Protein turnover and proliferation

Turnover kinetics associated with the elevation of 3T3-cell acid-proteinase activity and cessation of net protein gain

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1. At least 95% of the total protein of A31-3T3 cell cultures undergoes turnover. 2. First-order exponential kinetics were used to provide a crude approximation of averaged protein synthesis, K_s , degradation, K_d , and net accumulation, K_a , as cells ceased growth at near-confluent density in unchanged Dulbecco's medium containing 10% serum. The values of the relationship $K_a = K_s - K_d$ were : 5%/h = 6%/h – 1%/h in growing cells, and 0%/h = 3%/h – 3%/h in steady-state resting cells. 3. As determined by comparison of the progress of protein synthesis and net protein accumulation, the time course of increase in protein degradation coincided with the onset of an increase in lysosomal proteinase activity and decrease in thymidine incorporation after approx. 2 days of exponential growth. 4. After acute serum deprivation, rapid increases in protein degradation of less than 1%/h could be superimposed on the prevailing degradation rate in either growing or resting cells. The results indicate that two proteolytic mechanisms can be distinguished on the basis of the kinetics of their alterations. A slow mechanism changes in relation to proliferative status and lysosomal enzyme elevation. A prompt mechanism, previously described by others, changes before changes in cell-cycle distribution or lysosomal proteinase activity. 5. When the serum concentration of growing cultures was decreased to 1% or 0.25%, then cessation of growth was accompanied by a lower steady-state protein turnover rate of 2.0%/h or 1.5%/h respectively. When growth ceased under conditions of overcrowded cultures, or severe nutrient insufficiency, protein turnover did not attain a final steady state, but declined continually into the death of the culture.

Although the exact fraction of cell proteins that might be stable has not been determined (see below), the majority have been found to exist in a state of continual replacement (Eagle, 1959; Mandelstam, 1963). Thus cell growth is accompanied by an unbalanced state of protein turnover wherein the gross synthetic rate exceeds that of degradation. Conversely, the transition to quiescence involves the attainment of a steady state of equal synthesis and degradation, with resulting net protein gain of zero.

It was previously reported that growing 3T3 cells maintained a low activity of lysosomal proteinases, and that these enzymes were markedly increased in

association with the cessation of growth (Lockwood & Shier, 1977). Because alteration in the degradative rate of some proteins is well established (Mandelstam, 1963; Herskho *et al.*, 1971; Poole & Wibo, 1973), it seemed hypothetically possible that the control of protein degradation as well as synthesis could be involved in the control of net protein accumulation during growth transitions (Goldberg & St. John, 1976; Castor, 1977; Bradley, 1977; Warburton & Poole, 1977; Hendil, 1977; Holzer & Heinrich, 1980). Nonetheless, the consensus of several reports is that sustained changes in protein degradation do not contribute appreciably to the control of bulk protein accumulation during growth transitions (Bradley, 1977; Baxter & Stanners, 1978; Amenta *et al.*, 1980). By implication, the control of net protein accumulation during growth transitions would thus be exerted largely by changes

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in synthesis against a constant catabolic rate. Moreover, several studies have also implied that a considerable fraction of cell protein degradation takes place independent of the lysosomal system [reviewed by Dean (1980) and Goldberg *et al.* (1981)].

Thus far, studies on protein turnover kinetics have not accounted for the fact that cultured cell growth can cease under greatly varying experimental conditions. Firstly, if cells are plated at low density in Dulbecco's medium containing 6–10% serum, then cessation of growth at subconfluent density is due to selective depletion of limiting growth factor(s) from unchanged medium (Holley & Kiernan, 1968, 1974*a,b*; Bartholomew *et al.*, 1976). Such cultures can remain viable indefinitely and will resume proliferation on addition of fresh serum. However, if cells are cultured in unchanged Eagle's minimal essential medium instead of Dulbecco's enriched medium, they cease growing and die, due to depletion of synthetic components of the medium rather than serum factors. Secondly, if the Dulbecco's medium of growing cultures is changed to one containing very low serum concentrations (0.2–0.5%), then growth cessation and some cell death occurs within 1 day due to non-selective deprivation of unknown serum components. The distinction between a true growth-promoting factor and a survival or growth-permissive factor has been discussed (Gospodarowicz & Moran, 1976). Thirdly, a poorly understood mechanism limits 3T3-cell growth at high densities (Dulbecco, 1970). However, if the medium of high-density cultures is renewed, cells are stimulated to overgrowth, followed by acidification of the medium and eventual death.

