The Separation of Proteins Based on Their Age, for the Study of Protein Degradation in *Escherichia coli*

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Density labelling with $^2$H$_2$O has been used in association with isopycnic centrifugation to isolate proteins of known age from cultures of *Escherichia coli*. The physical properties of protein samples of known age have been examined to detect a correlation between specific properties and susceptibility to degradation. No evidence of a correlation between size, charge, thermodynamic properties or amino acid composition, on one hand, and susceptibility to degradation, on the other hand, was observed following step-down conditions of growth. However, the SH content of proteins in *E. coli* does appear to be correlated with their susceptibility to degradation.

**INTRODUCTION**

Protein degradation in exponentially growing *Escherichia coli* is relatively slow, probably less than 2% h$^{-1}$ (Mandelstam, 1957), and may be restricted to a small number of proteins variously estimated as 47 out of 188 examined (Mosteller *et al*., 1980) and 3 or possibly less out of 250 examined (Larrabee *et al*., 1980; Calandruccio & Larrabee, 1981). However, when growth rate is reduced by shortage of nutrients, protein degradation is enhanced (Mandelstam, 1957, 1960; Pine, 1972). The degradation is selective in the sense that some proteins are degraded more rapidly than others (Willetts, 1967) and soluble proteins are more labile than ribosomal or membrane proteins (St John *et al*., 1979). A number of studies (reviewed by Goldberg & St John, 1976) have suggested that the susceptibility to proteolytic attack is determined by the physical properties of the proteins rather than by the specificity of the proteolytic system. Thus the half-life of a protein is genetically determined and specified in the amino acid sequence, which prescribes the structural properties recognized by the proteolytic system.

Most of the evidence supporting this general hypothesis has been obtained with mammalian systems but its applicability to bacteria has been argued by Goldberg & Dice (1974) and Goldberg & St John (1976). Most of the evidence has been in the form of correlations between the *in vivo* rates of protein degradation determined by the double isotope method of Arias *et al.* (1969) and particular physical properties which enable proteins to be fractionated, e.g. size or charge. Physical properties which cannot be used as a basis for protein fractionation cannot be studied by this method. This technological restriction is philosophically unacceptable, and a better approach would be to separate proteins on the basis of their age. It would then be possible to see if change in any specific property was correlated with age. Thus, if the SH content of proteins is correlated with their rate of degradation, a population of 'aged' proteins should be poor in SH groups compared with a population of newly synthesized proteins, because SH-rich proteins will rapidly disappear.

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When cells are grown for a short time in $^2$H$_2$O, they form 'heavy' proteins which can be separated from 'light' proteins formed before and after the pulse of deuterated water (Boudet et al., 1975). This paper describes the application of this method to the isolation of proteins of known age from a growing population of E. coli and reports the results of experiments designed to test possible correlations between rates of protein degradation and the physical properties of the proteins.

METHODS

Organism and culture conditions. Escherichia coli strain BB, kindly provided by Dr C. Clarke, was grown in shake culture at 37 °C in the basal liquid medium of Weismeyer & Cohn (1960). Cultures (80 ml) were grown in conical flasks (250 ml) and when the optical density of the culture, measured at 550 nm, exceeded 1.0 ($4.4 \times 10^8$ cells ml$^{-1}$), air was bubbled through the medium. Under full nutrient conditions, growth was exponential with a doubling time of 48.5 min and cells contained 10.5 pg RNA and 103 pg protein (mg wet wt)$^{-1}$ (equivalent to $3.4 \times 10^8$ cells).

Growth in $^2$H$_2$O. Cells from a culture in exponential growth were harvested by centrifugation (18000 g for 5 min) and resuspended in a basal medium containing 92.5% (w/w) $^2$H$_2$O to give an optical density at 550 nm of 0.6. Air was bubbled through the cultures, which were maintained at 37 °C for 5 h, before the cells, which had increased eightfold in number, were collected by centrifugation.

