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Regulation of Intracellular Proteolysis in *Escherichia coli*

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Individual nitrogenous metabolites have been examined as regulating agents for the breakdown of intracellular proteins in *Escherichia coli*. Generally, NH_4^+ is the most effective regulator. Its depletion progressively increases the basal proteolytic rate to maximum in most strains when the doubling time is increased to 2 h. In *E. coli* 9723, the rate is further increased at longer doubling times. Amino acids have individual effects on intracellular proteolysis. The basal rate in amino acid-requiring auxotrophs of *E. coli* 9723 is stimulated weakly by starvation for histidine, tryptophan, or tyrosine, moderately by four other amino acid depletions, and more strongly by eight others. The degree of stimulation roughly correlates with the frequency of the amino acid in the cell proteins. Amino acid analogues that incorporate extensively into protein generally slightly inhibit intracellular proteolysis, except for selenomethionine, which is slightly stimulatory. Metabolic inhibitors were studied at graded concentrations. Chloramphenicol inhibits the basal level of intracellular proteolysis when protein synthesis is slightly or moderately inhibited, and stimulates proteolysis slightly at higher levels. Graded inhibition of ribonucleic acid synthesis with rifampin progressively stimulates intracellular proteolysis. Uracil depletion is also stimulatory. Inhibition of deoxyribonucleic acid synthesis with mitomycin C or by thymine starvation slightly inhibits intracellular proteolysis. Intracellular proteolysis is postulated to be regulated primarily by active ribosomal function. At 43 to 45 C, intracellular proteolysis becomes maximally induced and unresponsive to normal regulatory control by metabolites. Most regulation is directed towards the breakdown of the more stable cell proteins. Total proteolysis in all cell proteins is no more than doubled by the most effective conditions of starvation.

During active growth of *Escherichia coli*, intracellular proteolysis is kept to a minimum of 2.5% per h, and the process is confined to the repeated cycling of a small protein fraction variously estimated at 1 to 7% of the cellular total (12, 15, 17, 18, 22). When growth is stopped, the proteolytic pattern may remain unchanged (13, 17), it may become more uniform without increase in overall rate (16), or the rate may be stimulated (6, 10, 11, 13). To more clearly determine the underlying factors regulating these different proteolytic rates, a number of growth-inhibitory conditions and metabolite depletions have been examined in detail, and, whenever possible, at graded dosage. It appears that different methods of arresting growth can have an unanticipated variety of stimulatory and inhibitory proteolytic actions. A regulatory mechanism is proposed to account for the effects by a combination of enzymatic feedback and possible biosynthetic controls.

MATERIALS AND METHODS

The organisms used were *E. coli* B and the radiation-sensitive $B/_{s-1}$ (R. Hill); *E. coli* K_{12} strains A233-A1122 (Luria), requiring arginine, proline, histidine, and thiamine; 112-12-A15 (Mueller-Hill), requiring cystine and histidine, and a uracil- and histidine-requiring derivative of the mutant of Gerhart (5); *E. coli* W strains 113-3, requiring methionine, and M 83-8, requiring tyrosine (B. Davis); and strain ATCC 9723f, requiring phenylalanine. Requirements for additional amino acids were obtained in strains $B/_{s-1}$ and 9723f by mutagenesis with *N*-methyl *N*-nitroso-*N'*-nitroguanidine (1) and selection with penicillin (9).

The organisms were cultivated in a minimal medium of salts and glucose (16) with amino acids, purines, and pyrimidines at 30 μg/ml, and thiamine at 1 μg/ml when required. Cultures were grown overnight from a series of dilutions of the inoculum. When a suitable dilution reached optical density (A_{660nm}) of 0.2, 5 to 10 ml were labeled with 0.2 to 0.4 μCi of L-leucine-*1*-^{14}C, 50 Ci/mol. This was incorporated into protein within a few minutes (17). In experiments

where very early nutritional effects were followed, the leucine-*1*-^{14}C level was raised 20-fold. In following the effects of leucine starvation, 0.4 μCi of isoleucine-*1*-^{14}C, 10 Ci/mol was the label.

Shortly after these incorporations, the most unstable cell proteins decomposed and their label was reincorporated into the more stable cell proteins, whose proteolysis is more clearly subject to metabolic regulation (13, 14, 21). After 1 h, the cells were briefly washed at 37 C on a cellulose acetate filter with deficient medium. An eightfold dilution of the washed cells was provided with NH_4Cl at 1 mg/ml, amino acids at 100 μg/ml, and purines and pyrimidines at 30 μg/ml when required. Free amino acid-^{14}C arising from intracellular proteolysis was trapped with leucine at 500 μg/ml or isoleucine at 100 μg/ml. Flasks were provided with side-arm tubes if turbidity was to be determined. To determine the production of free leucine-*1*-^{14}C from cell protein, 1-ml samples were removed at timed intervals during growth or starvation into polyethylene counting vials with 0.05 ml of 75% citric acid. In short-term experiments when little free leucine was produced, protein was filtered out from the chilled acidified samples to reduce background activity in subsequent decarboxylations. After steaming briefly to remove residual CO_2, the samples were incubated at 37 C with 0.3 ml of 10% ninhydrin in methyl cellosolve, with shaking for 6 h at 200 rpm. The $^{14}CO_2$ evolved from the free leucine was collected with 0.1 ml of 1 N NaOH applied to a Schliecher & Schuell no. 26 glass filter pad which adhered within the underside rim of the vial cap. The cap was then transferred to a new vial and the pad was shaken into 10 ml of scintillant solution (2) and counted. In each experiment, a minor correction for yield was made with a standard leucine-*1*-^{14}C sample that was decarboxylated and compared to a sample directly spotted on a pad. A 0.1-ml sample of the cell suspension in 10% trichloroacetic acid was also filtered on a pad, washed, dried, and counted. Proteolysis was calculated as the percentage of total initial cellular radioactivity that becomes susceptible to decarboxylation. Some loss of free amino acid label occurs by deamination. It is significant only under NH_4^+ starvation (unpublished observations) amounting to 12 μg of leucine per ml decomposed to α-keto acid in 4 h, as judged from the production of ether-extractable material from leucine-*1*-^{14}C after acidification. The levels of carriers and the experimental conditions used reduce this loss to insignificance.

