975; Dich & Gluud, 1975) observed in normal hepatocytes. However, these discrepancies could have been due to methodological differences. Jeejeebhoy et al. (1975), using a more rigorous approach with more active isolated cells, demonstrated that insulin could maintain protein synthesis of albumin and of transferrin for up to 2 days of incubation.

1.4.5 Kidney protein synthesis

In uncontrolled experimental and human diabetes, the marked polyuria and glycosuria is associated with enlarged kidneys and increased glomerular filtration rates, abnormalities which can be reversed by prolonged insulin treatment (Mogenson & Anderson, 1973, 1975; Rasch, 1979).

On a subcellular level, these hypertrophied diabetic kidneys demonstrated a marked increase in capacity to incorporate amino acids in vitro. This difference was maintained when the kidney ribosomes were stripped of endogenous mRNA and artificial polyuridylic acid messenger was added (Peterson et al., 1971). This led them to suggest that the

effect of insulin deficiency in enhancing amino acid incorporation was mostlikely due to a post-transcriptional change in protein synthesis.

In vivo studies by Seyer-Hansen (1977) and Fox et al. (1977) all show strong correlations between kidney size, protein content and basement membrane thickening with plasma glucose values and thus, the severity of the disease. Renal hypertrophy of diabetic kidneys was associated with increased total protein, increased DNA to RNA ratios and thus cellular growth, and in the later stages of diabetes, increased DNA concentration or actual cell hyperplasia (Seyer-Hansen, 1976, 1977).

Work load hypertrophy has been suggested as a possible cause, with the diabetic kidney attempting to compensate for the increased demands of polyuria, glycosuria and ketonuria. Ross & Goldman (1970, 1971) and Seyer-Hansen (1978) have clearly established that compensatory renal hypertrophy caused by unilateral nephrectomy could be further enhanced by induction of diabetes. As in human diabetes, all these changes were reversed by insulin treatment.

In patients with diabetes of long duration, thickening of the renal glomerular basement membrane is a common finding and this form of microangiopathy is generally accepted to be a forerunner of the later more severe form of diabetic nephropathy (Reddi, 1978). This thickening of the basement membrane has been shown to correlate with increased protein synthesis. Basement membranes of glomeruli from diabetic rats demonstrated marked increases in incorporation rates of ^{14}C amino acids, which could be reduced to normal by insulin treatment (Cohen & Vogt, 1975; Cohen & Khalifa, 1977).

At this time no in vivo data were available on the actual rates of protein synthesis in kidneys of diabetic rats. Therefore in this study, fractional synthesis rates were measured in rats with acute and chronic diabetes by constant infusion (Section 6) and by single injection of a

large amount of labelled amino acid (Section 7).

1.4.6 Gut and lung protein synthesis

Although rates of protein synthesis in gut and lung were not measured in the present study, available data are presented here to complete the overall picture of the effect of diabetes on the various tissues and organs of the body.

In contrast to the catabolic effect of insulin deficiency in skeletal muscle, but similar to changes in the kidney, hypertrophy of the small intestine and particularly of the mucosa has been reported to be stimulated by diabetes. This hypertrophy has been suggested as resulting from an anatomic adaption of the gut to the marked hyperphagia which develops in experimental diabetes (Jervis & Levin, 1966). However, Miller et al. (1977) has reported that the increase in DNA synthesis in the mucosa that they observed was probably a more primary cause. The only in vivo measurements on synthesis rates of diabetic gut mucosa were reported by McNurlan & Garlick (1980a). By using a single large dose method developed to measure proteins with rapid turnover such as in liver and gut (McNurlan et al. 1979), no change in fractional synthesis rates of jejunal mucosa or serosa could be demonstrated in diabetic rats.

In a similar way, the in vitro rate of protein synthesis in the lung appears to be unaffected by diabetes (Rannels et al., 1979).

1.5 THE EFFECT OF DIABETES AND INSULIN ON PROTEIN BREAKDOWN

1.5.1 Muscle protein breakdown

In vivo studies: Indirect in vivo measurements of protein degradation in skeletal muscle of diabetic rats has been reported by Millward et al. (1976). By comparing the rate of protein synthesis with the rate of change of the protein mass of the muscle, a rate of protein breakdown

was obtained. They established that after 6 days of diabetes, both synthesis and breakdown rates were decreased, in contrast to the increase in proteolysis demonstrated in vitro if muscle was perfused in the absence of insulin (Jefferson et al., 1977).

In the present study, protein breakdown in skeletal and cardiac muscle was determined by a similar method to that used by Millward et al. (1976). Greater emphasis was placed on minimizing the errors involved in measuring changes in protein mass by doing so on a day-to-day basis immediately after withdrawal of insulin from diabetic rats (Section 7).

The only other in vivo study of protein degradation in muscle of diabetic rats is that of Dice et al. (1978), who used the double label technique of Arias et al. (1969) to differentiate between protein degradation rates of different proteins in the same muscle homogenate. As discussed earlier in Section 1.1.4.1, Dice et al. (1978) modified the basic technique to accommodate for measurement of protein turnover in the non-steady state of diabetes. The double labelled proteins from diabetic muscle homogenates were separated according to subunit molecular weight or according to isoelectric point. They found that the increase in protein breakdown in the diabetic or starved rat was quite different from that seen in normal protein degradation. In normal cells large molecular weight proteins are more rapidly degraded than smaller proteins and acidic proteins more rapidly than neutral or basic proteins. In muscles from diabetic and starved rats they found that the correlation between degradation and protein molecular size and isoelectric point was absent.

In vitro studies: Although there appears to be no direct evidence that all cellular protein breakdown is mediated by lysosomes, changes in the activity of the proteolytic enzymes in these organelles can be achieved by the presence or absence of insulin in perfused systems. In the

perfused hemikorpus (Jefferson et al., 1977) and heart (Rannels et al., 1975) of normal rats, the absence of insulin from the perfusing medium increased the rate of protein breakdown. This proteolysis was associated with greater fragility of lysosomes and with development of autophagic vacuoles (Jefferson et al., 1974). If perfused over a long period, large amounts of alanine and glutamine were released into the perfusate (Ruderman & Lund, 1972). The increase in proteolysis and decrease in protein synthesis in these perfused hemikorpus preparations could be prevented by addition of insulin (Jefferson et al., 1977).

In perfusions of normal cardiac muscle without insulin there was also a net release of amino acids and ammonia and a decreased latency of the lysosomal enzymes β -acetyl-glucosaminidase and cathepsin D (Rannels et al., 1975). In perfusions of diabetic hearts, Chua et al. (1979) have reported a decrease in both protein synthesis and breakdown rates.

The work of Fulks et al. (1975) with incubated diaphragm muscles from normal rats have indicated that insulin not only inhibits proteolysis and promotes protein synthesis, but does so in the presence of cycloheximide. This suggests that insulin by itself can prevent degradation independent of any effect on protein synthesis. A similar inhibition of proteolysis was observed if the three branched-chain amino acids and specifically leucine were added to the incubation method (Fulks et al., 1975). This restraining effect of the branched-chain amino acids on protein degradation of muscle was also demonstrated when diaphragm muscles of diabetic rats were incubated in the presence of these amino acids (Buse & Weigand, 1977).

1.5.2 Liver protein breakdown

In vivo studies: At the time of this study only one in vivo investigation of liver proteolysis had been reported by Dice et al. (1978). As was

shown in muscle with the double label technique, liver degradation was enhanced, but the correlation between protein size and degradative rate was abolished in proteins of liver obtained from diabetic rats.

In vitro studies: The detailed studies of Mortimore and co-workers have demonstrated increased proteolysis if normal livers were perfused without addition of insulin (Mortimore & Mondon, 1970). As shown in skeletal muscle, liver proteolysis was also associated with greater fragility of lysosomes (Neely et al., 1974) and the development of high density lysosomal particles. This was also observed by Pain et al. (1974) in livers of streptozotocin diabetic rats. Amherdt et al. (1974) also identified hepatic autophagic vacuoles in severely diabetic rats and showed that treatment of the rats with insulin could reverse these ultrastructural changes.

1.6 THE EFFECT OF DIABETES AND INSULIN ON PROTEIN OXIDATION

Diabetes and starvation are both associated with increased mobilization of amino acids for either gluconeogenesis or complete oxidation. Adibi et al. (1974) therefore described skeletal muscle as having a defensive mechanism in these states in that it functions as a substrate depot. By increasing amino acid oxidation, the protein of muscle can be utilized by the liver for gluconeogenesis.

The three branched-chain amino acids, i.e. leucine, isoleucine and valine share a common pathway of first a reversible transamination step which results in formation of α -keto acids. The next step of decarboxylation is irreversible and hence the amino acids are completely oxidized to CO_2 and H_2O (Adibi, 1976).

The relative rates of oxidation of various body tissues have been established by incubation studies in vitro, with kidney having the greatest oxidation capacity, followed in descending order by brain,

adipose tissue, skeletal muscle and liver. However, because skeletal muscle constitutes more than 43% of the total body mass, muscle degrades more than 50% of leucine (Odessey & Goldberg, 1972, 1979), providing energy to the muscle and probably amino groups for alanine and glutamine synthesis (Chang & Goldberg, 1978).

Most of the amino acid oxidation investigations have been done with branched-chain amino acids and for the purpose of this discussion, the oxidation of leucine in diabetic tissues will be considered since this was also the amino acid of choice in this study.

Leucine oxidation in diabetes: Skeletal muscle is highly adaptable to changes in nutrition, e.g. in starvation and diabetes the levels of branched-chain amino acids in plasma are increased (Carlsten et al., 1966; Aoki et al., 1976) and oxidation of these amino acids enhanced (Paul & Adibi, 1978), resulting in accelerated gluconeogenesis via the glucose-alanine cycle (Odessey et al., 1974).

The anabolic role of leucine in diabetes appears to be similar to that of insulin and in fact, as suggested by Buse & Weigand (1977), leucine can substitute up to a point for the lack of insulin in promoting protein synthesis in vitro. It is interesting to note that leucine is also a potent stimulator of insulin release (Milner, 1970) and can induce marked hypoglycaemia (Clark et al., 1968).

In vivo studies on leucine oxidation in diabetes have not been reported to date. Most of the investigations have measured oxidation after a single dose of ^{14}C amino acid and presented the rate of oxidation only as a proportion of the dose excreted as $^{14}\text{CO}_2$ (McFarlane & Von Holt, 1969; Meikle & Klain, 1972; Neale & Waterlow, 1974a,b). Without measuring the specific activity of the precursor at the site of oxidation, the absolute rate of oxidation cannot be obtained. Sketcher (1976) did measure the specific activity of leucine in the intracellular pool of

muscle when a plateau was reached in output of $^{14}\text{CO}_2$ during a constant infusion of $[1-^{14}\text{C}]$ leucine. This enabled him to calculate the absolute rate of protein synthesis from flux as described in Section 1.1.2. Sketcher (1976) showed that in protein depleted animals, the rate of protein oxidation was reduced. In the work to be presented, a similar approach was used for measuring the rate of oxidation of carboxyl labelled leucine from the output of $^{14}\text{CO}_2$ in expired air and from the specific activity of the leucine in both plasma and tissue free amino acid pools (Section 5).

The only data available on leucine oxidation in diabetes is that obtained by in vitro incubation studies on individual tissues. Buse and her colleagues have systematically studied the effect of diabetes on leucine oxidation in various tissues, and found increased rates of oxidation in rat hemidiaphragms (Buse et al., 1976a), sciatic nerves (Buse et al., 1976b) and rat retina (Frayser & Buse, 1978). In the hemidiaphragm, oxidation was increased by 46%, and could be brought down to near normal values by treatment of the diabetic rats with insulin (Buse et al., 1976a). Paul & Adibi (1978) established the rates of decarboxylation of leucine in homogenates prepared from liver, kidney and gastrocnemius muscle. In contrast to starvation in which leucine oxidation was enhanced in only muscle, in diabetes leucine oxidation was enhanced in liver, kidney and gastrocnemius muscle incubated in vitro.

In the work to be presented the rate of whole body protein oxidation was measured in diabetic rats 4 or 8 days after the streptozotocin injection and in insulin treated diabetic rats. $[1-^{14}\text{C}]$ leucine was given by constant infusion, flux was obtained and hence whole body protein synthesis and breakdown could be calculated (Section 5).

1.7 THE AIMS OF THIS WORK

- (a) To investigate the exact mechanism of the negative nitrogen balance present in uncontrolled, insulin dependent diabetic patients.
- (b) To follow the changes in protein metabolism in these patients during the initial stages of insulin therapy and again after several months of treatment.
- (c) To measure whole body protein synthesis, breakdown and oxidation in diabetic rats, and to determine the rate at which each of these parameters of protein metabolism could be normalized by insulin treatment.
- (d) To identify the effect of diabetes in animals at the tissue level, i.e. to measure changes in tissue composition and rates of protein synthesis and breakdown in acute and chronic diabetic rats and in diabetic rats well controlled by insulin.

SECTION 2

GENERAL EXPERIMENTAL TECHNIQUES

2.1 MATERIALS

Most of the reagents used in the following experiments were of Analar grade, obtained from BDH (Poole, Dorset, UK) or Sigma Chemicals Limited (Poole, Dorset, UK). All radioactive isotopes were purchased from The Radiochemical Centre (Amersham, Bucks., UK) and enzymes from Sigma Chemicals.

2.2 SYMBOLS

- S_A = specific activity of precursor amino acid
- S_B = specific activity of protein amino acid
- S_i = specific activity of intracellular free amino acid
- S_p = specific activity of plasma free amino acid
- k_s = fractional synthesis rate of protein
- k_d = fractional breakdown rate of protein
- k_g = fractional growth rate of protein
- Z = absolute rate of protein synthesis
- B = absolute rate of protein breakdown
- E = absolute rate of protein oxidation

2.3 RADIOACTIVITY COUNTING

Two liquid scintillation counters were used, namely a Beckman LS 150 and a Nuclear Chicago Delta 300. Samples were counted until 10,000 counts were accumulated (SE = 1%). Counting efficiency for ^{14}C

labelled samples varied from 80-85% and for ^3H samples from 19-23%. The counts were corrected for quenching by the automatic external standard (AES) method. Appropriate quench curves were constructed with commercial calibrated standards of [^3H] or [^{14}C]n-hexadecane (The Radiochemical Centre, Amersham, Bucks., UK), (Dyer, 1974).

Two scintillation cocktails were used :

- (a) 0.4% 2,5 di phenyloxazole (PPO) in toluene, for counting of $^{14}\text{CO}_2$ trapped in 3 ml of a hyamine hydroxide:ethanol (1:2) solution.
- (b) a Triton-X100/xylene based scintillant containing per litre: 3g 2,5 di phenyloxazole, 357 ml Triton, 37 ml ethanediol, 106 ml ethanol, 500 ml xylene (Fricke, 1975); for counting of radioactivity in all samples other than expired $^{14}\text{CO}_2$.

2.4 PRESENTATION OF RESULTS

All results in tables and figures are given as mean values \pm standard error of the mean (SEM). In the clinical work (Section 3) the mean difference between pre- and post-insulin values were obtained by paired comparison. Significance levels were assessed by the Student's 't' test and 'p' was the probability that any difference between two groups of values was due to chance alone, with 'p' values of 0.05 or less considered to be significant.

The sequence of experiments to be presented is not necessarily in the same order in which they were carried out. The animal experiments were done simultaneously with the clinical work which spanned 20 months.

SECTION 3WHOLE BODY PROTEIN TURNOVER IN DIABETIC PATIENTS MEASURED WITH
[¹⁵N] GLYCINE3.1 INTRODUCTION

The anabolic role of insulin in promoting protein synthesis and inhibiting protein breakdown has been demonstrated in vivo and in vitro in experimentally diabetic animals. A preliminary study in this department on two diabetic patients indicated a similar response, namely that uncontrolled diabetes is associated with very low rates of protein synthesis, which can be increased by treating these patients for 3 to 5 days with insulin (Waterlow et al., 1977).

The aim of the present study was to investigate further the effect of insulin on protein synthesis in patients with uncontrolled diabetes, by modified methods and protocols which had been perfected for this type of investigation in the two years following the first study. Whole body protein turnover measurements were made using an oral dose of [¹⁵N] glycine, with determination of the excretion of ¹⁵N in urinary ammonia. This method relies on a basic two-pool model discussed in the Introduction (see Section 1.1.1).

The protocol for the present study was approved by the Ethical Committee of King's College Hospital, Denmark Hill, London.

3.2 EXPERIMENT I3.2.1 Subjects

A total of six male diabetic patients participated in this investigation. They were selected for this study by a consultant or registrar at the Diabetic Clinic, King's College Hospital, during the

patients' routine 3 or 6 monthly visits. All six had been on oral hypoglycaemic agents (sulfonylureas) for at least 3 months but required insulin to achieve better diabetic control. Their symptoms at this time included hyperglycaemia, glycosuria, mild ketosis, some weight loss and generalized weakness. All six patients were within the normal weight range for their age and height. Details on age, duration of diabetes and weight are given in Table 3-1.

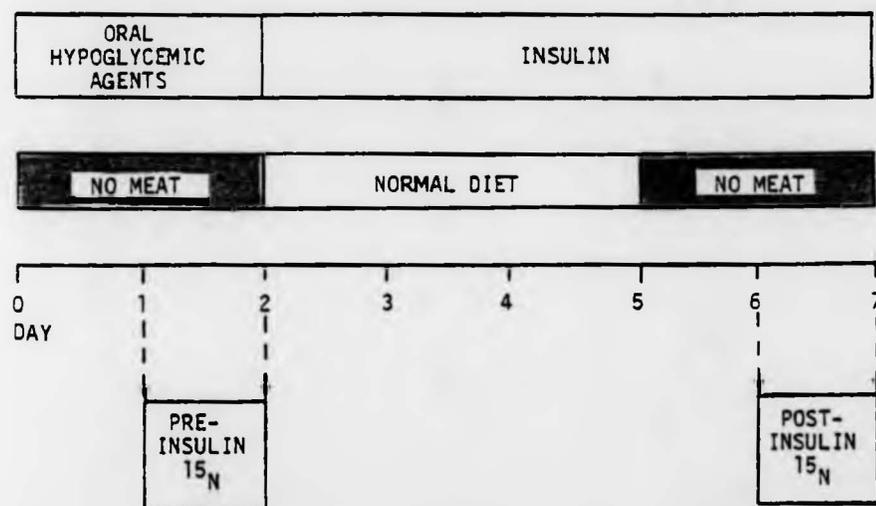
The basic details and aims of the study were explained to each patient at the clinic and if they agreed to participate, they were admitted to the diabetic ward of King's College Hospital for a period of at least 7 to 8 days. During this time protein turnover measurements were made using [^{15}N] glycine during the period before insulin treatment commenced and repeated 4 to 5 days later after insulin control had been established (Figure 3-1). At the time of measurement the patients were on controlled diets containing no meat.

The first four patients were studied using the following protocol (Experiment I), which was subsequently modified for the measurement of protein turnover in the last two patients (Experiment II).

3.2.2 Dietary protocol

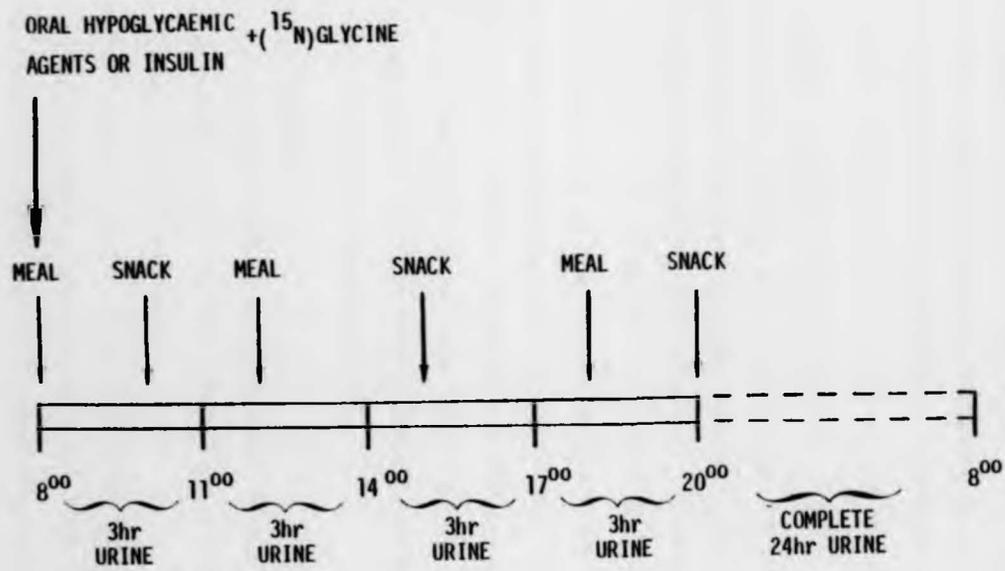
Each patient was admitted 24 hours before the actual [^{15}N]-glycine experiment for stabilization on a controlled diet prepared under supervision in the hospital diet kitchen. This diet excluded all meat products to permit measurement of 3-methyl histidine excretion in urine. Each diet contained 1 gram protein and 30 kcal (126 kJ) per kg body weight, provided as 3 meals and 3 snacks. The carbohydrate content and distribution was similar to the patients' own diet at home. Details of a 24 hour patient protocol are shown in Figure 3-2.

FIGURE 3-1 TREATMENT PROTOCOL DURING PROTEIN TURNOVER MEASUREMENTS



DIET: 1 g protein/Kg body weight
30 Kcal/Kg body weight

FIGURE 3-2 A 24 HOUR PATIENT PROTOCOL



3.2.3 ^{15}N glycine administration

An oral dose of 100 mg ^{15}N glycine (95.2 atoms percent excess, Prochem division of British Oxygen Company, London) was administered at exactly 08.00 hrs on the day of the study. The tracer was preceded by 100 ml of milk and was followed by breakfast 30 minutes later. The cumulative excretion of ^{15}N was determined in urinary ammonia during the first 12 hours (Exp. I) or 9 hours (Exps. II and III).

3.2.4 Urine and blood sample collection

A 24-hour urine collection was made on the day preceding ^{15}N -glycine and analyzed for total nitrogen and creatinine excretion. For the first 12 hours immediately after the oral ^{15}N dose, urine was collected at exactly timed intervals of 3 hours and thereafter one 12 hour collection was made to determine N-balance (Figure 3-2). The urine collection bottles contained a 2% solution of Hibitane (chlorhexidene gluconate) in 6N HCl, which effectively prevented bacterial growth in urine samples stored at room temperature.

Blood samples of 10 ml each were collected before each meal for plasma glucose and insulin determinations.

3.2.5 Sample analysis

Plasma glucose: Plasma glucose was determined in the Diabetic Clinic Laboratory using the glucose oxidase method as automated for a Technicon Autoanalyzer Colorimeter.

Plasma insulin: Plasma insulin was estimated by the radioimmunoassay method of Hales and Randle (1963), using a kit from the Radiochemical Centre, Amersham, U.K. This method is based on competitive binding between insulin in plasma and added radioactive insulin (^{125}I -insulin), with an antibody specific to insulin. The insoluble insulin-antibody complex which formed was then counted for radioactivity using a well-type, gamma scintillation counter (EKCO Electronics Limited, U.K.). The

level of radioactivity was related in an inverse manner to the amount of insulin present in the plasma.

Plasma urea: Boehringer Mannheim, (London) provided a urease kit for plasma urea measurement. The ammonium carbonate formed from urea, split by urease, was reacted with first phenol and then hypochlorite to give a coloured complex. The absorbance was read at 550 nm on a Gilford 240 spectrophotometer.

Urinary creatinine: Creatinine was measured in urine as an indication of the completeness of urine collections. For this purpose urine was reacted with alkaline picric acid and after colour development, the OD read at 520 nm.

3-Methyl histidine: The excretion of 3-methyl histidine was used as index of protein breakdown in muscle (Bilmazes et al., 1978a). The concentration of 3-methyl histidine in urine was determined with an automated amino acid analyzer (Locarte Co., London W14, U.K.). The samples were loaded onto a column (23 cm x 0.9 cm) containing a cation exchange resin (LA/49, Locarte Co., London W14, U.K.) and was eluted first with 0.2 M sodium citrate buffer, pH 3.44 for 75 minutes, then with 1.0 M sodium citrate buffer, pH 4.55 for 175 minutes, at a temperature of 45°C and flow rate of 30 ml per hour. A standard sample of 1 mM 3-methyl histidine was run each day with the urine samples. The analyzer was routinely operated by Mr Philip Broadbent.

Urinary nitrogen: Total nitrogen was assayed by the micro-Kjeldahl method by which the urine was digested in concentrated sulphuric acid plus a copper catalyst for 12-20 hours, followed by distillation with a Markham still into 2% (w/v) boric acid and titration to determine concentration.

Urinary ammonia and ^{15}N abundance: Ammonia was collected by aeration of strongly alkaline urine into saturated boric acid. After titration, ammonia was redistilled into 1 N HCl. The distillate was then assayed

for ^{15}N abundance by the method of Sprinson & Rittenberg (1949). A single collector MS 20 mass spectrometer (AEI Scientific Apparatus Limited, Manchester, U.K.) was used as described by Golden & Waterlow (1977) and was routinely operated by Miss Hilda Sheppard. A standard solution of $^{15}\text{NH}_4\text{Cl}$ (0.30 atoms percent excess ^{15}N) and of unlabelled NH_4Cl was measured in duplicate each day with each set of samples.

3.2.6 Calculation of protein synthesis and breakdown

Flux, which is the rate at which amino-nitrogen enters or leaves the metabolic pool, was measured after a single oral dose of [^{15}N] glycine (see Section 1.1.1 for detail of 2 pool-model and calculations). Flux was obtained by measurement of the area under the curve for ^{15}N excretion between time zero and 12 hours (t). From

$$Q = \frac{d^*}{\int_0^t S \cdot dt}$$

synthesis (Z) and breakdown (B) rates were calculated, since

$$Q = Z + E = B + I$$

Excretion of total amino nitrogen (E) was measured over 24 hours.

3.2.7 Results and Discussion - Experiment I

Effect of insulin treatment on diabetic control: On admission to the ward and whilst still on oral hypoglycaemic agents, all patients were in poor diabetic control as shown by the large amounts of glucose excreted in the urine (Table 3-2). After 4 to 5 days of insulin, glycosuria was considerably reduced, though it was still present in all four patients. Similarly, plasma glucose, which was elevated pre-insulin, was still above normal control values post-insulin (Table 3-3), although a mean glucose value of 10.6 ± 1.0 mmol/litre is not an uncommon finding in insulin treated diabetics.

Plasma insulin was assayed before the insulin treatment regime commenced. The mean values at 08.00, 15.00 and 18.00 hours were

TABLE 3-2 EXCRETION OF URINARY GLUCOSE AND CREATININE* PRE- AND POST-INSULIN (g/day)

Patient	Urinary glucose		Urinary creatinine	
	Pre-insulin	Post-insulin	Pre-insulin	Post-insulin
1. (R.H.)	14.1	3.5	1.32	1.19
2. (N.A.)	12.8	4.0	1.66	1.24
3. (A.P.)	18.2	16.4	1.28	1.27
4. (B.S.)	21.0	10.1	1.37	1.52
$\bar{x} \pm \text{SEM}$	16.5 ± 1.9	8.5 ± 3.0	1.41 ± 0.09	1.31 ± 0.07
Mean difference $\pm \text{SEM}$ (Post-insulin - pre-insulin)		-8.0 ± 2.1		-0.10 ± 0.12

*Creatinine used as test for completeness of urine collections.

TABLE 3-3 PLASMA GLUCOSE CONCENTRATION* (mmoles/l), NITROGEN BALANCE (g N/d) AND URINARY AMMONIA EXCRETION (mg/ 12 hours) BEFORE AND AFTER INSULIN TREATMENT

Patient	Glucose		Nitrogen balance		Ammonia	
	Pre-insulin	Post-insulin	Pre-insulin	Post-insulin	Pre-insulin	Post-insulin
1. (R.H.)	13.3	9.6	+ 0.9	+ 1.5	277	242
2. (N.A.)	16.5	11.2	- 7.8	+ 2.1	480	195
3. (A.P.)	13.4	13.0	- 0.1	+ 0.5	331	214
4. (B.S.)	15.4	8.5	- 1.5	- 1.4	446	293
$\bar{x} \pm \text{SEM}$	14.7 ± 0.8	10.6 ± 1.0	-2.1 ± 2.0	$+0.7 \pm 0.8$	383 ± 47	236 ± 21
Mean difference $\pm \text{SEM}$ (Post-insulin - pre-insulin)		-4.1 ± 1.4		$+2.8 \pm 2.4$		-148 ± 52

* Mean value for 3 measurements per day before each main meal.

14.7 \pm 6.6, 17.6 \pm 5.4 and 12.2 \pm 3.7 μ Units per ml. Although the mean values appear to be within the normal range, the usual response of insulin secretion to food intake was insignificant (Holman & Turner, 1977). This lack of response fits with the diagnosis of these patients, namely that they no longer responded to oral hypoglycaemic agent treatment and required insulin to restore diabetic control.

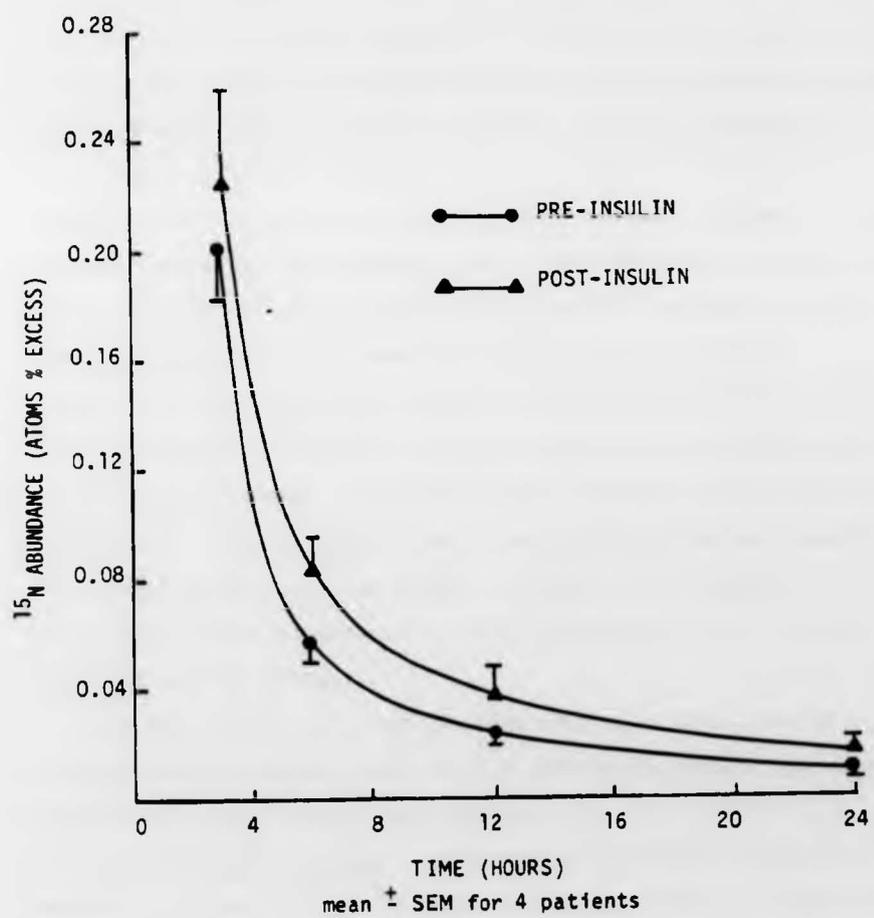
All the patients were in negative nitrogen balance before insulin treatment. The response to insulin was poor in that N-balance was improved in some but not all patients (Table 3-3). This slow response in normalization of N-balance was not unexpected, given that the work of Walsh et al. (1976) suggested that the accumulation of total body nitrogen lost during uncontrolled diabetes would require up to 8 weeks for restoration. Unfortunately the neutron activation analysis technique used by Walsh and co-workers does not allow expression of nitrogen changes in absolute terms and therefore one cannot predict what the amount of nitrogen accumulation per day could be in these patients after only 4 days of insulin treatment. Oddoye et al. (1979) have shown that a maximum nitrogen accretion of 1.6 g per day can be achieved on extremely high protein diets of 36g N per day.

