Evidence for heterogeneity of protein-turnover states in cultured cells

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Previous studies on L-cell cultures [Amenta & Sargus (1979) *Biochem. J.* **182**, 847–859] have suggested: (a) that degradation of slow-turnover proteins occurs in a distinct cell state (D-state); (b) that cells randomly enter the D-state with a first-order transition constant, rapidly degrade cell protein, and return to a quiescent G_0 -state. In the present study we have tested the hypothesis that the putative D-state exists as a substate within A-state (non-replicating) fibroblasts. Rat-embryo fibroblasts were prelabelled with [^{14}C]leucine and [^3H]thymidine, 'chased' for 24 h, and then placed in fresh growth medium containing either vinblastine ($10\mu\text{M}$) or colchicine ($25\mu\text{M}$) for three successive 24 h periods. Cells trapped in mitosis were separated from the residual non-replicating cells and rates of protein synthesis, degradation and net accumulation were measured in both populations. We observed that significant protein degradation occurred only in the non-replicating population, although both populations showed equally high rates of protein synthesis induced by fresh growth medium. These data support the hypothesis that degradation of slow-turnover protein is heterogeneous, occurring only in A-state cells. A model that proposes a separate D-state within G_0 -phase successfully accounts for these observations and previous reports on this cell line [Amenta, Sargus & Baccino (1978) *J. Cell. Physiol.* **97**, 267–283] showing no differences in degradation of the slow-turnover protein pool in growth-stimulated and stationary-phase fibroblast cultures.

Although the degradative mechanisms involved in basal turnover of cell proteins remain largely unknown, the degradative reactions are generally considered to be operating uniformly in all cells [for reviews see Goldberg & Dice (1974) and Goldberg & St. John (1976)]. Studies on rat fibroblasts from our laboratory (Amenta *et al.*, 1978a; Amenta & Sargus, 1979) suggest an alternative possibility: that cultured cells may exist in at least two turnover states, a quiescent state with relatively little protein degradation and an active state characterized by a rapid breakdown of cell proteins. We have hypothesized (a) that quiescent cells in culture randomly enter the active state, degrade a large fraction of the cell protein, and then return to the quiescent state; and (b) that this stochastic mechanism may account in part for the observed first-order decay kinetics of the slow-turnover-protein pool in these cells.

Experiments on cultured L-cells (Amenta & Sargus, 1979) suggest that a high-degradation state (D-state) exists outside of the replicative cycle, possibly as a subset of the cells existing within G_0 -phase. Although these reports suggest the existence of a stochastically derived subpopulation of cells with a high rate of protein degradation in

some cell cultures, the putative unique characteristics of protein turnover in L-cells (King *et al.*, 1960; Warren & Glick, 1968) may invalidate extrapolation of such observations to primary cell strains. Noteworthy in this regard, Poole *et al.* (1977) have observed that, at least under certain conditions, cellular proliferation and protein degradation in cultured fibroblasts appear to be inversely related, 'all-or-none', phenomena. Unfortunately, a reliable method for isolating and studying viable cells in this degradative state is yet to be developed. However, if slow-turnover proteins are degraded in a cell subpopulation that is exclusive of most of the proliferative cell cycle, then we should expect to find differences in protein turnover in replicating and non-replicating cells.

In the present study we have chosen to test this hypothesis by separating a slow-growing fibroblast culture into replicating and non-replicating subpopulations by means of the microtubular inhibitors vinblastine and colchicine. If the putative D-state exists as a subset of the G_0 -phase population, we would expect little or no protein degradation in the replicating population compared with the non-replicating G_0 -phase cells. Our data are consistent

with this prediction. We also unexpectedly found that, in growth-stimulated cultures, containing a mixture of proliferating and non-proliferating cells, both subpopulations show the same stimulation in protein synthesis.

Experimental

Methods

Stock cultures of rat-embryo fibroblasts were subcultured for 4 days in Eagle's minimum essential medium with 10% (v/v) foetal-calf serum containing [^{14}C]leucine (0.8 $\mu\text{Ci}/10\text{ml}$) and [^3H]thymidine (0.2 $\mu\text{Ci}/10\text{ml}$), as previously described (Amenta *et al.*, 1976, 1977a, 1978a; Amenta & Sargus, 1979). All cultures were placed in a non-labelled growth medium for 24 h before the experiment. At this stage these cultures were generally reaching a subconfluent stage, growing at a decreased exponential-growth rate, but susceptible to further proliferation with the addition of fresh growth medium. At zero time all cultures were rinsed three times with 5 ml of cold phosphate-buffer saline (Dulbecco & Vogt, 1954), then placed in either fresh growth medium with 15 mM-leucine or growth medium containing leucine and either 10 μM -vinblastine or 25 μM -colchicine. Vinblastine and colchicine at these concentrations have been previously shown to be non-toxic to this cell line, yet effective in inhibiting cell functions depending on microtubular activity (Amenta *et al.*, 1977b). After 24 h, cultures were chilled in a refrigerator at 4°C for 30 min, then shaken gently for 1 min until most of the rounded mitotic cells were lifted from the monolayer. The medium containing free-floating cells was decanted into 15 ml centrifuge tubes, centrifuged at 4°C for 10 min at 400 g, the clear supernatant decanted, and the cell pellet was washed twice with 2 ml of cold phosphate-buffered saline. Fresh media, as described above, were placed on the cell monolayers and the trapping procedure repeated for two additional 24 h intervals.