To estimate the total rate of proteolysis of all cell proteins (Fig. 8) the cells were grown from an overnight inoculum in 3 to 4 times the standard level of leucine-*1*-^{14}C with an excess of carrier leucine at 15 μg/ml, which labeled the proteins uniformly.

RESULTS

Effect of NH_4^+ starvation. NH_4^+ deficiency is not total under the conditions of radioactive measurement; removal of NH_4^+ specifically stimulates the deamination of the carrier amino acids to give doubling times of 4 to 11 h (unpublished data). Additions of alanine, arginine, aspartate, glutamate, methionine, or serine produce a variety of growth rates. Cell proteolysis under these conditions (Fig. 1) tends to decrease proportionately as growth accelerates from a doubling time of 2 to 1 h. At the 2-h doubling time, proteolysis appears maximal in *E. coli* strains B and W. Judging from a few determinations (not shown), the same is probably true for *E. coli* K-12. *E. coli* 9723 is distinctive in ultimately increasing proteolysis to higher levels at slower growth rates.

Amino acid depletion and antagonism. The effects of 15 amino acid deficiencies were compared in non-leaky amino acid auxotrophs derived from *E. coli* 9723. The effect of phenylalanine deficiency is shown in Fig. 2. Brief cell washing alone does not induce additional proteolysis. Phenylalanine depletion would spare general depletion of the nitrogenous cellular pools, thus when phenylalanine and NH_4^+ are both deficient, the increase in breakdown is limited to the level found with starvation of the amino acid alone. Deficiencies of the remaining amino acids produced significant differences in proteolytic rates which generally were evident by the first hourly determination and were maintained consistently thereafter, as in the curves shown in Fig. 2. Only the 4-h values are summarized (Table 1). Proteolytic rates under

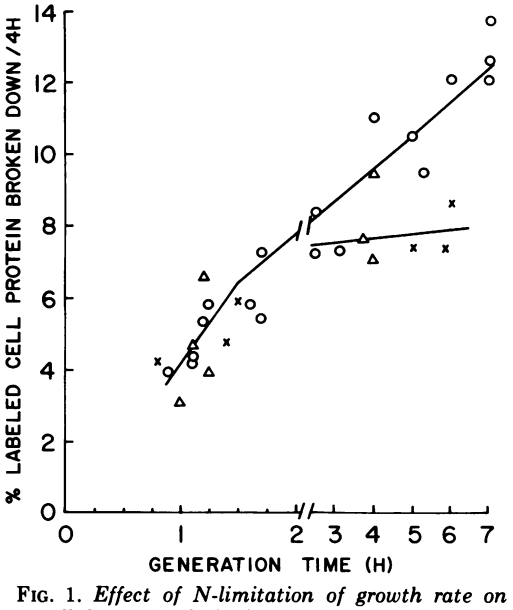

FIG. 1. *Effect of N-limitation of growth rate on intracellular proteolysis in E. coli. N sources at maximum generation times are leucine and required amino acids. Additional supplementations at other growth rates are NH_4^+, ala, arg, asp, glu, met, or ser. Symbols:* O, *E. coli 9723 phe*$^-$; ×, *E. coli B;* Δ, *E. coli W met*$^-$.

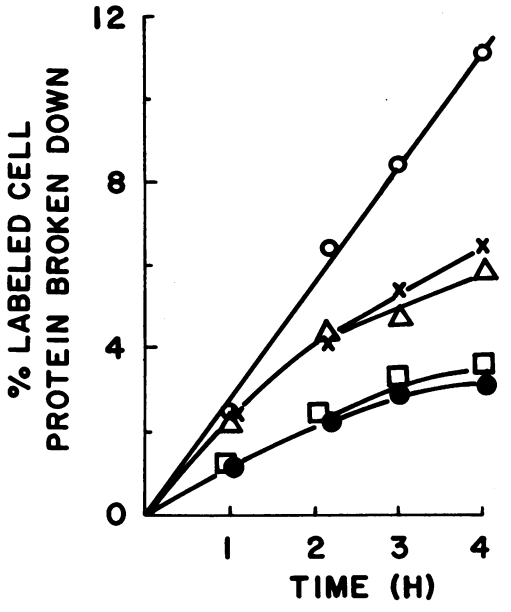

FIG. 2. *Effect of washing and of NH_4^+- and phenylalanine deficiencies on intracellular proteolysis in E. coli 9723 phe⁻. Symbols: ●, growth and washing; □, growth without washing; △, phe deficiency; ○, NH_4^+ deficiency; ×, combined phe- and NH_4^+ deficiency.*

