

# Roles of Amino Acid Activating Enzymes in Cellular Physiology

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INTRODUCTION.....	701
<i>Cellular Responses to Amino Acid Restriction</i> .....	701
<i>Key Position of Aminoacyl sRNA Synthetases</i> .....	703
MUTANTS WITH MODIFIED AMINOACYL sRNA SYNTHETASES.....	704
<i>Conditionally Expressed Mutations</i> .....	704
<i>General background</i> .....	704
<i>Temperature-conditional mutations</i> .....	704
<i>Streptomycin-conditional mutations</i> .....	704
<i>Methods for isolating conditional mutants</i> .....	705
<i>Mutations Conferring Analogue Resistance</i> .....	705
<i>Naturally Occurring Variants</i> .....	705
<i>Epi-regulatory Mutations</i> .....	705
GENETIC AND METABOLIC CHARACTERIZATION OF MUTANTS WITH MODIFIED SYNTHETASES.....	706
<i>Valyl sRNA Synthetase</i> .....	706
<i>Glycyl sRNA Synthetase</i> .....	708
<i>Histidyl sRNA Synthetase</i> .....	709
<i>Alanyl sRNA Synthetase</i> .....	709
<i>Arginyl sRNA Synthetase</i> .....	709
<i>Phenylalanyl sRNA Synthetase</i> .....	710
PHYSIOLOGICAL RESPONSES OF MUTANTS WITH MODIFIED SYNTHETASES.....	711
<i>Regulation of RNA Synthesis</i> .....	711
<i>Regulation of DNA Synthesis</i> .....	713
<i>Repression of Biosynthetic Enzymes</i> .....	714
<i>End-Product Inhibition of Biosynthetic Pathways</i> .....	715
<i>Catabolite Repression</i> .....	715
PHAGE-INDUCED MODIFICATION OF VALYL sRNA SYNTHETASE ACTIVITY.....	716
SUMMARY AND FUTURE GOALS.....	717
LITERATURE CITED.....	717

## INTRODUCTION

### *Cellular Responses to Amino Acid Restriction*

Cell growth is the orderly synthesis of protoplasm, and involves the highly coordinated formation of a great number of macromolecules. It is the task of cell physiology to account for this orderliness by discovering the special mechanisms that integrate hundreds of potentially independent biochemical reactions so effectively that a single functional unit is generated.

One simple but effective way to illustrate the operation of control devices in bacterial cells is to examine the network of controls that radiates out from individual amino acids, linking them to most of the cell's biosynthetic processes. This network can be revealed by the following simple experiment. Establish a culture in balanced growth in a chemostat with some amino acid as the restricting nutrient and then, while carefully monitoring several major cell processes,

suddenly reduce the rate of supply of the amino acid by a factor of two. Within 1 to 10 min, the cells of such a culture exhibit at least six major responses (Table 1). The rapidity of these responses in cells with perhaps a 60- to 100-min generation time indicates that they occur independently of any long-term effect of amino acid restriction, such as reduced enzyme levels.

(i) The first effect noted in Table 1 is really a metabolic consequence of amino acid restriction rather than a regulatory response: protein synthesis, within 1 min, is diminished by a factor of two. The postulated cause, a reduced level of aminoacyl soluble ribonucleic acid (sRNA), appears at first glance to be obvious and undeserving of further comment. Nevertheless, the molecular details of the consequence of an amino acid restriction are far from clear. Direct measurements of the *in vivo* state of charging of sRNA molecules in some instances show only a slight reduction in charging level upon complete amino

TABLE 1. *Early consequences of amino acid restriction in bacteria*

Cellular response	Probable or postulated cause
1. Decreased overall rate of protein synthesis	1. Decreased concentration of charged sRNA
2. Preferential decrease in rate of synthesis of ribosomal and (?) soluble and messenger RNA	2. Mechanism of regulation not known; faulty in relaxed cells
3. Restricted initiation of DNA replication	3. Decreased synthesis of protein "initiator" of DNA synthesis
4. Derepression of specific amino acid biosynthetic enzymes	4. Decreased concentration of a corepressor derived from that amino acid
5. Immediate acceleration of synthesis of restricted amino acid or its precursors	5. Release of end-product inhibition; less amino acid bound to allosteric enzyme
6. Total repression of enzymes susceptible to catabolite repression	6. Transient overproduction of repressing catabolites as a result of diminished utilization in biosynthetic pathways

acid starvation, and rarely show a complete discharge. Morris and De Moss (23) have recently investigated this phenomenon and ascribe it to an unusually high dependence of protein synthesis on the concentration of charged sRNA. Yegian and Stent (43) have presented evidence that a portion of sRNA is acylated with compounds other than single amino acids. An alternative possibility is that the complete discharge of one species of sRNA blocks protein synthesis and thereby prevents the discharge of any other species of sRNA bearing the same amino acid. Another interesting facet of amino acid deprivation is that it causes a rapid disappearance of polysomes (24). It is possible, therefore, that partial restriction of the supply of an amino acid (or its sRNA derivative) causes a proportional reduction in polysome content. If this were the case, then reducing the supply of an amino acid by a factor of two would halve the overall rate of protein synthesis by halving the number of growing polypeptide chains rather than by reducing the rate of growth of individual chains.

(ii) Within 1 min of imposing an amino acid restriction, the accumulation of RNA virtually ceases. Some synthesis of messenger RNA (mRNA) continues, perhaps at a reduced rate,

but there is no accumulation of ribosomal or soluble RNA for almost a generation time. Such a marked though transient decrease in total RNA synthesis as a result of a moderate reduction in amino acid supply is actually typical of many conditions that restrict protein synthesis in bacteria (27). The mechanism of this response is not known; contemporary theories postulate, alternatively, (i) that uncharged sRNA inhibits RNA polymerase (18, 37, 38), (ii) that a special protein must be made to permit RNA accumulation (1, 2), (iii) that reduced protein synthesis disaggregates polysomes and releases an inhibitor of RNA synthesis (23, 24), and (iv) that such polysome disaggregation disrupts the stripping off of ribosomal RNA from its deoxyribonucleic acid (DNA) template (36).

(iii) DNA synthesis continues at a gradually diminishing rate for perhaps 30 min, permitting a 40 to 50% increase, after which its velocity becomes adjusted to the new lower rate of growth. This behavior has been ascribed to a requirement for the synthesis of a specific protein to permit the initiation of new rounds of DNA replication. The experimental evidence for this picture and a specific model for the regulation of DNA replication have been presented by Lark (19).

(iv) Beginning 5 to 10 min after the shift down in amino acid supply, a marked derepression of the enzymes concerned with the biosynthesis of the restricted amino acid occurs, presumably as a result of a reduced intracellular concentration of a repressing derivative of the amino acid (39).

(v) Most amino acids have the ability to inhibit the activity of one of the early enzymes in its biosynthetic pathway. Amino acid restriction quickly releases this inhibition and permits a flow of carbon skeletons along the particular pathway (39).

(vi) A near-total repression of many catabolic enzymes begins within a few minutes of the amino acid shift down. The current view of this phenomenon postulates the following chain of events. Restriction of any amino acid slows down protein synthesis, causing a surplus of the 19 other amino acids [and adenosine triphosphate (ATP)], each of which shuts off its own biosynthetic pathway and thus contributes to a build-up of intermediary catabolites which are corepressors of catabolic enzymes (*see* 20).

There may be responses in addition to these six. Possibly, for example, protein degradation might be initiated. During unrestricted batch culture, the turnover of bacterial proteins is almost immeasurably slow. Upon total starvation for  $\text{NH}_4^+$  and an amino acid, a great increase in the rate of protein turnover ensues,

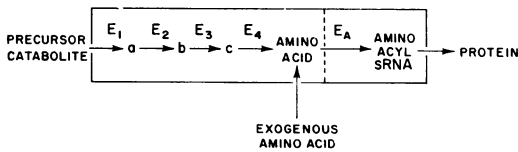


FIG. 1. Generalized features of the metabolism of a nondegradable amino acid.  $E_{1-4}$  are biosynthetic enzymes;  $a$ ,  $b$ , and  $c$  are intermediates in the biosynthetic pathway;  $E_A$  is the specific aminoacyl sRNA synthetase for the given amino acid.

attaining a level of 5% turnover per hour (21). It is not certain, however, that a mere halving of the supply of a restricting amino acid would initiate protein destruction.

Of the six known responses to amino acid restriction, only the first is a straightforward metabolic consequence of the imposed stress; the remaining five appear to be homeostatic responses, each of which reflects the operation of a separate device for physiological integration. The multiple regulatory involvements of each amino acid make all the more intriguing the question of their biochemical mechanism.

