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Replacement Perfusion of Cultured Eucaryotic Cells: A Method for the Accurate Measurement of the Rates of Growth, Protein Synthesis, and Protein Turnover

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The fractional rates of protein synthesis (k_s) and degradation (k_p) were studied in the myeloma cell line SP2/0-AG14 grown at different rates (k_g). Cells in spinner flask suspension cultures were maintained at constant cellular density for prolonged periods by replacement perfusion of labeling medium at a rate equivalent to the rate of growth. Total protein synthesis was calculated from the specific radioactivity of labeled L-leucine in the precursor (medium) and cellular protein. Fractional synthesis rates determined by approach to equilibrium labeling were the same as those determined by equilibrium-pulse labeling kinetics and pulse-chase kinetics. The rate of protein degradation was determined from the established relationship $k_g = k_s - k_p$. Protein synthesis rates remained constant over a threefold range in the rate of cell growth. At relatively slow growth rates (k_g = 0.017/hr) turnover represented a major fraction of total synthesis (k_p = 0.032/hr = 0.65k_s). At rapid growth rates (k_g = 0.058/hr) the value of k_p was less than 0.005/hr. No major difference was observed between the k_s determined for individual cellular proteins (separated by SDS-polyacrylamide (7.5%) gel electrophoresis) from rapid- and slow-growing cultures. Thus, with an invariable k_s, any change in growth rate is due to an inverse change in the rate of turnover. Since turnover is the balance between synthesis and degradation and since synthesis is unchanging, then changes in the growth rate of SP2/0-AG14 should be due to changes in the rate of protein degradation. Experiments were therefore performed to determine the origin of the degradative machinery, ie, cytosolic or lysosomal; autolysis of prelabeled cellular protein (in vitro) was observed only at acidic pH (4.2) and was totally inhibited by addition of leupeptin (10 μM) and pepstatin (2 μM), the specific inhibitors of lysosomal cathepsins B (&L) and D, respectively. Since growth rate appears to be regulated by the alterations in the rate of protein degradation and degradation (in vitro) in SP2/0-AG14 appears to be lysosomal, then one should be able to alter the rate of cellular growth by interfering with rate of lysosomal proteolysis. Indeed, when the lysosomotropic amine NH_4Cl (10 mM) is added to cells growing with a k_g of 0.018/hr $\pm$ 0.001 (k_s = 0.050/hr $\pm$ 0.002) the growth rate increased to 0.051/hr $\pm$ 0.002 without change in the rate of

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protein synthesis (k_s = 0.049/hr ± 0.003). It is suggested from our data that the cellular growth rate of SP2/0-AG14 is regulated by the lysosomal apparatus; whether this regulation is itself regulated by either a specific compartmentalization of the lysosomal proteinases and/or their substrates or by endogenous protease inhibitors, should prove to be an exciting area for future investigation.

Key words: cellular growth, protein synthesis, protein turnover, lysosomes, proteolysis, myeloma

All cellular proteins undergo a process of intracellular turnover which is characterized by continual protein synthesis and degradation. An independent alteration in either (or both) the synthesis or breakdown rate will lead to a change in intracellular protein levels [8,9,17] or in regard to cellular populations a change in the rate of growth. The relationship between these parameters has been clearly defined by the established equation $k_g = k_s - k_p$, where k_g, k_s, and k_p represent the fractional rates of growth, synthesis, and turnover, respectively [11,24]. The factors controlling or coordinating these three rates have been the object of numerous studies, yet they remain poorly understood. While previous studies have measured these rate parameters with a great precision, methodological problems [cf. 24] have yielded results with questionable accuracy.

Accurate measurement of any two of the three fractional rates will, by difference, give an accurate value for the third. A sensitive method for the accurate measurement of k_s in cultured cells has previously been reported [5]. Accurate measurement of k_p, on the other hand, is hampered in most instances by the reutilization of the tracer isotope; thus, even though conditions can be maintained in cellular cultures to keep reutilization to a minimum, the accuracy of this measured rate remains questionable. Part of this communication, therefore, reports on the use of the technique of "replacement perfusion" for the accurate measurement of the third rate parameter, k_g, in eucaryotic cultures. In addition to being an accurate means of measuring k_g, the replacement perfusion technique offers certain advantages over monolayer culture methods; these advantages are: (1) elimination of density-dependent changes in growth rate, (2) maintenance of a constant level and supply of fresh nutrients, and (3) continuous removal of and maintenance of constant levels of metabolites which may adversely alter growth rate.

Data is presented in this communication which correlate the rates of total protein synthesis and turnover over a wide range of controlled growth rates. Growth rate control is achieved by using the replacement perfusion technique with suspension cultures of the nonsecreting myeloma cell line, SP2/0-AG14. The ultimate goal is to extend the studies reported here to separate cellular proteins in an effort to evaluate how the turnover of individual cellular proteins is affected and coordinated under a variety of conditions. A preliminary report of the data has been published previously [20].

MATERIALS AND METHODS

Cells and Culture Medium

Nonsecreting SP2/0–AG14 cells [18] are maintained in DMEM (Gibco) containing 20% fetal bovine serum (KC Biologicals) supplemented with the following: 0.21% sodium bicarbonate, 0.35% glucose, 6 μg% folic acid, 1.1 mg% sodium pyruvate,

4.5 mg% gentamycin, 7.5×10^4 units penicillin/liter, 7.5×10^4 mcg% streptomycin, and 10 mcg% fungazone. Different growth rates are achieved by replacement of the fetal bovine serum with the following serums: horse serum, bobby calf serum, and gg-free fetal bovine (Gibco; Table I).

Cellular Growth and Labeling Conditions

The labeling protocol combined "equilibrium" and "pulse" labeling in medium containing radioactive leucine. Equilibrium was established by maintaining the cultures for 3 days (72 hr) in medium containing either [^{3}H] or [^{14}C] leucine as described below. The medium was replaced daily during the isotope equilibration period. Aliquots of cells were sampled at 6- to 20-hr intervals during "prepulse" labeling for determination of protein specific radioactivity [dpm ^{3}H or ^{14}C leucine/mg protein] and growth rates. Once equilibrium was established, and in no case less than 72 hr, cells were transferred into spinner flasks containing labeling media and grown to densities of 4–7 $\times$ 10^5 cells/ml. At this point, perfusion with labeling medium was initiated with a medium replacement rate adjusted to maintain cellular density constant (Equations 2,3). Cellular densities in the spinner flask were monitored for an additional 24 to 36 hr to insure that a stable balance between growth rates and perfusion rates had been established. The second or "pulse-labeling" isotope of leucine was then added to the spinner flask and perfusion medium and the cultures labeled for 4 hr. Aliquots (5–10 ml) of cells and labeling medium were withdrawn during "pulse-labeling" for analysis of isotope ratios in total protein and in leucine in the culture medium. Media replacement rate was adjusted after removal of each sample aliquot to compensate for the change in culture flask volume (see Equations 3,4, below).