comparable treatments tend to vary from experiment to experiment, usually correlating with poorer growth of fully supplemented mutants. Individual deficiencies can nevertheless be accurately compared in the same mutant, relative to other reference deficiencies. Thus, proteolysis remains nearly at the minimum with histidine, tryptophan, or tyrosine deficiencies (experiments 1–3). It can be stimulated either weakly by leucine deficiency (experiment 4) or moderately and to a more or less common level by either phenylalanine, methionine, or arginine deficiencies (experiments 5 and 6). With deficiencies of proline, glycine plus serine, threonine, lysine, cystine, isoleucine, or valine (experiments 6–13), stimulation is significantly greater. Additional differences are evident; for example, valine deficiency is more effective than isoleucine deficiency (experiment 9). Also, the proteolytic stimulation in threonine deficiency is characteristic of a double deficiency, for supplementation with isoleucine, which is derived from threonine, reduces the stimulation (experiment 10). The general order of effects of individual amino acid deficiencies is reproducible in repetitions of these experiments and with additional histidine, valine, isoleucine, and cystine mutants of *E. coli* 9723 (not shown). Also, from the mutant of experiment 6, six auxotrophs were derived with complete or partial (6-h doubling time) arginine requirements. In five of them, the rates of arginine- and phenylalanine-deficient proteolysis matched within 10% of each other, similar to the results of the arginine deficiency in experiment 5, while in one remaining nonleaky mutant, stimulation was somewhat greater, comparable to that in proline deficiency. The genetic locus of the deficiency could therefore sometimes influence proteolytic control. In a mutant similar to that in experiment 12 but with a fairly rapid doubling time of 2.5 h without lysine, proteolysis was still effectively stimulated, almost as much as by complete deficiency. In agreement with the results with *E. coli* 9723, proteolytic increases in other *E. coli* strains are slight with histidine and tyrosine deficiencies (experiments 14, 16, and 17) and greater with arginine, proline, cystine, lysine, and methionine deficiencies (experiments 14–16, and 18). The increases are, however, more limited, similar to those in NH_4^+ deficiency. In *E. coli* 9723, no amino acid deficiency is as effective as that of NH_4^+ except in the slow-growing mutant of experiment 13. Combined amino acid deficiencies sometimes have additive effects on proteolysis (experiments 4, 10, and 14). Usually they show varying antagonism (experiments 1, 2, 8, 11, 14, and 16).

Amino acid antagonists that can incorporate into protein, such as *p*-fluorophenylalanine (experiment 19), canavanine, azetidine-2-carboxylic acid (experiment 20), and norleucine (experiment not shown) slightly depress the basal rate of intracellular proteolysis, but thienylalanine (experiment 19) is markedly stimulatory, and selenomethionine is moderately so. Taken in the same order, these analogues sustain cell growth for at least 30 min, as judged by incorporation of excess amounts of leucine-*1*-^{14}C, at 34, 44, 24, 52, 4, and 90% of the rate for cells with normal amino acid supplementation. Thereafter, growth eventually stops at different times with each analogue. Because of poor incorporation, growth in thienylalanine is probably equivalent to phenylalanine deficiency in stimulating intracellular proteolysis. In contrast, selenomethionine is stimulatory to proteolysis even though it is used more effectively than any other amino acid analogue.

Inhibitors of protein biosynthesis. Chloramphenicol inhibits intracellular proteolysis slightly (Fig. 3) when either barely inhibiting growth (2 μg/ml) or suppressing growth to the level found in NH_4^+ deficiency (7 μg/ml). When protein biosynthesis is completely suppressed at 100 μg of chloramphenicol per ml, the reverse effect of proteolytic stimulation is noted.

TABLE 1. *Effects of metabolite deficiency and replacement on intracellular proteolysis in E. coli*

Expt	Mutant	Treatment	Hydrolysis of ^{14}C-protein (% per 4 h)	Expt	Mutant	Treatment	Hydrolysis of ^{14}C-protein (% per 4 h)
1	9723 *phe⁻his⁻*	−Phe	10.0	14	K-12 *arg⁻his⁻pro⁻*	−Arg, −Pro	7.0
		−His, −Phe	7.5			−Arg, −His, −Pro	6.1
		−His	5.8			−Arg	5.9
		None (growth)	5.0			−Arg, −His	5.8
2	9723 *phe⁻try⁻*	−Phe	6.4			−Pro	5.7
		−Phe, −Try	5.6			−Pro, −His	5.2
		−Try	4.3			−His	4.7
		None	3.5			None	4.6
3	9723 *phe⁻tyr⁻*	$-NH_4^+$	11.5	15	K-12 *cys⁻*	$-NH_4^+$	6.2
		−Phe	8.5			−Cys	5.8
		−Tyr	4.3			None	4.1
		None	4.1	16	B/S-1 *his⁻lys⁻*	$-NH_4^+$, −Lys	8.9
4	9723 *phe⁻leu⁻*	−Phe, −Leu	7.7			$-NH_4^+$	8.6
		−Phe	6.2			−Lys	8.5
		−Leu	4.6			−Lys, −His	6.1
		None	2.7			−His	5.8
5	9723 *phe⁻arg⁻*	−Arg	7.1			None	5.0
		−Phe	6.4	17	W *tyr⁻*	$-NH_4^+$	8.2
		None	3.9			−Tyr	4.7
6	9723 *phe⁻met⁻pro⁻*	$-NH_4^+$	12.3			None	3.5
		−Pro	10.4	18	W *met⁻*	$-NH_4^+$	10.8
		−Met	8.5			−Met	7.4
		−Phe	7.8			None	4.1
		None	4.5	19	9723 *phe⁻*	$-NH_4^+$	11.0
7	9723 *phe⁻ile⁻*	$-NH_4^+$	14.5			−Phe, +Thienyl ala	7.2
		−Ile	9.5			−Phe	6.4
		−Phe	8.8			None	3.3
		None	3.7			−Phe + *p*FPhe	2.2
8	9723 *phe⁻val⁻*	−Val	9.3			−Phe + *p*FPhe + Tyr	2.0
		−Phe, −Val	7.3			Phe, + Tyr	
		−Phe	7.1	20	9723 *phe⁻met⁻pro⁻ arg⁻*	−Phe, + Thienyl ala, + Tyr	6.2
		None	4.7			−Met, + Se Met	4.2
9	9723 *phe⁻ile⁻val⁻*	$-NH_4^+$	14.3			None	3.3
		−Val	14.3			−Arg, + canavanine	2.2
		−Ile, −Val	13.6			−Pro, + azetidine-2-carboxylate	2.1
		−Ile	10.9				
		−Phe	9.1	21	9723 *phe⁻ad⁻*	−Phe	9.6
		None	6.9			−Adenine	6.5
10	9723 *phe⁻thr⁻*	−Thr	11.6			None	4.6
		−Phe	9.9	22	K-12 *his⁻u⁻*	$-NH_4^+$	6.6
		−Thr, +Ile	8.3			−Uracil	5.9
		None	5.5			−Uracil, −His	4.9
11	9723 *phe⁻gly⁻ser⁻*	−Gly, −Ser	9.4			−His	4.6
		−Gly, −Ser, −Phe	8.7			None	4.4
		−Phe	7.1	23	B/r *his⁻thy⁻*	None	3.5
		None	6.4			−Thymine	2.8
12	9723 *phe⁻lys⁻*	$-NH_4^+$	14.4				
		−Lys	9.4				
		−Phe	7.8				
		None	4.4				
13	9723 *phe⁻cys⁻*	$-NH_4^+$	12.3				
		−Cys	9.0				
		−Phe	7.6				
		None	4.3				

