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Requirement for Protein Synthesis in the Regulation of Protein Breakdown in Cultured Hepatoma Cells†

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ABSTRACT: The modes of action of insulin and of inhibitors of protein synthesis on the degradation of labeled cellular proteins have been studied in cultured hepatoma (HTC) cells. Protein breakdown is accelerated upon the deprivation of serum (normally present in the culture medium), and this enhancement is inhibited by either insulin or cycloheximide. An exception is a limited class of rapidly turning over cellular proteins, the degradation of which is not influenced by insulin or cycloheximide. Alternative hypotheses to explain the relationship of protein synthesis to the regulation of protein breakdown, viz., control by the levels of precursors of protein synthesis, regulation by the state of the ribosome cycle, or requirement for a product of protein synthesis, have been examined. Protein breakdown was not influenced by amino acid deprivation, and measurements of valyl-tRNA levels in HTC cells subjected to various experimental conditions showed no correlation between the levels of charged $tRNA^{Val}$ and the rates of protein degradation. Three different inhibitors of protein synthesis (puromycin, pactamycin, and cycloheximide) suppressed enhanced protein breakdown in a similar fashion. A direct relationship was found between the respective potencies of these drugs to inhibit protein synthesis and to block enhanced protein breakdown. When cycloheximide and insulin were added following a prior incubation of HTC cells in a serum-free medium, protein breakdown was maximally suppressed within 15–30 min. Actinomycin D inhibited protein breakdown only after a time lag of about 90 min. It is suggested that the regulation of protein breakdown in hepatoma cells requires the continuous formation of a product of protein synthesis, in a manner analogous to the mode of the control of this process in bacteria.

In cultured mammalian cells, the rate of degradation of cellular proteins is subject to environmental and hormonal influences. Thus, the withdrawal of serum from the culture medium accelerates the breakdown of labeled cellular proteins in hepatoma cells (Hershko and Tomkins, 1971) and in fibroblasts (Hershko et al., 1971). This "enhanced" protein breakdown is readily slowed down to the "basal" rate upon the supplementation of either serum or insulin (Hershko et al., 1971; Gelehrter and Emanuel, 1974; McIlhinney and Hogan, 1974). Protein breakdown is also enhanced upon exposure of cultured cells to "conditioned" medium (Poole and Wibo, 1973) or following prolonged incubation with dexamethasone (Gelehrter and Emanuel, 1974). Furthermore, the breakdown of the inducible enzyme tyrosine aminotransferase is similarly regulated by the absence or presence of serum (Hershko and Tomkins, 1971).

Some insight into the mechanisms of the regulation of protein breakdown has been provided by the observation that cycloheximide, an inhibitor of protein synthesis, blocks the enhancement of protein breakdown evoked by serum deprivation (Auricchio et al., 1969; Hershko and Tomkins, 1971). On the other hand, the inhibitor has no further influence on the basal rate of protein breakdown that occurs in cells supplemented with serum or insulin. The action of the inhibitor of protein synthesis on enhanced protein breakdown might mean that the stimulation of protein breakdown requires concurrent protein synthesis. Alternatively, it is possible that cessation of protein synthesis causes the accumulation of a precursor (such as aminoacyl-tRNA) which in turn inhibits enhanced protein breakdown (Goldberg, 1971). It may also be that a specific alteration in the state of the protein synthesizing system, produced by the inhibitor, may influence protein breakdown. For example, the state of polysome aggregation (Stanners, 1966) and the availability of various translational factors or of other substances participating in protein synthesis may all be affected by cycloheximide, and these could in some way participate in the regulation of protein breakdown.