To verify the efficacy of this 'mitotic trap', we fixed and stained portions of the cell suspension and scored for mitotic figures. Cells in mitosis comprised 60–85% of the trapped population and this value generally correlated with the decrease in DNA specific activity observed for each time interval. Since the cell pellets obtained from the free-floating mitotic cells were small, pellets from three flasks at each time interval were combined for subsequent analysis. Also at each time interval cell monolayers from both the control group (growth medium only) and the experimental group (medium containing the microtubular inhibitor) were washed three times with 8 ml of cold phosphate-buffered saline and processed as described below.

During the course of these experiments we used

two different methods for processing the pellets and cell monolayers. In the initial experiment with vinblastine, cells were processed as follows: (a) cell pellets were precipitated in 0.25 ml of cold 8% trichloroacetic acid and centrifuged at 4°C for 20 min at 750 g. A portion of the clear supernatant was placed in 10 ml of ACS liquid-scintillation fluid (Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) and counted for acid-soluble radioactivity. The pellet was resuspended in 0.25 ml of water by sonication (Sonifier Cell Disrupter, Plainview, NY, U.S.A.) and a portion taken for assay of DNA (Fujimoto *et al.*, 1977). The remainder was digested in an equal volume of 1 M-NaOH at 37°C for 1 h and samples were taken to determine protein (Lowry *et al.*, 1951) and acid-insoluble radioactivity; (b) cell monolayers were precipitated with 6 ml of cold 8% trichloroacetic acid, scraped, and rinsed with 4 ml of trichloroacetic acid. The precipitated proteins were centrifuged in the cold and a portion of the supernatant was used to determine acid-soluble radioactivity. The pellet was resuspended in 0.5 ml of water by sonication and DNA, protein, and acid-insoluble radioactivity were measured as described above.

In the latter experiment with colchicine, cells were processed as follows: (a) cell pellets were placed in 2 ml of ice-cold 0.2 M-HClO₄ for 30 min and the precipitate centrifuged at 4°C for 10 min at 750 g. A portion of the clear supernatant was added directly to the counting fluid for assay of the acid-soluble radioactivity. The precipitate was then digested in 1 ml of 0.3 M-NaOH for 75 min at 37°C. Samples were taken for assay of cell protein (Lowry *et al.*, 1951) and acid-insoluble radioactivity; the remainder was used for assay of DNA (Robinson *et al.*, 1976); (b) the cell monolayers were precipitated with 6 ml of cold 0.2 M-HClO₄ for 30 min, scraped, and rinsed with an additional 4 ml of HClO₄. The precipitate was digested in 2 ml of 0.3 M-NaOH and the subsequent procedure for assay of DNA, protein and acid-insoluble radioactivity was as described for the cell pellet.

Acid-soluble radioactivity in the media was determined at each 24 h interval by precipitation of a media sample with an equal volume of 16% (w/v) cold trichloroacetic acid (Amenta *et al.*, 1978a). After centrifugation, a portion of the clear supernatant was placed in 10 ml of ACS liquid-scintillation fluid and counted for radioactivity.

Calculation of fractional-turnover rates

Fractional-synthesis rates (k_s), degradation rates (k_d) and growth rates (k_g) were determined from logarithmic plots of specific radioactivity, total radioactivity, and mass (Koch, 1962). We have previously discussed the application and limitations of this method (Amenta *et al.*, 1978a; Amenta &

Sargus, 1979) in studies of slow-turnover protein in cell cultures.