Growth under nitrogen limitation. Cells which had been grown in 92.5% $^2$H$_2$O for 5 h were harvested and resuspended in a nitrogen-free medium to give a fourfold dilution of cell number density. The nitrogen-free medium was prepared by replacing the (NH$_4$)$_2$SO$_4$ (2 g l$^{-1}$) of the basal medium with KH$_2$PO$_4$ (2 g l$^{-1}$). A growth rate of about 1 division per 35 h was obtained by pumping a solution of (NH$_4$)$_2$SO$_4$ (2 mg ml$^{-1}$) at a rate of 0.5 ml h$^{-1}$ into the aerated culture maintained at 37 °C. After 30 h the culture was diluted with an equal volume of nitrogen-free medium.

Extraction of soluble proteins. Cells were collected by centrifugation (18000 g for 5 min at 2 °C), resuspended in ice-cold water and repelleted. The pellets were frozen at −70 °C, then 300–500 mg (wet wt) were ground in a chilled mortar with alumina and extracted with 4 ml Tris/HCl buffer (10 mM, pH 7.5) containing MgCl$_2$ (10 mM), NH$_4$Cl (60 mM) and mercaptoethanol (6 mM). The extracts were incubated with deoxyribonuclease I (EC 3.1.21.1) (15 μg ml$^{-1}$) for 15 min at room temperature, and alumina and large cell fragments were then removed by centrifuging at 10000 g for 5 min at 2 °C. Ribosomes were largely removed from the supernatant by centrifuging at 15000 g for 15 min at 2 °C and the remaining ribosomes were eliminated by layering 4 ml extract on 2 ml of a solution containing 0.3 M sucrose in the extraction buffer and centrifuging in the Spincro 65 rotor at 200000 g for 60 min at 2 °C. After the run, the upper 5 ml of the supernatant was collected and passed through a column (1.2 × 25 cm) of Sephadex G-100, previously equilibrated with potassium phosphate buffer (pH 7.5, 50 mM) containing mercaptoethanol (6 mM). After the void volume, fractions containing molecules larger than about 12000 mol. wt were pooled and brought to 65% saturation with (NH$_4$)$_2$SO$_4$. The precipitate collected by centrifugation (13000 g for 20 min at 2 °C) was dissolved in the potassium phosphate buffer and desalted by passage through a column (1 × 10 cm) of Sephadex G-25, previously equilibrated with the same buffer. The desalted fractions were used for isopycnic centrifugation.

Isopycnic centrifugation. All centrifugation was done in a Beckman Spincro L2-65B preparative ultracentrifuge. Two types of KBr gradients were used. For protein samples up to 1 mg, the gradient described by Boudet et al. (1975) was used and the type 65 rotor spun at 45000 r.p.m. for 24 h at 15 °C. For protein samples between 1 and 6 mg, the protein, dissolved in 7.5 ml of a 66-6% saturated solution of KBr, was gently layered on 12.5 ml of a saturated KBr solution in polyallomer tubes for the type 70 Ti rotor, which were then filled with paraffin oil. All KBr solutions were in potassium phosphate buffer (pH 7.0-7.5, 50 mM) containing mercaptoethanol (6 mM). Samples were centrifuged at 49000 r.p.m. for 24 h at 15 °C. Fractions were then collected by inserting a fine tube to the bottom of the centrifuge tube and pumping out the solution. Fractions of 1.19 ml were collected, of which 0.065 ml was used to determine the refractive index, 0.125 ml was used for the assay of radioactivity and 1 ml was stored for the examination of physical properties. The refractive indices measured with an Abbé refractometer were converted to buoyant densities by the equation:

$$Q^{25} = (6.4786 \times \text{refractive index at } 25^\circ C) - 7.6431$$

Radioactivity was measured in an Intertechnique SL30 liquid scintillation spectrometer as described by Boudet et al. (1975). The fractions with buoyant densities between 1.3550 and 1.3265 kg l$^{-1}$ were pooled (three tubes gave about 15 ml) and desalted by passage through a column (1.5 × 40 cm) of Sephadex G-25 previously equilibrated with potassium phosphate buffer (pH 7.5, 50 mM).

Amino acid analysis. Protein samples (0.5–0.8 mg) were precipitated with TCA (final concentration 10%, w/v), and after centrifugation the pellet was washed once with 3 ml 10% TCA and twice with 3 ml ethanol/ether (1:1,
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The pellet was hydrolysed by heating at 110 °C in 1 ml 6 M-HCl for 20 h. Alternatively, to preserve tryptophan, hydrolysis was done with 1 ml 3 M-mercaptoethanesulphonic acid (Penke et al., 1974). The amino acids were separated with a Beckman amino acid analyser.

