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SYMPOSIUM ON 'THE INFLUENCE OF AMINO ACID SUPPLY ON POLYNUCLEOTIDE AND PROTEIN METABOLISM'

An appraisal of techniques for the determination of protein turnover in vivo

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Our knowledge of protein synthesis in vitro has increased appreciably over the last few years—by contrast the problems of protein turnover are little understood. Since the level of protein in living tissues is controlled by the process of turnover, several techniques for measuring this have been devised. This paper is mainly concerned with those methods which are used to assess turnover in whole tissues or even whole animals where a population of proteins occurs with a range of different turnover rates. Those methods which are more suited to determination of turnover rates of isolated pure proteins from tissues have been discussed in reviews by Schimke (1970) and Schimke & Doyle (1970), and those for plasma proteins in this symposium (Hoffenberg, 1972).

Turnover is the dynamic balance between the two opposing processes of protein synthesis from free amino acids present in the tissue, and protein breakdown, which liberates and returns them to the free amino acid pool. Only when the protein mass is in the steady state are these two processes equal, and a complete description of the turnover process requires that both synthesis and breakdown are independently measured. Because the two operate simultaneously, it is not, in general, possible to measure their rates from changes in the protein mass, even if they occur. In most instances, isotopic tracer techniques have been used and this discussion is confined to these.

Two approaches are possible. A labelled sample of protein loses label through breakdown; the rate of decay of label is used to determine rates of breakdown and, in certain circumstances, rates of synthesis. Alternatively, labelled free amino acids are incorporated into protein and their rate of accumulation can be used to calculate rates of synthesis. The two approaches, incorporation and decay, will be discussed separately, firstly as if the protein mass were homogeneous. Afterwards consideration will be given to the validity of the methods with heterogeneous mixtures of protein as in complete tissues.

The decay of isotope in labelled protein

Isotope can only be lost from protein by the breakdown process, but the synthesis of new unlabelled protein will alter its specific activity. Thus the rate of decay of total activity and specific activity in a sample are only equal in the steady state. When the protein mass is changing, the rate of decay of total activity is used to determine rates of breakdown, and the decay of specific activity for rates of synthesis (Koch, 1962). Protein breakdown is in general considered to be random (Schimke, 1970) and so the loss of isotope will be exponential. Thus the semi-logarithmic plot of total radioactivity against time is a straight line whose gradient is the rate of breakdown.

The above argument is only true if the amino acid incorporated into the protein during the experiment is unlabelled. Usually the process known as recycling or reutilization of label occurs, whereby isotope released by protein breakdown is reincorporated into newly synthesized protein. This decreases the apparent rate of loss of isotope from the protein. It is most serious when the protein is labelled by injection of such labelled amino acids as lysine, leucine or ^{75}Se-selenomethionine, when some of the more slowly turning over proteins may not even become maximally labelled for several days after injection, giving very slow rates of decay (Penn, Mandeles & Anker, 1957; Poole, 1971). In liver protein, [6–^{14}C]arginine is considered to be only minimally reutilized because of the presence of the enzyme, arginase. This enzyme removes the labelled guanidine group from arginine released by protein breakdown before it can be reincorporated into protein (Swick, 1958; McFarlane, 1963). In other tissues which do not contain this enzyme, the label is extensively reutilized. Labelling of arginine in protein can be achieved by injection of [6–^{14}C]arginine. This labels tissues such as muscle more highly than liver since removal of the label by arginase substantially reduces the specific activity of liver free arginine. Subsequently, arginine returns to the liver from highly labelled periferal tissues where some is reutilized, decreasing the apparent turnover rate of liver proteins. This can be avoided if liver protein is labelled by an injection of $Na_2{}^{14}CO_3$ which labels arginine in liver only, by way of the urea-cycle enzymes (McFarlane, 1963). Less reutilization occurs since arginine in periferal tissues does not become substantially labelled. Although other amino acids in liver also become labelled, they are not appreciably reutilized and chemical isolation of arginine from the protein is unnecessary (Millward, 1970*a*). Injection of $Na_2{}^{14}CO_3$ can also be used to label protein in other tissues since the carboxyl groups of aspartate and glutamate, which become labelled after the injection, are only minimally recycled. The activity of the transaminase and citric acid cycle enzymes in tissues such as muscle causes the specific activity of free aspartate and glutamate to fall to very low levels within hours after the injection (Millward, 1970*a*).

