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Effect of medium composition on protein degradation and DNA synthesis in rat embryo fibroblasts

(protein turnover/fibroblast growth factor/growth regulation/serum)

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ABSTRACT **Fibroblasts in medium deficient in serum, amino acids, phosphate, or glucose stop synthesizing DNA and increase the rate of degradation of their long-lived cellular proteins approximately 2-fold. There is no difference in the rate of degradation of short-lived proteins under these conditions. Insulin, dexamethasone, and fibroblast growth factor act synergistically to inhibit protein degradation and to stimulate thymidine incorporation to about the same extent as serum. When the medium content in serum or fibroblast growth factor is varied over a wide range, there is a close, inverse correlation between the rate of protein degradation and the extent of thymidine incorporation. When serum is added to cells that have been deprived of serum, the inhibition of protein degradation is immediate whereas the enhanced rate of protein degradation in serum-free medium is attained within 1 hr after serum removal. A 30-min exposure to serum followed by incubation in serum-free medium was as effective as continuous exposure to serum in stimulating thymidine incorporation after 8–24 hr.**

Nontransformed mammalian fibroblasts seeded at low density in appropriate medium normally divide repeatedly until they approach confluence, when the rate of division gradually decreases. Fibroblasts will survive, but will not divide, if the medium is deficient in serum (1, 2), amino acids, phosphate, or glucose (3). On the addition of the limiting factor, quiescent cells undergo a series of coordinated macromolecular changes that has been termed the pleiotypic response (4). Such changes include increased uptake of amino acids (5), nucleic acid precursors (6), phosphate (7), glucose (8), and divalent metal ions (9), and increased synthesis of RNA (10), protein (11), and DNA (12). Contradictory evidence exists concerning changes in cyclic nucleotide levels during the stimulation of quiescent cells (13, 14). Previous studies in this laboratory (15) and in other laboratories (16–18) have indicated that cells exposed to deficient medium show an increased rate of cellular protein degradation. Similar phenomena occur in bacteria (19). In this paper, we will examine in more detail the effects of medium composition on cell growth and on cellular protein degradation.

MATERIALS AND METHODS

Fibroblasts, isolated from trypsinized rat embryos, were cultivated in basal Eagle's medium supplemented with 10% fetal calf serum/10 mM $NaHCO_3$/7.5 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine(tricine)/penicillin and streptomycin (100 μg/ml each) in culture flasks (75 cm^2 growth surface, containing 30 ml of medium) or in Leighton tubes (4.8 cm^2 growth surface, containing 2 ml of medium) and used for experiments at cell confluence between the third and fifth passages. Unless otherwise indicated, all media were supplemented with 10% fetal calf serum. In experiments with labeled media, the cells were washed four times with Hanks' solution at each change of medium. At the end of each experiment the washed cells were dissolved in 0.1 M NaOH/0.4% sodium deoxycholate. Total radioactivity and 8% trichloroacetic acid-soluble radioactivity were measured in each sample of cells and medium as described (15).

DNA synthesis was measured by the thymidine incorporation method of Gospodarowicz (20). Protein was measured by the automated Lowry method (21). Fibroblast growth factor (FGF) was isolated from beef pituitary by the procedure of Gospodarowicz (20).

[^{3}H]- and [^{14}C]Leucine and [^{3}H]thymidine were purchased from Schwarz/Mann.

RESULTS

Table 1 shows the results of an experiment in which we measured the effects of medium composition on three cellular processes—degradation of cellular proteins with rapid turnover rates, degradation of cellular proteins with slow turnover rates, and the incorporation of labeled thymidine. In agreement with the results of previous studies (3), in the presence of serum, the omission of amino acids, glucose, or phosphate had a marked inhibitory effect on the incorporation of thymidine. In the absence of serum (see below) there was very little thymidine incorporation. In the presence of serum, the effects of these omissions on protein degradation were very different depending on the turnover rate of the proteins studied. The omissions had no obvious effect on the degradation of the ^{3}H-labeled proteins with rapid turnover rates, whereas the omissions resulted in a substantially greater rate of degradation of the ^{14}C-labeled proteins with slow turnover rates. The absence of serum alone resulted in a substantially greater rate of degradation of the proteins with long half-lives but had little effect on the degradation of the proteins with short half-lives. In the absence of serum, there was little further effect on the protein degradation rate when the other medium components were omitted.

