On the Measurement of Protein Turnover in Animal Cells*

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SUMMARY

The double isotope technique of Arias, Doyle, and Schimke (1969) J. Biol. Chem. 244, 3303) with radioactive leucine as precursor has been modified to yield accurate values for rate constants of protein degradation. The advantages of the method are that it is both rapid and reproducible in that the protein whose half-life is being measured is isolated only once from one experimental organism and that the values obtained for the rate constants of degradation are comparable to those measured with [guanidino-14C]arginine, and that unlike the latter precursor, radioactive leucine is extensively incorporated into liver protein.

The turnover of the population of total soluble proteins and six proteins isolated in homogeneous form from rat liver was studied by means of the double isotope method. The majority of liver proteins are degraded with half-lives between 2 and 8 days. There is a general correlation between subunit size and rate of degradation. The subunits of the multimeric proteins δ-aminolevulinate dehydratase, catalase, ferritin, and lactate dehydrogenase are degraded at the same rate as the protein in its quaternary configuration. No intermediate states in the degradation of the subunits were found in the biologically active proteins.

The importance of degradation as a mechanism for regulating the concentration of protein in mammalian cells is now well established and in recent years much information has been obtained concerning the cellular events involved in this process (1, 2). However, a major problem still complicating the study of protein turnover is the difficulty of measuring accurately the first order rate constant of degradation. Measurements of this constant of degradation. The rate depend in large part on isotopic methods which are laborious in that they require repeated isolations of a protein after it has been labeled with precursor. Furthermore, isotopic methods, with few exceptions (4, 5), are complicated by the effect of precursor reutilization. Arias, Doyle, and Schimke (3) used a simpler double isotope method to estimate a relative rate of protein degradation. In this technique one isotopic form of an amino acid (14C) is administered initially and is allowed to decay a definite length of time. Then, a second isotopic form (3H) of the same amino acid is given, and the animal is killed a short time later. The ratio of 3H:14C radioactivity in a protein isolated from the animal gives a relative indication of its rate of degradation. That is, among a population of proteins those with high 3H:14C ratios are being degraded at a faster rate than proteins with low 3H:14C ratios.

The experimental ease with which the ratio can be obtained makes the method one of great potential value in the study of protein degradation. However, it is difficult to assess the accuracy of the double isotope method compared to other methods since it yields only a ratio of 3H:14C radioactivity and not a rate constant of degradation.

In the present study we show that the double isotope method with leucine as precursor can be adapted to give rate constants of degradation; the values are comparable to those obtained with [guanidino-14C]arginine, a precursor that cannot be reutilized for protein synthesis because the guanidino carbon is hydrolyzed by arginase to urea (6, 7). The double isotope method then can be used with confidence to measure rates of protein degradation. By means of this method, we have calculated rate constants of degradation for homogenate, three cell fractions, the population of total soluble proteins, and six proteins isolated in homogeneous form from rat liver. The majority of liver proteins are degraded with half-lives of from 2 to 8 days. There is a general correlation between subunit size and rate of degradation (8) with large polypeptides being degraded at a faster rate than smaller polypeptides. The subunits of the multimeric proteins δ-aminolevulinate dehydratase, catalase, ferritin, and lactate dehydrogenase are degraded at the same rate as the protein in its quaternary configuration. No intermediate states in the degradation of the subunits were found in the biologically active proteins.

EXPERIMENTAL PROCEDURE

Animals—Female Sprague-Dawley rats weighing 120 to 140 g were used in all experiments. They were purchased from Zivic-
Miller Laboratories, Allison Park, Pa., and were maintained on a diet of Purina Laboratory Chow and water ad libitum.

