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Growth-Linked Instability of a Mutant Valyl-Transfer Ribonucleic Acid Synthetase in *Escherichia coli*[1]

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The valyl-transfer ribonucleic acid (tRNA) synthetase of *Escherichia coli* strain NP2907, previously described as having an elevated K_m for adenosine triphosphate and reduced stability in vitro compared to the wild type, was found to be conditionally thermolabile in vivo. The rate of inactivation of this enzyme at a particular temperature appears to be coordinated with the rate of growth; at 40 C this coordination results in equal rates of synthesis and destruction over a wide range of growth rates. In vitro studies showed that conditions favoring maintenance of the valyl-tRNA synthetase-valyl adenylate complex conferred complete protection against inactivation at 40 C, whereas the further addition of uncharged tRNA caused rapid, irreversible decay. We propose that the rate of inactivation of this mutant valyl-tRNA synthetase in vivo is a function of the ratio of deacylated to acylated $tRNA^{val}$ and that this ratio is a function of growth rate. The event which renders the valyl-tRNA synthetase susceptible to inactivation is likely to be the normal breakdown of the valyl-tRNA synthetase–valyl-adenylate complex during a cycle of aminoacylation of $tRNA^{val}$.

Escherichia coli strain NP2907, possessing an altered valyl-transfer ribonucleic acid (tRNA) synthetase (VRS), has been described (1). The unique feature of this strain is the apparent dependence of the intracellular valyl-tRNA synthetase concentration upon the temperature of cultivation of the cells. At a particular temperature, the level of detectable VRS which can be sustained is constant over a wide range of growth rates which are nutritionally set. At 40 C, the cells can apparently produce no VRS, and when they are shifted from 30 to 40 C the enzyme concentration declines at the same rate that protein is synthesized. The result is a dilution of VRS activity, and the enzyme level per milliliter of culture remains constant.

Two possibilities can be entertained to explain this phenomenon. The first is that the synthesis of VRS is temperature-sensitive and that VRS once formed is thermostable. At 40 C, the synthesis of new VRS would be precluded, and only preexisting enzyme would remain active, to be diluted out by continued protein synthesis. The second possibility is that the rates of synthesis and inactivation of VRS are coordinated in such a manner that the measurable concentration (units/milligram of total protein) of VRS is a net balance between the two rates; at 40 C these rates would be equal, resulting in no net accumulation.

The former possibility was advocated in a previous note (Anderson and Neidhardt, Bacteriol. Proc., p. 118, 1968), and there are several precedents in the literature for the temperature-sensitive synthesis of proteins. Sadler and Novick (16) reported a mutationally generated temperature-sensitive synthesis of the repressor protein of the *E. coli lac* operon; Kornberg and Smith (11) have isolated a temperature-sensitive synthesis mutant of the *E. coli* isocitrate lyase; and Condon and Ingraham (3) have discovered temperature-sensitive synthesis of the muconate lactonizing enzyme in a mutant of *Pseudomonas putida*. We found, as did these workers for their systems, that the

[1] The studies reported here are taken from a thesis submitted by James J. Anderson to Purdue University in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of some of these findings has appeared (14).

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VRS of strain NP2907 was apparently inherently stable at 40 C when media conditions precluded new VRS synthesis. This evidence, together with the demonstration that detectable cross-reacting material (to antibody prepared against wild-type VRS) did not accumulate at 40 C, suggested that our first conclusion was correct.

Unequivocal proof, however, required the positive identification of preexisting, thermostable VRS in a growth situation where de novo VRS synthesis should be halted. A convenient tool for this study was provided by the density shift technique originally suggested for proteins by Hu, Bock, and Halvorson (7), and recently employed by Chrispeels *et al.* (2), and Williams and Neidhardt (19) to resolve problems of aminoacyl-tRNA synthetase formation. Briefly, cellular protein is labeled with deuterium by growth in deuterium oxide under one set of conditions, thus labeling the enzyme of choice, and then shifting to the conditions of interest in H_2O, such that any new proteins synthesized will be unlabeled. The preexisting "heavy" and de novo "light" proteins are readily separable on CsCl density gradients after centrifugation to equilibrium. No prior purification of the enzyme is required.

We now report that new VRS is indeed synthesized at 40 C in strain NP2907. The evidence strongly suggests the bizarre coordination of synthesis and inactivation of VRS, the degree of which sets the maximum concentration of VRS the cells can sustain, and which concentration diminishes to zero as the temperature approaches 40 C.

Based on the in vitro stability characteristics of the mutant VRS, we propose a model which relates the rate of inactivation to the in vivo functional state of the VRS vis a vis its substrates and products.

MATERIALS AND METHODS

Organisms. *E. coli* strains NP2 (wild type), NP29 (a primary mutant of strain NP2), and NP2907 were described previously (1).

Media and methods of cultivation. Media were prepared as in the accompanying paper (1), with the following exception. The components of the minimal medium involved in deuterium-labeling studies were dissolved in 99.75% D_2O (Volk Radiochemicals) rather than H_2O. L-Valine (150 μg/ml) and L-isoleucine (50 μg/ml) were present in all minimal media unless specifically noted otherwise.

Shifts from D_2O to H_2O media were accomplished by quickly chilling the D_2O culture and centrifuging the cells at 9,000 $\times$ *g* at 0 C for 10 min. The pelleted cells were washed twice with H_2O medium at 0 C and then resuspended in a small volume of the cold medium. This suspension was then rapidly diluted at least ten-fold into prewarmed H_2O medium at the appropriate temperature. Growth was monitored by optical density at 420 nm with a Beckman model DB spectrophotometer.

Bacterial cultures were slowly adapted to D_2O by successive growth for at least five generations in 25, 50, 80, and then 95% D_2O minimal medium before use in experiments. Unless specifically noted, cultures were always sampled at cell densities which were consistent with balanced growth.