The present studies indicate a marked dependence of 3T3-cell protein-turnover kinetics on the experimental conditions used to manipulate cell growth. The following paper describes protein turnover kinetics in SV-3T3 (SV-40-virus-transformed 3T3) cells that fail to increase lysosomal proteinases and cease net protein accumulation (Lockwood & Minassian, 1982). A partial characterization of the lysosomal and non-lysosomal pathways of degradation in 3T3 cells (T. D. Lockwood & A. L. Goldberg, unpublished work) has been described preliminarily (Goldberg *et al.*, 1981).

Experimental

Cell culture

Clone A31-3T3 cells (Aaronson & Todaro, 1968) were provided by Dr. George Todaro to the M.I.T. cell-culture centre and cultured in Dulbecco's medium containing 10% (v/v) foetal-calf serum (Flow Laboratories) by standard procedures. In

extended experiments, dishes were kept in covered containers to maintain evaporative water loss below 2%. Growing cells were characterized in a transient 20–30 h culture period, during which time proliferation is maximal at a subconfluent density of 2×10^5 – 8×10^5 cells per 5 cm dish (Falcon). After a change of medium at an initial cell density of 2×10^5 cells per dish (Fig. 1), anchored growing 3T3 cells require approx. 20 h to adapt to and condition fresh medium so as to attain maximal proliferation. Various conditions of quiescent cultures are described below.

Protein synthesis

[4,5- ^3H]Leucine (40–60 Ci/mmol; 2 $\mu\text{Ci/ml}$; New England Nuclear) was added to the medium of quadruplicate plates simultaneously with 100 μl of 200 mM non-radioactive leucine (4 mM final concn.) for the indicated labelling times. Cells were then rinsed four times with Dulbecco's phosphate-buffered saline and harvested by scraping in 4 ml of 0.05% Triton X-100. Bovine serum albumin was added as carrier in a final concentration of 0.1%. The precipitate from 6% (w/w) trichloroacetic acid was washed twice more, redissolved in 1 ml of 0.1 M-NaOH at 37°C and reprecipitated with 6% trichloroacetic acid. This pellet was hydrolysed in 1 ml of 0.5 M-NaOH at 90°C for 2 h and adjusted to pH 5 with 1 ml of 1 M-sodium acetate buffer. Samples (0.5 ml) were counted for radioactivity in Aquasol (New England Nuclear). Beginning at 5 min after addition of label, the leucine incorporation rate per mg of protein was calculated after subtracting the radioactivity incorporated in 5 min.

The validity of the present procedures, including rapid and complete exchange of the intracellular and extracellular amino acid pools, has been verified by several studies (Poole & Wibö, 1973; Van Venrooij *et al.*, 1974; Hod & Herskho, 1976; Neff *et al.*, 1977; Robinson, 1977). The maximal possible depletion of the leucine from the medium during cell growth (see below) cannot appreciably alter the specific radioactivity of the large excess present during the pulse. Deviation of the leucine incorporation rate from linearity contributed a small error, as determined by use of different labelling times as shown below (Fig. 1).

The rate of protein synthesis per unit of protein was also determined by using the isotopic ratio of a pulse label to continuous label. An indication of the total amount of culture protein was provided by previously growing cells continuously in [^{14}C]leucine (0.05 $\mu\text{Ci/ml}$; New England Nuclear) over at least a 30-fold increase in cell number so as to label protein uniformly (see below). The rate of [^3H]leucine incorporation during a 2.5 h pulse time was determined as described above. In experiments where the ^{14}C -labelled medium was changed, a

portion of the initial medium was retained for the change of medium so as to maintain the identical ^{14}C specific radioactivity. Isotopic spillover was corrected by internal standardization. The double-label technique provided results that were identical with the $[^3\text{H}]$ leucine-incorporation rate per unit of measured protein.

Protein degradation

Protein degradation was measured over a 20 h or 30 h period (Lockwood & Minassian, 1982) by observing the decline in radioactivity of total cell protein that had been uniformly labelled by growing cells in medium containing $0.1\mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml over at least a 30-fold increase in cell number. At zero time the radioactive medium was replaced with either fresh or depleted unlabelled chase medium after dishes were rinsed three times with non-radioactive medium containing no serum (see the legend of Fig. 2). Then 6 mM non-radioactive leucine was added to the chase medium to prevent competitively re-incorporation of labelled amino acids after their release from cell protein. It was determined that the growth of a confluent monolayer in a 5 cm dish incorporated into protein approx. 5% of the 0.8 mM-leucine content of 5 ml of Dulbecco's medium. Therefore rinsing the dishes and increasing the leucine concentration from 0.8 to 6 mM maintained the specific radioactivity of the precursor at a negligible fraction of the original specific radioactivity in which cells were grown. The use of 3 mM non-radioactive leucine instead of 6 mM did not alter the results of other experiments. At the indicated times, remaining macromolecular radioactivity in the monolayer was determined as described above, in quadruplicate cultures.