#### Key Position of Aminoacyl sRNA Synthetases

One of the first questions one might ask is whether it is the amino acid itself or a derivative of it that plays each of these several physiological roles. For end-product inhibition it seems fairly certain that the amino acid itself is involved, whereas for regulation of DNA synthesis it appears that the amino acid requirement is a secondary consequence of the fact that a regulatory protein must be made. For the other regulatory devices the matter is less certain, and is a subject of much current interest.

Figure 1 briefly shows the major features of the metabolism of an (for simplicity's sake, nondegradable) amino acid: (i) a series of linked, enzymatic reactions (four are shown) converting a precursor catabolite into the amino acid; (ii) an alternative source of the amino acid, preformed, from the medium; (iii) activation of the amino acid and its attachment to one or more specific sRNA molecules by an appropriate aminoacyl sRNA synthetase; (iv) incorporation of the aminoacyl group into growing polypeptide chains via the polysome system.

The aminoacyl sRNA synthetase, or activating enzyme, is a particularly crucial element in the route leading from amino acid to protein. Because of the adaptor function of sRNA, the successful assembly of proteins depends on the fidelity with which each aminoacyl sRNA synthetase distinguishes one particular amino acid from 19 others and one particular sRNA from perhaps 40 others.

The reaction catalyzed by an aminoacyl sRNA synthetase is shown in Fig. 2. It is generally pictured as proceeding in two biochemically distinct steps. In the first step, the enzyme binds its cognate amino acid and ATP and, in the presence of  $Mg^{+2}$ , catalyzes the activation of the aminoacyl group by a pyrophosphorolytic split of ATP to form the aminoacyl adenylate and inorganic pyrophosphate. This reaction is reversible, and is the basis of the pyrophosphate ( $P^{32}$ -labeled)-ATP exchange assay for these enzymes. The hydroxamate assay also measures this first step, but by the formation, in the presence of hydroxylamine, of the aminoacyl hydroxamate derivative. Under physiological conditions, the aminoacyl adenylate formed in the first step does not dissociate from the enzyme; instead, a second reaction occurs in which the aminoacyl group is transferred to a specific sRNA molecule, releasing adenylic acid. The overall reaction is customarily studied by measuring the attachment of  $C^{14}$ -amino acid to sRNA. This assay technique certainly comes closest to measuring the physiological reaction, yet even it departs in one significant respect from biological reality. In the cell, the sRNA molecules do not vastly outnumber the synthetase molecules, whereas in the *in vitro* assay system the sRNA must of course be added in substrate-like rather than cofactor-like concentrations.

Until recently it has been assumed that there is but one aminoacyl sRNA synthetase for each amino acid, despite the knowledge that more than one species of sRNA exists for many amino acids. Re-examination of this question by use of some of the new techniques for protein fractionation [acrylamide gel electrophoresis, diethylaminoethyl (DEAE)-sephadex chromatography, and hydroxylapatite chromatography]

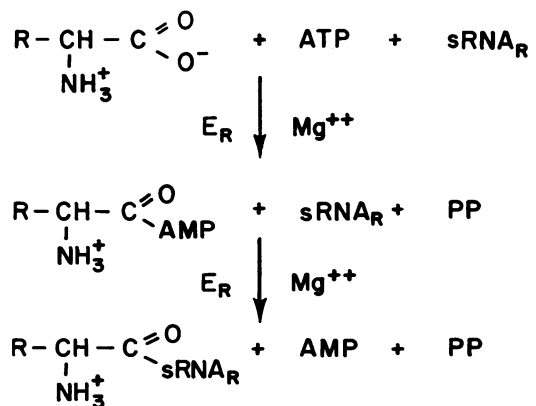


FIG. 2. Reaction catalyzed by an aminoacyl sRNA synthetase.

has suggested multiple synthetases for leucine in *Escherichia coli* (44) and for phenylalanine and aspartic acid in *Neurospora* (3). This evidence is strengthened in some cases by the fact that chromatographically separable activities can be shown to differ in sRNA specificity. On the other hand, the increasing number of cases (discussed below) where a single mutation can be shown to alter virtually all the measurable activity of a given aminoacyl sRNA synthetase (though not yet for the leucine enzyme) suggests the possibility that the separable fractions represent multiple forms of an enzyme, all of which share at least one polypeptide.

For several years we have been interested in the regulatory involvements of amino acids and have wondered whether the aminoacyl sRNA synthetases play as critical a role in regulatory phenomena as they do in protein synthesis. In this paper, I shall review the results we have obtained through the isolation and study of bacterial mutants with damaged aminoacyl sRNA synthetases and, in addition, shall discuss the related work on these enzymes that has been proceeding in five other laboratories.

#### MUTANTS WITH MODIFIED AMINOACYL sRNA SYNTHETASES

##### *Conditionally Expressed Mutations*

*General background.* One of the most powerful tools available to the student of bacterial physiology is the use of mutants deficient in one or another enzymatic activity. Mutant analysis has been of incalculable value in studies aimed at: (i) the elucidation of biosynthetic pathways leading to the subunits of protoplasm (amino acids, nucleotides, cofactors, etc.); (ii) the definition of the fine structure and functioning of genes; (iii) the establishment of the *in vivo* operation of the genetic code; and (iv) the clarification of how gene activity is governed by induction and repression.

Part of the power of mutant analysis lies in the fact that mutational alteration is an extremely selective way to inhibit a particular reaction. To establish whether aminoacyl sRNA synthetases have functions other than their obvious role in protein synthesis, it would, of course, be useful to be able to interfere selectively with their activity *in vivo*. Few suitable inhibitors are known (though as we shall see,  $\alpha$ -methyl-histidine seems to be one). However, the isolation of mutants lacking an active aminoacyl sRNA synthetase would appear impossible on theoretical grounds, because ordinary mutant technology can be used only when the blocked reaction can be bypassed nutritionally, either by

supplying to the cell the product (or end product) of the blocked reaction or by employing conditions where the product is not essential for growth. Aminoacyl sRNA preparations cannot be supplied exogenously to bacterial cells, and are indispensable.

In the past 3 years, much progress has been made in developing variations of mutant technology that offer the possibility of extending the usefulness of this tool to the study of indispensable enzymes. These advances are based on the concept of conditionally expressed mutations, that is, mutations that exert their full phenotypic effect under one environmental condition (the restrictive condition), but not under another (the permissive condition).

In the study of bacterial enzymes, two selective environmental components can now be used: temperature and streptomycin.

*Temperature-conditional mutations.* The biological activity of proteins depends on the tertiary and quaternary structure of their constituent polypeptide chains. The "native" or "active" configuration of a protein is just one of the many possible configurations for its polypeptides, and is presumably the most stable one *in situ*. Many of the forces contributing to this configuration are noncovalent and individually weak. Usually they can generate and maintain the appropriate folding only over a narrow temperature range, and the lower range of temperatures at which proteins are inactivated overlaps the upper range at which mesophilic bacteria, such as *E. coli*, can operate.

Inspired by the success of Edgar and his colleagues (9) in isolating a large number of conditionally expressed phage mutants using temperature as the restricting environmental parameter, we started a search 3 years ago for mutants with temperature-sensitive aminoacyl sRNA synthetases. At approximately the same time, Yanif, Jacob, and Gros (42) began similar searches, and other laboratories were using temperature-conditional mutations to aid in studying other cellular processes (14, 17, 28).

*Streptomycin-conditional mutations.* The phenotypic effects of some mutations are partially corrected by growth in the presence of subinhibitory concentrations of streptomycin. The correction is particularly effective in cells possessing certain streptomycin-resistance alleles, and appears to be the result of translational infidelity induced by the interaction of this antibiotic with the ribosome (16). This situation offers the possibility of isolating mutants that can produce a particular indispensable enzyme, such as an aminoacyl sRNA synthetase, in a functional form in the presence of streptomycin

but not in its absence. To date, however, no streptomycin-dependent cell has been identified as having an altered aminoacyl sRNA synthetase.

*Methods for isolating conditional mutants.* The task of finding conditionally expressed mutants in a particular enzyme may be divided into three processes. The first involves the production (usually by mutagenesis) and isolation (sometimes by penicillin selection) of mutants that grow poorly or not at all under one of the restricting conditions described above, but well under any other condition.

The second process involves the identification of the specific enzyme lesion in the mutants isolated by one of the above procedures. This process is difficult but not impossible, and much has been learned from the behavior of mutants already identified that will facilitate future identifications. In brief, conditional mutants are shifted from permissive to restrictive conditions, and the kinetics of RNA, DNA, and protein synthesis are followed. On the basis of their behavior in this test, mutants are then assigned to various classes. Appropriate additional tests (behavior in the presence of chloramphenicol, response to phage infection, effect of an RC (relaxed) gene, response to carbon and energy source shifts, and other nutritional stresses) are then made on each class to determine tentatively which mutants are likely to have primary lesions in protein synthesis. These are then surveyed for aminoacyl sRNA synthetase activities.