Polyacrylamide gel electrophoresis. Desalted samples of 14C-labelled proteins (0.5 mg) were lyophilized, then dissolved in 0.5 ml water and dialysed for 2 h against 200 ml sodium phosphate buffer (pH 7-2, 10 mM). SDS and mercaptoethanol were added to give 1% (w/v) final concentration of each and the solution was incubated at 100 °C for 2 min. Aliquots containing 100 μg protein were applied to 5% (w/v) polyacrylamide gels and electrophoresis was performed according to the method of Weber et al. (1972). At the end of the run, gels were sliced and the radioactivity was determined according to Aloyo (1979).

Thermal denaturation. This was measured at 280 nm in a Cary 14 spectrophotometer, equipped with a thermostatted cell holder and an electronic thermometer.

Guanidine denaturation. This was measured in a Hitachi-Perkin Elmer spectrofluorimeter, using 287 nm for excitation and 335 nm for analysis.

Ion-exchange chromatography. Protein samples (0.5 mg) obtained by isopycnic centrifugation were desalted and lyophilized, then dissolved in water and dialysed against 200 ml Tris/HCl buffer (pH 7.5, 10 mM) for 2 h. The samples were applied to columns (1 × 5 cm) of DE-52 cellulose previously equilibrated with the same buffer and eluted with a step-wise gradient of NaCl (0, 0.1, 0.2, 0.3, 0.5 and 1.0 M) in the same eluting buffer. Forty 0.8 ml fractions were collected and the radioactivity in each fraction was measured by scintillation counting.

Analytical methods. RNA was determined as described by Neiman & Paulsen (1963), protein by Lowry’s method and SH groups by the method of Steinert et al. (1974).

Chemicals. [1-14C]Leucine and N-ethyl[2,3-14C]maleimide were obtained from Amersham, Sephadex from Pharmacia and DE-52 cellulose from Whatman. All chemicals were of the best reagent grade.

Results

Toxicity of deuterium oxide

The isolation of deuterated proteins by isopycnic centrifugation calls for the maximum incorporation of deuterium, but since 2H2O is relatively toxic, a compromise concentration must be used. The rate of growth on 92.5% 2H2O was 50% of that on H2O but was adequate for the present investigation.

Isolation of proteins of known age

Cells from a culture in exponential growth were collected by centrifugation and resuspended in a complete growth medium containing 92.5% 2H2O. The cells were incubated and aerated at 37 °C for 5 h and, where required, [1-14C]leucine was added in the last 30 min. The cells were harvested, a portion was frozen with liquid nitrogen, and the protein was extracted and subjected to isopycnic centrifugation. The remaining cells were resuspended and diluted fourfold in a nitrogen-free medium, before being incubated in an aerated culture at 37 °C. A solution of (NH₄)₂SO₄ (2 mg ml⁻¹) was pumped in at 0.5 ml h⁻¹ to maintain a growth rate of 1 division per 35 h. Samples were removed at 30 h and 60 h, and protein was extracted and fractionated by isopycnic centrifugation. Data on the growth under nitrogen limitation are given in Table 1 and the results of isopycnic centrifugation in Fig. 1. Fractions from each gradient with

<table>
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<th>Time (h)</th>
<th>OD₅₅₀</th>
<th>Protein (mg per 100 ml)</th>
<th>RNA (mg per 100 ml)</th>
<th>Half-life for protein degradation* (h)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.3</td>
<td>17.5</td>
<td>7.05</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>17.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>30</td>
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<td>26</td>
<td>6.71</td>
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</tr>
<tr>
<td>60</td>
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<td>43</td>
<td>9.65</td>
<td>59.2</td>
</tr>
</tbody>
</table>

