PROTEIN TURNOVER IN PLANTS AND POSSIBLE MEANS OF ITS REGULATION

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INTRODUCTION

Protein turnover is now recognized as an important component in the regulation of biological systems. It provides a way of altering the general protein constituents as well as varying the enzymatic complement during differentiation, growth, and response to environmental conditions. The ability to alter protein and enzymatic complement is important to a plant's ability to compete both under optimum and unfavorable environmental conditions. When nutrients are limiting, for example, turnover of existing protein is probably the only mechanism available for providing amino acids to allow changes in the protein complement. The importance of the ability to inactivate induced enzymes has been pointed out by other investigators (52, 62, 132, 198). In bacteria, during exponential growth, the concentrations of particular proteins are altered mainly by changes in synthetic rates. Unlike bacteria, plants do not divide rapidly and outgrow their induced enzyme complements, but must regulate enzymes in other fashions, e.g. degradation, inactivation, or inhibition. In both plants and mammals, protein levels change with changes in the rate of synthesis or degradation of the protein.

Since Vickery et al (242) obtained evidence for turnover of protein in plants, most investigations for many years dealt with the turnover of total protein. Studies of total protein turnover, however, yield very little information on the intricate interaction of synthesis and degradation or activation and inactivation in controlling the specific biochemical sequences which are in turn reflected by physiological response. Analysis of these processes requires information on turnover of specific enzymes and proteins. Studies of the turnover of total protein are valuable, however, as they show that protein turnover is a common phenomenon, and provide information on turnover rates during different developmental stages of various plants. Also, information about cellular protein turnover in the whole organism might be informative in studies involving plant modeling or energy budgets. Because of experimental and conceptual difficulties, however, few of the reported rates of protein turnover have been accurate. Such studies have elucidated many of the problems involved and have shown how these affect the accuracy of determinations of turnover.

Turnover can now be considered on a molecular, organelle, cellular, or whole-organism level if one is conscious of the limitations that exist at each level and adapts the methodology accordingly.

The number of studies of turnover of individual proteins in animals and bacteria has increased greatly during the past ten years. Much circumstantial evidence has begun to appear for the turnover of specific plant proteins, though unequivocal evidence showing turnover or lack of turnover of individual proteins during specific phases of their induction is limited. Such information, although difficult to obtain, is vital to understanding internal regulation of the plant.

Terminology in this field has been somewhat confusing as turnover has been defined in many ways (188, 258). We shall define turnover as the *flux of amino acids through protein*. With this definition, protein will be in turnover even if both synthesis and degradation are occurring simultaneously so that no net change in protein concentration is observed. As discussed below, control in such situations
differs from that present when proteins are not simultaneously synthesized and degraded but change concentrations in response to germination, senescence, or environmental changes. In these cases, synthesis and degradation seem to occur independently. Sometimes it may be difficult to prove the occurrence of simultaneous synthesis and degradation if one of the two processes occurs at a much greater rate than the other (119).

Problems in terminology also arise with the level at which turnover is applied. Some studies involve the flux of amino acids through total cellular protein. Others involve the flux of amino acids through an enzyme or particular protein. The former often leads to a loose definition of turnover, e.g. in experiments involving either pulse incorporation of labeled amino acids into protein or loss of radioactivity after a pulse, heterogeneous mixtures of protein cannot be considered as a single protein having a turnover rate equal to the mean of the mixture (59, 119, 131, 226). The proteins turning over more rapidly receive more of the pulse dose, and the slope of the resulting decay curve is too steep. As a result, the apparent rate decreases with time.

Accurate measurements of turnover of protein are difficult when synthesis and degradation are occurring simultaneously. To obtain accurate rates of turnover, both the rate constant for synthesis \( (k_s) \) and the rate constant for degradation \( (k_d) \) must be determined. Various equations have been developed to describe the relationship between synthesis and degradation of protein (188, 258). A change in concentration per g of tissue can be described by:

\[
\frac{dP}{dt} = k_s - k_d P
\]

where \( P \) is the content of protein \([g (g tissue)^{-1}]\); \( k_s \) is the rate constant of synthesis, usually zero-order \([g (g tissue)^{-1} \text{ time}^{-1}]\) (198), but first-order \((\text{time}^{-1})\) in exponentially growing tissue (236); and \( k_d \) is the rate constant for degradation, which, with the known exception of hemoglobin of red blood cells, is a first-order process \((\text{time}^{-1})\) (198). At steady state, when \( dP/dt = 0 \), \( k_s = k_d P \), and \( P = k_s/k_d \), then the turnover rate can be estimated by determining either \( k_s \) or \( k_d \).

PROBLEMS OF METHODOLOGY

Because of the difficulties involved in accurately determining turnover, a description of the problems involved becomes important. Estimates of protein turnover rely strongly on isotopic methods. Determining the rate of turnover requires accurate measurements of the change in radioactivity in the protein and the precursor pool per unit time, as well as the loss of radioactivity from the product. Measurement of the precursor pools of amino acids represents the largest error and the major difficulty involved in studies of protein turnover. A great deal of evidence has accumulated to show a compartmentation of metabolites into active and relatively inactive pools within plant cells. Immediate precursor pools of amino acids may not be in rapid equilibrium with pools of stored amino acids; however, some amino acids—for example, alanine (19) and asparagine (219)—may bridge the separated com-
Evidence for this concept is well documented in an excellent review by Oaks & Bidwell (163). These authors point out that the linear increase of $^{14}$C-amino acid incorporation into protein product often precedes the saturation of the total extractable precursor (162, 220). In addition, the specific activity of the product may be greater than that of the total extractable precursor (23, 86, 130). This suggests the existence of separate pools of precursor (161, 220).

The lag time required before the increase of labeled precursor into protein becomes linear depends on the size of the precursor pool (23, 162). With this approach Oaks identified two leucine pools in maize roots, one metabolically active and the other an apparent storage pool. Holleman & Key (86) found evidence of two pools each for leucine and valine: a precursor pool closely related to protein synthesis; and a storage pool only remotely connected to protein synthesis. They also observed that the immediate leucine precursor pool turned over 20 times as fast as did the valine pool. The immediate precursor pools of valine in soybean hypocotyl (86) and leucine in maize root tips (161) are expandable to a degree, enlarging in response to increasing concentration of either amino acid.

Feeding experiments with labeled acetate or glucose (130) show that large amounts of individual amino acids are physically remote from the respiratory centers of the cell and that the amounts of amino acids and organic acids in turnover pools vary from tissue to tissue. Experiments with Neurospora crassa indicate that there are two ornithine pools which do not readily mix. One pool is supplied by exogenous sources, or by the catabolism of arginine, and serves as a protein precursor. The second pool is supplied by an endogenous source, i.e. N-acetylg glutamate, and serves as an arginine precursor (163).

Hellebust & Bidwell (82) determined the proportions of carbon coming either from soluble amino acid pools or by direct route from photosynthate in wheat leaves. More than half of the serine and glycine incorporated into protein was derived from newly assimilated CO$_2$, while glutamic acid, aspartic acid, and alanine incorporated into protein came more from the soluble pools.

Using carrot explants, Bidwell et al (19) and Steward & Bidwell (219) found further evidence of multiple pools. Exogenous sugar and glutamine were more direct sources of amino acids for protein synthesis than other exogenously supplied amino acids or internal pools of stored amino acids. Amino acids preferentially used in protein synthesis were derived from sugar and mixed but little with the stored amino acid pools. Alanine was the only amino acid investigated that was in fairly rapid equilibrium with stored pools. Asparagine accumulated in soluble pools that were metabolically inactive and contributed only slightly to metabolism, respiration, and protein synthesis, whereas exogenous glutamine was freely utilized in those processes. In contrast, Kemp & Sutton (112) reported that the total soluble leucine pool may also be the precursor pool for leucine incorporation into protein in cultured tobacco cells. The specific activity of the soluble leucine pool remained constant during the labeling period, whereas incorporation of labeled leucine was linear.

Since different amino acids apparently can come through different precursor pools with differing turnover rates, the determination made of protein turnover may vary with the labeled amino acid chosen for the study. Poole et al (174) have shown how
important those considerations are. They used three different substrates to determine the half-life of rat liver catalase: $^3$H-leucine, $t_{1/2} = 3.7$ days; guanidino-$^{14}$C-arginine, $t_{1/2} = 2.5$ days; and $^{14}$C-S-amino levulinic acid, $t_{1/2} = 1.8$ days.