Essentially analogous phenomena occur in bacteria where protein degradation is markedly accelerated upon the deprivation of some essential nutrients, such as a nitrogen source, a carbon source, or inorganic phosphate (Mandelstam, 1963; Pine, 1972; Shechter et al., 1973). Here again, inhibitors of protein synthesis prevent the enhancement of protein breakdown elicited by nutritional deprivation (Mandelstam, 1958; Schlessinger and Ben-Hamida, 1966). Our previous studies, utilizing mutant strains of *Escherichia coli* defective in their *rel* loci or containing temperature-sensitive aminoacyl-tRNA synthetases, have led us to the conclusion that in bacteria, the regulation of protein breakdown requires concomitant protein synthesis (Rafaeli-Eshkol and Hershko, 1974; Rafaeli-Eshkol et al., 1974).

Much less information concerning the mechanisms of regulation of protein breakdown is available in animal cells

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than in bacteria. In the present study, we examine in some detail the modes of action of insulin and of inhibitors of protein synthesis on protein breakdown in hepatoma cells in culture. We find no relationship between the levels of aminoacyl-tRNA and the rates of protein breakdown in various experimental conditions. On the other hand, several lines of evidence suggest that in mammalian cells, as in bacteria, there is a direct requirement for protein synthesis in the regulation of protein breakdown.

Materials and Methods

Cell Culture. Rat hepatoma (HTC) cells were obtained originally from Dr. G. M. Tomkins. The cells were grown in suspension culture in Swim's S77 medium (Grand Island Biological Co.) modified to contain 4 g/l. of glucose, 0.5 g/l. of $NaHCO_3$, 298 mg/l. of L-glutamine, 13.8 mg/l. of L-cystine, 0.05 *M* Tricine[1] (pH 7.6), and 10% (v/v) newborn calf serum (Bio-Lab Ltd., Jerusalem). The doubling time of cell growth was 20–24 hr, and the saturation density, 1–1.2 × 10^6 cells/ml. Periodic tests for mycoplasma contamination were negative.

Chemicals. L-[4,5-^{3}H]Leucine (55 Ci/mmol), L-[U-^{14}C]leucine (340 mCi/mmol), and L-[2,3-^{3}H]valine (30 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Crystalline bovine insulin, cycloheximide, actinomycin D, cordycepin, and puromycin were obtained from Sigma Chemical Co. Pactamycin was the generous gift of Dr. J. R. Swain, Upjohn International Co. All other chemicals used were of reagent grade or tested purity.

Degradation of Slowly Turning Over Cellular Proteins. HTC cells were suspended to a density of 4–5 × 10^5 cells/ml in fresh growth medium, and were incubated with L-[4,5-^{3}H]leucine (2 μCi/ml) at 37°C for 16–20 hr with continuous magnetic stirring. The label of relatively rapidly turning over proteins was then chased by a two-step treatment. First, unlabeled L-leucine was added to a concentration of 4 m*M* (which is approximately 20-fold higher than the concentration of this amino acid in the growth medium) and incubation was continued for 5–7 hr. Subsequently, the cells were centrifuged and resuspended in unlabeled growth medium to a density of 3–4 × 10^5 cells/ml and were further incubated for 18–22 hr. The breakdown of labeled proteins was then estimated by the release of trichloroacetic acid soluble radioactive material, in the presence of an excess of unlabeled L-leucine (to prevent the reutilization of the released labeled amino acid), as described earlier (Hershko and Tomkins, 1971). In brief, the cells were harvested by centrifugation, washed twice, and resuspended in the appropriate media containing 2 m*M* unlabeled leucine, and were incubated under conditions as described in the legends to the figures. Except where otherwise stated, all tested substances were added at the start of the incubation. At timed intervals, duplicate aliquots were withdrawn, treated with trichloroacetic acid, and repeatedly centrifuged; 0.7-ml portions of the clear supernatants were counted with 10 ml of toluene–Triton scintillation mixture (Turner, 1969). Zero-time values of trichloroacetic acid soluble radioactivities were subtracted from the released counts, and the results were expressed as the percentage of the radioactivity initially contained in cellular proteins.

