ENERGY COST OF PROTEIN TURNOVER: THEORETICAL CALCULATION AND EXPERIMENTAL ESTIMATION FROM REGRESSION OF RESPIRATION ON PROTEIN CONCENTRATION OF FULL-GROWN LEAVES

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Introduction

The concept of growth and maintenance respiration has been the subject of many reviews over the last ten years. The respiration rate for maintenance of plant biomass may be determined by various methods, but the outcome varies and depends to a large extent on interpretation of the data (Amthor 1986, 1989, Irving & Silsbury 1987, 1988, Lambers 1985, Lambers *et al.* 1983b, Penning de Vries 1975). In contrast to growth rate (dry matter accumulation) and ion uptake, rates of specific maintenance processes have not been related to respiration rate. Maintenance respiration is in fact an unexplained fraction of dark respiration in most analyses.

For maintenance of plant biomass about 20-60% of the photosynthates produced daily may be oxidized in respiration (Amthor 1989, Lambers 1985, Van der Werf *et al.* 1988). The molecular nature of maintenance processes is largely unknown. Protein turnover is generally regarded as one of the most important maintenance processes in terms of energy requirements, but quantitative data at the intact plant level are scarce and variable (Amthor 1989, Barneix *et al.* 1988, Davies 1982, Huffaker 1982, Lambers 1985, Penning de Vries 1975). While much is known about the *rate* of protein turnover in higher plants (*i.e.* protein half-life), little information is available on the *energy costs in vivo*. In calculating rates of energy use for maintenance, information is needed about the identity and rate of these processes, their specific costs and the efficiency of energy production (Penning de Vries 1975). Progress has been made in all of these three areas. Since the first extensive calculations on energy cost of maintenance by Penning de Vries (1975), new processes have been found that are involved in the maintenance of proteins. These processes control the orderly, thus energy-requiring, expression of genetic information in protein synthesis or regulate enzyme activity by protein modification. Indeed, proteins are not just products of random polymerization. First, evidence is increasing that protein degradation requires energy, *e.g.* in ubiquitination (Dalling 1987, Hershko 1988), in contrast to earlier data (Penning de Vries 1975). Second, Cramer & Freist (1987) have recently demonstrated a new and important energy-requiring process, *i.e.* amino acid recognition and error correction by aminoacyl-tRNA synthetases. This process increases the cost of protein biosynthesis by at least a factor of two (*cf.* Freist 1990), thus also affecting the cost of turnover. Third, translation of many mRNAs in eukaryotic cells

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yields pro-proteins which are longer than the final protein, containing presequences of 13-30 amino acid residues; these signal sequences ensure targeting of the proteins to one of the more than 30 compartments in the cell (Chrispeels 1991, Lehninger 1982). Fourth, protein turnover involves posttranslational modifications of proteins, *e.g.* by proteolysis, methylation, acetylation of amino groups, ADP-ribosylation and glycosylation (Beevers 1982, Chrispeels 1991, Lehninger 1982, Thompson & White 1991). Also, many proteins are (de)phosphorylated, *e.g.* in enzyme activity regulation (*e.g.* Lehninger 1982, Bennett 1991, Thompson & White 1991). These processes increase the cost of protein biosynthesis and turnover. The above data on the mechanisms of protein biosynthesis and biodegradation are used for calculation of the specific energy cost of protein turnover.

The respiratory *rate of ATP production* for protein turnover equals the product of the *specific ATP cost* and the *rate* of protein turnover (*cf.* Penning de Vries 1975). We are interested in quantifying the energy cost of leaf protein turnover *in vivo*, preferably on intact plants, to avoid artefacts. To that end, rates of ATP production and protein turnover *in vivo* should be determined simultaneously in leaves varying in protein turnover rate. This appears to be impossible with present-day techniques. As a first attempt, the relationship between dark respiration rate and protein concentration is examined in full-grown leaves, where respiration for growth and net ion uptake is negligible. The experimental estimates of energy cost of protein turnover in leaves of intact plants and leaf discs are compared with calculated values as obtained by Penning de Vries (1975) and as updated with the new biochemical data mentioned above.

Material and methods

Plant material. Bean (*Phaseolus vulgaris* L. cv. Berna) plants were grown in a growth room [daylength 16 h, PPFD 500 μmol m^{-2} s^{-1} (HPI), temperature 21°C, relative humidity 65/85%], on aerated, modified Hoagland nutrient solution (half-strength; 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 45 mM iron as Fe(III)EDTA; micronutrients 23 μM H_3BO_3, 4.6 μM $MnCl_2$, 0.4 μM $ZnSO_4$, 0.16 μM $CuSO_4$, 0.26 μM Na_2MoO_4; *cf.* Smakman & Hofstra 1982); nitrate was supplied daily as $Ca(NO_3)_2$ and KNO_3 in a ratio of 1:1, at four relative addition rates (0.05, 0.085, 0.115 and 0.15 day^{-1}; *cf.* Ingestad 1979). Potato plants (*Solanum tuberosum* L.) of 15 cultivars, Alcmaria, Alpha, Bintje, Civa, Désirée, Katahdin, Kennebec, Krostar, Maritta, Multa, Pimpernel, Saturna, Spunta, Veenster, Woudster) were grown in Hoagland nutrient solution (half-strength, with iron and micronutrients as given above; Smakman & Hofstra 1982) in a greenhouse at 18°C (Wageningen, spring 1988), or in the field on a sandy soil (Wageningen, June 1989). Ryegrass plants (*Lolium perenne* L., clones of the cvs Parcour, Pelo, Selectie I, and Splendor) were grown in a greenhouse at 19°C (Wageningen, spring 1988) on Hoagland nutrient solution as described above for bean plants, but with nitrate supplied daily at two relative addition rates, *i.e.* 0.05 and 0.15 day^{-1}. Winter barley plants (*Hordeum vulgare* L. cv. Hasso) were grown in pots filled with sandy loam under a glass roof (Wageningen, spring 1989) with varying amounts of nitrogen as described by Groot (1988). In all cases, youngest mature, *i.e.* full-grown (in terms of dry matter accumulation)

leaves were selected for measurements of dark respiration.

