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Regulation of protein accumulation in cultured cells

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1. A technique is described whereby protein synthesis, protein breakdown and net protein accumulation are measured separately in monolayer cultures of mammalian cells. All rates are expressed as μg of protein per 18h incubation. 2. Under most incubation conditions with either L6 rat myoblasts or T47D human breast carcinoma cells the rates of protein accumulation, determined directly, agreed with the rates obtained by subtracting protein breakdown from protein synthesis. 3. Foetal calf serum, human and bovine colostrum, human milk and insulin increased protein accumulation in both cell lines, mainly as a consequence of effects on protein synthesis. 4. NH_4Cl, in addition to inhibiting protein breakdown in both cell lines in the presence and in the absence of serum, stimulated protein synthesis in L6 myoblasts. 5. Leupeptin slightly inhibited protein breakdown without affecting protein-synthesis rates. 6. Cycloheximide almost completely inhibited protein synthesis, but restricted the net loss of cell proteins under most conditions because protein-breakdown rates were also decreased. 7. The assumptions, limitations and potential application of this technique for evaluating changes in protein turnover are described.

Growth and the concomitant increase in the protein content of cultured cells can be accelerated by agents that selectively stimulate protein synthesis, selectively inhibit protein breakdown or produce a non-selective response in which the net result is an increase in protein accumulation. Insulin, epidermal growth factor, the insulin-like growth factors, as well as mixtures such as serum and colostrum, increase the growth rate of cultured cells by mechanisms that may involve a co-ordinated stimulation of protein synthesis and inhibition of protein breakdown. Other compounds, especially lysosomotropic agents, which include weak bases and inhibitors of lysosomal cathepsins, clearly decrease rates of protein breakdown but may also affect rates of protein synthesis.

Although the effects of many agents on both protein synthesis and protein breakdown have been tested in different cell types (for reviews see Goldberg & St. John, 1976; Ballard, 1977; Amenta & Brocher, 1981), they have not hitherto been examined on both processes in a single cell type with the use of identical time periods and identical media. In the present paper I report details of a technique that permits separate measurements of protein synthesis and breakdown in monolayer cultures. In addition, the rate of protein accumulation over the measurement period can be compared with the predicted rate obtained by subtracting protein breakdown from protein synthesis. The method has subsequently been used to examine the responses produced by a wide range of effectors in rat L6 myoblasts and the T47D human breast carcinoma cell line.

Experimental

Materials

Rat L6 myoblasts and the T47D human breast carcinoma cell line were kindly provided by Dr. J. M. Gunn, Texas A & M University, College Station, TX, U.S.A., and Dr. T. J. Martin, Repatriation General Hospital, West Heidelberg, Vic., Australia, respectively. Foetal calf serum (batch 29 101 829) was purchased from Flow Laboratories, Stanmore, N.S.W., Australia; amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; Earle's salts and Hanks salts were from GIBCO, Grand Island, NY, U.S.A.; penicillin G and streptomycin sulphate were from Glaxo, Boronia, Vic., Australia; Fungizone was from E. R. Squib and Sons, Princeton, NJ, U.S.A.; gentamycin was from Schering Corp., Kenilworth, NJ, U.S.A. Leupeptin was kindly provided by Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14-23 Kamiosaki 3-Chome, Shinagawa-Ku, Tokyo, Japan. The insulin was

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Actrapid from Novo Industri A/S, Copenhagen, Denmark, and epidermal growth factor was a gift from Dr. J. Koch, C.S.I.R.O. Molecular and Cellular Biology Unit, North Ryde, N.S.W., Australia, and was equipotent in a radioreceptor assay with material obtained from Collaborative Research, Waltham, MA, U.S.A. Bovine colostrum (batch 2; Ballard *et al.*, 1982) was collected before suckling at the Northfield Experimental Station, S. Australia, Australia. Human colostrum was obtained on the day of birth, and milk, from the same mother, 45 days later. L-[4,5-^{3}H]Leucine (40–60 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. L-[3,5-^{3}H]Tyrosine (40–60 Ci/mmol) and NCS tissue solubilizer were purchased from Amersham Corp., Sydney, N.S.W., Australia.

Measurements of protein breakdown, protein synthesis and protein accumulation

All incubations were performed at 37°C under a humidified atmosphere of 5% CO_2 in air in sterile conditions. Cells were subcultured into 24-place Costar dishes. Each well contained 1 ml of Eagle's minimal essential medium (Eagle, 1959) containing 5% (v/v) foetal calf serum and antibiotics (Ballard *et al.*, 1980). When monolayers approached confluence the medium in half the wells was replaced with 1 ml of the same medium but with the leucine concentration lowered from 400 μM to 100 μM. Cells exposed to this 'sham-labelling' medium were subsequently used for protein-synthesis measurements. In order to prelabel cell protein for the degradation measurements, the medium in each remaining well was replaced with 1.0 ml of identical medium except for the addition of 1 μCi of [^{3}H]-leucine/ml. After 3 days cells to be used for both synthesis and breakdown measurements were washed twice with medium containing Hanks salts and incubated for 3 h in 1 ml of Eagle's minimal essential medium containing 5 mM-leucine. Zero time is taken as the end of this chase period.

Some wells, typically two from each synthesis multi-well dish and two from each degradation multi-well dish, were harvested at zero time. The harvesting procedure involved removal of medium, rapid washing of each monolayer with two changes of medium containing Hanks salts at 0°C, and fixing twice with 1 ml of 5% (w/v) trichloroacetic acid at 0°C, followed by a wash with 2 ml of water. The trichloroacetic acid was left in contact with the cells for at least 5 min. This washing and fixing procedure was performed as reproducibly as possible and with minimal disturbance of the cell monolayers. The five washings of each well that had been prelabelled with [^{3}H]leucine (the zero-time 'degradation' wells) were combined together with 200 μg of bovine serum albumin, and protein was precipitated with trichloroacetic acid at a final concentration of 10%. The pellet obtained after centrifugation of this mixture was termed the '0 h wash protein'. The pellet was dissolved in 1 ml of 0.5 M-NaOH and the radioactivity was determined by scintillation counting of 50 μl portions in 4 ml of a solution containing 4 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 70 ml of NCS solubilizer per litre of toluene. Radioactivity in the medium amino acid, medium protein and cell protein fractions, described below, was measured in the same way.

Washings were discarded from the wells corresponding to zero time in the dishes to be used for the measurement of protein synthesis. Monolayers washed and fixed at zero time were dissolved in 1 ml of 0.5 M-NaOH containing 0.1% Triton X-100 for the measurement of radioactivity in the degradation (prelabelled) wells and cell protein in both synthesis and degradation wells. These and subsequent procedures are summarized in Scheme 1.

