PROTEIN SYNTHESIS IN MAMMALIAN TISSUES WITH SPECIAL REFERENCE TO PRECURSOR POOLS.

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Abstract

1) The nature of the pool of free amino acids that act as immediate precursors for protein synthesis has been investigated under conditions *in vivo*.

2) Rats were infused with [U-^{14}C] glycine for up to 6 hours during which time the specific radioactivity of free glycine in plasma and tissue approached a constant value. Free serine also became labelled. The ratio of the specific radioactivities of serine and glycine in the total protein pool of liver, heart, diaphragm, gastrocnemius, kidney, brain and jejunum was compared with the ratio in the free amino acid pool of these tissues and also of plasma. In all cases the ratio in protein was closer to the ratio in the tissue free pool.

3) Infusion of L-[U^{14}C] serine resulted in labelling of free glycine. In almost every case the ratio of the specific radioactivity of glycine to serine in the tissue protein was closer to the ratio in the free amino acid pool of the tissue.

4) After infusion of [U-^{14}C] glycine, the relative specific radioactivities of serine and glycine in plasma albumin, liver ferritin and total liver protein were similar to the ratio in the liver free amino acids but differed from both arterial and hepatic portal plasma. However, the ratios in albumin, liver ferritin and total liver protein in themselves showed small but statistically significant differences.

5) Comparisons were made of the fractional synthesis rates of the tissue proteins from calculations which assumed either the tissue or the plasma free serine and glycine to be the precursor. These synthesis rates were also compared with others calculated after the infusion of L-[U^{14}C] tyrosine.

6) The results showed that the plasma amino acids were not utilised directly in protein synthesis but suggested that the precursor pool was an intracellular compartment. There were also indications that more than one intracellular pool may be involved in protein synthesis.

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" But I can do nothing unless I am in complete possession of the facts. Obviously you can't cook them unless you have them ".

W. Somerset Maugham : Cakes and Ales.

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SECTION 1 Introduction.

1.1. The Demonstration of Protein Turnover

Up to the early part of this century the dominant ideas on the metabolism of protein suggested that dietary proteins were much more active in metabolism than were proteins which formed part of the living structure of an animal (Munro, 1964; Neuberger & Richards, 1964). The concept that all proteins, both dietary and structural, participated in common metabolic exchanges was not considered until 1935 by Borsook and Keighley and was not generally accepted until 1942 as a result of the now classical experiments of Ratner, Rittenberg and Schoenheimer (Schoenheimer, 1942).

Schoenheimer and his colleagues were the first to apply the use of isotopes to the study of animal biochemistry. Their experiments involved feeding ^{15}N labelled amino acids to rats for a period of three days and then tracing their destination within the animal. Contrary to earlier belief, they found that the urine did not contain all, or even most, of the administered isotopes. In fact, in the case of ^{15}N leucine, only about 35% of the initial dose was excreted. By far the largest quantity of the retained label was traced to tissue protein. As the animals had not increased in body weight during the time of the experiment, growth of tissue mass seemed an unlikely cause for the extensive incorporation. A more reasonable explanation was that tissue proteins were not inert but in a continuous dynamic state, being constantly synthesised and degraded. This work of Schoenheimer and his colleagues illustrated some important qualitative concepts in the study of protein metabolism and has formed the foundation on which our present day understanding of the subject is based.

Since 1942 there have been great advances in our understanding of the mechanisms involved in protein metabolism, but much of this newly

acquired knowledge has been concerned with the molecular aspects of protein synthesis. Although questions still remain to be answered, the overall operation by which proteins are synthesised in a cell is now well documented (for a review see Lucas-Lenhard & Lipmann, 1971; Korner, 1964). In contrast, it is surprising to realise how little is known about the opposing process of protein breakdown (Schimke, 1970; Goldberg & Dice, 1974) and it is equally surprising to find that knowledge concerning the integration of these two processes at the cellular level of organisation is still very superficial. Because of these gaps in our information only limited progress has been made in appreciating the dynamic aspects and the control of protein metabolism. The extent of this progress is well represented by the remarks of McFarlane (1973):

"Since the work of Schoenheimer, it has taken more pedestrian workers practically a lifetime to try to introduce quantitative aspects into the subject of protein turnover. In particular, it has taken us nearly 30 years to demonstrate, even in one instance, and by independent isotopic techniques, the truth of a simple corollary of turnover, namely that in an adult animal in nutritional equilibrium the absolute amount of a substance synthesised in a given time must equal the absolute amount catabolised. I wonder how long it will take us to measure, in patients, the difference between these two rates."

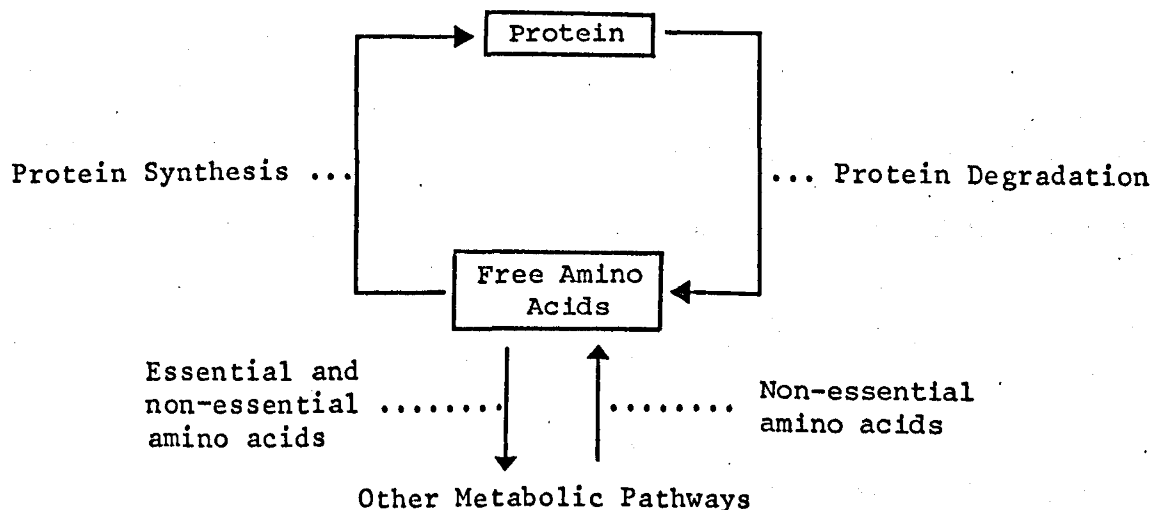
Much of what we know today concerning the rates of protein synthesis and degradation is based on several important assumptions. The validity of some of these assumptions is in question and it is these doubts that impede most of the progress in this field.

1.2. The Concept of Protein Turnover

It is commonly accepted that living systems are in a continuous dynamic state at all biological levels of organisation from whole organisms to populations of molecules within cells (Waelsch, 1962)

and that proteins are no exception to this (Goldberg & Dice, 1974). The reason why an organism or cell should continuously synthesise and degrade protein is at first sight difficult to appreciate, because "turnover" appears to be a futile operation, but it has been pointed out (Schimke, 1970; Goldberg & Dice, 1974) that it can serve at least three important functions. Turnover can aid in the removal of abnormal proteins resulting from mutations or transcriptional errors; it may increase the ability to adapt to changing environment and it may also give an organism the capability of utilising its own protein resources for energy or glucose production during periods of nutritional stress.

The components of protein turnover (metabolism) as it is understood today, can be illustrated by a very simple model (Halvorson, 1962).



Although simple, this model illustrates several of the main features and concepts. For instance, it is implicit from the above model that free amino acids are intimately involved in protein metabolism as they are the immediate substrate for synthesis and the final product of degradation (Simpson & Velick, 1954; Askonas et al., 1955; Loftfield & Harris, 1956; Schimke, 1970). It is also implicit that synthesis and degradation are two opposing processes which act on a common pool of protein. The size of this pool is therefore controlled by the relative

rates of these processes. When both are occurring at the same rate the size of the protein pool will remain constant. Under these conditions, protein metabolism is said to be in a "steady state". When the rate of synthesis differs from that of degradation, the mass of protein will not remain stable but will change by an amount reflecting the difference in the rates of these two processes. Thus, if more protein is being synthesised than degraded, the size of the protein pool will increase, and <u>vice versa</u>. In these situations protein metabolism is said to be in a "non-steady state".

In addition, the model shows that of the three components of protein turnover, i.e. the rate of synthesis, the rate of degradation, and the size of the protein pool, only two are independently variable. Consequently, to describe any change in the rate of turnover, only two of these parameters need to be measured.

Methods for measuring protein concentration are numerous and well documented (see Henry, 1966) and although the accuracy of many of these methods, in absolute terms, is dependent on the nature of the protein standard used for calibration, in relative terms they give very reliable results. Unfortunately, the same cannot be said for the methods of measuring the rates of either degradation or synthesis. In both cases practical and theoretical complications make it difficult not only to judge the accuracy of the results but also to measure both processes similtaneously in the same experimental animal. These problems are outlined and discussed below.

1.3. Methods for Measuring the Rate of Protein Breakdown

It is generally supposed that the kinetics of protein catabolism follow the principles of first order kinetics (Koch, 1962; Schimke, 1970; Tavill, 1972) implying that it is a random process in which all protein molecules carry the same susceptibility to degradation

irrespective of age. This is not true, however, for all proteins. Some, such as haemoglobin, exhibit "life span" kinetics (London, 1950) which are characterised by an age dependancy for degradation. In the case of haemoglobin, each molecule is maintained within the envelope of the red cell for 70-100 days before degradation occurs. It has also been suggested that muscle myofibrillar protein (Dreyfus, Kruh & Schapira, 1960) and rhodopsin (Hall, Bok & Bacharach, 1969) may behave in a similar manner.

Methods for measuring the rate of protein breakdown are, in general, based on the assumption that the decay of either biological activity or radioactivity from a population of protein molecules is exponential and that a semilogarithmic plot of total activity remaining against time will produce a straight line. The gradient of this line will give the fractional rate of breakdown.

There are two methods by which rates of protein degradation can be measured. The first is a non-isotopic method which is based on the rate of change in biological (enzymatic) activity of a single protein from one steady state to another. The changes in steady state levels can be brought about in three ways. In the first, the pool size of protein can be increased by the presence of a specific inducing agent; the rate of decay subsequent to the removal of the inducing agent is indicative of the rate of breakdown. This method was first used by Feigelson, Dashman and Margolis (1959) to determine the turnover rate of tryptophan pyrrolase. In the second case, protein synthesis is suppressed by inhibitors such as cyclohexamide or puromycin. This effectively increases the rate of degradation so that the protein pool decreases to a new level. The technique has been used to calculate the turnover rate of δ-amino levulinic acid synthetase (Marver _et al._, 1966). The last method relies on irreversibly inhibiting the activity of an enzyme and

and measuring the rate at which new enzyme is synthesised. Though simple, this method is limited in its use because it is confined only to enzymes. It is also limited by the availability of suitable inhibitors. The technique has been applied to the study of catalase turnover in rat liver by Price et al., (1962) and to the turnover of plasma cholinesterase (Neitleich, 1966) and brain monoamine oxidase (Barondes, 1966).

Although measuring the rate of decay of biological activity has the advantage of not requiring any prior isolation of the protein in question, it does depend on several assumptions. Among these are: 1) that the decay of activity represents the actual degradation of the enzyme molecule; 2) in the case of irreversible inhibition the return of activity represents synthesis of new protein rather than the return of activity to existing protein; 3) that the rate of degradation during the abnormal conditions of induction or inhibition is not different from that found in a normal physiological state; 4) that inducing agents can be removed abruptly; and 5) that any agent used to alter the rate of synthesis does not simultaneously affect the rate of degradation.

The alternative approach to measuring the rate of degradation involves the use of radio-isotopes. The protein pool of a tissue is initially labelled with a radioactive precursor, normally an amino acid, and the subsequent rate of isotope decay is used to calculate the rate of protein breakdown. This method overcomes the criticisms levelled at the previous technique, since it can be used under normal physiological conditions and no assumptions are made about the relationship of biological activity and protein concentration. However, it does require other equally important assumptions which can have a significant bearing on the results. The most important of these concerns reutilisation. After a pulse label, the decrease in total radioactivity in protein is assumed to depend only on the rate at which these proteins are degraded. If

labelled amino acids liberated through proteolysis are reincorporated, then the decay of total radioactivity will also be a function of the rate at which these free amino acids enter the pool from which proteins are synthesised. Thus, if reutilisation occurs, the observed rate of decay will be less than the true rate; the greater the reutilisation, the greater will be the difference. The overall effect will be to underestimate the actual rate of protein breakdown and therefore to prolong the apparent half-life of the protein. In principle, two forms of reutilisation can be distinguished. The first has been termed 'internal reutilisation' and represents the reincorporation of an amino acid, previously released from protein by degradation, within the same cell or tissue. The second is 'external reutilisation' and refers to the exchange of amino acids that have been released by protein degradation between different tissues. Thus radioactivity originally present in the protein or free amino acid pool of one tissue can be incorporated into the protein pool of another tissue. Both forms of recycling can be significant and there are several examples in the literature illustrating the magnitude of the errors due to the reutilisation of label. For example, Stephen and Waterlow (1966) have shown that reutilisation can cause a two- to five-fold increase in the apparent half-life of rat serum and liver protein. Similar increases due to reutilisation have also been reported by Arias, Doyle and Schimke (1969) and by Johnson and Velick (1972).

The problems of internal recycling can be overcome to some extent by using a specifically labelled amino acid. An example is the guanidine carbon of arginine (Swick & Handa, 1956). Because of the rapid turnover of the minute free arginine pool of the liver and the presence of the urea cycle enzymes, guanidino-^{14}C labelled arginine liberated from protein is immediately broken down to form ^{14}C urea and ^{12}C ornithine.

The opportunity of recycling of ^{14}C is therefore minimal. However, initially labelling the protein pool of the liver with ^{14}C labelled guanidino arginine can still give rise to appreciable external re-cycling (Swick & Ip, 1974) because the injected label is also taken up by other tissues, especially muscle, which have no arginase activity. Labelled arginine stored in these tissues can subsequently pass to the liver and be incorporated into protein. The effect of external re-cycling in the liver can be reduced if labelling of the guanidino carbon of arginine is achieved by a cellular metabolic sequence. It is fortunate in this instance that the liver alone is capable of fixing CO_2 into the guanidino group of arginine through the action of carbamyl synthetase and the enzymes of the urea cycle (Swick, 1958; McFarlane, 1963). Thus, when ^{14}C bicarbonate is injected into an animal it is only incorporated into free arginine within the liver.

The ability to fix $^{14}CO_2$ into other amino acids can also be used to minimise reutilisation in other tissues (as well as in liver). Injection of ^{14}C bicarbonate leads to appreciable incorporation of radio-activity into the carboxyl groups of aspartic and glutamic acid (Millward, 1970a). When these amino acids are released by protein degradation, they undergo rapid decarboxylation. The $^{14}CO_2$ released mixes with the large bicarbonate pool so that reincorporation is minimised. Millward (1970) has shown that the half life of muscle protein calculated from decay curves of these two amino acids was significantly lower than those calculated from other amino acids labelled with ^{14}C bicarbonate or from the injection of 6-^{14}C arginine. Sabri, Bone and Davison (1974) have also calculated that the half life of total brain protein in the rat is very much shorter when based on the decay of ^{14}C glutamate and ^{14}C aspartate (4 days) than on the decay of ^{14}C lysine (27 days).

Very recently a new approach circumventing the problem of reutilisation has been reported by Humphrey and Davies (1975). The method involves the use of tritiated water and is based on the principle that the exchange of hydrogen on the α-carbon of free amino acids is rapid as a result of high transaminase activity. In contrast, there is no exchange of this hydrogen after the amino acids have been incorporated into protein. If a pulse of tritiated water is given, protein becomes labelled and subsequently tritiated amino acids released by proteolysis tend to lose their radioactivity to the intracellular water. The authors applied this technique to measure the rates of protein turnover in plants <u>in vitro.</u> Their results show that leucine and tyrosine were most active in exchanging the hydrogen of the α-carbon atom.

Other reutilisable labels are known but the use of them is restricted to specific proteins. These include δ-aminolevulinic acid as a non-reutilisable label for measuring the rate of degradation of haemoprotein (Levin & Kuntzman, 1969). The major assumption in the use of these labels is that the prosthetic group does not dissociate from the apoprotein until the entire complex is degraded.

Even when methods have been used to minimise reutilisation, there is a second major problem associated with measuring the rate of protein degradation by decay of total degradation. This applies only to heterogeneous mixtures of proteins. When a semi-logarithmic plot relating decay of total radioactivity to time is constructed for a pure protein, it results in a straight line. The gradient of this line gives the fractional rate of degradation. When a similar plot is constructed for a heterogeneous mixture, it results in a curve. It is therefore impossible to obtain an accurate value for the gradient. Calculating the mean gradient from the curve is not satisfactory because the value obtained will vary with the duration of the experiment, becoming less as the

period of measurement becomes longer.

The problem can be met on theoretical grounds in two different ways (Garlick & Swick, 1975). Firstly, the heterogeneous mixture can be assumed to be divided into several components each with differing rates of turnover. The rate of decay of total radioactivity is determined by multi-exponential analysis. Secondly, a stochastic approach can be used in which no assumptions are made about the number or nature of individual protein components. The analysis is made by plotting the decay to infinite time on a linear graph, and then dividing the peak height of this curve by the area underneath it. For accuracy both methods require measurements to be made over a very long period (30-40 days) and are therefore not always practical.

A different approach and one which appears to have no reasonable explanation is based on empirical observations. If the initial point on a semilogarithmic plot of decay is joined to a point, on the same curve, corresponding to a time of between 3 and 4 half-lives (i.e. 10% of the initial radioactivity) the gradient of this line (the apparent degradation rate) coincides with the true mean degradation rate for the mixture of proteins (Garlick & Millward, 1972).

1.4. Methods for Measuring the Rate of Protein Synthesis

One method of measuring protein synthesis has much in common with that described for the measurement of degradation. When a pool of protein is radioactively labelled with a pulse dose, the decay of total radioactivity is a measure of the rate of degradation. The decay in specific radioactivity, however, is an indication of the rate of synthesis. If it is assumed that degradation is a random process, then after the initial pulse dose of radioactivity any amino acid newly incorporated into protein should ideally be unlabelled. Protein synthesis, therefore, will dilute the radioactivity already present in

the pool. When the rate of decay of specific radioactivity is plotted on a semilogarithmic graph, the gradient of the straight line will give the fractional rate of synthesis. However, this method is only applicable under ideal conditions; the disadvantages are identical with those which affect the measurement of degradation rates by decay of total radioactivity. Reutilisation and heterogeneity are both serious problems.

The second method, and the one most frequently used, measures the rate of incorporation of a radioactive precursor into protein. Although synthesis of a protein involves more than one step, it can be considered as a single reaction if all the synthetic intermediates rapidly reach the same specific radioactivity as the initial precursor. That this is valid is shown by the notable absence of a lag in incorporation of radioactivity seen in many studies (e.g. Halvorson & Cohen, 1958; Kipnis <u>et al.</u>, 1961) and from the fact that the half life of amino acyl transfer-RNA, the most obvious intermediate, is only between 1 and 3 seconds (Manchester, 1970; Airhart, Vidrich & Khairallah, 1974).

Differences in the rate of isotope incorporation need not reflect a difference in the rate of protein synthesis; therefore it is always necessary to relate the rate of incorporation to the actual quantity of protein synthesised. To do this the specific radioactivity of the precursor amino acids must be known, and this constitutes the major problem. It becomes much more difficult if the specific radioactivity of the precursor amino acid does not remain constant during the time over which the measurements of incorporation are made. For this reason, the methods by which the isotope is administered becomes an important consideration. In studies in whole animals there are essentially two ways in which the radioactive amino acid can be introduced; by a single pulse or by continuous infusion.

A good example of the changes in specific radioactivity resulting from a pulse dose is shown in the studies of Henriques, Henriques and

Neuberger (1955). They demonstrated that after a single injection of ^{14}C glycine the specific radioactivity of the free amino acid in plasma was initially very high but soon decreased very rapidly until after 6 hours it was only 1% of the value at 5 minutes. A similar pattern was also seen in the liver, but in this case the maximum labelling did not occur until 15-20 minutes after the initial pulse. In gastrocnemius the peak labelling occurred between 2-3 hours after the injection but remained relatively constant thereafter.

The changes in plasma and liver illustrate some of the difficulties in making allowances for the changing specific radioactivity in the pool of free amino acids and therefore, presumably, in the precursors for protein synthesis. Moreover, these changes cannot be assumed to be identical in all animals (Henriques, Henriques & Neuberger, 1955) nor equal under different conditions such as differing nutritional status of the animals (Haider & Tarver, 1969).

One way in which such rapid changes can be suppressed is to include, within the pulse, a large quantity of unlabelled amino acid. This results in more or less constant specific radioactivity for a sufficient time to enable the rate of incorporation to be measured (Henshaw, Hirsch, Morton & Hiatt, 1971).

Although this simplifies calculation of the synthesis rate, the use of substrate rather than tracer amounts of amino acids has the potential of producing abnormal metabolic changes.

The alternative method of administration, and one which is also capable of stabilising the specific radioactivity of the free amino acid, is the continuous infusion of isotope. This method was first used by Loftfield and Harris (1956). When an amino acid is continuously infused into an animal its specific radioactivity in plasma and in tissues rises to a constant level (plateau) which can be maintained for

many hours (Loftfield & Harris, 1956; Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick, 1969). If measurements of incorporation are made after equilibration has been established then there should be no variation in the precursor pool. Even if measurements are made before a plateau has been reached it is possible to make corrections for this because the initial rise in specific radioactivity can be expressed by a simple exponential (Waterlow & Stephen, 1968) without too much inaccuracy.

The use of the constant infusion method has emphasised the most important problem in measuring the rate of protein synthesis by isotopic incorporation. It has been shown by a number of people that although the specific radioactivity reaches a plateau in the plasma and in the tissues, the levels of the plateau in individual tissues are never the same. For instance, the plateau specific radioactivity of the free amino acid pool of liver is approximately 50% of that in the plasma (Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick, Millward & James, 1973) and in the case of brain it can be as low as 7% (Seta, Sansur & Lajtha, 1973). Consequently there is strong evidence for a considerable degree of compartmentation of the body's free amino acid pool. This illustrates the importance of knowing the exact location of the precursor pool from which proteins are synthesised. Without this knowledge the calculation of absolute rates of synthesis can carry appreciable margins for error.

Conclusion

The preceeding pages have briefly outlined the methods and problems of measuring the kinetics of protein turnover *in vivo*. Because techniques for measuring the rate of synthesis differ in many respects from those for measuring the rate of degradation, it is not possible to study both processes in the same animal or over the same time period. Therefore it

is a matter of choice which of the methods to use. This will nearly always be governed by the particular circumstances of the experiment. The factors influencing this choice are summarised below.

Measurements of decay rates have three significant advantages over measurements of incorporation. 1) It is possible to distinguish between "random" and "life-time" kinetics. 2) Rates of both synthesis and breakdown can usually be calculated from the experimental data. 3) The method avoids the problem of defining the pool of free amino acids which serve as precursors for protein synthesis.

There are several disadvantages, however. 1) Measurements have to be made over a much longer period of time compared with those for incorporation. Thus it is impossible to detect short term changes in turnover. 2) Re-utilisation is a major problem, which, although can be minimised in several instances, cannot be completely eliminated. 3) Perhaps the most important drawback is the difficulty encountered with heterogeneity. This is of course avoided with pure proteins, but it is sometimes of interest to study the collective response of all the proteins comprising a tissue. Under these conditions the interpretation of decay measurements becomes problematical. 4) In order to define accurately the shape of the decay curve several animals have to be used. The rate of degradation calculated from this curve is therefore the mean rate for the whole group of animals. When synthesis is measured by incorporation (continuous infusion) a rate can be calculated for each individual animal. The use of large numbers of animals in decay studies also results in a reduction of experimental precision, through biological variation between animals, and in an increase in experimental cost.

Many of these problems can be avoided by measuring the incorporation of radioactivity especially when one is dealing with short term changes in turnover, since in nutritional or hormonal studies there may be

rapid changes in protein metabolism. However, until we are able to define the specific radioactivity of the precursor amino acids, measurements of the rate of protein synthesis by incorporation must still be considered with some reservation. In spite of much research into this problem it is still not clear whether amino acids are incorporated after prior equilibration with a homogeneous intracellular pool, from a discrete intracellular pool or directly from an extracellular or intramembrane source. It is the purpose of this study to reinvestigate this question with particular referrence to protein synthesis under conditions *in vivo*.

1.5 Review of Studies on Precursor Compartmentation.

At the beginning of the 1950's, at a time when there was relatively little understanding of the mechanism of protein synthesis, there was much controversy over the nature of the immediate precursors for the biosynthesis of protein. The controversy was round the question whether proteins could be synthesised from pre-existing polypeptides or whether the free amino acids were necessary intermediates. It had arisen as a result of several studies which claimed to show that labelled protein or peptides were incorporated more rapidly into new protein than similarly labelled free amino acids. An example of such a study was that of Babson and Winnick (1954).

The question was finally resolved by several experiments, three of which are of particular importance.

By giving a mixture of five ^{14}C labelled amino acids and comparing their specific radioactivities in two different proteins of rabbit muscle, Simpson and Velick (1954) were able to show that the specific radioactivities of all five amino acids was consistently 1.8 times higher in muscle aldolase than in the second protein, glyceraldehyde-3-phosphate dehydrogenase. As these two proteins were known to have different amino acid compositions, Simpson and Velick concluded that both were synthesised from the same precursor pool of free amino acids. If incorporation of pre-existing polypeptides had occurred, or if free amino acids had exchanged with those bound in protein, by a process other than de novo synthesis, the specific radioactivities of the injected amino acids would not have been constantly related in the two proteins.

In 1955 Askonas et al. studied the synthesis of casein and β-lactoglobulin

in lactating goats. The technique involved a single injection of a mixture of ^{14}C amino acids into the animal and at various time intervals isolating these proteins from the milk. Each protein was subjected to partial hydrolysis. The results showed that the specific radioactivity of a given amino acid was the same in any peptide fragment from one single protein, indicating that each fragment had been synthesised from the same free amino acid pool.

In 1956 Loftfield and Harris used a completely different experimental procedure involving the induction of liver ferritin. By continuously infusing rats with ^{14}C leucine, isoleucine and valine via the femoral vein they were able to maintain a constant specific radioactivity in the free amino acid pool of the liver. The synthesis of ferritin was induced at the start of the infusion by the injection of iron, and samples of the protein were taken throughout the experiment. The results showed that the specific radioactivity of the branched chain amino acids in ferritin was very similar to that in the free amino acid pool of the liver. Their conclusions were in agreement with those of Simpson and Velick and of Askonas et al.

Subsequently the mechanisms of protein synthesis were better understood. The role of transfer RNAs and their specificity for single amino acids (see reviews by Simpson, 1962 and Novelli, 1967) added weight to the arguments that the free amino acids and not peptides were the precursors of proteins.

Today, some twenty years later, a different controversy exists which questions not the nature of the amino acids involved in protein synthesis, but the origin of them and their precise cellular location. The controversy is by no means new and has arisen as a result of the

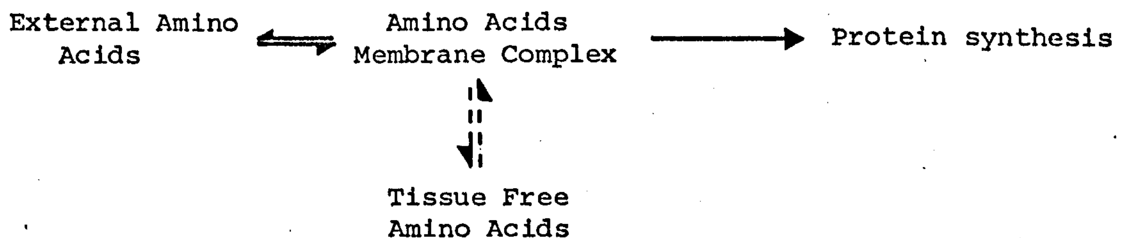
increasing use of radioactive isotopes for measuring rates of protein synthesis. The following account is a review of the relevant experiments and illustrates the differences in opinion regarding the site at which amino acids are utilised for protein synthesis. As the experimental systems used in the attempt to understand this problem have been so diverse, the review is divided into five main categories: single cell systems; plant tissues; isolated animal tissues incubated in vitro; perfused organs; and in vivo studies.

Single Cell Systems

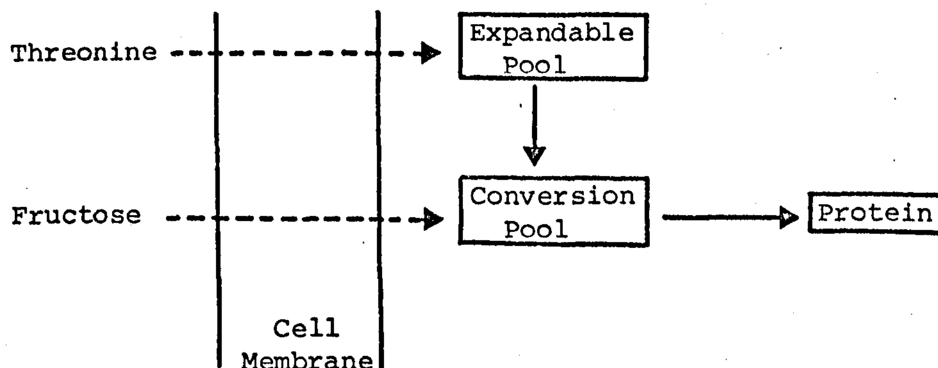
Populations of individual cells are the simplest of all systems used for studies of precursor-protein relationships. Their simplicity arises from the fact they are essentially composed of two pools, the intracellular pool and the medium. In multicellular systems an additional pool occurs as an intermediate between the medium and the intracellular water through which amino acids are obliged to pass before cellular transport can occur. The presence of this third pool in multicellular systems imposes additional difficulties in interpreting the experimental results. It is for this reason and, perhaps, also because of the practical ease with which experimental procedures can be carried out, that single cell systems have been extensively used in this field.

One of the earliest works dealing with the problems of locating the precursor pool was that of Halvorson and Cohen (1958), who investigated the incorporation of ^{14}C phenylalanine into protein of yeast cells. They observed that the rate of incorporation into protein was always linear, with no initial lag period. In contrast, the amount of isotope in the intracellular pool was not constant but increased rapidly with time. They argued that the total intracellular pool of free amino acids could

not be the precursor, since the rate of ^{14}C phenylalanine incorporation into protein would increase as the level of isotope in the intracellular pool increased. In a subsequent experiment they showed that when valine was added to the medium (10^{-2}M) the rate of incorporation of ^{14}C phenylalanine was only minimally affected while its uptake into the intracellular pool was very much decreased. This led them to propose a model in which amino acids destined for protein synthesis could originate directly from the medium and be incorporated into protein without first equilibrating with the intracellular pool.



In 1959 Cowie and McLure, working with Candida utilis, concluded that in this organism the intracellular pool was compartmented into two functionally distinct amino acid pools: a concentrating pool (expandable) and a conversion pool. The function of the expandable pool was to accumulate exogenous amino acids and consequently was only apparent when exogenous amino acids were present. Its physical size was reported to be related to the concentration of amino acid in the medium and it also exhibited sensitivity to osmotic shock. Another function of the expandable pool was to supply amino acids to the conversion pool which served as the site of de novo synthesis of amino acids from other carbon sources. The conversion pool differed from the expandable pool by having a constant size, in being unable to exchange with amino acids in the medium and by being insensitive to osmotic shock. Cowie and McLure suggested that it was this pool that provided precursor amino acids for protein synthesis.



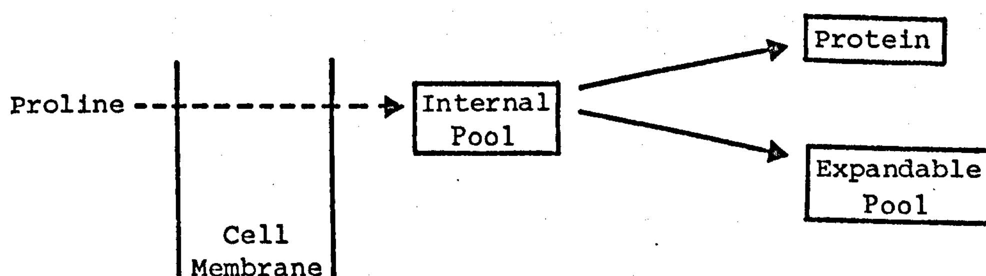
Much of this evidence was based on the differential incorporation of radioactivity into protein from ^{14}C threonine, ^{14}C arginine and ^{14}C fructose.

A very similar model was proposed by Klempner and Cowie (1960) to explain the differential incorporation of phenylalanine and its analogue, para-fluorophenylalanine into protein of E.coli and Candida utilis. The model differed slightly in that a third pool was defined, the internal pool which in itself acted as the precursor pool. This internal pool was supplied with amino acids from both the conversion pool and the expandable pool. The preferential incorporation of intracellular amino acids by E.coli has subsequently been reported by Britten and Mclure (1962) in studies using proline and valine and by Sercarz and Gorini (1964) investigating the contribution of exogenous and endogenous arginine to repressor formation in a super derepressed mutant of E.coli (strain BC28). The latter workers concluded that while exogenous arginine influenced the formation of repressor of arginine synthesis, the endogenous arginine was utilised for protein synthesis.

