Protein degradation during terminal cytodifferentiation

Studies on mammary gland in organ culture

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1. In mammary gland explants subjected to experimental manipulation, average rates (during 24 h periods) of degradation of fatty acid synthase, casein and cytosol-fraction proteins were measured by a double-isotope method. Rates of degradation of fatty acid synthase were also computed from measurements of changing enzyme amount and rate of synthesis. 2. During the period of most rapid enzyme accumulation there is a transient decrease in the computed rate of degradation of fatty acid synthase. Removal of hormones produces a rapid increase in the computed rate of degradation of the enzyme. 3. The average rate of degradation of fatty acid synthase measured by the double-isotope method is low in the presence of hormones, and increases on hormone removal. This increase in degradation rate is inhibited by adrenaline and further blocked by insulin. NH_4Cl (10 mM) also partially inhibits the increase in protein degradation on hormone removal. 4. The pattern of changes in the average rate of degradation of cytosol-fraction proteins is similar to that for fatty acid synthase alone. There is no relationship between subunit molecular weight and rate of degradation under all experimental conditions. 5. Isotope ratios for resolved cytosol protein mixtures are transformed logarithmically to make the standard deviations an estimate of heterogeneity of degradation rates. By this analysis, in some conditions there appears to be significant measurable heterogeneity of degradation rates. 6. Little degradation of casein is measured in the presence of hormones, but a marked increase in the rate of degradation can be measured when hormones are removed. Whereas at 24–48 h NH_4Cl (10 mM) has little effect on this enhanced rate of degradation, at 48–72 h it causes a large decrease in degradation rate. 7. Results are discussed in terms of a two-component degradation system in mammary gland explants.

In differentiating cells, the accumulation of a protein may depend not only on an increase in its rate of synthesis, but also on a concurrent decrease in its rate of degradation (Schimke & Doyle, 1970; Philippidis *et al.*, 1972; Every & Ashworth, 1975). Previous work has shown that, during hormonal stimulation of mammary gland explants in organ culture, a sustained increase in the rate of synthesis of fatty acid synthase, coupled with a transient decrease in its rate of degradation, results in a phase of rapid enzyme accumulation (Speake *et al.*, 1975, 1976; Paskin & Mayer, 1977; Mayer & Paskin, 1978).

It is not clear whether this transient change in the rate of degradation of fatty acid synthase reflects a general decrease in protein degradation in the tissue;

measurement of the rate of degradation of mammary-gland cytosolic proteins during hormonally-stimulated protein accumulation gave equivocal results when a single-isotope pulse-chase method was used (Speake *et al.*, 1975, 1976). In the experiments reported here, hormonally-manipulated changes in the rate of degradation of fatty acid synthase and other proteins in the cytosolic fraction are investigated with a double-isotope technique (Dice *et al.*, 1978), which offers a more sensitive method for measuring protein degradation (Zak *et al.*, 1979). This technique is also used to study the degradation of casein, a secretory protein whose rate of synthesis is regulated by hormones, but which is apparently not degraded to any great extent in the tissue (Al-Sarraj *et al.*, 1979). The results are discussed with reference to the heterogeneity of protein degradation rates and putative mechanisms of protein degradation in eukaryotic cells.

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Materials and methods

Materials

Mid-pregnant rabbits were obtained from the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. Adrenaline was from McCarthys Laboratories, Rainford, Essex, U.K. Antibiotics and other hormones were obtained from the sources described by Speake *et al.* (1975). Medium 199 was from Wellcome Research Laboratories, Beckenham, Kent, U.K. L-[4,5-³H]Leucine (sp. radioactivity 113 Ci/mmol), L-[U-¹⁴C]leucine (sp. radioactivity 351 Ci/mol) and L-[1-¹⁴C]glutamic acid (sp. radioactivity 50 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-[2,3-³H]Glutamic acid (sp. radioactivity 22.6 Ci/mmol) was from New England Nuclear, D-6072 Dreieich, W. Germany. The sources of all other chemicals were as described by Paskin & Mayer (1978).

Preparation and culture of mammary explants

Mammary explants were prepared from the lobulo-alveolar tissue of mid-pregnant rabbits by the method of Forsyth & Myers (1971). Groups of 30 explants were cultured at 37°C in O₂/CO₂ (19:1) as described by Speake *et al.* (1975) in the presence of hormones (insulin, 5 µg/ml; prolactin, 1 µg/ml; cortisol, 1 µg/ml). The medium was renewed and gassed every 24 h.