None of the patients showed any evidence of ketone excretion in the urine before or after insulin treatment. However, total ammonia excretion was decreased with insulin treatment (Table 3-3) in contrast to the very small drop in excretion reported by Waterlow et al., Ch. 17, 1978a, in the pilot study.

Effect of insulin treatment on ^{15}N excretion and protein turnover:

Figure 3-3 shows the time course of the cumulative excretion of ^{15}N in ammonia in the four patients before and after insulin treatment. The two excretion curves both have a sharp inflexion at about 12 hours, at which point the cumulative excretion of the label in the ammonia end-product had reached a maximum.

FIGURE 3-3 TIME COURSE OF EXCRETION OF ^{15}N IN
AMMONIA BEFORE AND AFTER INSULIN



The effect of insulin treatment on protein synthesis and breakdown rates is given in Table 3-4. In the pre-insulin period rates of protein synthesis were well within the normal range despite hyperglycaemia and negative N-balance. In the pilot study Waterlow et al. (1977) reported that protein synthesis rates were extremely low in the two diabetic patients measured during the pre-insulin period, i.e. 0.77 and 1.57 g protein/kg/day. In that study insulin treatment resulted in a highly significant increase in protein synthesis, but in the present study, protein synthesis in fact decreased on the average by 16%. This result was contrary to what one would expect, given the known anabolic role of insulin in promoting protein synthesis as demonstrated in diabetic patients (Waterlow et al., 1977) and in diabetic rats (Sections 6 and 7). Insulin, which is thought to inhibit protein degradation in perfused liver tissue (Mortimore & Neely, 1975), did in fact decrease the average rate of protein breakdown by 25%. However, the excretion of 3-methyl histidine did not differ significantly before (338 ± 28 $\mu\text{moles/d}$) and after (326 ± 29 $\mu\text{moles/D}$) insulin treatment. Therefore 3-methyl histidine gave no indication that there was a change on a tissue level of rates of muscle protein breakdown. The decrease in whole body protein breakdown suggests that, where there was an improvement in N-balance, it appeared to result mainly from a decrease in protein breakdown and not from any change in protein synthesis.

Possible explanations for these surprising results in protein synthesis were sought by comparing the differences between protocols used in the present and in the pilot study.

(a) Size of [^{15}N] glycine dose: In the pilot study large non-tracer amounts of 238 and 315 mg [^{15}N] glycine were administered compared to the 100 mg used in this experiment. Since a non-tracer dose leads to excess excretion of isotope (Waterlow et al., 1978b) this could explain

TABLE 3-4 PROTEIN FLUX*, SYNTHESIS AND BREAKDOWN (g protein/Kg/d) IN DIABETIC PATIENTS BEFORE AND AFTER INSULIN TREATMENT

Patient	Pre - insulin			Post - insulin		
	Flux	Synthesis	Breakdown	Flux	Synthesis	Breakdown
1. (R.H.)	4.0	3.1	3.0	3.4	2.4	2.3
2. (N.A.)	4.9	3.0	3.8	4.0	3.2	3.0
3. (A.P.)	5.6	4.6	4.6	3.8	2.8	2.3
4. (B.S.)	5.6	4.5	4.6	5.3	4.2	4.3
Mean difference \pm SEM. (Post-insulin - Pre-insulin)				-0.90 \pm 0.32	-0.65 \pm 0.43	-1.03 \pm 0.44

*Flux calculated from 12 hour excretion of ^{15}N , total N output was measured over 24 hours.

the very low pre-insulin values obtained in the pilot study for synthesis i.e. 1.2 g protein/kg/d and the higher rates of 3.5 g protein/kg/d with 100 mg [^{15}N]glycine in the present study.

(b) Time of [^{15}N] glycine administration: In this study the patients received their daily oral hypoglycaemic agent (or insulin injection in the post-insulin period) with 100 ml of milk 10 minutes before the oral ^{15}N dose, which was followed by breakfast 30 minutes later. In the pilot study the patients were also treated with insulin just before breakfast, but ^{15}N administration was delayed for 2 hours thereafter. However, it has since come to light that in the pilot study oral hypoglycaemics were actually withdrawn for 48 hours before the pre-insulin measurement. This did not result in very high glucose values (11 mmol/l), but probably insulin levels were lower than in the present study, since the sulfonylureas that were used are thought to increase β -cell function (Shen & Bressler, 1977a,b). This presents an alternative suggestion why the pre-insulin synthesis rates in the pilot study were so low, in that the speculated lower plasma insulin concentrations could have resulted in lower protein synthesis.

However, this fact was unknown at the time of this study and in a following study in one patient (Mr R.H.), only one change in protocol was tested, namely that of the time of [^{15}N] glycine administration.

10 months post-insulin follow-up study: Two measurements of protein turnover were made within 3 days of each other on Mr R.H. (patient 1, Table 3-1), who returned after 10 months of insulin treatment. At this time he was feeling well and his diabetes was well controlled. The two aims of this study were :

- (a) to establish the effect of 10 months of insulin treatment on protein turnover as compared with 4 days of insulin (Protocol as in Exp. I),

(b) to determine the effect of giving the oral dose of [^{15}N]glycine 2 hours after breakfast and his daily injection (Protocol II).

The results in Table 3-5 demonstrate that after 10 months of insulin treatment, synthesis rates were increased back to pre-insulin values and protein breakdown was now even further reduced by 57%. In the 10 month period he had gained 5 kg in body weight and was at the time of measurement in a highly positive nitrogen balance. Furthermore, it is clear that administration of [^{15}N] glycine 30 minutes before or 2 hours after breakfast had no different effect on protein turnover. This follow-up study therefore did not clarify in any way why the results of the pilot study differed from the present investigation.

A further two patients were studied using a modified dietary protocol (Experiment II). One of the patients was also investigated after 8 weeks of insulin treatment with again a further dietary modification (Experiment III).

3.3 EXPERIMENT II

3.3.1 Modified protocol

Diet: Two changes were made in the dietary intake and distribution of food :

- (a) The energy value of the diet was increased from 30 to 40 kcal/kg/day (126 to 168 kJoules). This was to test if the energy content of the diet could limit protein synthesis or its response to insulin.
- (b) An approximately continuous food intake and absorption was accomplished by feeding the total intake in 6 meals every 2 hours, each containing equal amounts of protein. Since completion of the first experiment, Garlick et al. (1980a) have demonstrated that protein synthesis fell rapidly after cessation of food given hourly over 12 hours. This dietary modification of a 6 meal

TABLE 3-5 PROTEIN SYNTHESIS AND BREAKDOWN RATES (g protein/Kg/day) AFTER 10 MONTHS OF INSULIN TREATMENT

PATIENT	PROTOCOL	SYNTHESIS	BREAKDOWN
1. (R.H.)			
Pre-insulin	I	3.1	3.0
4 days Post-insulin	I	2.4	2.3
10 months Post-insulin	I	3.2	1.3
10 months Post-insulin	II	3.0	1.3

Protocol I : Normal meal pattern, 30 Kcal/day
100 mg [¹⁵N] glycine 30 minutes before breakfast

Protocol II : Normal meal pattern, 30 Kcal/day
100mg [¹⁵N]glycine 2 hours after breakfast

pattern and its effect on protein turnover was tested in both pre- and post-insulin periods.

Isotope administration: [^{15}N] glycine was given at the same time as in the pilot study of Waterlow et al. (1977), i.e. 2 hours after breakfast, the time at which the patients also received their oral hypoglycaemics or insulin. Furthermore, the 100 mg [^{15}N] glycine was given as an oral dose in 500 ml water which the patient sipped slowly, so that influx of the isotope into the metabolic pool could be delayed.

Calculation of protein turnover: Calculation of flux was modified from that used in Experiment I, since urine collection times were now changed to encompass the total feeding time. The first 0-3 hour collection was started when [^{15}N] glycine was given and hence 2 hours after the first meal. The last collection was completed one hour after the 6th meal. Therefore the cumulative excretion of ^{15}N in ammonia was now measured over 9 hours, whereas in Experiment I excretion of isotope was measured over 12 hours and that of unlabelled amino nitrogen over 24 hours. In this experiment both isotope and unlabelled nitrogen were determined over 9 hours. This latter change required a modification in calculation of total amino nitrogen excretion (E in eqn 2, Experiment I). Since the urea pool of the body is large and turns over slowly, it could effectively delay nitrogen excretion. Hence, excretion (E) over 9 hours would not be an accurate estimation of the true excretion rate of amino nitrogen (Waterlow et al., 1978a). A correction factor for amino nitrogen was thus calculated from changes in the urea pool between 0 and 9 hours. Plasma samples for urea analysis were taken when ^{15}N was given and exactly 9 hours later. The size of the urea pool was calculated from total body water as percent of body weight, using a value of 60.2% (for males) of body water determined by urea (Scientific Tables, Documenta Geigy). The corrected amino nitrogen was then calculated as total

nitrogen excreted over 9 hours plus or minus the change in urea pool size over that time.

3.3.2 Results and Discussion - Experiment II

Two patients (No.5, F.E. and No.6, A.S. of Table 3-1) were studied using this modified protocol. A.S. responded to insulin in the same fashion as the patients in Experiment I, i.e. plasma glucose was reduced and the negative N-balance improved. Similarly, his protein synthesis rate was slightly reduced by insulin treatment (Table 3-6) and breakdown was decreased by 23%. Unfortunately the severe diabetic state of F.E. was not improved by only 4 days of insulin treatment and as will be discussed in Section 3.4, after 8 weeks his diabetes was still poorly controlled despite many changes in insulin type and dosage and investigations at the Diabetic Clinic. His negative N-balance and hyperglycaemia were hardly improved by 4 days of insulin and similarly, there was no change detectable in protein synthesis or breakdown rates.

In total, the synthesis and breakdown rates measured with this modified protocol differ strikingly from those of Experiment I, namely with synthesis rates of 6.6 g protein/kg per day versus much lower rates of 3.8 in Experiment I (Table 3-4). Possible explanations for this difference could be :

- (a) The practically continuous intake of food during the time that [^{15}N]glycine excretion was measured. Waterlow et al. (1978b) have reported a 34 to 48% decrease in synthesis with ^{15}N given without food as compared to ^{15}N given with food. In the present study rates of protein synthesis in Experiment I were decreased by 42% as compared with Experiment II.
- (b) Since ^{15}N and total nitrogen excretion were measured over only nine hours, the time in which all food was eaten, it is not surprising that the flux estimation would be larger and therefore the

TABLE 3 - 6 PROTEIN SYNTHESIS AND BREAKDOWN (g protein/Kg/day)
AND NITROGEN BALANCE (gN/d) IN DIABETIC PATIENTS
BEFORE AND AFTER INSULIN TREATMENT IN EXPERIMENT II

Patient	P r e - i n s u l i n		
	Synthesis (g protein / kg / d)	Breakdown (g protein / kg / d)	N-balance (gN/d)
5. (F.E.)	6.2	6.3	- 6.8
6. (A.S.)	6.9	6.4	- 2.5
	P o s t - i n s u l i n		
5. (F.E.)	6.0	6.3	- 6.6
6. (A.S.)	5.4	4.9	+ 0.5

Protocol: 6 meals with equal protein content 40 Kcal/Kg/day,
 $[^{15}\text{N}]$ glycine 2 hours after breakfast.

calculated synthesis and breakdown rates higher.

The results of Experiment II still did not change the conclusion reached with the first experiment, namely that insulin treatment of diabetic patients in poor control resulted in a slight decrease in protein synthesis and an even larger reduction in protein breakdown. A follow-up study was conducted in one of the patients and a further modification of caloric content was tested (Experiment III).

3.4 EXPERIMENT III

3.4.1 Protocol

Mr F.E. (patient No. 5 in Table 3-6) returned after 8 weeks on insulin treatment. At this point his diabetes was still very unstable although he had regained 5 of the 13 kg body weight loss with onset of diabetes 6 months previously. The protocol of Experiment II (6 meals given 2 hourly and [¹⁵N]glycine 2 hours after first meal) was reported in this patient to determine the effect of 8 weeks of insulin treatment. Three days later a further modified diet was tested, namely an increase of energy content of the diet which was increased from 40 to 60 kcal/kg/day.

3.4.2 Results and Discussion

Table 3-7 shows the effect of 4 days and 8 weeks of insulin treatment on protein turnover in this patient. Protein synthesis was slightly increased and breakdown reduced by 22%, as were breakdown rates in the first 4 patients studied. The increased calories did not appreciably affect protein synthesis or breakdown, therefore a lack of energy content of the diet could not have been the cause of the delayed response of synthesis to insulin or lack of weight gain.

The effect of insulin after 8 weeks on protein turnover (Table 3-7) and after 10 months (Table 3-5) appear to suggest that protein breakdown is more sensitive to long term insulin treatment than protein synthesis.

TABLE 3 - 7 PROTEIN SYNTHESIS AND BREAKDOWN RATES AND PLASMA
GLUCOSE VALUES AFTER 8 WEEKS OF INSULIN TREATMENT

Protocol	Patient No. 5 (F.E.)	Plasma glucose (mmole/l)	Synthesis (g protein / Kg / day)	Breakdown (g protein / Kg / day)
I	Pre-insulin	15.0	6.2	6.3
I	4 days Post-insulin	10.4	6.0	6.3
I	8 weeks Post-insulin	13.0	6.6	4.9
II	8 weeks Post-insulin	8.5	6.6	4.3

Protocol I : 6 meals with equal protein content,
40 Kcal/Kg/day, [¹⁵N] glycine 2 hours after
breakfast.

Protocol II : 6 meals with equal protein content,
60 Kcal/Kg/day, [¹⁵N] glycine 2 hours after
breakfast.

3.5 SUMMARY AND CONCLUSIONS

In conclusion, the data of the two experiments and follow-up studies suggest that short-term insulin treatment results in a slight decrease in protein synthesis rate and that the improved negative nitrogen balance is a result of a larger decrease in breakdown. These conclusions are not effectively changed by manipulation of dietary intake, meal pattern or [^{15}N] glycine administration. Insulin treatment for 8 weeks and longer results in a return of synthesis rate to pre-insulin values with a very large reduction in protein breakdown.

The results of this study not only contrast with that of Waterlow et al. (1977) on diabetic patients, but also with *in vivo* data on tissues from experimentally diabetic rats, i.e. that diabetes decreased protein synthesis and this could be normalized by insulin treatment of the animal (Pain & Garlick, 1974, Section 7).

At this time no published data were available on the effect of diabetes on whole body protein synthesis and breakdown in rats. The next step was therefore to design a suitable animal model which would enable comparison with the diabetic patients, i.e. a nonketotic, acutely diabetic rat, requiring insulin treatment, which could be studied also in the chronic phase of the disease. The following chapter describes the method followed in choice and dose of diabetogenic agent and the characterization of this model in terms of changes in body and tissue weight, food and water intake and lastly, tissue composition.

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SECTION 4

CHARACTERISTIC FEATURES OF THE STREPTOZOTOCIN DIABETIC RAT

4.1 INTRODUCTION

In all the studies, the choice of a diabetic animal model was governed by the fact that it had to facilitate comparisons with the clinical work that was being carried out simultaneously. The adult human diabetics were insulin-dependent, non-ketotic and of normal body weight at the time of protein-turnover measurements (see Section 3). These patient characteristics immediately excluded the use of genetically diabetic animals, because of the obesity factor which generally accompanies the diabetic gene (Herberg & Coleman, 1977). Alloxan was also rejected owing to its known effect of causing a ketotic form of diabetes (Mansford & Opie, 1968). Streptozotocin, a cytotoxic anti-bacterial agent was therefore chosen as a chemical agent for β -cell destruction, since it is thought to cause no other biochemical or metabolic abnormalities than hypoinsulinaemia and hyperglycaemia.

Streptozotocin, which is prepared from Streptomyces acromogenes, is composed of a cytotoxic moiety, 1-methyl 1-nitrosourea attached to carbon-2 of glucose. It can exist in the pyranose form as either an α or β anomer, with the α -anomer having a much greater β -cell destruction ability at lower doses (Rossini et al., 1977). The streptozotocin used in this study (Sigma Chemical Company Limited, Poole, Dorset, U.K.) contained an unknown distribution of anomers. However, Rossini et al. (1977) have shown that at high doses (> 50 mg/kg body weight) there is no difference in α or β anomer action on the β -cell.

4.2 METHOD

Male albino Wistar rats of 90-100g were used in all studies and were obtained from Charles River, Margate, Kent, U.K. On arrival they were housed in wire bottom cages, usually singly, and had free access to water and a cubed diet (Oxoid Limited, Basingstoke, U.K.), containing 23% (w/w) crude protein. The animal house lighting was set on 12 hours light - dark cycles whilst a thermoneutral temperature of 25°C (77°F) was maintained throughout.

Young growing rats are known to be resistant to streptozotocin (Turner & Heard, 1972) and the dosage was increased accordingly (Portha et al., 1974). However, even a high dose does not necessarily always destroy all β -cells and hence the success rate in each experiment was approximately 80%.

A dose of 130 mg streptozotocin per kilogram body weight was used. Since the weights of the rats were close enough to 95-100 gm, 13 mg of streptozotocin was dissolved in 0.3 ml of 0.05 M citrate buffer (pH 4.5), immediately before it was injected via the lateral tail vein of the rats. Because of the low pH, rats were anaesthetized with ether 30-60 seconds before the injection. In the first few studies, control rats were also anaesthetized and injected with citrate buffer to minimize the immediate effect of ether on food intake. However, this effect was found to be minimal and the procedure was not repeated in control groups of later experiments.

The effectiveness of this streptozotocin dosage to create an acceptable animal model was tested against known published criteria for plasma glucose and insulin, and changes in concentration of protein, RNA and DNA in various tissues.

4.3 EFFECT OF STREPTOZOTOCIN ON PLASMA GLUCOSE AND INSULIN

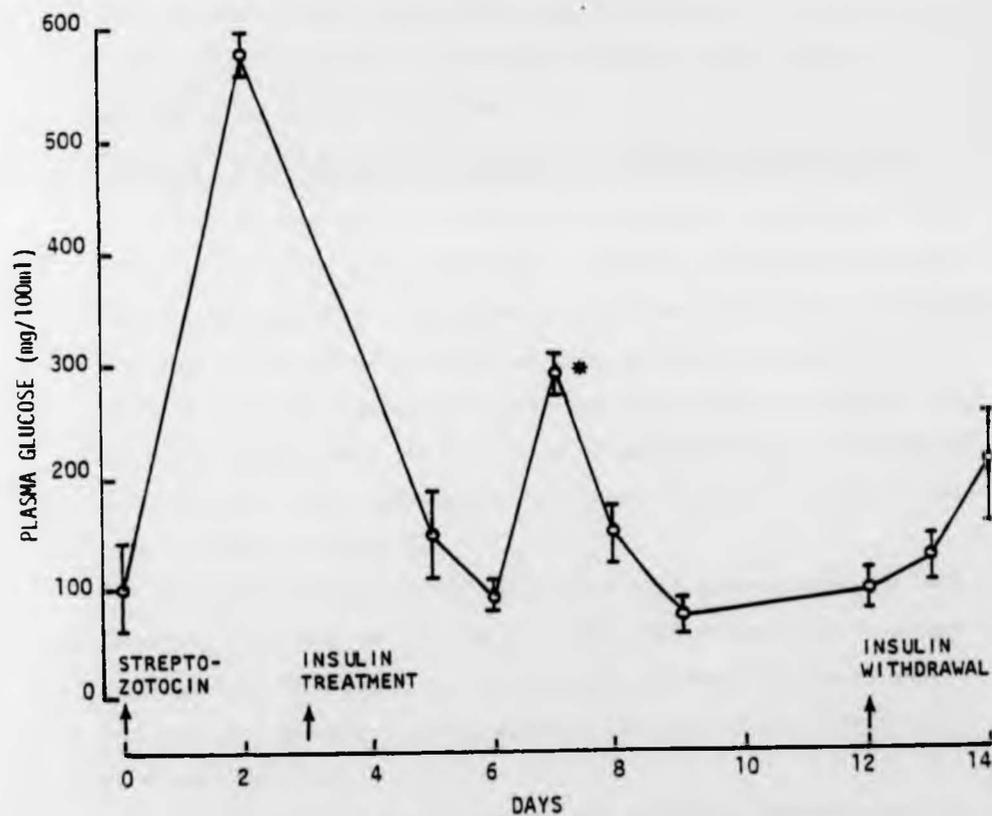
Base-line data on the physiological response to streptozotocin were obtained by measuring and analyzing blood and urine collected from rats placed in metabolic cages. Glucose was measured enzymatically using the glucose oxidase method and kit of Boehringer Corporation (London). Plasma insulin was estimated by the radioimmunoassay method of Hales & Randle (1963), using a kit from the Radiochemical Centre, Amersham, U.K. and following the same procedure as in determination of plasma insulin of diabetic patients.

By withdrawing small amounts (50 μ l) of blood from the tip of the tail, it was possible to show that rats were severely diabetic 48 hours after injection with streptozotocin (Figure 4-1). Rats with plasma glucose values below 350-400 mg/100 ml were rejected and this selection procedure resulted in mean plasma concentrations of 450-600 mg per 100 ml in all experiments. That streptozotocin did not destroy all β -cells in the Islets of Langerhans was apparent from the still measurable insulin in plasma. Insulin values were very low (2-6 μ Units/ml) in all experiments, but this appeared to be sufficient to maintain life, certainly up to 8 weeks after streptozotocin injection.

Severe diabetes was also reflected in the dramatically enhanced glucose excretion in the urine i.e. 6 ± 1.6 grams per day. Similarly, urine volumes were increased from control amounts of 14 ml to 66 ml per day. This polyuria resulted in a spontaneous increase in water intake i.e. from 24 ± 3 mls per day in controls to 94 ± 14 ml per day after 4 to 5 days of diabetes. Similar results on urine volume and water intake have been reported by Rasch et al. (1979).

The above mentioned symptoms of severe diabetes could all be reversed by an insulin treatment regime. Protamine Zinc Insulin (PZI) was chosen, since it is a long acting insulin (24-36 hours) and if

FIGURE 4-1 THE EFFECT OF STREPTOZOTOCIN
AND INSULIN ON PLASMA GLUCOSE



All rats treated with 4 U PZI/day except *received 3 U PZI/d on day 6. Mean \pm SEM for 5 rats per group

injected at 10.00 hrs each day, would probably achieve peak action at approximately 18.00 - 20.00 hrs and so coincide with the dark cycle of food intake by the rat.

A treatment regime of 4 units PZI per day, injected subcutaneously as a single dose, was found to be highly effective in restoring normal blood glucose values within 48 hours (Figure 4-1). There appeared to be no advantage in a split dose injected twice a day and 3 units was found to have a diminished effect, which is clearly illustrated in Figure 4-1. This figure also demonstrates the rate of onset of diabetes after insulin withdrawal.

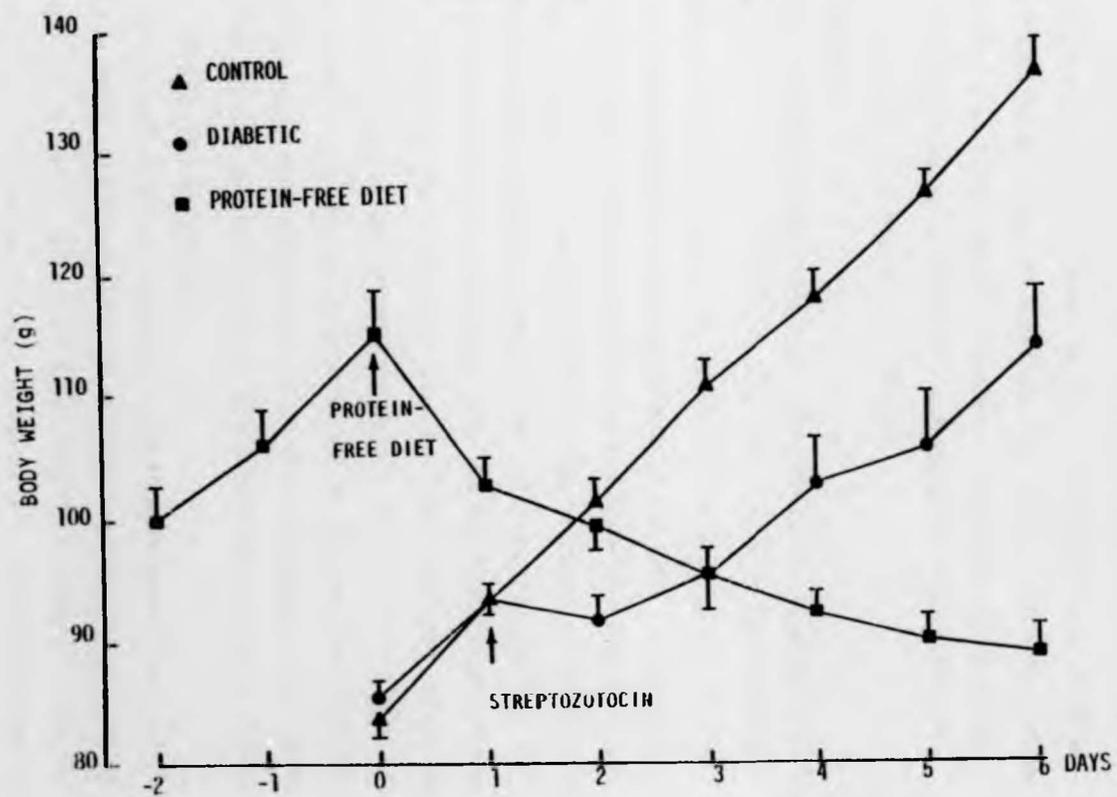
4.4 EFFECT OF STREPTOZOTOCIN AND INSULIN TREATMENT ON GROWTH RATES

Normal growth rates of each set of rats were established in the 5 - 8 days preceding each experiment. Therefore, rats were ordered at 50 - 60 grams and used in experiments only after reaching 90 - 100 grams in weight. Generally, growth rates were severely depressed in the first 24 - 48 hours immediately after the streptozotocin injection, but thereafter became stabilized at rates of approximately 3 - 4 grams per day in diabetic rats compared to 7 - 8 grams of growth in normal rats. This is shown in Figure 4-2.

For comparative purposes this figure also demonstrates the loss of weight which occurs in rats fed a protein free diet. Loss of weight in a streptozotocin diabetic rat will only be observed in older, non-growing rats, whereas in young diabetic rats growth will continue but at a much lower rate.

Treatment with insulin resulted in accelerated growth rates of 8 - 9 grams per day and control body weight was generally regained within 4 to 6 days of treatment. More detailed results will be illustrated in the actual experiments (see Section 7) where insulin treated rats were used as controls to insulin withdrawn diabetic rats.

FIGURE 4-2 THE EFFECT OF DIABETES AND A PROTEIN-FREE DIET ON BODY WEIGHT
(mean \pm SEM for 5 rats per group)



4.5 EFFECT OF STREPTOZOTOCIN DIABETES AND INSULIN TREATMENT ON FOOD INTAKE

Hyperphagia in diabetic and insulin treated rats has been reported by various workers (Booth, 1972; Panksepp et al., 1975). In our pilot studies the published degree of hyperphagia could not be demonstrated after 6 days of diabetes, the time at which most of the experiments that follow were terminated. A detailed study of the onset and magnitude of hyperphagia in diabetes and the effect of insulin treatment was therefore initiated.

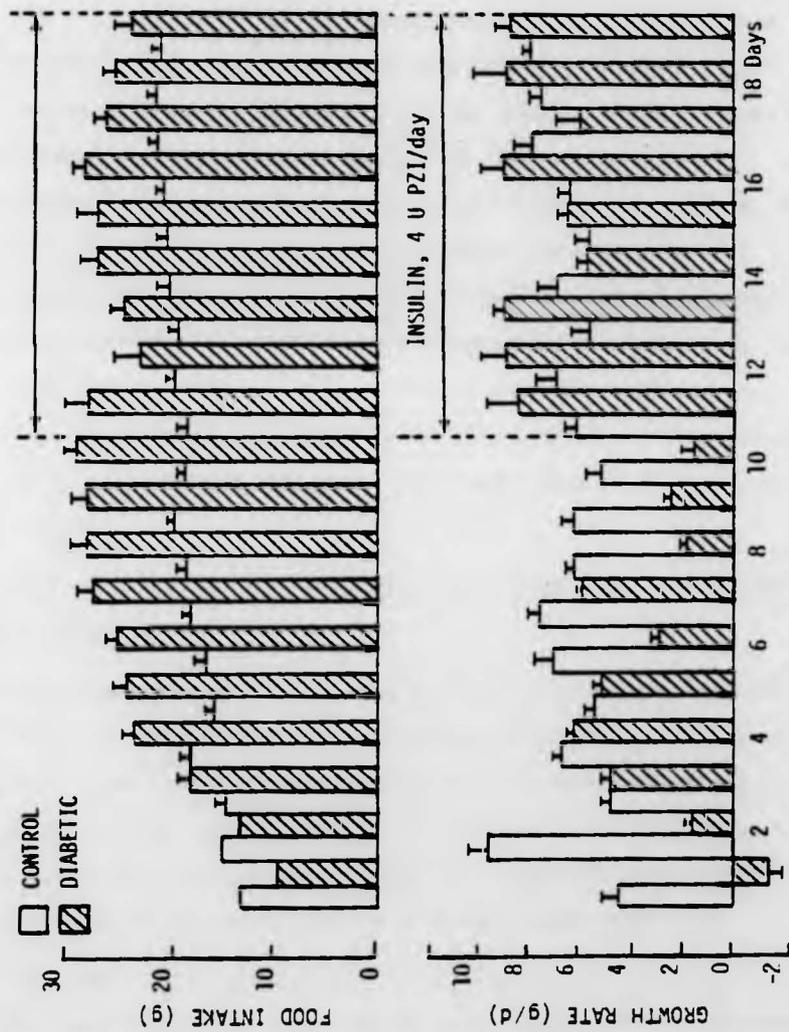
4.5.1 Method

90 - 100g male Wistar rats on a cubed diet were injected with 13mg streptozotocin. The rats were housed singly in wire-bottom cages with paper instead of wood chips in the bottom tray to facilitate weighing of spilled food. At approximately the same time each day the rats were weighed and food intake estimated from left-over diet and spillage. Because of the large urine volume produced by diabetic rats and their habit of partially chewing the pelleted diet (Booth, 1972), the much larger spillage from these rats was heavily contaminated with urine. Therefore, left-over food plus spillage from both control and diabetic rats were dried to constant weight in an oven at 110°C. Food intake, growth rate and plasma glucose values (as obtained from the tip of the tail) were determined for 10 days of diabetes, and thereafter for 10 days in which each rat was treated with 4 units PZI/day.

4.5.2 Results and Discussion

The influence of streptozotocin diabetes on growth rate and food intake is shown in Figure 4-3. The depressed growth rate caused by diabetes and marked acceleration after insulin treatment is clearly illustrated, as is the day-to-day variability of this measurement in control and experimental groups.

FIGURE 4-3 FOOD INTAKE AND GROWTH RATES OF CONTROL, DIABETIC AND INSULIN-TREATED DIABETIC RATS



mean \pm SEM for 5 rats per group

Immediately after injection with streptozotocin, food intake was generally suppressed, but as the disease state progressed, so food intake increased. At 4 days, food intake of diabetics equalled that of controls and thereafter hyperphagia became firmly established (26.2 ± 0.4 g diet/day in diabetic rats versus 19.1 ± 0.5 g/day in control rats) with only a slight decrease after insulin treatment.