Because of the apparent inverse correlation between the rate of degradation of proteins with long half-lives and the rate of thymidine incorporation, we studied the phenomenon in more detail. Fig. 1 shows the results of an experiment in which parallel tubes from the same culture were exposed to various amounts of serum in the medium. We measured the rate of release of label from prelabeled long-lived cellular proteins (protein degradation), the amount of cell protein accumulated after 24 hr in culture (cell growth), and the amount of labeled thymidine incorporated (DNA synthesis). It is clear from Fig. 1 that the dependence of the response to serum concentration was very similar for these three processes. The addition of even very small amounts of serum had an effect. Maximum response occurred at 3–5% serum concentration, and half-maximal response occurred at about 1% serum concentration. In Fig. 2, we have plotted the protein accumulation and thymidine incor-

Abbreviation: FGF, fibroblast growth factor.

Table 1. The effect of omitting various medium components on cellular protein degradation and DNA synthesis

Medium component omitted	Protein degradation*				DNA synthesis† [^{3}H]dT incorporation with serum
	Short-lived (^{3}H)		Long-lived (^{14}C)		
	With serum	Without serum	With serum	Without serum	
None	100	113.6 ± 3.9	100	228.6 ± 7.8	100
Amino acids	98.7 ± 5.7	114.9 ± 6.1	175.0 ± 9.4	281.8 ± 8.9	25.6 ± 10.7
Glucose	103.1 ± 4.8	112.3 ± 7.9	205.2 ± 6.3	210.4 ± 8.3	34.3 ± 3.8
Phosphate	103.9 ± 5.3	113.6 ± 6.1	165.1 ± 4.7	219.8 ± 5.7	22.8 ± 3.5

All values are expressed as a percentage of control value and represent the means of three experiments ± SD. These absolute control values were 22.8 ± 1.3%/2 hr for short-lived (^{3}H) protein degradation with serum, 1.92 ± 0.17%/2 hr for long-lived (^{14}C) protein degradation with serum, and 28,900 ± 3000 cpm for DNA synthesis with serum.

* Cells were labeled with [^{14}C]leucine (1 μCi/ml) for 50 hr, left for 15 hr in unlabeled medium, and then labeled for 1 hr with [^{3}H]leucine (10 μCi/ml). Washed cells were incubated in the media shown and the output of trichloroacetic acid-soluble radioactivity was measured after 2 hr.

† Cells were rendered quiescent by incubation in medium containing 0.25% serum for 48 hr. Washed cells were incubated for 24 hr in medium containing 10% dialyzed serum with various medium components omitted. Cells were labeled with [^{3}H]thymidine (1 μCi/ml) for 16 hr (8–24 hr after start of treatment). The incorporation of radioactivity into trichloroacetic acid-insoluble material was determined.

poration of Fig. 1 against the protein degradation in order to illustrate the closeness of the inverse correlation. These results suggest that serum contains a factor or factors that are effective at the same concentration on the degradation of long-lived cellular proteins and on promoting cell growth. However, serum is a very complex mixture and this correlation in dose response could be fortuitous. The close correlation is somewhat surprising since the rate of protein degradation was measured 2 hr after the beginning of the experiment while the thymidine incorporation and the cell protein accumulation were not measured until between 8 and 24 hr later. Consequently, it was of interest to determine the rate at which the transitions took place from the protein degradation rate with serum to that without serum and vice versa. This is shown in Fig. 3. In *A*, we see that when cells cultured in 10% serum were placed in serum-free medium, the transition to a stable, enhanced rate of protein degradation required at least 15 min. In contrast, as shown in *B*, when cells in serum-free medium were exposed to medium containing serum, the transition to the slower rate of protein degradation characteristic of medium containing serum was immediate, with no discernible lag.