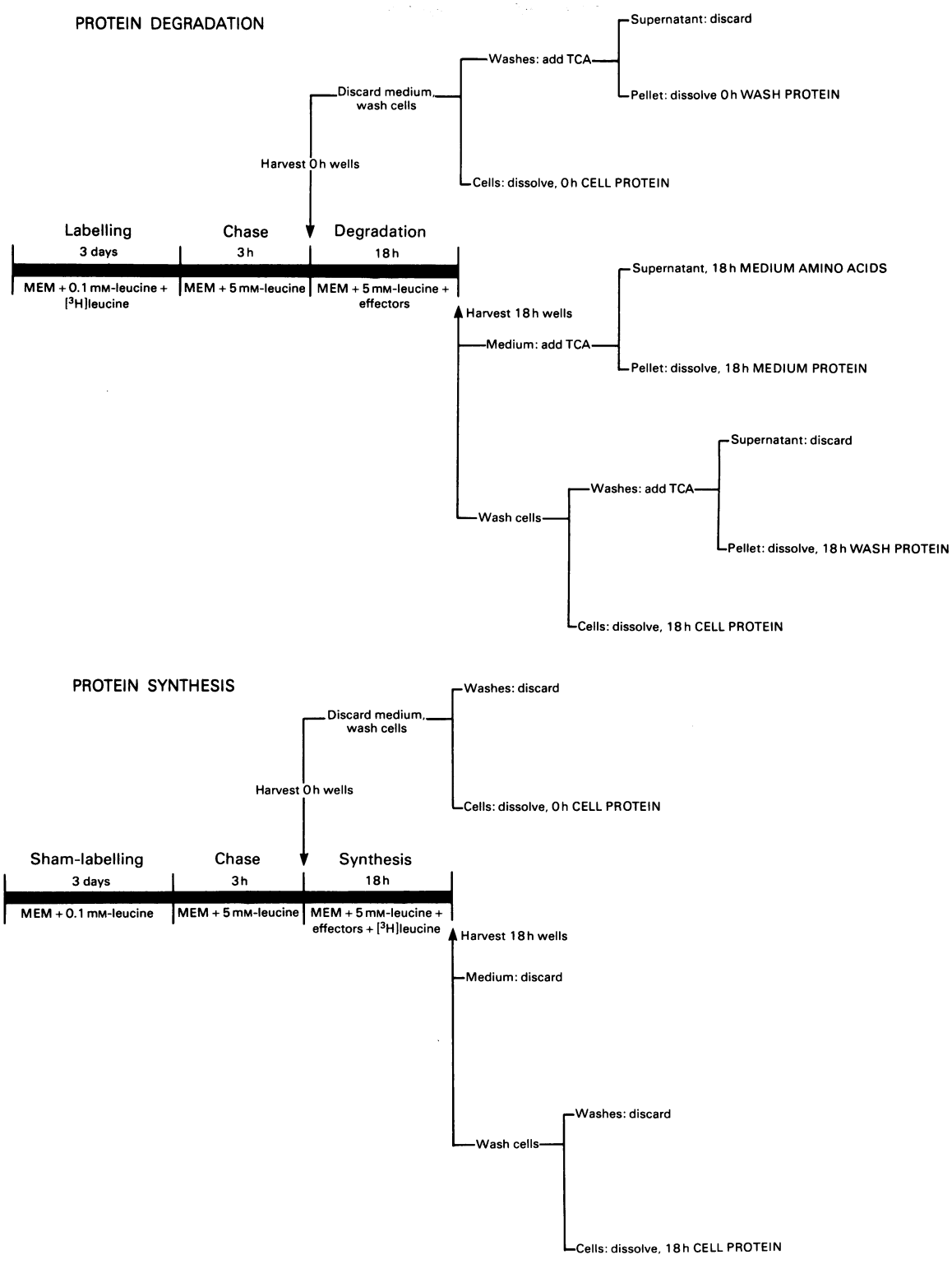
A 900 μl portion of Eagle's minimal essential medium containing 5% (v/v) of a 0.1 M-leucine solution was added at the end of the 3 h chase period to each of the wells other than those harvested at zero time. The only difference between the medium used for synthesis and degradation studies was that 2 μCi of [^{3}H]leucine was added per ml of the synthesis medium. Samples of this medium were taken for counting of radioactivity in order to measure the specific radioactivity of leucine. Putative modifiers were added at zero time to each well at 10 times the desired concentration in 100 μl of 0.9% NaCl. Contents of dishes were thoroughly mixed by swirling.

At the completion of the 18 h period the medium in the degradation wells was transferred to 5 ml tubes, 100 μg of bovine serum albumin was added to each solution and 'medium protein' was precipitated by the addition of trichloroacetic acid to a final concentration of 10%. The monolayers were washed and harvested as described above for the zero-time wells to obtain 18 h 'wash protein' and 18 h 'cell protein' fractions. The supernatant obtained after centrifugation of the trichloroacetic acid-treated medium was termed the 'medium amino acid' fraction. The residual medium obtained after removal of the supernatant was dissolved in 1 ml of 0.5 M-NaOH ('18 h medium protein').

At the completion of the 18 h period or at other times during time-course experiments, the medium in the synthesis wells was removed, and the monolayers were washed, fixed with trichloroacetic acid and dissolved in a manner identical with that described for the degradation part of the experiment except that washing solutions were discarded.

Protein measurements

All proteins that had been dissolved in 0.5 M-



Scheme 1. *Flow sheets for the measurements of protein synthesis and protein breakdown*
The protocols are described in the text. Abbreviations: TCA, trichloroacetic acid; MEM, Eagle's minimal essential medium.

NaOH containing 0.1% Triton X-100 were measured by using an automated technique based on the manual method of Dulley & Grieve (1975).

Leucine content of cell protein

L6 myoblasts and T47D cells were grown to confluence in Petri dishes containing Eagle's minimal essential medium containing 5% (v/v) foetal calf serum and antibiotics. Monolayers were washed twice with serum-free Eagle's minimal essential medium and left for 1h in this medium. The cells were washed with 0.9% NaCl and fixed with 5% trichloroacetic acid. Protein was scraped off the dishes in 5% trichloroacetic acid and centrifuged. Subsequently the protein was washed once with 5% trichloroacetic acid, twice with ethanol, once with 30% (v/v) ethanol in diethyl ether and twice with diethyl ether before being dried in air and stored at 0°C. The protein was dissolved in 0.5M-NaOH, and portions were taken for protein analysis, with bovine serum albumin as standard. Additional portions were hydrolysed in 6M-HCl for 24h *in vacuo* at 110°C. The hydrolysates were evaporated to dryness, reconstituted in 0.01M-HCl, and leucine was measured after chromatography on a JEOL 6AH amino acid analyser with buffers recommended by the manufacturer. Amino acids were detected by *o*-phthalaldehyde fluorescence. The amount of leucine was quantified by using the same 0.1M-leucine solution used to prepare the medium for protein-synthesis measurements as the standard. The amount of leucine present in protein hydrolysates was related to the amount of protein detected as bovine serum albumin equivalents. By this procedure the leucine contents of L6-myoblast protein and T47D-cell protein were 0.765nmol per μg and 0.788nmol per μg respectively.