* Determined by the method of Boudet et al. (1975).
Fig. 1. Isopycnic centrifugation of density-labelled proteins from *E. coli*. Cells were grown for 4.5 h in a medium containing 92.5% $^{2}$H$_2$O. [L-14C]Leucine [3 nmol (0-18 μCi) ml$^{-1}$] was added and, after a further 0.5 h, cells were harvested and transferred to a H$_2$O medium lacking nitrogen. (NH$_4$)$_2$SO$_4$ was supplied at the rate of 1 mg h$^{-1}$ and, after 30 h and 60 h, cells were harvested, and protein was extracted and separated by isopycnic centrifugation: aliquots of 6 mg protein were analysed in each KBr gradient (70 Ti type rotor). O. Cells grown for 5 h in 92.5% $^{2}$H$_2$O (total radioactivity in gradient 2220000 c.p.m.); A, cells subsequently grown for 30 h in a nitrogen-deficient medium (1134000 c.p.m.); ●, cells subsequently grown for 60 h in a nitrogen-deficient medium (708000 c.p.m.).

Buoyant densities between 1.3265 and 1.3550 kg 1$^{-1}$ were pooled to give protein samples of age 0, 30 and 60 h, designated $t_0$, $t_{30}$ and $t_{60}$, respectively.

**Purity of protein samples**

Since three protein samples of different ages were to be compared, it was important to know the 'age' purity of the samples. The sample at $t_0$ consisted of proteins formed in the 5 h period on $^2$H$_2$O, but may have been contaminated with 'older' proteins. The $t_{30}$ and $t_{60}$ samples consisted of proteins made in the 5 h period on $^2$H$_2$O, but may have been contaminated with 'younger' proteins made in the 30 or 60 h of subsequent growth, as well as by some 'older' protein.

To measure this contamination, cells were grown (A) in complete medium, (B) in complete medium with 92.5% $^2$H$_2$O and (C) in the nitrogen-deficient medium after growth in 92.5% $^2$H$_2$O. The cultures were supplied with [L-14C]leucine (59 μCi μm$^{-1}$; 2-18 MBq μm$^{-1}$) and, after 30 min, protein was isolated and the distribution of the isotope in the density gradient obtained by isopycnic centrifugation was examined (Fig. 2). The contamination of a sample by 'old' protein was determined by comparing the fraction of protein with density greater than 1.3265 kg 1$^{-1}$ in cells grown in complete medium (Fig. 2, A) with the corresponding fraction in cells grown in complete medium containing $^2$H$_2$O (Fig. 2, B), corrected for growth in $^2$H$_2$O. The contamination by 'new' protein was determined by comparing the fraction of protein with density greater than 1.3265 kg 1$^{-1}$ in cells grown in nitrogen-limiting conditions (Fig. 2, C), corrected for growth (Table 1), with the corresponding fraction in cells grown in $^2$H$_2$O (Fig. 2, B), corrected for degradation (Fig. 2, B; Fig. 1). These calculations give the contamination of the samples as follows: $t_0$ was contaminated by about 1% of 'old' protein; $t_{30}$ was contaminated by
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Fig. 3. Size distribution of protein sub-units in samples of differing ages. Proteins labelled with \( ^{3}H \) and \( ^{14}C \) were isolated from E. coli at 0, 30 and 60 h after synthesis. After SDS-polyacrylamide gel electrophoresis, gels were sliced and counted by scintillation. A 100 \( \mu \)g aliquot of protein from each sample was analysed, and the total radioactivity present in each gel was: \( t_{0} \), 22900 c.p.m.; \( t_{30} \), 15300 c.p.m.; \( t_{60} \), 11200 c.p.m. The data are presented as a cumulative percentage of total radioactivity (open symbols) and also by comparing the mean of the radioactivity in a slice and its two adjacent slices (expressed as % of total radioactivity) for the \( t_{0} \) sample with the corresponding value for the \( t_{30} \) and \( t_{60} \) samples (filled symbols). ○, Sample at \( t_{0} \); △, sample at \( t_{30} \); □, sample at \( t_{60} \); A, \( \ln \left( \frac{\text{Radioactivity at } t_{0}}{\text{Radioactivity at } t_{30}} \right) \); B, \( \ln \left( \frac{\text{Radioactivity at } t_{0}}{\text{Radioactivity at } t_{60}} \right) \). Arrow A, serum albumin (mol. wt 63000); arrow B, bromophenol blue.

about 1% of 'old' protein and 8% of 'new' protein; \( t_{60} \) was contaminated by about 1% of 'old' protein and 20% of 'new' protein.