Oaks (161) also reported that the uptake of an amino acid precursor can be decreased by other amino acids in the medium. Autoradiographic experiments showed that different plant cells accumulate amino acids at different rates (36, 169). These factors could well influence the specific activity of the precursor pool, especially in short-term experiments. Problems might also arise from any changes in the pool size that feeds protein synthesis during the experiment (193).

Reutilization of amino acids released from protein is another problem to be considered in studies involving protein turnover. This could be as important in plants as in animal systems, where up to 50% of the free amino acids in rat liver may be derived from protein catabolism (56). Reutilization of amino acids released from protein causes estimated rates of degradation to be less than the actual rates, the degree of error being greater with proteins that have high rates of degradation. Zielke & Filner (257) observed this problem in studies with tobacco cells, as did Zucker (260) with Xanthium discs. The error resulting from utilization of amino acids released from proteins can be decreased if a portion of the amino acid is removed from the site of protein synthesis. Bidwell et al (19) have shown that such removal may occur to a degree in some plant systems. They have evidence that part of the released amino acids can be removed by respiration in carrot explants, and estimate that amino acids released from protein can account for up to 25% of the total CO$_2$ released. In wheat leaves, however, they noted that protein accounted for very little of the respired CO$_2$ during the experiment (81). They also observed that amino acids released in carrot explants enter pools separate from those closely associated with protein synthesis (19, 219). The amount of reutilization apparently depends on the amino acid used as the label.

End-product repression must also be recognized as being partially responsible for competition among exogenously supplied sugars, amino acids, and endogenous amino acids as carbon sources for proteins (103, 163). Oaks (161, 162) presents evidence for both end-product repression and discrete pools of amino acids in maize roots.

When labeled amino acids are used in a turnover study, it is important to consider the various metabolic reactions that might be involved, including interconversions to other amino acids and nonamino-acid metabolites, which could affect the measured turnover rates. It therefore becomes important to consider where the labeled amino acid is located in the metabolic "family" sequence (79). Glutamic acid as the head of a family is readily metabolized (55, 190) and can label protein glutamate, arginine, ornithine, proline, and hydroxyproline. In contrast, proline and arginine, situated at the end of the family sequence, do not contribute carbon to other protein amino acids (1, 2). Bidwell (18), using wheat leaves, found that glutamate-$^{1}$C can also label aspartic acid. Aspartate, in turn, can donate carbon to the members of its family: Threonine, isoleucine, lysine, and methionine (55). To further complicate matters, Nair & Vining (157) observed direct conversion of phenylalanine to tyrosine in spinach leaves. It has also been found that fungi have a phenylalanine
ammonia lyase that converts phenylalanine$^{14}\text{C}$ to $^{14}\text{CO}_2$, thus resulting in loss of the label if phenylalanine is ring labeled. Catalysis of this reaction has been suggested as another possible role of phenylalanine ammonia lyase in plants (42, 152, 153). Joy & Folkes (103) observed that aspartate and glutamine in barley embryos yielded significantly more respiratory $\text{CO}_2$ than did other amino acids. In carrot explants, glutamic acid-L$^{14}\text{C}$ lost more $^{14}\text{CO}_2$ to respiration than did glutamic acid-3, 4-$^{14}\text{C}$. The latter labeled compound resulted in greater distribution of label into other amino acids than the former (19). Amino acids closely related to Krebs cycle intermediates are those most likely to be labeled from sucrose or glutamine and to rapidly lose their radioactivity (219). Hence it becomes important to know which carbon is labeled and how much of the radioactive carbon is reworked to other amino acids.

Another important facet to consider is that plants can readily refix some of the $\text{CO}_2$ released through respiration (8, 73, 98). With wheat leaves, $^{14}\text{CO}_2$ labeled all the protein amino acids that were studied by Bidwell (18). Since photosynthesis appears to furnish substrates directly for synthesis of amino acids in some plants (19, 219), refixation of labeled $\text{CO}_2$ lost from amino acids could also affect the specific activity of the precursor pool.

In addition to the problems discussed above, other factors must also be considered in a turnover study. An important consideration is the method of getting the label into the plant. If labeled amino acids or sugars are to be used, it may be necessary to utilize detached leaves because application to roots results in dilution, slow uptake, and translocation. Since detaching leaves may alter the turnover rates of proteins, labeling intact plants with $^{14}\text{CO}_2$ may be more desirable. However, $^{14}\text{CO}_2$ incorporation, as mentioned above, results in wide distribution of labeled products. In some cases, labeled substrate could be injected directly into the stem, allowing for rapid uptake in intact plants (87). Age and growth conditions of the plant and other factors could also affect the turnover rates observed. Even slight environmental changes at the beginning of or during the experiment could affect the turnover enzymes sensitive to such changes.

In summary, amino acid uptake, pool size, pool accessibility to protein synthesis, end-product repression, metabolic interconversions, and reutilization must all be carefully considered in turnover studies.

**METHODS FOR STUDYING PROTEIN TURNOVER**

*Single Administration or Pulse*

The method used most commonly in estimating degradation rates has been the single administration of a precursor, i.e. a pulse procedure. Interpretation is based on the loss of the radioactivity from the protein after removal of the exogenous labeled precursor. At a steady-state level of protein, when $k_s = k_dP$, the loss of radioactivity from the protein is an apparent first-order reaction and allows an estimation of the degradation rate. A major limitation of this method is that the released amino acids may be reutilized, resulting in an erroneous estimate of degradation rate (198). This is especially important if the turnover rate of the protein in
relation to the turnover rate of the precursor pools is great enough to supply significant amounts of labeled amino acids to the precursor pools.

As mentioned above, another problem with this method is that the half-life obtained can vary with the length of the exposure to labeled amino acid. The proteins of a plant organ are a mixture, having different half-lives. During short exposure to a labeled amino acid, protein having a high turnover rate will be highly labeled. More stable proteins will be less labeled during short exposure, and their turnover rates will not be revealed. A longer exposure allows labeling of the more stable proteins and results in an apparent increase in the half-life (131, 225, 236). Swick (225) found that the half-life of rat liver protein varied from 1.8 to 3.8 days depending on the length of exposure to labeled CO₂.

**Kinetic Analysis of Uptake**

This approach can and should be incorporated into many of the presently used methods. Oaks (161) has shown that much useful information can be obtained concerning estimation of equilibration times and relative sizes of precursor pools by following a time course for amino-acid incorporation into protein. Her results showed that when incorporation of labeled precursor into protein was linear, the precursor pool was saturated and the newly labeled protein had the same specific activity as that of the precursor pool. In addition, the use of several concentrations of labeled precursor allows determination of the concentration at which swamping of the precursor pool is approached, since in this limit the specific activity of the pool approaches that of the added label. Several investigators have shown that the incorporation of labeled precursor under these conditions is quite constant at several concentrations of the exogenous label (86, 161, 193). Nevertheless, the researcher must beware since, as pointed out (103, 163), there are dangers to swamping. When a tissue is supplied with a large amount of amino acid, inhibition of enzymes, amino acid antagonisms, and disruption of metabolic pathways can occur.

**Constant Infusion or Continuous Labeling**

This method involves continuous administration of a labeled amino acid (or other precursor) of constant specific activity. The degradative rate constant can be determined from the time course of the approach of the labeled protein's specific activity to that of the specific activity of the administered isotope (198). This eliminates the problem of major changes in the specific activity of the precursor pool early in the experiment; however, problems of reutilization become increasingly important as the specific activity of the protein increases. The difficulty with reutilization has been minimized by the interesting approach of Swick et al (226). By constant administration of ¹⁴C-carbonate to rats they achieved uniform labeling of the guanidine carbon of arginine. In rat liver ¹⁴C-carbonate went predominantly to the guanidine carbon of arginine, and because of the close relationship of cellular arginine to urea, the prevailing specific activity of the free liver arginine could be measured by determining radioactivity in the excreted urine (225). The turnover rate could then be calculated by measuring the specific activity of the guanidine-¹⁴C-arginine from the protein and the average specific activity of the urea during the exposure period (144, 226). The same theoretical approach was utilized in following ³⁸S-cystine labeling
of protein while monitoring the appearance of labeled inorganic sulfate in the urine as a measure of the specific activity of the endogenous pool (9).

