General Base Catalysis in the Reactions of Transfer Ribonucleic Acid Ligases*

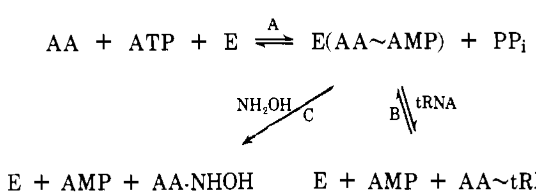
Robert B. Loftfield and Elizabeth Ann Eigner

ABSTRACT: Enzyme-bound aminoacyl adenylates react with hydroxylamine to form amino acid hydroxamates. Depending on the enzyme and substrate, the rate of the reaction is second order with respect to hydroxylamine over a 100-fold change of rate of hydroxamate formation. We infer that two molecules of hydroxylamine are involved in the rate-determining reaction. Of these two, one appears in the product and is clearly required for the synthesis of hydroxamate. The other molecule of hydroxylamine serves as a general base and may be substituted for by other general bases such as imidazole, pyridine, or phenanthroline. The relevance of the general base catalysis of amino acid hydroxamate formation to the physiological aminoacylation of transfer ribonucleic acid (tRNA) is suggested since several general bases such as Tris, imidazole, and ammonia stimulate the formation of aminoacyl-tRNAs as much as fivefold. Polycoordinate bases such as pyrophosphate and phenanthroline are markedly inhibitory. Bases which stimulate aminoacyl-tRNA synthesis or hydroxamate formation are uniformly inhibitory to adenosine triphosphate–inorganic pyrophosphate exchange.

Preparatory to incorporation into protein, amino acids react with adenosine triphosphate[1] in the presence of an appropriate "activating enzyme" or aminoacyl-tRNA ligase. As with the activation of several other carboxylic acids, the end products include inorganic pyrophosphate, adenylic acid, and a more "active" form of the amino acid (Stulberg and Novelli, 1962).

Equation 1 summarizes the generally accepted scheme for the sequence of reactions catalyzed by these enzymes.

Reaction 1A leads to the formation of enzyme-bound



(1)

aminoacyl adenylate and inorganic pyrophosphate which quickly dissociates from the enzyme. Since reaction 1A is readily and rapidly reversible in the presence of PP_i, the enzyme can be detected and studied by its ability to catalyze the incorporation of $[^{32}P]PP_i$ into ATP in the presence of the appropriate amino acid. There is considerable evidence that chemically or enzymatically synthesized aminoacyl adenylates will bind to these enzymes and, in the presence of PP_i, reaction 1A is reversed to yield ATP and AA (DeMoss *et al.*, 1956; Berg, 1957; Karasek *et al.*, 1958). The ability of the enzyme to select between closely related amino acids is great but not complete (Bergmann *et al.*, 1961).

Reaction 1B is presumed to be the universal second step in protein synthesis and constitutes the physiologically significant function of these enzymes. Unlike reactions 1A and 1C, it appears to be totally selective between closely related amino acids. Some of this specificity may result from allosteric effects (Loftfield and Eigner, 1965), some may be a consequence of differing stabilities of the enzyme–aminoacyl adenylate complexes (Norris and Berg, 1964; Baldwin and Berg, 1966), and some of the great specificity remains beyond accounting at this time (Loftfield and Eigner, 1966a).

The formation of amino acid hydroxamates (reaction 1C) provided the first evidence for the existence of these enzymes (Hoagland, 1955). Although the rates of formation of hydroxamates are generally low compared to the rates of incorporation of $[^{32}]PP_i$ into ATP, there are many advantages to the hydroxamate assay (Loftfield and Eigner, 1959, 1963a). This is especially true if a $[^{14}C]$amino acid is used, and when the enzyme preparation is relatively crude. As with the PP_i–ATP exchange and unlike the tRNA reaction, closely related amino acids react with the same enzyme (Loftfield and Eigner, 1966a).