Administration of Isotopes—L-[guanidino-14C]Arginine, specific activity 4.58 mCi per mmole, L-[U-14C]leucine, specific activity 262 mCi per mmole, and L-[4,5-3H]leucine, specific activity adjusted to 1.0 Ci per mmole, were purchased from New England Nuclear. In the double isotope experiments rats were fasted for 16 hours, and then, each rat was given an intraperitoneal injection of 25 µCi of [14C]leucine in a volume of 0.5 ml. Food was restored 4 hours later. Three or 10 days after the initial injection the fasting regimen was repeated, but the animals were injected with 75 µCi of [4,5-3H]leucine in a volume of 0.5 ml. In some cases both isotopic forms of leucine were administered simultaneously. The animals were killed 4 hours after the final injection of isotope. A 4-hour interval was chosen because this period of protein synthesis is short relative to the half-lives of the specific proteins and cell fractions, yet sufficiently long to clear the liver of secretory proteins which can complicate the analysis of membrane fractions (3). L-[guanidino-14C]Arginine also was given intraperitoneally in a volume of 0.5 ml, 25 µCi per rat, after the rats had been starved for 16 hours. Preparation of the material for counting varied according to experiment and specific isotope; details are given in the legends to tables and figures. Usually, proteins in crude fractions or purified enzymes were precipitated with 10% trichloroacetic acid and washed according to the procedure of Siekevitz (9). The proteins then were dissolved in 1 ml of Protosol (New England Nuclear) and counted in 10 ml of a toluene-based scintillation mixture (10). Proteins separated on polyacrylamide-sodium dodecyl sulfate gels were prepared for counting by first slicing the gel into 1-mm sections. Each slice was transferred to a scintillation vial containing 0.8 ml of 30% H2O2, and the vials were placed overnight in a 60° oven. Scintillation fluid containing Triton X-100 (11), 10 ml, was added to each vial. All counting was done in a Packard Tri-Carb liquid scintillation spectrometer. Channels were chosen such that there were no 3H-counts in the 14C channel while between 7 and 13% of the 14C counts were in the 3H channel. Sufficient counts were obtained to ensure a counting error of 5% or less. Efficiencies were determined with internal standards.

Cell Fractionation and Isolation of Specific Proteins—Animals were killed by decapitation, and the livers were perfused via the splenic vein with cold 0.85% NaCl. Livers then were removed and cut into 1-mm cubes on a McIlwain tissue chopper. Cubes from four to eight livers were combined, and a portion was removed for the isolation of catalase and lactate dehydrogenase isozyrne-5. The latter enzyme was assayed by recording the decrease in absorbance of NADH at 340 nm in the system described by Markert and Faulhaber (12). Lactate dehydrogenase was purified to homogeneity as judged by analytical disc gel electrophoresis (13, 14) according to the procedure of Hsieh and Vestling (15). Catalase was assayed as described by Ganschow and Schimke (16) and was partially purified through step six of the method of Price et al. (17) with no loss of total activity. The remaining liver cubes were homogenized in 0.25 M sucrose and the mitochondrial, microsomal, and supernatant fractions were obtained by standard techniques (18). Cytochrome b5 and NADPH-cytochrome c reductase were purified from the "washed microsomal fraction" of Omura et al. (19) by a modification of their procedure (3). Both proteins were homogeneous by the criteria of analytical disc gel electrophoresis.

Ferritin was partially purified from the high speed supernatant fraction of rat liver through the (NH4)2SO4 fractionation step of Drysdale and Munro (20) and was assayed as described elsewhere (20). δ-Aminolevulinate dehydratase was partially purified from the same high speed supernatant fraction through the (NH4)2SO4 fractionation step of Coleman (21) and was assayed as described previously (22). Both proteins were recovered through the partial purification in essentially 100% yield. δ-Aminolevulinate dehydratase, catalase, and ferritin were precipitated from the partially purified extracts with antisera specific for each protein. Antibody to mouse liver δ-aminolevulinate dehydratase was prepared in rabbits as described previously (22). Antibody to mouse liver catalase, prepared in goats, was kindly supplied by Dr. Roger Ganschow. Antibody to horse spleen ferritin was obtained from Nutritional Biochemicals. The cross-reactivity and specificity of each antiserum was determined by Ouchterlony double diffusion analysis and quantitative precipitin reactions (23). Protein was determined by the method of Lowry et al. with bovine serum albumin as standard (24).