Indirect estimation of rates of protein degradation

Rate constants of degradation for fatty acid synthase were calculated from measurements of rates of synthesis and amount of the protein, together with one estimate of the protein half-life. The rate of synthesis of fatty acid synthase was measured by the rate of incorporation of L-[4,5-³H]leucine into immunodetectable enzyme, and fatty acid synthase activity was assayed as described by Speake *et al.* (1975).

The conditions chosen for radiolabelling and measurement of radioactivity in fatty acid synthase and the method for calculating its rate of degradation are described by Paskin & Mayer (1978).

Estimation of protein degradation by the double-isotope method

Incubation of explants. At 0, 24 and 48 h in culture, groups of 30 explants were incubated at 37°C for 30 min in medium 199 containing either 15 µCi of one of the two labelled species of leucine or 15 µCi of one of the two labelled species of glutamic acid. Each incubation was stopped by the addition, at 4°C, of medium 199 (5 ml) containing 10 mM-leucine (or, where appropriate, 10 mM-glutamate) and the hormone regime to be used in the subsequent culture period. The groups of explants were

then washed at 4°C with 4 × 5 ml of this medium at 5 min intervals. The tissue was blotted and weighed with a torsion balance. At this point, the weight of tissue in each group of explants was made the same. These groups of explants were then sorted into pairs comprising one group labelled with L-[U-¹⁴C]leucine or L-[2,3-³H]glutamic acid (the 'non-decaying' labels) and another group labelled with L-[4,5-³H]leucine or L-[1-¹⁴C]glutamic acid (the 'decaying' labels). For the controls at each time point, both groups of explants were frozen at -75°C and not cultured again. For the test samples, only the group incubated with the 'non-decaying' label was frozen at -75°C and not cultured again. The other test group of explants, i.e. the group incubated with the 'decaying' label, was cultured for a further 24 h in medium containing a defined hormone regime. At the end of this culture period, these explants were also stored at -75°C until analysed. When degradation of casein was measured by using [³H]- and [¹⁴C]glutamic acid, explants were cultured after incubation in the presence and absence of glutamate (2 mM).

Preparation of tissue extracts. Equal weights of ³H- and ¹⁴C-labelled explants were combined and homogenized at 4°C in 0.7 ml of 0.25 M-KH₂PO₄ (adjusted to pH 7.0 with KOH) containing 1 mM-EDTA, 1 mM-dithiothreitol and 2 mM-phenylmethanesulphonyl fluoride (to inhibit serine proteinase activity; Paskin & Mayer, 1976). A particle-free supernatant and pellet was prepared by centrifugation for 60 min at 115 000 *g*_{av.}. A sample of this supernatant (0.2 ml) was taken for analysis by SDS/polyacrylamide-gel electrophoresis and the remainder, after addition of Triton X-100 (1%, v/v, final concentration) was used for the immunolysis of fatty acid synthase. The pellet was resuspended in 0.5 ml of phosphate buffer containing Triton X-100 (1%, v/v) and 5 mM-EGTA, and homogenized with a Polytron X-1020 (Intern. Laborat. App. GmbH., D-7801 Dottingen, W. Germany) for 30 s at setting 3. A second particle-free supernatant was prepared by centrifugation (60 min at 115 000 *g*_{av.}), and from this casein was immunisolated.

Immunolysis of fatty acid synthase and casein. An antiserum to purified fatty acid synthase from rabbit mammary gland was raised in sheep and was shown by double-diffusion and immunoelectrophoretic analyses to be monospecific (Paskin & Mayer, 1976; Paskin, 1978). IgG, containing antibodies only to fatty acid synthase, was further prepared by immunoaffinity chromatography with Sepharose 4B-fatty acid synthase. The Sepharose 4B-fatty acid synthase was made by covalently linking purified fatty acid synthase (Paskin & Mayer, 1976) to Sepharose 4B through imido-carbonate activation with CNBr (Porath *et al.*,