Preparation of extracts. Crude extracts were prepared from rapidly chilled culture samples as described previously (1). The washed cells were sonically disrupted in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.3, containing 0.001 M L-valine and 0.006 M 2-mercaptoethanol (TVM buffer), and centrifuged at 9,000 $\times$ *g* to remove whole cells and debris. The clarified extracts were assayed for VRS activity within 24 hr, except for the equilibrium centrifugation studies and the in vitro analysis of VRS stability, which required the following purification to remove nucleic acid.

Extracts of strains NP2 and NP2907 were further centrifuged at 100,000 $\times$ *g* for 2.5 hr in TVM buffer containing 0.01 M $MgCl_2$. The pellet was discarded, and the supernatant liquid was titrated with 10% streptomycin sulfate solution to a final concentration of 2% streptomycin. After centrifugation at 10,000 $\times$ *g* for 30 min, the precipitate was discarded, and the supernatant liquid was exhaustively dialyzed against TVM. The extracts were made 25% in glycerol and stored at 0 C where they retained activity for months. Immediately before use, samples were passed through columns of Sephadex G-25, equilibrated with TVM buffer, to remove small molecules. These preparations contained less than 1% RNA by ultraviolet absorption (280/260 nm).

Aminoacyl-tRNA synthetase assay. The measurement of the aminoacylation of tRNA (General Biochemicals, from *E. coli* K-12) has been described (1). In several experiments which measured the stability of VRS in incomplete reaction mixtures at varying temperatures, the assays were performed in the following way. A solution (0.5 ml) containing the usual proportions of Tris buffer, $MgCl_2$, KCl, glutathione, ^{14}C-L-valine, and bovine serum albumin was supplemented with 0.5 unit of inorganic pyrophosphatase (Worthington), nucleic acid-free crude extract of strains NP2 or NP2907, and combinations of adenosine triphosphate (ATP) and tRNA. At intervals of incubation at elevated temperature, including a zero point, 0.1-ml samples were taken and rapidly pipetted into 0.4 ml of a complete reaction mixture at 30 C. From the latter tube, 0.1-ml samples were periodically removed and pipetted into 3 ml of 5% trichloroacetic acid containing 1 mg of D,L-valine at 0 C. After 39 min, the precipitates were collected by filtration onto cellulose acetate filters (Schleicher and Schuell, B4) and washed with 5% trichloroacetic acid-valine (two 5-ml rinses) and finally with 5 ml of 67% ethanol. The filters were air dried and counted in a toluene-based scintillation fluid with a Packard liquid scintillation counter.

Isopycnic centrifugation in cesium chloride. The isopycnic centrifugation procedure used was

that of Williams and Neidhardt (19). Centrifugations were carried out in a Beckman-Spinco SW39L rotor with a Spinco model L-4 ultracentrifuge. The 5.0-ml centrifuge tubes contained the following: 1 ml of saturated CsCl (Harshaw) in water at 25 C; 2 ml of TVM buffer containing up to 1.5 mg of crude bacterial protein; and 1.5 ml of mineral oil layered on top. The tubes were centrifuged at 39,000 rev/min for 60 to 70 hr at 5 C. After centrifugation the tubes were pierced through the bottom and six-drop fractions were collected. Samples (10 μliters) were removed from each fraction and assayed in the usual aminoacylation reaction. The CsCl gradient was monitored by the refractive index of the remaining fraction volume with a Zeiss refractometer, and particular values were converted to density (grams/milliliter) by means of a standard curve.

Quantitative microcomplement fixation. The method of Wasserman and Levine (18) for microcomplement fixation was used without modification. Basically, the assay measures the formation of antibody-antigen complexes through their specific inactivation (fixation) of complement. The assay was performed in two steps. (i) A fixed quantity of antibody to VRS (a gift of M. Yaniv) was titrated with increasing concentrations of the antigen in the presence of fixed quantity of complement (guinea pig, Difco). Incubation to completion was permitted for 18 hr at 4 C. (ii) Unfixed complement was measured by adding sheep red blood cells which had been sensitized with anti-sheep hemolysin (Difco), and lysis was permitted at 37 C for 1 hr. The solubilized hemoglobin was measured spectrophotometrically at 413 nm. Within defined limits of the assay, the amount of hemoglobin released is an inverse measure of the quantity of antigen-antibody complex present. Results are expressed as $C'H_{50}$ units fixed versus antigen concentration, where 1 $C'H_{50}$ unit equals that dilution of complement which will cause 50% lysis of sensitized red blood cells.

Crude extracts of *E. coli* strains NP2 and NP2907 were the source of the VRS antigen; these extracts were prepared in 0.006 M potassium phosphate buffer (*p*H 7.3) containing 0.006 M 2-mercaptoethanol. All procedures for measurement of complement activity, sensitization of red blood cells, and preparation of reagents were taken from Kabat and Mayer (8).

Calculation of the rate of synthesis of VRS from density shift data. The unique properties of strain NP2907 allowed a simpler method of analysis than that employed by Williams and Neidhardt (19).

At 40 C the rates of synthesis, v_s, and inactivation, v_d, must be identical since the amount of VRS per milliliter of culture remains constant. That is,

$$v_s = -v_d \tag{1}$$

To measure the true rate of synthesis of new enzyme, therefore, it is sufficient to measure the rate of inactivation of preexisting VRS, which is simplified by the density shift method discussed above. If prelabeled enzyme is E_h (units/milliliter of culture), then

$$d(E_h)/dt = v_d \tag{2}$$

Where E_t = total enzyme measured. Integrating,

$$\ln E_{h2} - \ln E_{h1} = (v_d/E_t)(t_2 - t_1) \tag{3}$$

For the sake of convenience, since the rate of VRS synthesis relative to the previous rate at 30 C is of issue, let $E_{h1} = 1$. Then the relation is simplified to

$$\ln E_{h2} = v_d (t_2 - t_1) \tag{4}$$

If, during this interval, an amount Q of new enzyme is made,

$$Q = v_s (t_2 - t_1) \tag{5}$$

then, combining (1), (5), and (4),

$$\ln E_{h2} = -Q \tag{6}$$

Which relates the amount of new synthesis of VRS to the measurable loss of the old enzyme.