Respiration measurements. Carbon dioxide production was measured on leaves of intact plants of bean and ryegrass by infra-red gas analysis (open system; Louwerse & van Oorschot 1969) with computerized control and data processing. Oxygen uptake was monitored manometrically (Umbreit *et al.* 1957) on samples of 4 leaf discs (ø 1 cm) incubated on wet filter paper in 15 ml Warburg flasks, with 0.1 g $KMnO_4$ (ethylene scrubber) in the side arm and 0.2 ml of 0.9 M KOH with filter paper in the centre well (potato and barley), or polarographically (Yellow Springs Instrument Co., model 53; ryegrass, potato) on 1 cm (ø) leaf discs or 2mm leaf slices in 10 mM MES/50 mM HEPES buffer (pH 6.6) with 0.2 mM $CaCl_2$ according to Azcón-Bieto *et al.* (1983). Temperature was 20°C. Respiration rate was independent of the technique used, indicating that the respiratory quotient (RQ) in the leaves was close to unity.

Protein-N determination. Leaf tissue was boiled for one hour. Protein was precipitated overnight in 10% (w/v) TCA at room temperature. N content of the precipitate was determined by Kjeldahl analysis. In barley leaf discs, total N was determined with a CHN analyser (Heraeus); protein-N content was then calculated as (total-N content)*(protein-N/total-N ratio); this ratio is close to 0.8 for many species under most conditions (Mengel & Kirkby 1978), and is hardly affected by nutrient supply (Sørensen 1971) or age (Robson & Pitman 1983). The four ryegrass genotypes differ slightly in protein-N/total-N ratio of the leaf lamina (0.76-0.83, $P<0.05$; unpubl. data).

P/O ratio. Techniques for directly determining P/O ratio of leaves *in vivo* are not available. Methods for determination of the activity of the CN-resistant alternative pathway are well developed (Møller *et al.* 1988), and have been used to estimate P/O ratios (de Visser *et al.* 1986). For leaves collected in the light period, alternative path activity averages about 15% (8 species, Lambers *et al.* 1983a), but after several hours in the dark, this activity may drop to zero (Azcón-Bieto *et al.* 1983). Therefore, we assumed the P/O ratio in leaves to be close to 3 (*i.e.* 36 mol ATP produced mol^{-1} glucose).

Experimental estimation of ATP costs of protein turnover. The standard free energy of hydrolysis of ATP with the formation of ADP and inorganic phosphate (P_i), 30.5 kJ mol^{-1}, is used as the unit of free energy in the present analyses. Thus, the orthophosphate cleavage of one mol of ATP to one mol of AMP and two moles of P_i, is expressed as two moles of ATP. Ideally, both respiration rate and specific energy cost should be expressed in terms of ATP-equivalents.

For full-grown tissues where respiration for growth and ion uptake may be neglected, the rate of dark respiration (r) equals the rate of maintenance respiration (m). This rate may be considered as the sum of the maintenance coefficients (rates of respiration; m_i) of the various processes, *i.e.* protein turnover (m_p), compartmentation of ions and other solutes (m_c), export of assimilates (m_e) and unidentified processes (m_u):

$$r = \Sigma m_i = m_p + m_c + m_e + m_u \qquad (1a)$$

where r and m_i are generally expressed on a *dry weight basis* (*e.g.* nmol O_2 g^{-1} dry weight s^{-1}). The maintenance coefficients may be determined by analyzing the mul-

tiple regression of respiration rate on the rates of the processes i. Here, we attempt this for protein turnover by an indirect method.

If protein turnover is one of the most important energy-requiring processes in mature leaves (Penning de Vries 1975), the rate of ATP production in leaf respiration is expected to relate linearly to the concentration of leaf protein-N. Therefore, under the assumptions mentioned below, dark respiration rate (r; O_2 uptake, or CO_2 production when the respiratory quotient is 1.0; nmol g^{-1} dry weight s^{-1}) may be considered to be linearly related to the concentration of protein-N in the tissue (N_t; $mmol^{-1}$ N g^{-1} dry weight):

$$r = m_p + c = m_p' * N_t + c \quad (1b)$$

where m_p' is the maintenance coefficient of protein turnover expressed on a *protein-N basis* (nmol O_2 $mmol^{-1}$ N s^{-1}) and c is a constant fraction of respiration (nmol O_2 g^{-1} dry weight s^{-1}) not related to protein-N concentration. Similar equations have been proposed by Amthor (1986) and Kuperman & Khitrovo (1990). Assumptions regarding the tissues with different protein-N concentrations are: *a.* no net increase in biomass, *b.* constant biochemical composition of the protein fraction (protein turnover costs may depend on protein type), *c.* constant chemical composition of the non-protein fraction (or: protein turnover is by far the most important maintenance process), and *d.* other energy-requiring (maintenance) processes, *e.g.* solute compartmentation, are either constant or quantitatively insignificant (no data known to the authors). Therefore, full-grown, and non-senescing tissue in a steady state was chosen to satisfy the above conditions. Furthermore, the treatments creating variation in protein concentration were designed to meet the assumptions.