It was the purpose of this study to correlate these three

* From the Department of Biochemistry of the School of Medicine, The University of New Mexico, Albuquerque, New Mexico (mailing address), and the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts. *Received May 4, 1967.* This work has been supported by Grants CA 02387 and CA 08000 of the U. S. Public Health Service and constitutes Publication No. 1317 of the Harvard Commission on Cancer Research. Some of this material has been presented at the Federation Meetings (*Federation Proc. 24*, 216, 1965).

[1] Abbreviations used: ATP, adenosine triphosphate; AMP, adenylic acid; AA, free amino acid; AA~tRNA, amino acid bound to tRNA; AA~AMP, aminoacyl adenylate; AA-NHOH, amino acid hydroxamate; Hy, hydroxylamine; Im, imidazole; v_{cor}, corrected rate of reaction; tRNA free enzyme, activating enzyme containing no discernible tRNA; tRNA-bound enzyme, activating enzyme associated with homologous tRNA; amino acid catalyzed ATP–PP_i exchange, reaction 1A usually measured by determining the extent of incorporation of $[^{32}P]PP_i$ into ATP.

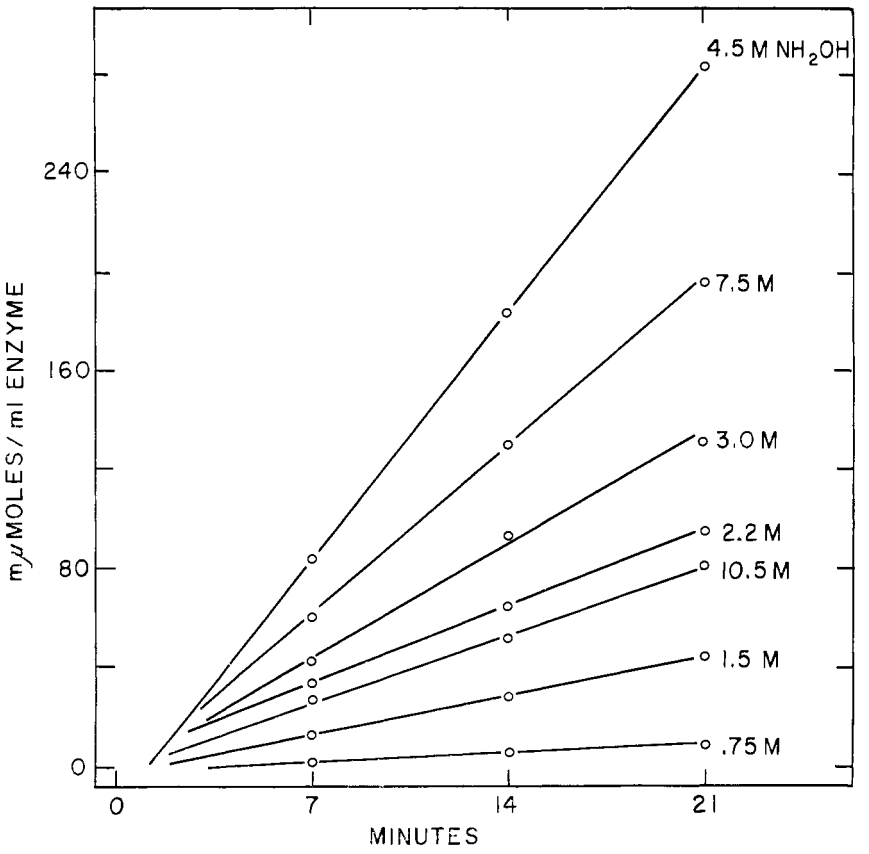


FIGURE 1: A typical experiment that shows the rate of isoleucine hydroxamate formation by isoleucine enzyme is constant for 21 min for all concentrations of NH_2OH from 0.75 to 10.5 M. Each reaction tube contained in 100 μl 10 mM ATP (Mg^{2+} salt), 7 mM [^{14}C]isoleucine, indicated concentrations of NH_2OH neutralized with HCl to pH 7.0, 7 μl of enzyme solution, 10 μg of albumin, and water. The reagents, except NH_2 OH, were combined at 25° and reaction was initiated by the addition of NH_2-OH. ^{14}C-labeled hydroxamate was separated from unreacted isoleucine and assayed as described in the text.

reactions so as to improve the enzyme assays, and to learn something of the mechanism of the reaction. It is our unanticipated observation that amino acid hydroxamates and aminoacyl-tRNAs are formed in a general base catalyzed reaction which may be somewhat different in mechanism from the ATP–PP_i-exchange reaction.