Measurement of the rate of synthesis of VRS at 30 C proceeds simply because the specific activity of the culture is not changing. Therefore, the amount of inactivation of preexisting VRS, which is simply measured by the density shift technique described above, can be added on to the measurable amount of new VRS made to obtain the true rate of synthesis of new enzyme.

RESULTS

In vivo stability of the mutant valyl-tRNA synthetase at 40 C. The experiments depicted in Fig. 1 and 2 were employed to examine the in vivo thermostability of the VRS of strain NP2907 in environments which would not permit protein (new VRS) synthesis. Figure 1 shows that, when cultures of strain NP2907 were shifted from growth at 30 C in rich broth to a 40 C salt solution, the VRS activity remained stable in the presence of external L-valine but declined gradually if the amino acid was omitted. The initially rapid rate of inactivation slows upon continued starvation, perhaps because of endogenous generation of intracellular L-valine from protein turnover. In the same figure, the ability of the two cultures to resume growth at 40 C when the original broth was reconstituted is illustrated. The ability to respond was directly proportional to the measurable amount of VRS remaining, confirming the previous observation that the VRS in this strain is limiting at 40 C in TGYE broth (1).

Protein synthesis was halted in other cultures by either tetracycline inhibition, L-isoleucine and L-tryptophan starvation, or levallorphan tartrate inhibition (Fig. 2). In the first two cases, there was but a slight decline in VRS level with time; however, levellorphan treatment, which causes a drastic decline in intracellular ATP (6), caused a dramatic inactivation of the VRS.

These experiments demonstrated that the

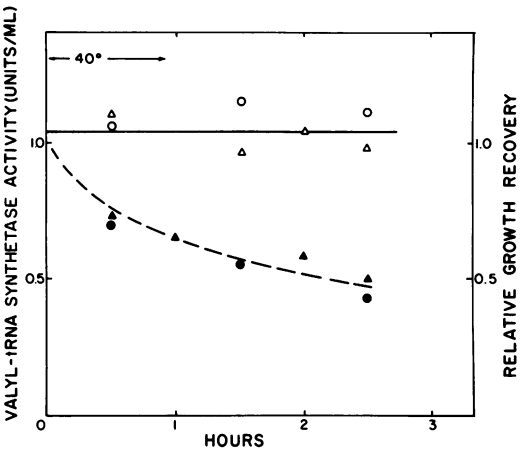

FIG. 1. *In vivo stability of the valyl-tRNA synthetase of strain NP2907 at 40 C during starvation. A culture of strain NP2907 growing at 30 C in TGYE broth (1) was harvested at 0 C, washed three times with ice-cold 0.7% NaCl, divided into two parts, and resuspended at 40 C in this solution either with 150 μg of* L-*valine per ml (open symbols) or without (closed symbols). Periodically, samples were removed for determination of valyl-tRNA synthetase activity (triangles), other samples were added to complete TGYE broth at 40 C, and the initial growth rate was monitored by optical density at 420 nm. Initial growth rate at each starvation time was then expressed relative to the initial growth rate (k = 1.95 hr^{-1}) in TGYE without starvation and plotted on the ordinate as relative growth recovery (circles).*

VRS level of strain NP2907 could be lowered by manipulating the L-valine and ATP levels of the cell; however, it is unlikely that the loss of either operates during growth. L-Valine is always present in excess in the medium, and this strain was shown to be permeable to L-valine under the conditions used for temperature shifts (*unpublished data*). Total starvation for energy sources at 40 C did not affect the level of VRS, so ATP level is probably not limiting during growth. Greene and Magasanik (6) have shown the difficulty of depressing the ATP pool by nutritional means.

These data suggested that under normal conditions of growth the VRS of strain NP2907 was thermostabile in vivo at 40 C and confirmed the findings of Yaniv and Gros (21) that L-valine and ATP confer stability upon VRS. We were tempted to conclude that the inability of this strain to accumulate VRS at 40 C was the result of an inability to synthesize VRS at this temperature. At least three different molecular bases for temperature-sensitive synthesis can be imagined. First, there might be a temperature-sensitive step in either the initiation of mRNA formation or the initiation of its translation. Second, the elongation of the VRS polypeptide chain might be halted by a temperature-induced conformational change of the folding polypeptide such that further chain elongation is inhibited. Third, newly made VRS might be more susceptible to heat inactivation than older enzyme due to a transitory lack of substrate-induced stable configurations.

We attempted to detect inactive VRS (which the second possibility would predict should accumulate at 40 C) by an immunochemical procedure. Figure 3 illustrates the immunochemical profiles of VRS antigen in crude extracts of the wild strain, NP2, of strain NP2907 grown in identical media at 30 C, and of strain NP2907 which had been al-

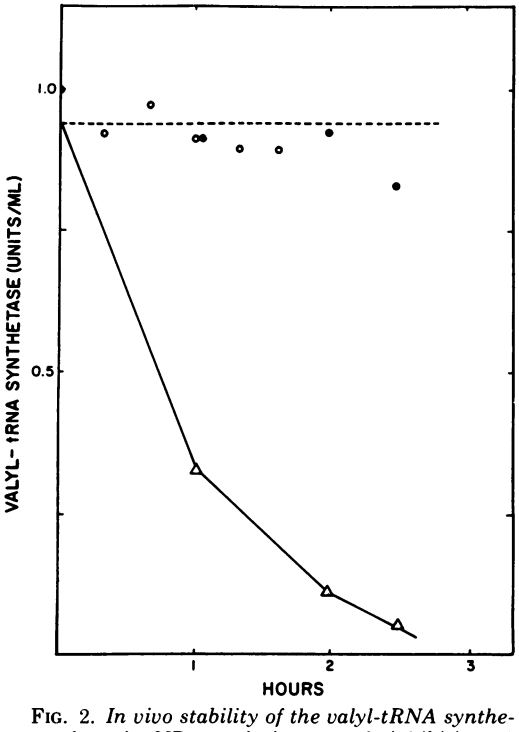

FIG. 2. *In vivo stability of the valyl-tRNA synthetase of strain NP2907 during growth inhibition. A culture of strain NP2907 growing at 30 C in minimal medium containing* L-*valine (150 μg/ml) and* L-*isoleucine (50 μg/ml) was harvested as described in Fig. 1, divided into three parts, and resuspended at 40 C in either: original medium plus 5 mM levallorphan tartrate (△), original medium plus 100 μg of tetracycline per ml (●), or minimal medium plus 150 μg of* L-*valine and 50 μg of 5-methyltryptophane per ml (○). Samples were taken at intervals and assayed for valyl-tRNA synthetase activity in the usual manner except that the crude extracts were exhaustively dialyzed before use.*