1973) under the conditions described for moderate activation. The resulting monospecific IgG was covalently linked to Sepharose 4B by the same method. Under these conditions, 2.25 mg of anti-(fatty acid synthase) antibodies was bound per ml of Sepharose, and this immunoadsorbent bound 0.96 mg of purified fatty acid synthase per ml of Sepharose. Samples (approx. 0.3 ml) of the first particle-free supernatant were incubated with 1 ml of immunoadsorbent for 1 h at room temperature (20°C) and 1 h at 4°C. The immunoadsorbent was then used to prepare columns in 2 ml plastic syringe barrels. A disc of Whatman glass-fibre paper (GF/A) was used to retain the Sepharose. Each column was washed with 2 ml of 0.25 M-KH₂PO₄ (adjusted to pH 7.0 with KOH) containing Triton X-100 (1% v/v) and then with 8 M-urea (2 ml) containing 2% (w/v) SDS. After 10 min equilibration the urea/SDS solution was collected and the columns were washed with a further 2 ml of urea/SDS. The combined urea/SDS washings were extensively dialysed against water (5 litres) at room temperature (3 days with five changes of water), and each dialysed preparation was freeze-dried. This freeze-dried material was taken up in 0.1 ml of 0.01 M-H₃PO₄ (adjusted to pH 6.8 with 5.0 M-Tris) containing 8 M-urea, 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol, heated at 100°C for 3 min and subjected to SDS/polyacrylamide-gel electrophoresis. Further analysis of the immunisolated material by SDS/polyacrylamide-gel electrophoresis eliminates the effect of non-specific binding to the immunoadsorbent.

Anti-casein IgG was prepared by immunoaffinity chromatography with a Sepharose-recombined casein immunoadsorbent, and was shown to be monospecific by double-diffusion and immunoelectrophoretic analyses (Al-Sarraj *et al.*, 1978). The immunoadsorbent (1.34 mg of IgG/ml of Sepharose) bound 125 µg of recombined casein per ml of Sepharose. Casein was immunisolated from the second particle-free supernatant (0.4 ml) with 2 ml of immunoadsorbent, and eluted and prepared for electrophoresis by the same method as described for fatty acid synthase.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out on 5% (or for immunisolated casein, 12.5%) (w/v) slab gels in the presence of SDS and urea, as described by Swank & Munkres (1971); 5% gels were run at 20 mA for 18 h, and 12.5% gels were run at 40 mA for 20 h. The gels were stained for protein with Coomassie Blue, and regions containing fatty acid synthase and casein were identified. These regions, and whole tracks containing resolved cytosol fractions, were cut into slices (2 mm thick), and processed for radioactivity determination as described by Paskin & Mayer (1978). Radioactivity

was measured with a Packard Tri-Carb 3255 liquid-scintillation spectrometer. Dual-isotope measurements were corrected for quenching by the external standard ratio method. Results are expressed as d.p.m.

Expression of data from double-isotope experiments. Average rates of degradation of immunisolated proteins and of protein mixtures during 24 h periods are expressed as the ratio of L-[U-¹⁴C]-leucine/L-[4,5-³H]leucine or of L-[2,3-³H]glutamic acid/L-[1-¹⁴C]glutamic acid radioactivity. Thus, higher ratios mean higher rates of degradation. Logarithmic transformation of the isotope ratios enables the degradation constant (K_D) of the protein or protein mixture to be calculated, and allows the variance of the transformed ratios for protein mixtures to be used as an estimate of heterogeneity (see below). As the isotope ratio for each protein sample may reflect unequal labelling of protein precursor pools and statistical errors of scintillation counting, control incubations were carried out for each period (see above). Therefore, rate constants of degradation are expressed as test/control ratios of the transformed isotope ratios. The relationship between the rate constant of degradation (K_D) and the ratio of the initial radioactivity to the radioactivity remaining in a protein after time t is given by the equation describing first-order decay (Glass & Doyle, 1972):

$$K_D t = \ln \frac{P_{(0)}}{P_{(t)}}$$

where $P_{(0)}$ is the initial number of radioactive molecules initially present, $P_{(t)}$ is the number of radioactive molecules remaining after time t , and K_D is the rate constant of degradation. In the double-isotope experiments described here data are expressed as test/control ratios, and K_D is given by:

$$K_D t = \ln \left(\left(\frac{\text{isotope 1}}{\text{isotope 2}} \right)_{\text{test}} \times \left(\frac{\text{isotope 2}}{\text{isotope 1}} \right)_{\text{control}} \right) \\ = \ln(\text{test isotope ratio}) - \ln(\text{control isotope ratio})$$

where isotope 2 is the decaying label. By this method K_D or t_1 (i.e. $\ln 2/K_D$) has been calculated.

Statistical analysis of isotope ratios of protein mixtures. To measure the extent of variation or heterogeneity in rates of degradation of proteins in the cytosol fraction, the variance of test $\ln(\text{isotope ratios})$ has been compared by the F -test with the variance of the control $\ln(\text{isotope ratios})$, which are considered to reflect only variations due to experimental error (see above).