Hellebust & Bidwell (83) used a method similar to constant infusion by supplying $^{14}$CO$_2$ in light for 11 1/2 hr each day for 6 days to primary and secondary leaves of wheat while concurrently measuring the specific activities of the precursor amino acids and the protein (described below). Trewavas (236) used a constant-labeling procedure with *Lemna* (described below), in which rate constants for synthesis and degradation were determined at times when the specific activity of methionyl-acyl-tRNA was constant and incorporation of label into protein and protein content were increasing linearly. The method of constant infusion may also suffer from the limitation that rate constants depend on the period of exposure to isotope. The seriousness of this problem can be reduced, however, by determining rate constants at various times during the experiment.

**Inhibitors of Protein Synthesis**

Theoretically, rate of degradation could be based on the decay of a protein after its synthesis has been halted by an inhibitor. A cautious approach is required, however, since inhibitors of protein synthesis have also been shown to alter the rates of loss of nitrate reductase (233), phenylalanine ammonia lyase (47), thymidine kinase (89, 90), UDP galactose polysaccharide transferase (223), and fatty acid synthetase enzymes (250). Glasziou et al (63) successfully used protein syntheses inhibitors to determine the turnover rate of invertase in sugar cane (described below). Price et al (183) and Rechcigl (186) went a step further and used allylisopropylacetamide, a compound which specifically blocks the synthesis of catalase without affecting total protein synthesis, to measure the degradation rate of rat liver and kidney catalase.

**Dual Labeling**

Recently developed dual labeling techniques show great promise both in ease of analysis and reliability. Arias et al (5) used a simple double isotope method for estimating rates of protein degradation in rat liver. The technique involved the incorporation of $^{14}$C-amino acid; then, after a given period for decay, a second injection was made of the same amino acid, this time labeled with $^3$H. A short time later, the animals were killed, the various protein fractions isolated, and the $^3$H:$^{14}$C ratios determined. The proteins with high $^3$H:$^{14}$C ratios were degraded faster than the proteins with low $^3$H:$^{14}$C ratios. [For a discussion on the assumptions and limitations of the procedure, see Arias et al (5).] Glass & Doyle (61) modified the dual-labeling method to give rate constants of degradation and half-lives of liver proteins comparable to those obtained by constant infusion of guanidino $^{14}$C-arginine (197).

**Density Labeling**

Since its introduction by Hu et al (92) density labeling has been used by many researchers to prove de novo synthesis of various enzymes (52). The organism is allowed to metabolize $D_2O$, $H_2^{18}O$, or $^{15}$N-nitrate under conditions which result in enzyme induction, i.e. germination, hormonal treatments, or substrate changes.
The density of the isolated protein is compared both before and after induction by isopycnic equilibrium centrifugation in CsCl. Even though density labeling has been frequently used in proving de novo synthesis, it has very seldom been applied to protein-turnover studies. Zielke & Filner (257) used $^{15}$N-density labeling successfully in conjunction with labeled amino acids to demonstrate both de novo synthesis and turnover of nitrate reductase (discussed further below).

**Kinetic Analysis of Changes in Enzyme Activities**

This procedure is less rigorous than those which require isolation of specific proteins but can give an indication of the relative rates of turnover. In this procedure, an increase of the enzyme level is induced by changes in diet (226, 228), hormones (200, 205, 228), etc. Measurements of $k_s$, $k_d$, and the half-life can then be made by following changes in enzymatic activity as the stimulus is given and later removed. This procedure has been useful in determining the turnover rates of several enzymes in animals (198). Even though significant changes can be induced in a wide variety of plant enzymes by such factors as light, temperature, hormones, and substrates, as well as by changes that occur during germination, aging, and senescence (52, 62, 132, 175, 261), kinetic means have not yet been applied to any of these enzymes to measure rate constants and turnover. One of the major problems involved in such studies is the need to rely solely on enzymatic activity to determine changes in enzymatic protein level.

Studies involving only changes in enzymatic activity are fraught with problems. Thus Joy (102) has shown that what was thought to be induction of glutamate dehydrogenase (101) was merely a change of enzyme from EDTA-sensitive to EDTA-insensitive state. Enzymatic specific activity of ribulose 1,5-di-P carboxylase increases with time as barley plants are left in darkness (171). Turnover estimates based on activity would then be partially masked. Loss of ornithine transcarbamylase activity was the result of the binding of an inhibitor protein and not loss of enzymatic protein (14). Loss of activity of both phenylalanine ammonia lyase in gherkin (261) and invertase in maize (100) may be due to the binding of inhibitor protein rather than degradation.

Because of these problems, it is almost imperative to purify or otherwise isolate the enzyme under investigation rather than relying on enzymatic activities or inhibitor studies to unequivocally prove turnover. In many cases enzyme purification procedures are laborious, time consuming, insufficiently quantitative, and often result in poor yields. To overcome these problems, many researchers have begun to use immunological methods which offer specific advantages: (a) they are highly specific and permit rapid analysis of a constituent in a mixture without chemical fractionation; (b) very small amounts of antigen are required for protein analysis by the Folin-Ciocalteau method; and (c) precipitates of antigen and antibody can be washed, dissolved, and analyzed for radioactive label in the antigen.

**TURNOVER OF TOTAL PROTEIN**

Most methods used in the past to estimate protein turnover suffered from inaccurate estimates of the precursor amino acid pool. Where the specific activity of the total
extractable pool was considered to be the specific activity of the precursor, inaccurate estimates of protein turnover were the likely results. As Oaks & Bidwell (163) pointed out, the general presence of multiple pools of metabolites has led many unwary workers astray. For this reason this presentation covers only studies that recognized the presence of precursor pools and attempted to deal with the problem.

Hellebust & Bidwell (83) used a method similar to constant infusion to help solve the problem of determining the specific activity of the precursor pool. Wheat seedlings were supplied with $^{14}$CO$_2$ in light for 1 1/2 hr each day for 6 days, during which time the specific activities of various amino acids and protein were determined. The object was to label all the possible precursors of protein carbon to the same extent and measure turnover rates as proteins accumulate radioactivity. The free amino acids and sugars attained about the same specific activity after the first day and the specific activities remained quite constant during the experiment. Incorporation of $^{14}$C into protein was approximately linear during the experiment, strongly indicating that the precursor pools had early reached a constant specific activity which remained almost constant throughout the six day experimental period. If the specific activity based on the total content of each amino acid is representative of the specific activities of the precursor pools, the turnover values obtained in this study seem fairly accurate. Turnover rates of about 0.4 to 0.5% per hour were obtained in rapidly growing secondary wheat leaves and 0.2 to 0.3% per hour in nongrowing primary wheat leaves. An important observation was that turnover was greatest during growth and decreased when growth stopped. Hence, turnover appears to be important in changing enzyme levels during biochemical differentiation.

The above authors (83) also applied the pulse-chase method using $^{14}$CO$_2$ to label tobacco leaf protein. After waiting until the soluble compounds had decreased to a low level of radioactivity, determinations of turnover were begun. The estimates of turnover were made difficult because the loss of label from soluble compounds was slow. The turnover rates calculated were 0.15 to 0.2% per hour for expanding tobacco leaves and little or none for fully expanded leaves.

Holleman & Key (86) used methods similar to those of Oaks (161) to estimate turnover in soybean hypocotyl. By following incorporation of labeled leucine and valine at increasing concentrations, they detected both concentrations and time at which swamping of the precursor pool was assumed to approach that of the exogenous amino acid (86). They calculated turnover rates of 2.5% per hour by determining the rate of incorporation of leucine and valine, their contents in the protein, and the steady-state amount of protein. They estimated the sizes of the precursor pools for leucine and valine by multiplying the output rate (in umoles/min) of the precursor pools by the lag time required before linear incorporation of $^{14}$C into protein occurred. This allowed calculation of the relative turnover rates of leucine and valine precursor pools, the former having 20 times the turnover rate of the latter.

Kemp & Sutton (112) used techniques involving constant infusion, a kinetic analysis of absorption of labeled precursor into the precursor pools, and incorporation of precursor into protein in tobacco callus. During the experiment the specific activity of the free amino acid pool remained constant between 0.5 and 7 hr while incorporation into protein was linear during the same time course. The results were
interpreted to mean that the soluble leucine pool was the precursor pool. However, leucine in the immediate precursor pool might also have been in rapid isotopic equilibrium with the other amino acid pools present in the tissue.

