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A Relationship between Protein-Degradation Rates *in vivo*, Isoelectric Points, and Molecular Weights Obtained by using Density Labelling

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1. Half-lives of five plant enzymes were estimated by rate-labelling with ^2H_2O on the assumption that loss of catalytic activity is equivalent to protein degradation. 2. This involved measuring band-broadening of activity profiles after isopycnic centrifugation. 3. Isoelectric points were determined by isoelectric focusing, and molecular weights were estimated by gel filtration. 4. The conclusion is drawn from the experimental evidence presented that a weak correlation exists between rates of degradation and isoelectric points ($r = 0.699$; $P > 0.10$; not significant). 5. A highly significant relationship exists between the logarithm of subunit size and half-life ($r = -0.939$; $P < 0.02$). 6. A literature survey confirmed the trends observed.

Rates of intracellular protein degradation are a function of composition and structure [for reviews, see Huffaker & Peterson (1974), Goldberg & Dice (1974) and Goldberg & St. John (1976)]. Parameters, apparently independent, influencing protein half-life are size (Dehlinger & Schimke, 1971; Dice & Goldberg, 1975*a*; Momany *et al.*, 1976), charge (Dice & Goldberg, 1975*b*; Dice *et al.*, 1979) and extent of glycosylation (see Dice *et al.*, 1978).

Two potential gaps would appear to exist in our present state of knowledge. Firstly, although prokaryotes and mammalian cells have undergone extensive study, plants have not. In fact, only one report of a relationship between protein size and degradation rate *in vivo* in a higher plant (Dice *et al.*, 1973) appears to exist. Secondly, the majority of studies in which data were obtained in the same laboratory used the 'double-labelling' procedure of Arias *et al.* (1969). The technical and theoretical limitations encumbent with this technique are well documented (see, e.g., Goldberg & Dice, 1974). It is therefore imperative that these relationships be tested in a single study by a labelling procedure as different as possible to that of Arias *et al.* (1969). The object of the present study was to provide this information.

We would emphasize that, in the present paper, turnover is defined as the flux of amino acids through protein (Huffaker & Peterson, 1974). For the sake of simplicity, we will equate half-life of enzyme activity with the half-time for protein degradation. Since the assumption inherent in this reasoning, namely that enzyme inactivation is the rate-limiting step of protein loss, may not be justified, we will refer to 'apparent' protein half-lives when these are determined by activity losses (see Segal, 1976). Some justification for the above assumption does, however, exist. Studies on pyridoxal- and NAD-dependent enzymes indicate that a reversible inactivation (conversion from the holo into the apo form) produces a susceptible form, and that this is both the rate-limiting and initiating step in the degradation of vitamin B_6-dependent enzymes *in vivo* (Katunuma, 1977). A similar change in the conformation of enzymes that lack a prosthetic group could make inactivation the rate-limiting step in other systems as well (Katunuma, 1977). The implications of the existence of long-lived (and degradable) unassayable forms of proteins (see, e.g., Johnson, 1977) will be discussed at the end of the present paper.

The technique chosen to determine half-lives was rate-labelling to saturation with ^2H_2O (density labelling). A very major advantage inherent in this choice is the facility to directly measure the degree of heterogeneity (or lack of) in labelling of a given protein pool. In practice, this is achieved by measuring parameters such as bandwidth (Filner & Varner, 1967; Acton *et al.*, 1974) or bandwidth with peak height (Betsche & Gerhardt, 1978) of activity profiles obtained after isopycnic centrifugation. Thus, under ideal or testable conditions, heterogeneity in labelling (in the present work band-broadening) is an index of apparent turnover time [for a theoretical discussion, see Acton *et al.* (1974)]. The choice of density labelling is further justified by the need to saturate tissue with label to overcome the likelihood of temporary storage in vacuoles [a serious drawback inherent with radioisotopes in plants; see Davies &

Humphrey (1978)] and the desirability of avoiding problems of recycling of individual amino acids (Davies & Humphrey, 1978) [see also Dice *et al.* (1978) for alternative approaches]. A slow rate of labelling of the amino acid pool(s) from which a protein is synthesized is automatically tested for; no band-broadening will arise if pool labelling is rate-limiting (Acton *et al.*, 1974). In addition, the requirement to test for transient changes (under steady-state conditions with regard to enzyme activity) in protein-turnover rates during the experimental period (see Dice *et al.*, 1978) can be met to a limited extent. The key to our argument is that, under steady-state conditions, a relatively even transition from a homogeneously unlabelled population, through heterogeneity to a homogeneously labelled population of protein molecules [see Tong & Schopfer (1976) for an example] primarily excludes the above potential artefact at least in the case of gross transient changes. An alternative approach is advocated by Johnson and co-workers (Johnson, 1977; Johnson & Smith, 1978), which relies on the parameter of density shift to estimate apparent enzyme half-life. This is to determine the time for labelling the enzyme pool to half-saturation.