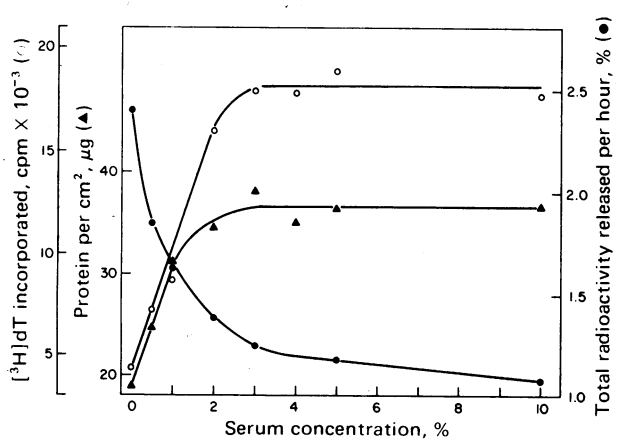

FIG. 1. The effect of serum concentration on DNA synthesis, protein content, and protein degradation. To measure DNA synthesis, we rendered cells quiescent by incubation in medium containing 0.25% serum for 48 hr. After washing, cells were incubated in fresh medium containing various serum concentrations for 24 hr. The cells were labeled with 1 μCi/ml [^{3}H]dT for 16 hr (8–24 hr after the addition of serum). The incorporation of radioactivity into trichloroacetic acid-insoluble material and the protein content of each culture were determined. To measure the protein degradation, we labeled cells with [^{3}H]leucine (1 μCi/ml) for 50 hr, and then left them in unlabeled medium for 15 hr. Washed cells were incubated in medium containing various serum concentrations, and the output of trichloroacetic acid-soluble radioactivity into the medium was measured after 2 hr.

This marked difference between the kinetics of acceleration (slow) and deceleration (immediate) suggested an experiment, the results of which are shown in Fig. 4. Cells cultured in the absence of serum and consequently degrading cellular proteins at a rate of almost 2.5% per hr were exposed to medium containing serum for 15 min (*A*) or for 1 hr (*B*) and then were replaced in serum-free medium. As would be expected from the results of Fig. 3, on addition of serum the rate of protein breakdown decreased immediately and continued at a uniform rate while the serum was present. Surprisingly, when the serum was removed after 1 hr (*B*), the lag period before the maximum rate of protein degradation was attained was almost 50% longer than it was in the experiment shown in Fig. 3 in which cells were exposed to serum for many hours before the serum was withdrawn. The lag period for recovery after 15 min exposure to serum (*A*) was even longer than it was after a 1-hr exposure.

The response of cells to serum depends on the synergistic action of a number of serum components (22). In many cell types, the effect of serum on cell growth is mimicked by the synergistic effect of dexamethasone, insulin, and the FGF isolated by Gospodarowicz (20) from beef pituitary. Consequently,

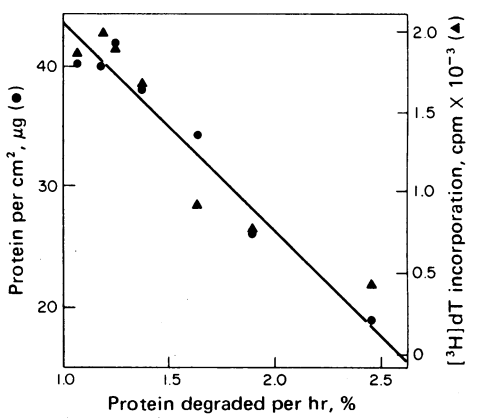

FIG. 2. Correlation diagram between protein degradation and cell growth. Data from Fig. 1.