Materials

L-[1-^{14}C]Amino acids were prepared by the Bucherer hydantoin method from $Ba^{14}CO_3$ (Loftfield and Eigner, 1966b; Loftfield, 1950). One sample of L-[^{14}C]phenylalanine was purchased from the New England Nuclear Corp. All [^{14}C]- and [^{12}C]amino acids were regularly assayed by column chromatography for impurities. [^{14}C]Phenylalanine was observed to have a high rate of deterioration, apparently radiation induced oxidation to tyrosine-like materials. All amino acids were prepared and used at specific activities between 20 and 30 mCi per mmole.

Salt-free hydroxylamine was prepared by neutralization of methanolic hydroxylamine hydrochloride with methanolic sodium hydroxide followed by two distillations (Loftfield and Eigner, 1966a). Imidazole, phenanthroline, ATP, collidine, lutidine, pyridine, amino acids, Tris, and inorganic compounds were commercial products of highest available purity.

E. coli activating enzymes specific for isoleucine, valine, leucine, and phenylalanine were prepared by minor modifications of the methods of Bergmann *et al.* (1961) and of Conway *et al.* (1962). The enzymes as finally eluted from a DEAE column were protected with 60 mg/100 ml of reduced glutathione and assayed by [^{32}P]PP_i–ATP exchange, hydroxamate formation, and aminoacyl-tRNA formation. In each case the specific activity was comparable to but somewhat less than the maximum activity reported by the above workers. tRNA was prepared by phenol extraction of *E. coli* strain B cells (Monier *et al.*, 1960) or was received as a gift from Schwarz BioResearch.

Methods

The hydroxamate assay was in conducted general using 10 mM ATP(Mg^{2+}), 2.5 M NH_2OH (adjusted to pH 7.0 with HCl), and appropriate amounts of [^{14}C]-amino acid and activating enzyme. Three or four aliquots were taken at intervals up to 40 min, and the formed [^{14}C]hydroxamate was separated from unreacted [^{14}C]amino acid on cation-exchange paper (Loftfield and Eigner, 1963a, 1966a).

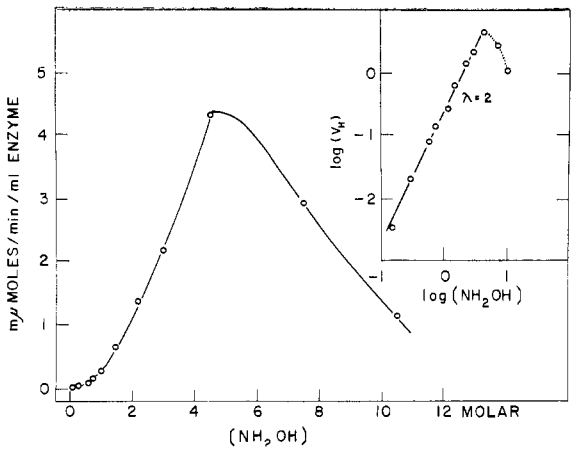


FIGURE 2: The data of Figure 1 and of another similar experiment are plotted as rate *vs.* NH_2OH concentration. The inset shows the same information plotted as the logarithm of the rate *vs.* the logarithm of the NH_2OH concentration. The drawn solid line in the inset has a slope of 2 indicating a second-order dependence of rate on NH_2OH concentration at concentrations of NH_2OH less than 4 M.

