Critical Assessment of Methods Used to Measure Protein Synthesis in Human Subjects

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The loss of body protein that frequently accompanies illness occurs through changes in protein synthesis and degradation. In human tissues, rates of protein synthesis can be assessed with stable isotopic tracer techniques and mass spectrometry. The basic principles of these methods are explained, and the advantages and drawbacks of the two main approaches, the constant infusion method and the flooding method, are described. Examples are given of the use of these methods to investigate protein synthesis and surgical trauma, pathologies of the gastrointestinal tract and the response of tumor growth to amino acid supplements.

INTRODUCTION

Injury and inflammation are frequently accompanied by loss of protein from body tissues, and one of the aims of treatment is to minimize this loss and promote its recovery by nutritional and other therapies. A better understanding of the processes that influence tissue protein gain and loss can be achieved by studies of protein synthesis and degradation in tissues. These two complex and opposing processes are controlled by a variety of hormonal, chemical and physical signals, resulting in the maintenance of protein balance in the healthy adult individual. An imbalance resulting from modulation of either synthesis or degradation is the cause of protein gain or loss, but in this article we will concentrate on measurements of protein synthesis. In particular, we aim to describe techniques and approaches suitable for in vivo studies in human volunteers and patients.

MEASUREMENT OF PROTEIN SYNTHESIS

Rates of protein synthesis have been measured for many years in cells and tissues in vitro and in animal models using radioactively labeled amino acids. These studies have made an invaluable contribution to our understanding of protein metabolism, but their relevance to human disease can only be assessed by direct measurements in humans. Risk of radiation limits the use of radioactive isotopes in humans, however, and they have now been largely replaced by their stable isotope counterparts. The relationships between the radioactive and stable isotopes of the relevant elements are shown in Table 1. The ones most commonly used are $^{13}$C, $^2$H and $^{15}$N, the latter being important because it is the only usable isotope of nitrogen, there being no radioactive analogue of practical significance. In most respects experiments with stable isotopes are the same as those with radioactive isotopes, except the term “isotopic enrichment” replaces the term “specific radioactivity.” The enrichment is the number of labeled molecules expressed as a proportion of the total number of molecules of the compound, and is measured with a mass spectrometer.
The limitations of tissue sampling in humans and the continual improvement of mass spectrometers have resulted in the development of a variety of approaches for measuring rates of protein synthesis and degradation in the whole body. The label is administered orally or intravenously, and measurements are made on samples of urine or blood and breath, which are readily available by relatively non-invasive techniques [1]. For example,

**Table 1. Stable and radioactive isotopes of elements commonly used in metabolic research**

| Element  | Normal | Radioactive | Stable     |
|----------|--------|-------------|------------|
| Hydrogen | $^1$H  | $^3$H       | $^2$H (0.015 percent) |
| Carbon   | $^{12}$C | $^{14}$C     | $^{13}$C (1.1 percent) |
| Nitrogen | $^{14}$N | -           | $^{15}$N (0.37 percent) |
| Oxygen   | $^{16}$O | -           | $^{17}$O (0.037 percent) |
|          |         |             | $^{18}$O (0.2 percent) |

The "Normal" isotope is the most abundant naturally occurring form. The "Stable" isotopes also occur naturally, and the average natural abundance is shown in parentheses. "Radioactive" isotopes are readily available for research for hydrogen and carbon. The radioactive isotopes of nitrogen and oxygen have extremely short half lives and can only be used with very special techniques and equipment.

intravenous infusion of [$1$-$^{13}$C]leucine for 6-12 hrs results in the attainment of a plateau in the enrichment of free leucine in the blood. This plateau value, together with an estimate of the amount of $^{13}$C expired in breath CO$_2$ can then be used to determine the total body turnover of leucine, and also its origin from dietary intake and body protein degradation and its disposition into oxidation and body protein synthesis [2, 3, 4]. These techniques have been used extensively (for reviews see [1, 5, 6]) and have given rise to much useful information, but suffer from the limitation that changes in whole body rates of protein synthesis cannot be ascribed with any certainty to any particular organ, tissue or body compartment. For example, animal studies have shown that inflammation resulting from subcutaneous injection of turpentine is associated with a fall in protein synthesis in skeletal muscle, but a rise in the liver [7]. Over the last decade or so, therefore, there has been an increasing interest in measurements of protein synthesis rates in individual tissues of human subjects.

