The Kinetics of Disappearance of Labeled Leucine from the Free Leucine Pool of Rat Liver and Its Effect on the Apparent Turnover of Catalase and Other Hepatic Proteins*

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SUMMARY

The specific radioactivity of free leucine has been measured in rat liver at various times after the injection of labeled leucine. The results of these measurements have served to calculate the effect of leucine reutilization on a previous measurement of the apparent turnover rate of catalase with labeled leucine as precursor. This effect was found to account entirely for the difference between the half-life of catalase observed in these experiments (3.5 days) and that determined with δ-aminolevulinate as precursor or by indirect methods (1.5 days).

It is shown that in general, when the specific radioactivity of proteins is measured between 1 and 10 days after the injection of labeled leucine, all proteins with half-lives between 0.1 and 2 days will show apparent half-lives between 3 and 4 days. The limitations of using leucine for studies on protein turnover are discussed, and some suggestions are made for optimizing the design of such experiments.

Tritiated leucine is frequently used as a precursor in studies of protein synthesis, transport, and turnover in rats. It is relatively inexpensive and it is not likely to be incorporated into macromolecules other than protein to any large extent. Moreover it is an essential amino acid in rats and consequently, variations in its endogenous synthesis will not contribute to variation in the extent of protein labeling. However, it has long been recognized that an appreciable fraction of the labeled leucine liberated from degraded protein is reincorporated into newly synthesized protein and that this process results in apparent protein half-lives longer than the true half-lives. Exactly how much longer than the true half-life the apparent half-lives are has not been known.

Koch (1) has made a theoretical study of a limiting case of precursor recycling where a small precursor pool is in dynamic equilibrium with the product pools and the nutritional input of precursor. He showed that when the input of precursor is small enough, all product pools will show essentially the same decay of radioactivity after a pulse label and no useful information can be obtained from such data.

Studies in this laboratory on the turnover rate of rat liver catalase have given a variety of half-lives depending on the methods used (2). The half-life of the heme prosthetic group seems to be about 1.5 days, while the apparent half-life of the protein, determined with labeled leucine, was 3.5 days. In this paper we show that this discrepancy can be accounted for entirely by leucine reutilization.

It has generally been assumed that, while the decay rates of labeled leucine in proteins of various half-lives will all be slower than the true turnover rates, proteins of different half-lives will show detectably different isotopic decay rates. We will see below that this assumption is not always valid.

In studies of the transport of newly synthesized protein within cells, after the injection of labeled leucine into rats, it has been assumed that the bulk of the incorporation of labeled precursor into proteins and the attainment of maximal labeling occurs very soon after injection. This turns out to be true only for proteins with half-lives in the order of hours.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 200 to 250 g (purchased from Charles River Breeding Laboratories, North Wilmington, Massachusetts) were maintained in a room with alternating 12-hour periods of light and darkness with food and water ad libitum. One to 1.5 mCi of L-[4,5-3H]leucine (6 Ci per mmole from Schwartz/Mann, Orangeburg, New York) was injected into them intraperitoneally at a time from 3 to 5 hours after the beginning of the light period. At various times thereafter they were killed by decapitation and their livers were perfused through the aorta with ice-cold 0.25 M sucrose. Free amino acids were extracted from the livers and concentrated by the method of Loftfield and Harris (3). At early time points when the specific radioactivity of the leucine was sufficiently high, it was separated on a Jeoleo AH-5 amino acid analyzer (Jeoleo U. S. A. Inc., Medford, Massachusetts) and the split stream was directed through a 1-ml volume anthracene packed flow cell in a

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Packard 3375 scintillation counter (Packard Instruments, Downers Grove, Illinois). At later time points the leucine was separated on a column of Aminex A-4 resin (Bio-Rad Laboratories, Richmond, California), the eluate was collected in fractions, and the ninhydrin reactive material and tritium were measured.