Simultaneous Determination of the Degradation of Rapidly and Slowly Turning Over Proteins. This was measured by a double-isotope method, in which slowly turning over proteins were first labeled by a procedure identical with that described above, except that [^{14}C]leucine (1 μCi/ml) was used in this step. At the end of the second chase period, the cells were exposed to [^{3}H]leucine (5 μCi/ml) for 60 min, and the degradation of the ^{14}C- and ^{3}H-labeled proteins was followed as described above. The results were corrected for the spill-over of the ^{14}C radioactivity into the ^{3}H channel and for quenching.

Assay for tRNA Charging Levels. The levels of charged $tRNA^{Val}$ were determined by a modification of the periodate oxidation method of Lewis and Ames (1972), as described previously (Rafaeli-Eshkol et al., 1974). Samples of 100–200 ml of HTC cell suspensions in serum-free medium (5 × 10^5 cells/ml) were treated with 10% trichloroacetic acid, and were rapidly chilled. The precipitate was collected by centrifugation in the cold and dispersed in 1 ml of a solution consisting of 0.25 *M* sodium acetate (pH 5.0), 1 m*M* EDTA, and 0.05% sodium dodecyl sulfate. Phenol extraction, sonication, and ethanol precipitation of RNA were carried out as described by Lewis and Ames (1972). Periodate and mock-periodate treatments and assay of amino acid acceptor activity of $tRNA^{Val}$ with an enzyme partially purified from *E. coli* were performed as described previously (Rafaeli-Eshkol et al., 1974). The assay was linear up to 1.6 $A_{260\,nm}$ units of HTC cell RNA in the reaction mixture. The specificity of the reaction was tested by the addition of 18 nonradioactive L-amino acids (except L-valine) at 0.02 m*M*, and this did not cause any measurable interference with the incorporation of [^{3}H]valine into HTC cell tRNA. The percentage of charged $tRNA^{Val}$ was calculated as the ratio of acceptor capacity of periodate to mock-treated samples. Accuracy of duplicate samples was within the range of 15% maximal deviation of each other.

Results

Characteristics of the Degradation of Rapidly and Slowly Turning Over Cellular Proteins. Measurements of total protein turnover show that different classes of cellular proteins are degraded at widely heterogeneous rates (Pine, 1972; Goldberg and Dice, 1974). In bacteria, nutritional deprivation accelerates the breakdown of relatively stable cellular proteins, but does not influence the degradation of a class of rapidly turning over proteins (Pine, 1966; Willetts, 1967; Nath and Koch, 1971). Similarly, in cultured rat fibroblasts, fresh medium inhibits the breakdown of proteins with long half-lives, while it has no effect on the degradation of proteins of short half-lives (Poole and Wibo, 1973). In order to define optimal experimental conditions for the study of the regulation of protein breakdown, and to further investigate the resemblance of the characteristics of protein breakdown in cultured animal cells to those of bacteria, we have first examined the influences of serum and insulin on the breakdown of classes of proteins differing in their degradation rates. In the experiment shown in Figure 1, relatively stable cellular proteins were first labeled by a prolonged exposure to [^{14}C]leucine, and following a period of chase, the rapidly turning over fraction was labeled by a short pulse of [^{3}H]leucine; breakdown rates were then estimated by the release of trichloroacetic acid soluble ^{3}H and ^{14}C radioactivities. The initial rate of the degradation of the rapidly labeled fraction was 3–4 times higher than that of slowly turning over proteins, and then decreased sharply with the time of incubation. On the other hand, the release of the label from the slowly turning over class proceeded in

[1] Abbreviation used is: Tricine, *N*-tris(hydroxymethyl)methylglycine.