The third process is mutant verification. When potent mutagens have been employed, it is essential to establish that the particular enzyme alteration detected *in vitro* is in fact the cause of the changed growth. This verification is done by infecting the mutant at low multiplicities with P1 phage previously grown on normal cells. Transductants are selected for ability to grow at the restricting temperature (in the case of temperature-conditional mutations), and then their levels of the altered enzyme are examined. Recombinants from appropriate bacterial mating experiments can be similarly examined for this purpose.

#### *Mutations Conferring Analogue Resistance*

The conditional mutants discussed above are isolated on the basis of the inability of the modified enzymes to permit protein synthesis and, hence, growth under the restrictive condition. In some cases, unconditional mutants with altered synthetases can be isolated. Some mutations affect an enzyme in such a way that it has a greatly diminished affinity (higher  $K_m$ ) for a particular analogue. Historically, it was this

fact that led to the first isolation of a mutant with an altered aminoacyl sRNA synthetase. A *p*-fluorophenylalanine-resistant mutant of *E. coli* was isolated by W. Fangman in our laboratory, and was discovered to have a phenylalanyl sRNA synthetase which could still competently attach phenylalanine to sRNA but which differed from the normal enzyme in having a greatly reduced ability to attach *p*-fluorophenylalanine to sRNA (11, 12).

#### *Naturally Occurring Variants*

A somewhat surprising finding was made in our laboratory by Lia Faiman (at that time, Lia Eidlic) during routine screening of parental and mutant strains for aminoacyl sRNA synthetase activities. The two wild strains most commonly used by us, strains K10 and KB, were found to differ over 100-fold in their glycyl sRNA synthetase activity. Additional investigation revealed that it was the strain with the low activity, KB, that was the unusual one, for several other common laboratory strains shared the high activity of strain K10 (though strain C seemed to be intermediate). Furthermore, the very low activity of strain KB can be increased by increasing the glycine concentration in the assay system. The apparent  $K_m$  for glycine is about 100 times higher for the enzyme of strain KB than that of strain K10, as measured in crude extracts (5).

Berg and Yanofsky (cited in 6) have discovered, in addition, that a survey of large numbers of laboratory strains reveals many with markedly altered glycyl sRNA synthetase activities.

Perhaps this natural genetic variation is unique to the glycine enzyme, but if not it may prove very useful in the future to examine many laboratory strains as a potential source of useful "mutants."

#### *Epi-regulatory Mutations*

As we shall see in the next section, there is one function of at least some aminoacyl sRNA synthetases that can be used to select specific mutants. Synthetases are needed to convert some amino acids into repressors. In these cases, some techniques for isolating mutants derepressed for biosynthetic enzymes yield cells with impaired activating ability. In some cases, these mutations affect the growth rate of the cell in addition to derepressing a biosynthetic pathway. Such mutations have been reported for the histidyl sRNA synthetase of *Salmonella typhimurium* (31) and of *E. coli* (25).

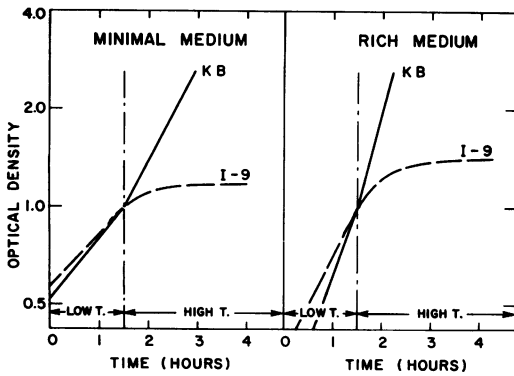


FIG. 3. Growth of a normal wild strain of *Escherichia coli* (KB) and a temperature-sensitive mutant (I-9) in minimal and rich media. The cultures growing in each medium were shifted from 30 to 40 C at the time indicated by the arrows. The optical density of the cultures is plotted as a logarithmic function of time. For methods, consult Eidic and Neidhardt (7).

#### GENETIC AND METABOLIC CHARACTERIZATION OF MUTANTS WITH MODIFIED SYNTHETASES

##### *Valyl sRNA Synthetase*

The first temperature-conditional synthetase mutant to be identified was *E. coli* I-9(7). The growth curves presented in Fig. 3 depict the behavior of a normal wild strain of *E. coli* KB, at 30 and 40 C in a minimal and in a rich medium. These cells grow approximately twice as fast at the higher temperature. Mutant strain I-9 was derived from strain KB by ethylmethane sulfonate mutagenesis, followed by counterselection with penicillin in rich medium at 40 C. As shown in Fig. 3, this mutant fails to grow at 40 C in either medium, and growth ceases fairly abruptly when the temperature is suddenly raised from the permissive to the restrictive level. The abruptness suggests inactivation of some vital enzyme rather than a failure to make it at the restrictive temperature.

The growth rate of strains KB and I-9 in minimal medium is plotted in Fig. 4 as a logarithmic function of the reciprocal of the absolute temperature (i.e., an Arrhenius plot). Growth of these strains is indistinguishable up to 28 C, but the growth rate of the mutant is not increased by raising the temperature further, and balanced growth is abruptly terminated between 35.0 and 35.5 C. The sharp temperature dependence of growth of the mutant in this narrow temperature range further supports the notion that an indispensable enzyme becomes thermally denatured *in situ* above 35 C.

The strategy outlined in the previous section yielded results that pointed to a lesion in protein

synthesis in I-9. Examination of the various aminoacyl sRNA synthetase activities in cell-free extracts of I-9 quickly revealed that this

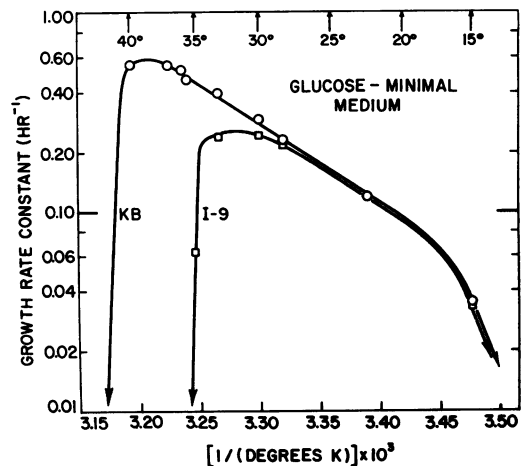


FIG. 4. Effect of temperature on the growth rate of a wild strain of *Escherichia coli* (KB) and a temperature-sensitive mutant (I-9) in glucose minimal medium. The specific growth rate constant,  $k$ , is plotted as a logarithmic function of the reciprocal of the absolute temperature (degrees K). At the top of the figure, equivalent temperatures in Celsius degrees are shown for ease of inspection. For methods, consult Eidic and Neidhardt (7). From Neidhardt and Earhart (27).

TABLE 2. *Valyl sRNA synthetase* activities of extracts of normal (KB) and mutant (I-9) strains of *Escherichia coli* grown at different temperatures

Cells	Growth temp <sup>a</sup>	Specific activity <sup>b</sup>		
		sRNA attachment	Pyro-phosphate exchange	Hydroxamate formation
	C			
KB	30	0.063	3.5	0.048
I-9	30	0.0002	1.4	0.006
KB	37	0.075	3.6	0.047
I-9	37	0.0001	1.6	0.005

<sup>a</sup> The temperature-sensitive mutant, where indicated, was exposed to 37 C for 2 hr before harvesting.

<sup>b</sup> The specific activities are expressed as micromoles of aminoacyl sRNA formed, pyrophosphate exchanged, or aminoacyl hydroxamate formed per hour per milligram of protein. The attachment and exchange assays were carried out at 30 C, the hydroxamate assay at 37 C. (From 30 to 37 C, the temperature had little effect on the results obtained by any of the assay procedures.) From Eidic and Neidhardt (7).

TABLE 3. Genetic correspondence between temperature sensitivity (*ts*) and valyl sRNA synthetase activity (*val-act*) in *Escherichia coli* strain I-9

Bacterial mating experiments <sup>a</sup>			
Hfr parent	F <sup>-</sup> parent	Recombinants ( <i>ts</i> <sup>+</sup> <i>str-r</i> )	
		<i>val-act</i> <sup>+</sup>	<i>val-act</i> <sup>-</sup>
K10 ( <i>ts</i> <sup>+</sup> <i>val-act</i> <sup>+</sup> <i>str-s</i> )	I-9 ( <i>ts</i> <sup>-</sup> <i>val-act</i> <sup>-</sup> <i>str-r</i> )	106	0

Phage P1kc transduction experiments <sup>b</sup>			
Donor	Recipient	Transductants ( <i>ts</i> <sup>+</sup> )	
		<i>val-act</i> <sup>+</sup>	<i>val-act</i> <sup>-</sup>
KB ( <i>ts</i> <sup>+</sup> <i>val-act</i> <sup>+</sup> )	I-9 ( <i>ts</i> <sup>-</sup> <i>val-act</i> <sup>-</sup> )	49	0

<sup>a</sup> From Eidlic and Neidhardt (7).