The general procedure for measuring tissue protein synthesis in vivo is to inject an amino acid labeled with the chosen isotope into the bloodstream and to assess its incorporation into tissue protein. To determine the rate of protein synthesis, two separate measurements must be made. Firstly, a tissue sample is taken at a specific time (t days) after the isotope is given, and the enrichment of the amino acid in protein ($E_p$) is measured. Secondly, the time course of enrichment of the free amino acid precursor of protein synthesis over the measurement period must be evaluated, and its average value ($E_p$) determined. The rate of protein synthesis ($k_s$) is then calculated from the following equation:

$$k_s = E_p \times 100 / E_f \times t$$

$k_s$ is the fractional rate of protein synthesis, that is the amount of protein synthesized expressed as a proportion of the amount of protein in that tissue.

The amount of label incorporated into protein can not only be measured directly on a tissue sample, but also it can be inferred from the disappearance of label from the blood (see Organ Balance Measurement, below). The assessment of the enrichment of the free amino acid that is being incorporated into protein is more difficult and has given rise to much debate. Theoretically this measurement should be made on the pool of amino acyl
tRNA in the tissue, but this is complicated by the extremely rapid rate of turnover of this pool and its small size, requiring large tissue samples. For most practical purposes the alternative is to use either the plasma or the tissue (intracellular) free amino acid. As will be seen below, the isotopic enrichment in these two compartments is not necessarily the same, which has led to uncertainty in the calculated values for protein synthesis and has been influential in determining the way in which the labeled amino acid is administered. Both of the procedures commonly used today were originally suggested by the work of R.B. Loftfield in the 1950s. When a radioactively labeled amino acid was given to rats by continuous intravenous infusion, the specific radioactivity in the plasma and tissues rapidly rose to constant (plateau) values, but as illustrated in Figure 1a, in the tissues the value remained substantially lower than in the plasma [8]. The interpretation made was that the intracellular pool was derived partially by transport of amino acid into the cell from the plasma and partially from the degradation of unlabeled protein. Moreover, it would be difficult to calculate rates of protein synthesis with confidence, because it was not known which, if either, of these two values would be appropriate as the precursor enrichment. Rates of protein synthesis were not therefore calculated, and instead a different method of label administration was devised, with the aim of making the intra- and extracellular enrichments the same, thus minimizing the ambiguity. This was achieved by injecting the labeled amino acid together with a large amount of unlabeled amino acid, sufficiently to dominate (flood) the small endogenous pool of unlabeled free amino acid [9]. This approach has become known as the "flooding method" and is illustrated in Figure 1b. Both of these approaches have been used extensively in animals with radioactively labeled amino acids [1, 10]: their use in human subjects will now be described in more detail.

**Constant infusion method**

The first studies with this method in humans involved measurement of protein synthesis in muscle with \([^{15}\text{N}]\)lysine [11] and in GI tissues, including tumors, with \([^{15}\text{N}]\)glycine [12]. Subsequently, the majority of constant infusion studies have used \([1,13\text{C}]\)leucine, which is moderately priced and relatively easily measured [13]. Infusions

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**Figure 1.** Curves illustrating the two main methods of measuring tissue protein synthesis with labeled amino acids given by constant intravenous infusion (a) and by flooding (b). The lines represent the time course of enrichment of the free amino acid in the plasma and the tissue pool over periods of time commonly used with these methods in humans. To determine rates of protein synthesis, a tissue sample is taken at the end of the time period and the enrichment of the amino acid in protein determined.
lasting between 4-12 h are generally primed [13], to achieve plateau labeling more quickly, and are followed by tissue sampling, which in the case of skeletal muscle, can be done by percutaneous needle biopsy. The enrichment of the amino acid in protein from the tissue biopsy has usually been measured by chromatographic isolation of the leucine from protein and decarboxylation followed by measurement of the enrichment of the CO₂ produced by gas isotope ratio mass spectrometry (GIRMS)\(^b\).