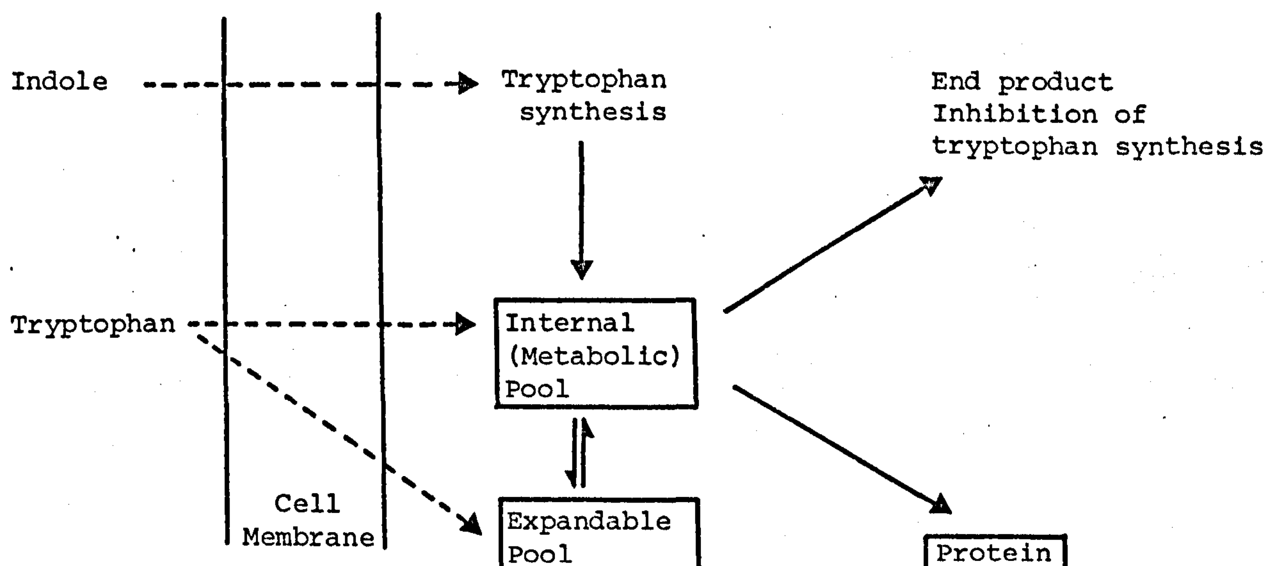
The involvement of the intracellular pool in protein synthesis has also been shown for the slime mould Dictyostelium discoideum, by Wright

and Anderson (1960). The main indication for this conclusion arose from the finding that high exogenous concentrations of methionine failed to inhibit the incorporation of pre-accumulated ^{35}S methionine.

Studies in Neurospora crassa (Zalokar, 1961; Matchett & Demoss, 1964; Brooks & DeBusk, 1973; Weiss, 1973) have all suggested compartmentation of the intracellular amino acids. Zalokar showed an initial lag in the incorporation of proline into protein which he explained was due to the presence of an intermediate pool of proline in the cell. This pool he termed the internal pool. He also postulated the existence of a second pool, an expandable pool, on the basis that the free proline pool of myceli was greater than the pool necessary to account for the lag in protein synthesis. The model put forward to explain the experimental findings indicated that exogenous proline enters the internal pool and is taken up into protein from here. Any exogenous proline not incorporated into protein is diverted into the expandable pool.



Matchett and Demoss studied the incorporation of tryptophan and indole into protein and suggested a model compatible with that of Zalokar and Cowie & Mclure.



Brooks and DeBusk suggested a three compartment model: a small endogenous pool of constant size; an expandable pool related to the exogenous amino acids; and a protein synthetic pool which was preferentially, but not exclusively, supplied with endogenous amino acids. Their conclusion, based on the differences in uptake and incorporation of phenylalanine and its analogue, p-fluorophenylalanine, in tyr-1 mutants, resembled that reported for <u>E.coli</u> and <u>Candida utilis</u> (Klempner & Cowie, 1960).

Weiss (1973) concluded that intracellular arginine and ornithine were contained in at least two distinct compartments. The first, which accounted for 90% of the tissue content of these amino acids, was attributable to a vesicular organelle. The remainder was present in the cytoplasm, and it was from this compartment that arginine was incorporated into protein. Weiss proposed that the vesicles sequestered arginine and ornithine in order to regulate the cellular metabolism of these amino acids.

One of the first attempts to investigate compartmentation in mammalian cells was that of Kipnis, Reiss and Helmreich (1961). Using isolated guinea pig and rabbit lymph node cells, they provided evidence which indicated heterogeneity of the intracellular pool. Like Halvorson and Cohen (1958)

their conclusion was based on the ability to show that apart from an initial lag, incorporation of ^{14}C leucine and ^{14}C glycine into protein was always linear with respect to time, whereas the radioactivity of the free amino acid pool of the cells rose exponentially. They speculated that the heterogeneity was achieved either by structural compartmentation within cellular organelles or simply by a concentration gradient without any structural partitions. In the latter case, they envisaged that the protein synthetic machinery occurred close to the site at which amino acids were transported into the cell and consequently the total free intracellular pool could not be the precursor pool for protein synthesis.

Pichler (1965) also concluded that incorporation of L-^{14}C glutamic acid into protein of mouse ascites tumour cells was independent of the total intracellular amino acid pool. The evidence for this conclusion was very similar to that of Kipnis _et al._, (1961) in that it illustrated both linearity and an absence of an initial lag in incorporation at a time when the intracellular glutamate pool was still increasing its radioactivity content. In addition, Pichler showed that the rate of $^{14}CO_2$ production did exhibit a significant lag before it becomes constant, indicating that this process was related to the intracellular glutamate pool.

Roscoe, Eaton and Chin Choy (1968) investigated cellular uptake and the incorporation of ^{14}C leucine and ^{14}C lysine into protein by Krebs 2 ascites cells and the inhibition of these processes by D- and L-phenylalanine. Their results showed no direct relationship between the total acid soluble pool and the rate of protein synthesis.

Berg (1968) proposed a model in which the source of amino acids destined for synthesis of protein was dependent on the exogenous concentrations of amino acids. Using ^{14}C valine, he studied its intracellular uptake

and incorporation into protein in various developmental stages of sea urchin. Uptake and incorporation rates both exhibited a non-linear relationship for different concentrations of exogenous valine but uptake was more responsive to the change. Berg interpreted his data as indicating that the intracellular amino acid pool was not an obligatory intermediate for the incorporation of exogenous valine but at low exogenous concentrations valine required for incorporation could be drawn partly from this pool. At high exogenous concentrations the intracellular pool participated very little in providing amino acids for incorporation as exogenous valine was incorporated preferentially. This same model was used by Winkler (1972) to explain his results on the incorporation and uptake of ^{3}H leucine by washed human leucocytes. Winkler proposed two intracellular pools, an expandable pool capable of rapid equilibration with the medium and an internal pool in much slower equilibration with the expandable pool. His results showed that extracellular amino acids are used for protein synthesis in preference to those in the internal pool but when the supply of extracellular amino acids was insufficient the internal pool supplemented the supply. Consequently he pointed out that interpretation of incorporation data could be significantly biased if the medium contained appreciable amounts of amino acids.

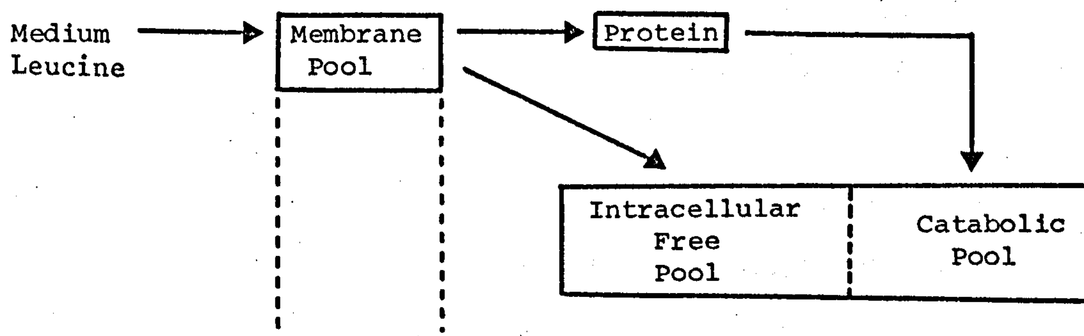
However, in 1970 Berg reported that his earlier interpretation (Berg, 1968) of ^{14}C valine incorporation into sea urchin embryo was untenable in the light of further investigations. He concluded that the total amino acids pool was an obligatory intermediate for incorporation of exogenous valine, and that his earlier work could be explained on the basis that the influx of ^{14}C valine rapidly expanded the intracellular pool, thereby raising its specific radioactivity almost to that of the external medium. Thus, his embryos appeared directly to incorporate exogenous amino acids. The suppression of incorporation of intracellular ^{14}C valine by the presence

of exogenous ^{12}C valine conversely reflected a decreasing specific radioactivity in the expandable pool due to an influx of the extracellular non-radioactive valine. Berg's reassessment of this data signifies an important aspect in the location of precursor amino acids. Many investigations have been concerned almost entirely with the kinetics of radioactivity alone, without relating them to actual specific radioactivities. As the rate of incorporation of radioactivity is intimately related to the specific radioactivity of the precursor pool and not to the concentration of radioactivity within it, measurements of the kinetics and concentration of radioactivity alone, can lead to false interpretations.

One study in which specific radioactivities were determined was that of Righetti, Little & Wolf (1971). Working with HeLa cells they attempted to demonstrate intracellular compartmentation by a technique earlier employed by Loftfield and Harris (1956). By comparing the specific radioactivity of lysine, leucine and phenylalanine in newly induced ferritin, they showed that although the specific radioactivity of lysine in ferritin was similar to that in the intracellular pool, the same was not true for leucine or phenylalanine. In the case of leucine the specific radioactivity in ferritin was approximately 30% less and with phenylalanine it was about 62% lower. As the relative specific radioactivities of the three amino acids were not similarly related in ferritin and in the intracellular pool they suggested that amino acids released from degradation were differentiately reutilised without having initially to mix with the total intracellular pool. Thus they implied that the precursor pool for synthesis was a discrete intracellular compartment.

In 1974 van Venrooij, Moonen & van Loon-Klaassen studied the source

of amino acids for protein synthesis in HeLa cells and concluded that the precursor amino acids were selected at a site on or near the plasma membrane in close conjunction with the amino acid transport system. They also suggested that amino acids released by protein degradation were not directly re-utilised under the conditions of the experiment but that reincorporation could only be brought about by the efflux and re-entry of these amino acids into the cell. The work was based on pre-labelling HeLa cell protein with ^{3}H leucine and subsequently incubating in ^{14}C leucine. Comparisons were then made of the $^{14}C/^{3}H$ ratio of the medium, the cellular pool and of leucyl t-RNA.



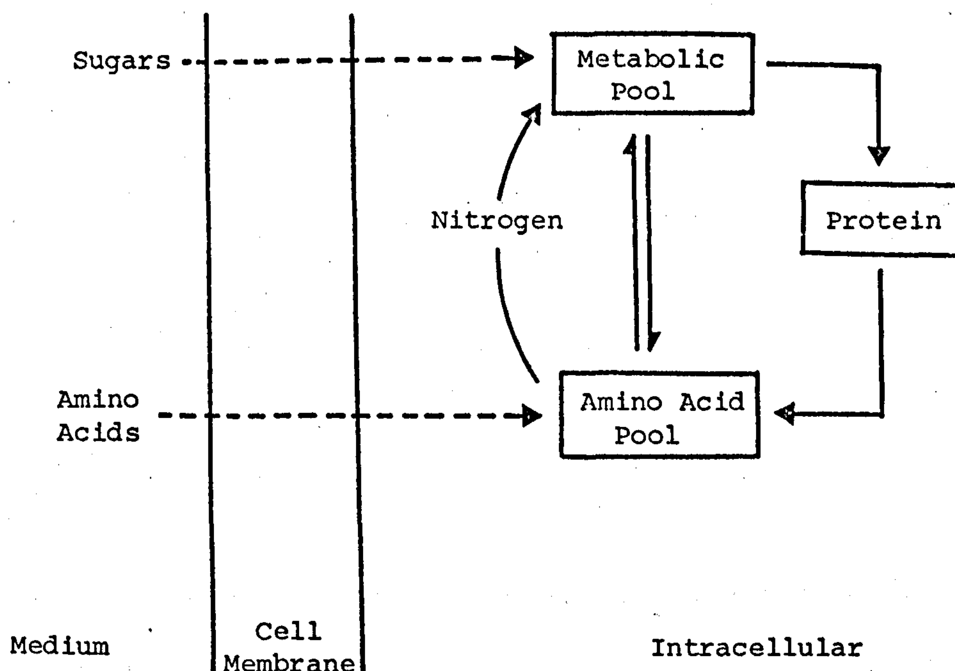
From the above description of the studies on single cell systems it is apparent that no clear understanding of the problem has emerged. Differences in the conclusions have probably arisen as a result of the large variations in the experimental conditions, especially in terms of the type of tissues studied and the nature of the amino acids used. The majority of investigations have favoured the incorporation from the tissue pool rather than the medium and many of these have suggested that this pool is further compartmented.

Higher Plant Tissues

Although plant tissues have seldom been used for investigating the problem of precursor pools in protein synthesis, two studies have examined the question in much detail. Because both anatomical and bio-chemical features of plants differ markedly in many instances from those of animals it is often supposed that little similarity exists between

the two forms with respect to protein metabolism. Nevertheless, it is important to realise the possibilities that exist in plants and to see how the demonstrated mechanisms of amino acid incorporation may relate to animal tissues.

The extensive studies of Bidwell, Barr & Steward (1964) have illustrated several points of importance. Working with explanted tissue from the storage root of carrot, they concluded that sugar from an exogenous source proved to be a more direct source of carbon for the amino acids in protein than either exogenously supplied or intracellular amino acids. It was also established that externally supplied amino acids tended to increase the use of ^{14}C carbon from sugar in protein synthesis, principally by providing a source of nitrogen. Any provision of carbon by these amino acids was thought to be of secondary importance. In addition they showed that in the case of glutamic acid, for instance, the α-carbon is largely decarboxylated and the remaining carbon atoms enter the metabolic pool to form acceptors for nitrogen before protein synthesis. The overall conclusion was that there are two pools of amino acids in the cell, one of which provides substrates for oxidation and is separate from the second pool, which provides amino acids for protein synthesis. Exchange of amino acids between these two pools is limited, with alanine being the most readily transported amino acid. Their proposed model is similar in many respects to that of Cowie and McClure (1959).



More recently Kemp and Sutton (1971) suggested that in tracer experiments incorporation of isotope is a function of the specific radioactivity of the _total_ soluble pool and that a simple differential equation could relate the specific radioactivity of this pool to measurements of the rate of protein synthesis. Their studies of ^{3}H leucine incorporation in tobacco callus _in vivo_ showed that although the rate of incorporation was inversely related to the size of the incubated tissue, so also was the specific radioactivity of the free leucine pool. Consequently, very similar rates of synthesis were obtained when the rate of incorporation was compared with the specific radioactivity of the free intracellular pool. Support for the involvement of the total intracellular pool came also from the finding that the calculated rate of protein synthesis in this tissue was very similar when either exogenous ^{3}H leucine or ^{14}C sucrose was the sole source of label. The ^{14}C sucrose in this system gave rise to ^{14}C labelled leucine.

In both the above studies intracellular amino acids were implicated as the precursor for protein synthesis although Bidwell, Barr & Steward (1964) proposed further compartmentation of the intracellular pool. In neither study was the extracellular fluid (medium) proposed as the precursor pool.

Incubated Mammalian Tissues *in vitro*

Many investigations involving the incubation of isolated tissues *in vitro* have strongly suggested compartmentation of the tissue free amino acids and several have further proposed that the intracellular pool is less closely related to the synthesis of protein than extracellular amino acids. However forceful some of these arguments may be, the *in vitro* incubation of isolated tissues is open to many serious artefacts that can give false impressions of the true state of compartmentation. Perhaps the most important criticism levelled against this type of work derives from doubt about the viability of the tissue preparation. As supply of nutrients and oxygen under these circumstances is solely accomplished by diffusion from the medium into the tissue it is likely that with large tissue masses cells in the centre are less able to remain viable than those at the surface. In terms of protein metabolism this suggests that cells in direct contact with the medium are likely to show higher rates both of incorporation of isotope and of protein synthesis than those in the interior. Support for this criticism has been given by Kemp and Sutton (1971) and van Venrooij, Poort, Kramer and Jansen (1972). One other objection to multicellular systems in general, which includes perfused organs and studies in the whole animal, is that no tissue is comprised of a homogeneous population of cells. If each cell type within a tissue exhibits different rates of amino acid transport and protein turnover, then the demonstration of different intracellular pools in the whole tissue does not necessarily imply

that more than one such pool exists in every cell. It is equally likely that each demonstrable pool is peculiar to one cell type.

Despite these objections, in vitro incubations have been valuable because they have exhibited compartmentation, and, even if these demonstrations have been the result of some misinterpretation, they have been responsible for much of the current interest in the cellular location of precursor amino acids.

The first important study in this field was that of Kipnis, Reiss and Helmreich (1961) who investigated the incorporation of ^{14}C proline in rat diaphragm. This work was done simultaneously with the work described earlier involving isolated guinea pig and rabbit lymph node cells. As before they showed that proline incorporation into diaphragm protein was linear with no measurable lag period, while the uptake of proline into the intracellular free pool followed a curvi-linear function. This they interpreted as indicating that the total intracellular pool was not involved in protein synthesis. They suggested that a small intracellular compartment in rapid equilibrium with the medium was responsible for the supply of amino acids for protein synthesis. A similar conclusion was reached by Rosenberg, Berman and Segal (1963) who suggested that in rat kidney cortex slices the total intracellular pool of glycine and lysine need not equilibrate with exogenous amino acids before linear rates of incorporation were achieved. In contrast their results on the kinetics of the appearance of $^{14}CO_2$ suggested that the oxidation of ^{14}C lysine was related to the specific radioactivity of the intracellular pool.

Heterogeneity of the intracellular pool was also suggested by Phang, Finerman, Singh, Rosenberg and Berman (1971) in foetal rat calvaria. By compartmentation analysis of collagen synthesis in this tissue they proposed that proline entering the cell must first pass through a pool

from which proteins are synthesised and that the size of this pool is related to the rate of proline transport. The model put forward also included an "equilibrated pool" which bore no relationship to the rate of proline influx into the cell. This pool derived its amino acids, in the steady state, only from the precursor pool.

In 1964 Guidotti, Ragnotti and Rossi, studying the incorporation of glycine into protein of rat liver slices, inferred that the intracellular pool was not homogeneous. Although their general conclusion was in agreement with the above studies, it is interesting that the evidence from which their conclusion was drawn differed significantly. While Kipnis et al., (1961) and Rosenberg et al., (1963) argued that an absence of a lag in the incorporation of radioactive amino acids into protein indicated intracellular compartmentation, Guidotti et al., argued that in their system the lag in incorporation of externally supplied label was of greater duration than would be predicted from the time taken for the total intracellular pool to equilibrate. Thus the precursor pool in this case was more slowly equilibrating than the total intracellular compartment.

A different approach to the problem was made by Hider, Fern and London (1969). In their experiments they first incubated isolated extensor digitorum longus muscle of small rats in medium containing either ^{14}C glycine or ^{14}C leucine. After 30 minutes the muscles were rapidly washed and some were then transferred to medium containing tritiated isotopes of these amino acids, and further incubated for 30 minutes. At the end the rates of uptake into the cells and of incorporation into protein were measured. Their results showed that during the first incubation ^{14}C incorporation was linear with the exception of a small initial lag in the incorporation of ^{14}C glycine. When the muscles were transferred to the tritiated medium, incorporation

of ^{14}C label stopped almost immediately in both cases although the intracellular pool still contained significant amounts of free ^{14}C leucine or ^{14}C glycine. Incorporation of label into protein during the second incubation followed essentially the same pattern as in the first incubation although now the amino acids were tritiated. This indicated two things; first, that the rate of protein synthesis did not change appreciably during the entire experimental period; second, and most importantly, that the extracellular amino acids appeared to be incorporated in preference to those in the intracellular pool. This led them to conclude that amino acids were incorporated directly from the extracellular pool. The model put forward was identical to that of Halvorson and Cohen (1958)

In a subsequent report Hider, Fern and London (1971a) looked at their system in more detail. Using a technique previously employed by Guidotti, Ragnotti and Rossi (1964) and Kostyo (1968) they were able to isolate glycine contained in the extracellular space of the muscle and measure and relate its specific radioactivity to the rate of isotope incorporation. The results showed that an extracellular amino acid pool could be defined, which was more closely related to protein synthesis than the intracellular pool. The results also showed that the defined extracellular pool contained leucine and glycine at higher chemical concentrations than were found in the medium.

Double isotope techniques were subsequently used in a number of studies, all of which concluded that extracellular amino acids were more intimately connected with the precursor pool than intracellular amino acids. For example, this conclusion was reached by Fern, Hider and London (1971) measuring leucine incorporation in rat jejunal segments, by Van Venrooij, Poort, Kramer and Jansen (1972) measuring leucine and lysine incorporation into rat pancreas fragments, by Van Venrooij, Kuijper-Lenstra and Kramer (1973) measuring leucine incorpora-

tion into rat submandibular gland, and by Adamson, Herrington and Bornstein (1972) measuring proline utilisation in embryonic chick cartilage. In all these studies it was shown that incorporation of one isotope into protein ceased immediately when the tissue was transferred to a second medium containing the same amino acid but a different isotopic label.

Adamson, Herrington and Bornstein (1972) also studied the effect of various agents, such as serum, ouabain and other amino acids known to modify proline uptake. From their data they suggested a model in which selection of amino acids for protein synthesis occurred while the amino acid was still in association with the transport system of the membrane. The model also envisaged that a proline molecule in the intracellular pool must become re-associated with a "membrane-complex" before incorporation could occur. This model agrees in principle with that put forward by Hendler (1962), who also suggested an intramembrane pool of precursor amino acids, and only differs from that of Phang _et al._ (1971) in the precise location of the precursor pool. Both Adamson _et al._ (1972) and Phang _et al._ have proposed that the total intracellular pool is not involved in protein synthesis.

The general emphasis from all the work on incubated tissues so far discussed has been the important contribution of the extracellular amino acids to protein synthesis, either directly or indirectly via a fast equilibrating intracellular pool. None of the studies have implied that the total intracellular pool is homogeneous, neither have they suggested that precursor amino acids originate from this pool. There are, however, two exceptions. In 1972 Alpers and Thier studied the accumulation of glycine by rat jejunal segments and its incorporation into protein. Their experimental data suggested that extracellular glycine was not the direct precursor for protein but that this

amino acid must first pass through some intracellular pool or pools. Their conclusion was based on two observations: first, that the addition of pyridoxal hydrochloride increased both cellular accumulation and incorporation into protein, thereby implying a relationship between the intracellular pool and protein synthesis; second, they demonstrated that when jejunal segments were initially incubated in ^{14}C glycine, incorporation was linear. On transferring the tissue to a medium containing only ^{12}C glycine incorporation of isotope did not cease immediately, as might have been expected from the data of Fern _et al._, (1971) but continued for at least a further 10 minutes. As they showed that the medium contained little radioactivity during the second incubation, they argued that further incorporation must have been at the expense of the intracellular pool.

In agreement, Li, Fulks and Goldberg (1973) also concluded, from studies of the incorporation of tyrosine into rat diaphragm protein, that the free intracellular tyrosine was the precursor for protein synthesis whereas the extracellular was not. In their experiments pieces of rat diaphragm were incubated in the same concentration of ^{3}H leucine but in different concentrations of ^{14}C tyrosine. The ^{3}H leucine was present to demonstrate a uniform rate of protein synthesis when the diaphragms were incubated in increasing concentrations of tyrosine. The results indicated that, although the incorporation rate of ^{14}C tyrosine increased as its chemical concentration was increased in the medium, the amount of tyrosine utilised for protein synthesis was constant when the incorporation rate was related to the intracellular specific radioactivity. Evidence for this conclusion was also provided by a "chase experiment" in which pieces of rat diaphragm were incubated initially in ^{3}H leucine and ^{14}C tyrosine and subsequently in ^{3}H leucine and ^{12}C tyrosine. Again, the amount of tyrosine utilised for protein

synthesis was constant when the rate of incorporation was related to the intracellular specific radioactivity during both incubations. The rate of ^{3}H leucine incorporation (c.p.m./mg protein) was constant throughout suggesting that the rate of protein synthesis did not change during the experiment.

The results of studies involving in vitro incubations of isolated tissues also do not agree on a satisfactory definition of a precursor pool, although many of the investigations have favoured the involvement of extracellular amino acids. As with the single cell systems, many different tissues and different amino acids have been used, together with varying sizes of tissue fragments and varying concentrations of amino acids in the incubation medium. For these reasons it is not altogether surprising that no consistant conclusions have emerged. However, it becomes clear that amino acid compartmentation is by no means simple or uniformly organised.

Perfused Organs

The use of perfused organs in this field is a relatively new approach. As a technique it has most of the advantages of in vivo methods and is able to overcome the major disadvantages accompanying incubations of isolated tissues in vitro. In particular, as the circulating system is intact metabolites and oxygen can penetrate the tissue much faster. Consequently there is less dispute about the viability of the preparation or the homogeneous distribution of the isotope. Nevertheless, the system is not ideal because although it mimics in vivo conditions to a considerable degree, it does not do so fully, with the result that often factors such as hormonal balances are disturbed. Despite these minor objections the technique is very useful and has made significant contributions to this problem.

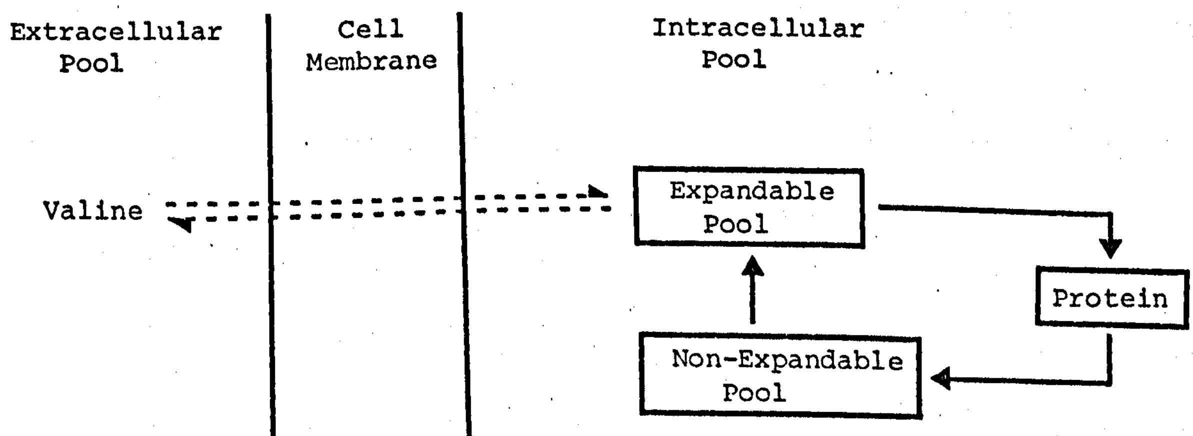
The most detailed study has been performed by Mortimore, Woodside and Henry (1972). Using a perfused liver system they initially

studied the relationship of the specific radioactivity of free intracellular valine to that of external (perfusate) valine by varying the concentration of this amino acid in the perfusate. At low concentrations (0.54-0.64 mM) they found that although the specific radioactivity of the intracellular pool equilibrated with the perfusate it only rose to half its value. On increasing the concentration of valine in the perfusate they were able to increase the specific radioactivity of the intracellular valine so that it became equal to that of the medium. This occurred at extracellular valine concentrations above 6 mM. During this experiment they also measured the incorporation of label into protein and calculated apparent rates of protein synthesis by assuming either the intracellular or extracellular valine to be the precursor. In neither case did the two assumed precursors give constant results except at extracellular valine concentrations above 5 mM. When the extracellular pool was assumed to be the precursor the calculated synthesis rate increased as the perfusate valine concentrations increased, and conversely, the rates calculated from the intracellular specific radioactivity fell as the perfusate valine levels were increased. At extracellular valine levels above 5 mM both pools gave very similar synthesis rates.

Assuming that the rate of valine incorporation was not influenced by variations in external valine concentration, Mortimore *et al.* explained the lack of constancy by proposing that intracellular valine was represented by two separate pools, an expandable pool which readily equilibrated with extracellular valine and a non-expandable pool which failed to equilibrate with label in the perfusate. They further suggested that the non-expandable pool was supplied with amino acids from protein degradation and that it emptied into the expandable compartment but without any significant equilibration occurring between the two. On the assumption that the expandable pool was the source of

precursor amino acids, the calculated rates of protein synthesis were uniform at all external valine concentrations. They further substantiated their model by measuring the rate of release of radioactivity from livers previously labelled with ^{14}C valine. When no valine was present in the perfusate the release of label was linear and this was taken to represent the rate of protein breakdown minus the rate of reincorporation (reutilisation) of label by protein synthesis. After 2 hours the perfusate was changed to contain 15 mM valine. They considered this concentration to be sufficient to prevent reincorporation of label released by proteolysis. Under the new conditions the rate of release of label increased to a new constant level which represented the absolute rate of protein breakdown. By subtracting the rate of release of label when the perfusate contained no valine from the rate when the perfusate contained 15 mM valine they derived the rate of reincorporation (i.e. the rate of synthesis). This they found to compare very favourably with the rate of synthesis calculated from the initial experiment.

Their overall conclusion therefore indicated that the precursor site was intracellular and that the specific radioactivity of this pool was higher than that of the total intracellular pool. The extent of the difference between the precursor pool and the total intracellular pool is governed by the relative sizes of the expandable and non-expandable compartments.



The involvement of the intracellular pool in protein synthesis was also investigated by Morgan, Earl, Broadus, Wolpert, Giger and Jefferson (1971) in a perfused rat heart preparation. Their kinetic data on the incorporation of lysine and glycine into protein suggested that the intracellular pool of these two amino acids was involved in protein synthesis. The evidence for their conclusion was based on the demonstration of a lag in incorporation of radioactivity into protein corresponding to the time taken for the intracellular pool to equilibrate. A relationship was also shown between the radioactivity of the intracellular pool and the rate of incorporation of label.

The same conclusion was reached by Mowbray and Last (1974) in perfused rat hearts. Their experimental protocol was designed to mimic the double isotope procedure of Hider et al., (1969). However, unlike Hider et al. they were unable to show a rapid decrease in the incorporation of ^{14}C glycine into protein when the label in the perfusate was changed from ^{14}C to ^{3}H. Because of this and also because they found an appreciable lag in the initial incorporation of glycine, they reported their results as being consistent with the existance of an intracellular precursor pool for glycine. They also studied the kinetics of leucine incorporation in an identical way, but because of the rapid exchange of this amino acid they were unable to show whether the precursor in this case was intra-or extracellular.

Finally, Rannels and Morgan (1973) investigated the problem in perfused rat hearts in a very similar manner to that of Mortimore et al., (1972) with the exception that the amino acid used in this case was phenylalanine. They concluded that the model best representing the experimental data was identical to that of Mortimore et al., (1972) but differed in the actual sizes of the expandable and non-expandable pools.

The studies of precursor-product relationships in perfused organs have all strongly implicated the intracellular pool in protein synthesis. This contrasts with the differing conclusions of the previous sections.

In vivo Systems

The main advantage of an *in vivo* method is that there is less danger of interfering artificially with the system under study and thereby investigating a tissue in abnormal conditions. In terms of maintaining the physiological and biochemical status of the tissue there is no better system. However, because it is difficult to preset specific conditions in an experiment it is also difficult to interpret the results. It is probably for this reason that little research has been concerned with the problem of defining a pool of free amino acids directly related to protein synthesis in the intact animal.

Compartmentation of amino acids has, however, been illustrated in a number of tissues. For example, Garfinkel and Lajtha (1963) demonstrated that in rat kidney the specific radioactivity of excreted hippuric acid was higher than the average intracellular value for glycine, its precursor. A computor analysis of their data led them to conclude that although compartmentation existed it could be adequately explained on the basis of "hindered diffusion" rather than distinct physical separation. Thus they implied that hippurate is synthesised at a cellular site, such as the plasma membrane, where the specific radioactivity of glycine is higher than that of glycine in more central areas of the cell. Similar evidence has been presented for the labelling of glutathione and glutamine (Garfinkel, 1966).

Compartmentation of amino acids has also been demonstrated in rat liver (Waelsch, 1962) and in different cellular fractions of this tissue (Portugal, Elwyn and Jeffay, 1970). Studying specifically the distribution of free lysine during a continuous infusion of ^{14}C lysine, Portugal

Elwyn and Jeffay showed the specific radioactivity to be lowest in the mitochondria and highest in the microsomal and cytoplasmic fractions. This was only true, however, for infusion times of up to one hour. Their results after infusing for three hours showed little difference between any of the fractions.

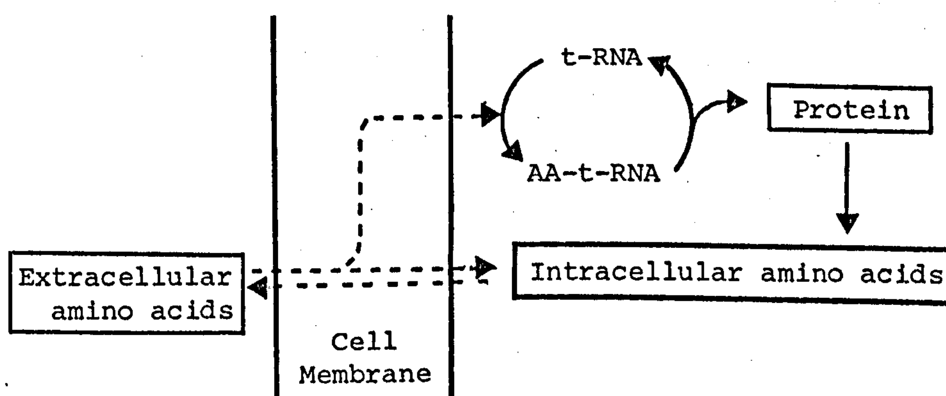
There are also several good reviews on the subject of amino acid compartmentation in the rat brain (Waelsch, 1962; Garfinkel and Hemmets, 1969; Berl and Clarke, 1969; Quastel, 1974).