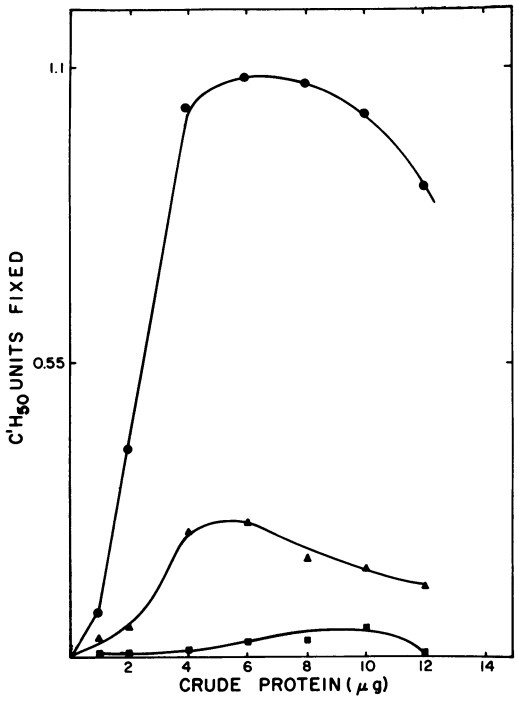

FIG. 3. *Complement fixation of valyl-tRNA synthetase in crude extracts of strains NP2 and NP2907. Each reaction tube contained, in a volume of 0.6 ml: 1.2 units C'H_{50} of guinea pig complement; 2.5 μg of antibody against wild-type valyl-tRNA synthetase; 0.6 mg of bovine serum albumin; and indicated quantities of crude bacterial protein from extracts of strain NP2 (●) and NP2907 (▲) grown in TGYE broth (1) at 30 C and strain NP2907 (■) grown in TGYE at 40 C for sufficient time to reduce assayable valyl-tRNA synthetase activity sixfold from the 30 C level. Each point represents a triplicate determination of residual complement corrected for nonspecific fixation by an antibody-negative control.*

lowed to grow at 40 C until the measurable level of VRS had fallen sixfold. The profiles generated by complement fixation are analogous to curves generated by the precipitin assay. It can be seen that the VRS of both strain NP2 and NP2907 have similar equivalence points, although (as would be predicted for an altered enzyme) the VRS of strain NP2907 differed fivefold in the extent of cross-reactivity to the anti-VRS antibody prepared against the wild-type enzyme. These data indicate that despite a twofold difference in the specific activity of the two extracts there are similar numbers of molecules of VRS in the two preparations. However, in the extract of strain NP2907 where the VRS level was reduced sixfold by growth at 40 C, the equivalence point was shifted somewhere to the right of the figure. The extent of this shift was not possible to estimate because extensive nonspecific complement fixation occurred at crude protein quantities beyond 10 μg. Theoretically, the eventual equivalence point should have the same magnitude as that of the 30 C strain NP2907 extract. Despite the lack of quantitation, the data were consistent with the possibility that inactive VRS did not accumulate at 40 C. Such immunochemical data, however, rely on the unproved assumption that inactivated VRS will retain cross-reactivity, which has been shown in other instances to be invalid (9). It was necessary to apply a more rigorous demonstration that synthesis of new VRS at 40 C did not occur.

Measurement of synthesis of VRS. The technique of detecting unlabeled de novo VRS by introducing a density label into preexisting enzyme was described above. Cultures of strain NP2907 adapted to D_2O minimal medium and cultures grown normally in H_2O minimal medium were harvested, and the extracts were tested for the recovery of VRS from CsCl. Figure 4 is a composite of three experimental CsCl gradients. One tube con-

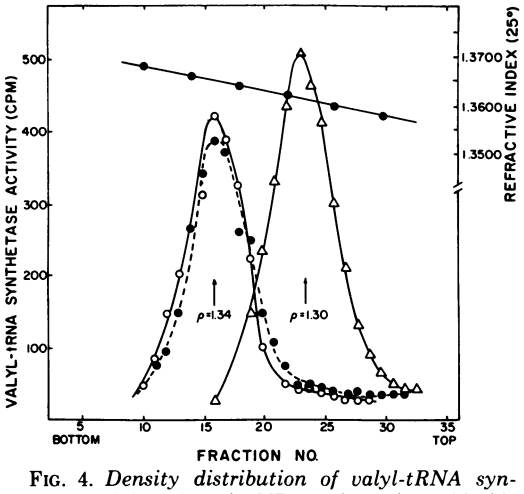

FIG. 4. *Density distribution of valyl-tRNA synthetase activity of strain NP2907 in cesium chloride gradients. Cultures of strain NP2907 were grown in minimal medium made with D_2O or H_2O at 30 C. Strain NP29 was grown in H_2O minimal medium at 30 C. Figure is a composite of three separate gradient tubes, as explained in the text. One contained 400 μg of crude protein from unlabeled strain NP2907 (△), one contained 300 μg of protein from deuterium-labeled NP2907 (○), and one contained both 300 μg of labeled NP2907 protein and 400 μg of unlabeled NP29 protein (●). Six-drop fractions were taken and assayed for refractive index and valyl-tRNA synthetase activity.*

tained only D_2O-labeled VRS and banded symmetrically at a density of 1.34 g/ml; another tube contained unlabeled VRS and banded at a density of 1.30. The third tube tested the effect of unlabeled proteins upon the banding of deuterium-labeled VRS by including an equal quantity of unlabeled crude extract of strain NP29, an isogenic strain of NP2907 which contains no detectable VRS activity in vitro. There was no effect of the latter upon the banding of the labeled VRS.