However, if we express food intake per gram of growth (Figure 4-4), the hyperphagia of the diabetic rat can be interpreted as a compensatory response to a lack of utilizable fuel. In contrast, the hyperphagia of the insulin treated rat appears to be a purely physiological adaptation to the highly accelerated catch-up growth. The sensitivity of this adaptation is illustrated in Figure 4-4. On day 14 the insulin dose was reduced from 4 to 3 units PZI per day. Plasma glucose immediately increased to 289 mg/100 ml and similarly, food intake was enhanced and growth rate suppressed on that and the following day.

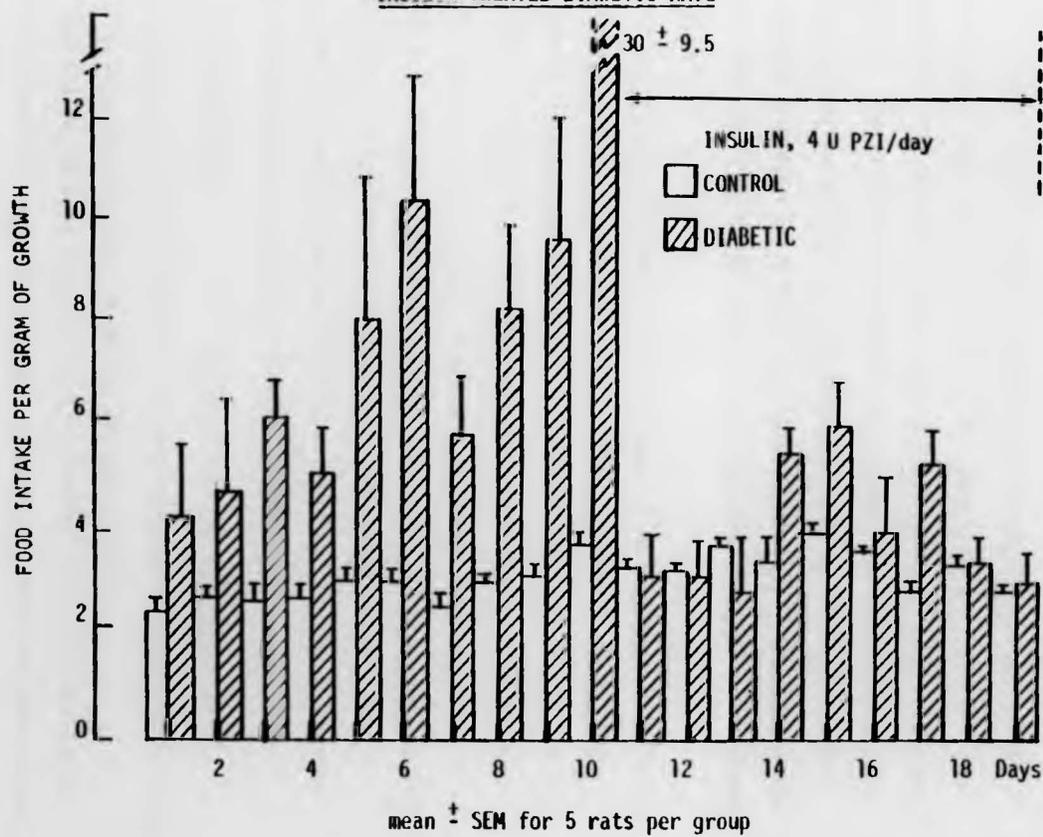
4.6 EFFECT OF STREPTOZOTOCIN AND INSULIN ON TISSUE PROTEIN, RNA AND DNA CONCENTRATIONS

The following investigation was a pilot study for the planned measurement of protein turnover by constant infusion of [^{14}C] tyrosine (Section 6). Rats were killed after acute and chronic diabetes, i.e. 5 and 56 days after streptozotocin. In addition, a further group of diabetic rats were treated with insulin for 4 days to test the effectiveness of insulin in normalization of these tissue results.

4.6.1 Methods

Rats were housed, fed and diabetes was induced with streptozotocin as described. At the appropriate days after streptozotocin, rats were decapitated and bled from the neck. The liver, kidneys and gastrocnemius muscles were quantitatively but rapidly removed, blotted,

FIGURE 4-4 FOOD INTAKE TO GROWTH RATIO IN CONTROL, DIABETIC AND
INSULIN-TREATED DIABETIC RATS



weighed and frozen in liquid nitrogen and stored at -15°C . The mixed venous blood was spun at $1200 \times g$ for 10 minutes at 4°C and the plasma removed for glucose and insulin assays.

The frozen tissues were homogenized in 2% (w/v) perchloric acid (PCA) using an ultrasonic tissue homogenizer (Polytron, The Northern Media Supply Limited, Hull, U.K.) in two 10-second periods at top speed, whilst keeping the tissue in the tube cold on ice. In later experiments, the frozen tissues were pulverized to a fine consistency by placing the tissue in a plastic bag between two aluminium blocks previously cooled on dry ice, the top of which was then hammered. Both methods achieved an effective tissue homogenate from which protein, RNA and DNA could be extracted, in the sequence of steps as described in Figure 4-5.

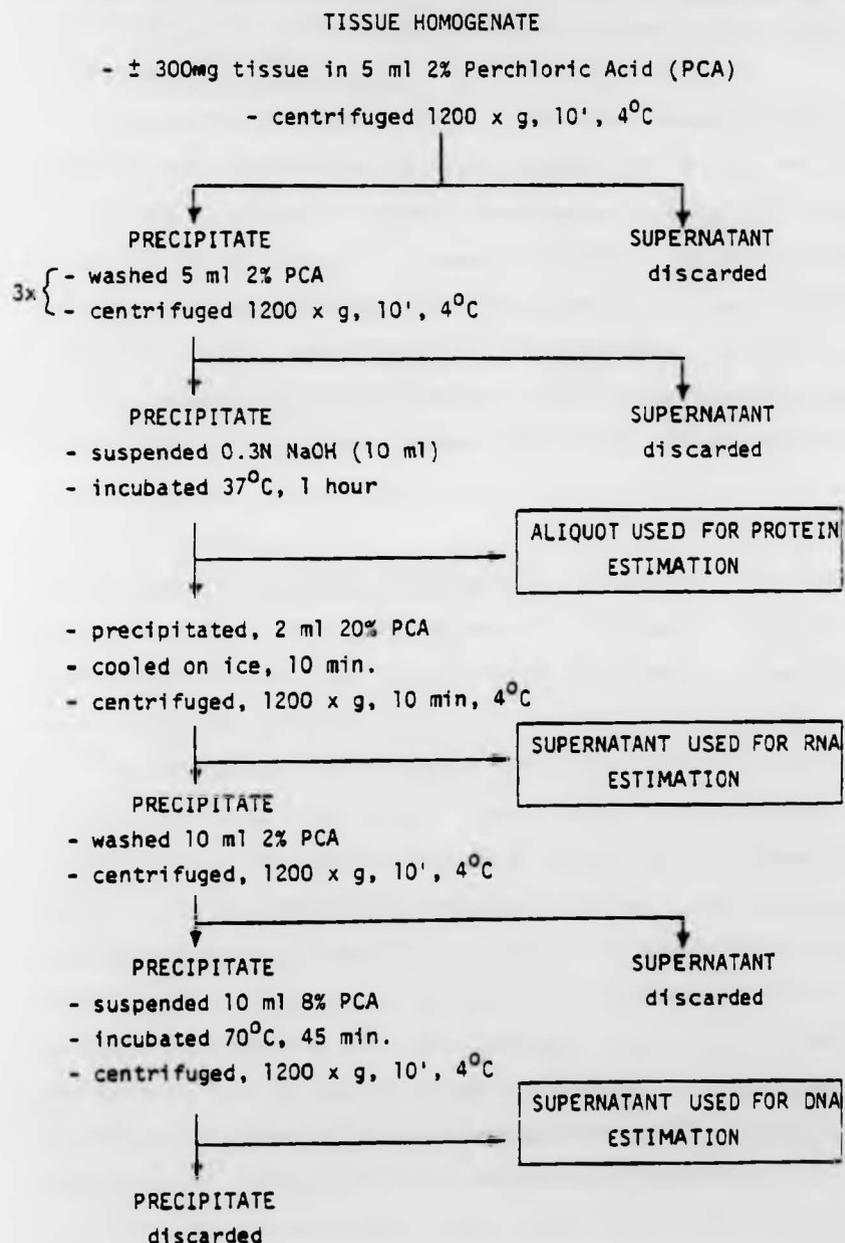
The procedure outlined in this figure is a modification of the method of Schmidt-Thannhauzer as described by Munro & Fleck (1969). The protein fraction was estimated by the automated procedure of Lowry et al. (1951), using a Technicon Autoanalyzer. Each set of protein assays were run with standard solutions of bovine serum albumin in 0.1N NaOH. The RNA concentration was determined by measuring optical absorption at 260nm using a Gilford 240 spectrophotometer. A correction for peptide contamination was made by reading absorption also at 232nm and applying the equation of Munro & Fleck (1969) :

$$\text{RNAphosphorus} = (3.4 \times \text{OD}_{260\text{nm}}) - (1.44 \times \text{OD}_{232\text{nm}}) \text{ ug/ml}$$

RNA is obtained from RNAphosphorus by multiplying by 10.53 (the average ratio by molecular weight of RNA to RNA phosphorus).

The DNA content of tissues was estimated by the diphenylamine method as modified by Giles and Myers (1965), in which the DNA extract is reacted with diphenylamine in glacial acetic acid to yield a blue colour after 18 to 24 hours. Optical absorption was read at 595nm and

FIGURE 4-5

EXTRACTION OF PROTEIN, RNA AND DNA FROM HOMOGENISED RAT TISSUE

corrected for possible turbidity by reading also at 700nm. DNA concentration was obtained from a standard curve using deoxyribonucleic acid (Type 1, calf thymus, Sigma Chemicals Limited, Poole, Dorset, U.K).

4.6.2 Results and Discussion

The presence of overt diabetes 5 days after induction with streptozotocin was characterized by hyperglycaemia (481 ± 21 v 148 ± 3.5 mg glucose/100 ml plasma in controls) and hypoinsulinemia (2.7 ± 0.7 v 19.5 ± 2.4 μ Units insulin/ml plasma in controls). This high plasma glucose value was maintained for 56 days (583 ± 12.4 mg/100 ml) despite even lower insulin production (1.8 ± 0.6 μ Units/ml). It would appear that the residual β -cell function was sufficient to prevent ketosis and death but could not maintain growth (Table 4-1). Severe weight loss of 50% was observed in the chronic diabetic rats and loss of 18% in the acutely ill rats.

Muscle composition changes with diabetes: On a tissue level similar changes were shown with a 35% decrease in total gastrocnemius weight in acute diabetes and an even greater reduction of 66% in weight after 56 days of diabetes (Table 4-1).

The decrease in gastrocnemius weight was paralleled by a drop in total protein and total RNA. Since in most tissues ribosomal RNA accounts for at least 80% of total RNA (Young, 1970), a change in RNA content or in the RNA:protein ratio may be a good index of the rate of protein synthesis (Millward et al., 1973). In this study the RNA to protein ratio of muscle (also defined as the capacity of protein synthesis, Millward et al. 1975) was unchanged in the chronic diabetic rats and nonsignificantly lower at 5 days of diabetes. A larger and significant decrease of 32% has been reported by Pain & Garlick (1974) and subsequent studies (see Table 4-4) have confirmed this.

Total DNA was decreased in both acute and chronically diabetic groups, with a very significant reduction in the protein to DNA ratio.

TABLE 4 - 1 THE EFFECT OF ACUTE AND CHRONIC DIABETES ON GASTROCNEMIUS
MUSCLE RNA, DNA AND PROTEIN CONCENTRATIONS

	Control (6 rats)	Acute diabetes (13 rats)	Control (5 rats)	Chronic diabetes (8 rats)
Total body weight (g)	138±2	113±4	421±13	212±28
Total tissue weight (g)	1.14±0.04	0.74±0.05	4.17±0.08	1.40±0.31
Protein (g/100g)	13.22±0.92	14.83±0.42	19.45±0.71	19.05±0.35
Total protein (mg)	151±11	110±13	811±30	267±38
RNA (mg/g)	1.05±0.14	0.97±0.04	0.92±0.03	0.89±0.03
Total RNA (mg)	1.20±0.11	0.72±0.13	3.84±0.71	1.25±0.22
RNA/Protein (mg/g)	7.9±1.0	6.5±0.3	4.6±0.3	4.8±0.2
DNA (mg/g)	0.89±0.05	1.10±0.06	0.31±0.02	0.49±0.09
Total DNA (mg)	1.01±0.09	0.81±0.09	1.29±0.13	0.69±0.08
Protein/DNA (mg/mg)	152±14	139±7	620±22	474±80

Mean values ± SEM. Acute diabetes 5 days post-streptozotocin and chronic diabetes 56 days post-streptozotocin. Protein, RNA and DNA concentrations mg/g wet tissue.

Cheek et al. (1971) defined a DNA-unit as an imaginary volume of cytoplasm managed by a single nucleus. The protein to DNA ratio would then be an indication of the size of these DNA-units, an increase in which would suggest cellular hypertrophy.

Liver composition changes with diabetes: The effect of short and long-term diabetes on liver is shown in Table 4-2. Both at 5 and 56 days, diabetes resulted in reduced tissue weight and protein, RNA and DNA content. The 34% decrease in RNA to protein ratio in acute diabetes and 21% in chronic diabetes is comparable to the results of Pain & Garlick (1974) in 6 day diabetic rats. This fall would appear to suggest a decrease in capacity of hepatic protein synthesis, as will be shown in Section 7 where rates of both cellular and secretory protein synthesis were measured in diabetic rats.

Kidney composition changes with diabetes: Enlarged kidneys with increased glomerular filtration rates have been reported to be associated with the polyuria and glycosuria of untreated diabetes in humans and rats (Mogenson & Anderson, 1973, 1975; Seyer-Hansen, 1977). The results of this study on kidney weight and composition is shown in Table 4-3. The enlarged kidneys and increased protein content in acutely diabetic rats confirm the published data of Seyer-Hansen (1976). The increase in RNA and DNA was much less than the protein changes and no change could be detected in the protein to DNA ratio in acute diabetes. However, a 28% increase in this ratio and hence, cellular growth, was observed after 56 days of diabetes. Similarly, Seyer-Hansen (1976) has reported a 20% enhancement of protein to DNA ratios after 42 days of diabetes.

Effect of insulin treatment on tissue weights and composition: Insulin treatment is known to prevent the onset of these kidney abnormalities or to reverse the development if already established (Seyer-Hansen,

TABLE 4 - 2 THE EFFECT OF ACUTE AND CHRONIC DIABETES ON LIVER RNA, DNA AND PROTEIN CONCENTRATIONS

	Control (6 rats)	Acute diabetes (13 rats)	Control (5 rats)	Chronic diabetes (8 rats)
Total tissue weight (g)	7.2±0.2	5.1±0.3	16.7±1.0	11.6±1.2
Protein (g/100g)	13.59±0.35	16.55±0.30	22.21±1.83	26.00±1.01
Total Protein (mg)	979±28	836±37	3716±171	3013±244
RNA (mg/g)	6.76±0.38	5.57±0.21	7.36±0.45	7.00±0.16
Total RNA (mg)	48.7±2.9	28.1±3.1	123.1±5.3	81.1±8.7
RNA/Protein (mg/g)	50.0±3.5	33.4±1.6	33.6±3.6	27.2±1.1
DNA (mg/g)	2.95±0.29	3.25±0.13	1.06±0.08	1.37±0.04
Total DNA (mg)	21.2±2.5	16.4±2.7	17.7±2.3	15.9±2.8
Protein/DNA (mg/mg)	48.4±4.7	51.9±2.2	209±7	179±11

Mean values ± SEM. Acute diabetes 5 days post-streptozotocin and chronic diabetes 56 days post-streptozotocin. Protein, RNA and DNA concentrations mg/g wet tissue.

TABLE 4 - 3 THE EFFECT OF ACUTE AND CHRONIC DIABETES ON KIDNEY PROTEIN, RNA AND DNA CONCENTRATIONS

	Control (6 rats)	Acute diabetic (13 rats)	Control (5 rats)	Chronic diabetic (8 rats)
Total tissue weight (g)	1.31±0.04	1.53±0.06	3.07±0.16	3.09±0.24
Protein (g/100g)	11.37±0.18	10.71±0.20	11.26±0.74	11.43±0.34
Total protein (mg)	149±8	164±9	348±6	359±7
RNA (mg/g)	3.34±0.26	3.09±0.12	3.09±0.21	2.28±0.16
Total RNA (mg)	4.38±0.11	4.73±0.09	9.86±0.12	7.10±0.17
RNA/Protein (mg/g)	26.3±3.3	29.0±1.1	27.6±1.2	19.8±1.1
DNA (mg/g)	5.52±0.33	5.19±0.22	4.48±0.30	3.72±0.37
Total DNA (mg)	7.23±0.71	7.94±0.58	13.71±0.82	11.52±0.63
Protein/DNA (mg/mg)	21.0±1.3	21.2±0.8	25.1±0.8	32.0±3.0

Mean values ± SEM. Acute diabetes 5 days post-streptozotocin and chronic diabetes 56 days post-streptozotocin. Protein, RNA and DNA concentrations mg/g wet tissue.

1976). This was examined in a study in which 4 day diabetic rats were treated for a further 4 days with PZI (4 units/day). As is shown in Table 4-4, this short duration of insulin treatment was ineffective in normalizing kidney protein and RNA content, despite increased growth rates and control plasma glucose values in the insulin treated rats. The gastrocnemius muscle responded to insulin with an increase in RNA content and RNA to protein ratios, but only partially so as was reported also by Pain & Garlick (1974). In contrast, the liver demonstrated a marked increase in total tissue weight, namely from 6.4 g in control and 4.9 g in diabetic rats to 9.1 g in insulin treated rats. This pronounced enlargement of the liver in response to insulin has been noted by other workers (Steiner & Williams, 1959; Peavy et al., 1978) and is probably partly due to an ~~transitory~~ accumulation of glycogen and fat in the liver since glycogen deposition, as measured by activity of UDPglucose glycogen glucosyl-transferase, is increased within hours of insulin administration (Steiner & King, 1964).

4.7 CONCLUSION

From this series of experiments it was concluded that in young (90-100g) rats, a streptozotocin dose of 13 mg effectively destroyed enough β -cells of the Islets of Langerhans to result in a severe diabetic state. The results of the effect of diabetes on growth, food intake, plasma glucose and tissue protein, RNA and DNA content, are all similar to published data. Furthermore, the insulin treatment regime as tested was found to be an efficient method of normalizing the physiological abnormalities caused by diabetes.

This diabetic model and insulin treatment regime was used throughout in the experiments to be discussed.

TABLE 4 - 4 THE EFFECT OF INSULIN TREATMENT ON PROTEIN AND RNA CONTENT OF LIVER, GASTROCNEMIUS AND KIDNEY

	Control (5 rats)	Diabetic (5 rats)	Insulin treated (4 rats)
Body weight (g)	137 ± 2	115 ± 4	128 ± 4
Plasma glucose (mg/100 ml)	178 ± 16	633 ± 26	108 ± 12
Total protein (mg) - Liver	1036 ± 36	1033 ± 46	1320 ± 73
- Gastrocnemius	222 ± 10	156 ± 13	164 ± 19
- Kidney	174 ± 10	198 ± 14	190 ± 13
Total RNA (mg) - Liver	55 ± 3	42 ± 3	66 ± 3
- Gastrocnemius	2.5 ± 0.1	1.3 ± 0.2	1.9 ± 0.2
- Kidney	5.9 ± 0.3	6.3 ± 0.5	6.0 ± 0.4
RNA/Protein (mg/g) - Liver	53.3 ± 1.1	40.8 ± 3.2	50.0 ± 3.6
- Gastrocnemius	12.4 ± 1.3	9.1 ± 0.6	11.9 ± 0.8
- Kidney	34.6 ± 2.1	31.9 ± 0.5	31.9 ± 1.4

Diabetic rats were 5 days post-streptozotocin.

Insulin treatment (4 U PZI / day) of 4 day diabetic rats for a further 4 days.

SECTION 5WHOLE BODY PROTEIN TURNOVER IN DIABETIC AND INSULIN TREATEDRATS5.1 INTRODUCTION

The investigation of protein metabolism in adult diabetics failed to show any differences between rates of whole-body protein synthesis before and after short periods of insulin treatment (Section 3), which contrasted with the large decrease in muscle-protein synthesis found in streptozotocin diabetic rats (Pain & Garlick, 1974; Millward et al., 1976, and Sections 6 and 7). Since no published data was available on whole-body protein turnover in diabetic animals, the following study was planned :

- (a) to determine whole-body protein synthesis and breakdown rates by constant infusion with L-[1-¹⁴C] leucine in diabetic (4 and 8 days post-streptozotocin), insulin treated diabetic and age control rats.
- (b) to determine whole-body protein oxidation rates in these three experimental groups and their controls during the same infusion by measurement of the proportion of the ¹⁴C label excreted in respiratory CO₂.

5.2 METHOD

From the two-pool model discussed in Section 1.1.1 it is evident that the loss of label of a ¹⁴C amino acid via oxidation and the excretory pathway can be determined from the output of ¹⁴CO₂. If measurements are made over a short time and so exclude re-entry of

tracer from the breakdown pathway of this model, the two main routes of disposal of amino acids are by synthesis and oxidation. Therefore, the proportion of label appearing in respiratory CO_2 should give an estimate of the relative flow of amino acids along the pathway of oxidation compared with that of synthesis to protein.

Most published in vivo data, using closed systems for $^{14}\text{CO}_2$ collection, have expressed amino acid oxidation only as proportion of $^{14}\text{CO}_2$ output, without measurement of flux which enables calculation of synthesis rates (McFarlane & von Holt, 1969 ; Meikle & Klain, 1972; Neale & Waterlow, 1974 a, b).

5.2.1 Calculation of protein turnover

Flux was determined from the specific activity of the free amino acid in plasma when a plateau was achieved during constant infusion with $[1-^{14}\text{C}]$ leucine. At plateau the input and outflow of radioactivity would be equal, hence

$$Q = \frac{i^*}{S_{\max}} \quad (1)$$

where i^* is the rate of isotope infused (dpm/minute), S_{\max} the specific activity of plasma at plateau (dpm/ μmole) and Q the flux ($\mu\text{moles/minute}$). Oxidation (E , $\mu\text{moles/minute}$) was obtained from

$$E = \frac{e^*}{S_{\max}} \quad (2)$$

where e^* is the rate of excretion of $^{14}\text{CO}_2$ (dpm/minute).

Protein synthesis (Z), oxidation (E) and breakdown (B) rates were obtained from

$$Q = Z + E = B + I \quad (3)$$

The calculation of breakdown required precise information on diet intake (I) and on the leucine content of the rat diet. From automated

amino acid analysis, the Oxoid diet was found to contain 1.17g leucine per 100 gram diet. Alternatively, breakdown was calculated from total body growth (G)

$$B = Z - G \quad (4)$$

where grams of body weight gained per day was converted to leucine, using a value of 88.6 μ moles leucine per gram body weight (Fern, 1975).

In all the experiments flux was determined from the plasma specific activity of leucine, with the plasma extracellular pool therefore used as a representative of the precursor pool from which protein synthesis and oxidation take place. In Section 5.4.4, a comparison will be made with the same results calculated from the tissue free amino acid pool.

All values for synthesis oxidation and breakdown were expressed as μ moles leucine per minute per 100g rat weight to accommodate for the difference in final body weight between diabetic and control groups.

5.2.2 Tissue and respiratory CO₂ collection and analysis

5.2.2.1 Apparatus for ¹⁴CO₂ collection

[1-¹⁴C] leucine was infused over a period of 4 hours and ¹⁴CO₂ in expired air collected by an apparatus designed to overcome the complexity and problems of a closed air collection system.

A diagram of the system used is shown in Figure 5-1. [¹⁴C]-leucine was infused into the rat via a tail vein by a slow speed constant infusion pump. The rat, wrapped in a restraining cloth, was placed in a glass tube (25cm x 6 cm) closed at one end with a rubber stopper pierced by an outlet bore hole. The outlet was connected to translucent polythene tubing (9.52 mm OD x 6.35 mm ID) leading to a system of scintillation vials containing 3 ml of a 1:2 (v/v) mixture of hyamine hydroxide and ethanol, a mixture which effectively trapped CO₂. At precisely timed intervals, air was sequentially bubbled

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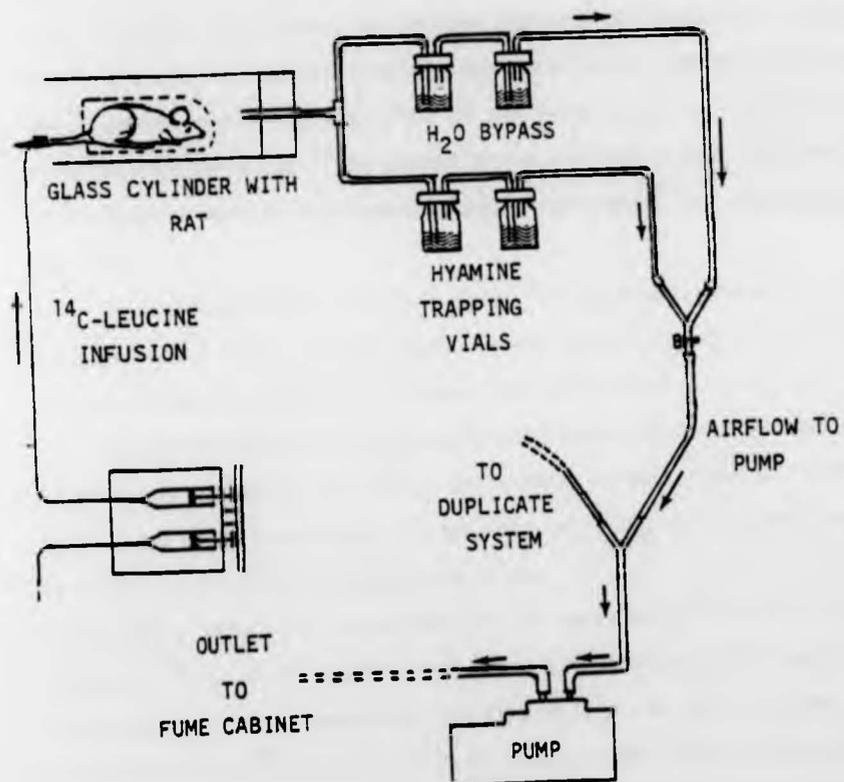
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FIGURE 5-1 APPARATUS FOR THE COLLECTION OF $^{14}\text{CO}_2$ DURING INFUSIONS WITH (1- ^{14}C)LEUCINE



through the two vials for exactly 60 seconds. The air pump was a Charles Austin diaphragm pump (Type MU 19/26) with a free air displacement of 16 litres per minute and 500 mm Hg vacuum capacity. Air was drawn through the scintillation vials via resistant thick walled polythene tubing (ID 2mm x OD 3mm) which passed through the vial caps. Two vials were used sequentially, since the first vial was 98 to 99% efficient in trapping $^{14}\text{CO}_2$. The second vial functioned as 'spillover' trap. Between the actual collection periods of 60 seconds, air was drawn through an identical bypass system of vials containing water, which maintained a constant flow of air from the glass cylinder and effectively prevented $^{14}\text{CO}_2$ accumulation in the system. Expired air was pumped directly to a fume cabinet to prevent $^{14}\text{CO}_2$ contamination of room air.

The radioactivity of the trapped $^{14}\text{CO}_2$ was determined by counting in a Beckman LS 150 liquid scintillation counter, with 10 ml of 0.4% 2,5 dinitrophenyloxazole in toluene as scintillant.

The final apparatus included a duplicate system which enabled simultaneous infusion and $^{14}\text{CO}_2$ collection of two animals. The efficiency of the apparatus in recovery of $^{14}\text{CO}_2$ was tested with $\text{Na H}^{14}\text{CO}_3$ and $[1-^{14}\text{C}]$ leucine infusions.

Bicarbonate infusion: Oxidation of ^{14}C amino acids or $\text{Na H}^{14}\text{CO}_3$ produces $^{14}\text{CO}_2$ which rapidly exchanges with the body bicarbonate pool from which it is released and excreted in expired air. In man, infusion of $\text{Na H}^{14}\text{CO}_3$ results in only 80% of the label being excreted as $^{14}\text{CO}_2$, with a 20% retention in the body (James et al., 1976). Sketcher (1976), using a closed system of $^{14}\text{CO}_2$ collection, established that in rats the label was quantitatively excreted when $\text{Na H}^{14}\text{CO}_3$ was infused.

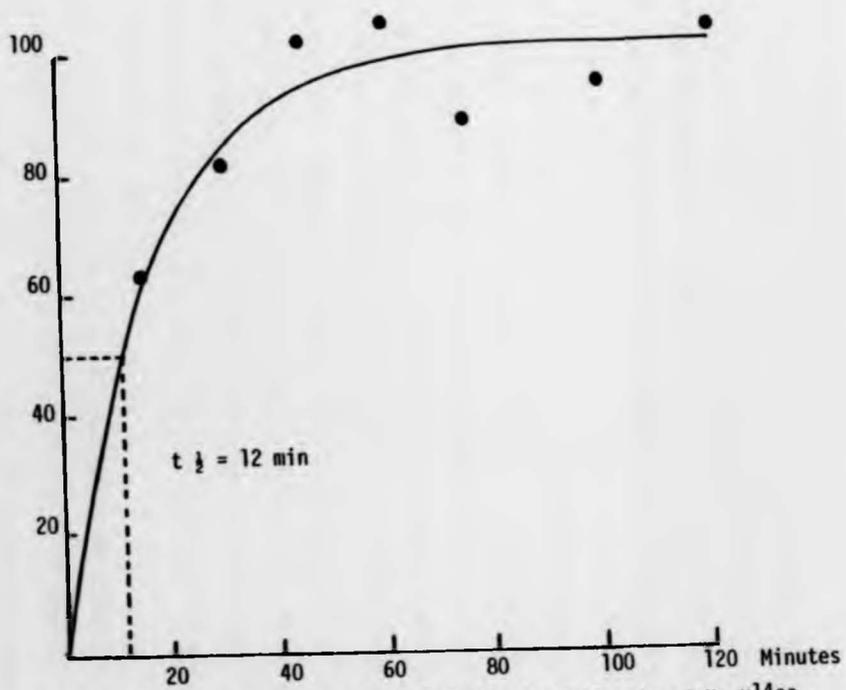
The present system was tested with infusing $\text{Na H}^{14}\text{CO}_3$ directly into a beaker containing 1 N HCl placed in the glass tube. 100% of

the label was recovered as $^{14}\text{CO}_2$. However when $\text{Na H}^{14}\text{CO}_3$ (2 $\mu\text{Ci/ml}$) was infused into animals, each rat quite surprizingly and consistently produced a $^{14}\text{CO}_2$ output of 150 - 249% of the infused dose! This indicated that $^{14}\text{CO}_2$ was accumulating in the glass tube between periods of $^{14}\text{CO}_2$ collection. At this stage of development the system did not include the bypass vials, hence airflow was restricted to a minimum between each collection period of 60 seconds. Therefore a change was made by building into the collection system two bypass vials containing water. Between $^{14}\text{CO}_2$ collection periods air would thus flow uninterrupted from the rat to the pump via these vials and so prevent accumulation. As a last precaution the pump-outlet was led directly to a fume cabinet to prevent any re-circulation of $^{14}\text{CO}_2$ in the room air.

With these adjustments made, further infusions of $\text{Na H}^{14}\text{CO}_3$ (2 $\mu\text{Ci/ml}$) into control male albino Wistar rats of 100 - 120g, gave the expected result of 100% excretion of the label (Figure 5-2). $^{14}\text{CO}_2$ output reached a plateau value within 60 minutes and was maintained throughout 2 - 3 hours of infusion. With no retention of ^{14}C in the bicarbonate pool, it was assumed that no correction factor would be required to estimate the absolute oxidation rate with [^{14}C] leucine infusions. The calculated half-life of the bicarbonate pool was 12 minutes (Figure 5-2) which agrees well with the 15 minutes reported by Sketcher (1976) and the 12.5 minutes of Millward (1970).