Whereas semi-logarithmic plots of radioactivity against time with homogeneous proteins are straight lines, with heterogeneous mixtures neither total nor specific activity gives a straight line (e.g. Millward, 1970*b*). This occurs because the initial labelling is greater for the most rapidly synthesized components and is approximately proportional to the turnover rate (Koch, 1962). Therefore the initial gradient is biased

towards the rapidly turning over proteins. At later times, most of the label in this rapidly turning over fraction has decayed, leaving only the label in the more slowly turning over components. If a line is drawn between the initial point and any subsequent point on the curve, its gradient decreases as the period of time over which it is measured increases. The question is, 'Which of these gradients gives us the correct mean turnover rate of the mixture?' In an attempt to discover this we have considered the decay of label in four different model systems of heterogeneous mixtures of protein, in which the true mean turnover rate is known (Garlick, Waterlow & Millward, 1972). These models were constructed so that they contained proteins which varied in quantity by a factor of 10 and in others there was a 350-fold difference in their turnover rates. One of these models (model 4) contained figures for actual turnover rates of different liver enzymes published by Schimke (1970). For each model we have plotted the apparent turnover rate, calculated from the gradients of the lines which join the initial point to a series of later points, against time expressed as a multiple of the true mean half-life of the mixture (Fig. 1). We wanted to find out the time at which the apparent rate coincided with the true mean turnover rate, and this has been marked on each curve by a triangle. With all four models, this point at which the true and apparent rates coincided, occurred at a time between three and four times the half-life of the mixture. Therefore, this

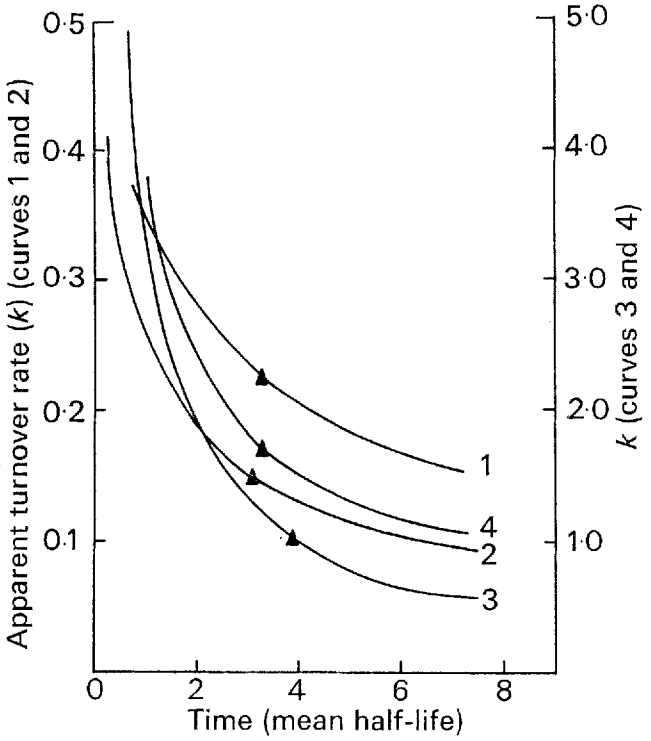

Fig. 1. Theoretical curves of the apparent turnover rate, measured by the decay of label over varying periods of time, in four different models of heterogeneous mixtures of protein. Time is expressed as a multiple of the true mean half-life of each mixture. A triangle (▲) marks the point on each curve where the apparent rate is equal to the calculated mean rate.

suggests that the best time for measuring the turnover rate of a tissue's proteins is that time corresponding to 3–4 half-lives for that tissue. This is not a completely circular argument because we have a good idea of the true mean half-life of a tissue's protein from measurements of synthesis in the steady state by incorporation experiments. By using this half-life value we can now estimate the time when the decay of labelled protein should be measured. This time can now be used in measurements of changing decay rates under different conditions and will not give undue bias to any particular group of proteins, such as would be given to the rapidly turning over fraction with short measurements, which may not behave like the rest of the mixture. Whereas this solution is by no means perfect, something of the sort is clearly necessary if the decay-rate technique is to be used for measuring turnover rates of heterogeneous mixtures.