Fractional protein degradation was also measured over shorter time intervals by determination of the rate of release of previously incorporated leucine into the culture medium as described by Poole & Wibo (1973). Total culture protein was labelled to uniformity as described above. Cultures were rinsed five times in non-radioactive medium containing 2 mM-leucine. After 7 min in the last rinse, cultures were rinsed four more times and permitted to remain for 7 min more in the rinsing solution. This procedure removed essentially all initially trichloroacetic acid-soluble radioactivity from the cultures. The percentage release of radioactivity into the medium was observed over the time periods indicated below. The value of that amount of acid-precipitable radioactivity at 14 min after the initial rinse was taken to be 100%. When the measured degradative period was begun after the same number of rinses over 7 and 21 min rinse times, results were indistinguishable from those with the 14 min rinse time. The medium was harvested separately from the monolayer and made 0.1% in bovine serum albumin.

Protein that had detached from the monolayer into the medium was precipitated from a final concentration of 6% trichloroacetic acid and rinsed twice. This radioactivity was added to that of the monolayer. The monolayer was processed as described above, except that protein was hydrolysed in 0.5 M-NaOH at 90°C overnight. The percentage of the initial trichloroacetic acid-precipitable radioactivity that was released per h into the medium in acid-soluble form was calculated after internal standardization.

Acid proteinase assay

Acid proteinase activity at pH 2.8 toward ^3H -radiolabelled haemoglobin (Hille *et al.*, 1970; Barrett, 1977) was determined as previously described (Lockwood & Shier, 1977). A unit of activity is equivalent to $1\mu\text{g}$ of haemoglobin hydrolysed to acid-soluble peptides/h.

The proteinase activity of 3T3-cell 0.05%-Triton-X-100 extracts exhibited no deviation from linearity at pH 2.8 under the present conditions. Thus any possible latent activity is either instantaneously activated under these conditions or remains unchanged during the assay. The activity was also directly proportional to a wide range of dilutions of cell extracts from growing cells with low activity and quiescent cells with induced activity.

Thymidine incorporation

The thymidine incorporation rate per cell was determined during a 2 h labelling period in quadruplicate cultures as previously described (Lockwood & Shier, 1977), except that precipitates were hydrolysed in 0.2 M-HClO₄ before radioactivity counting.

Determination of the rates of net protein accumulation and cell division

Protein was determined by the method of Lowry *et al.* (1951) in quadruplicate cultures. The amount of dried or precipitated serum protein surviving six rinses over 2 min was negligible in parallel cultures without cells.

The exponential rates of net protein accumulation, K_a , in units of %/h was calculated as the average over a 10 h interval by the relationship:

$$K_a = \left(\frac{P_t}{P_{t-10}} - 1 \right) \times 100$$

where P_{t-10} and P_t are the total culture protein contents at the beginning and end of the 10 h period. Over this time interval the protein doubling time, T_d , is described by the relationship: $T_d = \ln 2 / K_a$. Cell numbers were determined in quadruplicate or quintuplicate cultures with a Coulter Counter after monitoring each plate microscopically during trypsin treatment in EDTA to ensure that not more than

2–3% of the trypsin-treated cells were aggregated. The exponential rate of cell proliferation during a 10h period was determined as described for protein; these calculations are illustrated in Fig. 1 by the partition of the growth curve into 10h intervals. The times of all values are plotted so as to average the determinations of the midpoint of the measured period. The error associated with the calculations of the cell and protein accumulation rates at 10h intervals is consistent with the limits of the overall accuracy and variability of the present approximations (see below).

Results

Protein turnover kinetics as cells ceased net protein accumulation due to selective depletion of limiting growth factors from 6–10% serum

After a maximal growth stimulation under initial conditions described in Fig. 1, the limiting growth factors of unchanged medium were consumed by the production of 2×10^6 cells per dish after 4 days of proliferation. At this final density, cells were near-confluent; however, at least a tripling of this cell number can be caused by medium change.

Net protein accumulation. The maximal rate of protein accumulation of 50%/10h or 5%/h ($K_a = 0.05 \text{ h}^{-1}$) at approx. 20h was followed by a progressive decline toward zero over 4 days. The decline in the rate of protein accumulation was approximately paralleled by the decline in thymidine incorporation rate per cell and the decline in the rate of cell proliferation (Fig. 1).