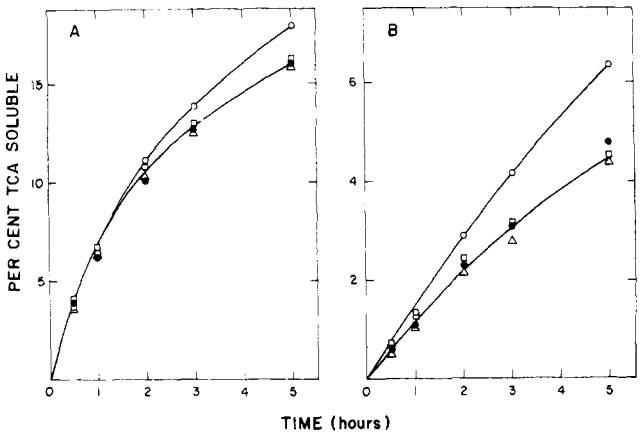

FIGURE 1: Influence of insulin and serum on the breakdown of proteins with rapid and slow turnover rates. The degradation of rapidly and slowly turning over protein classes was determined by the double-isotope method described under Materials and Methods. (A) Rapidly turning over ^{3}H-labeled fraction; (B) slowly turning over ^{14}C-labeled fraction; (○) control (S-77 medium without serum); (●) insulin (5 μg/ml) added; (□) calf serum (15%, v/v) added; (△) insulin and calf serum added.

a more linear fashion, indicating a greater uniformity of the degradation rates of this population of cellular proteins. Insulin and serum had virtually no influence on the rate of degradation of the rapidly labeled fraction until about 2 hr of incubation, and afterwards inhibited it only slightly. By contrast, the degradation of slowly turning over proteins was suppressed by insulin and serum at an earlier time and to a more marked extent (Figure 1). Similar results were obtained in control experiments in which the ^{3}H and ^{14}C labels were reversed.

In further experiments of similar design it was found that the inhibitor of protein synthesis, cycloheximide, suppressed the degradation of proteins with long half-lives, while it had very little influence on the breakdown of rapidly degrading proteins (data not shown). These findings indicate that the previously observed effects of serum, insulin, and cycloheximide on protein breakdown (Hershko and Tomkins, 1971; Hershko et al., 1971) are selective, and influence mainly the degradation of relatively slowly turning over cellular proteins. Therefore, all subsequent experiments were performed under experimental conditions that specifically assess the breakdown of slowly turning over proteins.

Effects of Amino Acid Deprivation and Aminoacylation of tRNA on Protein Breakdown in HTC Cells. We have now tested the possibility that the breakdown of slowly turning over proteins is regulated by the levels of precursors of protein synthesis, such as amino acids or aminoacyl-tRNA (Goldberg, 1971). The effects of the deprivation of a single essential amino acid (valine) or of all amino acids on protein degradation in HTC cells are shown in Figure 2. Deprivation of valine had virtually no influence on protein breakdown, while the deprivation of all amino acids slightly inhibited it. Furthermore, insulin slowed down protein degradation equally in the presence or absence of amino acids.

Next, we have measured the levels of charged tRNA in HTC cells. Preliminary experiments have revealed that in tRNA extracted from HTC cells, highest amino acid accepting activity was obtained with valine (among several amino acids tested), and therefore $tRNA^{Val}$ was chosen for further study. Table I shows the levels of charged valyl-tRNA in HTC cells incubated under various experimental conditions that influence the rates of protein breakdown. In

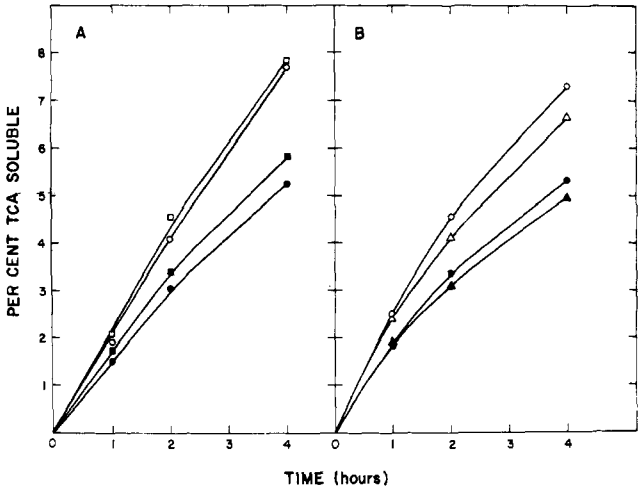

FIGURE 2: Influence of amino acid deprivation on protein breakdown in HTC cells. Degradation of slowly turning over cellular proteins was assayed as described under Materials and Methods, with the exception that prior to the measurement of the release of trichloroacetic acid soluble radioactivity, the cells were washed and resuspended either in serum-free S-77 medium ("control"), or in corresponding media from which valine or all amino acids were omitted. (A) Effect of the deprivation of valine; (B) effect of the deprivation of all amino acids; (○) control; (□) medium devoid of valine; (△) medium devoid of all amino acids; closed symbols, same as above but with the addition of insulin (5 μg/ml).