Calculations

Protein degraded. The amount (μg) of protein degraded was calculated by multiplying the average, corrected, amount of protein in zero-time wells from both synthesis and breakdown dishes by the fraction of labelled protein that was degraded over the 18h test period.

The average, corrected amount of protein at zero time ('0h μg of protein, corrected') was the mean value obtained after multiplying each 0h protein measurement by the following factor derived from the 0h degradation wells:

$$1+\frac{\text{radioactivity in wash protein at 0h}}{\text{radioactivity in cell protein at 0h}}$$

The fraction of labelled protein that was degraded over the 18h period was obtained for each well by using the expression:

$$\frac{\text{18h radioactivity in medium amino acids}}{\text{18h radioactivity in medium protein + wash protein + cell protein}}$$

Protein synthesized. The amount (μg) of protein synthesized was calculated from the leucine incorporation rate and the leucine content of cell protein after a correction and normalization procedure had been applied.

The leucine incorporation rate (nmol/well) equals:

$$\frac{\text{radioactivity in cell protein per well at 18h}}{\text{radioactivity per nmol of leucine in the labelling medium}}$$

Incorporation rates were corrected for protein lost or released during the 18h test period and protein dislodged during harvesting by multiplying each value by the following factor derived from the degradation wells exposed to the same test medium:

$$1+\frac{\text{18h radioactivity in wash protein + medium protein}}{\text{18h radioactivity in cell protein}}$$

Each corrected incorporation rate was normalized to allow for any differences between the amounts of protein in synthesis wells and amounts in degradation wells that had been exposed to identical media by multiplying the corrected incorporation rate by the following factor determined for each test medium:

$$\frac{\text{mean corrected protein content at 18h in synthesis and breakdown wells}}{\text{mean corrected protein content at 18h in synthesis wells}}$$

This corrected and normalized incorporation rate was divided by the amount (nmol) of leucine present in each μg of L6-myoblast protein or T47D-cell protein in order to obtain the amount (μg) of protein synthesized.

Protein accumulated. The amount (μg) of protein accumulated was calculated by subtracting the average corrected amount of protein at zero time ('0hμg of protein, corrected') from each corrected

protein content at 18h. To obtain this latter value the correction factor described above under protein-synthesis measurements was applied to 18h protein analyses from both synthesis and degradation wells.

Statistics

Values are given as means ± S.E.M. Comparisons between means were made by using unpaired *t* tests.

Results

The overall procedure used for the measurement of protein synthesis, degradation and accumulation is designed so that each experiment includes an equal number of synthesis and degradation wells for every treatment. Protein accumulation is measured in both synthesis and degradation wells. The media used for synthesis and degradation studies differ only in that [^{3}H]leucine is present during prelabelling in the degradation studies and during the 18h period for the synthesis studies. Since all other treatments are identical, the synthesis wells have been exposed to the same media and conditions throughout the experiment as have the degradation wells. This is evident from Scheme 1. Comparison between amounts of protein synthesized, degraded or accumulated is possible because all protein measurements are expressed as bovine serum albumin equivalents.

The measurement and correction factors needed to determine the amount (μg) of protein degraded, synthesized and accumulated for a single treatment (medium containing 5% foetal calf serum) are shown in Table 1.

This study, part of a larger experiment in which 15 other treatments were tested with three 24-place multiwell dishes for synthesis and three for degradation measurements, indicates the precision of the various analyses and the magnitudes of the corrections described in the Experimental section under 'Calculations'. It is evident that both the protein-degradation and protein-synthesis measurements are extremely reproducible; there is, however, more variation in the protein-accumulation results (Table 1). The reason for this latter variability is twofold. Firstly, although care was taken during the subculturing to add an equal number of cells to each well, up to 5% variation could be expected. Secondly, for some unexplained reason the amount of cell protein in wells used for degradation studies averaged about 5% lower than in wells used for synthesis measurements. The percentage differences become larger when calculated as protein increments between 0h and 18h. Nevertheless the variability in the rate of protein accumulation is not extremely large, and the resultant rate is very similar to that obtained by subtracting the protein-degradation rate from the protein-synthesis rate.

Leucine was added at a concentration of 5mM in measurements of both protein synthesis and protein breakdown in order to flood the intracellular site of protein synthesis. Provided that this aim can be

Table 1. *Protein synthesized, degraded and accumulated in L6 myoblasts incubated for 18h in medium containing 5% foetal calf serum*

Amounts and radioactivities are expressed per culture well. Values are given as means ± S.E.M. with the numbers of determinations in parentheses. Calculations and other details are described in the text.

Protein degradation	
18h cell protein (10^{-4} × d.p.m.)	139.7 ± 7.6 (4)
18h medium protein (10^{-4} × d.p.m.)	19.3 ± 0.9 (4)
18h wash protein (10^{-4} × d.p.m.)	6.1 ± 0.3 (4)
18h amino acids (10^{-4} × d.p.m.)	46.2 ± 2.1 (4)
Fraction of protein degraded	0.219 ± 0.002 (4)
Protein degraded (μg)	30.2 ± 0.3 (4)
Protein accumulation	
0h protein (μg)	133.9 ± 3.3 (12)
0h cell protein (10^{-4} × d.p.m.)	198.3 ± 7.2 (6)
0h wash protein (10^{-4} × d.p.m.)	6.1 ± 0.4 (6)
0h protein, corrected (μg)	138.0 ± 3.4 (12)
18h protein (μg)	160.4 ± 6.5 (8)
18h protein, corrected (μg)	189.8 ± 7.7 (8)
Protein accumulated (μg)	51.8 ± 7.7 (8)
Protein synthesis	
Leucine incorporated (nmol)	58.5 ± 1.6 (4)
Leucine incorporated, corrected (nmol)	69.2 ± 1.9 (4)
Leucine incorporated, normalized (nmol)	64.2 ± 2.8 (4)
Protein synthesized (μg)	83.9 ± 3.7 (4)
Protein synthesized − protein degraded (μg)	53.7

accomplished, the re-utilization of labelled leucine released during protein breakdown will be minimized, to produce a situation whereby measured protein-breakdown rates will not be underestimates of true rates.

If the specific radioactivity of the intracellular leucine pool used for protein synthesis approaches the extracellular specific radioactivity as the leucine concentration is increased at constant extracellular specific radioactivity, the amount of label incorporated should increase to a plateau. It can be seen from Fig. 1(*b*) that the leucine incorporation does increase as the concentration is raised to 1 mM for both L6 and T47D cells. A more gradual increase in leucine incorporation occurs at higher leucine concentrations. The observed rate of protein breakdown in L6 myoblasts is constant at leucine concentrations above 0.5 mM (Fig. 1*a*), but only half the rate is obtained if no leucine is included in the medium. This lowered rate in the absence of leucine is more pronounced in T47D cells, where it represents only 10% of the rate achieved at 0.1 mM or higher leucine concentrations. The degradation experiments are best interpreted as showing extensive re-utilization of label for both cell lines at low added leucine but effective flooding of the intracellular pools when 0.5 mM-leucine or a higher concentration is present.