Sufficient antiserum was added to the extracts to precipitate completely each of the proteins. The mixtures were incubated at 37° for 30 min and then at 4° for 12 hours. The resulting immune precipitates were collected by centrifugation and were washed three times with cold 0.85% NaCl. The supernantant fluids were assayed to ensure that all of the enzyme activity or ferritin had been precipitated. No immune precipitates were formed with control sera.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (25) in tubes (0.5 x 7 cm) containing 10% acrylamide as the separating gel. Large preparative gels (1.5 x 10 cm) were used to separate radioactively labeled proteins in the total soluble fraction of rat liver, to separate the subunits of lactate dehydrogenase and to separate the subunits of antigens and immunoglobulins in the immune precipitates. The separating gel again consisted of 10% acrylamide in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate. Large dehydrogenase-5 purified from 30 g of liver and immune precipitates containing either ferritin, catalase, or δ-aminolevulinate dehydratase from 1 to 2 g of liver were dissolved in 0.5 ml of 1% sodium dodecyl sulfate, 2% 2-mercaptoethanol in 0.01 M sodium phosphate buffer, pH 7.0. Soluble liver proteins were prepared for electrophoresis by adding 0.5 ml of 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol in 0.02 M sodium phosphate buffer, pH 7.0, to 0.5 ml of a 105,000 X g supernatant fraction of rat liver containing 8 mg of protein. Proteins or immune precipitates were dissociated into subunits by heating at 100° for 5 min. Samples then were mixed with a small amount of glycerol and tracking dye (bromphenol blue) and layered directly onto the separating gel. A current of 4 mA per gel was applied until the tracking dye had entered the gel. Then, the current was increased to 10 mA per gel. Electrophoresis was terminated when the tracking dye had migrated to about 1.5 cm from the bottom of the tube. The gels were either stained with Coomassie brilliant blue in 7.5% acetic acid and 5% methanol or frozen on dry ice and cut into 1-mm slices for counting. Gels were destained in 7.5% acetic acid and 50% methanol.

Small analytical gels and large preparative gels were calibrated with bovine serum albumin (68,000 daltons), liver alcohol dehydrogenase (41,000 daltons), trypsin (23,000 daltons), and cytochrome c (11,700 daltons) as standards.
RESULTS

Double Isotope Technique—The essentials of the double isotope technique are depicted schematically in Fig. 1, which shows the loss of radioactivity expected for five hypothetical proteins with different first order rate constants of degradation. It is assumed that the proteins in Fig. 1 were labeled by a short exposure to a precursor which is not reutilized. The loss of radioactivity from protein A with a half-life of 1 day would be much faster than that from protein E with a half-life of 6 days. Proteins B, C, and D have half-lives of 2, 3, and 4 days, respectively. The logarithm of the specific activity, \( \ln \left( \frac{P(t)}{P(0)} \right) \), at any time \( t \) has elapsed and \( k_d \) is constant, can be seen from the equation describing first order decay.

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

where \( P(0) \) is the initial number of molecules present, \( P(t) \) is the number of molecules remaining after time \( t \) has elapsed and \( k_d \) is the rate constant of degradation. The \( \ln \left( \frac{P(t)}{P(0)} \right) \) is equivalent to \( \ln \left( \frac{P(t)}{P(0)} \right) \) at any given time and represents the interval between first and second injections or precursor.

The loss of radioactivity from the protein remaining after time \( t \) has elapsed and \( k_d \) is constant, can be seen from the equation describing first order decay.

The In of \( \frac{P(t)}{P(0)} \) or \( \ln \left( \frac{P(t)}{P(0)} \right) \) against time should yield a straight line. Ideally, the slope of this line should give \( k_d \), the time interval between injections.

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( {^{3}H}:{^{14}C} ) Ratio</th>
<th>( t_1 )</th>
<th>( k_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate (3, 26)</td>
<td>5.0</td>
<td>0.14-0.16</td>
<td>0.28</td>
</tr>
<tr>
<td>Mitochondria (5)</td>
<td>4.2</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>Microsomes (3, 27)</td>
<td>6.3</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>Supennatant (3)</td>
<td>4.3</td>
<td>0.14-0.16</td>
<td>0.28</td>
</tr>
<tr>
<td>Cytochrome b5 (19, 27)</td>
<td>3.3</td>
<td>0.14-0.16</td>
<td>0.28</td>
</tr>
<tr>
<td>NADPH-cytochrome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c reductase (19, 27)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase (28)</td>
<td>2.5</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>Ferritin</td>
<td>720</td>
<td>2.5</td>
<td>0.28</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>1,200</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>( \delta )-Amino-levulinate dehydrotase</td>
<td>1,700</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

* The numbers in parentheses are reference numbers.