<sup>b</sup> From Böck, Faiman, and Neidhardt (4). The multiplicity of infection was approximately 1.0.

strain had a damaged valyl sRNA synthetase.

Results such as those presented in Table 2 disclosed two interesting properties of the valyl sRNA synthetase activity of I-9. First, of the three conventional assay procedures, the attachment reaction was the most severely damaged; the other assay systems indicated that considerable activation activity was retained by the modified enzyme. Second, valyl sRNA synthetase activity could only barely be detected by the attachment assay at 30 C, even in extracts of I-9 cells never exposed to the restrictive temperature.

The latter finding would be expected if the mutant enzyme were extremely labile in vitro. An alternative explanation would be that I-9 lacks a competent valyl sRNA synthetase at any temperature, and that the temperature-sensitive growth pattern and the altered enzyme activity are unrelated. Clearly it was of the utmost importance to resolve this question.

Both genetic and biochemical evidence was obtained that supported the interpretation that the mutant enzyme is temperature-sensitive in vivo and simply inactive in vitro at any temperature. Table 3 summarizes the results from many bacterial mating and viral transduction experiments (4). When K10 (*ts*<sup>+</sup> *val-act*<sup>+</sup> *str-s*) was mated to I-9 (*ts*<sup>-</sup> *val-act*<sup>-</sup> *str-r*) and *ts*<sup>+</sup> *str-r* recombinants were selected, all of them received the *val-act*<sup>+</sup> character. When the generalized transducing phage P1kc was grown on KB (*ts*<sup>+</sup> *val-act*<sup>+</sup>) and used to infect I-9 (*ts*<sup>-</sup> *val-act*<sup>-</sup>) at low multiplicities, transductants selected for *ts*<sup>+</sup> had all received *val-act*<sup>+</sup>. These data make it highly likely that the *ts* and *val-act* characters are the result of a single altered chromosomal gene.

The location of this gene has been determined, by means of interrupted matings, to be close to minute 87 on the Taylor-Thoman map (37a), as shown in Fig. 5. This map location is of interest because it shows that the *val-act* locus is not adjacent to the operon for the valine biosynthetic enzymes.

Biochemical evidence lends further support to the notion that the valyl sRNA synthetase of I-9 operates in vivo at 30 C but not at 40 C. We have measured the intracellular concentration of the product of this enzyme at both tem-

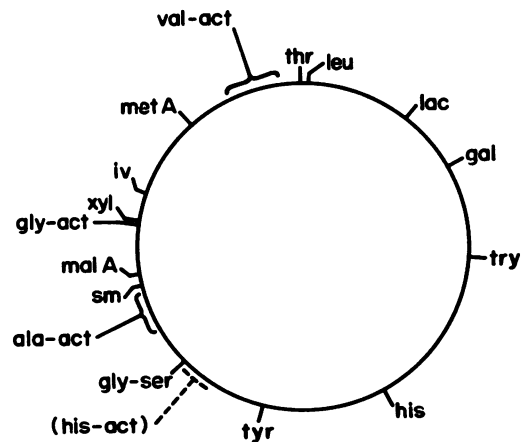


FIG. 5. Linkage map of *Escherichia coli* showing locations of structural genes for various aminoacyl sRNA synthetases. Consult text for references. The histidyl sRNA synthetase gene (*his-act*) shown in parentheses has not yet been mapped in *E. coli*, and the position shown is chosen by analogy with *Salmonella typhimurium*.

TABLE 4. *Effect of temperature and amino acid starvation on valyl sRNA charging in wild (KB) and mutant (I-9) strains of Escherichia coli<sup>a</sup>*

Strain	Medium	Temp	Specific acceptance activity <sup>b</sup>	Valyl sRNA charging
		C		%
KB	Minimal + aa <sup>c</sup>	30	2.06 (1.74)	85
KB	Minimal + aa	40	2.34 (1.77)	76
I-9 <i>iv</i> <sup>-</sup>	Minimal + aa	30	2.90 (2.48)	86
I-9 <i>iv</i> <sup>-</sup>	Minimal + aa - val	30 <sup>d</sup>	4.22 (0.30)	7
I-9 <i>iv</i> <sup>-</sup>	Minimal + aa	40 <sup>e</sup>	3.17 (0.73)	23

<sup>a</sup> From Böck, Faiman, and Neidhardt (4).

<sup>b</sup> Valine-accepting capacity of RNA isolated from each culture. Expressed as counts per minute per microgram of RNA. The number in parentheses is the accepting capacity after periodate oxidation of the sample. All samples were stripped enzymatically before final acceptance assay.

<sup>c</sup> aa = mixture of 20 L-amino acids, 50 µg/ml each.

<sup>d</sup> Incubation for 30 min in valine-deficient medium.

<sup>e</sup> Incubation at 40 C for 30 min.

peratures by means of the periodate method (4). Uncharged sRNA is readily inactivated by periodate oxidation; attachment of an aminoacyl group to the 2' or 3' position of the terminal adenosine residue affords protection from periodate oxidation. The data in Table 4 are the results of measurements of the percentage of total valyl sRNA acceptor activity that is resistant to periodate oxidation in KB and I-9 cells under different conditions. KB cells have only slightly less charged valyl sRNA at 40 C than at 30 C, whereas I-9 cells have much less. Other experiments showed that the decrease in valyl sRNA charging is very rapid when a culture of I-9 is shifted from 30 C to the restrictive range and in fact parallels the decrease in protein synthesis. At the same time, the charging of leucyl sRNA rises to near 100%, an expected consequence of the inhibition of protein synthesis.

We have identified one other temperature-conditional strain with a modified valyl sRNA synthetase, and Yanif, Jacob, and Gros (42) have identified several such strains. The frequency with which such mutants arise, relative to other synthetase mutants, is quite striking and must reflect the fact that valyl sRNA synthetase can readily be altered by any one of several point mutations so as to become thermally denatured in situ between 30 and 40 C. As yet the nature of these alterations is not known. Some of the Paris mutants resemble I-9 in possessing an enzyme that is very unstable in vitro even at low temperature, whereas others show more stability. Many of the mutant enzymes, like the one in I-9, continue to activate valine after they have lost the ability to attach it to sRNA. Genetic mapping with one of the Paris strains has indicated a *val-act* location similar to that found for the I-9 gene; whether the lesions are really at the

same location will require finer mapping, as by cotransduction.

#### *Glycyl sRNA Synthetase*

It was fortuitously discovered by L. E. Faiman in our laboratory that normal strains of *E. coli* differ in their glycyl sRNA synthetase activity. Extracts of strains B, W, and K10 were found to have approximately the same activity, whereas strains C and KB yielded extracts with only 15% and less than 0.1%, respectively, of this activity. A mixture (1:1) of extracts from KB and K10 exhibited only the expected 50% reduction in specific activity, indicating the absence of any free inhibitor in the KB extract. Increasing the amount of sRNA or of KB protein in the assay system did not increase the specific activity of KB extracts, but increasing the amount of glycine did. Determination of the  $K_m$  for glycine in crude, dialyzed extracts gave values of  $10^{-5}$  to  $5 \times 10^{-5}$  M for K10 extracts, and values 50 to 100 times higher for KB extracts.

Further study of strain KB by A. Böck (5) has revealed that one consequence of the poor enzyme is that the level of charged glycyl sRNA is abnormally low during growth of KB cells in minimal medium, and that supplementation of the medium with high concentrations of glycine increases the in vivo level of charging to that found in normal cells growing in minimal medium. Glycine supplementation does not affect the growth rate of either strain KB or K10.

Dr. Böck has been able to map very precisely the glycyl sRNA synthetase gene of KB by bacterial mating and by P1kc transduction (5). The gene is located at minute 69.5 on the Taylor-Thoman map (37a), closely linked to *xyl* between *xyl* and *malt* (Fig. 5).

Berg and Yanofsky (cited in 6) have found that



extracts of *E. coli* strains Ymel and Hfr  $R_{try}^-$  differ markedly in measurable glycyl sRNA synthetase activity, and that this difference affects the extent to which a missense suppressor gene can substitute a glycine residue for an arginine one in tryptophan synthetase A-protein (6). They have confirmed the map location for the glycyl sRNA synthetase gene.