The precursor enrichment is obtained from serial samples of blood taken during the infusion. The choice is between free leucine in blood or blood α-ketosiocaprico acid (KIC), which is the transamination product of leucine and is thought to be representative of the intracellular leucine in skeletal muscle [14]. The KIC enrichment is typically 70-80 percent of that of leucine [14]. It is often thought that the KIC gives the best estimate of the precursor enrichment, but this remains unproven. The KIC in blood might not be derived entirely from muscle, and at best it represents an average enrichment for all muscles, which might differ from the enrichment in the single muscle under study [15]. Moreover, there is the possibility that the enrichment of leucyl tRNA might sometimes be lower than that of the KIC or the intracellular leucine [16, 17]. Although the KIC has been used for non-muscle tissues such as liver [18], this is not advisable, as there is little reason to suppose that the KIC reflects the liver free leucine enrichment. For liver synthesized proteins (e.g., serum albumin), a far more reliable measure of the precursor enrichment can be derived from measurements of the ApoB protein of very low density lipoprotein in the blood. As this pool turns over very rapidly, it achieves the same enrichment as its own precursor within the liver after a few hours of infusion [19].

The constant infusion method has been used for many investigations of muscle protein synthesis in volunteers and patients [20]. Its advantages are that simultaneous measurement can also be made of whole-body protein turnover, and that the labeled amino acid is given as a tracer, unlike the flooding method, and therefore should not disturb metabolism. Its disadvantages are the need for a metabolic steady state during the infusion, which might preclude observations when acute metabolic changes are occurring (e.g., after a meal, in acute illness or during surgery), and the uncertainty regarding the precursor enrichment.

**Constant infusion during limb or organ balance measurement**

Arterio-venous differences in blood amino acid concentrations coupled with measurement of blood flow have often been used to measure the net balance of amino acids across a limb or organ [21]. When this procedure is performed during a constant infusion of a labeled amino acid, then rates of protein synthesis and degradation can also be obtained [22]. In this case, synthesis is not derived from the amount of label that appears in protein of a biopsy, but instead from the amount of label that disappears from the blood as it passes through the tissue. For an amino acid that is not metabolized in the tissue under study, e.g., phenylalanine in muscle, the rate of uptake of label from the blood must equal the amount incorporated into protein, if the tissue free amino acid pool does not expand or contract. In other respects, the assumptions are the same as those for the constant infusion method. In particular, a value for the precursor enrichment is needed, and this is usually taken from the free amino acid in the venous blood.

The major advantage of this method is that a value for protein degradation can also be obtained, by subtracting the net balance from the rate of synthesis. It should be recognized, however, that this is not an independent estimate. If there is any error in synthesis, e.g., arising from the precursor measurement, then the rate of degradation will also be in error. A recent modification of this technique employs a tissue biopsy at the end of the procedure [23]. This enables the rate of synthesis to be calculated from the intracellular enrichment, which is useful, as there is no metabolite of phenylalanine equivalent to KIC
which can be used as an indicator of intracellular enrichment. In addition, it is possible to calculate the rates of transport of the amino acid between the blood and the intracellular pools.

The balance method can only be done on a tissue or organ with a well defined arterial and venous vasculature. In addition to the disadvantages of the infusion method, there is also the need for arterial catheterization and precise blood flow measurement.