In either case the sample was applied to the column equilibrated with 0.2 M sodium citrate, pH 3.25, and elution with 0.2 M sodium citrate at pH 4.25 was begun immediately at 30°. This procedure produced improved separation of leucine from isoleucine.

In order to facilitate direct comparison of leucine specific activities in different rats in different experiments, all specific activities are expressed as labeling index, defined as the ratio of cpm per pmole of leucine (whether free or in protein) to the cpm injected per mg of whole rat. The leucine content of catalase was calculated to be 0.482 μmole per mg of protein, on the basis of the amino acid composition (4).

RESULTS

Decay of Radioactivity in Free Leucine Pool—Fig. 1 shows the decay of the specific radioactivity of free leucine in liver after the injection of labeled leucine. There is rapid decay during the first day after injection and then rather slow decay during the following 9 days. The solid line in Fig. 1 represents Equation 1, which can be seen to fit the experimental data reasonably well:

\[ F(t) = \frac{16}{0.25 + t} + \frac{1.02}{(0.055) + t} \] (1)

F(t) is the specific radioactivity of the free leucine pool, and t is the time after injection of the precursor, in days.

Influence of Reutilization of Leucine on Apparent Turnover of Catalase—Fig. 2 shows the manner in which the specific radioactivity of leucine in hepatic catalase decays between 1 and 10 days after the injection of tritiated leucine to the animals. Plotted semilogarithmically as they are here, the results conform reasonably well to a straight line, with a slope corresponding to a half-life of about 3.5 days.

This method of computation of the turnover rate of a protein is in general use and is based on two assumptions. First that substantially all the incorporation of labeled precursor into the protein in question occurs before the first specific activity measurement is performed. Second that thereafter the differential equation describing the decay of specific radioactivity of the protein has the form:

\[ \frac{dP(t)}{dt} = -kP(t) \] (2)

where P(t) is the specific radioactivity of the protein and k its rate of turnover. In integrated form Equation 2 becomes:

\[ P(t) = P(0)e^{-kt} \] (3)

However, if there is significant reutilization of precursor, the appropriate differential equation is:

\[ \frac{dP(t)}{dt} = k[P(t) - P(0)] \] (4)

where P(t) now represents the specific radioactivity of the precursor in the protein and F(t) defines the same quantity for the pool of free precursor as indicated above (see Equation 1), as a function of time.

Equation 4 was integrated stepwise with a digital computer, with F(t) given by Equation 1. The best fit with the data of Fig. 1 was obtained with k equal to 0.47 day⁻¹. As shown by the solid line in Fig. 2, this function fits the data very well. It corresponds to a true half-life of 1.47 days, in complete agreement with the results obtained with δ-aminolevulinate as precursor (2) or by indirect methods with inhibitors of catalase activity or...
Days after injection

FIG. 3. The change with time of specific radioactivity that would be shown by hepatic proteins of various half-lives, after the injection of labeled leucine.

Influence of Reutilization of Leucine on Apparent Turnover Rate as a Function of True Turnover Rate—It is clear from the example just studied that reutilization of label can have a profound influence on the apparent turnover rate of a protein when leucine is used as precursor. Since this amino acid is used extensively in studies of protein turnover and intracellular transport, it was of interest to consider the changes of specific radioactivity with time that would be shown by proteins of various half-lives. In Fig. 3 are plotted a number of such curves, computed from Equations 1 and 4 with various values of k. Some surprising features of these curves are immediately obvious. The initial increase in specific radioactivity is not immediate, but takes a substantial amount of time, depending on the half-life of the protein in question. This is shown more clearly in Fig. 4 where this time has been plotted against protein half-life. Even for a protein with a half-life of only 1 day, maximum labeling takes 18 hours to occur, while for a protein with a half-life of 5 days, this takes more than 2.5 days.