Although density-labelling has become a popular technique among plant biochemists [for a general review, see Chrispeels & Varner (1973)], the method has been little exploited for protein-turnover studies (Huffaker & Peterson, 1974; present Table 2). The reliability of both the density shift (see, e.g., Shepard & Thurman, 1973; Pitt, 1974) and bandwidth (Attridge *et al.*, 1974; Johnson, 1977) parameters of the technique has been questioned. We would respectfully refer the reader to the elegant data of Tong & Schopfer (1976) to dispel misgivings about technical limitations under adequately controlled conditions. Theoretical constraints on the approach adopted here include the necessity to rely on a non-destructive method of recognition (in the present work enzymic activity) in the light of misgivings by some workers (Boudet *et al.*, 1975) of the assumption inherent throughout the present study that enzyme inactivation be rapidly followed by bulk protein degradation. In addition, the procedure lacks precision (Davies & Humphrey, 1978), and ^2H_2O at the concentrations used causes physiological stress (Acton & Schopfer, 1974); thus all apparent half-life estimates presented are relative and not necessarily equivalent to values in water. Further, the technique relies on the knowledge (or assumption) that activity profiles are unlikely to be complicated by transient changes in isoenzymes (that is, heterogeneity in molecular weight). Given these constraints and considerations, it will be shown that relationships exist between turnover rates and isoelectric points and turnover rates and particle size for five plant enzymes under (with one exception) non-adaptive conditions. A preliminary report has appeared (Acton & Gupta, 1978), and a literature survey is given in the present Table 2.

Experimental

Materials

Chemicals. ^2H_2O (99.75 atom % of ^2H_2O) was obtained from the Australian Institute for Nuclear Science and Engineering, Lucas Heights, N.S.W., Australia. 'Ultra-pure' $(NH_4)_2SO_4$ and sucrose were supplied by Mann Research Laboratories, Orangeburg, NY, U.S.A. and bovine serum albumin (96–99%, fraction V, dry, crystalline) and alcohol dehydrogenase (alcohol–NAD^+ oxidoreductase, EC 1.1.1.1) (yeast) from Sigma, St. Louis, MO, U.S.A. Lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27) [ox heart, crystalline suspension in $(NH_4)_2SO_4$] and peroxidase (donor–H_2O_2 oxidoreductase, EC 1.11.1.7) (horseradish, A. grade) were obtained from Calbiochem, Carlingford, N.S.W., Australia, and catalase (H_2O_2–H_2O oxidoreductase, EC 1.11.1.6) (ox liver) from Boehringer-Mannheim, Melbourne, Vic., Australia. Ampholytes were either supplied by LKB, Bromma, Sweden, or were a gift from Dr. B. V. McCleary, Biological Chemical Research Institute, Rydalmere, N.S.W. 2116, Australia. Ultrodex (evaporation limit 39%) and Ultrogel AcA 34 were obtained from LKB. Sephadex was obtained from Pharmacia, Uppsala, Sweden; dichlorodimethylsilane and Triton X-100 from Ajax, Sydney, N.S.W., Australia, and glycine (AnalaR) from BDH Chemicals, Port Fairy, Vic., Australia. Other chemicals were either obtained from sources previously quoted (Acton *et al.*, 1974; Acton & Schopfer, 1974, 1975; Gupta & Acton, 1979) or were of the highest grade commercially available.

Plants. Lupins (*Lupinus albus* L.) were grown in darkness as previously described (Acton & Schopfer, 1974). Etiolated mustard (*Sinapis alba* L.) seedlings used to extract nitrate reductase (NADH–nitrate oxidoreductase, EC 1.6.6.1) (seeds from Asgrow Company, Freiburg-Ebnet, Germany; 1972 harvest) were grown in darkness from surface-sterilized seeds (aq. 5% NaOCl) at 25°C on five layers of germination paper with 150nM-Na_2MoO_4/100mM-KNO_3 (5.0ml/45 seeds) under aseptic conditions. For density-labelling of nitrate reductase, 36h-old etiolated seedlings were either transferred (dim green light) to 70 atom % ^2H_2O diluted with sterilized water (3.5ml/45 seedlings) or transferred to sterilized water alone (unlabelled controls; 3.5ml/45 seedlings). Growth of etiolated mustard seedlings (and labelling treatments) for extraction of other enzymes were as already described (Acton *et al.*, 1974; Acton & Schopfer, 1975; Gupta & Acton, 1979).

Methods

Preparation of extracts (3°C). Acid ribonuclease (ribonucleate 3′-oligonucleotidohydrolase, EC 3.1.4.23) was extracted from ribosomes as described by Acton (1974) and Acton & Schopfer (1974). Ascorbic acid oxidase (L-ascorbate–oxygen oxidoreductase, EC 1.10.3.3) and acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] were extracted from etiolated mustard cotyledons as described by Drumm *et al.* (1972), except that serum albumin was omitted. Preparations for isoelectric focusing and gel filtration on Ultrogel AcA 34 were further purified by saturation to 40% with $(NH_4)_2SO_4$, and the resultant precipitate was removed by centrifugation (29500*g*; $r_{av.}$ 8.3cm) and resuspended in 200mM-dipotassium hydrogen phosphate adjusted at 25°C to pH5.0 with 100mM-citric acid and containing 1mM-cysteine. L-Phenylalanine ammonia-lyase (EC 4.3.1.5) was extracted from etiolated mustard cotyledons as described by Acton & Schopfer (1975). For isoelectric focusing the enzyme was further purified by chromatography on L-phenylalanyl–Sepharose 4B and on Sephadex G-200 as previously described (Gupta & Acton, 1979). Enzyme that had been purified to homogeneity was used for gel filtration (Gupta & Acton, 1979). Nitrate reductase was extracted from etiolated mustard (*Sinapis alba*) cotyledons grown under aseptic conditions with molybdenum and nitrate. Freshly harvested cotyledons were ground in acid-washed sand in 25mM-potassium dihydrogen phosphate/25mM-dipotassium hydrogen phosphate, pH7.4, and containing 1mM-EDTA and 1mM-cysteine (2.0ml/45 cotyledon pairs), by using a pestle and mortar. Cell debris was removed by centrifugation (29500*g*; $r_{av.}$ 8.3cm), low-molecular-weight substances were removed by chromatography on Sephadex G-25, and the resultant effluent made to 40% saturation with $(NH_4)_2SO_4$. The precipitate obtained was removed by centrifugation (29500*g*; $r_{av.}$ 8.3cm) and resuspended in 100mM-potassium dihydrogen phosphate/100mM-dipotassium hydrogen phosphate, pH7.4, and containing 1mM-EDTA and 1mM-cysteine (0.4ml/45 cotyledon pairs).