Protein sub-unit size in relation to age

The distribution of sub-unit size in the population of proteins in each of the three \( ^{14}C \)-labelled samples was examined by SDS-polyacrylamide gel electrophoresis. Although the size distribution was highly reproducible within the gels, no simple relationship between sub-unit size and age was apparent (Fig. 3).

Thermal stability in relation to age

The protein samples were mixed with potassium phosphate buffer (pH 6.8, 0.15 M) to give an optical density of 0.1 at 280 nm. The increase in optical density at 280 nm was recorded while the samples were heated in a Cary 14 spectrophotometer equipped with a thermostatted cell holder and an electronic thermometer. The increase in optical density due to protein precipitation and consequent light scattering is shown in Fig. 4(a). There were no significant differences between the \( t_{0} \) and \( t_{30} \) samples, but there was some indication that the \( t_{60} \) sample was more heat stable. However, there were no significant differences between the samples when the intrinsic fluorescence of the proteins was measured at 335 nm with excitation at 287 nm in the presence of varying amounts of guanidine hydrochloride (Fig. 4b).

Protein charge in relation to age

The three protein samples, labelled with \( [L-^{14}C] \)leucine at \( t_{0} \), were placed on columns of DE-52 cellulose and eluted stepwise with increasing concentrations of NaCl. The proteins eluting at each concentration of NaCl were collected and the radioactivity was determined by scintillation
Fig. 4. Denaturation curves of protein samples of known age. (a) Thermal denaturation (measured by light scattering); (b) guanidine denaturation (measured by intrinsic fluorescence). For details see text. ○, Protein immediately after synthesis; ▲, protein synthesized 30 h earlier; ●, protein synthesized 60 h earlier.

Fig. 5. Ion-exchange chromatography of soluble proteins of known age. For details see text. Aliquots of 300 µg protein were analysed. □, Protein immediately after synthesis (total radioactivity in sample 61 000 c.p.m.); ■, protein synthesized 30 h earlier (37 500 c.p.m.); □, protein synthesized 60 h earlier (28 700 c.p.m.).

counting. The elution profiles of the three samples, compared in Fig. 5, show no simple correlation between age and charge.

Amino acid composition in relation to age

The three samples were hydrolysed in 6 M-HCl and another set was hydrolysed in 3 M-mercaptoethanesulphonic acid to preserve tryptophan. The results of the amino acid analyses are shown in Table 2.

Protein SH groups in relation to age

Samples of protein (25 µg) were incubated at 37 °C with 1 ml potassium phosphate buffer (pH 7.5, 50 mM) containing N-ethyl[2,3-14C]maleimide [25 nmol ml⁻¹, 10 µCi µmol⁻¹ (370 kBq µmol⁻¹)]. The reaction was stopped with an excess of mercaptoethanol (6 µmol) and the samples were filtered on cellulose membranes (Millipore HAMK 02412, 0.45 µm pore size).
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Table 2. Amino acid composition of soluble proteins from E. coli

The data are expressed as molar percentages of the total. HCl denotes hydrolysis in 6 M-HCl; MESA denotes hydrolysis in 3 M-mercaptoethanesulphonic acid.

<table>
<thead>
<tr>
<th>Sample age*</th>
<th>(t_0)</th>
<th>(t_{30})</th>
<th>(t_{60})</th>
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<tbody>
<tr>
<td></td>
<td>HCl</td>
<td>MESA</td>
<td>HCl</td>
</tr>
<tr>
<td>Asp</td>
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</tr>
<tr>
<td>Cysteic acid</td>
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</table>

Percentage of hydrophobic residues: 46.1 44.8 47.6 44.6 47.4 43.1

* The subscript number indicates hours from time of synthesis.
† Standard deviation for three samples.
‡ Hydrophobic amino acids.

Fig. 6. Titration of SH groups of soluble protein from E. coli. Protein samples (25 \(\mu\)g ml\(^{-1}\)) aged 0, 30 and 60 h were titrated with \(N\)-ethyl[2,3-\(^{14}\)C]maleimide (25 pmol) at 37 °C and the reaction was stopped by an excess of mercaptoethanol. O, \(t_0\) samples; \(\triangle\), samples 30 h after synthesis; \(\square\), samples 60 h after synthesis.