The gradual increase in leucine incorporation into protein that occurs as the amino acid concentration is increased above 1 mM (Fig. 1*b*) could be explained if leucine truly stimulated protein synthesis. This hypothesis was tested by adding leucine to a synthesis experiment in which [^{3}H]tyrosine replaced [^{3}H]leucine. The experimental protocol was identical with that described in the Experimental section except the tyrosine and control leucine concentrations were 0.5 mM and 0.4 mM respectively. It was observed that in L6 myoblasts 5 mM-leucine produces a 5% increase in protein synthesis in the absence of serum and an 8% increase when 5% foetal calf serum is present (Table 2). This increase approximates that observed as the leucine concentration was increased from 0.4 mM to 5 mM in the previous experiment (Fig. 1*b*). However, leucine did not alter rates of tyrosime incorporation into protein in T47D cells (Table 2).

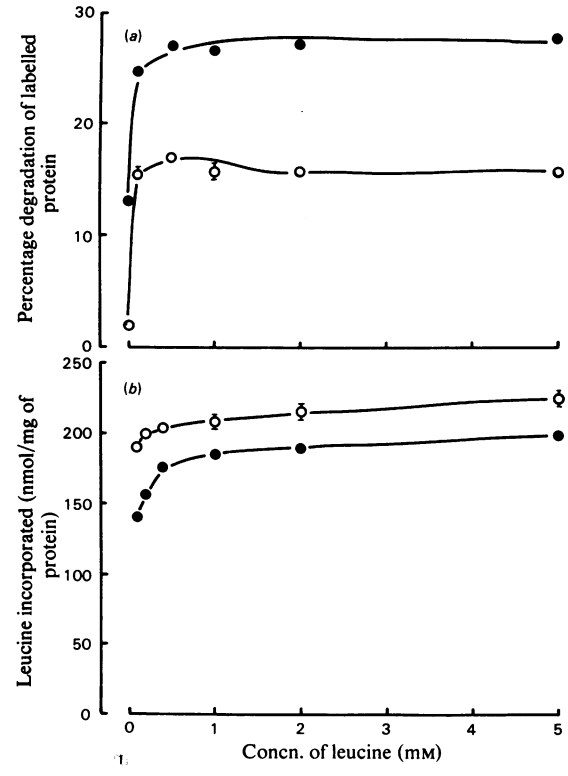


Fig. 1. *Effect of leucine concentration on protein synthesis and breakdown in L6 and T47D cells*

(*a*) Protein degradation was measured over 19 h in the presence of the indicated leucine concentrations added to leucine-free Eagle's minimal essential medium after prelabelling of cell protein with [^{3}H]-leucine. (*b*) Protein labelling was measured over 19 h in the presence of different leucine concentrations added to leucine-free Eagle's minimal essential medium. The specific radioactivity of [^{3}H]leucine was constant. Values represent means ± S.E.M. for four determinations at each leucine concentration with L6 myoblasts (●) or T47D breast cells (○). For full experimental details see the text.

Table 2. *Effect of 5 mM-leucine on tyrosine incorporation into cell protein and on the degradation of protein prelabelled with [^{3}H]tyrosine*

The measurement periods were 19 h. Other experimental details are given in the text. Values are means ± S.E.M. for three synthesis measurements and six degradation measurements.

Measurement	Addition(s)	L6 cells	T47D cells
Tyrosine incorporated (nmol/mg of protein)	None	9.1 ± 0.3	15.4 ± 0.3
	5 mM-Leucine	9.6 ± 0.1	14.9 ± 0.2
	5% serum	17.9 ± 0.1	14.6 ± 0.4
	5% serum, 5 mM-leucine	19.4 ± 0.3	14.5 ± 0.2
Percentage degradation of labelled protein	None	27.2 ± 0.2	15.5 ± 0.2
	5 mM-Leucine	25.7 ± 0.1	15.7 ± 0.2

Protein degradation, measured as the release of [^{3}H]tyrosine from prelabelled protein, was slightly diminished in L6 cells when leucine was increased from 0.4 mM in Eagle's minimal essential medium to 5 mM (Table 2). No effect could be detected in T47D cells. It should be noted that similar fractional degradation rates were obtained by this tyrosine method as reported in the preceding study with the leucine technique (compare Table 2 with Fig. 1*a*). On the other hand the incorporation of tyrosine into cell protein occurs at much lower rates than does leucine incorporation, presumably a reflection of the different contents of these amino acids in cell protein.

Effects of anabolic agents

A number of potentially anabolic agents have been tested for effects on protein metabolism in L6 myoblasts (Table 3) and T47D cells (Table 4). These two Tables include composite data from several experiments in which the amount of cell protein at zero time was different. Accordingly protein measurements have been normalized to the mean zero-time protein content for each cell line, namely 126.7 μg per well for L6 myoblasts and 32.5 μg per well for T47D cells. Since the protein-degradation values are based on the protein content at zero time, they are also normalized by this technique. The procedure cannot be extended to synthesis measurements because zero-time values are not involved in those calculations. Synthesis rates have been normalized to the mean rate for control medium (Eagle's minimal essential medium containing 5 mM-leucine) by multiplying each synthesis rate by the factor:

$$\frac{\text{mean } \mu\text{g of protein synthesized in all controls}}{\text{mean } \mu\text{g of protein synthesized in controls for each experiment}}$$

This normalization has also been applied to the data in Tables 5 and 6, thus explaining why control rates are consistent for each cell line.

Protein synthesis is markedly stimulated by foetal calf serum, bovine colostrum and insulin in L6 myoblasts (Table 3), whereas human colostrum and human milk show consistent but lower effects. Neither epidermal growth factor nor creatine alters rates of protein synthesis in this cell line. The responses to insulin and serum are partly additive. Protein breakdown is slightly inhibited by bovine colostrum, moderately inhibited by insulin and stimulated by 2% human colostrum. In general, the measured accumulation of protein over the 18 h experimental period is somewhat less than the value obtained by subtracting degradation rate from synthesis rate, especially under conditions when the synthesis rate is high.