Results

Vinblastine effected a loss of cells from the monolayer that approximated first-order kinetics, with a rate constant of 0.17 day^{-1} (Fig. 1a). Comparable control cultures showed a small cell loss of 0.015 day^{-1} . Cell loss could also be demonstrated by measuring the decreasing amounts of DNA remaining in the cell monolayer treated with vinblastine (Fig. 1b). Cell replication in the control cultures was evident from the increasing amount of DNA in the monolayer cells, particularly during the first 24 h period. Plots of the decreasing specific radioactivity of DNA, measuring DNA synthesis, confirmed that most of the cellular replication in control cultures occurred during the first 24 h of the experiment (Fig. 1c). In the vinblastine-treated cultures we observed a decrease in DNA specific radioactivity during the first 4–6 h, corresponding to the brief increase in DNA previously demonstrated (Fig. 1b). During this initial period at least, vinblastine did not inhibit DNA synthesis nor prevent these cells from remaining attached to the dish surface. The specific radioactivity of the [^3H]DNA remained relatively constant thereafter in the residual monolayer, suggesting that the vinblastine-induced cell loss involved the replicating population observed in the corresponding control cultures.

The control cultures, placed in fresh growth medium daily, showed a fractional growth rate for the cell protein of 0.18 day^{-1} , whereas the vinblastine-treated cultures maintained a constant amount of cell protein after the release of mitotic cells into the medium (Fig. 2a). This latter observation was unexpected, since we had anticipated an actual decrease in monolayer protein, reflecting the progressive loss of both daughter cells caught in the vinblastine trap. Analysis of the protein/DNA ratios, however, indicated that the residual cells in the monolayer (A-state) were actually increasing in size (measured as protein/DNA ratio) and effectively compensating for the loss of replicating (B-phase) cells (Fig. 2b). In the corresponding control cultures, new DNA synthesis produced a 30% decrease in the protein/DNA ratio at 24 h, but at later intervals these cells again approached the protein/DNA ratio observed at zero time.

We also compared the relative growth rates for cell protein attained by the entire population of cells and the residual fraction (A-state cells) in the vinblastine-treated cultures. By dividing the protein mass in each culture by the corresponding [^3H]DNA, we obtained a measure of the protein mass, adjusted for cell losses. The residual mono-

layer cells composed of non-replicating cells increased their protein mass for 48 h at virtually the same rate as the control cultures containing replicating and non-replicating cells. Only at 72 h was a significant difference noted in protein accumulation (Fig. 2c). These results suggested (a) that vinblastine, as expected, was releasing into the medium cells with low protein/DNA ratios, and (b) that the cells remaining on the monolayer, although not replicating DNA, were increasing their protein mass at the same rate as the replicating cells.

The fractional rate of protein synthesis in these cultures was not affected by vinblastine. By placing the control cells in fresh growth medium daily, a high and constant rate of protein synthesis, 0.47 day^{-1} , was maintained throughout the experiment (Fig. 3a). This was consistent with our previous reports showing that the normal rate of protein synthesis in non-proliferating cultures is approx. 0.30 day^{-1} and that fresh growth medium stimulates protein synthesis 50–60% (Amenta *et al.*, 1978a). In the cultures treated with vinblastine, the non-replicating cells remaining in the monolayer showed the same stimulated rate of protein synthesis as the corresponding control culture, which contained both A-state and B-phase cells. Apparently stimulation of protein synthesis occurred not only in the stimulated replicating cells in these cultures, but was equally evident in the so-called quiescent, non-replicating A-state cells. In the former case, augmented protein synthesis was associated with DNA synthesis and replication (Fig. 1b), and in the latter case with cellular hypertrophy (Fig. 2b).

In contrast, the degradation rate of ^{14}C -labelled proteins in the recovered A-state subpopulation differed from the degradation rate in the control cultures (Fig. 3b). The control cultures showed a steady degradation rate of 0.28 day^{-1} for the labelled protein. The cells of the residual monolayer in the vinblastine-treated group showed a slightly greater rate of protein degradation, averaging 0.31 day^{-1} . We have previously demonstrated that, in cultures maintained in a basal state of protein turnover, vinblastine has no effect on the basal degradation of labelled proteins (Amenta *et al.*, 1977a,b). Since total protein degradation in the vinblastine-treated fibroblast cultures remained at control rate, and since the non-replicating subpopulation showed a higher rate than average, we concluded that the replicating cells isolated in the medium by vinblastine were degrading protein at a lower-than-average rate. From Fig. 1(a), which indicated that cells were entering the B-state at a rate of 0.16 day^{-1} , we calculated that for this small fraction of cells to account for the observed difference in k_d values (Fig. 3b), the B-state cells must be degrading protein at a very low rate, namely $0.01\text{--}0.02\text{ day}^{-1}$. Degradation of slow-turnover pro-

Fig. 1. DNA and radioactivity in residual monolayer after removal of mitotic cells