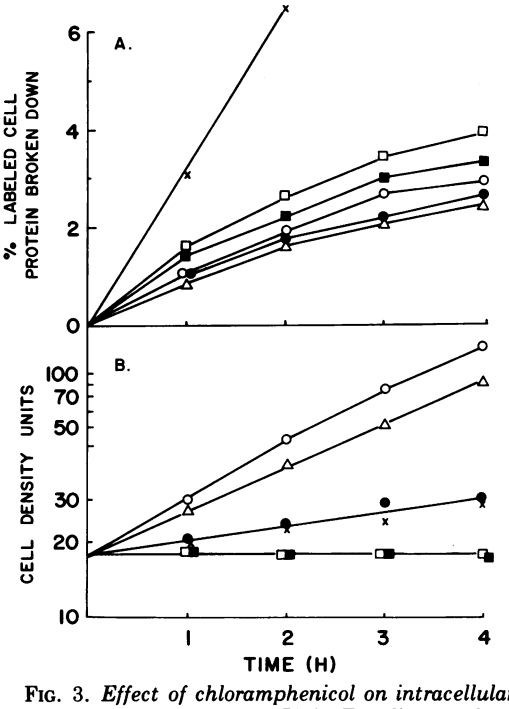

FIG. 3. *Effect of chloramphenicol on intracellular proteolysis (A) and growth (B) in E. coli 9723 phe⁻. Symbols: ○, growth (untreated); chloramphenicol at: △, 2 μg/ml; ●, 7 μg/ml, and □, 100 μg/ml; ×, NH_4^+ deficiency; ■, NH_4^+ deficiency with 100 μg/ml chloramphenicol.*

Chloramphenicol also antagonizes the stimulatory effect of NH_4^+ starvation upon proteolysis, reducing it slightly but reproducibly below the proteolytic level of drug-treated cells in complete medium (Fig. 3). At this level, chloramphenicol also antagonizes the stimulation of intracellular proteolysis by phenylalanine or glucose starvations (not shown). Pactamycin slightly stimulates the basal rate of intracellular proteolysis at concentrations that give partial to almost complete growth inhibition (3–7 μg/ml) and also at higher concentrations (11–300 μg/ml).

Inhibition of nucleic acid biosynthesis. Intracellular proteolysis is moderately stimulated by nonspecific inhibition of nucleic acid biosynthesis upon adenine depletion (Table 1, experiment 21) or by inhibition of ribonucleic acid (RNA) biosynthesis upon uracil depletion (experiment 22). The effect is again antagonized by simultaneous histidine depletion. Inhibition of RNA biosynthesis with rifampin also stimulates intracellular proteolysis, and at graded drug dosage the stimulation is roughly proportional to growth inhibition, without evidence of biphasic action (Fig. 4). In contrast, inhibition of deoxyribonucleic acid (DNA) biosynthesis, either by thymine starvation (Table 1, experiment 23) or with mitomycin C (Fig. 4), slightly inhibits intracellular proteolysis. In both cases inhibition is noted only after about an hour, when growth stops at low DNA-to-protein ratios. Similar proteolytic inhibition by mitomycin C at 8 μg/ml was also noted in *E. coli* K-12, which is lysed, and in a nonlysing derivative cured of λ phage. Lytic treatment alone with pencillin at 90 μg/ml does not alter intracellular proteolysis during the 1-h period of growth that precedes lysis.