Unlike L6 myoblasts, which do not accumulate significant amounts of protein unless growth factors are added, T47D cells increase their protein content in control incubations (Table 4) and have a relatively high basal rate of protein synthesis when mean zero-time protein content is taken into consideration. In T47D cells protein synthesis is increased by foetal calf serum, human milk and colostrum, epidermal growth factor and insulin, but especially by bovine colostrum. The responses to insulin and foetal calf serum are additive at the concentrations tested. Protein breakdown is inhibited by bovine colostrum, human milk and colostrum, insulin and epidermal growth factor, so that the net protein accumulation in T47D cells is a consequence of anabolic effects on both synthetic and degradative pathways. The measured accumulation rate of T47D-cell protein in

Table 3. *Effects of potential anabolic agents on protein synthesis, degradation and accumulation in L6 myoblasts*
Values are expressed as μg of protein per well and are given as means ± S.E.M. The numbers in parentheses are the numbers of replicate wells used for synthesis or degradation measurements. Accumulation values are based on twice those numbers. Other details are given in the text.

Treatment	Synthesis	Degradation	Synthesis − degradation	Accumulation
Control (14)	31.2 ± 0.6	26.7 ± 0.2	4.5	0.6 ± 2.5
Foetal calf serum (5%) (14)	90.2 ± 2.0	26.5 ± 0.2	63.7	51.7 ± 3.2
Bovine colostrum (1%) (6)	77.6 ± 1.1	24.5 ± 0.1	53.1	48.8 ± 4.0
Bovine colostrum (5%) (6)	97.1 ± 1.3	24.5 ± 0.2	72.6	69.6 ± 2.2
Human colostrum (2%) (4)	42.1 ± 0.7	32.0 ± 0.8	10.1	13.9 ± 5.2
Human milk (2%) (4)	37.6 ± 0.7	26.7 ± 0.4	10.9	11.2 ± 4.8
Insulin (1 μM) (14)	81.6 ± 2.1	22.9 ± 0.3	58.7	37.4 ± 3.7
Epidermal growth factor (10 nM) (4)	33.1 ± 0.6	25.8 ± 0.8	7.3	5.2 ± 3.8
Foetal calf serum (5%) + insulin (1 μM) (4)	114.0 ± 2.2	28.3 ± 0.8	85.7	63.6 ± 7.8
Foetal calf serum (5%) + epidermal growth factor (10 nM) (4)	92.3 ± 2.8	25.8 ± 0.4	66.5	51.6 ± 6.0
Creatine (0.5 mM) (4)	32.5 ± 1.2	26.2 ± 0.4	6.3	4.3 ± 6.7
Creatine (5 mM) (4)	29.9 ± 0.6	25.8 ± 0.3	4.1	0.1 ± 6.6

Table 4. *Effects of anabolic agents on protein synthesis, degradation and accumulation in T47D cells*
Values are expressed as μg of protein per well and are given as means ± S.E.M. Other details are given in the legend to Table 3 and in the text.

Treatment	Synthesis	Degradation	Synthesis − degradation	Accumulation
Control (10)	10.9 ± 0.1	4.7 ± 0.1	6.2	8.5 ± 1.4
Foetal calf serum (5%) (8)	17.2 ± 0.5	4.8 ± 0.2	12.4	13.8 ± 1.9
Bovine colostrum (0.5%) (4)	15.5 ± 0.3	4.1 ± 0.1	11.4	14.8 ± 2.2
Bovine colostrum (2%) (4)	22.0 ± 0.5	4.1 ± 0.2	17.9	21.0 ± 2.9
Human colostrum (0.5%) (4)	15.4 ± 0.5	4.1 ± 0.1	11.3	13.1 ± 1.9
Human colostrum (2%) (4)	16.4 ± 0.6	4.3 ± 0.1	12.1	15.6 ± 2.4
Human milk (0.5%) (4)	13.6 ± 0.4	4.1 ± 0.2	9.5	10.0 ± 1.6
Human milk (2%) (4)	16.5 ± 0.6	4.2 ± 0.1	12.3	14.7 ± 2.8
Insulin (1 μM) (10)	13.7 ± 0.2	3.8 ± 0.1	9.9	10.6 ± 1.4
Epidermal growth factor (10 nM) (4)	13.0 ± 0.5	4.4 ± 0.1	8.6	10.0 ± 2.1
Foetal calf serum (5%) + insulin (1 μM) (8)	21.6 ± 0.4	4.1 ± 0.1	17.5	20.9 ± 2.2
Foetal calf serum (5%) + epidermal growth factor (10 nM) (4)	19.5 ± 0.3	4.4 ± 0.1	15.1	20.6 ± 4.4

these experiments is greater than the difference between synthesis and breakdown, the opposite situation to that observed with L6 myoblasts.

The concentration-dependence of the effects of foetal calf serum on protein metabolism in L6 myoblasts is shown in Fig. 2. These results are from a single experiment without the normalizations used to obtain the data in Tables 3–6. Protein synthesis is stimulated half-maximally by 1% foetal calf serum, and a significant effect is noted at 0.1% serum. Protein breakdown is diminished by low concentrations of foetal calf serum to give a maximum effect at 1% serum. At higher concentrations protein-breakdown rates are increased, so that in the presence of 10% foetal calf serum the rate exceeds that found in the absence of serum. However, these changes in protein degradation produce only a minor effect on net protein accumulation. The accumulation curves are very similar whether obtained from direct protein measurements or from subtracting degradation rates from synthesis rates. Half-maximal responses on protein accumulation are produced by 1% serum.

It is evident from many reports on intracellular protein breakdown, where incubations of a few hours are used, that the rate of this pathway responds quickly to anabolic factors. The time course of protein synthesis in both L6 myoblasts or T47D cells shows similar rapid changes (Fig. 3).

It should be noted that the protein-synthesis rates in Fig. 3 are expressed per unit of cell protein in order to establish the time at which a new rate is achieved. Recalculation of the appropriate 18h values in Table 3 on a similar basis indicates an increase in the protein-synthesis rates to twice the control rate when 5% serum is added to L6 cells and to 1.9-fold in the presence of 1 μM-insulin. Since the magnitude of these changes is very close to those evident between 1h and 4h in Fig. 3(*a*), it is clear that the increased rates of protein synthesis are rapidly attained and sustained for at least 18h. The

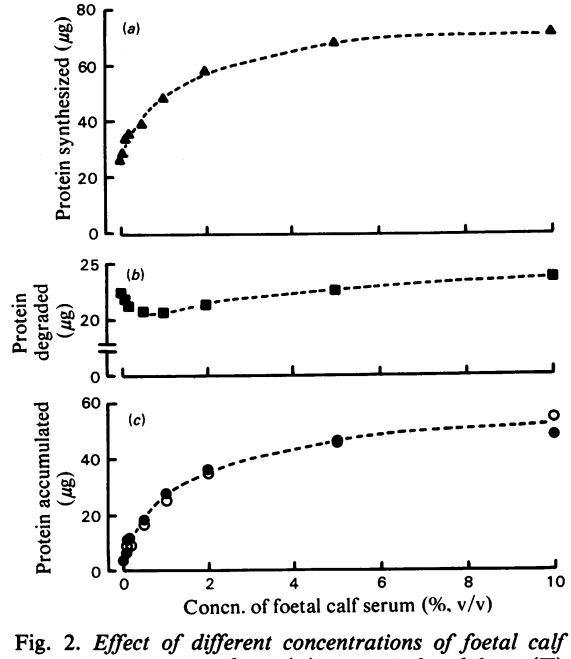


Fig. 2. *Effect of different concentrations of foetal calf serum on protein synthesis* (▲), *protein breakdown* (■) *and the increase in protein content* (●) *in L6 myoblasts*
Protein accumulation is also given as the difference between the synthesis rate and the degradation rate (○). Values are means for four determinations at each serum concentration for synthesis or degradation and for eight determinations for protein accumulation. For full experimental details see the text.