(a) Effect of vinblastine on ^3H DNA recovered in the monolayer. Fibroblasts were prelabelled with ^3H thymidine and ^{14}C leucine, as described under 'Methods.' At zero time, fresh growth medium with $10\mu\text{M}$ -vinblastine without labels was added to experimental group (□) and fresh growth media only to controls (○). After a 24 h interval, cells were separated into two subpopulations, as described under 'Methods' and the cells remaining in the monolayer processed. This procedure was repeated for two successive days with additional cultures. The ^3H -labelled DNA in the residual monolayer is plotted (ordinate) against time (abscissa). Each point represents the average of six values. The pooled s.e.m. was $\pm 2.0\%$ for control and 1.7% for vinblastine-treated groups. At zero time, radioactivity of ^3H DNA was 116800 d.p.m./flask. (b) Effect of vinblastine on DNA recovered in the cell monolayer. Cultures were processed as described in (a). In one experiment cell monolayers were also processed at 4 h. DNA in residual monolayer (ordinate) was plotted against time (abscissa). The average amount of DNA at zero time was $46.1\mu\text{g}$ /flask. Each value represents the average for six flasks. The pooled s.e.m. was 3.9% for control and 4.7% for vinblastine-treated groups. (c) Effect of vinblastine on specific radioactivity of DNA in cell monolayer. Cultures were processed as described in (a). Specific activity of DNA in residual monolayer (ordinate) was plotted against time (abscissa). The average specific radioactivity of DNA at zero time was 2550 d.p.m./ μg . Each value represents the

teins in the cell, unlike synthesis, appeared to be heterogeneous, occurring predominantly in A-state and not in B-phase cells.

To confirm this hypothesis, we assayed the radioactivity in cells recovered from the medium containing vinblastine. These cells, collected during each 24 h interval, showed a 30% higher $^{14}\text{C}/^3\text{H}$ ratio than that observed in the corresponding control monolayers at the same time (Fig. 3*b*). Alternatively, these replicating cells, trapped by vinblastine, showed a $^{14}\text{C}/^3\text{H}$ ratio that corresponded to the average $^{14}\text{C}/^3\text{H}$ ratio of the slow-turnover protein pool existing in the culture approx. 24 h before the assay. These results confirmed that the cells actually in the replicative cycle manifested a relative low degradation rate of cell protein. If we assume that the cell cycle is approx. 20–24 h in cultured rat fibroblasts (Baserga, 1976), these data suggest that degradation of slow-turnover proteins in the replicating cycle was near zero, as indicated previously. Since the A-state cells remaining in the monolayer are turning over cell proteins, the $^{14}\text{C}/^3\text{H}$ ratio at the beginning of each 24 h period is progressively lower. This in turn is reflected in the progressive decrease in the $^{14}\text{C}/^3\text{H}$ ratio in the vinblastine-trapped cells collected over each 24 h period. We also attempted to evaluate the specific radioactivity of the ^{14}C -labelled protein in this subpopulation and were not able to find any consistent difference between the specific radioactivities in monolayer and floating cells. However, the high protein content of the medium relative to the small size of the recovered pellet made the data relatively imprecise in this experiment, in spite of repeated washing of the pellet with buffer.

To check further the possibility that these effects of vinblastine were the result of segregation of cells into proliferating and quiescent cell subpopulations,

and not due to a direct toxic action of this agent on the metabolism of cellular proteins, we repeated these studies, substituting 25 μM -colchicine in place of vinblastine as the trapping agent. The results of this study closely paralleled the data obtained with vinblastine as the trapping agent. With the colchicine block, we were able to obtain, in the medium at 48 and 72 h, cells with 30–60% of the DNA specific radioactivity of the residual monolayer cells, indicating that cells blocked in mitosis were being released into the medium (Figs. 1*d–1f*). Again, the cells of non-replicating residual monolayer showed the same rate of protein synthesis (Fig. 3*c*) and of growth (Figs. 2*d–2f*) as found in the control cultures, whereas distinct differences were found in the rate of protein degradation (Fig. 3*d*). As with vinblastine, the colchicine-treated cultures were losing into the medium a subpopulation that showed little, if any, protein degradation (Fig. 3*d*).

Some minor differences, however, were apparent between the experiments with vinblastine and colchicine. In the latter experiments, for some unknown reason, we were able to stimulate more effectively replication throughout the 3-day experimental period with fresh growth medium (compare Figs. 1*b*, 1*c*, 1*e* and 1*f*). Small, but significant, cell losses were also observed in these experiments (Fig. 1*d*), most probably reflecting the weaker attachment of mitotic cells to the plastic surface during the media changes. This higher rate of replication, however, resulted in a larger fraction of cells in the mitotic trap, accentuating the differences in the fractional degradation rates between the two groups (compare Figs. 3*b* and 3*d*). Finally, release of cells from the monolayer appeared to be somewhat delayed with colchicine, since no difference in the [^3H]DNA specific radioactivity was evident at 24 h, whereas differences were found with vinblastine at