Flooding method

Preliminary experiments showed that intravenous injection of 4 g of [1-13C]leucine per 80 kg body weight resulted in almost complete equalization of enrichment in the plasma and intracellular pools in muscle of healthy volunteers over a two hour period (illustrated in Figure 1b, [24]). The rate of muscle protein synthesis is calculated from the enrichment of leucine in muscle protein at 90 min and the average value for precursor enrichment, derived from serial measurements on plasma leucine or KIC at intervals between 0 and 90 min [24]. The value obtained was 1.86 ± 0.12 percent/day when the precursor was taken to be the plasma leucine and 1.95 ± 0.12 percent/day when the plasma KIC was used as an index of intracellular labeling [24]. These values are expressed as fractional rates, in the units of percent per day, which represent the amounts of protein synthesized as percentages of the amount in that tissue.

Although the flooding method successfully equalizes the rates calculated from intracellular and plasma enrichments, these values are higher than those measured by constant infusion of [1-13C]leucine, e.g., 1.10 ± 0.07 percent/day [25]. This discrepancy has led to much debate about the validity of these two methods [10, 20], which is not yet resolved. There do not appear to be similar discrepancies in tissues of animals such as rats, or in human tissues other than skeletal muscle [10]. Apparently higher values for colon, liver and serum albumin synthesis obtained by flooding reported by Rennie et al. [20] can readily be explained. In liver, the longer period of labeling with infusion means that only non-secretory proteins are measured, whereas total synthesis is measured by flooding [10]. Similarly, with albumin there is turnover of the newly synthesized pool in the plasma during a long infusion, resulting in an underestimate of the synthesis rate [10]. For both the colon and the liver, Rennie et al. [20] quote values from patients with pathological conditions (liver protein synthesis in ulcerative colitis patients and protein synthesis in benign villous adenomas to represent colonic mucosa), in which rates of protein synthesis are known to be abnormally high [26].

Two possible reasons for the discrepancy in human muscle have been suggested. The first is the possibility that the enrichment of the precursor amino acid is not being accurately reflected by measurements of plasma leucine or KIC during a constant infusion. There is evidence that this can occur in cell culture [16], but confirmation that this can occur in human tissues in vivo is so far lacking (see [10]). If the enrichment at the site of protein synthesis were lower than the KIC, this might result in an underestimate of the rate of synthesis by constant infusion. Conversely, it has been suggested that the large amount of labeled amino acid given during flooding might itself stimulate muscle protein synthesis, thus giving rise to an artifically high measured rate by this method [20]. There is no direct proof that this can occur except from studies using the constant infusion approach [27], which does not provide independent evidence. Moreover, labeled phenylalanine and valine both give similar values to that with leucine, when given in flooding amounts, and injection of leucine does not alter the distribution of polyribosomes in muscle [28].

The advantages of infusion are that the label is given as a true tracer, which does not alter metabolism, and measurements of whole body protein turnover can be made simultaneously. The disadvantage is the ambiguity regarding the precursor enrichment and the need to establish a metabolic steady state in order to maintain plateau labeling of free
amino acid pools. We prefer the flooding method because it is of short duration (typically 45-90 min), so is suitable for observing acute changes, it is not dependent on a metabolic steady state, so is suitable for studies in the perioperative period, and minimizes problems of precursor measurement. The potential disadvantage is the lack of direct proof that protein synthesis is not altered by the "flood." Both methods have been used to investigate the control of protein synthesis in a variety of human tissues. Figure 2 illustrates the wide range of fractional rates of synthesis observed in various healthy tissues taken from volunteers and from patients during surgery. The remainder of this article will be devoted to examples of measurements of protein synthesis by the flooding method in human subjects in a range of metabolic and clinical states.

**MUSCLE PROTEIN SYNTHESIS AND SURGICAL TRAUMA**

In an attempt to elucidate the muscle wasting that takes place after surgical injury, measurements of muscle protein synthesis were made in cholecystectomy patients before and after surgery. A short period of anesthesia without surgery did not affect muscle protein synthesis, but immediately after the completion of surgery, there was already a 30 percent fall [29]. By day 3 after surgery, the decrease in protein synthesis had enlarged to 50 percent [30]. These data suggest that muscle wasting after surgery results at least in part from an immediate and sustained decrease in muscle protein synthesis. Further experiments were performed to see whether this change was dependent on the degree of trauma, and whether it could be modified by strategies designed to alleviate the wasting. Minor surgery for breast lump removal did not affect muscle protein synthesis [31]. Surprisingly,
Garlick et al.: Human protein synthesis

Figure 3. The response of muscle protein synthesis to surgery (cholecystectomy) by the conventional (open) technique and by laparoscopy. The rate of protein synthesis was measured by injection of a flooding amount of $[\text{2H}_{5}]$phenylalanine on two occasions in each patient: before surgery and one day after surgery. Data from [32].