Since protein turnover involves hydrolysis and formation of peptide bonds and modification of amino acid residues, *specific* energy costs of protein turnover must be expressed on the basis of number of peptide bonds, or number of proteinaceous amino acids, rather than on a protein-N or total-N basis (*cf.* Penning de Vries 1975).

The rate of protein turnover is characterized by exponential decay (constant half-life), with a degradation constant K_d (g protein g^{-1} protein s^{-1}, abbreviated to s^{-1}; $K_d = \ln 2/\text{half-life}$; Davies 1982). The maintenance coefficient for protein turnover expressing the respiratory cost on a protein-N and time basis (m_p', nmol O_2 $mmol^{-1}$ N s^{-1}), equals the product of *specific ATP cost* (E_{sp}, mol ATP mol^{-1} peptide bond) and *rate* of protein turnover (*cf.* Penning de Vries 1975):

$$m_p' = E_{sp} * K_d * k \quad (2a)$$

where k is a correction factor for transformation of units (see under equation 2b). Experimental values of *specific* ATP costs for protein turnover (E_{sp}, mol ATP mol^{-1} peptide bond), are calculated from m_p' using

$$E_{sp} = (m_p'/K_d) * (P/O_2) * N_p \quad (2b)$$

where m_p' is the maintenance coefficient of protein turnover (mol O_2 mol^{-1} N s^{-1}) as obtained from equation 1b, K_d is the degradation constant of protein turnover (s^{-1}), P/O_2 is the P/O ratio of oxidative phosphorylation (*cf.* Lambers 1985) ex-

Table 1. Specific energy cost of protein turnover (E_{sp}), calculated using biochemical information on processes involved. For details on processes, data, references and assumptions, see Material and methods.

Biosynthetic process	Specific energy cost (E_{sp}) mol ATP mol^{-1} peptide bond			
	Penning de Vries 1975		present paper	
	low	high	low	high
Protein biodegradation	1.0	1.0	1.0	2.0
Protein biosynthesis				
Amino acid activation	2.0	2.0	2.0	2.0
Error correction by aminoacyl-tRNA synthetases	–	–	5.0	8.0
Peptide bond formation and translocation	1.0	2.0	2.0	2.0
Tool maintenance	1.0	1.0	1.0	1.0
Signaling sequences (extra 5–10% biosynthesis and biodegradation of polypeptide)	–	–	0.6	1.9
Amino acid turnover (100 or 0% recycling)	0	3.5	0	3.5
Post-translational processing				
Methylation, acetylation, glycosylation, etc.	–	–	0.1	0.1
Phosphorylation [*e.g.* enzyme (de)activation]	–	–	0.1	0.3
Total	5.0	9.5	11.8	20.8

pressed as mol ATP mol^{-1} O_2, and N_p is a constant, being the average N content of the protein (mol N mol^{-1} peptide bond). Penning de Vries (1975) estimated the K_d of leaf protein at 0.12 day^{-1}. More recent experimental values of K_d also average about 0.12 day^{-1} under non-stress conditions (Davies 1982, Huffaker 1982, Simpson *et al.* 1981; *i.e.* $1.39*10^{-6}$ s^{-1}), although higher values, 0.28 to 0.42 day^{-1} have been reported (Barneix *et al.* 1988, Davies 1982).

Theoretical calculation of ATP costs of protein turnover (E_{sp}). Penning de Vries (1975) has calculated specific protein turnover costs from biochemical data. Here, these calculations are updated using new information on energy costs of some processes. Biodegradation of protein is a highly organized process requiring ATP (Dalling 1987, Huffaker 1982, Summers *et al.* 1988), *e.g.* in ubiquitin-mediated protein degradation (Hershko 1988, Jabben *et al.* 1989). No quantitative information is available for plants, but in animal systems the amount of ATP hydrolyzed per peptide bond probably ranges from 1 to 2, because of the different mechanisms of proteolysis involved (Summers *et al.* 1988).

Amino acid activation and error correction by aminoacyl-tRNA synthetases requires the hydrolysis of 3.4-5.0 mol ATP to AMP, *i.e.* hydrolysis of 7 – 10 mol ATP to ADP per peptide bond formed, depending on cellular conditions (Cramer & Freist 1987, Freist 1990). This is 5-8 mol ATP more than two ATP per peptide bond as known till now (Barneix *et al.* 1988, Penning de Vries 1975). Peptide bond formation and translocation require two mol ATP mol^{-1} peptide bond (Lehninger 1982). Costs for tool maintenance (ribosomes, mRNA, tRNA) are according to Penning de Vries (1975; Table 1).

Signaling sequences are 13-30 amino acid residues long and are removed by endopeptidases upon reaching the target compartment (proteolysis; Chrispeels 1991); assuming an average leaf polypeptide molecular weight of 25-45 kD (*cf.* Heitz *et al.*

1991, Hurkman *et al.* 1991, Nguyen-Quoc *et al.* 1990; including thylakoid membrane proteins; *cf.* Burkey & Wells 1991), the number of amino acids in these presequences amounts to 5-10% of those in the final polypeptides.