average for six flasks. The pooled S.E.M. was 3.0% for control and 3.8% for vinblastine-treated groups. (*d*) Effect of colchicine on [^3H]DNA recovered in the monolayer. Fibroblasts were prelabelled with [^3H]thymidine and [^{14}C]leucine as described under 'Methods'. Beginning at zero time and daily thereafter, fresh growth medium with 25 μM -colchicine was placed on monolayers of experimental group ($\square$) and fresh growth medium only to controls ($\circ$). At each 24 h interval, cells were separated into two subpopulations as described under 'Methods', and the cells in the residual monolayer processed. [^3H]DNA in the monolayer, as a percentage of the zero-time value (ordinate), is plotted against time (abscissa). Each point represents the average of six values; the zero-time value for [^3H]DNA radioactivity was 141200 d.p.m./flask. The pooled S.E.M. was 1.6% for controls and 1.3% for experimental groups. (*e*) Effect of colchicine on DNA recovered in the cell monolayer. Prelabelled fibroblast cultures were treated and processed as described in (*d*). DNA recovered in the monolayer, as a percentage of the zero-time value (ordinate), was plotted against time (abscissa). Each point is average of six values; the zero-time value for DNA was 53.6 μg /flask. The pooled S.E.M. was 3.7% for the control group and 3.9% for the colchicine-treated group. (*f*) Effect of colchicine on DNA specific activity in the monolayer cells. Prelabelled fibroblasts were treated and processed as described in (*d*). The specific radioactivity of DNA in residual monolayer, as a percentage of the zero-time value (ordinate) was plotted against time (abscissa). Each point is the average of six values; the zero-time value for DNA specific radioactivity was 2635 d.p.m./ μg . The pooled S.E.M. was 2.0% for control group and 2.1% for colchicine-treated group. Values for cells recovered in media (Δ) are presented for comparison with the monolayer.

Fig. 2. Protein in residual monolayer after removal of mitotic cells

(a) Effect of vinblastine on cell protein in monolayer. Fibroblasts were prelabelled as described under 'Methods'. Cells were placed in fresh growth medium daily. At zero time, $10\mu\text{M}$ -vinblastine was added to the experimental groups (□); the control cultures received only fresh growth medium (O). At each 24 h interval, cells in the monolayer were processed as described under 'Methods'. Protein (ordinate), calculated as a percentage of the zero-time value, was plotted against time (abscissa); the amount of protein at zero time was $1132\mu\text{g}/\text{flask}$. Each point represents the average of six values. The pooled S.E.M. was 2.6% for controls and 3.0% for vinblastine-treated groups. (b) Effect of vinblastine on cell size in residual monolayer. Fibroblasts were treated as described in Fig. 1(a) and in (a) above. Total protein divided by total DNA (ordinate) was plotted against time (abscissa). Values are given as percentage of zero-time values; the ratio at zero time was $24.6\mu\text{g}$ of protein/ μg of DNA. Each point represents the average result for six flasks. The pooled S.E.M. for controls was 3.2%, and 3.8% for the vinblastine-treated group. (c) Effect of vinblastine on cell protein in culture adjusted for cell loss. Cell cultures were treated as described in (a). Total protein from (a) divided by total [^3H]DNA from Fig. 1(a) was plotted on ordinate against time on abscissa. Values plotted are percentages of the zero-time value ($969\mu\text{g}/10^5\text{d.p.m.}$). Each point represents the average result for six flasks. The pooled S.E.M. for controls was 3.2%, and 4.3% for vinblastine-treated group. (d) Effect of colchicine on cell protein in monolayer. Prelabelled fibroblasts were treated and processed as described in Fig. 1(d). The total protein in the residual monolayer was plotted as a percentage of the zero-time value (ordinate) against time (abscissa). Each point is average of six values; the zero-time value was $1234\mu\text{g}/\text{flask}$. The pooled S.E.M. was 2.2% for control group and 3.4% for colchicine-treated group. (e) Effect of colchicine on protein/DNA ratio. Prelabelled fibroblasts were treated

this time (compare Figs. 1*c* and *f*). As noted below, this may be related to the ability of vinblastine to affect replicating cells at other than mitosis.