Table I: Effects of Amino Acid Deprivation and Insulin on the Aminoacylation of tRNA in HTC Cells.[a]

Incubation Conditions	Percent Valyl-tRNA Charged	
	Without Insulin	With Insulin
Expt 1		
Complete medium	82	84
minus valine	26	9
Expt 2		
Complete medium	89	91
minus all amino acids	81	84

[a] HTC cells were washed and resuspended either in serum-free S-77 medium ("complete medium"), or in media of similar composition, except that valine or all amino acids were omitted. Where indicated, insulin was added at a concentration of 5 μg/ml. The cell suspensions were incubated at 37°C with shaking for 2 hr in experiment 1, and for 4 hr in experiment 2, and the charging of $tRNA^{Val}$ was determined as described under Materials and Methods.

cells incubated in the complete medium, around 80–90% of $tRNA^{Val}$ was found to be aminoacylated. The omission of valine caused a marked drop in the levels of charged valyl-tRNA, whereas the deprivation of all amino acids decreased valyl-tRNA charging only slightly. This somewhat surprising finding is presumably due to the decreased utilization of valyl-tRNA for protein synthesis, which is inhibited more markedly when all amino acids are missing than when only valine is lacking. The addition of insulin did not increase significantly the levels of charged valyl-tRNA in cells incubated in the complete medium. When insulin was added to cells deprived of all amino acids or of valine, charged tRNA levels were not changed or were even somewhat depressed (Table I). Comparison of these results with the effects of amino acids and insulin on protein breakdown (Figure 2) shows that there is no relationship between valyl-tRNA charging and protein degradation. Thus, the deprivation of valine, which decreases tRNA charging, does not ac-

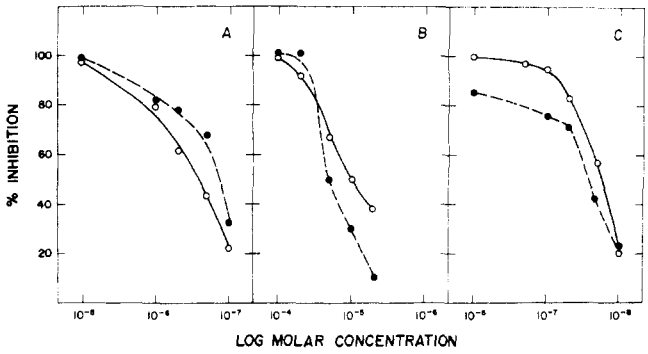

FIGURE 3: Dose-response relationship between the effects of inhibitors of protein synthesis on enhanced protein breakdown and on protein synthesis. *Protein breakdown:* The release of Cl_3CCOOH soluble radioactivity from the slowly turning over protein fraction (see Materials and Methods) was determined following 3 hr of incubation in serum-free S-77 medium, in the presence of the various inhibitors at concentrations as indicated in the figure. To each experiment two controls were added: one without inhibitor (a) for the estimation of enhanced protein breakdown, and another with a high concentration (0.1 m*M*) of cycloheximide (b) to give the value of "basal" protein breakdown. The percentage of inhibition of enhanced protein breakdown was calculated by subtracting the value in the experimental sample (c) from the untreated sample and dividing by the difference between untreated and 0.1 m*M* cycloheximide-treated values (% inhibition = $(a - c)/(a - b) \times 100$). *Protein synthesis:* HTC cells were suspended in serum-free S-77 medium to a density of 5×10^5 cells/ml in the presence of different inhibitors at concentrations as indicated in the figure. The cell suspensions were incubated at 37°C for 30 min and then [^{3}H]leucine (2 μCi/ml) was added and the incorporation of radioactivity into Cl_3CCOOH-precipitable material was measured during an additional 120-min incubation period. The results were expressed as the percentage of inhibition relative to the control sample, to which no inhibitor was added. (A) Cycloheximide; (B) puromycin; (C) pactamycin; (●- - -●) inhibition of enhanced protein breakdown; (○—○) inhibition of incorporation of [^{3}H]leucine into protein.