Amino acid turnover does occur and affects the energy cost of protein turnover. Experimental estimates of energy cost are scarce; here, it is assumed to range from 0-3.5 mol ATP mol^{-1} peptide bond, depending on the fraction recycled (100 to 0%; Penning de Vries 1975), which is probably close to 50% (Davies 1982).

Posttranslational processing affects the energy cost of protein biosynthesis and turnover. Modifications include many processes, *e.g.* proteolysis, methylation, acetylation, carboxylation, phosphorylation, glycosylation, ribosylation, carbohydrate and prosthetic group attachment (Lehninger 1982), but quantitative data are not available; it is assumed that 10% of the amino acid residues is phosphorylated, and 15% modified in any other way, requiring one mol ATP per mol amino acid processed. Phosphorylation of serine, threonine and tyrosine residues in proteins is accompanied by dephosphorylation (Lehninger 1982). In plants, it plays a role in light responses (Thompson & White 1991) and in regulation of enzyme activity and photosystem proteins in chloroplasts where half-times for (de)phosphorylation are in the order of minutes, and in many other aspects of growth and development (Bennett 1991). In the absence of quantitative data at the level of the intact leaf, it is assumed that about 10% of the amino acid residues is (de)phosphorylated 1-3 times per day.

The amino acid composition of total leaf protein is little affected by species, ontogeny or growth conditions (Beevers 1976, Lyttleton 1973, Mader *et al.* 1982), although protein composition may change, *e.g.* in response to N-supply (Evans & Terashima 1987). From this information, the following data were obtained: the average molecular weight of amino acid residues in leaf protein is 110 g mol^{-1}, and the N content of leaf protein is close to 1.26 mol N mol^{-1} amino acid residue or peptide bond (*cf.* Beevers 1976, Lehninger 1982).

Statistical analysis. Data were analyzed with the statistical package Genstat 5 (Payne 1987).

Results

Theoretical calculations. The results of the calculations described in Materials and Methods are summarized in Table 1. The total specific energy cost of leaf protein turnover (E_{sp}; mol ATP mol^{-1} peptide bond) ranges from 5.0 to 9.5 according to Penning de Vries (1975). More recent data (see Materials and Methods) indicate that the energy requirements are higher. Calculation of E_{sp} yields values ranging from 11.8-20.8 mol ATP mol^{-1} peptide bond when energy costs are considered of biodegradation, amino acid recognition and error correction, presequences and posttranslational processing (see Material and methods and Table 1).

Experimental data. The experiments were designed to meet the assumptions mentioned in Material and methods. Protein-N concentrations in the leaves were manipulated by factors that are unlikely to affect, in any systematic way, solute compartmentation ('ion transport'), another major maintenance process. These factors (sources of variation) were nitrogen supply (Figs 1 and 2), genotype (Fig 3) or both

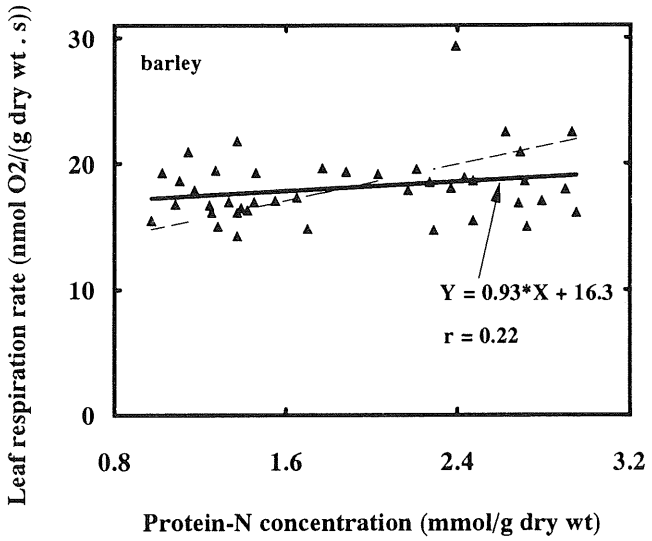

Fig. 1. Dark respiration rate *versus* protein N concentration of fully expanded flag leaves of *Hordeum vulgare* L. Plants were grown with four rates of nitrogen supply (see Material and methods). Solid and broken lines indicate the experimental and theoretical (maximum slope $m_p' = 3.8$ nmol O_2 mmol^{-1} N s^{-1}; Table 2) regression lines, respectively.

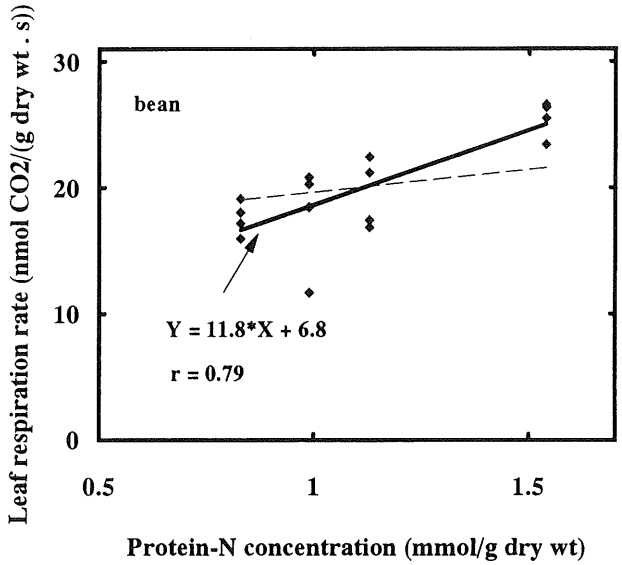

Fig. 2. Dark respiration rate *versus* protein N concentration of fully expanded leaves of *Phaseolus vulgaris* L. Plants were grown with four relative nitrogen addition rates (see Material and methods). For more information, see the legend to Fig. 1.