**Fig. 1** The loss of radioactivity expected for five hypothetical proteins having different half-lives and undergoing first order decay. Half-lives for the five proteins, A to E, are indicated by arrows. The numerical values indicate the ratios of the initial radioactivity to the protein remaining after 3 days of decay (dashed line).

**Fig. 2** The dependence of the rate constant of degradation on the logarithm of the ratio of initial radioactivity to radioactivity remaining after 3 days for the five proteins shown in Fig. 1.
Each of two rats received a simultaneous injection of 25 μCi of \(^{14}C\)leucine and 75 μCi of \(^{3}H\)leucine. Four hours later the animals were killed and the cell fractions were isolated and counted as described under "Experimental Procedure." There is a heterogeneity of ratios among the cell fractions and proteins with ferritin, initially followed 3 days later by \(^{3}H\)leucine. One, 3, and 5 days after injection, animals were killed and cell fractions, ferritin, and \(\delta\)-aminolevulinate dehydratase were isolated from liver as described under "Experimental Procedure." \(^{3}H\) and \(^{14}C\) radioactivity were determined on the same sample of protein from the same group of rats. Half-lives then were estimated from a semilogarithmic plot of radioactivity per mg of protein against time. Each point represented the radioactivity in a liver cell fraction, \(\delta\)-aminolevulinate dehydratase, or ferritin from two to five rats.

Fig. 3. A plot of the logarithm of the \(^{3}H\):\(^{14}C\) ratio against \(k_d\) as determined by \([\text{guanidino-}^{14}C]\text{arginine for cell fractions and proteins of rat liver; 3-day interval between injections of isotope.}\) The data from Table I together with \(^{3}H\):\(^{14}C\) ratios from two to four other experiments are plotted against \(k_d\) as compiled from the literature or determined as shown in Table III. The points represent the average of the ratio, while the bars represent the range of values. No bars indicate that the range of values was within the area of the point.

Initially followed 3 days later by \(^{3}H\)leucine. There is a heterogeneity of ratios among the cell fractions and proteins with ferritin having the highest ratio and \(\delta\)-aminolevulinate dehydratase and lactate dehydrogenase having the lowest. Table II shows a control experiment in which rats were given \(^{3}H\)leucine and \(^{14}C\)leucine simultaneously. This experiment indicates the degree of error inherent in the double isotope method, with fractions varying in ratio from 2.9 to 3.1 and also gives the isotope ratio to be expected without the effect of degradation, about 3.

Table I had ratios greater than 3 indicating that some degradation had occurred.

Included in Table I are rates of degradation, expressed as \(k_d\) and a half-life, reported in the literature for the cell fractions and three of the liver proteins. These values were determined by following the loss of the minimally reutilizable precursor \([\text{guanidino-}^{14}C]\text{arginine from each of the cell fractions or proteins.}\) When the logarithm of the double isotope ratio is plotted against the rate constant of degradation, \(k_d\), a smooth curve is obtained (Fig. 3). The only exception is the supernatant fraction, which with a ratio of 4.3, should have a half-life of about 4 days (\(k_d = 0.18\)) to fit on the curve. The value reported in the literature for this fraction is a 5.1-day half-life. When the curve in Fig. 3 was extended to include the double isotope ratio for ferritin, a value for the half-life of this protein of about 1.5 days was obtained.

To assess the accuracy of this value, we measured the rate of degradation of ferritin by following the loss with time of \([\text{guanidino-}^{14}C]\text{arginine and }^{3}H\)leucine after a single administration of both precursors to a group of rats. The results, presented in Table III, show that the double isotope technique gives a value for the half-life of ferritin (1.5 days) that is equivalent to the one obtained with \([\text{guanidino-}^{14}C]\text{arginine and }^{3}H\)leucine. The latter isotope is utilized for protein synthesis and calculation of a half-life from the slope of the first order decay curve yields values which are overestimated by a factor of about two. Included in Table III are values for the half-lives of the liver cell fractions. They are for the most part equivalent to those reported in the literature, but we obtained a half-life for the supernatant fraction of 8.7 days which is again