Protein synthesis. An absolute measurement of fractional protein synthesis would be subject to several sources of experimental uncertainty; however, an arbitrary indication of relative changes is provided by the rate of leucine incorporation per unit of protein. This measurement of the fractional synthetic rate is independent of possible changes in the total cell protein content during the growth transition. The rate of protein synthesis per mg of protein declined in parallel with, and in approximate proportion to, the initial 30% decline in the protein accumulation rate (Fig. 1). Then at 50h, the rate of protein synthesis attained a plateau, and the rate of net protein accumulation diverged and accelerated its decline toward zero at 90–100h. As determined by recovery of trichloroacetic acid-precipitable radioactivity from the medium of cells previously grown in [^3H]leucine, less than 1–2% of the total monolayer protein content is released during 10h by secretion or cell detachment; and the experimental design eliminates the error of cumulative protein detachment beyond a 10h period. Therefore the increasing difference between the rates of protein synthesis and net protein accumulation must be indicative of an increase in protein degradation.

Protein degradation. The trace describing 'whole cell' protein degradation versus time is theoretically a multicomponent curve; however, the progress of the hydrolysis of bulk protein to trichloroacetic acid-soluble form empirically approximated to a log-linear relationship (Fig. 2). The averaged rate of protein degradation, K_d , is obtained from the slope of the indicated traces by the relationship $K_d = \ln 2 / t_{\frac{1}{2}}$, where $t_{\frac{1}{2}}$ is the averaged protein half-life. As shown in Fig. 2, the rate of protein degradation was 1.0–1.2%/h ($t_{\frac{1}{2}} = 60\text{--}70 \text{ h}$) in the maximally growing cell, and this rate increased to 2.7–3.0%/h ($t_{\frac{1}{2}} = 23\text{--}26 \text{ h}$) in the quiescent cell. Thus the progressive increase in presumptive protein degradation shown by the divergence of synthesis and net accumulation in Fig. 1 corresponds to a measured increase in protein degradation as shown in Fig. 2.

To obtain an indication of the maximal fraction of intracellular and accumulated extracellular protein that does not undergo degradation, quiescent cultures were permitted to age in unchanged medium for 3 days after 4 days of growth before measurement of degradation. After a subsequent 9-day degradation period, 5% of the initial monolayer protein remained attached to the dish and 7% was recovered in trichloroacetic acid-precipitable form from the unchanged growth medium. Therefore the vast majority of intracellular 3T3-cell proteins undergo degradation, and results do not require correction for extracellular non-degraded protein or detached protein (see also Lockwood & Minassian, 1982).

Relationship between protein synthesis, degradation and net accumulation. The total culture protein, P_t , accumulated over a time interval of first-order exponential growth, Δt , can be empirically approximated by the equation: $P_t = P_0 e^{K_a \Delta t}$, where K_a is the net protein accumulation rate during the time interval. The net protein accumulation rate at any point in time can be approximately described by the synthetic rate, K_s , minus the degradative rate, K_d : $K_a = K_s - K_d$. A description of the instantaneous rate of cell protein turnover requires specification of the simultaneous rates of synthesis, degradation and net accumulation. If any two of the three rates, K_a , K_s and K_d , are known simultaneously, then the third can be calculated. Thus the absolute fractional synthetic rates in growing and resting cells can be calculated from the measured rates of fractional accumulation and fractional degradation without a measurement of fractional synthesis. The observed change in net accumulation is from 5%/h during maximal growth (doubling time, $T_d = 14 \text{ h}$) to zero net gain as cells equalize synthesis and degradation (Fig. 1). When the protein accumulation rate was 5%/h, the degradative rate was 1%/h; therefore the synthetic rate of the maximally growing population can be inferred to be

Fig. 1. DNA synthesis, protein synthesis, protein accumulation and lysosomal proteinase activity in A31-3T3 cells. At 2 days before the experiment, cells were plated at 10^5 per 5 cm dish in Dulbecco's medium containing 10% foetal-calf serum. Zero time corresponds to replacing the medium of growing cultures with 5 ml of Dulbecco's medium containing 10% serum. Thus the initial shock of trypsin treatment was avoided, and the experiment was begun with anchored cells in exponential growth. All measurements were undertaken in a single parallel experiment using the same initial batch of cultures. The trace describing the rate of increase in cell number shown in panel (a) is the result of the calculation illustrated by the partition of the growth curve (inset, and see the text). The values of all points shown at 10 h intervals are the results of averaging two or three additional interspersed points not shown for a total of 8–12 cultures per point. The rate of leucine incorporation in units of c.p.m./mg of protein is plotted so as to coincide at the maximum with the rate of protein accumulation. However, the absolute value of protein synthesis at the maximum and at quiescence can be shown to be approx. 6%/h and 3%/h respectively (see the text). (a): ●, Rate of increase in cell number; ▲, rate of thymidine incorporation/cell; inset, cumulative cell number versus time, 0–100 h. (b): ▲, Rate of net protein accumulation; ●, rate of leucine incorporation per mg of protein measured over a 4 h incorporation time; ○, cathepsin D activity per mg of protein. Also shown at 15, 45 and 85 h is the rate of leucine incorporation per mg of protein as measured with 2 h (■) and 6 h (□) labelling times.