#### *Histidyl sRNA Synthetase*

The possession by KB cells of a glycyl sRNA synthetase activity with a greatly decreased affinity for glycine does not appear to limit their growth rate. The opposite is true in the case of a derivative of strain KB that has a modification in another synthetase. During an examination of "leaky" histidine auxotrophs (bradytrophs), G. Nass discovered that one such mutant of *E. coli* possesses a damaged histidyl sRNA synthetase (25). This strain, designated  $his^-C$ , grows slowly in minimal medium, but normally when this medium is supplemented with histidine (40  $\mu$ g/ml). Crude extracts of this mutant proved to have a histidyl sRNA synthetase with a  $K_m$  for histidine approximately eightfold higher than normal. Apparently strain  $his^-C$  has a normal capacity for histidine biosynthesis, but the pool size of free histidine, which would be set by end-product inhibition on the first enzyme in the pathway, is insufficient to saturate the damaged histidyl sRNA synthetase. Preformed histidine in the medium can be pumped into the cell, elevating the internal pool, and thereby increasing the rate of histidyl sRNA formation and hence the growth rate. There is an upper limit, however, to the growth rate stimulation that can be caused by histidine. In a very rich medium, the presence of exogenous histidine can increase the growth rate of  $his^-C$  cells only to approximately 80% of the growth rate of wild-type cells (Table 5).

Mapping the histidyl sRNA synthetase structural gene in *E. coli* is still in progress in our laboratory, but what is apparently the same gene has already been mapped in *S. typhimurium*. Hartman and Ames and their collaborators have isolated four genetic groups of mutants in which the histidine operon is not fully repressed (31-33). All of the mutants were isolated by selection for resistance to the histidine analogue 1,2,4-triazole-3-alanine, a false corepressor of the histidine operon. This compound is inhibitory to the growth of *Salmonella* strains that already have a partial block in the histidine pathway, and resistance is caused by a damaged repression mechanism. One of the four classes of resistant mutants has an altered gene, termed *hisS*, that is the structural gene for histidyl sRNA synthe-

TABLE 5. Growth rate of wild (KB) and mutant ( $his^-C$ ) strains of *Escherichia coli* in different media

Growth medium	Growth rate constant <sup>a</sup>	
	PFP-10	$his^-C$
Glucose-minimal.....	0.49	0.17
Glucose-minimal + histidine (40 $\mu$ g/ml).....	0.49	0.46
Glucose-minimal + histidine (40 $\mu$ g/ml) + Casamino Acids (20 mg/ml).....	0.76	0.76
Tryptone-glucose-yeast ex- tract.....	1.65	1.18

<sup>a</sup> Specific growth constant,  $hr^{-1}$ . Strain  $his^-C$  has a modified histidyl sRNA synthetase;  $K_m$  for histidine of the KB enzyme:  $7.1 \times 10^{-4}$  M;  $K_m$  for histidine of the  $his^-C$  enzyme:  $5.0 \times 10^{-3}$ . From Nass and Neidhardt (25; in preparation).

tase. Mutants of the *hisS* class have a synthetase with changed kinetic constants, including a decreased affinity for histidine. This gene is linked to the *strB* and *quaA* loci of the *Salmonella* chromosome, quite distant from the histidine operon (Fig. 5).

#### *Alanyl sRNA Synthetase*

Yanif and Gros (41) have isolated one class of temperature-conditional mutants of *E. coli* that exhibit a strange behavior when placed at their restrictive temperature, 40 C. The cells continue to incorporate  $C^{14}$ -leucine into protein for 2 hr at a constant linear rate. Alanine incorporation diminishes steadily after the first 10 to 20 min of exposure to the high temperature. Analysis of two of these mutants has revealed that each possesses a temperature-sensitive alanyl sRNA synthetase. It is still not clear whether the continued leucine incorporation at 40 C represents synthesis of proteins that are low in, or lack, alanine, or whether abnormal polypeptide derivatives are formed. The alanyl sRNA synthetase gene affected in these mutants has been located between *str* and *thy* (Fig. 5).

#### *Arginyl sRNA Synthetase*

Hirshfield and Maas (*personal communication*) have used a clever selection to isolate an *E. coli* mutant with a modified arginyl sRNA synthetase. The arginine analogue, canavanine, is activated by extracts of *E. coli*, is incorporated into protein by these cells, and may act as a corepressor of the arginine biosynthetic enzymes. Resistant mutants usually have a damaged regulatory gene (R), are derepressed, and overproduce arginine in sufficient amounts to compete with

TABLE 6. Comparison of some properties of phenylalanyl sRNA synthetase from a *p*-fluorophenylalanine-sensitive strain (KB) of *Escherichia coli* and its resistant mutant (PFP-10)<sup>a</sup>

Enzyme	(molarity $K_m \times 10^7$ )		$K_i$ (molarity $\times 10^6$ ) of DL- <i>p</i> -fluorophenyl- alanine	Turnover no.	
	L-phenylalanine	DL- <i>p</i> -fluorophenyl- alanine		L-phenyl-alanine	DL- <i>p</i> -fluorophenyl- alanine
KB	6	910	12	8.1	8.1
PFP10	100	6,000	6,000	4.6	0.33

<sup>a</sup> The values were determined from Lineweaver-Burke plots of kinetic results obtained by the attachment assay system on 200-fold purified preparations of the enzymes. To calculate the turnover number (moles per mole of enzyme per minute at  $V_{max}$ ), these preparations were assumed to be pure and the value of 160,000 was used for the molecular weight of the enzyme. From Fangman, Nass, and Neidhardt (11).

canavanine for activation. Hirshfield and Maas started with a derepressed strain that had a leaky lesion in one of the arginine biosynthetic enzymes, causing it to produce arginine at a restrictive rate and therefore to be sensitive to canavanine. A canavanine-resistant mutant isolated from this strain proved to have a modified arginyl sRNA synthetase, possibly with an increased  $K_m$  for canavanine (and arginine). Genetic mapping is underway. Recently, mutants with modified synthetases have been isolated by direct selection for canavanine resistance starting with normally repressible ( $R^+$ ) bradytyphs.

#### Phenylalanyl sRNA Synthetase

Two different classes of phenylalanyl sRNA synthetase mutants have been isolated and extensively studied: analogue resistant mutants and temperature-conditional mutants.

The former class is by now a large one, because it is relatively simple to isolate mutants of *E. coli* that are resistant to *p*-fluorophenylalanine by virtue of an altered phenylalanyl sRNA synthetase (12). This analogue competes with phenylalanine as a substrate for the synthetase. Even though the  $K_m$  for the analogue is 150 times higher than the  $K_m$  for phenylalanine, the competition results in a massive substitution of the analogue for the natural amino acid in protein synthesis *in vivo*. This effect most likely results both from the ability of the cells to concentrate *p*-fluorophenylalanine and from the ability of this compound to decrease endogenous phenylalanine biosynthesis by end-product inhibition. Substitution of phenylalanine by its *p*-fluoro derivative results in the formation of false (enzymatically inactive) protein, and growth is severely inhibited.

Resistance can arise by several mechanisms. Some mutants overproduce phenylalanine, even in the presence of the analogue, and the natural

amino acid can then easily out-compete the analogue for the synthetase site. Other mutants have a defective concentrating mechanism and do not permit entry of the analogue. A third mechanism, however, is exhibited by mutants such as PFP-10. The phenylalanyl sRNA synthetase of this cell has been damaged in such a way that it has a 6-fold increase in  $K_m$  and a 25-fold decrease in  $V_{max}$  for *p*-fluorophenylalanine. The  $K_m$  for phenylalanine has also been increased, but there has been only a twofold reduction in  $V_{max}$  for this substrate (Table 6). No detectable change has occurred in either the molecular weight or the immunochemical properties of the enzyme as a result of the mutation (11).