![Figure 3](http://www.jbc.org/DownloadedFrom/.../197117.png)
TABLE IV

\[ ^{3}\text{H}:^{14}\text{C}} \text{ ratios of rat liver cell fractions and proteins; 10-day interval between injections} \]

Experimental details are exactly the same as given in the legends to Tables I and II and under “Experimental Procedure” except the interval between first and second injection of isotope was 10 days. The ratio for lactate dehydrogenase was the same for the enzyme as purified from 30 g of liver and the subunit as separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$^{3}\text{H}$</th>
<th>$^{14}\text{C}$</th>
<th>Ratio $^{3}\text{H}:^{14}\text{C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>17,400</td>
<td>1,600</td>
<td>11.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>9,000</td>
<td>1,400</td>
<td>6.2</td>
</tr>
<tr>
<td>Microsomes</td>
<td>13,100</td>
<td>800</td>
<td>16.7</td>
</tr>
<tr>
<td>Supernatant</td>
<td>10,543</td>
<td>1,030</td>
<td>10.0</td>
</tr>
<tr>
<td>Ferritin</td>
<td>14,190</td>
<td>304</td>
<td>50.0</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>7,430</td>
<td>1,240</td>
<td>6.2</td>
</tr>
<tr>
<td>( \delta )-Aminolevulinate dehydratase</td>
<td>4,400</td>
<td>659</td>
<td>6.7</td>
</tr>
</tbody>
</table>

consistent with the value obtained from the double isotope experiment.

The reproducibility of the double isotope method for determining rates of degradation is also illustrated in Fig. 3. The points for each protein or cell fraction in the figure represent the average of the ratios obtained in from three to five separate experiments. It is obvious that the ratios do not deviate much from the average provided that the experimental protocol is reasonably consistent from experiment to experiment.

By extending the interval between injections of precursor to 10 days, the rates of degradation of \( \delta \)-aminolevulinate dehydratase and lactate dehydrogenase can be measured. Double isotope ratios from the 10-day experiment for these two proteins, ferritin, and the liver cell fractions are presented in Table IV. A plot of the logarithm of the ratio for ferritin, homogenate, microsomal fraction, and supernatant fraction against the rate constant of degradation determined from \( \text{[guanidino-}^{14}\text{C]} \text{arginine decay yields a smooth curve (Fig. 4). Unlike the other fractions, the ratio for mitochondria in our hands is not independent of the length of the experiment. The ratio for this fraction with a 3-day interval indicates a half-life of 4 to 4.5 days while the ratio for a 10-day interval indicates a half-life of 6 to 7 days. These results mimic those obtained by Swick et al. (5), who measured the turnover rate of the mitochondrial fraction of rat liver by the incorporation of \( ^{14}\text{C]} \text{carbonate given continuously, a method that is not complicated by significant precursor reutilization. They found that the half-life varied from 4.3 days after a 2-day experimental period to as long as 6.8 days after 12 days of continuous administration of isotope. This change in the rate of protein degradation with time may be a function of the heterogeneous protein composition of mitochondria and the fact that some proteins are synthesized directly in the mitochondrion while others are synthesized in the cytoplasm. We have used a value of 6.5 days for the half-life of the mitochondria fraction \( (k_d = 0.1) \) and plotted this against the double isotope ratio for a 10-day interval in Fig. 4. The ratios for \( \delta \)-aminolevulinate dehydratase and lactate dehydrogenase when placed on the curve indicate half-lives of these proteins of 6 days and 6.5 to 7 days, respectively. The value for \( \delta \)-aminolevulinate dehydratase is in good agreement with the 5- to 6-day half-life determined for this protein from the loss of \( \text{[guanidino-}^{14}\text{C]} \text{arginine (Table III). The value for the rate of degradation of lactate dehydrogenase agrees with the 3.5- to 6-day half-life estimated by Kuehl and Sumsion (26), but is much shorter than the 16-day value determined by Fritz et al. (29). The reason for the discrepancy is not known.}