Discussion

By virtue of their ability to disrupt microtubular function, vinblastine and colchicine are effective trapping agents for cells that have entered the mitotic cycle (Marcus & Robbins, 1963; Johnson & Holland, 1965; Pfeiffer & Tolmach, 1971). In our cultures this can be observed under phase-contrast microscopy as a progressive increase in the number of cells withdrawing their cytoplasmic processes and rounding up. Although some of these cells spontaneously float in the medium, chilling of the culture, followed by gentle shaking, dislodges almost all of these rounded cells into the medium (Sinclair & Morton, 1963). We believe that most of these rounded cells recovered from the medium represent cells trapped in mitosis, based on our observations with the phase-contrast microscope, and the high mitotic index and decreased specific radioactivity of the DNA in these cells. Other workers have reported that vinblastine will also block cells near the G₁/S-phase junction (Baker, 1976; Resch *et al.*, 1977). Our experience with this agent also suggests that it may be the more toxic of the two, affecting B-phase cells at other than mitosis. We were never able to recover consistently a well-defined cell population from the medium in experiments with this agent. The small number of cells we did recover appeared to have [³H]DNA specific radioactivities closer to that found in the residual monolayers. These findings are consistent with the idea that vinblastine also inhibits in the pre-S-phase of the cell cycle. For purposes of the present study, whether the block is at the G₁/S-phase interface or within M-phase, or both, is not critical as long as most of the cells blocked in the B-phase by vinblastine are released into the medium. Colchicine, however, appeared to be more specific in its ability to separate replicating and non-replicating cells. For both agents, cells were removed from the monolayer at a net decay rate of approx. 0.16–0.17 day⁻¹ (Figs. 1*a* and 1*d*), obeying the first-order transition kinetics

proposed by Smith & Martin (1973). That this reflected DNA synthesis and replication in the second series of experiments was confirmed by the plots of total DNA (Fig. 1*e*) and DNA specific radioactivity (Fig. 1*f*) in the corresponding control cultures; the rate of DNA synthesis measured in the control cultures was almost exactly the rate of cell loss in the colchicine-treated cultures. With vinblastine the correspondence of the decay curve of the treated culture (Fig. 1*a*) and the synthesis curves of the control cultures (Figs. 1*b* and *c*) was less striking; most of the DNA synthesis appeared to occur within the first 24 h, yet cells were removed from monolayer by vinblastine at a relatively constant exponential rate for 72 h. It is possible that this was a peculiarity of the growth characteristics of these cultures and/or that vinblastine has significant toxic effects on cells not related to microtubules and mitosis. In support of the latter possibility, Creasey *et al.* (1971) have reported that vinblastine at this concentration can inhibit the incorporation of [¹⁴C]valine into cell proteins of isolated leucocytes, whereas this agent can stimulate autophagy in rat liver (Arstila *et al.*, 1974; Hirsimaki *et al.*, 1976) and in Ehrlich ascites-tumour cells (Hirsimaki *et al.*, 1975).

An important consideration in these studies is whether these agents, in addition to segregating two cell subpopulations, directly stimulate protein degradation. In this instance we are concerned with the slow-turnover protein pool, which in our cell system accounts for approx. 90–95% of the mass of cell protein (Amenta *et al.*, 1980). Most important for this study would be the possibility that these agents may be increasing protein degradation in the non-proliferating cells remaining in the residual monolayer (Figs. 3*b* and *d*). In previous studies we have not been able to detect any effect of these agents on basal proteolysis (Amenta *et al.*, 1976, 1977*a,b*), either stimulatory or inhibitory, though we would add that both agents have an immediate inhibitory effect on induced proteolysis occurring in autophagic vacuoles (Amenta *et al.*, 1978*b,c*; Amenta & Brocher, 1980). In these experiments, cells were carefully maintained in fresh growth medium with 10% (v/v) foetal-calf serum to minimize any activation of the autophagic system. Additionally,

and processed as described in Fig. 1*d*). The total protein in the monolayer was divided by the DNA in the monolayer, and the ratio was plotted as a percentage of the zero-time value (ordinate) against time (abscissa). Each point is the average of six values; the zero-time value was 23.0. The pooled s.e.m. was 3.4% for control group and 2.9% for colchicine-treated group. (*f*) Effect of colchicine on cell protein in culture, adjusted for cell loss. Cultures were prepared as described in Fig. 1*d*). The total protein in the monolayer was divided by [³H]DNA, and the ratio plotted as a percentage of the zero-time value (ordinate) against time (abscissa). Each point represents the average of six values; the zero-time value was 873 µg/10⁵ d.p.m. The pooled s.e.m. for controls was 1.5% and 2.2% for colchicine-treated group.