Speed of proteolytic adjustment to nutritional change. To determine whether the rate of intracellular proteolysis can be altered immediately and without adaptive lag, cultures were labeled, diluted, and grown for 4 h, and proteolysis was measured after a rapid 45-s

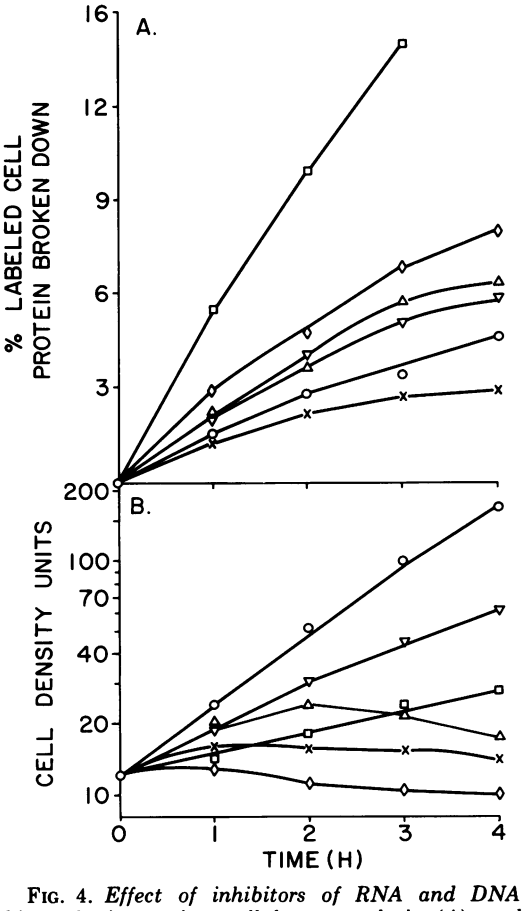

FIG. 4. *Effect of inhibitors of RNA and DNA biosynthesis on intracellular proteolysis (A) and growth (B) in E. coli 9723 phe⁻. Symbols: (○) growth (untreated); rifampin at: (▽) 6 μg/ml, (△) 12.5 μg/ml, and (◇) 32 μg/ml; (×) mitomycin C at 30 μg/ml; (□) NH_4^+ deficiency.*

wash in NH_4^+-deficient medium. Proteolysis appears to be immediately elevated in the deficient cells in comparison to those in which NH_4^+ is re-added (Fig. 5A). In an additional experiment not shown, the labeled cells were washed and suspended in either complete or deficient medium. Proteolysis was followed at 5-min intervals. The results were not substantially altered by this washing procedure, and after 20 min, additional adaptive increases were evident in NH_4^+-deficient cells. The amino acid analogues thienylalanine, azetidine-2-carboxylic acid, canavanine, and selenomethionine also alter intracellular proteolysis immediately, producing detectable changes within 4 or 8 min after their substitution for the normal amino acids.

Acquired proteolytic activity can also be rapidly lost, in part. NH_4^+-deficient intracellular proteolysis falls to normal if NH_4^+ is restored early, within 4 or 9 min. After 19 or 44 min, however, the proteolytic rate remains significantly above normal for about an hour after NH_4^+ restoration. Chloramphenicol at 100 μg/ml can reverse proteolytic increase at any time during NH_4^+ deficiency, to the same extent that it partly prevents it. *E. coli* 9723 *phe*$^-$*try*$^-$ was pulse labeled, grew for a further 2 h, and was then starved of NH_4^+ for 30 min,

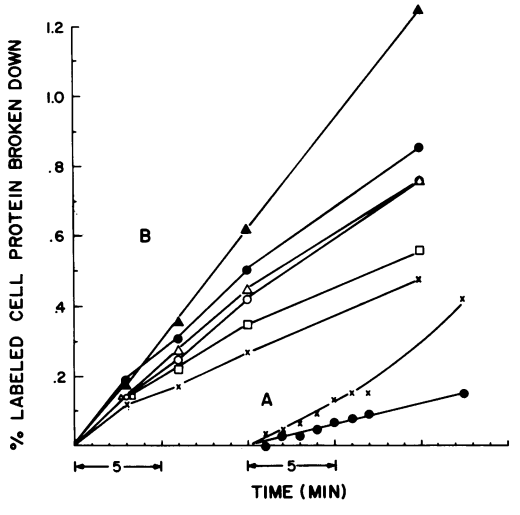

FIG. 5. *Early course of intracellular proteolysis in E. coli 9723 after nutritional change. A. Growing cells of E. coli 9723 phe*$^-$ *were depleted of* NH_4^+ (×) *or resupplemented with* NH_4^+ (●). *B. E. coli 9723 phe*$^-$*try*$^-$ *was starved of* NH_4^+ *for 30 min and restored to complete medium* (□), *with 4 µg of chloramphenicol per ml* (○); *to medium deficient in either* NH_4^+ (△), *tryptophan* (●), *or phenylalanine* (▲); *or with p-fluorophenylalanine replacing phenylalanine* (×).

which would have permanently increased the proteolytic capacity. The cells were rapidly washed in multiply deficient medium and resupplemented in several ways. Proteolytic changes are evident immediately in the most extreme treatments (Fig. 5B). These changes are reproducible, but their order is somewhat altered as compared to the response of the growing cell. NH_4^+ restoration initially lowers proteolysis, but only slightly. NH_4^+ restoration in the presence of *p*-fluorophenylalanine predictably lowers proteolysis even more, and a change to tryptophan or phenylalanine deficiencies stimulates proteolysis, but more effectively than continued NH_4^+ deficiency (Fig. 5B). These amino acids have become more effective regulators of intracellular proteolysis than NH_4^+. Addition of chloramphenicol at 4 μg/ml to NH_4^+-restored cells is stimulatory rather than inhibitory. Neither the rate of growth nor its sensitivity to chloramphenicol have been altered by the starvation procedure. This was determined from 5-min incorporations of excess ^{14}C-leucine with and without chloramphenicol. Incorporations in NH_4^+-restored and unstarved cells were virtually identical over a subsequent 30-min period. Thus, the opposing effects of chloramphenicol upon proteolysis depend on both drug concentration and the physiological state of the cell.