Incorporation of label into protein

The principle of this technique is to label the free amino acid pool of a tissue and to measure the rate of incorporation of this label into protein. If the rate of synthesis is to be calculated, the specific activity of the precursor free amino acid during the experiment must also be known. In practice the specific activity of the intracellular free amino acid is usually measured, but recent work (Hider, Fern & London, 1971) suggests that in incubated rat muscle the precursor pool is distinct from the remainder of the intracellular pool and is more rapidly in equilibrium with the extracellular pool. These experiments, however, only describe the rate of equilibration of this pool and its specific activity once equilibrium is reached is yet to be measured. At present it is probably wise to make measurements on both the total intracellular and the plasma free amino acid, and to interpret results with both of these possibilities in mind.

Several methods of labelling the free amino acids have been employed, the simplest of these being the single injection of a tracer-labelled amino acid (e.g. Haider & Tarver, 1969). After injection, the specific activity of the free amino acid in the plasma is initially high, and then falls rapidly. In the tissues it rises to a maximum and then falls also (Zilversmit, 1960). A single measurement at the end of the experiment is inadequate to describe the entire time-course of specific activity, and even killing large groups of animals at different time-points results in large standard errors because of variations between animals. The curves for plasma and intracellular specific activity are so different that serious errors in calculation of synthesis rates may occur if the site of the precursor amino acid is not correctly identified.

To avoid the rapid variations in specific activity after a single injection, several techniques have been devised which attempt to maintain the specific activity of the free amino acid in the tissues constant. The theory behind these is illustrated by Fig. 2. If the precursor specific activity is constant throughout, as with the line $a=a_{max}$, the calculation of synthesis rate is straightforward. In practice there is generally a period before the constant level is reached as shown by the rising curve, and one of two approaches is used. Either the measurement lasts so long that the

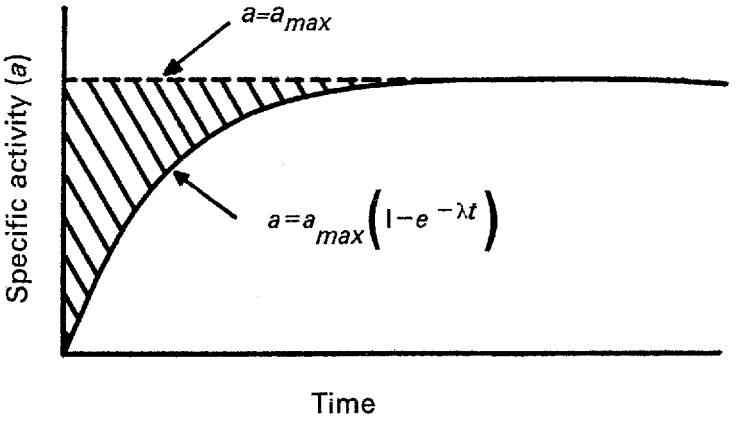

Fig. 2. An illustration of the extent of the error in the determination of protein synthesis rate caused by assuming the precursor specific activity to be constant ($a=a_{max}$) when in fact it rises exponentially to a constant level ($a=a_{max}(1-e^{-\lambda t})$). The amount of label incorporated into protein is approximately proportional to the area under the curve of precursor specific activity; therefore the error is determined by the relative sizes of the shaded area and the area under the curve for the duration of the experiment.

difference between the curves, illustrated by the shaded area, becomes negligible compared with the total area under the curve; or alternatively we attempt to make an allowance for the early part by means of an expression for specific activity such as that shown in the diagram. The advantage of all these methods is that a single measurement at the end of the experiment is sufficient to define the entire time-course of specific activity, and so the rate of synthesis can be calculated for each separate animal. Furthermore, differences between plasma and intracellular amino acid specific activity are not usually so pronounced as with single-injection techniques, and errors arising from incorrect identification of the precursor pool will not be so serious.