In contrast to other investigations, they found that the rate of incorporation of precursor into protein was independent of the exogenously supplied precursor. As long as the specific activity of leucine was followed in both the free amino acid pool and in the protein, the rate of incorporation was similar whether the source of label was sucrose or leucine. As mentioned above, other investigators, working with carrot explants, found that sucrose was a much better source of precursor for protein synthesis than were exogenously added amino acids.

Kemp & Sutton (112) estimated the turnover rate by determining the difference between the measured rate of protein synthesis and the measured rate of protein accumulation. A turnover rate of 1.1% per hour for protein was determined.

Trewavas (236), in an excellent study, overcame the problems listed above by using a combination of procedures including constant infusion, pulse labeling, dual-labeling techniques, and kinetic analysis. In both the constant-infusion and pulse-chase method, he used an amino-acyl-tRNA fraction for analysis of the specific activity of the immediate precursor pools. He solved the problems of determining low levels of the amino acid attached to tRNA by growing *Lemna* to isotopic equilibrium on $^{35}$SO$_4$. The plants were then transferred to $^3$H-methylmethionine. Isolation of the protein methionine from the exponentially growing plants and determination of the $^3$H/$^{35}$S ratio gave a direct measurement of the specific radioactivity of the proteins. The specific radioactivity of the methionine pool contributing to protein synthesis was determined by isolating methionyl-acyl-tRNA (methionyl-tRNA).

In the constant-labeling experiment, the specific radioactivity of methionyl-tRNA, the specific radioactivity of protein, and the concentration of protein were followed as a function of time. The rates of synthesis and degradation were determined at a time when the specific radioactivity of methionyl-tRNA had reached a constant value, the incorporation of label into protein was exponential, and the protein content was exponentially increasing.

In the second method, the protein was pulse-labeled, then chased with unlabeled precursor until the specific activity of the methionyl-tRNA was effectively zero. Estimates of the rates of synthesis, degradation, and turnover were in very good agreement with those obtained by constant labeling. A problem with both methods is that the methionyl-tRNA complex is relatively unstable (overnight storage at $-15^\circ$ resulted in some loss). Trewavas noted that, since losses may occur during extraction, the results probably do not represent in vivo rates of turnover.

From these methods the rate constants in cultures grown under good nutritional conditions were about 0.02 hr$^{-1}$ for protein synthesis and about 0.087 days$^{-1}$ for degradation. Under limiting nutritional conditions, i.e. H$_2$O instead of sucrose and salts, the rate of synthesis was decreased while the rate of degradation was increased. This same result occurred when nutritional elements were withheld one at a time (236, 237). Benzyl adenine (BA) increased the rate of synthesis without affecting the rate of degradation under growth conditions of sufficient nutrition (236, 237). Under
growth-limiting conditions, BA did not affect the rate of synthesis but decreased the rate of degradation. On the other hand, abscisic acid reduced $k_s$ while increasing $k_d$.

TURNOVER OF SPECIFIC ENZYMES AND PROTEINS

To our knowledge, nitrate reductase is the only specific plant protein for which incontrovertible evidence of turnover has been obtained. A great deal of circumstantial evidence, however, now indicates that quite a large number of specific enzymes are turned over in plants. As purification procedures are developed for these, the interaction of synthesis and degradation in their regulation will be worked out.

Nitrate Reductase

The synthesis of this enzyme is induced by its substrate nitrate. After induction, it reaches a steady-state level which is modulated by environmental conditions such as nitrate concentration (15, 71), temperature (15), light (33, 71, 233, 234), and moisture (95). When induced plants are placed under adverse or noninducing conditions, the enzyme is rapidly lost (15, 71, 104, 233, 234). The loss can be greatly decreased when induced plants are treated with inhibitors of protein synthesis or maintained at low temperatures (233). Such evidence strongly indicates the presence of a turnover system of nitrate reductase (NR) in several plants.

Zielke & Filner (257) provided unequivocal evidence for the turnover of NR in tobacco cells by using a procedure which involved triple labeling. Preexisting proteins were labeled with $^{14}$C-arginine and with $^{15}$N to increase the buoyant density. Cells were subsequently transferred to a medium containing $^{14}$N and $^3$H-arginine. The degradation of $^{15}$N-labeled proteins was followed after transfer of cells to the new medium. The effect of the preexisting pools of $^{15}$N-amino acids on the density of newly synthesized protein was determined by the $^3$H label. The labels were followed during induction of NR, during steady-state activity, and after a shift to noninducing conditions. During these times protein was extracted and isopycnic equilibrium centrifugation was carried out. The results showed that while the enzyme level remained constant, the buoyant density of NR decreased from that of $^{15}$N-NR toward that of $^{14}$N-NR, showing that both synthesis and degradation were occurring. Preexisting protein labeled with $^{14}$C and newly synthesized protein labeled with $^3$H both turned over.

These workers (257) showed that the enzyme was under constant turnover, being continuously synthesized and degraded during the induction phase, during the steady-state phase, and during the decay phase of noninducing conditions.

The turnover rate of NR in the tobacco cells was difficult to measure because released $^{15}$N-amino acids were apparently reused. The rapid turnover rate of proteins furnished a large amount of $^{15}$N-amino acids to the protein precursor pools. Taking into account the problem of reutilization of released amino acids, Zielke & Filner estimated the lower limit of turnover for NR to be 4.3 hr. On the basis of rate of loss of NR activity under noninducing conditions, half-lives have been reported of 4 hr for corn leaves (204), 2 to 3 hr for corn roots (164), and 9 to 12 hr for barley leaves (233).
The mechanism of induction of NR is still unknown; however, it is obvious that both synthesis and degradation are important during induction. Changes in either or both rates could result in net induction or net degradation. Zielke & Filner's (257) data show that the rate of turnover of NR is close enough to the turnover rate of the precursor amino acid pool in tobacco cells to make the estimates of $k_s$ and $k_d$ inaccurate. Hence, both the rate of turnover of the precursor pool and reutilization of released amino acids have been shown experimentally to confound the estimates of synthetic and degradative rates. Therefore, accurate rates of synthesis and degradation for NR are going to be difficult to obtain.

The rate of turnover of NR, as measured by loss of activity during noninducing conditions, can differ according to the treatment of induced plants. When nitrate was removed from the growth medium of tobacco cells, the turnover rate was about 6.5 hr (80). When casein hydrolysate was added to a growth medium containing nitrate, the half-life was 3.7 hr. Glucose in the incubation medium greatly retarded the loss of NR activity in darkness in barley leaves, giving a half-life of 30 hr, compared with 14.5 for the control lacking glucose (8). This study showed that respiration was required for a net induction and maintenance of NR activity. In darkness with glucose, respiration probably maintained the synthetic phase of the turnover system of NR at a higher level than in the control. Turnover during darkness can be greatly slowed by placing induced plants at a low temperature (233). An increase in temperature is accompanied by a more rapid loss of activity. As will be discussed below, the degrading system of NR itself appears to be turning over.

Inhibitors of protein synthesis such as cycloheximide and actinomycin D greatly retard the loss of NR in induced barley plants placed in darkness (233). One explanation for this is that both the synthesis of NR and of its degrading system are stopped. The degrading system itself may then be turned over, accounting for decreased rate of loss of NR. This postulated degrading system in corn appears more stable since cycloheximide has been reported to have no effect on the rate of loss of NR during noninducing conditions (164, 204).

Nitrate reductase activity is rapidly lost during heat stress (142, 166). Treatment with cycloheximide just prior to heat stress halved the rate at which NR activity is lost (166). Both the apparent rate constant for loss and the inhibitory effect of cycloheximide on loss of NR activity indicated that NR was being degraded under the unfavorable conditions of heat stress. Chloramphenicol has no effect on either induction (203) or loss (233) of NR activity. This indicates that both NR and its degrading system are not dependent on chloroplast protein synthesis but probably are synthesized on 80S cytoplasmic ribosomes.