Effect of temperature on intracellular proteolysis. In NH_4^+-deficient cells, temperature increases produce an expected exponential increase in the rate of proteolysis, as averaged from determinations for 1 and 2 h (Fig. 6B). Up to 37 C these values are 2.5 to 3-fold those of growing cells (Fig. 6C), and when rates are calculated from the terminal slopes of the breakdown curves at 4 h, the differences are greater, 6- to 9-fold. Above 37 C, however, the growth-imposed inhibition of proteolysis is progressively lost (Fig. 6B and C). This is somewhat paralleled by a decline in growth rates (Fig. 6A). The decreased growth rate may be due in part to a decline in the supply of certain precursor metabolites which in turn control proteolysis. The cells were therefore compared at 37 and 45 C under conditions that can produce proteolytic maxima and minima without extensive growth or consumption of metabolites (Fig. 7). Proteolysis at 45 C is only slightly lowered by chloramphenicol at concentrations that partially inhibit growth. In other experiments, glucose starvation, or substitution of *p*-fluorophenylalanine plus tyrosine for phenylalanine did not further alter the proteolytic responses. The growth behavior of *E. coli* at 45 C is not always predictable. In one repetition of the experiment

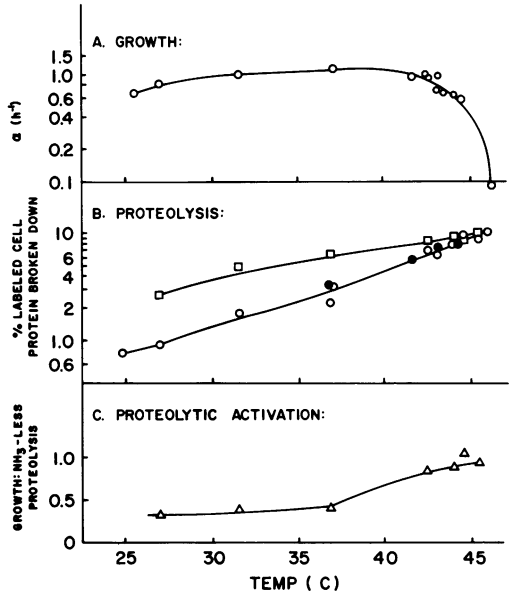

FIG. 6. *Effect of temperature on intracellular proteolysis in E. coli 9723 phe⁻. A, Cellular growth constant; B, proteolysis in growing cells labeled during growth at 25 C (●), and in growing cells (○) and NH_4^+-starved cells (□) labeled during growth at 37 C. Proteolysis is averaged from 1- and 2-h readings. C, Proteolytic activation (growth proteolysis: NH_4^+-deficient proteolysis) calculated from data of B.*

of Fig. 8 at this temperature, growth almost totally stopped (doubling time, 7 h). However, the addition of 4, 7, and 15 μg of chloramphenicol per ml still lowered proteolysis to the same extent shown in Fig. 7, and 2 μg/ml lowered proteolysis nearly as effectively. The three lowest concentrations did not alter the small amount of residual growth. Thus, the regulatory actions of chloramphenicol and other agents appear minimized at high temperatures regardless of whether growth is postulated to be limited by the biosynthesis of protein or its precursors, or by the metabolism of an alternate nonprotein component. By default, some intrinsic component of the system regulating intracellular proteolysis would presumably be lost or ineffective at high temperatures, making proteolysis unresponsive for the most part to its normal regulatory stimuli.

Determination of absolute proteolytic rates. For cell proteins totally labeled throughout growth, the initial proteolytic rates in NH_4^+ deficiency and combined NH_4^+ and glucose deficiency are 4 and 5%/h, respectively, at 37 C, and 7%/h during phenylalanine deficiency at 43 C (Fig. 8). At the latter temperature, the proteolytic rate is presumably characteristic for any condition of growth or starvation. None of the rates are genuinely linear even within an hour, and all fall off after several hours. No more than about 30% of the original cell protein can be broken down, even after several days. Although glucose starvation produces more proteolysis than NH_4^+ starvation, it stops more abruptly (Fig. 8).

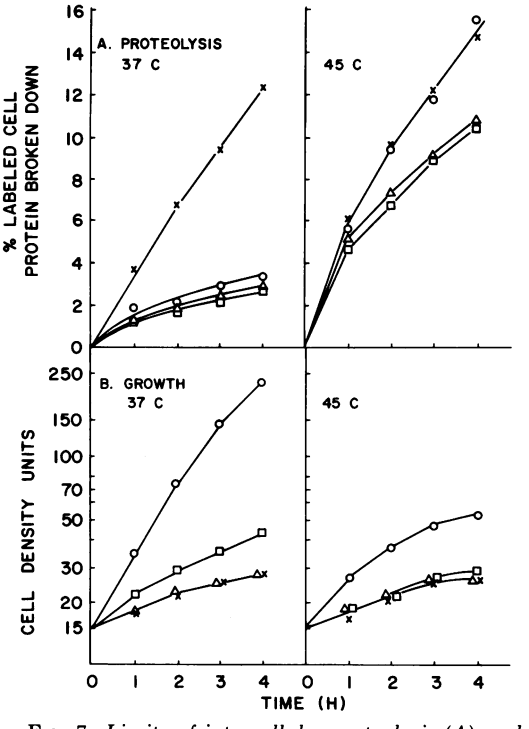

FIG. 7. *Limits of intracellular proteolysis (A) and growth (B) in E. coli 9723 phe⁻ at 37 and 45 C. Symbols: ○, complete medium; with (□) 4 and (△) 6 μg of chloramphenicol per ml; ×, NH_4^+-deficient medium.*