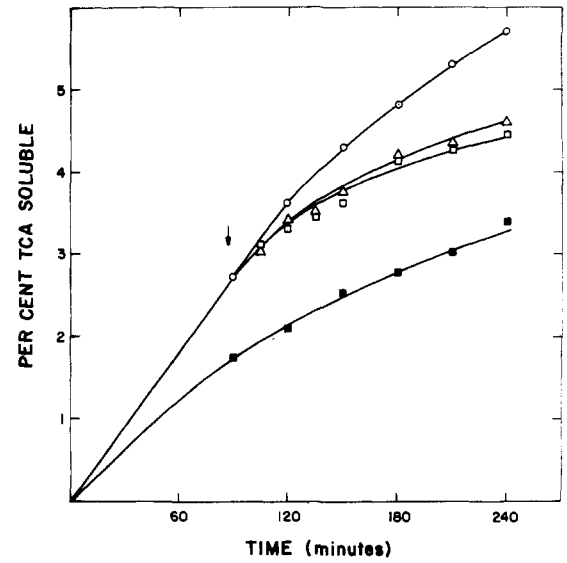

FIGURE 4: Kinetics of the effects of cycloheximide and insulin on enhanced protein breakdown. The degradation of slowly turning over proteins was measured as described in Materials and Methods. (○) Control (S-77 medium without serum); (■) cycloheximide 0.1 m*M* added at 0 time; (□) cycloheximide 0.1 m*M* added at 90 min (arrow); (△) insulin (5 μg/ml) added at 90 min.

celerate protein breakdown, whereas insulin suppresses cellular proteolysis without raising charged tRNA levels.

Effects of Inhibitors of Protein Synthesis on Protein Breakdown. Since protein breakdown did not seem to be influenced by the levels of precursors of protein synthesis, the action of cycloheximide to inhibit enhanced proteolysis also could not be explained by the accumulation of these substances. We have now considered the possibility that a specific perturbation in the state of the protein synthesizing machinery, produced by the inhibitor, might in some way influence protein breakdown. To test this interpretation, we have compared the influence on protein breakdown of various inhibitors of protein synthesis, which act at different sites of the ribosome cycle. These included pactamycin, that at low concentrations specifically inhibits the initiation of translation of HTC cells (Scott et al., 1972), puromycin that releases nascent polypeptide chains (Nathans, 1964), and cycloheximide that blocks both initiation and elongation (Obrig et al., 1971). As shown in Figure 3, all different inhibitors of protein synthesis suppressed enhanced protein breakdown. Moreover, detailed comparison of the concentration curves of these drugs required to inhibit protein synthesis and to block enhanced protein breakdown revealed that pactamycin, puromycin, and cycloheximide inhibited both protein synthesis and protein breakdown at roughly similar doses (Figure 3). It thus seems that inhibition of protein synthesis by any mechanism leads to an inhibition of enhanced protein breakdown at a degree which is directly proportional to the extent of inhibition of protein synthesis.