Fig. 3. *Specific radioactivity and labelling ratio in residual monolayer after removal of mitotic cells*

(a) Effect of vinblastine on protein synthesis. Cell cultures were processed as described in Fig. 1(a). The specific radioactivity of cell protein was determined as described under 'Methods' and plotted as a percentage of the zero-time value (160 d.p.m./ μ g). Fractional protein synthesis is given by slope of each line. Each point is average of six values from two separate experiments. The pooled s.e.m. for control was 1.3%, and 1.2% for the vinblastine-treated group. (b) Effect of vinblastine on protein degradation. In cell cultures described in (a), total [¹⁴C]protein in each monolayer was divided by [³H]DNA. This ¹⁴C/³H ratio was plotted as a percentage of the zero-time value (1.56). Fractional degradation is given by slope of each line. For cells maintained in fresh growth medium from day 0 to day 3, $k_d = 0.281$; for cells treated with vinblastine, $k_d = 0.314$. Differences between slopes were tested by using an analysis of covariance (Snedecor & Cochran, 1967). Mean square for difference between slopes was 0.00965 and mean square for residual error was 0.00112. Calculated F value was 8.616 ($P < 0.01$). The pooled s.e.m. for controls was 0.7%, and 0.6% for the vinblastine-treated group. Values for cells recovered in media (Δ) are presented for comparison with cells in residual monolayer ($\square$) in the vinblastine-treated group. Differences between means were tested by using a standard two-way analysis of variance ($P < 0.01$). (c) Effect of colchicine on protein synthesis. Fibroblast cultures were prepared as described in Fig. 1(d). Total [¹⁴C]protein was divided by total protein, and the ratio plotted, as a percentage of the zero-time value (ordinate) against time (abscissa). Each point represents the average of six values; the zero-time value was 108 d.p.m./ μ g of protein. Fractional protein synthesis is given by slope of lines. The pooled s.e.m. for the control was 1.0% and 1.2% for colchicine-treated groups. Values for cells recovered in colchicine-containing media (Δ) are presented for comparison. (d) Effect of colchicine on protein degradation. Fibroblast cultures were prepared as described in Fig. 1(d). The total [¹⁴C]protein was divided by total [³H]DNA and the ratio plotted as the percentage of the zero-time value (ordinate) against time (abscissa). Each point

we were not able to detect any consistent differences in the amount of acid-soluble ^{14}C accumulating in the control and inhibitor-containing media, even though one would expect at least a small difference, since the inhibitor-containing media prevented the small fraction of cells in the B-phase from returning to a G_0 -state in which proteins are being degraded. Nor have we been able to detect any effect of these agents on [^3H]leucine incorporation into cell protein (J. S. Amenta & M. J. Sargus, unpublished work). In these experiments, therefore, any difference in the metabolism of ^{14}C -labelled protein is likely to reflect metabolic differences attributable to the different subpopulations rather than any specific effect of vinblastine or colchicine on protein metabolism. Some unreported observations on the small numbers of cells isolated in the media of control cultures offer additional support for this conclusion. Although a complete analysis was not possible on these cells, the $^{14}\text{C}/^3\text{H}$ ratio, reflecting protein degradation, was within the range of values found in the mitotic cells isolated with colchicine. It is likely that here we were analysing a small number of mitotic cells recovered without the aid of a mitotic trapping agent, mitotic cells that were showing essentially the same low rate of protein degradation as observed in the colchicine-trapped cells.

Our data indicate that protein degradation (slow-turnover pool) occurs predominantly, if not totally, within A-state cells. (This, of course, does not mean that turnover, particularly of fast-turnover proteins directly associated with the replicative process, is not occurring in replicating cells.) That turnover of proteins in the slow-turnover pool occurs largely in A-state cells is also seen in cultures of growing and non-growing L-cells. These cells, when carefully maintained in a high growth state, show virtually no protein degradation, whereas cultures maintained at a high-density steady state, namely A-state cells, degrade cell protein rapidly (Amenta & Sargus, 1979; King *et al.*, 1960; Warren & Glick, 1968). Unlike normal fibroblast cultures, L-cells show little decrease in protein synthesis at high densities, thus magnifying the differences in degradation of cell proteins in A-state and B-phase cells. In addition, it appears that any L-cells returning to the degradative A-state can be easily separated from the proliferating subpopulation (Amenta & Sargus, 1979), facilitating the isolation of a relatively pure non-degradative B-state population. In contrast, normal fibroblasts, even under conditions of growth stimulation, appear to be mixtures of A-state and

B-phase cells (Pardee, 1974, 1978). Thus a critical difference between normal fibroblasts and L-cell cultures appears to be the ease with which one can study reasonably pure populations of A-state and B-phase cells; with L-cells this is easily achieved by modifying growth conditions and isolation techniques, whereas with normal fibroblasts this is attained only when specific separation techniques are used. In both cases, however, it appears that B-phase cells manifest little degradation of the slow-turnover pool, degradation occurring almost entirely within A-state cells. Though this, of course, does not resolve whether degradation is occurring uniformly in all A-state cells or in a subgroup, the hypothesized D-state cells, we would emphasize that failure to find a difference in protein degradation between A-state and B-phase cells would have been strong evidence against the hypothesis of a degradative D-state within the larger A-state population.