(Fig. 4). Respiration rate of mature, full-grown leaves was positively correlated with leaf protein concentration in bean plants grown with different nitrogen supply rates under controlled conditions ($P < 0.001$; Fig. 2), and nearly so in 15 potato cultivars

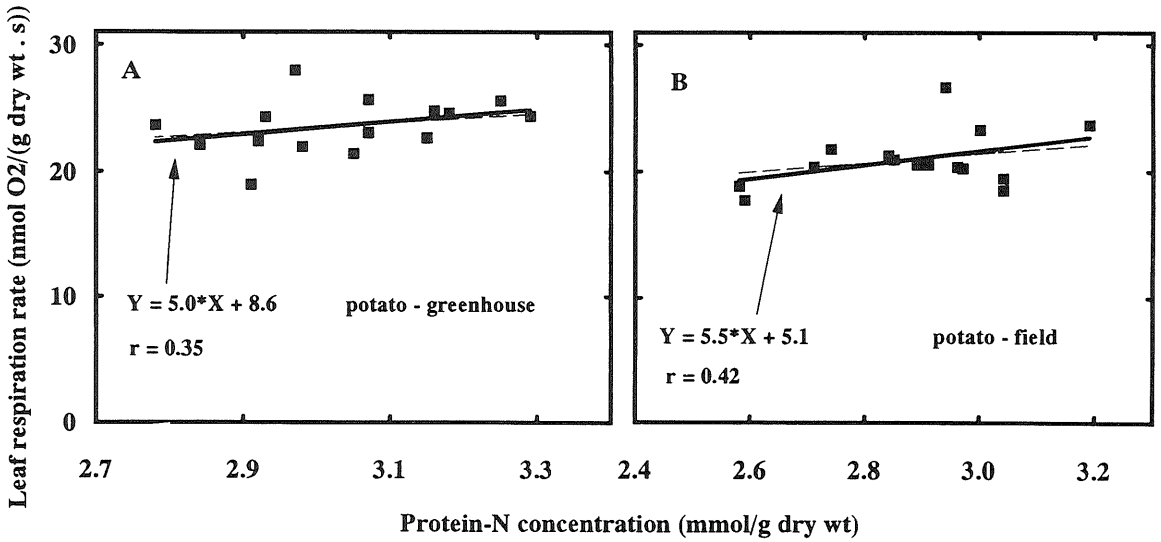

Fig. 3. Dark respiration rate *versus* protein N concentration of fully expanded leaves of 15 cultivars of *Solanum tuberosum* L. grown in a greenhouse (A, n = 6) or in the field (B, n = 8). For more information, see the legend to Fig. 1.

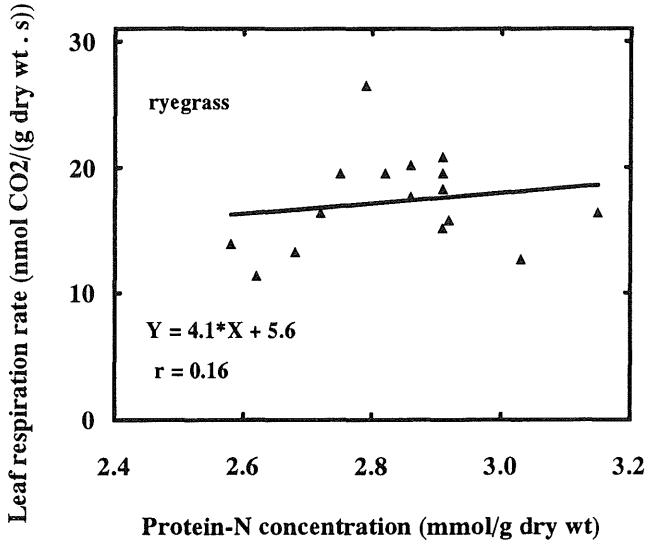

Fig. 4. Dark respiration rate *versus* protein N concentration of fully expanded leaves of four cultivars of *Lolium perenne* L. grown with two relative nitrogen addition rates (see Materials and methods). The experimental and theoretical regression lines overlap; *cf.* the legend to Fig. 1.

grown in the field ($P < 0.06$; Fig. 3B). Experimental (solid) and theoretical (broken) regression lines are shown in Figs 1-4. The genotypic variation in leaf respiration rate of potato was significant in both greenhouse and field experiment ($P < 0.01$; Bouma *et al.*, 1992). No significant correlation was found for the same 15 potato cultivars (Fig. 3A) and ryegrass plants (four genotypes and two N supply rates; Fig. 4) grown in a greenhouse, and for winter barley (flag leaf; four N supply regimes; Fig. 1) grown under a glass roof. However, slope and correlation coefficient in linear regression are mathematically related parameters {r = slope [SD(x)/SD(y)]}. Although not significant due to variation in the data, the low values for m_p' in Figs 2 and 3 are nevertheless in the range of values expected theoretically, *i.e.* up to 3.8 nmol O_2 $mmol^{-1}$ protein-N s^{-1} (Table 2; *cf.* maximum theoretical slope m_p' shown as a broken line in Figs 1 to 4). Respiration-axis intercepts are significantly different from zero in Figs 1 (barley; $P < 0.001$) and 2 (bean; $P < 0.01$). Moreover, the average experimental value of m_p' over the present five independent experiments is 5.5 (SE = 1.8) nmol O_2 $mmol^{-1}$ protein-N s^{-1} (*cf.* Table 2) which is significantly different from zero ($P < 0.05$).