Results

Rate of degradation of fatty acid synthase

Indirect estimation. The degradation profile for

fatty acid synthase was computed from measurements of changes in the rate of synthesis (K_s) and amount of the protein, together with one estimate of its half-life (Paskin & Mayer, 1978). The results of two experiments are shown in Fig. 1. In each case there was a transient decrease in degradation rate during the period of most rapid enzyme accumulation, although this phase did not occur at the same time in each experiment. Removal of hormones at 24 or 48 h produced a marked decrease in the rate of synthesis of fatty acid synthase and a rapid increase in its rate of degradation.

Fig. 1. Turnover of fatty acid synthase in mammary explants

The amount and rate of synthesis (K_s) of fatty acid synthase was measured during culture with hormones (—) and after removal of hormones (---). The results of two separate experiments are shown (a–c and d–f). Enzyme degradation profiles (K_d) were determined as described by Paskin & Mayer (1978), based on pre-stimulation steady-state half-lives for fatty acid synthase of 20 h (a–c) and 29 h (d–f).

Measurements with the double-isotope method.

The rate of degradation of fatty acid synthase is markedly lower in the presence of hormones than when explants are cultured in their absence (Table 1). Removal of hormones after 24 or 48 h causes a marked increase in the average rate of degradation of fatty acid synthase, which is inhibited by adrenaline (100 μ g/ml) and blocked further by insulin (5 μ g/ml). The double-isotope technique was designed to measure the average rate of degradation over a 24 h period. Nevertheless, it is clear that the pattern and relative magnitude of changes in the rate of enzyme degradation in the presence and absence of hormones are similar to those determined by analysis of the accumulation profile and rate of synthesis (Fig. 1). In some cases the calculated rate constant of degradation of fatty acid synthase is negative, a value which has no counterpart in real protein metabolism. These values are assumed to represent no measurable degradation during the period concerned. Several negative values for the K_d of fatty acid synthase were also obtained by indirect estimation (Paskin & Mayer, 1978). NH_4Cl (10 mM), which has been reported to inhibit autophagic protein degradation (e.g. Amenta *et al.*, 1978), also partially inhibits the increase in enzyme degradation on hormone removal (Table 1). Although the qualitative patterns for changes in degradation rates are similar at 24–48 h and 48–72 h in culture, the changes in the rate of degradation are smaller at 48–72 h.

Rates of degradation of proteins in the cytosolic fraction

Average rates of degradation of cytosol fraction proteins are expressed as mean rates of degradation (K_d). These are calculated from the means of logarithmically-transformed isotope ratios for gel slices, after the cytosol protein mixtures have been resolved by SDS/polyacrylamide-gel electrophoresis. Rates of degradation are also expressed as protein half-lives ($t_{1/2}$). In contrast with the results of other workers (Dice *et al.*, 1973, 1978; Goldberg & St. John, 1976), there is no correlation between protein subunit size and rate of degradation either in the presence or absence of hormones (Fig. 2). The pattern of changes in the average rate of degradation of proteins in cytosol fraction (Table 2) is similar to that for fatty acid synthase (Table 1); the average rate of degradation is high when freshly cut explants are cultured in the absence of hormones, and increases on removal of hormones at 24 or 48 h. Also, this increase is inhibited differentially by adrenaline (100 μ g/ml), adrenaline plus insulin and NH_4Cl . Changes in protein degradation are most marked at 24–48 h. The results indicate that changes in the average rate of degradation of fatty acid synthase (Table 1) reflect general changes in the

Table 1. *Degradation of fatty acid synthase during experimental manipulation of mammary explants*

Average rates of enzyme degradation during 24 h periods were measured by the double-isotope technique, with L-[U-¹⁴C]leucine and L-[4,5-³H]leucine as the protein precursors. The rate constant of degradation (*K_D*) and the protein half-life (*t_{1/2}*) are calculated from the logarithmically-transformed test and control isotope ratios (see the Materials and methods section). Where indicated, lipogenic hormones were removed at the beginning of the time period. *, *K_D* slightly negative; this is assumed to represent no degradation during the period concerned.