approx. 6%/h. In the particular case of zero net protein gain ($K_a = 0$), the synthetic component of turnover can be equated with the measured degra-

dativ rate that increased from 1%/h to 3%/h as cells became quiescent. Secondly, an absolute measurement of changes in the fractional synthetic

Fig. 2. Protein degradation in growing and resting A31-3T3 cells

The degradation rate of total cell protein in growing cultures (▲), quiescent cultures (●) and quiescent cultures in unchanged Eagle's medium (■) was determined as described in the text. The growing cultures had been transferred from prior growth, in identically radiolabelled medium, as described in Table 1. At 20h before zero time, the growth of already proliferating cultures was maximally stimulated by change of medium containing fresh 10% serum and the identical batch of radiolabelled medium in which cells had been previously growing. At the beginning of the measured degradation period cells were maximally growing at a density of 3×10^5 , and the indicated degradation period corresponds approximately to the period of 10–30h in Fig. 1. The non-growing cultures were quiescent at a slightly subconfluent density of 1.5×10^6 per dish after having been plated at the low density of 5×10^4 cells/dish and previously grown for 6 days in unchanged radiolabelled medium. To preserve the status of quiescent cultures during the degradative period, the depleted medium of non-radioactive cultures grown in parallel was used to replace the radioactive medium in which labelled cultures were grown. The half-life in four different experiments varied in the range of 60–70h in growing cells and 23–26h in resting cells. The half-life of suboptimally growing cultures was intermediate between the two traces (results not shown). The half-life of quiescent cell protein in unchanged depleted Eagle's medium was determined to be 50h after 2 days culture, and results shown are representative of two experiments.

rate is independently provided by placing the final steady-state leucine incorporation rate at the measured turnover rate of 3%/h ($t_{1/2} = 23$ h) in quiescent cells. The maximal leucine incorporation rate in growing cells was 80–100% higher than the steady-state value of 3%/h; therefore the maximal synthetic rate was approx. 6%/h. In summary, the approximate relationship $K_s = K_g - K_d$ in maximally growing cells is 5%/h = 6%/h – 1%/h, and in quiescent steady-state cells, 0%/h = 3%/h – 3%/h. The pre-

Fig. 3. Protein synthesis per unit of protein as cells ceased growth at overcrowded density and near-confluent density

An indication of the rate of fractional protein synthesis was provided by the $^3\text{H}/^{14}\text{C}$ ratio of pulse-labelled to uniformly labelled protein as described in the text. Cell protein was uniformly labelled before and during the experiment with the identical specific radioactivity of [^{14}C]leucine. In the experiment shown in (a), cultures had been previously grown in 10% serum to a nearly confluent density. At the beginning of the experiment, the medium was changed, and growth was stimulated to a high final density of 7×10^6 /dish. By 100h the pH indicator of the medium began to turn slightly orange, but cultures were viable. Within 1 day after the last point shown, the medium had been acidified and crowded cultures began to die. In (b), cells were cultured from a low initial density in unchanged medium as described in Fig. 1. Cells became quiescent at a slightly subconfluent density of 10^6 per dish. During the indicated time period the unchanged medium was not acidified and cultures remained viable. (a), Overcrowded cultures: ●, total culture protein content; ○, protein synthesis per unit of protein. (b), Subconfluent cultures: ▲, total culture protein content; △, protein synthesis per unit of protein.

sent kinetic model is limited to a close approximation, due to the overall errors introduced by protein detachment, protein that might not undergo turnover, and the use of a 10h time interval to measure net protein accumulation.