Leucine infusion: The objective of this pilot study was to determine (a) the rate of $^{14}\text{CO}_2$ excretion and (b) the time in which a plateau would be reached in $^{14}\text{CO}_2$ output in diabetic and control rats. Six days after diabetes was induced with streptozotocin, diabetic and control rats were infused for 4 hours with [$1\text{-}^{14}\text{C}$] leucine (5 $\mu\text{Ci/ml}$, 50 mCi/mmol). The rise to plateau of $^{14}\text{CO}_2$ in expired air as % of

Figure 5-2 $^{14}\text{CO}_2$ OUTPUT AS % OF INFUSED $\text{NaH}^{14}\text{CO}_3$



Output of $^{14}\text{CO}_2$ during constant infusion by tail vein of $\text{NaH}^{14}\text{CO}_3$
($2\mu\text{Ci/ml}$) at 0.5ml/hour into one 100g rat.

infused dose is shown in Figure 5-3. In control rats a plateau was reached within 2 hours and 12.5% of the label was recovered in CO_2 . In the diabetic rats a plateau was also reached and excretion of label increased up to 20.4%.

The results of the $\text{Na H}^{14}\text{CO}_3$ and leucine infusion pilot studies confirmed that the apparatus was an effective system for the measurement of expired $^{14}\text{CO}_2$ and hence oxidation rate.

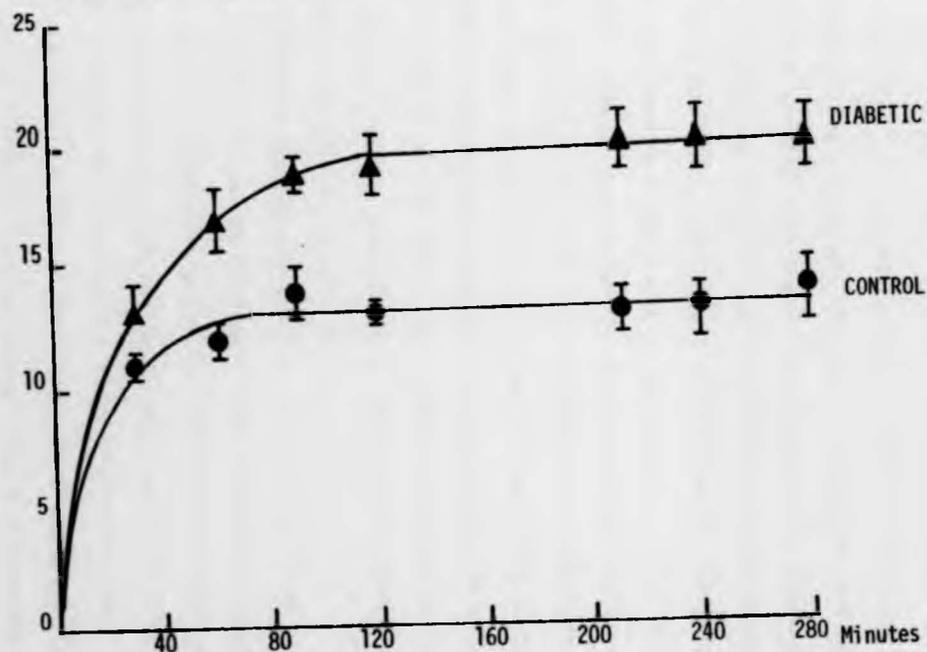
5.2.2.2 Constant infusion technique

Each rat was wrapped in a cloth before being placed inside the glass tube (see Figure 5-1). This restrained movement during the infusion and from previous experience in this laboratory, did not appear to seriously stress the rat or impair normal breathing. The rat's tail was washed in warm water and the tail veins further dilated by a coating with xylene. The lateral vein was cannulated with a 26G ($\frac{1}{2}$ inch) needle without its plastic ferrule. The needle was connected to a soft-walled polyvinyl cannula (ID 0.4mm) leading to a 1 ml syringe containing 0.9% (w/v) saline. The needle with cannula was held in place in the vein by adhesive tape around that section of the tail. The infusion was started by replacing the saline syringe with a 10 ml syringe containing the ^{14}C labelled amino acid. This 10 ml syringe was firmly clamped onto a constant speed slow infusion pump (Scientific and Research Instruments Limited, Croydon, Surrey, U.K.) set at a flow-rate of 0.46 ml per hour.

Each infusion lasted at least 4 hours with 60 second periods of $^{14}\text{CO}_2$ trapping every 30 minutes until a plateau was reached in $^{14}\text{CO}_2$ production. Thereafter the rate of $^{14}\text{CO}_2$ production was measured at intervals of 15 minutes.

Modification of infusion technique: Food intake data were collected in all experimental groups and their controls on the two days preceding the constant infusion of [^{14}C] leucine, whilst growth was measured

Figure 5-3 $^{14}\text{CO}_2$ OUTPUT AS % OF INFUSED (^{14}C)LEUCINE



Output of $^{14}\text{CO}_2$ during constant infusion of L(^{14}C)leucine ($5\mu\text{Ci/ml}$) at 0.5ml/hour in 5 diabetic and 5 control rats.

daily by weighing the rats at the same time each morning.

Due to the severe hyperphagia observed in the 8 day diabetic and insulin treated rats, a modification had to be made in Experiment II (Section 5.4.2) in the constant infusion technique. In a pilot study of PZI treated diabetic rats infused with [^{14}C] leucine, only 2 out of 5 rats survived the 4 hour period in the glass cylinder without access to food. The surviving rats had very low plasma glucose values, ie. 23 and 51 mg/100 ml. The absence of food combined with the still available PZI in the plasma was obviously responsible for severe hypoglycaemia which in some rats caused their death. It was therefore prudent to modify the existing system of $^{14}\text{CO}_2$ collection to enable all rats in Experiment II to have access to food during the infusion.

Instead of being wrapped and restrained by a cloth inside the glass tube, the rats were placed in wire restraining cages with free access to food and water, whilst being infused via the tail vein. The restraining cages consisted of a cylinder formed by 15 cm long wire needles protruding through a movable perspex foot-plate on one end and immovably attached to the other end. These wires enabled changes in both the inside diameter and length of the cage to accommodate the large control and small diabetic rats.

By constantly monitoring the rat in the restraining cage, it was possible to prevent the rat from pulling or chewing the cannula from its tail vein. It was also possible to observe and confirm that hyperphagic rats did in fact eat small meals at short intervals.

After 3 hours of [^{14}C] leucine infusion, the rats were gently removed from the wire cage, wrapped in a cloth and placed in the glass cylinder of the $^{14}\text{CO}_2$ collection system without dislodging the infusion cannulas. Having previously established that plateau $^{14}\text{CO}_2$ production was reached within two to three hours, adequate measurements of $^{14}\text{CO}_2$ excretion in this experiment were made during only the last

hour of the infusion.

5.2.2.3 Specific activity measurements

At the end of each 4 hour infusion, rats were decapitated and bled from the trunk vessels. The gastrocnemius muscles were rapidly removed and frozen in liquid nitrogen.

The mixed venous and arterial blood was spun at 1200 x g for 10 minutes and part of the plasma was used to determine plasma glucose. The rest of the plasma was precipitated with an equal volume of 5% sulphosalicylic acid and spun. The plasma supernatant containing the free amino acid fraction was then stored frozen and used to determine the specific activity of [^{14}C]leucine in plasma by automated amino acid analysis.

The muscle samples were prepared for amino acid analysis by pulverizing the frozen muscle between two aluminium blocks cooled on dry ice (see Section 4) and precipitated with 10% trichloroacetic acid. The acid was removed from the supernatant containing the free amino acids by three ether washes and the supernatant was then stored frozen for later analysis.

All specific radioactivities were determined with a Locarte amino acid analyzer (Locarte Company, London W12) fitted with a column effluent stream splitter which enabled quantitation of both amino acid and its radioactivity. The procedure followed was similar to the method described in Section 3 for 3-methyl histidine determinations, but with some modifications. The column was eluted with 0.2 M sodium citrate buffer (pH 3.44) at a temperature of 65°C, with a total amino acid analysis running time of 240 minutes per sample and flow rate of 30 ml per hour. Internal calibration standards of 1 mM norleucine and 2 mM cycloleucine were run with each sample, and at a few days intervals, a Pierce-H amino acid calibration standard (Pierce Chemical Company, Rockford, Ill, U.S.A.). An internal standard equivalent could then be

calculated and the specific activity determined of leucine in each sample from the counts in each fraction collected by the column effluent stream splitter. A split of 1:1 (fraction collector:amino acid analyzer) was used and 6 minute fractions were collected with an LKB Ultrarac (LKB Limited, South Croydon, Surrey, U.K.). The fractions were quantitatively transferred to scintillation vials and counted in a Triton X-100/xylene based scintillant.

The same system of automated amino acid analysis (without effluent stream splitter) was used to determine the leucine content of the cubed diet (Oxoid). This information was required to convert food intake to leucine intake and from this data, calculate breakdown as described in the calculation section. For amino acid analysis the cubed diet was hydrolyzed in 6N HCl for 24 hours in sealed tubes and evaporated to dryness to remove the acid. The procedure of analysis was the same as described above.

5.3 TREATMENT PROTOCOLS FOR EXPERIMENTS I AND II

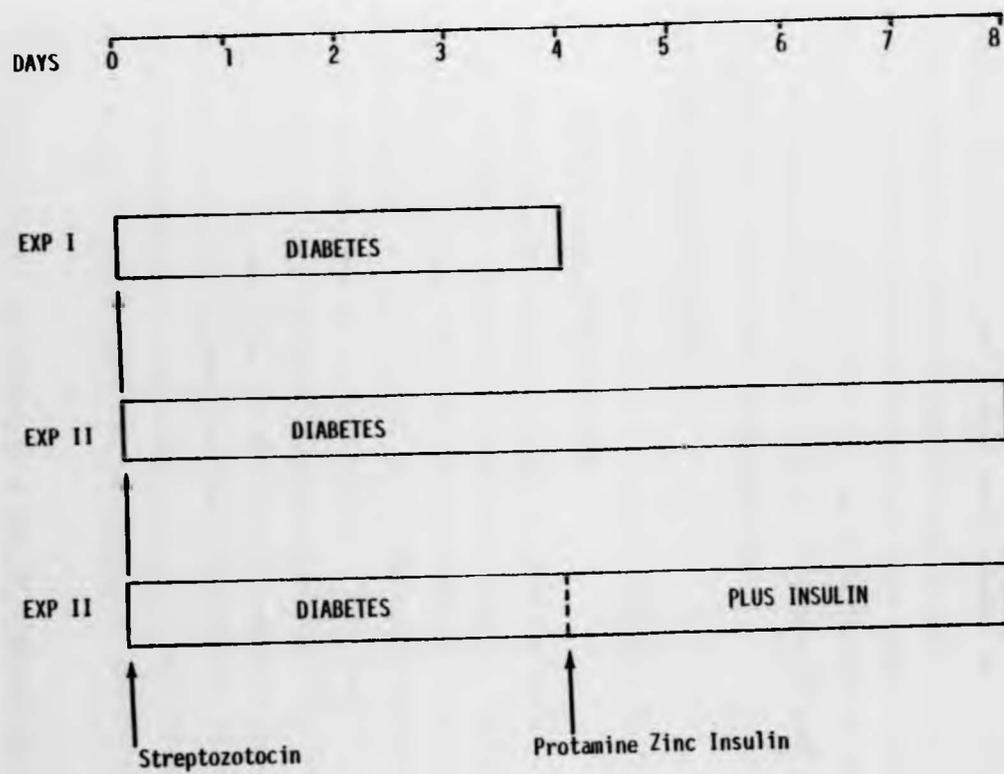
Experiments were planned to measure whole body protein turnover in rats with short and long duration diabetes and in diabetic rats treated with insulin to enable valid comparisons to be made with insulin treated diabetic patients. An outline of the protocol used is shown in Figure 5-4.

Male Wistar rats of 90 - 100 grams were used in all experiments. Diabetes was induced with 13mg streptozotocin as explained in Section 4. Food intake was monitored in all rats.

In Experiment I rats were infused with [1-¹⁴C] leucine after 4 days of diabetes at which time the food intake of the diabetic rats and their controls were equal. This experiment therefore excluded the effect of changes in food intake on the rate of leucine oxidation.

In experiment II both the diabetic and insulin treated rats were

FIGURE 5-4 TREATMENT PROTOCOL FOR PROTEIN TURNOVER STUDIES



hyperphagic compared with their controls. The diabetic rats were infused 8 days after diabetes was induced, at which time hyperphagia was well established. As shown in Section 4, insulin treatment results in a similar hyperphagic response as that observed in long duration diabetes. Rats diabetic for 4 days, were treated for a further 4 days with a single daily dose of 4 units PZI and infused on day 8 (Figure 5-4). In this experiment the rats were allowed access to food during the first 3 hours of the infusion as explained in Section 5.2.2.2.

The results of each set of diabetic or insulin treated rats were compared with it's own group of age controls.

5.4 RESULTS AND DISCUSSION

5.4.1 Experiment I: The effect of 4 days of diabetes on protein synthesis and oxidation rates

After 4 days of diabetes, protein synthesis rates were only slightly decreased (Table 5-1), whereas the rate of oxidation was significantly increased above that of the controls. Due to normal animal variation in resistance to β -cell destruction by streptozotocin, some of the rats were only mildly diabetic. This group differed from the age controls only in plasma glucose values and $^{14}\text{CO}_2$ production (Table 5-1). The two groups of diabetic rats showed a significant correlation ($p < 0.05$) between the degree of diabetes, ie. plasma glucose, and the rate of leucine oxidation (Fig.5-5). No difference was shown in rate of flux between the two diabetic groups and the control rats.

5.4.2 Experiment II: The effect of 8 days of diabetes and of insulin treatment on protein synthesis and oxidation rates

After 8 days of diabetes, plasma glucose and food intake were significantly increased above that of the control groups and growth

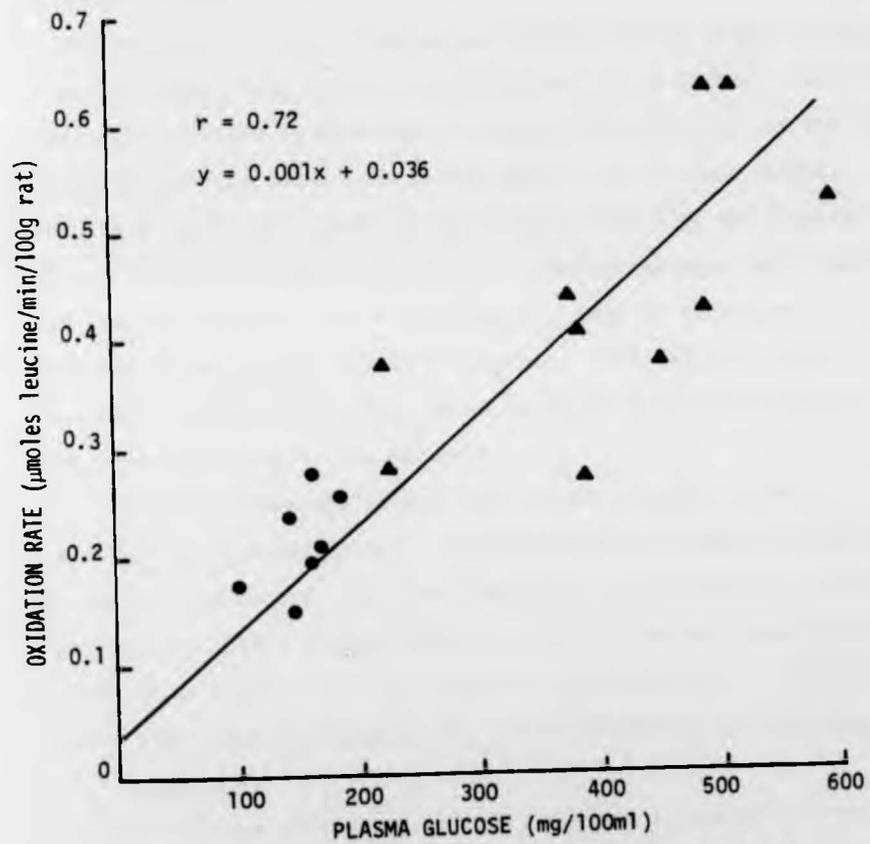
TABLE 5 - 1 THE EFFECT OF MILD AND SEVERE DIABETES (4 days) ON $^{14}\text{CO}_2$
OUTPUT AND RATES OF FLUX, OXIDATION AND SYNTHESIS

	Control (5 rats)	Mild diabetes (5 rats)	Severe diabetes (5 rats)
Food intake (g/day)	16.6±0.5	-	16.3±0.4
Body weight (g)	109±4	101±6	92±4
Plasma glucose (mg/100ml)	159±6	262±44	453±11
$^{14}\text{CO}_2$ output (dpm/min)	11,416±484	12,720±1148	21,656±1230
S_A Leucine (dpm/ μmole)	47,987±1404	55,709±6299	56,320±4430
Flux [†]	1.677±0.080	1.680±0.059	1.785±0.097
Oxidation [†]	0.222±0.018	0.231±0.009	0.428±0.035*
Synthesis [†]	1.455±0.064	1.450±0.067	1.358±0.074

All values mean \pm SEM for age controls and diabetic groups (both 4 days post-streptozotocin injection). Leucine specific activity (S_A) measured in plasma and used in estimating flux. Food intake mean value of 2 days preceding ^{14}C infusion. [†] μmoles leucine per min per 100 g rat.

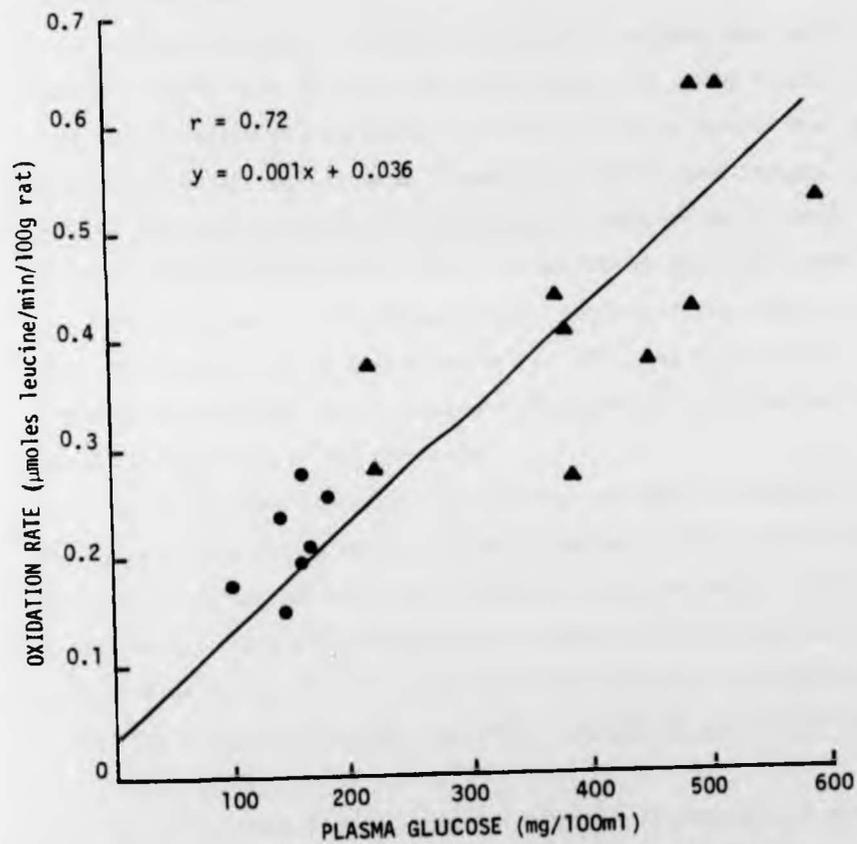
* significantly different from control $p < 0.001$.

FIGURE 5-5 CORRELATION OF PLASMA GLUCOSE WITH OXIDATION RATES MEASURED IN DIABETIC RATS



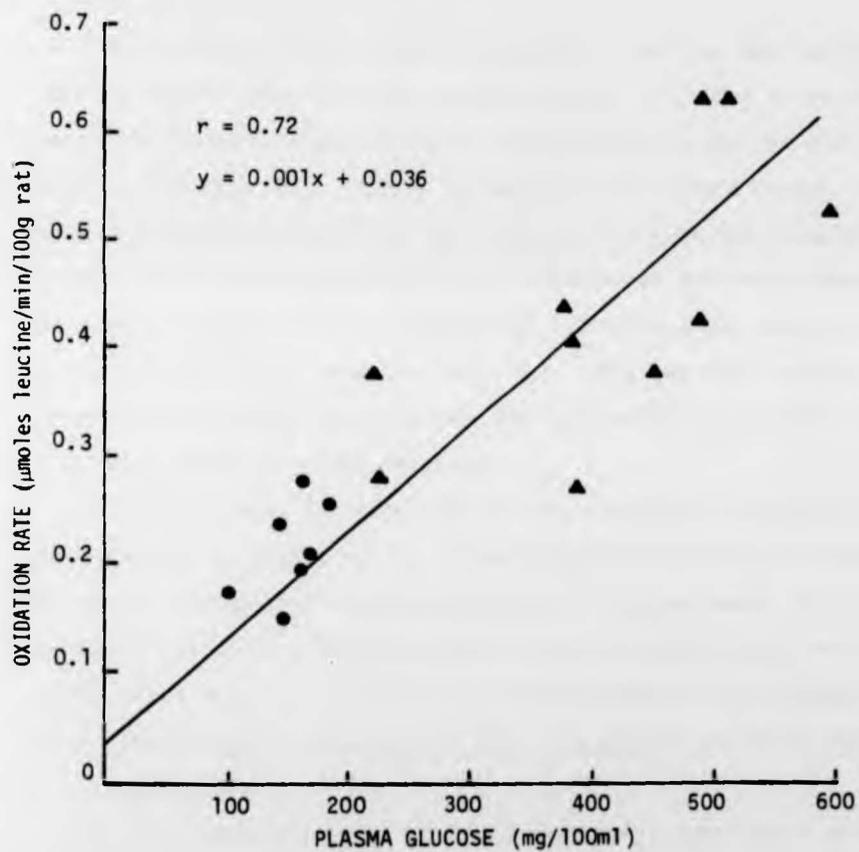
The regression line is that for glucose values of diabetic rats only (▲). Values for control animals (●) also lie close to this line.

FIGURE 5-5 CORRELATION OF PLASMA GLUCOSE WITH OXIDATION RATES MEASURED IN DIABETIC RATS



The regression line is that for glucose values of diabetic rats only (▲). Values for control animals (●) also lie close to this line.

FIGURE 5-5 CORRELATION OF PLASMA GLUCOSE WITH OXIDATION RATES MEASURED IN DIABETIC RATS



The regression line is that for glucose values of diabetic rats only (▲). Values for control animals (●) also lie close to this line.

severely depressed (Table 5-2). The longer duration of diabetes resulted in a significant decrease in synthesis ($p < 0.05$). Oxidation rates were even further increased ($p < 0.001$) above control levels, most probably as a result of the combined effect of the longer duration of the disease and the increase in food intake. Again no change in flux was observed.

The four day insulin regime successfully restored body weight to that of control rats by an accelerated growth rate. The insulin treatment also resulted in a hyperphagic state similar to that of the long duration diabetic rats, with an increase of 18% in food intake. Insulin effectively normalized the hyperglycaemia of the diabetic state. Plasma glucose values of 50 ± 8 mg/100 ml were much lower than the control values of 173 ± 6 mg/100 ml. Despite these seemingly hypoglycaemic conditions, no deaths occurred. This was most probably prevented by the rats having access to food and actually eating during the first few hours of the infusion.

Table 5-3 shows the effect of insulin treatment on protein oxidation and synthesis rates. Protein synthesis rates were restored to that of the control rats, but despite a large decrease, the rate of oxidation was still significantly ($p < 0.05$) elevated above the control value. Flux rates, as in all previous experiments were unchanged.

5.4.3 The effect of diabetes and insulin treatment on whole body breakdown rates

Although calculation of protein breakdown is possible in theory from the equations in Section 5.2.1, namely from either growth or food intake data, in practice the results are difficult to interpret. Figure 5-6 shows the rate of protein breakdown calculated from both growth and food intake of rats diabetic for 4 days (Section 5.4.1). Breakdown from growth data appears to indicate that although whole body

TABLE 5 - 2 THE EFFECT OF LONG DURATION DIABETES (8 days) ON ^{14}C OUTPUT AND RATES OF FLUX, OXIDATION AND SYNTHESIS

	Control (6 rats)	Diabetic (8 rats)
Body weight (g)	151 ± 2	103 ± 4
Plasma Glucose (mg/100 ml)	163 ± 5	507 ± 17
Food intake (g/day)	18.9 ± 0.9	24.6 ± 0.8
^{14}C Output (dpm/min)	11,679 ± 872	28,970 ± 1901
S _A Leucine (dpm/μmole)	32,103 ± 1456	43,051 ± 1601
Flux [†]	1.913 ± 0.089	2.055 ± 0.075
Oxidation [†]	0.242 ± 0.017	0.671 ± 0.056*
Synthesis [†]	1.671 ± 0.077	1.385 ± 0.057**

All values shown as mean ± SEM. Leucine specific activity (S_A) measured in plasma and used in estimating flux. Food intake mean value of 2 days preceding ^{14}C infusion. [†]μmoles leucine per min per 100 g rat.

* significantly different from control p < 0.001 and ** p < 0.05.

TABLE 5 - 2 THE EFFECT OF LONG DURATION DIABETES (8 days) ON $^{14}\text{CO}_2$ OUTPUT AND RATES OF FLUX, OXIDATION AND SYNTHESIS

	Control (6 rats)	Diabetic (8 rats)
Body weight (g)	151 \pm 2	103 \pm 4
Plasma Glucose (mg/100 ml)	163 \pm 5	507 \pm 17
Food intake (g/day)	18.9 \pm 0.9	24.6 \pm 0.8
$^{14}\text{CO}_2$ Output (dpm/min)	11,679 \pm 872	28,970 \pm 1901
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All values shown as mean \pm SEM. Leucine specific activity (S_A) measured in plasma and used in estimating flux. Food intake mean value of 2 days preceding ^{14}C infusion. [†] μ moles leucine per min per 100 g rat.

* significantly different from control $p < 0.001$ and ** $p < 0.05$.

TABLE 5 - 3 THE EFFECT OF INSULIN TREATMENT OF DIABETIC RATS ON RATES OF FLUX, OXIDATION AND SYNTHESIS[†]

	Control (6 rats)	Insulin treated diabetics (6 rats)
Flux	1.671 ± 0.139	1.698 ± 0.104
Oxidation	0.230 ± 0.021	0.314 ± 0.028*
Synthesis	1.441 ± 0.129	1.384 ± 0.091

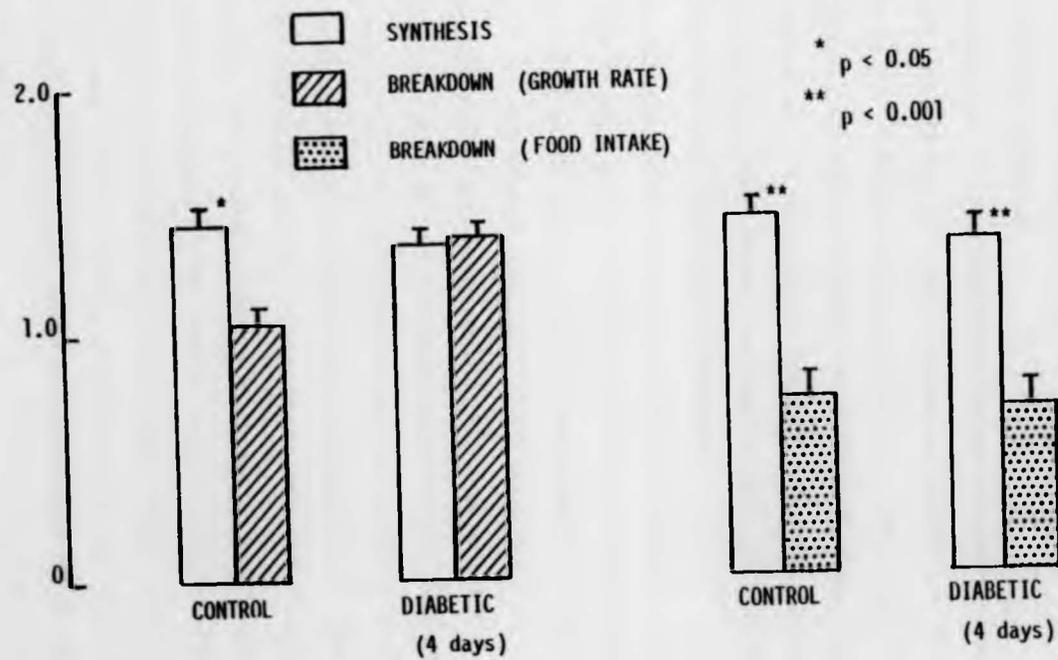
All values mean ± SEM calculated from plasma leucine specific activity.

* significantly different from control $p < 0.05$.

[†]umoles leucine per min per 100 g rat.

FIGURE 5-6

WHOLE BODY PROTEIN SYNTHESIS AND BREAKDOWN RATES ($\mu\text{moles leucine/minute/100g rat} \pm \text{SEM}$)



protein synthesis was not affected by 4 days of diabetes, protein breakdown was significantly increased. However, if we consider breakdown rates calculated from food intake data from these non-hyperphagic diabetic rats, the total picture is changed, with both synthesis and breakdown unaltered by diabetes.

The interpretation of breakdown rates becomes even more obscure if one considers the effect of 8 days of diabetes combined with hyperphagia (Figure 5-7). From growth data, breakdown is not significantly changed by diabetes, whereas from food intake data breakdown rates are now severely depressed. In the insulin treated rats, the accelerated growth rate and enhanced food intake resulted in very low breakdown rates as calculated from either growth or intake.

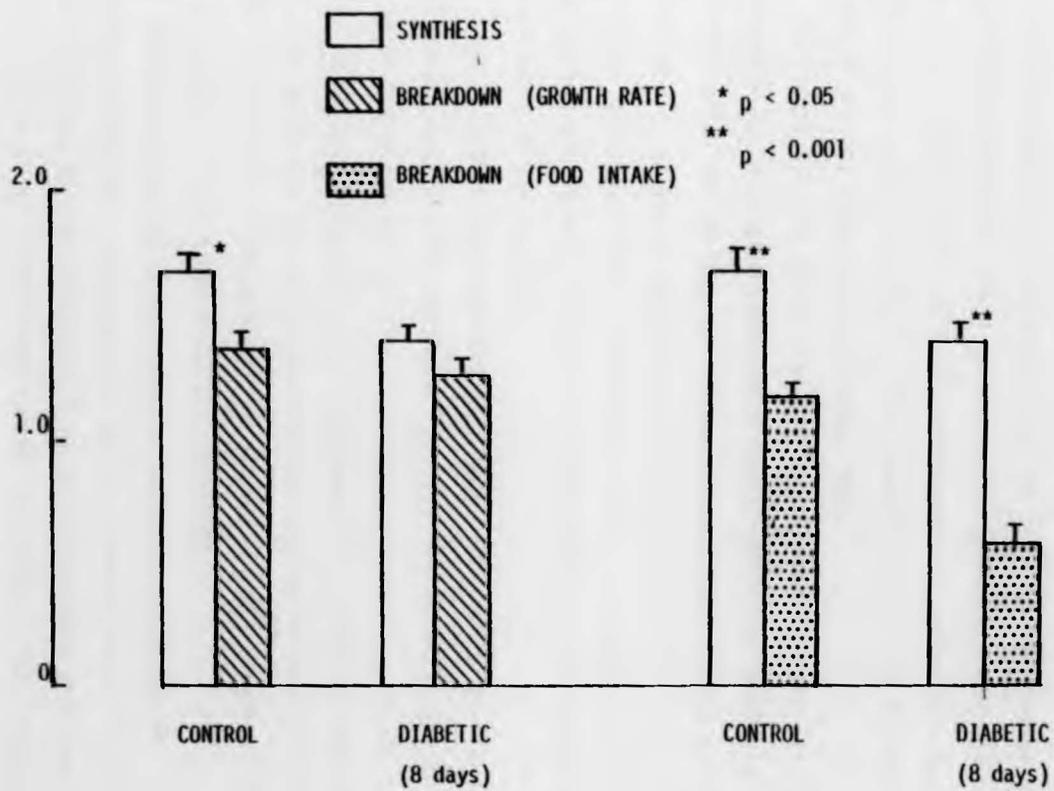
In humans the estimation of breakdown from $Q = Z + E = B + I$ is valid since the intake of food can be regulated to coincide with the time (9 or 12 hours) over which protein turnover measurements are made (see Section 3.3.1). In the animal experiments however, whole body protein turnover is measured during a 4 hour infusion and compared with the rate of whole body growth over 24 hours or the intake of food over 24 hours. Therefore, a mean value of food intake is applied in the calculation without knowledge of the rate of absorption of protein from food during the 4 hour infusion period. Furthermore, the actual measurement of growth and food intake cannot be exact and is highly variable, for example it is influenced by the time of day at which the rats and food are weighed or even conditions in the animal house such as lesser disturbance over weekends than during the week. The results of this study suggest that calculation of whole body breakdown rates are not valid in the animal model.