**Phenylalanine Ammonia Lyase**

Evidence is being accumulated to support the possibility that phenylalanine ammonia lyase (PAL) is also controlled by a turnover system, in some plants at least. In contrast to proteins under constant turnover, the induction of PAL synthesis and the induction of PAL inactivation may be sequential, the induction of the inactivating system occurring after that of the synthesis (260). After induction of the inactivation system, the enzyme is still apparently synthesized during a time when its
activity is rapidly disappearing (259). Regulation of the inactivation phase is becoming recognized as an important means of controlling the amount of PAL in the tissue (261). Zucker presented evidence for the turnover of PAL, and his results show the difficult problems which must be surmounted to identify the controls of this enzyme.

Zucker (260) induced PAL in _Xanthium_ leaf discs for 48 hr, pulse-labeled with \(^{14}\text{C}\)-leucine for 4 hr, then chased with \(^{12}\text{C}\)-leucine for 16 hr in both light and dark. The enzyme was then isolated by sucrose density-gradient centrifugation. In light, the specific radioactivity (cpm/million units of enzymatic activity) remained quite constant. If turnover were occurring, dilution of the \(^{14}\text{C}\)-labeled amino acids with the chase \(^{12}\text{C}\)-amino acids would be expected to result in a lower specific activity. In darkness both total radioactivity and enzymatic activity greatly decreased. The apparent lack of turnover in light indicates that the loss in darkness is due to a sequential rapid synthesis of a protein inactivator. Synthesis of the enzyme continued during inactivation as evidenced by the incorporation of \(^{14}\text{C}\)-labeled amino acids into PAL in darkness.

The mechanism of inactivation of PAL is not yet known. The kinetics for the disappearance of activity fit either degradation (turnover) (261) or combination with a protein inhibitor (46, 48, 261). Zucker's work (260) showed that although PAL activity and total amount of radioactivity were decreasing in darkness, the specific activity of the enzyme increased twofold. It was suggested that this might be due to extensive reutilization of released amino acids from PAL during turnover, which increased the proportion of radioactive amino acids in the precursor pools. Reutilization of released amino acids from a pool not equilibrated with \(^{12}\text{C}\)-amino acids might also have masked turnover of PAL in light under steady-state conditions. Since the specific radioactivity was based on enzymatic activity instead of enzyme protein, a decreased enzymatic activity per unit protein could also result in increased specific radioactivity. Hence, a combination of PAL protein with an inhibitor protein resulting in decreased PAL activity could also explain the twofold increase in specific radioactivity. Engelsma (48) reported results with gherkin seedlings which can be explained by the complexes of a protein inhibitor with PAL which is dependent upon temperature. The complex can be weakened by treatment at low temperature, and the active enzyme can appear again after return to normal temperature.

Similar to nitrate reductase, the inactivation of PAL can be largely prevented by cycloheximide in some plants (46, 261). At least two possible explanations exist: (a) that induction of the inactivating system of PAL is sequential to that of the synthesis of PAL; or (b) that synthesis of the degrading system is stopped and the degrading system itself disappears through turnover. Zucker (261) interpreted the available evidence in favor of the former on the basis that inactivation may be low or absent in light but increases greatly in darkness.

The pattern of PAL development and the inducing agents can vary depending upon the plant and whether excised or intact tissue (245) was used. The increase in PAL activity in excised bean axes (245), coleus and soybean callus (191), and asparagus discs (65) does not require light. Other differences in the manner of inactivation of PAL have also been reported. After induction, cycloheximide did not prevent the loss of PAL activity in asparagus discs (65).
Elucidation of the interaction of synthesis and inactivation in the control of the level of PAL and the type of inactivation await a purification of the enzyme protein. Indications are that if the enzyme is degraded, accurate rates of synthesis and inactivation will be difficult to obtain. The apparent rapid rate of inactivation of PAL in relation to the rate of turnover of the precursor pool and the possible extensive reutilization of released amino acids for the synthesis of PAL are complications difficult to surmount. These are the same problems encountered in studies of nitrate reductase, as mentioned above.

**Invertase**

Regulation of the enzyme invertase appears to vary in different plants. Available evidence indicates that regulation of its content in sugar cane may be by continuous synthesis and degradation (63). In a number of other plants, including corn (100), beets and sweet potatoes (181), and potato tuber (182), the activity seems, at least partially, controlled by a protein inhibitor.

Glasziou et al (63) used inhibitors of protein synthesis to determine the turnover rate of invertase in sugar cane. The addition of chloramphenicol, puromycin, or actinomycin D markedly decreased the activity of internodal invertase, presumably by inhibition of synthesis of invertase. Exogenous glucose also caused the same rate of loss of invertase activity, presumably by inhibiting the synthesis and allowing degradation of the enzyme. The rapid loss of activity indicated the presence of a degrading system. The like effect of glucose indicated that the presence of the inhibitors of protein synthesis did not alter the rate of degradation of invertase. No evidence was found in that study for the presence of low or high molecular-weight inhibitors of invertase activity. The apparent half-life was about 2 hr.

Seitz & Lang (206) observed a similar loss of invertase activity from lentil epicotyls in the presence of cycloheximide, and concluded that invertase was under continuous turnover. The estimated half-life of invertase in lentil epicotyl was 14 hr.

From Pressey & Shaw's work (182) it appears that both total invertase activity and an invertase inhibitor protein vary in concentrations in potato tubers according to their storage temperature. The changes in each are reversible when tubers are subjected to alternating temperatures. These results indicate that both invertase and the invertase inhibitor protein are turned over. Hence, two protein turnover systems seem involved in controlling invertase, one controlling its content and the other controlling its activity by regulating the content of inhibitor protein.

Future work on the interaction of these possible turnover systems in the control of invertase level and activity should be particularly rewarding. The protein inhibitor has been purified (180) and invertase at least partially purified (63). The time seems ripe for determination of the specific types of turnover and rates of turnover for these two proteins for further elucidation of the interaction of these two controls.

**Ribulose 1,5-Diphosphate Carboxylase**

The interaction of synthesis and degradation of ribulose 1,5-diphosphate carboxylase (RuDPCase) in the regulation of steady-state levels represents a different type of control. This protein is quite unique in that it comprises a large percentage of the total soluble protein of many plant leaves and therefore is probably a major storage
protein (43, 118, 184, 185) as well as a major catalyst for photosynthetic CO$_2$ fixation (168, 247). Its abundance and great solubility make it a major source of protein for animal life. Life ultimately depends on this protein since it catalyzes the net fixation of CO$_2$ during photosynthesis. The C4 pathway appears to be an adjunct of the Calvin cycle rather than a separate pathway (43); hence, RuDPCase and the Calvin cycle are required for the net reductive fixation of CO$_2$. The enzyme is located in chloroplasts, and its appearance is closely correlated with leaf (215) and chloroplast development (93, 94). Light is required for development of both the chloroplast and RuDPCase (94, 133). Several investigators have evidence that induction of RuDPCase may be related to a phytochrome response (51, 67). It has been shown unequivocally that de novo synthesis of the enzyme occurs during greening of barley seedlings (118). The enzyme is also rapidly degraded during aging of tobacco (39, 108, 110) and Perilla leaves (253) and in barley during extended periods of darkness (171). During aging of tobacco (111) and Perilla (105) leaves and extended dark treatment of barley leaves (171), 90% of the protein decrease is accounted for by RuDPCase. When barley plants were returned to light the synthesis of RuDPCase accounted for about 90% of the increase in soluble protein (171).

To determine whether the enzyme was being turned over in barley leaves while its concentration remained quite constant (in light), RuDPCase was labeled with $^{14}$C by introducing $^{14}$CO$_2$ when the leaves were rapidly synthesizing the enzyme. After removal of $^{14}$CO$_2$ the plants were put into $^{12}$CO$_2$, and the $^{14}$C content of RuDPCase and other soluble protein was followed with time. Analyses were begun after a steady-state level of RuDPCase had been achieved. The concentration of RuDPCase was determined by precipitation with a RuDPCase-specific rabbit antibody (117).

The specific radioactivity of RuDPCase remained constant during the 120 hr of the experiment, showing no turnover (neither synthesis nor degradation), while there was little change in its concentration. In contrast, dilution of label did occur with other soluble proteins. Their specific activity decreased significantly during the first 50 hr of the experiment, showing the occurrence of turnover of non-RuDPCase protein.

In barley leaves, RuDPCase was remarkably stable after its synthesis. Although it can be both degraded and synthesized, these processes seem not to occur simultaneously, but rather are induced independently by changing environmental conditions. Zucker (260) also observed the stability of RuDPCase in green Xanthium leaf discs. While significant amounts of radioactivity were incorporated into phenylalanine ammonia lyase, little was detected in fraction I protein. Abundant evidence shows that fraction I protein is crude RuDPCase (111, 118, 238, 241).