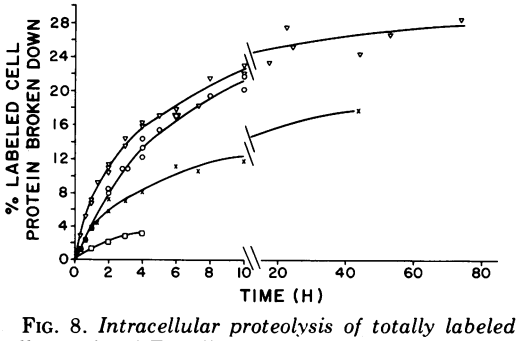

FIG. 8. *Intracellular proteolysis of totally labeled cell protein of E. coli 9723 phe⁻ leu⁻. Symbols: □, complete medium, 37 C; ○, NH_4^+-deficient medium, 37 C; ×, NH_4^+ and glucose-deficient medium, 37 C; ▽, phenylalanine-deficient medium 43 C.*

Validity of measurement of intracellular proteolysis. For reliable measurement of proteolytic rates under a variety of physiological conditions, the proteolytic system must be stable metabolically, and free leucine liberated by proteolysis must equilibrate adequately with the external carrier before it is reutilized for protein biosynthesis. Moreover, the equilibration must occur with a common component of the cellular pool, a condition that may not be fulfilled in some cellular systems (18). The speed of effective isotopic equilibration can thus only be arrived at empirically, but it is at least equal to or greater than the fastest proteolytic rate for any protein species of the system (18). Intracellular proteolysis was measured in *E. coli* 9723f at two physiological extremes: normal mid-exponential phase of growth, which would allow maximal reutilization of liberated free amino acid, and complete biosynthetic paralysis after 2 h of treatment with 100 μg of chloramphenicol per ml. The drug was lowered to 3 μg/ml to allow very low leucine incorporation, and raised to the original level in measuring proteolysis. This condition would be likely to maximize and slow decay of the proteolytic system and prevent cellular repair. The most unstable cell proteins were labeled by pulsed labeling for 1 min in mid-exponential phase cells and for 10 min in chloramphenicol-treated cells. Over a 1- to 10-min interval thereafter, 3.7 and 21% of the protein label, respectively, was converted to free amino acid, followed by slower proteolysis. These values were not experimentally different from those previously demonstrated with this and the remaining strains of these experiments, *E. coli* B, K_{12}, and W, and also with *E. coli* 1250-228, under a great variety of conditions of moderate to extreme growth arrestment (3, 12, 14, 16, 17). It thus appears that the complete proteolytic system is sufficiently rugged and that the carrier exchange is adequate for the measurements of the present experiments on comparatively stable cell proteins with half-lives in the order of hours. In addition, the amino acid supplements can not be affecting proteolysis by altering penetration of leucine carrier, because they do not affect the proteolytic rates of *E. coli* 9723f when added gratuitously to growing or phenylalanine-starved cells. A single exception to this is tyrosine which can stimulate proteolysis slightly in phenylalanine-starved cells. Since it does not, however, affect proteolysis in the double *phe⁻met⁻* mutant during growth or when methionine is starved for, it is unlikely that it is interfering with carrier transport. Rather, since the regulations of tyrosine and phenylalanine biosynthesis are so closely interrelated, this may more likely be a secondary regulatory effect on intracellular proteolysis.

DISCUSSION

Control of intracellular proteolysis by amino acids. A select number of amino acids appear to be the most potent single metabolites that control intracellular proteolysis, inhibiting the reaction when they are all present and stimulating it when individually deficient. Amino acid deficiencies may also be the regulating events that raise proteolysis in NH_4^+ and glucose deficiencies. Limitation of amino acid pools may also maintain proteolysis during growth in minimal media. Thus, chloramphenicol can either lower basal proteolysis or immediately reverse the stimulatory effects of glucose or NH_4^+ deficiency. Presumably, proteolysis would be depressed by blockage of the biosynthetic utilization of amino acids to an extent where the amino acids, both synthesized de novo and recycled from intracellular proteolysis, can fully load on transfer RNA (tRNA) and saturate the cell pools of proteolytic regulators. This interpretation would be generally in agreement with that of Goldberg (6). A rough correlation can be made between the capacity of an amino acid to regulate intracellular proteolysis and its abundance in *E. coli* cell protein. Thus, histidine, tyrosine, and tryptophan which, together with cystine are the rarest amino acids of the soluble protein (20), are poorer regulators of intracellular proteolysis than phenylalanine. Cystine may be superior because it is also a source of methionine and glutathione. Methionine, arginine, and proline, which are most similar to phenylalanine in absolute level, are similar or superior to phenylalanine as regulators. The remaining amino acids examined, which are more abundant in the soluble cellular protein, are generally superior regulators. Ribosomal conformation may thus be the more immediate regulator of intracellular proteolysis, individual amino acids regulating indirectly when they are deficient in proportion as unoccupied amino acid-accepting sites accumulate on the ribosomes. Inhibition of RNA synthesis by uracil or adenine starvation or by rifampin treatment should likewise stimulate proteolysis through limitation of messenger RNA (mRNA). Ribosomal inhibitors such as chloramphenicol should inhibit proteolysis by causing the accumulation of polysomes blocked in active configurations. Thienylalanine and selenomethionine, which are, respectively, the least and most actively incorporated amino acid analogues

tested, both stimulate intracellular proteolysis, perhaps to the extent that they fail to bind to tRNA as wuickly as their normal counterparts. The remaining amino acid analogues do incorporate more extensively than thienylalanine into protein and no doubt bind to tRNA more actively. Nevertheless, because of the conformational abnormalities in the proteins produced, growth does diminish to different extents with each analogue, presumably with the active polysomal configuration still intact. Selenomethionine, which can support exponential growth in *E. coli* (4), would produce relatively few protein abnormalities and other regulatory effects such as those due to amino acid limitation would predominate. The early effects of the amino acid analogues are characteristic only for the proteolysis of normal proteins, as distinguished from those proteins into which the analogues are incorporated. The latter are broken down more rapidly than usual, presumably as a result of proteolytic discrimination between normal and abnormal protein configurations (8, 16, 19). Although this effect would be part of the general toxic action of the analogues, it would not account for or correlate with their effects on the proteolysis of normal proteins.