The labeling protocol used in these studies combines "equilibrium" and "pulse" labeling with medium containing radioactive L-leucine as described in detail earlier [5]. Briefly, cells were labeled to equilibrium in medium that contained either 0.1 μCi/ml L-(U-^{14}C)-leucine (Amersham CFB.67; final specific activity of 0.15 mCi/nmole) or with 1.0-μCi/ml L-(4,5-^{3}H)-leucine (Amersham TRK.170; final specific activity of 1.5- mCi/nmole). "Pulse" labeling of the proteins was conducted in the presence of the "equilibrium" label with the isotope ratios in the "pulse" labeling medium adjusted as follows: When the equilibrium isotope was ^{14}C-leucine, pulse labeling was achieved by adding ^{3}H-leucine to the medium to give a final ^{3}H:^{14}C specific activity ratio of about 50:1. When ^{3}H-leucine was employed as the equilibrium label, pulse labeling was achieved by adding ^{14}C-leucine to give a final specific activity ratio of approximately 2.5:1 (^{3}H:^{14}C).

TABLE I. The Effect of Serum on Cellular Growth Rate*

	Serum source[a]		
	Fetal bovine	Bobby calf	1/2 Horse + 1/2 gg-free fetal bovine
T_g range (hours)	13.0 ± 1.4	22.9 ± 0.5	34.3 ± 5.5

*Cellular growth rate was varied by changing the source of the serum used to prepare the complete medium.

[a]Cells were grown in complete medium containing 20% serum as described in the text.

Analysis of Precursor and Protein Specific Radioactivity

The cellular density in each fraction of cells was determined by hemocytometer counting and protein was assayed using bovine serum albumin as reference protein [12]. Cells were separated from the labeling medium by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS) at pH 7.2 and then fixed in cold 5% trichloroacetic acid (TCA; 4°C). The precipitated protein was collected by aspiration onto glass fiber filters (Whatman GF/C) along with several rinses of cold 5% TCA. The trapped protein was dissolved in 1.0 ml of 1 N NaOH by heating at 100°C and its concentration was determined by the procedure of Lowry et al [12]. Radioactivity (^{3}H and ^{14}C) were determined by scintillation counting for estimation of specific radioactivities and isotope ratios. The ratio of the "pulse" and "equilibrium" isotopes in leucine in the culture medium was determined after isolation of leucine by thin layer chromatography as described previously [13]. Radioactivity in 25- to 200-μl samples placed in 7.5 to 10.0 ml of Scintisol scintillation cocktail (Isolab, Inc.) was read using appropriate window settings on a Packard Tricarb LSC, Model 3390. The ^{3}H counting efficiency was greater than 40% and the ^{14}C efficiency was greater than 70% with less than a 10% spillage of ^{14}C into the ^{3}H channel. Radioisotope ratios were computed from the dpm value determined by correction of the cpm in each sample for ^{3}H, ^{14}C, and ^{14}C-spillage.

Analysis of Endogenous Proteolytic Activity

Cells were labeled to equilibrium with ^{3}H-leucine at 1.0 μCi/ml as described above, collected by centrifugation, washed twice in ice cold PBS, and then treated with 0.1% triton X-100 in PBS. The cell lysate containing the equivalent of 10^6 cells was mixed with an equal volume of 100 mM acetate/phosphate buffer containing 2-mM dithioerythrotol (DTE) and 2 mM EDTA at either pH 7.0 or 4.2; the pH was adjusted as necessary. Leupeptin (10 μM) and pepstatin (2 μM) were also included in some reactions as indicated in the figure legends. The reaction mixture was incubated for 3 hr at 37°C then stopped by addition of an equal volume of ice-cold 10% TCA. Both the TCA soluble and insoluble radioactivity were determined with the relative proteolytic activity expressed as the percentage of total radioactivity released into the TCA soluble fraction.

Electrophoretic Procedures

Total cellular protein was prepared for electrophoretic analysis by solubilization of washed cell pellets with an equal volume of solubilization medium (4% sodium dodecyl sulfate (SDS); 20% glycerol; 0.5 M TRIS-HCl, pH 6.8; 0.002% bromophenol blue; 10% 2-mercaptoethanol). Solubilization was by heating for 10 minutes at 100°C. Samples from solubilized total cellular proteins were electrophoretically separated in one-dimensional tube gels by the neutral phosphate procedure of Weber and Osborn [22]; gels were composed of 7.5% polyacrylamide gels with a 37.5:1 acrylamide:bisacrylamide (BioRad) cross-link ratio. Gels were stained with Coomassie Blue R-250 as described previously [19] and stored in 7% acetic acid until sectioned for determination of radioactivity.

Isotope ratios (^{3}H:^{14}C) were determined in 1- to 2-mm sections (tube gel slicer, BioRad) of stained gels after elution of proteins into 15 ml of 4% NCS (Amersham), 4.2% Liquifluor (Amersham), and 91.8% toluene. The gel sections were rehydrated for 24 hr in 1.0 ml of 90% NCS, 10% water; 10 ml of 4.2% Liquifluor in toluene

were added and the gels extracted an additional 72 hr at room temperature. After extraction of labeled proteins from both single and two-dimensional gels, gel sections were removed from the scintillation fluid to avoid heterogeneity in sample quenching during scintillation spectroscopy.

Determination of Leucine-Specific Radioactivity

The specific activity of L-leucine was assayed both in the labeling medium and hydrolysates of total cellular protein. Total cellular proteins were hydrolyzed in 6 N HCl according to the procedure of Martin et al [13]. The specific radioactivity of L-leucine in the protein hydrolysate and of leucine isolated from labeling medium by TLC was determined by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method originally described by Airhart et al [1] and as modified by Everett and colleagues [7].

Calculations and Terminology

The rate data given in this report are expressed as fractional rate constants according to the terminology and notations that have been described in detail elsewhere [21,24]. A fractional rate is defined as the fraction of the total amount of each protein that is synthesized (k_s), broken down (k_p), or accumulated (k_g) per unit time. The fractional rate of protein synthesis is given by the equation

$$k_s = \frac{[P^*(t_2) - P^*(t_1)]}{\int_{t_1}^{t_2} F^*(t)dt - \int_{t_1}^{t_2} P^*(t)dt} = hr^{-1} \quad (1)$$

where P* is the amino-acid-specific radioactivity in the protein and F* is the specific radioactivity in the precursor. The derivation and application of this equation in the steady and nonsteady states is described in detail by Zak et al [24]. Since the specific radioactivities F* and P* were directly proportional to the isotope ratios (pulse/equilibrium) in the precursor and product, k_s could also be evaluated by substituting the isotope ratio in each compartment for F* and P* in Equation 1.

The fractional rate of protein accumulation or growth (k_g) was evaluated under two conditions (ie, with or without flow) by the standard equation describing exponential growth

$$k_g = k_{ga} = \frac{ln\left[\frac{x \text{ or } p \text{ @ } t_2}{x \text{ or } p \text{ @ } t_1}\right]}{(t_2 - t_1)} = hr^{-1} \quad (2)$$

where "x" and "p" are the biomass product concentration as cells/ml and as μg cellular protein/ml, respectively. k_g and k_{ga} represent the fractional rate of growth; the additional subscript "a" represents the "apparent" fractional growth rate during replacement perfusion (flow). During perfusion the k_{ga} is equal to zero, ie, there is no change in cellular density.