The samples were washed 10 times in Tris/HCl buffer (pH 7.8, 0.1 M), dried, and then counted for radioactivity. The \(N\)-ethylmaleimide concentration represented a 20- to 40-fold excess and the reaction was complete after 1 h (Fig. 6). A 50% reduction in the concentration of \(N\)-ethylmaleimide did not produce any change in the number of SH groups per mg protein.

The number of titratable SH groups was calculated from the specific activity of the radioactive \(N\)-ethylmaleimide added. Blank values, which were less than 5% of the samples, were subtracted. Yeast alcohol dehydrogenase was used as a standard; it gave 54 nmol SH per mg protein, which is in agreement with eight SH groups per mol (Steinert et al., 1974). The total...
Table 3. SH content of the soluble proteins from E. coli

The data are expressed as nmol SH (mg protein)⁻¹, ± S.D. for 5 samples.

<table>
<thead>
<tr>
<th>Sample</th>
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<td>Native protein</td>
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<td>27.8</td>
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<td>55.8</td>
<td>50.1</td>
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</table>

number of SH groups was estimated by carrying out the titration in the presence of urea (8 M) and EDTA (30 mM). The results presented in Table 3, which suggest a correlation between age and the SH content of proteins, should be considered in relation to the large increase in cysteic acid (presumably derived from cysteine and cystine) which was detected by amino acid analysis (Table 2).

DISCUSSION

Evidence of heterogeneity in rates of degradation of soluble proteins has been obtained from in vivo measurements of the rates of enzyme degradation (Dice & Goldberg, 1975a), by analyses of the decay curves of labelled proteins (Garlick et al., 1976), by studying catabolite inactivation in micro-organisms (Holzer & Heinrich, 1980), but mostly by the double isotope technique of Arias et al. (1969). The possibility that the heterogeneity of rates of degradation might reflect the heterogeneity of proteolytic enzymes has largely been discounted on the principle of parsimony. Many proteinases are known and some are highly specific, for example, the recA gene product from E. coli promotes proteolytic cleavage of bacteriophage λ repressor (Roberts et al., 1978), but only a few endoproteinases appear to be involved in the intracellular degradation of protein and in the case of yeast only two may be involved (Malano & Gancedo, 1974). However, work with mutant yeasts lacking one or other of the well-characterized proteinases has indicated the presence of up to 20 new endoproteinases (Achstetter et al., 1981; Wolf, 1982), and these findings invite reconsideration of the role of multiple proteases.

Evidence for the alternative view, that heterogeneity of degradation is due to the physical properties of the proteins, has been obtained by using the double isotope method (Arias et al., 1969) to measure the rate of degradation of protein fractions which have been separated on the basis of a particular physical property. The errors and limitations of the double isotope method discussed by Poole (1971), Zak et al. (1977, 1979), Davies (1980) and Russell et al. (1980) may explain discrepancies in reports concerning a possible correlation between sub-unit size and degradation. However, the dilemma is compounded when a modification designed to minimize these errors (NaH¹⁴CO₃ as the first isotope, [³H]arginine as the second) led Dice et al. (1978) to conclude that there is a correlation between protein size and degradation, whereas Russell et al. (1980) concluded that there is no such correlation!

The conflicting reports in the literature constitute a justification for a new approach to the problem of correlations between rates of degradation and various physical properties. We have restricted our study to soluble proteins since ribosomal proteins appear to be degraded as a unit (Tsurugi et al., 1974). The method described in this paper yields protein fractions of known age, but with some contamination by older and younger proteins (2–20%), which may mask some possible correlations. Thus, the reported absence of a correlation should be taken to mean only within the limitations of the method.