Time period in culture (h)	Treatment	[¹⁴ C]/[³ H]	ln([¹⁴ C]/[³ H])	<i>K_D</i> (day ⁻¹)	<i>t_{1/2}</i> (days)
0–24	Control	1.07	0.07	—	—
	Insulin + prolactin + cortisol	0.77	−0.26	*	*
	No hormones	1.41	0.34	0.28	2.50
24–48	Control	0.85	−0.16	—	—
	Insulin + prolactin + cortisol	1.02	0.02	0.18	3.80
	Hormones removed	1.76	0.57	0.73	0.95
	Hormones removed; adrenaline added	0.97	−0.03	0.36	1.90
	Hormones removed; adrenaline + insulin added	0.75	−0.29	*	*
	Hormones removed; 10 mM-NH ₄ Cl added	1.41	0.34	0.51	1.37
48–72	Control	0.97	−0.03	—	—
	Insulin + prolactin + cortisol	0.75	−0.29	*	*
	Hormones removed	1.39	0.33	0.36	1.90
	Hormones removed; adrenaline added	0.99	−0.01	0.02	∞
	Hormones removed; adrenaline + insulin added	0.87	−0.14	*	*
	Hormones removed; 10 mM-NH ₄ Cl added	1.11	0.10	0.14	4.96

Fig. 2. *Isotope ratios for cytosol protein mixtures resolved by polyacrylamide-gel electrophoresis*

Cytosol proteins from test (Δ) and control (●) groups of explants were resolved by SDS/polyacrylamide-gel electrophoresis and isotope ratios for the polypeptides in gel slices were calculated (see the Materials and methods section). Test manipulations were as follows: (a) 24–48 h + insulin, prolactin and cortisol; (b) 24–48 h, hormones removed, (c) 48–72 h + insulin, prolactin and cortisol; (d) 48–72 h, hormones removed.

Table 2. *Degradation of proteins in the cytosolic fraction during experimental manipulation of mammary explants*
Average rates of degradation during 24h periods were measured by the double isotope technique, with L-[U-¹⁴C]-leucine and L-[4,5-³H]leucine as the protein precursors. Test manipulations are as described in Table 1. Protein mixtures were resolved by polyacrylamide-gel electrophoresis. The rates of degradation (K_D) and protein half-life (t_1) are calculated from the means of logarithmically-transformed test and control isotope ratios (see the Materials and methods section). Transformed test isotope ratios that exhibit significantly greater variance from the mean than their respective transformed control isotope ratios are determined by the *F*-test; **P* < 0.05, ***P* < 0.01.

Time period in culture (h)	Treatment	Mean ([¹⁴ C]/ [³ H])	ln (Mean [¹⁴ C]/[³ H])	K_D (day ⁻¹)	t_1 (days)
0–24	Control	0.69	–0.42	—	—
	Insulin + prolactin + cortisol	1.19	0.16	0.58	1.19
	No hormones	2.07	0.66	1.09	0.64
24–48	Control	0.64	–0.50	—	—
	Insulin + prolactin + cortisol	0.97	–0.07	0.43	1.61
	Hormones removed	2.48	0.88	1.38	0.50
	Hormones removed; adrenaline added	1.23	0.22	0.72*	0.96
	Hormones removed; adrenaline + insulin added	0.91	–0.09	0.41	1.69
	Hormones removed; 10 mM-NH ₄ Cl added	1.87	0.59	1.09	0.64
48–72	Control	0.58	–0.56	—	—
	Insulin + prolactin + cortisol	1.28	0.23	0.79	0.88
	Hormones removed	1.62	0.45	1.01**	0.69
	Hormones removed; adrenaline added	1.60	0.46	1.02	0.68
	Hormones removed; adrenaline + insulin added	0.96	–0.07	0.49	1.41
	Hormones removed; 10 mM-Na ₄ Cl added	1.37	0.39	0.95	0.73

Table 3. *Degradation of casein during experimental manipulation of mammary explants*
Average rates of protein degradation during 24h periods were measured by the double-isotope technique, with L-[1-¹⁴C]glutamic acid and L-[2,3-³H]glutamic acid as the protein precursors (see the Materials and methods section). Test manipulations and the expression of results are as described in Table 1. Rates of degradation were measured in the absence and presence of 2 mM-glutamic acid.