time course shown for myoblasts in the presence of 2% bovine colostrum is similar to that found when 5% serum was added. Since this rate is intermediate between the synthesis rate found with 1% and 5% bovine colostrum (Table 3), it is likely that the increase in protein synthesis observed at 2h is also sustained throughout the total 18h incubation. Similar results were also found with T47D cells (Fig. 3*b*), although the smaller effects on protein synthesis lower the precision of any comparison between early effects and those apparent after 18h incubation.

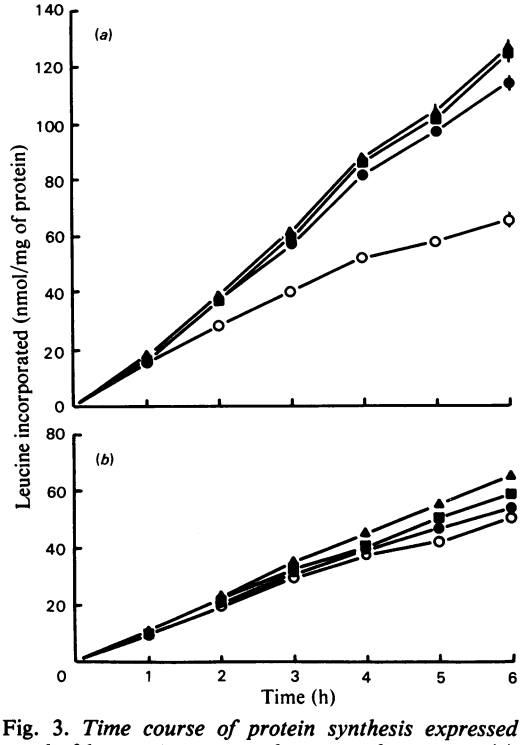


Fig. 3. *Time course of protein synthesis expressed as nmol of leucine incorporated per mg of protein in (a) L6 myoblasts and (b) T47D cells in response to the addition of 5% foetal calf serum (■), 2% bovine colostrum (▲) or 1 μM-insulin (●) at the beginning of the labelling period*
Values with control Eagle's minimal essential medium are also indicated (○). Symbols represent means of six determinations at each time point with standard errors of the means indicated when they are larger than the symbols. For full experimental details see the text.

Effects of lysosomotropic agents and cycloheximide

Ammonia, leupeptin and cycloheximide were tested for effects on protein metabolism both in the presence of control Eagle's minimal essential medium and when 5% foetal calf serum was also added. In L6 myoblasts ammonia produced a substantial inhibition of protein breakdown under all conditions (Table 5). This weak base, when added to control medium, produced increases in protein synthesis and protein accumulation appropriate for an anabolic agent that acted on both protein synthesis and protein breakdown. On the other hand the addition of ammonia with foetal calf serum did not affect protein synthesis, so that the increased rate of protein accumulation could be explained fully by a decrease in the rate of protein breakdown. Leupeptin did not alter rates of protein synthesis, and inhibited protein breakdown in myoblasts only when serum was present. Cycloheximide inhibited protein synthesis by at least 95% in L6 myoblasts under all conditions. This compound also inhibited protein degradation in control medium, but produced a significant increase in the degradation rate if serum was present. In general, the direct measurements of protein accumulation in the myoblasts gave higher values than if calculated by subtracting degradation rates from synthesis rates. This was true in all media containing cycloheximide, and especially so when cycloheximide was present together with serum.

Protein degradation in T47D cells was substantially inhibited by cycloheximide (by 40%) and

Table 5. *Effects of lysosomotropic agents and cycloheximide on protein synthesis, degradation and accumulation in L6 myoblasts*
Values are expressed as μg of protein per well and are given as means ± S.E.M. Other details are given in the legend to Table 3 and in the text.

Treatment	Synthesis	Degradation	Synthesis − degradation	Accumulation
Control (14)	31.2 ± 0.6	26.7 ± 0.2	4.5	0.6 ± 2.5
NH_4Cl (5 mM) (14)	40.9 ± 0.9	20.8 ± 0.4	20.1	18.6 ± 2.9
Leupeptin (50 μg/ml) (10)	32.7 ± 0.4	25.8 ± 0.5	6.9	7.1 ± 2.3
Cycloheximide (0.1 mM) (13)	1.0 ± 0.1	17.1 ± 0.1	−16.1	−8.6 ± 2.0
NH_4Cl (5 mM) + cycloheximide (0.1 mM) (4)	1.6 ± 0.1	15.9 ± 0.3	−14.3	−9.6 ± 8.8
Foetal calf serum (5%) (14)	90.2 ± 2.0	26.5 ± 0.2	63.7	51.7 ± 3.2
Foetal calf serum (5%) + NH_4Cl (5mM) (8)	92.8 ± 2.3	19.8 ± 0.3	73.0	57.7 ± 5.2
Foetal calf serum (5%) + leupeptin (50 μg/ml) (4)	89.7 ± 2.4	20.7 ± 0.2	69.0	56.2 ± 5.5
Foetal calf serum (5%) + cycloheximide (0.1 mM) (7)	2.5 ± 0.3	30.5 ± 0.4	−28.0	−12.4 ± 2.9
Foetal calf serum (5) + NH_4Cl (5 mM) + cycloheximide (0.1 mM) (4)	2.9 ± 0.3	19.8 ± 0.6	−16.9	−5.0 ± 9.1

Table 6. *Effects of lysosomotropic agents and cycloheximide on protein synthesis, degradation and accumulation in T47D cells*

Values are expressed as μg of protein per well and are given as means ± S.E.M. Other details are given in the legend to Table 3 and in the text.