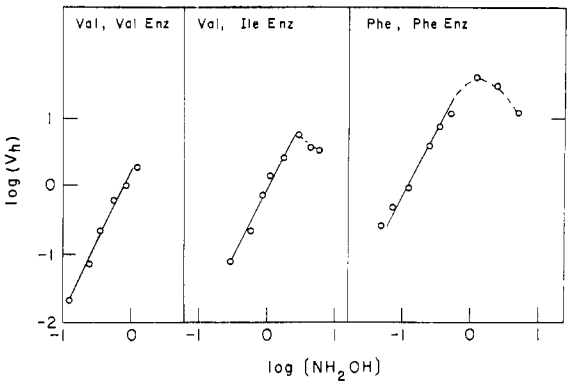


FIGURE 3: Plots of the logarithms of rate of hydroxymate formation *vs.* NH_2OH similar to the inset of Figure 2. From left to right the curves show hydroxmate formation from valine with valine enzyme, from valine with isoleucine enzyme, and from phenylalanine with phenylalanine enzyme. In each case the drawn solid line has a slope of two indicating a second-order dependence of rate on hydroxylamine concentration. The reaction conditions and assays are essentially those described in Figure 1.

Formation of aminoacyl-tRNA was measured by incubation of [^{14}C]amino acid, ATP(Mg^{2+}), Veronal buffer, purified ligase, and unpurified *E. coli* tRNA for periods up to 10 min. Aliquots were removed at different times, precipitated with trichloroacetic acid, collected on Millipore filters, washed, and counted in a low-background Nuclear-Chicago end-window Geiger counter (Loftfield and Eigner, 1967, 1966a). PP_i–ATP exchange was measured as previously described (Loftfield and Eigner, 1963b, 1966a; Berg, 1958).

It is a matter of definition that kinetics involves the determination of rates rather than the extent of conversion of substrate into product at a particular time point. Although enzymologists have long emphasized the need to determine initial reaction rates, there has been a deplorable tendency to make purported kinetic determinations from a single observation. Problems of substrate or product inhibition, approach to equilibrium, enzyme deterioration, nonenzymatic side reactions, etc., are often undetected as a result. It becomes especially important to establish true initial rates of reaction when inhibitors are being used as in this study. Unless initial reaction rates remain reasonably constant, there is a substantial possibility that the inhibitor functions not as a competitor for the substrate or by reversible modification of the enzyme but by progressive inactivation of the enzyme. Accordingly, every observation reported in this study is drawn from determinations of the extent of reaction at not fewer than three time intervals. Figure 1 is typical of all the reactions we have studied. Even when the reaction rate is substantially depressed as with 10 M NH_2OH in Figure 1, the rate of reaction remains constant during the period of observations.

Results and Discussion

The rate of hydroxamate formation has been reported to depend on the concentration of hydroxylamine (Loftfield and Eigner, 1963a). In every case studied, there has been an increase in rate with concentration up to a maximum in the range of 2–5 M NH_2OH with lower rates at higher concentrations. When this variation of rate with concentration is examined more closely, as in Figures 1 and 2, it is observed that the rate of hydroxamate formation is almost never a linear function of the hydroxylamine concentration. The concave shape of the curve suggests a second-order dependence, which is confirmed by the insert in Figure 2. When the logarithm of rate is plotted against the logarithm of the hydroxylamine concentration, we obtain a straight line with a slope of 2 as would be expected for a reaction second order in hydroxylamine. Above 4 M NH_2OH, the reaction is progressively inhibited although the enzyme remains active to 10 M as can be noted in Figure 1.

Substantially similar results are obtained with valine on the valine enzyme, valine on the isoleucine enzyme, and phenylalanine on the phenylalanine enzyme. Figure 3 shows that in each case the log–log plot has a slope of 2, the only difference being in the concentration of hydroxylamine at which inhibition begins. Thus, each of these four systems is second order in hydroxylamine, from which one infers that two molecules of hydroxylamine participate in the rate-limiting reaction.