During constant volume perfusion or replacement perfusion (ie, dV/dt = ∅ when media inflow = media and cell outflow) the true fractional rate of growth (k_g) is described by the following equation

$$k_g = k_f + k_{ga} \tag{3}$$

where k_f is the fractional rate of flow which is itself defined as follows

$$k_f = f/V = hr^{-1} \tag{4}$$

where "f" is the flow or pumping rate in ml/hr and "V" is the volume of the culture in milliliters.

RESULTS

Cells were maintained in spinner flasks at a constant rate of exponential growth by constant volume replacement perfusion of the culture medium [16] as described in Materials and Methods. The perfusion rate was adjusted to maintain a constant cellular density at levels between 4 and 7 × 10^5 cells/ml. Cellular growth and medium perfusion rates were equal when the cellular density remained constant; cellular density increased exponentially when the cultures were not being perfused (Fig. 1).

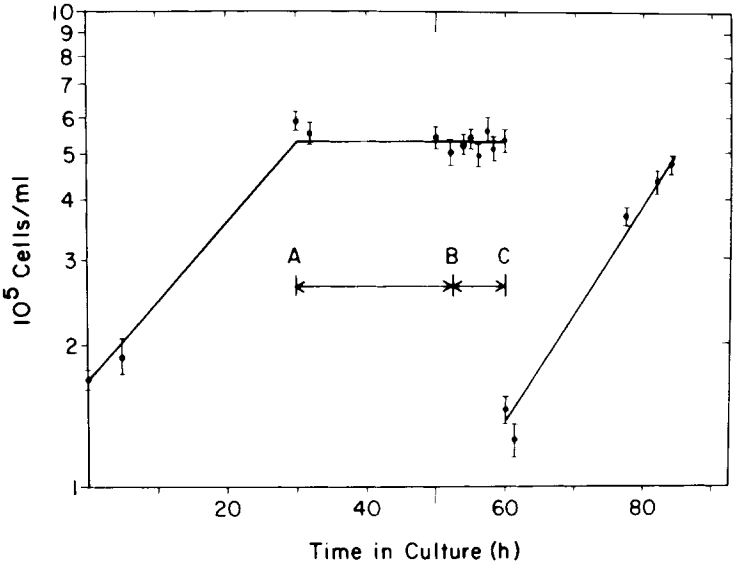

Fig. 1. The growth of SP2/0-AG14 in spinner flask culture. Cells are continuously labeled with the equilibrium isotope (either ^3H or ^{14}C-leucine) and are grown until they have reached maximal labeling (Figs. 2,3; Table II). The cells are then transferred to spinner flasks and allowed to grow to a density of from 4 to 7 × 10^5 cells/ml (to point A); in no case is this period less than 24 hr. The fractional rate of growth is determined (Equation 2) and replacement perfusion is initiated at point A; the replacement perfusion is initiated at a flow rate (k_f) to just balance the rate of growth thus maintaining cellular density constant (points A → C, text Equations 3, 4). Pulse labeling is usually performed between points B → C and is discussed in subsequent figures and tables. The data plotted to the right of point C represents a dilution of the culture after completion of analysis and is performed to demonstrate that the cellular cultures are still in exponential growth.

The rate of growth was varied between doubling periods (T_g of 12 to 40 hr by substitution of different serum supplements in the medium (Table I). Cellular growth rates were determined either by cell counting or by total protein; both methods gave identical results (Fig. 2).

Fractional rates of protein synthesis were determined in rapid- and slow-growing cultures from the rate of leucine incorporation into protein. The rate of leucine incorporation was independently determined by two labeling procedures: (1) the rate of equilibration of protein-specific radioactivity with the precursor (media free-leucine) during continuous labeling and (2) the rate of incorporation of leucine into protein during short pulse labeling. Both procedures require that the specific radioactivity of the labeled amino acid be determined in both the protein (product) and the precursor (medium or t-RNA). This was accomplished by labeling the cell cultures with two isotopic forms [^{3}H and ^{14}C] of the same amino acid. The first isotope was administered continuously until the protein-specific radioactivity reached a maximal value (Fig. 2) and was in "equilibrium" with the specific radioactivity of the precursor (Table II). The second isotope was then administered for a short

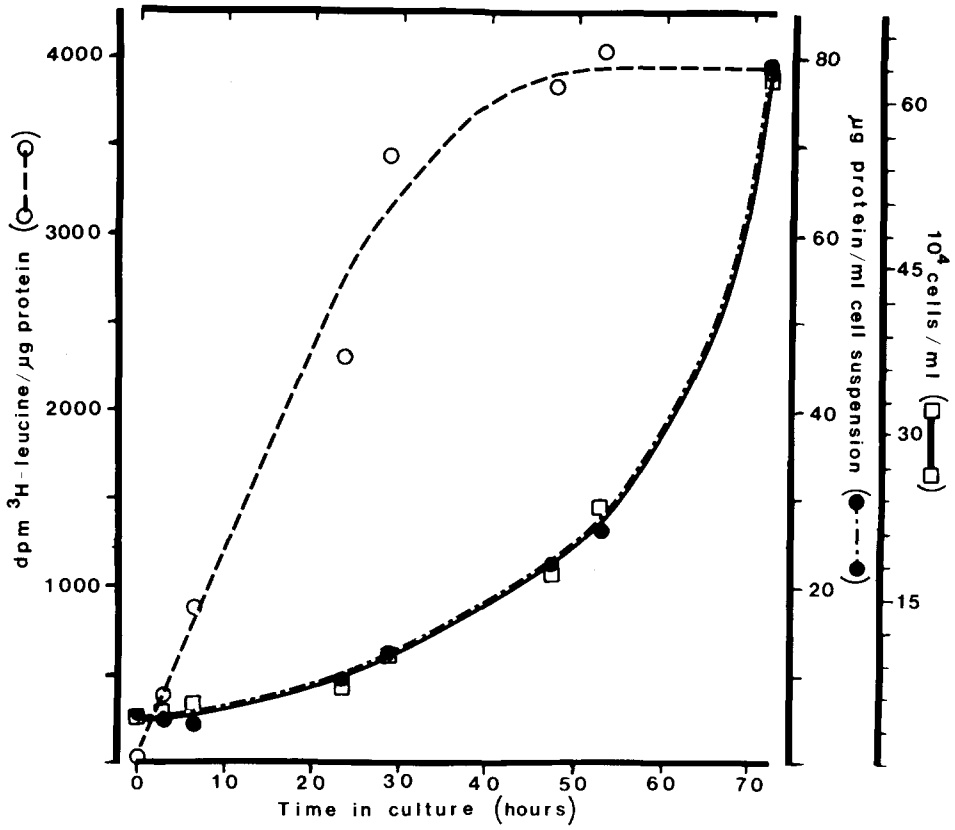

Fig. 2. Kinetics of equilibrium labeling during exponential growth. SP2/0-AG14 cultures were grown in medium containing ^{3}H-leucine (0.68 mM; 1.0 µCi/ml). Aliquots of the culture were removed at timed intervals for analysis of the amount of label incorporated into cellular protein (dpm ^{3}H-leu/µg protein; open circles). Cellular density was also determined from these aliquots and expressed in terms of cell number (open squares) and cellular protein (closed circles) using bovine serum albumin as standard.