Treatment	Synthesis	Degradation	Synthesis − degradation	Accumulation
Control (10)	10.9 ± 0.1	4.7 ± 0.1	6.2	8.5 ± 1.4
NH_4Cl (5 mM) (10)	12.5 ± 0.4	4.1 ± 0.1	8.4	8.3 ± 1.7
Leupeptin (50 μg/ml) (10)	12.1 ± 0.4	4.5 ± 0.1	7.6	8.9 ± 1.6
Cycloheximide (0.1 mM) (8)	1.3 ± 0.1	2.8 ± 0.1	−1.5	−0.1 ± 1.3
NH_4Cl (5 mM) + cycloheximide (0.1 mM) (4)	1.1 ± 0.1	2.4 ± 0.1	−1.3	2.4 ± 1.8
Foetal calf serum (5%) (8)	17.2 ± 0.5	4.8 ± 0.2	12.4	13.8 ± 1.9
Foetal calf serum (5%) + NH_4Cl (5 mM) (8)	19.1 ± 0.8	4.1 ± 0.1	15.0	17.7 ± 2.5
Foetal calf serum (5%) + leupeptin (50 μg/ml) (8)	18.8 ± 0.4	4.3 ± 0.1	14.5	18.8 ± 2.6
Foetal calf serum (5%) + cycloheximide (0.1 mM) (8)	0.9 ± 0.1	2.7 ± 0.1	−1.8	3.3 ± 1.0
Foetal calf serum (5%) + NH_4Cl (5 mM) + cycloheximide (0.1 mM) (4)	1.0 ± 0.1	2.4 ± 0.1	−1.4	0.7 ± 1.7

moderately by ammonia (by 15%) under all conditions, whereas leupeptin was only slightly inhibitory (Table 6). Ammonia stimulated protein synthesis both in control medium and in the presence of serum. Cycloheximide inhibited protein synthesis by about 90%. The direct values for protein accumulation in T47D cells were slightly higher than those obtained by the difference method. Unlike the situation with the myoblasts, there was reasonable agreement between the two methods of calculating protein accumulation in those incubations where cycloheximide was present.

Discussion

Assumptions and limitations of the technique

The methods reported in the present paper for quantifying rates of protein synthesis, breakdown and accumulation in cultured cells are based on several assumptions, the most important of which are: (*a*) that meaningful rates of protein synthesis can be obtained by assuming that extracellular leucine is the appropriate precursor pool for protein synthesis; (*b*) that labelled leucine is not metabolized before incorporation into cell protein; (*c*) that labelled leucine produced by the degradation of intracellular protein is quantitatively released into the medium as leucine or a non-volatile acid-soluble product; (*d*) that protein released from cells during the protein synthesis incubations or during washing of the monolayers is labelled to the same extent as intracellular protein.

Rates of protein synthesis calculated by using the specific radioactivity of an extracellular amino acid are sometimes lower than rates obtained by using the specific radioactivity of charged tRNA. This discrepancy even occurs when attempts are made to flood the precursor pool by adding up to 5 mM of labelled amino acid (Airhart *et al.*, 1981; Hildebran *et al.*, 1981; Schneible *et al.*, 1981). No attempts were made to measure the specific radioactivity of leucine-charged tRNA in the present study, but the experiments performed with different leucine concentrations (Fig. 1) are consistent with virtual equilibration of the amino acid pools at extracellular leucine concentrations above 0.5 mM. Also, the concordance between the measured synthesis rates and those obtained by adding rates of breakdown to rates of accumulation (see Tables 2–6) argues that any discrepancy must be small. Indeed, with L6 myoblasts the measured rates of protein synthesis are actually higher than those obtained indirectly by the summation technique, a result that is not consistent with the specific radioactivity of leucyl-tRNA being lower than the specific radioactivity of medium leucine. The second assumption appears to be valid because hydrolysis of protein that had been labelled with [^{3}H]leucine either during the 3-day period of prelabelling or during the synthesis measurements showed that at least 98% of the radioactivity was in leucine. The third assumption was tested by chromatographing deproteinized extracts of medium amino acids and collecting eluates for measurement of radioactivity. By this technique approx. 90% of the label that is not precipitated by 3% sulphosalicylic acid or 10% trichloroacetic acid is co-eluted with leucine when the medium is derived from 18 h myoblast incubations (F. J. Ballard, unpublished work). Most of the remaining radioactivity is not eluted until column regeneration and may be present in small leucine-containing peptides. A small amount eluted in the void volume and removed by freeze-drying is presumably ^3H_2O. The further metabolism of labelled leucine released during protein breakdown was assessed by examining the radioactivity present within the monolayers at the end of the 18 h period. For both L6 and T47D cells approx. 1% of this

radioactivity is soluble in trichloroacetic acid or sulphosalicylic acid, and largely represents residual [^{3}H]leucine not removed by aspiration of the medium. As mentioned above, the trichloroacetic acid-precipitable radioactivity is protein-bound leucine. The fourth assumption is important because dislodged radioactive protein from the 0h and 18h degradation wells is used to correct for losses in protein content. The key assumption made here is that the handling procedures are the same throughout. Although there is no doubt that medium protein and wash protein are correctly added to cell protein in order to obtain the total protein in each well, the correction may not be appropriate for measurements of protein synthesis. Thus the correction implies that wash protein and medium protein are labelled equally to cell protein. Should part of the dislodged protein fraction represent poorly labelled protein from dead or dying cells, the correction used would overestimate rates of protein synthesis. However, any overestimation is likely to be minor for most treatments, because at least part of the dislodged protein is surely labelled. Also, the correction factor is small except in the presence of cytotoxic substances such as cycloheximide.

One error in the technique is that rates of protein synthesis will be underestimated to the extent that some proteins labelled during the 18h incubation period will be degraded before the end of this time. An equal error is introduced in the degradation measurement because the labelling protocol is such that proteins synthesized and degraded during the 18h incubation period will not be labelled and accordingly not detected. Thus, although flux in the two pathways is underestimated, accumulation calculated by subtracting degradation from synthesis is correct.

A second unavoidable error is introduced under conditions when net growth is occurring because the protein-synthesis rate is based on an increasing protein content and consequently will be overestimated in relation to the breakdown rate. If we assume that protein increases linearly throughout the labelling period, the true amount of protein degraded can be calculated from the amount present midway through the 18h period. With the example outlined in Table 2, this modification would alter the degradation rate from 30.2μg of protein to 35.8μg of protein (21.9% of 163.9μg). Although this calculation is only an approximation because protein accumulation is exponential during cell growth, it does indicate the magnitude of the introduced error. I have chosen not to modify the data in Tables 2–6 with this correction in order to avoid interdependence of synthesis, degradation and accumulation rates. I note, however, that part of the discrepancy observed in L6 cells between accumulation rates calculated from protein content and accumulation by subtracting breakdown from synthesis rates would disappear if the correction was made. The error is greatest under conditions where the protein accumulation rate is the highest.

A third limitation of the technique is that synthesis and breakdown are measured in separate monolayers. The possibility of using [^{3}H]leucine to measure synthesis rates and [^{14}C]leucine to measure breakdown rates (or the reverse) was examined but discarded, because at least one measurement would involve quantitative measurement of ^{3}H-labelled material in the presence of a very large excess of ^{14}C-labelled protein or amino acid. It would have been possible to measure protein breakdown and synthesis sequentially in the same cultures, as has been reported by Ronning *et al.* (1981), but their method is not appropriate for the relatively long incubation periods needed for detecting changes in protein amount. Tischler *et al.* (1982) have described a method for measuring protein synthesis and calculating protein breakdown as the sum of synthesis and net tyrosine release. This method is especially useful for single muscles, where multiple replicates are not available from the same animal, but cannot provide measures of protein accumulation or loss independent of synthesis and breakdown rates.