Protein synthesis and degradation as cells ceased growth under conditions of overgrowth, decrease in total serum concentration, or under culture in unchanged minimal essential medium

When cells ceased growth under selective depletion of limiting growth factors from optimal 10% serum concentrations, the final plateau rate of protein synthesis per unit of protein in quiescent cells remained constant for at least the 10-day duration of the experiment shown. Because both the total culture protein content and the fractional synthetic rate remained essentially unchanged, it can be inferred that protein turnover was constant and stable during this time. However, when the medium of near-confluent cultures was changed to fresh 10% serum, the cultures grew to an overcrowded density before attaining a constant protein content (Fig. 3). Under these conditions, protein synthesis per unit of protein declined by 80% in viable cells just before evidence of acidification of the medium and culture death. The decline in fractional protein synthesis continued from 30 to 100h despite the almost constant culture protein content. This implies that the degradative component must also be declining during this time (see below). A phenomenon similar to that shown in Fig. 3 was observed to occur more slowly at subconfluent density if cells were cultured in unchanged Eagle's minimal essential medium containing 10% serum (results not shown).

When growth in Dulbecco's medium was stopped by decreasing the total serum concentration from 10% to 1% or 0.25%, fractional protein synthesis in slightly subconfluent cultures declined by approx. 60 and 75% respectively, before approaching an approximately constant value (Fig. 4). At minimal survival serum concentrations, cells do not remain viable indefinitely; however, under depleted serum concentrations of 2–5%, fractional protein synthesis approximated to a steady state at lower rates for periods of more than 2 days (results not shown). The final steady-state rate of leucine incorporation per unit of protein in medium containing 10% depleted serum in Fig. 4 represents a turnover rate of 3%/h ($t_1 = 23$ h) as shown in Fig. 2. Therefore the steady-state degradative rates in medium containing 1.0% and 0.25% depleted serum in Fig. 4 can be inferred to be approx. 1.5–2.0%/h and 1.0–1.5%/h respectively, because the observed decreases in steady-state synthetic rates are directly proportional to decreases in steady-state turnover rates.

The above culture conditions that led to either a decreased steady-state synthetic rate, or a con-

Fig. 4. Protein synthesis per unit of protein as cells ceased proliferation by selective depletion of limiting growth factors from 10% serum and by decrease in total serum concentration

The culture procedure in controls (■) was similar to that of Fig. 1, except that cells had previously been labelled to uniformity with ^{14}C leucine. Protein synthesis per unit of protein was determined in quadruplicate cultures by the double-label technique described in the text. At the time indicated by the arrow, the batch of cultures was divided. The control cultures were permitted to become quiescent in unchanged ^{14}C -labelled medium as described in Fig. 1. The media of two other groups of cultures were changed to fresh media containing serum concentrations of 1% (●) and 0.25% (▲), and the identical specific radioactivity of ^{14}C leucine. The change to decreased serum concentration was accompanied by the detachment of approx. 10–15% of the cells within 1 day; however, this does not affect the measurement during a short pulse. At 30h the control cultures were decreasing their rates of exponential growth and by 90h all three groups had ceased net protein gain. Therefore the different $^3\text{H}/^{14}\text{C}$ ratios at 90h are indicative of relative rates of steady-state protein turnover.

tinually declining synthetic rate, also led to a decline in protein degradation. After culture in unchanged Eagle's minimal essential medium with 10% serum for 2 days, the protein half-life of slightly subconfluent cultures was 50h as measured over 21h (Fig. 2). Quiescent cultures maintained in Dulbecco's medium containing 2% depleted serum exhibited a protein half-life of approx. 50h (results not shown). In moderately crowded viable cultures of 4×10^6 – 6×10^6 cells per dish after 1 day of quiescence, the half-life of cell protein in three experiments was 35, 38 and 45h (results not shown). Because both the synthetic and degradative components of turnover were independently observed to decline in proportion to nutrient insufficiency, overcrowding or decreased serum concentration, the bulk fractional

rate of protein replacement in quiescent cells appears to be markedly dependent on culture conditions.

Prompt changes in proteolysis associated with serum or insulin responsiveness compared with delayed changes in proteolysis associated with growth transitions

The kinetic alterations described in Fig. 1 occurred in relation to changes in thymidine incorporation and proliferation over a time period consistent with a change in the cell-cycle distribution of the population. A promptly changing proteolytic mechanism (Poole & Wibo, 1973; Gunn *et al.*, 1977) can be distinguished from the slowly changing mechanism on the basis of the kinetics of its experimentally induced alterations. When serum was withdrawn acutely, the rapidly changing proteolytic mechanism superimposed increases of approx. 0.5–1.0%/h upon the 1%/h proteolytic rate of growing cells or the 3%/h rate of quiescent cells (Table 1). This proteolytic increase could be reversed by re-addition of 10% serum or supraphysiological insulin concentrations of 1 μ M (Table 1; Hershko *et al.*, 1971). In one

experiment the rapid increase in proteolysis was not sustained for more than 5 h in resting cells before declining to rates below those of parallel controls in depleted medium (results not shown).