**Relationship of Subunit Size and Rate of Degradation**—The double isotope technique was used to measure the rates of degradation of the polypeptides composing the total soluble proteins of rat liver. The proteins in the 105,000 \( \times \) g supernatant fraction of rat liver were dissociated into subunits with sodium dodecyl sulfate. The subunits then were separated according to molecular size on sodium dodecyl sulfate-polyacrylamide gels (25, 30). The double isotope ratios for both a 3-day interval and a 10-day interval between administrations of isotope are presented in Fig. 5. In this figure the ratios have also been converted to absolute half-lives (refer to Figs. 3 and 4). The results show again the importance of the proper choice of interval between first and second injection of precursor to the measurement of the rate of degradation. With a 3-day interval the longest half-life that can be measured is about 5 days, because at this time the ratio approaches 3 indicating the absence of significant degradation. With a 10-day interval the shortest half-life that can be measured with confidence is about 2 days. The majority of the subunits forming the proteins of the soluble fraction of rat liver have rates of degradation of between 2 and 8 days.
It is obvious from Fig. 5 that there is a general relationship between the molecular weight of the subunit and the rate of degradation. That is, large subunits appear to be degraded at a faster rate than smaller subunits. This result confirms the original observation of Dehlinger and Schimke (8) on the relationship of size to rate of degradation for both total soluble and total membrane proteins of rat liver. This relationship also holds in general for individual proteins purified from the soluble and membrane fractions. Table V lists the molecular weights and the rates of degradation for two membrane proteins, cytochrome $b_6$ and NADPH-cytochrome c reductase and four proteins from the soluble fraction of rat liver. The membrane proteins exist as single polypeptides while the soluble proteins are all multimeric being composed of four (catalase and lactate dehydrogenase), six (δ-aminolevulinate dehydratase), and 20 to 24 (ferritin) identical subunits. There is a general correlation between subunit size and the rate at which the biologically active protein is degraded for five of the proteins. Ferritin is an exception; it has a relatively small subunit (mol wt 1,600), but the multimeric protein newly synthesized polypeptides (3H) would be degraded fairly rapidly.

A possible explanation for the relationship between 3H:14C ratio or the rate at which a protein is degraded and the length of the polypeptide chain might be that as a multimeric protein "ages" in the cell it accumulates "nicks" or breaks in its component polypeptides which do not alter multimeric structure or biological activity. In this case after dissociation of the multimeric protein newly synthesized polypeptides (PH) would be longer than "older" polypeptides (14C). The rates of degradation for the four soluble proteins presented in Table V are for the multimeric biologically active enzymes as isolated by either purification (lactate dehydrogenase) or in quantitative yield with their specific antibody (ferritin, catalase, or δ-aminolevulinate dehydratase). The degradation of the subunits of these proteins was examined by dissociating the antigen-antibody complex containing ferritin, catalase, or δ-aminolevulinate dehydratase with sodium dodecyl sulfate and measuring the rate constant of degradation of the isolated subunits. The antiserum to each of these proteins was relatively specific in that only a single line of immunoprecipitate was formed after reaction with crude extracts of rat liver (Fig. 6). When the immune precipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels, all of the 14C and 3H counts migrated with the mobility of the subunits of ferritin, catalase, or δ-aminolevulinate dehydratase (Fig. 7). In this experiment a 3-day interval between injections was used to label ferritin and catalase. The side wells in A and B contained equal amounts of 105,000 × $g$ supernatant fraction of rat liver. The center well in each case contained the antiserum to the respective protein. The upper and lower wells in A and B contained equal amounts of a partially purified (NH4)2SO4 fraction of ferritin and δ-aminolevulinate dehydratase, respectively. The upper and lower wells in C contained a sodium deoxycholate extract of total rat liver homogenate. The side wells contained a preparation of rat liver catalase purified through Step 6 of Price et al. (17).

![Graph](http://www.jbc.org/)

**Fig. 5.** 3H:14C ratios and rates of degradation of proteins in the supernatant fraction of rat liver as separated on sodium dodecyl sulfate-polyacrylamide gels; 3-day and 10-day intervals between injections. Proteins of the supernatant fraction, 8 mg, were prepared for electrophoresis and counting as described under "Experimental Procedure." Larger molecular weight polypeptides are located near the top of the gel (Fraction 1); while smaller polypeptides are near the bottom (Fraction 60). Ratios were converted to half-lives by reference to Figs. 3 and 4, and the expression $t_1/2 = 0.693/k_d$.