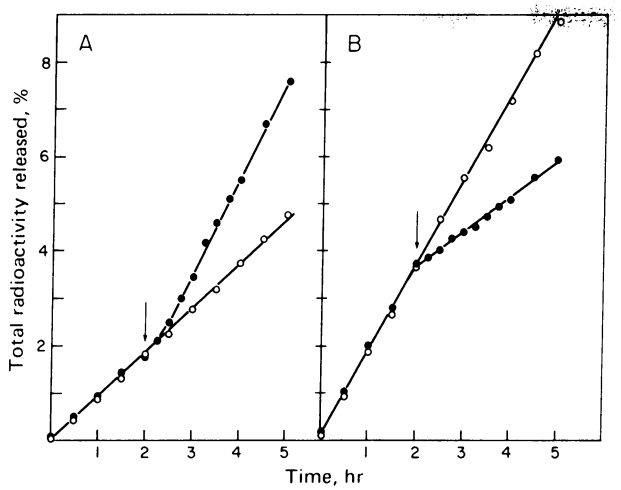

FIG. 3. The reversibility of the inhibition of protein degradation by serum. Cells were labeled for 50 hr with [^{3}H]leucine, and then left in unlabeled medium for 15 hr. (*A*) Output of trichloroacetic acid-soluble tritium: O, fresh control medium containing 10% serum changed after 2 hr (arrow); ●, fresh medium containing 10% serum, changed after 2 hr to fresh medium without serum (arrow). (*B*) Output of trichloroacetic acid-soluble tritium: O, fresh control medium without serum; ●, fresh medium without serum, changed to 10% serum after 2 hr (arrow).

it was of interest to determine what effect, if any, this mixture would have on protein degradation. Table 2 shows the results of an experiment in which the effects of FGF, insulin, and dexamethasone, individually and in combination, were measured on the degradation of long-lived proteins and on thymidine incorporation. None of these three substances alone had a very great effect either on thymidine incorporation or on protein degradation. In combination, however, they acted synergistically to inhibit protein degradation and to stimulate thymidine incorporation. The effect of this combination was almost, but not quite, as great as the effect of serum. Again, there was a close correlation between the effects of medium composition on protein degradation and on thymidine incorporation. Consequently, we investigated the effect of a wide range of concentrations of FGF on the two processes.

Table 2. Effect of insulin, dexamethasone, and FGF on protein degradation and DNA synthesis

	Protein degradation total trichloroacetic acid-soluble radioactivity released per hour,* %	DNA synthesis [^{3}H]dT incorporation into DNA,† cpm × 10^{-3}
Serum 10%	0.91 ± 0.06	28.9 ± 3.1
Serum 0%	2.01 ± 0.08	4.5 ± 1.1
Insulin (50 ng/ml)	1.92 ± 0.03	6.1 ± 0.6
Dexamethasone (0.4 μg/ml)	1.99 ± 0.05	5.2 ± 0.6
Insulin + dexamethasone	1.84 ± 0.08	8.4 ± 0.9
FGF (120 ng/ml)	1.69 ± 0.04	10.4 ± 0.9
Insulin + dexamethasone + FGF	1.24 ± 0.06	23.2 ± 1.8

Means of three experiments ± SD.

* Cells were labeled with [^{3}H]leucine (1 μCi/ml) for 50 hr, left in unlabeled medium for 15 hr, and finally exposed for 1 hr to serum-free medium. Washed cells were incubated in the media shown and the output of trichloroacetic acid-soluble radioactivity was measured after 2 hr. All media without serum contained 0.5% bovine serum albumin.

† Cells were rendered quiescent by incubation in medium containing 0.25% serum for 48 hr. Washed cells were incubated in the media shown for 24 hr. Cells were labeled with [^{3}H]dT (1 μCi/ml) for 16 hr (8–24 hr after start of treatment). The incorporation of radioactivity into trichloroacetic acid-insoluble material was determined.

Fig. 5 shows the results of this experiment. Both for the degradation of long-lived proteins and for the thymidine in-