Equilibrium sedimentation. Methods used to obtain, and data on, band-broadening of activity profiles of acid ribonuclease, ascorbate oxidase, acid phosphatase and L-phenylalanine ammonia-lyase have already been published (Acton *et al.*, 1974; Acton & Schopfer, 1974, 1975). Nitrate reductase is inactivated by salts generally used for self-forming gradients (Tischner & Hüttermann, 1978). However, the enzyme is stable in sucrose, and Hirschberg *et al.* (1972) claim to have 'banded' the protein to an isodensity position of 1.065kg/litre in preformed linear sucrose gradients. In the present work centrifugation was performed (at −2°C) in preformed linear (370–950mM) sucrose gradients. Extract (0.2ml; 1.3mg of protein) was mixed with 10μl of ten-times-diluted lactate dehydrogenase (external marker enzyme), layered over gradients (4.8ml), tubes filled with paraffin (kerosene), and proteins 'banded' (conditions are given in the legend to Fig. 1). After centrifugation, fractions (20μl) were collected (see Acton & Schopfer, 1974), and refractive indices determined at 20°C on every tenth fraction and converted into density units by using a calibration graph [data from Reid (1971)]. Bandwidths of activity profiles of external marker enzymes were taken as an index of consistency of centrifuging conditions throughout (see e.g. Tong & Schopfer, 1976). The buoyant densities obtained (approx. 1.1kg/litre) are lower than anticipated (1.3kg/litre) for proteins. This low value would appear to arise, at least in part, from protein interaction with the supporting solute, since increasing the time of centrifugation from 20 to 25h (Fig. 1) did not measurably move the peak to a higher density. Tischner & Hüttermann (1978) also observed an abnormally low value (1.23kg/litre) for nitrate reductase banded to equilibrium in metrizamide.

Isoelectric focusing (5°C). Column focusing was performed in linear 0–1.375M-sucrose gradients (LKB 8101 column freshly coated with dichlorodimethylsilane) in buffer solutions at 5mM (final concn.), containing 1% (w/v) glycine and 0.01% Triton X-100 (see the legend to Fig. 3 for details). Acid ribonuclease was focused in granulated gel (9.0mg of protein/4g dry wt. of Ultrodex) for 15h by using the procedure of Radola (1975) (LKB 2117 Multiphor) [5.0ml of ampholytes (pH3–10)/4g dry wt. of Ultrodex swollen in 5M-sodium acetate adjusted at 25°C to pH5.6 with acetic acid] and final settings of 1000V and 1mA. Each gel strip was eluted with water (for pH determination) and further eluted with 100mM-sodium acetate buffer, pH5.6.

Assays. (*a*) *Fractions.* Acid ribonuclease, ascorbate oxidase, acid phosphatase and L-phenylalanine ammonia-lyase were all assayed as previously described (Acton *et al.*, 1974; Acton & Schopfer, 1974, 1975), except that L-phenylalanine ammonia-lyase from the Ultrogel column was assayed by an isotopic method using ring-labelled L-phenylalanine (Attridge *et al.*, 1974). Nitrate reductase was assayed by the method of Hirschberg *et al.* (1972), except that NADH was supplied rather than produced *in situ*, and the reaction stopped by enzymic removal of excess NADH. In all cases, reaction velocities were approximately linear with time (plots of reaction velocity against time were slightly concave-downwards in nitrate reductase assays exceeding 90min). Recoveries of nitrate reductase from isopycnic centrifugation were 75%. ^2H-labelled and unlabelled enzymes were of similar stability.

(*b*) *General assays.* Proteins were precipitated with trichloroacetic acid (two cycles) and determined by

the procedure of Lowry *et al.* (1951), with dry crystalline bovine serum albumin as standard. Peroxidase was assayed (25°C) by the method of Evans (1970), catalase (25°C) by the procedure of Aebi (1974) with minor modification (Gupta & Acton, 1979), alcohol dehydrogenase (25°C) as described by Racker (1955) and lactate dehydrogenase (25°C) by the procedure of Kornberg (1955).

Computing and statistics. Data were analysed by using scattergram, multiple-linear-regression and partial-correlation subprograms of Program SPSS-10, University of Pittsburgh, Pittsburgh, PA, U.S.A. Bandwidths and density shifts were determined from linear plots of activity profiles (Acton *et al.*, 1974; Acton & Schopfer, 1974, 1975). For nitrate reductase, evidence for band-broadening was also obtained from probit plots of activity profiles (Fig. 1). All bandwidth changes and density shifts were from pooled data from a minimum of four gradients and were tested for significance (Acton & Schopfer, 1974, 1975) (Fig. 1). In the cases of ascorbate oxidase and acid phosphatase labelled in etiolated mustard cotyledons (Acton *et al.*, 1974), the estimated error (S.E.M. of pooled data) of bandwidth measurements was ±0.0005 (±1.6%) and ±0.0011 (±2.6%) kg/litre respectively. Maximum percentage assay errors in significant figures were 6, 2, 10, 8 and 6 for nitrate reductase, L-phenylalanine ammonia-lyase, ascorbate oxidase, acid phosphatase and ribonuclease (Kunitz assay) respectively.