Time period in culture (h)	Treatment	Glutamic acid present	[³ H]/ [¹⁴ C]	ln ([³ H]/ [¹⁴ C])	K_D (day ⁻¹)	t_1 (days)
24–48	Control	—	2.00	0.69	—	—
	Insulin + prolactin + cortisol	+	2.11	0.75	0.05	∞
	Hormones removed	—	3.78	1.33	0.64	1.09
	Hormones removed	+	3.07	1.12	0.43	1.63
	Hormones removed; 10 mM-NH ₄ Cl added	—	3.81	1.34	0.65	1.07
	Hormones removed; 10 mM-NH ₄ Cl added	+	3.42	1.23	0.54	1.29
48–72	Control	—	1.96	0.67	—	—
	Insulin + prolactin + cortisol	—	2.75	1.01	0.34	2.06
	Insulin + prolactin + cortisol	+	2.56	0.94	0.27	2.57
	Hormones removed	—	6.22	1.83	1.15	0.60
	Hormones removed	+	6.01	1.79	1.12	0.62
	Hormones removed; 10 mM-NH ₄ Cl added	—	2.91	1.07	0.39	1.77

average rate of degradation of cytosol fraction proteins (Table 2).

Heterogeneity of degradation rates of proteins in the cytosolic fraction

The variances of transformed ratios for test samples, compared by the *F*-test with those for control samples, are considered to reflect physiological variations over and above variations pro-

duced by the experimental manipulations. In two cases, at 24–48h when lipogenic hormones are removed and adrenaline added, and at 48–72h when hormones are removed, the proteins in the cytosolic fraction exhibit significant measurable heterogeneity of degradation rates (results not shown). In all other experimental conditions, the data suggest that heterogeneity in degradation rates may be due to variations introduced by the experimental mani-

pulations. However, the effects of averaging must be carefully considered in this regard (see the Discussion).

Rates of degradation of casein

The double-isotope method shows that there is little degradation of casein in the presence of hormones, but that on hormone removal at 24 or 48 h an increase in degradation occurs (Table 3). With the single-isotope pulse-chase method, no such increase in casein degradation could be detected (Al-Sarraj *et al.*, 1979). Unlike the effect of hormone removal on the degradation of fatty acid synthase and other proteins in the cytosolic fraction, the increase in casein degradation may be more pronounced at 48–72 h in culture. In addition, NH_4Cl (10 mM) has little effect at 24–48 h, but at 48–72 h it appears to inhibit completely the increase in degradation on hormone removal. These differing responses to hormone removal and NH_4Cl at 24–48 and 48–72 h may reflect the subcellular location of intracellular casein as culture proceeds.

The close agreement between rates of degradation measured with and without glutamate (2 mM) in the culture medium (see the Materials and methods section) indicates that there is no reutilization of this amino acid label (Table 3). Indeed, if reutilization were to occur, then it would be expected that the test/control ratio measured in the presence of glutamate would be greater, whereas, if anything, the opposite is the case. With fresh explants there was too little incorporation of radioactive label into casein for meaningful results to be obtained.

Discussion

Double-isotope method

The major advantage of the double-isotope method is that it can provide a sensitive index of relative rates of protein degradation in a tissue in a single experiment. This sensitivity depends to a large extent on the schedule of labelling with the two radiolabelled species and to a lesser extent on the half-life of the protein(s) under study (Zak *et al.*, 1979). In the present study, the choice of a 1 day culture period with the 'decaying' isotope must necessarily be a compromise between the limited life-span of mammary explants and the interval that gives optimum sensitivity (suggested to be no more than 1–3 times the protein half-life, Zak *et al.*, 1979). However, although the sensitivity of the method may change with the half-life of fatty acid synthase (Paskin & Mayer, 1978) and other proteins in the cytosolic fraction, the double-isotope technique can nevertheless indicate differences in degradation rates when the tissue is subjected to different treatments (Tables 1 and 2). The choice of a 30 min labelling period for the 'non-decaying' isotope is also a

compromise between the need to incorporate enough radioactivity for accurate measurements, and the loss of sensitivity that occurs as the labelling time increases (Poole, 1971; Zak *et al.*, 1977). Thus, these two time intervals determine the range or 'window' of protein degradation rates that is seen. 'Window' size must therefore be considered when heterogeneity of degradation rates is estimated. In differentiating mammary explants, proteins turn over rapidly (Paskin & Mayer, 1977, 1978), so in these experiments the choice of 'window' is for proteins with relatively short half-lives.

The double-isotope method does not obviate the problem of isotope reutilization. However, careful design of the labelling and washing procedures have minimized reutilization (Speake *et al.*, 1975, 1976; Mayer, 1979), to the extent that supplementing medium 199 with unlabelled leucine (10–50 mM) during the decay part of the experiment does not alter the isotope ratio (C. J. Wilde & R. J. Mayer, unpublished work). More recently we have replaced leucine, a tracer subject to considerable reutilization, at least in liver (Poole, 1971; Burgess *et al.*, 1978; Tauber & Reutter, 1978), with glutamic acid. In mammary gland tissue this amino acid is likely to be metabolized before reutilization can occur (Hardwick, 1966; Herzfeld & Greengard, 1971). This may of course make it difficult to ensure sufficient incorporation of label into protein, but when rabbit casein is the protein under study, this is offset by the fact that glutamic acid is its most abundant constituent amino acid (R. J. Mayer, unpublished work).