In tobacco leaves the ratio of fraction I protein to total protein reaches a maximum as leaf elongation is completed and then steadily declines (111). It has been proposed that the enzyme in tobacco leaves is under constant turnover and that the changing concentration of fraction I protein is due to changes in either the rates of synthesis or degradation. Since simultaneous synthesis and degradation of this protein have not yet been detected, a slow degradation after the synthetic phase has ceased would also explain the observed results. The appearance and disappearance
of fraction I protein in *Perilla* leaves follows a pattern similar to that in tobacco leaves (105, 253). Woolhouse interpreted these results in like manner, i.e. that synthesis is switched off and a slow degradation ensues.

**Enzymes for Chlorophyll Synthesis**

Chlorophyll synthesis in most plants is under light control. Placing plants in darkness or adding cycloheximide or chloramphenicol (13, 60, 156, 224, 261) will inhibit further accumulation of chlorophyll or protochlorophyllide. The phytochrome-induced response appears to be at the level of δ-aminolevulinic acid (δ-ALA) synthesis, probably δ-ALA synthetase (156, 224), although sites further on in the path of porphyrin biosynthesis have also been suggested (70). These phytochrome-controlled enzymes seem to be turned over very rapidly. In barley, half-life estimates range from 10 min (224) to about 1½ hr (156). The lifetime of the enzymes in a light-requiring mutant of *Chlorella* is estimated at 30 min (13). Since these estimates are arrived at from kinetic studies (time to stop protochlorophyllide synthesis or loss of enzymatic activities) they are subject to the limitations and considerations already discussed.

**Sulfate Permease**

Results from investigations of the sulfate permease and reduction system of *Neurospora* are particularly interesting for several reasons. *Neurospora* possesses many of the attributes of higher organisms, including chromosomes and nuclei. Because of ease of manipulation of this organism, types of control can be revealed which emulate the organization of higher systems. The permease in *Neurospora* develops by de novo synthesis (137). The turnover system of the permease is not present in dormant conidia but seems to develop soon after germination begins, the half-life of sulfate permease being about 2 hr. The turnover of sulfate permease is inhibited by cycloheximide, similar to nitrate reductase and phenylalanine ammonia lyase. The manner of the inhibition is not yet known.

The location of the permease in membranes is a strategic point for regulation of entire pathways by modulating the influx of substrates into intracellular pools. Rapid turnover of permeases then represents an effective means of cellular control. Permeases have not yet been proved to be present in roots of higher plants. Jackson et al (97) recently presented evidence for the possible induction of a nitrate permease system in corn seedlings. Such controls may yet be found in uptake of specific ions by higher plants.

**Other Evidence of Turnover**

Studies involving acrylamide gel electrophoresis of plant proteins from aging or metabolically changing organs indicate that significant reworking of the protein complement does occur during these processes. Major changes in protein banding have been observed during the transition of vegetative apices to floral shoots in *Xanthium* (159), violets (136), and tulips (11). There is a decrease in the number of protein bands with the aging of roots (187, 221), leaves (30, 34, 128), and coleoptiles (187). One exception is cinnamon fern leaves, where there was an in-
crease in the number of bands in the oldest leaves (30). Similar results on aging have been obtained with immunochemical procedures. Kawashima et al (109) have shown that seven antigenic components from tobacco leaves change significantly with age. Both seed germination (12, 129) and fruit ripening (21) result in significant changes in electrophoretic patterns. It has also been observed that the isoenzyme patterns of several enzymes not only vary from tissue to tissue (129) but also vary with changes in development or age (17, 34, 35, 113, 129, 189, 210, 256). Even though these observations are not solid proof of turnover per se, they do show that the plant’s protein complement is not static but is in a dynamic state of fluctuation. Changes in age, environment, or development can effectively change not only the quality but the quantity of the plant’s protein complement. For such changes to occur, it is necessary that proteins be turned over.

The circumstances surrounding the appearance and disappearance of a number of other enzymes have prompted various groups to suggest that protein turnover might be active in these systems. A common occurrence, already discussed in relation to NR and PAL, is inhibition of the loss of enzymatic activity by inhibitors of protein synthesis. In addition, there are other cases in which an enzyme is induced by environmental changes, its substrate, or as a consequence of the normal developmental cycle of the plant. Both the synthesis and subsequent loss of activity are delayed by inhibitors of protein synthesis. Such results have been documented in the case of the appearance and disappearance of thymidine kinase during DNA synthesis in the microspores of *Lilium* (89, 90) and wheat embryo (91), the accumulation and disappearance of UDP galactose polysaccharide transferase during cytokidifferentiation of *Dictyostelium discoideum* (222, 223), the loss of natural phosphatase in *Euglena* after induction (125), and the developmental time course of fatty acid synthetase enzymes in aging potato tuber slices (250).

Cytokinins are well known for maintaining protein and retarding senescence in many plants (194, 195, 213). Controversy exists as to whether cytokinins increase the rate of protein synthesis (213) or decrease the rate of degradation (121, 148, 229, 239). Trewavas’ work with *Lemna* is probably the most rigorous study showing the effect of benzyl adenine (BA) on turnover of protein (237). As mentioned above, the BA effect on turnover of protein can differ with the growth conditions. Under optimum growing conditions BA increased the rate of synthesis without affecting the rate of breakdown, while under growth-limiting conditions, BA did not influence the rate of synthesis but decreased the rate of degradation. Rigorous studies like the above are required to delineate the effects of cytokinins on protein content of the many plants studied. Particular attention should be paid to defining the effect of growth conditions and detachment of plant parts since it now appears that cytokinins can influence the rate of either protein synthesis or degradation.

Protein turnover has also been implicated as explaining various hormonal effects in plants. Hormonal treatments often result in increased protein synthesis, which is believed to mediate the response. Once the hormone is removed, the response generally stops. One explanation is that the enzymes or proteins synthesized in response to the hormonal treatment are rapidly turned over. Once the inducer is removed, synthesis decreases and the induced enzymes rapidly disappear, ending the
mediated response. Such mechanisms have been suggested to explain the auxin-induced growth response in Jerusalem artichokes (160, 231), stem elongation and deformability in pea stems (154, 160) and Avena coleoptiles (20, 160), and auxin control of cellulase activity in Alaska pea seedlings (49).

Rapidly turned over mRNA (127) also implicates the involvement of protein turnover. In detached soybean hypocotyl the mRNA has a mean life of 2 hr, and continued synthesis is required for growth of the excised tissue (114). The necessity for RNA synthesis in maintenance of growth may not be attributed directly to RNA synthesis, but rather to RNA-directed protein synthesis. If that is true, then the protein fractions limiting cell expansion in soybean hypocotyls may also be characterized by rapid turnover with a half-life of a few hours.

REGULATION OF PROTEIN BREAKDOWN

Although information on protein synthesis has increased greatly, little is known about the regulation of protein degradation systems. Even less is known about how synthesis and degradation are integrated to control turnover rates. The removal of unneeded proteins from the cell may occur on a very specific basis, with single enzymes disappearing independently of all others. In some cases the degradative systems responsible for the hydrolysis of certain enzymes might themselves be rapidly turned over. Coupled with this, the heterogeneity of degradation rate constants and the fact that those rate constants can be changed on an individual basis indicate that the plant's proteolytic system is very complex. Understanding the degradative system undoubtedly will be difficult, and future work probably will show the existence of many types of control.

A major difficulty in studies of this type is the selection of an appropriate substrate. Ideally, the protease's natural protein substrate should be used. In some cases specific proteins may be required to assay certain proteolytic enzymes. Thus assays using casein or hemoglobin as substrates are not expected to represent the in vivo activity, and therefore are not a good measure of the total activity. Some researchers have attempted to minimize this problem by using natural protein substrates (98, 214, 217), generally the seed's storage protein. Even then the results may not be clear. Skupin & Warchalewski (214) characterized a protease from wheat seed that has twice the activity toward casein as toward gluten, a storage protein isolated from the same wheat seeds. There are several possibilities for such a response (some discussed in succeeding sections). Since in the majority of proteolytic studies synthetic substrates and proteins have been utilized that are not natural substrates, it is difficult to assess the role the various enzymes play in protein degradation.