Results and Discussion

Half-life estimations

Plots of band-broadening and of labelling to half-saturation against time (Figs. 1 and 2) gave apparent half-life values of 1.8 and 3.4h respectively for nitrate reductase in 70 atom % of ^2H_2O in etiolated cotyledons, i.e. a mean value of 2.6h. This estimate is subject to two constraints. Firstly, labelling was necessarily performed under adaptive conditions [deviation from steady-state due to induction by nitrate at germination (R. Starr & G. J. Acton, unpublished work)]. However, the measured nitrate-mediated increase in assayable activity over 3h was around 20%. This is sufficiently small not to obviate the band-broadening method. The value of 2.6h is therefore likely to be an underestimate (unless maximal specific activity of the amino acid pool from which the reductase is synthe-

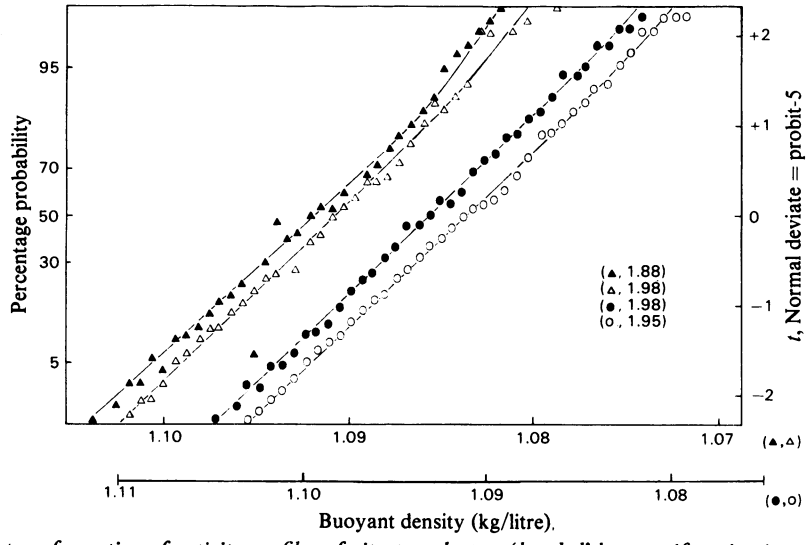

Fig. 1. *Probit transformation of activity profiles of nitrate reductase 'banded' by centrifugation in sucrose gradients*
Batches (45) of 36h-old etiolated mustard seedlings, grown on water as described in the Experimental section, were transferred to either fresh water or 70 atom % of ^2H_2O for 1.5 or 6.0h. Extracts (see the Experimental section) were mixed with commercial lactate dehydrogenase and layered on to preformed sucrose gradients. Filled tubes (see the Experimental section) were centrifuged in a Beckman SW-41 swinging-bucket rotor run at 195000*g* ($r_{av.}$ 13cm) for 20h (−2°C) without use of the brake. Duplicate gradients containing unlabelled enzyme and duplicate gradients containing ^2H-labelled enzyme were run in parallel in each experiment. A minimum of two independent experiments were run for each time point. Data are from a representative gradient from each treatment. Assay points: 1.5h H_2O (△), ^2H_2O (▲); 6.0h H_2O (○), ^2H_2O (●). The bottom axis has been shifted to permit the presentation of all data. Changes in profiles after labelling are indicated by deviation from the transformation plot of unlabelled treatments. The steepness of the lines is indicated in parentheses in units of 10^{-2} probits/litre per kg.

sized is reached slowly, in which case an overestimate may be obtained, particularly over the longer period required to label to half-saturation). The second constraint is imposed by accuracy of the procedures. Systematical error produced by developmental changes in substances that interfere with refractometer readings (Acton & Schopfer, 1975) are corrected for, in the case of density shifts, by running an external marker enzyme (in this case lactate dehydrogenase) in each gradient. A similar control is not possible in the case of band-broadening measurements (unless a marker enzyme bands over the same region as the test enzyme), so water controls are run in parallel in each centrifugation against which any changes are measured (Acton & Schopfer, 1975). In the present work, developmental changes caused native bandwidths to vary by more than the measured difference at 1.5h (Fig. 2) (G. J. Acton & S. Gupta, unpublished work). Providing that the necessary controls are run (see above) this does not obviate the technique (Acton & Schopfer, 1975; Johnson, 1977). The errors (based on S.E.M. of bandwidth and 'corrected' density shift) of the apparent half-life estimates are ±0.6 and ±0.4h for the band-broadening and half-saturation methods respectively. It is relevant to point out that the converse approach, namely to predict the apparent half-life of the reductase from multiple-regression analysis of data in Table 1, gives a value of 4.2h.