5.4.4 Flux measurements from plasma and tissue free precursor pools

The results presented to date were based on flux measurements from the specific activity of leucine in the plasma precursor pool.

FIGURE 5-7

WHOLE BODY SYNTHESIS & BREAKDOWN RATES ($\mu\text{moles leucine/minute/100 rat} \pm \text{SEM}$)



Waterlow et al. (1978a) have suggested that since the precursor pool is not homogenous and possibly compartmented, the pool probably lies somewhere between the intracellular (free tissue) and extracellular (plasma) pools.

The intracellular free amino acid pool (from gastrocnemius muscle) is therefore an equally valid choice and, as precursor pool for protein oxidation, probably more realistic since muscle is not only a major site of oxidation of leucine but also comprises about 50% of body weight in the rat (Odessey & Goldberg, 1972).

In Figure 5-8 the rates of oxidation are summarized for all the experiments as calculated from either the plasma or intracellular specific activities of leucine. Since the specific activity of leucine in plasma will always be higher, the calculated oxidation rate will be the lowest value possible. From Figure 5-8 it is clear that the choice of precursor pool does not change the interpretation or statistical significance of the results.

This is rather less apparent in Figure 5-9 which shows a summary of all whole body protein synthesis rates as calculated from either plasma or intracellular specific activities. Whereas synthesis rates appear to be normalized by insulin treatment with plasma as precursor pool, the synthesis rates of diabetic rats calculated from the intracellular pool are not brought back to the age control values, although the difference is not statistically significant.

The error involved in deciding on the correct pool can be minimized by giving a large dose of amino acid which would flood and nearly equalize the specific activities of all possible precursor pools (Henshaw et al., 1971; McNurlan et al., 1979). This approach was used in Section 7.

FIGURE 5-8
 PROTEIN OXIDATION RATES ($\mu\text{moles}/\text{min}/100\text{g rat} \pm \text{SEM}$)

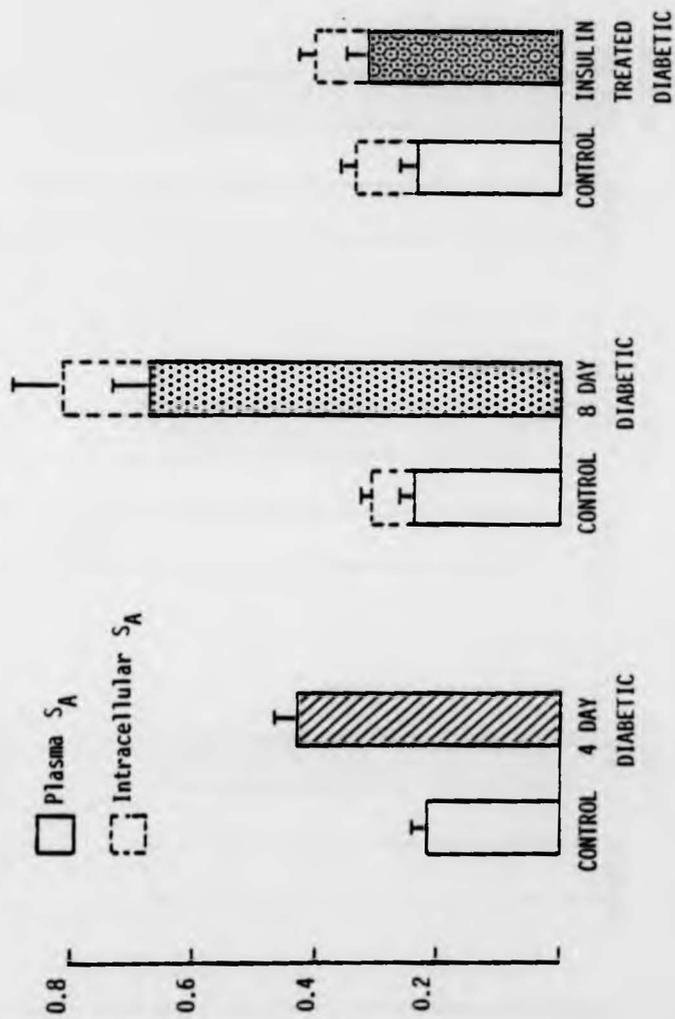
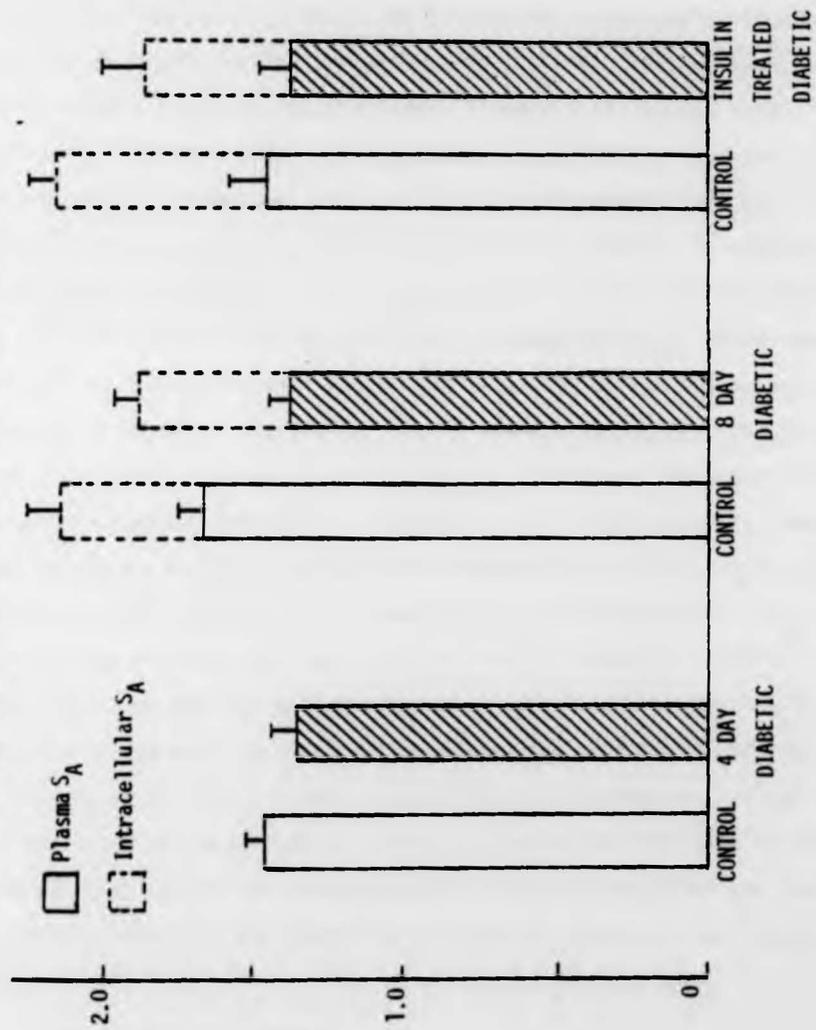


FIGURE 5-9
 PROTEIN SYNTHESIS RATES ($\mu\text{moles/minute}/100\text{g rat} \pm \text{SEM}$)



5.4.5 The effect of diabetes and insulin treatment on leucine pool size

The size of the free amino acid pool (plasma or intracellular) is influenced by the influx of amino acids from either recycling via breakdown or from dietary input, or by a block in protein synthesis and thus decreased outflow (Waterlow et al., 1978a). Millward et al. (1976) showed increased leucine levels in muscle of energy restricted, starved and diabetic rats. They suggested that this is not necessarily a consequence of breakdown only but also from decreased synthesis. In diabetes the results of Experiment II show that there is an expanded leucine pool (Table 5-4). This could possibly result from the decrease in protein synthesis combined with the increased dietary inflow into the pool in the hyperphagic state. It is possible that the expanded pool could enhance oxidation rate in an attempt to regulate the pool size. Changes in oxidation with starvation have been demonstrated by Goldberg & Odessey (1972) and similarly, Meikle & Klain (1972) showed that increases in in vivo oxidation rates were paralleled by elevated plasma leucine concentrations (from 0.19 to 0.75 μ moles/ml). On refeeding and therefore stimulation of insulin release, oxidation rates declined, but plasma leucine remained elevated, suggesting that the rate of dietary leucine intake exceeded the outflow or utilization of leucine by protein synthesis, oxidation or lipogenesis. In the present study the near-control values of protein oxidation in insulin treated rats (Table 5-4) and the drop in leucine concentration, appear to indicate that the increased rate of protein synthesis was probably sufficient to regulate the size of the plasma leucine pool.

5.5 SUMMARY AND CONCLUSION

The results of whole body oxidation rates appears to confirm the in vitro studies of Buse and co-workers on diabetic rats. The increased

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TABLE 5 - 4 PLASMA LEUCINE CONCENTRATION AND OXIDATION RATES IN DIABETIC AND INSULIN TREATED RATS

	Control ^a	Diabetic (8 days)	Insulin treated
Plasma free leucine pool (μ moles/ml plasma)	0.149 \pm 0.005	0.256 ^b \pm 0.017	0.136 \pm 0.024
Oxidation rate (μ moles leu/min/100 g rat)	0.236 \pm 0.013	0.671 ^c \pm 0.056	0.314 \pm 0.028

All values mean \pm SEM.

a - mean control values from Experiments I and II.

b - significantly different from control $p < 0.005$.

c - significantly different from control $p < 0.001$.

oxidation rates observed with incubation studies in hemidiaphragms (Buse et al., 1976a), retina (Frayser & Buse, 1978), sciatic nerves (Buse et al., 1976b) and in liver, kidney and gastrocnemius muscle (Paul & Adibi, 1978) could all be decreased to near normal values by prior treatment of the rats with insulin. The present work confirms that whole body protein oxidation rates are greatly enhanced in diabetes and also normalized by insulin treatment.

Buse et al. (1976a) furthermore suggested that in diabetes the increase in oxidation was paralleled by enhanced breakdown. Due to the inherent practical problems in calculating valid protein breakdown values, it is difficult to evaluate this suggestion. Although it should be possible in theory to calculate protein breakdown from this method, the results in the preceding section show that this is not feasible in the present animal model. A possible approach would be to maintain feeding via an intragastric tube throughout the 4 hour measurement of protein turnover.

The decrease in whole body protein synthesis rate caused by diabetes confirms the in vivo tissue measurements in diabetic rats (Pain & Garlick, 1974; Millward et al., 1976). Similarly, the normalizing effect of insulin treatment on whole body protein synthesis has also been reported for tissue synthesis rates (Pain & Garlick, 1974).

However, considering that these rats were severely diabetic, the drop of 17% in whole body synthesis was rather small compared to the 60-70% decrease measured in tissue synthesis (Sections 6 and 7). This may explain why the changes observed in whole body protein synthesis in humans (Section 3) could not be detected when measured before or after insulin treatment, since in these patients the severity of the diabetic state was much less than in the rats studied here.

The next investigations were therefore to determine the effect of diabetes on individual tissues from rats with acute and chronic diabetes.

SECTION 6TISSUE PROTEIN SYNTHESIS IN DIABETIC RATS INFUSED WITH [¹⁴C]-
TYROSINE6.1 INTRODUCTION

In diabetic rats the 17% decrease observed in rates of whole body protein synthesis (Section 5) was much smaller than the effect which had been demonstrated in some tissues of diabetic rats. Pain & Garlick (1974) reported a drop in synthesis of 69% in skeletal muscle and 44% in heart muscle. Similarly, Millward et al. (1976) demonstrated a decrease of 63% in synthesis rates of gastrocnemius muscle in rats not even severely diabetic (8 μ Units insulin per ml plasma were still present in the diabetic group). In contrast to muscle, protein synthesis in liver was found to be unaffected by diabetes as measured by the constant infusion technique (Hay & Waterlow, 1967; Pain & Garlick, 1974).

These studies were at the time the only published data on the in vivo effect of diabetes on protein synthesis in skeletal muscle, heart and liver.

The aim of this experiment was therefore to investigate protein synthesis in the liver, gastrocnemius muscle and kidney in rats (a) in the acute phase of the disease, ie. 5 days after injection with streptozotocin, (b) in the chronic phase, 56 days post-streptozotocin and (c) to follow the severity and progress of diabetes in rats with different initial body weights.

6.2 METHOD

Two groups of male Wistar rats weighing 70 and 100 grams were purchased from Charles River. They were housed individually and allowed free access to food and water until their body weights reached 90 and 120 grams respectively.

The animals were then divided into three experimental groups :

- (a) Acute diabetic group, initial weight 90 gram, infused 5 days post-streptozotocin (6 rats).
- (b) Acute diabetic group, initial weight 120 gram, infused 5 days post-streptozotocin (6 rats).
- (c) Chronic diabetic group, initial weights 90 and 120 gram, infused 56 days post-streptozotocin (7 rats).

The results of the three experiments were compared with three groups of age controls (6 rats each). Diabetes was induced by streptozotocin as described previously.

The constant intravenous infusion technique was similar to that in Section 5.2.2.2 with the following changes. [$U-^{14}C$]Tyrosine (The Radiochemical Centre, Amersham, U.K.) was infused for 6 hours at a concentration of 4 μ Ci per ml at a rate of 0.5 ml per hour. During this time the rats were restrained by being wrapped in a cloth.

6.2.1 Sample collection

After 6 hours the cannula was removed, the rats decapitated and mixed arterial and venous blood collected from the severed trunk vessels. The liver and both kidneys and gastrocnemius muscles were rapidly removed and weighed. Part of the liver, one gastrocnemius muscle and one kidney were immediately frozen in liquid nitrogen for subsequent analysis of protein, RNA and DNA content, by the procedure previously described (Section 4.6.1). Plasma was prepared by centrifugation of the heparinized blood collected at sacrifice. Aliquots

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6.2.1 Sample collection

After 6 hours the cannula was removed, the rats decapitated and mixed arterial and venous blood collected from the severed trunk vessels. The liver and both kidneys and gastrocnemius muscles were rapidly removed and weighed. Part of the liver, one gastrocnemius muscle and one kidney were immediately frozen in liquid nitrogen for subsequent analysis of protein, RNA and DNA content, by the procedure previously described (Section 4.6.1). Plasma was prepared by centrifugation of the heparinized blood collected at sacrifice. Aliquots

were taken for later analysis of glucose and insulin (Section 4). Approximately 1 gram of tissue was homogenized in ice-cold 10% (w/v) trichloroacetic acid (TCA) (liver 3 ml, kidney and muscle 2 ml). All the homogenized samples were then centrifuged and the supernatant containing the free amino acid removed and reserved. The protein precipitates were washed twice with 1 ml 10% TCA to further extract free radioactivity, with each wash being combined with the first supernatant. TCA was removed from these supernatants by three ether washes. Thereafter each supernatant was made up to a standard volume of 5 ml and stored frozen at -15°C for later analysis of specific radioactivity of free tyrosine. The protein precipitates were hydrolyzed in sealed tubes for 20 hours at 110°C with 5 ml 6N HCl ('constant boiling'), plus 3 drops of mercaptoethanol to prevent amino acid oxidation. The hydrochloric acid was removed by repeated heat evaporation and the hydrolyzed protein was subsequently taken up in 5 ml distilled water and stored frozen.

6.2.2 Tyrosine specific activity

The specific radioactivity of free and protein tyrosine was estimated as described by Garlick & Marshall (1972). Tyrosine in the samples was converted to tyramine by L-tyrosine decarboxylase (acetone powder of Streptococcus faecalis, Type 1, Sigma Chemical Company, London). The enzyme was first washed with citrate buffer (0.5M, pH 5.5) and then suspended at a dilution of 2 mg/ml in citrate buffer. 1 ml of each sample was incubated for 1 hour at 37°C with 1 ml of citrate buffer and 1 ml of buffered enzyme. The resulting tyramine was extracted from the enzyme solution into 10 ml ethyl acetate in the presence of 1g anhydrous sodium carbonate and 1g sodium chloride. The organic phase was removed and 5 ml chloroform plus 4.5 ml (3.0 ml for protein samples) sulphuric acid (1:2000) added, mixed and centrifuged at low speed. Aliquots (2 x 1 ml) of the upper aqueous phase containing

the tyramine were counted for radioactivity in a Beckman LS 150 liquid scintillation spectrometer using a Triton-X100/xylene based scintillant.

Duplicate 1 ml samples (10 - 20 μ l for protein hydrolysates) were assayed by the fluorimetric method of Waalkes & Udenfriend (1957). The tyramine in the sample was reacted with 1 ml 1-nitroso-2-naphtol (0.1% in ethanol) in the presence of 1 ml nitric acid reagent (0.05% sodium nitrite in 1:5 nitric acid), which formed a stable yellow compound during a 30 minute incubation at 55°C. Unchanged nitrosonaphtol reagent was extracted with 10 ml dichloroethane and after centrifugation, the supernatant aqueous layer was transferred to cuvettes. Fluorescence was measured at 570 $m\mu$ using a Locarte fluorimeter, (Locarte Company, London). Appropriate standard solutions of tyramine hydrochloride (0.01 mM) were assayed with each set of samples.

6.2.3 Calculation of protein synthesis rates

The results were calculated by the method of Waterlow & Stephen (1968), as modified and described by Garlick et al. (1973). As discussed in Section 1.1.3 fractional synthesis rate (k_s) for liver and kidney was obtained from

$$\frac{S_B}{S_i} = \frac{\lambda_p}{(\lambda_p - k_s)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-\lambda_p t})} - \frac{k_s}{(\lambda_p - k_s)} \quad \text{--- (1)}$$

and for tissues such as skeletal and cardiac muscle, from

$$\frac{S_B}{S_i} = \frac{R}{(R - 1)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-Rk_s t})} - \frac{1}{(R - 1)} \quad \text{--- (2)}$$

The value of k_s was derived by plotting curves of S_B/S_i and k_s at fixed values of t and R and λ_p . An approximate value of $\lambda_p = 80 \text{ days}^{-1}$ was used in eqn. 1 and $R = 400$ in eqn. 2 (Waterlow et al., 1978a).

6.3 RESULTS AND DISCUSSION

6.3.1 Effect of diabetes on tissue compositions

Table 6-1 shows that acute diabetes was characterized by low growth rates, high glucose and low insulin concentrations and increased water intake. The same symptoms were present in the chronically diabetic rats with more severe weight loss and lower insulin values. The difference in initial body weight had no apparent effect on the outcome and symptoms of acute diabetes. All the results shown for the chronic diabetic groups are in fact mean values for rats who had initially weighed 90 or 120 grams. Since there was hardly any difference in body weight and in tissue composition between the two chronic diabetic groups and the two control groups after 56 days, the results were combined to facilitate discussion and to enlarge the number of rats per group.

Table 6-2 documents the effect of acute and chronic diabetes on tissue weight and composition of the liver. The total weight was reduced by acute and chronic diabetes, but total liver protein was not significantly affected, as reported previously (Pain et al., 1978b). The reduction in RNA to protein ratio in the livers of diabetic rats resulted from greater decreases in the total RNA content of the liver. No significant change could be detected in DNA content or protein to DNA ratios between diabetic groups compared with their own age controls. Increases in total DNA or number of cells were observed in the older age controls compared with the 90 and 120 gram controls and in chronic diabetic rats versus acute diabetics.

Severe muscle wasting was present in the acute diabetic rats and this was even more pronounced in the chronic state (Table 6-3). Protein and RNA content was significantly reduced in all three diabetic groups as compared with their controls. The RNA to protein ratio was

TABLE 6 - 1 EFFECT OF ACUTE AND CHRONIC DIABETES ON GROWTH AND PLASMA CONCENTRATIONS OF GLUCOSE
AND INSULIN

		Number of Rats per group	Final Body weight (g)	Growth* Rate (g/d)	Plasma Glucose (mg/100ml)	Plasma Insulin (μ U/ml)	Food Intake (g/d)	Water Intake (ml/d)
Acute diabetic	90g	6	107 \pm 3	4.3	450 \pm 36	4.2 \pm 0.7	25 \pm 2	100
control	90g	6	123 \pm 3	6.7	148 \pm 4	10.7 \pm 1.9	24 \pm 1	28
Acute diabetic	120g	6	141 \pm 4	3.5	484 \pm 25	3.0 \pm 0.7	28 \pm 2	123
control	120g	6	168 \pm 3	7.8	171 \pm 4	19.4 \pm 3.8	26 \pm 1	35
Chronic diabetic		7	247 \pm 15	2.4	501 \pm 12	1.9 \pm 0.7	-	-
control		7	427 \pm 12	5.6	145 \pm 4	38.4 \pm 1.6	-	-

* Growth rate mean value of two days preceding infusion. All other values mean \pm SEM.

TABLE 6 - 2 EFFECT OF ACUTE AND CHRONIC DIABETES ON LIVER TISSUE WEIGHT AND COMPOSITION (MEAN \pm SEM)

		Total weight (g)	Total Protein(mg)	Total RNA(mg)	Total DNA(mg)	RNA/Protein (mg/g)	Protein/DNA (mg/mg)
Acute diabetic	90g	3.98 \pm 0.21	837 \pm 62	17.9 ^a \pm 3.46	12.04 \pm 1.40	21.6 ^a \pm 3.8	72.4 \pm 7.9
control	90g	4.85 \pm 0.15	748 \pm 27	27.36 \pm 2.40	11.74 \pm 1.73	36.5 \pm 2.6	73.6 \pm 2.5
Acute diabetic	120g	5.61 \pm 0.20	1186 \pm 56	27.16 \pm 2.67	12.95 \pm 1.18	23.1 \pm 2.4	94.5 \pm 8.5
control	120g	6.75 \pm 0.20	1288 \pm 51	33.38 \pm 4.00	13.89 \pm 1.06	27.7 \pm 3.8	82.9 \pm 4.1
Chronic diabetic		11.87 \pm 0.54	2993 \pm 161	52 \pm 8	27 \pm 1	17.3 \pm 2.3	112 \pm 5
control		14.68 \pm 0.86	3260 \pm 160	69 \pm 10	31 \pm 1	21.9 \pm 4.0	106 \pm 6

a - significantly different from control p < 0.05

TABLE 6 - 3 EFFECT OF ACUTE AND CHRONIC DIABETES ON GASTROCNEMIUS TISSUE WEIGHT AND COMPOSITION (MEAN \pm SEM)

		Total weight (g)	Total Protein(mg)	Total RNA(mg)	Total DNA(mg)	RNA/Protein (mg/g)	Protein/DNA mg/mg)
Acute diabetic	90g	0.90 ^c \pm .08	140 ^a \pm 7	0.99 ^b \pm 0.11	0.82 \pm 0.04	7.0 ^a \pm 0.6	170 ^a \pm 6
control	90g	1.25 \pm 0.07	169 \pm 9	1.58 \pm 0.11	1.18 \pm 0.06	9.3 \pm 0.5	144 \pm 7
Acute diabetic	120g	1.24 ^c \pm 0.15	178 ^a \pm 9	1.29 ^b \pm 0.84	1.07 \pm 0.04	7.3 \pm 0.5	167 \pm 7
control	120g	1.66 \pm 0.12	244 \pm 10	1.95 \pm 0.16	1.36 \pm 0.04	8.0 \pm 0.5	180 \pm 7
Chronic diabetic		1.98 ^c \pm 0.25	351 ^c \pm 37	1.26 ^c \pm 0.21	0.92 ^c \pm 0.10	3.5 \pm 0.3	382 \pm 19
control		5.44 \pm 0.59	913 \pm 71	3.39 \pm 0.51	2.12 \pm 0.12	3.8 \pm 0.4	433 \pm 33

a - significantly different from control $p < 0.05$

b - significantly different from control $p < 0.01$

c - significantly different from control $p < 0.001$

decreased in the 90 gram acutely diabetic rats, but not in the other two diabetic groups. Total DNA in muscle was higher in controls than diabetic groups and the DNA content of muscle in the chronic diabetic controls were higher than in the acute diabetic controls. Similarly the protein to DNA ratio was increased in the chronic diabetic control group, indicating increased cell size.

Total kidney weights were increased by approximately 10% in the diabetic rats. After 56 days the total kidney weights were no different from those of age controls, but expressed per 100 gram body weight, kidney weights of chronic diabetic rats were nearly 42% higher. Tissue composition data on the kidneys from these rats were unfortunately not available due to an experimental error, but there is no reason to believe that the kidney RNA, DNA and protein concentrations as measured previously (Section 4, Table 4-3) would not be applicable here.

6.3.2 Effect of diabetes on synthesis rates of liver, gastrocnemius and kidney

Fractional synthesis rate (k_s) defined as that percentage of tissue protein which is replaced daily by protein synthesis, is shown in Table 6-4 for liver, gastrocnemius and kidney. The fractional synthesis rates of 14.7 and 13.2% per day for gastrocnemius muscle in control rats (90 and 120 gram groups) compare well with the published rates of 13.6% per day for animals of the same strain and weight range (Pain & Garlick, 1974). Similarly, the decline in fractional synthesis rate with age as seen in the 56 day control rats has been reported (Millward, 1978). The rate of protein synthesis in gastrocnemius muscle was significantly decreased (40-50%) by diabetes, in confirmation of the 69% drop in synthesis reported by Pain & Garlick (1974) and the 63% observed by Millward et al. (1976).

The synthesis rates of liver appeared to be unaffected by diabetes

TABLE 6 - 4 THE EFFECT OF ACUTE AND CHRONIC DIABETES ON THE RATE OF PROTEIN SYNTHESIS IN THE LIVER, MUSCLE AND KIDNEY

		$S_B/S_i \times 10^{-3}$			k_s (percent per day)		
		Liver	Muscle	Kidney	Liver	Muscle	Kidney
Acute diabetic	90g	114 ± 6	15.5 ± 1.6	101 ± 7	51.1 ^c ± 2.7	7.3 ^a ± 0.7	44.7 ± 3.1
control	90g	167 ± 14	33.7 ± 2.4	100 ± 3	77.4 ± 7.2	14.7 ± 1.0	44.5 ± 1.6
Acute diabetic	120g	111 ± 4	11.0 ± 1.0	108 ± 6	49.7 ± 2.0	5.4 ^a ± 0.4	48.3 ± 3.0
control	120g	120 ± 3	30.0 ± 2.3	107 ± 5	53.8 ± 1.5	13.2 ± 1.0	47.7 ± 2.2
Chronic diabetic		114 ± 5	5.3 ± 0.5	159 ± 11	51.0 ± 2.1	2.9 ^b ± 0.3	73.3 ± 5.4
control		131 ± 5	11.8 ± 1.8	178 ± 15	58.9 ± 2.3	5.7 ± 0.8	82.8 ± 7.6

All values mean ± SEM, statistically different from controls a = p < 0.001; b = p < 0.005; c = p < 0.05

(Table 6-4), substantiating the findings of Pain & Garlick (1974) who used the same method of constant infusion. The difference in liver synthesis rate between the 90 gram acutely diabetic rat and its age control is difficult to interpret. The control value of 77% is probably wrong since it is very high compared to published data (Pain & Garlick (1974) and, furthermore, the individual k_s values in this group were highly variable. In contrast to the absence of an effect of diabetes on the fractional synthesis rate of liver proteins, the ratio of RNA to protein was decreased in all diabetic groups. Since the constant infusion method measures only fixed or cellular proteins and not secreted proteins, the results of this study do not exclude the possibility of diabetes affecting synthesis of other hepatic proteins.

The effect of diabetes on rates of protein synthesis in the kidney is also documented in Table 6-4. Despite an increase in total protein content, acute or chronic diabetes did not appear to have any effect on protein synthesis. The control values of 45 and 48% protein per day compare well with the 50% quoted by Waterlow et al. (1978a) and the 55% per day reported by Garlick et al. (1975) in rats of the same age and weight range. The very high rates of kidney protein synthesis, ie. 73% and 83% in chronic diabetic rats and their age controls have not been reported before.

6.4 SUMMARY AND CONCLUSIONS

Fractional synthesis rates of skeletal muscle responded markedly to insulin deficiency with decreases of 40 - 50%. The magnitude of the decline in synthesis was unaffected by the duration of diabetes or any difference in initial body weight when diabetes was induced. In contrast, synthesis rates in the kidney and the liver appeared to be relatively unchanged by either the acute or the chronic state of diabetes.

Since the constant infusion method could only give an estimate of changes in cellular hepatic proteins, a more appropriate method was used in the following experiment, which enabled determination of synthesis rates of total (cellular plus secretory) hepatic protein. In addition, rates of protein breakdown were estimated by the change in protein mass at the time at which synthesis was measured. Apart from the liver, kidney and gastrocnemius studied in this experiment, four other muscles were additionally investigated in the following experiment.

SECTION 7

SYNTHESIS AND BREAKDOWN RATES IN TISSUES OF DIABETIC AND INSULIN TREATED RATS AS MEASURED BY A LARGE DOSE OF LABELLED AMINO ACID

7.1 INTRODUCTION

The constant infusion technique used in the preceding study was an inappropriate method to measure both cellular and secretory hepatic proteins. Secondly, in that method the precursor of protein synthesis was assumed to be the free amino acid pool of the tissue. However, this pool is not homogenous; it is probably compartmented and during a constant infusion receives amino acids not only from intracellular proteolysis but also from the extracellular fluid.

In the present study a method was therefore employed which minimized the error involved in choice of precursor pool and secondly, enabled measurement of both cellular and secretory proteins. This method involved the use of a large dose of labelled amino acid and measuring incorporation of label over 10 minutes.

The aim of this study was :

- (a) to determine the effect of diabetes on synthesis of total (cellular and secretory) hepatic proteins.
- (b) to determine the effect of diabetes on the rates of protein breakdown in tissues.
- (c) in particular, to study rates of protein synthesis and breakdown within the first 24 hours of the onset of diabetic symptoms and to follow the changes in protein turnover as the disease progressed.

The large dose method employed, was first described by Henshaw et al. (1971) and more recently was modified by McNurlan et al. (1979). It has the following advantages :

(a) the amount of labelled amino acid injected is much larger than the normal free amino acid pool of the rat. Hence the specific activity of the intracellular and extracellular pools are approximately equalized during the short time of measurement. Furthermore, the decline in specific activity in plasma and tissue is very slow and can be assumed to be linear.

(b) the short time of measuring incorporation (10 minutes) enables determination of rates of synthesis of cellular and secretory proteins before they are transported from the liver.

(c) this short period of measurement also prevents return of label to the intracellular pool via recycled amino acids, a particular problem when tissues with rapid turnover (i.e. liver and gut) are measured by constant infusion.