When the specific activity (units/milligram of protein) of VRS in crude extracts of deuterium-labeled and unlabeled cultures of strain NP2 and NP2907 was measured, it was discovered that the labeled enzyme activity had increased relative to the unlabeled in both wild strain and mutant (Table 1). The arginyl-tRNA synthetase was not affected, eliminating trivial reasons for the discrepancy. Either the deuterium-labeled "heavy" VRS had greater catalytic activity than unlabeled "light" enzyme, or in the presence of D_2O there was an anomalous derepression of VRS synthesis. For the subsequent experiments, this difference could be compensated for, although further work is required to resolve it.

The recovery of an equal number of units of heavy and light VRS centrifuged through CsCl is shown in Fig. 5. The two peaks are significantly separated, and there is no significant difference in the recovery of the two species. The generation of a small peak of intermediate density was repeatable even with similar extracts of the wild-type VRS and is most likely the result of aggregation of VRS in CsCl, for

TABLE 1. *Effect of deuterium substitution upon aminoacyl-tRNA synthetase activity in crude extracts of strains NP2 and NP2907 at 30 C*[a]

Organism	Expt no.	Specific activity[b] (deuterated/unlabeled)	
		Valyl-tRNA synthetase	Arginyl-tRNA synthetase
Strain NP2	1	1.8	
	2	1.7	1.0
	3	1.56	
Strain NP2907	1	1.48	
	2	1.6	1.0
	3	1.6	

[a] Deuterated and unlabeled cultures were grown at 30 C as described in the text in a minimal medium containing L-isoleucine (50 μg/ml) and L-valine (150 μg/ml). The cells were sampled in balanced growth.

[b] Expressed as micromoles of aminoacyl-tRNA produced per milligram of protein per hour.

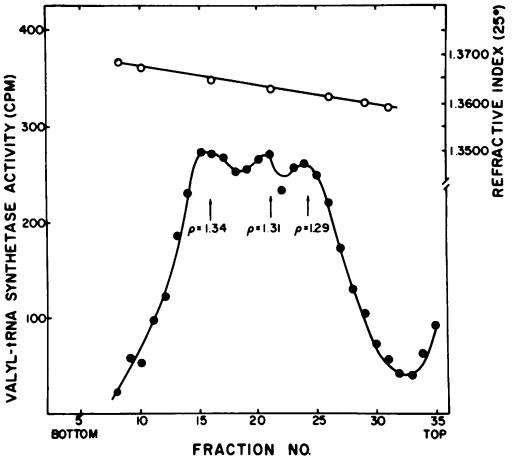

FIG. 5. *Density distribution of valyl-tRNA synthetase activity in a cesium chloride gradient of a mixture of D_2O- and H_2O-grown cultures of strain NP2907 at 30 C. Extracts of cultures of strain NP2907 grown in D_2O and H_2O minimal medium at 30 C were made, and 0.093 unit of valyl-tRNA synthetase activity of each was mixed and subjected to centrifugation in CsCl. Fractions were assayed for valyl-tRNA synthetase activity (●) or refractive index (○). Peak densities were determined from refractive index.*

which there is a precedent with β-galactosidase (7). The peak height was not affected by prolonged incubation of mixed extracts of heavy and light VRS in CsCl (not shown) and is therefore unlikely to be a result of subunit exchange. The magnitude of the peak (less than 10% of total activity) did not interfere with subsequent quantitation of individual peaks.

An experiment was designed to determine unequivocally whether new VRS was being synthesized at 40 C when net synthesis could not be demonstrated. A D_2O-adapted culture of strain NP2907 growing at 30 C was shifted to 40 C in H_2O minimal medium. After one mass doubling the culture was harvested, an extract was made, and a portion was subjected to isopycnic centrifugation in CsCl. Figure 6 depicts the resultant banding of VRS activity. A peak of light VRS corresponding to newly synthesized enzyme is clearly evident and means that a corresponding quantity of preexisting heavy enzyme was inactivated. The determination of the true rate of synthesis of new enzyme at 40 C, relative to the rate at 30 C, was determined according to the analysis presented above. It is evident from Table 2 that the actual differential rate of synthesis at 40 C is indistinguishable from the apparent rate of accumulation of VRS at 30 C.

Our previous report (1) established that the concentration of VRS which strain NP2907 could maintain was a function of temperature, even below 30 C. If the results presented above are consistent with an overall case of regulated synthesis and inactivation of VRS, then the 30 C level of VRS itself should reflect a balance between these two rates. Figure 7 illustrates the result of shifting a D_2O-adapted culture of strain NP2907 from 30 C back to 30 C in H_2O minimal medium. After one mass doubling, the ratio of heavy to light enzyme was 1.0, but the total amount of heavy VRS per milliliter of culture had fallen, and the true rate of synthesis of VRS must be adjusted upwards by a factor of 1.42 (Table 3).

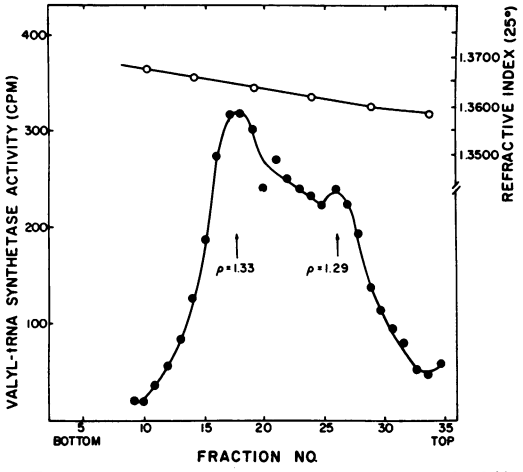

FIG. 6. *Effect of a D_2O-to-H_2O, 30-to-40 C shift upon the density distribution of valyl-tRNA synthetase activity of strain NP2907 in a CsCl gradient. A culture of strain NP2907 growing in D_2O minimal medium at 30 C was harvested, washed, and resuspended in H_2O minimal medium at 40 C as explained in the text. After one mass doubling, the culture was harvested, and an extract was made and subjected to centrifugation in CsCl (1.34 mg of protein were added). Valyl-tRNA synthetase activity* (●) *was measured, as well as refractive index* (○).