A model placing the bulk of protein turnover only in A-state cells predicts that cell cultures, stimulated to proliferate, should manifest a decreased rate of proteolysis. Our previously reported observations on this cell line under these exact conditions, however, appear to contradict this model: these cells maintained a constant rate of basal proteolysis during a brief stimulated growth period and during the subsequent stationary phase (Amenta *et al.*, 1978a). Castor (1977), using WI-38 fibroblasts, and Baxter & Stanners (1978), using rat and hamster fibroblasts, also observed a constant proteolysis rate as cultures shifted from growth to a non-growth high-density state. These observations appear to disfavor the idea that degradation of slow-turnover proteins occurs uniformly and contemporaneously in all A-state cells, but can be reconciled by hypothesizing that degradation of slow-turnover proteins occurs only in a stochastically derived subset of the A-state population, the hypothesized D-state. We have summarized these thoughts in Fig. 4, presenting two alternative hypotheses by which degradation of slow-turnover protein may occur in A-state cells.

With the D-state hypothesis (Fig. 4, model II), cells would leave the quiescent G_0 -state with first-order kinetics and face two alternatives: (a) enter the D-state directly, rapidly degrade a large fraction of cellular protein, and, as small cells, return to the quiescent G_0 -state; or (b) detour through the replicative cycle, then enter the D-state and return to the A-state. Quiescent cells, stimulated to replicate

represents the average of six values; the zero-time value was 0.94. The fractional degradation rate is given by slope of lines. The pooled S.E.M. for controls was 0.8%, and 1.0% for the colchicine-treated group. Values for cells recovered in colchicine-containing media (Δ) are presented for comparison.

Fig. 4. *Alternative models demonstrating degradation of slow-turnover protein in A-state cells*

Model I: all cells in G_0 -phase degrade protein (▨). To enter the proliferative cycle, cells leave G_0 -state via a transition probability (*), cycle through B-cycle (b_1 , b_2 , b_3) and either return to G_0 -state or remain in B-cycle for extended growth. Model II: slow-turnover protein is degraded only in D-state cells (▨). Cells leave G_0 -state via a transition probability, and either directly enter D-state for rapid degradation of cellular protein or detour through growth cycle (α , b_2 , β) before arriving at the D-state. At maximum growth rates, cells remain in B-cycle via the b_1 -shunt and minimize protein degradation. S signifies DNA synthesis and M, mitosis.

for a single cycle only (Amenta *et al.*, 1978a), would be expected to show about the same rate of proteolysis as found in non-proliferating control cultures. Proteolysis would be temporarily delayed for cells in the B-phase until such time as the cells returned to quiescent G_0 -state via the D-state. It is possible that our techniques, measuring cumulative proteolysis over 24 h, were not sensitive enough to detect this delay. Poole *et al.* (1977), using more sensitive methods for detecting both DNA synthesis and proteolysis, have reported this predicted complementary relationship between protein degradation and cell replication. The fact that some cells, e.g. rapidly growing L-cells, do not degrade protein implies, as proposed by Dorian *et al.* (1978), that cells completing mitosis may have the option of returning to the A-state or remaining in the B-phase via a G_1 shunt (b_1 in model II). These cells, by selecting this shunt, may thereby entirely avoid protein degradation occurring in the quiescent A-state.

Although this stochastic model explains many of these and other observations (Amenta *et al.*, 1978a, 1979), a direct test awaits the development of a method for isolating cells characterized by a rapid rate of cell protein degradation (model II) or alternatively the demonstration that degradation of slow-turnover proteins is occurring contemporaneously and approximately equally in all G_0 -state cells (model I).

Finally, the observation that, in growth-stimulated cultures, both the replicating B-phase cells and the non-replicating cells showed an equal increase in protein synthesis was unexpected. It is generally assumed that the increase in protein synthesis found in proliferating cell cultures is a characteristic of replicating, as distinct from the G_0 -state cells (Baxter & Stanners, 1978; Todaro *et al.*, 1965; Levine *et al.*, 1965; Ward & Plegmann, 1969). Some workers have also proposed that an increase in the rate of protein accumulation in the cell is sufficient to initiate the replicative cycle (Shilo *et al.*, 1978; Robinson *et al.*, 1976; Soliro & Amos, 1966). An important consequence suggested by our observations is that the control of general protein synthesis and/or accumulation does not alone regulate the transition from the quiescent to the replicating state. Experiments on L-cells (Amenta & Sargus, 1979) suggest that this accumulated extra protein mass in the non-proliferating cells is later quantitatively degraded within the vacuolar system.

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