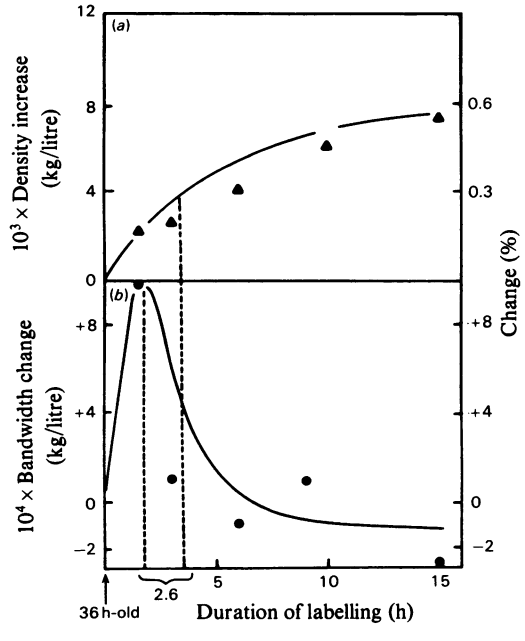

Fig. 2. *Time-course for density shifts and band-broadening of nitrate reductase labelled with ^2H_2O and 'banded' by centrifugation in sucrose gradients*

Batches (45) of 36-h-old etiolated mustard seedlings were transferred either to fresh water or to 70 atom % of ^2H_2O for the times indicated and the cotyledons then harvested (see the Experimental section for details). Partially purified extracts were centrifuged and density shifts (*a*) and bandwidths (*b*) measured. Bandwidth changes were indexed from bandwidths of unalabelled enzyme run in parallel in each centrifugation. This is necessary to prevent large systematical errors being introduced (see Acton & Schopfer, 1975). Results are means for at least four gradients. Density shifts (▲) at all time points and the bandwidth change (●) at 1.5h were all significant (comparison-of-two-means test, $P = 0.05$).

Similar plots of published data (cf. Fig. 2) for band-broadening and labelling to half-saturation of L-phenylalanine ammonia-lyase in 80 atom % ^2H_2O in mustard cotyledons under steady-state conditions (Acton & Schopfer, 1975; Johnson & Smith, 1978) give apparent half-life estimates of 3 ± 0.3 and 3.5h respectively (mean of 3.3h, Table 1). Rapid turnover ($t_{\frac{1}{2}}$ 3–4h) of the lyase in 70 atom % ^2H_2O in mustard was also reported by Tong & Schopfer (1976), who used annulled-induction pretreatments to give steady-state amounts of enzyme activity. Apparent half-lives of 30 ± 4.0 and 36 ± 8.6h were obtained via band-broadening for ascorbate oxidase and acid phosphatase in 80 atom % ^2H_2O in mustard under conditions that approximate to steady state (Acton *et al.*, 1974).

An apparent half-life in the region of 200 ± 31h is estimated for acid ribonuclease in 99 atom % ^2H_2O under steady-state conditions (Acton & Schopfer, 1974). This is calculated by assuming that heterogeneity in molecular weight in the published activity profiles (Acton & Schopfer, 1974) is essentially limited to two components, and on the basis that band-broadening is unlikely to exceed the 0.021kg/litre density value obtained under adaptive (light-treatment) conditions. Thus band-broadening of 0.008kg/litre over 3.5 days represents a 20% replacement (data from Acton & Schopfer, 1974).

Justification of these values is derived from the absence of aggregates or isoenzymes (that is, potential heterogeneity in molecular weight) in the cases of nitrate reductase (Table 1; Fig. 3*a*), L-phenylalanine ammonia-lyase (Schopfer, 1971; Gupta & Acton, 1979; Table 1; Fig. 3*b*) and ascorbate oxidase (Möller & van Poucke, 1970; Drumm *et al.*, 1972; Table 2; Fig. 3*d*). In addition, time courses for band-broadening showed relatively even transitions to a homogeneously labelled state. Thus artefacts such as gross transient changes in enzyme-turnover rates during the labelling period, or limitation of labelling by the rate of equilibration of the amino acid pool with ^2H_2O are excluded. This is consistent with the previous argument that maximal specific

Table 1. *Relationship between turnover time, isoelectric point, and molecular weight for five plant enzymes*
All half-lives were estimated after labelling with ^2H_2O and isopycnic centrifugation (see the Experimental section for details). Isoelectric points and molecular weights were determined for same tissue under same growth regime except that water replaced the ^2H_2O. Column isoelectric focusing was performed as described in the legend to Fig. 3. Details of flat-bed focusing of ribonuclease are given in the Experimental section. Molecular weights (multimeric form) were determined by gel-filtration chromatography on Sephadex G-100 for partially-purified ribonuclease (Acton *et al.*, 1970) or on Ultrogel AcA 34 for partially purified extracts. A column (440mm × 18mm) of Ultrogel was equilibrated in 100mM-Tris buffer adjusted to pH 8.6 (at 25°C) with 6M-HCl for L-phenylalanine ammonia-lyase or in 100mM-potassium dihydrogen phosphate/dipotassium hydrogen phosphate, pH 7.4 at 25°C, containing 1mM-EDTA and 1mM-cysteine for other enzymes. Extracts and marker proteins were loaded separately and eluted with the same buffer. Blue Dextran and Phenol Red were loaded in addition to determine the void volume (V_0) and total accessible space (V_t) respectively (see Gupta & Acton, 1979). Only one peak was obtained for all enzymes, except ribonuclease (see Acton *et al.*, 1970). For L-phenylalanine ammonia-lyase, enzyme purified down to a single homogeneous protein was used and the mol.wt. value (250000) was a mean of determinations from rate-zonal centrifugation, electrophoresis in a polyacrylamide gradient gel and chromatography on Ultrogel. Subunit composition was taken from the literature as follows: nitrate reductase, two large cytochrome *c* reductase subunits (4S) plus a small molybdenum-containing binding protein (see Wallace & Johnson, 1978); L-phenylalanine ammonia-lyase, tetrameric (Gupta & Acton, 1979); ascorbate oxidase, dimeric (Strothkamp & Dawson, 1974); acid phosphatase, dimeric (Kubicz, 1973); acid ribonuclease monomeric (see Wilson, 1978). Correlation coefficients (r) were determined for the linear function of isoelectric point and the logarithmic function of size. Correlations are shown in part (*b*) of the Table.