Examination of mammary explant tissue by electron microscopy shows that, when stimulated by hormones (insulin, prolactin and hydrocortisone) this system mimics the development *in vivo* of the tissue during pregnancy and lactation; removal of hormones during culture produces changes in cellular morphology similar to those seen at weaning (C. J. Wilde & R. J. Mayer, unpublished work). However, the concentrations of hormones used in these experiments bear no relation to those *in vivo*, and were chosen solely because they produce maximal changes in rates of protein turnover during the short period over which these preparations are studied (Speake *et al.*, 1975, 1976).

Experimental manipulation of mammary explants produces a non-steady state, that is, one in which the amounts of intracellular proteins are changing. Therefore, changes in the specific radioactivity of the protein(s) under study may reflect not only the loss of label due to protein degradation, but also a dilution effect due to changes in the total amount of protein present. Such a dilution effect is proportional to the increase in the rate of synthesis of the protein(s) under study. In the present experiments, the effect of increasing synthesis rates and

changing protein amount(s) is avoided by minimizing isotope reutilization and measuring total radioactivity in the protein(s) from equal weights of tissue taken at the beginning of the 24 h culture period. Thus, the isotope ratio is a measure of the rate of protein degradation and not of the rate of protein turnover, i.e. rate of synthesis and degradation.

The isotope ratios for control samples should be 1.00. In practice, however, these isotope ratios do vary from unity. As there is no evidence to suggest that these deviations represent real variations which have physiological significance, it is considered that they reflect errors of measurement (see the Materials and methods section). In the same way, these experimental errors will account for slightly negative K_D values.

Heterogeneity of degradation rates

Heterogeneity in rates of protein degradation has been shown either by collating measurements on single proteins in a tissue in a number of laboratories (Dice & Goldberg, 1975b), or by measuring degradation in resolved mixtures by using the double-isotope technique (Arias *et al.*, 1969; Dehlinger & Schimke, 1971; Dice *et al.*, 1973, 1978; Walker *et al.*, 1978). These data have led to suggestions that the apparently heterogeneous degradation of proteins may be related to several physicochemical properties, such as subunit molecular weight (Dice *et al.*, 1973; Dice & Goldberg, 1975a), net charge (Dice & Goldberg, 1975a; Dice *et al.*, 1978) and hydrophobicity (Segal *et al.*, 1976; Bohley *et al.*, 1977). In the present experiments, no correlation of degradation rate with subunit size is seen (Fig. 2), an observation that is supported by other recent data (Brinster *et al.*, 1979; Horst & Roberts, 1979; Russell *et al.*, 1980).

In the statistical analysis of resolved proteins of cytosol fractions, the mean and standard deviation of the transformed isotope ratios for each test and control sample are calculated separately. As the variance of the test and control ratios is always different, a direct comparison of the ratios from equivalent test and control gel slices is unwarranted. The variances of transformed test isotope ratios are used as estimates of heterogeneity of degradation rates, since this ratio reflects variation not due to that seen in the controls, i.e. due to heterogeneity of protein degradation.

By this method of analysis, proteins in the mammary gland cytosolic fraction exhibit heterogeneity of degradation rates under only a few experimental conditions (results not shown). The isotope ratio for each gel slice is the mean of the ratios for all the polypeptides in that slice; it has been suggested that the similarity of protein degradation rates seen in some systems (Roberts & Yuan,

1975; Tweto & Doyle, 1976) may be due to the masking of real differences by this averaging of isotope ratios (Horst & Roberts, 1979). However, even two-dimensional electrophoretic analysis of protein mixtures reveals great similarities in degradation rates for plasma-membrane proteins (Horst & Roberts, 1979). A further factor that mitigates against averaging in a single-dimensional electrophoretic analysis of protein mixtures is the unequal distribution of polypeptides along the gel, which reflects the size distribution of subunits of intracellular proteins (Darnall & Klotz, 1975). Therefore it seems unlikely that averaging can completely account for the similarity of isotope ratios for resolved protein mixtures.