![Graph](http://www.jbc.org/)

**Fig. 6.** Ouchterlony double diffusion analysis of antibodies to ferritin (A), δ-aminolevulinate dehydratase (B), and catalase (C). The center well in each case contained the antiserum to the respective protein. The upper and lower wells in A and B contained equal amounts of a partially purified (NH4)2SO4 fraction of ferritin and δ-aminolevulinate dehydratase, respectively. The upper and lower wells in C contained a sodium deoxycholate extract of total rat liver homogenate. The side wells contained a preparation of rat liver catalase purified through Step 6 of Price et al. (17).

**Table V**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Molecular weight of subunit</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b$_6$</td>
<td>16,800</td>
<td>16,800</td>
<td>5.1</td>
</tr>
<tr>
<td>Ferritin (32)</td>
<td>440,000-655,000</td>
<td>18,600</td>
<td>1.5</td>
</tr>
<tr>
<td>Lactate dehydrogenase (33)</td>
<td>150,000</td>
<td>55,000</td>
<td>6-7</td>
</tr>
<tr>
<td>δ-Aminolevulinate dehydratase (21, 34)</td>
<td>250,000</td>
<td>39,500</td>
<td>5.6</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (35)</td>
<td>57,700</td>
<td>57,700</td>
<td>2.5</td>
</tr>
<tr>
<td>Catalase (33)</td>
<td>250,000</td>
<td>60,000</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a* The numbers in parentheses are reference numbers.

We thank Dr. Robert T. Schimke for helpful discussions and particularly for pointing out to us this interpretation of the experiment shown in Fig. 7.
The method yields rates of degradation which are equivalent in accuracy to those which were used to construct the standard curve. The standard curves in the experiments reported here were constructed from proteins or cell fractions whose half-lives were determined from [guanidino-14C]arginine decay. Thus, the method yields values for the rate constant of degradation which are corrected for reutilization at least to the extent that [guanidino-14C]arginine is reutilized for protein synthesis and which are, therefore, equivalent to the values obtained by following the loss of [guanidino-14C]arginine from protein.

The double isotope method, moreover, can be used to estimate rates of degradation of liver proteins that are not present in sufficient amounts to be labeled with [guanidino-14C]arginine. The latter precursor is incorporated into liver protein to about 5% or less of the extent of incorporation of labeled leucine. Thus a protein has to be present in high concentration to get it sufficiently labeled with [guanidino-14C]arginine to be able to follow the loss of radioactivity with time.

It should be recognized that the cell fractions, homogenate, mitochondria, microsomes, and supernatant, are complex heterogeneous mixtures of proteins. It would be better to construct a standard curve with proteins purified to homogeneity from liver. Except for ferritin, this is not practicable. However, each cell fraction with the possible exception of mitochondria does turn over with pseudo first order kinetics whether the precursor is leucine or [guanidino-14C]arginine (Table III). As shown in Figs. 3 and 4, an adequate curve can be constructed from the ratios of the cell fractions.

As shown in experiments by Poole, Leighton, and DeDuve (28) [guanidino-14C]arginine is reutilized for protein synthesis to some extent in rat liver. When the rate constant of degradation of hepatic catalase was measured by following the loss of [1-14C]leucine, they obtained a half-life of about 4 days. The loss of [guanidino-14C]arginine indicated a half-life for catalase of 2.5 days. But, the loss of radioactivity from the homogeneity of catalase labeled in vivo with [guanidino-14C]arginine acid yielded a half-life of 1.8 days. The latter method is not subject to reutilization of precursor and gave a value for the half-life comparable to one obtained when the catalytic activity of catalase was inhibited irreversibly with the drug aminotriazole (16, 36). Thus, the values obtained for the rate constant of degradation by the double isotope method are overestimated to the same degree that [guanidino-14C]arginine is reutilized. If this precursor is reutilized by other liver proteins to the extent indicated for catalase, the curves in Figs. 3 and 4 should be adjusted accordingly by about 1 day.