Parameter ...	$t_{\frac{1}{2}}$ (h)	pI	Multimer size (10^{-4} × mol.wt.)	Subunit size (10^{-4} × mol.wt.)
(*a*) Enzyme				
Nitrate reductase*	2.6	4.6	21.5	10.3
L-Phenylalanine ammonia-lyase*	3.3	5.6	25.0	6.3
Ascorbate oxidase*	30	8.3	15.0	7.5
Acid phosphatase*	36	6.5	12.5	6.2
Acid ribonuclease†	200	8.5	2.5	2.5
(*b*) Correlation tested				
$t_{\frac{1}{2}}$ versus pI	$r = 0.699$ ($P > 0.10$)			
$t_{\frac{1}{2}}$ versus log(multimer size)	$r = -0.991$‡ ($P < 0.01$)			
$t_{\frac{1}{2}}$ versus log(subunit size)	$r = -0.939$‡ ($P < 0.02$)			

* From etiolated mustard cotyledons.
† From etiolated lupin hypocotyls.
‡ Significant.

activity of the amino acid pool need not be reached instantly. We argue that even when the speed of amino acid pool turnover is faster than enzyme turnover (i.e., band-broadening is obtained), the 'dead time' for the uptake and incorporation of ^2H into cellular metabolites could introduce a situation in which maximal specific activity is not reached within one amino acid-pool lifetime. The possibility that amino acid-pool turnover is slower than enzyme turnover is further excluded for enzymes in etiolated mustard seedlings by the data of Johnson & Smith (1978). In no instance did density shifts reach the 0.010kg/litre value observed for amino acid-pool ^2H-labelling by these authors within one measured enzyme half-life [the above results and those in Acton *et al.* (1974) and Acton & Schopfer (1975)].

The introduction of error into turnover estimates by isotopic stress is likely to be small. Amounts of assayable nitrate reductase and acid phosphatase were essentially unchanged by 70 atom % ^2H_2O (G. J. Acton & S. Gupta, unpublished work). Lyase activity was stimulated by 20% by 80 atom % ^2H_2O (which may reflect a wounding response) (Acton & Schopfer, 1975), and ascorbate oxidase was inhibited by up to 27% at this isotope concentration (Acton *et al.*, 1974), but this only occurred for a short time over the second phase of the time course for labelling. On the other hand, 100 atom % ^2H_2O prevented a late rise in ribonuclease activity (Acton & Schopfer, 1974), so that no effective estimate of this effect of isotopic stress is possible.

Isoelectric point and half-life

Isoelectric points of pH 4.6, 5.6, 8.3 and 6.5 were determined for nitrate reductase, L-phenylalanine ammonia-lyase, ascorbate oxidase and acid phosphatase respectively under (except nitrate reductase) non-adaptive conditions (Fig. 3). Acid ribonuclease gave a major peak at pH 8.5, with a minor peak in the region of pH 7.0 (results not shown). A preliminary report of an isoelectric point of pH 7.4 was incorrect (Acton & Gupta, 1978). Weak correlation was observed between degradative rate and isoelectric point ($r = 0.699$; Table 1). If ascorbate oxidase was

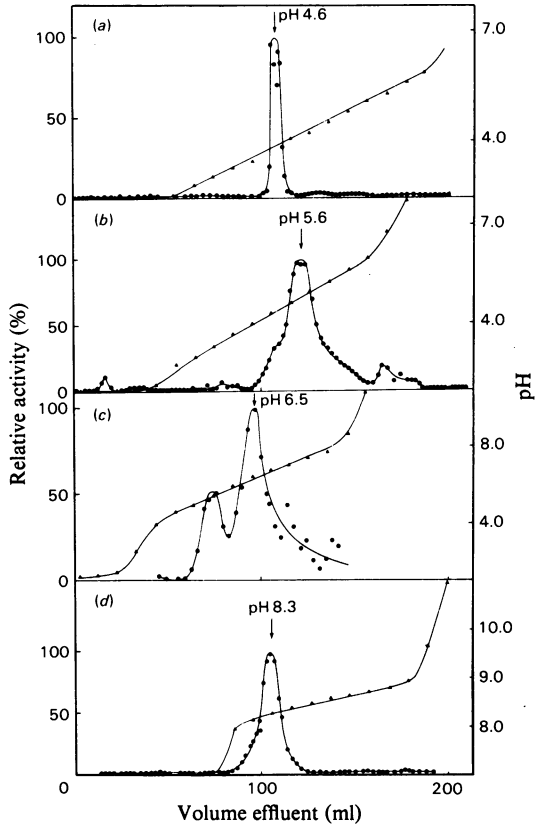

Fig. 3. *Column isoelectric focusing of four enzymes extracted from etiolated mustard cotyledons*