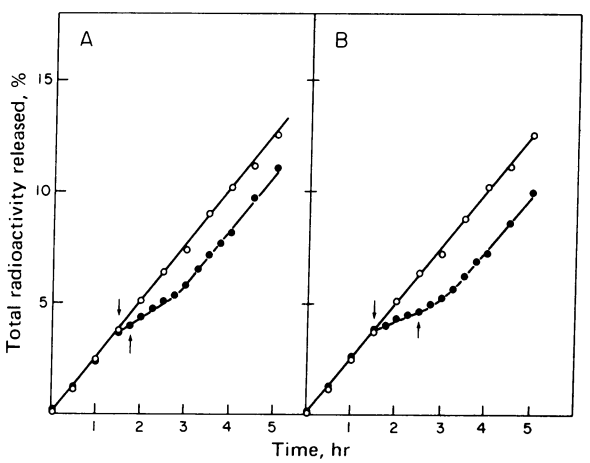

FIG. 4. The effect of a short exposure to serum on the release of trichloroacetic acid-soluble radioactivity into the medium. Cells were labeled for 50 hr with [^{3}H]leucine, and then left in unlabeled medium for 15 hr. (*A*) O, fresh control medium without serum, changed after 105 min (second arrow); ●, fresh control medium without serum, changed to 10% serum after 90 min (first arrow), changed to fresh medium without serum after 105 min (second arrow). (*B*) O, fresh control medium without serum, changed after 150 min (second arrow); ●, fresh control medium without serum, changed to 10% serum after 90 min (first arrow), changed to fresh medium without serum after 150 min (second arrow).

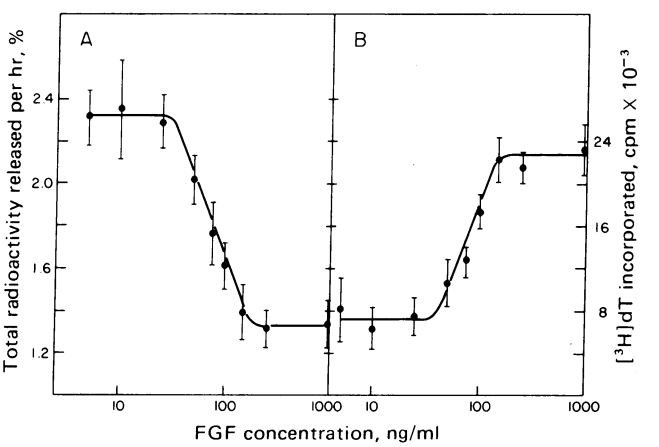

FIG. 5. Effect of FGF concentration on protein degradation and DNA synthesis. (*A*) Protein degradation. Cells were labeled with [^{3}H]leucine for 50 hr, left in unlabeled medium for 15 hr, and finally exposed for 1 hr to serum-free medium. After washing, the cells were incubated in fresh, serum-free medium containing 0.5% bovine serum albumin and various concentrations of FGF, insulin (50 ng/ml), and dexamethasone (0.4 μg/ml) for 2 hr. (*B*) Cells were rendered quiescent by incubation in medium containing 0.25% serum for 48 hr. After washing, the cells were incubated with varying concentrations of FGF, insulin (50 ng/ml), and dexamethasone (0.4 μg/ml) for 24 hr. During the last 16 hr of incubation, [^{3}H]thymidine (2 μCi) and thymidine (2.5 μg) were added to each tube.

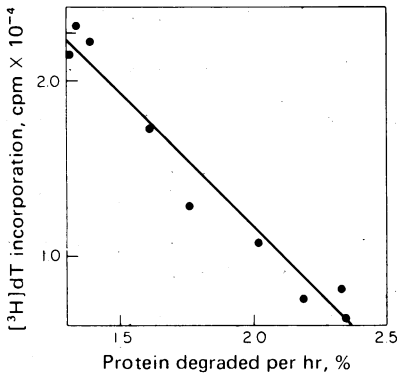

FIG. 6. Correlation between protein degradation and thymidine incorporation in fibroblasts exposed to various concentrations of FGF. Data from Fig. 5.

corporation, the concentrations of FGF required for zero, half-maximal, and maximal response were very similar. The closeness of the inverse correlation between thymidine incorporation and protein degradation is illustrated in Fig. 6 in which the two measurements have been plotted against one another.

Because it was seen from the data of Fig. 3 and 4 that the effect of serum on the degradation of long-lived protein was reversible at least an hour after serum removal, and because the incorporation of thymidine was measured many hours after exposure to serum, we investigated the effect of various durations of exposure to serum on subsequent thymidine incorporation. The results of this experiment are shown in Fig. 7. Even as short an exposure time as 15 min had almost as great an effect on thymidine incorporation as did exposure for 24 hr.