The differential rates of VRS synthesis in Tables 2 and 3 are comparable and indicate that new VRS was made during growth at 40 C at a rate only 24% less than at 30 C. As stated earlier, a reduction of synthesis can be understood if there exists a thermolabile state during maturation of the enzyme. What must be emphasized is that the rate of synthesis of VRS and the rate of its inactivation are coordinated.

From these studies it was possible to establish three characteristics of the VRS of strain NP2907. First, the measurable concentration of this enzyme is a balance between the rates of synthesis of the enzyme and its destruction. Secondly, the ratio of destruction to synthesis is a constant for a particular temperature and varies from a probable value of zero at temperatures under 20 C (the VRS level does not vary when growth takes place below 23 C) to a value of 1.0 at 40 C. Thirdly, since at a given temperature a variety of nutritionally set growth rates, and thus a variety of time rates of VRS synthesis, is possible (the differential rate of synthesis of VRS in units/milligram of

TABLE 2. *Differential rate of synthesis of valyl-tRNA synthetase (VRS) in strain NP2907 at 40 C*

Expt no.	Time[a] (generations)	Protein[b] (mg/ml)	Total VRS[b] (units/mg of protein)	Total VRS[c] (units/ml of culture)	Heavy VRS[d] (fraction of total)		Heavy VRS[e] (units/ml of culture)	Rate of synthesis[f] (units/mg of protein)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1	0.00	1.00	1.00	1.00	1.00	1.00	1.00	
	1.00	2.00	0.40	0.80	0.55	0.45	0.36	1.02
2	0.00	1.00	1.00	1.00	1.00	1.00	1.00	
	1.00	2.00	0.40	0.80	0.50	0.40	0.32	1.13

[a] Zero time is the time at which the cells were transferred to H_2O medium at 40 C. A second sample was taken when the culture had doubled (135 min).

[b] For ease of calculation and to facilitate comparison between experiments, the actual concentration of protein in the cultures (approximately 0.8 mg/ml in both experiments) and the specific activity of VRS (0.19 unit per mg of protein in both experiments) were normalized to a value of 1.00 at 0 time.

[c] Calculated as the product of columns 3 and 4.

[d] The fraction of VRS banding in the heavy peak was measured by means of a planimeter, and this value is presented in column 6. This value was then corrected by inflating the light peak by a factor of 1.5 to compensate for the difference in activity of deuterated and nondeuterated extracts (see Table 1). The corrected value for heavy VRS appears in column 7.

[e] Calculated as the product of columns 5 and 7.

[f] Calculated according to equation 6 in the text.

protein should not change at different growth rates), then the time rates of inactivation must likewise vary, coordinately. It will be recalled that at 40 C the amount of enzyme per milliliter of culture remained constant whether growth and protein synthesis were totally inhibited, whether growth occurred slowly in minimal medium, or when it occurred rapidly in rich medium (1). In the first case, the rate of VRS synthesis was zero, and the rate of inactivation must also have been zero. In the latter case, VRS synthesis occurred at significant rates but was exactly balanced by inactivation. That VRS synthesis should be proportional to protein synthesis is self-evident and dull, but to find that VRS inactivation should be proportional to protein synthesis is unexpected and deserving of explanation. We next investigated the possible connection that inactivation might have with the rate of growth, and specifically with protein synthesis.

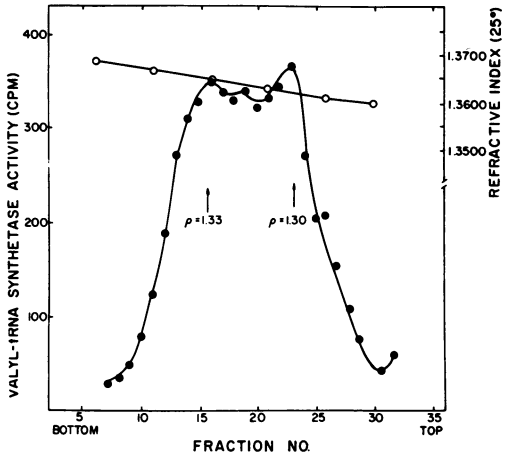

FIG. 7. *Effect of a D_2O-to-H_2O shift at 30 C upon the density distribution of valyl-tRNA synthetase activity of strain NP2907 in a CsCl gradient. Procedure was identical with that of Fig. 6, except that no shift was made from 30 C. After one mass doubling in H_2O minimal medium, the culture was harvested, extract was made, and 1.3 mg of protein was subjected to centrifugation in CsCl. Valyl-tRNA synthetase activity (●) and refractive index (O) were measured.*

Influence of substrates upon valyl-tRNA synthetase inactivation. The rate of inactivation of VRS in strain NP2907 clearly depends on some parameter set by the rate of growth. One approach to the solution of this problem was to attempt to mimic in vitro the paradox that VRS is stable under nongrowth conditions but unstable during maximal growth.

The series of experiments generating the data of Fig. 8 were performed. In one tube we placed a solution containing L-valine and ATP, but no tRNA, with the usual assay conditions which favor the maintenance of the VRS-valyl-adenylate complex. At intervals during incubation at 40 C, samples were removed and assayed for enzyme activity at 30 C. Figure 8 shows the result: the mutant VRS remained completely stable at 40 C. Yaniv and Gros (21) have reported similar effects of L-valine and ATP upon their mutant VRS in vitro. However, when tRNA was also included in the reaction mixture such that aminoacylation could occur at 40 C, the enzyme was drastically inac-

TABLE 3. *Differential rate of synthesis of valyl-tRNA synthetase (VRS) in strain NP2907 at 30 C*

Time[a] (generations)	Protein[b] (mg/ml)	Total VRS[b] (units/mg of protein)	Total VRS[c] (units/ml of culture)	Heavy VRS[d] (fraction of total)		Heavy VRS[e] (units/ml of culture)	Rate of synthesis[f] (units/mg of protein)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
0.00	1.00	1.00	1.00	1.00	1.00	1.00	
1.00	2.00	0.89	1.78	0.50	0.37	0.66	1.42

[a] One mass doubling took 240 min.