"pulse." Since the specific radioactivity of the equilibrium isotope was the same in the precursor and product, the specific radioactivity of the pulse label could be determined directly in both compartments from the ratio of the two isotopes (Fig. 4).

Protein-specific radioactivity (as disintegrations per min, dpm, per μg) in all cultures (ie, fast and slow growing) reached a constant value after 50 to 70 hr of continuous labeling in radioactive leucine (Figs. 2,3). During this labeling period, total cellular protein and cell number increased indentically (Fig. 2). Constant specific

TABLE II. Leucine-Specific Radioactivity at Equilibrium in the Culture Medium and in Total Cellular Protein*

Group	Specific radioactivity of L-leucine (dpm/nM)
Culture medium	1687.4 ± 57.0 (5)
Cellular protein	1628.3 ± 101.0 (4)

*SP2/0-AG14 cultures with doubling times of 24 hr (k_g = 0.0289 h^{-1}) were grown for 55 hr in medium containing 0.1 μCi/ml of L-[U^{-14}C] leucine. Total cellular protein from washed pellets were obtained at this time and hydrolyzed in 6.0 N HCl. Specific radioactivity of L-leucine in the media and hydrolyzed protein were determined by modification [7] of the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method [1]. Values listed are means ± standard error (number of determinations).

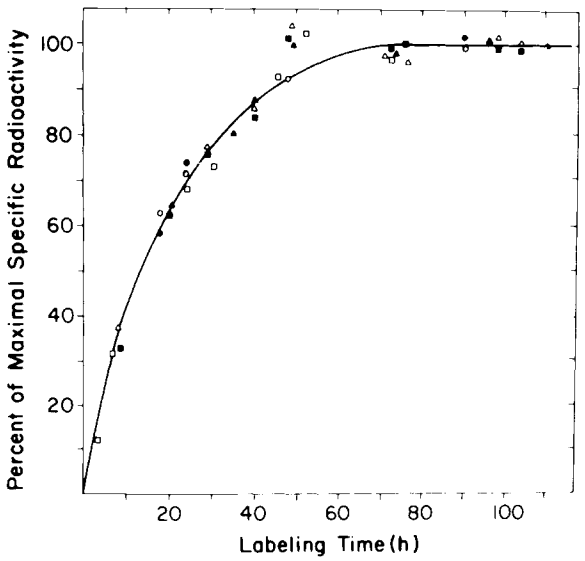

Fig. 3 Equilibrium labeling in SP2/0-AG14. Protein-specific radioactivity [P*(t)], expressed as dpm ^{3}H-leu or dpm ^{14}C-leu per μg cellular protein, is determined as described in the text and is presented as the % of maximal specific radioactivity [$P^*_{(max)}$]. The fractional rate of protein synthesis can be estimated from Equation 5 in the text. Symbols represent populations of SP2/0-AG14 having the following doubling times ($T_g = 0.693/k_g$ = hours): (○), 11.98; (●), 12.02; (□), 14.81; (■), 22.48; (△), 23.25; (▲), 28.60.

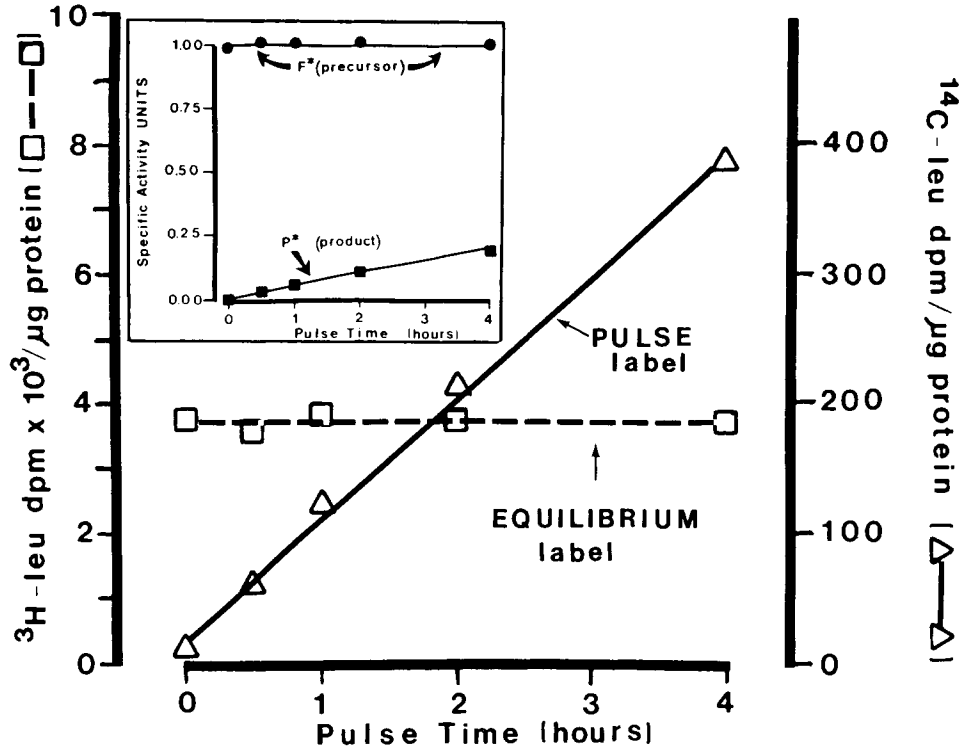

Fig. 4. Determination of specific radioactivity by the equilibrium-pulse labeling procedure. This graph represents the specific radioactivity (dpm/μg protein) in cellular protein at various times during the tracer pulse interval. The equilibrium label was ^{3}H-leu (0.68 mM; 1.0 μCi/ml) and was presented to the culture for at least 96 hr (eg, to point B in Fig. 1) prior to addition of the pulse label (^{14}C-leu at 0.68 mM; 0.4 μCi/ml). The collection of cellular samples for analysis are as described in the text. Insert: The insert represents our method for the normalization of the data in terms of specific activity units for comparison of the results from one set of data to another. This was accomplished by expressing the specific activity of the tracer amino acid in the product (P*, the ratio of the pulse/equilibrium specific radioactivity) as a fraction of the specific activity of the tracer amino acid in the precursor (F*). F* is the leucine-specific radioactivity (dpm "pulse"/dpm "equilibrium") in the medium and is normalized in this insert to a value of 1.0 at time zero of the tracer-pulse period (point B, Fig. 1). P* is the leucine-specific radioactivity in the cellular protein and is expressed in the insert as the fraction of F*. During the entire pulse interval (4 hr) F* remained constant in all experiments while P* increased in a linear fashion. Depending upon which isotopic species was used as the equilibrium isotope (ie, ^{14}C or ^{3}H) the prenormalized values for F* at zero time are found to be 40.0 and 0.6 for ratios of ^{3}H/^{14}C and ^{14}C/^{3}H, respectively.

radioactivity suggests that equilibrium exists, and we verified this by a direct comparison of the specific radioactivities of L-leucine in the culture medium and in total cellular protein (Table II). The fractional rate of protein synthesis (k_s) could then be determined by fitting the data to Equation 5 (below) by least-squares regression analysis and solving for k_s

$$P^*(t) = P^*_{(max)}(1-e^{-k_s t}) \quad (5)$$

where P*(t) is the protein specific radioactivity at time t, P*(max) is the specific radioactivity of the protein in "equilibrium" with the precursor, and k_s is the fractional rate of synthesis [5,24]. No significant difference was observed in the rate of equilibration of the protein with the precursor in the cultures with doubling periods (T_g) ranging from 12 to 40 hrs (Fig. 3, Table III).