A remaining possible explanation of the above experiments appeared to be that the formation of some product of protein synthesis may be required for enhanced protein breakdown. In this case, it will have to be assumed that the active substance itself is rather labile, since the effects of inhibitors of protein synthesis on enhanced protein breakdown are quite rapid. This conclusion is based on the experiment illustrated in Figure 4, in which HTC cells were first preincubated in a serum-free medium for 90 min, then cycloheximide or insulin was added and the precise kinetics of their action were examined. Following the addition of cycloheximide, protein breakdown continued at the "enhanced" rate for 15–30 min, and then abruptly slowed down to the "basal" rate (which was essentially identical with that obtained in cells incubated with cycloheximide from the start of the experiment). This shows that the effective lifetime of the product of protein synthesis which may be responsible for enhanced protein breakdown is rather short. Essentially similar kinetics were obtained following the addition of insulin (Figure 4).

Influence of Inhibition of RNA Synthesis. If a product of protein synthesis is required for the acceleration of protein breakdown, the question arises whether RNA synthesis is also necessary for this process. Figure 5 illustrates the time course of the influence of actinomycin D, an inhibitor of RNA synthesis, on protein breakdown in HTC cells incubated in a serum-free medium (the concentration used was sufficient to inhibit over 90% of the incorporation of labeled uridine into RNA). Addition of actinomycin D before the start of the incubation did not change the rate of protein breakdown for the first 90 min, but decreased it sharply afterwards, to a rate which was close to that obtained in the presence of cycloheximide. When actinomycin D was added after 2 hr of incubation, a similar time lag of about 90 min was observed preceding the suppression of enhanced proteolysis (Figure 5).

It may be asked whether the observed delayed inhibition of enhanced protein breakdown by actinomycin D was the consequence of the inhibition of RNA synthesis, or whether it reflects some other action of the drug that directly affects protein synthesis (Singer and Penman, 1972). We find that

the extent of the inhibition of enhanced protein breakdown by actinomycin D (after the initial lag period) is significantly higher than the inhibition of protein synthesis by this drug. Thus, following incubation of HTC cells with actinomycin D (1 μg/ml) for 3 hr, the incorporation of labeled leucine into protein was inhibited by about 50%, while enhanced protein breakdown was suppressed by more than 80% (calculated by the method described in Figure 3). These findings suggest that at least part of the effect of this inhibitor on protein breakdown might be exerted by interference with mRNA formation.

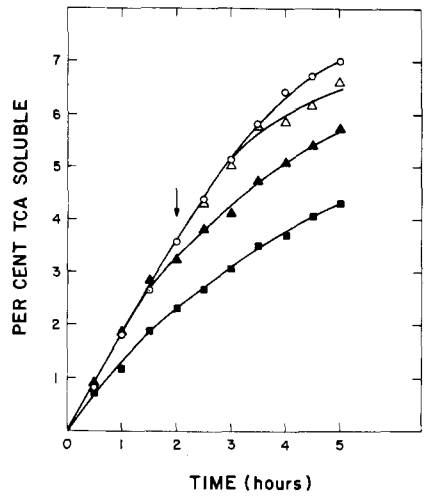

FIGURE 5: Influence of actinomycin D on enhanced protein breakdown in hepatoma cells. Protein breakdown (slowly turning over class) was measured as described under Materials and Methods. (○) Control; (■) cycloheximide 0.1 m*M* added from the start of the experiment; (▲) actinomycin D (1 μg/ml) added from the start of the experiment; (△) actinomycin D (1 μg/ml) added at the time indicated by the arrow.