Laparoscopic surgery did not produce a smaller response than conventional, open surgery (Figure 3), even though its beneficial effects on recovery are well accepted [32]. Moreover, postoperative total parenteral nutrition (TPN) did not diminish the decrease in protein synthesis [30]. These results suggest that new strategies, such as provision of glutamine in TPN [33], or treatment with growth hormone [34], might be needed to minimize protein loss after surgery and optimize recovery.

PATHOLOGIES OF THE GASTROINTESTINAL TRACT

The GI tract is accessible to study during surgery or endoscopy. Rates of protein synthesis in mucosa of the esophagus, stomach and colon are several fold higher than that in muscle, consistent with the rapid rate of cell proliferation and protein secretion of mucosal tissue (Figure 2). Samples of inflamed colonic mucosa taken during surgery for ulcerative colitis showed much higher rates of protein synthesis than those in healthy mucosa ($24.7 \pm 2.5$ percent/d, vs $9.4 \pm 1.2$ percent/d) [26], probably as a result of increased cell turnover and mucous synthesis. However, the inflammation in the colon is also accompanied by systemic changes, as shown by a 50 percent stimulation of protein synthesis in the liver ($35.4 \pm 2.3$ percent/d vs $20.7 \pm 1.9$ percent/d) [26]. This result in humans is consistent with information obtained in animal models of inflammation, such as subcutaneous injection of turpentine or interleukin-1B [7], and might result in part from a stimulation of acute-phase proteins synthesis.

Other diseases of the GI mucosa also lead to alterations in protein synthesis. The columnar lined esophagus of Barrett’s disease has been shown to have a lower rate of protein synthesis than adjacent squamous mucosa [35], in line with the reported lower rate of cell proliferation of the columnar cells [36]. By contrast, both malignant and benign tumors of the colon have rates of protein synthesis that are much higher than in normal
healthy colonic mucosa [26]. Not only do these tumors have high rates of mucous production, but they have high rates of cell proliferation and net growth. It has therefore been suggested that tumor protein synthesis might be used as an index of changes in tumor growth rate [37, 38]. Moreover, in tumor cells in culture, changes in cell growth are accompanied by changes in protein synthesis in the same direction [39-41]. This has led us to a series of experiments on the effects of amino acids on the growth of human tumors in vivo.

AMINO ACIDS AND TUMOR GROWTH

The question of whether feeding of the host stimulates the growth of the tumor led to measurements of protein synthesis in colorectal tumors of fasted and fed patients. During the 24 hrs prior to surgery, patients were either totally fasted or were given intravenous nutrition. [1-13C]Leucine was injected just before induction of anesthesia and a small sample of tumor was taken. The rate of protein synthesis was 80 percent higher in the patients given TPN [42], suggesting that the tumor was indeed sensitive to the supply of nutrients. However, when the TPN containing a standard mixture of amino acids was replaced with one containing a higher proportion of branched chain amino acids (BCAA) the stimulation of tumor protein synthesis was significantly diminished [43]. BCAA supplementation had previously been suggested to be beneficial to cancer patients, because it was shown to enhance whole-body protein synthesis [44]. However, in the experiment on colorectal cancer patients, the rate of protein synthesis in skeletal muscle was also lower in the BCAA supplemented group [43], suggesting that the BCAA did not favor host tissue metabolism over tumor growth.