Such behavior is reminiscent of the observations of Kirsch and Jencks (1964), Jencks and Carriluo (1960), and Caplow and Jencks (1962) on the hydrolysis of various esters, as well as Bruice and Bruno (1961) and Bruice and Benkovic (1964). In each case these workers found a term second order in imidazole or first order in a general base which, they concluded, resulted from a reaction in which one molecule of nucleophile attacked the ester, while a second molecule served as a general base. If, in the present case, one molecule of hydroxylamine is a nucleophile that attacks enzyme-bound aminoacyl adenylate, and the second molecule is acting only as a general base, we should expect that imidazole or other bases would catalyze the formation of hydroxamates. More specifically, we would expect that at

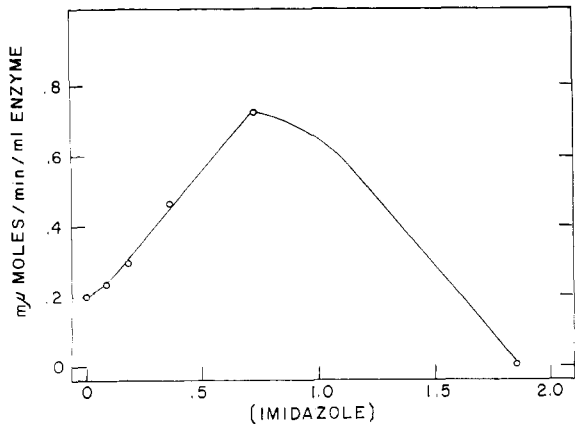


FIGURE 4: The formation of isoleucine hydroxamate in the presence of increasing amounts of imidazole. The reaction conditions and assay were as previously described (Figure 1) except that each tube contained 0.65 M NH_2OH and the indicated amount of imidazole, each adjusted with HCl to pH 7.0.

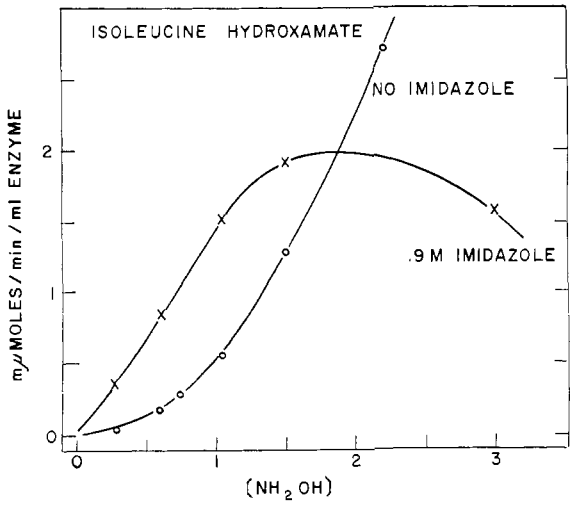


FIGURE 5: The effect of a constant concentration of imidazole on the rate of isoleucine hydroxamate formation. The reaction conditions and assay were as described in Figure 1 except that 0.9 M imidazole was added to one series of reaction tubes.

low fixed concentrations of hydroxylamine, increases in the rate of hydroxamate formation would be proportional to increases in imidazole concentration. Conversely, at a fixed and relatively high concentration of imidazole, the rate of hydroxamate formation would be *almost* proportional to hydroxylamine concentration. Figures 4 and 5 show this to be the case with the formation of isoleucine hydroxamate on the isoleucine enzyme. Imidazole appears to be about four times better a general base than hydroxylamine. If this is the case, one would predict that the rate of reaction would vary according to ([hydroxylamine])([hydroxylamine] + 4 [imidazole]), and that a log–log plot of rate *vs.* the above product should have a slope of 1. Figure 6 shows this to be the case for phenylalanine hydroxamate formation over a tenfold concentration range. A similar plot disregarding the participation of imidazole is not linear. Collidine and phenanthroline show similar effects when examined, except that neither is as effective as imidazole. Although imidazole stimulates isoleucine hydroxamate and phenylalanine hydroxamate formation two- to fourfold, the valine enzyme shows only a 25% stimulation (2.4 M imidazole) before inhibition results from higher concentrations. The fact that each of the combinations deviates from the second-order relationship at a different concentration of hydroxylamine emphasizes the multiplicity of competing effects. For instance, inhibition of hydroxamate formation at high concentrations of hydroxylamine may be due to conformational changes in the enzyme or general solvent effects. The initial catalysis by imidazole followed by inhibition at higher concentrations may be due to the formation of highly reactive acylimidazolium compounds which may react with hydroxylamine to form hydroxamates or with water to regenerate free amino acid. Alternatively, it is possible that imidazole may be inhibiting by forming a stable complex as postulated by Connors and Mollica (1965).