Although these studies indicate that amino acids are not uniformly distributed within a tissue or cell, they give no indication of the location or nature of amino acids serving as precursors for protein synthesis. One study which specifically attempted to investigate this was that of Alpers and Thier (1972). They infused ^{14}C leucine into the blood and ^{3}H leucine into the gut lumen of a rat and subsequently compared the $^{3}H/^{14}C$ ratio in mixed intestinal proteins with that of the free amino acids in three different compartments, namely the total tissue pool, the blood and the luminal pool. The results showed that the ratio in protein (10.5) was very similar to the total tissue pool (10.0) and significantly different from the blood (150) and the lumen (0.9). This strongly suggested that the intracellular pool was a good approximation to the true precursor pool.

Other investigations dealing with the definition of a precursor pool *in vivo* have all attempted to answer the question by a more direct approach. Although free amino acids can be considered as protein precursors they are not as immediately available to the synthetic apparatus as, for instance, those acylated to t-RNA. Consequently by isolating charged tRNA and measuring the specific radioactivity of the attached amino acid it is possible to determine the specific radioactivity of the precursor amino acids. Although this method is open to some practical objections, it is a very reasonable approach to the problem.

Henshaw, Hirsch, Morton and Hiatt (1971) injected large quantities of lysine into rats to achieve and maintain constant specific radioactivities in the free lysine pool of both liver and blood. Under these conditions the specific radioactivity of the free lysine in the liver was 80% of the plasma value. After isolating lysyl t-RNA and measuring the specific radioactivity of the bound lysine, they showed that it was very similar to the specific radioactivity of the free lysine in liver.

Airhart, Vidrich and Khairallah (1974) isolated valyl t-RNA from the liver of rats given a single pulse dose of ^{3}H valine. In contrast to Henshaw <u>et al.</u>, (1971) their results indicated that valine attached to tRNA had a specific radioactivity intermediate between that of the plasma and the calculated value for the intracellular pool. They interpreted this to indicate that the precursor pool was in the cell membrane. Their model was similar to those proposed by Hendler (1962) and by Adamson, Herrington and Bornstein (1972). They further suggested that the precursor pool could be supplied with amino acids from both intracellular and extracellular sources



In conflict with both previous studies Martin, Prior and Zak (1973) reported that in heart the specific radioactivity of ^{3}H leucine derived from leucyl t-RNA was close to that in the plasma. It may be significant that in the work of Airhart <u>et al.</u>, (1974) and of Martin

et al., (1973) only a tracer dose of amino acid was used, whereas that of Henshaw et al., (1971) involved appreciably higher quantities.

Recently there has been a report (Ilan and Singer; 1975) in which another direct approach has been employed involving the isolation of nascent polypeptide chains from newt liver. Subsequent analysis of these polypeptides revealed that the specific radioactivity of the incorporated leucine was between 7-9 times greater than that of the leucine in the free amino acid pool of the liver homogenate. As the authors did not measure the specific radioactivity of leucine in the plasma, it is difficult to assess whether the newly incorporated leucine was more closely related to the plasma or to the homogenate pool. However, considering the large differences in specific radioactivity between the nascent chain and the free pool in the liver, these results would imply that precursor amino acids are supplied more directly from the plasma.

The same lack of a uniform conclusion seen with previous methods is also shown by the experiments in vivo. Therefore the overall problem of characterising a precursor pool for protein synthesis has not been satisfactorily resolved.

1.6. Methods of Investigation

The most direct method for characterising the precursor pool is to measure the specific radioactivity of an amino acid incorporated into a protein in isotopic equilibrium, a nascent polypeptide or an amino acyl transfer RNA. Although these techniques are direct, they are, nevertheless, associated with certain practical difficulties. One important criticism of the study of amino acyl t-RNA is the fact that its concentration is very low and the turnover of the pool is very fast. The total t-RNA pool size in heart, for example, is less than 0.1% of the tissue wet weight (Earl & Korner, 1966; Wool et al., 1968) and the half-life

for charged t-RNA is in the region of 1-3 seconds (Manchester, 1970; Airhart et al., 1974). As a result, any alteration in the natural compartmentation of amino acids within the cell, such as occurs during homogenisation, may bring about a new level equilibration of the amino acyl t-RNA unrelated to that in its natural state. A second difficulty is that some amino acyl t-RNA species are unstable under the conditions of the procedures used for their isolation, and therefore investigations are restricted to the use of the relatively stable species such as valyl and leucyl t-RNA (Coles, Bukeberger & Meister, 1962; Sarin & Zamecnik, 1964; Gatcia et al., 1966; Davey & Manchester, 1969).

Similarly studies of complete pure proteins in isotopic equilibration often involves the induction of these proteins by agents which can themselves, directly or indirectly, influence the movement of amino acids within the cell. It has been shown, for instance, that ouabain and amino-oxyacetic acid can alter the relative labelling of glutamate and glutamine in the rat retina (Starr, 1974). When inducing agents such as cortisone (Berlin & Schimke, 1965) and L-tryptophan (Knox & Auerbach, 1955) are used specifically to increase the synthesis of protein, the normal distribution of radioactivity within the different compartments of the cell may also be changed. It is, of course, possible to circumvent the problems of induction by selecting a protein which has a naturally fast turnover e.g. δ -aminolevulinc acid synthetase ($T_{\frac{1}{2}}$=60-72 minutes; Marver et al., 1966) or tyrosine transaminase ($T_{\frac{1}{2}}$=2-4 hours, Kenney, 1967), but even in cases like these it is necessary to maintain the experimental system in isotopic equilibration for a period of at least 4-5 half lives.

The ability to isolate nascent polypeptide chains is a relatively new technique (Ganoza & Nakamoto, 1966; Slabaugh & Morris, 1970) which has

only recently been applied to the study of precursor pools in protein synthesis by Ilan & Singer (1975). These workers investigated this problem in the liver of the newt. As far as is known no such study has been applied to mammalian systems. Although theoretically the use of nascent polypeptide chains in this context should provide very useful information, its full potential has not yet been assessed.

Collectively, one significant criticism of the direct methods is that they appear to be restricted to certain specific tissues. Up to now no investigation has been concerned with tissues other than the liver (Loftfield & Harris, 1956; Henshaw et al., 1971; Airhart, 1974; Ilan & Singer, 1975), the heart (Davey & Manchester, 1969; Martin, Prior & Zak, 1973) or cultured HeLa cells (Righetti, Little & Wolf, 1971; Van Venrooij, Moonen & Van Loon-Klaassen, 1974). The extension of these techniques to other tissues is dependent on the presence of a sufficiently fast turning over protein, an inducible protein, or a high acceptor capacity of specific amino acyl t-RNA. This may not always be possible. For example Davey and Manchester (1969) have shown that the acceptor capacity of t-RNA in diaphragm was too low for specific radioactivity determinations to be made accurately.

An alternative approach is the more indirect study of the kinetics of pool labelling. While this method has contributed some valuable information, it only considers the dynamic aspects of the system and gives little knowledge about the actual specific radioactivity of the precursor pool. An example of this type of study is that of Kipnis et al., (1961) and Halvorson and Cohen (1958).

The experimental design of the present investigation has been based on the assumption that the ratio of the specific radioactivities of two amino acids in protein will reflect the ratio of the specific radio-activities of the same two amino acids in the precursor pool. Con-

sequently by measuring the ratio in protein and comparing it with the ratio in an experimentally defined free amino acid pool, it is possible to assess to what extent this defined pool mimics that of the precursor. As far as is known comparison of specific radioactivity ratios in the study of precursor-product relationships has been used only on four previous occasions (Simpson & Velick, 1954; Wu & Soeiro., 1971; Alpers & Thier, 1972; Van Venrooij et al., 1974).

1.7. Methods of Differentially Labelling Free Amino Acid Pools

As the controversy about precursor amino acid pools is principally concerned with whether they are of extracellular or intracellular origin, the initial aim of this study was to produce a contrast in specific radioactivities of two amino acids between the plasma and tissue free amino acid pools.

There are several possible ways in which this might be achieved. Firstly, differences can be created by infusing into the plasma two amino acids with the same isotopic label. As protein degradation results in isotopic dilution the intracellular specific radioactivity of each amino acid will always remain lower than in the plasma. However, while differences in specific radioactivity can be produced between these two pools, the relative specific radioactivities of the two amino acids within each compartment might be the same. For this method to succeed, therefore, there must be a difference in turnover of the intracellular pool of each amino acid.

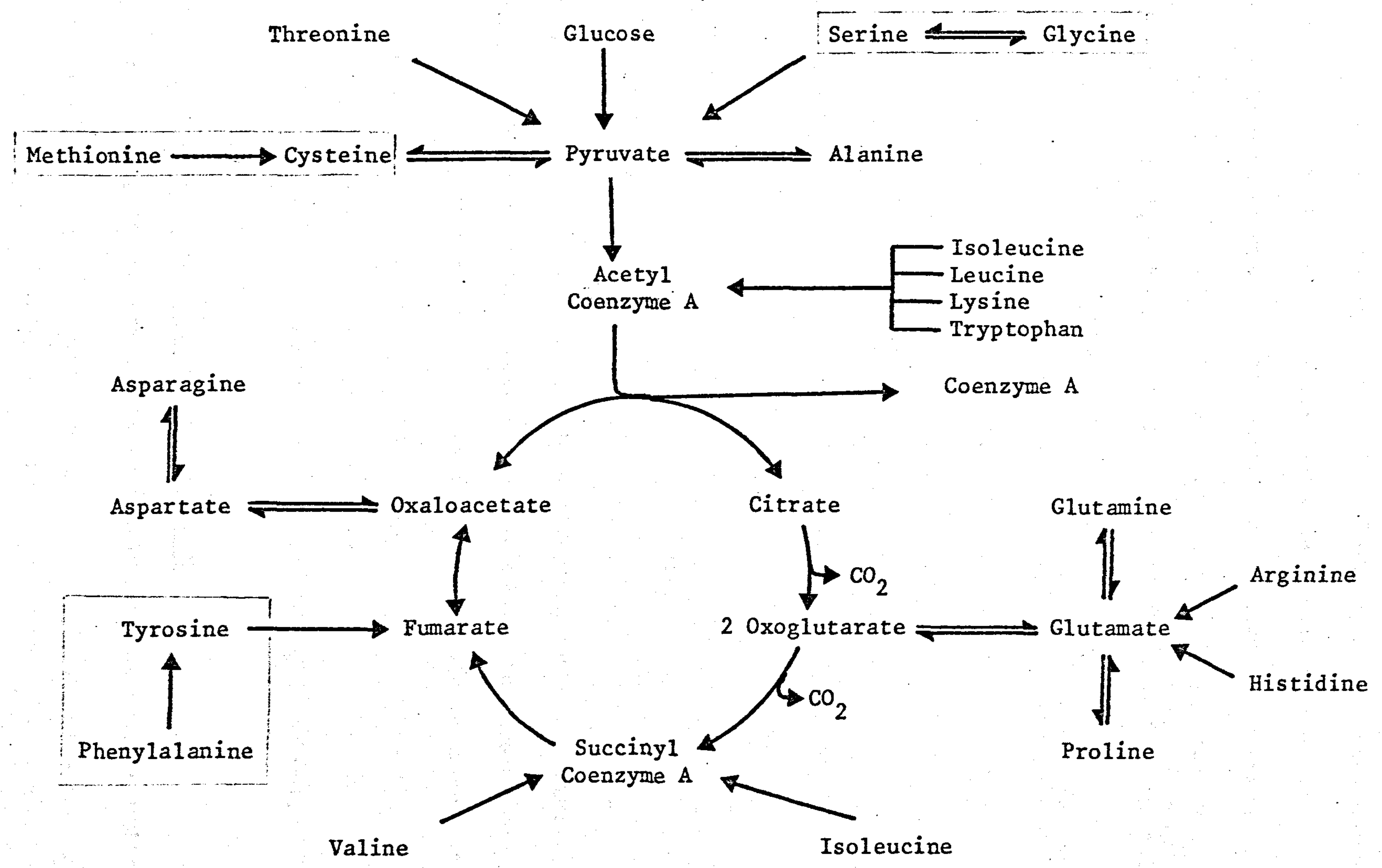
A more satisfactory method is to supply the intracellular pool with radioactive amino acids by two different routes. This is only practicable in certain tissues. In the gut, for example, amino acids can be infused via the plasma and also be perfused via the lumen. Alpers and Thier (1972) employed this technique in their study of precursor pools; they administered different isotopes of the same amino acid, leucine, into the blood and the gut lumen. This method can be used in studies of the brain,

where pentration of the tissue can be achieved both by intravenous and by intracisternal infusion.

Differential labelling can also be accomplished by relying on an intracellular metabolic sequence to alter either the distribution of isotope within the infused molecule or to convert it to an amino acid that can subsequently be incorporated into protein. Redistribution of isotope can, for example, be brought about by infusing both tritiated arginine and ^{14}C carbonate. The activity of the urea cycle in the liver will lead to the incorporation of $^{14}CO_2$ into arginine, predominately within the guanidino group (Swick, 1958; McFarlane, 1963). As a result the ratio of $^{14}C/^{3}H$ in liver arginine would be very much higher than in plasma. A similar technique could possibly be applied to methionine. Alternatively, there are many instances where amino acids can be synthesised directly from other amino acids or from carbohydrate sources. For instance, when diaphragm is incubated with ^{14}C aspartate appreciable amounts of radioactivity are incorporated into glutamate (Manchester & Young, 1959). Similarly these workers showed that radioactivity originally present in glucose or pyruvate could subsequently be located in alanine. Scheme 1 diagrammatically presents an outline of amino acid metabolism in which many additional examples are illustrated.

For the purpose of the present investigation the most practical method is that of metabolic interconversion, as this has the potential of producing extreme differences in specific radioactivity between the infused amino acid and the amino acid which is metabolically derived from it. Thus in plasma, the infused amino acid will have a specific radioactivity higher than that in the intracellular pool, whereas the derived amino acid will be the opposite way round, with a higher specific radioactivity in the intracellular pool than in the plasma. This will produce different specific radioactivity ratios in the tissue and in the plasma. The choice of amino acid for infusion is largely governed by the fact that acid

Scheme 1 . Diagrammatic Representation of Amino Acid Metabolism.



hydrolysis of protein leads to the degradation of glutamine and asparagine (to glutamate and aspartate respectively), with the result that it is impossible to determine accurately the specific radioactivity of these amino acids in protein. Enzymic hydrolysis can be used to overcome this problem but this in itself presents additional complications. Consequently the use of any amino acids in Scheme 1 that results in the formation of either aspartate or glutamate is not really suitable. The conversion of phenylalanine to tyrosine, of methionine to cysteine and of glycine to serine are the three remaining options. Of these the serine-glycine conversion is preferable for three significant reasons. Firstly, both serine and glycine are present in protein in relatively large quantities (See Table 3), whereas tyrosine, phenylalanine, methionine and cysteine are present in much smaller amounts. This is relevant if the technique is to be applied to situations where the total yield of protein is low. Secondly, the conversion of glycine to serine is reversible, giving the added advantage that the experimental system can be studied independently in both directions. Lastly, the use of glycine avoids possible errors due to contamination by D-amino acids. This is important because the method of analysis used in this study, i.e. automated ion exchange chromatography, does not differentiate between D- and L- forms. The possibility of errors caused by the presence of D-lysine was well illustrated by Waterlow and Stephen (1967).

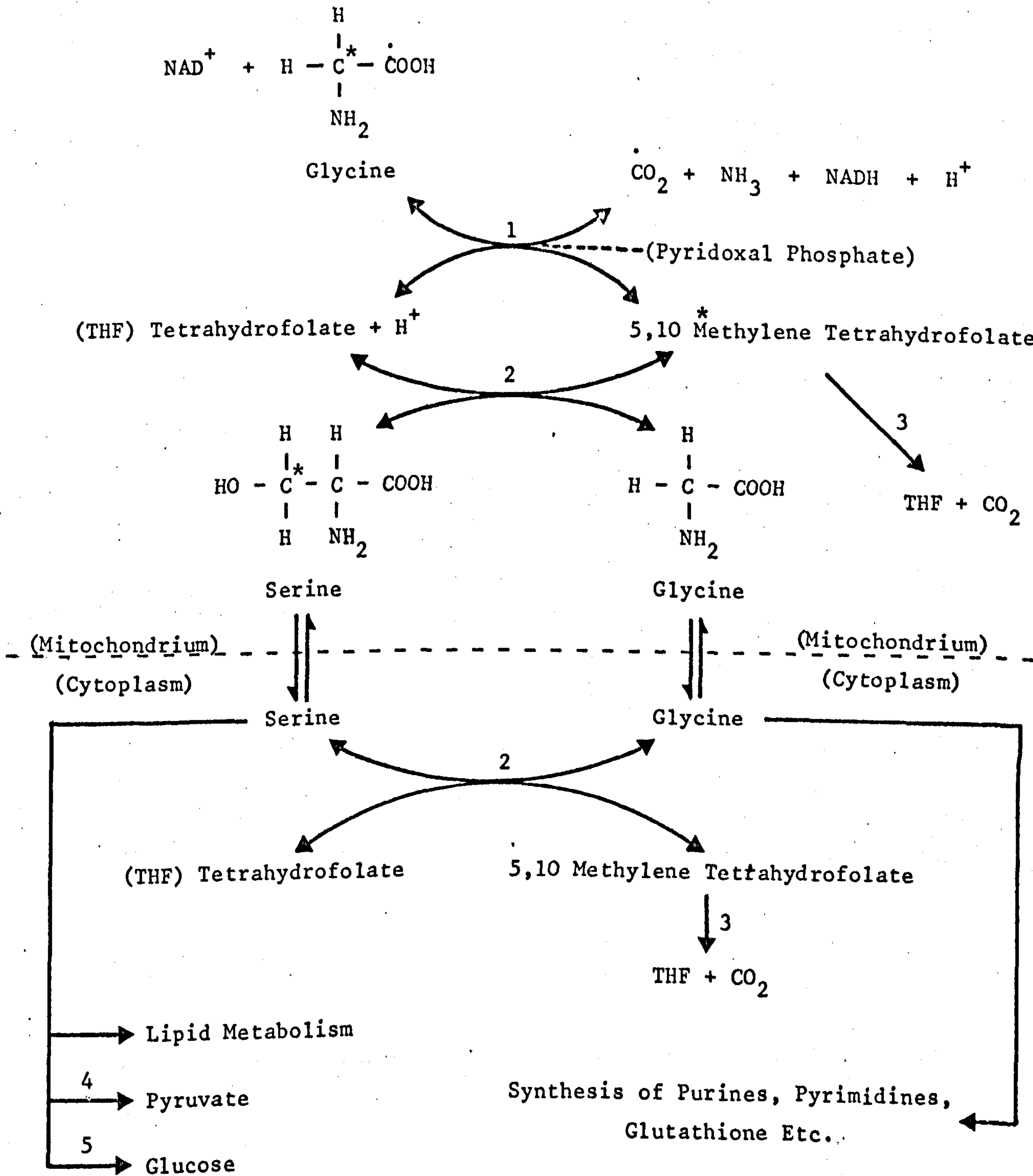
1.8. Mechanism of Interconversion of Glycine and Serine

Shemin (1946) first demonstrated the conversion of serine to glycine by the transfer of ^{15}N and ^{13}C isotopes from the amino and carboxyl groups of serine to hippurate in rats and guinea pigs. The reversible interconversion of glycine and serine was later shown by Sakami (1948) and by Winnick *et al.*, (1948). It is now known that the reaction involves a 'one carbon unit' represented by 5:10 methylene tetrahydrofolic acid

and that it is catalysed by serine hydroxymethyl transferase (E.C.2.1.2.1.). This enzyme occurs in both cytosol and in the mitochondria (Yoshida & Kikuchi, 1972; Fujioka, 1969). Glycine is also catabolised to carbon dioxide, ammonia and a 'one carbon unit' by a metabolic sequence referred to as the "glycine cleavage system". First demonstrated in bacteria by Sagers & Gunsalus (1961) and later in avian liver (Richert et al. 1962) and rat liver (Kawasaki et al. 1966), the cleavage is believed to be achieved by a complex series of reactions catalysed by four protein components and requiring pyridoxal phosphate and tetrahydrofolate as co-factors (Kikuchi, 1973). Its activity is thought to be located only within the mitochondria (Sato et al., 1969; Motokawa & Kikuchi, 1974).

The activity of serine hydroxymethyl transferase and the glycine cleavage system has been shown to be significant in liver and, to a much lesser extent, in kidney and intestine (Yoshida & Kikuchi, 1972). In other tissue, although activity can be demonstrated, it is very low by comparison. It has been suggested that the catabolism of serine in rats occurs mainly through the activity of serine hydroxymethyl transferase forming glycine and 5:10 methylene tetrahydrofolate and the major catabolism of glycine occurs by the activity of the glycine cleavage system (Yoshida & Kikuchi, 1973). The role of serine dehydratase in serine degradation is considered to be unimportant (Kikuchi, 1973) except under specific physiological conditions such as feeding of high protein diets (Cheung, 1969). This is in agreement with studies which have shown that the contribution made by serine to hepatic gluconeogenesis is relatively minor when compared with such precursors as alanine (Ishikawa, Aikawa & Matsutaka, 1972). Scheme 2 outlines the interconversion of serine and glycine together with some of the other catabolic fates of these amino acids.

Scheme 2 . Details of the Metabolism of Serine and Glycine



1. Glycine Cleavage System (Yoshida & Kikuchi 1972, 1973)
2. Serine Hydroxymethyltransferase (E.C. 2.1.2.1.)
3. Methylenetetrahydrofolate Dehydrogenase (E.C. 1.5.1.5.)
4. Serine Dehydratase (E.C. 4.2.1.13.)
5. Gluconeogenisis via 3 Phosphoglycerate

SECTION 2

Amino Acid Pools

In his review Munro (1970) pointed out that the supply of free amino acids to the tissues must be a central feature of protein metabolism as the synthesis and degradation of protein intimately involves these compounds. In order to appreciate the central role more fully it is necessary to consider the amounts of individual amino acids present in different pools and also the amounts participating in various physiological and metabolic processes. The purpose of this introductory section is therefore to show, in quantitative terms, the importance of each of the defined pools and each of the defined pathways. It is not intended to be a detailed analysis of the situation but merely to make an overall assessment of the magnitude of these processes in the rat. More specifically this section is concerned with these parameters in 100-130 g female hooded rats maintained on a high protein diet. These animals have been used almost exclusively throughout the turnover studies.

2.1. Experimental Methods.

Animals. Female hooded rats weighing 45 g were obtained from Animal Supplies (London) Limited (London N12, U.K.).. On arrival they were bathed in Tetmosol (tetraethylthiuram monosulphide - I.C.I. Ltd., Macclesfield, Cheshire, U.K.) to minimise parasitic infestation and were then housed three to a cage. They were allowed free access to water and to a powdered diet (details given below) until their body weight reached 130 g. Some control animals were used at 65 g. Gain in body weight was determined by weighing the rats daily and was estimated to be 3.3 ± 0.2 (S.E.M.) g/day. The measurements were made during growth between 50-130 g.

Diet. The powdered diet was prepared in the laboratory from constituents which were bought from the following suppliers: casein (Casumen; Prideaux Milk Foods, Evercreech, Somerset); maize starch and dextrinised starch (Corn Products Ltd., Manchester); solka floc (Cellulose; Johnson, Jorgensen and Wettre Ltd., London); arachis oil (Ground Nut Oil; British Drug Houses Ltd., Poole, Dorset); mineral salt mixture, B-vitamin mixture and fat soluble vitamins (Cooper Nutritional Products, Witham, Essex) and L-methionine (Sigma Chemicals Ltd., London). The diet, the composition of which is given in Table 1, was designed to have a ratio of energy supplied by utilisable protein: total metabolisable energy (NDP:E) of 10%. This gave maximum rates of body growth. Increasing the protein content of the diet did not increase the growth rate of the animals.

The diet was analysed for amino acid composition by hydrolysis as described for the hydrolysis of muscle protein (see below). 1 g samples were used in the procedure. The results are also given in Table 1.

Food Intakes. The daily food intake was measured by giving the animals a known weight of food and subtracting what remained at the end of 24 hours. Loss due to spillage was accounted for. The daily intakes of individual amino acids were calculated from the food intake and the amino acid composition of the diet. The intake of food was measured over a 7-14 day period immediately before the time the animals were used for experimentation.

Plasma Free Pool. Plasma samples were obtained by decapitation and subsequent collection of blood in heparinised tubes. After low speed centrifugation the plasma was mixed with an equal volume of 5% sulphosalicylic acid (SSA), left for 2 hours at $4^{o}C$ and finally spun to remove the protein precipitate. The resulting supernatant was stored for analysis.

Muscle Free Pool. Extensor digitorum longus muscles (50 mg) were

Table 1 .

a) Composition and Amino Acid Content of Diet

Composition of Diet (10% NDP:E)

Content (g/Kg diet)	
Casein(Casumen)	108.9
Maize Starch	425.6
Dextrinised Starch	272.2
Solka Floc (Cellulose)	90.7
Arachis Oil	45.5
Salt Mixture*	45.4
B-Vitamin Mixture*	10.0
Fat Soluble Vitamins*	0.91
L-Methionine	0.91

* Details given in Payne & Stewart (1972)

Energy Content = 15.6Kjoules/g diet
Nitrogen Content = 16.44mg N/gdiet
Water Content = 0.096g/g diet

Amino Acid Content	Mean ± S.E.M. (μmoles/g diet)
Aspartate + NH_2	42.0 ± 0.7
Threonine	28.6 ± 0.7
Serine	44.2 ± 1.1
Glutamate + NH_2	122.7 ± 2.2
Glycine	24.0 ± 3.0
Alanine	32.9 ± 1.1
Valine	50.3 ± 2.6
Methionine	27.9 ± 1.4
Isoleucine	30.9 ± 0.4
Leucine	63.0 ± 1.4
Tyrosine	28.6 ± 0.3
Phenylalanine	29.2 ± 0.1
Lysine	46.8 ± 1.6
Histidine	16.9 ± 0.5
Arginine	19.2 ± 0.5

b) Food Intake of Rats at 65g and 130g Body Weight

	Mean Rat weight (g)	Food Intake (g diet/day)	Energy Intake (Kjoules/g body weight$^{0.73}$)	N_2 Intake (mg N/g body weight$^{0.73}$)
"65g" Rat (n=20)	62.6 ± 1.1	8.26 ± 0.21	6.27 ± 0.16	6.63 ± 0.17
"130g" Rat(n=29)	111.9 ± 1.5	12.12 ± 0.28	6.02 ± 0.14	6.36 ± 0.15

dissected from both hind limbs and homogenised in an all glass hand homogeniser in 2 ml SSA containing norleucine (50 nannomoles/ml: Sigma Chemicals Ltd., London). After homogenisation the samples were stored at $4^{\circ}C$ for 2 hours before being centrifuged at 1000 x g for 10 minutes. The supernatant was decanted and stored for analysis.

Muscle Protein Pool. The precipitate from the above sample was washed x 3 in 4 ml 5% SSA (not containing norleucine). The washed precipitate was then sealed in a glass container with 4 ml "Constant Boiling" HCl and an atmosphere of nitrogen. Hydrolysis was carried out at $105^{\circ}C$ for 24, 48 and 72 hours. Finally the hydrolysates were evaporated to dryness, reconstituted in 1 ml lithium citrate buffer (pH 2.2, 0.3 M lithium, 0.1 M citrate) and stored for analysis. Decomposition of amino acids, such as serine, was corrected for in the final calculations.

Composition of Total Body Protein. Six female rats weighing approximately 100 g were killed and dried to constant weight in a $100^{\circ}C$ oven. The dried weight was calculated to be 30.0 ± 0.4 % of the body wet weight. After desiccation the carcasses were ground to a fine homogeneous powder from which a 2 g aliquot was taken and mixed with 7.5 ml. 'Constant Boiling' HCl. The resulting mixture was hydrolysed at $105^{\circ}C$ for 24 and 72 hours in an atmosphere of nitrogen. The hydrolysate was then dried down to a 2 ml volume and reconstituted to 50 ml with distilled water. A 10 µl sample of this was added to 1 ml pH 2.2 lithium citrate buffer and analysed as described in the section on amino acid analysis.

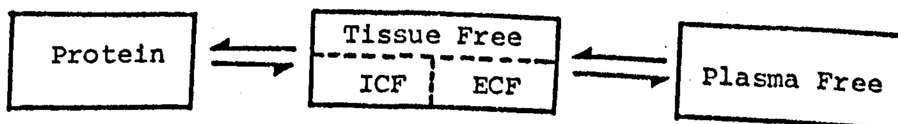
Amino Acid Analysis. Amino acid levels in plasma and tissue free pools, in muscle and total body protein and in the diet were determined by automated ion exchange chromatography with a Locarte Analyser (Floor Model - The Locarte Company, London). Amino acids were resolved

in a 50 cm x 0.9 cm glass column containing the ion exchange resin (8.25% cross linked; particle size 6-8 μ as supplied by The Locarte Company) by a gradient system comprising 3 buffers: 1) pH 2.8 : 0.3 M lithium : 0.09 M citrate, 2) pH 3.5 : 0.3 M lithium : 0.1 M citrate, 3) pH 4.5 : 1.2 M lithium : 0.1 M citrate. The three buffers were run for 120, 150 and 270 minutes respectively. At 120 minutes the temperature of the column was increased from 36°C to 55°C and maintained to the end of the run. After each sample run the column was regenerated with lithium hydroxide (0.3 M : 30 minutes) and the initial buffer (pH 2.8 : 90 minutes). The flow rate was maintained at a constant 30 ml/hour throughout. Detection of eluted amino acids was achieved by reaction with ninhydrin and subsequent measurement of the optical density at 570 nm. Quantitation of the individual amino acids was based on the presence of a known quantity (50 nannomoles) of nor-leucine in the sample and calculated by manual integration as described in Techniques in Amino Acid Analysis (1966). Results were corrected for the individual molar extinction constants for each amino acid.

Total Body Protein Turnover. This was measured from the rate of irreversible disposal of tyrosine from the plasma. Further details are given in the section on amino acid balance (see further on).

2.2. Results and Discussion of Amino Acid Pools

To illustrate the magnitude of some of the main amino acid pools in the experimental rats, measurements were made of plasma free, tissue free and protein bound amino acids. The relationship of these three pools is shown below:



ICF = Intracellular Fluid ECF = Extracellular Fluid

Free and protein bound amino acid concentrations were measured in skeletal muscle (extensor digitorum longus) because this tissue has been reported to account for as much as 80% of the total free amino acid pool of the body (Munro, 1970). No distinction has been made between muscles of different fibre types but additional studies on soleus muscle indicated that only the free glutamate and aspartate pools differed appreciably from those in extensor digitorum longus. Extensor digitorum longus is predominantly a white fibred muscle (65%) while soleus is composed of 80% intermediate fibres (Close, 1972).

Table 2 shows that the concentration of all amino acids in the three pools differed markedly. By far the largest quantity was contained in the protein compartment. In the case of many of the essential amino acids this compartment was 200-650 times as large as the corresponding free pool. For the non-essential amino acids this figure was much lower, being of the order of 15-100 times.

In addition the free amino acid concentrations in the plasma were consistently lower than in the tissue. The difference was most obvious in the case of the non-essential amino acids. For some of the essential amino acids such as leucine, valine and phenylalanine the difference between concentrations in the two pools was very small. The variation in the concentration gradients between tissue and plasma of the amino acids in Table 2 can be explained by differences in the mechanism of cellular transport (Oxender and Christensen, 1963; Christensen, 1969). Those amino acids that exhibit a strong affinity for the L system and a much weaker one for the A system have similar concentrations in both pools. Examples of such amino acids are leucine, valine,isoleucine, tyrosine and phenylalanine. In contrast, those that exhibit a much stronger affinity for the A system than they do for the L system e.g. glycine, serine, alanine and threonine, have very much higher concentrations in the tissue than in the plasma. The L system

Table 2 : Concentration of Amino Acids in the Free and Protein Bound Pool of Muscle and in the Free Pool of Plasma.

Amino Acid Pool Sizes. Mean ± S.E.M.

	Muscle Protein (n=6)	Muscle Free (n=6)	Plasma Free (n=6)	Muscle Protein / Muscle Free
	μmoles/g tissue wet weight		μmoles/ml plasma	
Aspartate + NH_2	109.9 ± 6.0	1.133 ± 0.065	0.075 ± 0.003	97.0
Threonine	60.2 ± 4.2	1.940 ± 0.110	0.497 ± 0.033	31.0
Serine	73.9 ± 6.5	1.960 ± 0.190	0.373 ± 0.012	37.7
Glutamate + NH_2	147.6 ±12.4	9.910 ± 0.710	0.605 ± 0.033	14.9
Glycine	116.5 ± 9.8	1.940 ± 0.100	0.209 ± 0.004	60.1
Alanine	110.6 ± 7.0	2.770 ± 0.070	0.695 ± 0.033	39.9
Valine	82.8 ± 2.8	0.305 ± 0.019	0.267 ± 0.010	271.5
Methionine	35.5 ± 1.8	0.158 ± 0.015	0.051 ± 0.004	224.6
Isoleucine	50.1 ± 1.9	0.164 ± 0.009	0.108 ± 0.008	305.5
Leucine	108.9 ± 3.7	0.196 ± 0.006	0.187 ± 0.012	555.6
Tyrosine	36.0 ± 1.4	0.136 ± 0.013	0.120 ± 0.011	265.7
Phenylalanine	45.2 ± 1.7	0.070 ± 0.004	0.061 ± 0.004	645.7
Lysine	58.2 ± 5.0	1.860 ± 0.150	0.606 ± 0.042	31.3
Histidine	26.1 ± 1.0	0.391 ± 0.026	0.089 ± 0.003	66.8
Arginine	66.6 ± 3.4	0.248 ± 0.005	0.109 ± 0.006	268.5

is characterised by kinetics which show a saturable, sodium- and energy-independent uptake, incapable of net transport against a concentration gradient. The A system differs in that it is both sodium- and energy-dependent and is capable of producing and maintaining a concentration gradient.