Replacement of depleted medium with medium containing fresh serum or insulin did not promptly cause the full decline to the degradative rate that was characteristic of maximally growing cells (Table 1); however, results were inexplicably variable. In most experiments when the medium of quiescent cultures that had previously been in 10% depleted serum was changed to medium containing 10% fresh serum, the degradative rate as measured from 0 to 4 h declined by approx. 0.5%/h in comparison with that in control cultures to which 10% depleted medium was added (Table 1). However, in six independent experiments, one showed no decrease in degradation relative to quiescent controls, and one showed a very large decrease, to almost the 1%/h degradative rate of growing cells (results not shown). The variability of these and other similar data is currently unexplained. Similarly variable results were obtained when degradation was measured as the amount of

Table 1. *Prompt alterations of protein degradation in growing and resting 3T3 cells*

Protein degradation was measured as the release of previously incorporated leucine from cell proteins into medium containing 2 mM-leucine over 3 h as described in the text. Results are representative of at least three separate experiments, including one with a 2 h degradation period. To stimulate maximal growth, the radioactive medium in which growing cells had been plated was replaced with medium containing the identical specific radioactivity of [3 H]leucine and 10% fresh serum 24 h before the observed degradation period. To label cell protein uniformly over a 40-fold increase in cell number, stock cultures were grown in a portion of the identical radiolabelled medium employed for the experiment. After the removal of radioactive growth medium and rinsing at 3×10^5 cells per dish, non-radioactive medium containing 10% fresh serum was added to one group of cultures (a), and medium without serum was added to the other group (b) (Expt. 1). In Expt. 2, cells were initially quiescent at 2×10^6 cells per dish in [3 H]-leucine-labelled medium after 6 days of culture with 6% serum without change of medium. The radiolabelled medium of group (c) was replaced with non-radioactive medium that had been depleted by simultaneous parallel growth of cultures in unlabelled medium. To group (d) was added fresh medium without serum and to group (e) was added medium with 10% fresh serum. A final concentration of 1 μ M-bovine insulin (Sigma, 24 units/mg) was added to group (f) in a 50 μ l volume. The degradative rates in the presence of fresh serum (e) and insulin (f) were not consistently different from each other in separate experiments.

	Protein degradation (%/h)
Expt. 1	
(a) Growing + 10% fresh serum	1.2 ± 0.19 (n = 6)
(b) Growing – serum	$1.7 \pm 0.23^*$ (n = 6)
Expt. 2	
(c) Quiescent + 10% depleted serum	3.1 ± 0.32 (n = 5)
(d) Quiescent – serum	$3.8 \pm 0.20^*$ (n = 6)
(e) Quiescent + 10% fresh serum	$2.6 \pm 0.16^*$ (n = 5)
(f) Quiescent + 1 μ M-insulin	$2.2 \pm 0.11^*$ (n = 5)

* Different from control, $P < 0.025$.

radioactivity remaining in monolayers from 0 to 10 h as described in Fig. 2 (results not shown).

Lysosomal proteinase activity and protein degradation

While 3T3 cells were proliferating maximally, acid proteinase activity was maintained at a constant low value (Fig. 1). At the time of divergence of protein

synthesis and net protein accumulation, the activity began a marked increase. In other experiments the activity approached a constant plateau as cells became quiescent; however, the maximal activity was not attained until approx. 1 day after cessation of growth (Lockwood & Minassian, 1982). An inverse relationship between thymidine incorporation and acid proteinase activity was also observed in early-passage embryonic mouse cells (results not shown).

The experiments of Fig. 5 were undertaken to compare the time course of elevation in proteinase activity with the prompt and delayed elevations in protein degradation; however, these experimental manipulations might not be indicative of induction conditions *in situ*. Upon replacement of medium containing serum with medium containing 0% or 0.25% fresh serum, the low acid proteinase activity of growing cells was not increased at 3 h (results not shown). Upon replacement of medium containing fresh serum with medium containing depleted serum, the elevation of acid proteinase activity in growing cells also did not occur promptly (Fig. 5). When slowly growing cultures with partially elevated proteinase activity were changed from medium containing 10% partially depleted serum to medium containing 1% depleted serum, a progressive acceleration in elevation of proteinase activity was observed over several hours; however, no elevation was observed promptly (Fig. 5). In separate experiments the time course of increase was variable; however, no increase was observed promptly (results not shown). Thus the rapid elevation in proteolysis observed in growing cells upon serum deprivation (Table 1) is not accompanied by simultaneous rapid increases in measured acid proteinase activity. The present results and others (Lockwood & Minassian, 1982) suggest that the increase in acid proteinases might be cell-cyclic-specific for a non-replicative phase. Under conditions of progressive over-crowding, lysosomal proteinase activity remained elevated while protein degradation declined as described above. Thus elevated lysosomal proteinase activity is not always associated with sustained maximal protein degradation in cultured cells, as measured by the average of total degradation.