Even the initial product of reaction with hydroxylamine is in some doubt since measurement of hydroxamate formation over very short time intervals sometimes shows a time lag. A similar lag was observed by Jencks (1958), who found that some acyl adenylates are first attacked by the oxygen atom of hydroxylamine to yield *O*-acyl hydroxamates. These may or may not react or rearrange to form stable *N*-acyl hydroxamates. Suffice it to say that for each system there is a range of hydroxylamine concentrations in which hydroxamate production is proportional to the square of the hydroxylamine concentration, that in every case a variety of bases accelerate the reaction and in at least one case this stimulation is exactly what would be predicted if the function of the one base (imidazole) is that of a general base and the hydroxylamine is serving both as a nucleophile and as a general base.

The most pertinent question is whether the formation of aminoacyl-tRNA follows a similar course. We have previously noted that the kinetics of tRNA labeling, of

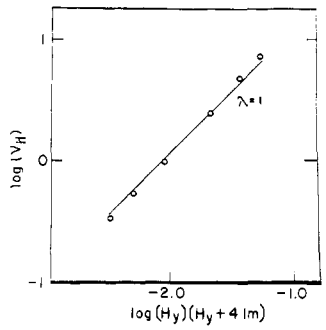


FIGURE 6: The dependence of phenylalanine hydroxamate formation on hydroxylamine concentration in the presence of 0.15 M imidazole (adjusted to pH 7.0). The units of the abscissa scale are log ([Hy])([Hy] + 4 [Im]). The rational for this transformation is that 1 mole of imidazole is approximately equivalent to 4 moles of hydroxylamine as a general base. The drawn straight line has a slope of 1 and its close fit to the data indicates the rate-limiting reaction involves one hydroxylamine molecule and either one hydroxylamine molecule or one imidazole molecule which is four times as effective as the hydroxylamine molecule.

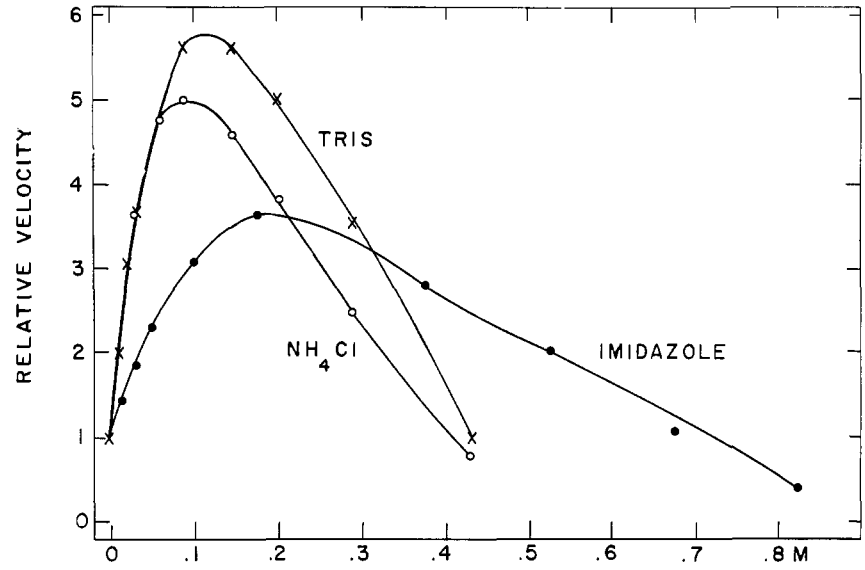


FIGURE 7: General base catalysis of valyl-tRNA synthesis. [^{14}C]Valine (10^{-4} M), 0.01 M ATP (Mg^{2+} salt), 0.001 M excess $MgCl_2$ 150 μg of *E. coli* tRNA, and 5 μl of a solution of purified valine-activating enzyme were combined in 175 μl of 0.01 M barbital buffer (pH 7.6) containing the indicated total quantities of base plus conjugate acid (also adjusted to pH 7.6). Three 50-μl aliquots were removed at intervals up to 10 min and the trichloroacetic acid isoluble [^{14}C]valyl-tRNA was determined in order to establish rates of aminoacylation.