Another property of the curves of Fig. 3 is that for proteins of half-life longer than about 1 day they all become very flat and very little different one from another a few days after injection of the labeled precursor. In view of this similarity, it seemed of interest to find out how closely the decay part of these curves would fit an exponential function of the form of Equation 2, and what kind of apparent half-life would be derived from a semilogarithmic plot of this sort. For this purpose a calculation was made of the results that would be obtained in a set of experiments in which specific radioactivities were determined daily from 1 to 10 days, the points were then fitted to a straight line on a semilogarithmic plot, and the half-life was calculated from the slope of this line. In Fig. 5 are plotted the results of these calculations. The F ratio plotted as a dotted line is the ratio of the variance of the data about the mean to that about the straight line fitted to the data. It is seen that this ratio is high (indicating a close fit to a straight line) only for proteins with half-lives between about 1 and 3 days. This fit is illustrated by the experimental data obtained on catalase, which has a half-life of 1.6 days (Fig. 2). Unfortunately, this advantage is offset by the low degree of correlation between apparent half-life and true half-life in the region of greatest linearity.

In experiments of this type where specific activity measurements begin only 1 day after the leucine injection, all proteins with half-lives less than 2 days will show half-lives between 3 and 4 days. The significance of these findings for the interpretation of turnover data will be examined further in the discussion.

Influence of Reutilization of Leucine on Relative Turnover Measured by Double Labeling Method—Arias, Doyle, and Schimke (6) have devised a method for the measurement of relative protein turnover on a single animal. In such experiments animals receive two injections of leucine labeled with different isotopes at different times. One injection is given shortly before the
animal is killed, the other some time earlier. Particular protein species or protein fractions are then isolated from the animal and their isotopic ratio is determined. Proteins of different half-lives should show different isotopic ratios if the times of the two injections are appropriately chosen. But it is clear from a close examination of Fig. 3 that the ability to distinguish small differences in half-lives of proteins depends very much on the choice of times of injection. From various solutions of Equation 4 we can calculate the results of such experiments. In particular we can search for the optimum times of injection in order to obtain the maximum difference in isotopic ratio between proteins of different half-lives. It turns out that the second injection should be given as soon as possible before the animal is killed, consequently the proteins in question will not have had time to reach their maximum specific activity. The optimum time of the first injection depends on the turnover rate of the proteins in question. We have searched for the time of injection that will produce the greatest difference between the isotopic ratios of proteins of very similar half-lives. The results of this search are presented graphically in Fig. 6. We see that the optimum time for the first injection is about 6 half-lives before killing the animal. We will not present data on the optimum time of injection to distinguish between all possible pairs of protein half-lives, but as a general rule, the best time is the average of the times indicated in Fig. 6 for the two proteins in question.

**Discussion**

In this study we have been careful to administer the injections of labeled leucine at the same time of day as they were administered in our previous studies of protein turnover (2). Lebouton and Handler (7) have reported a small diurnal rhythm in the extent of incorporation of labeled amino acids into the protein of rat liver. Thus the time of injection of label might change to some extent the early stages of decrease in free leucine specific activity. It would be unimportant for the later stages (past 1 day) since any diurnal effects would have been averaged out. We have not looked for diurnal variations in the specific radioactivity of free leucine in liver since published reports of diurnal variations in free leucine pool size (8, 9) have shown only minor changes, well within the noise level of our data. It remains possible that some of our general conclusions concerning temporal specific activity patterns of proteins with high turnover rates may be valid only for labeled leucine injected in the morning.

It is clear from the results presented above that the turnover rate of the protein part of the catalase molecule is the same, within experimental error, as that of the heme prosthetic groups. Hence there is no longer any evidence to support the hypothesis of independent turnover (2). Previously we had reported studies on the turnover rate of the protein components separated by chromatography from rat liver peroxisomes (2). We found no significant differences of half-life among these proteins and we concluded that peroxisomes are probably degraded as wholes within the liver cell. The calculations presented in Fig. 5 tend to weaken somewhat that conclusion. Proteins with a true half-life of about 1.5 days show apparent half-lives with labeled leucine of about 3.5 days, but the curve is rather flat in this region and any difference in true half-life produces only about half as much proportional difference in apparent half-life.