Discussion

Dependence of protein turnover kinetics on culture conditions used to manipulate cell growth

When non-crowded cells became quiescent in 10% depleted serum, the final steady-state synthetic rate of 3%/h was approx. 50% of the maximal 6%/h synthetic rate during cell growth. Under these conditions the degradative change from 1%/h to 3%/h contributes to the control of net accumulation in an amount almost equal to the synthetic change.

Fig. 5. Acid proteinase activity after acute decrease in concentration in maximally growing and slowly growing cultures

(A) Maximally growing cultures (squares). After 2 days growth from a low initial density, growth was stimulated by change of medium 15 h before the experiment when cells were one-tenth confluent, at 5×10^5 /dish. At time zero, the fresh medium containing 10% fresh serum was replaced with fresh medium containing 0.25% depleted serum. The replacement medium was prepared by diluting depleted medium containing 10% serum with fresh medium without serum. The mean activities $\pm$ s.d. of six parallel control cultures in unchanged medium are shown at 0 and 9 h (open squares). (B) Slowly growing cells (circles). After 3 days growth in unchanged medium, the cell population had begun to slow its rate of proliferation and had partially increased the averaged proteinase activity at a density of 9×10^5 cells per dish. The partially depleted medium containing 10% serum was replaced with medium containing 1% depleted serum. The mean activities $\pm$ s.d. of five parallel control cultures in unchanged partially depleted medium containing 10% serum are shown at 0 and 11 h (open circles). Results in both (A) and (B) were qualitatively similar when proteinase activity was expressed in terms of cell number instead of mg of protein.

The fact that the final 2.7–3.0%/h turnover rate can be sustained for at least 9 days in near-confluent quiescent cultures indicates that turnover either is stable under these culture conditions or is very slowly declining (Fig. 3). However, it appears that a factor or factors that are not depleted from 6–10% serum by cell growth are necessary to sustain a maximal 3%/h rate of quiescent-cell protein turnover (Fig. 4; see also Lockwood & Minassian, 1982). Thus when cell growth was manipulated by decreasing the serum concentration to 0.5 or 0.25%, then net protein accumulation ceased under an insufficiency of these unknown factors (Fig. 4); and the resulting experimental conclusion was that the cessation of net protein accumulation was accompanied by a large synthetic decrease and a small degradative increase. A similar conclusion resulted when turnover kinetics were observed in overcrowded cultures with presumably high concentrations of metabolic products, or under the severe nutritional insufficiency caused by culture in unchanged Eagle's minimal essential medium. These conclusions are similar whether final steady-state turnover rates under the various conditions are compared by observations of synthesis (Figs. 3 and 4) or degradation (Fig. 2). The present results might be relevant to the opposing reports that protein degradation was increased (Bradley *et al.*, 1976) or decreased (Kafatory *et al.*, 1978) in association with the senescence of WI-38 cells. Further, the present findings might also provide a partial explanation of the differences frequently observed in half-lives of identical individual proteins in cultured cells. Nonetheless, the exact relationship of any of the present culture conditions to the control of protein synthesis or degradation *in situ* is uncertain (Lodish, 1976; Ochoa & de Haro, 1979; Holzer & Heinrich, 1980; Nielsen & McConkey, 1980).

Prolonged cell survival under widely varying steady-state turnover rates (Figs. 2 and 4) requires co-ordination of protein degradation and synthesis; however, the identity of the proteolytic mechanism(s) involved remains uncertain. A large fraction of quiescent 3T3-cell protein degradation can be promptly inhibited in a dose-dependent manner by the glycolytic inhibitor 2-deoxyglucose, or by the inhibitor of oxidative phosphorylation, 2,4-dinitrophenol (T. D. Lockwood & A. L. Goldberg, unpublished work). Thus the presently observed decline in protein synthesis and degradation might be a co-ordinated response to an energy deficit resulting from nutritional and/or hormonal deprivation.

Growth-related changes in lysosomal proteinase activity and protein degradation

The present discussion is continued in the accompanying paper in relation to a study of protein

turnover in SV-3T3 cells that have lost the ability to increase lysosomal proteinases (Lockwood & Minassian, 1982).

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