hydroxamate formation, and ATP–PP_i exchange are so different as to make it unwise to assume that the tRNA-free enzyme is similar to the tRNA-bound enzyme (Loftfield and Eigner 1966a, 1965). Norris and Berg (1964) have noted that the isoleucine enzyme forms valyl adenylate while the isoleucine enzyme complexed with isoleucine tRNA hydrolyzes valyl adenylate. Recently Mitra and Mehler (1967) have reported that an arginine-activating enzyme possesses no PP_i–ATP-exchange activity or hydroxamate-forming activity in the absence of tRNA. Hence, it is wise to be circumspect in drawing analogies between reactions where tRNA is or is not present.

Nonetheless it is notable that the reaction of tRNA with valine in the presence of the valine enzyme and ATP is strongly catalyzed by a variety of monofunctional bases. (Inhibition of the reaction by polycoordinate bases will be the subject of a subsequent paper.)

Figure 7 shows the effect of adding increasing amounts of NH_4Cl, imidazole, or Tris to a reaction mixture buffered with veronal at pH 7.5. The rate of reaction is substantially increased by each of these bases and, in each case, the extent of increase is approximately proportional to the amount of added base at low salt concentrations. We (Loftfield and Eigner, 1967) have recently shown that the rate of aminoacylation of tRNA is sensitive to ionic strength according to eq 2.

$$\log v/v_{cor} = (-0.5)(18)(\sqrt{\mu}/(1 + \sqrt{\mu})) \qquad (2)$$

The observed rate (v) can be corrected by eq 2 to give the corrected rate (v_{cor}). Similarly, the amount of free NH_3, Tris base, or free imidazole can be estimated from the dissociation constants (pK = 9.7, 7.9, and 6.9, respectively) and the pH which is 7.5. In Figure 8, the rate corrected for ionic strength is plotted against estimated concentration of free base. In each case, much of the curve can be described by eq 3, where v_u is the

$$v_{cor} = v_u + [B]k \qquad (3)$$

uncatalyzed rate of reaction, [B] is the molarity of free base, and k is a constant expressing the relative effectiveness of the base catalyst. The values of k are 31,600

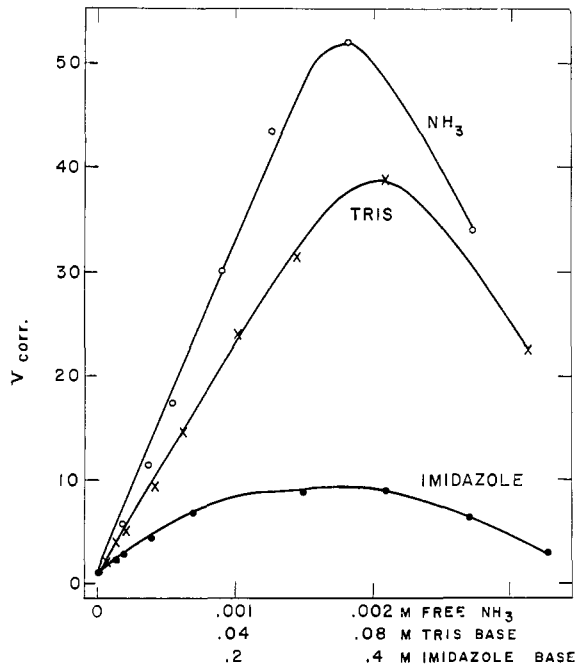


FIGURE 8: Rate of valyl-tRNA formation corrected for ionic strength effects. The rate data of Figure 7 have been corrected according to the equation $\log [v_{obs}/v_{corr}] = -9(\sqrt{\mu}/(1 + \sqrt{\mu}))$. The different scales on the abscissa indicate the estimated amounts for free base present in the reaction.