Discussion

The experiments reported in this paper support the notion that in cultured animal cells, the regulation of protein breakdown involves a continuous requirement for a product of protein synthesis. Alternative hypotheses, such as that protein breakdown is controlled by the levels of aminoacyl-tRNA (Goldberg, 1971) or by the state of the ribosome cycle, have met several experimental difficulties. We find that in HTC cells incubated in a serum-free medium either in the presence or absence of insulin, the charging of $tRNA^{Val}$ is nearly complete (around 80–90% by the periodate oxidation method). Other reports indicate that in various animal cells, such as in the livers of fed or fasted rats (Allen et al., 1969) or in HeLa cells growing in culture (Vaughan and Hansen, 1973), all aminoacyl-tRNA species are nearly fully charged. It might be that charged tRNA levels are higher in animal cells than in *E. coli* due to larger amino acid pool sizes (Holden, 1962; Eagle and Piez, 1962). Since $tRNA^{Val}$ charging is rather high and does not change in various experimental situations that influence protein breakdown (Table I), and since protein breakdown is not affected by a severe drop in the level of charged valyl-tRNA, caused by the single deprivation of valine, it does not seem likely that protein breakdown is influenced by aminoacyl-tRNA levels. It should be pointed out, however, that conclusions based on measurements of valyl-tRNA may not necessarily apply to charged tRNA in general. Furthermore, it cannot be ascertained that the bacterial enzyme used in our assay recognizes all species of hepatoma $tRNA^{Val}$. In addition, though amino acid deprivation does not influence protein breakdown in hepatoma cells (Figure 2) or in fibroblasts (Morhenn et al., 1974), the supplementation of amino acids at rather high concentrations does decrease the degradation rates of the enzymes tyrosine aminotransferase (Hershko and Tomkins, 1971; Lee and Kenney, 1971) and ornithine transcarbamylase (Hogan and Murden, 1974) and suppresses general proteolysis in the perfused liver (Woodside and Mortimore, 1972) and in the isolated diaphragm (Fulks et al., 1975). Since at least some of these results were obtained in experimental conditions comparable to those of the present study (viz., at maximal charging of tRNA), it seems that a high supply of amino acids can influence protein degradation in certain cases by a mechanism which presumably does not involve alteration of aminoacyl-tRNA levels.

The outcome of the experiments on the influence of various inhibitors of protein synthesis on protein breakdown seems to rule out the interpretation that the regulation of protein breakdown is coupled to a specific stage of the protein synthesizing system. Inhibition of initiation or elongation of protein synthesis by pactamycin or cycloheximide produced essentially similar effects on protein breakdown and, moreover, enhanced protein breakdown was also inhibited by puromycin, that allows both initiation and elongation reactions to proceed, but releases prematurely terminated peptides. In addition, a remarkable similarity was found between the dose–response curves of the effects of all three agents on protein synthesis and on enhanced proteolysis (Figure 3). This indicates that a product of protein synthesis is needed for the enhancement of protein degradation, rather than that the inhibition of protein breakdown is secondary to some specific perturbation of protein synthesis.

These suggestions are in agreement with previous conclusions concerning the involvement of protein synthesis in the regulation of protein breakdown in *E. coli* (Rafaeli-Eshkol and Hershko, 1974; Rafaeli-Eshkol et al., 1974). The remarkable analogy between the various characteristics of protein breakdown in animal cells and in bacteria has already been pointed out (Hershko et al., 1971; Hershko, 1973).

At present one can only speculate about the possible significance of the requirement for protein synthesis in the regulation of protein breakdown. It has been suggested that the formation of a labile polypeptide may be involved in the degradation of tyrosine aminotransferase (Kenney, 1967; Barker et al., 1971). It is possible that the continued synthesis of similar protein(s), which either stimulate or participate in enhanced protein breakdown, is necessary for the degradation of many other cellular proteins as well. If such a protein has a regulatory function, its lability may provide a rapid and flexible mechanism for the control of protein breakdown. On the other hand, the mRNA coding for such a regulatory protein may be more stable, since inhibition of RNA synthesis affected enhanced protein breakdown only after a lag of about 90 min (Figure 5).

According to this hypothesis, it may be assumed that insulin and serum inhibit enhanced proteolysis by repressing the formation of such a regulatory polypeptide. This assumption is consonant with the similarity of the actions of insulin and cycloheximide on all aspects of protein breakdown, including their selective effects on slowly degrading proteins and the kinetics of their action. Since the inhibition

of RNA synthesis affects protein breakdown only delayedly, it might be that insulin acts on the formation of the regulatory protein by a post-transcriptional mechanism. More direct testing of these ideas will be possible only with the reconstruction of an in vitro system of protein breakdown, which will also have to account for the other characteristics of this process, including its specificity and energy dependence (Hershko, 1973).

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