The rate of protein breakdown in tissues was estimated by an indirect method, i.e. as the difference between rates of synthesis and rates of change of protein mass of the tissue.

To accomplish the third aim of the study, i.e. to measure changes in protein turnover within 24 hours of onset of diabetes, the animal protocol as used in the previous experiments was modified. Streptozotocin diabetic rats were treated with insulin for 3 days and protein measurements were made after one or more days of insulin withdrawal. The results were compared with age control rats, which were diabetic rats receiving insulin treatment throughout the experiment.

Since large groups of rats were to be killed at various time points, a pilot study was planned to identify the rapidity and magnitude of response in different tissues as the severity of diabetes increased. In the pilot study, a large dose of [^3H]leucine was injected and

incorporation of label into tissue was measured over 10 minutes without taking into account the specific activity of the free amino acid. Since the large dose would tend to flood the free amino acid pool, incorporation rates of amino acids from this pool into protein would reflect a relative rate of synthesis.

In the actual study a large amount of [^3H]phenylalanine was used and incorporation measured also over 10 minutes. Fractional synthesis rates were obtained by measurement of specific activity of the free and of the protein-bound amino acid. [^3H]Phenylalanine was used instead of [^3H]leucine since an enzymatic method had recently been developed in this laboratory for the determination of the specific activity of this amino acid (P.J. Garlick, M.A. McNurlan and V.R. Preedy, to be published). This method therefore did not require the time consuming and expensive use of an automated amino acid analyzer.

7.2 METHODS

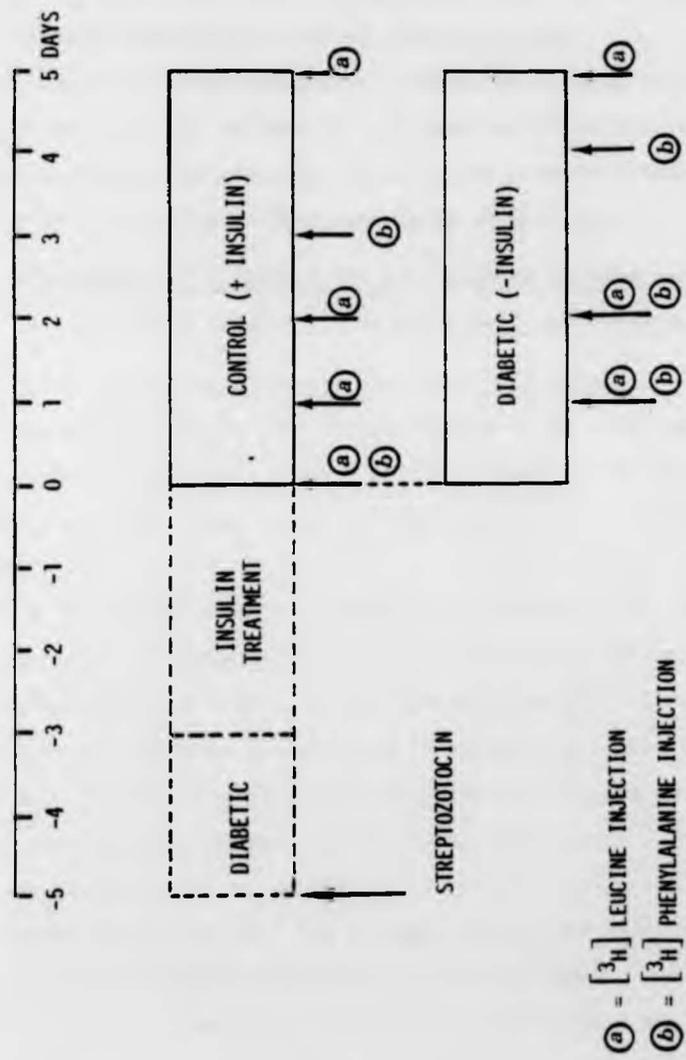
7.2.1 Animal treatment protocol

Male Wistar rats (90-100g) were housed and fed as described previously. The experimental protocol followed after the streptozotocin injection is outlined in Figure 7-1. Two days after induction of diabetes, blood samples from the tail vein were analyzed for glucose. Those with glucose values less than 400 mg/100 ml were excluded from the study. The rest of the animals were treated with 4 Units PZI per day. After 3 days of insulin, the animals were randomly divided into a control group, which continued on insulin treatment and a diabetic group, in which insulin was withdrawn. Measurements were made at various days after insulin withdrawal or continued insulin treatment, as will be indicated later for each study.

7.2.2 Measurements of incorporation of [^3H]leucine

Leucine incorporation measurements were made in diabetic (insulin

FIGURE 7-1 PROTOCOL FOR PROTEIN TURNOVER STUDIES USING A LARGE AMOUNT OF LABELLED AMINO ACID



withdrawn) and in control (insulin treated) groups of rats (5 rats per group). [^3H]Leucine was injected into the tail vein (100 μmoles unlabelled leucine and 40 μCi [^3H]leucine per 100 gram body weight). After 10 minutes the rats were decapitated, bled and the liver, heart, kidneys and gastrocnemius muscles rapidly removed. The tissues were then blotted, weighed and frozen in liquid nitrogen.

The protein and RNA content was determined in each tissue by the procedure described in Section 4-6. Incorporation of the label into tissue was measured by counting 100 μl of the protein aliquot (see Figure 4-5) in a Triton X-100/xylene based scintillant.

7.2.3 Measurement of incorporation of [^3H]phenylalanine and calculation of rates of protein synthesis and breakdown

7.2.3.1 Injection procedure and sample collection

Diabetes was induced with streptozotocin in 80 rats, of which only 60 were used in the actual study. (Mildly diabetic rats were rejected from the study and some died in hypoglycaemic coma following insulin treatment).

L-[4- ^3H]Phenylalanine (27 Ci/mmol) was combined with unlabelled phenylalanine to a concentration of 150 μmoles and 55 μCi per ml. Rats were injected by tail vein with 1 ml/100g body weight. Groups of rats were killed at 2 minutes (4 rats) and 10 minutes (6 rats) after isotope injection. At sacrifice blood was collected for glucose analysis and several tissues were removed rapidly but quantitatively. These were the liver, kidneys, heart, gastrocnemius, soleus, extensor digitorum longus (EDL) and diaphragm. The tissues were cooled rapidly in ice water, blotted, weighed and frozen in liquid nitrogen. The time between killing and cooling in ice water of each tissue was noted.

7.2.3.2 Sample preparation and analysis

Approximately 200 mg of tissue was homogenized in 2 ml of 2%

(w/v) perchloric acid (PCA). The supernatant containing the free amino acid was removed after centrifugation, and the precipitate was further washed with 2% PCA. Protein and RNA were separated and analyzed as described in Section 4-6, Figure 4-5. The PCA was removed from the supernatant by precipitation in the cold with 100 μ l of 5M KOH in a 1M potassium citrate buffer (pH 6.0). The supernatants were then frozen for later analysis of free phenylalanine specific activity. The precipitate containing the protein-bound amino acid, obtained after RNA extraction was hydrolyzed as described in Section 6.2.1. The protein fraction was resuspended in 2 ml sodium citrate buffer (0.5M, pH 6.3) and stored frozen.

7.2.3.3 Specific activity of phenylalanine

The specific activity of free and protein-bound phenylalanine was estimated by the enzymatic method described by P.J. Garlick, M.A. McNurlan and V.R. Preedy (to be published).

Phenylalanine was converted to phenylethylamine by incubating 0.5 ml of sample with 0.5 ml of buffered enzyme for 20 hours at 50°C. The enzyme solution contained per sample: 250 mUnits L-tyrosine decarboxylase (Type I, Sigma Chemical Company Limited, Poole, Dorset, U.K., containing phenylalanine decarboxylase as an impurity), 0.25 mg pyridoxal phosphate and 0.5 ml citrate buffer (0.5M, pH 6.3). 1.0 ml of 3M NaOH was added after the incubation and the phenylethylamine was extracted into 10 ml of a heptane:chloroform (3:1) solution. This procedure does not extract tyramine formed by reaction of tyrosine with tyrosine decarboxylase. The organic phase was then added to 4 ml sulphuric acid (1:2000) and 5 ml chloroform, shaken and centrifuged at low speed. An aliquot of the upper aqueous layer containing the phenylethylamine was counted in a Triton X-100/xylene scintillant.

Phenylethylamine in this aqueous layer was assayed by the fluorimetric method of Suzuki and Yagi (1976) as modified by Garlick et al.

(unpublished). 1.0 ml of sample was incubated for 60 minutes at 60°C with 2.5 ml phosphate buffer (0.5M, pH 8.8), 1.0 ml 50mM ninhydrin and 0.5 ml 2mM L-leucyl-L-alanine (Sigma Chemicals). This incubation yielded a highly fluorescent compound which was unstable in light and sensitive to changes in temperature. The 60 minute incubation and subsequent measurements of fluorescence were therefore carried out in a dark room. Samples were cooled on ice for 5 minutes after incubation and then kept at room temperature in a waterbath for a further 15 minutes. Fluorescence was measured using a Locarte fluorimeter with a flow cell. This enabled very rapid reading of samples, thus avoiding decay of fluorescence during the measurement. Standard solutions of β -phenylethylamine, ranging from 2 to 20 nmoles/ml were assayed with each set of free (0.5 to 1.0 ml) and protein-bound (20-50 μ l) samples.

7.2.3.4 Calculations of fractional rates of synthesis and breakdown

Fractional synthesis rates (k_s) in percent per day, were calculated as described by McNurlan et al. (1979) and as discussed in Section 1.1.3. The following equation was used :

$$k_s = \frac{S_B}{\bar{S}_A \cdot t} \times 100$$

where S_B is the specific activity of phenylalanine in protein at 10 minutes, \bar{S}_A the mean specific activity of the free phenylalanine pool between zero and 10 minutes and t , time in days.

The arithmetic average of S_A at zero and 10 minutes was determined by measuring the free phenylalanine specific activity in tissues of groups of rats killed at 2 and 10 minutes after injection. The results were plotted against the exact time at which each tissue was removed and cooled in ice water, i.e. when label incorporation was stopped.

After killing, these times to cooling were: for liver, 40 seconds; for all skeletal muscles, 30 seconds (the whole hind leg without skin was placed in ice water); for kidneys, 60 seconds; heart 90 seconds and the diaphragm, 120 seconds. The value for S_A at zero time was obtained by extrapolating the decline between 2 and 10 minutes back to zero.

Fractional breakdown rates (k_d) were estimated from the equation :

$$k_d = k_s - k_g$$

where k_g is the fractional growth rate of the protein mass (see Section 1.1.4). Growth was determined by measuring the mean rate of change in total protein mass of the tissue in diabetic and control animals on the days immediately before and after the time at which [3 H]phenylalanine was injected to measure fractional synthesis rates. The fractional growth rate (as percent per day) was obtained by dividing the mean change in protein mass per day by the total protein mass of the tissue on the day that synthesis was measured. To define the change in protein mass more accurately, a group of diabetic rats was measured after 6 days of insulin withdrawal and a group of controls after a further 6 days of insulin treatment (see general protocol, Figure 7-1). These latter two groups of 5 rats each were used for analysis of tissue protein content only. The growth rate of protein in tissues of the insulin-treated rats at day 3 was therefore calculated from the mean of growth between days 0 to 3 and days 3 to 6. For growth at day 0 of insulin treatment, only the growth rate between 0-3 days were used. The calculation of liver breakdown rate from a change in protein mass requires additional information, since the measured rate of fractional synthesis is that of both cellular and secretory proteins. By contrast, fractional growth rate represents changes in only the cellular protein mass. The rate of cellular protein synthesis was estimated from measurements of rates of albumin synthesis. Albumin synthesis as a

proportion of total hepatic protein synthesis was determined in liver homogenates in all the groups of rats, as described by Pain et al. (1978a). By assuming that the proportion of total hepatic protein synthesis devoted to the production of plasma proteins other than albumin ($\pm 20\%$) was unaffected by diabetes (Pain & Garlick, unpublished data), a rate of synthesis of cellular protein could be calculated. With this correction, fractional breakdown rates of liver protein were calculated as the difference between the rates of synthesis and growth of cellular proteins. An investigation is presently in progress to confirm the assumption that synthesis of plasma proteins other than albumin is not changed in diabetes, with rats subjected to the same protocol of insulin withdrawal. However, time does not permit the inclusion of results of this experiment in this study.

7.3 [³H]LEUCINE PILOT STUDY

7.3.1 Protocol

The general protocol is shown in Figure 7-1. The incorporation of [³H]leucine and content of RNA and protein were determined in tissues from control (insulin treated) rats measured on day 0, 1, 2 and 5. Diabetic rats were measured at 1, 2 and 5 days after insulin withdrawal. Each group contained 5 rats.

7.3.2 Results and Discussion

Hyperglycaemia was well established in the diabetic rats one day after insulin withdrawal as shown in Figure 7-2.

Gastrocnemius muscle: The effects of insulin withdrawal and insulin treatment on protein and RNA content and on [³H]leucine incorporation are illustrated in Figure 7-3. Both protein and RNA are shown as a percentage change in the content of the tissue from the value on day 0, the day on which insulin was withdrawn in the diabetic group. Both RNA and protein mass increased during the first day of insulin withdrawal

FIGURE 7-2 PLASMA GLUCOSE CONCENTRATIONS IN CONTROL (INSULIN TREATED) AND IN DIABETIC (INSULIN WITHDRAWN) RATS

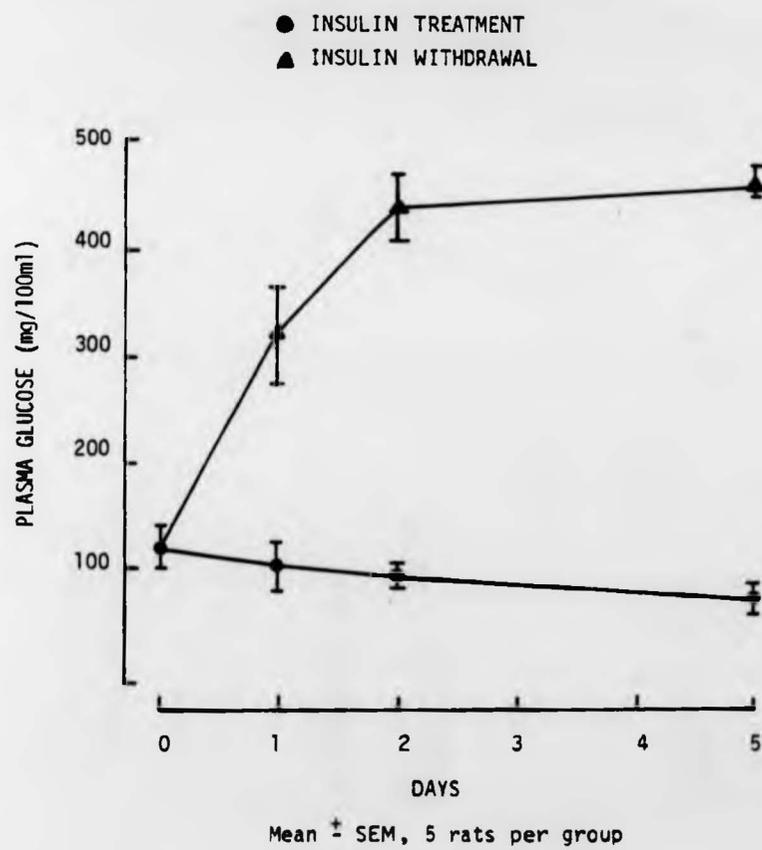
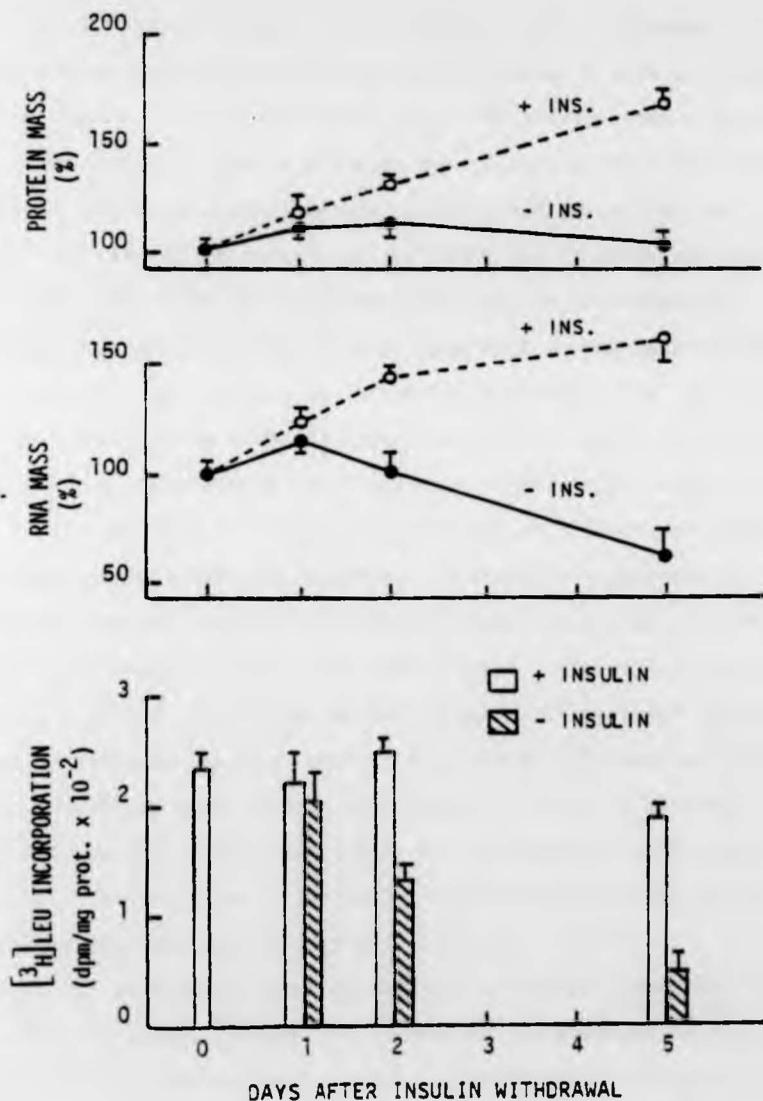


FIGURE 7-3 $[^3\text{H}]$ LEUCINE INCORPORATION AND PROTEIN AND RNA CONTENT IN GASTROCNEMIUS MUSCLE OF DIABETIC AND INSULIN-TREATED RATS



Protein and RNA mass as % change from value at day 0.

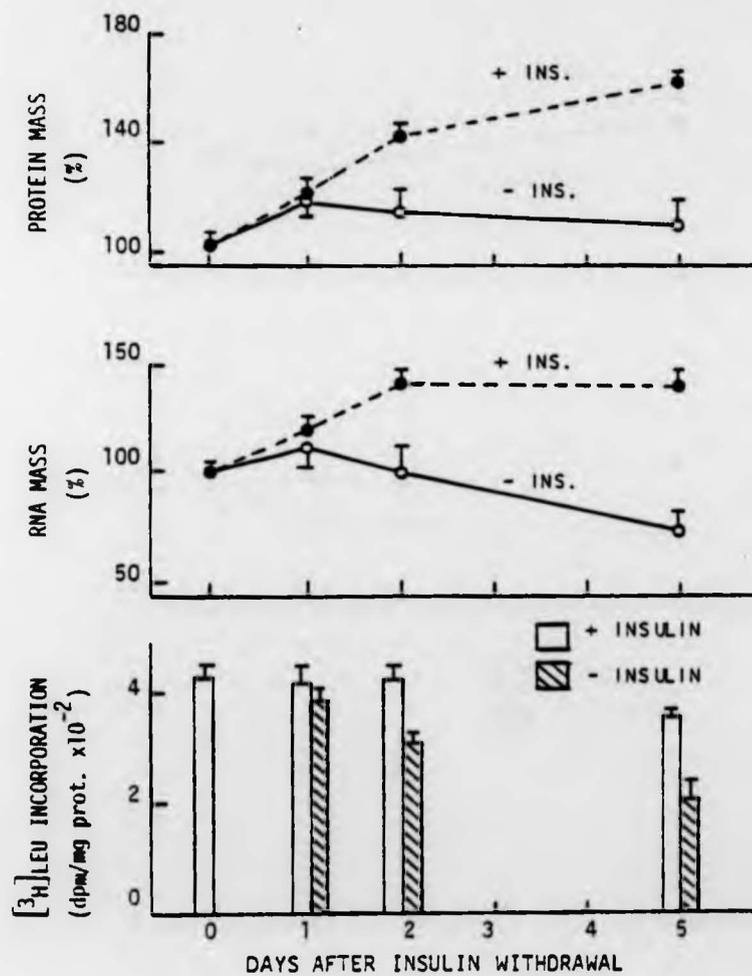
Mean \pm SEM for 5 rats per group

despite an increase in blood glucose from 123 ± 24 to 321 ± 50 mg%, but the increase in mass was lower than that in the insulin treated groups. Similarly, the relative rate of synthesis, expressed as the [^3H]leucine incorporated into protein over 10 minutes, was slightly, but insignificantly lower in the withdrawn group. However, the relative synthesis rate decreased rapidly after 2 days of diabetes. Protein mass of the gastrocnemius declined slowly after 2 days without insulin, but at 5 days of diabetes had reached a value 40% lower than that of the 5 day control group. In contrast to the decline in protein mass, RNA content decreased rapidly after the first day of diabetes and at 5 days the value was 62% lower than that of the controls.

Cardiac muscle: The decline in protein mass in the heart (Figure 7-4) followed the same pattern as in the gastrocnemius, i.e. an increase in protein mass in the diabetic group which was slightly less than that in the control group during the first day of insulin withdrawal followed by a slow decline. At 5 days a difference of 32% between control and diabetic protein mass was observed. Although the decrease in the RNA content of heart muscle from diabetic animals was similar to the decline in the gastrocnemius, the final RNA content differed from the controls on day 5 by 47%. This less marked response of heart RNA and protein mass to diabetes was also seen in the rate of [^3H]leucine incorporation i.e. a decrease after 2 days of diabetes. At 5 days, synthesis in the diabetic cardiac muscle was not so low as observed in the gastrocnemius. This relative resistance of heart muscle to the effect of diabetes was also reported by Pain & Garlick (1974).

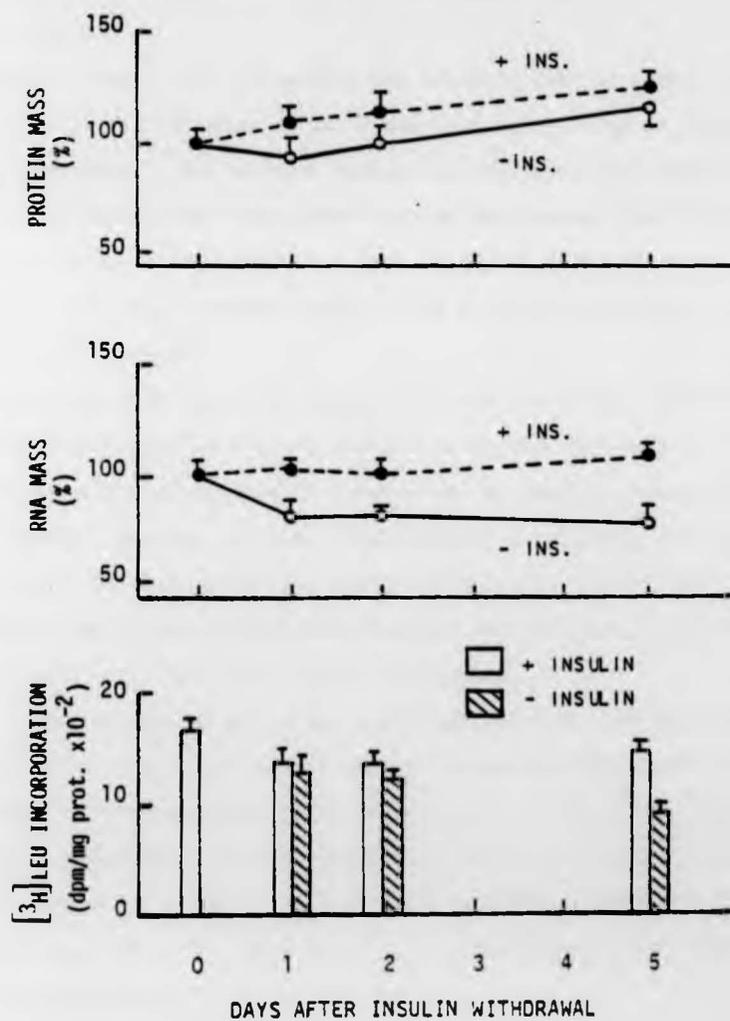
Liver: In contrast to muscle, the liver responded immediately to insulin withdrawal (Figure 7-5). Despite the presence of only a mildly hyperglycaemic state at day 1, protein mass decreased slightly but increased again after 2 days. Thereafter the growth of protein mass was approximately parallel to that of the controls. RNA mass

FIGURE 7-4 $[^3\text{H}]$ LEUCINE INCORPORATION AND PROTEIN AND RNA CONTENT IN CARDIAC MUSCLE OF DIABETIC AND INSULIN TREATED RATS



(See legend Figure 7-3)

FIGURE 7-5 $[^3\text{H}]$ LEUCINE INCORPORATION AND PROTEIN AND RNA CONTENT
IN LIVER OF DIABETIC AND INSULIN TREATED RATS



(See legend Figure. 7-3)

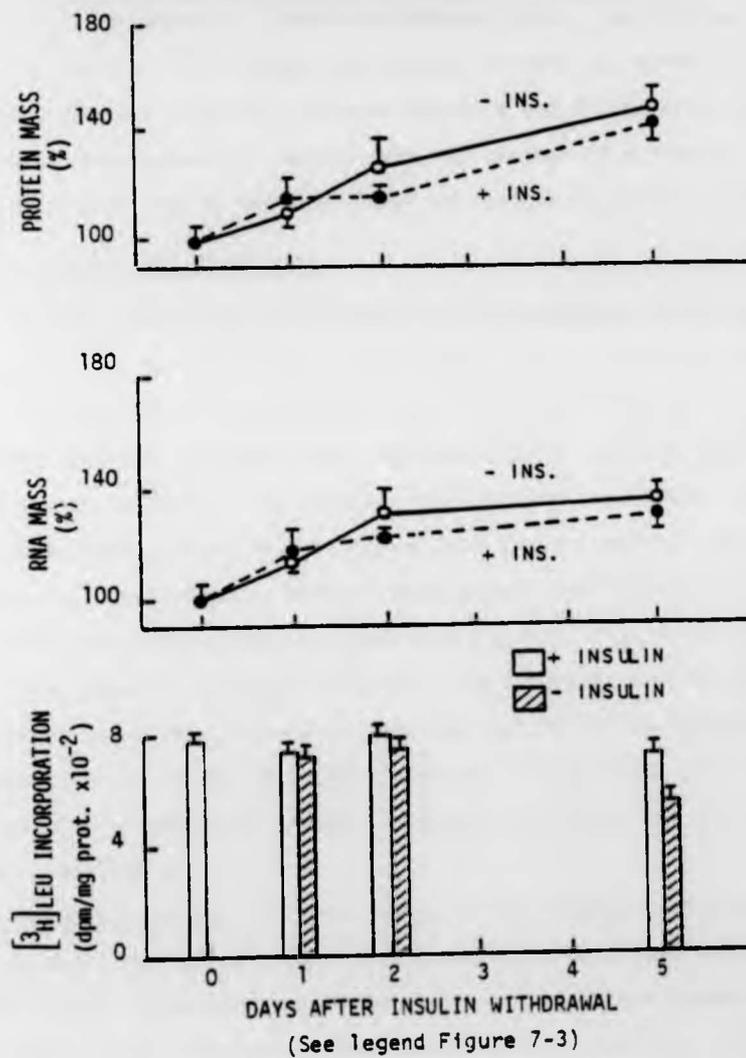
declined within the first 24 hours without insulin and this lower level was maintained up to 5 days of diabetes. In contrast to the findings of other studies (Hay & Waterlow, 1967; Pain & Garlick, 1974, and Section 6) where rates of liver protein synthesis appeared to be unaffected by diabetes when measured by constant infusion, incorporation from the single large dose of [^3H]leucine was reduced by diabetes. This decrease suggests that total liver protein synthesis was reduced.

Kidney: Figure 7-6 illustrates the relative lack of effect of diabetes on protein and RNA mass of the kidney and on the rate of [^3H]leucine incorporation. The relative rate of protein synthesis after 5 days of diabetes was however lower than that of the control insulin treated rats. In the [^{14}C]tyrosine constant infusion study (Section 6), no change in kidney synthesis rates could be observed after a similar period of diabetes.

Conclusions from the pilot study: The results of this pilot study demonstrated that in muscle, changes in protein mass and in the relative rates of synthesis appear after at least 48 hours of insulin withdrawal, whereas in liver these changes are already apparent within the first 24 hours after the onset of diabetes. In all four tissues studied the largest differences between diabetic and control rats were present 4 or 5 days after insulin withdrawal.

The results of this study therefore indicated the most appropriate days on which rates of protein synthesis should be measured and when changes in protein mass should be determined to enable estimations of protein breakdown. For measurements of changes in muscle protein turnover, the most important periods were from day 2 to day 5. For liver, the initial 24 to 48 hours of insulin withdrawal was the essential time for measurements.

FIGURE 7-6 $[^3\text{H}]$ LEUCINE INCORPORATION AND PROTEIN AND RNA CONTENT
IN KIDNEY OF DIABETIC AND INSULIN TREATED RATS



7.4 [³H]PHENYLALANINE STUDY

7.4.1 Protocol

[³H]Phenylalanine was injected as a large single dose in control (insulin treated) rats on day 0 and day 3 (see Figure 7-1 for general protocol). In diabetic (insulin withdrawn) rats, injection was on days 1, 2 and 4. Each group consisted of 10 rats, of which 4 rats were killed 2 minutes after the isotope injection and 6 rats after 10 minutes. One group of 5 control rats and another of 5 diabetic rats were studied on day 6 for measurement of changes in protein mass.

7.4.2 Results and discussion

Insulin treatment of the diabetic rats maintained normal glucose concentrations throughout the study (Table 7-1). The growth rate of 10.7 ± 0.7 g/d (mean rate between day 0 to day 3) in these insulin treated controls was higher than that observed in previous experiments with normal controls. The diabetes resulting from withdrawal of insulin was characterized by a fall in growth rate and an increase in plasma glucose within 24 hours. Marked hyperglycaemia was present only after 48 hours and blood glucose was maintained at this high level throughout.

The effect of diabetes on each of the 7 tissues studied will be discussed separately. Complete data for each tissue on changes in composition and rates of protein turnover will be shown in a table, followed by a summary of changes in protein synthesis and breakdown shown graphically.

Gastrocnemius muscle: The total weight of the muscle continued to rise during the first day of insulin withdrawal, but decreased subsequently (Table 7-2). There was a small decrease in the protein concentration and a much larger decrease in RNA concentration, resulting in a 23-26% fall in RNA to protein ratio after 4 days of diabetes. This was not as marked as the 32% decline reported by Pain & Garlick (1974) in

TABLE 7 - 1 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON BODY WEIGHT AND PLASMA GLUCOSE CONCENTRATION

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Initial body weight (g) on day 0	112 ± 6	109 ± 5	111 ± 4	101 ± 6	99 ± 3
Final body weight (g) at kill	112 ± 6	138 ± 5	118 ± 3	110 ± 6	107 ± 5
Plasma Glucose (mg/100ml)	106 ± 10	98 ± 11	275 ± 44	581 ± 35	644 ± 35

- (a) All values mean \pm SEM for 10 rats in each group.
- (b) CONTROL: These were diabetic rats pre-treated with PZI (4 U/d) for 3 days. Protein turnover was measured after these 3 days of pre-treatment (day 0 control) and after a further 3 days on insulin (day 3 control).
- (c) DIABETIC: These were diabetic rats pre-treated with PZI (4 U/d) for 3 days and measured on day 1, day 2 and day 4 of insulin withdrawal.
- (d) In all the following tables, total tissue weights are the combined weights of two gastrocnemius, soleus and EDL muscles and two kidneys. Synthesis/RNA (mg prot/mg RNA/d) was derived from dividing k_s by RNA/protein.