Experimental values of E_{sp} are calculated from the maintenance coefficient m_p' (the solid regression lines in Figs 1-4), using equation 2b (Table 2; see Material and methods). Leaf ATP production rates may be calculated from gas exchange data when the respiratory quotient (RQ; when measuring CO_2) and the respiratory P/O ratio *in vivo* are known. The RQ was 1.0 in mature leaves. The P/O ratio is assumed to be close to 3.0 (see Material and methods). The specific costs of protein turnover (E_{sp}) range from 5 to 64 (Table 2), averaging 30 (SE = 10) mol ATP mol^{-1} peptide bond in the present five independent experiments. The maintenance coefficient m_p as expressed on a dry weight basis, is also given as a percentage of leaf dark respira-

tion rate; the average rates of leaf dark respiration of the present species are easily read from the Figs 1-4. About 56% (SE = 12) of the respiration rate of full-grown leaves appears to be related with leaf protein content (average of the five present experiments).

Discussion

The specific energy cost of leaf protein turnover (E_{sp}), including biodegradation and posttranslational modification (Table 1), is calculated here to be about twice the cost estimated almost 20 years ago (Penning de Vries 1975). The major reason for this are new insights in the mechanisms of protein biosynthesis. Error correction in amino acid recognition by aminoacyl-tRNA synthetases is accompanied by hydrolysis of 5-8 mol ATP mol^{-1} peptide bond formed (Cramer & Freist 1987, Freist 1990; Table 1). As a result, the specific ATP cost of protein *biosynthesis* is 10-13 rather than 4 or 5 mol mol^{-1} peptide bond as is often used in calculations (*e.g.* Barneix *et al.* 1988, Van der Werf *et al.*, 1992; Table 1). When the energy costs are expressed per peptide bond of actually present protein, synthesis and degradation of signaling sequences have to be taken into account. These presequences of 13-30 amino acid residues function in protein targeting and are removed by endopeptidases cotranslationally or upon arrival of the protein in the organelle specified by the presequence (Chrispeels 1991). Thus, with some other new and well-established biochemical data on processes like protein biodegradation and post-translational modification (see Material and methods), total specific energy costs of protein turnover (E_{sp}) are most likely in the range of 12-21 mol ATP mol^{-1} peptide bond (Table 1). Using equation 2, this corresponds to a maintenance respiration rate for protein turnover (m_p') of about 69-120 mg glucose per g protein per day (Table 2), for $K_d = 0.12$ day^{-1} (*cf.* Davies 1982, Huffaker 1982, Simpson *et al.* 1981). Penning de Vries (1975) presents E_{sp} values of 0.22-0.43 g glucose per g protein, without or with 100% amino acid turnover, respectively (excluding turnover of other nitrogenous compounds). This equals 5.0-9.5 mol ATP mol^{-1} peptide bond (Table 1), yielding a m_p' of 27-52 mg glucose per g protein per day for the same K_d (assuming complete oxidation of glucose, P/O = 3 and 1 mol peptide bond per 110 g protein; Table 2). Penning de Vries (1975) concludes that these protein turnover costs plus the energy cost of maintaining ion concentrations are lower than indicated by the rate of maintenance respiration of crops, and suggests that this may be explained by higher rates of protein turnover (K_d) under field conditions compared to the constant, experimental conditions with low light intensities. However, this remains uncertain, since high K_d values have also been reported for plants grown under constant, mild conditions in growth cabinets (Barneix *et al.* 1988), and m_p' of potato leaves is similar under field and greenhouse conditions (Fig. 3; Table 2). Here, we present some evidence for an alternative explanation: the difference may also be caused by higher specific energy costs of protein turnover (E_{sp}; see Table 1).

When our theoretical estimates of E_{sp} (Table 1) are used to calculate the maintenance coefficient of protein turnover (m_p'), by multiplication with the degradation constant $K_d = 0.12$ day^{-1} using equation 2, theoretical m_p' values are obtained ranging from 2.2 to 3.8 nmol O_2 $mmol^{-1}$ N s^{-1}; this equals about 33% (range:

Table 2. Experimental and theoretical specific energy cost of protein turnover (E_{sp}, mol ATP mol^{-1} peptide bond) in full-grown leaves or whole plants, and the component of ('maintenance') respiration rate related to protein concentration, expressed on a dry weight (m_p) and protein-N basis (m_p'), for P/O = 3 and $K_d = 0.12\ day^{-1}$. N is protein-N. Values between brackets indicate SE. For more information, see Figs 1 to 4 and Material and methods.