The values for the tissue free amino acid pool in Table 2 have not been corrected for the presence of extracellular amino acids. Therefore they do not represent the size of the true intracellular pool in this tissue. In the majority of cases, however, correcting the results for 30% of tissue weight as extracellular water (Hider, Fern and London, 1971b) will not appreciably alter the values. The concentrations of amino acids in the free pool of muscle are similar to those reported by others (Millward, Nnanyelugo, James and Garlick, 1974; Munro, 1970)as are the values for plasma free amino acids (Adibi, Modesto, Morse and Amin, 1973; Christophe, Winard, Kutzner and Hebbelinck, 1971) and for protein bound amino acids (Adibi, Modesto, Morse and Amin, 1973).

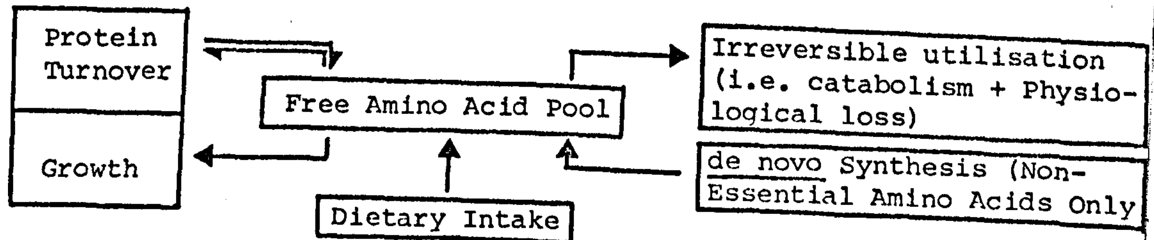
These results serve to illustrate the point that small changes in the size of the protein pool are capable of creating much larger changes in the tissue free amino acid pool and subsequently in plasma. They also show that the tissue free pool is not capable of supporting the synthesis of very much protein. In order to sustain the rate of synthesis during growth and normal protein turnover the precursor pool must itself turn over at a very high rate. As these measurements are of static pool sizes they will of course give no indication of the speed at which individual amino acids pass through these pools.

2.3. Results and Discussion of Amino Acid Balance.

In order to illustrate the magnitude of certain major pathways of amino acid metabolism an experimental protocol was designed to measure the rate of body growth, dietary intake, protein turnover, irreversible

utilisation of amino acids and changes in the free amino acid pool size during growth of rats from 65-130 g. These parameters were taken to be related as shown below:

Protein Metabolism



They were estimated in the following way:

Dietary Intake:-	Direct measurement (Table 1)
Amino Acids used for Growth:	Calculated from the daily change in body weight and the amino acid composition of the body (Table 3).
Changes in free Amino Acid Pool: of Muscle:	Direct measurement of difference between muscle pool at 65 and 130 g body weight. (Table 3).
Irreversible Utilisation:	Calculated as the difference between the amount supplied by the diet and the amount utilised for growth. For the esential amino acids this is valid because there is no <u>de novo</u> synthesis from other carbon sources. In effect the calculated rate of irreversible utilisation for each essential amino acid is a maximum value. For the non-essential amino acids the situation is different due to <u>de novo</u> synthesis from other carbon sources. The calculated rate of utilisation for these amino acids is consequently a minimum value.
Total Body Protein Turnover;	The amount of each amino acid turnover over in protein was calculated from the flux of free tyrosine from the plasma during an infusion of L-[$U^{14}C$]tyrosine.

During this infusion the specific radioactivity of free tyrosine in the plasma reached a plateau. Because equilibrium had been achieved the amount of radioactivity infused per unit of time is equal to the amount leaving the plasma. If the plateau specific radioactivity of the plasma is known then the molecular amount of tyrosine leaving this compartment can be calculated. This amount is referred to as the flux or rate of irreversible disposal from the plasma andis a measure of the rate an amino acid is utilised for protein synthesis and catabolism including oxidation. Flux is calculated according to Waterlow and Stephen (1967).

$$F = \frac{ISi}{Sp}$$

where

F = flux rate (µmoles/hr)
I = infusion rate (µmoles/hr)
Si = specific radioactivity of infused amino acid (DPM/µmole)
Sp = specific radioactivity of the plasma at plateau (DPM/µmole)

On the assumption that the flux is entirely the result of protein synthesis, the total body synthesis rate is then calculated from the expression:

$$\text{\% of Total Body Protein Synthesised per Day} = \frac{F.24.100}{CW}$$

F = flux rate (µmoles/hr/g body weight)
C = total body protein amino acid composition (µmoles/ g body weight)
W = rat weight (g)

This expression however may result in an underestimation of the true rate because it assumes that the free amino acid pool of the body is homogeneous and therefore that the plasma represents the specific radioactivity of the precursor. It is known that the free amino acid pool of the body is not homogeneous because the degradation of

unlabelled protein within the cell results in a lower specific radio activity of the intracellular pool. For instance the specific radioactivity in liver at equilibrium has been shown (Gan & Jeffay, 1967; Waterlow & Stephen, 1968) to be approximately half that of the plasma and the value for brain can be even lower (Seta, Sansur & Lajtha, 1973). The plateau value in muscle has been shown to be about 80% of that in plasma. (Garlick, Millward & James, 1973). If the tissue pool is the precursor, then the total body synthesis rate calculated as above will be in error by an amount which is difficult to assess accurately but is likely to be of the order of 25-30%. This is based on the relationship between percentage tissue mass and the extent of equilibration with the plasma.

Offsetting this error to some extent is the assumption that the flux rate is accounted for entirely by protein synthesis. Ignoring the quantity of amino acids utilised by catabolism leads to an overestimation of the rate. Again the degree of overestimation is difficult to assess but the results of Neale and Waterlow (1974) showed that, during the constant intravenous infusion of ^{14}C valine, leucine, lysine and phenylalanine, the radioactivity recovered as $^{14}CO_2$ was 16.2%, 11.9%, 15.8% and 8.8% respectively of the total infused dose of radioactivity.

Although the measurement of the rate of total body protein synthesis is not without problems, it must be emphasised that the object of it in this section is only to give an indication of the amounts of individual amino acids involved in protein turnover for comparison with the amounts involved in other metabolic processes. For this purpose the whole body turnover rate has been taken as 25% of total body protein per day. The measured value from the tyrosine infusion was in fact 31.4% per day. Both figures are in reasonable agreement with those reported by Garlick,

Millward and James (1973) using tyrosine, with Waterlow and Stephen, (1967) using lysine and with Garlick (1972) using glycine.

Table 1 shows the content and amino acid composition of the 10% NDP:E casein diet. On a molar basis the most abundant amino acid(s) are glutamate + glutamine which are present at twice the concentration of the next most abundant, leucine. The least abundant are the two basic amino acids histidine and arginine. When the compositions of the diet is compared with the amino acid composition of the total body protein (Table 3) it is apparent that the amino acid most rate limiting for protein synthesis is threonine. Glycine, alanine, arginine and aspartate are also present in low concentrations relative to the amount required for protein synthesis but these are capable of being synthesised intracellularly and are therefore not required to be provided by the diet. Also shown in Table 1 are the food intakes of 65 and 130 g rats. On a weight basis the larger rats consumed more food per day than did the smaller rats, but when this figure was standardised for surface area and volume of the animal (body weight $^{0.73}$) the intake of energy was very similar.

The size of the free amino acid pool in muscle has been given in Table 3 to show that, while some significant changes occurred during growth of the animals between 65 - 130 g body weight, in quantitative terms these changes were unimportant. For example, the biggest change occurred in the pool size of glycine which decreased from a value of 3.99 µmoles/g muscle to 1.94 µmoles/g muscle. This change occurred over a period of 18 to 19 days and therefore constituted a decrease of only about 0.11 µmoles/day, or less than 5% of the daily dietary intake. For many of the amino acids there was virtually no change in the pool sizes.

Table 3 also documents the various parameters of amino acid metabolism measured in 130 g animals and the next table (Table 4) shows the propor-

Table 3. Comparison of Amounts of Individual Amino Acids Participating in Various Physiological and Metabolic Processes.

	Intake in Food	Requirement for Growth	Irreversible Utilisation	Protein Turnover (25% / day)	Total Body Protein Composition	Free Pool Size (Muscle)	
	←(μmoles / g body weight / day)→				(μmoles/g body weight)	(μmole/g tissue)	
						65g Rat	130g Rat
Leucine*	5.88	2.27	3.61	22.15	88.6	0.14	0.20
Isoleucine	2.88	1.17	1.72	11.29	45.7	0.13	0.16
Valine*	4.69	1.72	2.98	16.78	67.1	0.26	0.29
Tyrosine*	2.67	0.54	2.13	5.28	21.1	0.15	0.14
Phenylalanine*	2.72	1.05	1.68	10.18	40.7	0.07	0.07
Methionine*	2.60	0.58	2.02	5.65	22.6	0.15	0.16
Threonine*	2.67	1.54	1.13	15.05	60.2	0.61	1.94
Lysine*	4.36	1.52	2.84	14.90	59.6	2.13	1.86
Histidine	1.58	0.80	0.78	6.75	27.0	0.52	0.39
Arginine	1.79	1.49	0.30	14.60	58.4	0.23	0.25
Serine	4.11	1.89	2.22	18.48	73.9	2.18	1.96
Glycine	2.24	5.58	- 3.34	54.40	217.6	3.99	1.94
Alanine	3.07	3.31	- 0.24	32.30	129.2	4.00	2.77
Aspartate + NH_2	3.92	2.68	1.24	26.20	104.8	1.36	1.14
Glutamate + NH_2	11.44	3.78	7.66	36.95	147.8	11.58	9.91

* denotes essential amino acid.

Table 4. Percentage of Total Metabolic Amino Acids Participating in Protein Turnover, Growth, Irreversible Utilisation and Dietary Intake.

	% of Total Metabolic Amino Acids.			
	Protein Turnover	Irreversible Utilisation	Growth	Dietary Intake
Leucine	79.0	12.9	8.1	21.0
Isoleucine	79.6	12.2	8.3	20.3
Valine	78.1	13.9	8.0	21.9
Tyrosine	66.4	26.8	6.8	33.6
Phenylalanine	78.9	13.0	8.1	21.1
Methionine	68.5	24.5	7.0	31.5
Threonine	84.9	6.4	8.7	15.1
Lysine	77.4	14.7	7.9	22.6

tions of the essential amino acids required for protein turnover, irreversible utilisation and growth. The results are expressed as a percentage of the sum total of each amino acid used in the three processes. It is evident from the table that protein turnover involves the largest quantity of amino acids, accounting for between 66% (tyrosine) and 84% (threonine) of the total amount metabolised. With the exception of threonine the greater proportion of the remaining amino acids was accounted for by irreversible utilisation. The range in this case was between 12.1% (isoleucine) and 26.8% (tyrosine). The smallest proportion, again with the exception of threonine, was related to the growth of the animals. The range extended from 6.8% (tyrosine) to 8.3% (isoleucine). Table 4 also indicates that under these specific dietary conditions, the quantity of amino acids supplied by the food was on average 19.2% of the total metabolic pool. All the values mentioned above concern only the present experimental animals on a normal protein diet. The rates of irreversible utilisation, growth and protein turnover will of course alter if the diet or dietary intake is changed in any significant way. For instance, the rate of growth and protein turnover has been shown to diminish if the protein content of the diet is reduced (Waterlow & Stephen, 1966; 1968; Stewart, 1973) and the rate of oxidation of some amino acids has also been shown to decrease after prolonged feeding of low protein diets (McFarlane & Von Holt, 1969; Sketcher, Fern & James, 1974).

One other purpose of this section was to try to calculate a renewal time for each amino acid within the precursor pool for protein synthesis. Theoretically it is not possible to do this as the pool has not been sufficiently defined to allow a reasonable estimation of its physical size to be made. Nevertheless it is possible to calculate a maximum renewal time if two assumptions are made: firstly, that the rates of

protein synthesis and growth given in Table 3 constitute the total amount of protein synthesised per day: secondly, that the precursor pool is homogeneous and equal to the size of the free amino acid pool in muscle. The renewal time in this instance is defined as the time required to deplete the precursor pool completely of each amino acid when protein synthesis is occurring at a constant rate. These calculated times are given in Table 5. The amino acid with the fastest turnover was phenylalanine with a renewal time of 9.0 minutes and the slowest was glutamate (+glutamine) with a renewal time just short of 6 hours. In general the majority of the amino acids were renewed in a time of less than 60 minutes. With the exception of lysine and threonine, this applied to all other essential amino acids. The values in this table are by definition maximal and consequently no real comparison can be made between different amino acids. These results, however, do serve the purpose of showing that the precursor pool must have a rapid turnover. If the assumption that the precursor pool is represented by the total free amino acid pool of the tissue is in error, and in fact it is a smaller distinct compartment, then the renewal times will be correspondingly even shorter. Thus if the extracellular fluid serves as the precursor then the renewal time for phenylalanine would be 2.3 minutes. This is based on the assumption that the extracellular fluid has the same phenylalanine concentration as plasma and that it comprises 30% of the tissue wet weight.

Table 5. Calculated Maximum Renewal Times for Precursor Pool Amino Acids.

	Renewal Time (min)
Phenylalanine	9.0
Leucine	11.8
Isoleucine	18.5
Arginine	22.4
Valine	22.6
Tyrosine	34.6
Methionine	37.0
Glycine	46.6
Aspartate + NH_2	56.8
Histidine	74.4
Alanine	112.0
Serine	138.6
Lysine	163.1
Threonine	168.4
Glutamate + NH_2	350.4

The data used for calculating these renewal times are taken from Table 3. See the text for the assumptions made in the calculations.

SECTION 3 PRECURSOR COMPARTMENTATION IN DIFFERENT TISSUES.

3.1. Experimental.

Animals.

Female hooded rats weighing 65 g were obtained from Animal Supplies (London) Limited (London N12, U.K.). On arrival they were bathed in Tetmosol (tetraethylthiuram monosulphide - I.C.I. Ltd., Macclesfield, Cheshire, U.K.), to minimise parasitic infestation and were then housed three to a cage. They were allowed free access to water and a powdered diet until their body weight had reached 100-110 g at which time they were used for experimentation. The average body weight gain during this period was 3.0 g per day.

Diet

The diet was identical to that previously used. The details have been given earlier in Section 2.

Chemicals

All chemicals used were of Analar grade and unless otherwise stated were obtained from British Drug Houses, Poole, Dorset, U.K. Amino acid calibration standards were purchased from Hamilton Micromeasure N.V., P.O. Box 205, The Hague, Holland. Carrier amino acids for the infusion solutions came from Sigma Chemicals Ltd., London, U.K. as did the Bovine Serum Albumin (Fraction V) used for standardisation of gel filtration columns. Bovine Serum Albumin was also obtained from Miles Laboratories, Inc., (Pentex) Slough, Bucks, U.K., as also was horse spleen Ferritin (2X cryst. - trace cadmium).

Sulphur free toluene (B.D.H.) and Triton X 100 (Lennig Chemicals Ltd., Croydon CR9, Surrey, U.K.) were used to prepare scintillation fluid and the scintillant (2,5-diphenyloxazole) was purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Ninhydrin (Merck Laboratory Chemicals, Anderman & Co. Ltd., East Moseley, Surrey, U.K.)

and 2-methoxyethanol (Reeve Angel Scientific Ltd., London SE1) were used in the automated amino acid analysis.

Radiochemicals

[U-^{14}C] Glycine (10 mCi/mmol.), L-[U-^{14}C] serine (10 mCi/mmol.) and L-[U-^{14}C] tyrosine (10 mCi/mmol) were all purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Methods

Constant Infusion of Isotope

Female hooded rats (100-110 g) were placed in conditions that restricted movement. The tail was washed in warm water and lightly coated with xylene. The lateral tail vein was then cannulated with a 0.5 x 16 mm syringe needle (minus ferrule) and connected by a thin soft walled polyvinyl cannula (i.d. 0.5 mm) to a 1 ml syringe containing 0.9% (w/v) saline. After cannulation had been achieved the tubing was disconnected from the 1 ml syringe and reconnected to a 10 ml syringe containing the radioactive amino acid and approximately 10 mM carrier amino acid in 0.9% saline. The 10 ml syringe was driven by a constant speed infusion pump (Scientific and Research Instruments Ltd., Croydon, Surrey, U.K. Cat. No. 5200). The infusion pump was adjusted to give a flow rate of 0.48 ml/h. The length of time of infusion varied from 2-6 hours. Each animal remained restricted throughout the infusion by being securely wrapped in a piece of material. This infusion technique is a modification of one described by Garlick & Marshall (1972).

Preparation of Tissues

At the end of the infusion period the animals were anaesthetised with diethyl ether and decapitated immediately the infusion needle was disconnected. When mixed venous and arterial blood samples were taken the sample was collected from the severed neck but otherwise the

abdomen was opened and samples of hepatic portal and arterial blood were taken with a heparinised syringe. Tissue samples were rapidly dissected out, washed free of blood in ice-cold 0.9% saline and immediately homogenised in 3% (w/v) sulphosalicylic acid. In the case of the jejunum the luminal contents were washed out before homogenisation. The heart was opened up before washing free any residual blood. After homogenisation the homogenate was left at 4°C for 2 hours to allow protein to precipitate before being centrifuged at 1000 g for 10 minutes. The resulting supernatant was decanted and stored for analysis. The protein precipitate was processed for the removal of ribonucleic acids and lipids by a method described by Hider <u>et al.</u>, (1971a). Protein hydrolysis was carried out for 24 hours at 105°C in sealed glass tubes containing approximately 10 mg protein, 1.5 ml 'constant boiling' HCl and an oxygen free nitrogen atmosphere. After hydrolysis the samples were evaporated to dryness to remove the HCl reconstituted to 1 ml in lithium citrate buffer (0.3M lithium 0.1 M citrate pH 2.2), and stored for analysis.

Blood samples were centrifuged and the plasma mixed with an equal volume of 3% (w/v) sulphosalicylic acid (SSA). After 2 hours at 4°C the precipitate was centrifuged and the supernatant containing the free amino acids were stored for analysis.

In all cases the tissue free amino acid pool was taken to be the supernatant after homogenisation and precipitation of protein with 3% SSA. The protein pool was always taken to be the precipitate from the above process after the removal of ribonucleic acids and lipids.

<u>Automated Amino Acid Analysis</u>

Specific radioactivity determinations were all performed on a Locarte Automated Amino Acid Analyser - Floor Model (The Locarte Company, London W14). The analyser was fitted with a split stream attachment

allowing the effluent to be both analysed and collected in fractions for radioactivity determinations. Two glass columns, containing the same ion exchange resin (8.25% cross linked; particle size 6-8 μ: as supplied by The Locarte Company) were used. The first column, measuring 23 cm x 0.9 cm, was used to measure the specific radioactivities of the free amino acids in plasma and tissues while the second (50 cm x 0.9 cm) was used for analysis of the protein hydrolysates. The split ratio (volume of column effluent to fraction collector : volume of column effluent to analytical system) was adjusted to be 1:1 for the free amino acids and 4:1 for the protein hydrolysates.

Both columns were eluted with a single buffer (0.3 M lithium, 0.09 M citrate, pH 2.8) containing the following (g/litre): lithium hydroxide 6.71; lithium chloride 5.94; citric acid 18.90; sodium n-octonoate 0.1; Brij 35 0.7; thiodiglycol 5 ml and approximately 10 ml of concentrated HCl. Total running time for one analysis was 240 mins (23 cm column) or 340 mins (50 cm column) at a resin temperature of 36°C and a flow rate of 30 ml/hour. Quantitative determinations of glycine and serine were based on two internal calibration standards, α-amino adipic acid (ADA) and α-amino-n-butyric acid (ANB). These particular standards were chosen in preference to norleucine for two reasons. Firstly they are well resolved from other physiological amino acids and secondly they are eluted from the ion exchange resin at a similar time to glycine. They are also not present in tissue or blood samples at levels that are detectable by this system.

Figures 1 and 2 show analytical traces of a Hamilton calibration standard and of a liver free amino acid sample. It can be seen that glycine is well resolved from both alanine and proline and serine is quantitatively resolved from threonine and well resolved from glutamate. The internal standard equivalent (optical density (570 mu) per mole of

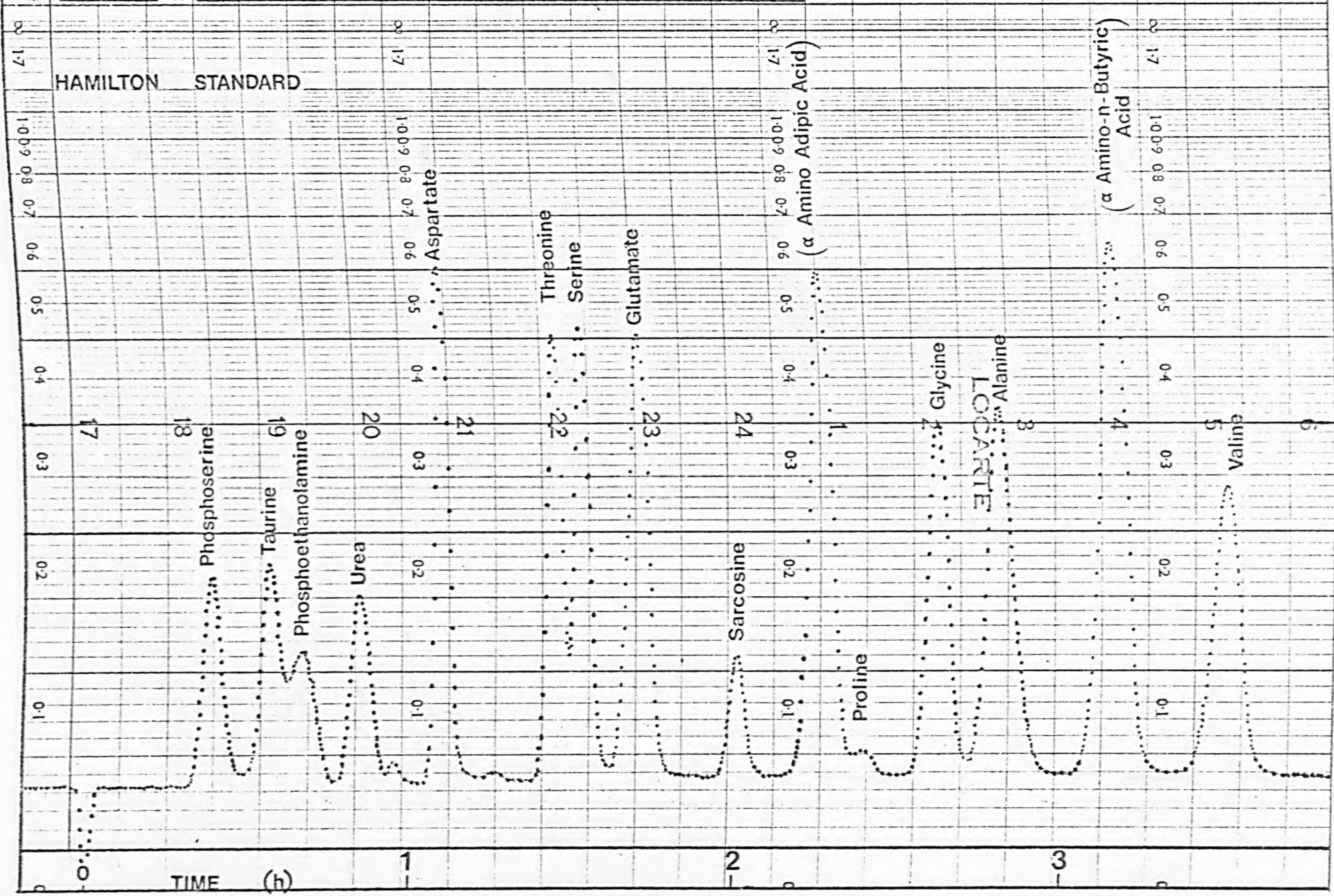
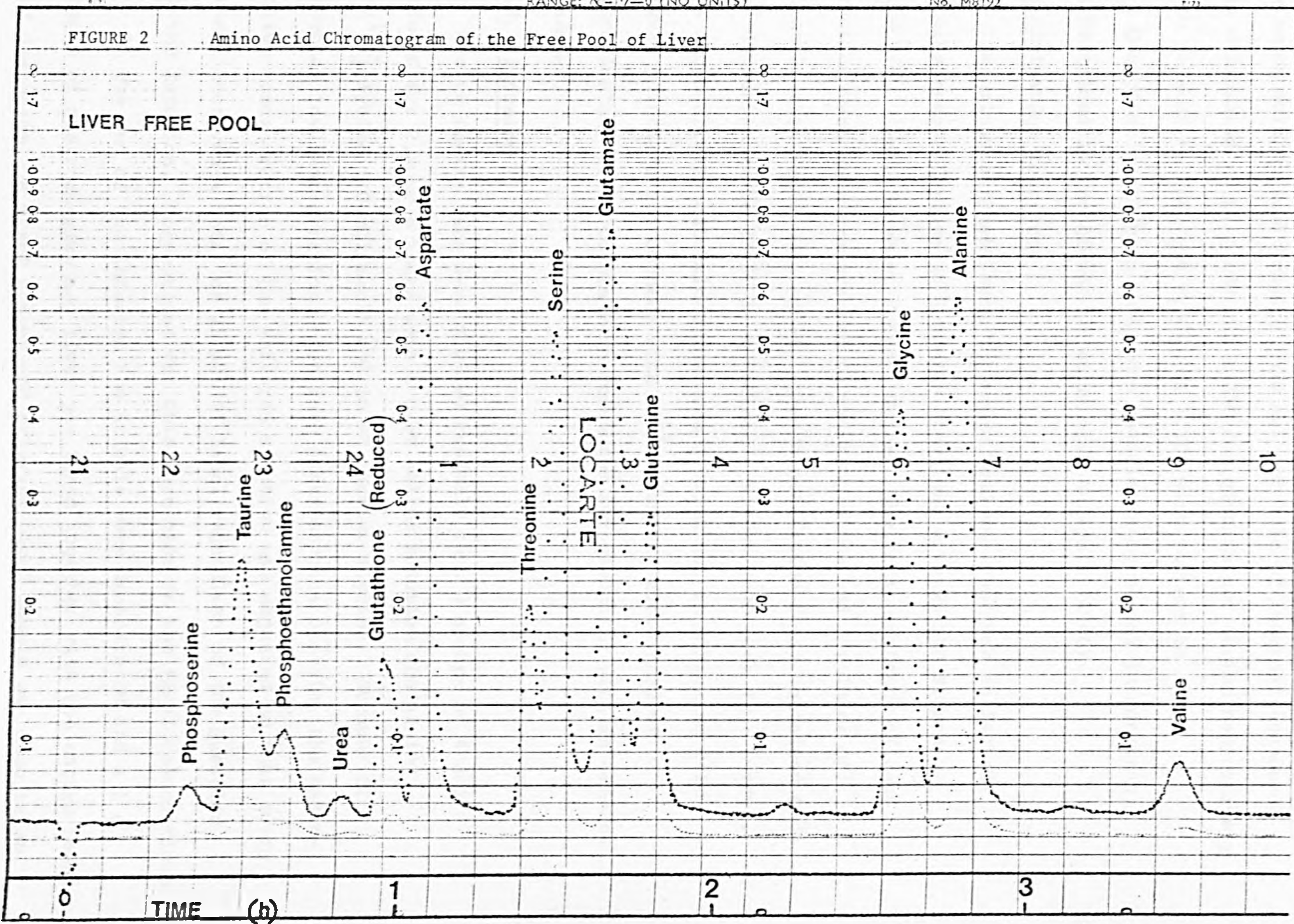


FIGURE 1 Amino Acid Chromatogram of a Standard Calibration Solution.

FIGURE 2 Amino Acid Chromatogram of the Free Pool of Liver



an amino acid/optical density (570 mu) per mole of internal standard) was calculated to be 1.198 and 1.250 for glycine and serine respectively with regards to ADA, and 1.055 and 1.105 with respect to ANB.

Quantitation of both peaks was calculated by manual integration as described in Techniques in Amino Acid Analysis (1966).

Scintillation Counting

The column effluent from the amino acid analyser was collected after stream splitting in 5 minute fractions with an LKB UltraRac (LKB Ltd., South Croydon, Surrey, U.K.). The total contents of each fraction were then transferred to counting vials and 5 volumes of scintillation fluid were added. The scintillation fluid contained 0.4% 2,5-diphenyloxazole (PPO) in a toluene - Triton X100 base (2 : 1 v/v). All radioactivity determinations were carried out in a Packard 2420 liquid scintillation counter with a ^{14}C counting efficiency of approximately 80%. Counts were corrected for quenching with an external standard.

3.2. Infusion 1

Twelve female rats were divided randomly into 3 groups of 4 and infused for 2,4 or 6 hours with a 0.9% saline solution containing [U-^{14}C]glycine (10 mCi/mmol) and 10 mM carrier glycine. Because of expected low incorporation rates into gastrocnemius protein, the radioactive concentration of the infusion solution was adjusted so that every animal received a total of 25 µCi of label regardless of the duration of the infusion. Corrections for this were made in the final calculations.

At the end of the infusion the animals were decapitated and a 1 g sample of gastrocnemius and liver was taken together with the entire heart and diaphragm. A mixed venous and arterial blood sample was also taken. Both tissues and blood were then treated as described in the methods section.

Figure 3 shows the changes in the specific radioactivity of free glycine in plasma and tissue pools. The specific radioactivity of free glycine in plasma rose to a plateau at approximately 2 hours and the level was maintained to the end of the infusion. Similar results have been obtained with the infusion of a number of different amino acids (Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick & Marshall, 1972; Seta _et al._, 1973). In liver and heart there was also little increase in the specific radioactivity of free glycine between 2 and 6 hours but in diaphragm and gastrocnemius muscle there was a continued rise throughout the infusion period. It was estimated by visual extrapolation, that in liver the plateau value reached 55% that of the plasma, in heart it was 65% and although the specific radioactivity was still rising, the plateau value in gastrocnemius and diaphragm was calculated to be 80% in both cases.

The fact that the specific radioactivity at plateau was different in the plasma and the various tissues is indicative of effective compartmentation. One reason for a lower specific radioactivity within the tissue is dilution of the intracellular pool by unlabelled amino acids (glycine) derived from proteolysis (Gan & Jeffay, 1967; Waterlow & Stephen, 1968). This is based on the observation that tissues in which proteolysis is thought to contribute extensively to the supply of intracellular amino acids show lower plateau values. In Figure 3 the liver plateau value is significantly lower than that of heart, diaphragm and gastrocnemius.

By contrast the specific radioactivity of free serine derived from the infused [$U^{14}C$] glycine was much higher in liver, the major site of glycine/serine interconversion (Yoshida & Kikuchi, 1972; 1973; Kikuchi, 1973) than in plasma (Figure 4). It can also be seen that the specific radioactivity of free serine rose above that of free glycine

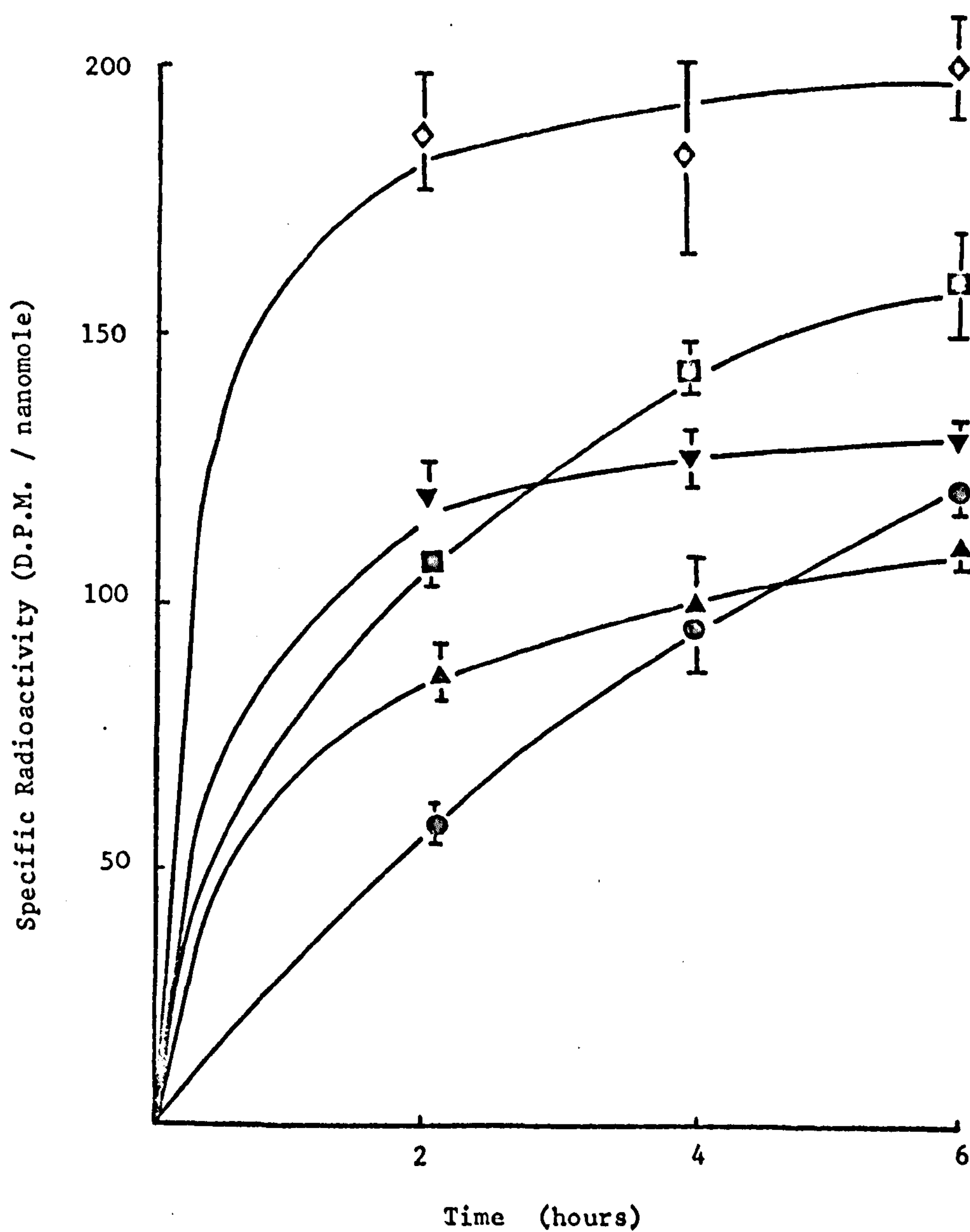


Figure 3 Change in Specific Radioactivity of Free Glycine in Tissue and Plasma Pools.