Effects of anabolic factors

Foetal calf serum, bovine or human colostrum, human milk and insulin all stimulate protein synthesis and protein accumulation in L6 myoblasts and T47D cells. Although the effects of these agents on protein breakdown are more variable, it is clear that with both cell lines the major anabolic response is on protein synthesis. Thus protein-synthesis rates in myoblasts expressed cumulatively per well over the 18h period (Table 3) or expressed per unit of cell protein (Fig. 3) are increased 2-fold or more by the anabolic agents. On the other hand the largest effect on protein breakdown is produced by insulin, but even this response is no more than a 20% inhibition (Tables 3 and 4).

The components of serum or colostrum that account for the anabolic effect on cell growth are not known. Certainly insulin is not the active factor, at least for the two cell lines studied in the present work. Insulin can be excluded, because its concentration in bovine colostrum is approx. 2nM (Ballard *et al.*, 1982), equivalent to 0.04nM in medium containing 2% colostrum. Neither T47D or L6 cells respond to insulin concentrations below 1nM (F. J. Ballard, unpublished work). It should be noted, however, that in H35 and MH_1C_1 hepatomas, cells that are exquisitely sensitive to insulin (Gunn *et al.*, 1977; Kelley *et al.*, 1978), the anabolic effects of bovine colostrum are lost if the colostrum is

treated with immobilized anti-insulin antibody (Ballard *et al.*, 1982). Since epidermal growth factor is present at approx. 10nM in human milk and 100nM in human colostrum (L. S. Read & F. J. Ballard, unpublished work), this growth factor could account for the moderate anabolic effect of these fluids in T47D cells. However, colostrum also stimulates protein synthesis in L6 myoblasts, cells that do not respond to epidermal growth factor (Table 3). A more likely alternative explanation is that one or other of the insulin-like growth factors accounts for the growth-promoting effects of milk, colostrum and foetal calf serum in cultured cells.

Creatine has been reported to stimulate protein synthesis in muscle-cell cultures (Ingwall *et al.*, 1972), although a re-examination by one of these authors failed to demonstrate any response to creatine over a wide concentration range (Fry & Morales, 1980). The present work confirms the latter finding in L6 myoblasts, because neither 0.5mM- nor 5mM-creatine altered rates of protein synthesis, breakdown or accumulation.

Effects of leupeptin and ammonia

Protein breakdown in T47D and L6 cell lines is only slightly inhibited by leupeptin (Tables 5 and 6), and, accordingly, any effects on protein accumulation are very small. A more substantial response was found when leupeptin was added together with serum to the myoblast cultures. In that experiment the decrease in the rate of protein breakdown was reflected by an increased protein accumulation, since the difference between the two treatments was $7.6 \pm 1.9\,\mu g$ of protein (mean $\pm$ S.E.M., $n = 8$). Many cell types (Knowles & Ballard, 1976; Hopgood *et al.*, 1981; Knowles *et al.*, 1981), including muscle (Libby & Goldberg, 1978), are sensitive to this microbial proteinase inhibitor. Additional measurements on the effect of leupeptin and related compounds on protein synthesis and accumulation, perhaps by using the technique described in the present paper, are appropriate because of the potential application of proteinase inhibitors to diminish muscle protein loss in dystrophies and other muscle-wasting conditions.

Many weak bases, including ammonia, methylamine and chloroquine, have been shown to lower rates of intracellular protein breakdown in isolated cells (Knowles & Ballard, 1976; Seglen, 1975, 1978; Amenta & Brocher, 1980; Grinde & Seglen, 1980; Wibo & Poole, 1974). Ammonia is effective in the two cell lines studied in the present work. It should be noted that in L6 and T47D cells ammonia is equally effective when added in the presence of 5% foetal calf serum, a result that conflicts with the concept that lysosomotropic agents decrease only the 'step-down' increment of protein breakdown that is associated with autophagy. Ammonia also increased the rate of protein synthesis in L6 myoblasts. In support of this finding, the protein-accumulation rate was increased to a greater extent than could be explained if the sole action of the weak base was to diminish protein breakdown. The response is surprising, because others have reported an inhibition rather than a stimulation of protein synthesis by ammonia (Seglen, 1978; Grinde & Seglen, 1980). It is possible that the increase in protein synthesis shown in Table 5 is an artifact of the technique used. Thus some additional labelling of cell protein is expected in the presence of ammonia as a consequence of the extra protein in each well that has accumulated because ammonia is inhibiting protein breakdown. However, even if it is assumed that all of the extra $5.9\,\mu g$ of protein ($26.7 - 20.8\,\mu g$ of protein degraded; Table 5) was present throughout the 18h labelling period, the resultant correction would only account for an additional $1.5\,\mu g$ of protein synthesized ($5.9\,\mu g$ divided by the protein content at zero time, $126.7\,\mu g$, multiplied by $31.2\,\mu g/18\,h$, the synthesis rate in control wells). Clearly, more extensive measurements of the effects of weak bases on protein metabolism are required to resolve the question as to whether weak bases stimulate protein synthesis.

Effects of cycloheximide

In addition to the almost complete inhibition of protein synthesis produced by cycloheximide in both L6 and T47D cells, protein breakdown was inhibited in all situations except when L6 cells were incubated with serum. Inhibitors of protein synthesis have been shown to lower rates of protein breakdown in many cell lines (Goldberg & St. John, 1976), and are thought to interfere with lysosomal proteolysis (Ballard, 1977; Amenta & Brocher, 1981). Accordingly, the effects of saturating concentrations of cycloheximide and ammonia should not be additive (Knowles & Ballard, 1976), a prediction consistent with the present results. Since poisons and other agents that kill cells also decrease protein breakdown to very low rates (Knowles & Ballard, 1976; Goldberg & St. John, 1976; Ballard, 1977), care must be taken in interpreting experiments when inhibitors of protein synthesis are used. The measurement of changes in protein content in cells exposed to cycloheximide may be subject to relatively large errors, because labelled protein in the medium or wash fractions can represent up to 30% of the total radioactivity. Accordingly, the factors generated for the correction of protein content and protein synthesis will be substantial. Protein-breakdown measurements are not subject to the same potential errors, since these are based on the protein content at zero time, i.e. before the cells are exposed to cycloheximide. Also, the possible error in the synthesis measurement is very small in absolute

terms, because synthesis rates are so low. Thus the net losses of protein indicated in Tables 5 and 6 for all incubations where cycloheximide was added are more reliable indices of protein status than are the values obtained by subtracting the corrected protein contents at 18h from the contents at zero time.

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