Partially purified extract (8–25mg of protein) was loaded into the column at a position corresponding to 0.69mM-sucrose and focused for 9–12h (1500V, 3mA final settings) at 3°C, and 0.5ml fractions collected. The anode is on the left of each panel. In (*a*), nitrate reductase was focused in 10mM-potassium dihydrogen phosphate/10mM-dipotassium hydrogen phosphate, pH7.4 at 25°C, containing 1.0mM-EDTA and 1.0mM-cysteine, in a pH4–6 gradient. In (*b*) L-phenylalanine ammonia-lyase was focused in 5mM-Tris, adjusted to pH8.6 at 25°C with 6mM-HCl, in a pH4–6 gradient. In (*c*), acid phosphatase was focused in 10mM-dipotassium hydrogen phosphate, adjusted to pH5.0 at 25°C with 5mM-citric acid and containing 1.0mM-cysteine, in a pH3–10 gradient. In (*d*), ascorbate oxidase was focused as in (*c*), but a pH8–10 gradient was used. For (*a*), (*b*) and (*d*), preliminary focusing experiments were carried out in pH3–10 gradients to establish approximate isoelectric points. Columns were coated, and buffers contained glycine and Triton X-100 (see the Experimental section) to combat interference of bands during unloading by precipitated proteins. Relative activity means that points are expressed as a percentage of maximum activity. ▲, Ampholine (pH) gradient; ● enzyme-activity profile.

omitted, linear-regression analysis on the remaining four point shows a stronger (and significant) correlation ($r = 0.938$; $P < 0.10$). Possible explanations for the relatively short half-life of ascorbate oxidase in relation to ionic charge would include the proposal of a high carbohydrate content (Stark & Dawson, 1962). Glycoproteins are degraded more rapidly than non-glycoproteins in other tissues (Dice *et al.*, 1978). We conclude therefore that either the lack of a highly significant correlation between half-life and charge (cf. Dice & Goldberg, 1975*b*) reflects the small sample size available, or that higher-plant proteins show a weaker correlation than do those of other eukaryotes.

Molecular weight and half-life

Molecular weights were already known (Acton *et al.*, 1970; Gupta & Acton, 1979) or were determined by gel filtration (see the legend to Table 1). Similar, highly significant, correlations existed between degradation rate and the logarithm of multimer size ($r = -0.991$; $P < 0.01$) and degradation rate and the logarithm of subunit size ($r = -0.939$; $P < 0.02$) (Table 1). When size is expressed arithmetically, these correlations are decreased, but remain significant (multimer: $r = -0.908$; $P < 0.05$; subunit: $r = -0.847$; $P < 0.10$). The above data show an unusually high correlation between half-life and size (cf. Dice & Goldberg, 1975*a*), but provide no evidence that subunit size (as opposed to multimer size) preferentially influences the rate of degradation (cf. Dehlinger & Schimke, 1970; Glass & Doyle, 1972; Dice *et al.*, 1973). Examination of partial correlation coefficients shows that when the contribution of ionic charge was held constant, a good correlation remained ($r = -0.887$) between the logarithm of subunit size and half-life. However, when the contributions of either the logarithm of subunit size or degradation rate were held constant, weak relationships were observed for the remaining factors ($r = 0.263$ when size held constant; $r = -0.068$ when half-life held constant). Thus size and ionic charge appear as independent factors influencing protein breakdown. A more conclusive demonstration of this is provided by Dice & Goldberg (1975*b*).

Literature survey

Published values for four of the enzymes under study, selected and treated as described in the legend to Table 2, showed essentially the same trends (cf. Table 1 with Table 2). A slightly improved (but not significant) correlation existed between turnover rate and isoelectric point ($r = 0.857$; $P > 0.10$). On the other hand, slightly weaker relationships existed between the logarithm of multimer and of subunit size and half-life ($r = -0.961$; $P < 0.05$ for multimer; $r = -0.929$; $P < 0.10$ for subunit). Thus the unexpected

Table 2. *Literature survey of half-lives, isoelectric points and molecular weights of four plant enzymes*

Criteria for inclusion of data are as follows. The survey is limited to higher plants. Data are only taken when both the isoelectric point and size of a protein have been reliably reported for a single plant. Subunit means the smallest particle size consistently reported. For nitrate reductase this was determined on the basis of a trimeric structure (8.5S) of two large equal cytochrome *c* reductase subunits (4S) (taken as the subunit) and molybdenum-containing protein (see Wallace & Johnson, 1978). L-Phenylalanine ammonia-lyase was taken as tetrameric [similar sized subunits, Havir & Hanson (1973)], high-molecular-weight ascorbate oxidase as dimeric [equally sized subunits, Strothkamp & Dawson (1974)] and low-molecular-weight enzyme as monomeric. Likewise, high-molecular-weight acid phosphatase was taken as dimeric [equally sized subunits, Kubicz (1973)] and low-molecular-weight forms as monomeric (Kubicz, 1973). Data on probable aggregates of the multimeric forms are not included. Ribonuclease has been omitted because of the wide range of estimates of half-life and size coupled with the extreme paucity of electrofocusing data. To overcome paucity of data on degradation rates, average values have been calculated from data from all known sources and correlation coefficients (r) determined for the linear function of the average of the isoelectric points and the logarithmic function of average of size against this single half-life value. In the case of nitrate reductase no difference existed in averaged half-lives from inducing conditions [$t_{\frac{1}{2}} = 5.6 \pm 1.71$ (S.E.M.) h (6)] and in the absence of nitrate [$t_{\frac{1}{2}} = 4.13 \pm 0.53$ h (8)]; thus all available data were pooled. In addition, the bulk of these data are derived from use of inhibitors of protein synthesis or enzyme assembly, which is likely to bias the value in the direction of an overestimation of the half-life (an underestimation of the turnover rate) (see the footnotes).