TABLE 7-2 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON GASTROCNEMIUS COMPOSITION AND
PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	1.019 ± 0.055	1.266 ± 0.063	1.134 ± 0.042	1.095 ± 0.069	0.990 ± 0.062
Protein (mg/g wet tissue)	153 ± 4	174 ± 5	160 ± 3	150 ± 5	147 ± 2
RNA (mg/g wet tissue)	1.497 ± 0.028	1.772 ± 0.087	1.628 ± 0.032	1.462 ± 0.032	1.104 ± 0.045
RNA/Protein (mg/g)	9.8 ± 0.3	10.2 ± 0.4	10.2 ± 0.3	9.8 ± 0.5	7.5 ± 0.3
S _i (dpm/nmole)	503 ± 4	472 ± 8	487 ± 8	470 ± 3	458 ± 7
S _B (dpm/nmole)	0.73 ± 0.05	0.59 ± 0.06	0.62 ± 0.02	0.35 ± 0.04	0.21 ± 0.05
k _S (%/d)	18.6 ± 1.1	16.8 ± 1.7	16.2 ± 0.5	9.8 ± 1.1	5.9 ± 1.3
Synthesis/RNA (mg prot/mg RNA/d)	18.3 ± 1.0	16.1 ± 1.7	16.2 ± 0.4	10.2 ± 0.9	7.8 ± 1.4
k _g (%/d)	+ 14.8	+ 6.7	+ 6.8	- 2.9	- 9.1
k _d (%/d)	3.8	10.1	9.5	12.7	15.0

See legend Table 7-1.

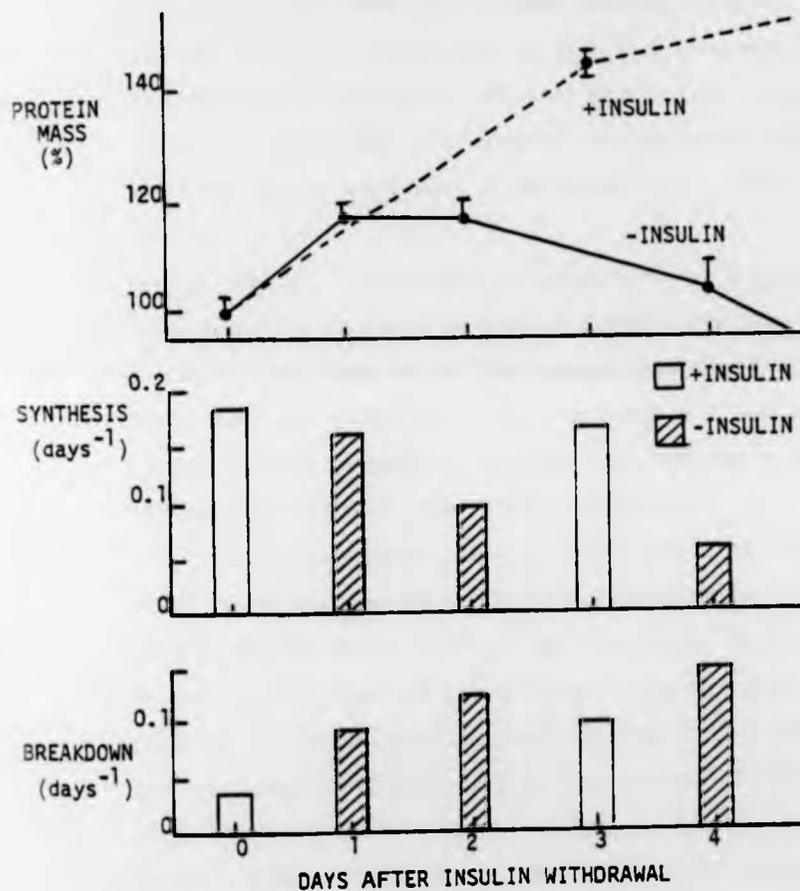
gastrocnemius muscle of 5 day diabetic rats.

The fractional synthesis rates of the day 0 insulin treated control rats are slightly higher than reported by others (Pain & Garlick, 1974; Millward et al., 1976) for normal controls (Table 7-2 and Figure 7-7). This higher synthesis rate most probably reflects a period of catch-up growth after induction of diabetes and treatment with insulin. The growth rate of 14.8%/day at day 0 is very rapid indeed, but by day 3 it had fallen to 6.7%/day which is similar to that observed in normal rats of this age (Section 6).

In the diabetic rats, fractional synthesis rates fell rapidly after 2 days without insulin and at 4 days reached a value only 32 to 35% of the synthesis rates in controls. This confirms the results of Pain & Garlick (1974) and those obtained by constant infusion (Section 6). The reduced rate of synthesis in the 4 day diabetic rats resulted from a small decrease in the number of ribosomes, as shown by the decrease in the RNA/protein ratio, and a much greater fall in the activity of those ribosomes remaining, as indicated by the low rates of synthesis expressed per mg RNA. However, the latter effect was observable almost immediately, while the former did not appear until 4 days of diabetes.

Figure 7-7 illustrates the change in total protein mass, the change in the fractional rate of protein synthesis and also the rates of protein degradation derived from these. The protein mass is the total protein content of two gastrocnemius muscles, expressed as mg protein per 100g initial (day 0) body weight, to take account of the fact that the different groups of rats began the experiment on day 0 with slightly different mean body weights. Each value was then expressed as a percent of the mean value for day 0. Growth rates obtained from the change in protein mass (k_g in Table 7-2) were initially depressed in the diabetic

FIGURE 7-7
PROTEIN TURNOVER IN GASTROCNEMIUS MUSCLE OF DIABETIC
AND INSULIN-TREATED RATS



Mean \pm SEM for 10 control (insulin treated diabetic) and 10 diabetic (insulin withdrawn) rats. Protein mass (mg protein/100g initial body weight on day 0) as % change from mean protein content at day 0.

rats and then became negative as protein was lost. The calculated rates of breakdown showed a progressive increase with onset of severe diabetes. This increase in protein breakdown rates measured in vivo in the diabetic animal has not been reported before. These results contrast with the 45% decrease in in vivo breakdown rates of diabetic skeletal muscle reported by Millward et al. (1976). However, the increase in breakdown rates observed in the present study does substantiate the in vitro work of Jefferson et al. (1974) and Fulks et al. (1975).

Cardiac muscle: The changes in composition and protein turnover of cardiac muscle are shown in Table 7-3 and Figure 7-8. The effect of diabetes on this muscle was less marked than in the gastrocnemius. Again there was a lag period of 2 days before tissue mass and protein content started to decrease. There was a decline in RNA concentration and RNA/protein ratio after 1 day of diabetes.

The higher turnover rate of cardiac muscle of control rats compared to skeletal muscle such as the gastrocnemius has been reported before (Garlick et al., 1975). The results in these diabetic rats further confirm that protein turnover in cardiac muscle is more resistant to change than the gastrocnemius (Pain & Garlick, 1974), i.e. the fractional synthesis rates fell to a value of 46% of the control rates by 4 days. The amount of protein synthesized per mg RNA was not markedly affected by diabetes, hence the decline in fractional synthesis rates were probably due to the decrease in amount of RNA. The present results confirm those obtained in vivo by Pain & Garlick (1974) and the more recent results of Chua et al. (1979), who observed a 38% reduction in protein synthesis by perfused diabetic hearts.

At one day of diabetes neither growth rate nor synthesis rate of protein were different from control values, but thereafter both rates fell. Similarly the derived breakdown rate at one day was very low,

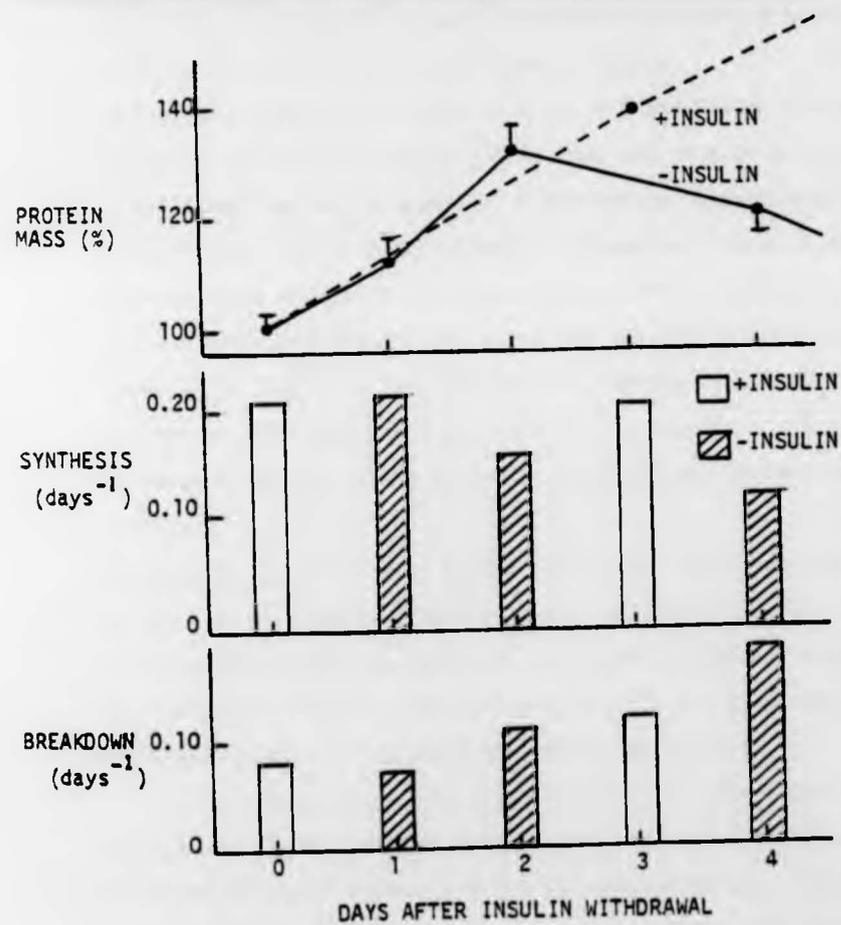
TABLE 7-3 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON HEART COMPOSITION AND PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	0.43 ± 0.02	0.53 ± 0.02	0.47 ± 0.01	0.44 ± 0.03	0.38 ± 0.02
Protein (mg/g wet tissue)	108 ± 2	118 ± 2	107 ± 2	129 ± 2	128 ± 3
RNA (mg/g wet tissue)	2.227 ± 0.031	2.383 ± 0.068	2.326 ± 0.049	1.893 ± 0.041	1.645 ± 0.051
RNA/Protein (mg/g)	20.7 ± 0.3	20.2 ± 0.5	22.9 ± 0.7	14.7 ± 0.4	12.9 ± 0.4
S _i (dpm/nmole)	482 ± 11	472 ± 5	469 ± 3	462 ± 5	434 ± 4
S _B (dpm/nmole)	0.83 ± 0.03	0.81 ± 0.04	0.86 ± 0.03	0.63 ± 0.04	0.45 ± 0.03
k _S (%/d)	20.9 ± 1.0	20.4 ± 1.1	21.2 ± 0.7	15.7 ± 0.9	12.0 ± 0.7
Synthesis/RNA (mg prot/mg RNA/d)	10.2 ± 0.4	9.9 ± 0.4	9.5 ± 0.5	10.6 ± 0.4	9.3 ± 0.2
k _g (%/d)	+ 12.9	+ 8.6	+ 14.1	+ 5.1	- 6.1
k _d (%/d)	8.0	11.8	7.1	10.6	18.1

See legend Table 7-1.

FIGURE 7-8

PROTEIN TURNOVER IN HEART OF DIABETIC AND INSULIN-TREATED RATS



(See legend Figure 7-7)

comparable to that in the day 0 control. Subsequently breakdown increased so that by day 4 it was 53% higher than the day 3 control. This increase in breakdown rate contrasts with the 17% reduction in degradation reported by Chua et al (1979). However, they used cardiac muscles obtained from alloxan-insulin treated rats. They perfused these muscles after 3 days of insulin withdrawal, at which time, according to the present study, breakdown rates would not be detectably different between control and diabetic groups.

Diaphragm: The results shown in Table 7-4 and Figure 7-9 confirm the suggestion of Pain & Garlick (1974) that the rate of protein turnover of the diaphragm muscle would be intermediate between that of the heart and gastrocnemius in its response to diabetes. After 2 days of diabetes, the weight of the diaphragm declined, protein concentration was unchanged and RNA concentration and the RNA to protein ratio were decreased. Similarly, as fractional synthesis rates declined, fractional breakdown rates were enhanced. Again, as in gastrocnemius, the number as well as the activity of remaining ribosomes were decreased.

Soleus and EDL: The total tissue weights of the soleus and EDL both declined with diabetes (Table 7-5 and 7-6 respectively). However, the actual weights shown are probably not quantitative and accurate enough and therefore changes in protein mass, protein growth and protein breakdown rates are not shown for these two muscles.

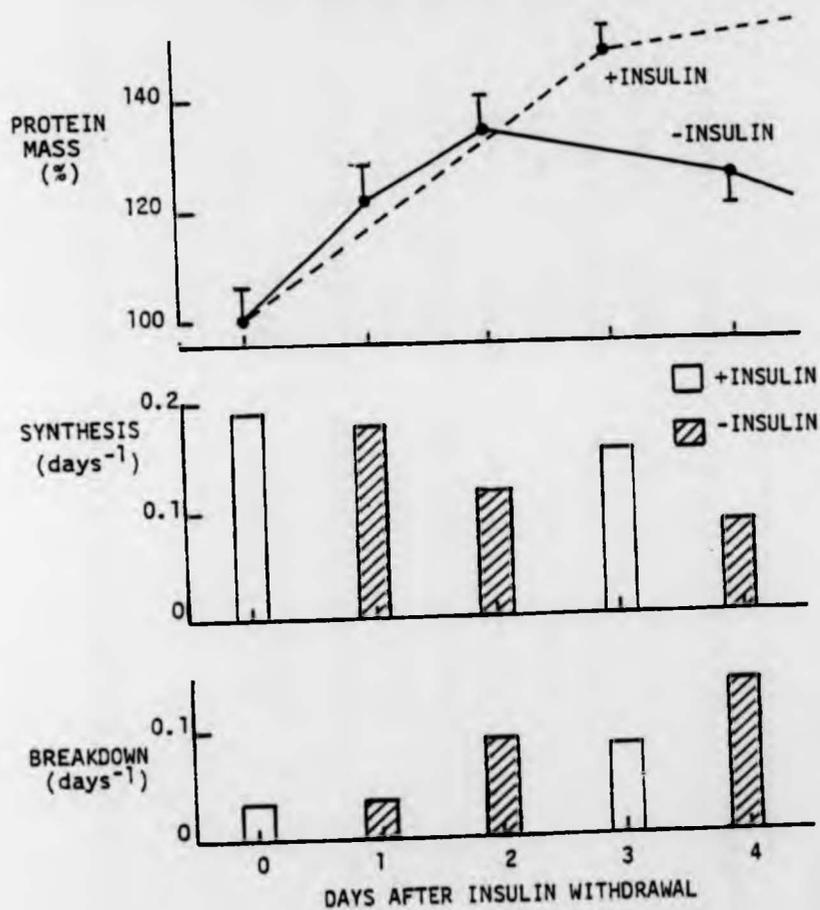
In both muscles, protein concentration was unaffected by diabetes and the decreases in RNA to protein ratios seen in these two muscles were brought about by the drop in RNA concentration. Fractional synthesis rates in both muscles declined at a steady rate after 2 days without insulin. These decreases in synthesis rates were probably due to the loss in number and activity of ribosomes as shown by the decline in synthesis rate per mg RNA in Tables 7-5 and 7-6. However, the 29%

TABLE 7-4 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON DIAPHRAGM COMPOSITION AND PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	0.312 ± 0.019	0.415 ± 0.015	0.370 ± 0.024	0.382 ± 0.072	0.346 ± 0.018
Protein (mg/g wet tissue)	113 ± 3	124 ± 3	117 ± 2	116 ± 2	114 ± 2
RNA (mg/g wet tissue)	1.377 ± 0.039	1.501 ± 0.063	1.417 ± 0.036	1.213 ± 0.053	0.975 ± 0.048
RNA/Protein (mg/g)	12.2 ± 0.3	12.2 ± 0.7	12.1 ± 0.3	10.5 ± 0.4	8.5 ± 0.3
S _i (dpm/nmole)	440 ± 2	455 ± 4	441 ± 3	449 ± 6	431 ± 7
S _B (dpm/nmole)	0.74 ± 0.05	0.60 ± 0.05	0.68 ± 0.03	0.46 ± 0.05	0.33 ± 0.04
k _s (%/d)	19.1 ± 1.2	15.4 ± 1.2	17.8 ± 0.8	11.8 ± 0.4	8.6 ± 0.9
Synthesis/RNA (mg prot/mg RNA/d)	16.0 ± 0.8	13.0 ± 0.3	15.2 ± 0.9	11.3 ± 0.8	9.7 ± 0.7
k _g (%/d)	+ 15.6	+ 6.9	+ 14.1	+ 2.9	- 5.6
k _d (%/d)	3.5	8.5	3.7	9.0	14.2

See legend Table 7-1.

FIGURE 7-9
PROTEIN TURNOVER IN DIAPHRAGM OF DIABETIC AND
INSULIN-TREATED RATS.



(See legend Figure 7-7)

TABLE 7-5 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON SOLEUS COMPOSITION AND PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	0.101 ± 0.003	0.124 ± 0.004	0.110 ± 0.003	0.101 ± 0.006	0.092 ± 0.005
Protein (mg/g wet tissue)	120 ± 3	118 ± 4	127 ± 5	142 ± 3	152 ± 2
RNA (mg/g wet tissue)	1.574 ± 0.032	1.480 ± 0.023	1.542 ± 0.056	1.341 ± 0.023	1.288 ± 0.051
RNA/Protein (mg/g)	13.2 ± 0.4	12.6 ± 0.2	12.3 ± 0.5	9.5 ± 0.3	8.5 ± 0.5
S _i (dpm/nmole)	531 ± 14	503 ± 3	525 ± 12	496 ± 8	502 ± 2
S _B (dpm/nmole)	0.75 ± 0.04	0.63 ± 0.02	0.65 ± 0.04	0.56 ± 0.06	0.37 ± 0.04
k _s (%/d)	19.3 ± 1.0	16.5 ± 0.6	15.6 ± 0.8	13.5 ± 1.2	9.6 ± 0.9
Synthesis/RNA (mg prot/mgRNA/d)	15.1 ± 0.8	13.2 ± 0.2	13.0 ± 0.5	14.0 ± 0.9	11.4 ± 0.5

See legend Table 7-1.

TABLE 7-6 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON EXTENSOR DIGITORUM LONGUS COMPOSITION AND PROTEIN TURNOVER

	CONTROL		DIABETIC		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	0.090 ± 0.004	0.113 ± 0.004	0.093 ± 0.003	0.104 ± 0.005	0.090 ± 0.007
Protein (mg/g wet tissue)	121 ± 4	112 ± 4	125 ± 4	127 ± 3	134 ± 6
RNA (mg/g wet tissue)	1.310 ± 0.025	1.292 ± 0.062	1.296 ± 0.030	1.071 ± 0.057	0.881 ± 0.046
RNA/Protein (mg/g)	10.7 ± 0.7	11.7 ± 0.6	10.5 ± 0.4	8.4 ± 0.4	6.7 ± 0.5
S _i (dpm/nmole)	486 ± 7	500 ± 3	496 ± 6	498 ± 2	506 ± 9
S _B (dpm/nmole)	0.59 ± 0.01	0.67 ± 0.04	0.61 ± 0.02	0.40 ± 0.04	0.26 ± 0.05
k _s (%/d)	15.7 ± 0.2	17.5 ± 1.1	15.8 ± 0.6	10.3 ± 0.9	6.8 ± 1.3
Synthesis/RNA (mg prot./mg RNA/d)	14.2 ± 0.8	14.4 ± 0.6	16.1 ± 0.7	11.8 ± 1.0	10.0 ± 1.5

See legend Table 7-1.

decrease in activity of the EDL synthesizing mechanism and the 14-16% decrease shown in that of the soleus is much less pronounced than the reduction of 51% observed in the gastrocnemius.

Kidney: Table 7-7 and Figure 7-10 illustrate the response of the kidney to insulin withdrawal and insulin treatment. As diabetes progressed, so tissue weight and total protein mass increased relative to the values for control animals. There was a small decrease in RNA concentration and RNA to protein ratio after 4 days of diabetes when compared with the day 0 and day 3 control values.

The fractional synthesis rates of 47 and 42% demonstrated in the control, insulin treated rats are very comparable to the values of 47% obtained by constant infusion with [14 C]tyrosine (see Section 6.3.2) in normal control rats. Similarly, the kidney again appeared to be relatively unaffected by diabetes. The fractional synthesis rates of 4 day diabetic rats were lower only if the values are compared to that of the day 0 controls, i.e. the group assumed to be in a phase of catch-up growth. The protein synthesis rates shown here and in Section 6 are to our knowledge the first published indication that synthesis rates of diabetic kidneys do not increase in a similar fashion as do kidney weight and glomerular filtration rates in response to diabetic hyperglycaemia, polyuria and glycosuria. The work of Seyer-Hansen (1976, 1977 and 1978) on the diabetic kidney described hypertrophy only in terms of increased protein, RNA and DNA content. In contrast, Peterson et al. (1971) reported that kidney ribosomes incorporated more amino acids into protein in vitro.

Protein breakdown rates in the kidney were depressed only on the initial day of insulin withdrawal. Thereafter breakdown rates increased so that by 4 days of diabetes there was little difference between the control and diabetic values.

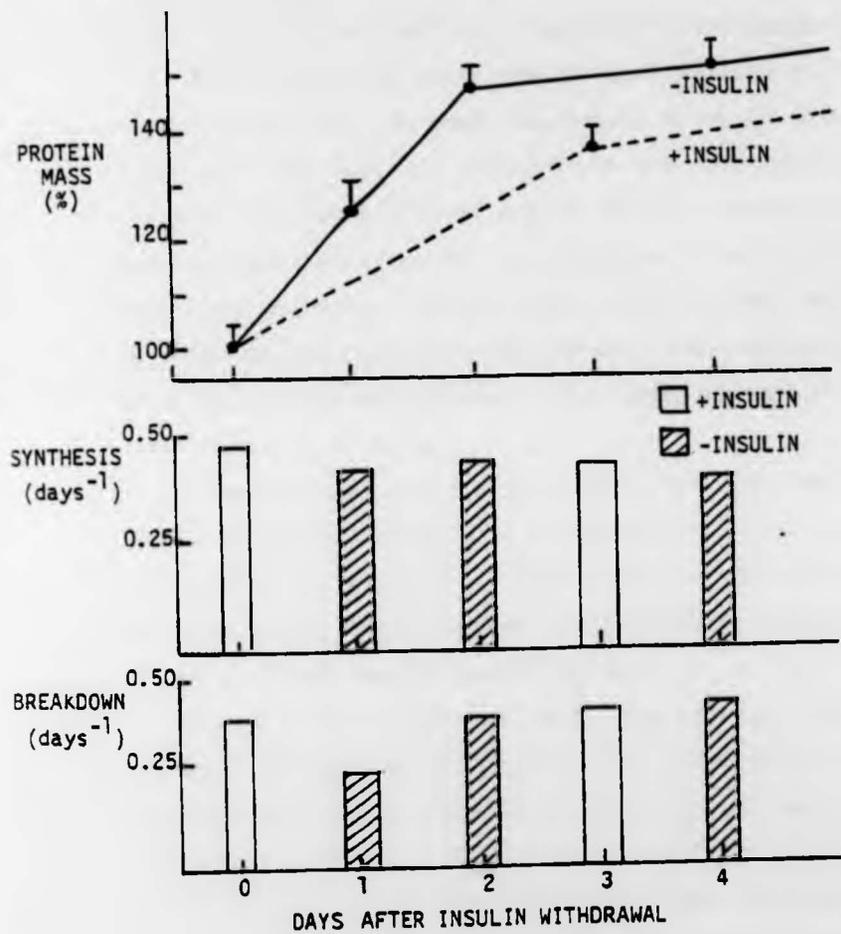
TABLE 7 - 7 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON KIDNEY COMPOSITION AND PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	1.25 ± 0.05	1.49 ± 0.05	1.40 ± 0.05	1.45 ± 0.06	1.58 ± 0.06
Protein (mg/g wet tissue)	91 ± 3	101 ± 2	95 ± 7	104 ± 5	97 ± 2
RNA (mg/g wet tissue)	4.076 ± 0.127	4.027 ± 0.052	4.137 ± 0.105	3.919 ± 0.060	3.655 ± 0.068
RNA/Protein (mg/g)	45.0 ± 0.8	39.7 ± 0.5	41.6 ± 1.4	38.4 ± 1.6	37.9 ± 0.7
S _i (dpm/nmole)	520 ± 11	525 ± 4	513 ± 7	510 ± 13	494 ± 12
S _B (dpm/nmole)	1.92 ± 0.04	1.70 ± 0.02	1.67 ± 0.04	1.74 ± 0.03	1.58 ± 0.08
k _s (%/d)	46.7 ± 1.2	42.0 ± 0.4	41.3 ± 1.1	43.2 ± 0.7	39.2 ± 1.8
Synthesis/RNA (mg prot/mg RNA/d)	10.3 ± 0.2	10.5 ± 0.2	10.3 ± 0.7	11.3 ± 0.7	10.2 ± 0.4
k _g (%/d)	+ 12.1	+ 5.5	+ 18.9	+ 8.3	+ 1.5
k _d (%/d)	34.6	36.5	22.4	34.8	37.7

See legend Table 7-1.

FIGURE 7-10

PROTEIN TURNOVER IN KIDNEY OF DIABETIC AND INSULIN-TREATED RATS



(see legend Figure 7-7)

Liver: The changes brought about by diabetes on liver composition and protein metabolism are shown in Table 7-8 and Figure 7-11. Total liver weight decreased within the first day of insulin withdrawal and then remained more or less constant. There was little change in composition.

The fractional synthesis rate of total (cellular plus secretory) hepatic protein also responded immediately to insulin withdrawal, despite the fact that this group of rats was only mildly hyperglycaemic after only 24 hours without insulin. The synthesis rate of total protein thereafter fell slowly and consistently as insulin withdrawal was prolonged. This decrease in total protein synthesis rates confirms the results of McNurlan & Garlick (1980a) who used the same large dose method and reported an even larger decrease in synthesis after 10 days of diabetes.

Albumin synthesis as a proportion of total liver protein synthesis showed a disproportionate fall, in confirmation of the results of Pain et al. (1978a) and Peavy et al. (1978). This decline in albumin synthesis was on its own however insufficient to account for the overall drop in total hepatic protein synthesis.

Pain & Garlick (unpublished data) have previously shown that the proportion of total hepatic protein synthesis devoted to the production of plasmaproteins other than albumin (approximately 20%) was not changed by diabetes. The synthesis rate of cellular proteins could then be calculated from the rate of synthesis of albumin and that of total hepatic proteins (Table 7-8). In diabetic rats a decrease in cellular synthesis rate was observed, but this was less marked than the decrease in rates of synthesis of total liver proteins.

Degradation of liver cellular proteins was elevated above day 0 and day 3 control values for only the first day of insulin withdrawal during the period of rapid protein loss (Figure 7-11). After 4 days of diabetes breakdown rates had however declined to levels below that

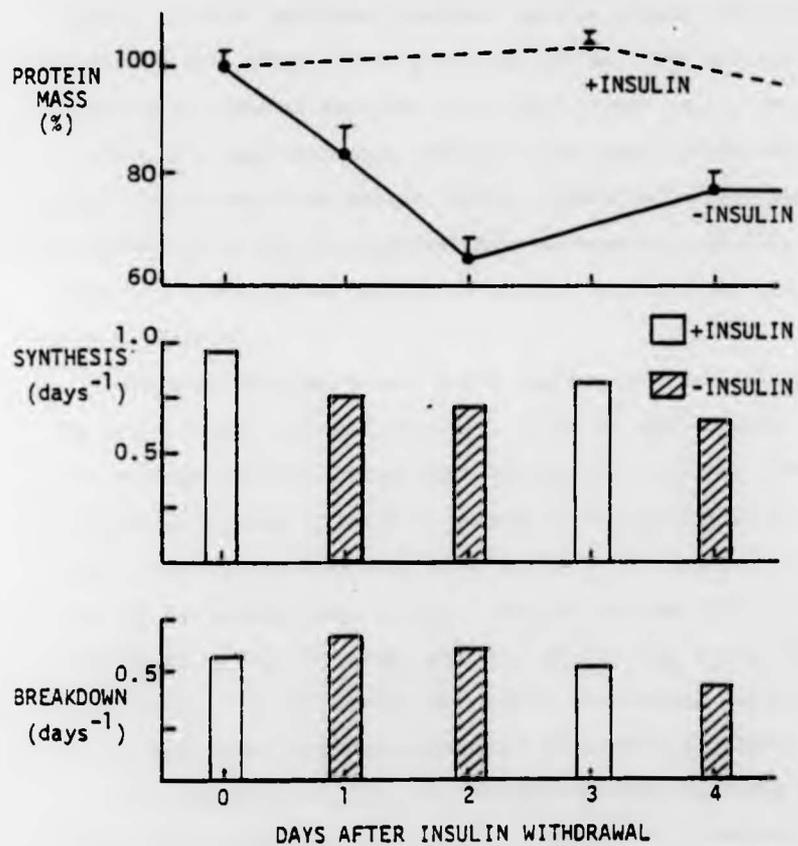
TABLE 7-8 THE EFFECT OF DIABETES AND INSULIN TREATMENT ON LIVER COMPOSITION
AND PROTEIN TURNOVER

	C O N T R O L		D I A B E T I C		
	Day 0	Day 3	Day 1	Day 2	Day 4
Total tissue weight (g)	7.41±0.39	6.45±0.25	5.48±0.16	5.00±0.25	5.22±0.20
Protein (mg/g wet tissue)	162±6	187±3	182±4	141±4	153±4
RNA (mg/g wet tissue)	7.840±0.221	8.238±0.158	8.751±0.179	7.675±0.103	7.611±0.201
RNA/Protein (mg/g)	48.6±1.1	48.1±0.8	44.0±0.8	54.7±1.5	48.6±2.0
S _i (dpm/nmole)	487±6	467±11	453±9	461±6	434±15
S _g (dpm/nmole)	3.70±0.07	3.10±0.12	2.85±0.05	2.72±0.10	2.52±0.16
k _s Total Protein (%/d)	96.4±2.7	82.5±2.7	75.7±0.8	71.5±2.2	65.3±3.2
k _s Cellular Protein (%/d)	59.7	49.5	46.7	47.2	42.8
Synthesis Albumin (% of total prot. synthesis)	18.1±0.6	20.0±0.6	18.3±1.0	14.0±0.6	14.5±1.5
Synthesis/RNA (mg prot/mg RNA/d)	19.3±0.5	19.1±0.6	15.7±0.2	13.3±0.6	14.3±0.6
k _g (%/d)	+ 1.2	- 1.7	- 20.3	- 9.8	+ 3.1
k _d (%/d)	58.5	51.2	67.0	57.0	39.7

- (a) k_s of total protein includes synthesis rates of cellular plus secretory proteins
- (b) k_s of cellular protein was calculated from synthesis of total protein minus synthesis of albumin and synthesis of other plasma proteins (± 20% of total liver proteins for both control and diabetic groups).
- (c) k_g reflects growth of non-secretory or cellular protein only, therefore k_d was calculated as the difference between k_s of cellular protein and k_g.
- (d) see legend of Table 7-1 for detail on control and diabetic groups.