Protein synthesis rates were also determined during short (0.5–4.0 hr) periods of "pulse" labeling by comparing the specific radioactivity of leucine in the culture medium with that in total protein. These specific radioactivities were determined from isotope ratios of the "pulse" and "equilibrium" labels using Equation I (Materials and Methods). P* and F* are the specific radioactivities of the amino acid in the product (cellular protein) and precursor (media free leucine), respectively; since the specific radioactivity of the equilibrium isotope was the same (Table II) in the precursor, (F*), and product, (P*), then the specific radioactivity of the "pulse" label can be determined in both compartments from the ratio (ie, pulse/equilibrium) of the two isotopes (Fig. 4, insert). Thus k_s could be evaluated by substituting the isotope ratio in each compartment for the values of F* and P* in Equation 1 (Materials and Methods). During pulse labeling, leucine incorporation appeared to be linear and began without any detectable lag after addition of label (Figs. 4,5). Fractional rates of protein synthesis determined from these data also indicated no significant difference between rapid- or slow-growing cultures and were in complete agreement with the data derived from equilibrium labeling described above (Table III). In addition, no significant alteration in the fractional rate of protein synthesis was observed in experiments designed so that the media-free leucine concentration was altered from approximately 0.5 to 1.5 times that of the standard culture medium (Table IV).

Since the equilibrium-pulse labeling protocol consists of a relatively short (4 hr) tracer pulse period, one might expect the pulse isotope to be unequally incorporated into some species of proteins especially in cultures with different rates of growth. Thus, experiments were designed to respond to this possibility: (1) fractional rates of protein synthesis were determined by performing an isotopic "chase" experiment just after the short pulse period, and (2) by determining the "pulse/equilibrium" isotope ratios for individual cellular proteins isolated from fast- and slow-growing cultures after sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

TABLE III. Comparison of Fractional Rates of Growth and Protein Synthesis in SP2/0-AG14

Expt.	T_g (hr)	k_g ($10^{-2}h^{-1}$)	k_s[a] ($10^{-2}h^{-1}$)	k_s[b] ($10^{-2}h^{-1}$)	k_p ($10^{-2}h^{-1}$)
1	11.9	5.8 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	−0.4
2	12.0	5.8 ± 0.2	4.8 ± 0.3	5.3 ± 0.1	−0.5
3	14.8	4.7 ± 0.2	4.3 ± 0.8	5.3 ± 0.2	+0.6
4	22.5	3.1 ± 0.4	5.0 ± 0.4	4.7 ± 0.4	+1.6
5	23.3	3.0 ± 0.3	5.3 ± 0.4	5.1 ± 0.2	+2.1
6	28.6	2.4 ± 0.1	4.4 ± 0.7	4.7 ± 0.2	+2.3
7	38.8	1.7 ± 0.1	N.D.	4.9 ± 0.2	+3.2

[a] k_s determined by the rate of protein equilibration during continuous labeling according to Equation 5 in text.

[b] k_s determined by the rate of incorporation of leucine into protein during the short *pulse* labeling period according to Equation 1 in text.

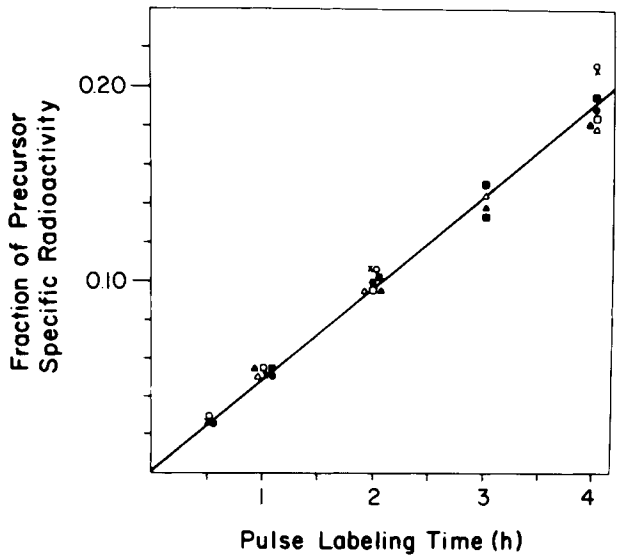

Fig. 5. Incorporation of L-leucine into total cellular protein. Rate of incorporation of isotope into the product expressed as a fraction of the specific activity in the precursor (media-free leucine); this rate of incorporation is presented as P*/F* as described in the legend to Figure 4. The data contained in this graph were independently obtained from all seven experimental growth rates investigated; the experimental doubling times (T_g = hours) of the SP2/0-AG14 are represented by the following symbols: (○), 11.98; (●), 12.02; (□), 14.81; (■), 22.48; (△), 23.25; (▲), 28.60; X, 39.80. Note that in all experimental groups, P* (representing the fractional rate of protein synthesis) is linear, constant, and equal.

TABLE IV. The Effect of Leucine Concentration on the Fractional Rate of Protein Synthesis (k_S)*

	Media free leucine		
	Specific radioactivity (dpm/nM)		
Concentration (mM)	Equilib isotope ^3H at equilib	Tracer pulse ^{14}C at time zero	$k_S \pm SE$ ($10^{-2}h^{-1}$)
0.39	3302 ± 273	1254 ± 48	5.26 ± 0.23
0.68[a]	3144 ± 191	1256 ± 31	4.94 ± 0.31
1.02	3089 ± 339	1252 ± 139	4.99 ± 0.32

*Fractional rates of synthesis (k_S, column 4) were determined in cultures growing with generation times (T_g) of 22.5 hours. Stock media containing a serum supplement was determined to be 0.39 mM leucine. Thus, the leucine concentration of the media was adjusted to either 0.68 or 1.02 mM by addition of appropriate amounts of GIBCO 100X L-leucine. The "[a]" refers to the concentration of leucine present in all other experiments described in this report. Cells grown in these media were allowed to reach equilibrium in the presence of ^3H-leu (in no case did it take more than 50 hr to reach equilibrium labeling). The ^3H-leu was added to the media such that the specific activity was the same at each leucine concentration (column 2). Once equilibrium was established, a tracer pulse of ^{14}C-leu was also added such that specific activity (dpm ^{14}C-leu/nM) was the same in all three groups (column 3). Pulse labeling was monitored for 4 hr and the ratio of the specific radioactivities of the *"pulse"* and *"equilibrium"* isotope in the precursor (F*, medium) and product (P*, cellular protein) was determined. The fractional rate of protein synthesis (k_s) was determined for each medium leucine concentration using Equation 1 in text (column 4).