DISCUSSION

The close inverse correlation between the dose response functions measured for the degradation of long-lived proteins and for thymidine incorporation makes it unlikely that these two different effects of medium composition are completely unrelated. The inverse correlation holds whether the medium components varied are serum concentration (Fig. 1), amino acid, glucose, or phosphate concentration (Table 1), or the

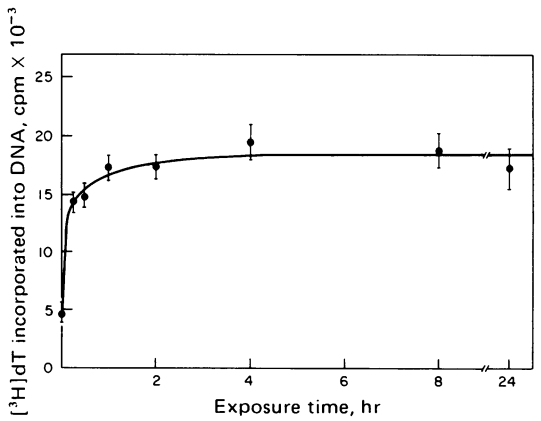

FIG. 7. DNA synthesis in quiescent fibroblasts after a short exposure to serum. Cells were rendered quiescent by incubation in medium containing 0.25% serum for 48 hr. After washing, cells were incubated in medium containing 10% serum for the indicated times. After further washing, cells were incubated in serum-free medium. Incubation was continued so that the cells were incubated for 24 hr after initiation of treatment. All cells were labeled with [³H]dT (1 μCi/ml) for 16 hr (8–24 hr after start of treatment). The incorporation of radioactivity into acid-insoluble material was determined.

Table 3. Correlation between protein degradation and thymidine incorporation

Data from	*n*	Correlation coefficient
Table 1	4	0.863
Fig. 1	7	0.973
		0.944*
Table 4	7	0.997
Fig. 5	9	0.982

* Correlation between protein degradation and protein accumulation.

content of the synergistic factors, insulin, dexamethasone, and FGF (Fig. 5). The correlation coefficients are given in Table 3. It should be stressed that the increased protein content in the growing fibroblasts depends on increased protein synthesis and is not merely the consequence of decreased degradation, although decreased degradation may contribute a substantial amount to this accumulation. In the experiment illustrated in Fig. 1, the decrease in protein degradation in the presence of saturating amounts of serum was only from 2.4 to 1.1% per hr. This 1.3%-per-hr difference applied to the initial 19 $\mu g/cm^2$ would have led to the accumulation of 7 $\mu g/cm^2$ of protein after 24 hr, while the cells treated with serum actually accumulated 17 $\mu g/cm^2$.

A peculiar feature of the close inverse correlation between the degradation of long-lived proteins and thymidine incorporation is the difference in time scale of the two phenomena. Inhibition of protein degradation by serum is immediate and it persists as long as serum is present and for a short period of time after its removal. For reasons that are not clear, the length of this period varies between 15 min, when previous exposure has been for several hours, to as much as 60 min after a 15 min exposure. Stimulation of thymidine incorporation, on the other hand, occurs only after a considerable lag (more than 8 hr after serum was added) and is almost unaffected by the presence of serum. It is practically the same whether serum is left in the medium or removed after as little as 15 min. In the latter case, therefore, DNA synthesis starts several hours after protein degradation has resumed its original high rate. Clearly, inhibition of protein degradation may be an early link in the chain of events leading to DNA synthesis, or it may be a side effect, but it certainly is not a necessary concomitant of the DNA synthesis itself.

Another curious feature of the correlation is its implication with respect to the possible all-or-none nature of the inhibition-sensitive protein degradation process. A given cell may conceivably degrade more or less protein, but it does not make more or less DNA. It either does or does not replicate its DNA. One is tempted to conclude that it similarly either does or does not switch from high to low levels of protein degradation and that this switch, even when reversed later, is linked with an irrevocable commitment to DNA synthesis. If such is not the case and inhibition of protein degradation can be partial and shared uniformly by all cells, then we must admit that the probability for individual cells to commit themselves to DNA synthesis several hours later is directly proportional to the extent to which their protein degradation is inhibited for a short period.

The clear dissociation between the effects of medium composition on the rates of degradation of cellular proteins with slow and with rapid rates of turnover shown in Table 1 is reminiscent of the difference found previously (15) between the sensitivity of the degradation of these two protein classes to fresh

and conditioned media. Clearly, the rate-limiting step in the degradation of the two classes of proteins must be different.

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