There have now been several different temperature-conditional mutants identified in our laboratory as having a modified phenylalanyl sRNA synthetase. They are similar in all important respects, and in some ways resemble the temperature-conditional valyl sRNA synthetase mutants. For example, extracts of mutant strain IV-4 appear devoid of phenylalanyl sRNA synthetase activity whether or not the cells have ever been exposed to the restrictive temperature (7). Also, considerable phenylalanine activation activity (as measured in the pyrophosphate-ATP exchange assay or the hydroxamate assay) remains despite the loss of sRNA attachment activity (Table 7). It appears, then, that the lesions in the two enzymes are similar, and that therefore the absence of attachment activity in extracts of IV-4 is simply a result of the extreme lability of the mutated phenylalanyl sRNA synthetase under *in vitro* conditions; the enzyme works *in vivo* at 30 C but not at 40 C, and fails to work at either temperature *in vitro*. In fact, by assaying extracts of IV-4 cells within minutes of preparing them (i.e., omitting dialysis), it is possible to demonstrate a phenylalanyl sRNA synthetase

TABLE 7. Phenylalanyl sRNA synthetase activities of extracts of normal (K10) and mutant (IV-4) strains of *Escherichia coli* grown at different temperatures

Cells	Growth temp <sup>a</sup>	Specific activity <sup>a</sup>		
		sRNA attachment	Pyrophosphate exchange	Hydroxamate formation
	C			
K10	30	0.030	1.6	0.040
IV-4	30	0.0001	0.9	0.002
K10	37	0.035	2.1	0.035
IV-4	37	0.0001	0.8	0.002

<sup>a</sup> See footnotes to Table 2. From Eidlic and Neidhardt (7).

activity that is rapidly decaying (Böck and Neidhardt, unpublished observations).

Genetic evidence supports the conclusion that the temperature-sensitive growth pattern of IV-4 cells is caused by the altered synthetase. When IV-4 cells are infected at low multiplicities with phage P1kc previously grown on normal cells, all transductants selected for ability to grow at 40 C yield extracts with normal wild-type phenylalanyl sRNA synthetase activity. Furthermore, if the transducing phage are grown on PFP-10 (the mutant with the reduced ability to activate *p*-fluorophenylalanine), most of the temperature-resistant transductants exhibit a PFP-10-like enzyme activity (unpublished observations).

It has therefore been quite surprising for us to find that, when IV-4 cells are shifted from 30 to 40 C and protein synthesis is inhibited, the intracellular level of phenylalanyl sRNA charging does not decrease (Böck and Neidhardt, unpublished observations). Starvation of a phenylalanine auxotroph causes the usual decrease in phenylalanyl sRNA level in the cell, but at 40 C strain IV-4 behaves as if its lesion in protein synthesis were *after* rather than *before* the step of phenylalanyl sRNA formation (Table 8).

We can suggest two possible explanations for this paradox. First, *E. coli* may possess a minor species of phenylalanine-specific sRNA that is not detected in the usual periodate oxidation assay for charged sRNA. The damaged IV-4 enzyme at 40 C in situ may then be unable to charge this hypothetical sRNA species, but still able to charge, at a very slow rate, the major species. Protein synthesis would halt upon exhaustion of the minor species, permitting accumulation of the acylated form of the major species.

A second explanation is that at 40 C in vivo

TABLE 8. Effect of temperature and of amino acid starvation on phenylalanyl-sRNA charging in a phenylalanine auxotroph of *Escherichia coli* with a temperature-sensitive phenylalanyl sRNA synthetase<sup>a</sup>

Medium	Temp	Specific acceptance activity <sup>b</sup>	Phenylalanyl sRNA charging
	C		%
Minimal + phenylalanine <sup>c</sup>	30	0.49 (0.46)	94
Minimal + phenylalanine <sup>c</sup>	40 <sup>d</sup>	0.64 (0.62)	97
Minimal.....	30 <sup>e</sup>	0.75 (0.03)	4
Minimal.....	40 <sup>f</sup>	0.70 (0.00)	0

<sup>a</sup> From Böck and Neidhardt, unpublished experiments.

<sup>b</sup> Phenylalanine-accepting activity of RNA isolated from each culture. Expressed as counts per minute per microgram of RNA. The number in parentheses is the accepting activity after periodate oxidation. For methods and media, see Böck et al. (4).

<sup>c</sup> 50 μg/ml.

<sup>d</sup> Incubation at 40 C for 1 hr.

<sup>e</sup> Starvation for phenylalanine for 1 hr at 30 C.

<sup>f</sup> Starvation for phenylalanine for 1 hr at 40 C.

the damaged enzyme charges the sRNA satisfactorily but fails to release the phenylalanyl sRNA complex for participation in protein synthesis on the polysomes.

In either case, one would have to assume that the enzyme undergoes a further transition in vitro, destroying its ability to charge any sRNA. At the moment, there is insufficient information to rule out either of these suggestions.

Table 9 lists the mutants with modified synthetases that are known at present, and Fig. 5 shows the current status of their mapping.

#### PHYSIOLOGICAL RESPONSES OF MUTANTS WITH MODIFIED SYNTHETASES

The existence of mutants for 6 of the 20 aminoacyl sRNA synthetases has made it possible to examine whether these enzymes are involved in regulatory phenomena, and, incidentally, to confirm the indispensable role they play in protein synthesis.

#### Regulation of RNA Synthesis

The temperature-conditional synthetase mutants for valine (7, 41), alanine (41), and phenylalanine (Fairman, Böck, and Neidhardt, unpublished observations) all show the same behavior when placed at their restrictive temperature: the accumulation of RNA ceases as abruptly

TABLE 9. Mutants of *Escherichia coli* and *Salmonella typhimurium* with modified aminoacyl sRNA synthetases

Strain designations	Aminoacyl sRNA synthetase	Nature of lesion	References
<i>E. coli</i>			
PFP-10; KB10R, PFP-12S, K10S, and K10F	Phenylalanine	Altered $K_m$ for <i>p</i> -fluorophenylalanine	10; Fangman and Neidhardt (unpublished)
IV-4; Cl-2; EV-5, and EV1-33	Phenylalanine	Temperature-sensitive	7; Eidlic and Neidhardt (unpublished); Earhart and Neidhardt (unpublished)
KB and others	Glycine	High $K_m$ and possibly other abnormalities	5; 6
I-9; 2E-17; T2, T7, T9, T16, T722, T141, T146, T536, and T537	Valine	Temperature-sensitive	7; Earhart and Neidhardt (unpublished); 41
his <sup>-</sup> C	Histidine	High $K_m$ for histidine	25
T65, T140	Alanine	Temperature-sensitive	41
K12 derivatives	Arginine	Not known	Hirshfield and Maas (unpublished)
<i>S. typhimurium</i>			
hisS 1520, 1210,	Histidine	High $K_m$ for histidine	33
hisS 1209, 1210, 1213, 1219, 1259	Histidine	Possibly high $K_m$ for histidine	33
hisS 1211	Histidine	Unknown	33

as, or more abruptly than, the synthesis of protein. The response of the valine mutant, I-9, is shown in Fig. 6. This result indicates that the amino acid-mediated regulation of RNA accumulation operates not with the free amino acid but with some derivative of it that can be

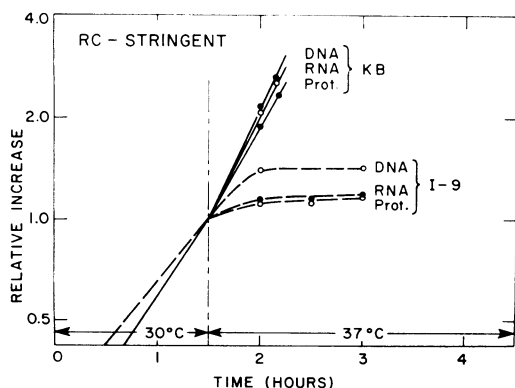


FIG. 6. Effect of a temperature shift on macromolecule synthesis in wild (KB) and mutant (I-9) strains of *Escherichia coli*. At the time indicated by the arrow, cultures of KB and I-9 growing in glucose-tryptone rich medium were shifted from 30 to 37°C. Samples were removed at intervals to determine the amount of DNA, RNA, and protein per milliliter in each culture by methods described elsewhere (7). All values are normalized to the value at the time of the shift, and are plotted as a logarithmic function of time. From Neidhardt and Faiman (unpublished observations).

made only with a competent aminoacyl sRNA synthetase. It suggests, but does not rigorously prove, that aminoacyl sRNA itself is the important factor in this control device. The same conclusion can be drawn from the fact that *p*-fluorophenylalanine will permit RNA accumulation in a phenylalanine auxotroph with a normal phenylalanyl sRNA synthetase but fails to do so in the mutant that cannot activate this analogue (13).

The dependence of RNA accumulation on aminoacyl sRNA synthetase activity holds true for cells with normal amino acid control (stringent), but not for cells lacking this control (relaxed). If the temperature-sensitive valyl or phenylalanyl enzymes are placed in cells with relaxed amino acid control, then an overproduction of RNA occurs at the restrictive temperature (4, 7). This behavior is illustrated in Table 10, which shows the response of a stringent strain (I-9) and a relaxed strain (Re24) which possess the same temperature-sensitive valyl sRNA synthetase. In the relaxed as well as the stringent cell, the level of acylated sRNA<sub>val</sub> falls markedly at 40°C, indicating that no other enzyme has assumed valyl sRNA synthetase function at 40°C in the relaxed cell. In the case of the temperature-sensitive phenylalanyl enzyme, as we have noted in a previous section, all of the detectable sRNA<sub>phe</sub> is acylated at the restrictive temperature whether the cell is relaxed or stringent.