[b] These values have been normalized as described in footnote *b*, Table 2; the actual values were: 1.10 mg of protein/ml of culture and 0.19 unit of VRS per mg of protein.

[c] Calculated as the product of columns 2 and 3.

[d] The fraction of VRS banding in the heavy peak was measured by means of a planimeter, and this value is presented in column 5. This value was then corrected by inflating the light peak by a factor of 1.5 to compensate for the difference in activity of deuterated and nondeuterated extracts (see Table 1). The corrected value for heavy VRS appears in column 6.

[e] Calculated as the product of columns 4 and 6.

[f] The differential rate of VRS synthesis (units of VRS made/mg of protein made) was calculated by adding the negative rate of heavy VRS decay ($\ln 0.66 = -0.42$) to the observed rate of accumulation of VRS at 30 C (1.00), yielding 1.42 units/mg of protein. This true rate of synthesis is expressed relative to the observed rate of accumulation and can be compared directly with the rates of synthesis at 40 C shown in Table 2.

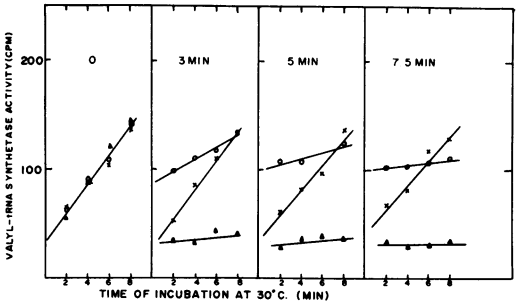

FIG. 8. *Effect of substrates upon valyl-tRNA synthetase activity of strain NP2907 in vitro at 40 C. Experimental design is described in the text. Intervals listed within the figure are times after incubation of a master mixture of nucleic acid-free extract of strain NP2907 in the usual aminoacylation assay with the following modifications: tRNA omitted* (×), *ATP omitted* (Δ), *complete* (○). *Samples were removed at the stated times and residual activity was assayed by means of the time course presented at 30 C (abscissa). Not shown: master mixture minus both ATP and tRNA. There was no detectable activity after 3 min at 40 C.*

tivated. This was not due to exhaustion of either ATP or L-valine, since both were in excess in the master mix. It was not due to the buildup of either tRNA (acylated) or some other inhibitor, since inclusion of fresh NP2907 VRS in the 30 C assay mix with the 40 C samples showed only additive effects (not shown). When ATP was omitted from the master mix at 40 C there was no protection, despite the presence of tRNA which Yaniv and Gros (21) found to stabilize VRS. These results were repeated at intermediate temperatures, at which the rate of inactivation of the enzyme in the presence of the complete reaction mixture was steadily reduced as the temperature was lowered (Fig. 9). In fact, VRS activity actually increased somewhat when incubated at 37 C in the absence of tRNA.

There was no influence of substrates upon the stability of the VRS of strain NP2 at the temperatures used above. The enzyme was completely stable and uniform in activity.

These findings suggested that the rate of inactivation of VRS in vivo in strain NP2907 may depend upon the particular state of the enzyme vis a vis its substrates and products, which in turn may be controlled by the overall growth rate of the cells.

DISCUSSION

The data we have presented from in vivo and in vitro experiments have suggested that the thermolability of the VRS of strain NP2907 is due to the particular state of the VRS as it is modified by the intracellular environment, which is itself altered by the growth rate of the cells. We believe that enough information about the in vitro properties of aminoacyl-tRNA synthetases is now available to propose a mechanism which can account for this unique phenomenon. We shall address ourselves to the influence of substrates and end products upon the functional state of the VRS.

It is clear that many aminoacyl-tRNA synthetases behave like allosteric enzymes, in that the binding of one substrate influences kinetic properties of the enzyme regarding other substrates and products and that the conformation of the enzyme can be profoundly altered by such interactions (15). Mehler and Mitra (13) have shown that $tRNA^{arg}$ must bind to the arginyl-tRNA synthetase before that enzyme can bind arginine and that this binding confers stability to the enzyme. Loftfield and Eigner (12) have reported changes in substrate specificity of the isoleucyl-tRNA synthetase by prior binding of $tRNA^{ile}$, and Yarus and Berg (22) have shown that isoleucine itself modifies the rates of release and binding of acylated and deacylated $tRNA^{ile}$. Yaniv and Gros (20) and George and Meister (5) have demonstrated protection of the SH groups of the *E. coli* VRS from oxidation by the binding of valyl-adenylate (val-AMP), and the former workers have also demonstrated increased thermostability of the VRS in this state. Doolittle and Yanofsky (4) have likewise shown that mutant tryptophanyl-tRNA synthetases are stabilized by tryptophanyl-AMP. Helene and Yaniv, as

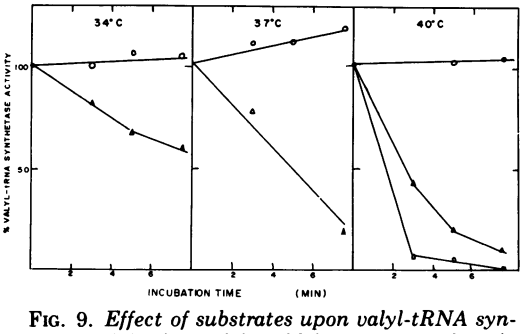

FIG. 9. *Effect of substrates upon valyl-tRNA synthetase activity in nucleic acid-free extracts of strain NP2907 incubated at various temperatures. Experimental design was identical to that of Fig. 8. Results are plotted here as per cent of initial valyl-tRNA synthetase activity, as measured by initial velocity from a time course of 8 min at 30 C. Abscissa is the time of incubation of a master mixture at the indicated temperature. The mixtures were complete* (Δ), *minus tRNA* (○), *or minus ATP* (□).

quoted in Yaniv and Gros (20), have found that $tRNA^{val}$ binding to VRS caused a change in intensity of tryptophan fluorescence, indicative of a conformational change of the protein.