Continuous giving of labelled amino acids (Swick, 1958, Schimke, 1964) and labelled carbonate (Swick, 1958) have been used to achieve constant precursor specific activity. The latter is particularly useful for liver protein since the specific activity of the arginine which becomes labelled can be determined from measurements on the urea excreted. A single injection of a large quantity of a labelled amino acid, unlike the tracer dose discussed above, results in a very slow fall in specific activity in the tissues, which can be considered constant over a period of 20 min (Henshaw, Hirsch, Morton & Hiatt, 1971). We do not know, however, how toxic this increased concentration of a single amino acid may be. Another technique employs repeated injections of [^{14}C]glucose after which the specific activity of aspartate and glutamate in rat brain has been shown to remain constant for several hours (Austin, Lowry, Brown & Carter, 1972).

Constant intravenous infusion of a tracer dose of a labelled amino acid can be used to determine synthesis rates either in the whole animal or in individual tissues (Waterlow & Stephen, 1968). During the infusion the specific activity of the free amino acid in the blood rapidly rises to a constant or plateau level from which the amount of amino acid leaving the free amino acid pool for protein synthesis (the

flux) can be calculated (Waterlow & Stephen, 1967). Although the plateau is maintained for many hours (Gan & Jeffay, 1967; Waterlow & Stephen, 1967), ultimately the specific activity rises once again because of recycling of label from protein breakdown. The values of flux for lysine (Waterlow & Stephen, 1967), glycine (Garlick, 1969) and tyrosine in rats (Garlick, unpublished) are appreciably different. On the basis of the total body amino acid composition, however, the rate of protein synthesis can be calculated, and each amino acid gives the same result.

For measurements in individual tissues we must know the time-course of free amino acid specific activity as well as the protein specific activity at the end of the infusion. In plasma this can be measured by taking serial samples of blood and it has been shown that the plateau is approached by a pathway which can be approximated to the single exponential expression shown in Fig. 2, where λ, the rate-constant, can be measured.

The intracellular free amino acid specific activity also reaches a plateau, but one which is lower than in the plasma because of dilution of the label by unlabelled amino acids from intracellular protein breakdown. This plateau is approached by a pathway which can also be approximated to a single exponential as in the blood, but with a different rate-constant. To use this expression in calculating protein synthesis rates, an approximate value for this rate-constant must be found. This has been shown to depend on the tissue and the amino acid infused and is a function of either the rate-constant for blood, or of the rate of protein synthesis in the tissue (Garlick *et al.* 1972). This expression for the precursor specific activity can then be used to derive a formula which relates the rate of protein synthesis in the tissue to the specific activity of the free and protein bound amino acid at the end of the infusion.

As with decay rates, apparent synthesis rates decrease as the period of labelling increases, but there is an important difference. The apparent rate equals the true mean rate at the beginning of the experiment. Koch (1962) analysed the results of Swick (1958) who labelled rat liver protein for a period of 8 d. After 1 d the apparent rate differed very little from the estimate of the true rate, but after 8 d the difference was about 40%. With constant infusion of [^{14}C]tyrosine into mice for periods of time between 0·5 and 2 h there was no appreciable alteration in the estimate of brain protein synthesis rate (Garlick & Marshall, 1972). Thus we are led to the conclusion that, if measurement of incorporation of label into mixed proteins is restricted in length to a few hours only, the apparent rate of synthesis of protein will not differ appreciably from the mean.

Conclusions

Protein turnover is generally measured by isotopic techniques which fall into two classes; rates of incorporation of label are used to determine synthesis rates, whereas rates of decay are used to measure both synthesis and breakdown. There is little to choose between the two approaches except for convenience. Because incorporation experiments are continued for only minutes or hours, they are more suited to measurements of rapid fluctuations in turnover, such as diurnal rhythms; on the

other hand decay is generally measured over a period of days and is more suited to long experiments where short-term variations may only mask the differences.

The disadvantages are evenly balanced. Lack of knowledge about the precursor specific activity affects only incorporation experiments, whereas the problems of measurement on heterogeneous mixtures in whole tissues are more serious with decay rates. These problems may not be so serious however, since measurements of synthesis by incorporation methods such as the constant infusion of labelled amino acids, on the whole agree very well with those from decay of ^{14}C-carbonate labelled protein (Garlick *et al.* 1972).

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