It is clear from Fig. 4 that data on the intracellular transport of newly synthesized protein labeled in vivo with leucine will be hard to interpret except in the case of those proteins of very short half-life. An injection of labeled leucine does not produce anything like a pulse of the synthesis of labeled protein. Our injections were performed intraperitoneally and this may have introduced some delay in absorption, but it is seen that the times of synthesis shown in Fig. 4 are of the order of hours to days, much longer than any such delay.

Fig. 5 tells a great deal about the utility of conventional turnover studies with labeled leucine. We must caution that this figure cannot be used in a simple way to convert to the true half-lives the apparent half-lives found in any turnover experiment with labeled leucine. It can be used as a general guide in the interpretation of such experiments when they have been performed over a time scale similar to the one chosen here. Clearly it will be impossible to determine precise values of the true rates of protein turnover from the results of such experiments. Within a certain range of turnover rates, with suitably designed experiments it may be possible to demonstrate differences in turnover rate between protein species or protein fractions, and the presence or absence of such differences is usually the most interesting conclusion from such studies.

We will consider three classes of proteins, those with half-lives shorter than 1 day, those with half-lives between 1 and 3 days, and those with longer half-lives. Let us examine first those proteins of shortest half-life. If these are to be studied by measuring the decrease with time of specific radioactivity after the injection of labeled leucine, the measurements must all be made in the first day, for proteins turning over very rapidly, in the first few hours. However, such proteins can be studied much better with the double injection technique of Arias et al. (6), providing the time of the first injection is chosen according to Fig. 6. In such experiments all comparisons are made between specific activities of proteins in the same animal and a great deal of noise is eliminated. For proteins of short half-lives the problem of contamination, to be discussed below, is not important.

For proteins of intermediate half-lives, the situation is more complex. As seen in Fig. 5, specific activity of these proteins shows a pseudo-exponential decay over a period of 10 days and the decay constant derived from such data does change with the
half-life of the protein. However, the relative change in the apparent half-life is considerably less than that of the true half-life. Consequently, small differences in half-life between proteins would be difficult to demonstrate with certainty. In the past too little attention has been paid to the statistical evaluation of turnover data. It is a simple matter to calculate the confidence level of any apparent difference of slope, but this is rarely done. Apparent differences, however intuitively convincing, may have no significance whatsoever. In theory the double injection technique would be a much more sensitive way to detect differences in turnover rate. In practice there is one serious difficulty. No preparation of a protein species is totally pure. If we compare the isotope ratios of preparations of two protein species which turn over at the same rate, and one of these preparations has a trace amount of contamination from some protein species of very rapid turnover, then we will find very different isotopic ratios, and conclude erroneously that the two protein species have different turnover rates. Similarly, a tiny amount of contamination could compensate for a true difference in isotopic ratio between two proteins and lead us to the erroneous conclusion that they had the same half-life. Thus differential measurements of turnover rate of proteins with half-lives between 1 and 3 days are theoretically possible, but fraught with practical difficulties.

For proteins with half-lives longer than 3 days all these difficulties become magnified. It is useless to measure specific radioactivity of these proteins at various times after the injection of labeled leucine, because there is very little change in specific activity and it would be completely obscured by noise in the data. In a double injection experiment, any amount of contamination, even by proteins of moderate half-life, will seriously bias the data. Consequently, we must conclude that leucine is not at all a suitable precursor for the study of the turnover of proteins with long half-lives. These proteins can be studied with guanidino-labeled arginine (10), but this precursor is very expensive, it is very inefficient as precursor, and there seems to be large individual variation in its extent of incorporation (2). The carbonate labeling method of Swick (10) may be the only practical technique.

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