The amino acid arginine has been studied extensively for its potentially beneficial effects on immunity, recovery from surgery, wound healing, and specifically on tumor growth [45]. An inhibitory effect of arginine supplementation on the growth of tumor cells in culture and in animal models of cancer has been reported in a variety of studies [45], but this effect is paradoxical, as in other studies a stimulation of tumor growth by arginine was demonstrated [46, 47]. However, none of these observations were made in human cancer patients. We therefore made measurements of tumor protein synthesis in breast cancer patients who had taken arginine supplements (30 g/d for 3 days) prior to surgery, compared with patients receiving no supplements [48]. The result is given in Table 2, showing that the arginine supplements, rather than inhibiting the tumor, actually stimulated. This implies that arginine supplementation of cancer patients cannot be used to inhibit the tumor, and might in fact enhance tumor growth. It reveals the need for more information on arginine’s effects on human tissues, before its potential for benefit in cancer and other diseases can be realized. Preliminary studies on a different human cancer, squamous

Table 2.

| Tumor type     | Protein synthesis (percent/d ± sem) |
|----------------|-------------------------------------|
|                | Control                             | Arginine            |
| Breast         | 10.0 ± 1.2                          | 22.6 ± 2.7*         |
| Head/neck      | 23.9 ± 2.0                          | 20.8 ± 2.4          |

Effect of dietary arginine supplements on rates of protein synthesis in two different human tumors. Patients were given dietary arginine supplements (30 g/d) for three days prior to surgery, and controls were given no supplement. At surgery rates of protein synthesis in the tumor were measured by injection of a flooding amount of [1-13C]leucine (breast) or [3H]phenylalanine (head and neck), followed by removal of a tumor sample for assessment of the incorporation of label. *p < .005. Data from [48, 49].
tumors of the head and neck, have shown that the stimulation by arginine is not common to all tumor types. As Table 2 shows, these tumors not are stimulated by arginine supplements in the same way as the breast tumors [49], giving hope that arginine might be of value in some types of cancer.

In the two studies described above, tentative conclusions regarding the effects of nutrients on tumor growth were made from changes in tumor protein synthesis. This measurement has the advantage that it can be performed in vivo in the cancer patient, in whom direct measurement of tumor growth is not usually possible, thus reducing the need to rely on evidence extrapolated from animal models to humans. Additional validation of this procedure was provided in the above studies by measurements of the expression of cell proliferation indices. The response of colorectal tumors to feeding with conventional or BCAA supplemented amino acids was the same when assessed by measurement of proliferating cell nuclear antigen [43]. Moreover, the stimulation of protein synthesis in breast tumors by arginine was also apparent as a large increase in the expression of Ki67 [48], another marker of cell proliferation. Measurement of tumor protein synthesis might therefore be a useful tool for evaluating the factors that determine human tumor growth.

CONCLUSIONS

These examples illustrate the range of studies of protein synthesis that can now be performed in human volunteers and patients. Until recently, these studies were somewhat limited by the need for large (~200 mg) tissues samples, to enable the enrichment in protein to be measured by gas isotope ratio mass spectrometry (GIRMS). This also made the analysis very expensive, as an additional instrument, a gas chromatography mass spectrometer (GCMS), was needed to measure the free amino acid enrichment. However, new advances in mass spectrometry have enabled these measurements to be made on tissue biopsies as small as 1 mg. We have been able to modify the GCMS procedure for the multiply labeled amino acid, [2H5]phenylalanine, to enable very low enrichments such as those found in muscle protein to be measured [50]. This method is proving very useful, as it is very sensitive, requires a relatively low cost GCMS instrument which can also measure the free amino acid enrichments, and involves much simpler preparation for analysis than the GIRMS procedure for [1-13C]leucine. The GIRMS method has also been modified to achieve better sensitivity and ease of use by interfacing with a gas chromatograph and combustion furnace [51]. These improvements will greatly expand the range of tissues that can be studied and will enable measurements to be made on isolated cells and purified proteins from humans, reducing the need to rely on experimental animal models, and leading to a better understanding of the pathophysiology of human illness and nutrition.

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