Not all of the present observations are consistent with a theory of ribosomal regulation of intracellular proteolysis. Leucine, an abundant amino acid, is a poorer proteolytic regulator than phenylalanine. When two amino acids are depleted simultaneously, intracellular proteolysis is frequently regulated to an extent characteristic of the rarer amino acid. Chloramphenicol inhibits intracellular proteolysis at low to moderate concentrations but is slightly stimulatory at very high concentrations. Such anomalies would not be unexpected since the parameters for exhaustion of aminoacyl tRNA and maintenance of active polysomal structure may not be consistent under all circumstances.

Intracellular proteolysis has been found to be altered conditionally in a mutant of *E. coli* with relaxed control of RNA synthesis (21) but it is not clear whether the process is directly regulated by the RC gene for stringent biosynthetic control or whether the described effect is a secondary one. Application of the present findings with individual amino acids to the regulation of RNA synthesis should provide an answer to this question. It is now obvious that no single mode of growth arrestment can give a representative estimate of the increase in intracellular proteolysis when growth stops. Thus, Nath and Koch (13) concluded that the basal proteolytic rate in *E. coli* B cannot be increased by growth arrestment. This was judged from starvation of glucose or histidine. It is evident from the present experiments, however, that proteolysis in this strain is increased in lysine and NH_4^+ deficiencies (Table 1, expt 4). Moreover, it is a peculiarity of this strain that intracellular proteolysis can be increased by glucose removal only if it is gradual (unpublished data).

In the present experiments, the proteolysis of the actively growing cell has been considered the proteolytic norm, and other activities must necessarily be compared to this level at different degrees of growth inhibition. Thus, chloramphenicol can both inhibit and stimulate proteolysis at low and at high growth-inhibitory concentrations, respectively. Also, most of the stimulation of proteolysis from nutritional depletion occurs when growth is inhibited by about 50%, as judged by graded NH_4^+ depletion or by lysine starvation in a leaky mutant. In other studies, in contrast to the present one, rifampin and chloramphenicol at high levels have been interpreted as proteolytic inhibitors rather than stimulants (14, 24). The NH_4^+-starved cell was considered the proteolytic norm, and proteolysis during growth was either not measured or not fully considered (11, 21). The apparent inhibitory effect of rifampin on intracellular proteolysis (21) would, in fact, appear to be an antagonism of the stimulatory effect of NH_4^+ starvation.

Mechanism of proteolytic control. The increases and decreases in intracellular proteolysis in metabolite-starved and -restored cells occur rapidly enough to be attributable to direct feedback effects of effectors, perhaps on the proteolytic enzymes themselves. In both adaptation and deadaption, however, there are slower further changes that may be attributed to the synthesis of new proteolytic enzymes and their destruction, but they might also be attributed to delayed changes in cellular pools or polysomes.

A number of protease inhibitors have been found to reduce the rate of intracellular proteolysis of starved *E. coli* to levels that are more characteristic of growing cells (7, 18). Two proteases, therefore, have been proposed: a basal enzyme not affected by the inhibitors and a starvation-induced enzyme that is inactivated. However, proteolytic changes by any toxic agent are equally attributable to regulatory effects. Thus, proteolysis in nitrogen- or carbon-starved cells can decrease if RNA or protein biosynthesis is also impaired as a toxic secondary effect, or if an ineffective regulator is depleted and spares an effective one. Starving cells may be especially vulnerable to deleterious pool changes, since some of the inhibitors are

toxic to starving cells (18) but allow growth of fully supplemented cultures (7).

The overall rate of proteolysis in *E. coli* B growing in glucose medium has been estimated at 2.5% per h (17). The overall range of proteolysis in totally labeled starved cells is initially 4 and 5% per h in NH_4^+ and glucose starvation, respectively, and then falls off. This decrease is partially compensated by recycling of the protein just synthesized during starvation, which contains components that are much more unstable than those synthesized during growth (3, 14). After an hour of NH_4^+ starvation, the cumulative recycling of the unstable proteins has been estimated (unpublished data) to increase intracellular proteolysis 80% above the rates as conventionally determined with cells originally labeled during growth. However, the rates would eventually fall below initial values, and during starvation of ineffective regulators the basal proteolytic rate would be exhausted even faster. When all sources of intracellular proteolysis are considered, the response of intracellular proteolysis to growth arrestment per se is to remain the same or to fall somewhat, provided effective regulators are not depleted. This is in keeping with past conclusions (13, 17) that minimal proteolysis in growing and starving cells, although qualitatively different, is quantitatively similar. In the event that certain effective negative regulators are depleted, however, total intracellular proteolysis is variably increased, but at most no more than twofold. For some proteins, however, the change in proteolysis may be greater. These differences in protein turnover rates with different amino acid deficiencies should have different consequences for cellular survival and adaptation.

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