Rats were continuously infused for 2,4 and 6 hours with [U-^{14}C] glycine. Each point is the mean of four animals (± S.E.M.). Specific radioactivity of free glycine in plasma ◆ ; liver ▲ ; heart ▼ ; diaphragm ■ ; gastrocnemius ● .

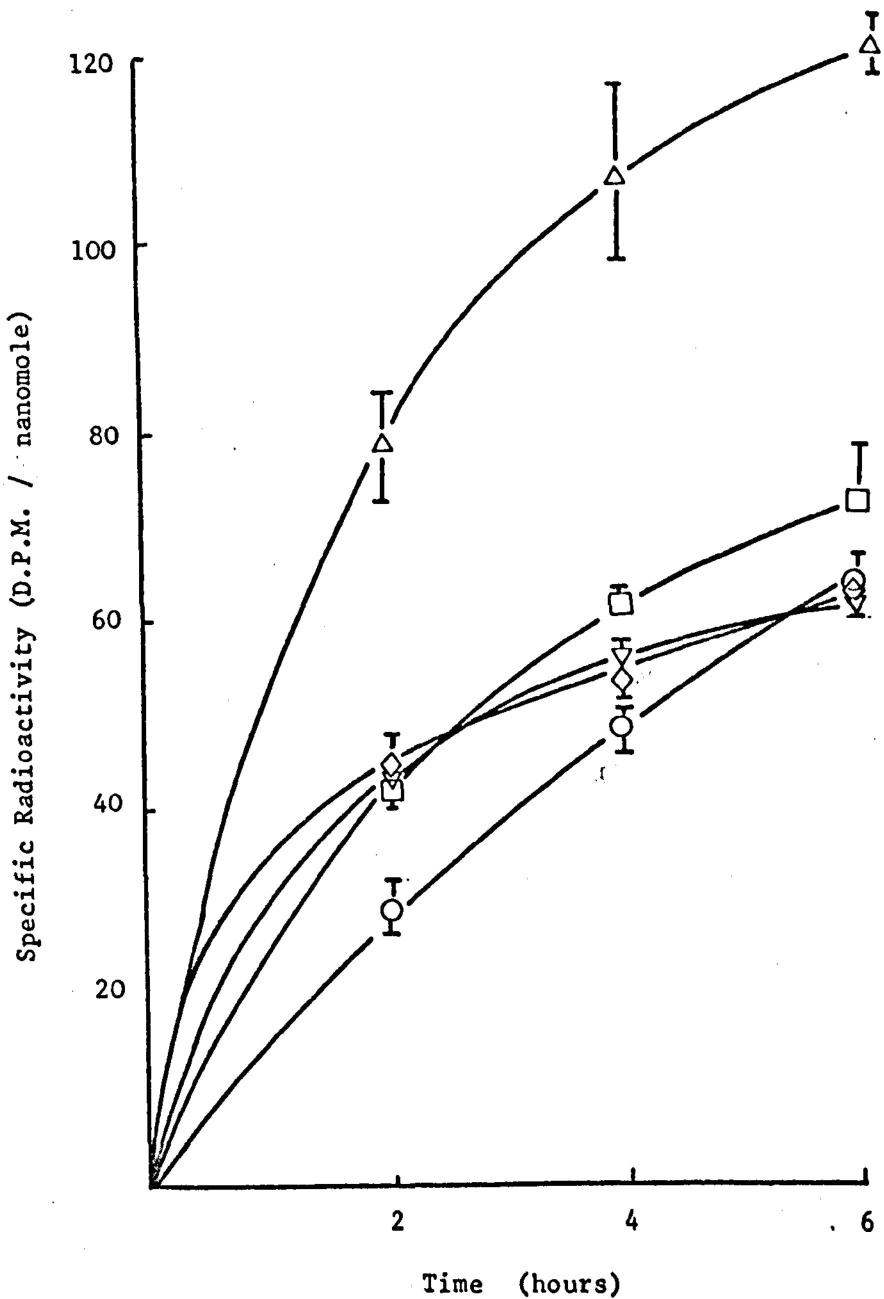


Figure 4 Change in Specific Radioactivity of Free Serine in Tissue and Plasma Pools.

Rats were continuously infused for 2,4 and 6 hours with [U-^{14}C] glycine. Each point is the mean of four animals (±S.E.M.). Specific radioactivity of free serine in plasma ◇ ; liver △ ; heart ▽ ; gastrocnemius ○ ; diaphragm □ ;

(Figure 3). Generally it is not possible for a product to have a higher specific radioactivity than its precursor but in this situation the anomally is explained by the fact that serine is a C_3 amino acid whereas glycine is a C_2 amino acid. Serine is formed by the addition of a carbon atom via methylene tetrahydrofolate to a glycine molecule. If glycine is uniformly labelled and the additional carbon atom is unlabelled both amino acids will have the same specific radioactivity at equilibrium. If, on the other hand, the additional carbon atom is labelled then the specific radioactivity of serine will be higher. The maximum theoretical ratio of the specific radioactivity of serine to glycine is therefore 1.5. In the liver the C_3 of serine is gradually becoming labelled because the methylene moiety of methylene tetrahydrofolate can be supplied by glycine as well (Yoshida & Kikuchi, 1972; 1973; Kikuchi, 1973). Thus the higher specific radioactivity of serine compared to glycine does not necessarily indicate compartmentation of glycine.

Figure 4 also shows that in heart, diaphragm and gastrocnemius the specific radioactivity of free serine was similar to that in plasma. In none of the tissues had the specific radioactivity of serine fully reached a plateau value.

The rates of incorporation of ^{14}C glycine and ^{14}C serine into protein of the various tissues are shown in Figures 5 and 6. Incorporation of both amino acids into liver protein was linear with respect to time without any apparent initial lag period. This absence of a lag period may well be explained in terms of heterogeneity of liver protein turnover. (Schimke, 1970). A lag period should have been evident as the specific radioactivity of free glycine and serine in liver and plasma did not reach a plateau value until about 2 hours after the start of the infusion. If fast turning over proteins compensate for slower turning over proteins then the effect would be to diminish any lag in incorporation.

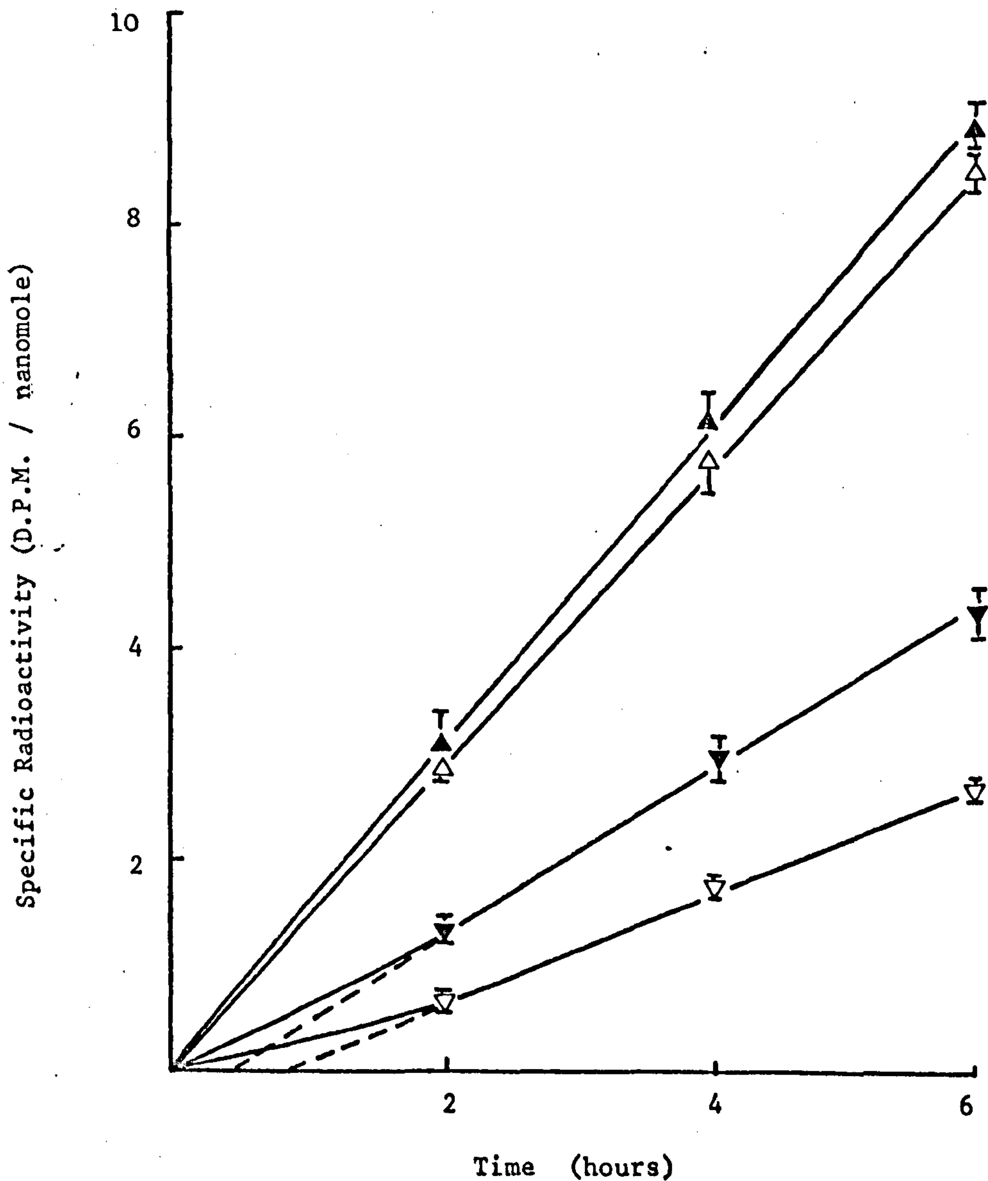


Figure 5 Change in Specific Radioactivity of Protein-Bound Glycine and Serine in Liver and Heart as a Function of Time.

Rats were continuously infused for 2,4 and 6 hours with [U-^{14}C] glycine. Each point is the mean of four animals (± S.E.M.). ▲ , Specific radioactivity of liver protein-bound glycine; △ , liver protein-bound serine; ▼ , heart protein-bound glycine; ▽ , heart protein-bound serine.

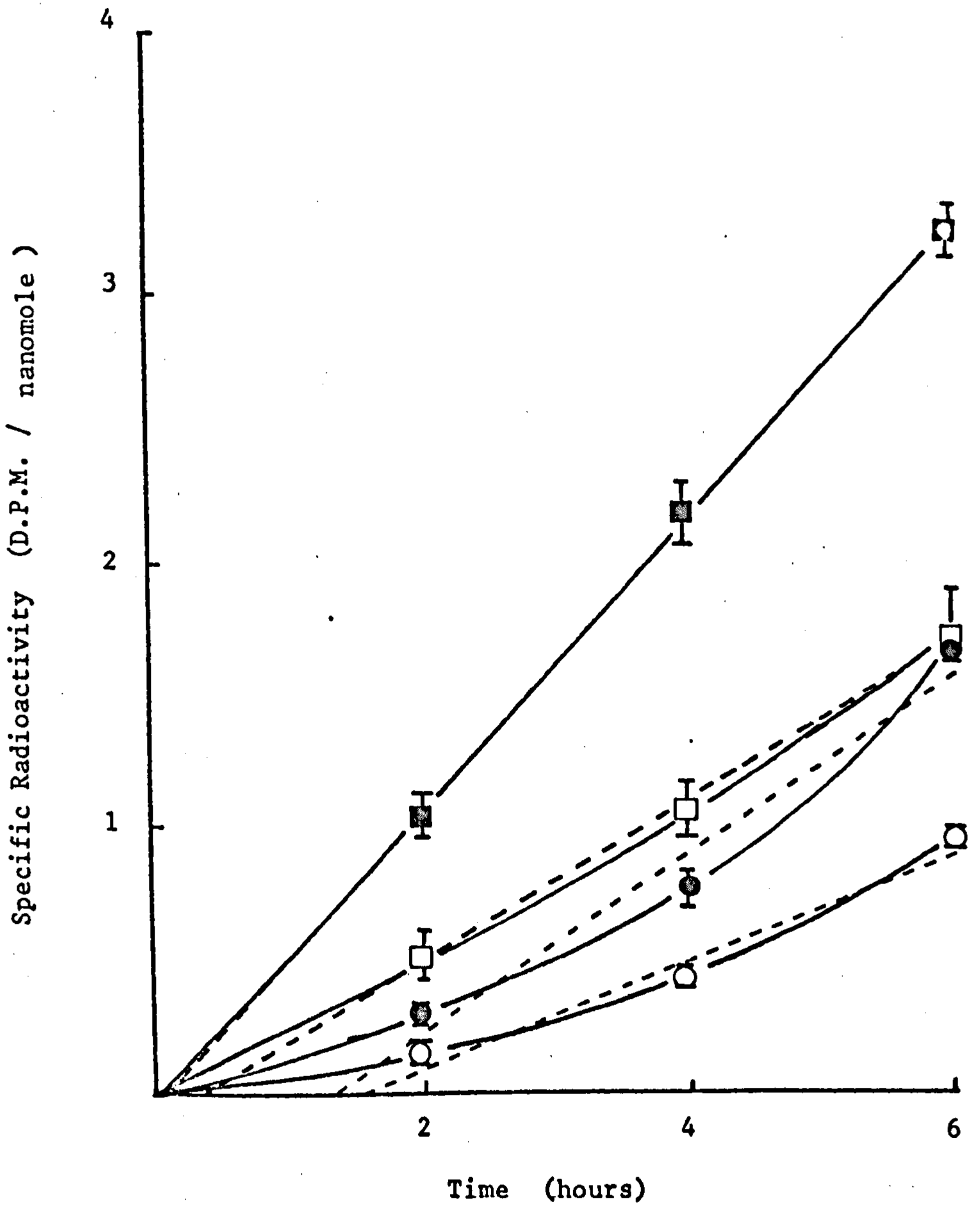


Figure 6 Changes in Specific Radioactivity of Protein-Bound Glycine and Serine in Diaphragm and Gastrocnemius as a Function of Time.

Rats were continuously infused for 2,4 and 6 hours with [U-^{14}C] glycine. Each point is the mean of four animals (± S.E.M.). ■ ,Specific radioactivity of diaphragm protein-bound glycine; □ , diaphragm protein-bound serine; ● , gastrocnemius protein-bound glycine; ○ , gastrocnemius protein-bound serine.

A time lag was however seen in the incorporation of both amino acids into protein of heart, diaphragm and gastrocnemius. In heart and diaphragm the lag period was between 25 - 45 minutes but subsequently the incorporation became linear. In contrast incorporation of glycine and serine in gastrocnemius remained non-linear throughout the 6 hours of infusion. While interpretation of kinetics of incorporation from measurements at only three time points must be made with care, these results on the gastrocnemius suggest that glycine and serine at the site of protein synthesis do not have the same specific radioactivity as in plasma. If one assumed that the free amino acids in plasma were the immediate precursors for protein synthesis the incorporation should have been almost linear for both amino acids, since the specific radioactivity of free glycine in plasma rose by only 10% between the second and sixth hour of infusion and that of serine by 40%. On the other hand if the assumption is made that the tissue free pool is the immediate precursor, then incorporation of both amino acids into gastrocnemius protein would have to follow curvi-linear kinetics because the specific radioactivity of free glycine in the tissue pool rose by 120% in the same period and that of serine by 130%. In Figure 6 the graphs depicting the incorporation into protein of gastrocnemius are better explained by a model that proposes that the tissue free pool is the precursor of protein.

In liver heart and diaphragm it is not possible to say with any certainty that the rate of increase of the specific radioactivity of protein is more consistent with incorporation from one or other of the free pools because the changes in the specific radioactivities of the free amino acids over the 4 hour period were much smaller than those seen in the gastrocnemius. In heart and liver for instance the changes in the specific radioactivity of free glycine and serine were similar to

those seen in the plasma, being of the order of 15 and 30% for glycine and 40 and 50% for serine over the 4 hour period. Even in diaphragm the changes were not totally different from that in plasma. In this tissue the specific radioactivity of free glycine rose by 55% and free serine rose by 75% between 2 and 6 hours.

Table 6 shows the ratio of the specific radioactivities of serine to glycine in the plasma free, tissue free and tissue bound pools. These results are also illustrated in Figure 7. The ratio of the free amino acids in every tissue was appreciably higher than that in the plasma owing either to the intracellular conversion of glycine into serine (Yoshida & Kikuchi, 1973) or to differential rates of cellular uptake of these amino acids (Henriques et al., 1955; Guidotti et al., 1971; Banos et al., 1971; Figures 1 and 2). The ratios in the various tissue proteins was also much higher than that in the plasma and was much nearer to those of the respective tissue pools. The minimum difference between the plasma ratio and the protein ratio was 75%, occurring in diaphragm after 6 hours of infusion. In comparison the maximum difference between the ratios in protein and the respective tissue free ratio was 26%. This occurred with heart after 2 hours of infusion.

The data presented above therefore seems to suggest that the specific radioactivity of the precursor pool for protein synthesis is not represented by that of mixed venous and arterial plasma. The extent to which the ratio in plasma varies with different sites of sampling may, of course, have a significant bearing on the interpretation of these results, but as was shown by Wolff and Bergman (1972) in sheep, and as will be shown in Infusion 4, any differences that occur are small and do not alter the interpretation of the results.

Table 6 . The Ratio of the Specific Radioactivity of Serine to Glycine in the Free and Protein-Bound Pool of Liver, Heart, Diaphragm and Gastrocnemius and in the Free Pool of Plasma after Infusion of [U-^{14}C]Glycine.

Tissue	Amino Acid Pool	$\frac{\text{Specific Radioactivity of Serine}}{\text{Specific Radioactivity of Glycine}}$ Time of Infusion (hours)		
		2	4	6
Plasma	Free	0.245 + 0.023	0.296 + 0.036	0.304 + 0.029
Liver	Free	1.052 + 0.006	1.080 + 0.037	1.108 + 0.020
	Protein	0.938 + 0.009	0.933 + 0.022	0.952 + 0.026
Heart	Free	0.387 + 0.011	0.466 + 0.026	0.487 + 0.043
	Protein	0.486 + 0.048	0.585 + 0.016	0.609 + 0.034
Diaphragm	Free	0.396 + 0.043	0.438 + 0.020	0.464 + 0.031
	Protein	0.472 + 0.049	0.482 + 0.019	0.533 + 0.027
Gastrocnemius	Free	0.518 + 0.100	0.519 + 0.030	0.530 + 0.021
	Protein	0.486 + 0.010	0.564 + 0.045	0.573 + 0.021

Each result is the Mean (± S.E.M.) of four animals.

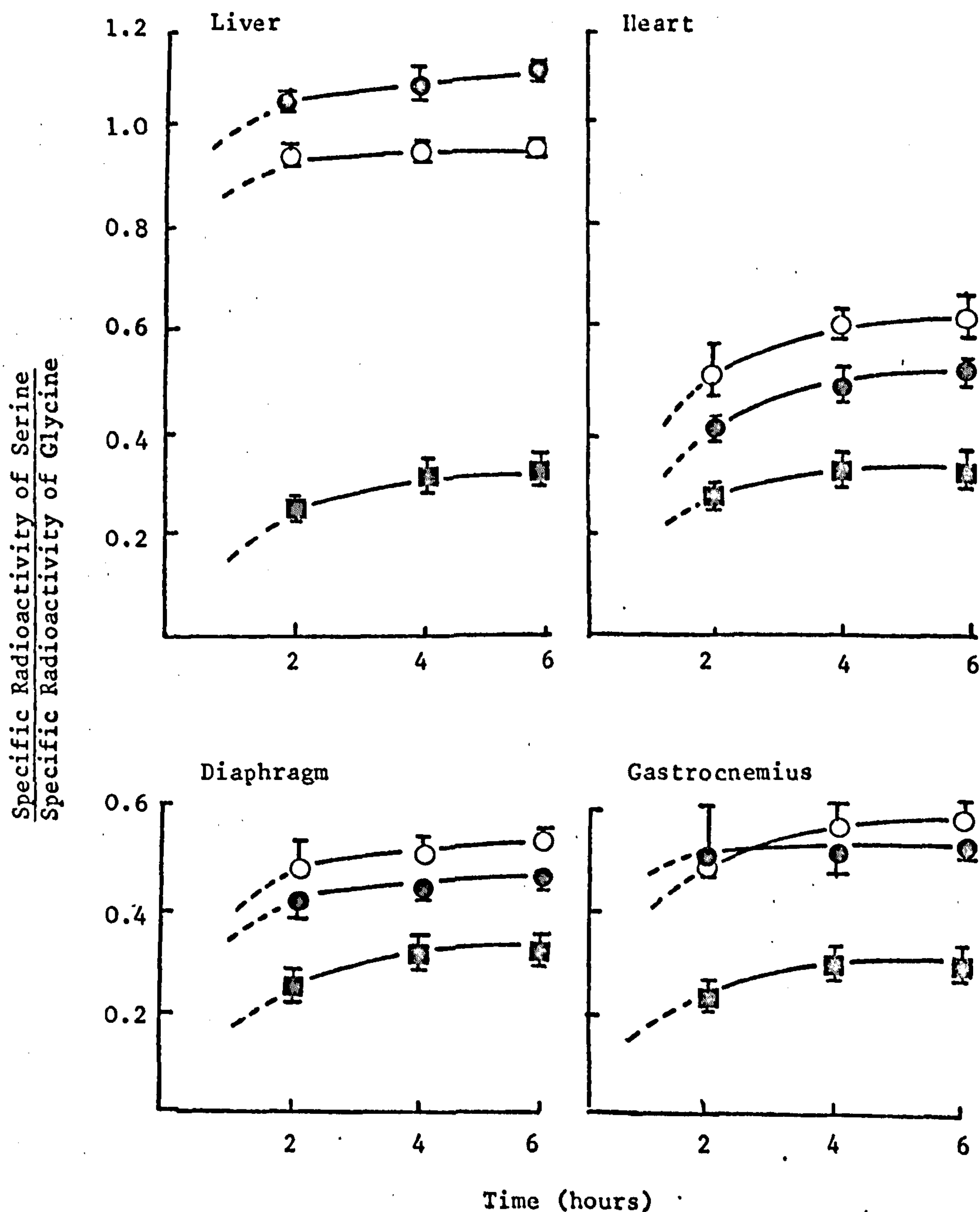


Figure 7 — Ratio of the Specific Radioactivity of Serine and Glycine in Plasma, Liver, Heart, Diaphragm and Gastrocnemius as a Function of Time.

Each point is the mean (± S.E.M.) derived from the constant infusion of four rats. □ , Ratio of specific radioactivity of free serine and free glycine in plasma; ○ , ratio of the specific radioactivity of free serine and free glycine in the tissue pool; ○, ratio of the specific radioactivity of protein-bound serine and protein-bound glycine of the tissue.

3.3. Infusion 2

In order to establish whether the findings of Infusion 1 extended to other tissues a further study was carried out involving the infusion of an identical [U-^{14}C] glycine solution. In Infusion 2 a total of four rats were infused, 2 for 2 hours and 2 for 6 hours. At the end of the infusion a mixed venous and arterial plasma sample was taken together with the whole brain and kidney. A 1.5 g sample of jejunum was also taken 15 cm from the pylorus after the luminal contents had been washed out with 0.9% saline. All tissues were prepared and analysed in the same way as in Infusion 1.

The choice of tissues in this investigation was governed by the need to study systems which differed in some important respects from those previously examined. The brain, kidney and jejunum were all chosen because of their known intricate anatomical structure which could consequently give rise to complexities in amino acid compartmentation. The kidney was also chosen because of its relatively high capacity for the interconversion of glycine and serine (Yoshida & Kikuchi,1973; Sato et al., 1969; Pitts et al., 1970), the brain for its inability to exchange amino acids with blood rapidly (Lajtha et al., 1959) and the jejunum for its high rate of protein turnover (Millward & Garlick, 1972).

Table 7 gives the results of this investigation. The specific radioactivity of free glycine and serine in plasma reached values similar to but slightly lower than those seen in Infusion 1. However the ratios of the specific radioactivity of serine to that of glycine were identical with those observed earlier. The specific radioactivity of free glycine in all three tissues remained appreciably lower than in plasma. After 6 hours of infusion it had achieved 80% of the plasma value in jejunum, 53% in kidney, and only around 6% in brain. The specific radioactivity of free serine in kidney and jejunum on the other

Table 7. The Specific Radioactivity of Serine and Glycine in the Free and Protein-Bound Pools of Kidney, Jejunum and Brain and in the Free Pool of Plasma After the Infusion of $[U-^{14}C]$ Glycine.

		Specific Radioactivity (DPM/nanomole)				Serine Specific Radioactivity / Glycine Specific Radioactivity	
		Serine		Glycine			
Time of Infusion (hours)		2	6	2	6	2	6
Plasma	Free Pool	23.0	44.5	105.3	150.2	0.218	0.296
Kidney	Free Pool	27.3	56.2	49.1	80.8	0.557	0.696
	Protein Pool	1.02	3.83	1.80	6.12	0.566	0.626
Jejunum	Free Pool	16.3	85.6	35.7	119.6	0.603	0.652
	Protein Pool	2.42	7.58	4.00	11.65	0.605	0.651
Brain	Free Pool	6.0	11.7	6.2	10.5	0.790	1.035
	Protein Pool	0.069	0.361	0.090	0.350	0.766	1.031

hand remained higher than in the plasma at the end of the infusion. In brain the value remained much lower, achieving only 25% of the plasma value.

The specific radioactivity of both amino acids in protein is also given in Table 7. As expected the values were very much less than in the respective tissue free pools. Even in jejunum, despite its high rate of protein turnover, the specific radioactivity of either amino acid had only reached about 10% of the free value. These figures also give some indication of the initial lag in incorporation. For comparative purposes the 6 hour value should be three times the 2 hour value. This is most closely approached in jejunum. As in the liver in Infusion 1, the absence of any lag may result from heterogeneity of protein turnover rates. In both kidney and brain the specific radioactivity of free glycine or serine in protein at 6 hours was greater than three times the 2 hour value.

Comparison of the specific radioactivity ratios of serine to glycine in protein with those in plasma and tissue free pools leads to the same conclusion as in Infusion 1. At both times the   ratio in protein of kidney, jejunum and brain was almost identical to that seen in the respective tissue pools and differed appreciably from the ratio in plasma. It is evident that in these tissues the free amino acids in plasma are not the direct precurors of tissue proteins and that those in the free pool are more immediately related to the protein synthetic machinery.

3.4. Infusion 3

As a logical sequal to the infusion of radioactive glycine and comparison of the ratio of the specific radioactivity of metabolically derived serine with that of the infused glycine, this study was designed to observe the system in the reverse direction i.e. by the infusion of radioactive serine and its metabolic conversion to glycine.

In this investigation two rats were infused for 2 hours and a further two for 6 hours with L-[U-^{14}C]serine. The conditions for the infusion and the subsequent treatment and analysis of the samples were identical to those of Infusions 1 and 2. The sample of plasma was taken, as before, from a mixed venous and arterial blood specimen. All tissues previously investigated were also studied in this infusion.

The analytical results are documented in Table 8. As the specific radioactivities of serine and glycine were only measured at two time points it is not feasible to discuss the kinetics or absolute value of the plateau specific radioactivity, but by relating the tissue free values to those of the plasma at each time point some interesting observations emerge. Firstly, the specific radioactivity of free serine in liver remained at about the same value relative to that of plasma (45%) as did the specific radioactivity of free glycine during the infusion of [U-^{14}C] glycine in Infusion 1 (55%). Tentatively this applies also to the jejunum. In contrast to this in certain other tissues the equilibration of serine between the tissue free pool and the plasma was appreciably more rapid than that observed for glycine. In heart, for example, the specific radioactivity of free glycine during the infusion of [U-^{14}C]glycine was 65% of the plasma level (Fig. 3). This was after 6 hours and after a plateau value had been achieved. In contrast after 6 hours infusion of L-[U-^{14}C] serine the tissue free serine was about 90% of the plasma level. This rapid equilibration of serine was also seen in Infusion 1 (Fig. 4) where the specific radio-activity of free serine derived from ^{14}C glycine remained insignificantly different from that in the plasma at 2, 4 and 6 hours. One explanation for this is that serine is much more rapidly exchanged across the plasma membrane than is glycine (Banos <u>et al.</u>, 1971) and the exchange is rapid enough to overcome the diluting effect of proteolysis. In heart this

Table 8. The Specific Radioactivity of Serine and Glycine in the Free and Protein-Bound Pool of Various Tissues and in the Free Pool of Plasma after the Infusion of L-[U-^{14}C] Serine.

		Specific Radioactivity (SR) (DPM / nanomole)				Glycine SR / Serine SR	
		Serine		Glycine			
	Time of Infusion (h) ...	2	6	2	6	2	6
Tissue	Amino Acid Pool						
Plasma	Free	65.2	90.5	13.4	23.8	0.206	0.263
Liver	Free	30.5	42.4	16.4	27.5	0.538	0.649
	Protein	1.27	3.63	0.55	1.93	0.433	0.532
Heart	Free	71.9	79.3	21.7	33.0	0.302	0.416
	Protein	0.88	2.75	0.22	0.90	0.250	0.327
Diaphragm	Free	70.3	88.6	16.2	29.8	0.230	0.336
	Protein	0.69	2.39	0.14	0.62	0.203	0.259
Gastrocnemius	Free	55.7	86.9	9.60	21.9	0.172	0.252
	Protein	0.36	1.18	0.06	0.26	0.167	0.220
Kidney	Free	28.9	73.3	16.7	33.5	0.284	0.457
	Protein	2.14	5.35	0.67	1.89	0.313	0.353
Jejunum	Free	40.2	75.7	13.6	33.6	0.338	0.444
	Protein	4.77	16.1	1.42	6.71	0.298	0.417
Brain	Free	12.7	17.8	5.70	7.60	0.375	0.426
	Protein	0.18	0.66	0.06	0.32	0.333	0.485

cannot be explained simply by differences in the intracellular pool sizes because the total tissue pool of free serine was about twice that of the glycine pool (see Table 13). It cannot also be entirely explained by interconversion of glycine and serine, because although interconversion does occur, it only does so to a limited extent. For example, Yoshida and Kikuchi (1973) measured the activity of serine hydroxymethyl transferase (E.C.2.1.2.1.) in rat heart and found it to be less than 5% of the activity in liver. Similarly Mowbray and Last (1974) working with perfused hearts reported that after a 60 minute perfusion with ^{14}C glycine, serine contained only 7.5% of the radioactivity found in glycine.

The same phenomenon was seen with the diaphragm and gastrocnemius. In Table 8 the tissue specific radioactivity of free serine in diaphragm after 6 hours of infusion was 98% of the plasma level and in gastrocnemius it was 96%. Similarly in Figure 4 the specific radioactivity of free serine derived from the infused glycine in both tissues is also not very different from the plasma value.

Some further evidence for the rapid equilibration of serine in gastrocnemius muscle is seen in Table 9, in which the results of rat hind limb perfusions are given. The experimental details of this procedure are given at the end of this section. After 2 hours perfusion with $[U-^{14}C]$ glycine, the tissue pool of free glycine in gastrocnemius achieved only 51% of the perfusate specific radioactivity. In contrast, when L-$[U-^{14}C]$ serine was perfused for 2 hours the specific radioactivity of free serine in gastrocnemius rose to 98% of the perfusate value.

It appears, therefore, that in muscle serine behaves in a manner different to that observed for several other amino acids. In practical terms this is very important because if the specific radioactivity of free serine is similar in the free pool of muscle as it is in plasma it implies one of two possibilities. The first is that the specific

Table 9 . Specific Radioactivity of Glycine and Serine in the Free Pool of Gastrocnemius and the Perfusate after Perfusion of Hindlimbs with either [U-^{14}C] Glycine or L-[U-^{14}C] Serine.

[U-^{14}C] Glycine Added to Perfusate	Specific Radioactivity (DPM/nanomole)				Specific Radioactivity of Serine / Spacific Radioactivity of Glycine	
	Gastrocnemius		Perfusate			
Perfusion	Serine	Glycine	Serine	Glycine	Gastrocnemius	Perfusate
1	12.0	79.7	9.1	175.5	0.151	0.052
2	14.4	77.0	13.5	137.1	0.187	0.098
3	13.1	84.0	12.0	159.4	0.156	0.075
Mean	13.2	80.2	11.5	157.3	0.165	0.075

L-[U-^{14}C]Serine Added to Perfusate	Specific Radioactivity (DPM/nanomole)				Specific Radioactivity of Glycine / Specific Radioactivity of Serine	
	Gastrocnemius		Perfusate			
Perfusion	Serine	Glycine	Serine	Glycine	Gastrocnemius	Perfusate
1	80.7	9.1	86.9	10.3	0.113	0.119
2	81.1	10.7	79.5	12.2	0.132	0.153
3	82.6	10.4	84.5	12.1	0.126	0.143
Mean	81.5	10.1	83.6	11.5	0.124	0.138

radioactivity of the precursor is effectively the same as that of the free pool of plasma and muscle. The second possibility is that the precursor pool is small and although it may in fact have a lower specific radioactivity, this is masked by the much larger intracellular pool. If the first possibility is true then the rate of protein synthesis in these tissues can be calculated with a reasonable degree of accuracy by relating the rate of isotope incorporation into protein to the specific radioactivity of the free amino acid in the tissue or plasma pool.