540, and 40 for NH_3, Tris, and imidazole, respectively, and are approximately proportional to the corresponding basic dissociation constants.

The above data would also be consistent with catalysis of the reaction by the conjugate acids of these bases That this is not likely the case is demonstrated by the fact that 0.1 M NH_4Cl increases the rate of formation of valyl-tRNA fourfold at pH 7.2 and tenfold at pH 7.8 in a Veronal buffer of constant ionic strength. The concentration of NH_4^+ is essentially constant. Only the concentration of NH_3 has been changed significantly (about threefold) and this exactly accounts for the increased catalysis.

The rate of amino acid catalyzed PP_i–ATP exchange is suppressed by the bases enumerated above and especially strongly suppressed (90% by 0.002 M phenanthroline) by polyfunctional bases which, as noted above, stimulate hydroxamate formation. From these observations, we conclude that the rate-determining reaction in PP_i–ATP exchange is different from the rate-determining reaction in hydroxamate or aminoacyl-tRNA formation. It is to be emphasized that the effectiveness of the base and the optimal concentration are different for each enzyme and amino acid further pointing up the complexity of these systems.

References

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 839.

Berg, P. (1957), *Fed. Proc. 16*, 152.

Berg, P. (1958), *J. Biol. Chem. 233*, 601.

Bergmann, F. H., Berg, F., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1735.

Bruice, T. C., and Benkovic, S. S. (1964), *J. Amer. Chem. Soc. 86*, 418.

Bruice, T. C., and Bruno, J. J. (1961), *J. Amer. Chem. Soc. 83*, 3494.

Caplow, M., and Jencks, W. P. (1962), *Biochemistry 1*, 833.

Connors, K. A., and Mollica, J. A. (1965), *J. Amer. Chem. Soc. 87*, 123.

Conway, T. W., Lansford, E. M., and Shive, W. (1962), *J. Biol. Chem. 237*, 2850.

DeMoss, J. A., Genuth, S. M., and Novelli, G. D. (1956), *Fed. Proc. 15*, 241.

Hoagland, M. B. (1955), *Biochim. Biophys. Acta 16*, 288.

Jencks, W. P. (1958), *J. Amer. Chem. Soc. 80*, 45.

Jencks, W. P., and Carriulo, J. (1960), *J. Amer. Chem. Soc. 82*, 675.

Karasek, M., Castelfranco, P., Krishnaswamy, P. R., and Meister, A. (1958), *J. Amer. Chem. Soc. 80*, 2335.

Kirsch, J. F., and Jencks, W. P. (1964), *J. Amer. Chem. Soc. 86*, 833.

Loftfield, R. B. (1950), *J. Amer. Chem. Soc. 72*, 2499.

Loftfield, R. B., and Eigner, E. A. (1959), *J. Amer. Chem. Soc. 81*, 4753.

Loftfield, R. B., and Eigner, E. A. (1963a), *Biochim. Biophys. Acta 72*, 372.

Loftfield, R. B., and Eigner, E. A. (1963b), *Acta Chem. Scand. 17*, S 117.

Loftfield, R. B., and Eigner, E. A. (1965), *J. Biol. Chem. 240*, PC 1482.

Loftfield, R. B., and Eigner, E. A. (1966a), *Biochim. Biophys. Acta 130*, 426.

Loftfield, R. B., and Eigner, E. A. (1966b), *Biochim. Biophys. Acta 130*, 449.

Loftfield, R. B., and Eigner, E. A. (1967), *J. Biol. Chem. 242*, 5355.

Mitra, S. K., and Mehler, A. H. (1967), *Fed. Proc. 26*, 866.

Monier, R., Stephenson, M. L., and Zamecnik, P. C. (1960), *Biochim. Biophys. Acta 43*, 1.

Norris, A. T., and Berg, P. (1964), *Proc. Nat. Acad. Sci. U.S. 52*, 330.

Stulberg, M. P., and Novelli, G. D. (1962), *Enzymes 6*, 401.