This assessment is further complicated when the proteolytic enzymes are removed from their natural cellular environment. Such factors as compartmentation, location, and microenvironment interactions could markedly alter the degradative system. Once removed from the cell some proteases may rapidly lose their activity, or changes in specificity may occur. These effects are not at all uncommon. During the curing of tobacco leaves, chloroplast protein was lost more rapidly than cytoplasmic protein. However, the in vitro proteolytic activity of the cytoplasm was many times
greater than that of the chloroplast (108). Results of Schimke et al (201) indicate that the rapid in vivo loss of rat liver tryptophan pyrrolase, as determined by immunological and enzymatic activity, cannot be duplicated in liver homogenates. Studies in both mammals (22, 84, 170) and bacteria (64, 131, 172, 202, 218, 251) have revealed the necessity of respiratory energy for active protein turnover to occur. It appears the energy is required to maintain a structural component of the protein catabolic system which is either important in the initiation or regulation or in the site of degradation. However, the protease involved in the catabolic process does not require respiratory energy in order to cleave the peptide bonds (22). Elucidation of these interactions may hold an important key to understanding the regulation of protein breakdown. This section presents some of the possibilities for modulation and control of the plant’s protein content. Many of the examples are taken from fields other than plant research and should be considered in that light. Siekevitz (211) has presented an interesting theoretical discussion probing the apparent necessity for protein turnover.

Proteolytic Enzymes

In a recent review, Ryan (192) documented the known proteolytic enzymes of higher plants. As yet there is no complete documentation of the numbers and types of proteolytic enzymes in any given plant tissue; in fact, the heterogeneity of isolation procedures and substrates used for assay makes difficult any definitive comparisons between results of two different groups working with the same tissue. The most extensive studies of proteases have been done with germinated barley seeds, mainly because of their importance in the malting industry. Accurate count of the number of different enzymes described is difficult because the information has come from a number of different laboratories. However, there appear to be at least five endopeptidase enzymes which hydrolyze gelatin, casein, and hemoglobin (24, 25, 44, 45), three carboxypeptidases (126, 147, 150, 243), and three aminopeptidases (120, 146, 178) with varying pH optima and substrate specificities. There are also several peptidases which hydrolyze various synthetic substrates (25–28, 176, 177). Several of these enzymes have been purified to homogeneity and characterized as to substrate specificity, pH optimum, active groups, and physical qualities.

A number of other plant proteases have also been purified and studied in detail (192). Some of these enzymes possess unusual substrate specificities. For example: an acid protease isolated from germinated sorghum seeds (58) specifically cleaves the peptide linkages involving the α-carboxyl group of either aspartic acid or glutamic acid, with release of the acyl portion of these acidic amino acids. It is necessary that the side-chain carboxyl groups of the two amino acids be unsubstituted. An acid protease from the insectivorous plant Drosera peltata preferentially splits peptide bonds on the carboxyl side of aspartic acid, alanine, and perhaps lysine. It also has limited ability to hydrolyze the peptide bond on the amino side of asparatic acid (3). Some of the carboxypeptidases (243), aminopeptidases (120), and peptidases (7, 146, 149) also have narrow substrate specificities. The proteases of soybean have been separated into six different proteolytic fractions by column
All fractions can hydrolyze casein, but differ in specificity toward synthetic substrates. The interesting observation was also made that there was no self-digestion in the purified fractions, nor did one fraction hydrolyze another (173). Thus within a given tissue there are a number of different types of proteolytic enzymes with various types of bond specificities and probably protein specificities.

The basic classification of proteolytic enzymes into serine proteinases, thiol proteinases, acid proteinases, and metallo proteinases (72) suggests basic methods by which the activity of the various groups might be controlled. Serine and thiol groups are highly reactive and will interact both reversibly and irreversibly with a large number of compounds (145). Acid proteinases require low pHs (from 3 to 4) for maximum activity. Thus compartmentation coupled with changes in pH could partially regulate their activity. The removal of metal ions from animal carboxypeptidase A (240) and B (54), aminopeptidase (85), and neutral proteases (50) results in an apoenzyme with no enzymatic activity. In most cases full activity can be restored with the addition of the metal ion. It is interesting that most of the carboxypeptidases and aminopeptidases of plants are not metallo proteases. Even so, some di and tripeptidases (6, 7, 26, 32), as well as endopeptidases (99, 176), have been isolated which require metals for activity. Several plant proteases have been sufficiently characterized to be classified as serine proteases (96, 135, 248, 249), sulfhydryl proteases (4, 45, 99, 120, 143, 212), or acidic proteases (3, 45, 57, 143, 216). Most others, however, have not yet been characterized sufficiently for classification.

The probability that the degradation of individual proteins might be carried out by specific proteases seems more likely today than it did a short time ago (198). There is good evidence that in at least three cases specific proteases react only with specific proteins or types of proteins. A single enzyme that proteolytically degrades insulin has been purified 1000-fold from rat skeletal muscle (41). The enzyme appears not to degrade insulin to its amino acid components, but it does cleave approximately nine peptide bonds, leading to loss in TCA or antibody-precipitable insulin. The enzyme is very specific for insulin and does not attack proinsulin. An enzyme that is specific toward the apoprotein of pyridoxal enzymes has been found in rat skeletal muscles and small intestines (107). This enzyme specifically degrades ornithine transaminase and tyrosine transaminase to smaller oligopeptides and is completely inhibited by the binding of pyridoxal phosphate to the apoprotein. A similar enzyme with activity toward the apoprotein of the NAD-dependent enzymes lactic dehydrogenase and glutamic dehydrogenase has been located in rat small intestines (106). Like the pyridoxal enzyme protease, this protease splits the apoenzymes to lower molecular weight compounds and the hydrolysis is prevented by NAD. Undoubtedly, more enzymes will be added to this list in time, perhaps some from the plant kingdom.

Circumstantial evidence for protein-specific proteases in plants which may bring about the breakdown of nitrate reductase, phenylalanine ammonia lyase, UDP-gal-polysaccharide transferase, and fatty acid synthetase enzymes has been alluded to in previous sections. The almost specific loss of RuDPCase protein when barley seedlings are placed in the dark (171) also suggests some degree of specificity.
Structural Considerations of the Protein Molecule as a Substrate

There are substantial differences in the rates at which proteins undergo inactivation by proteolytic enzymes (124). Proteolytic cleavage of a particular bond of a native protein is not likely if the bond is buried inside the molecule (232) or is included in a rigid region of the peptide chain. It is only in exposed regions characterized by a high degree of local flexibility that peptide bonds will be able to assume the position in the active center of the protease that allows them to be hydrolyzed (158). Such bonds in the native protein might be considered hypersensitive to proteolytic attack. This has been confirmed by findings that a series of proteolytic enzymes of different specificities can cleave methionyl-tRNA synthetase into very similar fragments (31) and that both the "hinge" region of immunoglobulin (196) and the peptide links between globular units of plasma albumin (252) are particularly susceptible to proteolysis. Therefore, only certain regions of the protein are attacked even though the peptide bonds of proper specificity are probably distributed along the entire polypeptide chain. In many instances proteins are much more susceptible to hydrolysis if they are denatured (165) or if certain peptide bonds are broken (167). Both cases generally result in destruction of the molecule's inherent stability, causing the peptide chain to unfold and expose more sites to proteolytic attack. Since protein molecules can exist in a number of different thermodynamic states resulting from conformational changes, it is not surprising that the degree to which a protein can be hydrolyzed varies under differing conditions. Thus the oxidized forms of cytochrome a (254) and cytochrome c (165) are much more sensitive to proteolysis than the reduced form. Studies with E. coli suggest that at least bacteria have the ability to recognize abnormal proteins and specifically degrade them (64). In those experiments unfinished polypeptides containing puromycin and abnormal proteins containing amino acid analogs were degraded more rapidly than normal cell proteins. It was suggested that normal proteins of E. coli share certain general conformational features that prevent their rapid hydrolysis. Deviations from these common morphological characteristics might then result in proteins that are more sensitive to the degradative system.