The increased transport of serine was also seen in the case of kidney and brain but there was still a marked difference between the intracellular specific radioactivity and that of the plasma.

With the exception of gastrocnemius and brain the specific radio-activity of free glycine derived from the infused serine was equal to or greater than that in plasma in all other tissues. This was true after both 2 and 6 hours of infusion. In gastrocnemius the specific radio-activity of free glycine was slightly lower than in plasma but brain only achieved 30% of the plasma value.

Table 8 also shows the ratio of the specific radioactivity of glycine to that of serine in plasma and tissue free pools and in the tissue protein. Except in gastrocnemius, the specific radioactivity ratio of the free amino acids in plasma was always lower than in the tissue. In the protein of liver, kidney, jejunum and brain the ratio was closer to that of the free amino acids in the tissue pool than in the plasma. This confirms the conclusions drawn from Infusions 1 and 2, that in these tissues the precursor pool for protein synthesis is more likely to be the free pool of the tissue than of the plasma.

With the three types of muscle the position is less clear. In gastrocnemius the ratio at 2 and 6 hours was almost the same in all three pools; protein, tissue free and plasma free amino acids. In

heart the ratio in protein was between that of the two free pools and in the protein of diaphragm it was closer to the free amino acid pool in the plasma. This experiment therefore does not exclude the possibility that the plasma free pool is the precursor for protein synthesis in diaphragm and perhaps gastrocnemius. It is unlikely that this can be the case in heart because the specific radioactivity ratio should always be higher in the precursor than in the protein. This seems to suggest that the plasma free pool is not the precursor for the synthesis of protein in heart muscle.

3.5. Rat Hemi-Corpus Perfusion

The purpose of the rat hemi-corpus perfusion was first, to demonstrate a more rapid equilibration of serine in comparison to glycine, between the perfusion medium and the free pool of gastrocnemius, and secondly to determine the extent of glycine-serine interconversion in peripheral tissue.

Six female hooded rats (180 g) were prepared for hemi-corpus perfusion by the procedure of Ruderman, Houghton and Hems (1971). Briefly, the operative procedure involved anaesthesia with an oxygen/ether mixture followed by a mid-line incision in the abdominal wall. The subsequent dissection involved the tying of ligatures around the tail, rectum and bladder and around the following blood vessels: the arteries to the body wall and skin, the inferior mesenteric artery, the ovarian arteries, the renal and suprarenal arteries and veins and the coelic and superior mesenteric arteries. All visceral tissues except for the small and large intestine and the liver were then removed from the animal. The cannula was inserted and tied in position in the descending aorta just below the level of the diaphragm. Finally, after the intestine and liver were removed the animal was transected above the level of the aortic cannula and perfusion of the tissue started. The preparation was perfused without perfusate recycling for 10 minutes before any

studies were attempted. The final preparation weighed 65 g (after perfusion) of which muscle and bone constituted 75% of the weight.

The perfusion medium was fundamentally a Krebs-Henseleit bicarbonate buffer (Umbreit, Burris & Stauffer, 1949), containing plasma levels of essential and non-essential amino acids at concentrations given in Table 2. The buffer was gassed with a mixture of oxygen/carbon dioxide (95:5) for 15 minutes. "Aged" human blood (21 days old) was centrifuged at 1,000 g for 5 minutes at 4°C. After aspirating the plasma the packed red blood cells were washed 3 times with the Krebs-Henseleit buffer before being finally centrifuged to give a packed cell preparation. The perfusate was prepared from these packed washed human erythrocytes (90 ml), dialysed bovine serum albumin Fraction V (60 ml of a 15% solution: w/v), Heparin (0.4 ml of a solution containing 1,000 U/ml) and D-glucose (1.0 ml of a 20% solution : w/v). The volume was adjusted to 200 ml with oxygenated Krebs-Henseleit buffer giving a final albumin concentration of 4.5%.

The perfusion system was basically the same as that described by Ruderman et al., (1971) with facilities for recycling of the perfusion medium. The perfusion pressure was adjusted to 60-80 mm Hg giving a flow rate of between 10-12 ml/minute. The ambient temperature was controlled at 37°C and evaporation was minimised by placing the hemicorpus preparation in a small sealed container.

After the initial 10 minutes of non-recycled perfusion [U-^{14}C] glycine and [U-^{14}C] serine was added as a pulse dose and the system altered so that the perfusate was subsequently recycled. Each rat was perfused for 90 minutes, at the end of which time samples of gastrocnemius and perfusion medium were taken and the specific radioactivity of free serine and glycine determined. The method of analysis was identical to that in infusions 1-3.

The hemi-corpus preparation remained viable throughout the 90 minutes of perfusion as judged by various criteria. Firstly, the physical appearance of the tissue, especially the feet, remained unchanged and showed no signs of cyanosis. There was also no evidence of oedema and the tissue water content at the end of perfusion was 73%. Secondly, the potassium concentration in the perfusion medium showed little signs of change and remained at approximately 4 m Eq/litre. Thirdly, the rate of glucose consumption and lactate production remained unchanged during the perfusion period.

The results of this study are given in Table 9 and the significance of them has already been discussed (Section 3.4). No measurements of isotopic incorporation into protein were made. Because of the low level of insulin used in the perfusion system (0.05 m U/ml) and the comparatively short duration of the experiment, the amount of incorporation of radioactivity into the protein was relatively small, and in the case of the metabolically derived amino acid it was too low to measure accurately.

3.6. Estimated Fractional Rates of Tissue Protein Synthesis

To show the relationship between the free amino acid pool of tissue and plasma and that of the precursor pool, the fractional rates of protein synthesis have been calculated from the data of the incorporation of both amino acids into protein. Theoretically when calculations of the rate of protein synthesis are based on the specific radioactivity of the true precursor pool the rates derived from one amino acid should not differ from that derived from any other amino acid.

The method of calculation was that of Seta *et al.*, (1973) in which the increase in specific radioactivity of each amino acid in the protein between 2 and 6 hours of infusion was related to the precursor specific radioactivity and the period of incorporation in the following way:

$$\text{Fractional Rate of Protein Synthesis} = \frac{P_6 - P_2}{S\ t}$$

where P_6 = Specific radioactivity of protein after 6 hours infusion

P_2 = Specific radioactivity of protein after 2 hours infusion

S = Specific radioactivity of the precursor pool

t = time of incorporation expressed as fraction of a day

The specific radioactivity of the precursor was taken to be that of the average value of the free amino acid either in the free pool of the tissue or in the plasma between the second and the sixth hour of infusion.

Table 10 gives the fractional rates of protein synthesis calculated from the results of infusions 1, 2 and 3. In each case the ratio is expressed as a percentage of the total tissue protein mass synthesised per day. If the results of each infusion are compared separately, it is evident that when the free amino acids in the plasma are assumed to be the precursor, the estimated rates of protein synthesis differ considerably. In contrast, the estimated rates of protein synthesis based on the tissue specific radioactivity of glycine and serine are very similar indeed. This is with the exception of heart, diaphragm and gastrocnemius in infusion 3. Here, there is little to choose between either amino acid or either precursor site. Reasons for this have been discussed earlier. In addition, in all the tissues except jejunum, synthesis rates calculated from the specific radioactivity of the tissue pool were similar irrespective of which amino acid was infused. The discrepancy in the rates of synthesis for jejunum between Infusions 2 and 3 remain unexplained.

3.7. Cellular Metabolism of Glycine and Serine

In order to appreciate the extent of metabolism of glycine and serine in various tissues of the rat, the percentage recovery of radioactivity of each amino acid was calculated. These results, shown in Tables 11 and 12,

Table 10 . Estimated Rates of Protein Synthesis for the Tissues Studied in Infusions 1, 2 and 3.

Tissue	Infusion	Infused Amino Acid	Fractional Rate of Protein Synthesis (%/day). Precursor Site: Tissue		Plasma	
			Glycine	Serine	Glycine	Serine
Liver	1	Glycine	35.9	33.1	18.0	60.6
	3	Serine	37.8	38.8	46.2	19.1
Heart	1	Glycine	15.3	23.0	9.6	22.3
	3	Serine	14.8	14.8	22.3	15.1
Diaphragm	1	Glycine	9.6	12.6	6.7	13.3
	3	Serine	12.4	13.0	15.8	13.8
Gastrocnemius	1	Glycine	9.2	10.5	4.2	8.9
	3	Serine	7.4	6.9	6.5	6.6
Kidney	2	Glycine	39.9	35.3	20.3	50.2
	3	Serine	29.2	29.1	40.6	25.9
Jejunum	2	Glycine	59.1	60.8	36.0	91.8
	3	Serine	134.0	118.6	176.6	92.1
Brain	2	Glycine	18.7	19.7	1.2	5.8
	3	Serine	26.0	19.3	8.8	1.3

are expressed as the percentage of the total counts, in either the free or in the protein bound pool, attributable to glycine and serine. They are calculated from Infusions 1, 2 and 3.

In the free pool of all tissues except gastrocnemius and diaphragm, glycine and serine together accounted for less than 70% of the total radioactivity. In many tissues this figure was less than 50% and in the extreme case of the brain (after infusion of [U-^{14}C]glycine) it was as low as 34%. The protein bound pool was similarly analysed for percentage recovery. Tables 10 and 11 show that, with one exception, over 90% of the total radioactivity of the protein bound pool was associated with serine and glycine. In several instances the recovery was much nearer 100%. This indicates that the radioactivity in the free pool which was not attributable to glycine or serine could not be contained in other amino acids to any appreciable extent.

3.8. The Pool Size of Free Glycine and Free Serine in Tissues

The total pool sizes of free serine and glycine were measured in plasma and tissues at the end of Infusions 1, 2 and 3. They were measured by an automated amino acid analyser by comparison with two internal calibration standards, α-aminoadipic acid and α-amino-n-butyric acid.

The levels are presented to show that in the tissues studied the amounts of serine and glycine within the free pool are not similar, nor do they bear a constant relationship to each other. They are also presented as data from which extracellular corrections can be calculated.

The results, shown in Table 13, are given separately for the infusion of each amino acid. The pool size of glycine, and to a lesser extent, of serine varied considerably from tissue to tissue. Plasma contained the lowest levels of both amino acids, whereas the highest level of glycine was seen in the kidney. The highest level of serine occurred in gastrocnemius during the infusion of serine. The relative amounts of serine to glycine

Table 11 Percentage of Total Radioactivity Associated with Glycine and Serine in the Tissue Free and Tissue Protein Pools after 6 Hours Infusion of $[U-^{14}C]$ Glycine.

Tissue	Fraction of Total Counts in Glycine and Serine (%)					
	Free Pool			Protein Pool		
	Glycine	Serine	Sum	Glycine	Serine	Sum
Plasma	38.8	16.5	55.3	-	-	-
Liver	28.8	19.5	48.3	56.9	42.2	99.4
Heart	30.1	29.3	59.4	72.1	32.1	104.2
Diaphragm	47.5	26.1	73.6	75.0	28.0	103.0
Gastrocnemius	62.6	20.6	83.2	69.8	30.8	100.6
Kidney	46.2	11.5	57.7	-	-	-
Brain	15.6	18.0	33.6	-	-	-
jejunum	33.0	13.1	46.1	-	-	-

Table 12 Percentage of Total Radioactivity Associated with Glycine and Serine in the Tissue Free and Tissue Protein Pools after 6 Hours Infusion of L-[$U^{14}C$] Serine.

Tissue	Fraction of Total Counts in Glycine and Serine (%)					
	Free Pool			Protein Pool		
	Glycine	Serine	Sum	Glycine	Serine	Sum
Plasma	5.3	53.1	58.4	-	-	-
Liver	20.2	29.7	49.9	30.5	55.2	85.7
Heart	9.6	59.8	69.4	23.0	72.3	95.3
Diaphragm	11.5	71.3	82.8	20.4	72.3	92.7
Gastrocnemius	16.4	72.9	89.3	19.1	81.3	100.4
Kidney	24.2	19.7	43.9	24.8	69.9	94.7
Brain	13.2	36.0	49.2	30.0	63.4	93.4
Jejunum	24.8	36.2	61.0	28.4	75.4	103.8

Table 13 . The Pool Size of Free Glycine and Serine in Plasma and Tissues after 6 Hours Infusion of Either $[U-^{14}C]$ Glycine (10 mM) or L-$[U-^{14}C]$ Serine (10 mM).

	Pool Size of Free Amino Acid (µmoles/g tissue or µmoles/ml plasma)			
Infused Amino Acid	Glycine (10mM)		Serine (10mM)	
	Serine	Glycine	Serine	Glycine
Plasma	0.46 ± 0.03 (12)	0.30 ± 0.01 (12)	0.61 ± 0.05 (4)	0.26 ± 0.03 (4)
Liver	1.31 ± 0.08 (12)	2.01 ± 0.11 (12)	2.34 ± 0.25 (4)	2.32 ± 0.22 (4)
Heart	1.01 ± 0.03 (12)	0.47 ± 0.02 (12)	1.23 ± 0.27 (4)	0.52 ± 0.02 (4)
Diaphragm	1.49 ± 0.06 (12)	1.12 ± 0.05 (12)	1.77 ± 0.03 (4)	1.01 ± 0.08 (4)
Gastrocnemius	1.48 ± 0.05 (12)	2.20 ± 0.09 (12)	2.18 ± 0.08 (4)	2.33 ± 0.25 (4)
Kidney	1.40 ± 0.04 (4)	4.20 ± 0.20 (4)	1.04 ± 0.04 (4)	3.49 ± 0.12 (4)
Brain	1.10 ± 0.06 (4)	0.98 ± 0.05 (4)	0.95 (2)	0.90 (2)

also varied from one tissue to another. In kidney, for instance, the glycine pool was three times larger than the serine pool, whereas in heart the serine pool was a little over twice the size of the glycine pool.

In general the concentration of glycine altered little in response to changing the infused amino acid from glycine (10 mM) to serine (10 mM) In contrast the free pool size of serine was larger in plasma, liver, heart, diaphragm, and gastrocnemius when serine was infused. The largest of these changes was in liver where the serine pool size showed an increase of 79%.

Section 4

4.1. Infusion 4

In infusions 1, 2 and 3 the general conclusion, that the specific radioactivity of the precursor is closer to that of the free amino acid in the tissue rather than in the plasma, was based on two assumptions. The first was that the plasma acted as a homogeneous pool in which the specific radioactivity of the serine and glycine was the same irrespective of the site of sampling. The second assumption was that the specific radioactivity ratio of the total mixed proteins of the tissue would be the same as the ratio in individual proteins from the same tissue. To test the validity of these assumptions Infusion 4 was designed to measure the specific radioactivity of serine and glycine in plasma sampled from different sites and to establish whether, in the liver, the specific radioactivity ratio in the total tissue protein was the same as in two purified liver proteins, albumin and ferritin.

Method

Twelve hooded female rats (108 g), maintained on standard 10% NDP:E diet, were infused with [U-^{14}C]glycine by the procedure described previously. The infusions were carried out for both 3 and 6 hours, six rats being infused at each time. At the end of the experiment the animals were anaesthetised with ether and the abdominal cavity opened by a mid-line incision. After the portal vein had been exposed, the infusion was stopped by the removal of the catheter. Immediately 2 ml of blood was withdrawn from the hepatic portal vein with a heparinised syringe. The syringe and needle were left located in the portal vein after the sample had been taken to prevent haemorrhage. In a similar manner a smaller blood sample (1 ml) was withdrawn from the left ventricle of the heart. After the blood samples had been collected the liver was dissected out

and rapidly washed with ice-cold saline. A sample of liver (0.5 g) was taken to determine the specific radioactivity of the tissue free and protein bound glycine and serine and the remainder of the liver was used for the isolation of ferritin.

<u>Isolation of Plasma Albumin</u>

Albumin was isolated from the hepatic portal plasma by a modification of the method of Debro, Tarver and Korner (1957) as described by Judah, Gamble and Steadman (1973). To one volume of plasma was added nine volumes of a 1% trichloroacetic acid/ethanol (w/v) solution at $0^{o}C$. The mixture was allowed to stand for 15 minutes before being centrifuged at 10,000 g for 20 minutes at $0^{o}C$. The supernatant was added to 20 volumes of ether and after the precipitate had formed the sample was recentrifuged at 10,000 g for 15 minutes. The supernatant was discarded and the residue reconstituted in Tris-HCl buffer (100 mM pH 7.8) to the same volume as the original plasma sample. The crude albumin isolated by this procedure is known to be contaminated with γ-globulin and α-globulin. In order to remove the globulin contaminants the crude albumin sample was subjected to chromatography on Sephadex G200. The Sephadex G 200 had been previously equilibrated for 3 days in the Tris-HCl eluent buffer at room temperature. The equilibrated beads were packed in a perspex column (50 cms x 1.6 cms) and the system operated with a hydrostatic pressure of 8cms and a flow rate of 9-10 ml per hour. The effluent was collected in 10 minute fractions using an LKB Minirac fraction collector.

Figure 8 illustrates the resolution of the column using a "Pentex" Albumin standard and Figure 9 shows the extent of separation of the albumin isolated by the above technique, from the globulin contaminants. Fractions relating to the hatched area of the albumin peak in Figure 9 were pooled and concentrated by ultrafiltration using a Amicon filter

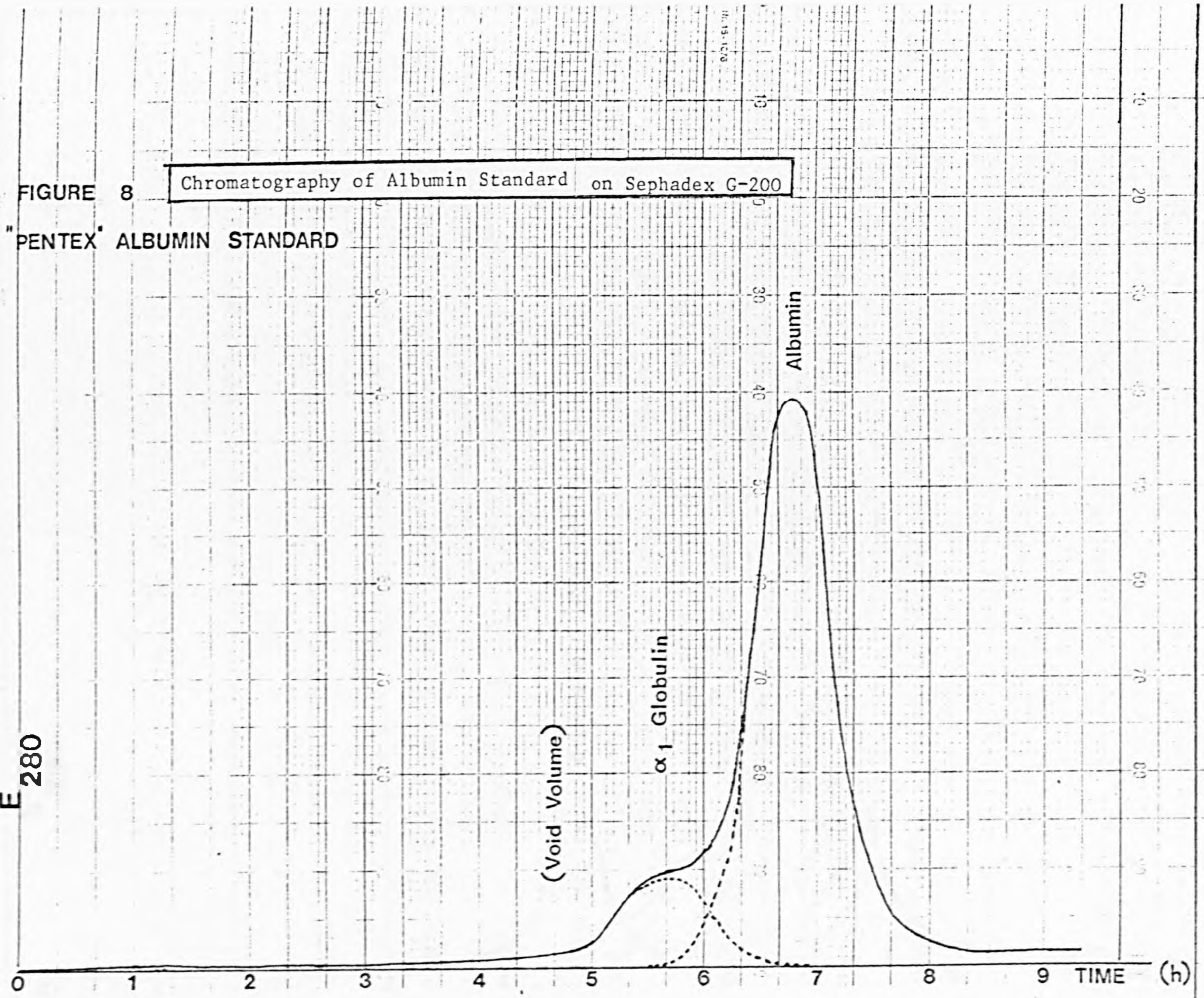


FIGURE 8
Chromatography of Albumin Standard on Sephadex G-200
"PENTEX" ALBUMIN STANDARD
Albumin
α 1 Globulin
(Void Volume)
E 280
0
1
2
3
4
5
6
7
8
9
TIME (h)

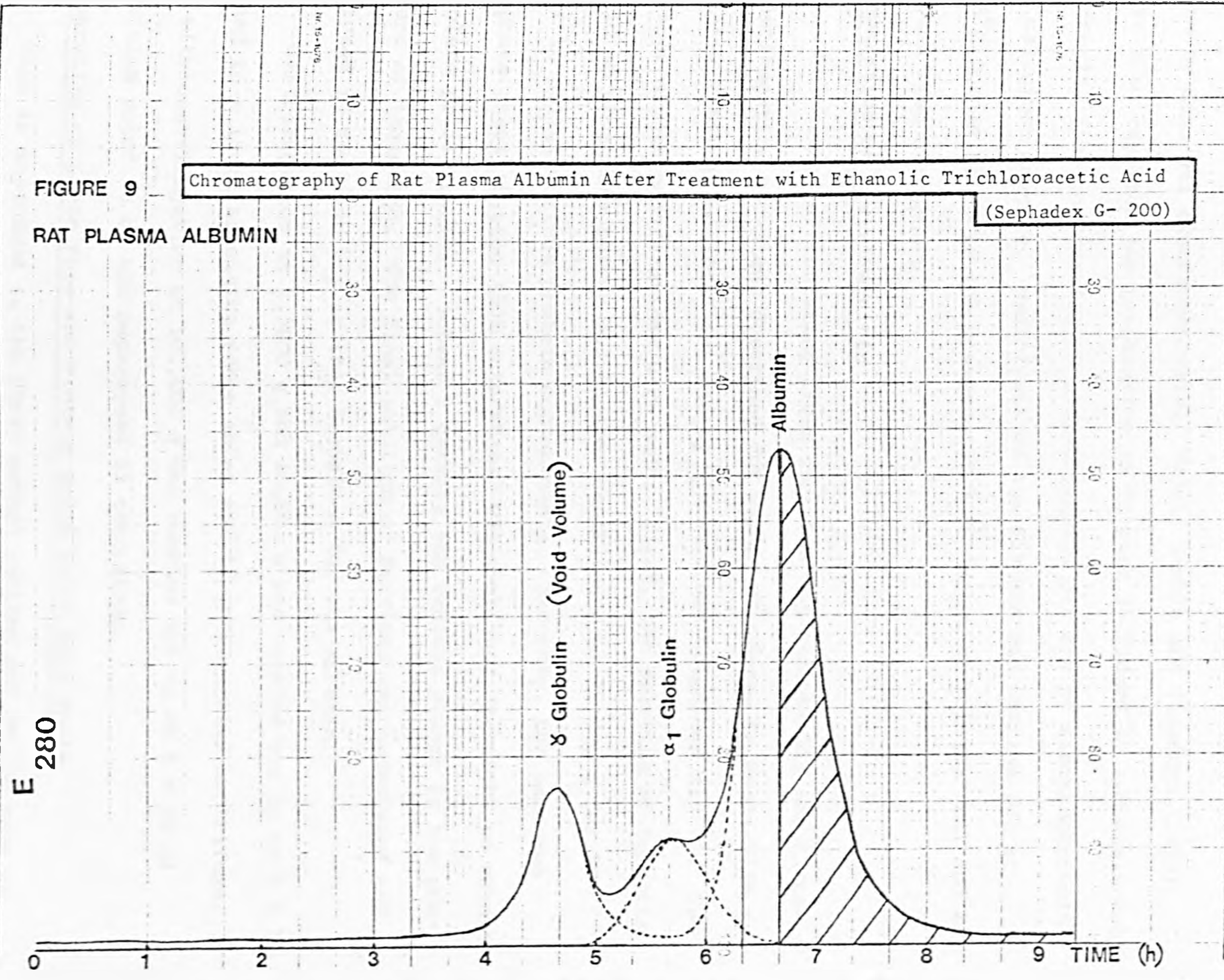


FIGURE 9

RAT PLASMA ALBUMIN

Chromatography of Rat Plasma Albumin After Treatment with Ethanolic Trichloroacetic Acid (Sephadex G- 200)

(Model 52 - Diaflo Filter PM10, 43 mm). The concentrated albumin obtained by this procedure was estimated to be approximately 97% pure by disc gel electrophoresis. Judah, Gamble and Steadman (1973) have also reported the preparation to be pure as judged by immunoelectrophoresis, electrofocussing on polyacrylamide gel and by electrophoresis on cellulose acetate. Hydrolysis of the albumin was carried out at 105°C for 24 hours in the presence of 2 ml "constant boiling" HCl and an oxygen free atmosphere.

Preparation of Liver Ferritin

The method of isolating liver ferritin was essentially the ultracentrifugation technique described for rabbit (Penders, De Rooij-Dijk and Leijnse,1968) as modified for rat by Bjorklid and Helgeland (1970). Two further slight modifications were made. First, the solution of ferritin in 10 mM phosphate buffer was further centrifuged at 10,000 g for 1 hour to pellet various protein contaminants. Secondly, the ammonium sulphate precipitation (50% saturation) was continued overnight to ensure complete precipitation. Scheme 3 details the various stages in the procedure of isolation. The final solution of ferritin was hydrolysed and analysed in the same way as that described for the albumin.

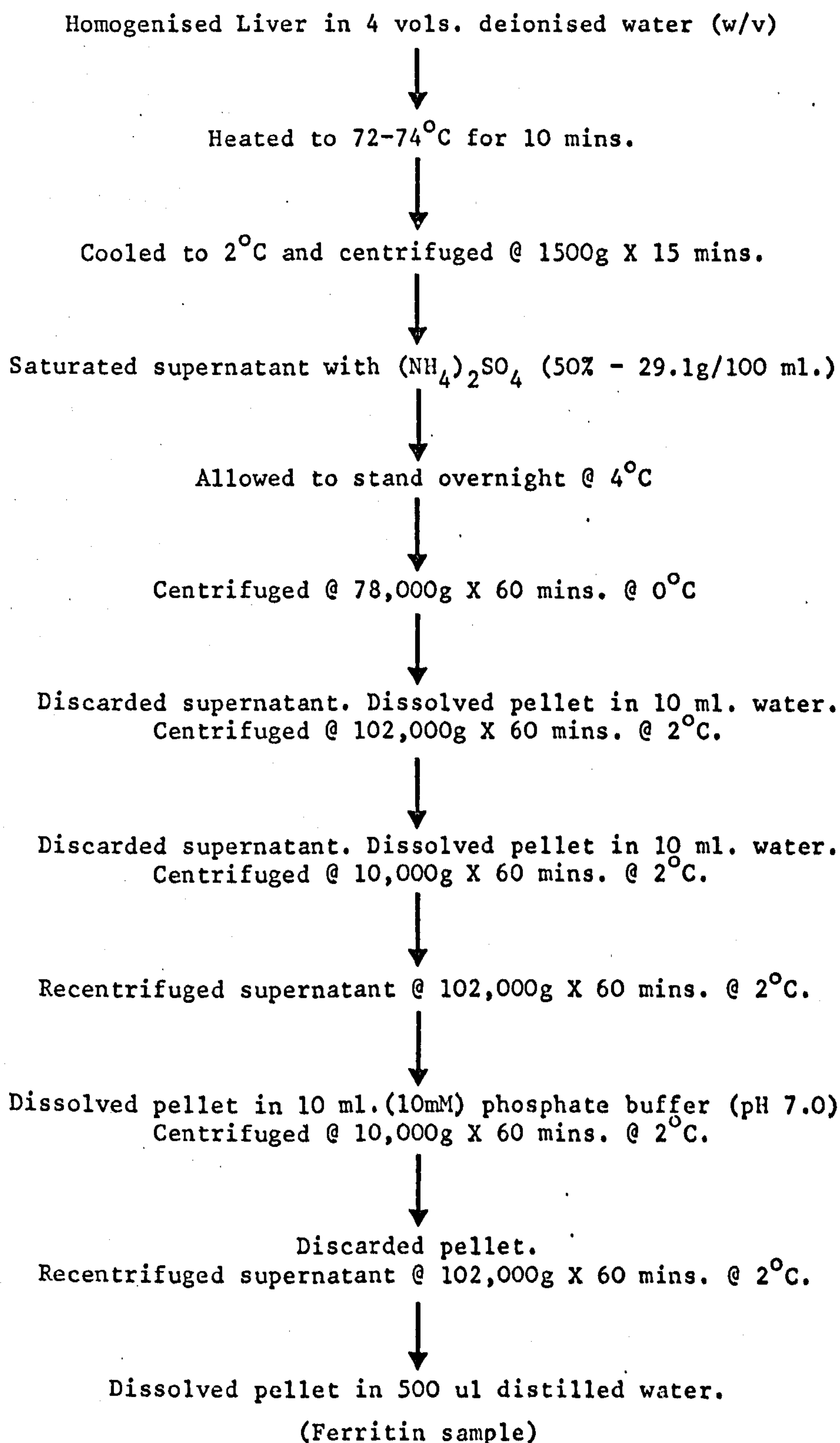
Centrifugation at 1,5000 g and 10,000 g was carried out in an 8 x 50 ml and 16 x 15 ml aluminium rotor in an MSE 18 refrigerated centrifuge. The ultracentrifugation at 102,000 g was carried out in an 8 x 50 ml aluminium rotor in an MSE Superspeed 65 centrifuge.

Preparation of Liver Free and Protein Bound Amino Acid Pools

This is described in the first method section and is the same as in the other infusions. Briefly, samples were obtained after homogenisation and precipitation in 3% SSA (w/v) and subsequent centrifugation. The free pool was taken as the supernatant and the protein bound pool as the

Scheme 3 . Isolation of Ferritin by Ultracentrifugation.

(Penders, De Rooij-Dijk & Leijnse 1968; Bjorkland & Helgeland 1970)



precipitate. In the case of this infusion, however, the protein precipitate was not treated by the method described by Hider, Fern and London (1971) but was simply washed three times in 3% SSA.

Preparation of Plasma Samples

Both heparinised hepatic portal and arterial plasma samples were initially spun in a bench centrifuge. The plasma was decanted, mixed with an equal volume of 3% SSA and stored at 4°C for 2 hours to ensure total precipitation. After centrifugation the supernatant was stored for analysis also at 4°C. The method for the determination of the specific radioactivity of glycine and serine has been described earlier.

Results and Discussion

a) Specific Radioactivity of Glycine and Serine Pools in Plasma

Table 14 contrasts the specific radioactivities of free glycine and serine in arterial and hepatic portal plasma. The results are shown for both 3 and 6 hours of infusion.

As this investigation was concerned with the liver, it would have been more satisfactory to determine the specific radioactivities of serine and glycine in the hepatic portal, hepatic venous and arterial plasma as this would have given some indication of the movement of these amino acids within this tissue. But, because of the small size of the experimental animals (108 g) and the inaccessability of the hepatic vein, there was considerable difficulty in obtaining samples of hepatic venous plasma. As a result plasma was taken only from the hepatic portal vein and the left ventricle of the heart (arterial). This is in effect a measurement of the arterio-venous difference across the intestine.

From the results (Table 14) it can be seen that in either plasma sample, although a plateau specific radioactivity of free glycine had not fully been reached after 3 hours of infusion, the increases during the second

Table 14 . The Specific Radioactivity of Glycine and Serine in the Free Pool of Arterial and Hepatic Portal Plasma After the Infusion of [U- ^{14}C] Glycine.

	Specific Radioactivity (DPM/nanomole)					
	Serine		Glycine		Serine Specific Radioactivity / Glycine Specific Radioactivity	
Time of Infusion (hours)	3	6	3	6	3	6
Arterial Plasma	34.2	44.6	130.3	142.2	0.266	0.314
	± 2.0	± 2.2	± 9.0	± 4.4	± 0.016	± 0.011
Hepatic Portal Plasma	32.0	43.6	103.4	125.4	0.319	0.351
	± 1.8	± 1.5	± 7.6	± 5.4	± 0.033	± 0.020

Each result is the Mean (± S.E.M.) of 6 animals at each time.

3 hour period were relatively small. For instance, the specific radioactivity of glycine in the hepatic portal plasma increased by 21% between 3 and 6 hours ($p = < 0.05$) and in the arterial plasma the increase was 9% (statistically not significant). Similarly, the specific radioactivity of free serine derived from the infused glycine had also not reached a plateau. The changes between 3 and 6 hours in this case were larger showing increases of 30% in the arterial plasma ($p = < 0.01$) and 36% in the hepatic portal plasma ($p = < 0.001$).