NH_4Cl is considered to be a lysosomotropic agent and as such is thought to inhibit specifically autophagic protein degradation (Seglen & Reith, 1976; Amenta *et al.*, 1978; Seglen *et al.*, 1979). In mammary explants NH_4Cl causes a partial inhibition of the increase in degradation of fatty acid synthase and cytosol proteins on hormone removal (Tables 1 and 2). These results suggest that the mammary degradation system may consist of two components, one of which is lysosomal autophagy. Similarly, inhibition by NH_4Cl of enhanced protein degradation under 'step-down' conditions in isolated rat hepatocytes (10 mM- NH_4Cl ; Seglen *et al.*, 1979) and cultured fibroblasts (20 mM- NH_4Cl ; Amenta *et al.*, 1978) has been used as evidence for a two-component system.

The two components of the degradation system may be studied by examining differences in the effects of various agents on the degradation of fatty acid synthase, an intracellular protein, and casein, a cell-specific secretory protein. The partial inhibition of the increase in fatty acid synthase degradation on hormone removal by NH_4Cl (Table 1) may indicate that, when this enzyme is destroyed at high rates, both components of the degradation system operate at greater rates. However, although the average rate of degradation of casein is also increased on removal of hormones, only at 48–72 h does NH_4Cl inhibit this effect (Table 3). This difference may be due to the different location of intracellular casein during the culture period. At 24–48 h most of the intracellular casein may be within the lumen of the endoplasmic reticulum or secretory endomembrane system (Morré & Ovtracht, 1977), into which it passes immediately after synthesis on membrane-bound polysomes (Craig *et al.*, 1978). Autoradiographic evidence shows that the majority of casein is rapidly secreted (K. Al-Sarraj, D. A. White and R. J. Mayer, unpublished work). On removal of hormones this casein may be degraded by luminal ammonia-insensitive proteinases of the endoplasmic reticulum or the related secretory endomembrane system. At 48–72 h, as the alveolar lumen becomes

filled with casein, it may accumulate intracellularly within membrane-limited secretory vesicles, and in this location become susceptible to lysosomally mediated autophagic degradation when hormones are removed. Thus, the increase in casein degradation at this time is inhibited by NH_4Cl (Table 3). Rapid intracellular degradation of the newly-synthesized form of another secretory protein, collagen, has also been demonstrated (Bienkowski *et al.*, 1978) and conceivably takes place lumenally in the intracellular endomembrane system. It is also possible that casein is degraded extracellularly; the presence of plasminogen in milk (Eigel *et al.*, 1979) raises the possibility that casein may be degraded by plasmin in the alveolar lumen on hormone removal. However, the differential inhibitory effect of NH_4Cl during different culture periods is not consistent with such a mechanism (Table 3).

On the basis of its adenylate cyclase response, rabbit mammary tissue does not appear to have glucagon receptors, but it is sensitive to adrenaline (Bär, 1973). Therefore, adrenaline has been tested to see if this hormone can mimic the stimulation of autophagy in rat liver by glucagon (Arstila *et al.*, 1972; Ayuso-Parrilla *et al.*, 1976). Adrenaline, in fact, inhibits the increase in degradation of fatty acid synthase and cytosol proteins on hormone removal; indeed, together with insulin, it completely blocks this increase in degradation. Insulin may block autophagic degradation (Knowles & Ballard, 1976; Dice *et al.*, 1978), possibly by inhibiting the formation of new autophagic vacuoles (Pfeifer, 1978). Therefore, as hormone removal may stimulate both the 'basal' and autophagic degradation systems, it would seem that at least part of the effect of adrenaline may be due to an action on the 'non-lysosomal' degradation system.

In conclusion, the present data support the concept of a two-component degradation system (Schimke, 1970; Ballard, 1978), although they do not indicate any great selectivity in the degradation of mammary cytosol proteins. The basal or 'non-lysosomal' component appears to be able to degrade proteins on either side of the endoplasmic reticulum membrane. The close relationship between protein synthesis and degradation in this and other experimental systems (Goldberg & St. John, 1976), together with the evidence that hormonal stimulation of mammary tissue also causes a large increase in the half-life of casein mRNA (Rosen *et al.*, 1979), suggests that the polysome-endoplasmic reticulum may play a central co-ordinating role in the balance between synthetic and degradative processes. The 'basal' component of protein degradation may degrade completely some proteins (Etlinger & Goldberg, 1977), and produce partial degradation of others, with subsequent destruction of these proteins by a process associated with

lysosomes (Novikoff & Shin, 1978; Ward *et al.*, 1979; Dean, 1979).

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