Fractional rates of protein synthesis were determined during a long "chase" period by incubating the cultures with "equilibrium media" free of the "pulse" isotope. The major difference between the "chase" protocol and the "pulse-labeling" protocol is in the relationship between the radiospecific activity of the precursor (F*) and the product (P*). In the "pulse" protocol, F* > > P*, whereas in the chase protocol, F* < < P*. Analysis of the rate of protein synthesis k_s in both the "pulse" and the "chase" procedure was by Equation I as described in Materials and Methods. Fractional rates of growth and synthesis derived by either the short-term pulse method or the longer-term chase method were not significantly different (Table V), suggesting that incorporation of tracer isotope into a pool of rapidly turning over proteins was insignificant as far as we could determine by our methodology; this appeared to be the case for both fast- and slow-growing cultures.

We examined the incorporation kinetics of our short-term pulse isotope into individual cellular proteins by SDS-PAG electrophoresis. When the "pulse/equilibrium" ratio of each gel slice is plotted as the fraction of the ratio determined for total cellular protein we observe no significant differences in rates of incorporation into individual proteins of either fast- or slow-growing cultures (Fig. 6).

Since the fractional rate of protein synthesis (k_s) did not change over a wide range of growth rates in SP2/0, it would seem that the observed alteration in the rate of protein accumulation (k_g) might be due to variation in the rate of protein degradation. A comparison was therefore made of the endogenous proteolytic activity (lysosomal and nonlysosomal) in rapid- and slow-growing cultures in order to determine whether changes in proteolytic activity could be correlated with rate of growth. Protease activities were determined from the hydrolysis of endogenously labeled protein in triton X-100 lysates of cultures incubated at either pH 4.2 or 7.0 (Fig. 7). Leupeptin and pepstatin, specific inhibitors of lysosomal cathepsin B and D [23], respectively, were added to further characterize the type of proteolytic activity mea-

TABLE V. Assessment of Fractional Rates of Protein Synthesis by Equilibrium-Pulse Labeling and Pulse-Chase Techniques*

Exptl Group	Experimental period (period duration)	Fractional rate ($10^{-2}h^{-1}$) ± S.E.	
		K_g	k_s
Rapid-growing culture	Pulse / (4 hr)	5.79 ± 0.52	5.26 ± 0.22
	Chase / (26 hr)	5.55 ± 0.27	4.84 ± 0.32
Slower-growing culture	Pulse / (4 hr)	3.08 ± 0.82	4.65 ± 0.80
	Chase / (24 hr)	4.08 ± 0.12	5.49 ± 0.41

*Fractional rates of protein synthesis (k_s) were determined by fitting the specific radioactivity ratios (ie, the specific radioactivity (dpm/μg protein) of the tracer *pulse* label to the *equilibrium* pulse label) of the precursor (F*) and product (P*) to Equation 1. During the 4-hr short-term pulse period the F* > > P*, whereas during the longer-period (24–26 hr) of the chase, the F* < < P*. The protocol for the *equilibrium-pulse* protocol is as detailed in the text. The protocol for the *pulse-chase* portion of this study is as follows: after the 4-hr pulse period the cells were centrifuged at low speed and the suspending media containing the pulse isotope removed. The cellular pellets were then resuspended and washed twice with media removed from matched pair cultures and which contained only the equilibrium isotope; this procedure took approximately 1.5 to 2 hr. Cells and media were harvested at various times during the next 24–26 hr to measure cellular densities and cellular and media radiospecific activities for subsequent determination of the fractional rates of growth and protein synthesis. No significant difference was observed between the *pulse* growth rate and the *chase* growth rate determined from either the slow- or the rapid-growing cultures.

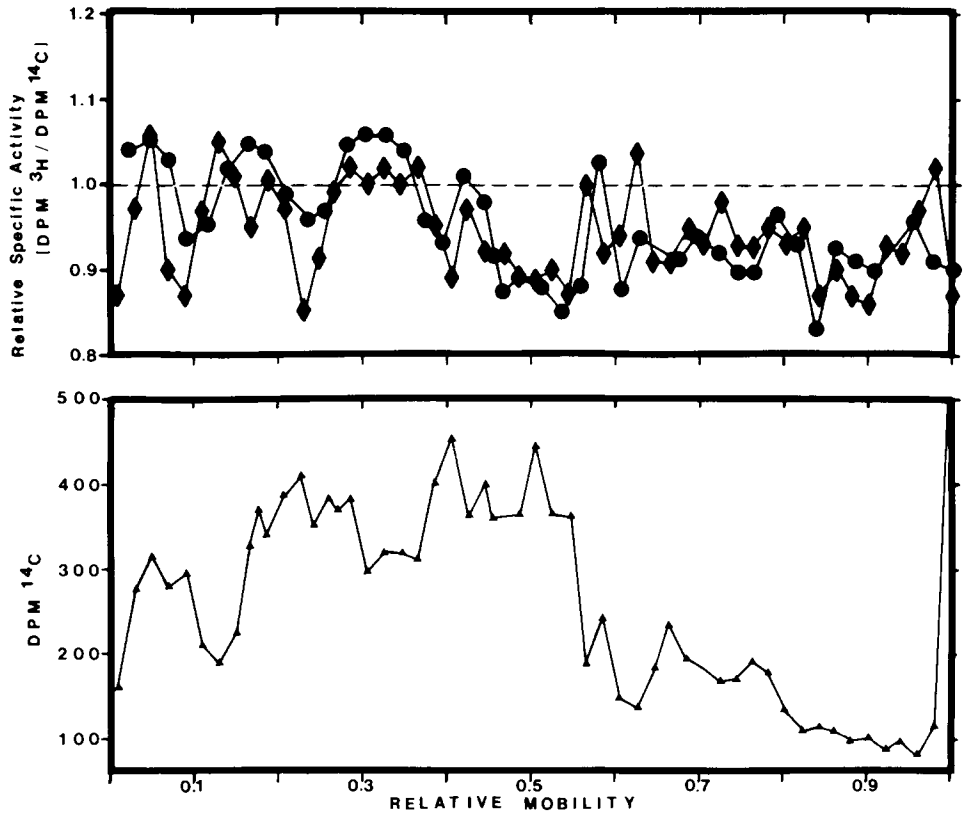

Fig. 6. Isotope ratios of individual cellular proteins after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cultures of SP2/0-AG14 grown at different rates were equilibrium/pulse labeled as described in the text. Protein fractions were prepared and separated on 7.5% polyacrylamide gels as described under Materials and Methods. The gels were cut and placed in NCS-Liquifluor as described and radioactivity of both ^{3}H and ^{14}C isotopes and their ratios determined. Top: Isotope ratios (pulse/equilibrium) are presented relative to the ratio of the isotope in total cellular protein. Thus a ratio of > 1.0 represents proteins with a rate of synthesis faster than the average cell protein, while a ratio of < 1.0 represents protein synthesis at a rate slower than the average population of protein. Since the slicing and length of the tube gels varied, the abscissa is presented as the relative mobility of the protein along the length of the gel (left, top of gel; right, bottom of gel). Isotope ratios are based on a single 4-hr pulse. Circles represent cultures growing with doubling times of 11.98 hr ($k_g = 0.05786$ h_r^{-1}) with a mean total protein specific activity ($P^* =$ dpm ^{3}H/dpm ^{14}C) of 7.55. Diamonds represent cultures growing with a T_g of 39.74 hr ($k_g = 0.01744$ hr^{-1}) with a P* of 8.15. Bottom: The data in the lower figure present the total dpm ^{14}C/slice; we have previously shown [5] that this represents the relative distribution of cellular proteins along the polyacrylamide gel.