An important observation from the results in Table 14 is that the specific radioactivity of free glycine in the arterial plasma was higher than in the hepatic portal plasma at both 3 and 6 hours. At 3 hours the arterial plasma was 26% higher ($p = < 0.05$; paired t-test) and at 6 hours it was 13% higher ($p = < 0.05$; paired t-test). These differences can be largely attributed to the diluting effect of protein breakdown within the jejunum. The ^{12}C glycine released from protein breakdown will dilute the specific radioactivity of the intracellular pool and, in turn, that of the plasma. The difference in specific radioactivity between the portal and the arterial plasma therefore gives an indication of the extent of the dilution of isotope by proteolysis. This is only true for relatively short infusion times. As the time of infusion increases, radioactivity in the faster turning over intracellular protein will be released by protein degradation and the free glycine so produced will contain increasing amounts of ^{14}C label. This is one reason why the difference between the two plasmas is larger at 3 hours (26%) than it is at 6 hours (13%).

In contrast, protein breakdown appeared to have no effect on the specific radioactivity of free serine. At both 3 and 6 hours there was no significant difference between the specific radioactivity of free serine in arterial plasma and hepatic portal plasma. Theoretically, if

proteolysis is sufficient to lower the specific radioactivity of glycine in the venous plasma, it should also be effective in lowering the specific radioactivity of free serine. If it fails to do so, then it is because the specific radioactivity of the serine being effluxed from the jejunum is equal to, or nearly equal to, that of the plasma. This may be brought about by the capacity of the jejunum to synthesise serine from glycine (Yoshida & Kikuchi, 1973; Table 7). While the rate of conversion is only about 27% of that in the liver, it is sufficient to maintain the plasma specific radioactivity despite dilution by proteolysis.

One of the purposes of this infusion was to determine the degree to which the specific radioactivity ratio of serine to glycine varied in plasma sampled at different sites. From Table 14 it can be seen that, although the ratios were higher in the portal blood, only the difference at 6 hours (11%) was statistically significant ($p=<0.05$; paired t-test). If the hepatic portal plasma does exhibit the highest ratio then the conclusions of the previous infusions are confirmed. However, because of the capacity of the liver to interconvert serine and glycine, the highest ratio is most likely to occur in the hepatic venous plasma. As this was not measured it is not possible to comment but the results of Wolff and Bergman (1972) indicate that during an infusion of [U-^{14}C] glycine in sheep, the ratio of the specific radioactivity of serine to glycine in the hepatic vein is only approximately 25% higher than in the hepatic portal and arterial plasma. If these results were applicable to the rat then the ratio in the hepatic venous plasma would be less than a value of 0.439.

b) Specific Radioactivity of Serine and Glycine Pools in Liver

In the liver homogenate the specific radioactivity of free glycine and serine had almost reached plateau at the end of the experiment. In both cases the difference between 3 and 6 hours was approximately 20%

(Table 15). The specific radioactivty ratio of free serine to free glycine in plasma, showed smaller differences of less than 5% between 3 and 6 hours of infusion. These ratios were very similar to those obtained in Infusion 1, as were the ratios for the total liver protein.

In this experiment, the highest rate of incorporation was seen in the albumin fraction, in which the specific radioactivity of glycine and serine had reached 8.3 - 10.3% of that of the free amino acid after 6 hours of infusion. Over the same period both amino acids in total liver protein had reached approximately 7% of the respective free values. In ferritin the values were 4.6% for serine and 9.0% for glycine.

The specific radioactivity ratio of serine to glycine in albumin was significantly lower ($p = <0.01$; paired t-test) than in total liver protein. The difference was approximately 16% at both times. Similar results were found with ferritin in which the specific radioactivity ratio was even lower than in albumin. However, for the purposes of this discussion the ferritin results will be disregarded for the following reasons. Firstly, because small animals were used in this experiment, very little ferritin was isolated from the liver. From the data of Bjorklid and Helgeland (1970) and Linder, Moor and Munro (1974) it was estimated that the initial liver homogenate contained between 1 and 2 mg of apoferritin. The difficulty in analysis did not arise only from the small chemical amount but to some extent also from the small amount of incorporated isotope. Secondly, the results of serine and glycine incorporation into ferritin (Table 15) indicate the possibility that non-specific binding of radioactivity had occurred. The results of the incorporation of serine appeared reasonable, in that the specific radioactivity after 6 hours was about twice that at 3 hours. The apparent incorporation of glycine, on the other hand, showed that the specific

Table 15 The Specific Radioactivity of Glycine and Serine in the Free Pool and the Total Protein Pool of Liver, in Liver Ferritin and in Plasma Albumin after the Infusion of [U-^{14}C] Glycine.

	Time of Infusion (h)	Specific Radioactivity (S.R.) (D.P.M. / nanomole) Serine	Glycine	Serine S.R. / Glycine S.R.	Serine Residues / Glycine Residues
Liver Free Pool	3	59.18 ± 3.36	64.90 ± 4.93	0.919 ± 0.020	-
	6	75.24 ± 4.46	77.91 ± 3.17	0.963 ± 0.031	-
Liver Total Protein	3	2.32 ± 0.09	2.71 ± 0.09	0.861 ± 0.038	-
	6	5.16 ± 0.16	5.76 ± 0.14	0.896 ± 0.018	-
Liver Ferritin	3	1.64 ± 0.14	4.82 ± 0.86	0.405 ± 0.101	0.774 ± 0.026
	6	3.47 ± 0.18	7.03 ± 0.47	0.509 ± 0.052	0.783 ± 0.017
Plasma Albumin	3	2.60 ± 0.09	3.51 ± 0.13	0.739 ± 0.007	1.409 ± 0.028
	6	6.22 ± 0.36	8.31 ± 0.42	0.748 ± 0.019	1.420 ± 0.025

Results are the Mean (± S.E.M.) of 6 animals at each time.

radioactivity at 3 hours was nearly 70% of the 6 hour value. This increased incorporation of glycine during the first half of the infusion was not seen either in total liver protein or in albumin. Both these explanations are consistent with the large standard errors of the specific radioactivity ratio in ferritin. For the 3 hour value the standard error was ± 25% and for the 6 hour value it was ± 10%. These were unusually high when compared with the results from either total liver protein or albumin (± 4.5% S.E.M.). The nature of this non-specific binding to ferritin was not determined but it seemed that it could not involve the free amino acid otherwise serine should have equally become bound. A more reasonable possibility was the binding of glutathione to the ferritin. Glutathione, a tripeptide containing equimolar quantities of glutamic acid, cysteine and glycine, becomes heavily labelled after administration of ^{14}C glycine (Henriques, Henriques & Neuberger 1955). When rats were infused with [U-^{14}C] glycine, the specific radioactivity of glycine in glutathione rose to 50-70% of that of the free glycine in the liver and 8.7 - 12.2 times higher than that of glycine in total liver protein (Table 16). Thus a small amount of contamination with glutathione could produce a large error in the apparent amount of radioactivity incorporated into protein.

Non-specific binding of the radioactivity could have occurred with ferritin because the isolation procedure is relatively mild, involving only ultracentrifugation, a heat precipitation and an ammonium sulphate precipitation. Furthermore, the isolated ferritin was hydrolysed as the complete protein rather than as apoferritin.

The difference between the specific radioactivity ratio of albumin and of total liver protein (Table 15) can be explained in two ways. The ratio in the total liver protein may not reflect the ratio in the precursor pool because of distortion by the heterogeneity of the protein sample itself (for a more detailed explanation see final discussion). Alternatively, albumin

Table 16 . The Relative Specific Radioactivity of Glutathione.

Rat No.	Time of Infusion (h)	Specific Radioactivity (SR) (DPM / nanomole) Glutathione Glycine (GSH)	Homogenate Free Glycine	Protein Glycine	Glutathione SR / Homogenate SR (%)
1	3	10.01	15.82	↑	63.3
2	3	6.54	13.14	0.632	49.8
3	3	6.60	12.80	↓	51.6
	Mean	7.72	13.92	0.632	54.9
4	6	14.56	17.68	↑	82.4
5	6	9.69	16.03	1.304	60.5
6	6	9.82	17.77	↓	55.3
	Mean	11.36	17.16	1.304	66.1

Female rats were continuously infused for either 3 or 6 hours with [U-^{14}C] glycine. At the end of the infusion the liver was prepared exactly as in previous experiments. The glutathione was isolated from the SSA soluble fraction by ion-exchange chromatography using an automated amino acid analyser and hydrolysed in the presence of 6N HCl and an oxygen free atmosphere. The hydrolysate was rechromatographed and the glycine peak quantitated for both chemical and radioactive concentration as described for Infusions 1,2,3 and 4. The final chromatogram of the hydrolysate showed the presence of glutamate, cysteine and glycine in equimolar amounts along with a variable quantity of aspartic acid. The aspartic acid was present as a contamination from the origonal seperation of glutathione. These four amino acids were the only ones present in the final run.

The specific radioactivity of the total liver protein was analysed at each time, as a pooled sample.

may be synthesised from a pool of amino acids which is distinct from that which serves as the precursor for the majority of proteins in liver. Unfortunately, because the ferritin results are not reliable it is difficult to assess the situation or to discuss it further. In order to be able to do this it must first be established that the specific radioactivity ratio given by albumin is representative of other pure proteins.

c) Rates of Protein Synthesis

Table 16 gives the rates of protein synthesis, calculated according to Seta *et al.*, (1973), for total liver protein, albumin and ferritin. Rates of synthesis have been calculated by assuming that the precursor was in either the liver free pool, the hepatic portal plasma or the arterial plasma. As in previous infusions it is evident that the rates of synthesis calculated from the specific radioactivity of serine and glycine in the free amino acid pool of liver are better related than those calculated from the plasma specific radioactivities.

The fractional synthesis rate for the liver (mean value 34% per day) is almost identical to that calculated from Infusion 1 (34.5% per day) and Infusion 3 (38.3% per day). The fractional synthesis rate for albumin calculated at 43.1% per day for serine and 53.8% per day for glycine is equivalent to an absolute rate of 6.0 mg/hour/300 g body weight (serine) and 7.5 mg/hour/300 g body weight (glycine). These figures have been calculated on the assumption that the amount of circulating albumin in a rat is 111.0 mg/100 g body weight (Jeejeebhoy *et al.*, 1973). They are, in fact, underestimates because no allowance has been made for loss of radioactive albumin from the intravascular to the extravascular compartment.

The fractional synthesis rate for ferritin of 21.8% per day from the incorporation of serine compares well with the rate calculated from incorporation of glycine (24.8% per day). This is surprising in view of the evidence presented above that the glycine data are in error. It

Table 17. Estimated Fractional Rates of Protein Synthesis of Total Liver Protein, Liver Ferritin and Plasma Albumin After the Infusion of [U-^{14}C] Glycine.

	Fractional Rate of Protein Synthesis (% tissue protein mass / day)					
Precursor Pool	Tissue Free		Hepatic Portal Plasma		Arterial Plasma	
Precursor Amino Acid	Serine	Glycine	Serine	Glycine	Serine	Glycine
Total Liver Protein	33.8	34.2	60.1	21.3	57.6	17.9
	(5.9)	(7.6)	(5.7)	(7.3)	(5.8)	(6.9)
Liver Ferritin	21.8	24.8	38.7	15.5	37.1	13.0
	(8.5)	(17.8)	(8.5)	(17.8)	(8.5)	(17.8)
Plasma Albumin	43.1	53.8	76.6	33.6	73.4	28.2
	(5.9)	(7.6)	(5.7)	(7.3)	(5.8)	(6.9)

Figures in parenthesis indicate the largest S.E.M. (given as a % of the mean) of the data from which the synthesis rates were calculated.

further supports the theory that there was non-specific binding of highly labelled glycine to the ferritin. If this bound material had a reasonably constant specific radioactivity, as with free glycine, the difference between the 3 and 6 hour values would be unaltered.

4.2. Infusion 5

Because of possible errors in measuring the specific radioactivity ratio in ferritin in Infusion 4, the aim of the next experiment was to repeat the previous infusion but this time incorporating several modifications into the experimental protocol in an attempt to increase the yield and purity of the protein in the final preparation. These modifications included the use of larger rats (180-220 g), inducing the synthesis of ferritin with iron and the addition of a further purification step to the isolation procedure.

Method

Two groups of six female hooded rats were used, both of which were fed the standard 10% NDP:E powdered diet. The first group, designated "normal", were maintained on the diet for a period of three weeks, after which time their body weight had reached 216 g. The gain in body weight during this period was 2.1 g/day. The second group was given a single subcutaneous injection of 10 mg iron sorbitol ("Jectofer"; Astra Chemicals Ltd.,) to induce the synthesis of ferritin. The injection was given 8 days after the start of the diet, when the rats weighed 175 g. The injection had an adverse effect on these animals, because during the following nine days they remained at approximately the same weight. However, the daily gain in body weight over the 4 days before infusion and during the first week before injection of the iron sorbitol was very similar to that of the normal group. Figure 10 shows the daily change in body weight for these two sets of animals. Sixteen hours before the start

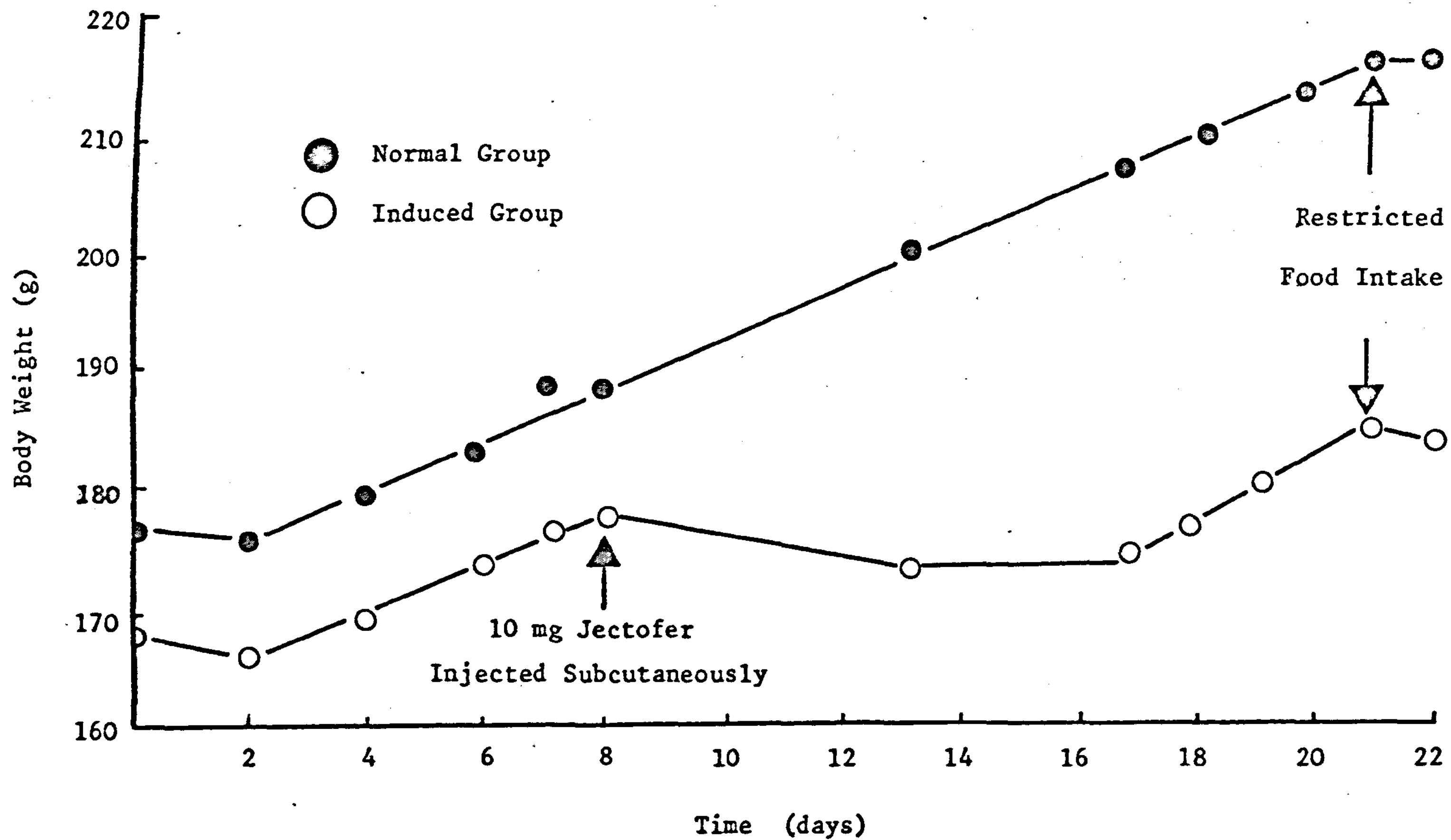


Figure 10 Daily Changes in Body Weight of Normal and Induced Rats

Each point is the mean of 6 animals. Coefficient of variation was less than 8%.

of the infusion both groups were given a restricted amount of food to ensure that absorption of food from the gut did not interfere with the rise in specific radioactivity of glycine during the infusion.

[U-^{14}C] glycine was infused by the tail vein into both groups of animals for a period of 6 hours. No carrier glycine was present in the infusate. Other details of the infusion and the methods for isolating liver free glycine and serine, liver total protein and albumin were identical to those described previously. The isolation of ferritin was also the same as before except that an additional step was included at the final stage to minimise non-specific binding of radioactivity. This entailed incubation of the ferritin in 1 mM dithiothreitol for 60 minutes at 37°C, followed by precipitation with 5% SSA. The precipitate was then washed a further three times with SSA, before being hydrolysed in 6N HCl at 105°C. Specific radioactivity was determined as described previously.

Results and Discussion

Due to the use of larger rats and the induction of synthesis, the isolation of ferritin in this infusion was quantitatively more satisfactory. On average the amount of ferritin isolated from the liver of the normal group was 160% greater than from the previous infusion, and the amount obtained from the livers of the induced rats (compared with the normal rats) was increased by 120%. These figures were calculated from the amino acid analysis of the ferritin hydrolysates.

Incubation of the ferritin with dithiothreitol resulted in a decrease in the number of residues of glycine relative to serine. In the previous infusion the ratio of serine residues to glycine residues in ferritin was calculated to be 0.778 ($\pm$ 0.021 S.E.M.). In this infusion, the ratio was 0.897 ($\pm$ 0.011) for the normal group and 0.878 ($\pm$ 0.007) for the induced group. These figures are in good agreement with those given for horse

spleen ferritin (0.908) by Crichton (1969) and 0.909 by Bryce and Crichton (see Crichton, 1971). To show that this difference was not simply the result of analytical variation, the ratio in albumin was almost identical in both studies i.e. 1.420 $\pm$ 0.025 (previous infusion) 1.430 $\pm$ 0.024 (normal group) and 1.405 $\pm$ 0.025 (induced group).

The results of the specific radioactivity of glycine and serine in the liver free amino acid pool, the total liver protein, albumin and ferritin are given in Table 18. Although the specific radioactivities are lower than in the previous infusion, their inter-relationship is very similar (compare with Table 15). This is with the exception of ferritin. The highest rate of incorporation was again shown by albumin in which the specific radioactivity of glycine or serine was between 7.1 - 10% of the free amino acid pool. The specific radioactivity of the total liver protein was between 7.0 - 8.1% of the free pool, and in ferritin, which showed the lowest rate of incorporation, the specific radioactivity of these amino acids was only around 2-3%. These figures apply to both the normal and the induced group.

With one exception, statistical comparison of the specific radioactivities of the free pool, the total protein, ferritin, and albumin in the normal group with those in the induced group showed no significant differences. The one exception was albumin in which the specific radioactivity of glycine was higher in the normal group ($p = 0.02$).

Table 19 shows the specific radioactivity ratios of serine to glycine in both groups. Statistical comparison within each group indicates that in both normal and induced animals the ratio in albumin was significantly different from that in the free pool, the total tissue protein and ferritin. In addition, in the induced group the specific radioactivity ratio in ferritin was significantly different from that of the total liver protein. Within each group no other comparisons were significant.

Table 18 . The Specific Radioactivity of Serine and Glycine in the Free Amino Acid Pool of Liver, in Liver Total Protein, in Liver Ferritin and in Plasma Albumin After 6 Hours of Infusion with [U-^{14}C] Glycine.

	Specific Radioactivity (DPM / nanomole)							
	Liver Free		Liver Total Protein		Liver Ferritin		Plasma Albumin	
	Serine	Glycine	Serine	Glycine	Serine	Glycine	Serine	Glycine
Normal Group	23.51 ± 0.38	25.30 ± 0.51	1.89 ± 0.10	1.77 ± 0.11	0.571 ± 0.040	0.554 ± 0.039	1.97 ± 0.11	2.53 ± 0.13
Induced Group	23.25 ± 0.59	23.44 ± 0.69	1.84 ± 0.07	1.78 ± 0.07	0.641 ± 0.048	0.677 ± 0.053	1.67 ± 0.09	2.01 ± 0.10
Statistical Comparison Between Normal and Induced Groups	NS	NS	NS	NS	NS	NS	NS	p = 0.02

Each result is the Mean (± S.E.M.) of 6 animals

(NS = not significant i.e. p = > 0.05)

Table 19. The Ratio of the Specific Radioactivity of Serine to Glycine in the Liver Free Pool, Total Liver Protein, Liver Ferritin and Plasma Albumin in Normal and Induced Rats After 6 Hours Infusion with [U-^{14}C] Glycine.

$$\frac{\text{Serine Specific Radioactivity}}{\text{Glycine Specific Radioactivity}}$$

	Liver Free	Liver Total Protein	Liver Ferritin	Plasma Albumin
Normal Group	0.981	1.076	1.035	0.783
	± 0.021	± 0.050	± 0.039	± 0.016
Induced Group	0.993	1.037	0.950	0.828
	± 0.016	± 0.031	± 0.025	± 0.016

Results are the Mean (± S.E.M.) of 6 animals

Statistical Comparison (Paired t-test)

	Values of 'p' Normal Group	Values of 'p' Induced Group
Albumin v Liver Free	< 0.01	< 0.001
Albumin v Liver Protein	< 0.01	< 0.01
Albumin v Ferritin	< 0.01	< 0.02
Ferritin v Liver Free	NS	NS
Ferritin v Liver Protein	NS	< 0.05
Liv. Free v Liver Protein	NS	NS

(NS = not significant i.e. p => 0.05)

The results of this infusion were originally intended to show that the specific radioactivity ratio would be identical in two pure proteins. When the ratio in albumin is compared with that in ferritin it becomes evident that this is not the case. In both the normal and induced groups the ratio in albumin was significantly lower than that in ferritin, by 24% ($p = < 0.01$) and 12.2% ($p = < 0.02$) respectively. If all proteins are synthesised from a single precursor pool then the ratio in these two pure proteins should be the same, provided the relative specific radioactivities of the two amino acids in the precursor pool remain constant. In Infusions 1, 2 and 4 it was shown that the relative specific radioactivities of serine and glycine in the free amino acid pool of the liver do remain almost constant after 2-3 hours of infusion.

The above results suggest that albumin and ferritin are not synthesised from the same free amino acid pool. This apparent compartmentation can be examined at three different levels; at the organ level, at the tissue level and at the cellular level. The first considers the possibility that plasma albumin is not synthesised exclusively by the liver. Although it is generally accepted that the liver is the sole site for the synthesis of albumin (e.g. Peters and Anfinsen, 1950; Miller & Bale, 1954; Peters, 1970) if a small fraction of plasma albumin was synthesised in extrahepatic tissues in which the specific radioactivity ratio of free serine to glycine in the precursor was very low, it could account for the lower ratios in albumin relative to liver ferritin. This is not a likely explanation because, if it assumed that the lowest ratio in any tissue is equal to that of plasma (0.300), then the fraction of total albumin synthesised by extrahepatic tissues would have to be considerable (very approximately 20-25%).

The second possibility is that the differences in the ratios in albumin and ferritin arise as a result of compartmentation at the tissue

level. The liver consists of several distinct cell types of which the parenchymal (hepatocyte) and the reticuloendothelial (Kupffer) cells together comprise more than 85% of the cell population and volume. (Daoust & Cantero, 1959; Weibel, Staubli, Gnagi & Hess, 1969). Although non-haem iron has been identified in both cell types, it has been suggested that, in the rat, liver ferritin is almost entirely contained within the hepatocyte (Van Wyk, Linder-Horowitz &Munro, 1971; Cook, Hershko & Finch, 1974). Both these studies have estimated that the hepatocytes account for over 95% of liver ferritin. Cook et al., (1974) suggested that the non-haem iron present in the reticuloendothelial cells is predominantly haemosiderin. The synthesis of albumin has also been attributed to the hepatocyte (Hamishima, Harter & Coons, 1964; Chandrasakharam, Fleck & Munro, 1967; Glaumann & Ericsson, 1970; East, Louis & Hoffenberg, 1973; Jeejeebhoy et al., 1975).

These observations therefore, suggest that any differences in the site of synthesis of albumin and ferritin must arise through compartmentation within the cell itself rather than from the tissue as a whole. But the situation is more complex than this because functional variations among populations of hepatocytes have been implied by cytologists for a long time. Electron microscopy has shown that in general hepatic parenchymal cells in the centrolobular region have smaller and rounded mitochondria, a limited rough endoplasmic reticulum, poorly developed Golgi complexes and only a few lysosomes. In contrast, hepatocytes in the perilobular areas have much larger and longer mitochondria, an increase in the amount of rough endoplasmic reticulum, well developed Golgi bodies and many more lysosomes (Tanikawa, 1968). Because of these variations, especially those which affect the rough endoplasmic reticulum and the Golgi complexes, it is possible that hepatocytes differ in the quality and quantity of individual proteins they synthesise. Thus those with increased

rough endoplasmic reticulum may synthesise more albumin than ferritin and vice versa (see later discussion). There is evidence to support the suggestion that an apparently homogeneous population of cells may not in fact synthesise the same spectrum of proteins. Barnhart and Anderson (1962) have shown that not all relevant cells contain prothrombin but that the number can increase drastically in response to a demand, from almost no cells to practically all. Lane (1968) reported that under certain conditions only 20% of hepatocytes stained for the presence of transferrin. In addition, Chandrasakharam et al., (1967) have shown that, in the rat, the number of hepatocytes containing albumin altered directly with the protein content of the diet and Hamashima et al., (1964) observed that only a small proportion of human liver cells contained both albumin and fibrinogen. Thus the difference in the specific radioactivity ratios between albumin and ferritin may result from compartmentation at the tissue level.

Another possibility is that compartmentation of protein synthesis is occurring at the cellular level. This can arise in many ways, as for example in the distribution of messenger-RNA, but the most obvious way is through localisation of ribosomes within different areas of the cell. It has been known for some years now that there are basically two forms of ribosomes; those that are attached to the membrane (rough endoplasmic reticulum) and those that exist free within the cytoplasm. The ratio of bound ribosomes relative to those that are free has been estimated at approximately 3 or 4 to 1 (Blobel & Potter, 1967). Functionally the membrane bound ribosomes were at first considered to be responsible for the synthesis of export (secreted) protein while free ribosomes were responsible for the synthesis of domestic (or non-secreted) proteins. (Howatson & Ham, 1955; Siekevitz & Palade, 1960), but this simple appreciation has in recent years been questioned because anomalies have been

found. Attardi et al., (1969), for instance, have demonstrated that in HeLa cells about 15% of the total ribosomes are bound to the membrane yet less than 2% of the synthesised proteins are secreted. Similarly Dallner et al., (1966) and Omura and Kuriyama (1971) have indicated that certain constituent proteins of the cell membrane are produced by membrane bound ribosomes. The situation has been confused even further by the suggestion that NADP-cytochrome c reductase (Ragnotti et al., 1969) and cytochrome c (Gonzalez-Cadavid & Saez de Cordova, 1974) are synthesised on both free and bound ribosomes.

According to current understanding (Rolleston, 1974) the ribosome population of a cell can be tentatively divided into three groups. The first are 'tightly' bound to the membrane and serve to synthesise proteins for secretion. The second group are also membrane bound but are only 'loosely' associated with this structure and can be removed relatively easily (Bleiberg, Zauderer & Baglioni, 1972) and the third group include those ribosomes which remain free in the cytoplasm unattached to any membrane. It is thought that the anomalies outlined above may in some cases be due to difficulties in differentiating the 'loosely' bound ribosomes from those more permanently bound and those occurring free in the cytoplasm.

In terms of the synthesis of albumin there are many studies which have indicated that this protein is almost exclusively synthesised by membrane-bound ribosomes (Takagi & Ogata, 1968; Hicks, Drysdale & Munro, 1969; Takagi, Tanaka & Ogata, 1970; Tanaka & Ogata, 1968; Uenoyama & Ono, 1972; Ikehara & Pitot, 1973). Rolleston (1974) has estimated that at least 90% of albumin is synthesised by the bound ribosomes. This calculation was based on the observation that on average 5 times more albumin is synthesised per mg of membrane-bound ribosomes than per mg of free ribosome and that the ratio of the amounts of bound to free ribosomes

is 3 or 4:1 (Blobel & Potter, 1967).

The situation regarding the synthesis of ferritin is by no means as clear. Studies by Hicks _et al._, (1969), Redman (1969) and by Pruro and Richter (1971) in rat liver have all agreed in showing a preferential, but not exclusive,synthesis of ferritin by free ribosomes.

Shafritz and Issalbacher (1974) have also found that ferritin synthesis in an _in vitro_ liver system using endogenous m-RNA, occurs predominately on the free ribosomes (approximately 80%). However, when the synthesis was compared in a rabbit reticulocyte system, there was almost no difference between the two ribosomal species in their ability to synthesise ferritin. This was interpreted as demonstrating that both free and membrane bound ribosomes have the potential for ferritin synthesis but under native conditions the translational activity of the bound ribosomes is inhibited in some way. Over the last three years there have been major developments in the biochemistry of ferritin, particularly with regard to its structure. For many years the structure of rat liver ferritin has been assumed to be very similar to that of horse spleen ferritin, consisting of a complex of 24 identical sub-units each with a molecular weight of 18,500 (Crichton, 1973). Recently, however, there have been reports that rat liver ferritin (and horse spleen ferritin) is comprised of two different sub-units (Konijn, Baliga & Munro, 1973; Niitsu, Ishitani & Listowsky, 1973; Linder, Moor & Munro, 1974). The exact value for the molecular weights of these subunits are in dispute but Linder _et al._, (1974) estimate them to be approximately 19,500 and 13,500. More relevant to this discussion is the report (Konijn _et al._, 1973) that free and bound ribosomes each synthesise only one sub-unit. Konijn and co-workers suggested that free ribosomes were responsible for synthesising the smaller protein units whilst the bound ribosomes were responsible for synthesising the larger ones. They further speculated that

these larger sub-units were synthesised in ribosomes that were loosely bound to the membrane. It has also been suggested (Konijn, et al., 1973; Linder et al., 1974) that both sub-units are not always synthesised in the same proportion. For instance, animals injected with ferric ammonium citrate showed an increase in the synthesis of both sub-units, but the increase in the number of the smaller sub-unit was much larger by comparison.

It is obvious from this discussion that there is still a considerable amount to be understood about the nature of ribosomes and their specificty for synthesis. The problems and potential of such studies are only just becoming apparent.

The results of the specific radioactivity ratios in Table 19 can tentatively be explained in terms of cellular compartmentation. The fact that the ratio in albumin was significantly lower than in the liver free pool, in total liver protein and in ferritin may be an indication that secreted proteins are synthesised from a precursor pool different from that used in the synthesis of domestic proteins. This explanation is strengthened a little by the fact that in the normal group the specific radioactivity ratio in ferritin was not significantly different from that in total liver protein. In the induced group, however, the ratio in ferritin was slightly lower than in the total liver protein by approximately 8% ($p = < 0.05$), but still not nearly as low as in albumin. The difference in the ratio of ferritin between the normal and the induced group may be related to the disproportionate synthesis of the smaller subunits after induction of synthesis with iron.

Although ribosomes have been differentiated into sub-groups for some time, it has only just been recently suggested (Ilan & Singer, 1975) that this differentiation can be extended to other components of protein synthesis. Their results showed that the specific radioactivity of leucine

in nascent peptide chains isolated from free ribosomes was double that of the leucine in nascent chains isolated from membrane bound ribosomes. As their experimental system was in an isotopic steady state, these results imply that there are at least two pools of free amino acids acting as precursors for protein synthesis. The results of this infusion can also be interpreted to show the existence of more than one precursor pool, but,as there are no other known studies on this particular aspect, the validity of these findings must await further investigation.

Appendix

The question of whether the ratio of the specific radioactivity of two amino acids in any pure protein will exactly reflect the ratio of the same two amino acids in the precursor pool, irrespective of the amino acid composition of the protein, can be considered mathematically.

Let k_s = the fractional synthesis rate

C_1 = the total amount of amino acid 1 in the protein

C_2 = the total amount of amino acid 2 in the protein

therefore, the amount of amino acid 1 incorporated at any time = C_1k_s

and the amount of amino acid 2 incorporated at any time = C_2k_s

If S_1 = the specific radioactivity of amino acid 1 in the precursor pool

S_2 = the specific radioactivity of amino acid 2 in the precursor pool

then the amount of radioactivity incorporated from amino acid 1 = $S_1C_1k_s$

and from amino acid 2 = $S_2C_2k_s$

The specific radioactivity of amino acids 1 and 2 in protein (P_1 and P_2) is then given by:

$$P_1 = \frac{S_1C_1k_s}{C_1} \qquad \text{and } P_2 = \frac{S_2C_2k_s}{C_2}$$

i.e.

$$P_1 = S_1k_s \qquad \text{and } P_2 = S_2k_s$$

Therefore the specific radioactivity ratio in protein $\frac{P_1}{P_2} = \frac{S_1k_s}{S_2k_s} = \frac{S_1}{S_2}$

Section 5

Infusion 6

Glycine and serine are both examples of amino acids that exhibit an active sodium dependant form of cellular uptake (Oxender and Christensen, 1963; Christensen, 1969). As a result their intracellular concentrations are very much higher than the concentrations in plasma (see Table 2). Because of the possibility that the conclusions of the preceeding infusions were influenced by the study of such actively transported amino acids, this infusion was primarily intended to compare the calculated rates of protein synthesis in the various tissues (Table 10) by using an amino acid whose mode of cellular uptake differed appreciably from that of glycine and serine. Of the possible amino acids that undergo transport by an inactive facilitated diffusion (Guroff & Udenfriend, 1960; Oxender & Christensen, 1963), tyrosine was chosen because it has the additional advantage that it can be assayed by an alternative method (Waalkes & Udenfriend, 1957) to the one used in Infusions 1, 2 and 3. Therefore it is possible to make an almost completely independant assessment of the validity of the preceeding conclusion.