We feel that the findings of Yarus and Berg (22) are particularly important for an understanding of the properties of strain NP2907. These workers pointed out that the rate-limiting step of aminoacylation of tRNA for the isoleucine synthetase (IRS) is most likely, from kinetic evidence, the release of isoleucyl-tRNA from the ternary complex, IRS-ile-AMP-ile-$tRNA^{ile}$, when ATP, isoleucine, and tRNA are saturating. The similarity in kinetic constants of the VRS and IRS (20) suggests that this is most likely true for the VRS as well. Therefore, during most in vivo conditions, the VRS exists with bound valyl-AMP.

This information has led us to propose a model with the following features. (i) The predominant functional state of the VRS in vivo and in vitro (usual assay conditions) is the ternary complex, VRS-val-AMP-valyl-$tRNA^{val}$. In vivo, under nongrowth conditions in which protein synthesis is halted but L-valine is present in excess, this state is probably the only one possible. As determined experimentally, the VRS must be completely thermostable. Drastically decreasing either the L-valine or ATP concentrations permits inactivation. (ii) The flux of valyl-tRNA in protein synthesis continuously regenerates $tRNA^{val}$. Since there is little difference in affinity in the binding of acylated and deacylated $tRNA^{val}$ to the VRS (20), the fate of the ternary complex is dependent upon the relative concentrations of the two species, that is, the ratio of deacylated to acylated $tRNA^{val}$. When the ratio of $tRNA^{val}$ to valyl-$tRNA^{val}$ approaches zero, exchange occurs as follows: VRS-val-AMP-valyl-tRNA $\rightleftarrows$ VRS-val-AMP + valyl-tRNA. Since the complex, VRS-val-AMP, remains intact, the resulting conformational change in the enzyme would be preserved. However, when the inverse ratio, valyl-$tRNA^{val}$ to $tRNA^{val}$, approaches zero, the ternary complex is entrained in the cycle of aminoacylation shown in Fig. 10, and the enzyme sustains continuous changes in conformation as the complex breaks down and reforms. This situation is approximated by the in vitro assay conditions, at least initially, wherein the VRS is extremely susceptible to inactivation at elevated temperatures, as experimentally noted. (iii) At intermediate values of $tRNA^{val}$/valyl-$tRNA^{val}$, the probability of inactivation of the VRS at a particular temperature is found between the two extremes:

$$v_d = f(tRNA^{val}/\text{valyl-}tRNA^{val}) \quad (7)$$

(iv) The overall rate of protein synthesis at a particular temperature determines the $tRNA^{val}$ to valyl-$tRNA^{val}$ ratio [since the ratio of total tRNA to total protein is nearly constant in *E. coli* (10)], where k = specific growth rate constant,

$$tRNA^{val}/\text{valyl-}tRNA^{val} = f(k) \quad (8)$$

Therefore, combining (7) and (8)

$$v_d = f(k) \quad (9)$$

The rate of inactivation of VRS is then coordinated with the rate of growth, as experimentally indicated.

The central feature to this model is that the mutant VRS undergoes inactivation as a consequence of its functioning. Specifically, the transition from state II to state III, as shown in Fig. 10, is viewed as the likely step in which a conformational change occurs which can lead to inactivation of VRS. Another instance of reaction inactivation has recently come to light. Singh and Srere (17) have found that the citrate lyase of *Aerobacter aerogenes* is inactivated in vitro as it functions. No information

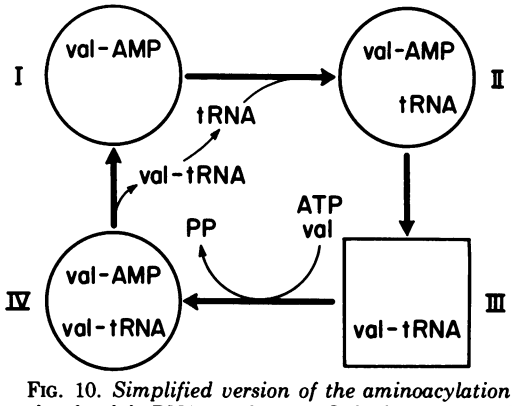

FIG. 10. *Simplified version of the aminoacylation cycle of valyl-tRNA synthetase. Only four states of the enzyme are shown. In state I, the enzyme (large circle) has valyl-adenylate (val-AMP) bound to it. In state II, a valine-specific tRNA has bound to the enzyme. This complex degrades into state III by the formation of valyl-tRNA, still bound to the enzyme, and the release of AMP. New molecules of ATP and valine next bind to the enzyme, forming state IV which has both valyl-adenylate and valyl-tRNA bound to it. Finally, dissociation of val-tRNA yields state I again. This latter step in the model of Yarus and Berg (22) for isoleucyl-tRNA synthetase is the rate-limiting one. We postulate that it is the change from II to III that leads to inactivation of the mutant VRS.*

is known about the behavior of this enzyme in vivo.

The cellular environment may also have a profound effect upon the stability of wild-type aminoacyl-tRNA synthetases in certain circumstances. Williams and Neidhardt (19) have reported that cycles of "feast and famine" during discontinuous feeding of *E. coli* in chemostat cultures limited by amino acid supply cause extensive inactivation of the respective aminoacyl-tRNA synthetase. Cells completely starved for amino acid lose synthetase activity at a far lower rate, and cells limited in growth by slow but continuous generation of intracellular amino acid do not lose synthetase at all. The first set of conditions generates a different pattern of substrate-enzyme complexes than those reported here, and these may contribute to extremely unfavorable oscillations of the aminoacyl-tRNA synthetases between unstable states.

The possible role that various functional states of aminoacyl-tRNA synthetases may play in intracellular regulatory mechanisms needs further exploration.

ACKNOWLEDGMENTS

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