FIGURE 7-11

PROTEIN TURNOVER IN LIVER OF DIABETIC AND INSULIN-TREATED RATS



(see legend Figure 7-7 and Table 7-8)

of the controls.

7.4.3 Summary and Conclusions

The gastrocnemius muscle appears to be highly sensitive to changes in insulin levels and responds with a rapid and pronounced decline in rates of protein synthesis within 24 hours of insulin withdrawal. Cardiac and other skeletal muscles showed a decrease in synthesis only after a 1 to 2 day lag period. The magnitude of response to diabetes appeared to be much higher in the white glycolytic muscles, i.e. gastrocnemius and EDL. The heart, soleus and diaphragm, which contain more red muscle fibres, seemed to be less affected by diabetes and on the average synthesis decreased by 40-45%, whereas the synthesis rates in the more white muscles decreased by 60-70% after 4 days of diabetes.

Protein breakdown rates in all muscles were severely depressed in the day 0 insulin treated controls, since the growth rates on this day were extremely high. These day 0 values of fractional growth probably represents a phase of catch-up growth in the muscles resulting from only 3 days of pre-treatment with insulin. After a further 3 days on insulin, the growth rates of day 3 insulin treated controls were comparable to that of normal controls of the same age in other experiments. In all muscles the effect of diabetes was shown as a steady elevation in protein breakdown as protein synthesis declined.

In contrast to muscle, the diabetic kidney increased in weight and protein content whilst rates of synthesis and breakdown were relatively unchanged.

Liver proteins responded immediately to insulin withdrawal with a decrease in rates of synthesis in both total hepatic proteins and of albumin as a proportion of total protein. Protein breakdown rates in the liver were only transiently elevated on the first day without

insulin, thereafter rates of protein breakdown below those of the controls were observed.

SECTION 8GENERAL DISCUSSION

The primary aim of this investigation was to examine the mechanism of the negative N-balance present in uncontrolled diabetes. In the preceding experimental section some results were presented for rates of protein synthesis and breakdown in diabetic animals that were not only new information, but contrasted with some published data obtained in vivo. In addition, the results observed in rates of protein synthesis in the diabetic subjects appeared to conflict with the anabolic function of insulin. If one is to accept these results as being a true reflection of changes in the diabetic state, the following questions should be considered :

- (a) did the methods that were used give reasonable results in the normal or control animal or human.
- (b) how were the rates of protein turnover altered by diabetes with these methods.
- (c) can one make any comparison between the results observed in the experimentally diabetic rat and the disease state as presented in the diabetic subjects.

In this general discussion, consideration will be given firstly to the control values obtained for protein turnover in the whole body in man and in the rat and also in tissues of the rat. Secondly, the changes observed in the diabetic state on a whole body and on a tissue level will be compared with the control values measured and with other published in vivo and in vitro investigations in diabetes.

8.1 PROTEIN TURNOVER IN NORMAL MAN AND CONTROL RATS

8.1.1 Whole body protein turnover in man

In the Introduction (Section 1.1.2) various methods were discussed which could be used to measure whole body protein turnover. These methods differ from each other by :

- (a) the use of either radioactively labelled ^{14}C amino acids or stable isotopes such as ^{15}N labelled amino acids.
- (b) by administration of the labelled amino acid by either constant infusion (^{14}C and ^{15}N amino acids), or by multiple or single oral doses (^{15}N amino acids).
- (c) by measurement of the excretion of [^{15}N] abundance in the urinary end products urea or ammonia if [^{15}N]glycine was used.
- (d) by measurement of excretion of the ^{14}C label as $^{14}\text{CO}_2$ if a ^{14}C amino acid was used.

In the present study, the stable isotope [^{15}N]glycine was chosen since the age group of the patients studied excluded on ethical grounds the use of ^{14}C labelled amino acids. It was more convenient in these patients to administer the [^{15}N]glycine as a single oral dose. It was also more realistic in the non-steady state of diabetes to measure [^{15}N] abundance in ammonia as end product since this enabled completion of a study within 9-12 hours. In contrast, 24-48 hours would have been required if urea had been used as urinary end product.

Rates of protein synthesis: In the diabetic patients the rates of whole body protein synthesis measured pre-insulin (3.8 ± 0.4 g protein/kg/day, Exp. I, Section 3) were not markedly different from the rates observed after 4 to 5 days of insulin treatment (3.2 ± 0.4 g protein/kg/day) or even after 10 months of insulin (3.0 g protein/kg/day). The post-insulin values were assumed to be representative of the controlled diabetic state since hyperglycaemia was reduced to near-

normal values and the negative N-balance improved.

Despite the fact that the choice of labelled amino acid, of end product and of route of administration all have some disadvantages as discussed in Section 1.1.2, the synthesis rate of 3.2 ± 0.4 gram protein/kg/day observed post-insulin in the 4 patients of Exp. I are quite comparable to that reported by others as shown in Table 8-1. In this table a summary is given of results reported by various research groups using both ^{14}C and ^{15}N labelled amino acids with different routes of administration and with measurements on urinary ammonia, urinary urea or plasma. Not only are the results obtained remarkably consistent between methods, but are comparable over a wide age-range.

In Experiment II of Section 3, much higher synthesis rates of 6.6 g protein/kg/d were observed in two patients. At the time of measurement this was thought to possibly result from the practically continuous feeding of protein meals at 2 hourly intervals during the 9 hours in which ^{15}N abundance was measured in urinary ammonia. The absorption of food would not only have delayed excretion of label from the metabolic pool, but food intake in itself could have maintained a high rate of protein synthesis, since Garlick et al. (1980a) have recently shown that the absence of feeding results in an abrupt decrease in synthesis. However, these high values of protein synthesis were not observed in control subjects receiving a similar dietary protocol (see Table 8 - 1, unpublished data, 3.0 g protein/kg/day, range: 1 - 5 g protein/kg/d). The reason for the high synthesis rates observed in these subjects in the present study is therefore not clear. Although these rates were measured in only two patients, it was a consistent finding in each patient before and after insulin treatment (Table 3-6) and this high rate was again observed in one patient after 8 weeks of insulin (Table 3-7).

TABLE 8-1 RATES OF PROTEIN SYNTHESIS IN ADULT MAN AS OBTAINED BY DIFFERENT METHODS

	Isotope	Method of administration	Measurements on	No.	Age	Synthesis
Steffee et al. (1976)	[¹⁵ N]glycine	repeated oral doses	urinary urea	6	20-25	3.0 ± 0.3
Crane et al. (1977)	[¹⁵ N]glycine	repeated oral doses	urinary urea	11	20-65	3.8 ± 0.7
Golden & Waterlow (1977)	[¹⁵ N]glycine	constant intragastric infusion	urinary urea	6	66-91	3.3 ± 0.3
Golden & Waterlow (1977)	[¹⁵ N]glycine	constant intragastric infusion	urinary ammonia	6	66-91	2.3 ± 0.6
Golden & Waterlow (1977)	[¹⁵ N]glycine	constant intragastric infusion	urinary total N	6	66-91	3.2 ± 0.5
Golden & Waterlow (1977)	[¹⁴ C]leucine	intragastric or intravenous infusion	plasma leucine	6	66-91	2.7 ± 0.7
O'Keefe et al. (1974)	[¹⁴ C]leucine	constant intravenous infusion	plasma leucine	4	52-75	4.0 ± 0.5
James et al. (1976)	[¹⁴ C]tyrosine	constant intravenous infusion	plasma tyrosine	6	30-65	4.6 ± 0.8
Halliday & McKeran (1975)	[¹⁵ N]lysine	constant intravenous infusion	plasma lysine	5	31-46	2.3 ± 1.2
Unpublished data*	[¹⁵ N]glycine	constant intravenous infusion	urinary ammonia	11	20-45	3.0 ± 1.1

Rates of protein synthesis are expressed as g protein/kg body weight/day, ± S.D.

The four results of Golden & Waterlow (77) were obtained by simultaneous infusion of [¹⁵N]glycine and [¹⁴C]leucine into the same six patients.

The rate of synthesis from [¹⁵N]lysine infusion was calculated from the flux minus the urinary N excretion (75). *Garlick, P.J., McNurlan, M.A., Fern, E.B., Tomkins, A. and Waterlow, J.C. Unpublished data of this Department.

Rate of protein breakdown: In the 4 patients studied in Experiment I (Section 3), rates of whole body protein breakdown of 3.0 ± 0.5 g protein/kg/day were measured (Table 3-4). These rates were quite comparable to the 3.9 and 3.7 g protein/kg/day observed pre- and post-operatively with repeated doses of [^{15}N]glycine and measurement of ^{15}N in urinary urea (Crane et al., 1977). Similarly, O'Keefe et al. (1974) reported values of 299 and 302 g protein/day (approximately 4 g protein/kg/day) for patients pre- and post-operatively as measured by constant infusion of [^{14}C]leucine.

In conclusion, it would appear that the rates of protein synthesis and breakdown measured post-insulin in the diabetic subjects of Experiment I were consistent with results obtained by others using different methods and different labelled amino acids.

8.1.2 Whole body protein turnover in control rats

Rates of protein synthesis: The rate of whole body protein synthesis was measured in control, diabetic and insulin treated diabetic rats by constant infusion of L-[^{14}C]leucine (Section 5). Inherent advantages of this method were the short time (2 hours) required for the specific activity of the free amino acid in the blood and in tissues to reach a plateau. Secondly, since the ^{14}C label was quantitatively excreted as $^{14}\text{CO}_2$, oxidation rates could be obtained as described in Section 1.1.2.

The synthesis rates measured as μmoles leucine per minute per 100g body weight could be converted to g protein per kg per day, by assuming that rat protein contains approximately 8% leucine (Fern, 1975). The values of 39 g protein per kg per day for control rats in this experiment is comparable with the 31 g protein/kg/day observed by McNurlan & Garlick (1980b). They injected a large amount of [^{14}C]leucine, measured label incorporation over 10 minutes and determined

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total body protein content and the specific activity of leucine in free and protein-bound tissue obtained by homogenizing the total rat carcass. The synthesis rates of this study were also consistent with the 42 g protein/kg/day reported by Garlick et al. (1975) who calculated the rate from flux observed by constant infusion of [^{14}C]-tyrosine and by assuming that body protein contained 3% tyrosine.

The vast difference in absolute synthesis rates between humans and rats measured here, i.e. of approximately 3-4 g protein/kg/day in man versus 30-40 g protein/kg/day in the rat has been noted before (e.g. Garlick, 1979) and appears to be related to the very high metabolic rate of the smaller species (Munro, 1969).

Rates of protein oxidation: The whole body oxidation rates measured *in vivo* in control rats, i.e. of 0.236 ± 0.013 $\mu\text{moles leucine/minute/100 g rat}$ were very similar to the values of 0.277 $\mu\text{moles leucine/minute/100 g rat}$ observed by Sketcher (1976) using a similar method.

Rates of protein breakdown: In contrast to the measurement of protein breakdown in humans as the difference between flux rates and food intake, in the rat this method has been shown not to be applicable (Section 5.4.3). Since flux was measured over 4 hours, comparison with either food intake or growth measured over 24 hours was shown not to be practical.

8.1.3 Tissue protein turnover in control rats

Growth rate of protein mass: The comparison between control rates of fractional synthesis in tissues, observed by either [^{14}C]tyrosine infusion or injection with a large amount of [^3H]phenylalanine and with other published data requires consideration of the fact that the controls of Section 7 were diabetic rats treated with insulin. In terms of plasma glucose values these animals were well-controlled at

day 0 (after 3 days pre-treatment with insulin) and at day 3 (after a further 3 days of insulin). At day 0 these rats not only had extremely rapid body growth rates (11 g per day), but also rates of growth in protein mass of 13-16%/d in skeletal and cardiac muscle (Section 7). These rates of growth in protein mass are extremely rapid compared to the 6-8%/d observed in normal rats of similar age and weight (e.g. Section 6). The high rates of growth of protein mass in controls at day 0 were associated with equally high rates of fractional synthesis (e.g. k_s of 19%/d was measured in the gastrocnemius muscle of day 0 control rats, Table 7-2).

Similar high rates of synthesis have been observed during the period of rapid catch-up growth in malnourished rats following refeeding (Millward et al., 1974, 1975). This rapid growth during rehabilitation was associated with not only increased protein synthesis, but also increased rates of protein breakdown. High synthesis and high breakdown rates have also been observed in skeletal muscle of very young rats during the period of rapid growth (Waterlow et al., Ch. 16, 1978a). In contrast, in the present study, after 3 days of insulin treatment (day 0 controls) rapid growth was associated with high rates of synthesis, but with extremely low rates of breakdown, (e.g. 3%/day as measured in the gastrocnemius), a finding consistent with the anabolic role of insulin.

Rapid growth of protein mass associated with high fractional synthesis rates have been observed in hypertrophied muscles following various surgical and mechanical interventions. Goldberg reported compensatory growth in the soleus and plantaris muscles after severing the connection of the gastrocnemius to the Achilles tendon. This hypertrophy after tenotomy was shown to occur not only in control rats, but also in hypophysectomized (Goldberg, 1967a) and in diabetic rats

(Goldberg, 1968), where one would expect the absence of growth hormone and insulin to prohibit rapid growth. Hypertrophy with high rates of protein synthesis and of breakdown measured in vivo has been demonstrated to occur in the wing muscle of the fowl following a long period of muscle stretch by weights attached to the wing (Laurent et al., 1978). Similarly, hypertrophy of the isolated diaphragm after denervation has been reported by Buse et al. (1965) and by Harris & Manchester (1966). Turner & Garlick (1974) also observed hypertrophy of diaphragm muscle after nerve section and by constant infusion of [^{14}C]tyrosine could determine that again both in vivo synthesis and breakdown rates were enhanced, a finding consistent with that of Millward et al. (1974, 1975) in rats during rehabilitation following malnutrition.

It would appear therefore that the control rats at day 0 of the experiment reported in Section 7, were still in an initial phase of rapid catch-up growth in response to the insulin treatment, which after a further 3 days of insulin treatment (day 3 controls), had settled down to a state more characteristic of normal rats. In all the experiments to be discussed, comparisons will therefore be made with either normal age control rats (Section 6, rats with similar initial body weight and age as experimental groups) or day 3 controls (Section 7, diabetic rats after a total of 6 days of insulin).

Tissue synthesis rates: In Table 8-2 control values are presented for fractional synthesis rates in some tissues as measured in this study by either constant infusion of [^{14}C]tyrosine or as a single injection of a large amount of [^3H]phenylalanine. It is clear that not only do the values compare well between the two methods, but also with other in vivo studies using different strains of rats, diets, controls and with different days of duration of diabetes. The table

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TABLE 8-2 FRACTIONAL SYNTHESIS RATES (k_s as %/d) IN SOME TISSUES OF CONTROL RATS AND DIABETIC RATS (100 - 120g)

REF.	CONTROL					DIABETIC				
	Gastroc-nemius	Heart	Kidney	Liver total	Liver cellular	Gastroc-nemius	Heart	Kidney	Liver total	Liver cellular
a	13	-	48	-	54	5	-	48	-	50
b	17	20	42	83	50	6	12	39	65	43
c	14	17	-	-	50	4	9	-	-	50
d	13	17	48	-	67	-	-	-	-	-
e	13	-	-	-	-	5	-	-	-	-
f	-	-	-	97	-	-	-	-	52	-

a = Section 6; [^{14}C]Tyr infusion, 120g age controls and 5 day diabetic rats.

b = Section 7; [^3H]Phe large dose, day 3 (insulin treated diabetic) controls and day 4 (insulin withdrawn) diabetic rats.

c = Pain & Garlick (1974); [^{14}C]Tyr infusion, age controls, 5 day diabetic rats.

d = Garlick et al. (1975); [^{14}C]Tyr infusion, age controls.

e = Millward et al. (1976); [^{14}C]Tyr infusion, age controls and 6 day diabetic rats.

f = McNurlan & Garlick (1980a); [^{14}C]Leu large dose, age controls and 10 day diabetic rats.

also confirms that the method of injecting a large dose of labelled amino acid does in fact measure synthesis of total hepatic proteins as suggested by the work of McNurlan et al. (1979). With this method McNurlan et al. (1979) investigated the possibility of the large dose affecting the rate of protein synthesis. They showed equal incorporation of [^{14}C]glycine with or without a large amount of [^{14}C]-leucine. The very small differences observed in the present study in synthesis rates of gastrocnemius, kidney and liver in controls measured by either constant infusion or by the large dose method would appear to confirm the assumption that the large dose of labelled amino acid would not adversely affect protein synthesis.

In Table 8-3 values are shown for the contribution of individual tissues to whole body protein turnover. Since synthesis rates were measured in only 5 muscles, this table does not clearly indicate that the contribution of skeletal muscle to whole body protein synthesis is in fact quite large. Despite very slow rates of turnover, skeletal muscle can contribute more than 19% of protein synthesis in the whole body if one assumes total body muscle mass to be 36% of the body weight of a 100 g rat. Although it has a very rapid turnover rate, the liver contributes not more than about 25% of total body protein synthesis, since it only contains approximately 5% of the body's protein mass.

In Table 8-3, the rates of synthesis in the tissues measured in the present study accounts for only 1.03 g protein/day of a whole body protein synthesis of 3.9 g/day. Of the 2.9 g protein/day not accounted for, a large contribution of 0.4 g/day or 10% of whole body synthesis will be made by the small intestine (McNurlan et al., 1979), and the rest by tissues such as smooth muscle, skin, bone, brain, lung and other visceral organs.

TABLE 8-3 RATES OF PROTEIN SYNTHESIS IN TISSUES OF CONTROL AND DIABETIC RATS (100-120g)

	C O N T R O L			D I A B E T I C		
	k_s (%/d)	Absolute Synthesis Rate		k_s (%/d)	Absolute Synthesis Rate	
		g/d	% of whole body		g/d	% of whole body
Gastrocnemius	16.8	0.034	0.94	5.9	0.009	0.27
EDL	17.5	0.002	0.06	6.8	0.001	0.02
Soleus	16.5	0.002	0.06	9.6	0.001	0.04
Diaphragm	15.4	0.007	0.19	8.6	0.003	0.10
Heart	20.4	0.012	0.33	12.0	0.006	0.18
Kidney	42.0	0.058	1.61	39.2	0.060	1.82
Liver (Total)	82.5	0.914	25.39	65.3	0.543	16.5
(cellular)	49.5	0.549	15.24	42.8	0.356	10.8
Whole body	-	3.900	100	-	3.300	100

- (a) All tissue values obtained by injecting a large amount of [^3H]phenylalanine and measuring incorporation over 10 minutes (Section 7)
- (b) Control values are those measured after 6 days of insulin treatment (4 U PZI/d) of diabetic rats, i.e. Day 3 controls of Table 7-1.
- (c) Diabetic values are those measured after 4 days of insulin withdrawal, i.e. Day 4 diabetic rats of Table 7-1.
- (d) Whole body synthesis rate was determined by constant infusion of [^{14}C]leucine (Section 5, Exp. II).
- (e) Absolute rates of protein synthesis as gram protein per day calculated by multiplying the fractional rate of protein synthesis (k_s , %/d) by the protein content of the tissue (which is approximate only for soleus and EDL muscles).

Tissue breakdown rates: The values obtained for fractional rates of protein breakdown by use of the indirect method are influenced by the inherent errors involved as discussed in Section 1.1.2, e.g. by deriving these breakdown rates from measurements of changes in protein mass over several days. In this investigation (Section 7) the day 3 control values of growth in protein mass (k_g) were obtained as the mean of the changes between day 0 and day 3 and between day 3 and day 6. The day 0 control k_g value was measured only from day 0 to day 3 and was assumed to be linear over this period.

At day 0 the control fractional rates of protein breakdown were extremely low in all muscles. This is to be expected since the rates were derived as the difference between the k_s and k_g values of each muscle, both rates which were highly elevated. The day 3 control rates are for reasons discussed previously more similar to those of normal growing rats. Rates of breakdown in gastrocnemius (10.1%/d), heart (11.8%/d) and diaphragm (8.5%/d) were comparable to the 9.4%/d reported by Turner & Garlick (1974) in hemidiaphragm muscle or values of 8-11%/d in skeletal muscle reported by Garlick et al. (1975) and Millward et al. (1976).

In conclusion, it appears not unrealistic to accept the values obtained for protein synthesis and breakdown in diabetic subjects treated with insulin and in control animals, since these rates are quite consistent with values obtained by other groups using similar or different methods. In the following discussion, results observed in the diabetic state in humans and in rats will be examined as measured by these methods.

8.2 PROTEIN TURNOVER IN DIABETIC SUBJECTS AND ANIMALS

8.2.1 Whole body protein turnover in diabetic adults

Rates of protein synthesis: The normal control rates of protein

synthesis measured pre-insulin and the slight decrease in synthesis rates observed in diabetic patients 4 days post-insulin (Section 3.2.7), not only contrasts with the results of the pilot study by Waterlow et al. (1977), but also with the expected anabolic function of insulin. Since there was a slight improvement in the negative N-balance in most patients, this could have only resulted from the more pronounced decrease in breakdown following insulin treatment. The small decrease in synthesis rate after 4 to 5 days of insulin was observed in 5 out of 6 patients and was not affected by manipulation of caloric content of the diet, the meal pattern itself or time of ^{15}N glycine administration. The apparently higher synthesis rates before insulin in the first 4 patients might, however, be a metabolic artifact resulting from the higher rate of NH_3 excretion in the diabetic state, even though there was no frank acidosis. The possibility exists that when the rate of urinary NH_3 excretion is increased, glycine might contribute a smaller proportion of N to the formation of NH_3 . The effect of this would be to lower the ^{15}N abundance in urinary NH_3 , and to increase the calculated values for flux and synthesis. However, in patients Nos. 5 and 6 where rates of protein synthesis were also higher in the pre-insulin period, ammonia excretion was unchanged in one patient and increased in the other. The difference between normal and pre-insulin values measured in this study and the very low synthesis rates observed pre-insulin in the pilot study (Waterlow et al., 1977) have been discussed in detail (see Section 3.2.7).

Rates of protein breakdown: The decrease in rates of whole body protein breakdown in these subjects following 4 days, 8 weeks or 10 months of insulin therapy fits with the role of insulin in inhibiting protein degradation. In contrast, no change in muscle protein breakdown could

be detected in these patients after insulin as indicated by excretion of 3-methylhistidine.

From the results of the present investigation in diabetic subjects one must conclude that large changes in whole body protein turnover could not be detected in these patients. It is possible that such changes may occur in diabetic patients with more severe biochemical abnormalities, e.g. in the newly diagnosed insulin dependent subject with severe weight loss, hyperglycaemia and glycosuria, or alternatively in a well-controlled diabetic subject after one or two days of insulin withdrawal.

8.2.2 Whole body protein turnover in diabetic rats

Rates of protein synthesis: The 17% decrease in whole body protein synthesis observed in diabetic rats (Table 8-3 and Section 5) was not as pronounced as the 60-70% decrease measured in fractional synthesis rates in skeletal muscle (Tables 8-2 and 8-3) of rats with similar periods of duration of diabetes and severity of hyperglycaemia. Since whole body protein synthesis in the severely diabetic rat decreased only slightly, it may possibly be a further explanation why hardly any change could be detected in whole body protein synthesis in the diabetic patients who were not so severely ill.

Rates of protein breakdown: Reasons have already been discussed why rates of whole body protein breakdown could not be calculated in control rats and therefore by implication also in diabetic rats. This is particularly unfortunate since no other published information is available on in vivo whole body protein breakdown rates in the experimentally diabetic animal.

Rates of protein oxidation: The highly elevated rates of oxidation measured in diabetic rats with either normal or increased food intake confirm the many observations by Buse and her colleagues of increased

oxidation rates in incubated diaphragms, retina or sciatic nerves from diabetic rats (Buse et al., 1976a & b, Frayser and Buse, 1978). The only other published data on whole body absolute oxidation rates in abnormal nutritional states are that of Sketcher (1976), who demonstrated decreased oxidation rates in protein depleted rats. Without measurement of the specific activity of the precursor pool, Meikle & Klain (1972) could show only an increase in proportional oxidation rate after starvation, i.e. as the amount of $^{14}\text{CO}_2$ excreted at plateau as a % of the infused isotope.

In the present study the increase in whole body rate of leucine oxidation was observed in diabetic rats whose intake of food was similar to that of controls. This would suggest that leucine was being mobilized from other tissues and hence a change in balance of synthesis and breakdown could have taken place in the diabetic state. This possibility will be analysed in the next section.

8.2.3. Tissue protein turnover in diabetes

Skeletal muscle: The decrease in fractional synthesis rate of the gastrocnemius muscle after 4 to 5 days of diabetes was similar when measured by either constant infusion or a large amount of labelled amino acid and is also comparable with the published in vivo work of Pain & Garlick (1974) and Millward et al. (1976) as shown in Table 8-2. The skeletal muscles appear to differ in their response to insulin deficiency (Table 8-3). Those with more white fibres such as the gastrocnemius and EDL were much more sensitive to the lack of insulin. They demonstrated a very marked decrease of 60 to 70% in both fractional and absolute rates of protein synthesis. In contrast, muscles with more red fibres such as the diaphragm and soleus responded less with a 33 to 47% decrease in rates of synthesis. During the course of this work, this relative resistance to insulin deficiency by muscles with a

greater proportion of red fibres was also reported by Flaim & Jefferson (1979). In their perfusion study, they showed that in diabetic muscle preparations the soleus with more red fibres was less susceptible to the development of the initiation block to peptide-chain synthesis which develops quite rapidly in psoas and gastrocnemius muscles which contain more white or mixed fibres.

The low in vivo rates of synthesis seen in all skeletal muscles from diabetic rats are consistent with in vitro data of Buse et al. (1976a) with incubated diaphragms and of Jefferson et al. (1972) with perfused psoas muscle of diabetic rats.

The increase in fractional breakdown rates measure in vivo in skeletal muscle after 4 days of diabetes has not been reported before. This steady and progressive elevation of protein breakdown was demonstrated in the gastrocnemius, diaphragm and heart muscles (Figures 7-7, 7-8 and 7-9).

This increase in rates of protein breakdown with diabetes does not confirm the data of Millward et al. (1976). They also used the method of indirect measurement of breakdown with constant infusion of [14 C]tyrosine to determine synthesis. They reported that both synthesis and breakdown were decreased in diabetes. The discrepancy between these two reports may be due to differences in measurement of growth rates of protein mass and of experimental diabetic models used. In the study of Millward et al. (1976), protein synthesis was measured 6 days after the rats were injected with streptozotocin, at which time their plasma insulin levels were however not very low (8 μ U/ml). In the present study, protein breakdown rates were determined in insulin withdrawn diabetic rats which were severely hyperglycaemic. Furthermore, Millward and co-workers calculated the mean growth in protein mass from values of protein content obtained only on day 0 and day 6

(Millward, personal communication). It is apparent from the figures in Section 7 that protein mass does not decline in a linear fashion over this period. In fact, there was a considerable lag period of 24 to 48 hours in all muscles before a drop in protein mass could be measured. In the present study protein mass was determined on each day before and after the measurement of protein synthesis to obtain a more accurate estimate of the change in rate of growth of protein mass with diabetes. This lag period followed by a decline in protein mass of skeletal muscle was observed not only in the actual study, but also in the pilot study.

The increase in breakdown rates measured in vivo in this study does confirm the in vitro results of various workers, who have shown increased proteolysis in hemi-corpus preparations of normal rats if perfused in the absence of insulin (Jefferson et al., 1974, 1977; Ruderman & Berger, 1974).

Cardiac muscle: The approximately 40% decrease in in vivo fractional synthesis rate as measured by the two methods (Table 8-2) resulting from diabetes is similar to the changes observed by Pain & Garlick (1974) in vivo and the recent perfusion studies of Chua et al. (1979). This decrease does not substantiate the suggestion of Rannels et al. (1970) that rates of protein synthesis in perfused diabetic cardiac muscle is not affected by insulin deficiency, since free fatty acids can substitute for insulin to prevent development of a block in peptide-chain initiation. Certainly the results in this study do suggest that the heart muscle, which contains predominantly red fibres, is like the soleus and diaphragm, less susceptible to the effects of diabetes.

As demonstrated in skeletal muscle, the in vivo rate of protein breakdown in cardiac muscle of diabetic rats progressively increased as insulin was withdrawn (Figure 7-8). Similar increases in protein breakdown have been observed in perfusion of diabetic cardiac muscle

(Chua et al., 1979) or if insulin was absent from the perfusing medium (Rannels et al., 1975).

Kidney: Hypertrophy of diabetic kidneys, as demonstrated by increased protein mass and total weight, was not associated with enhanced fractional synthesis rates as measured by either constant infusion or a single large amount of amino acid (Tables 8-2 and 8-3). However, since absolute rates of synthesis are based on the total amount of protein present, a slight increase in the absolute rate of synthesis was observed after 4 days of diabetes and an increase of 13% in the contribution of kidney protein synthesis as a % of whole body. Data on in vivo synthesis rates in diabetic kidneys have not been presented before. Protein breakdown rates were depressed on only the first day of insulin withdrawal, thereafter there was little difference between breakdown rates in insulin-treated or insulin-withdrawn groups (Figure 7-10).

Liver: With the constant infusion technique which measures only non-secretory or cellular proteins, not much of an effect could be demonstrated in fractional synthesis rate in diabetic livers as shown previously by Hay & Waterlow (1967) and Pain & Garlick (1974), (Table 8-2). With the large dose of labelled amino acid and measurement of incorporation over 10 minutes, there was no doubt that diabetes did in fact decrease total hepatic protein synthesis (Table 8-3) as observed previously by McNurlan & Garlick (1980a). Similarly the rate of albumin synthesis was also decreased after 4 days of diabetes as demonstrated by Pain et al. (1978b) and Peavy et al. (1978). However, the decrease in total liver protein synthesis was not nearly as marked as was seen in skeletal muscle.

Results of perfusion studies have demonstrated that liver degradation is accelerated if insulin is absent from the perfusate (Mortimore & Mondon, 1970). In the present investigation breakdown

rates were only transiently elevated after one day of insulin withdrawal (Figure 7-11).

Contribution of tissues from diabetic rats to whole body protein turnover:

In Table 8-3 absolute rates of protein synthesis were calculated for each tissue measured and the % contribution of each tissue to protein synthesis of the whole body. As discussed in the preceding chapter and as illustrated in Table 8-3, the effect of diabetes was much more pronounced on a tissue level. In terms of absolute synthesis rates, the 1.03 g protein/day contributed by the tissues measured in this study in control rats declined to 0.62 g protein per day in diabetic rats, whereas total body protein synthesis dropped only slightly from a value of 3.9 in controls to 3.3 g protein/day in the diabetic rats. This would suggest that the tissues that were not measured in this study were much less affected by diabetes, e.g. protein synthesis of the small intestine has been reported to be unchanged in diabetic rats (McNurlan & Garlick, 1980a), and similarly, synthesis in lung as measured in vitro (Rannels et al., 1979). No information is available on the effect of diabetes on protein synthesis rates in tissues such as smooth muscle, brain, spleen, bone or skin. One can only speculate that protein synthesis in some of these tissues is either not changed by diabetes or might even be enhanced. The small decrease in whole body protein synthesis in diabetic rats could then possibly be explained by the large decrease in protein synthesis in skeletal muscle being balanced by a small decrease in liver, plus hardly any change in kidney, gut and lung, and possibly, in some of the other body tissues not measured here.

8.3 CONCLUSION

The changes in protein metabolism resulting from diabetes presented here on a whole body and on a tissue level would suggest that in experimentally diabetic rats, the negative N-balance of uncontrolled diabetes

is probably brought about by the combined effect of a decrease in the rates of protein synthesis and an elevation in rates of protein breakdown. Whereas these changes were not observed in the diabetic subjects investigated in the present study, the possibility of similar protein abnormalities being present in the severely ill diabetic patient cannot be excluded and therefore should merit equal attention in criteria for establishing good control in the insulin treated diabetic patient.

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