Method

Twelve female rats, weighing approximately 108 gm and maintained on the standard 10% NDP:E diet were arbitrarily divided into two groups and infused with L-$[U^{14}C]$ tyrosine (3.5 µCi/ml; 10mCi/mmol), one group for three hours and the second for six hours. The infusion method was identical to that described previously with the exception that no 'carrier' tyrosine was present in the infusate. At the end of the infusion the rats were decapitated and a mixed arterio-venous blood sample collected. The tissues were immediately removed in the order: liver, jejunum, kidney, brain, heart, diaphragm and gastrocnemius. They were homogenised instantly in 3 ml ice-cold trichloroacetic acid (TCA 10% w/v) to precipitate

the protein, and stored at $0^{o}C$. The plasma proteins were precipitated with an equal volume of 10% TCA. All the samples were then centrifuged and the supernatants kept. With the exception of plasma, the protein precipitates were washed twice with 1 ml 10% TCA and the washings added to the original supernatants. The protein was hydrolysed with 5 ml 'constant boiling' HCl (6N) <u>in vacuo</u> for 20 hours at $105^{o}C$. Hydrochloric acid was removed after hydrolysis by heat evaporation and subsequently made to a final volume of 5 ml with distilled water.

The supernatant sampled from the plasma and tissues were washed twice with 5 ml diethyl ether to remove the TCA and then incubated for 1 hour at $37^{o}C$ to remove traces of ether. The volumes were adjusted to 5 ml with distilled water.

L-Tyrosine in protein and tissue free pools was estimated as the decarboxylated product, tyramine, after incubation with L-tyrosine decarboxylase (S-faecalis-Type 1-Sigma Chemical Company, London). 1 ml of each sample was incubated with 1 ml citrate buffer (pH 5.5; 0.5 M) and 1 ml of the decarboxylase enzyme suspension (2 mg/ml citrate buffer) for 1 hour at $37^{o}C$. The resulting tyramine was extracted into 10 ml ethyl acetate in the presence of 1 g sodium chloride and 1 g sodium carbonate (anhydrous). The organic phase was subsequently shaken with 5 ml chloroform and 4.5 ml (3.0 ml in the case of protein samples) sulphuric acid (1/500 v/v) and centrifuged at low speed. The upper aqueous portion, containing the tyramine, was removed after centrifugation and 2 x 1ml aliquots were counted in a Packard 2420 liquid scintillation counter. The scintillant contained 5 g 2,5-diphenyloxazole and 100 g Naphthalene made up to a final volume of 1 litre with 1,4-dioxan. Duplicate 1 ml sample (50 µl in the case of protein hydrolysates) were also assayed for tyramine by the fluorimetric method of Waalkes and Udenfriend (1957) as adapted for autoanalysis by Blau and Edwards (1971).

Results and Discussion

Table 20 shows the specific radioactivity of free tyrosine in plasma and tissues after 3 hours and 6 hours of infusion. Although in none of the tissues had an absolute constant value been achieved, in most cases the specific radioactivity at 3 hours was more than 75% of the specific radioactivity at 6 hours. The exceptions were liver and jejunum which attained 71% and 60% respectively. Some previous studies involving the constant infusion of tyrosine have reported almost complete isotopic equilibration in several tissues, including liver, within six hours of infusion (Garlick & Marshall, 1972; Garlick, Millward & James, 1973). A possible explanation for the small, but nevertheless increasing specific radioactivity of free tyrosine between 3 and 6 hours of infusion is the presence of food in the stomach at the beginning of the experiment. If this were the case, then the rate of rise of the specific radioactivity would be depressed initially by the absorption of ^{12}C tyrosine from the gut. As the amount of food present diminishes, the rate of absorption will decrease until finally the influx of ^{12}C tyrosine will cease to influence the equilibration of the isotope. As the experimental animals were fed ad libitum it is possible that the last meal was taken close to the start of the infusion. Although this absorption will effect the rate of rise of the specific radioactivity, it should not alter the final plateau value.

Table 20 also illustrates the relative specific radioactivities of the free amino acid in tissues compared with that in the plasma. As with infusion of serine and glycine, the liver specific radioactivity at 6 hours was about half the plasma value. It was also comparatively low in jejunum. The specific radioactivities in the remaining tissues ranged from 64%-83% of the plasma level. Included in this group is brain,

Table 20. The Specific Radioactivity of Tyrosine in the Free Pool of Tissue and Plasma after the Infusion of L-[U-^{14}C] Tyrosine.

	Specific Radioactivity (SR) (DPM / nanomole)		$\frac{\text{Tissue SR}}{\text{Plasma SR}}$ (%)		$\frac{\text{3 Hour SR}}{\text{6 Hour SR}}$ (%)
Time of Infusion ...	3	6	3	6	
Plasma	117.05 ± 8.20	134.04 ± 10.85	-	-	87.3
Liver	43.82 ± 4.32	61.86 ± 2.72	37.4	46.2	70.8
Heart	92.07 ± 7.03	111.35 ± 6.42	78.7	83.1	83.1
Diaphragm	76.20 ± 6.13	89.82 ± 2.60	65.1	67.0	84.8
Gastrocnemius	77.46 ± 6.84	97.83 ± 8.49	66.2	73.0	79.2
Kidney	75.25 ± 6.49	85.42 ± 9.03	64.3	63.7	88.1
Jejunum	44.34 ± 4.66	74.37 ± 8.07	37.9	55.5	59.6
Brain	72.92 ± 6.18	96.48 ± 5.78	62.3	73.0	75.6

Each result is the Mean (± S.E.M.) of 6 animals.

Table 21. The Specific Radioactivity of Tyrosine in the Protein-Bound Pool of the Tissues after the Infusion of L-[$U^{14}C$] Tyrosine.

	Specific Radioactivity (SR) (D.P.M. / nanomole)		Protein-Bound SR / Free SR (%)		6h Protein SR / 3h Protein SR
Time of Infusion (h) ...	3	6	3	6	
Liver	4.70 ± 0.41	9.43 ± 0.47	10.7	15.2	2.01
Heart	2.55 ± 0.29	4.91 ± 0.25	2.8	4.4	1.93
Diaphragm	1.82 ± 0.05	3.60 ± 0.22	2.1	4.0	1.98
Gastrocnemius	1.07 ± 0.03	2.04 ± 0.08	1.4	2.1	1.91
Kidney	4.79 ± 0.41	9.29 ± 0.24	6.4	10.9	1.94
Jejunum	10.30 ± 0.60	21.72 ± 0.47	23.2	29.2	2.11
Brain	1.65 ± 0.08	3.33 ± 0.15	2.3	3.5	2.02

Each result is the Mean (± S.E.M.) of 6 animals

Table 22 . Fractional Rates of Tissue Protein Synthesis Calculated from the Results of Infusion 6.

	Fractional Rate of Protein Synthesis (% tissue protein mass synthesised / day)	
Precursor Pool	Plasma Tyrosine	Tissue Tyrosine
Liver	30.1 (8.7)	71.5 (9.9)
Heart	15.0 (11.4)	18.5 (11.5)
Diaphragm	11.3 (8.1)	17.2 (8.0)
Gastrocnemius	6.2 (8.1)	8.8 (8.8)
Kidney	28.7 (8.1)	44.9 (10.6)
Jejunum	72.8 (8.1)	153.9 (10.9)
Brain	10.7 (8.1)	15.8 (8.5)

Figures in parenthesis indicate the largest S.E.M. (given as a % of the mean) of the data from which the synthesis rates were calculated.

which in earlier infusions reached very much lower specific radio-activities.

The specific radioactivity of protein bound tyrosine remained much lower than that of the free amino acid. The results are shown in Table 21. The highest specific radioactivity was seen in jejunum reaching 29% of that of the corresponding free pool. The lowest was in gastrocnemius (2.1%). In general, the 6 hour values were approximately twice the 3 hour figure. This would be expected if incorporation of radioactivity were linear.

The fractional rates of protein synthesis in the different tissues are shown in Table 22. As previously, they have been calculated according to Seta et al., (1973). It is obvious from the table that the fractional rates calculated from the specific radioactivity of free tyrosine in the plasma are lower than those calculated from the free tyrosine in the tissue. In a few of the tissues, such as heart and gastrocnemius, the differences were small, while in others such as liver and jejunum the rates differed by a factor of 2. As before, the tissue with the highest rate of synthesis was the jejunum followed by the liver and kidney. Heart, diaphragm and brain were intermediate with very similar rates and the slowest was gastrocnemius. The significance of these synthesis rates is considered in the final discussion.

SECTION 6 . Final Discussion

Compartmentation, or metabolic channelling as it is sometimes known, has become an important consideration in biochemistry mainly because it has made difficult attempts to measure accurate rates of metabolic reactions. Although conceptually its existance is well recognised, very little is known about this phenomenon, either in qualitative or quantitative terms. Davis (1972) adequately described the situation when he wrote: "Metabolic channeling is a seductive idea: it is easy to understand and hard to prove ..... even once demonstrated it is hard to explain in terms of cell structure".

Compartmentation is certainly no less of a problem in protein metabolism than in any other field of biochemistry if only because twenty different amino acids participate in the process and the extent of compartmentation may not be the same for all. The situation is made more complex in that the metabolism of protein is a cyclic system which involves free amino acids as both substrates and products. This immediately illustrates part of the difficulty, because in studies of isotope incorporation it is the condition of the experiment that amino acids acting as substrates are radioactively labelled while those released by protein degradation are not. Thus the design of the experiment itself highlights the distinction of separate pools.

Of the little that is known about amino acid compartmentation within the cell, the greater part has resulted from kinetic studies on single cell suspensions. This is because of their relative homogeneity. In studies on whole tissues the inevitable difficulty is that the results do not easily differentiate between compartmentation at the tissue level (due to the presence of many different cell types) and that at the cellular level. As mentioned in the introduction and in Infusion 5, the demonstration of different compartments in multicellular systems does not necessarily imply that all are present in each and every cell.

Because of the problems in interpretating results from experiments

in vivo and because of the practical need to understand the nature of the precursor pool under these conditions, the present method of investigation was designed on a very simple basis making virtually no assumptions about structural or metabolic aspects of the system. The only requirements of the method are 1) that one amino acid is capable of being converted to another within the system and 2) that the relative specific radioactivities of these two amino acids remain constant during the period over which the comparisons are made.

Validity of the Method.

If the above conditions are met then the method only assumes that the specific radioactivity ratio in a single protein is an accurate reflection of the ratio in the precursor pool at the time of synthesis. Although by definition this should be true (proof given in Appendix of Infusion 5) there may be discrepancies. If, for instance, there is a significant lag in the incorporation of radioactivity from the precursor into protein and, in addition, the specific radioactivity ratio in the precursor pool is still rising to a constant value (as occurs during the initial part of the infusion) then the ratio in a pure protein would be lower than in its precursor. As the time of infusion becomes longer this disparity will become smaller. This is not so much a problem in the case of glycine and serine because the ratios in the free and in the protein bound amino acids follow each other very closely and are almost constant after two hours of infusion (see Fig. 7).

A more serious difficulty may be that the assumption is not always valid for heterogeneous mixtures of proteins. In such mixtures there are three factors which will determine the ratio; first, the concentration of each protein species; secondly, the amino acid composition of each protein and thirdly, the individual rates of turnover. The overall specific radioactivity ratio in a sample of mixed proteins is in fact related to the *mean* of the individual specific radioactivities of each amino acid

in the different proteins present. Mathematically it can be expressed as follows:

$$\frac{\overline{P}_1}{\overline{P}_2} = \frac{\frac{\sum(MC_1P_1)}{\sum(MC_1)}}{\frac{\sum(MC_2P_2)}{\sum(MC_2)}} = \frac{\text{The Mean Specific Radioactivity of Amino Acid 1 in the Protein Mixture}}{\text{The Mean Specific Radioactivity of Amino Acid 2 in the Protein Mixture}}$$

where

M = the total amount of each individual protein within the mixture

C_1 = the concentration of Amino Acid 1 in each protein

C_2 = the concentration of Amino Acid 2 in each protein

P_1 = the specific radioactivity of Amino Acid 1 in each protein

P_2 = the specific radioactivity of Amino Acid 2 in each protein

In order to try to establish the extent to which the ratio in such a mixture would differ from that in the precursor pool a theoretical model of heterogeneity was devised comprising three proteins, each with varying concentrations, rates of turnover and composition of glycine and serine. The relative composition of serine to glycine in the three proteins was decided on the basis of the known composition of 40 mammalian proteins (Handbook of Protein Sequences 1973) the range of which is shown in Table 23. The composition chosen for the proteins in the model was the mean of this range (1.14) plus and minus two standard errors of this mean (0.86 and 1.42). The measured ratios of serine to glycine residues in the total protein of the tissues investigated in this present study do infact fall within the range given by two standard errors of the mean (0.900 : jejunum - 1.073 : brain).

The conditions set up for the model were as follows:

Mixture	Protein	% of Each Protein in the Mixture	Specific Radioactivity Relative to Precursor (%)	Serine/Glyci Composition
A	1	50	50	1.13
	2	25	100	0.85
	3	25	10	1.44
B	1	50	50	1.13
	2	25	100	1.44
	3	25	10	0.85

Table 23 . Glycine and Serine Content of Various Mammalian Proteins.

Protein	Source	Total No. of Residues	Serine Residues / Glycine Residues
Cytochrome c	Horse	105	0.00
Elastin	Bovine	861	0.02
Cytochrome c	Rabbit	105	0.08
Collagen	Bovine	953	0.09
Glycine Rich-Histone	Rat	118	0.12
Myoglobin	Bovine	153	0.46
Triose Phos. Isomerase	Rabbit	248	0.50
Milk Lysozyme	Human	130	0.55
Glycerald.3 Phos. Dehyd.	Pig	332	0.59
Glutamate Dehyd.	Bovine	500	0.62
Alcohol Dehyd.	Horse	375	0.68
Thymus Histone III	Calf	135	0.71
Basic Myelin Protein	Bovine	171	0.76
Crystalin	Bovine	165	0.86
Adrenodoxin	Bovine	118	0.88
Elastase	Pig	240	0.92
Chymotrypsinogen B	Bovine	245	0.95
ACTH	Bovine	39	1.00
Insulin	Rat	51	1.00
Epidermal Growth Factor	Mouse	53	1.00
Haemoglobin (β-chain)	Rabbit	146	1.00
Myosin	Rabbit	840	1.05
Pepsin	Bovine	341	1.16
α-Lactalbumin	Bovine	123	1.17
Cytochrome B5	Rabbit	98	1.17
Growth Hormone	Bovine	188	1.20
Chymotrypsinogen A	Bovine	245	1.22
Haemoglobin (α-chain)	Rabbit	141	1.33
Prolactin	Sheep	198	1.36
Trypsinogen	Bovine	229	1.36
Carboxypeptidase A	Bovine	307	1.41
Immunoglob. (λ-chain)	Mouse	215	1.41
Serum Albumin	Human	513	1.47
α-Lactoglobulin	Guinea Pig	123	2.00
Parathyroid Hormone	Bovine	84	2.00
Immunoglob. (K-chain)	Mouse	214	2.07
Ribonuclease	Rat	127	2.14
β-Casein (A2)	Bovine	209	2.20
Pituatry Growth Hormone	Human	188	2.25
Ribonuclease	Bovine	124	5.00

Mean Ratio 1.14 ± 0.14 (S.E.M.) (n=40)

Range of Ratio ± 2 S.E.M. = 0.86 - 1.42

Heterogeneous Mixture of Proteins 1,2 and 3

Mixture A	Serine Residues (No.)	Radioactivity in Serine (DPM)	Glycine Residues (No.)	Radioactivity in Glycine (DPM)
Protein 1	2650	132,000	2350	117,500
Protein 2	1150	115,000	1350	135,000
Protein 3	1475	14,750	1025	10,250
Total	5275	262,250	4725	262,750
Spec. Rad	49.72		55.61	

$\frac{\text{Serine}}{\text{Glycine}}$ Specific Radioactivity Ratio = 0.894 (Protein) (Precursor = 1.000)

Mixture B	Serine Residues (No.)	Radioactivity in Serine (DPM)	Glycine Residues (No.)	Radioactivity in Glycine (DPM)
Protein 1	2650	132,500	2350	117,500
Protein 2	1475	147,500	1025	102,500
Protein 3	1150	11,500	1350	13,500
Total	5275	291,500	4725	233,500
Spec. Rad.	55.26		49.42	

$\frac{\text{Serine}}{\text{Glycine}}$ Specific Radioactivity Ratio = 1.118 (Protein) (Precursor = 1.000)

Because it is impossible to assess the true extent of heterogeneity, it is difficult to estimate an accurate figure for the errors involved in comparing the specific radioactivity ratio in a mixture of proteins with that in the precursor pool. The errors indicated by the model (i.e. about 10-12%) are those likely to be encountered in biological systems. They are not necessarily extreme values. However, in order to invalidate the conclusions reached in Infusions 1-5, the errors would have to be at least 20%.

Definition of Precursor Pool

Although the results of this study are difficult to interpret in terms of defining the precursor pool, some evidence of its nature is given by comparing the fractional rates of protein synthesis calculated from infusions of glycine and serine with those from the infusion of tyrosine (Table 24). In calculating these synthesis rates from the free amino acid pool of the tissue, no allowance was made for amino acids contained within

Table 24 . Calculated Fractional Rates of Protein Synthesis from the Results of Infusions 1,2,3,4 and 6.

	Fractional Rate of Protein Synthesis (% / day)													
	Precursor Site													
	Plasma Free Pool							Tissue Free Pool						
Infused Amino Acid.....	Tyrosine (6)	Glycine (1&2)		Glycine (4)		Serine (3)		Tyrosine (6)	Glycine (1&2)		Glycine (4)		Serine (3)	
Precursor Amino Acid...	Tyr.	Gly.	Ser.	Gly.	Ser.	Ser.	Gly.	Tyr.	Gly.	Ser.	Gly.	Ser.	Ser.	Gly.
Liver	30.1	18.0	60.6	21.3*	60.1*	19.1	46.2	71.5	35.9	33.1	34.2	33.8	38.8	37.8
Heart	15.0	9.6	22.3	-	-	15.1	22.3	18.5	15.3	23.0	-	-	14.8	14.8
Diaphragm	11.3	6.7	13.3	-	-	13.8	15.8	15.9	9.6	12.6	-	-	13.0	12.4
Gastrocnemius	6.2	4.2	8.9	-	-	6.6	6.5	8.8	9.2	10.5	-	-	6.9	7.4
Kidney	28.7	20.3	50.2	-	-	25.9	40.6	44.9	39.9	35.3	-	-	29.1	29.2
Jejunum	72.8	36.0	91.8	-	-	92.1	176.6	153.9	59.1	60.8	-	-	118.6	134.0
Brain	10.7	1.2	5.8	-	-	1.3	8.8	15.8	18.7	19.7	-	-	19.3	26.0

Figures in parenthesis indicate the Infusion Number. Asterisk indicates rates calculated from the specific radioactivity of glycine and serine in hepatic portal plasma. All other 'plasma' rates calculated from mixed arterio-venous plasma.

the extracellular compartment. If such a correction is made, for liver, on the assumption that the extracellular fluid in this case has a volume of 27% of the tissue wet weight (Airhart et al.,1974) and that its composition is identical to that of plasma, some interesting observations emerge. Table 25 shows the corrected specific radioactivities of the "intracellular" free pool of glycine, serine and tyrosine in liver, as defined above, and also the synthesis rates calculated from these new values. An important point is that because of the large intracellular pool of free serine and glycine, the correction makes little difference to the measured specific radioactivity of these amino acids (differences are less than 7%). The specific radioactivity of free tyrosine, on the other hand, is reduced by between 40-55% as a result of the correction. These differences are reflected in the synthesis rates. The rate calculated from the "intracellular" glycine is only marginally increased (5.6%) and that from "intracellular" serine is only marginally decreased (3.6%). In contrast, the synthesis rate calculated from "intracellular" tyrosine is considerably increased (by 84%). This magnifies the discrepancy between the rates given by glycine and serine and those given by tyrosine. Table 25 shows that much better agreement is achieved if the fractional rate of synthesis is calculated from the specific radioactivity of free glycine and serine in either the homogenate or the corrected intracellular pool and from the specific radioactivity of free tyrosine in the plasma. This therefore questions the conclusions of Infusions 1 - 5.

The difference between tyrosine, on the one hand, and glycine and serine on the other, can reasonably be explained by a model originally proposed by Mortimore, Woodside and Henry (1972) to describe the incorporation of ^{14}C valine into protein in perfused liver.

For a given amino acid under a given set of conditions, the model supposes that the non- expandable pool (Pool B) receives amino acids from

Table 25 . Correction of Liver Homogenate for Extracellular Contamination.

a) Comparison of the Specific Radioactivity of Free Glycine, Serine and Tyrosine in Plasma and Liver Homogenate with those Calculated for the Liver Intracellular Pool (ICF).

The extracellular correction applied to the liver homogenate was based on the assumption that the extracellular volume was 27% of the liver wet weight and that the amino acid composition of this compartment was identical to that of the plasma. The necessary data for this correction was taken from Infusions 1 and 6 together with additional data from Tables 2 and 13.

Specific Radioactivity (DPM/nanomole)

Time of Infusion (h) ...	2			6		
	Plasma	Liver Homogenate	Liver ICF	Plasma	Liver Homogenate	Liver ICF
Glycine	184.9	83.3	78.0	200.4	109.7	104.8
Serine	45.2	78.7	81.1	63.8	121.1	126.1
Tyrosine	117.1	43.8	19.6	134.0	61.9	38.0

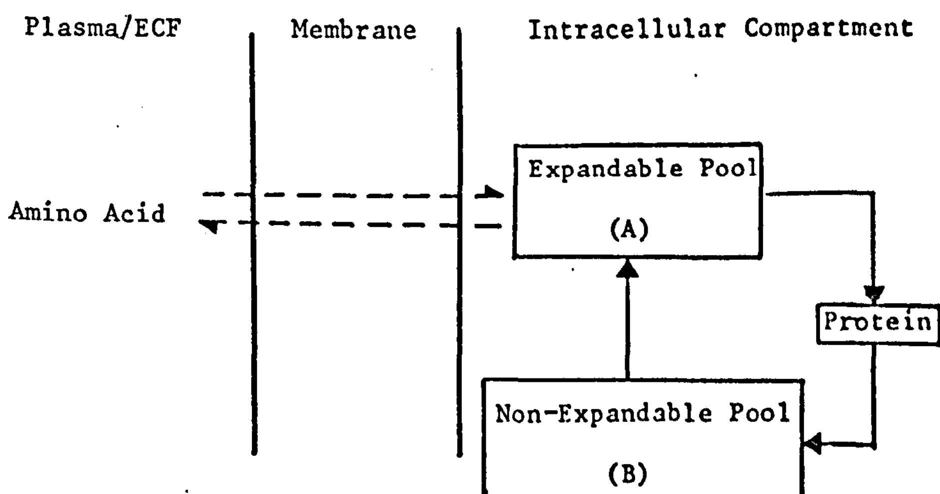
b) Fractional Rate of Protein Synthesis in Liver Calculated from the Above Specific Radioactivities and the Results of Incorporation Given in Figure 5 and Table 21.

The method of calculation was exactly as in previous infusions.

Fractional Rate of Protein Synthesis (%/day)

Precursor Pool ...	Plasma	Liver Homogenate	Liver ICF
Glycine	18.0	35.9	37.9
Serine	60.6	33.1	31.9
Tyrosine	30.1	71.5	131.4

the site of protein degradation and that its size remains constant. The size of the expandable pool (Pool A) is directly dependent on the concentration of the amino acid in the plasma or extracellular compartment and is therefore not constant. Pool B (the non-expandable pool) empties into Pool A (the expandable pool) but does not equilibrate with it. It is further proposed that the supply of precursor amino acids is the function of Pool A.



During an infusion of a radioactive amino acid the average specific radioactivity of the intracellular pool (after correction for the extra-cellular contamination) is then given by :-

$$\frac{C_a S_a}{(C_a + C_b)} \quad \text{where} \quad \begin{array}{l} C_a = \text{amount of amino acid in Pool A} \\ C_b = \text{amount of amino acid in Pool B} \\ S_a = \text{specific radioactivity of Pool A} \end{array}$$

(Pool B is considered to have negligible radioactivity as it derives its amino acids from protein breakdown. This is valid for short term experiments).

If C_a is very large relative to C_b then the average specific radioactivity of the intracellular pool will be almost the same as that of Pool A. This is likely to be the case for glycine and serine as they are both present in high concentrations within the cell. If on the other hand C_a is very much smaller than C_b then the average intracellular specific

radioactivity will be proportionally lower than that of S_a .

This model will explain the differences in the synthesis rates calculated from the average intracellular specific radioactivity of glycine, serine and tyrosine because, in the case of glycine and serine, the average intracellular specific radioactivity is very similar to that of Pool A, the precursor pool. For tyrosine, the specific radioactivity of Pool A is not the same as the average intracellular value because Pool A is smaller than Pool B. The specific radioactivity of Pool A is therefore higher than that given by the mean value for the intracellular pool. In order to obtain synthesis rates comparable to those estimated from the glycine and serine data (i.e. approx. 35%/day) the specific radioactivity of tyrosine in Pool A in Infusion 6 would have to be about 108 DPM/nanomole, which is only 14% lower than that of the mean value for free tyrosine in plasma (Table 25). From this it can be calculated that the size of Pool A is approximately 50% that of Pool B.

In other tissues,such as heart, diaphragm, brain and especially gastrocnemius, there is virtually no disagreement in the synthesis rates calculated from the specific radioactivity of the tissue free pool. This can also be explained in terms of the model if it is assumed that the size of Pool B (the pool of unlabelled amino acids derived from proteolysis) is directly related to the rate of protein degradation. In gastrocnemius the fractional rate of degradation is about 5.2%/day. This is calculated by subtracting the growth rate (3.3%/day) from the measured synthesis rate (mean of 8.5%/day). In liver the degradation rate, calculated in a similar manner, is 32%/day. Thus if the size of Pool B, in muscle, is smaller than in liver by a factor of 6, and if Pool A remains the same size in both tissues (which is reasonable as the plasma is common to both) then, in the case of tyrosine, Pool A in muscle will be about three times the size of Pool B. Consequently the average specific radioactivity of the intracellular free tyrosine will be much nearer that of Pool A in

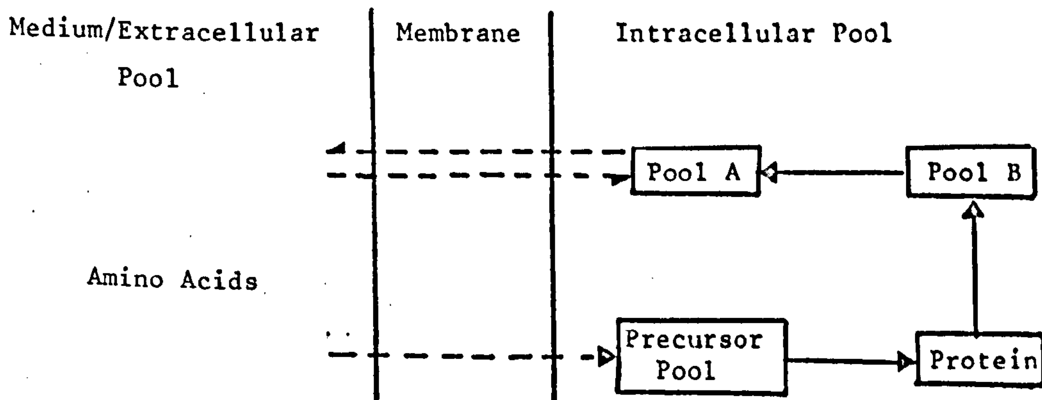
gastrocnemius than it will be in liver. If no extracellular corrections are made, the average specific radioactivity of the tissue homogenate will be even closer to that of Pool A.

In the above discussion it has been assumed that the fractional synthesis rates calculated from the incorporation of glycine and serine are accurate representations of the true rate in liver. This assumption is reasonable because all the rates calculated from the incorporation of these two amino acids are very similar (Table 24) despite the fact that the specific radioactivity gradient of the infused amino acid is in an opposite direction to that of the metabolically derived amino acid. Thus when glycine was infused the specific radioactivity of the free amino acid in plasma was twice that in the liver intracellular pool whereas the specific radioactivity of the derived free serine in plasma was only half that in the intracellular pool (see Table 25). In addition, the synthesis rates estimated from Infusions 1,2,3 and 4 are in good agreement with other studies in which rates were obtained by totally different methods. The results of such studies are collectively presented in Table 26.

While this model can explain the results of most studies involving whole animals or perfused organs, it is unable to explain why, in kinetic studies of radioactive uptake *in vitro*, the rate of incorporation into protein is linear almost from the beginning of the incubation whereas uptake into the free cellular pool shows curvilinear characteristics. The model also cannot account for the results of double isotope "chase" experiments in which the incorporation of one isotope rapidly ceases when the tissue is transferred to a second medium containing a different isotope. To explain such observations a very small and rapidly turning over pool of precursor amino acids would have to be proposed. This pool would have to be distinct from other free amino acids in the cell. One model capable of explaining the results of *in vitro* studies is shown overleaf.

Table 26. Fractional Rates of Tissue Protein Synthesis *in vivo* in the Rat as Measured by Other Methods.

	Fractional Rate of Synthesis (%/day)	Injected Precursor	Amino Acid Measured	Method of Measurement	Time of Measurement	Reference
Liver	73.0	^{14}C Lysine	^{14}C Lysyl t-RNA	Incorporation	20 mins	Henshaw *et al* (1971)
	51.0	^{14}C Na_2CO_3	^{14}C Amino Acids	Decay	7 days	Millward (1970)
	36.3	^{14}C Na_2CO_3	6-^{14}C Arginine	Decay	3-4 days	Stephen & Waterlow (1966)
	29.3-34.0	^{14}C $CaCO_3$	6-^{14}C Arginine	Incorporation	1 day	Swick (1958)
	24.8-26.8	"	"	"	4 days	"
	18.7-19.3	"	"	"	8 days	"
	25.0	"	1-^{14}C Glycine	"	4 days	"
	19.0	"	"	"	8 days	"
Muscle	11.5	^{14}C Na_2CO_3	^{14}C Glu. & Asp.	Decay	12 days	Millward (1970)
	8.0	^{14}C Lysine	^{14}C Lysyl t-RNA	Incorporation	20 mins	Henshaw *et al* (1971)
Brain	19.3	^{14}C Glucose	^{14}C Glutamate	Incorporation	1-8 hours	Austin *et al* (1972)
	17.3	^{14}C Glucose	^{14}C Glu. & Asp.	Decay	28 days	Sabri, Bone & Davison (1974)



Such a model could not account, however, for the data from perfusion studies or experiments *in vivo*.

Conclusions.

The results of this study can be summarised as follows :

1) In the case of glycine and serine the plasma does not appear to be the immediate pool from which these amino acids are incorporated into protein

2) The precursor pool for these amino acids is most likely to be an intracellular compartment, the specific radioactivity of which is similar to that of the tissue homogenate.

3) In the case of tyrosine no clear picture emerges. In liver, synthesis rates comparable to those found with glycine and serine were obtained only when the specific radioactivity of the precursor tyrosine was assumed to be similar to that in the plasma. For heart, diaphragm and gastrocnemius there was little difference in the synthesis rates calculated from the specific radioactivity of free tyrosine in either the plasma or in the tissue homogenate pool. Synthesis rates calculated from both pools were similar to those determined from incorporation of serine and glycine from the tissue pool.

4) There is good evidence to suggest that serine is a very satisfactory amino acid to use in the study of protein synthesis in muscle, especially

in the case of heart, because isotopic equilibration between the plasma and the heart is very rapid.

5) In liver, the results of the relative incorporation of serine and glycine into plasma albumin, liver ferritin and total liver protein tentatively indicate that more than one precursor pool may exist. This can be interpreted in terms of membrane bound and free ribosomes.

The technique of differential labelling used in this investigation has provided only limited evidence to illustrate the nature of the precursor pool. Its application has been relatively crude in so far as only the specific radioactivity of the entire molecule of serine and glycine has been measured. The method can be elaborated if, for instance, specifically labelled amino acids are used for infusion and comparisons are then made of the relative radioactivity of each carbon atom of the amino acid in both protein and in the free pool. This may enable only a single amino acid to be used and consequently the problems of heterogeneity, outlined in the previous part of this discussion, may be overcome.

While the direct methods of investigation, such as the isolation of nascent peptide chains, may prove to be more satisfactory in determining the actual specific radioactivity of the precursor pool, they are unlikely to provide sufficient information to be able to locate the precursor pool in any particular cellular structure. Evidence of this nature could be provided by differential labelling.

Finally, the conclusions of the present experiments are, as yet, only applicable to healthy rats on a diet of normal protein content. If other conditions, either nutritional or pathological, alter the relative sizes of the proposed intracellular pools (i.e. the expandable and the non-expandable pool) then new interpretations may have to be made.

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