These results prove that in a stringent cell both

TABLE 10. Incorporation of  $C^{14}$ -uracil by stringent (I-9) and relaxed (Re24) strains of *Escherichia coli* that possess the same temperature-sensitive valyl sRNA synthetase<sup>a</sup>

Time after temp shift	Uracil-2- $C^{14}$ incorporated			
	Cells not pre-labeled <sup>b</sup>		Pre-labeled cells <sup>c</sup>	
	I-9	Re24	I-9	Re24
min				
0	—	—	1.00	1.00
10	1.44	7.65	0.97	1.25
20	1.69	14.60	0.90	1.41
30	2.24	20.30	0.91	1.63
60	3.44	30.60	0.88	1.96

<sup>a</sup> From Böck et al. (4).

<sup>b</sup> Radioactive uracil was added at zero-time to cultures in minimal medium containing 50  $\mu$ g of uracil per ml. The data are expressed as millimicromoles of uracil incorporated by a 2-ml sample at an optical density of 1.0.

<sup>c</sup> These cultures had been pregrown in minimal medium containing uracil-2- $C^{14}$ . The data are normalized to the values at zero-time.

the amino acids and their synthetases must be present for RNA to accumulate. This requirement may be explained in any of several ways. First, it has been suggested that uncharged sRNA may inhibit RNA polymerization (18, 37, 38). In this case, the amino acid and the synthetase would both be required to neutralize the inhibitor. This possibility is made unlikely by the fact that the stringent phenylalanyl sRNA synthetase mutant ceases RNA accumulation at 40 C despite the absence of any detectable uncharged sRNA<sub>phe</sub> (though one might still postulate an undetectable sRNA<sub>phe</sub> species). Also, there is no difference between the RNA polymerases of stringent and relaxed cells, yet RNA accumulation proceeds in the latter despite the presence of unacylated sRNA. Morris and De Moss (23) and Ezekiel (10) have also described situations in which RNA regulation appears uncorrelated with the amount of uncharged sRNA.

If concomitant synthesis of a special protein is required for RNA accumulation, then the necessary participation of the aminoacyl sRNA synthetases would be obvious. This possibility, which appears so easy to test, has really not yet been eliminated. The critical experiment is to see whether RNA accumulation can occur in the total absence of protein synthesis. This situation has been approximated (e.g., with high concentrations of chloramphenicol), but it is of course difficult to establish that no protein molecules are being made under a given condition.

The recent discovery that polysomes disaggregate upon amino acid starvation (24, 30) has led to the suggestion that polysome integrity is required for RNA accumulation. This view has the advantage that it places importance on the availability of charged sRNA [and perhaps other components of protein synthesis such as guanosine triphosphate (GTP)] rather than the absolute amount of uncharged sRNA in the cell. It is supported by the findings that polysomes remain intact during amino acid starvation of relaxed cells (24, 30), and that high concentrations of chloramphenicol induce polysome re-aggregation in amino acid-starved cells (40). It has been suggested that polysome integrity is necessary either to bind an inhibitor of RNA synthesis (24) or to strip actively the nascent RNA chains off the DNA template (36). It is clear that all of the results with temperature-sensitive synthetase mutants are consistent with the view that polysome integrity rather than the absence of uncharged sRNA is the key factor. More critical information may become available when we learn what step is blocked in the phenylalanyl sRNA synthetase mutants and whether polysomes are present in them at 40 C.

One disturbing feature of all of these theories is that none are supported by compelling evidence of what is cause and what is effect. Cessation of protein synthesis may stop RNA synthesis, or vice versa; and disaggregation of polysomes may stop RNA synthesis, or vice versa. Furthermore, Gallant has recently presented evidence that the amino acid requirement for RNA formation operates by controlling uridine triphosphate (UTP) biosynthesis (Gallant and Cashel, *personal communication*).

It is clear then that amino acids must be acted upon by competent aminoacyl sRNA synthetases to permit RNA accumulation in stringent cells. It is not yet clear that the derivative made by the synthetase is identical to the aminoacyl sRNA products that participate in protein synthesis, and (whether or not it is the same) it is not clear by what biochemical mechanism RNA accumulation is halted in the absence of the amino acid or its competent synthetase.

#### Regulation of DNA Synthesis

The behavior of I-9 cells shown in Fig. 6 is typical of most temperature-sensitive synthetase mutants when placed at their restrictive temperature. It can be seen that DNA synthesis continues at the restrictive temperature after protein and RNA synthesis have halted. After increasing approximately 40%, DNA then is no longer made. This behavior shows that failure

to activate one amino acid is sufficient to interfere with DNA synthesis, and is consistent with the view that amino acids are required for sustained DNA synthesis only because they are required to make a special initiator protein, without which new rounds of DNA replication cannot be started though previously initiated strands can be completed (19).

#### Repression of Biosynthetic Enzymes

Repression of the enzymes of the valine-isoleucine pathway requires the simultaneous presence of all four of the products derived from this pathway: valine, isoleucine, leucine, and pantothenate (Fig. 7). A limitation of any one of the products derepresses both threonine deaminase (enzyme 1) which is involved only in isoleucine synthesis, and the four enzymes which participate in valine and isoleucine synthesis (enzymes 2, 3, 4, and 5). Reaction 9 is catalyzed by relatively nonspecific transaminases. The three specific enzymes leading from  $\alpha$ -keto-isovalerate to leucine (enzymes 6, 7, and 8) are repressed by leucine alone.

When I-9 cells (possessing a temperature-sensitive valyl sRNA synthetase) are shifted from 30 to 35.5 C, some protein synthesis occurs before growth stops. During this period, valine-controlled enzymes are derepressed. The results presented in Fig. 8 show a derepression of enzyme 2 under these conditions. No derepression occurs in the wild strain, and neither strain derepresses the leucine-controlled enzyme 6. Chloramphenicol blocks the increase in enzyme activity, indicating that the temperature shift does not simply activate preformed enzyme. Similar re-

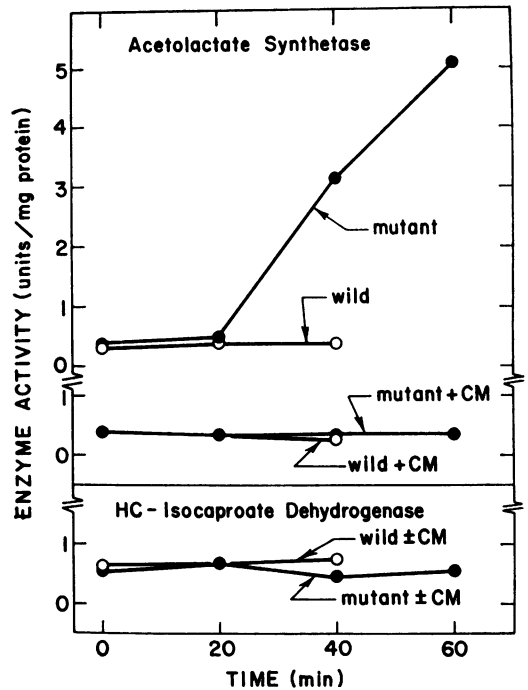


FIG. 8. Enzyme synthesis in wild (KB) and mutant (I-9) strains of *Escherichia coli* after a shift from 28 to 36 C. The cultures were grown in glucose-minimal medium supplemented with L-valine (100  $\mu$ g/ml), L-isoleucine (50  $\mu$ g/ml), L-leucine (50  $\mu$ g/ml), and pantothenic acid (1  $\mu$ g/ml). The specific activities of acetolactate synthetase (enzyme 2 of Fig. 7) and of  $\alpha$ -hydroxy- $\beta$ -carboxyisocaproate dehydrogenase (enzyme 6 of Fig. 7) are expressed as units of enzyme per milligram of protein, and plotted as a function of time after the temperature shift. From Eidlic and Neidhardt (8).