Parameter ... $t_{\frac{1}{2}}$ (h)	pI	Multimer size (10^{-4} × mol.wt.)	Subunit size (10^{-4} × mol.wt.)
(*a*) Enzyme and source			
(1) Nitrate reductase:			
Mustard (*Sinapis alba*) (the present study)	4.6	21.5	10.3
Spinach (*Spinacea oleracea*) (Notton *et al.*, 1972, 1977; Graf *et al.*, 1975)	3.5, 4.9	25.0, 24.0, 19.7	12.1, 11.5, 9.4
Maize (*Zea mays*) (Wells & Hageman, 1974; Hageman & Hucklesby, 1971; Roustan *et al.*, 1974)	4.7	27.0, 16.0	13.1, 7.5
2.6† (the present study)			
4.2, 3.5 (Schrader *et al.*, 1968)			
4.0, 6.0,6.0,8.0 (Aslam & Oaks, 1976)			
1.5 (Radin, 1974)			
3.7,6.5 (Heimer & Filner, 1971)§			
4.3† (Zielke & Filner, 1971)			
3.0,2.0 (Oaks *et al.*, 1972)			
12.4 (Travis *et al.*, 1969)			
(2) L-Phenylalanine ammonia-lyase:			
Mustard (the present study; Gupta & Acton, 1979; Schopfer, 1971)	5.6	25.0, 30.0	6.3, 7.5
Radish (*Raphanus sativus*) (Blondel *et al.*, 1973)	4.9	30.0	7.5
Maize (Havir *et al.*, 1971; Hanson & Havir, 1972)	5.1*	30.8	7.7
3.0† (Acton & Schopfer, 1975)			
4.0† (Tong & Schopfer, 1976)			
3.5† (Johnson & Smith, 1978)			
5.0† (Wellman & Schopfer, 1975)			
7.5*† (Betz *et al.*, 1978)			
28*† (Duchesne *et al.*, 1977)			

(3) Ascorbate oxidase:			
Mustard (the present study)	8.3	15.0	7.5
Squash (*Cucurbita pepo*) (Amon & Markakis, 1973; Dunn & Dawson, 1951; Lee & Dawson, 1973; Marchesini *et al.*, 1977)	5.3, 5.3*	3.5, 14.0, 14.0	3.5, 7.0, 7.0
Cucumber (*Cucumis sativus*) (Wills, 1952; Nakamura *et al.*, 1968; Amon & Markakis, 1973)	5.1, 6.9*, 6.7	13.2, 3.0	6.6, 3.0
	30† (Acton *et al.*, 1974)		
(4) Acid phosphatase:			
Mustard (the present study)	5.5, 6.5	12.5	6.2
Sweet potato (*Ipomoea batatas*) (Uehara *et al.*, 1974)	5.0	11.0	5.5
Potato (*Solanum tuberosum*) (Kubicz, 1973; Kubicz *et al.*, 1974; Lucas & Pitt, 1975)	6.0*	9.6, 4.8, 2.4	4.6, 4.8, 2.4
	36† (Acton *et al.*, 1974)		
(*b*) Correlation tested			
$t_{\frac{1}{2}}$ versus pI	$r = 0.857$ ($P > 0.10$)		
$t_{\frac{1}{2}}$ versus log (multimer size)	$r = -0.961$‡ ($P < 0.05$)		
$t_{\frac{1}{2}}$ versus log (subunit size)	$r = -0.929$‡ ($P < 0.10$)		

* Average of range given.
† Determined by labelling.
‡ Significant.
§ Interpreted by Zielke & Filner (1971).

bias towards size, as opposed to ionic charge, in determining susceptibility to degradation *in vivo* is not entirely removed by a larger amount of data (all averaged) within the limited group size (four or five enzymes) tested in Table 1.

Essential constraints on the present study include deficiencies in amounts of half-life data available (Table 2) and the undesirability of limiting analyses to five (or four) proteins and of determining half-lives under conditions of isotopic stress. On the other hand, the strength of the present study lies in the requirement to determine all three factors (degradation rate, size and ionic charge) in the same tissue under similar conditions and in the same laboratory (Table 1). Thus on the basis of the assumption made throughout the present paper, namely that enzyme inactivation is the rate-limiting step in degradation (Katunuma, 1977), we confirm the general trends elucidated in plant (Dice *et al.*, 1973; Naya *et al.*, 1977) and in other tissues (Dice & Goldberg, 1975*a*,*b*) by using an alternative technique. On the basis of this reasoning, the cross check provided by the present study adds weight to the evidence accumulating that criticisms levelled at the double-isotope technique procedure may be without foundation.

An alternative explanation, however, presents itself. This is that the consistent result obtained by the double-isotope and the density-labelling procedures is fortuitous, and that half-life estimates from the activity-loss measurements are too short. This could arise if pools of a relatively stable inactive form of an enzyme existed in a situation in which both the active and inactive forms were degraded at different rates and by different processes (Johnson, 1977). All that can be said at present is that such a proposal in the context of the mustard seedling appears to be speculative. This is because evidence for the existence of reversible-inactivation processes involving relatively stable unassayable forms of nitrate reductase and L-phenylalanine ammonia-lyase (Johnson, 1977) could not be confirmed in other laboratories (Acton & Starr, 1978; Acton & Schopfer, 1975; R. Starr & G. J. Acton, unpublished work; Tong & Schopfer, 1976).

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