Decreased digestibility of proteins in the presence of various ligands and changes in pH, ionic strength, and temperature have been frequently reported. In most cases the protective effect is considered to result from conformational changes that are less susceptible to attack; however, other models are possible. Several hypersensitive sites have been identified in cytochrome b2 which are sensitive to endogenous proteases as well as trypsin. The sensitivity of the various sites to hydrolysis is modulated by changes in ionic strength or the binding of ligands (heme and flavin). Such treatments resulted only in changes of the relative rates of hydrolysis of certain bonds and had no effect on the fragments being formed (158). The treatment of serum albumin with various ligands which will bind to the protein significantly reduced hydrolysis of the protein by several proteolytic enzymes. The binding of one mole of ligand per mole of protein was sometimes enough to slow digestion significantly (134). Similar effects were observed with Ca\(^{2+}\) and Mn\(^{2+}\) ions (66). Conalbumin was less susceptible to trypsin hydrolysis in the presence of iron (10) or substrate
cofactors (235). Homologous haptens significantly reduced the digestibility of rabbit antibody (134). The presence of substrate molecules also stabilizes enzyme proteins (69). Both in vivo and in vitro degradation of tryptophan oxygenase is decreased by tryptophan (199, 201), thymidine prevents degradation of thymidine kinase (116, 126), and the administration of iron stabilizes rat liver ferritin (40). When appropriate experimental controls were done it was shown that ligands, cofactors, activators, or substrates act on the protein substrate, not on the protease enzyme. However, it is possible that such factors may concurrently act on both the degraded and the degrading system to modulate the cell's protein components.

**Proteolytic Control Through Zymogen Activation**

The proteolytic enzymes produced by the mammalian pancreatic tissue are present as inactive zymogens. Limited proteolysis of these precursors results in the active protease. The activation of a staphylococcal protease, of mucus membrane prorennin, and the cascading series of proenzyme-enzyme transformation leading to the clotting of blood all involve limited proteolysis for activation (167). No plant protease has yet been shown unequivocally to be activated in a similar manner. However, the rapid increase in proteolytic activity during the germination of many seeds (192) might suggest such an origin for some enzymes. Some suggestive evidence has accumulated in favor of zymogen-type proenzymes in plants (53, 68, 179, 207). Shain & Mayer (207) have invoked an autocatalytic process involving a kind of trypsinogen-like form coupled with the removal of proteolytic inhibitors to account for the activation of a trypsin-like enzyme in germinating lettuce seeds. Ample evidence exists that a protease in yeast, protease-c, exists first as a proprotease which is later activated by limited hydrolysis or conformational changes (74–78). Another possible example of a proenzyme comes from germinating pea seeds. The enzyme amyllopectin 1,6-glucosidase is associated with a particulate fraction, and its release from that fraction requires proteolysis (208, 244) and results in activation of the enzyme.

**Lysosomes**

The catabolic nature of lysosomes has been implicated with a variety of the cell's processes (38) including intracellular digestion of food, autolysis of mitochondria and microsomes, and turnover of various cell components. The classical lysosomal organelle of animal cells is yet to be discovered in plants. It has been argued, however, that turnover of protein, cytoplasmic nucleic acids, and other plant cell constituents indicates that the lysosomes or lysosomal apparatus must be present in higher plant cells (140). Whether lysosomes have or will be discovered in plants probably depends on the definition of the organelle. Plant spherosomes (141), microsomes (37), vacuoles (16, 138), and aleurone grains (139, 255) have been implicated as lysosomes because of the presence of the various acid hydrolases. Important to our discussion here is the existence of proteolytic enzymes in these organelles. These have acid protease with a pH optimum between 3 and 4. Interestingly enough, most of the plant proteases have pH optima higher than this (192) and do not appear to be particulate-bound. In understanding the degradative process, it is not enough
to endow lysosomes with the sole responsibility, but it must somehow be determined how nonparticulate, nonacid proteases are involved. As Schimke & Doyle (198) pointed out, it is difficult to understand how the lysosome could function in the process, since protein degradation involves randomness, heterogeneity of degradation rate constants, and the rate constants that can be altered by various experimental treatments. Implication of the lysosome does not alter the problem of specificity but simply moves the problem to the lysosomes. Thus, some mechanism must be invoked for the recognition of protein molecules that are to be degraded and then specifically transported into the lysosome. It might be more reasonable to propose that the plant's lysosomal systems are important at a time when gross changes in the rates of protein degradation occur. However, the possibility of some role in protein turnover cannot be completely dismissed.

**Membranes**

A discussion of lysosomes logically leads to a consideration of the importance of membranes and how they might function in giving specificity to the turnover system. Practically no information is available in this area, even though it may be of major importance. A growing number of enzymes are suggested to be associated with membranes. It is possible that membrane binding, besides endowing activity, might also eliminate or reduce the chances of proteolysis. A possible example of this comes from work with NADPH-cytochrome c reductase in rats. Injecting rats with phenobarbital speeds synthesis and slows degradation of the microsomal membrane-bound enzyme. The slower degradation appears to be the result of the enzyme becoming more tightly bound to microsomal membranes in treated animals (211). Furthermore, studies indicate that newly synthesized NADPH-cytochrome c reductase is more susceptible to degradation than old membrane-bound enzyme (122, 123). It is suggested that newly synthesized enzyme molecules are first released from the ribosome into the cytoplasmic pool and then incorporated into a proper site on the membrane. While in the cytoplasmic pool, the enzyme is more unstable and thus more easily hydrolyzed than its membrane-bound counterpart.

The mechanism of membrane binding might also be applied as a method of activating, inactivating, or changing the specificity of proteolytic enzymes as well. Not much is known about the interaction of proteases and membranes; even in cases where proteases are associated with lysosomal-like particles, the interaction, if any, with the membrane has not been investigated. However, there is at least one example of a membrane protease in animals. A protease with some chymotrypsin-like activity has been isolated from human erythrocyte membranes (151, 155). It has a pH optimum of 7.4, and experiments indicate that the activity is a part of, or at least is bound to, the lipoprotein of the membrane.

**Proteolytic Control Through Endogenous Inhibitors**

Protein inhibitors from plants with activity against trypsin and chymotrypsin have been well documented (192). These trypsin inhibitors are widely distributed in higher plants but apparently have very little if any function in controlling the bulk of endogenous proteolytic activity. Yet controlling certain of the plant's proteolytic
enzymes by inhibitors could represent not only a simple but rapid and effective means of control. To date very little definitive information is available concerning endogenous inhibitors. Knowledge about this very interesting and important area of physiological control in plants is mostly circumstantial. Generally, evidence has come merely as sidelight observations during studies of proteolytic enzymes. The proteolytic activity of crude extracts frequently can be significantly enhanced by dialysis (29, 45, 115, 227) or chromatography (7, 44, 230, 246), suggesting the presence of endogenous inhibitors in the extracts.

Extracts of barley seeds (45) and seedlings (29) contain endogenous inhibitors of barley endopeptidase activity. The embryo of rice (88) and barley seeds (29, 115) contains the highest concentration of the inhibitors. There is a rapid decline in inhibitor activity during germination of barley (45, 115), rice (88), lettuce (209), and sorghum (57). Correlated with this loss is an increase in proteolytic activity. Haynes & Feeney (79) suggested that inhibitors in seeds may function by inhibiting proteolytic enzymes while storage proteins are being laid down. At the onset of germination the inhibitors may be removed, allowing proteolytic enzymes to hydrolyze the stored proteins. In this case, inhibitors may express control over the breakdown of storage proteins. In the same sense, inhibitors might influence not only the turnover rate but also the concentration of other plant enzymes.

CONCLUSIONS

Development of information on protein turnover in plants has lagged well behind that of animals and microorganisms. At present turnover or lack of turnover under specific conditions has been demonstrated for only two plant enzymes, nitrate reductase and ribulose-diP carboxylase. However, much recent circumstantial evidence indicates that a large number of plant enzymes might be turned over. Techniques are now more generally available to either minimize or overcome many of the procedural problems involved in proving the occurrence of turnover of plant proteins and in estimating turnover rates. The future will see more research efforts directed toward such studies.

A most intriguing aspect of turnover is the control of its occurrence and rate. What modulates the rates of synthesis and degradation during induction, during steady state levels, and during losses of specific enzymes? Relevant information is largely lacking on these subjects for living organisms. The study of such regulation is vastly complicated by the possibilities that the proteolytic enzyme(s) itself is rapidly turning over or is sequentially released, activated, or synthesized, and then degraded or inactivated when no longer required. Elucidation of these complex controls will be a major achievement.
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