Species	Tissue	Maintenance coefficient				Specific energy cost	Ref.
		m_p'		m_p		E_{sp}	
		nmol O_2 $mmol^{-1}$ N s^{-1}	mg glucose g^{-1} protein d^{-1}	nmol O_2 g^{-1} dry wt s^{-1}	% of total respiration	mol ATP mol^{-1} peptide bond	
Theoretical							
various	leaf	0.9 – 1.7	27 – 52	1 – 5	7 – 90*	5.0 – 9.5	Penning de Vries (1975)
various	leaf	2.2 – 3.8	69 – 120	2 – 11	11 – 60	11.8 – 20.8	present paper
Experimental							
various	plant	1.4 – 7.3	44 – 230	1 – 22	–	8 – 42	Amthor (1989)
various	plant	12.0	377	12 – 35	–	65	Irving & Silsbury (1987)
Maize	8th leaf	5.9	185	6 – 18	70	32	Kupermann & Khitrovo (1991)
Barley	flag leaf	0.9(0.7)	28	1 – 3	5 – 13	5(4)	present paper
Bean	leaf	11.8(2.5)	370	9 – 18	55 – 73	64(14)	present paper
Potato							
greenhouse	leaf	5.0(3.7)	158	14 – 17	60 – 66	27(20)	present paper
field	leaf	5.5(3.3)	165	14 – 18	75 – 76	30(18)	present paper
Ryegrass	leaf	4.1(6.8)	132	11 – 13	66 – 68	23(38)	present paper

* maintenance respiration rate.

10-60%) of leaf dark respiration rate in the species presented here (*cf.* Figs 1-4; Table 2). Experimental m_p' values, obtained by regression analysis of leaf dark respiration rate on leaf protein concentration, overlap with theoretically calculated values, covering a wide range, 1 to 12 nmol O_2 $mmol^{-1}$ N s^{-1}, or about 56% (range: 10-75%) of leaf dark respiration rate (Table 2). Thus, both biochemical information and experimental data indicate that protein turnover is a major maintenance process in leaves, responsible for 30-60% of leaf dark respiration.

Experimental E_{sp} values average 30 (SE = 10) mol ATP mol^{-1} peptide bond for the present four species, ranging from 5 (barley flag leaf, different N-supply) to 64 (bean, different N-supply) mol ATP mol^{-1} peptide bond in our experiments, in accordance with values we calculated from similar data in the literature (8 to 65 mol ATP mol^{-1} peptide bond; Table 2), using equation 2b and assuming P/O = 3, and $K_d = 0.12$ day^{-1} (*cf.* Davies 1982, Huffaker 1982, Simpson *et al.* 1981). It is unlikely that the low m_p' value for barley flag leaf can be explained by variation in protein-N/total-N ratio; even in the most extreme case of this ratio decreasing with age from 0.8 to 0.7 (Robson & Pitman 1983, Sørensen 1971; see Material and methods), m_p' would still be only 1.2. A possible explanation is, that the rate of protein turnover (K_d) increases with decreasing N-supply, as found for *Lemna* (Davies 1982). This results in higher rates of respiration at low protein-N contents (*cf.* Fig. 1). The high values of m_p' and E_{sp}, especially those for bean, are discussed in the last three paragraphs.

Most of the present experimental values of specific energy costs of leaf protein turnover (E_{sp}) are close to, or up to 50% higher than the highest theoretical estimate (Table 2). This may be caused by shortcomings of the present experimental method, in such a way that the present assumptions are not met, *i.e.* that activities of some other (maintenance) processes correlate with protein content (see below). Other explanations are that either actual specific costs of protein turnover (E_{sp}) are even higher than theoretically estimated here, or that the rate of protein turnover *in vivo* (K_d) is higher than experimentally determined (*e.g.* by dual labelling; Davies 1982). Also, E_{sp} and especially K_d may depend on environmental conditions (Cramer & Freist 1987, Davies 1982; see below). Clearly, more work needs to be done on turnover of amino acids and protein *in vivo*, both at the intact plant and the molecular level. Another possibility is that the P/O ratio *in vivo* is lower than 3 as may be deduced from the activity of the CN-resistant alternative path (*e.g.* de Visser *et al.* 1986, Møller *et al.* 1988), which is generally low in leaves (Azcón-Bieto *et al.* 1983). Other processes may lower the P/O ratio *in vivo*, *viz.* proton leakage over the inner mitochondrial membrane, H^+-ATPase idling, or oxidation of succinate or exogenous NAD(P)H (A.M. Wagner, pers. commun.). Also, futile cycles, *e.g.* in sucrose synthesis in leaves (Huber 1989), may decrease the apparent efficiency of respiratory energy utilization. Thus, the experimental values of the specific ATP cost of protein turnover (E_{sp}), determined from gas exchange measurements, may overestimate the actual costs. This stresses the importance of developing non-invasive techniques for determining the *in vivo* P/O ratio of leaves, *e.g.* ^{31}P-NMR (Roberts 1984).