The assumptions inherent in the use of the double isotope technique have been described in detail elsewhere (3). Briefly, they are (a) the isotope is not metabolized into other products which are incorporated into the protein or cell fraction. (b) The proteins follow exponential decay kinetics. (c) The rates of synthesis of the proteins of the liver are the same at the time when the first and second injections of isotope are given. (d) At the time the animal is killed the labeled proteins are undergoing isotopic decay. The first three assumptions, in fact, have been shown to be valid. Leucine is not metabolized in any significant amount to products which are incorporated into rat liver proteins (3); all of the cell fractions and proteins examined here have been shown to decay with apparent first order kinetics; and the amount of [1-14C]leucine incorporated into the liver cell fractions is the same whether this isotope is administered simultaneously with, 3 days after, or 10 days after [3H]leucine.

The fourth assumption is not strictly valid. Any isotopic...
technique for measuring rate constants of degradation which depends on a single administration of precursor requires that this administration be of short duration or a pulse. Injection of \( {\text{g}}unadinic-^{3}C \)arginine into an intact rat does mimic a pulse in that no free precursor can be found in the intracellular liver pool after 20 to 30 min (7). Most of the other amino acids cannot be administered as a pulse. For example, Poole (37) has shown that some \( {\text{H}} \)leucine is still present in the free pool as long as 10 days after administration. Thus, it is not valid to assume that a liver protein will be maximally labeled within a very short time after a single injection of this precursor. Actually, the time required to reach maximum specific activity is a function of the protein's rate constant of degradation, and the protein may not be undergoing complete isotopic decay when the animal is killed. It is this problem which leads to overestimates of half-lives when using a reutilizable precursor.

The double isotope technique obviates many of the problems associated with a single administration of labeled leucine. As shown in Figs. 3 and 4, the method yields accurate values for rates of degradation at least within a range of half-lives from about 1 to 7 days. Half-lives of less than 1 day could also be measured, but in this case it would probably be better to use \( {\text{g}}unadinic-^{3}C \)arginine for the first injection and \( {\text{H}} \)arginine for the second. Proteins with short half-lives are turning over rapidly and consequently would be synthesized rapidly. Hence, these proteins would have high specific radioactivities after a short pulse with \( {\text{g}}unadinic-^{3}C \)arginine, and the determination of their half-lives would not be complicated by a lingering pool of precursor. As shown in Fig. 5 most of the subunits forming the total soluble proteins of rat liver are turning over with half-lives of degradation of 2 to 8 days. Our results, in agreement with those of Dehlinger and Schimke (8), show a general correlation between subunit size and rate of degradation for total liver soluble proteins, for the soluble enzymes catalase, ferritin, and lactate dehydrogenase, and for the subunit size of subunits of these proteins, for the soluble enzymes catalase, ferritin, and lactate dehydrogenase. The correlation between molecular size and rate of subunit degradation for the multimeric proteins catalase, ferritin, and lactate dehydrogenase, and \( \delta \)-aminolevulinate dehydratase suggests that it is the subunit of these proteins that is being degraded in the cell. If this is correct, the subunits during degradation are removed from the biologically active protein in an all or none fashion. That is, if the subunits undergo partial cleavage to smaller molecular weight polypeptides as a first step in degradation, the products are not present in the active enzyme. This does not imply that partial degradation of subunits does not occur. Fragments may be present in the cell but not in the enzyme. If so, they are not recognized by the antibody to the multimeric protein, nor do they exchange with intact subunits.

Finally, the degradation of \( \delta \)-aminolevulinate dehydratase, catalase, ferritin, and lactate dehydrogenase is first order. First order kinetics implies that once the polypeptide is synthesized and released from the polyribosomes, it has an equal chance of being degraded as any other like polypeptide. In the double isotope experiments the \( {\text{C}} \) counts represent polypeptides which had been synthesized 3 or 10 days before the polypeptides represented by the \( {\text{H}} \) counts. The fact that both subunits have an equal chance of being degraded suggests that the rate of association-disassociation of subunits is much faster than the rate of subunit degradation, assuming again that the subunit must be free to be degraded in the cell.

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REFERENCES

37. Poole, B. (1971) J. Biol. Chem. 246, 6587
On the Measurement of Protein Turnover in Animal Cells
Richard D. Glass and Darrell Doyle


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