sured. Only minimal proteolytic activity was observed at pH 7.0 in lysates of both fast- and slow-growing cultures, whereas lysosomal protease activity at pH 4.2 was considerable in both these cultures ($P < 0.05$). The acidic proteolytic activity measured in lysates of rapid growing cultures was determined to be two-thirds (0.67) that of lysates of slow-growing cultures and was found to be not significantly different at a $P < 0.05$ based on the small number ($n = 3$) of experiments performed. Acidie proteolytic activity in both types of cells was reduced to background levels with the addition of leupeptin and pepstatin.

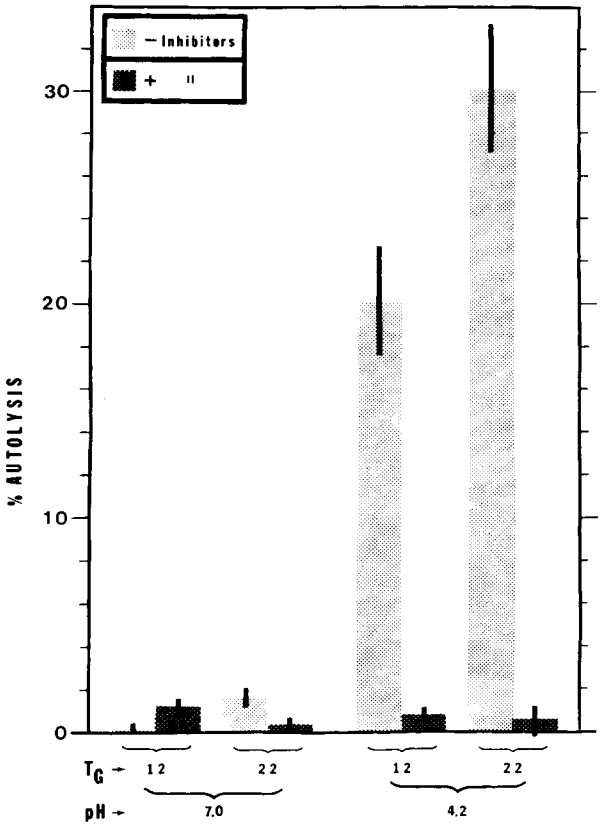

Fig. 7. Endogenous proteolytic potential of SP2/0-AG14 grown at different rates. The proteolytic activity in lysates of cultures maintained at two different rates of growth was determined. Proteolytic activity was measured using endogenous cellular proteins as substrate. The population doubling times of the two different cultures used in this study were 12 hr and 22 hr. Cellular cultures were prelabeled to equilibrium with [^{3}H]-leucine and lysates were prepared as described in text. The pH of the lysate was adjusted to either 7.0 or 4.2 with acetate/phosphate buffer and the mixture incubated for 3 hr at 37°C with and without the addition of lysosomal protease inhibitors (10 μM leupeptin and 2 μM pepstatin). The reaction was stopped by addition of an equal volume of 10% trichloroacetic acid (TCA) at 4°C. Autolytic activity is given by the percentage of the total radioactivity appearing in the TCA soluble fraction after 3 hr of incubation for each 10^6 cells. Data represent the mean ± the standard error for three separate experiments.

Previous data indicated that the fractional rate of protein synthesis remains constant at all measured rates of growth (Table III) and suggested that protein degradation (in vitro) appears to be primarily lysosomal in nature (Fig. 7). Thus, one theoretically could expect that inhibition of lysosomal proteolysis (in vivo) would lead to alteration in the rate of protein accumulation (k_g). With this in mind, an experiment was designed in an attempt to inhibit lysosomal proteolysis in a slow-growing culture by addition of the lysosomotropic amine NH_4Cl (Fig. 8). Addition of NH_4Cl to the culture did not alter the fractional rate of protein synthesis (k_s, Fig. 8, insert), but did increase the measured rate of protein accumulation, (k_g), or population doubling time (T_g) almost three-fold (Fig. 8). An additional observation revealed that the fractional