Leaf respiration rate is not *directly* proportional to leaf protein concentration (Figs 1 to 4). A large fraction of leaf dark respiration, 44% (range: 25-90%; Table 2), is not related to protein concentration. The Y-axis intercepts in Figs 1 and 4 are signifi-

cantly different from zero ($P < 0.01$ and $P < 0.001$, respectively). Both theoretical and experimental data show that protein turnover is an important energy-requiring process in leaves, but not the only component of (maintenance) respiration, as suggested by some authors claiming a proportional relation between respiration rate and protein-N content (*e.g.* Irving & Silsbury 1987). Data on a per plant basis (*cf.* Irving & Silsbury 1987) do not necessarily provide evidence for a correlation and may be misleading, especially where plant weight varies by more than a factor of 10. Even when assuming the highest theoretical estimate (E_{sp}) of 21 mol ATP mol^{-1} peptide bond, low regression coefficients are obtained using equation 2b ($m_p' = 3.8$ nmol O_2 $mmol^{-1}$ N s^{-1}), as well as positive Y-axis intercepts (Table 2; Figs 1 to 4; assuming $K_d = 1.39*10^{-6}$ s^{-1}, $P/O_2 = 6$ and $N_p = 1.26$ mol mol^{-1} peptide bond). The absence of a significant correlation between leaf respiration rate and protein-N content in some of the data is partly caused by the small values of the slope m_p', *i.e.* the relatively low rate of respiration for protein turnover. These data show that care should be taken in correcting maintenance coefficients for N-concentrations that deviate from the standard leaf tissue of Penning de Vries (1975), as is common use in crop growth simulation modelling (*e.g.* Van Keulen & Seligman 1987).

The present results of theoretical calculations and experimental determinations are similar and indicate that *i)* protein turnover *in vivo* requires more energy than generally assumed on the basis of older biochemical data (Penning de Vries 1975; Table 1), *ii)* protein turnover is responsible for about 30-60% of dark respiration of full-grown leaves, and *iii)* in some leaves (bean, Fig. 2; potato, Fig. 3) other, as yet unknown, energy-requiring reactions may take place which correlate with protein concentration. Nitrate content of plant tissues increases with N-supply (Beevers 1976, Mengel & Kirkby 1978). Therefore, maintenance of ion compartmentation might increase with N-supply. However, in the present potato cultivars, leaf respiration rate was *negatively* correlated with nitrate and potassium concentrations. Since we are dealing with non-growing tissues, biosynthesis for growth can be discarded. Another process possibly involved is carbohydrate export. Irving & Silsbury (1988) have suggested that the well-known dark-decay of leaf respiration rate is caused by the high energy cost of assimilate export during the normal dark period. Multiplying high carbohydrate export rates in leaves in the dark (10-20 mg carbohydrate g^{-1} dry wt h^{-1} after correction for a decrease due to respiration; Azcón-Bieto *et al.* 1983, Challa 1976) with known specific costs of phloem loading (1.1-1.4 mol ATP mol^{-1} sucrose; Giaquinta 1983), gives, for a protein-N content of 1-3 mmol g^{-1} dry wt and $P/O = 3$, oxygen uptake rates for carbohydrate export of 0.5-3.8 nmol O_2 $mmol^{-1}$ N s^{-1}, *i.e.* about 8-20% of leaf dark respiration rate in the present species. Therefore, assimilate export may be a major sink of respiratory energy, and variation in (the energy costs of) assimilate export may account for a part of the differences in m_p' and E_{sp} between species, or between calculations and experiments (Table 2). However, this cannot explain the results for bean, since leaf carbohydrate export was negligible (T.J. Bouma, unpubl. data).

The energy expenditure of leaves for the maintenance of proteins appears to be hardly affected by the growth conditions, since potato leaves show similar E_{sp} values when grown under greenhouse and field conditions (Fig. 3; Table 2). However, under some stress conditions protein degradation and synthesis may increase, *e.g.* upon heat-shock (Clarke & Critchley 1990), or infection by microorgan-

isms (fungi, Roby *et al.* 1991; viruses, Heitz *et al.* 1991). Therefore, it is likely that K_d is not constant, but sensitive to environmental growth conditions, as is confirmed experimentally (Davies 1982). Important factors affecting energy cost of protein turnover may be temperature, water stress and salinity, nutrient starvation, and dynamics of the growth conditions, since adaptive metabolic responses involve energy dependent reactions like protein biosynthesis (Penning de Vries 1975). There may be energy-requiring processes related to protein concentration that are not involved in protein turnover, but in regulation of enzyme activities, *e.g.* by (de)phosphorylation, ADP-ribosylation, and acetylation. The rates of these processes are not known at present. More research is needed on the *in vivo* rates, mechanisms and energy costs of biosynthesis and biodegradation of proteins and regulation of enzyme activity in plants, and on the (unknown) processes depending on the remaining 40-70% of respiratory energy. Ion compartmentation and carbohydrate export may be other important processes (Penning de Vries 1975), but quantitative experimental evidence is lacking.

Summarizing, theoretical calculations show that the specific energy cost of leaf protein turnover is about 12-21 mol ATP mol^{-1} peptide bond or 33% of leaf dark respiration. Using literature values for the half-life of leaf protein, experimental estimates of the energy cost of protein turnover of full-grown leaves *in vivo* are about 30 mol ATP mol^{-1} peptide bond or 56% of the ATP produced in leaf dark respiration. The present experimental method, regression of respiration rate on protein-N concentration, is sensitive to variation in the data and correctness of the assumptions, but may be improved by combining respiration measurements with determinations of the *in vivo* P/O ratio and the rates of protein degradation (or turnover), carbohydrate export and ion compartmentation. We conclude that protein turnover is an important energy-demanding process in leaves, responsible for 30-60% of leaf dark respiration and requiring more research.

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