The apparent absence of a correlation between sub-unit size and degradation may be due to heterogeneity within each slice of the gel as suggested by Roberts & Yuan (1975) and Tweto & Doyle (1976). However, as pointed out by Wilde et al. (1980), the distribution of polypeptides along the gel reflects the size distribution of protein sub-units and it therefore seems unlikely that the averaging within the slice can account for the lack of correlation. The lack of correlation agrees with the conclusion of Kemshead & Hipkiss (1976) that the size correlation is not valid for E. coli growing exponentially.
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The energy difference between folded and unfolded forms of proteins is low, and such forms are readily interconvertible (McLendon & Smith, 1978). The unfolding equilibrium constant cannot easily be determined but may be related to the rate of heat inactivation of enzymes and to the thermal denaturation curves of enzymes. Hopgood & Ballard (1974) found that the rate of inactivation of seven out of nine enzymes was correlated with their in vivo half-lives and a general correlation was also noted by Bond (1975). Similarly, McLendon & Radany (1978) observed a linear correlation between the thermal unfolding temperature and the in vivo half-lives for eight of the nine enzymes examined. No such correlation was observed in the present study—the hint of a possible correlation observed by thermal denaturation was not confirmed by guanidine denaturation. This negative conclusion is consistent with the reports of Kuehl & Sumsion (1970) and Johnson & Kenny (1973) that heat stability is not related to degradation. However, it should be noted that the techniques for detecting denaturation are relatively crude and may mask possible correlations.

Dice & Goldberg (1975b) and Dice et al. (1979) have noted a correlation between isoelectric point and degradation. We have used chromatography on DEAE-cellulose to separate proteins on the basis of their charge, which is a function of size and isoelectric point. A similar technique was used by Duncan et al. (1980) and Cooke et al. (1980) who concluded that acidic proteins degrade more rapidly than basic proteins; our results show no such relationships for E. coli.

The amino acid composition of proteins is closely related to their physical properties—for example, acidic proteins contain more acidic than basic amino acids (Duncan et al., 1980) and hydrophobic proteins may have excess hydrophobic amino acids, although their position within the protein may be critical. Furthermore, if any amino acid acts as a signal for protein degradation, then it would be expected to be in high concentration in proteins which turn over rapidly. Thus, for example, Robinson & Rudd (1974) have suggested that glutamyl and asparaginyl residues function as the molecular clocks of proteins. The methionine content of proteins may be a signal for degradation in E. coli (Mosteller et al., 1980) and a dramatic change in the lysine content of the proteins of Chlorella fusca occurs when the cells are transferred to a nitrogen-deficient medium (Richards & Thurston, 1980). We used acid hydrolysis for the determination of amino acid composition and consequently cannot comment on the deamidation theory of Robinson (1974), but lysine and methionine do not show significant changes. The possibility that hydrophobicity is related to degradation is suggested by the observation that two proteins with short half-lives are tightly bound by detergent affinity columns, whereas three proteins with long half-lives were hardly retained (Mann & Shah, 1979). Our results show no significant changes in the content of hydrophobic amino acids. However, if the correlation between hydrophobicity and degradation is due to binding to lysosomes, there is no rationale for such a correlation in bacteria!

The most obvious change to be seen in Table 2 is the large age-dependent increase in the cysteic acid content of protein, which is accompanied by a decrease in the SH content (Table 3). These observations suggest that SH-rich proteins are susceptible to proteolysis whilst SS-rich proteins are resistant. This is in accord with the observations of Varandani & Shroyer (1973) and Gustafson & Ryan (1976) that SS-rich proteins are resistant to in vivo degradation and with their conclusion that the number of SH groups may be a principal factor influencing susceptibility of proteins to degradation.

The view that selectivity of protein degradation is highly correlated with single physical properties such as size has been well argued by Goldberg & St John (1976). However, a number of recent reports have indicated the absence of such correlations (Walker et al., 1978; Brunster et al., 1979; Horst & Roberts, 1979; Russell et al., 1980; Wilde et al., 1980). The inclusion of bacteria in the generalization concerning the specificity of protein degradation seems to have been based on the degradation of abnormal proteins. More recently the absence of correlations between protein degradation in growing cultures of E. coli and size or charge of the proteins has been reported (Mosteller et al., 1980). Thus the lack of correlations noted in this paper joins the increasing evidence which suggests that the hypothesis is too simplistic. A complicating factor has been the assertion that size and charge are independent factors influencing protein half-life (Dice & Goldberg, 1975a). The evidence for this view was obtained by comparing data from
different animals and different organisms so that the conclusion must be treated with caution. It appears likely that the selectivity of protein degradation is determined by a number of physical properties and only in special cases will a single property dominate and show a strong correlation with degradation. The data presented in this paper suggest that the SH content of proteins may play a dominant role in determining the half-life of soluble proteins in E. coli.

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REFERENCES