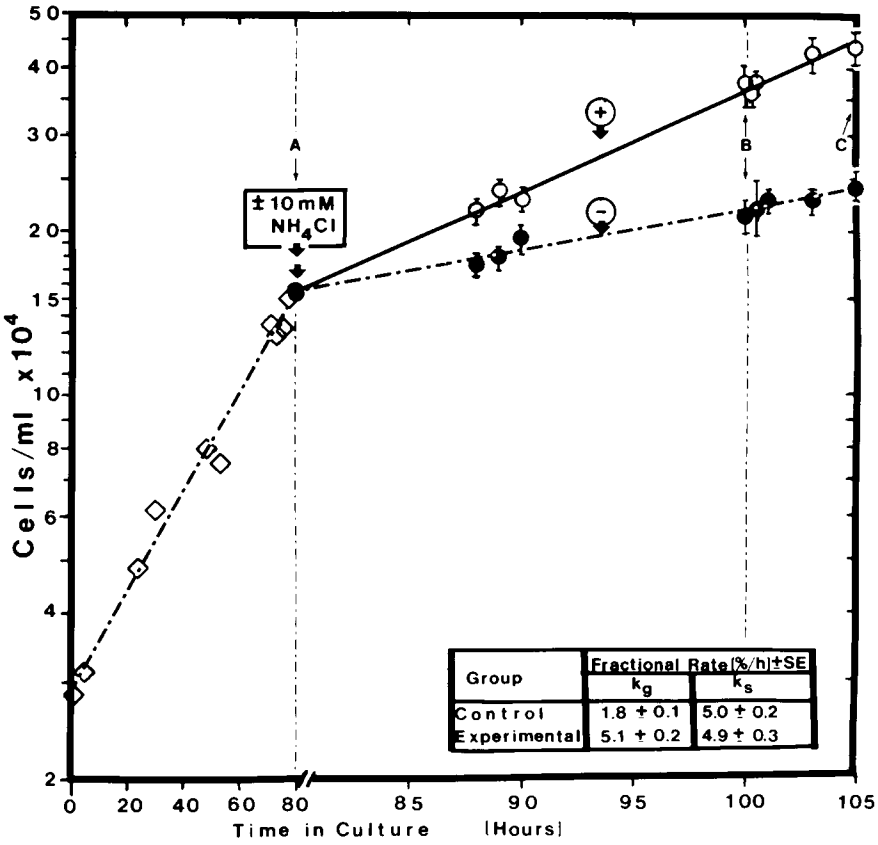

Fig. 8. The effect of a lysosomotropic amine, NH_4Cl, on cellular growth. Cultures of SP2/0-AG14 are grown in medium containing ^{14}C-leucine (0.68 mM; 0.1 μCi/ml) until equilibrium is established as described in text. During the 80 hr of isotope equilibration, the fractional rate of growth (k_g) is determined to be 0.018 hr^{-1} ± 0.002 (T_g = 38.2 hr). The culture is divided into two groups (point A), placed in media containing the equilibrium isotope either with (open circles) or without (closed circles) the addition of 10 mM NH_4Cl. The cultures are then spinner-incubated without reperfusion for an additional 20 hours (points A to B); replacement perfusion is not used in this experiment since the fractional flow rate (k_f; Equation 3) which is based on the fractional rate of growth (k_g; Equation 4) is unknown for the NH_4Cl-group past time point A. Aliquots of medium are removed at timed intervals (between points A and B) for determination of the rate of cell proliferation. A short (5 hr) pulse of ^3H-leucine to (0.68 mM; 5.0 μCi/ml) is given at point B and aliquots of cell suspension removed at intervals (points B to C) for determination of cellular growth and labeling kinetics. Fractional rates of growth and synthesis are determined as described in the text (Equations 1, 2) and are presented in the insert.

rate of growth determined for the NH_4Cl-treated culture changed to essentially the same as that observed in cultures grown in medium which facilitates rapid growth rates (Table III).

DISCUSSION

Regulation of the rate of cell division and net protein accumulation in the myeloma line SP2/0-AG14 appears to be mediated through changes in pathways for

protein degradation rather than changes in rates of protein synthesis. At maximum rates of cellular proliferation (T_g = 12 hr) the fractional rates of protein synthesis (k_s) and accumulation (k_g) are the same, which implies that there is virtually no protein degradation or turnover in these cells under conditions of maximal growth. Lower growth rates resulted by changing the serum supplement in complete medium, but the rate of protein synthesis remained the same. When cellular doubling periods were increased to 40 hr (ie, growth rate reduced), the estimated rate of protein degradation was almost two-thirds the rate of protein synthesis (0.65 k_s) and was found to be inhibited upon addition of the lysosomotropic amine NH_4Cl.

The validity of estimates of protein degradation rates are based on the measurements of rates of protein accumulation and synthesis. Under standard culture conditions the rate of cellular growth may be continually changing as cellular density and the rate of nutrient depletion increases. To overcome this difficulty and insure constant rates of cellular growth over prolonged periods, a system of medium perfusion in suspension cultures was employed. When the percent of the medium volume replaced per unit time equals the fractional rate of increase in cellular protein, then the density of cells and the concentration of nutrients and metabolites in the medium remain constant for as long as perfusion is maintained [16] Equations 3 and 4).

Fractional rates of protein synthesis were determined primarily by two separate procedures. In both cases, however, the rate of isotope incorporation into protein must be normalized to the specific radioactivity of the precursor for a determination of the fractional rate of protein synthesis [5,24]. In one procedure (approach to equilibrium labeling) it is assumed that the specific radioactivity of leucine in the culture medium closely approximates the specific radioactivity of the immediate precursor, leucyl-tRNA. A number of studies have compared the specific radioactivity of amino acids in the extracellular and aminoacyl-tRNA pools in vivo [7,13–15] and have concluded that at equilibrium these compartments exist at nearly identical specific radioactivities [7,14]. In tissue culture, however, there are several reports which indicate that the specific radioactivity of aminoacyl-tRNA is significantly less than that of the intracellular free pool as well [1]. A comparison of the data from the equilibrium and pulse labeling kinetics reported in this communication indicate that this is not the case in cultured SP2/0-AG14 myeloma cells. In determination of fractional synthesis rates by equilibrium labeling, the specific radioactivity of the precursor is determined from the specific radioactivity of the amino acid in the protein pool after it has reached equilibrium with the precursor (Figs. 2,3, Table II). Fractional synthesis rates determined by this method were in complete agreement with synthesis rates derived from pulse-labeling and pulse-chase data after normalization to the specific radioactivity of leucine in the culture medium (Tables III, V). Furthermore, the fractional synthesis rates determined by either procedure were not significantly altered when an increase in the rate of protein degradation occurred (Table III) nor when the rate of protein degradation was inhibited (Fig. 8). If amino acids derived from protein degradation were preferentially used for charging tRNA as was suggested by Airhart et al [1], then the difference between the specific radioactivity of leucine in the medium and in leucyl-tRNA would be expected to increase as the rate of protein degradation increased. This would have been indicated by an increased difference between the apparent fractional synthesis raes derived from data which had been normalized to either medium or equilibrium specific radioactivities; clearly, this did not occur in these studies (Table III). Varying the normal medium concentration

[14] of leucine (ie, 0.68 mM) from as low as 0.39 mM (normal plasma serum levels) to as much as 1.02 mM also had no significant effect on fractional synthesis rates determined by the equilibrium/pulse procedure (Table IV). The data presented in this report thus provide strong kinetic evidence that rapid and complete equilibration of leucyl-tRNA has occurred at a specific radioactivity level equal to the leucine in the medium. Data presented in Figure 6 demonstrate the similarity in the relative rate of synthesis of individual cellular proteins in a rapid- and slow-growing culture (T_g = 12 and 40 hr, respectively), suggesting that there is a similarity in the processes which coordinate the protein synthesis (accumulation) in these myeloma cells under very different growth conditions.

The data presented above indicate that cellular growth (protein accumulation) in SP2/O-AG14 under conditions of continuous nutrient replenishment, metabolite removal, and constant cellular density are primarily a function of the rate of protein degradation and more specifically the activity of the lysosomal system with its associated hydrolases. Thus, in rapid-growing cultures we see almost no detectable rate of turnover (Table III) whereas in slow-growing cultures with high rates of turnover we observe an enhancement of lysosomal proteolytic activity (Fig. 7) which is reversed by addition of the lysosomotropic amine NH_4Cl (Fig. 8). These observations appear to be in agreement with those reported by others [2–4,10,15] for confluent (slow) and exponentially (rapid) growing, anchorage-dependent cultures [2,6]. On the other hand, rapid-growing cultures of SP2/–0AG14 which exhibit essentially no measurable rate of turnover do contain a full complement of lysosomal proteases, as evidenced by their ability to degrade endogenous protein substrate when lysed with triton X-100 (Fig. 7). Taken as a whole, the data above suggest that in rapid-growing cultures the potential of the lysosomal proteases to degrade endogenous cellular proteins must be either wholly or partly masked. This masking could possibly be due to either some mechanism of compartmentation of the proteases and/or their respective substrates [17] or due to the presence of endogenous lysosomal protease inhibitors [19]. Distinguishing between these possibilities and analyzing the mechanism of their modulation by external stimuli should prove to be an important area for further investigation.

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