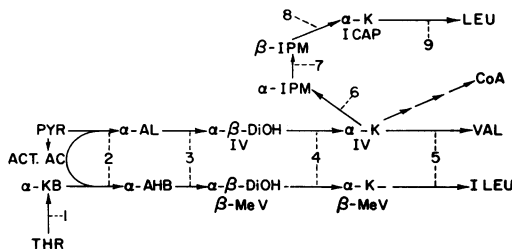


FIG. 7. Biosynthetic pathway of branched chain amino acids in *Escherichia coli*. Abbreviations used: THR, threonine;  $\alpha$ -KB,  $\alpha$ -ketobutyrate; PYR, pyruvate; ACT AC, active acetaldehyde;  $\alpha$ -AL,  $\alpha$ -acetolactate;  $\alpha$ -AHB,  $\alpha$ -acetohydroxybutyrate;  $\alpha$ - $\beta$ -DiOH IV,  $\alpha$ - $\beta$ -dihydroxyisovalerate;  $\alpha$ - $\beta$ -DiOH- $\beta$ -MeV,  $\alpha$ - $\beta$ -dihydroxy- $\beta$ -methylvalerate;  $\alpha$ -K IV,  $\alpha$ -ketoisovalerate;  $\alpha$ -K- $\beta$ -MeV,  $\alpha$ -keto- $\beta$ -methylvalerate; VAL, valine; ILEU, isoleucine; CoA, coenzyme A;  $\alpha$ -IPM,  $\alpha$ -isopropylmalate;  $\beta$ -IPM,  $\beta$ -isopropylmalate;  $\alpha$ -KICAP,  $\alpha$ -ketoisocaproate; LEU, leucine.

sults are obtained in either minimal or in amino acid-supplemented media, and enzyme 1 shows a similar response (8).

These results, confirmed in Paris (41), indicate that valine can participate in multivalent repression only when it can be acted upon by a competent valyl sRNA synthetase. The considerable ability of I-9's valyl sRNA synthetase to catalyze valyl adenylate formation in vitro suggests that the rate-limiting process at high temperature is the sRNA attachment function of the synthetase. Therefore, the possibility that the valyl-enzyme complex, or valyl adenylate-enzyme complex, acts as a repressor is not very likely. Rather, it would seem that to act as a corepressor valine must be attached by means of the synthetase to one or more of its cognate sRNA species, or to some still unknown apo-repressor.

Evidence is equally strong that histidyl sRNA synthetase is required to convert histidine into a repressing derivative. Schlesinger and Magasanik

(34) presented the first evidence by showing that  $\alpha$ -methyl histidine inhibits histidyl sRNA synthetase in *Aerobacter aerogenes* and in *E. coli*, leading both to the accumulation of histidine and a derepression of the histidine biosynthetic enzymes. More direct evidence is provided, in *Salmonella*, by the *hisS* mutants, previously described, that possess a damaged histidyl sRNA synthetase and are derepressed (31). In addition, another class of derepressed mutants has been identified as being deficient in sRNA for histidine (35). The latter class, termed *hisR*, are characterized by having only 50% of the normal sRNA acceptor activity for histidine, and elevated acceptor activities for several other amino acids. It is not clear what function the *hisR* gene plays in sRNA<sub>his</sub> formation, but the existence of these mutants further implicates sRNA in repression.

The *Salmonella* mutants were isolated as regulatory mutants. The *E. coli* mutant, *his*<sup>-</sup>C, was isolated as a histidine bradytroph, but it appears to share many of the properties of the *Salmonella hisS* mutants. The histidyl sRNA synthetase of *his*<sup>-</sup>C has an elevated  $K_m$  for histidine, and whenever it is placed in a medium in which its growth is subnormal (as a result of restricted histidine activation) it is derepressed for the histidine biosynthetic enzymes (25).

One feature of enzyme repression in synthetase mutants deserves special noting. Yanif and Gros (41) have observed that derepression of threonine deaminase (enzyme 1, Fig. 7) can be observed at temperatures slightly below that at which growth becomes detectably retarded. Some of the *S. typhimurium* mutants with a modified histidyl sRNA synthetase can be repressed by the addition of histidine to the minimal medium even though their growth rate is not stimulated (33). The *E. coli* histidyl sRNA synthetase mutant is almost maximally derepressed when growing in rich medium containing histidine, even though its growth rate is 80% of normal (25). Yanif and Gros (41) have pointed out that their results are consistent with the earlier chemostat studies by Gorini (15) in which it was found that repression of the arginine biosynthetic enzymes occurs only if the amino acid is supplied to the cell at a rate sufficient to saturate the demands of the protein-synthesizing machinery. This situation can be formally expressed by saying that the rate constant of a key reaction by which the activated amino acid participates in repression is smaller than the rate constant of the reactions leading to incorporation into proteins.

While the evidence indicates that at least two

amino acids, histidine and valine, must be activated to repress, all existing evidence suggests that the aromatic amino acid pathway may be regulated by a biochemically different mechanism. Ravel, White, and Shive (29) have shown that some analogues of tyrosine can repress tyrosine-controlled 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP) synthetase without being activated. No tyrosyl-sRNA synthetase mutants are available to test this system further, but we have examined the regulation of the phenylalanine-controlled DAHP synthetase in our mutants. Studies with the *p*-fluorophenylalanine-resistant mutant have led to equivocal results, but the temperature-sensitive phenylalanyl sRNA synthetase mutant has clearly demonstrated a lack of derepression at growth-restricting temperatures (Faiman and Neidhardt, *unpublished observations*). No conclusion can really be drawn, however, for it should be recalled that this mutant contains apparently fully charged sRNA<sub>phe</sub> at the restrictive temperature.

#### *End-Product Inhibition of Biosynthetic Pathways*

All information to date indicates that amino acids which participate in end-product inhibition do so as free amino acids, not as their aminoacyl sRNA derivatives. Fink and Berkowitz (cited in 33) have shown that histidyl sRNA is not active as an end-product inhibitor at physiological concentrations. Triazolealanine is activated and acts as a repressor of the histidine operon, but is inactive as an end-product inhibitor (22). Indirect evidence indicates that *p*-fluorophenylalanine still acts as an end-product inhibitor in vivo in a cell that cannot activate it (Fangman and Neidhardt, *unpublished data*).

#### *Catabolite Repression*

The repression of catabolic enzymes that ensues during amino acid restriction is an indirect effect, and is believed to be similar to the repression imposed by any biosynthetic restriction when catabolism is unimpaired. Restriction of any amino acid or nucleotide slows down protein (and RNA) synthesis, causing a surplus of the other amino acids, and nucleoside triphosphates. Each of these end products inhibits its own biosynthetic pathway, leading to the build-up in the cell of intermediary catabolites which are then converted into corepressors of catabolic enzymes. This chain of events is so long that one could not hope to learn much about the mechanism of catabolite repression by analyzing aminoacyl sRNA synthetase mutants. Nevertheless, temperature-conditional synthetase mu-





contribute useful information about synthetase structure and function in vivo.

#### SUMMARY AND FUTURE GOALS

Of the six processes of bacterial cells that are linked to amino acids, four of them, protein synthesis, DNA synthesis, RNA synthesis, and biosynthetic enzyme repression, require the interaction of the amino acid with the same specific aminoacyl sRNA synthetase. The other two processes, pathway regulation and catabolite repression, are mediated by the amino acid itself acting as an inhibitor of an early enzyme in its biosynthetic pathway.

Figure 10 summarizes our current knowledge of the network of regulatory devices linking each amino acid to a host of cellular processes. A precursor catabolite is shown being converted through three intermediates, *a*, *b*, and *c*, into an amino acid. The amino acid is acted upon by its specific aminoacyl sRNA synthetase and attached to one or more cognate sRNA species preparatory to being incorporated into a growing polypeptide chain. In addition, the synthetase is, at least for valine and histidine, responsible for making two derivatives of the amino acid: *aa-x*, the corepressor of the enzymes in the biosynthetic pathway; and *aa-y*, the "inducer" of RNA accumulation. It is possible that *aa-x* or *aa-y*, or both, are identical to one of the *aa-sRNA* species.

Much of the information we have about the in vivo role of aminoacyl sRNA synthetases has been obtained very recently by the use of defective mutants, many of them of the conditional type. To date, useful mutants have been obtained for 6 of the 20 synthetases, and there seems to be no reason why more cannot be obtained. Continued analysis of the mutants already at hand, and isolation of new ones, should help resolve many important questions about aminoacyl sRNA synthetases. Future goals should include the following: (i) discovery of the nature of *aa-x* and *aa-y*, the effectors in enzyme repression and RNA control, respectively, and clarification of the role of synthetases in repression by tyrosine and phenylalanine; (ii) elucidation of the fine structure of aminoacyl sRNA synthetases, including their subunit composition and the location of the amino acid and the sRNA recognition sites; (iii) resolution of the question of single versus multiple forms of synthetases for each amino acid; (iv) definition of the number of cistrons coding for each synthetase, their map location, and the nature of their products; (v) discovery of whether modified synthetases can suppress missense and nonsense mutations; (vi) description of the molecular details of synthetase

function, particularly the nature and extent of its association in the cell with sRNA; (vii) discovery of how the formation of aminoacyl sRNA synthetases is regulated; and (viii) elucidation of the nature and physiological significance of phage-induced modification of synthetases.

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